DOI : 10.5958/0974-0112.2020.00040.7

Indian J. Hort. 77(2), June 2020: 293-300

Species discrimination of mountain garlic based on the nrDNA ITS region sequence

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ABSTRACT

Mountain garlic is an edible perennial herb widely distributed in Asian, European, and Siberian regions, referring to a class of *Allium* species. In South Korea, mountain garlic mainly consists of two *Allium* species, *Allium ochotense* Prokh. and *A. microdictyon* Prokh. Due to having spicy garlic odor and nutrition contents, mountain garlic is used as a vegetable and traditional medicine in South Korea, Japan, and China. However, the genetic relationship between *A. ochotense* and *A. microdictyon* belonging to the subgenus *Anguinum* is still unclear. To understand the genetic diversity and phylogenetic relationships of mountain garlic populations in South Korea, we collected 25 mountain garlic materials from 8 geographical areas and analyzed their genetic diversity and relationships based on sequence variations of the nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region. The DNA alignment result showed that *A. ochotense* and *A. microdictyon* were clearly divided into two groups, sharing 11 respective, particular nucleotide variable sites. It suggested that the nrDNA ITS region could be used to discriminate among *Allium* intraspecies. This species identification method would be utilized to distinguish unclear mountain garlic materials. This work not only provides more sequence resources of mountain garlic, but also validates the efficiency of the molecular discrimination method based on the nrDNA ITS region sequence.

Key words: Genetic diversity, sequence variation, *Allium ochotense*, *Allium microdictyon*, nrDNA ITS.

INTRODUCTION

Mountain garlic is an edible perennial herb, widely distributed in Asia, Europe and Siberia (Hur *et al*., 7). Due to having especial spicy garlic odor, and rich nutrition contents, mountain garlic is considered as some famous *Allium* species. In South Korea, the leaves of mountain garlic are not only used as vegetables but also as a folk medicine for the treatment of gastritis and heart failures (Woo *et al*., 15). In Japan, it is the monks' favorite as a potential functional vegetable (Nishimura *et al*., 13). Recently, many studies show that mountain garlic has some potentially significant pharmacological properties, such as anti-arteriosclerotic (Kim *et al*., 8), anti-cancer (Lee *et al*., 11), anti-obesity (Choi *et al*., 2), anti-neuroinflammatory (Woo *et al*., 16), hepatoprotective, and nephroprotective effects (Kim *et al*., 10). Furthermore, Choi *et al*. (3) found that extracts of mountain garlic can be used as an antimicrobial and antioxidant in the food industry. All these establish mountain garlic's enormous medicinal values and economic benefits. However, because mountain garlic belonging to the genus *Allium,* one of the largest genus in the family of the Amaryllidaceae having more than 920 species (Herden *et al.*, 6), consists of several *Allium* species (*A. lusitanicum*, *A. senescens* subsp. *montanum*, *A. montanum* F.W. Schmidt, *A. ochotense* Prokh., and *A. microdictyon* Prokh., and others), their evolutionary and phylogeny are always discussed. Despite many previous studies have been done, there are still many gaps in our knowledge of their infrageneric taxonomy and evolution of the genus *Allium*.

In South Korea, mountain garlic mainly includes *A. ochotense* Prokh., *A. microdictyon* Prokh. naturally distributed over a mountainous area, including Jiriin Mountain, Odae Mountain, and the forest region of Ulleung Island. Due to the sampling distribution area, *A. victorialis* var. *platyphyllum* in South Korea, which is usually reported in previous studies (Kim *et al*., 9; Yoo *et al*., 18; Hur *et al*., 7), is a synonym for *A. ochotense* (Friesen *et al*., 5). Because the *A. victorialis* L. is only distributed in rocky places and mountain pastures along the mountain ranges of Europe and the Caucasus Mountains (Stearn, 14), *A. ochotense* has relatively wide distribution in the East

