



# KOREA SEED & VARIETY SERVICE

## Development of EST-SSR Markers of Lettuce and Application for Variety Identification

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KOREA SEED & VARIETY SERVICE

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# Lettuce(*Lactuca sativa* L.)

## ◎ Species & distribution

- Lettuce is a diverse group of plants with a global distribution
- Cultivated lettuce: *L. sativa* L.
- Wild species: *L. serriola* L. *L. saligna* L. *L. virosa* L.

## ◎ Consumption: “a SSAM(fresh vegetable wrap)”





# Plant Materials

## ◎ DUS testing field



(Candidate variety : Similar variety)

## ◎ Types of lettuce and 156 varieties used



Crisphead(10)

Leaf(140)

Romaine(6)



Butter head

Ratin

Stem

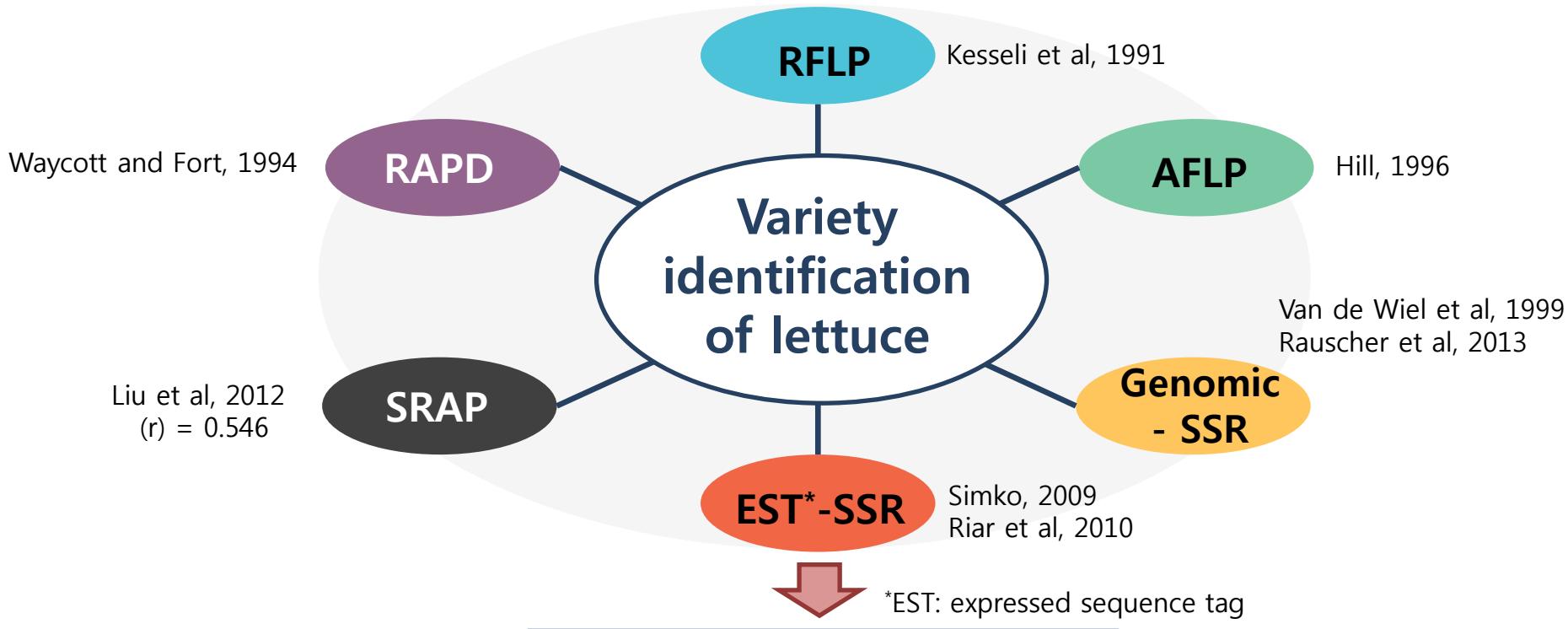
(Kristkova et al, 2008)

Number of PVP titles in vegetables	
1	Pepper (212)
2	Chinese cabbage (129)
3	Radish (109)
4	Lettuce (67)
5	Watermelon (66)

(2014.12.31.)



