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# Phylogenetic Analyses of Nuclear rDNA *ITS* Sequences of Korean *Allium* L. Subgenus *Rhizirideum* (Alliaceae)

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**Phylogenetic relationships among the Korean taxa of the genus *Allium* subgenus *Rhizirideum* and some related taxa were assessed on the basis of *ITS* sequences of nuclear ribosomal DNA. Twenty-eight accessions of the genus *Allium* L. consisting of subgenera *Rhizirideum* (19 taxa), *Allium* (5 taxa) and *Amerallium* (one taxon) were analyzed. The variation in the *ITS* region was informative at the levels of section except for sect. *Reticulato- bulbosa* which is known to be of multiple origin. The *ITS* 2 region was longer than the *ITS* 1 region, and all of the investigated *Allium* taxa were the same in length in the 5.8S region except for *A. monanthum*. *Allium cyaneum* var. *cyaneum* was the shortest (635 bp) and *A. victorialis* the longest (646 bp) among the investigated Korean taxa. The three morphologically similar taxa, *A. thunbergii*, *A. sacculiferum* that has been included in *A. thunbergii*, and *A. deltoide- fistulosum*, had the same *ITS* lengths of 641 bp, but were clearly distinguished in the phylogenetic analysis of their *ITS* sequences.**

*Allium* L. (Alliaceae) contains about 700-750 taxa in northern temperate region, including a number of important edible plants such as onion, green onion, garlic, leek and wild garlic (Hanelt et al., 1992; Berg et al. 1996; Mes et al., 1997; Klaas, 1998). The genus is characterized by perennials with alliaceous ordour and tunicated bulb. Leaves are basal, but their bases may form a flat or terete, sometimes fistular pseudostem. Inflorescences are umbel-like with few or many flowers, sometimes with bulbils. Tepals are six, and usually small, free or slightly united below. Stamens are six, and usually attached to the base of tepals. Filaments are often dilated at the base. Ovules are 1-10 in each locule, but often two. Seeds are broad and triangular in transection, with a thick phytomelan crust (Rahn, 1998).

The classification at intrageneric levels based on the morphological characters has been in dispute (Vvedensky, 1944; Bentham and Hooker, 1965; Hanelt et al., 1992). Recently, the genus was classified into five subgenera (*Allium, Amerallium, Rhizirideum, Melanocrommyum, Caloscordon*) according to the chromosome number and chloroplast DNA polymorphism (Samoylov et al., 1999).

Most of the Korean *Allium* except for *A. monanthum*, wild garlic and green onion belong to the subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo having a rhizome and basic chromosome number of 8 (Dubouzet et al., 1997; Klaas, 1998). Since Palibin (1901) had reported *A. japonicum*, Park (1949) recorded 21 species and two varieties including five cultivars, and Nakai (1952) summerized that there are 20 species, one variety and one forma in Korean *Allium*. Chung (1956, 1965) illustrated 11 species, and Kitagawa (1979) recorded 13 species, one variety, and one forma in Korean *Allium*. Lee (1996) summerized Korean *Allium* as 17 species, three varities, and two formas including cultivars. Yu et al. (1981) studied the taxonomy of the Korean *Allium*, and reported 12 species, two varieties, and one forma.

Many morphological or anatomical studies on *Allium* have been performed, but taxonomic studies based on flower, pollen, leaf, and seed did not reveal the gross morphological derivative characters among the genera, subgenera, and sections, due to the difficulty in identification of the minute characters of the dried specimen (Mes et al., 1997). Recently, molecular approaches such as isozyme, chloroplast DNA, and *ITS* sequence of nuclear rDNA have been applied to solve the morphological problems (Berg et al., 1996; Dubouzet et al., 1997; Mes et al., 1997; Dubouzet and Shinoda, 1998; Klaas, 1998).

This study is to reveal the phylogenetic relationships among taxa of Korean *Allium* subgenus *Rhizirideum* based on nuclear ribosomal DNA *ITS* sequence. Also, this study attempted to reveal the utility of *ITS* sequence data to taxonomic level such as section and species.

