Molecular Deversity and Characterization of Nitrite Reductase Genes from Continuous and Rotational Cropping Soybean

XIAO Cui-hong[1], SUN Dong-mei[1], TANG Hui[2], LIU Zhen-hua[1]

(1. College of Life Science, Heilongjiang August First Land Reclamation University, Daqing 163319, Heilongjiang; 2. College of Life Science, Hebei University, Baoding 071002, Hebei, China)

Abstract: Gene sequence analysis of nitrite reductases was performed on continuous cropping and rotational cropping soils of soybean to assess their incidence in different bacterial taxa and their taxonomical value. *NirS* gene fragments could be amplified from both soils. A total of 268 *nirS* clones were detected by restriction fragment length polymorphism (RFLP) analysis and obtained 29 and 34 distinct *nirS* clones in continuous cropping and rotational cropping soils respectively. A dominant *nirS* pattern, a single dominant *nirS* restriction pattern of rotational cropping, was observed in both samples. Beside above pattern, another two dominant patterns were also detected in continuous cropping soil. Many of the sequences belonged to dominant pattern were not closely related to previously observed genes and some phylogenetically related sequences were obtained from similar samples. The results indicated that the continuous cropping and rotational cropping soils contained novel *nirS* sequences, functional diversity of *nirS* genes changed in the two type soils.

Key words: Continuous cropping; Soybean; Rotational cropping; Nitrite reductase (*nirS*)

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连作与轮作大豆土壤反硝化细菌多样性与组成结构

肖翠红[1], 孙冬梅[1], 汤 晖[2], 刘振华[1]

(1. 黑龙江八一农垦大学 生命科学技术学院, 黑龙江 大庆 163319; 2. 河北大学 生命科学技术学院, 河北 保定 071002)

摘 要: 应用PCR-RFLP方法对连作与轮作大豆土壤中反硝化微生物种群和分类进行研究。结果2种土壤样品中均可检测到反硝化微生物存在。从2种土壤中获取268个 *nirS* 基因克隆, 46种基因型, 其中连作土壤中有29种基因型, 轮作土壤中有34种基因型, 分析结果显示2种土壤样品中均有优势菌群存在, 并且连作土壤样品中反硝化细菌的优势菌群略多于轮作土壤样品。序列同源性分析显示 *nirS* 基因克隆序列与已知反硝化微生物具有较远的亲缘关系。结果提示: 与轮作大豆相比, 连作大豆土壤中反硝化微生物的遗传多样性和组成有所改变, 类群的结构组成易受耕作方式影响。

关键词: 大豆; 连作; 轮作; 亚硝酸盐还原酶

Introduction

Heilongjiang of China is a major agriculture province, which crop acreage is 10.988 million hectares. The planting area of soybean has been enlarged and continuous cropping has become a serious problem[1]. The study showed that continuous cropping can increase secondary metabolites, which are secreted or released from soybean roots. Secondary metabolites, including the majority of autotoxic chemicals (vanillic acid, vanillin, and phydroxybenzoic acid), can induce changes in the composition of soil microbial community[2].

Denitrification, a part of the global nitrogen cycle, is primarily a bacterial respiratory process, in which oxidized nitrogen is used as an alternative electron acceptor for energy production. Advantage of denitrification, on the one hand, can monitor global nitrogen balance. On the other hand, nitrate-and nitrogen oxide-polluted water can be cleared by denitrification. Although favourable in nutrient removal from wastewater and bioremediation, denitrification is also responsible for nutrient loss in agriculture and a contribution to the greenhouse effect and the damage to the ozone layer[3]. Denitrification consists of four enzymatic reaction

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Biography: XIAO Cui-hong (1972-), female, lecture, teaching and research in physiology and ecology.

