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## Phylogeny of the Spiny African Daisies (Compositae, tribe Arctotideae, subtribe Gorteriinae) based on *trnL-F*, *ndhF*, and ITS sequence data

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## ABSTRACT

The tribe Arctotideae (African Daisies), of the flowering plant family Compositae (Asteraceae), is a diverse and interesting group with a primarily southern African distribution (ca. 13 genera, 215 species) and many species in the Cape Floristic Region. It is divided into two subtribes: Arctotidinae (ca. 5 genera, 85 species) and Gorteriinae (ca. 8 genera, 130 species). The monophyly of the genera within the subtribe Gorteriinae and their relationship to one another was investigated using 71 samples/212 sequences including 64/141 of which are newly reported from three phylogenetic markers, two from chloroplast DNA (*trnL-F* and *ndhF*) and one from the nuclear genome (ITS). The outgroup was composed of seven members from the sister subtribe. Results show the subtribe Gorteriinae to be divided into three monophyletic groups, the *Gazania-Hirpicium-Gorteria* group, the *Didelta* group, and the *Berkheya-Cullumia* group. Within these three groups are 13 sub-groups, one of which has sub-clades. The genus *Berkheya* Ehrh. is paraphyletic, falling into five different sub-groups. The two monotypic genera, *Cuspidia* and *Heterorhachis* are not nested within any of the *Berkheya* clades. *Hirpicium* and *Cullumia* each have most of their taxa in a monophyletic group, but they also have one or two taxa associated with other clades. Four of the five sub-groups of *Berkheya* have morphologically recognizable shared characters, such as habit and spines that have been recognized by past studies. However, the grouping of one species with *Didelta* is difficult to explain. Support for the major clades and most of the sub-groups is strong but the relationships among some of the terminal taxa are variable.

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## 1. Introduction

The Compositae family has the largest number of species of any family of seed plants (23,000–30,000) and today its members can be found on every continent except Antarctica. The advent of DNA sequence data from the chloroplast and nuclear genomes has changed Compositae systematics in dramatic ways. First, by identifying basal clades that turned the ideas about evolution within the family upside down (Jansen et al., 1991; Kim and Jansen, 1995) and more recently by revisions that have divided the family into 10 or more subfamilies and 35 tribes (Baldwin et al., 2002; Panero and Funk, 2002). The Arctotideae subtribe Gorteriinae is the subject of our current investigations; it is particularly interesting not only because it is more or less confined to southern Africa, but also because many of its species are endemic to the Cape Floristic Region (sensu Goldblatt and Manning (2000).

The tribe Arctotideae belongs to the re-defined subfamily Cichorioideae s.s. (Panero and Funk, 2002; Funk et al., 2005).

The subfamily contains four traditional, larger tribes: the Arctotideae (African daisies), Lactuceae (dandelions), Liabeae (Andean sunflowers), and Vernonieae (Ironweeds), as well as three small tribes containing one or two genera each: Eremothamneae (*Eremothamnus* O. Hoffm. and *Hoplophyllum* DC.), Gundeliaceae (*Gundelia* L., Crown of Thorns), and Moquineae (*Moquinea* DC. and *Pseudostiffia* H. Rob.) and two unplaced genera (*Platycarpha* and *Heterolepis*).

Data from a variety of sources (Cassini, 1816, 1821; Beauverd, 1915a,b; Robinson and Brettell, 1973; Norlindh, 1977; Robinson, 1992, 1994; Bremer, 1994; Herman et al., 2000; Leistner, 2000; Funk et al., 2004; Karis, 2006a, 2007) show a lack of characters, both morphological and molecular, to support the monophyly of the Arctotideae, however, the two main subtribes, Arctotidinae and Gorteriinae, are well supported (Funk et al., 2004; Karis, 2006a, 2007). A third monotypic subtribe the Gundeliaceae, is sometimes recognized, however, recent studies have shown that this monotypic genus does not belong in the tribe and that the most likely placement is as the sister group to the tribe Lactuceae (Karis et al., 2001; Panero and Funk, 2002; Funk et al., 2004). A short history of the classification within the tribe and a discussion on the morphology can be found in Funk et al. (2004).

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The only revision of the Gorteriinae was published by Roessler (1959). In his treatment only *Berkheya* was divided into Series. *Berkheya*, which had 72 species when treated by Roessler but now probably has over 75, is by far the largest genus in the subtribe. A recent paper by Karis (2006a) presented a cladistic analysis of the subtribe Gorteriinae using morphological characters, unfortunately many of the taxa used in the two studies are not the same. Future plans are underway to fill in all those gaps and to produce a combined analysis (Karis and Funk, personal communication).

The goals of this paper are: (a) to investigate the monophyly of the genera found within the subtribe Gorteriinae using molecular data, (b) to better resolve the subclades identified in Funk et al. (2004), (c) to use the phylogeny to help set up a sound basis for a generic, and possibly sub-generic classification of the tribe, (d) to examine past classifications in light of the phylogeny, and (e) to determine the direction of future studies. This is the final paper of the initial efforts to understand the overall patterns with the tribe Arctotideae that began in 1997 (Funk et al., 2004, 2007; Holland and Funk, 2006; Wortley et al., 2008).

## 2. Materials and methods

The outgroup was formed using members of the sister subtribe, Arctotideae and included five samples from *Haplocarpha*, one from the monotypic genus *Dymondia*, and one from *Arctotis* and it rooted the tree without major problems but by a long branch. Previous analyses (Funk et al., 2004, 2007) have shown that *Haplocarpha* is paraphyletic and that *Dymondia* and *Arctotis* are more highly nested. Both subtribes, as currently circumscribed, are monophyletic so the outgroup is shown as a monophyletic group.

For this study we consider the ingroup to include all taxa that were shown by Funk et al. (2004) to be members of the subtribe Gorteriinae (8 genera, ca. 130 species). All of the ingroup genera were sampled including 51 species with 64 samples (Tables 1 and 2). The data included 20 species (25 samples) from the largest genus *Berkheya* representing all but two of eight series recognized by Roessler (1959) in his monograph of the subtribe. Other genera included *Hirpicium* (7 species/7 samples), *Gazania* (8/10), *Cullumia* (5/7), *Gorteria* (5/6), *Didelta* (2/5), and the monotypic genera *Cuspidia* (1/2) and *Heterorhachis* (1/2). Most plant samples were collected in the field and stored in silica gel, however some samples were taken from herbarium specimens (see Table 2).

Taxon selection was guided by the Roessler revision (1959) and Table 4 lists the taxa sampled from each of the eight Series of *Berkheya*. One missing Series is the monotypic *Angustae*; we were unable to find *B. angusta* and there were no recent collections. Also missing is Series *Armatae*; we have collections of two species from this Series but were unable to get at least two of the three markers from either of them. Each marker was first analyzed individually, then the chloroplast data were analyzed together, and finally all the data were combined for analysis.

### 2.1. DNA amplification and sequencing

DNA extractions were performed using a Qiagen DNeasy Plant Mini Kit following the instructions supplied but with an extended incubation period (up to 40 min) for herbarium material. Primer ITS5A (Downie and Katz-Downie, 1996), based on White et al.'s (1990) fungal primer ITS5 and corrected at two positions for angiosperms, was substituted for ITS5 in this study. All primer sequences are given in Table 3. Primers used to amplify and sequence the *trnL*-F and downstream *trnL*-F spacer region of chloroplast DNA were designed by Taberlet et al. (1991) and those used for the 3' end of the *ndhF* region were designed by Jansen (1992).

**Table 1**

Genera of the subtribe Gorteriinae and the outgroup (information taken from Herman et al. (2000))

Genera	Total No. of species/ □#used in this study	Distribution
Outgroup: Subtribe Arctotideae		
<i>Haplocarpha</i> Less.	10/5	Africa, 5 in southern Africa
<i>Dymondia</i> Compton	1/1	South Africa, Western Cape
<i>Arctotis</i> L.	60+/1	Southern Africa & Angola; mostly in Eastern & Western Cape and Namaqualand
Ingroup: Subtribe Gorteriinae		
<i>Berkheya</i> Ehrh.	ca. 75/20	71 in South Africa, rest in tropical Africa
<i>Cullumia</i> R. Br.	15/5	South Africa: Northern and Western Cape
<i>Cuspidia</i> Gaertn.	1/1	South Africa: Northern, Western and Eastern Cape
<i>Didelta</i> L'Herit	2/2	Southern Namibia and South Africa: the western parts of the Northern and Western Cape
<i>Gazania</i> Gaertn.	17/9	Mainly Namibia and South Africa but one species reaching into Angola and Tanzania and one to Mozambique
<i>Gorteria</i> L.	3/4 (2 new to science)	Southern Namibia and Southern Africa: Northern Cape and along the west and southern coastal regions of the Western and Eastern Cape
<i>Heterorhachis</i> Sch. Bip. ex Walp.	1/1	South Africa: Northern and Western Cape
<i>Hirpicium</i> Cass.	12/7	Southern and tropical Africa; 8 in southern Africa

For the PCR amplification reactions, each 25 µl PCR cocktail contained 12.9 µl of sterile water, 2.5 µl of 10× PCR buffer A (Promega), 2 µl of 20 mM dNTPs (Pharmacia) in an equimolar ratio, 2.5 µl of 25 mM magnesium chloride, 0.5 µl of 10 mg/µl Bovine Serum Albumin (Sigma), 1 µl of a 10 µM concentration of the forward primer, 1 µl of a 10 µM concentration of the reverse primer, 0.1 µl of *Taq* DNA polymerase enzyme (5 U/µl from Promega), and 2.5 µl of sample DNA. The amount of template DNA was adjusted when necessary to generate sufficient PCR products for DNA sequencing.

The amplification reactions were conducted using thin-walled 0.2 ml PCR tubes in a GeneAmp PCR System 9700 (Perkin-Elmer). The PCR program consisted of an initial preheating at 94 °C for 2 min. Then, the first reaction cycle proceeded as follows: 1 min at 94 °C to denature the template DNA, followed by 1 min at 48 °C (54 °C for cpDNA) to allow primer annealing and 2 min at 72 °C for primer extension. Primer extension time was increased by 4 s (7 s for cpDNA) for each subsequent reaction cycle. After a total of 40 reaction cycles, an additional 7-min extension at 72 °C was allowed for completion of unfinished DNA strands. All PCR products were quantified by agarose gel electrophoresis with comparison of an aliquot of products with a known quantity of a 100-bp DNA ladder (GeneChoice; visualized with ethidium bromide). The remainder was stored at 4 °C until utilized.

