A phylogenetic analysis of the *Crinum rautanenianum* complex (Amaryllidaceae)

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Crinum macowanii , anonymous.

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Forord

Denne masteroppgaven markerer slutten på fem fine år på lektorstudiet ved Universitet i Oslo. Gjennom arbeidet med oppgaven har jeg fått et nytt syn på planteriket, og jeg gleder meg til å ta med elever ut i felt for å videreføre kunnskapen jeg har opparbeidet meg. Underveis i arbeidet har jeg fått god støtte fra mange, som jeg vil benytte anledningen til å takke.

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Abstract

Within genus *Crinum* L. there has been a lot of confusion, especially in the *Crinum rautanenianum* complex comprising *Crinum carolo-schmidtii*, *C. euchrophyllum*, *C. paludosum, C. rautanenianum*, and also a specimen referred to as sp. A in Flora of Tropical East Africa. Maximum parsimony and Bayesian inference analyses were conducted on two datasets: nuclear DNA region (ITS) and five plastid regions (*trn*L-F spacer, *trn*L intron, *rps*16 intron, *psb*A-*trn*H spacer, and *rps*12-*rpl*20 spacer). Additionally, morphological characters based on herbarium specimens, pictures from the field, type specimens, and protologues were studied to establish diagnostic characters within the complex. Results from phylogeny and morphological characters provide evidence for the reinstatement of *C. euchrophyllum*, recognition of *C. rautanenianum*, *C. carolo-schmidtii*, *C. paludosum*, and the description of a new taxon: *C. luangwense*.

Key words: *Crinum L*, pan species, phylogeny, morphology, new species.

1. Introduction

1.1 The genus *Crinum* L.

Crinum is a genus in the subtribe *Crininae* in Amaryllidaceae (Snijman and Linder, 1996). It was originally described by Linné (1753), who included four species in the genus. Today this pantropical genus includes 113 species (WCSP 2020), and is most species-rich in Africa (Nordal 1977). Despite that *Crinum* has been thoroughly studied, species circumscriptions are still discussed and new taxa are being described (Patel and Patel, 2019). Most *Crinum* species are geophytes and adapted to strong seasonality in savannas and pans. This seasonal cycle enables the plants to build up reserves during the wet season and shut down photosynthesis during the harsh, dry periods (Bjorå et al. 2009). This is an adaptation that will be increasingly important in the time of climatic change, where unpredictability in rainfall is increasing.

1.2 *Crinum rautanenianum* complex

One group in the genus that has caused a lot confusion is within the so-called "pan-species" (Bjorå et al. 2006; Kwembeya and Stedje, 2007). These species have developed adaptations to grow in seasonally flooded pans or depressions with clayey and poorly drained soils. This way of growth can make an impressive sight in big pans where thousands of *Crinum* flowers simultaneously (figure 1E). Although defined by habitat, they also share several morphological features which have resulted in nomenclatural confusion and numerous misidentifications in the past. I will here in particular focus on the "*Crinum rautanenianum* complex". Names from this group includes *C. carolo-schmidtii*, *C. euchrophyllum*, *C. paludosum, C. rautanenianum*, and a specimen referred to as sp. A in Flora of Tropical East Africa (FTEA, Nordal 1982). Current identifications and circumscriptions of these taxa are ambiguous because of conflicting treatments by several authors (figure 2) (Verdoorn 1973; Lehmiller 1997; Bjorå et al. 2006; Kwembeya 2006; Kwembeya et al. 2007; Kwembeya and Stedje, 2007; Zimudzi et al. 2008). Traditionally, Crinum has been divided into two sections depending on flower shape; radial star-shaped as *Stenaster* and zygomorphically bell-shaped as *Codonocrinum* (Herbert 1837; Baker 1898). All species in *C. rautanenianum* complex have bell-shaped flowers, and can be recognized by having mostly white tepals that turns pink to varying degrees with age. This is contrary to two related species, *C. macowanii* and *C. lugardiae*, both with a pink median stripe on the tepals, turning brownish with age. Another character that distinguishes the *C. rautanenianum* complex from their close relatives is the fruit. *Crinum macowanii* and

C. lugardiae have distinctly beaked fruits while the species within the complex only have a short beak or no beak at all.

Crinum rautanenianum was the first of these species to be described, with type specimen from Ovamboland in northern Namibia (Schinz 1896), followed by Dinter (1914) who described *C. carolo-schmidtii* from Hereroland in NE Namibia. In 1968, Verdoorn described *C. paludosum* as "the *Crinum* of the marshes" with type specimen from Natal, and mentioned *C. rautanenianum* as the sister-species. Verdoorn also described *C. euchrophyllum* in 1972 based on one population from eastern Zambezi region of Namibia.

In addition to all these species from southern Africa, another specimen from southern Tanzania was referred to as sp. A in FTEA, (Nordal 1982). She denoted that it resembles the closely related *C. paludosum* from Namibia and South Africa in number of flowers, but is probably closer to *C. euchrophyllum* in leaf arrangement (Nordal 1982). Verdoorn (1973) claimed that *C. euchrophyllum* was unique within the complex by having distichous leaf arrangement. Later, Lehmiller (1997) revised the genus for Namibia where he sunk Verdoorn's *C. euchrophyllum* into *C. rautanenianum* with the argument that the bulb was cultivated in a small pot so that it was stunted, which led Verdoorn to mistake the plant as a new species (Lehmiller 1997). Lehmiller (1997) also specified that *C. rautanenianum* differs from the rest of the pan species by its distichous, U-shaped leaves.

With respect to *Crinum carolo-schmidtii*, Verdoorn (1973) reported that this species stands out by having very narrow, flaccid leaves that are green and rosulate, as opposed to the glaucous and distichous ones of *C. euchrophyllum*. In contrast to other pan species, *C. rautanenianum* leaves are rather firm, suberect and deeply channelled. Bjorå et al. (2006), Kwembeya and Stedje (2007), and Kwembeya et al. (2007) all misinterpreted another specimen resembling *C.* sp. A sensu FTEA with *C. rautanenianum*. None of them included Lehmiller's (1997) former *C. euchrophyllum* in their analyses. Verdoorn (1973) also pointed out that *C. rautanenianum* and *C. carolo-schmidtii* have a restricted distribution in the northern regions of Namibia, *C. paludosum* is found in the centre of the province and eastwards to Zululand, whereas *C. euchrophyllum* was recorded from the eastern Zambezi region of Namibia. According to Flora Zambesiaca (FZ, Zimudzi et al. 2008) *C. rautanenianum* (where sp. A is included), grows in Botswana, Zambia, and in the Oshana, Omusati, Oshikoto, Ohangwena, and Kunene regions of Namibia.

