Long-term monoculture reduces the symbiotic rhizobial biodiversity of peanut

Shuai Shao\textsuperscript{a,c,1}, Mingna Chen\textsuperscript{b,1}, Wei Liu\textsuperscript{a,d,e}, Xiaoke Hu\textsuperscript{a,d,e}, En-Tao Wang\textsuperscript{f}, Shanlin Yu\textsuperscript{b,\ast}, Yan Li\textsuperscript{a,d,e,\ast}

\textsuperscript{a} Key Laboratory of Coastal Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai, 264003, China
\textsuperscript{b} Shandong Peanut Research Institute, Qingdao, 266100, China
\textsuperscript{c} Life Science College, Yantai University, Yantai, 264005, China
\textsuperscript{d} Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China
\textsuperscript{e} Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao, 2666071, China
\textsuperscript{f} Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Mexico City D.F, 11340, Mexico

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Long-term monoculture (LTM) decreases the yield and quality of peanut, even resulting in changes in the microbial community. However, the effect of LTM on peanut rhizobial communities has still not been elucidated. In this study, we isolated and characterized peanut rhizobia from 6 sampling plots with different monoculture cropping durations. The community structure and diversity index for each sampling site were analyzed, and the correlations between a peanut rhizohium and soil characteristics were evaluated to clarify the effects on peanut rhizobia communities. The competitive abilities among representative strains were also analyzed. A total of 283 isolates were obtained from 6 sampling plots. Nineteen recA haplotypes were defined and were grouped into 8 genospecies of \textit{Bradyrhizobium}, with \textit{B. liaoningense} and \textit{B. ottawaense} as the dominant groups in each sample. The diversity indexes of the rhizobial community decreased, and the dominant groups of \textit{B. liaoningense} and \textit{B. ottawaense} were enriched significantly with extended culture duration. Available potassium (AK), available phosphorus (AP), available nitrogen (AN), total nitrogen (TN) and organic carbon (OC) gradually increased with increasing monoculture duration. OC, TN, AP and AK were the main soil characteristics affecting the distribution of rhizobial genospecies in the samples. A competitive nodulation test indicated that \textit{B. liaoningense} presented an excellent competitive ability, which was congruent with its high isolation frequency. This study revealed that soil characteristics and the competitive ability of rhizobia shape the symbiotic rhizobial community and provides information on community formation and the biogeographic properties of rhizobia.

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Introduction

Rhizobia are gram-negative bacteria that have the ability to form nodules on legume hosts such as groundnut. In these nodules, the rhizobia reduce atmospheric nitrogen into ammonium to provide nutrients for the host [15]. This kind of symbiotic nitrogen fixation provides 40 million tons of combined nitrogen across the world per year [11]. Therefore, efficient rhizobia play a vital role in sustainable agricultural production, especially for commercial crops such as beans, soybeans and peanuts. Peanut or groundnut (\textit{Arachis hypogaea} L.) is an important oil and grain legume crop that originated from South America and has been cultivated since 350 BC [14]. This plant was introduced to China approximately 500 years ago [49], and now, China provides more than 16 million tons of peanut each year (http://faostat3.fao.org/), accounting for approximately 45% of the total production in the world. The three main producing regions for peanut in China are the Huanghuaihai (or Northern) Plain, the drainage area of the Yangtze River, and the southeast coastal region [3]. It has been reported that peanut mainly forms symbiosis with different slow-growing rhizobia classified as \textit{Bradyrhizobium} [2,8,28,31,38]. In addition to the 14 new candidate \textit{Bradyrhizobo-
bium species classified recently [3], more than 30 Bradyrhizobium species have been verified as microsymbionts of peanut. A few fast-growing rhizobia, such as Neorhizobium huautlense, Neorhizobium galegae, Pararhizobium giardini and Rhizobium tropici, were isolated from peanut nodules in Moroccan and Argentinean fields [7,28,39]. However, only slow-growing rhizobia were found to nodulate with peanut in different regions of China [3,49].

Due to factors ranging from its high profits to the requirement that regional agro-industrialization be intensified, peanut has been cultivated by LTM (long-term monocropping) on a large scale in China [24]. LTM not only resulted in lower productivity and quality for peanut [41] but also resulted in a decrease in soil bacterial diversity, especially for LTM together with chemical fertilization [26]. The abundance and diversity of the soybean rhizobial community was proven to be affected by long-term land use and crop management, and continuous LTM led to a decrease in the rhizobial diversity index [48]. However, information about the effects of continuous LTM on the abundance and diversity of peanut rhizobia is limited.

