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Research Article

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Genetic diversity analysis of azotobacter in cotton soil of Yangtze River Basin

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ABSTRACT

To research the effect of cotton soil microorganisms, the genetic diversity of 58 aerobic nitrogen-fixing bacteria isolated from cotton soils in Yangtze River were studied by ERIC-PCR and the 16S rDNA sequence analysis. The ERIC-PCR results showed that tested strains formed 9 clusters defined by 0.65 of Watson distance. The type strains of 6 cluster were identified as Enterobacter, Bacillus, Acinetobacter, Pseudomonas, Serratia, Yersinia by 16SrDNA sequencing analysis.

Key words: Cotton soil, Aerobic self-nitrogen-fixing bacteria, ERIC-PCR, Yangtze River, Genetic diversity

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INTRODUCTION

All living organisms require nitrogen (N), and one process-biological N fixation-accounts for more than 97% of natural N inputs to terrestrial ecosystems [1]. Biological N fixation plays the critical role in the global N cycle [2]. It appears that a significant reduction in the relative use of fertiliser-N can only be achieved if biological nitrogen fixation is made directly available to cereal crops in an effective associative system with some of the characteristics of the legume symbiosis. Experience since the plant-associating diazotrophs were discovered by Dobereiner and her coworkers [3] has shown that this objective will be elusive and that achievement of nitrogen fixation with cereals is still some distance away although there may be other benefits from inoculation of field crops [4]. Over geological time scales, nitrogen fixation is important for regulating fixed N concentrations in the soil and thereby sustain soil fertility [5].

Azotobacter is an independently nitrogen-fixing microorganisms conversed of dinitrogen (N2) gas into two molecules of ammonia [6], a important component of plant growth promoting bacteria in the rhizosphere. researchers have found that nitrogen-fixing ability of bacteria from raw dozens of species, which belong to different families and genera, in different climatic conditions, soil and waters are widely distributed. According to reports, the amount of nitrogen fixation in soil by Azotobacter be 60 kg • hm^{-2} • Y^{-1}, although relatively lower than compared to the amount of nitrogen fixation by symbiotic association. Azotobacter may have a more important role in nitrogen cycle of natural ecosystems, especially in the barren wasteland, because it has more good characteristics, such as it does not fit with the particular plant, has a large of total resources, widely distributed, strength adaptability.

Biological nitrogen fixation, a process utilized only by certain prokaryotes, is catalyzed by a two-component nitrogenase complex [7]. Nitrogen-fixing microorganisms colonize a wide variety of habitats and can be found freeliving in soils and water, in association with grasses, or in root-nodule symbioses with legumes. Azotobacter is diversity.

Commensal intestinal microflora have been monitored by cultivation-based techniques and molecular

detections[8-10]. Enterobacterial repetitive intergenic consensus (ERIC)-PCR uses oligonucleotides targeting short repetitive sequences that are dispersed throughout various bacterial genomes [9]. Commensal intestinal microflora can be identified at the genus, species and strain levels based on the electrophoretic pattern of amplified products [11,12]

Yangtze River Basin is one of Chinese major cotton-producing regions because of good in lighting conditions, rich in calories. Azotobacter in this region cotton soil showed rich diversity of genetic type because of the long interaction between cotton and cotton growing conditions. The purpose of this paper study those azotobacter genetic diversity by EPIC-PCR method and heir taxonomic status by sequencing full sequence of 16S rDNA.

EXPERIMENTAL SECTION

Mediums

Medium for isolation was Ashby nitrogen-free medium(Mannitol 5 g, KH_2PO_4 0.2 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, NaCl 0.2 g, $CaSO_4{\cdot}2H_2O$ 0.2 g, $CaCO_3$ 5 g, Distilled water 1.0 L, pH7.0-7.2). Medium for culture was TY broth medium (peptone 5 g, yeast extract 3 g, $CaCl_2$ or NaCl 0.7 g, distilled water 1.0 L).

