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Variations of SSU rDNA Group I Introns in Different Isolates of *Cordyceps militaris* and the Loss of an Intron during Cross-Mating[§]

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***Cordyceps militaris*, the type species of genus *Cordyceps*, is one of the most popular mushrooms and a nutraceutical in eastern Asia. It is considered a model organism for the study of *Cordyceps* species because it can complete its life cycle when cultured *in vitro*. In the present study, the occurrence and sequence variation of SSU rDNA group I introns, Cmi.S943 and Cmi.S1199, among different isolates of *C. militaris* were analyzed. Based on the secondary structure predictions, the Cmi.S943 intron has been placed in subgroup IC1, and the Cmi.S1199 intron has been placed in subgroup IE. No significant similarity between Cmi.S943 and Cmi.S1199 suggested different origins. Three genotypes, based on the frequency and distribution of introns, were described to discriminate the 57 surveyed *C. militaris* strains. It was found that the genotype was related to the stroma characteristics. The stromata of all of the genotype II strains, which possessed only Cmi.S943, could produce perithecium. In contrast, the stromata of all genotype III strains, which had both Cmi.S943 and Cmi.S1199, could not produce perithecium. Cmi.S1199 showed the lowest level of intra-specific variation among the tested strains. Group I introns can be lost during strain cross-mating. Therefore, we presumed that during cross-mating and recombination, intron loss could be driven by positive Darwinian selection due to the energetic cost of transcribing long introns.**

***Keywords*:** *Cordyceps militaris*, group I introns, occurrence, sequence variation, stroma characteristics

Introduction

Group I introns are mobile genetic elements characterized by conserved RNA primary and secondary structures that are essential for splicing and are often capable of "self-splicing" (Lambowitz and Belfort, 1993). Their characteristic RNA folds generally consist of 10 conserved, paired elements (P1 to P10) that can help in catalyzing a two-step, self-splicing transesterification reaction resulting in intron release and ligation of the exons. Based on conserved nucleotide sequences and secondary structure characteristics, group I introns are classified into 5 major groups (IA to IE), which can be subdivided further into subgroups (e.g., IA1 and IC1) according to the presence/absence of peripheral paired elements (Suh *et al.*, 1999). Frequently found in the nuclear ribosomal DNA (nrDNA) region of fungi, group I introns have been increasingly used as a molecular marker of strain identification (Coates *et al.*, 2002; Côté *et al.*, 2004) and genetic diversity (Machouart *et al.*, 2011; Takizawa *et al.*, 2011).

Cordyceps militaris is the type species of the genus *Cordyceps* in the family Cordycipitaceae (Sung *et al.*, 2007). This species, commonly known as orange caterpillar fungus, is easily cultured in both solid and liquid media. It has been increasingly viewed as a substitute for *Ophiocordyceps sinensis*, a suggested National Fungus of China (Zhang *et al.*, 2012) because of their similar chemical capacities and medicinal properties (Shrestha *et al.*, 2012). Because *C. militaris* can complete its life cycle when cultured *in vitro*, it is considered a model organism for the study of *Cordyceps* species (Shrestha *et al.*, 2012).

Cordyceps militaris is a bipolar heterothallic fungus. A molecular study has demonstrated that *C. militaris* possesses opposite mating-type idimorphs, MAT1-1 and MAT1-2 (Yokoyama *et al.*, 2006). Mating compatibility not only produces fertile perithecial but also affects developmental and morphological patterns of stromata. Stromata produced by successful mating are perithecial club-shaped whereas those produced without mating are non-perithecial (Shrestha *et al.*, 2004).

The variability of nuclear SSU-rDNA group introns has been studied mostly at the level of genus or above (Feau *et al.*, 2007; Hafez *et al.*, 2012). Few studies on the variation of nuclear SSU-rDNA group introns in intra-species have been published. Variations of SSU rDNA group I introns in *Monilinia fructicola* isolates confirmed the absence of a group I intron in some of the isolates (Côté, 2004). Studying group I introns can also help to advance understanding of the genetic differentiation within *Botryosphaeria dothidea* (Xu *et al.*, 2013).

