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GENETIC VARIATION AND PHYLOGENETIC RELATIONSHIP AMONG DIFFERENT LEAF LETTUCE (*LACTUCA SATIVA* L.) VARIETIES IN KOREA

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Abstract

The genus *Lactuca*, known as vegetables or oil crops, is widely cultivated in Canada, Australia, European and Asian countries. To satisfy people's demands of nutrition and taste on lettuces, abundant germplasm and hybrids have been bred by breeding programs or hybridized naturally. This makes variety identification of these germplasm and hybrids difficult, and baffles the following trait improvement and target selection by breeding programs. In this study, we selected 18 distinguished edible lettuce varieties, and evaluated their genetic divergence and phylogenetic relationship based on multi DNA markers to search for an effective method of variety discrimination. DNA alignment results suggested that the rRNA 5S gene was the most variable DNA marker among investigated markers, and the nrDNA ITS gene was not sensitive between *Lactuca* and *Brassica* genera. In the 5S phylogenetic tree, all materials were divided into two groups, and the grouping pattern was considered to be related with leaf color but not leaf shape. S3 and S5 had two nucleotide array deletions and one addition as comparison to other lettuces, sharing relatively nearer phylogenetic relationship compared to other lettuces. By analyzing multi DNA markers, the relations between grouping and leaf color and shape were further improved in the combined phylogenetic tree. This work could help to direct lettuce variety discrimination and target selection for breeding programs. The most informative DNA marker for lettuce variety discrimination could be applied in the variety discrimination of other plant species.

Key words: *Lactuca*, *Brassica*, Molecular identification, DNA markers, DNA barcoding.

Introduction

Lettuces belonging to genus *Lactuca*, family Compositae (Asteraceae), tribe Cichorieae, are important leafy fresh vegetables, native to the Mediterranean area and cultivated in Egypt as early as 4500 BC (Romani *et al*., 2002). People usually consume them as fresh salads, and fry or pickle them as mensal delicacies. Due to being rich in vitamins and minerals of human's essentials and other multiple health-beneficial components, more and more people pay attentions on lettuce. Approximately 100 species were included in the genus *Lactuca*, and all species are self-fertilizing diploids species (Simko, 2009). That is to say, there is possibility of mutual fertilization among different species. For instance, salad lettuce (*L. sativa* L.) could be sexually compatible with prickly lettuce (*L. serriola* L.), willow leaf lettuce (*L. saligna* L.), and bitter lettuce (*L. virosa* L.) Because of this, more hybrids and cultivated varieties of each *Lactuca* species have been naturally hybridized or bred by conventional hybridization methods, some of which have been popularized all around the world. Moreover, going with the increase of more and more people's needs for this crop, various cultivars and types with distinctive characters could be bred by manual intervention means, e.g. the mutation, hybridization, and other breeding methods (Mou, 2011). In addition, as the self-fertilizable crops, lettuces show relatively less limited genetic variability than those cross-pollinated or self-incompatible crops, that dose not favor germplasm improvement, species conservation, and variety/cultivar discrimination.

To clearly understand the variety provenance, many technologies have been developed and carried out, by which we could identify definitely which genus or variety one species belongs to, or what the phylogenetic relationships among several related species are. Among these technologies, the molecular technologies through measuring their genetic variations and genetic diversity at the DNA level were considered to be accurate, efficient, and time-saving (Joshi *et al*., 2004; Kress *et al*., 2005). To date, many biochemical and molecular technologies have been applied for lettuce variety discrimination, including isozymes, simple sequence repeats, amplified fragment length polymorphism, and random amplified polymorphic DNA (Simko, 2009; Simko *et al*., 2009; Rauscher & Simko, 2013). However, due to possessing limited genetic diversity, the DNA markers able to use for lettuce variety discrimination must be very informative. Among a wide variety of DNA markers, the nucleotide ribosomal DNA internal transcribed spacer (nrDNA ITS) region, and the rRNA 5S and 18S genes are the most widely used regions for species, variety, and population identification (Coleman, 2003; Karehed *et al*., 2008). In this study, we selected 17 *Lactuca sativa* (lettuce) and one *Brassica oleracea* (cabbage) materials from Korea to determine their genetic diversity and phylogenetic relationship. Because in the cooking culture of Korea, people love to eat roast meats with garlic or hot pepper which are together wrapped by the fresh lettuce leaves, there are many distinguished edible lettuce varieties. The cabbage material (*B. oleracea*) was used for the comparison. We amplified the nrDNA ITS, rRNA 5S and 18S genes from 18 materials successfully and evaluated the sequence variation. Genetic diversity

obtained by our results could clearly explain the phylogenetic relationship among these lettuce materials. This work will provide more obvious sequence sources which would be used to further understand the variety discrimination and phylogeneny among *Lactuca sativa* L. and plant breeding and variety conservation of lettuce.

