Bradyrhizobia associated with *Laburnum anagyroides*, an exotic legume grown in Poland

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**Abstract**

We isolated 18 rhizobial strains from root nodules of a leguminous shrub *Laburnum anagyroides* (common laburnum) grown in Southeast Poland as an exotic plant. With the use of BOX-PCR fingerprinting, the isolates were clustered into 2 main groups and one separate lineage, which was congruent with the ITS-RFLP results. The phylogenetic trees constructed based on 16S rRNA and combined atpD, dnaK, glnA, and recA gene sequence data separated the representative strains into three evolutionary lineages within the *Bradyrhizobium jicamae* supergroup, with *Bradyrhizobium algeriense* and *Bradyrhizobium valentinum* as the closest relatives. The *nodA* and *nifH* gene phylogenies proved that the *L. anagyroides* symbionts carry a symbiotic gene variant known as Clade IV, representing the symbiovar retamae. Phenotypic characteristics of the isolates and reference strains are also reported. Our study of the rhizobia nodulating *L. anagyroides* growing in Poland complements earlier few findings on the symbiotic associations of this Genisteae species.

**Keywords** Bradyrhizobium · *Laburnum anagyroides* · Symbiosis · Phylogeny · MLSA

1 **Introduction**

Bacteria of the genus *Bradyrhizobium* (Jordan 1982) and at least 11 other rhizobial genera participate in the process of N2-fixing symbiosis with legume plants classified in the family Fabaceae (Lindström et al. 2015; Shamseldin et al. 2017). They can establish symbiosis with all major legume lineages of Fabaceae spp. and even non-legume *Parasponia andersonii*, which suggests that bradyrhizobia may have been the ancestor of all rhizobia (Hungria et al. 2015; Parker 2015). *Bradyrhizobium* communities are also abundant in many soils where legumes are absent, often acting as endophytes of different plants (Vanlnsberghe et al. 2015). The studies have revealed that the genus *Bradyrhizobium* is separated into seven lineages, including symbiotic as well non-symbiotic bacteria (Avontuur et al. 2019; Ormeño-Orrillo and Martínez-Romero 2019). A large number of species in *Bradyrhizobium* has been recognized, however the list of species in this genus will probably be still growing due to the rapid development of molecular tools and the huge diversity of this genus (Stępkowski et al. 2018; de Lajudie et al. 2019).

Given the widespread deforestation and the need for increasing soil fertility and quality of poorly productive and anthropogenically affected soils, more importance is now being given to woody and shrubby legumes due to their beneficial properties (Adams et al. 2010; Castro et al. 2017). *Laburnum anagyroides* is a large shrub from the tribe Genisteae originating from Southern and Central Europe. The partnership of *L. anagyroides* with N2-fixing rhizobia gives this plant an ability to grow in nutrient deficient soils. The common laburnum was first introduced in Poland in 1805 and is now found in many parks, open spaces, and gardens as a decorative plant. Although this plant is widespread in Poland, there have been no studies characterizing its symbiotic bacteria. A few earlier studies identified the Spanish *L. anagyroides* microsymbionts as *Bradyrhizobium canariense* (Ruiz-Diez et al. 2009) and these from Belgium and Croatia as *Bradyrhizobium* spp. (De Meyer et al. 2011; Parker 2015); however, further investigations are necessary to provide better understanding of the *L. anagyroides* symbiosis.
microsymbiont diversity and to confirm their positive effects on plant growth.

2 Materials and methods

2.1 Isolation of rhizobia from root nodules

The bacterial isolates were obtained from effective (pink) root nodules of *L. anagyroides* Medic. plants growing in urban green areas in South-Eastern Poland (Table 1). The root systems of 50 plants were examined and about 850 nodules were obtained for further analysis. The bacteria were isolated from surface-sterilized nodules and after sterilization prepared and plated on solid Yeast Mannitol Agar (YMA) as previously described (Somasegaran and Hoben 1994). The bacteria isolated from the nodules were purified by streaking to obtain single colonies and pure cultures were used in further experiments.

