Fireball lilies of Africa: a molecular phylogeny of the genus *Scadoxus* Raf. (Amaryllidaceae)

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Illustration: Aasne Aarhus, 1976.

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Abstract

The species delimitation and phylogeny of the genus *Scadoxus* (Amaryllidaceae) have previously been based on morphological characters only. The aims of this study were to construct molecular phylogenies and test if the morphologically based phylogeny, species and subspecies delimitations were reflected by them. Maximum Likelihood and Bayesian Inference analyses were based on Sanger sequences of the nuclear ribosomal internal transcribed spacer (ITS1, 5.8S and ITS2) and six chloroplast regions (*trn*L-F, *trn*S-G, *psb*A-*trn*H, *rps*16, *rpl*20-5*rps*12 and *Mat*K) for 79 and 71 accessions, respectively. Additionally, Maximum Likelihood analysis was performed of whole chloroplast genomes retrieved by Ion Torrent sequencing for a subset of 22 *Scadoxus* accessions. All previously recognized species or subspecies were included in the molecular phylogeny except *S. longifolius.*

The molecular phylogenies largely support previous morphological conclusions regarding species delimitation. Morphological species or subspecies confirmed as monophyletic by the molecular phylogenetic analyses include: *Scadoxus nutans, S. cyrtanthiflorus, S. pole-evansii, S. cinnabarinus, S. pseudocaulus, S. multiflorus* subsp. *longitubus* and *S. multiflorus* subsp. *katherinae.* Morphological species not contradicted by the molecular phylogenetic analyses include *S. membranaceus.* Morphological species or subspecies contradicted by the molecular phylogenetic analyses include *S. puniceus, S. multiflorus,* and *S. multiflorus* subsp. *multiflorus* Two new combinations are proposed: *S. katherinae* (from *S. multiflorus* subsp. *katherinae)* and *S. abyssinicus* (from *S. multiflorus* subsp. *multiflorus*). *Scadoxus longifolius* is recommended to be reduced to synonymy under *S. cinnabarinus.* The previous division of *Scadoxus* into two subgenera was clearly not reflected by the molecular phylogenies.

This study detected incongruence between the phylogenies based on nuclear DNA and chloroplast DNA, which did not allow for a straightforward interpretation in all respects. Further, the basal branches of the phylogenies had low support. Including more nuclear DNA could possibly help resolve this issue. Still, this study has offered new and important information about the speciation and relations in a widespread and conspicous genus of Sub-Saharan Africa.

1 Table of Contents

1 Introduction

Amaryllidaceae is referred to the monocot order Asparagales (Chase et al., 2016). The name is taken from the genus *Amaryllis* L. and the family is commonly known as the Amaryllis family. Amaryllidaceae is divided in three subfamilies: Amaryllidoideae, Allioideae and Agapanthoideae, with more than 2250 accepted species in 81 genera (WFO, 2020). The family is widely distributed from temperate to tropical regions, and especially diverse in South Africa, Andean South America and the Mediterranean region (Judd et al., 2016).

1.1 The genus *Scadoxus*

Within the family Amaryllidaceae, *Scadoxus* Raf. falls into subfamily Amaryllidoideae and is a member of tribe Haemantheae, which based on morphology initially included the genera *Clivia* Lindl., *Cryptostephanus* Welw. ex Baker, *Haemanthus* L. and *Scadoxus* (Nordal and Duncan, 1984). Based on molecular analysis (Meerow and Clayton, 2004) *Gethyllis* L. and *Apodolirion* Baker were later included in the tribe.

The genus *Scadoxus* has close affinities with the genus *Haemanthus* (Figure 1) which included the former until it was raised as a separate genus by Friis and Nordal (1976). However, the name was initially given by Constantine Samuel Rafinesque (Rafinesque, 1838). The separation was justified by different basic chromosome numbers, different distribution patterns, together with several morphological differences. The *Scadoxus* species are distributed in Sub-Saharan Africa and the Arabian Peninsula and have 2n = 18 chromosomes, whereas the *Haemanthus* species are distributed only in Africa and have 2n = 16 chromosomes(Bjørnstad and Friis, 1972a). Vosa and Marchi (1980) demonstrated that two smaller chromosomes in the karyotype of *Scadoxus* are homologous to one larger chromosome in the complement of *Haemanthus*. Further distinguishing characteristics are the leaves and subterranean organ: *Scadoxus* species have thinner leaves in rosulate arrangement with a petiole developed,

Figure 1. *Haemanthus coccineus* L. Kerner (1805)

and *Haemanthus* species have thicker leaves without a petiole, distichous leaf arrangement leaves and bifarious bulbs (Friis and Nordal, 1976). The separation of the two genera have later been confirmed by molecular studies (Meerow and Clayton, 2004, Bay-Smidt et al., 2011, Rønsted et al., 2012).

Scadoxus species are rhizomatous or bulbous plants, and those with bulbs usually have distinct rhizomes as well. In some species the petioles are sheating to form a pseudostem (false stem) most often with red to purple spots. The scape can also be spotted and it appears either from pushing through the side of the pseudostem or from among the leaves. Leaves are thin, lanceolate with an acute apex and a prominent midrib. The inflorescence at the top of the scape is an umbel-like with many flowers and subtended by four or more free or partly fused involucral bracts. These persist during flowering in some species, whereas in others they wither before the flowers are fully developed. The flowers are pink to red on long pedicels, with a distinct narrowly cylindrical tube and spreading, linear to lanceolate perianth segments. Filaments are red or greenish and filiform, anthers are yellow and small. The inflorescence is most often upright, with the exception of *S. nutans* (Friis & I. Bjørnstad) Friis & Nordal where the scape bends downwards, and *S. cyrtanthiflorus* (C.H. Wright) Friis & Nordal where the open flowers are drooping. The fruit is an orange to red berry with one to three rather large seeds with pale testa (Nordal, 1982).

Scadoxus covers a range of habitats from the rainforest to the savannah and coastal areas. All the species grow terrestrial, while the three species *S. nutans*, *S. puniceus* (L.) Friis & Nordal (rarely) and *S. cyrtanthiflorus* grow as an epiphyte as well as on the forest floor (Bjørnstad and Friis, 1974, Demissew and Nordal, 2010, Hutchinson, 2014). There are two different phenological flowering patterns of *Scadoxus* seemingly related to habitat. Individuals may be synanthous (flowers and leaves emerging nearly simultaneously, Figure 2A) or hysteranthous (flowers emerging before the leaves, Figure 2B). The plants that grow in the drier savannah are commonly hysteranthous, whereas those growing in the more humid rainforest and mountains tend to be synanthous, taller and having bigger flowers than the hysteranthous plants. The widespread *S. multiflorus* Raf. and *S. puniceus* has both forms. It has not been tested molecularly if these forms represent different taxa, or it is only an ecological adaptation (Bjørnstad and Friis, 1974, Bjorå and Nordal, 2014).

Figure 2. *Scadoxus multiflorus* displaying different fenology. **(A)** Synanthous, Zimbabwe. Photo: Charlotte S. Bjorå. **(B)** Hysteranthous, Ethiopia. Photo: Kine Hals Bødker

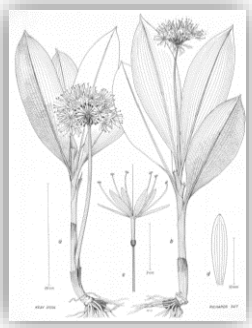
As within many of the amarylloids, the bulb of *Scadoxus* species is rich in alkaloids that are poisonous, and deaths have been reported following the ingestion. Nevertheless, this genus has been used to a variable extent in traditional medicine (Naidoo et al., 2015). Examples of uses include clearing skin conditions, treatment of kidney and urinary infections, gastro-intestinal problems, liver cancer, cardiovascular disease, microbial diseases, mental disorders, pneumonia, cure tonsillitis, and it is used as a pain reliever, detoxifying and energizing agent as well (Ndhlala et al., 2011, Pagning et al., 2016). It has also traditionally been used as part of a medicine taken regularly during pregnancy to ensure a safe delivery (Veale et al., 1992).

According to the World Checklist of Selected Plant Families there are currently nine accepted species of *Scadoxus* along with subspecies (WCSP, 2020). Their names, distribution, habitat types and illustrations are summarized in Table 1 and Figure 3 (Bjørnstad and Friis, 1972a, Bjørnstad and Friis, 1972b, Bjørnstad and Friis, 1974, Nordal, 1982, Nordal, 1997, Zimudzi et al., 2008, GBIF.org, 2020).

Table 1. Distribution and ecology of the *Scadoxus* species.

Name	Distribution and habitat	Name	Distribution and habitat
S. cinnabarinus **(Decne.) Friis & Nordal** 	From Sierra Leone to Uganda and Angola. · Lowland forest, riverine forest or mixed deciduous, high evergreen forest, from near sea level up to 1400 m.	***S. cyrtanthiflorus*** **(C. H. Wright) Friis & Nordal** 	Endemic to the Ruwenzori Mts, border between Democratic Republic of the Congo and Uganda. · In dense upland rainforest, ground or epiphytic; 1600-3200 m.
S. longifolius **(De Wild. & Th. Dur.)** 	Democratic Republic of the Congo; unknown location. · Rainforest.	***S. membranaceus*** **(Baker) Friis & Nordal** 	South Africa. · Coastal forest.
S. multiflorus **Raf. subsp. *multiflorus*** 	Throughout tropical Africa, from Senegal to Yemen and to South Africa and Namibia. · Wide range of habitats: grassland, wooded grassland, woodland, riverine vegetation, coastal rocks, montane forest and grassland.	**subsp. *longitubus*** **(C. H. Wright) Friis & Nordal** 	From Guinea to Ghana. · Lowland rainforest from sea level up to 850 m.
		subsp. *katherinae* **(Baker) Friis & Nordal** 	South Africa, Eswatini, Mozambique · Forest mosaic and coastal scrub and grassland, from sea level up to 750 m.

Table 1. Continued.

S. nutans **(Friis & I. Bjørnstad) Friis & Nordal** 	Endemic to Ethiopia. · Evergreen mountain rainforests, between 1000-2500 m.	***S. pole-evansii*** **(Oberm.) Friis & Nordal** 	Endemic to Nyanga, Zimbabwe. · Evergreen moist forest; 1800 m.
S. pseudocaulus **(I. Bjørnstad & Friis) Friis & Nordal** 	Nigeria, Equatorial Guinea, Cameroon and Gabon. · From sea level and up to 1400 m in regions of high rainfall - rainforest	***S. puniceus*** **(L.) Friis & Nordal** 	Ethiopia, Tanzania, Zambia, Malawi, Zimbabwe, Mozambique, South Africa. · Montane forest, woodland, on ground or epiphytic; 1000-2700 m.

Photos: Jonathan Hutchinson (*S. cinnabarinus*, *S. cyrtanthiflorus*, *S. membranaceus*, *S. multiflorus* subsp. *katherinae*. Curtis, W. (1878, *S. multiflorus* subsp. *longitubus*) Kine H. Bødker (*S. multiflorus* subsp. *multiflorus*, *S. nutans*, *S. pole-evansii*). Meise botanical garden (holotype *S. longifolius*). Richards, 3417, BM drawing of holotype reproduced in Bjørnstad and Friis, 1972b, p.218 (*S. pseudocaulus*), Charlotte S. Bjorå (*S. puniceus*).

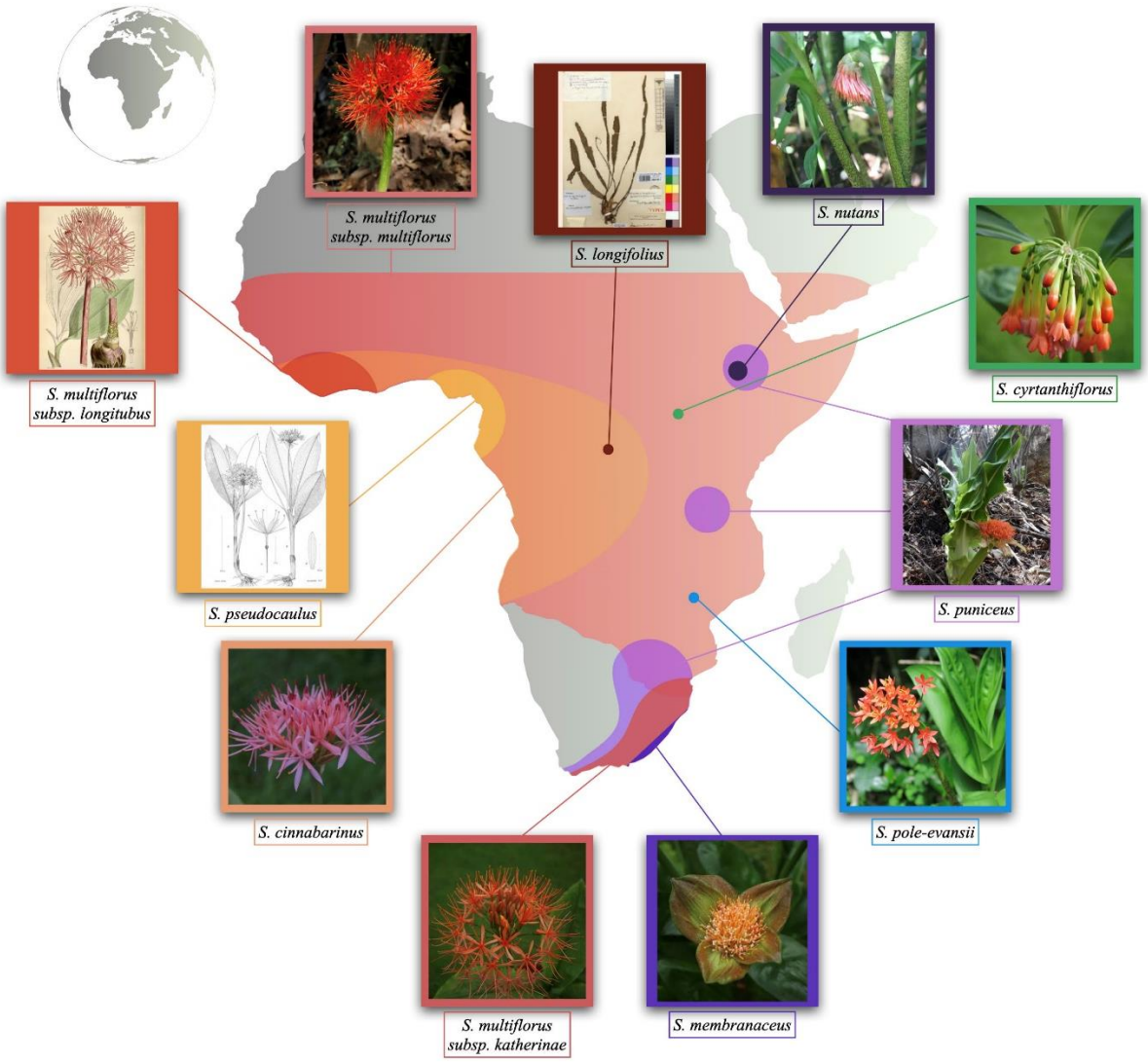


Figure 3. Overview of *Scadoxus* distributions. References to photos as in Table 1.

1.2 The phylogenetic history of *Scadoxus*

Nordal and colleagues conducted thorough studies of *Scadoxus* in the 1970-80s based on morphological, cytological, palynological and ecological data (Bjørnstad and Friis, 1972a, Bjørnstad and Friis, 1972b, Bjørnstad and Friis, 1974, Friis and Nordal, 1976, Nordal and Duncan, 1984). In addition to the division of *Scadoxus* from *Haemanthus*, new combinations of subgenera and sections were formally given (Friis and Nordal, 1976). *Scadoxus* was delimited into the subgenera *Demeusea* and *Scadoxus*. Subgenus *Demeusea* included sections *Demeusea* (De Wild. & Th. Dur.) Friis & Nordal, *Choananthus* (Rendle) Friis & Nordal and *Gamolepsis* (Friis & Bjørnstad) Friis & Nordal. Subgenus *Scadoxus* included sections *Scadoxus* and *Gyaxis* (Salisb.) Friis & Nordal (Table 2).

Table 2. Overview of the subgenera, sections and species of the genus *Scadoxus.*

Subgenus	Section	Species
Demeusea	*Demeusea* (De Wild. & Th. Dur.) Friis & Nordal	*S. cinnabarinus* *S. pseudocaulus* *S. longifolius*
	Choananthus (Rendle) Friis & Nordal	*S. cyrtanthiflorus*
	Gamolepsis (Friis & Bjørnstad) Friis & Nordal	*S. nutans*
Scadoxus	*Scadoxus* Raf.	*S. multiflorus* *S. pole-evansii*
	Gyaxis (Salisb.) Friis & Nordal	*S. membranaceus* *S. puniceus*

Further, cladistic analyses of *Haemanthus* and *Scadoxus* were performed, based on 25 characters using both parsimony and compatibility methods (Nordal and Duncan, 1984). The species *S. longifolius* (De Wild. & T. Durand) Friis & Nordal was omitted from the cladistics analyses, due to the questioning of warranting specific status by only being collected once, being closely related to *S. cinnabarinus* (Decne.) Friis & Nordal and not differing from that species in any character used in the analyses.

The resulting cladograms were different depending on which method used. However, the authors found it reasonable to conclude that the compatibility analysis gave the best reflection of the specific relations within *Scadoxus* (Figure 4), as this was also consistent with the subgeneric delimitation proposed by Bjørnstad and Friis (1972a) (Figure 5).

