Biodiversity assessment based on cpDNA and crossability analysis in selected species of Allium subgenus Rhizirideum

Article *in* Theoretical and Applied Genetics · November 2003

DOI: 10.1007/s00122-003-1335-8 · Source: PubMed

5 authors, including:

Leo van Raamsdonk
Wageningen University & Research
162 PUBLICATIONS **2,304** CITATIONS

SEE PROFILE

Adriaan W van Heusden
Wageningen University & Research
129 PUBLICATIONS **4,638** CITATIONS

SEE PROFILE

Theor Appl Genet (2003) 107:1048–1058
DOI 10.1007/s00122-003-1335-8

L. W. D. van Raamsdonk · W. Ensink · A. W. van Heusden · M. Vrielink-van Ginkel · C. Kik

Biodiversity assessment based on cpDNA and crossability analysis in selected species of *Allium* subgenus *Rhizirideum*

Received: 20 August 2002 / Accepted: 19 April 2003 / Published online: 25 June 2003

Abstract The chloroplast DNA diversity of 33 accessions belonging to 16 species of five sections in *Allium* subgenus *Rhizirideum* was studied by analysing the sequence of three fragments: the trnL-F intergenic spacer, the rps 16 intron and rbcL (rubisco large subunit). The three sections *Cepa, Schoenoprasum* and *Rhizirideum*, representing the majority of the included species, each possess a separate clade after phylogenetic analysis. Exceptions to this general rule are the placement of *Allium pskemense* (section *Cepa*) connected to *Allium senescens* (section *Rhizirideum*) and *Alium roylei*, taking an intermediate position between sections *Cepa* and *Schoenoprasum*. Both species were located in their own section after nuclear DNA analysis. A range of crossing experiments has been carried out. The different position of *A. roylei* when comparing cpDNA and nDNA diversity was not confirmed with the production of hybrid seeds after crossing *A. roylei* with species other than those of section *Cepa*. The different position of *A. pskemense* in the cpDNA and the nDNA tree can not be compared to its crossability, since only a few crossing experiments are reported for this species. The hypothesis that a shorter distance between two species in a cpDNA tree compared to their distance in a nDNA tree will indicate interfertility at a certain level, is neither confirmed nor rejected by the currently available results.

Keywords *Allium* subgenus *Rhizirideum* · *Allium cepa* · *Allium roylei* · Sequence analysis · trnL · rps16 · rbcL · Onion · Phylogeny reconstruction · cpDNA

Communicated by R. Hagemann

L. W. D. van Raamsdonk (✉) · W. Ensink · A. W. van Heusden · M. Vrielink-van Ginkel · C. Kik
Plant Research International, Business Unit Genetics and Breeding, P.O. Box 16, 6700 AA Wageningen, The Netherlands
e-mail: Leo.vanraamsdonk@wur.nl

L. W. D. van Raamsdonk
RIKILT Institute for Food Safety, P.O. Box 230, 6700 AE Wageningen, The Netherlands

Introduction

The genus *Allium* comprises approximately 700 species, arranged in four large subgenera with 100–300 species each (*Allium, Rhizirideum, Amerallium* and *Melanocrommyum*) and two much smaller subgenera (*Bromatorrhiza* and *Caloscordon*; Hanelt et al. 1992). The main cultivated species are found in the subgenera *Allium* (garlic, leek) and *Rhizirideum* (onion, shallot, bunching onion, chives, chinese chives), whereas a range of ornamentals originates from subgenus *Melanocrommyum*. Most research has been focused on the improvement of these cultivated species. Especially onion (*Allium cepa*), as the most important cultigen in the genus, received much attention with respect to the relationship with wild species (El-Gadi and Elkington 1977; Hanelt 1990; van Raamsdonk and de Vries 1992a) and to crossability (Saini and Davis 1967; McCollum 1971, 1974; Gonzalez and Ford-Lloyd 1987; van der Meer and de Vries 1990; van Raamsdonk et al. 1992; Keller et al. 1996). Diversity studies based on DNA variation have been carried out to study the position of *A. cepa* (Havey 1992; Wilkie et al. 1993; Bradeen and Havey 1995; Linne von Berg et al. 1996; Dubouzet et al. 1997; Mes et al. 1997, 1999; van Raamsdonk et al. 1997, 2000; Fritsch et al. 2001; Lilly and Havey 2001). Although several studies of selected sets of species of *Allium* subgenus *Rhizirideum* were based on chloroplast (cp) DNA, detailed sequence analyses of specified cpDNA fragments, or a comparison of cpDNA and nDNA based phylogenies of exactly the same set of species were rarely published (Friesen et al. 1999, 2000; Mes et al. 1999; Fritsch et al. 2001).

Chloroplast DNA is in general maternally inherited, as is the case in *Allium* (Havey 1995), and its lack of recombination makes it suitable for the study of hybridisation and introgression (Rieseberg and Soltis 1991; Rieseberg 1995). The importance of cpDNA for studying the aforementioned phenomena has recently been emphasized by Hollingworth et al. (1999). In *Allium* subgenus *Rhizirideum* the inheritance of nuclear (n) DNA variation in hybrids has been studied by means of

the Hybrid Distance approach (van Raamsdonk et al. 2000). A cpDNA study of the same set of species will give much more insight about events of hybrid speciation, will yield information on the crossability relationship to be expected and provides the opportunity to compare phylogenies based on biparentally and maternally inherited DNA.

In the present study, sequences of four cpDNA fragments will be used for studying the diversity in cpDNA subjected to cladistic analysis. The study includes equal amounts of accessions of the sections *Cepa, Rhizirideum* and *Schoenoprasum* in order to reach a balanced share in the resulting phylogeny. The use of more than one accession for most species of sections *Rhizirideum* and *Schoenoprasum* allows us to compare the infraspecific variation with the interspecific variation. Some species of section *Oreiprason* (three species), of section *Petroprason* (one) and of section *Reticulatobulbosa* (one) were added for comparison. *Allium tuberosum* will be used as an outgroup. The aim of the present study is: (1) to analyse the cpDNA diversity and unravel the maternally based relationships in *Allium* subgenus *Rhizirideum*, (2) to study the occurrence of introgression events and of hybrid speciation, and (3) to analyse whether a shorter distance between two species in a cpDNA tree compared to their distance in a nDNA tree will indicate interfertility.

Materials and methods

Plant material

The species and accessions included in the analysis are listed in the Appendix.

Vouchers are deposited in either the herbaria of Wageningen University (WAG), the Royal Botanic Gardens in Kew (K), the Institute für Pflanzengenetik und Kulturpflanzenforschung in Gatersleben (GAT), and the Plant Research international (formerly the Institute for Horticultural Plant Breeding, Wageningen, WAHO). All accessions labeled as "BG" originate from an originally wild population.

DNA-isolation

Genomic DNA was isolated from leaf material with a midi DNA preparation procedure (Beek et al. 1992) with some minor modifications: after hooking the DNA out of the isopropanol mixture, the DNA was washed overnight in 70% EtOH and 100 mM NH_4Ac, and resuspended in 200 μl or more-sterile TE (10 mM Tris-HCl pH = 8.0 and 1 mM EDTA). DNA concentrations were measured using a fluorometer. DNA could also be isolated with the mini prep DNA-isolation method: approximately 0.25 g of fresh leaf material was collected in Eppendorf tubes, frozen and ground in liquid nitrogen, and stored at –50 °C; 750 μl of DNA-isolation buffer (IB) with $Na_2S_2O_5$ (3.8 g/l) was added to the leaf material. This mixture was incubated for 60 min at 65 °C with occasional inverting of the tubes [IB = lysis buffer:extraction buffer:sarkosyl (5% w/v) = 2.5:2.5:1; lysis buffer = 0.2 M Tris-HCl pH = 7.5, 0.05 M EDTA, 2 M NaCl, 2% w/v CTAB; extraction buffer = 0.35 M Sorbitol, 0.1 M Tris HCl pH = 7.5, 5 mM EDTA]. The DNA was further purified by adding 750 μl of chloroform/isoamylalcohol (24:1), inverting the tubes (10–20 times) and centrifuging for 5 min at 14,000 rpm. After transfer of the supernatant to a new tube the DNA was precipitated by the addition of 400-μl isopropanol (–20 °C). The DNA could either be hooked out or it had to be pelleted for 5 min at 14,000 rpm. The DNA samples were washed once with 70% ethanol and re-suspended in 100 μl of TE.

