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Controversial taxonomy of Strumariinae (Amaryllidaceae) investigated by nuclear rDNA (ITS) sequences

1. *Hessea*, *Namaquanula*, *Kamiesbergia*, and *Dewinterella*

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Abstract. Two opposing opinions concerning the generic differentiation of Amaryllidaceae subtribe Strumariinae two taxonomic opinions were published in the last decade. According to Müller-Doblies and Müller-Doblies (1985, 1994) the Strumariinae includes eight genera, among them *Hessea*, *Namaquanula*, and *Dewinterella*. Snijman (1991) described the new genus *Kamiesbergia* and accepted *Namaquanula* (1992). Of the entire complex she recognized only *Hessea* (Snijman 1994). The section *Myophila* was simultaneously described as the genus *Dewinterella* by Müller-Doblies and Müller-Doblies.

The possible phylogenetic relationships of eight species belonging to these taxa are estimated from the sequences of the ITS regions of the 18S–25S rDNA. Two species of *Nerine* are used as the outgroup. The sequences of these taxa are analyzed with maximum parsimony, distance, and maximum likelihood methods. In all phylogenetic reconstructions *Namaquanula* is confirmed to be an independant clade aside from all other six species of the Strumariinae. In this group *Hessea* and *Dewinterella* turn out to be sister groups. *Hessea stenosiphon* (subgenus *Kamiesbergia*) is the sister taxon of *H. breviflora*.

Key words: Amaryllidaceae, Strumariinae. Phylogeny, ITS 1, ITS 2, 18S rDNA, 25S rDNA, maximum parsimony analysis, distance analysis, maximum likelihood analysis.

The cosmopolitan family Amaryllidaceae consists of nine tribes, some of them divided into subtribes (Dahlgren et al. 1985). The predominantly South African tribe Amaryllideae is the largest tribe of the family according to the number of genera (11–16 genera) and the second in size according to the number of species. It comprises two subtribes, Amaryllidinae (confined to Africa except for the subcosmopolitan genus *Crinum*) and Strumariinae (endemic to southern Africa).

Most genera of the Amaryllidinae have been revised, either provisionally or comprehensively, throughout the second and third quarters of this century: *Ammocharis* and

Cybistetes (Milne-Redhead and Schweickerdt 1939), *Brunsvigia* (Dyer 1950, 1951), *Nerine* (Traub 1967), and *Crinum* in South and East Africa (Verdoorn 1973, Nordal 1977) and Cameroun (Nordal and Wahlstrøm 1980).

Numerous classifications from the 19th century dealing with the *Hessea-Strumaria* group and its related genera can be found (Herbert 1837, Kunth 1850, Salisbury 1866, Baker 1888) which reveal a heterogeneous picture of this cluster. Not until the middle of this century, however, did several botanists start working on it again: Barker (1948), Leighton (1948), Obermeyer (1963), Traub (1963), and Goldblatt (1976). In 1985 Müller-Doblies and Müller-Doblies validated Traub's (1957) Strumariinae as a subtribe including seven genera: *Carpolyza* Salisb., *Namaquanula* D. & U. M-D., *Strumaria* Jacq., *Bokkeveldia* D. & U. M-D. (segregated from *Strumaria*), *Gemmaria* Salisb. (segregated from *Hessea*), *Hessea* Herb., and *Tedingea* D. & U. M-D. Nine years later another genus, *Dewinterella* D. & U. M-D., was added (Müller-Doblies and Müller-Doblies 1994), accommodating two isolated species of *Gemmaria*. The new genera were accepted by Gunn et al. (1992), Brummitt (1992), and Greuter et al. (1993). Two of them, *Tedingea* and *Namaquanula*, were also approved by Snijman and Perry (1987) and Snijman (1992). At the beginning of the nineties a new genus of the Strumariinae, *Kamiesbergia* Snijman, was published (Snijman 1991) which Müller-Doblies and Müller-Doblies had published as a new species of *Hessea* at the same time. Shortly afterwards *Kamiesbergia stenosiphon* was changed to *Hessea stenosiphon* (Müller-Doblies and Müller-Doblies 1992).

Snijman, (1994) who did not recognize the subtribe Strumariinae of Amaryllideae, divided it into two subclades, A^1 (= *Hessea* sensu Snijman) and subclade A^2 (= *Carpolyza* and *Strumaria* sensu Snijman). The subclade A^1 contains three subgenera, *Namaquanula*, *Kamiesbergia*, and *Hessea*. *Hessea* subgen. *Namaquanula* consists of two sections: section *Namaquanula* (formally) with the single species *H. bruce-bayeri* (D. & U. M-D.) Snijman and section *Myophila* with two species, *H. pulcherrima* (D. & U. M-D.) Snijman and *H. mathewsii* W. F. Barker (Fig. 1). When the latter sectional taxon was published in June 1994, the genus *Dewinterella* D. & U. M-D. with the same two species, *D. pulcherrima* (D. & U. M-D.) D. & U. M-D. and *D. mathewsii* (W. F. Barker), was already in print. As both taxa, the section *Myophila* and the genus *Dewinterella*, do not compete for priority, it is possible, therefore, to consider them as more or less simultaneously published.

