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Classification of genetic variation in garlic (*Allium sativum* L.) using SSR markers

Man Hyun Jo[1], In Ki Ham[1], Kyaw Thu Moe[2], Soon-Wook Kwon[2,3], Fu-Hao Lu[2], Yong-Jin Park[2,3], Woon Seob Kim[1], Mi Kyoung Won[1], Tae Il Kim[1], Eun Mo Lee[1]

[1]Chungcheongnam-do Agricultural Research & Extension Services, Yesan 340-861, Republic of Korea
[2]Department of Plant Resources, College of Industrial Science, Kongju National University, Yesan, 340-702, Republic of Korea
[3]Legume Bio-Resource Center of Green Manure (LBRCGM), Kongju National University, Yesan 340-702, Republic of Korea

***Corresponding author: manhyunjo@korea.kr**

Abstract

Garlic bulb is an important seasoning ingredient in many of the world's cuisines. However, clonal lineages within this species show a remarkably high degree of phenotypic diversity in bulb size and color. Present study classified the genetic variations of garlic. Seven selected simple sequence repeats (SSRs) revealed a total of 37 alleles across 120 garlic accessions, with an average of seven alleles per locus. The values for observed heterozygosity ranged from 0 to 0.99 (mean = 0.71). The average genetic diversity and polymorphic information content values were 0.586 and 0.518, respectively. Based on the 37 alleles obtained from the seven SSRs, a phylogram was constructed to understand the relationships among the 120 accessions. The garlic accessions were clustered into four main groups (G1–G4) in the phylogram. Group 1 consisted of accessions of 'Aomori', Group 2 consisted of 64 accessions, Group 3 consisted of 25 accessions, and Group 4 consisted of 20 accessions. Our results indicate that genetic diversity is correlated with geographical region. There may have been local selection pressure and differences in adaptability of the garlic to different geographical conditions. All of the tested loci deviated significantly ($P < 0.01$) from Hardy-Weinberg equilibrium. Thus, a number of disturbances occurred in the garlic population tested, including natural selection. Our findings will help explain the genetic relationships and population structure of garlic accessions.

Keywords: germplasm; genetic diversity; Hardy-Weinberg equilibrium; population structure.
Abbreviations: Simple sequence repeat (SSR), before the birth of Christ (BC), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphism (RFLP), genetic diversity (GD), polymorphic information content (PIC), polymerase chain reaction (PCR), Dimethyl sulfoxide (DMSO), Chuncheongnam-do Agricultural Research and Extension Services (CNARES), unweighted pair group method with arithmetic mean (UPGMA), Markov chain Monte Carlo (MCMC), log probability of data (LnP[D]).

Introduction

Garlic (*Allium sativum* L.) belongs to the family *Liliaceae* and genus *Allium*, which contains more than 600 species (Osman et al., 2007). Yamaguchi, (1983) pointed out that garlic was grown and consumed between 2780 and 2100 BC, during the building of the pyramids in Egypt. However, Vavilov, (1951) and Hong and Etoh, (1996) proposed that garlic, *Allium sativum* L. (Alliaceae), originated in central Asia. Garlic has been cultivated and consumed worldwide since ancient Egyptian period. Garlic bulb is an important seasoning ingredient in many of the world's cuisines, and the green flower stalks and young leaves are eaten fresh or cooked. Furthermore, large quantities of garlic are used for pharmaceutical purposes (Kik and Gebhardt, 2001), and garlic extract has been used as a traditional medicine for the prevention and treatment of cardiovascular disease (Ackermann et al., 2001). Garlic is a diploid obligate apomict that is primarily propagated asexually from cloves (Bradley et al., 1996). Clonal selection is the main breeding method for modern garlic, since plant sterility usually precludes crop improvement through cross-hybridization (Lampasona et al., 2003). For centuries, garlic has been propagated clonally, which may have resulted in a genetic bottleneck. However, clonal lineages within this species show a remarkably high degree of phenotypic diversity in bulb size and color, leaf length, growth habits, and agronomic traits such as stress and drought tolerance (Pooler and Simon, 1993; Bradley et al., 1996; Panthee et al., 2006; Stavělíková, 2008). As reported by Bozzini (1991), common garlic cultivars have a somatic chromosome number of 2n = 16 (with a karyotypic formula of six metacentric chromosomes, four submetacentric chromosomes, and six acrocentric chromosomes), although some garlic plants found in the Campania region of Italy were shown to be tetraploid (4n = 32), although some cultivars might be triploid. Chromosomal aberrations are common in garlic, due to multiple translocations that can involve eight or even ten

