

# Genetic Diversity among U.S. Garlic Clones as Detected Using AFLP Methods

Gayle M. Volk<sup>1</sup>, Adam D. Henk, and Christopher M. Richards

National Center for Genetic Resources Preservation, U.S. Department of Agriculture, Fort Collins, CO 80521

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**ABSTRACT.** Garlic (*Allium sativum* L.) has been clonally propagated for thousands of years because it does not produce seed under standard cultivation conditions. A single garlic accession frequently displays a high degree of phenotypic plasticity that is likely to be dependent upon soil type, moisture, latitude, altitude, and cultural practices. The diversity observed by collectors has occasionally led to the renaming of varieties as they are exchanged among growers and gardeners. As a result, there are numerous garlic varieties available both commercially and within the USDA National Plant Germplasm System (NPGS) that may be identical genotypically, yet have unique cultivar names. To address this possibility, we performed amplified fragment-length polymorphism (AFLP) analysis on a comprehensive selection of 211 *Allium sativum* and *Allium longicuspis* accessions from NPGS and commercial sources. We used several statistical approaches to evaluate how these clonal lineages are genetically differentiated and how these patterns of differentiation correspond to recognized phenotypic classifications. These data suggest that while there are extensive duplications within the surveyed accessions, parsimony and distance based analyses reveal substantial diversity that is largely consistent with major phenotypic classes.

Garlic (*Allium sativum* L.) is a vegetatively propagated crop that does not set seed under standard growing conditions. Clonal lineages within this species show a remarkably high degree of phenotypic diversity. New genotypes have not been obtained through hybridization, but through the selection of spontaneous mutations expressing traits of horticultural interest. More than 200 named garlic clones are commercially available in the United States. The U.S. National Plant Germplasm System (NPGS) maintains 193 main accessions (with PI, Plant Introduction, and W6 prefixes) at the Western Regional Plant Introduction Station (WRPIS) in Pullman, Wash. The diversity of these clones is described by a set of phenotypic and morphological descriptors known to be phenotypic and morphological descriptors known to be plastic (Al-Zahim et al., 1997; Ipek et al., 2003). Plant and bulb characteristics are determined by growing season, winter conditions, location, as well as nutrient and water availability. Most likely, many of the commercial garlic cultivars have been renamed as they have been traded among growers and gardeners for hundreds of years.

Maintenance of these accessions requires a substantial effort since all clones must be planted in the field each fall and bulbs are harvested the following summer. The collection is at risk because pathogens may easily invade field plots and the shelf life of most accessions is less than a year. Methods are being developed to backup the NPGS garlic collection cryogenically. Cryopreservation is time-consuming, expensive, and often genotype dependent. However, when reliable methods are developed, long-term preservation can be attained (Cho et al., 2003; Keller, 2002; Makowska et al., 1999).

Garlic has been cultivated for millennia, but the taxonomic origins of this domestication process have not been identified. *Allium*

*longicuspis*, which can be found in the wild in Central Asia, was once thought to be the living progenitor of *A. sativum*. However, recent molecular evidence indicates that *A. longicuspis* lies within the range of genetic diversity found within *A. sativum* (Etoh and Simon, 2002). Many classification systems for garlic have been described (Etoh and Simon, 2002). Most systems describe subspecies that are either bolting types or non and incomplete bolting types and are termed *ophioscorodon* and *sativum*, respectively (Etoh and Simon, 2002). Garlic growers have further classified garlic varieties based on phenotypic characters as exhibited in Okanogan, Wash. (Engeland, 1991, 1995) (Table 1). Since most commercially available garlic cultivars in the United States have been classified according to this scheme, we used this system to associate morphological features with our genetic analyses. Table 1 includes a column to convert the classification system proposed by Engeland (1991, 1995) to the subgroup classification system used in Germany and described by Keller (2002), Hanelt (2001), and Maass and Klaas (1995).

*Allium sativum* ssp. *sativum* includes the softneck garlic cultivars that are either nonbolting or produce only very weak stalks. In general, most softneck types have 12 to 20 cloves arranged in three to six layers within a bulb. Artichoke-type softnecks tend to have early maturing cloves with coarse, thick clove skins and yellow-green, horizontal leaves that emerge from all sides of the plant. During stressful conditions, asexual propagules called bulbils may be produced within the false stems, resulting in the classification of incomplete bolting types. In contrast, silverskin-type softnecks have nearly vertical, blue-green leaves that display a bilateral symmetry. The tight clove skin on silverskins and late maturation dates enable these types to be stored for long periods of time. Softneck cultivars often grow best under mild winter conditions (Engeland, 1991, 1995).

*Allium sativum* ssp. *ophioscorodon* includes the hardneck garlic types that produce scapes, or flower stalks. These types tend to prefer northerly U.S. climates with cold winters. Many of these types produce a single layer of cloves around the stalk within the bulb. Rocambole types exhibit one to three coils in the developing scape and have 6 to 11 rounded cloves. Bulbs store poorly since cloves have loose, easily peeled clove skins.

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<sup>1</sup>Corresponding author; e-mail gvolk@lamar.colostate.edu.

Table 1. Phenotypic types of garlic as described by Engeland et al. (1991, 1995).

Type	Scape curl	Clove skin	Bulbils	Leaf architecture	Leaf color	Clove no.	Clove arrangement	Corresponding subgroup <sup>z</sup>
Silverskin	none	tight	none	nearly vertical	blue-green	8–24	3–6 layers	<i>sativum</i>
Artichoke	none	coarse and thick	none or lower stem	nearly horizontal	yellow-green	12–16	3–6 layers	<i>sativum</i>
Rocamboles	1–3 coils	easily peeled	10–40, br, y, purple			3–14	single layer	<i>ophioscorodon</i>
Porcelain	random	tight	many tiny white			2–5	single layer	<i>sativum</i>
Purple Stripe	0.75 loop	easily peeled	>50 purple			8–12	single layer	<i>longicuspis</i>
Marble purple stripe	0.75 loop	easily peeled	20–60 purple			4–7	single layer	<i>longicuspis</i>
Asiatic	droops	tight	few, purple			4–8	single layer	<i>pekinse</i>
Turban	U downwards	coarse and thick	30–100 small purple			7–11	single layer	
Creole	U downwards	tight	>30 small-medium			4–8	single layer	

<sup>z</sup>According to the German classification system described by Keller (2002), Hanelt (2001), and Maass and Klaas (1995).

Rocamboles generally produce 10 to 40 brown, yellow, or purple bulbils in the umbels. A former classification included a category titled “continental”; varieties that had many tiny bulbils. This category has now been divided into the purple stripe (including purple stripes and marble purple stripes) and porcelain variety types. Both purple stripe and marble purple stripe garlic types have scapes that make a 270° loop and produce cloves that can be easily peeled. Purple stripe garlics have 8 to 12 tallish and crescent-shaped cloves, while marble purple stripe garlics have 4 to 7 rounded cloves per bulb. Porcelain garlic types have scapes that have random curling characteristics, tight clove skins, and four to six elongated cloves (Engeland, 1991, 1995).

