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Genetic Relationships of *Lactuca* spp. Revealed by RAPD, Inter-SSR, AFLP, and PCR-RFLP Analyses

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Abstract

RAPD, Inter-SSR, and AFLP markers were used to assess the genetic diversity of lettuce cultivars and the phylogenetic relationships in *Lactuca* spp. A total of 216 polymorphic bands from seven RAPD primers, four Inter-SSR primers, and five AFLP primer combinations were used to elucidate the genetic similarity among lettuce cultivars. Forty-four lettuce accessions were subdivided into discrete branches according to plant type: crisphead, butterhead, and stem type, with some exceptions. The leafy- and cos-type accessions were intermingled in other groups with no discrete branch indicating that these are more diverse than others. Three accessions, including the Korean cultivar 'Cheongchima', the Korean local landrace 'Jinjam', and the German cultivar 'Lolla Rossa' were classified as the most diverse accessions. Twenty bands were unique in specific cultivars. Among these, three were specific in a plant type; one in Korean leafy type, one in crisphead type, and one in cos type lettuce. In the phylogenetic analysis among *Lactuca* species, *L. saligna*, *L. serriola*, and *L. georgica* clustered in a sister branch of the *L. sativa* complex. Two *L. virosa* accessions show the highest intra-specific relationships. *L. perennis* outlied from all the other *Lactuca* species at a genetic similarity of 0.53 and clustered with two *Cichorium* species, C. *intybus* and C. *endivia*, with genetic similarity of 0.67. The phylogenetic tree was supported by data from polymorphism of chloroplast genome which was revealed by PCR-RFLP.

Key words: *Lactuca* SPP, chloroplast, genetic relationships

Introduction

DNA markers have been valuable tools for the preservation and analysis of germplasm diversity. They could also be useful for several aspects of breeding programs (Hill et al. 1996; Fang et al. 1997; Hongtrakul et al. 1997). Several types of DNA markers, such as RFLP, RAPD, Inter-SSR, AFLP, and transposon insertion polymorphism, were developed for this purpose (Vos et al. 1995; Yang and Park 1998; Yang et al. 2007; Zietkiewicz et al. 1994).

Lactuca is a widely-distributed genus including cultivated species *L. sativa* (lettuce) in the family Compositae. *L. sativa* belongs to a subsection of *Lactuca* that also includes three well-defined wild species, *L. serriola*, *L. saligna*, and *L. virosa*, which have nine chromosome pairs (Ryder and Whitaker 1976; Robinson et al. 1983). Lettuce spread from Egypt to western Europe where a distinct head lettuce first appeared and then to China where a stem type lettuce evolved (Whitaker 1969). It was introduced into Korea about 1,100-1,200 years ago through India, Tibet, Mongolia, and China. All Korean indigenous lettuce is a loose, leafy type because of the distinct Korean food custom called 'Ssam' in which fresh lettuce leaves are used to wrap rice and meat together.

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Recently, a well-defined lettuce genetic map was constructed using AFLP, insertion polymorphism of LTR retrotransposon, and the nucleotide binding site-leucine-rich repeat (NBS-LRR) family of disease resistance-associated genes (Syed et al. 2006). Genetic relationships among *Lactuca sativa* accessions and two other wild species, *L. indica*, and *L. perennis*, have been investigated using isozyme markers (Kesseli and Michelmore 1986), RFLPs (Kesseli et al. 1991), RAPD (Yu and Jang 2003), and AFLPs (Hill et al. 1996; Jansen et al. 2006). However, Korean leafy-type lettuce accessions were not included in these studies.

This study aimed to assess the genetic diversity and relationships among lettuce accessions, including several Korean leaf lettuce cultivars and landraces, to identify cultivar-specific markers and to determine phylogenetic relationships among *Lactuca* spp using various types of DNA markers.