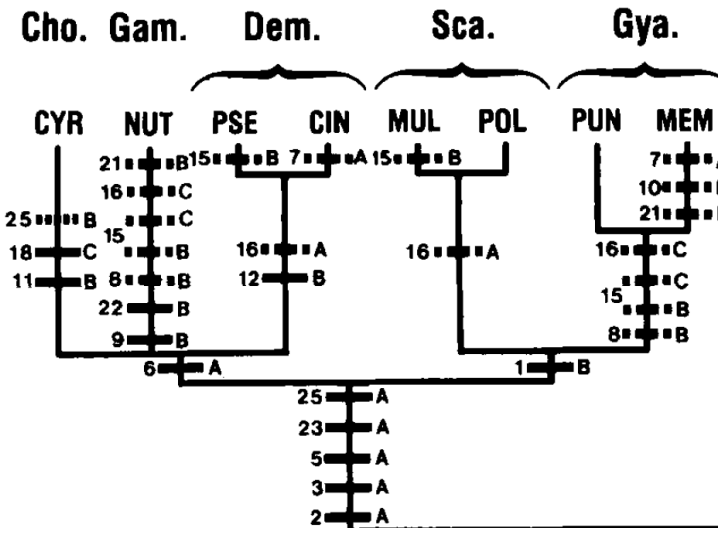


Figure 4. Changes in character states on the S*cadoxus* cladogram as proposed by the character compatibility analysis. Cho. = *Choananthus*, Gam. = *Gamolepsis*, Dem. = *Demeusea*, Sca. = *Scadoxus*, Gya. = *Gyaxis*, CYR = *S. cyrtanthiflorus*, CIN = *S. cinnabarinus*, PSE = *S. pseudocaulus*, POL = *S. pole-evansii*. MUL = *S. multiflorus*, PUN = *S. puniceus*, MEM = *S. membranaceus*, NUT = *S. nutans* (Nordal and Duncan, 1984 p.152).

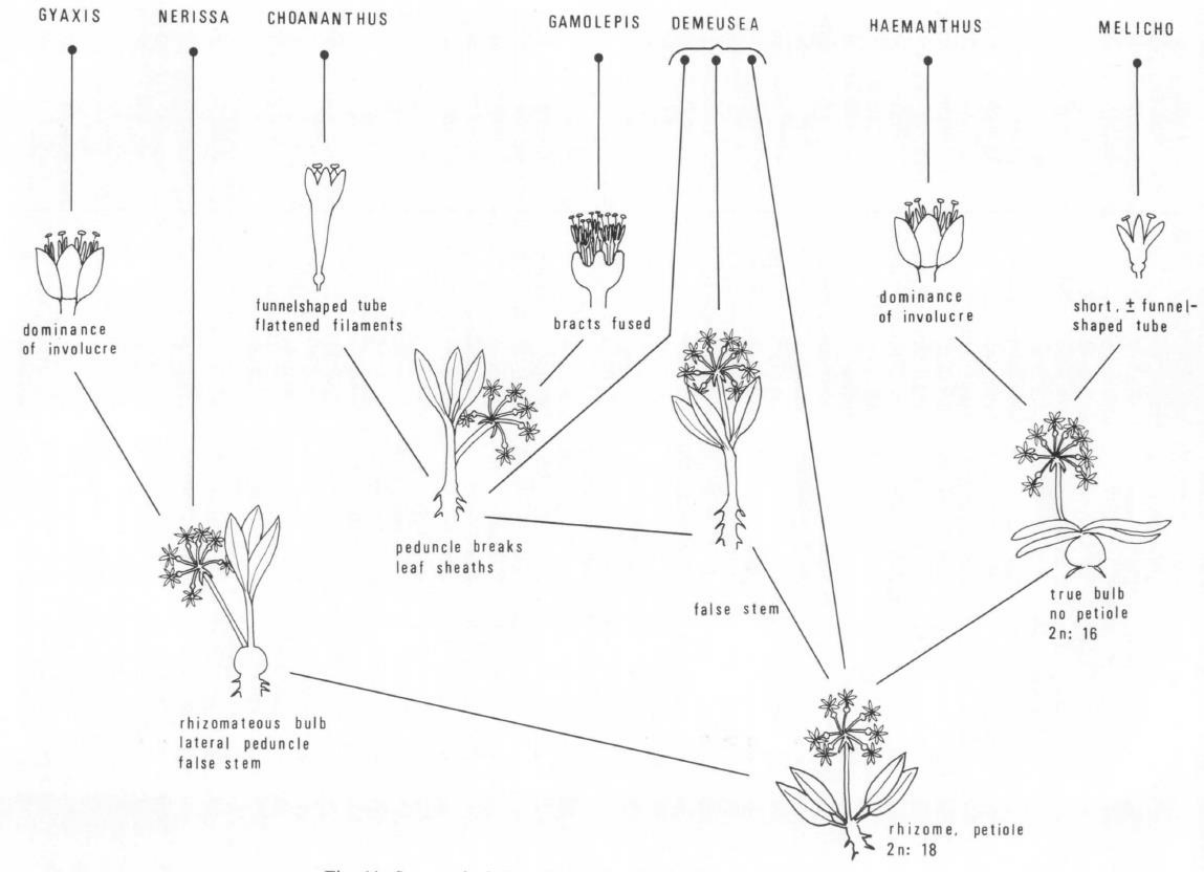


Figure 5. Suggested phylogenetic relationship between sections proposed by Bjørnstad and Friis (1972a p. 203). Section *Nerissa* is equivalent to section *Scadoxus*.

It has been hypothesized that the speciation of *Scadoxus* may have been due to vicariance, by which speciation occurs when populations become geographically isolated from each other so that gene flow is halted and new species may form (Coyne and Orr, 2004). Nordal (1984) hypothesized that speciation in *Scadoxus* is driven by ecological adaption. The two main subclades correspond thus to the rainforest species (subgenus *Demeusea*) and the savannah species (subgenus *Scadoxus*), within which subsequent speciation related to geography could also have been a strong driver (Figure 6). The subgenera differ significantly concerning phytogeography and ecology. *Demeusea* includes all the true rhizomatous rainforest taxa having the initiation of the scape among the leaves, as opposed to *Scadoxus* which includes the savannah taxa having rhizomatous bulbs with the scape outside the leaf cluster (Nordal and Duncan, 1984).

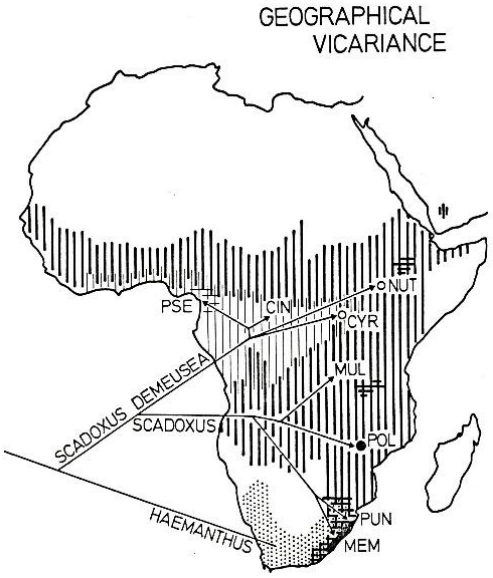


Figure 6. Hypothesized phylogeny based on geographical vicariance (Nordal, 1984).

Scadoxus multiflorus shows a considerable variation throughout its large, continuous distribution area. Many names have been reduced to synonyms of the species, and there is currently three accepted subspecies; the widely distributed subsp. *multiflorus*, the Southern African subsp. *katherinae* (Baker) Friis & Nordal and the West African subsp. *longitubus* (C.H. Wright) Friis & Nordal (Figure 7). The division into subspecies has been justified by regional geography, ecology and the following morphological characters: length of perianth tube, width of perianth segments and, to some extent, characters that express the general robustness. The latter can be evaluated on characters such as number of flowers and diameter of inflorescence, length of peduncle and diameter of corm (Bjørnstad and Friis, 1974).

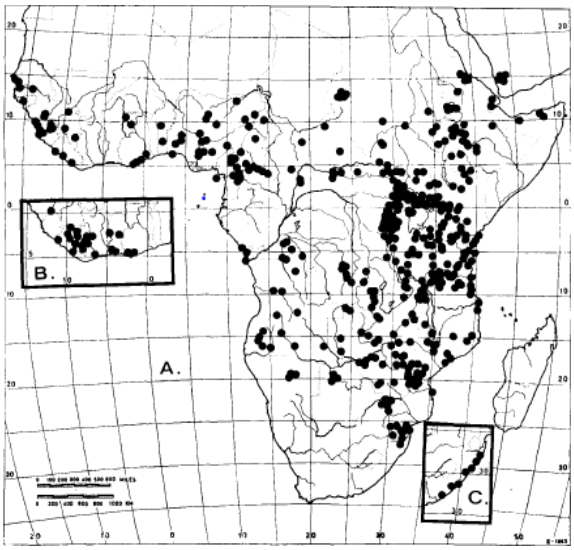


Figure 7. Map showing the distribution of *Scadoxus multiflorus* subspecies **(A)** subsp. *multiflorus*, **(B)** subsp. *longitubus*, and **(C)** subsp. *katherinae* (Bjørnstad and Friis, 1974, p. 263).

In Ethiopia, there is a common form of *S. multiflorus* subsp. *multiflorus* differing from forms found elsewhere, by having partly fused involucral bracts and an elongated, almost stolon-like, rhizomatous part under the bulb. This taxon has previously been named *Haemanthus bivalvis* Beck (Beck, 1888). The name was reduced to synonymy under *Scadoxus multiflorus* subsp. *multiflorus* based on the only existing documentation; an illustration representing the upper part of the peduncle and the inflorescence (the type material seemingly lost). Apart from the involucrum they concluded that the illustration agreed well with *S. multiflorus* in showing clearly spreading perianth segments. They also concluded that the specimen may represent a young form with the involucral bracts not yet reflexed and partly fused (Bjørnstad and Friis, 1974). Later is has been remarked that further studies should be undertaken to see whether this form deserves taxonomic recognition on the specific or subspecific level. Both this form and the "normal" form of *S. multiflorus* subsp. *multiflorus* exist in Ethiopia, and it is possible that the two forms meet. Since underground organs are rarely included in herbarium material, and the bract are often too withered to judge the degree of fusing, more field observations were encouraged to settle this problem (Demissew and Nordal, 2010).

Molecular studies of the whole genus have never been performed, although a few studies have included some species. Meerow et al. (1999) included *S. cinnabarinus* in a systematic study of Amaryllidaceae based on cladistic analysis of chloroplast DNA sequence data (*trn*L-F and *rbc*L). Further, Meerow and Clayton (2004) did studies of the tribe Haemantheae including three *Scadoxus* species using ITS and *trn*L-F, showing that *S. membranaceus* (Baker) Friis & Nordal and *S. puniceus* were more closely related to each other than to *S. cinnabarinus*. This study also confirmed the inclusion of the genera *Gethyllis* L. and *Apodolirion* Baker within tribe Haemantheae. Another phylogenetic study of Haemantheae (assessing alkaloid profiles as well) included the same three species as the former study as well as *S. multiflorus* (Bay-Smidt et al., 2011). Parsimony analysis (based on ITS and *trn*L-F) resulted in incongruence between the trees; *S. cinnabarinus* came out as sister to a polytomy of the other species in the nuclear ribosomal DNA (nrDNA tree). A larger study of the Amaryllidaceae phylogeny (Rønsted et al., 2012) using ITS, *mat*K, *trn*L-F and *nad*1 and the phylogenetic correlation with alkaloids and their biological activity included only *S. multiflorus* and *S. puniceus*, and resulted in no further resolution of the *Scadoxus* phylogeny. Lastly, a study of rare plant species in Saudi Arabia included one accession of *S. multiflorus* subsp. *multiflorus* (Al-Qurainy et al., 2013). These last mentioned accessions as well as two accessions from the former mentioned study are also included in this study.

1.3 Research aims and questions

This study will perform molecular phylogenetic analyses based partly on Sanger sequences and partly on chloroplast genome sequences. The objectives of this study are to test:

1. If the species delimitation based on morphology is confirmed by the molecular phylogeny.

2. If the division of *S. multiflorus* into subspecies is reflected in the molecular phylogeny.

3. If *Haemanthus bivalvis* and *Scadoxus multiflorus* are conspecific.

4. If the *Scadoxus* species have evolved as hypothesized by Nordal and Duncan (Figure 4).

2 Materials and methods

2.1 Sampling

Plant material used in this study was freshly collected specimens obtained during field work or retrieved from collaborators (both cultivated and wild plants), in addition to herbarium and greenhouse specimens. Altogether this study includes samples from all assumed species and subspecies of *Scadoxus* except *S. longifolius* (as the holotype and only collection is not available) and *Haemanthus albiflos* Jacq. used as an outgroup (Appendix Table A1). Since the phylogenetic relationships of the *S. puniceus* complex was designated as a separate master project, this study included mainly accessions from South Africa (the type location) as a reference. However, the chloroplast genome analysis (which included *S. puniceus* from all the major distribution areas) was shared with Ida E. Moe, who will discuss the *S. puniceus* complex in her thesis.

2.1.1 Field work

Field work was conducted in Zimbabwe and Ethiopia during January 2019 and February/March 2019, respectively. The localities were chosen according to information about previous collection sites found in floras and herbarium sheets. In Zimbabwe collection was mainly carried out in Matobo National Park and Nyanga National park. Samples were taken from eleven localities (Figure 8A). In Ethiopia, samples were collected from thirteen localities in the western regions (Figure 8B). The fieldwork provided in total 50 new collections, which included *S. multiflorus* subsp. *multiflorus* (both countries), *S. pole-evansii* (Oberm.) Friis & Nordal (Zimbabwe) and *S. nutans* (Ethiopia).

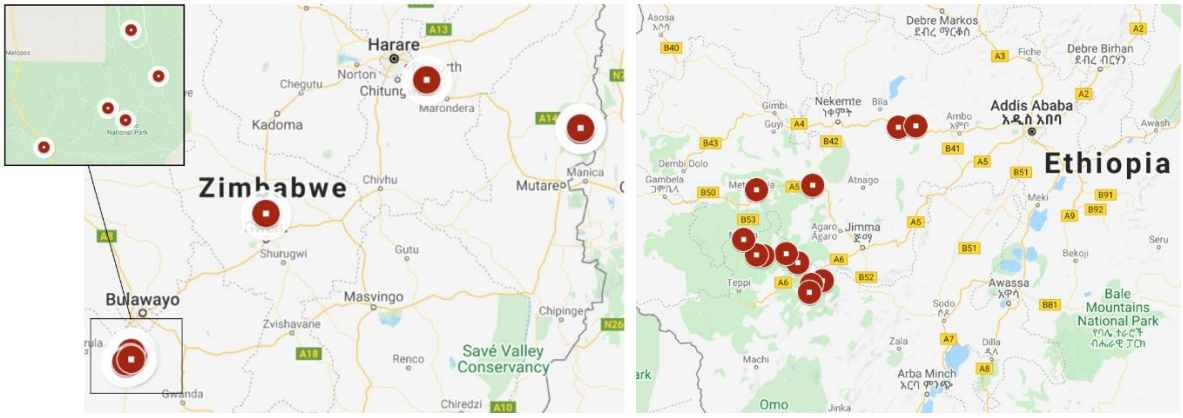


Figure 8. Collection localities in **(A)** Zimbabwe and **(B)** Ethiopia (Google, 2019). Some locations are hidden behind others due to near proximity within both Matobo National Park and Nyanga National Park.

In the field, each plant and its habitat were documented by photographs (Figure 9) and the youngest leaf was sampled and dried on silica gel for subsequent DNA extraction. Each sampled individual was pressed and distributed to either herbaria, botanical gardens or research institutes in the respective countries (Appendix Table A1). When correct species was difficult to determine due to lack of flowers, the bulb was taken to be grown by our collaborators and later identified to species.

Figure 9. Photos taken during field work**. (A-C**) The characteristic horizontally growing bulb, habitat and flower of *S. pole-evansii* in Nyanga, Zimbabwe. (**D**) *S. multiflorus* subsp. *multiflorus* in fruit (Zimbabwe). (**E-F)** Terrestrial and epiphytic growth forms of *S. nutans* in Ethiopia. (**G-H)** *S. multiflorus* subsp. *multiflorus* growing either in clusters or singularly (Ethiopia). Photos: Kine H. Bødker.

2.2 Laboratory work

All laboratory work was performed at the DNA lab at the Natural History Museum, University of Oslo, except for the Covaris E220 Focused-ultrasonicator (Covaris Inc., Woburn, MA, USA) used at the Department of Biosciences, University of Oslo.

2.2.1 DNA extraction

DNA was extracted from 10-30 mg dried plant material (leaf, flower or root) from each sample using the E.Z.N.A.® SP Plant DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the protocol with a few modifications. In the first step, where disruption of plant tissue was conducted, instead of grinding samples with a pellet pestle or freeze with liquid nitrogen, two tungsten-carbide beads were added with the plant material to a 2 ml LoBind microcentrifuge tube (Eppendorf, Hamburg, Germany) and placed into a Mixer Mill MM 301 (Retsch, Haan, Germany) at 45 Hz for 2 x 60 sec reversing the position of the adapters in between rounds. At step 4 in the protocol samples were incubated at 65°C for 60 min instead of 10 min, and samples were mixed several times during incubation. A volume of 50 µl Elution Buffer was used in two rounds, and instead of incubating in room temperature for 3-5 min, the samples were placed at 65°C for 5 min to possibly increase yields. In total 186 DNA extractions were performed, with some samples extracted several times due to unsatisfactory quality for next-generation sequencing requirements. Also, herbarium samples were extracted several times in an attempt to obtain high enough DNA quantity for Sanger sequencing.

2.2.2 Library prep and next-generation sequencing with Ion Torrent

All of the extracted DNA samples were analyzed prior to library preparation to assess DNA quantity and quality using NanoDrop One C (Thermo Fisher Scientific, Madison, USA), dsDNA BR (Broad Range) Assay Kit on a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and visualizing of DNA fragmentation by 0.8% agarose gel electrophoresis. Of the 24 spots available for library prep, 23 samples were selected based on the sample quality and to cover the full taxonomical and geographical range for making a phylogeny, including an outgroup. (Table 3). The last spot was given to a sample from the genus *Aloe* L. for another project and will not be discussed further.

Table 3. Specimens used for next-generation sequencing of the chloroplast genome.

