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ORIGINAL PAPER

Insight into the evolutionary history of symbiotic genes of *Robinia pseudoacacia* rhizobia deriving from Poland and Japan

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Abstract The phylogeny of symbiotic genes of *Robinia pseudoacacia* (black locust) rhizobia derived from Poland and Japan was studied by comparative sequence analysis of *nodA, nodC, nodH*, and *nifH* loci. In phylogenetic trees, black locust symbionts formed a branch of their own suggesting that the spread and maintenance of symbiotic genes within *Robinia pseudoacacia* rhizobia occurred through vertical transmission. There was 99–100% sequence similarity for *nodA* genes of *Robinia pseudoacacia* nodulators, 97–98% for *nodC*, and 97–100% for *nodH* and *nifH* loci. A considerable sequence conservation of *sym* genes shows that the symbiotic apparatus of *Robinia pseudoacacia* rhizobia might have evolved under strong host plant constraints. In the *nodA* and *nodC* gene phylograms, *Robinia pseudoacacia* rhizobia grouped with *Phaseolus* sp. symbionts, although they were not closely related to our isolates based on 16S rRNA genes, and with *Mesorhizobium amorphae*. *nifH* gene phylogeny of our isolates followed the evolutionary history of 16S rDNA and *Robinia pseudoacacia* rhizobia grouped with *Mesorhizobium* genus species. Nodulation assays revealed that *Robinia pseudoacacia* rhizobia effectively nodulated their native host and also *Amorpha fruticosa* and *Amorpha californica* resulting in a significant enhancement of plant growth. The black locust root nodules are shown to be of indeterminate type.

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Introduction

Soil bacteria collectively called rhizobia are able to induce nitrogen-fixing symbiotic association with roots of leguminous plants. An effective symbiosis requires several bacterial genes, including nitrogen-fixation (*nif*) genes that encode nitrogenase responsible for N_2 reduction and nodulation (*nod*) genes that encode Nod factors which trigger root hair deformations and cortical cell divisions (Debelle et al. 2001; Perret et al. 2000). Nod factors consist of β-1,4-linked N-acetyl glucosamine oligosaccharide backbone ranging in length from 3 to 5 residues that are substituted by an N-acyl chain on the non-reducing end and other chemical groups on the glucosamine residues. The backbone of the Nod factors is synthesized under control of the common *nod* genes, *nodA* (acyl transferase), *nodB* (deacetylase), and *nodC* (N-acetylglucosaminyl transferase), and next it is modified by different chemical groups (sulfate, fucosyl) that determine the specificity of rhizobium–legume interactions. The synthesis and transfer of chemical "decorations" to the backbone part of Nod factors is encoded by the host-specific nodulation (*hsn*) genes. In addition, the common *nod* genes contribute also to the bacterial host range. For example, NodA proteins recognize and transfer only specific fatty acids to the Nod core, the length of which determines NodC protein (Debelle et al. 2001; Downie 1998). Symbiotic *nod* and *nif* genes which are located on transmissible plasmids in *Rhizobium, Sinorhizobium,* and some *Mesorhiozbium* species or on genomic islands in *Mesorhizobium loti* and *Bradyrhizobium japonicum* may be transferred across divergent chromosomal lineages (González et al. 2003; Gottfert

et al. 2001; Mierzwa et al. 2009; Nandasena et al. 2007; Sullivan and Ronson 1998; Xu and Murooka 1995; Zhang et al. 2008; Zou et al. 1997). It was shown for agricultural rhizobial populations (Laguerre et al. 1996; Sullivan et al. 1995) and concluded by discordance between the genealogies of *nod* and housekeeping loci (Laguerre et al. 2001; Suominen et al. 2001; Vinuesa et al. 2005a; Young and Haukka 1996). Such gene transfer may adopt rhizobial populations to new host plant and enable symbionts with the same genetic background but different *nod* genes to nodulate distinct legumes as it was found for *R. leguminosarum* forming symbiosis with *Viciae, Trifolium,* and *Phaseoli* species plants (Martínez et al. 1990). By contrast to *nod* genes, the phylogeny of *nifH* genes, which encode dinitrogenase reductase, was reported to be congruent with 16S rDNA phylogeny (Hennecke et al. 1985; Wdowiak-Wróbel and Małek 2010; Young 1992; Zhang et al. 2008); however, in some cases, it followed that of *nodA* genes (Haukka et al. 1998; Laguerre et al. 2001; Vinuesa et al. 2005a, b).

Symbiotic promiscuity of *R. pseudoacacia* was examined by Han et al. (Han et al. 2008), Ulrich and Zaspel (Ulrich and Zaspel 2000), Wei et al. (Wei et al. 2009) and Mierzwa et al. (Mierzwa et al. 2009). Those studies showed black locust to be nodulated by *Rhizobium, Sinorhizobium*, and *Mesorhizobium* genera bacteria, however, the latter ones clearly predominated in root nodules. Despite taxonomic diversity, *R. pseudoacacia* rhizobia from North America, Germany, and China shared very similar sequences of *nodA, nodC,* and *nifH* genes (Wei et al. 2009). According to Wei et al. (Wei et al. 2009), local rhizobia from China were adopted to symbiosis with black locust by lateral transfer of *nod* genes from *R. pseudoacacia* nodulators derived from North America where this plant is native.

In this paper, we present the phylogeny of symbiotic *nodA, nodC, nodH*, and *nifH* genes of *R. pseudoacacia* rhizobia derived from Poland and Japan and microscopic structure of black locust root nodules.

