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Julian R. Starr
Université de Montréal, Montreal, Canada

Geneviève Gravel
Université de Montréal, Montreal, Canada

Anne Bruneau
Université de Montréal, Montreal, Canada

A. Muthama Muasya
East African Herbarium, Nairobi, Kenya

PHYLOGENETIC IMPLICATIONS OF A UNIQUE 5.8S nrDNA INSERTION IN CYPERACEAE

Julian R. Starr,[1,3,4] Geneviève Gravel,[1] Anne Bruneau,[1] and A. Muthama Muasya[2]

[1]*Institut de recherche en biologie végétale (IRBV), Université de Montréal, 4101 rue Sherbrooke est, Montréal, Québec H1X 2B2, Canada;* [2]*East African Herbarium, National Museums of Kenya, P.O. Box 45166-0100, Nairobi, Kenya*
[3]*Corresponding author (jstarr@uottawa.ca)*

ABSTRACT

The purpose of this study was to assess the phylogenetic utility of a large insertion (3 bp) in the 5.8S gene of nuclear ribosomal DNA (nrDNA) in Cyperaceae and selected Juncaceae. This was done by reconstructing the character evolution of the insertion on a phylogeny derived from *rbc*L sequences. Results suggest that the insertion was gained once at the base of Cyperaceae followed by multiple losses in its most-derived taxa. Despite several homoplastic losses (CI = 0.20), the pattern of insertion loss (RI = 0.88), and base pair variation within the insertion were useful for defining sedge clades at various taxonomic levels. For example, whereas a loss of the insertion appeared to characterize a major terminal clade within Cyperaceae, both an insertion loss and sequence variation were consistent with infrageneric clades previously discovered in an ITS phylogeny of *Eleocharis*. The presence/absence of the insertion also supported previous conclusions based on morphological and molecular data that tribe Scirpeae and *Scirpus* s.l. are polyphyletic. In the context of our current understanding of Cyperaceae relationships, evolutionary patterns related to this insertion provide additional support for groups defined in prior phylogenetic analyses. The present analysis also suggests that the controversial position of *Oxychloe andina* (Juncaceae) in previous *rbc*L analyses, as sister to Cyperaceae (Y12978) or as nested within Cyperaceae (U49222), is due to the fact that Y12978 is a Juncaceae/Cyperaceae chimera, whereas U49222 is the sequence of a Cyperaceae contaminant. When U49222 is excluded from analyses and the Cyperaceae portion of Y12978 is removed, Juncaceae and Cyperaceae are monophyletic with *Oxychloe* positioned within a Juncaceae clade of single-flowered genera.

Key words: 5.8S insertion, chimeric sequences, Cyperaceae, ITS region, Juncaceae, *Oxychloe*.

INTRODUCTION

Cyperaceae Juss. (sedges) are a large (ca. 5000 spp., 104 genera; Goetghebeur 1998) cosmopolitan family of mostly herbaceous, anemophilous perennials that occur in diverse habitats ranging from rain forests to tundra. The family is well known for a variety of economic uses including paper (*Cyperus papyrus* L.), construction materials (e.g., *Scirpus lacustris* L.), food (e.g., *Eleocharis esculentus* L.), and even medicines (e.g., *Carex arenaria* L.) (Le Cohu 1967; Simpson and Inglis 2001). The ecological importance of the family is also well recognized. Sedges are key species in the development of peat, in the prevention of erosion, and in the shelter and nourishment of wildlife (Le Cohu 1967; Crum 1988; Catling et al. 1990; Fox 1991; Simpson and Inglis 2001). Despite the economical and ecological significance of the family, evolutionary relationships within Cyperaceae are poorly known. This is mainly due to the family's great diversity and reduced morphology, which have made it difficult to establish homologies across the family (Goetghebeur 1986; Bruhl 1995; Muasya et al. 2000). As a result, traditional classifications have often been controversial, with debates between competing systems resting on a different interpretation of the family's anatomy and morphology (even in modern classifications, cf. Bruhl 1995 and Goetghebeur 1998).

Modern systematists have since recognized that morphol-

ogy alone will not provide a natural classification, and important contributions to the systematics of Cyperaceae in anatomy (e.g., Metcalfe 1971; Bruhl 1995), micromorphology (e.g., Schuyler 1971), and embryology (e.g., van der Veken 1965) have significantly improved contemporary family classifications (e.g., Bruhl 1995; Goetghebeur 1998). Nonetheless, many problems remain. Modern classifications differ considerably on the number of subfamilies and tribes that should be recognized (two subfamilies, 17 tribes, Bruhl 1995; four subfamilies, 14 tribes, Goetghebeur 1998), and even though much has been achieved in large, heterogeneous taxa such as *Scirpus* L. s.l. (200–300 spp.; e.g., Raynal 1973; Wilson 1981; Goetghebeur and Simpson 1991; Muasya and Simpson 2002), considerable circumscriptional problems remain at all taxonomic levels.

The modern ''holistic'' approach to Cyperaceae systematics has now begun to integrate molecular characters with other types of data. Within Cyperaceae, nuclear and chloroplast DNA sequence studies have examined relationships at the infratribal (e.g., tribe Cariceae, Yen and Olmstead 2000; Roalson et al. 2001; Starr et al. 2004, in press), and infrageneric (e.g., *Carex* L. and *Uncinia* Pers., Starr et al. 1999, 2003; *Eleocharis* R. Br., Roalson and Friar 2000; *Isolepis* R. Br. and *Cyperus* L., Muasya et al. 2001, 2002) levels. Similarly, *rbc*L sequences from the chloroplast genome have shed light on the ordinal, subordinal, and subfamilial relationships of the family (Chase et al. 1993; Duvall et al. 1993; Muasya et al. 1998, 2000; Simpson et al. 2007). In general, molecular makers have been very useful for evaluating past classifications and for circumscribing dif-

[4] Present address: Department of Biology, University of Ottawa, Gendron Hall, 30 Marie Curie, Ottawa, Ontario K1N 6N5, Canada
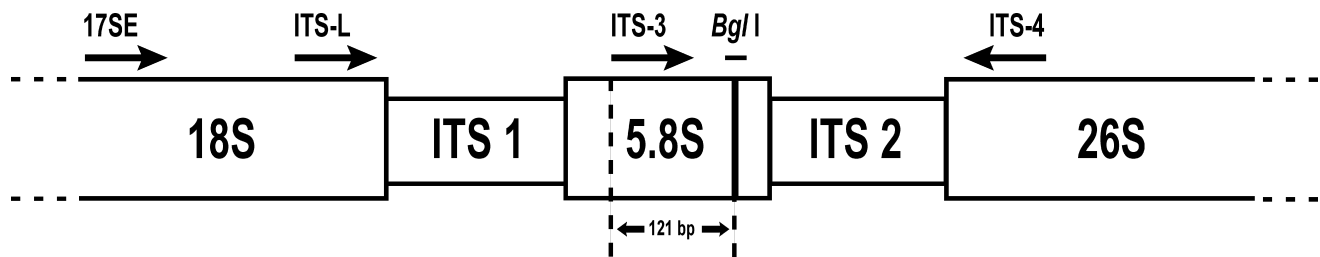
Fig. 1.—ITS nrDNA region in Juncaceae and Cyperaceae illustrating the relative positions of the primers used in PCR and sequencing. The position of the *Bgl* I restriction site is indicated by a vertical bar in the 5.8S gene. When the enzyme cuts an ITS-3/ITS-4amplicon containing the insertion, a 121 bp fragment is produced (see text).

ficult groups (e.g., Simpson et al. 2003). These studies have proposed many new hypotheses of relationship, but their conclusions have largely been uncontroversial when evaluated within the context of other data. One notable exception has been the position of the Juncaceae genus *Oxychloe* Phil. in *rbc*L phylogenies (Chase et al. 1993; Duvall et al. 1993; Plunkett et al. 1995; Muasya et al. 1998, 2000). Despite supporting traditional evidence (e.g., paracytic stomata, post-reductional meiosis, diffuse centromeres, onagrad embryo development) for a close relationship between Juncaceae and Cyperaceae, the unexpected position of *Oxychloe* in *rbc*L trees as either a member of Cyperaceae (Duvall et al. 1993; Plunkett et al. 1995) or as its sister (Muasya et al. 1998, 2000), has fueled doubts over the monophyly of these seemingly well-marked families. *Oxychloe* possesses an indehiscent fruit like Cyperaceae, and it shares a spiro- or ortho-distichous phyllotaxy with the Cyperaceae genus *Oreobolus* R. Br. (Dahlgren et al. 1985; Simpson 1995); however, numerous other characters such as solitary (Cyperaceae) vs. multiple ovules (Juncaceae, including *Oxychloe*), nuclear vs. helobial endosperm, pollen pseudomonads vs. tetrads at release, and the presence vs. absence of silica bodies suggest that *Oxychloe*'s affinities are with Juncaceae (Dahlgren et al. 1985; Simpson 1995).

