Theor Appl Genet (1995) 91:89–97

H. I. Maaß · M. Klaas

Infraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers

Received: 12 September 1994 / Accepted: 8 December 1994

Abstract Garlic (*Allium sativum* L.) is a sterile species of considerable variability with respect to morphological and physiological features. The crop presumably originated in West to Middle Asia from its progenitor *A. longicuspis* Regel and was transported from there to the Mediterranean and other areas of cultivation. In order to clarify older classification schemes, often based on small or biased collections, we used isozyme and RAPD markers to analyze and structure a collection of 300 accessions, many of which were gathered in Middle Asia close to the assumed center of origin. All of the accessions were first investigated with isozymes, and 48 were selected for a RAPD analysis. The resulting molecular markers were used to construct neighbor-joining dendrograms to group the accessions and to indicate the genetic distances between them. Based on the dendrograms and in conjunction with some morphological features, we propose an infraspecific classification of garlic with four major groups. In agreement with the results of other workers, *A. longicuspis* lies within the range of the species *A. sativum*. Numerous forms with varying degrees of domestication are part of our longicuspis group, from which presumably the more derived cultivar groups originated. The origin and spreading of the crop are discussed with respect to the geographical distribution and the genetic distances of the accessions.

Key words Garlic · Isozyme · RAPD · Crop evolution · Domestication

Introduction

Garlic, *Allium sativum* L., has been used since early times both as a spice and as a remedy for various ailments. Although seed sterile, it displays considerable variability, differing widely in morphological features like bulb size and color, scape height, flower characteristics, number and size of bulbils and cloves, as well as in physiological properties such as time of maturity, storability, and dormancy. Different cultivars are adapted to a wide range of climatic regions, some being grown in the tropics, others being frost-hardy. Thus, many strikingly diverse forms are known, and some of these have even been designated as botanical varieties. Helm (1956) recognized var. *sativum*, var. *ophioscorodon* (Link) Döll, and var. *pekinense* (Prokh.) Maekawa. Kazakova (1978) proposed a schema with two subspecies, ssp. *sativum* and ssp. *asiae-mediae* Kaz., with two forms each to classify a collection from the former Soviet Union. However, she did not delimit ssp. *asiae-mediae* against *A. longicuspis* Regel, the species considered to be either the closest wild relative or the ancestor of garlic, which is distributed from Tien-Shan, Pamir-Alai, Kopet-Dagh to Kurdistan, and South-east Anatolia.

More recently, other workers have distinguished between cultivar groups on the basis of morphological, physiological, and isozyme markers (Messiaen et al. 1993; Pooler and Simon 1993; Lallemand et al. 1994). However, the overall situation of infraspecific classification still remains problematic, since few markers or properties are unequivocally restricted to certain groupings and the different grouping systems have been based on limited accession material or biased collections. Thus, we decided to apply more readily scorable isozyme and random amplified polymorphic DNA (RAPD) markers in order to investigate and structure our comprehensive collection, which contains many accessions from Asia close to the assumed area of origin of the crop.

Furthermore, we wanted to address the following questions in this study: is there still considerably more variability close to the putative area of origin of the crop and how extensive does the loss in diversity concur with domestication, adaptation, and the spread to different climatic regions? How far can morphologically recog-

Communicated by G. Wenzel

H. I. Maaß (✉) · M. Klaas
Department of Taxonomy, Institute for Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany

nized types be similarly distinguished on the basis of molecular markers? Do isozyme and RAPD analyses yield comparable results with respect to the branching pattern in a dendrogram or the extent of genetic diversity in the different groups?

Isozyme polymorphisms of banding patterns in garlic have been reported for alcohol dehydrogenase (Siqueira et al. 1985), diaphorase (Pooler and Simon 1993; Lallemand et al. 1995), esterase (Siqueira et al. 1985; Chromátová et al. 1990; Pooler and Simon 1993; Lallemand et al. 1995), glucose-6-phosphate dehydrogenase (Pooler and Simon 1993), malate dehydrogenase (Lallemand et al. 1995), peroxidase (Etoh and Ogura 1981; Siqueira et al. 1985), phosphogluco-isomerase (Siqueira et al. 1985; Lallemand et al. 1995), and phosphoglucomutase (Pooler and Simon 1993). RAPD analysis (Williams et al. 1990) has been successfully applied to the infraspecific classification of a number of crop species; in the genus *Allium* close relations of *A. cepa* have already been analyzed with RAPDs (Wilkie et al. 1993).

In this study, 12 isozyme assays were tested and in total 22 loci were found, 10 of which were polymorphic. For the RAPD experiments, 15 arbitrary oligonucleotide primers were selected which resulted in 125 polymorphic bands.

Materials and methods

Plant materials

A collection of 300 garlic accessions from different countries was investigated by isozymes (Table 1), and 48 of these accessions were selected for RAPD analysis. Further information on the origin of the plant material is available on request.

Isozymes

The 12 isozymes analyzed were aconitate hydratase (aconitase) (ACO, E.C. 4.2.1.3), aspartate aminotransferase (AAT, E.C. 2.6.1.1), fructosebiphosphate aldolase (ALD, E.C. 4.1.2.13), glucose-6-phosphate isomerase (phosphogluco isomerase) (GPI, E.C. 5.3.1.9), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), leucine aminopeptidase (LAP, E.C. 3.4.11.1), malate dehydrogenase (MDH, E.C. 1.1.1.37), NADH dehydrogenase (diaphorase) (DIA, E.C. 1.6.99.2), phosphoglucomutase (PGM, E.C. 5.4.2.2), phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44), shikimate dehydrogenase (SKD, E.C. 1.1.1.25), and triosephosphate isomerase (TPI, E.C. 5.3.1.1).

