

Phylogenetic insights into New World *Cyperus* (Cyperaceae) using nuclear ITS sequences

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Abstract. We analyzed nuclear ITS sequences of 95 samples representing 85 taxa of *Cyperus* sensu lato under maximum likelihood and Bayesian frameworks. This work is exploratory in nature with the objectives of gaining insight into relationships among New World *Cyperus* and targeting problematic clades for future research. In our resulting phylogenetic hypothesis, the genera *Cyperus*, *Kyllinga*, *Oxycaryum*, *Pycurus*, and *Queenslandiella* were intermixed, confirming the paraphyletic nature of *Cyperus* as reported by other researchers. We recovered a strongly-supported clade whose members possess C₄ photosynthesis. The C₃/C₄ split has also been previously reported based on research employing different methods of phylogenetic estimation and different molecular markers. We provide preliminary evidence that *Cyperus* sections *Haspani*, *Laxiglumi*, *Strigosi*, *Thunbergiani*, and *Umbellati* are not monophyletic. *Cyperus* subgenus *Diclidium*, which is characterized by a unique mode of spikelet shattering, is monophyletic and highly derived in our analysis, but additional taxa are needed for a more robust assessment. We discuss hypothesized relationships and taxonomic implications for several other species and species complexes.

Key Words: *Cyperus*, Cyperaceae, nuclear ITS, phylogeny.

Even when treated in its most narrow taxonomic concept, *Cyperus* L. is the second largest sedge genus. Typical *Cyperus* (sensu stricto) is recognized as having an herbaceous habit with basally disposed leaves, a terminal anthelate inflorescence immediately subtended by leafy bracts, spikes clustered on often elongate peduncles (rays), laterally flattened spikelets with two-ranked floral scales, and flowers lacking a perianth. Most of these characteristics can be seen in Fig. 1 A–D. *Cyperus* includes several well-known and interesting members. *Cyperus papyrus* L. was an important source of paper in the early history of western civilization (Bryson & Carter, 2008). The cosmopolitan *Cyperus esculentus* L. (chufa or yellow nutsedge) possesses edible tubers. This species is also valued as an important wildlife food, while regarded in some settings as a weed (Bryson & Carter, 2008). *Cyperus*

rotundus L. (purple nutsedge) has been branded the world's worst agricultural weed (Holm et al., 1977). Many *Cyperus* species (and other sedges) are ecologically important and are valuable to wildlife.

The question “What is *Cyperus*?” has not always been easy to answer, as evidenced by the considerable variation in the circumscription of the group (Kükenthal, 1935–1936; Koyama, 1961; Haines & Lye, 1983; Goetghebeur, 1998). *Cyperus* sensu stricto, as in Goetghebeur's (1998) scheme, is paraphyletic (Muasya et al., 1998; Simpson et al., 2007; Muasya et al., 2009a, b; Larridon et al., 2011a, 2013). With *Cyperus* as its core genus, the well-supported *Cyperus* clade also includes the genera *Alinula* Raynal, *Androtrichum* (Brongn.) Brongn., *Ascolepis* Nees ex Steudel, *Courtoisina* Soják, *Kyllinga* Rottb., *Kyllingiella* R. Haines & Lye,



FIG. 1. Representative members of the *Cyperus* clade. **A.** *Cyperus strigosus*. **B.** *Cyperus odoratus*. **C.** *Cyperus haspan*. **D.** *Pycurus lanceolatus*. **E.** *Kyllinga odorata*. **F.** *Lipocarpha maculata*. **G.** *Oxycaryum cubense*. A, B, D–G by R. Carter, C by T. Jones.

Lipocarpha R. Br., *Oxycaryum* Nees *Pycreus* P. Beauv., *Queenslandiella* Domin, *Remirea* Aublet, *Sphaerocyperus* Lye, and *Volkiella* Merxm. & Czech (Muasya et al., 2009a). Some of these genera have been treated as infra-generic taxa of *Cyperus* in more inclusive classifications (e.g., Haines & Lye, 1983). Based on research to date, the only consistent division in the *Cyperus* clade is based on the C₃/C₄ photosynthetic pathways (Muasya et al., 2001; Muasya et al., 2002; Besnard et al., 2009; Larridon et al., 2011a, 2013). In their study of C₃ *Cyperus*, Larridon et al. (2011a) recovered a C₃ grade basal to a strongly supported C₄ clade. The division of *Cyperus* into two groups based on presence of Kranz anatomy, an anatomical characteristic of the C₄ pathway, was originally suggested by Rikli (1895). This division was advocated by Goetghebeur (1989), who proposed that *Cyperus* sensu stricto be split into two subgenera: *Anosporum* (Nees) C.B. Clarke, containing species lacking Kranz anatomy and possessing C₃ photosynthesis; and *Cyperus*, comprising species with Kranz anatomy and C₄ photosynthesis. This classification is also proposed by Larridon et al. (2011b, 2013).

Recent research supports a broader circumscription of *Cyperus*, with the *Cyperus* clade containing ca. 950 species (Muasya et al., 2009b; Larridon et al., 2013). Relationships among selected members of the *Cyperus* clade possessing C₃ photosynthesis were estimated by Larridon et al. (2011a). A phylogenetic study of C₄ segregates *Kyllinga* and *Pycreus* has been completed (Larridon et al., 2013), while a study of *Lipocarpha* is underway (K. Bauters, pers. comm.). The studies cited above include mainly Old World members of the *Cyperus* clade. The purpose of our study is to present an exploratory phylogenetic analysis of 85 in-group *Cyperus* s.l. taxa that are primarily New World natives, using sequences from the nuclear internal transcribed spacer (ITS) region. Specific objectives were to perform a preliminary assessment of the sectional classification presented in Kükenthal's (1935–1936) monograph of *Cyperus*, to evaluate relationships among species and species complexes represented, and to determine directions for more comprehensive work in the future.

