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Divergence Time Estimation of Aloes and Allies (Xanthorrhoeaceae) Based on Three Marker Genes

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Abstract: Aloes and allies are prominent members of African succulent vegetation and especially of the highly diverse Cape Flora. The main goal of this study was to obtain age estimates for alooids by calibrating a Bayesian phylogenetic analysis based on two chloroplast markers (the *trnL-trnF* spacer region and *rbc*L gene) and one gene marker (ITS) using a relaxed molecular clock. Seventy four species from all succulent genera of alooids were analysed with MrBayes to infer species relationships. We discuss the age estimates to address the question whether vicariance or dispersal could account for the diversification of Madagascan alooids. In the combined maximum clade credibility tree obtained from BEAST the succulent alooids have split from asphodeloids around 51.8 Mya in Early Miocene. Divergence time age estimation for succulent drought resistant alooids (late Oligocene to early Miocene) correspond well with dates identified for several other plant lineages in southern Africa and does match with the start of dry period in Miocene which triggered speciation and evolutionary radiation of these genera and families. All climbing aloes and some tree aloes which were recently split into new genera are amongst the early diverged group in alooids and the crown node of this group diverged around 16.82 (15.5–22.4) Mya. The oldest node age estimation for aloes from Madagascar (5.1 Mya) is in early Pliocene and our findings support the hypothesis that the Africa-Madagascan divergence is best explained by oceanic long-distance dispersal rather than vicariance. This study is one of the first to give age estimates for clades of alooids in Xanthorrhoeaceae as a starting point for future studies on the historical biogeography of this family of succulent plants which are important for ethnomedicine, and as ornamental and horticultural plants.

Keywords: Xanthorrhoeaceae; alooids; molecular phylogeny; divergence time; *rbc*L; *trnL_F*; ITS

1. Introduction

Alooids or the subfamily Alooideae sensu)Dahlgren, et al. 1985 ([1] are a well-known group of southern African and Cape rosette leaf succulents (ca. 530 species) adapted to life in dry areas; some members of *Aloe* occur on the Arabian Peninsula, Madagascar and the Mascarene Islands. Alooids are of interest from an ethnomedical, ornamental and horticultural perspective (Smith et al., 2000) [2]. *Aloe ferox* is the source of anthraquinones, which are used in medicine as a laxative. *Aloe vera* is also used as a laxative but also as a gel used for wound-healing, and skin care (van Wyk and Wink, 2017) [3]. In Africa, harvested species of *Aloe* come from east and southern Africa. During the 1990s, exports of wild-harvested exudate (Aloe) from Kenya sometimes exceeded 80 tons per annum (Oldfield, 2004) [4].

During the last 350 years, this group of plants has seen a number of diverse classifications. At the starting point of modern nomenclature, Linnaeus (1753) [5] had recognised a single genus *Aloe* to accommodate all aloes and close relatives. Until recently, six genera have been traditionally accepted:

Aloe L., *Astroloba* Uitewaal, *Gasteria* Duval, *Haworthia* Duval, *Chortolirion* A. Berger, and *Lomatophyllum* Willdenow (Smith and Van Wyk, 1998) [6]. Phylogenetic analyses have revealed a high degree of poly- and paraphyly among these genera (Treutlein et al., 2003; Ramdhani et al., 2011; Daru et al., 2012; Manning et al., 2014; Grace et al., 2015) [7–11]. *Lomatophyllum*, known as berried-aloes, includes around 14 species from Madagascar and some of the Mascarene Islands. This genus has recently been included in the genus *Aloe* [6]. Manning et al. (2014) [10] have confirmed the paraphyly of the former genus *Aloe* and suggested to split it into *Aloidendron* Klopper & Gideon F.Sm., *Kumara* Medik., *Aloiampelos* Klopper & Gideon F.Sm., *Aloe*, *Aristaloe* Boatwr. & J.C.Manning and *Gonialoe* (Baker) Boatwr. & J.C.Manning. The APG IV system (2016) [12] places *Aloe* and associated genera in the family Asphodelaceae, subfamily Asphodeloideae, one of the three subfamilies of the Xanthorrhoeaceae.

Alooids are characterised by rosulate and succulent leafs and synapomorphies like: Bimodal karyotype with four long and three short chromosomes, hemitropous ovules, a parenchymatous, cap like inner bundle sheath at the phloem poles, 1-methyl-8-hydroxyanthraquinones in the roots and anthrone-C-glycosides in the leaves (Treutlein et al., 2003) [7]. *Aloe* species are often pollinated by insects and birds but can also be autogamous. Moreover, the widespread occurrence of secondary growth might be added to these characters (Smith and Van Wyk, 1998) [6].

As the largest genus in the Asphodelaceae with approximately 530 species, *Aloe* has centers of diversity in southern Africa. It occurs widespread in Africa, Arabia, and on several island of the Western Indian Ocean Islands off the east coast of Africa, such as Madagascar, and Socotra (Klopper et al., 2010) [13]. The distribution of *Haworthia*, *Astroloba* and *Gasteria* are similar. The berry fruited *Lomatophyllum* is limited to Mascarene Islands.

Phylogenetic relationships within alooids have been analysed by sequencing a number of chloroplast and nuclear marker genes. These studies revealed that the genus *Aloe*, that includes *Lomatophyllum*, *Chortolirion* and *Haworthia*, appeared to be paraphyletic (Treutlein et al., 2003; Ramdhani et al., 2011; Daru et al., 2012; Manning et al., 2014; Grace et al., 2015) [7–11]. The complex generic relationships suggest reticulate evolution and multiple hybridization events (Viljoen, 1999; Ramdhani et al., 2011) [8,14]. Rapid speciation events are especially apparent in the polyphyletic genus *Haworthia* (Bayer, 1976, 1982, 1999; Treutlein et al., 2003; Ramdhani et al., 2011) [8,15–18].

