Theor Appl Genet (1999) 98: 422–433

J. G. Dubouzet · K. Shinoda

Relationships among Old and New World *Alliums* according to ITS DNA sequence analysis

Received: 29 July 1998 / Accepted: 13 August 1998

Abstract Phylogenetic analysis of ITS DNA sequences of various Old and New World *Alliums* suggests the reinclusion of *Nectaroscordum* and affirms the reestablishment of *Caloscordum* as subgenera of genus *Allium*. The results sanction the elimination of the Old World *Allium* species from subg. *Amerallium* Traub and endorse Wendelbo's definition of subg. *Molium* (excluding sect. *Porphyrason*).

Key words *Allium* · *Nectaroscordum* · *Caloscordum* · Subgenus *Amerallium* · Phylogeny · ITS sequence analysis

Introduction

Genus *Allium* (approx. 700 species) is distributed over all of the Northern Hemisphere. Two closely related genera, *Caloscordum* and *Nectaroscordum*, can be found in Europe and Asia. Both of these genera closely resemble *Allium* and also possess the latter's characteristic smell (Bryan 1989). North and South American plants classified under genus *Nothoscordum* resemble *Allium* but lack the latter's characteristic odor (Bryan and Griffiths 1995). Due to their close morphological similarities, the relationships between these genera have long been embroiled in taxonomic controversy.

Nothoscordum seems to be a well-circumscribed genus except for the misnomer *N. neriniflorum*, and no contemporary taxonomist doubts that it is a genus separate from *Allium*. Fay and Chase (1996) used *rbc*L sequence analysis to show that, despite its morphological similarity, *Nothoscordum* is a very distant relative of *Allium*. Its linkage to *Allium*, if established, could help clarify the origins of herbaceous boreotropical taxa.

The eastern Asia species *Caloscordum neriniflorum* Herbert has been included in genus *Allium* as *A. neriniflorum* (Herbert) Baker or in genus *Nothoscordum* as *N. neriniflorum* (Herb.) Benth. et Hook (Hanelt and Fritsch 1994). Kamelin (1980) placed this species under subg. *Melanocrommyum*, but Hanelt and Fritsch (1994) found sufficient basis to assign it to subg. *Caloscordum* Fritsch under genus *Allium*. Hence, their differences center on whether this species should be accorded subgeneric status in genus *Allium*.

East Mediterraenean plants classified under genus *Nectaroscordum* closely resemble *Allium* and also possess the characteristic garlic smell (Bryan 1989). *Nectaroscordum siculum* Lindl. was excluded from the genus *Allium* by the Gatersleben group (Fritsch 1992; Hanelt et al. 1992). Traub (1968) noted that the leaf vascular bundles of *Amerallium* and *Nectaroscordum* are arranged in one row, whereas those of the rest of *Allium* were arranged in a more complicated manner. Traub (1972), summarizing data from other researchers, reported that the laticifers in subgenera *Amerallium* and *Nectaroscordum* are hypodermal in the bulb scale, whereas these structures occur in the second layer below the epidermis (or deeper) in the rest of genus *Allium*. Dahlgren et al. (1985) included *Nectaroscordum* in genus *Allium*, and *rbc*L (Fay and Chase 1996) sequence analysis also affirmed that it was closely related to *Allium*. Internal transcribed spacer region (ITS) rDNA sequence analysis (Dubouzet and Shinoda 1998) indicated that *A. cernuum* (representing subg. *Amerallium*) was more closely related to *N. siculum* than to representatives of other *Allium* subgenera.

Traub (1968, 1972) proposed a classification system for *Allium* which divided the species into three subgenera, *Allium*, *Amerallium* Traub and *Nectaroscordum*

Communicated by R. Hagemann

J. G. Dubouzet (✉) · K. Shinoda
Hokkaido National Agricultural Experiment Station,
Hitsujigaoka 1, Toyohira-ku, Sapporo 062, Japan
Fax: + 81 011-859-2178
E-mail: joseph@cryo.affrc.go.jp

(Lindl.) Traub. The most recent classification of this genus is based on morphological, cytological, geographical, serological and anatomical studies (Hanelt et al. 1992). This classification largely accepted Traub's definition of *Amerallium* but rejected his ideas on the rest of the genus.

Traub (1968) defined subg. *Amerallium* to include *Allium* species found in the Old and New Worlds with one-nerved tepals, leaf vascular bundles arranged in one row, and, with few exceptions, a basic chromosome number of $x = 7$. Most (90 out of 130) of the species are found in North America while the rest are spread out, rather disjunctively, in the Mediterraenean and North African regions (Hanelt et al. 1992). Linne von Berg (1996) generated restriction fragment length polymorphisms (RFLPs) from chloroplast (cp) DNA of various *Alliums* using cpDNA from *A. tuberosum* as a probe, but they failed to completely separate the North American from the Mediterraenean *Ameralliums*. This failure must have been due to distant genetic relationship between the source of probe (*A. tuberosum*) and the *Ameralliums* (see Dubouzet et al. 1997). The geographic separation among the *Ameralliums*, however, was successfully reflected in phylogenetic trees derived from chloroplast RFLP profiles probed with specific gene fragments (Samoylov et al. 1995).

The separation between the Old and New World branches of subg. *Amerallium*, evidenced by geographical and molecular data, has fueled doubts in other researchers. According to McNeal (1992), US systematists do not fully subscribe to Traub's classification and prefer Ownbey's (see Saghir et al. 1966) loose 'Alliances' instead. McNeal and Ownbey (1973) pointed out major differences between some Old and New World species classified in Traub's *Amerallium* in terms of bulb morphology and morphogenesis. Unfortunately, Ownbey's classification of North American *Alliums* did not speculate on their relationship to the Old World *Alliums*. In addition, the term 'Alliance' has no status under the taxonomic code of nomenclature (McNeal 1992).

DNA sequence analysis of ITS of nuclear ribosomal DNA has provided sufficient evidence to largely confirm the current classification of subgenera in *Allium* and sections in subg. *Melanocrommyum* (Dubouzet and Shinoda 1998). In the study presented here, phylogenetic analysis of the ITS DNA sequences was performed to elucidate the position of various Old and New World *Allium* species classified under subgenus *Amerallium* in relation to representative species from the better circumscribed subgenera in the genus *Allium*. In addition, representative species from the closely allied genera *Caloscordum*, *Nectaroscordum* and *Nothoscordum* were also evaluated to determine their phylogenetic relationship to genus *Allium*. The ITS-based phylogeny was analysed and discussed in regard to current views on the infrageneric classification and evolution of *Allium*.

Materials and methods

The genetic materials listed in Table 1 were obtained from the *Allium* collection of the Hokkaido National Agricultural Experiment Station. DNA extraction, polymerase chain reaction (PCR) amplification of ITS sequences and cycle sequencing were performed as previously described (Dubouzet and Shinoda 1998). The PCR reaction was kept in ice water during mixing ('Cold-Mix') and the tubes were loaded onto a preheated (approx. 90°C) thermal cycler ('Hot Start'). Amplification was performed using 'Touchdown PCR' (Don et al. 1991). Replicate PCR reactions were pooled and purified to act as the template for the cycle sequencing reaction. Replicated, independent sequencing of the forward and reverse-primed reactions was also performed.

These ITS sequences were analysed along with sequences from *A. cernuum*, *A. giganteum*, *A. oreophilum*, *A. sativum*, *A. senescens* and *N. siculum* that were obtained from our previous study (Dubouzet and Shinoda 1998). *Nothoscordum gracile* was used as the outspecies for comparison. The sequences generated in this study were submitted to GenBank (NCBI, USA) under accession numbers AF055095-AF055113.

