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Comparative Study on Methods for Identification of Chloroplast DNA of Cultivated and Wild Species in Section *Cepa* of *Allium*.

Ken-ichiro YAMASHITA, Tomohiro OYAMA, Rumi NODA,
Tomoya MIYAZAKI and Yosuke TASHIRO
(Laboratory of Biotechnology and Plant Breeding)
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Summary

Several methods for the identification of chloroplast DNA (cpDNA) of the species in section *Cepa* of *Allium* were compared. Different types of RFLP analyses of cpDNA were performed in two cultivated species, *A. fistulosum* and *A. cepa*, and in four wild species, *A. altaicum*, *A. galanthum*, *A. oschaninii*, and *A. vavilovii*. RFLP analysis of purified cpDNA detected polymorphisms in all species combinations except for that of *A. cepa* and *A. vavilovii*. RFLP analysis of total DNA by Southern hybridization with the probe(pF1) developed from cpDNA of *A. fistulosum* detected polymorphisms in most of the species combinations. PCR-RFLP analysis of the *rbc*L-ORF106 region of cpDNA also detected polymorphisms in all species combinations except for that of *A. cepa* and *A. vavilovii*. All the methods performed in this study were found to be effective in the identification of cpDNA of reciprocal F_1 hybrids between *A. fistulosum* and *A. cepa*, and it was confirmed that cpDNA was of maternal inheritance. Because of the speed of detection, PCR-RFLP analysis of the *rbc*L-ORF106 region was considered to be the most efficient method for identification of cpDNA of the species in section *Cepa*.

Introduction

Allium fistulosum L. and *A. cepa* L., economically important cultivated species, belong to section *Cepa* of *Allium*[17),7)]. To improve these species, some researchers have attempted to introduce useful nuclear genes of the wild species in section *Cepa* into these cultivated species[28),29),15),16),18),34),32)]. To utilize the cytoplasm of the wild species, Yamashita and Tashiro[36)] substituted cytoplasm of *A. galanthum* Kar. et Kir. for that of *A. cepa* by continuous backcrossing and demonstrated that there was a possibility of developing a male sterile line of *A. cepa* with cytoplasm of *A. galanthum*. Hereafter, the cytoplasms of wild species will be utilized for breeding of cultivated *Allium* species. To confirm the cytoplasmic substitution, it is necessary to establish the method for identifying the cytoplasms of cultivated and wild species in advance. The cytoplasm of plant includes chloroplasts and mitochondria which have their own DNAs. Chloroplast DNA (cpDNA) is highly conserved for its nucleotide sequence[26)], but its sequence diversity has been observed among species in many plants [25),27),5)]. Restriction fragment length polymorphisms

(RFLPs) have been analyzed to detect the sequence diversity of cpDNA[2),3),20),22),24),9), 10)]. In this study, three types of RFLP analyses of cpDNA were compared with each other to evaluate the effectiveness of those methods in identifying cpDNA of cultivated and wild species in section *Cepa* of *Allium*.

Materials and Methods

Materials

Two cultivated species, *A. fistulosum* and *A. cepa*, and four wild species, *A. altaicum* Pall., *A. galanthum*, *A. oschaninii* O. Fedtsch., and *A. vavilovii* M. Pop. et Vved., and reciprocal F_1 hybrids between *A. fistulosum* and *A. cepa* were used for cpDNA analysis (Table 1).

Table 1. Plant materials used in this study.

Species	Cultivar name, accession number or cross combination
Allium fistulosum L.	'Kujyo'
A. cepa L. Aggregatum group	'Chiang mai'
A. altaicum Pall.	85003[a]
A. galanthum Kar. et Kir.	65447[a]
A. oschaninii O. Fedtsch.	78227[a]
A. vavilovii M. Pop. et Vved.	83010[a]
F_1 hybrid	*A. fistulosum* × *A.cepa*
F_1 hybrid	*A. cepa* × *A. fistulosum*

[a]Accessions from IVT (CPRO-DLO) in the Netherlands.