Materials and methods

Bacterial strains and growth conditions

For phylogenetic analysis of *R. pseudoacacia* rhizobium symbiotic genes, the following nodule isolates were chosen: RPP14, RPP20 (from Poland), RPJ3, RPJ5, RPJ6, and RPJ16 (from Japan; Mierzwa et al. 2009). Additionally, these strains and 16 other black locust rhizobia described in the paper of Mierzwa et al. (Mierzwa et al. 2009) were used in plant tests in order to determine their host range and the effectiveness of N_2 fixation. Rhizobium strains were maintained and grown on yeast extract-mannitol medium (YEM) as described earlier (Mierzwa et al. 2009).

PCR amplification and sequencing of amplicons

For each *R. pseudoacacia* nodulator, total genomic DNA was isolated, as described previously (Mierzwa et al. 2009), and used as PCR template. PCRs were performed with the ReadyMix^{TM}Taq kit according to the manufacturer's recommendations (Sigma). The 660-bp fragment of *nodA* gene was amplified and sequenced using forward primer nodA-1 (5′-TGCRGTGGAARNTRNNCTGGGAAA-3′) and reverse one nodA-2 (5′-GGNCCGTCRTCRAAWGT CARGTA-3′) according to the procedure described by Haukka et al. (Haukka et al. 1998). The *nodC* gene sequences were amplified and sequenced with forward primer nodCFu (5′-AYGTHGTYGAYGACGGITC-3′) and reverse primer nodCI (5′-CGYGACAGCCANTCKCTA TTG-3′) corresponding to the position 251–269 bp and 1160–1181 bp of *nodC* gene of *R. leguminosarum* bv. viciae, respectively. PCR was performed using the same cycling parameters as reported for this gene by Laguerre et al. (Laguerre et al. 2001). The 567-bp fragment of *nodH* gene was amplified and sequenced with forward primer TSnodH1 (5′-VTKGAGYAACGGTGARYTGCTCA-3′) and reverse one TSnodH2 (5′-GCGAAGTGAWSCCGCA ACTC-3′) under the following conditions: preheating at 95°C for 2 min; 35 cycles of denaturing at 95°C for 45 s, annealing at 53°C for 30 s, and extension at 72°C for 2 min; and a final extension at 72°C for 7 min. A large ~780-bp long fragment of *nifH* gene was amplified and sequenced with primers NifH1 (5′-CGTTTTACGGCAAGGGCGG-3′) and NifH2 (5′-TCCTCCAGCTCCTCCATGGT-3′). *nifH* sequences were amplified following the procedure described by Perret and Broughton (1998). PCR products were purified by using Montage PCR Filter Units (Millipore, Massachusetts, USA) as recommended by the manufacturer. Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle sequence kit (Applied Biosystems model 310 DNA sequencer). Sequences were deposited in the GenBank database under accession numbers listed in the phylograms.

Sequence analyses

Multiple sequence alignments were generated using ClustalX (Thompson et al. 1997) and manually corrected using GeneDoc software (Nicholas and Nicholas 1997). Phylogenetic trees were generated by maximum likelihood (ML) approach using PhyML 3.0 and the nucleotide substitution model selected by the Akaike information criterion, as implemented in MODELTEST3.6 (Posada and Crondall 1998). Robustness of tree nods was estimated by bootstrap analysis with 100 replicates of each sequence using PhyML. The trees were displayed by using TreeView (Page 1996).

Plant tests

R. pseudoacacia microsymbionts were tested for symbiotic interactions with their original host plant (*R. pseudoacacia*), *Trifolium repens*, *Lotus corniculatus*, *Medicago sativa*, *Ornithopus sativus*, *Phaseolus vulgaris*, *Glycine max*, *Astragalus glycyphyllos*, *Astragalus sinicus*, *Vicia sativa*, *Lupinus luteus*, *Amorpha fruticosa*, and *Amorpha californica*. Seeds of legumes were surface-sterilized, germinated, and plants inoculated with bacterial suspension were grown in nitrogen-free Hoagland's medium as described by Mierzwa et al. (Mierzwa et al. 2009). For each legume species–rhizobium interaction (ten strains from Poland and ten strains from Japan), three replicates were prepared. The symbiotic properties of *R. pseudoacacia* nodulators were assessed by the presence of nodules, shoot dry weight, and the acetylene-reducing activity (Turner and Gibson 1980). Non-inoculated plants were used as negative controls.

Light and electron microscopy

For the microscopy studies, a whole root nodules sampled 14, 28 days after inoculation (DAI), and nodule fragments sampled 91 DAI were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 2 h under air pressure of −0.06 MPa at room temperature. Next, the samples were treated as described earlier (Kalita et al. 2006), embedded in glycid ether 100 epoxy resin (SERVA), and the resin was polymerized at 60°C for 24 h according to the manufacturer's formula. Serial resin sections 2–4 μm thick (depending on the nodule size) and ultra-thin sections were cut and treated as described by Kalita et al. (Kalita et al. 2006). The anatomical observations and micrographs were done using light microscope Provis AX (Olympus) equipped with digital photo camera. The ultrastructure was observed under transmission electron microscope Morgagni 268 (FEI Company) operating at 80 kV. The images were adjusted using "Levels" or "Curves" tools in Photoshop 7.0 CE (Adobe) software.

