Daru, B.H. et al. (2013). Molecular and morphological analysis of subfamily Alooideae (Asphodelaceae) and the inclusion of Chortolirion in Aloe.
Taxon, 62(1): 62 – 76

Molecular and morphological analysis of subfamily Alooideae (Asphodelaceae) and the inclusion of *Chortolirion* in *Aloe*

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Abstract

Asphodelaceae subfam. Alooideae (Asparagales) currently comprises five genera, four of which are endemic to southern Africa. Despite their importance in commercial horticulture the evolutionary relationships among the genera are still incompletely understood. This study examines phylogenetic relationships in the subfamily using an expanded molecular sequence dataset from three plastid regions (*matK*, *rbcLa*, *trnH-psbA*) and the first subunit of the nuclear ribosomal internal transcribed spacer (ITS1). Sequence data were analysed using maximum parsimony and Bayesian statistics, and selected morphological traits were mapped onto the molecular phylogeny. *Haworthia* is confirmed as being polyphyletic, comprising three main clades that largely correlate with current subgeneric circumscriptions. *Astroloba* and *Gasteria* are evidently each monophyletic and sister respectively to *Astroloba* and *H.* subg. *Robustipedunculares*. *Chortolirion* is shown to be deeply nested within *Aloe* and is formally included in that genus. *Aloe* itself is clearly polyphyletic, with the dwarf species *A. aristata* allied to *Haworthia* subg. *Robustipedunculares*. The taxonomic implications of these findings are examined but branch support at critical lower nodes is insufficient at this stage to justify implementing major taxonomic changes.

Introduction

Asphodelaceae subfam. Alooideae (sensu APG II, 2003; Aloaceae sensu Smith & Steyn, 2004) are an Old World group comprising some 500 species characterised by more or less distinctly succulent leaves, often with prickly or toothed margins, and a markedly bimodal karyotype with the basic chromosome number $x = 7$ (Taylor, 1925; Smith & Van Wyk, 1998). Representatives of the subfamily also share some chemical characters, notably the presence of anthrone-C-glycosides in their leaves and 1-methyl-8-hydroxyanthraquinones in their roots (Smith & Van Wyk, 1998).

Modern taxonomy of the group begins with Linnaeus (1753), whose rather heterogeneous concept of *Aloe* L. encompassed several Asparagalian taxa with more or less tubular flowers and leathery or succulent leaves. Of the 16 names included by him in the genus, four are not members of Alooideae (three are now in *Sansevieria* Thunb. and one in *Kniphofia* Moench).

The remainder, grouped by flower size and shape, are currently segregated among *Aloe* (four species), *Gasteria* Duval (one species), *Astroloba* Uitewaal (one species) and *Haworthia* Duval (five species). Linnaeus's (1753) preliminary grouping of the alooid species was subsequently formalised, first at sectional level within *Aloe* by Salm-Dyck (1836–1863) and later by the recognition of the segregate genera *Gasteria*, defined by the moderately large, curved and often gasteriform flowers, and *Haworthia*, with very much smaller, whitish flowers (Duval, 1809). *Haworthia* was further split when those species with more or less actinomorphic flowers were separated from those with bilabiate flowers into the small genus *Astroloba* (Uitewaal, 1947). Three additional small genera have since been recognised, namely *Chortolirion* A. Berger, *Lomato-phyllum* Willd. and *Poellnitzia* Uitewaal but the current classification of the subfamily (reviewed in Klopper & al., 2010) includes *Lomatophyllum* in *Aloe*, and *Poellnitzia* in *Astroloba*, thus retaining the five genera *Aloe*, *Astroloba*, *Chortolirion*, *Gasteria* and *Haworthia* with a wide range of distinguishing features (Table 1).

Aloe, with approximately 400 species, is by far the largest genus in Alooideae and also the most widespread (Reynolds, 1966; Viljoen, 1999; Glen & Hardy, 2000; Klopper & Smith, 2007). It is distinguished from other Alooideae genera by many features including morphology, growth form and distribution (Table 1).

Gasteria with 23 species (all endemic to South Africa) resembles *Aloe* in its tubular, reddish flowers but is distinguished from most *Aloe* species by its inclined racemes of pendulous, curved flowers sometimes swollen at the base (Table 1). *Poellnitzia rubriflora* L. Bolus, from the Western Cape of South Africa, was recently transferred to *Astroloba* (as *A. rubriflora* (L. Bolus) Gideon F. Sm. & J.C. Manning) by Manning & Smith (2000) due to its close vegetative similarity to some species of *Astroloba*, with which it also shares similar lipophilic anthronoid aglycones (Manning & Smith, 2000). The species was essentially distinguished from *Astroloba* by its inclined racemes of secund, orange-red flowers with connivent tepals, apparently an adaptation to sunbird pollination (Manning & Smith, 2000). The six species of *Astroloba* are all endemic to the Western and Eastern Cape of South Africa (Smith, 1995a; Manning & Smith, 2000). *Astroloba* is vegetatively very similar to some species of *Haworthia* and the two genera are distinguished by floral symmetry: the flowers of *Astroloba* are actinomorphic with tepals spreading at the tips while those of *Haworthia* are more or less bilabiate.

Table 1. Comparison of major distinguishing features of currently recognised genera of Alooideae.

Currently recognised Alooideae genera	Number of species	Distinguishing features	Distribution	References
Aloe L.	ca. 400	Growth habit: trees, shrubs and stemless, sometimes geophytic perennials Leaves: tough, spiked or toothed margins with astringent/unpalatable juice, sunken stomata, distichous leaf arrangement, surface (smooth to verrucose) Flowers: vivid yellow to red, sometimes bicoloured, size (small to large), campanulate to tubular or gasteriform, radially symmetrical to bilabiate Seeds: flattened, wind-dispersed, sometimes fleshy	Africa, Arabian peninsula, Madagascar, islands in western Indian Ocean	Reynolds (1966), Viljoen (1999), Glen & Hardy (2000), Klopper & Smith (2007)
Astroloba Uitewaal	7	Growth habit: caulescent; multi-stemmed Leaves: hard, spirally arranged, pungent Flowers: tubular, orange-red, suberect, included anthers, secundly arranged on inclined racemes, actinomorphic, connivent tepals	Western and Eastern Cape of South Africa	Smith (1995a); Manning & Smith (2000)
Chortolirion A. Berger	3	Growth habit: acaulescent Leaves: grass-like, bulb-like swelling of leaf bases, weakly armed with small, white marginal teeth Flowers: small, bilabiate, included anthers, white/cream/greenish	Grasslands of Angola, Botswana, Namibia and the summer rainfall areas of South Africa	Obermeyer (1973); Smith (1991, 1995a, b); Smith & Van Wyk (1993); Fritz (2012)
Gasteria Duval	23	Leaves: triangular in cross section, firm-textured, margins (horny but never spiny), dark green with bands of whitish spots Flowers: tubular, reddish, arranged on inclined racemes of pendulous, curved flowers sometimes swollen at the base (gasteriform)	Eastern Cape of South Africa, southern Namibia	Van Jaarsveld (2007)
Haworthia Duval	ca. 61	Growth habit: small, mainly acaulescent succulents (sometimes caulescent) Leaves: a rosette; size and shape variable Flowers: small, mostly bilabiate, included anthers, white/cream/greenish	Western and Eastern Cape, Mpumalanga and Limpopo Provinces of South Africa; Swaziland, Mozambique and Namibia	Bayer (1999, 2002)

Haworthia includes approximately 61 species plus numerous infraspecific taxa (Bayer, 1999, 2002). Most species are highly localised and largely restricted to the winter rainfall parts of South Africa, with outliers extending northwards into Mpumalanga, Swaziland, Mozambique and Namibia.

Chortolirion is a small genus of three acaulescent species from summer rainfall grasslands with grass-like leaves swollen at the base and weakly armed with small, white marginal teeth (Smith & Van Wyk, 1993; Smith, 1995b; Fritz, 2012). The flowers of *Chortolirion* species closely resemble those of *Haworthia* species and it was included in the latter in the past (Obermeyer, 1973), but has generally been retained as distinct on the basis of the bulb-like swelling of the leaf bases, and its distribution and habitat (Smith, 1991, 1995b; Smith & Van Wyk, 1991, 1993) (Table 1). In leaf anatomy, *Chortolirion* resembles the grass-like species of *Aloe* sect. *Leptoaloe* A. Berger (Smith & Van Wyk, 1993).

Phylogenetic relationships among and within the genera of Alooideae are still incompletely resolved. Current generic circumscriptions are based on floral characters, namely the size, symmetry, shape and colour of the perianth, supplemented in some instances by vegetative characters, phytochemistry, cytology and nectar sugar composition (Smith & Steyn, 2004; Klopper & al., 2010). *Aloe* itself is poorly defined and lacking in synapomorphies.