The enzymes were extracted by grinding 150 mg fresh leaves in TRIS-HCl extraction buffer (Soltis et al. 1983) modified by the addition of 12% (w/v) PVP-40 000. Two to six plants per accession were tested. Chloroplasts from 8 accessions were isolated according to Katayama et al. (1991) to distinguish cytosolic and plastid isozymes. Percoll was replaced by sucrose (30, 40, 50%). The chloroplasts were extracted for enzyme analysis as described for the leaf material. Electrophoresis was carried out on 12.5% starch gels at 7 °C.

The AAT, DIA, GPI, and TPI banding patterns were resolved in a Lithiumborate/TRIS-citrate buffer system, and ACO, ALD, LAP, and PGD were detected in a Histidin-citrate buffer system (Raelson and Grant 1989). IDH, MDH, PGM, and SKD were analyzed in a TRIS-citrate buffer system (Soltis et al. 1983). The gels were stained according to Wendel and Weeden (1989: ALD, DIA, SKD) and Soltis et al. (1983).

Table 1 Accessions of garlic clones included in this study (*BG* Botanical Garden, *VRI* Vegetable Research Institute)

Origin (donor)	Gatersleben accession number	Isozyme type
Albania	K 9086	IIb
	K 9164, 9175	IIc
Australia	ALL 902	III
Belarus	ALL 684	Ic
Bulgaria	TAX 958	IIc
	ALL 142	Ia
China	ALL 116, 822	IIa
	ALL 859; K 9143	IVb[a]
	ALL 129	IVe
Cuba	ALL 802	IIa
Czech Republic	ALL 111, 115, 135, 144	III
France	—	IIb
Germany	ALL 264	Ic
	ALL 685	IIb
	ALL 92, 290, 493–511, 514, 516	III
	ALL 518–524, 526–527, 529–530	III
	K 8582; TAX 791	III
Georgia/Caucasus	ALL 766, 771, 774, 780, 790, 792,	Ia
	ALL 797–798; TAX 1743	Ia
	ALL 784, 787, 791, 794, 830	Ic
	ALL 765, 767, 775	IIa
	ALL 263, 658, 754–759, 761, 762,	IIc
	ALL 764, 768–770, 772, 777, 779,	IIc
	ALL 781–783, 788–789, 796, 825,	IIc
	ALL 829, 831–832, 834, 836–839,	IIc
	ALL 841, 846, 848, 851, 854,	IIc
	ALL 866–871, 873–874, 876;	IIc
	K 7995–7996	IIc
	ALL 753, 760, 763, 776, 778, 793,	IId
	ALL 795, 827, 853	IId
	ALL 824, 826, 840, 842–845, 847,	III
	ALL 852	III
	ALL 835	IVe
Greece	K 9082	IIc
India	L 380, 404, 412	Va
Indonesia	K 8999	IIa
	K 8977–8979; L 209	Vb
Iran	TAX 5130	IIa
Italy	ALL 651a; K 8591	Ic
	ALL 651b, 799, 804	IIc
Japan	TAX 452	IVb
	K 8831	IVb[a]
Kazakhstan	TAX 546	Id
	TAX 2336	IVc
	ALL 130–131, 877; TAX 3215	IVe
Korea	ALL 820	IIa
	ALL 936	IIc
	ALL 785–786, 818–819, 908, 937;	IVb[a]
	K 8007; L 225	IVb[a]
Kyrgyzia	TAX 1575, 2512, 3213	Ic
Maderia	L 513	IIa
Moldavia	TAX 2030, 2032	III
Nepal	L 163	Va
Poland	ALL 525	III
Romania	ALL 816; K 8593	IIb
	ALL 649, 652, 814–815, 817	III
Russia	ALL 253a, 850, 889	Ic
	ALL 849	IVe
Slovakia	ALL 655	Ic
	ALL 143, 654	IIc
	ALL 275, 292, 650, 653, 656–657	III
Spain	K 8792	IIb
	K 8590	IIc
Taiwan	K 8969–8970, 8972–8976, 8980,	IVb[b]
	K 8982, 8991, 8995–8998	IVb[b]
	K 8981, 8983, 8985–8990, 8992, 8994	Vb

Table 1 (Continued)

Origin (donor)	Gatersleben accession number	Isozyme type
Tajikistan	TAX 1337, 1413, 1429	Ib
	TAX 2533	Ic
	ALL 691–692	IIa
	TAX 1319	IVb
Thailand	K 8960–8961	IIa
	K 8965–8968	Vb
Tunisia	T 11, 81–82, 128, 226	IIa
Turkmenistan	ALL 261	IIc
	K 9140	IVb[a]
Ukraine	ALL 133	Ia
	ALL 149	Ic
USA	ALL 806, 893, 894, 907	IIb
	ALL 805, 807, 862, 864, 865;	IIc
	K 8006; TAX 1808, 1816	IIc
	ALL 881–888, 892, 895, 896, 898,	III
	ALL 899–901, 903–904, 906, 910;	III
	TAX 2060, 2600	III
	ALL 803	IVb[a]
	ALL 890; TAX 1793	IVc
Uzbekistan	ALL 136	Ia
	TAX 2739	Ib
	TAX 2740	Ic
	TAX 1125	IVb
Yugoslavia	ALL 808, 810, 812	IIb
(BG Alma-Ata)	K 8003	IVb
	K 8002	IVd
(BG Munich)	TAX 505	Ib
(BG Poznan)	TAX 912	IVc
(BG Rotterdam)	TAX 1437	IVa
(BG Vienna)	ALL 100a	III
	ALL 100b	IVb
(VRI Olomouc)	ALL 146	IVe