Results

Phylogeny of symbiotic genes of *R. pseudoacacia* rhizobia

The *R. pseudoacacia* microsymbionts, two from Poland and four from Japan which represented three different phenogroups (Mierzwa et al. 2009), were studied for the phylogeny of their symbiotic loci by comparative analysis of nodulation *nodA, nodC, nodH,* and dinitrogenase reductase *nifH* genes. These genes have been widely used in studies of symbiosis genealogy in rhizobia. *nodA* PCR amplification with nodA-1, nodA-2 primers yielded a single band of about 600-bp. ML analysis of 437-bp *nodA* sequences of black locust rhizobia derived from Poland and Japan (this study) and China, Germany, and North America (present in the GenGank database), as well as other nodule bacteria, is shown in Fig. 1. The best-fit model selected by Akaike Information Criterion (AIC) was Tamura-Nei (TrN + I + G). All *R. pseudoacacia* rhizobia of *nodA* sequence identity 99–100% were grouped together with 100% bootstrap support (BS) and in bigger highly validated monophyletic cluster (98% BS) with *R. giardini* bv. *giardini, R. gallicum* bv. *gallicum, R. tropici* (symbionts of *Phaseoli* sp.), and *M. amorphae* (symbiont of *Amorphae fruticosa*) as a basal bifurcation. NodA sequence similarities of these bacteria and black locust rhizobia ranged from 73 to 79%. Other rhizobia, with 57–73% *nodA* sequence identities to *R. pseudoacacia* nodule isolates, formed four clearly separated lineages. On the outskirt of this tree, *A. caulinodans* (57% sequence similarity) was located.

Next, we concentrated on *nodC* gene evolutionary history. Phylogenetic analysis was carried out on 837-bp *nodC* fragment amplified and sequenced in all six tested *R. pseudoacacia* microsymbionts (data not presented) and on a shorter 419-bp segment when black locust rhizobia from Germany, China, and the USA (GenGank database) were included into studies (Fig. 2). ML approach to *nodC* gene phylogeny gave in both cases very similar tree topologies in TrN + I + G model selected by AIC. *R. pseudoacacia* rhizobia from Poland, Japan, China, Germany, and North America, with 97–98% *nodC* nucleotide identities, formed monophyletic branch together with *M. amorphae, R. gallicum* bv. *gallicum, R. giardinii* bv. *giardinii, R. gallicum* bv. *phaseoli*, and *R. etli* (76–82% sequence similarity) which splits in two well-supported major clusters. One of them contains nine black locust rhizobia and *M. amorphae*, the second one harbors bean endosymbionts (Fig. 2). At the basal position of this clade, *Mesorhizobium* sp. (*Oxytropis*) N33, *M. septentrionale*, and *M. temperatum* were situated. In respect to *nodC* sequences of the tested rhizobia, they showed differences in 17–21% nucleotides. Other rhizobia which displayed 55–77% *nodC* nucleotide sequence similarity to black locust isolates spread into five clearly resolved lineages with *A. caulinodans* as outlier (55% identical nucleotides).

To uncover the phylogeny of dinitrogen reductase of *R. pseudoacacia* microsymbionts, we amplified and sequenced the 689-bp fragments of *nifH* genes in all six tested rhizobia. Phylogenetic analysis was carried out on the whole this amplicon and its 469-bp segment when black locust rhizobia from China, Germany, and North America (GenGank database) were included into studies. Both *nifH* phylograms, obtained under the best-fit General Time

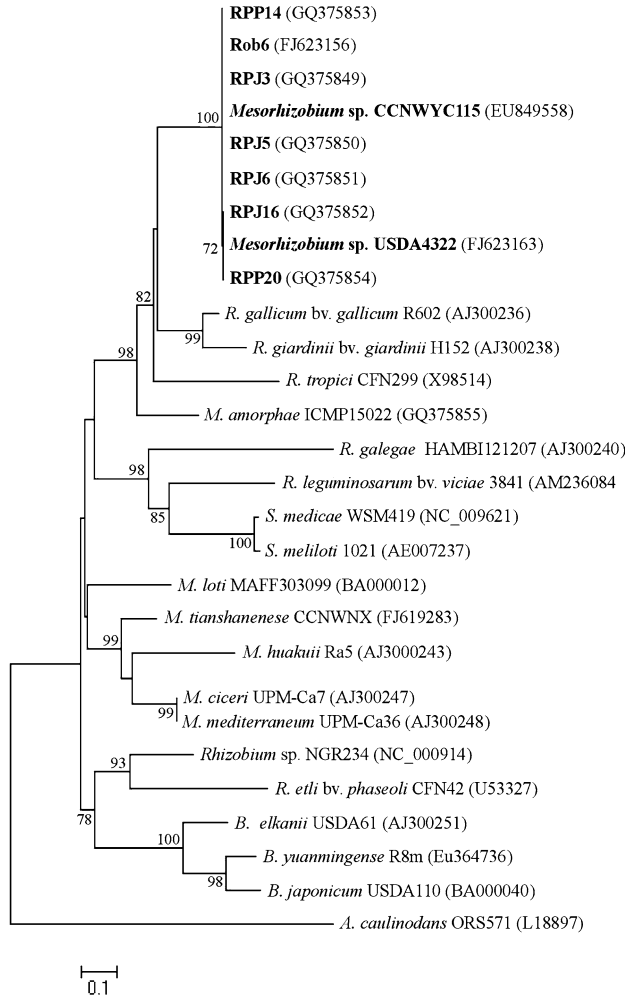

Fig. 1 Phylogenetic ML tree based on 437-bp nucleotide sequences of the *nodA* genes of *R. pseudoacacia* nodule isolates (shown in *bold*) and reference rhizobial strains. Only bootstrap probability values of ≥ 70% (for 100 pseudoreplicates) are indicated at the branching points. The *scale bar* indicates the number of substitutions per site. GenBank accession numbers are shown in parentheses