Materials and Methods

Plant materials: Seventeen different lettuce varieties were selected and used to evaluate the genetic divergence and phylogenetic relationship. In addition, we selected one more relatively close genera *B. oleracea* cabbage material as the comparison. The voucher specimens, variety or type name, and other detailed information about these 18 materials were listed in Table 1. Mature seeds were sown in plates containing clay and vermiculite with the ratio of 3:1 (v:v). After germinated, the seedlings were watered with 1/2 Hoagland nutrient solution (Hoagland & Arnon, 1950) on alternate days. Until some mature leaves came out from the lettuce seedlings, we sampled two healthy, mature leaves and used for DNA extractions.

DNA extraction, PCR amplification and sequencing: The modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987) were used to extract DNAs from lettuce materials. The nrDNA ITS genes of lettuces were amplified using universal primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') in 20 µl PCR reaction (White *et al*., 1990). The 5S rRNA genes were amplified using the P1 (5'-GATCCCATCAGAACTCC-3') and P2 (5'-GGTGCTTTAGTGCTGGTAT-3') primer set in 20 µl PCR reaction (Park *et al*., 2000). And the 18S rRNA genes were amplified using universal primers 18SF (5'-AACCTGGTTGATCCTGCCAGT-3') and 18SR (5'-TGATCCTTCTGCAGGTTCACCTAC-3') in 20 µl PCR reaction (Sogin, 1990). The effective PCR amplification conditions contain 1 µl of template DNA (500~1000 ng), 10 µl 2 × PCR Dye Master Mix (QIAGEN, Korea), and 0.1 µmol l^{-1} of each primer. The conditions for PCR amplification were based on the their respective annealing temperatures of primer sets, and the common PCR amplification programs were list as follows: one cycle of pre-denaturation at 95ºC for 10 min, 35 cycles of denaturation at 95ºC for 1 min, their respective annealing temperatures for 1 min, and a final extension step at 72ºC for 1 min, with the end of one cycle of extension at 72ºC for 10 min. The PCR amplification products were checked by electrophoresis through 1.0% agarose gel, and then purified using a Gel Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were sequenced at BGI in Beijing, China (http://www.genomics.cn/index).

Sequence editing and alignment: We used the software program DNAMAN version 6.0 (Lynnon Biosoft Corporation, USA, http://www.lynon.com/) to shear, edit, and assemble sequencing results. Using the assemble sequencing results to do the BLAST detection in NCBI servicer, we could make sure whether our sequencing results were the preconceived target genes. The analogical sequences obtained by BLAST showed our sequencing results were highly identical to the existing corresponding sequence resources from other plant species, that could make sure our sequencing results were the target gene sequences from our lettuce materials. Then, we used DNAMAN version 6.0 software to align all the sequencing results, to analyze their nucleotide polymorphisms.

Phylogenetic analysis: The genetic divergences were assessed according to the pairwise distance calculation method (Meyer & Paulay, 2005). Jaccard coefficients were calculated by similarity coefficient [Sj = a/(a+u)]. In gene sequences, having base variation marked '1' and no variation marked '0'; 'a' represented the number of the same bases and 'u' represented the number of different bases between two varieties. The phylogenetic relationships among 18 materials were estimated after the construction of a phylogram using the DNAMAN version 6.0 software.

Table 1. Species, common name and their varieties of cabbage samples investigated in this study.