[a] var. *pekinense*
[b] Morphological grouping was not possible because of the poor growth of these plants

RAPD analysis

Total DNA was isolated according to Saghai-Maroof et al. (1984) with slight modifications. Plant leaves were ground in liquid nitrogen in a mortar; the powder was extracted in 2% CTAB, 0.1 *M* Tris/HCl, pH 8.0, 1.4 *M* NaCl, 10 m*M* EDTA, 10 m*M* EGTA, 0.2% 2-mercaptoethanol for 20 min at 60 °C in a shaking waterbath. A dichloromethane/isobutanol (24:1) extraction was performed twice on the suspension, then the DNA was precipitated from the aqueous phase by the addition of 0.6 volume of isopropanol. The DNA was recovered by low-speed centrifugation (10 min at 4000 *g*), rinsed with 70% and 96% ethanol, dried, and redissolved in TE. After treatment with 10 µg/ml RNase A for 2 h at 37 °C, the DNA was purified in 3 ml CsCl gradients according to standard procedures (Sambrook et al. 1989). The purified DNA was dissolved and stored in TE buffer, and the concentration was determined fluorometrically.

Polymerase chain reactions (PCR) were carried out using 15 arbitrary primers obtained from Operon Technologies, Alameda, Calif. (Table 2). The amplification conditions were optimized according to Blanchard et al. (1993), and each template was amplified at two concentrations. The 25-µl reactions contained 10 m*M* Tris/HCl, pH 8.3, 1.8 m*M* $MgCl_2$, 50 m*M* KCl, 20 m*M* NH_4Cl, 0.4 µ*M* primer, 0.6 units *Taq*-polymerase (Boehringer Mannheim or Gibco BRL) 0.15 m*M* each of dATP, dGTP, dCTP, and dTTP, and either 50 ng or 100 ng of template DNA, overlayed with a drop of mineral oil. After the reactions were combined on ice, the tubes were placed in a preheated Grant Autogene waterbath-cycler for a 2-min denaturation at 95°C, followed by 36 cycles of 0.8 min at 37°C (annealing), 1.2 min at 72°C (elongation), and 0.8 min at 96°C (denaturation). One-third of the reaction mixtures was separated on 1.5% agarose gels in 0.5 TBE, followed by staining with ethidium bromide (Sambrook et al. 1989).

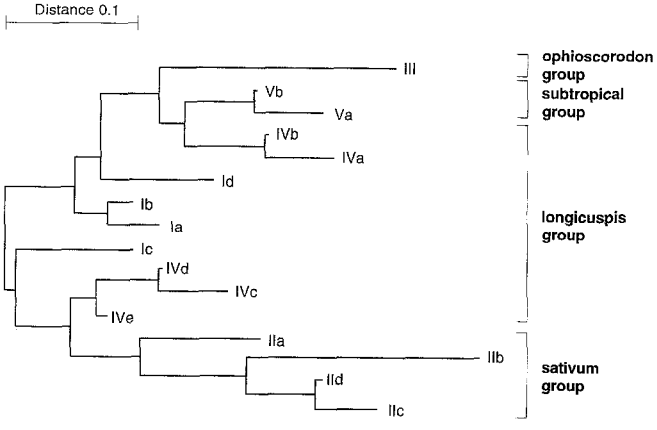
Table 2 RAPD primers and number of scored bands for each primer

Primers	Sequence (5′ to 3′)	Number of scored bands
OPC-5	GATGACCGCC	8
OPC-7	GTCCCGACGA	8
OPC-9	CTCACCGTCC	10
OPC-12	TGTCATCCCC	4
OPC-13	AAGCCTCGTC	6
OPD-1	ACCGCGAAGG	13
OPD-3	GTCGCCGTCA	8
OPE-11	GAGTCTCAGG	6
OPE-17	CTACTGCCGT	14
OPG-13	GTCTCCGCCA	8
OPG-14	GGATGAGACC	7
OPG-19	GTCAGGGCAA	2
OPAB-4	GGCACGCGTT	15
OPAB-18	CTGGCGTGTC	10
OPAC-2	GTCGTCGTCT	6

Data analysis

The gels were analyzed either directly (isozyme) or from enlarged photographic prints (RAPD). Bands were scored as present or absent. RAPD bands were scored as present or absent. For the isozyme analysis presence or absence of specific alleles were deduced from the banding patterns (Wendel and Weeden 1989). For each experiment, the results were combined in a 1/0 data matrix that was used as input file for the program package TREECON, version 3.0 (Van de Peer and de Wachter 1993). From both matrices, the pairwise distances between the 16 isozyme types and the 48 selected accessions (RAPD) were calculated as the fraction of differing 1/0 values with the option "no correction". From each distance matrix, a tree was constructed (Figs. 1, 2) using the neighbor-joining method (Saitou and Nei 1987).

