The *Scadoxus puniceus* complex; one or three species?

Ida Emilie Moe

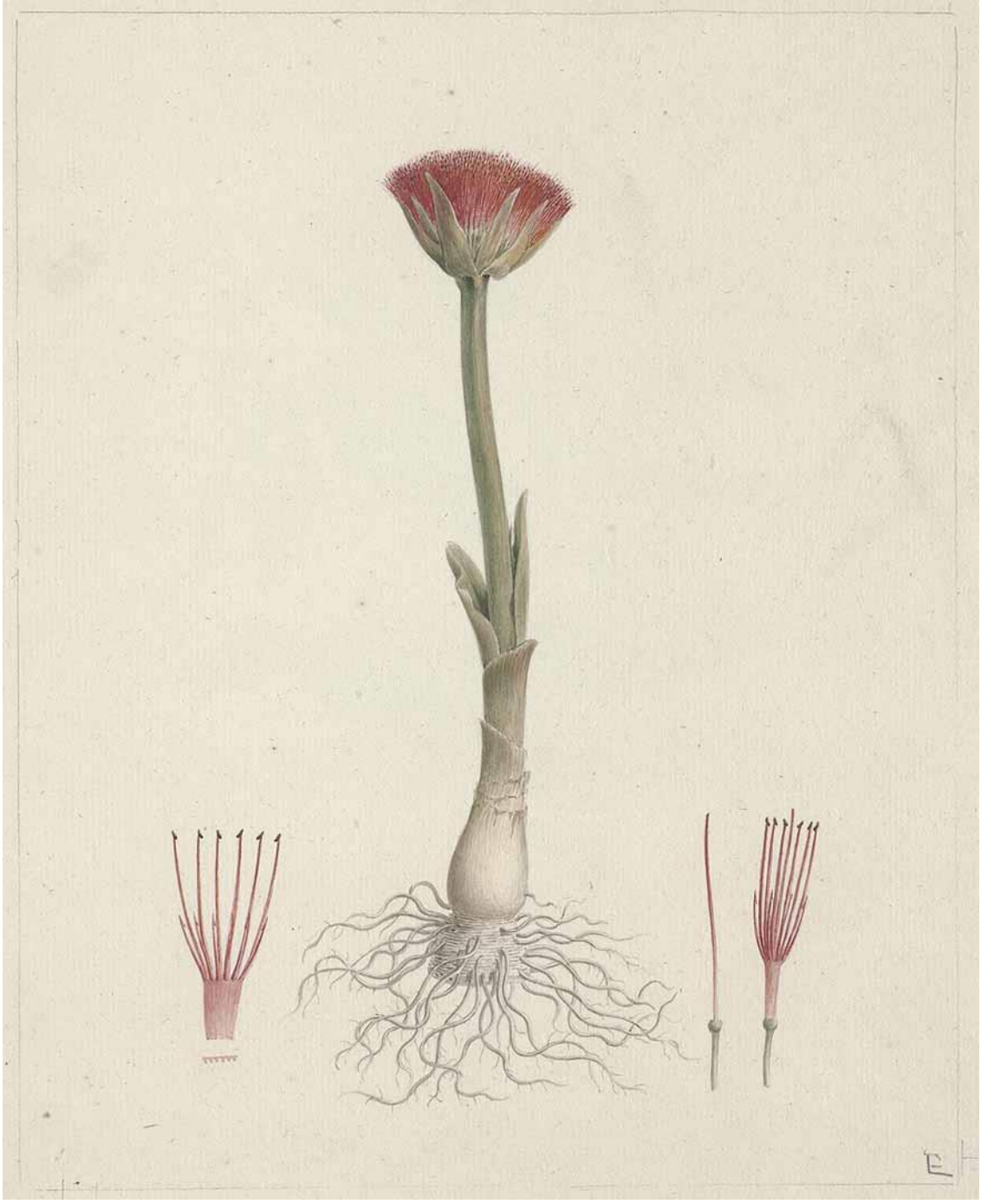

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Photo: *Scadoux puniceus* by Luigi Balugani 1737-1770

http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Abstract

The aim of this project is to evaluate if *Scadoxus puniceus* resolves in a monophyletic clade. *Scadoxus puniceus* displays a highly disjunct distribution, and is found in Ethiopia, Tanzania and Southern Africa. A thorough morphological study by Bjørnstad and Friis in 1974 concluded that these populations could not be separated based on morphological characters. Molecular analyses (Sanger sequencing for 43 specimens and whole chloroplast genomes for 24 specimens) were performed to reveal the phylogenetic relations. All the molecular analyses shows clearly that *S. puniceus* is not monophyletic. The type of *S. puniceus* comes from South Africa, so the Southern African clade will have priority for that name. The Tanzanian species will be recombined to ***Scadoxus goetzei*** **(**Harms) I.E.Moe & Bjorå and the Ethiopian species to ***Scadoxus fax-imperii*** (Cufod.) I.E.Moe & Bjorå

Acnowledment

I want to thank my supervisors, Charlotte S. Bjorå, Anne K. Brysting and Inger Nordal for all of your hard work during this project. Although you had more than one student you still made time for all of us and worked hard to give us feedback. Thank you for many hours spent of helping me and my fellow peers to finish our projects. Thank you Kine, to be so lucky to have such a wonderful and sweet person to share a common path with, has been a delight. A very special thank you to Jonathan Hutchinson for your enthusiasm and support. For making the fieldtrip to Zimbabwe an amazing botanical trip, although alas, without any successful capture of *Scadoxus puniceus* for us both to enjoy. All the thank you's to fellow colleagues and students in Zimbabwe. I especially want to thank the lab technicians at NHM, Jarl Andreas, Lisbeth and Audun, for the extraordinary help and making my days at the lab welcoming, fun and always a day full of experience. Also a thank you to the greenhouse gardeners in the botanical garden, NHM for taking care and cultivating our specimen. And a big thank you to Anders Krabberød for helping with the technicalities in the genome assembly.

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But most importantly, thank you Thea for being the very ground beneath my feat, for all the hours you have cared, and hours you still do.

Lastly, my greatest gratitude for Anders, who pushed me to start my scientific journey, my love for science, and to never quit. I would not be here today if it wasn't for you.

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Introduction

The genus *Scadoxus*

The genus *Scadoxus* Raf. belongs in the family *Amaryllidaceae* in the order Asparagales (Byng et al., 2016). The genus consists of herbaceous plants, with bulbs and well developed rhizomes. Leaves are lanceolate to ovate, glabrous, appearing during (synanthous) or after (hysteranthous) flowering. Petioles are sheathing and forming a distinct pseudostem. The scapes are solid, lateral or central among the leaves. The involucral bracts are four to many, soon drooping and withering or erect and conspicuous at anthesis. Flowers are many, red to pink with cylindrical tube and free segments. The fruits are globose, orange to red berries (Zimudzi et al., 2008).

In total, nine species are recognized within the genus and are distributed across Africa south of Sahara and into the Arabian Peninsula. They have a wide habitat range, from savannah to rainforests, some widely distributed throughout Africa, as *Scadoxus multiflorus* (Martyn) Raf., while others have a more restricted distribution like e.g. *S. pole-evansii* (Oberm.) Friis & Nordal, only growing in Nyanga National Park, Zimbabwe. The species in *Scadoxus* were referred to *Haemanthus* L. before Friis & Nordal (1976) split *Haemanthus* into two genera, based on morphological differences, as well as chromosome number. A cytological study by Friis & Nordal (1976) revealed that *Haemanthus* has 2n = 16 while *Scadoxus* has 2n = 18.

Scadoxus puniceus and Gyaxis

Scadoxus puniceus (L.) Friis & Nordal was first described by Linné (1753) in the genus *Haemanthus,* but was later recombined in the genus *Scadoxus* by Friis & Nordal (1976). *Haemanthus puniceus* L. (and after the recombination, *S. puniceus*) was referred to the section *Gyaxis* (Salisb.) Friis & Nordal, together with *Haemanthus membranaceus* Baker (*Scadoxus membranaceus* (Baker) Friis & Nordal). The sister section to *Gyaxis* was named *Nerissa* (Baker) Pax and Hoffman, but when later recombined in *Scadoxus*, the name of the section was changed to *Scadoxus* Raf. It consists of *S. multiflorus* and *S. pole-evansii* (Bjørnstad & Friis, 1972). Differences between these two sections were based on morphology (Fig. 1); in section *Gyaxis* all taxa have dominating involucral bracts, making the inflorescence conical and flowers compressed, i.e tepals +/- erect. In section *Scadoxus* the involucral bracts are early withering

and much less prominent. The inflorescence is globular to semi-globular in shape, with more prominent stellate flowers (Bjørnstad & Friis, 1974).

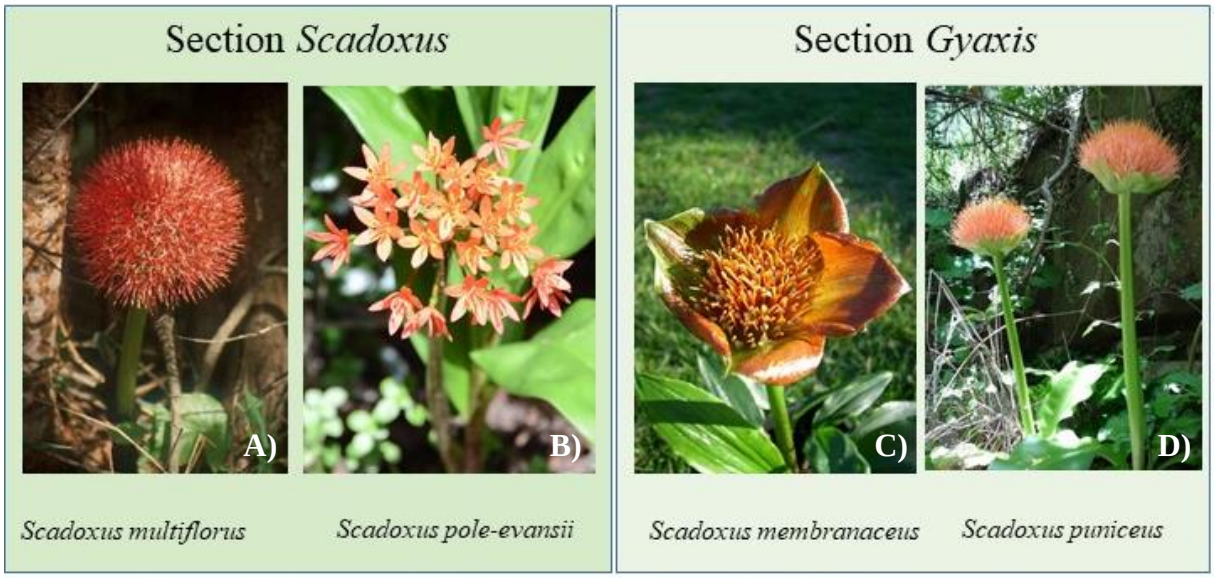


Figure 1 The species in the sections *Scadoxus* and *Gyaxis.* Photos: A) *S. multiflorus,* Malawi, by Bjorå B) *S. pole-evansii,* Zimbabwe, by Bjorå C) *S. membranaceus,* South Africa, by Hutchinson D) *S. puniceus,* South Africa, by Dressler.