# DNA markers for lettuce

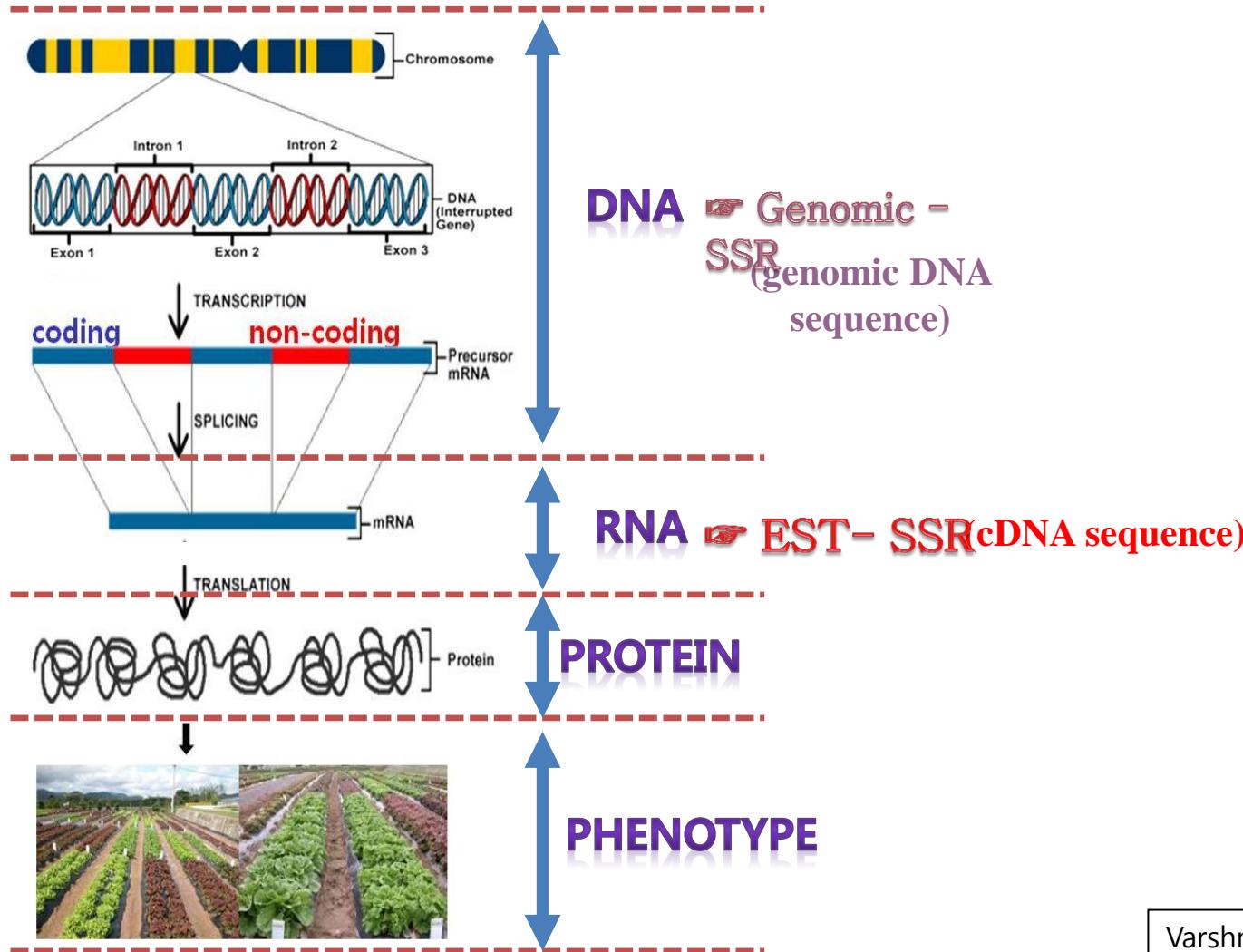


- ◎ **Level of polymorphism**  
: EST-SSR < Genomic-SSR
- ◎ **Expressed portion of the genome**  
: EST-SSR > Genomic-SSR
- ◎ **Interspecific transferability**  
: EST-SSR > Genomic-SSR
- ◎ **Development easiness**  
: EST-SSR > Genomic-SSR



# Two Types of SSR Markers

- ◎ SSR markers : Different number of repeats in microsatellite regions e.g.  $(AT)_{18}$ ,  $(TCT)_{12}$





# Objectives and Experiments

## Objectives

- **Development of useful markers for identification & similarity test of lettuce varieties**
  - used for infringement cases and other seed-related disputes, market managements, and various PVP purposes

## Experiments

- Experiment 1: Development of EST-SSR markers
- Experiment 2: Construction of DNA profile database and similarity test
- Experiment 3: Breeder's test and correlation estimation between EST-SSR markers and morphological distances



