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Short communication

Random amplified polymorphic DNA (RAPD) analysis and the nucleosides assessment of fungal strains isolated from natural *Cordyceps sinensis*

K. Feng[a], S. Wang[a], D.J. Hu[a], F.Q. Yang[a], H.X. Wang[b], S.P. Li[a,*]

[a] *Institute of Chinese Medical Sciences, University of Macau, Macao*
[b] *Department of Microbiology, China Agricultural University, Beijing 100094, PR China*

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ABSTRACT

Random amplified polymorphic DNA (RAPD) and high performance liquid chromatography (HPLC) were applied to investigate genetic and chemical variations of 2 natural *C. sinensis*, 16 fungal strains isolated from *C. sinensis*, and 2 fungal strains of *C. militaris*. Five of the 68 arbitrary decamer primers were available for discrimination of the investigated samples. As a result, 20 investigated samples were divided into three main clusters according to the genetic distance, and some fungal strains isolated from natural *C. sinensis* were obviously different. But according to the contents of nucleosides, including uracil, uridine, hypoxanthine, inosine, guanosine, adenosine, adenine, and cordycepin, natural and cultured *Cordyceps* were in two individual sub-groups, which suggested that chemical characteristics among cultured mycelia of different fungal strains isolated from natural *C. sinensis* were similar, but they were different from natural one.

1. Introduction

Cordyceps sinensis, also known as "DongChongXiaCao" (winter worm summer grass), is one of the most precious Traditional Chinese Medicines due to its multiple pharmacological activities [1]. It is rare and expensive because it is a typical caterpillar fungus only found in the soil of a prairie at an elevation of 3500–5000 m, mainly in the provinces of Tibet, Qinghai, Sichuan, Gansu and Yunnan of China. In order to resolve the shortage of natural *C. sinensis*, scientists have extensively developed substitutes of cultured *Cordyceps* in last decades. Up to date, more than 13 genera including 22 species fungi have been isolated from natural *C. sinensis* [2]. Several fungal strains, e.g. *Paecilomyces hepiali* and *Cephalosporium sinensis*, isolated from natural *C. sinensis* have been manufactured in large quantity by fermentation technology [3], and their products are commonly sold as health food or drug in the market. Interestingly, it was reported that cultured *Cordyceps* mycelial products had similar chemical characteristics though their fungus species were different [4–6]. Recently, molecular approaches including random amplified polymorphic DNA (RAPD) [7–11], ribosomal DNA internal transcribed spacers (rDNA ITS) [12–16] and PCR-single-stranded conformational polymorphism (PCR-SSCP) analysis [17,18] have been widely employed for elucidation of genetic characteristics of *C. sinensis* and authentication of its anamorph. Especially, RAPD analysis does not require any sequence information of the genome besides simple, rapid, reliable and cost-effective [10,11].

At present, *Hirsutella sinensis* has been authenticated as anamorph of *C. sinensis* [7,9,13,14,16]. In addition, nucleosides are believed to be the active components in *Cordyceps* [19], and adenosine has also been used as a marker for the quality control [20]. However, the products of cultured *Cordyceps* derived from variant fungal species [3]. To date, there is no report for the relationship of genetic divergence of *Cordyceps* species and their nucleosides contents.

In this study, the genetic relationship between natural *C. sinensis* and its isolated fungi, including 2 natural *C. sinensis* samples, 16 fungal strains related to natural *C. sinensis*, and 2 fungal strains of *C. militaris*, were analyzed by using RAPD. Their chemical characteristics, nucleosides, were also determined and compared.

2. Materials and methods

2.1. Materials and chemicals

The caterpillar (S01) and fruiting body (S02) of *C. sinensis* were collected from Huzhu County, Qinghai of China. The fungal strains from natural *C. sinensis* (S03–S018) and *C. militaris* (S19 and S20) were prepared in our lab, or collected from academic institution and manufacturers. In brief, the strains LCT-1 (S03), LZST-2 (S04) and LZST-4 (S05) were isolated from the caterpillar and fruiting

* Corresponding author. Tel.: +853 8397 4692; fax: +853 2884 1358.
E-mail addresses: LISHAOPING@HOTMAIL.COM, spli@umac.mo (S.P. Li).

