KCS Ⅳ-3

A molecular genetic study on the fruiting-body formation of *Cordyceps militaris*.

T.C. Wen[1], M.F.Li[2], J.C. Kang[1*], B.X. Lei[1]

[1] *The Research Center for Biochem-engineering at Guizhou, Guizhou University, Guiyang 550025, Guizhou Province, PR China*

[2] *College of Life Science, Guizhou University, Guiyang 550025, Guizhou Province, PR China*

Abstract: In the fungal genus *Cordyceps,* the type species *Cordyceps militaris* produces bioactive ingredients and exhibits medicinal effects as a Traditional Chinese Medicine (TCM). The fruiting bodies of *C.militaris* have now been mass-produced artificially and used as functional food and medicine in China. The unstable variation in forming fruiting body is however a key restrictive factor in industrial production. The genetic study on *in vitro* stromata formation of C. *militaris* has rarely been carried out. Here, we report the effects of genetic variation including the mating system on perithecial stromata formation of C. *militaris*. Monoconidial isolates which have both MAT1-1-1 and MAT1-2-1(genotype MAT1-1/2) could produce stromata. While the isolates only have either MAT1-1-1 or MAT1-2-1 (genotype MAT1-1 or MAT1-2) failed to produce stromata. Despite obvious heterothallism, homothallism was occasionally observed in a few isolates of *C. militaris*. High genetic variation was observed amongst the different monoconidial isolates of *C. militaris*. The unstable variation or lose of fruiting body formation was found to be caused by the inner-species high genetic variation of *C. militaris*. These results also indicated that C. *militaris* sexually behaved as both heterothallic and homothallic and required two mating type compatible in the same culture in order to produce regular clubshaped perithecial stromata.

Keywords: *Cordyceps militaris*; genetic variation; molecular phylogeny; homothallism; heterothallism; artificial fruiting

* Corresponding author. Jichuan Kang; email: bcec.jckang@gzu.edu.cn

INTRODUCTION

The genus *Cordyceps* (Fr.) Link (Clavicipitaceae, Hypocreales, Ascomycta) is a large, cosmopolitan genus, comprising of 460 to 500 species and varieties[1]. Most of its members are pathogenic to different insects, spiders, and few grow on hypogeal fungi of *Elaphomyces* spp. They are mainly distributed in the sub-tropical to temperate regions of the world. *Cordyceps* species are highly regarded as medicinal mushrooms in east Asia for thoudsands of years.

C. militaris is the type species of the genus *Cordyceps*. *C. militaris* has been recently used as functional food and medicine in China. C. *militaris* was characterized with the production of cordycepin that was found to be effective in antitumor and antivirus. *C. militaris* has been studied for the commercial production of bioactive compounds through their *in vitro* culture[2-5]. Artificial culturing of *C.militaris* is a good way to solve the insufficient resource in the nature. However in the process of artificial culturing of *C. militaris*, the isolates showed unstable variation in forming fruiting body. Most of the isolates failed to produce stromata or produced only few deformed ones. Other isolates, which used to produce good fruiting bodies, could not produce the same quality of fruiting body when their subcultures were used. The declining in forming fruiting body of the isolates is a key restrictive factor in industrial production. At present, there are only few reports on molecular study of genetic variation and mating system of *C. militaris*[7,8]. But conflicting types of mating system in *C. militaris* was reported [7,9].Sato *et al.*[9]considered that *C. militaris* has homothallism based on the research by lepidopteran pupae.Shrestha[7] reported that *C. militaris* behaved as a bipolar heterothallic fungus and requires two mating compatible strains in order to produce regular clubshaped perithecial stromata. The evidence mainly based on mono-ascospore strains was however insufficient due to lack of molecular biology techniques.

In this study, molecular genetics of *in vitro* stromata formation of *C. militaris* has been carried out based on monoconidial isolates. Mating system was determined by observing perithecial stromata formation and PCR assay. Herewith, we report the effects of genetic variation including the mating system on perithecial stromata formation and both heterothallic and homothallic sexual behavior of C. *militaris*.

MATERIALS AND METHODS

Fungal culture

Paecilomyces militaris isolates WWM04 (named YUANSHIJUNZHU) were originally isolated from mountainous Sichuan, China. The isolate was isolated from wild perithecial stromata and maintained on PDA medium at 25℃ at 15:9 L:D.

Twenty-two mono-conidium isolates (named SSP1 ~ SSP22) were established from the original isolates[11]. *Paecilomyces militaris* WWM04 was cultivated until sporulation was evident (approximately 10 days). Conidia and attached hyphae were removed with a loop and transferred to fresh medium. This was repeated for twelve successive in vitro sub-cultures (named TUIHUAJUNZHU). All cultures were stored at 2℃ until use in bioassays.

