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Bradyrhizobium canariense and *Bradyrhizobium japonicum* are the two dominant rhizobium species in root nodules of lupin and serradella plants growing in Europe

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ABSTRACT

Forty three *Bradyrhizobium* strains isolated in Poland from root nodules of lupin species (*Lupinus albus*, *L. angustifolius* and *L. luteus*), and pink serradella (*Ornithopus sativus*) were examined based on phylogenetic analyses of three housekeeping (*atpD*, *glnII* and *recA*) and nodulation (*nodA*) gene sequences. Additionally, seven strains originating from root-nodules of yellow serradella (*O. compressus*) from Asinara Island (Italy) were included in this study. Phylogenetic trees revealed that 15 serradella strains, including all yellow serradella isolates, and six lupin strains grouped in *Bradyrhizobium canariense* (BC) clade, whereas eight strains from pink serradella and 15 lupin strains were assigned to *Bradyrhizobium japonicum* (BJ1). Apparently, these species are the two dominant groups in soils of central Europe, in the nodules of lupin and serradella plants. Only three strains belonged to other chromosomal lineages: one formed a cluster that was sister to *B. canariense*, one strain grouped outside the branch formed by *B. japonicum* supergroup, and one strain occupied a distant position in the genus *Bradyrhizobium*, clustering with strains of the *Rhodopseudomonas* genus. All strains in nodulation *nodA* gene tree grouped in a cluster referred to as Clade II, which is in line with earlier data on this clade dominance among *Bradyrhizobium* strains in Europe. The *nodA* tree revealed four well-supported subgroups within Clade II (II.1–II.4). Interestingly, all *B. canariense* strains clustered in subgroup II.1 whereas *B. japonicum* strains dominated subgroups II.2–II.4.

Introduction

The genus *Lupinus* comprises some 275 species of annual and perennial herbs and shrubs, the majority of which are distributed in the New World, mainly in the western part of North America and the Andes. Only 15 species are native to the Old World, in areas surrounding the Mediterranean [1,16]. Lupins form highly effective nitrogen-fixing symbiosis with root nodule bacteria often in nutrient-poor soils. This enables their cultivation in soils where growth of more demanding crops is difficult [19]. Lupins have been cultivated since the antiquity, mainly, as green manure and important pulse crop [12]. Three cultivated in Poland lupin species (*Lupinus albus*, *L. angustifolius*, *L. luteus*), as well as pink serradella (*Ornithopus sativus*) were introduced from southern Europe in the first half of the XIX century. Extensive use of lupins as green manure was reported in the area around Poznań in 1840 [53]. In 1938, the area of 383,500 ha under lupins was recorded in Poland, whiles cultivation of serradella reached its peak in 1960 with 524,602 ha used for this legume crop. Since then the area under lupins and serradella declines constantly, so only about one tenth of it is used to grow this plants presently [13,53].

Lupins and serradella are nodulated by bacteria belonging to the genus *Bradyrhizobium*. Additionally, strains corresponding to other rhizobium genera inhabit lupin, but not serradella, nodules. These "fast growing" rhizobia, however, tend to form less effective nitrogen fixing symbiosis with lupin plants than bradyrhizobia [2,11,55]. Due to the highly specific symbiosis, serradella is often regarded as a surrogate hosts for lupins, which implies that serradella strains (all of which seem to belong to the genus *Bradyrhizobium*) nodulate lupins, whereas some lupin rhizobia are unable to infect serradella. Housekeeping gene phylogenies have revealed the considerable level of diversity among *Bradyrhizo-*

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bium strains nodulating lupins and serradella [5,18,40,49,50,59,60]. However, the majority of strains clustered with *B. japonicum sensu lato*, and only few grouped with *B. elkanii* [31,50].

Phylogenies based on nodulation gene sequences tend to display different patterns than non-symbiotic gene trees, a fact ascribed to lateral gene transfer affecting, primarily, the evolution of symbiotic loci that are under plant host selective pressure [18,29,34,51]. In trees generated on *nodA* gene sequences, the majority of lupin–serradella bradyrhizobia group within a discrete cluster named Clade II [33]. Clade II strains predominate among lupin–serradella *Bradyrhizobium* strains originating from Europe whereas in the Americas this clade strains appear less common. Higher sequence diversity of the European strains (with respect to the American strains) implicates a European, or precisely, a Mediterranean origin for Clade II bradyrhizobia [49,50].

Central Europe is essentially free of exotic legumes which are nodulated by bradyrhizobia. Cultivation of soybean requires application of commercial strains in order to establish effective symbiosis [27]. Unlike soybeans, lupins and serradella in Poland do not require rhizobium inoculants. A recent work has revealed the presence of lupin–serradella bradyrhizobia in 61 out of the 80 studied soils, finding them more common than rhizobium symbionts of widespread *Melilotus* spp. in Poland [30]. Given the similarity of the housekeeping and nodulation gene sequences, bradyrhizobia nodulating native to Poland Genisteae (and Loteae) species appear to constitute a natural pool of effective strains for the introduced lupins and serradella spp. [21,22]. To elucidate, to which extend *Bradyrhizobium* strains nodulating lupins and serradella in Poland resemble those in other parts of the world, we carried out phylogenetic analyses of *nodA* and three housekeeping genes. This study reveals similarity of our lupin and serradella isolates to Genisteae bradyrhizobia originating from the Mediterranean [50,57]. This indicates that the differences in climates have not constrained the spreading of these rhizobia from southern Europe to the north, which is a consequence of recent migration of their Genisteae hosts after the retreat of the ice shield [6].