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## Materials and Methods

### *Plant materials*

In order to evaluate phylogenetic relationships in the genus, we selected 28 accessions representing 25 taxa and the vouchers were kept in the herbarium of Ewha Womans University (EWH) (Table 1).

*Allium monanthum* of subgenus *Amerallium* and five taxa of subgenus *Allium* were selected as outgroup, because they are native or cultivated taxa in Korean Peninsula and closely related to the subgenus *Rhizirideum* that is problematic.

### *DNA extraction*

Total DNAs were extracted from leaves, either fresh or dried with silica gel using modified hexaadecyltrimethylammonium bromide (CTAB) extraction method (Doyle & Doyle, 1987). Fresh leaf materials were powerded in liquid nitrogen and kept in -70℃ deep freezer until DNA extraction.

### *DNA amplification*

Total DNA extracted by CTAB was further purified with Geneclean Kit II (BIO 101, Carlsbad, USA) for polymerase chain reaction (PCR). The *ITS* regions were amplified using primers, ITS 1, ITS 4 or ITS 4-1 which was modified from ITS 4 described by White et al. (1990) (Table 2). PCR was carried out in 100 μL final volume containing 0.5 μg template DNA, 2.5 unit Taq DNA polymerase (Bioline, Randolph, USA), 10X PCR buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, and 500 mM KCl, pH 8.3) 10 μL, 20 μM for each dNTP, 0.5 μM of each primer and 5-10% DMSO. Amplification reactions involved 3 min at 95℃ for pre-denaturation, 35 cycles consisting of 1 min at 95℃ for denaturation, 1 min at 52℃ for annealing, 45 sec at 72℃ for extension, with increasing 3 sec per cycle after first cycle, and a final extension of 7 min at 72℃.

### *Sequencing*

Amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or PEG treatment. For the sequence reaction, the cycle sequencing reaction was carried out using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA, USA) and DNA thermal cycler (Thermal cycler 9600, Perkin

**Table 1.** List of samples used for ITS sequencing

| Subgenus / section / species | Source / Locality / Voucher |
|---|---|
| **Subgenus *Allium*** | |
| Section *Allium* | |
| *A. sativum* L.. | CHOLLIPO 94-183 |
| *A. scorodoprasum* L.. | Cultivated (Y. N. Lee) *A. ampeloprasum* L.. RDA |
| *A. porrum* L.. | CHOLLIPO (unknown) |
| *A. sphaerocephalum* L.. | CHOLLIPO 88-49 |
| **Subgenus *Amerallium*** | |
| Section *Microscordon* | |
| *A. monanthum* Maxim. | EWH Mt. Chonma, N. S. Lee 9848 |
| **Subgenus *Rhizirideum*** | |
| Section *Anguinum* | |
| *A. victorialis* L. | EWH Ulleung Is., H. K. Woo 98630 |
| Section *Butomissa* | |
| *A. tuberosum* Rottl. ex Spreng | Cultivated |
| *A. ramosum* L. | CHOLLIPO (unknown) |
| Section *Cepa* | |
| *A. cepa* L. | Cultivated |
| *A. fistulosum* L. | Cultivated |
| *A. wakegii* | Cultivated |
| Section *Cyathophorum* | |
| *A. cyathophorum* Bureau et Franch. | N. Friesen 6622 |
| Section *Petroprason* | |
| *A. obilquum* L. | EBG E00077857, Turda, Transsilvania. |
| Section *Rhizirideum* | |
| *A. senescens* L. | EWH Ulreung Is., H. K. Woo 9871 |
| *A. senescens* L. | CHOLLIPO (unknown) |
| *A. nutans* L. | EBG 19280485, cultivated (Hardy Farm) |
| *A. togashii* Hara | Shososhima 16. Aug, 1963, Sirokitamura |
| Section *Reticulato-bulbosa* | |
| *A. cyaneum* var. *cyaneum* Regel | EBG China, Sichuan, Aba Zang 88916 |
| *A. cyaneum* var. *cyaneum* Regel | EWH Mt. Halla, N. S. Lee & H. K. Woo 98181 |
| *A. cyaneum* var. *deltoides* S. Yoo, W. Lee & S. Lee | EWH Mt. Kaya, H. K. Woo 98827 |
| *A. cyaneum* var. *deltoides* S. Yoo, W. Lee & S. Lee | EWH Mt. Sorak, Bisundae, H. K. Woo 98727 |
| *A. splendens* Willd. | EWH Mt. Mai, H. K. Woo 98625 |
| Section *Sacculiferum* | |
| *A. sacculliferum* Maxim. | EWH Jangbong Is., N. S. Lee 98319 |
| *A. thunbergii* G. Don. | EWH Mt. Hwangmae, H. K. Woo 98630 |
| *A. deltoide-fistulosum* S. Yoo, W. Lee & S. Lee | EWH Koksung, H. K. Woo 98830 |
| Section *Schoenoprasum* | |
| *A. schoenoprasum* L. | EBG E00077861, Spain, Pido. |
| Section *Tenuissima* | |
| *A. anisopodium* Ledeb. | EBG E00077854, Russia, Buriatia, 1988 |