Materials and Methods

Plant materials

A total of 53 morphologically-diverse *Lactuca* accessions including 44 *L. sativa* accessions, five wild species of *Lactuca*, and two *Cichorium* species, were examined (Table 1). DNA was extracted from leaves as described previously (Luo et al. 1992) with a slight

Table 1. Names, Origin, Leaf Types and their species

NO.[2]	Accession Names	Origin	Leaf type	Species
1	Salinas-88	USA	Crisp Head	*Lactuca sativa L.*
2	Arucadia	Japan	Crisp Head	
3	Urake	Japan	Crisp Head	
4	Malika	Netherland	Crisp Head	
5	Bravo	Philipin	Crisp Head	
6	Majestic	France	Crisp Head	
7	Texasgreen	USA	Crisp Head	
8	Emperor	USA	Crisp Head	
9	Patriot	USA	Crisp Head	
10	Vanguard 75	USA	Crisp Head	
11	Ithaca	USA	Crisp Head	
12	Salad crisp	USA	Crisp Head	
13	C1	USA	Crisp Head	
14	C-2-1-11	USA	Crisp Head	
15	D-3-22M	USA	Crisp Head	
16	C-2-1-1	USA	Crisp Head	
17	Sacramento	Japan	Crisp Head	
18	Great lakes 659	China	Crisp Head	
19	Wihu	China	Crisp Head	
20	Sioux	Germany	Crisp Head	
21	Ice Cube	USA	Crisp, mini	
22	Mini-Green	USA	Crisp, mini	
23	Blush	USA	Crisp, mini	
24	Green ball	Korea	Crisp Head	
25	Hajichungchukmyoun	Korea	Leafy	
26	Ddugsumjukchukmyoun	Korea	Leafy	
27	Jukchima	Korea	Leafy	
28	Chungchima	Korea	Leafy	
29	Sunghwajukchukmyoun	Korea	Leafy	
30	Jinjam	Korea	Leafy	
31	Lolla Rosa	China	Oak leaf	
32	C13	Unknown	Oak leaf	
33	Oak leaf	USA	Oak leaf	
34	Mammy	Japan	Leafy	
35	Mona	Germany	Leafy	
36	PI 342539	Netherland	Butterhead	
37	Shirley	Netherland	Butterhead	
38	Elvira rz	Netherland	Butterhead	
39	PI 342491	Netherland	Butterhead	
40	PI 358039	Yugoslavia	Cos	
41	Cos Lettuce	Japan	Cos	
42	Majestic RD	USA	Cos	
43	PI 342556	Netherland	Stem	
44	Celtus	Japan	Stem	
45	PI 251798	Italy	Wild relative	*L. saligna*
46	PI 253229	Turkey	Wild relative	*L. saligna*
47	PI 271938	USA	Wild relative	*L. virosa*
48	PI 261651	Porutucal	Wild relative	*L. virosa*
49	PI 206694	India	Wild relative	*L. serriola*
50	Unknown	England	Wild relative	*L. georgica*
51	PI 274415	Denmark	Wild relative	*L. perennis*
52	Sonsino		Root cichory	*Cichorium intybus*
53	D'inverno		Leaf cichory	*C. endivia*

[2]Numericals, instead of accession names, were used at Figs. 1, 2, 3, 5, and 6.

modification (Yang and Park 1998). DNA concentration was measured with a fluorometer (Hoefer Scientific Instrument, California).

RAPD and Inter-SSR analysis

Eleven primers (Table 2) were used for RAPD and Inter-SSR PCR analysis. All the methods followed were as previously described (Yang and Park, 1998). Primers were purchased from the Biotechnology Laboratory, University of British Columbia, Canada (UBC primer Kit #2, mtD, and #9). Each 25㎕ amplification reaction consisted of 200 uM of dNTP, 200 nM of primer, 1 unit of Taq polymerase (Bioneer, Korea), and 20ng of template DNA. Amplification was performed in a 96-well Perkin Elmer Thermocycler 9700 under the following conditions: 5 min at 94℃ for denaturation, followed by 45 cycles of 30 s at 94℃, 30 s at 36℃ or 52℃(for RAPD and for Inter-SSR, respectively), and 60 s at 72℃, and a subsequent incubation of 5 min at 72℃. Amplification products were separated on 1.5% agarose gels (FMC Co.).