There is some confusion with respect to the classification of the Asiatic, Creole, and Turban garlic types. Genetic analyses of Pooler and Simon (1993) classify these garlic types as softnecks, despite the fact that they occasionally make scapes. Asiatic garlic types have drooping scapes and six to nine plump, glossy skinned cloves per bulb with a few purple bulbils in an elongated, wrinkled bulbil capsule (Engeland, 1991, 1995). Turban-type garlics are similar to Asiatics, with a turban-shaped bulbil capsule and a very early maturation date, often sprouting by the October planting date. Creoles have vivid wrappers, tallish cloves with elongated tips and numerous tiny bulbils, and grow best in the southern United States. Creole scapes are generally short and weak (Engeland, 1991, 1995).

Most of the commercial garlic cultivars have been classified into one of the categories listed above. However, this sort of phenotypic classification is not available for most accessions maintained in the NPGS.

The purpose of this study is to examine the diversity of a collection of 211 commercial and federally held accessions using a set of amplified fragment-length polymorphism (AFLP) markers. Analysis of this data was subjected to several analytical techniques that allowed us to compare the molecular diversity of these accessions a phenotypic classification scheme. By including both commercial and federal collections in our dataset, we can identify both the structure of diversity and the extent of duplications within the collection. In addition, these data will be valuable in prioritizing representative accessions for further cryopreservation research.

### Materials and Methods

**PLANT MATERIAL FOR ANALYSIS.** Garlic cloves were provided by the WRPIS (Pullman, Wash.) and from commercial growers Walter Lyons ([www.thegarlicstore.com](http://www.thegarlicstore.com)) and Tom Cloud ([www.filareefarms.com](http://www.filareefarms.com)). Accession data are provided in Table 2. In the collection of 211 accessions in this data set, 20 named cultivars

were represented by 47 clones. These represent accessions that had the same cultivar name but were obtained from different sources. Accessions duplicated in this manner are identified in Table 2 with an appended number to the name, e.g., ‘Siberian-1’ and ‘Siberian-2’. Having these duplications allowed us to test whether cultivars bearing the same name but collected from different growers are genetically identical. Garlic cultivars were classified by the growers. NPGS accessions were classified as hardneck or softneck by identifying a stem in cross-sectional images of garlic accessions publicly available through GRIN (Germplasm Resources Information Network, <http://www.ars-grin.gov/npgs/>).

**MOLECULAR ANALYSIS.** DNA was independently extracted from shoot tips within two cloves from each accession using the DNeasy 96 Plant Kit (Qiagen, Valencia, Calif.) with some minor modifications. For each extraction, 50 mg of shoot tip tissue was frozen in liquid nitrogen and ground to a powder. Initial extraction volumes using the lysis buffer AP1 was doubled to 800 µL per individual. AFLP technique (Vos et al., 1995) was performed on each DNA sample as described by Myburg et al. (2000). In short, 500 ng of genomic DNA was digested with EcoRI and MseI restriction enzymes (New England Biolabs, Beverly, Mass., and Invitrogen, Carlsbad, Calif.). Adapters (Table 3) were ligated to the digested genomic DNA. The ligation products were diluted 1:10 with distilled water and preselective PCR amplification was performed using an MJ Research (Reno, Nev.) PTC200 or Dyad thermalcycler. Products were diluted 1:40 with low TE [0.1 mM EDTA, 10 mM Tris (Sigma-Aldrich Corp., St. Louis), pH 8.0] and selectively amplified using one of the primer sets listed in Table 3. Adapters and nonlabeled primers were synthesized by IDT (Skokie Ill.) and dye-labeled primers were synthesized by MWG Biotech (High Point, N.C.). Labeled products were separated with a LI-4200 DNA sequencer (LI-COR, Lincoln, Neb.) and digitally captured using the Saga 2.0 software package (LI-COR).

**DATA ANALYSIS.** TIFF images were retrieved and partially analyzed using SAGA 2.0 MX module (LI-COR). We manually selected the most informative and consistently reproducible markers for analysis in this data set. These 27 loci were manually scored for each of the two independent replicate samples from each accession. When one of the 27 loci was different among the replicate samples, the locus was omitted as unreconciled and a missing (ambiguous) data point replaced this locus in the consensus score. Samples with >4% (more than one ambiguous value for 27 loci scored) were removed from the dataset and not included in any analyses. In the data for this study, there were a total of 211 samples. Of these samples, 158 samples had a complete genotype at all 27 loci and 53 had one ambiguous value. These genotypes represent intraspecific diversity and as such were analyzed in a way to display the most conservative clustering. We used principle

Table 2. Names, phenotypic classification, and genetic classification of garlic clones.