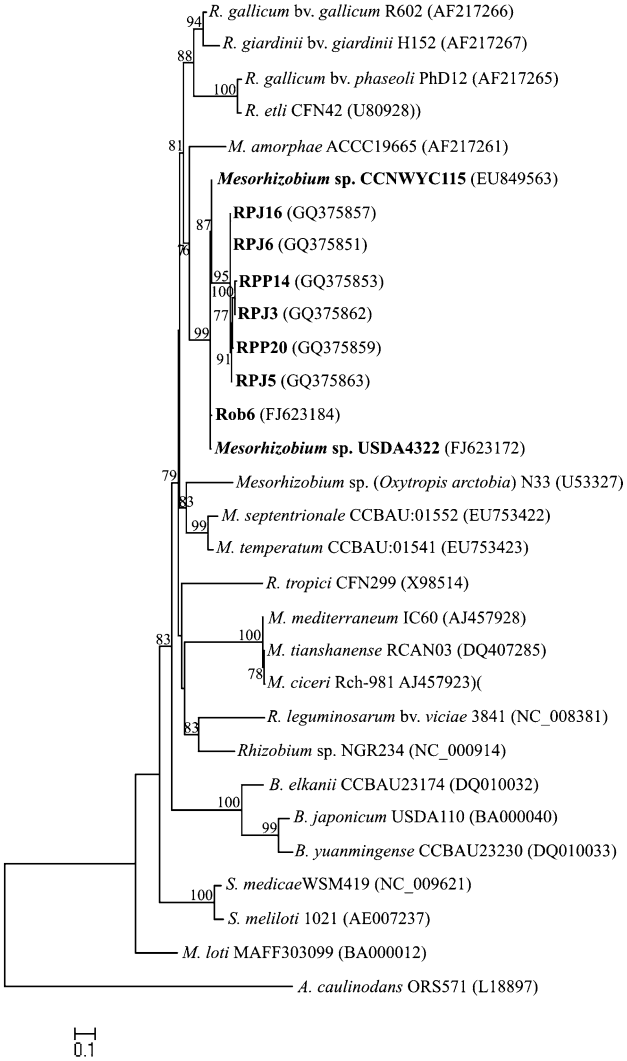

Fig. 2 ML phylogram based on 419-bp nucleotide sequences of the *nodC* genes of *R. pseudoacacia* nodule isolates (shown in *bold*) and reference rhizobial strains. Bootstrap values of ≥70% (for 100 pseudoreplicates) are indicated at the corresponding nodes. The *scale bar* indicates the number of substitutions per site. GenBank accession numbers are shown in parentheses

Reversible model of sequence evolution (GTR + I + G) have a very similar topology. Only the tree for shorter *nifH* gene fragment is presented in Fig. 3. All *R. pseudoacacia* rhizobia from Europe, Asia, and North America with 97–100% *nifH* sequence identity to each other lied in the same clade in 98% of bootstrap replicates. They grouped with *Mesorhizobium* species (except *M. loti*) within well-resolved clade and shared with them 88–94% *nifH* sequence identity. Outside this clade, on the sister branch, *M. loti* along with *R. tropici* was positioned. *nifH* sequences from these bacteria and black locust endosymbionts revealed identity from 89 to 92%. Other rhizobia were placed in three clearly resolved clusters and one single strain lineage (*R. etli*). *Bradyrhizobium* species and *A. caulinodans* formed a highly validated, well-resolved cluster at the basal position of the corresponding tree. All these bacteria differed from the tested *R. pseudoacacia* microsymbionts at 29–10% *nifH* sequences.

In our studies on symbiotic genes of *R. pseudoacacia* microsymbionts, we also focused on the *nodH* host-specificity genes involved in transfer of activated sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to the Nod factor core structure (Debelle et al. 2001; Perret et al. 2000). Five hundred and sixty-five–base pair fragment of *nodH* gene was amplified and sequenced in all six tested strains. The nucleotide identity for *nodH* genes among black locust rhizobia was 97–100%. Phylogenetic analysis of *nodH* partial sequences with best-fit Hasegawa-Kishino-Yano model (HKY + G) of nucleotide substitution resulted in ML tree presented in Fig. 4. *R. pseudoacacia* nodule