2.2 Phenotypic analysis

The culture characteristics were assessed in YM Broth (YMB) or on YMA plates. Mean generation times were determined with the spectrophotometric method (480 and 600 nm) after incubation of the bacteria in YM broth at 28 °C and pH 7.0. The growth temperature was determined by incubation of the cultures in YMA pH 7.0 from 4 °C to 45 °C. The growth pH range was determined by incubation of the cultures in YMA at 28 °C and pH from 4.0 to 10.0. Basal medium pH was adjusted to required pH with 1 M HCl or 1 M NaOH. The salt tolerance of the isolates was assayed by adding 0.5, 1, 2, and 3% NaCl to the medium. Utilization of 11 different amino acids or assimilation of 13 various carbohydrates at 1% (w/v) as a sole nitrogen or carbohydrate sources was investigated on a modified liquid YM broth, in which yeast extract or mannitol (respectively) was replaced by the different amino acids or the respective carbohydrates. The intrinsic antibiotic resistance was tested on YMA plates containing 14 different antibiotics at the appropriate dilution.

2.3 Plant nodulation tests

The plant test was carried out as previously described (Hoagland and Arnon 1950; Vincent 1970; Somasegaran and Hoben 1994). Briefly, the seeds were sterilized, prepared, germinated and planted on a solid N-free Hoagland’s medium (per jar). After 7–14 days, healthy seedlings were inoculated with 0.5 U McFarland standard (1.5 × 10^8 CFU/ml) cells of an individual strain grown in liquid YM broth, pelleted, and suspended in sterile water. The plants were grown for a minimum of 3 and a maximum of 6 weeks in a greenhouse under natural light supplemented with artificial light (14 h day/10 h night, at 24/19 °C). Then, the plants were examined for the presence of nodules, their number, size, and color, as well as appearance of the aerial parts. The tests were performed in triplicate and compared with the negative control (non-inoculated plants) and positive control (reinoculated *L. anagyroides*).

2.4 Rep-PCR fingerprinting, ITS-RFLP, and gene sequencing

The genomic DNA was isolated from the bacteria using a Genomic Mini AX Bacteria Spin Kit (A&A Biotechnology). For rep-PCR fingerprinting, DNA was amplified by PCR with the primer BOXA 1R (Versalovic et al. 1994) as described by Kaschuk et al. (2006). To confirm the reproducibility of the BOX-PCR fingerprinting protocol, the isolates were analyzed independently three times. A computer cluster analysis of polymorphic bands was carried out using a simple matching

| Strain       | Geographic origin         | BOX-PCR and ITS-RFLP genotype |
|--------------|---------------------------|-------------------------------|
| Ba-Z92, Z93, Z95, Z97, Z98, Z99, Z90 | Lublin, estate greenery 51°14′23″N 22°30′52″E | I                             |
| Ba-Z104, Z105, Z106, Z109 | Lublin, estate greenery 51°14′24″N 22°31′00″E |                              |
| Ba-Z88       | Lublin, park              51°14′19″N 22°30′52″E | II                            |
| Ba-Z30, Z44  | Sandomierz, botanical garden 50°40′28″N 21°44′26″E |                              |
| Ba-Z40, Z45, Z47 | Sandomierz, botanical garden 50°40′28.5″N 21°44′23″E | II                            |
| Ba-Z102      | Lublin, park              51°14′28.5″N 22°30′35″E | III                           |

The representative strains used for further phylogenetic analysis are marked in bold.

Table 1 *Bradyrhizobium* strains from root nodules of *L. anagyroides* considered in this study, their geographic origin and classification based on BOX-PCR and ITS-RFLP analysis
were able to nodulate in two cities of Southeast Poland (Table 1). All the strains – result, the big dark green plants developed an average of 8 pink root nodules per plant 3 with the studied isolates (Fig. 1). These plants were clearly *Glycine max* established with *Lupinus polyphyllus* (red nodules). No symbiosis was induced effective nodules on representative isolates Ba-Z45, Ba-Z95, and Ba-Z102 also different from the small non-inoculated control plants. Three isolates sampled from

In this study, 18 rhizobial strains were isolated from root nodules grown as a decorative plant *L. anagyroides* characterized of the representative isolates Ba-Z45, Ba-Z95, and Ba-Z102 also assimilated methionine, valine, and cysteine, histidine, isoleucine, leucine, proline, and serine, but not lysine and arginine. Additionally, two isolates, Ba-Z45 and Ba-Z102 did not grow in the presence of doxycycline (15 μg mL⁻¹) and erythromycin (15 μg mL⁻¹). Only Ba-Z45 isolate did not grow in the presence of doxycycline (30 μg mL⁻¹) and tetracycline (15 μg mL⁻¹). Differential characteristics of the representative *L. anagyroides* isolates in comparison to the relevant reference species of the *Bradyrhizobium* genus are described in Table 2.