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body of *C. sinensis*, respectively, from Huangzhong County, Qinghai of China; *H. sinensis* (S06), BNQM (S09), BNQM-K (S10), CNB (S11), *Tolypocladium sinense* (S12), *Gliocladium roseum* (S13), HS-1 (S14), and HS-2 (S15) were collected from Institute of Microbiology of Chinese Academy of Sciences; QHJ (S07) and QHJ-11 (S08) were provided by Qinghai Academy of Animal and Veterinary Science; CS-50542 (S16), CS-50562 (S17), CS-50677 (S18) and *C. militaris*-51762 (S19) were collected from Agricultural Culture Collection of China; *C. militaris*-ZJG (S20) was provided by Zhangjiagang City Zanglian Biotechnology Co., Ltd., Jiangsu Province, China. Sixty-eight arbitrary decamer primers and kit for PCR were purchased from Beijing Dingguo Biotechnology Co., Ltd., China. Patoto dextrose agar (PDA, each 40.1 g contains the extract of 300 g fresh potato, 20 g dextrose, 15 g agar and 0.1 g chloramphenicol), bacterial peptone, and mold liquid medium (each 16.6 g contains 5.0 g peptone, 10 g dextrose, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, and 0.1 g chloramphenicol) were purchased from Guangdong Huankai Microbiol Sci. & Tech. Co., Ltd., China. Bacto™ Yeast Extract was from Becton, Dickinson and Company (Sparks, USA), and KH_2PO_4 (A.R Grad) and $MgSO_4{\cdot}7H_2O$ (A.R Grad) were purchased from Tianjin Damao Chemical Factory, China. Milli-Q water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, USA). Reagents not mentioned here were from standard sources.

2.2. Isolation and culture of fungal strains

The peridium on caterpillar of fresh *C. sinensis*, after cleaning, was peeled off carefully. Then the caterpillar and fruiting body were wiped twice with cotton balls soaked with 75% ethanol and separated them at the end of the fruiting body under axenic condition. The fruiting body was rived longitudinally, and a piece of inner tissue was picked up and transferred on improved PDA media (40.1 g PDA, 5.0 g bacterial peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, and 2 g yeast extract in 1 L with Milli-Q water, pH 5.6) in tube slant. These isolated tissues were incubated at 15 °C for 1 month to culture the fungus. Similarly, the fungus from dead caterpillar was also obtained.

The fungal strains in tube slant were transferred and implanted into improved mold liquid medium (16.6 g mold liquid medium, the extract of 200 g fresh potato, and 2 g yeast extract in 1 L with Milli-Q water, pH 5.6). Conical flasks (500 mL) containing 150 mL of medium were inoculated with purified colony and incubated in an C24KC refrigerated incubator shaker (New Brunswick Scientific, USA) under 150 rpm at 16 °C until plentiful mycelia balls presented. Mycelia were harvested by centrifugation, washed twice with sterile PBS buffer, and stored at 4 °C until use.

2.3. RAPD analysis

2.3.1. Genomic DNA extraction

Genomic DNA of natural *C. sinensis* was extracted from its fresh caterpillar and fruiting body collected from Qinghai. The investigated fungi genomic DNA were extracted from their fresh cultured mycelia, which was performed according to the protocol of nucleic acid isolation for E.Z.N.A™ Fungal DNA Mini Kit (Omega, USA). In brief, the ground sample (about 150 mg) was put into a 1.5 mL