The last decade has seen the emergence of phylogenetic studies of nucleotide sequence data in assessing relationships within Alooideae (Adams & al., 2000; Chase & al., 2000; Treutlein & al., 2003a, b; Zonneveld & Van Jaarsveld, 2005; Ramdhani & al., 2011). Phylogenetic relationships in Asphodelaceae were first investigated by Chase & al. (2000), who analysed plastid DNA sequence data of a small sample of taxa, recovering a monophyletic Alooideae but demonstrating that Asphodeloideae were paraphyletic. Although this analysis placed *Haworthia* as sister to *Gasteria*, the limited taxon sampling prevented further analysis. Treutlein & al. (2003b), in their examination of a larger sample of species, using chloroplast nucleotide sequence data plus genomic DNA fingerprinting of Alooideae, inferred that *Haworthia* and *Aloe* are both polyphyletic as currently circumscribed. In their analysis, representatives of *Haworthia* subg. *Haworthia* formed a well-supported clade nested within *Aloe*, while *H.* subg. *Hexangulares* was placed in a separate clade that also included *Gasteria*, *Astroloba*, *Aloe aristata* and ×*Astroworthia* G.D. Rowley, a hybrid between *Astroloba* and *Haworthia* (Treutlein & al., 2003b). *Chortolirion* clustered with the grass-like species in *Aloe* sect. *Leptoaloe*, and *Lomatophyllum* was firmly nested within another unresolved clade of *Aloe*. A later study by Ramdhani & al. (2011) addressed phylogenetic relationships in the genus *Haworthia*, analysing relationships among 26 species from all three subgenera (*H.* subg. *Haworthia*, subg. *Hexangulares*, subg. *Robustipedunculares*) using DNA sequences from three gene regions. They confirmed the polyphyletic nature of *Haworthia* identified by Treutlein & al. (2003b). Sampling in both of these studies was still sparse in terms of number of species included, geographical coverage, and number of gene regions analysed.

Despite their shortcomings, most attempts at resolving relationships in Alooideae (e.g., Adams & al., 2000; Chase & al., 2000; Treutlein & al., 2003a, b; Zonneveld & Van Jaarsveld, 2005; Ramdhani & al., 2011) suggest that there is some degree of mismatch between current generic circumscriptions in the subfamily and available phylogenetic hypotheses. As yet, however, no adequately sampled or well-supported phylogenetic analysis exists on which to base an alternative classification.

In this study we use nucleotide sequences from the first sub-unit of the internal transcribed spacer (ITS1) of nuclear ribosomal DNA and three chloroplast regions (*matK*, *rbcLa*, *trnH-psbA*) to assess phylogenetic relationships and monophyly among the genera currently recognised within Alooideae. We include 150 taxa representing all five genera and 20 sections of *Aloe*, covering a wide range of vegetative and floral diversity in the subfamily (Fig. 1). We use these data to (1) examine phylogenetic relationships among the genera, (2) assess the monophyly of various groups within Alooideae, (3) evaluate the taxonomic value of diagnostic morphological traits, and (4) examine options for deriving a phylogeny-based classification based on reciprocally monophyletic taxa.

Materials and methods

Taxon sampling. — Representatives of all five accepted genera in subfamily Alooideae (including 150 taxa) were analysed for four gene regions, nuclear ITS1 and plastid *matK*, *rbcLa*, and *trnH-psbA*. We included 20 taxa of *Gasteria*, 68 of *Haworthia*, 57 of *Aloe* from 19 sections (including *Lomatophyllum*), four of *Astroloba* (including *Poellnitzia*) and one *Chortolirion* species in the analyses. Samples were collected from living material in private and national collections in South Africa (Sheilam Nursery, Robertson; Gariep Nursery, Pretoria; University of Johannesburg (JRAU); Kirstenbosch Botanical Gardens, Cape Town) (Appendix S1). Most of these accessions were originally wild-collected. Representatives of Anthericaceae (*Anthericum liliago* L.), Asphodelaceae: Asphodeloideae (*Asphodeline lutea* (L.) Rchb., *Bulbine fistulosa* (Chiov.) Baijnath, *B. frutescens* (L.) Willd., *B. semibarbata* (R. Br.) Haw., *Eremurus spectabilis* M. Bieb., *Kniphofia galpinii* Baker, *K. uvaria* (L.) Oken), Tecophilaeaceae (*Tecophilaea cyanocrocus* Leyb., *Zephyra elegans* D. Don), and Xanthorrhoeaceae (*Xanthorrhoea resinosa* Pers., *Xanthorrhoea* sp.) were selected as outgroups based on previous molecular and morphological studies within Asparagales (Smith & Van Wyk, 1991; Chase & al., 2000; Treutlein & al., 2003a, b; Devey & al., 2006). The outgroup samples were obtained from the DNA Bank at the Royal Botanic Gardens, Kew. Voucher specimen information and GenBank accession numbers are listed in Appendix S1. Taxonomic concepts in *Aloe*, *Gasteria* and *Haworthia* follow Glen & Hardy (2000), Van Jaarsveld (2007) and Bayer (1999), respectively.

DNA extraction, amplification and sequencing. — Total genomic DNA was extracted from either fresh or silica-gel dried leaf material using the 2× CTAB method described by Doyle & Doyle (1987). Polyvinyl pyrolidone (2% PVP) was added to reduce the effect of high polysaccharide concentrations in the samples. All samples were purified using QIAquick purification columns (QIAgen, Inc., Hilden, Germany) according to the manufacturer's protocol.

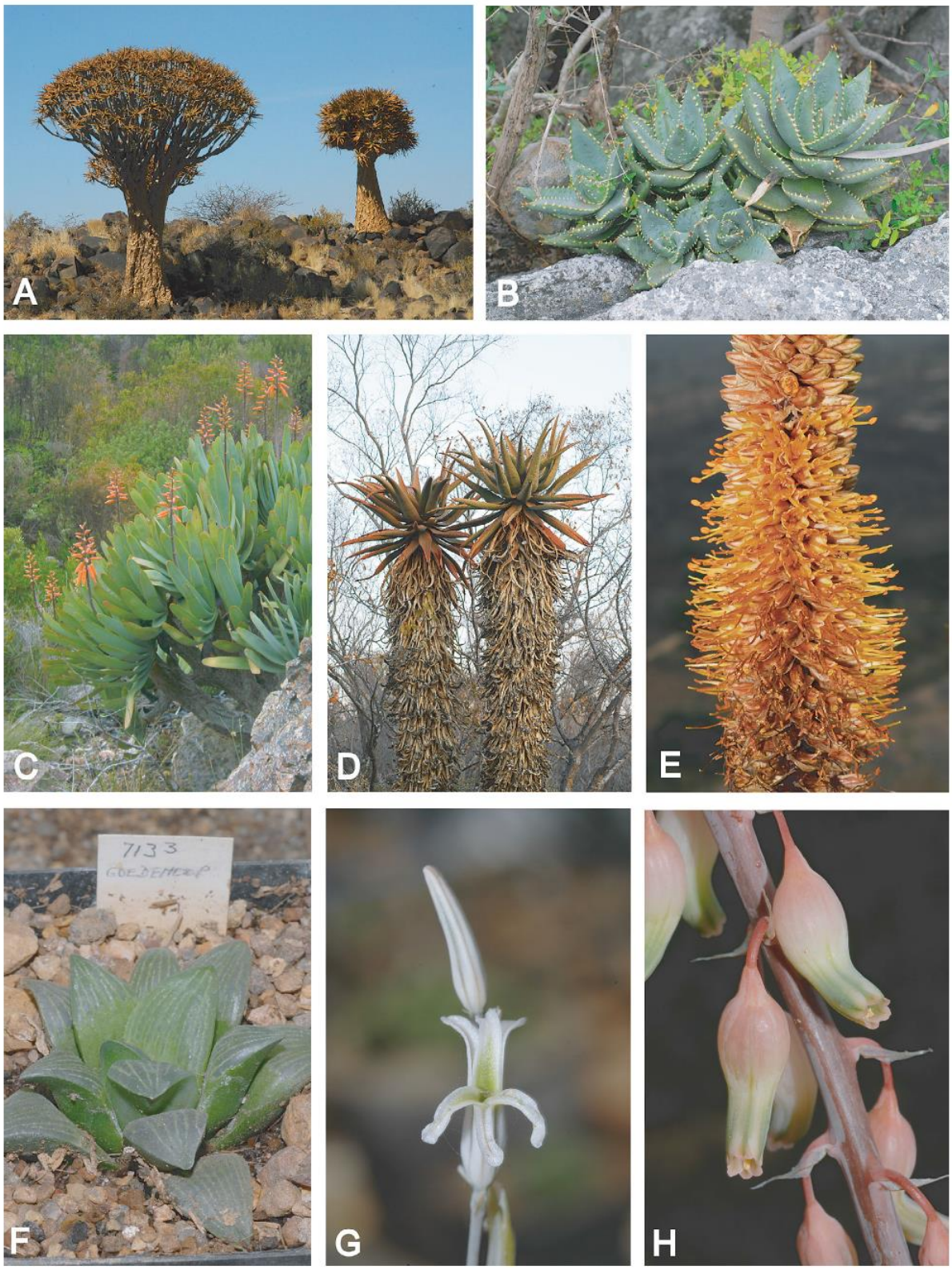


Fig. 1. Representatives of Asphodelaceae subfam. Alooideae showing variation in the subfamily. **A,** *Aloe dichotoma*; **B,** *A. perfoliata*; **C,** *A. plicatilis*; **D,** *A. ferox*; **E,** *A. spicata*, inflorescence; **F,** *Haworthia chloracantha*; **G,** *H. chloracantha*, inflorescence; **H,** *Gasteria obliqua*. — Photographs: A, B, D–H, courtesy O. Maurin; C, by S.R. Cousins.