In this study, we survey the distribution of the largest insertion (3 base pairs [bp]) yet discovered in the highly conserved 5.8S nrDNA gene for an angiosperm family (Starr et al. 1999), and we examine its phylogenetic significance relative to a Cyperaceae phylogeny reconstructed from *rbc*L sequences. Many studies have suggested that such unusual insertion/deletion (indel) events are strong indicators of monophyly (Lloyd and Calder 1991) since the mutational processes associated with these characters are less frequent (Saitou and Ueda 1994; Ophir and Graur 1997) and more complex than those associated with single base pair substitutions (Graham et al. 2000; de Jong et al. 2003). When such rare genomic changes are located in highly conserved regions of the genome they may provide important characters for resolving higher-level relationships. For example, in cases where sequence data have been equivocal, indels have provided strong evidence that liverworts represent the earliest branch of land plants (Qiu et al. 1998), that the prochlorophytes are the common ancestors of chloroplasts (Morden and Golden 1989), or that the eutherian tree may be rooted by a Xenarthra + Afrotheria clade (de Jong et al. 2003).

In the original study where this insertion was discovered, Starr et al. (1999) suggested on the basis of 5.8S sequences

from *Carex* and *Kobresia* Willd., that the insertion might be useful for delimiting the family, tribe Cariceae, or a clade within the tribe itself. Sequences from subsequent studies have shown, however, that the insertion is common to all Cariceae (Roalson et al. 2001; Starr et al. 2003, 2004), but not to all genera of Cyperaceae (Roalson and Friar 2000; Roalson et al. 2001). The objectives of this study were (1) to understand the evolution of the 5.8S insertion by sequence comparison, secondary reconstructions, and character analysis relative to an *rbc*L phylogeny, (2) to determine whether the insertion could serve as a useful taxonomic marker for resolving the limits of Cyperaceae and/or its infrafamilial groups, and (3) to clarify the phylogenetic position of the controversial genus *Oxychloe*.

MATERIALS AND METHODS

Taxon Sampling and Outgroup Choice

All *rbc*L sequences used in this analysis were downloaded from GenBank. Taxa were chosen to represent as closely as possible the sampling previously used in the *rbc*L Cyperaceae analysis of Muasya et al. (1998, 2000; Table 1). In order to broaden our taxonomic sampling, the presence/absence of the insertion was determined for all Juncaceae and Cyperaceae 5.8S sequences currently available on GenBank. In addition, several other Juncaceae taxa for which *rbc*L sequences were not available were also assayed for the presence/absence of the insertion. Phylogenetic analyses included both *rbc*L sequences for the controversial Juncaceae genus *Oxychloe* (i.e., Y12978 and U49222; see below). *Prionium serratum* was used as the outgroup in all analyses based on its sister position to a Juncaceae/Cyperaceae clade in previous molecular studies (e.g., Duvall et al. 1993; Plunkett et al. 1995; Muasya et al. 1998).

DNA Extraction, Amplification, Sequencing, and Restriction Enzyme Digests

Total DNA extractions, polymerase chain reaction (PCR), and sequencing protocols for the ITS region were performed as in Starr et al. (1999, 2003). PCR amplifications of the complete ITS region (3′-18S–5′-26S fragment) using primers 17SE (Sun et al. 1994) and ITS-4 (White et al. 1990; Fig. 1) were initially performed for ten taxonomically diverse taxa (Table 1) to assess ITS (ITS-1, 5.8S gene, ITS-2) sequence variation within Cyperaceae/Juncaceae, and to determine whether a compensatory insertion might have occurred in the 5′-26S sequence that is complementary to 3′-

