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Criteria for Sampling in *Allium* Based on Chloroplast DNA PCR-RFLP'S

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ABSTRACT. Variation of approximately 20 kb of non-coding regions of the chloroplast genome of 29 species of *Allium* and 7 species of related genera have been cladistically analyzed. Both restriction site mutations and length mutations have been included in these analyses. The cladograms obtained with both types of mutation are highly similar topologically and display similar levels of homoplasy. No indication of significant between-data-set incongruence was found. The results of Wagner, Dollo and weighted parsimony indicate that recognition of the monotypic *Nectaroscordum siculum* renders *Allium* paraphyletic. The two large subgenera *Rhizirideum* and *Bromatorrhiza* are probably not monophyletic. Two strongly supported clades, both of which comprise species of different subgenera, are not characterized by morphological synapomorphies when analyzed a posteriori. Furthermore, the position of *Allium tuberosum* (subg. *Rhizirideum*) and *A. fimbriatum* (subg. *Amerallium*) changes considerably with different types of analysis, which suggests that inclusion of these and affiliated taxa may strongly influence the relationships found among chloroplast types in the genus. The highly artificial nature of the subgenera (subg. *Bromatorrhiza* is also indicated to be para- or polyphyletic) is not unexpected given the taxonomic complexity of *Allium*. As a consequence, however, many more taxa have to be included in an analysis of the phylogenetic relationships of chloroplast DNA's in the genus. The *trnK*, *trnC* - *trnD*, *psbA* - *trnS*, and *rbcL* - *ORF106* regions are most suited for further use because these regions are easily amplified and exhibit a considerable level of sequence variation with a restricted degree of length variation.

The species of the circumboreal genus *Allium* (Alliaceae) are primarily found in humid, mesic, semi-arid and arid environments. Most of the approximately 700 species currently recognized (Hanelt et al. 1992) are hemicryptophytes or geophytes which are specifically adapted to different environments by means of bulbous or rhizomatous storage organs. Similar storage organs probably evolved several times independently (Cheremushkina 1992; Kamenetsky 1996).

Many of the infrageneric taxa are characterized by combinations of subtle differences which frequently can not be observed in dried specimens (Traub 1968). As a result, the genus has been considered taxonomically difficult (Traub 1968). Thirty-one species (grouped into three informal groups) were recognized by Linneaus (1753). Six sections were distinguished for the Old and New World Alliums by Regel (1875). The two latest taxonomic revisions of Kamelin (1973) and Hanelt et al. (1992) both distinguish six subgenera. The number of infrageneric taxa recognized in these studies showed a sharp increase to 30 sections and 13 subsections, and 46 sections and 11 subsections, respectively. Using the classification of Hanelt et al. as a reference, the genus is classified into four large subgenera each with 100–300 species (*Allium*, *Amerallium*, *Melanocrommyum*, and *Rhizirideum*), one subgenus with nine species (*Bromatorrhiza*) and a subgenus with two or three East Asian species (*Caloscordon*). All subgenera are mainly distributed in the Northern hemisphere. Many species have overlapping geographic distribution areas, one morphologically variable species, chive (*A. schoenoprasum*), even has a circumboreal distribution.

The increase in the number of infrageneric taxa as exemplified by the number of sections clearly reflects the problematic taxonomic delimitation of groups within the genus. Although to date no formal cladistic analyses of morphological or anatomical data have been conducted, large scale taxonomic studies using morphological data from flowers, embryos, leaves, and seeds (compiled in Hanelt et al. 1992), have not revealed any gross morphological synapomorphies for the large subgenera (Havey 1992). Some potential/putative

synapomorphies from anatomy, phenology and cytology have been reported for the large subgenera, for example for subg. *Melanocrommyum* (two rows of vascular bundles in the leaf blade instead of one, an extremely short developmental period, the presence of true palisade parenchyma; Hanelt et al. 1992) and *Amerallium* (base chromosome number $x = 7$ instead of $x = 8$ in nearly all species). In view of the lack of visible morphological synapomorphies, it is not surprising that many of the subgenera, sections and varieties have been hypothesized to be artificial (Havey 1992).

Despite the economic value of species such as *A. cepa* (bulb onion) and *A. sativum* (garlic), reliable information on the evolution of the genus is extremely scarce. Phylogenetic studies using molecular markers did not result in robust phylogenetic inferences either, primarily because an extremely small and biased number of species was used (Havey 1991, 1992). Probably, large numbers of species need to be included for a correct inference of the lineages in the genus.

The phylogenetic analysis of variation of PCR amplified segments of the chloroplast genome is expanding, as has been foreseen (Olmstead and Palmer 1994). Molecular phylogenies at the interfamilial (Tsumura et al. 1995), intergeneric (Liston et al. 1995), intrageneric level (Schwarzbach and Kadereit 1995), and at the intraspecific level (Ferris et al. 1993) have indeed demonstrated the value of the approach. It is clear from the literature (e.g., Gielly and Taberlet 1994) that different noncoding regions have evolved at different rates and under different constraints.

The relatively recent, strongly subdivided classification of Hanelt et al. (1992) is taken as a starting point for a preliminary analysis of the phylogenetic relationships based on PCR-RFLP's of non-coding regions of the chloroplast genome. The most important goal of this study is to identify natural groups and to provide criteria for further sampling within the genus.

Materials and Methods

The species studied were selected from the *Allium* working collection of the Taxonomy Department, IPK Gatersleben. This collection comprises more than 300 species, most with multiple accessions per species. Most accessions were collected in natural habitats. All species were carefully determined and are under permanent taxonomic supervision. Voucher specimens have been deposited in the Herbarium Gaterslebense (GAT) under the respective species name and accession number. Accession numbers and the sectional classification (Hanelt et al. 1992) of the species examined are indicated in Table 1.

