Evidence for the Polyphyly of *Haworthia* (Asphodelaceae Subfamily Alooideae; Asparagales) Inferred from Nucleotide Sequences of *rbc*L, *mat*K, ITS1 and Genomic Fingerprinting with ISSR-PCR

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Received: July 8, 2003; Accepted: August 21, 2003

Abstract: Four molecular markers have been studied to examine the phylogenetic position of the South African plant genus *Haworthia* Duval within the succulent Asphodelaceae. Sequence data of the chloroplast genes *mat*K and *rbc*L were compared to the nuclear markers ITS1 and ISSR (Inter Simple Sequence Repeat) analysis. Both lines of molecular data, chloroplast and nuclear DNA, indicate that *Haworthia* is polyphyletic, forming two distinct clades. Most taxa previously combined as *Haworthia* subgenus *Haworthia* branch off early in the alooid chloroplast trees forming a strongly monophyletic group, whereas subgenus Hexangulares forms a polyphyletic assemblage comprising other alooid genera. The nuclear markers ITS1 and ISSR fingerprinting support the two groups as distinctly different, therefore confirming the division seen in chloroplast DNA. The practical implication is that the generic concept of *Haworthia* may have to be restricted to *H.* subgenus *Haworthia* or alternatively, that the groups of *Haworthia* be treated as infrageneric taxa within a broadened (Linnaean) concept of *Aloe.*

Key words: *Haworthia,* Inter Simple Sequence Repeat (ISSR), nuclear internal transcribed spacer (ITS), maturase K (*mat*K), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbc*L), phylogeny.

Abbreviations:

ISSR-PCR	inter simple sequence repeat – PCR
ITS	internal transcribed spacer
*mat*K	maturase K
ML	maximum likelihood
MP	maximum parsimony
NJ	neighbour joining
PCR	polymerase chain reaction
*rbc*L	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

Introduction

Ever since pre-Linnean times the rosulate-leaved, succulent plant genus *Haworthia* Duval has had a complex taxonomic and nomenclatural history (Wijnands, 1983). In 1753, three of the species today classified as *Haworthia* were described by Linnaeus as aloes, namely *Aloe retusa* L., *Aloe viscosa* L. and *Aloe pumila* L. (Scott, 1985). Some 50 years later, Haworth (1804) split the genus *Aloe* into three distinct subunits, of which the Parviflorae represented the current generic concept of *Haworthia* and *Astroloba.* The genus *Haworthia,* as currently circumscribed, was established by Duval (1809) shortly thereafter.

After the creation of *Haworthia* (Duval, 1809), many of the species of *Haworthia* saw changes in rank or name and, at one time or another, over 400 different names have been used to refer to species, subspecies, varieties and forms (Uitewaal, 1947 a, b, 1949; Jacobsen, 1951, 1965; Scott, 1973, 1985; Bayer, 1982, 1999; Breuer, 2002). This trend carries on to the present time, as the genus is enjoying renewed taxonomic attention (see for example Breuer, 2002 and references therein). Predictably, the application of names in the genus remains largely dependent on the concepts of the individual researcher and it is debatable whether the current taxonomies do adequate justice to a classification reflecting inter- and intrageneric affinities.

The taxonomic and nomenclatural instability in *Haworthia* is also reflected at the generic level within the subfamily Alooideae. Over the years, various genera have been split off or combined; it is only in recent years that more rigorous studies have been done leading to new insights in relationships within the group (Smith and Van Wyk, 1991, 1998; Van Wyk et al., 1995; Chase et al., 2000; Smith and Meyer, 2000). In a recent study of generic relationships using molecular markers, we found evidence for the genus *Haworthia* not being monophyletic (Treutlein et al., 2003). This rather interesting result prompted a more detailed study to obtain additional evidence, the results of which are presented here.

Plant biol. 5 (2003): 513 – 521

ISSN 1435-8603

Table 1 Origin of plant samples, botanical garden accession numbers and EMBL database accession numbers for *rbc*L, *mat*K and ITS1 sequences. + = presence/– = absence of sequence/ISSR information. Comparison of chloroplast, ITS1 and ISSR groups of the species examined (molecular groups, column MG: I = *Haworthia* group I; II a = *Haworthia* group II a, II b = *Haworthia* group II b) and the morphological treatment of the taxa according to Bayer, 1999 (current classification of subgenera of the genus *Haworthia*, column SG; Hex = subgenus Hexangulares, Haw = subgenus *Haworthia*, * hybrid of garden origin, subgeneric classification of the parents unknown, ** *Haworthia* × *semiglabrata Haw*. is a hybrid between *Haworthia maxima* [Haw.] Duval and *Haworthia marginata* [Lam.] Stearn, both belong to the subgenus Robustipedunculares)