Primer pairs used for the polymerase chain reaction (PCR) amplification of *matK*, *rbcLa*, and *trnH-psbA* regions were Kim Ki-Joong-3F and Kim Ki-Joong-1R (CBOL Plant Working Group, 2009), rbcLa-F and rbcLa-R (CBOL Plant Working Group, 2009), and psbAF and trnHR (Sang & al., 1997), respectively. The ITS1 was amplified using the primer combination ITS18-ITS5 (Treutlein & al., 2003a). The PCR amplification primers were also used as cycle sequencing primers.

PCR amplification for *matK* and *rbcLa* was carried out at the Canadian Centre for DNA Barcoding (CCDB), Biodiversity Institute of Ontario of the University of Guelph in Canada. Details of the project including voucher information, GPS coordinates, images and DNA barcodes are available on BOLD (http://www.boldsystems.org; Ratnasingham & Herbert, 2007) within the project file 'Alooideae of Africa' (ALOAF). Sequencing of ITS1 and *trnH-psbA* as well as some additional *matK* and *rbcLa* samples was carried out at the African Centre for DNA Barcoding (ACDB) at the University of Johannesburg in South Africa. All PCR amplifications were performed using ReadyMix Master mix (Advanced Biotechnologies, Epsom, Surrey, U.K.). Bovine serum albumin (3.2%) was added to both nuclear and plastid reactions, whereas 4.5% dimethyl sulfoxide (DMSO) was added only to *matK* and ITS1 PCR amplifications. These additives serve as stabilisers for enzymes, reduce problems caused by secondary structure and improve annealing (Palumbi, 1996). PCR amplification was performed using the following programs: for *rbcLa* and *trnH-psbA*, pre-melt at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 1 min (for 28 cycles), followed by a final extension at 72°C for 7 min; for *matK*, the protocol consisted of pre-melt at 94°C for 1 min, denaturation at 94°C for 30 s, annealing at 50°C for 40 s, extension at 72°C for 40 s (for 35 cycles), and a final extension at 72°C for 5 min. We selected ITS1 for phylogeny reconstruction because of its value in previous studies in the subfamily (e.g., Treutlein & al., 2003a, b; Ramdhani & al., 2011) and also because it has been routinely used to infer phylogenetic relationships at various infrageneric levels in other plant groups (Hillis & Dixon, 1991; Baldwin & al., 1995; Small & al., 2004). A preliminary PCR amplification of Alooideae using ITS2 was unsuccessful. The ITS1 protocol consisted of pre-melt at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 48°C for 1 min, extension at 72°C for 3 min (for 26 cycles), followed by a final extension at 72°C for 7 min. Prior to cycle sequencing, PCR products were visualised on a 1.5% agarose gel and subsequently purified using QIAquick (Qiagen Inc.) silica columns according to the manufacturer's protocol.

Cycle sequencing reactions for all genes used in this study were performed using ABI PRISM BigDye Terminator v.3.1 Cycle Sequencing Kits (Applied Biosystems, Inc., Foster City, California, U.S.A.). Cycle sequenced products were precipitated in ethanol and sodium acetate to remove excess dye terminators before sequencing on an ABI 3130x1 genetic analyser.

Phylogenetic analyses and tree construction. — Complementary strands were assembled and edited using Sequencher v.4.8 (Gene Codes Corp., Ann Arbor, Michigan, U.S.A.). The sequences were aligned using multiple sequence comparison by log-expectation (MUSCLE v.3.8.31; Edgar, 2004) and the alignment finally adjusted manually in PAUP* (v.4.0b.10; Swofford, 2002) without difficulties. This is because of low levels of

insertions/deletions except for the *trnH-psbA* region, from which 15.5% of the region was excluded from the analyses due to alignment difficulties at positions 1–24, 123–202, 272–290, 830–839 of the aligned matrix. The aligned matrices are available as supplementary data.

The separate datasets were assessed for congruence using partitioned Bremer support (DeSalle & Brower, 1997) with 1000 heuristic searches in the program TreeRot v.3 (Sorensen & Franzosa, 2007) in combination with PAUP* (v.4.0b.10; Swofford, 2002) to find the nodes at which support increases upon concatenating the data partitions or identify the sites of incongruence. This avoids constraining all gene regions to fit a single topology, especially if gene regions differ in evolutionary histories. Thus, we determined congruency using Bremer support indices (Bremer, 1988) generated from TreeRot v.3 (Sorensen & Franzosa, 2007). Based on the congruence test, we carried out phylogenetic analyses using maximum parsimony (MP) and Bayesian inference (BI) methods.

MP analyses were performed on the ITS1, combined plastid, and total combined datasets whereas BI was employed only on the total combined dataset. MP analyses were performed using PAUP* v.4.0b.10 (Swofford, 2002). Tree searches were conducted using 1000 random sequence additions, retaining ten trees at each step, with tree-bisection-reconnection (TBR) branch swapping and MulTrees in effect. The resulting trees were then used as starting trees in a second search with the same parameters but without a limit for the number of trees per replicate (swapping to completion), in order to see if the shortest trees were found in the previous analysis. Delayed transformation (DELTRAN) character optimisation was used instead of acceleration of transformation (ACCTRAN) for calculating branch lengths because of reported errors with version 4.0b.10 of PAUP* (http://paup.csit.fsu.edu/problems.html). Branch support was estimated using bootstrap analysis (Felsenstein, 1985) with 1000 replicates, simple sequence addition, TBR swapping, with MulTrees in effect saving ten trees per replicate. Only groups of greater than 50% bootstrap support (BS) were reported. The following arbitrary scale for evaluating BS was applied: weak (50%–74%), moderate (75%–84%) or strong (85%–100%). BI (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) was performed using MrBayes v.3.1.2. For each matrix (ITS1, *matK*, *rbcLa*, *trnH-psbA*) the most appropriate model was selected based on Akaike information criterion (AIC) implemented in MODELTEST v.3.06 (Posada & Crandall, 1998) (Table 2).

We used 2,000,000 generations with a sampling frequency of 200. Partition analysis was run for the combined dataset. The log-likelihood scores were plotted to determine the point of stationarity, and all trees prior to stationarity were discarded as the "burn-in" phase (1000 trees). All remaining trees were used to produce a 50% majority-rule consensus tree showing the frequencies (posterior probabilities or PP) of all observed bi-partitions. The following scale was used to evaluate the PPs: below 0.95, weakly supported; 0.95–1.0, strongly supported. To map the bootstrap values (BS) and PP values (MrBayes tree) onto the tree, the nexus tree file from the BI analysis was rescaled using "ape" v.2.0-1 (Paradis & al., 2004) and "adephylo" v.1.1 (Jombart & Dray, 2010) packages implemented in R (R Development Core Team, 2011).

Coding of morphological characters. — A matrix of 20 morphological characters was prepared for the 150 taxa of Alooideae and outgroups included in the analyses. Most taxonomic studies in Alooideae identified these diagnostic characters (often at species level) to infer relationships within the subfamily (Reynolds, 1966, 1969; Jeppe, 1969; Bayer, 1982, 1999, 2002, 2009; Van Jaarsveld, 1992, 1994, 1998, 2001; Van Jaarsveld & al., 1994; Glen & Smith, 1995; Mössmer & al., 1995; Smith, 1995a, b; Smith & al., 1995; Meyer & Smith, 1998, 2001; Glen & Hardy, 2000; Van Wyk & Smith, 2003; Van Jaarsveld & Van Wyk, 2004, 2005, 2006; Smith & Steyn, 2005; Germishuizen & al., 2006; Gildenhuys, 2007; Klopper & Smith, 2007). These characters were scored as present or absent in a 1/0 matrix as indicated in Appendix S2. The patterns of evolution of these characters were examined by reconstructing them onto the majority-rule consensus tree produced by the BI analysis using Mesquite v.2.75 (Maddison & Maddison, 2011). Morphological characters and character-states are defined in Appendix S2 and the data matrix used for character reconstructions is presented in Appendix S3.

