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Chloroplast DNA restriction analysis and the infrageneric grouping of *Allium* (*Alliaceae*)

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Abstract: The utility of chloroplast DNA variation for checking a recently proposed infrageneric classification of the genus *Allium* was tested. cpDNA restriction patterns of 49 species representing the main subgenera, sections, and subsections of the existing classification were compared. 363 different fragments generated by 4 restriction enzymes were identified and analysed by UPGMA clustering. The resulting phenogram largely confirms the subgeneric classification based on an integration of morphological and other methods.

The genus *Allium* L. is a very variable group distributed over the whole northern hemisphere and comprises approximately 700 species, some of which are economically important crop plants. Many papers have been published in recent time dealing with the description of new species and other taxa (e.g., HANELT & FRITSCH 1994, BRULLO & al. 1991), but there are still many gaps in our knowledge of the geographical and evolutionary relationships and of the infrageneric taxonomy, since the last comprehensive monograph was published by REGEL (1875).

Since 1983 a collection of more than 2500 accessions currently representing approximately 300 taxa has been gathered and cultivated in Gatersleben. Based on morphological, geographical, cytological, anatomical, serological, and numerical studies, a new proposal for an infrageneric classification was published (HANELT & al. 1992). As some problems, particularly in regard to a possible polyphyletic nature of some groups, are still unsolved, a new methodical approach was desirable.

The molecular analysis of chloroplast DNA (cpDNA) has proved to be a very useful tool for the study of evolutionary differentiation (CLEGG & ZURAWSKI 1992, WEN-HSING LI & GRAUR 1991). Up to now molecular data from the genus *Allium* have been available only for a very small group of crop species and their relatives. KATAYAMA & al. (1991) constructed a physical map (155 kb) of the plastome of *Allium cepa* and located 16 genes. Interestingly, size and arrangement of genes resemble more the plastome of dicots, e.g., tobacco than that of gramineous plants. Based on restriction site variations in the plastomes, HAVEY (1991) presented a cladogram for *A. ampeloprasum* L., *A. sativum* L., *A. tuberosum*, *A. cepa*, *A. fistulo-*

Table 1. *Allium* species used in the experiments and their taxonomic classification according to Hanelt & al. (1992), Hanelt & Fritsch (1994), Friesen (1988) and Khassanov & Fritsch (1994). Details on the origin of the accessions are available on request

Subgenus	Section	Subsection	Species	Abbr.	Acc. no. (TAX)
Allium	*Allium*	*Allium*	*A. atroviolaceum* Boiss.	atvi	395
Allium	*Allium*	*Allium*	*A. scorodoprasum* L. subsp. *rotundum* (L.) Stearn	rotu	444
Allium	*Allium*	*Allium*	*A. sativum* L.	sati	144
Allium	*Allium*	*Oenoprason*	*A. sphaerocephalon* L.	sphc	114
Allium	*Allium*	*Oenoprason*	*A. vineale* L.	vine	176
Allium	*Codonoprasum*		*A. flavum* L.	flum	2202
Allium	*Codonoprasum*		*A. paniculatum* L.	pani	273
Allium	*Scorodon*		*A. caesium* Schrenk	caes	1077
Amerallium	*Amerallium*	*Canadense*	*A. canadense* L.	cana	2031
Amerallium	*Amerallium*	*Canadense*	*A. plummerae* Wats.	plum	1560
Amerallium	*Arctoprasum*		*A. ursinum* L.	ursi	1350
Amerallium	*Briseis*		*A. triquetrum* L.	triq	933
Amerallium	*Caulorhizideum*		*A. validum* Wats.	vali	1573
Amerallium	*Lophioprason*	*Cernua*	*A. cernuum* Roth	cern	275
Amerallium	*Molium*	*Molium*	*A. zebdanense* Boiss. & Noe	zebd	1653
Amerallium	*Molium*	*Molium*	*A. subhirsutum* L.	shir	1447
Amerallium	*Molium*	*Xanthoprason*	*A. moly* L.	moly	1356
Bromatorrhiza	*Bromatorrhiza*		*A. hookeri* Thw.	hook	2500
Bromatorrhiza	*Cyathophora*		*A. farreri* Stearn	farr	165
Melanocrommyum	*Acmopetala*	*Acmopetala*	*A. backhousianum* Rg. (*A. gulczense* B. Fedt.)	back	3337
Melanocrommyum	*Acmopetala*	*Spiralitunicata*	*A. suworowii* Rg.	suwo	3657
Melanocrommyum	*Kaloprason*	*Kaloprason*	*A. cristophii* Trautv.	cris	783
Melanocrommyum	*Kaloprason*	*Ligulifolia*	*A. nevskianum* Vved. ex Wdb.	nevs	2269
Melanocrommyum	*Megaloprason*	*Elatae*	*A. aflatunense* B. Fedt.	afla	1211
Melanocrommyum	*Megaloprason*	*Elatae*	*A. stipitatum* Rgl.	stip	436

