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Research Article

Evolutionary history of the arid climate-adapted *Helichrysum* (Asteraceae: Gnaphalieae): Cape origin and association between annual life-history and low chromosome numbers

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Abstract The basal grade of the large, widely-distributed *Helichrysum-Anaphalis-Pseudognaphalium* (HAP) clade (Asteraceae, Gnaphalieae) comprises exclusively southern African taxa. These species possess unusual trait combinations relative to the remaining species (a high proportion of annuals, unusual capitulum arrangement, and low base chromosome numbers). A time-proportional Bayesian phylogenetic hypothesis is generated from nuclear ribosomal sequences from 110 accessions. Ancestral area, life history, and base chromosome number are reconstructed using maximum likelihood, and correlations between life-history and chromosome number are tested in a phylogenetic framework. The results show that the HAP clade probably originated and experienced initial diversification in the Greater Cape Floristic Region in the Early to Middle Miocene. The ancestor of the HAP clade is inferred to have been perennial with $x = 7$ base chromosome number. Several independent acquisitions of the annual life-history are inferred, accompanied by reductions to $x = 4$ and 5. A single reversal to perennial life history is associated with a subsequent change back to the state of $x = 7$. Origin and early diversification within the HAP clade follows the pattern of multi-area seeded radiations within southern Africa, with subsequent migrations to the rest of Africa and the Northern Hemisphere. Occupation of drier habitats with shorter growing seasons may select for the acquisition of a shorter life-cycle, and our results indicate a strong association between short life-cycle and reduced chromosome number.

Key words: ancestral character state reconstruction, chromosome evolution, *Helichrysum*, life form, phylogeny, southern Africa.

1 Introduction

The interrelations between life history, DNA amount and ecological factors have been extensively studied. Authors have generally found that annual species have lower cellular DNA content than perennials (e.g., Labani & Elkington, 1987; Rayburn & Auger, 1990; Torrel & Vallès, 2001; Bancheva & Greilhuber, 2006; Leitch & Bennett, 2007; Andrés-Sánchez et al., 2013). Low DNA content favors shorter meiotic and mitotic cycles, and Bennett (1972) demonstrated shorter cell cycle durations in annuals. However, some authors (e.g., Albach & Greilhuber, 2004; Chrtek et al., 2009) have suggested that this correlation should be interpreted with caution. In many cases, it has been studied without a phylogenetic framework, and the correlation depends on the statistical analyses used. The relationships between genome size and ecological factors are less clear, but some studies suggest that aridity affects DNA amount (Ohri, 1998; Nevo, 2001; Torrell & Vallès, 2001; Hodgson et al., 2010). Several factors are thought to promote annuality in dry habitats: firstly, seeds are more resistant to aridity than live plants; secondly, low adult survival rates select for the annual habit; thirdly, desert habitats have more open spaces available for seedling germination and, finally, even after transient rainfall events the higher growth rates of annuals permit them to produce seed before moisture disappears (Schaffer & Gadgil, 1975; Evans et al., 2005; Verboom et al., 2012).

The winter-rainfall Greater Cape Floristic Region (GCFR, South Africa), more loosely known as the Cape region, is exceptional for its high levels of floristic species diversity and

endemism, and is known to have acted as a source area for plant radiations in southern Africa and other parts of the world, especially Australasia (Linder & Verboom, 2015). Additionally, many lineages from the Cape region experienced massive diversification based on diploid radiations during the Miocene and Pliocene (Oberlander et al., 2016) possibly favoured by contemporary cycles of aridification (Marlow et al., 2000; Linder, 2003; de Menocal, 2004). The large and taxonomically intractable paper-daisy genus *Helichrysum* Mill. (ca. 600 species, Anderberg, 1991; Ward et al., 2009) exemplifies these Cape lineages, with an apparent origin in the GCFR (Galbany-Casals et al., 2014; Nie et al., 2016) giving rise to high diversity in the Cape region (94 species) as well as 148 additional species in the rest of southern Africa, and seeding dispersal to, and radiation in, the rest of Africa, Madagascar, the Mediterranean and Asia. Many members of the early-diverging lineages of *Helichrysum* occur in the Cape region particularly in the more arid areas (Galbany-Casals et al., 2014). The taxa so far placed in these lineages are characterised by a high proportion of annuals, as well as by uniquely low base chromosome numbers (Galbany-Casals et al., 2014), and thus form an ideal study group to explore the association of life history with chromosome number evolution.

The most extensively-sampled molecular phylogeny of *Helichrysum* to date is that of Galbany-Casals et al. (2014), based on nuclear ribosomal and plastid DNA, and including about 24% of *Helichrysum* species. Despite poor resolution along the backbone of the tree, Galbany-Casals et al. (2014) recovered 17 subclades, all but two of them including southern African species of *Helichrysum*, usually with a mix of species from outside the region. Although several other genera (e.g., *Achyrocline* Less., *Anaphalis* DC., *Pseudognaphalium* Kirp.) are nested within *Helichrysum,* forming what Smissen et al. (2011) termed the HAP clade, a basal grade of three lineages [clades P and Q of Galbany-Casals et. al. (2014), and *H. lambertianum* DC.] comprised only *Helichrysum* species from southern Africa. A ribosomal DNA study of the entire Gnaphalieae (Nie et al., 2016) subsequently confirmed these findings, and established that an additional six exclusively southern African species belong in these early-diverging lineages. These 19 basal-grade species are mostly endemic to the winter-rainfall GCFR, but several occur in the arid to semi-arid, mostly summer-rainfall regions comprising large parts of the interior of southern Africa. We include the arid Kalahari and semi-arid Nama-Karoo regions in this categorisation, hereafter called the arid summer-rainfall region of southern Africa, or ASRSA. While the GCFR is well-studied due to its hyper-diverse, endemic-rich flora, the ASRSA has fewer species with lower endemism, and the evolutionary history of its flora is less well-understood.

In contrast to the early-diverging lineages, the core HAP radiation contains only five GCFR-endemic species and a single ASRSA species (*H. zeyheri* Less.). This strongly suggests an origin for the entire HAP clade in southwestern Africa, as proposed but not explicitly tested by Galbany-Casals et al. (2014). The biogeographic analysis of Nie et al. (2016) found good support for an origin for the HAP clade in southern Africa. Within this subregion, which includes the GCFR, *Helichrysum* species occupy a range of habitats, including the winter-rainfall GCFR, the summer-rainfall arid regions, and the summer-rainfall but cool growing-season habitats of the Afrotemperate grasslands. Here, we investigate the geographic origins of the HAP clade, exploring the role of these three main habitats in the early divergence and subsequent speciation events in the basal lineages. In addition, we aim to further elucidate the phylogenetic relationships of southern African *Helichrysum*, particularly the GCFR taxa, as previously-published phylogenetic trees have included only 41 of the 94 species that occur in the GCFR.

Of the 19 early-diverging southern African *Helichrysum* species, nearly half are obligate or facultative annuals (Fig. 1). Short-lived life-histories are otherwise extremely rare in the HAP clade: with the exception of the mostly-annual genus *Pseudognaphalium*, almost all other members, and almost all *Helichrysum* species outside the basal grade, are perennial herbs or shrubs. A large proportion of the early-diverging taxa also exhibit an unusual capitulum arrangement: while almost all HAP clade taxa have a corymbose or open paniculiform arrangement, 12 of the 19 sampled basal-grade species have capitula aggregated into synflorescences that loosely mimic secondary heads. These synflorescences comprise glomerules or rarely solitary capitula that are frequently surrounded by an involucre-like whorl of leaves, hereafter named leafy glomerules. This unusual trait is otherwise present in just a few species from the core HAP radiation (e.g., *H. selaginifolium* R. Vig. & Humbert). Furthermore, five of these 19 basal-grade species have their pappus bristles arranged in two, rather than the usual one, series: *H. zwartbergense* Bolus, *H. litorale* Bolus, *H. spiralepis* Hilliard & B.L.Burtt and *H. argyrosphaerum* DC. (Galbany-Casals et al., 2014) as well as *H. niveum* Less. (Nie et al., 2016). The biseriate pappus led Cassini (1822) to erect a novel genus, *Leontonyx* Cass., for some of these taxa, but they have since been reinstated in *Helichrysum* (Hilliard & Burtt, 1981), a decision supported by phylogenetic results (Galbany-Casals et al., 2014; Nie et al., 2016). Nevertheless, several species with a biseriate pappus have not yet been included in molecular analysis [*H. caespititium* (DC.) Sond., *H. paronychioides* DC., *H. lucilioides* Less., *H. lineatum* Bolus, and *H. tinctum* (Thunb.) Hilliard & B.L. Burtt].

Apart from unique patterns of geographic distribution, life-history and morphology, several species recovered in the early-diverging lineages of the HAP clade have unique and unusually small base chromosome numbers. While the typical base number for the HAP clade is $x = 7$ (occurring in diploid, tetraploid, hexaploid and octoploid series; Namur & Verlaque, 1976; Galbany-Casals & Romo, 2008; Galbany-Casals et al., 2014), four annual GCFR species in the basal grade are diploids with base numbers of $x = 4$ and $x = 5$ (Galbany-Casals & Romo, 2008; Galbany-Casals et al., 2009). Galbany-Casals et al. (2009) postulated a link between annuality, occupation of arid environments, and dysploidy for these taxa, but chromosome counts have been performed for only six of the 19 species of the early-diverging lineages. Lack of karyotype information, and sparse taxon sampling make it difficult to determine the ancestral base chromosome number, to infer patterns of chromosomal evolution, and to examine evolutionary interactions with other traits such as life-history.

Here, we aim to further elucidate the composition and biogeographic history of the exclusively GCFR and the ASRSA HAP lineages, and to explore evolutionary association, if any, between annual life history and low chromosome number. Although phylogenetic inference is ideally based on multiple independently-segregating DNA loci, incongruence between

Fig. 1. Flowering shoots of species from the HAP basal grade (all in clade P; see later). **A,** *Helichrysum tinctum* S. Andrés-Sánchez & N. Bergh SA809 (NBG). **B,** *H. spiralepis* S. Andrés-Sánchez & N. Bergh SA809 (NBG). **C,** *H. leontonyx* S. Andrés-Sánchez & S. Smuts SA761 (NBG). **D,** *H. stellatum* S. Andrés-Sánchez & S. Smuts SA765 (NBG). **E,** *H. pulchellum* DC. S. Andrés-Sánchez & S. Smuts SA769 (NBG). **F,** *H. cylindriflorum* S. Andrés-Sánchez & A. Sánchez Garciamadrid SA774 (NBG). **G,** *H. dunense* S. Andrés-Sánchez & S. Smuts SA771 (NBG). **H,** *H. alsinoides* S. Andrés-Sánchez & S. Smuts SA758 (NBG). **I,** *H. micropoides* Vanrhynsdorp, 30-9-2013, S. Andrés-Sánchez et al. (photo). **J,** *H. asperum* Jonaskop, 20-1-2014, S. Andrés-Sánchez et al. (photo). **K,** *H. herniarioides* S. Andrés-Sánchez et al. SA732 (NBG). **L,** *H. marmarolepis* S. Moore S. Andrés-Sánchez & S. Smuts SA766 (NBG).

loci generated with traditional Sanger-sequencing, which has been detected in Gnaphalieae (Ward et al., 2009; Galbany-Casals et al., 2010, 2014; Montes-Moreno et al., 2010; Bergh et al., 2011; Smissen et al., 2011; Bengtson et al., 2014; Bentley et al., 2014) presents analytical challenges. In Gnaphalieae, plastid loci tend to yield poorly-resolved and poorly-supported trees with poor correspondence to morphology (Bayer et al., 2000, 2002; Bergh & Linder, 2009; Galbany-Casals et al., 2010, 2014; Smissen et al., 2011), possibly due to lower species-fidelity of plastid DNA, particularly in outcrossing plant species (Petit & Excoffier, 2009). Here we use only nuclear DNA sequences (ETS and ITS) to construct a phylogenetic hypothesis for the HAP clade, with a focus on the early-diverging southern African lineages.