Cultivar identifier	WRPIS cultivar	Source	Type	Class	PCA	Group
W6-49	M89-39	WRPIS	Hardneck	Hardneck	I	A
W6-671	W6-671	WRPIS	Softneck	Softneck	I	D
W6-672	W6-672	WRPIS	Softneck	Softneck		D
W6-1861	Dunganski	WRPIS	Hardneck	Marble PS	IV	
W6-1862	Ferganski	WRPIS	Hardneck	Purple Stripe	I	H
W6-1880	<i>longicuspis</i>	WRPIS	Hardneck	<i>longicuspis</i>		
W6-1883	Maiski	WRPIS	Hardneck	Asiatic	I	H
W6-1885	Ferganski	WRPIS	Hardneck	Purple Stripe	IV	L
W6-1903	<i>longicuspis</i>	WRPIS	Hardneck	<i>longicuspis</i>	IV	
W6-2560	W6-2560	WRPIS	Softneck	Softneck	I	D
W6-2561	Arguni White	WRPIS	Softneck	Softneck		D
W6-2562	Arguni White	WRPIS	Softneck	Softneck	I	D
W6-2563	W6-2563	WRPIS	Softneck	Softneck	I	D
W6-8403	Aja rondena	WRPIS	Softneck	Softneck	I	D
W6-8404	Bhsto de chinchon	WRPIS	Softneck	Softneck		D
W6-8405	Blanco de bonda	WRPIS	Softneck	Softneck	I	D
W6-8406	Blanco de clenla	WRPIS	Softneck	Softneck	I	D
W6-8409	Blanco de valla dolid	WRPIS	Softneck	Softneck		D
W6-8410	Blanco gallego	WRPIS	Softneck	Softneck	I	D
W6-8411	Blanco vallelado	WRPIS	Softneck	Softneck	I	D
W6-8413	Labera del obispo	WRPIS	Hardneck	Hardneck	I	A
W6-8414	Manuel benitee	WRPIS	Hardneck	Hardneck		Q
W6-8415	Morado de pedronera	WRPIS	Hardneck	Hardneck	I	A
W6-8416	Rojo de banolas	WRPIS	Hardneck	Hardneck	I	Q
W6-8417	Rojo de castro	WRPIS	Hardneck	Hardneck		A
W6-8420	Rojo del Pais baza	WRPIS	Hardneck	Hardneck		A
W6-10473	E92-16	WRPIS	Hardneck	Hardneck	I	H
W6-10729	B92-16	WRPIS	Softneck	Softneck	I	D
W6-10734	B92-21	WRPIS	Softneck	Softneck		D
W6-10736	B92-23	WRPIS	Softneck	Softneck	I	D
W6-10737	B92-24	WRPIS	Softneck	Softneck		D
W6-10739	B92-222	WRPIS	Softneck	Softneck	I	D
W6-12816	WWE Italian garlic	WRPIS	Softneck	Silverskin	I	D
W6-12823	199-33	WRPIS	Hardneck	Hardneck		O
W6-12824	199-36	WRPIS	Hardneck	Hardneck	II	F
W6-12825	199-41	WRPIS	Hardneck	Hardneck		F
W6-12828	850910-2	WRPIS	Hardneck	Hardneck	II	F
W6-12829	851004-1	WRPIS	Hardneck	Hardneck	II	F
W6-12830	8510301-1	WRPIS	Softneck	Softneck	I	A
W6-12831	870529-01	WRPIS	Hardneck	Hardneck		A
W6-12832	870825	WRPIS	Hardneck	Hardneck	I	A
W6-12833	871210	WRPIS	Hardneck	Hardneck		F
W6-12836	French garlic	WRPIS	Softneck	Softneck	I	D
W6-12837	GSF-18	WRPIS	Hardneck	Hardneck	II	F
W6-12838	GSF-60	WRPIS	Hardneck	Hardneck	II	F
W6-12839	Gourmet Red	WRPIS	Hardneck	Hardneck	IV	J
W6-12840	Italian purple artichoke	WRPIS	Softneck	Softneck	I	D
W6-12842	Poodles	WRPIS	Hardneck	Hardneck	II	O
W6-12844	890609	WRPIS	Softneck	Softneck	I	D
W6-12912	Montana Roja	WRPIS	Hardneck	Hardneck	II	F
W6-17074	Chinese	WRPIS	Softneck	Softneck	I	H
W6-17281	W6 17281	WRPIS	Hardneck	Hardneck	I	H
W6-18723	1	WRPIS	Hardneck	Hardneck	IV	N
W6-18724	2	WRPIS	Softneck	Softneck	I	D
W6-18726	4	WRPIS	Softneck	Softneck	I	D
W6-18729	7	WRPIS	Softneck	Softneck		D
PI 383817	Domasen	WRPIS	Softneck	Softneck	IV	
PI 383819	Pristinski	WRPIS	Softneck	Silverskin	I	B
PI 383820	Prizrenski	WRPIS	Softneck	Softneck	I	B

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Table 2 (continued). Names, phenotypic classification, and genetic classification of garlic clones.

Cultivar identifier	WRPIS cultivar	Source	Type	Class	PCA	Group
PI 383821	Lokalen	WRPIS	Softneck	Softneck	I	E
PI 383822	Veleski	WRPIS	Softneck	Softneck	I	B
PI 383823	Ivankovski	WRPIS	Softneck	Softneck	I	C
PI 383824	Lokalen	WRPIS	Softneck	Softneck	I	P
PI 383831	Klisurski	WRPIS	Softneck	Softneck	I	E
PI 493097		WRPIS	Softneck	Softneck	II	O
PI 493106	61	WRPIS	Softneck	Softneck		P
PI 493114	Cinsky	WRPIS	Softneck	Softneck	I	B
PI 493115	Prim	WRPIS	Softneck	Silverskin	I	B
PI 493116	174	WRPIS	Hardneck	Hardneck	IV	
PI 493117	Tybet 1814	WRPIS	Softneck	Softneck		C
PI 493118	188	WRPIS	Softneck	Softneck		A
PI 493124	Chonan	WRPIS	Hardneck	Hardneck	I	C
PI 497942		WRPIS	Hardneck	Hardneck		
PI 497943		WRPIS	Softneck	Softneck	I	C
PI 497944		WRPIS	Hardneck	Hardneck	II	G
PI 497947		WRPIS	Softneck	Softneck	I	B
PI 515972	Music (rocambole type)	WRPIS	Hardneck	Rocambole	II	
PI 540314	<i>longicuspis</i>	WRPIS	Hardneck	<i>longicuspis</i>		Q
PI 540327	Mexicano-B	WRPIS	Hardneck	Hardneck	II	O
PI 540331	Horsky	WRPIS	Hardneck	Hardneck		A
PI 540333	Dugganskij-Mestnyj	WRPIS	Hardneck	Hardneck	II	F
PI 540334	Monsanskij	WRPIS	Hardneck	Hardneck	IV	J
PI 540335	Americky Maly	WRPIS	Hardneck	Hardneck	IV	J
PI 540336	Seversky Palicak	WRPIS	Hardneck	Hardneck	IV	
PI 540337	Adizanskij	WRPIS	Hardneck	Hardneck		
PI 540338	Starobelski belyj	WRPIS	Hardneck	Hardneck	IV	L
PI 540340	Jampol skij	WRPIS	Hardneck	Hardneck	III	I
PI 540343	W6 4177	WRPIS	Hardneck	Hardneck	II	F
PI 540344	W6-4178	WRPIS	Hardneck	Hardneck	II	G
PI 540346	W6-4180	WRPIS	Hardneck	Hardneck	II	
PI 540351	W6-4185	WRPIS	Hardneck	Hardneck	II	G
PI 540352	W6-4186	WRPIS	Hardneck	Hardneck	II	G
PI 540353	W6 4187	WRPIS	Hardneck	Hardneck	II	F
PI 540356	W6-4190	WRPIS	Hardneck	Hardneck	IV	
PI 540357	<i>longicuspis</i>	WRPIS	Hardneck	<i>longicuspis</i>	IV	L
PI 540359	Rocambole	WRPIS	Softneck	Softneck	I	
PI 540360	Spanish Red	WRPIS	Hardneck	Rocambole	II	F
PI 540361	De vivo	WRPIS	Hardneck	Rocambole	II	F
PI 540363	850904-09	WRPIS	Hardneck	Hardneck	II	G
PI 540365	Early Red	WRPIS	Hardneck	Hardneck	II	F
PI 540369	Creole	WRPIS	Hardneck	Softneck	I	B
PI 540370	S&H Italian purple	WRPIS	Softneck	Softneck	I	B
PI 543048	WKP-88-6	WRPIS	Softneck	Softneck	I	B
PI 543049	WKP-99-19	WRPIS	Hardneck	Hardneck		
PI 576914	<i>longicuspis</i>	WRPIS	Hardneck	<i>longicuspis</i>	IV	K
PI 615417	Gomecari	WRPIS	Softneck	Artichoke	I	E
PI 615418	Achaiami	WRPIS	Hardneck	Hardneck	II	F
PI 615419	W6-4264	WRPIS	Hardneck	Hardneck	IV	J
PI 615422	Blanco de huelma zamora	WRPIS	Softneck	Softneck	I	E
PI 615423	Blanco de huelma zamora	WRPIS	Softneck	Softneck	I	E
PI 615424	Fino de chincko ajofrin	WRPIS	Softneck	Softneck	I	B
PI 615425	B92-18	WRPIS	Softneck	Softneck	I	E
Ajo Rojo		Commercial	Hardneck	Creole	I	R
<i>Allium</i> sp.		WRPIS	Hardneck			
Bogaty-1		Commercial	Hardneck	Marble PS	IV	J
Bogaty-2		Commercial	Hardneck	Marble PS	IV	J
Bogaty-3		Commercial	Hardneck	Marble PS	IV	
Bogaty-4		Commercial	Hardneck	Marble PS		

