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RESEARCH

# Molecular phylogeny reveals new morphological and ecological insights into the *Cyperus margaritaceus–niveus* complex

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## Abstract

The *Cyperus margaritaceus–niveus* complex (Cyperaceae) is a group of nine C4 *Cyperus* species (*C. karlschumanii*, *C. kibweanus*, *C. ledermannii*, *C. margaritaceus*, *C. nduru, C. niveus* var. *leucocephalus*, *C. somaliensis*, *C. sphaerocephalus*, and *C. tisserantii*) from Sub-Saharan Africa and Madagascar united by the combination of a capitate inflorescence, white–yellow glumes and swollen bulb-like bases. Previous molecular studies hinted that this complex forms a natural group within the C4 *Cyperus* Clade, although not all taxa were included. In this study, we used the Angiosperms353 bait kit to generate sequence data for all taxa in the complex. The species complex was resolved as monophyletic, while inter-species relationships produced differing results. *Cyperus sphaerocephalus*, *C. kibweanus*, *C. ledermannii*, *C. somaliensis*, and *C. tisserantii* were resolved as monophyletic, while *C. karlschumannii* appears monophyletic except for a moderately resolved isolated lineage that is positioned elsewhere. *Cyperus niveus* var. *leucoephalus* was resolved as paraphyletic. *Cyperus nduru* was resolved as polyphyletic even after rechecking determinations of the specimens. The latter result appears to show correlation with habitat in which one clade comprises specimens associated with Miombo woodland and another clade that comprising specimens associated with bowal grassland. This ecological difference correlates with differences in underground organ morphology. *Cyperus margaritaceus* requires further study to delimit since a specimen identified as this species is found in an unusual position in the phylogeny, possibly representing a new species to science. Combining this phylogenomic dataset with previous studies on morphometrics demonstrates how such integrative and iterative taxonomic methods can be the optimal approach in resolving species complexes in species-rich genera such as *Cyperus*, especially in the rapid diversifying C4 Cyperus Clade.

**Keywords** Africa · Bowal grassland · C4 Photosynthesis · Cyperaceae · *Cyperus* · Integrative taxonomy · Miombo Woodland · Phylogeny · Species complex

## Introduction

*Cyperus* L. is the second largest genus in the Cyperaceae with 964 species (Larridon et al. 2021; Larridon 2022) and has a pantropical distribution, predominantly occupying wetlands, seasonally wet grasslands and the undergrowth of woodlands (Larridon 2011). Advances in molecular techniques have allowed new insights and re-assessments in our understanding of intra-generic relationships in *Cyperus*, following earlier phylogenetic studies that showed *Cyperus* to be non-monophyletic (e.g. Muasya et al. 2002). Phylogenetic studies by Larridon et al. (2011) revealed that *Cyperus* species using the C4 photosynthetic pathway—C4 *Cyperus*—formed a clade nested within a paraphyletic C3 *Cyperus*, which corroborates findings of C4 photosynthesis evolving only once in *Cyperus* (Besnard et al. 2009; Pereira-Silva et al. 2025). Larridon et al. (2013) showed this C4 clade to include ten other C4 lineages that were previously considered segregate genera (Goetghebeur 1998) (*Alinula* J.Raynal, *Ascolepis* Nees ex Steud., *Ascopholis* C.E.C.Fisch., *Kyllinga* Rottb., *Lipocarpha* R.Br., *Pycreus* P.Beauv., *Queenslandiella* Domin, *Remirea* Aubl., *Sphaerocyperus* Lye, and *Volkiella*

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Merxm. & Czech.). However, attempts to resolve relationships within C4 *Cyperus* have proven difficult due to many of them forming a polytomy, even when using fast mutating markers (Larridon et al. 2013). Larridon et al. (2020) sought to investigate the relationships within C4 *Cyperus* further by comparing the utility of two different targeted sequencing bait kits—the Angiosperms353 kit (Johnson et al. 2019) and a Cyperaceae-specific kit (Villaverde et al. 2020). This study marked the first use of phylogenomic techniques on *Cyperus.* One unexpected result from that study is the placement of the *Cyperus margaritaceus–niveus* complex: the focus group of this study

The *Cyperus margaritaceus–niveus* complex is a group comprising nine species (*C. karlschumanii* C.B.Clarke, *C. kibweanus* Duvign., *C. ledermannii* (Kük.) S.S.Hooper, *C. margaritaceus* Vahl, *C. niveus* var. *leucocephalus* Kunth (Fosberg), *C. nduru* Cherm., *C. somaliensis* C.B.Clarke, *C. sphaerocephalus* Vahl, and *C. tisserantii* Cherm.). Larridon et al. (2020) found that this complex forms a clade sister to the core C4 Cyperus Clade; after *Cyperus* section *Cuspidati* which forms the first lineage of the C4 Cyperus Clade (Larridon et al. 2011, 2013, 2020). Historically, the species of this complex have never constituted a formal group but rather have been linked together by dint of morphological similarity. The complex is united morphologically by a capitate inflorescence, composed of several spikelets with distichously arranged, white, off-white, or yellow glumes and swollen culm bases adapted for fire regimes (see Fig. 1). Two of these species, *C. sphaerocephalus*, and *C. niveus* var. *leucocephalus* have been experimentally shown to be insect-pollinated, which points to the characteristic glume colour as a key functional trait in the transition from wind to insect pollination in some members of the Cyperaceae (Wragg & Johnson 2011). The complex is distributed throughout Sub-Saharan Africa; *C. niveus* var. *leucocephalus* is the only taxon extending into Madagascar. The taxa are typically associated with two types of habitat, both of which are based on, or mainly comprise of, laterite (clay-based) soils. The first is Miombo woodland; a vegetation type characterized by frequent fires and strong seasonal rainfall, in which plants invest heavily in their culm bases which are covered in the hard basal sheaths from the previous year's growth that was burnt by fire. Once enough underground biomass is accumulated, the plants will rapidly produce flowering