Melanocrommyum	*Compactoprason*	*Erectopetala*	*A. elatum* RGL.	elat	2413
Melanocrommyum	*Melanocrommyum*	*Melanocrommyum*	*A. atropurpureum* WALDST. & KIT.	atpu	1651
Melanocrommyum	*Melanocrommyum*	*Melanocrommyum*	*A. nigrum* L.	nigr	515
Melanocrommyum	*Miniprason*		*A. karataviense* RGL.	kara	779
Melanocrommyum	*Porphyroprason*		*A. oreophilum* C. A. MEY.	oreo	115
Melanocrommyum	*Regeloprason*	*Regeloprason*	*A. lipskyanum* VVED.	lips	3118
Rhizirideum	*Anguinum*		*A. victorialis* L.	vict	419
Rhizirideum	*Butomissa*		*A. tuberosum* ROTTL. ex SPR.	tube	440
Rhizirideum	*Campanulata*		*A. barsczewskii* LIPSKY	bars	1330
Rhizirideum	*Cepa*	*Cepa*	*A. cepa* L.	cepa	2523
Rhizirideum	*Cepa*	*Cepa*	*A. galanthum* KAR. & KIR	gala	256
Rhizirideum	*Cepa*	*Phyllodolon*	*A. altaicum* PALL.	alta	1668
Rhizirideum	*Cepa*	*Phyllodolon*	*A. fistulosum* L.	fist	1120
Rhizirideum	*Oreiprason*		*A. carolinianum* DC.	caro	2906
Rhizirideum	*Oreiprason*		*A. globosum* M. BIEB. ex RED.	glob	3199
Rhizirideum	*Petroprason*		*A. obliquum* L.	obli	338
Rhizirideum	*Reticulato-bulbosa*		*A. montibaicalense* FRIESEN	moba	3390
Rhizirideum	*Reticulato-bulbosa*		*A. splendens* WILLD.	sple	3387
Rhizirideum	*Rhizirideum*		*A. nutans* L.	nuta	916
Rhizirideum	*Rhizirideum*		*A. senescens* L.	sene	47
Rhizirideum	*Sacculiferum*		*A. chinense* G. DON	chin	2015
Rhizirideum	*Schoenoprasum*		*A. schoenoprasum* L.	schp	390
Rhizirideum	*Schoenoprasum*		*A. karelinii* POLJAK.	kare	279
Rhizirideum	*Schoenoprasum*		*A. altyncolicum* FRIESEN	alty	42

sum, A. schoenoprasum with *A. cernuum* as an outgroup, confirming the traditional classification. A study of sect. *Cepa* showed a close relation between members of subsect. *Phyllodolon* (*A. fistulosum* and *A. altaicum*) and subsect. *Cepa* (*A. cepa*) (Havey 1992).