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Table 2 (continued). Names, phenotypic classification, and genetic classification of garlic clones.

Cultivar identifier	WRPIS cultivar	Source	Type	Class	PCA	Group
Brown rose		Commercial	Hardneck	Marble PS	IV	
Brown Tempest-1		Commercial	Hardneck	Marble PS	IV	J
Brown Tempest-2		Commercial	Hardneck	Marble PS		J
Brown Tempest-3		Commercial	Hardneck	Marble PS		
Burgundy		Commercial	Hardneck	Creole		A
CA Early		Commercial	Softneck	Artichoke		D
Carpathian		Commercial	Hardneck	Rocamboles		
Chamiskuri		Commercial	Softneck	Artichoke		
Chesnok Red-1		Commercial	Hardneck	Purple Stripe		
Chesnok Red-2		Commercial	Hardneck	Purple Stripe	IV	
Chet's Italian Red		Commercial	Softneck	Artichoke	I	D
Cichisdzhvari		Commercial	Hardneck	Porcelain		
Colorado Black		Commercial	Hardneck	Rocamboles	II	F
Creole Red		Commercial	Hardneck	Creole	I	C
Darcheli		WRPIS	Hardneck	Hardneck	IV	
DO-15		WRPIS	Hardneck	Hardneck		R
Dominics		Commercial	Hardneck	Rocamboles	II	F
DX-127		WRPIS	Softneck	Softneck		
DX-134		WRPIS	Hardneck	Hardneck		
DX-90		WRPIS	Softneck	Softneck		
French Rocamboles		Commercial	Hardneck	Rocamboles		F
Georgian Crystal-1		Commercial	Hardneck	Porcelain	III	I
Georgian Crystal-2		Commercial	Hardneck	Porcelain	III	I
Georgian Fire-1		Commercial	Hardneck	Porcelain	III	I
Georgian Fire-2		Commercial	Hardneck	Porcelain	III	I
German Brown		Commercial	Hardneck	Rocamboles	II	F
German Hardy		Commercial	Hardneck	Porcelain	III	I
German Porcelain		Commercial	Hardneck	Porcelain	III	I
German Red-1		Commercial	Hardneck	Rocamboles	II	F
German Red-2		Commercial	Hardneck	Rocamboles		
German White-1		Commercial	Hardneck	Porcelain	III	I
German White-2		Commercial	Hardneck	Porcelain	III	I
GSF 65		Commercial	Hardneck	Rocamboles	II	F
Inchelium-1		Commercial	Softneck	Artichoke	I	D
Inchelium-2		Commercial	Softneck	Artichoke	I	M
Italian Purple		Commercial	Hardneck	Hardneck	II	F
Japanese		Commercial	Hardneck	Asiatic	IV	K
Jerome's French Rose		WRPIS	Hardneck	Hardneck		
Keeper-1		Commercial	Hardneck	Asiatic	IV	K
Keeper-2		Commercial	Hardneck	Asiatic		A
Kilarney Red-1		Commercial	Hardneck	Rocamboles	II	F
Kilarney Red-2		Commercial	Hardneck	Rocamboles	II	F
Korean Red		Commercial	Hardneck	Hardneck	IV	
Leah 99		Commercial	Hardneck	Porcelain	III	I
Leningrad		Commercial	Hardneck	Porcelain		
Lorz Italian		Commercial	Softneck	Artichoke	I	D
Machashi		Commercial	Softneck	Artichoke		R
Marino		Commercial	Hardneck	Rocamboles	IV	J
Mchadidzhauri		Commercial	Softneck	Artichoke	I	D
Metechi-1		Commercial	Hardneck	Marble PS	IV	L
Metechi-2		Commercial	Hardneck	Marble PS	IV	
Mexican Red		Commercial	Hardneck	Purple Stripe	IV	
Music-1		Commercial	Hardneck	Porcelain	III	I
Music-2		Commercial	Hardneck	Porcelain	III	I
Music-3		Commercial	Hardneck	Porcelain	III	I
Nap's Mystery garlic		Commercial	Hardneck	Rocamboles	IV	J
Nootka Rose-1		Commercial	Softneck	Silverskin		A
Nootka Rose-2		Commercial	Softneck	Silverskin		A
Nootka Rose-3		Commercial	Hardneck	Silverskin	I	

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Table 2 (continued). Names, phenotypic classification, and genetic classification of garlic clones.