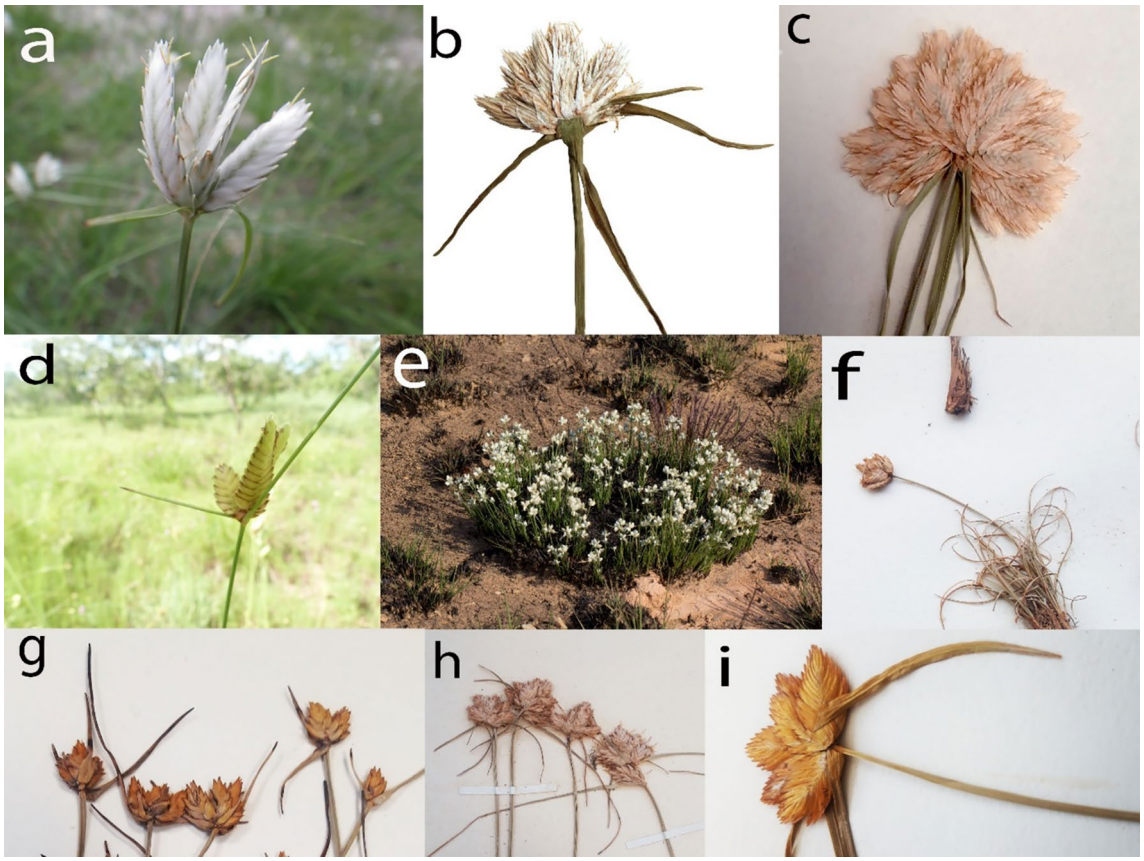



**Fig. 1** Representatives of the *Cyperus margaritaceus–niveus* complex: **a** *C. margaritaceus*, **b** *C. ledermannii,* **c** *C. niveus* var. *leucocephalus,* **d** *C. karlschumannii,* **e** *C. nduru,* **f** *C. somaliensis,* **g** *C. kibweanus,* **h** *C. tisserantii*, **i** *C. sphaerocephalus.* Photos: **a**, **d** and **e** X. van der Burgt; **b** J. Browning; **c**, **f**, **g**, **h** and **i** M. Xanthos

culms following the short intense rainfall period, giving them a small window of opportunity for pollination before other vegetation can dominate. The second habitat is known as bowal grasslands, which are characterized by the absence of trees due to the surface hardpan, which the roots cannot penetrate (Couch et al. 2019). The extreme edaphic and hydrological conditions of this habitat (Zwarg et al. 2012) have allowed for a specialized flora, in which members of the complex have been able to co-inhabit.

Species delimitation is challenging within the complex primarily due to many, if not all, of these species looking superficially similar to each other, and the subjective interpretation of the morphology has led to differing circumscriptions in regional floras. Xanthos et al. (2023) sought to test these circumscriptions by using morphometric analyses to study the complex across its entire geographic range (excluding *C. kibweanus*). In the Linear Discriminant Analysis (LDA), all taxa were represented as morphospecies, except for *C. niveus*, which failed to distinguish itself as a separate entity. In the Classification and Regression Tree Analysis (CART), six morphospecies were recognized; three of which were each split into two further groups while *Cyperus ledermannii, C. somaliensis,* and *C. niveus* all failed to separate as morphospecies. In the case of *C. niveus,* its type was assigned to the *C. obtusiflorus* group, the name of which was considered a variety of the Asian *C. niveus* by Fosberg (1977) who consequently found the correct name in the varietal rank. Thus, specimens from Africa named *C. niveus* or *C. obtusiflorus* are in fact *C. niveus* var. *leucocephalus*. In this study, we follow up on the results of Xanthos et al. (2020) to produce a molecular framework of the *Cyperus margaritaceus–niveus* complex, testing species limits in the complex as well as the relationship with the Asiatic *C. niveus.* We also aim to corroborate and expand on two key results from Larridon et al. (2020): (a) that the complex forms a monophyletic group; and (b) that the clade is sister to the core C4 Cyperus Clade. Our sampling includes all nine species, combining previously generated sequences from Larridon et al. (2020) with new sequences generated for this study

## Materials and methods

### Taxon sampling

Twenty-four leaf samples, representing all known species associated with the *C. margaritaceus–niveus* complex, and samples of *C. niveus* from Southeast Asia were newly collected from specimens held in the Kew Herbarium (K) for this study. In addition, 66 accessions from previously generated sequence data were included (Larridon et al. 2020). These 66 accessions represent species from the complex and various other C4 *Cyperus* species, including representatives of all former segregate genera except for *Volkiella*. In total, 90 accessions were included as the ingroup. For the outgroup, three *Ficinia* species were chosen, as previous studies have shown that the *Ficinia* Clade is sister to *Cyperus* (e.g. Muasya et al. 2002, 2009; Simpson et al. 2007; Larridon et al. 2020). These outgroup samples also represent previously generated sequences. A complete list of all accessions used in this study is provided in Online Resource 1.

### DNA extraction, library preparation, and sequencing

Total genomic DNA was extracted in the Jodrell Laboratory at the Royal Botanic Gardens, Kew, UK using a modified Cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1987) with 3 µL of 2-mercaptoethanol, followed by extraction with chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a new tube and 500 µL of Isopropanol was added to allow precipitation. Following a weeklong freeze period, the aqueous phase was decanted off, and the pellet washed with 750 µL of 70% $C_2H_5OH$. After decanting, the pellet was left to dry overnight, then resuspended with 100µL TE0.25 Buffer. Magnetic Ampure XP beads were used for cleaning, followed by washing with 200 µL of 80% Ethanol. Beads were eluted with of 60µL elution buffer (EB, Tris 10nMol). All DNA extracts were quantified with a Promega® Quantus™ Fluorometer (Madison, WI, USA) and ran on a 1% Agarose gel for quality control. Library preparation and sequencing was carried out externally, by Neogen Europe Ltd (Auchincruive, UK). DNA extracts were enzymatically fragmented to c. 500 bp. Paired-end genomic DNA libraries were constructed using the Kapa HyperPrep Kit (Roche Sequencing Solutions, Indianapolis, IN, USA) according to the manufacturer's instructions, with a 6-cycle PCR reaction. Libraries were analysed for size distribution using the 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA) and quantified using a Qubit 4 Fluorometer (Invitrogen, Waltham, MA, USA). Hybrid capture was performed in pools of 24 samples following the myBaits® kit manual. Samples were enriched with the Angiosperms353 enrichment panel v1 (Johnson et al. 2019) for 16 h at 65 ºC, followed by 10 cycles of PCR amplification. Library pools were assessed for quality using the TapeStation with HS DNA ScreenTape. Sequencing was performed on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using paired-end 150 bp (PE150) sequencing.

### Contig assembly, multi-sequence alignment, and bioinformatics

All phylogenomic analyses were carried out on the High Performance Computing (HPC) cluster at Royal Botanic

Gardens, Kew, UK. Demultiplexed raw reads (FASTQ files) were trimmed to remove residual adapters and low-quality sequences, using Trimmomatic v. 0.39 (Bolger et al. 2014) set to the following parameters: LEADING: 30 TRAILING: 30 SLIDINGWINDOW 4:2:30, MINLEN: 36. Contigs were assembled using HybPiper v. 1.3.1 (Johnson et al. 2016) using different target files in separate workflows in order to compare gene recovery rates. These were the Angio-353 DNA, Angio-353 AA, and the mega353 (McLay et al. 2021) target files. We used the Burrows--Wheeler Alignment tool (BWA) to map quality-filtered reads to the DNA and mega353 target files (Li and Durbin 2009) and BLASTx (Camacho et al. 2009) when mapping to the AA target file. Reads that successfully mapped to our target files were then assembled de novo in SPAdes (Bankevich et al. 2012), with cov_cutoff 4. We generated summary statistics with HybPiper to compare the gene recovery rates for all three target files, visualizing the results as heatmaps in R v. 4.5.1. (R Core Team 2024) using the pheatmap package (Kolde 2019) and its associated dependencies. We used the HybPiper retrieve command to consolidate the sequence-assembly results, by outputting single FASTA files containing retrieved contigs per gene. Contigs were aligned using MAFFT 7.471 (Katoh and Standley 2013), followed by cleaning using PhyUtility 2.7.1 (Smith and Dunn 2008). We experimented with the clean parameter settings, to test its effect on the resulting phylogeny with a maximum setting of 0.50. After each clean, we visually inspected the alignments using Geneious 2025.0.3 (https://www.geneious.com) to ensure no problematic gaps remained.

