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Genetic Relationships of Soft Rot Bacteria Isolated from Konjac in China by Amplified Fragment Length Polymorphism (AFLP) and 16S rDNA Gene Sequences

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Abstract

Twenty-three isolates of soft rot bacteria from konjac corms were examined for their diversity using 16S rDNAs and AFLP technology. Both methods clustered two groups, dependent on their biotype characterization of *Pectobacterium carotovora* subsp. *carotovora* (*P.c.c*) and *Pectobacterium chrysanthemi* (*P.ch*), respectively. Of all isolates, 17 (73.9%) belonged to *P. ch*, indicated as the main pathogenic bacteria of konjac producing areas in China. The genetic variation among isolates from the same biotype was also rich, not consistent with the distances of the geographic sources.

Keywords

Soft Rot Bacteria, Identification, 16S rDNA, AFLP, Genetic Diversity

1. Introduction

Amorphophallus konjac (konjac) has long been planted in Southeast Asia and East Asia as food source and traditional medicine [1]. Previous study has reported that konjac soft rot caused by the genus *Pectobacterium* is one major reason of limited konjac products [2]. Based on yield data from the farmer, the yield of konjac suffering from soft rot bacteria decreased 30% to 50%, and even more than 80% in some planting regions. Thus, soft rot is one of the most limiting factors in konjac production. At present, this kind of bacteria have been re-

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ported in other vegetables or ornamental crops, *i.e.* calla lilies [3] and potatoes [4] [5]. Waldee (1954) firstly reported this kind of pathogenetic bacteria, leading to soft rot, named as the genus *Pactobacterium* separated from the genus *Erwina* [6], and further divided it into two species, namely *P. chrysanthemi* and *P. carotovorum* [7].

With the development of molecular techniques, many methods, such as DNA-DNA hybridization [8] [9], ribotyping [10], ITS-PCR [11], 16S rDNA analyses [12] [13], and phenotypic techniques, including biochemistry [14] [15], have been used to research the genetic diversity of genus *Pectobacterium*, of which rDNA analysis is efficient as an important index of bacterial evolution and genetic relationship. Based on the differences of 16S rDNA sequences, the taxonomic status and genetic diversity of bacteria can be determined. Besides, amplified fragment length polymorphism (AFLP) as a genomic fingerprinting method has been described by Vos *et al.* [16]. AFLP analysis has proven to be an effective research tool for discriminating DNA samples from a variety of bacterial species and strains [16]-[18].

The aims of this study were to investigate the genetic diversity and taxonomic classification of soft rot isolates from Konjac corm using AFLP technique and 16S rDNA sequences at species and subspecies levels, and to type strains for epidemiological investigations.

2. Materials and Methods

2.1. Strains and Media

24 bacteria isolates were isolated from soft rot corm of *Amorphophallus konjac*, obtained from the main konjac producing areas in China and Myanmar (**Table 1**). All isolates have been inoculated and confirm the suscepti-

Table 1. Bacteria isolates from the soft rot corm of konjac used in this study.