3.2 BOX-PCR and RFLP analysis of the 16S–23S rDNA ITS

After DNA amplification with the BOX A1R primer, genonic DNA profiles consisting of 20 polymorphic bands sized from 200 to 2000 bp were obtained for all 18 strains. For some isolates the BOX A1R-PCR generated identical DNA profiles. Figure 2 shows a dendrogram based on the analysis of BOX A1R-PCR products, which were clustered at the similarity level of 41%. At 70% similarity, the studied strains grouped into two rep-PCR clusters consisting of 14 and 3 strains and 1 independent lineage (Fig. 2). Based on the 16S–23S rDNA ITS restriction fragment length polymorphism (RFLP) analysis, 3 ITS genotypes were distinguished among the 18 isolates (Table 1). Three isolates that represent different ITS RFLP and BOX-PCR types were used in the subsequent characterization.

3.3 Phenotypic characterization of *L. anagyroides* nodule endosymbionts

The studied strains were Gram-negative non-spore-forming rods. The appearance of non-mucous white colonies (<1.5 mm size) on the YMA plates incubated at 28 °C after 5–7 days and an alkaline reaction on the YMA plates supplied with bromothymol blue suggested that the studied bacterial isolates belong to the genus *Bradyrhizobium* (Adams et al. 2010). The optimal growth temperature of studied strains was between 28 °C and 35 °C. The optimum pH for growth ranged from 7.0 to 8.0. The isolates utilized erythritol, D-galactose, glycero, D-glucose, mannitol, sorbitol, starch, stevia, sucrose, Tween, and xylitol as carbon sources. Ba-Z102 strains also utilized xylose and D-fructose as a carbon source. All tested strains assimilated cysteine, histidine, isoleucine, leucine, proline, and serine, but not lysine and arginine. Additionally, two isolates, Ba-Z95, and Ba-Z102, also assimilated methionine, valine, and tyrosine as nitrogen sources. All studied microsymbionts were resistant to cefoxitin (110 μg mL⁻¹), ceftazidine (230 μg mL⁻¹), clindamycin (2 μg mL⁻¹), cloxacinil (5 μg mL⁻¹), meropenem (10 μg mL⁻¹), nalidixic acid (30 μg mL⁻¹), and piperacillin (30 μg mL⁻¹). The Ba-Z45 and Ba-Z95 strains were sensitive to streptomyacin (10 μg mL⁻¹). Ba-Z45 and Ba-Z102 did not grow in the presence of ampicillin (50 μg mL⁻¹), amoxicillin (25 μg mL⁻¹) and erythromycin (15 μg mL⁻¹). Only Ba-Z45 isolate did not grow in the presence of doxycycline (30 μg mL⁻¹) and tetracycline (15 μg mL⁻¹). Differential characteristics of the representative *L. anagyroides* isolates in comparison to the relevant reference species of the *Bradyrhizobium* genus are described in Table 2.
3.4 16S rRNA gene phylogeny

The sequences of 16S rDNA (1253 bp) isolates Ba-Z45, Ba-Z95, and Ba-Z102 shared 99.6–100% sequence similarity with each other. The analyzed sequences were most similar to the 16S rDNA sequence reported for *Bradyrhizobium algeriense* RST89T displaying 99.6–100% identity. Likewise, several 16S rDNA sequences of a type strain from other *Bradyrhizobium* species exhibited high identity values (99.5–99.9%) to 16S rDNA sequences of the studied isolates, including *Bradyrhizobium icense* LMTR13T, *Bradyrhizobium erythrophlei* CCBAU 53325T, *Bradyrhizobium embrapense* SEMIA 6208T, and *Bradyrhizobium viridifuturi* SEMIA 690T. Based on the phylogenetic analysis of 16S rDNA gene sequences, the isolates were clustered with the type strains representing the *Bradyrhizobium jicamae* supergroup (Fig. 3).

