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Phylogeny of *Allium* L. subg. *Melanocrommyum* (Webb et Berth.) Rouy based on DNA sequence analysis of the internal transcribed spacer region of nrDNA

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Abstract Sequence analysis of the ITS region of nuclear ribosomal DNA from subgeneric representatives of *Allium* L. produced phylogenetic trees which concurred with previous conclusions based on classical taxonomy. Phylogenetic analysis revealed a closer relationship between *Nectaroscordum siculum* and *Allium cernuum* (representing *Amerallium*) than between *A. cernuum* and the rest of the *Allium* species employed in this study. The phylogeny of subg. *Melanocrommyum* based on ITS sequences largely agreed with inferences made by previous researchers based on morphology or a restriction analysis of chloroplast DNA. However, the phylogenetic positions of *Allium protensum* and *Allium macleanii* based on ITS sequences did not correspond to their morphological similarity with *Allium schubertii* and *Allium giganteum*, respectively.

Key words *Allium* · *Nectaroscordum* · Subg · *Melanocrommyum* · Phylogeny · ITS sequence analysis

Introduction

There are approximately 700 *Allium* species distributed all over the northern hemisphere (Linne von Berg et al. 1996) and about 120 species have been assigned to the subgenus *Melanocrommyum* (Fritsch 1997). The species classified in this subgenus are spread out from Northern Siberia, Central Asia to North Africa in a wide variety of habitats. The latest attempt at a taxonomic classification of this subgenus was proposed by Fritsch (1997) who grouped the species into 16 sections to address the great morphological diversity among the species.

Some of the controversies in the classification of *Allium* can be traced to over-reliance on dried specimens and disagreements on the taxonomic importance of specific morphological traits (Khassanov and Fritsch 1994; Khassanov 1997). Recently, Linne von Berg et al. (1996) used chloroplast RFLPs to validate the Gatersleben approach to the infrageneric classification of *Alliums*. Dubouzet et al. (1997) used dot-blot analysis with randomly amplified DNA as a probe to characterize the phylogeny of the subgenus *Rhizirideum*. Both approaches are indirect measures of genetic similarity when compared to DNA sequence analysis.

DNA sequencing studies account for about 50% of all molecular investigations of systematics (Sanderson et al. 1993). DNA sequences coding for the large subunit of ribulose 1,5-biphosphate carboxylase (*rbc*L) are frequently used to deduce phylogenetic relationships at the plant family level (Palumbi 1996). *rbc*L sequences from *Allium altaicum* and *Allium subhirsutum* (Fay and Chase 1996) and from *Allium cepa* (Katayama and Ogihara 1996) are available from GenBank (National Center for Biotechnology Information, USA). Plant systematists have also used DNA sequences found between the 18S and 26S genes of nuclear (n) ribosomal (r) DNA for phylogenetic studies in many angiosperm families (reviewed by Baldwin et al. 1995). This section of nrDNA, known as the ITS region, has two variable sites called internal transcribed spacers 1 and 2 (ITS1 and ITS2) flanking a coding region for the 5.8S gene.

The present study has evaluated the utility of DNA sequencing and phylogenetic analysis of the ITS region of nrDNA for the clarification of genetic relationships among some species classified under the subg. *Melanocrommyum*, representative species from the subgenera

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Allium, *Rhizirideum* and *Amerallium*, and a representative species from the closely related genus *Nectaroscordum*. The molecular phylogenies were analysed and discussed vis-à-vis known relationships based on morphology and previously published work on molecular systematics.

Materials and methods

DNA extraction and amplification

Accessions used in this study were selected from the *Allium* collection maintained at the Hokkaido National Agricultural Experiment Station (HNAES). Species identity was verified by cross-referencing with available descriptors (e.g. Vvedenskii 1935; Wendelbo 1969; Stearn 1978; Davis 1984). DNA was extracted from leaves and bulbs of the species listed in Table 1 and their ITS regions were amplified using procedures described in our previous report (Dubouzet et al. 1998). The PCR solutions were kept in ice water during mixing and PCR was done with a manual hot start. Thermal cycling was performed using 'Touchdown PCR' (Don et al. 1991). We used ITSL (Hsiao et al. 1994) in place of ITS5 (White et al. 1990) as the forward primer, and ITS4 (White et al. 1990) as the reverse primer, for PCR amplification of the ITS region. The PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Germany). The concentration of the purified PCR products was estimated by fluorometry using DyNA Quant (Hoefer Pharmacia Biotech Inc., USA). Fifty nanograms from each taxon were used in 20-µl AmpliTaq FS dye terminator cycle sequencing reactions according to the standard protocol (Perkin-Elmer, USA). Forward and reverse sequencing reactions were performed at least twice for each taxon using the primers ITSL and ITS4, respectively. DNA sequences were analysed using the ABI 373A DNA sequencer according to the protocol of Perkin-Elmer, USA.