chromosomes. Some sterile cultivars have a normal karyotype (Sanai and Davis, 1967). Therefore, in garlic breeding programs, genetic variation can be increased only by somaclonal variation, mutagenesis, or genetic transformation (Novak, 1990; Burba, 1993; Kondo et al., 2000). Characterization of the garlic germplasm has largely been based on phenotypic characteristics; however, morphological characteristics can vary under different agroclimatic conditions. This situation complicates the characterization of garlic clones (Bradley et al., 1996; Al-Zahim et al., 1997). In recent years, molecular markers such as isozymes (Pooler and Simon, 1993; Lallemand et al., 1997; Ipek et al., 2003), random amplified polymorphic DNA (RAPD) (Maass and Klaas, 1995; Bradley et al., 1996; Ipek et al., 2003; Mario et al., 2008), and amplified fragment length polymorphisms (AFLP) (Lee et al., 2002; Lampasona et al., 2003; Volk et al., 2004; Ipek et al., 2003, 2005, 2008a) have been used to assess genetic diversity (GD) and the relationships among garlic clones, since they are not affected by environmental conditions. Simple sequence repeats (SSRs), or microsatellites, have been recognized as useful molecular markers in marker-assisted selection, the analysis of GD, and population genetic analysis in various species (Li et al., 2003; Agrama et al., 2007; Ma et al., 2009; Moe et al., 2010). SSRs are a small array of tandemly arranged bases (one to six) dispersed throughout the genome. SSRs as DNA markers have advantages over many other markers as they are highly abundant and polymorphic, inherited co-dominantly, analytically simple, and readily transferable (Weber, 1990). SSRs are reported to be more variable than restriction fragment length polymorphism (RFLP) or RAPD, and they have been widely utilized in plant genomic studies (He et al., 2003). Therefore, these markers would be useful for assessing genetic variation among asexually propagated garlic clones. The objective of this study was to understand the genetic variation among 120 garlic accessions from the Taean Lily Experiment Station of Chuncheongnam-do Agricultural Research and Extension Services (CNARES), Republic of Korea (Supplementary table 1). Present result will help explain the genetic relationships and population structure of garlic, which will in turn be useful for marker-assisted selection in garlic breeding.

Results

SSR polymorphisms

The SSR polymorphisms were measured in terms of the numbers of alleles, GD, and polymorphic information content (PIC) using PowerMarker 3.23 (Liu and Muse, 2005). Seven SSR markers revealed 37 alleles across the 120 accessions, with an average of 5.285 alleles per locus (Table 1). The allele size ranged from 171 (GB-ASM-080) to 410 (GB-ASM-072) bp. The allelic richness per locus varied widely among the markers, ranging from four (GB-ASM-078) to eight (GB-ASM-040) alleles. A total of four specific alleles were identified by three polymorphic markers (by GB-ASM-053 in Seosan-2-51, by GB-ASM-080 in Seosan-3-43, and by GB-ASM-072 in Geumsan-8 and Seosan-1-34). Thus, rare alleles (frequency ≤ 0.05) comprised 45.9% of all of the alleles. This result indicates that most alleles among the accessions studied exhibited a low frequency (Fig. 1 and Table 1). The values for H_O ranged from 0 in GB-ASM-109 to 0.991 in GB-ASM-035, with an average of 0.716. All loci deviated from HWE at the significance threshold ($P < 0.01$) (Table 1). The GD and PIC values ranged from 0.509 (GB-ASM-053) to 0.735 (GB-ASM-040) and from 0.441 (GB-ASM-080) to 0.696 (GB-ASM-040), with an average of 0.586 and 0.518, respectively. The major allele frequency per locus varied from 0.364 (GB-ASM-040) to 0.648 (GB-ASM-053), with an average of 0.530.

