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Phylogenetic relationships among cultivated *Allium* species from restriction enzyme analysis of the chloroplast genome

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Summary. The genus *Allium* contains many economically important species, including the bulb onion, chive, garlic, Japanese bunching onion, and leek. Phylogenetic relationships among the cultivated alliums are not well understood, and taxonomic classifications are based on relatively few morphological characters. Chloroplast DNA is highly conserved and useful in determining phylogenetic relationships. The size of the chloroplast genome of *Allium cepa* was estimated at 140 kb and restriction enzyme sites were mapped for KpnI, PstI, PvuII, SalI, XbaI, and XhoI. Variability at restriction enzyme sites in the chloroplast DNA was studied for at least three accessions of each of six cultivated, old-world *Allium* species. Of 189 restriction enzyme sites detected with 12 enzymes, 15 mutations were identified and used to estimate phylogenetic relationships. Cladistic analysis based on Wagner and Dollo parsimony resulted in a single, most-parsimonious tree of 16 steps and supported division of the species into sections. *Allium* species in section Porrum were distinguished from species in sections Cepa and Phyllodolon. Two species in section Rhiziridium, *A. schoenoprasum* and *A. tuberosum,* differed by five mutations and were placed in separate lineages. *Allium cepa* and *A. fistulosum* shared the loss of a restriction enzyme site and were phylogenetically closer to each other than to *A. schoenoprasum.* This study demonstrates the usefulness of restriction enzyme site analysis of the chloroplast genome in the elucidation of phylogenetic relationships in *Allium.*

Key words: RFLPs – *Allium* taxonomy – Onion – Chloroplast DNA

Introduction

Allium is a large genus of approximately 500 species and contains many economically important plants, including the bulb onion (*Allium cepa* L.), chive (*A. schoenoprasum* L.), Chinese chive (*A. tuberosum* Rottler ex Sprengel syn. *A. odorum*), garlic (*A. sativum* L.), Japanese bunching onion (*A. fistulosum* L.), and leek (*A. ampeloprasum* L. syn. *A. porrum* G. Don) (Fenwick and Hanley 1985). These cultivated *Allium* species are indigenous to the Eurasian continent. A detailed description of *Allium* in the Soviet Union was published by Vvedensky (1944), who classified the cultivated alliums into four sections, Cepa (bulb onion), Phyllodolon (Japanese bunching onion), Porrum (garlic and leek), and Rhiziridium (chive and Chinese chive). More recent classification schemes have also divided *Allium* into sections. Using morphological criteria, crossability, and karyotypes, Traub (1968) classified the cultivated alliums into four sections (Allium, Cepa, Fistulosa, and Rhiziridium), with further division into subsections.

The taxonomy of such a large genus as *Allium* is difficult because of no single uniting morphological trait (Stearn 1944), assimilation of complex chromosome aberrations (Ved Brat 1965), accumulation of unequal amounts of heterochromatin or spacer DNA (Jones and Rees 1969), and the probable loss of many species during the previous ice age (Traub 1968). The division of the genus into subgenera, sections, and varieties is artificial and is based on relatively few morphological characters (Stearn 1944). Karotypic analyses (Ved Brat 1965), numerical taxonomy (El-Gadi and Elkington 1977), and isozymes (Hadacova et al. 1981, 1983; Klozova et al. 1981; Rieseberg et al. 1987) have been used in *Allium* taxonomy. These analyses have resolved species relationships, supported the division of the genus into sections, and placed species that are crossable, albeit with difficulty, closely together. Badr and Elkington (1978) recommended that morphological, cytological, and chemical data be used together for a more informative classification of *Allium*.

Electrophoretic analysis of restriction enzyme (RE) digests of chloroplast DNA (cpDNA) of higher plants has proven useful in phylogenetic studies (Palmer 1986; Palmer et al. 1988). The linear array of genes in cpDNA is highly conserved between evolutionarily distant species (Palmer and Stein 1986). Analyses of variation at restriction enzyme sites in cpDNA have given unambiguous interpretations of the relationships between species that would not be possible using morphological traits or crossability studies (Palmer et al. 1988). The purpose of this research was to estimate phylogenetic relationships among the cultivated *Allium* species utilizing restriction fragment analysis of cpDNA. The results give a better understanding of the evolution of the cultivated alliums and the delineation of groups of *Allium* species within which interspecific hybrids potentially may be generated.