Inoculum preparation and fruiting

C. militaris was initially cultured on PDA in a petri dish, and then transferred to the seed culture by punching out 5 mm of the agar disc with a sterilized self-designed cutter. The seed culture was in a 250 ml flask containing 50 ml of basal medium, on a rotary shaking incubator, 26℃, 130 rev min^{-1} for 4 d. Fruiting medium of *C. militaris* was prepared by mixing 20 g of rice and 32 mL of liquid medium (20 g l^{-1} sucrose, 20 g l^{-1} peptone, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 1g l^{-1} K_2HPO_4 with 1000ml distilled water) in glass bottle and were autoclaved for 20 min at 121℃.Each glass bottle containing fruiting medium was inoculated with 5mL of liquid inoculum of *C. militaris* for *in vitro* fruiting. After inoculation, the bottles were incubated at 20℃ under dark for 10days,then under 14L:10D at 25℃ (light about 500 lux) and high humidity conditions (80-90%) for 50 days.All experiments were performed at least in duplicate (10 bottles for once) .

DNA extraction, PCR and sequencing

Reagent and DNA extraction

Taq enzyme and dNTP was obtained from Shanghai Sangon, An Agarose Gel DNA Purification kit ver 2.0 was from TRKARA Company. Fresh, sporulating cultures on Czapek agar were used for DNA extraction following Tigano-Milani *et al.*[10]; the extracted DNA is stored at -20℃.

PCR amplification and determination of DNA sequences

In the first preliminary experiments, when different DNA extraction methods were compared, RAPD-PCR amplifications were performed with an Gene Amp PCR system 9700(Bio-RAD) with a modified RAPD program (one cycle of 60 s at 95℃ followed by 40 cycles of 20 s at 94℃ , 60 s at 36℃ and 60 s at 72℃) . 50μL reaction system: 10× reaction buffer 5 μL, l0mM dNTP 0.66μL, random primer 2μL 25mM $MgCl_2$ 5μl,3μL of template DNA (50 ng/μl) ,Taq DNA polymerase 0.66μl and ddH_2O 33.68μL.

165 random 10-base oligonucleotide primers (Shanghai Sangon,Shanghai, China) were used in these experiments.

To amplify ITS1-5.8S- ITS2 rDNA, the following primers were used: ITS4 (5′-TCCTCCGCTTATTGATATGC- 3′). Polymerase chain reaction (PCR) amplification was performed according to the manufacturer's instructions, 50μL reaction system: 10× reaction buffer 5 μL, dNTP 1μL, primer ITS4 1μL, ITS5 1μL, Pfu buffer 0.5 μl, 2μL of template DNA and ddH_2O 39.5 μL. The amplification program : a first step of 94°C for 5 min; then 35 cycles consisted of 94°C for 40 s, 49°C for 40 s, and 72°C for 1 min; and a final step of 72°C for 10 min.

In the amplification of mating-type locus MAT1-1 a primer set [5′-GATGCGGAAC-GTTTATCTGG-3′ and 5′-CCCATCTC(A/G)TC(A/C)CGGAC(A/G)AA(C/G)GA-3′] was used[14]. PCR products were purified using Agarose Gel DNA Purification kit ver 2.0 according to its procedure (TAKARA Company), and purified DNA samples were sequenced using ABI PRISM 310 Genetic Analyzer (Perkin–Elmer).

Sequence alignment and phylogenetic analysis

Sequences were aligned by ClustalX ver.1.83, and adjusted to maximize homology. Then the phylogenetic tree was constructed using Neighbour-Joining (NJ) and Maximum Parsimony (MP) methods in PAUP 4.0b10. Confidence values for individual branches were determined by bootstrap analysis with 1000 replications.

RESULTS AND DISCUSSION

In the all twenty-four isolates, Two mono-conidium isolates SSP1 and SSP3, YUANSH- IJUNZHU and TUIHUAJUNZHU produced perithecial club-shaped stromata; The other twenty mono-conidium isolates SSP2 and SSP4~SSP22 produced either no stromata or only abnormal nonperithecial stromata.

All twenty-four isolates from the same original isolates differed significantly in their ability to form fruiting bodies, morphological characteristics and mycelium growth rate.In particular,the difference between the twenty no fruiting mono-conidium isolates SSP2 and SSP4 ~ SSP22 were also highly significant. These results confirmed the polymorphism in the anamorph of *C. militaris* [12,13]

RAPD polymorphism

Initial screening of 165 RAPD primers resulted in the identification of 25 primers that yielded unambiguously scorable bands with high reproducibility.These 25 selected primers amplified between 5

and 15 bands each. Most of the PCR products ranged between molecular weight of 200 bp to 2500 bp (Fig. 2).

The twenty-five oligonucleotide primers produced a total of 247 bands among the 6 *P.militaris* isolates including YUANSHIJUNZHU、TUIHUAJUNZHU、SSP19、SSP2、SSP7 and SSP21. Among them, 235 RAPD markers showed polymorphism (95.14%) (Table 2).The primers of S23, S3, S37, S46, S51, S61, S67, S80, S92, S103, S151, S153, S219 and S354 gave the highest polymorphism (100% of the bands) and the greatest number of genotypes.

Based on the analysis of all primers, average distances among YUANSHIJUNZHU and no fruiting isolates; among TUIHUAJUNZHU and no fruiting isolates; between YUANSHI- JUNZHU and TUIHUAJUNZHU, and among the all no fruiting isolates were 0.4048, 0.4027, 0.3026, and 0.4438, respectively. The average distance (0.4438) of no fruiting isolates showed higher diversity than those of others.