**Table 2**  
Source of sequence data and GenBank accession numbers

Tube#	Genus	Species	Authority	Locality	Date	Collector	No.	Herb	ITS	trnL	ndhF
128	<i>Berkheya</i>	<i>angolensis</i>	O. Hoffm.	Angola	23-Dec-30	Grossweiler	9572	US	<b>EU527205</b>	<b>EU527255</b>	<b>EU527305</b>
230	<i>Berkheya</i>	<i>annecteus</i>	Harv.	So. Afr., NC	26-Aug-04	Funk & Koekemoer	12524	US	<b>EU527211</b>	<b>EU527261</b>	<b>EU527308</b>
26	<i>Berkheya</i>	<i>bipinnatifida</i>	(Harv.) Roessler	Tanzania	6-May-87	Lovett, Keeley, & Lyser	2112	MO	<b>EU527198</b>	<b>EU527248</b>	<b>EU527298</b>
29	<i>Berkheya</i>	<i>canescens</i>	DC.	So. Afr., NC	30-Aug-99	Trinder-Smith	347	US	<b>EU527201</b>	<b>EU527251</b>	<b>EU527301</b>
274	<i>Berkheya</i>	<i>cardapatifolia</i>	(DC.) Roessler	So. Afr., NC	27-Aug-04	Funk & Koekemoer	12537	US	<b>EU527213</b>	<b>EU527263</b>	<b>EU527310</b>
18	<i>Berkheya</i>	<i>carlinopsis</i>	Welw. Ex O. Hoffm.	So. Afr., Transvaal	27-Mar-85	Bourell et al.	2689	MO	AY504709	AY504791	AY504751
224	<i>Berkheya</i>	<i>circisifolia</i>	(DC.) Roessler	So. Afr., Free State	11-Jan-03	Funk & Koekemoer	12402	US	<b>EU527207</b>	<b>EU527257</b>	None
17	<i>Berkheya</i>	<i>coddii</i>	Roessl.	So. Afr., Transvaal	26-Apr-84	Balkwill et al.	1460	MO	<b>EU527194</b>	<b>EU527244</b>	<b>EU527294</b>
99	<i>Berkheya</i>	<i>cruciata</i>	Willd.	So. Afr., WC	11-Oct-00	Koekemoer	2002	PRE	AY504712	AY504794	AY504754
126	<i>Berkheya</i>	<i>echinacea</i>	(Harv.) Burt Davy	S & SW Africa	31-Jan-59	Werdermann & Oberdieck	2099	US	<b>EU527204</b>	<b>EU527254</b>	<b>EU527304</b>
229	<i>Berkheya</i>	<i>eriobasis</i>	(DC.) Roessler	So. Afr., NC	29-Aug-04	Funk & Koekemoer	12546	US	<b>EU527210</b>	<b>EU527260</b>	<b>EU527307</b>
276	<i>Berkheya</i>	<i>eriobasis</i>	(DC.) Roessler	So. Afr., NC	29-Aug-04	Funk & Koekemoer	12544	US	<b>EU527214</b>	<b>EU527264</b>	<b>EU527311</b>
23	<i>Berkheya</i>	<i>fruticosa</i>	Ehrh.	So. Afr., NC	16-Sep-00	Koekemoer & Funk	1955	PRE	<b>EU527197</b>	<b>EU527247</b>	<b>EU527297</b>
27	<i>Berkheya</i>	<i>fruticosa</i>	Ehrh.	So. Afr., WC	19-Aug-99	Trinder-Smith	53	US	<b>EU527199</b>	<b>EU527249</b>	<b>EU527299</b>
231	<i>Berkheya</i>	<i>onobromoides</i>	O. Hoffm. & Muschler	So. Afr., NC	30-Aug-04	Funk & Koekemoer	12551	US	<b>EU527212</b>	<b>EU527262</b>	<b>EU527309</b>
226	<i>Berkheya</i>	<i>pannosa</i>	Hilliard	So. Afr., KZ-N	16-Jan-03	Funk & Koekemoer	12423	US	<b>EU527209</b>	<b>EU527259</b>	None
22	<i>Berkheya</i>	<i>pinnatifida</i>	(Thunb.) Thellung	So. Afr., NC	15-Sep-00	Koekemoer & Funk	1946	PRE	<b>EU527196</b>	<b>EU527246</b>	<b>EU527296</b>
168	<i>Berkheya</i>	<i>rhapontica</i>	(DC.) Hutchinson & Burt Davy	So. Afr.	1975	Bayliss	6989	S	<b>EU527206</b>	<b>EU527256</b>	<b>EU527306</b>
19	<i>Berkheya</i>	<i>rigida</i>	(Thunb.) Bolus & W-D	Australia, WA	12-Dec-99	Funk	12211	US	<b>EU527195</b>	<b>EU527245</b>	<b>EU527295</b>
225	<i>Berkheya</i>	<i>setifera</i>	DC.	Swaziland	20-Nov-02	Koekemoer	2527	PRE	<b>EU527208</b>	<b>EU527258</b>	None
106	<i>Berkheya</i>	<i>spinosa</i>	Druce	So. Afr., WC	24-Oct-00	Koekemoer	2041	PRE	<b>EU527202</b>	<b>EU527252</b>	<b>EU527302</b>
24	<i>Berkheya</i>	<i>spinossissima</i>	Willd.	So. Afr., NC	18-Sep-00	Koekemoer & Funk	1962	PRE	AY504710	AY504792	AY504752
28	<i>Berkheya</i>	<i>spinossissima</i>	Willd.	So. Afr., NC	30-Aug-99	Trinder-Smith	346	US	<b>EU527200</b>	<b>EU527250</b>	<b>EU527300</b>
125	<i>Berkheya</i>	<i>subulata</i>	Harv.	So. Afr., KZ-N	1-May-59	Sidey	3371	US	<b>EU527203</b>	<b>EU527253</b>	<b>EU527303</b>
16	<i>Berkheya</i>	<i>zeyheri</i>	(Sond. & Harv.) Oliv. & Hiern.	So. Afr., Transvaal	28-Apr-91	Balkwill et al.	6451	MO	<b>EU527193</b>	<b>EU527243</b>	<b>EU527293</b>
102	<i>Cullumia</i>	<i>aculeata</i>	(Houtt.) Roessler	So. Afr., WC	13-Oct-00	Koekemoer	2017	PRE	<b>EU527219</b>	<b>EU527269</b>	<b>EU527316</b>
36	<i>Cullumia</i>	<i>bisulca</i>	(Thunb.) Less.	So. Afr., Cape Prov.	4-Sep-78	Goldblatt, P.	4807	MO	<b>EU527217</b>	<b>EU527267</b>	<b>EU527314</b>
37	<i>Cullumia</i>	<i>bisulca</i>	(Thunb.) Less.	So. Afr., WC	14-Sep-00	Koekemoer & Funk	1935	PRE	AY504713	AY504795	AY504755
40	<i>Cullumia</i>	<i>bisulca</i>	(Thunb.) Less.	So. Afr., WC	8-Sep-00	Funk & Koekemoer	12271	PRE	<b>EU527218</b>	<b>EU527268</b>	<b>EU527315</b>
34	<i>Cullumia</i>	<i>decurrens</i>	Less.	So. Afr., EC	7-Sep-00	Funk & Koekemoer	12262	US	<b>EU527216</b>	<b>EU527266</b>	<b>EU527313</b>
32	<i>Cullumia</i>	<i>patula</i>	(Thunb.) Less.	So. Afr., WC	25-Sep-72	Bremer	217	MO	<b>EU527215</b>	<b>EU527265</b>	<b>EU527312</b>
39	<i>Cullumia</i>	<i>rigida</i>	DC.	So. Afr., NC	1-Aug-99	Trinder-Smith	182	US	AY504714	AY504796	AY504756
42	<i>Cuspidia</i>	<i>cernua</i>	(L. f.) B. L. Burt	So. Afr., WC	6-Oct-83	Retief & Reid	77	MO	<b>EU527220</b>	<b>EU527270</b>	<b>EU527317</b>
104	<i>Cuspidia</i>	<i>cernua</i>	(L. f.) B. L. Burt	So. Afr., EC	7-Oct-00	Koekemoer	1986	PRE	AY504715	AY504797	AY504757
52	<i>Didelta</i>	<i>carcosa</i>	Ait.	So. Afr., NC	1-Aug-99	Trinder-Smith	202	US	<b>EU527222</b>	<b>EU527272</b>	<b>EU527319</b>
50	<i>Didelta</i>	<i>carcosa</i>	Ait.	So. Afr., WC	14-Sep-00	Koekemoer & Funk	1943	PRE	AY504716	AY504798	AY504758
49	<i>Didelta</i>	<i>spinosa</i>	(L. f.) Ait.	So. Afr., WC	14-Sep-00	Koekemoer & Funk	1936	PRE	<b>EU527221</b>	<b>EU527271</b>	<b>EU527318</b>
51	<i>Didelta</i>	<i>spinosa</i>	(L. f.) Ait.	So. Afr., NC	21-Aug-99	Trinder-Smith	142	US	AY504717	AY504799	AY504759
275	<i>Didelta</i>	<i>spinosa</i>	(L. f.) Ait.	So. Afr., NC	27-Aug-04	Funk & Koekemoer	12536	US	<b>EU527223</b>	<b>EU527273</b>	<b>EU527320</b>
65	<i>Gazania</i>	<i>heterochaeta</i>	DC.	So. Afr., NC	24-Aug-99	Trinder-Smith	204	US	<b>EU527228</b>	<b>EU527278</b>	None
55	<i>Gazania</i>	<i>krebsiana</i>	Less.	So. Afr., EC	8-Sep-00	Funk & Koekemoer	12270	US	<b>EU527224</b>	<b>EU527274</b>	<b>EU527321</b>
62	<i>Gazania</i>	<i>krebsiana</i>	Less.	So. Afr., NC	19-Sep-00	Koekemoer & Funk	1969	PRE	AY504719	AY504801	AY504761
60	<i>Gazania</i>	<i>krebsiana</i>	Less.	So. Afr., NC	15-Sep-00	Koekemoer & Funk	1947	PRE	<b>EU527225</b>	<b>EU527275</b>	<b>EU527322</b>
61	<i>Gazania</i>	<i>lichtensteini</i>	Less.	So. Afr., NC	15-Sep-00	Koekemoer & Funk	1952	PRE	<b>EU527226</b>	<b>EU527276</b>	<b>EU527323</b>
63	<i>Gazania</i>	<i>longiscarpa</i>	DC.	USA, California	24-Oct-92	Ricketson & Schmidt	4906	MO	<b>EU527227</b>	<b>EU527277</b>	None
59	<i>Gazania</i>	<i>new species</i>		So. Afr., WC	13-Sep-00	Koekemoer & Funk	1929	PRE	AY504718	AY504800	AY504760
105	<i>Gazania</i>	<i>rigens var rigens</i>	(DC.) Roessler	Australia, WA	12-Dec-99	Funk	12210	US	<b>EU527229</b>	<b>EU527279</b>	<b>EU527324</b>
242	<i>Gazania</i>	<i>rigida</i>	(Burm. f.) Roessler	So. Afr., NC	27-Aug-04	Funk & Koekemoer	12540	US	<b>EU527230</b>	<b>EU527280</b>	None
64	<i>Gazania</i>	<i>temuifolia</i>	Less.	So. Afr., NC	18-Aug-99	Trinder-Smith	64	US	AY504720	AY504802	AY504762
137	<i>Gorteria</i>	<i>corymbosa</i>	DC.	So. Afr., Namaq.	29-Aug-83	Roux	2739	BOL	<b>EU527232</b>	<b>EU527282</b>	None
73	<i>Gorteria</i>	<i>diffusa</i>	Thunb.	So. Afr., WC	s.d.	Trinder-Smith	103	US	AY504722	AY504804	AY504763
71	<i>Gorteria</i>	<i>diffusa</i>	Thunb.	So. Afr., NC	15-Sep-00	Koekemoer & Funk	1945	PRE	<b>EU527231</b>	<b>EU527281</b>	<b>EU527325</b>
170	<i>Gorteria</i>	<i>new species A</i>		So. Afr., NC	23-Aug-04	Funk & Koekemoer	12496	US	<b>EU527234</b>	<b>EU527284</b>	<b>EU527327</b>
166	<i>Gorteria</i>	<i>new species B</i>		So. Afr., NC	1-Sep-04	Funk & Koekemoer	12556	US	<b>EU527233</b>	<b>EU527283</b>	<b>EU527326</b>
69	<i>Gorteria</i>	<i>personnata</i>	L.			Dodd	289	US	AY504721	AY504803	None
115	<i>Heterorhachis</i>	<i>aculeata</i>	(Burm. f.) Roessler	So. Afr., NC	11-Oct-58	Acocks	19737	BOL	<b>EU527235</b>	<b>EU527285</b>	<b>EU527328</b>