The ITS DNA sequences were too variable for manual alignment. Alignment was performed using the "old guide tree file" option, but the rest of the parameters used the default settings of CLUSTAL W (Thompson et al. 1994). The sequences were degapped in-between alignments. Alignment was reiterated three to four times until the shortest and most stable alignment was obtained. Jiggling with CLUSTAL W's defaults nor manual refinement of the alignment were not done to avoid introducing subjective bias. Two alignments were performed, with or without *Nothoscordum*.

Neighbor Joining genetic distances were calculated using the "exclude positions with gaps" and "correct for multiple substitutions" options of CLUSTAL W. Bootstrapping was performed 1000×. Kimura 2-parameter (K2P) and Maximum Likelihood (ML) genetic distances (GD) were calculated using DNADIST in PHYLIP (Felsenstein 1993). These are shown in Table 3. These genetic distances were then analysed by the Fitch-Margoliash Least Squares method in the programme FITCH (PHYLIP) using the Global rearrangement, Randomize sequence input and Jumble (11×) options and the program KITSCH (PHYLIP) using the Randomize sequence input and Jumble (11×) options. fastDNAml (Olsen et al. University of Illinois, USA) was used to calculate a maximum likelihood tree directly from the aligned sequences, using the Global rearrangement, Bootstrap (1000) and Jumble (11×) options. The alignments were also analysed using DNAMLK (PHYLIP) and its Global rearrangement option. Only two trees were generated by DNAPARS (PHYLIP) using the Search for Best Tree (default) and Randomize sequence input and Jumble 11× options. A consensus treefile was generated by CONSENSE (PHYLIP) from these two trees.

All treefiles were illustrated by NJPLOT (M. Guoy, University of Lyon, France). The aforementioned software programmes are available by anonymous ftp in the Internet. Site polymorphism analysis of the aligned ITS sequences of these species (excluding *Nothoscordum*) was implemented using SITES v1 (Hey and Wakeley 1997). Following Suh et al. (1993), evolutionary rates were calculated as $R = K/2T$ where R is the number of substitutions per site per year, K are the Kimura 2-Parameter distances and T is the time of divergence.

Results

The lengths of the ITS sequences shown in Table 1 are well within the range of our previous results. Many species had length polymorphisms which differed by

Table 1 HNAES accession numbers, origins and ITS length characteristics of some Old and New World *Alliums*. Classification based on Hanelt et al. (1992) and Fritsch (1992)

Accession no.	Source/origin	Subgenus/section	Species	Length (bp) of ITS			
				Total	ITS1	5.8S	ITS2
		Subg. *Amerallium* Traub					
65	Haiji Nursery	*Amerallium* (Traub) R. Kam.	*A. cernuum* Roth	659	246	164	249
350	AGS	*Amerallium*	*A. stellatum* Ker-Gawler	661	248	164	249
278	Onion Man	*Caulorhizideum* Traub	*A. goodingii* Ownbey	653	240	164	249
299	Robinett Bulb Farm (RBF)	*Caulorhizideum*	*A. validum* Wats.	655	243	164	248
316	Garlic	*Lophioprason* Traub	*A. amplectens* Torr.	645	239	164	242
341	Alpine Garden Society (AGS)	*Lophioprason*	*A. dichlamydeum* Greene	650	243	164	243
303	North American Rock Garden Society (NARGS)	*Lophioprason*	*A. hyalinum* Curran	645	238	164	243
57	Takii Seed Co	*Lophioprason*	*A. unifolium* Kell.	646	238	164	244
289	RBF	*Lophioprason*	*A. bolanderi* Wats.	645	237	164	244
297	RBF	*Lophioprason*	*A. sanbornii* Wood	625	211	164	250
46	Dai-ichi Engei	*Molium* G. Don ex Koch	*A. neapolitanum*[a] Cirillo	649	240	164	245
55	Yamato Nouen	*Molium*	*A. roseum* L.	648	240	164	244
245	NARGS	*Molium*	*A. subhirsutum* L.	628	241	164	223
49	Takii Seed Co	*Molium*	*A. zebdanense* Boiss. &Noe	627	240	164	223
54	Dai-ichi Engei	*Molium*	*A. moly* L.	660	243	164	253
998	NARGS	*Chamaeprason* Herm.	*A. chamaemoly* L.	649	242	164	243
42	Sakata Seed Co	*Briseis* (Salisb.) Stearn	*A. triquetrum* L.	660	244	164	252
		Related species					
1207	NARGS	Subg. *Caloscordum* (Herb.) R.M. Fritsch	*A. neriniflorum* Herb.	641	236	163	242
63	Dai-ichi Engei	Subg. *Melanocrommyum* (Webb et Berth.) Rouy	*A. oreophilum* C. Mey.	636	230	164	242
32	Takii Seed Co	Subg. *Melanocrommyum*	*A. giganteum* Regel	641	233	164	244
165	Uzbekistan	Subg. *Allium*	*A. sativum* L.	642	236	164	242
109	Sapporo, Japan	Subg. *Rhizirideum* (G. Don ex Koch) Wendelbo	*A. schoenoprasum* L.	632	233	164	235
79	Haiji Nursery	Subg. *Rhizirideum*	*A. senescens* L.	643	238	164	241
458	Sakata Seed Co	*Nectaroscordum* Lindl.	*N. siculum* (Ucria) Lindl.	652	242	163	247
454	HNAES	*Nothoscordum* Kunth	*N. gracile* Stearn	671	250	164	257

