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Research

Ecological speciation with gene flow followed initial large-scale geographic speciation in the enigmatic afroalpine giant senecios (*Dendrosenecio*)

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Summary

- Mountains have highly heterogeneous environments that generate ample opportunities for lineage differentiation through ecological adaptation, geographic isolation and secondary contact. The geographic and ecological isolation of the afroalpine vegetation fragments on the East African mountain tops makes them an excellent system to study speciation. The initial diversification within the afroalpine endemic genus *Dendrosenecio* was shown to occur via allopatric divergence among four isolated mountain groups, but the potential role of ecological speciation within these groups and the role of gene flow in speciation remained uncertain.
- Here we extend the sampling of *Dendrosenecio* and use phylogenomics to assess the importance of gene flow in the diversification of the genus. Then, population genomics, demographic modelling and habitat differentiation analyses are used to study ecological speciation in two sister species occurring on Mount Kenya.
- We found that two sympatric sister species on Mt Kenya occupy distinct microhabitats, and our analyses support that they originated *in situ* via ecological speciation with gene flow. In addition, we obtained signals of admixture history between mountain groups.
- Taken together, these results suggest that geographic isolation shaped main lineages, while ecologically mediated speciation occurred within a single mountain.

Introduction

The emergence of species, or separately evolving lineages (de Queiroz, 2007), depends on the emergence of barriers to gene flow, reducing the homogenising effects of recombination and promoting genetic, ecological or morphological divergence (Turelli *et al.*, 2001; Rundle & Nosil, 2005). In the presence of a geographic barrier, gene flow is expected to be absent or minimised, and the effects of selection, mutation and genetic drift would promote differentiation and, given sufficient time, result in geographic speciation (Pyron & Burbrink, 2010). After a period of geographic isolation, lineages may meet and, if reproductive isolation is incomplete, hybridise. Secondary contact may result in reversal of the speciation process (Seehausen *et al.*, 2008), or it may strengthen differentiation through processes such as reinforcement (Noor, 1999) or hybrid speciation (Mallet, 2007). In the absence of a geographic barrier, the presence of contrasting environments may promote the fixation of different alleles, each advantageous in one environment (Schluter, 2009). This adaptation to contrasting environments may reduce the viability of migrants adapted to alternative environments and enhance reproductive isolation (Nosil *et al.*, 2005). As a result, despite the presence of gene flow, divergent selection may facilitate lineage differentiation by making barriers to gene flow stronger with time, resulting in ecological speciation (Rundle & Nosil, 2005).

Mountains are dynamic regions that undergo tectonic, erosional and climatic processes, which often occur over a short geological time frame and provide ample opportunities for speciation (Rahbek *et al.*, 2019). High-altitude habitats, which are situated above the climatic forest limit, are highly heterogeneous at small spatial scales, showing large variation in temperature, water availability and wind exposure, as well as in topographic features such as ridges, valleys and lakes (Cortés & Wheeler, 2018). In addition, these habitats can be highly

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dynamic over time because of frequent climate-driven altitudinal range shifts during the Pleistocene glacial cycles (Flantua & Hooghiemstra, 2018). Such habitat heterogeneity and dynamics generate opportunities for lineage differentiation through ecological adaptation, geographic isolation and secondary contact (Rahbek *et al.*, 2019).

The tops of the widely scattered East African mountains provide an excellent system to study speciation (Hedberg, 1969a). Their heterogeneous high-altitude habitats are strongly isolated both geographically and ecologically, having an extreme diurnal climate with nightly frosts as opposed to the tropical climate at lower elevations (Hedberg, 1970). The afroalpine flora shows remarkable adaptations to this extreme climate and contains numerous endemic species (*c.* 70% of the flora; Hedberg, 1961, 1964, 1969a,b; Gehrke & Linder, 2014). However, most (85%) of the endemics are shared between two or more mountain regions (Gehrke & Linder, 2014), although the geographic and ecological isolation between these regions seem to have persisted even during glacial periods when the afroalpine plant communities shifted downward and expanded (Chala *et al.*, 2017; Kandziora *et al.*, 2024). The high number of endemics and other taxa shared among different mountains thus points to a prominent role of long-distance dispersal (LDD), rather than ecological connectivity (Brochmann *et al.*, 2022).

For this study, we chose one of the flagship genera of the afroalpine flora, the endemic giant senecios (*Dendrosenecio*), as an example of one of the few plant groups that diversified extensively in the East African mountains (Brochmann *et al.*, 2022; Gizaw *et al.*, 2022). The giant senecios are therefore particularly suited to study *in situ* speciation processes in this extremely fragmented and dynamic system. In earlier work, we showed that *Dendrosenecio* initially diversified by geographic speciation into four main lineages, one in each of four isolated mountain groups (Tusiime *et al.*, 2020; Gizaw *et al.*, 2022), but the possible role of gene flow among these lineages and the processes behind diversification within them were not fully clarified. In particular, we pointed out that two sister species that co-occur on a single mountain (Mt Kenya) may provide an example of ecological speciation, as they seem to be adapted to different microhabitats (Mabberley, 1973; Smith & Young, 1994; Knox, 2005).

To address these questions, we here extend the sampling of *Dendrosenecio* and use phylogenomics based on Hyb-Seq data, population genomics and demographic modelling based on ddRADseq data, and ecological analyses based on vegetation plot data and global environmental data. Our main aim is to test the hypothesis that two sister species on Mt Kenya, *Dendrosenecio keniensis* and *Dendrosenecio keniodendron*, originated via ecological speciation with gene-flow. We assess whether there are distinct habitat differences between these species that often occur in sympatry, and use demographic modelling to infer the extent of gene flow during the speciation process. In addition, we address the strength of the geographic barriers against gene flow across the strongly fragmented afroalpine system, predicting that there are only weak signals of admixture except between closely situated mountains.

Materials and Methods

Study group

The most recent taxonomic treatment of *Dendrosenecio* (Hauman ex Hedberg) B. Nord. recognizes 11 species (see Fig. 1; Knox, 2005), suggested to be uniformly decaploid ($n = 50$; on account of the base chromosome number in the tribe Senecioneae $x = 5$; Knox & Kowal, 1993). Eight of the 11 species are single-mountain endemics, and three are shared among two or more closely situated mountains. There are more than one species on each of four mountains/mountain ranges. Mount Kilimanjaro and the Rwenzori Mts each harbour two species, differing in their main altitudinal range. The closely situated Mt Kenya and the Aberdare Range each harbour two dominating species with similar altitudinal ranges, the sister species *D. keniensis* (Baker) Mabb. and *D. keniodendron* (R.E. Fr. & T.C.E. Fr.) B. Nord. on Mt Kenya, and the sister species *D. battiscombei* (R.E. Fr. & T.C.E. Fr.) E.B. Knox and *D. brassiciformis* (R.E.Fr. & T.C.E.Fr.) Mabb. in the Aberdare Range (Knox, 2005; Gizaw *et al.*, 2022). In addition, probably as a result of dispersal from the mountain where they originated, *D. keniodendron* is known from a single site in the Aberdare Range, and *D. battiscombei* has some occurrences on Mt Kenya (Knox, 2005).

In this study, we put particular focus on sister species on Mt Kenya, *D. keniensis* and *D. keniodendron*, to address ecological speciation, because they are conspicuously differentiated in morphology and appear to occupy distinct microhabitats, even if they often grow sympatrically intermingled at the same sites (Tusiime *et al.*, 2020; Gizaw *et al.*, 2022). In contrast to these two species, other closely related *Dendrosenecio* species also grow on the same mountain (*D. kilimanjari* (Mildbr.) E.B.Knox and *D. johnstonii* (Oliv.) B.Nord. on Mt Kilimanjaro, *D. battiscombei* and *D. brassiciformis* in the Aberdare Range, and *D. erici-rosenii* (R.E.Fr. & T.C.E.Fr.) E.B.Knox and *D. adnivalis* (Stapf) E.B.Knox in the Rwenzori Mts). However, in these cases there are altitudinal boundaries in their distribution, and their ecological and morphological differentiation is not so striking as those between the selected sister species pair from Mt Kenya (Knox, 2005). These species from Mt Kenya are restricted to the alpine zone, from 3300 to 4275 m in the case of *D. keniensis* and 3650 to 4350 m in the case of *D. keniodendron* (Knox, 2005). *Dendrosenecio keniensis* is a low-grown (up to 1.5 m tall), short-stemmed plant that branches close to the ground and frequently grows on constantly water-saturated soils; *D. keniodendron* is an erect giant (up to 7 m tall) with tall stems that branch high above the ground, and it frequently grows on well-drained soils. They are also clearly differentiated in reproductive traits and flowering phenology. While *D. keniensis* flowers every year with erect capitula composed of conspicuous, yellow ray flowers that produce sticky pollen, and also is able to reproduce clonally, *D. keniodendron* typically flowers synchronously every five (or more) years with rayless, nodding capitula with powdery pollen that are easily blown by wind (Mabberley, 1973; Smith & Young, 1994; Knox, 2005; Mizuno & Fujita, 2014). In addition, *D. keniensis*