(continued on next page)

Table 2 (continued)

Tube#	Genus	Species	Authority	Locality	Date	Collector	No.	Herb	ITS	trnL	ndhF
172	<i>Heterorhachis</i>	<i>aculeata</i>	(Burm. f.) Roessler	So. Afr., NC	30-Aug-04	Funk & Koekemoer	12550	US	<b>EU527236</b>	<b>EU527286</b>	<b>EU527329</b>
90	<i>Hirpicium</i>	<i>bechuanense</i>	(S. Moore) Roessler	Rhodesia	10-Jan-63	Wild	5978	MO	<b>EU527238</b>	<b>EU527288</b>	None
132	<i>Hirpicium</i>	<i>diffusum</i>	(O. Hoffm.) Roessler	Kenya, Nairobi	28-Jun-87	Faden & Ng'weno	87-36	US	<b>EU527241</b>	<b>EU527291</b>	None
25	<i>Hirpicium</i>	<i>echinus</i>	Less.	So. Afr., NC	19-Sep-00	Koekemoer & Funk	1966	PRE	AY504724	AY504806	AY504764
87	<i>Hirpicium</i>	<i>gazanioides</i>	(Harv.) Roessler	Namibia	9-Apr-87	Long & Rae	734	MO	AY504726	AY504808	AY504766
131	<i>Hirpicium</i>	<i>gorterioides</i>	(Oliv. & Hiern) Roessler	S & SW Africa	1-Mar-59	Werdermann & Oberdieck	2335	US	<b>EU527240</b>	<b>EU527290</b>	<b>EU527332</b>
89	<i>Hirpicium</i>	<i>integrifolium</i>	Less.	So. Afr., WC	19-Aug-99	Trinder-Smith	52	US	<b>EU527237</b>	<b>EU527287</b>	<b>EU527330</b>
130	<i>Hirpicium</i>	<i>pechuelii</i>	(O. Kuntze) O. Hoffm.	SW Africa	20-Apr-30	Sordah	6467	US	<b>EU527239</b>	<b>EU527289</b>	<b>EU527331</b>
	Outgroups										
243	<i>Arctotis</i>	<i>campanulata</i>	DC.	So. Afr., NC	25-Aug-04	Funk & Koekemoer	12519	US	<b>EU527192</b>	<b>EU527242</b>	<b>EU527292</b>
53	<i>Dymondia</i>	<i>margaretae</i>	Compton	So. Afr., WC	27-Feb-00	Trinder-Smith	197	US	AY504707	AY504789	AY504749
100	<i>Haplocarpha</i>	sp.	Less.	So. Afr., WC	14-Sep-00	Koekemoer	1941	PRE	DQ889641	DQ889657	DQ889672
139	<i>Haplocarpha</i>	<i>nervosa</i>	Beauverd.	Lesotho	14-Jan-03	Funk & Koekemoer	12417	PRE	DQ889643	DQ889659	DQ889674
76	<i>Haplocarpha</i>	<i>ruppallii</i>	(Sch. Bip.) A. Rich.	Kenya, Meru	16-Aug-85	Robertson, et al.	3960	MO	DQ889640	DQ889656	None
77	<i>Haplocarpha</i>	<i>scaposa</i>	Harv.	Lesotho	3-Feb-00	Trinder-Smith	191	US	AY504708	AY504790	AY504750
138	<i>Haplocarpha</i>	<i>scaposa</i>	Harv.	So. Afr., Free State	11-Jan-03	Funk & Koekemoer	12401	US	DQ889642	DQ889658	DQ889673

Newly reported sequence data have accession numbers in bold.

Table 3  
Primer sequences used for PCR and cycle sequencing

Name	Sequence (5' to 3')
ITS5A	GGA AGG AGA AGT CGT AAC AAG G
ITS4	TCC TCC GCT TAT TGA TAT GC
<i>trnL-F</i> C	CGA AAT CGG TAG ACG CTA CG
<i>trnL-F</i> F	ATT TGA ACT GGT GAC ACG AG
<i>ndhF</i> 1603	CCT YAT GAA TCG GAC AAT ACT ATG C
<i>ndhF</i> + 607	ACC AAG TTC AAT GYT AGC GAG ATT AGT C

PCR products used for sequencing were first purified for sequencing using an enzymatic PCR product pre-sequencing kit (USB). This procedure involved mixing 8 µl of the PCR product with 1 µl of each enzyme from the kit and then incubating the mixture first at 37 °C for 30 min, to degrade excess primers and dNTPs, and then raising the temperature to 80 °C for 15 min, to denature the enzymes themselves. This method of purification without loss of PCR products (no filtration, precipitation, or washes are necessary) is especially important for DNA extracted from herbarium vouchers, which is sometimes only weakly amplified and yields barely sufficient PCR product for sequencing.

The cycle sequencing reactions were done using 96-well microplates in a PTC-100 thermal cycler (MJ Research). Each one-eighth cycle sequencing reaction cocktail contains 50–150 ng of the purified PCR product, 2 µl of a 1 µM concentration of the sequencing primer, 0.6 µl of a 5× reaction buffer (400 mM Tris-HCl, 10 mM magnesium chloride at pH 9.0), and 1 µl of the reagent pre-mix from the BigDye (Version 2/3) dye terminator cycle sequencing pre-mix kit (Applied Biosystems). The cycle sequencing program consisted of an initial preheating at 96 °C for 30 s. Then, the first reaction cycle proceeded as follows: 10 s at 92 °C to denature the template DNA, followed by 15 s at 55 °C to allow primer annealing and 4 min at 60 °C for primer extension. Unincorporated dye terminators were removed by Sephadex (Sigma) gel filtration using MultiScreen plates (Millipore). The purified cycle sequencing products were then resolved by electrophoresis on a 5% polyacrylamide (MJ Research Kilobasepack) gel using a BaseStation 51 automated DNA sequencer (MJ Research). Sequences from both strands of each PCR product were examined, compared, and corrected using Sequence Navigator software (Applied Biosystems).

All *trnL-F*, *ndhF*, and ITS sequences were aligned visually, with the insertion of gaps where necessary. Maximum parsimony analysis and parsimony bootstrap analysis (with 1000 replicate runs, each with 10 random taxon additions, TBR branch swapping, and MULPARS in effect) of the aligned *trnL-F*, *ndhF*, and ITS sequences were performed (with and without the outgroups) for each marker and for the cpDNA data and the combined data sets via full heuristic searches with PAUP\* (Swofford, 2002). No character weighting was used. The likelihood ratio tests were done using the 'Tree Scores' function in PAUP\* under the likelihood criterion, the Hasegawa-Kishino-Yano model of sequence evolution (Hasegawa et al., 1985), and a gamma distribution of rate variation among sites (with the shape parameter estimated and with four rate categories).

### 3. Results

No matter what method was used to analyze the data, three major groups are resolved within the Gorteriinae: the *Didelta* clade, *Berkheya-Cullumia* clade, and *Gazania-Hirpicium-Gorteria* clade; *Berkheya* and *Hirpicium* are always paraphyletic (Figs. 1–7). To facilitate discussion the three major groups have been separated into 13 sub-groups: The *Gazania-Hirpicium-Gorteria* (HIR) clade contains four sub-groups one of which (Hir-2) may not be monophyletic: *Gazania* (Gaz), *Hirpicium*-1 (Hir-1), *Hirpicium*-2 (Hir-2), and *Gorteria* (Gor). The *Didelta* clade has two sub-groups: *Didelta*

**Table 4**

Taxon sampling based on classification by Roessler (1959)

Series Fruticosae: Type <i>B. fruticosa</i> (L.) Ehrh. Species 1–13 (5 of 13): 4. <i>B. fruticosa</i> ; 7. <i>B. spinosissima</i> ; 10. <i>B. carlinopsis</i> ; 12. <i>B. spinosa</i> ; 13. <i>B. canescens</i>
Series Angustae: Type <i>B. angusta</i> Schlechter Species 14 (none of 1): none
Series Cruciateae: Type <i>B. cruciata</i> (Houtt.) Willd. Species 15 (1 of 1): 15. <i>B. cruciata</i>
Series Armatae: Type <i>B. armata</i> (Vahl) Druce Species 16–21 (none of 6): none
Series Speciosae: Type <i>B. speciosa</i> (DC.) O. Hoffm. Species 22–30 (4 of 10): 22. <i>B. echinacea</i> ; 23. <i>B. rhapsontica</i> ; 28. <i>B. setifera</i> ; NEW. <i>B. pannosa</i>
Series Rigidae: Type <i>B. rigida</i> (Thunb.) Adams. & Salt. Species 31–44 (7 of 14): 31. <i>B. rigida</i> ; 34. <i>B. annectens</i> ; 37. <i>B. onobromoides</i> ; 39. <i>B. eriobasis</i> ; 40. <i>B. pinnatifida</i> ; 42. <i>B. bipinnatifida</i> ; 43. <i>B. cardopatifolia</i>
Series Subulatae: Type <i>B. subulata</i> Harv. Species 45–55 (4 of 11): 45. <i>B. zeyheri</i> ; 49. <i>B. subulata</i> ; 52. <i>B. coddii</i> ; 53. <i>B. angolensis</i>
Series Decurrentes: Type <i>B. decurrens</i> (Thunb.) Willd. Species 56–72 (1 of 17): 63. <i>B. cirsiifolia</i>

*Berkheya pannosa* was described in 1975 and was added where it keyed out using Roessler's key. Authorities for the taxa are in Table 2.

