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Phylogeny of *Allium* L. subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo according to dot blot hybridization with randomly amplified DNA probes

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Abstract Among 341 randomly amplified DNA sequences generated from 11 *Allium* species, 55 were purified by gel excision and subsequent reamplification by PCR. These were then used as probes in dot blot analysis to evaluate the relationships between 44 *Allium* accessions classified under the subgenus *Rhizirideum*. The hybridization signals were standardized and converted to Euclidean taxonomic distances. Unweighted Pair Group Mean analysis of the distance data generated a phyllogram which basically conformed to the classification system proposed by the Gatersleben (Germany) group. However, there was insufficient evidence to suppport the proposal to join *A. chinense* G. Don with *A. virgunculae* F. Maek. et Kitam. into sect. *Sacculiferum* or the recent suggestion to re-establish sect. *Phyllodolon*.

Key words *Allium* · *Rhizirideum* · Randomly amplified DNA probes · Dot blot analysis · Phylogeny

Introduction

There are 150–170 *Allium* species in subgenus *Rhizirideum* distributed throughout the warm temperate to subarctic northern hemisphere, from southwestern Europe to northeastern Asia and in north-east America (Hanelt 1990). The presence of a rhizome in various shapes is the unifying characteristic of this subgenus, but the species are quite variable in other morphological characteristics. Several conflicting classifications have been published, and the most recent was based on morphological, cytological, geographical, serological and anatomical studies (Hanelt et al. 1992).

Molecular taxonomy in *Allium* has been studied using restriction fragment length polymorphisms (RFLPs) obtained from chloroplast or nuclear DNA (e.g. Bradeen and Havey 1995; Havey 1991; Linne von Berg et al. 1996). However, RFLP analysis is limited by the low frequency of polymorphic sites (Havey 1991). Random amplified polymorphic DNA (RAPD) markers, which are easy to generate, have also been used for genetic analysis in *Allium* (e.g. Wilkie et al. 1993).

Both RAPD and RFLP analysis assume that bands with similar migration rates in an electrophoretic gel are homologous. However, genetic mechanisms such as homoplasy or convergence can produce non-homologous bands with similar electrophoretic mobility. Homoplasy has been reported in RAPD (Stammers et al. 1994) as well as RFLP (Havey 1992) analyses and it can lead to an overestimate of genetic similarity (Castagna et al. 1997).

Chromosomal aberrations such as insertions, deletions or inversions result in many RFLPs of uncertain homology that are not useful for phylogenetic analysis (Havey 1991). In the case of RAPD analysis, poor amplification of 'weak' priming sites due to competition can increase the number of 'polymorphisms' (Tinker et al. 1993). Both phenomena are likely sources of error in the calculation of genetic distance. Thus, a method that is less sensitive to homoplasy and chromosomal aberrations is necessary for the phylogenetic analysis of a variable group of species.

Dot blot analysis is used to determine the relative abundance of target sequences by hybridization of a specific gene probe to unfractionated DNA of various species (Brown 1995). With sufficient denaturation of the genomic DNA, hybridization will occur regardless

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of the position of the target sequence or the length of the DNA segment to which the target sequence is attached. However, it is difficult to obtain a large number of specific gene probes.

The aims of the study presented here were: (1) to evaluate the utility of randomly amplified DNA sequences as probes for dot blot hybridization and (2) to construct a phylogenetic tree of subgenus *Rhizirideum* based on the distance estimates derived from the hybridization signals.

Materials and methods

DNA extraction, amplification and probe preparation

Total DNA from the *Allium* species listed in Fig. 1 were extracted using a procedure reported by Dubouzet et al. (1997). In this study, the extraction buffer was mixed continuously over a heated (approx. 80°C) magnetic stirrer.

DNA from 11 species was amplified using single primers from Operon Industries (USA) (Table 1). The amplification reaction consisted of 10 µl DNA extract, 3.1 µl distilled, deionized water, 2.8 µl 25 m*M* $MgCl_2$, 2 µl 10 × buffer II, 1.6 µl 10 m*M* dNTP mix, 0.4 µl 10 µ*M* random decamer and 0.1 µl AmpliTaq DNA polymerase (Perkin Elmer, USA).