Figure 1: Examples of pan species in their habitats. A: *Crinum carolo-schmidtii*. B: *Crinum rautanenianum* on seasonally flooded clayey soil. C: *Crinum paludosum* in flower. D: *Crinum* sp. A. E: Pan with thousands of *C. paludosum*. Photographs: National Herbarium of Namibia (A); Charlotte Sletten Bjorå (B & D); Ezekeil Kwembeya (C & E).

	C. sp. A sensu FTEA	*C. rautanenianum* Schinz 1894	*C. euchrophyllum* 1972	*C. carolo-schmidtii* 1914	*C. paludosum* 1968
Verdoorn 1973		*C. rautanenianum*	*C. euchrophyllum*	*C. carolo-schmidtii*	*C. paludosum*
FTEA 1982	*C. sp. A*				
Lehmiller 1997		*C. rautanenianum*	*C. rautanenianum*	*C. carolo-schmidtii*	*C. paludosum*
Bjorå et al. 2006	*C. rautanenianum*	*C. rautanenianum*		*C. carolo-schmidtii*	*C. paludosum*
Kwembeya 2006	*C. rautanenianum*	*C. rautanenianum*	*C. rautanenianum*	*C. carolo-schmidtii*	*C. paludosum*
Kwembeya et al. 2007	*C. rautanenianum*	*C. rautanenianum*		*C. carolo-schmidtii*	
FZ 2008	*C. rautanenianum*	*C. rautanenianum*	*C. rautanenianum*	*C. carolo-schmidtii*	*C. paludosum*

Figure 2: Historic overview of treatment of taxa in the *Crinum rautanenianum* complex. Colour code follows the name, blank area if the taxon is not included.

1.3 Aims

In this thesis, I shall review the species belonging to the *Crinum rautanenianum* complex, using both morphological and molecular analyses. I shall also highlight morphological characters of diagnostic value separating the species.

2. Material and methods

2.1 Plant material

Plant material in this study was obtained from herbarium specimens held at Oslo (O), National Herbarium of Zimbabwe (SRGH), and University of Zimbabwe (CAH); silica-dried leaf samples collected during field work in Zimbabwe in Nov/Dec 2019; leaf samples collected from the greenhouse at Natural History Museum in Oslo, Norway; and silica-dried leaf samples held at O. A total of 42 specimens (table 1) were studied. Study of morphological characters were conducted on herbarium specimens, pictures taken in the field, and protologues. Pictures of type specimens were also analysed to establish morphological differences between the pan species.

2.2 DNA extraction, PCR amplification and DNA sequencing

Total genomic DNA was extracted from silica-dried leaf samples using E.Z.N.A SP Plant mini kit (Omega Bio-Tek, Atlanta, USA) following the manufacturer's instructions with following exceptions: dried samples were grinded with 2 tungsten-carbide beads in TissueLyser Adapter at 25 Hz for 1 minute at a time till samples were crushed to fine powder; incubation time were changed to 60-70 minutes; 60 µL Elution Buffer was added before the samples were placed in a 65°C heating cabinet.

Nuclear ribosomal internal transcriber spacer region (ITS) and five plastid regions were amplified and sequenced. Earlier molecular studies of *Crinum* (Bjorå et al. 2006; Kwembeya et al. 2007; Bjorå et al. 2009) shows low resolution for pDNA trees, which is why I included four more regions for this study. In addition to the previously *trn*L-F spacer, the following regions were added: *trn*L intron, *rps*16 intron, *psb*A-*trn*H spacer, and *rps*12-*rpl*20 spacer. For ITS, primers ITS5 and ITS4 were used for all samples, and internal primers ITS2 and ITS3 for samples where the former primers did not produce good results. All primers were obtained from White et al. (1990). For *trn*L intron and *trn*L-F spacer, c and f were used for all samples, and internal primers e and d for samples where primers c and d failed to bring out good results (all primers were obtained from Taberlet et al. 1991). For *rps*16, primers *rps*16F and *rps*162R were used (Oxelman et al. 1997), while primers from Hamilton (1999) were used for *psb*A-*trn*H and *rps*12-*rpl*20.

The regions were amplified from 1-3 µL diluted or undiluted DNA. A master mix that contained buffer, $MgCl_2$, dNTP (10 mM), BSA (1g/L), water, both primers (10 µM) and AmpliTaq (Applied Biosystems, Foster City, CA, USA) was made. This was mixed with the template before I performed amplifications under the following cycling conditions: 94°C for 2.5 min, 32 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 50 s; followed by 72°C for 4 min. PCR products were purified using 2 µL diluted ExoStar (GE Healthcare UK Limited) together with 8 µL PCR product, incubated at 37°C for 45 min followed by 80°C for 15 min. An amount of 10-30 µL MilliQ H_2O was added to the samples depending on the intensity of the PCR bands. The samples were prepared for sequencing in Axygen plates with a total amount of 10 µL. For normal and strong PCR products they were filled with 7.5 µL cleaned PCR product and 2.5 µL primer (same primes as used for the PCR). For weak PCR products the plates were filled with 1.3 µL primer, 1.2 µL MilliQ H2O and 7.5 µL of cleaned PCR product. Axygen plates were sent to Macrogen Europe in the Netherlands for sequencing.

Table 1: Table of voucher information (taxon name, herbarium, voucher identification, and country of specimen sample) and GenBank accession numbers for DNA sequences used in the present study. Sequences generated for the present study are marked with x. Abbreviations: *A*. = *Ammocharis*; *B*. = *Boophone*; C. = *Crinum*; Herb. = voucher-holding herbarium, n/a = not available.