EBG, Botanical Garden of Edinburgh; CHOLLIPO, Chollipo Arboretum, Korea; RDA, Rural Development Administration, Korea.

**Table 2.** Primers for amplifying and sequencing the *ITS* regions of nuclear rDNA from genus *Allium*.

| Primer | Sequence | Reference |
|---|---|---|
| ITS1 | 5′-TCCGTGGTGAACCTGCGG-3′ | White et al. (1990) |
| ATS2 | 5′-GCTACGTTCTTCATCGACAC-3′ | Designed by H. K. Woo |
| ATS3 | 5′-GTGTCGATGAAGAACGTAGC-3′ | 〃 |
| ITS4 | 5′-TCCTCCGCTTATTGATATGC-3′ | White et al. (1990) |
| ITS4-1 | 5′-CCTCCGCCTTATTGATATG-3′ | Designed by S. Kim |

Elmer-Cetus, Foster City, CA, USA). In addition to the PCR primers, internal primer sequencing primers (Table 2) were used to complete sequencing in both direction. Automated sequencing was employed with ABI 377 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA).

*Sequence alignment and phylogenetic analysis*

The boundaries of *ITS* 1, 5.8S and *ITS* 2 regions were determined by comparison with previous studies (Takaiwa et al., 1985; Schidbel and Hemleben, 1989; Unfried and Gruendler, 1990; Baldwin, 1992; Kim and Jansen, 1994). Sequences were aligned using Clustal X program (Thompson et al., 1997). Phylogenetic analyses were performed by maximum parsimony method using PAUP program (Ver. 4.0.1, Swofford, 1998). Searches for the most parsimonious tree were conducted using a heuristic search with Fitch parsimony. Pairwise nucleotide differences were calculated by Distance Matrix option of PAUP program. The G + C content of *ITS* region was calculated directly from raw data of each taxon. In bootstrap analysis (Felsenstein, 1985) of trees, 1000 replicates were performed. Neighbor-joining tree (Saitou and Nei, 1987) was obtained by PAUP on the basis of the distances were calculated using the Kimura's two-parameter method (Kimura, 1980). Rates for variable sites were assumed to be equal. The minimum evolution was chosen for the objective function and negative branch-length handling was set to zero.

