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Globally grown, but poorly known: species limits and biogeography of *Gazania* Gaertn. (Asteraceae) inferred from chloroplast and nuclear DNA sequence data

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Gazania is a popular horticultural subject worldwide and comprises 16 species, all from southern Africa. However, species delimitation is difficult as many species are morphologically variable. Here we present a phylogenetic analysis of 15 species of *Gazania*, based on DNA sequence data from four non-coding chloroplast regions (*trnL*-intron, *trnL-trnF* spacer, *psbA-trnH* spacer, *rps16* intron) and two nuclear spacers (Internal Transcribed Spacer, ITS; partial External Transcribed Spacer, ETS). The phylogenies derived from the plastid and nuclear datasets were not entirely congruent, but data combination was undertaken. Of the 15 species sampled, only 7 are supported as monophyletic. Most of the remaining taxa form a large, poorly resolved clade corresponding to a large, morphologically variable species complex. Using a range of ITS mutation rates, the diversification of the genus is estimated to have begun approximately 6.6 mya. The phylogeny, in conjunction with the distribution patterns, suggests that this genus arose in the semi-arid to arid Richtersveld/Namib regions of South Africa and Namibia.

KEYWORDS: Asteraceae, *Gazania*, molecular dating, phylogeny, southern Africa, species delimitation

INTRODUCTION

Gazania Gaertn. is a small genus of 16 species, all endemic to southern Africa. It is a member of the tribe Arctoteae, subtribe Gorteriinae, which includes seven other genera: *Berkheya*, *Gorteria*, *Cuspidia*, *Didelta*, *Heterorhachis*, *Cullumia* and *Hirpicium*. The genus was named in honor of Theodorus Gaza, a 15th-century Italian scholar and translator of the works of Theophrastus.

The early botanical exploration of the flora of southern Africa resulted in a number of genera being taken into cultivation in Europe which have subsequently been used in the horticultural trade across the globe. Examples include species of genera such as *Geranium* and *Pelargonium* (Geraniaceae), *Watsonia* (Iridaceae), *Clivia* (Amaryllidaceae) and *Gazania*. The latter genus is widely cultivated, being used as a ground cover and for their colorful floral display in gardens around the world. Many of these cultivars are patented, having been obtained from hybrids between currently recognized species (e.g., Egger & Beimel, 1990; De La Torre, 2005). In the wild, species of *Gazania* have yellow, orange or, less commonly, red ray florets (except for *G. jurineifolia* which has mainly white ray florets). However, cultivated hybrids can also have pink, deep red or mixed color ray florets.

Despite being such a common horticultural subject and a widespread and common genus across much of southern Africa, very little is known about the biology and evolutionary history of the genus. It is a major component of the annual spring time mass-flowering displays in the winter-rainfall southwestern Cape and Namaqualand regions of South Africa, and is also locally common in the arid summer-rainfall hinterland, where it flowers after rainfall events (i.e., the Succulent Karoo and Nama-Karoo biomes sensu Mucina & Rutherford, 2006; see Fig. 1). The genus is a mix of widely distributed species that are found throughout southern Africa, and narrow endemics (such as *G. caespitosa* and *G. schenckii*) that have been rarely collected. While some species of *Gazania* are found in the Fynbos biome of the southwestern Cape of South Africa, the genus is not an important element of this biome and cannot be considered as a typical "Cape clade" sensu Linder (2003). The genus does, however, belong to the "Greater Capensis" region as defined by Born & al. (2007), a region that includes both the Fynbos and Succulent Karoo biomes.

Taxonomy. — Despite the fact that *Gazania* is common both in the wild and cultivation, the taxonomy of the genus is in a state of confusion. The last species-level revision was carried out by Roessler in 1959, although it is clear from his treatment that he considered it to be a preliminary revision. Within the genus, two main growth-forms were described (Roessler, 1959). Eleven species have a shortened stem, with the leaves crowded into dense rosettes at the base, from which a leafless unbranched

