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Genetic relationship of *Aloe vera* 'Saengjang', a new forma, based on cpDNA and ITS sequence variation

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cpDNA와 ITS 염기변이에 근거한 신품종 생장알로에 유전적 상관관계

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ABSTRACT: This study was carried out to understand the genetic relationship of three *Aloe* spp. cultivated in Korea, *A. saponaria, A. vera* and *A. arborescens* and a new variant in Korea based on three plastid (*matK*, *trnL-F*, *rbcL)* and one nuclear (ITS regions) DNA barcode markers. A total of 2,420 bp sequence was amplified. Two indels were detected in the *trnL* region, and also several species specific nucleotide loci were detected in all 29 parsimonious informative sites, and 148 variable sites were detected among four taxa studied while 170 variable and 75 parsimonious sites were detected when other *Aloe* spp. in worldwide were used. An UPGMA phenogram with 10,000 bootstrap replication showed that the new variant was closest to *A. vera*. The variant was not morphologically and genetically concurrent with any reported species so far. The clustering of *Aloe* species were broadly in agreement with previously reported results.

Keywords: *Aloe vera* 'Saengjang', ITS & cpDNA regions, genetic relationship

적 요: 본 연구는 3개의 색소체 *matK, trnL-F, rbcL* DNA 염기서열과 1개의 핵 ITS DNA 염기서열을 근거로 한국산 *Aloe* 3종 *A. arborescens*, *A. vera* 그리고 *A. saponaria* 등과 하나의 변이종 알로에의 유전적 상관관계를 알고자 수행되었다. 전체 2,420 bp 서열이 증폭되었다. 두 개의 삽인-결실(indel)이 *trnL* 지역에서 확인되었고, 또한 여러 개의 종 특이적인 염기자위가 전체 29개의 최소변이 정보지역(parsimonious informative site)에서 확인되었다. 조사된 한국산 4 종간에는 148 염기변이 지역이 있었으며, 세계산 *Aloe* 종들을 포함한 비교해서는 170개의 변이지역 중 75개의 최소변이 지역이 확인되었다. UPGMA를 이용한 phenogram에서 새로운 변이종 알로에는 *A. vera*와 가장 가깝게 유집되었다. 변이종 알로에는 아직까지 보고된 어떤 종류의 *Aloe*속 내 종과 형태적 및 유전적으로 일치하지 않았다. 조사된 알로에 종들의 유집분석 결과는 기존의 연구결과와 일치하였다.

주요어: 알로에베라 '생장', ITS & cpDNA 지역, 유전적 상관관계

The genus *Aloe* L. belongs to Xanthorrhoeaceae and to the subfamily Alooideae (Smith and Steyn, 2004). It's a large genus comprising about 624 taxa (Grace et al., 2011) and is native to Africa, Madagascar and Arabia (Viljoenm et al., 1998). All species of *Aloe* have succulent, spiked/toothed leaves with or without speckles, acrid/unpalatable sap, sunken stomata, and bright yellow to red or sometimes bicolored flowers usually with exserted anthers and flattened, wind-dispersed seeds (Manning et al., 2014). The morphology and the taxonomic

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history of Alooideae are well summarized by smith and Van Wyk (1998) and Klopper et al. (2010). Though the medicinal properties of *Aloe* species have been used throughout history, only a few of the 624 species are actually used in traditional medicines (Kim et al., 2013).