Sequence alignment and analysis

SEQPUP v 0.6 (D. Gilbert of Indiana University, USA) was employed to format the data and generate the reverse complement sequence of the reverse-primed reactions. The sequence data were aligned using Clustal W v 1.7 (Thompson et al. 1994) by repeat iteration.

The ITS1, 5.8S and ITS2 regions were located according to Hsiao et al. (1993) and verified by comparison with the ITS regions of cotton (Mukhamedov et al. 1994), mung bean (Schiebel and Hemleben 1989), rice (Takaiwa et al. 1985) and tomato (Kiss et al. 1988). The DNA sequences of the ITS region were submitted to GenBank and given accession numbers from AF037607 to AF037624. DNA sequences were analysed using SITES v 1 (Hey and Wakeley 1997). The data in Tables 2 and 3 were generated mainly by SITES.

The DNA sequences of the ITS region and the combined ITS1 and ITS2 regions (excluding the 5.8S locus) were analysed using fastDNAml v 1 (Olsen et al. 1994). The ITS region was also analysed using DNAPARS and the trees generated by DNAPARS were consolidated using CONSENSE. DNAPARS and CONSENSE are included in PHYLIP v 3.5 (Felsenstein 1993).

Genetic distance files generated by CLUSTAL W, DNADIST (in PHYLIP) and PUZZLE v 3.1 (K. Strimmer and A. von Haeseler, Universität München, Germany) from the ITS region were analysed using the FITCH program in PHYLIP. All treefiles were illustrated using NJPLOT (M. Gouy, University of Lyon, France). These software programs are available by anonymous ftp in Internet.

Results

Boundaries and size of the ITS regions

PCR amplification of genomic DNA using the ITSL and ITS4 primers generated a single approximately 700-bp band according to agarose-gel electrophoresis. Cycle sequencing products ranged from 672 bp (*Allium protensum*) to 698 bp (*Allium cernuum*).

The aligned length of the consensus sequence from the *Tribus Allieae* species was 674 bp for the whole ITS region, 255 bp for both ITS1 and ITS2, and 164 bp for 5.8S; 21 indels in ITS1 and 11 indels in ITS2 were inferred in the consensus sequence (Fig. 1).

The values shown in Table 1 were obtained after de-gapping the ITS region of each species. The ITS1, 5.8S, and ITS2 regions ranged from 225 to 246 bp, 163 to 164 bp, and 238–249 bp, respectively. The larger range in ITS1 is due to the deletion (position 151–153, 156–161 in Fig. 1) in *A. protensum*. The GC content of the ITS region ranged from 38.7% to 51%.

Variability parameters of the ITS region

The ITS1 and ITS2 regions accounted for most (about 88%) of the phylogenetically informative sites in the ITS region (Table 2). Both ITS1 and ITS2 had almost similar D (estimate of non-neutrality of polymorphic-site distribution) and π (estimate of neutral mutation) values. However, ITS1 had twice more indels than ITS2. The 5.8S region had the lowest polymorphism values. Transitions accounted for most of the variability in the 5.8S region.

The number of base differences is a rough gauge of the genetic distance between two sequences (Hey and Wakeley 1997). The corresponding values for the ITS regions of these species are listed in Table 3.

Phylogenetic analysis

The phylogenetic tree generated by CONSENSE after DNAPARS analysis of the ITS region was topologically similar to that produced by fastDNAml (Fig. 2 A). Phylogenetic analysis of the distance files generated by CLUSTAL W (NJ method) and DNADIST (Kimura's two-parameter and Jukes-Cantor distances) by the FITCH program also produced dendrograms with topologies similar to Fig. 2 A. The trees generated by FITCH from the distance files created by PUZZLE using either a uniform or a two-rate (one fixed, one variable) model of rate heterogeneity had the same topology as the tree in Fig. 2 A. However, the trees generated by these programs differed in their branch-length values.

Phylogenies generated by fastDNAml from the ITS2 region were topologically similar to those in Fig. 2 B.

Discussion

Molecular variation in the ITS region

The values for ITS1, 5.8 S and ITS2 of the *Allieae* species shown in Table 1 are within the range cited by Baldwin et al. (1995). Length variation is negligible within the subg. *Melanocrommyum*. GC content is in the lower range of the values cited by Baldwin et al. (1995). This low GC content must be the reason for the ease with which the ITS regions of these *Allieae* species were amplified and sequenced.