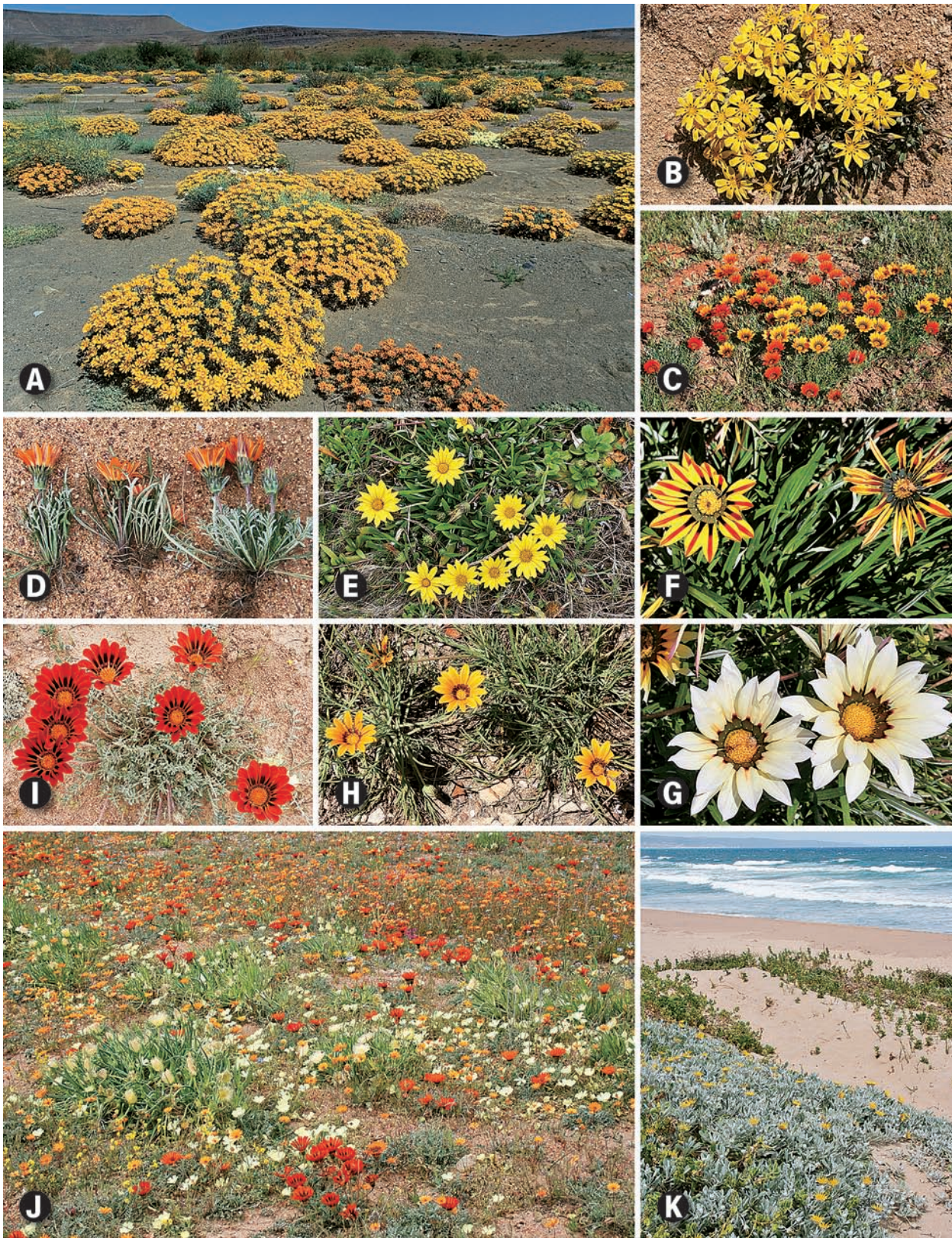

Fig. 1. A+B, *Gazania lichtensteinii* dominating sedimentary erosion surface on Hantam Plateau; C, *Gazania krebsiana* subsp. *krebsiana* in a road reserve in Olifants River valley; D, *G. serrata* collected in shale renosterveld shrublands of the upper Brede River; E, *G. rigens* var. *uniflora* from the sea splash-zone of the Eastern Cape coast near Port Elizabeth; F+G, ▶

stem arises bearing solitary inflorescences (Fig. 1D). The remaining five species possess a well-developed stem, with leaves produced more or less evenly along the stem. Roessler commented that the rosette growth-from was a characteristic of *Gazania* (but also observed in a few *Hirpicium* species), and the other growth-form a characteristic of *Hirpicium* (but also observed in a few *Gazania* species). The exact relationships between *Gazania* and *Hirpicium* are somewhat uncertain, and await further study (Funk & al., 2004; Karis, 2006).

Roessler (1959) recognized 16 species and ten subspecies. This treatment was based on morphological characters observed from herbarium specimens, in conjunction with geographical distribution of these samples. However, he never visited South Africa, and thus never saw the plants growing in the wild (P.O. Karis, Stockholm, pers. comm.). Some of Roessler's species are clearly morphologically distinct, while other species show overlap in both morphology and geography, a fact acknowledged by Roessler (1959) himself.

Biogeography. — There are now a considerable number of studies on the radiation of taxa from the Cape Floristic Region (CFR) or Fynbos biome (see for example synopses by Linder 2003, 2005, 2008), but to date there are few studies that focus on taxa from the Succulent Karoo Biome component of the "Greater Capensis." Preliminary data presented by Linder (2008) suggests that radiations of Greater Cape taxa are more recent in taxa and lineages from the semi-arid west coast flora, possibly in response to new habitat that became available following the onset cooler sea-surface temperatures that resulted in increasing aridity in the Middle Miocene. More studies on lineages centered in this region are required, and it is possible that *Gazania* is one such lineage.

Here we use DNA sequence data from four non-coding chloroplast regions and two nuclear regions to investigate species boundaries and relationships. The nuclear regions used are the Internal Transcribed Spacer (ITS) and partial External Transcribed Spacer (ETS). The four plastid regions used are the widely used *trnL*-intron and *trnL-trnF* spacer, the *psbA-trnH* spacer and the *rps16* intron. As a range of mutation rates of ITS are available for other taxa in the Asteraceae, the use of ITS permits us to conduct a preliminary molecular dating analysis, to obtain age estimates for key diversification events within *Gazania*. We then relate these dates to a biogeographic scenario in a narrative manner.

MATERIALS AND METHODS

Sampling, DNA extraction, amplification and sequencing. — Forty-three samples of *Gazania* representing fifteen species and ten subspecies of *Gazania* were obtained from the field (Appendix). Owing to the popularity of the genus as an ornamental, efforts were made to avoid collecting near human settlements. While we were unable to obtain material of *G. othonnites*, one of us (L.M.) found what is possibly a new species, referred to here as *Gazania* sp. nov. This latter taxon appears to be restricted to the Namaqualand region between Springbok and Port Nolloth, and possesses some unusual morphological characters (glabrous leaves and a slight succulence). Where possible, at least two samples were used per species or subspecies in order to test for taxon monophyly. All specimens were identified using Roessler's key to *Gazania* (Roessler, 1959). Two outgroup taxa, one each from *Hirpicium* and *Gorteria*, were also included. These two taxa are close relatives of *Gazania*, and are both from the subtribe Gorteriinae (Funk & al., 2004; Karis, 2006).

All samples were extracted using a modified CTAB DNA extraction protocol (Doyle & Doyle, 1987). The cpDNA regions were amplified and sequenced using the following primer combinations: the *psbA-trnH* with "psbA" and "trnH" (Sang & al., 1997); the *rps16* region with "rps16F" and "rps16R2" (Oxelman & al., 1997); the *trnL* intron and *trnL-trnF* spacer with "c", "d", "e" and "f" (Taberlet & al., 1991). The ITS region was amplified with "ITS18SF" and "ITS26SR" (Käss & Wink, 1997) and then sequenced with "Chromo5.8R" (Barker & al., 2005), "ITS1" (White & al. 1990), "ChrysITS4" (3′-TCCTCCGCTTATGGATATGC-5′) and "Chrys5.8F" (3′-GACTCTCGGCAACGGATATC-5′). ETS was amplified with "ETS18S" (Linder & al., 2000) and "ETS IntF" (3′-ACCAGCTGATGGACAAG-5′) designed for this study. Additional reverse sequences were obtained with "ETS IntR" (3′-ACCACCCGACTAGTAGCC-5′).

PCR amplifications were carried out using either a ThermoHybaid PCRSprint Temperature Cycling System or a Corbett Research PC-960G Microplate Gradient Thermal Cycler. The PCR products were cleaned using the PROMEGA Wizard SV Gel and PCR purification kit. Cleaned PCR product was sequenced using ABI prism BigDye Terminator v3.1 Ready Reaction Cycle sequencing kit (Applied Biosystematics), according to the manufacturer's instructions. Sequence data was checked and

► **two spectacular cultivars of *Gazania* from flower-beds of the Boschendal wine-farm (near Franschhoek); H, *G. ciliata* from saline loams of the lowlands south of Aurora (near Piketberg); I, *G. leiopoda* on coarse-sandy soils derived from granite in Skilpad Flower Reserve near Kamieskroon (Namaqualand); J, spring display of *Gazania leiopoda* (deep red), *Conicosia elongata* (yellow Mesembryanthema [Aizoaceae]), *Grielum humifusum* (light yellow creeping herb) and *Norlindhia amplectens* (yellow daisy) near Kamieskroon in Namaqualand; K, leeward slope of coastal dune on the coast near Groot Brak on the South Coast, dominated by *G. rigida* subsp. *leucolaena*. Photographers: A, K. Phillips; B–K, L. Mucina.**

edited using Sequencher (version 3.1.1; Gene Code Corporation 2004). Assembled sequences were exported from Sequencher, and imported into the alignment software MacClade (version 4.06; Sinauer Associates, Inc.) and aligned manually. GenBank accession numbers can be found in the Appendix.