Morphology

Scadoxus puniceus can grow up to 75 cm tall. At the lower part of the leaves, the colour varies from light green to milky green, with scarlet dots. The bulb has a distinct rhizome with roots. The leaves are long, the lamina lanceolate with a shiny cuticle. The colour is light green, with a prominent, central main vein in the lamina and 4-8 nerves on each side and ± parallel venation. The leaf arrangement is alternate, with old fleshy leaves making up the bulb, whereas the petiole form a pseudostem (Bjørnstad & Friis, 1974). *Scadoxus puniceus* is usually hysteranthous, meaning that the flower will emerge before the leaves (Fig. 2A1-2), but synanthous forms, with flowers and leaf appearing simultaneously, are not uncommon (Fig. 2B). *Scadoxus puniceus* has one inflorescence with many small flowers. Around 5-4 bracts support the inflorescence, often with reddish colour. The species epithet "*Puniceus*" refers to the reddish colour, phoenician purple. However, this varies between the geographical populations and among individuals, and flower colour can vary from bright red or pink to pale green (Fig. 3).

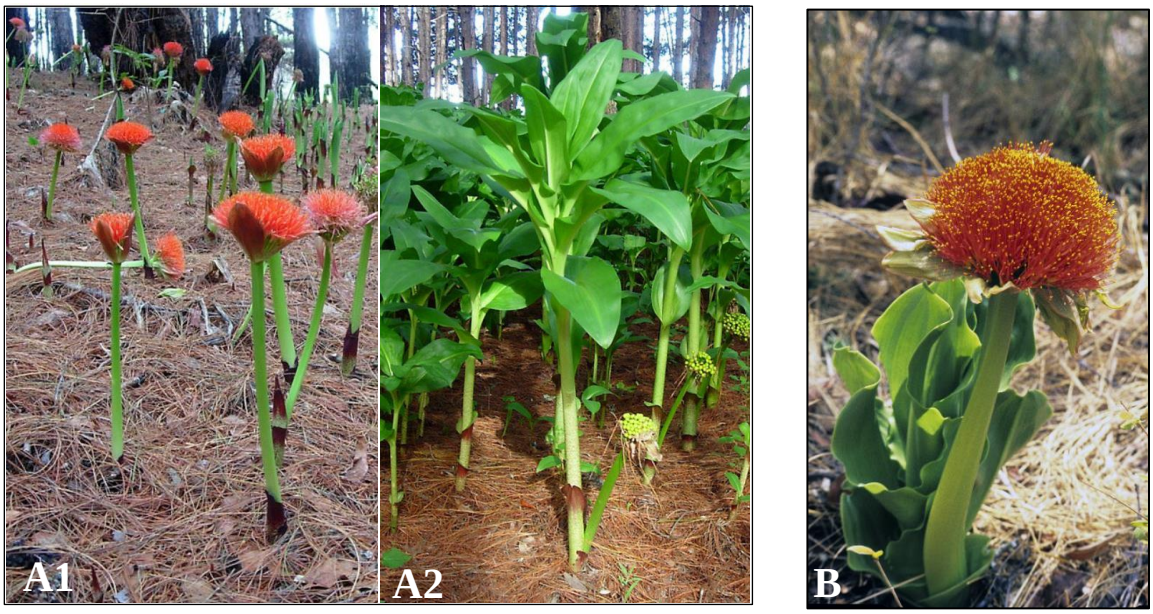


Figure 2 *Scadoxus puniceus* as A1-2) hysteranthous, B) Synanthous. Photos: A1-2. Rukwa by Blittersdorff., B. by Nordal.

The flowers produce nectar, which insects or birds consume, securing pollination (Butler & Johnson, 2020). The fruits are red berries and are bird dispersed (Butler & Johnson, 2020). It is common to observe leaves when berries are present as the leaves commonly emerge after anthesis.

Distribution

Scadoxus puniceus has a disjunct distribution, with isolated occurrences in Southern Africa, Tanzania and Ethiopia (Fig. 4). The geographical populations were first separately described as independent species and nine names have been reduced to synonymy (Table 1) by Bjørnstad and Friis (1974).

Table 1 Synonyms of *Scadoxus puniceus*, related to country from where they have been described (WCSP, 2020).

Synonyms of *Scadoxus puniceus* (L.) Friis & Nordal		
Ethiopia	**Tanzania**	**South Africa**
Haemanthus fax-imperii Cufod. 1939	*Haemanthus goetzei* Harms 1910	*Scadoxus puniceus* L. 1753 *Haemanthus orchidifolious* Salisb. 1796 *Haemanthus magnificus* (Herb.) Herb. 1841 *Haemanthus redouteanus* M.Roem. 1847 *Haemanthus insignis* Hook. 1853 *Haemanthus natalensis* Hook. 1863 *Haemanthus rouperi* Anon. 1875 *Haemanthus superbus* Baker 1888

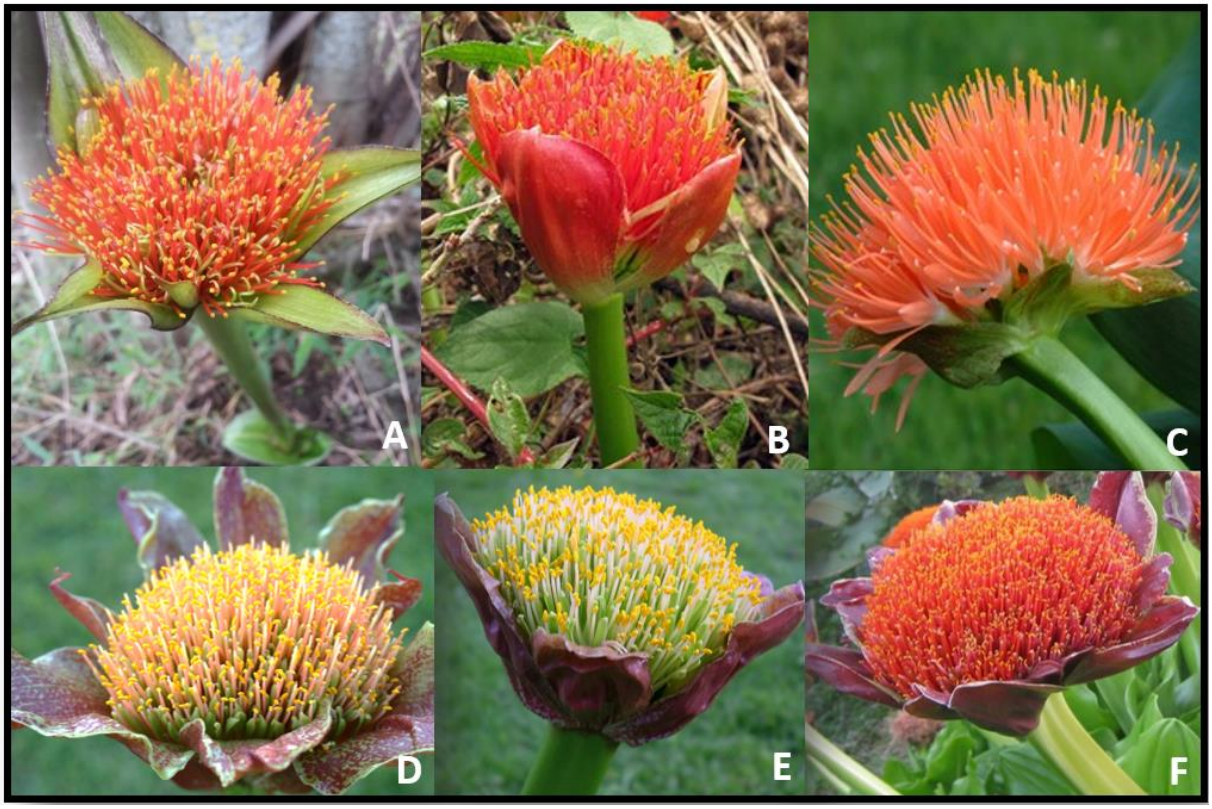


Figure 3 Variation in *Scadoxus puniceus,* A. Ethiopian plant with green, robust bracts, red flowers and reddish tipped filament; B. Tanzanian plant with red bracts, flowers and filament; C. South African plant with light green bract, pinkish flowers and flowers; the individual is from East Cape and shows a more delicate form than the more robust ones. D-F show three inflorescence with different color morphology (all are robust); D. bracts are spotted scarlet red, with green flowers and pink filament; E. bracts are scarlet red, green flowers and white filament; F bracts are scarlet red, with red flowers and red filament. Photos: A. Worku, B. Blittersdorff, C-F Hutchinson**.**

Morphological characteristics of the geographical populations

Ethiopia

The Ethiopian population consists of rather robust plants that often have green bracts, or green tinged (Sebsebe Demissew et al., 2003). The shape of the bracts and their colour variation are very similar to that found in the plants from South Africa. The plants are found from 1500 to 2000 m elevation, in deciduous woodland and grassland, on heavy black clay soils, often on basalt. They have been collected from areas around Shewa, Gonder, Harar and Meki. They are flowering from March to July in the rain season and can be either synanthous or hysteranthous (Bjørnstad & Friis, 1974; Sebsebe Demissew et al., 2003).

Tanzania

The Tanzanian material represents a rather slender, few-flowered form always with red bracts and flowers (Fig. 3B). The plants grow in humid areas at high altitudes, like mountain forests or swamps. They are recorded as epiphytes in forests, but more commonly found on the ground. The bracts are typically broad and greatly overlapping, though some forms have narrow not overlapping bracts, traits correlated with longer perianth tubes and pedicels (Bjørnstad & Friis, 1974). Bjørnstad and Friis (1974) indicated that this could be a possible result of introgression from *S. multiflorus*. Bracts and flowers have the same reddish colour with no variation as seen in Ethiopian and South African populations. Flowering period is from September to January, i.e. early in the rain season. It is found between 2000 and 2700 m elevation in south west Tanzania. Plants have been also been collected in adjacent parts of Zambia, Malawi and Mozambique, in Rukwa Mbizi Forest (Bjørnstad & Friis, 1974; GBIF, 2020).

Southern Africa

Populations from Transvaal are very robust and rich-flowered, while in East Cape the robustness of plants is varying (Fig. 3C, D&F). In Natal, forms with bracts that are relatively long and narrow often exceeding the perianth segments and filaments are more frequent than in other areas (Fig. 3E) (Bjørnstad & Friis, 1974). Colour of bracts alters from dark red to pure green, spotted or tinged with red. There is a strong correlation of bract and flower colour, usually with rich coloured bracts having inconspicuous greenish flowers (Fig. 3C&E) (Bjørnstad & Friis, 1974). Plants found in South Africa are reported to grow in forests and woodland, although plants from the lower parts of Eastern Cape are growing in coastal bush, at 10–1400 m elevation. It is found in the Free State, Northern Cape, Gautengs and further to Eastern Cape, Western Cap, Natal, Mpumalanga and Limpopo (Bjørnstad & Friis, 1974; POWO). In Zimbabwe it can be epiphytic as well as growing on the ground, in shaded areas as swamps, montane forests and stream valleys, found at 1200-1500 m elevation in Matobo National Park. It is also found further north in Zimbabwe and in Botswana (Zimudzi et al., 2008).