Results

Comparison of sequence partitions. — The statistics from the MP analyses for the single plastid analysis, combined plastid (*matK*+*rbcLa*+*trnH-psbA*), ITS1 and the total combined dataset are shown in Table 2. ITS1 had a significantly higher number of variable sites (40%) compared to the plastid regions combined (henceforth called plastid dataset). The number of potentially parsimony-informative characters for the plastid dataset within Alooideae (14%) is much lower than for ITS1 (22%). The average number of changes per variable site for ITS1 (2.1 changes per variable site; retention index RI = 0.92, and consistency index CI = 0.64) is higher than in the plastid dataset (1.6 changes per variable site; RI = 0.91; CI = 0.72). Analysis of each of the three plastid regions (*matK*, *rbcLa*, *trnH-psbA*) resulted in trees that were similar in topology. Trees resulting from the following analyses are presented: simplified tree topologies of the combined plastid regions (*matK*+*rbcLa*+*trnH-psbA*, Fig. 2A), ITS1 (Fig. 2B) and combined plastid and nuclear (*matK*+*rbcLa*+*trnH-psbA*+ITS1; Fig. 2C); combined plastid regions (*matK*+*rbcLa*+*trnH-psbA*) (Fig. S1), ITS1 (Fig. S2) and combined plastid and nuclear regions (*matK*+*rbcLa*+*trnH-psbA*+ITS1; Fig. 3).

Combined plastid data. — Individual plastid nucleotide sequence analyses (results not shown) were topologically consistent (negligible to zero incongruence), and for the purpose of the results and discussion were combined and treated as a single dataset. The statistics for MP analysis for the combined plastid data is presented in Table 2. From the heuristic search, we found 661 most parsimonious phylogenetic trees of which one is presented in Figure S1. Alooideae are supported as a monophyletic group, but resolution in the rest of the tree is low. Some groups in *Aloe*, *Haworthia* subg. *Haworthia*, *H.* subg. *Robustipedunculares* and *Gasteria* were moderately supported.

ITS data. — Summary statistics for the ITS1 data matrix is presented in Table 2. Analysis of the nuclear dataset also retrieved a monophyletic Alooideae, although there is some incongruence with the plastid data, it allows us to identify five major Alooideae groupings (Fig. S2) with moderate to strong support but poor resolution within each group: *Haworthia* subg. *Haworthia*, *Aloe* sect. *Macrifoliae*, *A.* sect. *Kumara*, a polytomy including the 'tree

Aloe' species plus *Aloe* s.str. (including *Chortolirion*), and a clade comprising *Astroloba*, *Gasteria*, *Haworthia* subg. *Hexangulares* and subg. *Robustipedunculares*, and *Aloe aristata*.

Table 2. Statistics from ModelTest and MP analyses obtained from separate and combined datasets.

	matK	*rbcLa*	*trnH-psbA*	Combined plastid	ITS1	Combined plastid+ITS1
No. of taxa included	172	172	157	172	156	172
No. of included characters	789	552	768	2109	409	2518
No. of constant characters	531	472	645	1648	244	1914
No. of variable sites	258 (33%)	80 (14%)	123 (16%)	461 (22%)	165 (40%)	604 (24%)
No. of parsimony informative sites	165 (21%)	55 (10%)	70 (9%)	290 (14%)	92 (22%)	376 (15%)
No. of trees (Fitch)	408	523	101	661	314	5020
No. of steps (Tree length)	408	120	194	752	346	1159
Consistency index	0.75	0.74	0.74	0.72	0.64	0.63
Retention index	0.92	0.91	0.92	0.91	0.92	0.89
Average number of changes per variable site (number of steps/number of variable sites)	1.6	1.5	1.6	1.6	2.1	1.9
Model selected by Akaike information criterion	TVM+G	HKY+G	TVM+I+G	TVM+I+G	TRN+G	GTR+I+G

Combined plastid and nuclear dataset. — Although the partitioned Bremer support test indicated some incongruencies between the nuclear and plastid datasets, visual inspection of the separate analyses (Fig. 2) shows that none of the strongly supported clades were mutually incompatible, and tests for incongruence have been shown to be of variable reliability (Reeves & al., 2001; Yoder & al., 2001). Based on the evident congruence between the two datasets, we therefore combined all data (Seelanan & al., 1997; Wiens, 1998) (Fig. 3). Statistics for the phylogenetic framework of the concatenated dataset is summarised in Table 2. A monophyletic Alooideae was recovered with high support (Fig. 3; BS = 93; PP = 1.00). The combined dataset resolved eight major groupings represented in different colours and capital letters in Figure 3: (A) the tree *Aloe* species, (B) *Aloe plicatilis*, (C) *Aloe* sect. *Macrifoliae*, (D) *Haworthia* subg. *Haworthia*, (E) the 'true' *Aloe* species, (F) the 'Haworthioid' clade (*H.* subg. *Robustipedunculares*+*Astroloba*+*Aloe aristata*), (G) *Haworthia* subg. *Hexangulares* and (H) *Gasteria*.

The 'tree aloes'. — The analysis retrieves most of the tree *Aloe* species (*A. barberae*, *A. dichotoma*, *A. eminens*) as a clade with strong support in the BI analysis (BS = 76; PP = 1.00). This lineage constitutes the earliest diverging elements in subfamily Alooideae (Fig. 3B). The remaining tree species, *A. plicatilis*, occupies an isolated position in an unresolved polytomy with *A.* sect. *Macrifoliae* and *Haworthia* subg. *Haworthia*.

***Haworthia* subg. *Haworthia*.** — This clade is well supported in both MP and BI analyses (BS = 98; PP = 1.00; Fig. 3B). The grass-like *Haworthia blackburniae* is resolved as sister to a well-supported (BS = 1.00; PP = 1.00) but internally unresolved clade including the other members of the subgenus.

The 'rambling aloes'. — This group, representing *Aloe* sect. *Macrifoliae* (*A. ciliaris*, *A. commixta*, *A. gracilis*, *A. striatula*, *A. tenuior*) was recovered in both analyses as a strongly supported clade (BS = 95; PP = 1.00; Fig. 3B).

The 'true aloes'. — This group, which comprises the majority of *Aloe* species plus *Chortolirion* (Fig. 3A) was retrieved with strong support in the BI (PP = 1.00). Several internal clades, none of which correspond exactly to current sections recognised in the taxonomy of the genus, were well supported in the BI analysis. Among these, the majority of the 'grass aloes', *A.* sect. *Leptoaloe*, grouped together (BS = 89; PP = 1.00) in a clade that included *Chortolirion angolense* (Baker) A. Berger, *A. kouebok-keveldensis*, *A. buhrii*, *A. lutescens*, *A. reynoldsii*, *A. spicata*, *A. striata* subsp. *komaggasensis* and *A. striata* subsp. *striata* albeit with weak BI support (PP = 0.60). In the MP tree this clade was recovered as part of a larger polytomy with no bootstrap support. A second clade comprised several of the 'single-stemmed aloes' (*Aloe* sect. *Pachydendron*) plus other species, with weak MP support (BS = 61) but strong support in the BI analysis (PP = 1.00). A third clade comprising a heterogenous assemblage of *Aloe* species (*A. excelsa*, *A. petricola*, *A. munchii*, *A. chabaudii*, *A. vryheidensis*) was well supported in the BI (BS = 53; PP = 0.99). A fourth clade recovered another heterogenous assemblage of *Aloe* species containing the type of the genus, *A. perfoliata*, with weak to moderate support (BS = 53; PP = 0.92). Two additional 'grass aloes', *A. albida* and *A. chortoliriodes*, were recovered as sisters with strong support (BS = 90; PP = 1.00). The remaining 'true aloes' are largely unresolved.

