

ORIGINAL ARTICLE

The winner takes it all: a single genotype of *Kalanchoe* × *houghtonii* is a global invader

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- **Background and Aims** Invasive alien plant species pose a global challenge, and their impact is amplified by globalization and the accelerating pace of climate change. In regions with mild climates, drought-tolerant invasive plants showing broad environmental tolerance have a competitive advantage. One example is *Kalanchoe* × *houghtonii* (Crassulaceae), popularly known as ‘mother of millions’. It is a hybrid resulting from the interploid cross between *Kalanchoe daigremontiana* and *Kalanchoe delagoensis*, both native to Madagascar. *Kalanchoe* × *houghtonii*, propagated as an ornamental plant, has emerged as a global invader in less than a century. Four morphotypes of this hybrid have been identified, with different ploidy levels and varying invasive capacities. Here, we aim to investigate the genomic variability behind the success of invasion of *Kalanchoe* × *houghtonii*.
- **Methods** We sampled 57 accessions of *Kalanchoe* × *houghtonii*, *K. daigremontiana*, *K. delagoensis* and closely related taxa, including old herbarium materials, from all over the world. We analysed the genome size and chromosome numbers, sequenced the whole genome, analysed the complete plastome sequence of each accession and studied the diversity of the ribosomal RNA genes. We also performed a detailed phylogenomic study using nuclear BUSCO genes.
- **Key Results** Our study reveals genetic and cytogenetic variability between morphotypes and shows that a single tetraploid genotype (morphotype A) dominates all populations, emerging as the first reported clonal hybrid capable of colonizing mild-climate regions worldwide. Morphotype A shows a striking genetic uniformity, high phenotypic plasticity and extremely high rates of vegetative reproduction, representing an example of a ‘general-purpose genotype’.
- **Conclusions** The astonishing reproductive capacity, broad adaptability and speed at which *K. × houghtonii* is colonizing new regions by clonal spread highlight the importance of understanding hybridization and polyploidy in the invasion of ecosystems. Our findings underscore the need to recognize and monitor the potential invasive risks of new hybrids developed through ornamental plant breeding.

Key words: Clonality, invasive alien plant species, phylogenomics, hybridization, genomics, ornamental plants, polyploidy.

INTRODUCTION

Invasive alien plant species are a global problem owing to their multiple environmental and economic impacts (Hulme and Shepherd, 2003; Bresch *et al.*, 2013) and are considered a major driver of recorded species extinctions (IPBES, 2023). Their expansion beyond their native range is caused by human activities and is often determined by socioeconomic drivers (Pyšek *et al.*, 2020), which promote their appearance even in protected or highly biodiverse ecosystems (Gioria *et al.*, 2023). Although some invasive alien plant species have been introduced accidentally, most introductions are intentional, in many cases linked to the globalized trade in ornamental plants

(Hulme, 2008; Pyšek *et al.*, 2011a; van Kleunen *et al.*, 2018). Their subsequent spread and establishment can threaten ecosystems, while causing economic impacts related to the cost of their management (Binggeli, 2001; Pyšek *et al.*, 2009).

The Mediterranean basin is recognized as a biodiversity hotspot (Mittermeier *et al.*, 2011), containing 7–10 % of the world’s plant diversity (Myers *et al.*, 2000; Thompson, 2020; Nieto Feliner *et al.*, 2023). The intense human activity and land degradation in this area (Hill *et al.*, 2008; Underwood *et al.*, 2009), together with climate change (MedECC, 2020; Herrando-Moraira *et al.*, 2022; Urdiales-Flores *et al.*, 2023), make the Mediterranean basin one of the most vulnerable

regions to be affected by invasive alien plant species (Gritti *et al.*, 2006; Cao Pinna *et al.*, 2021). According to Keller *et al.* (2011), ≥1780 non-native species have been established in natural ecosystems across Europe, including invasive alien plant species with high ecological and economic impact, such as *Ailanthus altissima*, *Cortaderia selloana* or *Robinia pseudoacacia* (Pyšek *et al.*, 2009), with most of them coming from the ornamental plant trade. Specifically, the Mediterranean region is harbouring ≥501 invasive taxa (Pyšek *et al.*, 2011b); however, this might be an underestimate, because a more recent study reports ~250 invasive alien plant species only in Italy (Galasso *et al.*, 2024). As the Mediterranean is becoming warmer and drier, representatives of drought-tolerant groups of plants, such as Aizoaceae, Cactaceae and Crassulaceae, typically succulent, are being established increasingly, e.g. several species of the genus *Kalanchoe* Adanson (Stinca *et al.*, 2021; Sakhraoui *et al.*, 2023).

Kalanchoe comprises ~140 species mainly native to Madagascar (Milad *et al.*, 2013; Kuligowska *et al.*, 2015). Many species of the genus are well known for their commercial value, mostly because of their ornamental and medicinal uses (Kolodziejczyk-Czepas and Stochmal, 2017; Stefanowicz-Hajduk *et al.*, 2020; Vargas *et al.*, 2022). Although natural hybrids are not common within the genus (Smith, 2020a; Smith and Figueiredo, 2020; Smith *et al.*, 2022), the ease with which hybrids can be created in cultivation has given rise to the description of several ornamental cultivars (Smith, 2020a, b, c, d, e, f, g; Smith and Figueiredo, 2020; Smith and Shtein, 2020).

Kalanchoe × *houghtonii* is an allegedly artificial hybrid complex resulting from the cross between the diploid species *Kalanchoe daigremontiana* ($2n = 2x = 34$) and the tetraploid species *Kalanchoe delagoensis* ($2n = 4x = 68$), both native

to Madagascar and growing sympatrically in some places on the island. *Kalanchoe* × *houghtonii* has been created synthetically at least twice, once in the USA by A. D. Houghton (1935) and again in Portugal by Resende and Warden (1954). Morphologically, *Kalanchoe* × *houghtonii* is a perennial erect succulent herb, generally monocarpic, that may reach ≤1.8 m. It has lanceolate leaves, which are serrate and mottled (Fig. 1). It flowers annually in late winter, with inflorescences that bear >100 pendulous dark-red flowers (Herrando-Moraira *et al.*, 2020). Shtein *et al.* (2021) reported the existence of four different hybrid morphotypes (named A, B, C and D). Plants of *K.* × *houghtonii* morphotype A are highly invasive, showing an extraordinary adaptive capacity to colonize and be naturalized in numerous mild-climate regions around the world (Herrando-Moraira *et al.*, 2020). Although it has been speculated that this morphotype could have been produced artificially more than once on different continents and that it could correspond to a fertile, probably tetraploid plant like the one Resende (1956) created in Portugal, the true origins and cytogenetic features of morphotype A are not yet known. Plants of morphotype B are significantly less invasive than those of morphotype A, although individuals representative of morphotype B have occasionally become naturalized in some places (e.g. in Spain; Shtein *et al.*, 2021). The original hybrid material created by Houghton (1935) is likely to correspond to morphotype B, which is triploid (Baldwin, 1949). Morphotype C is suspected to be a spontaneous hybrid between *K. daigremontiana* and *K. delagoensis* in Madagascar or a closely related non-hybrid taxon showing intermediate characters (Shtein *et al.*, 2021). Finally, morphotype D occurs naturally in Madagascar, where it is thought to result from the natural introgression between *K. daigremontiana* and native *K.* × *houghtonii* plants but can also

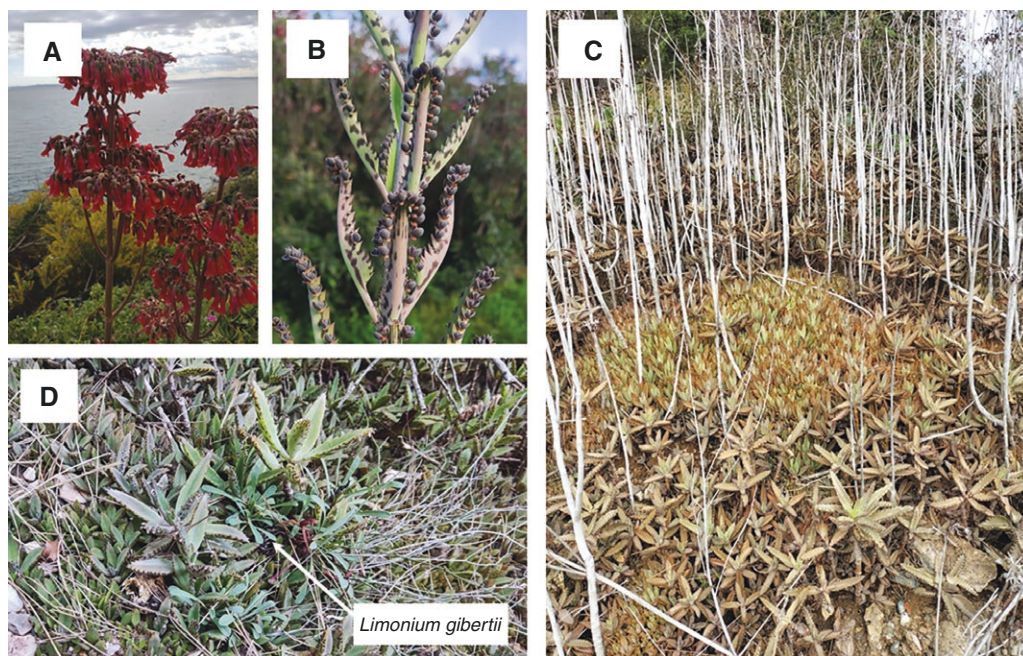


FIG. 1. Pictures of the invader *Kalanchoe* × *houghtonii* morphotype A. (A) Pictures of the plant flowering. (B) Details of adult leaves with plantlets emerging in the margins of the leaves. (C) Examples of habitats invaded by *K.* × *houghtonii*, becoming established and spreading by plantlet propagation. (D) Examples of *K.* × *houghtonii* displacing endemic *Limonium gibertii* in Montroig del Camp (Catalonia, Spain). Picture credits to: Jordi López-Pujol (A), Sònia Garcia (C) and Joan Pere Pascual-Díaz (B, D).

be found as a cultivar ('Parsel Tongue'), probably stemming from a back-cross of *K. daigremontiana* with the fertile cultivar *K. × houghtonii* 'J.T. Baldwin' (morphotype A) (Shtein *et al.*, 2021). As with morphotype A, the chromosome numbers and ploidy levels of morphotypes C and D have never been determined with certainty. The different hybrid morphotypes differ in leaf size and shape and in the number of plantlets produced along the leaf margin, a trait shared with their parental species. Specifically, morphotype A, the most invasive, produces a large quantity of foliar embryos along the leaf margins, similar to those found in *K. daigremontiana* (Shtein *et al.*, 2021).