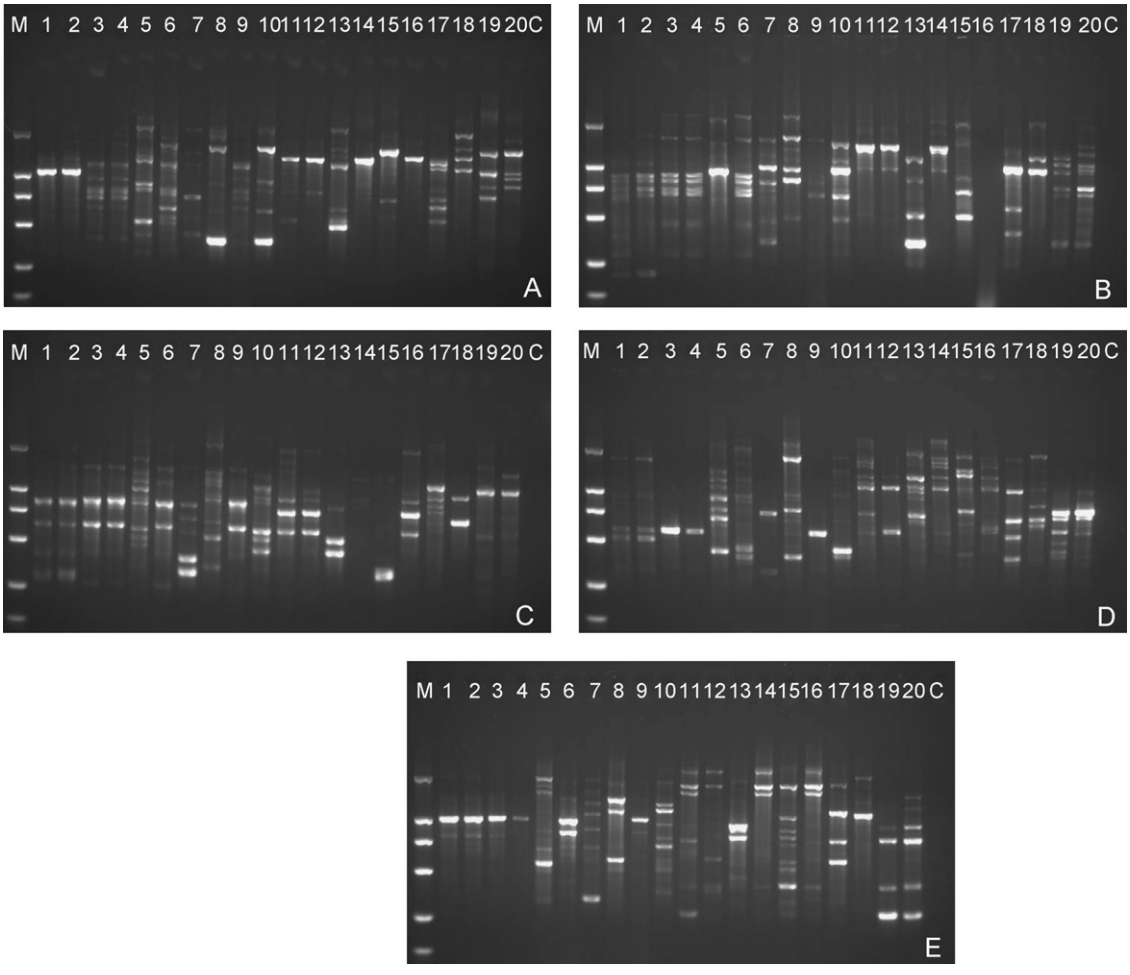

Fig. 1. RAPD patterns of genomic DNA from 20 investigated samples amplified using primer: (A) AP-A 20, (B) AP-D 18, (C) AP-G 05, (D) AP-H 18 and (E) AP-I 07. M, DNA marker-DL 2000 (2000, 1000, 750, 500, 250 and 100 bp); 1–20, S01–S20 same as in Table 1; C, blank control (without DNA template).

Eppendorf tube and added 1 mL buffer FG1 immediately, then the mixture was incubated at 65 °C for 30 min after vigorous vortex. Furthermore, buffer FG2 of 200 μL was added and vortexed to mix well before centrifugation at 13,000 × *g* for 5 min. Carefully aspirated the lysate to a new Eppendorf tube, and added 0.7 volume isopropanol and vortex to precipitate DNA, then centrifuged at 10,000 × *g* for 2 min to collect DNA. The obtained DNA was resuspended with 300 μL of 65 °C pre-heated sterile Milli-Q water, followed 4 μL RNase A at 65 °C. Then added 150 μL buffer FG3 followed by 300 μL absolute ethanol (−20 °C) to obtain a homogeneous mixture. The entire sample was applied to a HiBind® DNA column placed in a 2 mL collection tube and centrifuged at 10,000 × *g* for 1 min. Transferred the column bound DNA to a second collection tube and washed the column by adding 700 μL DNA wash buffer and centrifuged at 10,000 × *g* for 1 min twice. Transferred the dried column to a clean 1.5 mL tube and elution buffer was applied to the column to elute DNA twice. The purified genomic DNA was stored at 4 °C before use. The quality of genomic DNA was tested on 0.8% (w/v) agarose (USB, MB Grade, USA) gel, containing 20 μL/100 mL GelRed™ Nucleic Acid Stain (Biotium, USA), electrophoresis in TAE buffer, observed and photographed under UV light using a Molecular Imager ChemiDox XRS System (Bio-RAD, USA).