Materials and methods

Exclusive maternal inheritance of the *Allium* chloroplast genome has been demonstrated (Corriveau and Coleman 1988). The taxonomic unit in this study was an accession maintained by cross-pollinating several plants. Accessions of cultivated *Allium* species belonging to sections Cepa, Porrum, Phyllodolon, and Rhiziridium (Vvedensky 1944) were used (Table 1). Total DNA was isolated from freshly harvested green tissue by cTAB extraction (Murray and Thompson 1980). The DNA was digested with 12 restriction enzymes, BglII, BstEII, DraI, EcoRV, KpnI, PstI, PvuII, SacI, SalI, SmaI, XbaI, and XhoI, at 5 units/μg according to the manufacturer's recommendations. Electrophoresis of 3 μg of digested DNA was completed in 0.8% agarose gels in 1 × TBE (Maniatis et al. 1982) at 12 mA for 14 h. The DNA was then denatured by soaking in 0.4 N NaOH with 0.6 M NaCl for 20 min and blotted overnight to Zetaprobe (BioRad, Richmond, CA, USA) filters by capillary transfer (Southern 1975). The filters were rinsed in 0.1 × SSC (Maniatis et al. 1982) and dried by baking in a vacuum oven at 80 °C for 2–4 h.

Table 1. Origin of *Allium* accessions

Section	*Allium* species	P.I. number[a]	Origin
Cepa	*A. cepa*	172703	Turkey
	A. cepa	207456	Afghanistan
	A. cepa	227346	Iran
	A. cepa	260793	Fed. Rep. Germany
	A. cepa	293756	USSR
Phyllodolon	*A. fistulosum*	208733	Cuba
	A. fistulosum	223853	Japan
	A. fistulosum	433630	Japan
	A. fistulosum	491582	China
	A. fistulosum	491583	China
Porrum	*A. ampeloprasum*	256051	Afghanistan
	A. ampeloprasum	280574	USSR
	A. ampeloprasum	280575	USSR
	A. ampeloprasum	285545	Poland
	A. sativum	383820	Yugoslavia
	A. sativum	493104	USSR
	A. sativum	493105	Turkey
	A. sativum	497951	Syria
Rhiziridium	*A. schoenoprasum*	280578	USSR
	A. schoenoprasum	280580	USSR
	A. schoenoprasum	371880	USSR
	A. schoenoprasum	401719	Denmark
	A. tuberosum	372861	Egypt
	A. tuberosum	399173	Hong Kong
	A. tuberosum	483423	Switzerland
–[b]	*A. cernuum*	372503	Canada

[a] P.I. = USDA Plant Introduction Number. Accessions of *A. cepa* and *A. fistulosum* are available from P.I. station in Geneva, NY, USA and *A. ampeloprasum*, *A. cernuum*, *A. sativum*, *A. schoenoprasum*, and *A. tuberosum* from P.I. station in Pullman, WA, USA

[b] Not included in Vvedensky's (1944) classification

Clones from the chloroplast genome of the orchid *Oncidium excavatum* were used as probes (Chase and Palmer 1989). Individual clones or contiguous fragments representing 10–15 kb (fragments 12b and c; 13, 14, 15, and 16; 17 and 18; 19; 20, 21, 22 and 1; 2; 3 and 4; 6; 7 and 8; 9; 10; and 11 and 12a) were labelled with P^{32} by nick translation (Rigby et al. 1977) to a specific activity of 1×10^8 cpm/μg. Probe DNA was heat-denatured and hybridized to groups of 36 filters in 300 ml of hybridization solution (40% deionized formamide, 1 mM EDTA, 0.25 M Na_2HPO_4, pH 7.2, and 7% SDS) overnight at 42 °C. Filters were washed once for 5 min at 42 °C in 2 × SSC with 0.1% SDS, once for 15 min at 42 °C in 0.1 × SSC with 0.1% SDS, and once for 30 min at 65 °C in 0.1 × SSC with 0.1% SDS. Filters were rinsed in 0.1 × SSC, wrapped in plastic, and placed on X-ray film (Kodak XAR-5) with intensifying screens at −70 °C for 1–2 days.