Phylogenetic analyses. — Two datasets were obtained: a chloroplast dataset comprising all the data from the four non-coding regions (*trnL*, *trnL-trnF*, *psbA-trnH*, *rps16*), and a nuclear dataset comprising both the ITS and partial ETS data. Ambiguous base calls in the ITS and ETS sequences may represent paralogues, and were coded using the international ambiguity codes. Both datasets were subjected to parsimony and Bayesian analyses, as follows: Datasets were subject to random input analysis to ensure all islands of equally most parsimonious trees were found (Maddison, 1991) using PAUP* ver. 4.0b10 (Swofford, 2002). An initial heuristic search was conducted, with MAXTREES set to 100,000, using 1,000 random addition sequence replicates with TBR branch swapping, saving one tree no longer than the shortest tree length at each replicate, with the MULTREES and STEEPEST descent options in effect. A second heuristic search was then conducted using all the trees found by this method as starting trees, and allowed to swap to completion. A strict consensus tree was produced from the set of equally most parsimonious trees obtained. Bootstrap support values were calculated for 1,000 replicates with MAXTREES set to 1,000. Gaps were treated as missing data.

Prior to conducting a Bayesian analysis, datasets were partitioned by genetic region, as Bayesian analysis is based on explicit models of DNA evolution. MrModelTest (Nylander, 2004) was used to identify the model of DNA substitution that best fit each partition. The Bayesian analysis was conducted using MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001) as follows: four Markov chains, three heated and one cold, were run simultaneously for 5,000,000 generations and trees were saved every 100 generations. Each data partition was assigned model-specific Prset and Lset conditions as determined by MrModelTest. The starting tree was random, the branch lengths were saved and the first 4,000 trees were discarded as burn-in. The sumt function was used to generate a consensus tree.

Dataset combination. — The ILD test (Farris & al., 1994) as implemented in PAUP* (as the Partition Homogeneity Test) was used to investigate possible incongruence between the chloroplast and nuclear datasets, with the following options: heuristic search, simple addition, TBR branch swapping, 100 replicates, saving the 500 most parsimonious trees per replicate. The combined dataset was analysed as described above.

Molecular dating analysis. — To test if there is a constant mutation rate across all lineages, the Likelihood Ratio Test (Felsenstein, 1981) was conducted in PAUP*, comparing likelihoods obtained with and without an enforced molecular clock. Unfortunately, there are no fossils or geological events that can be used to calibrate any dating exercise. However, we can estimate the ages of key nodes using the average sequence divergence values between highly supported pairs of clades. Although we utilized sequence data from six different non-coding regions, mutation rates are only available for ITS data from a range of Asteraceae (e.g., Sang & al., 1995) and other angiosperms (Kay & al., 2008). We thus use only the ITS data for *Gazania* in this dating analysis, and present the results on the tree from the combined cpDNA and nrDNA dataset. The ITS mutation rates for herbaceous plants tend to be the faster (Kay & al., 2008), and additionally the rates for the Asteraceae tend to be fairly high. Since *Gazania* is a small herbaceous member of the Asteraceae that can go from seed to seed set in under a year, it is possible that it has a high mutation rate. The average Asteraceae mutation rate was calculated as 5.21×10^{-9} s/s/y (substitutions per site per year; based on an average calculated from Richardson & al.'s (2001) list of Asteraceae mutation rates) with a lower extreme of 3×10^{-9} s/s/y found in the Hawaiian Silverswords (Baldwin & Sanderson, 1988), and a higher extreme of 7.83×10^{-9} s/s/y in *Robinsonia* (Sang & al., 1995). These three rates (slow, average and fast) allow for an upper, lower and average age estimate of node ages.

The pairwise sequence divergence values between two sister groups were determined as the average of all pairwise sequence divergence values between taxa from the two clades, and was calculated as half of the divergence value divided by the rate of change in substitutions per site per year (Yuan & al., 2005). These average sequence divergence values were calculated from the uncorrected pairwise distance (obtained using PAUP*). As the assumption of a strict molecular clock was rejected, divergence dates were estimated using a relaxed clock with Bayesian inference and MCMC procedures implemented in BEAST 1.4.7 (Drummond & Rambaut, 2007).

RESULTS

Phylogeny reconstruction. — The levels of variability for each of the regions sequenced are shown in Table 1. The nuclear ITS and ETS regions were twice and three times as variable (respectively) as the most variable plastid region (*psbA-trnH*).

The parsimony and Bayesian phylogenetic analyses of the nuclear dataset retrieved very similar topologies. The Bayesian tree based on nuclear data is shown in Fig. 2, which is annotated with both parsimony bootstrap support values and Bayesian posterior probability values. These analyses show seven well supported lineages, six of

Table 1. Length, variability and informativeness of nuclear and chloroplast regions sequenced in this study.

	Aligned length	Bayesian analysis: selected model	Variable characters		Parsimony informative characters	
			Number	%	Number	%
cpDNA	**2,345**		**172**	**7.3**	**73**	**3.1**
trnL intron	489	F81	27	5.5	6	1.2
trnL-trnF	412	HKY+I	28	6.8	7	1.7
psbA-trnH	600	F81+I	68	11.3	40	6.7
rpS16	844	GTR+I	49	5.8	20	2.4
nrDNA	**1,439**		**408**	**28.4**	**212**	**14.7**
ITS	676	GTR+G	161	23.8	85	12.6
ETS	763	GTR+G	247	32.4	127	16.6