Taxon	Botanical garden accession number	EMBL database accession numbers			ISSR	MG	SG
		*rbc*L	*mat*K	ITS1			
Aloe arborescens Mill. 1	BG Puerto de la Cruz, Tenerife, Canary Islands	AY323645	AY323722	AY323680	–	true Aloes	–
Aloe arborescens Mill. 2	BG Marburg, Germany	AY323646	AY323723	AY323681	–	true Aloes	–
Aloe aristata Haw. 1	BG Heidelberg, Germany	AJ512319	AJ511407	AY323651	–	close to Haworthia II	–
Aloe aristata Haw. 2	BG Jena, Germany	AY323634	AY323713	AY323652	–	close to Haworthia II	–
Aloe barberae T.-Dyer	BG Heidelberg, Germany 9483	AJ512294	AJ511371	AY323661	–	treelike Aloes	–
Aloe capitata Baker var. *gneissicola* H. Perrier	BG Puerto de la Cruz, Tenerife, Canary Islands 0846 – 81	AY323643	AY323720	AY323677	–	true Aloes	–
Aloe ciliaris Haw. var. *ciliaris*	BG Puerto de la Cruz, Tenerife, Canary Islands 1182 – 70	AJ512287	AJ511379	AY323663	–	–	–
Aloe compressa H. Perrier var. *compressa*	BG Heidelberg, Germany 74679	AY323644	AY323721	AY323678	–	true Aloes	–
Aloe conifera H. Perrier	BG Heidelberg, Germany 72081	AJ512303	AJ511383	AY323679	–	true Aloes	–
Aloe deltoideodonta Baker var. *deltoideodonta*	BG Heidelberg, Germany	AJ512304	AJ511384	–	–	true Aloes	–
Aloe doei Lavranos	BG Heidelberg, Germany	AY323647	AY323724	AY323682	–	true Aloes	–
Aloe forbesii Balf. f.	BG Heidelberg, Germany 12610	AJ512308	AJ511389	AY323688	–	true Aloes	–
Aloe glauca Mill.	BG Heidelberg, Germany 3741	AJ512313	AJ511396	AY323670	–	true Aloes	–
Aloe humilis (L.) Mill.	BG Marburg, Germany	AY323642	AY323719	AY323675	–	true Aloes	–
Aloe inermis Forssk.	BG Heidelberg, Germany	AJ512288	AJ511387	AY323686	–	true Aloes	–
Aloe jucunda Reynolds	BG Heidelberg, Germany 9381	AY323641	AY323718	AY323674	–	true Aloes	–
Aloe juvenna Brandham and S. Carter	BG Heidelberg, Germany 11249	AY323640	AY323717	AY323673	–	true Aloes	
Aloe karasbergensis Pillans	BG Heidelberg, Germany	AJ512283	AJ511391	AY323669	–	true Aloes	
Aloe lineata (Aiton) Haw. var. *lineata*	BG Puerto de la Cruz, Tenerife, Canary Islands 0082-92	AJ511397	AJ511397	AY323671	–	true Aloes	–
Aloe niebuhriana Lavranos	BG Heidelberg, Germany 13382	AY323648	AY323725	AY323683	–	true Aloes	–
Aloe pillansii L. Guthrie	BG Heidelberg, Germany 100718	AJ512292	AJ511369	AY323659	–	treelike Aloes	–
Aloe plicatilis (L.) Mill.1	BG Heidelberg, Germany 9286	AY323613	AY323693	AY323662	–	–	–
Aloe plicatilis (L.) Mill 2	BG Tübingen, Germany	AY323614	AY323694	–	–	–	–
Aloe plicatilis (L.) Mill. 3	19503, ex Kirstenbosch	AY323615	AY323695	–	–	–	–
Aloe ramosissima Pillans	BG Heidelberg, Germany	AJ512293	AJ511370	AY323660	–	treelike Aloes	–
Aloe scobinifolia Reynolds and P. R. O. Bally	BG Heidelberg, Germany	AJ512307	AJ511388	AY323687	–	true Aloes	–
Aloe sinkatana Reynolds	BG Heidelberg, Germany 17353	AJ512306	AJ511386	AY323689	–	true Aloes	–
Aloe somaliensis W. Watson var. *somaliensis*	BG Heidelberg, Germany 8057	AY323639	AY323716	AY323672	–	true Aloes	–
Aloe striata Haw.	BG Heidelberg, Germany 100403	AJ512310	AJ511392	AY323668	–	true Aloes	–
Aloe suprafoliata Pole Evans	BG Puerto de la Cruz, Tenerife, Canary Islands 0086 – 92	AY323638	AY323715	AY323676	–	true Aloes	–
Aloe vera (L.) Burm. f. 1	BG Heidelberg, Germany 100677	AJ512309	AJ511390	AY323684	–	true Aloes	–
Aloe vera (L.) Burm. f. 2	BG Jena, Germany	AY323649	AY323726	AY323685	–	true Aloes	–
Astroloba spiralis (L.) Uitewaal	BG Jena, Germany	AY323636	AY323691	AY323658	+	II a	–
Astroloba spiralis (L.) Uitewaal cv. "Pentagona" Groen	BG Jena, Germany	AY323637	AY323692	–	+	II a	–
Bulbine frutescens (L.) Willd.	private collection of the authors	AJ512323	AJ511414	AY323650	–	–	–
Gasteria acinacifolia (Jacq.) Haw.	BG Heidelberg, Germany	AY323627	AY323706	AY323653	–	close to Haworthia II	–

