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Diploid *Allium ramosum* from East Mongolia: A Missing Link for the Origin of the Crop Species *A. tuberosum*?

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Diploid *Allium ramosum* from East Mongolia: A missing link for the origin of the crop species *A. tuberosum*?

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Abstract

In eastern Mongolia, a diploid close relative of the tetraploid (4x) crop species *Allium tuberosum* and its closest wild relative *A. ramosum* (4x) was found and characterized by karyotype analysis and with molecular marker techniques. An earlier analyses revealed *A. ramosum* to be sister of the crop but excluded it as its progenitor. At that time a putative diploid cytotype of *A. ramosum* was hypothesized as a potential progenitor taxon of the tetraploids. New phylogenetic analyses of chloroplast and nuclear DNA sequences including the recently found cytotype (*A.* aff. *tuberosum*) together with *A. tuberosum* and *A. ramosum* accessions revealed a sister group relationship of both species, with *A.* aff. *tuberosum* having sequences very similar or identical with *A. ramosum*. Two fingerprint analyses (RAPD, SCoT) resulted in phylogenetic trees where aff. *tuberosum* grouped basal to *A. ramosum*, although the two taxa are morphologically and ecologically clearly differentiated. We conclude that East Mongolian aff. *tuberosum* is not the progenitor of *A. tuberosum* but that it might belong to a stock of ancient lineages that gave rise to both tetraploid taxa.

Key words: *Allium ramosum*, *Allium tuberosum*, Chinese chive, domestication, karyology, phylogeny

Introduction

Chinese chive (*Allium tuberosum* ROTTLER ex SPRENG.) is the second-most economically important crop species of the onion genus *Allium* in Eastern Asia, and is widely cultivated throughout China, Korea, Vietnam, and Japan. The closest relative wild species to Chinese chive is *A. ramosum* L., which occurs in steppes and dry meadows of southern Siberia, Mongolia and northern China. Wild and cultivated forms are slightly distinct with respect to morphology and flowering time. Both taxa are tetraploid species with 2n = 32. The species have a somewhat complicated taxonomic and nomenclatural history, which has been first carefully worked out by STEARN (1944). *Allium tuberosum* has frequently been misnamed *A. odorum* and under this latter name has been confused with the wild species *A. ramosum*. For *A. ramosum*, HANELT (1988) reported substantial morphological variability and, particularly in populations from Mongolia, the occurrence of morphologically transitional types. SANČIR (1992) divided *A. ramosum* in Mongolia in four varieties: var. *ramosum* (var. *odorum* KAZ.), var. *mongolicum* KAZ., var. *violacenerve* SANČIR. (= *A. kerulenicum* DASCHNJAM, nom. invalid), and var. *uliginosum* (G. DON) SANČIR. Unfortunately, he wrongly interpreted the variations described by KAZAKOVA, 1978: i.e. var. *odorum* KAZ. = *A. tuberosum* and var. *mongolicum* KAZ. = *A. ramosum*. However, the division of these taxa remained controversial, and in his latest accounts on these *Allium* species Hanelt (1988, 2001) subsumed all forms within *A. ramosum*.

Allium tuberosum was for the first time reported for Mongolia by botanists of the Soviet-Mongolian floristic expedition (GUBANOV et al. 1990), who collected it in the Hingan Mountains in 1987. For his revision of the genus *Allium* in Mongolia, FRIESEN (1995) did not see all herbarium materials from eastern Mongolia and disclaimed *A. tuberosum* from the Mongolian Flora.

Chromosome numbers for *A. tuberosum* are mostly given as tetraploid, 2n = 32 (MATHUR & TANDON 1965, GOHIL & KAUL 1981, ZOU & JIA 1985, XU & KAMELIN 2000); only one publica-

tion for wild populations of *A. tuberosum* from Shaanxi, China reported a diploid number (YANG et al. 1998), and also triploid populations were found inside *A. tuberosum* (HUANG et al. 1985). *Allium ramosum* is mostly tetraploid (FRIESEN 1988) but in China also populations of diploid together with tetraploid plants were found (ZOU & JIA 1985), while in the Shaanxi Province only diploid populations were reported (SCHANG et al. 1997).