3'-end of 5.8S rDNA

```
                     140                150    Bgl I      160
                      .                  .                 .

                                        GCCNNNNNGGC

 1.  Uncinia uncinata        AGGGCACGCCTGCCTCACGGGCGTTAGAA  ┃
 2.  Hypolytrum nemorum      . . . . . . . . . . . . . . TAT . . . . . . . . . . .  ┃
 3.  Oreobolus kuekenthalii  . . . . . . . A . . . . . . CAT . . . . . . . . . . .  ┃
 4.  Eleocharis dulcis       . . . . . . . . . . . . . . CTT . . . . . . . . . . .  ┃  Cyperaceae
 5.  Eleocharis interstincta . . . . . . . . . . . . . . CTC . . . . . . . . . . .  ┃
 6.  Eleocharis marginulata  . . . . . . . . . . . . . . - - - . . . . . . . . . .  ┃
 7.  Cyperus papyrus         . . . . . . . . . . . . . . - - - . . . . . . . . . .  ┃
 8.  Juncus dregeanus        . T . . . . . . . . . . . . . - - - . . . . . C . TTT  ▌ Juncaceae
 9.  Oryza sativa            . . . . . . . . . . . . . . . - - - . . . . . C . CGC
10.  Cucumis melo            . . . . . . . . T . . . . . . - - - . . . . . C . CGC
11.  Vicia faba              . . . . . . . . T . . . . . . - - - . . . T . . C . C . T
12.  Pinus contorta          . . . . . . . . T . . . T . . - - - . . . . . CGC . T
13.  Osmunda regalis         . . . . . . T . . . . . . . . - - - . A . . . . CCT - C
14.  Mnium rugicum           . . . . . . TTT . C . TTA - - - . A . . . . C . C - C
15.  Chlamydomonas reinhardtii . . A . . . T . T . . . . . . - - - CA . . . . CG . GT
16.  Sclerotinia sclerotiorum  G . . . . T . . . . TTC - - - . A . . . . C . TTT
17.  Phytophthora megasperma   G . A . T . T . . . . TA . - - - CA . T . . CC . T .
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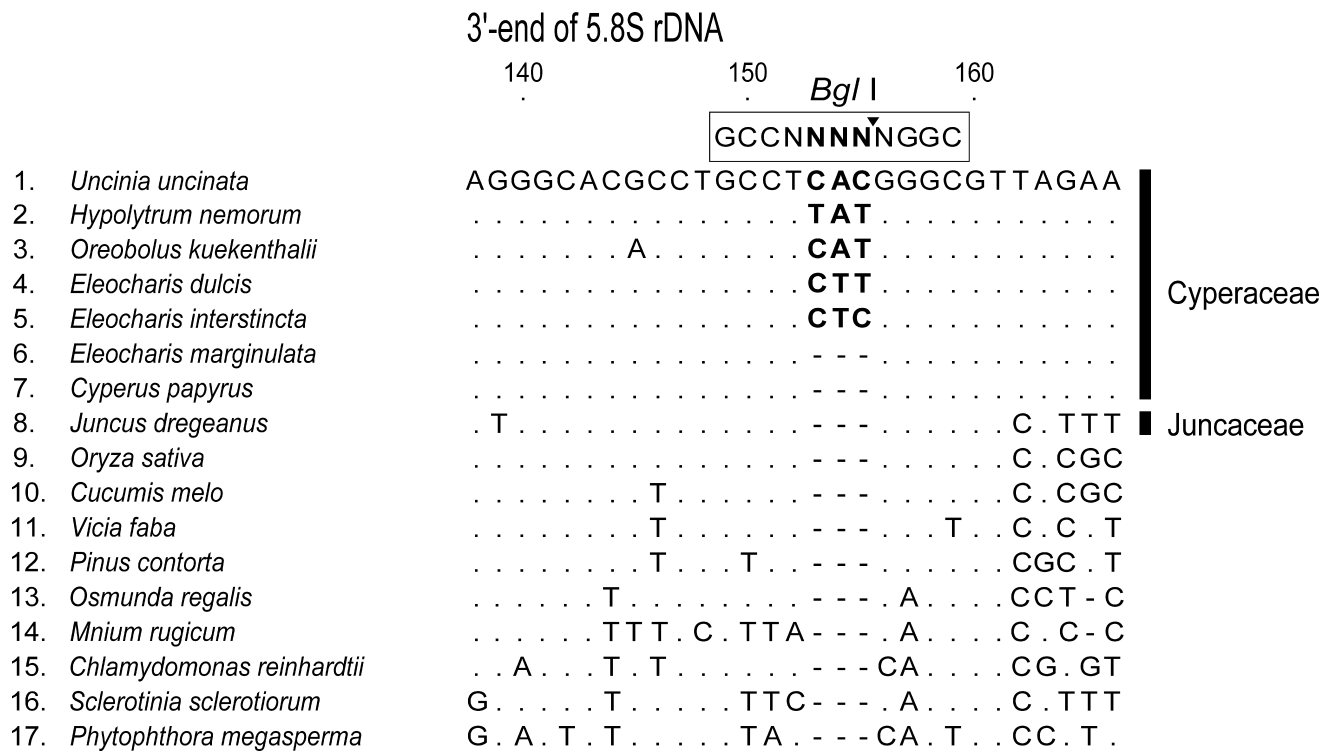
Fig. 2.—The aligned 3'-end of the 5.8S gene of selected land plants (1–14), a green alga (15), a fungus (16), and an Oomycete (17) illustrating the highly conserved region flanking the three base pair insertion (5' > YWY < 3') unique to Cyperaceae p. p. The sequence recognized by the restriction enzyme *Bgl* I is boxed above the alignment with the arrowhead indicating the point at which the enzyme cuts. Note that a BLASTN search of all Juncaceae and Cyperaceae 5.8S sequences revealed only 3 of 386 records where the insertion would not have been detected because of a point mutation in the *Bgl* I recognition site. GenBank numbers for taxa 4–5 and 9–17, which were not sequenced in this study, are as follows: (4) AF190604; (5) AF190611; (9) M16845; (10) M36377; (11) X17535; (12) U23956; (13) X63199; (14) X13432; (15) X65621; (16) M96382; (17) X75632.

5.8S nrDNA (Takaiwa et al. 1985). These ten taxa were sequenced using primers ITS-L (Hsiao et al. 1995) and ITS-4. All PCR reactions contained betaine in order to facilitate the amplification of high G/C content ITS sequences (see Starr et al. 2003), and to reduce the chance of amplifying nonfunctional paralogs (Buckler and Holtsford 1996; Buckler et al. 1997). After a diagnostic restriction enzyme for the 5.8S insertion was identified (see below), all PCR products were amplified with primers ITS-3 (White et al. 1990) and ITS-4, and digested with the restriction enzyme *Bgl* I (New England BioLabs, Inc., Beverly, Massachusetts, USA). Restriction reactions (500 ng of DNA, 0.7 units of *Bgl* I, 1× NEBuffer 3 [50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9]) were performed for 16 hr at 37°C prior to a 20 min enzyme inactivation step at 65°C. When necessary, amplicons were sequenced in a single direction with ITS-3 to confirm the presence/absence of the insertion (see below).

Method and Strategy for Determining the Presence/ Absence of the Insertion

To gauge the level of conservation in the area of the insertion, 5.8S sequences from the present study were initially aligned with sequences from taxa representing the major land-plant groups and selected kingdoms. These alignments were then subjected to the ''cut map'' function in Sequencher™ 3.0 (Gene Codes Corporation, Ann Arbor, Michigan,

USA) to determine whether a diagnostic restriction enzyme could be found for the insertion. This procedure identified a single restriction enzyme, *Bgl* I (5'–GCCNNNNNGGC–3'), which cuts 5.8S nrDNA only when the insertion is present (Fig. 2). When *Bgl* I digests an ITS-3/ITS-4 PCR amplicon containing the insertion, a diagnostic 121 bp fragment (Fig. 1) is always produced because of the highly size-conserved nature of the 5.8S gene in angiosperms (almost always 163 or 164 bp; Baldwin et al. 1995). The presence/absence of this diagnostic, 121 bp fragment was determined on 2% agarose gels by comparing the banding pattern of *Bgl* I digested ITS-3/ITS-4 amplicons to those of a standard containing the insertion, *Fimbristylis complanata*. This taxon was chosen as a standard because complete sequencing of its ITS region revealed that *Bgl* I cuts a *Fimbristylis complanata* ITS-3/ ITS-4 amplicon only once at the point of the insertion, producing two bands with the smallest being the diagnostic 121 bp fragment. In cases where restriction fragments were ambiguously aligned with the standard, the presence/absence of the insertion was confirmed by sequencing with ITS-3.

Although initial kingdom-wide alignments suggested that the recognition sequence for *Bgl* I was highly conserved, a BLASTN search (Altschul et al. 1997) of all publicly available Juncaceae and Cyperaceae 5.8S sequences revealed three (AF027473, AF284961, AF190617) of 386 records where *Bgl* I would not have identified the insertion because of a point mutation within the enzyme's recognition se-