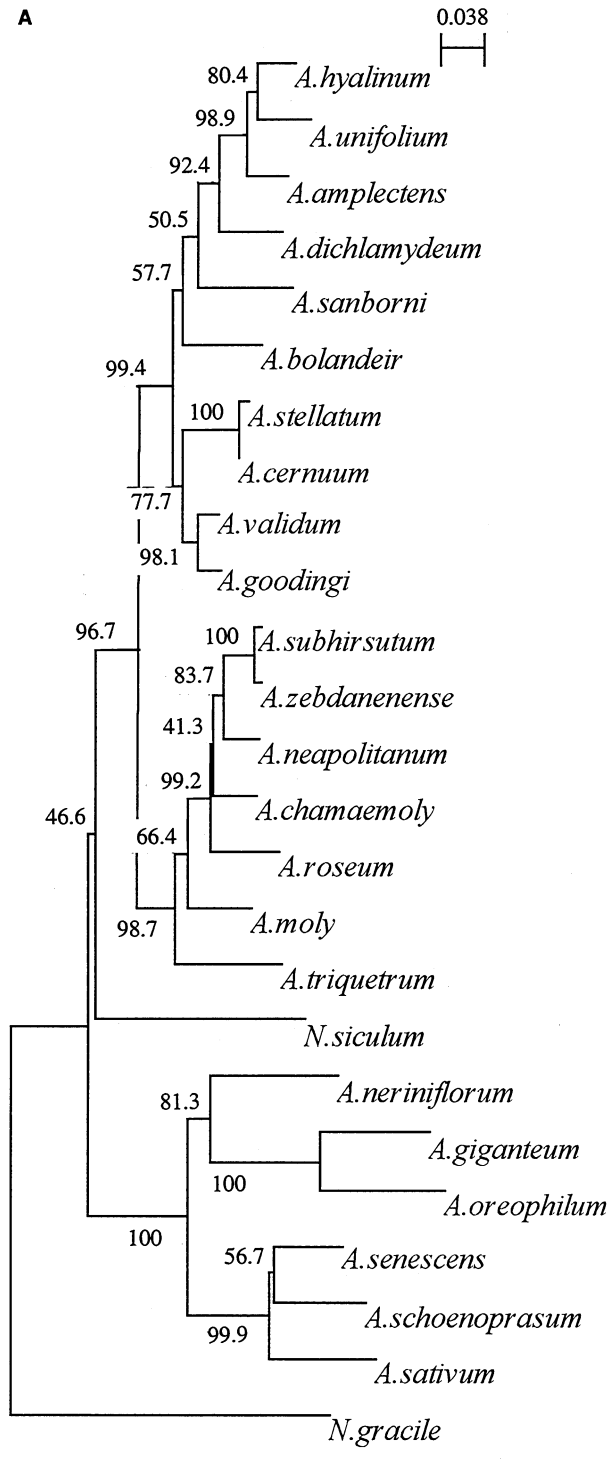
[a] Sold in Japan as *A. cowanii*

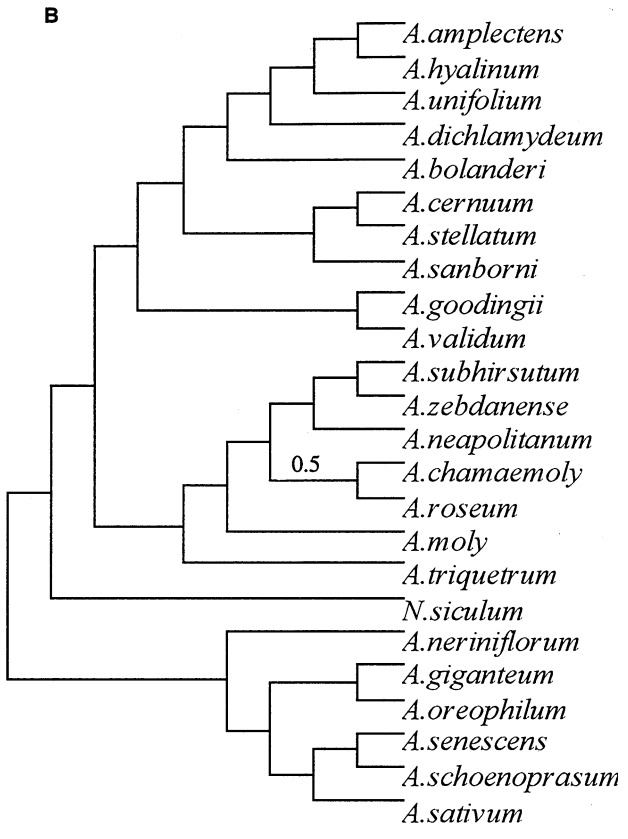
less than 5 bp, a difference which cannot be detected by agarose gel electrophoresis. *A. sanbornii* had the shortest ITS region (625 bp) and *N. gracile* had the longest (671 bp), SITES detected 146 indels, 434 transitions, 317 transversions and 409 informative positions. GC content analysis performed by SITES indicated that the average length of the ITS region was 645.5 bp with 50.9% GC content.

Sequence alignment was performed in the presence/absence of *Nothoscordum*. The dendrogram in Fig. 1A was generated by NJPLOT using a treefile produced by CLUSTAL W's implementation of NJ analysis. The high bootstrap values indicate the confidence level associated with each node. Figure 1B is one of only two trees found by DNAPARS. These trees required a total of 1608 steps. The two trees differed only in the presence/absence of a node uniting *A. chamaemoly* and *A. roseum*. Figure 1C was generated by NJPLOT from a treefile produced by fastDNAml. The Likelihood Ratio Test values indicate the confidence level of each branch (Table 2). Except for the presence/absence of *Nothoscordum*, Fig. 1A, B and C share practically the same topology.

The genetic distance (GD) is a relative estimate of the 'divergence time' between genotypes (Felsenstein 1993). The mean $\pm$ standard deviation (SD) of the Kimura 2-parameter (K2P) GD values between *N. gracile* and the rest of the species was 0.63 ± 0.07 (data not shown); this level of genetic divergence, as well as the absence of the typical *Allium* odor, justified its use as the outgroup species for NJ analysis. *N. siculum* is interposed between subg. *Amerallium* and the rest of the Old World representatives of the other subgenera. The subg. *Amerallium* is evenly split between its Old and New World members. *A. neriniflorum* is grouped with the representatives of subg. *Melanocrommyum*. *A. schoenoprasum*, the only circumpolar *Allium* species in this study, is clustered with *A. senescens*.

Maximum Likelihood genetic distances (MLGD) were also calculated with or without *Nothoscordum*.





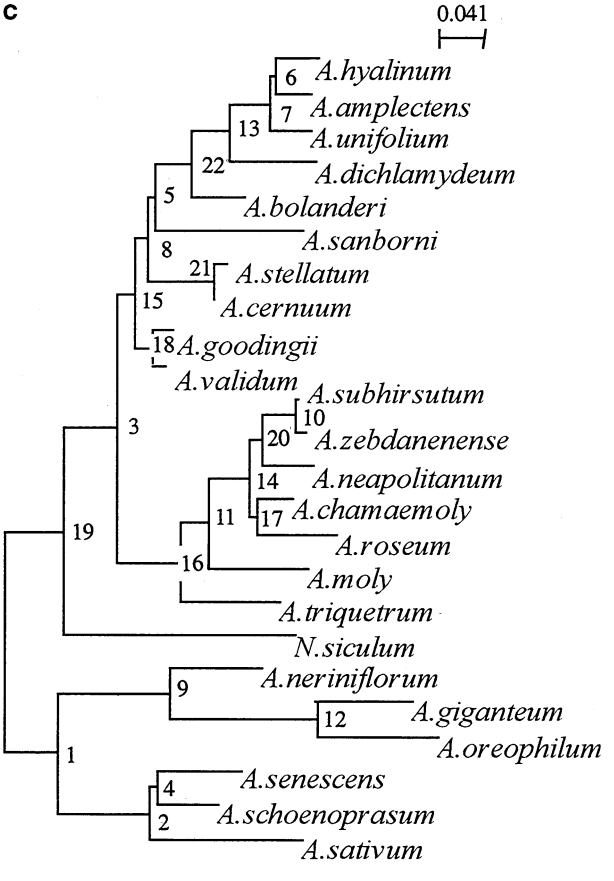


Fig. 1A–C Phylogeny of some Old and New World *Alliums* based on ITS sequence analysis. Dendogram **A** was generated by CLUSTAL W by NJ analysis of genetic distances. Bootstrap values are indicated in percentages. Dendogram **B** was generated by DNAPARS (PHYLIP). The *number* corresponds to the decay index of the only variable branch. Dendogram **C** was generated by FastDNAml. *Numbers* correspond to nodes

Table 2 Maximum Likelihood parameters of nodes and branch lengths among various Old and New World *Allium* species in Fig. 1C. Data generated by fastDNAml (*LRT* likelihood ratio test)