Separation of Azotobacter in Cotton Soil

Soil samples collected from Continuous culture cotton soil in Yangtze River Basin. Dilution of soil were prepared by ten-fold dilutions method. 0.1 mL dilutions from three soil dilutions 10^{-2}, 10^{-3}, 10^{-4} was inoculated on on the plate, three replicates of each dilution. 0.1 mL sterile water instead of soil dilutions was inoculated as control. All plate was cultured at 28 centidegree for 3-5 day. Pure cultures were obtained with one or more further subculturing steps on Ashby nitrogen-free medium.

ERIC-PCR fingerprinting Analysis of Azotobacter

Azotobacter were cultured in TY medium at 28 centigrade for 24-72 h, and cells were collected at 10000 r.p.m. at 4 centigrade for 10 min. Total DNA was extracted using the conventional phenol-chloroform method [13]. Specific primers for ERIC-PCR selected ERIC1R (5'-ATGTAAGCTCCCTGGGGATTCAC-3 ') and ERIC2L (5'-AAGTAAGTGACTGGGGTGAGCG-3'), synthesized by the Shanghai Sangon Technical Services Limited synthesis. The total reaction system was 25 µL (10 × Buffer (Biorule) 2.5 µL; $MgCl_2$ (25 mmol/µL) 2.0 µL; dNTP (2.5 mmol / L) 2.0 µL; ERIC1R (20 pmol / µL) 0.5 µL ; ERIC2L (20 pmol / µL) 0.5 µL; adding 13 µL of ddH2O; adding template total DNA (40-80ng) 4.0 µL). ERIC-PCR reaction procedure as follows: add the sample into the PCR amplification of denaturation at 95°C 7 min; remove PCR tubes quickly added 0.5 µL 2.5 UTaq enzyme (Biorule), then back into the PCR amplification mix instrument , 30 cycles (94°C 1 min; 52 °C 1 min; 65 °C 8 min; 65 °C 16 min); 4 °C forever. Reflecting the PCR tubes removed after 1% agarose gel 3.5 h, using the gel imaging system takes and stores the mapping device . Gels by a computer after the scan process, a band in the same position is "1 " , not as " 0 ." A tree diagram based on ERIC-PCR fingerprinting by using the unweighted pair-group method with arithmetic means (UPGMA) method of DPS software [14] was constructed.

16S rDNA Sequencing and Phylogenetic Analysis of Azotobacter

Azotobacter were cultured in TY medium at 28 centigrade for 24-72 h, and cells were collected at 10000 r.p.m. at 4 centigrade for 10 min. Total DNA was extracted using the conventional phenol-chloroform method [13]. 16S rDNA primers used P1 (5'-CGGGATCCAGAGTTTGATCCTGGCTCAGAACGAACGCT-3 ') and P6 (5'-CGGGATCCTACGGCTACCTTGTTACGACTT CACCCC-3'), synthesized by Shanghai Biological Engineering Technology Services Limited. 16S rDNA amplified product of isolates sequenced by the Shanghai Biological Engineering Technology Services Limited. Sequence analysis and comparison were conducted using Cluster X software. The nucleotide sequences of 16S rRNA genes of related bacteria used for comparison were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nib.gov/). A phylogeny tree was built using the program Phylogeny of MEGA 4.0 (Molecular evolutionary genetics analysis) [15].

RESULTS

ERIC-PCR Fingerprinting of Azotobacter

From different continuous culture cotton soil of Yangtze River Basin, 58 azotobacter strains were isolated and purified. ERIC-PCR fingerprinting of those strains were gained by amplificating. The ERIC-PCR fingerprinting of partial azotobacter was showed in figure 1. Spectrum band number of ERIC-PCR fingerprinting of tested azotobacter strains showed from 5 to 14, ranging from about 100 bp ~ 2500 bp, indicated that these strains obvious genetic differences.