# Experiment 1. Development of EST-SSR markers

## ① Download of 81,330 *L. sativa* ESTs from NCBI

## ② Sequence assembly

- Software : **CAP3** (Huang and Madan, 1999)

## ③ SSR detection

- Di(6 times repeat), Tri(5), Tetra(5), Penta(4), Hexa(4)
- Software : **MISA** (<http://pgrc.ipk-gatersleben.de/misa/>)

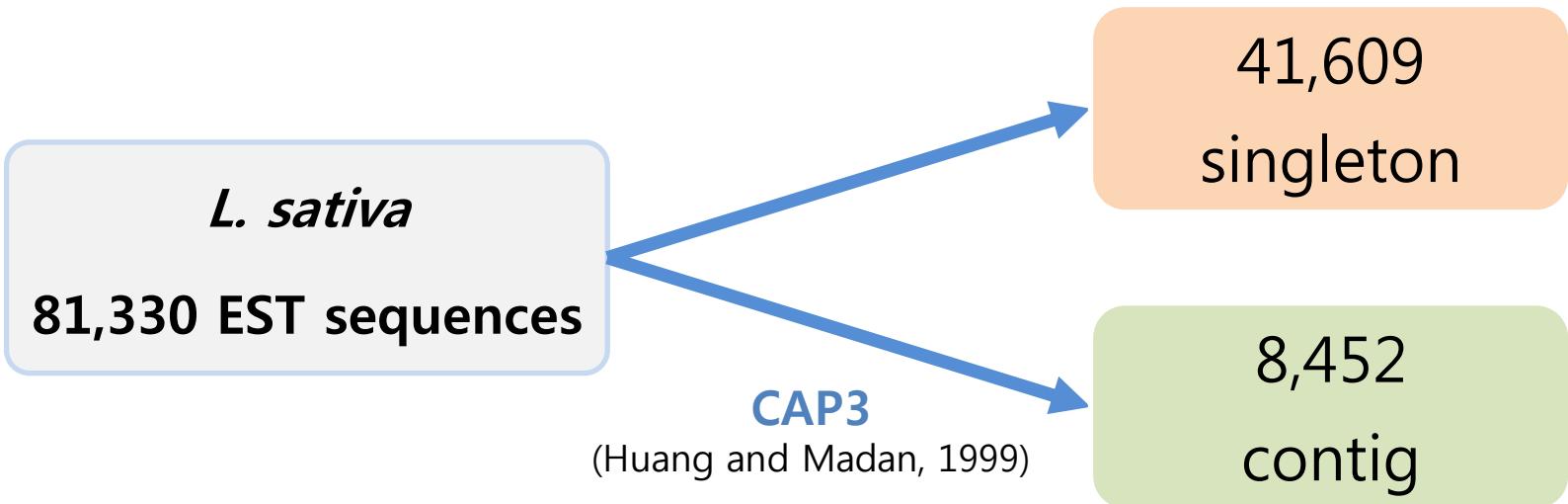
## ④ EST-SSR primer design

- Parameters : 18-27 nucleotides with product size 100-300bp, annealing temperature of 50°C-60°C, GC content 20 °C-80 °C
- Software : **Primer3** (Rozen et al, 2000)



# Sequence Mining & Assembly

EST Sequence data from public domain





# *in silico* SSR Detection in EST Seq.

## ◎ For singletons sequence

Parameters used in screening	Data generated by MISA
Total number of sequences examined	41,609
<b>Total number of identified SSRs</b>	<b>4,229 (10.2%)</b>

## ◎ For contigs sequence

Parameters used in screening	Data generated by MISA
Total number of sequences examined	8,452
<b>Total number of identified SSRs</b>	<b>807 (9.5%)</b>



# EST-SSR Primer Design

- ◎ Number of primers and SSR motifs designed from SSR sequences

SSR sequences source	Primer design	SSR motif	
Singleton 4,229 SSR	471	Dinucleotide Trinucleotide Tetranuclotide Pentanucleotide Compound formation	175 68 1 1 226
Contig 807 SSR	710	Dinucleotide Trinucleotide Tetranuclotide Pentanucleotide Hexanucleotide Compound formation	202 374 13 28 39 54
<b>Total</b>		<b>1,181</b>	



# Experiment 2. PCR amplification and DNA profile database construction

Classification	SSR marker selection	SSR profile database
Varieties	8~23 varieties	156 varieties + 16 germplasm
Primer	<b>352 primers</b> searched markers(81) + developed markers(271)	<b>High reproducibility and peak clearance</b>
Genotyping	6% polyacrylamide gels, silver staining	<b>Genetic analyzer(ABI3130xl)</b>
Data analysis	<ul style="list-style-type: none"><li>• Level of polymorphism : Polymorphism information content(PIC) (Anderson et al, 1993)</li><li>• Genetic relationship : NTSYS-PC version 2.01(Rohlf, 1998), Jaccard's coefficient</li><li>• Blast analysis between markers and proteins : BLASTX (Altschul et al, 1990)</li></ul>	