Phylogenetic analysis (BLATTNER & FRIESEN 2006) of crop (*A. tuberosum*) together with wild accessions (*A. ramosum*) led to the conclusion that the wild progenitor of *A. tuberosum* cannot be the tetraploid *A. ramosum*. The crop was not nested within the wild species but showed a sister group relationship, meaning that both species shared a common ancestor from which they developed as two independent lineages. One diploid accession of *A. tuberosum* of unclear origin was placed in between the two clear sister clades in the molecular study of BLATTNER & FRIESEN (2006), indicating that the progenitor of *A. tuberosum* should be searched in wild diploid populations of *A. ramosum* or *A. tuberosum*.

In summer 2010 we collected high plants (*c*. 1 m), in the Hingan Mountains (East Mongolia) on a floodplain meadow, which were morphologically different from *A. ramosum* and more similar to *A. tuberosum* (informally named *A.* aff. *tuberosum* (fig. 1). Typical *A. ramosum* (fig. 1, 4) was also found in Hingan Mountains, but only on tops and slopes of hills within steppe vegetation.

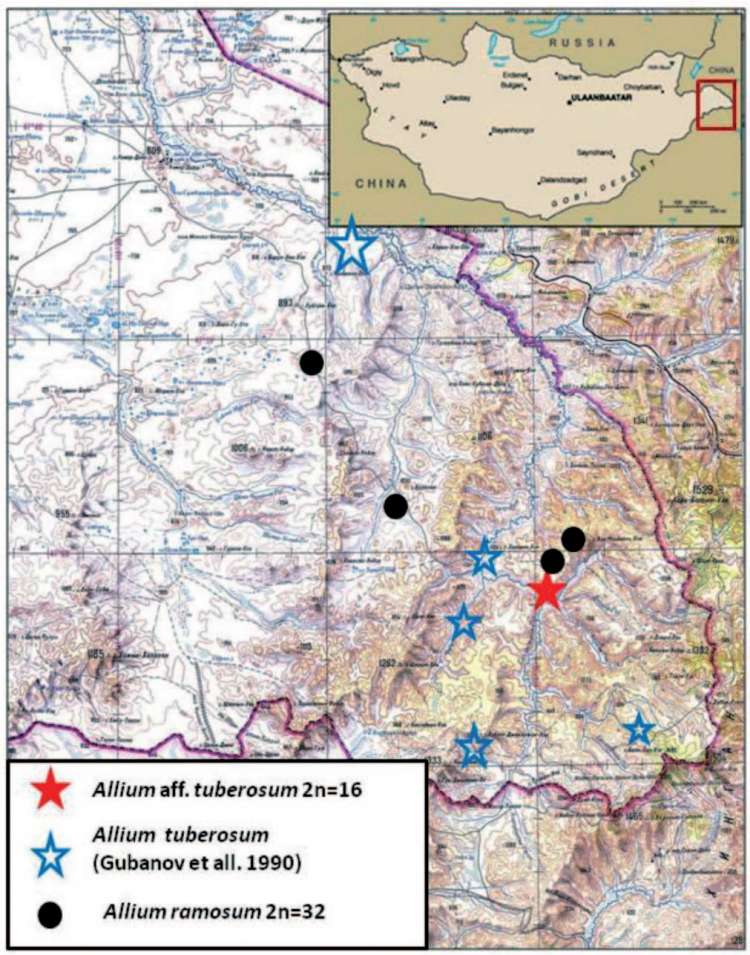

Fig. 1: Collection area in eastern Mongolia for the analyzed accessions.