continued →

Table 1 continued

Taxon	Botanical garden accession number	EMBL database accession numbers			ISSR	MG	SG
		*rbc*L	*mat*K	ITS1			
Gasteria bicolor Haw. var. *bicolor* 1	BG Marburg, Germany	AJ512282	AJ511401	AY323654	–	close to Haworthia II	–
Gasteria bicolor Haw. var. *bicolor* 2	BG Jena, Germany	AY323626	AY323705	AY323655	–	close to Haworthia II	–
Haworthia aristata Haw.	BG Heidelberg, Germany 142418	–	–	–	+	I	Haw
Haworthia attenuata Haw. var. *britteniana* (Poelln.) Poelln.	BG Heidelberg, Germany 142441	AJ512315	AJ511403	AY323728	+	IIa	Hex
Haworthia cf. *maughanii* Poelln.	BG Heidelberg, Germany 59150	AY323619	AY323690	–	+	I	Haw
Haworthia chloracantha Haw. var. *denticulifera* (Poelln.) M. B. Bayer	BG Heidelberg, Germany 142428	AJ512295	AJ511372	–	+	I	Hex
Haworthia coarctata Haw.	BG Heidelberg, Germany	AY323635	AY323714	–	+	IIa	Hex
Haworthia coarctata Haw. var. *coarctata* forma *greenii* (Bak.) M. B. Bayer	BG Heidelberg, Germany 142431	AY323630	AY323709	–	+	IIa	Hex
Haworthia coarctata Haw. var. *tenuis* (G. G. Smith) M. B. Bayer	BG Heidelberg, Germany 4385	AY323628	AY323707	–	+	IIa	Hex
Haworthia cooperi Baker var. *cooperi* 1	BG Jena, Germany	–	–	–	+	I	Haw
Haworthia cooperi Baker var. *cooperi* 2	BG Heidelberg, Germany 74528	AY323617	AY323697	–	+	I	Haw
Haworthia cymbiformis (Haw.) Duval	BG Jena, Germany	AY323621	AY323699	AY323666	+	I	Haw
Haworthia cymbiformis (Haw.) Duval var. *cymbiformis*	BG Jena, Germany	AY323620	AY323700	AY323667	+	I	Haw
Haworthia cymbiformis (Haw.) Duval var. *obtusa* (Haw.) Baker	BG Heidelberg, Germany 142417	AY323616	AY323696	–	+	I	Haw
Haworthia cymbiformis (Haw.) Duval var. *transiens* (Poelln.) M. B. Bayer	BG Heidelberg, Germany 142414	AJ512296	AJ511373	–	+	I	Haw
Haworthia fasciata (Willd.) Haw forma *fasciata*	BG Heidelberg, Germany 4859	AY323629	AY323708	–	+	IIa	Hex
Haworthia geraldii C. L. Scott	BG Heidelberg, Germany 142450	AJ512317	AJ511405	–	+	IIa	Haw
Haworthia glabrata Baker	BG Jena, Germany 981243	AJ512316	AJ511404	AY323656	+	IIa	Hex
Haworthia glauca Baker var. *herrei* (Poelln.) M. B. Bayer	BG Heidelberg, Germany 142437	AJ512318	AJ511406	AY323729	+	IIa	Hex
Haworthia glauca Baker var. *herrei* (Poelln.) M. B. Bayer forma *armstrongii* (Poelln.) M. B. Bayer	BG Heidelberg, Germany 8479	AY323633	AY323712	–	+	IIb	Hex
Haworthia gracilis Poelln. var. *gracilis*	BG Heidelberg, Germany 41010	AY323623	AY323702	–	+	I	Haw
Haworthia limifolia Marloth var. *stolonifera* Resende	BG Heidelberg, Germany 45587	AY323632	AY323711	AY323727	+	IIb	Hex
Haworthia maraisii Poelln. var. *notabilis* (Poelln.) M. B. Bayer	BG Heidelberg, Germany 30224	AY323624	AY323703	–	+	I	Haw
Haworthia mirabilis (Haw.) Haw. var. *triebneriana* (Poelln.) M. B. Bayer	BG Jena, Germany 11283	AY323618	AY323698	–	+	I	Haw
Haworthia reinwardtii (Salm-Dyck) Haw. var. *reinwardtii* forma *reinwardtii*	BG Jena, Germany	AY323631	AY323710	AY323657	+	IIa	Hex
Haworthia × *resendiana* Poelln.	BG Jena, Germany	AY323622	AY323701	AY323665	+	I	Hex
Haworthia × *rigida* (Lam.) Haw.	BG Heidelberg, Germany 142445	–	–	–	+	IIa	*
Haworthia × *ryderiana* Poelln. 1	BG Heidelberg, Germany 45928	AJ512299	AJ511377	AY323664	+	I	Haw
Haworthia × *ryderiana* Poelln. 2	BG Heidelberg, Germany 142232	AY323625	AY323704	–	+	I	Haw
Haworthia × *semiglabrata* Haw.	BG Heidelberg, Germany 142459	–	–	–	+	–	**