2.3.2. DNA amplification

Polymerase chain reaction (PCR) was performed in a total volume of 25 μL solution containing 20 ng template DNA, 2.5 μL PCR buffer (10×), 19.5 μL double distilled water, 1 μL primer (8 pmol/μL), 0.5 μL dNTP (10 mmol/L), and 0.5 μL *Taq* DNA polymerase (5 U/μL, Invitrogen, USA). PCR reactions were performed in a thermocycler (Gene Amp® PCR System 9700, Applied Biosystems, USA) and programmed as follows: initial denaturation at 95 °C for 5 min, followed by a total of 40 cycles consisting of a denaturation step at 94 °C for 1 min, an annealing step at 36 °C for 1 min, and an extension step at 72 °C for 2 min, and final 5 min extension at 72 °C. RAPD analysis for each primer was repeated at least once, and a blank control (without DNA template) was run in each of the PCRs.

PCR products were separated using 1.5% (w/v) agarose gel, containing 20 μL/100 mL GelRed™ Nucleic Acid Stain, electrophoresis in TAE buffer and observed and photographed under UV light using a Molecular Imager ChemiDox XRS System.

2.4. HPLC analysis

2.4.1. Sample preparation

Natural *C. sinensis* and cultured *Cordyceps* mycelia were dried using natural air drying and freeze drying, respectively. Dried powder of *Cordyceps* (0.2 g) was mixed with 10 mL Milli-Q water in a glass tube with stopper. After weighting accurately, the mixture was kept at room temperature (25 °C) for 18 h, and then boiled on Syncore Reactor (BUCHI-Syncore, Flawil, Switzerland) for 10 min [21]. The lost weight was made up with Milli-Q water, then centrifugation at 4500 rpm (Centrifuge 5415D, Eppendorf, Germany) for 15 min. The supernatant was filtered through a 0.45 μm Econofilter (Agilent Technologies, Palo Alto, CA, USA) before HPLC analysis.

2.4.2. Chromatograph condition

HPLC separations were performed on an Agilent Series 1100 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an auto-sampler, and a diode array detector (DAD) system, connected to a Agilent ChemStation software. A Zobax SB-AQ column (250 mm × 4.6 mm i.d., 5 μm) with a Zobax SB-C_{18} guard column (20 mm × 3.9 mm i.d., 5 μm) was operated at 20 °C. Solvents that constituted the mobile phase were (A) deionized water and (B) acetonitrile. The separation was achieved using gradient elution of 0–10 min with 0% B, 10–40 min

Table 1
Genetic distance based on the shared RAPD bands.

	S01	S02	S03	S04	S05	S06	S07	S08	S09	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20
S01	0.0000																			
S02	0.0270	0.0000																		
S03	0.4872	0.4737	0.0000																	
S04	0.5263	0.5135	0.0256	0.0000																
S05	0.8750	0.9149	0.9592	0.9583	0.0000															
S06	0.6087	0.6000	0.3617	0.3913	0.7857	0.0000														
S07	0.8636	0.8605	0.7333	0.7273	0.8148	0.7692	0.0000													
S08	0.8039	0.8400	0.8077	0.8039	0.7049	0.6949	0.7544	0.0000												
S09	0.7059	0.6970	0.3714	0.4118	0.9545	0.5714	0.8000	0.8298	0.0000											
S10	0.7917	0.8298	0.8367	0.8333	0.6897	0.7857	0.8148	0.5738	0.8182	0.0000										
S11	0.8537	0.8500	0.8571	0.8537	0.7647	0.7959	0.7447	0.8519	0.8378	0.8824	0.0000									
S12	0.7778	0.7714	0.8378	0.8333	0.7826	0.8636	0.8571	0.7959	0.9375	0.8261	0.3846	0.0000								
S13	0.8095	0.8537	0.8605	0.8571	0.8077	0.7600	0.7917	0.7455	0.8421	0.6923	0.7778	0.9000	0.0000							
S14	0.8333	0.8286	0.9459	0.9444	0.6957	0.8182	0.9048	0.7143	0.9375	0.8696	0.4872	0.4118	0.8500	0.0000						
S15	0.7619	0.7561	0.7674	0.8095	0.7308	0.8000	0.8750	0.9273	0.7895	0.7692	0.8667	0.8000	0.7826	0.7500	0.0000					
S16	0.8182	0.8125	0.8824	0.8788	0.8140	0.9024	0.8974	0.9565	1.0000	0.9070	0.3889	0.2258	0.8378	0.4839	0.7838	0.0000				
S17	0.7619	0.7561	0.7674	0.7619	0.7308	0.7600	0.8750	0.7455	0.6842	0.7692	0.7333	0.7000	0.8696	0.8000	0.8696	0.8378	0.0000			
S18	0.8333	0.8286	0.8919	0.8889	0.6957	0.8636	0.9048	0.7551	0.8750	0.8696	0.8462	0.8235	0.7500	0.7647	0.8000	0.8710	0.8000	0.0000		
S19	0.8095	0.8049	0.8140	0.8095	0.8462	0.8800	0.6250	0.8182	0.8421	0.8462	0.6444	0.7500	0.7826	0.7500	0.7826	0.7838	0.7826	0.8500	0.0000	
S20	0.9091	0.9070	0.9556	0.9545	0.8889	0.9615	0.6800	0.7895	0.9500	0.8519	0.7021	0.7619	0.8333	0.8095	0.8750	0.8462	0.8333	0.9048	0.2917	0.0000