Table 1. Classification and voucher data for taxa used in phylogenetic analyses and in restriction enzyme assays for the presence/absence of the 5.8S nrDNA insertion. Taxa are listed in alphabetical order according to the taxonomic hierarchy. The suprageneric classification of Cyperaceae follows Goetghebeur (1998). Samples whose DNAs were not assayed for the presence/absence of the insertion are provided with their GenBank accession numbers only. GenBank numbers for taxa that were sequenced for the complete ITS region are given in parentheses; all other accession numbers represent previously published *rbc*L sequences used in phylogenetic reconstructions. Individuals sampled from the same taxon are numbered (1), (2), and (3). The insertion was scored as absent in *Prionium serratum* and *Juncus effusus* on the basis of unpublished 5.8S sequences (E. Jones pers. comm.). Taxa sequenced to confirm the presence/absence of the insertion are marked by (§).

Taxon	Voucher	5.8S Insertion presence (+)/ absence (−)	GenBank accession numbers
Family Cyperaceae			
I. Subfamily Caricoideae Pax			
Tribe Cariceae Kunth ex Dumort.			
Carex conferta A. Rich.	KENYA: *Muasya 1055* (K)	+	Y12999
Carex echinochloe Kunze	KENYA: *Muasya 1051* (K)	+	Y12997
Carex monostachya A. Rich.	KENYA: *Muasya 1052* (K)	+	Y12998
Uncinia uncinata Kük.	NEW ZEALAND: *de Lange s. n.,* Acc. 226837 (AK)	+	AY242054
II. Subfamily Cyperoideae Suess.			
Tribe Abildgaardieae Lye			
Abildgaardia ovata (Burm. f.) Kral	KENYA: *Muasya et al. 684* (EA, K)	+	Y12985
Bulbostylis atrosanguinea (Boeck.) C. B. Clarke	KENYA: *Muasya 1037* (EA, K)	+	Y12992
Bulbostylis hispidula (Vahl) Haines	KENYA: *Muasya 1025* (EA, K)	+	Y12944
Fimbristylis complanata (Retz.) Link	KENYA: *Muasya 1029* (EA, K)	+	Y13009 (AY242051)
Fimbristylis dichotoma (L.) Vahl	KENYA: *Muasya 1006* (EA, K)	+	Y13008
Nemum spadiceum (Lam.) Desv. ex Ham.	WEST AFRICA: *Baldwin 9766* (K)	+	Y12945
Tribe Cypereae Dumort.			
Ascolepis capensis (Kunth) Ridl.	KENYA: *Muasya 1009* (EA, K)	−	Y13003
Ascolepis protea Welw.	CONGO: *Fay 2700* (K)	−	Y13002
Cyperus dichroostachyus A. Rich.	KENYA: *Muasya 976* (EA, K)	−	Y12965
Cyperus involucratus Rottb.	MADAGASCAR: Acc. 6136603 (K)	−	Y12967 (AY242052)
Cyperus kerstenii Boeck.	KENYA: *Muasya 984* (EA, K)	−	Y13018
Cyperus longus L.	EUROPE: *Chase 2276* (K)	−	Y13015
Cyperus papyrus L.	CHAD: *Hepper 4213* (K)	−	Y12966 (AY242048)
Cyperus rigidifolius Steud.	KENYA: *Muasya s. n.* (K)	−	Y13016
Ficinia gracilis Schrad.	TANZANIA: *Grimshaw 93939* (K)	−	Y12963
Ficinia nodosa (Rottb.) Goetgh., Muasya & D. A. Simpson	AUSTRALIA: *Stind 21216* (K)	−	Y12984
Ficinia striata (Thunb.) Kunth	SOUTH AFRICA: *Hanekon 1244* (K)	−	Y12964
Isolepis costata A. Rich.	KENYA: *Muasya 1049* (EA, K)	−	Y12981
Isolepis graminoides (Haines & Lye) Lye	KENYA: *Muasya 986* (EA, K)	−	Y12960
Isolepis keniaensis Lye	KENYA: Cabolt plant 'A' (K)	−	Y12980
Isolepis setacea (L.) R. Br.	KENYA: *Muasya 1059* (K)	−	Y12962 (AY242053)
Kyllinga appendiculata K. Schum.	KENYA: *Muasya 991* (EA, K)	−	Y13007
Kyllinga bulbosa P. Beauv.	KENYA: *Muasya 1020* (EA, K)	−	Y12979
Kyllingiella polyphylla (A. Rich.) Lye	TANZANIA: *Wingfield 497* (K)	−	Y13013
Lipocarpha microcephala (R. Br.) Kunth	AUSTRALIA: *Wilson et al. 3383* (K)	−	Y12991
Lipocarpha nana (A. Rich.) J. Raynal	KENYA: *Muasya 972* (EA, K)	−	Y12990
Oxycaryum cubensis (Poepp. & Kunth) Lye	ZAMBIA: *Richards 13318* (K)	−	Y13006
Pycreus flavescens (L.) Rchb.§	KENYA: *Muasya 1022* (EA, K)	−	Y13005
Pycreus nuerensis (Boeck.) S. S. Hooper	TANZANIA: *Muasya 940* (EA, K)	−	Y13004
Scirpoides burkei (C. B. Clarke) Goetgh., Muasya & D. A. Simpson	SOUTH AFRICA: *Hargreaves 3361* (K)	−	Y13001
Scirpoides holoschoenus (L.) Soják	SOUTH AFRICA: *Acocks s. n.* (K)	−	Y12994
Sphaerocyperus erinaceus (Ridl.) Lye	TANZANIA: *Faden et al. 96/338* (K)	−	AJ404699

Table 1.   Continued.

Taxon	Voucher	5.8S Insertion presence (+)/ absence (−)	GenBank accession numbers
Tribe Eleocharideae Goetgh.			
Eleocharis atropurpurea (Retz.) Presl	KENYA: *Muasya et al. 752* (EA, K)	+	Y13012
Eleocharis marginulata Steud.	KENYA: *Muasya 1039* (EA, K)	−	Y13011 (AY242056)
Tribe Fuireneae Rchb. ex Fenzl			
Actinoscirpus grossus (L. f.) Goetgh. & D. A. Simpson§	MALAYSIA: *Simpson 2660* (K)	−	Y12953
Bolboschoenus maritimus (L.) Palla	BOTSWANA: *Smith 2452* (K)	+	Y12996
Bolboschoenus nobilis (Ridl.) Goetgh. & D. A. Simpson	SOUTH AFRICA: *Leistner 144* (K)	+	Y12995
Fuirena ciliaris (L.) Roxb.	TANZANIA: *Muasya 951* (EA, K)	+	Y12971
Fuirena welwitschii Ridl.	KENYA: *Muasya 1024* (EA, K)	+	Y12993
Fuirena Rottb. sp.	BRAZIL: *Thomas et al. 10404* (NY)	+	Y12970
Schoenoplectus articulatus (L.) Palla	TANZANIA: *Muasya 947* (EA, K)	−	Y12987
Schoenoplectus junceus (Willd.) J. Raynal	KENYA: *Muasya et al. 775* (K)	−	Y12952
Tribe Schoeneae Dumort.			
Caustis dioica R. Br.	AUSTRALIA: *Chase 2225* (K)	+	Y12976
Cladium P. Browne sp.§	BRAZIL: *Thomas et al. 10403* (NY)	+	Y12950
Oreobolus kuekenthalii Steenis	MALAYSIA: *Simpson 2659* (K)	+	Y12972 (AY242047)
Pleurostachys Brongn. sp.	BRAZIL: *Kallunki et al. 513* (NY)	+	Y12989
Rhynchospora nervosa (Vahl) Boeck. subsp. *ciliata* (Vahl) T. Koyama	BRAZIL: *Kallunki et al. 512* (NY)	+	Y12977 (AY242050)
Schoenus nigricans L.	ARABIA: *Edmondson 3382* (K)	+	Y12983
Tribe Scirpeae Kunth ex Dumort.			
Eriophorum vaginatum L.	BRITISH ISLES: *Beyer et al. 2* (K)	+	Y12951
Scirpus polystachyus F. Muell.	AUSTRALIA: *Pullen 4091* (K)	+	Y12974
Trichophorum caespitosum (L.) Hartm.	BRITISH ISLES: *Nelmes 954* (K)	+	Y12969
Trichophorum clintonii A. Gray§	CANADA: *Baldwin 4856* (K)	−	Y12982
III. Subfamily Mapanioideae C. B. Clarke			
Tribe Chrysitricheae F. Lestib. ex Fenzl			
Chorizandra cymbaria R. Br.§	AUSTRALIA: *Wilson KLW9738* (NSW)	+	
Chrysitrix capensis L.§	SOUTH AFRICA: *Muasya SA 103* (K)	+	AJ419938
Hellmuthia membranacea (Thunb.) Haines & Lye§	SOUTH AFRICA: *Weerderman et al. 269* (K)	−	Y13000
Lepironia articulata (Retz.) Domin.	MALAYSIA: *Simpson 1236* (K)	+	Y12957
Tribe Hypolytreae Presl ex Fenzl			
Capitularina involucrata Kern	NEW GUINEA: *Johns 8725* (K)	+	
Hypolytrum nemorum (Vahl) Spreng.	MALAYSIA: *Simpson 1379* (K)	+	Y12958 (AY242046)
Mapania cuspidata (Miq.) Uittien§	BRUNEI: *Marsh 4* (K)	+	Y12955
Mapania meditensis D. A. Simpson§	BRUNEI: *Simpson et al. 2515* (K)	+	Y12954
Scirpodendron bogneri S. S. Hooper	MALAYSIA: *Simpson 2650* (K)	+	Y12946