Nikoh and Fukatsu (2001) studied the group I introns in nuclear ribosomal RNA genes of the genus *Cordyceps* and reported group I introns inserted at positions 943 and 1199 in the SSU rDNA of *C. militaris*. However, the structure and

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variation of the introns among the different isolates were not defined.

In the present study, both the occurrence and sequence variation of group I introns among different isolates of *C. militaris* were studied, and it was found that the variation was related to the cultivation characteristics. Furthermore, group I introns could be lost during strain cross-mating.

Materials and Methods

Fungal strains and growth conditions

Isolates of *C. militaris* were studied, and their mating type and origin are listed in Table 1. All isolates were maintained on potato dextrose agar at 4°C as stock. The identification of the strains was confirmed by means of morphological and molecular methods.

Single ascospore strains were isolated from strains 40 (CGMCC 3.16322) and 90. Freshly collected stromata were attached to the inner side of the lid of a Petri dish containing 1.5% water agar and left at 22°C under light. Single ascospores were randomly isolated from Petri dishes containing discharged ascospores using an inverted microscope (Axio observer A1, Zeiss, Germany) with a sterile pin and inoculated on potato dextrose agar at 20°C.

PCR amplification, reverse transcription PCR (RT-PCR) and sequencing

Genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The primers NS1 (White *et al.*, 1990) and FS2 (Nikoh and Fukatsu, 2001) were used to amplify partial SSU fragments. Primers MAT111F/R, MAT112F/R, and MAT121F/R (Supplementary data Table S1) were used to identify the mating type by PCR.

Total RNA was extracted from strains 13 (CGMCC 5.699),

Table 1. Strains used in this study

Strains No.	Locality	Mating type		Presence (+) or absence (–) of insertions in SSU		Genotype
		MAT1-1-1	MAT1-2-1	Cmi.S943	Cmi.S1199	
10	Liaoning	+	–	+	+	III
12	Guangdong	+	–	+	+	III
13	Shanxi	+	+	+	+	III
14	Liaoning	+	–	+	+	III
15	Liaoning	+	–	+	+	III
16	Liaoning	+	–	+	+	III
19	Liaoning	+	+	+	-	II
30	Liaoning	+	–	+	+	III
40	Liaoning	+	+	+	–	II
41	Shandong	+	+	+	–	II
42	Shandong	+	+	+	–	II
43	Shandong	+	+	+	–	II
44	Shandong	+	+	+	–	II
45	Inner Mongolia	+	–	+	+	III
57	Beijing	+	–	+	+	III
71	Shandong	+	–	+	–	II
81	Liaoning	+	–	+	+	III
85	Liaoning	+	–	+	+	III
86	Liaoning	+	–	+	+	III
90	Shandong	+	+	+	–	II
91	Shandong	+	+	+	–	II
136	Hubei	+	–	+	+	III
137	Sichuan	+	–	+	+	III
138	Sichuan	+	–	+	+	III
139	Jiangsu	+	–	+	+	III
141	Beijing	+	+	+	+	III
142	Beijing	+	+	+	+	III
145	Shanxi	+	–	+	+	III
153	Liaoning	+	–	+	+	III
154	Liaoning	+	–	+	+	III
155	CBS178.59	+	+	+	+	III
156	Shandong	+	–	+	+	III
159	Cross strain	+	–	+	+	III
175	Shandong	+	–	+	–	II
183	Jiangsu	+	+	+	–	II

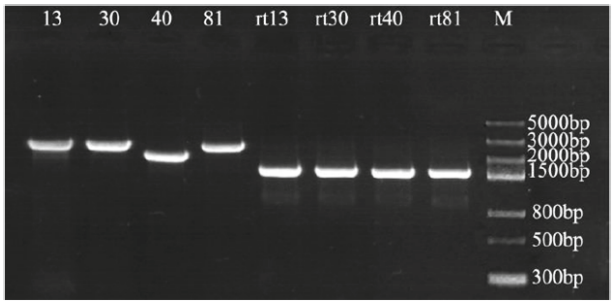

Fig. 1. PCR and RT-PCR analysis of the SSU rDNA for different *C. militaris* isolates to demonstrate the *in vivo* splicing of the introns. Lane 9 (labeled M) contains the Marker III DNA ladder (Solarbio Science & Technology Co., Ltd, China), lane 1–4 (labeled 13, 30, 40, and 81, respectively) represent a standard PCR reaction using primers NS1 and FS2 with genomic DNA of strains 13, 30, 40, and 81 as a template, while lane 5–8 (labeled rt13, rt30, rt40, and rt81, respectively) contain amplicons derived from the RT-generated cDNA template of 13, 30, 40, and 81. The genomic DNA generated a 2.3- or 1.9-kb PCR fragment, indicating the presence of introns, while the cDNA template generated a 1.5-kb PCR fragment, indicating that all introns were spliced out.