Fig. 1 Neighbor-joining dendrogram of isozyme types. The 16 types are based on the different allelic configurations inferred from the isozyme banding pattern of the 300 garlic accessions listed in Table 1



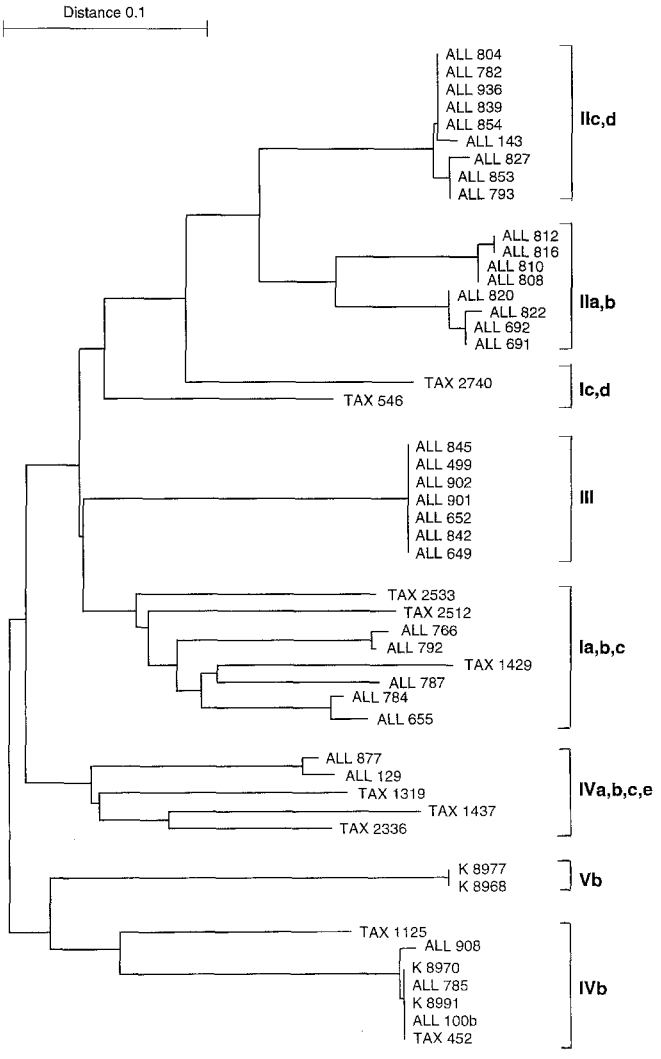


Fig. 2 Neighbor-joining dendrogram of 48 garlic accessions, based on 125 polymorphic RAPD markers. The *Roman numbers* designate the isozyme type to which the plants of the indicated branch of the dendrogram belong

Results

Isozyme banding patterns

Twenty-two loci were scored, 3 each for AAT, MDH, and TPI, 2 each for ACO, DIA, GPI, PGD, and 1 each for ALD, IDH, LAP, PGM, and SKD. Of these, 12 loci were invariant: *Aat-1*, *Aat-2*, *Aat-3*, *Ald*, *Dia-2*, *Gpi-1*, *Idh*, *Mdh-1*, *Mdh-2*, *Pgd-1*, *Pgd-2*, and *Tpi-1*. Three alleles were observed for the locus *Aco-2*, and 2 alleles were found for each of loci *Aco-1*, *Dia-1*, *Gpi-2*, *Lap*, *Mdh-3*, *Pgm*, *Skd*, *Tpi-2*, and *Tpi-3*. Not all of the expected banding patterns were observed, so none of the assumed homozygous phenotypes were found in *Dia-1a*, *Gpi-2b*, *Mdh-3a*, and *Skd-a*. ACO, DIA, LAP, PGM, and SKD had patterns characteristic of monomeric enzymes, while the GPI, MDH, and TPI patterns were characteristic of dimeric enzymes. The enzyme proteins of loci *Aat-1*, *Dia-2*, *Gpi-1*, *Pgd-1*, *Mdh-3*, and *Tpi-1* were found in enriched chloroplast fractions.

Accessions with the same banding pattern were combined into 1 isozyme type, and overall 16 types were registered (Table 3). These were combined into 5 larger units on the basis of the occurrence of some common alleles.

The neighbor-joining dendrogram derived from the isozyme data shows the relationships between the isozyme types and the taxonomical groups (Fig. 1). The uppermost long branch, type III, represents garlics that were determined to be var. *ophioscorodon*. Types Va/b and IVa/b are closely related. However, from the small number of investigated isozymes, type V could not be separated as a group of its own. Furthermore, there is no clear hierarchical structure between type I and IV. Consequently, these were combined into the longicuspis group based on their morphological features. This group includes also var. *pekinense* as a part of group IVb. Types IIa–IIc form together a well-defined group.

RAPD analysis

Two hundred arbitrary dekamer primers were screened for their suitability in PCR reactions with *A. sativum* DNA templates, and 15 were selected for this study (Table 3). The reactions were reproducible in both different cycler runs and in different reactions during the same run. CsCl purification of the templates and the exact determination of the DNA concentration were crucial for the reproducibility, since mucilagenous and other plant compounds in less thoroughly prepared samples rendered the PCR reactions incomplete. Two to fifteen bands from 400 to 3000 bp in size were scored as being present or absent per primer reaction. Only those bands which were scorable for their presence/absence in all 48 DNA templates and which were detectable at both template concentrations were considered as markers. Some primers gave a rather polymorphic pattern of reaction products with different templates, while other primers mainly produced bands common to all accessions. Only bands which gave at least one distinction between the accessions were scored.