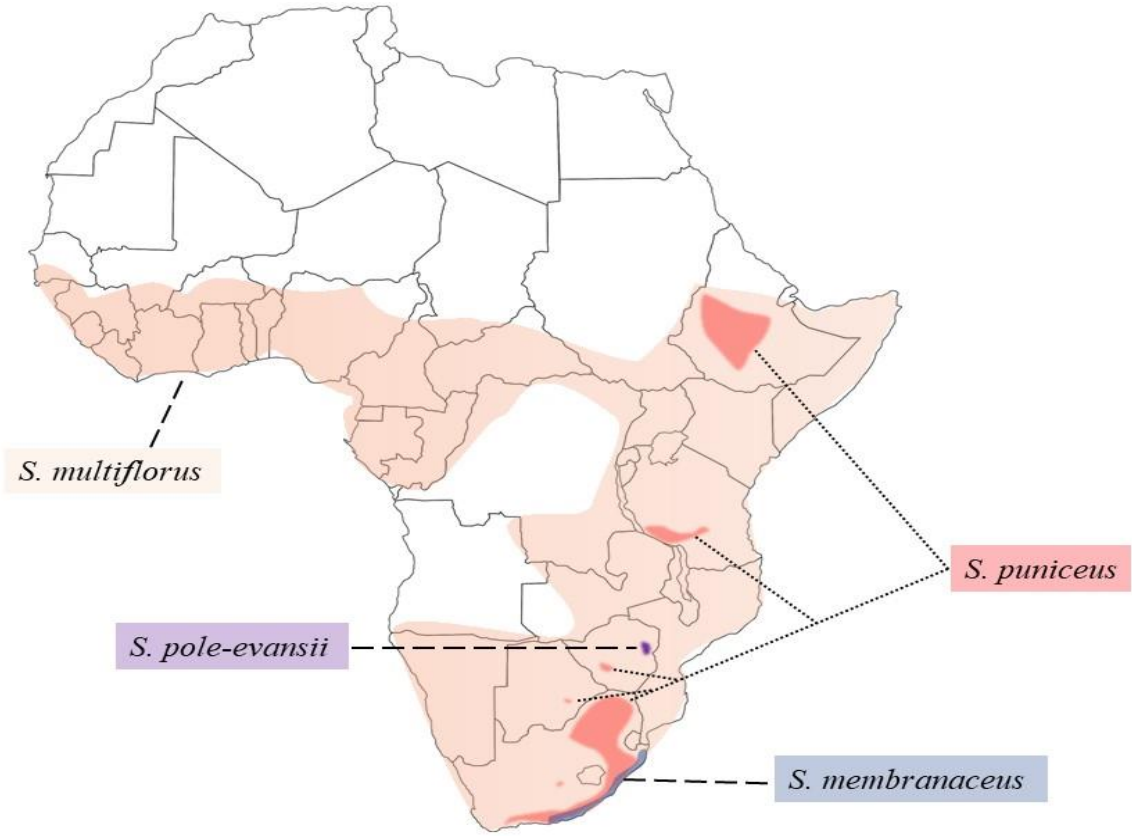


Figure 4 Distribution of species in the sections *Scadoxus* and *Gyaxis*: *Scadoxus multiflorus* complex (pale peach), *S. puniceus* (pink), *S. pole-evansii* (violet) and *S. membranaceus* (grey blue). Based on GBIF (preserved specimen) and Bjørnstad and Friis (1974).

Hypotheses for the disjunct distribution of *Scadoxus puniceus*

Bjørnstad and Friis (1974) suggested several competing hypotheses for the disjunct distribution that *S. puniceus* displays (Fig. 4). One obvious explanation was that the current distribution represents relict populations, and that earlier during more humid periods, *S. puniceus* was more continuously distributed across the eastern part of Africa. Climatic changes might have eliminated the species in the lower and drier tropical parts of the former distribution area.

A second explanation might be that *S. puniceus* is a polyphyletic species that originated independently in three areas. The transition from the widespread *S. multiflorus* in sect. *Scadoxus* may not involve a huge genetic change. A notation from flora Ethiopia and Erithea commented the difficulties of distinguishing the two when flowers were absent (Sebsebe Demissew et al.,

2003). Bjørnstad and Friis (1974) further hypothesized that the development of dominating involucral bracts was pollinator-driven. Lastly, the disjunct distribution could be a result of long-dispersal of seeds, probably by birds, with the centre of origin probably be in South Africa (Bjørnstad & Friis, 1974). Bjørnstad and Friis (1974) concluded, after very thorough morphological studies, that the geographical populations could not be separated based on morphological characters. They suggested that the distribution of the species represented a relict from an earlier wide distribution ranging from Ethiopia to East Cape.

Conservation and ethnobotany

Scadoxus puniceus is not considered endangered with the species delimitation of today, however, some of the geographical populations (Fig. 4) might deserve special attention. *Scadoxus puniceus* is amongst the most highly traded medicinal plant species for use in South African traditional medicine (Naidoo et al., 2017). Overharvesting has proved to result in poor productions of bulbs, which can result in a diminishing of the overall population (Batten, 1986; Dold & Cocks, 2002; Veale et al., 1992). Although the bulb is considered poisonous and deadly in high doses, it is commonly used in traditional medicine to treat gastrointestinal problems, coughs and for safe deliveries during pregnancies (Dold & Cocks, 2002; Koorbanally et al., 2000; Veale et al., 1992). Besides local folk medicinal usage, it is also commercial sold as an ornamental worldwide due to its bright coloured flowers, which is reflected by some of its vernacular names “fireball lily”, “red paintbrush” or “blood lily” (Van Wyk, 2011).

Alongside the worldwide trend of an increasing human population, suitable habitats are diminishing. The plant likes shady areas like ravines and forest, and human activity like deforestation is a potential and likely threat. A study of habitat loss in South Africa confirmed that 65% of the vegetation types in the study area was threatened by transformation due to mining, agriculture, mining and urban areas (Ntshane & Gambiza, 2016). In 2012 SANBI (South African National Biodiversity Institute) reported in the document *LIFE State of south Africa’s biodiversity* that habitat loss of natural landscapes in Gauteng, KwaZulu-Natal and North West Province, was prominent, and further warned that if the current rate of change due to cultivation, mining and urban expansion continued, these provinces would lose their natural habitat left outside protected areas by 2050 (BODATSA, 2020).

There are no documentation for use of the plant in Tanzania, however bathing with root infusion of *S. multiflorus* is used to treat mental illness (Chhabra et al., 1987). As the two species are

very alike before and after anthesis (Bjørnstad & Friis, 1974; Sebsebe Demissew et al., 2003), the plant might be used mistaken for the other.

Molecular phylogenetic analyses

A preliminary molecular phylogenetic analysis by Bjorå and Nordal (2014) using chloroplast and ITS sequences, showed very low genetic variation and poor resolution of phylogenetic relationships between the *S. puniceus* populations. In my study I have therefore supplemented with multiple region, as well as the full plastid genome in an attempt to resolve the phylogeny. The genome size of *Scadoxus* is large (Sakowicz et al., 1994; Zonneveld et al., 2005), preventing sequencing of the full genome as part of this thesis. However, inspired by Manzanilla et al. (2018), we used chloroplast enrichment to obtain full plastid genome sequencing for several *Scadoxus* species. This method has been applied in recent years with an overall success (Cai et al., 2006; Parks et al., 2009; Wysocki et al., 2016). The resulting molecular phylogenies will be used to discuss taxonmic and phylogeographic aspects of the *S. puniceus* complex.

Aims

The main focus of this study is to use the molecular phylogeny to 1) establish if section *Gyaxis* is monophyletic, 2) evaluate if *Scadoxus puniceus* is monophyletic and 3) discuss if the geographical populations should be recognized as own taxa, and in case, at which rank.

Materials and Methods

Sampling

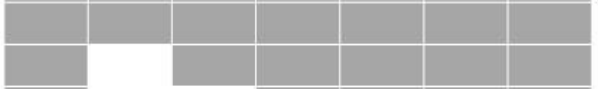
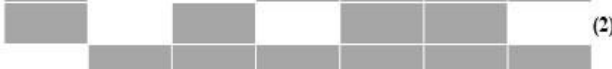
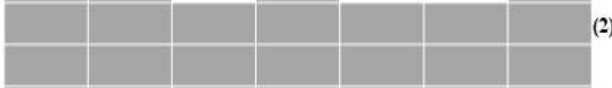
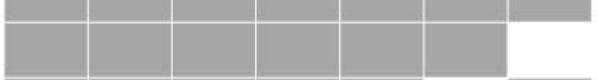
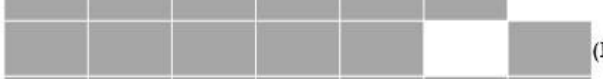
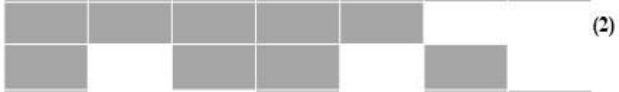
Samples were obtained from fieldtrips, garden collections and herbaria (for details see Table 2). When collecting tissue for DNA-extraction, fresh, healthy-looking leaves, not contaminated by soil, was preferred.

Fieldtrips were arranged in Zimbabwe (January–February 2019), mainly in Matobo National Park and Nyanga National park, and in Ethiopia (February–March 2019) in the regions of Kefa, Weleda and Shewa, as a part of the NORPART project, of which this study is part. Species collected were *S. puniceus, S. nutans* (Friis & I.Bjørnstad) Friis & Nordal (Ethiopia), *S. multiflorus* and *S. pole-evansii* (Zimbabwe). The localities for species collection were based on local observations, floras and herbarium material. In total 43 specimen were obtained for the Sanger sequencing, of which 39 successfully amplified and suitable to be analyzed. A total of 43 specimens was used for Sanger and 24 chloroplast genome sequencing.

Table 2 Samples included in the study with voucher information; taxon name, voucher identification, country, collection year, and list of regions acquired for individual species. Dark shaded areas show successful amplification and sequencing of a primer region and lack of data is illustrated by white colored areas. The different specimen are numbered corresponding to their place in the phylogenetic tree in the results. Abbrevation: n/a = not available, Bold numbers and X = species present in chloroplast genome sequencing, Italic numbers = Specimen used in ITS and chloroplast plasmid sequencing, bold an italic means both. Chip number shows correspondent chip the specimen was included on and their barcode number.

*National Plant Collection of the Royal Horticultural Society, UK

Taxon and species	Herb	Voucher specimen	Prominance	Collection Year	ITS	tmLF	tmS-tmG	psbA-tmH	rps16	rpl20-5'	MatK	Genom: Chip & number
Haemanthus albiflos Jacq.		004_H_albi, Bjorå & Nordal cult s.n,	South Africa	2019								(1) #7
Scadoxus cinnabarinus (Decne.) Friis & Nordal **(1)**	K	2016043*	Gabon	n/a								(2) #2
S. cinnabarinus **(2)**	O	2016055*	West Africa	n/a								(1) #2
*Scadoxus cyrtanthiflorus (*C.H.Wright) Friis & Nordal ***(*)***	O	2009030*	Uganda/Congo	n/a								(1) #1
Scadoxus membranaceus (Baker) Friis & Nordal ***(1)***	O	2004052*	South Africa	n/a								(1) #5
S. membranaceus (2)	O	2006052*	South Africa	n/a								
S. mul. ssp. *Katherinae* (Baker) Friis & Nordal ***(1)***	O	2018040*	South Africa	n/a								
S. mul. ssp. *katherinae (2)*	O	2016036*	South Africa	n/a								(2) #11
S. mul. ssp. *katherinae (3)*	O	HB,	South Africa	2019								
Scadoxus mul. ssp. *multiflorus* Raf. ***(1)***	ETH	2968A, Awas	Ethiopia	2019								(2) #12
S. mul. ssp. *multiflorus* ***(2)***	ETH	2965A, Awas	Ethiopia	2019								(2) #4
S. mul. ssp. *multiflorus (3)*	ETH	84A, Worku N.	Ethiopia	2019								
S. mul. ssp. *multiflorus* ***(4)***	EA	488, Wabuyele	Kenya	2019								(2) #5
S. mul. ssp. *multiflorus (5)*	O	1063, Bjorå	Tanzania	2010								
S. mul. ssp. *multiflorus* ***(6)***	O	1062, Bjorå	Tanzania	2010								(1) #6
S. mul. ssp. *multiflorus (7)*	SRGH	1623, Bjorå	Zimbabwe	2019								
S. mul. ssp. *multiflorus (8)*	SRGH	1750, Bjorå	Zimbabwe	2019								
S. mul. ssp. *multiflorus (9)*	SRGH	1613, Bjorå	Zimbabwe	2019								
S. mul.ssp. multiflorus **(10)**	SRGH	1590, Bjorå	Zimbabwe	2019								(2) #7
S. mul.ssp. multiflorus **(11)**	SRGH	1801, Chapano	Zimbabwe	2019								(1) #10
S. mul.ssp. multiflorus **(12)**	ETH	2973, Awas	Ethiopia	2019								(1) #8
S. mul.ssp. multiflorus **(13)**	ETH	2967, Awas	Ethiopia	2019								(1) #11