30 (CGMCC 3.16321), 40 (CGMCC 3.16322), and 81 (CGMCC 3.16324) using TRIzol reagent (Invitrogen, USA). Then, the RNA was treated with RQ1 RNase-free DNase (Promega, USA). RT-PCR was performed using the Prime Script® RT-PCR kit (TaKaRa, Japan) with primers NS1 and FS2. Amplification was performed for 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 30 sec at 50°C, 1.5 min at 72°C and a final extension for 10 min at 72°C.

Sequencing was conducted by the Beijing Genomics Institute (Beijing, China). Nucleotide sequences were assembled and analyzed using the ContigExpress program (Invitrogen).

Intron analyses and secondary structure prediction

The location of each intron insertion site was determined by comparison with the orthologous nuclear SSU-rDNA gene in *Escherichia coli* (accession no. J01695; Gutell, 1993), and the introns were named according to the proposed nomenclature by Johansen and Haugen (2001). To verify the position, PCR amplification was carried out using two sets of primers (Fig. 2): NS5 (White *et al.*, 1990)/18SD (Perotto *et al.*, 2000) and NS23 (Gargas and Taylor, 1992)/FS2.

Secondary structure models were predicted following the conventions for group I introns defined by Burke *et al.* (1987) and according to the models proposed by Cech (1988) and Michel and Westhof (1990). The intron structural elements (P1–P9 stem-loops) were individually identified by comparison with available group I intron sequences from the Comparative RNA web site (http://www.rna.icmb.utexas.edu/) and then folded using the Mfold web server at http://mfold.rna.albany.edu/?q=mfold (Zuker, 2003). The final structures were drawn using RnaViz2.0 (Rijk *et al.*, 2003).

Variations of SSU rDNA group I introns

The SSU sequences were aligned using ClustalX, and Kimura 2-Parameter (K2P) distances were computed using Mega5 (Tamura *et al.*, 2011). The average intra-specific distance and coalescent depth were calculated to evaluate the intra-specific variation using the K2P model.

The loss of group I introns during strain cross-mating

Two strains at the same proportion were simultaneously inoculated into seed medium for strain crossing. The fruit bodies were then cultivated according to the method of Zhan *et al.* (2006). The different pairs of isolates, 81 and 137 (genotype III), 40 and 90 (genotype II) and 30 (genotype III) and 90 (genotype II), were used for strain crossing. At least 10 bottles were cultured for every crossing. When the fruit body was mature after 60 d, 10 stromata were randomly collected and used for DNA extraction. PCR with primers NS1 and FS2 was used to detect the genotype.

To verify the loss of the intron and the occurrence of mating during the crossing, the single ascospore strains 40d-26 (Mating 1-2) and 90d-9 (Mating 1-2) were crossed with

