# Population Structure and Genetic Diversity of Garlic in Korea by ISSR Marker

Man Kyu Huh* · Jung Sook Sung[1] · Joo Soo Choi · Young-Kee Jeong[2] · Eun-Ju Rhu[3] and Kyung Tae Chung[4]

*Department of Molecular Biology, Dong-eui University, Busan 614-714, Korea*
[1]*Department of Ginseng & Medical Crops, National Institute of Crop Science, RDA, Suwon 441-857, Korea*
[2]*Department of Biotechnology, Dong-A University, Busan 604-714, Korea*
[3]*Department of Cosmetology, Hanseo University, Seosan 356-706, Korea*
[4]*Department of Life Science and Biotechnology, Dong-eui University, Busan 614-714, Korea*

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Garlic is a perennial herb primarily distributed throughout the world. These plants are regarded as a medically and agricultural important crop in the world. The genetic relationships between cultivated and wild species were investigated at the population levels by constructing tree based on ISSR (inter-simple sequence repeats) markers. In addition, ISSR analysis was also conducted to estimate genetic diversity and population structure of these species. Three wild garlic populations in Korea were found to have more alleles per locus (mean 1.672 vs. 1.510), higher percent polymorphic locus (67.2 vs. 51.0), and higher diversity (0.250 vs. 0.198) than three cultivated populations. The cultivated and wild species in Korea are well separated from each other at phylogenetic trees. Although there is not direct evidence that *A. victorialis* is an ancestor of Korean *A. sativum*, there is a possibility that cultivated *A. sativum* in Korea has evolved from wild *A. victorialis* in Korea. Populations of *A. victorialis* may be useful in germ-plasm classification and evolutionary process.

**Key words** – Garlic, ISSR analysis, phylogenetic trees

## Introduction

Wild garlic, *Allium victorialis* L. within the family Liliaceae comprising diploid species (2n=16) or tetraploid (36) distributes mainly in northeastern Asia[15]. Cultivated garlic, *Allium sativum* L. is economically important for leaves and bulbs, which historically were used in Korea for spices and condiments of Korean food as well as medicine crops. Garlic is the second widely consumed Allium next to the common onions (*A. cepa* L.) in the world[14]. The garlic as health reinforcing food, is the functional food with alicine and azoen which is effective for digestive disorder, insomnia, hypertension, cancer, cholesterol and stamina reinforcement[11,13]. Recently, it is processed into the highly concentrated garlic beverage, which removes the bad smell of garlic and keep the nutritious ingredients intact[13].

Garlic is most likely originated from western and central Asia and it was cultivated as early 3,000 B.C. in ancient Egypt[6,17,23]. With its various morphological and physiological features, garlic can be distributed to a wide range of regions in the world. As that result, distinctively various features have appeared, resulting in designations of new botanical various[17]. The genus *Allium* is comprised of about 14~15 species in Korea[15]. The taxonomy of *Allium* has processed mainly through morphological characteristics. However, morphological characteristics are restricted their resolving power mainly because of the small number of variables available. Efficient methods to clarify the taxonomic status of several species are much needed.

Over the past century, crop evolutionists have employed a diverse arsenal of techniques to unravel these and other mysteries surrounding the origin and evolution of domesticated plants. Classical taxonomy, biogeography, cytology, archaeology, and classical genetics have all made important contributions. Although much has been learned, the origins of many crops, even some of the most important ones, remain obscure[5].

Electrophoretic analysis of allozyme is cost-effective and can be applied without extensive technical development and allozyme exhibit Mendelian inheritance. Nevertheless, there are several reasons to apply other types of markers. For instance, attempts to measure gene flow at small spa-

*Corresponding author
Tel : +82-51-890-1521, Fax : +82-51-890-1529
E-mail : mkhuh@deu.ac.kr

tial scale by allozyme alleles are frequently frustrated by the limited variability of allozymes[2]. The development of molecular makers has provided powerful tools that may overcome such limitations. ISSR (inter-simple sequence repeats) analysis is quick, robust and requires minimal preliminary work[11]. Thus, we successfully assess the genetic relationships among the local populations *A. sativum* and *A. victorialis* species in Korea.