Cultivar identifier	WRPIS cultivar	Source	Type	Class	PCA	Group
Northern White		Commercial	Hardneck	Porcelain	III	I
Oregon Blue		Commercial	Softneck	Artichoke	I	D
Orting		WRPIS	Hardneck	Hardneck		
Persian Star-1		Commercial	Hardneck	Purple Stripe	IV	L
Persian Star-2		Commercial	Hardneck	Purple Stripe	IV	L
Polish Hardneck-1		Commercial	Hardneck	Porcelain	III	I
Polish Hardneck-2		Commercial	Hardneck	Porcelain	III	I
Pskem		Commercial	Hardneck	Marble PS		
Purple glazer		Commercial	Hardneck	Glazed PS	IV	N
Purple tip		Commercial	Hardneck	Hardneck		
Pyong Vang Korean		Commercial	Hardneck	Asiatic	IV	K
Randl Colorado White		Commercial	Softneck	Softneck	I	D
Red Italian-1		Commercial	Softneck	Softneck	I	D
Red Italian-2		Commercial	Softneck	Artichoke		D
Red Janice-1		Commercial	Hardneck	Turban	I	H
Red Janice-2		Commercial	Hardneck	Turban		
Red Toch		Commercial	Softneck	Artichoke	I	M
Red/Purple Italian		Commercial	Softneck	Artichoke	I	D
Romanian Red		Commercial	Hardneck	Porcelain	III	I
Russian Red-1		Commercial	Hardneck	Rocambole	II	F
Russian Red-2		Commercial	Hardneck	Rocambole	II	F
Russian Red-3		Commercial	Hardneck	Rocambole	II	F
Samarkand		Commercial	Hardneck	Hardneck	IV	L
Siberian-1		Commercial	Hardneck	Marble PS	IV	
Siberian-2		Commercial	Hardneck	Marble PS	IV	J
Silverwhite		Commercial	Softneck	Silverskin	I	C
Sonora		Commercial				H
Spanish Roja-1		Commercial	Hardneck	Rocambole	II	F
Spanish Roja-2		Commercial	Hardneck	Rocambole		F
Spanish Roja-3		Commercial	Hardneck	Rocambole	II	F
Susanville		Commercial	Softneck	Artichoke	I	D
Vladivostok #1		WRPIS	Hardneck	Hardneck	I	
Western Rojo		Commercial	Hardneck	Hardneck		F
Yugo Red		Commercial	Hardneck	Rocambole	II	F

Table 3. AFLP primers and number of bands scored for each selective primer.

Adapters			
<i>Eco</i> RI adapter		5'-CTC GTA GAC TGC GTA CC CAT CTG ACG GAT GGT TAA-5'	
<i>Mse</i> I adapter		5'-GAC GAT GAG TCC TGA GTA CTC AGG ACT CAT-5'	
Pre-amplification primers			
<i>Eco</i> RI		5'-GAC TGC GTA CCA ATT CA	
<i>Mse</i> I		5'-GAT GAG TCC TGA GTA AC	
Selective amplification primer sets		scored (no.)	Bands
A41		5'- <b>IR</b> -GAC TGC GTA CCA ATT CAC T 5'-GAT GAG TCC TGA GTA ACT AA	3
A42		5'- <b>IR</b> -GAC TGC GTA CCA ATT CAC T 5'-GAT GAG TCC TGA GTA ACT AG	5
B21		5'- <b>IR</b> -GAC TGC GTA CCA ATT CAC G 5'-GAT GAG TCC TGA GTA ACA AG	6
C11		5'- <b>IR</b> -GAC TGC GTA CCA ATT CAG C 5'-GAT GAG TCC TGA GTA ACA CG	7
E21		5'- <b>IR</b> -GAC TGC GTA CCA ATT CAC C 5'-GAT GAG TCC TGA GTA ACA AG	6
<b>Total</b>			27

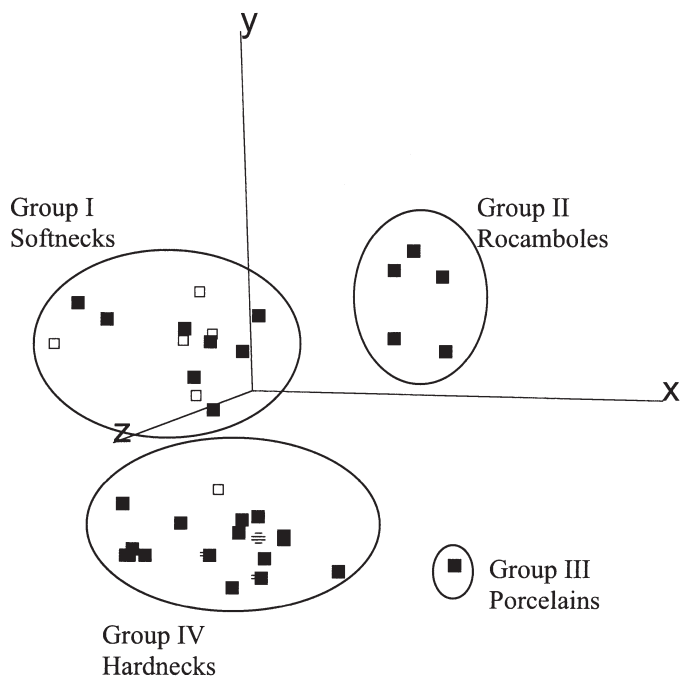
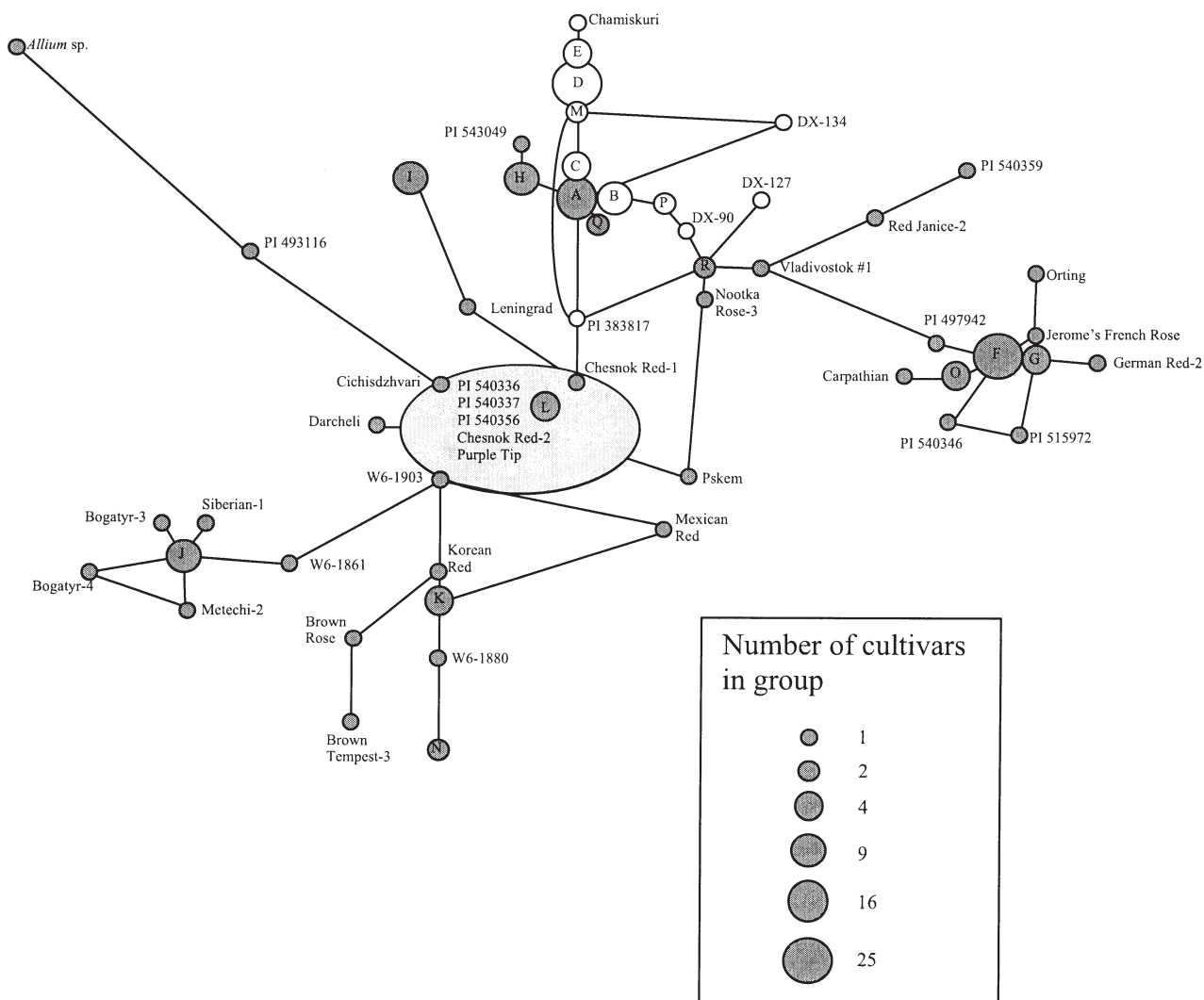