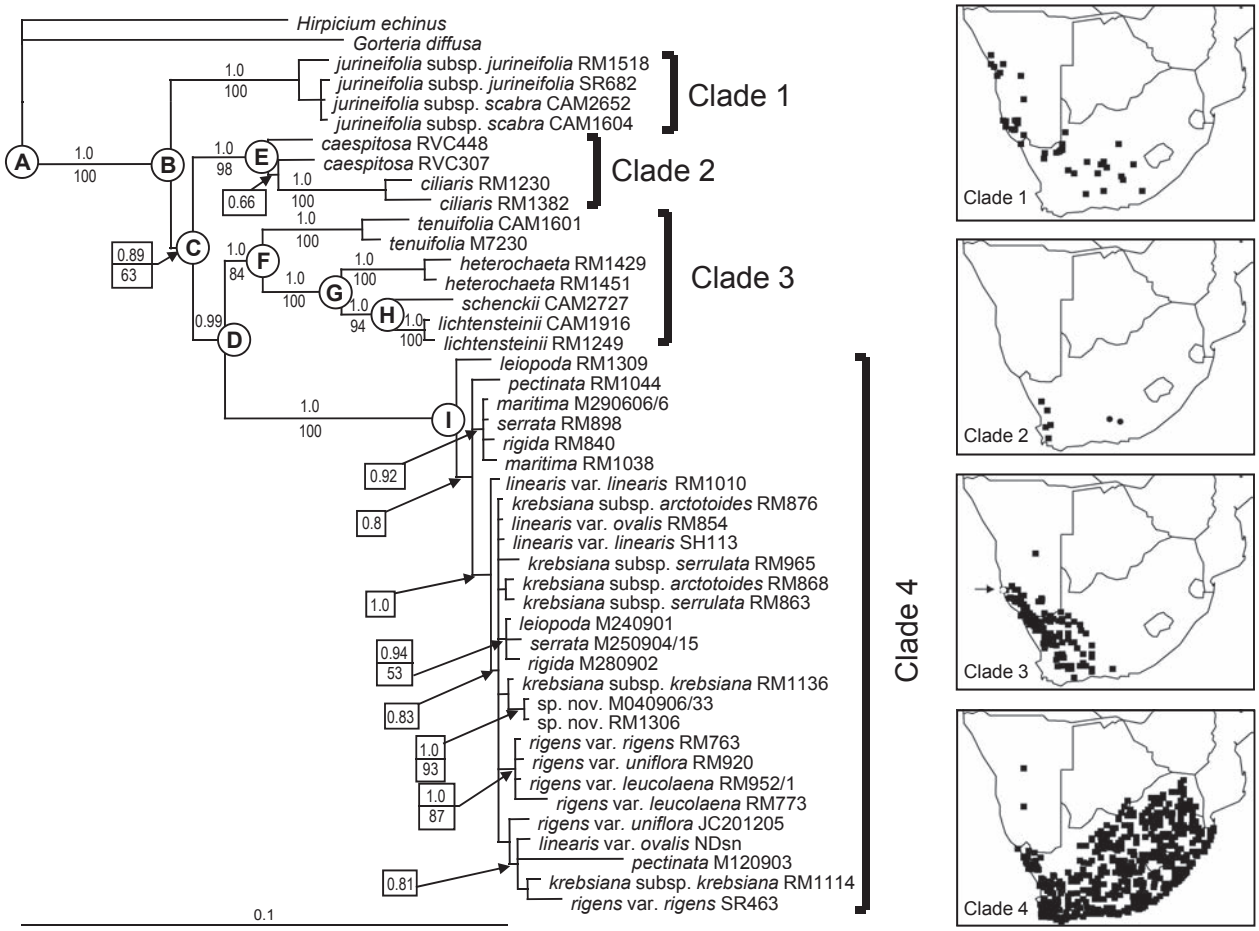

Fig. 2. Bayesian tree from analysis of nuclear dataset (ITS and ETS). Numbers above the branches indicate Bayesian posterior probabilities, numbers below the branches are bootstrap percentages based on a parsimony bootstrap analysis. The four clades discussed in the text are indicated, along with maps indicating the distribution of each clade. In the map of Clade 2 the dots represent the distribution of *Gazania caespitosa*, the squares *G. ciliaris*. In the map of Clade 3, the open circle (arrowed) represents the distribution of *G. schenckii*. Letters indicate nodes for which estimated ages are provided in Table 2.

Table 2. Estimated ages of nodes A–I (annotated in Fig. 2) based on a range of mutation rates of other Asteraceae as estimated using BEAST.

Node	Age estimate in Myr (maximum – average – minimum)
A	11.0 – **6.6** – 4.3
B	7.9 – **4.6** – 3.1
C	7.7 – **4.4** – 2.9
D	7.3 – **4.2** – 2.8
E	2.4 – **1.4** – 0.9
F	5.0 – **2.8** – 1.9
G	4.2 – **2.4** – 1.6
H	2.8 – **1.6** – 1.1
I	1.0 – **0.6** – 0.4

which correspond to the species identified as *G. ciliaris*, *G. lichensteinii*, *G. schenckii*, *G. tenuifolia*, *G. heterochaeta* and *G. jurineifolia* (the two subspecies of which are resolved here as non-monophyletic). Furthermore, the two representative samples of *G. caespitosa* are resolved as a paraphyletic grade, thus preventing it from being defined as a lineage. The eighth and largest clade includes representatives of all the remaining taxa, and is termed here the *G. krebsiana–G. rigens* species complex (or "K-R complex"). These lineages are resolved into four well-supported clades, labeled Clade 1–4 in Fig. 2, which also provides an indication of the distribution range of each clade.

The Bayesian and parsimony analyses of the plastid dataset (Fig. 3A, B) were not completely congruent, and retrieved fewer well supported branches when compared to the nuclear phylogeny. The parsimony analysis shows only five lineages (one of which—*G. tenuifolia*—is embedded in a large polytomy that includes the K-R complex and other taxa). The Bayesian analysis resolves four clear lineages arising from a basal polytomy.

The ILD test indicated that the nrDNA and cpDNA datasets were highly incongruent with each other ($P = 0.01$) indicating that they should not be combined. Upon examining the trees from the two datasets (Figs. 2, 3) it can be seen that the relative positions of Clades 1 and 3 are incongruent, but support at many nodes in the plastid topology is poor or non-existent, suggesting that the cpDNA contains limited signal in these regions of the topology. Furthermore, as many samples in the analysis were placed in a large, poorly resolved K-R clade, we tested if these samples were responsible for the ILD test result by sequential removal of randomly selected terminal taxa from each of the two datasets. This removal process revealed that the incongruence was almost entirely due to the effects of the large sample size of this poorly resolved clade, as the ILD test indicated dataset congruence once this clade had been reduced. The datasets were thus combined and analyzed.

The Bayesian tree from the combined dataset is shown in Fig. 4. This analysis once again resolves eight lineages corresponding to seven of Roessler's (1959) previously described taxa: *G. caespitosa* (now indicated as monophyletic), *G. ciliaris*, *G. lichtensteinii*, *G. schenckii*, *G. tenuifolia*, *G. heterochaeta*, *G. jurineifolia* and the K-R complex. The same set of four larger clades (Clades 1–4) that were resolved by the nuclear data are also retrieved in the combined analysis.