For a first survey of molecular markers in the genus *Allium* cpDNA restriction patterns of species belonging to the main taxa were compared and analysed by computer programs. Comparable studies have been published, e.g., for *Vicia faba* L. (Raina & Ogihara 1994) and *Plantago* (Hogglander & al. 1993). The resulting grouping was compared with the infrageneric classification of the genus *Allium* favoured in our department (Hanelt & al. 1992, Hanelt & Fritsch 1994). It was of special interest to confirm the monophyletic nature of the main infrageneric groups, and to clarify the relationships between American and European species. Additionally, we wanted to settle the taxonomic position and ranking of hitherto dubious groups, and to assess the correlation of molecular data with the phenotypical and morphological diversity.

Material and methods

Plant material. Material (Table 1) was taken from the living collection in the field or in the greenhouse. Voucher specimens are kept in the Gatersleben herbarium.

Chloroplast DNA isolation and restriction endonuclease analysis. Chloroplast DNA was extracted from green leaf material according to Holford & al. 1991 and Bookjans & al. (1984); modified by the addition of 10 mM EGTA [Ethylene Glycol-bis(β-aminoethyl Ether) N,N,N,N′-Tetraacetic Acid] to the extraction buffer. Digestions with the endonucleases Ban I, Ban II, Eco RI and Eco RV were carried out according to the instructions of the suppliers. The products were separated by electrophoresis on horizontal agarose slab gel (0.8%) in TAE buffer (Maniatis & al. 1982). Hybridization experiments with cpDNA probes from *A. tuberosum* were carried out according to the instruction for Dioxygenin-labelling and luminescent detection (Boehringer Mannheim Biochemica).

Data analysis. The fragment patterns of cpDNA from 49 species were compared. Assuming that all fragments of the same mobility are identical, presence or absence of 363 different fragments were recorded in a +/– matrix. The identity of 7 selected fragments were tested by hybridization with Ban I restriction fragments from *A. tuberosum* cpDNA as probes. With the aid of the program system NTSYS-PC (Rohlf 1993) a phenogram was constructed (Fig. 1: simple matching, UPGMA). RFLP data and the resulting matrix are available on request.

Results

UPGMA phenogram. In the UPGMA phenogram (Fig. 1) the distances corresponded to the similarities of cpDNA restriction patterns calculated by the simple matching procedure (100% similarity = 1.0). The bifurcation points indicate the levels of similarity.

Main groups. In the phenogram four main groups can be distinguished. The bifurcation (similarity level 0.7) separates a complex of species belonging to the subgenera *Bromatorrhiza* (*A. hookeri* and *A. farreri*) and *Amerallium*. The next steps first divide subg. *Allium* and then subg. *Melanocrommyum* and *Rhizirideum*. In a similar UPGMA phenogram (data not shown), *Nothoscordum bivalve* (L.) Britt. (*Alliaceae*) was included as outgroup and separated from the genus *Allium* at the similarity level 0.65.

