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A mis flores *Rolf* y *Florian*

Abstract

With over 840 species *Erica* L. is one of the largest genera of the Ericaceae, comprising woody perennial plants that occur from Scandinavia to South Africa. According to previous studies, the northern species, present in Europe and the Mediterranean, form a paraphyletic, basal clade, and the southern species, present in South Africa, form a robust monophyletic group. In this work a molecular phylogenetic analysis from European and from Central and South African *Erica* species was performed using the chloroplast regions: *trnL-trnL-trnF* and *5´trnK-matK*, as well as the nuclear DNA marker ITS, in order i) to state the monophyly of the northern and southern species, ii) to determine the phylogenetic relationships between the species and contrasting them with previous systematic research studies and iii) to compare the results provided from nuclear data and explore possible evolutionary patterns. All species were monophyletic except for the widely spread *E. arborea*, and *E. manipuliflora*. The paraphyly of the northern species was also confirmed, but three taxa from Central East Africa were polyphyletic, suggesting different episodes of colonization of this area. The inheritance path of chloroplast markers and the mitochondria region *cox3* from the analysis of a controlled hybrid and its parents was tested. The maternal inheritance of organelles is described for the first time in *Erica*, enabling the hypothesis that incongruences between chloroplast and ITS marker trees may be the result of a reticulate evolution within the genus. An approximation of nuclear DNA amount by the quantification of the relative genome amount via flow cytometry with DAPI was assayed for selected samples, all revealing to have a similar genome size, with the exception of *E. bauera* that seemed to present a higher ploidy level. The vitality test of pollen grains after storage from two *Erica* species was carried out using a FDA solution and applying UV-light fluorescence microscopy techniques with a positive yield of up to two living cells within the pollen tetrad after finalizing the storage period.

Zusammenfassung

Erica L. ist mit mehr als 840 Arten eine der umfangreichsten Gattungen der Ericaceae. *Erica* ist eine immergrüne Pflanze, die sich von Europa bis zu nach Südafrika ausbreitet hat. Bisherige Studien zeigen, dass die nördlichen *Erica*-Arten, d.h. die in Europa und im Mittelmeerraum vorkommenden Arten, eine paraphyletische Gruppe bilden, währenddessen die südlichen Arten als eine monophyletische robuste Gruppe dargestellt werden. In dieser Arbeit wurde eine molekular phylogenetische Analyse der europäischen und zentral- und südafrikanischen *Erica*-Arten durchgeführt. Dabei wurden sowohl chloroplastische als auch nukleare DNA-Marker eingesetzt. Die entsprechenden untersuchten Regionen sind *trnL-trnL-trnF* und *5´trnK-matK*, und ITS. Die Analyse beabsichtigte i) die Feststellung der Monophylie der nördlichen und südlichen *Erica*-Arten, ii) die Bestimmung der phylogenetischen Beziehungen zwischen den Arten und Vergleich gegenüber ehemaligen Studien und iii) die Gegenüberstellung der nuklearen Information, um neue mögliche evolutive Muster zu untersuchen. Alle Arten waren monophyletisch mit der Ausnahme von *E. arborea* und *E. manipuliflora*. Die Paraphylie der nördlichen Arten wurde bestätigt. Allerdings waren drei Taxa aus Zentralafrika polyphyletisch. Dies lässt vermuten, dass verschiedene Besiedlungsperioden stattgefunden haben könnten. Der Vererbungspfad der Chloroplasten-Marker sowie der Mitochondrien-Region *cox3* aus einem kontrollierten Hybrid wurden getestet. Dabei wurde zum ersten Mal die maternale Vererbung der Organellen bei *Erica* beschrieben. Dies ermöglicht die Hypothese, dass Inkongruenzen der Chloroplasten und ITS-Bäume das Resultat einer netzähnlichen Evolution innerhalb der Gattung sein könnte. Die relative Genomgröße wurde via Durchflusszytometrie mit DAPI für selektierte Proben kalkuliert. Alle untersuchten Proben zeigten eine ähnliche Genomgröße mit der Ausnahme von *E. bauera*. Diese hat vermutlich einen höheren Ploidiegrad. Die Vitalität der Pollenkörner nach der Lagerung wurde via Fluoreszenzmikroskopie mit einer FDA-Lösung getestet. Dies ergab ein positives Ergebnis von ca. zwei lebendigen Zellen innerhalb der Pollentetrade nach der Lagerung.