Chip 1 #	Taxa [a]	Chip 2 #	Taxa [a]
1	*S. cyrtanthiflorus* (2)	1	*S. puniceus* (4)
2	*S. cinnabarinus* (6)	2	*S. cinnabarinus* (3)
3	*S. nutans* (5)	3	~~*Aloe*~~ [b]
4	*S. pole-evansii* (1)	4	*S. multiflorus* subsp. *multiflorus* (29)
5	*S. membranaceus* (8)	5	*S. multiflorus* subsp. *multiflorus* (7)
6	*S. multiflorus* subsp. *multiflorus* (9)	6	*S. puniceus* (10)
7	*Haemanthus albiflos* (1)	7	*S. multiflorus* subsp. *multiflorus* (21)
8	*S. multiflorus* subsp. *multiflorus* (32)	8	*S. puniceus* (12)
9	*S. puniceus* (7)	9	*S. puniceus* (11)
10	*S. multiflorus* subsp. *multiflorus* (20)	10	*S. puniceus* (1)
11	*S. multiflorus* subsp. *multiflorus* (14)	11	*S. multiflorus* subsp. *katherinae* (4)
12	*S. pseudocaulus* (3)	12	*S. multiflorus* subsp. *multiflorus* (15)

[a] Multiple accessions of the same species are numbered according to Appendix Table A1. [b] Not part of this study.

Due to the extremely large genome size of 43.20 Giga base pairs (Gbp) found in *Scadoxus* (Zonneveld et al., 2005) and inspiration from Manzanilla et al. (2018), the NEBNext® Microbiome DNA Enrichment Kit (New England Biolabs, Ipswich, Massachusetts, USA) was used to reduce genome complexity by separating nuclear genomic DNA from organelle DNA. The kit uses IgG1 fused to the human methyl-CpG-binding domain, together making up MBD2-Fc (composed of the methylated CpG-specific binding protein MBD2, fused to the Fc fragment of human IgG), to pull down a methyl-CpGenriched fraction from a bead-associated element, leaving a methyl-depleted fraction in the supernatant. This methyl-depleted fraction should contain the organelle DNA, including our targeted chloroplast DNA. Input DNA was 1000 ng for all samples with the exception of *S. pseudocaulus* (182 ng) and *Haemanthus albiflos* (920 ng).

The manufacturer's recommendations were followed with smaller exceptions: the non-methylated DNA fractions were purified using 1.0X volume of AMpure XP beads (Beckman Coulter, Brea, CA, USA) and eluted in 50 µl 10 mM Tris-HCl buffer. The NEBNext® Microbiome DNA Enrichment Kit was initially applied on six samples for which quality

control in terms of size, purity and concentration of both the methylated and the non-methylated fractions were measured using a Fragment Analyzer (Advanced Analytical Technologies Inc., USA) with a DNF-488-33 HS dsDNA Reagent Kit. As an additional quality check to see if the separation of the fractions was successful, a qPCR was run on the same six samples. The setup included negative and positive controls using a chloroplast marker (region trnL-F: forward/reverse primer: e/f; Taberlet et al. (1991) and a nuclear marker (region ITS2: forward/reverse primer: ITS3/ITS4; White et al. (1990), for both enriched DNA (organelle) and host DNA (nuclear genomic). After the results showed a successful separation, the NEBNext Microbiome DNA Enrichment Kit was applied on the 18 remaining samples.

The organelle DNA fraction was subsequently sheared to ~200 base pairs (bp) fragments using a Covaris E220 Focused-ultrasonicator (Covaris Inc., Woburn, MA, USA) before using the NEBNext® Fast DNA Library Prep Set for Ion Torrent™ for end repair and adapter ligation of all the sheared DNA (50 µl). The samples were indexed using the IonXpress Barcode Adapter kit (Thermo Fischer, Waltham, MA, USA). After adapter ligation, the 24 samples were pooled into two libraries each labelled with IonXpress adapters 1-12; Library 1: Sample 1-12 (Table 3) and Library 2: Sample 13-24 (Table 3). Instead of size selection using AMPure XP beads according to the instruction manual, the libraries were size selected with BluePippin (Sage Science, Beverly, MA, USA). The aim was to select fragments in the size 210-300 bp using the 2% agarose 100-600 bp cassettes with internal standards, yielding 40 µl adaptor ligated DNA. Another deviation from the protocol was that size selection was performed after, instead of before, cleanup of adaptor ligated DNA, leaving 30 µl for BluePippin and 2 µl for the Fragment Analyzer.

In order to assess the number of PCR cycles in the library amplification, DNA concentration of the two adapter ligated pools was measured using Qubit® 2.0 Fluorometer. Based on the concentrations given, 20 µl (~10 ng DNA) of each library and 12 PCR cycles were used for the amplification (leaving one backup pool). The NEBNext® Fast DNA Library Prep Set for Ion Torrent™ library amplification protocol was followed exactly, before three rounds of cleanup of the amplified library using 1.2X volume of AMPure XP beads, with elution after the second and third cleanup steps in 30 µl 0.1X TE buffer.

Because of the relative high DNA yield, re-amplification of the remaining 20 µl (remaining after BluePippin) was done using only eight PCR cycles. This reduction aimed to reduce the

likelihood of PCR bias in the read distribution. Amplification and cleanup were performed in the same manner as previously described. Both libraries were run in the Fragment Analyzer in triplicates. Based on the results, the library run resulting from eight PCR cycles was used for sequencing, and the following steps were performed by lab manager Jarl Andreas Anmarkrud at the DNA lab, Natural History Museum, Oslo.

Based on the concentrations (nM) estimated in the Fragment Analyzer, the libraries were diluted to 45 pM. The purified, amplified and diluted libraries were then loaded on the sequencing chips using the Ion 540 Kit-Chef and sequenced on an Ion GeneStudio™ S5 System (Thermo Fisher Scientific, Waltham, MA, USA) using Ion Torrent 540 Chips and the Ion 5S Sequencing Kit. Sequencing reads were demultiplexed into FASTQ files using Torrent Suite version 15.12.1.

2.2.3 Polymerase Chain reaction (PCR) and Sanger sequencing

Regions attempted amplified for Sanger sequencing included the nuclear ribosomal internal transcribed spacer region (ITS1, 5.8S and ITS2) and nine chloroplast regions (Table 4). Initially efforts were made to amplify all regions for a small subset of samples. The following unsuccessful regions were excluded from further studies; *rbc*L, *psb*B-*psb*F and *at*103. Efforts were made to amplify the remaining regions for all of the 108 specimens. For a subset of 33 herbarium specimens, for which amplification of all other regions was unsuccessful, separate amplification of shorter regions within the ITS region (ITS1, ITS2) and *trn*L-F (*trn*L intron, *trn*L-F spacer) was attempted.

Table 4. Overview of the amplified regions and primers used for Sanger sequencing.

Region	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ITS	ITS5 mod[a]: GGAAGGAGAAGTCGTAACAAGG	ITS4[a]: TCCTCCGCTTATTGATATGC
ITS1	ITS5 mod[a]: GGAAGGAGAAGTCGTAACAAGG	ITS2 mod[a]: GCTACGTTCTTCATCGATGC
ITS2	ITS3[a]: GCATCGATGAAGAACGCAGC	ITS4[a]: TCCTCCGCTTATTGATATGC
*trn*L-F	c[b]: CGAAATCGGTAGACGCTACG	f[b]: AATTGAACTGGTGACACGAG
*trn*L intron	c[b]: CGAAATCGGTAGACGCTACG	d[b]: GGGGATAGAGGGACTTGAAC
*trn*L-F spacer	e[b]: GGTTCAAGTCCCTCTATCCC	f[b]: AATTGAACTGGTGACACGAG

Table 4. Continued.

*trn*S-G	trnS(gcu)[c]: AACTCGTACAACGGATTAGCAATC	trnG(ucc)[c]: GAATCGAACCCGCATCGTTAG
*psb*A-*trn*H	psbA[d]: CGAAGCTCCATCTACAAATGG	trnH[d]: ACTGCCTTGATCCACTTGGC
*rpl*20-5*rps*12	rpl20[d]: TTTGTTCTACGTCTCCGAGC	5rps12[d]: GTCGAGGAACATGTACTAGG
*rps*16	rps16-F[e]: GTGGTAGAAAGCAACGTGCGACTT	rps16-R2[e]: TCGGGATCGAACATCAATTGCAAC
Maturase K	MatK-XF[f]: TAATTTACGATCAATTCATTC	MatK-5R[f]: GTTCTAGCACAAGAAAGTCG
*psb*B-F	psbB[d]: GTTTACTTTTGGGCATGCTTCG	psbF[d]: CGCAGTTCGTCTTGGACCAG
*rbc*L	rbcL1-F[g]: TTGGCAGCATTYCGAGTAACTCC	rbcLB-R[g]: AACCYTCTTCAAAAAGGTC
*at*103	at103-F[h]: CTTCAAGCCMAAGTTCATCTTCTA	at103-R[h]: TTGGCAATCATTGAGGTACATNGTM ACATA

Regions and primers shaded in grey were attempted on subsets, while the rest were attempted on all samples. Primers developed by: [a] White et al. (1990), [b] Taberlet et al. (1991), [c] Shaw et al. (2005), [d] Hamilton (1999), [e] Oxelman et al. (1997), [f] Ford et al. (2009), [g] Palmieri et al. (2009), [h] Li et al. (2008).

All extracted DNA samples were set up in 10 µl PCR reactions containing 0.5 µl DNA extract, either diluted (most often for silica samples) or undiluted (most often for herbarium samples), with 1 µl 1 mg/ml BSA, 1 µl 25 mM MgCl2, 1 µl 10X PCR buffer, 1 µl 10 mM dNTP, 0.4 µl 10 µM of each forward and reverse primer, 0.08 µl of AmpliTaq polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 4.62 µl ultrapure water Milli-Q water (Merck, Darmstadt, Germany).

PCR was performed on a T100™ Thermal Cycler (Bio-Rad, California, USA) with the following protocol to amplify all regions: pre-denaturation at 95°C for 2.5 min followed by 32 cycles with denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, synthesis at 72°C for 50 sec and a final elongation step at 72°C for 4 min, before an infinite hold at 10°C.

Amplified PCR products were quality checked with electrophoresis on 1 % agarose gels run at 90V for 30-40 min. For successful PCR reactions that yielded product, 7 µl PCR product was purified with 0.2 µl illustra™ ExoStar 1-step (GE Healthcare, Chicago, USA) combined with 1.8 µl Milli-Q water. Samples were incubated on a thermal cycler at 37°C for 45 min, followed by 80°C for 15 min for inactivation of the hydrolytic enzymes.

Cleaned PCR products were diluted with either 10 µl Milli-Q water per sample (for weak bands shown on the agarose gel), or 20 µl Milli-Q water (strong gel bands). In new plates, 7.5 µl PCR product and 2.5 µl of either forward or reverse primer were added, before the plates were labeled and sent off for sequencing. Macrogen Europe (Amsterdam Zuid-Oost, The Netherlands) performed the Sanger sequencing.

2.3 Analyses of data from Ion Torrent sequencing

Processing and assembly of the Ion Torrent sequences were performed by Anders Kristian Krabberød from the Centre for Ecological and Evolutionary Synthesis (CEES) at the University of Oslo.

Adapter trimming and quality filtering of reads were done using Trimmomatic v0.38 (Bolger et al., 2014) with a sliding window of 15 bp and an average Phred threshold of 20. Low-end quality bases below a Phred score of 20 were removed, and only reads longer than 100 bp were retained. MITOBim v1.91 (Hahn et al., 2013) was used for assembly of the single-end Ion Torrent reads using iterative mapping with in silico baiting using the reference chloroplast genome of *Lycoris radiata* (Zhang et al., 2019). The chloroplast genomes were annotated using Geneious v2019.0.4 (https://www.geneious.com) (Kearse et al., 2012), and annotations of exons and introns were manually checked by alignment with their respective genes in the same annotated species genome in a few of them.

The matrix for phylogenomic analyses consisted of complete aligned chloroplast genomes, and the global alignment was done using MAFFT v7.429 (Katoh et al., 2002) with local re-alignment, and manual adjustments where necessary. The full chloroplast genomes of the closest relatives of *Scadoxus* were identified from being blasted against the non-redundant GenBank NCBI database (Sayers et al., 2019). Inverted repeats and ambiguous portions of the assembly were compared to the previously obtained Sanger sequences (this was done by the author) before the sequences were sent back to A. K. Krabberød for realignment and further analysis. Four genomes were retrieved and used as outgroups in addition to *Haemanthus albiflos* which was sequenced in this study.

Relationships from the nucleotide matrix were inferred using Maximum Likelihood (ML). The dataset was analyzed using RAxML v8.2.12 (Stamatakis, 2014). RAxML searches used the substitution model GTR+G+I. Tree searches and bootstrapping were conducted

simultaneously using the bootstrap convergence criteria autoMRE whichs allows for automatically determining a sufficient number of bootstrap replicates. The tree was displayed with FigTree v1.4.4 (tree.bio.ed.ac.uk/software/figtree/), and final visualization was done using iTOL (Letunic et al., 2016) and Keynote v10.0, Apple Inc.

2.4 Analyses of data based on Sanger sequences

In addition to the sequences obtained from the laboratory work, an additional seven sequences were acquired from previous preliminary analysis by Bjorå and Nordal (2014), as well as three sequences downloaded from GenBank (Sayers et al., 2019) (Appendix Table A1). Sequence assembly and editing were performed using Geneious Prime v2020.1.2 (Kearse et al., 2012). All sequences were manually inspected and edited. Multiple alignments were made using MUSCLE v3.8.425 (Edgar, 2004) within Geneious Prime v2020.1.2, under default parameter settings. All alignments were inspected and manually adjusted when necessary. Sequences found to be considerably divergent were blasted against GenBank NCBI database (Sayers et al., 2019) using Megablast (Morgulis et al., 2008), and removed when confirmed to be very different taxa than anticipated, i.e fungi.

Preliminary analyses were performed in Geneious Prime 2020.1.2 using FastTree v2.1.11 (Price et al., 2009, Price et al., 2010) which infers approximately-maximum-likelihood phylogenetic trees. As the individual chloroplast trees showed low resolution, alignments of sequences from the chloroplast regions were concatenated for further analyses. This resulted in a concatenated chloroplast alignment (*trn*L-F, *trn*S-G, *psb*A-*trn*H, *rps*16, *rpl*20-5*rps*12, *MatK*) of 71 accessions, and an ITS alignment of 79 accessions.

Alignments were inspected for insertion–deletion mutations (indels). The concatenated chloroplast alignment showed considerable cases of indels and gaps were subsequently coded as binary characters using simple indel coding (Ochoterena and Simmons, 2000) in SeqState v1.4.1 (Müller, 2005). The best-fit model of nucleotide substitution for all of the seven individual alignments was selected using jModelTest v2.1.10 (Guindon et al., 2003, Diego et al., 2012) under the Akaike Information Criterion (AIC). The general time-reversible model including a proportion of invariable sites (GTR+I) was determined as the best substitution model for all alignments.

The alignments were analysed using maximum likelihood (ML) and Bayesian inference. Maximum Likelihood (ML) analyses were conducted using the Randomized Axelerated Maximum Likelihood (RAxML) v8.2.11 (Stamatakis, 2014) as implemented in Geneious v2020.1.2. Rapid Bootstrapping and search for best-scoring ML tree algorithms were used, and bootstrap analyses were performed with 1000 replicates. Bayesian inference was performed using MrBayes v3.2.7a (Huelsenbeck and Ronquist, 2001, Ronquist and Huelsenbeck, 2003). Analyses were started using a random starting tree and run for four million generations, sampling every 1000 generations. Two Markov runs were conducted with four chains per run. To check whether the Markov Chain had converged well before finishing the analysis, the standard deviation of split frequencies (SDSF) was monitored to be below 0.01. The output file was inspected in Tracer v1.7.1 (Rambaut et al., 2018), and the 1000 first generations (25 %) were discarded as burn-in. The remaining trees were used for calculation of posterior probabilities and building a 50% majority-rule consensus tree. The trees were displayed with FigTree v1.4.4 (tree.bio.ed.ac.uk/software/figtree/), and final visualization was done using iTOL (Letunic et al., 2016) and Keynote v10.0, Apple Inc.

3 Results

3.1 Ion Torrent sequencing, assembly and alignment

The sequencing runs rendered quite different result for the two chips run. Even though 1 µg DNA was used as input per sample on both chips, the run of the first chip rendered 8.8 Gbp and 54 million reads (43 % usable reads), while the second chip rendered 16.6 Gbp and almost 94 million reads (69 % usable reads). For the first chip the read numbers ranged from 504,125 to 22,538,915 per sample, and for the second chip from 629,357 to 17,769,581 (Table 5). Complete lengths of the assembled genomes ranged from 158,155 bp to 165,359 bp. The chloroplast assemblies covered the entire circular chloroplast genome for all accessions with low levels of undetermined nucleotides (< 1 %), with the exception of the accession *S. pseudocaulus* with 35.9 % of missing data. This was the only sample with DNA extracted from herbarium material as opposed to silica dried material. The comparison of the chloroplast Sanger sequences to the chloroplast genomes confirmed the genome assemblies for the six regions, although it revealed some places where the genome included double copies of sequence parts next to each other (especially for *trn*S-G and *trn*L-F). These parts were accordingly removed.

Table 5. Summary information for the sequenced and assembled chloroplast genomes**.**

Chip # [a]	Taxa [b]	Number of reads	Genome length (bp)	% N [c]
Chip 1 - 1	*S. cyrtanthiflorus* (2)	1,345,742	158,778	0.1
2	*S. cinnabarinus* (6)	3,433,085	158,845	0.1
3	*S. nutans* (5)	1,599,670	159,028	0
4	*S. pole-evansii* (1)	2,478,783	163,281	0.4
5	*S. membranaceus* (8)	2,450,981	163,174	0.3
6	*S. multiflorus* subsp. *multiflorus* (9)	2,439,320	159,659	0.1
7	*Haemanthus albiflos* (1)	6,114,896	164,578	0.1
8	*S. multiflorus* subsp. *multiflorus* (33)	22,538,915	158,840	0.2
9	*S. puniceus* (7)	8,238,970	165,359	0.4
10	*S. multiflorus* subsp. *multiflorus* (21)	957,006	159,205	0

Table 5. Continued.

