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Rhizobium anhuiense as the predominant microsymbionts of *Lathyrus maritimus* along the Shandong Peninsula seashore line

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ABSTRACT

Beach pea [*Lathyrus maritimus* Bigelow, or *Lathyrus japonicus* subsp. *maritimus* (L.) P.W. Ball] is a wild legume distributed on the seashore line, and the rhizobia nodulating with this plant have been reported only rarely. In order to reveal the diversity of beach pea rhizobia on the seashore line of Shandong Peninsula, China, a total of 124 bacterial strains were isolated from the root nodules of beach pea plants collected from five sites. All the isolates were divided into five *recA* types after screening by *recA* gene sequence analysis and they consisted of *Rhizobium anhuiense* covering 122 symbiotic isolates in three *recA* types, as well as two single isolates *Rhizobium* sp. and *Rhizobium lusitanum* representing distinct *recA* types. The *recA* genotype III of *R. anhuiense* (103 isolates) represented by strain YIC11270 was dominant at all five sampling sites. Identical symbiotic genes (*nodC* and *nifH*) were detected in the three *recA* genotypes of *R. anhuiense* isolates that were closely related to those of the pea and faba rhizobia. This study clarified that *R. anhuiense* was the main symbiont for beach pea rhizobia on the seashore line of Shandong Peninsula. The low level genetic diversity of beach pea rhizobia revealed by both MLSA and the symbiotic genes might be related to the strong selection pressure produced by the saline–alkaline environment and the host plants.

Introduction

Beach pea or sea pea (*Lathyrus maritimus* or *Lathyrus japonicus* var. *maritimus*) is a herbaceous perennial legume, mainly spread along the sand beach of a coast line or seashore line in the northern regions of Asia, Europe and North America [1,6]. As a halophyte [33], beach pea is resistant to saline conditions. It can also endure low temperature and can spread in arctic and subarctic regions, where it is considered to be a cold-climate crop [9]. It has been used for conservation and restoration of foreshores and dunes in order to prevent seashore line erosion [1]. As a leguminous plant, its aerial parts are rich in protein and can be used as fodder [6,10]. Indeterminate nodules and high nitrogen fixation ability, especially nitrogen fixation in relatively low temperature conditions, have been recorded for this plant [4,7,9]. To date, little information on the diversity of beach pea rhizobia has been available. Most of beach pea rhizobia isolated from Quebec, Canada, the Hebei Province in China, and Japan had high similarities with *Rhizobium leguminosarum*, while a few strains were closely related to *R. pisi* [1,8,21]. The genus *Lathyrus* with species such as *Lathyrus davidii* and *L. odoratus* were reported to nodulate with *R. leguminosarum*, *R. multihospitium*, *R. tropici* and other rhizobial species in China [10]. However the rhizobial diversity of beach pea in China has been studied insufficiently, although this plant is widely spread along the seashore line of China [33]. It is known that the rhizobia have a geographic distribution and legume–rhizobia symbiosis progression is affected by the interaction of the host plant, rhizobia and environment factors [5,32], and the beach pea rhizobial populations in China may be different from those on the seashore lines of Canada and Japan. In addition, newly determined species related to *R. leguminosarum* have been described recently [31], which made the previous definition of beach pea rhizobia uncertain [1,8,21]. Based on the background mentioned above, the aims of the present study were: (1) to determine the community position of beach pea rhizobia on the seashore line of Shandong Peninsula, China; and (2) to compare the beach pea rhizobial populations in Shandong Peninsula with those from seashores in other countries.

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Materials and methods

Soil-nodule sampling and rhizobia isolation

Five soil samples were collected in the summers of 2013 and 2014 from beach pea plants growing in Longkou (37° 44′ N, 120° 31′ E), Yantai (37° 27′ N, 121° 42′ E), Rongcheng (37° 21′ N, 122° 38′ E), Haiyang (36° 35′ N, 120° 58′ E) and Qingdao (36° 14′ N, 120° 40′ E) (Fig. 1), which are located along the Shandong Peninsula coast line in China. Root nodules of *L. maritimus* were carefully collected from uprooted roots and transferred into sterilized tubes filled with dehydrated silica gel particles for preservation until the isolation of rhizobia. Approximately 50–80 nodules were collected from 15 to 20 plants at each sample site. Before isolation, nodules were immersed in sterile deionized water overnight at 4 °C, then the rehydrated nodules were surface-sterilized in 4% NaClO (w/v) solution using a standard protocol [29,32]. The surface-sterilized nodules were crushed and the liquid extract was streaked onto yeast mannitol plates (YMA) (yeast extract, 3.0 g; mannitol, 10.0 g; KH_2PO_4, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.1 g; distilled water, 1.0 L; pH 7.0). All the inoculated plates were incubated at 28 °C for 3–7 days. The bacterial colonies obtained were repeatedly streaked onto the same medium two to three times in order to purify the isolates. The purified cultures were then maintained in 20% (v/v) glycerol at −80 °C for long-term preservation [29,32].