The polymerase chain reaction (PCR) was performed in a Perkin Elmer 9600 thermal cycler. The thermal cycle consisted of a denaturation step of 98°C for 1.5 min followed by 45 cycles of (94°C 15 s, 36°C 15 s, 72°C 60 s) and a final extension step at 72°C for 5 min. Amplified bands ranging from 500 to 2000 bp were excised from the 0.8% agarose gels and melted in 200 µl water. From this mixture 10 µl aliquots were used in 20 µl re-amplification reactions. Reactions from which only one major reamplification product was obtained were used as probes. The size of the reamplified product was estimated using GELREADER 2.01 from the National Center for Supercomputing Applications at the University of Illinois at Urbana-Champaign (USA).

Dot blot hybridization

The 100 µl DNA samples were adjusted to 1 ng/µl based on UV-spectrophotometric data and then blotted on Hybond N + nylon membranes according to protocols described by Brown (1995). We used 1 µl of reamplified product per 30 µl labelling solution. Hybridization, washing and detection by autoradiography were done according to the manufacturer's recommendations (Amersham). A stringent wash was followed to reduce heterologous pairing. Hybridization was performed twice for each probe.

Densitometry and data analysis

The X-ray films were digitized using a NSF-1200CL color scanner, and densitometry was performed on a Macintosh Performa 5320 computer using the NIH IMAGE v. 1.61 program (written by W. Rasband at the US National Institutes of Health). The integrated density value, which is the sum of the gray values of the selected area (0.8 cm^2) after background subtraction, was calculated from the uncalibrated optical density value.

The hybridization signals were transformed by centering and standardization with the species mean and standard deviation, respectively. The Euclidean distance between species 1 and 2 was calculated from the standardized data (x_{ni}) as (Randerson 1993):

$$D_{1,2} = \sqrt{\sum_{i=1}^{m} (x_{1i} - x_{2i})^2}$$

The distance values were analyzed using the Unweighted Pair Group Method Analysis program in PHYLIP v. 3.572 (Felsenstein 1993). The treefile was illustrated using the TREEVIEW program (Page 1996). GELREADER, NIH IMAGE, PHYLIP and TREEVIEW are obtainable by anonymous ftp at the Internet.

Results and discussion

Evaluation of randomly amplified DNA sequences as probes

Among 341 amplified bands generated by 11 random decamers from 11 *Allium* species (Table 1), 235 were excised and reamplified but only 96 produced single major bands. About 53% of the reamplification reactions, especially those obtained from the longer (> 1000 bp) bands, generated the target band and a few shorter bands observed in the original amplification profile. Multiple priming sites in the longest amplified sequence can explain this observation (Tinker et al. 1993). A few of the re-amplified DNA sequences also generated longer bands probably through concatenation.

When used as probes, the 'polymorphic' amplified bands often hybridized with genomic DNA of species whose RAPD profiles 'lacked' such bands. This incongruence may be due to: (1) chromosomal aberrations that can produce various 'length polymorphisms' sharing a basic nucleotide sequence, (2) competition for priming sites in the genome that can lead to poor amplification of less common complementary sequences during PCR (Hallden et al. 1996) and/or (3) differing sensitivities between ethidium bromide staining and chemiluminescence (Tinker et al. 1993).

Analysis of blot hybridization signals

Dot blot analysis is free from the problems associated with homoplasy and 'polymorphisms' generated from chromosomal rearrangements which are the main limitations of RAPD and RFLP analyses. The method is highly conservative, if interpretation is based on the occurrence or absence of hybridization. For example, only 1 of the 55 random probes was species-specific (658-bp probe generated by OPA17 from *A. virgunculae*).

A 830-bp probe generated by OPA11 from *A. cepa* (Table 1) hybridized only with the DNA of other species classified under sect. *Cepa*. Such random probes could indicate gene coding sequences that determine traits that characterize the members of the section (Brown 1995).