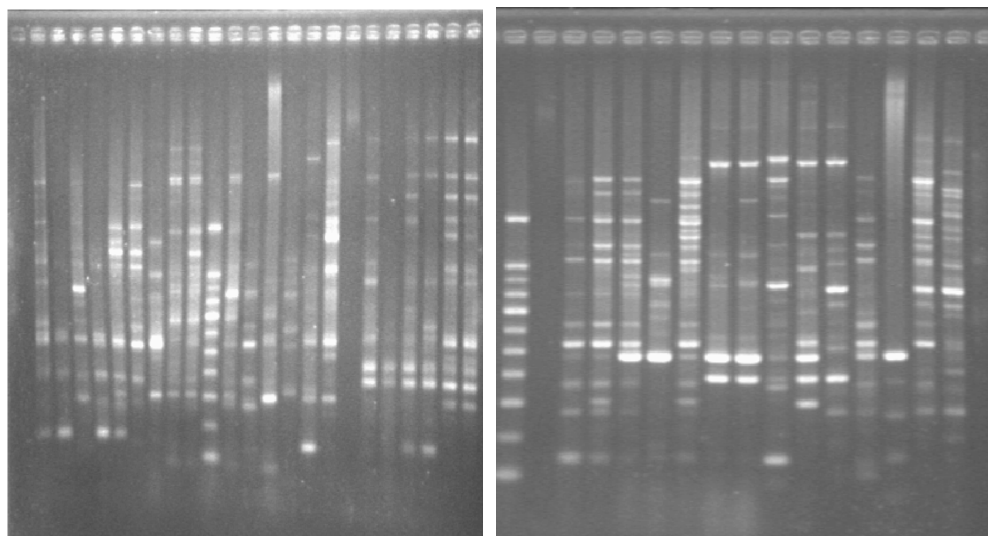

Fig.1 the ERIC-PCR fingerprinting of partial azotobacter

Dendrogram of twenty-six tested strains based on ERIC-PCR fingerprinting by using the unweighted pair-group method with arithmetic means (UPGMA) method of DPS software was constructed (Figure 2). All the tested strains were been together at 0.83 of Watson distance, and further divided into nine groups at 0.65 of Watson distance, named A, B, C, D, E, F, G, H, I, number of isolates in each group were 5, 8, 5, 23, 3, 7, 2, 2, 3.

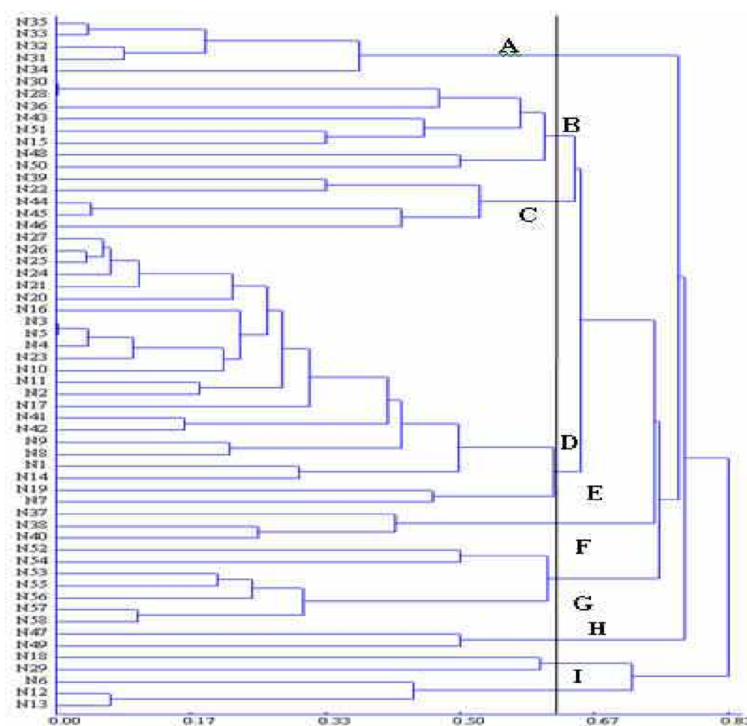

Fig.2 ERIC –PCR Dendogram of tested azotobacter

Phylogenetic Relationships among Azotobacter and Related Typed Strain Based on 16S-rDNA Gene Sequences.

Phylogenetic tree of twenty-six tested strains based on 16S rDNA gene sequences by Neighbor-Joining of Mega4 software was constructed (see Figure 3). Tested strains and their high similarity known typed strains can be divided into Enterobacter, Bacillus, Acinetobacter, Pseudomonas four branches, including Enterobacter, Bacillus, Acinetobacter, Pseudomonas, Yersinia, Serratia 6 genera.

