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Diverse *Mesorhizobium* bacteria nodulate native *Astragalus* and *Oxytropis* in arctic and subarctic areas in Eurasia

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ABSTRACT

Rhizobia nodulating native *Astragalus* and *Oxytropis* spp. in Northern Europe are not well-studied. In this study, we isolated bacteria from nodules of four *Astragalus* spp. and two *Oxytropis* spp. from the arctic and subarctic regions of Sweden and Russia. The phylogenetic analyses were performed by using sequences of three housekeeping genes (16S rRNA, *rpoB* and *recA*) and two accessory genes (*nodC* and *nifH*). The results of our multilocus sequence analysis (MLSA) of the three housekeeping genes tree showed that all the 13 isolates belonged to the genus *Mesorhizobium* and were positioned in six clades. Our concatenated housekeeping gene tree also suggested that the isolates nodulating *Astragalus inopinatus*, *Astragalus frigidus*, *Astragalus alpinus* ssp. *alpinus* and *Oxytropis revoluta* might be designated as four new *Mesorhizobium* species. The 13 isolates were grouped in three clades in the *nodC* and *nifH* trees. ^{15}N analysis suggested that the legumes in association with these isolates were actively fixing nitrogen.

Introduction

Nitrogen (N) is considered a limiting nutrient for sustainable plant growth in many ecosystems. In N poor soils legumes have an advantage as they are able to fix nitrogen biologically from the atmosphere through symbiotic association with soil bacteria collectively called rhizobia. This symbiotic interaction between legumes and their associated rhizobia is presently considered to be the main global contributor of biologically fixed N to the terrestrial ecosystem [6,11]. To exploit the benefits of this association, a lot of research has been done to understand the fundamentals of the interaction and methods developed to estimate the amount of fixed N contributed to the ecosystem. Despite a great wealth of knowledge gained over the decades, there is still very little information on nodulation, root nodule bacteria and the amount of nitrogen fixation by native legumes in northern temperate regions.

Legumes that are principally native to the northern hemisphere include species of the genera *Astragalus* and *Oxytropis*. *Astragalus* species are common in the northern hemisphere and extend into the arctic and subarctic regions while *Oxytropis* spp. are distributed throughout the northern temperate, the subarctic and arctic regions [14,23,28]. As the largest genus in the plant family Fabaceae, *Astragalus* has over 2500 species distributed over more than 100 subdivisions, but *Oxytropis* has relatively fewer species of about 400 [1,29]. These two genera are phylogenetically related [24] and are also generally thought to share rhizobia [14,25].

Most studies aimed at identifying or characterizing rhizobia associated with *Astragalus* spp. or *Oxytropis* spp. were largely carried out in North America and China. *Mesorhizobium* has been identified as the predominant genus that nodulates plants in the genera *Astragalus* and *Oxytropis*, whereas the genera *Rhizobium*, *Ensifer* and *Bradyrhizobium* have been found as minor symbionts of these plant genera [5,8,14,17,34,35].

Ampomah et al. [3] suggested, based on evidence from light and transmission electron microscopy of nodules and the presence of *nifH* that indigenous *Astragalus* and *Oxytropis* species in Sweden have the potential of fixing N_2. Otherwise, no information about how much nitrogen is fixed by these genera or the identity of their microsymbionts has been reported. Multilocus sequence analysis (MLSA) is a straightforward approach to determine bacterial phylogeny including rhizobia [18–21] and is assumed to reflect a robust bacterial phylogeny. In this study we used the MLSA approach to determine the phylogeny of rhizobia that are associated with some native *Astragalus* species in Sweden and a species in Yakutsk, Siberia. We also included isolates from *Oxytropis* spp. which we had

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the opportunity to sample from the southern part of Kamchatka, Russia. According to the Köppen – Geiger climate classification [13], the Swedish sites have a subarctic climate (Dfc), Yakutsk has an extremely continental climate (Dfd) and the Kamchatka sites have a subarctic climate (Dfc) at low altitudes and tundra climate (ET) at higher altitudes. Measurements of ^{15}N natural abundance have been used to prove capacity for N_2 fixation in the natural setting. To our knowledge, this is the first report to document potential N_2 fixation of naturally growing *Astragalus* and *Oxytropis* species in Northern Europe, Siberia and Kamchatka and to identify rhizobia associated with them.

Materials and methods

Plant sampling and nodule collection

The plant species studied were *Astragalus alpinus* L. ssp. *alpinus*, *A. alpinus* L. ssp. *arcticus* (Bunge) Lindm., *Astragalus frigidus* (L.) A. Gray, *Astragalus inopinatus* (Boriss.), *Oxytropis lapponica* (Wahlenb.) Gray, *Oxytropis revoluta* Ledeb and *Oxytropis* sp. Nodulated plants were sampled from different geographic sites (Table 1, Fig. 1). Where possible, legumes were sampled at the same time as nearby non-N_2-fixing reference plants for ^{15}N analysis. Depending on the abundance of the legume species at a site, samples were taken from up to eight randomly selected plants within an area of about 10 – 15 m^2 together with the reference plants growing in close proximity. Briefly, plants were dug out from the soil with a small spade to a depth of about 15 cm, and the soil clump with the intact plant transported in plastic bags to the laboratory or kept cold overnight. Reference plants growing nearby the legume species were also collected and handled in a similar manner. Mature green leaves were detached from the plants, kept in paper bags, and dried at 60 °C during 24 h. At this developmental stage of the plants seeds were not yet mature and could not be collected. Nodules from the legume roots were detached and preserved over silica gel until nodule bacteria were isolated [27].