IV. Subfamily Sclerioideae C. B. Clarke			
Tribe Bisboeckelereae Pax ex L. T. Eiten			
Becquerelia cymosa Brongn.§	BRAZIL: *Thomas et al. 10284* (NY)	+	Y12948
Diplacrum africanum C. B. Clarke§	TANZANIA: *Vollensen 3967* (K)	+	
Tribe Cryptangieae Benth.			
Lagenocarpus Nees sp.	BRAZIL: *Mayo 259* (K)	+	
Tribe Sclerieae Kunth ex Fenzl			
Scleria distans Poir.	KENYA: *Muasya 1023* (EA, K)	+	Y12968
Scleria foliosa A. Rich.	TANZANIA: *Muasya 939* (EA, K)	+	Y12986 (AY242049)
Scleria terrestris (L.) Fassett§	MALAYSIA: *Simpson 2658* (K)	+	Y12947

Table 1. Continued.

Taxon	Voucher	5.8S Insertion presence (+)/ absence (−)	GenBank accession numbers
Tribe Trilepideae Goetgh.			
Coleochloa abyssinica (A. Rich.) Gilly	ETHIOPIA: *Vollesen 80/2* (K)	+	Y12975
Family Juncaceae			
Distichia acicularis Balslev & Laegaard		n.a.	AJ419944
Oxychloe andina Philippi	(1) CHILE: *Wickens et al. 95* (K)	−	Y12978
	(2) No voucher information	n.a.	U49222
Juncus articulatus L.	(1) NEWFOUNDLAND: *CH000907-2* (MT)	−	
	(2) NEWFOUNDLAND: *NDC 00-1390* (MT)	−	
	(3) NEWFOUNDLAND: *NDC 00-1396* (MT)	−	
Juncus dregeanus Kunth	KENYA: *Muasya 1047* (EA, K)	−	(AY242055)
Juncus effusus L.	No voucher information	−	L12681
Juncus kraussii Hochst.	No voucher information	n.a.	AY216609
Juncus novaecambriae Gand.	No voucher information	n.a.	AY216643
Juncus repens Michx.	No voucher information	n.a.	AY216627
Juncus trifidus L.	No voucher information	n.a.	AY216618
Juncus vaginatus R. Br.	No voucher information	n.a.	AY216608
Luzula multiflora (Ehrh.) Lej.	(1) IRELAND: *Simpson 2667* (TCD)	−	
	(2) No voucher information	n.a.	AJ419945
Luzula multiflora (Ehrh.) Lej. subsp. *multiflora*	(1) LABRADOR: *NDC 99-952* (MT)	−	
	(2) LABRADOR: *NDC 99-498* (MT)	−	
Luzula nivea (Nath.) DC.	No voucher information	n.a.	AY216650
Luzula purpureosplendens Seub.	No voucher information	n.a.	AY216654
Luzula sylvatica (Huds.) Gaudin	IRELAND: *Simpson 2666* (TCD)	−	
Marsippospermum grandiflorum (L. f.) Hook. f.	No voucher information	n.a.	AJ419946
Rostkovia magellanica (Lam.) Hook. f.	No voucher information	n.a.	AJ419947
Family Thurniaceae			
Prionium serratum J. F. Drege	No voucher information	−	U49223

quence (data not shown). To account for a possible mutation within the *Bgl* I recognition sequence, amplicons were sequenced with ITS-3 whenever presence/absence data deviated from a general pattern of a single basal gain in Cyperaceae followed by a single loss in derived taxa. This pattern was detected in preliminary analyses.

Phylogenetic Analysis and Character Evolution

Heuristic maximum parsimony (MP) searches using PAUP* vers. 4.0b10 (Swofford 2002) were conducted on equally weighted characters for 10,000 replicates of a RANDOM addition of taxa. The MULTREES (save all minimal trees) and TBR (tree-bisection-reconnection) commands were used in searches for optimal trees. Clade support was assessed by bootstrap (BS) analyses (heuristic searches, TBR branch swapping, SIMPLE stepwise addition, 1000 replicates; Felsenstein 1985).

Heuristic maximum likelihood (ML) searches were also performed using TBR branch swapping and a random addition of taxa for 15 replicates. A general time reversible (GTR) model incorporating an estimate of the proportion of invariable sites (I) and a correction for rate heterogeneity among sites (i.e., a gamma distribution, Γ; Yang 1993) were used during these searches (model parameters are available upon request). This model was identified by Modeltest vers.

3.06 (Posada and Crandall 1998) as the best evolutionary model for the data as assessed by the Akaike Information Criterion.

Initial MP and ML analyses included only one (Y12978) of the two *rbc*L sequences for the controversial taxon *Oxychloe andina* since a previous study (Muasya et al. 1998) had considered the other (U49222) to be the sequence of a Cyperaceae contaminant. After significant topological differences were detected between MP and ML trees for *Oxychloe andina* Y12978, two additional analyses were conducted: (1) the distribution of homoplasy on the long terminal branches for Y12978 was examined, and (2) heuristic searches that included both Y12978 and U49222 in a reduced matrix of 22 selected taxa from Juncaceae and Cyperaceae were performed with the first 722 bp, then the last 677 bp of the matrix excluded (the exclusion point was determined from analysis 1). These two analyses suggested that the *rbc*L sequence Y12978 for *Oxychloe andina* was a Juncaceae/Cyperaceae chimera, whereas *rbc*L sequence U49222 was a Cyperaceae contaminant. Consequently, the original data set, minus *Oxychloe andina,* was reanalyzed under ML and MP as above.

The history of character change for the 5.8S insertion was inferred in MacClade vers. 4.0 (Maddison and Maddison 2000) using trees from Cyperaceae analyses and from the

ITS ML phylogeny of *Eleocharis* (Roalson and Friar 2000), the only infrageneric analysis currently available where the insertion is both present and absent amongst species. All taxa were scored for the presence (1) or absence (0) of the insertion based on 5.8S sequence or restriction fragment analysis. Character state changes were unordered and equally weighted. The evolution of the insertion in both Cyperaceae and *Eleocharis* was inferred from DELTRAN optimizations since other character reconstructions required the unlikely scenario of parallel gains of the insertion. Polytomies were resolved randomly during character reconstructions. DELTRAN optimization was also used to infer the history of base changes within the insertion of *Eleocharis* when character tracings were ambiguous.