Diversity analysis

A GD-based analysis was performed by calculating the shared allele frequencies among the 120 accessions, and an unrooted phylogram (Fig. 2) was computed using PowerMarker 3.23 and Mega 4 (Tamura et al., 2007). Colors were applied according to our model-based cluster analysis results. In the phylogram, all of the accessions were clustered into four main groups (G1–G4). Group 1 consisted of eight accessions of 'Aomori'. Group 2 consisted of 64 accessions (Green in Fig. 2; nine cultivars, including seven accessions of 'Geumsan', seven accessions of 'Namdo', eight accessions of 'Nonsan', eight accessions of 'Seosan-1', seven accessions of 'Seosan-2', six accessions of 'Seosan-3', seven accessions of 'Taean', seven accessions of 'Taean-1', and seven accessions of 'Taean-2'). Group 3 consisted of 25 accessions (Blue in Fig. 2; nine cultivars, including three accessions of 'Geumsan', three accessions of 'Namdo', two accessions of 'Nonsan', two accessions of 'Seosan-1', three accessions of 'Seosan-2', three accessions of 'Seosan-3', three accessions of 'Taean', three accessions of 'Taean-1', and three accessions of 'Taean-2'). Group 4 consisted of 20 accessions (Yellow in Fig. 2; two cultivars, including 10 accessions of 'Daeseo' and 10 accessions of 'Cheonwoon'). In other words, G1 consisted of eight accessions from Japan, G2 and G3 together consisted of 89 accessions from Korea and China, and G4 consisted of 10 accessions of 'Daeseo' from Spain and 10 accessions of 'Cheonwoon' obtained by crossing fertile garlic in Kazakhstan.

Population structure analysis

The model-based clustering method was applied to all 120 garlic accessions and seven SSR markers. However, inferring the exact value of K (gene pool) was not straightforward because the estimated LnP(D) values increased until K = 6, although an abrupt change in LnP(D) occurred between K = 2 and K = 3; after K = 3, the increases in LnP(D) diminished (Fig. 3A). The ad hoc quantity ΔK (Evanno et al. 2005) was also used to determine the real K value. The largest value of ΔK for the 120 accessions was found at K = 4 (Fig. 3B). An analysis of these data identified the major substructure groups when the number of clusters was set at four with a high value of ΔK and a high probability of accessions assigned to one specific cluster (Maccaferri et al. 2005). The relatively small value of the alpha parameter ($\alpha = 0.029$) indicated that most accessions originated from a single primary ancestor, with a few admixed individuals (Ostrowski et al. 2006). At K = 4, all 120 accessions were divided into four discrete clusters (by their inferred genome fraction value [> 75%]; Fig. 4). Three accessions showed evidence of mixed ancestry. Cluster 1 consisted of eight accessions ('Aomori'), whereas cluster 2 consisted of 64 accessions (including seven of 'Geumsan', seven of 'Namdo', eight of 'Nonsan', eight of 'Seosan-1', seven of 'Seosan-2', six of 'Seosan-3', seven of 'Taean', seven of 'Taean-1', and seven of 'Taean-2'). Cluster 3 consisted of 25 accessions (including three of 'Geumsan', three of 'Namdo', two of 'Nonsan', two of 'Seosan-1', three of 'Seosan-2', three of 'Seosan-3', three of 'Taean', three of 'Taean-1', and three of 'Taean-2'). Cluster 4 consisted of 20 accessions (including 10 of 'Daeseo' and 10 of 'Cheonwoon'). Two accessions of 'Aomori' and one accession of 'Seosan-3' were identified as admixtures (red type in Fig. 2 and Supplementary Table 1).

Table 1. Total number of alleles and genetic diversity index for seven simple sequence repeat (SSR) loci in the 120 garlic accessions.