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1 Introduction

Erica L. is a flowering plant (angiosperm) genus that taxonomically belongs to the calcifuge family Ericaceae (Oliver 1989; Luteyn 2002) (Table 1-1). Comprising around 125 genera and 4500 species, Ericaceae is a heliophilic family of acid loving edaphic conditions, presenting a worldwide geographic allocation and hence a corresponding diverse climatic distribution pattern among the temperate and cool tropical regions of all continents, excluding Antarctica (Luteyn 2002). With approximately 840 species, *Erica* is the second largest genera within its family preceded by ca. 1000 *Rhododendron* species including *Azalea*, and followed by *Vaccinium* with ca. 450 species, *Cavendishia* with ca. 130 species and *Gaultheria* with only around 115 species (Luteyn 2002). In present time the genus *Erica* is grouped together with other 23 genera in the monophyletic subfamily Ericoideae (Kron and Chase 1993), which includes five tribes: Bejarieae, Phyllodoceae, Empetreae comprising the genus *Corema* D. Don, Rhododeae, and Ericeae (Kron et al. 2002). The last one containing the genera commonly known as true heaths or heathers: *Calluna* Salisb., *Daboecia* D. Don and *Erica* L. (Oliver 1989). Early classification by Bentham (1839) subdivided the Ericeae tribe in two subtribes: Salaxideae and Euriceae where *Calluna* and *Erica* were included, but not *Daboecia*, which was categorized in the Rhododendroideae subfamily of Ericaceae due to the variance shown to the anatomical ericoid model such as deciduous corolla and septicidal capsule (Oliver 1991). While *Calluna* and *Daboecia* are occurring as monotypic genera, *Erica* presents the largest number of species of the whole subfamily (Fagúndez et al. 2010).

Table 1-1. Taxonomy of the Genus *Erica* (Oliver 2000).

Kingdom	Plantae
Order	Ericales
Family	Ericaceae
Subfamily	Ericoideae
Tribe	Ericeae
Genera	*Erica*, *Calluna*, *Daboecia*

Erica is distributed in whole Europe comprising the Atlantic islands, the Middle East: Lebanon, Syria and Turkey, Yemen in the south-western corner of the Arabian Peninsula, and all the way through the African continent including Madagascar and the Mascarene Islands, showing mainly a vertical dispersion line from Norway to the Cape of Good Hope (Oliver 1989, 1991 and 2000). Twenty different *Erica* species are disseminated in the European and nearby areas including the Middle East and Arabian Peninsula (Ojeda et al. 1998; Fagúndez et al. 2010).

A diversification of around 750 species, meaning an approximately 95% of the total species, is found in a ca. 90.000 km^2 land area of the south western Cape Province of South Africa between latitudes 31° and 34°30´S, with a high level of endemism and considered to be the epitome of floral biodiversity of the fynbos biome (Oliver 1991; Oliver 2000; Goldblatt and Manning 2002). By this the South African Cape flora is assumed to be a biodiversity hot spot outside of the tropics (Segarra-Moragues and Ojeda 2010) and the only place in the world with such a high number of diverse Ericaceae species (Goldblatt and Manning 2002), constituting an extraordinary and unique distribution event in the plant kingdom.

In addition to the described northern and southern heather species, there are several hybrids produced as a consequence of interspecific fertilization when parent plants overlap their growing habitat or geographical distribution. Natural hybrids are already described in South Africa as *E.* × *vinacea* L. Bol. (*E. fastigiata* L. × *E. fervida* L. Bol.) (Oliver 1986), and in the European region: *Erica* × *stuartii* M. T. Masters (*E. tetralix* L. × *E. mackayana* Bab.), *Erica* × veitchii Bean (*E. arborea* L. × *E. lusitanica* Rudolphi.), *Erica* × watsonii Benth. (*E. ciliaris* L. × *E. tetralix* L.), *Erica* × *williamsii* Druce. (*E. vagans* L. × *E. tetralix* L.), and *Erica* × *nelsonii* (*E. tetralix* L. × *E. cinerea*) (Fagúndez 2006, 2012b; Rose 2007; Nelson, 2012). The biogeography of these hybrids comprises mostly western Ireland and Galicia in north-western Spain, Asturias in northern Spain, southern England, north-western France, and the Lizard Peninsula in Cornwall in England, respectively (Fagúndez 2006; Nelson 2012). The artificial hybrid *Erica* × *darleyensis* (*E. carnea* L. × *E. erigena* R. Ross.) is normally a sterile plant but there is also new evidence that some clones are able to produce viable seeds after deliberate cross pollination making it a naturalized hybrid, that is an artificial plant now capable of growing by itself (Nelson 2012).

A linkage of *Erica* species between the European and the African continent is given nowadays mainly by: *Erica arborea*, *Erica australis*, *Erica scoparia*, *Erica ciliaris*, *Erica*

erigena, *Erica multiflora*, *Erica umbellata*, *Erica sicula* and *Erica terminalis* (Oliver 1991, 2000; Amor et al. 1993, Desamoré et al. 2011). The first one trespasses the Sahara desert occurring from Malawi, in the south, to Yemen, in the east, as well as in the Emi Koussi summit of the Tibesti Mountains, a volcanic group in the central Sahara desert located in northern Chad (Oliver 1991). Hansen (1950) already suggested *Erica arborea* as the most primitive European species of its genus and as a possible common ancestor of the whole group. *Erica australis* grows in north and west heathlands of the Iberian Peninsula, comprising Portugal and Spain, and it is also contemporary in northwest Morocco (Amor et al. 1993). *Erica scoparia* is distributed within Macaronesia, south-western Europe and north western Africa (Desamoré et al. 2011).