Heuristic ML searches (as above; GTR + I + Γ) were also performed using a constraint tree that forced all Cyperaceae that lacked the insertion to be monophyletic. This was done to determine whether the data could reject the initial hypothesis of a single gain at the base of Cyperaceae followed by a single loss in its most derived taxa. Topological tests (Kishino and Hasegawa 1989, KH; Shimodaira and Hasegawa 1999, SH) were then performed using a GTR + I + Γ model to assess whether optimal MP and ML trees were significantly different from constrained trees. Both tests (one-tailed) were performed in PAUP* by generating a test distribution from 100,000 bootstrap replicates using the resampling estimated log-likelihood (RELL) method of Kishino et al. (1990).

All matrices used in phylogenetic reconstructions and in character analyses are available online from TreeBASE (*www.treebase.org/treebase/*).

RESULTS AND DISCUSSION

Chimeras, Contaminants, and the Controversial Position of the Genus Oxychloe *in* rbcL *Analyses*

Since the first molecular analyses to include Juncaceae and Cyperaceae, the phylogenetic position of the Juncaceae species *Oxychloe andina* has been controversial. Molecular analyses have placed the two, highly dissimilar *Oxychloe andina* rbcL sequences U49222 and Y12978 as either nested in Cyperaceae (U49222; Chase et al. 1993; Duvall et al. 1993; Plunkett et al. 1995; Drábková et al. 2003), as sister to Cyperaceae (Y12978; Muasya et al. 1998, 2000), or as part of a polytomy with Juncacaeae and Cyperaceae (Y12978; Drábková et al. 2003). Such analyses have suggested that *Oxychloe* might either be an unusual member of Cyperaceae (Plunkett et al. 1995) or that Juncaceae and Cyperaceae may be paraphyletic (Muasya et al. 1998). Even though *Oxychloe* shares indehiscent fruits with Cyperaceae and spiro- or orthodistichous leaves with *Oreobolus* (Cyperaceae), a convincing suite of other characters (Dahlgren et al. 1985) and the results of morphological cladistic analyses (Simpson 1995), strongly suggest that *Oxychloe* is a member
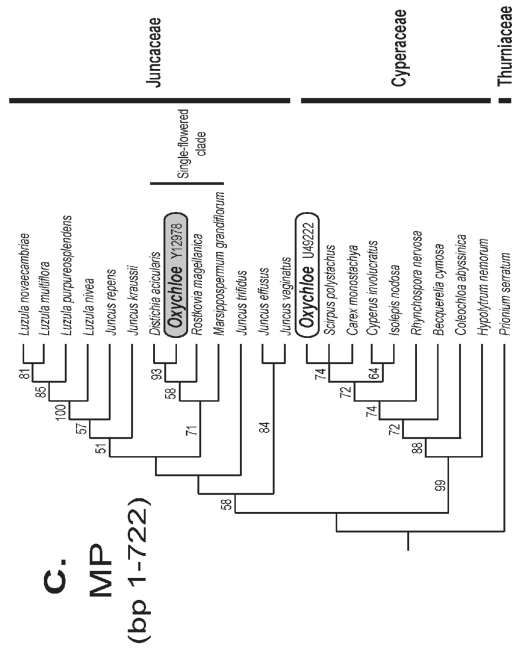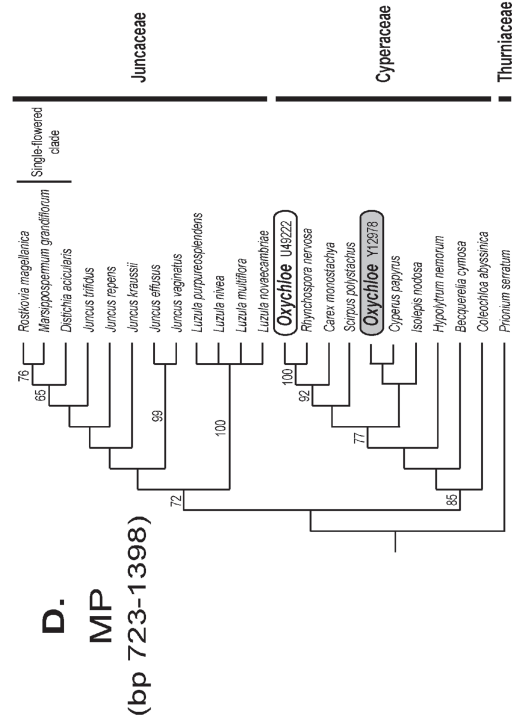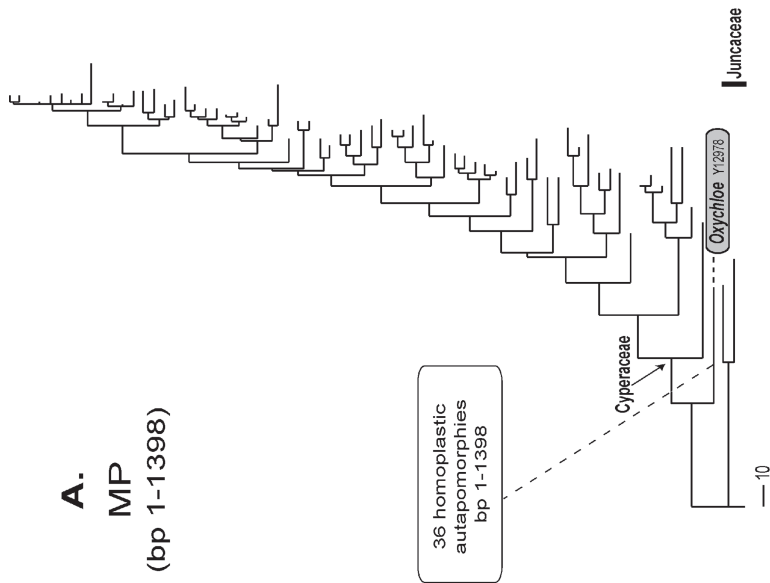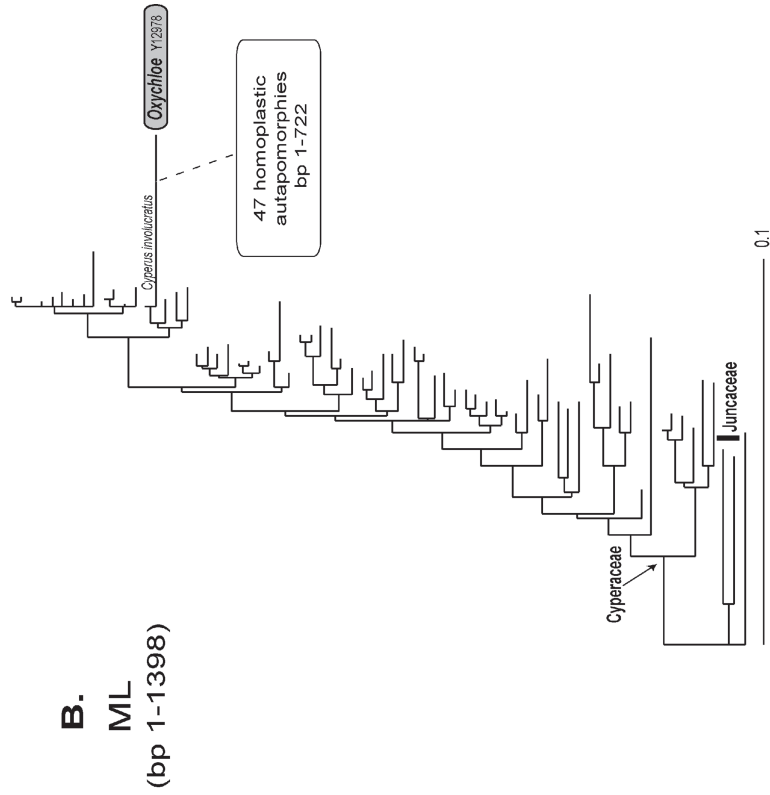
of Juncaceae and closely related to a small group that includes the single-flowered genera *Distichia* Nees & Meyen, *Marsippospermum* Desv., and *Rostkovia* Desv. (Drábková et al. 2003). The present *rbc*L analyses suggest that the unexpected positions of the two *rbc*L sequences for *Oxychloe andina* in previous analyses are due to the fact that Y12978 is a chimera, combining halves from Juncaceae and Cyperaceae, whereas U49222 is the sequence of a Cyperaceae contaminant. When U49222 is excluded from analyses and the Cyperaceae portion of Y12978 is removed, Juncaceae and Cyperaceae are monophyletic, with *Oxychloe* positioned within a Juncaceae clade of single-flowered genera. These conclusions are strongly supported by the *rps*16/*trn*L–F analysis of Jones et al. (2007).

In our initial MP analyses (Fig. 3A), *Oxychloe andina* Y12978 was placed in a basal position sister to Cyperaceae, whereas in ML analyses it was nested within the family next to *Cyperus involucratus* (Fig. 3B). An examination of homoplasy across the long terminal branches for Y12978 showed that all homoplastic autapomorphies in ML trees were confined to the first 722 bp, whereas in MP analyses they were evenly distributed across the molecule (Fig. 3A, B). These results suggested that Y12978 could represent a chimeric sequence since both a basal position in MP analyses (McDade 1992) and an uneven distribution of homoplasy in ML analyses are consistent with the properties of a hybrid sample. Subsequent MP analyses of a reduced, 22-taxon Juncaceae/Cyperaceae data set confirmed this initial hypothesis. When the last 677 bp of the matrix was excluded, analyses placed *Oxychloe* Y12978 as sister to *Distichia* (93% BS) in a Juncaceae clade of single-flowered genera (Fig. 3C), whereas analyses that excluded the first 722 bp positioned *Oxychloe* Y12978 within Cyperaceae sister to *Cyperus papyrus* (100% BS; Fig. 3D). Such analyses suggest that Y12978 is the product of a PCR-mediated recombination event where the first half of the molecule probably represents a ''true'' *Oxychloe andina* sequence, whereas the second half is derived from a Cyperaceae contaminant. In contrast, the same analyses suggest that the *rbc*L sequence U49222 that is currently attributed to *Oxychloe andina* is entirely derived from a sedge contaminant (Muasya et al. 1998). Evidence such as low sequence divergence with closely allied Cyperaceae (ca. 1.0%), a consistent phylogenetic placement within the sedge family (Chase et al. 1993; Duvall et al. 1993; Plunkett et al. 1995; Drábková et al. 2003; this study), and an even distribution of absolute differences (AD) across the molecule when compared to its nearest allies (bp 1–722 = 10 AD, bp 723–1398 = 9 AD), all suggest that U49222 is the sequence of a contaminant from Cyperaceae (Muasya et al. 1998).

Sometimes the greatest advantage of PCR, its sensitivity, can also be its major drawback since even small amounts of extraneous DNA can lead to the amplification of a contam-

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Fig. 3.—MP and ML trees resulting from *rbc*L analyses that included the putative chimeric (Y12978; circles with gray background) and contaminate (U49222; circles with white background) sequences for *Oxychloe andina*.—A. One of 10 MP trees, 1108 steps long (CI = 0.48; RI = 0.70) with all characters included.—B. ML tree (−*ln*L = 8404.97346) with all characters included.—C. Single MP tree, 294 steps long (CI = 0.63; RI = 0.76) with positions 1–722 included (i.e., the Juncaceae portion of Y12978).—D. Strict consensus of 8 MP trees, 277 steps long (CI = 0.65; RI = 0.72) with positions 723–1398 included (i.e., Cyperaceae portion of Y12978).

A.
MP
(bp 1–1398)

36 homoplastic autapomorphies bp 1–1398

B.
ML
(bp 1–1398)

47 homoplastic autapomorphies bp 1–722

C.
MP
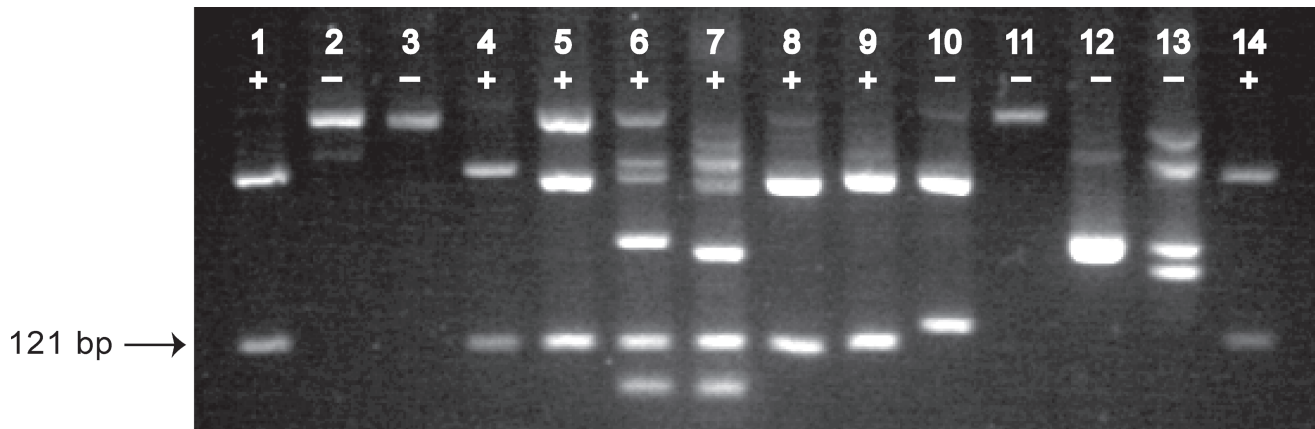(bp 1–722)

D.
MP
(bp 723–1398)