Materials and methods

A total of 95 ingroup sequences representing 85 taxa and 14 outgroup taxa were included in our analysis (listed in Table I). Materials for most ingroup taxa were collected by the authors from the southeastern United States (Louisiana, Florida, Georgia, and Texas) in 2010 and 2011, and during an expedition to northeastern Argentina in February of 2011. Materials of several taxa were provided by other botanists. Sequences of some ingroup and most outgroup taxa were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>).

While a broader circumscription of *Cyperus* is warranted by previous work (reviewed above), generic limits in this paper follow the more exclusive system of Goetghebeur (1998), to provide maximum contrast in evaluating our phylogenetic hypothesis. Genera included in the ingroup are *Cyperus*, *Kyllinga*, *Lipocarpha*, *Oxycaryum*, *Pycreus*, and *Queenslandiella* (Table I). Images of representatives of these genera, with the exception of *Queenslandiella*, are presented in Fig. 1. Outgroup taxa include Cyperaceous genera basal to tribe Cypereae, as well as *Juncus* L. and *Oxychloe* Phil. (Juncaceae).

Materials collected by the authors were placed in silica gel upon collection for drying. Voucher specimen information and GenBank accession numbers are provided in Table I. DNA extractions were carried out using the DNeasy Plant Minikit (Qiagen, Germantown, MD, USA). The ITS region (ITS 1 + 5.8S rDNA + ITS 2) was amplified by polymerase chain reaction (PCR) using forward primer *ser 17* (5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3') and reverse primer *ser 26* (5'-TAGAATTCCCGGTTCGCTCGCCGTTAC-3') (Sun et al., 1994). This region was amplified with a touchdown protocol using the parameters 95°C, 3 min; 9X (95°C, 1 min; 55°C, 1 min, reducing 0.5°C per cycle; 72°C, 1 min + 4 sec per cycle); 19X (95°C, 1 min; 55°C, 1 min; 72°C, 1 min); 72°C, 7 min; 4°C until stopped. Each PCR reaction consisted of 0.5 µL MasterAmp™ *tfl* polymerase (Epicentre Technologies, Madison, WI), 12.5 µL buffer GN, 8 µL sterile water, 1 µL of each primer,

TABLE I
Taxa studied with collection localities, voucher specimen information, and GenBank accession numbers.

Taxon	Collection Locality	Voucher Specimen	GenBank Accession No.
<i>Bulbostylis densa</i> (Wall.) Hand.-Mazz.			AB261663.1
<i>Carex decomposita</i> Muhl.			AY757411.1
<i>Kyllinga</i> sp.	Dade County, Florida	Carter 18739 (VSC)	KF146651
<i>Cyperus acuminatus</i> Torr. & Hook.	Sabine Parish, Louisiana	Reid 6171 (LSU)	KF146652
<i>Cyperus aggregatus</i> (Willd.) Endl. (Louisiana)	Allen Parish, Louisiana	Reid 7620 (LSU)	KF146653
<i>Cyperus aggregatus</i> (Willd.) Endl. (Argentina)	Corrientes, Argentina	Reid 7806 (LSU)	KF193566
<i>Cyperus amuricus</i> Maxim.			JX644852.1
<i>Cyperus articulatus</i> L.	Plaquemines Parish, Louisiana	Reid 7487 (LSU)	KF150538
<i>Cyperus cayennensis</i> Willd. ex Link	Corrientes, Argentina	Reid 7815 (LSU)	KF150539
<i>Cyperus cephalanthus</i> Torr. & Hook. (Louisiana)	Vermilion Parish, Louisiana	Reid 7058 (LSU)	KF150540
<i>Cyperus cephalanthus</i> Torr. & Hook. (Texas)	Brazoria Co., Texas	Reid and Rosen 7510 (LSU)	KF193567
<i>Cyperus compressus</i> L. (Georgia)	Lowndes Co., Georgia	Reid and Carter 7761	KF193575
<i>Cyperus compressus</i> L. (Louisiana)	St. Charles Parish, Louisiana	Reid 7580 (LSU)	KF150541
<i>Cyperus corymbosus</i> Rottb.	Corrientes, Argentina	Reid 7878 (LSU)	KF150542
<i>Cyperus croceus</i> Vahl	Tangipahoa Parish, Louisiana	Reid 7501 (LSU)	KF150543
<i>Cyperus cuspidatus</i> Kunth	Lowndes County, Georgia	Reid and Carter 7760 (LSU)	KF150544
<i>Cyperus cyperoides</i> (L.) Kuntze			AB261665.1
<i>Cyperus dichrostachyus</i> Hochst. ex A. Rich.			JX566744.1
<i>Cyperus difformis</i> L.	St. Charles Parish, Louisiana	Reid 7579 (LSU)	KF150545
<i>Cyperus digitatus</i> Roxb.	Chambers County, Texas	Reid and Rosen 7505 (LSU)	KF150546
<i>Cyperus distinctus</i> Steud.	Camden County, Georgia	Reid and Carter 7744 (LSU)	KF150547
<i>Cyperus echinatus</i> (L.) Alph. Wood (typical)	Cherokee County, Georgia	Reid and Carter 8044 (LSU)	KF150548
<i>Cyperus echinatus</i> (L.) Alph. Wood ("var. sphaericus")	Tangipahoa Parish, Louisiana	Reid 7504 (LSU)	KF150549
<i>Cyperus elegans</i> L.	St. Tammany Parish, Louisiana	Reid 7722 (LSU)	KF150550
<i>Cyperus eragrostis</i> Lam.	Harris County, Texas	Reid 7514 (LSU)	KF150551
<i>Cyperus erythrorhizos</i> Muhl. (north Louisiana)	Morehouse Parish, Louisiana	Reid 7530 (LSU)	KF193571
<i>Cyperus erythrorhizos</i> Muhl. (south Louisiana)	St. Charles Parish, Louisiana	Reid 7578 (LSU)	KF150552
<i>Cyperus esculentus</i> L. var. <i>leptostachyus</i> Boeck.	Plaquemines Parish, Louisiana	Reid 7481 (LSU)	KF150553
<i>Cyperus esculentus</i> var. <i>macrostachyus</i> Boeck.	Plaquemines Parish, Louisiana	Reid 7630 (LSU)	KF193572
<i>Cyperus filiculmis</i> Vahl	Beauregard Parish, Louisiana	Reid 7627 (LSU)	KF150554
<i>Cyperus flaccidus</i> R. Br.			JX644855.1
<i>Cyperus fuscus</i> L.	St. Charles Parish, Louisiana	Reid 7788 (LSU)	KF150555