From the dendrogram based on RAPD data, three clearly distinct groups are found that are internally rather homogeneous (Fig. 2). All accessions of the morphologically defined sativum group fall together (ALL 804 to ALL 691, from top downward, corresponding to isozyme group II). Two subdivisions are apparent, one containing non-bolting cultivars mainly from Georgia/Caucasus and the Mediterranean (ALL 804 to ALL 793), the other accessions are bolting plants from East and Middle Asia and from the Balkans (ALL 812 to ALL 691).

Still more set apart and genetically homogeneous are the garlics of var. *ophioscorodon* (ALL 845 to ALL 649), even though the accessions have been collected from rather disparate sources in East Germany, Romania,

Table 3 Polymorphic isozyme bands in garlic

Locus	Allele	Isozyme types														
		Ia	Ib	Ic	Id	IIa	IIb	IIc	IId	III	IVa	IV	IVc	IVd	IVe	Va	Vb
Aco-1	*a*	–[a]	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–
	b	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+
Aco-2	*a*	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–
	b	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+
	c	–	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–
Dia-1	*a*	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–
Gpi-2	*b*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Lap	*a*	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+
	b	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–
Mdh-3	*a*	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–
Pgm-2	*a*	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–
	b	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Skd	*a*	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	–
Tpi-2	*a*	–	–	+	–	+	+	+	+	–	–	–	+	+	+	–	–
	b	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	+
Tpi-3	*a*	–	–	+	–	+	+	+	+	–	–	–	+	+	+	–	–
	b	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	+
Number of accessions		12	5	19	1	18	14	68	9	86	1	32	4	1	8	4	18

Some rare alleles can serve as diagnostic markers for special taxonomical groups, e.g. *Dia-1a*, *Gpi-2b*, and *Pgm-a*. Type I is characterized by alleles *a* and *c* for *Aco-2* and type IV by allele *b* for *Lap*. In type II, allele *a* was observed for *Aco-1*. For more differentiation in types IIa/b and types IIc/d, the alleles *a* for *Aco-2* and *Dia-1* in the heterozygous state can be used. Type III is mainly characterized by alleles *a* for Pgm and Skd, while allele *b* for *Gpi-2* was only found in type V

[a] +, Band present; –, band absent

and overseas (Table 1). A third well-separated group are the subtropical accessions (K 8977, K 8968), though only 2 cultivars were analyzed. These are distantly related to accession TAX 1125, determined as *A. longicuspis* sensu Regel.

Compared with the ophioscorodon and sativum groups, the remaining accessions are genetically highly diverse, and the RAPD data do not suggest a common origin. Three major subdivisions appear, comprising the accessions TAX 2533–ALL 655, ALL 877–TAX 2336, TAX 1125–TAX 452. These are internally rather heterogeneous and seem less well-defined than the presumably more derived forms of ophioscorodon or the sativum groups. The accessions of var. *pekinense* (ALL 785, ALL 908) also associate with the longicuspis group, however, they are not genetically set apart from the other longicuspis group accessions (see bottom branch of the dendrogram, Fig. 2).

Discussion

A large collection of 300 garlic accessions was first analyzed with isozymes, which resulted in their differentiation into 16 types. Accessions of each type were then chosen for a RAPD analysis in order to compare the classifications resulting from the two approaches. Due to their large number and random distribution, RAPD markers should give a good approximation of the overall genetic distance between the compared taxa. Nevertheless, the much smaller number of isozyme loci gave a nearly identical branching pattern in the resulting phenogram. Thus, for an initial screening and structuring of a large collection, isozyme analysis is a valuable tool, especially after establishing the system, new accessions can be fitted in readily on the basis of the testing of a few diagnostic enzymes. RAPD analysis provides a more detailed and quantitative representation of the genetic diversity and distances in a smaller group to be investigated more thoroughly.

Infraspecific classification

An infraspecific classification of the species into four groups is proposed. In addition to our isozyme and RAPD data, morphological features are also taken into account for a more comprehensive description. Since we had the opportunity to investigate accessions analyzed by Lallemand et al. (1995) and Pooler and Simon (1993), we were able to compare our analysis with their results.

The sativum group

Morphologically, the group is divided into bolting and non-bolting cultivars. Based on isozyme and RAPD analysis, we found three major subdivisions corresponding to isozyme types IIa and IIb (bolting and coiling), and IIc/d (incomplete- or non-bolting).

Type IIa/b plants bolt later than other types, and the flowers do not open under our conditions. The accessions of type IIa have been collected from East and South-east Asia, Tajikistan, Georgia/Caucasus, Iran, Tunisia, and Madeira. Our type IIa corresponds to Messiaen's group V of southern Mediterranean and tropical cultivars (Messiaen et al. 1993). In these plants, several isozymes are present in a heterozygous state (*Aco-1*, *Aco-2*, *Mdh-3*, *Tpi-2*, *Tpi-3*), which could be an explanation for their adaptability to rather diverse climates. Type IIb has a more limited area of cultivation, i.e. the Mediterranean and the Caucasus. It corresponds to Messiaen's group I. Known cultivars are 'Bai Pi Suan' (China, isozyme type IIa), 'Goulurose' (France, type IIb), and 'Creole' (USA, type IIb).

Isozyme types IIc and IId show only little variability on the isozyme and RAPD level. The plants are mostly non-bolting, some are incomplete-bolting (25–50 cm high) with drooping inflorescences. The scape is usually shorter than the sheath of surrounding leaves so that the few pea-sized bulbils disrupt the surrounding tissue. Occasionally flowers were observed, but they were always severely deformed. These two types correspond to group III from Messiaen et al. (1993) and to a large extent to the "non-bolting sativum type" of Pooler and Simon (1993). The cultivars have mainly been collected in Georgia/Caucasus under the name "kartuli niori" and in the Mediterranean. Known cultivars are 'Thermidrome' (France), 'California Early' and 'Italian Purple' (USA).