The aim of this study is to analyze the phylogenetic position of *A.* aff. *tuberosum* in relation to *A. tuberosum* and *A. ramosum*. We report chromosome numbers of all studied accessions and analyze the nuclear ribosomal DNA internal transcribed spacers region (ITS), together with three different noncoding regions of the chloroplast genome (*trn*Q-*rps16* intergenic spacer; *trn*L-*rpl32* intergenic spacer and *rps16* intron). To analyze the genetic structure of *A. tuberosum* and *A. ramosum* we use also two anonymous marker approaches: Random Amplified Polymorphic DNA – RAPD (WILLIAMS et al. 1990) and Start Codon Targeted polymorphisms – SCoT (COLLARD & MACKILL 2009) to screen larger parts of the genome for taxon differences and similarities.

Material and methods

Origin of plant material

Herbarium specimens used for DNA sequencing are given in table 2. For cytological analysis we used living plants, which were collected 2010 in East Mongolia and grown in the collections of *Allium* in the Botanical Garden of Osnabrück University.

Karyotype analysis

Excised roots were kept in distilled water on ice overnight. They were then transferred to room temperature for 20 min and pre-treated for 2 h at room temperature in an aqueous 0.1 % solution of colchicine. The tissue was fixed in a freshly prepared solution of 96 % ethanol/glacial acetic acid (3:1). Meristems were hydrolyzed in 0.1 N HCl for 8 min at 60°C, dissected on a slide in 45 % carmine acetic acid and squashed under a cover slip. Chromosome nomenclature follows LEVAN et al. (1964).

DNA sequencing

Total genomic DNA was sampled from herbarium specimens listed in table 1 using the InnuPREPP Plant DNA Kit (Analytic Jena AG) according to the instructions of the manufacturer. Amplification and sequencing primers for ITS are given in FRIESEN et al. (2006). Primers and PCR conditions for the chloroplast regions were as follows: for the *trn*Q-5-*rps*16 and *trn*L-*rp*L32 regions as described in SHAW et al. (2007), and for *rps*16 intron as described in OXELMAN et al. (1997). Amplicons were purified and cycle sequenced with the ABI BigDye technology on an ABI 377XL automated DNA sequencer (Applied Biosystems). Forward and reverse sequences from every individual were manually edited in CHROMAS Lite 2.1 (Technesylum Pty Ltd) and combined in single consensus sequences. The sequences of all samples were aligned with CLUSTAL X (THOMPSON et al. 1997), and the alignment was subsequently corrected manually in MEGA 5 (TAMURA et al. 2011). Sequences were submitted to the EMBL nucleotide database and can be accessed under accession numbers HE774695 – HE774732.

Phylogenetic analyses

Allium oreoprasum SCHRENL *has been chosen* as *outgroup b*ased on earlier analyses of FRIESEN et al. (2006) and LI et al. (2010). Parsimony analysis was performed with PAUP* 4.0b10 (SWOFFORD 2002) using heuristic searches with TBR branch swapping and 100 random addition sequences. Bootstrap support (BS; FELSENSTEIN 1985) was estimated with 100 bootstrap replicates, each with 100 random addition sequence searches. Bayesian analyses were implemented with MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003). Sequence evolution models were evaluated using the Akaike information criterion in Modeltest 3.7 (POSADA & CRANDALL 1998). Two independent MCMC runs with eight chains each for 2 million generations sampling trees every 100 generations were conducted. Burn-in was set to discard the initial 25 % of trees. The remaining 25,000 trees were combined and a majority-rule consensus tree and Bayesian posterior probabilities were calculated in MrBayes 3.1.2.

Fingerprint analyses

RAPD – The entire set of accessions (table 1) was RAPD-analyzed using six Operon primers (B03, D05, D20, H07, H13 and R06) with amplification conditions as described by BLATTNER & FRIESEN (2006). Bands were separated and visualized by standard 1.5 % agarose gel electrophoresis. Clearly visible bands were scored manually for presence (1) or absence (0), using enlarged prints of the gels. From the resulting binary data matrix pairwise distances and phenograms were calculated using UPGMA (unweighted pair group method using arithmetic averages) clustering in PAUP*.