Taxon/Specimen No.	**Herb.**	**Voucher ID**	**Country**	**ITS**	***trn*L-F**	***rps*16**	***psb*A-*trn*H**	***rps*12-*rpl*20**
A. nerinoides (Baker) Lehmiller	O, WIND	Kwembeya et al. 186	Namibia	x	x	x	x	n/a
B. disticha Herb.	O	R 2020-19 O	South Africa	x	x	x	x	x
C. aurantiacum Lehmiller	O, WIND	Kwembeya et al. 138	Namibia	x	x	x	x	x
C. buphanoides Welw. ex Baker (1)	O, WIND	Kwembeya et al. 195	Namibia	x	x	x	x	x
C. buphanoides (2)	O, WIND	Kwembeya et al. 139	Namibia	x	x	x	x	x
C. buphanoides (3)	O, SRGH	Kwembeya et al. 35	Zambia	x	x	x	x	x
C. campanulatum Herb.	FTG	Meerow 2337	South Africa	AF373088.1	n/a	n/a	n/a	n/a
C. carolo-schmidtii Dinter	FTG	Meerow 2340	Namibia	AY139125.1	n/a	n/a	n/a	n/a
C. crassicaule Baker (1)	O, SRGH	Kwembeya & Nordal 15	Zambia	DQ386438	DQ388361	n/a	n/a	n/a
C. crassicaule (2)	SRGH	Chapano et al. 1858	Zimbabwe	x	x	x	x	x
C. crassicaule (3)	SRGH	Chapano et al. 1872	Zimbabwe	x	x	x	x	x
C. euchrophyllum I. Verd. (1)	O, SRGH	Kwembeya & Nordal 33	Zambia	x	x	x	n/a	x
C. euchrophyllum (2)	CAH	Zimudzi 603	Botswana	x	x	x	n/a	n/a
C. firmifolium Baker	M	Bogner 2915	Madagascar	x	n/a	n/a	n/a	n/a
C. glaucum A. Chev	O, P, YA	Nordal 971	Cameroon	x	x	n/a	n/a	n/a
C. jasonii Bjorå & Nordal	O	Nordal & Bjorå 5032	Zambia	x	x	x	x	x
C. kirkii Baker (1)	O	C. S. Bjorå 695	Tanzania	x	x	x	x	x
C. kirkii (2)	O	Nordal & Bjorå 5007	Tanzania	x	x	x	x	x
C. lugardiae N. E. Br.	CAH	Zimudzi B03	Botswana	x	x	x	x	x
C. ***macowanii*** Baker (1)	SRGH	Chapano et al. 1816	Zimbabwe	x	x	x	x	x
C. macowanii (2)	SRGH	Chapano et al. 1802	Zimbabwe	x	x	x	x	x
C. macowanii (3)	O, WIND	Kwembeya 137	Namibia	x	x	x	x	x
C. minimum Milne-Redh.	O	Nordal & Bjorå 5036	Zambia	x	x	x	x	x

... continued on the next page

Taxon/Specimen No.	**Herb.**	**Voucher ID**	**Country**	**ITS**	***trn*L-F**	***rps*16**	***psb*A-*trn*H**	***rps*12-*rpl*20**
C. moorei Hook.f.	O	R2020-35 O	South Africa	x	x	x	n/a	n/a
C. ornatum (L.f.) Herb.	O	74-608	Kenya	x	x	n/a	n/a	n/a
C. paludosum I. Verd.	O	R2020-36 O	South Africa	x	x	x	x	x
C. papillosum Nordal	O	Nordal & Bjorå 5069	Tanzania	x	x	x	x	x
C. purpurascens Herb.	O, P, YA	Nordal 902	Cameroon	x	n/a	n/a	n/a	n/a
C. rautanenianum Schinz (1)	O, WIND	Kwembeya et al. 175	Namibia	x	x	x	x	x
C. rautanenianum (2)	O, WIND	Kwembeya et al. 172	Namibia	x	x	x	n/a	x
C. sp. A (1)	O	Nordal & Bjorå 5060	Zambia	x	x	x	x	x
C. sp. A (2)	O	Nordal & Bjorå 5048	Zambia	x	x	x	n/a	x
C. stuhlmannii Baker	SRGH	B 403	Zimbabwe	x	x	x	x	n/a
C. variabile Herb. (1)	O	R2020-37 O	South Africa	x	n/a	n/a	n/a	n/a
C. variabile (2)	FTG	Meerow 2331	South Africa	AF373090.1	n/a	n/a	n/a	n/a
C. verdoorniae Lehmiller (1)	O, SRGH	Kwembeya & Nordal 31	Zambia	x	x	x	x	x
C. verdoorniae (2)	O, SRGH	Kwembeya & Nordal 23	Zambia	EF111003	EF119726	n/a	n/a	n/a
C. verdoorniae (3)	O, SRGH	Kwembeya & Nordal 26	Zambia	x	x	x	x	x
C. walteri Overkott (1)	CAH	Zimudzi 703	Botswana	x	x	n/a	n/a	n/a
C. walteri (2)	SRGH	Chapano et al. 1866	Zimbabwe	x	x	x	x	x
C. walteri (3)	SRGH	Chapano et al. 1859	Zimbabwe	x	x	x	x	x
C. welwitschii Baker	WIND	Kwembeya et al. 151	Namibia	x	x	x	x	x

2.3 Alignment and phylogenetic analysis

Geneious prime (https://www.geneious.com, Kearse et al. 2012) were used to automatic trim the low quality ends, assembling of the forward and reverse sequences using De Novo Assembly, and aligning of sequences. MUSCLE algorithms (Edgar 2004) were used to align all sequences in the different regions. All settings were default. Manual editing of alignments were performed in BioEdit 7.2.6.1 (Hall 1999). Insertions/deletions (indels) were coded as present/absent following the simple indel coding of Simmons & Ochoterena (2000). Sequences were analysed in two partitions: (1) ITS with 42 accessions; and (2) plastid DNA (pDNA) with 36 accessions. The datasets were analysed using maximum parsimony and Bayesian inference phylogenetic methods. For maximum parsimony analysis the alignments from BioEdit were manually formatted before the analyses were performed in TNT (Goloboff et al. 2003). *Boophone disticha* was chosen as outgroup. Heuristic searches were performed with 2000 replications of Wagner trees with maxtrees set to 10,000 and TBR as swapping algorithm. Otherwise default settings were used. The same approach and settings were used for both alignments. Jackknife (Farris et al. 1996) and bootstrap resampling (Felsenstein, 1985) studies were performed with 1000 replicates and with a cut-off value of 50%. For the Bayesian phylogenetic analyses, MrBayes v3.2.7 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) was used with prior models of nucleotide substitution set according to the AIC output of jModelTest v2.1.10 (Guindon and Gascuel 2003; Darriba et al. 2012). The analyses were run for 4,000,000 generations in four chains, one cold and three heated, saving trees every 1000th generation. Number of runs were set to 2 to check for an average standard deviation of split frequencies below 0.01. Burn-in was set to 25%. Maximum parsimony trees were congruent with trees for Bayesian inference, although the latter displayed more resolved trees.