The second subgenus, *H.* subgen. *Kamiesbergia*, is monotypic with *Hessea stenosiphon* (Snijman) D. & U. M-D.

The subgenus *H.* subgen. *Hessea* includes nine species. Three of them, *H. breviflora*, *H. stellaris*, and *H. cinnabarina*, are analyzed in the present paper of which the latter species deserves a taxonomic note. It was not recognized by Snijman and was treated as synonymous with *H. stellaris*. The same problem occurred with *H. longituba* which was placed into the synonymy of *H. breviflora*.

Thus within the subclade A^1 none of the three additional genera published between 1985 and 1994 are recognized, not even the genus *Kamiesbergia* of 1991. A single genus, *Hessea*, is left (Fig. 1).

There are some molecular studies of the Amaryllidaceae and closely related families, such as Liliaceae (Shinwari et al. 1994, Fay and Chase 1996). Snijman (1994) presented a scheme of the phylogenetic relationships of the Strumariinae based on morphological and anatomical data. Because of the controversial discussion concerning the phylogeny of the Strumariinae, it is useful to look for a new source of information for the phylogenetic reconstruction, based on the results in several publications dealing with ITS sequence data as a successful tool to reconstruct the phylogeny of certain plant groups (e.g. Poaceae by Hsiao et al. 1994, 1995a, b; Fabaceae by Wojciechowski et al. 1993; Asteraceae by Baldwin 1992, 1993), this region of the nuclear rDNA,

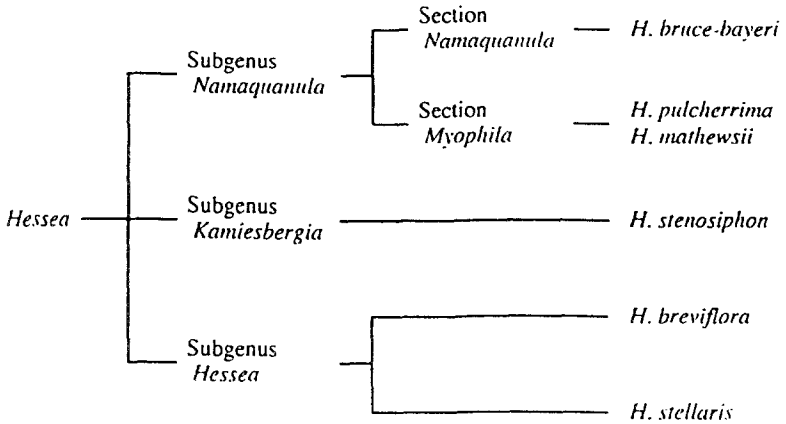

Fig. 1. Snijman's dendrogram containing the modified subclade A^1

the internal transcribed spacer (ITS) with the intervening 5.8S gene, was chosen. Therefore, it is hoped that this study provides a new and conclusive phylogenetic scenario of the Strumariinae.

Material and methods

Taxon sampling. Ten species, all arranged in the tribus Amaryllideae of Amaryllidaceae, were used for the sequence determination of the 5.8S gene and flanking transcribed spacers (ITS 1 and ITS 2 regions): *Dewinterella pulcherrima*, *Hessea breviflora*, *H. cinnabarina*, *H. longituba*, *H. stellaris*, *H. stenosiphon*, *Namaquanula bruce-bayeri*, *N. etesionamibensis*, *Nerine humilis*, and *N. sarniensis*. Both species of *Nerine* were chosen as the outgroup, because they belong to the Amaryllidinae and are therefore closely related to the genera of the Strumariinae. Moreover, *Nerine* was used as the outgroup in Snijman's work (1994), so that the results are well comparable.

This study was based on living materials of cultivated plants from wild provenance. The plants were collected in southern Africa and grown as flowers in a greenhouse of the Institute of Ecology of the Technical University of Berlin. The species, their abbreviations, taxonomic positions, cultivation numbers, collectors, collection numbers, grid numbers, and localities are listed in Table 1.

The leaves of two individual plants of each species from different locations – when available – were harvested and stored at −30 °C until DNA extraction.