With regards to species richness alooids are not alone in the Cape Floristic Region (CFR) (Linder et al., 1992; Sauquet et al., 2009) [19,20], which shows a high degree of endemism including 30% of the succulents plants of the world (Schnitzler et al., 2011) [21]. With the exception of the Karoo flora which diversified as a result of recent radiation during the late Miocene or Pliocene (Verboom *et al.*, 2003 [22], molecular phylogenies indicate that the radiation of several African plant lineages took place over much of the Neogene and had started earlier than the climatic changes in the late Miocene (Bakker et al., 2005; Schrire et al., 2003; Goldblatt et al., 2002) [23–25].

Although representatives of the subfamily Asphodeloideae (including Aloeae) are supposed to have been around since the early Cretaceous (Smith and Van Wyk, 1991) [26], only few dated phylogenies has been published for this diverse complex of succulent plants (Grace et al., 2015) [11].

In the current study, we have analysed nucleotide sequences of 77 taxa, comprising all genera of alooids and three genera of non-succulent Asphodeloids. These data are used to carry out an age estimation for the main clades of alooids (including a diversification of Madagascan aloes), using a "relaxed" molecular clock that permits variation of the molecular rate among lineage in two chloroplast markers (*trnL-trnF* spacer and *rbc*L) and one highly repeated nuclear ITS region. Since there are many different hypotheses including dispersal and extinction or vicariance and peripheral isolation in the speciation process of aloes, we investigated the hypothesis of vicariance vs. dispersal as explanations for the origin of Madagascan aloes.

2. Materials and Methods

2.1. Taxon Sampling

Data were compiled for 74 species from all succulent genera of alooids including *Lomatophyllum* (with three individuals each) and for all of these species new sequences for three gene regions were generated. Only three outgroups were additionally obtained from Genbank for non-succulent genera (asphodeloids which are the sister group of alooids) in the subfamily Asphodeloideae (family Xanthorrhoeaceae). Most of *Aloe* samples were collected from plants of wild provenance kept in the collection of Gariep Plants in Pretoria. Other genera came from the Botanical Garden of Heidelberg University and the Palmengarten in Frankfurt. Details of GenBank accession numbers and DNA voucher specimens which were deposited at IPMB (Heidelberg University) are presented in Table 1.

Table 1. Origins of samples. List of specimens, distribution of plant samples, morphological information, number in the herbarium of the Institute of Pharmacy and Molecular Biotechnology (IPMB) and GenBank accession numbers (from our own sequence analyses) listed in this order: *rbc*L, *trnL_F* and ITS. *Aloe* life forms are according to Carter et al. (2011) [27]: A = grass aloes, B = maculate aloes, C = stemless aloes (in small clumps, flower stems few-branched), D = stemless aloes (in small clumps, flower stems multi-branched), E = stemless aloes (in large clumps, flower stems few-branched), F = stemless aloes (in large clumps, flower stems multi-branched), G = pendulous or sprawling aloes, H = shrubby aloes (flower stems few-branched), I = shrubby aloes (flower stems multi-branched), J = tree aloes. Subgeneric classification of *Haworthia* is based on Bayer (1999) [17]: HA = subgenus *Haworthia*, HE = subgenus *Hexangulares* and RO = subgenus *Robustipendunculares*. Distribution abbreviations: SA = South Africa, Uga = Uganda, Mad = Madagascar, Mal = Malawi, Ken = Kenya, Yem = Yemen, Moz = Mozambique, Zim = Zimbabwe, Tan = Tanzania, Sud = Sudan, Eth = Ethiopia, Som = Somalia, Zam = Zambia, Bot = Botswana, Ang = Angola, Zan = Zanzibar, Swa = Swaziland, Eri = Eritrea, Nam = Namibia, Oma = Oman.

IPMB Number	Taxon	Distribution	*Aloe* Life Forms & *Haworthia* Subgenera	GenBank Accession Numbers
P4716	*Aloe arborescens* Mill.	SA	J	KF013362, KF013435, KF013255
P7612	*Aloe aculeata* Pole-Evans	SA	D	KF013363, KF013436, KF013256
P7613	*Aloe africana* Mill.	SA	J	KF013364, KF013437, KF013257
P7615	*Aloe aristata* Haw.	SA	F	AY323634, KF013438, KF013258
P7614	*Aloe amudatensis* Reynolds	Uga	B	KF013365, KF013439, KF013259
P7617	*Aloe barberae* Dyr.	SA	J	AJ512294, KF013444, KF013264
P8195	*Aloe bellatula* Reynolds	Mad	E	KF013367, KF013442, KF013262
P7624	*Aloe castellorum* J.R.I.Wood	Yem	C	KF013371, KF013447, KF013267
P8196	*Aloe capitate* Baker	Mad	D	AY323643, KF013448, KF013268
P7628	*Aloe ciliaris* Haw.	SA	H	AJ512287, KF013453, KF013273
P310	*Aloe conifer*a H. Perrier	Mad	C	AJ512303, KF013449, KF013269
P7630	*Aloe commixta* A. Berger	SA	H	KF013372, KF013450, KF013270
P7626	*Aloe chabaudii* Schönland	Zim	F	KF013374, KF013452, KF013272,
P7632	*Aloe confuse* Engl.	Tan	G	KF013375, KF013454, KF013274
P8197	*Aloe deltoideodonta* Baker	Mad	C	AJ512304, KF013459, KF013279
P7641	*Aloe elegans* Tod.	Eth	D	KF013381, KF013462, KF013282
P4567	*Aloe ellenbeckii* A. Berger	Som	B	KF013378, KF013457, KF013277
P8198	*Aloe erythrophylla* Bosser.	Mad	C	KF013382, KF013463, KF013283
P7642	*Aloe eminens* Reynolds & P.R.O.Bally	Som	J	KF013384, KF013465, KF013285
P7649	*Aloe fleurentiniorum* Lavranos & L.E. Newton	Yem	D	KF013388, KF013469, KF013289
P7654	*Aloe grandidentata* Salm-Dyck	SA	B	KF013389, KF013470, KF013290
P7653	*Aloe globuligemma* Pole-Evans	SA	F	KF013390, KF013471, KF013291
P7657	*Aloe helenae* Danguy	Mad	J	KF013392, KF013473, KF013293
P7658	*Aloe isaloensis* H.Perrier	Mad	I	KF013395, KF013476, KF013296
P7659	*Aloe juddii* van Jarssv.	SA	H	KF013396, KF013477, KF013297
P7662	*Aloe kilifiensis* Christian	Ken	B	KF013398, KF013480, KF013300
P7661	*Aloe karasbergensis* Pillans	SA	D	AJ522183, KF013481, KF013301
P317	*Aloe lavranosii* Reynolds	Yem	D	AY323647, KF013458, KF013278
P7666	*Aloe macroclada* Baker	Mad	C	KF013400, KF013483, KF013303

