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Putative novel *Bradyrhizobium* and *Phyllobacterium* species isolated from root nodules of *Chamaecytisus ruthenicus*☆

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ABSTRACT

In this study, the diversity and the phylogenetic relationships of bacteria isolated from root nodules of *Chamaecytisus ruthenicus* growing in Poland were investigated using ERIC-PCR fingerprinting and by multilocus sequence analysis (MLSA). Two major clusters comprising 13 and 3 isolates were detected which 16S rRNA gene sequencing identified as *Bradyrhizobium* and *Phyllobacterium*. The results of phylogenetic analysis of individual and concatenated *atpD*, *gyrB* and *recA* gene sequences showed that the studied strains may represent novel species in the genera *Bradyrhizobium* and *Phyllobacterium*. In the phylogenetic tree based on the *atpD-gyrB-recA* concatemers, *Bradyrhizobium* isolates were split into two groups closely related to *Bradyrhizobium algeriense* STM89^T and *Bradyrhizobium valentinum* LmjM3^T. The genus *Phyllobacterium* isolates formed a separate cluster close to *Phyllobacterium ifriqiyense* LMG27887^T in the *atpD-gyrB-recA* phylogram. Analysis of symbiotic gene sequences (*nodC*, *nodZ*, *nifD*, and *nifH*) showed that the *Bradyrhizobium* isolates were most closely related to *Bradyrhizobium algeriense* STM89^T, *Bradyrhizobium valentinum* LmjM3^T and *Bradyrhizobium retamae* Ro19^T belonging to symbiovar retamae. This is the first report on the occurrence of members of symbiovar retamae from outside the Mediterranean region. No symbiosis related genes were amplified from *Phyllobacterium* strains, which were also unable to induce nodules on *C. ruthenicus* roots. Based on these findings *Phyllobacterium* isolates can be regarded as endophytic bacteria inhabitating root nodules of *C. ruthenicus*.

Introduction

The nodules formed on the roots of many plant species of the Leguminosae family are usually viewed as habitats for bacteria commonly known as rhizobia, which are capable of fixing atmospheric nitrogen and establishing symbiotic interaction with their host. In addition to the classical rhizobia belonging to the genera *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and *Rhizobium*, recent studies have also identified nodule symbionts belonging to several other genera of *Alphaproteobacteria* (e.g. *Bosea*, *Devosia*, *Microviga*, *Ochrobactrum* and *Phyllobacterium*) and *Betaproteobacteria* (*Cupriavidus* and *Bulkholderia*). Moreover, root nodules of legumes can be inhabitated by non-nodulating bacteria (e.g. *Bacillus*, *Klebsiella*, *Micromonospora*, *Pantoea*, *Pseudomonas*) which may exhibit plant growth promoting properties and may enhance efficiency of nodulation and nitrogen-fixation [34,50].

Until now the phylogenetic position of *Chamaecytisus ruthenicus* root nodule isolates has not been studied thoroughly. There are two reports published by Baymiev et al. [3,4] where genus affiliation of five strains isolated from root nodules of *C. ruthenicus* growing in the Southern Urals was determined as *Bradyrhizobium* and *Phyllobacterium*. The genus *Chamaecytisus* is regarded as a monophyletic taxon or it is included into the genus *Cytisus* [5,47]. *C. ruthenicus* native range is Eastern Poland (where it reaches its western limit of geographic distribution) to Kazakhstan [58]. It belongs to the tribe Genisteae of which more than 70 species have been studied for their root nodule inhabitants [48]. *Genisteae* species are nodulated predominantly by *Bradyrhizobium* strains and in recent years seven new bradyrhizobial species were described among Genisteae root nodule isolates [1,6,7,14,21,40,52]. Although currently the genus *Bradyrhizobium* comprises 50 described species [48] the presumed number of species of this genus in nature may

☆ The GenBank accession numbers for sequences generated in this study are as follows: MN134545 – MN134560 (16S rRNA), MN159344 – MN159359 (*atpD*), MN159328 – MN159343 (*gyrB*), MN159360 – MN159375 (*recA*), MN159302 – MN159314 (*nodC*), MN159316 – MN159327 (*nodZ*), MN380483 – MN380495 (*nifD*), MN380496 – MN380508 (*nifH*).

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be from 750 to 800 as indicated in the latest analyses of the *glnII* and *recA* gene sequences and complete genomes of *Bradyrhizobium* available in public databases [37].

To explore the diversity and taxonomic position of 16 *C. ruthenicus* root nodule isolates we used PCR-based DNA fingerprinting technique (ERIC-PCR) and multilocus sequence analysis (MLSA). Rhizobial genes involved in the plant nodulation and nitrogen fixation (*nodC*, *nodZ*, *nifD*, and *nifH*) were used to determine symbiotic relationship of the studied bacteria.