Materials and methods

The list of strains used in this study is shown in Table 1. Yeast extract mannitol agar medium [58] was used for growth and maintenance of strains. All *Bradyrhizobium* strains were grown at 28 °C. *Bradyrhizobium* strains originated from lupin nodules collected near Poznań, western Poland, in 9 locations (Fig. 1). These strains were further examined for their growth rate and medium alkalisation ability. 23 slow-growing, medium alkalising strains were selected for detailed phylogenetic analyses. Additionally, we included 17 strains originating from pink serradella (*Ornithopus sativus*) plants growing near Parczew (eastern Poland), and seven strains originating from yellow serradella (*O. compressus*) plants from Asinara Island, Italy. Italian isolates have been characterized by [44,45]. Pink serradella strains were obtained from Professor Hanna Skorupska, University of Maria Curie Skłodowska in Lublin, Poland. All lupin and pink serradella strains were authenticated on their respective hosts in five replicates for each strain as described elsewhere [48]. Elite strains WSM1996 and VK7 were used as positive controls in plant test experiments.

Molecular techniques

For PCR-sequencing experiments, total genomic DNA was isolated using the SDS-proteinase K lysis procedure described by [33], or using the QIAGEN QIAamp DNA Mini Kit following the manufacturer's recommendations. The primers used in this study are listed in Table 2. All PCR amplification reactions were made following the procedures described previously [49]. In brief, PCR samples were denatured at 95 °C for 2 min followed by 35 cycles of 95 °C for 45 s, 58 °C (*atpD*, *glnII* and *recA*) for 30 s, 72 °C for 1.5 min (2.5 min for *nodA*), and a final elongation step of 10 min at 72 °C, as recommended for the FastStart High Fidelity PCR System® by the manufacturer (Roche Diagnostics GmbH, Germany). The annealing temperature for amplification of *nodA* was 53 °C [49]. PCR products were purified using the QIAquick gel extraction kit (QIAGEN, Germany) and sequenced with the BigDye® Terminator v3.1 cycle sequencing kit on an ABI3100 Automated Capillary DNA Sequencer (Applied Biosystems, USA). The accession numbers of sequences generated in this study are listed in Table 1.

Phylogenetic analyses

All sequences were aligned using clustalX [54] and edited manually under Genedoc [35]. Phylogenies were produced by Maximum Likelihood (ML) using PAUP4 [52] or phyML using the Phylogeny.fr website [10]. Node robustness was assessed using aLRT [3] and bootstrapping replicates (100) under phyML.

Results

Amplification and sequencing of three housekeeping and nodulation nodA genes

All strains in this study were selected taking into account their ability to form effective nodules on their respective lupin and serradella hosts and slow-growth and medium alkalisation phenotypes (data not presented). Finally, we characterized 50 *Bradyrhizobium* strains, out of which 26 strains originated from root nodules of three lupin species while 24 strains were isolated from pink and yellow serradella species.

Using primer sets and amplification protocols described previously [49] we amplified and sequenced *atpD* gene (in 43 strains), *glnII* (in 44 strains), *recA* (in 49 strains), and nodulation *nodA* gene (in 44 strains). We did not include 16S rRNA gene in our study due to limited resolution which characterize this gene sequences in the *Bradyrhizobium* genus [62]. The length of the alignments were as follows: 559 bp for *recA*, 485 bp for *atpD*, 519 bp for *glnII*, and 633 bp for *nodA*. For the combined phylogenies of *atpD*, *glnII* and *recA* gene, the missing data due to the lack of amplification in some strains or data absent from reference species (*recA* of *B. pachyrhizi* and *B. jicamae*) were filled with N in the alignments and subsequently treated as missing data in PAUP and phyML.

Single housekeeping gene phylogenies are shown as Supplementary data (Figs. S1–S3). Conflicting phylogenetic signals were detected among the housekeeping genes studied: strain UPP347 grouped with the fast-growing rhizobium genera on *atpD* tree and with the *Bradyrhizobium* branch in *recA* tree; strain UPP233 *atpD* sequence showed limited similarity to other *Bradyrhizobium* sequences; strain UPP332 showed conflicting placement in *glnII* and *recA* trees. These three strains were deducted from the concatenated dataset but can be seen in Supplementary material Fig. S1. Other recombination events were detected, as UPP331 grouping with *B. japonicum* on *atpD* and *glnII* trees clustered with *B. yuanmingense* in *recA* tree. In such cases, the conflicting gene-fragment was omitted and replaced by a series of "N" in the concatenated alignment.

Bradyrhizobium canariense and B. japonicum are the most common species among serradella and lupin isolates

Phylogenetic analyses based on single partial sequences of *recA*, *atpD* and *glnII* genes confirmed the affinity of our lupin–serradella isolates to the *Bradyrhizobium* genus (See Supp. material Fig. S1). A phylogeny was produced using mixed data of all three housekeep-