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Use of chloroplast DNA polymorphisms for the phylogenetic study of *Allium* subgenus *Amerallium* and subgenus *Bromatorrhiza* (Alliaceae) II.

Figuren

Summary

The utility of the chloroplast DNA (cpDNA) restriction mapping of 18 *Allium* species of the subgenera *Amerallium*, *Melanocrommyum*, *Allium* and *Bromatorrhiza* have been cladistically analysed, according to the recently proposed paraphyletic origin of the subgenera *Bromatorrhiza* and *Amerallium*. The size of the restriction fragment and the precise fragment order of *A. zebdanense* cpDNA were determined for 10 enzymes. The parsimony analysis confirms strongly the subdivision of the genus *Allium* in two main groups with different basic chromosome numbers (x = 7 and x = 8), which had been earlier proposed on the basis of morphological, karyological, anatomical and other molecular markers. Subgenus *Amerallium* (x = 7) itself is distinctly differentiated into two main subgroups, representing the Eurasian and the American taxa. The subgeneric status of *Bromatorrhiza* has to be cancelled, its members must be subordinated as sections to subgenus *Amerallium* (sect. *Bromatorrhiza*) and to one of the Eurasian subgenera with x = 8 (probably to the subgenus *Allium* or *Rhizirideum*).

Zusammenfassung

Die Methode der Chloroplasten-DNA (cpDNA) Restriktionskartierung wurde mit 18 *Allium*-Arten der Untergattungen *Amerallium*, *Melanocrommyum*, *Allium* und *Bromatorrhiza* in Hinblick auf den gegenwärtig diskutierten paraphyletischen Ursprung der Untergattungen *Bromatorrhiza* und *Amerallium* analysiert. Die Größe der Restriktionsfragmente und die genaue Fragmentreihenfolge wurden für 10 Enzyme charakterisiert. Die Unterteilung der Gattung *Allium* in zwei Hauptgruppen mit verschiedenen Chromosomenbasiszahlen (x = 7 und x = 8), die schon anhand morphologischer, karyologischer, anatomischer und anderer molekularer Marker vorgeschlagen wurde, wird durch die Parsimony-Analyse gestützt. Die Untergattung *Amerallium* (x = 7) selbst ist in zwei klare Hauptgruppen differenziert, die die eurasischen sowie die amerikanischen Arten repräsentieren. Der Status der Untergattung *Bromatorrhiza* muß aufgehoben werden, deren Taxa müssen als Sektionen der Untergattung *Amerallium* (sect. *Bromatorrhiza*) und einer der eurasischen Untergattungen mit x = 8 (wahrscheinlich zur Untergattung *Allium* oder *Rhizirideum*) zugeordnet werden.

Introduction

Within the large genus *Allium* two main groups can be distinguished which differ in the primary basic number of chromosomes (x = 7 or x = 8), in anatomical characters of the leaves and the scape and in phytogeographical respect (HANELT et al. 1992). This subdivision was supported by a chloroplast DNA restriction site analysis (LINNE VON BERG et al. 1996): restriction patterns, obtained by the application of 4 enzymes distinctly separated the x = 7 species of subgenus *Amerallium* from various other subgenera of the x = 8 taxa of the genus. To the x = 7 branch however two species of the then poorly known subgenus *Bromatorrhiza* were grouped, one of them with x = 7 *(A. hookeri)*, the other with x = 8 *(A. farreri)*.

An extended analysis of 16 species of these two subgenera of *Allium* by means of a

combination of 10 restriction enzymes and 9 heterologous DNA probes confirmed the strong similarity of the European taxa of subgenus *Amerallium* including those of subgenus *Bromatorrhiza* (SAMOYLOV et al. 1995). The latter, however, were placed within the dendrogram on different branches: one species pair (*A. hookeri*, *A. wallichii*, x = 7) clustered within the Old Word species assemblage of *Amerallium*, the other pair (*A. farreri*, *A. mairei*, x = 8) constituted the sister group to the Old World species of subgenus *Amerallium*. In general Old and New World taxa of these subgenus were significantly separated from one another. In that paper no further species of the x = 8 subgenera were analysed, that means without an outgroup analysis no final decision could be achieved on the taxonomic position of different *Bromatorrhiza* sections and the relations of subgenus *Amerallium* in general.

