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Genetic Distance of *Allium* Section *Cepa* by DNA Fingerprint

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ABSTRACT: Identification of compatible parental line is of great importance in introduction of useful characters to onion breeding program, beyond the severe hybridization barrier. Phylogenic analysis of *Allium* section Cepa was conducted through PCR by URPs, repeated sequences of *A. fistulosum*, and microsatellite markers. Totally 76 accessions originated from 21 countries were clustered into five groups at a 0.84-simi1arity level: group I ; *A. cepa* and its wild relatives and *A. cepa* ssp. *ascalonicum*, group II ; *A. cepa* ssp. *wakegii*, *A. cepa* ssp. *proliferum* and Samcheung-pa, group III ; *A. fistulosum* and *A. altaicum*, group IV ; *A. galanthum*, group V ; Soekkori-pa. Samcheung-pa and Soekkori-pa, Korean local varieties, shared band type of both Cepa group and Altaicum group, indicating that those are derived from interspecific hybridization between *A. fistulosum* and *A. cepa*.

Keywords : *Allium*, Section Cepa, PCR, URPs, Microsatellite

The genus *Allium* comprises 750 species within six subgenera, 46 sections and 11 subsections, and is distributed in wide geographical and ecological areas (Hanelt *et al.*, 1992; Stearn, 1992). *Allium* section *Cepa* belongs to Subgenus *Rhizirideum*, one of the largest subgenera in *Allium* (Hanelt, 1990). Japanese bunching onion (*A. fistulosum*) was separated from section *Cepa* and classified into section *Phyllodolon* (Vvedensky, 1944) or section *Fistulosa* (Tranb, 1968). Hanelt (1990) combined section *Cepa* and *Phyllodolon* into a single section *Cepa* with three informal alliances: *Galanthum* (*A. galanthum*), *Oschaninii* (*A. cepa*), and *Altaicum* (*A. fistulosum*). Section *Cepa* includes two economically important cultivated species, i.e., *A. cepa* (bulb onion) and *A. fistulosum* (Japanese bunching onion), as well as several wild species (Jones & Mann, 1963). *A. cepa* L. divided into three groups: the Common onion group, the Aggregatum group (potato onion, ever-ready onion and shallot), and the Proliferum group (top onion). Though both *A. cepa* ssp. *wakegii* and *A. cepa* ssp. *proliferum* have the same parental background, the parental strain of *A. cepa* is different. *A. cepa* ssp. *wakegi*, an allodiploid, is the hybrid between *A. cepa* ssp. *ascalonicum* (Aggregatum group) and *A. fistulosum* (Tashiro, 1984). While *A. cepa* ssp. *proliferum* (top onion) is believed to be a natural hybrid between *A. cepa* (common onion group) and *A. fistulosum* (Schubert *et al.*, 1983; Friesen & Klaas, 1998).

Introduction of useful characters from *A. fistulosum* is of great concern in onion breeding program. Since there is strong nuclear-cytoplasmic incompatibility between *A. fistulosum* and *A. cepa*, and hybrids show F1 sterility, embryo rescue for the inter-specific hybridization is needed. Identification of closely related species is highly recommended to introduce useful characters from wild relatives to cultivated *Allium*, beyond its hybridization barrier (Van der Valk *et al.*, 1991; Khrustaleva & Kik, 1998). Inter-specific hybridization of *Allium* was reported in several species by embryo rescue, protoplast fusion (Ohsumi *et al.*, 1993; Nomura *et al.*, 1994; Keller *et al.*, 1996; Buiteveld *et al.*, 1998).

URPs (universal rice primers) were designed by Kang *et al.* (1997) from repeated sequences of Korean wild rice. The main difference of URP-PCR from RAPD or AP-PCR (arbitrarily primed-polymerase chain reaction) is the use of relatively long (20 bp) primers, designed for fingerprinting any organism at a relatively high annealing temperature. Thus, high PCR reproducibility is expected (Wu *et al.*, 1991). It has been successfully applied for inter-specific classification of plants as well as fungi, bacteria and fishes (Kim *et al.*, 2001). This study investigates the phylogenic relationship of *Allium* section *Cepa* by PCR DNA fingerprint using URPs and further by repeated sequence based primers and microsatellite primers.