Locus name	GeneBank Accession no.	Size Range	specific alleles[a]	rare alleles[b]	N_A[c]	M_{AF}[d]	Ho[e]	GD[f]	PIC[g]
GB-ASM-035*	EU909132	286-313	0	1	4	0.495	0.991	0.574	0.484
GB-ASM-040*	EU909133	290-310	0	4	8	0.364	0.830	0.735	0.696
GB-ASM-053*	EU909134	228-302	1	2	5	0.648	0.567	0.509	0.450
GB-ASM-072*	EU909136	237-410	2	5	7	0.457	0.990	0.625	0.553
GB-ASM-078*	EU909137	206-232	0	2	4	0.538	0.866	0.565	0.480
GB-ASM-080*	EU909138	171-223	1	3	5	0.612	0.765	0.520	0.441
GB-ASM-109*	EU909139	216-223	0	0	4	0.596	0	0.574	0.522
Total			4	17	37				
Mean					5.285	0.530	0.716	0.586	0.518

[a] Allele found only in one accession, , [b] Number of alleles that <5 allele frequency, [c] Number of alleles, [d] Major allele frequency, [e]Observed Heterozygosity, [f] Gene diversity, [g] Polymorphic information content. * Locus which deviate from Hardy-Weinberg Equilibrium.

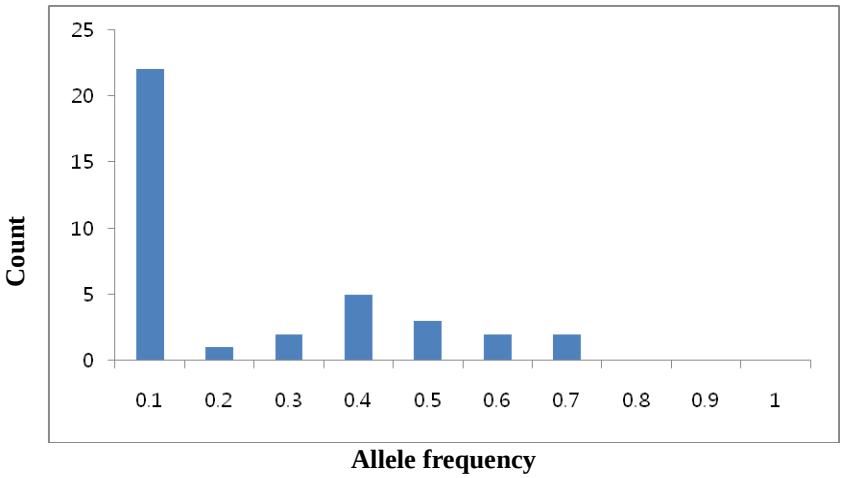


Fig 1. Histogram of allele frequencies for all 37 alleles in the 120 garlic accessions.

Discussion

Traditional methods for evaluating garlic diversity rely on resolving differences in morphological characters. However, the information provided by this approach is limited since the expression of such characters may differ under varying environmental conditions. Because of phenotypic flexibility and the occurrence of mutations, the identification and systematic classification of garlic is difficult. Correlation analysis revealed that allelic richness was significantly and positively associated with GD ($r = 0.78$, $P < 0.01$) and PIC ($r = 0.79$, $P < 0.01$) (data not shown). Allele size range was insignificantly associated with the number of alleles per locus. Our results are in contrast to those of Ma et al. (2009), who demonstrated that SSR loci with a large allele range showed great variation. Ipek et al. (2005, 2008b) reported the presence of more than two alleles per gene in the diploid garlic genome, and they suggested that duplication may be common in the large garlic genome. During SSR genotyping, we also found more than two alleles per SSR locus in some garlic accessions.