3. Results

3.1 Alignments and phylogenetic analysis

The lengths for the aligned genetic regions were: ITS 741; *trn*L intron and *trn*L-F spacer 967; *rps*16 967; *psb*A-*trn*H 590; and, *rps*12-*rpl*20 795. Estimated best fit models of nucleotide substitution were: GTR+G for ITS; GTR+G for *trn*L intron and *trn*L-F spacer; HKY+G for and *trn*H-*psb*A; HKY+I for *rps*16; and GTR+I for *rps*12-*rpl*20. Tree statistics from the maximum parsimony analyses of the two datasets were: (1) ITS – 4 most parsimonious trees (MPTs) with length 283, rescaled consistency index (RC) = 0.9, and consistency index (CI) = 0.85; and, (2) pDNA – 7000 MPTs with length 143, and with RC = 0.77, and CI = 0.78. In the Bayesian analysis of the ITS and pDNA datasets, the average standard deviation of split frequencies (ASDSF) had decreased to 0.004298 and 0.004854 by termination.

Bayesian 50% majority rule tree had a higher resolution then MPTs. Therefore, all results presented are based on Bayesian analysis, but with jackknife results from maximum parsimony included. Jackknife consensus tree and bootstrap consensus tree were congruent (not shown). The Bayesian analysis on the ITS dataset was congruent with the maximum parsimony analysis, although the former was more resolved than the latter (not shown). For pDNA, Bayesian vs. maximum parsimony were not fully congruent (not shown), but this only accounts for a few nodes with very low support. The same applies to ITS vs. pDNA datasets (Figure 3 a and b; clades discussed in the following are indicated with corresponding capital letters and Latin numerals).

The nuclear ITS rendered a more resolved tree than the tree based on pDNA dataset. The two trees are largely congruent, with only two minor conflicts; in the ITS tree the accessions of *Crinum aurantiacum* and *C. papillosum* resolves in the clade i, while in the plastid tree the former resolves in the clade with *C. rautanenianum* (low support) and the latter with *C. jasonii* (well-supported). In general, the supported clades in my analyses are in concordance with generic subdivision shown in previous molecular analyses of *Crinum* (Bjorå et al. 2006; Kwembeya et al. 2007).

The nuclear tree is divided into two main clades; clade A that are the most species-rich, and clade B that consists of the subgroups "West-African *Codonocrinum*" (PP 1), "East-African *Codonocrinum*" (PP 1), and one representative of "Western *Stenaster*" (figure 3a). Clade A is

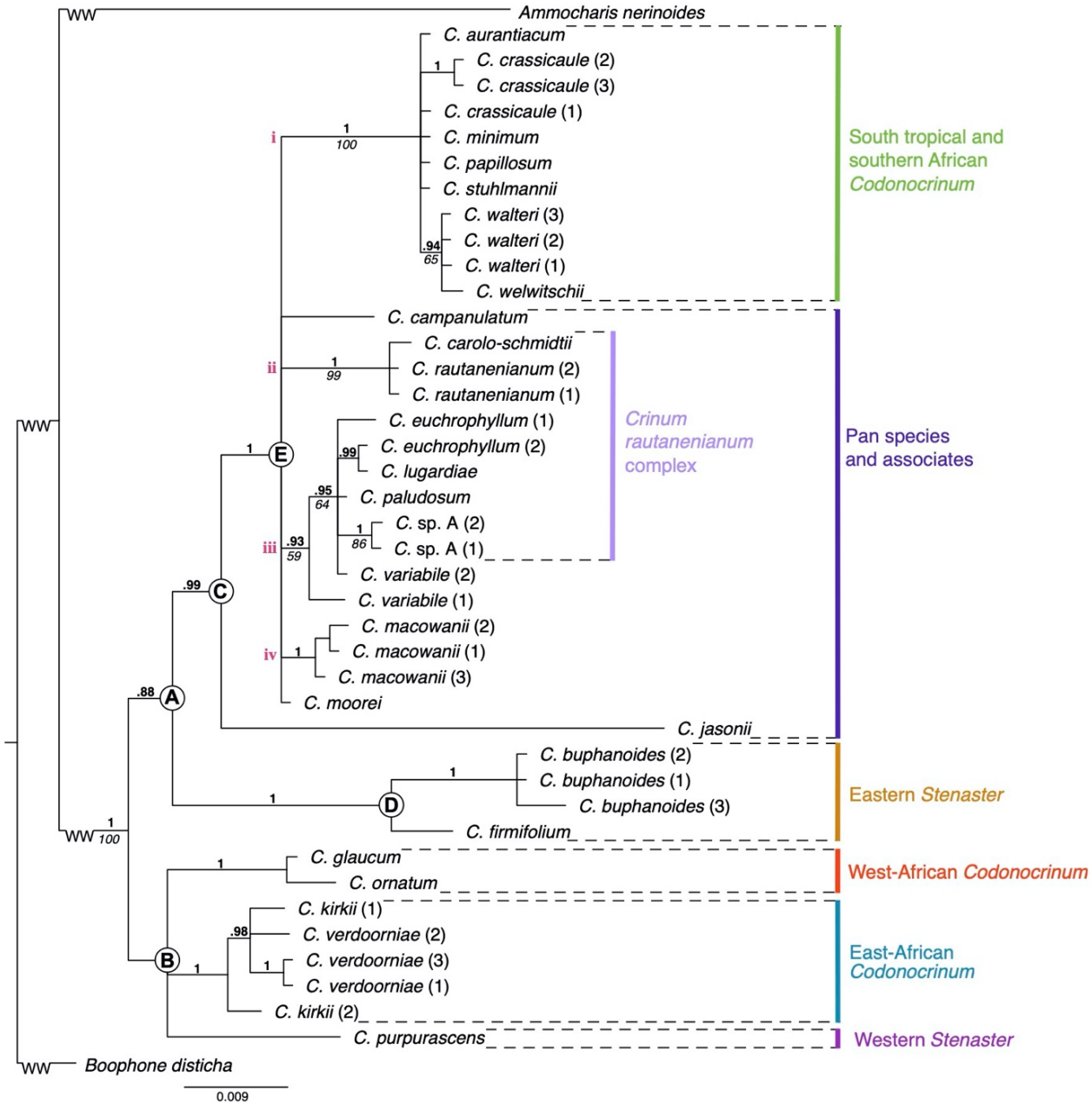
then split into two clades, the well-supported "Eastern *Stenaster*" (D; PP 1) and the large well-supported clade C (PP 0.99). *Crinum jasonii* resolves as a sister to the rest. Clade E displays a polytomy with larger subclades. One well-supported subclade (i; PP 1, JK 100) consists of species referred to as "South tropical and southern African *Codonocrinum*". The rest of this polytomy are the pan species and associates, while a part of this polytomy is the *C. rautanenianum* complex.