Even considering its likely recent origin, this complex (particularly morphotype A) exhibits an extraordinary invasive capacity. Moreover, according to preliminary species distribution models, the hybrids exhibit a larger potential distribution area in comparison to its parental species (Ojea-Jiménez *et al.*, 2024). As pointed out earlier, the invasiveness of *K. × houghtonii* is facilitated by its successful vegetative propagation through foliar embryos. These plantlets emerge profusely from the leaf margins (Akulova-Barlow, 2009) at densities of 1000–2000 individuals m⁻² when falling to the ground (Herrando-Moraira *et al.*, 2020). This feature earned them the popular names of 'mother of thousands' or 'mother of millions' (Fig. 1). The early history of the invasion of *K. × houghtonii* is poorly known, but it has reached a nearly worldwide distribution in only 80 years, currently being present in all continents except Antarctica (Herrando-Moraira *et al.*, 2020). Domestic gardens are the most apparent source of incursions into the wild, at least in the USA (Ward, 2006), Australia (Queensland Government, 2016) and Europe (Guillot *et al.*, 2014; Sáez *et al.*, 2017). The first confirmed wild record of the hybrid complex was in Queensland (Australia) in 1965, quickly followed by another in Oceania and then in North and South America in the 1970s. By the 2000s, an apparent 'outbreak' occurred worldwide, possibly owing to a much increased detection efficiency of the hybrid thanks to the emergence of citizen science platforms (Herrando-Moraira *et al.*, 2020). In the Mediterranean basin, the first naturalized record of the hybrid was reported in Calp (València, eastern Spain) in 1993 (Guillot-Ortiz *et al.*, 2015; voucher VAL-930300, initially misidentified as *K. daigremontiana*).

Kalanchoe × *houghtonii* poses a threat to native plant diversity, particularly in coastal areas, including some European Union habitats of conservation interest. One of the most affected habitats is the Mediterranean cliffs with endemic *Limonium* species (Fig. 1D), given the preference of *K. × houghtonii* for rocky substrates (Herrando-Moraira *et al.*, 2020). *Kalanchoe* × *houghtonii* is increasingly present in checklists of invasive flora in some Mediterranean countries, such as Algeria (Sakhraoui and Thomson, 2024) and Italy (Galasso *et al.*, 2024). It also appears in the Spanish checklist of 'Allochthonous species liable to compete with native wildlife, alter their genetic purity, or disrupt ecological balances' (Spanish Government, 2024). The recent appearance of this taxon in several such lists highlights the current concern of this problematic hybrid, for which management strategies are still lacking. Moreover, it has already been classified as invasive in Australia (Randall, 2007; Queensland Government, 2016) and the USA (Florida Exotic Pest Plant Council, 2017).

Although several studies have explored the ecological impact of *K. × houghtonii* in introduced areas (Herrera and Nassar, 2009; Herrera *et al.*, 2012, 2016, 2018; Herrando-Moraira *et al.*, 2020; Vargas *et al.*, 2022), the genetic and genomic mechanisms underlying the invasiveness of this hybrid complex remain largely unexplored. Hybridization can create a pool of genetic diversity, driving adaptive evolution and facilitating the emergence of novel genotypes through previously unexplored allele and gene combinations (Bock *et al.*, 2016; Smith *et al.*, 2020). It may also increase the structural genomic variability of taxa, including alterations in ploidy level, chromosomal rearrangements and variations in the activity or abundance of transposable elements (McClintock, 1984). Hybridization and polyploidy can also positively influence the invasion process, without necessarily increasing the genomic diversity, by enhancing asexual reproduction or clonality and by increasing phenotypic plasticity of individuals, i.e. the 'general-purpose genotype' strategy (Baker, 1955, 1974; Coughlan *et al.*, 2017; Bricker *et al.*, 2018; Goad *et al.*, 2021). The global invasion by *K. × houghtonii* raises questions about the role of hybridization and polyploidy in its high adaptability. It is unclear whether these processes contribute to increased genetic variability within the species complex or lead to the emergence of specific genotypes with higher fitness and environmental plasticity. Understanding this could shed light on how invasive alien plant species successfully adapt to diverse environments, outcompeting the native species.

Considering the extremely rapid and worldwide expansion of *K. × houghtonii*, we aimed to investigate the genomic variability behind its invasion success. To reach this goal, we analysed whole-genome sequencing data together with genome size estimations, chromosome counts and ploidy levels of 57 accessions of this hybrid complex, including samples from all morphotypes and the parental species. In particular, we aimed to: (1) determine the cytogenetic and genomic variability within the samples obtained from the Mediterranean basin, America and Australia through comparison of the broadly invasive morphotype A and the less invasive morphotype B; (2) disentangle the evolutionary origin of the morphotypes that occur naturally in Madagascar (i.e. morphotypes C and D); and (3) understand the relationship of specific cytogenetic and genomic features to its invasiveness.

MATERIALS AND METHODS

Sampling and whole-genome sequencing

Samples were field-collected from representative populations of *Kalanchoe* × *houghtonii*, *K. daigremontiana* and *K. delagoensis* across the Mediterranean basin and stored in the living collection of the Botanical Institute of Barcelona, IBB (CSIC-CMCNB) (Table 1; Fig. 2). Sampling consisted of 30 individuals of *Kalanchoe* × *houghtonii*, 5 of *K. delagoensis* and 4 of *K. daigremontiana*, all from different populations. Moreover, we sequenced 14 samples from herbarium vouchers across the world, including a paratype (a specimen mentioned in the protologue other than the holotype, isotype or syntype) and a topotype (a specimen collected from the type locality but not cited in the protologue) of *K. × houghtonii*, in addition to the first globally reported herbarium voucher of this

TABLE 1. *Information on all Kalanchoe populations sampled.*

Sample	Morphotype	Wild (W)/cultivar (C)	Location	Year
KDA (Bahamas)	Parental	W	Bimini, Bahamas	1975
KDA (Israel 3)	Parental	W	Haifa, Israel	2022
KDA (Israel 2)	Parental	W	Tel Aviv, Israel	2020
KDA (Tunisia 2)	Parental	W	Tunis Bizerte, Tunisia	2019
KDA (Spain 1)	Parental	W	València, Spain	2019
KDE (Tunisia 1)	Parental	W	Le Kram, Tunisia	2019
KDE (Madagascar 2)	Parental	W	Behara, Madagascar	2022
KDE (Madagascar 1)	Parental	W	Antananarivo, Madagascar	2022
KDE (Israel 1)	Parental	W	Tel Aviv, Israel	2020
KDE (Catalonia 1)	Parental	W	Tossa de Mar, Catalonia, Spain	2019
KH (Australia 1)	A	W	Chinchilla, QLD, Australia	1970
KH (Australia 2)	A	W	Ipswich, QLD, Australia	1970
KH (Australia 3)	A	W	Ipswich, QLD, Australia	1966
KH (Florida 1)	A	W	Monroe Co., FL, USA	2009
KH (Florida 2)*	A	W	Fort Myers Beach, FL, USA	2000
KH (Florida 3)**	A	W	Merritt Island, FL, USA	2004
KH (Ecuador)	A	W	Mitad del Mundo, Ecuador	1988
KH (Venezuela 2)	A	W	Churuguara, Venezuela	1980
KH (Dominican Republic)	A	W	San José de Ocoa, Dominican Republic	1993
KH (Spain 5)	A	W	Calp, Spain	1993
KH (Catalonia 4)	A	W	Garraf, Catalonia, Spain	2003
KH (Greece)	A	W	Athens, Greece	2019
KH (France 2)	A	W	Banyuls, France	2023
KH (Italy 3)	A	W	Camerota, Italy	2023
KH (Morocco)	A	W	Casablanca, Morocco	2019
KH ('Palamós')	A	C	In cultivation	—
KH (Italy 2)	A	W	Formia, Italy	2023
KH (Spain 2)	A	W	Almeria, Spain	2019
KH (Italy 4)	A	W	Genova, Italy	2023
KH ('Jaws of Life')	A	W	Cultivar	—
KH (Italy 7)	A	W	Lipari, Aeolian Islands, Italy	2023
KH (Portugal)	A	W	Lisboa, Portugal	2018
KH (Italy 8)	A	W	Livorno, Italy	2023
KH (Malta)	A	W	Mosta, Malta	2019
KH (France 1)	A	W	Cap d'Ail, France	2023
KH (Italy 5)	A	W	Ostia, Italy	2023
KH (Spain 3)	A	W	Alacant, Spain	2019
KH (Italy 6)	A	W	La Maddalena, Sardegna, Italy	2023
KH (Catalonia 3)	A	W	Tarragona, Catalonia, Spain	2023
KH (France 3)	A	W	Toulon, France	2023
KH (Spain 4)	A	W	Tórrox, Spain	2022
KH (Tunisia 2)	A	W	Tunis Bizerte, Tunisia	2019
KH (Australia 4)	B	W	Byrnestown, QLD, Australia	1965
KH (Australia 5)	B	W	Gracemere, QLD, Australia	1968
KH ('Pink Butterflies')	B	C	Cultivar	—
KH (Catalonia 2)	B	W	Cap de Creus, Catalonia, Spain	2021

TABLE 1. Continued

Sample	Morphotype	Wild (W)/cultivar (C)	Location	Year
KH ('Hybrida')	B	C	Cultivar	–
KH (Italy 1)	B	W	Ostia, Italy	2023
KH ('Linear leafed')	C	C	Cultivar	–
KH (Madagascar 3)	D	W	Makay Massif, Madagascar	2022
KH (Madagascar 4)	D	W	Tulear, Madagascar	2022
KH ('Parsel Tongue')	D	C	Cultivar	–
<i>Kalanchoe</i> ('RS574')	–	C	Cultivar	–
<i>Kalanchoe laetivirens</i>	–	W	Tulear, Madagascar	2022
<i>Kalanchoe sanctula</i>	–	W	Taolagnaro, Madagascar	2022
<i>Kalanchoe × descoingsii</i>	–	W	Arboretum Antsokay, Madagascar	2022

Paratype (*) and topotype (**) vouchers are indicated. Abbreviations: KDA, *Kalanchoe daigremontiana*; KDE, *Kalanchoe delagoensis*; KH, *Kalanchoe × houghtonii*.