2 Material and Methods

2.1 Taxon sampling

Although no full taxonomic treatment exists for the ca. 600 species of *Helichrysum*, Hilliard (1983) proposed 30 informal morphological groups for the 242 southern African species, based largely on capitulum characters. Most of the 19 species recovered in the basal grades (Galbany-Casals et al., 2014; Nie et al., 2016) belong to Hilliard's (1983) groups 12, 14 and 15. We accordingly focused our sampling on the remaining species from these groups, also using morphology and, to a lesser extent, geographic distribution to determine which other unsampled species to include. We were able to sample 74 individuals from 48 species, often including multiple accessions, including a total of 25 previously-unsampled species. The core HAP clade radiation was represented in our analysis by 26 species from all of the subclades (A through O) recovered by Galbany-Casals et al. (2014). Outgroups were represented by non-HAP clade members of the gnaphalioid "crown radiation" (*Craspedia* G. Forst., *Filago* L., *Syncarpha* DC. and *Vellereophyton* Hilliard & B. L. Burtt), while the more distant SIM clade ('*Stoebe-Ifloga-Metalasia* clade') of Bergh et al. (2015) was represented by *Dolichothrix* Hilliard & B. L. Burtt. Representatives of the *Relhania* clade (*Athrixia* Ker Gawl., *Leysera* L. and *Relhania* L'Hér.) were included as the most distant outgroup, as indicated by previous phylogenetic

analyses. Voucher information for all samples is provided in Appendix I.

2.2 DNA isolation, amplification and sequencing

Leaf material was either collected directly into silica gel in the field, obtained from dried specimens kindly provided by M. Koekemoer (PRE), or taken from other herbaria with the permission of the relevant curators. For field-collected material, at least one sheet was deposited at BOL, NBG, PRE or SALA (acronyms according to Thiers, 2018, continuously updated). Total genomic DNA was isolated from pulverized dried leaf material (ca. 25 mg) using the Qiagen DNeasy plant extraction kit (Qiagen Sciences, Valencia, California, USA) and GenElute Plant Genomic DNA Miniprep (Sigma-Aldrich Co. LLC., St. Louis, USA). DNA extracts were checked on 1% TAE-agarose gels. The nuclear ribosomal internal transcribed spacer (ITS) repeat unit (ITS1, 5.8S and ITS2) was amplified in a single reaction using primers ITS5 and ITS4 (White et al., 1990), while the entire external transcriber spacer (ETS) region was amplified using primers ETS1f (Linder et al., 2000) and 18S-ETS (Markos & Baldwin, 2001). Reaction mixtures consisted of 12.8 μL nuclease-free H_2O, 2.5 μL of 10x buffer (Kapa Biosystems Inc., MA, USA), 1.5 μL of 25 μmol/L $MgCl_2$, 1 μL dNTP mix at 0.2 μmol/L each dNTP, 0.5 μL 100% DMSO, 1.25 μL of each primer at 10 μmol/L, 0.2 μL of Taq DNA polymerase (Kapa Biosystems Inc., MA, USA) and 4 μL of template DNA at various dilutions. The PCR was performed in an Applied Biosystems 2720 thermal cycler (Applied Biosystems CA, USA) with the following thermal profile: initial denaturation for two minutes at 94 °C; 35 cycles consisting of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 2 min; and a final extension step of 72 °C for 8 min. Successfully amplified PCR products were cleaned and sequenced in both directions using the amplification primers, by the Central Analytical Facility at Stellenbosch University (South Africa) using BigDye terminator cycling and a 3130XL Genetic Analyzer/3730 Genetic Analyzer, or by Macrogen (Macrogen Inc, Korea) using BigDye terminator cycling and an ABI Automated Sequencer 3730XL (Life Technologies Corporation, Carlsbad, California, USA). Chromatograms were examined, corrected where necessary and assembled using Geneious Pro v 5.4.4 (Biomatters Ltd., Auckland, New Zealand). Initial automated alignment was performed with the program Clustal X v.2.0.10 (Thompson et al., 1997) with subsequent visual inspection and manual revision in BioEdit v 7.1.3.0 (Hall, 1999). Our final alignment comprised 110 accessions representing 73 species. Aligned matrices are deposited on TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S23293).

2.3 Phylogenetic analyses

Indels were treated as missing data in all analyses. Congruence of the ETS and ITS DNA regions was tested under the parsimony criterion with the Incongruence Length Difference test (ILD, Farris et al., 1995a, 1995b) implemented with the INCTST script (Goloboff et al., 2008) of TNT v1.1 (Goloboff et al., 2003–2005), running 1000 replicates. Maximum Parsimony analyses (MP) and Bayesian Inference (BI) of phylogeny were performed as described below for each region independently and, in the absence of supported incongruence, for both regions combined. Parsimony clade support was assessed using the bootstrap (Felsenstein, 1985) performed in PAUP* v.4.0b10 (Swofford, 2002) with 1000 replicates, each saving a maximum of 500 trees, 20 random addition sequences per replicate, and NNI branch swapping. Trees were rooted on *Athrixia phylicoides* DC. from the *Relhania* clade.

Bayesian inference (BI) was performed in MrBayes v.3.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best-fitting model of molecular evolution for each region (GTR + I + G for both ITS and ETS) was determined using Akaike's (1973) Information Criterion (AIC) as implemented in jModelTest v. 2.1.4 (Guindon & Gascuel, 2003; Darriba et al., 2012). The two regions were analysed separately and in combination, within a mixed-model framework. For each phylogenetic analysis, two independent runs were performed, each with four Metropolis-Coupled Monte Carlo Markov Chains starting from different random trees and running for 5×10^6 generations, sampled every 500 generations. The resulting samples (10 000 per run) were examined in Tracer v 1.6 (Rambaut et al., 2014) to check for convergence and to confirm the adequacy of sample sizes associated with each parameter estimate. The first 2000 samples of each run were discarded as the burn-in fraction, which amply ensured the exclusion of trees that might have been sampled prior to the convergence of the Markov chains. The tree sample was visualised by computing the 50% majority-rule consensus tree with mean branch lengths in MrBayes. Posterior probability support (PP) is deemed significant only for nodes with $PP \geq 0.95$.

2.4 Divergence time estimation

Divergence times were estimated using an uncorrelated relaxed lognormal Bayesian clock as implemented in BEAST v 1.8.4 (Drummond et al., 2012), the input data being configured using BEAUti v 1.8.4 (Drummond et al., 2012) and using the best-fitting models of molecular evolution as for the MrBayes analysis. For samples of the same species found to be monophyletic in MrBayes analysis, only a single accession was included. As a result, the data set used for divergence time estimations contained 85 samples. In the absence of available fossil calibrations within Gnaphalieae, a secondary calibration procedure was employed, with calibrations assigned to three nodes using the full confidence intervals of dates obtained by Nie et al. (2016), who applied a relaxed Bayesian clock in the context of five independent calibration priors, including one macrofossil, two pollen fossils, and two geological events. The calibration nodes used in the present study are: (i) the crown node of the *Relhania* clade [I: mean posterior estimate in Nie et al. (2016) of 23.4 Ma, and 95% HPD of 17.8-28.9 Ma]; (ii) the crown node of the HAP clade (II: mean posterior estimate of 12.7 Ma, and 95% HPD of 9.3-16.6 Ma); and (iii) node III, representing the most recent common ancestor (MRCA) of the clade comprising *Pseudognaphalium beneolens* (Davidson) Anderb., *H. foetidum* (L.) Moench and *H. wilmsii* Moeser, with a mean posterior estimate of 6.7 Ma, and 95% HPD of 5.8-8.7 Ma. To account for error associated with the secondary calibrations, we employed normal calibration priors in which the mean and 95% CI of the calibration density corresponded to the mean and 95% HPD estimates reported by Nie et al. (2016). Two MCMC chains were each run for 2×10^7 generations and sampled every 1000 generations. Convergence was analyzed as for the MrBayes analyses using Tracer v

1.6 (Rambaut et al., 2014). A 10% burn-in was removed and the maximum clade credibility (MCC) tree, annotated with median node ages, was extracted using TreeAnnotator v 1.8.4 (Drummond et al., 2012).

2.5 Ancestral area reconstruction

Our geographic range scheme comprises nine biogeographic regions. Since our major interest is in the evolutionary history of the lineages within southern Africa, this region was more finely divided, with areas corresponding to subregions representing major habitat types. Ranges outside of southern Africa were coded more broadly, the intention being to capture only major dispersal events. Species distributions were coded using range description data in Hilliard (1983) and by examining collection localities from the Botanical Database of Southern Africa (BODATSA; South African National Biodiversity Institute, 2016; doi to be assigned). The regions are: (A) the Greater Cape Floristic Region (GCFR), representing both semi-arid and mesic winter-rainfall shrublands in the southwest part of South Africa, and including a small extension into southwestern Namibia; (B) the arid to semi-arid summer rainfall regions of southern Africa (ASRSA), encompassing the Kaokoveld, the Desert and Nama-Karoo Biomes in South Africa and Namibia, and parts of the Savanna and Grassland Biomes of South Africa; (C) the cool growing-season afroalpine and afromontane regions of South Africa, corresponding to the mesic high-altitude portion of the Grassland Biome, generally coincident with the greater Drakensberg region; (D) Tropical Africa; (E) Madagascar; (F) the Mediterranean region; (G) the Americas; (H) Asia; and (I) Oceania. Range evolution was examined using both parsimony-based dispersal-vicariance analysis (S-DIVA; Yu et al., 2010) and likelihood-based dispersal-extinction-cladogenesis (DEC) inference (Ree & Smith, 2008). Both methods were implemented in RASP 2.0 (Yu et al., 2015) using the default settings. The input treefile comprised 18 000 trees randomly selected from the BEAST output, as well as the BEAST MCC tree. The maximum number of allowable ancestral areas per node was set to three for both analyses.

2.6 Morphological studies and ancestral trait reconstruction

All sampled species were scored for three morphological characters: life-history, synflorescence organisation and pappus bristle arrangement. To assess variability in these traits, both within and between species, ca. 1300 sheets in BOL, NBG and PRE were examined, in addition to field observations. Life-history was coded as (i) obligately annual (producing seeds and dying in the first year), (ii) facultatively annual (producing seeds in the first year, but able to persist for several years given appropriate conditions), or (iii) perennial (usually taking several years to flower). Synflorescence organisation was coded as (i) capitula in leafy glomerules, or (ii) capitula arranged in corymbiform or paniculiform synflorescences and lacking the pseudo-involucre. Finally, pappus bristle arrangement was coded as (i) pappus absent, (ii) pappus present with bristles arranged in one row, or (iii) pappus present with bristles arranged in two rows. Based on this scoring, the three traits were reconstructed on the BEAST MCC tree using the single-rate maximum likelihood (Mk1) model as implemented in Mesquite 3.31 (Maddison & Maddison, 2015).

2.7 Base chromosome number

We attempted chromosome counts for 14 species, but ultimately obtained good metaphase counts for just six species, due to non-germination of seeds and difficulty of determining chromosome numbers for specimens with relatively large chromosomes that overlapped on the slides. We used root and shoot tips of one- or two-day old seedlings, germinated in petri dishes at room temperature from field-collected seeds (population voucher information provided in Table 1) and fixed in Carnoy's solution (three parts absolute ethanol to one part glacial acetic acid; Sharma & Sharma, 1965) for two days at 4 °C, after which they were stored in 70% ethanol at 4 °C. Root and shoot tips were dissected off the fixed material, stained in 2% acetic orcein (La Cour, 1954) and squashed between a glass slide and a cover slip in a drop of 45% acetic acid. Chromosomes were viewed using a Nikon Eclipse 50i compound light microscope at 100x magnification, and images recorded with a Nikon DS Camera Control Unit DS-U2 and DS-5M Camera head using NIS Elements Documentation and Digital 3D Imaging Software (Nikon Instruments Europe B.V., Amsterdam). Two to four different cells, from between two and four different individuals, were counted for each population (Table 1).