Materials and methods

Root nodule collection and isolation of rhizobia

Chamaecytisus ruthenicus plants grown in Lublin Province (51°16′58″N, 22°38′28″E) were used for root nodule collection and nodule endosymbionts isolation. Root nodules were surface disinfected and rhizobia were isolated according to the procedure described elsewhere [54]. Yeast extract mannitol agar (YEM) was used for purification of the bacterial strains. The purified strains were kept in YEM medium at 4 °C and at −70 °C in YEM medium with 20 % (v/v) glycerol.

ERIC-PCR genome fingerprinting

Bacterial DNA fingerprints were obtained with the ERIC-PCR method using primers and PCR conditions described by Gnat et al. [18]. The experiments were performed three times using the same DNA samples. Only informative and reproducible products of the genomic fingerprinting reactions were analyzed. The PCR products were separated in 1.5 % (w/v) agarose gel, stained with ethidium bromide, and visualized under UV light. The DNA profiles were analyzed using BIO-GEN program version 11.01 (Vilber-Lourmat). The matrix reflecting the similarity of the studied isolates was calculated using the Nei and Li coefficient [36]. Cluster analysis of the similarity matrix was performed using the unweighted pair group method with arithmetic averages (UPGMA) in NTSYSpc (Exeter Software).

Amplification and sequencing of core and symbiotic genes

Fragments of the following loci were amplified using primers and the PCR conditions described elsewhere: 16S rDNA [55], *atpD* [17], *gyrB* [33], *nodC* [28,45], *nodZ* [35], *nifD* [39], *nifH* [28]. All PCR amplification reactions were carried out with REDTaq ReadyMix PCR reaction mix (Sigma Aldrich) according to the manufacturer's recommendations. The amplified products were purified with Clean-Up purification columns (A&A Biotechnology) or an Agarose-Out DNA purification kit (EurX). The purified fragments were sequenced with a BigDye Terminator Cycle sequencing kit using an 3500 Genetic Analyzer according to the manufacturer's procedures (Life Technologies).

Phylogenetic analysis

The sequences obtained in this study were used as queries in the BLAST [2] program and the GenBank database was used to search for homologous sequences. The sequences of individual loci (16S rDNA, *atpD*, *gyrB*, *recA*, *nodC*, *nodZ, nifD, nifH*) and concatenated genes (*atpD-gyrB-recA* and *nodC-nodZ-nifD-nifH*) were aligned independently with ClustalX [29] software using default settings. The resulting alignments were corrected manually in BioEdit [23] software. The 5′ and/or 3′ ends of longer sequences were truncated to adjust their lengths to the shortest sequence in the alignment. The alignments were used to find the best-fit nucleotide substitution model with AIC (Akaike's Information Criterion) in jModelTest [10]. The information on the model used is given in the phylogenetic tree legends. PhyML 3.1 software was used to infer phylogenetic trees with the maximum likelihood approach [22]. The reliability of internal branches of the resulting phylograms was assessed with nonparametric bootstrap using 1000 replicates. Sequence identity values for single genes and concatenated loci were calculated in BioEdit.

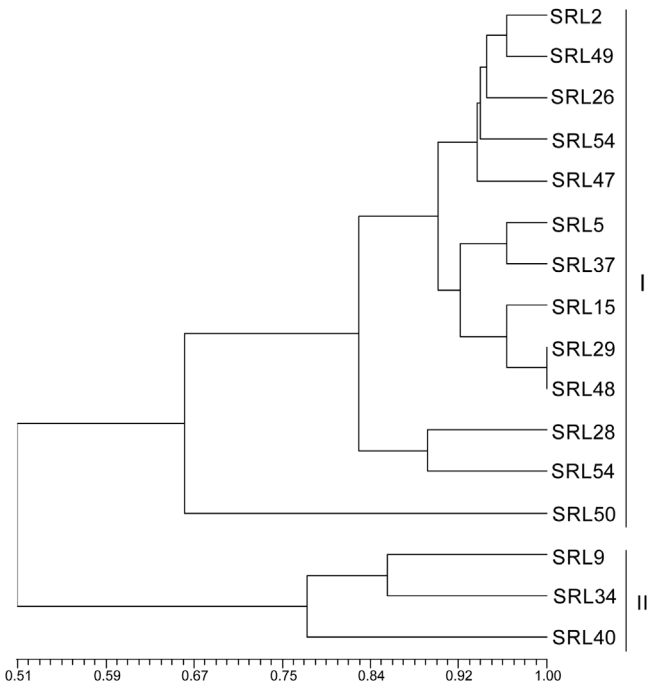

Fig. 1. UPGMA dendrogram based on BOX-PCR data of 16 *Chamaecytisus ruthenicus* root nodule isolates. Nei and Li coefficient was used for similarity measure.