In this study, we collected peanut nodules and soil samples that were disposed of during continuous LTM of peanuts at the Laixi experimental field of the Shandong Peanut Research Institute (120°44′28″E, 36°86′36″N). The longest continuous monoculture plot was continuously cultivated from 2006 to 2017. Rhizobia were isolated from root nodules to evaluate their genetic diversity, and the factors shaping the community were also analyzed.

Materials and methods

Root nodule and soil samples

In the present study, the sampling site (Laixi County) was the breeding and cultivation research base of Shandong Peanut Research Institute, and the cultivar (A. hypogaea L. Huayu 29), planting system, land use pattern and fertilizer inputs were the same for all the plots, e.g., peanut monoculture with chemical fertilizer (approximately 300 kg ha⁻¹ of urea, 750 kg ha⁻¹ of calcium magnesium phosphate, and 225 kg ha⁻¹ of potassium chloride were applied as fertilizer) at the same dose annually. Root nodules and soil samples were collected synchronously on September 2015, 2016 and 2017 from plots with monoculture histories of 1, 2, 3 and 10, 11 and 12 years, respectively. In brief, for each plot, at least five separate peanut plants were uprooted, and the root nodules were carefully collected and transferred into sealed tubes filled with dehydrated silica gel particles for preservation at room temperature until isolation. Soil samples were collected from the areas around five plants from each plot at depths of 0–20 cm. The sampled soils from the same plot were mixed in a sterile bag and preserved in an ice-cold box for transportation to the laboratory where they were air-dried for physicochemical characterization.

Rhizobial isolation and soil characterization

For rhizobial isolation, the dehydrated nodules from each plot were selected randomly, immersed in sterile saline solution (0.85%) and rehydrated at 4 °C for 6 h. Then, they were surface sterilized and crushed, and the juice was streaked on yeast mannitol agar (YMA) plates for incubation at 28 °C for 7–30 days [50]. Then, the single colonies were streaked on the same medium several times until pure cultures (identical colonies) were obtained. Finally, the pure cultures were maintained in YM broth supplied with 20% (w/v) glycerol at −80 °C.

The air-dried soil samples were sieved by a 2-mm mesh screen and used to determine the physicochemical properties as described previously [20,45]. In brief, the pH value was determined with a soil-water (1:5, w/v) suspension using a pH meter [5]. The organic carbon (OC) content was assayed using a wet-oxidation method [43]. The total nitrogen (TN) was measured using the titration method [32]. The available nitrogen (AN) was determined by quantifying the alkali-hydrolysable nitrogen [36]. The available phosphorus (AP) was detected by the colorimetry method using a spectrophotometer [43]. The available potassium (AK) was measured using a flame photometer at a wavelength of 767 nm [36].

PCR amplification of housekeeping, IGS, and symbiotic genes

The genomic DNA of each isolate was extracted using the TIANGEN genomic DNA extraction kit (TIANGEN, China) for bacteria, which was used as template to amplify the recA sequence from all the isolates using the primers recA41F and recA640R and the corresponding protocol [40]. The amplicons were sequenced directly by Beijing AuGCT DNA-SYN Biotechnology Co., Ltd. using Sanger methods [35]. All the recA sequences obtained in this study were aligned using Clustal W software [48], and then the haplotype was determined using DNASP v5 [25]. Since recA sequence analysis has been used as an effective marker to select representative strains of both slow- and fast-growing rhizobia [20,21,48], a representative sequence and the corresponding isolate for each recA haplotype were randomly selected for further study.

Housekeeping genes possess both conserved and variable regions that could provide more distinctive phylogenetic information, and combined analysis of housekeeping genes could provide more accurate phylogeny than 16S rRNA gene sequences [33]. For the representative strains, the housekeeping genes atpD, glhII, dnaK, gyrB and rpoB were amplified using the primers atpD255F/atpD782R [40], glhII2F/glhII689R [50], gryB343F/gryB1043R [50], TsDNAk3/TsDNAk2 [37] and rpoB454F/rpoB1364R [39] and the corresponding protocols, respectively. The IGS sequence was amplified using primers IGS-FGSP1490/IGS-FGSP132 [50], nodC was amplified using primers nodC540F/nodC1160R [50], and the nifH sequence was amplified using primers nifH/C/R [50]. All the PCR products were sequenced directly as described for recA sequencing. The obtained sequences were deposited in the GenBank database and were blasted in the GenBank database to search for homologous reference sequences. For each gene, a phylogenetic tree was reconstructed using MEGA software version 7.0 [16] based on neighbor-joining methods (Kimura 2-parameter model), and the topology of all the phylogenetic trees was evaluated using the bootstrap method with 1000 replications.

