Finding the silver lining in a crisped margin

– *A study of the prostrate species in* Chlorophytum *(Asparagaceae)*

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Chlorophytum flower. Photo by: Gry S. Hoell

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Forord

Denne oppgaven markerer slutten på et femårig studieløp ved Universitetet i Oslo. Det føles veldig godt, men samtidig litt vemodig. Tiden på Blindern, og senere NHM, har vært helt fantastisk. Men en ting er sikkert: denne tiden hadde ikke betydd halvparten så mye uten alle de fine menneskene jeg har blitt kjent med. En spesiell takk rettes til Malin og Karianne. Ikke bare har vi kommet oss gjennom et langt studieløp, men vi har blitt gode venner på veien. Dere har gjort studietiden til en tid jeg vil se tilbake på med stor glede.

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Martine,
15 juni 2020

Denne masteroppgaven er skrevet på artikkelform og er tilpasset retningslinjene til tidsskriftet Plant Ecology & Evolution med unntak av at tekstoppsettet er noe justert og figurer/tabeller er satt inn i teksten for å øke leservennligheten.

Content

ABSTRACT

Background and aims – The species delimitations within the prostrate species with condensed inflorescence in *Chlorophytum* are ambiguous and the taxonomic rank of several of the species involved are disputed. This study aims to evaluate the circumscription of the taxa and clarify what morphological characters that are diagnostic or most informative to distinguish between the them. **Methods** – Total genomic DNA was extracted from both herbarium and silica-dried specimens. One nuclear (ITS) and five plastid regions (*trn*L-F spacer; *trn*L intron; *psb*A-*trn*H spacer; *rps*12-*rpl*20 spacer; *rps*16 intron) were PCR amplified and sequenced. Subsequent phylogenetic analyses were conducted following both a Bayesian and parsimonious approach. Molecular methods were supplemented with morphological studies. Seeds of 11 accessions were photographed by a scanning electron microscope. **Key results** – *Chlorophytum latifolium* is reinstated at species level. Two different seeds are recorded for *C. pusillum*, indicating hidden diversity. An identification key is included to distinguish between the prostrate species in *Chlorophytum*. The variation within the different monophyletic groups within the prostrate species in *Chlorophytum* is not yet fully comprehended and require further studies.

Keywords – species delimitation, phylogeny, identification key, SEM, *C. geophilum*, *C. pusillum*, *C. stenopetalum*, *C. latifolium*.

INTRODUCTION

Chlorophytum Ker Gawl. is a genus within the family Asparagaceae in the order Asparagales. Currently, 180 species are included in *Chlorophytum* (Bjorå et al. 2017). The genus is distributed in Africa, Asia and Oceania, but the centre of diversity is in Africa south of Sahara with more than 120 species (Bjorå 2008). Systematic studies of the genus *Chlorophytum* has revealed some uncertainties about the delimitations of the prostrate species with short peduncle and condensed inflorescence. The taxonomic rank of several of these taxa are disputed (Kativu et al. 2008; Meerts & Bjorå 2012). The following taxa are part of this group: *Chlorophytum geophilum* Poelln., *C. pusillum* Schweinf. ex Baker, *C. stenopetalum* Baker, *C. latifolium* Engl. & K.Krause and *C. blepharophyllum* ssp. *rubropygmaeum* sensu Bjorå & Nordal. In the latest revision of these taxa, *C. blepharophyllum* ssp. *rubropygmaeum* did not get any taxonomic recognition and *C. latifolium* was reduced to a variety of *C. stenopetalum* (Meerts & Bjorå 2012; Meerts 2015). The representatives in this group are shown in fig. 1, and their distribution are depicted in fig. 2.

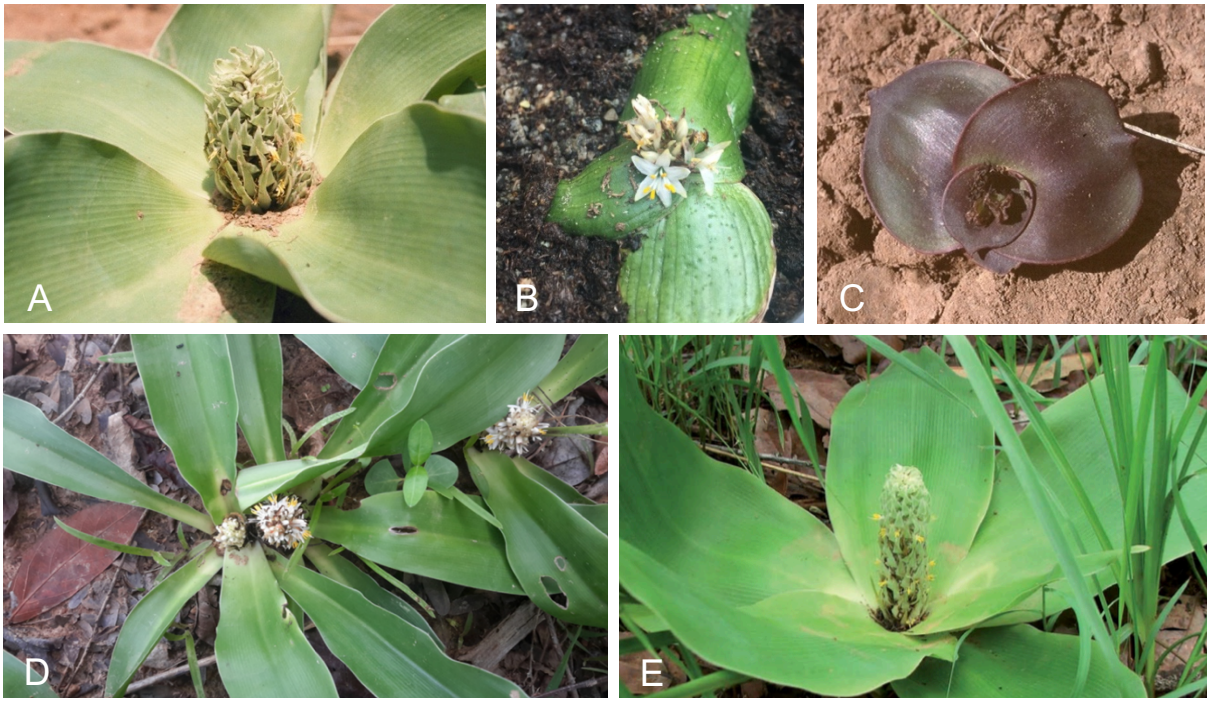

Figure 1 – All five representatives of the prostate species within *Chlorophytum*. **A**. *Chlorophytum geophilum* sensu Kativu et al. (2008); **B**. *Chlorophytum pusillum*. **C**. *Chlorophytum blepharophyllum* ssp. *rubropygmaeum* sensu Bjorå et al. (2008). **D**. *Chlorophytum stenopetalum*; **E**. *Chlorophytum stenopetalum* var. *latifolium* sensu Meerts & Bjorå (2012). Photographs by: A, Gry S. Hoell; B, Martine Haukland Nyrud; C, Brita Stedje; D, Blandine M. I. Nacoulma; E, J. Piqueray. Picture D is from African Plants – A Photo Guide www.africanplants.senckenberg.de