Results and discussion

Genetic diversity assessed by ERIC-PCR genome fingerprinting

Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences belong to conserved elements dispersed in bacterial genomes [51]. DNA profiling by ERIC-PCR has been widely usedy for the evaluation of the genetic diversity of bacterial strains isolated from root nodules of different legumes [11,18,19,46,53,56]. We applied the ERIC-PCR genome fingerprinting technique to assess the genetic diversity of 16 strains isolated from root nodules of *C. ruthenicus*. The primers used in PCR generated 8–12 DNA fragments ranging from 100 bp to 3700 bp. The results of the cluster analysis based on the DNA patterns are presented in Fig. 1. All the *C. ruthenicus* isolates grouped at DNA profile similarity level of 51 %. Two separate groups can be distinguished, one encompassing 13 strains grouped at 0.66 similarity coefficient (cluster I) and cluster II comprising three isolates forming a common group at 0.77 similarity coefficient (Fig. 1). The level of DNA pattern similarity among the isolates in the separate clusters ranged from 59 to 100 % in cluster I, and from 74 to 85 % among strains from cluster II. Although the ERIC-PCR results revealed the presence of 15 distinct DNA patterns among the studied bacteria and only two strains from cluster I – SRL29 and SRL48 had identical DNA profiles, most isolates of the cluster I were highly homogeneous and differed from each other by the presence or lack of a single PCR product. The SRL50 strain of cluster I was significantly different from other members of this common group and formed a separate branch in the ERIC-PCR based UPGMA dendrogram (Fig. 1). To reveal taxonomic position and phylogenetic relationship of stud-

ied strains with reference rhizobial species, all *C. ruthenicus* root nodule isolates were used in further analyses described below.

Phylogenetic analysis of 16S rRNA gene sequences

Based on the comparative 16S rRNA gene sequence analysis using BLAST tool and NCBI GenBank database, 13 strains of the ERIC-PCR cluster I were classified as *Bradyrhizobium*. They had 16S rRNA gene sequences similar to each other in the range from 99.6% to 100%. The 16S rDNA nucleotide sequences of *C. ruthenicus* root nodule bradyrhizobia were most similar to *Bradyrhizobium algeriense* RST89^T (99.7–100%), *Bradyrhizobium embrapense* SEMIA6208^T, *Bradyrhizobium erythrophlei* CCBAU53325^T, *Bradyrhizobium viridifuturi* SEMIA690^T (99.8–99.9%), and *Bradyrhizobium valentinum* LmjM3^T (99.5–99.7%). In the 16S rRNA phylogenetic tree, 12 *Bradyrhizobium* isolates were placed in a common group with the type strain of *Bradyrhizobium algeriense* and the *Bradyrhizobium* sp. SRL50 strain formed a separate branch (Fig. 2).

The other group of *C. ruthenicus* root nodule isolates, encompassing three strains of the cluster II shown in ERIC-PCR DNA pattern dendrogram (Fig. 1), was closely related to reference bacteria in the genus *Phyllobacterium*. The strains SRL9, SRL34, and SRL40 shared 99.3–100% 16S rDNA sequence similarity. These three isolates had 16S rRNA gene sequences most similar to the 16S rDNA sequences of *Phyllobacterium ifriqiyense* STM370^T (99.3–100%) and *Phyllobacterium catacumbae* CSC19^T (98.8–99.5%). These two reference species and *Phyllobacterium* strains isolated from root nodules of *C. ruthenicus* clustered together in the 16S rRNA phylogenetic tree with bootstrap value of 81 % (Fig. 2).

The genera *Bradyrhizobium* and *Phyllobacterium* have previously been isolated from root nodules of *C. ruthenicus* growing in the southern Urals [4]. Although Baymiev et al. [4] used fewer reference strains, in the 16S rRNA phylogenetic tree, the Russian *Bradyrhizobium* from *C. ruthenicus* root nodules were placed close to *Bradyrhizobium elkanii* [4]. In our analysis, the *Bradyrhizobium* sp. C1 isolated by Baymiev et al. [4] had the 16S rDNA nucleotide sequence most similar to Polish isolates (99.5–99.7%) and *B. algeriense* RST89^T (99.7 %), and it was grouped togheter with these strains in the 16S rDNA phylogram (Fig. 2). The Russian *Phyllobacterium* strains C2A and C2B isolated from root nodules of *C. ruthenicus* were identified by Baymiev at al. [4] as *Phyllobacterium trifolii* and in the 16S rRNA phylogram they were placed in a cluster separate from Polish isolates (Fig. 2), with which they shared 98–98.8% 16S rDNA sequence similarity.