AFLP analysis

AFLP was performed essentially according to the instruction manual of AFLP analysis system I of GibcoBRL (Invitrogen Co, USA) with minor modifications. Genomic DNA was digested with both *Eco*RI and *Mse*I. Adaptors for the restriction enzymes sites were ligated to the restriction fragments and preselective amplification using 1:10 diluted ligation mixture performed with *Eco*RI+A and *Mse*I+C. Preselectively amplified DNA was diluted to 1:50 with TE buffer and 5㎕ was used as template DNA of selective amplification. Selective amplification was performed with 0.2㎕ unlabelled *Eco*RI primer, 4.8㎕ of *Mse*I primer containing dNTPs, 1 unit of Taq polymerase (Promega Co. Madison), and 1x PCR buffer. The touch-down cycle profiles were 30 s at 94°, 30 s at 65°, 60s at 72° for 1 cycle; during the next 11 cycles, the annealing temperature was lowered 0.7 ° in each cycle and followed by 32 cycles with an annealing temperature of 56° Amplification products were mixed with a half volume of loading buffer (98% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue) and heated for 5 min at 90° T-he resulting mixtures were run on a 5% polyacrylamide gel under standard conditions of sequencing gels. Bands were detected by following the technical manual of Silver Sequence DNA Sequencing System (Promega Co. Madison).

PCR-RFLP

Two chloroplast genomic regions, *psb*A gene, and one intergenic region between tRNA-Leu and tRNA-Thr, were analyzed to detect polymorphism among *Lactuca* species. Primers were synthesized by Bioneer Co. of Korea based on previous information (Havey 1995; Tsumura et al. 1995; Cho et al. 2006). as follows: SM1 (5'-tct acc gat ttc gcc ata tc-3'); SM2 (5'-cat tac aaa tgc gat gct ct-3'); *psb*A1 (5'-tac gtt cgt gca taa ctt cc-3'); psbA2 (5'-cta gca ctg aaa acc gtc tt-3'). Amplification was carried out under the same conditions with Inter-SSR, with a slight increase in annealing temp to 52 °C and an increase in total volume to 50㎕. PCR reactions were directed used for digestion using four restriction enzymes; *Hae*III, *Hinf*I, *Taq*I, and *Msp*I (Takara Co. Japan).

Data analysis

Each band was treated as a unit character. Pair-wise genetic similarity coefficients among cultivars were quantified based on Nei's formula (1987). Cluster analysis was carried out by UPGMA using NTSYS-pc software (Rohlf 1992).

Results and Discussion

RAPD, Inter-SSR and AFLP analysis among *Lactuca* spp

A total of 112 RAPD bands were generated by 11 primers (Fig. 1). Among them, 111 and 69 bands were polymorphic among Lactuca spp. and within Lactuca sativa, respectively. Even though Inter-SSR markers are known to yield more bands than RAPDs because of their higher redundancy in the plant genome, similar numbers of scorable bands were obtained by random decamer primers and Inter-SSR primers, a result likely caused by the low resolution of agarose gel electrophoresis for separation of similar size of Inter-SSR PCR fragments (Table 2). Although more than 25 inter-SSR bands could be clearly counted by polyacrylamide gel electrophoresis, only two bands were scored by agarose gel electrophoresis. Fang et al. (1997) obtained 36 to 91 reproducible Inter-SSR bands by separation on PAGE.