SCoT – Start Codon Targeted (SCoT) polymorphism is a novel fingerprint technique for generating gene targeted markers (COLLARD & MACKILL 2009). DNA markers are produced by polymerase chain reaction (PCR) using single 18 bp primers that are designed from the short regions flanking the ATG start codon, which is conserved for most genes. This technique is in principle similar to RAPD or ISSR but longer primers and the targeted gene regions should result in more reliable results in comparison to these latter fingerprint techniques. Prior to the analysis of the entire set of accessions (table 1), 12 SCoT primers were tested on a small set of plants. Final amplifications were carried out using six primers (SCoT17, SCoT19, SCoT17, SCoT21, SCoT22, SCoT24) with amplification conditions described by COLLARD & MACKILL (2009). Bands were visualized with 1.5 % agarose gel electrophoresis. Clearly visible bands were scored manually for presence (1) or absence (0), using enlarged prints of the gels. From the resulting binary data matrix, phenograms were prepared using UPGMA in PAUP*. For a combined dataset derived from RAPD and SCoT characters a Neighbor-Joining (NJ) cluster analysis (SAITOU & NEI 1987) was conducted in PAUP* using Nei-Li pairwise distances. Bootstrap support values were calculated with 1000 data re-samples.

Results

Karyotype analysis

Chromosome numbers of the accessions are shown in table 1. All accessions of *A. ramosum* and *A. tuberosum* are tetraploids (2n = 4x = 32), only the *A.* aff. *tuberosum* from Numrug National Park in eastern Mongolia is diploid (2n = 16). Karyotype was analyzed only for the plants from

Table 1: Accessions of A*llium tuberosum* and *A. ramosum* used in the study

Species name	Origin	Herbarium voucher [1]	Chromosome number	Accession No.
Allium oreoprasum	Kyrgyzstan	OSBU 15359	2n = 16	B1
Allium ramosum	Russia, Altay	OSBU 18057	2n = 32	B3
Allium tuberosum	Japan	OSBU 21482	2n = 32	B4
Allium ramosum	Mongolia, Hentey	OSBU 20277	2n = 32	B5
Allium ramosum	Mongolia, Onon	OSBU 20394	2n = 32	B6
Allium ramosum	Mongolia, Chingan	OSBU 20695	2n = 32	B7
Allium aff. *tuberosum*	Mongolia, Chingan	OSBU 20642	2n = 16	B8
Allium ramosum	Mongolia, Kerulen	OSBU 20157	2n = 32	B9
Allium ramosum	Russia, Yacutia	GAT 1836	2n = 32	B10
Allium tuberosum	Korea	GAT1970	2n = 32	B11
Allium tuberosum	India, Agra	GAT 2454	2n = 32	B12

[1] OSBU = Osnabrück University, GAT = IPK Gatersleben

Numrug National Park (B7 and B8 in table 1): *Allium* aff. *tuberosum* (B8): K_{2n} = 16 = 2x = 14M + 2St (fig. 3). *Allium ramosum* (B7): K_{2n} = 32 = 4x = 28M + 4St (fig. 5). The karyotype of the B7 accession of *A. ramosum* is similar to previous published karyograms of *A. ramosum* (FRIESEN 1988, HANELT 1988).

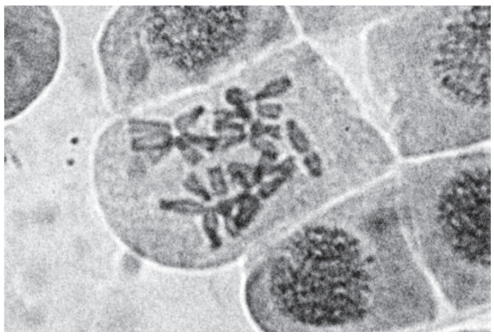

Fig. 2: Metaphase chromosomes of *Allium* aff. *tubero sum* (Acc. B8), Numrug National Park, 2n = 16.