The phylogenetic analysis of 16S rDNA sequences allowed us to unambiguously estimate taxonomic position of the studied bacteria at the genus level. However, the highly conservative nature of the 16S rRNA gene sequences of *Bradyrhizobium* and *Phyllobacterium* bacteria identified in our analysis is a limitating factor for drawing conclusions on species affiliations. The widely acccepted 98.7 % 16S rDNA sequence similarity value used for bacterial species demarcation [57] cannot be applied in the case of *C. ruthenicus* root nodule isolates since their 16S rRNA gene sequences are highly similar (>99 %) to more than one reference species.

Analysis of individual and concatenated housekeeping gene sequences

The phylogenetic analysis based on single and concatenated *atpD*, *gyrB*, and *recA* gene sequences was performed to elucidate the species position of *C. ruthenicus* root nodule isolates in relation to reference strains of the genera *Bradyrhizobium* and *Phyllobacterium*.

Twelve out of 13 *Bradyrhizobium* isolates shared 99.7–100% *atpD* gene sequence similarity and were most similar to *B. algeriense* (95.6–95.9%). The *Bradyrhizobium* SRL50 strain was less similar to other bradyrhizobial *C. ruthenicus* isolates (94.6–94.8%) than to *B. algeriense* (96.4 % *atpD* gene sequence similarity). The *atpD* gene sequence similarity of the studied bradyrhizobia to other reference *Bradyrhizobium* species ranged from 89.7 % (*B. denitrificans* LMG8443^T) to 94.8 % (*B. valentinum* LmjM3^T). The *Phyllobacterium* sp. SRL isolates were similar to each other at 99.7–100% and shared 91–91.2% *atpD* gene sequence identity with *P. ifriqiyense* LMG27887^T. The studied *Phyllobacterium* strains had *atpD* gene sequences similar to other *Phyllobacterium* species in the range from 82 to 89.1 %. In the *atpD* phylogenetic tree the analyzed *Bradyrhizobium* strains were distributed into two clusters. One group supported by 100 % bootstrap value comprised 12 of 13 *Bradyrhizobium* isolates. The other cluster encompassed *Bradyrhizobium* SRL50, *B. valentinum*, and *B. algeriense* strains confirming that the SRL50 isolate is more closely related to these reference species than to other bradyrhizobial strains specific to *C. ruthenicus* (Supplementary Fig. S1). *Phyllobacterium* sp. SRL strains formed a separate cluster in relation to other species of this genus. They were positioned closest to *P. ifriqiyense* and *P. catacumbae,* all of them grouped in the *atpD* phylogram with 89 % boostrap support (Supplementary Fig. S1).

Sequence data from *gyrB* and *recA* gave similar results to the *atpD* gene. The *gyrB* and *recA* gene sequences of 12 of the 13 *Bradyrhizobium* strains were most similar to *B. algeriense* (94.3 % and 93.4–94.2%, respectively) and they shared 95.6 % of *gyrB* and 93.7–94.7% of *recA* gene sequence similarities with *Bradyrhizobium* sp. SRL50, which was most similar to the *gyrB* gene sequence of *B. algeriense* (96.1 %) and the *recA* gene sequence of *B. valentinum* (94.4 %). In the *gyrB* and *recA* phylograms all studied *Bradyrhizobium* isolates except for the SRL50 strain formed a common group supported by 99 % and 98 % bootstrap values. The *Bradyrhizobium* SRL50 strain formed a separate branch in both *gyrB* and *recA* phylograms (Supplementary Figs. S2 and S3). Both *gyrB* and *recA* gene sequences of *Phyllobacterium* SRL strains were most similar to the *gyrB* and *recA* gene sequences of *P. ifriqiyense* LMG27887^T (90.8–91% and 90.2–90.5% respectively). In the *gyrB* and *recA* phylogenetic trees *Phyllobacterium* isolates grouped together as a distant cluster in relation to reference *Phyllobacterium* species (Supplementary Figs. S2 and S3).

In the phylogram reconstructed on the basis of the multiple sequence alignment made of concatenated partial sequences of the three housekeeping genes (1329 nt), all the studied *C. ruthenicus* root nodule isolates grouped similarly to the single gene phylogenetic trees. *Bradyrhizobium* sp. SRL isolates were distributed into two groups. One group comprised 12 of the 13 strains and was supported by 99 % bootstrap value. The *Bradyrhizobium* SRL50 strain was positioned close to *B. algeriense* and *B. valentinum* (Fig. 3). The major group of the studied bradyrhizobia had concatenated *atpD-gyrB-recA* gene sequences most similar to the sequences of reference strain of *B. algeriense* (94.7–95%). Nucleotide identity of three housekeeping genes of the SRL50 isolate and type strains of reference *Bradyrhizobium* species ranged from 88.7 (*Bradyrhizobium daqingense*) to 95.6 % (*B. algeriense*). The *Bradyrhizobium* SRL50 shared 94.7–95.1% nucleotide identity of concatenated *atpD-gyrB-recA* gene sequences with other *Bradyrhizobium* sp. SRL root nodule isolates. The presented results suggest that the strain SRL50 and other bradyrhizobia isolated from root nodules of *C. ruthenicus* do not belong to the same species and that they represent two putative novel species of the genus *Bradyrhizobium*. Although the results of comparative sequence analysis of a few protein encoding genes and phylogenetic analysis based on these sequences, cannot be used as the sole evidence for species delineation it can strongly indicate that the studied strains may represent new taxon. For example, in many recently published studies on *Bradyrhizobium*, the nucleotide identity of concatenated housekeeping gene sequences of these bacteria equal to or lower than 97 % was used as an indicator that the studied strains belong to new species. In all these cases, inferences