The tribe Ericeae can be botanically referred as a group of woody perennial shrubs or subshrubs of 0.2-1.5 m high, although some *Erica* species such as *Erica arborea*, *Erica terminalis* and *Erica scoparia* found in southern Europe and tropical regions of Africa may form tree plants that can reach up to 20 m high (Oliver 1991 and 2000). European *Ericas* grow typically in heathlands and moorlands in the north or in maquis and garigue in the south of the continent. Heathlands or moorlands are highly dynamic habitats (Fagúndez 2012a) developed, as a consequence of breeding grazing animals and as a result of the collection of wood and scrub for fire since the Mesolithic age (Rees 1996; Rackham 2003; Simmons 2003). These ecosystems still play an important role in the traditional European cultural landscape (Fagúndez 2012a). Maquis and garigue are shrubland regions along the shore of the Mediterranean and thus much affected by drought (Small and Wulff 2008). These landscapes with characteristic sclerophyllous shrub vegetation present a high canopy mat that is a high content of organic humus, with characteristic acid and low nutrient soil properties, and with low presence of tree plants. As reported by Oliver (1991) *Erica* is sometimes the only shrub capable of growing under such extreme conditions of pure organic matter.

In South Africa, concentrated in the Cape Town Region, *Erica* species are mainly confined to grow in acidic sandstone, quartzitic habitats or humic calcareous soils (Oliver 1991) with arid or semi-arid climatic states at high altitudes under the presence of dense fog, as well as other plants of the Ericaceae family in the Andean Neotropics: Ecuador and Colombia, occurring at Andean altitudes and open moist environment (Luteyn 2002).

According to their geographical habitat, different characteristic botanic features can be noticed for *Erica* and its tribe Ericeae. For example where European *Erica* species have the

faculty to resprout from lignotubers after a fire event occurs; this surviving capacity is rather unusual for South African *Erica* species (Ojeda et al. 1998) which are more prone to be seeders as a post fire response for survival (Segarra-Moragues and Ojeda 2010). Parameters such as predilection for acidic soils, pubescence and plant height can be related to temperate, Mediterranean and Atlantic heaths respectively (Ojeda et al. 1998), being a morphological manifestation of the chorological pattern diversity of heathers in the European territory (Ojeda et al. 1995).

Descriptive morphological aspects of heathers are their typical leaves, inflorescence and fruits. Leaves are 'ericoid' and determined by a narrow, needle like shape and sulcate abaxial surface, with a whorled arrangement, and a waxy layer in the upper epidermis (Oliver 2000; Oliva et al. 2009). Of all heathers only *Erica* presents a rolling-in of the leaf margins, with exception of some species that grow in moist locations and thus present a flat orbicular leaf (Oliver 2000). This kind of leaf morphology is one of many other xerophytic characteristics of plant organs, among other traits, for prevention of water loss for example through transpiration and high light radiation damage (Oliva et al. 2009).

The hermaphrodite flowers in *Erica* species are arranged in inflorescences and consist principally of ovaried flowers with stamens containing usually eight not fused anthers as described by Oliver (2000). Anthers present two appendages, and are so variable that are an important characteristic to discriminate between different species (Schumann et al. 1995). Pollen grains inside the anthers are described either as trizonocolpate or as trizonocolporate and are arranged in tetrads or monads with viscin threads lacking, presenting a range size between 17.4 μm and 82 μm (Oliver 2000).

In *Erica* corollas are persistent and vary in shape, colour and texture (Stevens 1971). While European *Ericas* have bell shape flowers, the South African ones developed a high variation in form (Schumann et al. 1995), what can be associated with a particular pollination syndrome. Tubular, flask, globose, urn, bell, bottle and cup shape, as well as white, red or yellow colours, are all distinctive corolla characteristics that can be found (Schumann et al. 1995). *Ericas* are self-pollinated, cross pollinated or both depending on the species itself, and there are three pollination syndromes described: insect, bird and wind pollinated flowers, leading to marked morphological characteristics (Hagerup and Hagerup 1953; Oliver 1991). As described by Schumann et al. (1995) entomophilous pollinated plants comprise around 80% of the South African species. Flowers tend to be flask, urn, egg, or conical shaped, and

the anthers present appendages. Bird pollinated ones have straight or curved tubes, and bi or tricolour flowers. The wind pollinated plants are considered to be 5% of the total South African species and have small, rounded egg or open mouth corollas.

In the genus *Erica* the fruits are complete dehiscent capsules, with exception of a few species with partially dehiscent or indehiscent fruits (Oliver 2000). The endocarp is sclerified and responsible for the dehiscence behaviour of the fruit (Oliver 2000). Seeds contained in the capsule have been much more studied for the European than the South African *Erica* species. They are normally dark brown to black and may have a size range between 0.3-1.5 mm long x 0.26-0.5 mm wide (Fagúndez and Izco 2004a, 2004b and 2010), where their morphological descriptive characters such as seed coat anatomy can be implemented for taxonomic purposes to explain phylogenetic relations at a subgeneric level.