Seven SSRs revealed considerable GD among 120 garlic accessions of diverse origins, with GD ranging from 0.509 to 0.735, with an average value of 0.586. The high level of genetic variation observed in this study is consistent with results from previous studies of garlic carried out using different molecular markers (Bradley et al., 1996; Ipek et al., 2003, 2005, 2008a; Lampasona et al., 2003; Volk et al., 2004), thereby confirming the great diversity among garlic accessions. Menezes-Sobrinho et al. (1999) characterized a germplasm comprising 89 garlic accessions from Brazil and found 13 clusters. Lallemand et al. (1997) evaluated 65 garlic accessions, and found six clusters on the basis of morphological characters, which were verified by isozyme analysis. Interestingly, although they had collected the germplasm from 25 different countries, they came up with only six groups. In our study, although the garlic accessions were collected from five different countries, we identified four distinct groups. Only two accessions from Japan (‘Aomori’) and one accession from Korea (‘Seosan-3’) (the admixtures) suggested close ancestry. According to the UPGMA dendrogram inferred from the shared allele frequencies and our model-based cluster analysis results for the 120 garlic accessions, Group 1 consisted of eight accessions of ‘Aomori’ introduced from Japan and one accession of ‘Seosan-3’. Group 2 consisted of 64 accessions, mainly from South Korea, and one accession from China. Group 3 consisted of 22 accessions from South Korea and three from China (except for two accessions of ‘Aomori’), while Group 4 consisted of 10 accessions each from Kazakhstan and Spain (Fig. 2 and Fig 4). Moreover, Chungcheongnam-do Province in Korea includes Geumsan, Nonsan, Seosan, and Taean. Our results indicate that GD in garlic is correlated with geographic distribution. Local selection pressure and differences in the adaptability of garlic to diverse geographical conditions may explain these results.

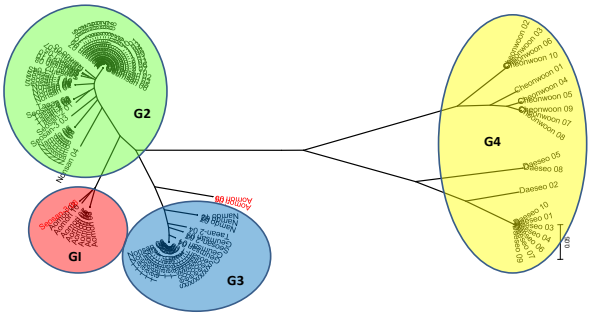
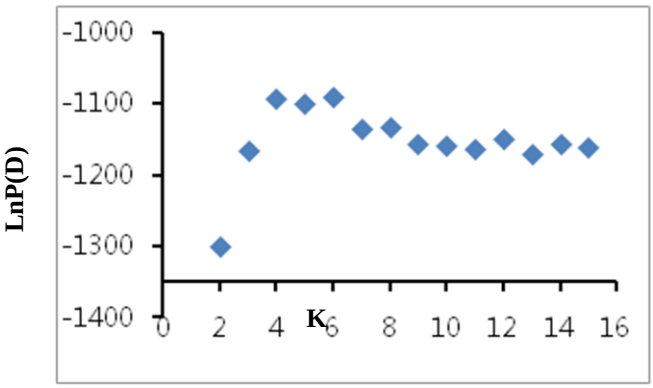
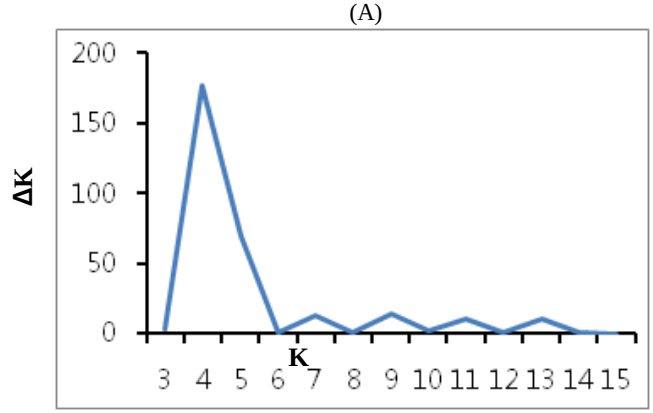


Fig 2. UPGMA dendrogram based on a genetic distance matrix among 120 garlic accessions from Korea, China, Japan, Spain, and Kazakhstan. The colors of the branches correspond to those of the model-based populations (POP1–POP4). Three admixed accessions are indicated in red type.



(A)



(B)

Fig 3. (A) Log-likelihood of the data (n = 120), L (K), as a function of K (number of groups used to stratify the sample). (B) Values of ΔK, with its modal value used to detect true K of the group (K = 4) For each K value, at least three independent runs were considered and averaged over the replicates.