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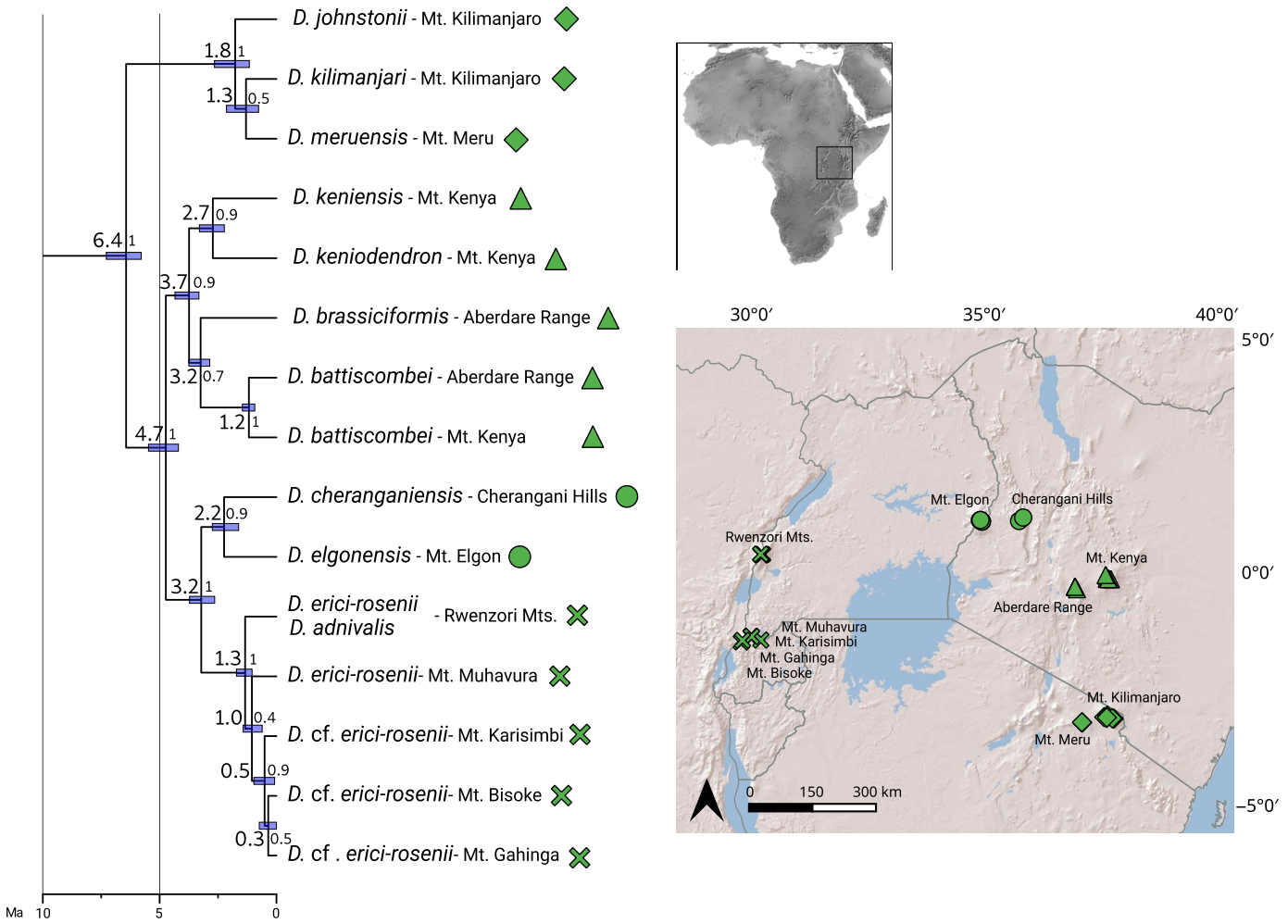

Fig. 1 Divergence time estimation of *Dendrosenecio* based on the multi-allele nrDNA phylogeny. Numbers in large font are median ages, numbers in small font are local posterior probabilities. The 95% confidence interval of the highest posterior density is shown by horizontal blue bars. The map shows sampled populations and the four mountain groups with different symbols.

has big intercellular spaces in roots, which are significantly smaller in *D. keniodendron*, indicating adaptation to water-saturated soils (Beck *et al.*, 1981, 1992). Notably, occasional hybrids between the two species have been reported (*Senecio* × *saundersii* W. Sauer & E. Beck; Hedberg, 1957; Beck *et al.*, 1992; Young & Peacock, 1992; Smith & Young, 1994), thought to solely represent first-generation hybrids (Knox, 2005).

Sampling, library preparation and sequencing

With the aim to evaluate the presence and extent of admixture across the geographic distribution of the genus, we extended the sampling of Gizaw *et al.* (2022) with 27 new samples, including three to four individuals for each of five populations and newly collected populations from Mounts Muhavura, Gahinga, Bisoke and Karisimbi in Uganda and Rwanda (Supporting Information Table S1). The Rwanda material lacked inflorescences but were referred to *D.* cf. *erici-rosenii* based on geography (Knox, 2005). The same four species within Senecioneae used in Gizaw *et al.* (2022) were included as outgroup taxa. DNA extraction, genomic library preparation and bait hybridization to generate targeted nuclear and off-target plastid sequence data followed the methodology of Gizaw *et al.* (2022). Targeted nuclear loci correspond to the Asteraceae conserved orthologous set (Compositae1061; Mandel *et al.*, 2014). The additional 27 samples were sequenced on an Illumina (San Diego, CA, USA) NovaSeq 6000 SP at IAB (Olomouc, Czech Republic).

For the purpose of inferring genetic differentiation and demographic processes on Mt Kenya, the sampling of the two sister species *D. keniodendron* and *D. keniensis* was extended to include five to ten individuals per population. In addition, a putative (morphology-based) hybrid sample was included, and the sampling of the *D. battiscombei* populations from Mt Kenya and the Aberdare Range was also expanded. We selected a total of 81 samples from 10 populations (Table S1), with a distance of at least 4 km between conspecific populations, for double-digest restriction-site-associated DNA sequencing (ddRADseq; Peterson *et al.*, 2012). Libraries for ddRAD sequencing were prepared following Peterson *et al.* (2012) as modified by Piálek *et al.* (2019). We used a combination of two Type II restriction endonucleases,

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SphI (GCATG/C recognition site) and MluCI (/AATT recognition site), and chose a narrow fragment size selection of 450–550 base pairs (bp) to obtain adequate numbers of informative sites from the large genomes of *Dendrosenecio* (1C = 15–17 Gbp, relative genome size estimated from flow cytometric data; see Methods S1; Table S2; Fig. S1). ddRADseq libraries of 83 samples, including two technical replicates, were pooled for sequencing on an Illumina HiSeq 4000 at the Genomics Core Facility at EMBL (Heidelberg, Germany).

Phylogenomic analyses, admixture and divergence time estimation based on Hyb-Seq data

For data processing and analysis of targeted nuclear and off-target plastid DNA we followed the methodology of Gizaw *et al.* (2022). Paralogy of targeted nuclear loci was accounted for using ParalogWizard (Ufimov *et al.*, 2022); the minimum and maximum pairwise sequence divergence of 9.14% and 21.96%, respectively, was chosen. The HybPhyloMaker workflow (Fér & Schmickl, 2018) was used to reconstruct species trees for both nuclear (nrDNA) and plastid DNA (cpDNA) data using ≤ 50% missing data per accession. We generated a coalescent-based nrDNA phylogeny using ASTRAL v.5.7.4 (Zhang *et al.*, 2018), collapsing branches with bootstrap support (BS) < 30% to account for gene tree estimation error (Sayyari & Mirarab, 2016). In addition, we built a multi-allele species tree (Rabiee *et al.*, 2019) in which accessions were forced into single tips to represent the geographic distribution of lineages and species according to Gizaw *et al.* (2022). Likewise, concatenated maximum likelihood phylogenies for both nrDNA and cpDNA were generated using RAxML-NG v.8 (Kozlov *et al.*, 2019) with the best substitution model for each partition for nrDNA and the GTR + GAMMA model for cpDNA.