Total DNA isolation was according to Maass & Klaas (1995). Primers for non-coding regions of the chloroplast genome, which together cover approximately 20 kb of the chloroplast genome, were from Taberlet et al. (1991), Arnold et al. (1991), Tsumura et al. (1995), Demesure et al. (1995), Savolainen et al. (1995) and Liston and Wheeler (1994). Amplification was performed in a water cooled thermocycler (Autogene) with 4.5 min denaturation at 92°C, 35 cycles of 24 sec at 92°C, 2 min annealing at 55.5°C and 24 sec extension at 72°C. Water temperature was 30.0°C. Using PCR-RFLP's, it is possible to rapidly survey a number of informative restriction site changes in a relatively large number of species. Digested amplification products were separated on 1.5% agarose gels. After amplification, a chloroform extraction, precipitation with ethanol, restriction digestion, and separation of digested products on 1.5% agarose gels with subsequent ethidium bromide staining was performed to detect polymorphisms. Only enzymes which cut the chloroplast DNA relatively infrequently were used (i.e. *BamHI, BanII, BclI, BglII, BstEII, ClaI, DraI, EcoRI, EcoRV, FokI, HaeIII, HindIII, HpaII, KpnI, PvuII, PstI, SalI, XbaI*, and *XhoI*, used singly or in combination) in order to ensure homology of restriction sites. No four base cutter enzymes with 50% AT content in their recognition sequence were used. The conservative approach resulted in a number of enzyme-probe combinations which did not reveal any polymorphisms.

Representatives of each of the six subgenera of Hanelt et al. (1992) were included. Relatively few representatives of the subgenera *Melanocrommyum* (3) and *Allium* (4) were investigated. The former subgenus has been indicated to be genetically rather homogeneous (Samoylov et al. 1995; Linne von Berg et al. 1996). No such evidence is at hand for subgenus *Allium*, and therefore this subgenus is probably underrepresented. For comparison of results with those of previous studies, some economically important species were included as well [*A. sativum, A. cepa, A. ampeloprasum* (leek), *A. schoenoprasum* (chive)]. The other species represent 24 sections. Outgroup species were selected from genera of Alliaceae and Amaryllidaceae as circumscribed by Fay and Chase (1996). In that study, the genus *Allium* occupies an isolated position in

TABLE 1. Plant material of *Allium*, *Nectaroscordum* and outgroup taxa studied.

Genus	Subgenus	Section	Species	Accession number
Allium L.	*Allium*	*Allium*	*A. sativum* L.	TAX4839
		Allium	*A. ampeloprasum* L.	TAX5173
		Scorodon Koch	*A. obtusiflorum* DC.	TAX3101
		Scorodon Koch	*A. tchihatchewii* Boiss.	TAX3063
		Vvedenskya Kamelin	*A. kujukense* Vved.	TAX3625
	Amerallium Traub	*Amerallium* Traub	*A. drummondii* Regel	TAX0200
		Arctoprason Kirschl.	*A. ursinum* L.	TAX9002
		Caulorhizirideum Traub	*A. validum* Wats.	TAX1779
		Lophioprason Traub	*A. cernuum* Roth	TAX0682
		Molium G. Don ex Koch	*A. roseum* L.	TAX3078
		Molium G. Don ex Koch	*A. subhirsutum* L.	TAX1447
		Narkissoprason (F. Herm.) Kamelin	*A. narcissiflorum* Vill.	TAX2628
		Rhophetoprason Traub	*A. fimbriatum* Watson	TAX3487
	Bromatorrhiza Ekb.	*Bromatorrhiza* Ekb.	*A. hookeri* Thw.	TAX2506
		Bromatorrhiza Ekb.	*A. wallichii* Kunth	TAX2510
		Cyatophora R. M. Fritsch	*A. cyathophorum* Bur. et Franch. var. *farreri* (Stearn) Stearn	TAX2824
	Caloscordum (Herb.) R. M. Fritsch	*Caloscordum* Herbert	*A. neriniflorum* (Herb.) Baker	TAX2797
	Melanocrommyum (Webb *et Berth.) Rouy*	*Acmopetala* R. M. Fritsch	*A. collis-magni* Kamelin	TAX3730
		Compactoprason R. M. Fritsch	*A. giganteum* Regel	TAX2936
		Porphyroprason Ekb.	*A. oreophilum* C. A. Mey.	TAX0348
	Rhizirideum (G. Don ex Koch) Wendelbo	*Anguinum* G. Don ex Koch	*A. tricoccum* Sol. in Ait.	TAX2582
		Anguinum G. Don ex Koch	*A. victorialis* L.	TAX2673
		Butomissa (Salisb.) Kamelin	*A. tuberosum* Rottl. ex Spr.	TAX2453
		Cepa (Mill.) Prokh	*A. cepa* L.	TAX4722
		Oreiprason F. Herm.	*A. globosum* M. Bieb. ex Red.	TAX1605
		Petroprason F. Herm.	*A. obliguum* L.	TAX0338
		Reticulata-bulbosa Kamelin	*A. cyaneum* Regel	TAX3849
		Schoenoprasum Dumort.	*A. schoenoprasum* L.	TAX3176
		Tenuissima (Tzag. Hanelt)	*A. tenuissimum* L.	TAX3249
Nectaroscordum Lindl.			*N. siculum* (Ucria) Lindl.	TAX2192
OUTGROUPS:				
Nothoscordum Kunth			*N. gracile* (Ait.) Stearn	TAX0191
Triteleia Douglas ex Lindle			*T. hyacynthina* (Lindl.) Greene	TAX1795
Tulbaghia L.			*T. violacea* Harv.	TAX2164
Dichelostemma Kunth			*D. pulchellum* (Salisb.) Heller	TAX2470
Bloomeria Kellog			*B. crocea* (Torrey) Coville	TAX2697
Agapanthus L'Herit.			*A. campanulatus* Leighton	TAX9004

Alliaceae, its closest relative being the Himalayan species *Milula spicata* Prain. As a result of this isolated position, we felt it important to include several outgroups. Unfortunately, neither fresh nor herbarium material of *Milula* Prain. species was available. Only one representative of the outgroup genera used in this study was included in a broader study of Lilianae which also supported affiliation

between Amaryllidaceae and Alliaceae (Chase et al. 1995).

Both restriction site and length variation were scored and all characters were unordered. Restriction site data was analyzed both separately and together with length-mutation data. For every region, only length polymorphisms shared by different combinations of taxa were scored.