Soil sampling and characterization

A 1 kg soil sample was taken at each site from the root zone (0–20 cm depth), air-dried, passed through a 2–mm-mesh screen and used to determine the chemical properties. Soil pH was determined in a soil:water (1:2.5 w/v) suspension [29]. The soil organic carbon (OC) content was measured by the wet-oxidation method with $K_2Cr_2O_7$-concentrated H_2SO_4 [29]. The available nitrogen (AN) was determined by means of quantifying the alkali-hydrolysable N content [20]. The available phosphorus (AP) content was determined by means of a colorimetric method [29]. The available potassium (AK) content was measured by means of NH_4OAc-extraction and the flame photometer method at a wavelength of 767 nm [20]. Total nitrogen (TN) content was measured with the standard acid titration method [29]. The fertility level was evaluated for each soil sample according to the National Norma of China (http://www.soil17.com/news_more/1663.html).

Phylogenetic analyses of housekeeping and symbiotic genes

The genomic DNA of each isolate was extracted by using the TIANGEN genomic DNA extraction kit (TIANGEN, China) for bacteria. All the purified isolates were designated in order to amplify and sequence the *recA* gene, using the primers recA41F/recA640R [14]. All the *recA* sequences acquired in this study were aligned by using Clustal W software and the similarity between each sequence pair was calculated by using MEGA 5.05 software. Strains possessing identical *recA* sequences were identified as a single *recA* genotype, and one representative strain from each *recA* genotype was randomly selected for further phylogenetic study. For each representative strain, the 16S rRNA gene, housekeeping genes (*atpD* and *glnII*) and symbiotic genes (*nodC* and *nifH*) were amplified with the primer pairs 27F/1492R [23], atpD255F/atpD782R [26], glnII12F/glnII689R [26], nodC540/nodC1160 [19] and nifHF/nifHR [12], respectively. All the amplicons were sequenced directly with the same primers by the Beijing AuGCT DNA-SYN Biotechnology Co., Ltd using the method of Sanger et al. [18].

All the sequences obtained in this study were deposited in the GenBank database and were blasted in GenBank to search for homologous reference sequences. Phylogenetic trees were reconstructed for each gene using the neighbor-joining method with Kimura's two-parameter model in MEGA software version 5.05 [17,22]. The topology of each phylogenetic tree was evaluated by the bootstrap method with 1,000 replicates.

Multilocus sequence analysis (MLSA) for housekeeping genes has been shown to possess greater discriminatory abilities than that of a single gene [16,25], and can determine the accurate phylogenetic position of rhizobial species [5]. Thus, MLSA was performed with combined sequences of the three housekeeping genes (*recA*, *atpD* and *glnII*), and the sequence similarities with reference strains were calculated as described above. Genospecies were defined based on the MLSA relationship using a 97% sequence similarity threshold, as suggested previously [5,14].

Genomic fingerprinting by BOX-PCR

To evaluate the genomic diversity within the rhizobial populations, BOX-PCR was performed with the primer BOX-AIR (5′-CTACGGCAAGGCGACGCTGACG-3′) and the PCR procedure described by Versalovic et al. [24]. The analysis of PCR patterns was visualized and compared according to the same study [24].

Analysis of symbiotic properties

The nodulation abilities of representative strains were determined under laboratory conditions with previously described procedures [27], including pretreatment of *L. maritimus* seeds with concentrated sulfuric acid, surface sterilization in 4% NaClO (w/v) solution, and germination on 0.6% water–agar plates at 28 °C in the dark for approximately 48 h. One germinated seedling was transferred to a Leonard jar that contained sterilized vermiculite irrigated with nitrogen free nutrient solution, and inoculated with 1 mL of a rhizobial suspension in distilled water (10^8 cells mL^{-1}) prepared from fresh rhizobial culture grown in YM broth at the exponential phase [30]. Three replicates for each strain were used, and a blank control set was inoculated with 1 mL distilled water. All the plants were grown at 26 °C in an automated plant-growth room with a daylight illumination period of 12 h [14,15]. After 60 days growth, all the plants were harvested and the effective (nitrogen fixing) root nodules were identified by the red color of the root nodules and the dark-green leaves of the plants.

Results

Soil properties of different sampling sites

As shown in Table 1, all the soil samples were neutral to slightly alkaline with pH 7.06–8.07. The concentrations of the main mineral nutrients in dry soils were (mg kg^{-1}) 0.03–0.42 for AN, 0.33–3.23 for AP, 17.86–30.36 for AK, 1.41–8.50 for OC and 3.08–6.52 for TN. According to the National Norma of China, most of the AN, AP and AK content levels in the samples were "extremely poor" (the lowest level), except for AP and AK in Longkou that were "very poor".

Diversity and composition of beach pea rhizobial populations

A total of 124 rhizobial strains were isolated from the root nodules of beach pea. The number of isolates from each sampling site is shown in Table 1. *recA* sequence analysis divided the isolates into five *recA* genotypes in three clusters (Table 1, and detailed information available as Supplementary Fig. S1): three *recA* types covering 3, 16 and 103 isolates, respectively, were identical or very similar to that of *Rhizobium anhuiense*, while the another two *recA* types each contained only one isolate that were either distantly or closely related to *R. lusitanum* PL-7^T.