There are three aspects that can be distinguished where *Erica* plays an important role: the social, the environmental and the economic aspect. Until the eighteen century and even nowadays heathers had primarily a significant value as a basic commodity for human use. They were implemented as a natural resource for the manufacture of brooms and brushes, construction of roofs, as mattress stuffing, source of nectar for honey and beeswax, as fuel for cooking and heating, and for flavouring beverages and foods (Small and Wulff 2008), proving to be of important help as a tool for household and work duties.

The environmental aspect of *Erica* includes the constitution of moorland among other ecosystems, habitat for birds and insects, and also as an important constituent of the food chain as a source of nutrients such as minerals for the heathland fauna (Rees 1996; Small and Wulff 2008). Some species of *Erica* are also considered to be metallophyte or capable of tolerating polluted soils, due to associated mycorrhiza in their root systems (Bradley et al. 1982), such as *Erica andevalensis* and *Erica australis* (Abreu et al. 2008; Oliva et al. 2009). *Erica* may serve as a natural source of decontamination of polluted areas.

Nowadays heathers have also an economic aspect to be considered. They are being domesticated and cultivated for gardening purposes as flowering ornamental plants due to their hardiness, that is their characteristic resistance to survive drought and colder periods as well as nutrient poor soil environments, making them so a considerable option adequate for horticulture (Small and Wulff 2008). Due to their gardening properties heathers have developed a major economic importance as ornamental crops. Thus breeding and production

of heaths has become a gain factor of the horticultural industry. These plants will be breed and produced for the local market in Germany as well as for export in the international market with 110 million *Calluna* and 10 million *Erica* plants cultivated for economic purposes nationwide in the year 2012 (Sondergruppe Azerca, personal communication).

Since the classical review performed by Stevens (1971) there have been important advances about the knowledge of the phylogenetics and systematics of the family Ericaceae. Among them, the use of molecular markers to assess evolutionary relationships has clarified its phylogenetics, leading to a new systematic approach of the family (Kron et al. 2002) and to the differentiation of several taxonomic groups within it like the Ericoideae (Gillespie and Kron 2010). *Erica* L., one of the largest genus of the family, was first investigated by McGuire and Kron (2005) and later in an extensive study by Pirie et al. (2011).

Considering the above mentioned qualities and important facts of *Erica* as a crop as well as a natural component of the environment, it is of biological as well as of horticultural breeding relevance to learn and understand more deeply about the interspecific phylogenetic relationships, among other qualities such as genome DNA quantification and pollen storage to facilitate a possible interspecific cross pollination between European and South African species. This becomes even more important when the systematics of the genus *Erica* is still unresolved (Fagúndez and Izco 2008).

To accomplish the desired analysis of the plants a four step approach was followed in this project. First a molecular phylogenetic analysis of the different *Erica* species was done via standard PCR amplification and subsequently sequencing of chloroplast and nuclear DNA data. Specific primers for markers located in the large single copy region of the chloroplast genome of high evolution rate were used: the *trnL* gene, a group I intron, with a conserved secondary structure that is homologous along land plants, together with the *trnL-trnF* intergenic spacer region that differs in its length and base pair composition (Gielly and Taberlet 1994; Hao et al. 2009), and the *5´trnK-matK*-intron, a group II intron, and *matK* gene, a rapidly evolving and ubiquitous open reading frame located within the *trnK* intron (Hilu et al. 2003; Hausner et al. 2006). Universal primers were implemented for amplification of the ribosomal nuclear marker region ITS, an internal transcribed spacer, consisting of two spacers with valuable characters suitable for phylogenetic studies (Baldwin et al. 1995). The obtained matrices were then implemented for the calculation of the phylogenetic trees to

develop an interpretive framework, based on the evolutionary history of encoded DNA marker regions, and so to depict the relationships of the contemplated species.

Since a hypothesis based on a hybrid origin for some species is presented, in the second step the parental inheritance of mitochondria and chloroplast was tested by analysing one artificial hybrid and its respective parents through standard PCR amplifications and further sequencing of mitochondrial marker region *cox3*, a cytochrome c oxidase subunit III gene, useful for phylogenetic differentiation with high amplification efficiency (Duminil et al. 2002; Tian et al. 2013), together with the already above mentioned chloroplast markers.

In the third step the intact cell nuclei of leaves were isolated, stained and then measured by means of flow cytometry methods to obtain the quantification of the relative total plant nuclear genome. This procedure enables a fast estimation and comparison of differences in genome DNA amount between the given species by analysing fluorescence intensities between the samples (Arumuganathan and Earle 1991). This should enable to indirectly speculate which plants are more prompt to be cross pollinated when their genome size show to be similar.