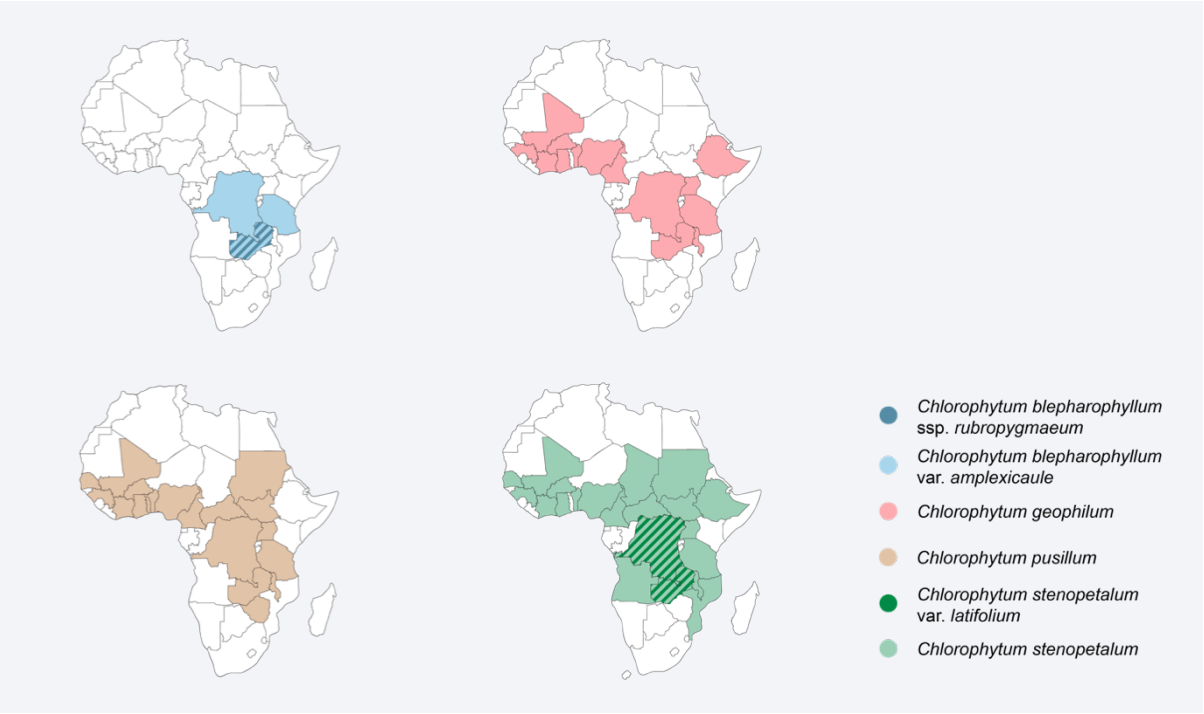

Figure 2 – Map showing the distribution of prostrate species with a condensed inflorescence in *Chlorophytum* on the African continent. The distribution of *C. blepharophyllum* ssp. *rubropygmaeum* sensu Bjorå et al. (2008) is highlighted in dark blue while the distribution of *C. blepharophyllum* var. *amplexicaule* is marked in light blue. The distribution of *C. geophilum* and *C. pusillum* are marked in pink and beige respectively. The distribution of *C. stenopetalum* var. *latifolium* is marked with dark green while the distribution of *C. stenopetalum* is highlighted in light green. The countries concerned are based on the species descriptions found in Nordal et al. (1997), Bjorå et al. (2008), Kativu et al. (2008), Meerts & Bjorå (2012), Meerts (2015) and GBIF (2020).

Many *Chlorophytum* species appears with the first rain and are consequently prone to grazing. Keeping the leaves and reproductive parts of the plant close to ground is probably a strategy to avoid herbivory. Selection towards a shortening of the peduncle might also influence the pedicel length (Inger Nordal, pers. com.). The location of the pedicel joint is a character that is often is used to distinguish between species (Nordal et al. 1997; Kativu et al. 2008). A reduction of the pedicel makes it difficult to interpret the location of the pedicel joint.

The taxa concerned are morphologically similar which has led to much confusion. Several floras state that *Chlorophytum geophilum* and *C. pusillum* are difficult to separate from each other (Nordal 1997; Kativu et al. 2008; Meerts 2015) considering both species have a reduced peduncle and prostrate leaves (Meerts 2015). In his treatment from Dem. Rep. Congo in 1956, Troupin included a description of *C. pusillum* that later was synonymized under *C. geophilum* by Meerts & Bjorå (2012). In Flora Zambesiaca (Kativu et al. 2008), *C. latifolium* is treated as an uncertain species with an unclear delimitation to *C. geophilum*, while it is reduced to a variety under *C. stenopetalum* in Meerts & Bjorå (2012) and Meerts (2015). *Chlorophytum stenopetalum* var. *latifolium* has sometimes been confused with *C. geophilum* because of its prostrate leaves, but according to Flore d'Afrique Centrale (Meerts 2015) it is distinguished in particular by an elongated cylindrical raceme and very short pedicels. Further, in Flora of Tropical East Africa (Nordal et al. 1997) the monophyly of *C. stenopetalum* is questioned as it is suggested that *C. stenopetalum* might represent a polyphyletic assemblage.

The current identifications and circumscriptions of these taxa are ambiguous due to conflicting treatments by various authors. Figure 3 provides an historic overview of the prostrate species in *Chlorophytum* with a condensed inflorescence and the species delimitation in different treatments.

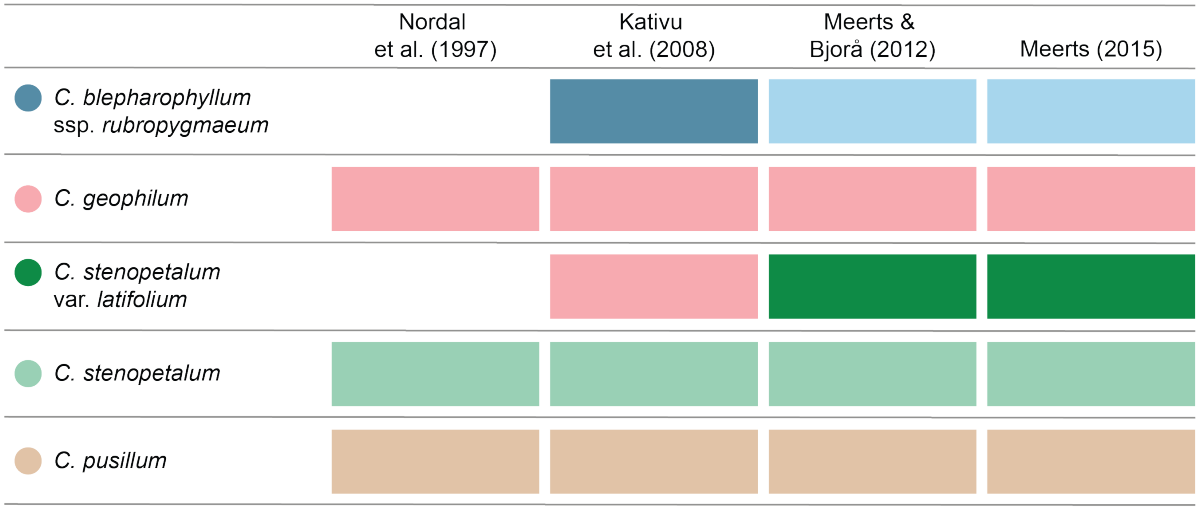

Figure 3 – displays the species delimitation of the taxa concerned in different treatments. The different treatments can be seen in the top row and each taxa is highlighted with its own colour: *C. blepharophyllum* ssp. *rubropygmaeum* in dark blue, *C. blepharophyllum* var. *amplexicaule* in light blue, *C. geophilum* in pink, *C. stenopetalum* var. *latifolium* in dark green, *C. stenopetalum* in light green and *C. pusillum* in beige. The colour coding depicts how the taxa have been treated differently in recent literature.