Number	Strain	Geographical origin	Species or subspecies	Accession no.	Cluster	
					16S rDNA	AFLP
1	BaDongI	Enshi, Hubei, China	*P.c.c*	FJ906788	Ib	Ib
2	BaDongHuII	Enshi, Hubei, China	*P.c.c*	HM622350	Ia	Ia
3	SichuanI	Mianyang, Sichuan, China	*P.c.c*	FJ906790	Ia	Ia
4	SichuanII	Mianyang, Sichuan, China	*P.c.c*	FJ906791	Ib	Ib
5	MyanmarI	Myanmar	*P.ch*	HM590190	IIa	IIa
6	MyanmarIII	Myanmar	*P.ch*	HM590192	IIa	IIa
7	AnK2	Ankang, Shanxi, China	*P.ch*	FJ463869	IIc	IIb
8	ShanXiI	Ankang, Shanxi, China	*P.ch*	FJ906792	IIc	IIb
9	ShanXiII	Ankang, Shanxi, China	*P.ch*	FJ906793	IIa	IId
10	NKYHuaYa	Wuhan, Hubei, China	*P.c.c*	HM622348	Ia	Ia
11	NKYC	Wuhan, Hubei, China	*P.c.c*	FJ463871	Ia	Ia
12	ZhXB	Zhuxi, Hubei, China	*P.ch*	FJ463870	IId	IIa
13	HuaII	Wuhan, Hubei, China	*P.ch*	FJ906794	IId	IIa
14	WuDaHuaYaI	Wuhan, Hubei, China	*P.c.c*	HM622345	Ia	Ia
15	CHQ	Chongqing, China	*P.ch*	FJ463865	IId	IId
16	AnKangIII	Ankang, Shanxi, China	*P.ch*	FJ906796	IId	IId
17	LiChuanII	Lichuan, Hubei, China	*P.ch*	FJ906795	IId	IIa
18	NKYA	Wuhan, Hubei, China	*P.ch*	FJ463863	IIb	IIc
19	WuFengII	Wufeng, hubei, China	*P.ch*	FJ906797	IId	IIa
20	YunNan	Yunnan, China	*P.ch*	HM590189	IIb	IIc
21	ESJSX	Enshi, Hubei, China	*P.ch*	FJ463864	IIb	IIc
22	ESH II	Enshi, Hubei, China	*P.ch*	FJ463867	IIb	IIc
23	ESHI	Enshi, Hubei, China	*P.ch*	FJ463866	IIc	IIb
24	ESH III	Enshi, Hubei, China	*P.ch*	FJ463868	IIb	IIc

Note: *P.c.c*. and *P.ch* are the abbreviation of *Pectobacterium carotovora subsp. carotovora* and *Pectobacterium chrysanthemi*, respectively.

bility (data not shown), and stored in the Luria-Bertani (LB) medium containing 30% (v/v) glycerol at −80˚C. Before using in this study, bacteria were grown in LB broth at 28˚C (200 r/min) for 14 - 16 hours.

2.2. Extraction of Bacteria Genomic DNAs

Genomic DNA was extracted from bacteria samples by using TIANamp Bacteria DNA Kit (TIANGEN, Co., China). These DNA templates were store at −20˚C for using. DNA concentrations were determined by measuring the A_{260} with a Uvikon model 940 spectrophotometer.

2.3. Phylogenetic Analysis on the Basis of Partial 16S rDNA Gene Sequencing

For 16S rDNA gene sequencing, the primers were used in this study: P_1 (5'-AGACTTTGATCCTGGCTCAG-3') and P_2(5'-CGGCTACCTTGTTACGACTTC-3') [19]. Each PCR reaction mixtures (25 µl) contained 10 pM of each primer, 0.5 mM dNTPs (Dingguo Ltd., China), 0.4 mM $MgCl_2$, 1 U of Taq polymerase and ~10 ng template-DNA. PCR amplifications were performed with the parameters: initial denaturation (3 min at 95˚C), 35 cycles of amplification (30 s at 95˚C, 30 s at 55˚C, 90 s at 72˚C), and a final elongation of 8 min at 72˚C. All amplifications were performed in a Lifepro Thermal Cycler (Hangzhou Bioer Technology Co., Ltd). The PCR products were checked by electrophoresis on 1.0% agarose gels (Sigma), and then the corresponding PCR products were purified with PureLink™ Quick kit (Invitrogen) following the recommended manufacturer. Purified products were further ligated into pGEM-T vectors (Promega Co., China), and then transformed into *E. coli DH-5a* cells. Positive colonies were selected for sanger sequencing by blue-white screening procedure [20]. Using these sequences, the phylogenetic tree was constructed by MEGA6.0 with the Neighbor-Joining method [21]. Bootstrap analysis with 1000 replicates was performed to calculate the support of branches. All 16S rDNA sequences in this study have been doposited to the NCBI Genbank databases (**Table 1**).