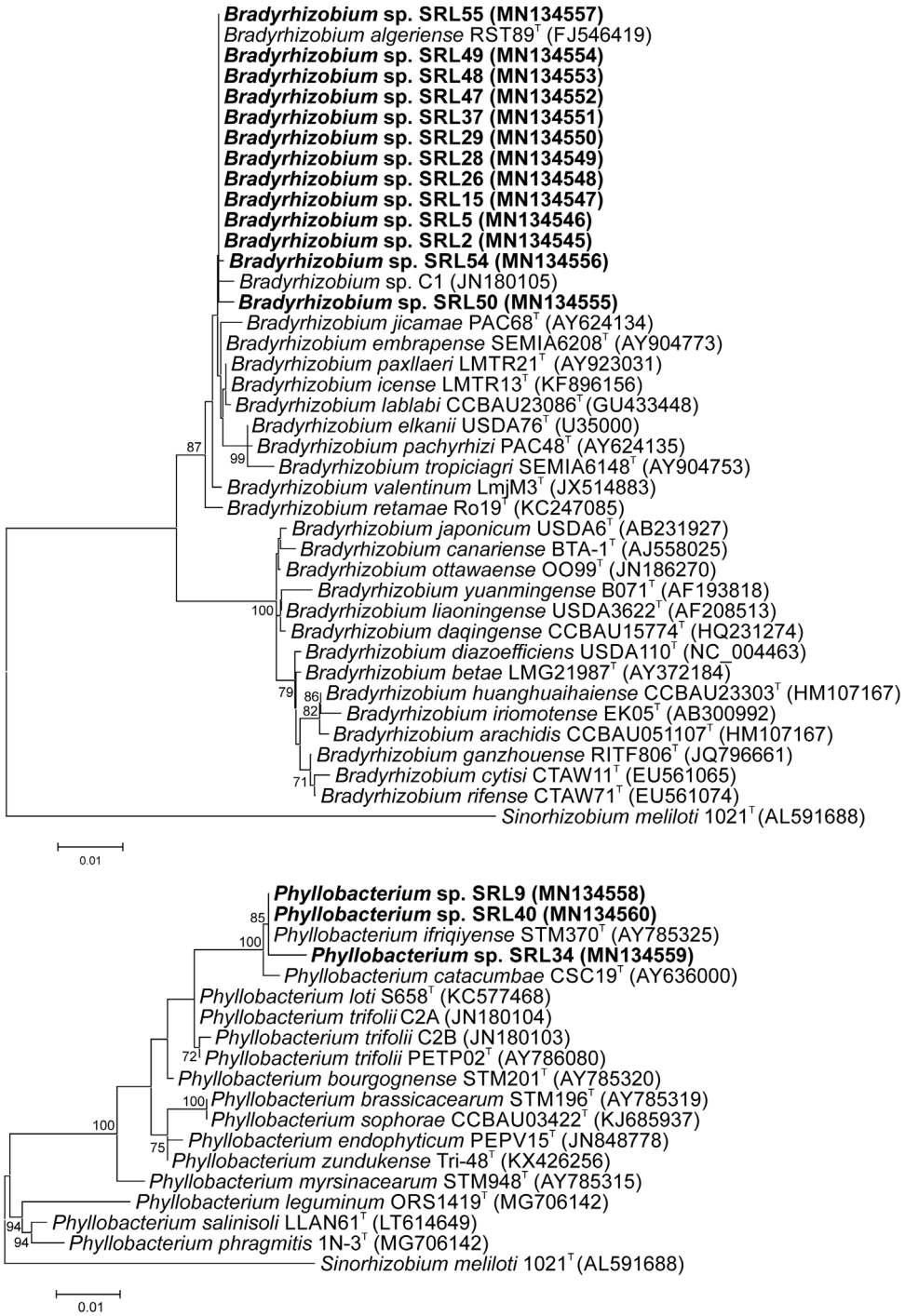

Fig. 2. The phylogenetic trees of 16S rDNA sequences of the *Chamaecytisus ruthenicus* root nodule isolates (shown in bold) and reference *Bradyrhizobium* (top) and *Phyllobacterium* (bottom). The phylogenies were inferred by using the Maximum Likelihood method based on TrN + I+Γ evolutionary model. Bootstrap values ≥70 % are given at the branching points. The scale bar indicates the number of substitutions per site. GenBank accession numbers are given in parentheses. The corresponding sequence from *Sinorhizobium meliloti* 1021 was used as outgroup.

from MLSA were subsequently supported by analysis of genomic relatedness using average nucleotide identity (ANI) or DNA-DNA hybridization [8,9,13,20,24].