# Polymorphism of Screened SSR Markers

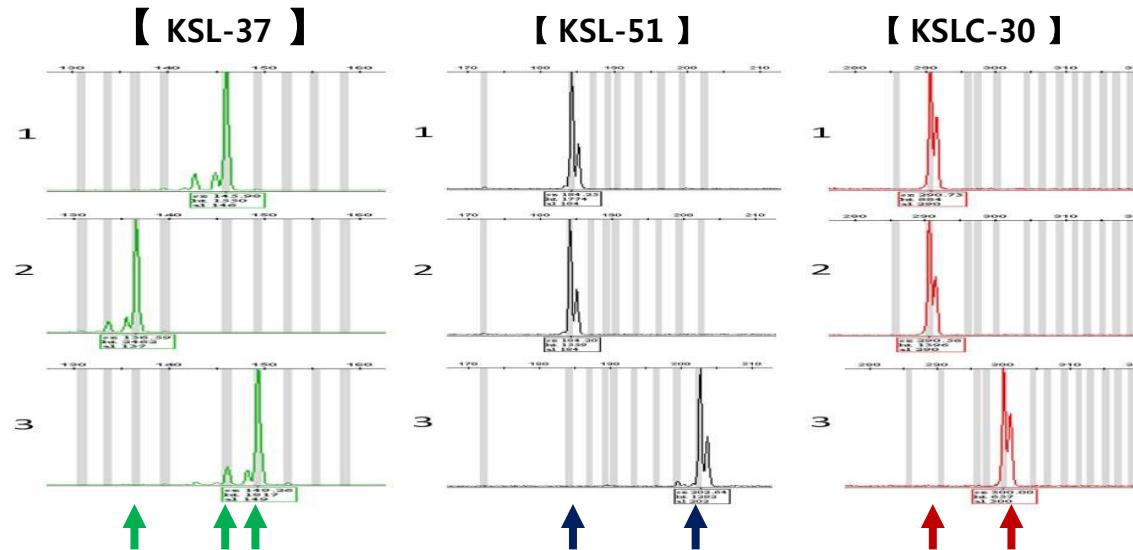
- Pre-screening of SSR markers using polyacrylamide gel electrophoresis.

	Number of screened markers (A)	Polymorphic markers (B)	Polymorphism % (B ÷ A)	SSR marker source
EST-SSR	20	1	5%	Jeuken et al. (2008)
	61	30	49.2%	Simko (2009)
	271	47	17.3%	In this study
	<b>352</b>	<b>78</b>	<b>22.2%</b>	

- Finally **60 SSR markers** were selected and used for the analysis of 156 lettuce varieties.