Table 1. *Cont.*

IPMB Number	Taxon	Distribution	*Aloe* Life Forms & *Haworthia* Subgenera	GenBank Accession Numbers
P7669	*Aloe megalacantha* Baker	Eth	I	KF013401, KF013484, KF013304
P7668	*Aloe marlothii* A.Berger	Bot	J	KF013402, KF013485, KF013305
P7667	*Aloe maculata* All.	SA	B	KF013404, KF013487, KF013307
P7674	*Aloe mudenensis* Reynolds.	SA	B	KF013405, KF013488, KF013308
P7675	*Aloe munchii* Christian	Zim	J	KF013406, KF013489, KF013309
P7676	*Aloe mzimbana* I.Verd. & Christian	Mal	E	KF013407, KF013490, KF013310
P327	*Aloe niebuhriana* Lavranos	Yem	E	AY323648, KF013491, KF013311
P7685	*Aloe pillansii* L.Guthrie	SA	J	AJ512292, KF013494, KF013314
P8202	*Aloe parallelifolia* H.Perrier	Mad	H	KF013408, KF013492, KF013312
P8193	*Aloe prostrata* (*H.Perrier*) *L.E.Newton & G.D.Rowley*	Mad	C	KF013361, KF013434, KF013254
P7693	*Aloe retrospiciens* Reynolds & P.R.O.Bally	Som	J	KF013410, KF013495, KF013315
P7697	*Aloe schweinfurthii* Baker	Sud	F	KF013412, KF013497, KF013317
P7702	*Aloe speciosa* Baker	SA	J	KF013413, HQ646844.1, KF013318
P7703	*Aloe spicata* L.f.	SA	J	KF013414, KF013499, KF013319
P7701	*Aloe somaliensis C.H.Wright ex W.Watson*	Som	D	AY323639, KF013501, KF013501
P331	*Aloe scobinifolia* Reynolds & P.R.O.Bally	Som	D	AJ512307, KF013502, KF013322
P345	*Aloe sinkatana* Reynolds	Sud	D	AJ512306, KF013503, KF013323
P7705	*Aloe striatula* Haw.	SA	H	KF013415, KF013500, KF013320
P7713	*Aloe tulearensis* T.A.McCoy & Lavranos	Mad	I	KF013420, KF013509, KF013329
P295	*Aloe vera* L.	NEA (North East Africa)	E	AJ290289.1, KF013511, KF013331
P4570	*Aloe viguieri* H.Perrier	Mad	G	KF013422, KF013512, KF013332
P7639	*Aloe vryheidensis* Groenew.	SA	J	KF013380, KF013461, KF013281
P7724	*Aloe yemenica* J.R.I.Wood	Yem	G	KF013426, KF013516, KF013336
P4635	*Haworthia blackburniae* W.F.Barker	SA	HA	AJ512300, HQ646793, KF013337
P8192	*Haworthia cymbiformis* Duval	SA	HA	AJ512296, KF013517, KF013338
P4587	*Haworthia mirabilis* Haw.	SA	HA	AY323618, KF013518, KF013339
P8189	*Haworthia tortuosa* Haw.	SA	HA	KF013427, KF013519, KF013340
P4605	*Haworthia reticulate* Haw.	SA	HA	KF013428, KF013520, KF013341
P4565	*Haworthia cooperi* Baker	SA	HA	AJ512275, KF013521, KF013342
P8190	*Haworthia truncate* Schönland	SA	HA	KF013429, KF013522, KF013343
P4593	*Haworthia gracilis* Poelln.	SA	HA	AY323623, KF013523, KF013344
P4604	*Haworthia fasciata* Haw.	SA	HE	AY323629, HQ646824.1, KF013345
P4599	*Haworthia glauca* Baker	SA	HE	AJ512318, HQ646827.1, KF013346
P8177	*Haworthia coarctata* Haw.	SA	HE	AY323635, KF013524, KF013347
P4582	*Haworthia reinwardtii* Haw.	SA	HE	AY323631, HQ646829.1, KF013348
P8191	*Haworthia venosa* Haw.	SA	HE	KF013430, KF013526, KF013349
P8175	*Haworthia attenuata* Haw.	SA	HE	AJ512315, KF013527, KF013350,
P4580	*Haworthia icosiphylla* Baker	SA		AJ512316, KF013528, KF013351
P6429	*Haworthia minima* Baker	SA	RO	KF013431, HQ646836.1, KF013352
P6427	*Haworthia pumila* Duval	SA	RO	KF013432, HQ646837.1, KF013353,
P8173	*Gasteria brevifolia* Haw.	SA		KF013433, KF013529, KF013354
P8172	*Gasteria batesiana* G.D.Rowley	SA		AJ512324, KF013530, KF013355
P4557	*Gasteria bicolor* var. *liliputana* (Poelln.) van Jaarsv.	SA		AJ512282, KF013531, KF013356
P306	*Lomatophyllum purpureum* T. Durand & Schinz	SA		AJ512301, KF013532, KF013357
P4632	*Astroloba foliosa* Utiwaal	SA		AJ512278, KF013534, KF013359
P378	*Asphodelus aestivus* Brot.	SA		AJ512314, KF013535, KF013360
	Bulbine Wolf			AJ512323.1, AJ290294.1, AY323650.1
	Kniphofia uvaria (L.) Hook			AJ512330.1, AJ290301.1, EU707283.1