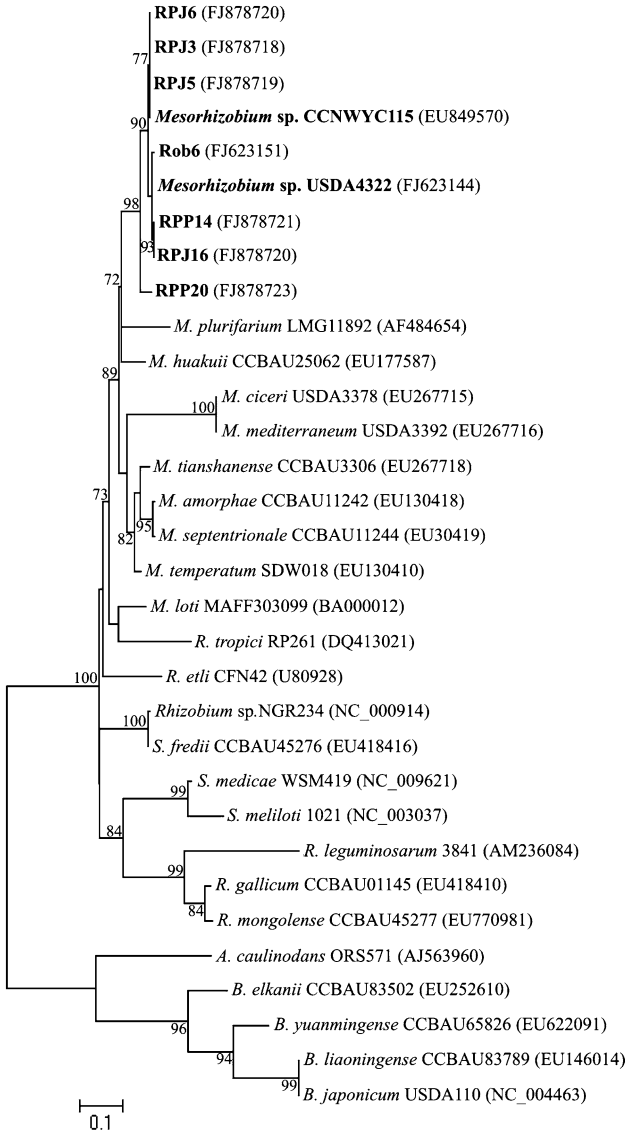

Fig. 3 Phylogenetic ML tree based on 469-bp nucleotide sequences of the *nifH* genes of *R. pseudoacacia* nodule isolates (shown in *bold*) and reference rhizobial strains. Only bootstrap probability values of ≥70% (for 100 pseudoreplicates) are indicated at the branching points. The *scale bar* indicates the number of substitutions per site. GenBank accession numbers are shown in parentheses

isolates from Poland and Japan clustered together at 100% bootstrap support and they grouped well with *Mesorhizobium* sp. (*Oxytropis arctobia*) N33 strain (80–81% sequence identity) and *Sinorhizobium* sp. BR816 (77% identity), suggesting that they might have been of monophyletic origin. The nucleotide sequence similarity of *nodH R. pseudoacacia* rhizobia, and *Methylobacterium nodulans* was 75–76%. Compared with the *nodH* sequences of *Sinorhizobium meliloti* strains and *Rhizobium* sp. LPU83, our isolates indicated a lower percentage of identical nucleotides, i.e., in the range from 73 to 74%. On the outskirts of *nodH* gene tree, *R. tropici* and *Burkholderia phymatum*

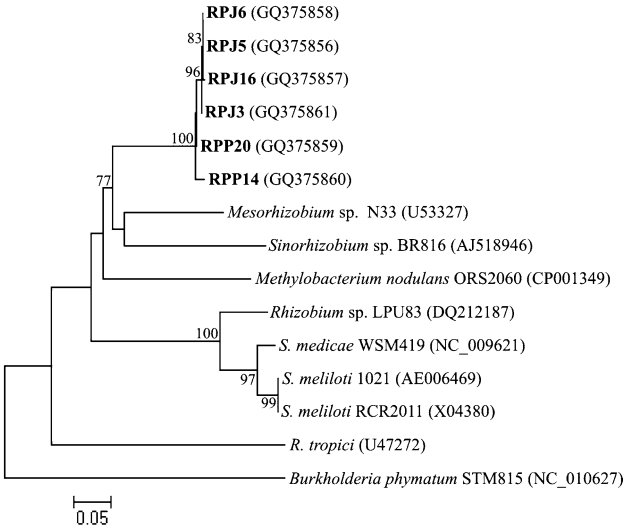

Fig. 4 ML phylogram based on 565-bp nucleotide sequences of the *nodH* genes of *R. pseudoacacia* nodule isolates (shown in *bold*) and reference rhizobial strains. Bootstrap values of ≥70% (for 100 pseudoreplicates) are indicated at the corresponding nodes. The *scale bar* indicates the number of substitutions per site. GenBank accession numbers are shown in parentheses

were positioned with 68 and 66% sequence similarity, respectively.

Host plant associations

R. pseudoacacia microsymbionts derived from Poland and Japan were able to form nitrogen-fixing symbiosis not only with the host from which they were isolated but also with *A. fruticosa* and *A. californica*. None of our isolates nodulated: *Trifolium repens*, *Lotus corniculatus*, *Medicago sativa*, *Ornithopus sativus*, *Phaseolus vulgaris*, *Glycine max*, *Vicia sativa*, *Lupinus luteus, Astragalus cicer, Astragalus glycyphyllos,* and *Astragalus sinicus* (Mierzwa et al. 2009). After 6 weeks postinoculation, the dry matter of green parts of nodulated legumes was 2–3 times higher than that of uninoculated ones showing that the used bacteria fix N_2 and supply it to the symbiotic partner (Table 1). Symbiotic interactions between the tested rhizobia and *R. pseudoacacia, A. fruticosa,* and *A. californica* were also evaluated for nitrogenase activity by the acetylene reduction test. The highest level of enzyme activity *R. pseudoacacia* rhizobia showed in association with their original host, and next in descending sequence with *A. fruticosa* and *A. californica*, although these bacteria were good diazotrophs in symbiosis with all three legumes (Table 1).

Microscopic structure of *R. pseudoacacia* root nodules

The root nodules of *R. pseudoacacia* were initiated within deep layers of root primary cortex and became infected *via*

Table 1 Nitrogen-fixing activity of *Robinia pseudoacacia* rhizobia in symbiosis with different legumes

Host plant for *Robinia pseudoacacia* nodule isolates	Symbiotic effectiveness			
	Shoot dry weight (mg/plant)		Nitrogenase activity (nmol ethylene/h/plant)	
	Average[a]	SD	Average[a]	SD
Robinia pseudoacacia	28.8	3.7	625.7	46.6
	NC 11.2	1.7	NT	
Amorpha fruticosa	13.9	1.8	410.4	29.3
	NC 5.6	0.8	NT	
Amorpha californica	11.9	1.7	325.8	27.4
	NC 4.9	0.7	NT	

SD standard deviation, *NC* negative control (uninoculated plant), *NT* not tested (uninoculated plants were not checked for acetylene reduction)