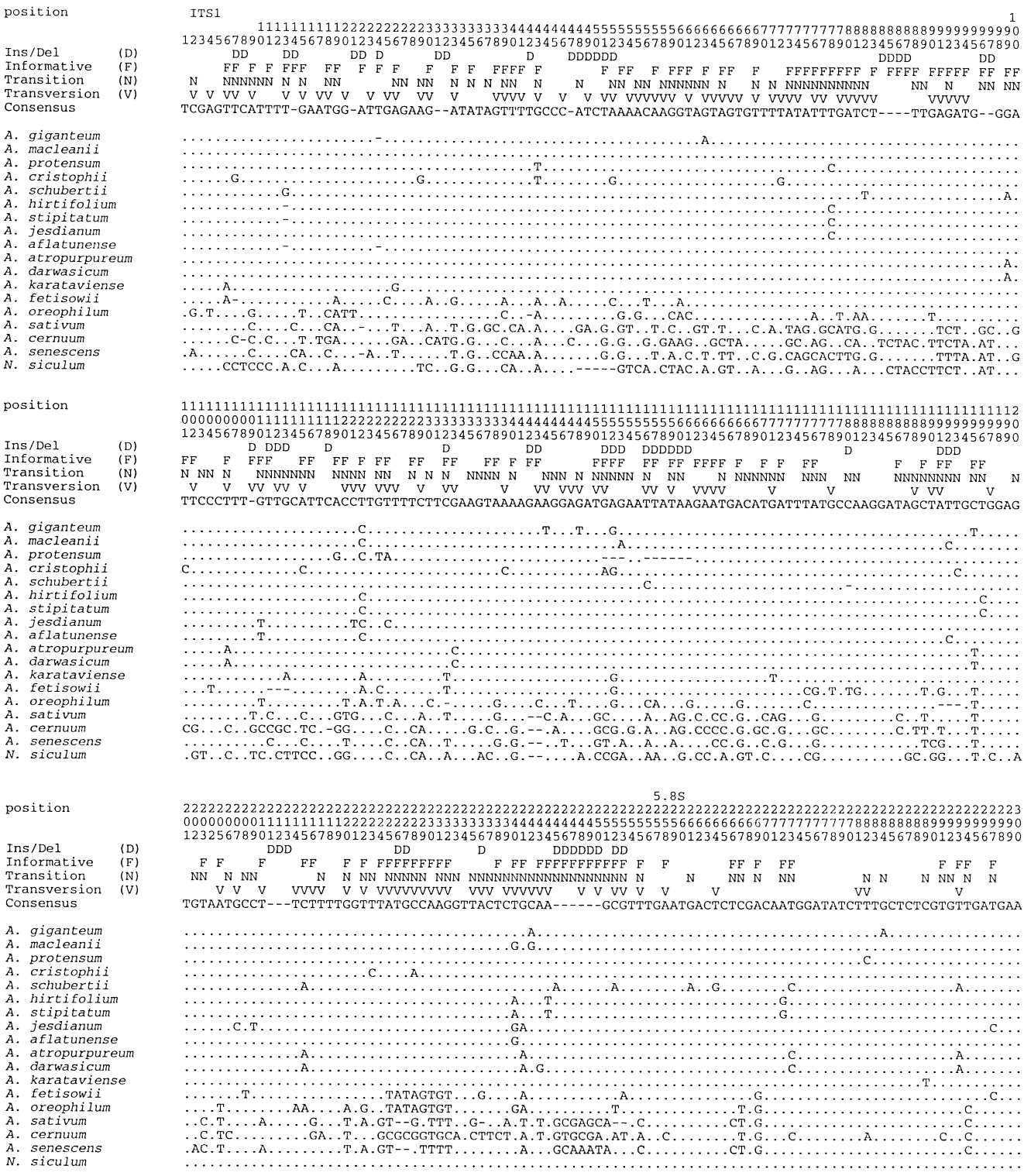

Fig. 1 See page 545 for legend

The combined ITS1 and ITS2 regions had 7.7 × more phylogenetically informative (F) sites, 42.5 × more indels, 6.1 × more transitions and 14.8 × more transversions than the 5.8S region (Table 2). These data affirm the heterogeneity of mutation rates in the ITS region. Rate heterogeneity can produce misleading inferences when maximum-likelihood models are used to investigate phylogenies (Hillis et al. 1996).