We used maximum likelihood estimation (MLE) to infer gene trees from the filtered alignments in IQ-TREE 2.0.6 (Nguyen et al 2014), applying 1000 ultra-fast bootstrap replicates. To produce the total-evidence species tree, we concatenated the alignments prior to running IQ-TREE. To negate the impact of gene tree discordance on the inferred MLE tree, we ran TreeShrink 1.3.5 (Mai and Mirarab 2018) on the concatenated tree to remove long branches. These branches most likely represent outlier taxa, which can result from sequencing errors, misalignments, or species misidentification, among other causes. For the multi-species coalescent approach, individual MLE trees were inferred in IQ-TREE, with settings as above, cleaned with TreeShrink to improve the accuracy of species tree inference, and then concatenated to run ASTRAL. We then ran ASTRAL III (Zhang et al. 2018) to infer the MSC species tree from the TreeShrink output, using '−t2' to generate quartet support values that summarize gene tree conflict. All trees were visualized in R (R Core Team 2024) using the packages ggtree (Yu et al. 2017), treeio (Wang et al. 2020), ggimage (Yu 2023), gridExtra (Augie 2017), readxl (Wickham and Bryan 2023), ape v.5.8 (Paradis and Schliep 2019), and their corresponding dependencies.

### Phylogenetic networks

Hybridization is well documented in the Cyperaceae (e.g. Marcks 1972, 1974; Waterway 1990, 1994; Hedrèn, 1997; Gehrke et al. 2010; Košnar et al. 2010), although some of these earlier studies relied on allozyme data and chromosome counts to detect gene flow between species. Recently, the use of plastid and nuclear markers have helped identify new hybrid species (Maguilla and Escudero 2016; Torimaru et al. 2021). The results of Larridon et al. (2020) indicated that some morphospecies within the complex are non-monophyletic. Such incongruences between recovered phylogenies and morphological classifications have been investigated in other plant groups (e.g. bamboos), and hybridization has been suggested as a possible cause (Triplett and Clark 2010, 2021; Yang et al. 2013). To anticipate similar incongruences in our dataset, we utilized the software package, PhyloNetworks (Solís-Lemus et al. 2017) to detect the presence of reticulation events, such as hybridization and horizontal gene transfer. We inferred an ASTRAL tree using just the accessions of the *C. margaritaceus--niveus* complex and converted this into a.tre file in FigTree v1.4.4 (Rambaut 2018) to use in the PhyloNetworks script as the starting point. PhyloNetworks was run in Julia (Bezanson et al. 2024) implementing SNaQ (species networks applying quartets; Solís-Lemus and Ané 2016), which performs maximum pseudolikelihood estimation of phylogenetic networks on subsets of 4 taxa at a time. Concordance factors were calculated for each quartet of taxa, which are a measure of genomic support for alternative topologies for each quartet. Phylogenetic networks were visualized in IcyTree (Vaughan 2017). All HPC and R scripts are available in Online Resources 2a & 2b respectively.

## Results

### Gene recovery and data quality

All samples were successfully quality filtered, except for CC-00022 (*C.niveus* from India). Recovery statistics are available in Online Resource 3. Gene recovery rates were as follows: 36.37% for the DNA target file, 56.28% for the AA target file and 62.81% for the mega353 target file. In the first two cases, no genes were recovered for the following samples: CC-00004 (*C. nduru*), CC-00023, and CC-00024 (*C. niveus*), while genes were recovered for CC-00004 with the mega353 file. This meant that across all target files, no data was available for the Asian *C. niveus*. Figure 2 shows the heatmap for gene recovery on the mega353 file. MAFFT performance was considerably better on the AA and MEGA353 target file with all 353 contigs aligned compared with the DNA file, where only 279 contigs were successfully