These various treatments, based on morphological similarity between the species, results in an unclear species delimitation between the prostrate species with compressed inflorescence in *Chlorophytum*. In this study, I will test the morphologically defined species delimitation through molecular analyses, and evaluate what morphological characters that are diagnostic or most informative. Based on these answers, I will suggest names for the taxa and produce a key for the prostrate taxa with condensed inflorescence in *Chlorophytum*.

MATERIALS AND METHODS

Plant material and sampling

Plant material used in this study constitutes of both herbarium specimens and silica-dried leaf samples. Freshly collected samples were provided during field work in Zimbabwe in November/December 2019, and a portion of the collected material is obtained through the NORPART project (NORPART 2016/10013: Collaborating learning in biodiversity, excellence in education through two-way North-South mobility). Herbarium specimens were obtained from the following herbaria: ETH, BR, BRLU, O and SRGH. The taxa included in this study are mostly species from genus *Chlorophytum*, but species from genera *Anthericum* and *Paradisea* are included to serve as outgroup in the subsequent phylogenetic analyses.

Scanning electron microscope (SEM)

Seed shape and testa characters have previously been proven to be important to distinguish between similar-looking taxa within the genus *Chlorophytum* (Bjorå 2008). Seed surfaces of 11 accessions have been photographed using a Hitachi S-3600N Scanning Electron Microscope (SEM) operated at 20 Pa and 15 Kv using a backscattered electron detector with 3D mode activated. The analysis were performed at low pressure and the samples were therefore not coated.

In total, 53 specimens were studied. Table 1 provides a full summary of the samples and sequences used in this study as well as voucher-holding herbarium, voucher ID, locality and GenBank accession numbers. Here it is also indicated which accessions that were photographed by scanning electron microscope.

Laboratory work

Total genomic DNA was extracted from both herbarium and silica-dried specimens using the E.Z.N.A SP Plant mini kit (Omega Bio-Tek, Atlanta, USA) with some minor deviations from the manufacturer's protocol: SP1 buffer, RNase A and powdered tissue sample were incubated for 1 hour; samples were incubated at 65 ºC for 5 minutes to increase yield during elution. Further, we PCR amplified and sequenced several regions including both nuclear and plastid DNA. Amplification of the ITS region was done using the ITS4-ITS5 primer pair. We used internal primers ITS2 and ITS3 as well as raising the template concentration for samples we struggled getting adequate results from. All primers from White et al. (1990). Altogether five plastid regions were targeted including *trn*L-F spacer, *trn*L intron, *psb*A-*trn*H spacer, *rps*12-*rpl*20 spacer and *rps*16 intron. Primers c and f from Taberlet et al. (1991) were used to amplify the *trn*L-F spacer and *trn*L intron. To yield better results for non-successful PCR amplifications we increased the template concentration and used internal primers e and d, also from Taberlet et al. (1991). For the intergenic spacer regions *psb*A-*trn*H and *rps*12-*rpl*20 we used primers from Hamilton (1999). For the *rps*16 intron the *rps*16F and *rps*162R primer pair from Oxelman et al. (1997) was used.

Table 1 – Overview of taxon name, herbarium, voucher ID, locality and GenBank accession numbers for DNA sequences used in this study. The taxa included are species from *Chlorophytum*, *Anthericum* and *Paradisea*. Accessions that were SEM photographed are marked with an X on the far right. Species from the genera *Anthericum* and *Paradisea* will serve as outgroup in the phylogenetic analyses. Herbaria acronyms and species author name from Index Herbariorum (2020) and IPNI (2020) respectively. Abbreviations: Herb. = voucher-holding herbarium, - = not available.

Taxon	**Herb.**	**Voucher ID**	**Locality**	**ITS**	***trn*L-F**	***rps*16**	***psb*A-*trn*H**	***rps*12-*rpl*20**	**SEM**
Anthericum ramosum L.	O	Bjorå 855	Cult.	KU88778	KU880877	KU880823	X	X	-
Chlorophytum affine Baker	O	Nordal & Bjorå 4552	Zambia	EF999985	EU000019	KU880830	X	X	-
C. andongense Baker	SRGH	Chapano et al. 1852	Zimbabwe	X	X	X	-	X	-
C. blepharophyllum ssp. *rubropygmaeum* Bjorå & Nordal	O	Nordal 4578	Zambia	X	X	X	-	-	-
C. blepharophyllum var. *amplexicaule* (Baker) Meerts (1)	O	Hoell & Nordal 134	Zambia	X	X	X	-	-	-
C. blepharophyllum var. *amplexicaule* (2)	BRLU	Meerts 36	D. R. Congo	X	X	X	-	-	-
C. blepharophyllum var. *blepharophyllum* Schweinf. ex Baker (1)	O	Hoell & Nordal 94	Zambia	KU880785	KU880882	KU880832	X	X	-
C. blepharophyllum var. *blepharophyllum* (2)	SRGH	Chapano et al. 1846	Zimbabwe	X	X	X	-	X	-
C. clarae Bjorå & Nordal	O	Nordal 4542	Zambia	X	-	X	-	-	-
C. comosum (Thunb.) Jacques	O	Nordal 3162	Zimbabwe	EF999993	EU000027	KU880840	X	X	-
C. filipendulum Baker (1)	O	Poulsen 956	Uganda	EF999994	EU000028	EU128968	-	-	-
C. filipendulum (2)	O	Nordal 3219	Zimbabwe	X	X	X	X	X	-
C. filipendulum ssp. *amaniense* (Engl.) Nordal & A.D.Poulsen	BR	19840800	Cult.	X	X	X	X	X	-
C. gallabatense Scweinf. ex Baker	O	Hoell & Nordal 25	Zambia	EF999996	EU000030	EU128971	X	X	-
C. cf. *galpinii* (Baker) Kativu	SRGH	Chapano et al. 1879	Zimbabwe	X	X	X	X	X	-
C. geophilum Peter ex Poelln. (1)	O	Hoell & Nordal 26	Zambia	EF999998	EU000032	EU128972	X	X	-
C. *geophilum* (2)	ETH	Hermann 36	Ethiopia	KU880798	X	KU880847	X	X	-
C. geophilum (3)	O	Bidgood 1332	Tanzania	X	X	X	X	X	-
C. geophilum (4)	O	Hoell & Nordal 79	Zambia	X	X	X	-	X	-
C. geophilum (5)	WAG	Sinsin 3636	Benin	X	-	-	X	-	-
C. geophilum (6)	WAG	Wilde 3139A	Cameroon	X	X	X	X	-	X
C. geophilum (7)	O	Peter 35704	Tanzania	-	-	-	-	-	X
C. geophilum (8)	WAG	Akoègninou 3531	Benin	-	-	-	-	-	X
C. lancifolium Welw. ex Baker	O	Nordal 4576	Zambia	X	X	X	X	-	-
C. longifolium Schweinf.	O	Nordal 1507	Zimbabwe	EU000001	EU000034	X	X	X	-
C. macrosporum Baker	SRGH	Chapano et al. 1815	Zimbabwe	X	X	X	X	X	-