11	*S. multiflorus* subsp. *multiflorus* (15)	504,125	158,841	0.1
12	*S. pseudocaulus* (3)	588,250	158,155	35.9
Chip 2 - 1	*S. puniceus* (4)	4,444,939	162,983	0.4
2	*S. cinnabarinus* (3)	8,490,960	159,590	0
4	*S. multiflorus* subsp. *multiflorus* (30)	17,769,581	165,307	0.1
5	*S. multiflorus* subsp. *multiflorus* (7)	17,358,373	162,714	0.3
6	*S. puniceus* (10)	12,546,918	163,296	0.1
7	*S. multiflorus* subsp. *multiflorus* (22)	4,243,613	162,526	0.2
8	*S. puniceus* (12)	2,404,435	161,386	0.2
9	*S. puniceus* (11)	3,413,445	160,353	0
10	*S. puniceus* (1)	6,593,129	163,357	0.5
11	*S. multiflorus* subsp. *katherinae* (4)	5,025,632	161,337	0.1
12	*S. multiflorus* subsp. *multiflorus* (16)	629,357	158,667	0.8

[a] Chip number refers to the two ion Torrent chips on which the genomes were sequenced. [b] Multiple accessions of the same species are numbered according to Appendix Table A1. [c] Percentage of undetermined nucleotides (N) in the assembled genomes.

The final matrix of the aligned genome sequences had a total length of 203,815 bp for a total of 27 individuals (including five outgroup taxa). Based on the alignment, the percentage of identical sites was 43.4% and average chloroplast genome pairwise identity was 92.8%, i.e the average percent identity over the alignment when looking at all pairs of bases at the same column.

3.2 Sanger sequencing, alignment and phylogenetic analysis

Apart from the regions initially attempted amplified and failed (*rbcl*, *psb*B-*psb*F, *at*103), the other seven regions were amplified successfully for most of the samples. Quite a few samples needed several attempts, especially for amplification of *trn*S-G and *rps*16. *Scadoxus cinnabarinus* was particularly difficult to obtain sequences from, even from freshly collected leaf material dried on silica. All regions proved especially difficult to amplify from old, dried herbarium material. Sequences were obtained from only five individuals of the 33 herbarium

samples attempted (all regions). Out of the 108 specimens attempted initially, the final phylogenetic analyses were therefore based on sequences successfully obtained from all or part of the following regions: ITS, *trn*L-F, *trn*S-G, *psb*A-*trn*H, *rps*16, *rpl*20-5*rps*12 and *Mat*K (Appendix Table A1).

Of 541 obtained sequences, the number obtained for individual taxa were: *S. cinnabarinus* 40; *S. cyrtanthiflorus* 20; *S. longifolius* 0; *S. membranaceus* 59; *S. multiflorus* subsp. *katherinae* 34; *S. multiflorus* subsp. *longitubus* 11; *S. multiflorus* subsp. *multiflorus* 218; *S. nutans* 58; *S. pole-evansii* 28; *S. pseudocaulus* 5; *S. puniceus* 56; *S. sp* 12; *H. albiflos* 7. The length in bp of the final aligned genetic regions were: ITS 700; *trn*L-F 912; *trn*S-G 1086; *psb*A-*trn*H 628; *rps*16 814; *rpl*20-5*rps*12 794; *Mat*K 917; concatenated chloroplast regions 5151 (Table 6).

Table 6. Details of matrices included in this study.

Matrix	Number of accessions	Number of aligned characters
ITS	79	700
trn*L-F*	71	912
trn*S-G*	57	1086
psb*A*-trn*H*	69	628
rps*16*	69	814
rpl*20-5*rps*12*	70	794
Mat*K*	67	917
Concatenated chloroplast	71	5151

Simple indel coding of the concatenated chloroplast alignment gave 74 coded indels. Resulting trees with and without simple indel coding were not entirely congruent. As the former had an overall higher resolution and branch support, all results presented herein were based on the indel coded analyses. In the Bayesian analysis of the ITS and concatenated chloroplast datasets, the standard deviation of split frequencies (ASDSF) fell to 0.005896 and 0.005625 at termination respectively.

3.3 Phylogenetic reconstruction

The following trees include accessions of *Scadoxus puniceus* to a varying extent. As previously mentioned the ITS and concatenated chloroplast analyses only included *S.*

puniceus accessions from South Africa (the type location) as a reference, whereas the chloroplast genome analysis included more *S. puniceus* accessions because it was also part of another master thesis focusing on *S. puniceus*.

Clades discussed in the following are indicated with corresponding capital letters in the figures. To simplify the discussion of monophyletic groups or sister relations, letters are given sequentially from the larger clades to smaller subclades. To simplify the discussion further, accessions from these countries are classified as follows: West Africa includes samples from Ghana, Gambia, Cameroon, Gabon, Nigeria; East Africa includes samples from Tanzania, Kenya, Zambia and Ruwenzori Mts.

3.3.1 Chloroplast genome phylogeny

The phylogeny based on the chloroplast genomes (gDNA tree) is represented by the best scoring ML tree (Figure 10) from the RAxML analysis of 27 accessions (of which five were outgroup taxa) and 203,815 characters. The tree had a very long branch leading to *S. pseudocaulus* (as expected due to a high amount of missing data), meaning that the position of this taxon is uncertain. However, there was no change in the tree topology if the taxon was removed.

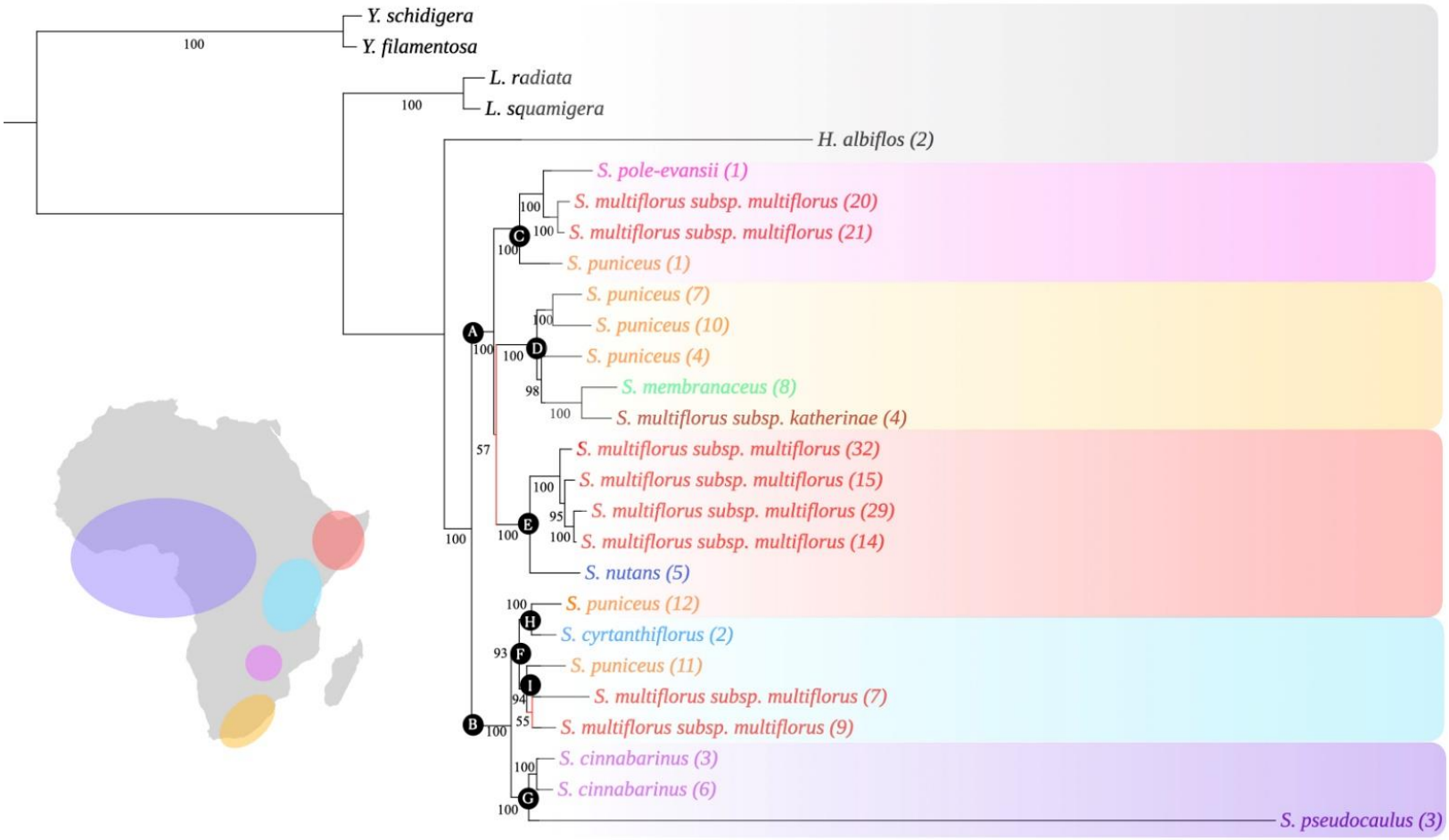


Figure 10. The best scoring Maximum Likelihood (ML) tree from RAxML analysis of 27 accessions and 203,815 characters. The RAxML bootstrap support (BS) values of at least 50 % are reported below or above branches. Branches lacking high support have red lines. Accessions are numbered according to Appendix Table A1. Abbreviations: Y. = *Yucca*, L. = *Lycoris*, H. = *Haemanthus* S. = *Scadoxus*. The clades discussed in the text are marked with capital letters. Shade colors in the tree correspond to: outgroups (grey), Zimbabwe (pink), South Africa (orange), Ethiopia and Saudi Arabia (red), East Africa (blue) and West Africa (purple).

The tree generally consisted of clades with high support and strong geographic patterns. Accessions were separated in two highly supported (bootstrap support, BS 100) main clades (Figure 10: clade A and B). The first main clade was divided into three clades (C, D, E), each with high support (BS 100), and with a geographic pattern. Clade C included all Zimbabwean accessions, clade D included all South African accessions and clade E included almost all Ethiopian accession (except *S. puniceus*). The relatedness between these three clades was, however, not resolved as the support for a sister relation between South Africa (D) and Ethiopia (E) was weak (BS 55). The second main clade (B) was divided into two highly supported clades (F: BS 90/G: BS 100), which also showed a geographic pattern, however, not as strongly as in clade A. Clade F included clade H (BS 100) with accessions from Ethiopia and Ruwenzori Mts, as well as clade I (BS 94) with only East African accessions. Lastly were the West African accessions included in clade G (BS 100).

Due to the strong geographic pattern, there was consequently low support for monophyly of widely distributed species. Accessions of *S. multiflorus* were, thus, not monophyletic and found in four separate clades (C, D, E, I) representing four out of the five main geographic areas. West African *S. multiflorus* was not included in this analysis. Within clade C, Zimbabwean subsp. *multiflorus* constituted a monophyletic group (BS 100) with *S. pole-evansii* as sister. Within clade D, the single accession of subsp. *katherinae* from South Africa had *S. membranaceus* as closest sister. Within clade I, East African subsp. *multiflorus* accessions (BS 55) were sister to Ethiopian *S. puniceus*.

Scadoxus puniceus was not monophyletic either. Accessions from Zimbabwe, South Africa, Ethiopia and Tanzania appeared in four different clades (C, D, H and I). Within clade C, *S. puniceus* was sister to the rest of the Zimbabwean taxa. Within clade D, the three accessions of South African origin did not even form a monophyletic group, as one of the accessions was sister to a clade consisting of *S. membranaceus* and *S. multiflorus* subsp. *katherinae*. Within clade H, Ethiopian *S. puniceus* was closest sister to *S. cyrtanthiflorus* and within clade I, Tanzanian *S. puniceus* was sister to East African *S. multiflorus* subsp. *multiflorus*.

Within clade G, a monophyletic group of *S. cinnabarinus* (BS 100) was sister to *S. pseudocaulus*. The monophyly of *S. pole-evansii, S. membranaceus, S. multiflorus* subsp. *katherinae, S. nutans, S. cyrtanthiflorus* and *S. pseudocaulus* could not be assessed as only

one accession was included of each taxa (but see the phylogenetic trees based on Sanger sequences).

3.3.2 Phylogenies based on Sanger sequences

Concatenated chloroplast phylogeny

The phylogeny (cpDNA tree) in Figure 11 is the 50 % majority-rule consensus tree from the Bayesian analysis of the concatenated matrix of six chloroplast regions (*trn*L-F, *trn*S-G, *psb*A-*trn*H, *rps*16, *rpl*20-5*rps*12 and *Mat*K). The topology was congruent with the Maximum Likelihood tree (not shown, but bootstrap values were inserted on the Bayesian consensus tree).

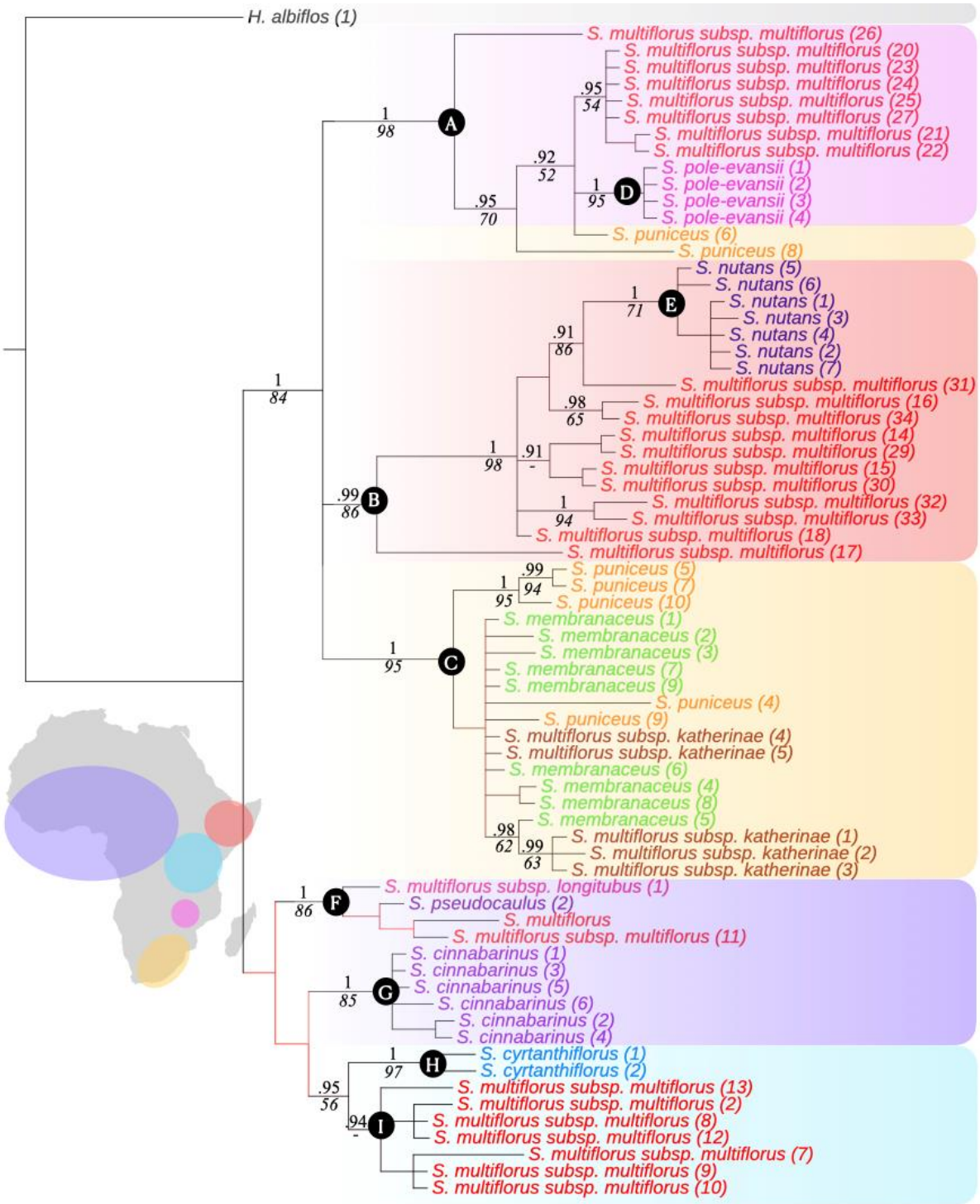


Figure 11. The 50 % majority-rule consensus tree from Bayesian analysis of the concatenated matrix of six chloroplast regions (*trn*L-F, *trn*S-G, *psb*A-*trn*H, *rps*16, *rpl*20-5*rps*12, *Mat*K) of 71 accessions and 5151 characters (incl. 74 coded indels). Bayesian posterior probability values (PP) of at least 0.9 are reported above branches, whereas RAxML maximum likelihood bootstrap support (BS) values of at least 50 % are reported in italics below branches. Branches lacking high support have red lines. Multiple accessions of the same species are numbered according to Appendix Table A1. Abbreviations: *S.* = *Scadoxus*, *H.* = *Haemanthus*. Shade colors in the tree correspond to: outgroups (grey), Zimbabwe (pink), South Africa (orange), Ethiopia and Saudi Arabia (red), East Africa (blue) and West Africa (purple). The clades discussed in the text are marked with capital letters.

The backbone of the tree had low resolution and several clades were part of a polytomy. The largest clade (PP 1/BS 84) showed strong geographic patterns and were further divided into three clades (A, B, C) with unresolved relations between each other. Clade A (PP 1/BS 98) included mainly Zimbabwean accessions, with the exception of two South African *S. puniceus* accessions. Clade B (PP 0.99/BS 86) included all Ethiopian accessions and clade C (PP 1/BS 95) included all South African accessions. The remaining clades also showed a geographic pattern, however less strongly supported. The West African accessions were split into two clades (clade F: PP 1/BS 86, and clade G: PP 1/BS 85) and the East African accessions formed a clade (H-I: PP 0.95/BS 56).

Again, corresponding with the strong geographic pattern, there was low support for monophyly particularly for species with wide distributions. Monophyletic species groups included *S. pole-evansii* (clade D, PP 1/BS 95), *S. nutans* (clade E, PP 1/BS 71), *S. cyrtanthiflorus* (clade H, PP 1/BS 97) and *S. cinnabarinus* (clade G, PP 1/BS 85). Accessions of *S. multiflorus* were not monophyletic, however, and were part of five separate clades with an evident geographic pattern, where four of the clades comprised *S. multiflorus* subsp. *multiflorus*. Within clade A Zimbabwean subsp. *multiflorus* was not monophyletic as both a couple of *S. puniceus* accessions and the *S. pole-evansii* clade were nested within the larger clade. Neither within the Ethiopian clade (B) was the subspecies monophyletic as *S. nutans* was nested within the *S. multiflorus* subsp. *multiflorus* accessions. Clade C comprised an unresolved polytomy where neither *S. multiflorus* subsp. *katherinae, S. puniceus* nor *S. membranaceus* were monophyletic. The East African subsp. *multiflorus* did, however, form a monophyletic group (clade I, PP 0.94/BS < 50) with *S. cyrtanthiflorus* as sister. The last clade comprised the West African accessions of subsp. *multiflorus,* subsp. *longitubus* as well as *S. pseudocaulus,* hence West African *S. multiflorus* was neither monophyletic. The monophyly of *S. pseudocaulus* could not be assessed as only one accession was included.