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Asian and the Southeast of Russian. To understand the genetic diversity among different populations of mountain garlic, Yoo *et al*. (18) had investigated the morphological information of mountain garlic varieties in South Korea. The results suggested that there was no difference in morphological traits between Jiriin Mountain variety and Odae Mountain variety, but not with Ulleung Island variety. Kim *et al*. (9) utilized randomly amplified polymorphic DNA-PCR method to analyze their genetic variations, and also supported that the Ulleung Island variety was discrete from the other two varieties. The detailed taxonomic phylogeny of *Allium* species was described based on the internal transcribed spacer (ITS) region sequences of nuclear ribisomal DNA (nrDNA) at sectional level (Friesen *et al*., 5), and *A. microdictyon* Prokh. and *A. victorialis* L. was newly divided into Subgenus *Anguinum* of Section *Anguinum* from traditional Subgenus *Rhizirideum* of Section *Anguinum*. However, unfortunately, the phylogeny and evolution of *A. ochotense* was not included in all these phylogenetic studies (Friesen *et al*., 5; Li *et al*., 12). Later, Herden *et al*. (6) manly focused on the phylogeny analysis of subgenus *Anguinum*, consisting of ten species and some varieties. According to the nrDNA ITS and chloroplast *rps16* intron, *rbc*L-*atp*B spacer, and *rpl*32-*trn*L spacer sequences, *A. ochotense* and *A. microdictyon* were divided into one monophyletic group, with *A. victorialis* as its sister group. Until now, the intraspecific phylogenetics between *A. ochotense* and *A. microdictyon* was still indistinct.

With the continuous development of science and technology, molecular marker technology develop rapidly compared to the morphological classification method. Molecular markers have many advantages over the morphological markers, including (i) abundance, (ii) phenotypic neutrality, (iii) co-dominance, and (iv) tissue and environment independent expression. Currently, there are dozens of DNA molecular markers, such as chloroplast DNA *rbc*L, *trn*H-*psb*A, *trn*L-*trn*F, *mat*K, 5S, 16S, 18S, and the internal transcribed spacer (ITS) nuclear ribosomal DNA (nrDNA) genes (Agarwal *et al*., 1). Among them, ITS region, between 18S ribosomal RNA (rRNA) and 28S rRNA, is widely used in the species classification of plants, especially intraspecific varieties of plants.

In the present study, we collected 25 different mountain garlic samples from 8 geographical areas of South Korea, which contained two main *Allium* species of mountain garlic. Their genetic diversity and phylogenetic relationships were evaluated based on the nrDNA ITS sequence analysis. Our result indicated that *A. ochotense* materials could be clearly divided from *A. microdictyon* materials. This study would provide more information about ITS sequences of mountain garlic, and validate the efficiency of the molecular discrimination method based on the nrDNA ITS region sequence. This result also could provide us a more efficient molecular method to determine which species some mountain garlic sample belongs to. Using this molecular classification method, the mountain garlic sample (GCAoG) which species is unclear or undetermined has been divided to *A. microdictyon* group. According to the resolution degree, we could basically conclude GCAoG is *A. microdictyon*.

MATERIALS AND METHODS

Twenty-five samples of mountain garlic were collected from eight different geographical areas in South Korea, including two *Allium* species. Fresh leaves were collected from healthy growing plants and immediately stored in liquid nitrogen. The specimen vouchers of samples investigated in this study and their collection geographical areas were listed in Table 1.

Total genomic DNAs were extracted using a plant DNA isolation mini kit (DNeasy Plant Mini Kit, Qiagen, Hilden, Germany). The stored leaf samples of mountain garlic in liquid nitrogen were ground into fine powder for total DNA extraction. The universal primers ITS5 (5'-GAA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 17) were used to amplify the nrDNA ITS region. Polymerase Chain Reaction (PCR) was performed in a 20 µl reaction volume with the following reaction components: 2 µl 10× PCR buffer, 20 mg template DNA, 0.20 mM dNTPs, 0.5 µM forward and reverse primer, 0.025 U i-star max DNA polymerase (Intron Sequence editing and alignment: Biotechnology, Seongnam, Korea), and sterile distilled water. The PCR amplification was programmed for pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. In addition, a final extension step was performed at 72°C for 10 min. The PCR products were checked by 1.0% agarose gel electrophoresis, and each product was purified with a Gene All DNA purification kit (Seoul, South Korea). The purified products were sequenced by an ABI4000 sequencer (ABI Inc., Foster City, CA, USA).