One of the subclade includes *Crinum rautanenianum* and *C. carolo-schmidtii* and are highly supported (ii; PP 1, JK 99). Unfortunately, I was unable to produce plastid sequences of *C. carolo-schmidtii*. In the plastid tree, *C. rautanenianum* resolves as sister to *C. aurantiacum* with very low support. The next clade (iii; PP 0.93, JK 59) includes *C. euchrophyllum*, *C. lugardiae*, *C. paludosum*, *C.* sp. A and *C. variabile*. Within this subclade, *C. euchrophyllum* (2) and *C. lugardiae* forms one group (PP 0.99), and *C.* sp. A another (PP 1, JK 86).

3.2 Morphology

The morphology studies revealed that the plants in the *Crinum rautanenianum* complex showed variation in the following characters: leaf orientation (rosulate vs. distichous), leaf width (1-6 cm), number of flowers (1-11), anther colour (yellow vs. black), and fruit (having no beak vs. having a short beak). See table 2 for how the different characters apply to the different taxa. Leaf orientation proved to be a character difficult to trust. This because plants in several populations are documented as distichous in young plants, and turning more rosulate with age (figure 4). In addition, leaf orientation can be difficult to interpret from pressed material. Cross section of mature leaves of *Crinum rautanenianum* and *C.* sp. A were also studied to clarify their differences (figure 5).

(a)


Ammocharis nerinoides
C. aurantiacum
C. crassicaule (2)
C. crassicaule (3)
C. crassicaule (1)
C. minimum
C. papillosum
C. stuhlmannii
C. walteri (3)
C. walteri (2)
C. walteri (1)
C. welwitschii
C. campanulatum
C. carolo-schmidtii
C. rautanenianum (2)
C. rautanenianum (1)
C. euchrophyllum (1)
C. euchrophyllum (2)
C. lugardiae
C. paludosum
C. sp. A (2)
C. sp. A (1)
C. variabile (2)
C. variabile (1)
C. macowanii (2)
C. macowanii (1)
C. macowanii (3)
C. moorei
C. jasonii
C. buphanoides (2)
C. buphanoides (1)
C. buphanoides (3)
C. firmifolium
C. glaucum
C. ornatum
C. kirkii (1)
C. verdoorniae (2)
C. verdoorniae (3)
C. verdoorniae (1)
C. kirkii (2)
C. purpurascens
Boophone disticha
0.009
i
ii
iii
iv
A
B
C
D
E
South tropical and southern African Codonocrinum
Crinum rautanenianum complex
Pan species and associates
Eastern Stenaster
West-African Codonocrinum
East-African Codonocrinum
Western Stenaster

(b)

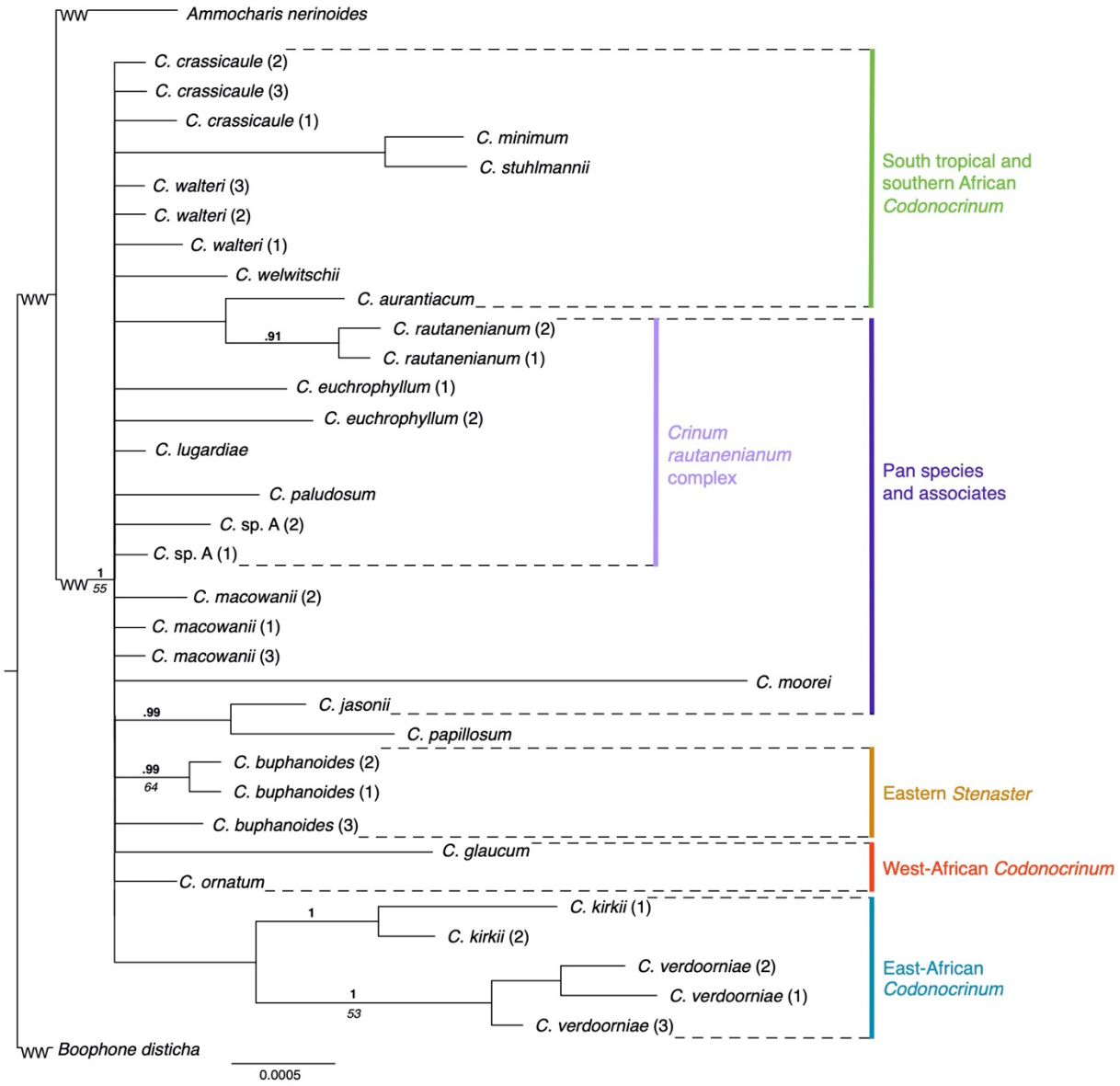


Figure 3: The 50% majority rule consensus phylogram for members of *Crinum* from Bayesian analyses of (**a**) ITS matrix with 42 accessions and 741 characters, and (**b**) the concatenated matrix of five plastid DNA regions (*rps*16, *trn*L intron, *trn*L-F spacer, *psb*A-*trn*H and *rps12-rpl20*), a total of 26 accessions with 3319 characters. Groups are named according to previous studies (Bjorå et al., 2006; Kwembeya et al., 2007) and the clades discussed in the text are marked with capital letters in a ring or Latin numerals. Bayesian posterior probability (PP) of at least 0.9 are included in bold above branches, whereas maximum parsimony jackknife support (JK) of at least 50% are included in italics below the branches. Multiple accessions of the same species are numbered according to Table 2. Abbreviations: *C.* = *Crinum*. Zigzag branch in the tree represents a manual shortening of long branches to reduce the width of the figure.