Multilocus sequence analysis has not been previously used as supporting evidence in the description of new species of the genus *Phyllobacterium*. The only phylogenetic marker commonly used to delineate the species within this genus was *atpD* [25,30,31,44]. In the paper describing *Phyllobacterium zundukense* the comparative analysis of the *atpD*, *glnII*, and *recA* gene sequences is presented, but MLSA was not performed and a phylogenetic tree based on these three markers was not constructed [42]. In our study we used the gene sequences of three loci from the genomes of *Phyllobacterium* species available in the NCBI Genome database. The studied markers of the two closest reference species of our strains, i.e. *P. catacumbae* LMG22520^T and *P. ifriqiyense* LMG27887^T were sequenced in this study as they are absent from the GenBank and NCBI Genome databases.

Nucleotide identity of *atpD-gyrB-recA* genes of *Phyllobacterium* SRL9, SRL34, and SRL40 isolates with type strains of described *Phyllobacterium* species ranged from 78.2 (*Phyllobacterium salinisoli*

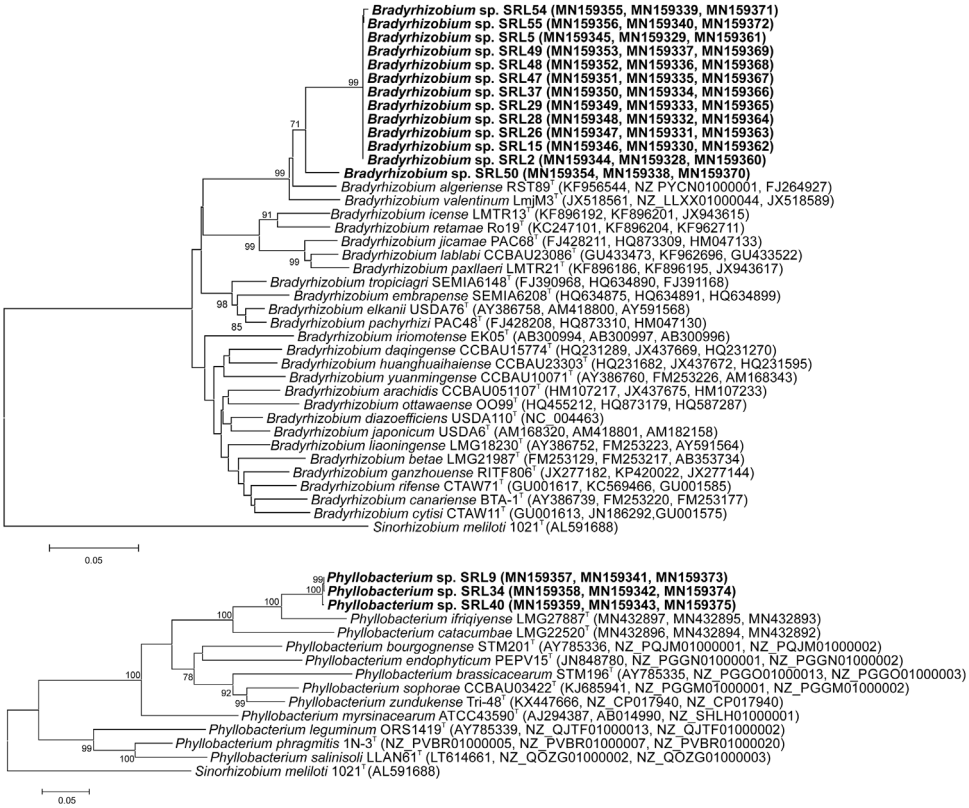

Fig. 3. The phylogenetic trees of concatenated *atpD, gyrB* and *recA* gene sequences of the *Chamaecytisus ruthenicus* root nodule isolates (shown in bold) and reference *Bradyrhizobium* (top) and *Phyllobacterium* (bottom). The phylogenies were inferred by using the Maximum Likelihood method based on GTR + I+Γ evolutionary model. Bootstrap values ≥70 % are given at branching points. The scale bar indicates the number of substitution per site. GenBank accession numbers are given in parentheses. The corresponding sequences from *Sinorhizobium meliloti* 1021 were used as outgroup.