# Detection of Allele & Construction of Profiles



## Amplification patterns:

3 EST-SSR markers for 3 varieties of (1)Joara; (2)Evergreen; (3)Hotred.

## ◎ Construction of SSR profile database

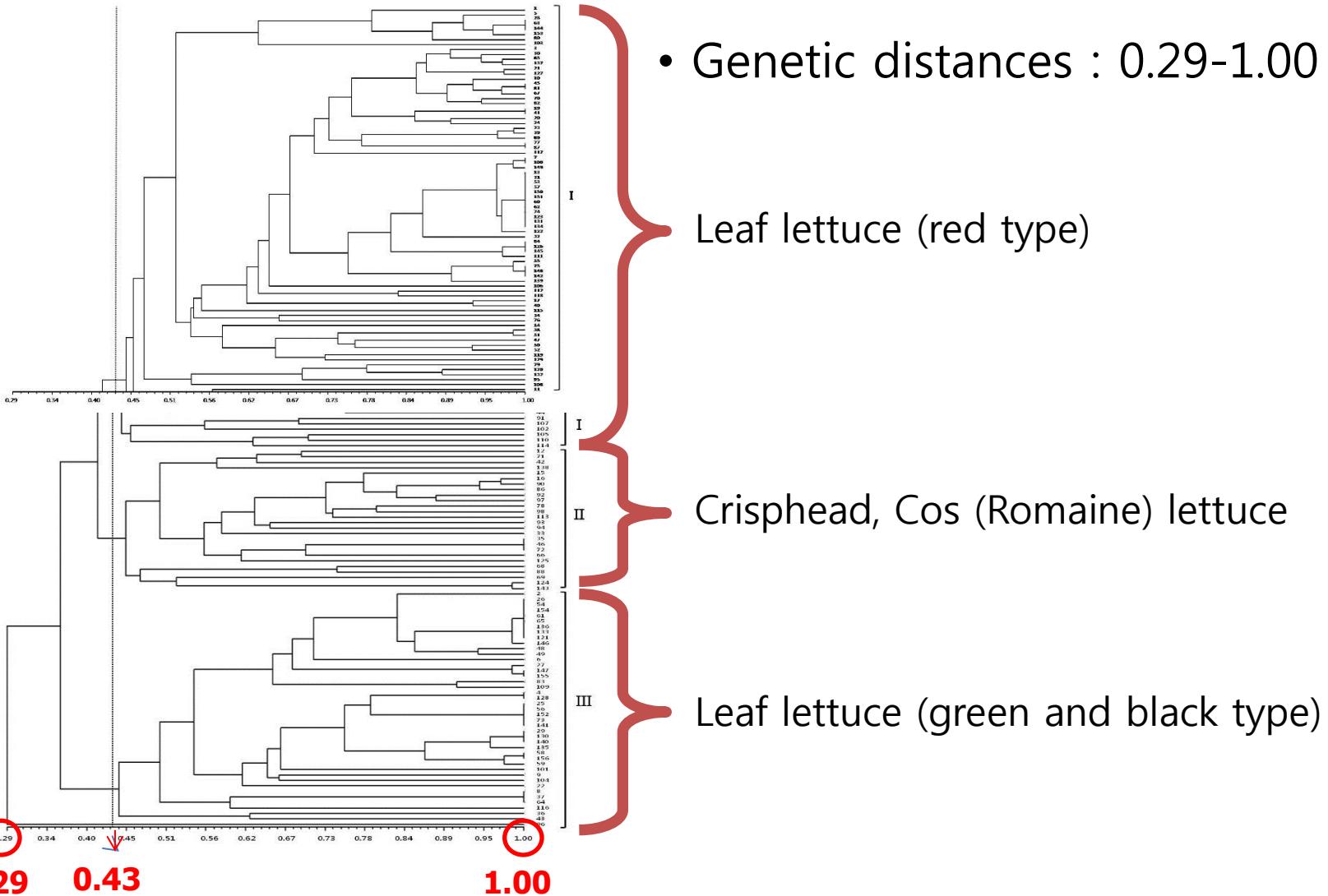
Size	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
SML-001	176	1	1	1	0	1	1	1	1	0	0	1	1	0	0	1	0	1	1	1	0	1	1	1	1	0	0	0	0
	180	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	182	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
	193	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	198	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SML-002	171	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	204	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	210	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	219	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
	222	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
SML-003	225	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
	114	0	0	1	1	0	0	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	0	1
	119	1	1	0	0	1	1	0	0	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0
	184	1	0	1	0	1	0	1	0	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	0	0
	196	0	1	0	1	0	1	0	1	1	0	1	1	1	1	1	1	0	1	0	0	1	0	1	1	1	1	0	1
SML-007	202	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	265	0	1	0	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1
	268	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	270	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	276	1	0	0	1	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
SML-013	254	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	0	1	0
	259	1	0	1	0	1	0	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
	269	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	263	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	267	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SML-015	163	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
	168	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0
	223	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	227	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	228	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