Table 1 Primers used for the amplification of three cpDNA fragments

rps16-R2:	5′-TCGRGATCGAACATCAATTGCAAC
rps16-F:	5′-GTGGTAGAAAGCAACGTGCGACTT
rps16-1A:	5′-GGGGGGGCGAATTTAGGG
rbcL-2:	5′-GATATCTTGGCAAGCATTCCGAG
rbcL-4:	5′-CGATTAGCTRCTGCACCAGGYGC
trnL (UAA) 5′ exon:	5′-CGAAATCGGTAGACGCTACG
trnL (UAA) 3′ exon:	5′-GGGGATAGAGGACTTGAAC
trnL-F-e:	5′-GGTTCAAGTCCCTCTATCCC
trnL-F-f:	5′-ATTTGAACTGGTGACACGAG

cpDNA sequencing

Four sequences of the cpDNA genome were amplified by specific primers: the trnL-F intergenic spacer and the trnL exon (Taberlet et al. 1991), the rps 16 intron (Oxelman et al. 1997) and the rbcL (rubisco large subunit). Four rbcL primers were designed from three *Allium* sequences available in the EMBL databases. The best results were obtained with primers 2 and 4, amplifying a sequence of approximately 1,100 nucleotides. Primers are listed in Table 1.

The PCR reaction mixture (total volume 25 μl) contained 25 ng of template DNA, 25 ng of the primers (Table 1), 10 mM of each dNTP, 0.1 μl (0.5 units) of super*Taq* polymerase, 50 mM of KCl, 10 mM of Tris-HCl, 1.5 mM of $MgCl_2$ and 0.01% gelatine (pH = 8.3). The mixture was covered by two drops of mineral oil. PCR was performed with a thermal cycler (Perkin Elmer) as follows: 35 cycles at 94 °C for 1 min (denaturation), annealing for 2 min, from 35 °C to 72 °C with a ramp of 1 °C/5 s, 72 °C for 2 min (extension); and one final cycle at 72 °C for 10 min. Optimal annealing temperatures were 60 °C for the trnL-F intergenic spacer, 55 °C for the trnL exon, 50 °C for the rps 16 intron and 60 °C for the rbcL sequence. Electrophoresis was performed through 1.5% 1× TBE agarose gels, for 6 to 8 h at 4 V/cm, stained with Ethidium bromide. Small fragments of the gel containing the DNA fragment were cut out and the DNA was extracted from the gel using the Qiaex protocol (QIAGEN corporation). The DNA amount was measured and 150–400 ng of DNA, depending on the size of the fragment, was used for the sequencing reaction. The fragment was amplified with a sequence mix, containing fluorescence-labelled nucleotides, and one of the primers in a PCR reaction of 25 cycles. The amplified fragment was sequenced in an ABI sequencer.

Scoring of data

All sequences were aligned manually. The resulting raw-data matrix consisted of 369 nucleotide positions for the trnL-F intergenic spacer, 339 for the trnL exon, 861 for the rps 16 intron (between primers R2 and 1A) and 1,128 for the rbcL (between primers 2 and 4). All differences were scored as 0, 1, 2 etc. depending on the number of states per mutation site. A mutation could either consist of a single nucleotide (single-site mutation) or a deletion or duplication of a stretch of nucleotides (indel). The direction of all mutations was estimated on the basis of the situation found in the outgroup (*A. tuberosum*).

Data analysis

All analyses are based on a matrix with digits (0, 1, 2, etc.) indicating independent states of each character per accession. All

Table 2 Sequences for 33 accessions of *Allium* subgenus *Rhizirideum* of the trnL-F intergenic spacer. Mutations are indicated by capitals (nucleotide changes) or by lower-case letters (duplications or deletions) in the bottom rows of the table. Letters in the sequences are given according to the IUB codes: A: adenosine, C: cytidine, G: guanosine, T: thymidine, R: A or G, Y: C or T, K: G or T, M: A or C, and *n*: any base

```
                1         10        20        30        40        50        60        70        80        90       100
Jumbo           CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
Stuttgarter     CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
Polar           CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
95081 vav       CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
206 osch        CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
103 gal         CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
207 pske        CAKTAnAAARSCCnYTTTACTTCTTTACTATAATTCTCCTTTTTTTTTTATAATTATAAGTGGTTCAAARAAAA      TGGTTCAAARAAAA     T
102 fist        CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T
201 alta        nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn nnnnnnnnnnTAAGT      GGTTCAAAGAAAA          CAAAGAAAA          T

104 royl        CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCCTTTTTTTTTTATAAKT      GGTTCAAARAAAA          CAAARAAAA          T

All 911 schoe   CAGTAAAAAGCCCGTTTTACTTCTTTAMTATAATTCTCCTTTTTTTTTTATAATT      GGTTCAAARAAAA          CAAATAAAA          T
93007 schoe     CAGTAAAAASCCGKnTTnAnTTCTTTACTATAATTCTCCTTTTTTTTTTATAATn      GGTTCAAAAAAAA          CAAATAAAA          T
Tax 1605 scho   CAGTAAAAAGCCCGTTTTACCTCTTTAAnATAATTCTCCTTTTTTTTTTATAAKT      GGTTCAAARAAAA          CAAATAAAA          T
Tax 42 altync   CAGTAAAAAGCCCGTTTTATTTTTTTAnTATAATTCTCCTTTTTTTTTTATAAGT      GGTTCAAAGAAAA          CAAATAAAA          T
93008 altync    CAnTAAAAAnCCCGCCTTAnnTCTTTACTATAATTCTCCnTTTTTTTTTTATAAGn      GGTTCAAAGnAAA          CAAATAAAA          T
Tax 536 karel   CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCCTTTTTTTTTTATAATT      GGTTCAAAAAAAA          CAAATAAAA          T

All 1122 sen    CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCCTTTTTTTTTTATAAKT      GGTTCAAARAAAA      TGGTTCAAARAAAA         T
89010 sen       CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCCTTTTTTTTTTATAAKT      GGTTCAAAAAAAA      TGGTTCAAAAAAAA         T
93005 sen       CAGTAAAAGSCCGCTTTTACCTCTTTACTATAATTCGCYYTnCTTTTTTATAAGT      GGTTCAAAGAAAA      TGGTTCAAAGAAAA         T
89008 nut       CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTC  TTTTTTTTTTATAAGT      GGTTCAAAGAAAA      TGGTTCAAAnAAAA         T
89009 nut       CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTTATAAGT      GGTTCAAAnAAAA      TGGTTCAAAGAAAA         T
89011 nut       CAGTAAAAAGCCCGTTTTACTTCTWTACnACAATTCTCC TTTTTTTTTTATAAGT      GGTTCAAAGAAAA      TGGTTCAAAGAAAA         T
93012 nut       CAGTAAAAASCCGCCTTnAnTTCTTTACTATAATTCTC  TTTTTTTTTTATAAGn      GGTTCAAAGnAAA      TGGTTCAAAGAAAA         T
Tax 256 ang     CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTTATAAGT      GGTTCAAAGAAAA      TGGTTCAAAGAAAA         T

Tax 2335 lin    CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTTATAAGT      GGTTCAAAnAAAA      TGGTTCAAAnAAAA         T

All 1123 glob   CAGTAAAAAGCCCGTTTTACTTCTTTACTATAATTCTCC TTTTTTTTTTATAAGT      GGTTCnAAnAAAACnATAnGTGGTTCAAAnAAAACCTnnAACAT
Tax 632 saxa    CAGTAAAAAGCCCGTTTTACnTCTTTACTATAATTCTCC TTTTTTTTTTATAAGT      GGTTCAAAGAAAACAATAAGTGGTTCAAAGAAAACAAAGAAAAT
Tax 750 hymen   CAGTAAAAAGCCCGTTTTACTTCTnTACGATAATTC  CACTTTTTTTTTATAAGT      GGTTCAAAGAAAA           CAAAGAAAA          T

Tax 94 obliq    CAGTAAAAAGCCCGTTTTACTTCTnTACTATCATnCTCC TTTTTTTTTATAAGT      GGTTCAAAGAAAACAATAAGTGGTTCAAAGAAAACAAAGAAAAT

86001 tub       CAGTAAAAAGCCCGTTTTACTTCTTTACTATA TTCTCC  TCTTTTTTATAAGn      GGTTCAAAGAAAA                          T
89006 tub       CAGTAAAAAGCCCGTTTTACTTCTTTACTAnA TTCTCC  TCTTTTTTATAAGT      GGTTCAAAGAAAA                          T
89007 tub       CAGTAAAAAGCCCGTTTTACTTCTTTACTATA TTCTCC  TCTTTTTTATAAGT      GGTTCAAAGAAAA                          T
Tax 1830 tub    CAGTAAAAAGCCCGTTTTACTTCTTTACTATA TTCTCC  TCTTTTTTATAAGT      GGTTCAAAGAAAA                          T

                                          A  B          C
                                             a      bc             d                 e      f    g        h
```