We analysed admixture history based on Hyb-Seq nrDNA utilizing a single nucleotide polymorphism (SNP)-based approach using DSUITE v.0.5 (Malinsky *et al.*, 2021) and a gene-tree-based approach using the Discordant Count Test and the Branch Length Test (DCT-BLT; Suvorov *et al.*, 2022). These two approaches to estimate admixture history allowed us to verify the consistency of the results. A subset of data with two accessions per species was used in both approaches, and in cases where species occupy more than one mountain two accessions per mountain were included. Due to the limited sampling of outgroup taxa, all being distant from *Dendrosenecio*, none of them could be used as a reference for SNP calling. Therefore, the ancestral sequence for the crown node of *Dendrosenecio* was estimated using RAxML-NG v.8 with the –ancestral command. Then, nrDNA loci were concatenated, and SNPs were called using SNP-SITES v.2.3.3 (Page *et al.*, 2016) and employing the ancestral sequence for the crown node of *Dendrosenecio* as reference. We filtered SNPs using BCFTOOLS v.1.17 (Li, 2011) setting 20% as missing data threshold. To retain unlinked SNPs, the resulting VCF file was thinned taking the first SNP in each locus. The filtered and thinned VCF file was used as input in DSUITE for estimating the *D* statistic (Patterson *et al.*, 2012). The *P*-value was adjusted using the False Discovery Rate (FDR) method, using the STATS v.3.6.3 package in R, to control the family-wise error rate, reduce false positives and minimise false negatives (Jafari & Ansari-Pour, 2019). An adjusted *P*-value below 0.01 was used to determine significance based on the value established in the description of the method (Malinsky *et al.*, 2021). Admixture history estimated using DCT-BLT was employed with the ASTRAL phylogeny previously used for the analyses with DSUITE as input together with the 1140 gene trees used to construct it. DCT-BLT allows to estimate an introgression proportion (γ) which is comparable to the statistics estimated with DSUITE. The *P*-value was also adjusted using the FDR method in R. An adjusted *P*-value below 0.05 was used to determine significance based on the value established in the description of the method (Suvorov *et al.*, 2022).

A molecular dating approach based on penalised likelihood was employed using TREEPL v.1.0 (Sanderson, 2002; Smith & O'Meara, 2012) and the analysis guidelines of Maurin (2020). Due to the lack of available fossil records for ancestors of *Dendrosenecio* or close relatives, secondary calibrations had to be used even though the accuracy and consistency of estimates obtained using this type of calibration is debatable (Schenk, 2016; Powell *et al.*, 2020). Two secondary calibration points were used based on the 95% confidence interval of the highest posterior density (HPD) values previously estimated for the stem age (30.73–15.18 million years ago (Ma)) and the crown age (16.77–2.07 Ma) of *Dendrosenecio* (Kandziora *et al.*, 2022). Using a TREEPL wrapper script (https://github.com/tongjial/treepl_wrapper), the nrDNA multi-allele species tree previously generated was used for the optimization of parameters and for cross-validation analyses. To infer confidence intervals for node ages, 105 nrDNA bootstrapped phylogenies with the same topology as the multi-allele species tree were dated using the calibration, optimization and cross-validation values previously calculated. The resulting dated trees were combined using TREEANNOTATOR v.2.6.4 (Suchard *et al.* 2018).

ddRADseq analysis of the decaploid Mount Kenya dataset

Raw reads were processed using the STACKS v.2.5 pipeline (Catchen *et al.*, 2011; Rochette *et al.*, 2019; see Methods S1 for details). The catalogue file, matches files and summary statistics for loci resulting from the STACKS pipeline were imported to the POLYRAD v.2.0 package (Clark *et al.*, 2019) in R v.3.5 (R Core Team, 2018). Polyploid genotype calling was done using the POLYRAD package and setting the inheritance mode to autopolyploid with ploidy equal to 10, according to chromosome counts that have been reported (Knox & Kowal, 1993). The threshold for the minimum number of individuals per locus was set to 15, the minimum number of individuals with reads for the minor allele was set to 10, and the rate of contamination between samples was set to 0.001. We tested for Mendelian segregation at each marker using the H_{ind}/H_E statistic (Clark *et al.*, 2022), only keeping loci under decaploid segregation ($H_{ind}/H_E \leq 0.9$; 99% of the loci met this requirement). Overdispersion was estimated and used to iteratively estimate genotypes, accounting for both population structure and linkage disequilibrium.

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The VCF file generated including all 10 sampled populations (full-dataset-10× hereafter) was filtered using BCFTOOLS v.1.17, with a minimum threshold for minor allele frequency (MAF = 0.05) and minimum depth coverage (DP > 20) over all individuals. Also, sites with at least 80% completeness were retained and one random SNP per locus was kept. The filtered VCF file included 823 SNPs and was used for principal component analysis (PCA, to be described later). As the number of retained loci was low, to perform Bayesian clustering analysis and estimate population genetic statistics (to be described later) a less strict filtering approach was taken. Only a mean DP ≥ 20 threshold was set and one random SNP per locus was kept, leaving 1509 SNPs that were exported as discrete genotypes. Likewise, a second dataset including only the *D. keniensis* and *D. keniodendron* populations (DkenDked-dataset-10× hereafter) was generated and exported as VCF for the demographic modelling analyses. The samples of *D. battiscombei* were excluded from this dataset because this species is more closely related to *D. brassiciformis* in the Aberdare Range. For this dataset, filtering was done using BCFTOOLS v.1.17; only sites with 70% completeness were retained, the minimum mean DP over all individuals was set to 20, a single SNP per locus was kept, and no minor allele frequency threshold was set to keep rare variants. Before filtering, the 'DkenDked-dataset-10×' was subsetted into population pairs for stringent comparison (to be described later).

ddRADseq analysis of the diploidized Mount Kenya dataset

We also analysed the ddRADseq data using the STACKS v.2.5 pipeline and VCFTOOLS v.1.6 (Danecek *et al.*, 2011) to obtain a diploidized dataset (full-dataset-2× hereafter; see Methods S1 for details). This diploidized dataset was used to test if biologically meaningful genetic groups, congruent with those obtained from estimates using the real ploidy, could be inferred using genetic clustering analysis and to generally test the robustness of the two approaches.

Population genetic analyses of Mount Kenya datasets

Genetically homogenous groups were identified using model-based Bayesian clustering with STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000). We performed clustering analyses ranging from $K = 1$ to $K = 10$ with 10 replicates per K, a burn-in period of 1×10^5, and with 1.1×10^6 iterations. We used a total of 1509 and 6416 SNPs in the decaploid (full-dataset-10×) and diploidized (full-dataset-2×) dataset, respectively. The optimal number of clusters was selected based on the rate of change in the log probability of data between successive K values (ΔK) as proposed by Evanno *et al.* (2005). Postprocessing and graphical representation of the results was performed using CLUMPAK v.1.1 (Kopelman *et al.*, 2015). To evaluate the robustness of the clustering analyses, a PCA was performed using the ADEGENET v.2.1.10 package (Jombart, 2008) in R. For the decaploid dataset (full-dataset-10×), excluding the putative hybrid sample, population differentiation was evaluated calculating rho (an F_{st} analogue suitable for polyploids; Ronfort *et al.*, 1998) in GENODIVE v.3.0 (Meirmans, 2020), and deviation from neutral evolution was estimated using Tajima's D (Tajima, 1989) based on 100 Poisson subsamples of each population's site frequency spectrum (SFS).

Habitat differentiation on Mount Kenya

Because the populations of *D. keniensis* and *D. keniodendron* grow only on Mt Kenya, often in the same sites, evaluating habitat differentiation based on niche modelling using global environmental data (climate and soil data) did not provide enough resolution (see Methods S1 for details). The collection of fine-scale environmental data in the field was not possible due to time and technical limitations and, to our knowledge, such data is not available from previous studies. Therefore, we used vegetation plot data as a proxy to assess habitat differences between *D. keniodendron* and *D. keniensis*. We sampled presence/absence data for a selection of 37 vascular plant species accompanying *Dendrosenecio* individuals. This dataset was collected from 90 plots on Mt Kenya, evenly distributed from 2200 to 4700 m, during the period from June 16 to June 30, 2019. Various volumes of the Flora of tropical East Africa were consulted for species identification (Turrill, 1956; Jonsell, 1982; Taylor, 1989; Verdcourt, 1994; Milne-Redhead, 2000; Knox, 2005; Paton *et al.*, 2009). The presence/absence data was filtered to keep only plots with presence of at least one of the two *Dendrosenecio* species, leaving 51 plots with a total of 26 vascular plant species (Table S3). The correlation in the (co-)occurrence of the species was assessed with PCA using the VEGAN v.2.6-4 package (Oksanen *et al.*, 2022) in R. A redundancy analysis (RDA) using the *Dendrosenecio* species as response variables and the remaining 24 species as explanatory variables was performed to determine the significance of interspecific differentiation in the surrounding vegetation composition. Model optimization was performed based on Akaike Information Criterion (AIC) using a stepwise algorithm. This stepwise algorithm performs permutation tests for automatic model building using the optimal number of explanatory variables based on AIC. Then, the goodness of fit of the optimized and full models was compared with the adjusted R^2. Collinearity was evaluated by calculating the variance inflation factor (VIF), and the significance of the model, the axes and each explanatory variable was determined by analysis of variance (ANOVA). RDA analysis was computed using the VEGAN package in R.

