Genetic Characterization of an Unknown Chinese Bulbous Leek-like Accession and its Relationship to Similar *Allium* Species

Borut Bohanec,¹ Marijana Jakše, and Predrag Šesek

University of Ljubljana, Biotechnical Faculty, Centre for Plant Biotechnology and Breeding, Jamnikarjeva 101, 1111 Ljubljana, Slovenia

Michael J. Havey

Agricultural Research, U.S. Department of Agriculture, Department of Horticulture, 1575 Linden Drive, University of Wisconsin, Madison, WI 53706

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Abstract. Bulbous leek-like plants are a poorly defined group usually assigned to the *Allium ampeloprasum* complex. Studies were initiated to determine the origin of an unusual bulbous accession received in Shanxi province in China, where it was used in diet as garlic but propagated by seeds, and to genetically compare this accession with morphologically similar plants from Europe. Genetic analyses included karyotypes and genomic in situ hybridization, pollination to leek, genome size determination and nuclear rDNA and plastid DNA polymorphisms. Results revealed that this agriculturally interesting accession from China is a so far unknown variant within tetraploid *A. ampeloprasum* cultivated taxa. We also observed that great-headed garlic did not share derived states in the chloroplast with leek, revealing that this cultivated plant does not possess the cytoplasm of leek or garlic, while its 1C genome size was 17% bigger than those of studied leek and bulbous-leek accessions.

The genus *Allium* L. contains about 700 species, several of which are edible and are among man's most ancient cultivated crops. The edible alliums and their areas of cultivation have been recently reviewed by Fritsch and Friesen (2002). The large number of *Allium* species and the diversity among the cultivated forms, which are spread across diverse climates throughout the Northern hemisphere, permits for the occasional discovery of new cultivated forms, not yet well described. Most botanical classifications of species were based on morphological characterizations, however modern genetic techniques can provide new insights regarding phylogenetic relationships.

Major crops within subgenus *Allium* section *Allium* are *A. sativum* L. (garlic) and the various cultivated forms of *A. ampeloprasum* L. According to Mathew (1996), *A. ampeloprasum* is widespread throughout the Mediterranean region and Middle Eastern countries; however, populations have been recorded in other areas possibly due to ancient introductions. Using the botanical terminology of Mathew (1996), wild *A. ampeloprasum* is thought to be the progenitor form of crop plants *A. porrum* L. (leek) and *A. kurrat* Schweinf., both cultivated for their leaves. A third variant, termed great-

headed garlic, is cultivated for its bulbs and asexually propagated. Mathew (1996) states that great-headed garlic closely resembles *A. ampeloprasum* and does not warrant classification as a separate species. Mathew (1996) also described *A. commutatum* Guss. and *A. bourgeauii* Rech. as closely related to *A. ampeloprasum*. Similarly Brewster (1994), following the classification proposed by Hanelt (1990), cites two major crop species within subgenus *Allium* section *Allium*: *A. sativum* (garlic) and *A. ampeloprasum* classified into the four horticultural groups of leek, kurrat, great-headed garlic and pearl onion. Based on their use, Messiaen et al. (1993) classified leek-like plants as *A. polyanthum* Schultes, *A. ampeloprasum* (*sensu stricto*), *A. commutatum* and *A. scorodoprasum* L.. These authors mention that wild *A. ampeloprasum* var. *bulbiferum* (from Yeu Island, off Western France) and Great Headed Garlic are chemically closer to garlic.

The ploidy levels of bulbous leek-like plants are highly variable. As recently reviewed by Mathew (1996), the chromosome numbers of *A. ampeloprasum* ranged from 16 to 56, *A. porrum* from 32 to 48, *A. kurrat* at 32, *A. commutatum* from 16 to 48, and *A. polyanthum* at 32. The predominant ploidy level among these species is tetraploid (32), while other ploidies are less frequent and may be associated with vegetative propagation. *Allium ampeloprasum*, *A. porrum* and *A. commutatum* may possess variable numbers of B chromosomes.

Our studies focused on the genetic characterization of economically useful forms of bulbous alliums with leaf morphologies

similar to leek. We concentrated on an unusual accession received in Shanxi province, China, where it is seed propagated but used in diets as garlic. Studies included the majority of morphologically similar alliums, using karyotype analysis, genomic in situ hybridization (GISH), pollination, genome size determination and molecular markers using nuclear rDNA and plastid cpDNA probes.