Scadoxus pseudocaulus (Friis & I.Bjørnstad) Friis & Nordal (*)	K	178, Hambler	Cameroon	1957		(1) #12
Scadoxus nutans (Friis & I.Bjørnstad) Friis & Nordal (*)	ETH	2971, Awas	Ethiopia	2019		(1) #3
Scadoxus pole-evansii (Oberm.) Friis & Nordal *(1)*	SRGH	1695, Bjorå	Zimbabwe	2019		(1) #4
S. pole-evansii (2)	SRGH	1696, Bjorå	Zimbabwe	2019		
Scadoxus puniceus (L.) Friis & Nordal *(1)*	ETH	86A , Worku N.	Ethiopia	2019		
S. puniceus (2)	ETH	86B, Worku N.	Ethiopia	2019		
S. puniceus ***(3)***	ETH	86C, Worku N.	Ethiopia	2019		(2) #8
S. puniceus (4)	ETH	87A, Worku N.	Ethiopia	2019		
S. puniceus (5)	ETH	87B, Worku N.	Ethiopia	2019		
S. puniceus (6)	ETH	87C, Worku N.	Ethiopia	2019		
S. puniceus (7)	ETH	85A, Worku N.	Ethiopia	2019		
S. puniceus (8)	O	1319, Bjorå?	Tanzania	-		
S. puniceus ***(9)***	O	2006099,	Tanzania	-		(2) #9
S. puniceus ***(10)***	ETH	2917, Nordal I.	Zimbabwe	1994		(2) #10
S. puniceus ***(11)***	O	Veksthus 1,	South Africa	2019		(2) #1
S. puniceus (12)	O	1999163*	South Africa	-		
S. puniceus (13)	O	2004144* Truter J.	South Africa	-		
S. puniceus (14)	O	2004145* Truter J.	South Africa	-		
S. puniceus ***(15)***	O	2016086, (L.) Friis & Nordal	South Africa	2019		(1) #9
S. puniceus (16)	O	2008055* Wash M.	South Africa	2019		
S. puniceus (17)	O	1999081* Brickell C.	South Africa	2019		
S. puniceus ***(18)***	O	1999011* Bayliss M.	South Africa	2019		(2) #6
S. puniceus (19)	O	2003028* Jeans M.	South Africa	2019		
S. puniceus (20)	NU	93A-E, Hannah C. Butler	South Africa	2019		

Laboratory Work

All laboratory work was undertaken at the laboratory of the Natural History Museum, University of Oslo, except for the sonication of organelle DNA into fraction, which was done at Department of Biosciences, University of Oslo.

DNA Extractions

DNA extractions was performed using the EZNA SP plant mini kit by Omega Bio-tek and following the manual with small modifications; instead of crushing the material in liquid nitrogen, two tungsten beads were added to a 2 ml Eppendorf tube with plant material, and crushing was done in a TissueLyser at 45 Hz twice for 60 seconds. Amount of plant material used per sample was approximately a 0.5 cm^2 chip of either dry leaf or flower. Flower material was only used from herbarium material when no other option was possible. Incubation time was set to 1 hour instead of 10 min. A total of 43 samples was extracted and stored in DNA LowBind tubes at 4°C. Some of samples (typically herbarium material) were extracted more than once, as they proved difficult to obtain enough DNA from. Quantitative and quality check of the samples was done by Nanodrop and electrophoresis using 1% agar gel with FastRule Low Range DNA ladder.

PCR and Sanger Sequencing

First step in the process of Sanger Sequencing is the amplification of targeted DNA. This is to ensure enough quantitative material. A standard protocol was used for the PCR. Each PCR reaction contained 0.5 µl DNA extract, 1 µl 1 mg/ml BSA, 1 µl $MgCl_2$, 1 µl buffer (10x), 1 µl dNTP, 0.4 µl 10 µM of each forward and reverse primer, 0.08 µl AmpliTaq polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and 4.62 µl ultrapure water Milli-Q water (Merck, Darmstadt, Germany). Total amount of master mix was prepared before individually pipetted into 8-strip 0.1 ml microcentrifuge tubes, resulting in 9.5 µl volume of master mix and 0.5 µl DNA per tube. All samples were vortexed and spun before placed in a thermal cycler. The amplification program initiated with pre-denaturation at 94°C for 2.5 min, then repeating 32 cycles each consisting of denaturation at 94°C and annealing at 53°C both for 30 sec, followed by 72°C synthesis for 50 sec, and then the final elongation step at 72°C for 4 min.

I amplified region ITS (nuclear ribosomal internal transcribed spacer), and six chloroplast regions: *trn*L-F, *trn*S-G, *psb*A, *rps*16, *rpl*20, and MatK (Table 3). As herbarium material proved difficult to amplify, I used alternative ITS and trnL-F primers that amplified smaller fragments: ITS1, ITS2, *trn*L-intron and *trn*F-spacer. However, this was not successful. A total of 157 samples were successfully amplified as was confirmed by electrophoresis on 1 % agarose gels run at 90V for 30-40 min.

Table 3 Regions and primers (including both forward and reverse) used in this study and their corresponding publication. From the top:

Region	Forward	Reverse	Publication
ITS	ITS5 mod: GGAAGGAGAAGTCGTAACAAGG	ITS4: TCCTCCGCTTATTGATATGC	White et al. 1990
trnL-F	c: CGAAATCGGTAGACGCTACG	f: AATTGAACTGGTGACACGAG	Taberlet et al. 1991
trnS-trnG	trnS(gcu): AACTCGTACAACGGATTAGCAATC	trnG(ucc): GAATCGAACCCGCATCGTTAG	Shaw et al. 2007
psbA-trnH	psbA: CGAAGCTCCATCTACAAATGG	trnH: ACTGCCTTGATCCACTTGGC	Sang et al. 1997
rps16	rps16-F: GTGGTAGAAAGCAACGTGCGACTT	rps16-R2: TCGGGATCGAACATCAATTGCAAC	Oxelman et al. 1997
rpl20-5rps12	rpl20: TTTGTTCTACGTCTCCGAGC	5rps12: GTCGAGGAACATGTACTAGG	Hamilton 1999
Maturase K	MatK-XF: TAATTTACGATCAATTCATTC	MatK-5R: GTTCTAGCACAAGAAAGTCG	Chase et al. 2005

The PCR products were cleaned for unused dNTP and primers, ensuring no residue were left, and then transferred to Axygen 96-well plates. A volume of 20 µl of the enzymes ExoSAP-IT™ PCR Product Cleanup Reagent and Exosap-IT (Thermofisher) was added to 180 µl dH_2O and distributed evenly into two plates for forward and reverse sequencing, resulting in 2.5 µl purified PCR product, 2.5 primer and 5 µl MilliQ water per well. Strong PCR products were diluted 20 times and weak PCR products ten, with MilliQ. The mix was incubated at 37°C on a thermocycler for 45 min and at 80°C (thus stopping the enzyme activity) for 15 min. Samples were sent to Macrogen Europe in Amsterdam for sequencing. Macrogen Europe is a DNA sequencing service that offers targeting sequencing procedures. A total of 43x7 samples were analyzed, with mixed results as can be seen in Appendix, Table A1.

Ion Torrent library preparation and sequencing

As mentioned, the genus *Scadoxus* has a large genome size 43.20 Gbp (Sakowicz et al., 1994; Zonneveld et al., 2005) so we decided to used Microbiome Enrichment kit (New England Biolabs, NEB, Ipswich, Massachusetts, USA) to separate non-methylated, enriched microbial DNA (chloroplast) and nuclear, methylated host (genomic). The kit is designed for human DNA, where IgG1 attaches to the human methyl-CpG-binding domain by MBD2-Fc bound magnetic beads. Thus, the procedure ends with a supernatant including both genomic DNA and cholorplast DNA, which are separated. Only the latter was used in this study. Genomic DNA was discarded and not used. The Microbiome Enrichment kit is designed for 24 samples. A total of 22 samples were included as representing the whole genus of *Scadoxus*. The samples were selected based on obtained DNA quantity and quality. In addition, the outgroup *Haemanthus albiflos,* and a sample from the genus *Aloe* L., to be used in another project, were also included, see Table 2. Six samples of *S.* p*uniceus* were included, with one sample from each country, except for South Africa, from which three samples were included to represent different regions and morphological traits.

The quantity (1 µg DNA) and quality (fragment sizes of < 15 kb) recommendations of the manufacturer were followed, except for two samples with lower DNA quantities, for *S. pseudocaulus* 182 ng DNA used and for *H. albiflos* 920 ng. Quality and quantity was determined by agarose gel electrophoresis, NanoDrop One C (Thermo Fisher Scientific, Madison, USA) and Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). The kit was first used, following the manufacturer's protocol, on a run of six samples as the method was not guaranteed success. The removal of genomic DNA was done with ethanol precipitation. The chloroplast DNA was rinsed by 1.0X AMpure XP beads and eluted in 50 µl 10 mM Tris-HCl buffer. A qPCR was run to ensure that capture of chloroplast DNA was successful the first six samples. A setup for both genomic (ITS) and organelle (trnL-F) DNA was run. ITS primers were ITS2 (forward and reverse), ITS3/ITS4 (forward and reverse). For trnL-F, forward primer **e** and reverse primer **f** were used. Success of the trial initiated round two using the same procedure for the Microbiome Enrichment kit for the remaining 18 samples.

As the desired DNA fragment length for IonTorrent sequencing is ~200 bp, we used Covaris E220 Focused-ultrasonicator (Covaris Inc., Woburn, MA, USA) at Blindern, University of Oslo at the Institute of Bioscience, for sonication of the organelle DNA. For end repairs and adapter ligation, NEBNext® Fast DNA Library Prep Set for Ion Torrent™ was used. The 24 samples

were divided into two groups, for each of which the organelle DNA fractions were indexed by IonXpress Barcode Adapter kit (ThermoFischer, Waltham, MA, USA). This resulted in two libraries with samples 1-12 in the first, and samples 13-24 in the second, see table 2. Final elution volume of each library was 32 µl (2 µl stored for later fragment analyzer runs; 30 µl used for size selection). A deviation from the protocol was that we used BluePippin (Sage Science, Beverly, MA, USA) for size selection instead of AMPure XP beads, and that it was performed before we cleaned the DNA from the adaptors. Aimed fragment size was 210-300 bp, and 40 µl adaptor ligated DNA and 2% agarose, dye-free, w/internal standards 100-600 bp casettes (SageScience) were used. For end repairs of the DNA and adapter ligation, NEBNext® Ultra™ DNA Library Prep Kit for Illumina was used.

In order to deduce the number of PCR cycles during library amplification, we measured DNA concentration of the two adapter ligated pools using Qubit® 2.0 Fluorometer: library 1 contained 0.669 ng/µl and library 2 0.543 ng/µl. Based on this, we used 20 µl (~10 ng DNA) and twelve PCR cycles in the amplification of library 1, leaving 20 µl as backup. However, as this resulted in high values of inference and likely over-amplification, library 2 got a shorter incubation time with only eight cycles to reduce the likelihood of PCR bias in the read distribution. The NEBNext® Fast DNA Library Prep Set for Ion Torrent™ protocol for the library amplification was followed, and subsequently the samples were cleaned with 1.2X AMpure XP beads in three rounds before eluted, after the second and third cleanup steps in 30 µl 0.1X TE buffer (provided in the kit). Another round of amplification and cleanup were performed in the same manner as previously described. Both libraries were run in the Fragment Analyzer in triplicates, see Appendix Figure A1 for result.