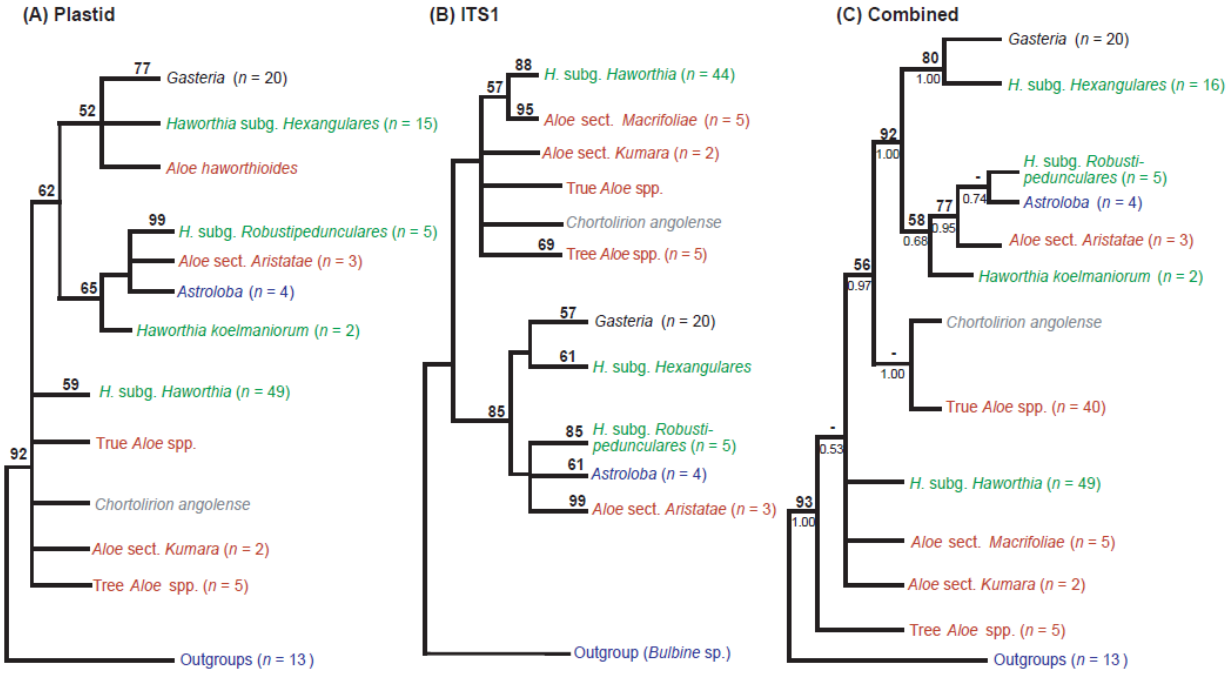
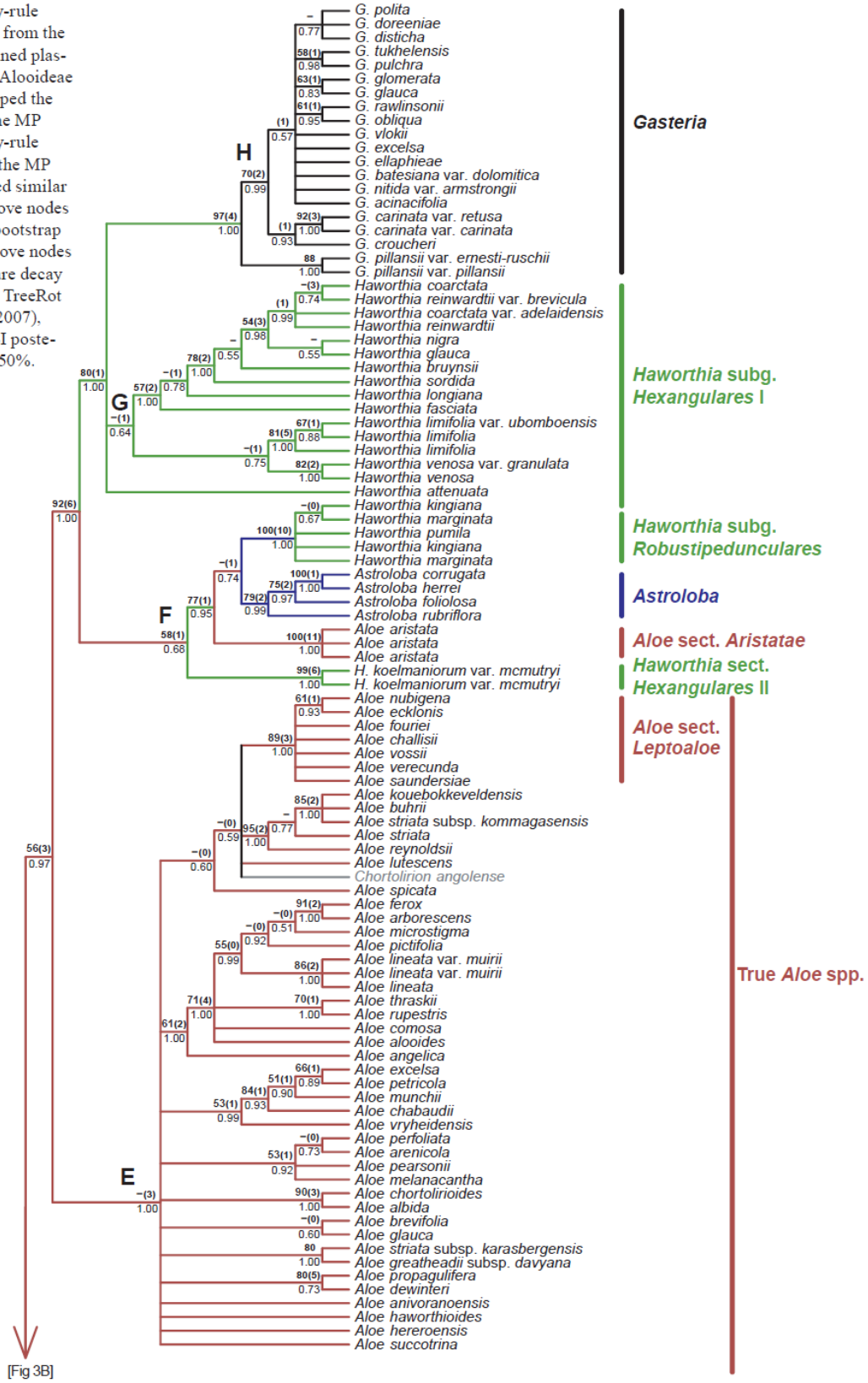


Fig. 2. Cladograms summarising relationships of major taxonomic groupings within Alooideae. Numbers above the branches are MP bootstrap support values and ones below are BI posterior probabilities.

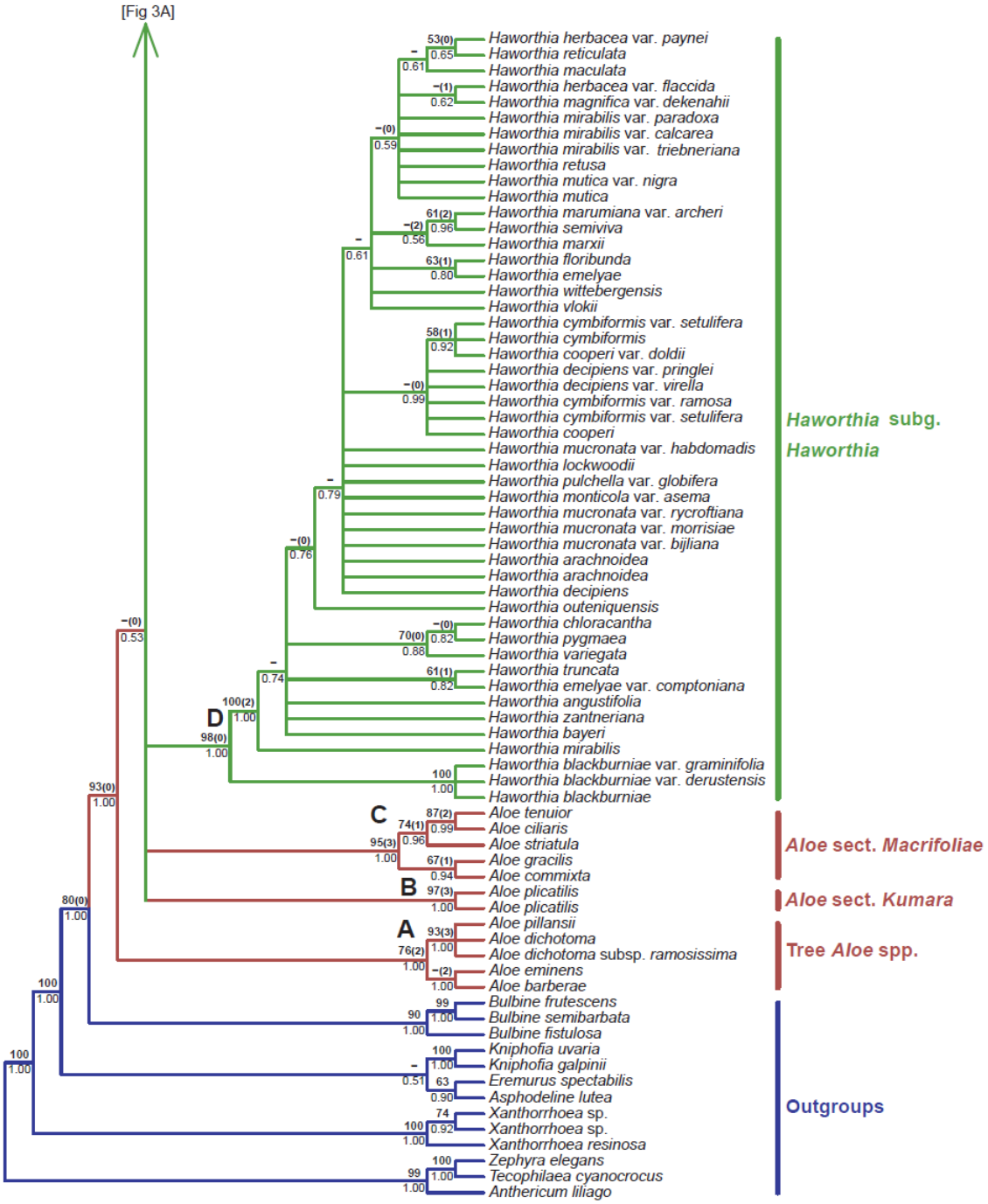
Fig. 3. The 50% majority-rule consensus tree obtained from the BI analysis of the combined plastid and ITS1 dataset for Alooideae plus outgroups. We mapped the bootstrap values from the MP tree onto the BI majority-rule consensus tree because the MP and BI analyses produced similar topologies. Numbers above nodes are nonparametric MP bootstrap support values, those above nodes but within parentheses are decay indices calculated using TreeRot (Sorensen & Franzosa, 2007), those below nodes are BI posterior probabilities above 50%.