Corresponding author: SUN Dong-mei (1970-), female, professor, teaching and research in microbiology. E-mail: lzwsdm@yahoo.com.cn.

steps, catalysed by four metalloproteins: nitrate reductase, nitrite reductase, nitric-oxide reductase and nitrous oxide reductase. Dissimilatory nitrite reduction is catalysed by two types of nitrite reductases: the copper-containing *nirK* and the cytochrome *cd nirS* gene products[4].

To investigate how continuous cropping can affect denitrifying communities, the diversity of *nirK* and *nirS* genes was determined for continuous cropping and rotational cropping. Our results indicate that *nirS* clones represent novel sequences and tillage manners appear to have affected denitrifying communities.

1 Materials and methods

1.1 Field site and soil sampling

The soybean field located in Lindian, Daqing, Heilongjiang Province, P. R. China, which was compartmentalized into two plots and separated by 5-miter-wide block. Each plot was divided into three smaller blocks (three replicates). The soil type was black loam soil. The pH of the soils and the initial levels of nitrate, ammonium nitrogen, available organic matter, phosphorus, potassium, calcium, and magnesium were similar originally. The only difference between these two plots was farming model. One was the rotational cropping (soybean-corn-soybean, RC), and the samples was taken during the soybean crop cultivation. Another was the continuous cropping (CC) over 5 years. Three replicates were established in each plot.

Rhizosphere soil sample (depth 5-15 cm) in the RC or CC soybean field was collected in September 2010 (harvest stage). For each plot, fresh soil sample was collected from 9 points (3 points per replicate). At each point, five plant roots were chosen and the loosely attached soil removed. The adhering soil of the five plants was pooled and considered as the rhizosphere soil. The samples of each replicate were mixed and sieved (< 2 mm). Aboveground plant materials and stones were removed, roots and soil samples were then conserved for further analysis. Conventional standard methods[5] in soil samples analysis were used to measure pH, total carbon, N-NO_3^-, and N-NH_4^+ concentrations. Total inorganic nitrogen (TIN) was calculated from the sum of the amounts of nitrate and ammonia. The physicochemical characteristics of the soil samples at the end were summarized in Table 1.

1.2 Extraction and purification of DNA from soil

Total soil DNA was directly extracted from the soil samples with an Soil Master DNA Extraction Kit (EPICENTRE, Madison, Wisconsin), then purified with Soil DNA Purification Kit (GENMED SCIENTIFICS INC. U. S. A). DNA quality and integrity was checked by electrophoresis on 0.8% agarose ethidium bromide gel.

1.3 RFLP-PCR

The oligonucleotide primers of *nirS* and *nirK* gene fragments were described by Braker[4], The PCR products were electrophoresed on 2.0% agarose to ascertain their size and quality, then were cloned in pMD18-TR linear plasmid vector (TaKaRa, China). Clones were selected for positive *nirS* fragments by PCR using the primers M13/pUC Sequencing Primer (−20) and M13/pUC Reverse Primer (−26). Two microliters of each PCR product were directly used for restriction enzyme cleavage using *Taq* Ⅰ and *Msp* Ⅰ (TaKaRa, China). Digested DNA samples were analyzed by electrophoresis in a 5% polyacrylamide gel (19∶1) (Bio-Rad). This procedure was repeated at least two times for each sample to verify the consistency of the patterns. DNA fragment sizes were estimated by comparison to molecular weight standards (500 bp DNA ladder, Promega). Clones were grouped into phylotypes according to banding patterns. Selected different phylotype clones (i. e., phylotypes containing more than one clone) were completely sequenced by SHANGHAI SANGON.

1.4 Data analysis

Soil chemical and physical properties were subjected to analysis of variance (ANOVA) using the general linear model procedure in SAS. Unique phylotypes were defined as OTUs with <95% *nirS* sequence similarity as determined by GCG BestFit software, and the data from unique phylotypes were analyzed with EstimateS version 8.0[6]. The diversity between RC and CC was evaluated by calculating the Shannon Diversity index[7]. The percent coverage (C) of the clone libraries was used as a measurement of captured diversity, and C was calculated according to the equation $C = [1 - (n_i/N)] \times 100$[8], where n_i is the number of unique clones as determined by RFLP analysis and N is the total number of clones in the library. To estimate species richness, the nonparametric Chao 1 estimate was calculated with log-

linear transformed confidence intervals at 95%[9].