Fig. 4.—DNA fragments resulting from the digestion of amplified ITS-3/ITS-4 PCR products with the restriction enzyme *Bgl* I. The presence of the 3 bp insertion at the 3′-end of the 5.8S gene is detected by a 121 bp fragment. Positive or negative signs below lane numbers indicate the presence or absence of the insertion. Lanes from left to right: (1) Standard (*Fimbristylis complanata*); (2) *Juncus articulatus*; (3) *Oxychloe andina*; (4) *Scirpodendron bogneri*; (5) *Schoenus nigricans*; (6) *Rhynchospora nervosa* subsp. *ciliata*; (7) *Carex monostachya*; (8) Standard; (9) *Bolboschoenus maritimus*; (10) *Hellmuthia membranacea* (11) *Isolepis costata*; (12) *Kyllingiella polyphylla*; (13) *Cyperus longus*; (14) Standard.

inant. This analysis demonstrates that both *rbc*L sequences currently available for *Oxychloe andina* on GenBank do not represent this species, but are due to the introduction of contaminant DNA during DNA extraction or PCR amplification (no misidentification; see Muasya et al. 1998; Drábková et al. 2003). This has led to chimera formation (Y12978) and the sequencing of a completely foreign *rbc*L gene (U49222). PCR-mediated recombination leading to in vitro chimera formation is a serious problem that must be addressed when amplifying nuclear genes, especially when dealing with polyploids and multigene families (e.g., Judo et al. 1998; Cronn et al. 2002). However, it is interesting to discover here that even when amplifying a haploid, single-copy cpDNA gene like *rbc*L, systematists must also consider chimera formation a legitimate concern. This is particularly true when DNAs are difficult to amplify or when target sequences are particularly long (e.g., Yang et al. 1996; Cronn et al. 2002) since contaminants have a greater chance of being amplified. How widespread chimeric sequences may be in past phylogenetic analyses is difficult to assess, but systematists should be conscious that topological differences between MP and ML analyses combined with long branches and an unequal distribution of homoplasy could be signs of PCR-mediated recombination. Moreover, as was the case for *Oxychloe andina* in this analysis, basally positioned sequences for controversial taxa should be scrutinized as potential chimeras since hybrid samples are predicted to be basal to the clade that includes their most derived parent (McDade 1992).
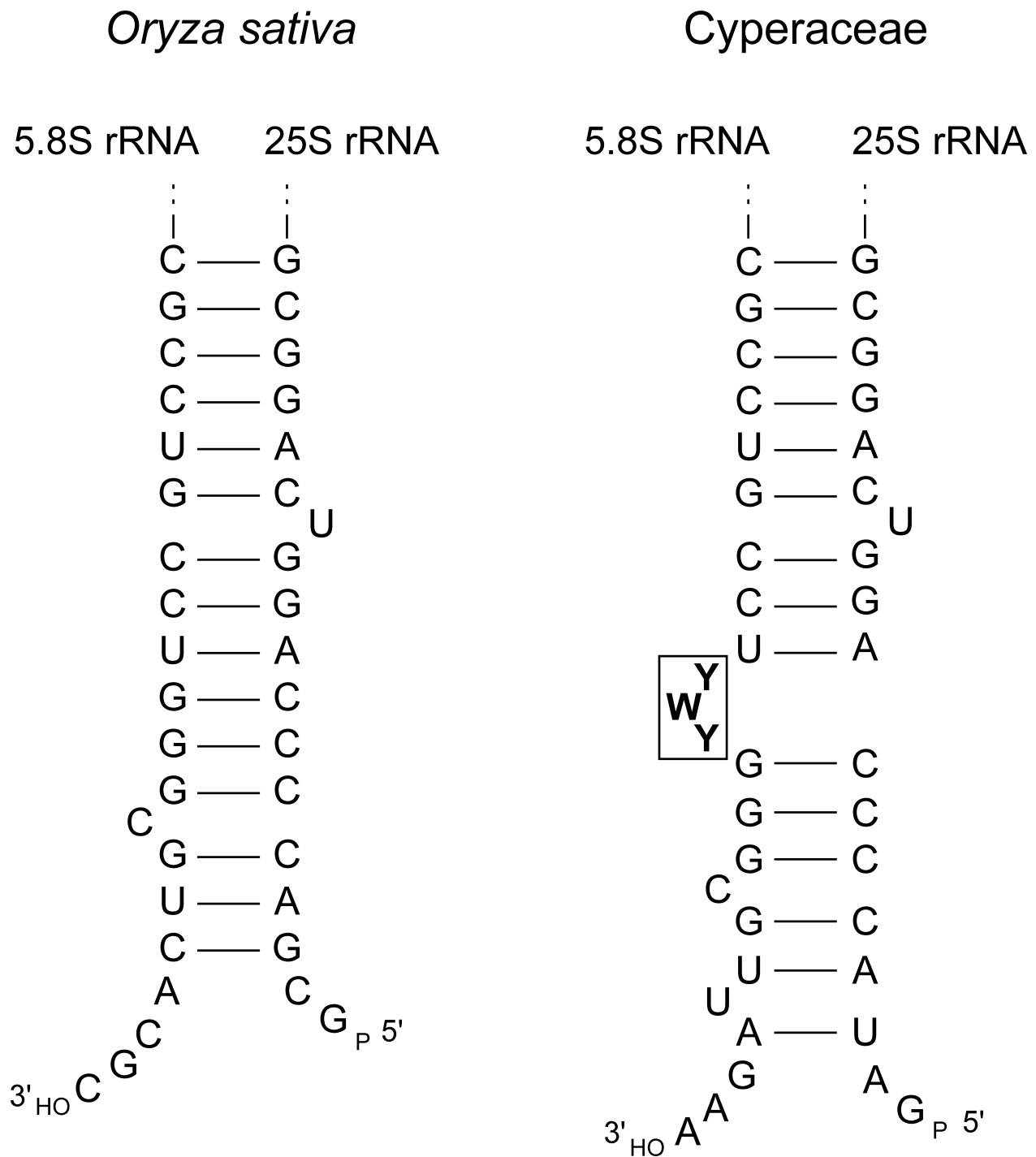
Phylogenetic and Taxonomic Significance of the 5.8S Insertion

Indels are now recognized as a valuable source of phylogenetic information (e.g., Kelchner 2000; Simmons and Ochoterena 2000). Nonetheless, the evolutionary significance of particular indels (Rokas and Holland 2000) and even whether certain indels ought to be included in analyses should be evaluated within the context of the sequence's conservation level (de Jong et al. 2003), and knowledge of the processes that generate such mutational events (Kelchner

2000; de Jong et al. 2003). For instance, short indels are more frequent than long indels (de Jong and Rydén 1981; Pascarella and Argos 1992; Gu and Li 1995), insertions are much rarer than deletions (de Jong and Rydén 1981, Saitou and Ueda 1994; Gu and Li 1995; Ophir and Graur 1997), and higher levels of homoplasy are expected in repeat regions (e.g., Levinson and Gutman 1987; Graham et al. 2000; Kelchner 2000) and at intron/exon junctions (e.g., de Jong et al. 2003). In contrast, indel homoplasy should be low in functionally or structurally important areas of a molecule like the stem regions of RNA stem-loop secondary structures since Watson-Crick base pairing may restrict mutations that compromise the stability of the stem (Takaiwa et al. 1985; Kelchner 2000).

In this study, the presence of the insertion was confirmed by DNA sequencing or restriction site analysis in 44 of the 75 taxa sampled from 43 Cyperaceae genera and four species from three genera (*Juncus* L., *Luzula* DC., *Oxychloe*) of Juncaceae (Table 1). An example of the banding patterns observed in restricted PCR products is shown in Fig. 4. All 44 taxa possessing the insertion were confined to Cyperaceae. Sequence characteristics, such as the highly conserved regions flanking the insertion (Fig. 2), the lack of repeated elements in the insertion area, the large size of the insertion for the 5.8S gene, and its position within a key stem region where 5.8S and 26S rRNA interact to form the large ribosomal subunit (Fig. 5; Takaiwa et al. 1985), are all evidence that this is an unusual and unique mutation to Cyperaceae.

Character reconstructions in MacClade (Fig. 6) broadly suggest that the insertion was gained once at the base of Cyperaceae, followed by a single loss in the most-derived clade in the family (Chrysitricheae II/Cypereae; Fig. 6). Three other losses appear to have occurred: autapomorphic losses in *Trichophorum* Pers. and *Eleocharis,* and a synapomorphic loss in *Schoenoplectus* (Rchb.) Palla and *Actinoscirpus* (Ohwi) R. W. Haines & Lye. Topological tests (KH and SH) support a multiple vs. a single-loss hypothesis within the family (*P* < 0.01). Because the basal portion of the tree is fully resolved beyond the point where the inser-

Oryza sativa

Cyperaceae

5.8S rRNA   25S rRNA

5.8S rRNA   25S rRNA

Fig. 5.—A reconstruction of rRNA secondary structures involving Cyperaceae 3′–5.8S and 5′–26S rRNA. Reconstructions are based on the model proposed for *Oryza sativa* L. (Takaiwa et al. 1985). The three boxed-in base pairs indicate the position of the insertion in Cyperaceae 5.8S nrDNA. Note that for all species that were sequenced for the ITS region, no reciprocal insertion was detected in the 26S gene when an insertion was present in 5.8S nrDNA.

tion is lost, the inferred character evolution for the insertion is unaffected by the choice of any of the 790 MP trees or the ML tree (MP and ML trees were not significantly different, $P = 0.28$, SH; $P = 0.20$, KH). Moreover, the tree topologies of Muasya et al. (1998, 2000) and Simpson et al. (2007) are broadly similar to the present analysis and suggest the same pattern of character evolution for the insertion, al-