Phylogenetic analyses of restriction site changes were performed on PAUP 3.1.1. (Swofford 1993) and involved Dollo, Wagner and weighted parsimony. For the first two types of analyses, TBR branch swapping, steepest descent, and mulpars were used. Weighted parsimony (1.1 to 1.3 weight ratio's for gains over losses, Albert et al. 1992) was implemented with SPR branch swapping, mulpars, steepest descent and mulpars and 40 random additions of taxa. The length mutations were also analyzed cladistically as unordered characters with NNI branch swapping, steepest descent, MULPARS, and 1,000 random additions of taxa (with a maximum of 100 trees per random addition of taxa). Statistical support of internal branches by means of a decay analysis was assessed with SPR branch swapping, steepest, mulpars and 500 random additions of taxa.

Cladistic analyses were performed on restriction site changes only, on length mutations only, and on length and restriction site changes combined. Prior to performing these analyses however, we determined the degree of incongruence within and between the data sets. The data sets have an identical evolutionary history because both are derived from the same, non-recombining genome. Significant levels of incongruence would therefore indicate technical difficulties such as problems of scoring length variation of the amplified regions instead of a different history of different partitions. Nevertheless, incongruence could still be taken to indicate that the data sets must not be combined (Huelsenbeck et al. 1996). The incongruence indices of Mickevich and Farris and Miyamoto (Swofford 1991) were calculated. Because in all analyses more than one most parsimonious tree was found, strict consensus trees were calculated and used as a constraint for the other data set.

The constrained analyses in which infrageneric groups were forced into monophyly were designed to determine the number of extra steps to obtain monophyly. These analyses were conducted using Wagner parsimony on the combined data set of restriction site changes and length polymorphisms. Search options were identical to those of the analyses of combined restriction sites and length mutations described above. Pairwise absolute distances (PAUP 3.1.1.) between species were calculated from the data matrix using both restriction site changes and polymorphisms to determine the degree of variation between taxa. The complete list of site and length mutations is available upon request.

Results

Inclusion of different combinations of outgroup species did not seem to alter the phylogenetic relationships within *Allium* (with *Nectaroscordum siculum* included in the ingroup) for the restriction site changes, the length mutations or the combined analysis. Therefore, only analyses with all outgroup species included are presented. 85 informative restriction site changes, and 19 unique restriction site changes were scored. Wagner parsimony of the informative restriction sites for all amplified regions found 476 trees with 231 steps, a consistency index of 0.41, and a retention index of 0.62. The strict consensus tree (Fig. 1) indicates that many relationships are unresolved.

Dollo parsimony of the informative restriction sites for all amplified regions found 24 trees with 314 steps, a consistency index of 0.30, and a retention index of 0.88 (not shown). The most important difference with the Wagner analysis of restriction sites is that the clade comprising *A. obliquum*, *A. globosum*, *A. cyathophorum*, *A. tchihatschewii*, *A. obtusiflorum*, *A. schoenoprasum*, *A. cepa*, *A. tenuissimum*, *A. cyaneum*, *A. sativum*, and *A. ampeloprasum* (clade A) is not recovered. The Dollo analysis indicated that the American and Eurasian species of subg. *Amerallium* are sister taxa.

Thirty-three informative length mutations, and four unique length mutations were scored. Wagner analysis of the length mutations resulted in more than 32,000 minimal length trees of 88 steps (Fig. 2, consistency index 0.48, retention index 0.69). Monophyly of *Allium* (*Nectaroscordum siculum* included in *Allium*), as well the detection of clade A agree with the analyses of restriction site mutations. These findings suggest a similar topology calculated from length polymorphisms on the one hand and restriction site mutations on the other.

The incongruence indices of Miyamoto, and Mickevich and Farris index (Table 2) indicated moderately low values of incongruence. The tests do not lend themselves to rigorous conclusions