The *nirS* sequenced sequences, as determined by the highest sequence similarity during BLAST analysis, were subsequently aligned using ClustalX version 1.81[10] with cultured and environmental denitrification bacteria. Phylogenetic trees were calculated by the Kimura two-parameter model and the neighbor-joining algorithm using the MEGA software[11]. A total of 1,000 bootstraps were performed to assign confidence levels.

The *nirS* gene sequences reported in this study were submitted to GenBank/NCBI database. Sequence accession numbers were deposited in the GenBank database under accession numbers GQ397298 to GQ397360.

2 Results

2.1 Physicochemical characteristics of the CC and RC soil samples

The physicochemical characteristics were different between CC and RC. The soil pH generally ranged from 7.69 under CC to 7.94 under RC (Table 1). There was higher soil total carbon in CC (26.95 $g \cdot kg^{-1}$) compared to RC (25.84 $g \cdot kg^{-1}$). But the ammonium and nitrate concentration were higher in RC than in CC, with significant different ($p \leqslant 0.05$) ammonium concentration between two soil samples (18.97 $mg \cdot kg^{-1}$ and 15.15 $mg \cdot kg^{-1}$). Compared two samples, CC had lower concentration ammonium (from 18.97 mg kg^{-1} to 15.15 mg kg^{-1}), nitrate (from 7.83 $mg \cdot kg^{-1}$ to 6.17 $mg \cdot kg^{-1}$), available N (from 23.56 $g \cdot kg^{-1}$ to 21.49 $g \cdot kg^{-1}$), and pH, and higher total carbon. And RC had approximately 1.2 fold more ammonium than CC.

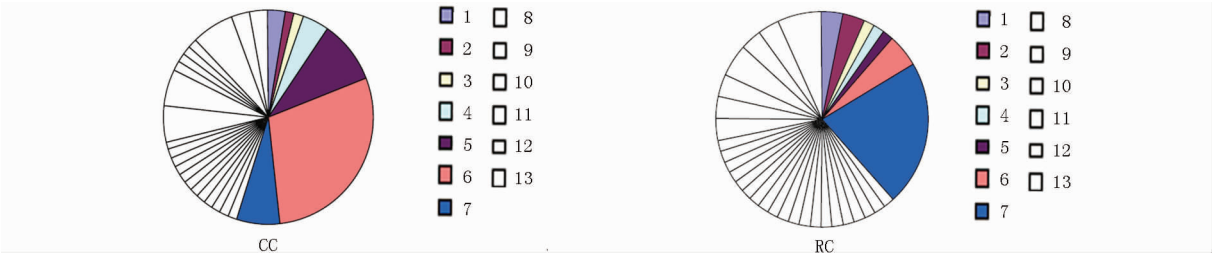
Table 1 Soil physicochemical characteristics of RC and CC collected on August 2010

Sample	pH	NH_4^+ /$mg \cdot kg^{-1}$	NO_3^- /$mg \cdot kg^{-1}$	Available N /$mg \cdot kg^{-1}$	Total carbon /$g \cdot kg^{-1}$
Rotational cropping	7.94	18.97[a]	7.83[a]	23.56[a]	25.84[a]
Continuous cropping	7.69	15.15[b]	6.17[b]	21.494[b]	26.95[b]

Values followed by the same letter within a column do not differ significantly at the 95% confidence level.