Aloe spp. are known for their health promoting and medicinal properties (Watt and Breyer-Brandwjik 1962; Grace et al., 2008) and have been shown to have anticancer, hypoglycemic, immunomodulatory, gastroprotective and wound healing properties (Reynolds and Dweck, 1999; Vogler and Ernst, 1999, surjushe et al., 2008; Feily and Namazi et al., 2009). The mucilaginous gel in the leaf pulp of *Aloes* has been used for various curative purposes for a long time. Among the *Aloe* spp., *Aloe vera* (L.) Burm. f. (=*Aloe barbadensis* Mill.), *A. saponaria* (Aiton) Haw., *A. arborescens* Mill are globally rated as the most important medicinal species in the genus *Aloe* (Amoo et al ., 2012; Kim et al., 2013). Among these three species of *Aloe, A. vera* and *A. arborescens* are widely cultivated around the world (Gutterman and Chauser-Volfson 2000). In particular the use of *A. vera* has been recorded throughout the ancient history, and also while *A. vera* is no longer known to occur in the wild several other species of *Aloe* are wild harvested for natural products (Grace 2009). Both *A. vera* and *A. arborescens* are used in foodstuffs in Asia (Grace 2011). *Aloe saponaria* also known as soap *Aloe* or African *Aloe* is used for their UV protective, antioxidative, wound healing activity (Silva et al., 2014).

Aloe spp. are non-native to Korea and are only cultivated in farms and tropical gardens. *Aloe* spp. where introduced into Korea since 1970's even though it is not clear when they were occasionally introduced into Korea (personal communication). Three species of *Aloe*, *A. arborescens*, *A. saponaria* and *A. vera* are mainly cultivated for commercial purposes in Korea. They have easily been identified by the several morphological features. *Aloe arborescens* has well developed stem up to 2-3 meters, but the others not. *Aloe vera* hardly develops the stem and the fleckled dots in leaf but some white fleck on leaf. *Aloe saponaria* is stemless and has broad and storied white flecked leaves. In addition, a new variant here studied, characterized by both stemless and non-storied white fleckled leaves, has increasingly been cultivated, but its taxonomic study has not yet conducted. It would be problematic if the variant would be developed as medical and functional foods without clarifying the taxonomic confirmation compared with other species.

The aim of this study was to confirm the taxonomic position of a variant that we describe here for the first time, and also to know the generic relationship of *Aloe* spp. cultivated in Korea to other closely related *Aloe* spp. occurring in worldwide.

Table 1. Morphological features of *Aloe* spp. cultivated in Korea.

Taxa	Features
A. arborescens	Well-developed stem, grayish green with white flecked leaves and sharp yellow teeth
A. saponaria	Stemless, pale green with white flecked leaves and sharp dark brown teeth
A. vera	Stemless, grayish green with thick white flecked leaves and small grayish teeth
A. vera 'Saengjang'	Stemless, pale green leaves with non-storied white speckled leaves and small yellowish teeth

Material and Methods

We sampled three plants each from *Aloe vera* (L.) Burm. f. (=*Aloe barbadensis* Mill.), *A. saponaria* (Aiton) Haw., *A. arborescens* Mill., and the new variant found in Korea for DNA isolation. The plants where collected from around Jeong-eup and Yong-jin myeon, Wanju-Gun areas in Jeollabuk-do province. They were transplanted in the plant growth facility of Chonbuk National University. We mainly used fresh plant materials for morphological and molecular investigations, because herbarium specimens of *Aloe* spp. are difficult to work with due to their succulent nature. The morphological features of the plants are summarized in Fig. 1.

Fig. 1. Photographs of Korean *Aloe* spp. (A) *A. vera* showing stemless or very short-stem, thick and white fleck leaves; (B) *A. arborescens* showing well developed stem up to 2~3 m, narrow and some white fleck leaves; (C) *A. saponaria* showing stemless, broad and storied white speckled leaves; (D) *A. vera* "Saengjang" showing stemless, narrow and thick and non-storied white speckled leaves.

Table 2. Genes, primer sequences, annealing temperature for PCR used in this study.