MATERIALS AND METHODS

Plant materials and DNA extraction

Allium section *Cepa* germplasm, comprising 11 subspecies, 76 accessions from 21 countries, was used for this study (Table 1). Leaves from three plants of each accession that was over wintered in plastic houses in Suwon, Korea were collected for DNA extraction. Genomic DNA was extracted and purified by using Plant Mini DNeasy kit (Qiagen, Germany).

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Table 1. List of *Allium* L. section *Cepa* accessions.

No.(76)†	No.(33)	Species	Origin	Cultivar
1		*A. cepa*	Philippines	Bagio, PHL
2		*A. cepa*	Yugoslavia	PI 370340
3		*A. cepa*	Jamaica	PI 414931
4		*A. cepa*	Mexico	Local
5		*A. cepa*	Korea	Nongwoodaego
6	1	*A. cepa*	Korea	Chenjuwhang-yangpa
7	2	*A. cepa*	United Kingdom	Ori
8	3	*A. oschaninii*	Former Soviet Union	PI 292163
9	4	*A. vavilovii*	Former Soviet Union	PI 281727
10	5	*A. dictyoprasum*	Former Soviet Union	PI 280558
11		*A.* sp.	Mongolia	Darkhan, MNG
12		*A.* sp.	Mongolia	Wild
13	6	*A. cepa* ssp. *ascalonicum*	Thailand	Din Daeng, THL
14	7	*A. cepa* ssp. *ascalonicum*	Vietnam	Ninhbinh, VNM
15		*A. cepa* ssp. *ascalonicum*	Vietnam	Ninhbinh, VNM
16		*A. cepa* ssp. *ascalonicum*	Vietnam	Hanoi, VNM
17		*A. cepa* ssp. *ascalonicum*	Vietnam	Hanoi, VNM
18	8	*A. cepa* ssp. *ascalonicum*	Mongolia	Local
19	9	*A. cepa* ssp. *ascalonicum*	Russia	Local
20	10	*A. cepa* ssp. *ascalonicum*	Germany	Local
21	11	*A. cepa* ssp. *ascalonicum*	United States of America	Yellow Potato
22		*A. cepa* ssp. *ascalonicum*	Czechoslovakia	Cervena
23	12	*A. cepa* ssp. *ascalonicum*	Czechoslovakia	Sumperska
24		*A. cepa* ssp. *ascalonicum*	United States of America	Frog's Legs Shallot
25	13	*A. cepa* ssp. *ascalonicum*	Netherlands	Pikant
26		*A. cepa* ssp. *ascalonicum*	Georgia	Sirakula
27		*A. cepa* ssp. *ascalonicum*	Georgia	Cvrili ohachvi
28	14	*A. cepa* ssp. *ascalonicum*	Georgia	Ravalsviliani
29	15	*A.* sp.	Korea	Samcheung-pa
30	16	*A.* sp.	Korea	Soekkori-pa
31	17	*A. cepa* ssp. *proliferum*	Italy	Local
32	18	*A. cepa* ssp. *wakegii*	Korea	Mooju local
33		*A. cepa* ssp. *wakegii*	Korea	Boryeong local
34	19	*A. cepa* ssp. *wakegii*	Korea	Pyeongchang local
35		*A. cepa* ssp. *wakegii*	Korea	Iksan local
36	20	*A. cepa* ssp. *wakegii*	Korea	Buahn local
37	21	*A. cepa* ssp. *wakegii*	Korea	Haenam local
38		*A. cepa* ssp. *wakegii*	Korea	Haenam local
39	24	*A. fistulosum*	Korea	Koyang local
40		*A. fistulosum*	Korea	Jinahn local
41		*A. fistulosum*	Korea	Pyeongchang local
42		*A. fistulosum*	Korea	Garang-pa
43	22	*A. fistulosum*	Korea	Gol-pa
44		*A. fistulosum*	Korea	Seoulbaek-pa
45	23	*A. fistulosum*	Japan	Ishikura
46		*A. fistulosum*	Korea	Keumjangyeadae-pa
47		*A. fistulosum*	United States of America	Kujyo-futo
48		*A. fistulosum*	United States of America	Aigarashu
49		*A. fistulosum*	Cuba	Cuba
50		*A. fistulosum*	United States of America	Everygreen Bunching
51		*A. fistulosum*	Bhutan	Kyoto
52	27	*A. altaicum*	Mongolia	Wild
53		*A. altaicum*	Mongolia	Wild
54	26	*A. altaicum*	Mongolia	Wild
55		*A. altaicum*	Mongolia	Wild
56		*A. altaicum*	Mongolia	Wild
57		*A. altaicum*	Mongolia	Wild
58		*A. altaicum*	Mongolia	Wild
59		*A. altaicum*	Mongolia	Local
60		*A. altaicum*	Mongolia	Local
61		*A. altaicum*	Mongolia	Local