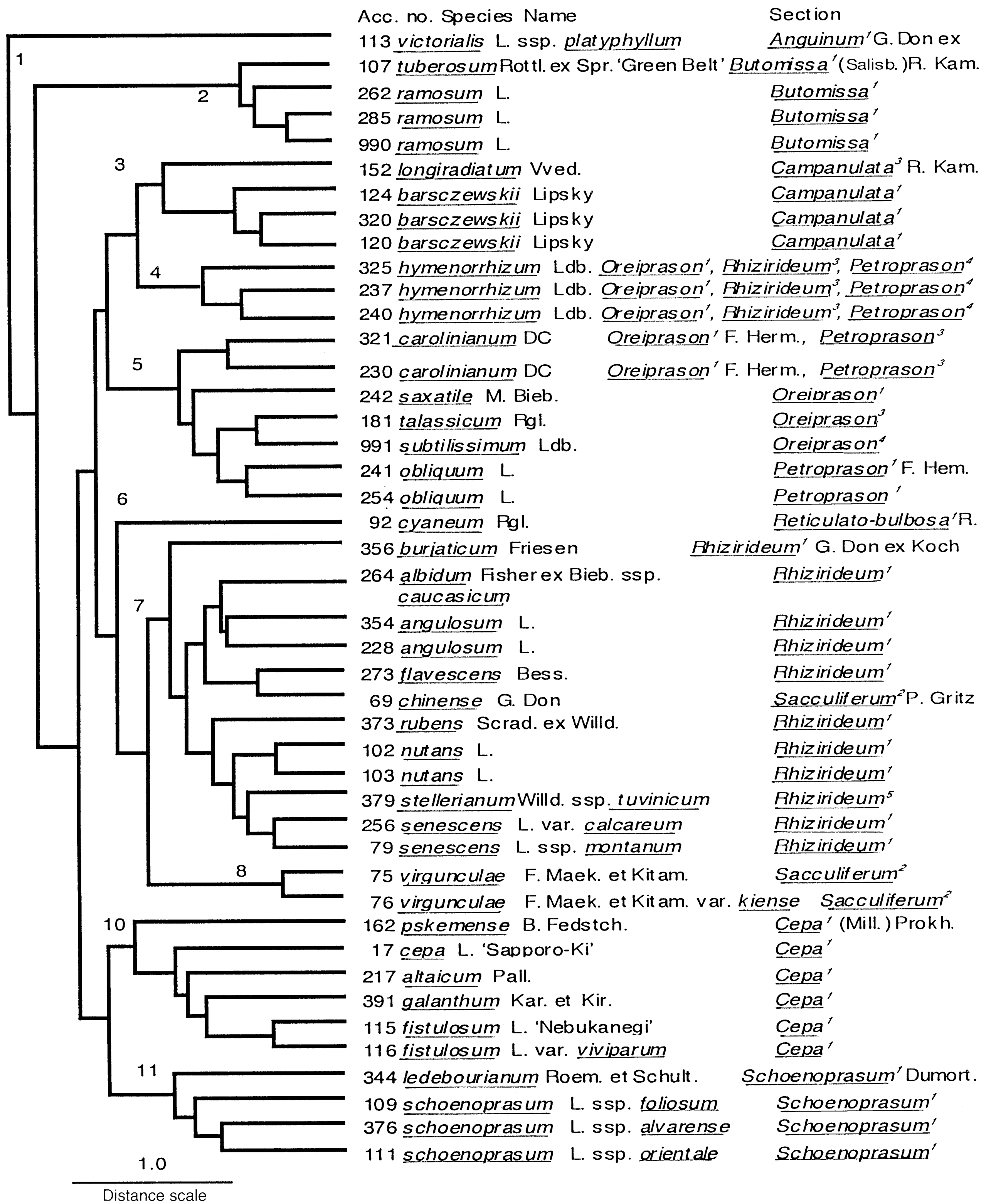

Fig. 1 Phylogeny of subgenus *Rhizirideum* according to Unweighted Pair Group Method Analysis of Euclidean taxonomic distances based on the hybridization of genomic DNA with randomly amplified DNA probes. *Superscript numbers* after section names correspond to the following references: [1] Fritsch 1992, [2] Hanelt and Fritsch 1993, [3] Khassanov 1992, [4] Sancir 1992