→ 156 varieties

Alleles: 1, present; 0, absent



# Genetic Relationships between Varieties



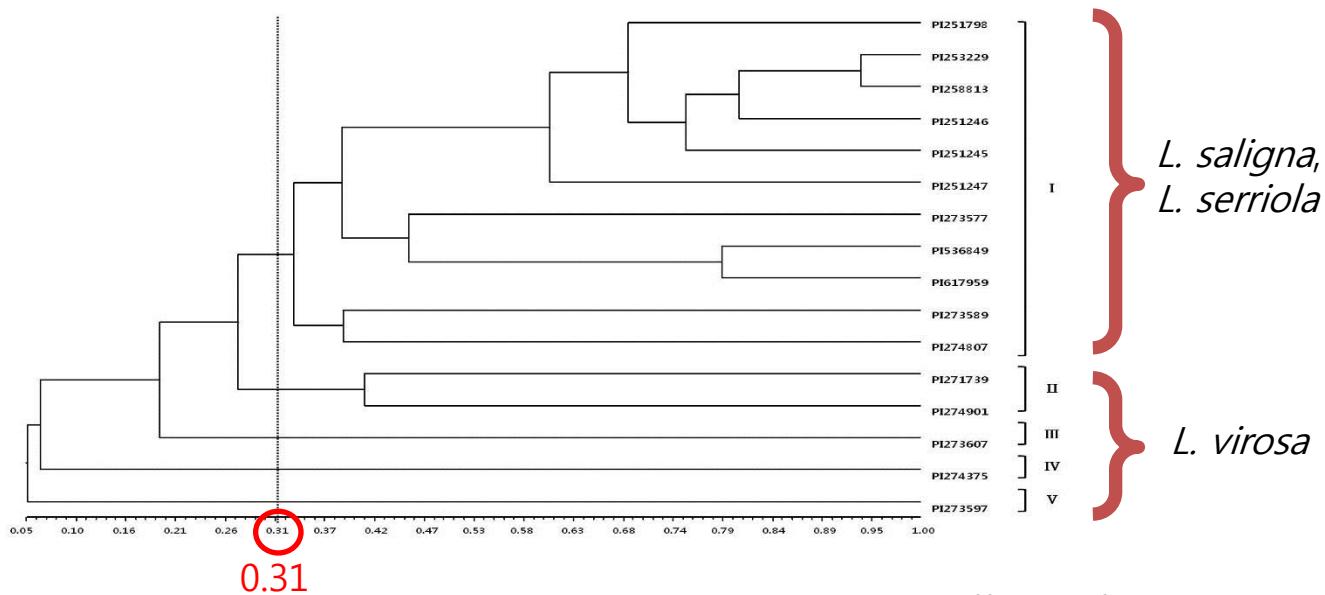
Dendrogram constructed based on Jaccard's similarity coefficient from 60 EST-SSR data on 156 lettuce varieties.



# Interspecific Transferability



Agarose gel electrophoresis of PCR amplification for 5 EST-SSR markers in 16 lettuce varieties and 16 germplasms



Dendrogram constructed based on Jaccard's similarity coefficient from 30 EST-SSR data on 16 wild accessions.



# Putative Functions of the Marker Regions

## ◎ Homology search for 18 EST-SSR markers by Blast X

No.	Primer name	EST ID	Putative function	Species	E-value
1	KSL-1	CLSS10849	TCP transcription factor 11	<i>Solanum lycopersicum</i>	4.00E-40
2	KSL-37	CLSM1424	Predicted protein	<i>Populus trichocarpa</i>	4.00E-72
3	KSL-271	CLSS8197	Flap endonuclease GEN-like 1	<i>Vitis vinifera</i>	1.00E-70
4	KSL-357	CLSS10992	Hypothetical protein MTR_1g089750	<i>Medicago truncatula</i>	3.00E-49
5	KSL-87	CLSY5704	Remorin, putative	<i>Ricinus communis</i>	5.00E-29
6	KSL-173	CLSM444	SEC14 cytosolic factor-like	<i>Vitis vinifera</i>	7.00E-114
7	KSL-245	CLSM513	Uncharacterized protein LOC100262360	<i>Vitis vinifera</i>	4.00E-58
8	KSL-7	CLSS10499	RING finger and WD Repeat domain-containing protein	<i>Medicago truncatula</i>	2.00E-27
9	KSL-26	CLSM14994	Hypothetical protein VITISV_044433	<i>Vitis vinifera</i>	2.00E-52
10	KSL-32	CLSM14764	Predicted protein	<i>Populus trichocarpa</i>	1.00E-64
11	KSL-43	CLSM1373	Unnamed protein product	<i>Vitis vinifera</i>	4.00E-37
12	KSL-75	CLSY7906	La-related protein 6-like	<i>Vitis vinifera</i>	4.00E-67
13	KSL-83	CLSY6646	Os01g0886200	<i>Oryza sativa Japonica group</i>	2.00E-08
14	KSL-92	CLSY517	14-3-3 protein, putative	<i>Ricinus communis</i>	4.00E-126
15	KSL-97	CLSY4815	Hypothetical protein RCOM_0927570	<i>Ricinus communis</i>	2.00E-24
16	KSL-115	CLSM11208	DNA binding protein, putative	<i>Ricinus communis</i>	5.00E-27
17	KSL-119	CLSM10279	Abhydrolase domain-containing protein FAM108C1-like	<i>Glycine max</i>	4.00E-93
18	KSL-123	CLSL2393	60S ribosomal protein L7a, putative	<i>Ricinus communis</i>	2.00E-48