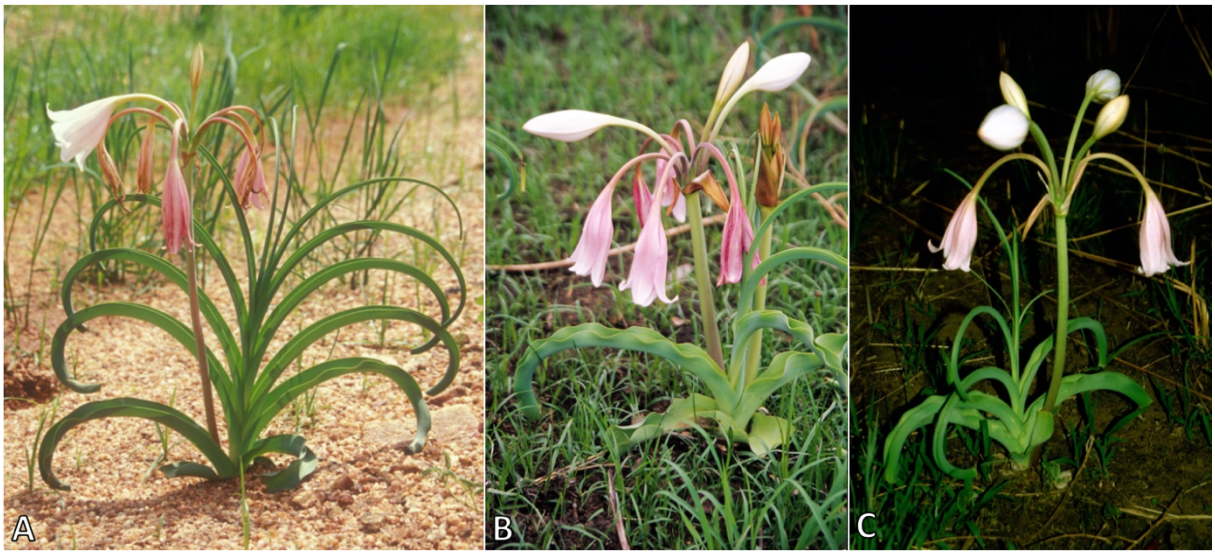


Figure 4: Different specimens of *Crinum* sp. A illustrating how leaf orientation can vary. A) is clearly distichous with oldest leaves somewhat turned. B) appearing rosulate. C) distichous in young leaves and more rosulate in older leaves. Photographs: Charlotte Sletten Bjorå.

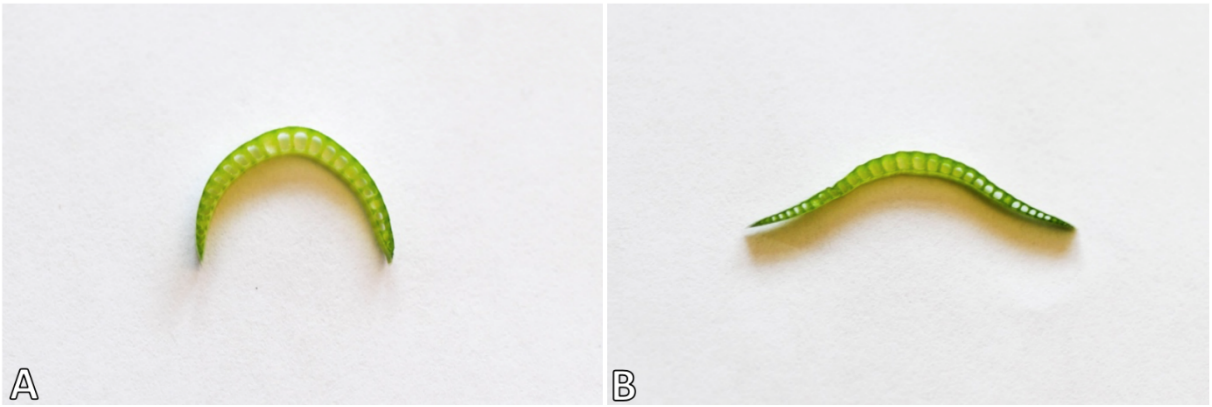


Figure 5: A) Cross section of a mature leaf of *Crinum rautanenianum*; B) Cross section of a mature the leaf of *Crinum* sp. A. Photographs: Charlotte Sletten Bjorå.

4. Discussion

4.1 Species delimitation in the *Crinum rautanenianum* complex

The tree based on ITS shows that the *Crinum rautanenianum* complex is not resolved as a monophyletic group, but in two different subclades (ii and iii) also including *C. variabile* and *C. lugardiae*. It is evident that *C. rautanenianum* from its type locality is not conspecific with *C.* sp. A, as the two taxa resolves in two different subclades, clade ii and iii, respectively. This is also supported by several morphological characters (table 2). The authors of FZ had not seen *C. rautanenianum* from the type locality (figure 1B) when they included *C.* sp. A (figure 1D) in *C. rautanenianum*, but based their information on the type and the protologue. The type of *C. rautanenianum* indeed gives the impression of being distichous and not very different from *C.* sp. A. However, as already mention (section 3.2), leaf orientation is a character that can be difficult to interpret from a pressed specimen.