TABLE I Continued

Taxon	Collection Locality	Voucher Specimen	GenBank Accession No.
<i>Cyperus grayoides</i> Mohlenbr.	Bienville Parish, Louisiana	Reid 7703 (LSU)	KF150556
<i>Cyperus haspan</i> L.	Allen Parish, Louisiana	Reid 7525 (LSU)	KF150557
<i>Cyperus hystricinus</i> Fernald (north Louisiana)	Bienville Parish, Louisiana	Reid 7705 (LSU)	KF193573
<i>Cyperus hystricinus</i> Fernald (south Louisiana)	Allen Parish, Louisiana	Reid 7520 (LSU)	KF150558
<i>Cyperus imbricatus</i> Retz.	Corrientes, Argentina	Reid 7807 (LSU)	KF150559
<i>Cyperus insularis</i> Heenan & de Lange	New Zealand	_____	DQ385560.1
<i>Cyperus iria</i> L.	Corrientes, Argentina	Reid 7799 (LSU)	KF150560
<i>Cyperus isocladus</i> Kunth	Florida	Abbott 24946 (FLAS)	KF150561
<i>Cyperus lancastrimensis</i> Porter	Dawson County, Georgia	Reid and Carter 8043 (LSU)	JX661627.1
<i>Cyperus lecontei</i> Torr. ex Steud.	Florida	Abbott 23732 (FLAS)	KF150562
<i>Cyperus ligularis</i> L.	Florida	Abbott 23681 (FLAS)	KF150563
<i>Cyperus lupulinus</i> (Spreng.) Marcks	Bienville Parish, Louisiana	Reid 7711 (LSU)	KF150654
<i>Cyperus luzulae</i> (L.) Rottb. ex Retz.	Corrientes, Argentina	Reid 7808 (LSU)	KF150565
<i>Cyperus macrocephalus</i> Liebm.	Tamaulipas, Mexico	Reid 7161 (LSU)	KF150566
<i>Cyperus microiria</i> Steud.	_____	_____	JX644858.1
<i>Cyperus nipponicus</i> Franch. & Sav.	_____	_____	JX644859.1
<i>Cyperus ochraceus</i> Vahl	Brazoria County, Texas	Reid and Rosen 7512 (LSU)	KF150567
<i>Cyperus odoratus</i> L. (Argentina)	Corrientes, Argentina	Reid 7798 (LSU)	KF150568
<i>Cyperus odoratus</i> L. (Louisiana)	Cameron Parish, Louisiana	Reid 7266 (LSU)	KF150569
<i>Cyperus orthostachyus</i> Franch. & Sav.	_____	_____	JX644860.1
<i>Cyperus ovatus</i> Baldw.	Hamilton County, Florida	Reid and Carter 7765 (LSU)	KF150570
<i>Cyperus oxylepis</i> Nees ex Steud.	Plaquemines Parish, Louisiana	Reid 7476 (LSU)	KF150571
<i>Cyperus pacificus</i> (Owhi) Owhi	_____	_____	JX644861.1
<i>Cyperus papyrus</i> L.	_____	_____	AY242048.1
<i>Cyperus pilosus</i> Vahl	Tangipahoa Parish, Louisiana	Reid 7575 (LSU)	KF150572
<i>Cyperus planifolius</i> Rich.	Dade County, Florida	Mears 5495 (LSU)	KF150573
<i>Cyperus plukenetii</i> Fernald	Taylor County, Georgia	Reid and Carter 8039 (LSU)	KF150574
<i>Cyperus prolifer</i> Lam.	Florida	Abbott 25162 (FLAS)	KF150575
<i>Cyperus prolixus</i> Kunth	Corrientes, Argentina	Reid 7902 (LSU)	KF150576
<i>Cyperus pseudovegetus</i> Steud.	Lanier County, Georgia	Reid and Carter 8035 (LSU)	KF150577
<i>Cyperus pulchellus</i> R. Br.	_____	_____	JX566736.1
<i>Cyperus reflexus</i> Vahl	Corrientes, Argentina	Reid 7869 (LSU)	KF150578
<i>Cyperus retroflexus</i> Buckley	Bienville Parish, Louisiana	Reid 7707 (LSU)	KF150579