For restriction site mapping of *A. cepa* cpDNA, total DNA from USDA Plant Introduction (P.I.) 227346 was singly digested with KpnI, PstI, PvuII, SalI, XbaI, and XhoI and doubly digested with PstI or XbaI and each of the above enzymes. The DNA was electrophoresed through 0.5%, 0.8%, 1.0%, and 1.3% agarose gels and blotted as previously described. Filters were hybridized with the same orchid chloroplast fragments as before. Fragment sizes were estimated from autoradiographs by a computer program interpolating from a standard curve of end-labelled fragments from the HindIII digest of the bacteriophage lambda (Schaffer and Sederoff 1981). Fragment order and the positions of inversions were determined following the protocol of Palmer (1986).

Cladistic analyses of phylogenetic relationships were completed using Wagner and Dollo parsimony by the program Phylogenetic Analysis Using Parsimony (PAUP, D. Swofford, State Natural History Survey Division, Champaign, IL, USA). Wagner parsimony treats equally the loss or gain of RE sites; Dollo parsimony takes into account that convergent RE site losses are more common than gains because a loss requires a mutation at only one of six nucleotides (for the restriction enzymes used in this study). For a convergent RE site gain, a change at the same nucleotide(s) must occur in two separate species (deBry and Slade 1985). The most parsimonious trees were generated and Felsenstein's (1985) bootstrap method estimated confidence intervals for the phylogenetic relationships.

Allium cernuum Roth was used as the outgroup for polarization of character states. This species is indigenous to North

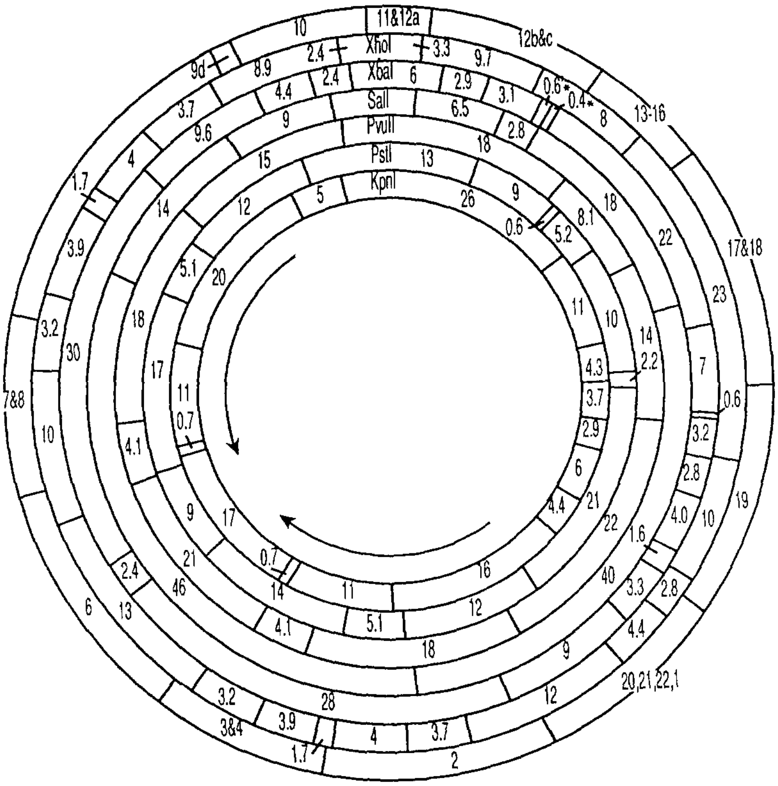

Fig. 1. Approximate fragment sizes in kilobases from digestion of chloroplast genome of *A. cepa* PI227346 with restriction enzymes KpnI, PstI, PvuII, SalI, XbaI, and XhoI. Positions of fragments with *asterisks* could not be unequivocally determined. *Arrows* show positions of inverted repeat. *Outer circle* indicates the approximate positions of complementary orchid clones

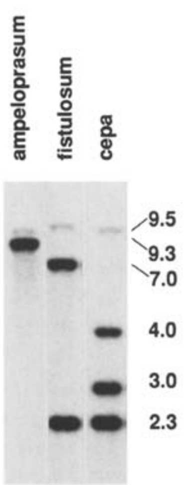

Fig. 2. Variation at two EcoRV sites in the chloroplast DNA of *Allium ampeloprasum*, *A. cepa*, and *A. fistulosum* detected by probing with fragments 12b and c of orchid chloroplast DNA. See "Materials and methods" for description of chloroplast clones and Table 2 for fragment size differences

Table 2. Polymorphic restriction enzyme sites detected in the *Allium* chloroplast genome