Due to high DNA yield a re-amplification of the remaining 20 µl of eight cycles of PCR was initiated for sequencing, the reduction aimed to reduce PCR bias in read distribution. Based on the estimated concentrations from the Fragment analyzer, the libraries were diluted to 45 pM, loaded into Ion Torrent 540 Chips according to the manual for the IonChef 540 Kit-Chef and sequenced on an Ion GeneStudio™ S5 System (Thermo Fisher Scientific, Waltham, MA, USA) using the Ion 5S Sequencing Kit. This last step was performed by lab manager Jarl Andreas Anmarkrud at the DNA lab, Natural History Museum, Oslo. The results were demultiplexed into FASTQ files using Torrent Suite version 15.12. For all information on Chip reads, see Appendix, Table A2. The distribution of reads among the samples showed uneven numbers,

which suggests that normalization should have been performed more precisely. The only normalization preformed was by starting with 1 µg DNA extract. Optimally, qubit analyses on the "organelle fraction" should have been performed to obtain DNA yield after non-methylated DNA enrichment, and then followed by normalization based on these fractions.

IonTorrent run summary and alignment of chloroplast genome

IonTorrent chip 2 produced double amount of usable reads (16.6 G of total bases, 69% usable reads) compared to chip 1 (8.8 G bases, 43% usable reads), rendering a more normalized read distribution (Fig. 5).

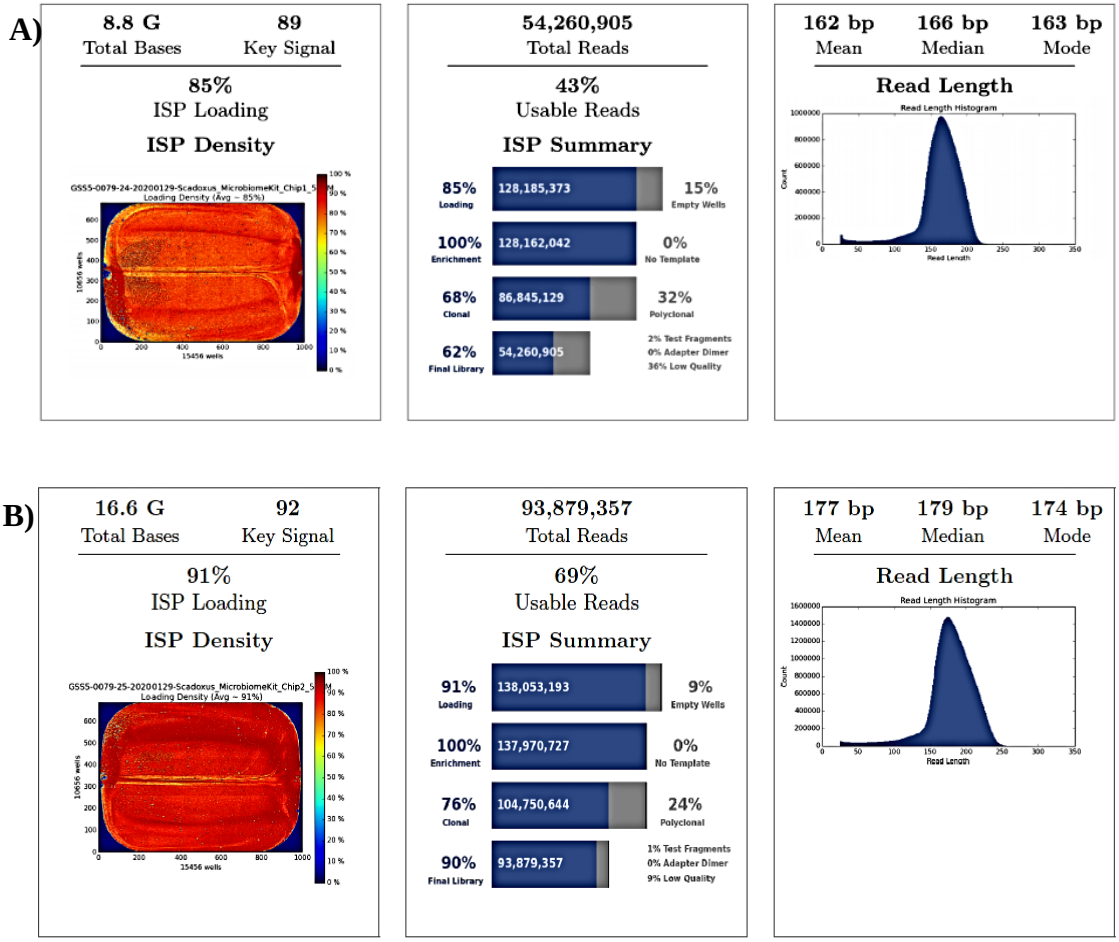


Figure 5 Run summary for Ion Torrent chip 1 (A.) and chip 2 (B). Left: summary of total bases and the ISP (Ion Sphere™ Particle) density and loading. The "heat map" shows ISP success to fill a well in the chip. Red areas are full with ISP, yellow areas have few and blue none. Middle: summary of total reads collected from the run. Right: average read length (bp). Figures generated by Torrent Suite software.

Both chips successfully removed template with 100% enrichment, and the percentage of polyclonal beads, mixed signals that could not be analyzed, was lower in chip 2 than in chip 1, indication of a better yield in chip 2. The percentage of wells filled with ISP (Ion Sphere™ Particle) was 85 % on chip 1 and 91% on chip 2, the latter almost maximum amount a chip can be loaded with.

Alignment and phylogenetic analysis

Sanger analysis

A total of 198 sequences of ITS and chloroplast were manually assembled forward and revers, by using Geneious Prime 2020.1.2 (Kearse et al., 2012) and further aligned by using MUSCLE *3.8.425* (Edgar, 2004). The chloroplast alignments were concatenated by using Geneious Prime. The data were analyzed using maximum parsimony and Bayesian analysis. For both the ITS and the chloroplast alignments indels were added as additional characters using the program SeqState (Müller, 2005) and simple indel coding of Simmons & Ochoterena (2000). The number of indels coded for ITS were ten, and for the concatenated partitioned dataset 92. The program used for maximum parsimony analyses was TNT (Goloboff & Catalano, 2016) applying the heuristic search option with 1000 replicates and max-trees set to 1000, adding option sub-pruning-regrafting (SPR) for Wagner start tree, otherwise default settings. Parsimony jack-knifing (JK) and bootstrap analyses were undertaken with 1000 replicates in both with a cut off at 50, otherwise using default settings. For the Bayesian inference, the program MrBayes 3.1.2 (Huelsenbeck et al., 2001; Ronquist & Huelsenbeck, 2003) was used, adjusted to appropriate nucleotide substitution models established by jMODELTEST "PhyML" (Guindon & Gascuel, 2003; Posada, 2008) and a 50% majority-rule consensus tree. Posterior probability (PP) values were determined by running one cold and three heated chains for 4 million generations, saving trees every 1000th generation. A discard of 25% of the sampled trees was done for burn-in, and otherwise default settings were used. By theory, a good convergence is indicated with an average standard deviation of split below 0.01 between two runs, which was obtained. The trees were configured in FigTree v1.4.4 (tree.bio.ed.ac.uk/software/figtree/), web tool iTOL (REF) and manually edited for final result.

Chloroplast genome analysis

Genomic assembly was done by Anders Kristian Krabberød, Centre for Ecological and Evolutionary Synthesis (CEES), at the University of Oslo. Assembly of the chloroplast genome pursued the same procedure as Manzanilla et al. (2018) and global alignment was completed by using MAFFT version 7.429 (Katoh et al., 2002) with adjustments of sequences as necessary. To obtain a reference for the assembly, the species with highest affinity to the *Scadoxus* chloroplast genomes were used, determined by blast against GenBank NCBI. This resulted in seven genomes used as outgroups: *Lycoris squamigera, L. radiata, Yucca schidigera, Y. filamentosa* and *Y. queretaroensis*. Ultimately, *L. squamigera* was used as reference in the chloroplast assembly for the *Scadoxus* species. The final matrix resulted in a total length of 159 556 bp for the 27 individuals. To check the de novo assembly, sanger sequences were aligned against the respective, assembled genome. The resulting sequences were aligned using MAFFT. Since the DNA yield of *S. pseudocaulus* was low the whole sample was used during library preparation and nothing was left for sanger sequencing. A Maximum likelihood (ML) analysis was performed in RAxML v8.2.12 (Stamatakis, 2014) using the GTR+G+I model, tree search and bootstrap convergence criteria autoMRE, that includes sufficient numbers of bootstrap replicas automatically (BL). The tree was configured in FigTree v1.4.4 (tree.bio.ed.ac.uk/software/figtree/), web tool iTOL (REF) and manually edited for final result.

Results

Alignment and phylogenetic analyses of Sanger sequencing

The lengths in base pairs of the aligned regions were: ITS 723, psbA-trnH 594, Mat-K 913, rpl20 770, trnS-trnG 1001, rps16 808, and trnL-F 888. The estimated best fit models of nucleotide substitution were: HKY+G for ITS; GTR+I for psbA-trnH, Mat-K and rps16; GTR+I+G for trnLF; F81+I for rpl20; and F81+G for trnS-trnG. The parsimony strict consensus trees with and without simple indel-coding were congruent. As the former had an overall better resolution and branch support, all results herein are based on the indel-coded analyses.

In the parsimony analysis of the ITS alignment, two most parsimonious trees were retained, with a length of 141 steps, a consistency index (CI) of 0.89, and a retention index (RI) of 0.94. In the parsimony analysis of the concatenated cpDNA alignment, 84 most parsimonious trees were retained, with a length of 294 steps, a consistency index (CI) of 0.71, and a retention index (RI) of 0.72. In the Bayesian analyses, the standard deviation of split frequencies descended to 0.002411 for ITS and 0.005878 for cpDNA at termination of the analysis. The parsimony and Bayesian analyses of both alignments were congruent in topology but with slightly different support values. The plastid regions separately rendered congruent topologies (not shown). Also the parsimony vs. Bayesian analyses of all separate plastid datasets were congruent, but resolved to different extents (not shown). In the following, trees based on ITS, concatenated chloroplast regions (cpDNA) and chloroplast genomes (gDNA) are described and compared. To ease the comparison, selected clades are marked with capital letters.

Review of concatenated chloroplast genome (gDNA)

Of practical reasons some of my samples were included in a bigger dataset with more *Scadoxus* species for the sequencing of the chloroplast genome. I will therefore not give much attention to accessions of *S. cinnabarinus* and *S. pseudocaulus* in my results and discussion. The first division in the gDNA tree (100 BS, bootstrap support, Fig. 6) separates the Ethiopian *S. puniceus*, the West and East African *Scadoxus*, *S. cinnabarinus* and *S. pseudocaulus* in one clade (A), and the accessions from Southern Africa and all accessions of *Scadoxus* from Ethiopia except *S. puniceus* in another clade (B), rendering the Ethiopian accessions polyphyletic. Within group A, *S. pseudocaulus* and *S. cinnabarinus* constitute a clade that is sister to the remaining accessions from Tanzanian, Kenyan, Ethiopia and Uganda/Congo. The

Tanzanian and Kenyan *S. multiflorus* ssp. *multiflorus* accessions are monophyletic, with Tanzanian *S. puniceus* as closest sister (94 BS). Ethiopian *S. puniceus* is sister to *S. cyrtanthiflorus* from Uganda/Congo with 100 BS support.