TABLE I Continued

Taxon	Collection Locality	Voucher Specimen	GenBank Accession No.
<i>Cyperus retrorsus</i> Chapm. (North Carolina)	Buncombe County, North Carolina	Carter 19850 (VSC)	KF150580
<i>Cyperus retrorsus</i> (south Louisiana)	Tangipahoa Parish, Louisiana	Reid 7502 (LSU)	KF193569
<i>Cyperus retrorsus</i> (north Louisiana)	De Soto Parish, Louisiana	Reid 6144 (LSU)	KF193570
<i>Cyperus rigens</i> J. Presl & C. Presl	Corrientes, Argentina	Reid 7885 (LSU)	KF150581
<i>Cyperus rigens</i> subsp. <i>serrae</i> (Boeck.) Pedersen	Corrientes, Argentina	Reid 7868 (LSU)	KF193568
<i>Cyperus rotundus</i> L.	Florida	Abbott 23635 (FLAS)	KF150582
<i>Cyperus spiralis</i> Larridon	Africa	Wingfield 497 (K)	JX566740.1
<i>Cyperus squarrosus</i> L.	Plaquemines Parish, Louisiana	Reid 7554 (LSU)	KF150583
<i>Cyperus strigosus</i> L. (Louisiana)	St. Charles Parish, Louisiana	Reid 7581 (LSU)	KF150584
<i>Cyperus strigosus</i> L. (Texas)	Chambers County, Texas	Reid and Rosen 7508 (LSU)	KF193574
<i>Cyperus surinamensis</i> Rottb.	Plaquemines Parish, Louisiana	Reid 7478A (LSU)	KF150585
<i>Cyperus tenuispica</i> Steud.			JX644863.1
<i>Cyperus tetragonus</i> Elliott	Glynn County, Georgia	Reid and Carter 7752 (LSU)	KF150586
<i>Cyperus thyrsoiflorus</i> Jungh.	Brazoria County, Texas	Reid and Rosen 7511 (LSU)	KF150587
<i>Cyperus ustulatus</i> A. Rich.	New Zealand		DQ385561.1
<i>Eleocharis tuberculosa</i> (Michx.) Roem. & Schult.			FJ826559.1
<i>Ficinia nodosa</i> (Rottb.) Goetgh., Muasya, & D.A. Simpson			DQ385568.1
<i>Fimbristylis autumnalis</i> (L.) Roem. & Schult.			AB250627.1
<i>Fuirena robusta</i> Kunth	Corrientes, Argentina	Reid 7814 (LSU)	KF150588
<i>Hypolytrum nemorum</i> (Vahl) Spreng.			AY242046.1
<i>Isolepis carinata</i> Hook. & Arn. ex Torr.	Catahoula Parish, Louisiana	Reid 7962 (LSU)	KF150589
<i>Juncus effusus</i> L.			AY727793
<i>Kyllinga brevifolia</i> Rottb.	Glynn County, Georgia	Reid and Carter 7755 (LSU)	KF150590
<i>Kyllinga odorata</i> Vahl	Glynn County, Georgia	Reid and Carter 7753 (LSU)	KF150591
<i>Kyllinga pumila</i> Michx.	Glynn County, Georgia	Reid and Carter 7754 (LSU)	KF150592
<i>Lipocarpha humboldtiana</i> Nees	Corrientes, Argentina	Reid 7852 (LSU)	KF150593
<i>Lipocarpha maculata</i> (Michx.) Torr.	Charlton County, Georgia	Reid and Carter 7742 (LSU)	KF150594
<i>Lipocarpha micrantha</i> (Vahl) G.C. Tucker	St. Charles Parish, Louisiana	Reid 7577 (LSU)	KF150595
<i>Oreobolus venezuelensis</i> Steyerm.			DQ450479.1
<i>Oxycaryum cubense</i> (Poepp. & Kunth) Palla	Iberia Parish, Louisiana	Reid 7796 (LSU)	KF150596
<i>Oxycaryum cubense</i> (GB) (Submitted to GenBank as <i>Cyperus blepharoleptos</i> Steud.)			JX566741.1
<i>Pycreus filicinus</i> (Vahl) T. Koyama	Jefferson Parish, Louisiana	Reid 7632 (LSU)	KF150597
<i>Pycreus flavescens</i> (L.) P. Beauv. ex Rchb.	Caddo Parish, Louisiana	Reid 7576 (LSU)	KF150598
<i>Pycreus lanceolatus</i> (Poir.) C.B. Clarke	Iberville Parish, Louisiana	Reid 7698 (LSU)	KF150599

TABLE I Continued

Taxon	Collection Locality	Voucher Specimen	GenBank Accession No.
<i>Pycreus macrostachyos</i> (Lam.) J. Raynal	Corrientes, Argentina	Reid 7819 (LSU)	KF193576
<i>Pycreus polystachyos</i> (Rottb.) P. Beauv.	Hamilton County, Florida	Reid and Carter 7764 (LSU)	KF150600
<i>Pycreus sanguinolentus</i> (Vahl) Nees ex C.B. Clarke	Camden County, Georgia	Reid and Carter 7747 (LSU)	KF150601
<i>Queenslandiella hyalina</i> (Vahl) Ballard	Dade County, Florida	Carter 18737 (LSU)	KF150602
<i>Rhynchospora chinensis</i> Nees & Meyen ex Nees	_____	_____	AB261680.1
<i>Schoenoplectus pungens</i> (Vahl) Palla	_____	_____	DQ385591.1
<i>Scleria muehlenbergii</i> Steud.	Allen Parish, Louisiana	Reid 7288 (LSU)	KF150603

and 2 μL template (diluted to 10^{-1}), totaling 25 μL . The ITS region of *Isolepis carinata* Hook. & Arn. ex Torr. was amplified using the same PCR cycle protocol but with the reaction consisting of 0.25 μL Phusion[®] polymerase (New England Biolabs[®], Inc., Ipswich, MA), 5 μL buffer HF, 0.5 μL MgCl_2 , 14.25 μL sterile water, 1 μL of each primer, and 2 μL template. Amplification of the ITS region with Phusion polymerase failed for the vast majority of taxa with which it was attempted, but for some reason was very successful with *Isolepis carinata*, while the *tfl* polymerase reaction was not successful with *I. carinata*. Gel electrophoresis was performed to confirm success of PCR reactions. PCR products were shipped on dry ice to Beckman Coulter Genomics, Danvers, MA, for sequencing using the amplification primers. Returned sequences were subjected to a BLAST search (Altschul et al., 1990) to verify that the sequences belonged to the target organism and not a contaminant. Sequences were edited using Sequencher v. 4.2.2 (Gene Codes Corporation). Sequences were aligned with MAFFT v. 7.017 (Katoh et al., 2002) using the MAFFT plug-in of Geneious v. 6.1.5 (Drummond et al., 2010) using default settings. The multiple sequence alignment of nrITS sequences was partitioned into ITS1, 5.8S, and ITS2 and analyzed under maximum likelihood and Bayesian frameworks. The maximum likelihood search was performed in RAxML v. 7.2.6 (Stamatakis, 2006) using the generalized time-reversible (GTR) model of sequence evolution with node support assessed