2.2. Molecular Methods

DNA was isolated from fresh leaves based on a modified CTAB method (Doyle and Doyle, 1990) [28]. Only the epidermal part of the leaves was used in DNA extraction due to large amounts of secondary metabolites in the mucilaginous part. Extracted DNA was dissolved in TE buffer and the concentration was measured by UV spectrophotometry.

The *rbc*L region was amplified using primers *rbc*L-N and *rbc*L-1R (reverse). Polymerase chain reaction (PCR) for *rbc*L was carried out using the protocol of Treutlein et al. (2003) [7]. A final volume of 50 µL contained 0.5–1 µg DNA, 5 µL 10× PCR buffer, 12.5 pmol primer, 1.5 µL dNTPs (10 mM), 0.75 U Taq polymerase, 1 µL DMSO and 1 µL 20 mg/mL BSA. PCR cycle for *rbc*L were 2 min at 94 °C, then 30 cycles with 45 s at 94 °C, 90s at 45 °C, 90 s at 72 °C and finally 5 min at 72 °C.

The *trnL-F* spacer was amplified using trnF and E pair primers (Taberlet et al., 1991) [29]. A final volume of 50 µL contained 0.5–1 µg DNA, 5 µL 10× PCR buffer, 12.5 pmol primer, 1.5 µL dNTPs (10 mM), 0.75 U Taq polymerase and 1 µL 20 mg/mL BSA. The initial denaturation at 94 °C for 2 min was followed by 28 cycles comprising denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min and a final extension of 7 min at 72 °C.

The internal transcribed spacer (ITS1 & 2 and 5.8srDNA) regions were amplified with the primers ITS4 and ITS5 of White, et al. [30] and the same PCR protocol of Adams, et al. [31] with addition of 4% DMSO to the PCR reaction. The following PCR was applied: 26 cycles of 97 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min, followed by a final extension at 72 °C for 7 min.

For sequencing, PCR products were precipitated following Gonzalez, et al. [32]. Sequencing was performed using an ABI 3730 automated capillary sequencer (ThermoFisher Scientific, Darmstadt, Germany) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 and was carried out by STARSEQ GmbH (Mainz, Germany). Accession number of plants and DNA sequences are provided in Table 1.

2.3. Sequence Editing and Alignment

Non-coding regions such as *trnL_F* spacer and ITS are known to contain more substitutions than coding sequences and also carry insertions/deletions (indels). A high occurrence of indel mutations of varying lengths makes sequence alignment problematic (Small et al., 2004) [33]. Because of problems confounding alignment of these regions, all alignments were done manually using BioEdit (Hall, 1999) [34] and gaps corresponding to indels were positioned to minimise the number of nucleotide differences among sequences. To facilitate alignment most of problematic regions in terms of alignment were omitted, which resulted in a fragment of 415 bp for *trnL_F* spacer, 535 bp for ITS region and the final aligned matrix for *rbc*L was 907 bp long. A sequence alignment can be obtained from the first author on request and sequences are deposited in the GenBank (Table 1).

2.4. Phylogenetic Analyses

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar, et al., 2016) [35]. Phylogenetic reconstruction was performed using maximum likelihood (ML) in MEGA v.7 with the Kimura two-parameter model (Kimura 1980) [36]. MEGA7 was used to estimate the best substitution model: Kimura's two parameter model corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites. Under a general time reversible nucleotide substitution model (Tavaré, 1986) [37], one thousand inferences were run using among-site rate variation modelled with a gamma distribution. Subsequently, 1000 non-parametric bootstraps were performed under the partition data mode, and bootstrap support values were drawn on the ML tree.

In addition to ML analyses, Bayesian inference (BI) was used implemented in MrBayes v3.2.6 (Ronquist et al., 2012) [38]. To determine the best-fit model of DNA substitution for each loci with Akaike information criterion, MrModeltest v.2.3 [39] was used (for both *rbc*L and *trnL_F*: GTR + I + G, and GTR + G for ITS). We used GTR model in MrBayes and BEAST and K2P in MEGA 7 because we wanted to test the consistence between the models. Moreover, K2P is a nested model which is a special case of more general model such as GTR.

Two parallel runs of four chains of the Markov Chain Monte Carlo (MCMC) were executed for 7,000,000 generations, sampled every 1000 generations.

All parameters were stationary after 500,000 generations. All trees prior to the stationary point were discarded as "burn-in" from the compilation of posterior probabilities (PP). Strongly supported clades have posterior probabilities above 0.90. Phylogenetic trees were reconstructed for single and combined gene data and visualised using FigTree v1.3.1 [40].

2.5. Estimating Divergence Times

The estimates of divergence time of alooids were conducted using combined chloroplast and nuclear datasets.

BEAST v1.8. was used for the Bayesian MCMC inferred analyses of the nucleotide sequence data and BEAUti (Bayesian Evolutionary Analysis Utility) v1.6.1 [41] was utilised to generate initial xml files for BEAST.

A Yule (Yule, 1924) [42] process of speciation ('a pure birth' process) was used as a tree prior for all the tree model analyses and a relaxed uncorrelated log-normal clock (Drummond et al., 2012) [43] in BEAST v1.8. was applied.