(Did) and *Berkheya*-1 (Ber-1). Finally, the *Berkheya-Cullumia* (BER) clade contains seven sub-groups one of which (Cul) may not be monophyletic: *Cuspidia* (Cus), *Berkheya*-2 (Ber-2), *Heterorhachis* (Het), *Berkheya*-3 (Ber-3), *Berkheya*-4 (Ber-4), *Berkheya*-5, and *Cullumia* (Cul).

Table 2 gives the GenBank numbers for the sequences used in this study, 141 are newly reported and 71 were taken from our previously published papers (Funk et al., 2004, 2007). All samples have internal transcribed spacer (ITS) and *trnL*-F sequences, but 11 are missing *ndhF* sequence data.

### 3.1. ITS

ITS nuclear sequence data were gathered for all ingroup and outgroup taxa. The analysis produced 5177 trees ( $L = 845$ ,  $\pi = 242$ ,  $CI = .560$ ,  $RI = .872$ ). The three main clades are strongly supported and all but two of 13 sub-groups are present. Fig. 1 is the strict consensus tree, note that the *Didelta* clade (DID) contains two samples of *Berkheya spinosissima* (Ber-1) and that the DID clade is sister to the BER clade. The relationships among the *Cullumia* taxa and *Berkheya*-4 and -5 are unresolved. Species of *Hirpicum* are found in two sub-groups: Hir-1 is sister to Hir-2 + *Gorteria*. The strict consensus had the same clades when the 11 taxa missing *ndhF* sequence data were removed from the analysis.

### 3.2. Chloroplast datasets

Neither of the chloroplast datasets, *ndhF* and *trnL*-F, had sufficient information to resolve the trees, however, the two datasets were combined to produce a larger number of characters that provided some overall structure although the tree continued to have unresolved areas. Eleven taxa were missing *ndhF* sequence data and were not included in the chloroplast analysis (1308 trees,  $L = 262$ ,  $\pi = 117$ ,  $CI = .790$ ,  $RI = .929$ ). The strict consensus tree for the chloroplast data (Fig. 2) shows that it is in basic agreement with the ITS tree, with two exceptions. First, the DID clade is sister to the HIR clade rather than the BER clade and second, Gaz is the sister group to the Gor + Hir-2 clade.

### 3.3. Combined data analysis

Data from all 71 samples (7 of which are part of the outgroup) were used for the combined data analysis (*trnL*-F, *ndhF*, ITS). Prior to examining the results of the rooted analyses, it is useful to examine an unrooted phylogram because it provides an unbiased look at the relationships among the ingroup taxa. Fig. 3 is one of the equally parsimonious trees of a run with all of the data and without the outgroup (544 trees;  $L = 753$ ,  $\pi = 289$ ,  $CI = .659$ ,  $RI = .914$ ). In separate runs the outgroup was placed on the tree using all the data (combined), the ITS data and the chloroplast data to determine what was causing the differences in the results shown in Figs. 1 and 2. Fig. 3 shows that the position of the outgroup attachment is

changing. Using only ITS data, the outgroup attaches so that DID is sister to BER (subsequent bootstrap runs show good support for this grouping). Using the chloroplast data, DID is sister to HIR (weak support). The combined analysis agreed with the ITS data, placing DID as the sister group to BER but with slightly less support than found in the ITS data. This figure shows clearly why the two areas of the phylogeny are showing conflicting patterns in the ITS and chloroplast trees. Likewise there are different attachments of the DID + BER clade to the HIR clade. The ITS data show Gaz as the sister to the other three sub-groups and the chloroplast data show Hir-1 as the sister to the other three sub-groups.

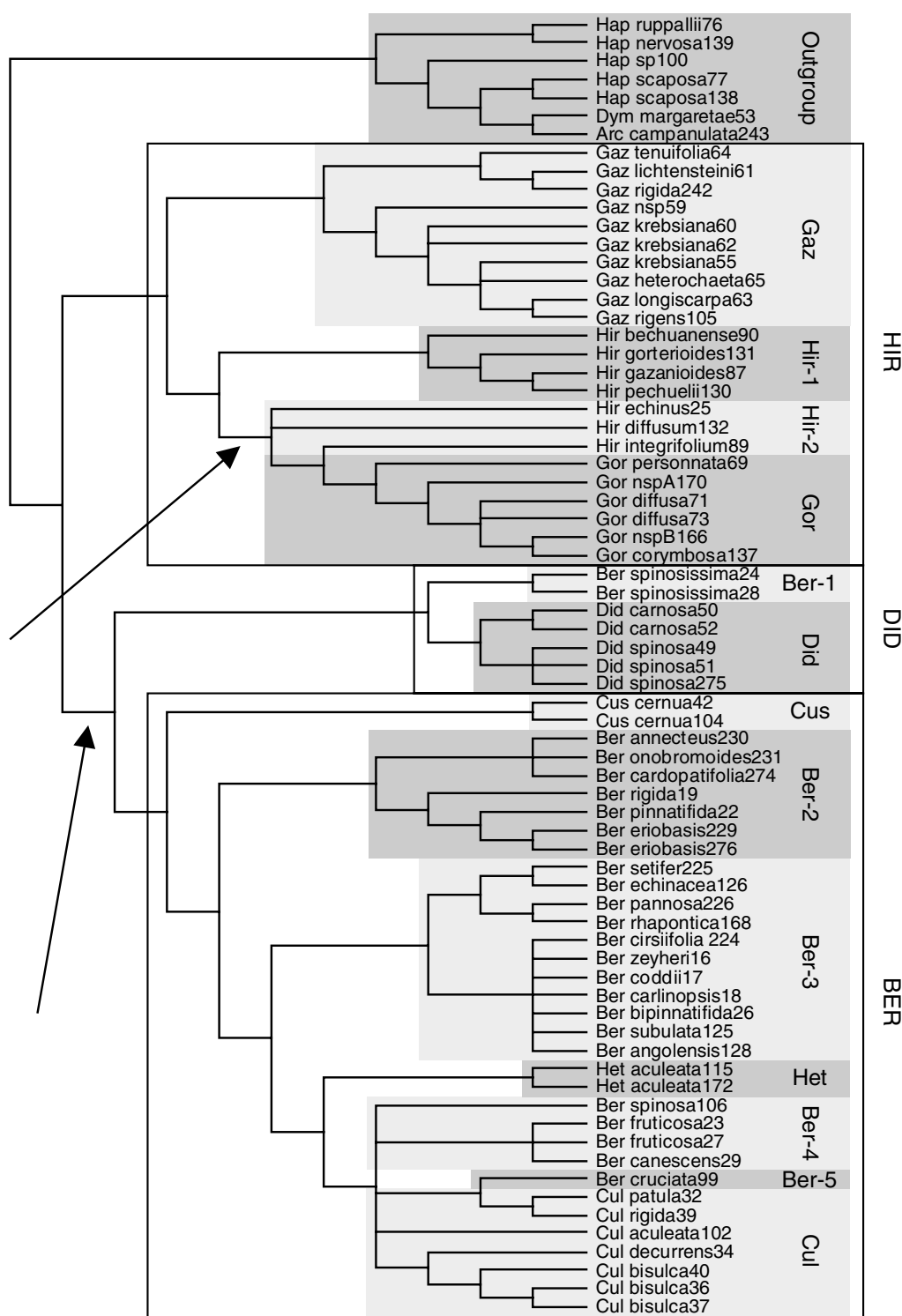
The parsimony analysis of all taxa and outgroups yielded 272 trees ( $L = 1130$ ;  $\pi = 365$ ;  $CI = .607$ ;  $RI = .882$ ), the strict consensus tree is shown in Fig. 4. Most of the equally parsimonious trees are the result of movement among closely related taxa in Ber-3. For instance, if one removes from Ber-3 three of the highly nested taxa that are part of a large polytomy (*Berkheya zeyheri*, *B. coddii*, and *B. carlinopsis*), then an analysis results in only eight equally parsimonious trees. An analysis run with all data but without the 11 taxa missing *ndhF* produced a strict consensus tree with the same basic structure.

Several taxa are present more than one time in the analysis. Except for *Gazania krebsiana* Less., all of the samples from the same species grouped together. In the case of *G. krebsiana* the three samples did not group with one another, but this is a variable species with many varieties and so it is no surprise that it would actually be several separate taxa. However, it is beyond the scope of this study to describe new taxa, especially when there are other varieties in this species and other species in this genus that were not sampled.

A bootstrap analysis was run on a reduced dataset that contained: one outgroup, no redundant taxa (except *Gazania krebsiana* and monotypic genera), without the three *Berkheya* species explained above (*B. zeyheri*, *B. coddii*, *B. carlinopsis*), and without any remaining taxa that were missing *ndhF* sequencing data. In the resulting bootstrap tree (Fig. 5) of 42 taxa, most branches were well supported including the DID + BER clade. However, three areas of the phylogeny had poor support. In the HIR clade, the Hir-2 + Gor clade grouped with Gaz rather than Hir-1 but with a bootstrap value of 65%; the position of *Heterorhachis* (Het) lost resolution; and several nodes in BER had values in the 60's, including the grouping of *B. cruciata* with *Cullumia*. There are two long branches belonging to the two monotypic genera; the same structure with similar values persisted when *Heterorhachis* was removed from the analysis. When *Cuspidia* was removed, there were four well supported branches in the BER clade in a polytomy (Het, Ber-2, Ber-3, and Ber 4 + 5 + Cul). When both *Heterorhachis* and *Cuspidia* were removed there were three well supported branches in the polytomy. In all of these alternatives, the connection between Ber 5 and Cul was poorly supported.