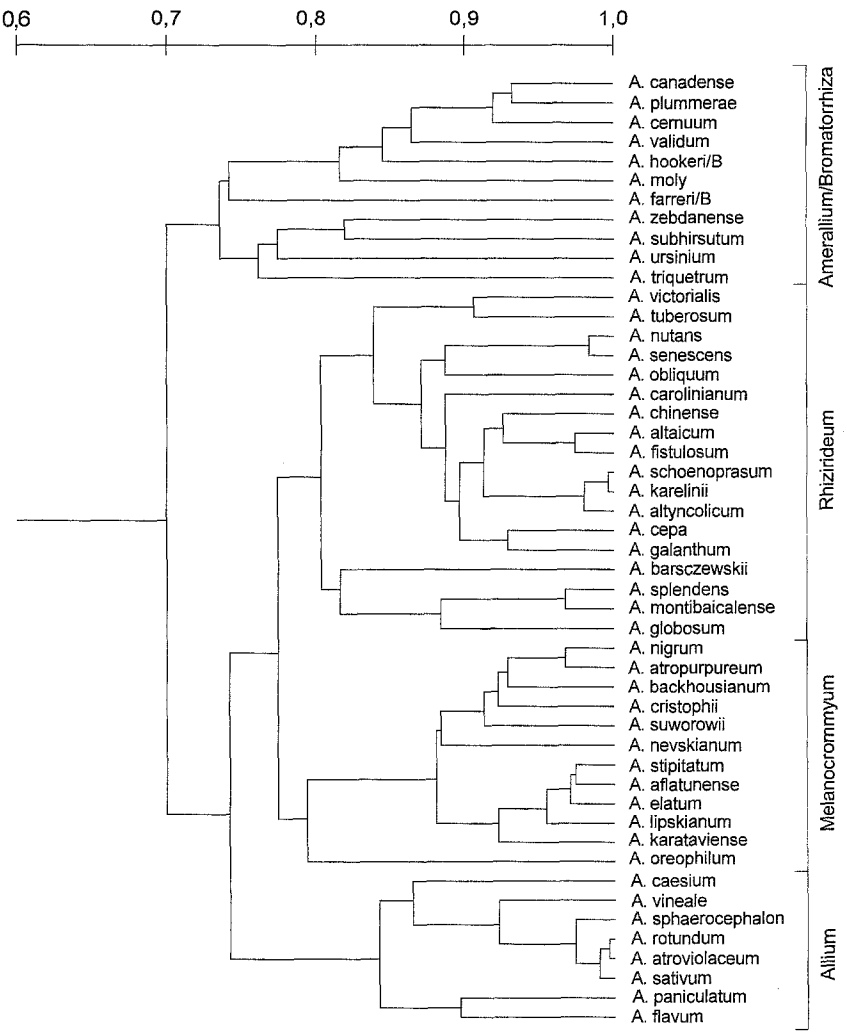

Fig. 1. UPGMA dendrogram showing the genetic relationships between 49 *Allium* species based on the chloroplast RFLP data

Subgenera *Amerallium* and *Bromatorrhiza*. The upper part of the dendrogram comprising the subgenera *Amerallium* and *Bromatorrhiza* is characterized by branching at rather low similarity levels. The first bifurcation at the level 0.735 generates two subgroups. The first one (from *A. triquetrum* to *A. zebdanense*) includes only European species of subg. *Amerallium*; the second one is more heterogenous and comprises two representatives of subg. *Bromatorrhiza* (*A. hookeri, A. farreri*), one European species (*A. moly*) of subg. *Amerallium,* and several species of American origin (from *A. validum* to *A. canadense*). The bifurcations in the first subgroup are between similarity levels of 0.764 and 0.821. The last bifurcation differentiates species of the same subdivision (*A. subhirsutum* and *A. zebdanense,* sect. *Molium* subsect. *Molium*).

As mentioned above, the second subgroup is rather heterogenous. *A. farreri*

(subg. *Bromatorrhiza* sect. *Cyathophora*) is separated at a low similarity level (0.744). The next branching steps differentiate first the European species *A. moly* (sect. *Molium* subsect. *Xanthoprason*) and then *A. hookeri* (subg. *Bromatorrhiza* sect. *Bromatorrhiza*) from the remaining species of American origin, which represent two different sections. Only *A. plummerae* and *A. canadense* (sect. *Amerallium* subsect. *Canadense*) belong to the same subdivision and are differentiated at level 0.932.

Subgenus *Allium*. The second main group (lower part) containing only species of subg. *Allium* is subdivided into three groups corresponding to the three sections *Allium, Codonoprasum* (*A. flavum* and *A. paniculatum*) and *Scorodon* (*A. caesium*). These branches deviate at levels 0.843 and 0.866, respectively. Within sect. *Allium* two subsections are represented. The representatives of subsect. *Allium* are clustered closely (bifurcations between levels 0.989 and 0.995), while the species of subsect. *Oenoprason* are separated at level 0.921.

Subgenus *Melanocrommyum*. The third main group comprises subg. *Melanocrommyum*. At the very low similarity level 0.796 *A. oreophilum* (sect. *Porphyroprason*) deviates from the remaining species divided into two blocks (level 0.879). In the first block sects. *Miniprason* (*A. karataviense*), *Regeloprason* (*A. lipskyanum*), *Compactoprason* (*A. elatum*) and *Megaloprason* subsect. *Elatae* (*A. aflatunense* and *A. stipitatum*) are combined. The bifurcations are between similarity levels 0.920 and 0.974. The two species of subsect. *Elatae* and one of sect. *Compactoprason* are close together (values are between 0.970 and 0.974).