Character number	Orchid clone[a]	Enzyme	Fragment sizes
1	3&4	BglII	4.0 = 3.0 + 1.0
2	12b&c	BglII	4.0 = 3.5 + 0.5
3	12b&c	EcoRV	9.3 = 7.0 + 2.3
4	12b&c	EcoRV	7.0 = 3.0 + 4.0
5	12b&c	PstI	20.0 = 11.0 + 9.0
6	12b&c	SacI	16.0 = 10.0 + 6.0
7	13–16	XhoI	6.0 = 4.0 + 2.0
8	17	BglII	3.1 = 1.8 + 1.3[b]
9	17	BglII	2.0 = 1.6 + 0.4
10	19	BglII	4.4 = 2.2 + 2.2
11	19	KpnI	9.0 = 6.0 + 3.0
12	6	BglII	4.4 = 3.0 + 1.4
13	11&12a	DraI	3.0 = 1.6 + 1.4
14	11&12a	BglII	7.0 = 6.0 + 1.0
15	22	XbaI	4.3 = 3.8 + 0.5

[a] For description of orchid clones, see "Materials and methods"

[b] *Allium ampeloprasum* and *A. sativum* possess a 0.6-kb insertion in the 1.8-kb fragment

America and has 14 chromosomes; the other *Allium* species studied herein are native to the Eurasian continent and possess 16 chromosomes. *Allium cernuum* was chosen because it would not likely be placed within phylogenetic lineages containing the old-world alliums.

Results

The size of fragments from RE digestions and their approximate linear order in the chloroplast genome of *A. cepa* were determined for six enzymes (Fig. 1). The molecule is approximately 140 kb in size and contains the inverted repeat common to most angiosperms (Palmer

Table 3. Presence or absence of restriction enzyme sites in chloroplast genome of cultivated and wild *Allium* species

Allium species	Accession[a]	Character number[b]														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A. ampeloprasum	256051	+	+	−	−	−	+	+	+	−	−	+	−	+	−	−
A. ampeloprasum	280574	+	+	−	−	−	+	+	+	−	−	+	−	+	−	−
A. ampeloprasum	280575	+	+	−	−	−	+	+	+	−	−	+	−	+	−	−
A. ampeloprasum	285545	+	+	−	−	−	+	+	+	−	−	+	−	+	−	−
A. cepa	172703	−	−	+	+	+	−	−	+	+	+	+	+	−	−	+
A. cepa	207456	−	−	+	+	+	−	−	+	+	+	+	+	−	−	+
A. cepa	227346	−	−	+	+	+	−	−	+	+	+	+	+	−	−	+
A. cepa	260793	−	−	+	+	+	−	−	+	+	+	+	+	−	−	+
A. cepa	293756	−	−	+	+	+	−	−	+	+	+	+	+	−	−	+
A. cernuum	372503	+	+	+	−	−	−	−	+	+	+	+	+	−	−	+
A. fistulosum	208733	−	+	+	−	+	−	−	−	+	+	+	+	−	−	+
A. fistulosum	223853	−	+	+	−	+	−	−	−	+	+	+	+	−	−	+
A. fistulosum	433630	−	+	+	−	+	−	−	−	+	+	+	+	−	−	+
A. fistulosum	491582	−	+	+	−	+	−	−	−	+	+	+	+	−	−	+
A. fistulosum	491583	−	+	+	−	+	−	−	−	+	+	+	+	−	−	+
A. sativum	383820	+	+	−	−	−	+	−	+	+	−	+	−	−	−	−
A. sativum	493104	+	+	−	−	−	+	−	+	+	−	+	−	−	−	−
A. sativum	493105	+	+	−	−	−	+	−	+	+	−	+	−	−	−	−
A. sativum	497951	+	+	−	−	−	+	−	+	+	−	+	−	−	−	−
A. schoenoprasum	280578	+	+	+	−	+	−	−	+	+	+	+	+	−	+	+
A. schoenoprasum	280580	+	+	+	−	+	−	−	+	+	+	+	+	−	+	+
A. schoenoprasum	371880	+	+	+	−	+	−	−	+	+	+	+	+	−	+	+
A. schoenoprasum	401719	+	+	+	−	+	−	−	+	+	+	+	+	−	+	+
A. tuberosum	372861	+	+	+	−	−	−	−	−	+	+	−	+	−	−	−
A. tuberosum	399173	+	+	+	−	−	−	−	−	+	+	−	+	−	−	−
A. tuberosum	483423	+	+	+	−	−	−	−	−	+	+	−	+	−	−	−