Evolutionary shifts in base chromosome number were reconstructed on the BEAST MCC tree pruned of all but the 18 species for which karyological information is available. For species not counted here, chromosome numbers were obtained from Galbany-Casals et al. (2014) and the online Index to Chromosome Numbers in Asteraceae (Watanabe, 2016). Base chromosome number was coded as four discrete, unordered states ($x = 4$, 5, 6 and 7). *Achyrocline satureioides* (Lam.) DC. has recorded base numbers of both $x = 6$ and $x = 7$ (Watanabe, 2016). In order to avoid coding this species as polymorphic, we performed the reconstruction twice, coding *Ac. satureioides* differently in each reconstruction. Ancestral state reconstruction was performed in R using the ape package (Paradise et al., 2004; R Core Team, 2018) and a one-rate model (all rates set to be equal).

We then tested whether changes in base chromosome number associate evolutionarily with life history, using the evolutionary contingency test of Sillén-Tulberg (1993). Specifically, we tested the hypothesis that reductions in base number from the inferred ancestral value of $x = 7$ are more frequently associated with branches reconstructed as having an annual life history (whether obligate or facultative) than expected by chance. The correlation was tested assuming two reductions from higher ($x = 6$ or 7) to lower values ($x = 4$ or 5), with two subsequent reversions to from lower to higher base numbers (in the ancestor of the *H. stellatum* subclade, and in *H. dunense* Hilliard), according to the results of the ancestral trait reconstructions.

3 Results

3.1 Phylogenetic analyses

The separate ITS and ETS trees (trees not shown) had very similar topologies, with the ILD test being non-significant ($p = 0.51$), so we concatenated these regions and performed a combined analysis. Parsimony statistics and alignment characteristics are provided in Table 2. We present the

Table 1 Chromosome counts generated from field-collected material with voucher details (herbarium acronyms according to Thiers, 2018, continuously updated)

Taxon	Clade	Chromosome number (2*n*)	Base chromosome number (*x*)	Number of cells counted	Voucher specimen, and herbaria
Helichrysum alsinoides	P1	8	4	3	South Africa, Western Cape Province, Vanrhynsdorp. *Andrés-Sánchez et al. SA758* (BOL, NBG, SALA).
H. cylindriflorum	P1	12	6	3	South Africa, Western Cape Province, Matroosberg Nature Reserve. *Andrés-Sánchez et al. SA824* (NBG).
H. dunense	P1	14	7	4	South Africa, Western Cape Province, Lambert's Bay. *Andrés-Sánchez et al. SA780* (BOL, NBG, SALA).
H. micropoides	P2	8	4	2	South Africa, Northern Cape Province, between Port Nolloth and Alexander's Bay, 80 km north of Port Nolloth. *Andrés-Sánchez et al. SA735* (BOL, NBG, SALA).
H. obtusum	P4	12	6	2	South Africa, Northern Cape Province, Lekkersing. *Andrés-Sánchez et al. SA738* (BOL, NBG, SALA).
H. stellatum	P1	14	7	4	South Africa, Northern Cape Province, between Kotzerus and Groenriviermond. *Andrés-Sánchez et al. SA765* (BOL, NBG, SALA).

Bayesian consensus topology with the addition of parsimony bootstrap support (BS) values in Fig. 2. Our estimated combined topology (Fig. 2) recovers the HAP clade as monophyletic with high support (PP = 1.00; BS = 96%). The HAP crown node supports an initial trichotomy consisting of *H. lambertianum* (clade S), clade Q with six species (PP = 1.00; BS = 99%) and clade R housing the remaining HAP species (PP = 1.00; BS = 75%). Clade R in turn comprises two well-supported sister lineages: clade P (32 species; PP = 1.00; BS = 98%) and clade A-O, incorporating clades A through O of Galbany-Casals et. al. (2014) and supported by PP = 1.00 and BS = 90%. Clades P, Q and S correspond to the "basal grade" of southern African taxa identified by Galbany-Casals et al. (2014). Clade P comprises species exclusively from Hilliard's (1983) southern African *Helichrysum* groups 12, 14 and 15. All sampled members of Hilliard's (1983) group 14 fall into clade P, although here they do not form a monophyletic group. Most sampled members of group 12 also fall into clade P, although others are recovered in clades Q and A-O. Most sampled members of group 15 also fall into clade P, although some are placed in clade Q. This clade comprises species from Hilliard's (1983) groups 12, 15, 17 and 26. In total, seventeen previously unsampled *Helichrysum* species fall into clades P and Q, increasing the membership of these two early-diverging lineages to 41 (from 19).

Clade P shows considerable internal structure, with the resolution of four well-supported subclades that we name P1 through P4 (Fig. 2). The largest of these, with 17 species, is P1 (PP = 1.00; BS = 84%), in turn comprising several subclades that possess clear morphological synapomorphies, and a

Table 2 Alignment characteristics for the ITS, ETS and concatenated matrices

		ITS	ETS	ITS +ETS
	Number of taxa	110	110	110
	Shortest and longest unaligned sequence length (bp)	587 (*Helichrysum lambertianum*) to 636 [*Dolichothrix ericoides* (Lam.) Hilliard and *Syncarpha mucronata* (P.J. Bergius) B. Nord.]	981 (*H. tinctum*) to 1540 (*H. micropoides*)	
	Number of indels (and length range in bp)	21-35 (1-47)	23-40 (1-177)	
	Aligned length (bp)	663	712	1375
Parsimony analyses	No. of parsimony informative characters	228 (34.4%)	341 (47.9%)	569 (41.4%)
	No. of trees retained	7050	52530	23090
	Best score	953	1464	2431
	Consistency index (CI)	0.4707	0.5112	0.5200
	Homoplasy index (HI)	0.4683	0.4888	0.4800
	Retention index (RI)	0.7827	0.7929	0.7891

The number of most parsimonious trees (MPTs) and indices were calculated in PAUP* v.4.0b10 (Swofford, 2002).

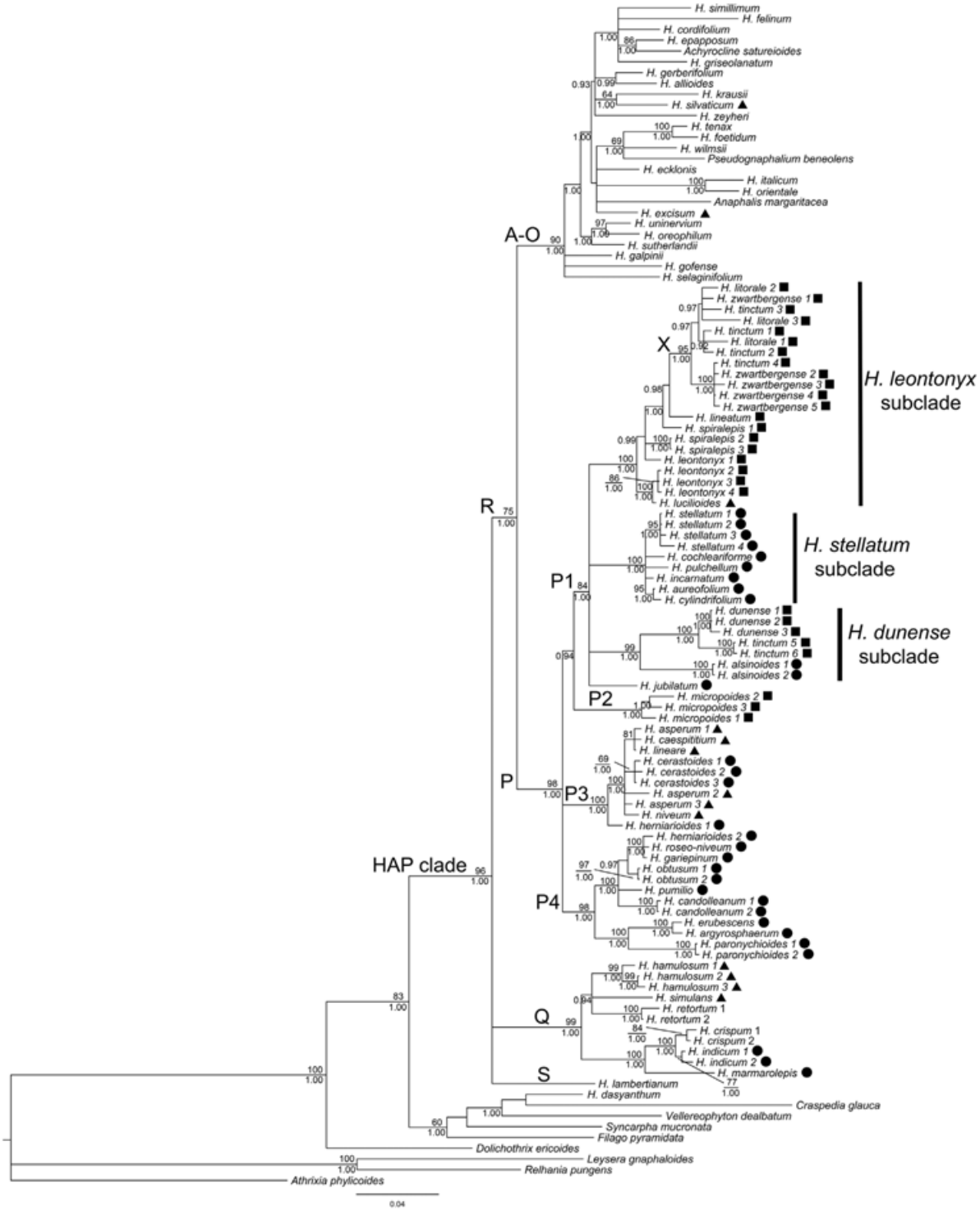

Fig. 2. Bayesian 50% majority rule consensus tree from MrBayes analysis of the ETS + ITS nuclear regions. Branch lengths are the average length from all post burn-in trees in which the branch is present. Numbers above branches represent parsimony bootstrap support values ≥60% while those below branches are the Bayesian posterior probabilities ≥0.90. Letters indicate node names as in Galbany-Casals et al. (2014). Hilliard's (1983) informal morphological groups are indicated after the taxon name: Group 12 (▲), Group 14 (■), and Group 15 (●).

single unplaced species (*H. jubilatum* Hilliard). The relationships amongst these subclades are unresolved, although the monophyly of each is highly supported (all with PP = 1.00 and BS ≥ 99%). For clarity, we have named these subclades the *H. leontonyx* subclade (7 species), the *H. stellatum* subclade (6 species), and the *H. dunense* subclade (3 species). Clade P2 (PP = 1.00 but no BS support; Fig. 2) is monotypic, comprising the three accessions of *H. micropoides* DC. (group 14) and being sister clade P1.