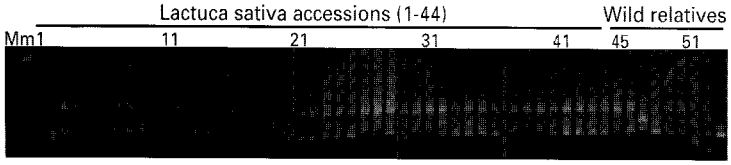

Fig. 1. RAPD profiles of 53 accessions of *Lactuca* spp. Including 44 *L. sativa* accessions and 9 wild relatives generated by primer UBCmt2. M and m: DNA size markers, PCR marker and λHindIII/EcoRI marker, respectively. Numericals denote the numbering of each accession in Table 1.

A total of 257 AFLP bands were obtained with five primer pair combinations and 147 of these bands were polymorphic (Fig. 2). Up to 73 bands were obtained by one primer combination and most bands were highly reproducible.

Table 2. Numbers of RAPD, Inter-SSR, and AFLP bands per each primer

	Primers	Nucleotide sequence (5' to 3')	Total No. of Bands	No. of Polymorphic Bands Withinz	Inter Species
	u103	gtg acg ccgc	8	2	8
	u115	ttc cgc gggc	5	4	5
	umt2	gct aaa taa gct aac agg ttc at	15	6	15
RAPD	umt4	tac aat tta tcg cct aaa ctt cag cc	14	14	14
	umt10	ttg att ttt tgg tca tcc aga agt	6	2	6
	umt13	aat atg gca gat tag tgca	12	6	11
	umt14	ggt caa aca att gag tct att tga ac	17	15	17
	u834	$(ag)_8$yt	8	4	8
Inter-	u842	$(ga)_8$yg	7	3	7
SSR	u847	$(ca)_8$rc	16	10	16
	u865	$(ccg)_6$	4	3	4
		Mid sum	112	69	111
	#1	EcoRI-aac / MseI-cac	25	5	-
	#2	EcoRI-aac / MseI-cac	73	47	-
AFLP	#3	EcoRI-aca / MseI-cag	57	34	-
	#4	EcoRI-acg / MseI-cta	66	47	-
	#5	EcoRI-agc / MseI-cta	36	14	-
		Mid sum	257	147	
		Total	369	216	

[z] Polymorphic bands among accession of *Lactuca sativa*

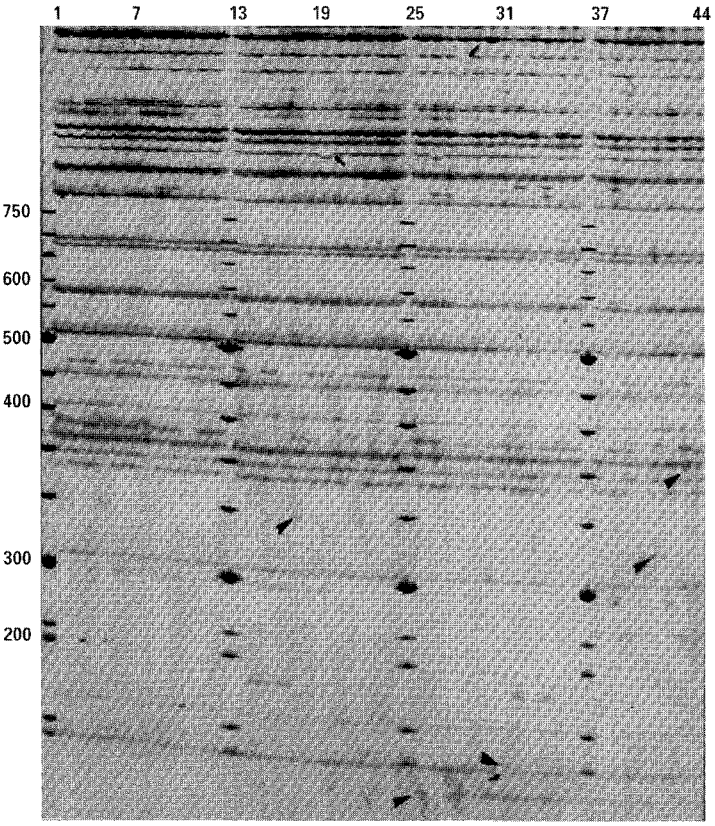

Fig. 2. AFLP profiles of 44 L. sativa accessions that generated by the primer combination, #4, GibcoBRL E-ACG/M-CTA. M: molecular size marker (50 bp ladder). Numericals denote the numbering of each accession in Table 1. Arrowheads and arrows indicate variety-specific bands; present and absent, respectively.