Genetic distances among sites

The genetic distances were calculated among the 6 isolates, based on null allele frequencies within each isolate (Table 3). There was great variation in the genetic distances matrix. The longest genetic distance, 0.4890, was encountered between SSP2 and TUIHUAJUNZHU, followed by SSP2 and SSP7 (0.4889) and SSP19 and SSP7 (0.4777). The shortest distance, 0.2834, was between SSP19 and TUIHUAJUNZHU, followed by YUANSHIJUNZHU and TUIHUAJUNZHU (0.3026). These genetic distances and phenotypes were positively correlated. For example, YUANSHIJUNZHU and TUIHUAJUNZHU (0.3026) have the same colony and both of them can produce stromata.SSP19 and YUANSHIJUNZHU (0.3765), SSP19 and TUIHUAJUNZHU (0.2834) also have shorter distance because of their same colony characteristics.

Cluster analysis

A dendrogram was constructed from a UPGMA cluster analysis based on matrix of genetics distance among the 6 isolates (Figure 3). YUANSHIJUNZHU, TUIHUAJUNZHU, SSP19, and SSP7 together form one cluster, and SSP2 and SSP21 form another. Within these two clusters, SSP19 and TUIHUAJUNZHU are the most similar or related sites. This result is in good agreement with phenotype similarity coefficients (also have same colony characteristics and failed to produce fruit body)

Table 2. Polymorphism provided by the RAPD primers.

Primers codes	Nucleotide sequence	Number of scorable PCR products	Number of ploymorphic PCR products
S3	CATCCCCCTG	15	14
S6	TGCTCTGCCC	12	10
S20	GGACCCTTAC	5	4
S23	AGTCAGCCAC	8	8
S26	GGTCCCTGAC	7	6
S30	GTGATCGCAG	9	9
S37	GACCGCTTGT	10	10
S46	ACCTGAACGG	12	12
S51	AGCGCCATTG	11	11
S61	TTCGAGCCAG	8	8
S67	GTCCCGACGA	9	9
S79	GTTGCCAGCC	9	8
S80	ACTTCGCCAC	12	12
S90	AGGGCCGTCT	10	9
S92	CAGCTCACGA	10	10
S103	AGACGTCCAC	11	11
S136	GGAGTACTGG	11	10
S140	GGTCTAGAGG	12	10
S151	GAGTCTCAGG	11	11
S153	CCCGATTCGG	12	12
S216	GGTGAACGCT	8	7
S219	GTCCGTATGG	13	13
S301	CTGGGCACGA	14	13
S354	CACCCGGATG	8	8
S360	AAGCGGCCTC	10	9
总计		247	235

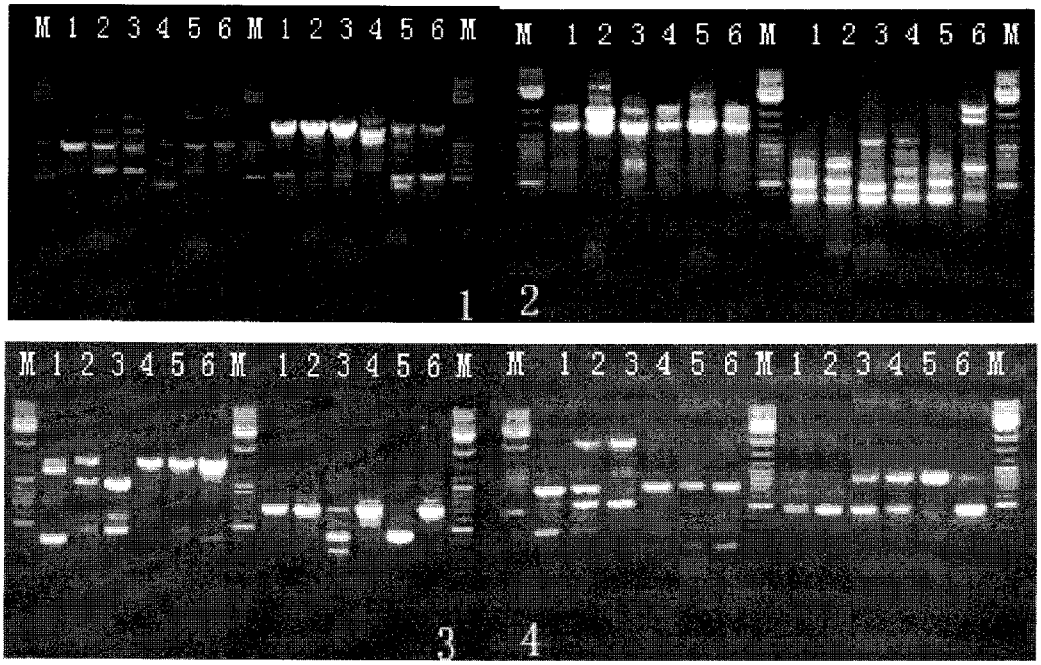

Fig. 2. Comparison of amplification patterns obtained by random amplified polymorphic DNA with selected 8 primers(1,S151 and S153 ; 2,S140 and S301 ; 3,S37 and S61 ; 4,S23 and S20) from genomic DNAs of 6 isolates (from left to right): 1,YUANSHIJUNZHU; 2,TUIHUAJUNZHU; 3,SSP19; 4,SSP2;5,SSP7;6,SSP21; M: molecular weight marker (SM0331 mix DNA ladder; Fermentas, Burlington, Canada).