Gene	Sequences	Length amplified	Annealing temperature	Reference
matK	Mat Kx TAATTTACGATCAATTCATTC Mat K5 GTTCTAGCACCAGAAAGTCG	868 bp	48ºC	www.kewgardens.org/barcode/update
trnL-F	trnL GGTTCAAGTCCCTCTATCCC trnlF ATTTGAACTGGTGACACGAG	481 bp	58ºC	Taberlet et al.,1991
*rbcL*a	rbcLa-f ATGTCACCACAAACAGAGACTAAAGC rbcLa-r GTAAAATCAAGTCCACCRCG	537 bp	58ºC	Kress and Erickson (2007)
nrITS	ITS4 TCCTCCGCTTATTGATATGC ITS5 GGAAGTAAAAGTCGTAACAAGG	680 bp	56ºC	white et al 1990

Table 3. Features of DNA sequence amplified in this study.

DNA region amplified	Aligned size (bp)	Avg GC (%)	Variable sites	Parsimonious informative sites
trnL-F (Plasmid)	481	29.1	45	25
rbcL (Plasmid)	537	43.2	16	8
matK (plasmid)	868	30.7	39	18
ITS (Nuclear)	680	62.7	81	27
Plastid combined	1740	33.6	90	48
Plastid + Nuclear	2420	41.6	170	75

Total genomic DNA was extracted from frozen leaves (100 mg), ground in liquid nitrogen. Their DNA was isolated using a modified CTAB method (Doyle and Doyle, 1990). The quality of the isolated DNA was checked on 0.8% agarose gel stained with ethidium bromide. The primers used in this study, their sequence information and annealing temperatures are in Table 2. Three plastid regions, *matK*, *trnL-F*, *rbcL* and one nuclear region, nrITS DNA, were amplified. The PCR reaction was carried out in 20 μL reaction containing 25 ng of DNA, 1X PCR reaction buffer, 2.5 mM dNTP's, 20 pmoles of primers, and 1 unit of tenuto Taq DNA polymerase (Enzynomics, Korea). The PCR reaction was carried out using a GeneAmp PCR system 2700 thermal cycler. The PCR cycling condition was as follows, an initial denaturation at 95ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing for 30 sec, and extension at 72ºC for 1 min, and a final extension step at 72ºC for 10 min. The PCR products were resolved on an agarose gel and stained with ethidium bromide. A 1-kb DNA ladder was used as size marker. The bands were then eluted from the gel, and sequenced.

Sequencing was carried out on an ABI prism 3700 sequencer. The sequence chromatogram was edited and assembled using Sequencher (ver. 4.1.1; Genecodes Corporation Inc, USA). The sequences retrieved from the Genbank database the same as in Table 4. After assembling the sequences of all the regions

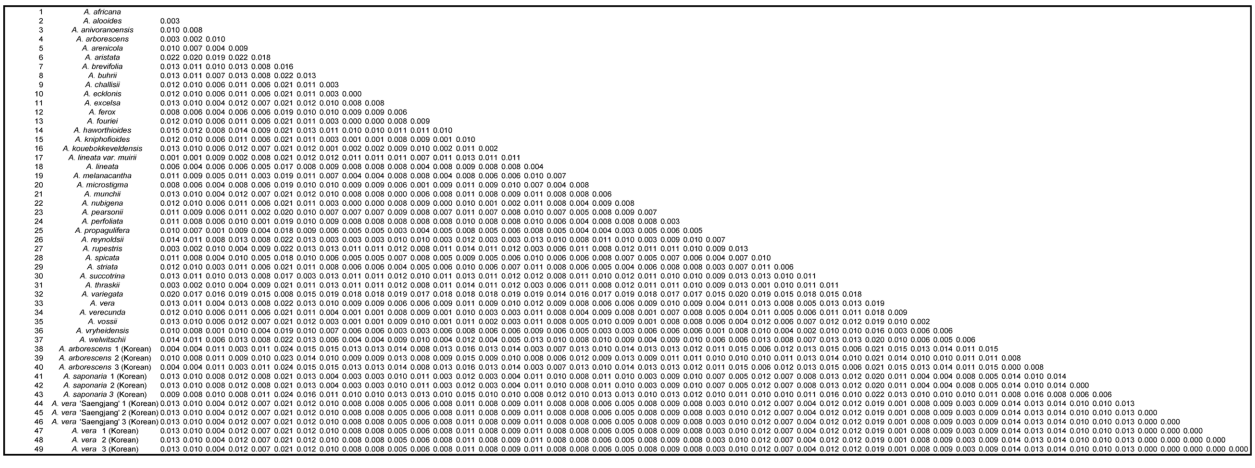

Fig. 2. Estimates of evolutionary distance. The numbers of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 49 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 2095 positions in the final dataset.