[a] The values are the mean of 60 plants inoculated separately with 20 strains (ten from each geographic region), in three replicates

an infection thread originating from the curled root hair. Up to 28–35 DAI, the root nodules were round with two strands of loose parenchyma on their surface (data not shown). Later, the nodules took an elongate shape (Fig. 5), and among 91 DAI nodules, bifurcated ones were found. Microscope observations proved the root nodules of *R. pseudoacacia* to be of indeterminate type, with persistent apical meristem, bacteroid tissue consisting of infected and uninfected cells and multilayered nodule cortex with a system of vascular bundles (Fig. 5). Within the bacteroid tissue, the following developmental zones were discernible: (1) infection thread penetration zone, (2) symbiosome multiplication zone, (3) differentiated zone, (4) senescent zone, and (5) saprophytic zone. The nodule meristem was narrow. Electron-dense precipitate or globules were observed in vacuoles. The organelles were relatively sparse and the plastids contained small starch grains. In 91 DAI nodules, the plastids within the nodule meristem, as well as the infection thread penetration zone, accumulated large amounts of ferritin (Fig. 6 and the insert). The infection threads, within the whole bacteroid tissue, contained a non-typical electron-empty layer between the infection thread wall and matrix. Starch accumulation was evident in the non-infected cells of differentiating bacteroid tissue, while in the infected cells, starch grains were small or not evident.

In the infected cells of differentiated bacteroid tissue, a large vacuole occurred at the centrally positioned nucleus (Fig. 7 and the insert). Plastids were devoid of starch grains. Together with mitochondria, they formed tight clusters at the intercellular spaces. Several bacteroids were observed in some symbiosomes.

The senescence of bacteroid tissue-infected cells started with widening of peribacteroid space and fusion of symbiosomes (data not shown). In some symbiosomes, the peribacteroid membrane fused with the tonoplast. In such cases, the vacuolar lytic system could participate in the degradation of bacteroids, but most bacteroids were degraded within their symbiosomes. The degradation of the host organellae followed the degradation of symbiosomes. The infection thread wall became visibly loosened. In the non-infected cells, the size of starch grains increased, the vacuoles decreased, but the cytoplasm loose its density.

After the infected cell's degradation was complete, some of the dead cells became populated by large number of rhizobia (Fig. 5), which released themselves from infection threads and never started the transformation into bacteroids. Such colonies of saprotrophic rhizobia were present also within numerous intercellular spaces.

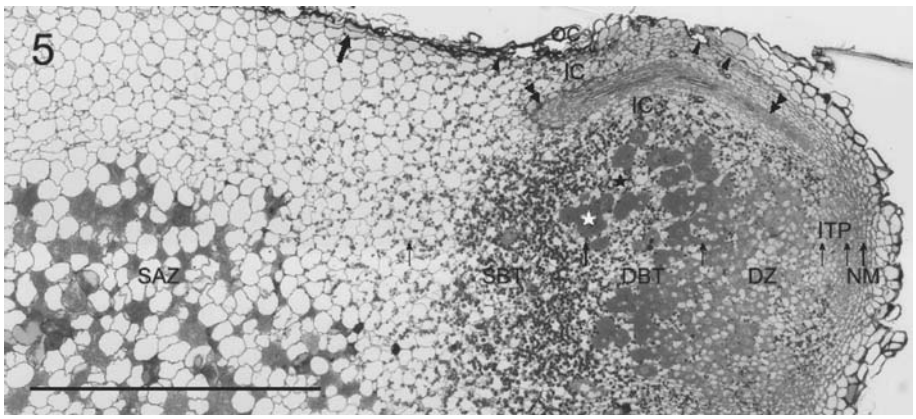

Fig. 5 Anatomy of *Robinia pseudoacacia* root nodules, 91 DAI. The approximate boundaries of developmental zones are marked with *thin arrows*. *NM* nodule meristem, *ITP* infection thread penetration zone, *DZ* differentiating zone, *DBT* differentiated bacteroid tissue, *SBT* senescent bacteroid tissue, *SAZ* saprophytic (= saprotrophic) zone, *OC* outer cortex, *IC* inner cortex, *white* and *black asterisks*—infected and non-infected cells in the bacteroid tissue, respectively, *arrowheads*—cortical endodermis, *double arrowheads*—vascular bundle, *arrow*—cell of the meristemoid producing the "periderm". *Bar* 600 µm

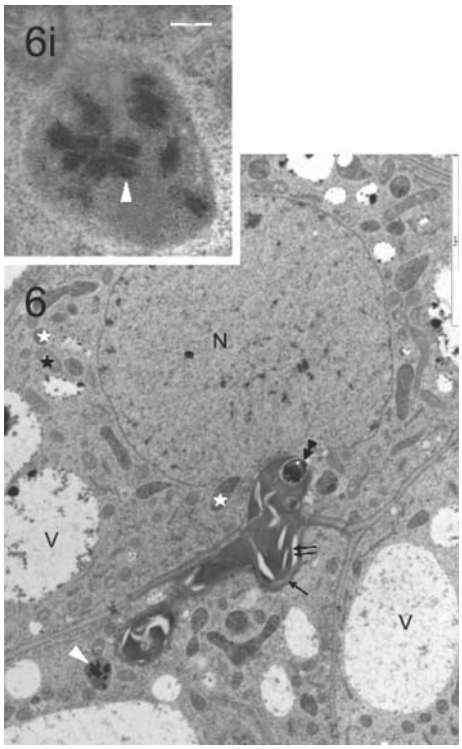

Fig. 6 Ultrastructure of infected cell from the infection thread penetration zone in *Robinia pseudoacacia* root nodules. *N* cell nucleus, *V* vacuole (note the electron-dense material), *thin arrow*—infection thread wall, *double arrow*—electron-empty layer between the infection thread wall and thread matrix, *double arrowhead*—rhizobium cell to be released from the infection thread, *black asterisks*—mitochondria, *white asterisks*—plastids, *white arrowhead*—a plastid with heavy accumulation of ferritin (shown enlarged in the insert). *Bars* 5 μm and 0.2 μm, respectively