Table 1. Continued

No.(76)†	No.(33)	Species	Origin	Cultivar
62		*A. altaicum*	Mongolia	Local
63		*A. altaicum*	Mongolia	Local
64		*A. altaicum*	Russia	I-5886
65		*A. altaicum*	Russia	I-5887
66	28	*A. altaicum*	Russia	I-5888
67		*A. altaicum*	Russia	I-5889
68		*A. altaicum*	Russia	I-5890
69		*A. altaicum*	Former Soviet Union	PI 280549
70		*A. altaicum*	Former Soviet Union	PI 280550
71	29	*A. altaicum*	Former Soviet Union	PI 280552
72		*A. altaicum*	Former Soviet Union	PI 280664
73	30	*A. chinense*	Mongolia	Local
74	31	*A. chinense*	Mongolia	Local
75	32	*A. galanthum*	Former Soviet Union	Wild
76	33	*A. galanthum*	Former Soviet Union	Wild
	25	*A. fistulosum/A. altaicum*	Korea/Mongolia	Hybrid

†No. 1-76 for Fig. 1; No. 1-33 for Fig. 2.

Table 2. List of primers used for PCR amplification.

	Primer	Sequence (5' to 3')	Reference
URPs	2R	CCCAGCAACTGATCGCACAC	Kang *et al.*(1997)
	4R	AGGACTCGATAACAGGCTCC	
	9F	ATGTGTGCGATCAGTTGCTG	
	13R	TACATCGCAAGTGACACAGG	
	30F	GGACAAGAAGAGGATGTGGA	
	41F	GGTTGTAGGCCGATATTGTC	
Repeated sequences of *A. fistulosum*	1L	CAACATCTGCGGTGCAAGGT	Designed from Irifune *et al.* (1995)
	2L	GCGGTGCAAGGTGCAACATT	
	3R	ACCTTGCACCGCAGATGTTG	

Table 3. List of microsatellite primers used for PCR reaction.

Primer	Annealing protocol	Microsatllite motif	Forward and reverse primer (5' to 3')
AMS03	56°C 35 fold	(GT)21	TAA CCC TAG GAT GAG TTG AG GGA TTT CCT CTT GAG ATG A
AMS07	67-65-63-62°C 08-03-03-35 fold	GTTTCTGTTT (CTT)6 (TC)2 TTT(CTT)2	TGC GAA TGT GAG GTT TTC TGC CGA CCC GGA AAT TTC GAT C
AMS09	54°C 40 fold	(AT)9 (GT)19	ACA ACT TTC AAT TGC ATT C CGT GGA CTA ACT TAC TAT CTA TC
AMS16	62-60-58-56-58°C 08-05-03-02-35 fold	(CA)20 (TA)2	CTG CAT TAA AAC AAC CAA ACT TG GAG CTC CAC TTC TTC CAA ACT AG
AMS18	56-55-54-53-54°C 03-03-03-03-36 fold	(CA)20(TA)3	ACT CGG GTG TTA TTC CAT CCA ATC AGA CAT ACC ATA CAA TC
AMS19	56-55-54-53-54°C 06-04-03-02-40 fold	GAAAAGAAGAAAGAGA AA(GAA)5 CAGAA	GCT CTG ATA CCA AAT GTA ACG A CGA ATG TGA GGT TTT CTG C
AMS20	58-56-55-54-56°C 05-03-03-02-36 fold	(GT)24	TTG AGC AGC AGA ACC AGA C ATT CGG ACG CAA CAC ATC
AMS21	59-58-56°C 06-05-40 fold	(CA)25	GGT TGT TTC CAC TAC ACT TGA G CGT CCT TGG TAT TCT TGT GC