Demographic modelling of sympatric population pairs on Mount Kenya

To test for ecological speciation (i.e. speciation with gene flow) against geographic speciation followed by secondary contact, we used the diffusion approximation method of ∂a∂i v.2.1.2 (Gutenkunst *et al.*, 2009). The filtered and subsetted 'DkenDked-dataset-10×' VCF files were used to generate a folded two-dimensional joint site frequency spectrum (2D-JSFS) for each of the two *D. keniensis* – *D. keniodendron* sympatric population pairs. Splitting the dataset into population pairs allowed us to have two biological replicates. One pair consisted

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of populations (DE013 and DE014) from a high-elevation site (4245 m), hereafter referred to as the 'high-elevation pair', and one pair of populations (DE016 and DE017) from a somewhat lower site (4190 m), hereafter referred to as the 'mid-elevation pair'. This sympatric population pair approach was taken to ensure that populations that likely underwent the same dynamics during past climate-driven altitudinal range shifts were compared. Each 2D-JSFS was used independently to examine 14 models that differed in assumptions related to migration rates, periods of isolation and population size changes, following the ∂a∂i-pipeline developed by Portik *et al.* (2017). This way, models assuming isolation during divergence with or without secondary contact represent geographic speciation, while models where continuous migration during divergence is assumed represent ecological speciation. SFSs were projected down to 50 alleles for *D. keniensis* and 50 alleles for *D. keniodendron* to maximise the number of sampled individuals and minimise levels of missing data. These values were kept equal for all of the population pairs analysed to ensure they are qualitatively similar. Considering that a small number of diploid individuals (at least 3) is sufficient when examining patterns of divergence between species with a long history of separation (*c.* 2.8 Ma; Robinson *et al.*, 2014; O'Dea *et al.*, 2016; McLaughlin & Winker, 2020), our approach does not compromise model selection or parameter estimation accuracy. The high-elevation pair dataset consisted of 5097 SNPs, which were projected down to 2668 segregating sites, and the mid-elevation pair dataset consisted of 4166 SNPs, which were projected down to 2004 segregating sites.

A linear extrapolation was used with a sequence of grid sizes of 90, 100 and 110. The optimization routine consisted of an initial round of threefold perturbed random starting parameters, followed by two rounds of twofold and another two of onefold perturbed parameters; these five rounds included 10, 20, 30, 40 and 50 replicates, respectively. The Nelder–Mead simplex algorithm was used as optimizer. To avoid issues due to a given model getting stuck in local optima, each model optimization was repeated six times. Convergence was verified by plotting log-likelihood scores across optimization rounds in R. The results of the optimization for each model were compared using the AIC, and the replicate with the highest likelihood for each model was used to calculate ΔAIC scores, relative likelihoods and Akaike weights (ω_i; Burnham & Anderson, 2002) using the QPCR v.1.4 package (Spiess, 2018) in R. The parameter values of the best-fit model for each population pair were used to perform a multinomial comparison between the model and the data.

Results

Phylogenomic analyses, admixture and divergence time estimation based on Hyb-Seq data

In the nrDNA phylogenies, the main splits were consistent with geography, and the species were supported as monophyletic except for *D. adnivalis* and *D. erici-rosenii* from the Western Rift Mountains (Figs 1, S2–S5). The individual populations were not recovered as fully monophyletic. The cpDNA phylogeny showed some discordance with the nrDNA phylogenies, in particular for *D. cheranganiensis* and some accessions of *D. battiscombei* and *D. brassiciformis* (Table S4; Figs S3, S4, S6). The analysis of admixture history using DSUITE was based on 1100 SNPs, and *D* statistic values were calculated for 4060 trios (Table S5). Five cases showed statistically significant admixture (adjusted *P*-value < 0.01) with *D* statistic values ranging from 0.80 to 0.53. In all cases, admixture was detected between species from different mountain groups (*D.* cf. *erici-rosenii* – *D. johnstonii*, *D. cheranganiensis* – *D. keniodendron*, *D.* cf. *erici-rosenii* – *D. cheranganiensis*, and *D. battiscombei* – *D. adnivalis*). Admixture history using DCT-BLT consisted in the analysis of 4060 triplets (Table S6), and resulted in two statistically significant (adjusted *P*-value < 0.05) admixture events. An admixture proportion of 9% was estimated between two species from different mountain groups (*D. keniodendron* – *D. kilimanjari*), and a value of 1% was estimated between two species from the same mountain group (*D. cheranganiensis* – *D. elgonensis*). However, in the latter case the number of gene trees that support the first alternative topology was slightly higher than the number of gene trees that support the species tree topology. If this is considered, the distance between the alternative topologies was not significantly different and admixture in this triplet was not supported according to the BLT test.

Diversification of the genus *Dendrosenecio* was estimated to have started 6.4 Ma (HPD: 7.3–5.8 Ma; Fig. 1). The divergence times within the four main lineages were estimated to 3.7 Ma (HPD: 4.3–3.3 Ma; the Mt Kenya/Aberdare lineage), 2.2 Ma (HPD: 2.7–1.6 Ma; the Mt Elgon/Cherangani Hills lineage), 1.8 Ma (HPD: 2.6–1.2 Ma; the Mt Kilimanjaro/Mt Meru lineage), and 1.3 Ma (HPD: 1.7–1.0 Ma; the Western Rift lineage). The origin of *D. battiscombei* and *D. erici-rosenii* was dated to < 1.5 Ma.

Population genomic analyses

In the population genomic analyses of the decaploid ddRADseq dataset (full-dataset-10×), the optimal number of clusters was three ($K = 3$) based on ΔK (Figs 2, S7), each representing one of the described species. Notably, at $K = 3$, the accessions of *D. battiscombei* from Mt Kenya and the Aberdare Range belonged to the same cluster, which was split according to geographic distribution at $K \geq 4$ (Fig. S7). The putative hybrid between *D. keniodendron* and *D. keniensis* showed the expected mixed ancestry. Population differentiation between the three species (estimated as rho) was > 0.6 (Table S7). Within species (among-population) differentiation was higher in *D. keniensis* (0.08) than in *D. keniodendron* (0.01), and showed large variation (0.04–0.55) in *D. battiscombei*. Tajima's *D* values were close to zero in six of the nine populations, suggesting near-neutrality (Table S7). In the population genetic analyses of the diploidized dataset (full-dataset-2×), the optimal number of clusters was four (Fig. S8). Three of them corresponded to the groups inferred for $K = 3$ in the decaploid dataset. The fourth cluster only occurred as intermixed within the other three clusters, possibly representing a bias