The sativum group was possibly developed a long time ago in the Mediterranean area. In ancient reports on garlic cultivation [the "scorodon" of Theophrast (390–305 BC) according to Helm (1956)], already several morphological and physiological types are mentioned, e.g. early and late forms of different sizes. An early cultivation, which means vegetative propagation in the absence of wild relatives native to Middle East/Central Asia, could explain the relative genetic homogeneity of the type II cultivars.

The ophioscorodon group

Isozyme type III is specific to the morphologically distinct variety *ophioscorodon* (Link) Döll, which mainly includes middle and eastern European accessions. Our accessions have been collected from East Germany, East Europe, and the USA. This group with 86 analyzed accessions is genetically homogeneous and corresponds with the "early senescing, non-fertile flowering type" of Pooler and Simon (1993) according to their PGM pattern 2 and is probably synonymous with group IV from Messiaen et al. (1993). Known cultivars are 'Bzenecký palicák' (Czech Republic), 'Thuma Rocambole' and 'Trail of Tears' (USA).

Overall morphological differences among these accessions are slight. All plants are bolting and coiling, the color of spathae is whitish-green. The flowers are often deformed, being four-merous instead of three-merous. The inner filaments usually have two lateral teeth and the outer filaments have four. Only yellow anthers are produced, indicating pollen sterility (Etoh 1986). In many cultivars the bulbs and flowers remain completely covered by a non-dehiscent spatha, which might be a part of the domestication process.

Transcaucasia and the region to the north of the Black Sea is the probable origin of this group, since type III cultivars do not grow well under Mediterranean conditions (CM Messiaen, personal communication). This group possibly derives from the longicuspis group and developed a long time ago in more temperate climates.

The longicuspis group

This group contains bolting and coiling plants that are defined as *A. longicuspis* sensu Regel (1875) in agreement with additional descriptions of Vvedenskij (1935), Wendelbo (1971), and Kollmann (1984). Morphologically the plants are mainly recognizable by small bulbils, numerous flowers with exserted anthers, and inner filaments with two or four lateral teeth. Many accessions still produce more-or-less fertile flowers, though the formation of open flowers with purple anthers depends on favorable growth conditions, like the hot summer of 1994. In this group isozyme types I and IV are combined. The major split into these subdivisions is also recognized by the RAPD analysis, though the internal heterogeneity of these factions becomes more apparent from the RAPD-based dendrogram (Figs. 1, 2). The longicuspis group is internally considerably more heterogeneous than the other groups discussed above. According to our analysis of the same accessions, Pooler and Simon's (1993) "fertile, pollen-shedding ophioscorodon types" correspond to our types Ia and IVe, whereas their "yellow-anthered pollen-sterile ophioscorodon types" are part of our type Ic. Known cultivars are 'Andizhanskij' (Uzbekistan, isozyme type Ia), 'Dunganskij' (Kazakhstan, type IVe), and 'Jampol' skij' (Ukraine, type Ic).

The subspecies *asiae-mediae* (Kazakova 1978) contains plants of this group. However, Kazakova also includes non-bolting plants that are restricted to isozyme type IIc/d. This subspecies is not supported by our data.

Generally, the longicuspis group might be considered to be an assortment of spontaneous or subspontaneous forms of comparatively primitive (still fertile to some extent) and more domesticated cultivars. These forms

have probably developed under less stringent selection conditions than, for instance, the more derived cultivars from the tropics or the temperate climate of Middle to East Europe. In agreement with Pooler and Simon (1993) our data do not suggest the placement of *A. longicuspis* as a species outside of *A. sativum*. Crossings possibly did still occur in the not-too-distant past, either between cultivars or with weedy relatives from the area of origin of the crop, thus engendering the rather diverse genetic background among these accessions.

Interestingly, var. *pekinense* accessions (ALL 785, ALL 908 in Fig. 2, bottom branch) are genetically very close to other isozyme type IVb plants (e.g. ALL 100b) even though they are morphologically distinct (40–75 cm high, relatively broad leaves, non-coiling inflorescence, few relatively large bulbils, a very long and often non-opening spatha). Contrary to the other earlier recognized botanical varieties (Helm 1956), the morphological distinctions of these plants do not reflect their genetic distance to other forms according to our isozyme and RAPD data. Therefore, we consider the pekinense plants to be only a subgroup within the longicuspis range. Their distinctive morphological features can be interpreted as part of the domestication process while the underlying genetic background has not yet changed accordingly. A known cultivar is 'Asian Tempest' (USA).

The subtropical group

Type V is clearly separated from all other types by both isozyme and RAPD markers even though only 2 DNAs have been analyzed by RAPDs. It possibly originated from the longicuspis group a long time ago in India after acquiring special adaptations (e.g. for growth and storage conditions) necessary for its spread through the tropics. This assumption is substantiated by early reports of garlic use in a Sanscrit manuscript (Titterington 1993). No stalks and flowers were observed due to the poor growth of these accessions under our conditions, so evaluation of morphological characteristics was not possible.