The phylogenetic analyses show that *Crinum euchrophyllum* and *C. rautanenianum* are not conspecific. As seen in figure 2, Lehmiller (1997) included *C. euchrophyllum* in *C. rautanenianum*. When the taxon was described, Verdoorn (1972) emphasized that *C. euchrophyllum* differed from the rest of the pan species by being distichous. This trait has in recent circumscriptions (FZ) also been a character for *C. rautanenianum*. However, at the type locality all specimens were clearly rosulate, and the inclusion of this character for *C. rautanenianum* is probably a consequence of the too wide circumscription of this taxon. Another character that separates the taxa is colour of the anthers; both *C.* sp. A and *C. euchrophyllum* have black anthers, while *C. rautanenianum* have yellow anthers. Additionally, they both have wider leaves than *C. rautanenianum*, are distichous at least in young individuals, and the cross section of the leaves are different (figure 4 A and B). It is therefore natural to reinstate Verdoorn's (1972) *Crinum euchrophyllum* at species level.

The taxa here denoted as *Crinum* sp. A (figure 1D) was misinterpreted as *C. rautanenianum* (figure 1B) in several earlier studies (Bjorå et al. 2006; Kwembeya 2006; Kwembeya et al. 2007; Kwembeya and Stedje, 2007; Zimudzi et al. 2008). *Crinum* sp. A differs clearly from the rest of the *C. rautanenianum* complex in morphology (table 2) and distribution. *Crinum* sp. A (sensu FTEA) is found in southern Tanzania and can be connected to *C.* sp. A in Eastern Zambia through the Luangwa arid corridor described by Huntley & Walker in 1982. The rest of the species in the *C. rautanenianum* complex are mainly found in Namibia and

adjacent areas. Based on molecular analysis combined with morphology and distribution, it is clear that *C.* sp. A is a separate taxon (see section 6. Taxonomic treatment).

Despite having added four plastid regions, the resolution did unfortunately not improve significantly. As seen in table 1, some accessions lack one or several of the plastid regions. This may have had influence on the resolution of the pDNA tree. The resolution within clade ii and iii in the ITS tree is not very good. To fully understand the relation in this complex it is of great value to include more powerful genetic tools. *Crinum carolo-schmidtii* and *C. rautanenianum* are genetically very similar, and they share many morphological characters, but they differ in number of flowers and possibly anther colour (further discussed under section 4.2). Consequently, the relationship between these two taxa would be an interesting topic for further investigation.

4.2 Diagnostic characters

A part of this study included a closer inspection of morphological characters based on types, protologues, herbarium material, and live specimens. It was clear that several characters were ambiguous and had been scored differently in various studies. This has contributed to the uncertainty and the many misidentifications. Another problem is that several characters can be problematic to study in the herbarium as flowers and fruits are often absent or not well preserved (Bjorå et al. 2006). This applies to e.g. anther colour and leaf orientation, which is difficult to see on herbarium specimen like the types. Anther colour has been regarded to be of diagnostic value (Nordal pers. com.). This character is rarely mentioned in protologues, and as it is difficult to evaluate from old herbarium specimens, it has led to a lot of confusion. This applies particularly to *C. carolo-schmidtii* and *C. rautanenianum*. Verdoorn (1973) includes anther colour in drawings, but not in the text. For *C. carolo-schmidtii*, drawings show black anthers (Verdoorn 1973), while FZ describes them as yellow. Regarding *C. rautanenianum*, anthers are drawn yellow (Verdoorn 1973), but FZ says black. Yellow anthers can also turn brownish after anthesis, which can be misinterpreted as black (Nordal, pers. com.). Figure 4 from the type locality of the new species, *C.* sp. A, shows that leaf orientation may not be a good character for determination of species, as it looks like young plants have distichous leaves, and that the early leaf fan gets slightly twisted with age making it more rosulate. This might be the case also for other pan species, maybe except for the real *C. rautanenianum* that seems to be rosulate at all times. The cross section of the mature leaves of *C. rautanenianum* (A) and *C.* sp. A (B)

clearly differ from each other (figure 5). *Crinum rautanenianum* has a distinct U-shaped leaf (Lehmiller 1997), while the leaf of *C*. sp. A is more flat. They both have aerenchyma, as they grow in water.

The circumscription of *C. rautanenianum* in FZ was altered after *C. euchrophyllum* and *C*. sp. A were included. This has led to a misleading description of *C. rautanenianum* having black anthers and distichous leaf orientation. These characters can change with age. Therefore, it is of great advantage to study these in the field. Verdoorn (1973) mentions in her revision from 1973 that of *C. rautanenianum* "very few specimens have been preserved in South African herbaria". There are still not many herbarium specimens of these taxa, and good preserved specimens, preferably in flower and fruit, is vital to get information about the distribution and biology of this complicated group, where the misidentifications have been so common. This again proves that more specimens should be collected and studied further, and characters like leaf arrangement and anther colour should be specified on the label.

Looking at table of different characters in the *Crinum rautanenianum* complex (table 2), it can be interpreted that *C*. sp. A are closer to *C. euchrophyllum* and *C. paludosum*. The former because of broad leaves and number of flowers, the latter because of distichous leaves and black anthers. This is consistent with what Nordal (1982) wrote about *C*. sp. A sensu FTEA (section 1.2). The same pattern can be interpreted from the tree, as they all are a part of subclade iii, while the other species in the complex are a part of subclade ii (figure 3a).

Of the characters I have studied, those that seem to be most important in deciding the species are anther colour, number of flowers, and width of leaves. Leaf orientation as a descriptive character should be treated with caution.

Before this study was conducted, the *Crinum rautanenianum* complex had three accepted species; *C. rautanenianum*, *C. paludosum*, and *C. carolo-schmidtii*. Based on study of morphological characters and molecular analyses, I now expand the group to five species; *C. rautanenianum*, *C. paludosum*, *C. carolo-schmidtii*, *C. euchrophyllum*, and *C*. sp. A. Because of this, *C. euchrophyllum* and *C.* sp. A should be removed and description, distribution, material examined, and the conservation status of *C. rautanenianum* should be revisited.

Table 2: Character states of some important traits that can distinguish species from the *Crinum rautanenianum* complex from each other. Abbreviations: FTEA = Flora of Tropical East Africa.

	***C. rautanenianum* (Schinz 1896)**	***C. carolo-schmidtii* (Dinter 1914)**	***C. paludosum* (Verdoorn 1968)**	***C. euchrophyllum* (Verdoorn 1972)**	***C.* sp. A of FTEA (Nordal 1982)**
Leaf orientation	Rosulate	Rosulate	Rosulate	Distichous	Distichous in early stages, turning more rosulate with age
Leaf width	2-3 cm broad	Around 1 cm broad	3-5 cm broad	Under 2.5 cm broad	Up to 6 cm
Number of flowers	Up to five-flowered umbels	1-3	5-11	1-2	5-8
Anther colour	Yellow	Conflicting information *	Yellow	Black	Black
Fruit	Not beaked	Not beaked	Not beaked	Not beaked	Short beak

* Conflicting information because of inadequate pictures, type material is difficult to interpret, possible misidentification, light brown colour could be noted as black, and not included in protologue.