S01 (caterpillar of *C. sinensis*); S02 (fruiting body of *C. sinensis*); the strains from natural *C. sinensis*: S03 (LCT-1), S04 (LZST-2), S05 (LZST-4), S06 (*Hirsutella sinensis*), S07 (QHJ), S08 (QHJ-11), S09 (BNQM), S10 (BNQM-K), S11 (CNB), S12 (*Tolypocladium sinense*), S13 (*Gliocladium roseum*), S14 (HS-1), S15 (HS-2), S16 (CS-50542), S17 (CS-50562), S18 (CS-50677); the strains from *C. militaris*: S19 (*C. militaris*-51762) and S20 (*C. militaris*-ZJG).

with 0–25% B. The flow rate was 0.8 mL/min, and the injection volume was 10 μL. The analytes were monitored at 260 nm.

2.5. Data analysis

The bands of RAPD products were appointed as binary digit "1" and "0" which stand for band present and band absent, respectively [9,11]. The genetic distance (D) between two samples was calculated using the following formula:

$$D = 1 - F$$

F is an estimation of similarity calculated as $2Nxy/(Nx + Ny)$, where Nxy is the number of shared amplified DNA fragments in both samples x and y, Nx is the total number of fragments scored in sample x, and Ny is the total number of fragments found in sample y [11]. Hierarchical clustering analysis was done by SPSS 14.0 for windows (SPSS Inc., Chicago, IL, USA). A method named as average linkage between groups was applied, and Cosine distance was selected as measurement.

3. Results and discussion

3.1. RAPD polymorphisms of investigated samples

For RAPD analysis, 68 arbitrary decamer primers were screened with the template of natural *C. sinensis* (S02) to determine which primers can generate obvious bands for RAPD analysis. Then the selected 8 primers were employed for RAPD analysis of the investigated samples, and 5 primers, AP-A 20 (5′-GTTGCGATCC-3′), AP-D 18 (5′-GAGAGCCAAC-3′), AP-G 05 (5′-CTGAGACGGA-3′), AP-H 18 (5′-GAATCGGCCA-3′) and AP-I 07 (5′-CAGCGACAAG-3′), were finally employed because they could produce bands for all samples. Fig. 1 showed RAPD patterns of genomic DNA from 20 investigated samples amplified using 5 selected primers. Total 124 bands produced by 5 different arbitrary primers were scored and analyzed using SPSS 14.0 for windows. The genetic distance and molecular phylogenetic tree were shown in Table 1 and Fig. 2A, respectively. The results indicated that two external standard samples of S19 and S20, *C. militaris*, were closer in spite of they were from different regions. For natural *C. sinensis*, the genetic distance between samples S01 and S02, the caterpillar and fruiting body from the same origin, was the closest. Similarly, samples S03 and S04 also had the closest genetic distance, and they were similar to sample S06 (*H. sinensis*). These samples were grouped into a cluster, which suggested that the isolated fungus may be *H. sinensis*, anamorph of natural *C. sinensis* [7,9]. Indeed, they also have same strains appearance. The difference between natural *C. sinensis* and their isolated fungi may attribute to the different origins, which is in accordance with the previous report [11]. The other investigated fungal strains were significantly different from natural *C. sinenis* except sample S09 based on the distributions of RAPD polymorphisms though they all derived from natural *C. sinenis* (Fig. 2A).