Fig. 1 (above). Principle component analysis of 158 garlic clones. Hardneck (■) and softneck (□) types are identified.

component analyses (PCA) to look for broad patterns of differentiation between hardneck and softneck classes. We used maximum parsimony analysis with boot strapped support values to refine the clustering and finally produced a minimum spanning network to show the general pattern across all 211 accessions. Networks are appropriate for intraspecific data because they are better able to represent alternate tree topologies (depicted as reticulations within the network) especially where the number of samples is large and the distances between individuals are small.

For construction of a minimum spanning network, AFLP fingerprints of all 211 accessions were merged into groups of individuals that had Euclidean distances of zero using MacClade 4.0 (Sinauer Associates, Mass.). Each group, and single accessions that did not group together, were plotted as nodes in a minimum spanning network using the computer algorithm Minspnet (Excoffier et al., 1992, <http://cmpg.unibe.ch/services/software.htm>). The minimum spanning tree was manually drawn using computer outputs. The number of accessions grouped within a node is reflected in the diameter of the node.

Fig. 2 (below). Minimum spanning distance diagram illustrates the genetic relatedness among 211 garlic clones. Node size represents the number of genetically identical accessions within a lettered node (key to letters is provided in Table 4). Accessions and nodes that are primarily softneck (open circles) or primarily hardneck (filled circle) are identified. The central region (shaded circles) is comprised of multiple accessions with many reticulations between them.



In addition, we investigated the effect of adding additional AFLP loci to differentiate accessions that had been merged together as a single group or node in the minimum spanning network. Using a smaller subset allowed us to increase the number of consistent AFLP characters and compare the genetic similarity among these accessions at higher genetic resolution. We sampled 34 accessions from four groups and compared their genotypes at 80 additional AFLP markers.

Each individual fingerprint was represented by an array of presence/absence scores for the 27 characters. A pairwise distance matrix using Jacquard's distance coefficient (using presence data only) was generated in NTSyspc ver. 2.1 (Exceter Software, N.Y.) using the set of 158 samples with complete genotypes. Principle component analyses were performed using the first three eigenvectors of the distance matrix. Parsimony analysis for the set of 158 complete genotypes was conducted with PAUP 4.0 (Sinauer Associates). Statistical support for parsimony clustering was obtained using 1000 bootstrap replications using one tree per

replication. Analysis of molecular variance (AMOVA) among clades identified in parsimony was conducted using Arlequin v. 2.0 (Schneider et al., 2000).

## Results

Of the 333 pairs of original replicate samples, 122 pairs of replicates were discarded from any analyses since a consensus score could not be determined. The remaining 211 samples were included in the minimum spanning distance analyses. Of those 211 samples, 158 samples had no missing data. These samples were used for parsimony, PCA, and AMOVA. Table 2 lists the garlic accessions that were included in the analyses.

Principle component analyses were performed for the 158 accessions that contained no missing data. The first, second, and third principle components accounted for 37%, 21%, and 12%, respectively, of the total variation and were plotted (Fig. 1). Softneck and hardneck garlic types can be distinguished (Group

Table 4. Groups of identical accessions as identified by MinSpnet.

<b>A</b>	<b>D (continued)</b>	<b>F Rocamboles</b>	<b>H</b>	<b>K Asiatic</b>
Burgundy	Oregon Blue	Colorado Black	Red Janice-1	Japanese
Keeper-2	Randl Colorado White	Dominics	Sonora	Keeper-1
Nootka Rose-1	Red Italian-1	French Rocamboles	W6-1862	Pyong Vang Korean
Nootka Rose-2	Red Italian-2	German Brown	W6-1883	PI 576914
W6-49	Red/Purple Italian	German Red-1	W6-10473	
W6-8413	Susanville	GSF 65	W6-17074	<b>L Purple Stripe</b>
W6-8415	W6-671	Italian Purple	W6-17281	Metechi-1
W6-8417	W6-672	Kilarney Red-1		Persian Star-1
W6-8420	W6-2560	Kilarney Red-2	<b>I Porcelain</b>	Persian Star-2
W6-12830	W6-2561	Russian Red-1	GA Crystal-1	Samarkand
W6-12831	W6-2562	Russian Red-2	GA Crystal-2	W6-1885
W6-12832	W6-2563	Russian Red-3	GA Fire-1	PI 540338
PI 493118	W6-8403	Spanish Roja-1	GA Fire-2	PI 540357
PI 540331	W6-8404	Spanish Roja-2	German Hardy	
	W6-8405	Spanish Roja-3	German Porcelain	<b>M</b>
	W6-8406	Western rojo	German White-1	Inchelium-2
<b>B</b>	W6-8409	Yugo Red	German White-2	Red Toch
PI 383819	W6-8410	W6-12824	Leah 99	
PI 383820	W6-8411	W6-12825	Music-1	<b>N</b>
PI 383822	W6-10729	W6-12828	Music-2	Purple glazer
PI 493114	W6-10734	W6-12829	Music-3	W6-18723
PI 493115	W6-10736	W6-12833	Northern White	
PI 497947	W6-10737	W6-12837	Polish Hardneck-1	<b>O</b>
PI 540369	W6-10739	W6-12838	Polish Hardneck-2	W6-12823
PI 540370	W6-12816	W6-12912	Romanian Red	W6-12842
PI 543048	W6-12836	PI 540333	PI 540340	PI 493097
PI 615424	W6-12840	PI 540343		PI 540327
	W6-12844	PI 540353	<b>J Marble Purple Stripe</b>	
<b>C</b>	W6-18724	PI 540360	Bogatyr-1	<b>P</b>
Silverwhite	W6-18726	PI 540361	Bogatyr-2	PI 383824
PI 383823	W6-18729	PI 540365	Brown Tempest-1	PI 493106
PI 493117		PI 615418	Brown Tempest-2	
PI 493124	<b>E</b>		Marino	<b>Q</b>
PI 497943	PI 383821	<b>G Rocamboles</b>	Nap's Mystery garlic	W6-8414
Creole Red	PI 383831	PI 497944	Siberian-2	W6-8416
	PI 615417	PI 540344	W6-12839	PI 540314
<b>D Artichoke</b>	PI 615422	PI 540351	PI 540334	
CA Early	PI 615423	PI 540352	PI 540335	<b>R</b>
Chet's Italian Red	PI 615425	PI 540363	PI 615419	Ajo Rojo
Inchelium-1				Machashi
Lorz Italian				DO-1
Mchadidzhauri				