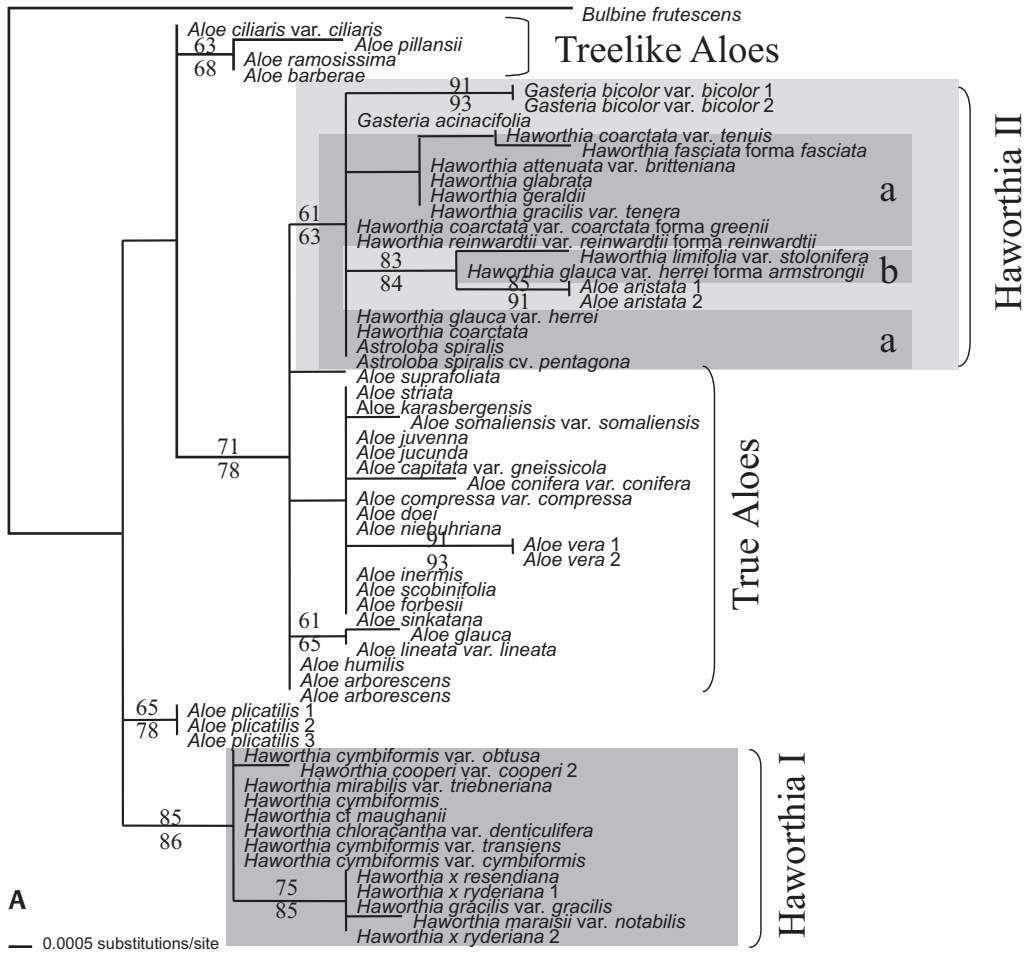


Fig. 1 A

Fig. 1 GTR + G + I ML phylograms. MP bootstrap values written above, NJ bootstrap values below the branches. Only values > 50% are shown. Branch lengths are proportional to genetic distances and represent the genetic distance under the GTR+G+I model of substitution, and can be compared to the distance bar. *Bulbine* is presented as outgroup for presentation purposes only. (**A**) *rbc*L phylogram of two best trees found. The -Ln likelihood was – 2153.1530. Substitution rate matrix: AC = 1.575442, AG = 3.913689, AT = 0.212397, CG = 1.442876, CT = 8.342613, GT = 1.000000; Assumed proportion of invariable sites = 0.701433; Shape parameter (alpha) = 2094.04, equalling 0.005 substitutions per site. MP bootstrap values above, NJ bootstrap values below the branch. Only values > 50% are shown (**B**) *mat*K phylogram-Ln likelihood of the two best trees found: 4386.6175; Substitution rate matrix: AC = 0.664916, AG = 1.327406, AT = 0.161452, CG = 0.829101, CT = 0.867127, GT = 1.000000; Shape parameter (alpha) = 0.901806; Assumed proportion of invariable sites = 0.426537 (**C**) ITS1-phylogram. -Ln likelihood of 6 best trees found: 1213.38418; shape = 1.02371; pi = 0,0748972; substitution rate matrix: AC = 0.474329, AG = 1.803093, AT = 0.878590, CG = 0.358055, CT = 2.526,601, GT = 1.000,000.

Since the establishment of PCR and DNA sequencing in the 1970s and 1980s, sequence analyses have become routine in plant systematics and identification, and are applied in this study to unravel the disputed phylogenetic structure of the genus *Haworthia.* Additionally ISSR fingerprinting, a genome-wide multilocus tool that has only recently been developed (Zietkiewicz et al., 1994; Wink et al., 1998, 2001), was used to corroborate the groups resulting from nucleotide sequences.

Materials and Methods

Sample origin and sequence database accession numbers

Altogether 30 accessions of *Haworthia* and 38 additional samples of allied succulent alooid genera (Table **1**) were sequenced and analysed by ISSR fingerprinting. Additionally, *Bulbine* was analysed for outgroup purposes.