[a] For description of accessions, see Table 1

[b] + and − represent the presence and absence, respectively, of a restriction site. For description of character numbers, see Table 2

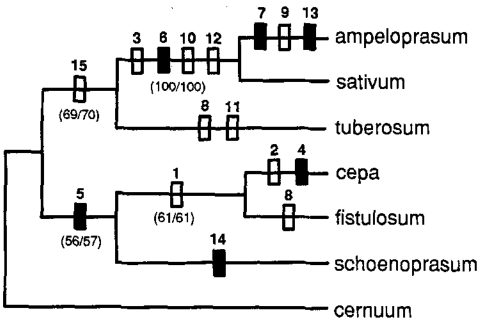

Fig. 3. Cladogram from Wagner and Dollo parsimony analysis of changes at restriction enzyme (RE) sites in cultivated *Allium* species using *A. cernuum* as the outgroup. *Open* or *solid boxes* designate the loss or gain, respectively, of a RE site. Numbers above boxes represent RE sites listed in Table 2. The two numbers below the boxes represent the frequency at which species were placed at each branch point for 100 independently generated, most-parsimonious Wagner and Dollo trees, respectively

and Stein 1986). This size estimate was slightly less than the 145 kb reported for *A. cepa* cpDNA by Chase and Palmer (1989).

There were 189 RE sites identified using the 12 enzymes, representing 1,134 nucleotides or 0.8% of the chloroplast genome. Loss or gain of a RE site was easily scored because two smaller fragments added to the size of one larger fragment (Fig. 2). Fifteen mutations at RE sites were discovered (Tables 2 and 3). Differences in fragment sizes due to insertions, deletions, or inversions were detected but were not used in the phylogenetic analyses. These differences were observed among conspecific accessions and occassionally gave complex banding patterns. Given the uncertain homology of fragment length changes (Palmer et al. 1983), estimations of phylogenetic relationships based soley on mutations at RE sites are more conservative.

Analyses using either Wagner or Dollo parsimony resulted in an identical, single, most-parsimonious tree with a length of 16 (Fig. 3). None of the 15 mutations distinguished the outgroup *A. cernuum*. Two different lineages were detected representing sections Porrum and Cepa-Phyllodolon. *Allium schoenoprasum* and *A. tuberosum*, both in section Rhiziridium, were placed in separate lineages with species in sections Cepa-Phyllodolon and Porrum, respectively. The consistency index (ratio of number of character states over number of steps in tree) of 0.938 reflected the convergent loss of character 8 in *A. fistulosum* and *A. tuberosum* (Fig. 3; Table 3). The bootstrap technique was used to calculate the frequency at which species were placed at each branch point for 100 independently generated, most-parsimonious trees. Only the clade containing *A. ampeloprasum* and *A. sativum* was found in all independently generated trees (Fig. 3). The lower frequencies observed for the other clades were the result of single mutations distinguishing groups of species.

Three equally parsimonious trees were observed with a length of 17 (trees not presented). A strict consensus of the four trees of 16 and 17 steps resolved only a single clade representing *A. ampeloprasum* and *A. sativum*. By allowing an additional step, the phylogenetic relationships between *A. cepa, A. fistulosum, A. schoenoprasum,* and *A. tuberosum* were unresolved.

Discussion

Analysis of the *Allium* cpDNA supported the division of cultivated species into different sections and resulted in estimation of phylogenetic relationships consistent with previous taxonomic classifications. For the species analyzed, two distinct groups were identified based on the 15 RE site mutations corresponding to Vvedensky's (1944) section Porrum and sections Cepa and Phyllodolon. This study indicates that the species of section Porrum are phylogenetically distant from *A. cepa*. The most-parsimonious tree required 12 RE site changes in the cpDNA between *A. ampeloprasum* and *A. cepa*. Although there may be no inherent relationship between diversity in the chloroplast genome and crossability, species possessing a similar chloroplast genome are phylogenetically closer and may have accumulated fewer structural changes between the chromosomes, e.g., translocations or varying amounts of heterochromatin. Therefore, gene transfer between *Allium* species with a similar chloroplast genome may be easier. Sexual gene transfer between *A. ampeloprasum* and *A. cepa* may be difficult, which is unfortunate because *A. ampeloprasum* shows resistance to white rot (Coley-Smith and Esler 1983), a major disease of the bulb onion.

