Classification of Genetic Variation in Garlic (*Allium sativum* L.) Revealed by RAPD

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Abstract. RAPD analysis was employed to assess genetic variation in named cultivars of garlic (*Allium sativum* L.), and to examine the relationships between cultivated garlic varieties and the wild progenitor *Allium longicuspis*. Twenty-seven accessions were subjected to RAPD analysis using 26 oligonucleotide primers. Of a total of 292 bands, 63 (21%) were polymorphic. Cluster analysis revealed groupings that in part reflected patterns of morphological variation. All bolting forms (including wild and cultivated) grouped separately from the nonbolting cultivars. *A. longicuspis* and var. *ophioscorodon* grouped together, indicating close taxonomic affinity. Based upon relative levels of variation within different groups, we suggest potential relationships within the *A. sativum*/*A. longicuspis* complex.

Although garlic cultivars are sterile and propagated only vegetatively by cloves, they exhibit morphological variation between cultivars and can be classified into two botanical varieties: *ophioscorodon* and *sativum* (Helm, 1956). Variety *ophioscorodon* (bolting garlic; also known in agriculture as top setting or hard neck) is distinguished by the production of a tall flower stalk (scape) that is initially coiled but is later upright and carries a sterile umbel. The other variety *sativum* (known as soft neck), does not bolt under normal conditions (Engeland, 1991).

Vvedensky (1944) proposed that all domesticated garlic has evolved from the wild species *Allium longicuspis*, which was originally distributed from southern Turkmenia to Tien Shan and is now confined to central Asia. This wild species cannot be easily distinguished from the cultivated var. *ophioscorodon* (Engeland, 1991) except by exerted anthers (Jones and Mann, 1963), which are rarely observed in cultivated material.

While morphological differences between cultivars can be observed in the extent of bolting, the number, size, shape, and color of the cloves, and time of maturing, only a few morphological traits are used for grouping garlic and these can be affected by environmental conditions (Engeland, 1991; Hanelt, 1991). Therefore, classification of garlic has proved to be difficult and is still tentative. Some success has been reported in correlating isozyme alleles with morphological traits (Pooler and Simon, 1993; Siqueira et al., 1985), whereas Maass and Klaas (1995) applied both isozyme and RAPD analysis to the study of the origin of garlic with respect to the geographi-

cal distribution and genetic variation of unclassified germplasm.

In our study, 27 garlic accessions were subjected to RAPD analysis. Most of these accessions, initially collected from different parts of the world, had been grown for several years and tentatively classified while growing under the same environmental conditions in north central Washington State, U.S.A. (Engeland, 1991), and some of the accessions are designated with PI numbers. The cultivars used were carefully selected so that they represented the major classification groups as determined by Engeland (1991, 1992). Our objective was to determine whether such groupings could be observed when an assessment of molecular variation was made using information from RAPD markers, and to provide a new classification framework from named, recognizable cultivars, on which further studies of this cultigen could be based.

The 27 garlic cultivars selected for this study (Table 1) represent the three major groups of garlic, namely *A. sativum* var. *sativum* (11 named cultivars), var. *ophioscorodon* (11 named cultivars) and *A. longicuspis* (five named selections from material collected wild) (after Engeland, 1992). The cultivated material used also represents other morphological groupings that have been described, namely Rocambole and Continental within both *A. longicuspis* and *A. sativum* var. *ophioscorodon*, and Artichoke and Silverskin within *A. sativum* var. *sativum* (Table 1).

A modification of a combination of two methods (Gawel and Jarret, 1991; Li et al., 1994) was used for DNA extraction. DNA was extracted from 0.2 g leaf tissue according to Gawel and Jarret (1991), and then precipitated using 1:20 sodium acetate : 95% ethanol. Two μ L of 10 mg·mL⁻¹ Dnase-free RNase A were added to the DNA resuspended in 200 μ L TE and incubated at 65 °C for 12 min. The DNA was loaded onto a column containing 0.7 mL Sephacryl S1000 (Li et al., 1994) and centri-

fuged for 2 min at 150 rpm. Three hundred μ L STE was added to the column, which was centrifuged again. Two hundred μ L of 20% PEG 800/1.2 M NaCl was added to the eluted DNA solution, which was incubated on ice for 20 min and centrifuged at 1300 rpm for 5 min. The DNA pellet was washed with 1 mL 95% ethanol and dissolved in 200 μ L TE. Concentrations of DNA in extracts were estimated by comparison of ethidium bromide-stained band intensities with λ DNA standards following electrophoresis of samples in 0.7% agarose gels.