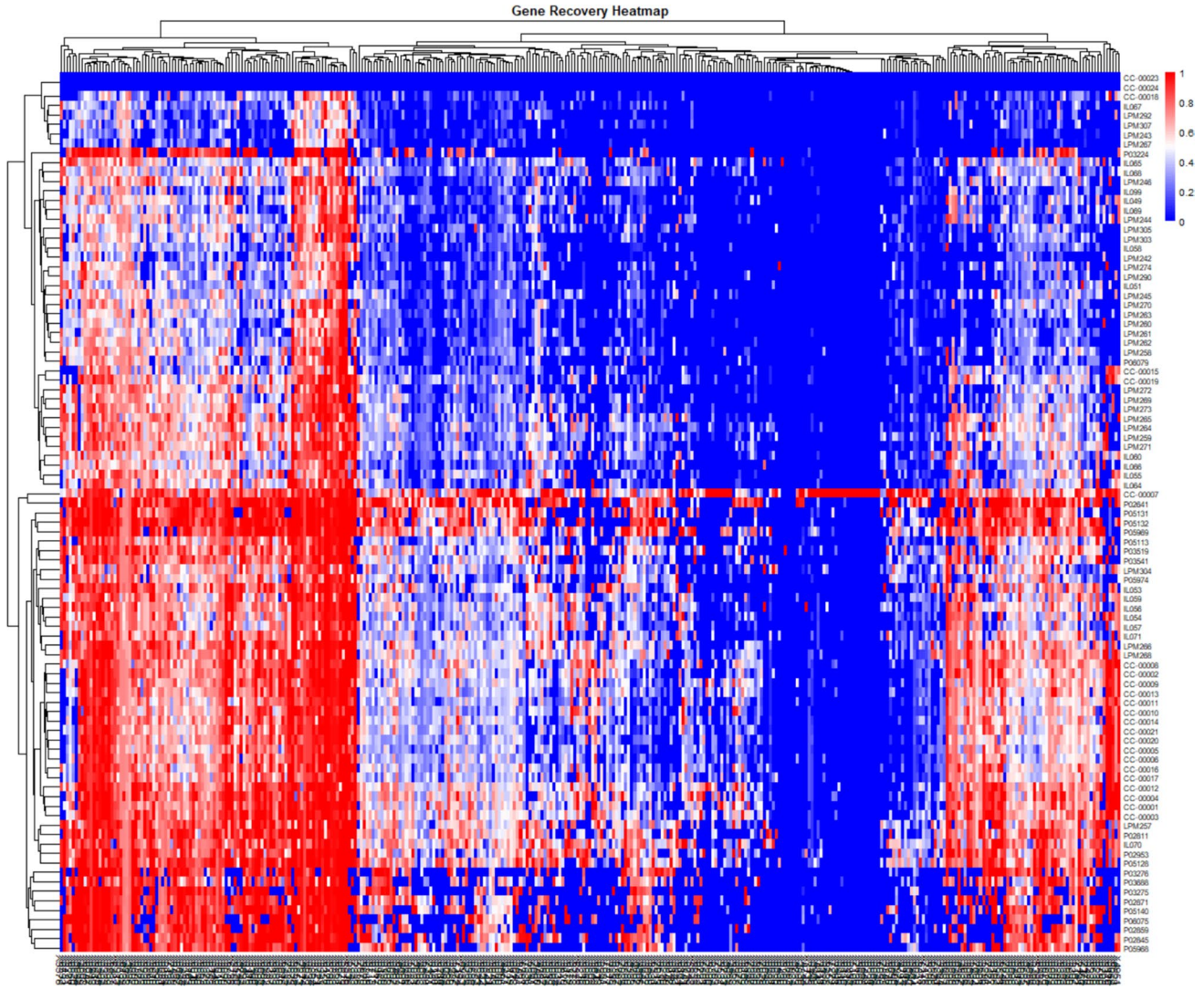


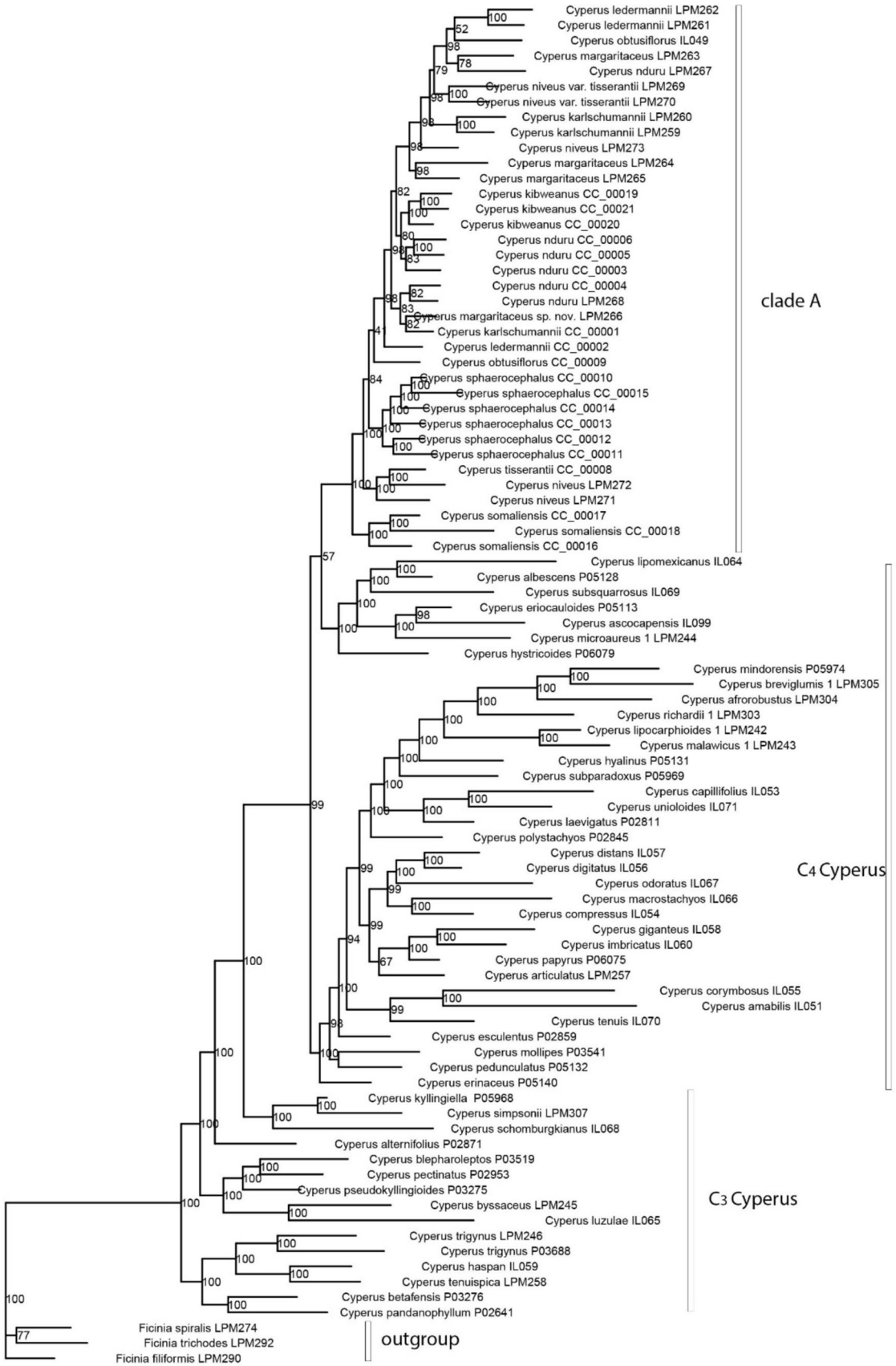

**Fig. 2** Heatmap showing the gene recovery rate for *Cyperus* accessions based on the mega353 target file. Individual genes shown along the bottom. Accessions shown on the right. *Red* maximum recovery; *blue* zero recovery

aligned. Following alignment cleaning, the DNA target file workflow retrieved 275 out of the 279 genes for downstream analysis, whereas the AA and mega353 target file pipelines retrieved 344 and 350 genes, respectively, out of the 353 genes that contained sequence data. Alignments with less than 3 sequences/gene were also removed, resulting in 326 genes for tree building, using the AA target file and 334 genes using the mega353 file. Since the mega353 file produced the highest gene recovery, we focus the rest of our results on the workflow using the mega353 file.

## Phylogenetic relationships

The results of the total-evidence MLE and the MSC species trees are shown in Figs. 3 and 4, respectively. Both the MLE and MSC trees showed the *C. margaritaceus–niveus* complex to form a monophyletic group (clade A), sister to the core C4 *Cyperus* clade showing 100% ultra-fast bootstrap (UFB) support, and 1.00 local posterior probability (LPP), respectively.

The MLE and MSC species trees show various incongruences with respect to clade A. The total-evidence MLE tree receives maximum (100% UFB) support for just over half of the nodes in clade A and medium (78–84% UFB support) to strong (98% UFB support) for many of the other nodes. Three of the groups in this clade were resolved as monophyletic: *C. kibweanus*, *C. sphaerocephalus*, and *C. somaliensis*. The ASTRAL tree showed similarly high levels of support within clade A and resolved monophyly for these three groups, although some nodes within these clades showed weak support and the *C. sphaerocephalus* clade appears nested within a *C. niveus* var. *leucocephalus* clade.


Cyperus ledermannii LPM262
Cyperus ledermannii LPM261
Cyperus obtusiflorus IL049
Cyperus margaritaceus LPM263
Cyperus nduru LPM267
Cyperus niveus var. tisserantii LPM269
Cyperus niveus var. tisserantii LPM270
Cyperus karlschumannii LPM260
Cyperus karlschumannii LPM259
Cyperus niveus LPM273
Cyperus margaritaceus LPM264
Cyperus margaritaceus LPM265
Cyperus kibweanus CC_00019
Cyperus kibweanus CC_00021
Cyperus kibweanus CC_00020
Cyperus nduru CC_00006
Cyperus nduru CC_00005
Cyperus nduru CC_00003
Cyperus nduru CC_00004
Cyperus nduru LPM268
Cyperus margaritaceus sp. nov. LPM266
Cyperus karlschumannii CC_00001
Cyperus ledermannii CC_00002
Cyperus obtusiflorus CC_00009
Cyperus sphaerocephalus CC_00010
Cyperus sphaerocephalus CC_00015
Cyperus sphaerocephalus CC_00014
Cyperus sphaerocephalus CC_00013
Cyperus sphaerocephalus CC_00012
Cyperus sphaerocephalus CC_00011
Cyperus tisserantii CC_00008
Cyperus niveus LPM272
Cyperus niveus LPM271
Cyperus somaliensis CC_00017
Cyperus somaliensis CC_00018
Cyperus somaliensis CC_00016
clade A
Cyperus lipomexicanus IL064
Cyperus albescens P05128
Cyperus subsquarrosus IL069
Cyperus eriocauloides P05113
Cyperus ascocapensis IL099
Cyperus microaureus 1 LPM244
Cyperus hystricoides P06079
Cyperus mindorensis P05974
Cyperus breviglumis 1 LPM305
Cyperus afrorobustus LPM304
Cyperus richardii 1 LPM303
Cyperus lipocarphioides 1 LPM242
Cyperus malawicus 1 LPM243
Cyperus hyalinus P05131
Cyperus subparadoxus P05969
Cyperus capillifolius IL053
Cyperus unioloides IL071
Cyperus laevigatus P02811
Cyperus polystachyos P02845
Cyperus distans IL057
Cyperus digitatus IL056
Cyperus odoratus IL067
Cyperus macrostachyos IL066
Cyperus compressus IL054
Cyperus giganteus IL058
Cyperus imbricatus IL060
Cyperus papyrus P06075
Cyperus articulatus LPM257
Cyperus corymbosus IL055
Cyperus amabilis IL051
Cyperus tenuis IL070
Cyperus esculentus P02859
Cyperus mollipes P03541
Cyperus pedunculatus P05132
Cyperus erinaceus P05140
C4 Cyperus
Cyperus kyllingiella P05968
Cyperus simpsonii LPM307
Cyperus schomburgkianus IL068
Cyperus alternifolius P02871
Cyperus blepharoleptos P03519
Cyperus pectinatus P02953
Cyperus pseudokyllingioides P03275
Cyperus byssaceus LPM245
Cyperus luzulae IL065
Cyperus trigynus LPM246
Cyperus trigynus P03688
Cyperus haspan IL059
Cyperus tenuispica LPM258
Cyperus betafensis P03276
Cyperus pandanophyllum P02641
C3 Cyperus
Ficinia spiralis LPM274
Ficinia trichodes LPM292
Ficinia filiformis LPM290
outgroup