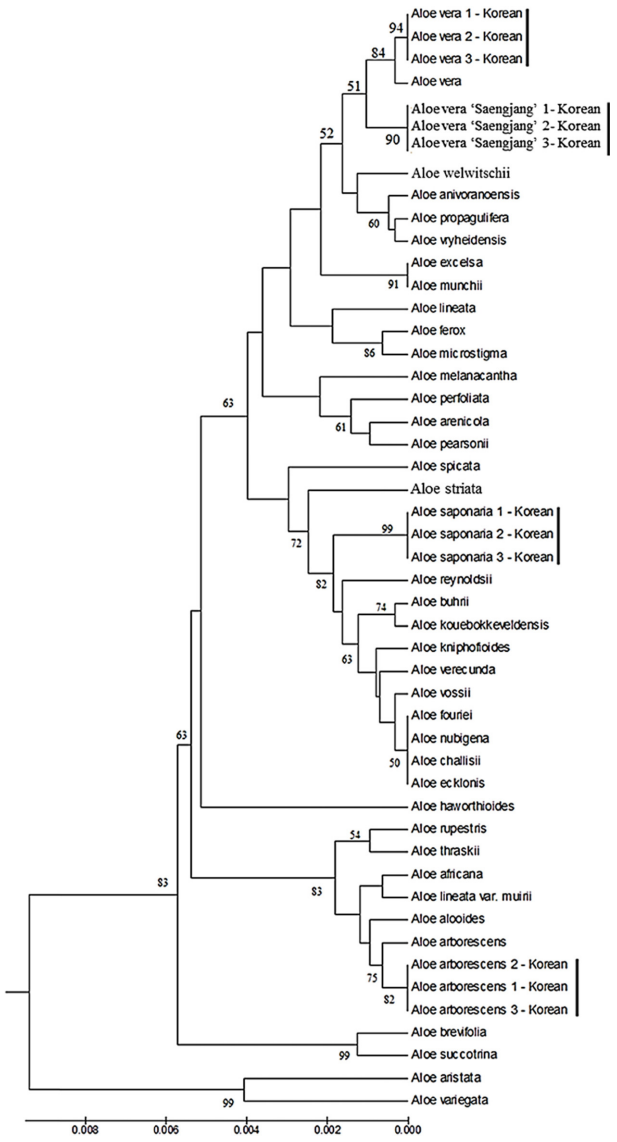

Fig. 3. A phenogram showing the genetic relationship among the *Aloe* spp. cultivated in Korea and other closely related species occurring worldwide. The optimal tree with the sum of branch length = 0.08405051is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Only bootstrap values < 50 are shown.

into one for each species, the sequences along with outgroup were aligned using CLUSTAL X (Thompson et al., 1997) and manually edited using BioEdit (Hall, 1999). A UPGMA tree was generated based on Kimura 2-parameter matrix (Kimura 1980) which was found to be the best model for analysis using MEGA 5 (Fig. 3) (Tamura et al., 2011). Statistical confidence on the UPGMA tree nodes was computed by 10,000 bootstrap permutations. A combined nuclear and plastid region analysis was done after no significant incongruence was detected.