Material and Methods

Plant material. In Shanxi province, China, in 1996, local agronomists gave B. Bohanec and M. Jakše two accessions of an *Allium* described as seed-forming garlic. No further information on the origin of these accessions could be obtained. These accessions obtained in Shanxi province in China will be referred to as Chinese bulbous leek. Plants were raised from seeds and maintained in the experimental field of the Biotechnical Faculty in Ljubljana. Sensory evaluation was done by 10 panelists, each of whom was given bulbs of garlic and Chinese bulbous leek. The taste attributes (aroma intensity, crispness, and pungency (scale 1 = low, 5 = high intensity) were assessed immediately after peeling and cutting samples.

Leaves and bulb tissues were obtained from *A. polyanthum*—two accessions (the first from St. Mandrier sur Mer, France and the second from Ile d'Yeu, France—for the second, determination of species was noted as not certain), *A. commutatum* (from Corsica), two accessions of great-headed garlic (from Spain and from India), and three forms of bulbous leeks—one from Ile d'Yeu (France), one from Apt (France) and one from Guadeloupe were kindly provided by C.M. Messiaen (Le Teil, France), from his personal collection. Additional samples that were included in studies were bulb onion, leek, garlic, bunching onion and *A. tuberosum* Rottl., which were either commercial varieties or accessions used in other genetic studies at the University of Wisconsin.

Chromosome preparations. The root tips were excised from 1- to 2-cm-long roots obtained after germinating on moist filter paper at 25 °C. They were then treated by a 1% (v/v) solution of monobromonaphthalene for 24 h at 4 °C or by 2 mM 8-hydroxyquinoline for 0.5 to 2 h at room temperature followed by storing for a further 0.5 to 2 h at 4 °C. The tissue was fixed in Farmer's fixative at for at least 24 h at 4 °C. Root tips were digested in 4% cellulase (Onozuka) and 1% pectolyase (Y23) for 45 min before squashing in 45% acetic acid. Cover slips were removed after freezing at –80 °C and the slides were dried. For karyotype analysis chromosomes were stained with 2 µg·mL⁻¹ 4'-6-diamidino-2-phenylindole (DAPI). The best quality preparations were used for chromosome counting and arranging into karyograms on the basis of increasing chromosome length. Measurements of chromosome length were performed using the program Image-Pro Express 4.0.

Genomic in situ hybridization. Total genomic DNA was isolated from young leaves using standard cationic detergent cetyl-tri-

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¹Corresponding author; e-mail borut.bohanec@bf.uni-lj.si.

methylanmonium bromide (CTAB) method. Total genomic DNA from leek, bulbous leek, garlic and great-headed garlic was labeled with biotin using BioNick labeling kit (Life Technologies) according to the supplier's instructions. Total genomic DNA of garlic, leek and of the undetermined *Allium* species was fragmented by autoclaving for 6 min and used as blocking DNA. Following standard RNase and pepsin treatments, in situ hybridization and probe detection were performed according to a procedure modified from Puizina et al. (1999). Briefly, chromosomes were denatured in 70% formamide in 2× SSC for 2 min at 70 °C, dehydrated through a cold ethanol series and air dried. The probe mixture, containing 40 to 200 ng of biotinylated genomic DNA, 20 to 60 times that amount of blocking DNA (when used), 50% deionized formamide, 10% dextran sulphate, salmon sperm DNA and 2× SSC, was denatured at 80 °C for 10 min, and then immediately put on ice for 5 min. The denatured probe mixture was applied on the denatured chromosome preparation. Hybridization was carried out overnight in a humid chamber at 37 °C. After the hybridization step the slides were washed 2× for 5 min in 50% formamide in 2× SSC and 1× 5 min in 2× SSC at 42 °C (37 or 60 °C were used for different stringency), followed by 10 min in 2× SSC at room temperature. The slides were then washed 5 min in detection buffer containing 4× SSC and 0.2% Tween-20 and incubated for 30 min at 37 °C with detection reagent containing 3% bovine serum albumin (BSA), 4× SSC and 0.2% Tween-20. For detection of hybridization sites slides were incubated with streptavidin-conjugated Cy3 (2.5 µg·mL⁻¹) in detection reagent for 60 min at 37 °C. After that the slides were subsequently washed in detection buffer 3× for 5 min at room temperature. The slides were mounted in antifade solution containing 0.2 µg·mL⁻¹ of DAPI. Photographs were taken with a Zeiss MC 80 camera mounted on a Jenalumar 250 (Carl Zeiss) fluorescence microscope with an appropriate filter system using Fuji 1000 color dia film. All in situ hybridizations were repeated, varying proportions of labeled DNA and blocking DNA and using a different temperature of stringency washing, for a more reliable result.