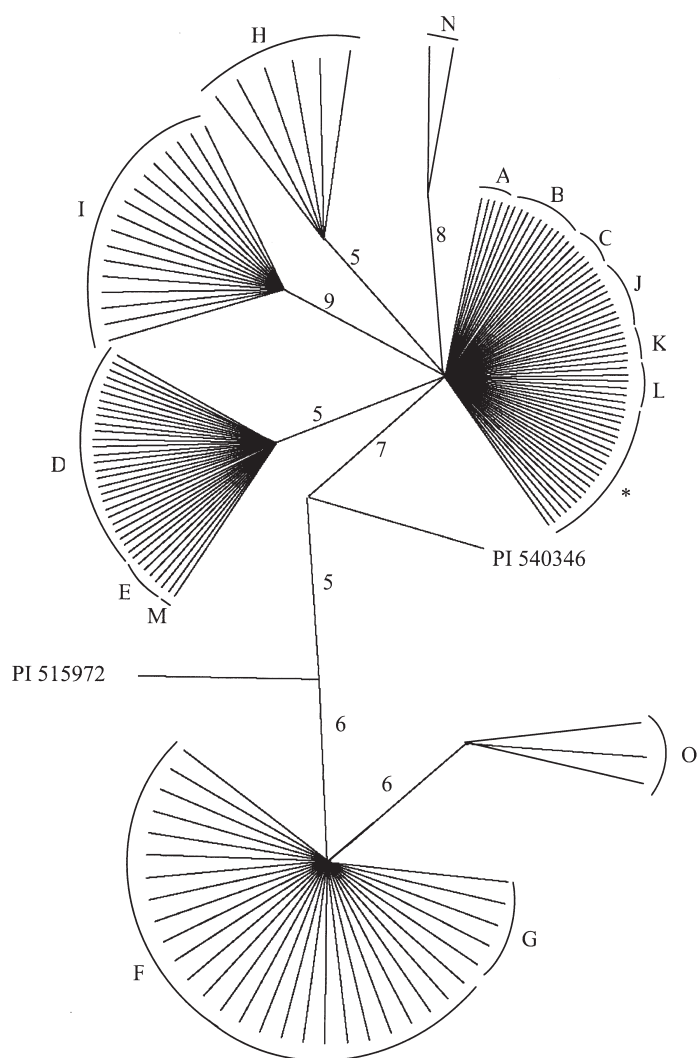


Fig. 3. Parsimony analysis (1000 bootstrap replicates) of 158 garlic clones. Letters refer to groups of genetically identical accessions defined in Table 4.

I vs. Groups II, III, and IV). Clusters of rocambole (Group II) and porcelain (Group III) accessions can be identified within the hardneck grouping. Accessions in each cluster are identified in Table 2. Softneck garlic types represent less genetic diversity than the hardneck types (Fig. 1) since they tend to be restricted to one cluster in principle component space.

The minimum spanning tree (Fig. 2) shows structure among the complete set of 211 accessions. An accession of *Allium* sp. (W6-12827) was included as the outgroup in the minimum spanning tree. Scape formation and classification data were overlaid on the minimum spanning tree to determine if phenotypes could be correlated with genetic distances. Many of the regions of the minimum spanning tree could be assigned to the grower classification categories. Our ability to assign phenotypic categories to all the samples included in the network is limited since most of the NPGS garlic collection have not been classified in this manner. The major classification groups of artichoke, rocambole,

porcelain, purple stripe, marble purple stripe, and Asiatic each included a set of genetically identical accessions (Table 4). In most cases, all the accessions within a group of genetically identical accessions belonged to the same phenotypic classification category. However, rocambole types 'Marino' and 'Nap's Mystery Garlic' clustered with the marble purple stripes (Tables 2 and 4). The most reticulations in the network were displayed in the purple stripe region (L). This region also is the basal region of the network as it shared the most identity with the out-group. To simplify the display, the nodes within this region were grouped in an ellipse with the component accessions listed. Overall, we identified 18 groups of genetically identical accessions using our 27 AFLP markers (Table 4). Replicate accessions of 'Keeper' and 'Metechi' did not cluster together in our analyses.

Using parsimony analysis on the set of 158 complete genotypes, we tested some of the statistical support for the structure revealed in the minimum spanning tree. The topology of the parsimony tree, while statistically supporting genetic differentiation among some accessions of marble purple stripes, porcelains, artichokes, and rocamboles, collapsed many accessions into large clades (Fig. 3). This condensing of data is not unexpected in this type of phylogenetic analysis given the structure of the number of accessions (158) and the number of informative characters (27) and underscores the uncertainty of the phylogenetic history of these clonal lineages using these 27 loci. Despite this, the parsimony and genetic distance based approaches revealed many common attributes in the diversity among these accessions.

While our phylogenetic analysis of the genetic relationships of U.S. garlic varieties suggests that phenotypic categories correlate with genetic distance data, some categories, such as turbans and creoles, require additional examination. We also believe that some diverse accessions may not be easily classified directly into the current commercial categories. For instance, *A. longicuspis* and creole types do not show any single genetic identity to other groups in the data set. We used AMOVA techniques to generate pairwise  $F_{st}$  to measure genetic differentiation between accessions in nine phenotypic classes listed in Table 2. These accessions include artichoke, asiatic, creole, *A. longicuspis*, marble purple stripe, porcelain, purple stripe, rocambole, and silverskin. The significance of these  $F_{st}$  estimates was tested using a permutation algorithm in Arlequin. The results showed that while seven of the nine groups were significantly differentiated, creole types and *A. longicuspis* were not (data not shown). Creole and *A. longicuspis* classification types may not be robust and may not reflect their true taxonomic origin. Of the 118 NPGS accessions, 24 had unique genotypes and of the 75 commercial accessions, 19 had unique genotypes. Overall 64% of the WRPIS and 41% of the commercial accessions were duplicates as determined by this method.