Results and Discussion

The three *Aloe*, *A. arborescens*, *A. vera* and *A. saponaria* cultivated in Korea, can be easily identified morphologically. *Aloe arborescens* has well developed above ground stem reaching to 2-3 m tall, and its leaves are relatively narrow and small in comparison with those of the other *Aloe* spp. *Aloe vera* has hardly developed above ground stem, and its leaves are bigger than those of the other spp. and also have white wax fleck on leaf. The above ground stem is absent in *A. saponaria*. Its leaves are relatively broad and thin in comparison to those of other *Aloe* spp. and develop white and well storied speckled dot. On the other hand, the overall morphology of the variant, called *A. vera* 'Saengjang' studied for the first time here, is at large similar to *A. vera*. The variant is, however, different from *A. vera* in the point of size, only half the size of *A. vera* and they grow relatively more slowly. In addition, the variant has developed with white non-storied speckled dot in leaf unlike in *A. vera* (Fig. 1).

The total length of the sequence amplified from *matK*, *trnL-F*, *rbcL* and ITS region was 2420 bp in *Aloe* spp. cultivated in Korea Its average GC content was 41.6% and had 170 variable and 75 parsimonious informative sites, but between Korean *A. vera* and *A. vera* 'Saengjang' there were only 6 variable and 5 parsimonious informative site. The features of the amplified regions are summarized in Table 2.

In order to find the genetic relationship of the *Aloe* spp. cultivated in Korea to other reported *Aloe* spp., the sequence information of closely related *Aloe* spp. was downloaded from Genbank database (Table 4) and used in this study. The evolutionary distance was calculated using Kimura's 2 parameter model (Fig. 2). The average distance between all pairs of accession was 0.013 (Fig. 2). The least similarity was between *A. variegate* and *A. rupestris*. An UPGMA phenogram was drawn based on the Kimura's 2 parameter distance matrix (Fig. 2). The outer most cluster was that of *A. variegate*, and *A. aristata* this was similar to what was observed by Manning et al. (2014). *A. vera*, *A. vera* "Saengjang", and *A. saponaria* were part of the same major cluster while Korean *A. arborescens* was part of the other cluster. The previously reported *A. vera* grouped outside the Korean *A. vera*, clearly showing that their divergence is more recent. The Korean *A. arborescens* was closest to a previously reported *A. arborescens*, while Korean *A. saponaria* was closest to *A. striata*. *A. saponaria* and *A. striata* are known to hybridize in the wild. And the hybrid *A. saponaria* x *striata* is a sought

Table 4. GenBank accession number of the closest *Aloe* spp. used for the analysis.