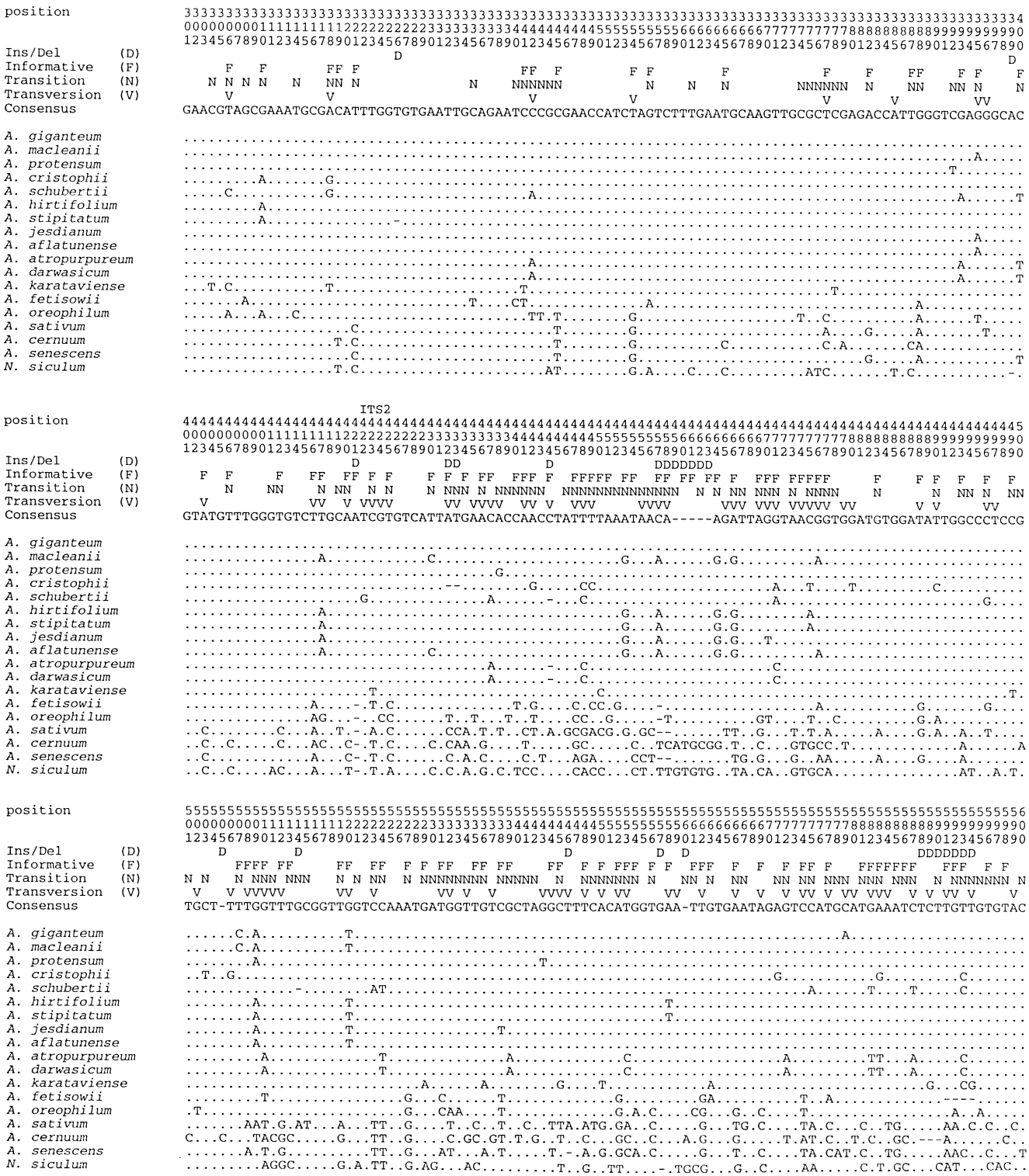

Fig. 1 See page 545 for legend

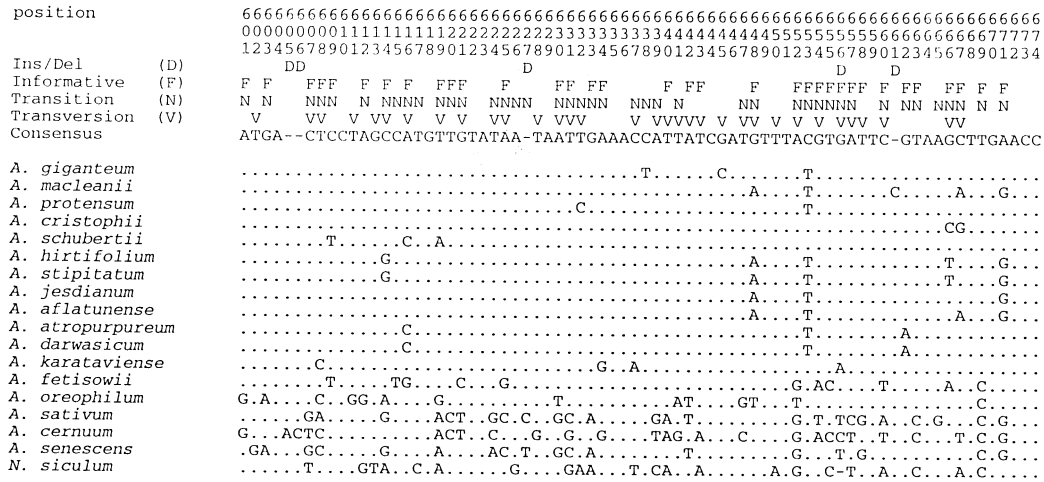

Fig. 1 DNA sequence of the ITS region of 18 *Allieae* species. *Dots* indicate identity with the consensus sequence whereas *dashes* denote gaps