The sequencing results were edited and assembled using the software program DNAMAN version 6.0 (Lynnon Biosoft Corporation, USA, www.lynon.com). The correctness of our assembled results were confirmed through Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) server. After confirming the correctness of our sequencing results, they were submitted and

Table 1. The detailed information about specimen vouchers, collection geographical areas, and NCBI accession number of mountain garlic samples investigated in this study.

Sample No.	Specimen voucher	Populations	Species	Collection geographical areas	NCBI accession No.
1	GCAoU-1	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Chuncheon City	MF599168
2	GCAoG	Gariwang-M	unclear or undetermined*	Gangwon Do Chuncheon City	MF599169
3	GCAoC	China	*Allium ohotense* Prokh.	Gangwon Do Chuncheon City	MF599170
4	GCAoU-2	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Chuncheon City	MF599171
5	GPAoU-1	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Pyeongchang County	MF599172
6	GPAoO-1	Odae-M	*Allium microdictyon* Prokh.	Gangwon Do Pyeongchang County	MF599173
7	GPAoU-2	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Pyeongchang County	MF599174
8	GPAoU-3	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Pyeongchang County	MF599175
9	GPAoO-2	Odae-M	*Allium microdictyon* Prokh.	Gangwon Do Pyeongchang County	MF599176
10	GHAAoU	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hwacheon County	MF599177
11	GHAAoO	Odae-M	*Allium microdictyon* Prokh.	Gangwon Do Hwacheon County	MF599178
12	GHOAoU-1	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hongcheon County	MF599179
13	GHOAoU-2	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hongcheon County	MF599180
14	GHOAoU-3	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hongcheon County	MF599181
15	GHOAoU-4	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hongcheon County	MF599182
16	GHOAoO	Odae-M	*Allium microdictyon* Prokh.	Gangwon Do Hongcheon County	MF599183
17	GHOAoU-5	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hongcheon County	MF599184
18	GYAoU-1	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Yanggu County	MF599185
19	GYAoU-2	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Yanggu County	MF599186
20	GGAoU-1	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Gangneung County	MF599187
21	GGAoO	Odae-M	*Allium microdictyon* Prokh.	Gangwon Do Gangneung County	MF599188
22	GGAoU-2	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Gangneung County	MF599189
23	GIAoU-1	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Inje County	MF599190
24	GIAoU-2	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Inje County	MF599191
25	GHEAoU	Ulleung-I	*Allium ohotense* Prokh.	Gangwon Do Hoengseong County	MF599192

No. means number. 'I' means Island, and 'M' means Mountain. *This material was naturally grown in Gariwang-Mountain (Gariwant-M) of South Korea, but cultivated in Gangwon-Do Chuncheon-City, South Korea. Because the morphologic traits and its taste are very different from both populations from Ulleung-I and Odae-M, it suggests the population from Gariwang-M is not belonging to *A. ohotense* Prokh. or *A. microdictyon* Prokh. However, there has been no determined species of this population from Gariwang-M, so for this population, the species was marked as unclear or undetermined.

logged in the NCBI GenBank database, with the accession numbers listed in Table 1. Phylogeny reconstruction was carried out using DNAMAN 6.0 based on the neighbor-joining method. Genetic distance (GD) value was calculated by MEGA 5.0.

RESULTS AND DISCUSSION

Twenty-five samples of mountain garlic were collected from 8 different geographical areas in South Korea, among which 18 samples were Ulleung Island species belonging to *A. ohotense* Prokh., 5 samples were Odae Mountain species belonging to *A. microdictyon* Prokh., 1 was Chinese species belonging to *A. ohotense* Prokh., and 1 was Gariwang Mountain species (Table 1). The species of Gariwang Mountain sample was not unclear or undetermined, but the local botanist used it as mountain garlic. Ulleung Island species showed wide oval leaves (Fig. 1A) and light green bulbs (Fig. 1C), while Odae Mountain species showed tall and slender leaves (Fig. 1B), and reddish brown bulbs (Fig. 1D).