Within clade P1, all members of the *H. leontonyx* subclade have leafy glomerules (Fig. 3). With the exception of *H. lucilioides*, all members are obligate or facultative annuals, and most have a biseriate pappus, this characterizing the former members of the genus *Leontonyx* (*H. litorale*, *H. spiralepis*, *H. lucilioides* and *H. tinctum*). In contrast to the *H. leontonyx* subclade, the *H. stellatum* subclade comprises exclusively perennials (Fig. 3) with open, corymbiform or paniculiform synflorescences lacking the subtending pseudo-involucre of leaves. All members of this subclade were included in Hilliard's (1983) group 15. Finally, the small *H. dunense* subclade is another clade of strict or facultative annual species with leafy glomerules. This *H. dunense* subclade comprises *H. alsinoides* DC. from group 15, *H. dunense* from group 14, and two accessions from the Kamiesberg massif that, according to the key of Hilliard (1983), belong in *H. tinctum*. This is a widespread species that occurs throughout the GCFR, from the Northern Cape to the Eastern Cape province. However, four other accessions of *H. tinctum*, from across the range, are distantly placed in the *H. leontonyx* subclade, and the two Kamiesberg specimens clearly require closer investigation. All members of the *H. dunense* subclade, as in the *H. leontonyx* subclade, have involucral bracts arranged in four or fewer series, but unlike the *H. leontonyx* subclade, all *H. dunense* subclade species possess a uniseriate pappus (Fig. 3).

Clade P3 (PP = 1.00; BS = 100%) comprises six species, all possessing leafy glomerules and all except *H. herniarioides* DC. being perennial (Figs. 2, 3). A second accession of *H. herniarioides* is placed outside clade P3, in clade P4, calling one of these placements into question. In contrast to other species of clade P3, *H. caespititium* and *H. niveum*, have a biseriate pappus. Clade P3 includes four species from Hilliard's (1983) group 12 [*H. asperum* (Thunb.) Hilliard & B.L.Burtt, *H. caespititium*, *H. lineare* DC. and *H. niveum*] and two from group 15 (*H. cerastoides* DC. and *H. herniarioides*).

Clade P4 (PP = 1.00; BS = 98%) houses nine species, all from Hilliard's (1983) group 15. Although it comprises a mix of annual and perennial species, all its members possess leafy glomerules. Within clade P4, *H. argyrosphaerum* and *H. paronychioides* possess a biseriate pappus, while the remaining species are uniseriate. Of the species with a biseriate pappus, those placed by Hilliard (1983) in group 14 fall into the *H. leontonyx* subclade within clade P1, while those classified in her group 15 fall into clades P3 and P4 (Fig. 3).

3.2 Ancestral trait reconstruction

Maximum likelihood reconstructions suggest that the ancestor of the HAP clade was perennial with a uniseriate pappus, and the synflorescence arranged in a corymb or open panicle lacking subtending leaves (Table 3; Fig. 3). A perennial life history was inferred as ancestral in all clades except for clade P1 + P2, although the exact point of the transition from perennial to annual is unclear. The ancestor of clades P1 + P2 has only a 0.45 relative probability of being perennial, which drops to 0.30 in the ancestor of clade P1. The ancestor of the *H. leontonyx* subclade, however, has 0.05 relative probability of being perennial, with P = 0.80 for being a facultative annual (Table 3; Fig 3). A corymbose or paniculiform synflorescence with no pseudo-involucre was inferred as the ancestral state for all clades except clade P and its constituent clades, for which the ancestral state was the presence of leafy glomerules (Table 3; Fig. 3). This type of synflorescence was thus inferred to have evolved twice, first in the perennial ancestor of clade P, coincident with an inferred colonisation of the ASRSA (Fig. 3), and later in a single species within clade Q (*H. simulans* Harv. & Sond.). The descendants of clade P retain the leafy glomerules, with the exception of the unusual *H. stellatum* subclade of clade P1, which is anomalous within clade P because of a uniformly perennial life-history, coincident with a reversion to the ancestral synflorescence arrangement.

The ancestral state of the pappus was inferred to be uniseriate (P = 1.00) in all clades except for the *H. leontonyx* subclade, where it was inferred to be biseriate (Table 3; Fig. 3). Taxa with biseriate pappus are clearly not monophyletic, and this trait exhibits a fairly high degree of lability. We infer a reversion to the uniseriate state in *H. leontonyx* DC. within the *H. leontonyx* subclade, as well as two independent shifts to the biseriate from a uniseriate ancestor: once within clade P4 (in the ancestor of *H. argyrosphaerum*, *H. erubescens* Hilliard and *H. paronychioides*), and again in *H. niveum* within clade P3.

3.3 Divergence time and biogeographic history

The time-proportional tree obtained from BEAST (Fig. 3) shows a very similar topology to the MrBayes tree. Although our analysis places the ancestor of the HAP clade (node II), and the early branching events in clades P, Q, R and S in the Early to Middle Miocene, most divergence events in the well-sampled clades P–S occurred in the Plio-Pleistocene. The ancestors of the entire crown radiation, of the HAP clade, and of clades P, Q, R and Q + S are inferred to have occurred in the GCFR (Fig. 3 and Table 4). Within clade Q + S, all descendants remained in the GCFR. In clade P, while many species currently occur in the GCFR, there were several subsequent occupations of the ASRSA during the Late Miocene to early Pliocene, as well more recent dispersals to the afrotemperate Drakensberg region. Although sparse sampling will lead to poorer inferences regarding clade A-O, our analysis indicates that this lineage expanded first to the Drakensberg, which may have acted as a springboard for dispersal to the north, and subsequently colonised the northern Hemisphere (Fig. 3; Table 4).

The crown node of clade P is dated as Late Miocene (8.6 Ma; 95% HPD 5.7–11.4), and both methods of ancestral area reconstruction placed this node with high probability in the GCFR (cumulative probability, P = 1.00 for S-DIVA and Lagrange), although in both cases there was also some probability of occurrence in the ASRSA (Fig. 3; Table 4). Clade P1 has an estimated age of 6.4 Ma (95% HPD 4.2–8.8 Ma) and its ancestor is inferred to have occurred exclusively within the GCFR (P = 100 for S-DIVA and Lagrange). Clade P3, by contrast, harbours species from both the summer and winter rainfall zones of South Africa, and ancestral area reconstructions

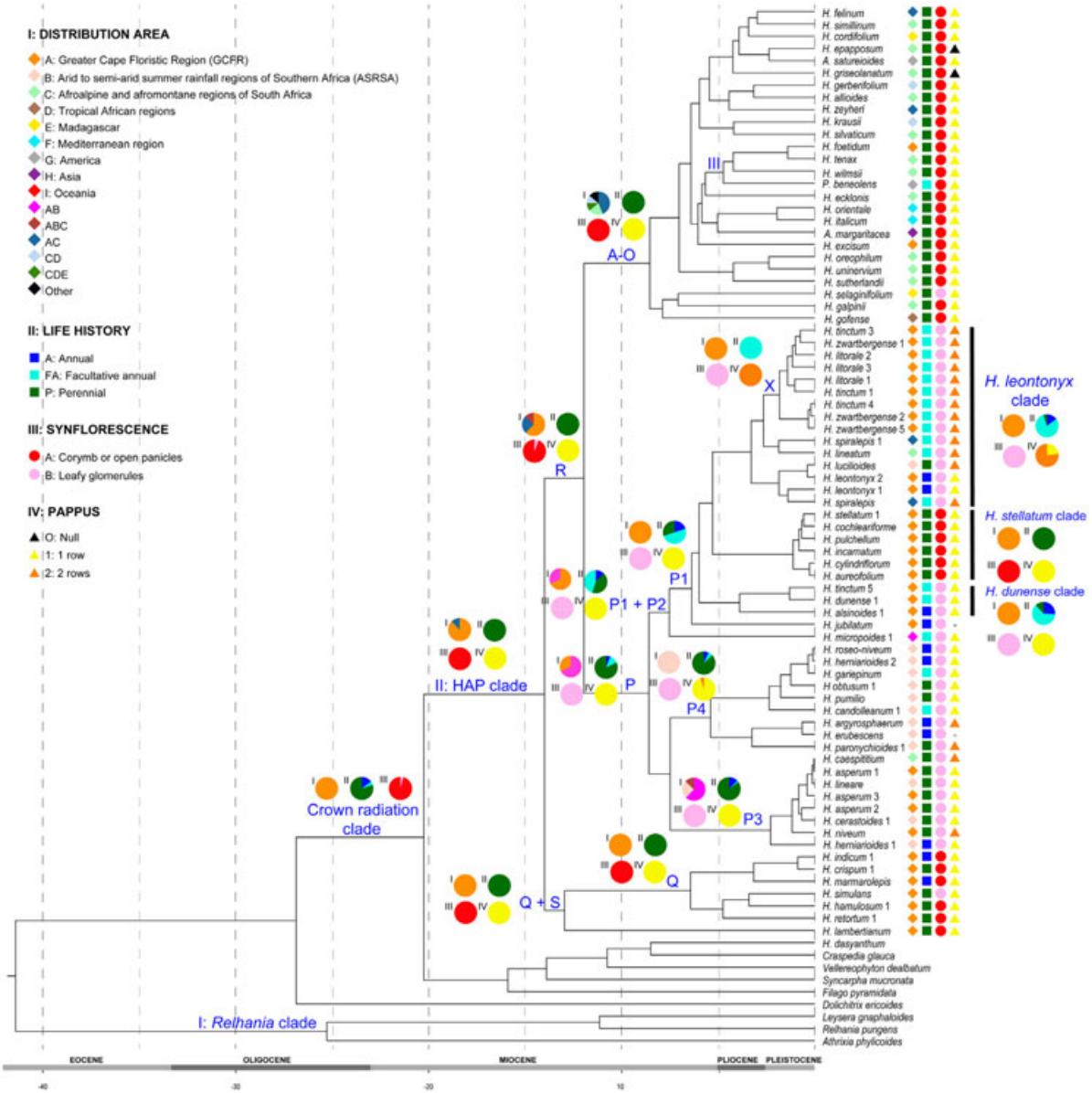

Fig. 3. Time-proportional maximum clade credibility tree obtained with BEAST v 1.8.4. Distribution area, life history, synflorescence type and pappus arrangement of extant species are mapped at the tips, on the right of each taxon name. The three secondary calibration nodes are indicated by roman numerals. Names of the main clades found in Fig. 2 are indicated in blue close to the node. Relative probabilities of ancestral reconstructions for selected nodes are shown as pie charts: I) ancestral area; II) life history; III) synflorescence arrangement; and IV) pappus arrangement. Probabilities of ancestral reconstructions are given in Table 3. Values of node ages estimates and ancestral area reconstruction [the statistical dispersal-vicariance analysis (S-DIVA) and the DEC model] are indicated in Table 4.

suggest an origin in both the GCFR and ASRSA (Fig. 3; Table 4) at the Pliocene-Pleistocene boundary (2.3 Ma, 95% HPD 1.1–4.0 Ma). Clade P4 is an endemic ASRSA clade, inferred to have both originated and radiated in this area at about 5.4 Ma (95% HPD 3.2–7.9 Ma).