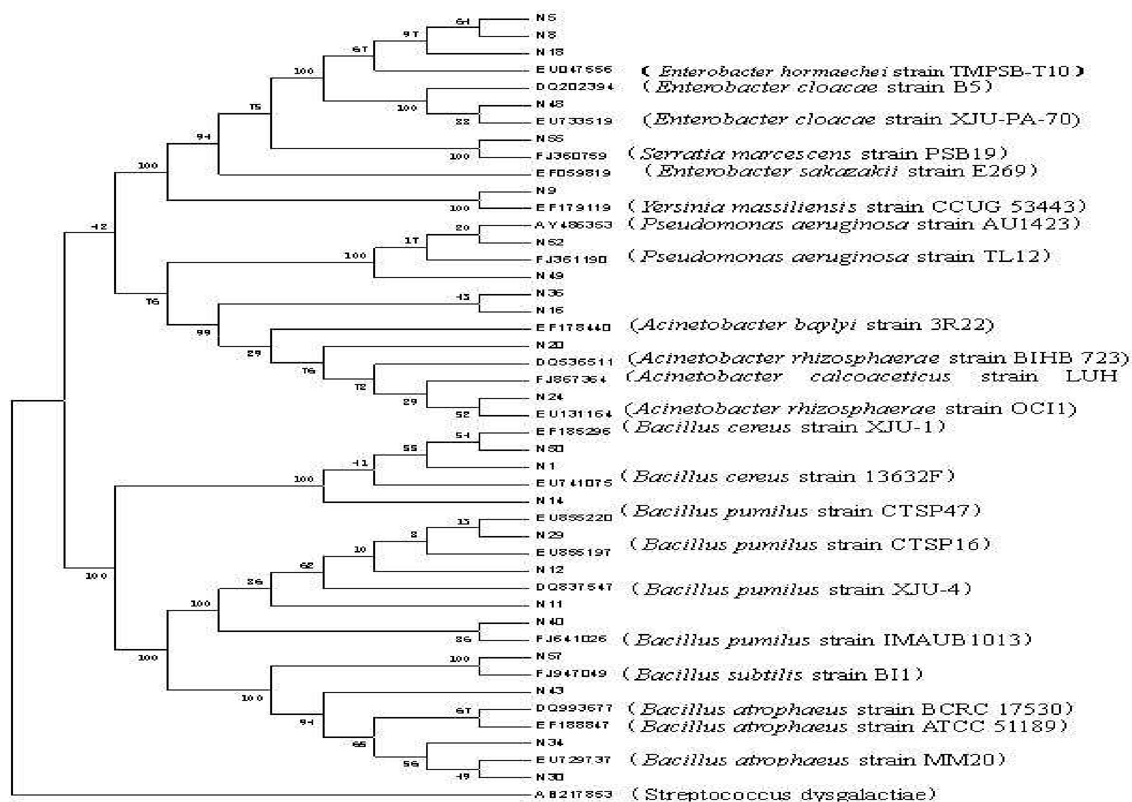

Fig.3 Dendogram of tested aerobic self-nitrogen-fixing bacteria 16S rDNA sequences

Eleven tested strains including N11, N29, N12, N14, N40, N30, N34, N43, N50, N57, N1 together constitute the genus *Bacillus*. Four (N11, N29, N12, N40) of 11 strains had close genetic relationship with *Bacillus pumilus*. Strains N57 had closer relationship with *Bacillus subtilis*. Strains N30, N34,and N43 clustered into a small branch with *Bacillus atrophaeus*. Strains N14, N50, and N1 gathered in a branch of *Bacillus cereus*. Strains N52 and N49 jointly vested *Pseudomonas*. Strains N9 was identified as *Yersinia*. Strains N55 was identified as *Serratia*. Four strains (N5, N18, N48, N8) jointly attributed to *Enterobacter*. Other four strains (N36, N16, N20, N24) closer relationship jointly vested in *Acinetobacter*. *Bacillus* was predominant, followed by *Enterobacter* and *Acinetobacter* in all tested strains in the phylogenetic tree.