Two independent simultaneous runs of 20,000,000 generations were completed, sampling one out of every 1000 trees in BEAST v 1.8. Log files were tested for ESS estimations with Tracer v1.6. (Rambaut & Drummond 2009) [44], LogCombiner v1.6.1 was used to combine the log files from the independent BEAST runs. Using TreeAnnotator v1.4.8, BEAST trees were summarised with a burn-in value of 25% and mean node heights (BEAUti, LogCombiner, and TreeAnnotator are all part of the BEAST software bundle).

The calibration mean date for the outgroup of alooids in Xanthorrhoeaceae or the genus *Bulbine* in Asphodeloids (51 Mya) and the date for the root of Xanthorrhoeaceae (61 Mya) used in the current study were taken from Wikström et al. (2001) [45].

Using fossils as calibration points, the ages and error estimates for over 75% of all angiosperm families were calculated in these studies, and these estimations were mainly compatible with the fossil record (Wikström et al., 2001) [45]. Moreover, in a recent revision of the age estimation and diversification of angiosperms using BEAST software (Bell et al., 2010) [46] the age estimation for the most cases overlapped the range published by Wikström et al. (2001) [45].

Since the Bayesian methods produce divergence time estimates which are dependent on priors and the model parameters, we tested the impact of using different settings in BEAST and a normal distribution was also applied with the mean value fixed at 61 Mya and a standard deviation of one.

Moreover, we compared our Bayesian estimates with those made using penalised likelihood approach previously applied in Hyacinthaceae, which is also a family in Asparagales, as described by Buerki et al., 2012 [47].

3. Results

Phylogenetic trees reconstructed from plastid and nuclear data showed almost identical topologies; therefore, the cpDNA and ncDNA datasets were combined. Partition homogeneity test in PAUP* 4.0 Beta was used and the *P*-value ($P = 0.0571$) indicates a congruency. Maximum Likelihood and MrBayes analyses recovered almost the same phylogenetic relationships for the combined data set. A ML phylogram is shown in Figure 1 and posterior probability values from MrBayes analysis and ML bootstrap numbers are provided at the nodes in this tree.

A BEAST analysis was used to reconstruct phylogeny and to estimate divergence times (Figure 2). The mean ages (with 95% HPD intervals) are given for the well supported nodes; similar estimates have also been reported in previous phylogenetic studies (Table 2). The mean coefficients of variation (σ_γ) under the relaxed clock model accounted for more than 1. This shows that a significant level of rate heterogeneity exists between lineages (Drummond et al., 2007) [48]. The 95% HPD intervals for the evolutionary root age of the outgroups was similar to those of the study of Wikström et al., (2001) [45]. In the combined maximum clade credibility tree obtained from BEAST, the mean age of the root of the tree for non-succulent asphodeloid members of the subfamily Asphodeloideae is about 51.8

(47.0–55.5) Mya. The succulent alooids have split from asphodeloids much later around 22.7 (20.3–24.1) Mya in Early Miocene. The result from penalised likelihood method were similar to the BEAST and the two different setting BEAST analyses provided similar values for node age estimates.

The combined dataset resolved four major clades within alooids labelled A–D in Figures 1 and 2: (A) some tree aloes from *Aloe* sect. *Aliodendron*, *Dracoaloe* and all shrubby aloes from *Aloe* sect. *Macrifoliae* (B) *Haworthia* subg. *Haworthia*, (C) the 'Haworthioid' clades including (C1): *Haworthia* subg. *Robustipedunculares* + *Astroloba* + *Poellnitzia* + *Aloearistata*; C2: *Haworthia* subg. *Hexangulares* and *Gasteria*, (D) all other 'true' *Aloe* species including *Lomatophyllum*.

(A) Shrubby and Tree aloes: In congruence with previous studies (Treutlein et al., 2003; Ramdhani et al., 2011; Daru et al., 2012) [7–9]. This group is now regarded as a new genus *Aloiampelos* Klopper & Gideon F. Sm. (Grace et al., 2015) [11]. Similarly, the tree aloes have been elevated to generic rank, as *Aloidendron* (A. Berger) Klopper & Gideon F. Sm. for the species with branched stems and rosulate leaves.

(B) *Haworthia* subgen. *Haworthia*: In the present study and in all previous phylogenetic reconstructions (Treutlein et al., 2003; Ramdhani et al., 2011; Daru et al., 2012; Manning et al., 2014) [7–10] these acaulescent Haworthias have always grouped as a monophylum with a high bootstrap and posterior probability values (PP = 1; BS = 99%). Although the mean age of the stem of this monophyletic group is about 12.59 (7.4–16.6) Mya (Table 2), the youngest well supported node amongst all Haworthias can be found in this subgenus with a mean age of about 1.81 (0.03–4.5) Mya.

(C) Haworthioids: The mean age of the stem node for two other subgenera in *Haworthia* and other small leaf rosulate taxa (*Astroloba* and *Aloe aristata*) is about 13.0 (8.6–17.7) Mya.

(C1) *Haworthia* subgen. *Robustipedunculares*, *Astroloba* and *Aloe aristata*: This clade displays almost the same topology (posterior probability; PP = 1) in the current and most previous studies (Ramdhani et al., 2011; Daru et al., 2012; Manning et al., 2014) [8–10] as a monophyletic sister clade to the other taxa of the subgenus *Haworthia* and *Gasteria* (clade C2).

This clade diverged around 9.8 (5.4–13.5) Mya; the divergence time of the well supported monophyletic members of *Haworthia* subgen. *Robustipedunculares* is in the late Miocene about 7.78 (3.8–11.4) Mya. These groups have now been raised to generic rank (or reinstated as genera), namely *Tulista* Raf. (for *H.* subgen. *Robustipedunculares*) and the monotypic *Aristaloe* Boatwr. & J.C. Manning (to accomodate *Aloe aristata*) (Manning et al., 2014) [10].