Although it is important to gain knowledge of the genetic variation for conservation purposes, detailed information on the levels and distribution of this variation, as well as population structure, are not available for most plant taxa in Korea[12]. The aims of this study were; 1) to estimate how much total genetic diversity is maintained in the *A. sativum* and *A. victorialis* species, 2) to describe how genetic variation is distributed within and among species, and to elucidate the suitability and efficiency of the ISSR (inter-simple sequence repeats) analyses to assess the phylogenetic relationships between the related species in Korea.

## Materials and Methods

### Plant Materials and DNA Extraction

All of the six populations of two garlic species, *A. sativum* and *A. victorialis* were collected from populations in Korea (Table 1). One young leaf per plant was sampled. Fifteen plants were randomly collected from each population.

The genomic DNA of the 90 samples was extracted from fresh leaves using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol.

Table 1. Code and locations of the garlic populations in this study

| Code of population | Locality of populations |
|---|---|
| *A. victorialis* | |
| ULL | Mt. Seoninbong, Ulleng-gun, Gyeungbuk Prov., Korea |
| SOB | Mt. Soback, Youngpung-gun, Gyeungbuk Prov., Korea |
| GIR | Mt. Giri, Sancheon-gun, Gyeongnam Prov., Korea |
| *A. sativum* | |
| NAM | Namhae-gun, Gyeongnam Prov., Korea |
| SEO | Seosan-gun, Chungnam Prov., Korea |
| SEC | Seocheon, Chungnam Prov., Korea |

### ISSR Analysis

Eleven arbitrarily chosen primers of Bioneer Technologies (Korea) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses (Table 2).

Amplification reactions were performed in 0.6 ml tubes containing 2.5 $\mu$l of the reaction buffer, 10 mM Tris-HCl (pH 8.8), 1.25 mM each of dATP, dCTP, dGTP, dTTP, 5.0 pM primer, 2.5 units Taq DNA polymerase, and 25 ng of genomic DNA. A 100 bp ladder DNA marker (Pharmacia) was used in the end of for the estimation of fragment size. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light using Alpha Image TM (Alpha Innotech Co., USA).

### Statistical Analyses

All ISSR bands were scored by eye and only unambiguously scored bands were used in the analyses. Because ISSRs are dominant markers, they were assumed that each band corresponded to a single character with two alleles, presence (1) and absence (0) of the band, respectively.

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh et al.[25]: the percentage of polymorphic loci ($P_p$), mean numbers of alleles per locus ($A$), effective number of alleles per locus ($A_e$), Nei's genetic diversity ($H$) [20], and Shannon's information index ($I$)[16].

To elucidate the organization of variability within *A. sativum* and *A. victorialis*, we examined the genetic variation by the differentiation among species and the number of migrants per generation ($Nm$) using the Nei's genetic diversity statistics[19].

The degree of polymorphism was quantified using Shannon's index of phenotypic diversity[3]:

$H_O = - p_i \log p_i$

where $p_i$ is the frequency of a particular phenotype $i$. $H_O$ can be calculated and compared for different populations[22]. Let

$H_{POP} = 1/n \; H_O$

be the average diversity over the different species and let

$H_{SP} = - p \log p$

be the diversity calculated from the phenotypic frequencies $p$ in all populations considered together. Then the proportion of diversity present within populations, $H_{POP}/H_{SP}$,

can compared with that of between species ($G_{ST}$), ($H_{SP}$ - $H_{POP}$)/$H_{SP}$.

The estimation of genetic similarity (GS) between genotypes was based on the probability that an amplified fragment from one individual will also be present in another[20]. GS was converted to genetic distance (1-GS).