On the other hand, Ipek et al. (2003, 2008a), Volk et al. (2004), and Mario et al. (2008) found no relationship between GD and site of collection. However, our results are in accordance with those of Hwang (1993) from a morphology-based analysis. Cultivars 'Seosan-1', 'Seosan-2', and 'Seosan-3' were grown more than 30 years ago by a farmer in Seosan, while 'Taean-1' and 'Taean-2' were cultivated more than 80 years ago in Taean County. However, 'Geumsan' was grown about 47 years ago by a farmer in the Geumsan area. 'Namdo' (introduced from Shanghai, China, in 1976) has been cultivated since 1983. 'Daeseo' (introduced from Spain in 1983) has been cultivated since 1986 (Lee, 1994; Song, 2001). 'Cheonwoon' (Kim, 2007), obtained by crossing fertile garlic, was introduced to Kazakhstan in 1998. The ecological distribution of garlic includes two types: Southern and Northern. Among the 12 cultivars we analyzed, 'Namdo' and 'Daeseo' were of the Southern type while the other 10 cultivars were of the Northern type. Our present results agree with those of Choi et al. (2003), who showed that the majority of 25 local cultivars in Korea had strong GD. All tested loci deviated from HWE and showed significant linkage disequilibrium. Thus, a number of disturbances occurred in the garlic population, including natural selection and limited population size or generational overlapping. Our data indicate that some accessions harbored specific alleles, which may lead to genetic improvement. The genetic relationships and detailed garlic population structure shown in this study will be useful for future garlic improvement programs.

Materials and methods

Plant materials and DNA extraction

A total of 120 accessions of 12 cultivars from five different countries, including the Republic of Korea (80), China (10), Japan (10), Kazakhstan (10), and Spain (10), were tested in this study. All plant materials were obtained from the Taean Lily Experiment Station of CNARES, Republic of Korea. A description of the garlic collection used in this study is given in Supplementary table 1. Total DNA was extracted from all accessions using an iNtRON DNA extraction kit (Daejeon, Republic of Korea). The relative purity and concentration of the extracted DNA was estimated using NanoDrop 2000 (www.nanodrop.com). The final DNA concentration was adjusted to 40 ng μL^{-1}.

SSR genotyping

A total of seven SSR markers were taken from a study by Ma et al. (2009). A three-primer system (Schuelke, 2000) was used that included a universal M13 oligonucleotide (TGTAAAACGACGGCCAGT) labeled with a fluorescent dye (6-FAM, VIC, NED, or PET), to allow the PCR products to be tetraplexed during electrophoresis. A special forward primer composed of the concatenation of the M13 oligonucleotide and specific forward primer was used with the normal reverse primer for SSR PCR amplification. PCR amplification was performed in a total volume of 20 µL containing 2 µL of 40 ng genomic DNA, 10× DiaStar Taq buffer, 10 mM each of dNTP mix and M13 forward primer, and 5 U Taq DNA polymerase, normal reverse primer, M13 fluorescent dye (FAM, VIC, NED, and PET), DMSO, and double-distilled water. The reaction conditions were as follows: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, the appropriate annealing temperature (60 or 61°C) for 45 s, and 72°C for 60 s, with a final extension at 72°C for 15 min. The products for the four microsatellites were mixed together at a ratio of FAM:VIC:NED:PET = 1:1:1:1, which was varied depending on the amplification intensity for individual markers as determined using an Applied Biosystems 3500 Genetic Analyzer (Hitachi, Tokyo, Japan). The PCR product mixture (1.5 µL) was combined with 8.25 µL of Hi-Di formamide, 0.25 µL of installation buffer, and 0.5 µL of an internal size standard. The samples were denatured at 95°C for 3 min, chilled for 3 min, and analyzed in an Applied Biosystems 3500 Genetic Analyzer. The molecular weights, in