PCR and data analysis

PCR conditions were adopted from those reported by Kang *et al.* (1997) and Kim *et al.* (2001). Sequences of URP (universal rice primer) are listed in Table 2. Amplification of *Allium* genomic DNA using URPs was performed in a PTC-100 thermal cycler (MJ Research, USA) programmed for 4 min at 94°C and 36 cycles of 1 min at 94°C, 2 min at 55, and 2 min at 72. PCR was carried out in 50 of reaction solution composed of 70~100 ng template DNA, 2.5 mM dNTP,

10X buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 20mM $MgCl_2$, 0.1% gelatin (Sigma)), 100 ng URPs, and 2.5 unit of DNA polymerase with combination ratio of 30 *Taq* (Takara, Japan) and 1 native *Pfu* (Stratagen Co.). Amplified DNA products were electrophoresed on 1.5% agarose gel on 70~90V for 7 hours and followed by staining with EtBr.

Individual URP-PCR products of each accession were scored for their presence (value=1) or absence (value=0). The phylogenic analysis was done by Nei method (Nei, 1987). The similarity coefficient (F) was calculated as the fraction of shared fragments between pairs of the accessions. For accessions, x and y, F=2Nxy/(Nx +Ny), where Nxy is the number of DNA fragments shared by accessions X and Y, while Nx and Ny are the number of fragments scored from the accessions X and Y, respectively. On the basis of the similarity coefficient, a dendrogram was constructed with the statistical program NTSYSpc (version 2.0, Exter Software, Setauket, NY) using the unweighted pair-group method with arithmetic mean (UPGMA).

Besides URPs, other primers were applied to obtain further information on phylogenic relationship of hybrids between *A. cepa* and *A. fistulosum*. Three primers were designed from repeated sequences of *A. fistulosum* (Irifune *et al.*, 1995) and amplified by the same methods in URPs with a little modification (Table 2). Eight sets of microsatellite primers were preselected from totally 30 sets of onion microsatellites (Fischer and Bachmann, 2000, Table 3). PCR was performed with the corresponding primer pairs: A sample of 32 μl containing 0.53 μM of each primer, 0.22 mM dNTP, 0.75 U *Taq* Polymerase (Takara, Japan), about 30 ng template DNA in a buffer with a concentration of 50 mM sodium and 2 mM magnesium ions. The three holds of these programs were 5 sec at 94°C, 45 sec at the cycle dependent variable annealing temperature and 60 sec at 72°C. PCR was preceded by two minutes of pre-denaturation at 94°C and followed by five minutes post-synthesis at 72°C. The apparent annealing temperatures and numbers of cycles are given in Table 3. Amplified DNA products from both repeated-sequence-derived primers and onion microsatellites were electrophoresed on 4% acrylamide gel followed by silver staining.

RESULTS AND DISCUSSION

DNA amplification by six URPs that were pre-screened from 12 primers performed from 76 accessions of section *Cepa*. Totally 111 polymorphic bands were produced from URP-2R, 4R, 9F, 13R, 30F and 41F. The genetic similarity index calculated from the PCR fingerprinting bands amplified by URPs was used to estimate the phylogenic relationship among *Cepa* accessions. Based on the URP-PCR fingerprint data, the genetic distance was used to construct a dendrogram for the *Cepa* accessions analyzed. Fig. 1 shows the genetic relationships of the accessions.