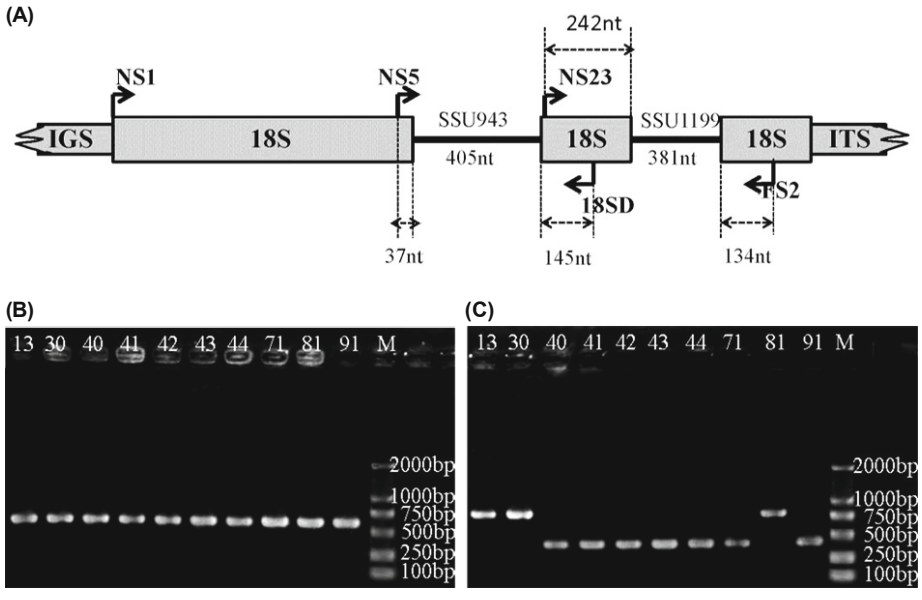

Fig. 2. PCR analysis of the SSU rDNA for different *C. militaris* isolates to verify the position of introns. (A) Diagrammatic representation of the primers used in this study and the relative locations of group I introns in the nuclear SSU-rDNA gene of *C. militaris*. (B) PCR examination of Cmi.S943. (C) PCR examination of Cmi.S1199.