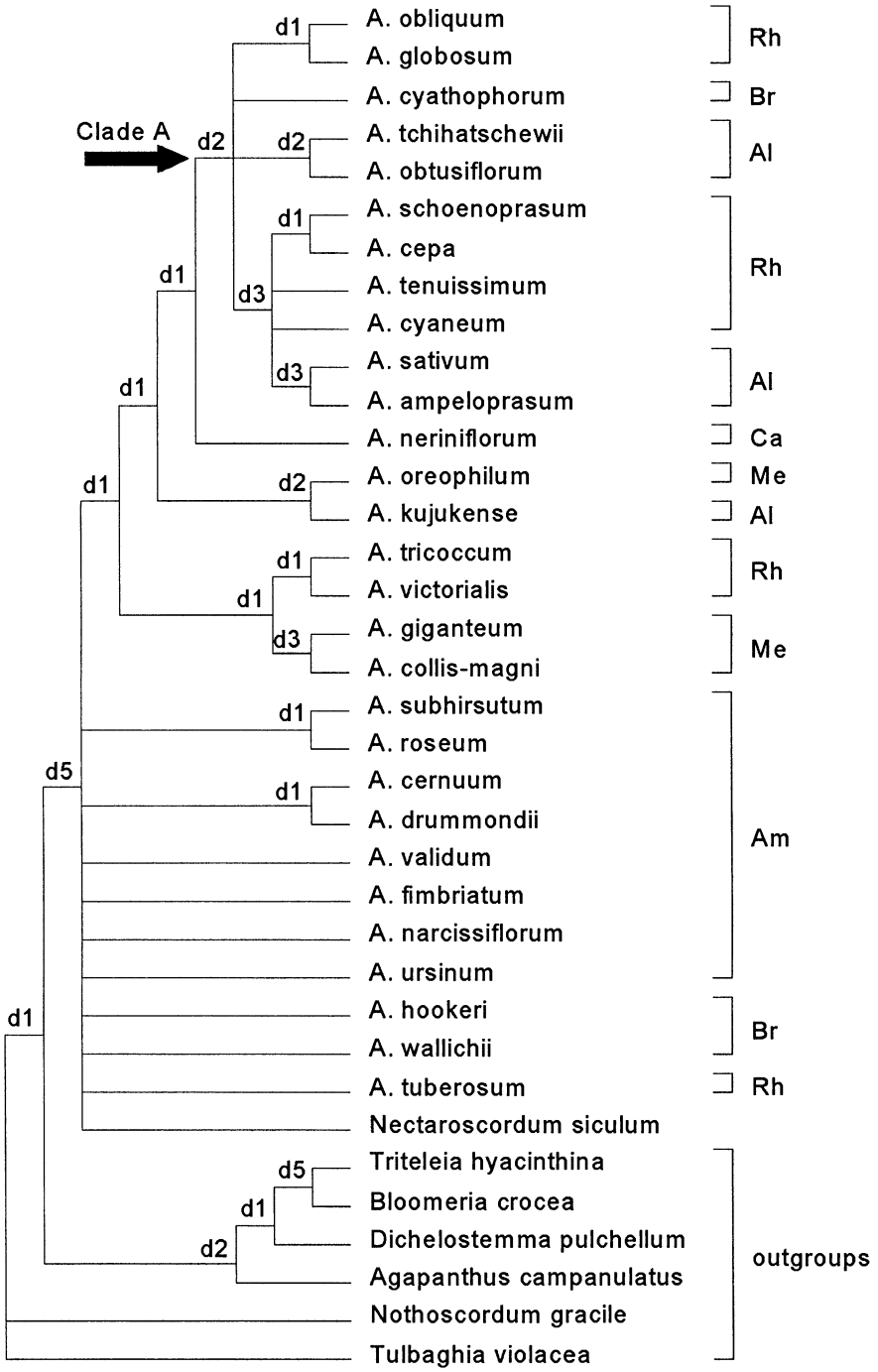

FIG. 1. Strict consensus Wagner tree based on only restriction site changes. Values preceded by a 'd' are decay values. Acronyms for the subgenera are: Al: *Allium*. Am: *Amerallium*. Br: *Bromatorrhiza*. Ca: *Caloscordum*. Me: *Melanocrommyum*. Rh: *Rhizirideum*.

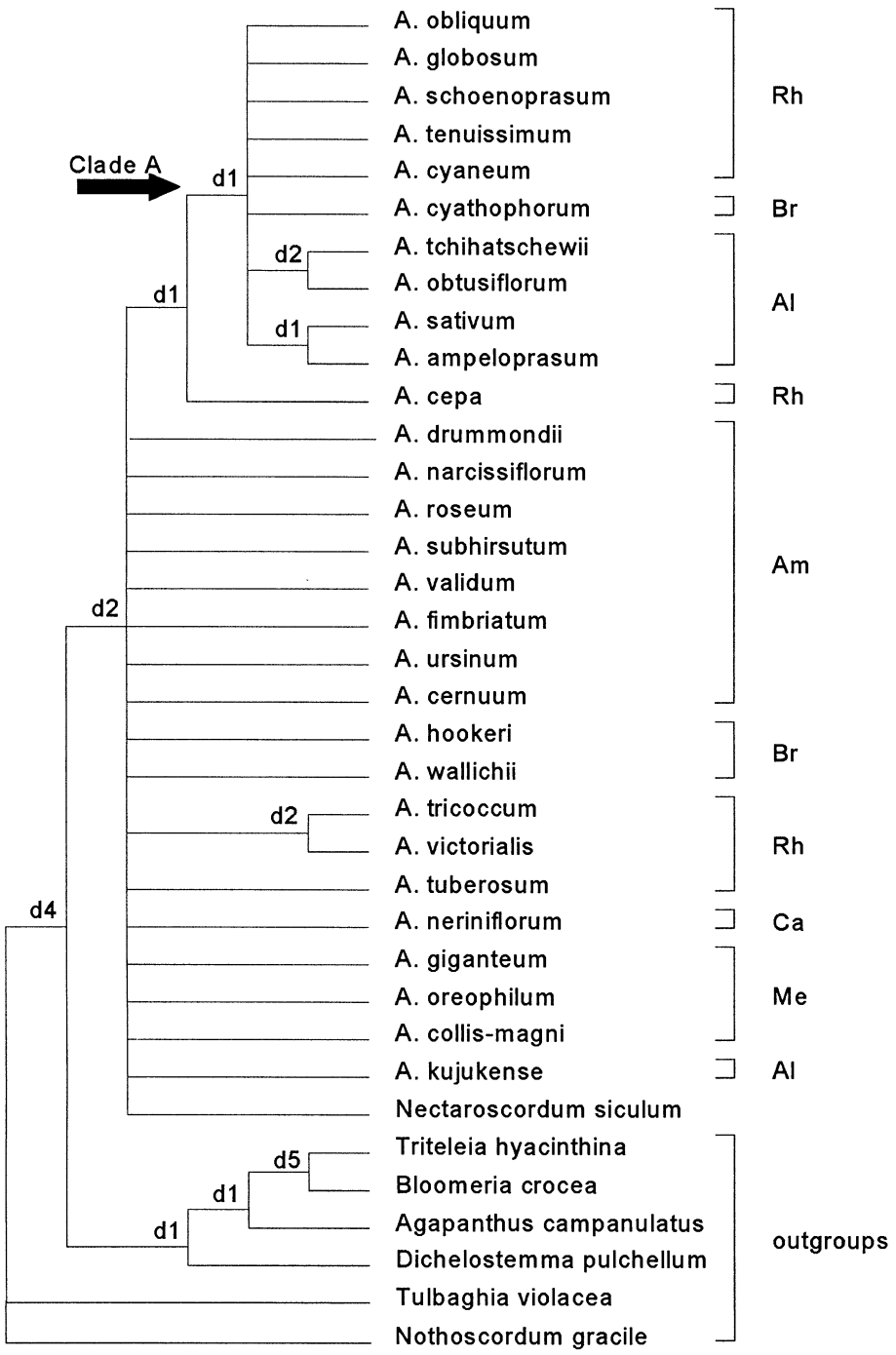

FIG. 2. Strict consensus of Wagner trees based on length mutations. Values preceded by a 'd' are decay values. Acronyms for the subgenera are as those listed for Fig. 1.

TABLE 2. Character incongruence between the *Allium* data sets as determined by the Mickevich and Farris' and Miyamoto's indices of incongruence. Strict consensus trees were used to summarize topologies based on the two individual data sets. c = number of phylogenetically potentially informative characters. s = number of steps of the most parsimonious trees. e = number of extra steps. e* = number of extra steps when data set is applied to tree based on the alternative data set. I_W = within-data set incongruence ($I_W = \Sigma e$). I_B = between data set incongruence ($I_B = I_T - I_W$). I_T = total incongruence for the Mickevich and Farris index ($I_T = s - c$ for combined data sets). I_T^* = total incongruence for the Miyamoto index ($I_T^* = \Sigma e^*$). I_M = Miyamoto index ($I_M = (I_T^* - I_W^*)/I_T^*$). I_{MF} = Mickeviche and Farris index ($I_{MF} = I_B/I_T$).