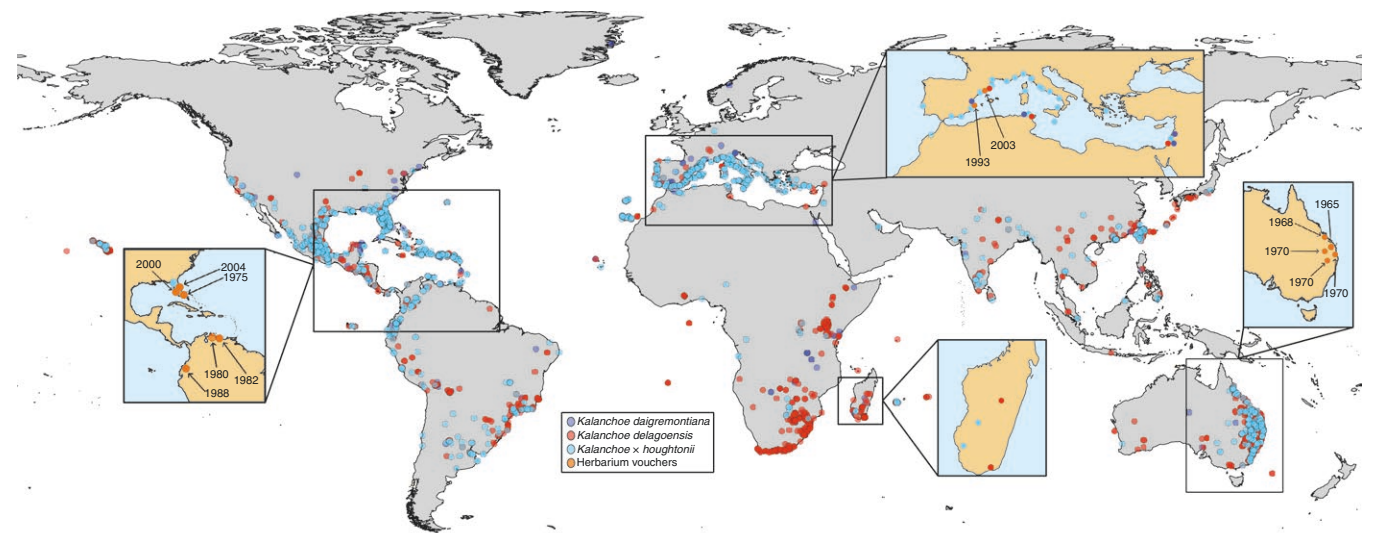


FIG. 2. Map of the global distribution of the hybrid *Kalanchoe × houghtonii* (light blue) and its parental species, *Kalanchoe daigremontiana* (dark blue) and *Kalanchoe delagoensis* (magenta). Data correspond to observations from 1950 to 2023 [GBIF.org (19 June 2024) GBIF occurrence download: <https://doi.org/10.15468/dl.asc2hx>]. Sampling for the present study is indicated in zoomed and coloured maps. Herbarium vouchers are indicated in orange.

hybrid (Australia). We also sampled four individuals from other *Kalanchoe* taxa (including *K. × descoingsii*, *K. laetivirens* and *K. sanctula*) closely related to *K. daigremontiana* and *K. delagoensis*. Herbarium vouchers of all specimens are deposited at the Botanical Institute of Barcelona (BC herbarium).

Total DNA was isolated from fresh leaves using two different modified Cetyltrimethylammonium bromide (CTAB) protocols, one general protocol for fresh leaf material (Doyle and Doyle, 1987) and another for samples from ancient herbarium vouchers (Hale et al., 2020). The quality of each sample was checked by spectrophotometry with the NanoDrop 1000 (PeqLab, Erlangen, Germany) and the DNA concentration by fluorometry with Qubit Fluorometric Quantification (Thermo Fisher Scientific, Waltham, MA, USA). The genomic DNA was sheared randomly into short fragments and sequenced by NovoGene Europe (Cambridge, UK). Libraries of the whole

genome with an average insert size of 450 bp were sequenced on an Illumina NovaSeq Platform (Illumina, San Diego, CA, USA). For all accessions, ~2.68–13.3 Gbp of raw data (equivalent to ~10× their respective genome size) were generated with paired-end 150 nt read length (Supplementary Data Table S1). We generated 375 Gb of whole-genome sequencing data for 57 *Kalanchoe* accessions (with one sample being replicated to determine the batch sequencing error in downstream analyses), with an emphasis on sampling *K. × houghtonii* from across its range in the Mediterranean basin ($n = 20$; Fig. 2).

Ploidy level and genome size estimation using flow cytometry

Ploidy level and genome size estimations were carried out with a flow cytometer (CyFlow® Space; Sysmex-Partec,

Norderstedt, Germany), coupled with the software FloMax (Partec GmbH, Münster, Germany). The internal standards used were *Petunia hybrida* cv. ‘PxPc6’ (obtained from the living collection at the greenhouses of the IBB), with a 2C genome size of 2.85 pg, and *Solanum lycopersicum* cv. ‘Stupické polní rané’, with a 2C genome size of 1.96 pg (Temsch et al., 2022). Fresh young leaves were chopped using a razor blade in general purpose buffer (GPB) (Loureiro et al., 2007) and stained by adding 40 µL of 1 mg mL⁻¹ propidium iodide solution. Nuclei suspensions were then incubated for ~20 min on ice prior to analysis. We measured three replicates for each population, with a minimum of 500 nuclei per fluorescence peak in each analysis.

Karyological observations

For the karyological observations, root tips of *K. × houghtonii*, *K. daigremontiana* and *K. delagoensis* were sampled from adult plants early in the morning, after generous watering of the adult plant 1 or 2 days earlier. Root tips were pretreated with ice-cold water for 24 h, following recommendations for species with very small chromosomes, then fixed in Farmer’s solution (3:1 ratio of absolute pure ethanol and glacial acetic acid). After fixation, acid hydrolysis with 1 N HCl at 60 °C for 10–15 min, followed by staining with 1 % acetic orcein (for ≥1 h), was conducted. Subsequently, individual root tips were selected with a magnifying glass, and the radical meristem was cut, discarding the rest of the tissue. The sample was then placed on a slide with a drop of 45 % acetic acid:glycerine (9:1) solution, squashed with tweezers or a scalpel and covered with a coverslip, following Olanj et al. (2015). Finally, preparations were observed under the optical microscope (Zeiss Axioplan) and photographed with a coupled AxioCam HRm camera.

Plastome assembly and phylogenomics

Plastomes were assembled from raw sequencing data, using NOVOPlasty v.4.2.1 (Dierckxsens et al., 2017) with default parameters and previously removing adapters Illumina reads, as recommended by the author. Chloroplast genome assemblies were done using as seed the *rbcL* gene of *K. daigremontiana* (NCBI GenBank: L11189) and, if the assembly was incomplete, the process was repeated using a different seed sequence [the *matK* gene (NCBI GenBank: AF274619) or the entire chloroplast sequence of *K. daigremontiana* (NCBI GenBank: MT954417)]. The resulting contig options were aligned to the pre-existing *K. daigremontiana* plastome genome (NCBI GenBank: MT954417) using MAFFT (Katoh and Standley, 2013) and manually rearranged such that the short single copy and inverted repeat were in the same orientation for each individual using Geneious Prime v.2023.2.1 (Kearse et al., 2012). Then, filtered whole-genome sequencing reads with a minimum quality score of 30 were mapped to the consensus plastome sequence using *bwa* (Li and Durbin, 2009), and the resulting consensus, with a minimum coverage of 20 reads per site, was adjusted manually for further phylogenetic analyses. All sites not supported by 90 % of the mapped reads were replaced by Ns (ambiguous bases that could not be confidently identified). No ambiguities were allowed, because the inheritance of the plastome is determined by only one parental individual. For four

herbarium accessions where the quality of reads was not good enough to allow the *de novo* reconstruction of the plastome, a reference-based reconstruction was performed using reads with a minimum quality score of 30 (using *fastp* pipeline; Chen et al., 2018). Herbarium voucher Australia 1 was excluded from the phylogenetic analysis owing to the high proportion of missing data (Supplementary Data Table S2). Each resulting plastome was annotated using the software GeSeq (Tillich et al., 2017) included in the platform MPI-MP CHLOROBOX (<https://chlorobox.mpimp-golm.mpg.de/>, accessed 11 February 2024), selecting the options to perform ARAGORN v.1.2.38 and BLAT, as recommended by authors for plastomes.

Plastome phylogenomics were inferred without one of the inverted repeats, because the inverted repeats within a plastome can recombine with each other, they are generally identical, and including them effectively inflates the weight assigned to those positions (Palmer, 1985; Blowers et al., 1989). Plastomes were subsequently partitioned based on their transfer RNA, ribosomal RNA, coding sequences (CDS) (separate introns and exons) and non-coding regions. The resulting sequences were aligned using MAFFT (Katoh and Standley, 2013). Then, we used PartitionFinder2 (Lanfear et al., 2017) to fit the best nucleotide substitution model for all the different partitions. A Bayesian inference (BI) phylogenomic tree was conducted using the software MrBayes v.3.2.6 (Ronquist et al., 2012), where two independent Markov chain Monte Carlo algorithms were run for 3 000 000 generations, with tree sampling every 1000 generations. The average standard deviation was confirmed to be <0.01, and the potential scale reduction factor was near 1.0 in all parameters. The first 25 % of the trees were discarded as ‘burn-in’, and the posterior probability was estimated by constructing the 50 % majority-rule consensus tree. Phylogenomic trees were visualized with FigTree v.1.4.5 (<http://tree.bio.ed.ac.uk/software/figtree>), using as outgroup *Kalanchoe humifera* (GenBank SRA: SRR32150939), according to Rodewald et al. (2025).

Ribosomal DNA reconstruction through the TAREAN pipeline

Ribosomal DNA (rDNA) identification and reconstruction by similarity-based clustering of Illumina paired-end reads was performed following the TAREAN pipeline (Novák et al., 2017). Initially, Illumina FASTQ files were filtered to avoid the presence of adapters, reads with indeterminate bases (N) and a minimum quality score of 30 using the *fastp* pipeline. After converting filtered FASTQ reads to interleaved FASTA files, clustering analyses were performed on these data using the following settings: minimum overlap = 55 and cluster size threshold = 0.01 %. Chloroplast and mitochondrial reads were removed before downstream analyses. For chloroplast reads, we mapped the filtered reads to the previously reconstructed plastome sequences. For mitochondrial reads, we mapped filtered reads of all accessions to *Sedum plumbizincicola* mitochondrial genome (NCBI GenBank: OP588116). The total number of reads used as input for the individual clustering analyses corresponds to 0.5× of the genome coverage for each accession. Then, individual TAREAN analyses were carried out for each accession, allowing the cluster merging (--merge_threshold 0.1) and the automatic filtering of abundant

satellite repeats (--automatic_filtering) to allow more reads to be analysed and increase the chances of reconstructing the rDNA. For the 35S and 5S rDNA phylogenetic network analyses, we extracted the consensus sequence identified in the TAREAN analyses. Once all consensus sequences were obtained, the different regions constituting the rDNA units were determined by BLAST using as reference the 35S (NCBI GenBank: [X52322](#)) and 5S (NCBI GenBank: [ATHRR5S](#)) rDNA genes of *Arabidopsis thaliana*. Afterwards, we mapped the quality trimmed Illumina reads from each sample to their respective 35S and 5S rDNA consensus sequences using *bwa*. We considered the potential intragenomic variability allowing the presence of ambiguous bases (>10 % of the reads) in the final consensus sequence. For the 35S rDNA arrays, genes were discarded for downstream analysis, because there was the presence of DNA contamination from other organisms (e.g. fungi, human DNA) probably owing to poor DNA preservation in some herbarium vouchers. Consensus sequences of all samples were aligned initially using MAFFT, then we reconstructed a Neighbor-net (Bryant and Moulton, 2004) by transforming sequence divergence to uncorrected phylogenetic distances and handling ambiguous characters as average states using SplitsTree (Huson and Bryant, 2024) (Supplementary Data Fig. S1).