characters were extracted from the original data matrix with nucleotide positions. The phylogenetic reconstruction was carried out using the PAUP software package 3.1 (Swofford 1993). Data were treated as unordered and equally weighted. Consensus trees were based on all most-parsimonious trees of equal length after a heuristic search of trees using the TBR and MULPARS options. Bootstrap analysis has been carried out with 500 replicates.

Hybridisation experiments

Flowers of the female parents were emasculated by hand prior to anthesis. Mature pollen was transferred from anthers of the pollen donor to the stigma of the female parent by means of a paint brush. All pollination was carried out in insect-free glasshouses. Ripe F1 seeds were harvested during summer and sown at late autumn. Seedlings were raised during winter in frost-free glasshouses. All presumed hybrids were checked on their hybrid background by using RAPDs.

Results

cpDNA diversity

Single nucleotide mutations or duplications/deletions were found in three of the four sequences investigated.

The trnL-F intergenic spacer showed 15 single nucleotide mutations, and 12 duplications and deletions of stretches longer than one nucleotide (Table 2). Only mutations that appeared to be unambiguous after checking the original sequence data are indicated and used for phylogenetic analysis. This spacer between the trnL intron and the 3′ end of the trnF gene includes 369 nucleotide positions in *Allium* subgenus *Rhizirideum*. All species except *A. tuberosum* share a duplication of nine nucleotides at position 87 in Table 2 (CAAAnAAAA). The representatives of sections *Cepa* and *Schoenoprasum* except for *Allium pskemense* share the absence of any further duplication in that region, whereas e.g. the species of section *Rhizirideum* share a further duplication of five nucleotides (TGGTT) in front of the first-mentioned duplication. It is unlikely that these two duplications arose independently, since independent duplications would imply that the five nucleotides TGGTT were shifted nine positions to the right after the duplication of the nine nucleotides. It is more likely to assume a duplication of 14 nucleotides in section *Rhizirideum* and at least one other duplication of nine nucleotides in the sections *Cepa* and *Schoenoprasum*. In this explanation it is not possible to

Table 2 (continued)

```
                    110        120        130        140        150        160        170        180        190        200
Jumbo               TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
Stuttgarter         TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAARAAAGTCTTC
Polar               TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
95081 vav           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
206 osch            TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGCGCATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
103 gal             TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
207 pske            TCAATATCTTTCTTATTCATTTTACTATTTCACAAATAGACCCAAAGTGAATTATT    CAYAGCCCATCTCATTTTCCTTACATTCACAAARAAAGYCTTC
102 fist            TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGCGCATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
201 alta            TCAATATCTTTCTTATnCATTTTACTCTTTCACAAATAGACCCAAAGCGCATTATT    CACAGCCCATCTCATTTTCCTTAnWTTCACAAAGAAAGTCTTC

104 royl            TCAATATCTTTCTTATnCATTTTACTCTTTCACAAATAAACCCAAAKTTAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAARAAAGTCTTC

All 911 schoe       TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAATTCTTC
93007 schoe         TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAAACCCAAAnnGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAARAAnnCTTC
Tax 1605 scho       TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATARACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAATTCTTC
Tax 42 altync       nCAATATCTTTCTTATTCATTTTACTCTTTCACAAATARACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAATTCTTC
93008 altync        TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGnGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGnATTC
Tax 536 karel       TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAAACCCAAATTGAATTATT    CACAnCCCATnTCnTTTTCCTTACnTTCnCAAARAAATTCTnC

All 1122 sen        TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATARACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAARAAATTCTTC
89010 sen           TCAATATCTTnCTTATnCATTTnACnCTTTCACAAATAAACCCAAAKTnAATTATT    CACAGCCCATCTCATTTnCCTTACnTTCACAAARAAATTCTnC
93005 sen           TCAATATCTTTCTTATTCATTTTRCTCTTTCRCAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCRCAAAGAAAGTCTTY
89008 nut           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
89009 nut           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
89011 nut           TCAATATCTTTCTTATTCATTTTACTCTTTCACCAATAGACCCAAAGTGAATAATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
93012 nut           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
Tax 256 ang         TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC

Tax 2335 lin        TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC

All 1123 glob       TnnATnTCTTTCTTATTCATACTACTCTTnCTCAAATnnACCCCAAnTGAATAAnTnTTCnYnSCCCATCTCATTTTCCTTnCnTnCACAAAGAAAGTCTTC
Tax 632 saxa        TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAAnAATTATTCACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
Tax 750 hymen       TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAATTATT    CACAGYCCATCTCATTTTACTTACATTCACAAAGAAAGTCTTC

Tax 94 obliq        TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATAGACCCAAAGTGAAGAATTATTCACAGCCCATCTCATTTTCCTTACATTCACAAAnAATGTCTTC

86001 tub           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATGGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
89006 tub           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATGGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
89007 tub           TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATGGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC
Tax 1830 tub        TCAATATCTTTCTTATTCATTTTACTCTTTCACAAATGGACCCAAAGTGAATTATT    CACAGCCCATCTCATTTTCCTTACATTCACAAAGAAAGTCTTC

                                                  D          EFG                                  H                  I
                                                                 i
```

prove whether the duplication in section *Cepa* is the same as found in section *Schoenoprasum* or not. There is at least one difference between these sections at the fifth nucleotide of the nine-nucleotide duplication (position 91 in Table 2: G or T, respectively). Duplications at position 75 and at 162–164 show synapomorphisms between species of section *Oreiprason* (*Allium globosum* and *Allium saxatile*) and a species of section *Petroprason* (*Allium obliquum*). A duplication of 25 nucleotides is found in the sequences of *A. cepa* and of *Allium vavilovii* at position 249–273 (Table 2).