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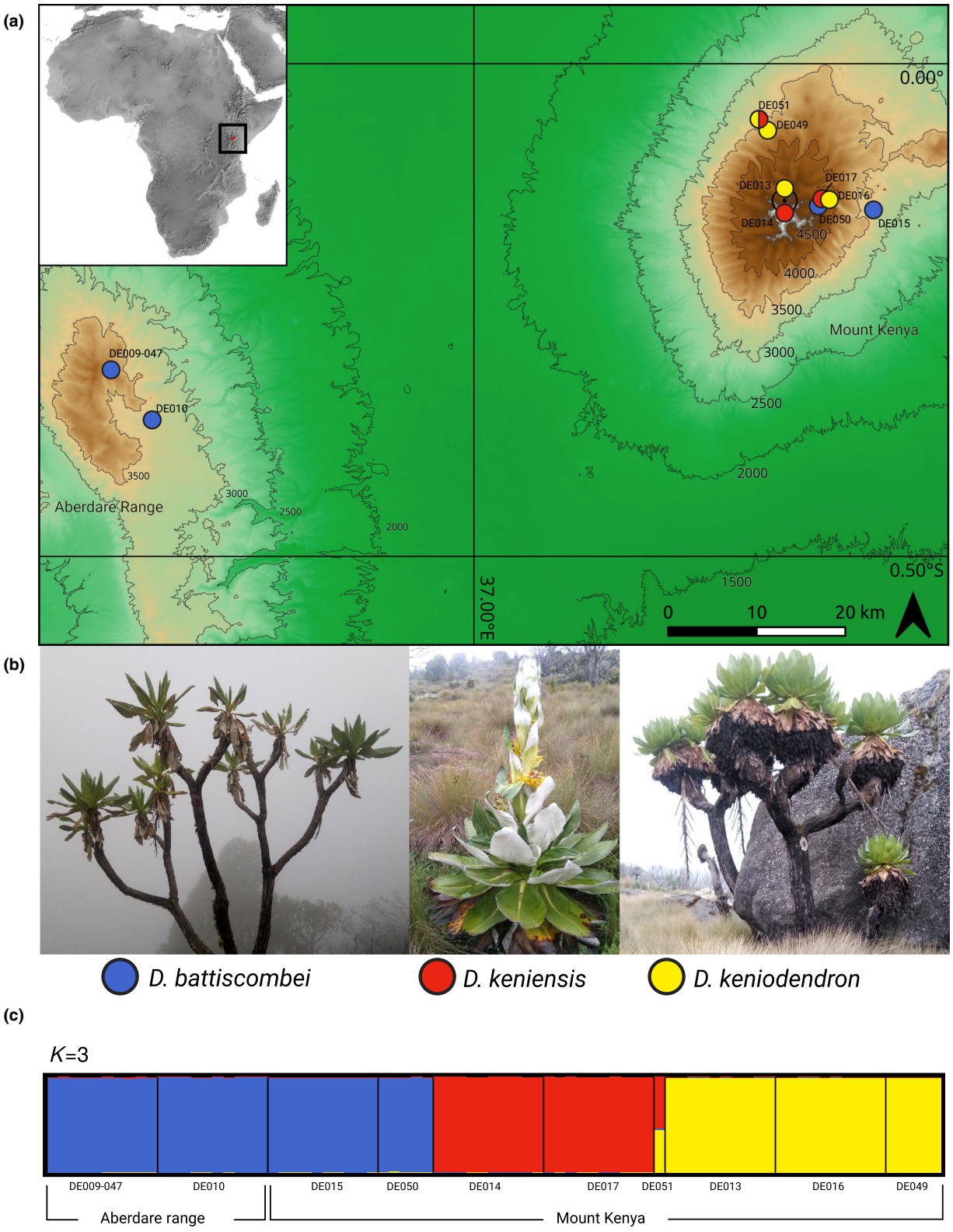

Fig. 2 Population sampling and genetic structure in *Dendrosenecio* in the Mt Kenya/Aberdare group based on ddRADseq data. (a) Map showing sites of the 10 sampled populations with colours corresponding to genetic cluster assignment; (b) Photos of the three species sampled illustrating differences in growth form and morphology; (c) Genetic structure for the inferred optimal number of clusters ($K = 3$) based on decaploid variant calling in 81 individuals. Photos: Abel Gizaw.

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in the analysis caused by erroneously called allele frequencies due to the wrong ploidy.

Habitat differentiation analyses

The vegetation plot-based habitat differentiation analysis used the species composition of 51 vegetation plots, of which 37% only contained *D. keniodendron*, 28% only contained *D. keniensis*, and 35% contained both species together. In the PCA, the two *Dendrosenecio* species were clearly differentiated both along axis 1 and axis 2 (Fig. 3). The optimized RDA model, which included six explanatory variables, showed a better fit (adjusted R^2 = 0.57) than the full RDA model (adjusted R^2 = 0.45). The plot of the optimized model showed the two *Dendrosenecio* species at distant and opposite positions along the first axis (RDA1), which explained 52.4% of the variation. VIF for all response variables was below 3, indicating no collinearity, and the ANOVA revealed strong significance (P-value ≤ 0.001) of the model, RDA1 and the explanatory variables *Carex monostachya* and *Arabis alpina* (Table S8).

Habitat differentiation based on niche modelling failed to detect significant differences between species niches, which showed an intermediate overlap (Methods S1; Table S9). This niche overlap was highest when using soil data alone (73% overlap) and lowest when using climatic data alone (57% overlap). A higher resolution of the soil data, which allowed us to increase the number of occurrences, gave reduced niche overlap values (66% overlap). The niche of *D. keniodendron* was almost fully within that of *D. keniensis*, while in contrast *c.* 60% or less of the niche of *D. keniensis* overlapped with that of *D. keniodendron*.

Demographic modelling

The demographic modelling of the two population pairs gave similar results (Fig. 4; Table S10) and the final optimizations converged on similar log-likelihood scores between replicates (Fig. S9). For the high-elevation pair, a model of divergence with continuous asymmetric migration (Fig. 4a) was strongly supported (ω_i = 0.99; Table S10), whereas the best alternative model differing by symmetric migration showed much lower support. In the best fitting model, continuous asymmetric migration was highest from *D. keniodendron* to *D. keniensis* (m12 = 5.91, m21 = 0.24), similar effective population sizes were obtained for both *D. keniensis* (nu1a = 0.01) and *D. keniodendron* (nu2a = 0.02), and a continuous divergence period was estimated (T = 0.01). For the mid-elevation pair, a model of divergence with continuous symmetric migration (Fig. 4b) was favoured (ω_i = 0.99; Table S10), and the best alternative model, differing by asymmetric migration, showed much lower support. In the best fitting model, the two species showed continuous migration (m = 3.70), similar effective population sizes were obtained for both *D. keniensis* (nu1a = 0.01) and *D. keniodendron* (nu2a = 0.02), and a continuous divergence period was estimated (T = 0.01).

True estimates of these population demographic parameters in the form of biologically relevant units are highly speculative for

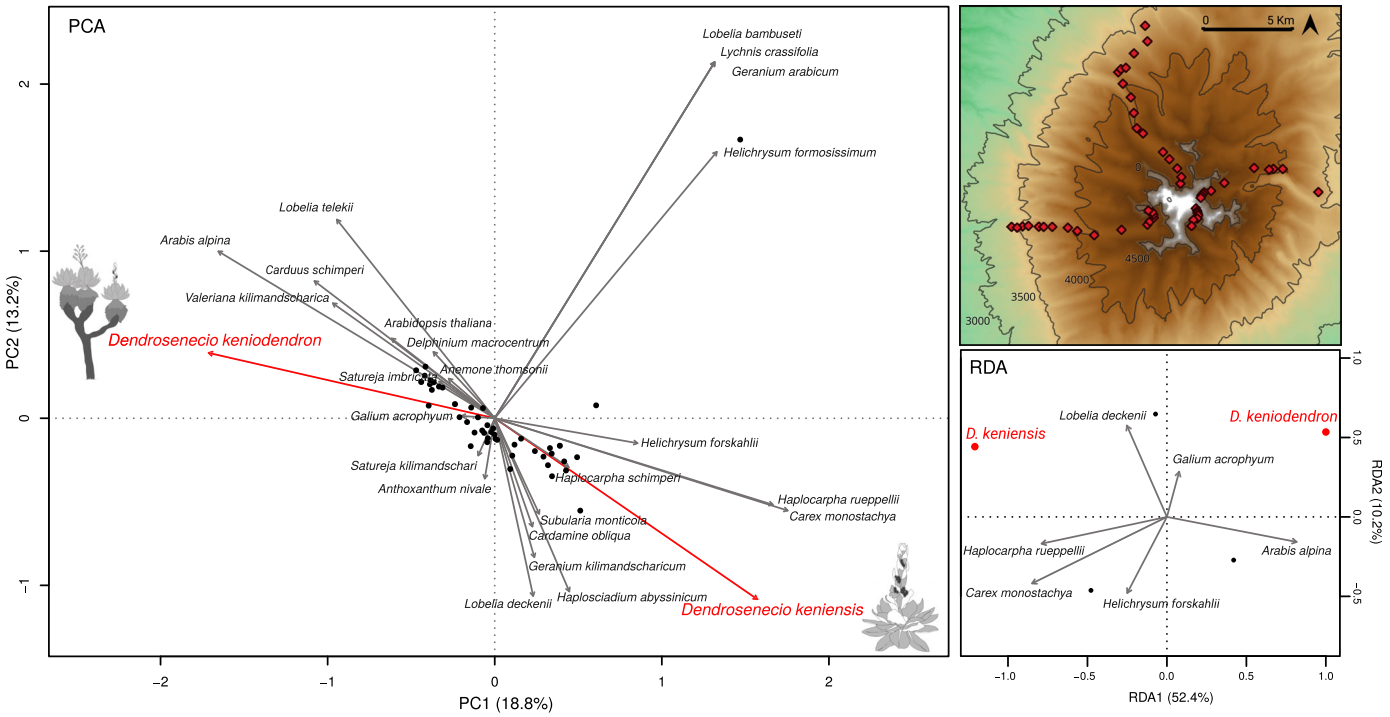

Fig. 3 Principal component analysis (PCA) and Redundancy analysis (RDA) of the vegetation plot data. Vectors show the direction of the species and sites are represented by points (sites overlap in the RDA plot). The two target *Dendrosenecio* species are highlighted in red. The red squares in the map show the location of the 51 plots analysed.