Pollination studies and confirmation of hybrid origin. The fertility of chinese bulbous leek was tested using pollen from 'Elefant' leek. When fertile chinese bulbous leek was used as the female parent, hand emasculatation was performed on about 100 flowers followed by a hand pollination with leek pollen. Hybrid origins of progenies were tested by the RAPD method using primers OPA-01, OPA-19, and OPA-20 as previously reported (Campion et al., 1995).

Genome size determination. Genome size was determined using flow cytometry. Nuclei were isolated by chopping young leaf tissue from all studied accessions and standard species (*Allium cepa* L. or *Vicia faba* L.) in an ice-cold LB-01 buffer (Dolezel et al., 1989), passed through 30 µm nylon filter and stained with 50 µg·mL⁻¹ RNase and 50 µg·mL⁻¹ propidium iodide. A generally accepted (Dolezel

et al., 1998) genome size for onion (33.5 pg per nucleus) was used as standard from which others including *Vicia faba* were calculated. For measurements, samples of the studied accession were mixed with species used as standard, data are based on four repetitions, and analysis was completed within 3 to minimize seasonal variation effect.

Hybridizations using nuclear rDNA and cpDNA probes. Variation was assessed at previously characterized polymorphisms in the chloroplast and nuclear 45 s ribosomal (r) DNAs, which can distinguish *A. ampeloprasum* and *A. sativum*. The nuclear 45s rDNAs were distinguished using polymorphisms at or between *Dra*I, *Eco*RI, *Eco*RV, and *Xba*I (Havey, 1992). The cytoplasms were differentiated using chloroplast characters 7, 9, and 13 (Havey, 1991). Genomic DNAs were isolated

using CTAB procedure as described by Saghai-Marouf et al. (1984). Digestions, transfers to filters, hybridizations, autoradiography, and scoring of polymorphisms were as previously reported (Havey, 1991, 1992).

Results

Morphological characteristics of an accession of chinese bulbous leek. Chinese bulbous leek formed, partially depending on growth conditions, more or less typical leek-like pseudostems. At the end of the first vegetation year a single bulb (round) was formed (Fig. 1A). At the end of the second year of vegetative growth (Fig. 1B), bulbs were composed of several cloves (Fig. 1C and D). Flower stalks were formed in the second growth year and during later years, forming at first leek-like

Fig. 1. Morphological characteristics of an accession of chinese bulbous leek: (A) round bulbs formed after first growth year, (B) plants at maturity after second growth year, (C) cross section of bulb, (D) separated individual cloves, (E) immature and mature inflorescences, and (F) seeds from a single plant

leafy spathes and later large inflorescences (Fig. 1E) which after cross pollination produced large number of fertile seeds (Fig. 1F).

Organoleptic properties of the bulbs were similar to those of garlic but with some distinctive differences. Average scores were 2.94 and 2.33 for aroma, 2.60 and 4.40 for crispness and 4.80 and 1.45 for pungency for garlic and chinese bulbous leek respectively.

Chromosomal studies. Chromosome counting revealed that chinese bulbous leek possessed the tetraploid chromosome number ($2n = 4x = 32$). In the majority of chromosome spreads, there were 2 to 4 minor (B) chromosomes. Figure 2A and B shows typical karyotype and karyogram of metaphase spreads, including 4 B-chromosomes.

GISH analysis of chinese bulbous leek. GISH was used to assess the degree of cross hybridization among four putative related species—leek, a European accession of bulbous leek, great-headed garlic and garlic. Hybridization to chinese bulbous leek with DNA from leek or the European accession of bulbous leeks produced strong and uniform hybridization signals, while hybridizations with DNA from great-headed garlic and garlic produced only faint and fragmented signals. Examples of strong and faint hybridization signals are shown in Fig. 3A and B.