	Site mutations	Length mutations	Combined mutations
Site mutations	c = 85, s = 231, e = 146	c = 85, s = 236, e* = 151	
Length mutations	c = 33, s = 101, e* = 68	c = 33, s = 88, e = 55	
Combined mutations			c = 118, s = 329, I_W = 201, I_B = 10, I_T = 211, I_T^* = 219, I_M = 0.085, I_{MF} = 0.047

however because null distributions for the test statistics have not yet been developed (Swofford 1991), and the use of strict consensus trees to summarize many most parsimonious trees probably renders these incongruence tests less powerful. Nevertheless, no strong indication for incongruence was found.

When the length polymorphisms were added to the restriction site data 81 trees were found with 329 steps, a consistency index of 0.41 and a retention index of 0.62 (Fig. 3). The consistency and retention indices in this combined analysis are identical to those obtained in the restriction site analysis. No indications for multiple islands of most parsimonious trees for the individual data sets were found as determined by tree-to-tree distances (Maddison 1991). Weak support is found for monophyly of subg. *Amerallium* (clade C). Two strongly supported clades were found. One comprises *A. oreophilum*, *A. kujukense*, *A. giganteum*, *A. collis-magni*, *A. tricoccum*, *A. victorialis*, and *A. neriniflorum* (clade B) and the other clade comprised *A. obliquum*, *A. globosum*, *A. cyathophorum*, *A. tchihatchewii*, *A. obtusiflorum*, *A. cyaneum*, *A. schoenoprasum*, *A. tenuissimum.*, *A. cepa*, *A. sativum*, and *A. ampeloprasum* (clade A). These analyses also indicate that the small subg. *Bromatorrhiza* is artificial. Relationships in subg. *Amerallium* are unresolved.

The Dollo analysis of sites and indels resulted in 12 trees with 417 steps, a consistency index of 0.32, and a retention index of 0.86. The strict consensus tree is to a large extent unresolved (not shown) and the underlying topological changes are restricted to a single species, i.e. *A. tuberosum*. The 12 most parsimonious trees belonged to two distinct islands of trees. In one island of six trees, *A. tuberosum* is sister to *Nectaroscordum siculum* whereas in the other island, it is sister to *A. oreophilum*, *A. kujukense*, *A. collis-magni*, and *A. giganteum*. The variable position of *A. tuberosum* is reflected in the Adams consensus tree of Fig. 4 as well as in weighted analyses. The position of *A. tuberosum* varies from sister to *A.* subg. *Rhizirideum* and subg. *Allium* (weight ratio 1.1 and 1.2), to sister to all other species of the genus (weight ratio 1.3).

Many of the subgenera of *Allium* are indicated to be artificial, although only a small sample of taxa has been analyzed for most subgenera. Table 3 indicates the number of additional steps required to obtain the monophyly of some traditionally recognized taxa of *Allium*. For the monophyly of most subgenera a relatively small number of extra steps is needed. However, the forced monophyly of the subgenera *Bromatorrhiza* required an additional 11 steps and that of *Rhizirideum* required an additional 16 steps.

In all analyses, *Nectaroscordum siculum* is included in the genus *Allium*. Although strong statistical support for the paraphyly of the genus *Allium* was not found (Table 3), *Nectaroscordum siculum* was never found to be sister to *Allium* in any of the analyses.

The position of *A. fimbriatum* (subg. *Amerallium*) in the Wagner analyses of site data is highly variable. It constitutes a position distinct from the other species of subg. *Amerallium* in the 50% majority rule Wagner tree of the combined analysis (not shown) and it is the putative sister species of the other species of *Amerallium*, *Nectaroscordum siculum*, *A. wallichii*, *A. hookeri* in the Wagner analyses of the combined sites and indel data, as well as in several weighted (1.1 and 1.2) analyses. In the Dollo analyses however, this species is terminal in a clade containing the other species of subg. *Amerallium*, subg. *Bromatorrhiza* and *Nectaroscordum siculum*.