Genetic similarity among *L. sativa* accessions

Phylogenetic analysis was conducted among 44 *L. sativa* accessions by separation with wild species to assess genetic diversity within cultivating lettuce species. A total of 216 polymorphic bands including 69 RAPD and inter-SSR bands and 147 AFLP bands were used for phylogenetic analysis because trees derived from separate data of RAPD and AFLP were not consistent with each other. All the cultivars were classified with a genetic similarity ranging from 0.664 to 0.945 as shown by the dendrogram (Fig. 3). Three accessions,

Table 3. DNA markers which are presented distinctly in one or a few cultivars

Accessions	Specific bands[z]	Accessions	Specific bands[z]
Arucadia	Umt2-400(+), Umt14-350(+)	PI34249	#4-50(-), Umt2-550(+)
Malika	Umt13-740(-)	Cos lettuce	#4-275(+)
Bravo	Umt2-410(+)	PI 342556	#4-370(+), #2-370(+), #4-2200(-)
Wihu	#2-180(-)	Celtuce	#2-505(+), #2-350(+)
Cheongchima	#4-1300(-)	Korea leaf lettuce[y]	#2-80(+)
Lolla Rosa	#4-150(+), #4-440(+)	Stem lettuce[x]	#2-150(+)
Mammy	#4-110(+)	Crisp head[w]	#2-70(+)
Elvira rz	#3-1000(-)	Cos type[v]	#2-68(+)

[z] Primer number, band size, and (): +, band presence; -, absence. #2, #3, and #4 indicate the primer combinations of E-aac/M-cac, E-aca/M-cag, and E-acg/M-cta, respectively.
[y] The band presented in Korean loose leafy lettuce cultivars (Haji-Cheong, Jukchima, Cheongchima, Seonghwa-Jeogchugmyeon, Jinjam), [x] in stem lettuce cultivars (PI342556, Celtus), [w] in most of crisp head cultivars (Arucadia, Urake, Malika, Bravo, Majestic, Texasgreen, Emperor, Patriot, Banguard 75, Ithaca, Salad Crisp), [v] in cos and romaine type cultivars (PI358039, Cos lettuce).