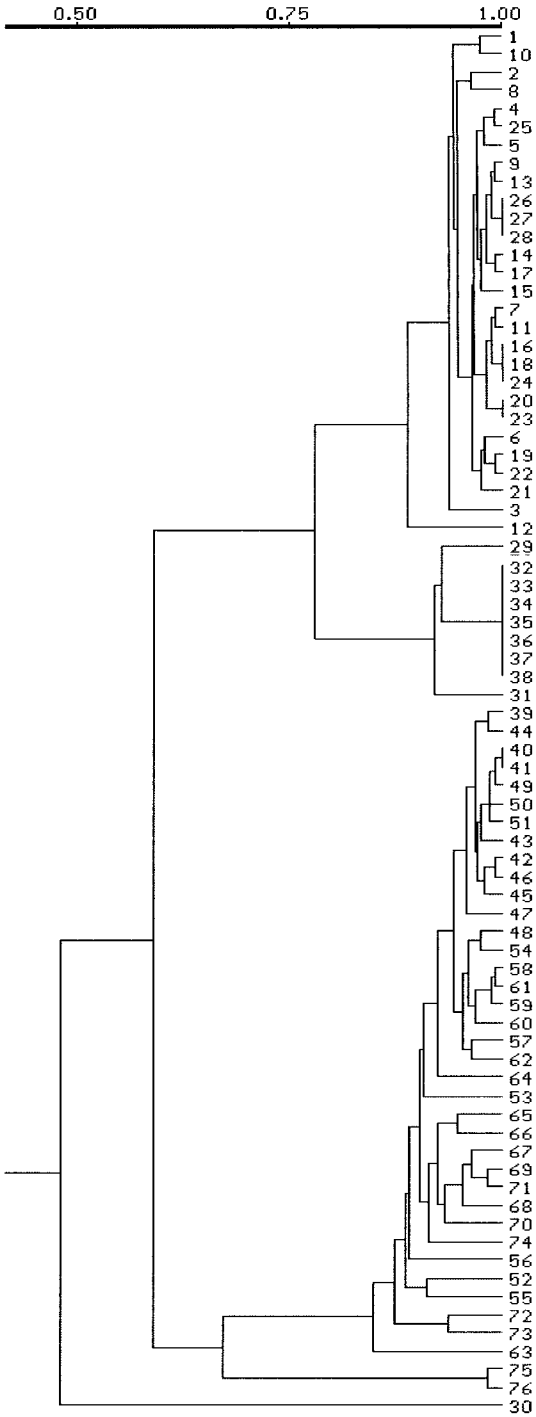

Fig. 1. Phylogenic relationship of 76 accessions of genus *Allium* L. section *Cepa* by URPs.

Allium section *Cepa* accessions are clustered into three groups at 0.67-similarity level (Fig. 1). Section Cepa-Oschanii (both cultivated and wild onions and shallots; Cepa group) is separated as one group. Section Cepa-Altaicum

(both cultivated and wild Japanese bunching onion; Altaicum group) and section Cepa-Galanthum (*A. galanthum*; Galanthum group) combined as one group. Soekkori-pa, Korean local variety, is separated from former two groups.

At 0.84-similarity level, *Cepa* accessions are clustered into five groups. Group I comprised common bulb onion, *A. cepa* ssp. *ascalonicum* (shallot) and wild relatives of onion, such as *A. vavilovii*, *A. oschanii* and *A. dictyoprasum* at a 0.88 similarity level. Three wild relatives of bulb onion and shallot, comprising 16 accessions from 8 countries, located within the intra-specific variation of *A. cepa*. This result coincides with the former report that shallot is close to the constitution of onion genome (Wilkie *et al.*, 1993).

Seven accessions of *A. cepa* ssp. *wakegii*, Korean landraces (Korean common name; Jjok-pa) were allotted in group II. As they are known as hybrid of *A. cepa* ssp. *ascalonicum* and *A. fistulosum*, they shared band patterns of both *A. cepa* group and *A. fistulosum* (No. 18-21 in Fig. 2). But they belong to onion group at 0.78-similarity level (No. 29-38 in Fig. 1). This group also contains morphologically different two accessions. One is Samcheung-pa, a unique Korean local cultivar, which forms bulbils (topsets) of third floor (No. 29 in Fig. 1). Another is *A. proliferum* (No. 31 in Fig. 1), originated from Italy. Samcheung-pa is morphologically similar to *A. cepa* ssp. *proliferum* (top onion). It (No. 29 in Fig. 1) produces swollen bulbs on bottom and several bulbils on the top of flower stalk. In some individuals, one to three flowers of fertile pollen were observed. The bulbils sprout and form the topset. Normally third floor topsets are observed, so it called Samcheung-pa, meaning third-floor Japanese bunching onion. Obviously it seems that Samcheung-pa is inter-specific hybrid between *A. fistulosum* and *A.*