Table 1 Probes generated by 11 random decamers from 11 *Allium* species

Acc. no.	Species	A01[a]	A03	A05	A08	A11	A12	A13	A16	A17	A18	A19
320	*A. barsczewskii* Lipsky		994	745					988		884	
17	*A. cepa* L. 'Sapporo-Ki'			1156		830		1004	1098		1116	992
69	*A. chinense* G. Don		1097		752 1165	910		905				
92	*A. cyaneum* Rgl.		983			994					710	1098
115	*A. fistulosum* L. 'Nebukanegi'	1190[b]	1014				975	732				958
254	*A. obliquum* L.		996		635 932	779		911	2035			
242	*A. saxatile*		1054	884	936							842
111	*A. schoenoprasum* L. ssp. *orientale*	1400	839	964								794
94	*A. senescens* L. ssp. *montanum*		830		747	780	842	831	874	1101		
107	*A. tuberosum* Rottl. ex Spreng.		735		422		1022		662	941		
113	*A. victorialis* ssp. *platyphyllum* Hult.				860			942 1092	1010	658		

[a] Primer sequences (5′-3′) as follow: A01 – CAGGCCCTTC, A03 – AGTCAGCCAC, A05 – AGGGGTCTTG, A08 – GTGACGTAGG, A11 – CAATCGCCGT, A12 – TCGGCGATAG, A13 – CAGCACCCAC, A16 – AGCCAGCGAA, A17 – GACCGCTTGT, A18 – AGGTGACCGT, A19 – CAAACGTCGG
[b] Numbers in each cell correspond to the length (in base pairs) of the purified band after reamplification

The majority of the probes hybridized with a probe-dependent set of species classified under several sections. This implies that most of the randomly amplified DNA sequences in this study were complementary to relatively well-conserved regions in the basic genomic complement of this subgenus. In general, the intensity of the hybridization reaction was directly proportional to the degree of taxonomic similarity among the accessions. Hence, analytical procedures for quantitative data were followed because the variation in hybridization signals was continuous, rather than binomial. Standardization of the hybridization signals based on the species mean and standard deviation minimized the effects of variation in the amount of DNA blotted onto the membrane (Randerson 1993).

Phylogeny of subgenus Rhizirideum based on dot blot hybridization

The validity of the analytical procedures described in the preceding sections is shown by the phylogenetic tree in Fig. 1. Hanelt et al. (1992) noted that the members of sections *Anguinum* and *Butomissa* were isolated from the rest of the subgenus based on morphological and serological traits. For example, among the species shown in Fig. 1, only *A. victorialis* ssp. *platyphyllum* has uniovulate locules while members of the sect. *Butomissa* have more than two ovules/locule; the rest of the species in the subgenus have biovulate locules (Hanelt 1992). Both sections (clusters 1 and 2) are also clearly separated from the rest of the subgenus in Fig. 1.

Fritsch (1992) reported that sections *Campanulata*, *Oreiprason*, *Petroprason* and *Reticulato-Bullbosa* form a peculiar group within the subgenus based on the general similarities of their excretory canals. This similarity is also reflected in the grouping of the species in Fig. 1 (clusters 3–6). The species in clusters 3 and 4 have similar leaf growth sequences (Kruse 1992). Members of sect. *Campanulata* are clearly grouped in cluster 3, whereas cluster 4 consists of *A. hymenorrhizum* accessions. Authorities disagree on the classification of *A. hymenorrhizum* (see notes in Fig. 1), but its current placement in cluster 4 partially supports the opinion of the Gatersleben group.

Hanelt et al. (1992) noted that the related sections *Oreiprason* and *Petroprason* have similar morphology, adaptation and distribution. These two sections are grouped in cluster 5 in Fig. 1. Although Khassanov (1992) classified *A. carolinianum* under sect. *Petroprason*, the position of the two *A. carolianianum* accessions in Fig. 1 indicates that they are more similar to sect. *Oreiprason* than to sect. *Petroprason*. This is another validation of the infrageneric classification proposed by the Gatersleben group.

Species classified under sect. *Rhizirideum* are grouped around cluster 7. Hanelt (1990) recommended further subdivision of this huge assemblage of species. The distinct subclusters under cluster 7 also affirm the need to propose new sections. However, *A. chinense* seems to be the major irregularity in this cluster.

