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Evolution and taxonomy of native mesorhizobia nodulating medicinal *Glycyrrhiza* species in China

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ABSTRACT

Previously, 159 bacterial strains were isolated from the root nodules of wild perennial Glycyrrhiza legume species grown on 40 sites in central and north-western China, in which 57 strains were classified as "true symbionts" belonging to the genus *Mesorhizobium* based on amplified fragment length polymorphism (AFLP) genomic fingerprinting and partial sequences of the 16S rRNA gene [20]. In the present work, the phylogeny of *Glycyrrhiza* nodulating mesorhizobia was further examined by multilocus sequence analysis (MLSA). The concatenated gene tree of three housekeeping genes (16S rRNA, *recA*, and *rpoB*) of 59 strains including the 29 mesorhizobial test strains and 30 type mesorhizobial species, was constructed applying the maximum likelihood method and Bayesian inference. In the concatenated gene tree, the 29 test strains were distributed in seven separate clades. Seventeen test strains clustered with *Mesorhizobium tianshanense*, *Mesorhizobium temperatum*, *Mesorhizobium muleiense*, and *Mesorhizobium alhagi* with high bootstrap support (BS > 85%). Eight test strains did not cluster with any of the described *Mesorhizobium* species. Based on the results, we proposed these eight test strains might belong to a putative new species of the genus *Mesorhizobium*. The sequences of three accessory genes (*nodA*, *nodC*, and *nifH*) of the test strains were also analyzed and were compared with those of representatives of the 30 described mesorhizobial species. The results showed that mesorhizobia involved in symbiosis with *Glycyrrhiza* plants probably have acquired some genetic material from other rhizobia in co-evolution with *Glycyrrhiza* and other legume species.

Introduction

The plant genus *Glycyrrhiza* belongs to the family Fabaceae/Leguminosae and consists of approximately 20 species, among which six species produce a sweet saponin glycyrrhizic acid (glycyrrhizin). Roots and rhizomes of *Glycyrrhiza glabra*, *Glycyrrhiza uralensis* and *Glycyrrhiza inflata* are considered as licorice, and are used extensively in food, tobacco, cosmetics, and medicine industries [17,20]. *Glycyrrhiza* species can be found on all continents but Antarctica; and are mostly endemic to Eurasia. *G. glabra* is a Mediterranean species which grows also in Iran, Iraq, Central Asia and the north-western part of China, whereas the species *G. uralensis* is found in China, Central Asia, and Mongolia. *Glycyrrhiza* spp. grow on dry grassy plains and mountainsides in the north-western provinces of China. Even though medicinal properties of *Glycyrrhiza* spp. were studied extensively, over 1500 reports exist on the compound glycyrrhizin, the biology and ecology of the plant species are not well known [14,15,20,33].

Glycyrrhiza spp. can establish symbiotic associations with a wide range of root-nodule bacteria, which in symbiosis reduce atmospheric dinitrogen gas (N_2) to ammonia. At least three symbiotic rhizobial genera, *Mesorhizobium*, *Ensifer* (syn. *Sinorhizobium*) and *Rhizobium* were found to be associated with *Glycyrrhiza* spp. [20]. The genera *Rhizobium* and *Ensifer* are classified in the family *Rhizobiaceae* whereas the genus *Mesorhizobium* consisting of 30 species is

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the member of the family *Phyllobacteriaceae* (http://www.bacterio.net).

Li et al. [20] studied 159 endophytic bacterial isolates form nodules of wild *Glycyrrhiza* growing on 40 sites in central and north-western China. The results of amplified fragment length polymorphism (AFLP) genomic fingerprinting, and partial sequences of the 16S rRNA gene as well as nodulation tests indicated that out of 159 endophytic strains 115 represented *Alphaproteobacteria*, involving the genera *Mesorhizobium* (57 strains), *Rhizobium* (25), *Ensifer (Sinorhizobium)* (11), *Phyllobacterium* (6) and *Agrobacterium* (16). Thus, at least, representatives of all these genera can colonize *Glycyrrhiza* nodules in China. Based on symbiotic properties, five *Mesorhizobium* groups (with 57 strains) represented the true symbionts of *G. glabra* and *G. uralensis*. Since the 16S rRNA gene is too conserved to allow close species separation [22–24], and only partial sequences of 16S rRNA gene (*rrs*) of representative strains of each AFLP subgroups were studied by Li et al. [20], further phylogenetic studies were required to resolve the taxonomy of these isolates. Multilocus sequence analysis (MLSA) method is considered as a straightforward method to study the phylogeny and taxonomy of rhizobia [22–25].

Aiming at determining the accurate taxonomic position of 29 mesorhizobial strains collected from nodules of *Glycyrrhiza* spp. growing in central and north-western China, we performed multilocus sequence analysis (MLSA) for 59 mesorhizobial strains, including 29 *Glycyrrhiza* test strains and 30 type strains of the *Mesorhizobium* species, since multilocus sequence analysis (MLSA) method is considered as a straightforward method to study the phylogeny and taxonomy of rhizobia [22–25]. Three housekeeping genes, namely 16S rRNA (*rrs*), *recA* and *rpoB*, coding for 16S ribosomal RNA, recombinase A, and RNA polymerase beta subunit, respectively were used in this study. Furthermore, we also investigated the phylogeny of three symbiotic genes of all the 59 mesorhizobial strains, involving nodulation genes *nodA* and *nodC* and the nitrogenase encoding gene *nifH*.