◂**Fig. 3** Phylogenetic reconstructions using IQ-TREE tree of the relationships within the *Cyperus margaritaceus–niveus* complex and its relationship to the core C4 *Cyperus* clade. Branch values represent Bootstrap (BS) support values

Two of the groups, *C. karlschumannii* and *C. ledermannii,* were resolved as polyphyletic in the MLE tree, which contrasts with the MSC tree, which resolved both groups as monophyletic, showing 1.00 LPP support. Between these two clades in the MSC tree lies a monophyletic clade corresponding to *C. niveus* var. *tisserantii.* consisting of two accessions (LPM269 and LPM270). The two accessions are from Ivory Coast and Ethiopia, and their names are based on the species *C. tisserantii* being reduced to a variety of *C. niveus* in many African floras. However, both sheets have *C. tisserantii* as their most recent determination. A third accession, CC_00008, identified as *C. tisserantii* from Kenya, is determined on the sheet as *C. niveus* var. *tisserantii.* This accession comes out in a clade with *C. niveus* var. *leucocephalus,* sister to *C. somaliensis.* The MLE tree showed similar placements for the three accessions of *C. tisserantii,* though the LPM269-LPM270 clade (100% UFB support) is placed elsewhere.

Three further taxa are retrieved as non-monophyletic in both the MLE and the MSC species trees: *C. margaritaceus, C. nduru*, and *C. niveus* var. *leucocephalus*. The first two are retrieved as polyphyletic, and in the case of the *C. nduru* accessions, CC_00006 (Tanzania), CC_00005 (Zimbabwe) & CC_00003 (Democratic Republic of Congo) form a clade, showing 83% UFB support and 0.87 LPP). In the MSC species tree, this clade was sister to LPM273 (*C. niveus*), LPM267 (*C. nduru* from Zambia) and LPM263 (*C. margaritaceus*), while for the total-evidence MLE tree, the clade is sister to another *C. nduru* clade comprising of two specimens (CC_00004 and LPM268) from Guinea. This smaller clade is also present in the MSC tree but is placed elsewhere, separate from the other nduru clade. The remaining *C. nduru* accession, CC_00007 from Zambia, is resolved in the MSC species tree as an isolated lineage but was absent from the MLE tree after removal by Treeshrink. Earlier iterations of the MLE tree, based on both DNA and AA target files indicated that CC_00007 caused long-branch attraction, which led to its removal in the analysis using the mega353 file.

The correct name for the accessions named as *C. niveus* and *C. obtusiflorus* in our phylogeny is *C. niveus* var. *leucocephalus*, except for LPM273 (*C. niveus* from Tanzania), which is a misidentification of *C. margaritaceus*. The following accessions, LPM272, CC_00008, and LPM271 were re-identified as *C. niveus* var. *leucocephalus* and form a clade (100% UFB and 1.00 LPP support values) sister to the *C. sphaerocephalus* clade in the MLE tree. In the MSC tree, the *C. sphaerocephalus* clade is sister to CC_00009; an isolated lineage of *C. niveus* var. *leucocephalus* from Madagascar. Hence, *C. niveus* var. *leucocephalus,* in the MSC tree, appears to be paraphyletic but is poorly resolved receiving 0.47 LPP support. The accession IL049 (*C. niveus* var. *leucocephalus* from Madagascar) is also resolved as an isolated lineage in the MSC tree and is placed next to the *C. sphaerocephalus* clade.

### Phylogenetic networks

The first phylogenetic network generated, implementing SNaQ in PhyloNetworks, is shown in Online Resource 4. A table of corresponding concordance factors is given in Online Resource 5. Only one potential hybridization event was identified between CC-00001 (*C. karlschumanii*) and the clade consisting of *C. sphaerocephalus* and elements of *C. niveus* var. *leucocephalus*. This is an unusual result since the MSC species tree shows *C. karlschumannii* to be monophyletic and *C. sphaerocephalus* to also be monophyletic and sister to the *C. niveus* var. *leucocephalus* clade. We expected hybridization to occur between *C. nduru* and *C. margaritaceus* since these two taxa display polyphyly in the ASTRAL tree. However, considering ecological differences that exist in these two taxa (see Discussion), it is possible that the separate clades are genetically distinct enough that hybridization is ruled out as a possible cause. Other explanations for polyphyly can be sought such as polyploidy events, and although chromosome variation has been extensively studied in the Cyperaceae (Márquez-Corro et al. 2019), no chromosome counts have thus far been conducted on our species complex.

## Discussion

### Integrative taxonomy and the nine clades of the *Cyperus margaritaceus–niveus* complex

The description and classification of new species based on morphological data—alpha taxonomy, is in essence, a subjective practice, since morphology can be interpreted in different ways by different observers. Over the course of the twentieth century, systematists have sought to objectify the taxonomic process by utilizing datasets that are less prone to conjecture e.g. biochemistry, palynology and most recently molecular data, but in doing so have upheld each data source in turn, as the ultimate tool for resolving classification. The integration of multiple data sources to frame species limits, otherwise known as integrative taxonomy (Yeates et al. 2011) has gained traction in recent years and has already helped in resolving difficult taxonomic groups (e.g. Shah et al. 2023; Pastori et al. 2018). We adopt this integrative approach for our complex to explain the molecular results