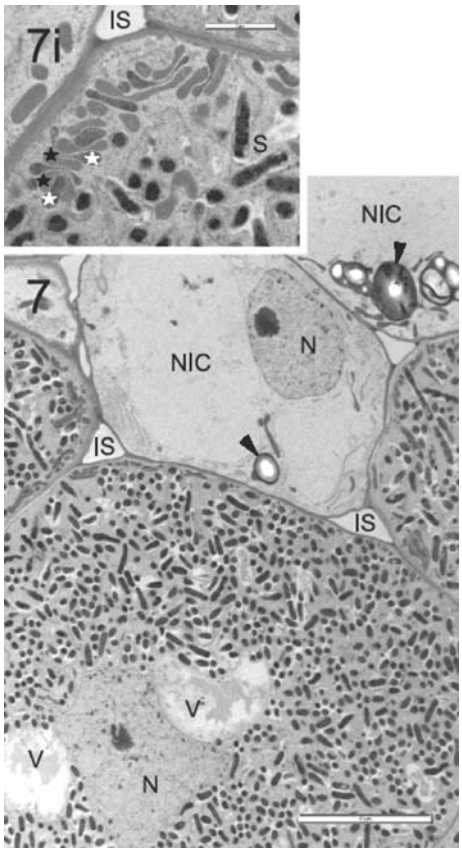

Fig. 7 Ultrastructure of differentiated bacteroid tissue in *Robinia pseudoacacia* root nodules. *IS* intercellular space, *N* cell nucleus, *NIC* non-infected cells of the bacteroid tissue, *S* symbiosomes, *V* vacuole with a fine-fibrillar material, *black asterisks* in insert—mitochondria, *white asterisks* in insert—plastids (note the close arrangement of plastids and mitochondria and their positioning at the IS), *arrowheads*—starch grains in NIC's amyloplasts. *Bars*10 μm and 2 μm, respectively

Within the nodule cortex of young nodules, three typical layers were differentiated (Fig. 5): the multilayered inner cortex with the boundary layer, the monolayered cortical endodermis, and the outer cortex of loose parenchyma. In some parts of the older nodules, the inner cortex cells adjoining the cortical endodermis dedifferentiated and formed meristemoids that produced parenchymatous cells in regular files, resembling the arrangement of periderm cells (Fig. 5).

Discussion

R. pseudoacacia rhizobia originating from Poland and Japan, which were identified as *Mesorhizobium* genus bacteria by phenotypic, genomic, and 16S rDNA phylogenetic analyses (Mierzwa et al. 2009), were now studied for evolutionary history of nodulation (*nodA, nodC, nodH*) and nitrogen-fixation (*nifH*) genes. The products of all these symbiotic genes have well-defined functions. The NodA determines the type of N-acyl substitution of the Nod factor and therefore it plays a crucial role in host plant recognition; NodC controls the length of chitin oligosaccharide in Nod factor biosynthesis, NodH is the host range determinant and catalyzes the transfer of sulfate from PAPS to Nod factor, and NifH is the Fe–S protein subunit of nitrogenase (Debelle et al. 2001; Perret et al. 2000). Phylogenetic analysis of both *nodA* and *nodC* sequences of *R. pseudoacacia* rhizobia provided compelling evidence for their monophyletic origin and a strong support for the hypothesis that these bacteria form a sister lineage to *Phaseolus* sp. symbionts known to be taxonomically distant and to *M. amorphae*, symbiont of *Amorpha fruticosa* (Figs. 1, 2). We also suggest that these bacteria inherit *nod* genes from their common ancestor. Black locust rhizobia studied in this work as well as those from China, Germany, and North America have very similar or even identical *nod* sequences without mosaicism and without biogeographic patterns (Figs. 1, 2). The clustering of all *R. pseudoacacia* rhizobia in a single, tight clade of their own may suggest that dissemination of *nod* genes within *R. pseudoacacia* rhizobia

lineage occurred mainly through vertical transmission and that lateral transfer of nod genes between these bacteria and other rhizobia is rather a rare event. We also suggest that an extremely low rate of *nodA* and *nodC* divergence noted for black locust symbionts is required to their proper function in symbiotic association with the host plant and that plant strongly affected evolution of common *nod* genes by selecting against changes preventing host nodulation. Several rhizobiologists discussed the role of leguminous plant as the key evolutionary force that provides cohesion to rhizobia by structuring of nodulation genes (Aguilar et al. 2004; Bena et al. 2005; Wernegreen and Riley 1999). Bipartition separating *R. pseudoacacia* symbionts and those of *A. fruticosa* (*nodC* gene tree) and *Phaseolus* sp. (*nodA* tree) may correspond to speciation events that guarantee optimal cooperation of both symbiotic partners. Extreme divergence of *nodA* and *nodC* sequences of *Azorhizobium caulinodans* in relation to those of the tested rhizobia that form stem and root symbiosis with plants of the same tribe Robinieae, i.e., *Sesbania* sp. and *Robinia pseudoacacia*, respectively (Perret et al. 2000), allows us to hypothesize that stem nodulation requires a specific adaptation of *nod* genes controlled by leguminous host.