Phylogenetic analysis

Trees created by CLUSTAL W, DNAPARS, fastDNAml (from DNA sequences) and FITCH (from the diverse distance files produced by CLUSTAL W, DNADIST and PUZZLE) were topologically similar. This indicates that: (1) base differences and indels are the main determinants of tree topologies generated from the ITS region, (2) rate heterogeneity in the ITS region, though pronounced, does not significantly affect tree topology, (3) sequence analysis of the ITS region, including or excluding the 5.8S gene, produces essentially similar tree topologies, and (4) sequence differences in the intronic (ITS1 and ITS2) regions in these *Allieae* species is the determining factor of overall tree topology (Fig. 2 b). The near unanimous results from such diverse analytical methods affirm the reliability of the phylogeny in Fig. 2 A. Hence, in this study, the phylogenetic information in the ITS sequences, rather than the various analytical methods, was the major determinant of tree topology.

Phylogeny of the *Allieae*

The unrooted trees in Fig. 2 segregate the entries into three major branches containing (1) *Nectaroscordum siculum* and *Allium cernuum*, (2) *Allium sativum* and *Allium senescens*, and (3) all the species currently classified under the subg. *Melanocrommyum*.

N. siculum Lindley (syn. *Allium siculum* Ucria) has elicited a great deal of controversy among taxonomists. Vvedenskii (1935) included it as a section (equivalent to a subgenus in current terminology) in the genus *Allium*. Traub (1968) also considered *Nectaroscordum* as a subgenus in *Allium*. *A. siculum* Ucria produces a garlic or onion smell when crushed (Mathew and Baytop 1984). *Amerallium* and *Nectaroscordum* have a similar number and orientation of vascular bundles in the leaf blade (Traub 1968). The main characteristic that separates *Nectaroscordum* from *Allium* is the number of veins in the tepals: *Allium* has only one whereas *Nectaroscordum* has 3–5 (Vvedenskii 1935; Traub 1968; Mathew and Baytop 1984). *Nectaroscordum* is excluded from recent classifications of the genus *Allium* (Stearn 1978; Davis 1984; Hanelt et al. 1992).

N. siculum was included in this study as a possible outspecies. Fig. 2 shows that both *N. siculum* and *A. cernuum* must have diverged from the rest of the species way back in evolutionary time. Both species are almost equidistantly related to the other *Allium* species (Fig. 2). The relatively low number of base differences between *A. cernuum* and *N. siculum* (165 in Table 3) and the sympatry of *N. siculum* with the Old World *Alliums* indicate that mechanisms other than geographic isolation may have induced the primordial divergence of *A. cernuum* (and *N. siculum*) from the rest of the *Allium* species.

Phylogenetic analysis of the partial *rbc*L sequences of some *Alliaceae* species submitted by Fay and Chase (1996) and Katayama and Ogihara (1996) to GenBank also confirm the closer relationship between *Nectaroscordum* (represented by *N. siculum*) and *Amerallium* (represented by *A. subhirsutum*) than between *Amerallium* and *Rhizirideum* (represented by *A. altaicum* and *A. cepa*). These results indicate the need for a re-evaluation of the true classification of *Nectaroscordum* (and *Amerallium*) relative to the genus *Allium*.

The relationships between *A. senescens*, *A. sativum* and members of the subg. *Melanocrommyum* shown in Fig. 2 can be explained from the perspective of plant-habit evolution. Bulbous species evolved from rhizomatous species, i.e. the subg. *Allium* and the subg. *Melanocrommyum* are derived from the subg. *Rhizirideum* (Hanelt et al. 1992). Figure 2 indicates that the

Table 1 HNAES accession numbers, origins and characteristics of the ITS region of 18 *Allieae* species