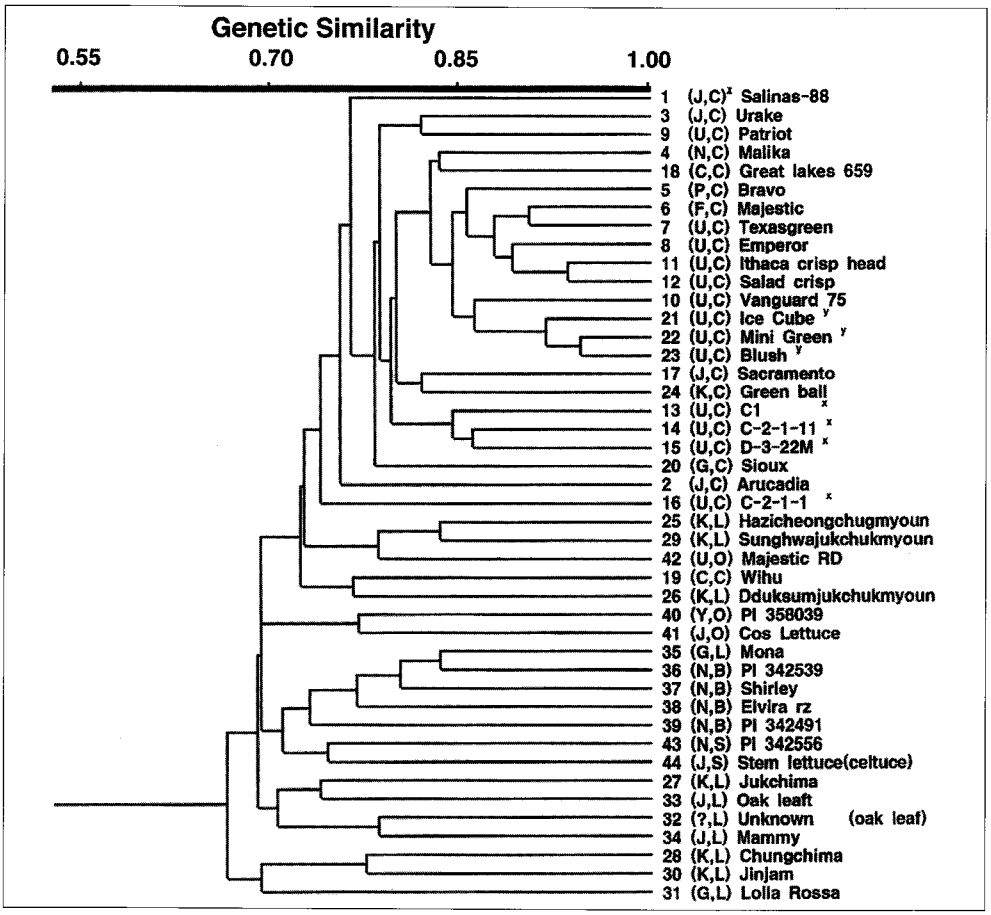

Fig. 3. Phylogenetic tree based on cluster analysis (UPGMA) of genetic similarity estimates for lettuce accessions from 202 polymorphic RAPD and AFLP bands. Z (Origin of country, Leaf type), y Miniature crisphead lettuce cultivars derived from the same cross of 86-1024 x Salinas, x breeding lines from same cross of 56679E x Vanguard75.

including the Korean cultivar 'Cheungchima', the Korean local race 'Jinjam', and the German cultivar 'Lolla Rossa', were distinguished from the remaining 41 accessions at a genetic similarity of 0.664.

The 44 accessions of *L. sativa* were subdivided into discrete branches according to plant type: crisphead, butterhead, and stem type, with some exceptions. Leaf- and cos-type accessions were not clearly separated and their genetic background seemed to be more diverse than the other types. This could be explained by the genetic system controlling the leafy type. Leafy- and cos-type lettuce can be derived from outcrossing of the crisphead type because heading is controlled by several recessive genes or quantitatively (Robinson et al. 1983). Loose leaf- and cos-types were more predominant than heading- or half-heading-type plants in the segregating population of most cross combinations. All the crisphead-type accessions were clustered together except for the Chinese cultivar 'Wihu'. It is assumed that 'Wihu' has a different genetic background than other crisphead cultivars. It might be useful as a parental line for the enlargement of genetic diversity for the development of an elite crisphead cultivar. The leafy-type German cultivar 'Mona' was clustered with butterhead-types, and seems to have a similar genetic background with butterhead-type cultivars. Stem-type cultivars also seem to have similar genetic background with the butterhead-type cultivars.

Genetic distance is consistent with the lineage of accessions whose breeding histories are known. Three miniature crisphead lettuce cultivars, 'Ice Cube', 'Mini Green', and 'Blush', clustered together with a high level of genetic similarity indicating they have similar genetic background. In fact, these were derived from one cross between '86-1024' and an elite cultivar 'Salinas' of which the 86-1024 is a very early flowering and dwarf mutant line induced by EMS treatment. Four accessions, C-1, C-2-1-11, D-3-22M, and C-2-1-1, were derived from one cross between '56679E' and 'Vanguard 75' of which the mutant line 56679E (E = early flowering) has a very early flowering characteristic which is governed by two independent dominant genes, *Ef*-1 and *Ef*-2. (Ryder 1988). These four lines grouped in the crisphead-type cluster and three of these showed very high genetic similarity.