Sample No.	Species	Variety or isolate	Common name	Voucher specimen	NCBI GenBank accession		
					nrDNA ITS	rRNA 5S	rRNA 18S
1.	*Lactuca sativa* L.	Dduk seom Chuk Myeon	Leaf Lettuce	S1	KT074384	KT074430	-
2.	*Lactuca sativa* L.	Lolla	Lettuce	S2	KT074385	KT074431	-
3.	*Lactuca sativa* L.	ALE-040301	Lettuce	S3	KT074386	KT074432	-
4.	*Lactuca sativa* L.	Mini Cup	Heading Romaine Lettuce	S4	KT074387	KT074433	-
5.	*Lactuca sativa* L.	Bowl Green	Lettuce	S5	KT074388	KT074434	-
6.	*Lactuca sativa* L.	Bowl Red	Leaf Lettuce	S6	KT074389	KT074435	KT225374
7.	*Brassica oleracea* L. var. *gem*	Asia King Ssam	Brussels Sprouts	S7	KT074390	KT074436	-
8.	*Lactuca sativa* L. var. *longifolia* Lam.	Caesar Red	Cos Lettuce	S8	KT074391	KT074437	KT225375
9.	*Lactuca sativa* L.	Saeng Chae	Lettuce	S9	KT074392	-	KT225376
10.	*Lactuca sativa* L.	Salad Express	Lettuce	S10	KT074393	-	KT225377
11.	*Lactuca sativa* L.	Caesar Green	Cos Lettuce	S11	KT074394	-	KT225378
12.	*Lactuca sativa* L.	Asia Ice Queen	Leaf lettuce	S12	KT074395	-	KT225379
13.	*Lactuca sativa* L.	Asia Yeoreum Jeok Chi Ma	Leaf Lettuce	S13	-		-
14.	*Lactuca sativa* L.	Asia Jeok Oak	Lettuce	S14	KT074396	KT074438	KT225380
15.	*Lactuca sativa* L.	Oaklin	Lettuce	S15	KT074397	KT074439	KT225381
16.	*Lactuca sativa* L.	Jeok Sam Gak Chu	Lettuce	S16	KT074398	KT074440	KT225382
17.	*Lactuca sativa* L.	Cheong Chi Ma	Leaf Lettuce	S17	KT074399	KT074441	KT225383
18.	*Lactuca sativa* L.	ALE-040302	Lettuce	S18	-	-	-

No. means number; var. means variety; - means not detected

Results and Discussion

PCR amplification and sequence analysis: Multi DNA markers including the nrDNA ITS, rRNA 18S, and rRNA 5S genes, which are widely used for species and variety identification (Luo & Liu, 2019; Huang *et al.*, 2020; Khaw *et al.*, 2020) were used for variety identification of Korean leaf lettuce in this study. The nrDNA ITS genes were successfully amplified from 16 of all 18 lettuce cabbage materials, with about 700 bp in length. The other both materials, S13 and S18, could although be amplified using the universal primer set, the amplification quality did not satisfy the demands of sequencing because both PCR products possessed multiple different repeats in this region. The rRNA 5S and 18S genes were amplified from 12 and 10 from all 18 materials investigated in this study, respectively (Table 1). The fail PCR amplification was caused by PCR condition unconformity, or/and multiple repeat appearance when sequencing.

To make sure the analogue of our sequences, we blasted all sequencing results using BLAST on NCBI server. The analogue results of ITS sequencing suggested that our sequencing result from *Lactuca sativa* L. (S1) material was 99% identity with *L. serriola* ITS gene sequences (NCBI GenBank accession: AB742455, AB742457, KF850588), and *L. sativa* ITS gene sequences (NCBI GenBank accession: KT249801-3, AJ633337), *Salvia cynica* (NCBI GenBank accession: DQ667332), *Salvia cyclosteqia* (NCBI GenBank accession: KC473234), 96% with *L. saligna* ITS gene sequences (NCBI GenBank accession: AJ633336, HQ161969), and *L. virosa* ITS gene sequence (NCBI GenBank accession: AJ633335), and 94% with *L. viminea* ITS gene sequence (NCBI GenBank accession: AJ633333), and *L. orientalis* ITS gene sequences (NCBI GenBank accession: KF485659).

The analogue result of 5S sequencing indicated that it was 100% identity with *Atractylodes lancea* 5S rRNA gene sequences (NCBI GenBank accession: GQ995228-9, GQ995231), 94% with *Aralia kingdom-wardii* 5S rRNA gene sequence (NCBI GenBank accession: AY304586), and 95% with *Solanum lycopersicum* 5S rRNA gene sequence (NCBI GenBank accession: KF156910). This analogue result proved that our sequencing results were rRNA 5S gene sequence, however, there was no existing rRNA 5S sequence resource from *L. sativa* in NCBI GenBank database to follow. The same situation of no existing sequence resource in NCBI GenBank database as reference occurred in *L. sativa* rRNA 18S sequencing results. The rRNA 18S sequencing result from S1 were 99% identity with *Brassica oleracea* rRNA 18S gene sequence (NCBI GenBank accession: KM538957), *B. rapa* rRNA 18S gene sequence (NCBI GenBank accession: KP099063-4, LC009534-6,), *Camelina sativa* rRNA 18S gene sequence (NCBI GenBank accession: FN599860), *Sisymbrium orientale* and *Arabidopsis thaliana* rRNA 18S gene sequences (NCBI GenBank accession: AB856329, CP002686, AY056114). All analogue results indicated that our sequencing results were just right to the target genes which we intended to amplify. Then, we submitted all the sequencing results in NCBI GenBank database (with accession numbers of KT074384-KT074399 for nrDNA ITS gene, KT074430-KT074441 for rRNA 5S, and KT225374-KT225383 for rRNA 18S, Table 1).