LLAN61^T) to 90.8 % (*P. ifriqiyense* LMG27887^T). In the *atpD-gyrB-recA* phylogram, *Phyllobacterium* SRL strains grouped in a clearly distinct cluster supported by 100 % bootstrap value. The closest relatives of the studied isolates were *P. ifriqiyense* LMG27887^T and *P. catacumbae* LMG22520^T (Fig. 3). Pairwise comparison between *atpD-gyrB-recA* concatemers from 11 reference *Phyllobacterium* type strains revealed the highest nucleotide identity of the studied genes between *P. zundukense* Tri-48^T and *P. sophorae* CCBAU03422^T (92.2 %), which is higher than previously mentioned 90.8 % of sequence similarity estimated between *C. ruthenicus* root nodule isolates and *P. ifriqiyense* LMG27887^T. The presented results suggest that *Phyllobacterium* SRL strains belong to a new species within the genus *Phyllobacterium*.

Phylogenetic analysis of symbiosis related genes

To infer the symbiotic relationship of *C. ruthenicus* root nodule isolates with other rhizobia, comparative and phylogenetic analyses of the *nodC* and *nodZ* gene sequences were used. The *nodC* gene encodes N-acetyl-glucosaminyltransferase and belongs to common nodulation genes determining the core structure of Nod factors which play a key role in bacteria – plant interactions. It was demonstrated that the *nodC* gene is related to the host range of rhizobia [28,41]. The *nodZ* gene codes for fucosyltransferase involved in fucosylation of glucosamine at the reducing end of the Nod factor. The presence of the *nodZ* gene is commonly described among *Bradyrhizobium* and *Rhizobium* [15,27,35,38]. Our studies were also based on the comparative sequence analysis of the *nifD* and *nifH* genes, which code for α-subunit of dinitrogenase and dinitrogenase reductase, and are the essential components of the nitrogenase enzyme complex present in diazotrophic microorganisms [59].

We were unable to amplify studied symbiosis related genes with the use of standard primers for our *Phyllobacterium* strains. Moreover, the studied isolates were negative in nodulation test with *C. ruthenicus* (data not presented). Some other authors also did not find symbiotic genes in *Phyllobacterium* strains [25,42]. In other studies the authors reported isolation of *Phyllobacterium* strains from root nodules but the isolates were unable to form nodules in their host plant [16,32]. On the other hand, it was reported that two species, *P. trifoli* and *P. sophorae,* are able to induce effective nodules on their host legumes [25,49]. The presence of *nifD* and *nifH* genes, showing high homology to the analogous genes described for the *Bradyrhizobium* strains, was detected in a single *Phyllobacterium* strain isolated from root nodules of *C. ruthenicus* growing in the southern Urals [4]. The absence of *nod* and *nif* genes and lack of ability to induce nodules in *C. ruthenicus* by the studied *Phyllobacterium* strains indicated that they should be regarded as endophytic non-nodulating bacteria.

Contrary to the above findings for *Phyllobacterium* strains, all the *Bradyrhizobium* SRL bacteria were able to nodulate their host legume *C. ruthenicus* and we amplified all the studied symbiosis related genes for the *Bradyrhizobium* isolates. Their *nodC* gene sequences were 100 % identical and they shared 99.8–100% of *nodZ*, *nifD*, and *nifH* gene sequence similarities. The *nodC*, *nodZ*, *nifD*, and *nifH* gene sequences of the studied bradyrhizobia were most similar to *B. algeriense* RST89^T (99 %, 98.7 %, 99.6–99.7%, 99.8–100%), *B. valentinum* LmjM3^T (98.5 %, 98.7 %, 99.6–99.7%, 99.6–99.8%), *B. retamae* Ro19^T (98.5 %, 98.3 %, 98.7–98.8%, 98.9–99%), and *B. icense* LMTR13^T (98.1 %, 94.9 %, 97.4–97.7%, 98.3–98.4%). In the phylogenetic trees of individual *nodC*, *nodZ*, *nifD*, and *nifH* genes *Bradyrhizobium* SRL isolates grouped together with *B. algeriense* RST89^T, *B. valentinum* LmjM3^T, and *B. retamae* Ro19^T in clusters