R. pseudoacacia rhizobia were found to harbor *nodH* genes suggesting that these bacteria produce Nod molecules that bear a sulfate, although the functionality of these genes was not proved for them. The almost identical *nodH* sequences of black locust rhizobia are the most similar to the corresponding genes of *Mesorhizobium* sp. (*Oxytropis arctobia*) N33 and *Sinorhizobium* sp. BR816 strains. On the *nodH* gene tree, all these bacteria grouped together in a single clade (Fig. 4) suggesting that their *nodH* genes have a common evolutionary history. The inferred phylogeny seems to be not reliable due to a very low number of *nodH* sequences available and included into this study. Sulfation of Nod signal molecules was demonstrated in several rhizobium species. For the first time in *S. meliloti,* where it is required for alfalfa nodulation (Lerouge et al. 1990), and subsequently in *Mesorhizobium* sp. (*Oxytropis arctobia*) N33 which forms nodules on both arctic and temperate legumes such as *Astragalus, Onobrychis*, and *Oxytropis* species (Debelle et al. 2001), *R. tropici*, a broad host range bacterium that is able to nodulate *Phaseolus vulgaris, Macroptilium atropurpureum,* and *Leucaena leucocephala* (Poupot et al. 1993), *M. huakuii*, symbiont of *Astragalus sinicus* (Yang et al. 1999), *Sinorhizobium* sp. BR816, a heat-tolerant tropical strain isolated from *Leucaena leucocephala* that also nodulate common bean (Laeremans et al. 1997), and *Methylobacter nodulans* ORS 2060, symbiont of *Crotalaria podocarpa* (Renier et al. 2007).

In this work, we generated also *nifH* sequences of the tested *R. pseudoacacia* rhizobia and studied their evolutionary history. Analysis revealed that *R. pseudoacacia* is nodulated by rhizobia with very similar *nifH* genes despite the fact that some were from Poland and some from Japan. In the *nifH* tree (Fig. 3), black locust symbionts formed its own highly supported lineage associated with *Mesorhizobium* species in a robust, monophyletic cluster reflecting that nitrogenase Fe–S proteins of these bacteria have a common evolutionary history. The observed clustering of *R. pseudoacacia* rhizobia was congruent with that expected from the previous work on 16S rDNA phylogeny (Mierzwa et al. 2009) and compatible with the view of many authors that *nifH* gene phylogeny closely follows 16S rDNA evolutionary history (Hennecke et al. 1985; Wdowiak-Wróbel and Małek 2010; Zhang et al. 2008). The common evolutionary patterns of *nifH* and 16S rRNA genes and tight clustering of *R. pseudoacacia* rhizobia on its own branch may suggest that *nifH* genes of these bacteria require for optimal functioning a correct chromosomal background and that they evolved to a large degree parallelly with bacteria which carry them without a significant gene exchange with other bacteria.

In conclusion, the results from molecular analysis of symbiotic genes of *R. pseudoacacia* rhizobia suggest that: (1) common *nod* and *nifH* genes have independent evolutionary histories, i.e., *nifH* genes evolved to a large degree in a similar fashion as the bacteria that carry them, whereas the *nodA* and *nodC* gene phylogeny did not follow that of the core genes, (2) the studied symbiotic genes are well conserved, and (3) *sym* gene inheritance seems to be dominated by vertical descent.

Nod metabolites, encoded by common and host specificity nodulation genes of rhizobia, are key molecules in eliciting nodules in legume roots/or stems (Perret et al. 2000). Rhizobia vary significantly in their nodulation specificity. Some of them nodulate only a particular leguminous plants as for example *R. leguminosarum* bv. *trifolii* that forms symbiosis with *Trifolium* sp., and others are promiscuous and infect plants from various tribes and even various subfamilies as, for example, *Rhizobium* sp. NGR234 that infects 232 legume species of 112 genera tested and even forms nodules on non-legume *Parasponia andersonii* belonging to Ulmaceae family (Pueppke and Broughton 1999).

Microsymbionts of *R. pseudoacacia* represent a restricted range of host specificity. They were fastidious in the their choice of a symbiotic partner and formed effective associations with the host from which they were isolated but also with plants of *Amorpha* genus, i.e., *A. fruticosa* and *A. californica* (Table 1). It is worth noting that vice versa *A. fruticosa* also formed N_2-fixing associations with *R. pseudoacacia* (data not presented).

Root nodules of the tribe Robinieae are generally of indeterminate type (Sprent 2001), although morphologically determinate nodules are also reported (Corby 1988).

Here, it is confirmed in *R. pseudoacacia*. In this species, young nodules, if not examined in serial anatomical sections, may be mistaken for determinate ones due to their spherical shape together with the characteristic "lenticels" (data not shown). However, the older nodules developed a cylindrical shape typical for indeterminate nodules (Fig. 5). Also, as showed our observations under the light microscope (Fig. 5), the general anatomy of *R. pseudoacacia* nodules is similar to the well-studied indeterminate nodules of *Medicago* sp. (Timmers et al. 2000; Vasse et al. 1990) and e.g., *Trifolium* sp. (Łotocka et al. 1997). However, at the level of ultrastructure, some traits are observed that are specific for *R. pseudoacacia* nodules (data not shown). Among them, the most significant seems to be the absence of the II/III interzone at the proximal face of the differentiating bacteroid tissue. In the II/III interzone, as described by Vasse et al. (Vasse et al. 1990) in *Medicago* sp. and later observed in the other genera, the amyloplasts of the infected cells are positioned at the intercellular spaces and they rapidly accumulated especially large starch grains. In our work on *R. pseudoacacia*, very little or none starch was observed in the infected cell plastids (Figs. 6, 7), also at their late differentiation stage, and thus no II/III interzone can be discerned in the nodules of this species.

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