# Experiment 3 : Breeder's test and correlation analysis

- ① **Varieties** : 7 group containing 18 varieties(genetic similarity 0.93~1.0)
- ② **Testing place(year)** : Suwon(2011 & 2012)
- ③ **Traits** : 29 traits among 33 leaf-trait of Korean lettuce TG
- ④ **Breeder's test (Blind test): Distinctness**
  - 4 breeders: Kwononong Seeds (1), Nongwoo Bio. (1), Asia Seeds(2)



- ⑤ **Correlation between genetic and morphological distances**
  - Use of 30 EST-SSR markers result
  - NTSYS-PC version 2.02 (Rohlf, 1998), Mantel (1967) matrix



# Distinctness est

## ◎ Variety comparison between similar varieties

Classification	Group 1	Group 2
Phenotype		
Genetic similarity	0.96	0.93
KSVS	No Distinctness	<b>Distinctness</b> (Intensity of color of outer leaves, bolting day)
Breeder's test	No Distinctness	<b>Distinctness (3)</b> <b>No Distinctness (1)</b>
Distinctness traits	-	Intensity of color of outer leaves, Intensity of anthocyanin coloration, glossiness



# Distinctness test

## ◎ Variety comparison between similar varieties

Classification	Group 3	Group 4
Phenotype		
Genetic similarity	1.0	1.0
KSVS	Distinctness(bolting day)	No Distinctness
Breeder's test	Distinctness (2) No Distinctness (2)	Distinctness (2) No Distinctness (2)
Distinctness traits	Leaf thickness, Intensity of anthocyanin coloration	Intensity of anthocyanin coloration, Leaf blistering