Methods

1. RFLP analysis of purified cpDNA

The methods reported by Katayama et al.[14)]and Holford et al.[13)] were modified and adopted to extract and purify cpDNA. About 20g of fresh leaves were homogenized in 160ml of extraction buffer (50mM Tris-HCl, 10mM EGTA, 0.35M sorbitol, 0.2% BSA, 0.05% cysteine, pH 8.0). The homogenate was filtered through four layers of gauze and one layer of Miracloth, and the filtrate was centrifuged at 200×g for 10min. The supernatant was then centrifuged at 1500×g for 10min to pellet the chloroplasts. The chloroplasts were resuspended in the extraction buffer, and the suspension was layered on the continuous gradient of Percoll. To prepare the gradient, the extraction buffer with 40% Percoll was centrifuged previously at 40000×g for 40min. After centrifugation of the layered suspension at 9000×g for 15 min, intact chloroplasts were collected from the lower of two bands. The chloroplasts were resuspended again in the extraction buffer and were centrifuged at 1500×g for 10min. $MgCl_2$, $CaCl_2$, and DNase I were added to the resuspended chloroplasts to final concentrations of 10mM, 2mM, and 200 μg/ml, respectively. The mixture was incubated at 4°C for 1hr and centrifuged at 1500×g for 10 min. The chloroplast pellet was resuspended in lysis buffer (50mM Tris-HCl, 10mM EDTA, 2% N-laurylsarcosine, 0.02%

Proteinase K, pH 8.0) and incubated at 37°C for 1hr. After RNase treatment (final concentration, 25 μg/ml), the cpDNA was purified with two times of phenol-chloroform treatments and collected by ethanol precipitation. The purified cpDNA was digested with five restriction enzymes, *Bgl* II, *Kpn* I, *Pvu* II, *Sma* I, and *Xho* I, and electrophoresed on 0.7% (*Bgl* II, *Kpn* I and *Xho* I) or 0.4% (*Sma* I and *Pvu* II) agarose gel at 4hr. The separated cpDNA was stained with ethidium bromide, and the pattern was observed on a UV transilluminator.

2. RFLP analysis using the probe

Tashiro et al. [30] reported the effectiveness of the cpDNA probe (pF1, 3.3kbp) developed from cpDNA of *A. fistulosum* for the identification of paternal and maternal parents of *A. wakegi* Araki. In this study, the pF1 was applied to identification of cpDNA of the species in section *Cepa*. Total DNA was extracted from 2g of fresh leaves using the method reported by Murray and Thompson[21]. The extracted total DNA was digested with *Bgl* II and *Bam*H I. Digested total DNA was electrophoresed on 0.7% agarose gel and transferred to a nylon membrane, Hybond-N+ (Amersham). Hybridization and detection of signal were carried out according to the DIG user's guide (Boehringer Mannheim).

3. PCR-RFLP analysis

The region between *ribulose-1,5-bisphosphate carboxylase gene* (*rbc*L) and open reading frame 106 (ORF106) of cpDNA was analyzed. Total DNA was extracted from 0.04g of

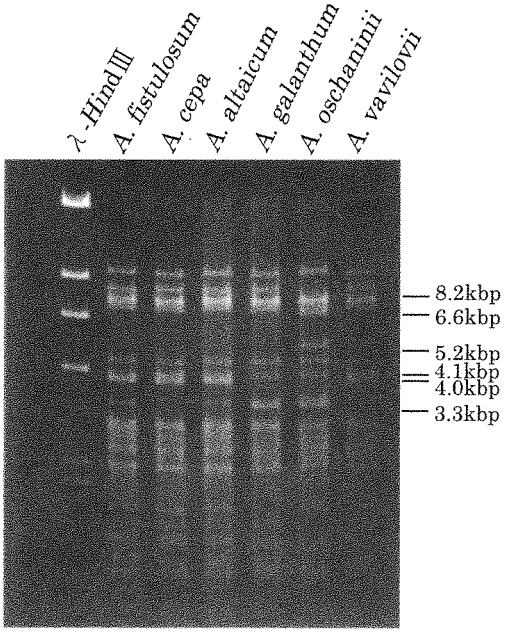

Fig. 1 Restriction patterns of *Bgl* II digested cpDNA in *A. fistulosum*, *A. cepa*, *A. altaicum*, *A. galanthum*, *A. oschaninii*, and *A. vavilovii*.