## Results

*ITS sequence analysis*

The sequences of *ITS* 1, *ITS* 2 and 5.8S coding regions of nuclear ribosomal DNA were generated from 28 accessions of subgenus *Rhizirideum* and outgroups in the genus *Allium* (Fig. 1). The *ITS* regions among the Korean taxa examined vary in length from 635 bp (*A. cyaneum* var. *cyaneum*) to 646 bp (*A. victorialis*). *ITS* 2 is longer than *ITS* 1 in the most of the taxa examined in this study, except for *A. tuberosum* and *A. ramosum*. The total length of *ITS* region of *A. thungergii, A. sacculiferum* and *A. deltoides*, which are difficult to distinguish by morphological characters and belong to section *Sacculiferum*, was the same (641 bp), and the length of their *ITS* 1, *ITS* 2 and 5.8S regions were all the same. The total *ITS* length of *A. cyaneum* var. *deltoides* was longer (639 bp) than *A. cyaneum* var. *cyaneum*, and the length of *ITS* 1 of *A. cyaneum* var. *cyaneum* was longer than *A. cyaneum* var. *deltoides*. The length of *ITS* 1 and *ITS* 2 regions fell within the reported values (187-298 bp and 187-252 bp) of the angiosperms (Baldwin et al., 1995). The shortest *ITS* 1 length among the Korean taxa was shown in *A. cyaneum* var. *deltoides* and *A. schoenoprasum* (233 bp) and the longest in *A. tuberosum* (241 bp). The shortest *ITS* 2 was shown in *A. cyaneum* var. *cyaneum* (236 bp), the longest in *A. fistulosum* (243 bp), and those of *A. victorialis, A. cepa, A. schoenoprasum, A. cyaneum* var. *deltoides, A. thungergii, A. sacculiferum* and *A. deltoide-fistulosum* were the same (242 bp). Sixty-six indel were observed in the region of *ITS* 1 and *ITS* 2, 43 bp indel in *ITS* 1, 23 bp indel in *ITS* 2 (Fig. 1). The 5.8S region was all the same (164 bp) for all the taxa except for one of outgroups, *A. monanthum* (163 bp), which had one deletion.

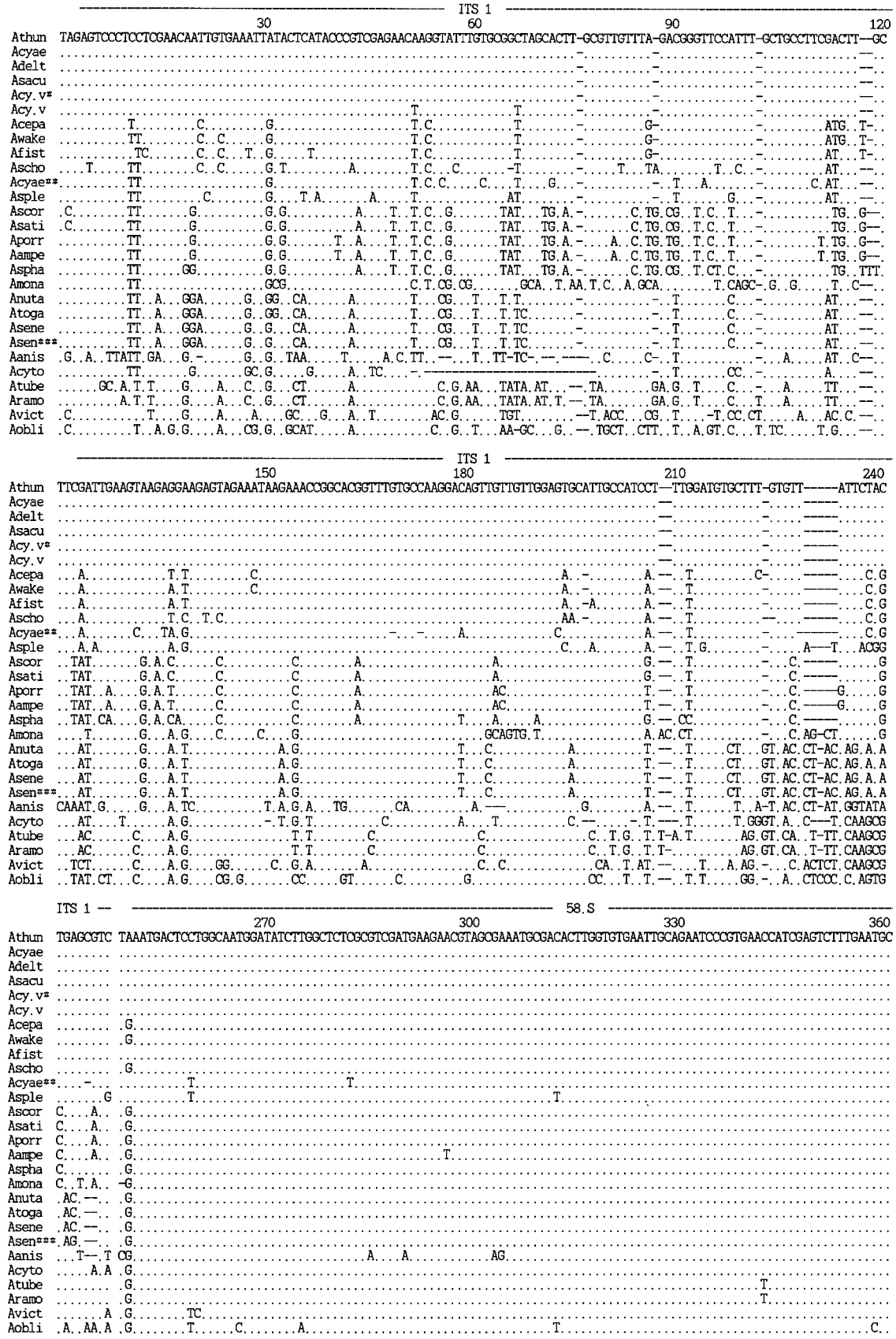
The G + C contents in *ITS* 1 varied from 41.8% (*A. senescens*) to 50% (*A. monanthum*), while *ITS* 2 varied from 43.6% (*A. senescens*) to 51% (*A. monanthum*). Among *A. thungergii, A. sacculiferum* and *A. deltoide-fistulosum* which are similar in morphology, the G + C contents in *ITS* 1 were 44.7%, 45.5%, and 45.1%, respectively, while the values in *ITS* 2 varied from 48.3% (*A. deltoide-fistulosum*) to 48.8% (*A. thungergii* and *A. sacculiferum*). The G + C contents in *ITS* 1, *ITS* 2 and 5.8S of *A. cyaneum* var. *deltoides* were consistently higher than those of *A. cyaneum* var. *cyaneum*.