DNA extraction and sequencing. To avoid mislabeling or cross-contamination of DNA, DNA's of different species and individuals were isolated on different days.

About 1 mg fresh or frozen leaves were homogenized in 400 µl Wilson-buffer (100 mM Tris, 10 mM EDTA, 100 mM NaCl, 0.1% SDS, pH 8.0) with a pestle. The homogenate was treated with RNAse, proteinase K (50 µg/ml), and 1% SDS for 2 h at 55 °C. Proteins and cell debris were extracted once with a phenol/chloroform/isoamylalcohol mix (25 : 24 : 1), once with a chloroform/isoamylalcohol mix (24 : 1) and precipitated in ethanol (Sambrook et al. 1989).

The investigated DNA region was amplified with primers described by Hsiao et al. (1995a, b). The primer ITSL (5′-TCGTAACAAGGTTTCCGTAGGTG-3′) anneals to the 3′ end of the 18S rDNA near the ITS 1 border, and the primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) is complementary to the 5′ end of the 25S rDNA near the ITS 2 border. ITSL and ITS4 cover the entire ITS region which is divided by the 5.8S gene into the ITS 1 and ITS 2 regions.

To confirm each sequence, the DNA of two different individuals were sequenced and compared. The sequences of each individual were also done in both directions by using complementary strands.

The PCR was carried out in a thermal cycler (Biometra) and set for initial 93 °C for 3 min (denaturation), followed by 37 cycles at 93 °C for 30 sec (denaturation), 59 °C for 30 sec (primer annealing), and 72 °C for 30 sec (polymerization). An elongation of the PCR products by 72 °C for 5 min completed the reaction. About 10 ng template DNA, 400 nM primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 10x buffer (100 mM TrisHCl, pH (25 °C) 9.0, 500 mM KCl, 15 mM $MgCl_2$, 1% TritonX100, 2 mg/ml BSA or gelatin, 70 °C), and 5

Table 1. Species, their abbreviations, taxonomic positions, cultivation numbers, collectors, collection numbers, grid numbers, and localities of 10 Amaryllidaceae included in this study

Taxonomic position Species/Abbreviation	Cultivation number	Collector	Collection number	Grid number	Locality
Strumariinae					
Dewinterella pulcherrima D. & U.	2032/5	U. and D. M-D.	78042e	3119DA	type coll.
M-D./	6748/3	"	86045d	3119BC	near type loc.
D. pulch.					
Hessea breviflora Herb./	6201/2	E. G. H. Oliver	EGHO 8584	3118 DC	Nardousberg
H. brev.	8421/3	"	EGHO s.n.	3018DB	Kliprand
H. cinnabarina D. & U. M-D./	3919/1	U. and D. M-D.	80077b	3019CC	type coll.
H. cinn.	3919/5	"	"	"	"
H. longituba D. & U.M-D./	4105/5	"	80096v	2917BB	"
H. long.	7195/1,1	"	88084b	2917BD	N Concordia
H. stellaris (Jacq.) Herb./	1825/A	M. B. Bayer	MBB 837a	3319DA	NE Worcester
H. stel.	3380/2	U. and D. M-D.	79237a	3118DB	S Vanrhynsdorp
H. stenosiphon (Snijman) D. & U.	8472/4	"	90109a	3018AC	± type loc.
M-D./	8472/6	"	"	"	"
H. sten.					
Namaquanula bruce-bayeri D. &	2808/5	"	79112a	2816BC	type coll.
U. M-D./	7445	"	88142f	2716CB	Rooiberg,
N. b-b.					Sperrgebiet
N. etesionamibensis D. & U. M-D.	7138/6	"	88070c	2817AA	type coll.
/N. etes.	7138/21	"	"	"	"
Amaryllidinae (outgroup)					
Nerine humilis (Jacq.) Herb./	852/1,1	"	74043m	3420AA	W Swellendam
Ne.hum.	5131/1,4	"	82113a	3218DB	Piekenierskloof Pass
	873/3,6	"	74052a	3418BD	Betty's Bay
N. sarniensis (L.) Herb./					
Ne. sarn.	3382/3	"	79240a	3318DB	Riebeek Kasteel

U/100 Taq-polymerase (Appligene, Heidelberg) were used.

The amplified DNA fragment was purified by electrophoresis through 1% agarose gel in TAE buffer (0.4 M Tris, 0.2 M NaAc, 0.01 M EDTA, pH 8.0). QIAquick Gel Extraction Kit (Qiagen, Hilden) was used to purify the gel slice containing the DNA fragment. The final yield was stored at 30 °C.

The purified double-stranded PCR products of all species were directly sequenced on a 373 DNA sequencer (Applied Biosystems, ABI) using Taq polymerase and dye-terminators according to the ABI manufacturer's instructions.