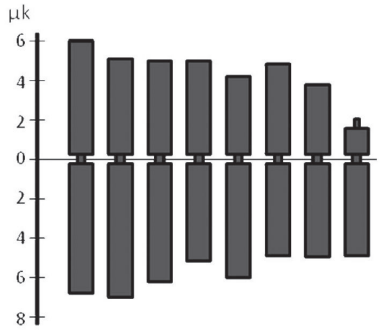

Fig. 3: Karyogram of diploid *Allium* aff. *tuberosum* (Acc. B8), Numrug National Park, 2n = 16.

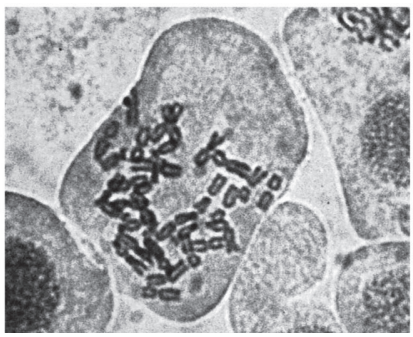

Fig. 4: Metaphase chromosomes of Allium ramosum (Acc. B7), Numrug National Park, 2n = 32.

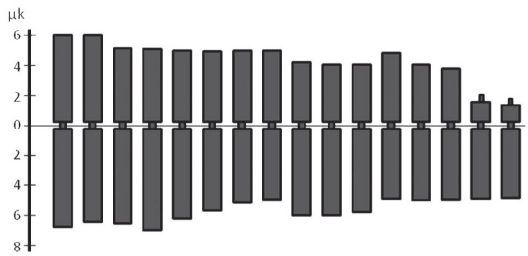

Fig. 5: Karyogram of tetraploid Allium ramosum (Acc. B7), Numrug National Park, 2n = 32.

Phylogenetic analyses

ITS sequence data – In the ITS analysis we included in addition to our 9 sequences also 9 sequences from GenBank (*A. tuberosum*: AJ411914, AJ250293, GQ412257, FJ980277; *A. ramosum*: GQ412229, GQ181079, AJ250295, EU096168 and *A. oreoprasum* – AJ411933). The alignment of combined ITS1 and ITS2 sequences including the 5.8 rRNA gen generated a matrix of 650 characters, of which 51 were parsimony informative. Unweighted parsimony analysis of the 18 sequences resulted in a single most parsimonious tree of 51 steps (CI = 1.0; RI = 1.0). For the Bayesian analysis, the substitution model HKY was chosen by AIC in Modeltest 3.7. Both methods resulted in an identical tree where all accession of *A. tuberosum* formed a clade (85 % bootstrap support and 1.0 Bayesian posterior probability) that is a sister group to the clade consisting of all accessions of *A. ramosum*, including the diploid accession of *A.* aff. *tuberosum* (fig. 6). The ITS sequences of diploid accession of *A.* aff. *tuberosum* are identical with the ITS sequences of all *A. ramosum* accessions.

CpDNA sequence data – Phylogenetic analyses were conducted separately for each cpDNA region sequenced. The alignments generated matrices of 809 basepairs (bp) length for the rps16 intron with six (0.74 %) parsimony informative characters; 795 bp for the *trn*L-*rpL32* region with 10 (1.25 %) parsimony informative characters and 840 bp for the *trn*Q-*rps16* region with 14 (1.67 %) parsimony informative characters.