The maximum likelihood (ML) tree (Fig. 6) has three areas of interest. It agrees with the bootstrap tree (Fig. 5) but not with the strict consensus tree (Fig. 4) in having Hir-1 as the sister taxon

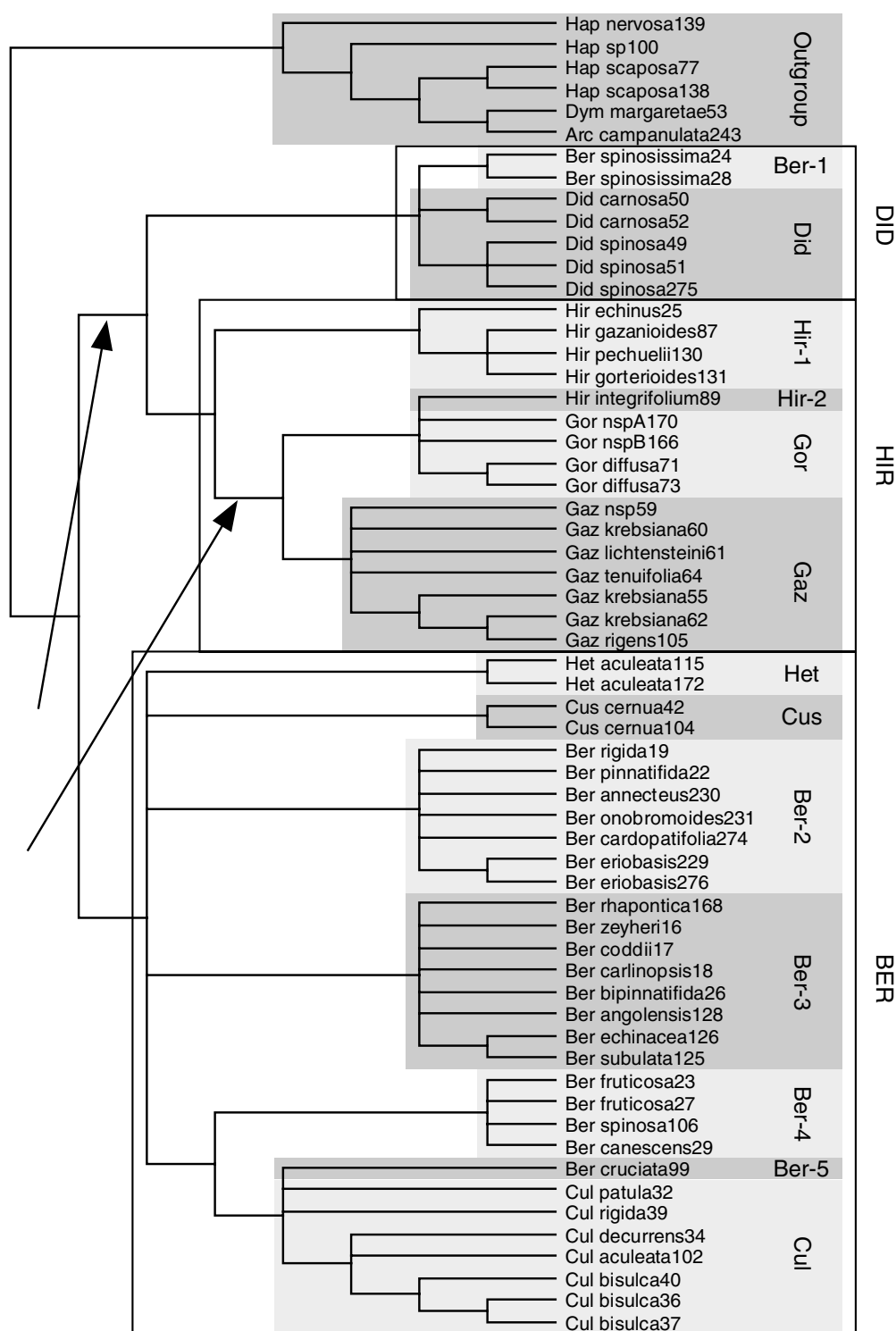




**Fig. 1.** A strict consensus tree based on ITS sequence data. Three main clades and 13 sub-groups are indicated on the diagram. The three main clades are: *Didelta* (DID), *Berkheya-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 sub-groups are: *Berkheya-1* (Ber-1), *Berkheya-2* (Ber-2), *Berkheya-3* (Ber-3), *Berkheya-4* (Ber-4), *Berkheya-5* (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium-1* (Hir-1), and *Hirpicium-2* (Hir-2). The analysis produced 5177 trees (L = 845, pi = 242, CI = .560, RI = .872). The arrows indicate the placement of the DID clade and the paraphyletic nature of *Hirpicium*.

of the rest of the HIR clade. New in the ML tree is the grouping of *Cuspidia* with Ber-2 rather than standing as the sister taxon to the rest of the BER clade as it is in the bootstrap and the parsimony trees. Finally, there is a weak grouping between Het + Ber-3 and Ber-2 + Cus; this grouping was not found in any other analysis. The latter two unique placements are on short branches in the ML tree.

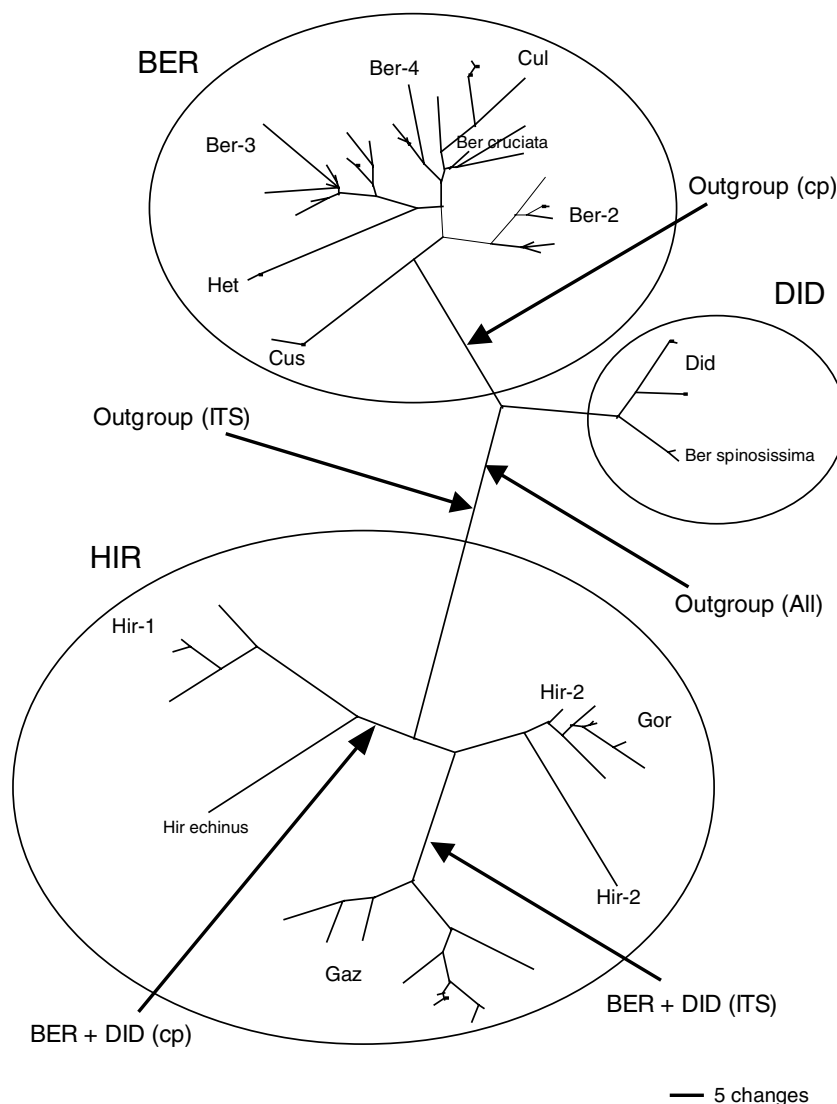
Table 5 gives the size of the aligned matrices, the number of gaps added in order to align the sequences, the number of informative characters, and the percent of sequence divergence (calculated using PAUP 4.0 pairwise distance option) for all three markers. When applicable, the figures were calculated for both the ingroup alone (ingroup) and for the ingroup plus the outgroup (all). This table shows that the percent sequence divergence is low in the



**Fig. 2.** A strict consensus tree based on *trnL-F* and *ndhF* (chloroplast) sequence data. Three main clades and 13 sub-groups are indicated on the diagram. The three main clades are: *Didelta* (DID), *Berkheya-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 sub-groups are: *Berkheya-1* (Ber-1), *Berkheya-2* (Ber-2), *Berkheya-3* (Ber-3), *Berkheya-4* (Ber-4), *Berkheya-5* (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium-1* (Hir-1), and *Hirpicium-2* (Hir-2). The analysis produced 1308 trees ( $L = 262$ ,  $\pi = 117$ ,  $CI = .790$ ,  $RI = .929$ ). The arrows indicate the grouping of Hir-2 + Gor with Gaz and the grouping of DID with HIR.

cpDNA (2.3–3.3% for *trnL-F* and 4.9–5.6% for *ndhF*) and higher in the ITS sequences (15.8–20.9%) all of which are in the normal range for these markers. The ITS, as usual, had the highest number of informative characters 193 and 242 which represented 28–36% of the total number of ITS characters. There were no alignment problems. The greatest distance in the ITS data—for just the in-

group—was found between *Gazania rigida* (see Table 2 for authorities) and *Berkheya spinosa*, in the *ndhF* data it was between *Berkheya bipinnatifida* and *Hirpicium echinus*, and in the *trnL-F* it was between *Hirpicium gazanioides* and three *Berkheya* species: *B. fruticosa*, *B. spinosa*, and *B. canescens*. As one might expect, the greatest distance is always between two long branches, one in



