Analysis of Genetic Diversity Among Chinese *Cyclocbe chaxingu* Strains Using Combined ISSR and SRAP Markers

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Abstract

Background. *Cyclocybe chaxingu* is an edible and medicinal fungal species commonly cultivated in China.The major problems currently facing growers of *C. chaxingu* is the random labeling of strains and the introduction into different regions of identical strains under different designations. Therefore, precise identification and classification of commercial *C. chaxingu* strains for Chinese and overseas markets.

Methods. In present study, the widely cultivated strains were collected from the main producing areas of China, and the genetic diversity analysis was performed. DNA polymorphism among 24 Chinese *C. chaxingu* strains was analyzed using inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers.

Results. Eight ISSR primers amplified a total of 75 DNA fragments of which 61 (81.33%) were polymorphic. Fifteen SRAP primer combinations amplified 166 fragments of which 132

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(79.52%) were polymorphic. Cluster analysis showed that the *C.chaxnigu* strains fall into five groups with a genetic distance values ranging from 0.06 to 0.60 by ISSR analysis, while the SRAP analysis divided the test strains into four groups within the range of genetic distance from 0.03 to 0.57. The results of the present study reveal that a high level of genetic diversity among the widely cultivated *C.chaxingu* strains. And strain JAUCC 0727 has a unique genotype, could be an excellent parent for breeding.

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Introduction

Cyclocybe chaxingu (N.L. Huang) Q.M. Liu, Yang Gao & D.M. Hu is an edible and medicinal species, which commonly cultivated in Jiangxi, Yunnan and Fujian provinces of China [1-3]. *C. chaxing*u is well received by consumers for its nutritional properties such as high protein, low fat and sugar, and its pharmacological effects such as anti-oxidation, anti-aging, anti-tumor and agglutination of red blood cells [4-6]. Now, it has been popularized and cultivated on a large scale and the estimated output of *C. chaxingu* in 2019 was almost 0.9 million tons in China [7]

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Commented [U11]: Agglutination of red blood cells may be dangerous to life so how consumers may prefer commercial strains of *Cyclocybe chaxingu with properties of red blood cells agglutination?*

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The cultivars of *C. chaxingu* are the foundation of the development of *C. chaingu* genetic and breeding in both theory and practice. Incorrectly designating cultivars or strains caused chaos of *C. chaxingu* industry, which resulted in huge economic losses. Therefore, it is very important to collect and evaluate the germplasm resources of Chinese *C. chaxingu*.

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Depends on its properties such as independence of environmental parameters and the high levels of detectable polymorphism, DNA molecular markers has played an irreplaceable role in biological germplasm identification and innovation [8, 9]. In previous studies, inter-simple sequence repeat (ISSR), and sequence-related amplified polymorphism (SRAP) techniques have been widely used in the analyses of genetic diversity. ISSR uses semi-arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite, which was a new molecular marker developed on the basis of simple sequence repeats (SSR) [10]. And SRAP uses two sets of positive and negative primers to amplified the Open Reading Frame (ORF), including the intron and promoter region [11]. These two markers have been utilized for the genetic diversity analyses of much many mushrooms species such as Auricularia auricula-judae [12], Auricularia polytricha [13], Lentinula edodes [14], Lepista nuda [15], Pleurotus citrinopileatus [16], Pleurotus eryngii [17] and Pleurotus pulmonarius [18]. Genetic diversity analysis combined ISSR and SRAP markers has been proven to be reliable and effective [19].

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In this study, combined ISSR and SRAP markers were adopted for 24 strains of *C. chaxingu* cultivated in China to analyze the genetic diversity and the relationships among the test strains.

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Materials & Methods

Mushroom Strains

A total of twenty-four strains of *C. chaxingu* strains collected from the main producing areas of China (Table 1), which were deposited in the Culture Collection of Jiangxi Agricultural University (JAUCC), were used in this study. The previous study based on ITS1SSU and RPB2 phylogenetic analysis showed that all the test strains belonged to *C. chaxingu* [20].