with 500 bootstrapped pseudoreplicate datasets. For the Bayesian analysis, the appropriate model of sequence evolution was selected for each data partition using jModelTest v. 2.1.3 (Guindon & Gascuel, 2003; Darriba et al., 2012). Models selected using the Akaike Information Criterion (AIC) were GTR + Γ + I for ITS1 and ITS2, and HKY + Γ + I for the 5.8S region. Bayesian analysis was conducted using MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003) via the CIPRES Science Gateway (Miller et al., 2010). The Bayesian analysis consisted of 4 parallel runs using one heated (temp=0.5) and three cold chains per run for 30,000,000 generations sampling every 1,000 generations. All parameters of sequence evolution models were unlinked across partitions. Convergence of each run was assessed by ensuring that potential scale reduction factors (PSRF) reported in MrBayes were close to 1 and effective sample size (ESS) values were greater than 200 in Tracer v. 1.5 (Rambaut & Drummond, 2007). A Bayesian majority-rule consensus tree was produced from the stationary distribution after discarding the first 25% of the sampled trees as burn-in. We produced a tree summarizing both analyses by mapping posterior probabilities and bootstrap proportions onto the Bayesian consensus tree using SumLabels v. 1.0.0, part of the DendroPy Phylogenetic Computing Library v. 3.12.0 (Sukumaran & Holder, 2010). The tree was inspected and edited using Fig Tree v. 1.3.1 (Rambaut, 2006–2009) and prepared for publication using Adobe[®] Illustrator[®] CS4.

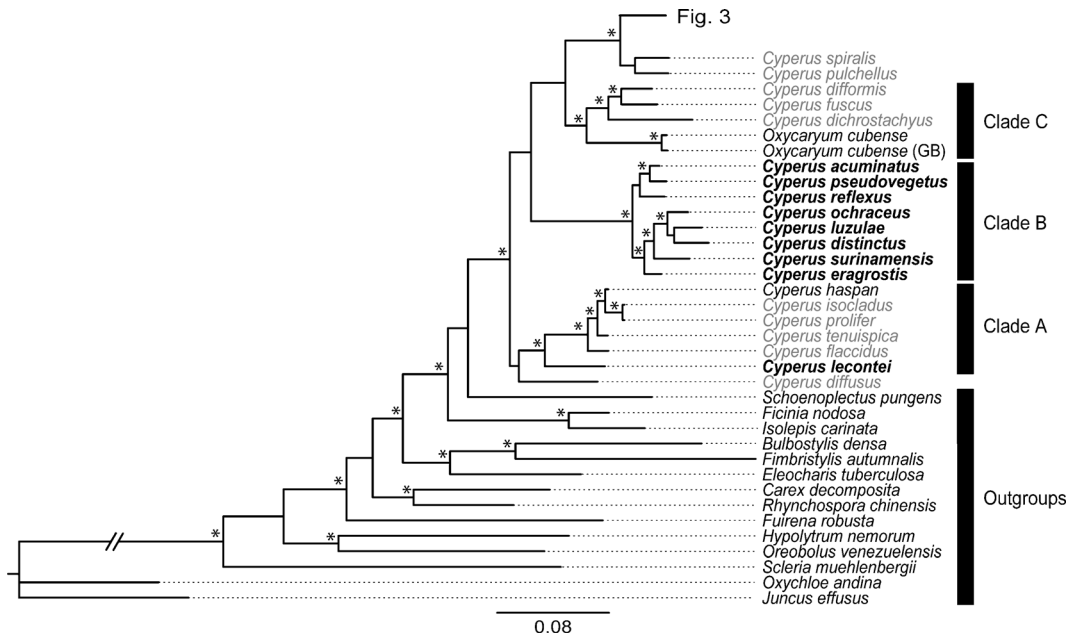


FIG. 2. Outgroups and C₃ grade. Bayesian majority-rule consensus tree based on maximum likelihood and Bayesian analyses of nuclear ITS sequences of *Cyperus*. Nodes with bootstrap proportions ≥ 75 or posterior probabilities ≥ 0.85 are labeled with an asterisk (*). Taxon name font color/style for ingroup taxa is according to native geographic distribution. Old World taxa are in gray font, pantropical taxa are in regular black font, and New World taxa are in bold black font. Native ranges of taxa were determined by consulting Schippers et al. (1995), Tucker (2002), Tucker et al. (2002), Lunkai et al. (2010), and eMonocot Team (2013). Lettered clades are discussed and exact support values are reported in text.