'Haworthioid' clade (*Haworthia* subg. *Robustipedunculares*, *Astroloba*, *Aloe aristata*, *H. koelmaniorum*). — A moderately supported 'haworthioid' clade (BS = 58; PP = 0.68) was retrieved as sister to the *Gasteria-Hexangulares* clade with strong support (BS = 92; PP = 1.00). *Haworthia koelmaniorum* var. *mcmurtryi* (*H.* subg. *Hexangulares*) was retrieved with weak MP and BI support (BS = 58; PP = 0.68) as the earliest-diverging lineage in the clade. There is moderate to strong support (BS = 77; PP = 0.95) for a sister relationship between *A. aristata* and *H.* subg. *Robustipedunculares*+*Astroloba*. The remaining species of *H.* subg. *Robustipedunculares* were recovered with strong support (BS = 100; PP = 1.00) but are only weakly supported in the BI (PP = 0.74) as sister to a moderate to well supported (BS = 79; PP = 0.99) *Astroloba*.

***Haworthia* subg. *Hexangulares*.** — *Haworthia* subg. *Hexangulares* (but excluding *H. koelmaniorum*; Fig. 3A) was recovered in both MP and BI analyses with moderate to strong support (BS = 80; PP = 1.00) in a clade with *Gasteria*. Relationships among *H. attenuata*, the rest of *H.* subg. *Hexangulares* and *Gasteria* remain unresolved.

***Gasteria*.** — *Gasteria* species form a well-supported clade in both analyses (BS = 97; PP = 1.00; Fig. 3A). The clade is recovered in all trees as one element of a moderately to well supported trichotomy (BS = 80; PP = 1.00) that includes the species of *Haworthia* subg. *Hexangulares*.



Evolution of morphological traits. — All 20 morphological traits that were analysed are shown to be homoplasious, namely habit, leaf insertion, leaf margins, leaf maculation and tuberculation, leaf apex, inflorescence branching and orientation, inflorescence length relative to pedicel, inflorescence shape and colour, perianth orientation at anthesis, flower arrangement on peduncle, perianth symmetry and colour, perianth shape and curvature, pedicel length, tepal connation, and stamen length (Appendix S3). The distribution of nine characters of particular importance in generic and infrageneric classification within Alooideae is summarised in Table 3 (see also Figs. S4–S6).

Habit. — Acaulescence is plesiomorphic within Alooideae, with certain lineages characterised by secondary caulescence and arborescence (Fig. S3A). Caulescence has developed several times in the subfamily, including *Aloe* itself (notably *A.* sect. *Macrifoliae*) and in some of the segregate genera, notably *Astroloba* and some species of *Gasteria* and *Haworthia* subg. *Hexangulares*. Arborescence is uncommon in the subfamily and is characteristic of species in *Aloe* sect. *Aloidendron* and *A.* sect. *Dracoaloe*, including *A. eminens* (Fig. S3A).

Leaf insertion. — Polystichous leaf insertion is common within the subfamily but distichous leaf insertion is rare, occurring several times in *Aloe*, once in *Haworthia* and possibly once in *Gasteria* (Fig. S3B).

Leaf tuberculation. — The presence of white tubercles on the leaves is largely restricted to *Gasteria* and *Haworthia* subg. *Hexangulares* and subg. *Robustipedunculares* but tubercules are also developed in some *Aloe* species (e.g., *Aloe aristata*, *A. haworthioides*, *A. verecunda*) and in *Chortolirion* (Fig. S3C).

Table 3. Character states for nine selected traits which have been employed in previous Alooideae systematics.

Character	Character state	Species
Growth habit	Stemless	Most *Gasteria*; half of *Haworthia* subg. *Hexangulares*; *H.* subg. *Robustipedunculares*
	Caulescent	*Gasteria obliqua*; half of *Haworthia* subg. *Hexangulares*; all *Astroloba* (including *Poellnitzia*)
	Arborescent	*Aloe plicatilis* (*A.* sect. *Kumara*); *A.* sect. *Pachydendron* and *A.* sect. *Dracoaloe*
Leaf insertion	Spiral/polystichous	Most Alooideae
	Distichous	*Gasteria obliqua*; *Aloe nubigena*, *A. plicatilis*, *A. verecunda* and *Haworthia truncata*
Leaf maculation	Immaculate	Most Alooideae
	Maculate	All *Gasteria*; several *Aloe* species, e.g., *A. aristata* and *A.* sect. *Leptoaloe*
Flower orientation at anthesis	Spreading	*Chortolirion*, *Astroloba*, *Haworthia* and a few *Aloe*
	Pendulous	Most *Aloe* and *Gasteria* species
Perianth symmetry	Actinomorphic	*Haworthia* subg. *Robustipedunculares* and several *Aloe*, e.g., *A. albida*
	Weakly bilabiate	*Gasteria* species
	Strongly bilabiate	*Haworthia* subg. *Haworthia* and subg. *Hexangulares* plus *Chortolirion angolense*
Perianth curvature	Straight	*Astroloba*, *Chortolirion*, *Haworthia* subg. *Hexangulares* and subg. *Robustipedunculares* and majority of *Aloe*
	Curved	*Gasteria*; *Haworthia* subg. *Haworthia*
Perianth	Cylindrical and tapering into pedicel	Most *Aloe*; *Astroloba*; *Chortolirion*; *Haworthia* subg. *Haworthia* and subg. *Hexangulares*
	Inflated basally or truncated	*Gasteria* and several *Aloe* species
Stamen inclusion	Exserted	Most *Aloe* species
	Inserted	*Chortolirion*, *Gasteria* and the three *Haworthia* subgenera
Tepal connation	Both whorls connate basally	The three *Haworthia* subgenera; *Astroloba* and some *Aloe* species
	Both whorls connate 1/2 or more of length	*Gasteria* and some *Aloe* species
	Only outer whorls connate 1/2 or more of length	A few *Aloe* species

From our analysis (not shown), vivid yellow, orange or reddish flowers are clearly plesiomorphic within Alooideae. However, *Chortolirion*, *Haworthia* and *Astroloba* (excluding *Poellnitzia*) and several species of *Aloe*, especially in *A.* sect. *Leptoaloe*, are characterised by whitish or greenish perianth.

The *Haworthia*-type flower. — Usually white and sometimes with strongly zygomorphic perianths, and inserted anthers are characteristic of the genus *Haworthia* and *Chortolirion*. Character reconstruction suggests that this flower type is homoplasious, and that it has evolved independently at least three and possibly four times (once each in *Chortolirion* and *H.* subg. *Haworthia* and once or twice in *H.* subg. *Hexangulares*/subg. *Robustipedunculares* with possible reversals in *A. aristata*, *A. haworthioides* and *Gasteria*) (Fig. S4A–C).

The *Gasteria*-type flower. — Flask-shaped flowers with inflated bases occur not only in *Gasteria*, but also in many *Aloe* species and have evidently evolved several times (Fig. S5A), although within *Aloe*, the stamens are often but not always exserted (Fig. S5B). Similar flowers are characteristic also of *Astroloba* (≡ *Poellnitzia*) *rubriflora* but here are uniquely held erect on an inclined raceme. A well-developed perianth tube formed by the fusion of both tepal whorls has evolved independently in *Gasteria*, *Astroloba* and *Aloe kouebokkeveldensis* (Fig. S5C).

Tepal connation. — The perianth in Alooideae comprises six tepals in two whorls, variously connate into a short or prominent tube. Basally connate tepals represent the plesiomorphic condition. In several of the ornithophilous *Aloe* species, however, the outer tepal whorl is connate in the basal half, forming a distinct perianth tube (Fig. S5C). Flowers with both whorls connate for half or more of their length are diagnostic for *Gasteria*, *Astroloba* (*Poellnitzia*) *rubriflora*, and *A. koue-bokkeveldensis* and appear to have evolved independently in these three lineages from ancestral types with tepals connate at the base only (Fig. S5C).