3.5 Housekeeping gene phylogeny

The sequences of *atpD* (491 bp), *dnaK* (236 bp), *glnII* (480 bp), and *recA* (412 bp) of the representative isolates exhibited 94–96%, 98–99%, 96–98%, and 94–95% similarity to each other, respectively. Compared to the type strains of recognized *Bradyrhizobium* species, these sequences were
Growth at:

+: growth or resistant; −: no growth or sensitive; w: weak growth or variable; ND: not determined

| Characteristic/strainsa | 1 | 2 | 3 | 4b | 5c | 6 |
|------------------------|---|---|---|----|----|---|
| Generation time (h) in YM broth | 8–9 | 8–9 | 9–10 | >20 | 11–12 | >6 |
| Growth at: | | | | | | |
| 1.0% NaCl | − | − | − | + | + | |
| pH 4.0 | w | − | w | + | − | + |
| pH 5.0 | + | + | + | ND | ND | + |
| pH 8.0 | + | + | + | ND | + | + |
| pH 9.0 | + | + | + | ND | + | + |
| pH 10.0 | w | w | w | − | + | +4d |
| 37 °C | + | + | − | − | − | + |
| Resistance to (μg/mL−1): | | | | | | |
| Ampicillin (50) | − | + | − | + | ND | + |
| Erythromycin (50) | − | − | − | − | − | + |
| Streptomycin (10) | − | + | − | − | − | − |
| Tetracycline (5) | − | + | + | + | − | + |
| Utilization of carbon sources: | | | | | | |
| D-galactose | + | w | + | + | ND | + |
| D-glucose | + | w | + | + | ND | + |
| D-fructose | − | − | − | + | + | + |
| Mannitol | + | + | + | + | − | + |
| Sucrose | + | + | − | − | − | + |
| Utilization of nitrogen sources: | | | | | | |
| Cysteine | + | + | − | ND | + |
| Histidine | + | − | + | + | ND | + |
| L-proline | + | + | + | ND | + | + |

a: Species/strains: L. anagyroides nodules isolates: 1. Ba-Z45; 2. Ba-Z95; 3. Ba-Z102; 4. B. algerienser RST89T; 5. B. icense LMTR13T; 6. B. elkanii USDA 76T
b: Ahnia et al. 2018; Durán et al. 2014; d This result is not consistent with that reported by Durán et al. 2014

The bacterial strains analyzed here are rhizobia obtained from nodules of L. anagyroides growing in Poland. To get a tentative insight into the genetic diversity of the isolates we performed rep-PCR fingerprinting with the BOX A1R primer, which had been shown to be a reliable typing method used for differentiation and preliminary characterization of rhizobial strain collections (Koueth et al. 1995; Kaschuk et al. 2006; Menna et al. 2009; Cardoso et al. 2017). The BOX-PCR analysis provided the basis for separating the isolates into three distinct groups (Fig. 2, Table 1). In order to confirm the diversity pattern of the tested strains obtained by rep-PCR, PCR-based 16S–23S rDNA RFLP analysis was carried out. Previous studies have shown relevant results for searching nodule bacterial biodiversity, and selection of strains for further experiments can be provided by restriction analysis of the ITS region (e.g. Menna et al. 2009; Sachs et al. 2009; Yao et al. 2014). We identified three different RFLP-PCR profiles of 16S–23S rDNA in the analysis of all tested L. anagyroides nodule isolates, corroborating the rep-PCR results (Table 1).

Phenotypic characterization of L. anagyroides nodule isolates was performed by assessment of properties that were previously described as useful for Bradyrhizobium species differentiation (Chang et al. 2011; Guerrouj et al. 2013). The three L. anagyroides strains Ba-Z45, Ba-Z95, and Ba-Z102, i.e. representatives of each rep-PCR and ITS-RFLP cluster, did not behave phenotypically as a homogenous group (Table 2). In contrast to strains of B. algerienser RST89T (Ahnia et al. 2018) and B. icense LMTR13T (Durán et al. 2014), all isolates studied had a shorter generation time, grew well at 37 °C and 42 °C but poorly at 45 °C, and utilized sucrose as the sole carbon source (Table 2).