Vvedensky (1944) placed the Japanese bunching onion (*A. fistulosum*) into section Phyllodolon. Although hybrids between *A. cepa* and *A. fistulosum* have been generated (Emsweller and Jones 1935), three differences at RE sites were detected between these species. Only a single example of homoplasy was observed in this study, the shared loss of character 8 in *A. fistulosum* and *A. tuberosum*. However, these two species were separated by four unique changes (characters 1, 5, 11, and 15).

Analysis of variation at RE sites in cpDNA has given an unbiased estimate of phylogenetic relationships among cultivated *Allium* species. The relationships are similar to Vvedensky's (1944) classification and are potentially useful in resolving the phylogeny of species in the complex section Rhiziridium. Because the progenitors of many cultivated alliums are unknown, analysis of RE sites in the cpDNA of *Allium* species will be helpful in identifying closely related wild *Allium* species potentially useful in the genetic improvement of the cultivated forms.

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References

Badr A, Elkington T (1978) Numerical taxonomy of species in *Allium* subgenus *Molium*. New Phytol 81:401–417

Chase M, Palmer J (1989) Chloroplast DNA systematics of lilioid monocots, feasibility, resources, and an example from the Orchidaceae. Am J Bot 76:1720–1730

Coley-Smith J, Esler G (1983) Infection of cultivars of onion, leek, garlic, and *Allium fistulosum* by *Sclerotium cepivorum*. Plant Pathol. 32:373–376

Corriveau J, Coleman A (1988) Rapid screening method to detect biparental inheritance of plastid DNA and results from over 200 angiosperm species. Am J Bot 75:1443–1458

deBry R, Slade N (1985) Cladistic analysis of restriction endonuclease cleavage maps within maximum-likelihood framework. Syst Zool 34:21–34

El-Gadi A, Elkington T (1977) Numerical taxonomic studies on species in *Allium* subgenus *Rhiziridium*. New Phytol 79:183–201

Emsweller S, Jones H (1935) An interspecific hybrid in *Allium*. Hilgardia 9:265–273

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fenwick G, Hanley A (1985) The genus *Allium*. Crit Rev Food Sci Nutr 22:199–271

Hadacova V, Klozova E, Hadac E, Turkova V, Pitterova K (1981) Comparison of esterase isozyme patterns in seeds of some *Allium* species and in cultivars of *Allium cepa* L. Biol Plant Prague 23:376–380

Hadocova V, Svachulova J, Klozova E, Hadac E, Pitterova K (1983) Use of esterase isoenzymes revealed by gel isoelectric focusing as an aid in chemotaxonomical study of the genus *Allium*. Biol Plant Prague 259:36–42

Jones R, Rees H (1969) Nuclear DNA variation in *Allium*. Heredity 23:591–605

Klozova E, Turkova V, Hadacova V, Svachulova J (1981) Serological comparisons of seed protein of some *Allium* (L.) species belonging to the subgenus *Rhiziridium* (G. Don ex Koch) Wendelbo. Biol Plant Prague 23:376–380

Maniatis T, Fritsch E, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Murray M, Thompson W (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4231–4325

Palmer J (1986) Isolation and structural analysis of chloroplast DNA. Methods Enzymol 118:167–186

Palmer J, Stein D (1986) Conservation of chloroplast genome structure among vascular plants. Curr Genet 10:823–833

Palmer J, Shields C, Cohen D, Orton T (1983) Chloroplast DNA evolution and the origin of amphidiploid *Brassica* species. Theor Appl Genet 65:181–189

Palmer J, Jansen R, Michaels H, Chase M, Manhart J (1988) Chloroplast DNA variation and plant phylogeny. Ann Mo Bot Gard 75:1180–1206

Rieseberg L, Peterson P, Soltis D, Annable C (1987) Genetic divergence and isozyme number variation among four varieties of *Allium douglasii* (Alliaceae). Am J Bot 74:1614–1624

Rigby P, Dieckmann M, Rhodes C, Berg P (1977) Labeling DNA to high specific activity in vitro by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Schaffer H, Sederoff R (1981) Improved estimation of DNA fragment lengths from agarose gels. Anal Biochem 115:113–122

Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Stearn W (1944) Notes on the genus *Allium* in the Old World. Herbertia 11:11–37

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

Ved Brat S (1965) Genetic systems in *Allium*. Chromosoma 16:486–499

Vvedensky A (1944) The genus *Allium* in the USSR. Herbertia 11:65–218