Between	And	Length	Approximate confidence limits	LRT
9	12	0.13832	(0.10101, 0.17811)	**
12	*A. oreophilum*	0.11276	(0.08143, 0.14582)	**
12	*A. gigantetum*	0.08924	(0.06097, 0.11892)	**
9	1	0.04902	(0.02296, 0.07626)	**
1	2	0.08792	(0.05763, 0.11984)	**
2	*A. sativum*	0.14275	(0.10924, 0.17825)	**
2	4	0.00688	(zero, 0.01861)	
4	*A. schoenoprasum*	0.05766	(0.03630, 0.07982)	**
4	*A. senescens*	0.07796	(0.05332, 0.10366)	**
1	19	0.10589	(0.07305, 0.14066)	**
19	*N. siculum*	0.21810	(0.17424, 0.26544)	**
19	3	0.04985	(0.02547, 0.07527)	**
3	16	0.06139	(0.03762, 0.08614)	**
16	*A. triquetrum*	0.09194	(0.06531, 0.11982)	**
16	11	0.02612	(0.01101, 0.04163)	**
11	*A. moly*	0.09145	(0.06528, 0.11883)	**
11	14	0.03735	(0.01883, 0.05647)	**
14	17	0.00659	(zero, 0.01577)	*
17	*A. roseum*	0.07577	(0.05321, 0.09921)	**
17	*A. chamaemoly*	0.03421	(0.01909, 0.04972)	**
14	20	0.01142	(0.00119, 0.02183)	**
20	*A. neapolitanum*	0.04683	(0.02904, 0.06516)	**
20	10	0.03137	(0.01656, 0.04656)	**
10	*A. zebdanenense*	0.00918	(0.00133, 0.01714)	**
10	*A. subhirsutum*	0.00217	(zero, 0.00672)	
3	15	0.01701	(0.00227, 0.03212)	**
15	18	0.01762	(0.00580, 0.02968)	**
18	*A. validum*	0.01061	(0.00224, 0.01910)	**
18	*A. goodingii*	0.01838	(0.00757, 0.02939)	**
15	8	0.01103	(0.00085, 0.02139)	**
8	21	0.06330	(0.04248, 0.08488)	**
21	*A. cernuum*	0.00000	(zero, 0.00302)	
21	*A. stellatum*	0.01110	(0.00292, 0.01940)	**
8	5	0.00842	(zero, 0.01968)	*
5	*A. sanbornii*	0.13845	(0.10638, 0.17234)	**
5	22	0.03296	(0.01634, 0.05007)	**
22	*A. bolanderi*	0.04924	(0.03065, 0.06842)	**
22	13	0.03472	(0.01785, 0.05209)	**
13	*A. dichlamydeum*	0.08386	(0.05975, 0.10900)	**
13	7	0.04089	(0.02314, 0.05918)	**
7	*A. unifolium*	0.03597	(0.02072, 0.05914)	**
7	6	0.00468	(zero, 0.01158)	
6	*A. amplectens*	0.03230	(0.01790, 0.04706)	**
6	*A. hyalinum*	0.03856	(0.02287, 0.05468)	**
9	*A. neriniflorum*	0.08596	(0.05710, 0.11629)	**

*,** Significantly positive at $P < 0.05$ and $P < 0.01$, respectively
Ln Likelihood = − 6999.20085

The average MLGD value was 20% higher when *Nothoscordum* was included in the calculations (data not shown) though FITCH and KITCH generated trees which were topologically similar to Fig. 1A. MLGD values calculated from an alignment excluding *Nothoscordum* are shown in Table 3. The MLGD values of *N. siculum* indicate that it is more similar to species under subg. *Amerallium* (0.36 ± 0.03) than to representatives of other *Allium* subgenera, viz. *A. sativum* (subg. *Allium*), *A. giganteum* (subg. *Melanocrommyum*) and *A. senescens* (subg. *Rhizirideum*) (0.49 ± 0.04). The MLGD values among the Old and New World members of subg. *Amerallium* are much lower (0.20 ± 0.07) than those among the Old World representatives of the other subgenera and the Old World species classified under subgenus *Amerallium* (0.36 ± 0.15). The MLGD values among New World *Amerallium* (0.36 ± 0.15). These relationships are more simply summarized in the dendrograms in Fig. 1.

The phylogenetic tree generated by KITCH using the molecular clock hypothesis was significantly different from the tree produced by FITCH that did not assume a molecular clock ($F_{22,231} = 10.93$**). This means that some of the assumptions of the molecular

Table 3 Maximum Likelihood genetic distances between ITS DNA sequences of some Old and New World *Alliums*

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*A. neriniflorum*																							
2	*A. giganteum*	0.35																						
3	*A. oreophilum*	0.38	0.22																					
4	*A. senescens*	0.29	0.37	0.39																				
5	*A. schoenoprasum*	0.31	0.44	0.46	0.15																			
6	*A. sativum*	0.35	0.50	0.51	0.20	0.20																		
7	*N. siculum*	0.44	0.51	0.55	0.47	0.50	0.51																	
8	*A. triquetrum*	0.38	0.51	0.54	0.44	0.46	0.50	0.40																
9	*A. moly*	0.37	0.49	0.53	0.42	0.43	0.47	0.35	0.17															
10	*A. chamaemoly*	0.34	0.50	0.52	0.41	0.43	0.46	0.34	0.19	0.13														
11	*A. roseum*	0.40	0.51	0.56	0.44	0.44	0.47	0.41	0.21	0.16	0.11													
12	*A. neapolitanum*	0.39	0.50	0.54	0.44	0.44	0.47	0.37	0.19	0.15	0.10	0.12												
13	*A. subhirsutum*	0.36	0.50	0.52	0.40	0.40	0.42	0.34	0.18	0.13	0.08	0.12	0.07											
14	*A. zebdanenense*	0.36	0.50	0.52	0.41	0.40	0.42	0.34	0.18	0.13	0.08	0.11	0.07	0.00										
15	*A. cernuum*	0.34	0.45	0.49	0.39	0.40	0.42	0.33	0.24	0.20	0.22	0.24	0.23	0.22	0.21									
16	*A. stellatum*	0.35	0.46	0.50	0.40	0.41	0.43	0.34	0.25	0.21	0.22	0.25	0.24	0.22	0.22	0.00								
17	*A. goodingii*	0.35	0.43	0.47	0.38	0.39	0.42	0.32	0.21	0.20	0.20	0.22	0.22	0.20	0.20	0.10	0.10							
18	*A. validum*	0.35	0.44	0.46	0.37	0.38	0.41	0.31	0.20	0.19	0.20	0.21	0.21	0.19	0.19	0.09	0.10	0.04						
19	*A. sanborni*	0.40	0.49	0.51	0.45	0.44	0.45	0.37	0.29	0.26	0.26	0.27	0.26	0.25	0.25	0.17	0.17	0.17	0.16					
20	*A. bolanderi*	0.40	0.45	0.50	0.42	0.44	0.45	0.37	0.27	0.26	0.26	0.28	0.26	0.24	0.24	0.15	0.16	0.12	0.13	0.21				
21	*A. dichlamydeum*	0.41	0.51	0.52	0.45	0.45	0.46	0.41	0.31	0.28	0.28	0.29	0.29	0.28	0.28	0.20	0.21	0.18	0.18	0.18	0.17			
22	*A. unifolium*	0.45	0.53	0.54	0.47	0.47	0.50	0.40	0.33	0.29	0.31	0.32	0.31	0.29	0.30	0.21	0.21	0.19	0.19	0.17	0.17	0.16		
23	*A. amplectens*	0.42	0.51	0.52	0.47	0.48	0.48	0.38	0.30	0.26	0.27	0.29	0.27	0.26	0.26	0.20	0.21	0.18	0.18	0.21	0.18	0.16	0.09	
24	*A. hyalinum*	0.43	0.53	0.54	0.46	0.48	0.49	0.38	0.33	0.28	0.30	0.30	0.30	0.29	0.29	0.20	0.20	0.18	0.18	0.21	0.17	0.15	0.09	0.09

clock hypothesis have been violated. DNAMLK produced a phylogeny which was slightly different (in some clusters) from that generated by fastDNAml.

Discussion

PCR factors and ML genetic distances

The rather high genetic distances (in Table 3) calculated by Maximum Likelihood analysis of the ITS sequences in *Allium* must be justified in light of known procedural limitations of PCR amplification and sequencing of ITS sequences. The high copy number (up to 30 000 per cell) of rDNA in plant genomes allows easy amplification, but it also increases the possibility of amplifying divergent paralogues from deactivated rDNA sites (Buckler et al. 1997). This is a major limitation when sequencing rDNA genes from individual clones. Fortunately, the small error rate of *Taq* polymerase dictates that the predominant signal in direct sequences of pooled PCR products is likely to represent DNAs that were accurately replicated (Baldwin et al. 1995). They recommended sequencing both strands of the ITS region from products of different PCR reactions to reduce the possibility of generating artifacts from PCR 'drift', 'jumping', 'selection' and 'chimera formation'. Hence, in line with the recommendations of Baldwin et al. (1995), our procedures involve pooling (at least) two independent PCR reactions and repeating both forward and reverse sequencing reactions at least twice.