Dating analysis. — There are a number of important caveats and limitations to the use of clocks based on sequence data, including substitution rate heterogeneity among lineages (which the LRT tests for), uncertainties regarding clock calibration, and unknown but presumably large estimation errors (Seelanen & al., 1997). In this analysis, the molecular clock hypothesis was rejected by the Likelihood Ratio Test (2,285.0211 vs. 2,250.2555, $P < 0.0084$), and node ages are determined using a range of possible mutation rates, so the results of this analysis are offered with no claims to veracity or accuracy. Dates (based on a range of mutation rates) for the major nodes labeled A to I in Fig. 2 are given in Table 2.

DISCUSSION

Taxonomic implications. — Marrying the existing species as defined by Roessler (1959) with our results presents some difficulties. Our results suggest that *Gazania* comprises only seven genetically well-defined species and an additional highly variable species complex comprising at least some species that are known to hybridize readily. The seven distinct species are *G. ciliaris*, *G. lichtensteinii*, *G. schenckii*, *G. tenuifolia*, *G. heterochaeta*, *G. jurineifolia* (which may comprise two subspecies) and *G. caespitosa*, which the ITS data suggest may be paraphyletic. The morphological distinctness of each of these species, along with molecular data indicating the substantial genetic uniqueness of each of these lineages (as indicated by branch lengths in the Bayesian trees), can be construed to be strong evidence that they are indeed "species" from both a genetic species concept perspective and a typological perspective, as these species are readily diagnosable on the basis of morphological features.

However, within the K-R complex, there is little clear morphological or genetic distinctions between the species, although there appears to be some evidence for the recognition of previously recognized taxa, such as *G. rigens*, although not all of the samples identified as such are placed in a monophyletic lineage (Figs. 2–4). The plastid data (some of which is from regions known to be variable at and below the species level) fails to provide

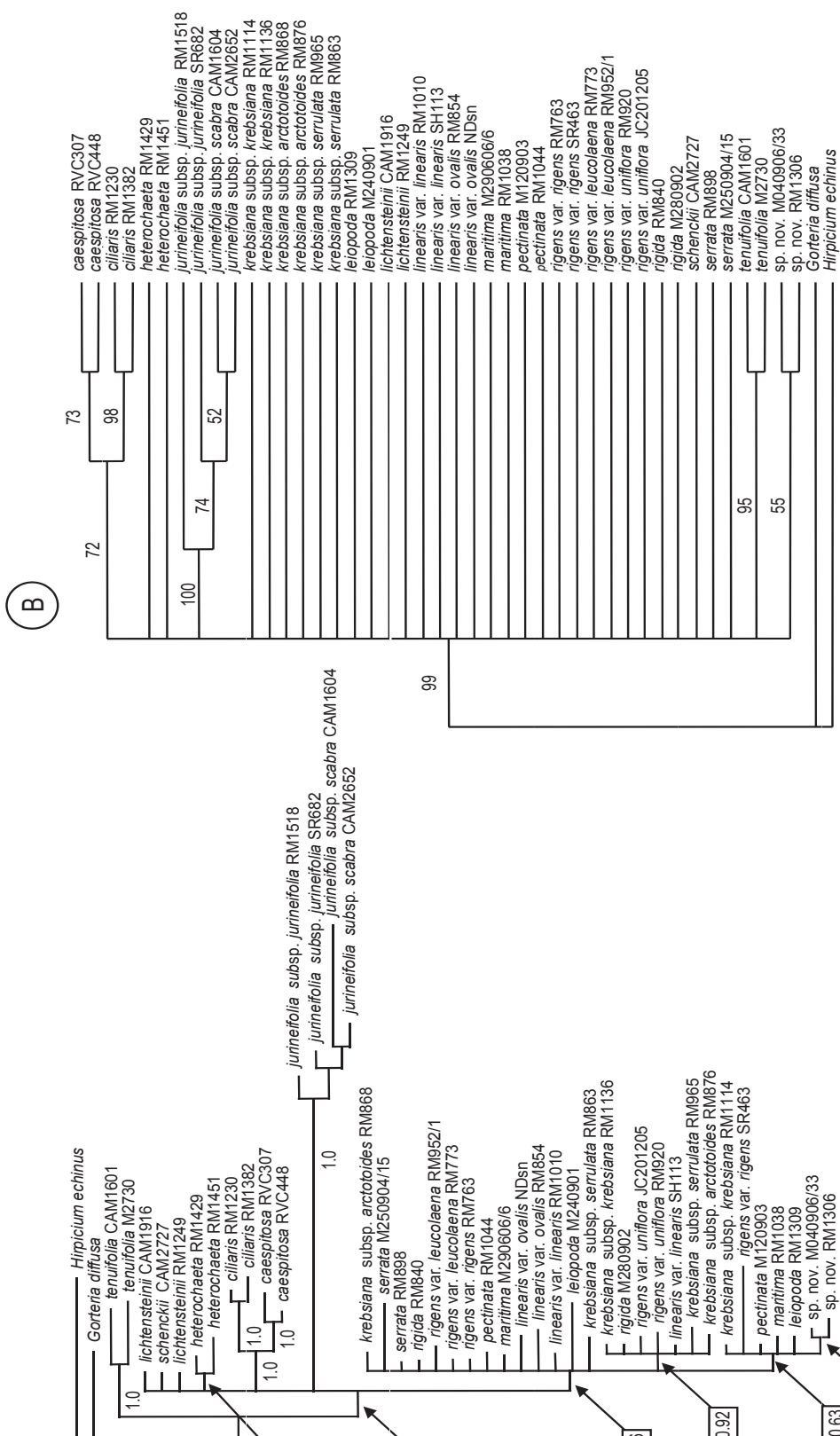

Fig. 3. Phylogeny obtained from analysis of the cpDNA data set. A, Bayesian tree, with numbers below the branches indicating Bayesian posterior probabilities; B, parsimony tree with numbers above the branches indicating bootstrap support values.