MES et al. (1998) on the basis of variation of non-coding regions of the chloroplast genome of 29 species of genus *Allium* and seven species of related genera showed the paraphyletic origin of the subgenus *Bromatorrhiza*: species with a chromosome base number x = 7 were clearly placed in the clade to subgenus *Amerallium,* and those with a base number of x = 8 in a clade with subgenera *Rhizirideum* and *Allium*. But the high number of mutations and the high level of homoplasy did not allow to resolve the relationships in the clade comprising the subgenera *Amerallium* and *Bromatorrhiza*. These problems are the concern of this new approach by means of a restriction site analysis.

Materials and methods

Species belonging to subgenera *Amerallium* (ten accessions), *Bromatorrhiza* (four accessions), *Melanochrommyum* (two accessions) and *Allium* (two accessions) were taken from the living collection at the Department of Taxonomy of IPK Gatersleben (Table 1). Total DNA was isolated from freshly harvested green leaves by a modified CTAB extraction (MAASS & KLAAS 1995). All DNAs were further purified by cesium chloride gradients. The DNA was digested with 11 restriction enzymes *Cla*1, *Bcl*1, *Bgl*II, *Xba*1, *Pst*1, *Hind*III, *Bam*HI, *Eco*R1, *Eco*RV, *Xho*1, *Sal*G1 according to the manufacturer's instructions. The restriction fragments were separated in 0.6–1.2% agarose gels in 1× TAE buffer. The DNA was then denatured by soaking in 0.4 N NaOH with 0.6 M NaCl and blotted overnight to nylon membranes (Hybond N+, AMERSHAM) by capillary transfer. The filters were washed in 1× SSC, dried and immobilized with UV crosslinking according to standard procedures (SAMBROOK et al. 1989).

Nine cloned cpDNA restriction fragments of *Oncidium excavatum* (CHASE & PALMER 1989) were used as heterologous probes in filter hybridizations to discover systematically useful variation in the cpDNA of *Allium* species. The *O. excavatum* clone bank kindly supplied by M. HAVEY (with permission of J. D. PALMER) was used to map restriction sites for ten enzymes which cut a typical chloroplast genome 35–80 times each. Individual clones were labelled with a random prime labelling kit according to suppliers' instruction (MBI Fermentas). Probe DNA was heat-denatured and hybridized to filters in 50 ml of hybridization solution [5× SSC, 5× Denhardt's solution, 0.5% (w/v) SD] overnight at 65°C. Following hybridization, filters were washed by incubation in 2× SSC, 0.1% (w/v) SDS at room temperature twice for 10 minutes, and once for 15 minutes at 65 °C with 1× SSC, 0.1% (w/v) SDS. Filters were wrapped in plastic and analyzed by Fosforimager. After a round of hybridization, the probes were stripped from the filters by 3 washes of boiling 0.1× SSC. These filters went through all rounds of hybridizations and were still in usable condition. A restriction site map for 10 enzymes was constructed for *A. zebdanense* (Fig. 1) using the mapping strategy of PALMER (1986). Total *A. zebdanense* DNA was singly digested with *Cla* 1, *Bcl*1, *Bgl*II, *Xba*1, *Pst*1, *Hind*III, *Bam*H1, *Sal*g1 and doubly digested with *Pst*1, *Xba*1 or *Sal*g1 together with each of the above enzymes.

Fragment sizes were estimated from autoradiography by standard curve of end-labeling fragments from the *Hind*III digest of the bacteriophage lambda and a 1kb DNA ladder (Gibco BRL). Both restriction site mutations and length mutations were used for this study.

From the resulting 1/0 data matrix distance and character based analyses were conducted. Wagner parsimony trees were calculated with PAUP 3.1.1 (SWOFFORD 1993), using the heuristic search algorithm with MULPARS, ACCTRAN, and TBR branch swapping.

Results

The size of the restriction fragments and the precise fragment order of *A. zebdanense* cpDNA were determined for 10 enzymes.

Table 1
The origin of the investigated accessions and its taxonomical position (accession numbers of the Department of Taxonomy of the Institut for Plant Genetic and Crop Plant Research, Gatersleben)