Buckler et al. (1997) noted that, in some species, ITS sequences generated using standard PCR conditions turned out to be divergent paralogues. They hypothesized that the secondary structure of the functioning rDNA inhibited proper denaturation such that the 'mutant' paralogues that did not possess secondary structures were preferentially amplified. If these 'divergent paralogues' do not show secondary structure, priming and background polymerization could well occur during the mixing phase. Buckler et al. (1997) also reported that DMSO increased the fidelity of the PCR by ensuring that both standard and divergent sequences were 'denatured' starting from mixing to PCR proper. The main role of DMSO is to lower the annealing temperature (Tm) of the primer, but it may also theoretically increase the effective denaturation temperature (Chester and Marshak 1993). Like many other researchers, we obtained disappointing results in our preliminary attempts to perform PCR in the presence of DMSO.

We used the 'Cold-Mix' and manual 'Hot Start' procedures to reduce the possibility of unwanted priming and background polymerization under non-denaturing conditions (during mixing up to the first denaturation step) and ensure that PCR starts at a denaturing temperature. Initial denaturation was performed at 94°C for 3 min. 'Touchdown PCR' was used to ensure that the initial annealing of the primers occurs at the highest possible temperatures, e.g. 65°C. 'Touchdown PCR' is especially helpful if mutations associated with the non-standard ITS paralogues occur in the priming site.

These procedures generated clean, relatively muddle-free forward and reverse sequences that were easily aligned in each species. The average GC content (50.9%) of the sequences is well within the range of GC contents reported in other species (Baldwin et al. 1995). This moderately high GC content also affirms the veracity of the sequences generated in this study because, according to Buckler et al. (1997), divergent paralogues usually show higher AT values (through deamination).

Sequence alignment and ML genetic distances

Genetic distance values, regardless of method, are basically determined by the sequence alignment. Manual alignment of clearly heterogenous sequences is not only very difficult but also quite prone to subjective bias and error. The most popular alignment programme, CLUSTAL W, has many features which allow researchers to 'fine-tune' the alignments. Such fine tuning, if done without sufficient theoretical justification, leads to doubts regarding objectivity.

Iterative alignment was performed using the "old guide tree file" option. This means using the NJ tree file generated in the previous alignment to realign the sequences in the following iteration. Up to a point, retaining the gaps between alignments resulted in ever-increasing final lengths of the aligned sequences. In addition, CLUSTAL W has the unfortunate predeliction of adding unnecessary gaps when this option is used. On the contrary, resetting gaps between alignments resulted in, up to a point, lower final lengths of the aligned sequences. Although differences in specific genetic distances were noted between these two modes of alignment, the overall average MLGD differed by only 4%, and the trees generated by both procedures also showed the same topology (data not shown). Mindful of the artificiality of gaps in DNA sequences, we present and discuss only the shorter alignment in this report.

ML genetic distances and outspecies considerations

The use of an *Allium* look-alike such as *Nothoscordum* as an outspecies confirms the aphorism that 'appearances can be misleading'. *Nothoscordum* was used as an outspecies also because it is primarily an American species and, hence, it would have been quite useful in establishing an American connection to the genesis of *Allium*. Unfortunately, the generally high K2P genetic

distance estimates associated with this entry do not justify any discussion on probable linkages.

Fay and Chase (1996) had also shown by means of *rbc*L sequence analysis that *Nothoscordum* is quite distantly related to *Allium*. This is a well-known weakness of ITS sequence analysis – that it is not very useful at higher taxonomic levels. Hence, we cannot recommend ITS sequence analysis to determine relationships beyond the genus level in the *Alliae*.

Sequence alignment and the consequent genetic distances obviously depend on the homogeneity of the genetic makeup of the sequences. Removal of *Nothoscordum* from the alignment reduced the average genetic distance among species by 20.2%, but this did not dramatically change the topology of the tree. In view of this, the genetic distances in Table 3 were calculated from sequences that did not include *Nothoscordum*.

Phylogenetic implication of large ML genetic distances

The large ML distances shown in Table 3 only leads to one conclusion: *Allium* is an old genus that was most probably already well-differentiated in the early Tertiary period (Hanelt et al. 1992). Fossilized pollen from *Liliaceae* have been dated back to the Eocene (Tiffney 1985), approximately 34–53 mya. Hanelt et al. (1992) speculated that *Allium* was already well-differentiated at the time when the Tethys sea link still connected Europe to North America. This land bridge in the North Atlantic broke up in the early Eocene (Tiffney 1985).

Allium could be one of the many herbaceous plants that, according to Tiffney (1985), formed part of the floor or disturbed sites of the boreotropical forests that covered the northern latitudes during the early Tertiary. Some *Alliums* (e.g. *A. triquetrum* and *N. siculum*) still possess some of the characteristics (e.g. tolerance to shade or damp sites) needed to survive in disturbed sites in the forest floor. *Allium* also consists of many species with weed-like properties – short life cycles, profuse seed production and efficient distribution. The mild temperate to subtropical conditions prevailing in the northern latitudes during the Paleocene and Eocene Eras (Tiffney 1985) would have promoted rapid world-wide colonization. Asexual propagules (bulbs, bulbils, bulblets, rhizomes, stolons, etc.) became an important mode of propagation later, probably in response to climatic fluctuations starting in the Oligocene (Cheremushkina 1992; Tiffney 1985).

Mode of reproduction and estimates of nucleotide substitution rates

Allium exhibits prolific sexual reproduction and a short generation time that, combined with sexual recombination among sympatric taxons, can introduce an upward bias in estimates of the 'mutation' rates of gene sequences (see Baldwin et al. 1995; Buckler et al. 1997).

The rapid spread to new habitats and elimination of unfit types increase genetic diversity. Fixation of the genotype by various modes of asexual propagation present in genus *Allium* must have assured survival in the fluctuating climates of the mid-Tertiary. Increasing reliance on asexual propagules obviously reduces genetic variance and effectively results in lower estimates of mutation rates.

This dual mode of reproduction probably confounded the estimates of sequence divergences because KITCH generated a phylogenetic tree that was significantly different (by the *F*-test) from those made by FITCH. DNAMLK and fastDNAml also generated trees with noticeably different topologies. These results should be borne in mind whilst interpreting nucleotide substitution rates (which can be derived from K2PGD estimates) and the consequent calculations on divergence time.

ITS phylogeny and taxonomic classification of *Allium* subgenera

Traub (1968, 1972) proposed classifying the 600 or so *Allium* species under three subgenera, viz. *Allium*, *Amerallium* and *Nectaroscordum*. In Traub's classification, *N. siculum* was the type species of subg. *Nectaroscordum*; subg. *Amerallium* united the North American species with the Mediterranean *Alliums* classified under sect. *Molium* Endl. and species that are now classified under sections *Arctoprason* Kirschl. and *Briseis* (Salisb.) Stearn (Hanelt et al. 1992); and the rest of the species were lumped together in subg. *Allium*. The species in Fig. 1 are distributed in accordance with Traub's tri-partite division of the genus.

Traub's definitions of subg. *Allium* and subg. *Amerallium* are apparently too heterogenous with respect to other taxonomic characters deemed important by present-day *Allium* specialists. Hanelt et al. (1992) collated sufficient evidence to reassemble the species pooled by Traub in subg. *Allium* into five subgenera viz. *Allium*, *Bromatorhiza*, *Caloscordum*, *Melanocrommyum* and *Rhizirideum*. RFLP analysis of chloroplast DNA subsequently suggested the elimination of subg. *Bromatorrhiza* and the redistribution of some of its members into the Old World group of subg. *Amerallium* (Samoylov et al. 1995). Representative species of subgenera *Allium*, *Caloscordum*, *Melanocrommyum* and *Rhizirideum* are separated in distinct branches in Fig. 1.