Results

Sequence length in our matrix ranged from 539 bp to 624 bp. Including gaps, the aligned matrix had 685 characters. Sequencing was straightforward, in that sequences required minimal use of ambiguous character states. Of the 74,665 cells in the matrix, 39 (~0.5%) were occupied by ambiguous character states. The phylogenetic tree resulting from our analysis is presented in Figs. 2 and 3. Ingroup taxa compose a well-supported clade (bootstrap proportion (bsp)/posterior probability (pp) = 75/1.00). Ingroup taxa possessing C₃ photosynthesis form a grade (Fig. 2) that is basal to a strongly-supported (bsp/pp = 94/1.00) C₄ clade (Fig. 3). Resolution is better and branch lengths are longer in the C₃ basal grade (Fig. 2). Resolution at deep to intermediate levels within the C₄ clade is poor as evidenced by several polytomies (Fig. 3). The pantropical *Cyperus cuspidatus* Kunth is sister to the remainder of the C₄ clade (Fig. 3), the same

phylogenetic position as reported in other studies (Muasya et al., 2002; Larridon et al., 2011a, 2013).

Kyllinga, *Lipocarpha*, *Oxycaryum*, *Pycurus*, and *Queenslandiella* are embedded within *Cyperus* (Figs. 2, 3). *Kyllinga* is monophyletic with strong support (Fig. 3; bsp/pp = 99/1.00). *Lipocarpha* is paraphyletic in our ITS tree (Fig. 3) and in the study of Larridon et al. (2013) where it fell within a clade also containing *Ascolepis* and *Volkiella* (not sampled here). *Lipocarpha* (Fig. 1F) has generally not been included within *Cyperus*, but consistently falls within it in molecular studies (Muasya et al., 1998; Muasya et al., 2001; Muasya et al., 2002; Simpson et al., 2007; Muasya et al., 2009a, b; Larridon et al., 2013). Prior to the availability of molecular evidence, Koyama (1961) was apparently the only author to have treated *Lipocarpha*, which has highly reduced spikelets, within *Cyperus*.

Oxycaryum cubense (Poepp. & Kunth) Palla is sister to a clade containing *Cyperus*

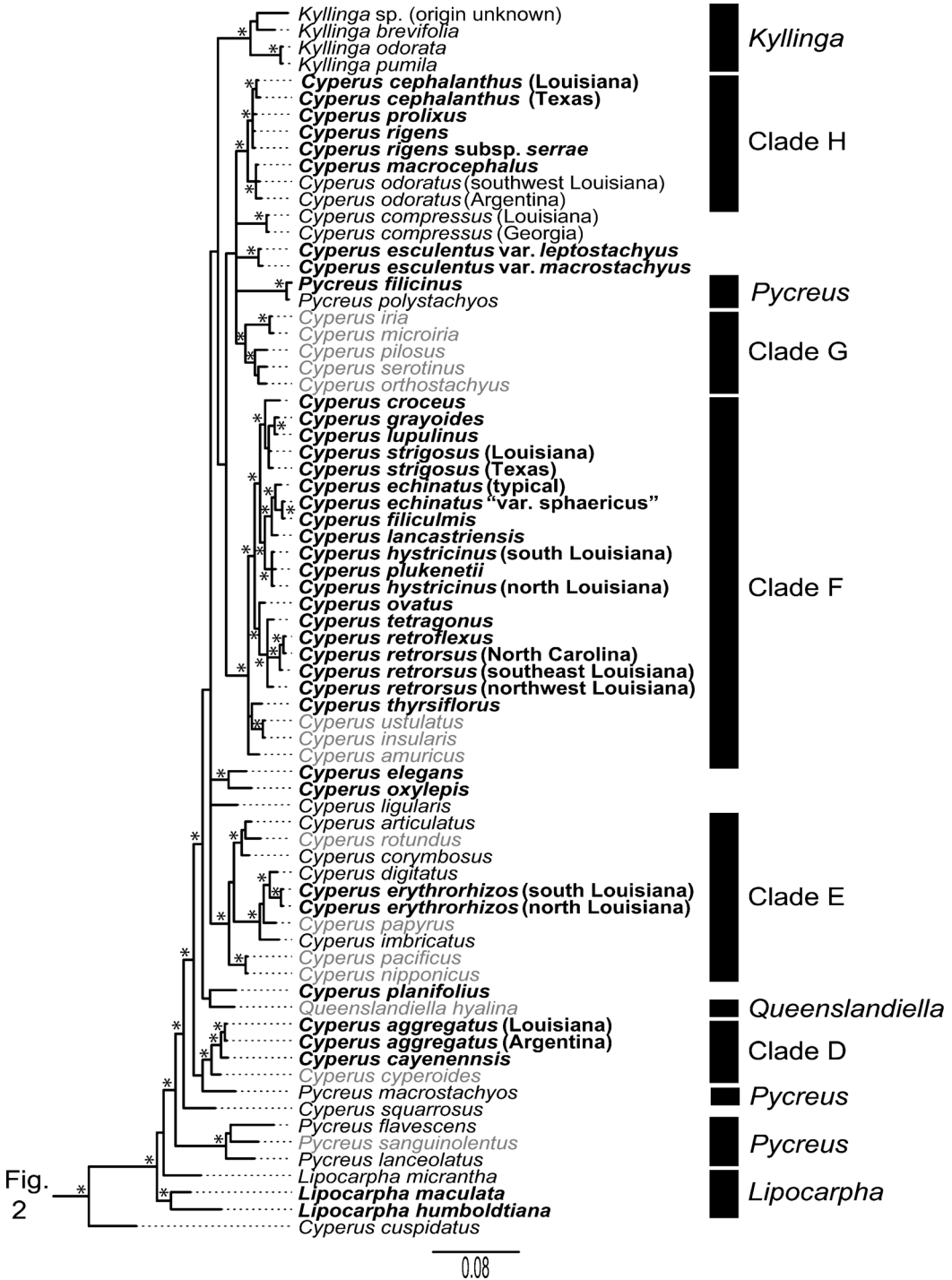


FIG. 3. C₄ clade. Bayesian majority-rule consensus tree based on maximum likelihood and Bayesian analyses of nuclear ITS sequences of *Cyperus* (continued).

dichrostachyus Hochst. ex A. Rich., *C. difformis* L., and *C. fuscus* L. which are members of section *Fusci* (Kunth) C.B. Clarke (Kükenthal, 1935–1936) (Clade C, Fig. 2). Clade C is well-supported (bsp/pp = 94/1.00). *Pycurus* is polyphyletic, with representatives associated with three widely separated clades (Fig. 3). *Pycurus* was also polyphyletic in the analysis of Larridon et al. (2013). *Queenslandiella* is monotypic, represented by *Q. hyalina* (Vahl) Ballard (= *Cyperus hyalinus* Vahl). The phylogenetic position of *Q. hyalina* is not well resolved in our ITS tree (Fig. 3).