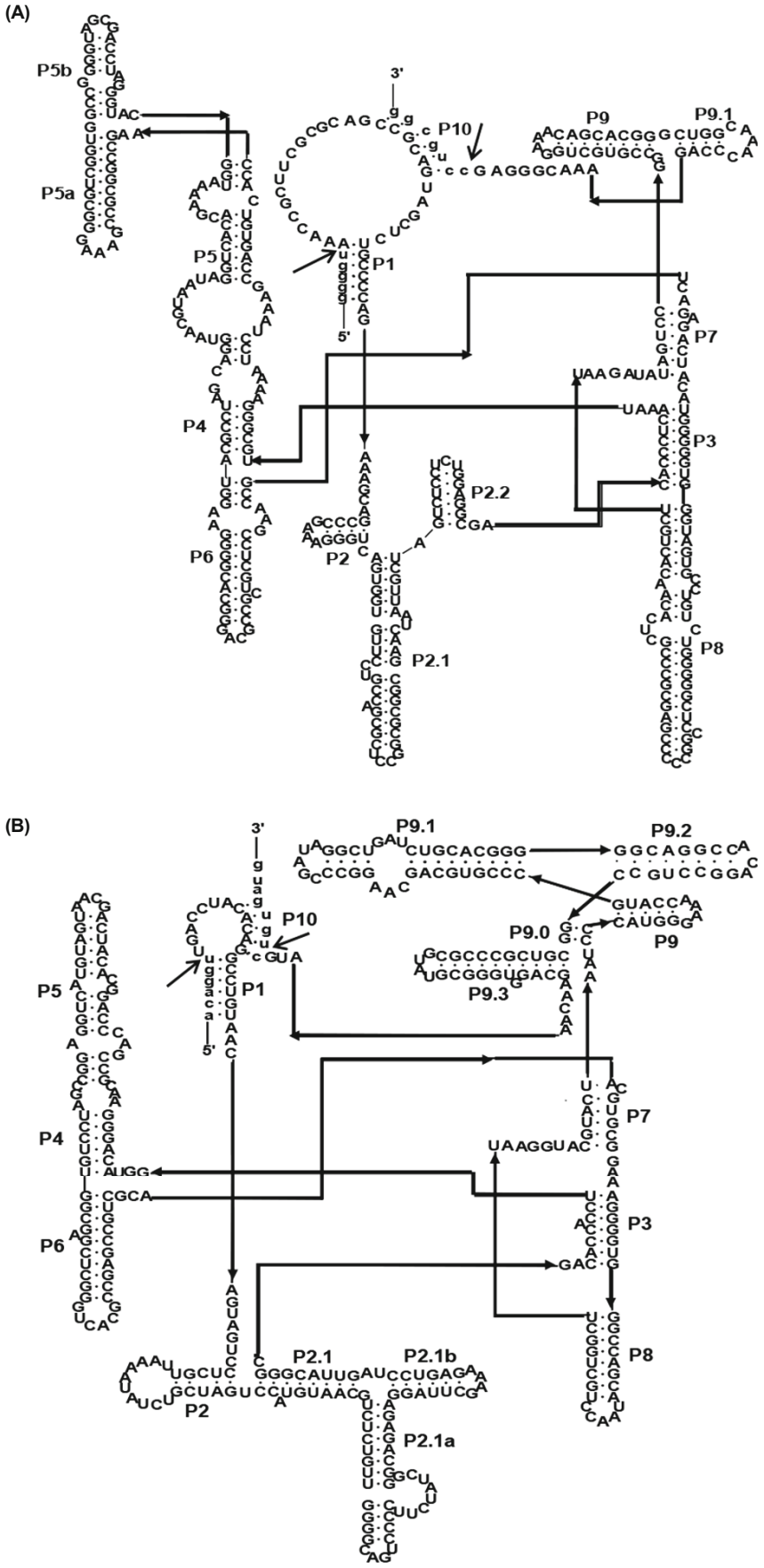

Fig. 3. Secondary structures of introns within the SSU rDNA from *C. militaris*. (A) Cmi.S943 and (B) Cmi.S1199. Intron sequences are in upper-case letters, and exon sequences are in lower-case letters. The 10 pairing regions (P1–P10) are indicated. The solid arrowheads indicate the intron-exon junctions (putative 5 and 3 splicing sites) (designed using RnaViz2.0).

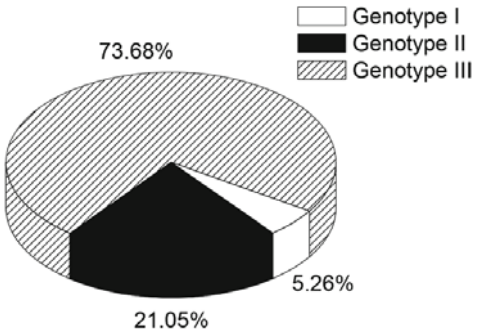

Fig. 4. Distribution of introns among different *C. militaris* isolates. The three different, filled patterns represent the three genotypes (Genotype I: no intron, Genotype II: Cmi.S943, Genotype III: Cmi.S943 + Cmi.S1199) divided by the presence or absence of introns. The ratios of the three genotypes are marked at their relevant positions of the pie chart.

strain 30 (Mating 1-1). Primers MAT111F/R, MAT112F/R, and MAT121F/R (Supplementary data Table S1) were used to identify the mating type and NS1/FS2 was used to detect the genotype by PCR.

Results

Identification of the intron sequences in the SSU-rDNA

The size of the amplified fragment when using primers NS1 and FS2 with genomic DNA as template was expected to be approximately 1.5 kb. However, longer PCR products (1.9 and 2.3 kb) were obtained (Fig. 1). Among the four isolates tested, three gave a 2.3-kb PCR product, and one isolate gave an amplicon of approximately 1.9 kb in size. RT-PCR with the cDNA template of all tested strains generated a 1.5-kb PCR fragment, indicating the presence of introns in the genomic DNA. The analysis also showed that introns in the SSU-rDNA of *C. militaris* would be removed from the pre-rRNA during rRNA maturation.

Based on the 1,542-bp-long reference 16S rDNA sequence of *E. coli* (accession number J01695), the intron positions were determined to be at nucleotides 943 and 1199. To verify these positions, PCR amplification was carried out using two sets of primers: NS5/18SD and NS23 /FS2. Analysis of the PCR products of all tested strains showed one specific band of 587 bp when using primer NS5/18SD on a 1.5% agarose gel (Fig. 2B), indicating an intron of 405 nt at nucleotide 943 when compared with the 16S rDNA sequence of *E. coli* (accession number J01695). The same analysis with primer NS23/FS2 showed bands of 757 bp (strain 10, 30, and 81) or 376 bp (the other tested strains), indicating an intron of 381 nt at nucleotide 1199 in some strains (Fig. 2C). Sequencing and alignment demonstrated the existence of introns (Supplemental Alignment). The group I introns that were found in this fungus were named Cmi.S943 and Cmi.S1199, respectively, following Johansen and Haugen's proposal (2001).