2.4. AFLP Analysis of Soft Rot Isolates

Two endonucleases (*Eco*RI and *Mse*I) were used for AFLP analysis in this study, and the reactions were performed as described previously with minor modification [16]. Firstly, bacterial genomic DNA (~0.2 µg) was digested with 10 U each of restriction endonucleases in 1 × EcoR I buffer (Thermo Scientific) for 3 h at 37˚C, and then 3 h at 65˚C. MseI and EcoRI adapters (**Table 2**) were ligated to the digested DNA in a total volume of 10 µl using T4 DNA ligase (Promega) for 12 h at 16˚C. After ligation, the ligated products were amplified by PCR using nonselective primers (EcoRI-00, *Mse*I-00, **Table 2**) in a total volume of 20 µl. Each reaction mixture contained 2 µl of the ligation mixture, 0.5 mM Mg^{2+}, 0.8 mM dNTPs, 1U of Taq polymerase and 10 pmol each of the *Eco*RI-00 and *Mse*I-00 primers. The PCR was performed under the following conditions: 36 cycles of 30 s of denaturing at 94˚C, 30 s of annealing at 56˚C, and 1 min of extension at 72˚C. All amplifications were performed in a Lifepro Thermal Cycler (Hangzhou Bioer Technology Co., Ltd). After PCR reaction, 10 µl of gel loading buffer (94% formamide, 10 mM EDTA, 0.5 mg of xylene cyanol FF ml^{-1}, 0.5 mg of bromophenol blue ml^{-1}) was added. Samples were heated to 95˚C for 5 min and immediately cooled on ice. PCR products were electrophoresed using 6% polyacrylamide denaturing gel. Gels were stained with silver nitrate, following the protocol described by Han *et al.* [22].

Polymorphic AFLP bands were manually scored as binary data for the presence (1) or absence (0) of fragments ranged from 100 and 500bp. A table containing binary information was used to calculate Jaccard's pair

Table 2. List of oligonucleotide primers and adapters.

Adaptor name	Adaptor sequence (5'-3')
EcoRI	5'-CTC gTA gAC TgC gTA CC-3' 5'-AAT Tgg TAC gCA gTC-3'
MseI	5'-gAC gAT gAg TCC TgA g-3' 5'-TAC TCA ggA CTC AT-3'
Primer name	**Primer sequence (5'-3')**
EcoRI-00	gAC TgC gTA CCA ATT C A
MseI-00	gAT gAg TCC TgA gTA A C

wise coefficients of similarity. All 24 bacteria isolates were clustered using the Unweighted Pair Group Method with Arithmetic averages (UPGMA) method and the SHAN clustering program by NTSYS [23].

3. Results and Discussion

In this study, a total of 24 bacteria isolates were used for diversity analysis using AFLP technology and 16S rDNA sequences. These bacteria were isolated from Myanmar (2 isolates) and 5 provinces in China (22 isolates), respectively (**Figure 1**). Before performing Molecular experiments, all isolates have been re-inoculated to normal konjac corm, and confirmed their susceptibility rDNA was an important index of bacterial evolution and genetic relationship. Based on the variation of 16S rDNA sequences, the taxonomic status and genetic diversity of bacteria can be determined. Thus, we firstly compared 16S rDNA sequences of 24 isolates with Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast) in NCBI. The results shown soft rot bacteria of konjac conclude two biotypes: *Pectobacterium carotovora* subsp. v*carotovora* (*P.c.c*) and *Pectobacterium chrysanthemi* (*P.ch*). Of which *P.ch* is mainly pathogenic bacteria (17/24, 71%). Besides, The isolates located at the same region (*i.e.* Wuhan and Enshi) can exist two biotypes (*P.c.c* and *P.ch*), indicated the infection complexity of konjac soft rot. Based on 16S rDNA sequences, a phylogenetic tree was obtained, and showed a high of genetic diversity across 24 isolates (**Figure 2**). This results support the heterogeneous taxonomic structure of the genus Pectobacterium. The phylogenetic dendrogram included two groups (I and II), Group I (all *P.c.c* types) was further divided into two subgroups (Ia and Ib). Group II (all *P.ch* types) contained 4 subgroups (IIa, IIb, IIc and IId).