2.2 RFLP analysis of *nirS* gene fragments

In all samples, amplification of the *nirS* gene gave PCR products of the expected sizes and a total of 268 *nirS* clones were detected. Percent coverage for all two *nirS* gene clone libraries was 93.6%, with RC's 94.8% and CC's 93.7% (Table 2). Twenty-nine and 34 unique restriction patterns were screened from two soil samples. Seven patterns shared in two soil samples. Among clone libraries, there were two dominant recovered clones CC14 and RC6. Clone CC14 accounted for 18% of the total *nirS* library and comprised 28% and 5% respectively of the CC and RC. And clone RC6, accounted for 14% of the total two clone libraries, and 22%, 6% respectively in RC and CC (Fig. 1). The CC sample had approximately 77% unique RFLP patterns that were not observed in RC, and 68% unique RFLP patterns of RC were not in CC. Most *nirS* clones were unique to the respective sample and had little overlap, except for the predominant patterns. A total of 63 operational taxonomic units (OTUs, i.e. unique patterns) were unique to two soil samples. In comparison to CC, RC displayed one predominant pattern and had high diversity, even though CC had three dominant pattern (Table 2).



Each color is a unique sequence, the same color shared between two sites is the same sequence. Pie segments depicted as white represent unique sequences observed only at the respective site.

Fig. 1 The clone distribution and overlap among two sites for the *nirS* gene sequences.

Table 2 Statistical analysis of *nirS* gene clone libraries in RC and CC

Sample	Number of clones	Chao 1 mean	Shannon mean	Coverage/%
Rotational cropping	146	62.25	3.46	94.8
Continuous cropping	122	87.03	3.82	93.7

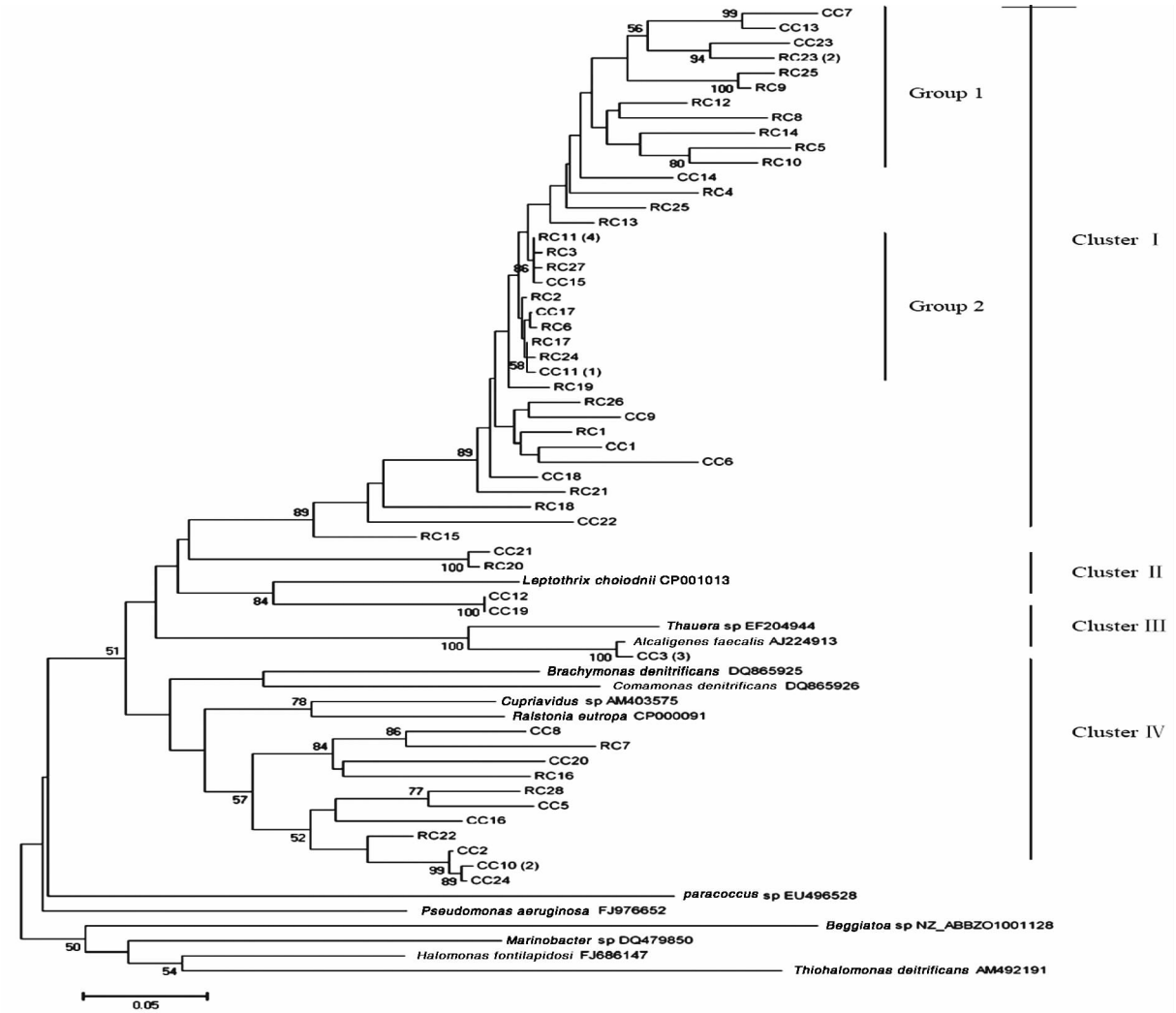
1. Sources of data were from the following libraries: Rotational cropping (RC); Continuous cropping (CC).