CONCLUSION AND DISCUSSION

Rich Genetic Diversity of Azotobacter in Cotton Soil of Yangtze River Basin

Experience in this paper indicated that azotobacter in cotton soil of Yangtze River Basin had abundant genetic diversity. The ERIC-PCR results showed that tested strains isolated from cotton soil of Yangtze River Basin formed 9 clusters defined by 0.65 of Watson distance. The type strains of 6 cluster were identified as *Enterobacter*、*Bacillus*、*Acinetobacter*、*Pseudomonas*、*Serratia*、*Yersinia*.

ERIC-PCR Analysis - an Effective Method of Genetic Diversity of Bacteria

Genetic diversity were detected at different levels. Morphology method was the initial detection of genetic diversity that people are starting. Molecular biology method and molecular cloning techniques has brought a series of more direct approach of genetic diversity from 1980s. Currently, DNA analysis, any genome and any DNA fragment, had become the most effective method of genetic diversity analysis. In this study, ERIC-PCR analysis well reflected the genetic diversity of strains isolated from cotton soil of Yangtze River Basin in genome-wide angle. The ERIC-PCR results showed that all tested strains were been together at 0.83 of Watson distance, and clustered into nine groups at 0.65 of Watson distance. ERIC-PCR fingerprints from the different strains were significantly different.

Depth Study of Azotobacter in Cotton Soil of Yangtze River Basin

This study only analyzed genetic diversity of azotobacter in cotton soil of Yangtze River Basin. The variation of species and quantity of azotobacter in cotton soil of Yangtze River Basin with continuous culture year and cotton growing period is unclear. Azotobacter in cotton coil of Yangtze River Basin based on this study were very rich, but

what kind of azotobacter can better play the role of nitrogen fixation in the cotton soil? In addition to nitrogen fixation, if there are other ways to promote the growth of cotton by azotobacter? Depth study and To solve these problems is important for a healthy, balanced microecosystem of cotton fields and sustainable production of cotton of Yangtze River Basin.

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REFERENCES

[1] Galloway, J.N., Townsend A.R., Erisman J.W., et al. *Science.* **2008,** (320):889–892.
[2] Townsend, A.R., Martinelli L.A., Howarth R.W. The global nitrogen cycle, biodiversity, and human health. In: Sala OE, Meyerson LA. *Biodiversity change and human health*. Washington, DC:Island Press, **2009**.
[3] Baldani V, Baldani J. and Dobereiner J. *Can. J. Microbiol*. **1983**, 29:924–929.
[4] Okon Y. and Labandera-Gonzolez C.A. *Soil Biol. Biochem*. **1994**, 26:1591–1601.
[5] Tschapek, M. and Giambiagi N. *Archiv für Mikrobiologie*. **1955,** 21:376-390.
[6] Benavides, M., Agawin, N. S. R., Aristegui, J., et al. *Aquat. Microb.Ecol*. **2011**, 65:43–53.
[7] Schrock, R.R. *Proc Natl Acad Sci* USA. **2006**, 103(46):17087.
[8] Finegold, S.M., Attebery H.R., Sutter V.L. *Am J Clin Nutr*. **1974**, 27: 1456-1469.
[9] Versalovic J., Koeuth T., Lupski J.R. *Nucleic Acids Res*. **1991**, 19: 6823-6831.
[10]Hulton, C.S.J., Higgins C.F., Sharp P.M. *Mol Microbiol*. **1991**, (5):825-834.
[11]Rodriguez-Barradas, M.C., Hamill R.J., Houston E.D., et al. *J Clin Microbiol*. **1995**, 33:1089-1093.
[12]Sampaio, J.L., Viana-Niero C., de Freitas D., et al. *Diagn Microbiol Infect Dis.* **2006**, 55:107-118.
[13]Ausubel, F.M., Brent, R., Kingston, R.E., et al. *Short Protocols in Molecular Biology*(3rd edn). New York, NY: John Wiley & Sons Inc. **1995**.
[14]Tang, Q.Y., Feng M.G. *Experimental design, statistical analysis and data mining of DPS data treatment system*. Beijing: Science Press. **2007**.
[15]Tamura, K., Dudley J., Nei M., et al. *Molecular Biology and Evolution,* **2007**, 24:1596-1599.