The universal primer set ITS4/ITS5 (White *et al*., 17) was used to amplify the total nrDNA ITS region of mountain garlic in this study. The total ITS regions were successfully amplified and submitted

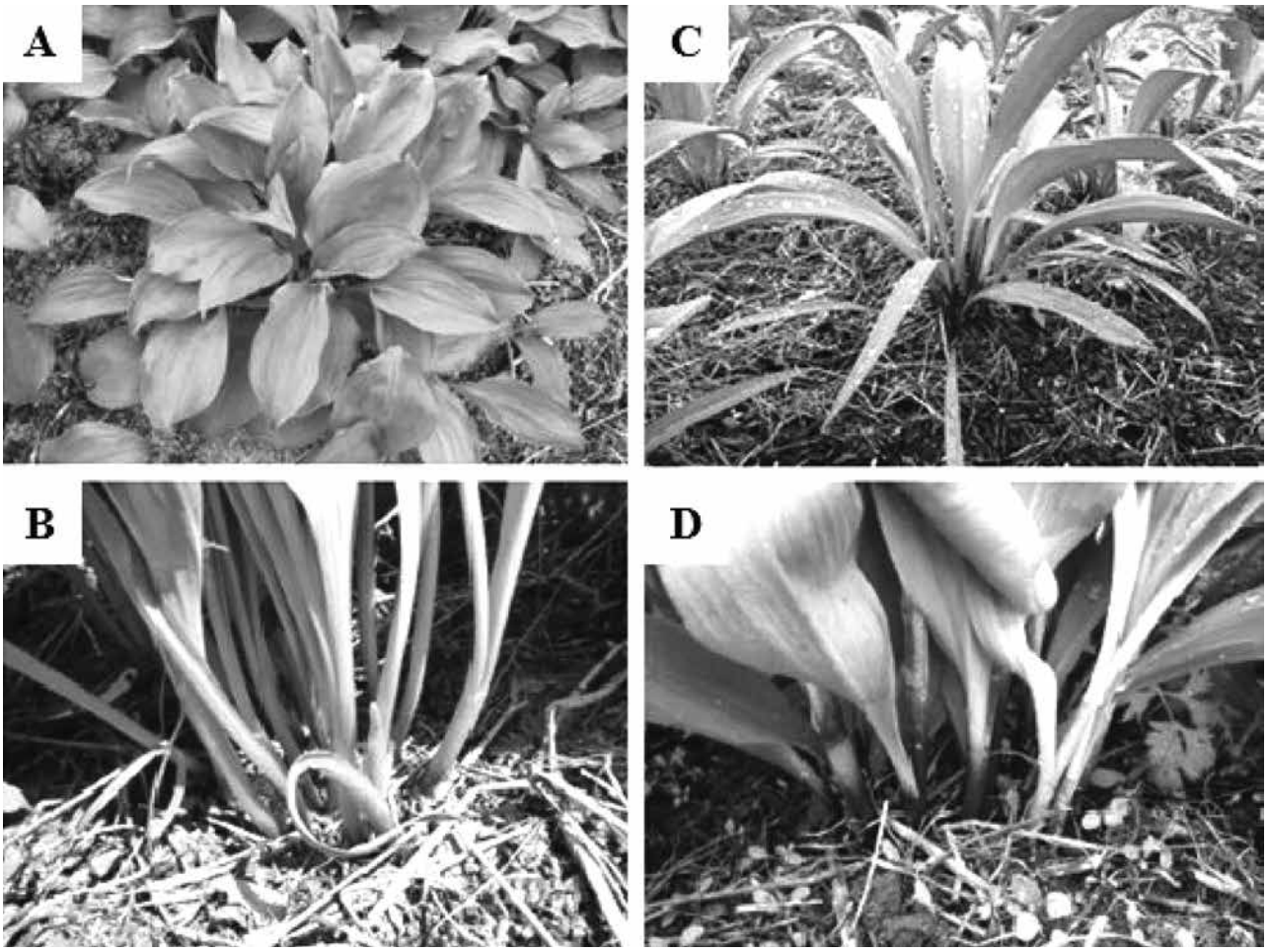

Fig. 1. Leaf (A and C) and bulb (B and D) morphological information of Ulleung Island species (*Allium ohotense* Prokh., A and B) and Odae Mountain species (*Allium microdictyon* Prokh., C and D).