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72 DNA Extraction

The mycelia grew on PDA at 25 for 8 days were used to extract DNA for molecular marker analysis. Genomic DNA was extracted from 100 mg of dry mycelia using a modified cetyltrimethyl ammonium bromide (CTAB) method [21, 22]. The purity and quality of the genomic DNA were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and electrophoresis on a 1.0% agarose gel. DNA samples were diluted to 50 ng/¿l for PCR amplification.

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80 ISSR and SRAP analyses

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A total of 23 primers or primer pairs (Biomed, Beijing, China) that produced clearly distinguishable and reproducible fragments were selected and used in this study for ISSR and SRAP analyses (Table 2). All of the amplification reactions were performed in a PCR Amplifier (BIO-RAD T100TM Thermal Cycler) in 25-¿L reaction mixtures.

For ISSR analyses, the reaction mixtures contained 12.5 ¿L of 2× Taq PCR Master Mix (Vazyme, Nanjing, China), 1 ¿L of primer (10 ¿M/L), 1 ¿L of template DNA, and 10.5 ¿L of ddH_2O. Amplification program was: 4 min of denaturing at 94 #, 35 cycles of 35 s at 94 #, 45 s at 46-55 # (see Table 2 for primer annealing temperature), 2 min at 72 # and followed by a final extension of 10 min at 72 #.

For SRAP analyses, the reaction mixtures contained 12.5 ¿L of 2× Taq PCR Master Mix (Vazyme, Nanjing, China), 1 ¿L of each primer (10 ¿M/L), 1 ¿L of template DNA, and 9.5 ¿L of ddH2O. The amplification included an initial denaturation at 94ÚC for 5 min, 5 cycles of 94 # for 1 min, 35 # for 1 min and 72 # for 1 min, followed by 35 cycles of 1 min at 94 ÚC, 1 min at 50 ÚC, and 1 min at 72 ÚC, and a final extension of 10 min at 72 ÚC.

Amplified products were fractionated by electrophoresis in 2% (w/v) agarose/TAE gels, visualized under UV after staining with TS-Gelred (Tsingke Biotechnology Co., Ltd., Beijing, China), and documented using a gel documentation and image analysis system (GenoSens 2000, Shanghai).

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100 Data analysis for genetic diversity

Qualitative scoring of bands was done from gel photographs obtained from ISSR and SRAP analyses with “1” for presence and “0” for absence to generate a binary matrix. Only those bands amplified consistently were considered. Smeared and weak bands were excluded from the analyses. For data structures with only 0 and 1, Jaccard similarity coefficient is generally adopted [23]. The genetic distances were estimated based on Jaccard similarity coefficient. A cluster analysis was performed based on the genetic distances using the Unweighted Pair Group Method of Arithmetic Average (UPGMA) by R Statistical Software [24] and packages vegan [25]. The goodness of fit of the clustering to the data matrix was calculated. And the optimal grouping strategy is to select the number of groups corresponding to the maximum average contour width [26]. Genetic diversity analysis was performed using the POPGENE program (version 1.32) [27]. The number of amplified loci (N), the percentage of polymorphic loci (PPL), the number of effective alleles (Ne), the Nei’s gene diversity (H) and the Shannon information index (I) were calculated for each primer and among all primers [28, 29]. In addition, a Principal Coordinate

Analysis (PCoA) [30] was performed using the R Statistical Software to obtain a graphical representation of the relationship between the 24 test genotypes.

Results

In total, 8 ISSR and 15 SRAP primers or primer pairs gave reproducible results that were further considered for data analysis. Table 3 shows the total number of bands and the percentage of polymorphisms for each primer or primer pair.

Genetic diversity based on ISSR marker

A total of 24 primers were initially screened to produce polymorphic patterns and only 8 of them were selected which gave reproducible and distinct polymorphic amplified products. For representational purposes, the extent of polymorphism revealed by primer P10 is shown in Figure 1. The data collected from inter-simple sequence repeat (ISSR), detected total of 75 loci in 24 strains, out of which 61 (81.33%) were polymorphic, with an average of 9.38 polymorphic fragments per primer (Table 3). The ISSR primer P1 and P2 gave the highest polymorphism (100%), while the lowest polymorphism (71.43%) was detected by the P4 primer. The values of Ne, H and I were 1.547, 0.312 and 0.459, respectively.