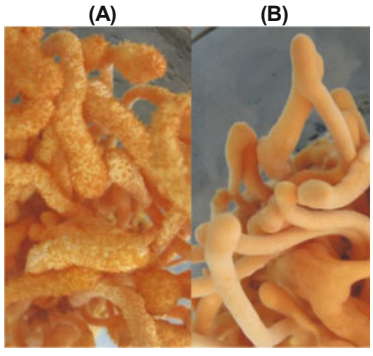

Fig. 5. Fruit bodies of *C. militaris*. (A) Fruit bodies with perithecium from genotype II strain, and (B) fruit bodies without perithecium from genotype III strain

Table 2. Analysis of intra-specific divergence among *C. militaris*

	Alignment length	Number of sequences	Average intra-specific distance	Coalescent depth[a]
Cmi.S943	405	27	0.0042±0.0008	0.040±0.009
Cmi.S1199	381	22	0.0036±0.0014	0.024±0.007
SSU	1457	30	0.0056±0.0008	0.033±0.005

[a] Coalescent depth is the maximum intra-specific distance.

Predicted secondary structures of group I introns

Based on secondary structure predictions, intron Cmi.S943 was placed in subgroup IC1 (Michel and Westhof, 1990) (Fig. 3A), and intron Cmi.S1199 was placed in subgroup IE (Suh *et al.*, 1999) (Fig. 3B). Sequence examination of the central core of the IE introns revealed that they belonged to the IE3 subgroup for the GNRA tetra loop in P9 and branched P2.1 with a large bulge within P2.1a according to the classification developed by Li and Zhang (2005).

Genotyping of *C. militaris* strains

Thirty-five isolates from our laboratory (Table 1) and 22 sequences from GenBank (Supplementary data Table S2) were analyzed. Three genotypes based on the frequency and distributions of the introns were described to discriminate the 57 surveyed *C. militaris* strains: 3 strains (genotype I, 5.26%) had no intron in the SSU, 12 strains (genotype II, 21.05%) possessed only Cmi.S943, and the remaining 42 strains (genotype III, 73.68%) had both Cmi.S943 and Cmi.S 1199 (Fig. 4).

Moreover, the stromata of the genotype II strains from 35 isolates from our laboratory could produce perithecium (Fig. 5A). Two mating-type idiomorphs, MAT1-1 and MAT1-2, were detected among most of the genotype II strains. None of the genotype III strains could produce perithecium (Fig. 5B).

Sequence variations of SSU rDNA group I introns

The length of Cmi.S943 varied from 390 to 405 nt, and its length in most of the strains (81.48%) was 405 nt (Supplementary data alignment). The length of Cmi.S1199 of all the tested strains was 381 nt (Supplementary data alignment). Nucleotide variations were observed among the sequences of the introns of the different *C. militaris* isolates. None of the nucleotide variations occurred in the conserved P, Q, R or S elements; and most of the variations did not occur in the pairing (P) segments that are responsible for the secondary structure of the group I intron.

Two parameters, average intra-specific difference (K2P distance) and coalescent depth, were employed to characterize the intra-specific variation of the SSU rDNA group I introns in *C. militaris* (Table 2). Here, Cmi.S1199 showed the lowest level of intra-specific variation, and the differences between individuals ranged from 0 to 0.024 (22 sequences), with an average of 0.0036. While Cmi.S943 exhibited the highest level of variation, differences between individuals ranged from 0 to 0.040 (27 sequences), with an average of 0.0042, followed by SSU that ranged from 0 to 0.033 (30 sequences), with an average of 0.0056.

Pairwise comparison was made of the two insertions; however, no significant similarity was found. BLASTN analyses of the Cmi.S943 sequence showed the closest homology (100% query coverage and 100% identity) to *Cordyceps taishanensis* (JN411084) and *Cordyceps kyushuensis* (AY465958) followed by *Cordyceps brongniartii* (JN941759, 100% query coverage and 92% identity). The sequence of Cmi.S1199 showed the closest homology (100% query coverage and 99% identity) to *Cordyceps kyushuensis* (AY465958), followed by *Torrubiella flava* (AB100609, 85% query coverage and 91% identity).