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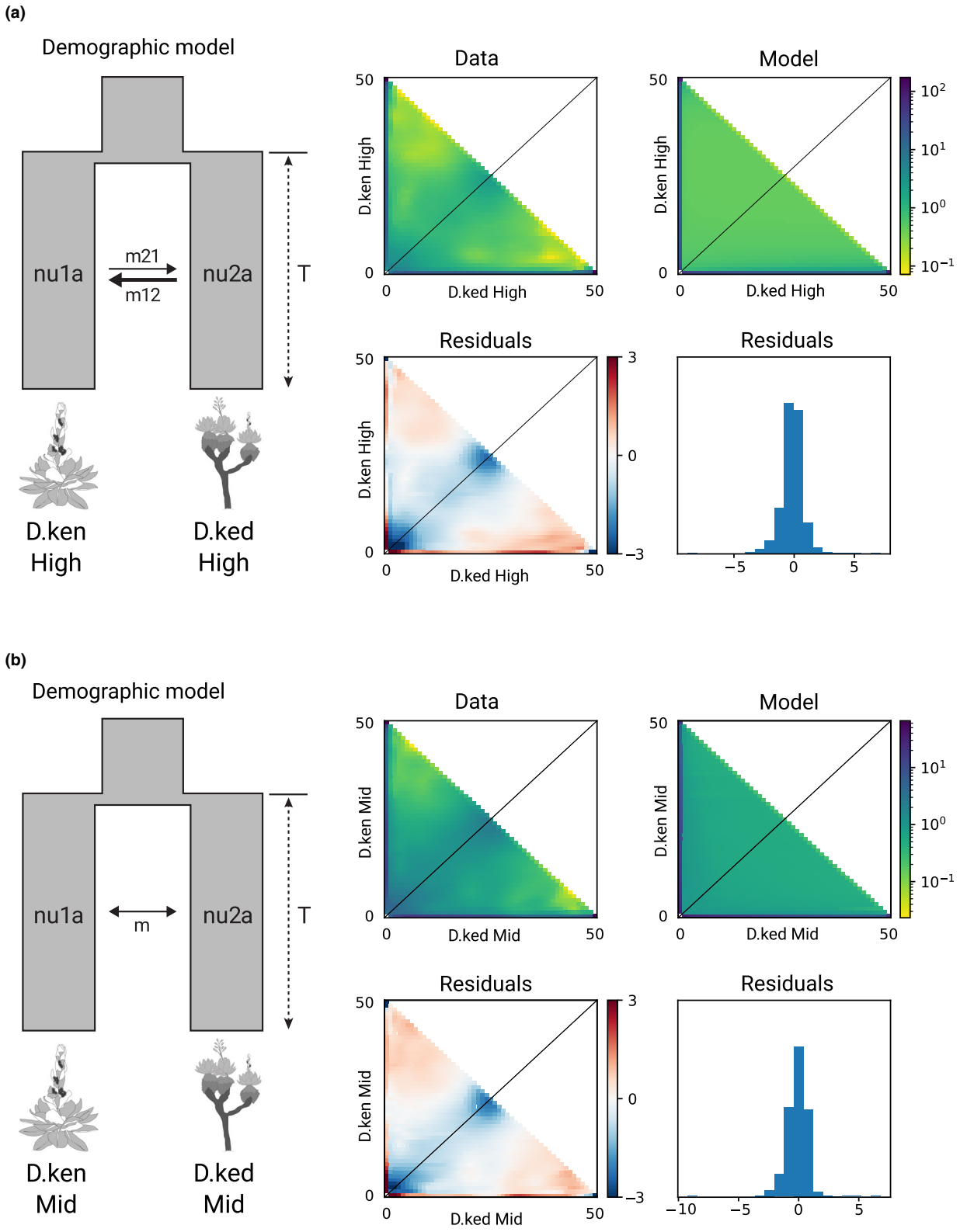

Fig. 4 Best fitting demographic models using the two-dimensional joint site frequency spectrum (2D-JSFS) for two sympatric population pairs of the sister species *Dendrosenecio keniensis* (D.ken) and *Dendrosenecio keniodendron* (D.ked). (a) High-elevation pair, including populations DE014 (n = 50) and DE013 (n = 50); (b) Mid-elevation pair, including populations DE017 (n = 50) and DE016 (n = 50). A schematic representation of the best-fit model is shown, along with multinomial comparisons of the 2D-JSFS for the data, model and resulting residuals. m, symmetric migration rate; m12, migration rate from population 2 to population 1; m21, migration rate from population 1 to population 2; nu1a, nu2a, effective population sizes; T, unscaled time.

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these two *Dendrosenecio* species. If we use a mutation rate of 7.83 × 10^{-9} (mutation rate estimated for *Robinsonia*, a genus that also belongs to Senecioneae; Sang *et al.*, 1995), the reference population size would be 83191.18 for the high-elevation pair and 71559.32 for the mid-elevation pair, after correcting for polyploidy (i.e. each decaploid individual is represented by five diploid individuals). Using these reference population size values to estimate ancestral population sizes would result in 1064.85 for *D. keniensis* and 1372.65 for *D. keniodendron* in the high-elevation pair, and 1037.61 for *D. keniensis* and 1109.17 for *D. keniodendron* for the mid-elevation pair. The proportion of migrants per generation would be 3.55 × 10^{-5} (from *D. keniodendron* to *D. keniensis*) and 1.46 × 10^{-6} (from *D. keniensis* to *D. keniodendron*) in the high-elevation pair, and 2.59 × 10^{-5} in the mid-elevation pair. Divergence between the species would be estimated to 2445.82 generations ago in the high-elevation pair and 1502.75 generations ago in the mid-elevation pair. Considering that in *D. keniodendron* plants < 1.5 m tall are usually unbranched (Smith & Young, 1982), evidencing that first flowering did not yet occur, and that the growth rate is *c.* 2.5 cm per year (Hedberg, 1969b; Beck *et al.*, 1984), the generation time for this species would be *c.* 60 years. Similar estimates cannot be performed in the case of *D. keniensis* because of a lack of information. Taking into account the uncertainty in the generation time, a mean generation time between 20 and 60 years would give a divergence time of 49 to 147 thousand years ago (Ka) in the high-elevation pair and 30 to 90 Ka in the mid-elevation pair.

Discussion

Role of gene flow in the differentiation of the main *Dendrosenecio* lineages

Differentiation of populations in the presence of a physical barrier that prevents migration, that is geographic speciation, seems to be the most common speciation mode in plants because most sister species have nonoverlapping ranges (Hernández-Hernández *et al.*, 2021). In *Dendrosenecio*, the extended Hyb-Seq-based phylogenomic analyses presented here are congruent with previously published phylogenies (Gichira *et al.*, 2021; Gizaw *et al.*, 2022) and point to geographic isolation as an important factor for diversification. A few signals of historical admixture between mountain groups were detected using DSUITE and DCT-BLT, but results were inconsistent between the two approaches. Although an insufficient number of SNPs or low variation among conserved Hyb-Seq loci could cause the inconsistency between results, the geographic isolation scenario in which rare gene flow occurred via LDD goes in line with previous studies in *Dendrosenecio* (Tusiime *et al.*, 2020; Gizaw *et al.*, 2022). The importance of geographic isolation in *Dendrosenecio* is consistent with the pattern observed for the afroalpine flora, where floristic similarity is explained by geographic isolation rather than environmental filtering (Gehrke & Linder, 2014). Examples of strong isolation with occasional intermountain gene flow via LDD have also been shown in several other afroalpine plant species such as *Arabis alpina*, *Carduus schimperi* and *Trifolium cryptopodium* (Assefa *et al.*, 2007; Wondimu *et al.*, 2014; Brochmann *et al.*, 2022).