Nuclear phylogenomics based on BUSCO genes

To gain a better understanding of the hybridization process and the genetic diversity exhibited by *K. × houghtonii*, we examined the composition of nuclear polymorphisms using Benchmarking Universal Single-Copy Ortholog genes (BUSCO; [Seppey et al., 2019](#)). The list of BUSCO genes used was obtained from the *Kalanchoe fedtschenkoi* partially assembled genome (*Kalanchoe fedtschenkoi* v.1.1; [Yang et al., 2017](#)), setting eudicots_odb10 as the lineage database. The resulting 1896 BUSCO genes were used as targets for bait sequence using HybPiper ([Johnson et al., 2016](#)). For each sample, we filtered each one of the BUSCO genes that hold three or more paralogues using the implemented HybPiper script 'paralogs_retriever.py', to avoid excessive heterozygosity and coverage, for both polyploid and diploid samples ([Bohutínská et al., 2023](#)). According to HybPiper statistics (reads mapped and gene recovery), populations Australia 1, Australia 2, Australia 3, Ecuador, Venezuela 2 and Dominican Republic were discarded from downstream analyses (Supplementary Data Table S3). Then, following [Pokorný et al. \(2024\)](#), the max_overlap.R script ([Shee et al., 2020](#)) was used to identify under-represented, incomplete and unevenly distributed sequences, where genes with less than two-thirds of the median coverage score values (computed by the above-mentioned script) were also discarded from downstream analyses. Herbarium vouchers (including population Florida 1) exhibited a lower gene recovery than the field-collected samples (Supplementary Data Table S4), but given that they were important to understand the hybrid complex (e.g. they include the paratype and the topotype), we kept them for downstream analyses.

MAFFT was used to generate alignments for individual BUSCO genes using the default parameters. Multiple sequence alignment summary statistics were then computed with AMAS ([Borowiec, 2016](#)) to assess quality (Supplementary Data Table

S5). BUSCO genes were excluded if the number of taxa or the proportion of parsimony-informative sites was less than one-third of the median value across all genes, or if the percentage of missing data was more than one-third of the median value across all genes. Resulting multiple sequence alignments were used to infer exploratory trees with FastTree2 ([Price et al., 2010](#)) for automated outlier removal with TreeShrink ([Mai and Mirarab, 2018](#)) in 'per-species' mode for various levels of false-positive tolerance (α), which controls outlier detection (-q '0.01 0.05 0.5'). Pre- and post-automated outlier removal FastTrees were inspected visually with Geneious Prime to check TreeShrink performance. Outlier-filtered data matrices (0.01 threshold) were realigned (using MAFFT), and summary statistics were computed as above (keeping a total of 1122 filtered BUSCO genes). Output multiple sequence alignments were refined with trimAl ([Capella-Gutiérrez et al., 2009](#)), using lax gap and conservation thresholds (-gt 0.3 -cons 30) to prevent the massive loss of data and phylogenetic signal (proportion of parsimony-informative sites).

Gene trees for each one of the remaining BUSCO genes were estimated with IQ-TREE v.1.5.5 ([Nguyen et al., 2015](#)) using ModelFinder Plus ([Kalyaanamoorthy et al., 2017](#)) to select the best-fitting model and continued with maximum likelihood (ML) tree inference and using both UFBoot (Ultrafast bootstrap; [Minh et al., 2013](#)) and SH-like ([Guindon et al., 2010](#)) approximation, to compute 1000 bootstrap replicates. The resulting gene trees had bipartitions collapsed under various bootstrap support (BS) thresholds ('i & b < '\$bs\$') using the newick_utils set of programs ([Junier and Zdobnov, 2010](#)). These variously collapsed BUSCO gene trees were used as input to estimate the nuclear-based species trees with ASTRAL III v.5.6.3 ([Zhang et al., 2018](#)), which was run with extensive Newick annotations (-t 2). As recommended by [Zhang et al. \(2018\)](#), the selected final tree was the one showing collapsing bipartitions with extremely low support ('i & b < 0'), because this strategy can improve accuracy substantially.

Singl nucleotide polymorphism filtering and variant analyses

Good-quality Illumina reads were mapped to each of the filtered BUSCO genes for every population with good gene recovery using *bwa*. Following [Bohutínská et al. \(2023\)](#), Genome Analysis Toolkit (GATK; [McKenna et al., 2010](#)) was used to remove duplicated sequences or PCR technical errors (MarkDuplicates), which can inflate the sequencing depth. Then, two separate variant-calling (HaplotypeCaller) datasets were conducted for each population: one considering the ploidy level (using -ploidy option), and one without considering it. Then, within each dataset, variant call files were combined (CombineGVCFs) and genotyped (GenotypeGVCFs), reaching a total of 148 908 unfiltered single nucleotide polymorphisms (SNPs) for the non-ploidy dataset and 34 893 SNPs for the ploidy dataset. BCFTOOLS v.1.11 ([Li, 2011](#)) was used to remove SNPs within 20 bp of an indel or other variant type, keeping only bi-allelic SNPs (72 684 SNPs for the non-ploidy dataset and 24 009 SNPs for the ploidy dataset). Then, SNPs were marked by VariantFiltration and excluded with SelectVariants if they met with one or more of the following criteria: (1) quality by depth (QD) < 10.0; (2) Fisher strand bias

(FS) > 60.0; (3) strand odds ratio (SOR) > 3.0; (4) root mean square (RMS) mapping quality (MQ) < 40; (5) $-2.5 < \text{mapping quality rank sum test (MQRankSum)} > 2.5$; (6) depth (DP) > 1426.2 (two times average DP); and (7) read position rank sum test (ReadPosRankSum) > 2.5. Then VCFTOOLS v.0.1.15 (Danecek et al., 2011) was used to convert individual genotypes to missing data when the genotype quality ($-\text{minGQ}$) was < 30 and the depth of coverage ($-\text{minDP}$) was < 10. Ultimately, the filtering resulted in 44 055 SNPs for the non-ploidy dataset and 9763 SNPs for the ploidy dataset.

The non-ploidy dataset of SNPs was used to perform a principal component analysis. For this, PLINK (Purcell et al., 2007) was used to generate eigenvectors and eigenvalues, previously pruning SNPs to remove high linkage disequilibrium (LD) ($-\text{indep-pairwise } 50 \ 5 \ 0.7$), keeping a total of 11 966 SNPs. Then, principal component PC1, PC2 and PC3 (Fig. 5C; Supplementary Data Fig. S2) outputs were visualized using the R package *ggplot2* (Wickham, 2016). Moreover, the non-ploidy dataset was used to assess the clonality of populations from morphotypes A, B and D (excluding morphotype C because only one population for this morphotype is available). Initially, the shared heterozygosity (SH) method (Yu et al., 2023) was used to identify clonemates, based on the number of heterozygous sites relative to the number of heterozygous sites observed in the individual with the higher count (Fig. 5A). Furthermore, identity-by-descent (IBD) was also used to define the clonal relationship for pairwise comparisons among individuals (Fig. 5B). The IBD analysis calculated the proportion of the SNPs with zero, one or two shared IBD alleles, to evaluate genetic similarity among individuals. For this, PLINK was used to calculate IBD values for pairwise comparisons among individuals, previously pruning SNPs to remove high LD ($-\text{indep-pairwise } 50 \ 5 \ 0.7$), and we considered pairs of individuals to be a clonal relationship if they had an IBD value of > 0.95 (Myles et al., 2011; Migicovsky et al., 2017; Liang et al., 2019; Bonfante et al., 2021; Cong et al., 2022; Zou et al., 2023).

The ploidy dataset of SNPs was used to identify nuclear genetic clusters of the different morphotypes using ENTROPY v.2.0, designed for quantifying population structure in autopolyploid and mixed-ploidy individuals using genotype-likelihood data (Shastry et al., 2021). As recommended by the authors, for each genetic cluster (*K*) we spawned three simultaneous Markov chain Monte Carlo algorithms to assess convergence. The maximum *K* in the genotype likelihoods was six (i.e. the sum of parental species and hybrid morphotypes), and the optimal *K* was determined by the deviance information criterion (DIC) and the log-pointwise predictive density (Supplementary Data Table S6).

Finally, the intragenomic polymorphisms present in the nuclear data were analysed to assess the hybridization and gene flow between samples. For this, we generated consensus sequences for all filtered BUSCO genes by mapping (with *bwa* v.0.0.17) the good-quality FASTQ files to the selected BUSCO genes with good coverage and with low missing data (total of 1122 genes). Then, consensus sequences were aligned and refined using MAFFT and trimAl, respectively, obtaining a final alignment of 4 000 577 bp. The obtained matrix was used as input for SplitsTree, reconstructing a Neighbor-net, transforming sequence divergence to uncorrected phylogenetic distances, and handling ambiguous characters as average state.

RESULTS

Ploidy level and genome size estimations from flow cytometry measurements and chromosome counts

The measurements of nuclear genome size (2C values) and ploidy level estimation were conducted on 42 samples (Table 2), consisting of 4 *K. daigremontiana*, 5 *K. delagoensis* and 33 *K. × houghtonii* populations. In all samples, endopolyploidy was common, depending on the plant material used for the measurements. *Kalanchoe daigremontiana* exhibited a genome size ranging from 0.54 to 0.58 pg, corresponding to a diploid ploidy level. *Kalanchoe delagoensis* had a genome size ranging from 1.05 to 1.16 pg, corresponding to a tetraploid ploidy level. For the *K. × houghtonii* hybrid complex, we found three different ploidy levels: (1) morphotype A had a genome size ranging from 1.04 to 1.18 pg, corresponding to a tetraploid; (2) morphotypes B and C had a genome size ranging from 0.79 to 0.85 pg, corresponding to a triploid; and (3) morphotype D had a genome size ranging from 0.53 to 0.59 pg, corresponding to a diploid. Chromosome counts confirmed $2n = 2x = 34$ for *K. daigremontiana* (population ‘Israel 2’), $2n = 4x = 68$ for *K. delagoensis* (population ‘Catalonia 1’), $2n = 3x = 51$ for *K. × houghtonii* morphotype B (population ‘Catalonia 2’), and $2n = 4x = 68$ for *K. × houghtonii* morphotype A (population ‘Palamós’). Pictures are shown in Supplementary Data Fig. S3.