In the last step the pollen vitality was tested after storage of pollen grains under cold conditions and subsequently staining them for ultraviolet light exposure for the fluorescence microscopic analysis (Pinillos and Cuevas 2008). This method should allow the definition of a possible viable storage time period of the pollen grains and by this to pursue a possible cross pollination between *Erica* species, especially between European and South African species with different seasonal flowering time during the year.

The present work has the aim to create useful information as an approach to clarify the phylogenetic relations within as well as between European and South African *Erica* species. That is to try to explain the north to south origin and the connections of the plant genus. This will provide information for biological purposes for better understanding of the natural environments involved, and on the other hand, in an economic level to directly support cross breeding within and between these plant species, supporting at the same time the sustainability of plant breeders, and so enabling the development of new *Erica* varieties available in the market in the future time.

2 Materials and Methods

2.1 *Erica* Species

For the current study a total of 244 plants of 20 different European, 160 different South African, and three Central East African *Erica* species were analysed. Species names, origin of the sample collection and voucher accession numbers are reported in Table 2-1, 2-2 and 2-3 for the European, South and Central East African plant samples respectively. *Calluna vulgaris* and *Daboecia cantabrica* were used as outgroup of the genus *Erica*, data comprised in Table 2-4. Plant samples were obtained from the collections of: Dr. Jaime Fagúndez from the University of Santiago de Compostela in Spain, the Bundesgarten-Belvedere Vienna in Austria, Kirstenbosch National Botanical Garden from Cape Town in South Africa, Botanic Gardens of the Rheinische Friedrich-Wilhelms-Universität Bonn in Germany, and from the Gartenbauzentrum Straelen in Germany. Voucher specimens are deposited in the SANT herbarium of the University of Santiago de Compostela and are accessible through GBIF (global biodiversity information facility).

Table 2-1. *Erica* species from Europe and Middle East analyzed in this work.

Species	Collection Place / Altitude [m] / Year	Voucher Specimens SANT herbarium / Collector
Erica andevalensis Cabezudo & Rivera	Huelva, Spain/1999	Fagúndez
Erica arborea L.	León, Spain/640 m/2009	Fagúndez
Erica arborea L.	Jaén, Spain//730 m/2009	Fagúndez
Erica arborea L.	Vizcaya, Spain/2004	Fagúndez
Erica arborea L.	Dalmatia, Croatia/2007	Fagúndez
Erica arborea L.	Ciudad Real, Spain/ 640 m/2010	Fagúndez
Erica arborea L.	Ciudad Real, Spain/ 730 m/ 2010	Fagúndez
Erica arborea L.	Thasos, Greece/2007	Carni
Erica arborea L.	La Gomera, Spain/1999	Fagúndez
Erica arborea L.	Madeira, Portugal/2004	Fagúndez
Erica arborea L.	Turkey	AAD 17714
Erica arborea L.	Tenerife, Spain/2010	Kuppler
Erica australis L.	León, Spain/1130 m/ 2009	Fagúndez
Erica australis L.	Jaén, Spain/740 m/ 2009	Fagúndez
Erica carnea L.	Italy/2006	Andrés
Erica carnea L.	BBG	
Erica ciliaris L.	A Coruña, Spain/290 m/ 2009	Fagúndez
Erica ciliaris L.	Lugo, Spain/415 m/ 2009	Fagúndez
Erica ciliaris L.	A Coruña, Spain/280 m/ 2009	Fagúndez
Erica ciliaris L.	Cádiz, Spain/2002	Fagúndez, Reyes
Erica cinerea L.	Lugo, Spain/465m/ 2009	Fagúndez
Erica cinerea L.	A Coruña, Spain/280 m/ 2009	Fagúndez
Erica cinerea L.	A Coruña, Spain/280 m/ 2009	Fagúndez
Erica cinerea L.	Connemara, Ireland/ 40 m/ 2009	Fagúndez
Erica cinerea L.	Connemara, Ireland/ 110 m/ 2009	Fagúndez
Erica erigena R. Ross	A Coruña, Spain/290 m/ 2009	Fagúndez