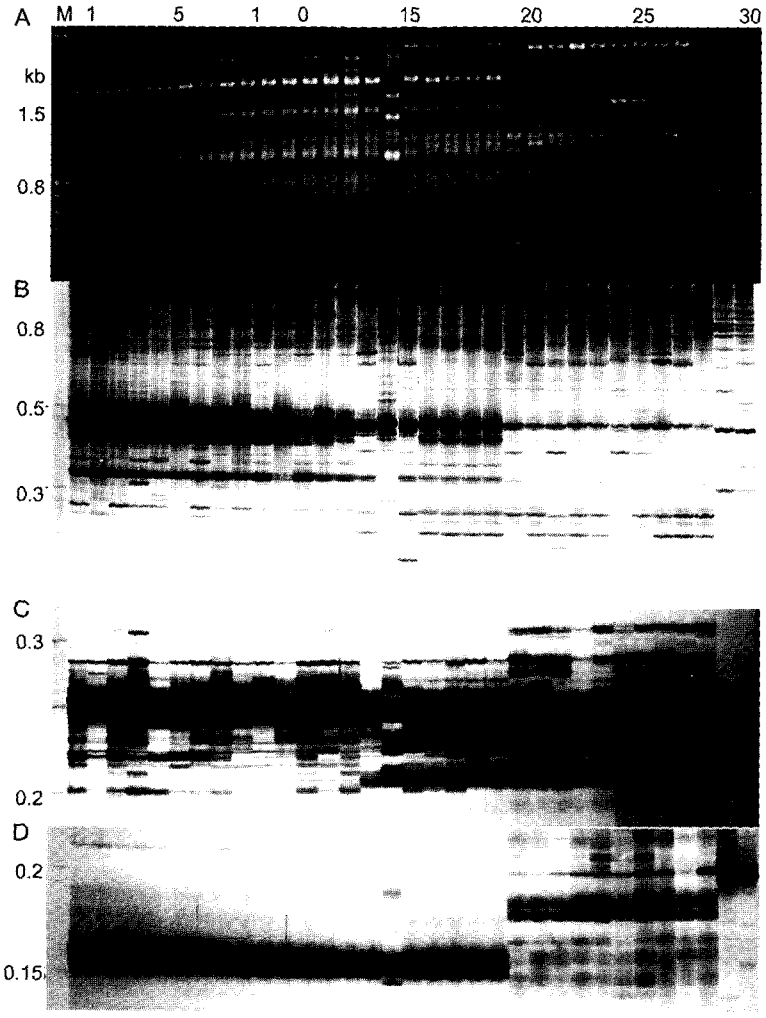

Fig. 2. Amplified DNA profiles from 33 accessions of *Allium* section *Cepa* by URP-2R(A), repeated sequence-based 1L(B) and microsatellite AMS-16(C) and AMS-18(D) primer. 1-2; *A. cepa*, 3; *A. oschaninii*, 4; *A. vavilovii*, 5; *A. dictyoprasum*, 6-14; *A. cepa* ssp. *ascalonicum*, 15; Samcheung-pa, 16; Soekkori-pa, 17; *A. prolifrum*, 18-21; *A. cepa* ssp. *wakegii*, 22-24; *A. fistulosum*, 25; hybrid(*A. fistulosum*/*A. altaicum*), 26-29; *A. altaicum*, 30-31; *A. chinense*, 32-33; *A. galanthum*.

cepa (No. 15 in Fig. 2). Its maternal line is similar to *A. fistulosum* in natural conditions. It has been cultivated in some local areas of Korea since early 1970s (personal communication with local people). In AFLP (amplified fragment length polymorphism) analysis of section *Cepa*, Samcheung-pa showed dissimilar band pattern to the both groups and had so many bands (Data not shown). Samcheung-pa can be used as a bridge cross for inter-specific hybridization between onion and Japanese bunching onion, as it has fertile pollen as well as is located between onion group and Japanese bunching onion group.