The loss of group I introns during strain cross-mating

When the two strains of genotype II (strain 40 and 90) were crossed by hyphal anastomosis, the F1 progeny was still genotype II (Fig. 6B); and the same result occurred when two strains of genotype III were crossed (strain 81 and 137, Fig. 6A). However, when a strain of genotype II (strain 90) and a strain of genotype III (strain 30) were crossed, the F1 progeny belonged to genotype II (Fig. 6C). For each condition, the 10 samples that were randomly selected showed the same result. It seemed that the Cmi.S1199 intron had been lost during the cross.

To verify this interesting result, several sets of strains of genotype II and III were selected for crossing, and the same result was obtained (Supplementary data Fig. S1). However, we could not judge whether mating between the strains did occur. Therefore, we separated the single ascospore isolates from genotype II strains 40 (CGMCC 3.16322) and 90. The single ascospore isolate, 40d-26, genotype II and mating 1-2, was used to cross with strain 30 (Mating 1-1, genotype III). The F1 progeny was genotype II for mating 1-1 and 1-2 (Supplementary data Fig. S1). The mating type identification ascertained that the cross-mating was successful (Supplementary data Fig. S2). Then, the PCR product from using primer NS1/FS2 was sequenced and aligned with other SSU sequences of *C. militaris* (Supplementary data alignment), and the expected intron lost was confirmed. The same result occurred when the single ascospore isolate 90d-9 (separated from strain 90, genotype II and mating 1–2) was crossed with strain 30 (Mating 1-1, genotype III, Supplementary data Figs. S1 and S2).

Discussion

The discovery of introns has led to many studies investigating their origin, spread, and functions in eukaryotic genomes. In the present study, the occurrence and sequence variation of group I introns among different isolates of *C. militaris* were studied. Based on the distribution of these introns among the 57 *C. militaris* strains, 3 genotypes were described. It was found that the genotype was related to the stromata characteristics. The loss of group I introns may occurred during the strain cross. This was the first report on the variation of group I introns among different isolates of *C. militaris* and on the loss of group I introns during strain crossing.

The two insertions exhibited the characteristic features of group I introns, i.e., (1) similar position as other group I introns, (2) paired elements P1–P10, and (3) the last exon base U located immediately upstream of the 5′ intron splice site and the last intron base G preceding the 3′ intron splice site (Cech, 1988; Michel and Westhof, 1990). Based on secondary structure predictions, Cmi.S943 and Cmi.S1199 were found to belong to subgroups IC1 and IE, respectively.

In filamentous fungi, nuclear rDNA genes are present in tandem repeats. It should be noted, therefore, that group I