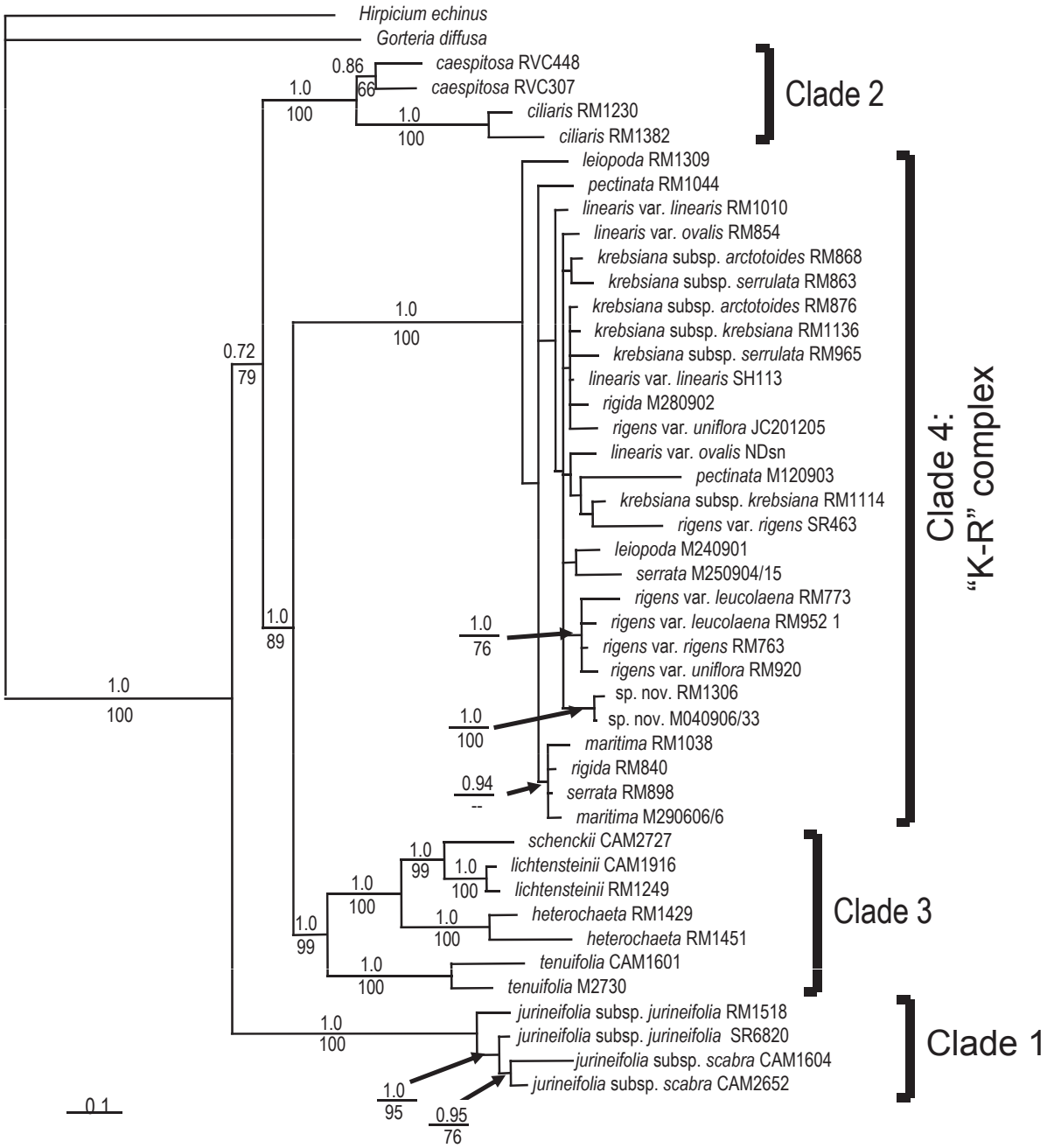

Fig. 4. Bayesian tree derived from analysis of the combined cpDNA and nrDNA datasets. Numbers above the branches indicate Bayesian posterior probabilities, numbers below indicate bootstrap support.

any resolution, while the more variable ITS and ETS data provides a more resolved topology, but with limited node support within the K-R complex. It would thus be expected that these rapidly evolving regions would be able to resolve hierarchical and non-reticulate relationships between closely related species. The lack of such a result may suggest recent or ongoing reticulation (through hybridization, from which many ornamental varieties are derived) or incipient speciation in which morphological diversity has not been matched by genetic divergence. At least some of the species in the K-R complex are known to hybridise readily, and Roessler (1959) considered that the intergradation of the morphological features of these species was a consequence of hybridization. It is thus possible that the K-R complex could be one large hybridizing species complex. However, there is a paucity of data on the reproductive biology of the genus, so hypotheses involving reproductive aspects such as hybridization are

just that: hypotheses. For this reason, we are hesitant to publish a definitive key to the species of *Gazania*, which awaits further investigations, both molecular and morphological, in order to determine the taxonomic status of lineages within the K-R complex. However, we present a modified version of Roessler's key in which the seven lineages that correspond to species are retained, and reduce the remaining taxa to the K-R clade, which, owing to the variability within it, appears in numerous places in this key.

1 Stem developed with leaves spaced along entire length.
2 Leaves linear (< 2 mm wide) . . . . . ***G. caespitosa***
2 Leaves obovate/obovate-lanceolate/lanceolate (> 2 mm wide).
3 All leaves deeply pinnatifid (1–5 lacinae) . . . . . **K-R complex**
3 Leaves entire or some few leaves pinnatifid (1–3 lacinae).
4 Leaf margin entire . . . . . **K-R complex**
4 Leaf margin dentate/denticulate.
5 Involucre tomentose, older stems woody . . . . . ***G. schenckii***
5 Involucre glabrous, stem not woody . . . . . ***G. lichtensteinii***
1 Stem shortened with leaves crowded at base in rosette.
6 Abaxial leaf surface glabrous, leaves succulent or glaucous . . . . . **K-R complex**
6 Abaxial leaf surface tomentose.
7 Leaf margins dentate/denticulate . . . ***G. lichtensteinii***
7 Leaf margins entire or ciliate.
8 Multiple rows of linear parietal scales upwards from truncate involucre base . . . . . ***G. tenuifolia***
8 Most involucral scales terminal, some single, parietal.
9 Inner involucre scales greatly and finely acuminate (> 8 mm long).
10 Inner involucral scale margins entire . . . . . **K-R complex**
10 Inner involucral scale margins ciliate . . . . ***G. ciliaris***
9 Inner involucre scales < 8 mm long.
11 Outer involucral scales < 4 mm long, inner > 4 mm long. Ray florets white . . . . . ***G. jurineifolia***
11 Outer involucral scales > 4 mm long, inner < 4 mm long. Ray florets yellow/orange.
12 Leaf obovate . . . . . ***G. heterochaeta***
12 Leaf linear/lanceolate . . . . . **K-R complex**

Molecular dating and biogeographic interpretation. — We acknowledge that the dating approach we use here is preliminary and based on a number of assumptions. Our results suggest that *Gazania* diverged from *Gorteria* and *Hirpicium* anywhere from 4.3 to 11.4 mya, with an average estimate of 6.6 ± 0.5 mya. This period (Late Miocene) coincides with the onset of aridity in the southwestern Cape associated with strong upwelling of the cold Benguela Current waters along the West coast of South Africa, a period that affected many plant lineages in the "Greater Capensis" region (Linder, 2003, 2005, 2008).