(C2) *Haworthia* subgen. *Hexangulares* and *Gasteria*: This clade includes a heterogeneous group of Haworthias from the subgenus *Hexangulares* which is apparently polyphyletic (Ramdhani et al., 2011; Daru et al., 2012; Manning et al., 2014) [8–10] as a sister clade of a strongly supported monophyletic *Gasteria* with a weak posterior probability (PP = 0.80; BS = 60). It has diverged around 10.6 (5.6–14.8) Mya from the other subclade of Haworthioids. The well supported monophyletic *Gasteria* diverged around 8.9 (4.3–13.2) Mya from *Haworthia* subgen. *Hexangulares*, which is now treated as the genus *Haworthiopsis* G.D. Rowley (Rowley 2013; Manning et al., 2014) [10,49].

(D) True aloes and *Lomatophyllum*: The rest of the species of aloes including some tree aloes were found to be in one clade (PP = 0.90; BS = 69%), which diverged about 15.8 (11.5–19.6) Mya. Only some internal groups are possibly monophyletic. For example, most samples from Yemen and North East Africa appeared in a single clade with moderate support value (PP = 0.90; BS = 59%) which has diverged only 4.1 (1.5–6.3) Mya. Moreover, most Madagascan aloes were found in a poorly supported internal clade of aloes with stem node divergence time about 5.3 (2.6–7.2) Mya. *Lomatophylum* is unlikely to be monophyletic.

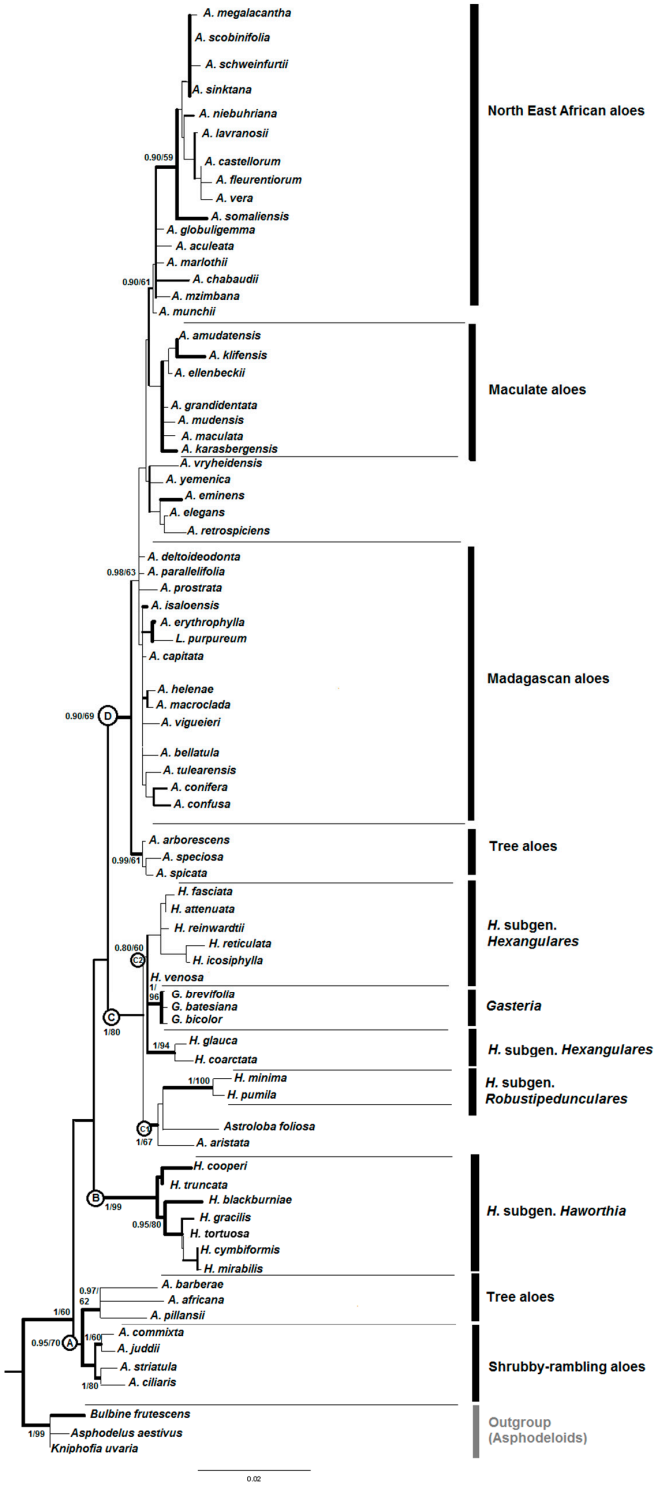

Figure 1. Phylogeny reconstruction of aloes using ML. Numbers at nodes in the ML phylogram refer to posterior probability values from MrBayes and ML bootstrap analyses. The branches in bold indicate a Bayesian posterior probability >0.95. Major lineages are highlighted by the letters A to D: (A) Shrubby and Tree aloes, (B) *Haworthia* subgen. *Haworthia*, (C) Haworthioids, (C1) *Haworthia* subgen. *Robustipedunculares*, *Astroloba* and *Aloe aristata*, (C2) *Haworthia* subgen. *Hexangulares* and *Gasteria*, (D) True aloes and *Lomatophyllum*.