Taxon	Accession number and origin of plants
Subgen. *Amerallium* TRAUB	
Sect. *Molium* G. DON ex KOCH	
A. moly L.	Tax 0506 (BG München-Nymphenburg)
A. roseum L.	Tax 0057 (BG Milano)
A. subhirsutum L.	Tax 1447 (BG Liverpool)
A. zebdanense BOISS. & NOË	Tax 1583 (BG Budapest)
Sect. *Briseis* (SALISB.) STEARN	
A. triquetrum L.	Tax 0933 (BG Liège)
Sect. *Narkissoprason* HERMANN	
A. insubricum BOISS. & REUT.	Tax 0230 (BG Marburg)
Sect. *Amerallium* (TRAUB) KAMELIN	
A. canadense L.	Tax 1441 (Gesellsch. Staudenfreunde)
Sect. *Arctoprason* KIRSCHL.	
A. ursinum L.	Tax 0914 (Hakel, Germany)
Sect. *Caulorhizideum* TRAUB	
A. validum WATS.	Tax 1779 (California, USA)
Sect. *Rhopetoprason* TRAUB	
A. kunthii G. DON	Tax 2158 (Chihuahua, Mexico)
Subgen. *Bromatorrhiza* EKBERG	
Sect. *Bromatorrhiza* EKBERG	
A. hookeri THWAITES	Tax 2013 (Kunming, Yünnan, China)
A. wallichii KUNTH	Tax 2510 (Yulong-shan, NW-Yünnan, China)
Sect. *Coleoblastus* EKBERG	
A. mairei LEV.	Tax 2104 (BG Zürich)
Sect. *Cyathophora* R. M. FRITSCH	
A. cyathophorum var. *farreri* (STEARN) STEARN	Tax 2824 (BG Oslo)
Subgen. *Melanocrommyum* (WEBB & BERTHEL.) ROUY	
Sect. *Melanocrommyum* WEBB & BERTHEL.	
A. nigrum L.	Tax 0515 (BG Leipzig)
Sect. *Porphyroprason* EKBERG	
A. oreophilum C. A. MEYER	Tax 0115 (BG Halle)
Subgen. *Allium* L.	
Sect. *Allium* L.	
A. longicuspis REGEL	Tax 1125 (Chatkal Mts, Uzbekistan)
Sect. *Codonoprasum* RCHB.	
A. flavum L.	Tax 0469 (Mt. Sibillini, Italy)

From these results the restriction fragment order of *A. zebdanense* cpDNA was proved to be similar to that of *O. excavatum* cpDNA in terms of Southern hybridization. All *O. excavatum* probes hybridized well with *Allium* cpDNA only probe 7 had a week signal or in some cases did not hybridize at all. Based on the molecular size of individual restriction fragments and their copy number, the genome size was estimated to range from 155.7 *Pst*1 digest to 156.6 *Sal*g1 digest and 156.1 *Pst/Sal* digests. From these values, the total chloroplast genome size of *A. zebdanense* was estimated to be approximately 156 kb (Fig. 1). This size