*Sequence divergence and phylogenetic analysis of the ITS region*

A total of 405 variable sites among 664 sites were detected from the *ITS* 1 and *ITS* 2 regions. 264 sites were phylogenetically informative; 126 sites (47.7%) were in *ITS* 1, 138 sites (52.3%) in *ITS* 2. Parsimony analysis of the *ITS* sequences using equally weighed character states resulted in three equally parsimonious trees of 870 steps. The consistency index (CI) for the trees was 0.686, and the retention index (RI) was 0.776.

The subgenus *Rhizirideum* composes one clade with 64 synapomorphic characters and it was supported by 100% bootstrap value. The sections *Sacculiferum, Reticulato-bulbosa, Cepa* and *Schoenoprasm* of subgenus *Rhizirideum* were grouped with *Allium monanthum* of subgenus *Amerallium* as an outgroup, sharing 16 synapomorphic characters. The sections *Rhizirideum, Tenuissima, Butomissa* and *Anguinum* made the other clade.

The taxa of section *Sacculiferum* in subgenus *Rhizirideum* formed a single clade having one synapomorphic characters and it was supported by 71% bootstrap value. However, the accessions of section *Reticulato-bulbosa* did not form a monophyletic clade. One of the three equally parsimonious trees was


ITS 1
30
60
90
120
Athun TAGAGTCCCTCCTCGAACAATTGTGAAATTATACTCATACCCGTCGAGAACAAGGTATTTGTGCGGCTAGCACTT-GCGTTGTTTA-GACGGGTTCCATTT-GCTGCCTTCGACTT-GC
Acyae
Adelt
Asacu
Acy.v*
Acy.v
Acepa
Awake
Afist
Ascho
Acyae**
Asple
Ascor
Asati
Aporr
Aampe
Aspha
Amona
Anuta
Atoga
Asene
Asen***
Aanis
Acyto
Atube
Aramo
Avict
Aobli
ITS 1
150
180
210
240
Athun TTCGATTGAAGTAAGAGGAAGAGTAGAAATAAGAAACCGGCACGGTTTGTGCCAAGGACAGTTGTTGTTGGAGTGCATTGCCATCCT—TTGGATGTGCTTT-GTGTT-----ATTCTAC
ITS 1 — 58.S
270
300
330
360
Athun TGAGCGTC TAAATGACTCCTGGCAATGGATATCTTGGCTCTCGCGTCGATGAAGAACGTAGCGAAATGCGACACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAATGC

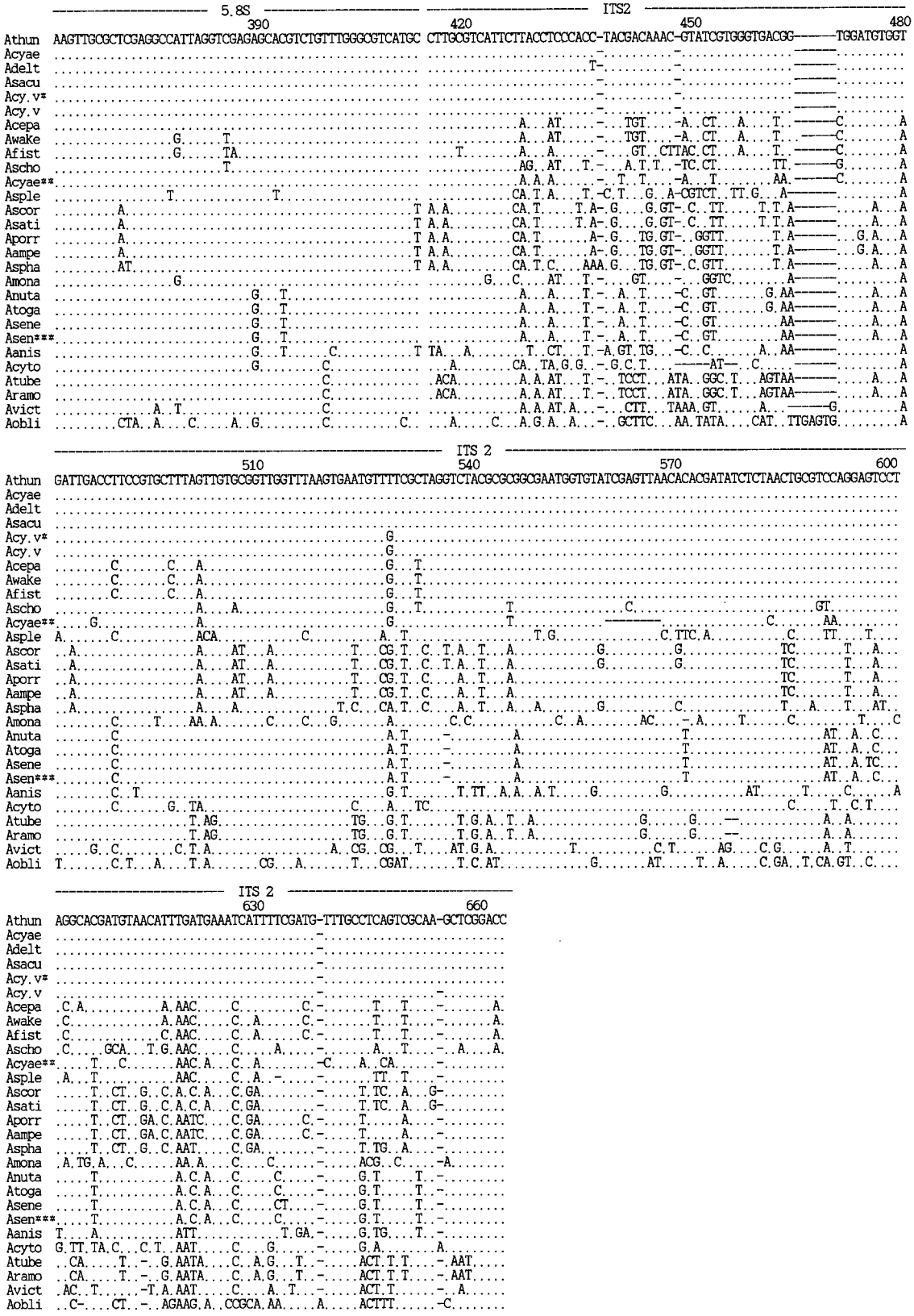



**Fig. 1.** Aligned sequences of *ITS* regions from 28 accessions of subg. *Rhizirideum* and the outgroup taxa. Dashes (−) are gaps required for alignment and dots (.) indicate matched sequences to the first taxon. *, plant from Mt. Sorak, Korea; **, plant from Sichuan, China; ***, cultivated plant from Chollipo Arboretum, Korea (see Table 1 for detail).