though taxonomic sampling for the insertion will need to be increased to confirm this hypothesis.

Within the context of the phylogeny, the gain of the insertion at the base of the family would appear to support the monophyly of Cyperaceae. In addition, the complete absence of the insertion in *Prionium* E. Mey. (E. Jones pers. comm.) and in Juncaceae (five genera, 16 spp. sequenced by E. Jones
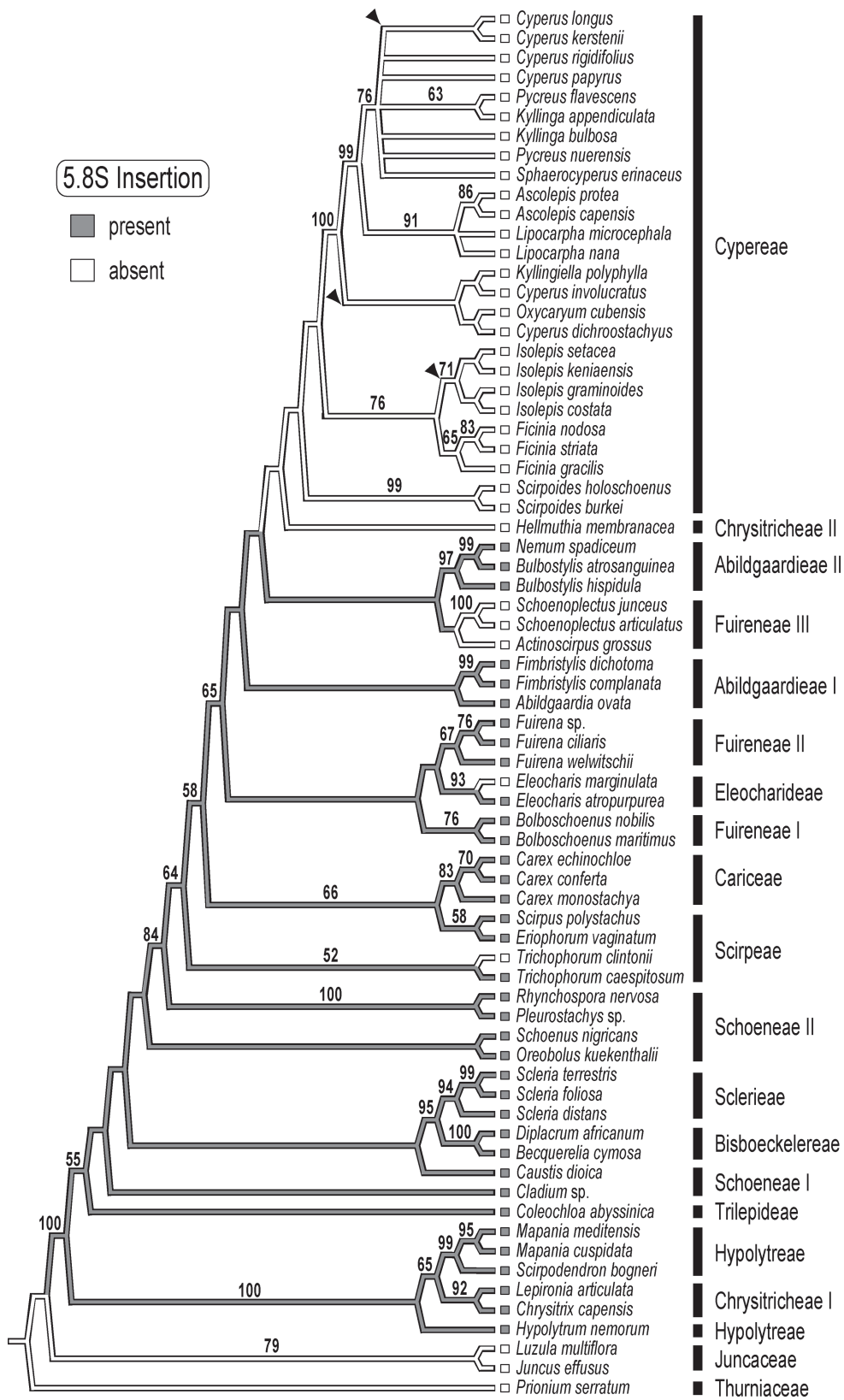
Fig. 6.—One of 790 MP trees, 1068 steps long (CI = 0.50; RI = 0.71). The tree is accompanied by bootstrap values (above branches) and a graphical representation of the inferred evolution of the 5.8S insertion (gray branches = present; white branches = absent). Those clades that collapse in the strict consensus tree are distinguished by arrowheads. The matrix consisted of 1398 characters of which 416 were variable and 244 were parsimony informative.
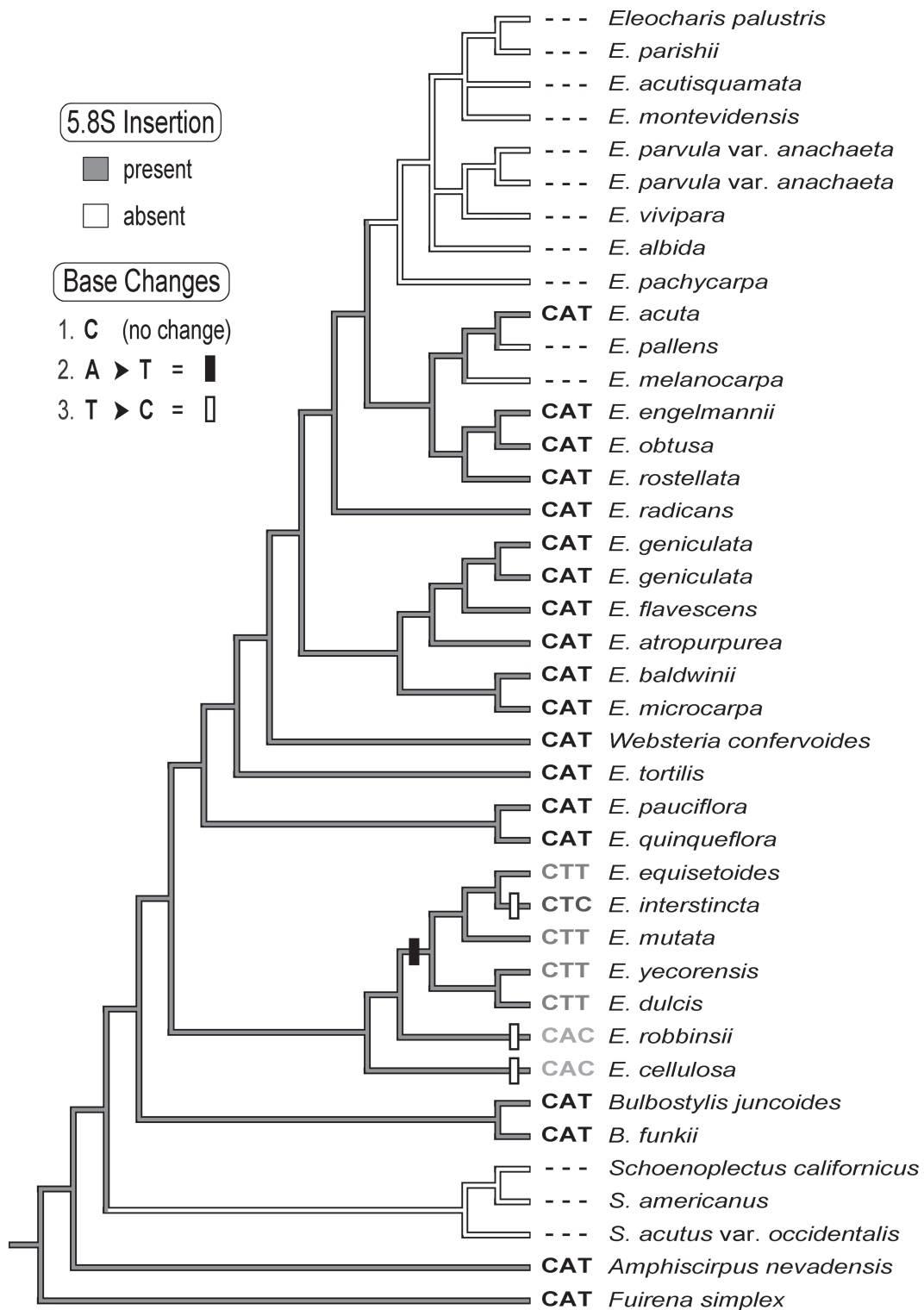
Fig. 7.—ITS phylogeny of *Eleocharis* (Roalson and Friar 2000) showing the inferred evolution of the 5.8S insertion (gray branches = present; white branches = absent) and its DNA sequence (closed boxes = A → T; open boxes = T → C). The distribution and base sequence of the insertion in individual taxa are plotted to the right of the tree. Dashed lines indicate the absence of the insertion.

pers. comm.; this study), combined with its ubiquitous presence in the basal clades of Cyperaceae, are all strong evidence that this mutation marks a monophyletic Cyperaceae.

Nonetheless, even this unusual character is homoplastic, a result that has been seen in other studies that have exam-ined seemingly rare indel events involving losses (e.g., Downie et al. 1991, 1998; Qiu et al. 1998). In this case, however, the reason why the 5.8S insertion is homoplastic is readily understood within the context of secondary recon-structions (Fig. 5) since no compensatory insertion was de-

tected for any taxon in the complementary stem region of the 5′–26S gene. As such, the three inserted bases in Cyperaceae 3′–5.8S sequences should form a secondary side loop whose reversal to the plesiomorphic state probably increases the stability of the stem structure.

Even though the insertion character itself is highly homoplastic (CI = 0.20) due to losses, it would appear that these losses are phylogenetically informative (RI = 0.88) at several different taxonomic levels. For example, losses appear to support the monophyly of the large (>900 spp.) terminal Chrysitricheae II/Cypereae clade that has been detected in all previous molecular analyses (Muasya et al. 1998, 2000; Simpson et al. 2007; Fig. 6), and a close relationship between *Schoenoplectus* and its segregate *Actinoscirpus* (Goetghebeur and Simpson 1991; Fig. 6). Moreover, optimizing the distribution of the insertion and its base sequence on the ITS tree for *Eleocharis* indicates that both a point mutation and an intron loss are congruent with minor clades within this genus (Fig. 7). Although our data indicate that losses may have occurred within several genera such as *Eleocharis* and *Trichophorum,* the pattern of loss, whether it is within a genus or across the family, is not chaotic. Indeed, it would appear that our failure to find losses in groups such as Cariceae is not due to inadequate sampling. In those Cyperaceae tribes where the 5.8S gene has been widely sequenced, the insertion has been detected in all Cariceae (5/5 genera, 256 spp. in GenBank), Abildgaardieae (3/6 genera, 36 spp., K. Ghamkhar pers. comm.), and Chrysitricheae/Hypolytreae (9/13 genera, 16 spp., E. Jones pers. comm.) except *Hellmuthia membranacea.* However, the absence of the insertion in *Hellmuthia* Steud. is consistent with other strong morphological and molecular evidence (e.g., Bruhl 1995; Muasya et al. 1998, 2000, 2001; Simpson et al. 2003, 2006) that *Hellmuthia* is not a member of a basal Chrysitricheae/Hypolytreae group, but a terminal taxon within a Chrysitricheae II/Cypereae alliance. Such examples indicate that insertion losses can be useful characters for defining clades when they occur, but as in the case of any single phylogenetic character, they are only useful within the context of other data.

On a broader scale, the presence/absence of the insertion lends support to data sources such as embryo types (van der Veken 1965), fruit epidermal silica bodies (Schuyler 1971), and morphological (Goetghebeur 1986, 1998; Bruhl 1995) and molecular (Muasya et al. 1998, 2000; Muasya and Simpson 2002) phylogenies that indicate that *Scirpus* s.l. (200–300 spp.) and tribe Scirpeae sensu Bruhl are unnatural and should be divided into several disparately related genera (e.g., Raynal 1973; Wilson 1981; Goetghebeur and Simpson 1991) and tribes (Goetghebeur 1998). Whereas, *Scirpus* s.s. (ca. 20 spp.; Goetghebeur and Simpson 1991) and several *Scirpus* s.l. segregate genera such as *Fimbristylis* Vahl, *Bolboschoenus* (Asch.) Palla, and *Eriophorum* L. possess the insertion, while other segregate genera such as *Isolepis, Oxycaryum* Nees, and *Schoenoplectus* do not (Fig. 6). Moreover, the presence of the insertion in *Bolboschoenus* and its absence in *Schoenoplectus* may be considered as additional support to morphology (Wilson 1981; Goetghebeur and Simpson 1991) for maintaining these two genera as separate.

Note added in proof.–Since this article was submitted in August 2004, two papers relevant to the results presented herein have been published: (1) Yano et al. (2004) *J. Pl. Res.* **117**: 409–419, and (2) Kristiansen et al. (2005) *Syst. Bot.* **30**: 284–289. The chimeric nature of the *Oxychloe andina rbc*L Y12978 sequence was first detected in December 2003, resulting in the removal of its Cyperaceae portion from GenBank concurrent with the submission of this article. The revised version of this manuscript was submitted in March 2005, prior to the publication of the second paper.

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