Hanelt and Fritsch (1994) amended sect. *Sacculiferum* to include *A. thunbergii*, *A. chinense* and *A. virgunculae* based on similarities in morphology and intercrossability using *in vitro* techniques. Crossability as a decisive parameter in classification may not be entirely valid in this case since *A. chinense* has been successfully crossed with *A. ampeloprasum*, *A. cepa* and

A. fistulosum using similar techniques (Nomura et al. 1994). According to Fig. 1, *A. chinense* is more similar to the other members of sect. *Rhizirideum* and quite distantly related to *A. virgunculae* and *A. virgunculae* var *kiiense*. *A. chinense*, along with many members of sect. *Rhizirideum*, is widely distributed in continental northeast Asia, whereas *A. virgunculae* is native only to southern Japan (Davies 1992).

Members of the sections *Schoenoprasum* and *Cepa* have fistular leaves and scapes, but sect. *Schoenoprasum* is mesophilic while members of sect. *Cepa* have xerophytic characteristics (Hanelt et al. 1992). Despite this difference in adaptive characters, the basic similarity between these two groups was confirmed by dot blot analysis with randomly amplified DNA probes, as shown by their positions in Fig. 1 (clusters 10 and 11). Vosa (1976) also reported that members of sections *Cepa* and *Schoenoprasum* have heterochromatic regions that are distally located, insensitive to cold, and show reduced fluorescence when stained with Quinacrine. However, members of sect. *Schoenoprasum* (cluster 11) are clearly separated from members of sect. *Cepa* (cluster 10), in accordance with previous reports (El-Gadi and Elkington 1977; Hanelt et al. 1992).

Sect. *Cepa* is the most important group in this subgenus so it has generated much attention and conflicting reports from a host of researchers. Vvedenskii (1944) used morphological characters to group *A. cepa*, *A. galanthum* and *A. pskemense* under sect. *Cepa* and assign *A. fistulosum* and *A. altaicum* into sect. *Phyllodolon*. The main criterion used to differentiate these two sections was the presence or absence of small nectaries at the base of the ovaries (Vosa 1976).

Hanelt (1990) united the sections *Cepa* and *Phyllodolon* into a single sect. *Cepa* and grouped the member species in three alliances according to morphology and geographical distribution. Havey (1992) could not find any supporting evidence from RFLP analysis of chloroplast (cp) DNA for these alliances. The Gatersleben group subsequently proposed the subdivision of sect. *Cepa* into subsect. *Cepa* (*A. cepa* and *A. galanthum*) and *Phyllodolon* (*A. altaicum* and *A. fistulosum*) based on morphological, geographical, cytological, anatomical, serological and numerical methods (Hanelt et al. 1992). Bradeen and Havey (1995) reported that nuclear RFLP analysis supported the triple alliance concept. Recently, the Gatersleben group suggested the re-establishment of sect. *Phyllodolon* based on cpDNA RFLP data (Linne von Berg et al. 1996).

However, the differences detected by the randomly amplified DNA probes used in this study were not large enough to justify the re-establishment of sect. *Phyllodolon* (Fig. 1). El-Gadi and Elkington (1977) evaluated 49 morphological and cytological characters and 38 volatile chemical characters by cluster and principal component analyses, and they also did not find any justification for the placement of *A. fistulosum* in a separate section (*Phyllodolon*). This is also borne out by inter-crossability among the species assigned to sect. *Cepa* (Hanelt 1990).

Dot blot analysis using randomly amplified DNA probes did not reveal enough basis for the subdivision of sect. *Cepa* into subsections *Cepa* and *Phyllodolon*. Data obtained from chloroplast (Havey 1992) and nuclear (Bradeen and Havey 1995) RFLPs also do not support the creation of these subsections.

The preceding discussion shows that randomly amplified DNA probes, in conjunction with dot blot analysis, are useful as genetic markers for the phylogenetic analysis of a variable group of species. Regarding the classification of subgenus *Rhizirideum*, the results from dot blot hybridization of genomic DNA with randomly amplified DNA probes generally concur with the infrageneric grouping proposed by the Gatersleben group. However, the status of sections *Sacculiferum* and *Phyllodolon* needs further study.

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