Multilocus sequence analysis (MLSA) was conducted with the concatenated housekeeping gene (recA, atpD, glhII, dnaK, gyrB and rpoB) and IGS sequences, and the sequence similarities between the obtained sequences in this study and related reference sequences were calculated by MEGA 7.0. The thresholds of 97.3% sequence similarity in MLSA [27] and 95.5% in the IGS phylogeny [44,50] were used to classify the representative strains into genospesies.

Diversity evaluation and correlation analyses

Peanut rhizobial diversity, species richness and evenness in different plots were evaluated by using the three commonly used alpha ecological indexes: the Shannon–Wiener index indicates species richness for a plot, the Simpson index explains the species dominance, and the Pielou index shows species evenness in a community [12]. The biodiversity indexes were calculated in the Vegan Package (version 2.5-6) by using R statistical language (version 3.6.1; http://www.r-project.org/). The correlation between peanut rhizobia and soil characteristics was calculated by using CANOCO software 4.5 [17]. The length of the gradient (first axis) was 1.36 in
Fig. 1. Phylogenetic tree of MLSA based on concatenated sequences of recA (381 nt), atpD (429 nt), gyrB (579 nt), rpoB (714 nt) and dnaK (448 nt). Taxa and GenBank accession numbers in bold font were determined in this study. The tree was reconstructed by the neighbor-joining methods, and bootstrap values greater than 50% are provided at the nodes. The scale bar represents 1% nucleotide substitution.

DCA; thus, RDA was selected to analyze the relationship between soil characteristics and rhizobial species.

Nodulation test

All representative strains were employed in the nodulation test. In brief, the peanut seeds were surface sterilized and germinated on a 0.6% agar-water plate in the dark at 28 °C for 2–4 days. One seedling was transferred to a Leonard jar filled with fertilized vermiculite mixed with low-nitrogen nutrient solution. An aliquot of 1 mL (10^8 cells) of rhizobial suspension in sterilized water was inoculated into each Leonard jar, and the plants were cultured in an automatic greenhouse with day/night cycles of 12 h at 25 °C for 45 days. The plants with green leaves and pink round nodules were regarded as effectively nodulated. For the competitive nodulation test, an equal bacterial density (10^8 cells mL^-1 in sterilized water) and volume from each bacterial suspension of all the representative strains were mixed together, and then 1 mL of mixed suspension was inoculated onto a seedling. Twenty plants were inoculated for each of the biological triplicate experiments. The plants inoculated with 1 mL sterilized water were used as blank controls. The nodules were harvested 45 days post inoculation. At least fifty nodules randomly collected from 10 plants (5–6 nodules per plant) were used for isolating rhizobia as mentioned above for each triplicate,
one strain was obtained from each plate, and the reisolated bacteria were identified through recA gene sequence analysis.

**Results**

**Physicochemical properties of the soil samples**

With the increase in monoculture duration, the contents of available nitrogen (AN), available phosphorus (AP), available potassium (AK), total nitrogen (TN) and organic carbon (OC) increased gradually (Table 1) and reached their highest levels after continuous cultivation for 12 years, with increases of 76.87%, 114.53%, 29.08%, 25.00% and 36.36%, respectively. However, the pH values of the soil samples were relatively stable, ranging from 6.99 to 7.07.

**Isolation and classification of peanut rhizobia**

A total of 280 isolates were obtained in this study. Among them, 40, 49, 39, 38, 50, and 64 were isolated from continuous monoculture plots with 1, 2, 3, 10, 11, and 12 years, depicted as Y1, Y2, Y3, Y10, Y11 and Y12, respectively, in the subsequent text (Table 1 and Table S1). According to recA sequence analysis, all the isolates were classified into 19 recA haplotypes, and thus, 19 strains representing them were randomly selected (Table S1).