Nuclear ribosomal ITS phylogeny

The phylogeny (nrDNA tree) in Figure 12 is the 50 % majority-rule consensus tree from Bayesian analyses of ITS. The topology was congruent with the Maximum Likelihood tree (not shown, but bootstrap values were inserted on the Bayesian consensus tree).

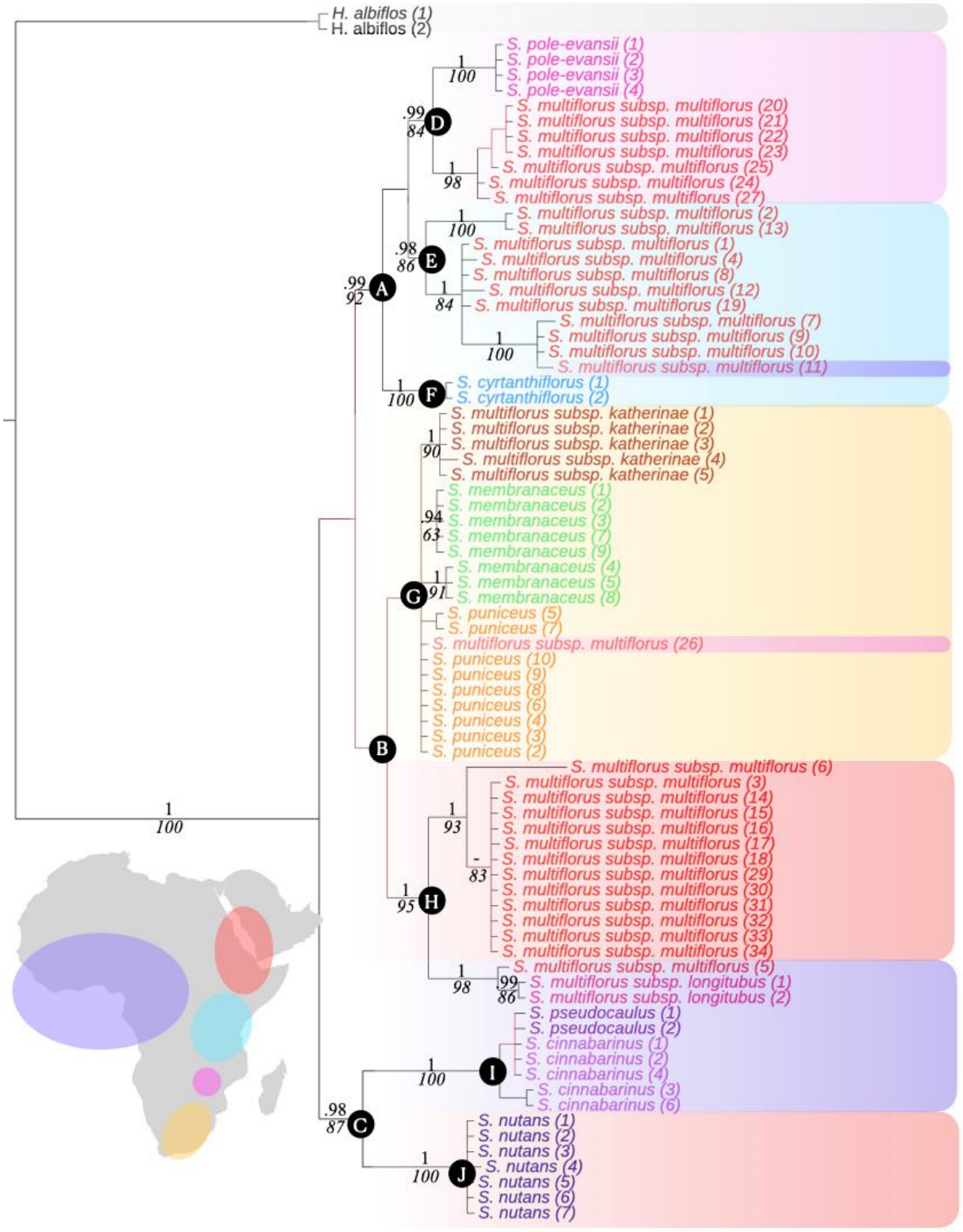


Figure 12. The 50 % majority-rule consensus tree from Bayesian analyses of the ITS matrix with 79 accessions and 700 characters. The Bayesian posterior probability values (PP) of at least 0.9 are reported above branches, whereas RAxML maximum likelihood bootstrap support (BS) values of at least 50 % are reported in italics below branches. Branches lacking high support have red lines. Multiple accessions of the same species are numbered according to Appendix Table A1. Abbreviations: *S.* = *Scadoxus*, *H.* = *Haemanthus*. Shade colors in the tree correspond to: outgroups (grey), Zimbabwe (pink), South Africa (orange), Ethiopia and Saudi Arabia (red), East Africa (blue) and West Africa (purple). The clades discussed in the text are marked with capital letters.

The backbone of the tree had low resolution and thus several clades were in essence part of a polytomy. The three main clades included the highly supported clade A (PP 0.99/BS 92), the low supported clade B (PP < 0.9/BS < 50) and the highly supported clade C (PP 0.98/BS 87). Clade A (PP 0.99/BS 92) showed a strong geographic pattern; two East African accessions of *C. cyrtanthiflorus* (PP 1/BS 100) were sister group to a larger clade consisting of a Zimbabwean clade (D, PP 0.99/BS 84) and a clade East African *S. multiflorus* subsp. *multiflorus* (E PP 0.98/BS 86), which also included a West African accession. The South African accessions, constituting a clade with low support (G, PP < 0.9/BS < 50), were in essence part of the backbone of the tree. The Ethiopian accessions were split between two clades; *S. multiflorus* subsp. *multiflorus* (H, PP 1/BS 93) and *S. nutans* (J, PP 1/BS 100). West African accessions were also split in two clades. *Scadoxus multiflorus* (part of clade H, PP 1/ML 98) as sister to Ethiopian *S. multiflorus* subsp. *multiflorus* (plus one Saudi Arabian accession). The other West African clade (I, (PP 1/ML 100) of S. *cinnabarinus* and *S. pseudocaulus* was sister to Ethiopian *S. nutans.*

Monophyly was supported for most species with multiple accessions except for a few where rather a geographic pattern among accessions was evident. The monophyletic groups of species included *S. pole-evansii* (part of clade D, PP 1/BS 100), *S. cyrtanthiflorus* (clade F, PP 1/BS 100), and *S. nutans* (clade J, PP 1/BS 100). The monophyletic groups of subspecies included *S. multiflorus* subsp. *katherinae* (part of clade G, PP 1/BS 90) and subsp. *longitubus* (part of clade H, PP 0.99/BS 86). Clade I (PP 1/BS 100) comprised *S. cinnabarinus* and *S. pseudocaulus* as part of an unresolved polytomy. Two subclades within clade G (PP 0.9/BS -, PP 1/BS 91) comprised accessions of *S. membranaceus*. This neither confirmed nor disproved monophyly as the clades were part of a polytomy also including *S. puniceus* (paraphyletic) and the previously mentioned *S. multiflorus* subsp. *katherinae*. Accessions of *S. multiflorus* were not monophyletic, however, but part of four separate clades with an evident geographic pattern. Within clade D Zimbabwean subsp. *multiflorus* was monophyletic (PP 1/BS 98) with *S. pole-evansii* as sister. An exception was the subsp. *multiflorus* (26) found in a polytomy in clade G with *S. puniceus* (paraphyletic), *S. membranaceus* and *S. multiflorus* subsp. *katherinae.* The East African clade was also monophyletic (E: PP 0.98/BS 86) as sister to the Zimbabwean clade D. An exception here was subsp. *multiflorus* (11) from Gambia found among the East African accessions. Clade H (PP 1/BS 96) included a subclade with the Ethiopian accessions as well as one Saudi Arabian accession (PP 1/BS 92), and a subclade (PP 1/BS 98) including West African subsp.

multiflorus and two accession of subsp. *longitubus*. The fourth clade of *S. multiflorus* was the previously mentioned monophyletic group of subsp. *katherinae* within clade G.

3.3.3 Comparison of the three phylogenies

The three resulting phylogenies of this study (Figure 10, 11, 12) resembled each other in many aspects. Although to different extents, they all showed evident geographic patterns with five main geographic areas present: West Africa, East Africa, Ethiopia, Zimbabwe and South Africa. The species or subspecies, which in all trees were monophyletic, or at least not disproved monophyletic, included *S. pole-evansii*, *S. nutans*, *S. cyrtanthiflorus, S. cinnabarinus, S. pseudocaulus, S. membranaceus, S. multiflorus* subsp. *katherinae* and subsp. *longitubus*. Non-monophyletic groups of species included *S. puniceus* and *S. multiflorus* both as a species and as subsp. *multiflorus.*

Similar topologies were found between the phylogenies based on chloroplast DNA (Figure 10, 11), but there were however a few exceptions related to the different number of accessions and characters included in the two analyses. The cpDNA tree included overall more accessions than the gDNA tree (70 vs. 22 ingroup accessions) representing all species and subspecies, including West African *S. multiflorus* with subsp. *multiflorus* and subsp. *longitubus*. The gDNA tree did not include West African *S. multiflorus*. It did, however, include three accessions of *S. puniceus* from Ethiopia, Tanzania and Zimbabwe as opposed to only South African *S. puniceus* in the cpDNA tree. Further there was a better resolved topology of the gDNA tree.

The nrDNA and cpDNA tree showed several incongruences (especially with regard to sister relations of several clades (Figure 11, 12). *Scadoxus multiflorus* subsp. *katherinae* was confirmed as monophyletic in the nrDNA tree but not in the cpDNA tree, while *S. cinnabarinus* was confirmed as monophyletic by the cpDNA tree but not in the nrDNA tree. The monophyletic group of *S. nutans* (nrDNA: clade J; cpDNA: clade E) attained incongruent positions. In the cpDNA tree *S. nutans* was more closely related to paraphyletic Ethiopian *S. multiflorus* subsp. *multiflorus,* while in the nrDNA tree *S. nutans* was sister to the clade of *S. cinnabarinus* and *S. pseudocaulus* (clade I). Further, in the cpDNA tree, *S. pseudocaulus* was not sister to *S. cinnabarinus*, but rather found among West Africa *S.*

multiflorus. In the nrDNA tree, the monophyletic group of *S. cyrtanthiflorus* (clade F) was sister to the clade consisting of *S. pole-evansii* and *S. multiflorus* subsp. *multiflorus* from Zimbabwe and East Africa respectively (clade D and E). In the cpDNA tree, *S. cyrtanthiflorus* (clade H) was, however, sister only to the East African *S. multiflorus* subsp. *multiflorus*. Two accessions of *S. puniceus* (6 and 8) attained incongruent positions as well. In the nrDNA tree, they were both part of clade G together with the other *S. puniceus* accessions, whereas in the chloroplast tree they were found in clade A amongst *S. pole-evansii* and Zimbabwean *S. multiflorus* subsp. *multiflorus*. One Zimbabwean *S. multiflorus* subsp. *multiflorus* (26) was grouped together with the other Zimbabwean accessions in the cpDNA tree, but was part of a polytomy with *S. puniceus* in the nrDNA tree. Apart from these incongruent patterns, the nrDNA and cpDNA topologies supported by PP of at least 0.9 or BS of at least 50 % were congruent, but resolved to different extents and in different parts of the trees.

4 Discussion

The main aim of this study was to use molecular phylogenies to investigate *Scadoxus,* and further reveal if species delimitation and sister relations based on morphology were reflected in the molecular analyses. The available sequence data for *Scadoxus* has been substantially increased with this study, both in terms of the number of newly sequenced taxa as well as in the number of DNA regions analyzed. As part of the study whole chloroplast genomes have been produced for all currently recognized *Scadoxus* species except *S. longifolius*.

The resulting phylogenetic trees (Figure 10, 11, 12) detected some incongruence between the chloroplast trees and the nrDNA tree.

Generally, such incongruence could be caused by either hybridization, allopolyploidy, sequence convergence, lineage sorting, introgression (i.e chloroplast caption) or paralogy of nrDNA (Rieseberg and Soltis, 1991). Chloroplast capture has been detected in a great number of plant groups, where the native chloroplasts of the cytoplasm are displaced by a foreign one through hybridization followed by repeated backcrossing (Stegemann et al., 2012, Rieseberg and Soltis, 1991, Rieseberg et al., 1996). This phenomenon could explain great discrepancies between phylogenies based on nuclear and chloroplast DNA, respectively (Tsitrone et al., 2003, Stegemann et al., 2012). Such horizontal genome transfer could occur frequently within or between species that are reproductively compatible and have a sympatric distribution, resulting in the maternally inherited chloroplast DNA phylogeny often correlating with geography rather than morphology and nuclear DNA (Fehrer et al., 2007, Acosta and Premoli, 2010, Whittemore and Schaal, 1991). This is especially relevant for *S. membranaceus, S. puniceus* and *S. multiflorus* subsp. *multiflorus* in South Africa, for which distribution areas and flowering periods overlap. The aforementioned conditions also concern *S. nutans* and *S. multiflorus* subsp. *multiflorus* in Ethiopia. On the other hand, there are disadvantages of using the nuclear ribosomal internal transcribed spacer (ITS) as well. This includes its limited use for inferring phylogenetics between taxa that have recently diverged, and also in the case of incomplete lineage sorting or hybridization events where it can be a challenge to determine sequence paralogy and orthology due to concerted evolution (Soltis et al., 1991, Fehrer et al., 2007, Bailey et al., 2003).

The phylogenetic incongruence between the chloroplast trees and the nrDNA tree in my study does not allow for a straightforward interpretation, and investigating these matters

further are beyond the scope of this study. Including more nuclear DNA, possibly the whole genome, could help resolve the basal branches of the phylogeny by adding stronger support. However, as previously noted an extremely large genome size of 43.20 Gbp is found in *Scadoxus* (Zonneveld et al., 2005), and that was why the decision to use the much shorter chloroplast genome (~160 000 kb) for next-generation sequencing was made.

Despite some poor resolution and discrepancy in the basal relationships between the trees, there are still several common features in terms of clades supporting certain species and species groups, as well as an overall geographic structure. This is the foundation for the following discussion.

4.1 Species delimitation and morphology

This section concerns the first aim of this study: to test if the species delimitation based on morphology is confirmed by the molecular phylogeny. The monophyly of each species will be discussed separately (when possible) taking into consideration the species delimitation based on morphology concluded by Bjørnstad and Friis (1972a, 1972b, 1974). See section 4.2 for the discussion of the subspecies.

Scadoxus pole-evansii – The four accessions of *S. pole-evansii* constitute a monophyletic group with high support in both the nrDNA tree (Figure 12, part of clade D) and cpDNA tree (Figure 11, clade D). This result is highly expected as this is a morphologically very distinct species, endemic to the highlands of eastern Zimbabwe. It has a narrow distribution area within Nyanga National Park, compared to the wide distribution of its habitat, namely the afromontane forest. Morphologically it is distinct in several independent flower characters giving it an unmistakable appearance; length of perianth tube (less than 5 mm), width of perianth segments (more than 5 mm broad), length of filaments being shorter than the perianth segments and long anthers (4.5-7 mm) (Bjørnstad and Friis, 1974). Field observations revealed a further character not mentioned in the key or description to the taxa. All specimens collected revealed a horizontally growing rhizomatous bulb (Figure 9A). As the species is described based on herbarium sheets only, this may be a character that has not previously been assessed. The rank of species for *S. pole-evansii* is confirmed by the molecular phylogenetic analyses.

Scadoxus nutans – All the seven accessions of *S. nutans* constitute a monophyletic group with high support in both the nrDNA tree (Figure 12, clade J) and the cpDNA tree (Figure 11, clade E). This result is also expected as this is another morphologically distinct species endemic to the Kefa and Illubabor floristic regions in Southwestern Ethiopia. It is native to moist afromontane forest where it grows both on the forest floor and as an epiphyte on tree trunks (Hutchinson and Wondafrash, 2011). Morphologically it is very distinct by having short and partly fused inflorescence bracts supporting a dense nodding inflorescence (the scape is bended, from where is has the name "nutans", meaning "nodding" in Latin). When the seeds are mature however, the infrutescence turns upwards (scape straightens out). It has further a pseudostem through which the inflorescence and new shoots pierce through during development (Figure 14). The subterranean organ is an elongated rhizome, sometimes with stolons (Demissew and Nordal, 2010). *Scadoxus nutans* has a distinct pollen morphology as well, with regards to the exine pattern; a very narrow lumina compared to the other species and broad muri all over the grain (Bjørnstad and Friis, 1972a).

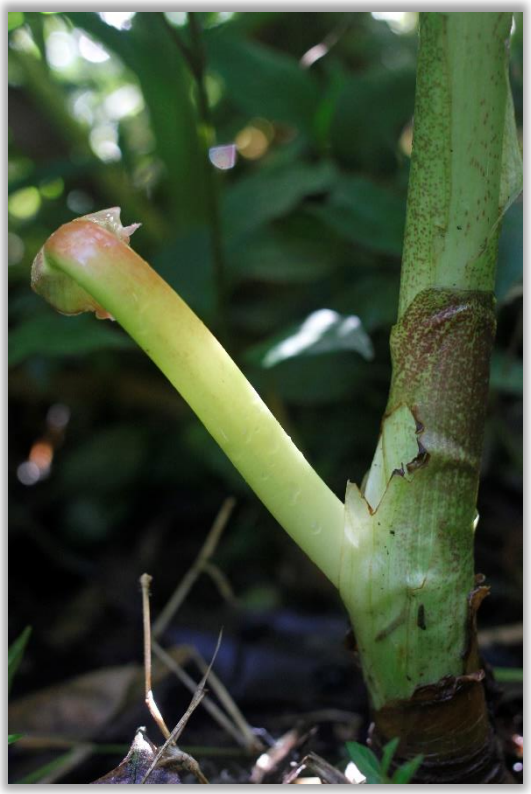


Figure 14 Scape breaking through the pseudostem in a young *S. nutans.* Photo: Kine H. Bødker.