Table 2-1 continued

Species	Collection Place / Altitude [m] / Year	Voucher Specimens SANT herbarium / Collector
Erica erigena R. Ross	A Coruña, Spain/280 m/ 2009	Fagúndez
Erica erigena R. Ross	Connemara, Ireland/ 40 m/ 2009	Fagúndez
Erica lusitanica subsp. cantabrica Rudolph.	Oviedo, Spain/2004	Fagúndez
Erica lusitanica subsp. cantabrica Rudolph.	Guipuzkoa, Spain/2004	Fagúndez
Erica lusitanica subsp. lusitanica Rudolph.	Ciudad Real, Spain/2000	Fagúndez
Erica lusitanica subsp. lusitanica Rudolph.	Huelva, Spain/1999	Fagúndez, Reyes
Erica mackayana Bab.	Lugo, Spain/465m/ 2009	Fagúndez
Erica mackayana Bab.	A Coruña/280 m/ 2009	Fagúndez
Erica mackayana Bab.	Connemara, Ireland/ 70 m/ 2009	Fagúndez
Erica mackayana Bab.	Connemara, Ireland/ 70 m/ 2009	Fagúndez
Erica mackayana Bab.	Connemara, Ireland/ 70 m/ 2009	Fagúndez
Erica maderensis (Benth.) Bornm.	Madeira, Portugal/1850 m/2004	Fagúndez
Erica maderensis (Benth.) Bornm.	Madeira, Portugal/1800 m/2004	Fagúndez
Erica manipuliflora Salisb.	Dalmacia, Croatia/2007	Fagúndez
Erica manipuliflora Salisb.	Cyprus/230m/2009	Fagúndez
Erica manipuliflora Salisb.	Mar Roukos, Lebanon/2010	Fagúndez, Bou-Daguer
Erica manipuliflora Salisb.	BGV	
Erica multiflora L.	Sicily, Italy/95m/2009	Fagúndez
Erica multiflora L.	Sicily, Italy/640m/2009	Fagúndez
Erica multiflora L.	Valencia, Spain/2000	Fagúndez
Erica platycodon (Webb & Berthel.) S.Rivas-Martínez subsp. *maderincola* (D.C. McClin.) S. Rivas-Martínez et al.	Madeira, Portugal/750 m/2004	Fagúndez
Erica platycodon (Webb & Berthel.) S.Rivas-Martínez subsp. *maderincola* (D.C. McClin.) S. Rivas-Martínez et al.	Madeira, Portugal/1850 m/2004	Fagúndez

Table 2-1 continued

Species	Collection Place / Altitude [m] / Year	Voucher Specimens SANT herbarium / Collector
Erica platycodon (Webb & Berthel.) S.Rivas-Martínez subsp. *platycodon*	Tenerife, Spain/1999	Fagúndez
E. scoparia L. subsp. *azorica* (Hochst.) D. A. Webb	Açores, Portugal/2009	Pene
E. scoparia L. subsp. *azorica* (Hochst.) D.A. Webb	Açores, Portugal/2009	Pene
Erica scoparia L. subsp. *scoparia*	Cádiz, Spain/2002	Fagúndez, Reyes
Erica scoparia L. subsp. *scoparia*	Ciudad Real, Spain/ 730 m/ 2010	Fagúndez
Erica sicula Gussone subsp. *sicula*	Sicily, Italy/160 m/ 2009	Fagúndez
Erica sicula Gussone subsp. *sicula*	Sicily, Italy/250 m/ 2009	Fagúndez
Erica sicula Gussone subsp. *sicula*	Sicily, Italy/240 m/ 2009	Fagúndez
Erica sicula Gussone subsp. *libanotica*	Kyrenia, Cyprus/630 m/ 2009	Fagúndez
Erica sicula Gussone subsp. *libanotica*	Kyrenia, Cyprus/390 m/ 2009	Fagúndez
Erica sicula Gussone subsp. *libanotica*	Kyrenia, Cyprus/420 m/ 2009	Fagúndez
Erica sicula Gussone subsp. *libanotica*	Nahr Ibrahim, Lebanon/ 2010	Fagúndez, Bou-Daguer
Erica sicula Gussone subsp. *libanotica*	Aaqoura, Lebanon / 1440 m/ 2010	Fagúndez, Douaihy
Erica sicula Gussone subsp. *libanotica*	Nahr Ibrahim, Lebanon/2010	Fagúndez, Bou-Daguer
Erica sicula Gussone subsp. *cyrenaica*	Libya	Guichard
Erica sicula Gussone subsp. *libanotica*	Antalya, Turkey/ 1992	Turland
Erica spiculifolia Salisb.	Serbia/2000 m/2005	Lazarevic
Erica spiculifolia Salisb.	Romania/2005	Bita-Nicolae
Erica spiculifolia Salisb.	BGV	
Erica terminalis Salisb.	Valencia, Spain/ 2002	Izco
Erica terminalis Salisb.	Málaga, Spain/ 2002	Fagúndez
Erica tetralix L.	A Coruña, Spain/290 m/ 2009	Fagúndez
Erica tetralix L.	Connemara, Ireland/ 40 m/ 2009	Fagúndez

Table 2-1 continued

Species	Collection Place / Altitude [m] / Year	Voucher Specimens SANT herbarium / Collector
Erica tetralix L.	Connemara, Ireland/ 70 m/ 2009	Fagúndez
Erica tetralix L.	Connemara, Ireland/ 70 m/ 2009	Fagúndez
Erica tetralix L.	Connemara, Ireland/ 110 m/ 2009	Fagúndez
Erica tetralix L.	Ciudad Real, Spain/ 2000	Fagúndez
Erica umbellata L.	Lugo, Spain/465 m/ 2009	Fagúndez
Erica umbellata L.	Toledo, Spain/700 m/ 2000	Fagúndez, Zuazua
Erica vagans L.	A Coruña, Spain/280 m/ 2009	Fagúndez
Erica vagans L.	León, Spain/2010	Fagúndez
Erica × *stuartii* M. T. Masters	Connemara, Ireland/ 70m/ 2009	Fagúndez

BGV: Belvederegarten Vienna

Table 2-2. *Erica* species from South Africa analysed in this work.