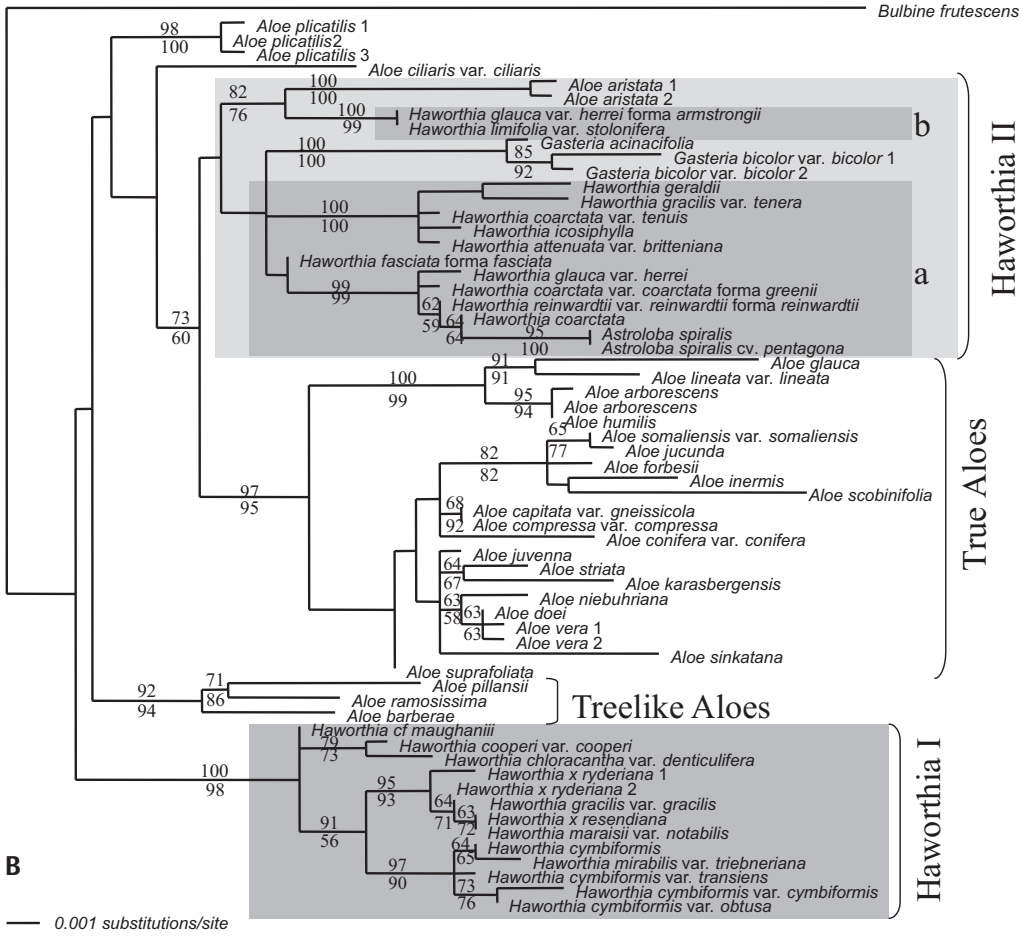
Extraction of DNA

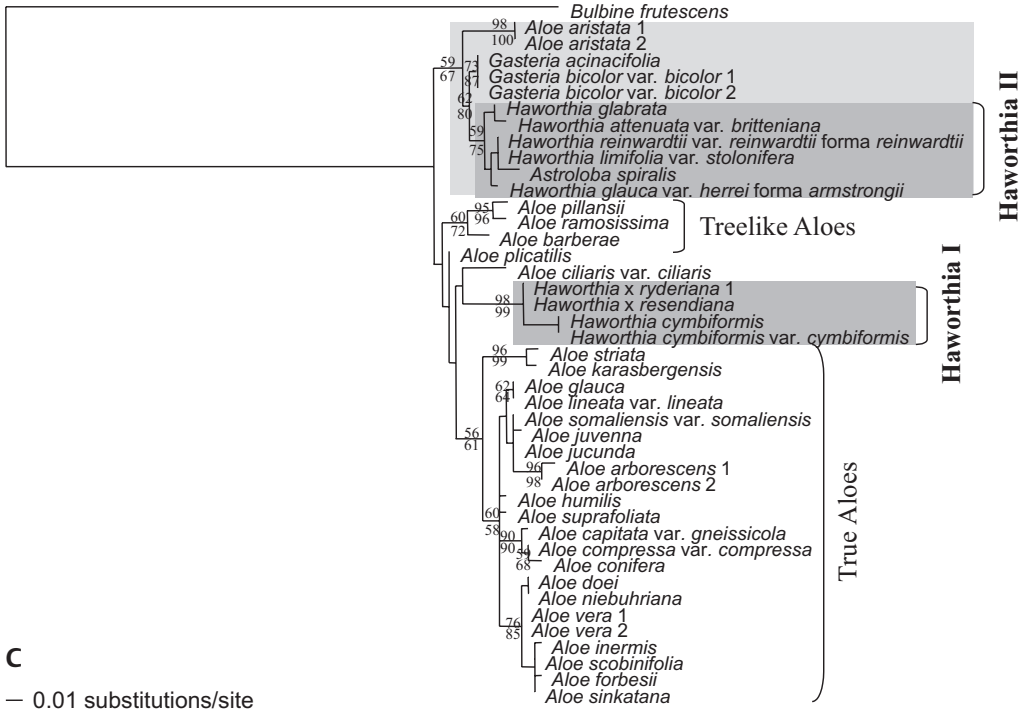
DNA was isolated from fresh leaves using the CTAB method (Doyle and Doyle, 1990) with minor modifications. These include small-scale isolation in Eppendorf tubes specific to the succulent habit of the plants: about 0.5 cm^2 of the outer green parenchymatic tissue was sliced off the leaf, avoiding the transparent inner pulp and homogenised with mortar and pestle without freezing.

ISSR-PCR

Amplification, electrophoresis conditions and detection of ISSR-amplification products: For amplification, 15 ng of total DNA were used as template, plus 3 pmol primer 5′-GAC AGA CAG ACA GAC A-3′, 1.5 mM $MgCl_2$, 0.1 mM of dGTP, dCTP, and dTTP, 0.075 mM dATP, 1 µCi [α-^{33}P]-dATP, 1.25 µl of 10 × ampli-

Figs. 1 B, C


Bulbine frutescens
Aloe plicatilis 1
Aloe plicatilis2
Aloe plicatilis 3
Aloe ciliaris var. ciliaris
Aloe aristata 1
Aloe aristata 2
Haworthia glauca var. herrei forma armstrongii
Haworthia limifolia var. stolonifera
b
Gasteria acinacifolia
Gasteria bicolor var. bicolor 1
Gasteria bicolor var. bicolor 2
Haworthia geraldii
Haworthia gracilis var. tenera
Haworthia coarctata var. tenuis
Haworthia icosiphylla
Haworthia attenuata var. britteniana
Haworthia fasciata forma fasciata
Haworthia glauca var. herrei
Haworthia coarctata var. coarctata forma greenii
Haworthia reinwardtii var. reinwardtii forma reinwardtii
Haworthia coarctata
a
Astroloba spiralis
Astroloba spiralis cv. pentagona
Haworthia II
Aloe glauca
Aloe lineata var. lineata
Aloe arborescens
Aloe arborescens
Aloe humilis
Aloe somaliensis var. somaliensis
Aloe jucunda
Aloe forbesii
Aloe inermis
Aloe scobinifolia
Aloe capitata var. gneissicola
Aloe compressa var. compressa
Aloe conifera var. conifera
Aloe juvenna
Aloe striata
Aloe karasbergensis
Aloe niebuhriana
Aloe doei
Aloe vera 1
Aloe vera 2
Aloe sinkatana
True Aloes
Aloe suprafoliata
Aloe pillansii
Aloe ramosissima
Aloe barberae
Treelike Aloes
Haworthia cf maughaniii
Haworthia cooperi var. cooperi
Haworthia chloracantha var. denticulifera
Haworthia x ryderiana 1
Haworthia x ryderiana 2
Haworthia gracilis var. gracilis
Haworthia x resendiana
Haworthia maraisii var. notabilis
Haworthia cymbiformis
Haworthia mirabilis var. triebneriana
Haworthia cymbiformis var. transiens
Haworthia cymbiformis var. cymbiformis
Haworthia cymbiformis var. obtusa
Haworthia I
B
0.001 substitutions/site