For the 27 cultivars, initially 35 random decanucleotides produced either by Alta Bioscience (Birmingham Univ., U.K.) or Operon Technologies (Alameda, Calif.) were used as single primers to direct PCR amplification. Twenty-six of these primers (from the Operon G, J, M, N, and O kits) were used subsequently because they resulted in efficient PCR amplification and in repeatable banding patterns between at least two separate replicate DNA extractions, and showed polymorphism. The conditions reported by Williams et al. (1990) for producing RAPD markers were optimized for use with *A. sativum* DNA. The optimum reaction mix for 25 μ L comprised 12 ng template, 1 unit of Taq polymerase (Bioline, U.K.) and 1 $\times$ reaction buffer, 0.3 μ M of the primer, 200 μ M of each of dNTPs, and 1.75 mM MgCl₂. The reaction mixture was overlaid with 35 μ L of mineral oil. Amplification was achieved in a thermocycler (Hybaid Omnigene, U.K.) programmed as follows: an initial 3 min at 94 °C; 45 cycles of 1 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C; and a final extension at 72 °C for 5 min. Fifteen μ L of the reaction mix was then subjected to electrophoresis in a 1.5% (w/v) agarose gel, and the DNA bands were stained by 50 ppm ethidium bromide. A DNA ladder of 1 kb was used as molecular standard, and bands were visualized under UV light and photographed using an IS 500 video computer system (IS-500 Digital Imaging System, Flowgen, U.K.).

Only strong and clear reproducible (between replicates) polymorphic RAPD bands were scored as present (1) or absent (0) for each of the primer-cultivar combinations. Pairwise comparisons of the garlic cultivars, based on the presence or absence of unique and shared bands, were used to generate similarity coefficients (Simple Matching and Jaccard's), which were then utilized to generate a dendrogram by the UPGMA method (Rohlf, 1992).

Results

A general morphological comparison between the two botanical varieties of domesticated garlic *sativum* and *ophioscorodon* and their presumed ancestral wild species *longicuspis* is summarized (Table 2).

Twenty-six of the 35 primers resulted in clear, reproducible, and polymorphic banding profiles (Alta: 2, 6, 9, 12, 14; OPG 2, 11, 12, 20; OPJ 13; OPM 17; OPN 1, 3, 7, 8, 13, 14, 16, 17, 19; OPO 1, 4, 8, 9, 10, 18). Among the 292 bands produced, 63 (21%) were polymorphic

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Table 1. Garlic cultivars, botanical varieties, species, and their origins.

No.	Cultivar/wild selection name (PI number) (abbreviation)	Variety/species	Group ^a	Origin
1	L.K.Manns C751 (Mann)	<i>longicuspis</i>	Roc	USSR
2	Wild Rocambole (Wild)	<i>longicuspis</i>	Roc	Poland
3	Persian Star (Pers)	<i>longicuspis</i>	Con	Samarkand
4	Blazer (PI250662) (Blaz)	<i>longicuspis</i> ^b	Con	USSR
5	Marble Horn (PI493096) (Marb)	<i>longicuspis</i>	Con	Moldova
6	Spanish Roja (Span)	<i>ophioscorodon</i>	Roc	Greece
7	Russian Red (Russ)	<i>ophioscorodon</i>	Roc	USSR
8	Yugoslavian (Yugs)	<i>ophioscorodon</i>	Roc	unknown
9	French (Rocambole) (Fren)	<i>ophioscorodon</i>	Roc	France
10	Korean Rocambole (Kore)	<i>ophioscorodon</i>	Roc	South Korea
11	Purple Glazer (Purp)	<i>ophioscorodon</i>	Con	Rep. of Georgia
12	Burgundy (Burg)	<i>ophioscorodon</i>	Con	unknown
13	Lorz Italian (Lorz)	<i>sativum</i>	Art	Italy
14	California Early (Earl)	<i>sativum</i>	Art	California
15	Chinese sativum (Chin)	<i>sativum</i>	Art	China
16	Argentine (Arge)	<i>sativum</i>	Art	Argentina
17	Gomecari (Gome)	<i>sativum</i>	Art	Rep. of Georgia
18	Taia Pink (Taia)	<i>sativum</i>	Art	Rep. of Georgia
19	Nootka Rose (Noot)	<i>sativum</i>	Sil	Washington
20	Belgian Red (Belg)	<i>ophioscorodon</i>	Roc	unknown
21	Celaya Purple (Cela)	<i>ophioscorodon</i>	Roc	Mexico
22	Broadleaf (Bro)	<i>ophioscorodon</i>	Con	Czech
23	Polish Hardneck (Poli)	<i>ophioscorodon</i>	Con	Poland
24	Cicamora (Cica)	<i>sativum</i>	Art	Rep. of Georgia
25	Zeda (Zeda)	<i>sativum</i>	Art	Rep. of Georgia
26	(PI497947) (Poli)	<i>sativum</i>	Sil	Poland
27	St. Helen (Hele)	<i>sativum</i>	Sil	Washington

^aRoc = Rocambole, Con = Continental, Art = Artichoke, and Sil = Silverskin.