Table 3. Estimation of matrix genetic distances between the 6 isolates with enset clones studied using RAPD.

	YUANSHIJUNZHU	TUIHUAJUNZHU	SSP19	SSP2	SSP7	SSP21
YUANSHIJUNZHU	0.0000					
TUIHUAJUNZHU	0.3026	0.0000				
SSP19	0.3765	0.2834	0.0000			
SSP2	0.4656	0.4890	0.4534	0.0000		
SSP7	0.3865	0.4374	0.4777	0.4889	0.0000	
SSP21	0.3906	0.4009	0.4656	0.3684	0.4089	0.0000

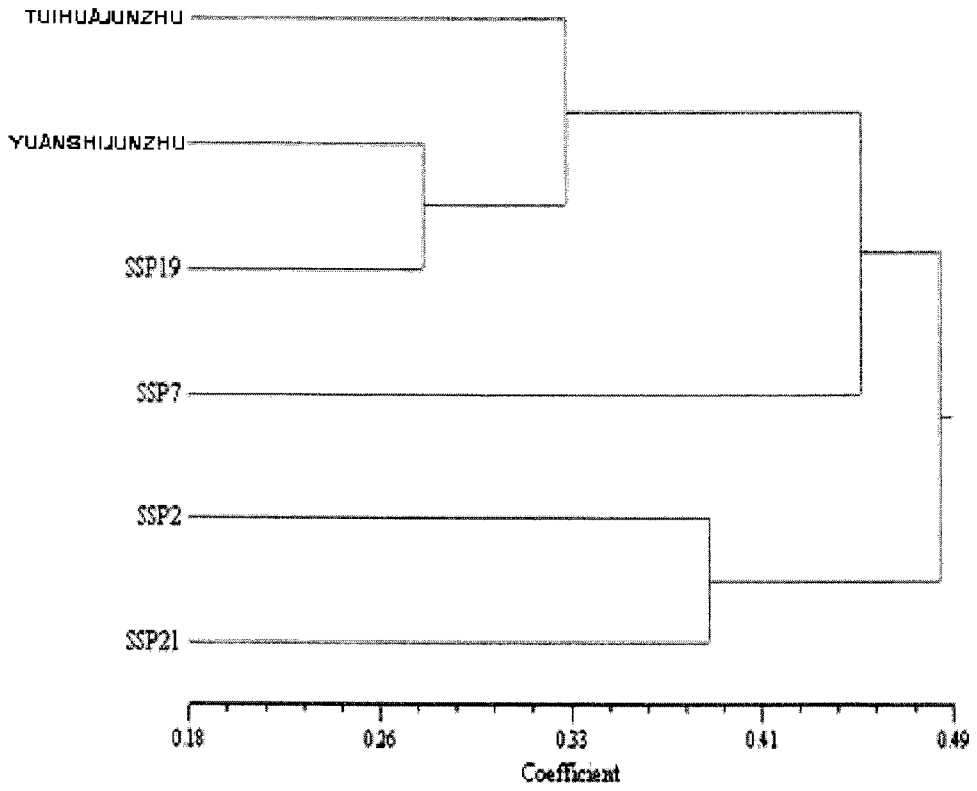

Fig. 3. UPGMA dendrogram calculated from RAPD profiles in enset, based on matrix genetic distances among 6 isolates.

PCR-based assay of the genotype MAT1-2 and fruiting

The primer sets (MAT1-1-1 and MAT1-2-1) designed in this study could amplify the MAT1-1-1 gene of the 11 isolates and the MAT1-2-1 gene of the 13 isolates of *Paecilomyces militaris* with a molecular weight of 220 bp to 250 bp (Table 4, Figure 4). These results indicated that YUANSHIJUNZHU、TUIHUAJUNZHU、SSP1 and SP3 which had both MAT1-1-1 and MAT1-2-1 as the genotype MAT1-1/2 could produce stromata. While the other isolates which only had either MAT1-1-1 or MAT1-2-1 as the genotype MAT1-1 or MAT1-2 could not produce stromata.

YUANSHIJUNZHU and TUIHUAJUNZHU which were not derived from single ascospore or conidium but from a mass of ascospores and conidium with a heterothallic mixture of MAT1-1 and MAT1-2 cells possess both MAT1-1-1 and MAT1-2-1. SSP1 and SP3 which were derived from a single conidium also possess both MAT1-1-1 and MAT1-2-1. There could be three reasons. The first reason could be that *P. militaris* SSP1 and SSP3 are homothallic (genotype is MAT1-1/2). The second reason might be the disomic (or diploid) condition, in which a nucleus (or nuclei) in mono-conidiums contained one extra chromosome of opposite mating type locus. The third reason might be due to mating

heterokaryotic condition, in which mono-conidiums consisted of more than one nucleus with opposite mating type locus; and like *Candida albicans*, *Botrytinia fuckeliana* and *Chromocrea spinulosa* that contained both homothallic and heterothallic mating system [6, 15].