Bulbine frutescens
Aloe aristata 1
Aloe aristata 2
Gasteria acinacifolia
Gasteria bicolor var. bicolor 1
Gasteria bicolor var. bicolor 2
Haworthia glabrata
Haworthia attenuata var. britteniana
Haworthia reinwardtii var. reinwardtii forma reinwardtii
Haworthia limifolia var. stolonifera
Astroloba spiralis
Haworthia glauca var. herrei forma armstrongii
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Aloe barberae
Treelike Aloes
Aloe plicatilis
Aloe ciliaris var. ciliaris
Haworthia x ryderiana 1
Haworthia x resendiana
Haworthia cymbiformis
Haworthia cymbiformis var. cymbiformis
Haworthia I
Aloe striata
Aloe karasbergensis
Aloe glauca
Aloe lineata var. lineata
Aloe somaliensis var. somaliensis
Aloe juvenna
Aloe jucunda
Aloe arborescens 1
Aloe arborescens 2
Aloe humilis
Aloe suprafoliata
Aloe capitata var. gneissicola
Aloe compressa var. compressa
Aloe conifera
Aloe doei
Aloe niebuhriana
Aloe vera 1
Aloe vera 2
Aloe inermis
Aloe scobinifolia
Aloe forbesii
Aloe sinkatana
True Aloes
C
0.01 substitutions/site

fication buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl, 5% Triton X-100) and 0.4 units Taq polymerase (Amersham Pharmacia Biotech) in a total volume of 12.5 µl. After an initial denaturation (120 s at 94 °C), 33 cycles of 60 s at 94 °C, 120 s at 55 °C, and 120 s at 72 °C were performed on a Biometra thermocycler, then at 72 °C for 4 min, followed by 4 °C for storage. PCR products were subjected to electrophoresis on 0.2 mm denaturing polyacrylamide gels at 65 W for 3 h (size 45 × 30 cm). After drying, the gel was exposed to Kodak Hyperfilm for several hours and developed. The film was scanned and synapomorphic bands were marked. ISSR fingerprinting was repeated several times to ensure reproducibility of the pattern.

PCR amplification and DNA sequencing parameters

*rbc*L gene was amplified using the PCR primers *rbc*L-N (forward): 5′-ATG TCA CCA CAA ACA GAR ACK AAA GC-3′, *rbc*L-R (reverse): 5′-TAT CCA TTG CTG GGA ATT CAA ATT TG-3′, and *rbc*L-1 R (reverse): 5′-GGG TGC CCT AAA GTT CCT CC-3′. For *rbc*L sequencing the forward primers Leg3-cy: 5′-TGC GTT GGA GAG ACC GTT TC-3′ and Leg4-cy: 5′-ACT TTA GGY TTT GTT GAT TT-3′, and the reverse primers Leg2-cy: 5′-ATT CGC AAA TCT TCC AGA CG-3′ and Leg7-cy: 5′-TTC GCA TGT ACC CGC AGT AGC A-3′ were used. *Mat*K was amplified by PCR using the primers *mat*K-724 F: 5′-CGC ACT ATG TAT CAT TTG ATA AC-3′ (forward) and *mat*K-2303 R: 5′-CAT TTA GAA AAT CTA AGA ATG AAT C-3′ (reverse). PCR conditions: a final volume of 50 µL contained 0.5 – 1 µg DNA, 5 µl 10 × Taq buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton × 100, pH 9.0), 3 µl 25 mM $MgCl_2$, 12.5 pmol primer, 1.5 µl dNTPs (10 mM), 0.75 U Taq-Polymerase (Amersham Pharmacia Biotech) and 1 µl 20 mg/ml BSA. PCR cycle: 2 min at 94 °C, then 30 cycles with 45 s at 94 °C, 90 s at 70 °C and 90 s at 45 °C, and finally 5 min at 72 °C. PCR products were further amplified by cycle sequencing using the "ThermoSequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP" (Amersham Pharmacia Biotech) according to the protocol of the manufacturer. Products were sequenced with an automatic sequencer ALFexpress II (Amersham Pharmacia Biotech). Cycle sequencing of the *mat*K region was performed using the following Cy5-labelled forward primers: *mat*K-AloeF4cy: 5′-GTA AGG ATT CAA ATG TTA GAG AAT T-3′, *mat*K-724Fcy: 5′-CGC ACT ATG TAT CAT TTG ATA AC-3′, makK-F1/D1170Fcy: 5′-AKA ATT TAC GAT CAA TTC ATT CAA-3′, *mat*K-F2/K1756Fcy: 5′-AGG ATC CAT ATA AAC CAA TTA TC-3′, *Mat*F3cy: 5′-GAA ATC TTT CTC ATT ATC ACA G-3′, and the reverse primers *mat*K-AloeR3cy: 5′-CGT AYT GTA CTT TTA TGT TTA CGA G-3′, *mat*K-R1/K1303Rcy: 5′-TRG AGA AAG AAT CGT AAT AAA TG-3′, AmatRcy: 5′-GTA CAA AAT TTA GCT TTA GAC-3′. PCR of ITS1 was done using the primers ITS18: 5′-GTC CAC TGA ACC TTA TCA TTT AGA GG-3′ and S5: 5′-TTC GGG CGC AAC TTG CGT TC-3′, sequencing primer was Cy5-labelled S7: 5′-AAG GAG AAG TCG TAA CAA GGT TTC CG-3′ and a fluorescently labelled version of PCR-primer S5.

Sequences were aligned manually and analysed using the phylogeny program PAUP* version beta 10 (Swofford 2003). Molecular phylogenies were reconstructed using Maximum Likelihood (ML) only. The ML search was conducted under the GTR+G+I model (6 substitution types) in a heuristic search manner. Branch swapping using the tree bisection and reconnection swapping algorithm was done on a starting tree built under the parsimony criteria. All three data sets were additionally bootstrapped (500 replicates) with Maximum Parsimony (MP) and distance with Neighbour Joining (NJ) algorithm (Tamura-Nei algorithm in the case of protein coding chloroplast genes; p-distance algorithm with ITS1). These values are referred to in the text as MP/NJ. Bootstrap values above 50% were written to the groups present in the ML phylogram (MP above branches; Distance/NJ below branches).