Phylogenetic relationships inferred from whole-plastome sequence analysis

The whole plastome was completely reconstructed for all accessions, including those from old herbarium vouchers. The length of the plastome sequences differed between accessions: all *K. delagoensis* accessions and the morphotype C (3x) of *K. × houghtonii* were 150 018 bp long, except for the *K. delagoensis* population ‘Madagascar 1’, which had a length of 149 975 bp; all *K. daigremontiana* accessions and the morphotype A (4x) of *K. × houghtonii* were 150 062 bp long; and all morphotype B accessions of *K. × houghtonii* (3x) were 150 056 bp long. Morphotype D and the other *Kalanchoe* taxa studied exhibited a range of lengths between 149 923 and 150 173 bp (for further details, see Supplementary Data Table S2).

The analysis of phylogenetic relationships based on plastome sequences (124 637 bp alignment, with 85.8 % identical sites after removal of one of the inverted repeats) is depicted in Fig. 3. The whole-plastome tree is rooted to *K. humifica*, and an early-diverging major clade is constituted by all samples of *K. delagoensis*, in addition to those of *K. × houghtonii* morphotypes C and D. In this clade, *K. × houghtonii* ‘Madagascar 3’ is the first-splitting sample, followed by the subclade constituted by *K. × houghtonii* ‘Parsel Tongue’ and ‘Madagascar 4’ (i.e. samples of morphotype D). All *K. delagoensis* and *K. × houghtonii* ‘Linear leafed’ (i.e. morphotype C) samples constitute another subclade, where *K. delagoensis* ‘Madagascar 1’ diverges first, and the rest of the samples together form a polytomy (*K. delagoensis* ‘Catalonia 1’, ‘Israel 1’, ‘Madagascar 2’, ‘Tunisia 1’ and *K. × houghtonii* ‘Linear leafed’). The other major clade of the tree is constituted by samples of *K. sanctula*, *Kalanchoe* ‘RS574’, *K. daigremontiana*, *K. laetivirens*,

TABLE 2. List of all *Kalanchoe* populations whose genome size and ploidy level estimations have been analysed.

Sample	Genome size (pg)	CV (%)	Ploidy level estimation
KDA (Israel 3)	0.56	13.53	Diploid
KDA (Israel 2)	0.58	11.31	Diploid
KDA (Tunisia 2)	0.54	11.57	Diploid
KDA (Spain 1)	0.58	12.39	Diploid
KDE (Tunisia 1)	1.16	9.37	Tetraploid
KDE (Madagascar 2)	1.08	10.16	Tetraploid
KDE (Madagascar 1)	1.05	9.15	Tetraploid
KDE (Israel 1)	1.06	12.99	Tetraploid
KDE (Catalonia 1)	1.10	8.92	Tetraploid
KH (Greece)	1.05	7.71	Tetraploid
KH (France 2)	1.08	10.80	Tetraploid
KH (Italy 3)	1.04	18.00	Tetraploid
KH (Morocco)	1.08	10.96	Tetraploid
KH ('Palamós')	1.14	9.43	Tetraploid
KH (Italy 2)	1.06	8.81	Tetraploid
KH (Spain 2)	1.07	6.51	Tetraploid
KH (Italy 4)	1.14	10.67	Tetraploid
KH ('Jaws of Life')	1.12	5.89	Tetraploid
KH (Italy 7)	1.04	6.94	Tetraploid
KH (Portugal)	1.10	6.58	Tetraploid
KH (Italy 8)	1.13	4.69	Tetraploid
KH (Malta)	1.13	5.63	Tetraploid
KH (France 1)	1.13	9.20	Tetraploid
KH (Italy 5)	1.13	5.63	Tetraploid
KH (Spain 3)	1.17	8.43	Tetraploid
KH (Italy 6)	1.17	8.03	Tetraploid
KH (Catalonia 3)	1.15	8.49	Tetraploid
KH (France 3)	1.12	4.88	Tetraploid
KH (Spain 4)	1.18	8.32	Tetraploid
KH (Tunisia 2)	1.10	8.24	Tetraploid
KH ('Pink Butterflies')	0.85	8.15	Triploid
KH (Catalonia 2)	0.79	11.28	Triploid
KH (Italy 1)	0.83	11.94	Triploid
KH ('Hybrida')	0.84	7.42	Triploid
KH ('Linear leafed')	0.84	5.41	Triploid
KH (Madagascar 3)	0.59	12.31	Diploid
KH (Madagascar 4)	0.58	6.30	Diploid
KH ('Parsel Tongue')	0.53	9.60	Diploid
<i>Kalanchoe</i> ('RS574')	0.80	5.61	Triploid
<i>Kalanchoe laetivirens</i>	1.59	3.91	Hexaploid
<i>Kalanchoe sanctula</i>	0.57	6.67	Diploid
<i>Kalanchoe</i> × <i>descoingsii</i>	1.30	3.74	Pentaploid

The amount of DNA is expressed as the 2C-value and in picograms. Abbreviations: KDA, *Kalanchoe daigremontiana*; KDE, *Kalanchoe delagoensis*; KH, *Kalanchoe* × *houghtonii*.

K. × descoingsii and *K. × houghtonii* morphotypes A and B. This major clade splits into two further clades: one constituted by *K. sanctula* and *Kalanchoe* ‘RS574’, appearing as the sister clade of a second larger clade consisting of all samples of *K. daigremontiana*, *K. laetivirens*, *K. × descoingsii* and those of *K. × houghtonii* morphotypes A and B. Three main subclades are nested within this second clade, one including *K. laetivirens* and *K. × descoingsii* samples, the second including all *K. × houghtonii* morphotype B samples, and the third encompassing all sampled populations of *K. daigremontiana* and all of *K. × houghtonii* morphotype A. The morphotype A and *K. daigremontiana* subclade includes all samples collected in the Mediterranean basin identified as morphotype A and historical vouchers, such as the paratype and the topotype of *K. × houghtonii* from Florida (USA), the first record of this hybrid in the Mediterranean region, and other herbarium accessions from Australia, Ecuador, Bahamas, Florida, Venezuela and the Dominican Republic, all together forming a polytomy, with the exception of the population Australia 3, which is slightly separated from the rest without support (posterior probability = 0.93). The morphotype B subclade, which includes populations from Spain (population from Cap de Creus, Catalonia), Italy, herbarium accessions from Australia (including the first globally reported herbarium voucher identified as *K. × houghtonii*) and the cultivars ‘Hybrida’ and ‘Pink butterflies’, also forms a polytomy.

Phylogenetic relationships inferred from ribosomal DNA

The phylogenetic relationships among samples of *K. × houghtonii* complex based on 35S and 5S rDNA sequences are presented in [Supplementary Data Fig. S1](#). The alignment of 35S rDNA sequences consisted of 3574 bp, excluding the 18S, 5.8S and 26S genes. For the 5S rDNA, 467 bp were aligned, including the 5S gene and the non-transcribed spacer. For the 35S rDNA, 96.0 % of sites were identical, and for the 5S rDNA, 86.3 % of sites were identical.

Both 35S and 5S rDNA networks exhibited the same overall topology. Parental accessions (*K. daigremontiana* and *K. delagoensis*) were distant from each other, without sharing direct reticulations. For both 35S and 5S rDNA networks, all samples from *K. daigremontiana* were grouped, and for *K. delagoensis* all samples were grouped except for one population from Madagascar, which was more closely related to the hybrid morphotype A in the 35S rDNA network. Samples from morphotypes A and B were closely related to each other, and in the 5S rDNA network, morphotype B nested inside the morphotype A cluster. Morphotype C also shared reticulations with both parental species, but the extent of connections differed between 5S and 35S rDNA networks. For the 35S rDNA, morphotype C was more connected to *K. daigremontiana*, whereas for the 5S rDNA it was closer to *K. delagoensis*. Finally, morphotype D was more closely related to the *K. daigremontiana* cluster, being its closest morphotype in both 35S and 5S rDNA networks.

Regarding the other *Kalanchoe* taxa (*K. × descoingsii*, *K. laetivirens* and *K. sanctula*), both 5S and 35S networks exhibited similar topologies. In the 35S rDNA network, all these other taxa were placed into a new branch closely related to *K.*

delagoensis samples, with long branches. In contrast, for the 5S rDNA both *K. × descoingsii* and *K. laetivirens* were more related to morphotype A and *K. daigremontiana*, but *K. sanctula* was separated by a long branch from the rest of the studied taxa. In contrast, the unidentified *Kalanchoe* sample ‘RS574’ was closely related to the *K. delagoensis* group in both 35S and 5S rDNA.

Nuclear genetic variation and structure within *Kalanchoe* × *houghtonii*

The phylogenomic relationships based on nuclear DNA were inferred using data from 1122 BUSCO genes across 47 populations ([Supplementary Data Fig. S4](#)). The tree is rooted to *K. humifica*, with *K. sanctula* positioned as the earliest-divergent species. The first major clade contains *Kalanchoe* ‘RS574’, diverging in a first branch from a subclade constituted by *K. delagoensis* and *K. × houghtonii* ‘Linear leafed’ (i.e. morphotype C). Within this subclade, *K. delagoensis* population ‘Madagascar 1’ diverges first, followed by *K. × houghtonii* ‘Linear leafed’ and the remaining *K. delagoensis* populations that are grouped together. In the other major clade of the tree, *K. × descoingsii* appears in a sister position to the rest of the samples, clustered in two large subclades. The first of these subclades encompasses all the samples from *K. × houghtonii* morphotype B, and the other large subclade includes samples from *K. laetivirens*, *K. × houghtonii* morphotype D, *K. daigremontiana* and *K. × houghtonii* morphotype A. Finally, this last subclade splits into two distinct groups: (1) a group containing all populations of *K. daigremontiana* and *K. × houghtonii* morphotype D, with *K. laetivirens* placed in a sister position to morphotype D samples; and (2) a group containing all *K. × houghtonii* morphotype A populations, except for ‘Florida 1’, which is separated from the rest of morphotype A without support (support = 0.52).

According to the analysis of population structure (ENTROPY), the optimal number of genetic clusters (*K*) for all samples is two, based on the model DIC and the log-pointwise predictive density (see [Supplementary Data Table S6](#)). The clustering revealed the potential contribution of each parental species to the nuclear genomic composition of the morphotypes ([Fig. 4A](#)). For each morphotype, the average contributions of *K. daigremontiana* and *K. delagoensis* genetic clusters were, respectively: morphotype A (4x), 52.97 and 47.03 %; morphotype B (3x), 35.17 and 64.83 %; morphotype C (3x), 32.19 and 67.81 %; and morphotype D (2x), 68.50 and 31.50 %.