3.2. Chemical characteristics of investigated samples

The contents of nucleosides, including uracil, uridine, hypoxanthine, inosine, guanosine, adenosine, adenine, and cordycepin, in natural and cultured *Cordyceps* were determined, and the results were summarized in Table 2. Hierarchical cluster analysis for the tested 17 samples of *Cordyceps* was performed based on the contents of tested nucleosides. As a result, they were divided into two groups, cluster I and cluster II (Fig. 2B). Cluster I was

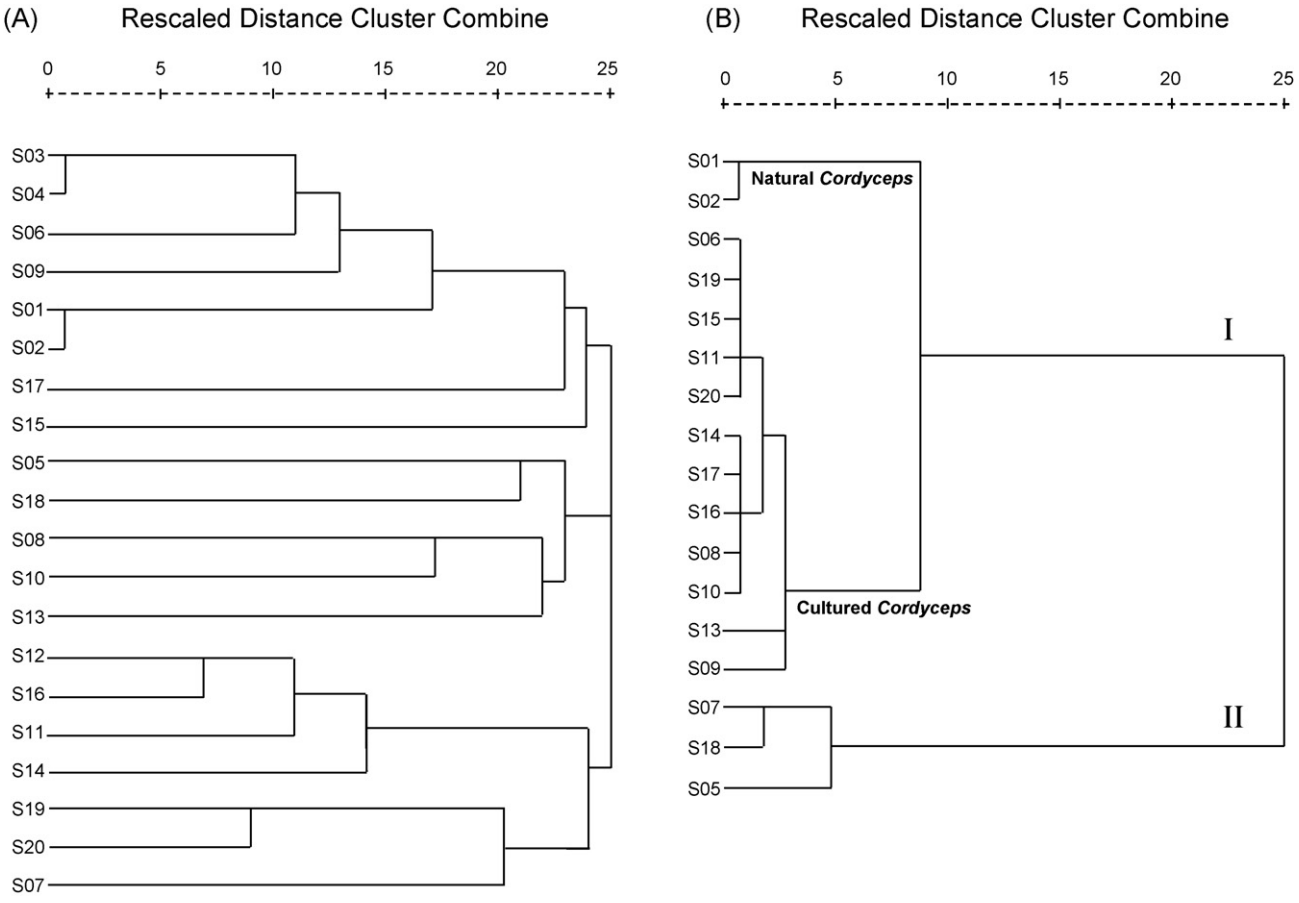

Fig. 2. Dendrograms of investigated samples based on RAPD polymorphisms (A) and peak areas of eight investigated compounds (B). The hierarchical clustering was done by SPSS software. A method named as average linkage between groups was applied, and Cosine distance was selected as measurement. S01–S20, same as in Table 1.

Table 2
Contents (mg/g) of eight investigated compounds in natural and cultured *Cordyceps*.

	Sample no.	Analytes							
		Uracil	Uridine	Hypoxanthine	Inosine	Guanosine	Adenosine	Adenine	Cordycepin
Natural *Cordycep sinensis*	S01	0.11[a]	2.22	0.18	1.29	1.50	0.06	0.06	–[b]
	S02	0.07	2.35	0.18	1.28	1.54	0.07	0.06	–
Cultured *C. sinensis*	S05	4.45	0.07	2.69	0.24	0.27	–	–	–
	S06	0.07	6.56	0.09	0.07	5.92	4.34	0.09	–
	S07	2.65	1.29	1.40	0.21	0.29	0.19	–	–
	S08	0.11	5.42	–	0.13	9.41	6.77	–	–
	S09	0.16	7.27	0.06	0.08	3.25	4.34	0.59	–
	S10	0.03	2.22	–	0.54	3.58	2.65	–	–
	S11	0.10	3.50	–	0.23	3.01	2.65	0.10	–
	S13	0.82	1.24	0.07	0.33	1.78	1.42	–	–
	S14	0.06	5.02	–	0.19	5.72	4.94	–	–
	S15	0.31	4.61	–	0.20	3.77	3.08	0.12	–
	S16	0.06	4.58	–	0.12	5.41	4.21	–	–
	S17	0.09	2.91	±[c]	0.16	3.35	2.93	–	–
	S18	2.18	1.43	1.19	–	0.42	0.82	0.20	–
Cultured *C. militaris*	S19	0.05	2.78	–	0.04	2.57	2.02	–	–
	S20	±	6.97	–	0.12	5.14	3.92	0.08	±

Note: S01–S20, same as in Table 1.
[a] Average of duplicates.
[b] Under the limited of detection.
[c] Not sure.