For further confirmed diversity of these isolates, AFLP analysis was done, and a total of 115 polymorphic DNA fragments ranging from 100 to 500 bp were detected, and used to analyze the diversity of 24 bacteria stain isolates. Based on binary data, a Phylogenetic tree was constructed according to UPGMA method and the SHAN clustering program by NTSYS version 2.20 k. The results showed the genetic similarity varied from 0.51 to 0.98. The dendrogram consisted of two major groups (**Figure 3**). Group I included all *P.c.c* isolates, and further clustered into two subgroups (Subgroup Ia, Ib) at the cutoff of the coefficient of 0.63. Subgroup Ib con-

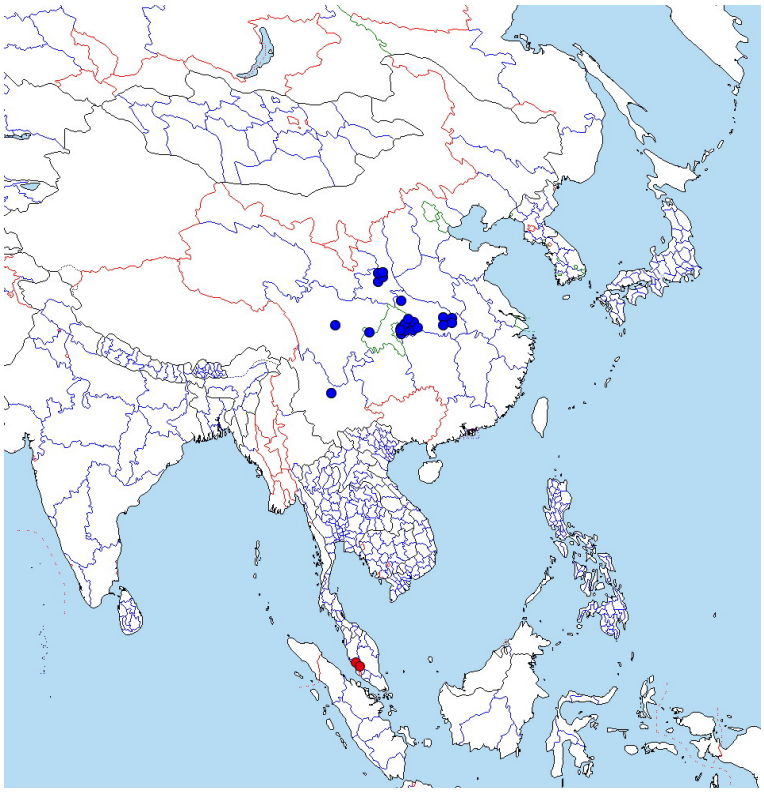

Figure 1. These bacteria were isolated from Myanmar (2 isolates) and 5 provinces in China (22 isolates).

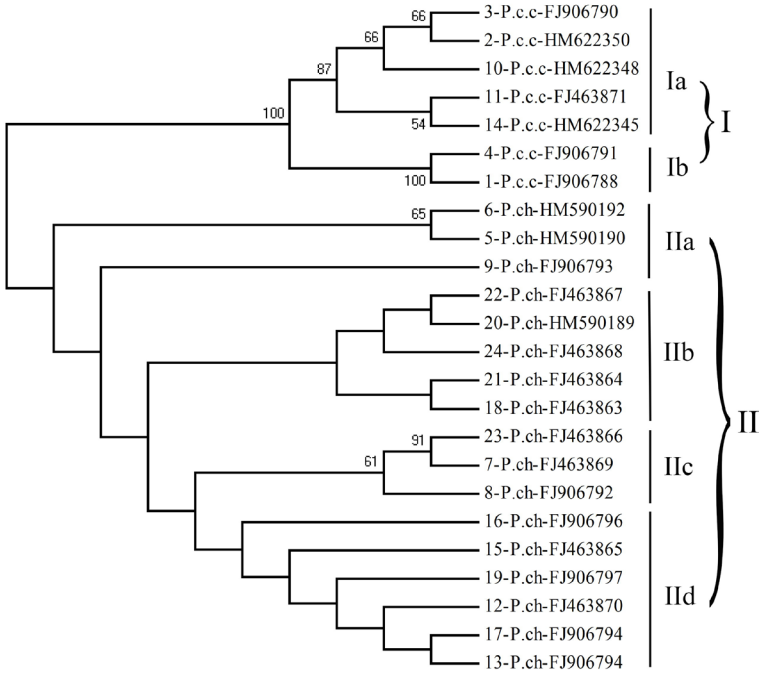

Figure 2. Phylogenetic dendrogram based on 16s rDNA gene sequences for soft rot isolates. The branching pattern was generated by the neighbor-joining method using MEG6.0. The numbers at the nodes indicate the levels of bootstrap support, and only the values more than 50 are given.