Genetic variation: DNA alignment was performed using the software DNAMAN version 6.0. ITS alignment showed the ITS sequences had high similarity with each other, even between *L. sativa* and *B. oleracea*. This result suggested that species discrimination using the nrDNA ITS gene was not sensitive between *Lactuca* and *Brassica* genus, but it did not suit for other genera or variety discrimination. The effectiveness of variety discrimination had been proved in *B. campestris* variety in our previous study (Sun *et al*., 2016).

Compared to the nrDNA ITS gene sequence, the rRNA 5S gene sequences showed high nucleotide variations, particularly in the NTS. There were a 'GGGTTTTTTTC' 11 bp-array deletion and a 'CGGAGG' 6 bp-array deletion appearing in the upstream and middle of the NTS of S3 and S5, respectively. And also in the NTS of S3 and S5, there was a 'GG' 2 bp-array addition. This result determined S3 and S5 showed nearer phylogenetic relationship at a certain extent. The rRNA 5S gene sequence of S7 did not show any specific nucleotide sites to differ from other lettuce materials. Because of the length of 18S sequencing result, the dissimilarity of this ribosomal RNA marker was relatively lower than the other rRNA 5S gene. S6 and S12, and S15 and S17 showed higher sequence similarity with each other than other both varieties.

Phylogenetic relationship: According to the DNA alignment result, the phylogenetic tree was constructed based on the nrDNA ITS gene, and the rRNA 5S, and 18S gene sequences. There was no any effective sequencing obtained from S13 and S18, so both lettuce materials did not go in for genetic divergence analysis. Using the nucleotide variable 5S gene sequence, 12 materials including the cabbage material (S7) were divided into two major groups, with S1, S2, S6, S14, S15, and S7 forming one group, the others forming the other (Fig. 2). Both groups shared 63% identity with each other. Interestingly, this grouping was certainly related with leaf color of lettuces. The leaf colors of S1, S2, S6, and S14 divided into one group were all purple, or brown red, while almost green leafy lettuces except S15 were divided into another group (Figs. 1, 2). Although the leaf colors of S8 and S16 were also purple, or brown red, they showed relatively far phylogenetic relationship from green leafy lettuce (Fig. 2). The thin, green leafy lettuce S15 in the colored lettuce group was the farthest from other lettuces. However, the grouping result might not be correlated with leaf morphological characteristics according to the rRNA 5S gene sequences. S1 and S2 absolutely had analogous leaf shape to S3, but they were separated into different groups (Fig. 1). And the same situation also appeared between S6, S14, S15 and S5, S16. This was the first finding about the correlation with leaf color and the rRNA sequence.