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Taxon	**Herb.**	**Voucher ID**	**Locality**	**ITS**	***trn*L-F**	***rps*16**	***psb*A-*trn*H**	***rps*12-*rpl*20**	**SEM**
C. macrophyllum Asch.	ETH	Hermann 102	Ethiopia	X	X	X	X	X	-
C. minor Kativu	O	Hoell & Nordal 77	Zambia	X	X	X	X	X	-
C. pauper Poelln. (1)	O	Hoell & Nordal 11	Zambia	X	X	X	-	X	-
C. pauper (2)	O	Hoell & Nordal 13	Zambia	X	X	X	-	-	-
C. pauper (3)	SRGH	Chapano et al. 1817	Zimbabwe	X	X	X	X	X	-
C. polystachys Baker	SRGH	Chapano et al. 1884	Zimbabwe	X	X	X	X	X	-
C. psammophilum Engl. & Gilg	O, SRGH	Kativu 321	Zimbabwe	KU880918	KU880935	KU880927	-	-	-
C. pusillum Schweinf. ex Baker (1)	O	Hoell & Nordal 5	Zambia	X	X	X	X	X	-
C. pusillum (2)	O	Nordal & Bjorå 4567	Zambia	EU000007	EU000040	EU128979	X	X	-
C. pusillum (3)	BRLU	Meerts sn.	D.R. Congo	X	X	X	X	X	-
C. pusillum (4)	O	Schmidt 2532	Tanzania	-	-	-	-	-	X
C. pusillum (5)	WAG	Akoègninou 3590	Benin	-	-	-	-	-	X
C. pusillum (6)	WAG	Essou 2785	Benin	-	-	-	-	-	X
C. rubribracteatum (De Wild) Kativu	O	Bjorå 657	Zambia	KU880808	KU880904	KU880860	X	X	-
C. silvaticum Dammer	O	Nordal & Bjorå 4621	Kenya	EU000008	EU000041	X	X	X	-
C. stenopetalum Baker (1)	WAG	Morton sn.	Ghana	X	X	X	X	X	X
C. stenopetalum (2)	O	Hoell & Nordal 166	Zambia	X	X	X	X	X	-
C. stenopetalum (3)	WAG	Sinsin 2250	Benin	-	-	-	-	-	X
C. stenopetalum (4)	O	Nordal 4563A	Zambia	-	-	-	-	-	X
C. stenopetalum (5)	WAG	Essou 2981	Benin	-	-	-	-	-	X
C. stenopetalum (6)	WAG	Tchouto 2421	Cameroon	-	-	-	-	-	X
C. stenopetalum var. *latifolium* (Engl. & K.Krause) Meerts	O	Hoell & Nordal 20	Zambia	X	X	X	X	X	-
C. subpetiolatum (Baker) Kativu (1)	O	Hoell & Nordal 15	Zambia	X	X	X	-	-	-
C. subpetiolatum (2)	SRGH	Chapano et al. 1832	Zimbabwe	X	X	X	X	X	-
C. suffruticosum Baker	O	Nordal 5014	Tanzania	KU880921	KU880938	KU880930	X	X	-
C. viridescens Engl.	O	Bjørnstad 265	Kenya	X	X	X	X	X	-
Paradisea Liliastrum Bertol.	O	Bjorå 852	Cult.	X	X	X	X	X	-

The genetic regions were amplified from 1–3 µL DNA template in 12.5 µL reactions using AmpliTaq DNA polymerase buffer II kit (Applied Biosystems, Foster City, CA, USA) constituting of dNTP (10 mM), bovine serum albumen (1 g/L), primer (10 µM), buffer, $MgCl_2$ and 5.1–7.1 µL milliQ H_2O depending on the volume of the template DNA. All amplifications were performed under the same reaction conditions: denaturation at 94ºC for 2.5 minutes followed by 32 cycles of 94ºC for 30 s, 53ºC for 30 s, 72ºC for 50 s and finishing with a 72ºC elongation step for 4 minutes.

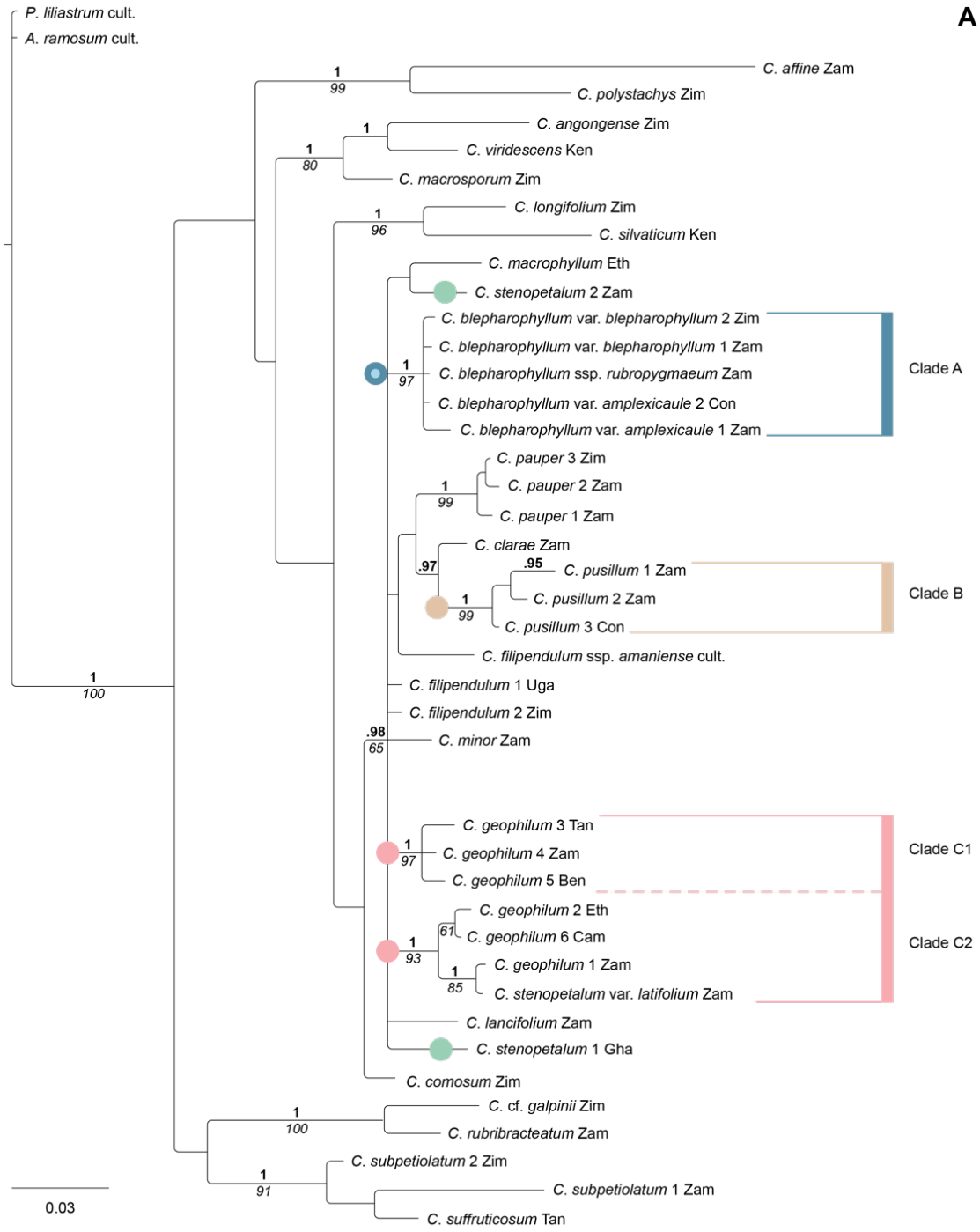
We used 2 µL diluted ExoSTAR (GE Healthcare UK Limited) together with 8 µL PCR product for the enzymatic cleanup of the PCR products. This was incubated at 37ºC for 45 minutes followed by 80ºC for 15 minutes. 10-30 µL MilliQ H_2O were added to the samples depending on the intensity of the PCR bands. The prepared samples for sequencing of normal and strong PCR products contained 7.5 µL cleaned PCR product and 2.5 µL primer. Amplicons of weak PCR product contained the same amount of purified PCR product, 1.3 µL primer and 1.2 µL MilliQ H_2O for a total volume of 10 µL. Sanger sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands).