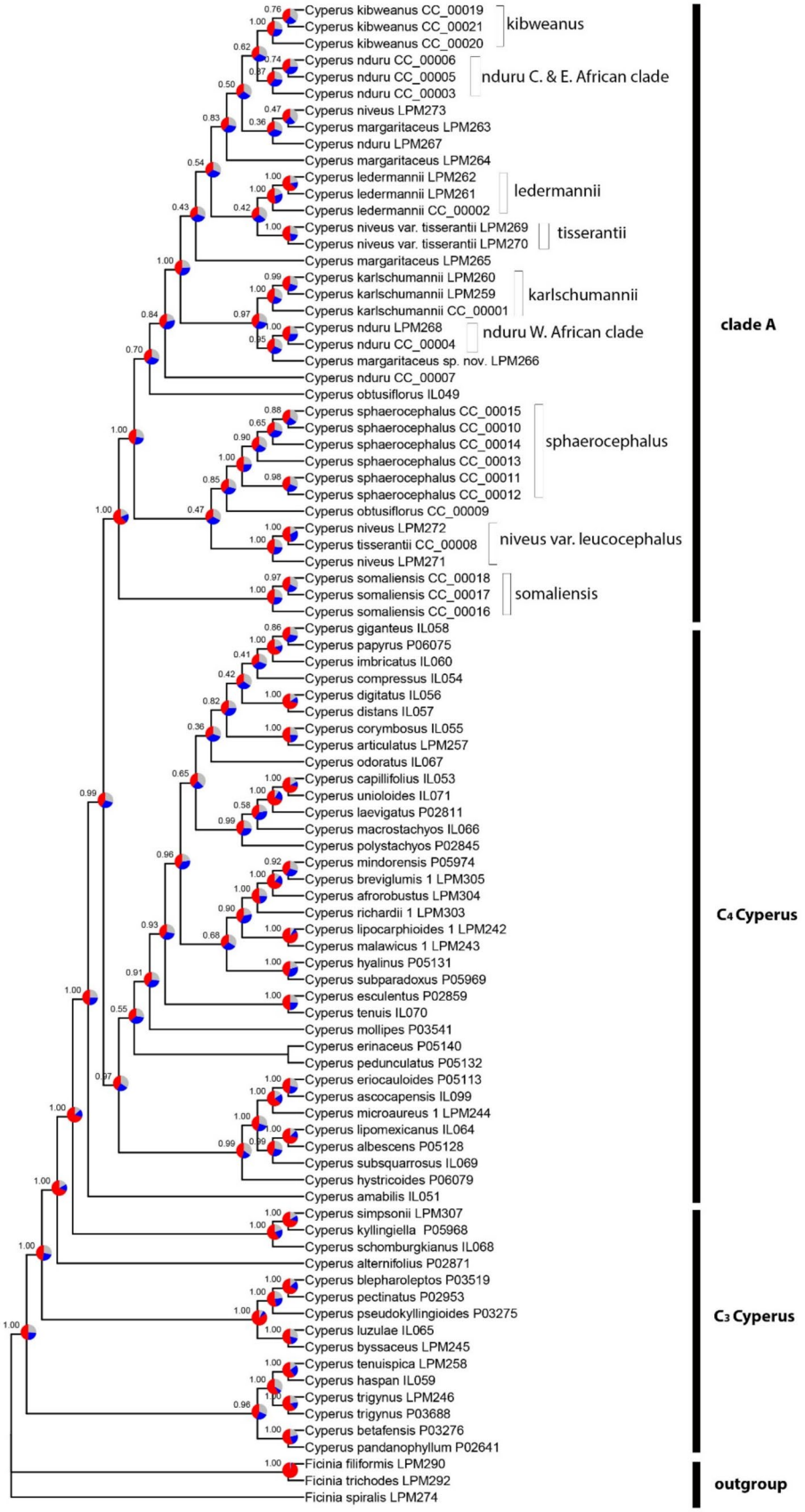



**Fig. 4** Phylogenetic reconstructions using ASTRAL of the relationships within the *Cyperus margaritaceus–niveus* complex and its relationship to the core C4 *Cyperus* clade. Branch values represent Local Posterior Probability (LPP) values. Pie charts visualizing quartet support values at the nodes (*red* main topology for that branch; *blue* 1st alternative topology; *grey* 2nd alternative topology.

in the context of previous analyses, i.e. morphometrics and morphological re-examination of specimens.

The complex, referred to as clade A in Fig. 4, is a well-supported monophyletic group with both 100% UFB support & 1.00 LPP support, and is sister to the core C4 *Cyperus* clade that includes the former segregate genera. This is a significant result given that it corroborates the findings of Larridon et al. (2020), which did not include all representatives of the complex, notably *C. kibweanus, C. sphaerocephalus*, and *C. somaliensis*, which to our knowledge is the first time that these species have been included in a molecular study. This finding also decreases the likelihood that the monophyly of this complex is a sampling artefact as postulated by Larridon et al. (2020). This, however, does not imply that the 'white-glume' trait evolved once even in C4 *Cyperus,* as several species not allied with the complex also have white glumes e.g. *C. angolensis*, as well as species in former segregate genera such as *Kyllinga, Lipocarpha*, and *Pycreus.* The incongruences relating to clade A between the IQ-TREE and ASTRAL tree is not an uncommon scenario (Tonini et al. 2015), and incomplete lineage sorting is a well-understood cause even occurring between gene trees (e.g. Yang et al. 2013; Badia et al. 2025). For the ASTRAL tree, six of the taxa in clade A are resolved as monophyletic, while *C. niveus* var. *leucocephalus* is resolved as paraphyletic and both *C. nduru* and *C. margaritaceus* are resolved as polyphyletic. Of the six monophyletic clades, only *C. karlschumannii*, *C. tisserantii*, and *C. sphaerocephalus* were also assigned as morphospecies (Xanthos et al. 2023). For the polyphyletic groups, *C. margaritaceus* and *C. nduru*, both were also assigned as morphospecies. Although Xanthos et al. (2023) found that each of these morphospecies were split into two groups, we found it hard to find clear morphological differences between them, despite the characters chosen by the CART analysis to separate them. Nor was there a clear geographical separation between the two groups for either morphospecies. In our phylogeny, clearer patterns are revealed regarding the placement of accessions for these two taxa and these are discussed below in conjunction with the nine clades that are recovered here as part of clade A.

## The kibweanus clade

This well-supported clade (100% UFB support and 1.00 LPP) consists of a single species, *C. kibweanus*, first described by Duvigneaud and Denaeyer-De Smet (1963) characterized by its yellow spikelets, filiform leaves, deeply furrowed glumes, and its close affinity with copper-rich soils. It was regarded by its authors as a 'little-known species' probably due to a lack of herbarium material and observational data. The three accessions used in this phylogeny represent all known specimens of this taxon, excluding the type. The species appears to be endemic to the Katanga Region of the Democratic Republic of the Congo and only six specimens, including the type, have been traced so far. Despite its characteristic yellow spikelets, the authors of the species allied it with the *C. margaritaceus* complex without providing further explanation. Nevertheless, our results show not only that *C. kibweanus* forms a clade but is part of the clade that unites the species in this complex. We therefore conclude that this taxon is part of the *C. margaritaceus* complex.

## The nduru clade

This clade is comprised mostly of *C. nduru* accessions from Central and Eastern Africa and is poorly supported in the MSC tree (0.50 LPP). It is divided into two smaller clades, one of which consists of three *C. nduru* accessions, CC_00006 (Tanzania), CC_00005 (Zimbabwe) & CC_00003 (Democratic Republic of Congo), has moderate support (0.87 LPP) and is here referred to as the C. & E. African *nduru* clade. Its sister clade is poorly resolved (0.36 LPP) and includes two accessions (LPM273; *C. niveus* from Tanzania and LPM263; *C. margaritaceus* from Zambia) we believe to be misidentified. *Cyperus nduru* is characterized morphologically by forming large clumps with hard basal sheaths, accrescent culms, and very short involucral bracts (Chermezon 1931). Poorly developed leaves were also cited as a diagnostic character, although well-developed leaves have been seen on specimens by the first author. Based on this combination of characters, we re-identify the accession, LPM263, as *C. nduru*, although the involucral bracts are longer than found for this species. LPM273 is re-identified as *C. margaritaceus* and is discussed later. The other *C. nduru* clade in the MSC tree consist of two accessions from Guinea receiving maximum support (1.00 LPP). Floristic treatments have circumscribed this taxon differently. Hooper and Napper (1972), in the Flora of West Tropical Africa, who retained it at species level, while Haines and Lye (1983) in their 'Sedges & Rushes of East Africa' reduced it to a variety of *C. margaritaceus*, though using the same characters to distinguish it at varietal level as Chermezon (1931) did at species level. Morphometric analysis by Xanthos et al. (2023) revealed that *C. nduru* was potentially comprised of two taxa based on glume width, but a re-examination of the specimens used in that study similarly did not reveal any other discernable differences between the two groups, nor was there a clear geographical correlation between the two groups, although the 'nduru2' group had more specimens from East and South Tropical Africa. The MSC species tree, however, shows a clear separation between the nduru C. & E. African clade and the nduru W. African clade, except for CC_00007 from Zambia, which receives moderate support (0.84 LPP) as an isolated lineage. The placement of these 'nduru' accessions in the phylogeny appears to show

an ecological correlation where the accessions in the nduru C. & E. African clade all occur in Miombo woodland and all have a very substantial culm base; in some accessions up to 1.5 cm thick. The Miombo typically stretches from Angola to Mozambique extending into Tanzania and Democratic Republic of the Congo (White 1983; Malmer 2007), and this habitat range overlaps with this clade. Whereas the accessions in the nduru W. African clade occur in bowal grassland and have less substantial, more flattened, culm bases. This is reflected in the ecology where natural fires are far less commonplace than they are in Miombo, hence there is less of a need for plants to invest in underground bulbous bases. The accession, CC_00007, occurs in Zambia and thus occurs in Miombo habitat. Unfortunately, the specimen is of poor quality with the basal parts missing.