The nuclear Neighbor-net, constructed using uncorrected phylogenetic distances with nuclear genomic data ([Fig. 4B](#)), supported the results obtained from the population structure analysis, in addition to the other phylogenomic trees generated in this study ([Fig. 3](#); [Supplementary Data Fig. S1](#)). The network clearly separated each hybrid morphotype and the parental populations, linking them through reticulated connections. Among the parental species, there was more genomic variation within *K. delagoensis* than within *K. daigremontiana* samples, because all populations of the latter split from the same node in the phylogenomic network. Regarding the *K. × houghtonii* hybrid complex, the samples of each morphotype were grouped in distinct clusters. In morphotype A, the group with the largest

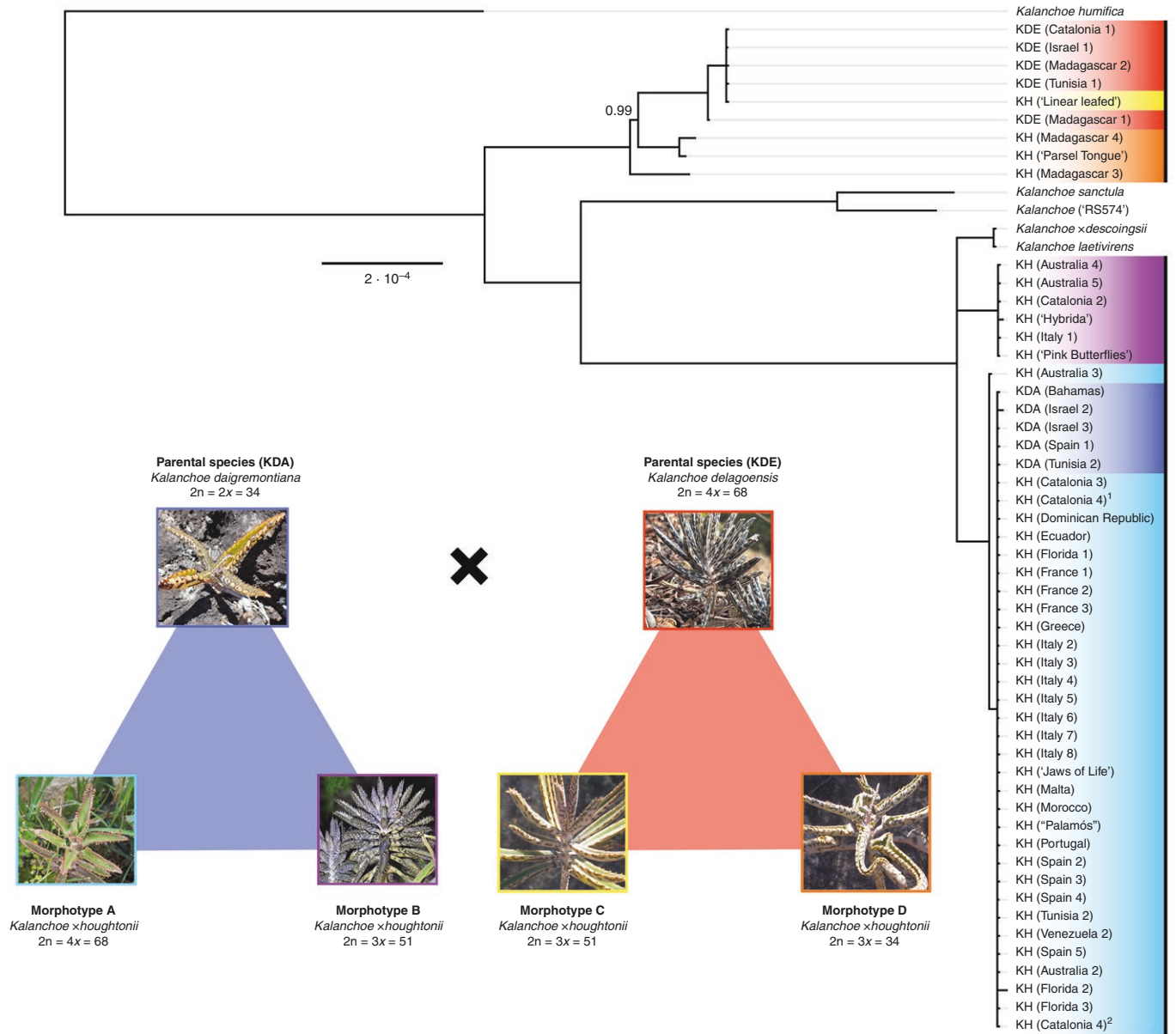


FIG. 3. Phylogenomic relationships of 58 samples of the *Kalanchoe × houghtonii* hybrid complex based on 125 386 bp of the whole plastid genome (with one inverted repeat removed) using Bayesian inference approach. Samples of *Kalanchoe × houghtonii* morphotype A are indicated in light blue, morphotype B in purple, morphotype C in yellow and morphotype D in orange. Samples of parental species are indicated in dark blue for *Kalanchoe daigremontiana* and magenta for *Kalanchoe delagoensis*. Nodes without support values are considered maximum supported (posterior probability = 1). Technical replicates are indicated with superindex 1 and 2. The whole-plastome sequence of *Kalanchoe humifera* is used as an outgroup. Picture credits: KDA to Robin White, KDE to Steve K., KH morphotype A to Piambri, KH morphotype B to Chris Bentley, and KH morphotype C and morphotype D to Solofo Eric Rakotoarisoa.

number of samples, the nuclear genomic variation was extremely low, because all samples emerged from the same node, indicating minimal genetic distance between the different populations. However, population ‘Spain 5’ and a replicate of the population ‘Catalonia 4’ from morphotype A, both obtained from herbarium vouchers, are slightly separated from the rest of populations. Morphotype B, although showing reticulations with *K. delagoensis*, clearly constitutes an independent group. Morphotype C was separated from the other three *K. × houghtonii* morphotypes, also being closely related to *K.*

delagoensis. Conversely, all samples from morphotype D appeared more closely related to *K. daigremontiana*.

The principal component analysis largely confirmed groupings by morphotype, supporting our previous results. The first and second PC axes explained 23.6 and 18.9 % of the variation in the data, respectively (Fig. 4C), and adding a third component meant that the first three PC axes accounted for 55.40 % of the variation in the data (Supplementary Data Fig. S2). In the resulting principal component analysis, the parental populations were respectively distributed in the top-centre and the bottom-left

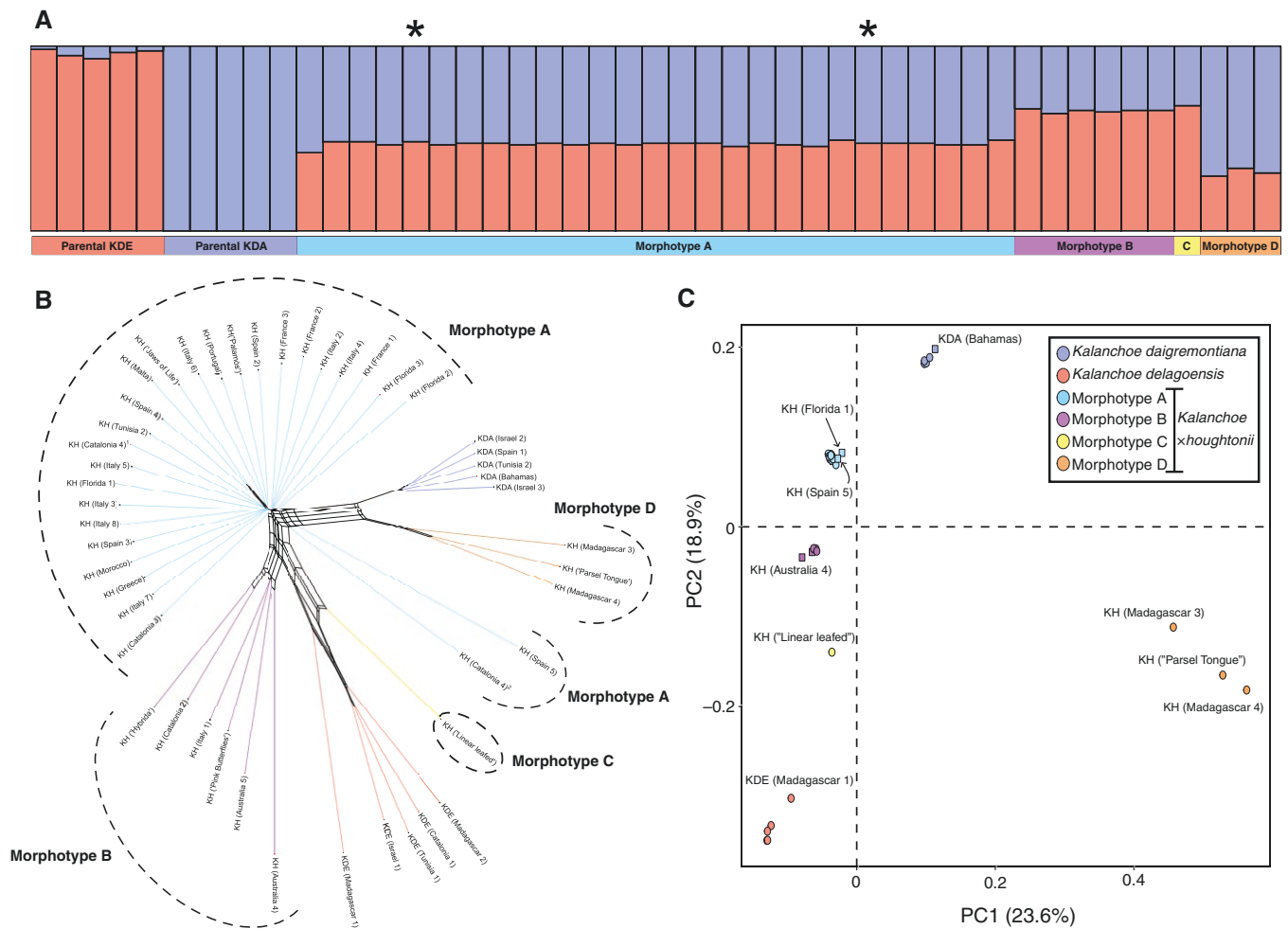


FIG. 4. Nuclear genetic variation and structure within the *Kalanchoe* × *houghtonii* hybrid complex. (A) Assignment to genetic clusters is shown for $K = 2$ based on 9763 bi-allelic SNPs, considering the differential allele dosage in polyploid samples. Technical replicates are indicated with an asterisk. (B) Phylogenetic network based on 1027 BUSCO genes in 47 *Kalanchoe* samples. (C) A principal component analysis across the first two axes based on 11 966 unlinked bi-allelic SNPs. Parental species and the different *Kalanchoe* × *houghtonii* morphotypes are represented in different colours. Herbarium vouchers are indicated with square symbols.

sides of the plot, with the different hybrid taxa falling between them. All samples from morphotype A were placed in the top-left quadrant, clearly separated from the rest of *K. × houghtonii* morphotypes. Samples from morphotypes B and C were more closely related to *K. delagoensis* (bottom-left quadrant), and samples from morphotype D were clearly distanced from the rest of the morphotypes and parental populations (bottom-right quadrant). All samples from each morphotype and parental species were grouped closely, consistently with the results from the population structure and the Neighbor-net analyses. However, some herbarium voucher samples of morphotype A (Florida 1 and Spain 5), morphotype B (Australia 4) and the parental *K. daigremontiana* (Bahamas) were slightly separated from their core group, probably owing to degradation associated with poor DNA preservation. Altogether, these results indicated very low genetic variation within each group, except for *K. delagoensis*, where a sample from Madagascar was clearly separated from the rest, and *K. × houghtonii* morphotype D, where populations appear well separated.