2. Chao 1 estimates were followed by log-linear transformed confidence intervals at 95%.

2.3 Sequence analysis of *nirS* clones

Partial sequenced sequences were determined from all clones having distinctive restriction patterns. Nucleotide identity was determined by highest sequence similarity during BLAST analysis. Many of the *nirS* sequences were more closely related to one another than to previously known genes, only one clone, CC3, had higher than 93% nucleotide identity with a known *nirS* gene, *Alcaligenes faecalis*. All *nirS* OTUs (n = 63) were compared with previously known genes, the identity values ranged from 70% to 93%. Almost 5% of the OTUs had ⩾ 90% sequence identity to previously known genes. Twenty-three percent of the OTUs had 80% to 90% identity with known sequences, the remaining OTUs were 70% to 80% identical to cultured or previously reported sequences.

The *nirS* dendrogram could be divided into four primary clusters (Ⅰ to Ⅳ) and cluster Ⅰ included two novel group (group1 and group 2), and this cluster contained majority of the OTUs of the RC and CC, which



The tree is based on a neighbor-joining method and the numbers in parentheses denote the numbers of additional*nirS* clones identified in this study that share ⩾95% nucleotide similarity with the corresponding representative sequence. Bootstrap values (1000 replicates) were given for nodes with ⩾50% support.

Fig. 2 Phylogenetic relationship of all unique cloned *nirS* sequences from CC and RC and reference sequences from the GenBank database

accounted for 60% of total OTUs. And in this cluster, there were no sequences had closed relationship to known sequences (Fig. 2). At same time, this cluster was also dominated by recovered OTUs from RC sample. Group 1 contained four predominant clones: CC1, CC18, CC14 and RC6, which counted for 47% of total clones. Group 1 and group 2 had above 95% identity with each other sequences. The clone cluster Ⅱ, which contained two sequences, had 94% sequence identity with the *Leptothrix cholodnii nirS* gene. Cluster Ⅲ only included one sequence CC3, which had 98% similarity with the *Alcaligenes faecalis* sequence, had 84% nucleotide identity with the *Thauera sp* gene. Cluster Ⅳ contained eleven recovered sequences with 70 to 80% identity to known references. The cluster Ⅱ and Ⅲ were not inclusively observed from the RC sample and cluster Ⅳ had at least 64% sequences belonged to CC.