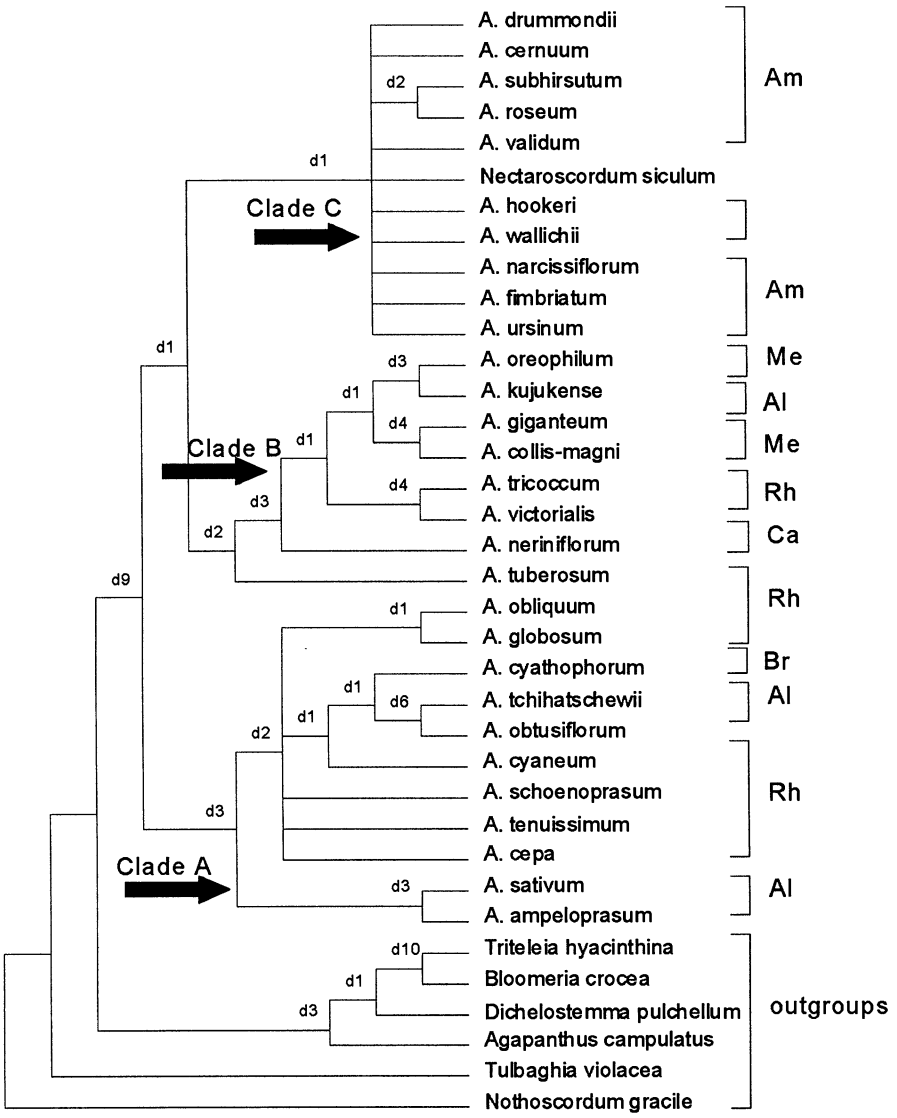

FIG. 3. Strict consensus of Wagner trees based on both restriction site changes and length. Values preceded by a 'd' are decay values. Acronyms for the subgenera are as those listed for Fig. 1.

The Wagner strict consensus tree using only site changes indicates that many relationships are unresolved as a result of the lack of synapomorphies in *Allium*. The addition of the unordered length mutations results in higher support for some clades. Likewise the consistency in this combined analysis does not deviate from the analysis using only site changes.

The number of autapomorphic restriction site mutations (aut *), the synapomorphic restriction site changes shared by two or more species (syn *), autapomorphic indels (aut p), and synapomorphic indels (syn p) are presented in Table 4. The divergence among the species of clades A, B, and C was calculated. The maximum number of absolute differences and the mean number of mutations between any pair of species in these three groups is 19 and 10.0 in clade A, 17 and 11.2 in clade B, and 21 and 14.0 in clade C, respectively. These data suggest that divergence is highest in the clade C which comprises representatives of the subgenera *Ameral-lium*, *Bromatorrhiza*, and *Nectaroscordum siculum*.

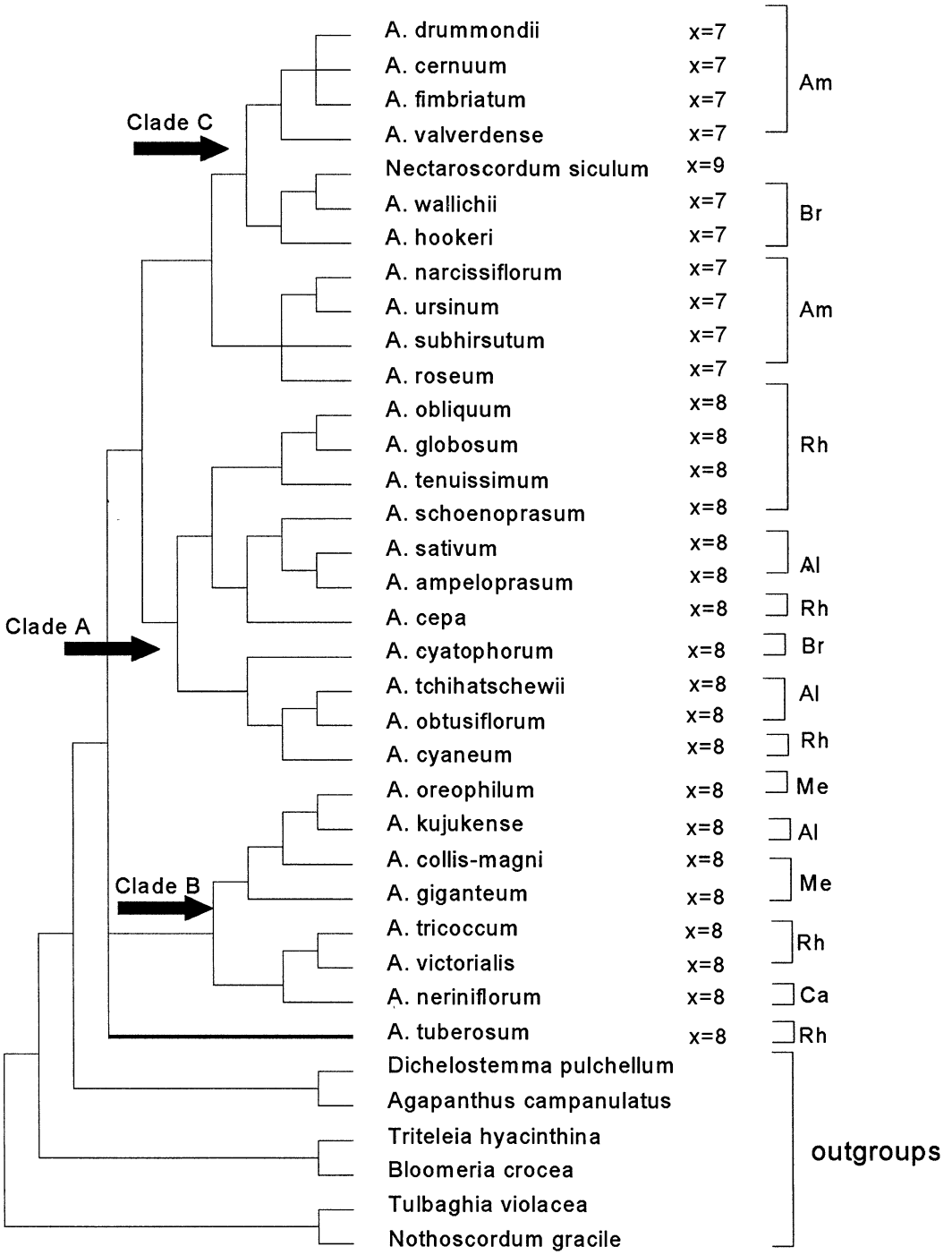

FIG. 4. Adams consensus tree of 12 most parsimonious Dollo cladograms of 417 steps. The trees comprised two topologically distinct islands in which the position of *A. tuberosum* varied considerably. The base chromosome number and subgeneric classification according to Hanelt et al. (1992) are indicated at the right. Acronyms for the subgenera are as those listed for Fig. 1.