3.4 Evolution of base chromosome number

Successful chromosome counts were obtained for two to four seedlings of each of six clade P species (Table 1; Fig. 4). We document for the first time in *Helichrysum* the base number of $x = 6$ and the chromosome number $2n = 12$, obtained for *H. cylindriflorum* (L.) Hilliard & B. L. Burtt and *H. obtusum* Moeser. All the species counted are diploid, and the base numbers inferred from the counts we obtained are $x = 7$ (*H. dunense* and *H. stellatum* Less.), $x = 4$ (*H. alsinoides* and *H. micropoides*) and $x = 6$ (*H. cylindriflorum* and *H. obtusum*). To date, the base number $x = 4$ is unique to clade P in the HAP clade (Galbany-Casals & Romo, 2008; Galbany-Casals et al., 2009). In addition, all available observations indicate that clades P and Q are exclusively diploid, while di-, tetra-,

Table 3 Relative probabilities of ancestral states at relevant nodes (refer to Fig. 3 for node labels) for life history, synflorescence structure, and number of pappus series, inferred using the one-rate ML reconstruction model on the BEAST MCC tree

Node	Life history	Synflorescence	Pappus series
I: *Relhania* clade	P: 0.56	A: 0.92	-
	FA: 0.22	B: 0.08	
	A: 0.22		
Crown radiation clade	P: 0.80	A: 0.96	-
	FA: 0.05	B: 0.04	
	A: 0.15		
II: HAP clade	P: 1.00	A: 98	1: 1.00
		B: 0.02	
Q + S	P: 0.98	A: 0.99	1: 1.00
	FA: 0.01	B: 0.01	
	A: 0.01		
Q	P: 0.98	A: 1.00	1: 1.00
	FA: 0.01		
	A: 0.01		
R	P: 0.98	A: 0.94	1: 1.00
	FA: 0.01	B: 0.06	
	A: 0.01		
P	P: 0.82	A: 0.2	1: 1.00
	FA: 0.12	B: 0.98	
	A: 0.06		
P1 + P2	P: 0.45	A: 0.01	1: 1.00
	FA: 0.43	B: 0.99	
	A: 0.12		
P1	P: 0.30	A: 0.02	1: 1.00
	FA: 0.50	B: 0.98	
	A: 0.20		
Helichrysum leontonyx subclade	P: 0.05	B: 1.00	1: 0.23
	FA: 0.80		2: 0.77
	A: 0.15		
X	FA: 1.00	B: 1.00	2: 1.00
H. stellatum subclade	P: 1.00	A: 1.00	1: 1.00
H. dunense subclade	P: 0.12	B: 1.00	1: 1.00
	FA: 0.62		
	A: 0.26		
P3	P: 0.85	B: 1.00	1: 1.00
	FA: 0.02		
	A: 0.13		
P4	P: 0.87	B: 1.00	1: 0.95
	FA: 0.06		2: 0.05
	A: 0.07		
A-O	P: 1.00	A: 1.00	1: 1.00

Abbreviations for life history: P = perennial, FA = facultative annual, and A = obligate annual. For synflorescence structure: A = corymb or open panicle while B = leafy glomerules. Number of pappus series is represented by numbers.

hexa- and octoploids have been observed in clades A-O (Galbany-Casals et al., 2014). Ancestral reconstruction of base number suggests that the ancestral value for the HAP clade and for clades A-O, P, Q and R is $x = 7$ (Fig. 5). Our reconstruction (Fig. 5) indicates a minimum of six chromosomal evolution events in the early-diverging clades P and Q. A reduction from $x = 7$ to $x = 4$ probably occurred somewhere between nodes P and P1, with the most parsimonious option being that the ancestor of subclades P1 + P2 already had $x = 4$, so that a second reduction is not required to explain this base number in *H. micropoides* (P2). However, there is still a moderately high relative probability of $x = 7$ in the ancestor of clade P1, and even in the ancestor of the *H. leontonyx* + *H. stellatum* subclades, so this may have occurred later. While the ancestor of clade P1 most likely had $x = 4$, subsequent reversions to $x = 7$ occurred twice within this clade, once in the ancestor of *H. dunense* and again in the common ancestor of *H. cylindriflorum* + *H. stellatum*. *Helichrysum cylindriflorum* then demonstrates a secondary reduction, from $x = 7$ to $x = 6$. A second, independent reduction from 7 to 6 is evident in the ancestor of *H. obtusum* in clade P4, while a unique reduction from 7 to 5 haploid chromosomes is inferred for the ancestor of *H. indicum* (L.) Grierson in clade Q (Fig. 5). Overall, the inferred changes involve four decreases from $x = 7$ (resulting once in $x = 4$, twice in $x = 6$, and once in $x = 5$), and two increases, both from $x = 4$ back to $x = 7$.

3.5 Association between chromosome number and life-history

Of the five perennial species from clades P and Q for which chromosome counts are available, all have base numbers of $x = 6$ or $x = 7$ (Fig. 5). By contrast, six of the seven short-lived taxa have base numbers of $x = 4$ or $x = 5$. The sole exception is the facultative annual *H. dunense*, with $x = 7$. Of the four inferred reductions in base chromosome number, the two involving reduction from $x = 7$ to $x = 5$ or fewer are associated with a shift from the perennial to the annual life-history, while the other two, involving the loss of a single chromosome pair (from $x = 7$ to $x = 6$), both occurred in the context of a perennial lineage (Fig. 5). Of the two inferred increases from $x = 4$ to $x = 7$, one is associated with a reversion from annuality to perenniality (in the *H. stellatum* subclade), while the other occurs in the facultative annual *H. dunense*. Assuming that $x = 6$ or $x = 7$ represents "higher" base numbers, and $x = 4$ or $x = 5$ "lower" numbers, the evolutionary contingency test of Sillén-Tulberg (1993) confirmed that reductions in base number are significantly ($p = 0.036$) more frequently associated with branches reconstructed as having a short-lived life-history (5 branches, 2 showing chromosomal reductions) than with branches reconstructed as having a perennial life-history (19 branches, 0 with chromosomal reductions).

4 Discussion

Increased phylogenetic representation of GCFR *Helichrysum* species (from less than half to two-thirds of the 94 species now represented), demonstrates that 17 previously-unsampled species of *Helichrysum* fall into the basal HAP grade (clades P, Q, and S), bringing the total known species-richness of these clades to around 40 (Galbany-Casals et al., 2014; Nie

Table 4 Node ages and ancestral area reconstruction for nodes labelled as in Fig. 3

Node	Mean age (Ma)	95% HPD (Ma)	Probabilities of ancestral areas (S-Diva)	Cumulative probability of ancestral areas (S-Diva)	Probabilities of ancestral areas (Lagrange)	Cumulative probability of ancestral areas (Lagrange)
I: *Relhania* clade	23.4	17.8-29.0	GCFR: 100	GCFR: 100	GCFR and afroalpine and afromontane regions of South Africa: 28	Afroalpine and afromontane regions of South Africa: 91
					Afroalpine and afromontane regions of South Africa: 19	GCFR: 79
					GCFR, ASRSA and afroalpine and afromontane regions of South Africa: 19	ASRSA: 19
					Others: 34	
II: HAP clade	12.7	9.3-16.6	GCFR: 99 Others: 1	GCFR: 100	GCFR: 88 GCFR and afroalpine and afromontane regions of South Africa: 12	GCFR: 100 Afroalpine and Afromontane regions of South Africa: 12
III	6.7	5.8-8.7	Afroalpine and afromontane regions of South Africa: 84	Afroalpine and afromontane regions of South Africa: 100	Afroalpine and afromontane regions of South Africa and America: 46	Afroalpine and afromontane regions of South Africa: 100
			Afroalpine and afromontane regions of South Africa and America: 16	America: 16	Afroalpine and afromontane regions of South Africa: 35	America: 84
					GCFR, Afroalpine and afromontane regions of South Africa and America: 19	GCFR: 19
Crown radiation clade	20.3	14.0-27.6	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
Q + S	13.0	0-0	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
Q	6.5	4.0-9.4	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
R	12.0	8.8-15.4	GCFR and afroalpine and afromontane regions of South Africa: 33	GCFR: 84	GCFR: 62	GCFR: 100
			GCFR, ASRSA and afroalpine and afromontane regios of South Africa: 33	Afroalpine and afromontane regions of South Africa: 72	GCFR and afromontane regions of South Africa: 25	Afroalpine and afromontane regions of South Africa: 38
			GCFR, afroalpine and afromontane regios of South Africa and tropical african regions: 6	ASRSA: 39	GCFR, ASRSA and afromontane regions of South Africa: 13	ASRSA: 13
			GCFR and tropical african regions: 6	Tropical african regions: 12		
			GCFR, ASRSA and tropical african regions: 6			
P	8.6	5.7-11.4	GCFR and ASRSA: 60 GCFR: 40	GCFR: 100 ASRSA: 60	GCFR and ASRSA: 65 GCFR: 35	GCFR: 100 ASRSA: 35

Continued

Table 4 Continued

Node	Mean age (Ma)	95% HPD (Ma)	Probabilities of ancestral areas (S-Diva)	Cumulative probability of ancestral areas (S-Diva)	Probabilities of ancestral areas (Lagrange)	Cumulative probability of ancestral areas (Lagrange)
P1	6.4	4.2-8.8	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
Helichrysum leontonyx subclade	3.3	2.0-4.7	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
X	1.8	1.1-2.8	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
H. stellatum subclade	1.1	0.5-1.9	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
H. dunense subclade	3.9	2.3-5.9	GCFR: 100	GCFR: 100	GCFR: 100	GCFR: 100
P1 + P2	7.5	5.0-10.3	GCFR: 100	GCFR: 100	GCFR: 67 GCFR and ASRSA: 33	GCFR: 100 ASRSA: 33
P3	2.3	1.1-4.0	GCFR and ASRSA: 100	GCFR: 100 GCFR: 100	GCFR and ASRSA: 63 ASRSA: 24 GCFR, ASRSA and afromontane regions of South Africa: 13	ASRSA: 100 GCFR: 76 Afromontane regions of South Africa: 13
P4	5.4	3.2-7.9	ASRSA: 100	ASRSA: 100	ASRSA: 100	ASRSA: 100
A-O	8.5	6.2-11.3	Afroalpine and afromontane regios of South Africa and tropical african regions: 50	Afroalpine and afromontane regios of South Africa: 95	GCFR and afroalpine and afromontane regios of South Africa: 44	GCFR: 58
			Afroalpine and afromontane region of South Africa and Madagascar: 37	Tropical african regions: 63	Afroalpine and afromontane region of South Africa: 20	Afroalpine and afromontane region of South Africa: 100
			Afroalpine and afromontane regios of South Africa, tropical african regions and Madagascar: 8	Madagascar: 53	Afroalpine and afromontane region of South Africa and tropical african regions: 11	Tropical african regions: 22
			Tropical african regions and Madagascar: 5		Afroalpine and afromontane regios of South Africa, tropical african regions and Madagascar: 11 Others: 14	Madagascar: 11

Probabilities of ancestral areas (columns 4 and 6) represent the unique probability of occurring in ONLY that area and no other, and sum to 100. Cumulative probabilities (columns 5 and 7) represent the summed probability of occurrence in an area, including all incidences in which an area was inferred, whether alone or in combination with other areas, and so may sum to >100.

et al., 2016). Our results confirm that all of the basal-grade HAP are located in southern Africa, with the vast majority (92%) in the GCFR or the ASRSA (the western part of southern Africa). The remaining three species extend only as far as the Afroalpine and afromontane Drakensberg region, which is also within southern Africa, but in the eastern half of the subcontinent.