Sequence alignment and data analysis. The sequence data of the roughly 660 bp amplified DNA of the investigated species were aligned using CLUSTAL V multiple sequence alignment program (Higgins et al. 1992).

The parsimony analysis was conducted with the computer program PAUP, version 3.1.1 (Swofford 1993) using the branch and bound search options to find the most parsimonious tree for the amplified DNA. All uninformative characters were ignored, and gaps were treated as missing or as a "fifth base". Bootstrap values were determined from 500 replications.

The distance analysis of the same aligned sequences was conducted with the computer program MEGA, version 1.01 (Kumar et al. 1993). Gaps were excluded by the pairwise deletion option. Both the Tajima-Nei distance (Tajima and Nei 1984) and the Kimura 2-parameter distance (Kimura 1980) were used for the reconstruction of the MEGA neighbor-joining tree (Saitou and Nei 1987). Bootstraps were done with 100 replications.

Maximum likelihood trees were constructed by the PHYLIP-DNAML program, version 3.5c (Felsenstein 1993).

The comparison of tree length was done by the MacClade 3 program (Maddison and Maddison 1992). To be able to compare the tree length of Snijman's dendrogram (1994) and the DNA dendrogram it was necessary to bring both dendrograms into agreement concerning the investigated species. Therefore only those species represented in both studies were included the calculation of the tree length, so that the number of taxa had to be diminished. Seven taxa were then left: *Dewinterella pulcherrima*, *Hessea breviflora*, *H. stenosiphon*, *H. stellaris*, *Namaquanula brucebayeri*, and both species of *Nerine*.

Results

Sequence variation of ITS region. All in all the aligned sequences yielded 662 characters, including the 3′ end of the 18S gene and the 5′ end of the 25S gene. The endpoints of the regions of the ITS regions and genes were based on comparative analysis (Fig. 2).

Table 2 gives information about the total character of the different regions of the amplified DNA and their variable and informative positions.

The length of the complete ITS region of the 10 studied Amaryllidaceae species ranged from 638 to 640 nucleotides. The ITS 1 region varied from 242 to 245 base pairs (bp) in length, and the ITS 2 region spanned from 231 to 232 bp. The length of the 5.8S gene, 164 bp, was constant in all species (Fig. 2; Table 3).

Table 2. Sequence variation in the ITS region and variable and informative sites of 10 Amaryllidaceae subtribe Strumariinae and Amaryllidinae

Region	Range from ··· to	Total character	Variable sites	Informative sites
Total character	1 ··· 662	662	165	124
Total ITS	7 ··· 648	642	164	123
ITS 1	7 ··· 252	246	68	52
5.8S	253 ··· 416	164	18	14
ITS 2	417 ··· 648	232	78	57

Note: Sites refer to aligned positions in Fig. 2.

Table 3. Sequence length of total character and total ITS and single ITS 1, 5.8S, and ITS 2 regions and G+C percentage of total ITS in 10 Amaryllidaceae subtribe Strumariinae and Amaryllidinae

	Total character	Total ITS		ITS 1	5.85	ITS 2
Species		length (in bp)	%(G+C)	length (in bp)	length (in bp)	length (in bp)
D. pulch.	658	638	60	242	164	232
H. brev.	659	639	56	244	"	231
H. cinn.	660	640	57	"	"	232
H. long.	659	639	56	"	"	231
H. stel.	660	640	57	"	"	232
H. sten.	659	639	56	"	"	231
N. b-b.	660	640	55	"	"	232
N. etes.	"	"	55	"	"	"
Ne. hum.	"	"	60	245	"	231
Ne. sarn.	"	"	"	245	"	"

For species abbreviations see Table 1.

The exact sequence length of the different regions and the G+C percentages of the entire ITS regions for all studied species are given in Table 3. Hsiao et al. (1994) found the same length of the 5.8S gene in the species of Poaceae and similar G+C percentages, ranging from 57.6% to 64%.

Whereas most of the sequence variation occurred in the spacer regions, there was little variation within the 5.8S subunit. All in all only 18 variable nucleotide positions could be found there (Fig. 2).

Of the 10 taxa examined, the ITS sequences of the two species of *Nerine* were identical. Obvious similarities could be found in all sequences of the species of *Hessea*, of which the sequences of *Hessea cinnabarina* and *H. stellaris* showed a difference in 11 nucleotide positions. The sequences of *H. breviflora* and *H. stenosiphon* varied in 8, the sequences of *H. breviflora* and *H. longituba* in 6 nucleotide positions. There was also considerable congruence between the two species of *Namaquanula*, which differed in 18 nucleotide positions. The sequence of the only species of *Dewinterella*, *D. pulcherrima*, resembled the sequences of the species of *Hessea* more than those of *Namaquanula* (Fig. 2).