Alignment and phylogenetic reconstructions

Forward and reverse sequences were trimmed, assembled and manually edited in Geneious Prime 2020.0.5 (http://www.geneious.com, Kearse et al. 2012). Subsequent multiple sequence alignment were also performed in Geneious Prime using the Muscle algorithm (Edgar 2004), followed by manual inspection in BioEdit 7.0.9.0 (Hall 1999). The alignments were adjusted when necessary. Furthermore, the alignments were gap coded manually treating indels as absent/present following the simple indel coding approach of Simmons & Ochoterena (2000). Maximum parsimony analyses were performed in TNT (Goloboff et al. 2008) using a heuristic search. Branch setting strategy were set to tree bisection reconnection (TBR), number of replicates to 2000 and maxtrees to 10 000. Clade support values were calculated through bootstrap analyses (Felsenstein 1985) using 1000 replicates and the cut off set to 50. Bayesian analyses were conducted in MrBayes v3.2.7 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) using the general time reversal model with an additional gamma parameter (GTR + G) as prior. The evolutionary model were set according to calculations made by the Akaike information criterion (AIC) in jModelTest (Guindon & Gascuel 2003; Darriba et al. 2012) where ‘Number of substitutions schemes’ were set to 3 and ‘Base tree for likelihood calculations’ to ‘Fixed_BIONJ-JC’. Posterior probabilities were calculated by running the analyses for 4.3 million generations in four chains sampling trees for every 1000th generation. Burn-in was set to 25%.

RESULTS

The lengths of the final nuclear and plastid alignments were: ITS 665; *trn*L-F 777; *psb*A-*trn*H 364; *rps*16 853; *rps*12-*rpl*20 775. For the nuclear dataset I found 18 most parsimonious trees with a tree length of 540. The plastid datasets resulted in 2713 most parsimonious trees with a tree length of 654. Figure 4 presents the majority rule consensus tree generated in MrBayes with posterior probabilities (PP) of at least 0.9 and parsimony bootstrap support (BS) of at least 50%. PP are shown in bold above branches and BS values in italic below branches.



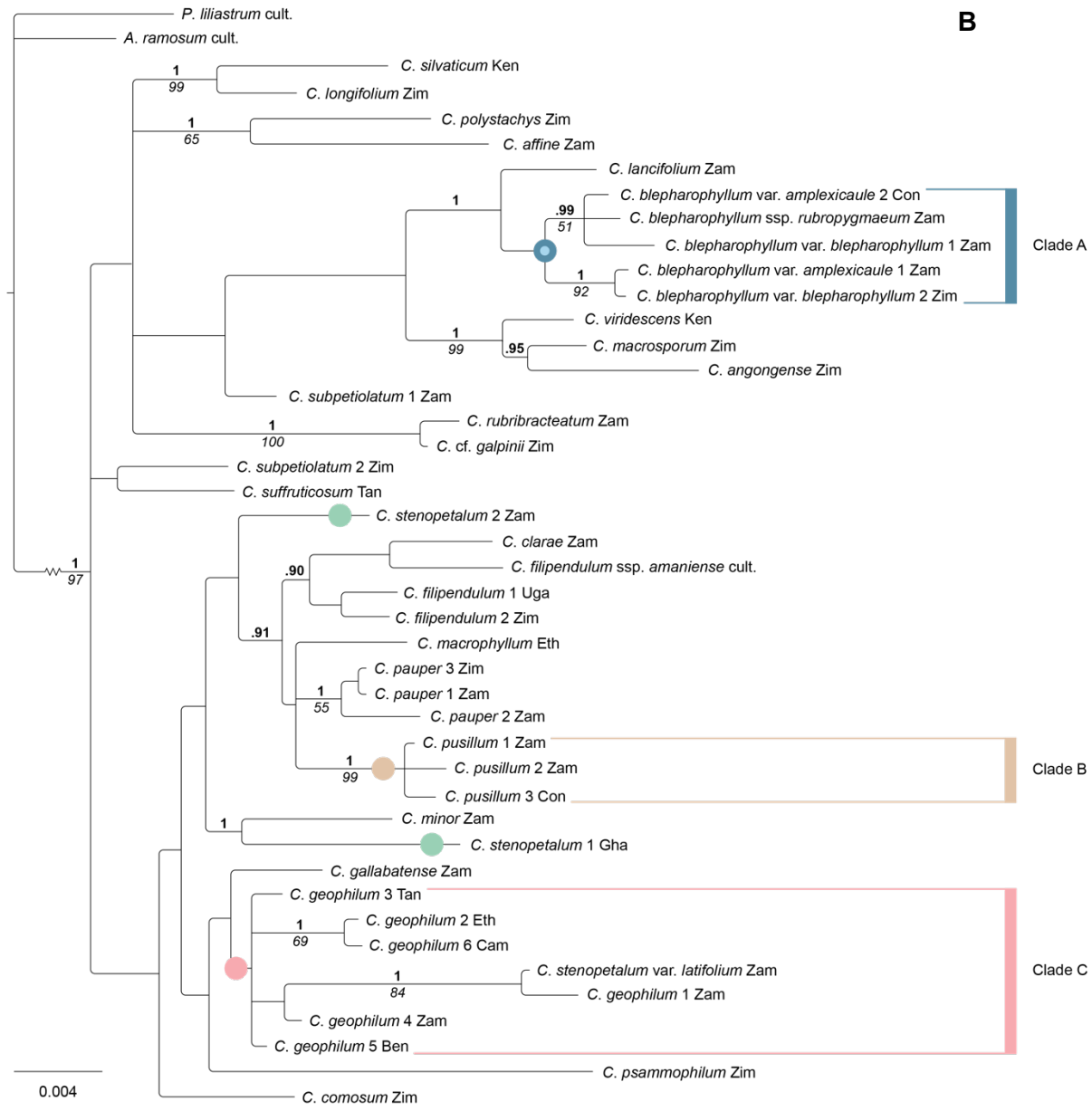


Figure 4 – Phylograms obtained from the Bayesian analyses of **A**. the ITS dataset with 44 accessions and 664 characters and **B**. the plastid datasets with 44 accessions and altogether 2769 characters. The taxa of interest are indicated by a coloured dot. Monophyletic groups are depicted with a bar on the right, following the same colour coding as in the introduction. Abbreviations: Ben = Benin, Cam = Cameroon, Con = Dem. Rep. Congo, cult. = cultivar, Eth = Ethiopia, Gha = Ghana, Ken = Kenya, Tan = Tanzania, Uga = Uganda, Zam = Zambia, Zim = Zimbabwe. All taxa with multiple accessions are marked with a number which corresponds with the numbering in the material list (see table 1).