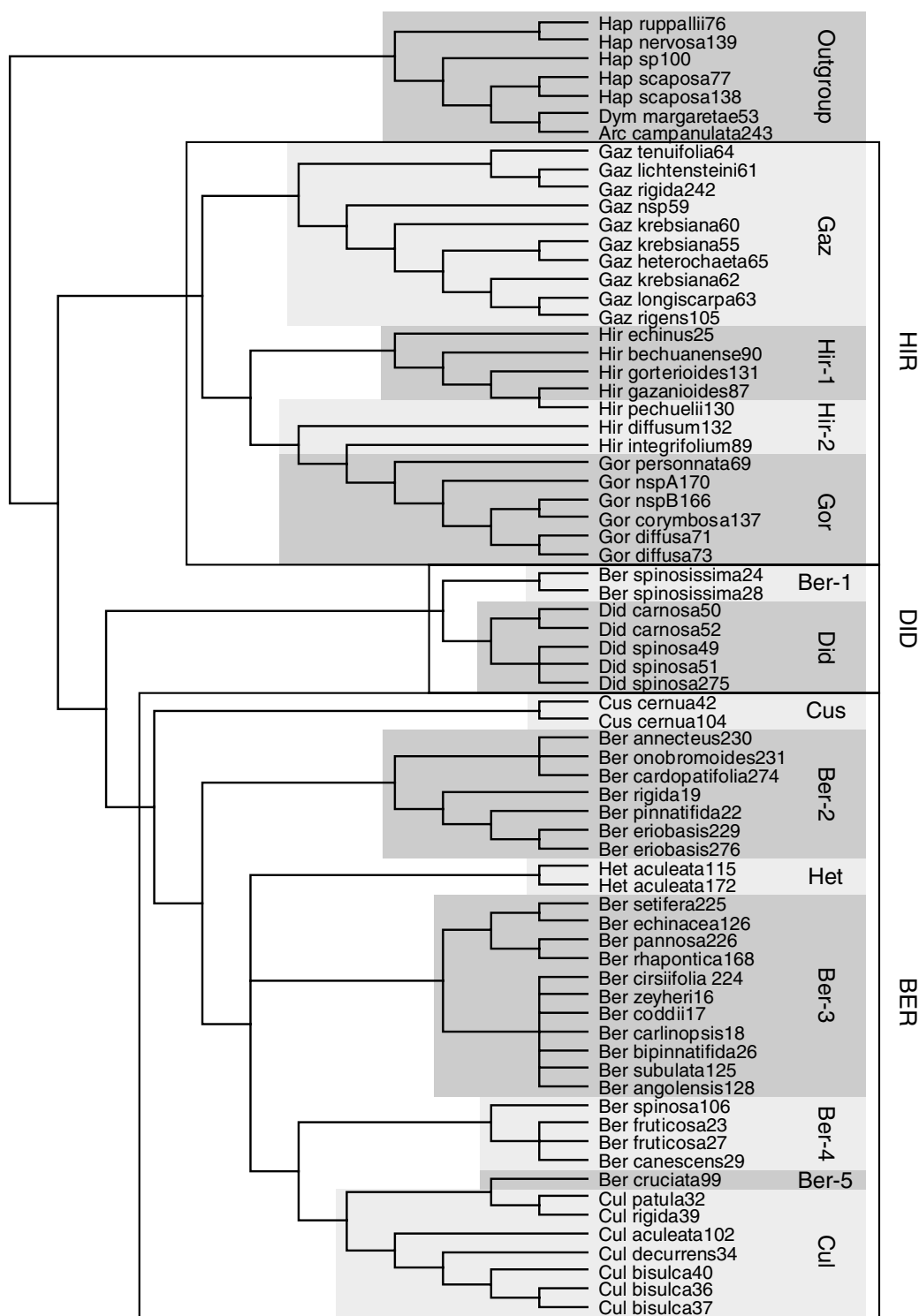
**Fig. 3.** One of the unrooted phylograms for the ingroup. The positions of the outgroup are indicated on the diagram. The three main clades are: *Didelta* (DID), *Berkheya-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 sub-groups are: *Berkheya-1* (Ber-1), *Berkheya-2* (Ber-2), *Berkheya-3* (Ber-3), *Berkheya-4* (Ber-4), *Berkheya-5* (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium-1* (Hir-1), and *Hirpicium-2* (Hir-2). Note the three possible points of attachment for the OG and for the BER + DID clades and the two placements for the BER + DID clades. The analysis produced 544 trees ( $L = 753$ ,  $\pi = 289$ ,  $CI = .659$ ,  $RI = .914$ ).

HIR and the other in BER. When the outgroup is included in the evaluation of the pairwise distances *Haplocarpha* sp. is often the most distant taxon from a species in *Gazania* or one highly nested in *Berkheya*. The distances within the ingroup are the same or slightly greater than the distances when all of the taxa are used in the analysis and this is in contrast to the analysis of the tribe that was published in 2004 (Funk et al., 2004) where the distances involving the outgroup were much larger.

Initial runs of the parsimony analyses were performed with the taxa in alphabetical order. However, after these first runs the data matrix was reorganized to reflect the 13 sub-groups. The data matrix was then scanned to look for insertions and deletions and diagnostic areas. Overall there were two visible lines in the data matrix, one between the outgroup and ingroup taxa and a second one between the *Gazania-Hirpicium-Gorteria* clade (HIR) and the rest of the taxa. It is interesting to note that the *Berkheya-Cullumia* (BER) clade shares more of what are apparently symplesiomorphic areas with the outgroup than does the HIR clade. In the first 180 bases of the ITS 1 region, there are numerous single base substitutions,

insertions, and deletions that were easy to recognize. Also of interest are several larger regions of insertions and deletions. One indel was in the ITS, a four base deletion (beginning at position 615) that supports the HIR clade. Two indels were in the *trnL-F* with a six base deletion supporting Gor + Hir-2 (beginning at position 232), and a six base insertion (beginning at position 324) supporting Gaz. The *trnL-F* spacer had three indels, an eight base deletion (beginning at position 30) supporting the HIR clade, and a 46 base deletion plus a six base insertion (beginning at position 398) supporting the monophyly of *Heterorhachis*. Finally, near the end of the *ndhF* spacer area there was a 150 base insertion for the two samples of the monotypic *Cuspidia*. This last insertion was not used in the analyses, however, it was included in the GenBank submissions. Of course the positions of all indels are approximate because of the retention in the aligned matrix of space for indels present in the larger Cichorioideae data project (Funk and Chan, unpublished data). None of the indels were coded separately in the data analysis because they supported groups that were already clearly indicated in the results.





**Fig. 4.** The strict consensus tree for the parsimony analysis using all data and taxa. The three main clades are: *Didelta* (DID), *Berkheya-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 sub-groups are: *Berkheya-1* (Ber-1), *Berkheya-2* (Ber-2), *Berkheya-3* (Ber-3), *Berkheya-4* (Ber-4), *Berkheya-5* (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium-1* (Hir-1), and *Hirpicium-2* (Hir-2). The analysis produced 272 trees ( $L = 1130$ ,  $\pi = 365$ ,  $CI = .607$ ,  $RI = .882$ ). Note that the DID clade is sister to the BER clade.

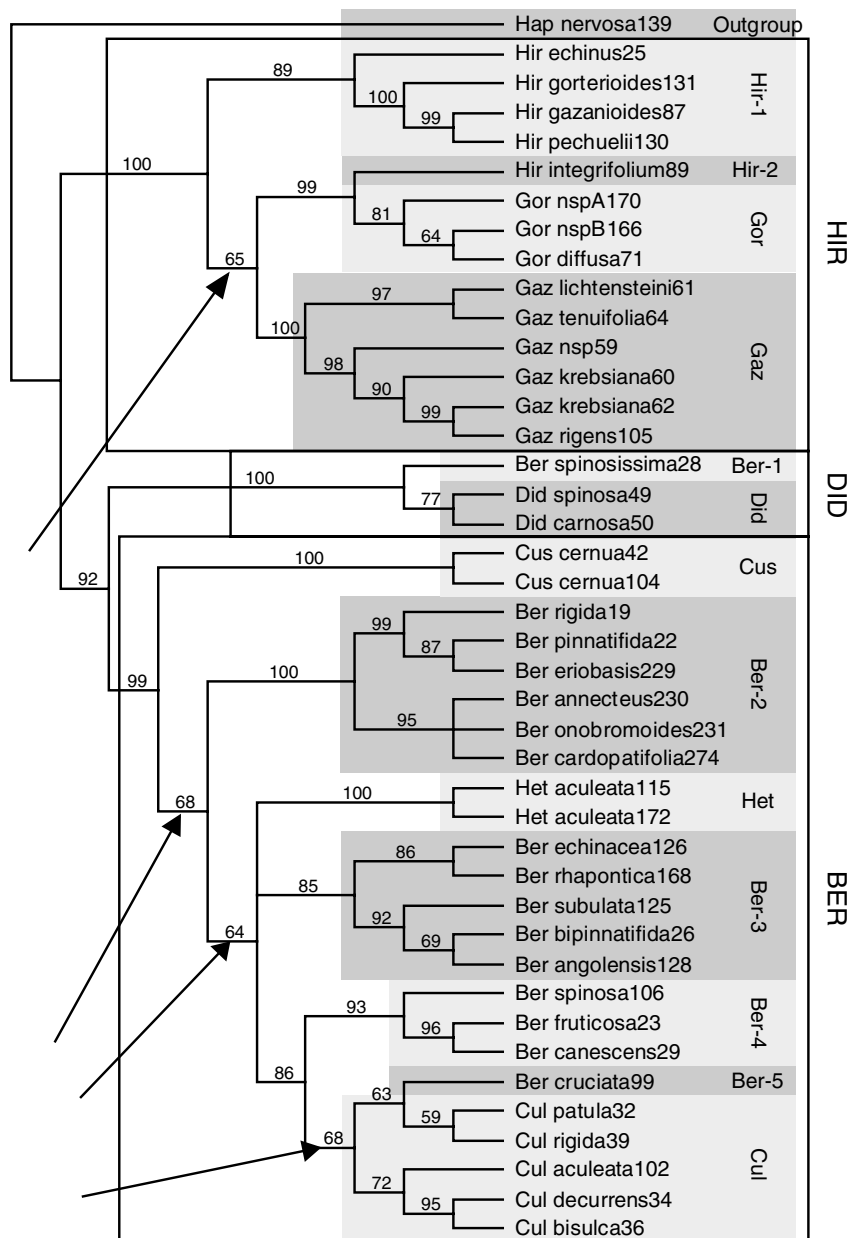
## 4. Discussion

### 4.1. Molecular

An examination of the cladograms shows several areas that do not agree with one another. These areas of disagreement can be most easily examined by asking several questions.

### 4.2. What is the sister group of *Gazania*, and are the *Hirpicium* species in *Hir-2* + *Gorteria* hybrids?

There are three genera in the HIR clade. *Gazania* and *Gorteria* are both well supported genera and they are each distinct morphologically while *Hirpicium* is not easy to define (Funk et al., 2004; Karis, 2006a,b). The seven (out of 12) species of *Hirpicium* that were