For the 24 C. chaxingu strains, the genetic distances estimated based on Jaccard coefficient using ISSR data (see Table S1) varied from 0.06 (JAUCC 2192 and JAUCC 2196) to 0.60 (JAUCC 0727 and JAUCC 1927), with an overall mean of 0.40. The co-phenetic correlation for the ISSR dendrogram was estimated at 0.93, corresponding to a good fit. A dendrogram constructed from the Jaccard distances matrix using the UPGMA method was shown in Figure 2 (a). All the test strains were grouped into five main clusters by calculating the maximum average contour width value. Cluster I and Cluster III each comprised a single genotype (JAUCC 0727 and JAUCC 1927), while Cluster II, Cluster IV and Cluster V were delineated into two sub-clusters. Within Cluster II, Cluster IV and Cluster V, JAUCC 1925, JAUCC 2119 and JAUCC 1926 appeared to be distinct from the other genotypes, respectively. Interestingly, most strains cultivated in Yunnan Province were clustered together at the distance level of 0.45. Groupings identified by UPGMA analysis were confirmed by PCoA data (Figure 2 (b)). The two most informative PCoA components accounted for 44.21% of the variation observed. Lane numbers correspond to the 24 strains listed in Table 1. Lane M: DL2000 molecular size markers (Tsingke Biotechnology Co., Ltd., Beijing, China)

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Genetic diversity based on SRAP marker

Among the 48 SRAP primer pairs tested in the study, 15 primer pairs were further used to characterize C. chaxingu strains. A representative set of amplification profiles obtained with primer combination me3 + em7 is shown in Figure 3. The present study showed that out of 166 loci, 132 (79.52%) loci were polymorphic showing an average of 11.07 polymorphic loci per primer pairs tested (Table 3). The maximum polymorphic loci were generated by primer pairs em6+me3 (94.45%). Among the primers pairs studied, primers pairs em6+me3 generated the

highest 18 loci, while primer combination em6+me2 and em3+me6 generated the lowest 8 loci. The values of Ne, H and I were 1.506, 0.289 and 0.425 based on SRAP marker.

With the cluster analysis on SRAP molecular marker, the two most closely related strains were found to be JAUCC 1847 and JAUCC 1851 (genetic distance was 0.03), and the two most distantly related strains were JAUCC 1847 and JAUCC 2924 with lowest similarity index (genetic distance was 0.57). Genetic distance estimated based on Jaccard coefficient obtained by SRAP profile with an average value of 0.39. Cluster analysis of SRAP data (see Table S2) based on the distance matrix generated a dendrogram with four major groups in a maximum average contour width value (Figure 4 (a)). The co-phenetic correlation for the SRAP dendrogram was estimated at 0.96, which showed a strong goodness of it. Within Cluster I and Cluster III, JAUCC 1918 and JAUCC 0727 were each comprised a single genotype. In the SRAP analysis, strain JAUCC 0727 showed a closer relationship with the other strains than in ISSR analyses. Similarly, there was a tendency to get together within most strains form Yunnan Province (JAUCC 19201JAUCC 19211JAUCC 19221JAUCC 19251JAUCC 1927), at genetic distance level of 0.38. Principal coordinate analysis (PCoA) data based on the genetic distance

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matrix are shown in Figure 4 (b). These revealed similar groupings to UPGMA, and confirmed the genetic uniqueness of genotypes JAUCC 0727. The two most informative PCoA components accounted for 63.81% of the variations observed. Lane numbers correspond to the 24 strains listed in Table 1. Lane M: DL2000 molecular size markers (Tsingke Biotechnology Co., Ltd., Beijing, China).