Within the C₃ grade (Fig. 2), Clade A (bsp/pp = 93/1.00) consists almost entirely of members of section *Haspani* (Kunth) C.B. Clarke, with the lone exception being *C. flaccidus* R. Br. of section *Graciles* Benth. (Kük.). *Cyperus isocladus* Kunth is treated as a synonym of *C. prolifer* Lam. by Govaerts and Simpson (2007), and our results support this treatment. (Clade A, Fig. 2). Clade B (bsp/pp = 100/1.00) consists of members of section *Luzuloidei* (Kunth) C. B. Clarke. This clade will be discussed more fully below.

Within the C₄ clade (Fig. 3), clades D - H have strong support. For Clade D, bsp/pp = 86/0.99. Several taxonomic sections are represented in Clade E (bsp/pp = 83/1.00), which includes species with pantropical distributions such as *Cyperus articulatus* L., *C. corymbosus* Rottb., and *C. digitatus* Roxb., several strictly Old World taxa such as *C. nipponicus* Franch. & Sav., *C. pacificus* (Ohwi) Ohwi, *C. papyrus*, and *C. rotundus*, and the New World *Cyperus erythrorhizos* Muhl. The majority of taxa in Clade F (bsp/pp = 95/1.00) are native to North America, with the exception of *Cyperus insularis* Heenan & de Lange and *C. ustulatus* A. Rich. which are both endemic to New Zealand, and the east Asian *C. amuricus* Maxim. Members of Clade G (bsp/pp = 67/0.93) are all Old World natives. Clade H (bsp/pp = 82/0.99) comprises two sub-clades. The sub-clade containing *Cyperus macrocephalus* Liebm. and *C. odoratus* L. corresponds to subgenus *Diclidium* (Schrader ex Nees) C. B. Clarke. Support for the *Cyperus odoratus*-*C. macrocephalus* clade is strong (bsp/pp = 83/1.00). The other sub-clade within Clade H has moderately strong support (bsp/pp = 81/0.81) and shows a close relationship

between *Cyperus cephalanthus* Torr. & Hook. and *C. rigens* J. Presl & C. Presl, members of the *C. rigens* complex that also includes *C. impolitus* Kunth, and several other primarily South American taxa (Pedersen, 1972).

Discussion

Our results support an expanded concept of *Cyperus* and resolve a C₃/C₄ split, adding support to the work of other researchers (Muasya et al., 1998, 2002, 2009a, b; Simpson et al., 2007; Larridon et al., 2011a, 2013). Short branch lengths and poor resolution, especially at intermediate depths, are evident in the C₄ clade in our phylogenetic hypothesis. Resolution is improved closer to tips of the tree (Fig. 3). Similar results were reported by other workers (Larridon et al., 2013). Our limited taxon sampling does not allow us to rigorously evaluate sectional taxonomy and biogeography, but does represent an important step toward that end. There are apparent relationships among several ingroup taxa at smaller scales that are worthy of discussion.

In the C₃ grade, section *Luzuloidei* is monophyletic (Clade B, Fig. 2). This group was also reported by Larridon et al. (2011a) to be monophyletic in their Bayesian and maximum likelihood analyses of the chloroplast markers *rpl32-trnL* and *trnH-psbA* and nuclear ETS1. A synapomorphy uniting the members of section *Luzuloidei* represented in our study is the presence of basally two-keeled floral scales (Denton 1978). Denton's (1978) view that her circumscription of the *Luzulae* group, consisting of the New World representatives of section *Luzuloidei* sensu Kükenthal, is natural is supported by our results and those of Larridon et al. (2011a). Our ITS phylogeny, which includes eight of the 10 species treated by Denton (1978) in a phenetic study involving clustering and discriminant analysis, supports her conclusion that *C. ochraceus* Vahl and *C. distinctus* Steud. are closely related. However, our analysis did not confirm close relationships between *C. luzulae* (L.) Rottb. ex Retz. and *C. pseudovegetus* Steud. and between *C. acuminatus* Torr. & Hook. and *C. eragrostis* Lam., which were supported by Denton's (1978) phenetic data.

Cyperus subgenus *Diclidium* (formerly *Torulium* (Desv.) Kük.) is represented in our study by *C. macrocephalus* and two

samples of *C. odoratus*. These species are considered conspecific by Tucker (1984). Tucker et al. (2002) report eight species in this group. Members of subgenus *Diclidium* are diagnosed by having rachillas that disarticulate at the base of each floral scale, the spikelets thus splitting into one-fruited segments (Tucker et al., 2002). In our analysis, subgenus *Diclidium* is monophyletic, highly derived, and apparently has some very close relatives not possessing the spikelet-shattering characteristic of *Diclidium*. Provided that greater taxon sampling continues to support the monophyly of *Diclidium*, a reduction in rank from subgenus to section may be appropriate.