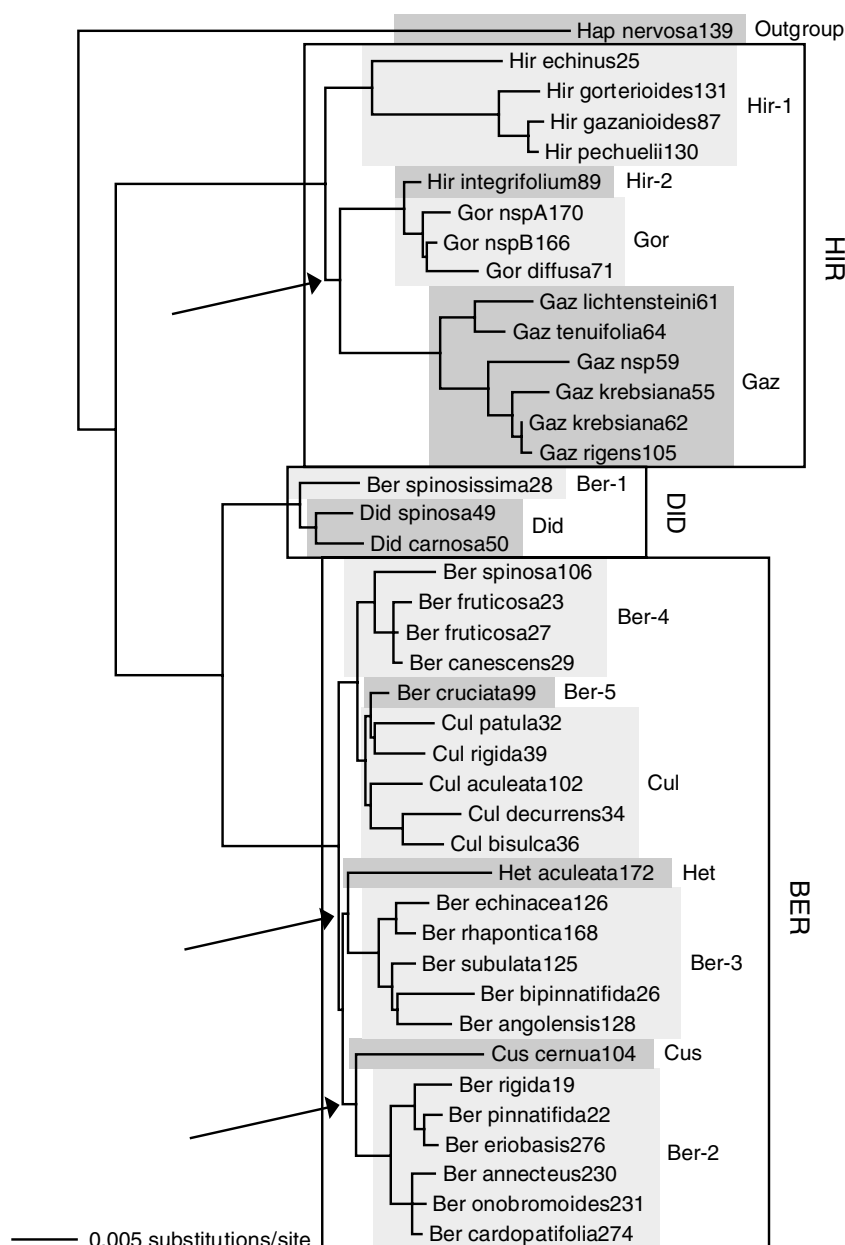
**Fig. 5.** Bootstrap tree (1000 reps) with values placed on the branches that had over 50% support. The tree was constructed using all of the data but with a pruned set of taxa. The three main clades are: *Didelta* (DID), *Berkhैया-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 sub-groups are: *Berkhैया-1* (Ber-1), *Berkhैया-2* (Ber-2), *Berkhैया-3* (Ber-3), *Berkhैया-4* (Ber-4), *Berkhैया-5* (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium-1* (Hir-1), and *Hirpicium-2* (Hir-2). The main nodes with poor support are indicated by arrows.

sampled fell into two groups, a monophyletic Hir-1 which had 4–5 species and the paraphyletic assemblage at the base of the *Gorteria* clade, Hir-2, which had 2–3 species. A detailed analysis of these three latter taxa involved an examination of the placement of *Gazania*. In the ITS and combined analyses (Figs. 1 and 4) *H. echinus* groups with Hir-2 + Gor and *Gazania* is the sister group. In the chloroplast analysis, *H. echinus* is found in the basal position in Hir-1 and *Gazania* groups with *Gorteria*. *Gazania* is allied with *Gorteria* as in most of the analyses: chloroplast parsimony, bootstrap, maximum likelihood, and when the Hir-2 taxa were removed. In fact the only time Hir-1 groups with *Gorteria* + Hir-2 is in the parsimony analysis of ITS and combined data. The other two *Hirpicium* taxa that are in Hir-2 (when present), *H. diffusum* and *H. integrifolium*, are consistently paraphyletic at the base of *Gorteria*. The cause of this movement is illustrated in Fig. 3, the

unrooted phylogram; it is the attachment of the BER + DID clade to the HIR clade. The movement of *H. echinus* suggests that it might be of hybrid origin (Yoo et al., 2002). However, the remaining taxa that have not been checked need to be added to the analysis before one can be sure.

#### 4.3. Is *Berkhैया spinosissima* really related to *Didelta*? What is the proper placement for the DID clade?

*Didelta* contains two species, in one of the species, *D. carnososa*, the receptacle starts out alveolate but when it matures it breaks into 3–5 outer parts and sometimes one central part, each of which has black spines and each of which encloses an achene, the outer parts are adnate to one of the involucre bracts becoming thickened and lignified while the central part becomes membranous; this species is a small sub-shrub with milky sap. The other species,



**Fig. 6.** Maximum Likelihood tree (8058.8508). The three main clades are: *Didelta* (DID), *Berkheya-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 subgroups are: *Berkheya*-1 (Ber-1), *Berkheya*-2 (Ber-2), *Berkheya*-3 (Ber-3), *Berkheya*-4 (Ber-4), *Berkheya*-5 (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium*-1 (Hir-1), and *Hirpicium*-2 (Hir-2). The arrows indicate the position of the Hir-2 + Gor clade and the two monotypic genera.

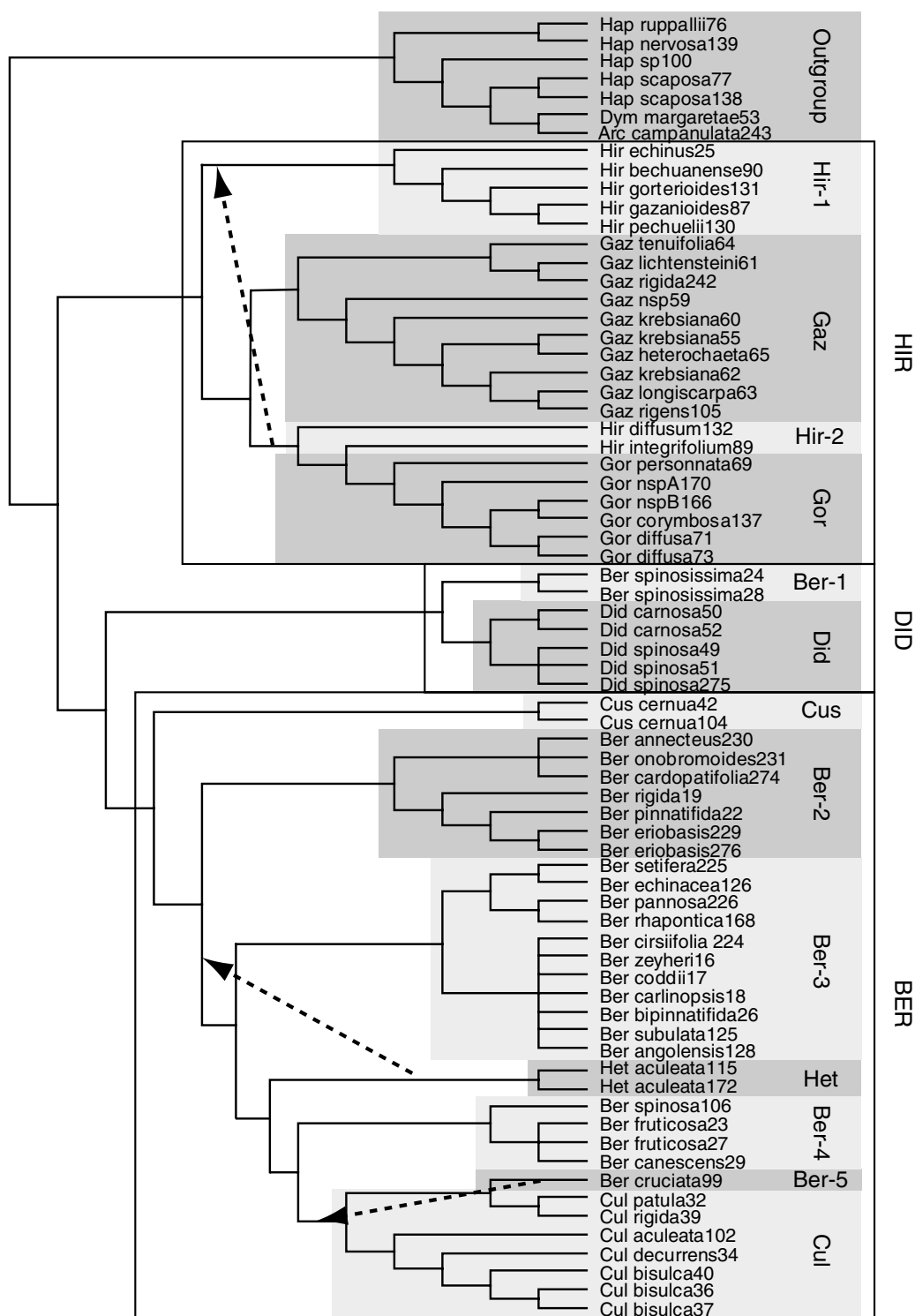
*D. spinosa*, looks similar but has a glabrous alveolate receptacle, is a large to medium sized shrub, and does not have milky sap. *Berkheya spinosissima* has an alveolate receptacle but the alveoleae are much deeper and the margins have scales; is a large to medium shrub and does not have milky sap. It does not look like either species of *Didelta*, however, it was strongly grouped with *Didelta*. This relationship was so surprising that we included in the analysis 2–3 populations of the two species of *Didelta* and *Berkheya spinosissima*.

In the ITS and combined parsimony analyses (Figs. 1 and 4) the DID clade was sister to BER with strong support. In the chloroplast analysis (Fig. 2) DID grouped with HIR with weak support; *B. spinosissima* always grouped with *Didelta*. If *B. spinosissima* is removed from the analysis the position of *Didelta* remains the same in all three analyses. If you remove *Didelta* from the analysis, *B. spinosissima* is nearly always the sister taxon to HIR but with weak support. So, *Didelta* has some affinity for both placements while *B. spinosiss-*

*ima* is more inclined to group with HIR. The bootstrap (93% support; Fig. 5) and maximum likelihood analyses (Fig. 6) place the DID clade as sister to BER. The unrooted phylogram (Fig. 3) shows that the movement of DID is actually caused by the placement of the outgroup. It seems best at this time to place the DID clade as sister to the BER clade but it is obvious that the chloroplast data are responsible for the alignment of DID with HIR. It is possible that other species of *Berkheya* may also fall into this clade so many additional taxa are needed to really understand this group.

#### 4.4. Why is *Heterorhachis* changing positions?

*Heterorhachis* and *Cuspidia* are two monotypic genera that are always found in the BER clade. The unrooted phylogram (Fig. 3) shows that they are both on long branches and not too far apart, but the attachment of the outgroup places *Cuspidia* at the base of



**Fig. 7.** Summary tree. The three main clades are: *Didelta* (DID), *Berkheya-Cullumia* (BER), and the *Gazania-Hirpicium-Gorteria* clade (HIR). The 13 sub-groups are: *Berkheya*-1 (Ber-1), *Berkheya*-2 (Ber-2), *Berkheya*-3 (Ber-3), *Berkheya*-4 (Ber-4), *Berkheya*-5 (Ber-5), *Cullumia* (Cul), *Didelta* (Did), *Cuspidia* (Cus), *Gazania* (Gaz), *Gorteria* (Gor), *Heterorhachis* (Het), *Hirpicium*-1 (Hir-1), and *Hirpicium*-2 (Hir-2). This tree is identical to the Bootstrap tree. It is also identical to the parsimony strict consensus tree with one exception, Gor + Hir-2 is the sister group of Gaz. Alternative placements of major taxa are indicated with arrows.