Clade B has 100 BS support and consists of three well-supported (100 BS) subclades, the Zimbabwean accessions (clade C), an Ethiopian clade (clade D), and a South African clade (clade E). The two latter are sister clades with low support (57 BS). The South African accessions of *S. puniceus* are paraphyletic, as one of them is sister group to *S. membraniceus* and *S. multiflorus* ssp. *katharinae* with 93 BS support. Within the Ethiopian clade, the *S. multiflorus* ssp. *multiflorus* accessions form a monophyletic group (95 BS support), with accessions of *S. bivalvis* and *S. nutans* as successive sisters (100 BS support). Within the Zimbabwean clade the *S. multiflorus* ssp. *multiflorus* accessions form a monophyletic group, with *S. puniceus* and *S. pole-evansii* accessions as successive sisters (100 BS support).

Comparison of gDNA and cpDNA

The cpDNA tree (Fig. 7) includes less *Scadoxus* taxa, but more accessions of *S. puniceus* than the gDNA tree. Despite these difference the overall topological patters are congruent between the two trees, though resolved to a different extent.

The same two main sister clades, A low supported and B (PP 0.92), are also found in the cpDNA tree reflecting a clear geographical separation. The Ethiopian *S. puniceus* is found in clade A, whereas other Ethiopian accessions (*S. multiflorus* ssp. *multiflorus*) are placed in clade B. Within clade A Ethiopian *S. puniceus* is split into two clades. Further, Tanzanian *S. puniceus* does not form a monophyletic clade with Tanzanian *S. multiflorus* ssp. *multiflorus* as is the case in the gDNA tree (Fig. 6A). However, there is generally low branch support and resolution within clade A. Clade B splits into a polytomy of three clades C, D and E, all with high support. Clade C contains Ethiopian *S. multiflorus* ssp. *multiflorus*, corresponding to the Ethiopian clade in the gDNA tree (Fig. 6D). Clade D and E correspond to the Zimbabwean and South African clades in the gDNA tree (Fig. 6C&E), respectively, except that two South African *S. puniceus* accessions are nested within the Zimbabwean clade (Fig. 7D). In both the gDNA and cpDNA tree, the *S. puniceus* accessions do not form a monophyletic group within the South African clade (Fig. 6-7, clade E).

Comparison of ITS and cpDNA

Despite being resolved to a different extent and not fully congruent, the ITS and cpDNA topologies do show several similarities considering overall geographical patterns of several clades. As in the cpDNA tree, the South African accessions of *S. puniceus* are paraphyletic in the ITS tree (Fig. 8). Three accessions form a monophyletic clade (clade A1), though not supported, which is sister group to all the remaining *Scadoxus* accessions (clade A2). Clade A2 is a polytomy consisting of the remaining South African *S. puniceus* accessions, a well-supported minor clade with the three *S. multiflorus* ssp. *katharinae* accessions, also from South Africa, and a larger, though unsupported, clade B with all remaining accessions. Within this clade are found some well-supported geographical clades corresponding partly to those of the cpDNA tree (Fig. 7).

Alike with the cpDNA tree, Ethiopia *S. multiflorus* ssp. *multiflorus* constitutes a monophyletic group (Fig. 8, PP 1, 100 JK, clade B). All the Ethiopia *S. puniceus* accessions form a monophyletic clade (clade D) with high support (PP 0.99, JK 99), whereas in cpDNA tree they are separated into two clades. In addition, the ITS tree contains a well-supported Tanzanian/Kenyan clade (PP 0.99, 71 JK, clade E) where *S. puniceus* is sister group to *S. muliflorus* ssp. *multiflorus*. In the cpDNA tree Tanzanian and Kenyan *S. multiflorus* ssp. *multiflorus* constitutes a low supported group, which is sister to Ethiopian *S. puniceus*, whereas the Tanzanian *S. puniceus* is part of the basal polytomy of clade A. Finally, the *S. pole-evansii* and S. *multiflorus* ssp. *multiflorus* accessions from Zimbabwe form a monophyletic group. In the cpDNA tree, one Zimbabwean *S. puniceus* accession and two Ethiopian *S. puniceus* accessions are grouping together with these Zimbabwean accession, but in ITS tree they are part of the larger unresolved polytomy (clade A2).

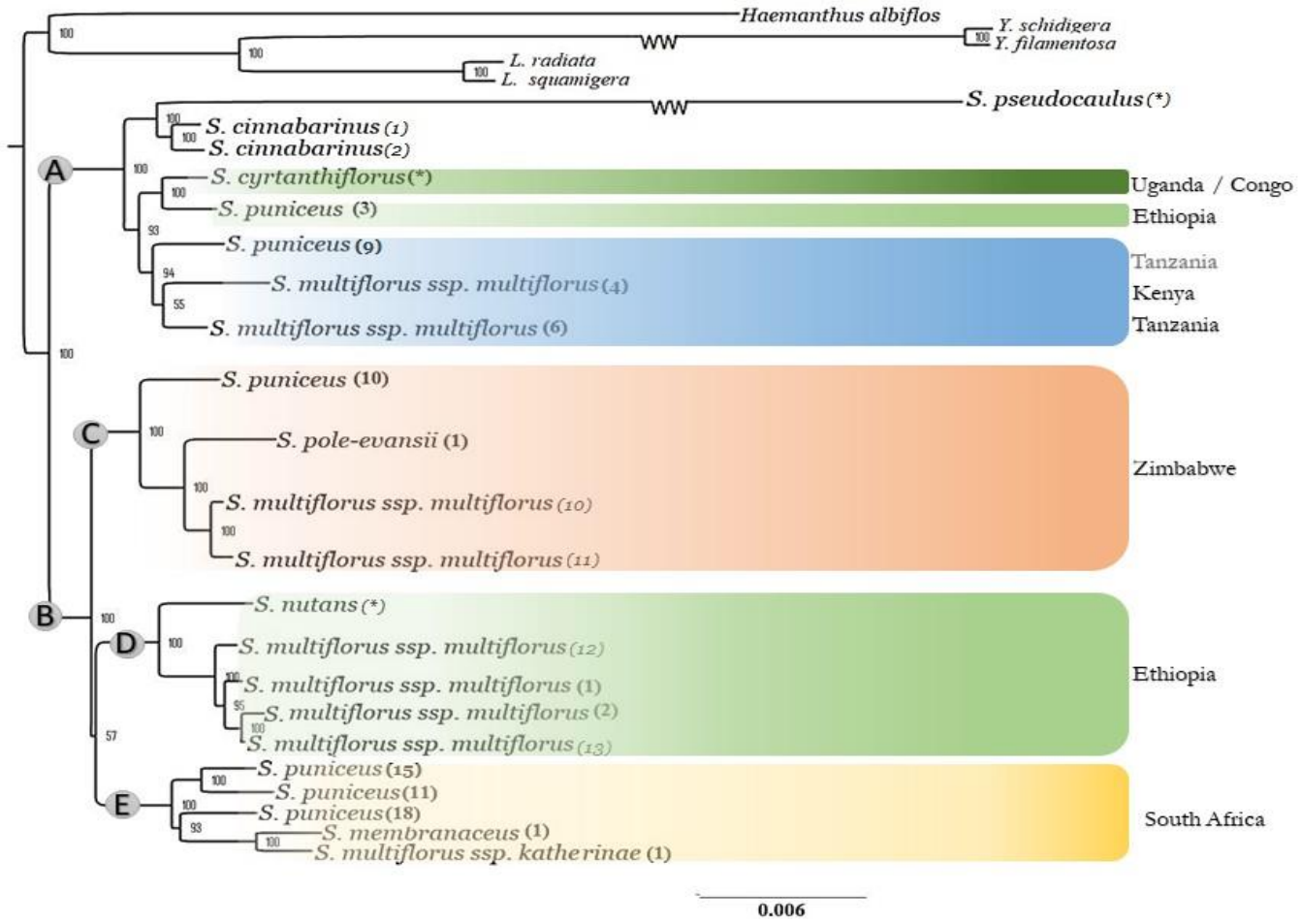


Figure 6 RAxML of the chloroplast genome concatenated from IonTurrent. The RAxML analysis consist of 27 accessions and 5151 characters, maximum likelihood bootstrap values (ML) of at least 50 % at nodes. Numbers correspondent to the species in both ITS and cpDNA trees. Clades discussed in text marked with capital letters at nodes. The zigzag are shortening of long branch to reduce the size. Abbrevations: L = *Lycorice*, S= *Scadoxus*, Y =*Yucca*, numbers corresponding material list Table 2&4, bold numbers also included in sanger sequencing analysis Table 2&4.

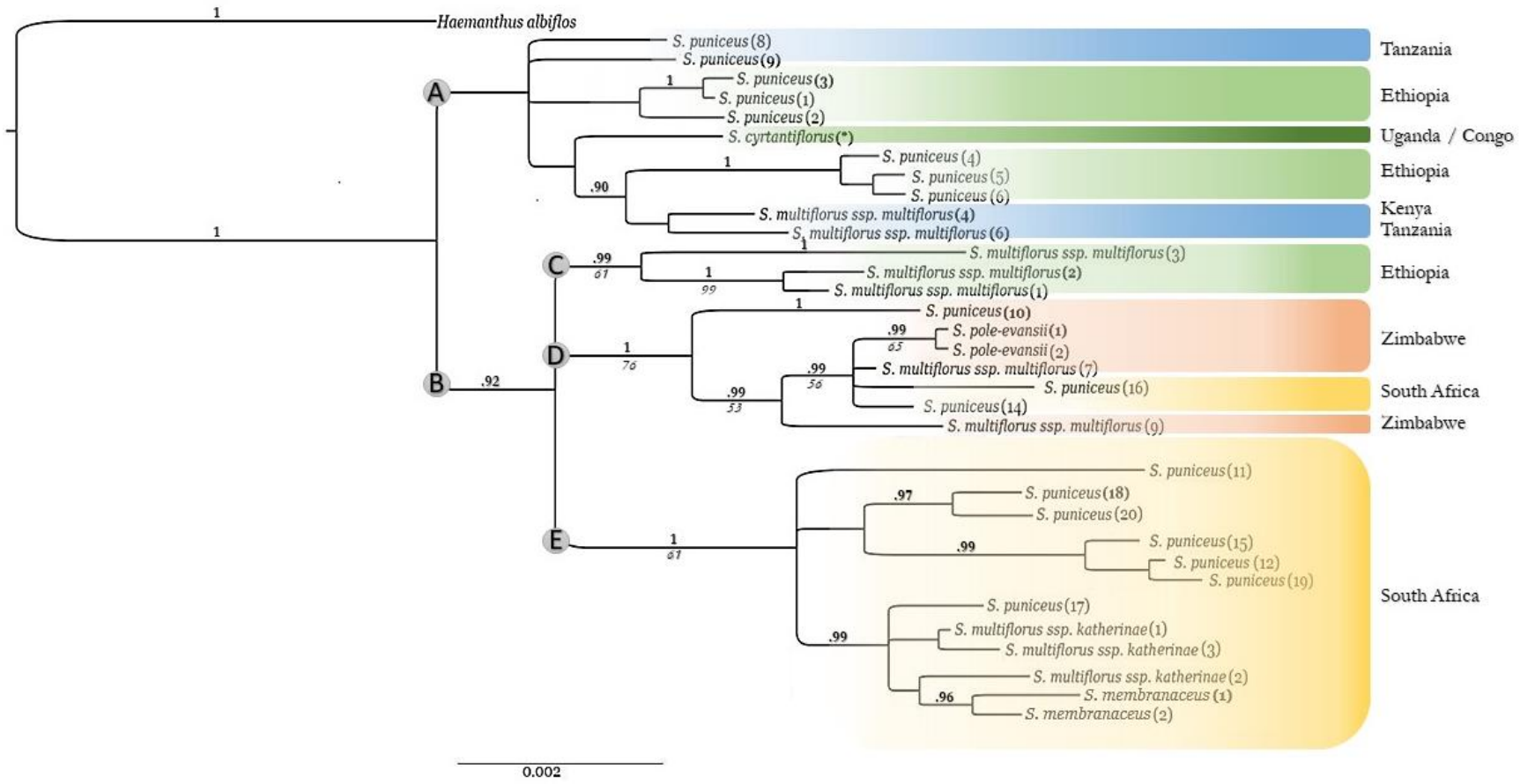


Figure 7 The 50 % majority rule consensus phylograms for the Bayesian analyses of the concatenated matrix of six plastids (psbA-trnH, Mat-K, rpl20, trnS-trnG, rps16 and trnL-F) DNA regions, 38 accessions and 5249 characters (incl. 99 coded indels). Maximum parsemony jack-knife support of at least 50% displayed in italic below branches and the Bayesian posterior probability values of at least 0.9 placed above. Abbreviations: S = Scadoxus, numbers corresponding material list Table 2, bold numbers also included in chloroplast genome analysis Table 2&4