in the NCBI GenBank database. The validity of PCR amplification was checked through the BLAST of the NCBI server. Taken our sequencing result of Sample 1 as example, it showed 100% identity rate with the existing ITS sequence report from *A. ochotense* (GQ412223, and GQ412224), 99% with *A. ochotense* (LN812896), 98% with *A. microdictyon* (GQ412216, and LN812905), and *A. victorialis* (KF419383, and LN812894). It suggested that our sequencing results were nrDNA ITS region sequences from *Allium* species, as expected.

The sequence lengths of the total ITS region of 25 samples were 683-697 bp (Table 2). The length of ITS1 ranged from 262 bp (Sample 24) to 270 bp (Sample 6, 7, 9, 11, 13, 16, and 21). The G+C content of the ITS1 region ranged from 47.04% (Sample 7) to 48.31% (Sample 2), with an average content of 47.57%. The length of ITS2 ranged from 253 bp (Sample 4, and 6) to 267 bp (Sample 18), and the G+C content ranged from 43.61% (Sample 4) to 47.57% (Sample 18), with an average content of 47.14%. The 5.8S regions were the same length because of the high conservation of this region (Cullings and Vogler, 4).

According to the alignment results, the 25 samples shared 98% similarity. The genetic distance (GD) value was calculated using MEGA software. The highest value (0.023) appeared between Sample 2 and Sample 3, and between Sample 2 and Sample 8. There are many varieties that showed the lowest GD value, 0, with each other, such as between Sample 1 and Samples 4, 5, 7, 10, 12, 13, 14, 17, 18, 19, 20, 22, 23, 24, 25, and between Sample 6 and Samples 9, 11, 16, 21. The typical variable nucleotide sites were also analyzed and are shown in Table 4. There were 15 variable nucleotide sites obtained in the total ITS region, including 1 site deletion, 2 site insertions, and 12 site substitutions. Among them, 11 variable sites were specific between *A. ochotense* Prokh. and *A. microdicyton* Prokh. Sample 2, 6, 9, 11, 16,

Table 2. Sequence length and G+C content (%) of the ITS region from 25 samples investigated in this study.

Sample NO.	Length range (bp)			(G+C) content (%)		
	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2
1	266	164	266	47.37	50.00	47.37
2	267	164	265	48.31	49.39	47.17
3	268	164	263	47.39	50.00	47.15
4	266	164	253	47.37	50.00	43.61
5	266	164	266	47.37	50.00	47.37
6	267	164	265	48.15	49.39	47.17
7	267	164	253	47.04	50.00	45.85
8	264	164	264	47.73	50.00	47.35
9	267	164	266	48.15	49.39	47.37
10	266	164	266	47.37	50.00	47.37
11	267	164	266	48.15	49.39	47.37
12	266	164	266	47.37	50.00	47.37
13	267	164	266	47.41	50.00	47.37
14	266	164	266	47.37	50.00	47.37
15	266	164	266	47.37	50.00	47.37
16	267	164	266	48.15	49.39	47.37
17	266	164	266	47.37	50.00	47.37
18	266	164	267	47.37	50.00	47.57
19	266	164	266	47.37	50.00	47.37
20	266	164	266	47.37	50.00	47.37
21	267	164	266	48.15	49.39	47.37
22	266	164	266	47.37	50.00	47.37
23	266	164	266	47.37	50.00	47.37
24	262	164	266	47.33	50.00	47.37
25	266	164	266	47.37	50.00	47.37

and 21, belonging to *A. microdicyton* Prokh. or *A.* cf. *microdicyton* Prokh., showed same nucleotide sequences which differed from others, belonging to *A. ochotense* Prokh. at 54 bp, 81 bp, 140 bp, 156 bp, 222 bp, 226 bp, 250 bp, 390 bp, 555 bp, 642 bp, and 645 bp (Table 4).