The ITS and pDNA topologies supported by BS of at least 50 % or PP of at least 0.9 were congruent but resolved to different extents and in different parts of the trees (fig. 4A, B). In general, there is little support in the basal nodes. One accession, *Chlorophytum clarae*, attained incongruent positions in the ITS vs. pDNA trees. In the ITS tree it resolves as sister to the *C. pusillum* clade (PP 0.97), while in the pDNA it is in a clade together with *C. filipendulum* Baker and *C. filipendulum* ssp. *amansiense* (Engl.) Nordal & A.D.Poulsen (PP 0.90). Otherwise, the topologies are congruent with previous clades following Bjorå (2008).

The prostrate species with condensed inflorescence in *Chlorophytum* do not display monophyly, but rather a polyphyletic relationship in both the nuclear and plastid phylogeny. The *C. geophilum* specimens make up two well-supported monophyletic clades in the nuclear tree: clade C1 (PP 1, BS 97) and C2 (PP 1, BS 93) (fig. 4A). These two clades show no clear geographical pattern and are part of a wide polytomy. The *C. stenopetalum* var. *latifolium* accession is included in clade C2 (fig. 4A) and is together with *C. geophilum* (Hoell & Nordal 26) sister to *C. geophilum* (Hermann 36) and *C. geophilum* (Wilde 3139A). In the pDNA tree all the *C. geophilum* specimens groups together with *C. stenopetalum* var. *latifolium* (Hoell & Nordal 20) in a poorly supported monophyletic group (fig. 4B, clade C). The internal structure of this clade supports *C. geophilum* (Hoell & Nordal 26) as sister to *C. stenopetalum* var. *latifolium* (Hoell & Nordal 20) (PP 1, BS 84). Both the plastid and the nuclear regions rendered tree topologies were the *C. pusillum* (fig. 4, clade B) constitutes a well-supported monophyletic clade (nDNA PP 1, BS 99; pDNA PP 1, BS 99). Further, the monophyly of *C. stenopetalum* was not supported in either phylogenies. In the nuclear tree, both the *C. stenopetalum* accessions were unresolved in the wide polytomy. In the pDNA tree, *C. stenopetalum* (Morton sn.) is resolved as sister to *C. minor* (PP 1) while *C. stenopetalum* (Hoell & Nordal 166) has an unresolved position. In both phylogenies, all accessions from the *C. blepharophyllum* complex forms a strongly supported monophyletic clade (clade A) (nDNA PP 1, BS 97; pDNA PP 1). The plastid tree divides clade A in two clades. One clade consisting of *C. blepharophyllum* var. *amplexicaule* (PM36), *C. blepharophyllum* ssp. *rubropygmaeum* (Nordal 4578) and *C. blepharophyllum* var. *blepharophyllum* (Hoell & Nordal 94) (PP 1, BS 51), and the other clade is made up of *C. blepharophyllum* var. *amplexicaule* (Hoell & Nordal 134) and *C. blepharophyllum* var. *blepharophyllum* (Chapano et al. 1846) (PP 1, BS 92).

Scanning electron microscopy (SEM)

In total, 11 accessions were photographed by SEM. Seed shape and testa of accessions that are congruent with the findings of Meerts & Bjorå (2012) are not shown, apart from the seed of *Chlorophytum pusillum* (Schmidt 2532) (fig. 5A), used for comparison of the two different seed forms that were recorded for *C. pusillum*. Accessions from Benin has a folded seed shape and a seed testa ornamentation that display distinct papilla (fig. 5B), in contrast to the *C. pusillum* seed in fig. 5A. Here, the seed is relatively flat with a more or less flattened seed testa ornamentation. One of the *C. geophilum* accessions show both a rounded and a more angled seed testa ornamentation (fig. 5C).

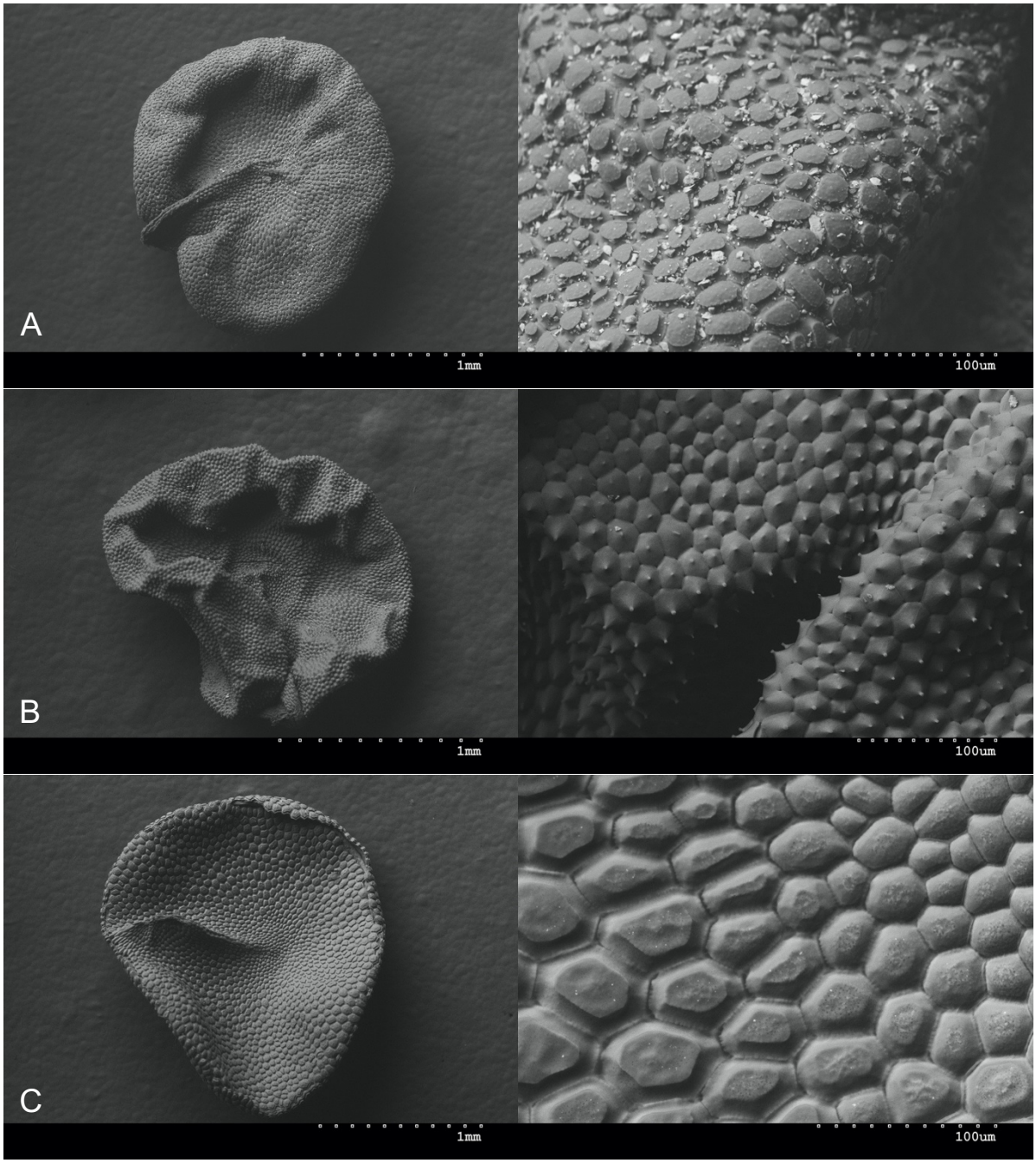


Figure 5 – Scanning electron micrographs of seed testa and shape. **A**. Seed characteristics of *C. pusillum* congruent with Meerts & Bjorå (2012), here represented by *C. pusillum* (Schmidt 2532). **B**. Seed shape and testa of an *C. pusillum* accession from Benin. Here represented by *C. pusillum* (Akoègninou 3590). **C**. *Chlorophytum geophilum* (Peter 35704) show variation of seed testa within one seed.