The intron of the rps16 gene sequenced with the R2 primer showed good results, but the reverse amplification with the F primer stopped at a region with nine G nucleotides. A new primer (20-mer) was designed covering the G region, which successfully amplified the remaining part of the rps16 intron sequence. This part, 175 nucleotides shorter than the part of the intron enclosed by R2 and F, showed seven single-site mutations and 15 duplications or deletions (sequences not shown). A duplication of 43 nucleotides is found in the sequences of *A. cepa* and of *A. vavilovii*, which is supported by the duplication of 25 nucleotides in the sequences of the trnL-F intergenic spacer of the same species. Agarose-gel electrophoresis showed that the intron of the rps16 gene is present in a large and a small copy, whereas *Allium fistulosum* and *Allium altaicum* show two copies of shorter size (Fig. 1). Sequence analysis showed that the main but smaller copy in these two species is 160 nucleotides shorter than the main copy in the other species. The short copy of *Allium schoenoprasum* accession 96152 possesses the same deletion of 160 nucleo-

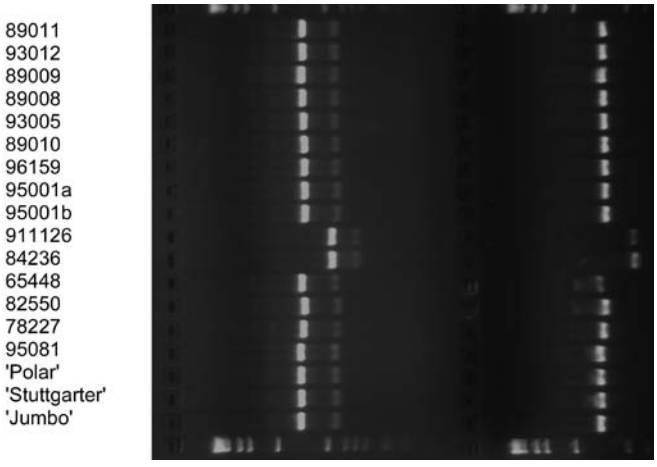

Fig. 1 Results of amplification of the rps16 intron for a range of species. *Left*: amplification at 55 °C, *right*: amplification at 60 °C. Note the shorter fragment in *A. fistulosum* and *A. altaicum*

Table 2 (continued)

```
                  210        220        230        240        250        260        270        280        290        300
Jumbo             TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGTAAAAATTAGGGAATAGCTCAGTTGTGTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
Stuttgarter       TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGTAAAAATTAGGGAATAGCTCAGTTGTGTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
Polar             TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGTAAAAATTAGGGAATAGCTCAGTTGTGTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
95081 vav         TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGTAAAAATTAGGGAATAGCTCAGTTGTGTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
206 osch          TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGT                                GTAnAGCAGAGGCTTGAAAATCCTTGTGTCACCG
103 gal           TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGT                                GTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
207 pske          TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTARAGCARAGGCTTGAAAATCCTTGTGTCACCG
102 fist          TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGT                                GTAnAGCAGAGGCTTGAAAATCCTTGTGTCACCG
201 alta          TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAnTTGT                                GTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG

104 royl          TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAKTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTGTCACCG

All 911 schoe     TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAKTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTGTCACCG
93007 schoe       TTTTTGAAAATCGGA  AAAATTAGGGAATAnCTCAnTTnT                                nnAAAnCAAAGGCTTGAAAATCCTTGTnnCACCG
Tax 1605 scho     TTTTTGAAAATCGGA  AAAATTAGGGAATACCTCAKTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTTTCACCG
Tax 42 altync     TTTTTGAAAATCGGA  AAAATTAGGGAATACCTCAKTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTTTCACCG
93008 altync      TTTTTGAAAATCGGA  AAAATTAGGGAATAGCTCAGTTGT                                GTAnAGCAnAGGCTTGAAAATCCTTGTGTCACCG
Tax 536 karel     TTTTTnAAAnTCGGA  AAAATTAGGGAATACCTCATTTnT                                TTAAAnCAAAGGCTTnAAAATCCTTnTTTCnCCG

All 1122 sen      TTTTTnAAAATCGAA  AAAATTAGGGAATACCTCAKTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTGTCACCG
89010 sen         TTTTTnAAAATCGAA  AAAATTAGGGAATACCTCATTTnT                                TTAAAnCAAAGGCTTnAAAATCCTTnTTTCACCG
93005 sen         TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAGAGCAnAGGCTTGAAAATCCTTGTGTCACCG
89008 nut         TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTGTCACCG
89009 nut         TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTnAAnCAAAGGCTTGAAAATCCTTGTGTCACCG
89011 nut         TTTTTGAAAATCGAA  AAAATTAGGGAATATCTCAGTTGT                                GTAAAGCAGAGGCTTGAAAATCCTTGTGTCTCCG
93012 nut         TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAnAGCAnAGGCTTGAAAATCCTTGTGTCACCG
Tax 256 ang       TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAnAGCAAAGGCTTGAAAATCCTTGTGTCACCG

Tax 2335 lin      TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAAAGCAAAGGCTTGAAAATCCTTGTGTCACCG

All 1123 glob     TTTTTGAAAATCGAA  TnTATTAGGAAAnRnCTCATTTGT                                GTnnnnnnnnnnnTCGAAnnTCCTCnnnnnnnnn
Tax 632 saxa      TTTTTGAAAATCGAA  AAAATTAGGAAATAnCTCAGTTGT                                GTAAAGCAnAnACTTGAAAATCCTCGTGTCnCCG
Tax 750 hymen     TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAAAGCAGAGGCTTGAAAATCCTTGTGTCACCG

Tax 94 obliq      TTTTTGAAAATCAAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAnAnCAnAGGCTTGAAAATCCTCGTGTCACCG

86001 tub         TTTTTGAAAATCGAA  AAAATTAGGGAATAGCTCAGTTGT                                GTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
89006 tub         TTTTTGAATGT  AAGGAAAATTAGGGAATAGCTCAGTTGT                                GTAGAnCAGAGGCTTGAAAATCCTTGTGTCACCG
89007 tub         TTTTTGAATGT  AAGGAAAATTAGGGAATAGCTCAGTTGT                                GTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG
Tax 1830 tub      TTTTTGAATGT  AAGGAAAATTAGGGAATAGCTCAGTTGT                                GTAGAGCAGAGGCTTGAAAATCCTTGTGTCACCG

                        J   KL   M
                           j   k                          l
```

tides. A duplication of seven nucleotides is erratically found in a range of different species. This set of seven nucleotides is included at one end of the already mentioned duplication of 43 nucleotides in *A. cepa* and *A. vavilovii*. The seven-nucleotide duplication is found at five locations in the tree of Fig. 2 (character 31), which indicates that it apparently does not influence the structure of the tree. It either might have arisen simultaneously in several events or it can be the remnant of a basic, larger duplication, that vanished in most species for its larger part during evolution. The large subunit of the rubisco gene (rbcL) is very conservative, and at the level of sections and species hardly any mutations were found. Four single-site mutations were recovered. The trnL exon with a size of 339 base pairs did not show any polymorphisms between the accessions included in the dataset.

Phylogeny

The phylogenetic analysis of the dataset including 53 mutations for 33 accessions resulted in 52 most-parsimonious trees of 78 steps with a consistency index (CI) = 0.805 ($CI_{uninformative}$ = 0.754) and a retention index (RI) = 0.915. One of the most-parsimonious trees is shown in Fig. 2. Section *Cepa* is basically divided in three clades. One consists of *Allium galanthum*, a second one of *A. cepa* and *A. vavilovii*, the last clade combines *A. fistulosum*/*A. altaicum* with *Allium oschaninii*. The species *Allium pskemense* of section *Cepa* is connected to the branch of one of the accessions *Allium senescens* (section *Rhizirideum*), but it shares some characters with certain branches of section *Cepa*, e.g. character 31 and 32b (Fig. 2). *A. obliquum* shares two duplications of three and seven nucleotides respectively in the trnL-F intergenic spacer with *A. globosum* and *A. saxatile*. A duplication of seven nucleotides in the rps16 intron is shared by *A. saxatile* and *Allium hymenorrhizum*. The 80% majority rule consensus tree based on the 52 most-parsimonious trees is shown in Fig. 3. The polytomies are caused by the synapomorphisms as indicated by the tree in Fig. 2. Sections *Cepa* and *Schoenoprasum* are closely connected, together with *Allium roylei*. The species of section *Rhizirideum* do not share a synapomorphism unambiguously, although some synapomorphisms are indicated in the individual tree of Fig. 2. Sections *Petroprason* and *Oreiprason* are not clearly separated. Bootstrap analysis