5. Conclusion

To conclude, one new species will be described and one species, *Crinum euchrophyllum*, has been reinstated. The new taxon is described in section 6. Taxonomic treatment. As a result of these new species delimitations, some corrections are needed for key specimens used in previous studies and floras. These are included in a table of corrections as an appendix. This study has pinpointed some interesting topics for further studies: The two clades within *C. rautanenianum* complex (ii and iii in figure 3a) need more examination with an increased amount of accessions. It would be especially interesting to study the relationship between *C. carolo-schmidtii* and *C. rautanenianum*, and to study the relationship between *C.* sp. A, *C. euchrophyllum*, and *C. paludosum*.

6. Taxonomic treatment

I have concluded that the circumscription of *Crinum rautanenianum* has been too wide, and that a new taxon has to be described. The taxon known as *C.* sp. A from FTEA deserves taxonomic recognition at the level of species and is formally described below.

Crinum luangwense **Uttgaard & Bjorå** ***sp. nov.*** Crino rautaneniano affinis sed foliis latioribus, non canaliculatis, margine undulato, foliis novis distichis, postea leviter plees mineesve roseelatis, floribus juvenibus albis, maturis roseis, antheris atris, fructibus rubellatis rostellatis. Typus: Zambia: E; Katete Dist.: few km W of Katete between Chipata and Petauke, 14° 05' 05" S 31° 58' 64" E, alt. 1030, in floodplain, [withheld] (holoypus O!)

Bulb 7-10 cm diameter, narrowed to a neck at apex, up to 13 cm long. Leaves green to glaucous, distichous in early stages turning more rosulate later, rather firm with thin woolly fibres, young leaves deeply channelled, older leaves undulate, width up to 6 cm, length and width strongly depended on age, margin scabrous, most leaves necrotic apically, exude hyaline sap. Scape reddish below, erect, (25) 40-65 cm long; spathe valves 5-6 x 1.8-2.5 cm, soon becoming papery and reflexed. Flowers (3) 5-8 (10), white in bud and at anthesis, turning pink after anthesis, subpetiolate. Pedicels 0.5 cm. Perianth tube 9-12 cm long; segments 9-12 cm long, the inner 3-3.5 cm broad, the outer narrower, up to 2.6 cm wide, connivant into a bell with only the apices reflexed. Stamens declinate; filaments ca. 7 cm long; anthers black. Fruits reddish, 4-6 cm long, ca. 4 cm wide, with a short beak. Seeds smooth, with a greyish water-repellent outer layer, floating in water.

DISTRIBUTION. Zambia, Central: Katete District: Luangwa valley; East: South Luangwa national park. Tanzania T4. Ufipa District: near Lake Sundu.
MATERIAL EXAMINED. Zambia. E: Katete Dist.: few km W of Katete between Chipata and Petauke, 14° 05' 05" S 31° 58' 64" E, alt. 1030, in floodplain, 7. Dec 2004, *Nordal & Bjorå* 5060 (O); South Luangwa National Park, Checheli road after Mbombosa Wakumba 13° 07' 12" S 31° 41' 20" E, alt 535m, 4. Dec 2004, pan in water, *Nordal & Bjorå* 5048. Tanzania. Ufipa District: Lake Sundu T4., 11. Nov 1958, *Richards* 10296!, grasslands on sandy soils, 1500 m.
HABITAT. Floodplain
CONSERVATION STATUS. *Crinum luangwense* is so far only known from three populations. One population was documented in Ufipa District in 1958, but no further information is known.

The two last populations are found in Central and Easter Zambia. The population in Central Zambia is found within South Luangwa National Park, this should hopefully prevent the population from habitat destruction. The largest population is found a few km W of Katete and contains 100 000 mature specimens. Considering the lack of information about the different populations, it is not possible to assess this taxon with IUCN criteria at this moment. On the basis of few known populations and where it is found, I would consider it vulnerable.

Declaration of Competing Interests

No interests to declare.

Highlights

- Updated species boundaries within the *Crinum rautanenianum* complex
- Correction of the far too wide circumscription of *Crinum rautanenianum*
- Identification of morphological characters of diagnostic value in treating the species complex
- *Crinum euchrophyllum* is reinstated
- One new species is recognised and described

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Appendix

Table 1: Overview of earlier mistakes in light of *Crinum rautanenianum*, *C.* sp. A, and *C. luangwense* included publication where the mistakes been made, voucher identification, name applied in the publication, the correct name for it today and comment where necessary.

Publication	**Voucher ID**	**Name applied**	**Corrected name**	**Comment**
FTEA 1982	Richards 10296	*C.* sp. A	*C. luangwense*	*C.* sp. A now included in the description of *C. luangwense*
Lehmiller 1997		*C. rautanenianum*	*C. euchrophyllum*	
Bjorå et al. 2006	Nordal & Bjorå 5060	*C. rautanenianum*	*C. luangwense*	Flow cytometry 2n = 22 Genbank: DQ386447 DQ388367
Kwembeya 2006	Kwembeya & Nordal 33	*C. rautanenianum*	*C. euchrophyllum*	*C. euchrophyllum* a part of the circumscription of *C. rautanenianum*
Kwembeya 2006	Nordal & Bjorå 5060	*C. rautanenianum*	*C. luangwense*	*C. luangwense* a part of the circumscription of *C. rautanenianum*
Kwembeya & Stedje, 2006 *		*C. rautanenianum*	*C. luangwense*	Figure 7A
Nordal et al. 2006 **	Nordal & Bjorå 5060	*C. rautanenianum*	*C. luangwense*	Seed
Kwembeya et al. 2007	Nordal & Bjorå 5060	*C. rautanenianum*	*C. luangwense*	
FZ 2008	Kwembeya & Nordal 33	*C. rautanenianum*	*C. euchrophyllum*	*C. euchrophyllum* a part of the circumscription of *C. rautanenianum*
FZ 2008	Nordal & Bjorå 5060	*C. rautanenianum*	*C. luangwense*	*C. luangwense* a part of the circumscription of *C. rautanenianum*

* Kwembeya, E. G., Stedje, B., 2006. The genus *Crinum* (Amaryllidaceae) – its taxonomy, distribution and conservation status in Namibia. In: Kwembeya (Ed.)The genus *Crinum* (Amaryllidaceae) – its taxonomy, phylogeny and conservation in Southern Tropical Africa. Faculty of Mathematics, University of Oslo, V.

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