A phylogenetic tree was constructed by the neighbor-joining (NJ) method[24] using the NEIGHBOR program in PHYLIP version 3.57[7].

## Results

From the 11 decamer primers used for a preliminary ISSR analysis, nine primers of them produced good amplification products both in quality and variability, while the remaining two (ISSR-03; -$(CA)_8$G- and ISSR-09; -GCGA $(AC)_8$-) none amplification (Table 2). Overall, 64 fragments were generated among the tested garlic array. The fragments ranged from 5-9 per primer.

In a simple measure of intraspecies variability by the percentage of polymorphic bands, the *A. victorialis* exhibited more variation (67.2%) than *A. sativum* (51.0%) (Table 3). Mean number of alleles per locus (*A*) ranged from 1.641 to 1.703 with a mean of 1.672 for the wild species and 1.510 for the cultivated species. The effective number of alleles per locus (*Ae*) was 1.430 for wild species and 1.343 for cultivated species.

The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*H*) within populations. Although the typical populations of *A. victorialis* were small, isolated, and patchily distributed for natural populations, they maintained a high level of genetic diversity for nine polymorphic primers. The mean *H* was 0.250 across species, varying from 0.240 to 0.268. In *A. sativum*, *H* was 0.198. Shannon's information index of phenotypic diversity (*I*) of *A. victorialis* (0.372) was higher than that of *A. sativum* (0.292). The both groups showed significant difference for all measures of genetic variability (paired *t* test).

On a per locus basis, the proportion of total genetic variation due to differences among species ranged from 0.074 for ISSR-04 to 0.159 for ISSR-05 with a mean of 0.106, indicating that about 89.4% of the total variation was within species.

An assessment of the proportion of diversity present within species, $H_{POP}/H_{SP}$, indicated that about 9.5% the total genetic diversity was between species. Thus, the majority of genetic variation (90.5%) resided within species (Table 4). The estimated *Nm* was slightly high among populations of two species (mean *Nm* = 4.214).

A similarity matrix based on the proportion of shared fragments (GS) was used to evaluate relatedness among species. The estimate of GS ranged from 0.940 to 0.980 (Table 5).

Table 2. List of decamer oligonucleotide utilized as ISSR primers, their sequences, and associated polymorphic fragments amplified in the *A. sativum* and *A. victorialis*

| No. of Primer | Sequence(5′ to 3′) | No. of fragments detected |
|---|---|---|
| ISSR-01 | -AGAGAGAGAGAGAGAGG- | 9 |
| ISSR-02 | -$(CT)_8$G- | 6 |
| ISSR-03 | -$(CA)_8$G- | 0 |
| ISSR-04 | -$(TC)_8$RA- | 5 |
| ISSR-05 | -GGAGAGGAGAGGAGA- | 8 |
| ISSR-06 | -$(GA)_8$GT- | 6 |
| ISSR-07 | -$(GA)_8$CG- | 7 |
| ISSR-08 | -$(GA)_8$TC- | 9 |
| ISSR-09 | -GCGA$(AC)_8$- | 0 |
| ISSR-10 | -GCGA$(CA)_8$- | 8 |
| ISSR-11 | -CCGG$(AC)_8$- | 6 |

Table 3. Measures of genetic variability for ISSRs generated among garlic populations

| Species | NP | $P_P$ | A | Ae | H | I |
|---|---|---|---|---|---|---|
| *A. victorialis* | | | | | | |
| ULL | 45 | 70.3 | 1.703 | 1.469 | 0.268 | 0.395 |
| SOB | 41 | 64.1 | 1.641 | 1.417 | 0.243 | 0.360 |
| GIR | 43 | 67.2 | 1.672 | 1.404 | 0.240 | 0.360 |
| Mean | 43.0 | 67.2 | 1.672 | 1.430 | 0.250 | 0.372 |
| *A. sativum* | | | | | | |
| NAN | 31 | 48.4 | 1.484 | 1.309 | 0.181 | 0.270 |
| SEO | 34 | 53.1 | 1.531 | 1.369 | 0.211 | 0.309 |
| SEC | 33 | 51.6 | 1.516 | 1.352 | 0.201 | 0.296 |
| Mean | 32.7 | 51.0 | 1.510 | 1.343 | 0.198 | 0.292 |
| t-test | 7.112** | 7.142** | 7.142** | 3.245* | 4.209* | 4.891** |
| Total | 49 | 76.6 | 1.766 | 1.419 | 0.246 | 0.371 |