HNAES acc.no.	Source	Subg./sect.	Species	Length (bp) of ITS region G + C				
				Total	ITSI	5.8S	ITS2	(%)
		subg. *Melanocrommyum* (Webb et Berth.) Rouy						
196	Kazakhstan	*Acmopetala* R.M. Fritsch	*A. fetisowii* Rgl.	632	230	164	238	39.6
32	Takii Seed Co.	*Compactoprason* R.M. Fritsch	*A. giganteum* Rgl.	641	233	164	244	39.5
27	Takii Seed Co.	*Compactoprason*	*A. macleanii* Bak.	643	234	164	245	40.6
197	Kazakhstan	*Kaloprason* C. Koch	*A. protensum* Wendelbo	633	225	164	244	40.8
231	North American Rock Garden Society	*Kaloprason*	*A. cristophii* Trautv.	640	234	164	242	42.3
40	Sakata Seed Co.	*Kaloprason*	*A. schubertii* Zucc.	640	234	164	242	39.2
29	Takii Seed Co.	*Megaloprason* Wendelbo	*A. hirtifolium* Bois.	641	233	164	244	40.6
1164	Kairyouen	*Megaloprason*	*A. stipitatum* Rgl.	640	233	163	244	40.6
117	Takii Seed Co.	*Megaloprason*	*A. jesdianum* Bois. et Buhse	642	234	164	244	40.0
22	Sakata Seed Co	*Megaloprason*	*A. aflatunense* B. Fedstch. 'Purple Sensation'[a]	640	232	164	244	40.5
1163	Kairyouen	*Melanocrommyum* Webb et Berth.	*A. atropurpureum* W. et K.	641	234	164	243	38.7
143	Uzbekistan	*Miniprason* R.M. Fritsch	*A. karataviense* Rgl.	642	234	164	244	39.6
198	Uzbekistan	*Regeloprason* Wendelbo	*A. darwasicum* Rgl.	641	234	164	243	38.8
63	Dai-ichi Engei	*Porphyroprason* Ekb.	*A. oreophilum* C.A. Mey.	636	230	164	242	41.2
		Related species						
165	Uzbekistan	subg. *Allium*	*A. sativum* L.	642	236	164	242	46.9
65	Haiji Nursery	subg. *Amerallium* Traub	*A. cernuum* Roth.	659	246	164	249	51.0
79	Haiji Nursery	subg. *Rhizirideum* (G. Don ex Koch) Wendelbo	*A. senescens* L.	643	238	164	241	44.3
458	Sakata Seed Co	*Nectaroscordum* Lindl.	*N. siculum* (Ucria) Lindl.	652	242	163	247	46.6
			Average	642.1	234.5	163.9	243.8	41.9

[a] Friesen et al. (1997) used genomic in situ hybridization to show that 'Purple Sensation' belongs to *A. hollandicum* R. Fritsch

Table 2 Polymorphic site analysis of the ITS region of 18 *Allieae* species

Site	Aligned length (bp)	Mean GC content (%)	Informative (F)	Indel (D)	Transition (N)	Transversion (V)	Tajima's D parameter	Nei's π parameter
ITS1	255	38.1	130	57	149	123	− 1.656	49.90
5.8S	164	48.4	33	2	49	16	− 1.229	13.52
ITS2	255	40.8	123	28	152	113	− 1.527	52.00
ITS region	674	41.7	286	87	350	252	− 1.563	115.42

subg. *Melanocrommyum* and the subg. *Allium* (represented by *A. sativum*) took divergent evolutionary paths. This is also shown by the average number of base differences between members of the subg. *Melanocrommyum* and *A. senescens* or *A. sativum* (175.5 and 200, respectively) and between *A. senescens* and *A. sativum* (110).

The relatively close genetic relationship between the subgenera *Allium* and *Rhizirideum* is also indicated by the fact that, to-date, successful subgeneric hybrids in *Allium* have been obtained only between these two subgenera (Ohsumi et al. 1992; Keller et al. 1996).

Phylogeny of the subg. *Melanocrommyum*

The classification of the species under the subg. *Melanocrommyum* in Table 1 follows that proposed by Fritsch (1997). Species under the subg. *Melanocrommyum* are clustered as a monophyletic group in Fig. 2. However, substantial genetic differences in the ITS region shown in Figs. 1 and 2 confirm the taxonomic isolation of *Allium oreophilum* from the rest of the subgenus (Fritsch 1992 a; Hanelt et al. 1992; Linne von Berg et al. 1996). There was an average of 117.3 base differences between *A. oreophilum* and the other species of the subgenus (Table 3). This is higher than the number of base differences between *A. senescens* and *A. sativum*!

Ekberg (1969) established the sect. *Porphyroprason* with *A. oreophilum* as its type species. He noted some similarities in bulb characteristics with some *Melanocrommyum* species but he classified this section under the subg. *Molium*. Badr and Elkington (1977) reported that fluorochrome and Giemsa C-banded karyotypes of *A. oreophilum* and *Allium paradoxum* (classified under sect.

Table 3 Number of base differences (excluding indels) in the ITS regions of 18 *Allieae* species. Numbers on the diagonal correspond to the length of the ITS region in base pairs; numbers above the diagonal denote the number of bases used for comparison, while the numbers below the diagonal signify the frequency of differences in base composition between two sequences