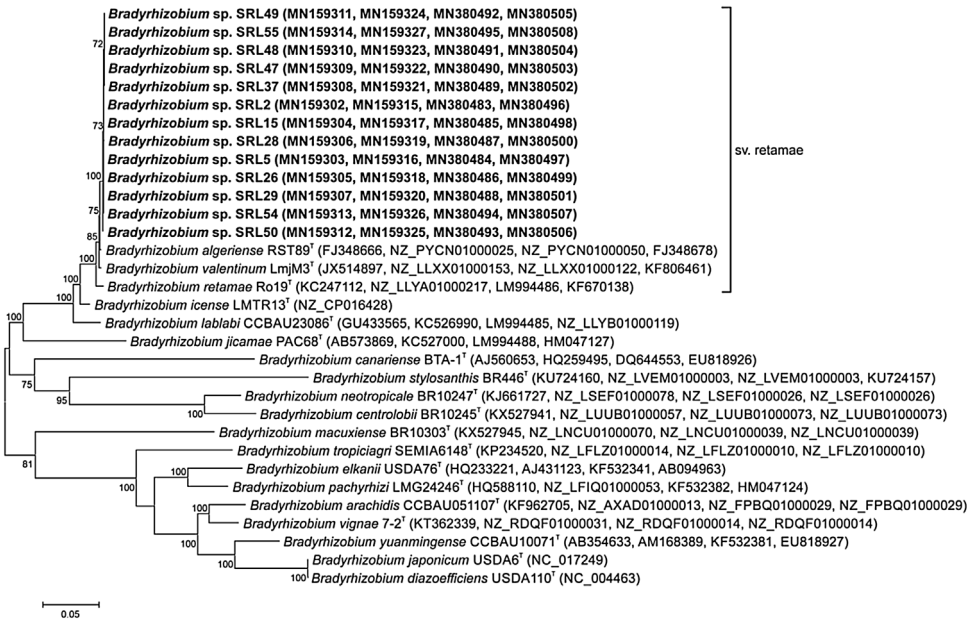

Fig. 4. The phylogenetic tree of concatecated *nodC, nodZ, nifD* and *nifH* gene sequences of the *Chamaecytisus ruthenicus* root nodule isolates (shown in bold) and reference *Bradyrhizobium*. The phylogeny was inferred by using the Maximum Likelihood method based on GTR + I+Γ evolutionary model. Bootstrap values ≥70 % are given at branching points. The scale bar indicates the number of substitution per site. GenBank accession numbers are given in parentheses.

supported by 91 %, 98 %, 98 %, and 95 % bootstrap values (Supplementary Figs. S4–S7). All these reference strains belong to recently described symbiovar retamae and were isolated from root nodules of the tribe Genisteae plants [1,14,21] to which *C. ruthenicus* also belongs. Phylogenetic analysis based on concatenated *nodC-nodZ-nifD-nifH* gene sequences confirmed the close position of *C. ruthenicus* bradyrhizobia to reference strains of the symbiovar retamae. All *Bradyrhizobium* SRL strains formed a separate group within a larger cluster encompassing type strains of *B. algeriense*, *B. valentinum*, and *B. retamae* supported by 100 % bootstrap value (Fig. 4). Nucleotide identity of *nodC-nodZ-nifD-nifH* concatemers of *Bradyrhizobium* SRL isolates with the representatives of symbiovar retamae ranged from 98.9 to 100%. *C. ruthenicus* root nodule isolates were distantly related to *B. canariense* BTA-1^T (83–83.1% *nodC-nodZ-nifD-nifH* sequence similarity) representing symbiovar genistearum, comprising bradyrhizobia effectively nodulating other plants of the tribe Genisteae (Fig. 4). In our previous paper we described bradyrhizobia isolated from root nodules of *Cytisus scoparius*, *Cytisus ratisbonensis*, *Genista germanica*, and *Genista tinctoria* growing in Poland and belonging to the Genisteae plants. All these bradyrhizobia were identified as *B. japonicum* and were classified to symbiovar genistearum based on the *nodC* and *nifH* phylogenies [26]. These findings together with the current study clearly indicate that *Bradyrhizobium* strains isolated from the Genisteae in Poland belong to two distinct symbiovars which are members of two clades, Clade II (encompassing symbiovar genistearum) and Clade IV (comprising symbiovar retamae) identified among *Bradyrhizobium* strains on the basis of *nodA* and *nifD* phylogeny [48]. Bacteria of the symbiovar retamae have been isolated from the Genisteae growing in Mediterranean region encompassing Spain, Morocco, and Algeria [1,14,21,43], thus our report is the first describing members of this symbiovar in Central Europe, providing evidence for the wide geographic distribution of *Bradyrhizobium* belonging to Clade IV.

The presented results lead to the following conclusions: the root nodules of *C. ruthenicus* growing in Poland are cohabitated by *Bradyrhizobium* and *Phyllobacterium*; the isolated bacteria belong to putative novel species of *Bradyrhizobium* and *Phyllobacterium*; the latter strains are non-nodulating endophytes of *C. ruthenicus*; the studied *Bradyrhizobium* bacteria are members of symbiovar retamae, one of two identified so far symbiovars of rhizobia effectively nodulating the tribe Genisteae plants. Further studies based on the comparative analysis of the genome sequences, which is recommended as one of minimal standards for the description of new species of rhizobia [12] should provide conclusive evidence whether our isolates represent new taxons in the genera *Bradyrhizobium* and *Phyllobacterium*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2020.126056.

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