further divided into 2 sub-groups, natural *Cordyceps* and cultured *Cordyceps* including cultured *C. militaris* (S19 and S20). The results show that natural *Cordyceps* are different from cultured one, which is in accordance with the previous reports [4–6]. Actually, the chemical characteristics of stromata and dead caterpillar from natural *C. sinensis* are very similar [22]. In addition, cordycepin is a rich specific component of *C. militaris*, but little can be found in its cultured mycelia. So the difference of nucleosides between natural and cultured *Cordyceps* mostly attributes to nucleosides variation during the growth of fungus. Indeed, the metabolite profiles may be highly variable during the ontogeny of a given fungal organism [23].

3.3. Correlation of genetic distance and chemical characteristics of investigated Cordyceps samples

Considering the genetic difference and chemical variation (Fig. 2), it is interesting that chemical characteristics of nucleosides in cultured *Cordyceps* mycelia are similar although their genetic distance may be far, even if different species of fungus. The causes may be: (1) nucleosides are primary metabolites, which are similar in most organisms; (2) some chemical compounds only can be produced at certain stage of fungal growth [23,24]. Therefore, it is crucial to develop cultured *Cordyceps* with the chemical characteristics of natural *C. sinensis* during the industrial fermentation.

4. Conclusions

The developed RAPD analysis is feasible to distinguish the fungal strains isolated from natural *C. sinensis*. The investigated nucleosides including uracil, uridine, hypoxanthine, inosine, guanosine, adenosine, adenine, and cordycepin are similar in cultured *Cordyceps* mycelia, even if they are different species of fungus. The results suggest that quality control of culture *Cordyceps* should be further carefully studied.

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