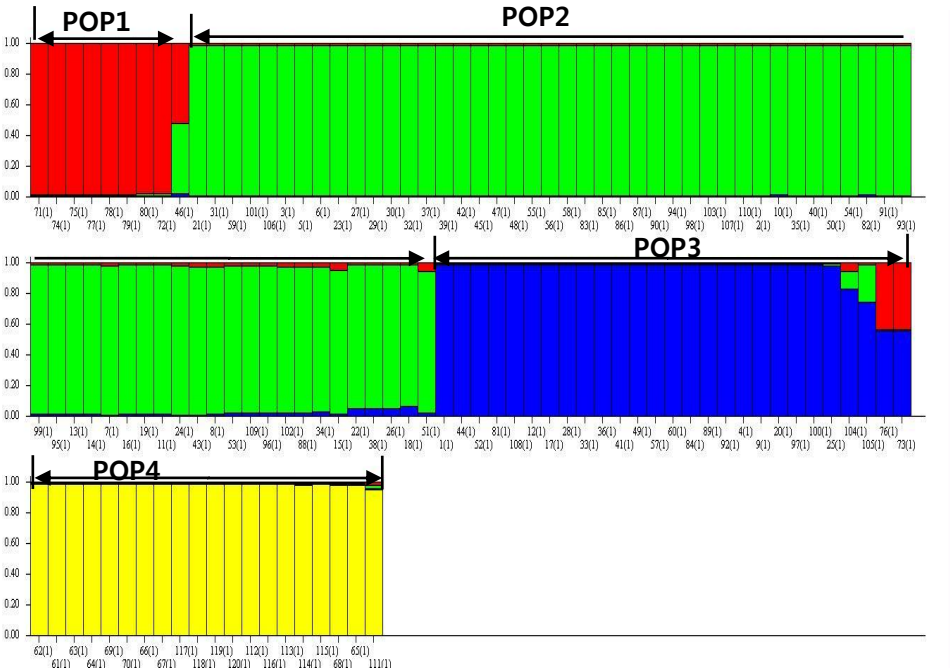


Fig 4. Model-based membership of 120 garlic accessions based on results of the Structure program. Colors represent model-based populations based on the four inferred clusters. (Red = Pop 1, Green = Pop 2, Blue = Pop 3, Yellow = Pop 4).

base pairs (bp), of the microsatellite products were estimated using GeneMapper ver. 4.1 (Applied Biosystems). The individual fragments were identified as alleles of the appropriate microsatellite loci with GeneMapper ver. 4.1 (Applied Biosystems).

Statistical analysis

Variability at each locus was measured in terms of the number of alleles, observed heterozygosity (H_O), major allele frequency (M_{AF}), GD, and polymorphic information content (PIC) using PowerMarker 3.25 (Liu and Muse, 2005). The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm was used to construct an unrooted phylogram from a distance matrix using MEGA4 software (Tamura et al., 2007) embedded in PowerMarker. The population structure was determined using the model-based software program Structure 2.3.3 (Pritchard et al., 2000; Falush et al., 2003). In this model, several populations (K) are assumed to be present, each of which is characterized by a set of allele frequencies for each locus. Individuals in the sample are assigned to populations (clusters) or jointly to more populations if their genotypes indicate that they are admixed. All loci are assumed to be independent, and each K is assumed to follow Hardy-Weinberg equilibrium (HWE). Posterior probabilities were estimated using the Markov chain Monte Carlo (MCMC) method. The MCMC chains were run for 100,000 burn-in period lengths, followed by 200,000 iterations using a model allowing for admixtures and correlated allele frequencies. At least five runs of Structure were performed with K values ranging from 2 to 15, and an average likelihood value, L (K), across all runs was calculated for each value of K. The model choice criterion to detect the most probable value of K was ΔK, which is an ad hoc quantity related to the second-order change in the log probability of data (LnP[D]) with respect to the number of clusters inferred by Structure (Evanno et al. 2005). An individual was assigned to a group if > 75% of its genome fraction value was derived from that group.

Conclusion

The ecological distribution of garlic includes two types: Southern and Northern. Among the 12 cultivars we analyzed, ‘Namdo’ and ‘Daeseo’ were of the Southern type while the other 10 cultivars were of the Northern type. Although the garlic accessions were collected from five different countries, we identified four distinct groups. Only two accessions from Japan (‘Aomori’) and one accession from Korea (‘Seosan-3’) (the admixtures) suggested close ancestry. A number of disturbances occurred in the garlic population, including natural selection and limited population size or generational overlapping.

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