Table 2 (continued)

```
                  310        320        330        340        350        360        370
Jumbo             GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC
Stuttgarter       GTTCAAATTTGGnTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAnCAGAGGACTGAAAATC
Polar             GTTCAAATTTGGnTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAGAGGACTGAAAATC
95081 vav         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC
206 osch          GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAnTTGGTAGAGCAGAGGACTGAAAATC
103 gal           GTTCAAATTTGACTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC
207 pske          GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTARAGCAGAGGACTGAAAATC
102 fist          GTTCAAATTTGGGTCCTGAATTGTTGGGATAGCTCAGTTGGnAGAGCAGAGGACTGAAAATC
201b alta         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC

104 royl          GTTCAAATTnGGCTCCTGAATTGTTGGGATAGCTCAKTTGGTAAAGCAAAGGACTGAAAATC

All 911 schoe     GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAKTTGGTAAAGCAAAGGACTGAAAATC
93007 schoe       GTTCAAATTTGGCTCCTGAATTGTTGGGATAnCTCAnTTGGTAAnnnnnnnnnnnnnnnnnnn
Tax 1605 scho     GTTCAAATTTGGCTCCTGAATTnTTGGnATASCTCATTTGGTAAASCAAAGGACTGAAAATC
Tax 42 altync     GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAKTTGGTAAAGCAAAGGACTGAAAATC
93008 altync      GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAnAGGACTGAAAATC
Tax 536 karel     GTTCAAATTTGGCTCCTGAATTGTTGGGATASCTCAnTTGGTAAAnCAAAGGACTGAAAATC

All 1122 sen      GTTCAAATTTGGCTCCTGAATTGTTGGGATACCTCATTTGGTAAAnCAAAGGACTGAAAnTC
89010 sen         GTTCAAATTTGGCTCCTnAATTnTTGGGATAnCTCATTTGGTAAAnCAAAGGACTGAAAATC
93005 sen         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAnAGGACTGAAAATC
89008 nut         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAAAGGACTGAAAATC
89009 nut         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAnTTGGTAnAGCAAAGGACTGAAAATC
89011 nut         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAnAGGACTGAAAATC
93012 nut         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCnnnnnnnnnnnnnnnn
Tax 256 ang       GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAnAGGACTGAAAATC

Tax 2335 lin      GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAAAGGACTGAAAATC

All 1123 glob     nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnn
Tax 632 saxa      GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAnCAAAGGnnTGAAAATC
Tax 750 hymen     GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAnAGCAnAGGACTGAAAATC

Tax 94 obliq      GTTCAAATTTGGGTCCTGAATTGTTGGGATAGCTCAGTTGGnAnAGCAnAGGAnTGAAAATC

86001 tub         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC
89006 tub         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC
89007 tub         GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAnCAnAGGACTGAAAATC
Tax 1830 tub      GTTCAAATTTGGCTCCTGAATTGTTGGGATAGCTCAGTTGGTAGAGCAGAGGACTGAAAATC

                                  N                                   O
```

showed weak support to some clades as indicated in Fig. 3. Although the separate position of *A. hymenorrhizum*, the clade with *Allium pskemense*/*A. senescens* and the position of *A. galanthum* at the root of the Cepa clade are weakly supported, these positions are all supported by more than 80% of the most-parsimonious trees. The final, conclusive tree is exclusively based on branches supported after bootstrap analysis (Fig. 4b).

Crossability analysis

Twenty one crosses are carried out in 16 combinations between eight species. The results are listed in Table 3. No hybrid seeds have been obtained in the current crossability program, which might be caused by the fact that almost all crosses involve combinations of species belonging to different sections.

Table 3 Results of crossing experiments between species of *Allium* subgenus *Rhizirideum*

Parents	Flowers	Seeds
Crossing Experiments in 1997		
roylei × *senescens* All 1122	10	0
roylei × *altyncolicum* Tax 42	13	0
roylei × *karelinii* Tax 536	7	0
roylei × *tuberosum* 86001	18	0
roylei × *obliquum* Tax 94	16	0
roylei × *schoenoprasum* Tax 1605	16	0
tuberosum 86001 × *karelinii* Tax 536	18	0
tuberosum 86001 × *roylei*	15	0
tuberosum 86001 self poll.	40	43
Crossing experiments in 1998		
cepa 96211 × *senescens* All 1122	120	0
cepa 96211 × *schoenoprasum* Tax 1605	140	0
cepa 96211 × *tuberosum* 86001	250	0
roylei 95001 × *altyncolicum* Tax 42	53	0
roylei 95001 × *schoenoprasum* Tax 1605	46	0
roylei 95001 × *tuberosum* 86001	51	0
roylei 95001 self poll.	120	99
senescens All 1122 × *cepa* 96030	60	0
senescens All 1122 × *roylei* 95001	73	0
senescens All 1122 × *tuberosum* 86001	78	0
altyncolicum Tax 42 × *senescens* All 1122	37	0
tuberosum 86001 × *senescens* All 1122	41	0
tuberosum 86001 × *schoenoprasum* Tax 1605	35	0
tuberosum 86001 × *altyncolicum* Tax 42	67	0

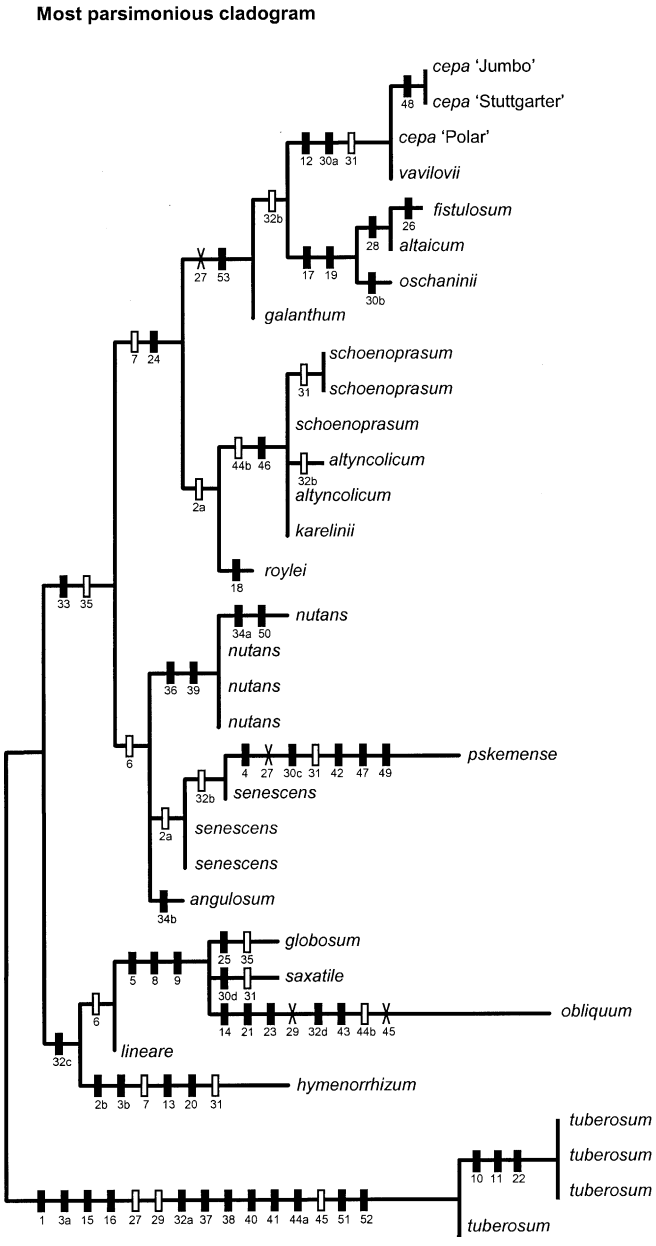

Fig. 2 One of the 52 most-parsimonious trees of 78 steps-long of representatives of *Allium* subgenus *Rhizirideum* (CI = 0.808; CI uninformative = 0.756; RI = 0.911). The *indices* of the bars indicate the position of the mutation: 1–12: deletions/duplications in the trnL-F intergenic spacer; 13–27: single-site mutations in the trnL-F intergenic spacer; 28–42: deletions/duplications in the rps16 intron; 43–49: single-site mutations in the rps16 intron; 50–53: single-site mutations in the rubisco large subunit