Based on our extended phylogenomic analyses, the estimated crown age of the genus (6.4 Ma) is younger than those inferred from previous dated phylogenies using nrDNA (10.6 Ma; Gizaw *et al.*, 2022), but older than those estimated from cpDNA (2.3 Ma; Gichira *et al.*, 2021). The latter discrepancy could be because cpDNA is highly conserved and has a slower evolutionary rate (Robbins & Kelly, 2023). The difference between nrDNA estimates could be due to our more complete sampling, to the use of a multi-allele phylogeny, which accounts for the impact of polymorphisms within species (Rabiee *et al.*, 2019), or to inconsistencies related to the use of secondary calibrations (Schenk, 2016). Nevertheless, our results confirm that *Dendrosenecio* started to diversify during the Late Miocene, as inferred by Gizaw *et al.* (2022), a period in which alpine habitats were likely available in East Africa (Hedberg, 1970; Gehrke & Linder, 2014; Wichura *et al.*, 2015). This estimate is congruent with the increased frequency of *in situ* diversification during that time period estimated for a large fraction of afroalpine plants (Kandziora *et al.*, 2022). The main *Dendrosenecio* lineages that evolved independently in the four geographically isolated mountain groups seem to have diverged between 6.4 and 3.2 Ma. While the uplift of some mountains (the Aberdare Range, Mt Elgon or the Rwenzori Mts) predates the estimated origin of *Dendrosenecio* lineages, the crown age estimates of the lineages from Mt Kilimanjaro/Mt Meru (1.8 Ma) and Mt Kenya (2.7 Ma) are similar to the orogeny estimates for these mountains (Gehrke & Linder, 2014). Subsequent diversification within mountain groups points to a Pleistocene origin of *Dendrosenecio* species. Similar ages have been estimated for afroalpine *Lychnis*, which diversified between the Late Miocene and the Late Pliocene (Gizaw *et al.*, 2016a), while the diversification of most *Alchemilla* dwarf shrubs and afroalpine *Helichrysum* has been estimated to the Pliocene – Pleistocene (Gehrke *et al.*, 2016; Blanco-Gavaldà *et al.*, 2023).

Studies of adaptive introgression in natural populations have estimated that divergence times between species that hybridise usually are < 2 Ma (Schmickl *et al.*, 2017). In the afroalpine flora, gene flow has been detected between species of *Festuca* that diverged 1.06–1.85 Ma (Mairal *et al.*, 2021). Thus, the low levels of gene flow detected between the four main lineages of *Dendrosenecio* can be explained by a reduced ability to introgress because of long-term divergence in combination with geographic isolation. Crossing experiments are needed to determine the strength of postzygotic reproductive isolation between *Dendrosenecio* species from different mountain groups, as well as between species of other afroalpine plant groups. The three genetic (ddRADseq) clusters we inferred correspond to the three described species and agree with our phylogenomic and species delimitation results (Gizaw *et al.*, 2022). The lower level of differentiation of *D. battiscombei* populations on Mt Kenya compared to those in the Aberdare Range suggests a more recent origin of Mt Kenya populations. The origin of the Mt Kenya populations could be the result of intermountain LDD, possibly facilitated by migration corridors during Pleistocene glacial periods (Chala *et al.*, 2017; Brochmann *et al.*, 2022). Intermountain migration, facilitated

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during Pleistocene cold periods, has been suggested for afroalpine species such as *Koeleria capensis* (Masao *et al.*, 2013) and *Festuca abyssinica* (Mairal *et al.*, 2021).

Ecological speciation on Mount Kenya

Using demographic modelling, we found strong support for the hypothesis that the two sister species of *Dendrosenecio* on Mt Kenya, *D. keniensis* and *D. keniodendron*, which are endemic or near-endemic to this mountain, originated via ecological speciation with gene flow. We also found that the two species occupy distinct microhabitats, although they frequently occur at the same sites, supporting that they originated by strong divergent ecological selection in the face of gene flow. We confirmed the suspected hybridization between the two species based on our genetic data. However, whether gene flow between both species is ongoing or species are reproductively isolated could not be determined with our data.

Our demographic modelling analyses of sympatric populations of *D. keniensis* and *D. keniodendron* supported a scenario of continuous migration (Fig. 4). Demographic parameter accuracy is strongly influenced by sample size, and only large sample sizes allow evaluating complex models and recent demographic histories (Robinson *et al.*, 2014). Given the sample sizes necessary to estimate accurate results (Robinson *et al.*, 2014; McLaughlin & Winker, 2020), we consider our sample size, after projecting down (50 diploid individuals), appropriate given the complexity of the models and the time of divergence between the studied species. Speciation in the face of gene flow was historically considered a rare phenomenon, especially in plants (Papadopulos *et al.*, 2011), however, recent studies have shown that it could be more common than initially assumed (Nosil, 2008; Campbell *et al.*, 2018). In fact, cases of continuous gene flow since divergence have been estimated in other alpine plant lineages such as *Lupinus* from the Andes (Nevado *et al.*, 2018) and *Populus* from the Qinghai–Tibet Plateau (Li *et al.*, 2021). The process of divergence with ongoing gene flow within *Dendrosenecio* likely occurred on top of Mt Kenya (possibly in sympatry), given that both species nowadays grow in sympatry and diverged recently in an evolutionary timescale (*c.* 2.7 Ma, given our divergence time based on Hyb-Seq data, or 150–30 Ka, given our divergence time based on RADseq data). This difference in divergence times depending on the molecular markers used is consistent with the older estimates obtained using targeted conserved loci compared to those using RAD loci (Ferrer Obiol *et al.*, 2021). However, uncertainties related to the use of secondary calibrations in the phylogenomic analysis and in the mutation rate and generation time in the demographic analyses could be accentuating the difference in divergence times. Also, unmodelled changes in population size may result in wrong divergence time estimates (Momigliano *et al.*, 2021). In either case, these species were estimated to diverge during the Pleistocene, a period marked by glacial–interglacial cycles (Lisiecki & Raymo, 2005) that influenced population demographic dynamics by enhancing population expansion–contraction during cold–warm periods (Hooghiemstra & Van der Hammen, 2004). Even though the demographic modelling approach used here does not consider these continuous cycles of population expansion–contraction, because the models would have been too complex for the amount of data available, Pleistocene climatic cycles likely influenced the divergence process. In this context of demographic dynamics, during population expansion in glacial periods, the higher frequency of rare alleles could have promoted the colonisation of new niches by certain individuals. Subsequently, divergent selection in different environments would have led to the fixation of different alleles facilitating lineage differentiation despite gene flow. As a result, both *Dendrosenecio* species followed opposite evolutionary trajectories adapting to contrasting environments.

Environmental differences are among the main causes for which ecologically based divergent selection evolves barriers to gene flow and results in ecological speciation (Rundle & Nosil, 2005). One of the processes which contributes to ecological speciation is the reduced probability of mating (premating reproductive isolation) that can arise via immigrant inviability (lower adaptation of one species in the other's environment) and/or assortative mating (Nosil *et al.*, 2005; Schluter & Conte, 2009). It is evident from our analyses of the vegetation plot data that the two sister species occupy distinct niches (Fig. 3), even if they often occur intermingled at the same sites. This is clearly illustrated by the positive correlations between the occurrences of *D. keniensis* and *Carex monostachya*, a species typical of permanently moist habitats such as bogs and swamps (Gizaw *et al.*, 2016b), and between the occurrences of *D. keniodendron* and *Arabis alpina*, a species typical of well-drained and rocky habitats (Hedberg, 1962, 1986; Mizuno, 2005). While the low resolution of global environmental data did not allow us to fully assess niche overlap, around half of the niche of *D. keniensis* seems to be unique. These findings are in agreement with the climatic and edaphic differences between these two *Dendrosenecio* species reported by Beck *et al.* (1981). Edaphic factors have been suggested as a main driver of genetic structuring in alpine plants (Alvarez *et al.*, 2009), and edaphic preferences have been found to be among the traits most likely to diverge between sister plant species when accounting for range overlap (Anacker & Strauss, 2014). For instance, shifts between dry and wet habitats are associated with diversification in *Dubautia* species from the Hawaiian archipelago (Baldwin, 1997). Furthermore, significant ecological differences suggest an important role of ecological adaptation in speciation among endemic *Dubautia* species from the island of Maui (Friar *et al.*, 2006).