TABLE 3. The number of additional steps needed to obtain the monophyly of several traditionally recognized subgenera of *Allium*, and the genus *Allium*. The most parsimonious trees based on the Wagner analysis of the combined data sets of restriction site and length mutations were used as a reference.

Monophyly of:	Number of additional steps needed
genus *Allium* (*Nectaroscordum siculum* sister to species of *Allium*)	3
subgenus *Allium*	3
subgenus *Amerallium*	2
subgenus *Bromatorrhiza*	11
subgenus *Melanocrommyum*	4
subgenus *Rhizirideum*	16

DISCUSSION

Our study has implications for further analysis of the evolutionary relationships in the large genus *Allium*. *Nectaroscordum*, which has been excluded from *Allium* on the basis of floral characteristics and the shape of the spathe, should be included in comparative investigations of *Allium*. The results of cpDNA RFLP's agree with a study of seed surface morphology where *Allium* and *Nectaroscordum* are distinct from other genera such as *Nothoscordum*, *Agapanthus*, *Triteleia*, *Dichelostemma* and *Milula* (Kruse 1994) and with a study of rbcL sequence variation in *Alliaceae*, *Amaryllidaceae*, and *Agapanthoideae* (Fay and Chase 1996) in which species of *Allium* and *Nectaroscordum siculum* form a clade. Second, the subgenera *Rhizirideum* and *Bromatorrhiza* are artificial. The number of additional steps needed to achieve monophyly of these two subgenera in the Wagner analyses using sites and indels is 16 and 11 steps, respectively. Apart from the lack of a well-supported and well-resolved chloroplast phylogeny of the genus, the role of reticulation in the evolution of the genus is not known. Therefore, the evolution of some characters considered crucial for taxonomic grouping of *Allium*, such as rhizomes or bulbs, the ancestral number of chromosomes, and the geographic origin of the genus remain to be established.

Given the high frequency of unnatural groups within the genus, two suggestions can be made. First, future experiments specifically designed to study the entire genus need to include a high number of species of subg. *Amerallium* because of

TABLE 4. Characteristics of the amplified regions of the chloroplast genome and the restriction enzymes used in this study of *Allium*. Length variation in this sample of species of *Allium*, the length variation and the number of different types of length- and site changes after digestion with the various restriction enzymes are indicated. The mutations are differentiated into the numbers of unique restriction site changes (aut *), the restriction site changes shared by two or more species (syn *) and indels (aut p and syn p).

Region	aut *	syn *	aut p	syn p	Restriction enzymes used	Length (bp) and length variation
trnK	8	14	1	10	EcoRI, EcoRV, BamHI, HindIII, HaeIII, XbaI, XhoI, DraI, BanII, BglII, ClaI, FokI, PvuII, Pst, HpaII, BclI, BstEII, KpnI, XhoI/SalI	2900, ±100
trnT-trnF	0	8	2	8	EcoRI, EcoRV, BamHI, HindIII, DraI, ClaI, XbaI, PstI/PvuII, BanII	1500, 300
trnC-trnD	5	18	1	9	EcoRI, EcoRV, BamHI, DraI, ClaI, HindIII, XbaI, HaeIII, BanII, BglII, BclI, PstI, BstEII	3500, ±100
psbC-trnS	3	7	1	1	EcoRI, DraI, BamHI, CfoI, HpaII, HaeIII, BanII, HindIII, XbaI, EcoRV/ClaI, BglII/SalI, PvuII/PstI, BclI	1500, ±100
rbcL-orf106	12	17	1	6	HpaII, DraI, BamHI, HaeIII, EcoRV, EcoRI, BglII, HindIII/XbaI, PvuII/PstI, ClaI, BanII	3200, 200
rbcL-atpB	3	3	0	1	HpaII, HaeIII, DraI, BanII, EcoRI/BglII, EcoRV/HindIII	900, ±50
psbA-trnS	6	12	0	0	EcoRI, EcoRV, HindIII, BglII, ClaI, XbaI, DraI, BamHI, BclI, HaeIII, HpaII, BanII, PstI/PvuII, SalI/XhoI	3100, ±100
rpoCI-rpoCII	4	15	0	4	EcoRI, HaeIII, EcoRV, HpaII, XbaI, BglII, PstI, PvuII, ClaI, BamHI, BanII, SalI/XhoI, BclI, KpnI	4200, ±200
trnD-trnT	2	3	0	5	XhoI/BglII/SalI, BclI, HindIII, ClaI, XbaI, BamHI, EcoRV, EcoRI, HaeIII, HpaII	1200, ±100

the high number of mutations and the high level of homoplasy in the subgenus (see position of *A. fimbriatum* and unresolved relationships in the clade C). Morphologically this subgenus is one of the most variable (Hanelt. et al. 1992). Second, increased sampling needs to be performed on *A. tuberosum* and its allies due to the variable position of this species in different analyses.

The *trnK, trnC - trnD, rbcL - ORF106* and the *psbA - trnS* regions seem most promising for further studies in *Allium*. Both restriction site mutations and length polymorphisms are present in these regions, and the interpretation of these mutations is not hampered by excessive length variation of the region as a whole.

One of the possible causes of the incongruence between the infrageneric classification and the mutations in the chloroplast genomes of the species of *Allium* is reticulation. It will therefore be necessary to use nuclear DNA markers for an independent estimate of the evolution of the genus.

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LITERATURE CITED

ALBERT, V. A., B. D. MISHLER, and M. W. CHASE. 1992. Character-state weighting for restriction site data in phylogenetic reconstruction, with an example from chloroplast DNA. Pp. 369–403 in *Molecular systematics of plants*, eds. P. S. Soltis, D. E. Soltis, and J. J. Doyle. New York: Chapman and Hall.

ARNOLD, M. A., C. M. BUCKNER, and J. J. ROBINSON. 1991. Pollen-mediated introgression and hybrid speciation in Louisiana irises. Proceedings of the National Academy of Sciences of the U.S.A. 88: 1398–1402.