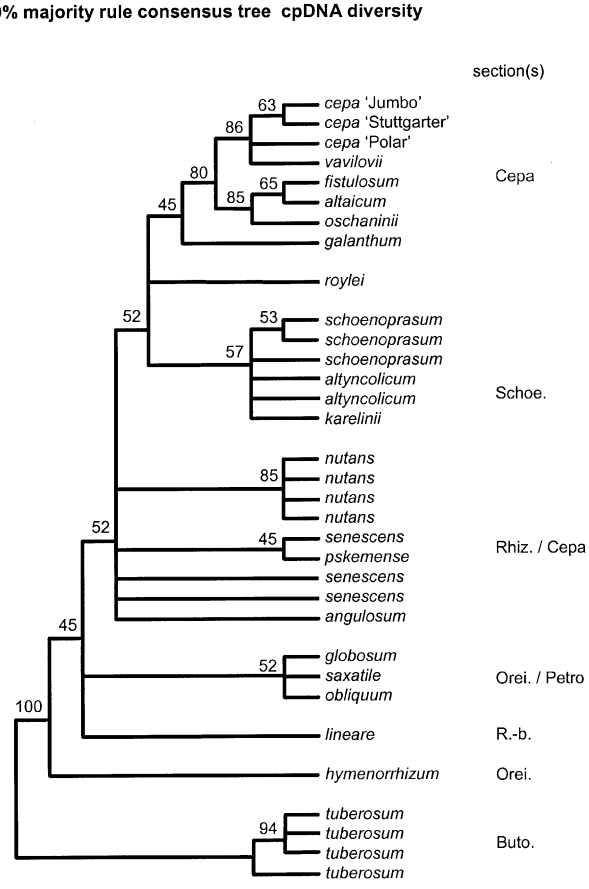

Fig. 3 Eighty percent majority rule consensus tree of 52 most-parsimonious trees showing the evolutionary position of 33 accessions of *Allium* subgenus *Rhizirideum*. Bootstraps values are indicated at the branches. Section names are abbreviated: *Cepa*: section *Cepa*; *Rhiz*: section *Rhizirideum*; *Schoe*: section *Schoenoprasum*; *Orei*: section *Oreiprason*; *Petro*: section *Petroprason*; *R-b*: section *Reticulato-bulbosa*; *Buto*: section *Butomissa*

Discussion

Phylogeny

The division of the included species in clades is generally in agreement with the accepted taxonomic division based on morphology, cytogenetics or chemical compounds (El-Gadi and Elkington 1977; Hanelt 1990; Hanelt et al. 1992; van Raamsdonk and de Vries 1992a; Hanelt and Fritsch 1994). The combination of species of sections *Oreiprason* and *Petroprason* in one cluster is also found by Dubouzet et al. (1997). This position is supported by the close resemblance of the species of these two sections in morphology and geographical distribution (Hanelt et al. 1992).

All species included with more than one accession (i.e. belonging to sections *Schoenoprasum* and *Rhizirideum*) appear to be monophyletic; or else due to a lack of discriminating characters monophyly was neither confirmed nor rejected (*A. cepa*/*A. vavilovii* and *A. schoenoprasum*/*Allium altyncolicum*). The same conclusion has been reached for all wild species of section *Cepa*, but cultigens were intermixed with their immediate ancestor (van Raamsdonk et al. 1997). Monophyly of species was also found after nDNA analysis except for one accession of *A. senescens* that was placed in one clade with the only accession of *Allium angulosum* (van Raamsdonk et al. 2000). These results agree with the results of Dubouzet et

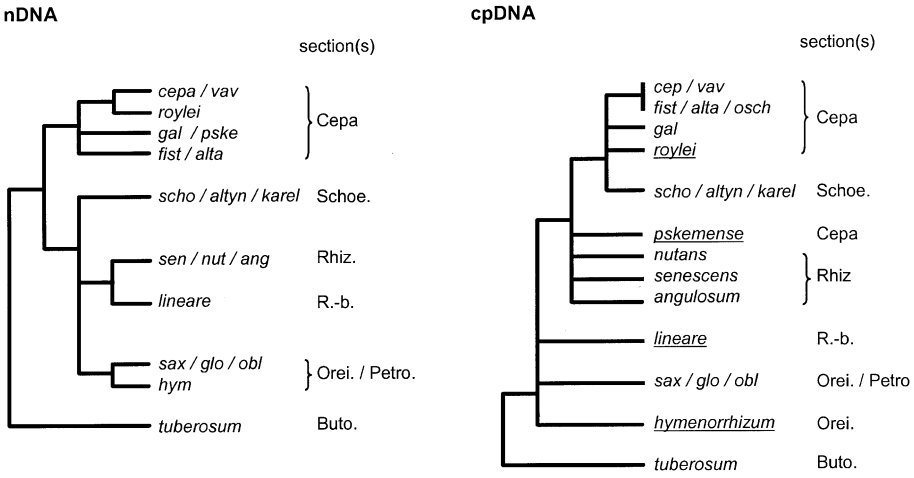

Fig. 4 Comparison of the nDNA phylogeny (van Raamsdonk et al. 2000) with the cpDNA phylogeny (current results) of a set of species of *Allium* subgenus *Rhizirideum*

al. (1997) where all accessions per species were placed in the same cluster.

The three alliances in section *Cepa* as proposed by Hanelt (1990) cannot completely be recognised in the current results (Figs. 2 and 3). The three groups, i.e. *A. fistulosum* and *A. altaicum, A. galanthum* and *A. pskemense*, and *A. cepa, A. vavilovii* and *A. oschaninii*, do arise after morphological analysis (van Raamsdonk and de Vries 1992a), and after analysis of nDNA variation (Bradeen and Havey 1995; van Raamsdonk et al. 2000). The species with a deviating position are *A. oschaninii* and *A. pskemense*. The position of *A. oschaninii* close to *A. fistulosum* and *A. altaicum* is in concordance with chromosome C-banding patterns (de Vries and Jongerius 1988), but disagrees with other data (van Raamsdonk 1992b). A distant position of *A. oschaninii* was also found after cpDNA analysis (Havey 1992; Lilly and Havey 2001). The current results support the main division between the clade of *A. cepa*/*A. vavilovii* and *A. fistulosum*/*A. altaicum* ("subsection Phyllodolon"). The distinction of the *A. galanthum*/*A. pskemense* alliance is not supported by the consensus tree since *A. pskemense* possess an *A. senescens*-like cpDNA type with some modifications and one notable reversal that is shared with other members of section *Cepa* (Fig. 2).

Each of the four main subgenera of the genus *Allium* posses a separate cluster after phenetic analysis of cpDNA diversity in the study of Linne von Berg et al. (1996). Species of sections *Cepa* and *Schoenoprasum* are located in the same cluster in their results, whereas the two species included in their study belonging to section *Rhizirideum* were placed at a larger distance. This seems to be in concordance with the present results, but different data sets can not be reliably compared by using different approaches, i.e. phylogenetic and phenetic analyses.

A phylogenetic analysis of section *Cepa*, including *A. roylei*, was carried out by Havey (1992). *A. oschaninii* and *A. pskemense* were placed at the root of the clade of section *Cepa*. Nothing can be said about the relationship between *A. pskemense* and any representative of section *Rhizirideum*, since none of the latter were included in his analysis.

It is most important for a proper comparison of differently based phylogenies that the same set of material is used in the datasets to be compared (e.g. Sanderson and Donoghue 1989). Such a comparison was carried out using the cpDNA dataset of Havey (1992) and the RAPD-nDNA dataset of van Raamsdonk et al. (1997) for the species exclusively belonging to section *Cepa*. There appeared to be a striking correlation between the cpDNA tree (Havey 1992) and the crossability dendrogram (van Raamsdonk et al. 1992), but differences in the level of crossability should be correlated with differences in species position between nDNA and cpDNA phylogenies, because of the different modes of inheritance of these types of DNA (van Raamsdonk et al. 1997). In order to extend a view on the crossability of *A. cepa* and *A. roylei* beyond the border of section *Cepa*, it is important to have different phylogenies available based on the same set of material.