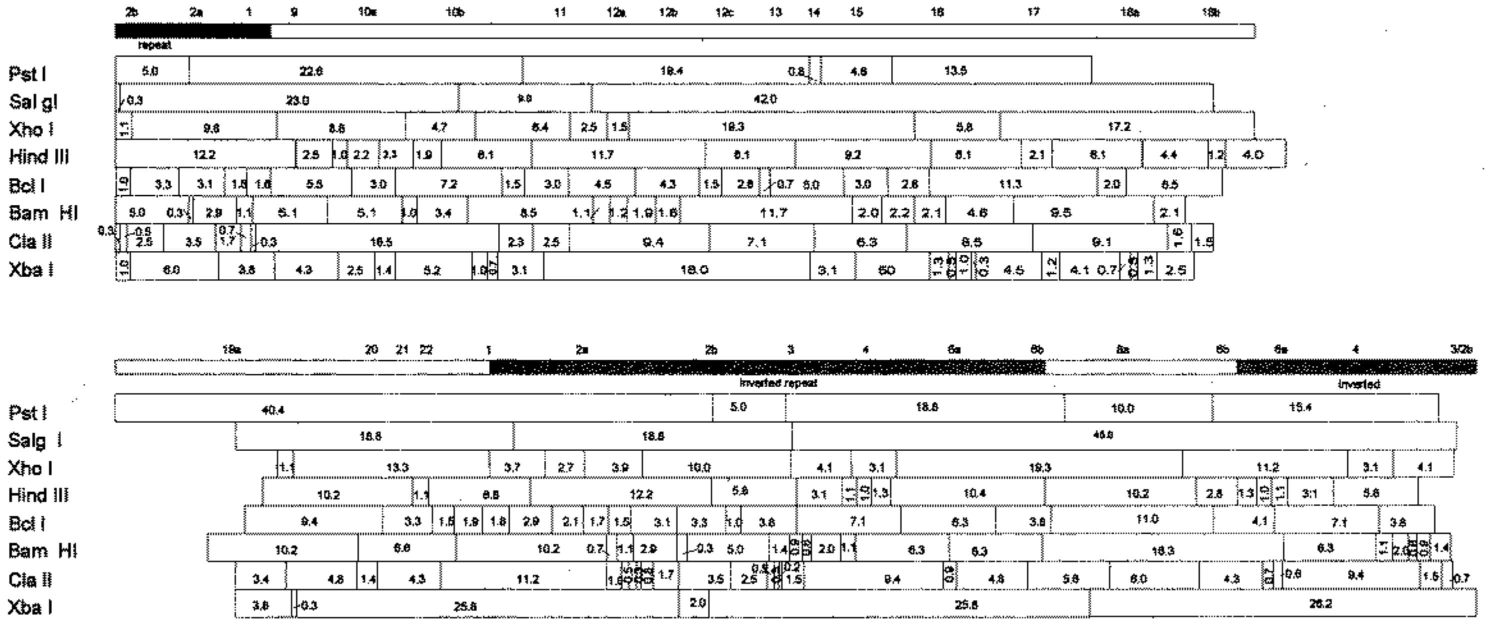

Fig. 1
The cpDNA map of the restriction fragments and the precise fragment order for 10 enzymes of *A. zebdanense*

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is very close to the genome size of *A. cepa* (155 kb) estimated by KATAYAMA et al. (1991) and about 10 to 15 kb larger as other estimations for the *A. cepa* genome (CHASE & PALMER 1989; HAVEY 1991).

On the basis of results obtained the genome of *A. zebdanense* cpDNA can consequently be divided into three parts: 82 kb large single copy region, a 20.4 kb small single copy region, and two inverted repeat regions of approximately 26.5 kb each. It is similar to the structure found for *A. cepa* (KATAYAMA 1991; CHASE & PALMER 1989) and most cpDNAs of vascular plants which show a typical genome structure having 20–26 kb of IR, 10–20 kb SSC, and 80–100 kb of LSC (PALMER 1985). Approximately 300 restriction sites were mapped for each of the eighteen species analysed. Eighty three site mutations were shared by two or more species and used in our study. Differences in fragment sizes due to insertions, deletions or inversions were also detected and used in the phylogenetic analyses. The most parsimonious single tree based on all 1031 cpDNA RFLP characters (948 length variation and 83 restriction sites) is shown in Fig. 2. Num-