Discussion

Relationships within Alooideae. — The monophyly of Alooideae was first demonstrated by Chase & al. (2000) using the two plastid regions *rbcL* and *trnL-F*, and more recently by Treutlein & al. (2003a, b), using *matK* and genomic fingerprinting. This finding is strongly supported by our study, using significantly greater taxon sampling and a combined analysis of three plastid regions (*matK*, *rbcLa*, *trnH-psbA*) plus the nuclear region ITS1. Although the monophyly of the subfamily is no longer in question, this cannot be accepted for all of the genera. It has become increasingly clear that neither *Aloe* nor *Haworthia* are monophyletic as currently circumscribed (Treutlein & al., 2003a, b; Ramdhani & al., 2011). Our analysis of the combined plastid and nuclear DNA datasets (Fig. 3) confirms that both *Aloe* and *Haworthia* are polyphyletic as currently circumscribed. We identify eight primary monophyletic lineages in Alooideae that largely correspond to the following currently recognised generic and infrageneric groups: (1) *Aloe* sect. *Dracoaloe* + *A.* sect. *Aloidendron*; (2) *A.* sect. *Kumara*; (3) *A.* sect. *Macrifoliae*; (4) *Haworthia* subg. *Haworthia*; (5) *A.* sect. *Aristatae* + *H.* subg. *Robustipedunculares* + *Astroloba*; (6) *H.* subg. *Hexangulares*; (7) *Gasteria*; (8) remaining

species of *Aloe*. This topology is congruent with that of Treutlein & al. (2003b), which had much reduced sampling. Among the smaller segregate genera, both *Astroloba* and *Gasteria* are monophyletic but *Chortolirion* is deeply embedded among true *Aloe* species.

Seven of the eight primary lineages are endemic to southern Africa, with only *Aloe* s.str. extending beyond the region. This suggests that the early diversification of the subfamily took place in the subcontinent, which is also the main centre of distribution for the subfamily (Smith & Van Wyk, 1998).

Relationships of the 'tree aloes'. — Our analysis does not retreive the 'tree aloes' sensu Van Wyk & Smith (2003) as a monophyletic group but as two separate lineages. *Aloe* sect. *Aloidendron* + *A.* sect. *Dracoaloe* (including *A. eminens* from Somalia) emerges as possibly one of the early-diverging lineages within the subfamily (Fig. 3B). The immediate relationships of the remaining tree *Aloe*, *A. plicatilis* (*A.* sect. *Kumara*) are unresolved.

Relationships of *Aloe* sect. *Macrifoliae*. — *Aloe* sect. *Macrifoliae*, the 'rambling aloes' (sensu Van Wyk & Smith, 2003), comprising five closely related species (*A. ciliaris*, *A. commixta*, *A. gracilis*, *A. striatula*, *A. tenuior*), is recovered as a strongly supported clade but its relationship to the remaining species in the subfamily remains unresolved. The section is defined vegetatively by its cane-like stems, and slender, sheathing, unspotted and mesophytic leaves with minute marginal teeth, and florally by the more or less entirely connate outer tepals (Glen & Hardy, 2000).

Relationships within the 'true aloes'. — The remaining species of *Aloe* (excluding *A. aristata*) are retrieved as a clade but relationships among them are poorly resolved. Although some of the currently recognised sections may be monophyletic, others are not, and a much more extensive sampling of species and additional gene regions is required to evaluate taxonomic and evolutionary relationships among them.

Although *Chortolirion* is deeply embedded within this group, as part of a clade including most of the grass *Aloe* species, its precise relationships are still unclear. Close morphological similarity to species such as *A. bowiea* and *A. inconspicua* in vegetative parts, namely the grass-like leaves with bulb-like swelling, and in the small, bilabiate flowers, suggest a close relationship to part of *A.* sect. *Leptoaloe*.

Relationships of *Gasteria*. — *Gasteria* forms a strongly supported clade sister to *Haworthia* subg. *Hexangulares*. It is defined by several morphological synapomorphies, notably the unarmed, verrucose leaves and inclined secund inflorescences of pendulous, gasteriform flowers with a well-developed floral tube (Van Jaarsveld, 2007), and was unsurprisingly one of the earliest segregates of *Aloe* to be recognised. Our phylogenetic analysis places the genus sister to *Haworthia* subg. *Hexangulares*, which includes species with remarkably similar leaves. The unique *Gasteria*-type flowers are most parismoniously interpreted as a reversion to bird-pollination from the entomophilous *Haworthia*-type flower

with included stamens. It is hardly surprising then that *Gasteria* flowers are not precisely matched by any bird-pollinated flowers in *Aloe*.

Relationships of *Astroloba*. — Species of *Astroloba* are retrieved as a clade sister to *Haworthia* subg. *Robustipedunculares*. The genus is morphologically defined by its caulescent habit with stiff, imbricate leaves, and small, actinomorphic flowers with included stamens.

Relationships within *Haworthia*. — Our analysis accords with previous studies indicating that *Haworthia* is not monophyletic but rather represents three lineages corresponding to the three subgenera proposed by Bayer (1976, 1999). Species of *H.* subg. *Haworthia* comprise a strongly supported clade, defined morphologically by the basally triangular perianth, obclavate flowers and upcurved style (Bayer, 1976, 1999). Additional support for this alliance comes from Smith & al. (2001), who reported the occurrence of hexose-poor nectar (less than 50% sucrose equivalents) in *H.* subg. *Haworthia* in contrast to hexose-rich nectar (more than 60% sucrose equivalents) in *H.* subg. *Hexangulares* and *H.* subg. *Robustipedunculares*. The latter is a small group of four species that is well-supported as monophyletic and sister to *Astroloba*. It is defined morphologically by its more or less straight perianth abruptly joined to the pedicel (Bayer, 1976, 1999). The flower type found in *H.* subg. *Robustipedunculares* is not dissimilar to that in *Astroloba*, differing essentially by its slight zygomorphy. Members of *H.* subg. *Robustipedunculares* are often robust with attenuate leaves, often scabrid and patterned with white tubercles (Bayer, 1999). A sister relationship between *Astroloba* and *H.* subg. *Robustipedunculares* is supported by similarities in nectar sucrose concentrations (Van Wyk & al., 1993).

The dwarf *Aloe aristata* is sister to *H.* subg. *Robustipe-dunculares*+*Astroloba*. This morphologically unusual species is unique in *Aloe* in having "*Haworthia*-like" leaves with dry, awn-tipped apices and white tubercles and distinctive, downcurved flowers with basal swelling (Glen & Hardy, 2000). The close vegetative similarity between this species and some members of *H.* subg. *Robustipedunculares* is consistent with a close relationship between them, and the primary difference between the two groups is evidently the large, orange flowers of *A. aristata*. This flower type is associated with bird pollination and has arisen several times within the subfamily. Alone, it is therefore not necessarily an indication of relationships.

Unlike the other two subgenera of *Haworthia*, subg. *Hexangulares* is possibly polyphyletic with the inclusion of *H. koelmaniorum*, which occupies an isolated position sister to *H.* subg. *Robustipedunculares*+*Astroloba*+*Aloe aristata* but with only weak support. The geographical distribution of this species is well north of most other species of *Haworthia* with the exception of *H. limifolia*. When first described (Obermeyer, 1967), *H. koelmaniorum* was treated in *H.* sect. *Margaritifera* (now *H.* subg. *Robustipedunculares*) but later transferred to *H.* subg. *Hexangulares*, where its relationships appear to lie with *H. limifolia* (Bayer, 1999). Further evidence for a final decision on its position is required. Species of *H.* subg. *Hexangulares* display the largest vegetative diversity in *Haworthia*, with some species closely resembling members of *Astroloba* and *H.* subg. *Robustipedunculares* in their vegetative morphology, especially the presence of tubercles on the leaves.

Evolution of selected characters in Alooideae. — Our reconstruction of the nine morphological characters onto the BI majority-rule consensus tree revealed that none is unique to a single clade identified in this study.

Habit. — Previous classifications (e.g., Reynolds, 1966; Holland, 1978) are implicit in treating arborescence in *Aloe* as a derived state. Brandham (1983) proposed that scandent *Aloe* species with usually relatively mesophytic leaves, e.g., *A. tenuior* and *A. ciliaris* (A. sect. *Macrifoliae*), represent the primitive state in *Aloe* species but Smith & Van Wyk (1991) argued that both small, highly succulent taxa and arborescent forms were derived from a mesophytic, comparatively acaulescent taxon. Our analysis supports this hypothesis, indicating that arborescence in *Aloe* is found not only in early diverging lineages but also in others deeply embedded within *Aloe*, and that the small, stemless grass *Aloe* (A. sect. *Leptoaloe*) are derived.

Leaf insertion. — Distichy is evidently the juvenile condition, present in all *Aloe* and *Gasteria* seedlings, and its persistence in adult plants is best interpreted as neoteny.

Leaf tuberculation. — Tuberculation is certainly a derived condition, as hypothesised by Smith & Van Wyk (1991). In *Gasteria*, Van Jaarsveld (1994) has proposed that its evolution was driven by the absence of the bitter constituent typical of *Aloe* species, implying that the rigid tubercles may make the leaves less palatable.

Perianth colour. — The bright yellow, orange or reddish flowers typical of most Alooideae are strongly associated with ornithophily. The whitish or greenish perianth characteristic of *Chortolirion*, *Haworthia, Astroloba* (excluding *Poellnitzia*) and *Aloe* sect. *Leptoaloe*, appears to be a derived adaptation to entomophily (Botes & al., 2008; Hargreaves & al., 2008).