Allium species in Fig. 1C are arranged from bottom to top as follows: node 1 – Old World (Asia and Europe) *Allium* representatives from subg. *Caloscordum*, *Melanocrommyum*, *Allium* and *Rhizirideum*; node 19 – sole representative of subgenus *Nectaroscordum* that is distributed in Southern Europe and the

Mediterranean; node 3 – Old and New World *Ameralliums*: node 16 – Old World *Ameralliums*; and node 15 – New World *Ameralliums*. All these nodes are distinctly present in all the trees generated by the various analytical programmes mentioned above.

Hanelt et al. (1992) noted that subg. *Amerallium* and *Rhizirideum* share many basic morpho-physiological properties which may reflect a common ancestry, a close relationship and the common rather ancestral state within the genus. However, the position of *Rhizirideum* (represented by *A. senescens* and *A. schoenoprasum*) vis-a-vis the *Ameralliums* in Fig. 1 indicates that *Nectaroscordum* and the *Ameralliums* took an ancient fork down the evolutionary road, separating from the rest of the genus *Allium*, i.e. the ancestral relationship may be common but not close. Figure 1 clearly indicates that *Ameralliums* have more in common with *Nectaroscordum* than with *Rhizirideum*.

Phylogenetic relationship between *Nectaroscordum* and genus *Allium*

With (Fig. 1A) or without (Fig. 1B, C) *N. gracile* as outspecies, *N. siculum* is clearly interposed between subg. *Amerallium* and the rest of genus *Allium*. It is nearer to the *Ameralliums* than subg. *Caloscordum* (0.36 ± 0.03 vs. 0.39 ± 0.03). The MLGD between *N. siculum* and the non-*Ameralliums* is 0.49 ± 0.07. The relative isolation of this species from the rest of *Allium* in Fig. 1 indicates that it can be considered as a subgenus in genus *Allium*.

Nectaroscordum was excluded from the infrageneric classification of genus *Allium* proposed by the Gatersleben group (Hanelt et al. 1992). Fritsch (1992) found that the septal nectaries of *Nectaroscordum* are too different from the rest of *Allium*. This difference, however, has to be weighed in relation to its chemo-morphological similarities to genus *Allium*. Traub (1968) noted that the vascular bundles of *Nectaroscordum* and *Amerallium* (with few exceptions) are laid out in one row. Later, he (Traub 1972) added that the laticifers in subgenera *Amerallium* and *Nectaroscordum* are hypodermal in the bulb scale, whereas these structures occur in the second layer below the epidermis (or deeper) in the rest of genus *Allium*.

In terms of MLGD of the ITS sequences (Table 3), the average MLGD between *N. siculum* and the rest of the entries in Table 3 is 0.40 ± 0.07. Old and New World *Ameralliums* are more closely related to *Nectaroscordum* (0.36 ± 0.03) than they are to the rest of the Old World *Allium* representatives (0.49 ± 0.04). These data lead to only one conclusion – include *Nectaroscordum* as a subgenus in *Allium*. Hence our results confirm conclusions made by Traub (1968, 1972), Dahlgren et al. (1985) and Fay and Chase (1996) suggesting the reinclusion of *Nectaroscordum* in genus *Allium*. Stearn (1978) noted that the nomenifer *Allium* subg. *Nectaroscordum* (Lindl.) Asch.&Graeb (1905) is 63 years older than *Allium* subg. *Nectaroscordum* (Lindl.) Traub.

ITS phylogeny and *Amerallium* biogeography

Assuming an Asian origin, Hanelt et al. (1992) postulated that the *Ameralliums* ($x = 7$) spread to North America via the Bering land bridge, but these authors were silent on the origin of the Mediterranean *Ameralliums*. From the putative Asian center, the Old and New World *Ameralliums* could have split up, with the first group migrating to America via the Bering land bridges while the remainder later moved west and ended up in the Mediterranean. This interpretation has a rather wide time window starting from the Pre-Tertiary to the late Miocene (from 90 to 5 mya). Traub (1968) opined that the genus *Allium* had already attained circumpolar and circumboreal distribution in the Miocene about 25 mya. In such an old genus, opposite migratory directions would dictate a great divergence between the Old and New World *Ameralliums*. One objection to this hypothesis is the relative dearth of species in continental Asia (between the Mediterranean and North America) that are unquestionably *Ameralliums*.

The alternate hypothesis is a predominantly unidirectional migration via the land bridges at Bering and North Atlantic. Unidirectional migration would also explain the monophylly of the Old and New World *Ameralliums* (Fig. 1C, node 3), the prevalence of 'primitive' characters (e.g. rhizomes) in the New World *Ameralliums* (McNeal and Ownbey 1973) and their corresponding rarity in the Old World *Ameralliums* (Mann 1960; de Wilde-Duyfjes 1976). This unidirectional theory, however, assumes that the genus *Allium* was already well-differentiated (into the $x = 7$ and $x = 8$ forms) before the break-up of the North Atlantic bridge in the Early Eocene.

Axelrod (1975) postulated that gene flow between the Mediterranean and North America could have continued in the early Oligocene (34 mya) until the end of the Paleogene (25 mya), when a drop in sea level provided a means of migration via microcontinents and volcanic island stepping stones. According to Stearn (1978) *Allium* bulbs can float unimpaired for a long time in salt water. This may also explain the predominantly coastal distribution of Mediterranean *Moliums*, e.g., the natural habitat of *A. chamaemoly* includes sands and rocks by the sea, in salt marches and in salt meadows (de Wilde-Duyfjes 1976). If this was the case, then the time window of the unidirectional hypothesis extends up to 25 mya.

The average MLGD between the Old World and New World *Ameralliums* is 0.25 ± 0.04 and, assuming a divergence time of approximately 50–25 mya this would result in nucleotide substitution rates equal to

approximately, $2.55–5.09 \times 10^{-9}$ per site per year. Sang et al. (1994) provided an estimate of approximately $3.90–6.06 \times 10^{-9}$ per site per year for the ITS region of *Dendroseris*. Considering the short generation time in *Allium* (relative to *Dendroseris*) and the confounding effects of asexual propagation, the higher estimate (5.09×10^{-9}) would probably be nearer to the true rate among *Amerallium*s.

Hanelt et al. (1992) postulated that *A. schoenoprasum*, a circumpolar species, is a recent postglacial migrant to North America. However, the MLGD from *A. senescens* is 0.15, which dates the divergence between the two species during the Miocene, between approximately 14 and 28 mya. Hence, this species could have migrated into North America through the Bering land bridge during warm intervals that, according to Wolfe (1978), occurred during the early to middle Miocene (approximately 17–15 mya) and the late Miocene to early Pliocene (6–5 mya). A comparison between the North American and Siberian forms of this species would give a more exact date of this migration. Future research must include more North American *Amerallium* species in order to elucidate the possible role of the Rocky Mountains and, hopefully, provide a more specific time frame for the evolution of these species.

Controversial species in subg. *Melanocrommyum*

A. neriniflorum was assigned by Kamelin (1980) into subg. *Melanocrommyum*. Hanelt and Fritsch (1994) cited sufficient differences in morphology, phenology and distribution to justify ranking *Caloscordum* as an *Allium* subgenus beside subg. *Melanocrommyum*. Friesen (1995) insisted that *C. neriniflorum* belonged to an independent genus, but more recently Friesen (personal communication) affirmed that *A. neriniflorum* clearly belongs to the genus *Allium*, near subg. *Melanocrommyum*.