The conspicuously different root morphology of the two *Dendrosenecio* species, with *D. keniensis* having big intercellular spaces in the roots (much smaller in *D. keniodendron*), is apparently an adaptation to waterlogged and poorly aired soils (Beck *et al.*, 1981, 1992). The uniqueness of *D. keniensis'* niche compared to that of *D. keniodendron* obtained in our niche overlap estimation could be explained to some extent by this adaptation. Moreover, previous studies showed that *D. keniodendron* plants naturally growing or transplanted into water-soaked soil have lower fitness than plants growing in their typical habitats (Beck *et al.*, 1981; Smith & Young, 1994). By contrast, it has been hypothesised that *D. keniodendron* habitats, where water

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accumulation is prevented by soil composition or by terrain steepness and where low-temperature soil conditions are prolonged, will hinder water uptake in *D. keniensis* (Beck *et al.*, 1981). Adaptation to local environments differing in abiotic factors such as soil composition or moisture can contribute to reproductive isolation and facilitate ecological speciation (Waser & Campbell, 2004). Thus, the difference between environments of alpine *Dendrosenecio* species on Mt Kenya and their adaptation to them supports immigrant inviability, which could have contributed to strengthen reproductive barriers and eventually have led to ecological speciation. In fact, immigrant inviability was among the strongest ecologically based reproductive barriers among *Senecio lautus* peripatric populations (Richards & Ortiz-Barrientos, 2016). Similarly, selection against immigrants played an important role in ecological reproductive isolation between *Mimulus guttatus* populations adapted to either summer drought or year-round soil moisture (Lowry *et al.*, 2008). In *Dendrosenecio*, adaptation to different elevations occurred independently in the different mountain groups (Tusiime *et al.*, 2020; Gizaw *et al.*, 2022). Except for the case of Mt Kenya addressed here, altitudinal boundaries between species growing on the same mountain are apparent, which could imply an important role of geographic isolation in speciation. Evaluating the role of geographic speciation against ecological speciation in other *Dendrosenecio* species pairs, which could not be addressed with our sampling, would give insights into the evolutionary trajectory of each mountain. Similarly, differences in habitat preference between closely related *Lobelia* species (Beck *et al.*, 1981; Knox & Palmer, 1998) and the candidate genes identified to be potentially involved in adaptation to different environments (Zhao *et al.*, 2016) make *Lobelia* another interesting plant group to further study ecological speciation within the afroalpine.

Alpine *Dendrosenecio* species from Mt Kenya also show differences in sexual reproductive traits and flowering phenology. *Dendrosenecio keniodendron* flowers synchronously at intervals of five (or more) years and produces pollen compatible with wind pollination, whereas *D. keniensis* flowers every year (with a different proportion of individuals from year to year) and produces pollen suitable for animal pollination (Smith & Young, 1982, 1994; Beck *et al.*, 1992). Years in which both species shared flowering periods have been documented (Smith & Young, 1994), however the differences in phenology make cross-pollination rather unlikely. The reduced chances for cross-pollination together with the differences in pollen traits likely contribute to assortative mating. This nonrandom mating among species will subsequently strengthen premating reproductive barriers and enhance the process of ecological speciation. For instance, divergence in flowering time, possibly associated with soil type, enhanced speciation between *Howea forsteriana* and *H. belmoreana* (Savolainen *et al.*, 2006). Pollinator isolation likely contributed to divergence between the sister species *Mimulus lewisii* and *M. cardinalis* (Ramsey *et al.*, 2003). Also, reproductive isolation through assortative mating has been reported in animals, such as Darwin's finches (Grant & Grant, 1979; Podos *et al.*, 2013). The directionality of gene flow differed between the *Dendrosenecio* population pairs analysed: while in the mid-elevation pair a model with symmetric migration was favoured, in the high-elevation pair stronger gene flow from *D. keniodendron* to *D. keniensis* was estimated. Pollinator abundance and flower visits decline with increasing elevation and decreasing temperatures (Totland, 2001; Inouye, 2020) among other factors. This suggests that wind pollination could be advantageous over animal pollination at higher elevations, which together with the higher abundance of *D. keniodendron* at these elevations (Smith & Young, 1994) explains the directionality of gene flow estimated for the high-elevation pair. However, further conclusions cannot be taken without knowledge of the mating system of these species. The absence of admixture (with the exception of the single hybrid individual) detected in our genetic clustering analysis could reflect gene flow getting reduced with time as a result of divergent selective pressures. The single admixed individual detected appears to be an F_1 hybrid. The meiosis in hybrids between *D. keniensis* and *D. keniodendron* seems to proceed normally (Beck *et al.*, 1992), but whether these hybrids are at least partly fertile and may backcross to their parents is not clear and cannot be fully assessed with our limited sampling. Given our estimated time of divergence between the parental species, the time typically needed to obtain pronounced hybrid sterility (> 4 Myr; Levin, 2012), excluding cases such as hybrid and polyploid speciation, has not been reached yet. However, the total absence of admixture (except for the apparent F_1 hybrid) between the two genetic clusters in our analysis (Fig. 2), the low proportion of developed achenes in the hybrids compared with their parents (Beck *et al.*, 1992), and the lower germination of seeds of the hybrids (*c.* 2%) than of *D. keniodendron* (1.8–23.7%; Smith & Young, 1994) suggest that, even though meiosis appears to proceed normally, hybrid fertility is reduced. Hybrid fitness is an important measure of postzygotic reproductive isolation (Campbell, 2004). For instance, reduced germination rates in F_1 hybrids contributed to reinforce reproductive barriers between *Mimulus lewisii* and *M. cardinalis* (Ramsey *et al.*, 2003). Also, low-hybrid fertility played an important role in the ecological speciation process in sticklebacks (Hatfield & Schluter, 1999).

In conclusion, our results support that geographic isolation shaped main lineages in *Dendrosenecio*, and ecologically mediated divergence promoted speciation between two species on Mount Kenya. Geographic isolation and ecological factors contributing to divergence in plants from mountainous regions have been pointed out for groups such as giant lobelias in the East African mountains (Knox & Palmer, 1998; Brochmann *et al.*, 2022), *Espeletia* (Cortés *et al.*, 2018; Pouchon *et al.*, 2021), *Lupinus* (Nevado *et al.*, 2018) and Lobelioideae (Lagomarsino *et al.*, 2016) in the Andes, and *Taxus* from the Himalaya-Hengduan Mountains (Liu *et al.*, 2013). The notion of ecological speciation as a major mode of plant speciation is gaining importance (Funk *et al.*, 2006; Givnish, 2010). We found support for distinct habitat preference in *Dendrosenecio* sister species from Mt Kenya enhancing ecological speciation despite ongoing gene flow. Adaptation to contrasting environments characterized by soil waterlogging or well-drained soil likely contributed to ecologically based reproductive isolation via immigrant inviability. Also, divergence in reproductive traits and flowering phenology could have contributed to reproductive

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isolation. However, quantitative data on these traits would be very valuable to determine the strength of reproductive barriers and their role in ecological speciation. The case of ecological speciation with gene flow between *Dendrosenecio* alpine species on Mt Kenya shows that high-altitude mountainous regions like the afroalpine are important systems to study speciation.

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Competing interests

None declared.

Author contributions

RS and JMG conceived and designed the research. DC, AG, FMT, ALSG, CB and their teams collected most of the plant material and vegetation plot data. LP provided training on ddRADseq library preparation. EZ provided assistance with ddRADseq library preparation and ddRADseq and demographic modelling analyses. JMG processed the data, performed the analyses and wrote the manuscript. RS supervised the analyses and the manuscript preparation. RS, CB and FK provided financial, logistic and infrastructure support.

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Data availability

Raw data are publicly available at the Sequence Read Archive under the BioProject number PRJNA1116128 with accessions nos. SRR29219294-SRR29219320 for the Hyb-Seq data and SRR29190900-SRR29190980 for the ddRADseq data. The data that support the findings of this study are openly available in the Open Science Framework (OSF) repository at doi: 10.17605/OSF.IO/AVKWM.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Histograms of relative fluorescence intensities obtained using flow cytometry.

Fig. S2 Coalescent-based multi-allele nuclear DNA phylogeny based on Hyb-Seq data.

Fig. S3 Coalescent-based nuclear DNA phylogeny based on Hyb-Seq data.

Fig. S4 Concatenated nuclear DNA phylogeny based on Hyb-Seq data.

Fig. S5 Divergence time estimation based on the multi-allele nuclear DNA phylogeny.

Fig. S6 Concatenated plastid DNA phylogeny based on Hyb-Seq data.

Fig. S7 Genetic structure based on ddRADseq decaploid variant calling.

Fig. S8 Genetic structure based on ddRADseq diploid variant calling.

Fig. S9 Log-likelihood scores between replicate rounds in the demographic modelling.

Methods S1 Additional details of methods used.

Table S1 Information on plant material of newly sequenced accessions.

Table S2 Relative genome size estimates obtained with flow cytometry.

Table S3 Information about vegetation plot localities and presence/absence data of target species.

Table S4 Summary statistics obtained in the processing of nuclear and plastid DNA data.

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Table S5 Summary statistics obtained in the admixture analyses using DSUITE.

Table S6 Summary statistics obtained in the admixture analyses using DCT-BLT.

Table S7 Population genetic statistics of the decaploid ddRADseq data.

Table S8 Redundancy analysis (RDA) significance tests of the optimized model.

Table S9 Similarity between ecological niche hypervolumes.

Table S10 Demographic models and parameter values for each population pair.

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