Scadoxus cyrtanthiflorus – The clade comprising the two accessions of *S. cyrtanthiflorus* is highly supported in both the nrDNA (Figure 12, clade F) and the cpDNA tree (Figure 11, clade J) and clearly monophyletic. This species is yet another narrowly distributed species of *Scadoxus*; native to the Rwenzori Mountains on the border between the Democratic Republic of the Congo and Uganda. In the rainforest it grows either on forest floor or epiphytic on tree trunks (Hutchinson, 2014). Morphologically it is distinct by having an inflorescence with funnel-shaped perianth tubes considerably longer than the perianth segments, flattened filaments, and with individual flowers facing downwards due to their long bended pedicel. It has further a pseudostem with a swollen base through which the inflorescence and new shoots

pierce through during development. The subterranean organ is an elongated rhizome (Bjørnstad and Friis, 1972a, Friis and Nordal, 1976).

Scadoxus cinnabarinus* and *S. pseudocaulus – In the cpDNA tree the six accessions of *S. cinnabarinus* constitute a monophyletic group with high support (clade G, Figure 11). The two accessions included in the gDNA tree also constitute a highly supported monophyletic group (Figure 10, part of clade G). The five accessions included in the nrDNA tree, however, form a highly supported clade only when to accessions of *S. pseudocaulus* are included (Figure 12, clade I). Within this clade two of the *S. cinnabarinus* accessions (3, 6) constitute a highly supported subclade whereas the remaining accessions are part of an unresolved polytomy. The separation of the two accessions from the rest could possibly be related to geography, however too little information is available to draw such a conclusion. *Scadoxus cinnabarinus* has an extensive distribution centered on the Congo basin, stretching as far as Sierra Leone in the north, Uganda in the east and Angola in the south. Two *S. cinnabarinus* accessions (1, 2) were from Cameroon, one from Gabon (3), while the remaining accessions were from cultivation. Accounts from cultivators suggest that the majority of *S. cinnabarinus* in cultivation is of the same origin (unknown) (Hutchinson, 2014).

The lack of separation of the two species in the nrDNA tree may be due to different sequence lengths of the internal transcribed spacer; the *S. cinnabarinus* sequence included ITS1, 5.8S and ITS2 (725-749 bp), while the *S. pseudocaulus* sequences included only ITS1 (230-236 bp) because of difficulty obtaining longer PCR products from the old herbarium material. Obtaining and including full sequences of *S. pseudocaulus* would probably be needed to resolve the nrDNA tree.

The two species, which both belong to section *Demeusea*, are separated by the presence or absence of a pseudostem and a few other morphological characters. *Scadoxus pseudocaulus* has a pronounced pseudostem 15-45 cm long (from which the name is derived), the scape is initiated within the false stem/central among the leaves, although in some cases it pierces and emerges from the pseudostem, appearing lateral. The height of the plant reaches 80 cm and number of flowers can be up to 50. *Scadoxus cinnabarinus* is lacking a pseudostem, and the scape arises centrally among the leaves. The plant can reach up to 60 cm with umbels consisting of up to 100 flowers, although few-flowered umbels are more common. The inflorescence seldom reaches above the leaves, and the pedicels droop at the fruiting stage

(Bjørnstad and Friis, 1972b). In addition it has been noted that cultivation requirements are quite different. *Scadoxus pseudocaulus* tolerates lower winter temperatures, but it has been reported a reluctance to flower (Hutchinson, 2014). Altogether, it is confirmed that both *S. cinnabarinus* and *S. pseudocaulus* deserves taxonomic recognition at the specific level.

Scadoxus longifolius – This species is only known from one incomplete specimen collected in 1891 with an unknown location other than country (Democratic Republic of the Congo). The genus *Demeusea* was based on this specimen by De Wildeman & Durand (1900). The small flowers, short perianth tubes, and short filaments that were given weight by the authors to distinguish the new genus from *Haemanthus* were all features of a young specimen. The rhizome, central peduncle, and lack of false stem show a close relation to *S. cinnabarinus*. The key that separates S. *longifolius* from *S. cinnabarinus* is based on leaf form; the specimen's broadest lamina is about 10 times longer than broad while the narrowest lamina found in *S. cinnabarinus* are never more than 5 times longer than broad. It is a very imperfectly known species, and the type specimen has been suspected to be a depauperate, incomplete and young individual of *S. cinnabarinus* (Bjørnstad and Friis, 1972b).

It has not been possible to sequence and include material from the type specimen in the phylogenetic analyses of this study, and neither been able to find any other reliable characters to confirm the species' uniqueness. I will therefore recommend that it will be reduced to synonym under *S. cinnabarinus* (see taxonomic conclusion).

Scadoxus membranaceus – The eight accessions of *S. membranaceus* form two separate clades, both highly supported in the nrDNA tree (Figure 12), with five and three accessions, respectively. *Scadoxus membranaceus* is therefore not monophyletic according to this region. The two clades are part of a larger polytomy (clade G) comprising *S. puniceus* (paraphyletic) and *S. multiflorus* subsp. *katherinae* (separate monophyletic group). The cpDNA tree, which includes the same accessions, shows almost the same story with the same three South African taxa part of a polytomy (Figure 11, clade C), although without any highly supported *S. membranaceus* clades as part of the polytomy. In the gDNA tree (Figure 10) clade D containing these three South African taxa are better resolved with high support for the subclades. Only one accession each of *S. membranaceus* and *S. multiflorus* subsp. *katherinae* are included in the gDNA tree, but still the results suggest that it is necessary to include more sequence data in order to get a full resolution. Preferably more sequences from other nuclear

DNA regions would be needed (due to previously discussed nuclear DNA vs. chloroplast DNA incongruence.)

Morphologically it is nonetheless clear that *S. membranaceus* is very distinct and thus warrant status at the specific level. Although it is similar in appearance to *S. puniceus*, it is smaller in size and does not produce a pseudostem. Further, the persistent bracts are usually four in number (as opposed to more than four), they are more prominent and more or less equally sized. These bract characters consequently result in a better support for the remarkable showy fruits. Also, the total distribution of *S. membranaceus* is restricted to the eastern coast of South Africa, between 27°S and 33°S, indicating that it is ecologically bound to the coast (Bjørnstad and Friis, 1974, Hutchinson, 2007). In regard to *S. multiflorus* subsp. *katherinae*, the morphology of this taxon is discussed later.

Scadoxus puniceus – *S. puniceus* is clearly not monophyletic. Accessions from Zimbabwe, South Africa, Ethiopia and Tanzania appear in four different clades throughout the gDNA tree (Figure 10). In the cpDNA (Figure 11), which includes South African accessions only, they are part of a polytomy (clade C) including *S. membranaceus* and *S. multiflorus* subsp. *katherinae* as mentioned above. Three accessions of *S. puniceus* (5, 7, 10) form, however, a subclade as sister to the rest of the South African clade. The two remaining *S. puniceus* accessions (6, 8) appear in clade A among Zimbabwean *S. multiflorus* subsp. *multiflorus* and *S. pole-evansii.* Within the nrDNA tree none of the accessions form separate clades but are part of a polytomy (Figure 12, clade G) also including *S. membranaceus* and *S. multiflorus* subsp. *katherinae* (which both do form separate clades) and one accession of Zimbabwean *S. multiflorus* subsp. *multiflorus* (26).

The morphological delimitation of *S. puniceus* is based on having more than four free, ascending involucral bracts persistent during the flowering period. Further it has a pseudostem present, rhizomatous bulb and scape-initiation outside the leaf cluster (Bjørnstad and Friis, 1974). *Scadoxus puniceus* will as previously mentioned be assessed in a separate thesis by Ida E. Moe, and thus the species delimitation of *S. puniceus* will not be discussed further here.

Scadoxus multiflorus – *S. multiflorus* as one species is clearly not monophyletic, but rather polyphyletic. The forty-three accession included from Gambia, Guinea and Ghana in the West, to Saudi Arabia, Ethiopia, Kenya, Tanzania, Zimbabwe, Zambia, Mozambique and

South Africa in the east and south rendered five clades with an evident geographic pattern: 1) **Zimbabwean accessions** (nrDNA, part of clade D; cpDNA, part of clade A; gDNA, part of clade C. 2) **Ethiopian accessions** plus one Saudi Arabian accession in the nrDNA tree (nrDNA, part of clade H; cpDNA, part of clade B; gDNA, part of clade E). 3) **East African accessions** plus one Gambian in the nrDNA tree (nrDNA, clade E; cpDNA, clade I; gDNA, part of clade I). 4) **West African accessions,** not included in the gDNA tree (nrDNA, part of clade H; cpDNA, part of clade F). 5) **South African accessions** (nrDNA, part of clade G; cpDNA, part of clade C; gDNA, part of clade D).

This result is probably not so inexplicable, as *S. multiflorus* shows considerable morphological variation throughout its large, continuous distribution area extending throughout tropical Africa, from Senegal to Yemen and to South Africa and Namibia.

The species was lastly revised after the examination of approximately 750 specimens, where Bjørnstad and Friis (1974) reduced 32 names to synonymy due to morphological continuities when considering material from all regions of the distribution area. Variation was especially found in general size and robustness, i.e characters such as length of peduncle, number of flowers, diameter of corm and diameter of inflorescence. Notable variation was in addition found in time of leaf development in relation to flowering (hysteranthous vs. synanthous), width of perianth segments, length of pedicel and length of perianth tubes (Figure 15).

Figure 15. Morphological variation within *S. multiflorus* from **(A)** Malawi, **(B)** Tanzania, **(C)** Zimbabwe, **(D)** Tanzania, **(E-F)** Zimbabwe, **(G)** Zambia. Photos: Charlotte S. Bjorå (A-F), Inger Nordal (G).

Specimens referred to the species were, however, united by the following characters: false stem, inconspicuous involucral bracts withering early during flowering period, semi-globose to globose inflorescence with spreading perianth segments narrower than 4 mm (rarely up to 5 mm), perianth tubes longer than 5 mm, mature filaments longer than the perianth segments with 1-3 mm long anthers (Bjørnstad and Friis, 1974).

This study concludes that the genetic diversity in relation to geographic patterns should be further analyzed in *S. multiflorus.*

See below for further discussion of the placement of the *S. multiflorus* subspecies in the molecular phylogenies and its relation to morphology

4.2 Division of *S. multiflorus* into subspecies

This section concerns the second aim of this study: to test if the division of *S. multiflorus* into subspecies is reflected in the molecular analyses. The monophyly of each subspecies will be discussed separately taking into consideration the subspecies delimitation based on morphology concluded by Bjørnstad and Friis (1974).

Scadoxus multiflorus subsp. multiflorus – *S. multiflorus* subsp. *multiflorus* as a subspecies is clearly not monophyletic, but rather polyphyletic forming four separate clades with an evident geographic pattern.

A highly supported clade in all trees contains the **Zimbabwean accessions**. In the nrDNA tree seven accessions are grouped together with high support (Figure 12, part of clade D). Although with lower support, the same was apparent in the cpDNA tree (Figure 11, part of clade A) including the same seven accessions. An exception from the mentioned grouping was *S. multiflorus* subsp. *multiflorus* (26), which appears in quite other parts of the tree compared to the other accessions (23, 24, 25) collected in the same area (Matobo National Park). In the nrDNA tree it is found as part of a large polytomy (clade G), while in the cpDNA tree it sister to the rest of clade A.

The **Ethiopian accessions** are grouped together in the nrDNA tree in a clade (Figure 12, part of clade H), which also included one Saudi Arabian accession which geographically is rather close. In the gDNA tree (Figure 10, part of clade E) the Ethiopian accessions are grouped with high support as well. In the cpDNA tree, however, Ethiopian subsp. *multiflorus* is paraphyletic with *S. nutans* nested within the clade (Figure 11, clade B).

The **East African accessions** also formed a separate clade (cpDNA: clade I), although not as highly supported as the Ethiopian clade. The same accessions are better supported as a group in the nrDNA tree (Figure 12, clade C), which includes an additional accession from Zambia (4) and one West African accession from Gambia (11) as well.

This latter accession is, however, part of a **West African clade** in the cpDNA tree (Figure 11, clade F), which further includes one accession of subsp. *longitubus* from Guinea and one Ghanaian *S. multiflorus* not determined to subspecies. The only accession of *S. pseudocaulus* is included here as well, but since the alignment of this accession consists of only three out of the six chloroplast regions, this placement is uncertain. In the nrDNA tree, the West African

clade (Figure 12, part of clade H) includes one Ghanaian subsp. *multiflorus* as sister to two subsp. *longitubus*.

This study clearly shows the polyphyletic nature of the subspecies. As one of the central controversies in contemporary taxonomy and systematics revolves around whether to accept or to reject paraphyletic taxa (Brummitt, 2002, Hörandl, 2006, Schmidt-Lebuhn, 2012), the author of this study does not feel comfortable with disintegrating the taxa without a more thorough approached inferred. Another reason is that it is not known where the exact geographical boundaries between the taxa would be. This is owing to the fact that specimens from all distribution areas are not included, and accessions from West Africa are scarce. Morphologically subsp. *multiflorus* has been delimited to consist very small up to robust plants with perianth tubes usually less than 15 mm long, perianth segments usually less than 2.5 mm broad, with a distribution from Senegal to southern Arabia and to KwaZulu-Natal, avoiding the lowland rainforest area (Bjørnstad and Friis, 1974). The conclusion about continuous morphology was, however, based on the regions the authors decided. Consequently, after sampling the whole distribution area it may be possible to find morphological differences if rather the boundaries formed by the molecular phylogenetic trees are followed

As this subspecies is very widespread and varies greatly morphologically, it is concluded that genetic diversity in relation to geographic distance should be further analyzed. An exception from this conclusion is the Ethiopian subsp. *multiflorus,* which has morphological traits differing from other subsp. *multiflorus.* See section 4.3 – "Are *Haemanthus bivalvis* and *S. multiflorus* conspecific?"

Scadoxus multiflorus* subsp. *katherinae – The molecular phylogenetic analyses confirm that subsp. *katherinae* deserves a taxonomic rank separate from other *S. multiflorus* subspecies. In the nrDNA tree the five subsp. *katherinae* accessions are found as a highly supported monophyletic group (Figure 12, part of clade G) located far apart from the other *S. multiflorus* accessions in the tree. They are not confirmed as monophyletic in the cpDNA tree (Figure 11, clade C) where three accessions form a subclade and the other two are part of a polytomy with the other South African accessions of *S. membranaceus* and *S. puniceus*. However, in the gDNA tree (Figure 10, clade D) the separation of these three taxa is evident, with subsp. *katherinae* as highly supported sister to *S. membranaceus.*

Subsp. *katherinae* was initially described in 1877 as *Haemanthus katherinae* Baker by the Kew botanist J.G. Baker from material collected by Katharine Saunders, a botanical illustrator and plant collector in KwaZulu-Natal, South Africa (Duncan, 2013). Later it was reduced to synonym of *H. multiflorus* as a subspecies (Bjørnstad and Friis, 1974), before being combined into *S. multiflorus* subsp. *katherinae* (Friis and Nordal, 1976). The recognition of subsp. *katherinae* within *S. multiflorus* was justified by regional geography, ecology and the following morphological characters: length of perianth tube, width of perianth segments and a general high robustness. The subspecies grows up to 1.20 m, has a high number of flowers (40-200), a long perianth tube (1.2-2.2 cm) and broad perianth segments (2.2-4.0 mm). It is distributed in the coastal bush areas from sea level up to 750 m, from southern Eswatini and Mozambique to KwaZulu Natal and the East Cape of South Africa (Duncan, 2013, Bjørnstad and Friis, 1974).

A recent study on pollination, which included subsp. *katherinae* and subsp. *multiflorus* from South Africa found that both are specialized for butterfly-wing pollination (Butler and Johnson, 2020). They are, however, pollinated by markedly different butterfly species; subsp. *katherinae* is mainly visited by forest taxa, while subsp. *multiflorus* is mainly visited by savanna taxa. Butler and Johnson (2020) suggested the existence of a pollination system specialization as the visiting butterflies represented only a subset of all butterflies active in the areas. In addition to previously mentioned morphological discriminating characters, they noted that the flowers of the two subspecies differ significantly in terms herkogamy. They also observed that subsp. *katherinae* has a high degree of self-incompatibility indicating an almost complete dependence on pollinators for cross-pollination and seed production. Self-incompatibility was not assessed for subsp. *multiflorus*. It has also been noted that subsp. *katherinae* has a flowering period from January to March, which is later than subsp. *multiflorus* which flowers in October in that part of Africa (Bjørnstad and Friis, 1974, Butler and Johnson, 2020). This is another fact that may explain why the two subspecies are kept separate despite being distributed in adjacent areas, i.e they are sympatric taxa.

Based on the molecular phylogeny of this study as well as regional geography, ecology and morphological characters discussed here, the conclusion is that *S. multiflorus* subsp. *katherinae* is so distinct that it should be re-instated at at the level of species, as *Scadoxus katherinae* (see taxonomic conclusion).

Scadoxus multiflorus* subsp. *longitubus – The clade comprising the two accessions of subsp. *longitubus* is highly supported in the nrDNA tree (Figure 12, part of clade H) and clearly monophyletic with a West African subsp. *multiflorus* as sister. In the cpDNA tree (Figure 11, clade F) the only subsp. *longitubus* accession included is part of a polytomy with a West African subsp. *multiflorus* and a Ghanaian *S. multiflorus* not determined to subspecies. The only accession of *S. pseudocaulus* is included here as well, but as previously mentioned the placement of this accession is uncertain due to missing data.

The recognition of subsp. *longitubus* within S. *multiflorus* was originally justified by regional geography, ecology and the following morphological characters: length of perianth tube, width of perianth segments and a general robustness (Bjørnstad and Friis, 1974). The subspecies is medium robust growing up to 65 cm with a long perianth tube (more than 1.5 cm) and broad perianth segments (1.4-3.5 mm). The subspecies is distributed in the lowland rainforest from sea level up to 850 m, from Guinea to Ghana.