The average MLGD between *A. neriniflorum* and the rest of the *Allium* (including *Nectaroscordon*) species is 0.37 ± 0.04. It is closer to the non-*Amerallium*s (0.33 ± 0.04) than it is to the *Amerallium*s (0.39 ± 0.03). Figure 1 places *Caloscordum* roughly between representatives of *Melanocrommyum* and *Allium*/*Rhizirideum*. Hence our data also affirm the status of *Caloscordum* as a subgenus in genus *Allium* and its closer relationship with *Melanocrommyum*.

We (Dubouzet and Shinoda 1998) suggested the need to verify the position of *A. oreophilum* vis-a-vis sections *Molium* and *Briseis* and subg. *Melanocrommyum*. *A. oreophilum* is clearly associated with subg. *Melanocrommyum* (represented by *A. giganteum*) in Fig. 1.

Relationships among Old and New World *Amerallium*s

The circumscription of subg. *Amerallium* Traub is based on chromosome number, structure of the leaf vascular bundles and tepal nerves (Traub 1968, 1972); serological studies and electrophoretic profiles of seed proteins (Hanelt et al. 1992; Maas 1992); and by chloroplast RFLP analysis (Samoylov et al. 1995; Linne von Berg et al. 1996). However, McNeal and Ownbey (1973) cited bulb characters such as the origin of the resistant layer, the order of development of the increase and renewal bulbs and the lack of a prophyll in some *Alliums* in the western United States to debunk their purported relationship to the Mediterranean *Moliums*.

Figure 1 illustrates the basic relationship between Old and New World species of Traub's *Amerallium*, and it graphically affirms previous results based on RFLP analysis of cpDNA (Samoylov et al. 1995) that clearly classified the Old and New World species in different clusters. Moreover, as in Fig. 1, Samoylov et al. (1995) also showed that the ranges of genetic variation (indicated by branch lengths) in both New and Old World clusters are almost similar. The average MLGD among the Old World *Amerallium*s is 0.13 ± 0.05, while those among the New World *Amerallium*s is 0.16 ± 0.05.

The data in Table 3 suggest that there may be sufficient molecular diversity to warrant the elevation of the Old and New World branches to subgenus level. The GD value between *A. sativum* (type species of subg. *Allium*) and *A. senescens* (type species of subg. *Rhizirideum*) is 0.20, whereas the average MLGD value between the Old and New World *Amerallium*s is 0.26 ± 0.04. This diversity is graphically illustrated by their separation into different nodes (15 and 16) in Fig. 1C.

Conferring separate subgenus status to the Old and New World *Amerallium*s may help resolve the seeming conflict in Traub's subg. *Amerallium* posed by the disjunctive distribution and the distinct differences in bulb morphology and morphogenesis of its component species.

Review of subg. *Amerallium* Traub

Restricting Traub's subg. *Amerallium* to the North American *Alliums* basically implies elevating Traub's subsections to section status. The New World *Alliums* are distributed in two distinct branches in Fig. 1C. Node 18 includes representatives of sect. *Caulorhizirideum* Traub. Species clustered in from node 8 to node 6 belong to sect. *Lophioprason* Traub. True to form, however, Traub's definition of sect. *Lophioprason* encompasses a large diversity of species that Ownbey originally distributed to five different 'Alliances', viz. *AA. acuminatum*, *campanulatum*, *canadense*, *cernuum*, *falcifolium* and *sanbornii* (see Saghir et al. 1966). MLGD estimates can be used as a simple and objective yardstick to justify the establishment or repudiation of such controversial taxonomic groups.

For example, the genetic distance between *A. senescens* (type species of sect. *Rhizirideum*) and

A. schoenoprasum (type species of sect. *Schoenoprasum*) is 0.15. Out of the 45 possible pair-wise combinations among the New World *Ameralliums*, 34 are equal to or greater than 0.15. *Caveat*: the use of genetic distance as a taxonomic indicator should be used only after the factors that influence these estimates (see above) have been thoroughly addressed.

The genetic distances in Table 3 and the topology in Fig. 1 reveal that, similar to sections *Caulorhizideum*, Traub's subsections *Acuminata* (node 13), *Cernua* (node 21) and *Sanborniana* (node 5) can be accorded section status. These subsections correspond to Ownbey's *AA. acuminatum*, *cernuum* and *sanbornii* 'Alliances' (D.W. McNeal, personal communication). Subsect. *Bolanderiana* (node 22) Traub, which he extracted from Ownbey's *A. acuminatum* 'Alliance', may also deserve to be raised to section status.

The molecular diversity among this albeit small sample of North American *Alliums* reveals that there is sufficient genetic variation in the New World *Alliums* to justify the redistribution of these species into new sections and subsections. The molecular relationships among a more comprehensive set of North American *Alliums* will provide a clearer picture on the exact groupings.

McNeal (1992) reported several traits such as bulb and seed coat morphology that could be useful in reexamining the classification of these species. Saghir et al. (1966) also found extensive variation in the composition of volatile chemicals in these species. Closer examination of live specimens, as Khassanov and Fritsch (1994) demonstrated for subg. *Melanocrommyum*, will reveal additional morphological differences among these New World *Alliums* that may help justify reclassification.

A reasonable alignment between the current Gatersleben classification and Traub's subsections or Ownbey's 'Alliances' may be obtained by a comparison of genetic distances generated from representative sections and subsections of the well-studied subg. *Allium*, *Melanocrommyum* and *Rhizirideum*.

Subgenus *Molium* (Koch) Wendelbo in retrospect

Wendelbo (1969) pooled sect. *Molium* G. Don ex Koch and sect. *Briseis* (Salisb.) Stearn in subg. *Molium* (Koch) Wendelbo. According to de Wilde-Duyfjes (1976), sect. *Molium* G. Don ex Koch includes species that were previously classified under sections *Chamaeprason* Herm. and *Xanthoprason* Herm. Species classified under *Molium* and *Brieseis* are well separated in the cluster containing the Old World *Ameralliums* in Fig. 1. Wendelbo's doubts about the inclusion of sect. *Porphyrason* in this subgenus turned out to be well founded since *A. oreophilum* is more closely related to subg. *Melanocrommyum* (Fig. 1; Linne von Berg et al. 1996).

Species under sect. *Molium* are clustered at nodes 11 and 14. *A. moly* (Fig. 1C, node 11) is distinctly separated from the rest of the species in agreement with the establishment of subsect. *Xanthoprason* (Herm.) Traub (node 11) and subsect. *Molium* (G. Don ex Koch) Traub (node 14) by Hanelt et al. (1992).

A. chamaemoly is cytologically and morphologically distinct from the rest of subg. *Molium*. Hence, most authorities on *Allium* taxonomy classify *A. chamaemoly* in sect. *Chamaeprason* (Hanelt et al. 1992; Kollmann 1984; Stearn 1978). Traub (1972) assigned *A. chamaemoly* in subsect. *Chamaeprasa* (Herm.) Traub of sect. *Molium* Endl. As mentioned above, de Wilde-Duyfjes (1976) included this species in sect. *Molium* G. Don ex Koch.

Figure 1 indicates that changes in the ITS DNA sequence of *A. chamaemoly* did not keep pace with the extreme karyological divergence shown by this species (Tzanoudakis 1992) relative to the other species in subg. *Molium* (Koch) Wendelbo. This asynchrony suggests that karyotype changes that usually have dramatic effects on morphology do not have the same effect on the rate of random mutations, which is the presumptive force behind variation in intronic DNA sequences. From the taxonomic viewpoint, mere aneuploidy, which can be accompanied by drastic morpho-physiological changes, does not automatically confer section status to a given taxon. The MLGD values between *A. chamaemoly* and the other species in sect. *Molium* (Koch) Wendelbo are too low to confer section status to this species. Hence, our results confirm the decision of de Wilde-Duyfjes (1976) who included *A. chamaemoly* in sect. *Molium* (Koch) Wendelbo.