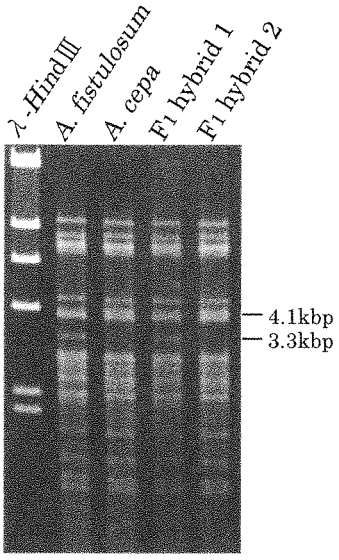

Fig. 2 Restriction patterns of *Bgl* II digested cpDNA in *A. fistulosum*, *A. cepa*, and their reciprocal F_1 hybrids. F_1hybrid 1: *A. fistulosum* × *A. cepa*, F_1hybrid 2: *A. cepa* × *A. fistulosum*.

Table 2. Presence or absence of polymorphic fragments in purified and *Bgl* II digested cpDNA in six species.

Species	Polymorphic fragment (kbp)					
	8.2	6.6	5.2	4.1	4.0	3.3
A. fistulosum	+	−	−	−	+	+
A. cepa	+	−	−	+	+	−
A. altaicum	+	−	−	−	+	+
A. galanthum	+	−	−	+	−	+
A. oschaninii	−	+	+	+	−	+
A. vavilovii	+	−	−	+	+	−

+ : Present, − : Absent.

Table 3. Types of restriction patterns of purified cpDNA in six species.

Species	*Bgl* II	*Kpn* I	*Pvu* II	*Sma* I	*Xho* I
A. fistulosum	1	1	1	1	1
A. cepa	2	2	2	2	2
A. altaicum	1	1	3	1	3
A. galanthum	3	1	4	3	4
A. oschaninii	4	3	2	4	5
A. vavilovii	2	2	2	2	2

fresh leaves using the method described by Hong et al.[12]. Sequences of the primers for amplification were 5'-ATGTCACCACAAACAGAAACTAAAGCAAGT-3' (*rbc*L) and 5'-ACTACAGATCTCATACTACCCC-3' (ORF106) described by Arnold et al.[1]. Polymerase chain reaction (PCR) was carried out under the condition of Arnold et al.[1] with minor modifications: reaction mixture (50μl) contained 10mM Tris-HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.1mM of each dNTP, 36pmol of each primer, 128ηg of template DNA, and 1.25unit of *Taq* DNA polymerase (TAKARA). Amplification was carried out for 1 min at 92°C, 1min at 55°C, and 4 min at 70°C, with a final 7 min at 70°C on a thermal cycler PC800 (ASTEC). Amplified product was digested with five restriction enzymes, *Alu* I, *Ase* I, *Hinf* I, *Taq* I, and *Pst* I, and electrophoresed on 1.5% agarose gel including ethidium bromide. The digestion pattern was observed on a UV transilluminator.

Results

1. RFLP analysis of purified cpDNA

In digestion patterns of purified cpDNA with *Bgl* II, *Kpn* I, *Pvu* II, *Sma* I, and *Xho* I, different types were observed among six species: four types with *Bgl* II, three types with *Kpn* I, four types with *Pvu* II, four types with *Sma* I, and five types with *Xho* I (Fig. 1, Table 2, 3). In all enzymes used, *Xho* I detected polymorphisms in the most species combinations in section *Cepa* (Table 8). This method detected polymorphisms in all species combinations except for that of *A. cepa* and *A. vavilovii*. The reciprocal F_1 hybrids between *A. fistulosum* and *A. cepa* had the restriction patterns identical to those of their seed parents when their cpDNA were digested with *Bgl* II (Fig.2).