As this study only included one or two accessions of subsp. *longitubus* in the phylogenetic trees, and also few accessions of West African *S. multiflorus* subsp. *multiflorus,* I should be careful to conclude about this taxa. However, it does seem to constitute a monophyletic group separate from other West African *S. multiflorus*, and is best kept as a subspecies as of now. It is in need further assessment together with the rest of the *S. multiflorus* complex.

4.3 Are *Haemanthus bivalvis* and *S. multiflorus* conspecific?

This study includes eleven accessions *S. multiflorus* subsp. *multiflorus* (14-18, 29-34 Appendix Table A1) collected on a field trip in Ethiopia, which morphologically most often resembled the taxon previously named *Haemanthus bivalvis* by Beck (1888). This included having an elongated stolon-like rhizomatous parts under the bulb, and partly fused involucral bracts. They often grew together in clusters, which the other *S. multiflorus* subsp. *multiflorus* specimens without stolons did not do (Figure 9, Figure 16).

Figure 16. Common characteristics of the Ethiopian *S. multiflorus* subsp. *multiflorus*, including **(A-B)** persistent two-parted bracts and **(C)** stolons.

In the molecularly phylogenies, there is no support for a new combination of *Scadoxus bivalvis*. The gDNA tree includes two *S. multiflorus* subsp. *multiflorus* accessions of the "bivalvis" form and two of the "normal" form, which are grouped together (Figure 10, part of clade E) with a mix of both forms. The same is seen in the cpDNA tree comprising all accessions (Figure 11, part of clade B). All Ethiopian *S. multiflorus* subsp. *multiflorus* accessions are part of a polytomy in the nrDNA tree (Figure 12, part clade H) with no further division into subclades.

Even though the *H. bivalvis* does not warrant resurrection, there is clearly evidence for raising Ethiopian S. *multiflorus* subsp. *multiflorus* to specific level. *Scadoxus multiflorus* subsp. *multiflorus* found in Ethiopia was named *Haemanthus abyssinicus* Herb. based on a collection from 1810 by Salt. The type clearly shows the typical form that occurs when the bracts are a bit more prominent than in other subsp. *multiflorus,* leading to a more upheld inflorescence. A new description of this taxon includes that the species is most often found

with prominent subterranean stolons and involucral bracts fused into two valves. See taxonomic conclusion.

4.4 Phylogenetic inference: morphology vs. DNA

In this section the discussion will contemplate the aim regarding sister relations in *Scadoxus*, and specifically if the species have evolved as hypothesized by Nordal and Duncan (1984). The result from their character compatibility analysis of *Scadoxus* and *Haemanthus* was based on 25 characters, where 16 characters remained if excluding characters identical for all *Scadoxus* species included. All species, except *S. longifolius*, were included in the analysis. The characters, which were phylogenetic informative, will be discussed in the following with regard to the molecular phylogenies resulting from this study (Figure 17).

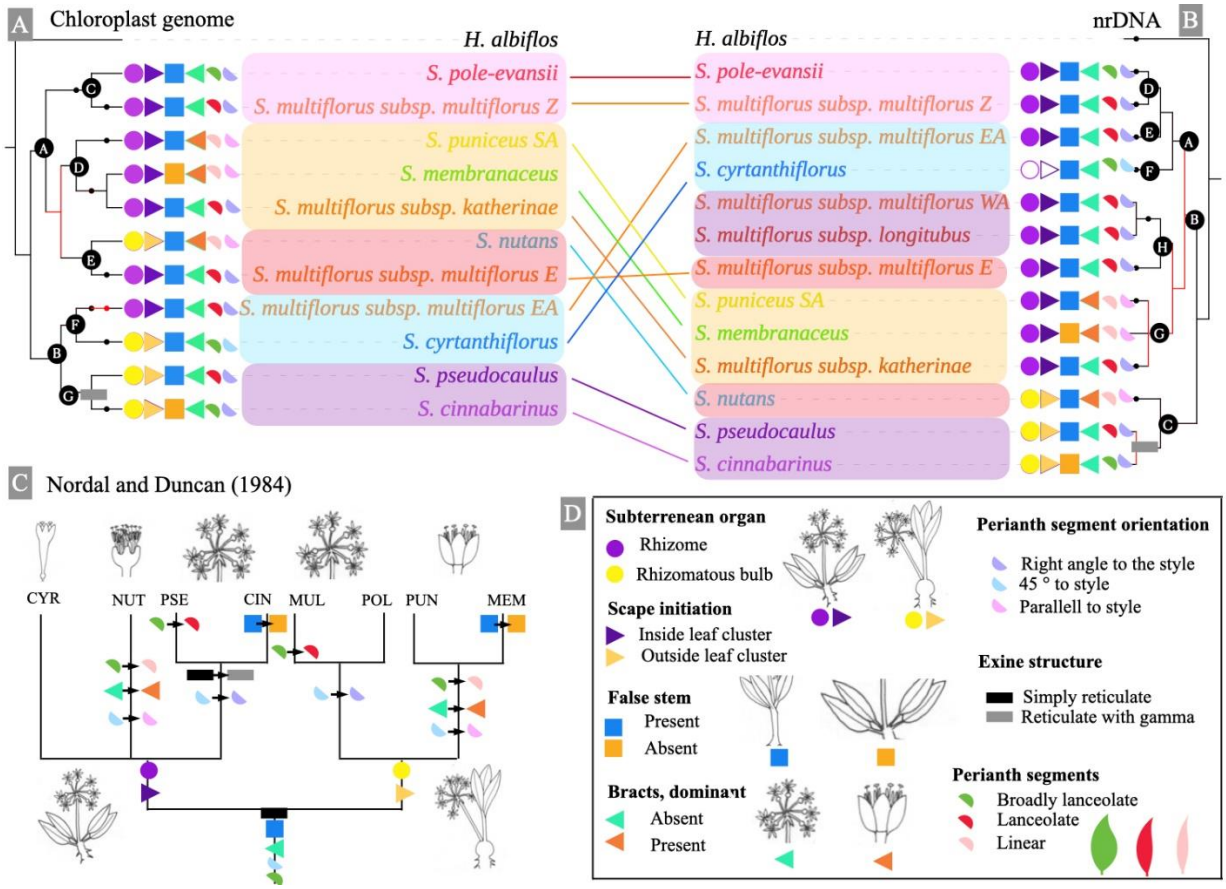


Figure 17. Pruned trees from **(A)** chloroplast genome and the **(B**) nrDNA. **(C)** The phylogeny from Nordal and Duncan (1984). **(D)** Phylogenetic informative characters used in their study. Illustrations from Bjørnstad and Friis 1972a, p. 203.

Subgenera - The division of *Scadoxus* into the two subgenera was based on the significantly different phytogeography and ecology (Nordal and Duncan, 1984). The subgenus *Demeusea*, also termed the rainforest group, includes all the true rhizomatous rainforest taxa from the Guineo-Congolean lowland rainforests, as well as some taxa from restricted areas of mountain rainforests of East Africa. All *Demeusea* taxa have the scape initiated centrally among the leaves, which may break the leaf sheats during development in forms with a false stem present. The subgenus *Scadoxus,* also termed the savannah group, includes all the savannah species with a rhizomatous bulb from primarily the Sudano-Zambezian region. These species have the initiation of the scape outside the leaf cluster.

Subgenus *Demeusea* – The character compatibility analysis could not fully resolve the phylogeny between the sections of subgenus *Demeusea* resulting in a polytomy comprising *S. nutans*, *S. cyrtanthiflorus* and section *Demeusea.* (Figure 17).

Section *Demeusea*: *S. cinnabarinus* and *S. pseudocaulus* – *Scadoxus longifolius* of section *Demeusea* will not be discussed here (see 4.1 Species delimitation and morphology).

The cladistic analysis based on morphology grouped *S. cinnabarinus* and *S. pseudocaulus* as sisters based on both having a distinct pollen morphology with gemmate sculpturing on the tectum, and both having the segment oriented at a right angle to the style, which both are characters not found in the other taxa of the subgenus (Nordal and Duncan, 1984).

The nrDNA tree (Figure 12) confirms a close relationship between the two taxa although not perfect sister relations owing to low resolution and an unresolved polytomy (previously discussed). However, the sister relations were shown in the gDNA tree (Figure 10).

Altogether, it is concluded here that *S. cinnabarinus* and *S. pseudocaulus* are sister taxa, confirming previous conclusions, which was based on morphology.

Section *Gamolepsis*: S. *nutans* – In this study the nrDNA and chloroplast trees rendered very different sister relations regarding the monophyletic group of *S. nutans.* The nrDNA tree (Figure 12) clearly shows that *S. nutans* is sister to a monophyletic clade (clade I) comprising *S. cinnabarinus* and *S. pseudocaulus*, with strong support. This result agrees well with Nordal and Duncans (1984) phylogeny based on morphology, suggesting a typical rainforest taxa as ancestor.

In the chloroplast trees, however, Ethiopian *S. multiflorus* subsp. *multiflorus* and *S. nutans* (also Ethiopian) are closest relatives. In the gDNA tree (Figure 10) this sister relation is highly supported, whereas in the cpDNA tree (Figure 11) *S. nutans* is nested within *S. multiflorus* subsp. *multiflorus* (clade B). Ethiopia and the other countries in the Horn of Africa are possessing the richest biodiversity of the continent and also host two of the 35 biodiversity hotspots in the world (Mittermeier et al., 2011, Wang et al., 2020, Friis et al., 2005). The argument could be made for *S. nutans* originating from Ethiopian *S. multiflorus* subsp. *multiflorus* before subsequently becoming adapted for the rainforest and thus attaining a very different morphology. However, the congruent result agrees well with studies of other plant groups where they have found that phylogenetic relationships based on chloroplast DNA often correlate with geography rather than morphology and nuclear DNA (Stegemann et al., 2012). Thus, the previously mentioned phenomenon of chloroplast caption is probably a better explanation as the species have overlapping distribution areas and flowering periods. This could lead to pollen of *S. multiflorus* subsp. *multiflorus* being transferred to the stigma of *S. nutans*.

Section *Choananthus*: *S. cyrtanthiflorus* – As previously mentioned, the cladistics based on morphology could not resolve the phylogeny between this species, *S. nutans* and section *Demeusea.* (Figure 17). In this study the nrDNA and chloroplast trees again rendered quite different results regarding sister relations. With high support the nrDNA (Figure 12) clearly shows *S. cyrtanthiflorus* as sister to a monophyletic clade comprising the Zimbabwean *S. multiflorus* subsp. *multiflorus* and *S. pole-evansii* (clade D), as well as East African *S. multiflorus* subsp. *multiflorus* (plus one Gambian *S. multiflorus* subsp. *multiflorus*, clade E). In the cpDNA tree, the sister clade of *S. cyrtanthiflorus* is East African *S. multiflorus* subsp. *multiflorus* (clade I). However, in the gDNA tree, where accessions of *S. puniceus* from multiple countries are included, *S. cyrtanthiflorus* is closest sister to Ethiopian *S. puniceus* (clade H). Again, phylogenies based on chloroplast DNA seem to be more correlated with geography.

If a conclusion of the relations of *S. cyrtanthiflorus* can be drawn, it is that the polyphyletic *S. multiflorus* is a close relative. This result is interesting as they differ substantially in morphology. Typical characters of *S. cyrtanthiflorus* include: rhizome, scape initiation central among the leaves, but breaking through the false stem with a swollen base during development, flattened filaments and funnel-shaped perianth tube on flowers facing

downwards. Characteristics of *S. multiflorus* in contrast include: rhizomatous bulb, scape initiation outside leaf cluster, subulate filaments and cylindrical perianth tube on upright flowers. However, as *S. cyrtanthiflorus* is found only on the Ruwenzori Mountains between 1600-3200 m in evergreen montane forest, one could argue that it might be a relict, which was previously more widespread but now restricted to a smaller area. Typical traits of a relict population in a micro-refugia include being restricted to small areas, show low genetic diversity, clonal growth and low dispersal (Hampe and Jump, 2011, Keppel et al., 2012). However, as only isolation and restriction to a small area can be acertained for *S. cyrtanthiflorus,* it is not possible to conclude about these matters. Possibly a more probable explanation is that the species could result from a population previously part of the widespread *S. multiflorus* before diverging and getting ecologically adapted to the mountain area to which it is now confined. To conclude about the sister relations of *S. cyrtanthiflorus*, analyses of more data are needed.

Subgenus *Scadoxus* – The character compatibility analysis (Nordal and Duncan, 1984) resulted in a sister relation between section *Gyaxis* (comprising *S. puniceus* and *S. membranaceus*) and section *Scadoxus* (comprising *S. multiflorus* and *S. pole-* evansii) (Figure 17).

Section *Gyaxis*: *S. puniceus* and *S. membranaceus* – The analysis based on morphology grouped *S. puniceus* and *S. membranaceus* together based on both having an inflorescence surrounded by separate involucral bracts, ascending and dominating during the flowering period. Additionally by having linear and 1-nerved perianth segments oriented in parallel to the style (Nordal and Duncan, 1984).

S. puniceus – The sister relation to South African *S. puniceus* are unclear in all trees, but with *S. membranaceus* and *S. multiflorus* subsp. *katherinae* closely related. In the nrDNA tree (Figure 12, clade G) all accessions of *S. puniceus* are part of a polytomy where *S. membranaceus* and *S. multiflorus* subsp. *katherinae* form further subclades. In the cpDNA tree (Figure 11, clade C) the relations are even more unresolved with all previously mentioned taxa found at the base of the polytomy. Also, two accessions of *S. puniceus* (6, 8) appear among Zimbabwean *S. multiflorus* subsp. *multiflorus* and *S. pole-evansii* (clade A).

In the gDNA tree (Figure 10, clade D) South African *S. puniceus* has unresolved relations to *S. membranaceus* and *S. multiflorus* subsp. *katherinae,* which are nested within a clade of *S.*

puniceus. However, Zimbabwean *S. puniceus* is sister to Zimbabwean *S. multiflorus* subsp. *multiflorus* and *S. pole-evansii*, Ethiopian *S. puniceus* is sister to *S. cyrtanthiflorus*, and Tanzanian *S. puniceus* is sister to East African *S. multiflorus* subsp. *multiflorus*.

The results show that the polyphyletic *S. puniceus* consequently has different sister taxa, and thus that the morphologically based phylogeny is not supported. These issues will be further discussed in the thesis of Ida E. Moe as previously mentioned.

***S. membranaceus* –** As previously disclosed, *S. membranaceus* has an unresolved relation to *S. puniceus* and *S. multiflorus* subsp. *katherinae* in the trees based on Sanger sequences (Figure 11, 12). The gDNA tree (Figure 10, clade D), however, shows that *S. membranaceus* and *S. multiflorus* subsp. *katherinae* are sisters with high support. This is quite interesting as these taxa are morphologically very dissimilar. *Scadoxus membranaceus* does not have a false stem, has four persistent bracts during the flowering period and linear 1-nerved perianth segments, which are oriented in parallel to the style. In contrast, *S. multiflorus* subsp. *katherinae* has a false stem, more than four bracts not persistent during flowering and lanceolate perianth segments oriented at a right angle to the style. The latter is further a more robust species in terms of being at least double as tall, having higher number of flowers and broader inflorescences and corm.

They are, however, distributed in the same area on the Eastern coast of South Africa. As previously mentioned, the phylogenetic relationships based on the chloroplast genomes have been found to correlate with geography rather than morphology (both in this and other studies). Further research is thus needed to conclude about the sister relations of the South African taxa, preferably with a higher amount of nrDNA sequences.

Section *Scadoxus*: *S. multiflorus* and *S. pole-evansii* – The character compatibility analysis based on morphology grouped together *S. multiflorus* and *S. pole-evansii* based on both having similar vegetative characters. This includes the characters uniting the subgenus, as well as both having a false stem. They further have inflorescences where the involucral bracts are descending and never dominating during the flowering period. In addition, perianth segments are oriented at a right angle to the style (Nordal and Duncan, 1984).

In this study the accessions of *S. pole-evansii* are found to be sister to Zimbabwean *S. multiflorus* subsp. *multiflorus* in all trees with high support. In the nrDNA (Figure 12, clade D) the clades are supported as sisters, when disregarding one accession of subsp. *multiflorus*

(26) found within the polytomy (clade G) with *S. puniceus*, *S. membranaceus* and. *S. multiflorus* subsp. *katherinae*. Also the gDNA tree (Figure 10) showed a highly supported sister relation (clade C). In the cpDNA tree (Figure 11) they are found to be closely related as well (clade A), with the exception of two accessions of South African *S. puniceus* (6, 8) being included among them.

Due to the congruent results of both the nuclear DNA and the chloroplast DNA, it is concluded that *S. pole-evansii* is indeed sister to Zimbabwean *S. multiflorus* subsp. *multiflorus.* This is further in part congruent with the result of compatibility analysis based on morphology (Nordal and Duncan, 1984). However, as they included *S. multiflorus* as one species, the results of both these studies are only congruent when taking into account the exception of *S. multiflorus* being polyphyletic and *S. pole-evansii* only sister to the Zimbabwean *S. multiflorus* clade.

Rainforest group and savannah group – As the sister relations have been revealed, it is also clear that the separation of the savannah group (subgenus *Scadoxus*) and the rainforest group (subgenus *Demeusea*) were not confirmed by the molecular phylogenetic analyses. The two characters separating them include the subterranean organ and the position of the scape initiation. The initial hypothesis regarding the subterranean organ was the rhizome being the ancestral character state and rhizomatous bulb the derived character state (Bjørnstad and Friis, 1972a). An ancestor more or less like *S. cinnabarinus* was then regarded ancestral within the tribe Haemantheae, but as *Haemanthus* was later found to be sister to *Scadoxus* it seems more probable that a bulbous subterranean organ is ancestral. As the four taxa with a rhizome appear in three separate clades, it seems not to be a synapomorphic character (shared, derived character), but rather a homoplasious character (independently evolved). Such homoplasy could in general be caused by evolutionary reversal, convergent evolution or parallel evolution (Futuyma et al., 2013). In this case it seems to be due to parallel evolution as the same character state is found in closely related lineages and thus probably based on modification of the same developmental pathways. The rhizome probably evolved as the bulbous taxa existing on the drier savannah advanced into the less dry rainforests, and subsequently the need for a water storing bulb diminished.