Conclusions

DNA sequence analysis of the ITS region is a useful tool in the intrageneric classification of *Alliums*. Our results prove that it provides sufficient resolution of *Allium* phylogeny and classification from the subgenus down to the section level. It can be used to establish or verify taxonomic relationships among taxons with few (or many) obvious morphological differences. It can be an important tool in the alignment of incongruent classifications such as Ownbey's 'Alliances' and the current subgenus/section classification system. Conversely, it can be used as an objective reference when evaluating the phylogenetic importance of specific morphological characters.

However, phylogenetic analysis of ITS sequences may not always concur with classifications primarily based on morphology and karyology, as in the case of *A. chamaemoly*.

Our data support Traub's proposal to include *Nectaroscordum* as a separate subgenus in *Allium*. On the other hand, as other *Allium* taxonomists even before Traub had surmised, the taxons included under his definition of subg. *Allium* had sufficient genetic variation that warranted subclassification. The same

situation holds true for his definition of subg. *Amerallium*. The distinct separation of the Old and New World *Amerallium* indicates the justifiability of limiting the subgenus to its American members. Our data indicate that sect. *Lophioprason* Trab includes too many divergent species that need to be redistributed, probably in line with Ownbey's original 'Alliances'. The Old World *Amerallium*s can be accommodated by resurrecting subg. *Molium* (Koch) Wendelbo.

Acknowledgments The authors would like to extend their gratitude to Ms. E. G. Dubouzet for her expert assistance in DNA sequencing and proofreading and to Ms. N. Murata for her logistic support. We also thank Drs. N. Friesen and D. McNeal for sharing their valuable insights on the taxonomic classification of *Allium*. This research was financed through a post-doctoral fellowship granted to J.G. Dubouzet by the Research and Development Corporation of Japan.

References

Axelrod DI (1975) Evolution and biogeography of Madrean-Tethyan sclerophyll vegetation. Ann Mo Bot Gard 62:280–334

Baldwin BG, Sanderson MJ, Porter JM, Wojciechoski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Mo Bot Gard 82:247–277

Bryan JE (1989) Bulbs. Timber Press, Portland, Ore.

Bryan J, Griffiths M (1995) The new Royal Horticultural Society dictionary: manual of bulbs. Timber Press, Portland, Ore.

Buckler IV ES, Ippolito A, Holsford TP (1997) The evolution of ribosomal DNA: divergent paralogues and evolutionary implications. Genetics 145:821–832

Cheremushkina VA (1992) Evolution of life forms in subgenus *Rhizirideum* (Koch) Wendelbo, genus *Allium* L. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. IPK, Gatersleben, Germany, pp 27–34

Chester N, Marshak DR (1993) Dimethyl sulfoxide-mediated primer Tm reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction. Anal Biochem 209:384–290

Dahlgren RMT, Clifford HT, Yeo PF (1985) The families of the monocotyledons: structure, evolution, and taxonomy. Springer, Berlin Heidelberg, New York

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Dubouzet JG, Shinoda K (1998) Phylogeny of *Allium* L. subg. *Melanocroimmyum* (Webb et Berth.) Rouy based on DNA sequence analysis of the internal transribed spacer region of rDNA. Theor Appl Genet 97:541–549

Dubouzet JG, Shinoda K, Murata N (1997) Phylogeny of *Allium* L. subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo according to dot blot hybridization with randomly amplified DNA probes. Theor Appl Genet 95:1223–1228

Fay MF, Chase MW (1996) Resurrection of *Themidaceae* for the *Brodiaea* alliance, and recircumscription of *Alliaceae*, *Amaryllidaceae* and *Agapanthoideae*. Taxon 45:411–451

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle, Wash.

Friesen N (1995) The genus *Allium* L. in the flora of Mongolia. Feddes Repert Z Bot Taxon Geobot 106:59–81

Fritsch RM (1992) Septal nectaries in the genus *Allium* – shape, position and excretory canals. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. IPK, Gatersleben, Germany, pp 77–85

Hanelt P, Fritsch R (1994) Notes on some infrageneric taxa in *Allium* L. Kew Bull 49:559–564

Hanelt P, Schultze-Motel J, Fritsch R, Kruse J, Mass HI, Ohle H, Pistrick K (1992) Infrageneric grouping of *Allium* – the Gatersleben approach. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. IPK, Gatersleben, Germany, pp 107–123

Hey J, Wakeley J (1997) A coalescent estimator of the population recombination rate. Genetics 145:833–846

Kamelin RV (1980) Novye taksony roda *Allium* (*Alliaceae*). Bot Z 65:1459–1465

Khassanov FO, Fritsch RM (1994) New taxa in *Allium* L. subg. *Melanocrommyum* (Webb et Berth.) Rouy. Linzer Biol Beitr 26:965–990

Kollmann F (1984) *Allium* L. In: Davis PH (ed) Flora of Turkey. Edinburgh University Press, Edinburgh

Linne von Berg G, Samoylov A, Klaas M, Hanelt P (1996) Chloroplast DNA restriction analysis and the infrageneric grouping of *Allium* (*Alliaceae*). Plant Syst Evol 200:253–261

Mass HI (1992) Electrophoretic study of storage proteins in the genus *Allium*. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. IPK, Gatersleben, Germany, pp 183–189

Mann LK (1960) Bulb organization in *Allium*: some species in section *Molium*. Am J Bot 47:765–771

McNeal DW (1992) Taxonomy of North American Species of *Allium*. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. IPK, Gatersleben, Germany, pp 195–204

McNeal DW, Ownbey M (1973) Bulb morphology in some western North American species of *Allium*. Madroño 22:10–24

Saghir ARB, Mann LK, Ownbey M, Berg RY (1966) Composition of volatiles in relation to taxonomy of American *Alliums*. Am J Bot 53:477–484

Samoylov A, Klaas M, Hanelt P (1995) Use of chloroplast DNA polymorphisms for the phylogenetic study of the subgenera *Amerallium* and *Bromatorrhiza* (genus *Allium*) Feddes Repert Z Bot Taxon Geobot 106:161–167

Sang T, Crawford DJ, Kim S-C, Stuessy TF (1994) Radiation of the endemic genus *Dendroseris* (Asteraceae) on the Juan Fernandez Islands: evidence from sequence of the ITS regions of nuclear ribosomal DNA. Am J Bot 81:1494–1501

Stearn WT (1978) European species of *Allium* and allied genera of *Alliaceae*: a synonymic enumeration. Ann Musei Goulandris 4:83–198

Suh Y, Thien LB, Reeve HE, Zimmer EA (1993) Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. Am J Bot 80:1042–1055

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tiffney BH (1985) The Eocene North Atlantic land bridge: its importance in Tertiary and modern phytogeography of the Northern Hemisphere. J Arnold Arbor Harv Univ 66:243–273

Traub BH (1968) The subgenera, sections and subsections of *Allium* L. Plant Life 24:147–163

Traub HP (1972) Genus *Allium* L. – subgenera, sections and subsections. Plant Life 28:132–137

Tzanoudakis D (1992) Karyotype variation and evolution in the Greek *Allium*. In: Hanelt P, Hammer K, Knupffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. IPK, Gatersleben, Germany, pp 305–320

Wendelbo P (1969) New subgenera, sections and species of *Allium*. Bot Not 122:25–37

de Wilde-Duyfjes BEE (1976) A revision of the genus *Allium* L. (*Liliaceae*) in Africa. Meded Landbouwhogesch Wagningen 76, no 11

Wolfe JA (1978) A paleobotanical interpretation of Tertiary climates in the Northern Hemisphere. Am Sci 66:694–703