Recently, heterothallism in *C. takaomontana* have been reported and the mating type loci of *C. takaomontana* have been sequenced [16,17], although it's mating system is still unknown. Besides Clavicipitaceae family, mating systems of members of other orders and classes of filamentous Ascomycetes have been described and mating type loci have been sequenced [7]. Until now, it has not been fully understood why certain single ascospore or conidial strains of heterothallic filamentous ascomycetous species behave as self-fertile. In *Neurospora crassa,* bisexuality has been reported, but most of the cases might be due to simple mixtures of ascospores during isolation and further growth. Mating type heterokaryosis and self-fertility have been recently reported in *Cryphonectria parasitica* [18]. Similarly, a mating system with multiple mating type alleles has been reported in the filamentous ascomycete *Glomerella cingulata* [7].

Table 4. Fruiting-body formation and mating type test of C. *militaris*.

Strain	Mating type	Fruit body formation*
YUANSHIJUNZHU	MAT1-1/2	10/10
TUIHUAJUNZHU	MAT1-1/2	8/10
SSP1	MAT1-1/2	8/10
SSP3	MAT1-1/2	6/10
SSP5	MAT1-1	No fruiting
SSP6	MAT1-1	No fruiting
SSP8	MAT1-1	No fruiting
SSP9	MAT1-1	No fruiting
SSP10	MAT1-1	No fruiting
SSP12	MAT1-1	No fruiting
SSP14	MAT1-1	No fruiting
SSP2	MAT1-2	No fruiting
SSP4	MAT1-2	No fruiting
SSP7	MAT1-2	No fruiting
SSP11	MAT1-2	No fruiting
SSP13	MAT1-2	No fruiting
SSP15	MAT1-2	No fruiting
SSP16	MAT1-2	No fruiting
SSP19	MAT1-2	No fruiting
SSP21	MAT1-2	No fruiting

*Fruit body formation was examined in 10 trials.