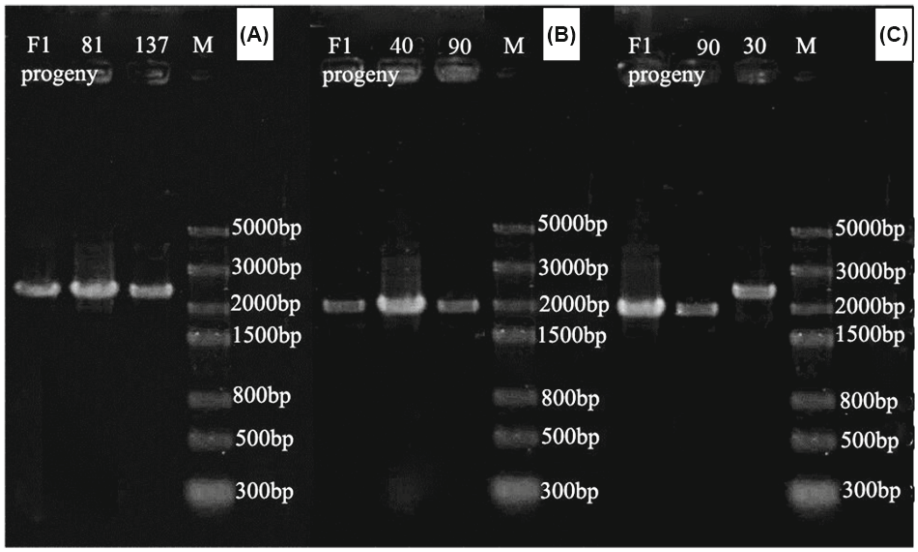

Fig. 6. Analysis of the SSU rDNA from hybrid strains. (A) Amplification of the SSU rDNA hybrid strain from two genotype III strains. Lanes: 1, represents the PCR product of the hybrid strain; 2–3 represent two different genotype III strains (strains 81 and 137); and 4 contains a marker. (B) Amplification of the SSU rDNA hybrid from two genotype II strains. Lanes: 1, represents the PCR product of the hybrid strain; 2–3 represent two different genotype III strains (strain 40 and 90); and 4 contains a marker. (C) Amplification of the SSU rDNA hybrid from genotype II and III strains. Lanes: 1 represents the PCR product of the hybrid strain; 2–3, respectively, represent genotype II (strain 90) and genotype III strains (strain 30); 4 contains a marker. In each condition, the result of 1 of the 10 randomly selected stromata is shown.

introns may not necessarily be found in all rDNA repeats within the genome (Perotto *et al*., 2000). However, we did not observe multiple bands when amplifying rDNA from several samples using different primers in this study.

Both Cmi.S943 and Cmi.S1199 were present in 42 of 57 *C. militaris* isolates (73.68%), which indicated that insertion was common. The group I intron, CgSSU, was present in 61 of 70 *Cenococcum geophilum* isolates (Shinohara *et al*., 1996); however, in the phylogenetically close species *Beauveria bassiana* and *B. brongniartii*, introns were present in 35 of 112 isolates (31.25%), which suggested that insertion frequency may be species-specific and dependent upon the time since acquisition or mobility.

Size polymorphisms of group I introns generated by the insertion of a mobile element, such as homing end nuclease genes (HEG), have been described (Xu *et al*., 2013). However, in this study, there was little length variation among strains, and no open reading frames of considerable size were identified in the group I introns in the nuclear rDNAs of *C. militaris*.

No significant similarity was found between Cmi.S943 and Cmi.S1199. BLASTN analyses showed that introns inserted at the same site were closely related to each other in *Cordyceps* fungi, whereas introns inserted at different sites were phylogenetically distinct, even in the same species. This finding suggested that Cmi.S943 and Cmi.S1199 might have different origins.

Three genotypes were established according to the patterns of intron distribution in the *C. militaris* isolates. It is interesting that the genotype was related to the stromata characteristics. A similar phenomenon of a genotype being related to some characteristics or geographic locations has been reported in *Exophiala dermatitidis* (Machouart *et al*., 2011), *Phialophora verrucosa* (Takizawa *et al*., 2011), and *Botryosphaeria dothidea* (Xu *et al*., 2013). In *E. dermatitidis*, the invasive genotype A and B strains from the natural environment were differentiated by the distribution of ribosomal introns. In *P. verrucosa*, 34 strains were classified into 5 genotypes according to intron distribution, and some correlation between genotypes and geographic location was inferred. For *B. dothidea*, 4 genotypes were established according to the patterns of intron distribution and a correlation between genotypes (based on SSU intron) and hosts or geographic locations was observed.

Nikoh and Fukatsu (2001) reported that, in *Cordyceps* fungi, introns have principally been immobile and vertically transmitted, and, in the course of vertical transmission, the introns have repeatedly been lost in a number of independent lineages. Our result suggested that cross-mating may cause repeated loss of introns in *C. militaris.*

Three models have been proposed for the mechanism of intron loss in eukaryotic evolution: the reverse transcriptase (RT), genomic deletion and double-strand-break repair models (Zhu and Niu, 2013). Among these, reverse transcription is proposed as the main mechanism of intron loss in many species. However, in the present study, intron loss did not occur when two strains of genotype II or III were crossed, which suggests that the reverse transcriptase model is not suitable here. We presumed during the cross-mating and recombination that intron loss may be driven by positive Darwinian selection due to the energetic cost of transcribing long introns. In fact, one case of positive selection associated with intron loss in a population of *Drosophila teissier* has also been reported (Llopart *et al*., 2002). In this case, the intron-loss allele was less strongly transcribed, and individual intron loss alleles were subject to selection.

Acknowledgements

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