No	Species	trnL	rbcL	matK	ITS
1	*A. africana*	HQ646845.1	JX518056.1	JX572268.1	HQ646951.1
2	*A. alooides*	JX630286.1	JX518239.1	JX572270.1	JQ025325.1
3	*A. anivoranoensis*	JX630285.1	JX517497.1	JX572271.1	JQ025371.1
4	*A. arborescens*	AB113160.1	JX518144.1	JQ412310.1	JQ025326.1
5	*A. arenicola*	JX630328.1	JQ024111.1	JQ024487.1	JQ025268.1
6	*A. aristata*	JX630272.1	JX518089.1	AJ512319.1	JQ025312.1
7	*A. brevifolia*	JX630273.1	JQ024117.1	JQ024493.1	JQ025314.1
8	*A. buhrii*	JX630257.1	JQ024118.1	JQ024494.1	JQ025263.1
9	*A. challisii*	JX630283.1	JX517888.1	JX572276.1	JQ025355.1
10	*A. ecklonis*	JX630277.1	JX517611.1	JX572280.1	JQ025307.1
11	*A. excelsa*	JX630302.1	JF270640.1	JF265284.1	JQ025301.1
12	*A. ferox*	JX630259.1	JX518209.1	JX572282.1	AF234338.1
13	*A. fouriei*	JX630281.1	JX517684.1	JX572283.1	JQ025358.1
14	*A. haworthioides*	JX630186.1	JQ024139.1	JQ024513.1	JQ025357.1
15	*A. kniphofioides*	KC985128.1	JX517649.1	KC960550.1	KC880128.1
16	*A. kouebokkeveldensis*	JX630245.1	JQ024144.1	JQ024518.1	JQ025264.1
17	*A. lineata var. muirii*	JX630262.1	JQ024148.1	JQ024521.1	JQ025321.1
18	*A. lineata*	JX630263.1	JQ024145.1	JQ024519.1	JQ025320.1
19	*A. melanacantha*	JX630274.1	JQ024150.1	JX572287.1	JQ025267.1
20	*A. microstigma*	JX630253.1	JQ024151.1	JQ024524.1	JQ025323.1
21	*A. munchii*	JX630282.1	JX517965.1	JX572289.1	JQ025302.1
22	*A. nubigena*	JX630239.1	JX518145.1	JX572290.1	JQ025356.1
23	*A. pearsonii*	JX630325.1	JQ024154.1	JQ024526.1	KC893736.1
24	*A. perfoliata*	JX630322.1	JQ024155.1	JQ024528.1	JQ025315.1
25	*A. propagulifera*	JX630284.1	JX517367.1	JX572294.1	JQ025359.1
26	*A. reynoldsii*	JX630246.1	JQ024160.1	JQ024532.1	JQ025265.1
27	*A. rupestris*	JX630280.1	JX517584.1	JX572295.1	JQ025317.1
28	*A. spicata*	JX630301.1	JF270642.1	JF265286.1	KC893739.1
29	*A. striata*	JX630256.1	AJ511392.1	AJ512310.1	KC893739.1
30	*A. succotrina*	JX630266.1	JQ024167.1	JQ024539.1	JQ025266.1
31	*A. thraskii*	JX630261.1	JQ024170.1	JQ024542.1	JQ025319.1
32	*A. variegata* .	KC985127.1	KC893718.1	JQ024543.1	KC880127.1
33	*A. vera*	AJ290289.1	AY323726.1	AJ512309	KC893746.1
34	*A. verecunda*	JX630271.1	AJ511395.1	AJ512312.1	JQ025346.1
35	*A. vossii*	JX630276.1	JX518216.1	JX572299.1	JQ025347.1
36	*A. vryheidensis*	JX630279.1	JX517863.1	JX572300.1	JQ025308.1
37	*A. welwitschii*	JX630278.1	AJ511393.1	JQ024552.1	JQ025344.1
38	*A. arborescens* 1 (Korean)	KP072695	KP072707	KP072719	KP072731
39	*A. arborescens* 2 (Korean)	KP072696	KP072708	KP072720	KP072732

Table 4. Continued.

No	Species	trnL	rbcL	matK	ITS
40	*A. arborescens* 3 (Korean)	KP072697	KP072709	KP072721	KP072733
41	*A. saponaria* 1 (Korean)	KP072698	KP072710	KP072722	KP072734
42	*A. saponaria* 2 (Korean)	KP072699	KP072711	KP072723	KP072735
43	*A. saponaria* 3 (Korean)	KP072700	KP072712	KP072724	KP072736
44	*A. vera* 'Saengjang' 1 (Korean)	KP072701	KP072713	KP072725	KP072737
45	*A. vera* 'Saengjang' 2 (Korean)	KP072702	KP072714	KP072726	KP072738
46	*A. vera* 'Saengjang' 3 (Korean)	KP072703	KP072715	KP072727	KP072739
47	*A. vera* 1 (Korean)	KP072704	KP072716	KP072728	KP072740
48	*A. vera* 2 (Korean)	KP072705	KP072717	KP072729	KP072741
49	*A. vera* 3 (Korean)	KP072706	KP072718	KP072730	KP072742

after garden plant. The groupings of the species where broadly in agreement to those reported by Treutlein et al. (2003), Grace et al. (2013), and Manning et al. (2014). *Aloe vera* 'Saengjang' studied here seems to have risen by natural mutation as it was found to be closest to the *A. vera* cultivated in Korea and was not concurrence with any *Aloe* spp. previously reported in sense of both morphological and genetic information.

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