The cluster did not reflect the country of origin, a result likely due to exchange of germplasm among countries. However, Korean leafy lettuces such as 'Chungchima' and 'Jinjam' were subdivided at the lowest genetic similarity, a finding likely due to the distinct preference of Korean in leafy-type lettuce.

Twenty bands were uniquely associated with specific cultivars (Table 3). Fourteen cultivars in Table 4 could be distinguished from all other cultivars based on the presence or absence of the specific band. Presence of the 400 bp band generated by primer Umt2 indicates that the cultivar might be 'Arucadia'. As to unique bands, one is uniquely amplified in most Korean leaf lettuces, one is unique in most crisphead-type cultivars, one is unique in cos- and romaine-type cultivars, and one is unique in most loose leafy-type cultivars. These bands could be very valuable for fingerprinting, identification, and varietal protection of lettuce cultivars (Hwang et al. 2002).

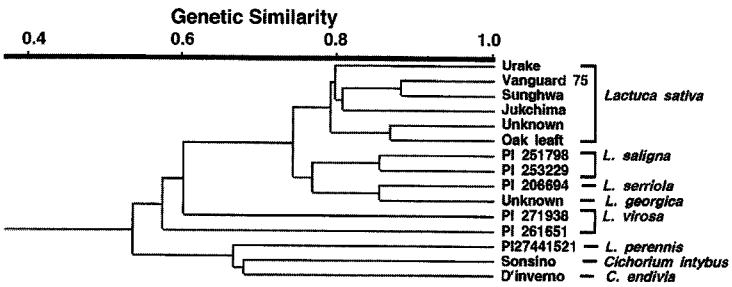

Fig. 4. Phylogenetic tree of Lactuca spp. and Cichorium spp based on 75 polymorphic RAPD bands

Phylogenetic relationship in Lactuca species

We conducted phylogentic analysis among 15 accessions in *Lactuca* genus including six *Lactuca sativa,* seven wild *Lactuca* species, and two Cichorium species. The genetic similarity was 0.53-0.88 based on an analysis using a total of 111 polymorphic bands

(Fig. 4). The resulting dendrogram grouped all species as distinct taxa and was consistent with previously defined inter-specific relationships, though a slight difference was noticeable (Hill et al. 1996; Kesseli and Michelmore 1986; Kesseli et al. 1991). All accessions of *L. sativa* clustered at the genetic similarity of 0.79. The accessions of *L. saligna, L. serriola, and L. georgica* clustered on a sister branch of the *L. sativa complex.*

Three possibilities were suggested for the elucidation of the origin of cultivated lettuce: (a) two species, *L. sativa* and *L. serriola*, might have originated from hybrid populations and then diverged into two groups by domestication: *L. sativa* cultivated by man, and *L. serriola* adapted to man-made waste habitats; (b) the ancestors of *L. sativa* might have been hybrids between *L. serriola* and a third species; (c) *L. serriola* might be a pimilarity of 0.87. *L. serriola* PI 206694 and *L. georgica* also clustered as a distinct unit of genetic similarity of 0.87. Two *L. virosa* accessions, PI 271938 and PI 261651, showed the highest intra-specific relationships with a genetic similarity of 0.58, even though two other *L. virosa* accessions, UC83UKI and LS85, clustered as a distinct unit with a relatively low mean intra-specific genetic distance of 0.16±0.05 in a previous report (Hill et al. 1996). The genetic distance of *L. sativa* with *L. virosa* is higher than with *L. serriola* and *L. saligna*, which is in keeping with the results of Lindqvist (1960). This conclusion is partially explained by the distinct polymorphic RFLP pattern in *V. virosa*, PI 271938, for a specific chloroplast gene, *psb*A (arrow in Fig. 5). All the species, including