Within *Gazania*, further divergences (such as the divergence of the ancestor of *G. jurineifolia* from the remaining taxa; Clade 1 in Fig. 2) may correspond to the boundary between Late Miocene and the Early Pliocene at 5 mya. This period was accompanied by a peak in global cooling which occurred in response to a pulse in Antarctic Ice Sheet growth (Lindesay, 1998). Further divergences within the four major clades could correspond with a period of continental uplift about 2.5 mya, a period that Linder (2008) suggests could have seen the radiation of several plant groups distributed along the west coast. During this period, the southeast regions of the southern African subcontinent rose by 600–900 m, while the south rose by 200 m and the west by 100 m. This uplift was accompanied by an altitude-related decline in temperatures in these newly elevated regions, and both sediment evidence and faunal species compositions point to wetter conditions in the mid-Pliocene (3.5 mya) followed by increasingly episodic rainfall and drier conditions after 2.5 mya when cooler conditions prevailed (Lindesay, 1998; Linder, 2008).

With the exception of Clade 2, all lineages occur in the semi-arid Namaqualand and arid Namib regions (Fig. 2), concentrated in what has been termed the East Gariep Centre of endemism by Jürgens (1991), or Gariep Centre of Endemism by Van Wyk & Smith (2001). This centre has long been recognized by phytogeographers (as summarized by Van Wyk & Smith, 2001), and while it includes the Richtersveld, it does extend beyond this area and includes the Namib Desert, as far north as Lüderitz on the Namibian coast.

Pickford (2004) noted that the isolation of the Namib promoted a high degree of isolation of the Namibian gene pool, and once having adapted to conditions in the Namib arid areas, these arid adapted entities would have been pre-adapted for expansion into neighboring areas as these, in turn, became arid during climate cycles, or with the onset of summer aridity. The latter scenario has been proposed for the origin of the CFR lineage of *Zygophyllum* (Bellstedt & al., 2008), and may also explain the origin of the Clade 2 of *Gazania*, which is found in the CFR. Efforts to determine the ancestral area of *Gazania* using a Dispersal-Vicariance approach as implemented in the software DIVA (Ronquist, 1997) failed to provide unequivocal results (results not shown), and must await a wider (sub-tribal) analysis.

To our knowledge there are no other phylogenetic studies on plants from this region that have included dating analyses. However, Touloumenidou & al. (2007) present a

phylogeny for *Monsonia* (including *Sarcocaulon*; Geraniaceae) that, when combined with distribution data, indicates that numerous Namib taxa form a basal grade to species with a more mesic distribution, a pattern we find in *Gazania*. Similarly, Bellstedt & al. (2008) interpreted branch lengths to show that within *Zygophyllum* (Zygophyllaceae), subgenus *Zygophyllum* (limited to CFR and Australia) underwent a more recent radiation than subgenus *Agrophyllum* which is generally restricted to the more arid regions of Namibia and the Northern Cape.

It is thus tempting to predict that a pattern of a basal arid clade or grade will be found repeatedly in plant groups associated with the semi-arid interior or more mesic Fynbos regions of southern Africa. This implies that the broader region comprising Namaqualand, Gariep region (lower Orange River valley) and the Namib Desert is an ancient center of diversity and possibly origin that contains palaeo-endemics of many southern African plant taxa. This region thus requires careful monitoring and conservation, especially in the light of predicted changes in the face of the current climate crisis.

The crown group diversification within the genetically discrete species and the K-R complex appears to date to the Quaternary—certainly our estimates for the K-R complex (node I in Fig. 2) suggest this. The Pleistocene dates for these more recent divergences correlate to what has been described as an important time for genetic diversification and speciation, based on the premise that Quaternary climatic conditions, characterized by a sequence of glacial cycles, fostered the isolation of populations and, in some instances, allopatric speciation (Willis & Niklas, 2004).

While southern Africa was not glaciated during the Pleistocene, the effects of the glacial climates nonetheless affected the biota, and a scenario of species being restricted to refugia, followed by subsequent expansion, secondary contact and possible hybridization can be postulated. This may be especially likely for the K-R complex, where there has been substantial recent radiation into a plethora of morphological entities that Roessler recognized as species. These "species" may have arisen as a consequence of strong selection (possibly in small populations that experienced genetic bottlenecks) during periods of isolation in refugia during the Pleistocene climate cycles, a mechanism advocated by Prance (1982) for driving diversification in the Neotropics. The K-R clade may thus comprise incipient or very young species, or perhaps these taxa might be considered merely as locally adapted and occasionally interbreeding ecotypes.

In summary, this study has partially resolved taxonomic issues within *Gazania*, and it seems appropriate to recognize only seven species in the genus: *G. caespitosa*, *G. ciliaris*, *G. lichtensteinii*, *G. schenckii*, *G. tenuifolia*, *G. heterochaeta*, *G. jurineifolia* (which may comprise two subspecies) and the large morphologically variable K-R species complex. Together with a phylogenetic framework and preliminary molecular dating estimates (and acknowledging the caveats associated with our dating method), a scenario of origin and diversification in the semi-arid and arid western regions of southern Africa, associated with climate changes is proposed. The accuracy and generality of this hypothesis awaits further testing by means of congruence with other similarly distributed plant groups.

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Appendix. Specimens used in the phylogenetic analysis. All voucher specimens deposited in the Selmar Schonland Herbarium (GRA).

Species, collection, locality, GenBank accession number: *trnL* intron, *trnL-trnF* spacer, *psbA-trnH* spacer, *rps16*, ITS, ETS

Gazania caespitosa Bolus, *Clark 307*, South Africa, Koudeveldberge, EF556458, EF556504, EF556366, EF556412, EF556320, EF556274; *Clark 448*, South Africa, Koudeveldberge, EF556459, EF556505, EF556367, EF556413, EF556321, EF556275. ***Gazania ciliaris*** DC., *McKenzie 1230*, South Africa, Paarlberg, EF556460, EF556506, EF556368, EF556414, EF556322, EF556276; *McKenzie 1382*, South Africa, Piketberg, EF556461, EF556507, EF556369, EF556415, EF556323, EF556277. ***Gazania heterochaeta*** DC., *McKenzie 1429*, South Africa, Steytlerville, EF556462, EF556508, EF556370, EF556416, EF556324, EF556278; *McKenzie 1451*, South Africa, Steinkopf, EF556463, EF556509, EF556371, EF556417, EF556325, EF556279. ***Gazania jurineifolia*** DC. subsp. ***jurineifolia***, *McKenzie 1518*, South Africa, Conway, EF556464, EF556510, EF556372, EF556418, EF556326, EF556280; *Ramdhani 682*, South Africa, Gamoep, EF556465, EF556511, EF556373, EF556419, EF556327, EF556281. ***Gazania jurineifolia*** subsp. ***scabra*** (DC.) Roessler, *Mannheimer 1604*, Namibia, Karas, EF556466, EF556512, EF556374, EF556420, EF556328, EF556282; *Mannheimer 2652*, Namibia, Aus, EF556467, EF556513, EF556375,

Appendix. Continued.