Material and methods

Bacterial strains and DNA isolations

59 mesorhizobial strains studied here (Table S1) included 29 mesorhizobial strains isolated from *Glycyrrhiza glabra* (10 strains), *G. uralensis* (14) and unidentified *Glycyrrhiza* species (5), and 30 type strains of the *Mesorhizobium* species, in which *Mesorhizobium camelthorni* HAMBI 3020^T, *M. muleiense* HAMBI 3264^T, *Mesorhizobium qingshengii* HAMBI 3277^T, *Mesorhizobium robiniae* HAMBI 3082^T, *Mesorhizobium sangaii* HAMBI 3318^T and *Mesorhizobium shangrilense* HAMBI 3050^T were obtained from the HAMBI Culture Collection, University of Helsinki. The bacterial samples were grown on yeast mannitol agar medium at 28 °C for two-three days [27]. For DNA isolation, single colonies of the bacteria were cultured in 5 ml of tryptone-yeast extract broth. Genomic DNA was isolated from each strain using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) and was preserved at −20 °C.

PCR amplification and gene sequencing

Three housekeeping genes (*rrs-recA-rpoB*) and three symbiotic genes (*nodA*, *nodC* and *nifH*) of the *Glycyrrhiza* test strains and the *rpoB* gene of six reference strains (not available in database) were sequenced in the current work. PCR amplification and sequencing were performed by using the primers listed in Table S2. The sequences of the mentioned genes for most of the type strains of *Mesorhizobium* species were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). The accession numbers of the studied housekeeping and symbiotic gene sequences are listed in Table S1.

Phylogenetic analyses of the sequences

The sequences were aligned by MUSCLE [5] software at EMLEBI [9] and were edited with program BioEdit version 7.0.5.3 [10]. The best-fit nucleotide substitution model of each gene was selected applying MEGA6 [29]. The concatenated gene tree (*rrs-atpD-rpoB*) of altogether 59 mesorhizobial strains was constructed using maximum likelihood (ML) and Bayesian interference. The ML tree of the combined genes (*rrs-atpD-rpoB*) was constructed with 1000 bootstrap replicates in MEGA6. The mean distance between the groups of the test and reference strains and the distances between the species were computed by MEGA6. The algorithm Metropolis-coupled Markov chain Monte Carlo (MCMCMC) for 2×10^6 generations was run twice with MrBayes 3.2 for the combined sequences dataset [26]. The generated Bayesian traces and trees were visualized by Tracer v1.5 (http://tree.bio.ed.ac.uk/software/tracer/) and FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). The ML individual gene trees of the 16S rRNA, *nodA*, *nodC*, and *nifH* loci were constructed with 1000 bootstrap replicates in MEGA6.

Results

Analyses of the housekeeping genes

The phylogeny of the *rrs* gene sequence (1247 bp) of 59 mesorhizobial strainsdemonstrated that the 29 mesorhizobial test strains were placed in five different clusters (Fig. S1). The ML tree of the combined three housekeeping genes (*rrs-recA-rpoB*) (Fig. 1) positioned the 29 test strains in seven separate clades. In addition, the Bayesian analysis returned similar topologies (not shown).

Eight strains isolated from Xinjiang region (Chahan, Hejing, and Ziniquan towns) formed a distinct clade (clade C) with a high bootstrap support (BS = 100%) that accommodates none of the reference strains. Seventeen test strains clustered with *M. tianshanense*, *M. temperatum*, *M. muleiense*, and *M. alhagi* with high confidence (BS > 85%), representing the groups A, D, E, and G respectively. The strain NWGS09 was placed close to *M. tianshanense* and *M. gobiense*. Based on the sequences of two protein-coding housekeeping genes (*recA-rpoB*), the pairwise ANI between eight *Mesorhizobium* species were over 96%. The strain NWSX24 was placed close to the clade that accommodates *M. temperatum* and three test strains (clade D). The strain NWNX05, isolated from *Glycyrrhiza* sp. in Ningxia, was grouped with *M. gobiense* supported by 100% of bootstrap value. Strain NWGS05, isolated from *Glycyrrhiza* sp. in Lingtai, Gansu, was clustered with *Mesorhizobium amorphae* (BS = 100%). Based on the sequences of two protein-coding housekeeping genes (*recA-rpoB*), the pairwise ANI between the following four pairs of species, *M. qingshengii* and *Mesorhizobium huakuii*, *Mesorhizobium silamurunense* and *Mesorhizobium shonense*, *M. tianshanense* and *M. gobiense*, and *M. alhagi* and *M. camelthorni* were over 96%.

Analyses of the individual accessory genes

Based on the analyses of the *nodA* gene sequences (Fig. S2 and Table 1), 28 test strains were placed in four main groups (A, B, C and E). Interestingly, the strains of clade B were placed separately from the reference strains. The *nodA* sequences of the species *M. tianshanense* and *M. temperatum* were grouped with 15 test strains, though neither of the clusters was strongly bootstrapping supported (BS < 75%). In the *nodA* gene tree, the species *M. alhagi* formed a well-supported clade (E) with five test strains alongside the species *M. camelthorni*.