In prokaryotes, the 16S rRNA gene sequence is a key phylogenetic marker, in most cases allowing fast identification of new isolates to at least the genus level (Stackebrandt et al. 2002; Tindall et al. 2010). It is generally accepted that nearly complete 16S rRNA gene sequence identity of two bacterial strains <95% and <98.7% is strong evidence for distinct genera and distinct species, respectively (Yarza et al. 2014). To classify our isolates to the genus level, we sequenced the 16S rRNA gene from three representative strains. The

4 Discussion

The nodA sequences of the L. anagyroides Bradyrhizobia isolated in Poland shared 98% similarity to each other and clustered with those of B. algerienser RST89T, B. valentinum LmjM3T, and Bradyrhizobium retamae R019T, displaying 97–99%, 96–97%, 96–97%, 95–97%, and 93–95% sequence identity, respectively (Fig. 5). The nifH gene sequences of three tested isolates shared 99% similarity. In the nifH phylogram, the L. anagyroides microsymbionts are grouped together with B. algerienser RST89T and B. valentinum LmjM3T, displaying 99–100% and 99%, sequence identity, respectively (Fig. 6).

3.6 nodA and nifH gene phylogeny

The nodA sequences of the L. anagyroides Bradyrhizobia isolated in Poland shared 98% similarity to each other and clustered with those of B. algerienser RST89T, B. valentinum LmjM3T, Bradyrhizobium retamae R019T, and Bradyrhizobium lablabi CCBAU 23086T displaying 97–99%, 96–97%, 96–97%, 95–97%, and 93–95% sequence identity, respectively (Fig. 5). The nifH gene sequences of three tested isolates shared 99% similarity. In the nifH phylogram, the L. anagyroides microsymbionts are grouped together with B. algerienser RST89T and B. valentinum LmjM3T, displaying 99–100% and 99%, sequence identity, respectively (Fig. 6).
Fig. 3 Maximum likelihood tree showing the phylogenetic relationships of the *L. anagyroides* symbionts studied (bolded) with *Bradyrhizobium* type strains of recognized species based on 16S rDNA sequences. Bootstrap values based on 1000 replicates >50% are indicated at the branching points.
Fig. 4 Maximum likelihood tree showing the phylogenetic relationships of the *L. anagyroides* symbionts studied (bolded) with *Bradyrhizobium* type strains of recognized species based on concatenated *recA*, *atpD*, *glnII*, and *dnaK* gene sequences. Bootstrap values based on 1000 replicates >70% are indicated at the branching points. The scale bar presents the number of nucleotide substitutions per site. The GenBank accession numbers for all used gene sequences are given in Fig. S1–S4.

Fig. 5 Maximum likelihood tree showing the phylogenetic relationships of the *L. anagyroides* symbionts studied (bolded) with *Bradyrhizobium* type strains of recognized based on *nodA* gene sequences. Bootstrap values based on 1000 replicates >70% are indicated at the branching points. The scale bar presents the number of nucleotide substitutions per site.
phylogenetic analysis of the 16S rDNA sequences (Fig. 3) showed that the tested \textit{L. anagyroides} nodule isolates were members of the \textit{Bradyrhizobium} genus with affinity to strains referred to as the \textit{B. jicamae} supergroup (Avontuur et al. 2019). The previous studies have shown that the \textit{B. jicamae} supergroup strains are relatively rare and their geographic range covers mainly hot and arid areas all over the world (e.g. Durán et al. 2014; Ahnia et al. 2018; Boulila et al. 2009). In contrast, the sequences of \textit{L. anagyroides} endosymbionts from Belgium (De Meyer et al. 2011) and Spain (Ruiz-Diez et al. 2009) are grouped with 16S rDNA sequences of \textit{Bradyrhizobium japonicum} supergroup, pointing to their phylogenetic separateness from the bradyrhizobia isolated in Poland (Fig. 3). Likewise, the prevalence of the \textit{B. japonicum} supergroup strains among Genisteae bradyrhizobia derived from Central and Northern Europe was indicated in earlier studies (Stepkowski et al. 2018). Therefore, identification \textit{L. anagyroides} symbionts inhabiting areas of the temperate climate, which markedly differs from the Mediterranean climate, as members of the \textit{B. jicamae} supergroup, suggest that the legume host, but not the climate type, is a crucial element in the process of the dissemination of the studied bacterial strains. It has been established that human-mediated transport of legume plants, especially seeds, plays an increasingly important role in the process of spreading particular groups of rhizobia, although it was earlier concluded that the wide geographic range detected for many bradyrhizobia might also result from natural processes of dispersal (Parker 2015).