Nodule bacteria isolation and DNA extraction

To isolate nodule bacteria, nodules stored over silica gel were rehydrated in water and kept at 5 °C overnight [27]. Surface sterilization and crushing of nodules was done as described in Ampomah and Huss-Danell [2]. Isolates spread on yeast mannitol agar (YMA) plates were kept at 28 °C and observed for 7 – 10 days for the appearance of colonies. Well isolated colonies typical of rhizobia were picked and streaked out on new YMA plates. Subsequent purifications were made until pure cultures of isolates were obtained. Pure cultures of isolates were stored at −80 °C in yeast mannitol liquid medium containing 20% (v/v) glycerol.

DNA from the isolates was obtained by lysis of single colonies streaked out on tryptone yeast medium [26]. Colonies suspended in 100 μl of 0.05 M NaOH were boiled for 4 min to lyse the cells. 900 μl of sterile ultrapure water was added to the lysate and then stored at −20 °C prior to PCR.

Authentication test

For the authentication of our nodule isolates we used seeds of *A. alpinus* ssp. *alpinus* L. from Storfjord, Skibotn (69 22′37″N 20 16′19″E, 3 m, Norway) and seeds of *Lotus corniculatus* purchased from Olssons frö AB, Helsingborg, Sweden because we were unable to obtain seeds for the other host species. The seeds were soaked in concentrated H_2SO_4 for 5 – 10 min and then rinsed 5 – 10 times in sterile water on a shaker before being placed on autoclaved tryptone soy agar (TSA) in petri dishes. Germinated seeds were moved to sterilized growth pouches (CYG, Mega International, Minneapolis, USA) and watered (sterile water or dilute N-free nutrient solution) as needed. Selected isolates were inoculated either on the roots of *A. alpinus* ssp. *alpinus* or *L. corniculatus* seedlings growing in the pouches. Nodulation was observed over a period of 6 weeks.

PCR amplification and sequencing of housekeeping and accessory genes

The 16S rRNA gene (*rrs*) was amplified by using the primer pair fD1 and rD1 in PCR as described by Weisburg et al. [32]. For *recA*, *rpoB* and the symbiotic genes *nodC* and *nifH*, PCR amplification and sequencing were performed according to Aserse et al. [4] and Mousavi et al. [20]. The *rrs* PCR products were purified using the Nucleospin Extract II column (Machery-Nagel, Duren, Germany) according to the manufacturer's recommendation and sequenced at Macrogen Inc., the Netherlands. The PCR products of *recA*, *rpoB*, *nodC* and *nifH* were purified with AMPure XP (Beckman Coulter, Inc.), sequenced using BigDye Terminator Chemistry v.3.1 and analyzed on an ABI 3010 Sequencer (Life Technologies) at the Sequencing and Genomics Lab., Institute of Biotechnology, University of Helsinki. The sequences of the *Mesorhizobium* reference strains were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank).

Accession numbers

The nucleotide sequences obtained in this study were deposited in the Genbank under accession numbers from KF034924 to KF034936 for the 16S rRNA fragments (1092 bp), KU729796 to KU729808 for the *recA* (483 bp), KU729809 to KU729821 for the *rpoB* (818 bp), KU729783 to KU729795 for the *nodC* (426 bp) and KU729770 to KU729782 for the *nifH* (355 bp). All accession numbers of the sequences used in this study are listed in Appendix A Supplementary Table S1.

Phylogenetic analyses

The sequences of the 16S rRNA gene were aligned using MUSCLE [7] software at EML-EBI [9]. ClustalW [16] in BioEdit version 7.0.5.3 [10] was used to align the *recA*, *rpoB*, *nodC*, and *nifH* gene sequences. The best-fit model of nucleotide substitution for the dataset of each locus was selected by Akaike information criterion (AIC) applied in MEGA6 [30]. The general time reversible plus gamma distribution plus invariable sites (GTR + G + I) nucleotide substitution model was used for the concatenated housekeeping genes (*rrs-recA-rpoB* and *recA-rpoB*), and Tamura 3-parameter plus gamma distribution plus invariable sites (T92 +G +I) was used for the phylogenetic analyses of the accessory genes (*nodC* and *nifH*) and the 16S rRNA gene. Maximum-likelihood phylogenetic trees for the three and two concatenated housekeeping loci (*rrs-recA-rpoB* and *recA-rpoB*) and the accessory genes *nodC* and *nifH*, were constructed with 1000 replicates using MEGA6. The mean distance between the groups was computed by MEGA6.

^{15}N analysis

Leaf samples were finely ground in a ball mill. Analysis of leaf N concentration and ^{15}N abundance were done at the Department of Forest Ecology and Management, SLU, Umeå, Sweden and at the Waikato Stable Isotope Unit at University of Waikato, New Zealand. Several samples were duplicated and sent to both laboratories. Very similar results of the analyses were obtained in the two laboratories.