Phylogenetic analysis. The phylogenetic analysis of the investigated species is based on the alignment shown in Fig. 2.

Figure 3 presents the most parsimonious tree (PAUP) obtained from the entire ITS regions (ITS 1 and 2) with a tree length of 158 (123 informative positions). The consistency index (CI) value for this tree is 0.854, which is relatively high and therefore indicated a strong phylogenetic signal in the data. Gaps were treated as missing data.

The species of *Hessea* clustered with a bootstrapping value of 98%. In this cluster *Hessea cinnabarina* and *H. stellaris* were separated based on a bootstrap confidence of 99%. The other three species of *Hessea*, *H. longituba*, *H. breviflora*, and *H. stenosiphon*, were grouped with a 100% bootstrap of which *H. breviflora* and *H. stenosiphon* were separated from *H. longituba* by a bootstrap value of 67%. The separation of *Hessea* and *Dewinterella* was supported by a bootstrap

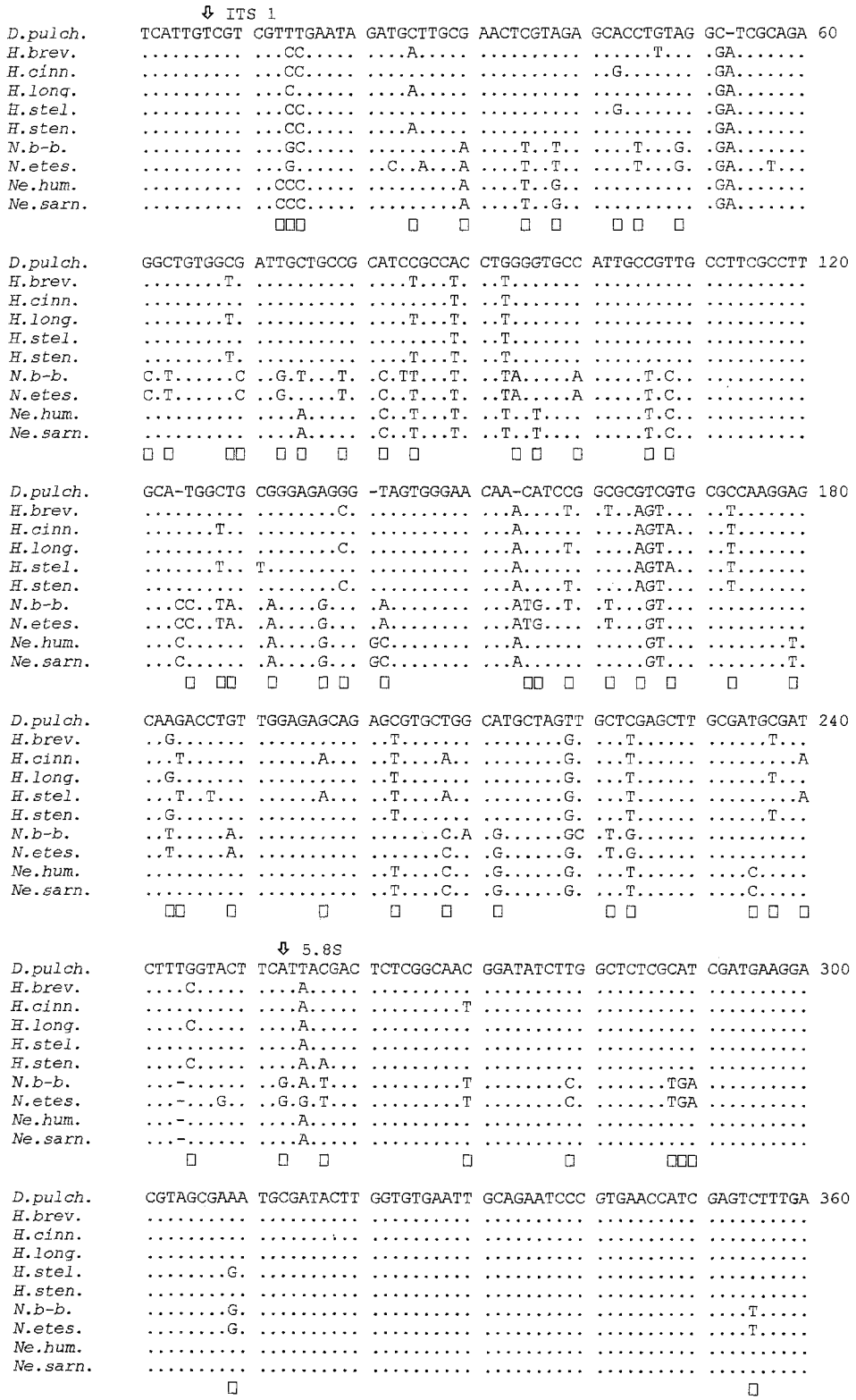

Fig. 2. Aligned sequences of 10 taxa of Amaryllidaceae subtribe Strumariinae and Amaryllidinae. Numbers indicate the succeeding positions of 1 to 662 from the end of the 18S region to the beginning of the 25S region; arrows mark the beginning of ITS 1, 5.8S, and ITS 2 regions; dashes denote gaps; dots indicate identity to *Dewinterella pulcherrima*; blank rectangles mark informative sites

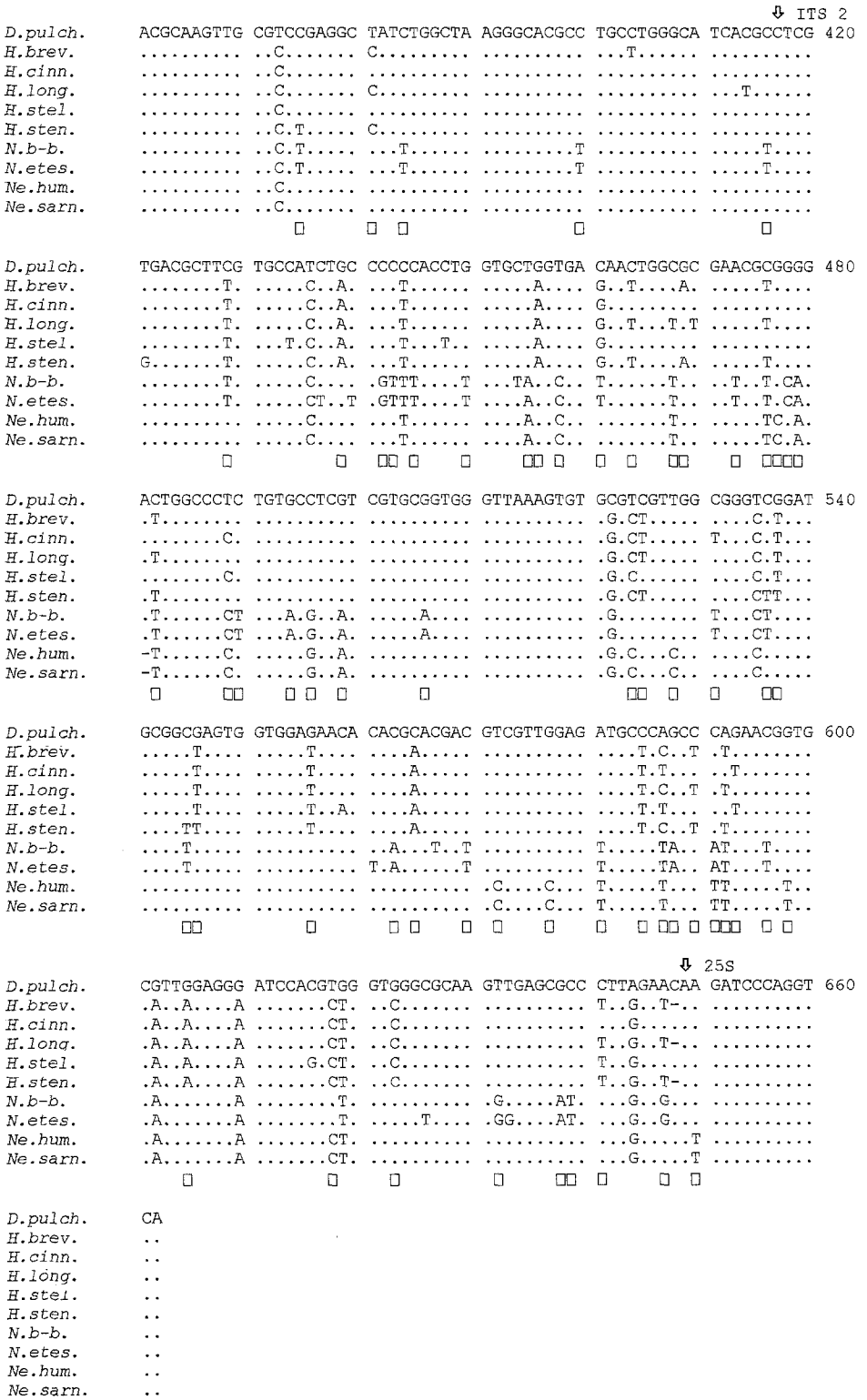

Fig. 2 (continued)