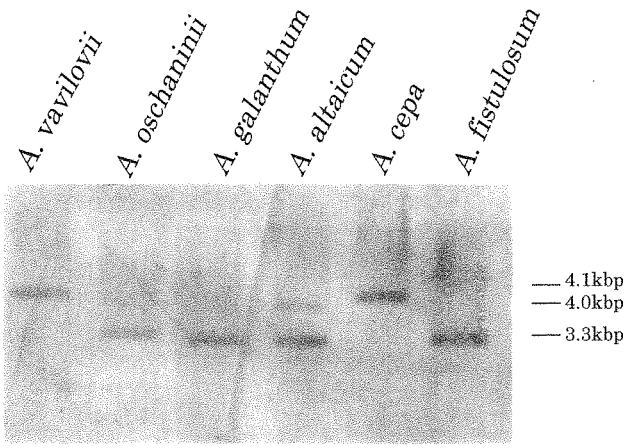

Fig. 3 Hybridization patterns of *Bgl* II digested total DNA, hybridized with the probe pF1, in *A. fistulosum, A. cepa, A. altaicum, A. galanthum, A. oschaninii, and A. vavilovii.*

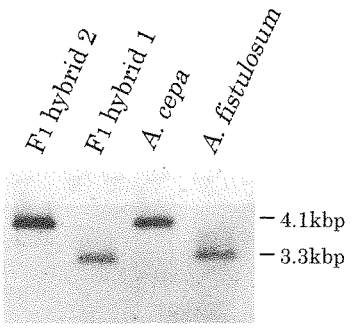

Fig. 4 Hybridization patterns of *Bgl* II digested total DNA, hybridized with the probe pF1, in *A. fistulosum, A. cepa,* and their reciprocal F_1 hybrids. F_1 hybrid 1: *A. fistulosum* × *A. cepa,* F_1 hybrid 2: *A. cepa* × *A. fistulosum.*

Table 4. Presence or absence of polymorphic fragments in *Bgl* II digested total DNA, hybridized with the probe pF1, in six species.

Species	Polymorphic fragment (kbp)		
	4.1	4.0	3.3
A. fistulosum	−	−	+
A. cepa	+	−	−
A. altaicum	−	+	+
A. galanthum	−	−	+
A. oschaninii	−	−	+
A. vavilovii	+	−	−

Table 5. Types of hybridization patterns of total DNA, hybridized with the probe pF1, in six species.

Species	*Bgl* II	*Bam* H I
A. fistulosum	1	1
A. cepa	2	1
A. altaicum	3	1
A. galanthum	1	1
A. oschaninii	1	2
A. vavilovii	2	1

2. RFLP analysis using the probe

In the hybridization patterns of *Bgl* II and *Bam* H I digested total DNA with the probe pF1, different types were observed among six species: three types with *Bgl* II and two types with *Bam* H I (Fig.3, Table 4, 5). The combination of pF1 and *Bgl* II was superior to that of pF1 and *Bam* H I for identification of cpDNA of the species in section *Cepa* (Table 8). The pF1 detected polymorphisms in all species combinations except for those of *A. fistulosum* and *A. galanthum* and of *A. cepa* and *A. vavilovii.* The reciprocal F_1 hybrids between *A. fistulosum* and *A. cepa* showed the hybridization patterns identical to those of their seed parents when their total DNA were digested with *Bgl* II (Fig.4).

3. PCR-RFLP analysis

The size of the amplified region between *rbc*L and ORF106 was about 3.2kbp. In digestion patterns of the region with *Alu* I, *Ase* I, *Hinf* I, *Pst* I, and *Taq* I, different

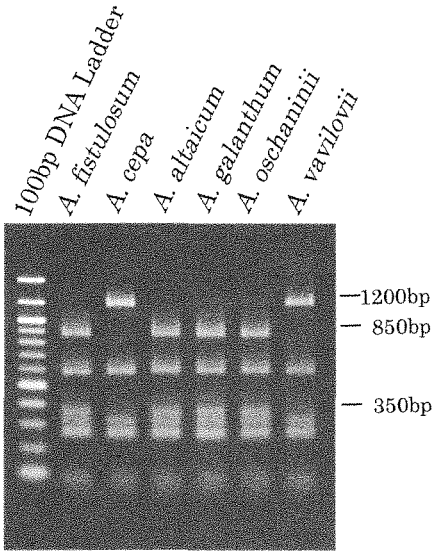

Fig. 5 Restriction patterns of *Alu* I digested *rbc*L-ORF106 region of cpDNA in *A. fistulosum*, *A. cepa*, *A. altaicum*, *A. galanthum*, *A. oschaninii*, and *A. vavilovii*.