Comparison of nDNA and cpDNA phylogenies and the analysis of introgression events

The set of species used in the current cpDNA study is identical to the set of species as used in the nDNA study of van Raamsdonk et al. (2000) except for *A. oschaninii*, due to the fact that most probably the pre-amplification of the nDNA of *A. oschaninii* did not work out well. As in the nDNA consensus tree (van Raamsdonk et al. 2000), sections *Cepa, Schoenoprasum* and *Rhizirideum* each possess a separate position in the tree (Figs. 3 and 4). The clade with *A. globosum, A. saxatile* and *A. obliquum*, combining species of sections *Oreiprason* and *Petroprason*, is also recognisable in both trees (Fig. 4). The relationships at the level of sections is not congruent between the nDNA and cpDNA-based phylogenies. For instance, the relatively close relationship between the sections *Cepa* and *Schoenoprasum* after cpDNA analysis (current results: Fig. 3) was not found after nDNA

analysis (van Raamsdonk et al. 2000). The relative distances between the different sections in the nDNA tree were almost equal to each other, as was shown in the dendrogram after phenetic analysis (van Raamsdonk et al. 2000: Fig. 4).

The main differences between the cpDNA and the nDNA-based phylogenies (Fig. 4) are the remote positions of *A. roylei, A. pskemense, A. lineare* and *A. hymenorrhizum*. It is clear that *A. pskemense* possesses the *A. senescens*-like cpDNA, notwithstanding the fact that *A. pskemense* shares two mutations with accessions of the section *Cepa*. Considering the situation that the cpDNA genome is not recombined during gametogenesis, some sort of chloroplast-capture occurred during the evolution of *A. pskemense*, followed by a re-establishment of a nDNA composition that fits with those of the species of section *Cepa*. The same conclusion might be applied to the situation of *A. roylei* at the root of section *Schoenoprasum*, since no sharing of mutations is found with representatives of the clade of section *Cepa* (Fig. 2). The differences between section *Cepa* and section *Schoenoprasum*, together with *A. roylei*, however, are too weak to be supported by bootstrap analysis (Fig. 3). The same situation might explain the position of *A. lineare* and *A. hymenorrhizum*, but their position in the final cpDNA tree is not supported after bootstrap analysis (Fig. 4b). The relatively separated position of *A. hymenorrhizum* found after cpDNA as well as nDNA analysis, is comparable to the results of Dubouzet et al. (1997).

Several other analyses are based on cpDNA as well as nDNA. These studies exclude most species of section *Cepa* (Mes et al. 1999; Friesen et al. 2000) or are exclusively based on species of section *Cepa* without an outgroup from outside the section (Friesen et al. 1999). A correlation between haplotypes and geographic distribution has been found in several species of *Allium*, indicating either parallel adaptation or gene flow. Incongruence between datasets could not be tested for the structure of the ITS dataset (Mes et al. 1999). The nDNA and the cpDNA phylogenetic trees in the study of Friesen et al. (2000) differ only for the position of *Allium kingdonii*. Hybridisation as well as sample errors are presumed as a cause (Friesen et al. 2000).

Crossability and the prediction of interfertility

The currently presented crossability study is the first one that covers a range of species belonging to different sections of the subgenus *Rhizirideum* without using artificial pollination methods. Earlier studies focused primarily on the combination of *A. cepa* with other species. Hybrid plants with *A. galanthum, A. fistulosum, A. vavilovii* and *A. roylei* were obtained, whereas crosses of *A. cepa* with *A. pskemense, A. oschaninii, A. angulosum, A. senescens* and *A. schoenoprasum* failed (Saini and Davis 1967; McCollum 1971, 1974; Gonzales and Ford-Lloyd 1987; van Raamsdonk et al. 1992). Hybrids between *A. cepa* and a range of species are being produced by means of embryo-rescue techniques (Keller et al. 1996). All the currently known results are summarised in Fig. 5.

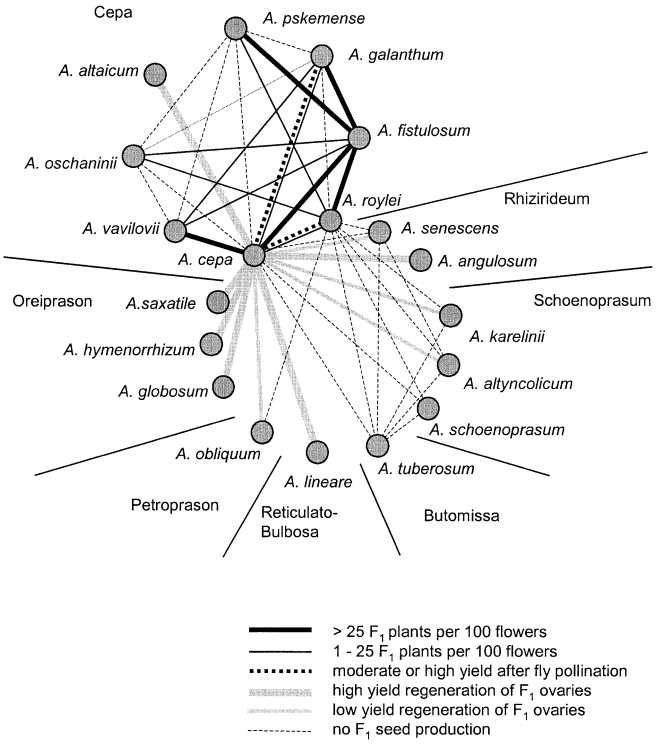

Fig. 5 Overview of the currently known crossability results between species of sections *Cepa, Rhizirideum, Schoenoprasum, Oreiprason, Petroprason, Reticulato-bulbosa* and *Butomissa*

The hypothesis is proposed that a shorter distance between two species in a cpDNA tree compared to their distance in a nDNA tree will predict inter-fertility at a certain level. On the other hand, no conclusion can be drawn from a larger distance in the cpDNA tree (van Raamsdonk et al. 1997). This hypothesis is based on the situation that chloroplast capture, i.e. a short distance in a cpDNA tree, is the result of a certain level of effective inter-fertility. Introgression between species as basic mechanism can result in replacement of the cytoplasmic DNA of the recipient parent, whereas only a small portion of donor nDNA will finally be included in the recipient gene pool. The differences between the effect of introgression of nuclear and cytoplasmic-types of DNA are due to the absence of recombination in the latter (van Raamsdonk et al. 1997; compare Rieseberg and Brunsfeld 1992). The present comparison of nDNA- and cpDNA-based phylogenies with the same set of species and accessions allows a detailed analysis of the differences between bi-parentally and maternally inherited genomes, and can be used for the prediction of inter-fertility. Validation of the hypothesis depends on the availability of crossing results. The position of *A. roylei* (nDNA: section *Cepa*) close to section *Schoenoprasum* in the cpDNA phylogeny should indicate a certain level of crossability with representatives of this section, but the currently

available crossing results neither support not reject this assumption (Fig. 5). Band sharing of the nDNA profiles of *A. roylei* with those of other species revealed a larger percentage of bands shared with representatives of section *Rhizirideum* than of section *Schoenoprasum* (van Raamsdonk et al. 2000). Most probably a considerable secondary evolution after the putative hybridisation and introgression event took place in *A. roylei*. Therefore, the effect of parallel evolution can not completely be ruled out. *A. roylei* is close to an *A. cepa* accession with the S-type cytoplasm (Lilly and Havey 2001). This CMS onion might have been originated after introduction of an alien cytoplasm from a *A. roylei*-like plant relating to some representative of section *Schoenoprasum*. The position of *A. pskemense* close to *A. senescens* in the cpDNA tree might also predict some interfertility between this species and *A. senescens*. However, this prediction can not be approved by any crossability result of *A. pskemense* with a representative of section *Rhizirideum* (Fig. 5). Further crossability results are necessary in order to find supportive evidence for the hypothesis that a shorter distance between two species in a cpDNA tree compared to their distance in a nDNA tree might indicate a certain level of inter-fertility.

Acknowledgements We thank our late colleague Dr. J.H. Sandbrink from Plant Research International for critically reading this manuscript and Dr. W. Koopman for assisting in the analyses. Dr. J. Keller (IPK, Gatersleben, Germany) kindly provided a range of different accessions from the Gatersleben collection. This research was supported by the EC-FAIR Programme (FAIR CT95-465) and by the Dutch Ministry of Agriculture, Nature Management and Fisheries.