3 Discussion

In this study RFLP-PCR approach was used to identify the diversity of thenitrite reductase bacteria in five years continuous soybean cropping. The study focused on the analysis of *nirS* genes since no *nirK* products were obtained from the investigated soils. Other authors reported that there were difficulties in amplifying of the nitrite reductase gene (*nirS* and *nirK*) from certain ecosystems. For example, Olaya was not detected *nirK* genes from constructed wetland sample in Girona, Spain[12], but *nirK* gene was detected in marine sediments from Puget Sound, Washington, USA, and from the River Colne estuary, in the UK[13-14], and from cultivated denitrifiers[15]. However, Yan et al. observed *nirS* and *nirK* genes from five contaminated groundwater sites. Neither forested upland soil, nor rhizosphere of cultivated grain and legumes soils *nirS* gene were amplified[16]. This apparent preference of both types of nitrite reductase bacteria for certain environments might be due to this kind of denitrifying microorganism better adapted to particular environmental conditions. Whereas, the absence of *nirK* organisms in our researched samples could not be completely ruled out from the analyses reported here, and other studies should be employed in those soils.

The *nirS* gene library of CC had different RFLP patterns compared with RC, a higher occurrence of CC14, and PCA did not group CC14 with the other sequences. For the RC clone library, RC6 was not grouped with the other clones. The comparisons of the unique *nirS* sequences indicated that CC14 and RC6 were phylogenetically distinct. In general, less *nirS* diversity was observed in CC than RC samples and different sequences appeared predominant in each site. The *nirS* phylogenetic analysis clustered all clones into four main clusters. Cluster Ⅰ contained most of the clones (60%) including four predominant clones: CC1, CC18, CC14, RC6 and those novel clone sequences in this cluster were predominantly from RC sample (71% of total cluster Ⅰ). All of cluster Ⅰ clones less closely related to known sequences, which suggested that the diversity of *nirS* would increase as new environments and conditions. Our study also showed that cluster Ⅱ, Ⅲ, Ⅳ comprised almost 40% of the entire *nirS* clonal library. The clone of the cluster Ⅱ and cluster Ⅲ had 94% and 98% sequence identity with the *Leptothrix cholodnii* and *Alcaligenes faecalis nirS* gene, respectively. Clones belonged to above clusters could be detected uniquely in CC, which might represent presumptive *nirS* containing denitrifiers acclimating to such environments (low pH, ammonium, nitrate and nitrogen, high total carbon). Unfortunately, there are presently no other reports on *nirS* diversity in CC dataset.

Different geochemical characteristics soils (CC and RC) were sampled. CC had lower pH, ammonium, nitrate and available N concentration, but higher total carbon levels than RC sample. A cropping model with RC would more likely accumulate ammonium, nitrate and available N concentration as compared to CC. The major difference between CC and RC appeared to be the nitrate levels[17] and ammonium/nitrate ratio and had a similar relationship OTUs based on RFLP pattern measurements. These results suggested that the ammonium and nitrate levels might be major factor on presumptive denitrifiers with *nirS* gene. But we also reminded that OTUs distribution was not always a sole function of nitrate and ammonium levels, for that the data detected were complexity and microbial community structure was most likely affected by many different abiotic (secondary metabolites) and biotic variables. Future inquiries into denitrifiers in the continuous cropping soil should address the relative abundance, activity, and interactions of important groups of microbes.

4 Conclusion

In summary, our study indicated the results as followed: (1) the novel *nirS* sequences were identified between CC and RC, and sequence analysis revealed higher nitrite reductase gene (*nirS*) diversity among two samples. (2) Diversity indices displayed lower *nirS* gene diversity in CC than in RC. (3) Principal component analysis implied that each sites had their own predominant sequences. (4) We can presume that the different tillage management affected the communities of the nitrite reductase bacteria. Future study is needed to characterize the relationship between the occurrence and distribution of sequence and the physicochemical characteristics of the soil, and cultivation-dependent should be employed to investigate the *nirS* functional gene.

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