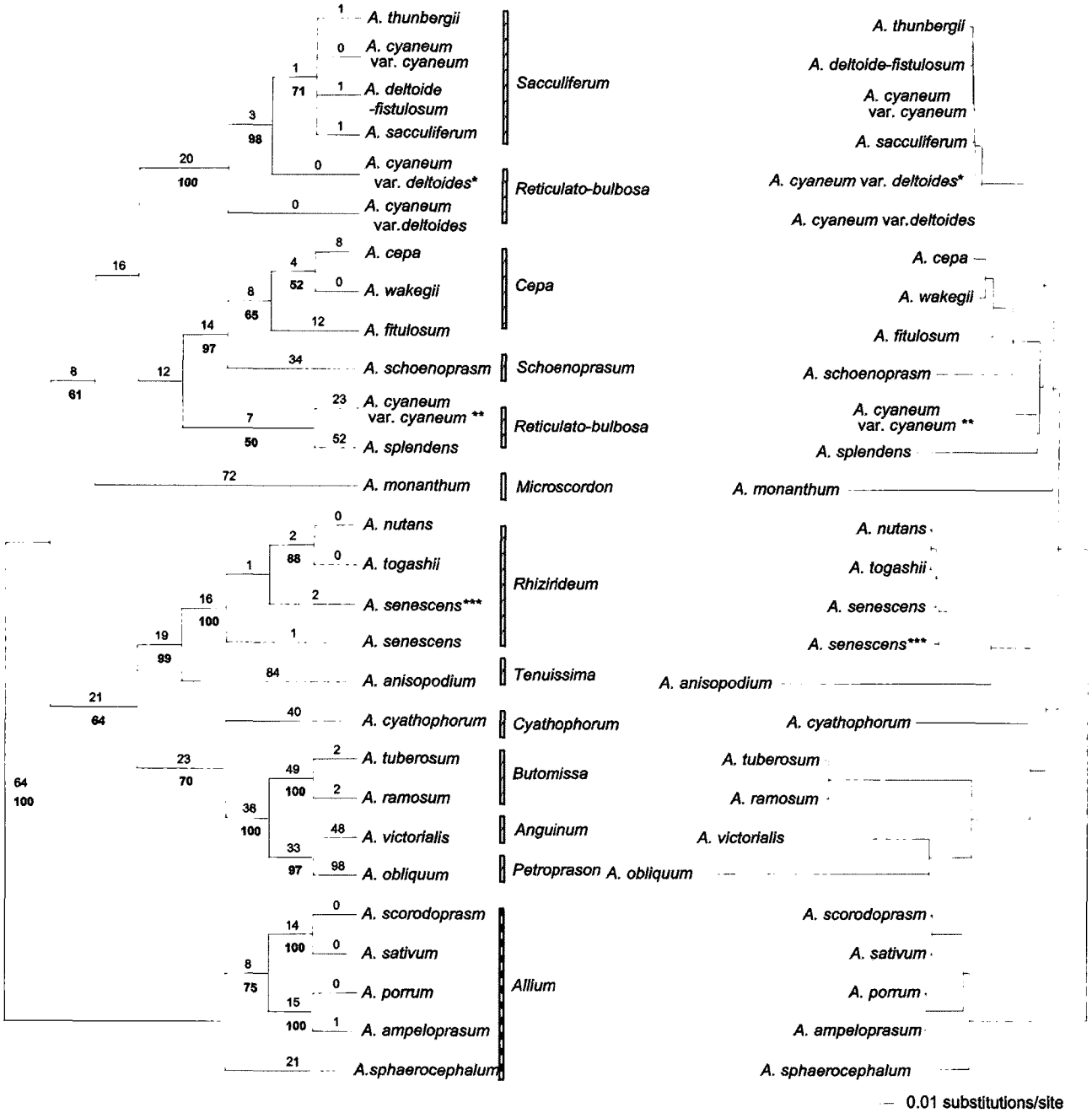



**Fig. 2.** A most parsimonious tree (left) and neighbor-joining tree (right) of *Allium* based on *ITS* sequences. Numbers above branches represent nucleotide subtitutions. Numbers below branches are corresponding bootstrap values. *, plant from Mt. Sorak, Korea; **, plant from Sichuan, China, *** cultivated plant from Chollipo Arboretum, Korea (see Table 1 for detail).

basically identical to the neigbor-joining tree (Fig. 2).