As the phylogenetic trees for the single cpDNA regions did not produce contradictory results (trees not shown) and all loci are within a single coupling group, we combined the cpDNA sequences, generating a combined matrix of 2444 characters, of which 30 (1.2 %) were parsimony informative. Parsimony analysis resulted in three most parsimonious trees of 612 steps (CI = 1.0, RI = 1.0). For the Bayesian analysis, the substitution model HKY was selected by AIC in Modeltest 3.7. The resulting phylogenetic tree (fig. 7) is compatible with the strict consensus of the parsimony analysis. In this tree the sister group relationship between the clade of *A. tuberosum* on the one side and the clade of *A. ramosum* accessions (including diploid *A*. aff. *tuberosum*) on the other is supported by high support values. The cpDNA sequences of diploid *A.* aff. *tuberosum* are mostly identical with cpDNA sequences of *A. ramosum* accessions, only in the *trn*L-*rpL32* spacer we found two substitutions different to *A. ramosum* and identical to *A. tuberosum*.

RAPD data – In the dataset including the outgroup *A. oreoprasum*, we found 86 polymorphic RAPD bands, of which 79 were polymorphic for the ingroup. UPGMA analysis of these bands resulted in the tree shown in fig. 8a. The UPGMA tree resulted in a sister group relationship between *A. tuberosum* and *A. ramosum* accessions. It is interesting that *A.* aff. *tuberosum* shows a much higher degree of polymorphism in comparison to *A. ramosum* and *A. tuberosum*, and is placed between *A. ramosum* und *A. tuberosum* accessions, though as sister to the *A. ramosum* group which has, however, weak bootstrap support.

SCoT data – In the dataset including the outgroup *A. oreoprasum*, we found 73 polymorphic SCoT bands of which 53 were polymorphic for the ingroup. UPGMA analysis of these markers resulted in the tree shown in Figure 8b. The UPGMA tree results in sister group relationship between *A. tuberosum* and *A. ramosum* accessions. As in the RAPD analysis, *A.* aff. *tuberosum* shows a much higher degree of polymorphism in comparison to *A. ramosum* and *A. tuberosum*, and is placed between *A. ramosum* und *A. tuberosum* accessions, basal in the *A. ramosum* group.

Combined fingerprint data – Neighbor-Joining analysis based on a combined dataset of both fingerprint methods resulted in the tree shown in the fig. 8c. *Allium tuberosum* and *A. ramosum* accessions form sister groups with *A.* aff. *tuberosum* being placed between both taxa as a sister to *A. ramosum*.

Discussion

All sequencing and fingerprint analyses clearly support the former conclusion of BLATTNER & FRIESEN (2006) that the wild progenitor of *A. tuberosum* cannot be the tetraploid *A. ramosum*, as the crop is phylogenetically not nested within the wild species. Diploid *A*. aff. *tuberosum* from East Mongolia does not group with *A. tuberosum* but has chloroplast and nuclear sequences identical with *A. ramosum* (figs. 6-7). This also excludes this taxon as a progenitor of domesticated Chinese chive, as we would then expect its chloroplast and/or ITS type to be found in the crop. In the fingerprint analyses (RAPD and SCoT, figs. 8a-c) the basal position of diploid *A*. aff. *tuberosum* in the *A. ramosum* clade shows that the diploid plants might belong to the progenitor lineage of tetraploid *A. ramosum* and, thus, could belong to the initial diploid stock of lineages that were the starting point for tetraploid formation in *A. ramosum* and possibly also *A. tuberosum*. However, as the population found in East Mongolia was not the direct progenitor of *A. tuberosum* it seems necessary to characterize other diploid populations in more detail, particularly those reported from Shaanxi in China. As diploid plants of *A. tuberosum* (YANG et al. 1998) and