Although the nduru clade is difficult to characterize morphologically, our results suggest that *C. nduru* from Miombo is distinct from *C. nduru* from Bowal grassland and hence may justify a taxonomic reappraisal of this species. Such ecological factors may also affect the morphology of other taxa in this complex e.g. *C. margaritaceus*. Haines and Lye (1983) postulated on their circumscribed varieties of *C. margaritaceus* as merely ecological forms of *C. margaritaceus* and that fire experiments in the field would determine their taxonomic position. Our results suggest two ecotypes of this species that can be separated based on their ecology and underground components. Further examination of specimens from both these vegetation types will help to confirm whether *C. nduru* can be separated into two taxa.

## The ledermannii clade

The taxonomic status of *C. ledermannii,* has remained in a state of flux since Kükenthal (1936) originally described the taxon as a variety of *C. obtusiflorus,* citing the longer and multi-flowered spikelets as distinguishing characters. Hooper (1972) elevated the taxon to species level stating that "this taxon seems as worthy as others in the *C. obtusiflorus-margaritaceus* group of specific recognition" on account of the same characters used by Kükenthal, although later adding 'flattened confluent spikelets' in the flora of west tropical Africa (Hooper and Napper 1972). Haines and Lye (1983) then reduced this species to a variety of *C. niveus* but without citing any specimens and stating that it was only recorded from Tanzania. Hooper (1972) stated that 'its occurrence in East Africa seems probable', although no specimens from this flora area have been seen for this study. Morphometric analysis by Xanthos et al. (2023) failed to distinguish this taxon as a separate morphospecies, at least using the CART analysis, with most of the specimens assigned, perhaps unsurprisingly, to *C. obtusiflorus.* This result alone lends itself to support the circumscriptions of Kükenthal (1936) and Haines & Lye (1983). However, due to the strong phylogenetic support in the ASTRAL tree (1.00 LPP), we are inclined to support the treatment of *C.ledermannii* as a distinct taxon at the rank of species.

## The tisserantii clade

This clade, sister to the ledermanii clade, is named as *Cyperus niveus* var. *tisserantii* receiving maximum support (1.00 LPP). *C. tisserantii* was originally described, along with *C. nduru*, by Chermezon (1931), who distinguished it from *C. nduru*, by its less clump-forming appearance, shorter & filiform stems, more numerous and well-developed leaves, longer involucral bracts, more numerous but smaller spikelets and longer glumes. Kükenthal (1936) then reduced it to a variety of 'margaritaceus' but without providing an explanation and this varietal change was adopted by Haines and Lye (1983). The name, as it appears in the phylogeny, comes from its taxonomic re-circumscription, according to several east African floras (Browning et al. 2020; Hoenselaar et al. 2010; Lye 1997), who again without explaining their reasoning, made it a variety of niveus. A re-examination of the specimens suggests that, given the suite of characters outlined by Chermezon (1931) that *C. tisserantii* is morphologically distinguishable from either species and our results show, with two of the accessions forming a clade that the taxon could be considered at species level. The third accession with this name in our phylogeny, comes out in an altogether different clade with two accessions named *C. niveus.* Re-examination of these three specimens suggests misidentification of these accessions. CC_00008 is a specimen from Uganda identified as *C. niveus* var. *tisserantii* but the plant base is a series of connected culm bases, akin to *C. margaritaceus*, and the inflorescence consist of at least 10 (probably more) spikelets, while the leaves are more flat than filiform. Therefore, we deduce that this specimen should be identified as *C. niveus* var. *leucocephalus.* The implications of this in relation to its position in the phylogeny are discussed below.

## The karlschumannii clade

This clade consists of two sub-clades, a monophyletic *C. karlschumanii*, and the nduru W. African clade, which includes the accession LPM266 receiving strong support (0.95 LPP). Although the karlschumannii sub-clade receives full support (1.00 LPP) there remains the accession LPM264, which is resolved as an isolated lineage and morphologically resembles *C. karlschumannii,* based on its very long spikelets and broad boat-shaped glumes. It is difficult to explain the placement of this accession given the moderate support it receives in the MSC tree (0.83 LPP). *Cyperus karlschumanii* is a morphologically distinct species, confined to West Tropical Africa and distinguished by the characters set out above. This species was also recognized

in the morphometric analysis (Xanthos et al. 2023) as a distinct entity and has undergone very little taxonomic flux since it was first described by Charles Clarke (Clarke 1906), although Kükenthal (1936) considered it a variety of *C. margaritaceus.* Based on the characters used to define the species as presented by Hooper and Napper (1972), combined with the morphometric analysis and the strongly supported sub-clade, it is hard to currently justify that this taxon may be polyphyletic. Until more sampling is carried out, we are therefore inclined to maintain this taxon at species level.

### The sphaerocephalus clade

This clade receives maximum support in both the MLE and MSC tree (100% UFB and 1.00 LPP) and is sister to a paraphyletic *C. niveus* var. *leucocaephalus* clade. The two species have a sympatric distribution in South Africa and superficially look similar; *C. sphaerocephalus* distinguished by its golden-yellow inflorescence and is, hence, commonly known as the 'golden-headed sedge'. Previous authors, however, have shown that differences are not confined to flower colour and have maintained both as independent species (Hilliard and Burtt 1986; Gordon-Gray 1995). Morphometric analysis on *C. sphaerocephalus* (Xanthos et al. 2023) had split the taxon into two morphospecies, based on glume width, but on re-examination of the two groups of specimens we do not find this to be a reliable character difference, although there was a geographical split between one group containing mostly specimens from South Africa and the other group containing specimens from Tanzania down to Zimbabwe, i.e. Miombo habitat. However, there does not appear to be clear morphological differences between the two groups even in the underground organs. The accessions used in our phylogeny came from various African countries, and since the taxon is resolved here as monophyletic, we do not postulate any potential splitting of this taxon. Our results, therefore, corroborate previous circumscriptions.

### The niveus var. leucocephalus clade

This clade with strong phylogenetic support from both the MLE and MSC tree consists of three specimens, LPM272, CC_00008 & LPM271, wrongly identified in the phylogeny. All three accessions have a compact head of up to 30 flattened spikelets, which place them morphologically under *C. niveus* var. *leucocephalus.* Xanthos et al. (2023) showed in their morphometric analysis, not only that *C. niveus* var. *leucocephalus* was a distinct entity, but that the type of *C. niveus* from India was assigned to the 'obtusiflorus.crt' group. *Cyperus niveus* var. *niveus* was not included in the morphometric study but it does have subtle morphological differences that appear to set it apart from the African complex. According to Fosberg (1977), the differences lay in the number of involucral bracts and the shape and colour of the spikelets. It is therefore unfortunate that the failure of the Asiatic samples in our methods leaves us unable to ascertain the phylogenetic position of the typical variety relative to the variety *leucocephalus* associated with Africa. Nevertheless, *C. niveus* var. *leucocephalus* is morphologically distinct from other members of the complex, primarily due to its inflorescence of up to 50 spikelets. However, in our phylogeny, the taxon is resolved as paraphyletic due to the clade being sister to the accession CC_00009, which is correctly identified as belonging to this species. This result is unexpected, as this accession does not bear any significant morphological differences to its sister clade, so we would've expected it to resolve itself within the *C. niveus* var. *leucocephalus* clade. The node at which both *C. sphaerocephalus* and *C. niveus* var. *leucocephalus* originate is poorly resolved (0.47 LPP), thus the proposal of treating both taxa as synonymous, to maintain monophyly of *C. niveus* var. *leucocephalus,* is hard to justify at present. More sampling is required, therefore, to resolve the paraphyly of this taxon.