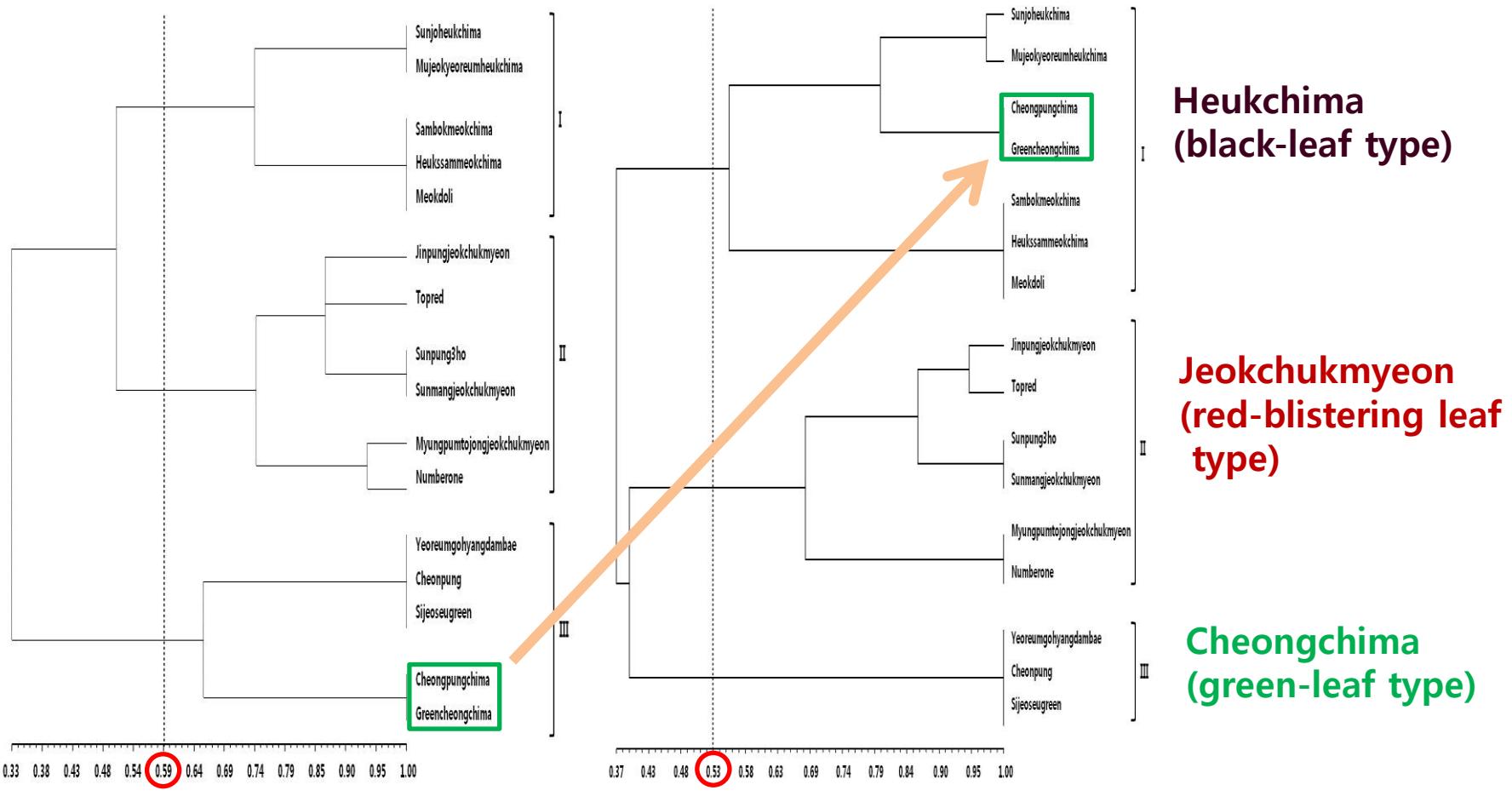
# Distinctness test

## ◎ Variety comparison between similar varieties

Classification	Group 5	Group 6	Group 7
Phenotype	  9 10  11	  12 13  14	  15 16
Genetic similarity	1.0	1.0	1.0
KSVS	No Distinctness	No Distinctness	No Distinctness
Breeder's test	No Distinctness	No Distinctness	No Distinctness

# Genetic Relationship of Morphological Characteristics

- Association of 18 lettuce varieties and comparison between morphological and genetic distances

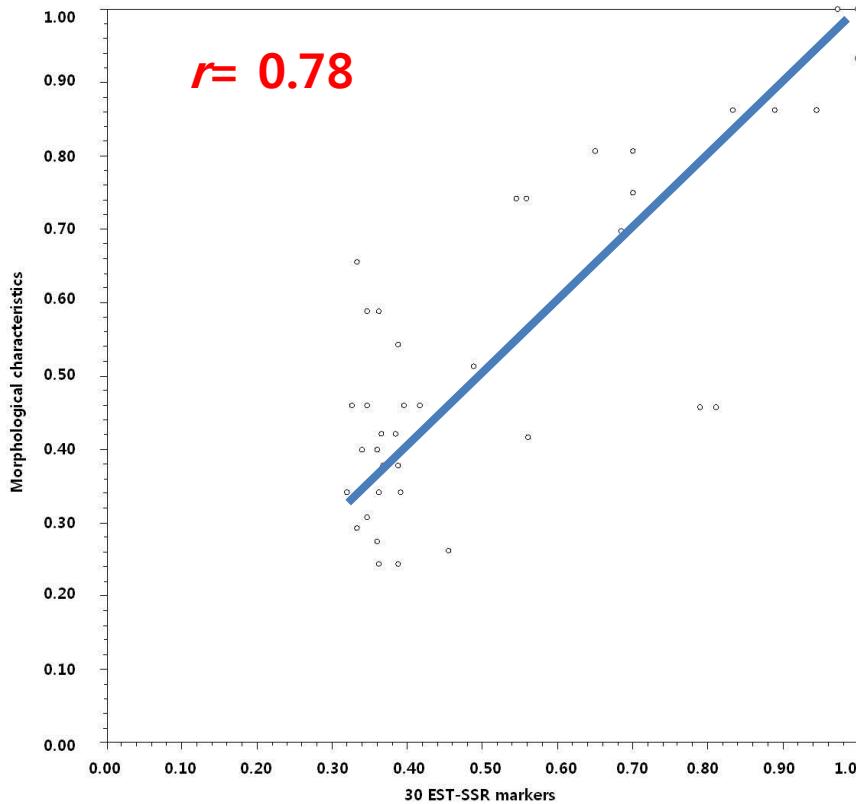


• Morphological distances : 0.33-1.00

• Genetic distances : 0.37-1.00



# Relationship between EST-SSR Markers and Morphological Characteristics



- Correlation coefficient : 0.78



Possible application  
for Option 2

Comparison between SSR markers and morphological distances using the Mantel test.



# Conclusions & Considerations

1. The total of 1,181 EST-SSR primers were developed from genic region of lettuce genome.
2. The marker set of 60 EST-SSR constructed a profile of 156 lettuce varieties and 115 varieties were discriminated.
3. Parts of the marker sequences showed a certain homology with known or predicted proteins.
4. Based on Breeder's test, the EST-derived SSR markers in lettuce showed relatively high correlations between genetic and morphological distances, indicating useful application for BMT Option in lettuce.
5. Further experiments are necessary for wider range of variety types to enhance reliability and usefulness.
6. The usefulness of EST-derived SSR markers can be considered in other species for BMT Options.



# KOREA SEED & VARIETY SERVICE

*Thank you for  
your attentions!!!*

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