Since the diversity in the 16S rRNA gene sequences of bradyrhizobia is too low to detect differences among species and distinguish them, the \textit{recA}, \textit{dnaK}, \textit{atpD}, and \textit{glnA} genes were sequenced subsequently to provide more discriminatory analysis of the tested \textit{L. anagyroides} endosymbionts. It is generally accepted that these protein-coding genes with faster evolution rates than those of the 16S rRNA gene are alternative taxonomic markers for easy and reliable identification of bacterial isolates as well as species delineation replacing DNA:DNA hybridization (Stackebrandt et al. 2002; Glaeser and Kämpfer 2015). Multilocus sequence analysis (MLSA) encompassing combined analysis of several housekeeping genes has been widely used in studies with bradyrhizobia (e.g. Stepkowski et al. 2005; Menna et al. 2009; Lindström et al. 2015; Kalita and Malek 2017). In our studies, the
taxonomic affiliations of the tested strains were analyzed by MLSA according to the scheme introduced by Menna and Hungria (2011) to establish the phylogenetic relations of diverse collections of Bradyrhizobium strains. Phylogenies were inferred for each atpD, dnaK, glnII, and recA loci separately (Fig. S1–S4) and for combined gene sequences (Fig. 4). In the case of housekeeping genes, their similarity values for the tested strains were much lower compared to those of the 16S rRNA gene sequences, which were identical or almost identical. However, it was earlier found that 16S rDNA could be identical among closely related species from the genus Bradyrhizobium (Degefu et al. 2018). Based on the high-level similarity of housekeeping gene sequences and the independent phylogenetic position of the tested strains along with the B. algeriense RST89T, recovered by MLSA, it is reasonable to regard B. algeriense as the closest phylogenetic neighbor of the tested isolates. However, MLSA revealed that the strains Ba-Z45, Ba-Z95, and Ba-Z102, representatives of the three groups delineated by BOX-PCR and ITS-RFLP, represent distinct evolutionary lineages, well differentiated from the currently named species. In fact, the values of the nucleotide identity of the concatenated sequences of recA, dnaK, atpD, and glnII between each other and B. algeriense RST89T (95–96%) were not higher than those between the type strain of the other recognized species, e.g. B. icense LMTR 13T/B. retamae Ro19T (95%), B. viridifluri SEMIA 690T/B. tropiciagri SEMIA 6148T (96%), and B. embrapense SEMIA 6208T/B. tropiciagri SEMIA 6148T (96%). Therefore, the tested L. anagyroides isolates might form a novel Bradyrhizobium species, but this should be confirmed by genomic data. In fact, due to the growing availability of genomes for rhizobia, MLSA has now been superseded by ANI comparisons between genomes, which has been proven to be adequate for taxonomic purposes (de Lajudie et al. 2019). Nevertheless, MLSA supports many phylogenetic relationships recovered in the genome-based phylogeny for Bradyrhizobium (Avontuur et al. 2019). Unfortunately, there is no defined MLSA threshold to identify bacteria at the species or genus level in rhizobia, in contrast to some other groups of bacteria, e.g. Xenorhabdus (Tailliez et al. 2010) or Bacillus (Liu et al. 2017). However, progress is expected in the next few years related to development of an online database of MLSA allowing comparison of genomic sequences for fast phylogenetic and taxonomic studies of bradyrhizobia (Azevedo et al. 2015).

The studied strains were confirmed to be closely associated with B. algeriense and B. valentinum, i.e. two species comprising nodule endosymbionts of Genistaeae legumes Retama sphaerocarpa and Lupinus mariae-josephae, respectively, which grow solely in the Mediterranean (Durán et al. 2014; Ahnia et al. 2018). Additionally, a tight relationship was also found between the tested isolates and Bradyrhizobium strains nodulating wild genistid legume shrubs Calicotome spinosa and Spartium junceum growing in the same region (Cardinale et al. 2008; Salmi et al. 2018). In our plant nodulation tests, the isolates were able to establish effective symbiosis with the native host L. anagyroides as well as C. arborescens and L. polyphyllus, but not with G. max, which was in line with the report by Ahnia et al. (2018). The data described indicate that these bacteria are not strictly specialized towards nodulation of specific plant hosts. In fact, the promiscuous type of symbiosis, widespread among bradyrhizobia, is regarded to be more ancestral than the host-limited symbiosis restricted to specific niches, which may be a result of coevolutionary relationships between microsymbionts and the host plant (Perret et al. 2000).