DISCUSSION

Monophyly of the prostrate species with condensed inflorescence in *Chlorophytum* is not supported (fig. 4), suggesting that the prostrate habit and reduced peduncle are analogous characters. This might be an ecological adaptation to avoid attention by herbivores (Inger Nordal, pers. com.), and has arisen several times independently.

Chlorophytum geophilum and *C. pusillum* have been regarded as very closely related (Meerts & Bjorå 2012) and difficult to separate from each other (Nordal 1997; Kativu 2008; Meerts 2015). Both taxa are relatively small plants raising only a few centimeters above the ground. Their inflorescence is dense and the leaves are prostrate, oblanceolate and glabrous (Nordal et al. 1997). However, the two taxa display clear differences regarding their root characteristics: *C. pusillum* have proximately swollen roots with elongated tubers, while *C. geophilum* have narrow roots with distal tubers. Differences can also be seen in their leaves. The leaves in *C. geophilum* are petiolate in contrast to the broad-based, membranaceous leaves present in *C. pusillum* (Nordal et al. 1997; Kativu et al. 2008). These morphological differences correspond with the phylogenetic analyses where the two taxa clearly make up separate clades (fig. 4) and are not as closely related as earlier treatments suggested.

Previous studies has shown that morphological similar taxa within *Chlorophytum* can be distinguished by their seed characteristics, as in the case of separating *C. macrophyllum* Asch. and *C. clarae* Bjorå & Nordal which previously were assumed to be conspecific (Bjorå 2008). Obtained SEM photographs show that seed characteristics seem to have little diagnostic value when it comes to distinguishing between the prostrate species in this study. However, the SEM photographs shows that the seed testa ornamentation of *C. geophilum* can vary from a rounded pattern to a more angled ornamentation (fig. 5C). This has not been previously recognized. Another interesting observation is that the SEM photography revealed that two Beninese *C. pusillum* accessions have very distinct seed shape and testa ornamentation (represented by *C. pusillum* (Akoègninou 3590) in fig. 5B). These characteristics strongly deviates from the seeds of *C. pusillum* (Schmidt 2532) (fig. 5A) and *C. pusillum* (Billiet & Jadin 4151) presented in Meerts & Bjorå (2012, fig. 6B p. 401). Both of the West African *C. pusillum* accessions are morphologically similar to *C. pusillum*, but the dissimilarity seen in the seeds indicate that these accessions might represent not yet understood diversity. In addition, *C. pusillum* (Akoègninou 3531) also lack the swollen proximal roots, which is a character used to key *C. pusillum* (Nordal et al. 1997; Kativu et al. 2008; Meerts 2015). Based on these findings I strongly recommend to sample more material, especially from West Africa, to include in future phylogenetic analyses.

The monophyly of *Chlorophytum stenopetalum* is not supported (fig. 4A, B) as indicated by Nordal et al. (1997). Morphological examination of the *C. stenopetalum* accessions reveals that they somewhat deviate from the flora circumscriptions of the taxon by having an elongated peduncle. This, in addition to resolving as non-monophyletic suggests that the name *C. stenopetalum* is poorly understood. This may have led to lumping of morphological similar specimens into the *C. stenopetalum* concept, where the morphological similarities probably are due to convergent evolution rather than a common ancestor. The circumscription of this taxon

can therefore be questioned. To know the right application of the name, material from the type locality or area should be included. Unfortunately this was not possible to obtain for this study. Even though no material from the type locality of *C. stenopetalum* is included in the current molecular analyses, one can still argue that there is little molecular support for recognizing the variety of *C. stenopetalum* defined by Meerts & Bjorå (2012). In the present molecular analyses *C. stenopetalum* var. *latifolium* (Hoell & Nordal 20) make a up well-supported clade with *C. geophilum* accessions in the nDNA tree (fig. 4A, clade C2) which clearly does not support a close relationship to *C. stenopetalum*.

The division of the *Chlorophytum geophilum* accessions into two clades (C1 and C2, fig. 4A), made it necessary to study their morphological features. Closer inspection of the accessions revealed that there are morphological characters that do distinguish the two clades. Clade C1 display crisped leaf margins, while clade C2 display smooth margins. In addition, available field photographs and herbaria material reveal that members in the C2 clade have dominating bracts with white margins. In the material available, these dominating bracts were not seen for the members in clade C1. As there are both molecular and morphological characters that separate two clades C1 and C2, it is reasonable to assume that they represent two different taxa. Their overlapping distribution argues against recognizing them as subspecies or varieties as these terms normally implies geographical separation (Jonsell 2004). I therefore prefer to treat these two clades as separate species. Characters found in clade C2 are consistent with the protologue of *C. latifolium*. Careful comparisons with the *C. latifolium* type specimen supported by my molecular analyses, makes it reasonable to reestablish *C. latifolium*. Reinstating *C. latifolium* consequently leads to a wider distribution of the taxon, which previously was restricted to Zambia.

The morphological and molecular variation within *Chlorophytum latifolium* (fig. 4A, clade C2) is interesting. Both of the accessions from Zambia, *C. geophilum* (Hoell & Nordal 26) and *C. stenopetalum* var. *latifolium* (Hoell & Nordal 20), both have broad-based leaves and a cylindrical inflorescence (fig. 6A, B), while accessions *C. geophilum* (Hermann 36) and *C. geophilum* (Wilde 3139A), from Ethiopia and Cameroon respectively, are petiolate and has a more globous inflorescence (not shown) – traits they have in common with *C. geophilum* accessions in clade C1. The smooth leaf margin however, is a trait that remains constant throughout the C2 clade. Figure 7 provides a detailed drawing of the difference in leaf margins and shape regarding *C. geophilum* and *C. latifolium*. There is certainly a need for more studies to fully comprehend both the variation within *C. latifolium* as well as determining the distribution of the taxon.

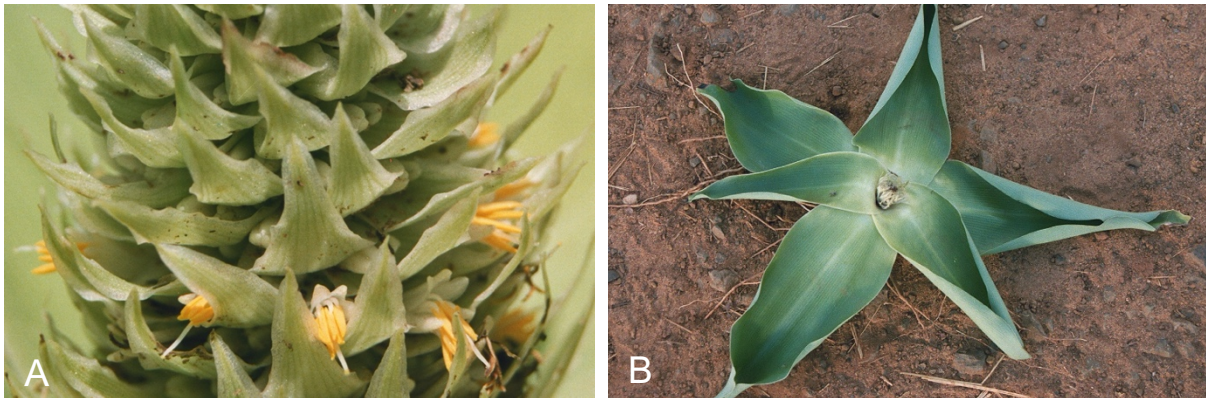


Figure 6 – **A**. The cylindrical inflorescence of *C. latifolium* sensu Nyrud (2020) with large dominating bracts almost covering all the individual flowers. Here the white margins on the bracts are clearly visible. **B**. *Chlorophytum latifolium* sensu Nyrud (2020) with broad-based, non-petiolate leaves. Photographs: Gry S. Hoell.