The assessment of clonality is described in Fig. 5, using the shared heterozygosity (SH) index and the IBD analysis. According to the SH index (Fig. 5A), morphotypes A and B showed high signs of clonality (SH index values > 0.90), with the exception of population Florida 1 from morphotype A, for which pairwise comparison values are below the clonality threshold. All pairwise comparisons between populations from morphotype D are below the clonality threshold, showing no evidence of clonality according to SH index. IBD analysis showed similar results (Fig. 5B). The results indicate that all populations of morphotypes A and B would be clonal (IBD values 0.96–0.98 and 0.97–0.99, respectively), excluding Florida 1 from morphotype A (IBD value of 0.94), a sample with significantly degraded DNA signal. Populations of morphotype D have IBD values ranging from 0.79 to 0.85, indicating no clonal origin. Clonality was also detected in both parental species, where all *K. daigremontiana* populations have an IBD value of 0.99, and in *K. delagoensis*, clonality was detected among populations ‘Israel 1’, ‘Tunisia 1’, ‘Madagascar

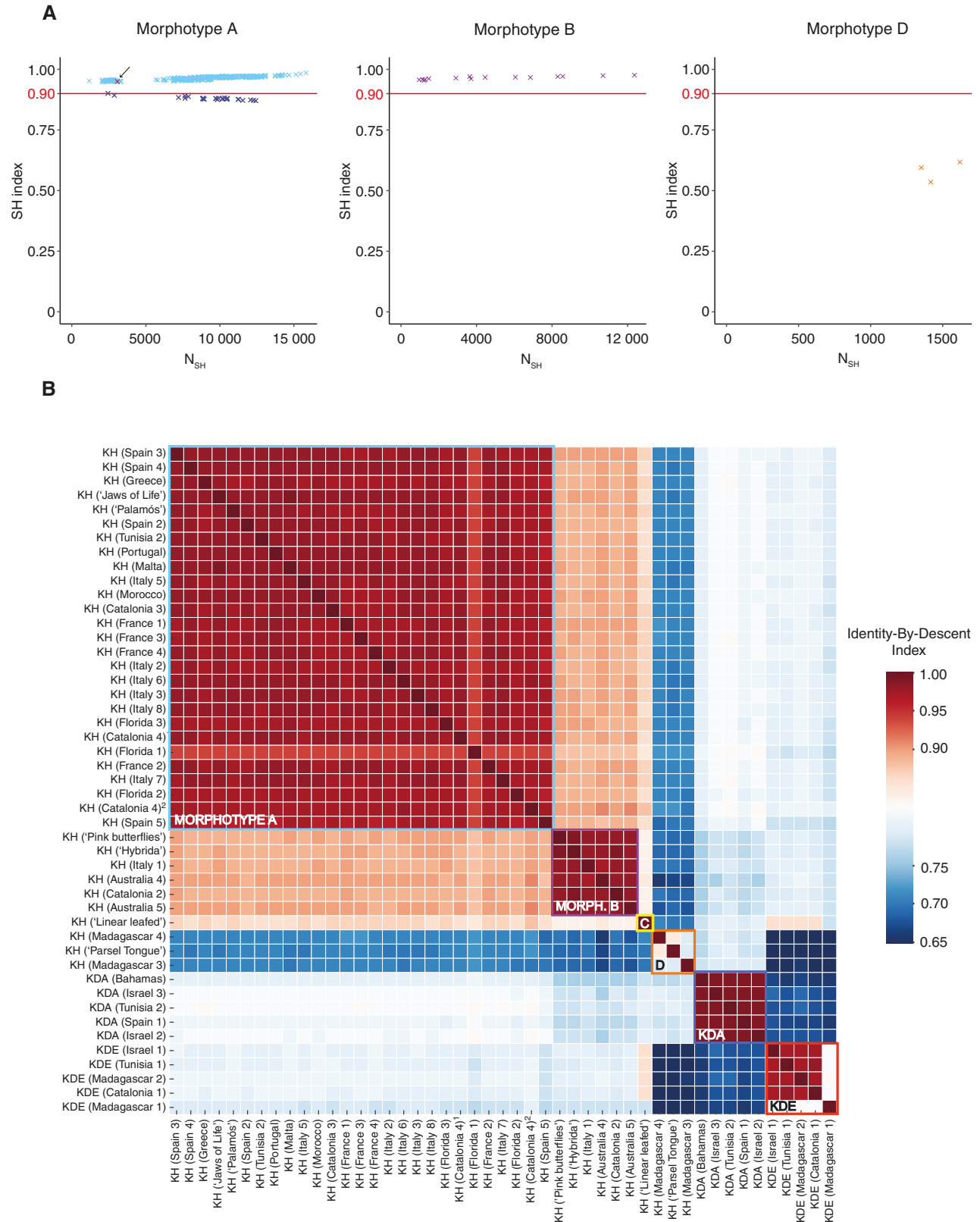


FIG. 5. (A) Detection of clonemate pairs using the shared heterozygosity (SH) index based on 44 055 bi-allelic SNPs. Sample pairs representing technical replicates are marked with red dots and an arrow. In morphotype A, pairs involving population KH (Florida 1) are indicated in dark blue. (B) Clonemate pairs were also detected by the identity-by-descent (IBD) index based on 11 966 unlinked bi-allelic SNPs. The empirical IBD cut-off value of 0.95 is defined to consider clonality between pair comparisons.

2' and 'Catalonia 1' (IBD values 0.97–0.98), whereas in population 'Madagascar 1' clonality was not detected (IBD values 0.82–0.83).

DISCUSSION

Artificial hybridisation in *Kalanchoe × houghtonii*

According to Shaw (2008) and Shtein *et al.* (2021), both morphotypes A (4x) and B (3x) could have been produced artificially more than once on different continents. Various cultivars have also been described for each of these reportedly artificial morphotypes (Guillot *et al.*, 2014; Shtein *et al.*, 2021). In theory, the multiple origins and diverse cultivars within these morphotypes could result in substantial genomic and cytogenetic variability of wild populations. However, our findings suggest that the genetic variability present in populations corresponding to the reportedly artificial morphotypes A and B is surprisingly low. Specifically, plastome sequences (Fig. 3) and nuclear genomic data (Fig. 4; Supplementary Data Fig. S1) indicate that all analysed populations across the Mediterranean Basin, Australia and America from morphotype A [tetraploid, as hypothesized by Shtein *et al.* (2021) and confirmed cytogenetically in this study for the first time (Table 2)] share the same genotype, confirming its presence in the wild at least since 1966 (Australia). To validate these results, we included two technical replicates of population 'Catalonia 4' of morphotype A in our analyses, with the results suggesting that the slight genetic differences observed between populations of morphotype A are probably non-significant and can be attributed to sequencing errors and/or somatic mutations. Our findings are consistent, on a global scale, with those obtained by Guerra-García *et al.* (2015) in a population genetics study on four invasive Mexican populations of *K. × houghtonii*. Using microsatellite markers, these authors found that a single genotype of the hybrid had been introduced successfully and expanded further in the region by clonal growth. In our present study, using genome-level markers and a global sampling, we show that a single clone of *K. × houghtonii* has been able to colonize and invade mild-climate regions in four continents.

Genetic variation and evolution are expected to play an important role in the success of invasive species (Dlugosch and Parker, 2008). Despite the hypothesis that invasive species might experience decreased genetic variation owing to population bottlenecks during colonization events (Hollingsworth and Bailey, 2000; Allendorf and Lundquist, 2003; Poulin *et al.*, 2005; Wang *et al.*, 2005; Li *et al.*, 2006; Roman and Darling, 2007; Lambertini *et al.*, 2010), some invasive alien plant species harbour high genetic diversity (Pappert *et al.*, 2000; Meekins *et al.*, 2001; Maron *et al.*, 2004; Genton *et al.*, 2005; Gutierrez-Ozuno *et al.*, 2009). However, in *K. × houghtonii*, the widespread and aggressive invader morphotype A of our study lacks genomic variability and only a single clonal genotype has been observed in all sampled populations of this morphotype (Figs 4 and 5; Supplementary Data Fig. S1). These findings match the hypothesis of the 'general-purpose-genotype' (Baker, 1955, 1974; Ferrero *et al.*, 2015), which suggests that the most successful colonizer would be the one to thrive without genetic variation, relying instead on a single 'best genotype' capable

of colonizing a wide variety of environments (Dlugosch *et al.*, 2016). Shtein *et al.* (2021) reported that the apparent morphological differences between the cultivars 'Jaws of Life' and 'J.T. Baldwin' of morphotype A disappeared when grown in the same conditions. In our genomic study, we have observed that samples named 'Garbí' and 'J.T. Baldwin' of morphotype A (i.e. those growing wild in Europe or in America, respectively) correspond to the same genotype. Therefore, the reported morphological differences among these cultivars are probably attributable to the wide phenotypic plasticity of this invasive genotype.