EF556421, EF556329, EF556283. ***Gazania krebsiana*** Less. subsp. ***krebsiana***, *McKenzie 1114*, South Africa, Bedford, EF556468, EF556514, EF556376, EF556422, EF556330, EF556284; *McKenzie 1136*, South Africa, Venterstad, EF556469, EF556515, EF556377, EF556423, EF556331, EF556285. ***Gazania krebsiana*** Less. subsp. ***arctotoides*** (Less.) Roessler, *McKenzie 868*, South Africa, Kenhardt, EF556470, EF556516, EF556378, EF556424, EF556332, EF556286; *McKenzie 876*, South Africa, Bloemfontein, EF556471, EF556517, EF556379, EF556425, EF556333, EF556287. ***Gazania krebsiana*** Less. subsp. ***serrulata*** (DC.) Roessler, *McKenzie 965*, South Africa, Elliot, EF556472, EF556518, EF556380, EF556426, EF556334, EF556288; *McKenzie 863*, South Africa, Olifantshoek, EF556473, EF556519, EF556381, EF556427, EF556335, EF556289. ***Gazania leiopoda*** (DC.) Roessler, *McKenzie 1309*, South Africa, Kamiesberg, EF556474, EF556520, EF556382, EF556428, EF556336, EF556290; *Mucina 240901*, South Africa, Nieuwoudtville, EF556475, EF556521, EF556383, EF556429, EF556337, EF556291. ***Gazania lichtensteinii*** Less., *Mannheimer 1916*, Namibia, Lüderitz, EF556476, EF556522, EF556384, EF556430, EF556338, EF556292; *McKenzie 1249*, South Africa, Wuppertal, EF556477, EF556523, EF556385, EF556431, EF556339, EF556293. ***Gazania linearis*** (Thunb.) Druce var. ***linearis***, *McKenzie 1010*, South Africa, Pondoland, EF556478, EF556524, EF556386, EF556432, EF556340, EF556294; *Howis 113*, South Africa, Stutterheim, EF556479, EF556525, EF556387, EF556433, EF556341, EF556295. ***Gazania linearis*** var. ***ovalis*** (Harv.) Roessler, *McKenzie 854*, South Africa, Bathurst, EF556480, EF556526, EF556388, EF556434, EF556342, EF556296; *Devos s.n.*, South Africa, The Haven, EF556481, EF556527, EF556389, EF556435, EF556343, EF556297. ***Gazania maritima*** Levyns, *Mucina 290606/6*, South Africa, Diaz Beach, EF556482, EF556528, EF556390, EF556436, EF556344, EF556298; *McKenzie 1038*, South Africa, Bredasdorp, EF556483, EF556529, EF556391, EF556437, EF556345, EF556299. ***Gazania pectinata*** (Thunb.) Spreng., *Mucina 120903/5*, South Africa, Piketberg, EF556484, EF556530, EF556392, EF556438, EF556346, EF556300; *McKenzie 1044/3*, South Africa, Bredasdorp, EF556485, EF556531, EF556393, EF556439, EF556347, EF556301. ***Gazania rigens*** (L.) Gaertn. var. ***rigens***, *McKenzie 763*, South Africa, Kasouga, EF556486, EF556532, EF556394, EF556440, EF556348, EF556302; *Ramdhani 463*, South Africa, Tugela River mouth, EF556487, EF556533, EF556395, EF556441, EF556349, EF556303. ***Gazania rigens*** (L.) Gaertn. var. ***leucolaena*** (DC.) Roessler, *McKenzie 773*, South Africa, Plettenberg Bay, EF556488, EF556534, EF556396, EF556442, EF556350, EF556304; *McKenzie 952_1*, South Africa, Cape St. Francis, EF556489, EF556535, EF556397, EF556443, EF556351, EF556305. ***Gazania rigens*** (L.) Gaertn. var. ***uniflora*** (L. f.) Roessler, *McKenzie 920*, South Africa, Cannon Rocks, EF556490, EF556536, EF556398, EF556444, EF556352, EF556306; *Cockburn 201205*, South Africa, Lupatana, EF556491, EF556537, EF556399, EF556445, EF556353, EF556307. ***Gazania rigida*** (Burm. f.) Roessler, *McKenzie 840*, South Africa, Caledon, EF556492, EF556538, EF556400, EF556446, EF556354, EF556308; *Mucina 280902/9*, South Africa, Oudtshoorn, EF556493, EF556539, EF556401, EF556447, EF556355, EF556309. ***Gazania schenckii*** O. Hoffm., *Mannheimer 2727*, Namibia, Lüderitz, EF556494, EF556540, EF556402, EF556448, EF556356, EF556310. ***Gazania serrata*** DC., *McKenzie 898*, South Africa, Robertson, EF556495, EF556541, EF556403, EF556449, EF556357, EF556311; *Mucina 250904_15*, South Africa, Simonstown, EF556496, EF556542, EF556404, EF556450, EF556358, EF556312. ***Gazania tenuifolia*** Less., *Mannheimer 1601*, Namibia, Arras, EF556497, EF556543, EF556405, EF556451, EF556359, EF556313; *Mucina 7230/2*, South Africa, Goegap Nature Reserve, EF556498, EF556544, EF556406, EF556452, EF556360, EF556314. ***Gazania* sp. nov.**, *Mucina 040906/33*, South Africa, Hondeklipbaai, EF556499, EF556545, EF556407, EF556453, EF556361, EF556315; *McKenzie 1306*, South Africa, Kleinzee, EF556500, EF556546, EF556408, EF556454, EF556362, EF556316. ***Gorteria diffusa*** Thunb., *McKenzie 1349*, South Africa, Springbok, EF556501, EF556547, EF556409, EF556455, EF556363, EF556317. ***Hirpicium echinus*** Less., *McKenzie 1324*, South Africa, Springbok, EF556503, EF556549, EF556411, EF556457, EF556365, EF556319.