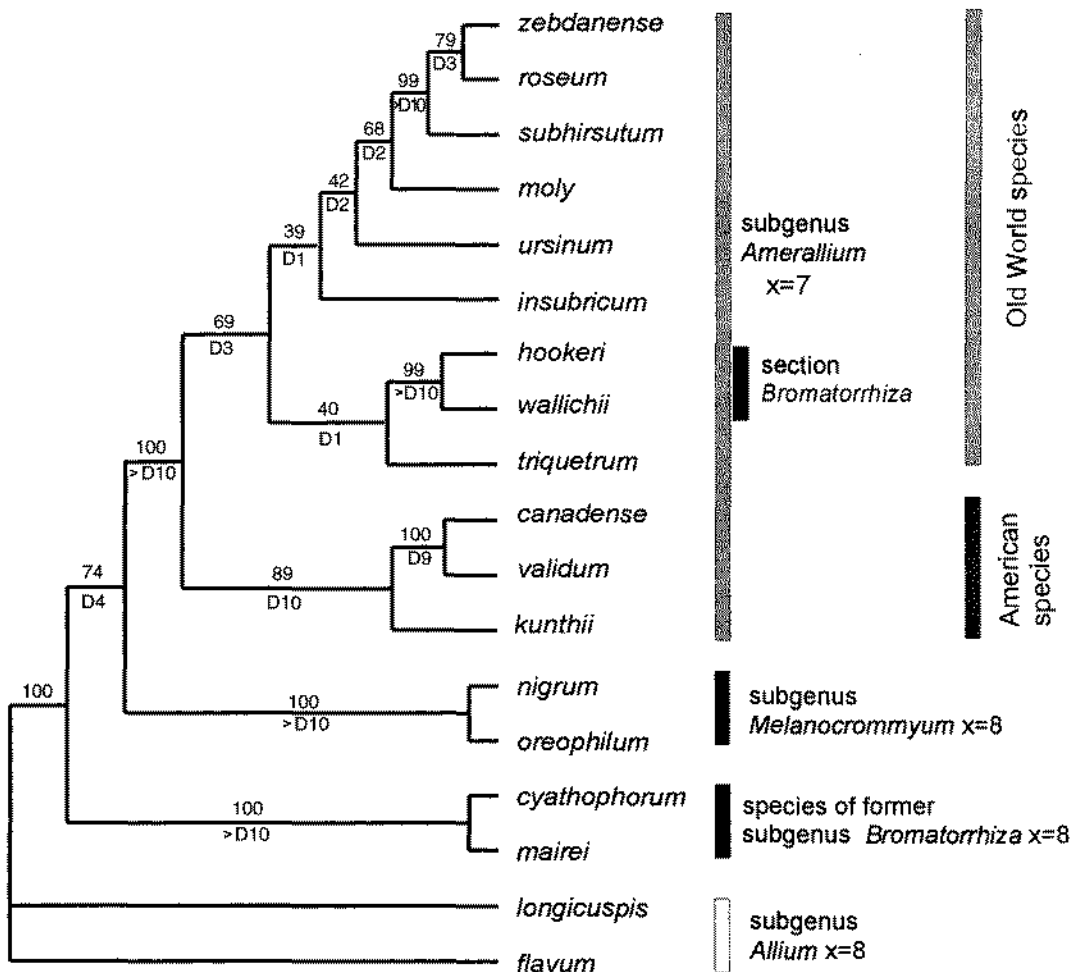

Fig. 2
Single most parsimonious tree, based on all 1031 cpDNA RFLP characters (restriction sites and length variation). Numbers above the brunches indicate bootstrap values (5000 resamples), numbers below are decay values.

bers above branches indicate bootstrap values (5000 resamples), numbers below branches show decay indices. In this tree the clade consisting of species of subgenus *Amerallium* (including *A. hookeri* and *A. wallichii*) is a strongly supported monophyletic group (Bootstrap values 100% and decay index >D10). *Allium farreri* and *A. mairei* constitute the sister group to the species of subgenus *Allium* and subgenus *Melanocrommyum*. American species form a monophyletic unit inside the subgenus *Amerallium* (Bootstrap values 89%, D10).

Discussion

The results confirm strongly the subdivision of the genus *Allium* in two main groups with different basic chromosome numbers (x = 7 and x = 8), which had been earlier proposed on the basis of morphological, karyological, anatomical and other molecular markers.

Subgenus *Amerallium* (x = 7) itself is distinctly differentiated into two main subgroups, representing the Eurasian and the American taxa, respectively. Within the Old World group the species belonging to the Mediterranean section *Molium (A. zebdanense, A. roseum, A. subhirsutum, A. moly)* constitute a somewhat separated assemblage of which *A. moly* corresponding to its status as subsection is somewhat less related to the remaining taxa.

The European species *A. ursinum* (sect. *Arctoprasum*), *A. insubricum* (sect. *Narkissoprason*) and *A. triquetrum* (sect. *Briseis*) although phenotypically explicitely differentiated by several distinct characters cannot be resolved because the bootstrap value and decay index are very weak. Additionaly, it is strongly underlined that sect. *Narkissoprason* is closely related to the Mediterranean sections of subgenus *Amerallium*, and its species would be misplaced if enclosed in subgenus *Rhizirideum* because of the shared possession of a rhizome.

Taxa of the former subgenus *Bromatorrhiza* are widely separated on the dendrogram: (1) species with x = 7 (*A. wallichii*, *A. hookeri*, from South and East Asia) are incorporated as a branch within the Eurasian subgroup of subgenus *Amerallium*, more closely to the unresolved taxa mentioned above than to the Mediterranean sect. *Molium*. (2) Species with x = 8 (*A. mairei*, *A. cyatophorum* var. *farreri*) are placed as a closely related species pair within the Old World branch of the x = 8 group and constitute here a sister group of subgenus *Allium*.

Thus the subgeneric status of *Bromatorrhiza* has to be cancelled and its members must be subordinated as sections to subgenus *Amerallium* (sect. *Bromatorrhiza*) and to one of the Eurasian subgenera with x = 8 (probably to the subgenera *Allium* or *Rhizirideum*). Our data do not allow an exact conclusion in this respect because: (1) the subgenera *Rhizirideum* and *Allium* are not monophyletic (MES et al. 1998), (2) the species selection of the analysis described shortly in this paper may thus not be representativ for the entire genus (several groups with x = 8 have not been analysed).

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