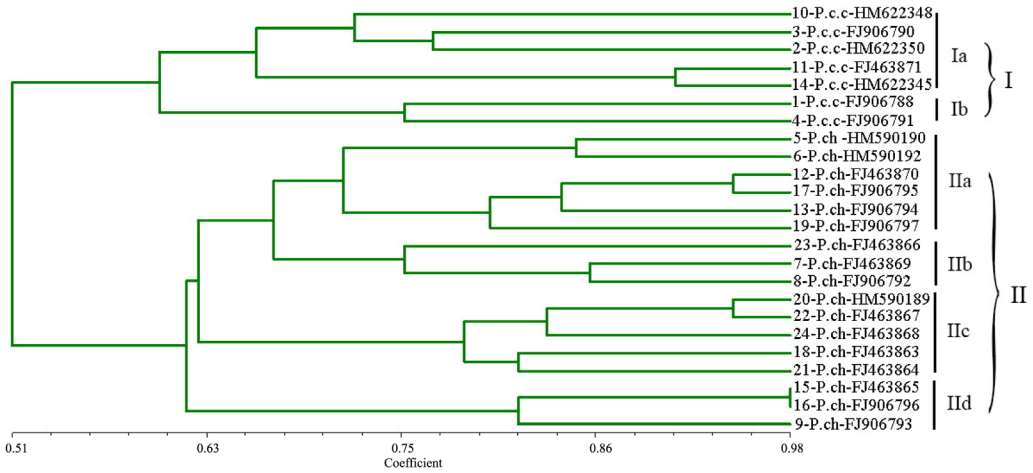

Figure 3. Dendrogram based on UPGMA clustering using AFLP data from the 24 soft rot isolates. The scale at the bottom indicates the degree of genetic relatedness between strains.

tained two isolates from Hubei and Sichuan province, China, respectively. Subgroup Ia comprised five isolates, of which 3-*P.c.c*-FJ906790 is the same geographic origin with 4-*P.c.c*-FJ906791 from subgroup Ib. All *P.c.c* isolates were clustered into Group II that further clustered into three subgroup (IIa, IIb, IIc and IId). Subgroup IIa included 6 isolates from Myanmar and Hubei, China, respectively. Subgroup IIb contained two Shanxi isolates and one Hubei isolates. Subgroup IIc possessed one Yunnan isolates and 4 Hubei isolates. Subgroup IId included 2 Shanxi and 1 Chongqing isolates. Obviously, based on the analysis of AFLP, the results support the heterogeneous taxonomic structure of genus *Pectobacterium*. And the genetic differences among isolates were not dependent on the resources of geographic area. The isolates from different province can be clustered into one group.

At present, konjac cultivars resistant to the soft rot disease have not been reported. This disease is conventionally controlled by cultivation measures (such as crop rotation, intercropping) and chemical control [24]. Chemical control is usually inappropriate because pathogens can develop resistance and also pesticides can pollute the environment. Therefore, researchers pay much attention to the biological control. In this study, two kinds of pathological bacterial on konjac (*P.c.c* and *P.ch*) were found in China, based on genetic relationships of konjac soft rot bacteria by AFLP and 16S rDNA sequences. This should contribute to understand pathological bacterial on konjac soft rot and research biological methods against Pectobacterium spp.

4. Conclusion

For konjac production, the soft rot is the most serious factor affecting its yield. To further understand constitution and diversity genetics of konjac soft rot bacteria, AFLP technique and 16S rDNA gene sequences were used to analyze the genetic diversity of 24 isolates collected from Malaysia and different regions in China. Through the 16S rDNA analysis, two kinds of pathogenic bacteria (*P.c.c* and *P.ch*) were identified, which was similar to previous reports. Meanwhile, there were two types of pathogens found in the same region, which indicated the complexity of the soft rot of konjac. In the phylogenetic tree, two clades were constructed depending on *P.c.c* and *P.ch* species, respectively; the genetic diversity within species was rich, and genetic differentiation among isolates was not based on the resource of geographic area.

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