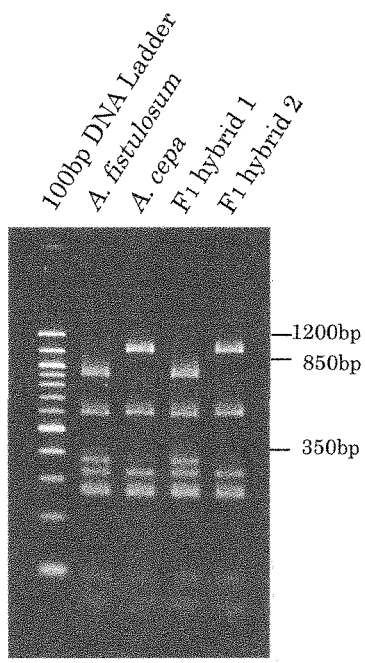

Fig. 6 Restriction patterns of *Alu* I digested *rbc*L - ORF106 region of cpDNA in *A. fistulosum*, *A. cepa*, and their reciprocal F_1 hybrids.
F_1hybrid 1: *A. fistulosum* × *A. cepa*,
F_1hybrid 2: *A. cepa* × *A. fistulosum*.

types were observed among six species : two types with *Alu* I, two types with *Ase* I, three types with *Hinf* I, three types with *Pst* I, and four types with *Taq* I (Fig.5, Table 6, 7). In all enzymes used, *Taq* I detected polymorphisms in the most species combinations in section *Cepa* (Table 8). In this method, it was possible to detect polymorphisms in all species combinations except for that of *A. cepa* and *A. vavilovii*. The reciprocal F_1 hybrids between *A. fistulosum* and *A. cepa* had the restriction patterns identical to those of their seed parents when their amplified products were digested with *Alu* I (Fig.6).

Table 6. Presence or absence of polymorphic fragments in *Alu* I digested *rbc*L-ORF106 region of cpDNA in six species.

Species	Polymorphic fragment (bp)		
	1200	850	350
A. fistulosum	−	+	+
A. cepa	+	−	−
A. altaicum	−	+	+
A. galanthum	−	+	+
A. oschaninii	−	+	+
A. vavilovii	+	−	−

+ : Present, − : Absent

Table 7. Types of restriction patterns of *rbc*L-ORF106 region of cpDNA in six species.

Species	*Alu* I	*Ase* I	*Hinf* I	*Taq* I	*Pst* I
A. fistulosum	1	1	1	1	1
A. cepa	2	2	2	2	1
A. altaicum	1	1	1	3	1
A. galanthum	1	1	1	3	2
A. oschaninii	1	1	3	4	3
A. vavilovii	2	2	2	2	1

Table 8. Distinguishability of cpDNA between six species.

Combination	Method[a] 1					2		3				
	Bgl II	*Kpn* I	*Pvu* II	*Sma* I	*Xho* I	*Bgl* II	*Bam*H I	*Alu* I	*Ase* I	*Hinf* I	*Taq* I	*Pst* I
A. fistulosum-*A. cepa*	+	+	+	+	+	+	−	+	+	+	+	−
A. fistulosum-*A. altaicum*	−	−	+	−	+	+	−	−	−	−	+	−
A. fistulosum-*A. galanthum*	+	−	+	+	+	−	−	−	−	−	+	+
A. fistulosum-*A. oschaninii*	+	+	+	+	+	−	+	−	−	+	+	+
A. fistulosum-*A. vavilovii*	+	+	+	+	+	+	−	+	+	+	+	−
A. cepa-*A. altaicum*	+	+	+	+	+	+	−	+	+	+	+	−
A. cepa-*A. galanthum*	+	+	+	+	+	+	−	+	+	+	+	+
A. cepa-*A. oschaninii*	+	+	−	+	+	+	+	+	+	+	+	+
A. cepa-*A. vavilovii*	−	−	−	−	−	−	−	−	−	−	−	−
A. altaicum-*A. galanthum*	+	−	+	+	+	+	−	−	−	−	−	+
A. altaicum-*A. oschaninii*	+	+	+	+	+	+	+	−	−	+	+	+
A. altaicum-*A. vavilovii*	+	+	+	+	+	+	−	+	+	+	+	−
A. galanthum-*A. oschaninii*	+	+	+	+	+	−	+	−	−	+	+	+
A. galanthum-*A. vavilovii*	+	+	+	+	+	+	−	+	+	+	+	+
A. oschaninii-*A. vavilovii*	+	+	−	+	+	+	+	+	+	+	+	+

[a]See text.
+ : Distinguishable.
− : Indistinguishable.