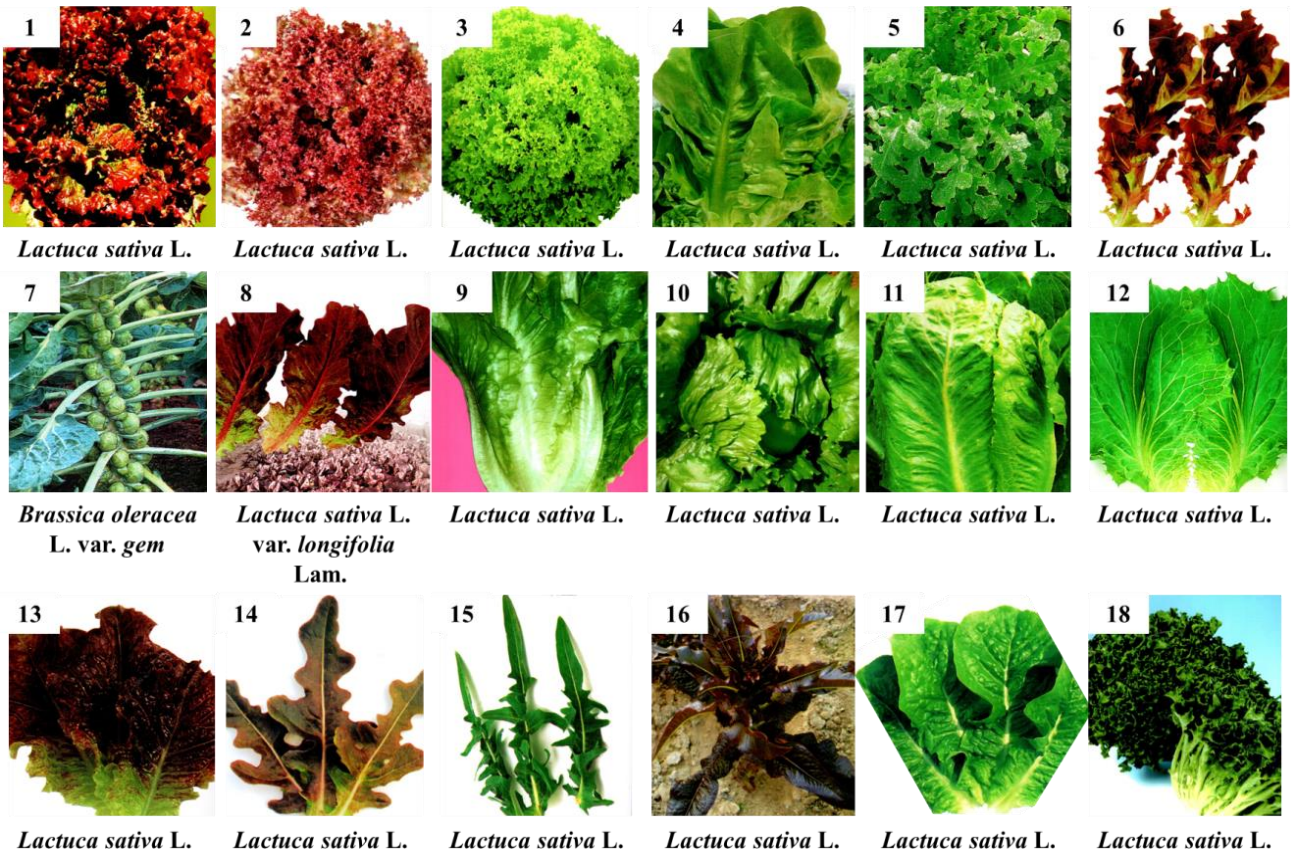

Fig. 1. The profiles of lettuce and cabbage materials investigated in this study.

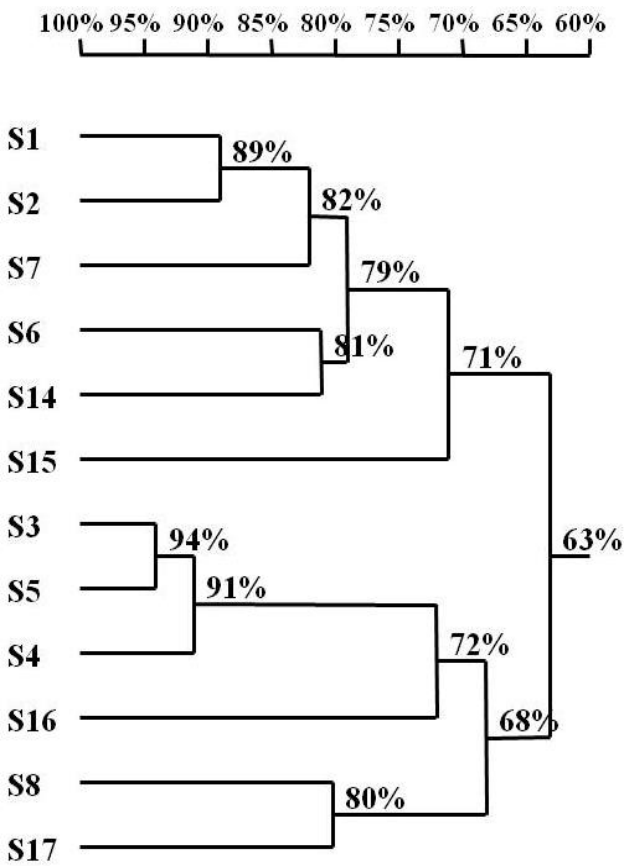

Fig. 2. Phylogenetic relationship of 11 lettuce and one cabbage materials based on the rRNA 5S gene sequences.