## Discussion

*ITS* sequence analyses showed variation at the sectional level. Three taxa of sect. *Sacculiferum, A. sacculiferum, A. thunbergii* and *A. deltoide-fistulosum,* formed a sinlge clade, but their phylogenetic relationships were not resolved at the species level. It is also very difficult to distinguish them based on morphological characters. It supports the taxonomic treatment that *A. sacculiferum* is included in *A. thunbergii,* in spite of their difference of leaf cross-section, length of leaf sheath, and filament of stamen (Wang and Tang, 1978). *Allium deltoide-fistulosum* which was described by Yu et al. (1981) was not separated from either *A.*

*thunbergii* or *A. sacculiferum*. With respect to the leaf cross-section and filament of stamen, the morphological characters of *A. deltoide-fistulosum* were overlapped with those of *A. thunbergii*. *ITS* and morphological data imply that *A. deltoides* could be a variety of *A. thunbergii* or should be included in *A. thunbergii*. This result suggests that the other phylogenetic methods such as isozyme, RAPD or ISSR are helpful in revealing the variation and the taxonomic delimitation among taxa of this section. It was reported that *ITS* 1 is longer than *ITS* 2 in many plants (Baldwin, 1992; Baldwin et al., 1995), but *ITS* 1 in Poaceae is longer than *ITS* 2 (Baldwin et al., 1995). *ITS* 2 in the taxa examined in this study is longer than *ITS* 1, except for *A. tuberosum* and *A. ramosum* of section *Butomissa* in subgenus *Rhizirideum*.

Some accessions of section *Reticulato-bulbosa*, *A. cyaneum* var. *cyaneum* from Mt. Halla and *A. cyaneum* var. *deltoides* from Mt. Kaya and Bisundae of Mt. Sorak, formed a clade with section *Sacculiferum*, which was separated from the clade, *A. cyaneum* var. *cyaneum* of China and *A. splendens*. It suggests that section *Reticulato-bulbosa* is not a monophyletic group (Fig. 2). *Allium anisopodium* that was described at Bisundae of Mt. Sorak by Yu et al. (1981) was considered as *A. cyaneum* var. *deltoides*, because the morphological characters were different from the taxonomic descriptions (Vvedensky, 1944; Wang and Tang, 1978) and it was grouped with *A. cyaneum* var. *deltoides* by having 3 synapomorphic characters in *ITS* sequences (Fig. 2).

*Allium victorialis* of sect. *Anguinum* and *A. senescens* of section *Rhizirideum* were separated from the other clade of the Korean subgenus *Rhizirideum*. It is consistent with the morphological characters such as leaf shape and the number of ovules per locule. It is congruent with the previous study showing that section *Angunum* is far from the other section of the subgenus in dot blot analysis for 10 sections of the subgenus *Rhizirideum* (Dubouzet et al. 1997).

*Allium monanthum* used as one of outgroups was different from the other Korean taxa of subgenus *Rhizirideum* with respect to morphological characters and the reproduction by bulblets or bulbils. However, *A. monanthum* nested in the clade with the other ingroup taxa of the subgenus *Rhizirideum*. This is congruent with the previous results from cpDNA analysis and dot blot examination claiming that the subgenus *Rhizirideum* could be polyphyletic (Dubouzet, 1997; Mes et al., 1997).

In the phylogenetic examination of subgenus *Rhizirideum* and related taxa of *Allium* with *ITS* sequences, the most parsimonious and neighbor-joining trees presented similar topologies in spite of very different algorithms by two methods. The study demonstrates that *ITS* sequence analyses are very useful in addressing phylogenetic questions on the section and subgenus, and more comprehensive sampling will certainly enhance our understandings on the phylogeny of *Allium*.

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