Discussion

This study inclusively demonstrated that three types of RFLP analyses of cpDNA using suitable enzyme, probe, and primer were so effective for identifying cpDNA of six species in section *Cepa*. All the methods performed successfully identified cpDNA of reciprocal F_1 hybrids between *A. fistulosum* and *A. cepa* and confirmed that cpDNA was of maternal inheritance in *Allium*, corresponding to a previous report[6]. Therefore, these methods are available for confirming cytoplasmic substitution between wild and cultivated species. In a comparison of three methods performed, the first and second methods were time-consuming in cpDNA purification, hybridization or signal detection. On the other hand, the third method allowed quick identification of cpDNA. Therefore, the third method was considered to be the most efficient one for identification of cpDNA in section *Cepa*.

No polymorphism in cpDNA was detected between *A. cepa* and *A. vavilovii* with any of the methods used in this study. This indicates that the homology of cpDNA is remarkably high between these species. Morphological studies[35),7),33)], crossability tests[34)], nuclear DNA[4)], and cpDNA and mitochondrial DNA analyses[10),11)]of *A. cepa* and *A. vavilovii* showed a close phylogenetic relationship between these species. Therefore, detailed investigations are essential to detect cpDNA polymorphism between these species. Polymorphisms observed in different specific regions of cpDNA of several plants have been successfully used for phylogenetic studies[31),37),8),19),23)]. Mes et al. [19)] reported that *trn*K, *trn*C-*trn*D, *psb*A-*trn*S, and *rbc*L-ORF106 regions of cpDNA were most suited for phylogenetic studies of *Allium* because these regions were easily amplified and exhibit a considerable

level of sequence variation with a restricted degree of length variation in twenty nine species of *Allium* (including *A. cepa* as a representative of the species in section *Cepa*) and seven species of related genera. Our study demonstrates that the PCR-RFLP analysis of the *rbc*L-ORF106 region is useful for identifying cpDNA of the species in section *Cepa*. Further investigations on the combinations of specific regions of cpDNA and restriction enzymes seem to make possible to distinguish between cpDNA of *A. cepa* and *A. vavilovii*.

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ネギ属*Cepa*節栽培種および野生種の葉緑体DNAの同定方法の比較検討

山下　謙一郎・大山　知泰・野田　ルミ・
宮崎　智也・田代　洋丞
(生物工学講座)

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摘　　要

ネギ属*Cepa* 節栽培種および野生種の葉緑体DNA（cpDNA）の有効な同定方法を検討するため，栽培種として*Allium fistulosum*および*A. cepa*，野生種として*A. altaicum*，*A. galanthum*，*A. oschaninii*および*A. vavilovii*を供試して，種々のRFLP分析法の比較を行った．葉から精製したcpDNAのRFLP分析では，*A. cepa*と*A. vavilovii*の組合せを除くすべての種間において多型が検出された．また，葉から全DNAを抽出し，*A. fistulosum*のcpDNAより開発したプローブ（pF1）を用いてサザンハイブリダイゼーション法によりRFLP分析を行った結果，多くの種間において多型が検出された．さらに，cpDNAの*rbc*L-ORF106領域のPCR-RFLP分析では，*A. cepa*と*A. vavilovii*の組合せを除くすべての種間において多型が検出された．これらの方法を*A. fistulosum*と*A. cepa* の正逆雑種のcpDNAの同定に応用した結果，いずれの方法でもcpDNAの同定が可能であり，cpDNAが母性遺伝することが確認された．本研究の結果から，これらの三つのRFLP分析法のいずれもが*Cepa* 節の種間のcpDNAの同定に有効であることが明らかになったが，*rbc*L-ORF106領域のPCR-RFLP分析法は，その迅速性から*Cepa* 節のcpDNAの同定に最も効率的であると考えられた．