Genome sizes. The genome size of chinese bulbous leek was compared to morphologically similar *Allium* species (Table 1) and was close to those measured previously for leek, garlic and great-headed garlic (Walters, 1992). The genome sizes for bulbous leeks, *A. polyanthum* and *A. commutatum* have not been previously reported. Our sample of *A. commutatum* was, according to its genome size, hexaploid, which has not been previously reported for this species. Accessions formed three distinct groups. Chinese bulbous leek, a commercial variety of leek and two European accessions of bulbous leeks formed the first group (average 13.223 pg), great-headed garlic formed the second group (average 15.482 pg), which was close to *A. polyanthum* and *A. commutatum* (average 15.626 pg), and a commercial variety of garlic (17.118 pg) formed the third group. Our results indicate that chinese bulbous leek is more closely related to accessions of bulbous leeks grown in Europe belonging to *A. ampeloprasum* group than to other alliums in this study. This group differed significantly from garlic, great-headed garlic, and the two wild species *A. polyanthum* and *A. commutatum*. On the other hand, great-headed garlic was very close in genome size to the two wild species *A. polyanthum* and *A. commutatum*, but different from garlic (Table 1). This is a new finding.

Polymorphisms in the nuclear ribosomal and chloroplast DNAs. The accession of chinese bulbous leek was identical to *A. ampeloprasum* for polymorphisms 7, 9, and 13 (Havey, 1991) in the chloroplast DNA (Fig. 4). Maternal phylogenies estimated using polymorphic restriction enzyme sites in the chloroplast genome support a well defined clade for *A. ampeloprasum* and *A. sativum* (Havey, 1991). These three chloroplast characters distinguishing *A. ampeloprasum* and *A.*

sativum were unique (autapomorphies) to *A. ampeloprasum*. Because these autapomorphic characters represent the derived, and not the progenitor, states, these chloroplast analyses strongly support the classification of chinese bulbous leek as a cultivated form of *A. ampeloprasum*, and not of garlic.

Polymorphisms in the nuclear 45s rDNA agreed with the chloroplast evaluations. The accessions of chinese bulbous leek possessed the same sized fragments as leek and bulbous leek for all fragments in the nuclear 45s rDNA generated by *Dra*I, *Eco*RI (Fig. 5), *Eco*RV, and *Xba*I digests (Havey, 1992). Highly conserved

Fig. 2. Root tips metaphase spread of chinese bulbous leek chromosomes: (A) karyotype and (B) karyogram

Fig. 3. GISH analysis (left DAPI and right CY3 fluorescence) on chromosomes of chinese bulbous leek hybridized with (A) european bulbous leek accession from Apt and (B) 'Thermidrome' garlic.

Table 1. Genome sizes of studied *Allium* species as revealed by flow cytometry.

Species	Total genome size (pg)	Estimated ploidy level and 1C value (pg)
Chinese bulbous leek*	52.598 ± 0.289	4n – 13.149
'Ardea' leek*	52.719 ± 0.438	4n – 13.180
Bulbous leek (Guadeloupe)*	52.634 ± 0.976	4n – 13.158
Bulbous leek (Apt)*	53.625 ± 0.245	4n – 13.406
<i>Allium polyanthum</i> (St. Mandrier sur Mer)*	61.060 ± 0.706	4n – 15.265
<i>Allium commutatum</i> (Corsica) **	92.222 ± 0.889	6n – 15.370
Great-headed garlic (Spain)**	92.774 ± 0.703	6n – 15.462
Great-headed garlic (India)**	93.021 ± 0.608	6n – 15.503
'Thermidrome' garlic*	34.236 ± 0.582	2n – 17.118

*Standard *Allium cepa* (33.5 pg).**Standard *Vicia faba* (25.412 pg).

Fig. 4. Autoradiogram showing polymorphism 9 (Havey, 1991) in the chloroplast DNA of the alliums listed across the top. Sizes in kilobases of polymorphic fragments listed on right.

*Eco*RI sites in the 18s and 26s coding regions produce a 3.6-kb fragment shared among all alliums analyzed to date (Havey, 1992). Variation in the sizes of the intergenic spacer (fragments larger than 3.6 kb in Fig. 5) or additional *Eco*RI sites (2.6-kb fragments in Fig. 5) generated RFLPs among the *Allium* species (Havey, 1992).