CHASE, M. W., M. R. DUVALL, G. G. HILLIS, J. G. CONRAN, A. V. COX, L. E. EGUIARTE, J. HARTWELL, M. F. FAY, L. R. CADDICK, K. M. CAMERON, and S. B. HOOT. 1995. Molecular phylogenetics of Lilianae. Pp. 109–137 in *Monocotyledons: systematics and evolution*, eds. P.J. Rudall, P. J. Cribb, D. F. Cutler, and C. J. Humphries. Kew: Royal Botanic Gardens.

CHEREMUSHKINA, V. A. 1992. Evolution of life forms of species in subgenus *Rhizirideum* (Koch) Wendelbo, genus *Allium* L. Pp. 107–123 in *The genus* Allium-*taxonomic problems and genetic resources*, eds. P. Hanelt, K. Hammer, and H. Knupffer. Germany: Gatersleben.

DEMESURE, B., N. SODZI, and R. J. PETIT. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Molecular Ecology 4: 129–131.

FAY, M. F. and M. W. CHASE. 1996. Resurrection of Themidaceae for the Brodiaea alliance, and recircumscription of Alliaceae, Amaryllidaceae and Agapanthoideae. Taxon 45: 441–452.

FERRIS, C., R. P. OLIVIER, A. J. DAVY, and G. M. HEWITT. 1993. Native oak chloroplasts reveal an ancient divide across Europe. Molecular Ecology 2: 337–344.

GIELLY, L. and P. TABERLET. 1994. The use of chloroplast DNA to resolve phylogenies: non-coding versus rbcL sequences. Molecular Biology and Evolution 11: 769–777.

HANELT, P., J. SCHULZE-MOTEL, R. M. FRITSCH, J. KRUSE, H. MAASS, H. OHLE, and K. PISTRICK. 1992. Infrageneric grouping of *Allium*—the Gatersleben approach. Pp. 107-123 in *The genus* Allium - *taxonomic problems and genetic resources*, eds. P. Hanelt, K. Hammer, and H. Knupffer. Germany: Gatersleben.

HAVEY, M. J. 1991. Phylogenetic relationships among cultivated *Allium* species from restriction enzyme analysis of the chloroplast genome. Theoretical and Applied Genetics. 81: 752–757.

———. 1992. Restriction enzyme analysis of the chloroplast and nuclear 45S ribosomal DNA of *Allium* sections *Cepa* and *Phyllodolon* (Alliaceae). Plant Systematics and Evolution 183: 17–31.

HUELSENBECK, J. P., J. J. BULL, and C. W. CUNNINGHAM. 1996. Combining data in phylogenetic analysis. Trends in Ecology and Evolution 11: 152–158.

KAMELIN, R. V. 1973. *Florogeneticeskij analiz estestvennoj flory gornoj Srednej Azii*. Leningrad: Nauka.

KAMENETSKY, R. 1996. Somatic evolution in the genus *Allium*: ecological and geographical aspects. *Proceedings of the 7th international symposium on flower bulbs*. Israel: Hezzliga.

KRUSE, J. 1994. Rasterelektronenmikroskopische Untersuchungen an Samen der Gattung *Allium* L. IV. Feddes Repertorium 105: 457–571.

LINNEAUS, C. 1753. *Species plantarum*. Stockholm: Holmiae.

LINNE VON BERG, G., A. SAMOYLOV, M. KLAAS, and P. HANELT. 1996. Chloroplast DNA restriction analysis and the infrageneric grouping of *Allium* (Liliiflorae: Alliaceae). Plant Systematics and Evolution 200: 253–261.

LISTON, A. and J. A. WHEELER. 1994. The phylogenetic position of the genus *Astragalus* (Fabaceae): evidence from the chloroplast genes rpoC1 and rpoC2. Biochemical Systematics and Ecology 22: 377–388.

———, ———, and J. W. KADEREIT. 1995. Chloroplast DNA evidence for introgression and long distance dispersal in the desert annual *Senecio flavus* (Asteraceae). Plant Systematics and Evolution 197: 33–41.

MAASS, H. I. and M. KLAAS. 1995. Infraspecific differentiation of garlic (*Allium sativum* L.) by isozyme and RAPD markers. Theoretical and Applied Genetics 91: 89–97.

MADDISON, D. R. 1991. The discovery and importance of multiple islands of most parsimonious trees. Systematic Zoology 40: 315–328.

OLMSTEAD, R. G. and J. D. PALMER. 1994. Chloroplast DNA systematics: a review of methods and data analysis. American Journal of Botany 81: 1205–1224.

REGEL, E. 1875. Alliorum. Monographia Acta Horti Petropolythanii 3: 1–266.

SAMOYLOV, A., M. KLAAS, and P. HANELT. 1995. Use of chloroplast DNA polymorphisms for the phylogenetic study of the subgenera *Amerallium* and *Bromatorrhiza* (genus *Allium*). Feddes Repertorium 106: 161–167.

SCHWARZBACH, A. E. and J. W. KADEREIT. 1995. Rapid radiation of North American desert genera of the Papaveraceae: evidence from restriction site mapping of PCR-amplified chloroplast DNA fragments. Plant Systematics and Evolution (supplement) 9: 159–170.

SAVOLAINEN, V. R. CORBAZ, C. MONCOUSIN, R. SPICHIGER, and J.-F. MANEN. 1995. Chloroplast DNA variation and parentage analysis in 55 apples. Theoretical and Applied Genetics 90: 1138–1141.

SWOFFORD, D. L. 1991. When are estimates from molecular and morphological data incongruent. Pp. 295–333 in *Phylogenetic analysis of DNA sequence*, eds. M. M. Miyamoto and J. Cracraft. New York: Oxford Univ. Press.

SWOFFORD, D. L. 1993. *PAUP: phylogenetic analysis using parsimony, version 3.1.1*. Champaign: Illinois Natural History Survey.

TABERLET, P., L. GIELLY, and J. BOUVET. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17: 1105–1109.

TRAUB, H. 1968. The subgenera, sections and subsections of *Allium* L. Plant Life 24: 147–163.

TSUMURA, Y, K. YOSHIMURA, N. TOMARU, and K. OHBA. 1995. Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. Theoretical and Applied Genetics 91: 1222–1236.