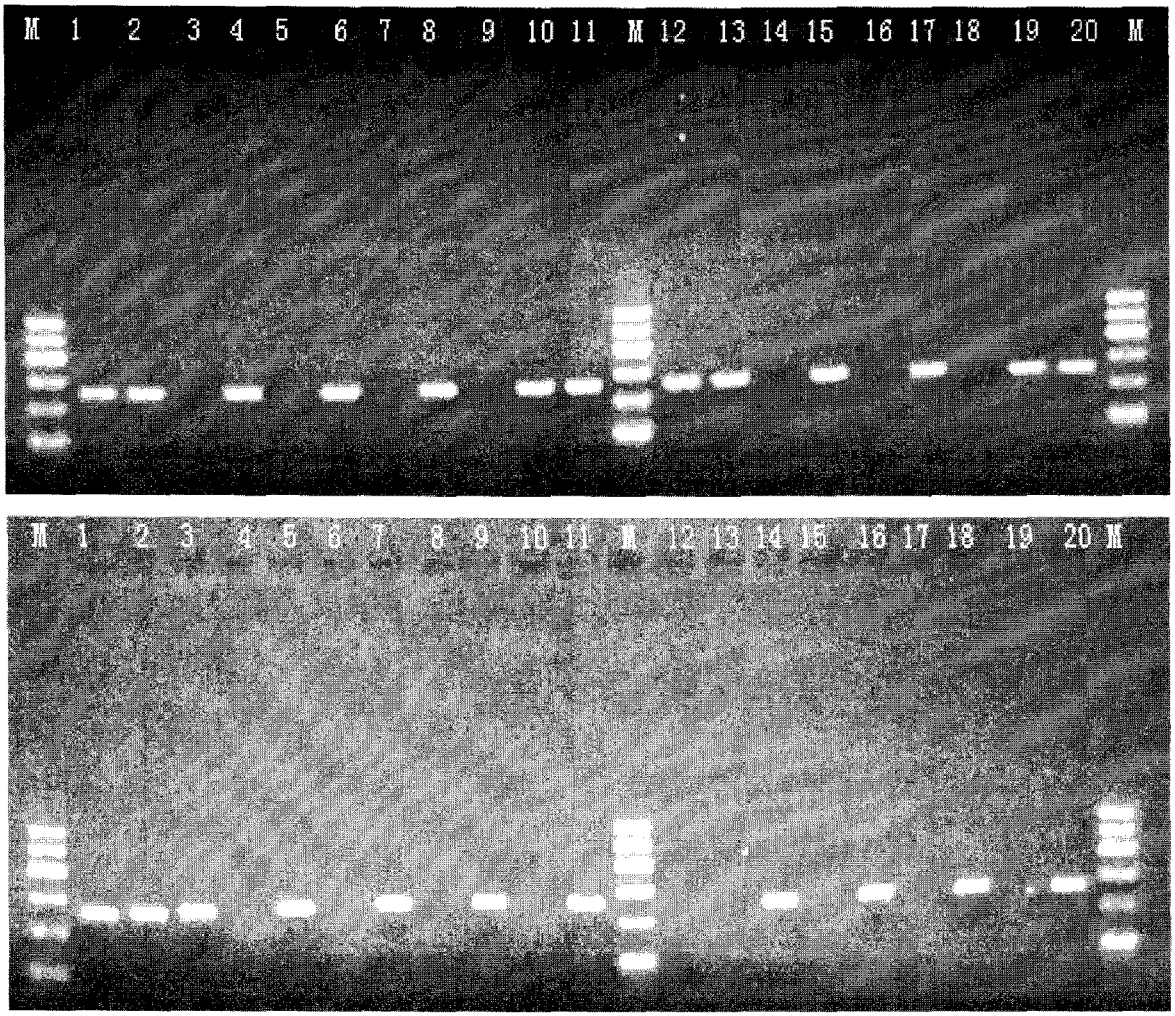

Fig. 4. Results of PCR assay for the *MAT1-2-1*(the upper part), *MAT1-1-1* (the latter part).PCR products were electrophoresed on a 1.5% agarose gel. Lane M is a 100bp DNA ladder (Fermentas, Burlington, Canada). Lanes 1 to 20 show the PCR products from *C. militaris* YUANSHIJUNZHU, TUIHUAJUNZHU, SSP9, SSP2, SSP6, SSP4, SSP5, SSP7, SSP10, SSP16, SSP1, SSP11, SSP13, SSP12, SSP15, SSP8, SSP19, SP14, SSP21, SP3, respectively.

Phylogenetic analyses of ITS rDNA and the mating-type genes sequences

ITS rDNA sequences are widely used in the phylogenetic analysis of *Cordyceps* [19][20], and has a benefit that both mating-type strains can be simultaneously examined. The mating-type genes MAT1-1-1 and MAT1-2-1 are the most conserved genes in the mating-type loci MAT1-1 and MAT1-2, respectively [21]. We performed the phylogenetic analyses of mono-conidium isolates and sub-culturing isolates of *C. militaris* using ITS rDNA sequence and MAT1-2-1 by the neighbor joining and maximum-parsimony methods (Fig.5, 6). To evaluate the diversity amongst mono-conidium isolates and sub-culturing isolates of *C. militaris*, we chose isolates YUANSHIJUNZHU, TUIHUAJUNZHU, SSP1, SSP2, SSP3, SSP4, SSP7, SSP11, SSP13, SSP15, SSP16, SSP19 and SSP21. In the alignment of ITS rDNA sequences, the dataset comprised 522 sites including 7 parsimony informative characters (PIC). While in the MAT1-2-1 sequences alignment, the dataset comprised 229 sites, 6 parsimony informative characters (PIC).

The two phylogenetic trees (Fig. 5 and Fig. 6) showed similar results.The phylogenetic tree of mating-type genes (Fig.6) showed better resolutions than that of ITS rDNA gene. In the two trees, TUIHUAJUNZHU and SSP1 as genotype MAT1-1/2 which could produce stromata formed one cluster, and SSP2 and SSP21 formed another. This result is in agreement with the RAPD dendrogram. The genetic distances showed in the two trees correlated with phenotypes positively.The genetic distance shows that diversity among no fruiting strains is very high.The diversity between YUANSHIJUNZHU(original strain) and other strains is higher than the diversity among no fruiting strains.These results indicated that genetic diversity among inner-species of *C.militaris* is very high.

In this study, high genetic variation of *C. militaris* from different monoconidial isolates and their serial sub-culture was found. In contrast, Wang *et al.* [22] reported that the genetic diversity of *C.militaris* (inter-species) was extremely small and did not correlate with geographical origins and types. It was concluded, therefore, that the reason of *C. militaris* showed unstable variation in fruiting-body formation was dued to high genetic variation of inner-species. Furthermore, the unstable variation in fruiting-body formation could represent go with their lose genotype of MAT1-1/2 in a same culture after serial sub-cultures of the same isolate.