Group III consists of *A. fistulosum* and its wild relatives, such as *A. altaicum* and *A. chinense*, 13, 21 and 2 accessions, respectively. *A. altaicum* is highly related to cultivated *A. fistulosum* and easily hybridized to *A. fistulosum*, thus believed to be an ancestor (Inada & Iwasa, 1983). Friesen and Pollner (1999) concluded that *A. fistulosum* originated monophylotically from an *A. altaicum* progenitor. But Bradeen & Havey (1995) proposed polyphyletic origin. Dubouzet *et al.* (1997) reported *A. galanthum* to be the sister taxon of *A. fistulosum*. Totally 21 accessions of *A. altaicum* produced slightly swollen small bulbs in Suwon area, Korea, not in the case of original habitat. The species were collected in Northern Mongolia and Siberia, where wild plants are still grown. High temperature and long day length may influence the bulbing of this species. In this study genetic diversity of *A. altaicum* was a little bit broader than that of cultivated *A. fistulosum*, as in the case of former studies (Inada & Endo, 1994; Maass, 1997; Friesen & Pollner, 1999). Intra-specific similarity index of *A. altaicum* and *A. fistulosum* was 0.85 and 0.96, respectively. Decrease of genetic diversity in domestication process is probably due to severe genetic bottleneck and localization (Zohary, 1996). Two accessions of *A. chinense*, rakkyo (No. 30-31 in Fig. 2) belong to intra-specific variation of *A. altaicum*.

Two accessions of *A. galanthum* (No. 32-33 in Fig. 2; Galanthum group) are separated as group IV. They showed different band pattern from those of both Cepa group and Altaicum group, indicating that these two groups were separated long time ago. From the principal component diagram based on 21 aerial morphological characters, van Raamsdonk & de Vries (1992) classified the *Cepa* section into three groups: Cepa group (*A. cepa*, *A. vavilovii* and *A. oschaninii*), Altaicum group (*A. fistulosum* and *A. altaicum*), Galanthum group (*A. galanthum* and *A. pskemense*). They stated that *A. oschaninii* might originate from *A. galanthum*, primitive species. It evolved to *A. vavilovii* and *A. cepa* by developing a different karyotype. *A. altaicum* and *A. fistulosum* may have originated from Galanthum group along a different evolutionary line. In crossability test, no seeds were produced between *A. oschaninii* and *A. vavilovii*, *A. galanthum* or *A. fistulosum* (van Raamsdonk *et al.*, 1992). Bradeen & Havey (1995) classified the Galanthum group between Altaicum group and Cepa group from RFLP (restriction fragment length polymorphism) of chloroplast DNA. This study reveals that Galanthum group is closely related to Altaicum group than Cepa group (similarity level between *A. galanthum* and Altaicum group and Cepa group is 0.67, 0.59, respectively).

Cepa group (onion and wild relatives and shallot) and Altaicum group (Japanese bunching onion and relatives) are clearly separated at a 0.77-similarity level. And their inter-specific hybrid, *A. cepa* ssp. *wakegii* and relatives (group II in Fig. 1) are more related to Cepa group (group I in Fig. 1) than Altaicum group (group III in Fig. 1). The genome size measured by flow cytometry was consistent to this observation. Mean 2C DNA content of the Cepa group (*A. cepa*; 67, *A. oschaninii*; 66, *A. cepa* ssp. *ascalonicum*; 61) is reportedly larger than that of Altaicum group (*A. fistulosum*; 53, *A. altaicum*; 53), and that of Galanthum group (*A. galanthum*; 59 pg) is intermediate (Ohri *et al.*, 1998).

Soekkori-pa, Korean local variety, meaning cow-tail Japanese bunching onion, is separated as group V, at a 0.48-similarity level. This variety has prostrate leaves and produces irregular shaped numerous bulbs. Fig. 2 shows DNA profiles of 33 accessions of Cepa section from URP, repeated sequences of *A. fistulosum*, and microsatellite primers. Though Soekkori-pa (No. 16) shared both Cepa group (No. 1-14 in Fig. 2) and Altaicum group (No. 15-31), it has dissimilar band pattern to two groups. More detailed study, including genomic *in situ* hybridization (GISH) is recommended.

The tandemly repeated sequence (380 bp) was cloned and sequenced from genomic DNA of *A. fistulosum*, which was homologous (80%) to the 375 bp sequence of *A. cepa* (Barnes *et al.*, 1985; Irifune *et al.*, 1995). Primers were designed (Table 2) from repeated sequences of tandem duplication in *A. fistulosum* (Irifune *et al.*, 1995) and amplified (Fig. 2-B). PCR by microsatellite primers (Fig. 2-C, D) produced highly dense amplicons, possibly due to tandem duplication. Tandem duplication of chromosomal segments is believed to be an important factor in the evolution of *Allium*, a large genome species (King *et al.*, 1998).

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