The topologies of phylogenetic trees constructed for each of the housekeeping genes recA, atpD, dnaK, gyrB and rpoB (Figs. S1–S5) were nearly the same as those of the IGS (Fig. S6) and MLSA (Fig. 1) phylogenetic trees. According to the MLSA results, the 19 representative isolates were identified as 8 genospecies, corresponding to: (I) *B. liaoningense*, covering 193 isolates (represented by 60,890, 62,318, 65,078 and 65,090), that shared similarities 98.3–99.6% among them and 97.6–98.7% with *B. liaoningense* LMG 18230T; (II) *B. arachidis*, covering 25 isolates represented by 60,894, 62,303, 62,250, 62,311 and 62,325 that represented similarities 99.1–99.9% with each other and 99.2–99.8% with *B. arachidis* CCBAU 051107T; (III) *B. ottawaense*, containing strains 60896, 60,904 and 65,059 that shared similarities 99.4–99.7% among them and 99.2–99.5% with *B. ottawaense* O009T; (IV) *B. guangdongense*, composed of isolates 61,102 and 61,103 that presented similarities 98.8% between them and 98.8%/100% with *B. guangdongense* CCBAU 51649T; (V) *B. yuanningsense*, covering two isolates represented by 65,010 that shared 100% similarity with *B. yuanningsense* CCBAU 10071T; (VI) *Bradyrhizobium* sp. I, covering five isolates represented by 60,954 and 65,054 that shared 99.2% similarity between them, and their most related reference strain was *B. ottawaense* O009T with 95.7% similarity; (VII) *B. huanghuaiaihense*, containing 65,027 and another isolate, which shared 98.5% similarity with *B. huanghuaiaihense* CCBAU 23303T; (VIII) *B. stylosanthis*, containing 61077, which shared 98.2% similarity with *B. stylosanthis* BR446T. The IGS phylogenetic relationships were the same as those in the MLSA phylogenetic tree, and the genospecies classification results at the threshold of 95.5% similarity were the same as those of the MLSA.

**Diversity and composition of rhizobial populations in different monoculture plots**

In general, *B. liaoningense* was the most abundant genospecies in all plots, accounting for 68.9% (193/280) of all the isolates. The second most abundant group was the *B. ottawaense* genospecies, which was also found in all plots and accounted for 17.5% (49/280) of all the isolates. *B. arachidis* was only found in the plots with 1, 2 and 3 years of continuous monoculture history (Fig. 2a, Table 1 and Table S1). The Shannon–Wiener diversity index for plot Y3 was the highest (1.23), and 6 genospecies were isolated. The following species were isolated from Y1 (1.09) and Y2 (0.96), while Y12 showed the lowest diversity, and only two genospecies were isolated from it. The Simpson index results were nearly the same as the Shannon–Wiener index results. The highest Pielou index (0.79) was found in Y1, followed by Y12 (Table 1). These results indicated that the diversity and genospecies composition of the peanut rhizobial community varied with monoculture duration and that the diversity indexes decreased with increasing monoculture time.

The RDA results indicated that OC, TN, AP, AK and AN were the main factors affecting the distribution of peanut rhizobia in this study because their length was long and the OC was the longest (Fig. 3). According to the length and angles among the factors and rhizobial genospecies, *B. liaoningense*, *B. stylosanthis* and *B. ottawaense* were positively correlated with OC, TN, AP, AK and AN; however, *B. arachidis* and *Bradyrhizobium* sp. I showed opposite results with the above genospecies. *B. yuanningsense* and *B. huanghuaiaihense* showed positive correlations, but *B. arachidis* showed negative correlations with pH.

**Phylogenies of nodC and nifH sequences**

Unlike the phylogenetic tree of the housekeeping genes, the nodC phylogenetic tree indicated that all the sequences obtained from the representative strains were grouped into two clusters. Cluster I included 12 representative strains grouped with *B. arachidis* CCBAU 051107T at 96.8–100% similarity. Cluster II included 3 strains grouped with *B. yuanningsense* NBRC 100594T at 93.7–100% similarity (Fig. S7). For nifH sequences, three clusters were identified: cluster I incorporated 13 strains and *B. arachidis* CCBAU 051107T that shared 99.3–100% similarities; cluster 2 included 3 isolates and *B. yuanningsense* CCBAU 10071T that shared 94.6–100% similarities; cluster 3 was composed of 4 isolates and *B.
Table 1
Distribution of different rhizobial genospecies and diversity indexes at 6 sampling sites. Y1, Y1, Y2, Y3, Y10, Y11, and Y12 correspond to sampling sites under monoculture for one, two, three, ten, eleven and twelve years, respectively.