Great-headed garlic was clearly differentiated from tetraploid *A. ampeloprasum* both in the chloroplast genome (Fig. 4), as previously reported by Havey and Leite (1999), and nuclear 45s rDNA (Fig. 5). In the chloroplast genome, great-headed garlic possessed the progenitor states for all three polymorphisms. Spanish great-headed garlic shared a 2.6-kb fragment, produced by an *Eco*RI site in the rDNA intergenic spacer (Havey, 1992), with bunching onion (*A. fistulosum* L.) and one accession (from Ile d'Yeu) of *A. polyanthum* (Fig. 5). The largest fragment in great-headed garlic revealed by hybridization of the rDNA probe to *Eco*RI digests was clearly larger than the largest fragment in leek. Although great-headed garlic has been classified as an *A. ampeloprasum* (Mathew, 1996), the leeks and great-headed garlic shared none of the evaluated polymorphisms. The accession of *A. polyanthum* from Ile d'Yeu (France) was identical to the Spanish accession of great-headed garlic for all evaluated polymorphisms in the chloroplast and nuclear 45s rDNAs (Figs. 4 and 5).

Pollination studies. An umbel of chinese bulbous leek pollinated with leek produced ten seeds, four of which germinated. RAPD analysis of individual parent plants compared to the progeny confirmed their hybrid origin (Fig. 6).

Discussion

Chromosomal studies of our accession of chinese bulbous leek showed high homologies to previously published descriptions of *A. ampeloprasum* (Koul and Gohil, 1970; Jones, 1990). Super-numerary chromosomes, which occur in some *Allium* species, often termed B-chromosomes, are small and variable in number. Jones (1990) reported zero to three additional B-chromosomes in leek. B-chromosomes were found in up to 30% of commercial leek samples and their presence may slightly enhance seed germination. Additional B-

Fig. 5. Autoradiogram showing *Eco*RI polymorphisms in the nuclear 45s ribosomal DNA among the alliums listed across the top. Sizes in kilobases of smaller fragments listed on right.

chromosomes were reported for triploid *A. ampeloprasum* var. *truncatum* (Kollmann, 1971); Guern et al. (1991) found one B-chromosome in *A. polyanthum* and Bothmer (1982) found one to two B chromosomes in *A. commutatum*.

Our results unambiguously demonstrate that our accession of chinese bulbous leek is not garlic, but a previously undescribed variant within the *A. ampeloprasum* group. The cpDNA hybridizations revealed shared autapomorphic characters with *A. ampeloprasum*. Because the accession of chinese bulbous leek was used locally as garlic, it is possible that long-term selection shifted the morphological characteristics in the direction of garlic-like cloves. However the taste was not the same as that of garlic, which was more pungent and had lower crispness. We also demonstrate that this accession of bulbous leek resembles closely the bulbous leek forms indigenous to Europe.

In contrast to several previously published classifications (Hanelt, 1990; Fritsch and Friese, 2002; Mathew, 1996; Messiaen et al., 1993; Van der Meer, 1997), it seems likely that studied accessions of great-headed garlic are not closely related to the tetraploid forms of *A. ampeloprasum* group. On average the 1C value of genome size of great-headed garlic was 17% bigger than those of the studied leek and bulbous leek accessions. Chloroplast DNA polymorphisms demonstrated that great-headed garlic possessed same progenitor states as *A. polyanthum* and *A. commutatum* and did not share the derived states with the *A. ampeloprasum* group (leek and its tetraploid bulbous forms). RFLPs in the nuclear 45s ribosomal DNA showed similar genetic relationships. This very preliminary result showed that great-headed garlic may be more closely related to tested wild *Allium* species than to *A. ampeloprasum* and *A. sativum*. However a comparison to a wider range of wild *Allium* relatives and to *A. ampeloprasum* accessions of various ploidy levels should be included in further studies to reveal its origin.

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Fig. 6. Amplification of pattern RAPD primer OPA-20. BL = chinese bulbous leek, female parent, 1 to 4 hybrid progeny, L = 'Ardea' leek, male parent. Arrows indicate bands present in pollen donor and in progenies but not in the female parent.

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