^bOriginally supplied as *A. longicuspis*, but subsequently found as *A. sativum* var. *ophioscorodon* within the USDA collection.

Table 2. Comparison of morphological traits of the three forms of garlic (after Engeland, 1992).

Trait	<i>Allium longicuspis</i>	<i>A. sativum</i> var. <i>ophioscorodon</i>	<i>A. sativum</i> var. <i>sativum</i>
Bolting/nonbolting	Bolting	Bolting	Nonbolting
Anthers	Exserted	Deformed	Nonexserted
Bulbs			
Color	Blotchy or striped purple	Purple/porcelain	White
Layers	1-2	2	4
Cloves			
Number	8	8	15
Color	Brown	Brown	White
Maturity	Late	Late	Early
Further subgroupings			
Scape	Rocambole: tightly coiled Continental: loose coil	Rocambole: tightly coiled Continental: loose coil	N/A
Bulb scales			Artichoke: coarse Silverskin: fine and smooth

with an average of 2.42 polymorphic bands per primer.

An association between polymorphic marker bands and morphological classification could be observed. For example, with primer OPG-20 a strong band at the 1150 bp position was present in 14 out of 16 bolting cultivars (nine var. *ophioscorodon* and all five *A. longicuspis*) and absent from all nonbolting (var. *sativum*) cultivars except no. 26.

The 63 polymorphic RAPD bands could be used to generate a dendrogram (Fig. 1) that grouped all 27 accessions in a manner generally resembling the classification of garlic based on morphology, and which was essentially the same when either Simple Matching or Jaccard's coefficients were used. Accessions were separated into two major clusters. The first cluster included bolting garlic of the variety *ophioscorodon* and the five wild ac-

cessions of species *longicuspis*. Although six Rocambole types within this group did cluster, generally there was no clear distinction between Rocambole, Continental, and wild *A. longicuspis* within this major cluster.

All cultivars in the second major cluster had been classified as nonbolting (variety *sativum*) except for 'Burgundy'. The origin of this cultivar is largely unknown, but it is the only cultivar previously classified as var. *ophioscorodon* to have a weak flower stalk and very small bulblets (Engeland, 1992). Therefore, this cultivar may be in variety *sativum*, as is indicated by the RAPD classification.

According to the clove color and shape, Engeland (1992) divided var. *sativum* into two groups: Artichoke and Silverskin. All eight Artichoke cultivars clustered at a relatively high level of similarity, indicating a close

relationship between these cultivars. In contrast the three cultivars identified as Silverskin did not form a single cluster and were substantially less similar to each other.

Discussion

RAPD markers have proved to be more useful in detecting variation between garlic cultivars than any other method. In a study carried out by Pooler and Simon (1993), four isozyme systems were employed to group garlic cultivars into 17 different electrophoretic phenotypes. Although some of those groups were associated with some of the morphological groups, none of the five accessions of *A. longicuspis* they examined fell into a unique isozyme group, and two of these accessions fell in the same group as nonbolting *sativum* cultivars. Other attempts to correlate specific isozyme patterns with particular morphological traits in garlic have had little success (Chomatova et al., 1990; Etoh and Nakamura, 1988). Siqueira et al. (1985) found that most of the 70 cultivars they examined fell into one of two isozyme groups. One group contained the medium maturity cultivars while the other contained early and late maturity cultivars. More recently Lallemand et al. (1995) correlated some isozyme patterns with the geographical origin of some cultivars.

In the most recent study conducted by Maass and Klaas (1995), 48 accessions of unclassified garlic germplasm were subjected to RAPD analysis. The 15 selected primers produced a total of 135 bands, and of these, 125 were polymorphic, giving more than 90% polymorphism. Such variation would seem to be extremely high even for a fertile plant species. When Wilkie et al. (1993) applied RAPD analysis to onion and to other *Allium* species, only 7 of the 20 primers used revealed polymorphism between cultivars of onion, but nearly all primers detected variation between species. Furthermore, in the Maass and Klaas (1995) study, none of the seven *ophioscorodon* accessions could be separated, whereas all *longicuspis* accessions grouped separately from *ophioscorodon*.

In comparison with these studies, and as the first conclusion, our work has demonstrated that morphological variation can be clearly related to the variation detected as RAPD polymorphism. By analyzing relationships and variation among named cultivars of garlic, all of which are either accessible commercially, or (with PI numbers) from the USDA collection, we now provide a cultivar and variety reference point for other researchers studying garlic germplasm.