|                | Y1    | Y2    | Y3    | Y10   | Y11   | Y12   | Total |
|----------------|-------|-------|-------|-------|-------|-------|-------|
| Monoculture time | 2015  | 2015–2016 | 2015–2017 | 2007–2016 | 2006–2016 | 2006–2017 |       |
| Sampling time   | 2015.09 | 2016.09 | 2017.09 | 2016.09 | 2016.09 | 2017.09 |       |
| B. liaoningense | 22    | 30     | 23     | 28     | 40     | 50     | 193   |
| B. arachidis    | 11    | 13     | 1      | 8      | 9      | 14     | 25    |
| B. ottaenaense  | 5     | 5      | 8      | 8      | 9      | 14     | 49    |
| B. guangdongense| 1     | 1      | 2      | 2      | 2      | 2      | 3     |
| B. yuanmingense | 2     | 3      | 3      | 1      | 1      | 1      | 6     |
| Bradyrhizobium sp. I | 2 | 3 | 3 | 1 | 1 | 1 | 6 |
| B. stylosanthis | 2     | 3      | 3      | 1      | 1      | 1      | 6     |
| In total       | 40    | 49     | 39     | 38     | 50     | 64     | 280   |
| Shannon–Wiener | 1.09  | 0.96   | 1.23   | 0.71   | 0.57   | 0.53   |       |
| Simpson        | 0.60  | 0.54   | 0.60   | 0.60   | 0.41   | 0.33   | 0.34  |
| Inverse Simpson | 2.52 | 2.19   | 2.49   | 1.69   | 1.49   | 1.52   |       |
| Pielou index   | 0.79  | 0.70   | 0.69   | 0.64   | 0.51   | 0.76   |       |

B. guangdongense and B. yuanmingense demonstrated that these species are common peanut rhizobia in China and in other countries [3,6,14,23,28,29,38,42]. However, B. ottaenaense, B. stylosanthis and a candidate novel genospecies Bradyrhizobium sp. I identified in the present study were newly recorded as peanut rhizobia and their biogeographic distribution was first reported here. These results demonstrated that (1) the main peanut rhizobia are restricted to the Bradyrhizobium species, which belong to the B. japonicum supergroup or superclade, which was classified recently [1,31]; (2) the peanut nodulating bradyrhizobia have been diversifying in different regions under the selection of soil conditions; and (3) the community structure of peanut bradyrhizobia is also shaped by the monoculture history, competitive ability, and soil properties.

Symbiotic genes and nodulation test

In the present study, the failure to amplify both nodC and nifH from B. huanghuaihaiense 65,027 and 65,047 is consistent with their failure in nodulation; thus, they might be nonsymbiotic endophytes for peanut, similar to the other nonsymbiotic bacteria isolated from Lathyrus maritimus, Caragana jubata and Oxytropis ochrocephala [21,46]. However, the reason for the failure of amplification of nodC from B. guangdongense 61,102, B. yuanmingense 65,010, Bradyrhizobium sp. 160,954 and 65,054 and the failure of nifH amplification in Bradyrhizobium sp. 160,954 (Table S3) is unclear since they formed effective nodules on peanut. Some of these strains harboring nifH but not nodC may indicate that they nodulate with peanut using a nod-independent mode, as previously reported [10]. Therefore, in contrast to B. huanghuaihaiense, the other 7 genospecies are effective microsymbionts for peanut. The division of two nodC clusters (25 haplotypes) and three nifH clusters (5 haplotypes) demonstrated that the symbiotic genes in peanut rhizobia have been diversified or have distinct origins, as suggested previously [3,22]. Furthermore, the strains of different genospecies within the same nodC cluster (I. B. arachidis covering 5 recA haplotypes, B. liaoningense representing 3 haplotypes, B. ottaenaense with 2 haplotypes, B. stylosanthis and B. guangdongense; II. B. yuanmingense, B. liaoningense, B. ottaenaense) and in the same nifH clusters (Fig. S7 and S8) might be evidence for the horizontal transfer of the symbiotic genes among the Bradyrhizobium species, similar to the estimations in previous studies [3].