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Genetic diversity combined ISSR and SRAP markers

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A total of 23 primers or primer pairs used for the analyses of combined ISSR and SRAP data, genetic distance among all the test strains ranged from 0.06 (JAUCC 2192 and JAUCC 2196) up to 0.54 (JAUCC 1851 and JAUCC 1920). A total of 241 loci in 24 strains, out of which 193 (80.08%) were polymorphic, with an average of 10.48 polymorphic fragments. The co-phenetic correlation for the combined ISSR and SRAP dendrogram was estimated 0.97, corresponding to a very good fit. Dendrogram by using UPGMA and Jaccard coefficient grouped the 24 test strains into five main clusters with a maximum average contour width value (Figure 5 (a)). Within Cluster IV, JAUCC 0727 appeared as a single genotype once again. Strains cultivated in Yunnan Province clustered together (genetic distance was 0.4) same as using ISSR or SRAP molecular marker alone.

The PCoA based on ISSR and SRAP data revealed that the strains belonging to a particular cluster were grouped together in the PCoA plot (Figure 5 (b)). Groupings identified by UPGMA analyses and Jaccard coefficient were confirmed by PCoA data which also revealed that the strain JAUCC0727 was genetically very distinct from the other genotypes. The two most informative PCoA components accounted for 61.01% of the variation observed.

Discussion

As one of the most important edible fungi in China, the research on cultivation of *C. chaxingu* is relatively intensive, but there is a lack of research on genetic diversity. Therefore, efforts have been made in the present study to characterize twenty-four strains of *C. chaxingu* collected from different part of China, using inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers

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The host and geographical diversity were the root causes of the genetic diversity of *C. chaxingu*. Wild strains of *C. chaxingu* mostly occurs on decayed and dead wood of broadleaf trees such as *Camellia oleifera*, *Populus* spp. and *Salix* spp.. It is mainly distributed in temperate and subtropical regions of China, such as Jiangxi, Fujian, Hunan, Sichuan and Yunnan Provinces [31]. In the previous study, the genetic diversity of eight *C.chaxingu* varieties in Hunan Province was analyzed using amplified fragment length polymorphism (AFLP) technique, the percentage

of polymorphic amplified by AFLP primers [32] was 94.10% higher than characterized by ISSR and SRAP (81.33% and 79.52%) in this study. It could be that ISSR and SRAP techniques target different parts of the genome. Since each DNA maker system has its own advantages, it is important to use more than one DNA marker system in the analysis of genetic diversity [17, 30].

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ISSR, SRAP and ISSR + SRAP dendrograms of the 24 test strains generally exhibited highly similar clustering patterns. For example, strains JAUCC 1920, JAUCC 1921, JAUCC 1922, JAUCC 1925 and JAUCC 1927 from the Yunnan Province in China clustered together in each case. The strain JAUCC 0727 which collected from the campus of Jiangxi Agricultural University always form a separate branch and more closely related to strains from Yunnan. These results possibly indicating that cultivated strains in Yunnan, with a narrow genetic basis, had been domesticated from wild-type strains. In fact, the findings indicated that Intra-strain ITS heterogeneity with positional double peaks was identified in most *C. chaxingu* strains [20, 33], suggested that most of the strains cultivated in China may have been obtained through hybridization. We learned that there are a lot of farmers who were using the random labeling of strains and the introduction into different regions of identical strains under different designations to grow *C. chaxingu* for economic benefit, especially in Jiangxi and Fujian provinces. The lack of new germplasm and the haphazard-introduction of species from one region to another may

impede breeding efforts. Therefore, explore more wild resources and breeding good character, high yield and stable *C. chaxingu* strains is conducive to the development of *C. chaxingu* industry. The success of strain selection depends on the investigation of genetic diversity [34].

In summary, DNA maker system is an efficient tool to reveal genetic relationship among different genotypes through the numbers of polymorphisms detected [35]. Surveying the genetic variation through ISSR and SRAP analyses could be useful in the selection of parental strains for breeding purposes. Our data showed that the 24 *C. chaxingu* strains cultivated in China showed high levels of genetic diversity. Strain JAUCC0727 has a unique genotype, it may be a high quality parent material in breeding.

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Acknowledgements

The authors would like to thank the members of Bioengineering and Technological Research Centre for Edible and Medicinal Fungi, Jiangxi Agricultural University (Nanchang, China) for their technical support

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