Geographical distribution

Garlic has long been known in ancient Mesopotamia and Egypt. Records in Sumerian and Akkadian cuneiform texts date back approximately to 2600 BC (Bottéro 1980), and the earliest plant remains from Egypt were discovered in the tomb of Tutankhamun from 1337 BC (Germer 1989). In East Asia garlic cultivation has a tradition of no more than 1500 years (Prokhanov 1930).

The opinions of different authors vary considerably about the origin of garlic or its place of domestication. Ford-Lloyd and Jackson (1986) speculated that garlic was originally grown in China and Europe, whereas Hems (1951) assumed that "garlic was carried from the lands of the Scythians along the well-trodden caravan routes leading to Asia Minor." Burkill (1966) noted that garlic has been cultivated in India from remote times and spread from there further to the east. According to Vavilov (1935), *Allium sativum* and *A. longicuspis* belong to the Inner-Asiatic center of origin that includes Northwest India, Afghanistan, Tajikistan, Uzbekistan as well as western Tien-Shan. For the large garlic form of the Mediterranean area, however, he postulated a secondary center of origin.

The geographical distribution of our accessions from the Old World is shown in Fig. 3. Our results indicate that the more derived forms of garlic originated from the heterogeneous longicuspis group. Some accessions with purple anthers were still found spontaneous or subspontaneous in western Tien-Shan (Kyrgyzia), the Karatau

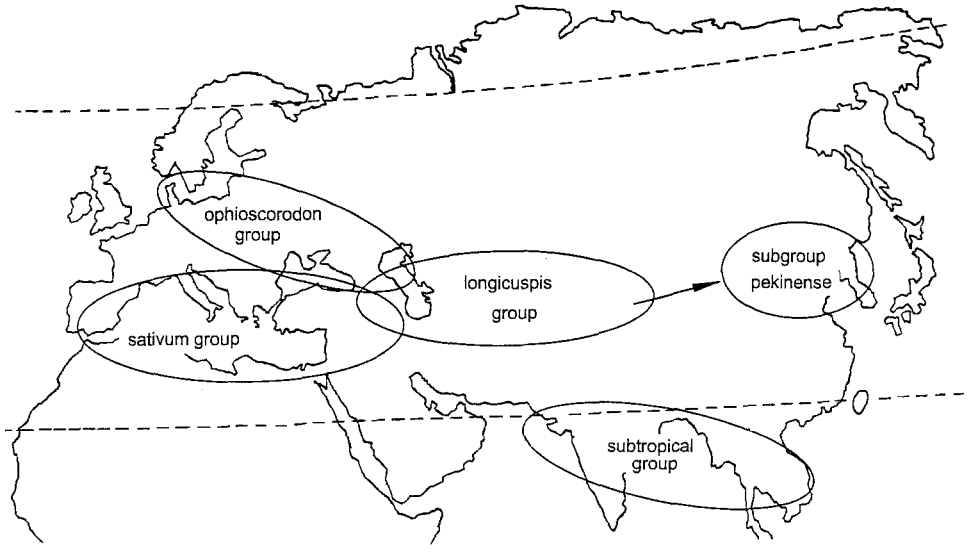


Fig. 3 Geographical distribution of the investigated garlic groups

mountain range (Kazakhstan), and the Chatkal mountains (Uzbekistan). According to Etoh (1986), the western Tien-Shan is the most likely original habitat of garlic. The most primitive cultivars of our collection with fertile flowers unobstructed by the spatha had isozyme type Ia. These accessions were collected from Uzbekistan (near Andizhan) and from West and South Georgia/Caucasus. According to information from local farmers, the Georgian accessions of type Ia were possibly introduced from Middle Asia. The fertile garlics collected in the Caucasus (Etoh et al. 1992) might be of the same origin. It is remarkable that such accessions were not gathered from the more isolated Inner-Georgian districts. Type IVe is another isozyme type containing accessions with purple anthers. Four of our 8 accessions were collected in Kazakhstan, the most likely area of origin.

Subgroup pekinense is genetically still quite close to the middle Asian longicuspis forms of isozyme type IVb. According to ancient Chinese literature, garlic was introduced from Iran or Turkmenistan to North China no more than 1500 years ago.

In contrast to the pekinense subgroup, the sativum group probably derived from longicuspis forms in West Asia more than 3000 years ago. Sativum plants have been grown in the Mediterranean area since ancient times and have spread from there throughout the world during the last 500 years. Ophioscorodon group accessions are distributed from Central and eastern Europe to the Caucasus. Many cultivars, however, are today grown in temperate America and Australia. The garlic accessions from subtropical regions of South and Southeast Asia are not connected to any of the other varieties of the crop. Our results suggest that these forms originated independently a long time ago from the longicuspis group, perhaps in northern India.

Studies on the origin and development of crops like garlic will become increasingly difficult since plants are often spread from private collections, botanical gardens, and even genebanks without proper documentation, so that the true origin of an accession may be uncertain. Furthermore, old cultivars are disappearing at an increasingly rapid pace, being replaced by newly imported varieties, especially if the crop is grown for the international market. Our collection is comparatively comprehensive in accessions from East Germany, East Europe, Middle Asia, and North Korea, and were gathered before these countries were opened to the world market of crops. In a future study, it would be important to include more landraces from Turkey, Syria, Iran, Iraq, Afghanistan, Nepal, and India, since Asia Minor and the Middle East is one of the oldest areas of cultivation of garlic.