Appendix: list of plant material used

Name	Source and origin	CPRO ref. no.
Section Cepa		
A. cepa 'Jumbo'		
'Stuttgarter'		
'Polar'		96147
A. vavilovii	Grown from 83010: H.B.Chorog, wild origin	95081
A. oschaninii	H. B. Budapest	78227
A. galanthum	Grown from 79147: USDA Beltsville	82550
A. pskemense	H. B. Alma-Ata	65448
A. fistulosum	Grown from 76201: H. B. Odessa	84236
A. altaicum		911126
A. roylei	Grown from 79150: USDA Beltsville C 502 originally from India	95001
Section Schoenoprasum		
A. schoenoprasum	IPK Gatersleben s.n.	93007
	IPK: All 911	96158
	IPK: Tax 1605	96152
A. altyncolicum	IPK: Tax 42	96150
	IPK Gatersleben s.n.	93008
A. karelinii	IPK: Tax 536	96154
Section Rhizirideum		
A. senescens	All Union Res. Inst. Veg. USSR	89010
	IPK Gatersleben s.n.	93005
	IPK: All 1122	96159
A. nutans	All Union Res. Inst. Veg. USSR	89008
	All Union Res. Inst. Veg. USSR	89009
	All Union Res. Inst. Veg. USSR	89011
	IPK Gatersleben s.n.	93012
A. angulosum	IPK: Tax 256	96149
Section Oreiprason		
A. globosum	IPK: All 1123	96151
A. saxatile	IPK: Tax 632	96157
A. hymenorrhizum	IPK: Tax 750	96153
Section Petroprason		
A. obliquum	IPK: Tax 94	96156
Section Reticulato-bulbosa		
A. lineare	IPK: Tax 2335	96155
Section Butomissa		
A. tuberosum	Origin: Thailand	
	All Union Res. Inst. Veg. USSR	89006
	All Union Res. Inst. Veg. USSR	89007
	IPK: Tax 1830	96148

References

Beek JG van der, Verkerk R, Zabel P, Lindhout P (1992) Mapping strategy for resistance genes in tomato based on RFLPs between cultivars: Cf9 (resistance to *Cladosporum fulvum*) on chromosome 1. Theor Appl Genet 84:106–112

Bradeen JM, Havey MJ (1995) Randomly amplified polymorphic DNA in bulb onion and its use to assess inbred integrity. J Am Soc Hort Sci 120:752–758

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

El-Gadi A, Elkington TT (1977) Numerical taxonomic studies on species in *Allium* subgenus *Rhizirideum*. NewsPhytol 79:183–201

Friesen N, Pollner S, Bachmann K, Blattner FR (1999) RAPDs and noncoding chloroplast DNA reveal a single origin of the cultivated *Allium fistulosum* from *A. altaicum* (Alliaceae). Am J Bot 86:554–562

Friesen N, Fritsch R, Pollner S, Blattner FR (2000) Molecular and morphological evidence for an origin of the aberrant genusd *Milula* within Himalayan species of *Allium* (Alliaceae). Mol Phylog Evol 17:209–218

Fritsch R, Matin F, Klaas M (2001) *Allium vavilovii* M. Popov et Vved. and a new Iranian species are the closest among the known relatives of the common onion *A. cepa* L. (Alliaceae). Genet Res Crop Evol 48:401–408

Gonzalez LG, Ford-Lloyd BV (1987) Facilitation of wide-crossing through embryo rescue and pollen storage in interspecific hybridization of cultivated *Allium* species. Plant Breed 98:318–322

Hanelt P (1990) Taxonomy, evolution and history. In: Rabinowitch HD, Brewster JL (eds) Onion and allied crops, volume I. Botany, physiology and genetics. CRC press, Boca Raton, Florida, pp 1–26

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, Maass HI, Ohle H, Pistrick K (1992) Infrageneric grouping of *Allium* – the Gatersleben approach. In: Hanelt P, Hammer K, Knüpffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, pp 107–123

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

Havey MJ (1995) Cytoplasmatic determinations using the polymerase chain reaction to aid in the extraction of maintainer lines from open-pollinated populations of onion. Theor Appl Genet 90:263–268

Hollingworth ML, Bailey JP, Hollingworth PM, Ferris C (1999) Chloroplast DNA variation and hybridization between invasive populations of Japanese knotweed and giant knotweed (*Fallopia*, Polygonaceae). Bot J Linnean Soc 129:139–154

Keller ERJ, Schubert I, Fuchs J, Meister A (1996) Interspecific crosses of onion with distant *Allium* species and characterization of the presumed hybrids by means of flow cytometry, karyotype analysis and genomic in situ hybridization. Theor Appl Genet 92:417–424

Lilly JW, Havey MJ (2001) Sequence analysis of a chloroplast intergenic spacer for phylogenetic estimates in *Allium* section *Cepa* and a PCR-based polymorphism detecting mixtures of male-fertile and male-sterile cytoplasmic onion. Theor Appl Genet 102:78–82

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

McCollum GD (1971) Sterility of some interspecific *Allium* hybrids. J Am Soc Hort Sci 96:359–362

McCollum GD (1974) Chromosome behavior and sterility of hybrids between common onion, *Allium cepa*, and the related wild *A. oschaninii*. Euphytica 23:699–709

Mes THM, Friesen N, Fritsch RM, Klaas M, Bachmann K (1997) Criteria for sampling in *Allium* based on chloroplast DNA PCR-RFLPs. Syst Bot 22:701–712

Mes THM, Fritsch RM, Pollner S, Bachmann K (1999) Evolution of the chloroplast genome and polymorphic ITS regions in *Allium* subg. *Melanocrommyum*. Genome 42:1–12

Meer QP van der, Vries JN de (1990) An interspecific cross between *Allium roylei* Stearn and *Allium cepa* L., and its backcross to *A. cepa*. Euphytica 47:29–31

Oxelman B, Liden M, Berglund D (1997) Chloroplast rps16 intron phylogeny of the tribe *Sileneae* (Caryophyllaceae). Plant Syst Evol 206:393–410

Raamsdonk LWD van, Vries T de (1992a) Systematics and phylogeny of *Allium cepa* L. and allies. In: Hanelt P, Hammer K, Knüpffer H (eds) The genus *Allium* – taxonomic problems and genetic resources. Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany, pp 265–270

Raamsdonk LWD van, Vries T de (1992b) Biosystematic studies in *Allium* L. section *Cepa*. Bot J Linnean Soc 109:131–143

Raamsdonk LWD van, Wietsma WA, Vries JN de (1992) Crossing experiments in *Allium* L. section *Cepa*. Bot J Linnean Soc 109:293–303

Raamsdonk LWD van, Smiech MP, Sandbrink JM (1997) Introgression explains incongruency between nuclear and chloroplast DNA-based phylogenies in *Allium* section *Cepa*. Bot J Linnean Soc 123:91–108

Raamsdonk LWD van, Vrielink-van Ginkel M, Kik C (2000) Phylogeny reconstruction and hybrid analysis in *Allium* subgenus *Rhizirideum*. Theor Appl Genet 100:1000–1009

Rieseberg LH (1995) The role of hybridization in evolution: old wine in new skins. Am J Bot 82:944–953

Rieseberg LH, Brunsfeld SJ (1992) Molecular evidence and plant introgression. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 151–176

Rieseberg LH, Soltis DE (1991) Phylogenetic consequences of cytoplasmic gene flow in plants. Evol Trends Plants 5:65–84

Saini SS, Davis GN (1967) Compatibility in some *Allium* species. Proc Am Soc Hort Sci 91:401–409

Sanderson MJ, Donoghue MJ (1989) Patterns of variation in levels of homoplasy. Evolution 43:1781–1795

Swofford D (1993) Phylogenetic analysis using parsimony, version 3.1

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17:1105–1109

Vries JN de, Jongerius MC (1988) Interstitial C-bands on the chromosomes of *Allium* species from the section *Cepa*. Proc 4th Eucarpia Allium Symp, Wellesbourne, United Kingdom

Wilkie SE, Isaac PG, Slater RJ (1993) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor Appl Genet 86:497–504