A homology tree was constructed using DNAMAN software based on the nrDNA ITS sequence of all 25 mountain garlic samples (Fig. 2). The results of the homology tree showed that all 25 samples were divided into two groups that shared a similarity of 98% with each other: Samples 2, 6, 9, 11, 16, and 21 formed Group I, while the others formed Group II. Expectedly, the 6 samples of Group I mainly belonged to *A. microdicyton* Prokh., except of Sample 2, while the other 19 samples of Group II all belonged to *A. ochotense* Prokh. Within each group, samples were divided into two subgroups (Fig. 2). Between subgroups, they showed a 99% similarity rate. In Group I, Sample 2, *A.* cf. *microdicyton* Prokh. sample, formed the respective subgroup from other 5 *A. microdicyton* Prokh. samples. In Group II, sample 3 and 8 formed one subgroup from other *A. ochotense* Prokh. samples. This suggested that the nrDNA ITS sequence could be used to discriminate between these both species. However, the monophyletic clade of *A. ochotens*e and *A. microdictyon* in phylogenetic tree by a Bayesian analyses of the nrDNA ITS sequences in the Herden *et al*. (6) study indicated that the nrDNA ITS region might not suitably differentiate these both species. Despite *A. ochotense* could be separated from *A. microdictyon* in the phylogenetic tree by combined chloroplast DNA markers, but *A. victorialis* was categorized as one group with *A. ochotense* which was not completely accurate according to the ancestral range reconstruction (Herden *et al*., 6). As early as 2000, the phylogeny and biogeography of Chinese *Allium* species were studied based on the nrDNA ITS and chloroplast *rps16* intron sequences (Li *et al.*, 12). The phylogenetic analyses indicated that the genus *Allium* was found to be monophyletic, however some subgenera were not. *A. microdictyon* and *A. victorialis* were separated into the same subgenera *Anguinum* and the same section *Anguinum*, which was monophyletic but showing two distinct groups (the Eurasian-American alliance and the East Asian alliance, Friesen *et al*., 5; Li *et al*., 12; Herden *et al*., 6). The sequence analysis results suggested that chloroplast DNAs could not be sensitive between the Eurasian-American alliance from the East Asian alliance of *A. victorialis*, but not the nrDNA ITS.

In Group I, Sample 2 belonging to the Gariwang Mountain species, and others belonging to the Odea Mountain species shared a 99% similarity rate, and the typical variable nucleotide sites were absolutely the same as the Odea Mountain species. This suggests that the Gariwang Mountain species and the Odea Mountain species might belong to the same species, *A. microdicyton* Prokh. However, they showed different leaf shapes, and the leaves of Sample 2 were longer and thinner than that Odea Mountain species. The different morphological characteristics between Gariwang Mountain species and Odea Mountain species might be explained by the long-term environmental evolution and adaptation. In Group II, Samples 7 and 8 were the same species and collected in the same region, Pyeongchang County Gangwon Do South Korea, but Sample 7 and 8 were located in different subgroup because of having several different variable sites. Sample 7 had a single base

Table 4. Typical variable nucleotide sites in the total ITS region of 25 samples investigated in this study.

Sample	19 bp	20 bp	48 bp	54 bp	81 bp	140 bp	156 bp	222 bp	226 bp	250 bp	390 bp	555 bp	642 bp	644 bp	645 bp
1	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
2	A	T	--	G	C	A	A	G	T	A	T	T	A	--	G
3	G	A	--	A	T	G	--	A	A	T	C	C	T	--	T
4	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
5	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
6	A	T	--	G	C	A	A	G	T	A	T	T	A	--	G
7	A	T	T	A	T	G	--	A	A	T	C	C	T	--	T
8	A	A	--	A	T	G	--	A	A	T	C	C	T	--	T
9	A	T	--	G	C	A	A	G	T	A	T	T	A	--	G
10	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
11	A	T	--	G	C	A	A	G	T	A	T	T	A	--	G
12	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
13	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
14	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
15	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
16	A	T	--	G	C	A	A	G	T	A	T	T	A	--	G
17	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
18	A	T	--	A	T	G	--	A	A	T	C	C	T	C	T
19	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
20	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
21	A	T	--	G	C	A	A	G	T	A	T	T	A	--	G
22	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
23	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
24	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T
25	A	T	--	A	T	G	--	A	A	T	C	C	T	--	T