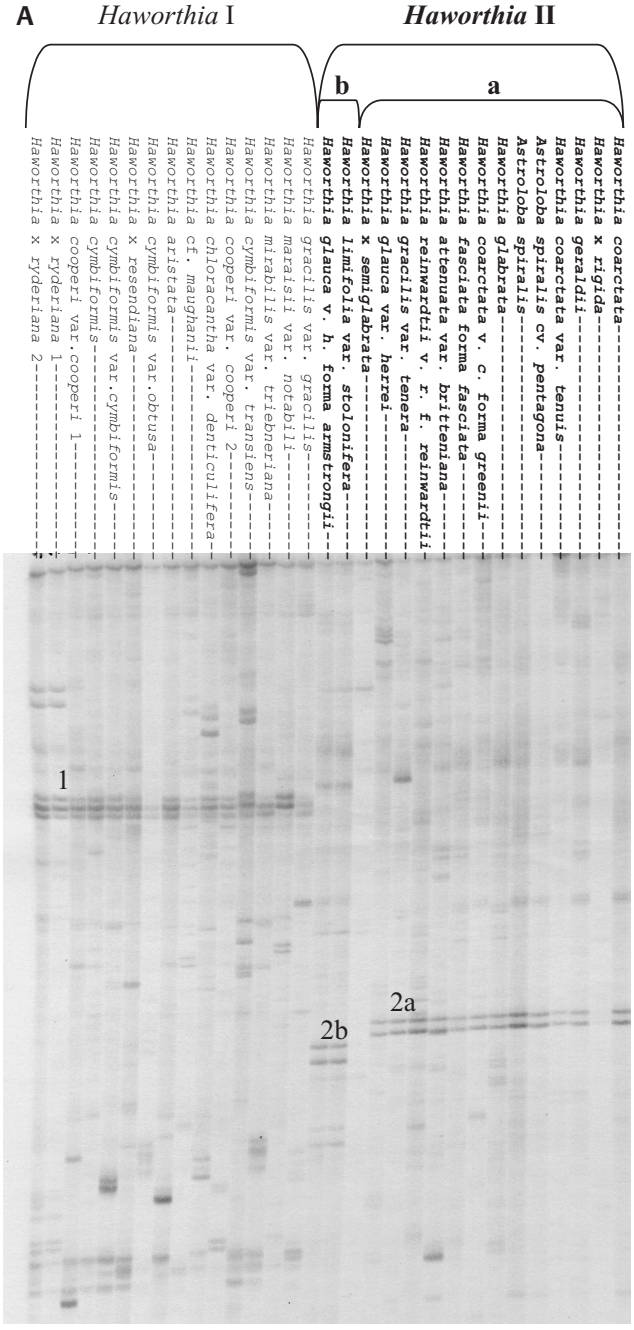

Fig. 2 (**A**) Autoradiogram showing the ISSR patterns amplified by primer $(GACA)_4$ in 29 accessions of the genus *Haworthia* and two accessions of *Astroloba.* Species classified as belonging to *Haworthia* subgenus *Haworthia* standard, those belonging to *Haworthia* subgenus Hexangulares bold. (**B**) Application of ISSR to further species of *Aloe* and *Gasteria* shows that patterns 1 and 2a are limited to the two groups of *Haworthia*/*Astroloba* and cannot contribute to their phylogenetic placement.

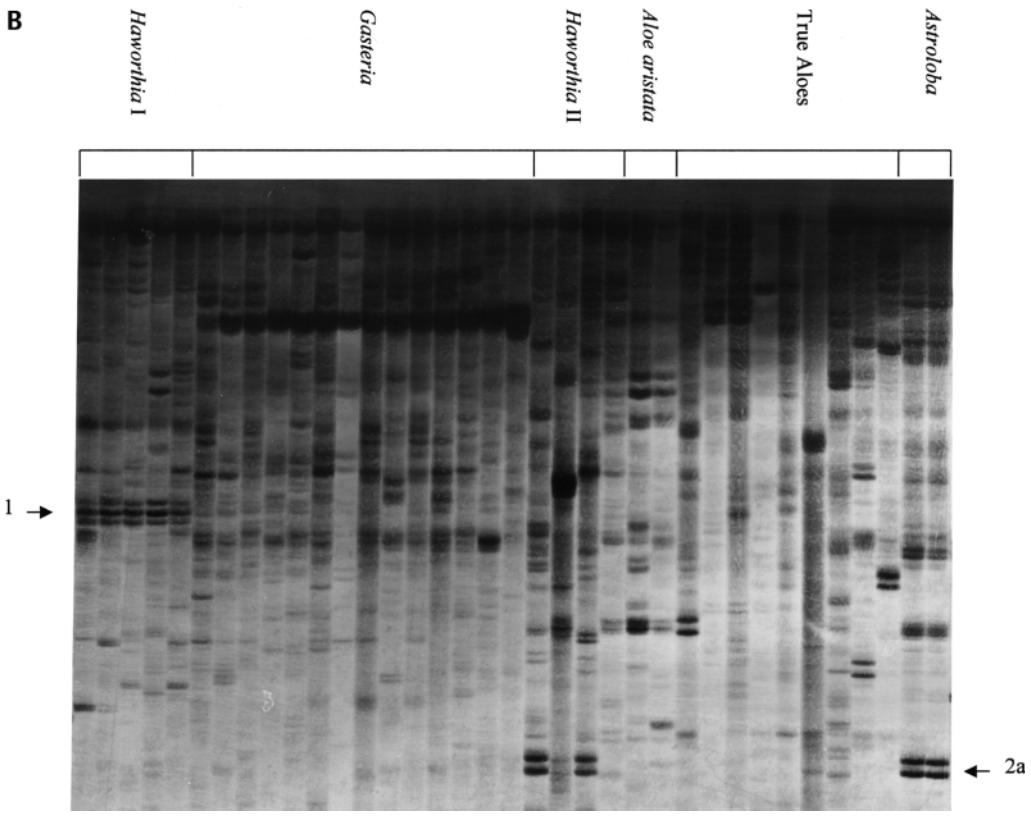

Fig. 2 B

Results and Discussion

Placement of Haworthia *in the phylogeny of the genes* rbc*L,* mat*K and ITS1*

The phylogenetic trees (Figs. **1 A – C**) illustrate the relationships of *Haworthia* within other genera of succulent Asphodelaceae. *Bulbine* was used as outgroup, being the closest living relative of the Alooideae (Chase et al., 2000; Treutlein et al., 2003). In the Alooideae, four distinct groups were invariably found in all three phylogeny reconstructions: a) the tree-like aloes, b) *Haworthia* I (Figs. **1 A – C, 2**, standard), c) the true *Aloe* cluster and the d) *Haworthia* II (Figs. **1 A – C, 2**, bold). The latter group is allied to *Gasteria/Aloe aristata* and *Astroloba.* The congruence of the three independent data sets, together with the strong differences in their ISSR patterns (Fig. **2**) and reasonable bootstrap support of the two groups provide evidence that the genus *Haworthia* is polyphyletic, consisting of one strongly monophyletic group and another which is intermixed with other alooid taxa.