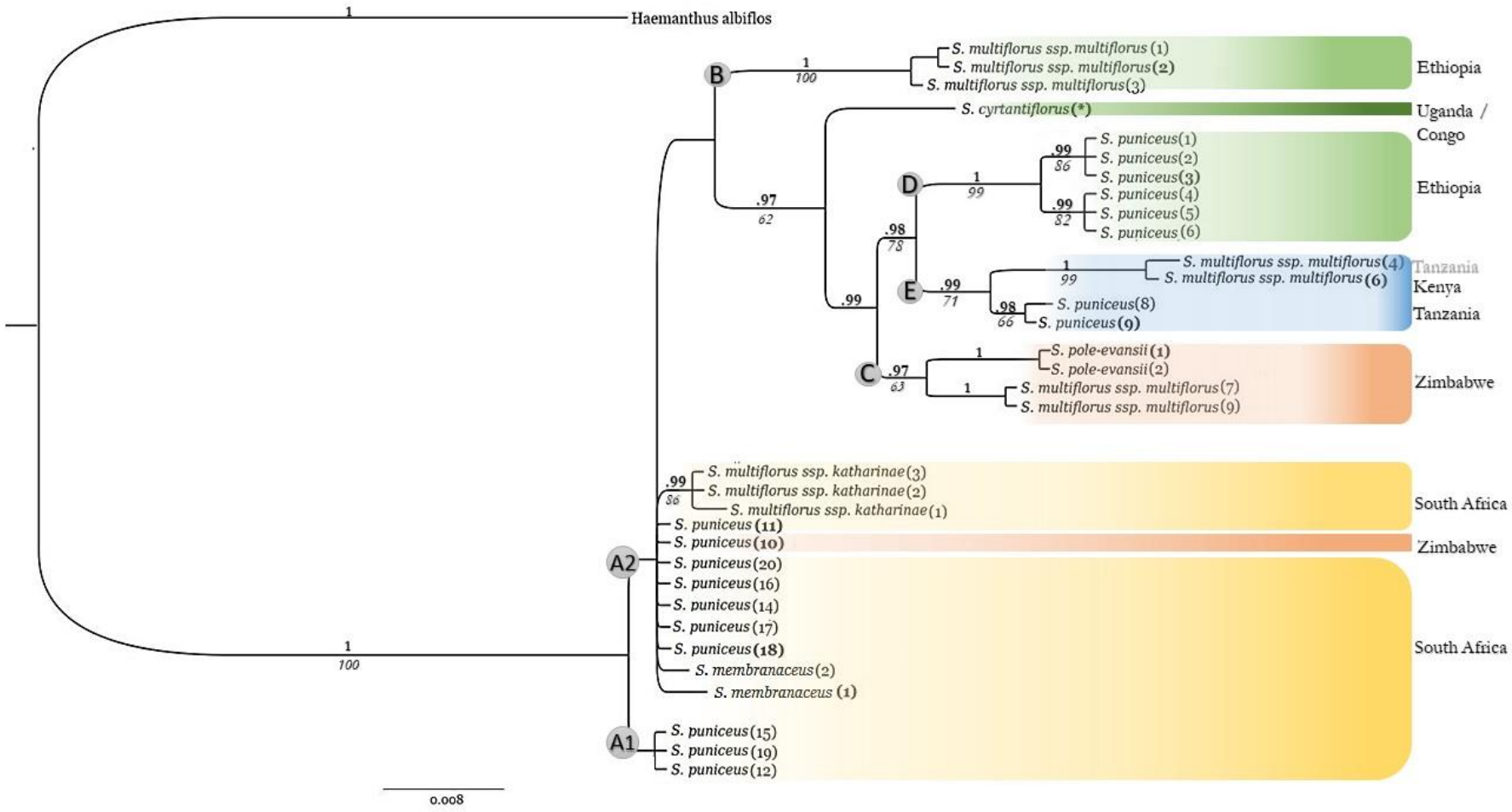


Figure 8 Illustrating the 50% majority rule consensus phylogram from the Bayesian analysis ITS. Total 40 acessions and 713 characters (including coded indels) in matrix. Maximum parsemony jack-knife support of at least 50% displayed in italic below branches and the Bayesian posterior probability values of at least 0.9 placed above. Abbrevations: S = *Scadoxus*, numbers corresponding material list Table 2, bold numbers also included in chloroplast genome analysis Table 2&4.

Discussion

The main focus of this study is to 1) establish whether *Gyaxis* is monophyletic using molecular phylogenetic methods, 2) evaluate whether *Scadoxus puniceus* is monophyletic using molecular phylogenetic methods and 3) analyse whether the geographical populations of *Scadoxus puniceus* should be recognized and in case at which rank.

Section *Gyaxis.* The morphologically based section *Gyaxis* (Bjørnstad & Friis, 1974) is not supported in my molecular analyses (Fig. 6-8), as the two species that make up the section, *S. puniceus* and *S. membranaceus*, do not form a monophyletic clade. *Scadoxus membranaceus* forms a monophyletic clade together with *S. multiflorus* ssp. *katherinae* in all topologies, highly supported in the cpDNA and chloroplast genome trees, and fairly supported in the ITS tree. *Scadoxus puniceus* resolves as polyphyletic in the cpDNA and chloroplast genome trees. The most important morphological trait defining section *Gyaxis* is the dominating involucral bracts that are persistent during anthesis, and give the flower a conical shape (Bjørnstad & Friis, 1974). The molecular analyses indicate that this trait has evolved more than once independently. Similarly biased production of genetic variation in close relatives may give way for parallel evolution (Dolph Schluter et al., 2004) but could also be an evolutionary change that increases the fitness for both/all lineages that have this change (Wichman et al., 1999).

Scadoxus puniceus. All trees (Fig. 6-8) show that *S. puniceus* is not monophyletic, but resolves in three to four subclades. The molecular phylogenetic analyses do not support the delimitation of Bjørnstad and Friis (1974) that was based on morphology. The subclades supported in the ITS and cpDNA analysis show a strong geographical pattern and almost all individuals are assigned to one of these geographical subclades. The major geographical subclades correspond to the geographical populations that are described in Bjørnstad and Friis (1974) and Friis and Nordal (1976), namely: Southern Africa, Tanzania and Ethiopia. In the chloroplast genome tree an additional region resolved as a separate clade, Zimbabwe (Fig. 6C).

In their revision Bjørnstad and Friis (1974) suggested three hypotheses for the disjunct distribution; 1) that *S. puniceus* is a relict from a once widely distributed species that diminished due to climatic change, 2) that the disjunct distribution is due to dispersal of seeds by birds, or 3) that it is of polyphyletic origin and has arisen independently in three different location.

Firstly, if the disjunct distribution pattern of today is a result of the splitting of a former more continuous distribution area, one would expect the *S. puniceus* complex to be monophyletic, which the molecular phylogenetic analyses have disproved.

Secondly, if long-dispersal of seeds by birds created the distinct pattern, one would also expect the *S. puniceus* complex to be monophyletic, with no sister relation to geographical adjacent species. This is clearly not the case in the molecular phylogenies, so also the bird dispersal hypothesis should be rejected. Bird dispersal might, however, play an important role for local distribution patterns (Voigt et al., 2009), which could explain the varying resolution within the topologies, e.g. among the South African specimens.

Thirdly, the phylogenetic analyses clearly indicate that the hypothesis of independent speciation in different geographical regions is the most likely explanation for the pattern observed. This could be due to pollinator driven evolution that has occurred more than once. Such parallel phenotypic evolution explains similar, but independent, evolutionary changes in traits among closely related species usually as a response to environmental or ecological changes (Haldane, 1932). Haldane (1932) proposed that new genetic variation could be due to underlying phenotypic traits biased in some directions, which might apply for the common inflorescence structure many of the *Scadoxus* species hold. A recent article by Butler and Johnson (2020) studied independent origin of butterfly-wing pollination in several lineages of South African Amaryllidaceae. They focused on *S. multiflorus* ssp. *multiflorus* and ssp. *katherinae* and established that *S. multiflorus* ssp. *katherinae* is genetically self-incompatible and therefore reliant on pollinators for seed production. They further predicted this mechanism for another nine species within South African Amaryllidaceae, generally plants with cone- or brush-like shaped inflorescences. Butler and Johnson (2020) emphasized that typical butterfly pollinated species have exerted reproductive parts. The anthers of *S. puniceus* are long, usually reaching above the petals and bracts. This matches well with the discovery by Bjørnstad and Friis (1974) that stamen filaments are longer than perianth segments during anthesis, and the style a little longer than filaments when mature. Butler and Johnson (2020) made further an interesting observation: the pollen did not mainly stick to proboscis and wings, but rather to the underside of the wings which gently touched the protruding dichogamous styles and stigmas. If that is so, it might be efficient for the plant to have the flowers connected in a cone (as in the *S. puniceus* complex) rather than spread in a globular fashion (as in the *S. multiflorus* complex). The pollen will in the first case be more concentrated in space corresponding to where the butterfly wings

might touch. However, Butler and Johnson (2020) suggested in their conclusion that *S. puniceus* most likely is pollinated by birds and not butterflies, but emphasized more studies on the area. Bjørnstad and Friis (1974) suggested the same in their revision, due to the form of the inflorescences and color and robustness of the bracts (Bjørnstad & Friis, 1974; Butler & Johnson, 2020).

The geographically separated clades of *S. puniceus* clearly deserves taxonomic recognition. As, explained above, *S. puniceus* is not monophyletic, and the geographical clades can therefore not be recognized as sub-species. A better alternative would be to recognize them at the species level, however the question remains as to how many of the clades that should be recognized.

As mentioned earlier there are four clades in the chloroplast genome tree, and three clades in ITS and cpDNA trees. In the chloroplast genome tree, the Zimbabwean *S. puniceus* is part of a highly supported Zimbabwean clade while South African *S. puniceus* is part of a highly supported South African clade. When adding more accessions (Fig. 7D, 8A2 & C) the Zimbabwean and the South African clades are still highly supported in the cpDNA tree, though two of the South African *S. puniceus* accessions are now included in the Zimbabwean clade, and one Zimbabwean accession in the South African clade. However, in the ITS tree (Fig. 8) both Zimbabwean and South African *S. puniceus* are part of the basal polytomy most likely as a result of insufficient data to provide a fully resolved phylogeny. The Zimbabwean *S. puniceus* is represented only by one accession in the phylogenetic analyses, and has never been regarded as a taxon in its own right. I find it premature to give it separate taxonomic recognition. Further analyses with wider sampling should be done to evaluate its position. Therefore, until further studies, I suggest to refer all Southern African accessions to the same name.