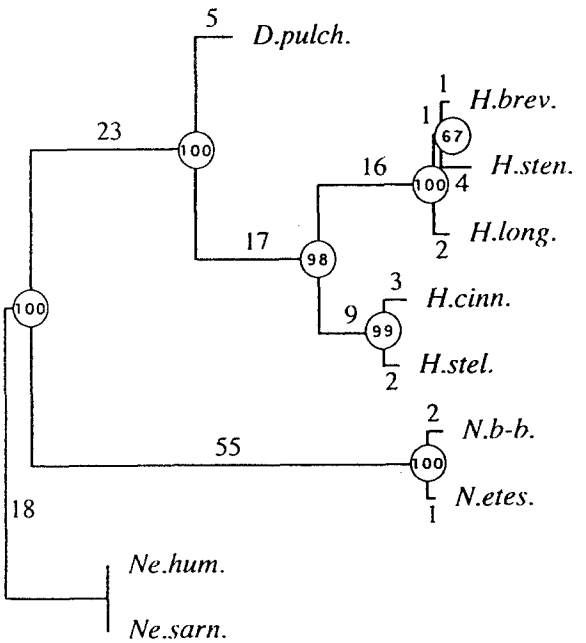

Fig. 3. Most parsimonious tree obtained from the entire ITS region. Numbers in circles denote bootstrap percentages; numbers above the branches indicate the inferred branch-length excluding uninformative characters

confidence of 100%. The monophyly of *Namaquanula* was confirmed by a 100% bootstrap confidence. The separation of *Hessea/ Dewinterella* and *Namaquanula* from a common ancestor was sustained by a 100% bootstrap.

The same tree topology gained from the whole ITS region occurred when gaps were treated as a "fifth base".

The MEGA neighbor-joining trees based on the analysis of the entire ITS region revealed exactly the same tree topology compared to the tree obtained from the parsimony analysis. Both trees (not shown), the one done with the Tajima-Nei distance and the other one done with the Kimura-2-parameter distance, showed that the species of *Hessea* were grouped with a bootstrapping value of 98% or 99%. *H. stellaris* and *H. cinnabarina* on the one hand and *H. breviflora*, *H. stenosiphon*, and *H. longituba* on the other hand, each clustered with a bootstrap confidence of 100%. The separation of *H. breviflora* and *H. stenosiphon* from *H. longituba* was sustained by a bootstrap value of 74% or 82%. *Hessea* and *Dewinterella* were separated, supported by a 100% bootstrap. The same bootstrap value indicated the monophyly of *Namaquanula* which turned out to be a sister group of *Hessea/Dewinterella* with a 100% bootstrap confidence.

The PHYLIP maximum likelihood tree (not shown) further confirmed the topology of the parsimony tree.

The most parsimonious trees (PAUP) obtained only from the single ITS 1 and ITS 2 regions both had a high CI value (0.894 and 0.813) which pointed to a strong phylogenetic signal in the data. Both trees turned out to be identical in their tree topology compared to the other trees. Only in some species of *Hessea* did both trees present polytomies.

Tree length analysis. The comparison of the tree length between the two different dendrograms (Figs. 1, 3) showed that 28 additional steps would be necessary to impose Snijman's tree topology on the molecular data.

Discussion

According to Snijman's subclade A^1 *Hessea mathewsii* and *H. pulcherrima* (= *Dewinterella pulcherrima*) are presented as a sister group of *H. bruce-bayeri* (= *Namaquanula bruce – bayeri*) (Fig. 1). The DNA phylogeny, however, showed that *Dewinterella* is a sister group of *Hessea*, indicated by a 100% bootstrap confidence. *Namaquanula* turns out to be the sister group to *Dewinterella* and *Hessea* which is also sustained by the maximum bootstrap value of 100% (Fig. 3). The result of this parsimony analysis was confirmed by the same results gained from the distance analysis and maximum likelihood trees.

This result indicates that the generic rank should be used instead of the subgeneric and sectional rank for all three taxa.