Percentage of polymorphism (Pp), mean number of alleles per locus (A), effective number of alleles per locus (Ae), Nei's genetic diversity (H), and Shannon's information index (I). * = $p < 0.05$ ** = $p < 0.005$.

Table 4. Partitioning of the genetic diversity into within and between garlic species using ISSR markers

| | $H_{pop}$ | $H_{sp}$ | $H_{pop}/H_{sp}$ | $(H_{sp}-H_{pop})/H_{sp}$ |
|---|---|---|---|---|
| *A. victorialis* | 1.899 | 2.100 | 0.937 | 0.063 |
| *A. sativum* | 1.762 | 2.100 | 0.855 | 0.145 |
| Total | 1.831 | 2.100 | 0.905 | 0.095 |

Table 5. Similarity matrix (above diagonal) of nine populations based on RAPD and genetic distances (below diagonal)

| Pop | ULL | SOB | GIR | NAM | SEO | SEC |
|---|---|---|---|---|---|---|
| ULL | - | 0.9566 | 0.9435 | 0.9520 | 0.9522 | 0.9529 |
| SOB | 0.0444 | - | 0.9623 | 0.9535 | 0.9698 | 0.9610 |
| GIR | 0.0582 | 0.0384 | - | 0.9404 | 0.9667 | 0.9589 |
| NAM | 0.0492 | 0.0476 | 0.0615 | - | 0.9679 | 0.9706 |
| SEO | 0.0490 | 0.0307 | 0.0339 | 0.0326 | - | 0.9798 |
| SEC | 0.0483 | 0.0398 | 0.0420 | 0.0298 | 0.0204 | - |

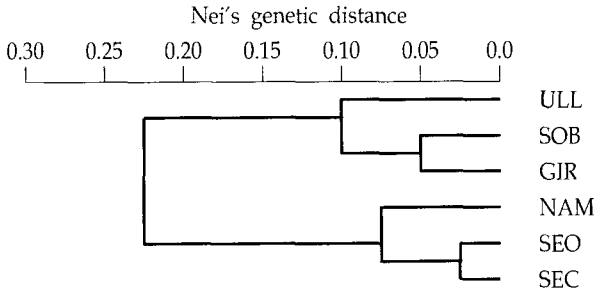


Fig. 1. A phylogenetic tree for garlic based on ISSR analysis. Codes are same as in Table 1.

Clustering of populations, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 1). The phylogenic tree showed two distinct groups Korean species, *A. sativum* and *A. victorialis* and six populations of both species were well separated each other. In three wild populations, geographically closed populations and species were situated in close positions in the dendrogram.

## Discussion

In ISSR analysis, two garlic species, *A. victorialis* and *A. sativum*, maintain a moderate or higher than average level of genetic diversity compared with other plant species, although there is the difference in methodology (e.g., dominant marker and co-dominant marker) that may preclude meaningful comparisons. For example, their values of genetic diversity (0.250 for *A. victorialis* and 0.198 for *A. sativum*) are higher than that for temperate-zone species (0.146), dicots (0.136), species with a sexual reproduction mode (0.151), and those with a perennial herbaceous (0.098)[10]. The percentage of polymorphic loci at the species level for *A. victorialis* is 67.2%, which is also higher than that for species with temperature-zone distributions (48.5%), dicots (44.8%), species with a sexual reproduction mode (51.6%), perennial herbaceous (39.6%), and temperate-zone species (48.5%).