Haworthia group I was completely composed of species that are morphologically placed in the subgenus *Haworthia* (Table **1**, bootstrap support *rbc*L: 85/86%, *mat*K: 100/98% and ITS1: 98/99%). This is the largest subgenus within the *Haworthia* and was established by Uitewaal (1947 a, b). He divided the genus *Haworthia* into two main sections (Triangulares and Hexangulares), the former including the subgenus *Haworthia.* He already noted the distinct discontinuity in flower morphology of the two groups, and his separation was further corroborated by the recent discovery that the nectar sugar composition of the two groups differs substantially (Smith et al., 2001). Table **1** compares the current morphological classification (Bayer, 1999) in relation to the genetic groupings.

The second bulk of *Haworthia* species (*Haworthia* II) cluster in an assemblage that further includes the genera *Gasteria, Astroloba* and *Aloe aristata* (bootstrap support *rbc*L: 61/63%, ITS1 59/67%, *mat*K: lower than 50%) demonstrating the close relationship between them and *Haworthia* subgenus Hexangulares.

In general, the morphological classification in the subgenera *Haworthia* and Hexangulares (Table **1**) is supported by DNA data, although they are genetically more strongly separated than previously thought. An exception in the current classification was found with the sister species *H. geraldii* and *H. gracilis* var. *tenera:* genetically they belong to group II, whereas morphologically they show affinities to the subgenus *Haworthia* (represented by group I).

ISSR-PCR: a new tool in Haworthia *research*

Unlike nucleotide sequences, ISSR markers describe the allelic states at several genome loci and thus avoid the use of gene trees as representative of species trees (Martin and Salamini, 2000). Banding patterns obtained by the tetranucleotide primer $(GACA)_4$ are presented in Figs. **2 A, B**, indicating the presence of microsatellites of this type in all taxa examined. The polyphyly of *Haworthia* seen in the sequence trees (Figs. **1 A – C**) is strongly supported by the ISSR banding patterns. $(GACA)_4$ amplification results in two genetically separat-

ed groups, which share no common amplification product, indicating that they are only distantly related. ISSR bands 1 and 2 are both universal motives in the genus *Haworthia,* even if band 2 is not present in all the species of group II. Extension of the ISSR method to further species of the genera *Aloe* and *Gasteria* (Fig. **2B**) shows that ISSR bands 1 and 2a are limited to the two groups of *Haworthia* and that no affinity to *Aloe* or *Gasteria* can be detected based on the two patterns.

The species of *Haworthia* subgenus *Haworthia* are characterised by a unique ISSR band 1 complex, those of the subgenus Hexangulares by patterns 2a or 2b, or none of the two patterns (Figs. **2A, B**). *Haworthia geraldii* and *Haworthia gracilis* var. *tenera* are sister species according to *rbc*L (Fig. **1A**) and *mat*K (Fig. **1B**). Contrary to their previous morphological classification (Bayer, 1999), they are clearly grouped in *Haworthia* subgenus Hexangulares by both molecular markers.

ISSR banding patterns do not show strong interspecific differences, with the exception of *Haworthia limifolia* var. *stolonifera* and *Haworthia glauca* var. *herrei* forma *armstrongii* (group II). These two species are characterised by an electrophoretic shift of band 2 to lower molecular weight, which may originate from a deletion event. In congruence, both species are also closely related according to the chloroplast markers *mat*K (Fig. **1B**) and *rbc*L (Fig. **1A**) and form the sister group to *Aloe aristata* (bootstrap support *rbc*L 83/84%, *mat*K 82/76%, not supported by ITS1).

Conclusions

The present study unequivocally shows that molecular evidence conflicts with the current treatment of *Haworthia* as a single genus. In order to rule out misleading phylogenies of *rbc*L and *mat*K due to chloroplast capture, we used nuclear ITS1 and ISSR genomic fingerprinting to examine the genetic relationships based on the nucleom. It is obvious that both data sets support the dichotomy of *Haworthia* at the present stage, and that the taxonomy of the genus *Haworthia* must be revisited. More species of both groups need to be examined to determine their phylogenetic relationship before taxonomic consequences should be drawn.

In addition, the strong genetic separation between the two *Haworthia* groups is reflected by morphological discontinuities (Uitewaal, 1947 a, b) and differences in the nectar sugar composition of the subgenera of *Haworthia* (Smith et al., 2001). From the molecular point of view, *Haworthia* section *Haworthia* (except *H. geraldii* and *H. gracilis* var. *tenera*) is convincingly monophyletic, whereas *Haworthia* section Hexangulares is intermixed with species of *Gasteria, Astroloba* and *Aloe aristata.* There are two possibilities for revising the generic classification of *Haworthia.* One option would be to treat the various groups of *Haworthia* as genera (which may be problematic in view of the lack of monophyly of section Hexangulares). The other, and perhaps more realistic possibility, is to consider *Haworthia* as comprising various subgenera within an enlarged generic concept of *Aloe.*

Acknowledgements

We thank Dr. Klaus Kramer (Botanical Garden Heidelberg, Germany) and the Botanical Gardens of Jena and Tübingen, Germany, for plant material, and Prof. F. Salamini for helpful suggestions on the manuscript.

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