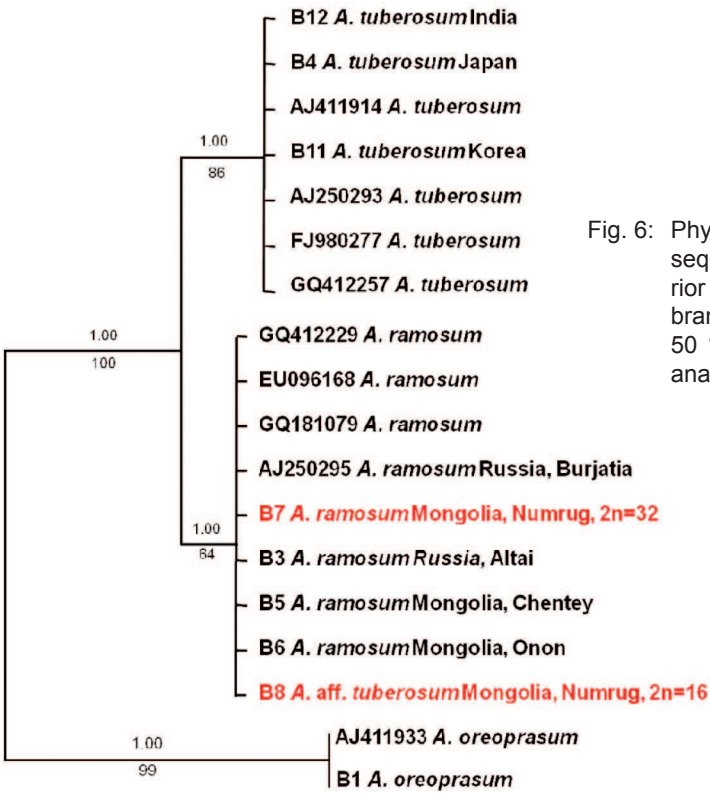

Fig. 6: Phylogenetic tree based on ITS sequences. Bayesian posterior probabilities are given above branches, bootstrap support over 50 % from maximum parsimony analysis below branches.

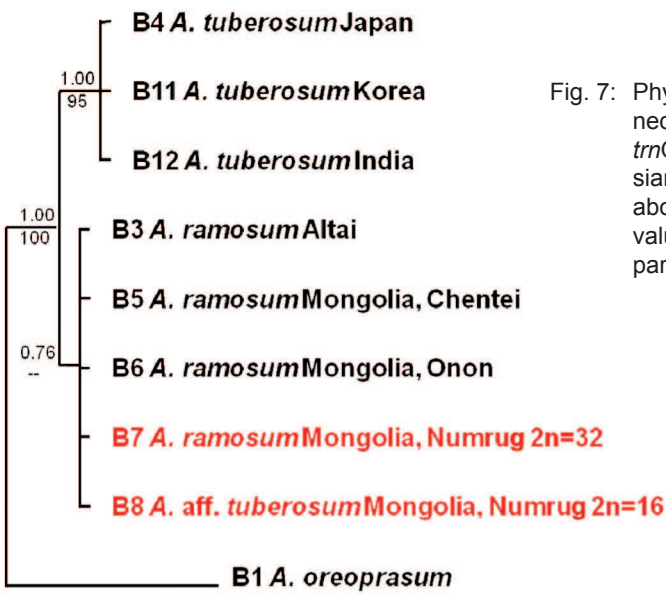

Fig. 7: Phylogenetic tree based on a combined cpDNA data set of *trn*L-*rpl*32, *trn*Q-*rps16* and *rps16* regions. Bayesian posterior probabilities are given above branches, bootstrap support values over 50 % from maximum parsimony analysis below branches.