Acknowledgements We thank P. Hanelt and G. Linne von Berg for their valuable comments on the manuscript. The technical assistance of T. Tölle and B. Wohlbier is gratefully acknowledged. Furthermore, we thank D.-E. Lesemann, BBA Braunschweig, for the South Asian material. This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

References

Blanchard MM, Taillon-Miller P, Nowotny P, Nowotny V (1993) PCR buffer optimization with uniform temperature regimen to facilitate automation. PCR Methods Applic 1:234–240

Bottéro J (1980) Knoblauch. In: Edzard DO (ed) Reallexikon der Assyriologie und vorderasiatischen Archäologie vol 6 (in French). Gruyter, Berlin, pp 39–41.

Burkill IH (1966) A dictionary of the economic products of the Malay Peninsula, 2nd edn. Ministry Agric Co-op, Kuala Lumpur

Chromátová S, Turková V, Klozová E (1990) Protein complex and esterase isoenzyme patterns of *Allium sativum* L. cultivars and clones-regenerants. Biol Plant 32:321–331

Etoh T (1986) Fertility of the garlic clones collected in Soviet Central Asia. J Jpn Soc Hortic Sci 55:312–319

Etoh T, Ogura H (1981) Peroxidase isozymes in the leaves of various clones of garlic, *Allium sativum* L. Mem Fac Agric Kagoshima Univ 17:71–77

Etoh T, Kojima T, Matsuzoe N (1992) Fertile garlic clones collected in Caucasia. In: Hanelt P, Hammer K, Knüpffer H (eds) The genus *Allium*–taxonomic problems and genetic recources. (Proc Int Symp.) IPK, Gatersleben, Germany, pp 49–54

Ford-Lloyd B, Jackson M (1986) Plant genetic recources: an introduction to their conservation and use. Edward Arnold, London

Germer R (1989) Die Pflanzenmaterialien aus dem Grab des Tutanchamun. In: Eggebrecht A, Schmitz B (eds) Hildesheimer Ägyptologische Beiträge. Pelizaeus-Museum, Hildesheim, no 28:1–94

Helm J (1956) Die zu Würz- und Speisezwecken kultivierten Arten der Gattung *Allium* L. Kulturpflanze 4:130–180

Hems J (1951) Garlic through the centuries. Veg News 30:100–101

Katayama H, Sasakuma T, Ogihara Y (1991) Physical map of chloroplast DNA of the onion *Allium cepa* L., showing the location of photosynthesis-related genes. Jpn J Genet 66:421–431

Kazakova AA (1978) Česnok (*Allium sativum* L.) In: Brezhnev DD (ed) Kul'turnaja flora SSSR, vol 10 (in Russian). Kolos, Leningrad, Russia, pp 71–77

Kollmann F (1984) *Allium*. In: Davis PH (ed) Flora of Turkey and the East Aegean islands, vol 8. University Press, Edinburgh, pp 98–211

Lallemand J, Messiaen CM, Briand F, Etoh T (1995) Delimitation of varietal groups in garlic (*Allium sativum* L.) by morphological and physiological characters, verified by the isozyme method. In: First Int Symp Edible Alliaceae. (Abstr, p 15). Acta Hortic 137 (in press)

Messiaen CM, Cohat JC, Leroux JP, Pichon M, Beyries A (1993) Les allium alimentaires reproduits par voie végétative. INRA, Paris

Pooler MR, Simon PW (1993) Characterization and classification of isozyme and morphological variation in a diverse collection of garlic clones. Euphytica 68:121–130

Prokhanov JI (1930) About culinary alliums of China and Japan – a critical literature view (in Russian). Bull Appl Bot Genet Plant Breed 24:123–183.

Raelson JV, Grant WF (1989) An isoenzyme study in the genus *Lotus* (Fabaceae). Experimental protocols and genetic basis of electrophoretic phenotype. Theor Appl Genet 77:595–607

Regel E (1875) Alliorum adhuc cognitorum monographia. Acta Horti petrop 3, 2:1–266

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Saitou N, Nei M (1987) The neigbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Habor Laboratory Press, Cold Springer Harbor, N.Y.

Siqueira WJ, Medina Filho HP, Salles Lisbão R, Fornasier JB (1985) Caracterização isoenzimática e morfológica de clones e introduções de alho. Bragantia 44:357–374

Soltis DE, Haufler CH, Darrow DC, Gastony GJ (1983) Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. Am Fern J 73:9–27

Titterington R (1993) Zestful garlic. The Garden 118:502–503

Van de Peer Y, de Wachter R (1993) TREECON: a software package for the construction and drawing of evolutinary trees. Comput Appl Biosci 9:177–182

Vavilov NI (1935) The phyto-geographical basis for plant breeding [first published in Russian, translated by Löve D (1992)]. In: Dorofeyev VF (ed) Vavilov NI Origin and geography of cultivated plants (English edn). University Press, Cambridge, pp 316–366

Vvedenskij AI (1935) *Allium* L. In: Komarov VL (ed) Flora SSSR, vol 4. Izd Akademija Nauk, Leningrad, pp 112–280

Wendel JF, Weeden NF (1989) Visualisation and interpretation of plant isozymes. In: Soltis DE, Soltis PS (eds) Isozymes in plant biology. Dioscorides Press, Portland, Ore., pp 5–45

Wendelbo P (1971) Alliaceae. In: Rechinger KH (ed) Flora Iranica. Akad. Druck- und Verlagsanstalt, Graz, cont no 76, pp 1–100

Wilkie SE, Isaac PG, Slater RJ (1993) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor Appl Genet 86:497–504

Williams GK, Kubelik AR, Livak KL, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535