Based on my phylogenetic analysis the Ethiopian and Tanzanian clades deserves to be recognized at species level. In all trees the Ethiopian *S. puniceus* is separated from Ethiopian *multiflorus* ssp. *multiflorus* (Fig. 6A & D, 7A & C, 8B & D). Tanzanian *S. puniceus* show the same pattern, with a well-supported separation of *S. puniceus* and *S. multiflorus* ssp. *multiflorus* in both ITS and chloroplast genomic tree (Fig. 8E & 7A), but with low support in cpDNA (Fig. 7A). By conclusion there are three clades to be recognized. Of the ten names (synonyms of *S. puniceus*) that are available (Table 1), some must be recombined in *Scadoxus*. The oldest name for the three areas are *Haemanthus fax-imperii* Cufod. for Ethiopia, *Haemanthus goetzei* Harms for Tanzania and *S. puniceus* for Southern Africa. The three new taxa will be recombined and reinstated at specific level in the taxonomical conclusion.

Conclusion

The main aim of my project is to investigate the specific delimitation of the *S. puniceus* complex. The results from the molecular phylogenies do not support subgenus *Gyaxis* as monophyletic, as *S. puniceus* and *S. membranaceus* are not sister species. There is neither support for monophyly of *S. puniceus, as it resolves in several* separate and well-supported geographical clades. The hypothesis of independent origin of the populations in Southern Africa, Tanzania and Ethiopia is thus clearly supported. When it comes to Southern Africa, the relation between the South African and the Zimbabwean populations is not concurrent in all the analyses, and should be further investigated.

Implication for conservation

Based on my results the once widespread *Scadoxus puniceus* have changed to three species with smaller distribution. Particularly has the Tanzanian species a narrow distribution (Fig. 4), and therefore it is major importance to evaluate the conservation status for all the three species. They grow in different countries and environment and are probably subjected to different threats, further studies is encouraged.

Taxonomic implications

Scadoxus fax-imperii (Cufod.) I.E.Moe & Bjorå **comb. nov.**

Basionym: [Withheld]. Miss. Biol. Borana, Racc. Bot., Angiosp.-Gymnosp.: 326 (1939) - Type: Ethiopia - Arero, Meta Gafersa, 1600m, 4. july 1937 (holotype FT; isotype W).

Syn: *Scadoxus puniceus* (L) Friis & Nordal pro parte.

Scadoxus goetzei **(**Harms) I.E.Moe & Bjorå **comb. nov.**

Basionym: [Withheld], Bot. Jahrb. Syst. 30: 276 (1901) - Type: Tanzania - Yawuaegerberg, 13 September 1899 (holotype B; isotype P).

Syn: *Scadoxus puniceus* (L) Friis & Nordal pro parte.

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Appendix

Table A1 List of all samples during this study and their Nanodrop and Qbit for extraction of DNA. Many are repeated since DNA yield, Nanodrop or Qbit had too low values for either Sanger sequencing or NextGen analysis.

CollNo	Country	**Nanodrop**			**Qubit**	
		ng/uL	A260/A280	A260/A230	ng/uL	ng (80 ul)
						80
1-Veksthus	South Africa	19.600	1.809	4.546	18.70	1496
1999163	South Africa	163.249	1.457	0.709	16.70	1336
1999163_2	South Africa	3.174	5.702	39.17	4.33	
2004144	South Africa	67.194	1.705	1.517	49.40	3952
2004144_2	South Africa	23.381	1.707	0.873	8.15	
2004145	South Africa	104.223	1.612	0.841	35.10	2808
2016086	South Africa	130.873	1.491	0.796	27.80	2224
2016086_2	South Africa	4.611	2.14	1.239	3.35	
2008055	South Africa	52.115	1.69	1.196	29.30	2344
1999081	South Africa	134.823	1.57	0.853	43.80	3504
1999081_2	South Africa	104.940	1.506	0.644	7.19	
1976-345	South Africa					
1999011	South Africa?	48.739	1.65	0.964	22.50	1800
2003028	?	36.846	1.643	1.033	16.60	1328
2014001	?	32.887	1.706	1.15	20.00	1600
2018053	?	24.034	1.65	0.892	7.39	591.2
2018054	?	28.022	1.756	1.726	24.00	
2917	Zimbabwe	43.082	1.776	1.59	31.90	2552
1590	Zimbabwe	58.163	1.809	2.301	47.30	3784
1597	Zimbabwe	29.468	1.924	3.583	28.00	2240
1613	Zimbabwe	71.942	1.844	2.476	56.70	4536
1622	Zimbabwe	138.673	1.766	1.646	93.00	7440
1623	Zimbabwe	55.558	1.816	2.251	46.40	3712
1625	Zimbabwe	35.707	1.828	2.645	33.00	2640
1319	Tanzania	34.448	1.701	0.849	11.40	
2006099	Tanzania	21.253	1.641	0.651	3.25	
2006099_2	Tanzania	9.853	1.477	0.821	3.24	
2006099_4	Tanzania	29.386	1.867	1.85	25.1	
86A	Ethiopia	29.386	1.867	1.85	25.1	
86A_1	Ethiopia	3.940	1.613	0.681	1.13	
86A_2	Ethiopia	29.678	1.523	0.732	5.54	
86A_3	Ethiopia	33.168	1.85	1.911	23.50	
86B_2	Ethiopia	166.599	1.816	2.248	144.00	

86C_2	Ethiopia	84.256	1.824	2.019	68.30	
87A	Ethiopia	16.157	1.583	0.597	0.02	
87A_2	Ethiopia	10.742	1.461	1.159	6.20	
87A_3	Ethiopia	23.054	1.773	1.62	17.80	
87A (DZ)	Ethiopia	29.678	1.523	0.732	5.54	
87A_2(DZ)	Ethiopia	33.168	1.85	1.911	23.50	
87B_2	Ethiopia	26.402	1.788	1.651	21.90	
87C_2	Ethiopia	14.975	1.79	1.425	11.50	
85A (Emb)	Ethiopia	166.599	1.816	2.248	144.00	
85A (Emb)	Ethiopia	117.54	1.757	1.546	71.20	
85A_2	Ethiopia	33.422	1.569	0.898	12.00	
85A_2	Ethiopia	84.256	1.824	2.019	68.30	
800_1	Ethiopia	16.157	1.583	0.597	0.02	
800_2	Ethiopia	10.742	1.461	1.159	6.20	
821	Ethiopia	23.054	1.773	1.62	17.80	
853_1	Ethiopia	26.402	1.788	1.651	21.90	
853_2	Ethiopia	14.975	1.79	1.425	11.50	
855	Ethiopia	117.54	1.757	1.546	71.20	
871	Ethiopia	33.422	1.569	0.898	12.00	
1245	Tanzania					
93A-E_1	South Africa	26.477	1.713	1.254		
93A-E _2	South Africa	16.559	1.654	1.043		
93A-E _3	South Africa	21.563	1.725	1.223		
93A-E _4	South Africa	24.713	1.734	1.187		
93A-E _5	South Africa	5.909	1.792	0.535		

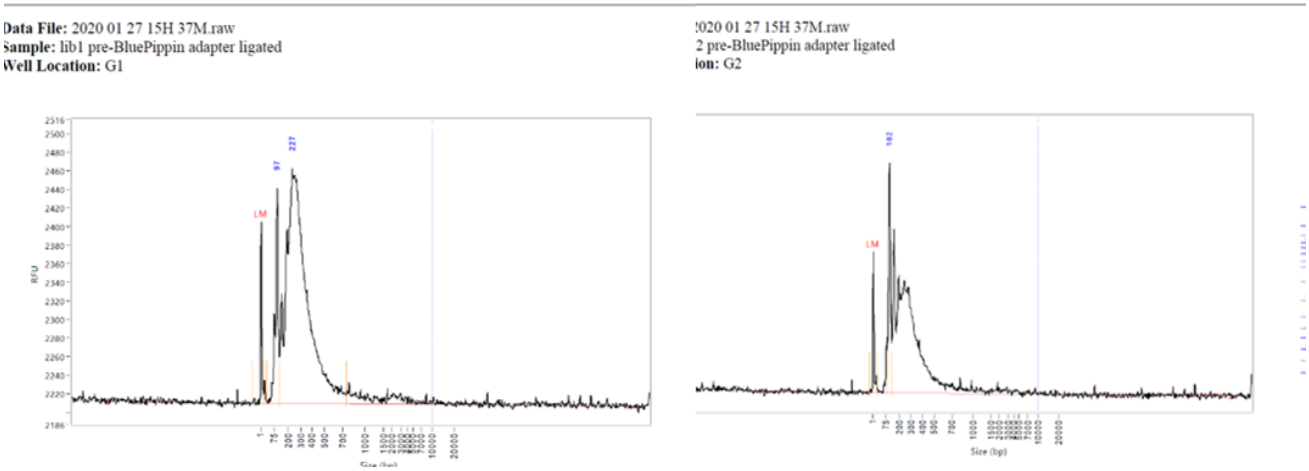

***Figure A1*:** Fraction Analyzer results showing trace of the adapter ligated pools, after BluePippin. Library 1 (left) and library 2 (right) both peaks illustrating aimed fragment size 210-300 bp.

Table A2 Summary of data from Chip 1 and 2. The total reads per sample listed besides it average base pair size. Results provided by IonTurrent

ID	Chip	IonXpress barcode	Sample name	Bases	>Q20	Reads	Mean size (bp)
	1		*n.a*	*220,056,080*	*188,350,789*	*1,568,373*	*140*
1	1	1	2005062	217,575,041	199,232,814	1,345,742	161
2	1	2	2016055	562,017,651	516,203,124	3,433,085	163
3	1	3	2971C	262,501,892	241,504,310	1,599,670	164
4	1	4	1695	411,145,989	377,261,153	2,478,783	165
5	1	5	2004052	399,128,345	365,469,450	2,450,981	162
6	1	6	1062	403,994,420	370,228,601	2,439,320	165
7	1	7	004_H_albi	1,010,222,575	923,256,301	6,114,896	165
8	1	8	2973A	3,650,174,817	3,324,468,474	22,538,915	161
9	1	9	2016086	1,347,286,594	1,237,098,799	8,238,970	163
10	1	10	1801	154,204,418	140,566,243	957,006	161
11	1	11	2967A	80,597,446	73,686,167	504,125	159
12	1	12	178	85,776,896	79,327,626	588,250	145
	2		*n.a*	*485,085,094*	*423,600,221*	*2,794,927*	*173*
13	2	1	Veksthus	796,658,727	716,697,020	4,444,939	179
14	2	2	2016043	1,494,238,999	1,347,093,827	8,490,960	175
15	2	3	EF22	1,461,964,955	1,313,983,994	8,159,198	179
16	2	4	2965A	3,126,424,673	2,809,143,061	17,769,581	175
17	2	5	488	3,036,344,409	2,734,861,630	17,358,373	174
18	2	6	1999011	2,241,023,514	2,009,804,592	12,546,918	178
19	2	7	1590	760,652,668	683,294,054	4,243,613	179
20	2	8	86C	418,104,139	374,874,954	2,404,435	173
21	2	9	2006099	605,178,259	543,473,128	3,413,445	177
22	2	10	2917	1,183,057,421	1,063,055,586	6,593,129	179
23	2	11	2018040	898,207,780	808,080,859	5,025,632	178
24	2	12	2968A	108,876,814	98,848,242	629,357	172