4.1 Age and geographic origin

Our results point conclusively to an origin for the HAP clade in southern Africa, and specifically in the GCFR, in the Late Oligocene to Early Miocene, which is close to the estimated age of establishment of GCFR summer arid conditions around the Middle Miocene (Dupont et al., 2011; Dupont et al., 2013; Hoetzel et al., 2013). The GCFR also appears to be the place of initial diversification in the HAP clade, although there is evidence for recruitment into the southern African afrotemperate zone (the greater Drakensberg region) by the Middle to Late Miocene. This was likely facilitated by the similar cool growing-season conditions of both the southern part of the GCFR and the afrotemperate zone, intensified by the appearance of new habitats due to the Miocene-Pliocene

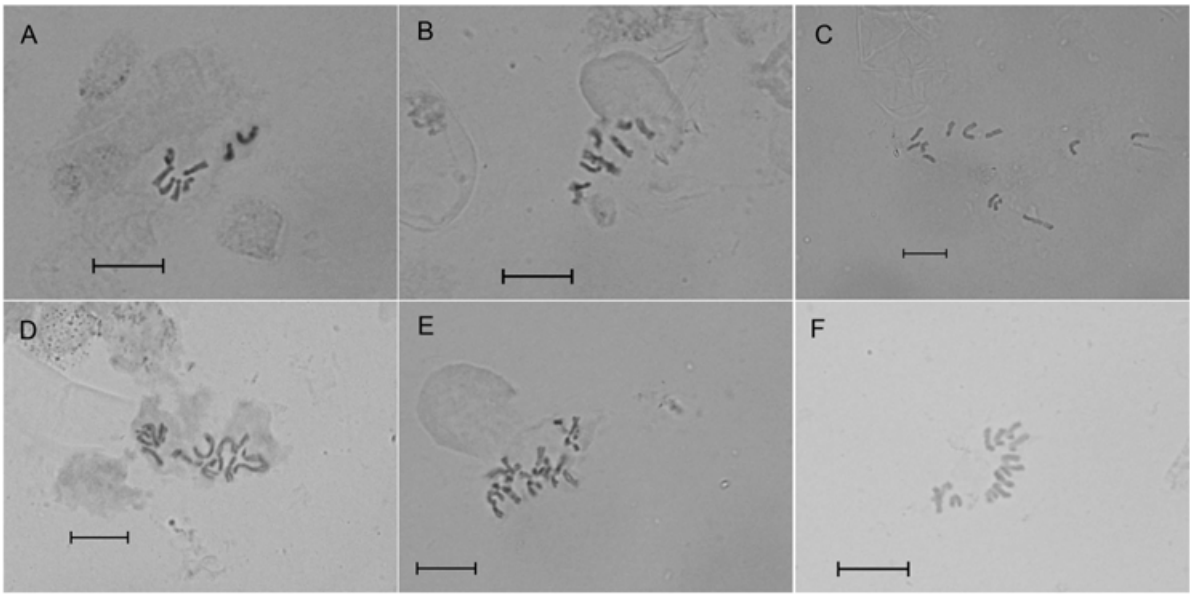

Fig. 4. Mitotic metaphases obtained in this study for members of clade P. **A,** *Helichrysum alsinoides* ($2n = 8$). **B,** *H. micropoides* ($2n = 8$). **C,** *H. obtusum* ($2n = 12$). **D,** *H. cylindriflorum* ($2n = 12$). **E,** *H. dunense* ($2n = 14$). **F,** *H. stellatum* ($2n = 14$). Scale bars indicate 10 μm.

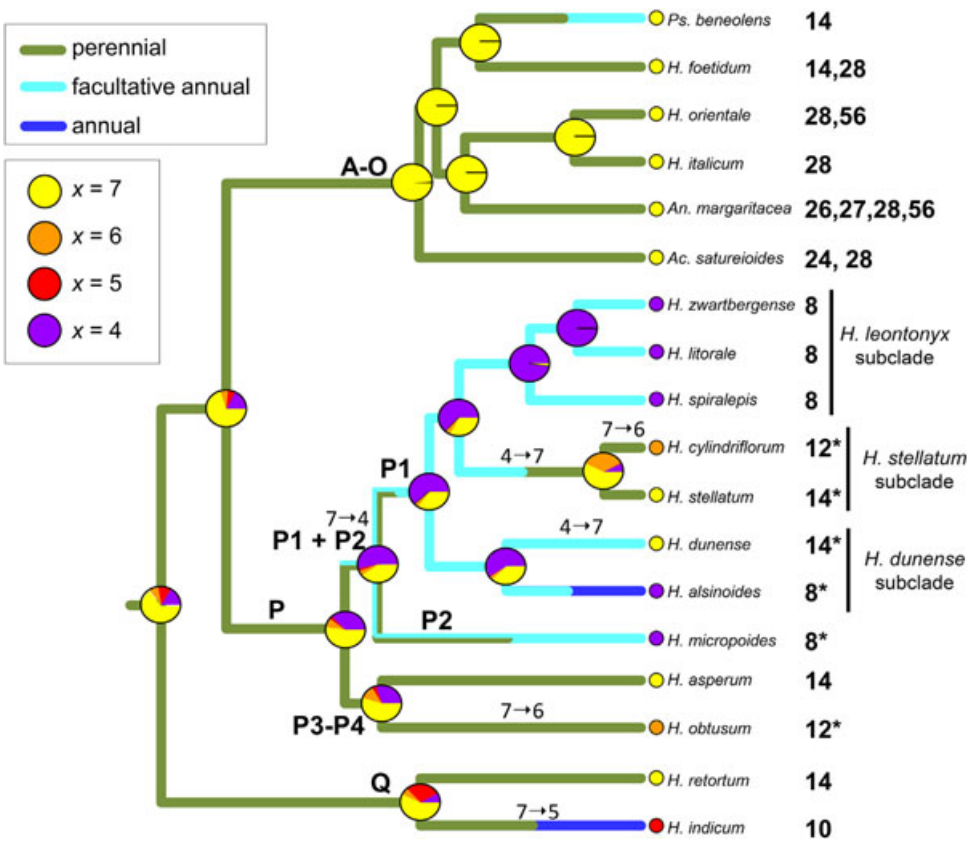

Fig. 5. Maximum likelihood reconstruction of base chromosome number, with *Achyrocline satureioides* coded as $x = 7$. Pie charts at nodes represent the relative probability of each state at that node. The reconstruction is presented on the BEAST MCC tree, pruned to include only those taxa for which chromosome numbers are known, and with branches coloured by inferred ancestral life-history according to the most probable state from the ML reconstruction in Mesquite (Fig. 3). Chromosome counts are indicated following the species name, with asterisks indicating counts made for the present study. Note that coding *Ac. satureioides* as $x = 6$ does not change the strong inference of a base number of $x = 7$ for the ancestor of clade A-O.

Drakensberg uplift (Moore, 1999; Carbutt & Edwards, 2004; Moore & Blenkinsop, 2006; Partridge et al., 2010; Bentley et al., 2014).

The arid summer-rainfall zone was independently colonised at around the same time, but diversification within the ASRSA did not lead to subsequent dispersal out of the region, although these findings are based on limited sampling. It appears from our reconstruction that dispersal to the afrotemperate zone may have facilitated the subsequent enormous diversification of the HAP clade coincident with colonisation of tropical Africa, Madagascar, the greater Mediterranean region and Asia. The evidence for this is that the ancestor of clade A-O has a high probability of being located in this zone (Fig. 3), possibly with a range that also encompassed GCFR habitats, and certainly with several re-colonisations of the GCFR, a pattern already noted in other lineages (Galley et al., 2007; Devos et al., 2010; Bentley et al., 2014: Linder & Verboom, 2015). In addition, at least three recent incidences of colonisation of the afrotemperate region from the GCFR in clade P (in *H. spiralepis*, *H. lineatum* and *H. caespititium*) indicate that this type of dispersal and/or range expansion is ongoing.

Our findings accord well with a late, cross-seeded set of floristic radiations in the southern African subregion, following the pattern found by Linder & Verboom (2015). We also demonstrate that the large, widely-distributed HAP clade is a "Cape lineage" in a historical sense, originating and experiencing both early and ongoing diversification in the Cape region, despite later dispersal and radiation in other parts of the world, and the current concentration of species diversity outside the GCFR.

Unlike the large HAP radiation clades A-O, almost all the descendants of the initial HAP divergence events (clades P, Q and S) remained in the GCFR or ASRSA, with the exception of the few, very recent expansions into the afrotemperate zone mentioned above. The early-diverging lineages thus demonstrate an ongoing link with arid habitats, due either to aridity during the summer months, or all-year dry conditions. In particular, the ancestors of clades P3 and P4 appear to have been specialised for the expanding ASRSA habitat, caused by more widespread aridification since the Miocene (Marlow et al., 2000; Linder, 2003; de Menocal, 2004) and possibly exacerbated by the rain-shadow effect of eastern uplift (Sepulchre et al., 2006). This migration route following the west coast of South Africa is hypothesized for other plant lineages such as Zygophyllaceae (Bellstedt et al., 2008) or *Moraea* (Goldblatt et al., 2002). Evolutionary specialisation for survival in arid conditions might explain why the descendants of these lineages (clades P3 and P4) were unable to disperse out of southern Africa; it may also be the reason for linkage with the suite of unusual traits prevalent in these taxa.

4.2 Annual life-history and low base chromosome number

The only other study to reconstruct life-history in the Gnaphalieae (Bergh & Verboom, 2011) inferred an annual ancestor for the clade, but was based on very sparse sampling, with only six HAP clade species. Inferences based on the more representative sample in the current study are likely to be more realistic. We find a perennial ancestor for the HAP clade, with several independent shifts to short-lived life-cycles in the basal grade. We also demonstrate a strong association between reduction in chromosome number and evolution of the short-lived life-history. Mas de Xaxars et al. (2016) hypothesized that dysploidy could be related to adaptation of plants to extreme habitats. Galbany-Casals et al. (2009) previously argued that dysploidy could be an adaptation to aridity in *Helichrysum*. Several other angiosperm lineages show the pattern of annual species having lower chromosome numbers or smaller C-values than closely-related perennials. For example, within other Cape Gnaphalieae, low chromosome numbers of $x = 4$ and $x = 5$ are known only in the six annual or biennial members of the *Relhania* clade, all the other species being perennials with $x = 7$ (Bergh et al., 2018). A correlation between low base number and annuality has also been observed in other groups within Asteraceae (García-Jacas et al., 1996; Watanabe et al., 1999; Garnatje et al., 2004). Gustafsson (1948), in a global survey, found that annual angiosperms generally show low basic chromosome numbers relative to their perennial relatives, and keep to the diploid or low-polyploid state. The present study, however, is the first to demonstrate a statistically significant evolutionary link between short life-history and low chromosome number.

One of the strongest ecological correlates of annual life-history is aridity (Schaffer & Gadgil, 1975; van Rooyen, 1999), and patterns in southern African *Helichrysum* support this, with the annuals occurring either in the more arid parts of the GCFR, or on coastal sands, or in the drier habitats of the ASRSA. Some authors consider this correlation to relate to the severity of the dry season (e.g., Evans et al., 2005) although in the context of the GCFR, Verboom et al. (2012) showed that annuality is more likely a response to a shorter growing season, creating a requirement for faster relative growth rates in order to flower and set seed within the short window of favourable conditions. Plants with smaller genomes have faster cell division cycles and so higher intrinsic growth rates (Chooi, 1971; Bennett, 1972; Rayburn & Auger, 1990; Knight et al., 2005), so smaller genome sizes undoubtedly offer selective advantages in annuals occurring in the short growing-season environments of the GCFR and ASRSA. Since most of the basal grade members of the HAP clade (including the annuals) are diploid, future examinations of C-values would be valuable in testing whether short-lived taxa with low chromosome number also have smaller genome sizes.

4.3 Synflorescence architecture

There appears to be an association between the leafy glomerules and the occupation of arid habitats, given the coincidence of the evolution of this architecture and the inferred habitat shift in the ancestor of clade P. Also, although the leafy glomerules are prevalent in GCFR species, the GCFR represents a range of habitats, from mesic to arid, and most species with leafy glomerules occur in the more arid habitats within this region. We are unable to explain the link, although speculate that it may be related to pollinator-limitation in arid environments, with the leafy glomerules possibly presenting a more compact floral unit for pollinators (e.g., Figs. 1A–1C versus 1D–1F). Several authors (Cronquist, 1981; John, 1996; Stevens, 2001; Katinas et al., 2008) argued that the structure of the synflorescence is a key feature behind the diversification of the family Asteraceae. The aggregation of two or more capitula into secondary inflorescences is considered a derived evolutionary feature (Katinas et al., 2008). Stebbins (1967)

proposed several advantages for secondary inflorescence aggregation, including greater attraction to insect pollinators or better protection for the seeds. In particular, the formation of secondary heads or clusters provides a mechanism for increasing the size of the inflorescence (Stebbins, 1967). Additionally, the general correlation between annuality and selfing (Stebbins, 1970; Lloyd, 1980; Barrett et al., 1997) seems not to hold in the arid southern African context, as Ueckermann & van Rooyen (2000) identified insect-mediated outcrossing in four species of Namaqualand (arid GCFR) daisies.