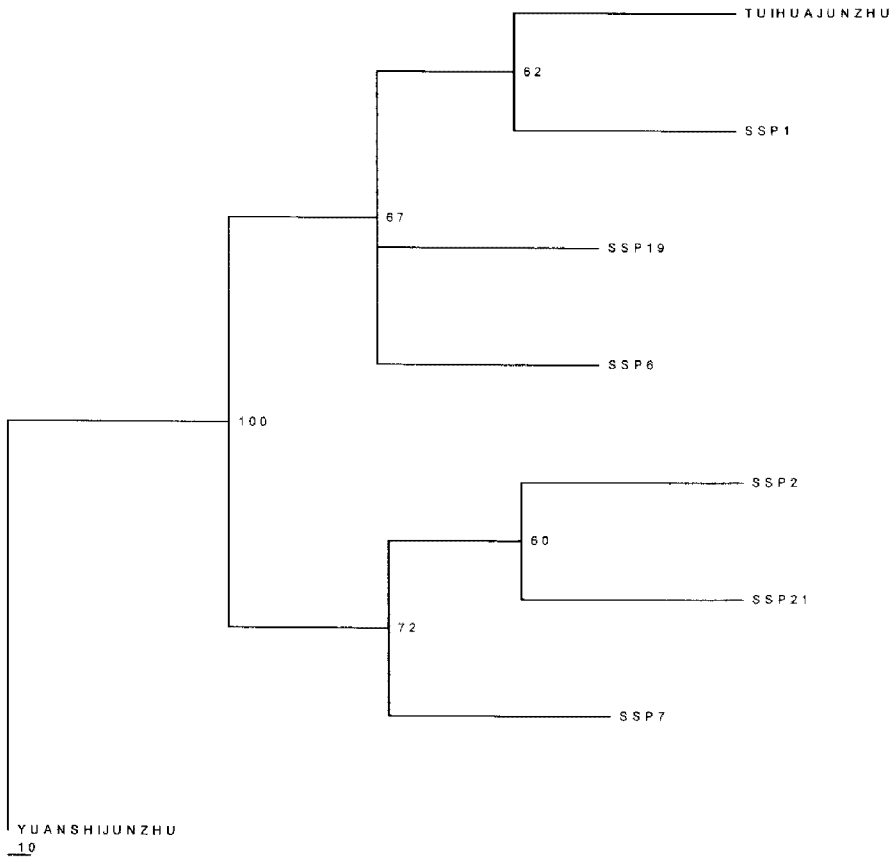

Fig. 5. Unooted phylogenetic tree of *C. militaris* tester strains generated from ITS sequence data.

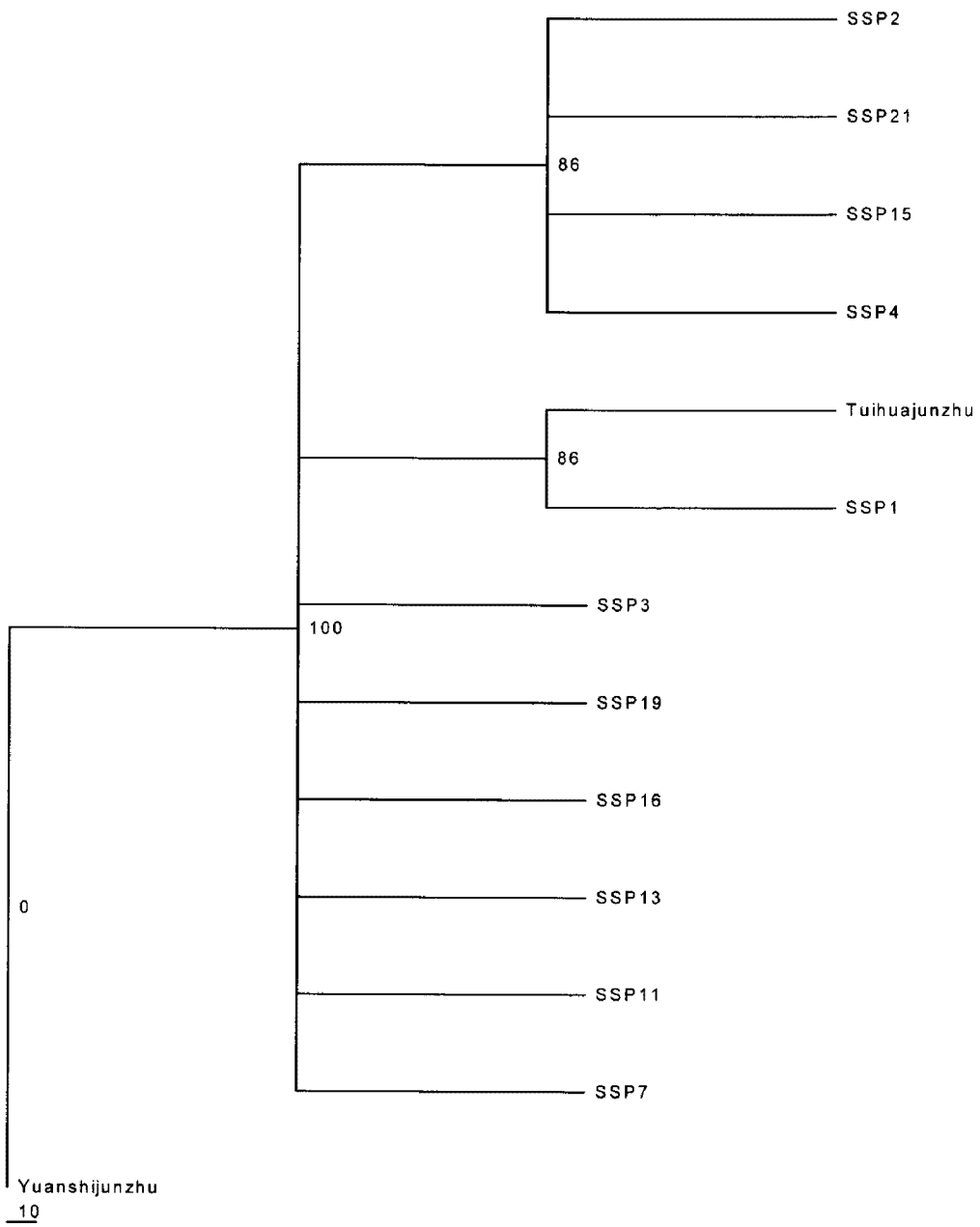

Fig. 6. Unooted phylogenetic tree of thirteen *C. militaris* test strains generated from M 1-2 gene sequence data

ACKNOWLEDGEMENTS

This work was supported by NSFC (NO.30870009) and the Modernization of Traditional Chinese Medicine program of Guizhou province (Study on the Good Quality Mycelia and Cordycepin Production Technique of *Cordyceps militaris*, NO. [2007]5012).