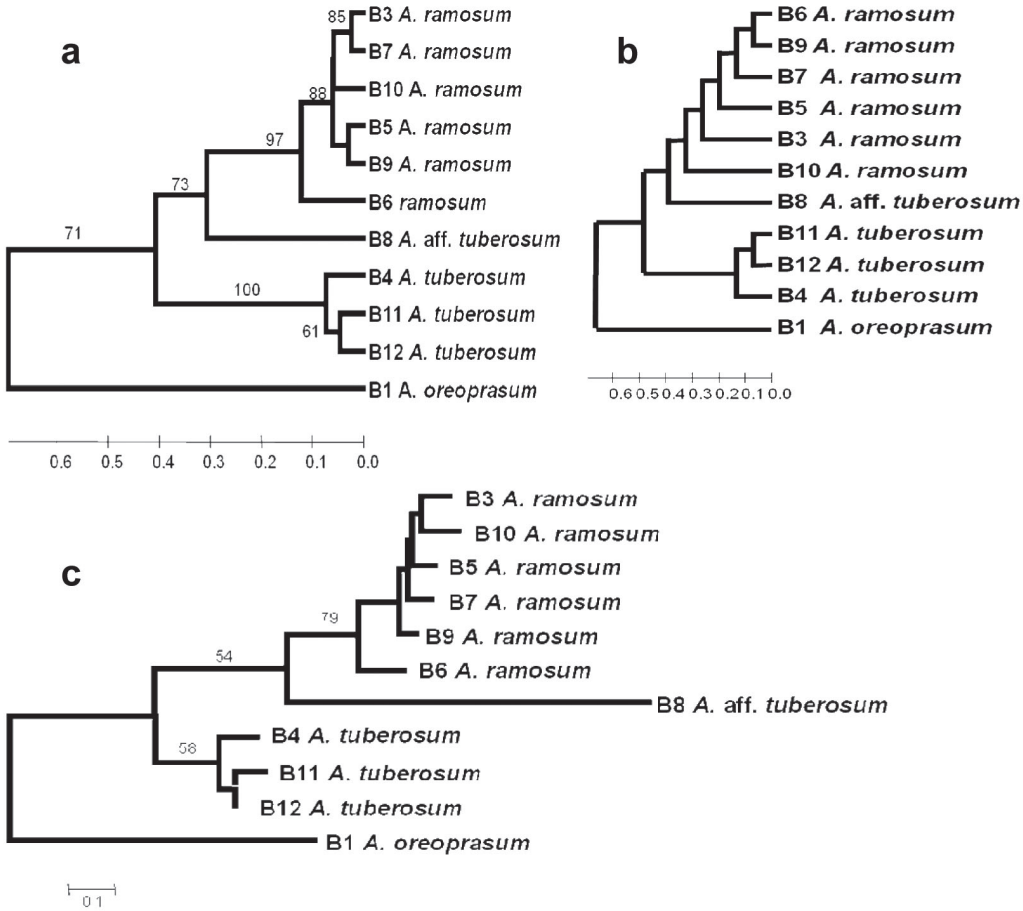

Fig. 8: **a** – UPGMA phenogram derived from the analysis of 86 RAPD characters of accessions of wild *A. ramosum* and the crop plant *A. tuberosum* together with the closely related *A. oreoprasum* as outgroup taxon. **b** – UPGMA phenogram derived from the analysis of 73 SCoT characters of 9 accessions of wild *A. ramosum* and the crop *A. tuberosum* together with the closely related *A. oreoprasum* as outgroup taxon. **c** – Neighbor-Joining tree based on combined RAPD and SCoT characters of 9 accessions of wild *A. ramosum* and the crop *A. tuberosum* together with the closely related *A. oreoprasum* as outgroup taxon.

A. ramosum (SCHANG et al. 1997) were reported for Shaanxi, it is possible that either this area harbors the diploid progenitors of both species or that a single taxon was affiliated with different names.

Taxonomical remarks

Clear morphological, cytological and ecological differences between tetraploid *A. ramosum* and diploid *A.* aff. *tuberosum* lead us to consider that the diploid is perhaps an independent taxon. Before embarking on a formal taxonomic description, we assume it is necessary to know the relationships between the Mongolian diploid plants and such from Shaanxi.

Due to the complicated nomenclature of *A. tuberosum* and *A. ramosum* providing a new name for *A.* aff. *tuberosum* is not an easy task (see also comments on *A. tuberosum* in XU & KAMELIN

2000). We think that the most suitable name for our diploid *A.* aff. *tuberosum* is one of the synonyms of *A. tuberosum*, i.e. *Allium uliginosum* G. Don. (Don, G. (1832): A monograph of the genus *Allium.* – Mem. Wern. Nat. Hist. Soc. (Edinburgh) **6**: 60).

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