Species	Source / Voucher Specimens SANT herbarium
Erica abietina subsp abietina L.	KBG 156/94
Erica acuta Andr.	KBG/ 1402/82
Erica albens L.	KBG/ 790/07
Erica amoena Wendl.	KBG/ 488/94
Erica aneimena Dulfer	KBG/ 974/86
Erica annectens Guth. & Bol.	KBG/ 479/90
Erica banksii Andr.	KBG/ 174/79
Erica bauera Andr.	GBZS/ 40
Erica baueri Andr.	KBG/ 335/05
Erica bergiana L.	BGV/ 83
Erica blancheana L. Bolus	KBG/ 398/88
Erica blandfordia Andr.	KBG 192/04
Erica blenna Salisb.	KBG/ 487/03

Table 2-2 continued

Species	Source / Voucher Specimens SANT herbarium
Erica bolusiae Salter	KBG/ 1466/84
Erica brachialis Salisb.	KBG / 517/85
Erica caffra L.	GBZS / 42
Erica calycina L.	GBZS / 30
Erica cameronii L. Bolus	KBG / 125/72
Erica canescens	KBG / 701/05
Erica capitata L.	KBG / 1467/84
Erica caterviflora Salisb.	KBG / 159/95
Erica cerinthoides L.	BBG / 16917
Erica chamissonis Klotzsch ex Benth.	KBG / 227/04
Erica chrysocodon Guth. & Bol.	KBG / 41/82
Erica clavisepala Guth. & Bol.	KBG / 395/88
Erica coarctata Wendl.	KBG / 611/86
Erica coccinea L.	KBG / 211/75
Erica conica Lodd.	BGV / 92
Erica conspicua Soland.	KBG / 283/72
Erica corifolia L.	KBG / 100/04
Erica cruenta Soland.	BGV / 93
Erica cubica L.	KBG / 342/84
Erica curviflora L.	BGB / 22593
Erica curvirostris	KBG / 177/98
Erica cyanthiformis Salisb.	BGV / 95a
Erica cyanthiformis Salisb.	BGV / 95d
Erica cyrilliflora Salisb.	KBG / 141/07
Erica deflexa Sincl.	KBG / 697/83
Erica demissa Klotzsch ex Benth.	KBG / 241/89
Erica densifolia Willd.	KBG / 157/09
Erica denticulata L.	KBG / AH 1009

Table 2-2 continued

Species	Source / Voucher Specimens SANT herbarium
Erica diaphana Spreng.	KBG / 348/04
Erica dichrus Spreng.	GBZS / 39
Erica discolor Andr.	KBG / 411/03
Erica dodii Guth. & Bol.	KBG / 794/07
Erica doliiformis Salisb.	BBG / 22592
Erica duthieae L. Bolus	KBG / 163/07
Erica elimensis L. Bolus	BGV / 99
Erica empetrina L.	KBG138/95
Erica erasmia	KBG / 426/03
Erica eugenea Dulfer	KBG / 131/94
Erica fairii H. Bolus	KBG62/71
Erica fascicularis L.	BGV / 101
Erica fasciculata Thunb.	GBZS / 27
Erica ferrea Berg.	KBG / 457/82
Erica fimbriata Andr.	KBG / 792/07
Erica foliacea Andr.	KBG / 720/82
Erica fontana L. Bolus	KBG / 61/77
Erica formosa Thunb.	BGV / 102
Erica fourcadei L. Bolus	KBG / 290/07
Erica georgica Guth. & Bol.	BBG / 22594
Erica gibbosa Klotzsch ex Benth.	BGV / 104
Erica glabella subsp. glabella	KBG / 624/75
Erica glandulosa Thunb.	BGV106
Erica glauca var. glauca Andr.	KBG / 94/04
Erica glomiflora Salisb.	KBG / 795/07
Erica gracilis Wendl.	BGV / 107
Erica grandiflora L.	BGV / 108
Erica grata Guth. & Bol.	GBZS / 23

Table 2-2 continued

Species	Source / Voucher Specimens SANT herbarium
Erica haemastoma Wendl.	KBG / 460/82
Erica haematocodon Salter	KBG / 526/87
Erica halicacaba	KBG / 136/07
Erica hebecalyx Benth.	BGV / 110
Erica heleogena Salter	KBG / 140/07
Erica heliophila Guth. & Bol.	GBZS / 25
Erica hirtiflora Curtis	BGV / 112
Erica humifusa Hibbert ex Salisb.	KBG / 813/07
Erica imbricata L.	KBG / 194/04
Erica inflata Thunb.	KBG / 45/09
Erica infundibuliformis Andr.	KBG / 183/07
Erica insolitanthera H. A. Baker	KBG / 242/94
Erica laeta Bartl.	GBZS / 33
Erica leptopus var. leptopus Benth.	KBG / 714/83
Erica leucantha Link	KBG / 864/89
Erica leucotrachela H. A. Baker	KBG / 988/77
Erica longifolia Ait.	BGV / 114
Erica lowryensis H. Bolus	KBG / 482/03
Erica lutea Berg.	KBG / 158/95
Erica mammosa L.	GBZS / 32
Erica margaritaceae Soland.	BGV120
Erica massonii L.	KBG / 47/98
Erica melanthera L.	KBG / 290/94
Erica modesta Salisb.	KBG / 462/87
Erica mollis Andr.	GBZS / 21
Erica monadelphia Andr.	KBG610/83
Erica multumbellifera	KBG / 405/98