Two alleles including ISSR-05-03 and ISSR-10-04 loci were found in only wild species, whereas none allele was specific to cultivated species. At multilocus analysis, the allelic composition of the cultivated species was a subset of that for the wild species. For all Korean garlic where number of alleles per polymorphic loci was calculated, wild species had more alleles than cultivated species. Korean garlic have been saved from artificial distribution. Periodical collecting have often been moved from mountains to nearby farmhouse for the purpose of medicine during the past several decades of years. Thus, domestication process via artificial selection have eroded the levels of genetic diversity in cultivated garlic. The result in this study is in general accord with the concept that most crops show a reduction on levels of polymorphism as compared with their presumed progenitor[5]. Although there is not direct evidence that *A. victorialis* is an ancestor of Korean *A. sativum*, there is a possibility that cultivated *A. sativum* in Korea has evolved from wild *A. victorialis* in Korea. Thus the hypothesis of the artificial selection speciation from wild populations of neighbor species needs to be tested by future work.

A striking feature of this study is the lacking of intra-populations variation. Only 9.5% variation was found between species and about 95.5% within populations. In particular, the species in Korea are less differentiated than the other insect-pollinated outcrossing or selfing species[4]. The mean ISSR $G_{ST}$ was 0.095 (Table 4). The mean value for outcrossing species was 15.5% and the mean for inbreeding species was 59.6%[4]. The value for *A. sativum* is similar with data summarized from AFLP for species with a mixed mating system[14].

Typical populations of *A. victorialis* are small and distributed in patches. Until a recent date, much of the Korean forest has been disturbed by the cutting of trees and herbs for medicine in rural areas[12]. The main concern of persistence of *A. victorialis* is continued habitat destruction and fragmentation. Consequently, wild *A. victorialis* populations have suffered loss individuals, loss and reduction of populations and fragmentation of remaining populations by human activities such as over-gathering medicinal plants. Thus Insights into the relative gene diversity among and within wild populations of *A. victorialis* would be useful in plant breeding and also for the development of strategies for *ex situ* conservation of plant genetic resources. Information on genetic variation and pop-

ulation structure is critical to the conservation of threatened taxa[1]. Genetic analyses can provide valuable insights into the process influencing extinction, while genetic data are used to define units for conservation management and for inferring changes in population structure and dynamics[18,21]. The mean 6.3% genetic differentiation coefficient of *A. victorialis* from ISSR suggests that those species are of a higher genetic diversity among populations than other endemic species[8,9]. However, the damage to their habitats is a main reason they are so rare. Thus, the best strategy to protect is *ex situ* conservation for endemic species.

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## 초록 : 산마늘의 지역적 변이와 종다양성 연구

허만규* · 성정숙[1] · 최주수 · 정영기[2] · 류은주[3] · 정경태[4]
(동의대학교 분자생물학과, [1]농촌진흥청 인삼 약초과, [2]동아대학교 응용생물공학부, [3]한서대학교 미용학과, [4]동의대학교 응용생명과학과)

마늘은 전 세계적으로 분포하는 다년생 초본이다. 마늘은 약리적, 경제적으로 중요한 작물이다. 야생종과 재배종의 유전관계를 ISSR 마커로 조사하였다. 또 ISSR 분석으로 이들 종의 유전적 다양도와 집단구조를 실시하였다. 한국의 세 야생 집단은 분리되어 있고 패치 분포를 보이지만 재배종에 비해 높은 유전적 다양성을 유지하고 있었다. ISSR 마커로 야생종과 재배종의 계통관계는 잘 분리되었다. 비록 한국내 재배종 마늘이 산마늘에서 진화하였다는 직접적 증거는 없지만 본 연구 결과 그런 가능성은 시사된다. 또한 야생종 산마늘 집단은 생식질 동정과 재배종 마늘의 진화과정에서 유익하게 쓰일 수 있다.