REFERENCES

[1]Liu ZY.Studies on relationship between *Cordyceps spp.* and their anamorphs [D]. Huazhong agricultural university.1999.

[2]Basith, M. and M. E Madelin (1968) Studies on the production of perithecial stromata by *Cordyceps militaris* in artificial culture. Can. J. Bot. 46: 475-480.

[3]Pen, X. (1995) The cultivation of *C. militaris* fruitbody on artificial media and the determination of SOD. Acta Edulis.Fungi 2: 25-28.

[4]Wen TC,Lei BX,Kang JC,*et al.*(2009)Enhanced production of mycelial and cordycepin by submerged culture using additives in *Cordyceps militaris*.Food and Fermentation Industries.35(8): 162-166.

[5]Wen TC,Kang JC,Li GR,et al.(2008)Effect of culture condition on the fruit body and cordycepin production in *Cordyceps militaris* in solid-state fermentation.Guizhou Agricultural Sciences.36(4): 92-94.

[6]Coppin E, Debuchy R, Arnaise S, *et al.*(1997)Mating types and sexual development in filamentous ascomycetes. Microbiology and Molecular Biology Reviews,61(4): 411-428.

[7]Shrestha B, Kim HK, Sung GH, *et al.* (2004)Bipolar heterothallism, a principal mating system of *Cordyceps militaris* in vitro. Biotechnol Bioprocess Eng 9: 440–446.

[8]Yokoyama E, Yamagishi K, and Hara A. (2005) Structures of the mating-type loci of *Cordyceps takaomontana*.Appl. Era:iron. Microbiol. 69: 5019-5022.

[9]Sato, H. and M. Shimazu (2002) Homothallism in *Cordyceps militaris*, pp. 3I 1. Book of Abstracts of the 7th International Mycological Congress. August 11 - 17. Oslo, Norway.

[10]Tigano-Milani MS, Samson RA, Martins I, *et al.*(1995)DNA markers for differentiating isolates of *Paecilomyces lilacinus*. Microbiology (Reading, U.K.), 141: 239–245.

[11]Liang ZQ, Roland T.V.Fox.(1997)Vegetative compatibility of inter-and intra-monoconidium strains of anamorph of *cordyceps pruinosa*.Mycosystema.16(3): 216-223.

[12]Liang ZQ, Roland T.V.Fox.(1990)Anamoph of *cordyceps militaris* and artificial culture of its fruitbody.Southwest China Journal of Agricuitural Sciences.3(2): 1-6.

[13]Liang ZQ, Roland T.V.Fox.(1998)The polymorphism in the anamorph of *cordyceps militaris*.Mycosystema.17(1): 57-62.

[14]Yokoyama, E, Yamagishi, K. and Hara, A. (2004) Development of a PCR-based mating-type assay for Clavicipitaceae. FEMS Microbiol. Lett. 237,205-212.

[15]Kevin Alby.(2009)Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*.Nature. 460: 890-893.

[16]Yokoyama, E., K. Yamagishi, and A. Hara (2005) Structures of the mating-type loci of *Cordyceps takaomontana*.Appl. environ. Microbiol. 69: 5019-5022.

[17]Eiji Y, Kenzo Y, Akira H. (2005)Heterothallism in *Cordyceps takaomontana*. FEMS microbiology letters, 250:11, 145-150.

[18]McGuire, I. C, P,. E. Marra, and M. G. Milgroom (2004) Mating-type heterokaryosis and selfing in *Cryphonectria parasitica. Fung. Gen. Biol.* 41: 521-555.

[19]Kang, J. C., Liang, Z. Q., Liu, A.Y. *et al.*(2000)Molecular evidence of polymorphism in Cordyceps based on 5.8S rDNA and ITS2 sequences. Mycosystema.19 (4): 492-497.

[20]Liu Z, Liang Z, Liu A, *et al.*(2002) Molecular evidence for teleomorph-anamorph connections in Cordyceps based on ITS-5.8S rDNA sequences. Mycol Res 106:1100–1108.

[21]Turgeon BG, Yoder OC.(2000) Proposed nomenclature for mating type genes of filamentous ascomycetes. Fungal Genet Biol 31: 1–5.

[22]Wang, L., Zhang, W.M., Hu, B.*et al.* (2008). Genetic variation of *Cordyceps militaris* and its allies based on phylogenetic analysis of rDNA ITS sequence data. Fungal Diversity 31: 147-155.