The position of the scape initiation, which is a character that always follows the subterranean organ type, is however not as easily explained. The connection could be due to both characters being based on of the same developmental pathways, where modification of one

character leads to modification of the other. It is interesting, however, that in the rhizomatous taxa where the scape is initiated centrally among the leaves, in three of the four taxa (all except *S. cinnabarinus*) the scape breaks through the false stem and appear lateral. Thus, a lateral scape seems to be the preferable state either way.

Pollination is another factor that probably has been an important driver in the evolution of the *Scadoxus* species. It has long been hypothesized that pollinators play a key role in the evolution of the flowering plants diversity, and evidence indicates that pollinators have the potential to drive diversification at separate levels of the evolutionary processes (Darwin, 1877, Crepet, 1984, Dodd et al., 1999, Vamosi and Vamosi, 2010, Van der Niet et al., 2014). Information about pollination of the *Scadoxus* species and from exact data from experimental studies have been very scarce until recently. Pooley (1998) recorded that sunbirds and weaver birds feed on the nectar of *S. puniceus* in South Africa. Duncan (2013) further noted that the brush-like inflorescences of *S. puniceus* and *S. membranaceus* are also visited by honey bees, while the nodding flowers of *S. nutans* and the pendent flowers of *S. cyrtanthiflorus* contain abundance of nectar and are probably bird-pollinated. Another explanation of the evolutionary background for the downward facings inflorescence in these rainforest taxa could be protection of the flowers from heavy rains (Demissew and Nordal, 2010). However, as indicated by Hutchinson (2014), in the case of *S. nutans* at least it blooms at a relatively dry period of the year, and thus the reason instead could be benefitting a specific pollinator from below. This could perhaps be a non-flying mammal as the flowers fulfill some of the criteria for mammal pollination by having exserted styles and stamens, but lacks some of the criteria, i.e robust flowers with dull coloration and copious amounts of nectar (Carthew and Goldingay, 1997, Johnson et al., 2001)

The previous discussion of pollination is nevertheless all based on anecdotal evidence or relying on theories of pollination syndromes, which concern how suites of convergent floral traits have evolved to different pollen vectors in response to natural selection (Vogel, 1954). Two recent studies have assessed the pollination of some *Scadoxus* species; the previously mentioned study by Butler and Johnson (2020) on the subspecies of *S. multiflorus* found in South Africa, as well as the assessment by Mertens et al. (2020) of changes in pollinator community of *S. cinnabarinus* along its elevational range on Mount Cameroon. Both studies

confirmed the prediction of a psychophilious pollination syndrome predicted by Vogel (1954) (Figure 18). For *S. cinnabarinus* butterflies significantly contributed to the pollination, and bees were found to probably serve as secondary pollinators at high elevation where a lower abundance of butterfly visitors were found. Both subspecies of South African were visited by large butterflies, but pollination was mediated by the wings and not by the head as the syndrome hypothesizes. Butler and Johnson (2020) further indicated that several species of *Scadoxus* may also be pollinated via butterfly wings given their similar open brush floral morphology. More studies of pollination within this genus is needed.

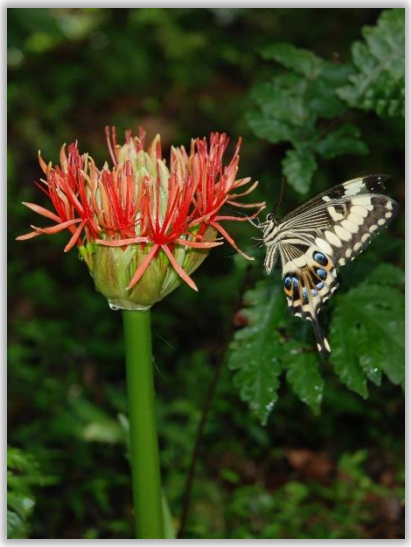


Figure 18. Butterfly visiting a young *S. multiflorus* in Tanzania. Photograph: Charlotte S. Bjorå.

Altogether, it seems likely that an ancestor of this genus was more or less similar to the widespread *S. multiflorus.* Different populations may have moved from the savannah into the forests, where speciation was driven further by ecological adaption with divergent pollinators as a driving factor. Local adaptation has been more important than dispersal, resulting in parallel evolution of diverse characters in the genus, e.g. reduction from rhizomatous bulb to rhizome, development of persistent bracts leading to differing floral morphologies as well as down-facing flowers.

Further research

To better understand the phylogeny of *Scadoxus*, a study including more material representing a wider geographical range (especially West African material) should be performed. This is especially relevant to fully understand what underlies the morphological and ecological differentiation of *S. multiflorus.* Preferably including more nuclear DNA, possibly the whole genome, could help resolve the basal branches of the phylogeny by adding stronger support. Further studies of pollination biology should be undertaken as this has only been studied for a few of the species. Only *S. pole-evansii* has been assessed in the IUCN red list, thus assessment of the other species should be undertaken. This is especially relevant for *S. nutans* and *S. cyrtanthiflorus*, which have restricted distributions.

5 Conclusion

The molecular phylogenies largely support previous morphological conclusions regarding species delimitation. Morphological species or subspecies confirmed as monophyletic by the molecular phylogenetic analyses include:
- *Scadoxus nutans, S. cyrtanthiflorus, S. pole-evansii, S. cinnabarinus, S. pseudocaulus, S. multiflorus* subsp. *longitubus* and *S. multiflorus* subsp. *katherinae.*
Morphological species not contradicted by the molecular phylogenetic analyses include:
- *S. membranaceus.*
Morphological species or subspecies contradicted by the molecular phylogenetic analyses include:
- *S. puniceus, S. multiflorus*, and *S. multiflorus* subsp. *multiflorus.*
Two new combinations are proposed: *S. katherinae* (from *S. multiflorus* subsp. *katherinae)* and *S. abyssinicus* (from *S. multiflorus* subsp. *multiflorus*).
Scadoxus longifolius is recommended to be reduced to synonym under *S. cinnabarinus.*

This study detected incongruence between the phylogenies based on nuclear DNA and chloroplast DNA, which did not allow for a straightforward interpretation. The basal branches of the phylogeny had low support in all molecular phylogenies. Despite some poor resolution and discrepancy in the basal relationships in the trees, there were still some common features in terms of clades for some species and groups, as well as an overall geographic structure. The previous division of *Scadoxus* into two subgenera was clearly not reflected by the molecular phylogenies.

Nevertheless, this study has offered new and important information about speciation and relations in a widespread and conspicuous genus of Sub-Saharan Africa. Further, it has transformed the way in which the genus *Scadoxus* should be viewed, and has provided a good foundation for further studies to help reveal more about the genus.

Taxonomic conclusions

Scadoxus cinnabarinus (Decne.) Friis & Nordal.
(Friis & Bjørnstad 1976: 64)
Basionym: [Withheld]. (Decne., FI. des Serres, 12: 27, Tab. 1195 (1857);
–*Type* Table 1195 of Flore des Serres, Ser. II, 2 (1857).

Scadoxus longifolius (De Wild. & Th. Dur) Friis & Nordal, **syn. nov.**
(Friis & Nordal 1976: 64)
Basionym: [Withheld]. De Wild. & Th. Dur
(De Wild. & Th. Dur Bull. Soc. Bot. Belg. 39 p. 78 1900)
- *Type*: F. *Demeuse* s.n., Congo sine loco 1891 (BR).

Scadoxus katherinae (Baker) Hals & Bjorå, **comb. nov.**
Basionym: [Withheld]. Baker, Gard. Chron. N. Ser. 7 p. 656. 1877. (Baker 1877)
Scadoxus multiflorus Raf. subsp. *katherinae* (Bak.) Friis & Nordal
- *Type*: Natal, Saunders s. n., March 1877; Natal: Tongaat N of Durban, (K, lectotype);
Sanderson s. n. 1869 (K, syntype)

Scadoxus abyssinicus (Herb.) Hals & Bjorå, **comb. nov.**
Basionym: [Withheld]. Herb. – (Herbert 1837 p. 232).
Synonym: *Haemanthus bivalvis* Beck. – (Beck 1888 p. 452).
- *Type:* Ethiopia, Salt in Herb. Lambert (BM, holotype).

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Appendix

Appendix table A1. Overview of specimens included in the phylogenetic analyses of this study.

Species	Specimen ID	Herb.	Country	Coll. year	Sequences obtained							
					ITS[b]	*trn*L-F	*trn*S-*trn*G	*psb*A-*trn*H	*rps*16	*rpl*20-5*rps*12	*Mat* K	cpDNA genome
Haemanthus L.												
H. albiflos Jacq. (1)	Bjorå cult s.n.	O	South Africa	2019								Chip1:7
H. albiflos (2)	Nordal cult s.n[g]	O	South Africa	2013								
Scadoxus Raf.												
S. cinnabarinus (Decne.) Friis & Nordal (1)	Nordal 5806	O	Cameroon	2007								
S. cinnabarinus (2)	Chase 549[h]	K	Cameroon	1990								
S. cinnabarinus (3)	Cult. 2016043[a]	O	Gabon	2019								Chip2:2
S. cinnabarinus (4)	Cult. 2017048[a]	O	West Africa	2019								
S. cinnabarinus (5)	Cult. 2015041[a]	O	West Africa	2019								
S. cinnabarinus (6)	Cult. 2016055[a]	O	West Africa	2019								Chip1:2
S. cyrtanthiflorus (C.H. Wright) Friis & Nordal (1)	Cult. 2009030[a]	O	Uganda	2019								
S. cyrtanthiflorus (2)	Cult. 2005062[a]	O	Uganda	2019								Chip1:1
S. membranaceus (Baker) Friis & Nordal (1)	Cult. 2003040[a]	O	South Africa	2019								
S. membranaceus (2)	Cult. 2006052[a]	O	South Africa	2019								
S. membranaceus (3)	Cult. 2015022[a]	O	South Africa	2019								
S. membranaceus (4)	Cult. 2018050[a]	O	South Africa	2019								
S. membranaceus (5)	Cult. 2018051[a]	O	South Africa	2019								
S. membranaceus (6)	Butler 89	NU	South Africa	2019								
S. membranaceus (7)	Cult. 2010063[a]	O	South Africa	2019								

S. membranaceus (8)	Cult. 2004052[a]	O	South Africa	2019								Chip1:5
S. membranaceus (9)	Cult. 2016016[a]	O	South Africa	2019								
S. multiflorus Raf.												
S. multiflorus	Wieringa 9494	WAG	Ghana	2019								
subsp. *katherinae* (Baker) Friis & Nordal (1)	Cult. 2008081[a]	O	South Africa	2019								
subsp. *katherinae* (2)	Cult. 2010064[a]	O	South Africa	2019								
subsp. *katherinae* (3)	Cult. 2016036[a]	O	South Africa	2019								
subsp. *katherinae* (4)	Cult. 2018040[a]	O	South Africa	2019								Chip2:11
subsp. *katherinae* (5)	Butler 90	NU	South Africa	2019								
subsp. *longitubus* (C.H. Wright) Friis & Nordal (1)	Jongkind 10389	WAG	Guinea	2011								
subsp. *longitubus* (2)	Schmidt 2047	O	Ghana	1996								
subsp. *multiflorus* Raf. (1)	Bjorå 863[g]	O	Tanzania	2010								
subsp. *multiflorus* (2)	Hemp 5047	UBT	Tanzania	2009								
subsp. *multiflorus* (3)	Nordal 1011[g]	O	Ethiopia	1982								
subsp. *multiflorus* (4)	Richards 1927[h]	K	Zambia	-								
subsp. *multiflorus* (5)	Jongkind 2136[h]	K	Ghana	1996								
subsp. *multiflorus* (6)	Al-Quarainy[e]	-	Saudi Arabia	2012								
subsp. *multiflorus* (7)	Wabuyele 488	EA	Kenya	2019								Chip2:5
subsp. *multiflorus* (8)	Cult. 2006100[a]	-	Tanzania	2019								
subsp. *multiflorus* (9)	Bjorå 1062	O	Tanzania	2010								Chip1:6
subsp. *multiflorus* (10)	Bjorå 1063	O	Tanzania	2010								
subsp. *multiflorus* (11)	Wieringa 9223	WAG	Gambia	2019								
subsp. *multiflorus* (12)	Hemp 5057	UBT	Tanzania	2009								
subsp. *multiflorus* (13)	Hemp 5062	UBT	Tanzania	2009								

subsp. *multiflorus* (14)	Awas 2967	ETH	Ethiopia	2019								Chip1:11
subsp. *multiflorus* (15)	Awas 2968	ETH	Ethiopia	2019								Chip2:12
subsp. *multiflorus* (16)	Awas 2981	ETH	Ethiopia	2019								
subsp. *multiflorus* (17)	Worku 84	ETH	Ethiopia	2019								
subsp. *multiflorus* (18)	Worku 88	ETH	Ethiopia	2019								
subsp. *multiflorus* (19)	Bjørnstad 588[c]	O	Tanzania	1971								
subsp. *multiflorus* (20)	Chapano 1801	SRGH	Zimbabwe	2019								Chip1:10
subsp. *multiflorus* (21)	Bjorå 1590	SRGH	Zimbabwe	2019								Chip2:7
subsp. *multiflorus* (22)	Bjorå 1597	SRGH	Zimbabwe	2019								
subsp. *multiflorus* (23)	Bjorå 1613	SRGH	Zimbabwe	2019								
subsp. *multiflorus* (24)	Bjorå 1622	SRGH	Zimbabwe	2019								
subsp. *multiflorus* (25)	Bjorå 1623	SRGH	Zimbabwe	2019								
subsp. *multiflorus* (26)	Bjorå 1625	SRGH	Zimbabwe	2019								
subsp. *multiflorus* (27)	Bjorå 1645	SRGH	Zimbabwe	2019								
subsp. multiflorus (28)	Bjorå 1750	SRGH	Zimbabwe	2019								
subsp. *multiflorus* (29)	Awas 2965	ETH	Ethiopia	2019								Chip2:4
subsp. *multiflorus* (30)	Awas 2969	ETH	Ethiopia	2019								
subsp. *multiflorus* (31)	Awas 2970	ETH	Ethiopia	2019								
subsp. *multiflorus* (32)	Awas 2973	ETH	Ethiopia	2019								Chip1:8
subsp. *multiflorus* (33)	Awas 2974	ETH	Ethiopia	2019								
subsp. *multiflorus* (34)	Awas 2982	ETH	Ethiopia	2019								
S. nutans (Friis & I. Bjørnstad) Friis & Nordal (1)	Cult. 2009014[a]	O	Ethiopia	2019								
S. nutans (2)	Cult. 2005008[a]	O	Ethiopia	2019								
S. nutans (3)	Awas 2964	ETH	Ethiopia	2019								

S. nutans (4)	Awas 2966	ETH	Ethiopia	2019								
S. nutans (5)	Awas 2971	ETH	Ethiopia	2019								Chip1:3
S. nutans (6)	Awas 2972	ETH	Ethiopia	2019								
S. nutans (7)	Cult. 2014029[a]	O	Ethiopia	2019								
S. pole-evansii (Oberm.) Friis & Nordal (1)	Bjorå 1695	SRGH	Zimbabwe	2019								Chip1:4
S. pole-evansii (2)	Bjorå 1696	SRGH	Zimbabwe	2019								
S. pole-evansii (3)	Bjorå 1697	SRGH	Zimbabwe	2019								
S. pole-evansii (4)	Bjorå 1698	SRGH	Zimbabwe	2019								
S. pseudocaulus (I. Bjørnstad & Friis) Friis & Nordal (1)	Lowe 2612[ch]	K	Nigeria	1973								
S. pseudocaulus (2)	Bipinde 3719[cd]	BR	Cameroon	1908								
S. pseudocaulus (3)	Hambler 178[h]	K	Cameroon	1957								Chip1:12
S. puniceus (L.) Friis & Nordal (1)	Nordal 2917	SRGH	Zimbabwe	1994								Chip2:10
S. puniceus (2)	Rønsted 321[f]	NU	South Africa	-								
S. puniceus (3)	Rønsted 313[f]	NU	South Africa	-								
S. puniceus (4)	Cult. NHM	O	South Africa	2019								Chip2:1
S. puniceus (5)	Cult. 1999163[a]	O	South Africa	2019								
S. puniceus (6)	Cult. 2004145[a]	O	South Africa	2019								
S. puniceus (7)	Cult. 2016086[a]	O	South Africa	2019								Chip1:9
S. puniceus (8)	Cult. 2008055[a]	O	South Africa	2019								
S. puniceus (9)	Cult. 1999081[a]	O	South Africa	2019								
S. puniceus (10)	Cult. 1999011[a]	O	South Africa	2019								Chip2:6
S. puniceus (11)	Cult. 2006099[a]	O	Tanzania	2019								Chip2:9
S. puniceus (12)	Worku 86	ETH	Ethiopia	2019								Chip2:8

GenBank accessions as outgroup for genome analysis													
Yucca schidigera Roezi ex Ortgies	[i]	-	USA	2016									KX931469
Y. filamentosa L.	McKain 101[i]	GA	USA	2016									KX931467
Lycoris radiata (L'Hér.) Herb	[j]	-	South Korea	2018									NC045077
L. squamigera Maxim	[k]	-	South Korea	2018									MH118290

Shaded squares represent obtained sequences, where the first seven columns represent Sanger sequences and the last column represent the chloroplast genome sequences from Ion Torrent sequencing. Chip number and place within chip are marked in the relevant rows. Abbreviations: Herb = Herbarium (herbaria abbreviation according to Index Herbariorum), Coll. year = collection year, Cult. = Cultivated, NHM = Natural History Museum, Oslo.

[a] Accession numbers from the National Plant Collections of the Royal Horticultural Society, UK.
[b] The nuclear ribosomal internal transcribed spacer (ITS1, 5.8S and ITS2).
[c] ITS1 only.
[d] *trn*L-F intergenic spacer.
[e] GenBank accession: KC311154 (Al-Qurainy et al, 2013),
[f] Rønsted 321/313. GenBank accession: HM140813/HM140814 (Bay-Smidt,et al., 2011)
[g] Acquired from previous preliminary analysis by Bjorå & Nordal (2014)
[h] DNA samples retrieved from Royal Botanic Gardens Kew DNA Bank.
[i] McKain et al., 2016. sett in referanser her når dokument merges
[j] Zhang et al., 2019.
[k] Jin et al., 2018.