Although our results prove that morphotype A has a single origin, its exact source remains unclear. Initially, it had been proposed that morphotype A could have been created by Houghton (1935) or by Resende (1956). Our genomic data shed light on this question, with all genotyped samples of morphotype A displaying the same plastome haplotype found in all analysed *K. daigremontiana* populations, revealing that this species was the maternal species in the cross. Houghton (1935) also reported that the maternal species in the cross between the parental species was *K. daigremontiana*. Yet, clones originating from Houghton's crossings, described as 'identical plants', corresponded to morphotype B. Moreover, samples from Houghton's gardens in San Fernando (California) were later determined cytogenetically as triploids by Baldwin (1949). Resende (1956) reported a single tetraploid clone resulting from the cross between *K. delagoensis* and *K. daigremontiana*, with *K. delagoensis* as the maternal genome donor. Therefore, our results reject the hypothesis that the origin of morphotype A is either the cross reported by Houghton (1935) or by Resende (1956). The source of this morphotype is still a mystery and might be either of artificial (as a result of some crossbreeding that has not been reported) or of natural origin (arising from spontaneous crossings in native or non-native areas where both parentals coexist; see Ward, 2006; Herrando-Moraira *et al.*, 2020), as occurred in Madagascar with morphotypes C and D (see below). The fact that there has not been a report, to our knowledge, of morphotype A plants in Madagascar (neither registered in local herbaria, nor in GBIF or iNaturalist), despite both parental species coexisting in certain areas, argues against the possibility of a natural origin. Besides, crosses between plants of different ploidy levels would often result in failure of endosperm development (Birchler, 2014). Morphotypes B and C are triploid ($2n = 3x = 51$), the most likely expected outcome from a cross between a diploid and a tetraploid. The cytogenetic composition of morphotype A, a tetraploid hybrid ($2n = 4x = 68$), is even more exceptional, because it implies necessarily the presence of a non-disjunct gamete from the diploid genome donor; the resulting hybrid has $n = 34$ from *K. daigremontiana* ($2n = 2x = 34$, $n = 34$ gametes) and $n = 34$ from *K. delagoensis* ($2n = 4x = 68$, $n = 34$ gametes). However, although artificial crossing could have made it easier to produce and select tetraploid plants, the possibility of natural hybridization in localities where both parentals coexist cannot be discarded.

Origin of natural hybrid forms in *Kalanchoe × houghtonii*

Plants representing morphotype C (3x) are difficult to distinguish from *K. delagoensis*, and they occur naturally in

Madagascar, for instance, along the Onilahy River, where the ranges of *K. daigremontiana* and *K. delagoensis* overlap (Shtein et al., 2021). Based on morphological and distribution data, plants of this morphotype have been proposed either to be a closely related non-hybrid taxon showing intermediate characters between the two parental species or to represent a natural hybrid. Our genomic population analysis indicates that plants of morphotype C show the genomic composition expected for a triploid cytotype derived from the cross between these parental species (i.e. one-third of the genome representing the diploid *K. daigremontiana* and two-thirds representing the tetraploid *K. delagoensis*) (Fig. 4A). In morphotype C, the cross between the parental species is reversed in comparison to plants representing morphotypes A and B, where *K. delagoensis* is the maternal genome donor and *K. daigremontiana* the paternal donor. The direction in which a hybrid cross is made has strong consequences for the transcriptional programme of offspring (Joseph et al., 2015; Flood et al., 2020). Maternal effects are based on physiological properties expressed in the mother plant, which are passed on to their progeny (Botet and Keurentjes, 2020). Hence, this switch in the crossing between both parents could explain why plants representing morphotype C are rather morphologically similar to *K. delagoensis* in comparison to plants of morphotype A and B, for which the maternal donor is *K. daigremontiana*.

The last described variant of *K. × houghtonii* is morphotype D, for which we report the diploid ploidy level for the first time (Table 2). According to Shtein et al. (2021), plants assigned to *K. × houghtonii* morphotype D are highly variable, ranging from plants almost indistinguishable from *K. daigremontiana* to plants showing intermediate characters between *K. daigremontiana* and *K. delagoensis*. These plants occur naturally in Madagascar, or in cultivation (e.g. cultivar ‘Parsel Tongue’). Our results suggest that the morphological variability found in plants representative of morphotype D could be related to their genetic and genomic diversity. We detected two different plastome sequence haplotypes corresponding to the parental species *K. delagoensis*, which is the maternal donor of this morphotype. The genetic diversity within the nuclear genome also shows significant differentiation among samples and confirms that *K. daigremontiana* is the major contributor to the genome composition of this morphotype (Figs 4 and 5). Besides, clonality assessments (Fig. 5) confirm that, unlike the other morphotypes, populations of morphotype D are unlikely to have a clonal origin. Hence, both plastome and nuclear data support the hybrid origin of this morphotype. Because of the morphological similarity between *K. daigremontiana* and morphotype D samples, it has been suggested that this morphotype (specifically, the cultivar ‘Parsel Tongue’) might represent a back-cross of *K. daigremontiana* with a fertile cultivar of *K. × houghtonii* ‘J.T. Baldwin’ (Shtein et al., 2021). However, considering that plants of morphotype A are tetraploid (including the cultivar ‘J.T. Baldwin’) and that *K. daigremontiana* plants are diploid, the direct combination of gametes from the tetraploid and the diploid would lead to triploid or tetraploid progeny. Furthermore, the inheritance of the plastome sequence must be considered, because morphotype A samples have *K. daigremontiana* as the maternal plastome donor, whereas plants representing morphotype D have *K. delagoensis* plastome instead. Therefore, the hypothesis of

morphotype D originating as a back-cross of *K. daigremontiana* with a fertile cultivar of *K. × houghtonii* morphotype A can be rejected, because of its diploid ploidy level and the plastome inheritance. According to our results, introgression between *K. daigremontiana* (2x) and an unknown hybrid taxon carrying *K. delagoensis*-like plastome (such as morphotype C, which shows connections with morphotype D in nuclear phylogenomic networks and coexists with it in Madagascar) might be a plausible origin of morphotype D.

Invasiveness of the *Kalanchoe* × *houghtonii* hybrid complex and beyond

Kalanchoe × *houghtonii* is an excellent model to study the effects of hybridization and polyploidy on the emergence of invasiveness. The hybridogenic nature of this complex has been clearly shown; however, this is also common in the genus (Kuligowska et al., 2015), in many cases involving *K. delagoensis* as a parental species (Shtein et al., 2021). For instance, *K. × descoingsii*, a recent hybrid taxon found in the Antsokay Arboretum of Madagascar, has been suggested (based on plant morphology) to be a cross between *K. delagoensis* and *K. laetivirens* (Shtein et al., 2021). This is evident in the ribosomal DNA networks (Supplementary Data Fig. S1), where the 5S rDNA corresponds to that of *K. laetivirens*, whereas the 35S rDNA is closely related to *K. delagoensis*. Besides, our results derived from the plastome sequence analyses suggest that *K. laetivirens* is the maternal genome donor species of *K. × descoingsii* (Fig. 1). This hybrid can also produce many plantlets, in a similar manner to *K. × houghtonii* (Shtein et al., 2021). Based on the ploidy level estimations provided in this study (Table 2), *K. × descoingsii* might be a pentaploid and *K. laetivirens* a hexaploid, consistent with the expected cross between *K. delagoensis* (4x) and *K. laetivirens* (6x).

In the context of global change, with rapidly changing environments and increasingly frequent extreme climatic events, generalist species with high phenotypic plasticity hold a significant biological advantage over specialist species (Clavel et al., 2010). As already proposed in some cases, such as *Spartina anglica* (Ainouche et al., 2009), *Reynoutria japonica* (Wang et al., 2025a, b) and *Reynoutria × bohemica* (Bzdega et al., 2016), or in the invasive algae *Caulerpa taxifolia* (Arnaud-Haond et al., 2013), our study shows that hybridization and polyploidy can be important sources of generalist genotypes, which might eventually displace more specialist taxa (for a review on the genomic mechanisms behind environmental robustness of polyploid species, see Shimizu, 2022). The interaction between clonal growth and climate change has also begun to attract interest (Yu et al., 2016), with the most problematic invasive alien plant species in this context being those that propagate clonally (Pyšek, 1997; Cadotte et al., 2006; Roiloa, 2019). Clonal reproduction, polyploid genomes and absence of genetic diversity seem to be a recipe for successful invasiveness.

Concluding remarks

Altogether, the *K. × houghtonii* complex exemplifies a model for understanding hybrid plant invasions, especially in the context of global change marked by drought, erosion and

rising temperatures (Seneviratne *et al.*, 2021). Its multiple cytotypes, with varying colonizing capacities, high morphological plasticity and resilience to drought and heat, contribute to its invasiveness. Its high rates of clonal reproduction, but, in particular, the presence of a single ‘general purpose genotype’ responsible for its global spread, add to a suite of traits enhancing its invasiveness. The case of the *K. × houghtonii* hybrid complex, whose origin is linked to horticultural practice, advocates for a ‘climate-smart invasive species management’ as suggested by (Colberg *et al.*, 2024). Mitigating the spread of both hybrids and parental species by restricting their sale and transport, implementing preventive actions and establishing a regulatory framework (particularly in countries more sensitive to their invasion, mostly, but not limited to, those of Mediterranean climate) could be crucial for addressing the spread of this complex, which has reached all continents except Antarctica in less than a century.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Table S1: information of all *Kalanchoe* species and populations sampled in this study, including the collectors, the herbarium vouchers, the SRA codes and the GenBank accessions of the plastome and the ribosomal DNA sequences. Table S2: descriptive statistics of the number of recovered genes, guanine–cytosine percentage, mapping details and GenBank accessions of all plastome reconstructions of each *Kalanchoe* population sampled. Table S3: detailed table summarizing target gene recovery efficiency from HybPiper analyses. Table S4: detailed table summarising the statistics from max_overlap pipeline of capture coverage of target sequences rescued by HybPiper. Table S5: detailed alignment statistics provided by AMAS for the preliminary alignment and the subsequent corrected alignment using TreeShrink with two different false-positive tolerance levels (0.01 and 0.05). Table S6: information about the deviance information criterion (DIC) and the log-pointwise predictive density for each cluster (*K*) tested with the ENTROPY analyses. Figure S1: evolutionary relationships based on rDNA among the different *Kalanchoe* × *houghtonii* morphotypes, their parentals and other closely related species and hybrid complexes. Figure S2: principal component analysis across the first and the third axes, with the genetic group coloured based on the *Kalanchoe* taxa. Figure S3: light microscope photographs of metaphase chromosomes of: (a) *K. daigremontiana* ($2n = 4x \approx 34$); (b) *K. × houghtonii* (triploid, $2n = 3x \approx 51$); (c) *K. × houghtonii* (tetraploid, $2n = 4x \approx 68$); (d) *Kalanchoe delagoensis* ($2n = 4x \approx 68$). Figure S4: phylogenomic tree of 50 samples of *Kalanchoe* based on 1122 filtered BUSCO genes, using as outgroup the species *Kalanchoe humifica*.

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AUTHOR CONTRIBUTIONS

S.G. and D.V. conceived the study. J.P.P.-D., J.L.-P., N.N., I.P.L., R.S. and S.G. collected samples. J.P.P.-D., N.B. and L.V. carried out laboratory work. J.P.P.-D., N.B., S.G. and D.V. analysed data. J.P.P.-D., S.G. and D.V. wrote the first version of the manuscript. All authors edited and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY

The data that support the findings of this study are openly available in NCBI at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA944709> number PRJNA944709. All ribosomal RNA genes and the entire plastome sequences are openly available in the NCBI repository GenBank (see [Supplementary Data Table S1](#)).

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