BER and *Heterorhachis* is nested higher in the phylogeny. *Heterorhachis* has two different placements, one as sister to a clade composed of Ber-4 + Ber-5 + Cul (ITS, some chloroplast, some combined, bootstrap, and maximum likelihood), and the other is below Ber-3 and the group listed above (some chloroplast and some combined trees). Essentially Ber-3 and Het are switching

places. This conflict results in a polytomy in the combined parsimony analysis, but in reality the first option is the best supported and that is the one reflected in the final phylogenetic tree (Fig. 7), however, the alternative position is indicated. If the analyses are performed without Het the only difference is that Ber-4 is more strongly linked to Ber-5 + Cul rather than unresolved.

**Table 5**

Size of aligned matrices, number of gaps added, number of informative characters, and percent sequence divergence for *trnL-F*, *ndhF*, and ITS

Markers	Size of aligned matrices	# of gaps added □ (ingroup/all)	# of informative characters □ (ingroup/all)	% sequence divergence (ingroup/all)
<i>trnL-F</i>	893	43–56/42–56	46/57	0.023/0.033
<i>ndhF</i>	667	0–5/same	50/66	0.049/0.056
ITS	678	34–41/same	193/242	0.158/0.209
Total	2238	76–102/77–102	292/526	n/a

Figures usually given for both “only the ingroup” and for “ingroup plus all of the outgroups”; single outliers have been removed.

#### 4.5. Is *Berkheya cruciata* most closely related to *Cullumia*? Is *B. cruciata* changing the position of *C. patula* and *C. rigida*?

*Berkheya cruciata* and the species of Ber-4 and *Cullumia* are unresolved in both the ITS and the chloroplast data. In the combined analysis (Fig. 4) Ber-4 is sister to Ber-5 + Cul but *Cullumia* is paraphyletic. However, the bootstrap support (Fig. 5) for the Ber-5 + Cul clade (68%) is not strong and support for the grouping of *B. cruciata* with the two species of *Cullumia* also is weak (63%). The maximum likelihood tree (Fig. 6) placed Ber-4 as the sister group to Ber-5 + Cul. In the analyses without *B. cruciata* some of the resolution of the BER clade is lost. As it stands now, there are three placements for *B. cruciata*: sister to *Cullumia*, in a polytomy with *Cullumia*, or nested within *Cullumia*.

The final phylogenetic tree (Fig. 7) supports three main clades of the subtribe and brings into question the monophyly of at least two genera, *Hirpicium* and *Berkheya*. The phylogeny is nearly identical to the results of the bootstrap analysis (Fig. 5) and the strict consensus tree (Fig. 4) with one exception: based on the discussion above it seemed best to place the Gaz clade as the sister clade of the Hir-2 + Gor group. In Fig. 7 the alternative placements for taxa have been indicated.

Taxa of the outgroup have long branches that separate the ingroup from the outgroup taxa. Fig. 3 shows that there are other taxa on long branches, for example, *Heterorhachis* and *Cuspidia* (the two monotypic genera), and *Berkheya bipinnatifida*, *Hirpicium gorterioides*, *Cullumia decurrens*. Also, the major clades in the ingroup have well supported branches. Within the HIR clade the branches are often long inside of the sub-groups, however within the BER clade they are often short. This could indicate more recent radiations within those parts of the BER clade.

#### 4.6. Morphology

A detailed study combining this molecular work and the work of Karis (2006a) is underway (Karis & Funk). However, based on the literature (Cassini, 1816, 1821; Lessing, 1832; Robinson and Brettell, 1973; Norlindh, 1977; Robinson, 1992, 1994; Bremer, 1994; Herman et al., 2000; Karis, 2006a) and from personal observations, the morphology can be briefly examined in light of the final phylogeny (Fig. 7). Most of the vouchers are located at the US National Herbarium (US) and were examined for this study.

The members of the subtribe Gorteriinae are defined as having laticifers (the cells that normally contain latex); latex is actually recorded as being present in *Gazania* and some *Berkheya* but it is likely that it is present in other taxa; **involucral bracts are connate at least at the base forming a cup; receptacles are more or less deeply alveolate and enclosing at least the base of the achenes; the lobes of the ray florets often have 5 veins and 4 lobes (when lobes are present)**; the rays are sterile; the central florets are deeply lobed; the anthers are sagittate but not tailed; the swollen portion of styles is either not present or

not well-developed; and the achenes are without well-developed ribs or wings. Many of these characters are symplesiomorphies, and those that are potential synapomorphies for the subtribe are in boldface. The *Gazania-Hirpicium-Gorteria* (HIR) clade comprises annual or perennial herbs with strongly fused involucre bracts, the *Berkheya-Cullumia* clade comprises a mixture of subshrubs, shrubs, and annual and perennial herbs that are usually spiny in some manner, and the *Didelta* clade consists of subshrubs or shrubs with one of the *Didelta* species having heads that break apart.

Because Roessler (1959) divided *Berkheya* into five series, we can pose an interesting question; are there any morphological characters that separate the five groups and/or link the species to other taxa such as *Didelta* and *Cullumia*?

The sub-group Ber-1 consists of two populations of *Berkheya spinosissima* and it groups closely with *Didelta*. *Didelta* is described above. *B. spinosissima* is a sub-shrub or small shrub with deeply divided leaves with spines on the margins of the leaves and on the involucre, similar to many other species in *Berkheya*. The sister group relationship between *B. spinosissima* and *Didelta* is inexplicable at this time. Ber-2 consists of perennial herbs or sub-shrubs with small spiny leaves and spiny involucre. Ber-3 has several different types of plants, those found in the *B. setifera* clade have no stiff spines and ovate leaves. Species found in the *B. subulata* clade are of three types, those with no stiff spines and elongate leaves (e.g., *B. subulata*), large herbs with many stiff spines and large heads (e.g., *B. cirsiifolia*) and those with a moderate amount of stiff spines (e.g., *B. carlinopsis*). Although *B. cirsiifolia* is in a polytomy on the consensus trees, in most other trees, it is the sistergroup to the rest of the *B. subulata* clade. Ber-4 consists of small to medium shrubs that have holly-like leaves with spines on the margins and on the involucre but it also has spines at the nodes of the branches. Ber-5 has only *B. cruciata* which is an odd looking plant with a single stem and closely packed leaves with very long thick spines.

When Roessler's classification (1959) is compared to the phylogeny (Fig. 7), *Gazania*, *Gorteria*, *Didelta*, *Heterorhachis*, *Cuspidia*, and most of *Hirpicium* and *Cullumia* are monophyletic. Two species of *Hirpicium* are more closely related to *Gorteria* and one species of *Berkheya* is either at the base of *Cullumia* or nested within it. *Berkheya*, however, is found in five groups. Roessler had eight Series in *Berkheya* and we have data for species from six of them; Table 4 gives Roessler's classification. All but one of Roessler's Series shows some consistency with the phylogeny. The single member of Series *Cruciatae* is closely related to two *Cullumia* species and although the relationship is not stable, this species never falls within any of the other *Berkheya* sub-groups, so the phylogeny supports Roessler's recognition of this species as separate from the rest of the genus. The species from sub-group Ber-2 are found in Series *Rigidae*. Sub-group Ber-3 has members in three series but there is some pattern. The Ber-3 clade breaks down into three parts, the clade containing *B. setifera*, the clade containing *B. subulata*, and *B. cirsiifolia* which is often found as the sister taxon to the rest of the *B. subulata* clade (not shown in any of the figures). Series *Speciosae* contains members of Ber-3 from the *B. setifera* clade. Series *Subulatae* contains species from the *B. subulata* clade and Series *Decurrentes* has the species *B. cirsiifolia*. However, there are two *Berkheya* species in Ber-3 that do not fit Roessler's classification. Roessler (1959) placed *B. bipinnatifida* in Series *Rigidae* which contains all of the sampled taxa that are in Ber-2 and he placed *B. carlinopsis* in Series *Fruticosa* which contains all of the sampled taxa that are in Ber-4 and one from Ber-3. Series *Fruticosa* also contains the type of the genus and has all three taxa from the Ber-4 clade but it also has *B. spinosissima* that is grouped with *Didelta* in our study. *Berkheya* is the subject of a dissertation by a South African student and no doubt some of these clades will be raised



to genus level when more taxa have been added to the analysis, but all in all, Roessler's classification is sound.

The final phylogenetic tree (Fig. 7) shows a pattern that was also noticed in all our work on this tribe, the clades (genera and their sub-groups) are usually well defined but the placement of these clades in relation to one another is sometimes difficult usually because the ITS and chloroplast data were attaching the outgroup at a different location. The same situation was found in our study of the subfamily Cichorioideae s.s. in that either the tribes or subtribes were well-defined but their relationships to one another were not (Funk et al., 2004). Also, in some of the highly nested clades the taxa are well separated (e.g., Hir-1) while others (those with more species such as *Arctotis* and *Berkheya*) have few molecular differences and form polytomies (e.g., Ber-3). One can speculate that these are old lineages and that some have undergone recent radiations.

In conclusion, we can address the goals of this paper stated in the introduction. Most of the genera are either monophyletic or can be made monophyletic by the relocation of one or two taxa with the exception of *Berkheya* and to a lesser extent *Didelta* which need further work. Within *Berkheya* there are at least five subclades that have been identified and new genera need to be described. However, before that can happen more taxa need to be added to the molecular analysis and the morphological analysis needs to be completed. The subclades of the Funk et al. (2004) paper along with additional ones are well supported and will provide ideal groups for more detailed ecological and biogeographical studies. The past classifications did a fairly good job on recognizing and describing diversity within the subtribe with the notable exception of some of the series within *Berkheya*. Finally, future studies include a detailed analysis of *Didelta* and its relationship to *Berkheya*; for instance, it is possible that *D. spinosa* is a hybrid between *D. carnosa* and *Berkheya spinosissima*? Just about every clade in the tribe has now been claimed by someone as a research project; we will be working on *Didelta* and *Cullumia*, Karis and Funk are collaborating on a combined morphology–molecular analysis within the next year (personal communication) and others are working on the remainder. It is a good bet that in a few years most of this tribe will have been investigated down to the species level, including revisions for all the genera. The results of all these studies should provide a means for a detailed investigation of the evolution of this southern South African tribe.

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