The second block contains species of sect. *Kaloprason* subsect. *Kaloprason* (*A. cristophii*) and subsect. *Ligulifolia* (*A. nevskianum*), sect. *Acmopetala* subsect. *Acmopetala* (*A. backhousianum*) and subsect. *Spiralitunicata* (*A. suworowii*) and two species of sect. *Melanocrommyum* subsect. *Melanocrommyum* (*A. atropurpureum* and *A. nigrum*). These two species are differentiated between levels 0.885 and 0.927, not strictly in accordance to their sectional position.

Subgenus *Rhizirideum*. The last main group is made up by subg. *Rhizirideum* and subdivided into two groups. The first subgroup (from *A. globosum* to *A. barsczewskii*) is an assemblage of species from different sections. *A. barsczewskii* (sect. *Campanulata*) deviates at a rather low level (0.819) from this subgroup. The next step separates *A. globosum* (sect. *Oreiprason*) at level 0.887. The last species, *A. montibaicalense* and *A. splendens*, belong to sect. *Reticulato-bulbosa* and are divided at a high similarity level (0.969).

The structure of the second subgroup is characterized by multiple bifurcations at very different similarity levels. Generally, the branching between similarity level 0.839 and 0.926 differentiates between different sections (Table 1), the branching at higher levels between representatives of the same section or subsection (e.g., *A. senescens* and *A. nutans* of sect. *Rhizirideum; A. altyncolium, A. karelinii, A. schoenoprasum* of sect. *Schoenoprasum; A. fistulosum* and *A. altaicum* of sect. *Cepa* subsect. *Phyllodolon; A. cepa* and *A. galanthum* of sect. *Cepa* subsect. *Cepa*). Subsections *Cepa* and *Phyllodolon* of sect. *Cepa* are located on different branches (bifurcation at level 0.898).

A phenogram based upon the neighbour joining procedure had a branching structure almost identical to that of the UPGMA graph, however, with some changes of the orientation of the branches (results not shown).

Discussion

The phenograms demonstrate the variation of the cpDNA restriction fragment patterns between all species selected, and its taxonomic significance. The two main branches of the phenogram separate the groups of *Allium* species with a different primary basic chromosome number: x = 7 (upper part; subg. *Amerallium,* for exceptions see below) and x = 8. The lower part of the UPGMA dendrogram with its three subbranches exactly matches the three subgenera *Rhizirideum, Melanocrommyum* and *Allium* (from top to bottom) of the traditional classification.

In many cases even the more detailed taxonomic subdivisions according to HANELT & al. (1992) are reflected in the phenograms. Species belonging to the same section or even subsection (e.g., *A. canadense/A. plummerae*) are grouped together. An example for subdivisions at the sectional level is subg. *Allium*, where the three branches represent the sects. *Scorodon, Allium* and *Codonoprasum.* Generally, in all cases where species are regarded as closely related (e.g., *A. nutans/A. senescens, A. altaicum/A. fistulosum;* subg. *Allium* sect. *Allium* subsect. *Allium* and subg. *Rhizirideum* sect. *Schoenoprasum*, respectively) they are clustered at very high similarity levels suggesting a short time of independent evolution.

On the other hand, phenotypically distinct species (e.g., *A. ursinum* and *A. sativum*) are placed in rather distant branches of the phenogram. The positions of species which are taxonomically isolated from their groups are verified, as for *A. ursinum* (FRITSCH 1988) and *A. oreophilum* (HANELT & al. 1989).