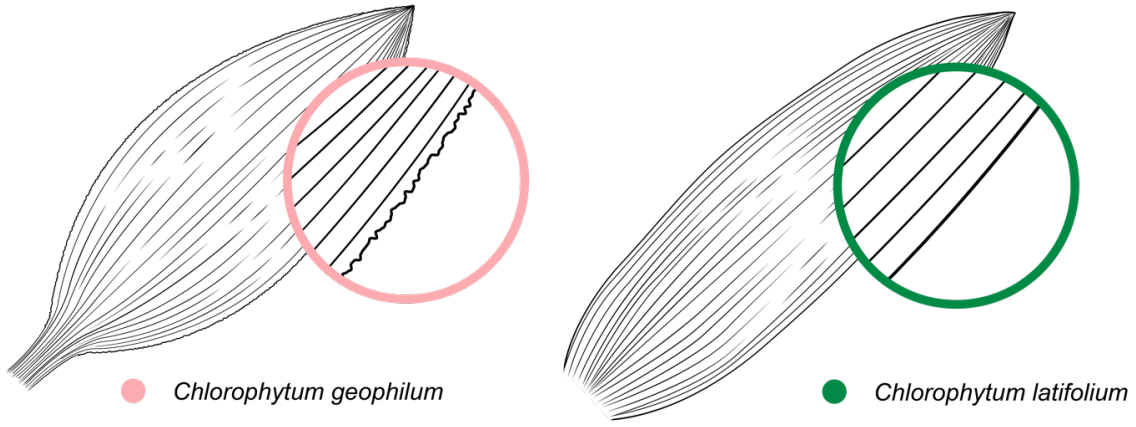


Figure 7 – Detailed drawing of crisped and smooth leaf margins found in clade C1 and C2 respectively (see fig. 4A). Crisped leaf margins is a trait found in *C. geophilum*, while smooth leaf margins seems to be a character that applies to *C. latifolium*. Petiolate leaves can also appear in clade C2 (not depicted). Illustration by Petter Tangen.

The monophyly of the accessions from the *Chlorophytum blepharophyllum* complex (clade A) is highly supported in both the nuclear and plastid phylogenies. In accordance with Meerts & Bjorå (2012) there is no support for the conclusion of Kativu et al. (2008) to recognize *C. amplexicaule* at a species rank nor *C. blepharophyllum* ssp. *rubropygmaeum*. Results from the molecular analyses in this study does not support the current understanding of the *C. blepharophyllum* complex as there is no obvious internal structure within clade A (fig. 4) that supports the recognition of the two varieties within the complex. The interpretation of the variation seen in this complex seems not to be fully understood. This study does, however, not provide sufficient material to make any certain species delimitations within this complex. It is therefore of vital importance to collect and include more samples and further investigate the species delimitation within the *C. blepharophyllum* complex.

Lastly, an identification key is provided to help distinguish between all the prostrate species in *Chlorophytum*. The key is based on all material available.

Key to prostrate species with compressed inflorescence in *Chlorophytum*

1. Leaves more than 6 times longer than broad; inflorescence narrow and cylindrical……..……..……..*C. stenopetalum*

   Leaves less than 4 times longer than broad; inflorescence broadly cylindrical to sub-glabrous……...…..……2

2. Pedicels apparently not articulated or articulated at the apex; leaves green; fruit as wide as long……..……..3

   Pedicels distinctly articulated; leaves reddish; fruit longer than wide…….....*C. blepharophyllum* var. *amplexicaule*

3. Roots swollen proximately, thin and branched distally; leaves non-petiolate……..…….*C. pusillum*

   Roots fibrous with distal tubers; leaves petiolate or not.…….…….…….4

4. Leaves petiolate with crisped, hairy margins; bracts not dominating with hairy margins……..……...……*C. geophilum*

   Leaves usually not petiolate, margins smooth and not crisped; large bracts with a distinct white smooth margins……..……..……..*C. latifolium*

By applying the key produced in this study, in addition to my molecular analyses, I was able to identify what I believe are misinterpreted herbaria sheets from both Meise Botanic Garden (BR), Naturalis (WAG) and Addis Ababa University (ETH). Appendix 1 provides an overview.

CONCLUSION

This study has reestablished *Chlorophytum latifolium* at species level as well as identifying bracts and leaf margin as important informative characters to discriminate *C. latifolium* from *C. geophilum*. The present study also show that the distinction between *C. geophilum* and *C. pusillum* is supported by both molecular and morphological evidence. However, the monophyly of *C. stenopetalum* remains unsupported and the variation within the *C. blepharophyllum* complex is still not fully comprehended. Moreover, this study has identified an interesting observation regarding variation in seed characteristics of *C. pusillum* accessions, which might indicate hidden diversity. Further studies with extensive collections of the prostrate species of *Chlorophytum* are strongly encouraged.

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Appendix 1

Table A1 – Overview of herbaria sheets I believe are misinterpretations. The table provides where the specimens are obtained, collector, locality, taxon and what it should be corrected to, as well as the reason why it should be corrected.

Herbarium	Collected by	Locality	Taxon	Corrected to	Reason
ETH	Hermann 36	Ethiopia	*C. geophilum*	*C. latifolium*	Leaf margins not crisped. Present molecular analyses.
WAG	Mrs. J. Ash 539	Ethiopia. Sandy soil, volcanic rocks. In a group on bare earth between chumps of grass. Alt.: 1768.	*C. geophilum*	*C. latifolium*	Leaf margins not crisped.
WAG	R. Letouzey 6347.	Cameroon, Maroua. In shade, on sandy clay soil. Savanna.	*C. geophilum*	*C. latifolium*	Leaf margins not crisped.
WAG	W. J. J. O. de Wilde and B. E. E. de Wilde-Duyfjes 3139A	Cameroon, 10 km S of Dogba. Alt.: 500 m. In moist sandy soil, in shade, among boulders.	*C. geophilum*	*C. latifolium*	Leaf margins not crisped. Present molecular analyses
WAG	R. K. Brummit and R. M. Polhill 13711	Zambia, Northern Province, Mbala district. Alt.: 1560 m. Brachystegia-Uapaca woodland.	*C. geophilum*	*C. pusillum*	Non-petiolate leaves, crisped margins, no distal tubers. Might be proximal tubers.
WAG	D. K. Harder 2681.	North Western Zambia. Along Kifubwa river in forest and on shallow soils in woodland.	*C. geophilum*	*C. latifolium*	Non-petiolate leaves, margins not crisped, long cylindrical inflorescence.
BR	Oumorou and Lejoly 605.	Benin.	*C. pusillum*	*C. geophilum*	Petiolate leaves, distal tubers.
BR	A. Noirfalise 748.	Dem. Rep. Congo. Parc National de la Garamba. Grass savanna	*C. pusillum*	*C. geophilum*	Petiolate leaves, no proximal tubers, crisped margins