Effects of long-term continuous monoculture on the peanut rhizobia community

Previously, it has been proven that the microbial community, including bacteria and fungi, was correlated with long-term mono-

Fig. 3. Correspondence analyses between the 8 genospecies and the soil characteristics evaluated by CANOCO. AN, available N; AP, available P; AK, available K; OC, organic carbon; TN, total N.

guangdongense CCBAU 51649T that shared 99.3%–100% similarities (Fig. S8).

Nodulation test and competitive nodulation

Most of the representative strains formed effective nodules on peanut hosts, except B. huanghuaihaiense 65,027, which did not form any nodules on peanut. Thus, a total of 7 genospecies were identified in this study as peanut symbionts. In competition tests, only three haplotypes of B. liaoningense were reisolated from the nodules. B. liaoningense 60,890 showed excellent competitive abilities, with approximately 81.1% nodule occupation, while B. liaoningense 62,318 and 65,078 accounted for approximately 13.9% and 4.9% of the nodule occupation, respectively (Fig. 2b).

Discussion

Unique community structure of peanut rhizobia in the sampling plots

In this study, all 280 isolates of peanut rhizobia were classified through the MLSA and phylogeny of IGS sequences (Fig. 2 and Fig. S6) as Bradyrhizobium spp., which is consistent with the results of previous studies on peanut rhizobia isolated from China and Argentina [3,29,31]. The identification of most of the isolates into genospecies corresponding to B. liaoningense, B. arachidis,
culture and fertilization types [4,26,51]. Except in Y3, the rhizobial diversity decreased with increasing monoculture duration, which is explained by the two species B. yuanmingense and B. huanghuaihaiense representing only two isolates in Y3. Soil characteristics such as AN, carbon content and others are also thought to play a role in determining rhizobial communities [9]. Rhizobia could provide ammonium to the host plant through nitrogen fixation; however, a high nitrogen concentration in the soil could result in nitrogen-dependent inhibition and decrease nodule number and size and even nitrogen-fixation activity [30,34]. Furthermore, recent studies have indicated that the long-term application of nitrogen-only chemical fertilizer and even nitrogen containing compound chemical fertilizer can negatively affect rhizobial biodiversity [47]. The decrease in peanut rhizobial diversity is consistent with the observation that long-term monoculture resulted in a decrease in soybean rhizobial diversity and also consistent with the long-term application of chemical fertilizer decreasing the biodiversity of soybean rhizobia [47,48]. In the present study, the AN, AP, AK and OC tended to increase gradually with the extension of culture duration (Table S3), and OC was the main determinant because its length was greatest in CANOCO analysis (Fig. 3). The accumulation of AP, AK, OC, AN and TN in long-term monocultured plots was also positively correlated with the dominance of B. liaoningense and B. ottawaense (Fig. 3). Although the community varied in different plots, B. liaoningense was dominant in all sampling plots, which was consistent with its excellent competition abilities (Fig. 2b). Thus, soil characteristics and rhizobial competitive abilities shaped the peanut rhizobial communities in the continuously monocultured plots. B. arachidis disappeared as the monoculture cultivation extended, and the minor isolation frequencies of B. guangdongense, B. yuanmingense, Bradyrhizobium sp. I and B. stylosanthes may be due to their low abundance in the soil and low competitive abilities.

Peanut secretes phytotoxic substances such as phenolic acids to the rhizosphere. Long-term monoculture might result in the accumulation of such phytotoxic substances in soils [13], which could increase pathogenic microbes, decrease beneficial microbes, and even decrease microbial diversity in long-term peanut monoculture soils [19,26]. The change in the peanut rhizobial community is correlated with the decrease in bacterial diversity [26], even the fungal community change in long-term monoculture [18].

Conclusion

Our study clearly demonstrates that long-term monoculture of peanut can decrease symbiotic rhizobial diversity. The soil characteristics and competitive abilities of rhizobia are the major factors shaping the rhizobial community. These findings provide novel information about the evolution of peanut rhizobial populations with regard to adaptations in response to changes in soil properties, land use, crop management and even the formation of biogeographic phenomena across rhizobial communities. It will also be interesting to uncover the relationship between the peanut rhizobial community and the overall microbial community through metagenomics in future studies.

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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.syamap.2020.26101.

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