Figure 4 compares of Snijman's modified dendrogram (Fig. 1) on the left hand side and

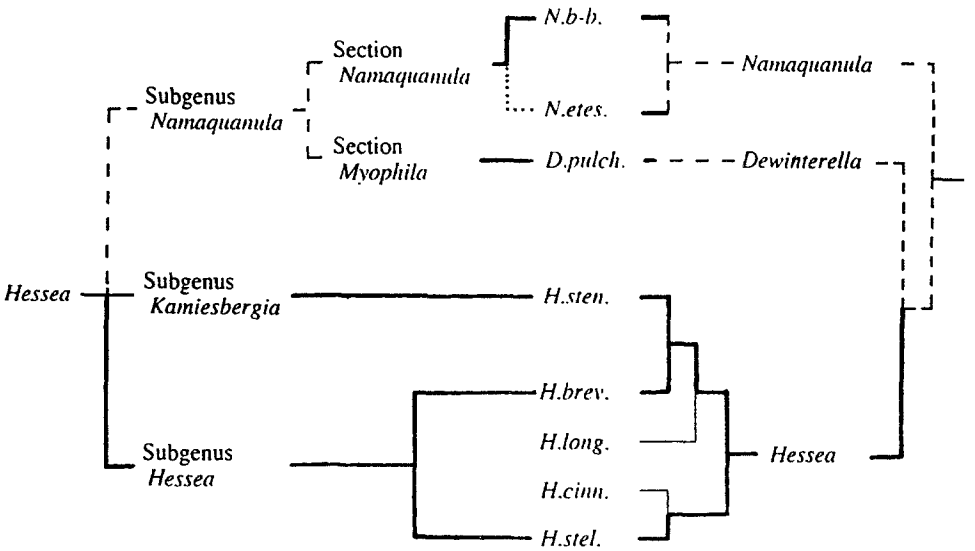

Fig. 4. Comparison of Snijman's dendrogram on the left hand side and the dendrogram based on the DNA phylogeny on the right hand side. Bold lines mark the species investigated on both sides; bold lines also unite the taxa *Hessea* subgen. *Hessea* and subgen. *Kamiesbergia* on the left and the taxon *Hessea* s.str. (sensu Müller-Doblies and Müller-Doblies) on the right side; broken line shows H. subgen. *Namaquanula* on the left and *Namaquanula* and *Dewinterella* on the right side; dotted line indicates that this species is represented in Snijman's work but not described by her; asterisks indicate species which are recognized by Müller-Doblies and Müller-Doblies but not by Snijman

the dendrogram based on the DNA phylogeny (Fig. 3) on the right hand side. Different types of lines are used which link taxa in Snijman's dendrogram and correspond to the taxa in the DNA dendrogram so that another fact becomes obvious. Either the monotypic subgenus *Kamiesbergia* has to be sunk into *Hessea* s.str. because the DNA analysis proved *Hessea stenosiphon* to be the sister taxon of *H. breviflora*, or the genus *Hessea* needs to be subdivided into at least four subgenera just to accommodate the five investigated species of *Hessea*.

Despite the conviction that *Hessea breviflora* and *H. stellaris* are sister taxa (Snijman 1994; Fig. 1), the DNA phylogeny does not support this proposal, for these two species do not cluster as a sister group. All molecular analyses showed that there are two groups of the studied species of *Hessea*, *H. breviflora*, *H. stenosiphon*, and *H. longituba* on the one hand and *H. cinnabarina* and *H. stellaris* on the other hand (Fig. 3).

In this context the question has to be raised which of the two patterns uniting the three genera, *Hessea*, *Dewinterella*, and *Namaquanula*, is to be preferred, the dendrogram of the DNA analysis or the one based on Snijman's suggestion (Figs. 3, 1). To begin with, the strong signals in the DNA (e.g. high bootstrap values, the high number of informative sites, high consistency index) strongly support the reconstructed phylogeny. Furthermore, the comparison of the tree lengths of both dendrograms on the basis of the sequences proved the DNA dendrogram to be shorter and therefore more probable. If one also takes the vegetative characters, bulb structures, and floral characters of these three genera into consideration, the affinities are very clear. *Hessea* and *Dewinterella* have more characteristics in common, e.g. two foliage leaves and a sheathing cataphyll, than *Dewinterella* and *Namaquanula*, whose foliage leaves differ in number from one to five and which lack a sheathing cataphyll (Table 4). The only

Table 4. Morphological comparison between three genera *Hessea*, *Dewinterella*, and *Namaquanula*

	Hessea and *Dewinterella*	*Namaquanula*
Vegetative characters		
Foliage leaves	2	1–5
Leaf in cross section	canaliculate	elliptic
Bulb structure		
Prophyll	usually present	absent
Sheathing cataphyll	present	absent
Floral characters		
Perigon	stellate (when fully expanded)	funnel-shaped
Perigon tube	absent or pterotube	eutube

common characters between *Namaquanula* and *Dewinterella* are the filament appendages and the adjacent papillae at the filament bases (Müller-Doblies and Müller-Doblies 1985, 1994). This morphological comparison stresses the result obtained from the DNA phylogeny, namely that there is a closer relation between *Hessea* and *Dewinterella* than between *Dewinterella* and *Namaquanula*.

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