The *Haworthia*-type flower. — Small, spreading flowers with a whitish, more or less bilabiate perianth and included anthers are diagnostic of *Haworthia* and *Chortolirion* (Bayer, 1999). Bayer (1976) identified small floral differences in the three subgenera of *Haworthia*, which is consistent with the independent evolution of this flower type in this genus. The convergence in this flower syndrome in *Chortolirion* provides clear evidence that such a flower type can evolve independently from an ornithophilous ancestor. Relatively short-tubed, whitish or cream-coloured flowers in some *Aloe* species such as *A. inconspicua* have been shown to be an adaptation to insect pollination (Botes & al., 2009). Although still recognisably '*Aloe*-like', the flowers of bee-pollinated *Aloe* species (sensu Botes & al., 2009) such as *A. linearifolia* and *A. minima* display several characteristics of the *Haworthia*-type flower apart from reduced size, namely their nearly horizontal orientation, whitish and weakly bilabiate perianth, and sometimes included stamens. Smith & Van Wyk (1991) suggested that floral zygomorphy in *Astroloba, Chortolirion* and *Haworthia* represents an advanced state derived from the plesiomorphic actinomorphic pattern, which is supported by our analysis.

The *Gasteria*-type flower. — The so-called gasteriform flower, curved and flask-shaped with an ovoid, inflated tube at least half as long as the perianth, and included or shortly

exserted stamens, is characteristic of the genus *Gasteria*. The flowers are also often bicoloured, with greenish tips to the tepals, and are borne secund on inclined racemes. This unique flower type possibly represents an adaptation to bird-pollination from an insect-pollinated ancestor.

Tepal connation. — Floral syndromes indicate that *Astroloba* (≡ *Poellnitzia*) *rubriflora, Gasteria* and several *Aloe* species are bird pollinated and our reconstruction suggests that ornithophily in *Gasteria* and *Astroloba* are secondary adaptations from an ancestral entomophilous, *Haworthia*-type flower rather than derived from a more typical *Aloe*-type flower.

It is increasingly evident that the differences in floral morphology that were used as the primary characteristic justifying the recognition of these various lineages as segregate genera represent syndromes associated with shifts in pollination systems from bird to insect and back. Historically, most of the segregate genera were erected before information on the full variation in *Aloe* was known, and certainly before the significance of floral syndromes in pollination was appreciated. In African Iridaceae in particular, where numerous specialist pollination systems have been documented, several erstwhile 'genera' have been shown to represent artificial associations of species based on floral characters associated with pollination systems (Goldblatt & Manning, 2006).

Implications for taxonomy. — Our results are essentially congruent with those of earlier systematic studies (e.g., Treutlein & al., 2003a, b; Ramdhani & al., 2011). The current classification of the subfamily into five genera (see review by Klopper & al., 2010) has been informed by evidence from cladistic studies of morphological traits (Smith & Van Wyk, 1991; Smith & Steyn, 2004; Klopper & al., 2010), as well as cytological (Taylor, 1925) and chemical data (Viljoen & al., 1998; Viljoen, 1999). These studies, although unable to resolve the relationships within the subfamily, provided a working hypothesis for this study.

Treutlein & al. (2003b) described four possible 'scenarios' to deal with the results of their preliminary phylogenetic analysis of Alooideae. These are summarised here for convenience.

1. *Scenario 1.* – The phylogenetic tree represented a gene tree. This is highly unlikely to apply to our combined analysis, which is based on four genes (three plastids and one nuclear region).

2. *Scenario 2.* – Retain the status quo. This scenario requires the acceptance of paraphyly in both *Aloe* and *Haworthia*. As Treutlein & al. (2003b) rightly point out, the current subfamilial classification does not reflect available evidence from phylogenetic analyses, and the further it departs from the phylogenetic evidence the more difficult it will be to integrate practice and theory.

3. *Scenario 3.* – A taxonomic 'splitter's' approach through the recognition of additional smaller genera within Alooideae in order to retain all or most of the currently accepted genera

as monophyletic. Although we are now in possession of a well-sampled tree, the poor support along the backbone places the same constraints upon us. Until these basal nodes are better supported we remain unable to propose an alternative classification. *Aloe aristata* is clearly misplaced with the other aloes and our current analysis suggests that it may be necessary to recognise an additional three genera from within *Aloe* in order to maintain currently recognised genera, with the exception of *Chortolirion*. It is now unquestionable that *Chortolirion* is deeply embedded within *Aloe* and cannot be retained without major and unprecedented fragmentation of *Aloe*. We therefore formally include it within *Aloe* as a separate section.

Branch support is, however, adequate to argue for the recognition of two additional genera for *Haworthia* subg. *Hexangulares* and *H.* subg. *Robustipedunculares*.

Our sampling of *Aloe* includes a large proportion of the morphological and geographical variation in the genus, notably among the southern African taxa where the early radiations appear to have occurred, and we regard it as unlikely that the inclusion of additional unusual species will necessitate the recognition of further genera.

3. *Scenario 4*. – A taxonomic 'lumper's' approach, in which all members of Alooideae are included in a single large genus *Aloe*. In this scenario additional infrageneric taxa are necessary to reflect the morphological data and the results of the phylogenetic analyses. Although unwilling to adopt this solution formally, Treutlein & al. (2003b) proposed informal taxonomic groupings that indicate unequivocally that they favour this solution. Their articulated objection to adopting it turns on the practical difficulties of implementing a hierarchical classification should the evolution of the subfamily be shown to be reticulate. The high congruence between the plastid and nuclear trees, especially at the lower nodes, in our analysis is a clear indication that reticulate evolution is not a significant problem at these levels. Although Ramdhani & al. (2011) have suggested that hybridisation is rife within *Haworthia*, due to the high levels of incongruence observed between the plastid and the nuclear trees, their study utilised multiple accessions within a genus in which species boundaries are notoriously uncertain. Their conclusions therefore have little bearing on the issue here. This option achieves maximum nomenclatural stability but the information content is reduced at the generic level, although the recognition of infrageneric taxa at the level of subgenera and sections will retain this.

On morphological grounds there is little to preclude implementing this option since *Aloe* already includes most of the variation evident in the subfamily. Branch support at the lower nodes in our analysis is not high enough to predicate the adoption of this option, and sequencing of additional gene regions might still group the tree and rambling aloes with the true aloes. Until a suitably well-supported topology is available on which to base a phylogenetic classification it seems best to refrain from major formal taxonomic and nomenclatural adjustments.

Nomenclature

Aloe sect. ***Chortolirion*** (A. Berger) Boatwr. & J.C. Manning, **stat. et comb. nov.** ≡ *Chortolirion* A. Berger in Engler, Pflanzenr. IV(38, III): 72. 1908 – Type: *Chortolirion angolense* (Baker) A. Berger [= *Aloe subspicata* (Baker) Boatwr. & J.C. Manning]

4. ***Aloe aestivalis*** Boatwr. & J.C. Manning, **nom. nov.** ≡ *Chortolirion latifolium* Zonn. & G.P.J. Fritz in Bradleya 28: 32 (figs. 4–6). 2010, non *Aloe latifolia* (Haw.) Haw. – Holotype: South Africa, [Free State], Bloemfontein near airport, 2009, *Fritz 1025* (PRE).

5. ***Aloe subspicata*** (Baker) Boatwr. & J.C. Manning, **comb. nov.** ≡ *Haworthia subspicata* Baker in Bull. Herb. Boissier, ser. 2, 4: 998. 1904 – Holotype: South Africa, [Gauteng], Modderfontein, 9 Sep 1897, *Conrath 645* (Z; isotype: K).
= *Haworthia angolensis* Baker in Trans. Linn. Soc. London, Bot. 1: 263. 1878 ≡ *Chortolirion angolense* (Baker) A. Berger in Engler, Pflanzenr. IV(38, III): 73. 1908, non *Aloe angolensis* Baker – Holotype: Angola, Huilla, Nov 1895, *Welwitsch 3756* (BM).

6. ***Aloe tenuifolia*** (Engl.) Boatwr. & J.C. Manning, **comb. nov.**
≡ *Haworthia tenuifolia* Engl. in Bot. Jahrb. Syst. 10: 2,
t. 1. 1888 ≡ *Chortolirion tenuifolium* (Engl.) A. Berger in Engler, Pflanzenr. IV(38, III): 73. 1908 – Holotype: South Africa, [Northern Cape], near Kuruman, Feb 1886, *Marloth 1049* (B; isotype: PRE).

Acknowledgements

We would like to thank Bruce M. Bayer, Sean Gildenhuys, Ernst van Jaarsveld, and the Molecular Systematics section of Jodrell Laboratory at the Royal Botanic Gardens, Kew (Laszlo Csiba and Felix Forest) for samples. The National Research Foundation (NRF) South Africa and the Royal Society of London are greatly acknowledged for funding. This project was also partly funded by the Government of Canada through Genome Canada and the Ontario Genomics Institute (2008-OGI-ICI-03). We appreciate two anonymous referees who provided useful comments on an earlier draft of the manuscript.

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