### The somaliensis clade

This clade represents the earliest diverging clade of the *C. margaritaceus–niveus* complex and is resolved as monophyletic in both the MLE and MSC tree (100% UFB support and 1.00 LPP support). The morphometric analysis (Xanthos et al. 2023) did not support this species as a distinct entity with most of the specimens assigned to *C. tisserantii* and *C. nduru.* When Clarke (1895) first described the species, he did not provide a comparison between it and other taxa except to say that it was 'near *C.* leucocephalus' meaning *C. niveus* var. *leucocephalus*. The two species differ from each other in several morphological respects and one reason for the morphometric analysis assigning specimens to other morphospecies is the similarity of the short leaves and small spikelets with thin glumes; characters it shares with *C. nduru* and *C. tisserantii.* However, *C. somaliensis* is a considerably smaller plant than either species and has papery basal sheaths compared to the hard basal sheaths typical of the complex. Because of the strong phylogenetic support in our analysis, we confidently assess that this taxon is worthy of species level status. The taxon is not to be confused with *C. pseudosomaliensis*, also described by Clarke in the same paper, which belongs to the 'Mariscus' group of *Cyperus* and has never been associated with other members of the complex.

### *Cyperus margaritaceus* and polyphyly

The four accessions that are either correctly identified, re-identified or most closely resembling *C. margaritaceus* are LPM273 (Tanzania), LPM265 (Botswana), LPM266

(Angola) and IL049 (Zimbabwe). West African specimens of *C. margaritaceus* are, unfortunately, not represented in our phylogeny due to the accession LPM264 (Guinea) being an apparant misidentification of *C. karlschumannii*. However, the four accessions above from East and South Tropical Africa show subtle morphological differences between each other that may explain their positions in the MSC tree and may represent different taxa.

Amongst these four accessions, LPM273 (Tanzania) and IL049 (Zimbabwe) are the most morphologically typical of the species; contiguous culm bases, and an inflorescence of relatively few narrowly ovate spikelets per head, yet these two accessions do not form a clade and may possibly warrant further taxonomic investigation.

LPM265 from Botswana, has clearer morphological differences from the typical species in the size and number of spikelets. Other morphologically similar accessions, at K, seen from neighbouring countries, e.g. Mozambique, Zimbabwe, South Africa also have larger inflorescences atypical for *C. margaritaceus*, and while this name is currently applied to these specimens, some of them were previously determined as *C. pseudoniveus.* This name was originally applied to a specimen from Namibia (Schinz 1887) by Johann Boeckeler, although no distinction was given in the protologue between it and whichever taxon it most closely resembled according to Boeckeler. The protologue states that it 'approaches *C. niveus* Retz.' but does not mention *C. margaritaceus*, which on morphological grounds it more closely resembles. It is unlikely that Boeckeler, a cyperaceae specialist, would not have seen *C. margaritaceus*, but since the original publication of *C. pseudoniveus* formed part of an expedition summary by Schinz, we cannot be sure of what Boeckeler's concept was for this taxon. Nevertheless, this name was subsequently reduced to variety by Clarke (1902) under *C. margaritaceus,* then synonymised under this name in subsequent African floras (Hoenselaar et al. 2010; Browning et al. 2020). Gordon-Gray (1995), however, retained the name as a variety of *C. margaritaceus* for South Africa. Further examination of specimens is needed to determine whether LPM265 is part of this 'pseudoniveus' group and whether the name could be resurrected at varietal or species level.

LPM266, an interesting collection from Angola, named in the phylogeny as *C. margaritaceus,* but with morphological differences that set it apart from other species in this complex. The specimen has morphological affinities with *C. karlschumannii*, but differs in several respects, including considerably larger and wider spikelets, wider leaves and a woody rhizome—as opposed to a series of connected culm bases. Moreover, its habitat differs from members typical of the complex that grow in Miombo or bowal grassland. LPM266 was growing on highly leached Kalari sand that forms the dominant substrate of Central & Eastern Angola (Goyder et al. 2018). This leads to extremely nutrient-poor soils that exclude woody plants and are replaced by a unique flora predominated by geoxylic suffrutices (White 1977). While our specimen does not belong to this growth habit per se, the woody rhizome is a differential feature since the complex typically consists of underground parts that are either a series of connected culm bases giving the appearance of a rhizome, or a pseudobulbous base. The spikelets are also considerably larger than even those of *C. karlschumannii.* No other species from the complex was found from this catchment area (Goyder et al. 2018) and its phylogenetic placement, which puts it as part of the complex, makes it an unusual addition in terms of its morphology and habitat. We therefore postulate that this accession could represent a new species.

## Conclusion

This study marks the first attempt to understand this striking complex of species at the phylogenomic level. Our studies show that there is a singular origin to this group, and while some species within it have been resolved as clades, the relationships of others remain unclear, although the placement of *C. nduru* accessions reveals ecological differences and subtle morphological characters that can be used to split this taxon. An important limitation of our study is the low level of sampling, particularly the lack of representation for *C. margaritaceus*. Nevertheless, the placement of the *C. margaritaceus* accessions in the MSC tree have highlighted morphological differences in the taxon that may warrant further species delimitation. In addition, the relationship of the Asiatic species, *C. niveus* var. *niveus* is important, as it appears morphologically distinct from all other taxa, despite morphometric analysis assigning its type to *C. niveus* var. *leucocephalus* (Xanthos et al. 2023). It was therefore necessary to include *C. niveus* var. *niveus* in this study, and it's unfortunate that these accessions failed in our protocols. Future molecular studies of the complex should therefore include this Asiatic species to determine its relationship. It is also possible that the use of the Cyperaceae-specific kit would increase resolution of some of the taxa in our dataset, particularly that of *C. karlschumannii* and the apparent polyphyly of *C. niveus* var. *leucocephalus*, although data generated with family-specific kits do not necessarily have more power than those generated with universal kits (Larridon et al. 2020).

Despite the limiting factors outlined above, we have begun to build a clearer picture as to the relationships within this striking group of species, especially when utilizing the integrative taxonomy approach. We have also shown, in the case of *C. nduru*, that hitherto hidden ecological differences can be revealed from the phylogeny, which in turn highlight

subtle morphological differences that were not otherwise detectable through previous morphological observations. Even though the potential exists for incongruences between datasets, such integrative approaches are, we believe, an integral approach to understanding other species complexes in the rapidly diversifying C4 Cyperus Clade.

Electronic supplementary material consists of a dataset of all the accessions used in the phylogeny, scripts used in the bioinformatics and R analysis, an excel table of the recovery statistics for all target files used, and the results of Phylonetworks.

## Information on Electronic Supplementary Material

**Online Resource 1.** Dataset of all the accessions used in the phylogenetic analysis.
**Online Resource 2. a** Scripts used for the bioinformatic analyses on the HPC. **b** R scripts used or visualization of trees and heatmap.
**Online Resource 3.** Recovery statistics for gene lengths of all accessions and gene recovery percentages using DNA, AA and mega353 target files.
**Online Resource 4.** The first phylogenetic network generated implementing SNaQ in PhyloNetworks.
**Online Resource 5.** Table of concordance factors and related parameter estimates used to infer the first phylogenetic network shown in Online Resource 4.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00606-025-01983-w.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Conflict of interest** The authors declare no competing interests.



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