- \*
  - P
  - Q
  - R
  - Ajo Rojo
  - Brown Rose
  - Bogatyr-3
  - Chesnok Red-2
  - Darcheli
  - Korean Red-3
  - Metechi-2
  - Mexican Red
  - Nootka Rose-3
  - Siberian-1
  - Vladivostok #1
  - PI 383817
  - PI 493116
  - PI 540356
  - PI 540359
  - W6-1903
  - W6-1861

Table 5. 107 loci were scored on the groups of accessions that were identified based on 27 loci.

Group	Unique cultivars (no.)	Loci scored (no.)	Identity (%)
D	9	107	99.6
F	10	107	99.1
I	10	107	99.7
J	5	107	100.0

## Discussion

Genetic analysis using AFLP in garlic was a challenging endeavor. Garlic has an unusually large genome size of  $>3 \times 10^{10}$  basepairs (Ranjekar et al., 1978) and even though the selective primer used four additional random bases for refining the selection of amplification products, comparisons made across hundreds of individuals resulted in reduced accuracy due to amplification instability. To solve this, we reduced the number of scorable markers to a verifiable subset and tested each of these markers for accuracy by genotyping two independent genomic DNA extractions from each accession.

We scored an additional 80 bands on 34 duplicate accessions to determine if the inclusion of additional loci would differentiate the samples that we identified as duplicates. As shown in Table 5, we had  $>99\%$  identity among consensus scores for samples that were deemed identical based on 27 loci. Ipek et al (2003) initially scored their accession data for 80 loci, and then increased the number of loci to 183 to determine their confidence in their assessment of duplication. Ipek et al (2003) were able to differentiate additional clones as they increased the number of scored loci in their dataset. These authors pooled multiple clones of a given accession for their analyses, but there is no indication that replicate samples were analyzed. Bradley et al. (1996) also identified uniquely named accessions with identical genotypes.

Matus et al. (1999) measured 17 phenological characters of 66 garlic clones and performed PCA to classify these accessions. These authors plotted the first and third principle components and could distinguish clones with flower stalks from those with incomplete and nonflowering phenotypes. Using RAPD analyses, Bradley et al. (1996) also separated flowering and nonflowering or incomplete flowering garlic accessions.

Our PCA analysis distinguished between many of the hardneck and softneck garlic types. We note that hardneck varieties that group with softneck varieties in our PCA have similar clustering patterns in the network analysis. Similar trends were identified by Maass and Klaas (1995), who used neighbor joining analyses to analyze isozyme data for 300 garlic clones. They could differentiate early senescing, nonfertile flowering clones from softneck clones from *A. longicuspis* clones collected from central Asia. Maass and Klaas (1995) used RAPD analyses to further distinguish softneck types from the Caucasus and Mediterranean regions (incomplete bolting artichoke types) from softneck types from east and middle Asia. Al Zahim et al. (1997) correlated morphological variation with RAPD patterns and could differentiate rocambos and most softnecks from other hardnecks.

We chose to present our genetic distance data as a minimum spanning tree, rather than a dendrogram. This method creates the smallest network length based on genetic distance values and several equally short networks are shown as reticulations in the network where a node has more than two connections. It also

presents identical accessions as nodes within the tree, which we felt was suitable since there were many examples of duplicate accessions within our dataset.

Since garlic phenotypes are known to vary according to environmental conditions, the descriptions of the classes of garlic in Table 1 are only relevant in the growing conditions in Okanogan, Wash. Most of the commercial clones of garlic have been classified according to their phenotypes at this location. Clones that fall within a given category are phenotypically similar. In most cases, clones that are phenotypically similar are also genotypically similar, as determined by our molecular analyses. Additional studies are needed to determine which phenotypic traits can accurately describe the phenotypic categories regardless of location.

Our data identify distinct groups of artichoke, rocambos, marble purple stripe, asiatic, porcelain and purple stripe garlics. There are some hardneck accessions that do not genetically align with the described hardneck categories. Many of these are NPGS accessions that are phenotypically unclassified.

Our data place the Asiatic type within the hardnecks, as opposed to the softneck category where it was classified by Pooler and Simon (1993). Our genetic results place one replicate of 'Keeper', phenotypically an Asiatic, within the softneck group. 'Keeper', a hardneck, was isolated after multiple selections from a population of softnecks (W. Lyons, personal communication), so it is not surprising that it is genetically more similar to softnecks than hardnecks. We have identified some phenotypic Asiatics that do not align with the other Asiatics. Perhaps the Asiatic phenotype extends to a number of genetically distinct accessions.

Our study included only a few turban and creole garlic types. Since the number of accessions is limited, and phenotypically similar accessions do not align, we can not speculate on the genetic relationships between and among turban and creole accession types.

Al-Zahim et al. (1997), determined that the clone 'Burgundy' (type creole) clusters with nonbolting-type garlic clones by RAPD analysis, even though it has been classified as a bolting-type garlic. Similar results were reported by Maass and Klaas (1995). Our studies also suggest that 'Burgundy' clusters with nonbolting types of garlic.

Ipek et al. (2003) and Pooler and Simon (1993) have generated dendrograms for 48 and 100 garlic accessions, respectively, based on isozyme, RAPD, and AFLP data. Nine accessions were common between our dataset and the phylogeny generated by Ipek et al. (2003). There is some agreement between the placements by Ipek and the placements reported in this study. Softneck accessions PI 383823, PI 383819, PI 497943, and PI 383824 colocalized with softneck types in both studies. Hardneck accession PI 493116 localized with other hardnecks in both studies.

Our phylogeny suggests that *A. longicuspis* is indistinguishable from *A. sativum*, as was found by Pooler and Simon (1993), Ipek et al. (2003), and Al-Zahim et al. (1997). Ipek et al. (2003) and Pooler and Simon (1993) were unable to correlate the geographical origin of their samples to phylogenetic relationships. Many of the garlic accessions in the U.S. collection may have secondary source data, rather than original wild collection data associated with them. Due to the difficulty of tracing garlic clones to geographical origins and the lack of correlation between banding patterns and geographical origin, we did not perform any geographic analyses.

This study determined that the uniquely named NPGS and commercial collections are 64% and 41% duplicate accessions, respectively. Unique commercial and NPGS accessions were

identified. When NPGS accessions are classified according to phenotype using a scheme similar to the one proposed by Engeland (1991, 1995) it would be useful to determine if these classifications are supported by our genetic analyses. Seventy-nine NPGS accessions were not included in these studies since we could not generate a consensus score with <4% error rate. We can prioritize the genotyped portion of the NPGS garlic collection for cryopreservation by assuming that a single accession representing each genetic group of accessions as well as the genetically unique accessions should have priority over duplicate samples.

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