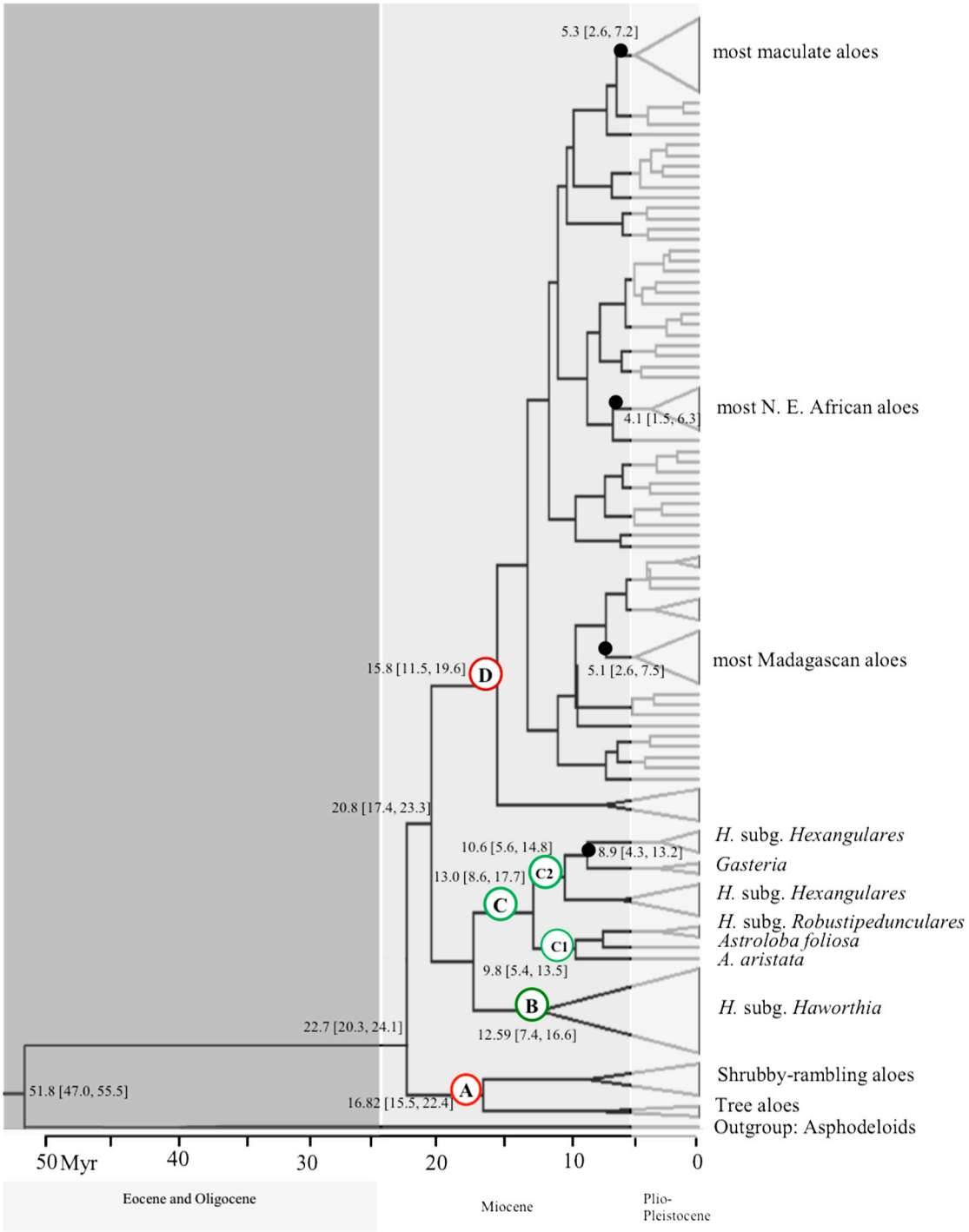

Figure 2. Estimation of divergence times in alooids. The combined maximum clade credibility tree was obtained from BEAST v1.8. Major lineages are highlighted by the letters A to D: (A) Shrubby and Tree aloes, (B) *Haworthia* subgen. *Haworthia*, (C) Haworthioids, (C1) *Haworthia* subgen. *Robustipedunculares*, *Astroloba* and *Aloe aristata*, (C2) *Haworthia* subgen. *Hexangulares* and *Gasteria*, (D) True aloes and *Lomatophyllum*.

Table 2. Node age with the posterior probability densities are shown for important clades and outgroups (asphodeloids).

Clades	Mean Age (Node, Mya)	95% HPD [a] (Mya)
Asphodeloids	51.8	47.0–55.5
Alooids	22.7	20.3–24.1
Shrubby and Tree aloes clade A	16.82	15.5–22.4
Haworthia subgen. *Haworthia* clade B	12.59	7.4–16.6
Haworthioid clade C1	9.8	5.4–13.5
Haworthioid clade C2	10.6	5.6–14.8
Gasteria	8.9	4.3–13.2
True aloes & *Lomatophyllum* clade D	15.8	11.5–19.6
Most North East African aloes	4.1	1.5–6.3
Most Madagascan aloes	5.1	2.6–7.5

[a] Represent lower–upper 95% HPD intervals, respectively. The 95% HPD is regarded as a Bayesian representation of confidence interval.

4. Discussion

4.1. Formation of Arid Habitats in Africa

A combination of the post African I erosion cycle (5–24 Mya), Post African II uplift event at the Pliocene and the glacial-interglacial cycles in the Pleistocene triggered a rapid speciation of many southern African plants (Siesser, 1978; Goldblatt, 1997) [50,51]. Through the Miocene (5.5–24 Mya) arid habitats became abundant in Africa (Coetzee, 1993; Axelrod and Raven, 1978) [52,53]. In this period falls the divergence of succulent alooids (22.7 Mya), corresponding with the diversification of many other South African lineages such as Iridaceae (Goldblatt and Manning, 2002) [25], *Pelargonium* (Bakker et al., 2005) [23], and *Ehrharta* (Verboom et al., 2003) [22].

The mid-Miocene Climatic Optimum (ca. 15 Mya) has led to the development of wide open ecosystems and the start of the radiation of the present hyperdiverse clades of the Cape flora. Moreover, the aridity of Southwestern Africa increased around 14 Mya (Siesser, 1978) [50] through the development of the proto-Benguela current off the coast of SW Africa as a result of the spread of the Antarctic ice sheet. This event led to the radiation of succulent life forms (Goldblatt, 1997) [51], among them alooids.