Unfortunately, not all L. anagyroides symbionts isolated earlier were included in the phylogenetic analysis due to the lack of complete sequence data. However, as illustrated in the dnaK and recA phylogenetic trees (Fig. S2, S3), the tested isolates, especially Ba-Z95, showed phylogenetic affinity to Bradyrhizobium spp. strains isolated from nodules of L. anagyroides in Croatia, in contrast to endosymbionts isolated from L. anagyroides nodules growing in Belgium and Spain, classified as B. canariense and Bradyrhizobium sp., respectively (Fig. 3).

Symbiotic genes are usually studied in polyphasic rhizobial research, since they allow verification of the nodulation and nitrogen fixation ability. Bacteria in the genus Bradyrhizobium have symbiotic genes displaying a high level of diversity and a different evolutionary history from their housekeeping genes, which suggests their acquisition by lateral gene transfer (LGT) (Menna and Hungria 2011; Lindström et al. 2015; Hungria et al. 2015). It has been reported that sequence analysis of nodA and nifH genes can be useful to examine the symbiotic diversity of Bradyrhizobium strains and elucidate the role of LGT in inheritance of the symbiotic loci (Degefu et al. 2018). The phylogenetic studies of nodA and nifH symbiotic genes, which revealed 16 major clades in the genus Bradyrhizobium, have facilitated the development of the phylogeographic framework for this group of bacteria (Beukes et al. 2016; Stepkowski et al. 2018). In our study the clustering results of the nodA sequences of the tested isolates were essentially similar to those obtained from the nifH gene sequence analysis (Figs. 5 and 6), but slightly different from the results of the analysis of housekeeping genes (Fig. 4). Following the classification scheme described for symbiotic genes of Bradyrhizobium symbionts, the strains nodulating L. anagyroides in Poland, like all other B. jicamae supergroup strains, appear to belong to a group referred to as Clade IV (Beukes et al. 2016).

Moreover, the phylogenetic analysis of nodA and nifH gene sequences allowed a conclusion that the studied L. anagyroides nodule isolates are part of the symbiovar retamae. This symbiovar is represented by B. retamae, B. algeriense, and B. valentinum nodulating Retama
spp., R. sphaerocarpa, and L. mariae-josephae, respectively (Guerrouj et al. 2013; Durán et al. 2014; Ahnia et al. 2018). The differences in the symbiotic and core gene phylogenies as well as the average nucleotide identity (ANI) values suggest that the symbiotic genes of rhizobia from the symbiovar retamae have been horizontally transferred among different species (Ahnia et al. 2018). Multiple symbiovars, distinguishing symbiotic capability (ANI) values suggest that the symbiotic genes of gene phylogenies as well as the average nucleotide identity (ANI) values suggest that the symbiotic genes of rhizobia from the symbiovar retamae have been horizontally transferred among different species (Ahnia et al. 2018). Multiple symbiovars, distinguishing symbiotic capabilities of rhizobia, presented within the same host suggest the promiscuity of legume species. Despite the lack of full sequence data of the symbiotic genes from other L. anagyroides symbiotic strains, it can be concluded that in its root nodules, L. anagyroides can harbor endosymbionts belonging to different symbiovar types, at least glycinearum (Bradyrhizobium sp. R-46310), genistearum (B. canariense), and retamae (the strains isolated in Poland). It was also reported by De Meyer et al. (2011) that L. anagyroides, like most of the other exotic legumes growing in Belgium, is a highly promiscuous host plant engaged in symbioses with indigenous rhizobia nodulating native legumes. This promiscuity could increase its ability to spread into new habitats; hence, L. anagyroides seems to be a good candidate for revegetation purposes. Together, our findings provide useful information on identification of the rhizobial partner of L. anagyroides, a legume originating from Southern Europe that has expanded its range into Poland. This is a necessary step for further research addressing ecological and evolutionary processes that shape these particular mutualistic relationships.

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