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 *A. fetisowii*	632	631	632	623	630	629	631	630	632	630	631	631	632	627	623	625	625	622
2 *A. giganteum*	86	641	641	632	639	638	640	639	641	640	640	640	641	634	630	633	632	630
3 *A. macleanii*	92	24	643	633	640	639	641	640	642	640	641	641	642	635	631	634	633	631
4 *A. protensum*	85	21	26	633	631	630	632	631	633	631	632	632	633	626	622	625	624	622
5 *A. cristophii*	92	42	48	38	640	637	639	638	640	638	639	639	640	633	629	632	631	629
6 *A. schubertii*	98	44	49	41	50	640	638	637	639	637	639	639	639	632	629	632	631	629
7 *A. hirtifolium*	94	28	18	26	46	49	641	640	641	640	640	640	641	634	630	633	632	630
8 *A. stipitatum*	94	28	18	26	46	49	0	640	640	639	639	639	640	633	629	632	631	629
9 *A. jesdianum*	91	30	17	27	52	52	19	19	642	640	641	641	642	635	631	634	633	631
10 *A. aflatunense*	90	25	4	25	46	46	16	16	13	640	639	639	640	633	629	632	631	629
11 *A. atropurpureum*	95	37	44	37	49	27	45	45	45	42	641	641	641	634	630	633	632	630
12 *A. darwasicum*	96	38	45	38	50	28	46	46	46	43	1	641	641	634	630	633	632	630
13 *A. karataviense*	85	40	47	39	52	52	48	48	50	45	52	53	642	635	631	634	633	631
14 *A. oreophilum*	124	123	125	118	127	131	125	125	121	121	130	131	133	636	626	628	628	625
15 *A. sativum*	194	198	201	189	205	206	197	197	196	199	206	206	205	201	642	635	640	632
16 *A. cernuum*	209	218	216	210	222	224	220	220	216	213	217	217	222	220	202	659	636	644
17 *A. senescens*	174	174	175	168	183	180	176	176	174	175	175	176	178	173	110	197	643	633
18 *N. siculum*	210	219	222	212	222	229	226	226	226	220	225	225	223	225	224	165	216	652