4.2. Divergence in Aloes

An early divergence of shrubby aloes (or their ancestors) around 16.82 (15.5–22.4) Mya had already been suggested by Holland (1978) [54], who had supposed that these succulents represent the original ancient lineage for other aloes during the desertification of Africa.

The early occurrence of fynbos aloes such as *Aloe commixta* and *Aloe juddii* (or their ancestors) is in good agreement with the age of many fynbos endemic lineages such as the African Restionaceae (Linder and Hardy, 2004) [55], *Moraea* (Goldblatt et al., 2002) [56], *Muraltia* (Forest et al., 2007) [57], *Ehrharta* (Bouchenak-Khelladi, 2007) [58], *Schoeneae* (Bremer, 2002) [59], and *Zygophyllum* (Verboom et al., 2003) [22]. It is remarkable that the divergence of these fire tolerant (Van Wyk and Smith, 2003) [60] rambling aloes agree with the age of the fynbos flora sensu Goldblatt P. and J.C. (2000) [61] in the Early Miocene, ca. 19.5 Mya.

Despite the assumption of an early radiation in southern Africa around the Early Miocene or earlier, most modern African species have radiated in contemporary climatic conditions and have evolved during the Pliocene–Pleistocene (Linder, 1992) [19]. A second Pliocene uplift event in Africa (Partridge and Maud, 2000) [62] caused extensive aridification by changing the ocean currents (Krammer et al., 2006) [63]; this was a period of rapid speciation in many clades such as in *Phylica* (Richardson et al., 2001) [64], semi-desert ice plants (Aizoaceae) (Klak et al., 2004) [65] and *Gladiolus*

(Rymer et al., 2010) [66]. The estimated mean crown age of many nodes within alooids also fall in this period (around 5 Mya).

From 2.5 Mya (i.e., the Quaternary) onwards, the climatic instability associated with glacial-interglacial cycles in the Northern hemisphere stimulated further diversification in South Africa (Cowling et al., 2009) [67]. The extensive speciation of many plants such as *Kniphofia* (Bakker et al., 2005) [23] and *Haworthia* subgen. *Haworthia* (Bayer, 1999) [17] falls in this period. Although the Haworthias diverged in the mid-Miocene, the youngest internal nodes within alooids are found in this group. This may be considered as further evidence for the recent speciation with in this subgenus of *Haworthia* as postulated by Ramdhani and co-workers (2011) [8]. The results of Manning et al. (2014) [10] confirm an early separation of the clade.

4.3. Divergence on Madagascar

A high diversity of *Aloe* and *Lomatophyllum* species was detected on Madagascar which represents the "hottest hotspot" of biodiversity of plants species of the world (Myers et al., 2000) [68]. Only grass aloes have not been found there (Reynolds, 1966) [69]. The oldest node age estimation for aloes from Madagascar (5.1 Mya) is in early Pliocene and apparently much later than the separation of this island during Gondwana from both the mainland of Africa (165–121 Mya) and India (88–63 Mya).

The divergence time of most Madagascan aloes correspond with other greatly diverse plants in Madagascar such as scaly tree ferns (Janssen et al, 2008) [70] and Indian Ocean Daisy Trees (*Psiadia*) (Strijk et al, 2012) [71]. Due to climatic alternations in the Pliocene (Coetzee, 1993) [52] resulting in habitat disintegration and repetitive decrease and increase of limited forest refugia, it has been assumed that these plants experienced fast geographical parallel diversification spurts in Madagascar. Our findings in aloes support the hypothesis that the Africa-Madagascan divergence is best explained by oceanic long-distance dispersal rather than ancient vicariance.

4.4. Speciation Processes in Aloes

Despite the strong influence of climate on plant diversification, it is very unlikely that climate alone is the cause for these levels of plant diversification especially in Cape Flora (Goldblatt and Manning, 2002) [25]. It has been proposed that speciation and endemism in alooids are associated with many other factors. Most species of alooids occur in extremely restricted areas, which are naturally isolated, thus showing a 'mosaic distribution' (Holland, 1978) [54]. It is assumed that specific microclimatic preferences of species had enhanced endemism in aloes (Kamstra, 1971) [72]. Therefore, the drivers of high endemism and speciation of alooids were mainly sought in mechanisms that lead to geographically isolated populations, and so to allopatric speciation (Schluter, 2001) [73]. From several proposed selective forces, a speciation in alooids might have been driven by a change of pollinators as well as by a slight differentiation in flowering times permitting the survival of new forms which enable a greater number of *Aloe* species to coexist (Rowley, 1976; Botes et al., 2008) [74,75].

In the Cape flora (including *Aloe*), parapatric sister-species limited to the diverse territories are known as a result of edaphic specialization (Goldblatt et al., 2001; Kurzweil et al., 1991) [76,77], leading to different life forms or ecomorphotypes that are described as different species today (Holland, 1978) [54].

Even though the real biological traits that have influenced speciation of alooids are unclear at this stage, the hypothesis of contemporary speciation and ongoing hybridization (Ramdhani et al. 2011) [8] in non-monophyletic genera of alooids (such as *Haworthia* sensu lato) should also be considered as an explanation for the complex taxonomy and the abundance of habitat restricted species.

5. Conclusions

In conclusion, although age estimations are dependent on fossil calibrations and monocots do not fossilise well, we hope that this phylogenetic study of alooids, in which we aimed to sample most sections of *Aloe* and many of its allied genera, will throw light on the causes of the high

diversity in alooids and the timing of their speciation. We suggest that future studies focus on increased taxon sampling to conclude more comprehensive age estimates for this important group of African succulents.

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