4.4 Taxonomy and morphology

The present study, based on improved sampling for the basal lineages relative to previous studies, confirms the lack of congruence between recovered clades and the informal infrageneric groups proposed by Hilliard (1983), although the basal grade of the HAP clade comprise species placed mostly in Hilliard's (1983) groups 12, 14 and 15. Although the informal groups are not monophyletic, morphology is not completely misleading amongst the HAP basal lineages. The features that most strongly define the subclades of Clade P are life-history, the presence of leafy glomerules or not, and the arrangement of the pappus bristles into one or two rows, all being morphological features that are otherwise unusual or absent within the HAP clade (Figs. 1, 3).

Despite being a difficult character to see in the field, the arrangement of the pappus bristles into two rows was greatly emphasised by Cassini (1822). He erected a new genus *Leontonyx* to house biseriate species *H. spiralepis* (*Leontonyx tomentosa* Cass.) and *H. tinctum* (*Leontonyx colorata* Cass.). Candolle (1838) added two more biseriate species, viz. *H. litorale* (*Leontonyx angustifolius* DC.) and *H. pumilio* (O. Hoffm.) Hilliard & B.L. Burtt (*Leontonyx pusillus* Less.), but this destroyed the macromorphological coherence of *Leontonyx*. Hilliard & Burtt (1981) did not support the separation of *Leontonyx*, because several former members have a uniseriate pappus (*H. micropoides*, *H. leontonyx* and *H. lineatum*). Our analyses demonstrate that while all species with biseriate pappus bristles fall into clade P, they are not monophyletic, and a separate genus is clearly not warranted for these taxa. It is not known whether the biseriate pappus is adaptive, and if so, how.

Several species included in the present study, e.g., *H. asperum*, *H. herniarioides*, *H. litorale*, *H. tinctum* or *H. zwartbergense*, show some degree of intraspecific sequence variation and non-monophyly of multiple accessions. Some of them also exhibit a large range of morphological variation, and particularly with regards to the *H. tinctum* samples from the Kamiesberg, it is possible that unrecognized taxa exist within them. Further studies should focus on the taxonomy of these species.

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Appendix I. Species included in the molecular analyses with voucher information and Genbank or EMBL accession number (ITS, and ETS). An asterisk indicates sequences previously published. Each entry is arrayed as follows: ***Taxon***; **country**, locality, collector and collector number, herbarium acronym according to Thiers (2018, continuously updated), Genbank or EMBL accession numbers (ITS, ETS), asterisks indicate the new sequences of this work.

Achyrocline satureioides (Lam.) DC. **ARGENTINA**, Entre Ríos, Departamento Colón, Gutiérrez et al. 658 (LP) (HG797713, HG797976). ***Anaphalis margaritacea*** (L.) Benth. & Hook.f. **CANADA**, West, Blanco et al. s.n. (BC) (FN645827, FN645632). ***Athrixia phylicoides*** DC. **SOUTH AFRICA**, Eastern Cape Province, between Mount Fletcher and Rhodes, Romo et al. 14395 (BC) (FN645816, FN645634). ***Craspedia glauca*** Spreng. **AUNTRALIA**, Tasmania, Eaglehawk Neck, Ford et al. 21/03 (CHR565520) (EF187655, EF187629). ***Dolichothrix ericoides*** (Lam.) Hilliard. **SOUTH AFRICA**, Swartberg Pass, Skelmdraai, Romo et al. 14514 (BC) (FN645828, FN645622). ***Filago pyramidata*** L. **SPAIN**, Balearic Islands, Ibiza, Sta. Agnès, Galbany et al. s.n. (BCN6124) (AY445190, FN645589). ***Helichrysum allioides*** Less. **SOUTH AFRICA**, Eastern Cape Province, Wodehouse District, Bester B7360 (PRE844007) (MH810349*, MH829994*). ***Helichrysum alsinoides*** DC. (1) **SOUTH AFRICA**, Western Cape Province, Holrivier, Andrés-Sánchez et al. SA756 (BOL, NBG, SALA) (MH810350*, MH829995*); (2) **SOUTH AFRICA**, Western Cape Province, Vanrhynsdorp, Andrés-Sánchez et al. SA758 (BOL, NBG, SALA) (MH810351*, MH829996*). ***Helichrysum argyrosphaerum*** DC. **SOUTH AFRICA**, Free State Province, Koekemoer M3532 (BC) (HG797730, HG797998). ***Helichrysum asperum*** (Thunb.) Hilliard & B.L. Burtt (1) SOUTH AFRICA, Eastern Cape Province, between Nieu-Bethesda and Aliwal North, Bergh 1077 (NBG) (MH810352*, MH829997*); (2) **SOUTH AFRICA**, Western Cape Province, Romo et al. 14526 (BC867744) (FJ211470, FJ211528); (3) **SOUTH AFRICA**, Western Cape Province, Knolfontein, Jardine 1476 (NBG272818) (MH810353*, MH829998*). ***Helichrysum aureofolium*** Hilliard. **SOUTH AFRICA**, Western Cape Province, Knolfontein, Swartruggens, 60 Km NE of Ceres, Jardine 1906 (NBG) (MH810354*, MH829999*). ***Helichrysum caespititium*** (DC.) Sond. **SOUTH AFRICA**, Free State Province, between Sasolburg and Parys, Koekemoer M3531 (PRE771641) (MH810355*, MH830000*). ***Helichrysum candolleanum*** Buek (1) **MOZAMBIQUE**, Gaza Province, Burrows 8560 (Buffelskloof herbarium) (HG797736, HG798009); (2) **NAMIBIA**, 88 Km S of Helmeringenhausen, Koekemoer M3525 (PRE802790) (MH810356*, MH830001*). ***Helichrysum cerastoides*** DC. (1) **NAMIBIA**, 12 KM S Otavi, Koekemoer M3528 (PRE802630) (MH810357*, MH830002*); (2) **SOUTH AFRICA**, Mpumalanga Province, Burrows 8504 (Buffelskloof herbarium) (HG797738, HG798011); (3) **SOUTH AFRICA**, Northern Cape Province, Roggeveld Escarpment between Middelpos and Calvinia, Bergh 1728 (NBG) (MH810358*, MH830003*). ***Helichrysum cochleariforme*** DC. **SOUTH AFRICA**, Western Cape, Velddrift, Rocher Pan Nature Reserve, Bergh 2279 (BOL, NBG, SALA) (MH810360*, MH830005*). ***Helichrysum cordifolium*** DC. **MADAGASCAR**, Antananarivo Province, Bayer et al. (MAD04003, CANB660340) (HG797744, HG798020). ***Helichrysum crispum*** (L.) D. Don (1) **SOUTH AFRICA**, Western Cape Province, Romo et al. 14532 (BC867748) (HG797746, HG798022); (2) **SOUTH AFRICA**, Western Cape Province, Struisbaai, Koekemoer M3728 (PRE847061) (MH810361*, MH830006*). ***Helichrysum cylindriflorum*** (L.) Hilliard & B.L. Burtt **SOUTH AFRICA**, Western Cape Province, Ceres, Andrés-Sánchez et al. SA787 (BOL, NBG, SALA) (MH810362*, MH830007*). ***Helichrysum dasyanthum*** (Willd.) Sweet. Ex J. Bot. Mar i Murtra Blanes (BCN6107) (HG798181, HM445678). ***Helichrysum dunense*** Hilliard (1) **SOUTH AFRICA**, Northern Cape Province, SE of Island Point, Helme 4029 (NBG207964) (MH810363*, MH830008*); (2) **SOUTH AFRICA**, Northern Cape Province, Hondeklipbaai, Andrés-Sánchez et al. SA762 (BOL, NBG, SALA) (MH810364*, MH830009*); (3) **SOUTH AFRICA**, Western Cape Province, Lambert's Bay, Andrés-Sánchez et al. SA780 (NBG, BOL, SALA) (MH810365*, MH830010*). ***Helichrysum ecklonis*** Sond. **SOUTH AFRICA**, Eastern Cape Province,