-- means nucleotide deletion.

insertion at 48 bp, while Sample 8 had a single base substitution at 20 bp, as shown in Table 4. This result made us speculate that genetic variation was liable to occur for adapting the environment of Pyeongchang County. The segregative grouping of *A. ochotense* and *A. microdictyon* was also reported in previous study (Herden *et al*., 6). Based on the comprehensive anlysis of divergence time estimates and ancestral range reconstruction for the subgenus *Anguinum*, *A. ochotense* from East Asian was monophyletic, with A. microdictyon as its sister group. However, this could not change the fact that *A. ochotense* and *A. microdictyon* from East Asian was grouped together.

In conclusion, the total sequences of the nrDNA ITS region were successfully amplified using the universal primer set, ITS4 and ITS5. Sequence analysis of the ITS region could completely discriminate *A. microdicyton* from *A. ochotense* which were sampled from different geographical areas in South Korea. The sequence analysis result suggested that the ability of ITS intraspecific discrimination between *A. microdicyton* and *A. ochotense* was very strong. It all depended on important and specific 11 variable nucleotide sites between both species. Sample 2 whose adscription was unclear had been suggested to be *A. microdityon* Prokh. This could be utilized to more precisely distinguish *Allium* species, especially *A. microdicyton* and *A. ochotense*.

ACKNOWLEDGEMENT

This study was supported by the Rural Development Administration (Gangwon Provincial Agricultural Research & Extension Service,

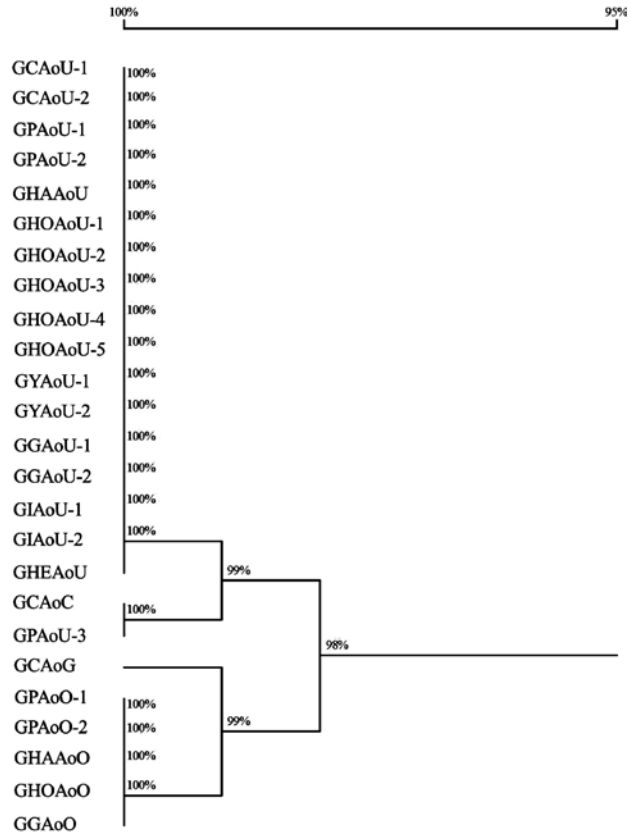

Fig. 2. Homology tree constructed by the total ITS region sequences of 25 samples investigated in this study. The names are marked by the sample number and its specimen voucher.

Chuncheono: Project on extending regional collaboration model [industry – academy – government research institution] for local specialty crops).

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Received : December, 2019; Revised : May, 2020;
Accepted : May, 2020