Our ITS tree provides preliminary evidence that the C_4 section *Thunbergiani* (C. B. Clarke) Kük. is artificial, because its representatives in our study, *Cyperus cephalanthus*, *C. insularis*, *C. planifolius* Rich., *C. rigens*, and *C. ustulatus*, do not form a clade. Section *Thunbergiani* is diagnosed by a robust, usually tall, habit, biennial or perennial lifespan, long leaves and bracts, numerous spikelets per spike, and floral scales that are often reddish (Clarke, 1897). Morphologically, the group seems well-defined, but our results suggest it is artificial. Suspicious about the position of *Cyperus planifolius*, we repeated DNA extraction and amplification from silica dried material and directly from the voucher specimen and recovered identical sequences.

In addition to members of section *Thunbergiani*, the strongly supported Clade F (Fig. 3) comprises members of several other mariscoid sections, fide Kükenthal (1935–1936), including *Laxiglumi* (C. B. Clarke) Kük., *Strigosi* Kük., *Tetragoni* Kük., and *Umbellati* (C. B. Clarke) Kük., suggesting their artificial nature as suspected by Carter and Jones (1997) based upon morphological incongruities. Members of Clade F are mostly New World species centered in North America with two taxa, *Cyperus thyrsoiflorus* Jungh. and *C. croceus* Vahl, also extending into South America (Tucker et al., 2002). The presence of New Zealand endemics *Cyperus ustulatus* and *C. insularis* and the Asian *C. amuricus* is of interest. If these taxa are truly closely related to the American taxa in Clade F, long distance dispersal could explain this

situation, as sedges, with their small fruits, can be dispersed by animals as well as on ocean currents (Kern 1974).

Cyperus plukenetii Fern. and *C. hystricinus* Fern. are remarkable among sedges of the southeastern United States in their adaptation to excessively well-drained sandy soils, and Clade F (Fig. 3) suggests a close relationship between these taxa previously supported only by morphological and ecological evidence (Carter, 1984). Other species in Clade F that are adapted to dry, sandy soils include *C. grayoides* Mohlenbr., *C. lupulinus* (Spreng.) Marcks, and *C. filiculmis* Vahl, all members of section *Laxiglumi*.

Clade F (Fig. 3) shows a close, but not sister, relationship between *Cyperus echinatus* (L.) Alph. Wood “var. *sphaericus*” [= *C. ovularis* (Michx.) Torr. var. *sphaericus* Boeck.] and the typical expression of *C. echinatus*. *Cyperus echinatus* “var. *sphaericus*” is sister to *C. filiculmis* in our analysis, and their ITS sequences are actually identical. In the southeastern United States, *Cyperus echinatus* “var. *sphaericus*” and *C. filiculmis* are characteristic species of sandy soils in fire-driven *Pinus palustris* Mill. woodlands. The latter usually inhabits slightly drier, sandier sites than the former. Conversely, typical *C. echinatus* is more of a weedy plant generally associated with mesic, finer-textured soils. In our study, *C. echinatus* “var. *sphaericus*” and *C. filiculmis* were collected from different sites on different dates, and PCR amplification was conducted in different batches on different dates, so cross-contamination does not seem a likely explanation of these taxa having identical ITS sequences. Additional sequencing of the ITS region in all three taxa in this clade will be carried out to determine consistency of sequences. It is possible that *C. echinatus* “var. *sphaericus*” is a result of hybridization between typical *C. echinatus* and *C. filiculmis*. If the hybridization hypothesis is correct, PCR could have amplified an ITS copy of *C. filiculmis* in material of “var. *sphaericus*”. The use of bacterial cloning when working with ITS, as done by Siripun and Schilling (2006) in *Eupatorium* L. (Asteraceae), plus sequencing markers from other genomes, would be helpful in elucidating the true identity and

proper taxonomic placement of “var. *sphaericus*”.

Cyperus ovatus Baldwin has an essentially Floridian distribution, extending westward along the Gulf coast into Louisiana and northward along the Atlantic coast to North Carolina. It was segregated by Small (1933), albeit under different names (*Cyperus pollardii* Britton, *C. winkleri* Britton & Small). Subsequently it was maintained as a species by Carter (1984) and Tucker et al. (2002). Presumably because of superficial similarity in gross features of inflorescence form, some workers (e.g., Godfrey & Wooten, 1979; Wunderlin & Hansen, 2003) have treated *C. ovatus* as a synonym of *C. retrorsus* Chapm. despite a number of differences between the two taxa, including floral scale posture (ascending vs. appressed) and color (yellow-stramineous vs. reddish brown), achene shape (elliptic vs. oblong), and inflorescence bract posture (divaricate vs. ascending). The position of *C. ovatus* vis-à-vis *C. retrorsus* in Clade F supports its treatment as a distinct taxon at the rank of species.

The poor resolution of deeper nodes in the C_4 *Cyperus* clade has been referred to as a hard polytomy (Reynders et al., 2010; Larridon et al., 2013). Larridon et al. (2013) regard C_4 photosynthesis as a key innovation which allowed invasion of hot, dry, high-irradiance sites, and sites experiencing fire or chemical stress, leading to rapid diversification. Perhaps the C_4 clade can be better resolved using large data sets generated by next generation sequencing technology, such as 454 sequencing.

We plan to sample putative relatives of members of Clade F which have more westerly distributions such as *Cyperus hermaphroditus* (Jacq.) Standley, *C. pseudothyrsiflorus* (Kük.) J. R. Carter & S.D. Jones, *C. lentiginosus* Millsp. & Chase, *C. floribundus* (Kük.) J. R. Carter & S. D. Jones, *C. manimae* Kunth, *C. hypopitys* G. C. Tucker, to see if Clade F continues to expand. Many members of Clade F grow in drier habitats. We plan to sample additional species of dry sandy soils such as *Cyperus schweinitzii* Torr., *C. houghtonii* Torr., and *C. grayi* Torr., which are members of section *Laxiglumi*, which is represented by several taxa in Clade F. In all future research,

additional markers from nuclear and plastid regions will be employed to increase the confidence of phylogenetic hypotheses.

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