As a second conclusion, cultivars of var. *ophioscorodon* closely resemble the germplasm of *A. longicuspis* morphologically, as well as in RAPD profiles, falling within the same major cluster as *A. longicuspis*. They are, therefore, indistinguishable morphologically and in terms of molecular profile from each other, and this raises the question as to whether var. *ophioscorodon* should be classified as belonging to *A. longicuspis*, or alternatively, whether *A. longicuspis* should be considered

Fig. 1. Dendrogram of 27 garlic accessions generated by similarity clustering of coefficient values (Jaccard's) computed from pairwise comparisons using 63 RAPD bands. For abbreviations see Table 1.

as con-specific with *A. sativum*. In contrast, the botanical variety *sativum* is quite distinct from the varieties *ophioscorodon* and *A. longicuspis*, both at the molecular and morphological levels, and needs to be recognized as a separate taxon.

The question still remains as to how much of the extensive morphological variation between cultivars was selected while *A. sativum* or its ancestors were still sexual, and how much has arisen after garlic became vegetatively propagated (Havey, 1995). Our results showing that *ophioscorodon* strains are closely related to *longicuspis* might suggest that some of the variation in these cultivated forms could have arisen through some residual sexual reproduction in this group. However, based upon our cluster analysis dendrogram, the *sativum* cultivars are at least fractionally more diverse than those of *ophioscorodon/longicuspis*

(grouping at a similarity of 0.55), indicating that considerable selection and/or genetic change during vegetative propagation must also have occurred.

Finally, there is sufficient measurable molecular DNA variation between garlic cultivars to enable varietal identification and fingerprinting to take place.

Literature Cited

- Chomatova S., V. Turkova, and E. Kozova. 1990. Protein complex and esterase isozyme patterns of *Allium sativum* L. cultivars and clone-regenerants. *Biol. Plant.* 32:321-331.
- Engeland, R.L. 1991. Growing great garlic. *Filaree Production*, Okanogan, Wash. p. 11-30.
- Engeland, R.L. 1992. *Filaree farm: Descriptions of named strains*. *Filaree Production*, Okanogan, Wash. p. 21-40.
- Etoh, T. and N. Nakamura. 1988. Comparison of the peroxidase isozymes between fertile and sterile

clones of garlic. *Proc. Fourth Eucarpia Allium Symp.*, 6-9 Dec. 1988, Wellesbourne, Warwickshire, U.K., p. 115-199.

Gawel, N.J. and R.L. Jarret. 1991. A modified DNA extraction procedure for *Musa* and *Ipomoea*. *Plant Mol. Biol. Rpt.* 9:262-266.

Hanelt, P. 1991. Research project on taxonomy of the genus *Allium*. *Allium Improvement Nwsltr.* 1:19-21.

Havey, M.J. 1995. Onions and other cultivated alliums, p. 344-350. In: J. Smartt and N.W. Simmonds (eds.). *Evolution of crop plants*. Longman, Harlow.

Helm, J. 1956. Die zu wurz—Und speisewecken kultivierten Arten der Gattung *Allium* L. *Die Kulturpflanze*. 4:30-80.

Jones, H.A. and L.K. Mann. 1963. *Onions and their allies*. Inter. Science, New York.

Lallemant, J., C.M. Messiaen, F. Briand, and T. Etoh. 1995. Delimitation of varietal groups in garlic (*Allium sativum* L.) by morphological and physiological characters, verified by the isozyme method, p. 15. In: *First Intl. Symp. Edible Alliaceae*. (Abstr.)

Li, Q., Q. Cai, and C.L. Guy. 1994. A DNA extraction method for RAPD analysis from plants rich in soluble polysaccharides. *Plant Mol. Biol. Rep.* 12(3):215-220.

Maass, H.I. and M. Klaas. 1995. Interspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. *Theor. Appl. Genet.* 91:89-97.

Pooler, M.R. and P.W. Simon. 1993. Characterization and classification of isozyme and morphological variation in a diverse collection of garlic clones. *Euphytica* 68:121-130.

Rohlf, F.J. 1992. *NTSYS-PC: numerical taxonomy and multivariate analysis system*. Exeter Software, New York.

Siqueira, W.J., H.P.M. Filho, R.S. Lisboa, and J.B. Fornasier. 1985. Morphological and electrophoretic characterization of garlic clones. *Bragantia* 44(1):357-374.

Vvedensky, A. 1944. The genus *Allium* in the USSR. *Herbertia* 11:65-218.

Wilkie, S.E., P.G. Isaac, and R.J. Slater. 1993. Random amplified polymorphic DNA (RAPD) markers for genetic analysis. *Theor. Appl. Genet.* 86:497-504.

Williams, G.K., A.R. Kubelik, K.L. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6535.