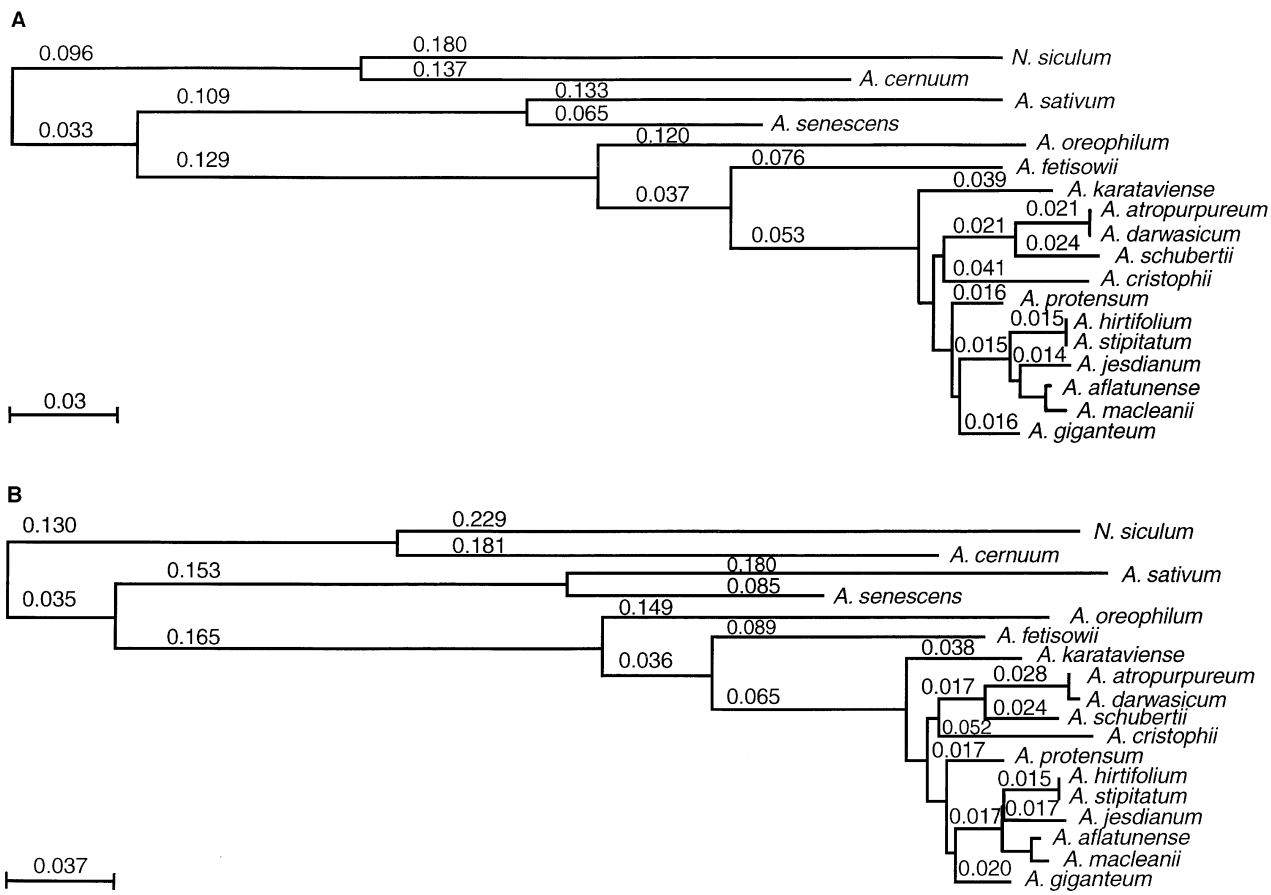

Fig. 2 Phylogenetic trees generated by fastDNAml from DNA sequences of **A** the whole ITS region and **B** combined ITS1 and ITS2 regions. *Numbers* indicate branch lenghs

Briseis) were very similar. Recently, data from restriction analysis of the chloroplast genome clearly grouped *A. oreophilum* in the subg. *Melanocrommyum* (Linne von Berg et al. 1996). Considering these conflicting reports and our current data, it is clear that the position of *A. oreophilum* can only be resolved by a study including representatives from the sections *Molium*, *Briseis* and the subg. *Melanocrommyum*.

Most species of sect. *Acmopetala* (represented here by *Allium fetisowii*) are restricted to the Tian-Shan and

Pamir Alai mountain systems (Fritsch 1997). The species classified under sect. *Acmopetala* are distantly related to other tall-growing taxa under sections *Megaloprason*, *Compactoprason*, and *Melanocrommyum* (Khassanov and Fritsch 1994). This distant relationship is also indicated by significant changes in the sequence of the ITS region (Fig. 1) and by the position of *Allium fetisowii* in the resultant phylogenetic tree (Fig. 2).

Allium karataviense is classified under the sect. *Miniprason* (Fritsch 1992 a). Its distance from *Allium atropurpureum* confirms Fritsch's decision to remove *A. karataviense* from the sect. *Melanocrommyum*. The other species under the subg. *Melanocrommyum* are grouped in two tight clusters, comparable to the results obtained by Linne von Berg et al. (1996).

The differences between *A. atropurpureum* (sect. *Melanocrommyum*) and *Allium darwasicum* (sect. *Regeloprason*) were negligible (one base difference in Table 3). This is rather surprising in view of their morphological differences.

The species in sect. *Kaloprason* (*Allium schubertii*, *Allium cristophii*, and *Allium protensum*) are distributed from Tian-Shan in Central Asia to the deserts of North Africa (Fritsch 1997). Wendelbo (1969) found sufficient morphological differences to classify *A. schubertii* under the sect. *Kaloprason* and *A. cristophii* under the sect. *Acanthoprason* Wendelbo. These morphological differences are echoed in the large number of base differences (50) between *A. schubertii* and *A. cristophii* (Table 3).

Khassanov (1997) classified *A. schubertii* in the subsect. *Schubertia* R. Kam. and grouped *A. cristophii* and A. *protensum* under the subsect. *Cristophiana* Tscholok. Data in Table 3 and Fig. 1 also shows that *A. protensum* is more similar to *A. cristophii* than to *A. schubertii*. However, the DNA sequence of the ITS region of *A. protensum* was substantially more similar to the members of the sect. *Megaloprason* than to *A. cristophii* or *A. schubertii* (Table 3).

Excluding *A. protensum*, the lowest cluster of both trees in Fig. 2 includes species that Wendelbo (1969) assigned to the sect. *Megaloprason*. Fritsch (1992 a,b) noted distinct morphological characters shared by *Allium giganteum* and *Allium macleanii* that justified the establishment of the sect. *Compactoprason*. The position of *A. giganteum* in Fig. 2 may justify the establishment of a separate section but Fig. 2 does not warrant lumping the two, albeit morphologically similar species, in one section.

Fritsch (1992 b) accepted *Allium aflatunense*, *Allium jesdianum* and *Allium stipitatum* in the sect. *Megaloprason*. Davies (1992) noted that *Allium hirtifolium* has a phenotype which is very similar to *A. stipitatum*. Fritsch (1997) concluded that *A. hirtifolium* is a synonym of *A. stipitatum*. DNA sequence data from the ITS region indicate that they differ only in a single base deletion at position 326 (5.8S gene region in Fig. 1).

Conclusions

DNA sequencing and phylogenetic analysis of the ITS region of nrDNA has confirmed many of the taxonomic conclusions based (primarily) on morphological comparisons in the genus *Allium* and, more specifically, in the subgenus *Melanocrommyum*. The data from this study largely confirm the results of a restriction analysis of the chloroplast genome. However, the present study has shown that, in some cases, changes in the ITS region are asynchronous with changes in phenotype. This conflict between morphological and nrDNA sequence analyses, which is not uncommon in molecular systematics, may require the concurrent evaluation of other phylogenetically useful traits for complete resolution.

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