Romo et al. 14485 (BC867712) (HG797751, HG798027). ***Helichrysum epapposum*** Bolus **SOUTH AFRICA**, Mpumalanga Province, Sterkspruit Nature Reserve, Koekemoer M3504 (PRE847850) (MH810366*, MH830011*). ***Helichrysum erubescens*** Hilliard **NAMIBIA**, Kaokoveld, Orupembe, Hall 380 (NBG) (MH810367*, MH830012*). ***Helichrysum excisum*** (Thunb.) Less. **SOUTH AFRICA**, Western Cape Province, Koekemoer M3433 (BC) (HG797754, HG798031). ***Helichrysum felinum*** Less. **SOUTH AFRICA**, Western Cape Province, Franschhoek Pass, Koekemoer M3737 (PRE847044) (MH810368*, MH830013*). ***Helichrysum foetidum*** (L.) Moench Ex Dresden Bot. Gard. (BCN8219) (AY445221, HG798036). ***Helichrysum galpinii*** N.E. Br. **SOUTH AFRICA**, Mpumalanga Province, Romo et al. 14569 (BC867776) (HG797762, HG798042). ***Helichrysum gariepinum*** DC. **NAMIBIA**, between Noordoewer and Rosh Pinah, Koekemoer M3516 (PRE847847) (MH810369*, MH830014*). ***Helichrysum gerberifolium*** Sch.Bip. ex Hochst. **SOUTH AFRICA**, Mpumalanga Province, along the Weltevreden Road between Makobulaan and Buffelskloof S of Lydenburg, Koekemoer M3401 (PRE) (MH810370*, MH830015*). ***Helichrysum gofense*** Cufod. **ETHIOPIA**, Bale Mountains plateau, Aldasoro et al. 10336 (BC) (HG797764, HG798047). ***Helichrysum griseolanatum*** Hilliard **SOUTH AFRICA**, Eastern Cape Province, between Rhodes and Naudesnek, Koekemoer M3447 (PRE768542) (MH810371*, MH830016*). ***Helichrysum hamulosum*** E. Mey ex DC. (1) **SOUTH AFRICA**, Western Cape Province, Bergh 1319 (NBG) (MH810372*, MH830017*); (2) **SOUTH AFRICA**, Western Cape Province, between Montagu and Matroosberg, Koekemoer M3480 (PRE768570) (MH810373*, MH830018*); (3) **SOUTH AFRICA**, Western Cape Province, Romo et al. 14540 (BC867751) (HG797767, HG798051). ***Helichrysum herniarioides*** DC. (1) **SOUTH AFRICA**, Western Cape Province, Tankwa Guest Farm, Koekemoer M3133 (PRE757959) (MH810374*, MH830019*); (2) **SOUTH AFRICA**, South West of Kakamas on the farm Droëgrond. Near highest trig beacon, Koekemoer M2930 (PRE) (MH810375*, MH830020*). ***Helichrysum italicum*** (Roth) G. Don **BOSNIA-HERZEGOVINA**, Herzegovina, Redžić et al. s.n. (BCN20756) (FJ211422, FJ211480). ***Helichrysum incarnatum*** DC. **SOUTH AFRICA**, Western Cape Province, top of Dasklip Pass, Bergh 1802 (NBG) (MH810376*, MH830021*). ***Helichrysum indicum*** (L.) Grierson (1) **SOUTH AFRICA**, Western Cape Province, Paardeberg, Nicolson et al. 622 (NBG269235) (MH810377*, MH830022*); (2) **SOUTH AFRICA**, Western Cape Province, Romo et al. 14547 (BC867758) (HG797771, HG798055). ***Helichrysum*** cf. ***jubilatum*** Hilliard **SOUTH AFRICA**, Northern Cape Province, Lekersing, Andrés-Sánchez et al. SA740 (NBG) (MH810359*, MH830004*). ***Helichrysum kraussii*** Sch.Bip. **ANGOLA**, Huila Province, Humpata, Koekemoer M3674 (PRE847830) (MH810378*, MH830023*). ***Helichrysum lambertianum*** DC. **SOUTH AFRICA**, Western Cape Province, Romo et al. 14556 (BC) (FJ211472, FJ211530). ***Helichrysum leontonyx*** DC. (1) **SOUTH AFRICA**, Western Cape Province, Vanrhynsdorp, Andrés-Sánchez et al. SA747 (BOL, NBG, SALA) (MH810379*, MH830024*); (2) **SOUTH AFRICA**, Northern Cape, N7 roadside ~ 14 km N of Garies, Bergh 1660 (NBG) (MH810380*, MH830025*); (3) **SOUTH AFRICA**, Northern Cape Province, Garies, Brakfontein, Koekemoer M3515 (PRE847845) (MH810381*, MH830026*); (4) **SOUTH AFRICA**, Northern Cape Province, Namakwa National Park, Koekemoer M3009 (PRE752393) (MH810382*, MH830027*). ***Helichrysum lineare*** DC. **SOUTH AFRICA**, Eastern Cape Province, Rhodes, Koekemoer M3544 (PRE851091), (MH810383*, MH830028*). ***Helichrysum lineatum*** Bolus **LESOTHO**, between Oxbow and Mahlasela Pass, Koekemoer M2895 (PRE594241) (MH810384*, MH830029*). ***Helichrysum litorale*** Bolus (1) **SOUTH AFRICA**, Western Cape Province, Cape of Good Hope, Koekemoer M3476 (PRE771646) (MH810385*, MH830030*); (2) **SOUTH AFRICA**, Eastern Cape Province, Romo et al. 14500 (BC867722) (HM244706, HG798062); (3) **SOUTH AFRICA**, Western Cape Province, Yzerfontein, Andrés-Sánchez et al. SA773 (BOL, NBG, SALA) (MH810386*, MH830031*). ***Helichrysum lucilioides*** Less. **SOUTH AFRICA**, Western Cape Province, along the Groot Graffwater turn-off from the N7, Koekemoer M3637 (PRE) (MH810387*, MH830032*). ***Helichrysum marmarolepis*** S. Moore **SOUTH AFRICA**, Western Cape Province, Vanrhynsdorp, Boucher 6768 (NBG191256) (MH810388*, MH830033*). ***Helichrysum micropoides*** DC. (1) **SOUTH AFRICA**, Western Cape Province, Vanrhynsdorp, Andrés-Sánchez et al. SA752 (BOL, NBG, SALA) (MH810389*, MH830034*); (2) **SOUTH AFRICA**, Northern Cape, between Port Nolloth and Steinkopf, Bergh 1676 (NBG) (MH810390*, MH830035*); (3) **SOUTH AFRICA**, Northern Cape Province, between Port Nolloth and Alexander's Bay, 80 Km north of Port Nolloth, Andrés-Sánchez et al. SA735 (BOL, NBG, SALA) (MH810391*, MH830036*). ***Helichrysum niveum*** Less. **SOUTH AFRICA**, Western Cape Province, Stilbaai, Koekemoer M3425 (PRE771649) (MH810392*, MH830037*). ***Helichrysum obtusum*** Moeser (1) **SOUTH AFRICA**, Northern Cape, Richtersveld National Park, Helshoogte Pass, Bergh 2108 (NBG) (MH810393*, MH830038*); (2) **NAMIBIA**, between Noordoewer and Rosh Pinah, Koekemoer M3571 (PRE802645) (MH810394*, MH830039*). ***Helichrysum oreophilum*** Klatt **SOUTH AFRICA**, Mpumalanga Province, Long Tom Pass, between Lydenburg and Sabie, Koekemoer M3389 (PRE802821) (MH810395*, MH830040*). ***Helichrysum orientale*** (L.) Gaertn. **GREECE**, Crete, ex Roy. Bot. Gard. Kew (BCN6098) (AY445205, FJ211567). ***Helichrysum paronychioides*** DC. (1) **SOUTH AFRICA**, Gauteng Province, Klerksoord, Rosslyn, Pretoria, Bester s.n. (PRE755137) (MH810396*, MH830041*); (2) **SOUTH AFRICA**, North-West Province, Klerksdorp, Bester s.n. (PRE764790) (MH810397*, MH830042*). ***Helichrysum pulchellum*** DC. **SOUTH AFRICA**, Northern Cape Province, Garies, Kotzerus, Andrés-Sánchez et al. SA769 (BOL, NBG, SALA) (MH810398*, MH830043*). ***Helichrysum pumilio*** (O. Hoffm.) Hilliard & B.L. Burtt **SOUTH AFRICA**, Northern Cape Province, Bushmanland, Desmet et al. 3711 (NBG249959) (MH810399*, MH830044*). ***Helichrysum retortum*** (L.) Willd. (1) **SOUTH AFRICA**, ex Silverhill Seeds (BCN6112) (AY445222, HG798096); (2) **SOUTH AFRICA**, Western Cape Province, Hermanus, Koekemoer M3719 (PRE847037) (MH810400*, MH830045*). ***Helichrysum roseo-niveum*** Marloth & O. Hoffm. **NAMIBIA**, Kuiseb River bed, Bergh 1469 (NBG) (MH810401*, MH830046*). ***Helichrysum selaginifolium*** R. Vig. & Humbert **MADAGASCAR,** Antananarivo Province, Mt. Ibity, Bayer et al. (MAD04074, CANB660411) (HG797798, HG798101). ***Helichrysum silvaticum*** Hilliard **MOZAMBIQUE**, Licuati Sand Forest, McMurtry 11424 (Buffelskloof herbarium) (HG797799, HG798103). ***Helichrysum simillimum*** DC. **SOUTH AFRICA**, KwaZulu-Natal Province, Matatiele, Koekemoer M3443 (PRE768546) (MH810402*, MH830047*). ***Helichrysum simulans*** Harv. & Sond. **SOUTH AFRICA**, Northern

Cape Province, Holrivier, Andrés-Sánchez et al. SA755 (NBG) (MH810403*, MH830048*). ***Helichrysum spiralepis*** Hilliard & B.L. Burtt (1) **SOUTH AFRICA**, Kwazulu-Natal Province, Romo et al. 14372 (BC867645) (FJ211477, FJ211535); (2) **SOUTH AFRICA**, Eastern Cape Province, Elliot District, Bester B7433 (PRE843883) (MH810404*, MH830049*); (3) **SOUTH AFRICA**, Eastern Cape, Barkley East, L.A.P.A. Munnik Pass, Bergh 1495 (NBG) (MH810405*, MH830050*). ***Helichrysum stellatum*** Less. (1) **SOUTH AFRICA**, Western Cape Province, Vredenburg, Saldanha Peninsula, Goldblatt 13571 (NBG) (MH810406*, MH830051*); (2) **SOUTH AFRICA**, Northern Cape Province, between Soutfontein and Nariep, Kokemoer M3513 (PRE771643) (MH810407*, MH830052*); (3) **SOUTH AFRICA**, Northern Cape Province, Koekemoer M3513 (BC) (HG797801, HG798107); (4) **SOUTH AFRICA**, Western Cape Province, between Graafwater and Trawal, Andrés-Sánchez et al. SA757 (BOL, NBG, SALA) (MH810408*, MH830053*). ***Helichrysum sutherlandii*** Harv. Davy **LESOTHO** below Moteng Pass, Koekemoer M3713 (PRE) (MH810409*, MH830054*). ***Helichrysum tenax*** M.D. Hend. **LESOTHO**, between Sehlabathebe and Ramatseliso's border post, Koekemoer M3557 (PRE772162) (MH810410*, MH830055*). ***Helichrysum tinctum*** (Thunb.) Hilliard & B.L. Burtt (1) **SOUTH AFRICA**, Northern Cape, Spektakel Pass, W of Springbok near Naries Guest House, Koekemoer 3648 (PRE) (MH810411*, MH830056*); (2) **SOUTH AFRICA**, Eastern Cape Province, Port Elizabeth, Kragga Kamma, Andrés-Sánchez et al. SA808 (NBG, SALA) (MH810412*, MH830057*); (3) **SOUTH AFRICA**, Western Cape Province, between Clanwilliam and Graafwater, Ysterfontein, Andrés-Sánchez et al. SA779 (BOL, NBG, SALA) (MH810413*, MH830058*); (4) **SOUTH AFRICA**, Eastern Cape Province, Kouga Mountains, Euston-Brown 1397 (NBG274203) (MH810414*, MH830059*); (5) **SOUTH AFRICA**, Northern Cape Province, Leliefontein, Andrés-Sánchez et al. SA760 (BOL, NBG, SALA) (MH810415*, MH830060*); (6) **SOUTH AFRICA**, Northern Cape Province, Leliefontein, Andrés-Sánchez et al. SA760 (BOL, NBG, SALA) (MH810416*, MH830061*). ***Helichrysum uninervium*** Burtt Davy **SOUTH AFRICA**, Mpumalanga Province, Three Rondavels Viewpoint, Koekemoer M3605 (PRE802826) (MH810417*, MH830062*). ***Helichrysum wilmsii*** Moeser **SOUTH AFRICA**, Mpumalanga Province, Buffelskloof Nature Reserve, Koekemoer M3496 (PRE769046) (MH810418*, MH830063*). ***Helichrysum zeyheri*** Less. **SOUTH AFRICA**, Western Cape Province, Romo et al. 14542 (BC867754) (FJ211478, FJ211536). ***Helichrysum zwartbergense*** Bolus (1) **SOUTH AFRICA**, Western Cape Province, Zwartberg Pass, Andrés-Sánchez et al. SA815 (BOL, NBG, SALA) (MH810419*, MH830064*); (2) **SOUTH AFRICA**, Western Cape, Groot Swartberg, Bergh 1781 (NBG) (MH810420*, MH830065*); (3) **SOUTH AFRICA**, Western Cape Province, Romo et al. 14520 (BC867739) (HM244707, HG798119); (4) **SOUTH AFRICA**, Western Cape Province, between Oudtshoorn and Prince Albert, Koekemoer M3474 (PRE771644) (MH810421*, MH830066*); (5) **SOUTH AFRICA**, Western Cape Province, Zwartberg Pass, Andrés-Sánchez et al. SA828 (BOL, NBG, SALA) (MH810422*, MH830067*). ***Leysera gnaphalodes*** (L.) L. **SOUTH AFRICA**, Western Cape Province, Romo et al. 14546 (BC867757) (FN645815, FN645636). ***Pseudognaphalium beneolens*** (Davidson) Anderb. **UNITED STATES OF AMERICA**, California, San Diego Co, Rebman 10825 (RSA705579) (HG797813, HG798283). ***Relhania pungens*** L'Hérit. **SOUTH AFRICA**, Western Cape Province, N of Riversdale, Garcia's Pass, Koekemoer M3427 (BC) (FN645814, FN645635). ***Syncarpha mucronata*** (P.J. Bergius) B. Nord. **SOUTH AFRICA**, Western Cape Province, southern slopes of Swartberg Pass, Romo et al. 14511 (BC867732) (FJ211421, FJ211479). ***Vellereophyton dealbatum*** (Thunb.) Hilliard & B.L. Burtt **SOUTH AFRICA**, Western Cape Province, between Ashton and Montagu, Romo et al. 14549 (BC) (FN645832, FN645631).