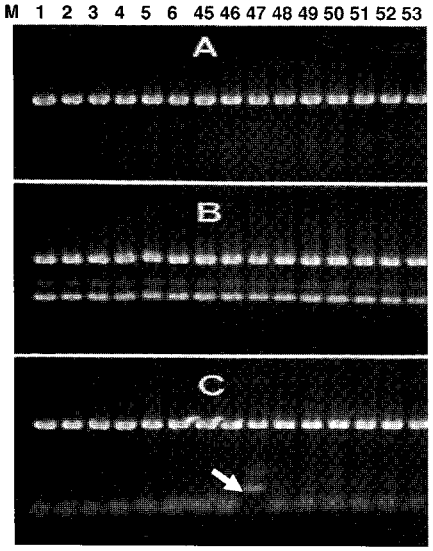

Fig. 5. RFLP patterns of PCR-amplified psbA gene of chloroplast genome. A, B, and C are uncut, HinfI-digested, and HaeIII-digested psbA gene, respectively. Numericals denote the numbering of each accession in Table 1. Arrow indicates polymorphism detected in one L. virosa accession, PI 271938. M, PCR marker (Promega Co.)

two *Cichorium* species, showed the same band pattern for the PCR-RFLP of the *psb*A gene, but PI 271938 (one of two *L. virosa* accessions) showed distinct polymorphism by digestion with *Hae*III (Fig. 5 C). The accession PI 271938 has a distinct molecular profileand several important characteristics such as a biennial life cycle (bolt-resistance) and resistance to several diseases such as soft rot, corky root rot, lettuce drop, powdery mildew, cucumber mosaic virus, broad bean wilt virus, big vein, root knot nematodes, and the leaf aphid (R.W. Robinson: personal communication).

L. sativa is incompatible with three wild species, *L. serriola, L. saligna* and *L. virosa* which also have nine pairs of chromosomes. Interspecific crosses between *L. sativa* and *L. virosa* usually yield sterile F_1 plants even though great efforts, via biotechnology as well as conventional breeding, were made to introgress the agriculturally-important characteristics into elite lettuce cultivars from the wild relative. Only one cultivar, 'Vanguard', has ever been developed from an interspecific cross and subsequent colchicine treatment. However, 'Vanguard' does not have a biennial life cycle and disease resistance (Thompson and Ryder 1961).

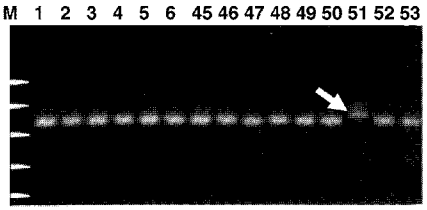

Fig. 6. PCR profile of the analyzed plants. Primers targeted into inter-genic space region between tRNA-Leu and tRNA-Thr of chloroplast genome. Numericals denote the numbering of each accession in Table 1. Arrow indicates the product of L. perennis. M, PCR marker (Promega Co.)

L. perennis, PI 274415, clustered as the most diverse species from all other Lactuca species with a genetic similarity of 0.53, which is well in accordance with the previous report (Hill et al. 1996). This conclusion is also well supported by PCR results regarding one specific chloroplast region, the intergenic space between tRNA-Leu and tRNA-Thr (Fig. 6). Only *L. perennis* showed approximate distinct 750 bp polymorphic band (arrow Fig. 6). Two *Cichorium* species, *C. intybus* and *C. endivia*, clustered at the genetic similarity of 0.69. Two species formed a sister branch with *L. perennis* at a genetic similarity of 0.67. However, they did not show any polymorphism at two chloroplast s with *Lactuca* species (Figs. 5 and 6) indicating they are closely related to the genus *Lactuca*.

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