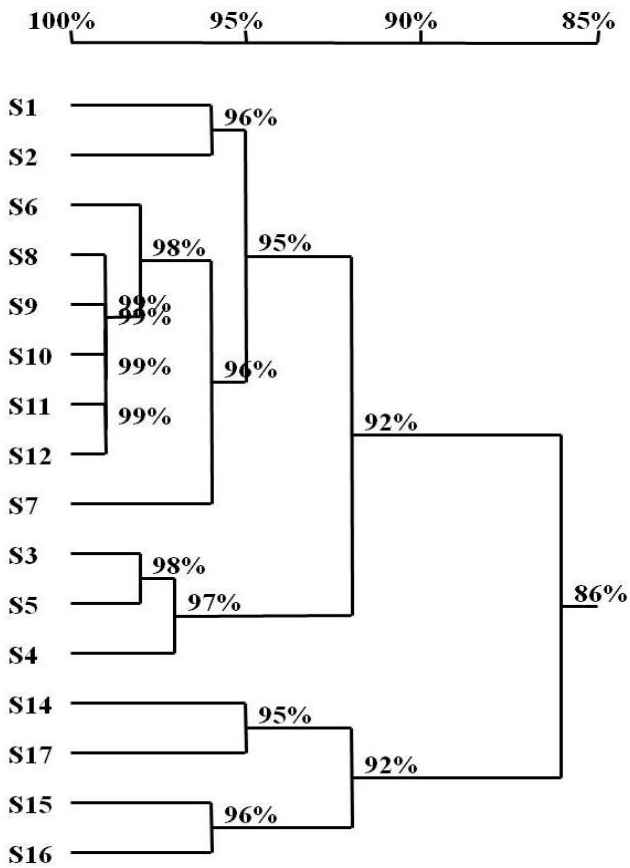

Fig. 3. Phylogenetic relationship of 16 lettuce and one cabbage materials based on the nrDNA ITS, rRNA 5S and 18S region sequences.

To add to analyze the genetic variation of S9, S10, S11, and S12 with other materials, we combined the nrDNA ITS gene, rRNA 5S, and 18S gene sequences and constructed the phylogenetic tree (Fig. 3). As the incompletion of all three markers, the analysis result of DNA alignment might be influenced by the sequence length at a certain extent. However, the observed divergency method had been able to reduce the influence degree. For example, six lettuce materials including S6, S8, and S14-S17 with three complete sequencing results, were divided into three groups, that was enough to tell these markers could be used for variety discrimination. And S1-S5 all having ITS and 5S sequencing results were separated into two groups (Fig. 3). Seen from the combined sequences, the grouping showed some relations with leaf shape, color, and morphological characteristics. Based on the combined sequences, S6 and S8 were nearer to S7, and S1 and S2 in phylogenetic tree as compared to S3-S5, and S14-S17 (Fig. 3). But, all head lettuces in our investigated lettuce materials (S9-S12) were considered to be closely phylogenetic and nearer to S8 (*L. sativa* L. var. *longifolia* Lam). Thus, we conjectured that the origins of S8 would have relations with head lettuce. This result would assist to conduct lettuce breeding program and trait improvement.

Conclusions

In the present study, we selected 18 distinguished, popular edible lettuce materials in Korea and evaluated their genetic variation and phylogenetic relationships. Some materials were very rare, and the relative investigation was few as well. So, our study was very reference valuable for *Lactuca* phylogenetic studies. In this study, we selected one *Brassica* species (S7) as the comparison. Our results suggested that the rRNA 5S gene was the most variable marker among investigated markers, while the nrDNA ITS gene was not sensitive among related genus. Both materials, S3 and S5 showed two nucleotide array deletions and one addition, as comparison to other lettuces. In the 5S phylogenetic tree, materials were divided into two groups, and S3 and S5 showed nearer phylogenetic relationship, that was mainly caused by the nucleotide array deletions and nucleotide addition. In addition, the grouping in the 5S phylogenetic tree was also considered to relate with leaf color. Seen from the phylogenetic tree constructed by combined gene sequences, it further proved the relations between grouping and, leaf color and shape. The present work would provide more sequence resource and lettuce phylogenetic evidences, which would use to further evaluate genetic relationship of *Lactuca* lettuce vegetables. Our result will direct trait improvement and plant breeding program of *Lactuca* species.

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