The correspondence of the main taxonomic groups of the genus to the main branches of the phenogram strongly supports the concept of the monophyletic character of the four subgenera; this status will be finally clarified by DNA sequence analysis, being in progress just now. There is, however, one exception with regard to subg. *Bromatorrhiza*, described by EKBERG (1969), a rather heterogeneous group sharing mainly the development of thick fleshy roots as storage organs instead of rhizomes or bulbs (HANELT & FRITSCH 1994). The true status of this subgenus had been questioned earlier (HANELT & al. 1992), and it was considered reasonable to eliminate this taxon and to distribute its species into different other groups. Its artificial nature is confirmed by our molecular data, because the two species of subg. *Bromatorrhiza* are positioned within subg. *Amerallium.* However, these two species (*A. hookeri* and *A. farreri*), represent rather unrelated branches, supporting our former idea of the diphyletic nature of subg. *Bromatorrhiza,* based, e.g., on leaf anatomy (FRITSCH 1988). The integration of these taxa into subg. *Amerallium* is strongly supported by our and further supplementing data (SAMOYLOV & al. 1995).

A minor discrepancy at the sectional level refers to the two species of sect. *Oreiprason* analysed (*A. globosum* and *A. carolinianum*) which are widely separated within the subg. *Rhizirideum* branch. This contradiction of morphological and molecular data has to be examined further.

The phenotypical variability of the subgenera is closely reflected by branchings at many different similarity levels. This is very nicely illustrated for the morphologically, anatomically, and ecologically very diverse subgenera *Amerallium* and *Rhizirideum.* With exception of the isolated position of *A. oreophilum* (HANELT & al. 1989), however, the striking morphological variability of the species within

subg. *Melanocrommyum* is hardly reflected in the molecular data. Thus, the detailed taxonomic classification recently proposed by KHASSANOV & FRITSCH (1994) seems to be unjustified by our data of cpDNA variation. This conflict may be explained by the assumption that subg. *Melanocrommyum* is an ancient offspring from rather primitive groups of the genus, but that its astonishing radiation in Middle Asia is a more recent phenomenon, connected with the formation of many new ecological niches after the upheaval of the Tien-Shan/Alai mountain range and the accessibility for colonization of territories of the former Tethys sea.

The similarity of American and Old World members of subg. *Amerallium* in regard to important karyological, anatomical and serological characters is confirmed by their RFLP patterns. Species of both geographical groups are united into the upper main branch of the phenogram, and in its first subgroup even the Mediterranean *A. moly* and the Himalayan/West Chinese *A. hookeri* + *A. farreri* are clustered together with American taxa like *A. canadense* and *A. cernuum*. This branching pattern demonstrates close relationships between geographically diverse members of this subgenus and implicitly also their long common evolutionary history. The branching at a low similarity level within the *Amerallium* clade may indicate a rather high phylogenetic age of the subgroups which is partly true also for subg. *Rhizirideum*. Presumably an ancestral group of subg. *Amerallium* established itself in America where it could evolve and radiate into numerous species. This might explain the rather close clustering and high similarity levels of the American species in contrast to the European ones.

With the exception of subg. *Bromatorrhiza*, the ranking of the subgenera of the traditional classification is thus confirmed by the cpDNA phenogram. The same is true for most of the sections from which at least some taxa have been analysed. The more separate position of *A. altaicum* and *A. fistulosum* (hitherto sect. *Cepa* subsect. *Phyllodolon*) from taxa of sect. *Cepa* s.str (*A. cepa, A. galanthum*), however, suggests the reestablishment of *Phyllodolon* as a section of its own (as formerly described by PROKHANOV 1931). The RFLP data also suggest a closer relationship between the subgenera *Melanocrommyum* and *Rhizirideum* than supposed earlier, and a greater affinity of sects. *Allium* and *Scorodon* (subg. *Allium*), both only distantly related to sect. *Codonoprasum* (formerly thought to be closer to sect. *Scorodon* (HANELT & al. 1992).

In this first application of a molecular approach to the whole genus, the analysis of RFLP patterns of total cpDNA has proven to be a useful tool to reveal relationships within *Allium* at several taxonomic levels. In future studies, more sensitive methods of cpDNA analysis like restriction site variations, hybridization with specific plastid probes, or DNA sequencing will be used in order to obtain more detailed information about selected taxonomic and evolutionary problems.

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