Table 2-2 continued

Species	Source / Voucher Specimens SANT herbarium
Erica nabea Guth. & Bol.	KBG / 564/06
Erica nana Salisb.	KBG / 115/74
Erica nevillei L. Bolus	KBG / 86/04
Erica oatesii Rolfe	KBG / 823/89
Erica oblongiflora Benth.	KBG / 4/84
Erica oreotragus E. G. H. Oliv.	KBG / 796/07
Erica ostiaria Compton	KBG / 147/79
Erica pageana L. Bolus	GBZS / 26
Erica parilis Salisb.	KBG / 97/04
Erica parvula Guth. & Bol.	KBG / 544/88
Erica patersonia Andr.	BBG / 112675
Erica patersonii L. Bolus	KBG/ 4/89
Erica peltata Andr.	KBG / 348/09
Erica penicilliformis Salisb.	KBG340/06
Erica perspicua Wendl.	BGV / 123
Erica peziza Lodd.	KBG / 169/79
Erica physodes L.	KBGAH 2311
Erica pillansii H. Bolus	BGV / 124
Erica pubescens L.	BGV / 125
Erica pyxidiflora Salisb.	KBG / 130/07
Erica quadrangularis Salisb.	KBG / 164/07
Erica quadrisulcata L. Bolus	KBG / 543/87
Erica recta H. Bolus	KBG / 211/73
Erica regia subsp. regia Bartl.	KBG / 1613/70
Erica retorta Montin	KBG / 336/84
Erica rubens Thunb.	BGV / 126
Erica scabriuscula Lodd.	KBG / 428/03
Erica serpifolia Andr.	GBZS / 20
Erica sessiliflora L.	BGV / 129

Table 2-2 continued

Species	Source / Voucher Specimens SANT herbarium
Erica shannonii Lodd.	KBG / 607/83
Erica sitiens Klotzsch	KBG / 49/05
Erica sonderiana Guth. & Bol.	KBG / 804/07
Erica sparrmanii L.	KBG / 180/92
Erica sparsa Lodd.	BGV / 130
Erica spectabilis Klotzsch ex Benth.	KBG / 193/84
Erica stokoei L. Bolus	KBG / 62/83
Erica strigilifolia Salisb.	KBG / 249/89
Erica stylaris Spreng.	KBG / 721/83
Erica subdivaricata Berg.	KBG / 655/84
Erica taxifolia Bauer	KBG / 58/82
Erica tenuis Salisb.	KBG/ 446/90
Erica toringbergensis H. A. Baker	KBG / 45/92
Erica totta Bartl.	KBG / 156/95
Erica tragulifera Salisb.	KBG / 349/04
Erica transparens Berg.	KBG / 801/07
Erica transparens Berg.	BGV / 135
Erica triflora L.	BGV / 136
Erica tumida Ker-Gawl.	KBG / 98/04
Erica turgida Salisb.	BGV / 134
Erica uberiflora E. G. H. Oliv. ex. *Simocheilus multiflorus* Klotzsch	KBG / 250/89
Erica urna-viridis H. Bolus	KBG / 192/93
Erica uysii H. A. Baker	KBG / 2/84
Erica vallis-aranearum E. G. H. Oliv.	KBG / 545/84
Erica ventricosa Thunb.	GBZS / 34
Erica verecunda Salisb.	KBG / 230/06
Erica versicolor Andr.	KBG / 197/08
Erica verticilata Berg.	BBG / 17637
Erica vestita Thunb.	KBG / 176/05
Erica viridescens Lodd.	GBZS / 28

Table 2-2 continued

Species	Source / Voucher Specimens SANT herbarium
Erica viridiflora Andr.	KBG / 505/02
Erica viscaria Ait.	KBG / 91/04
Erica walkeriana Sweet	BGV / 140
Erica winteri H. A. Baker	KBG / 343/84
Erica zwartbergensis	KBG / 361/09

BBG: Bonn Botanical Garden; BGV: Belvederegarten Vienna; BZS: Gartenbauzentrum Straelen; KBG: Kirstenbosch South Africa; GBZS: Gartenbauzentrum Starelen

Table 2-3. Analyzed *Erica* species from Central East Africa.

Species	Collection Place / Altitude [m] / Year	Voucher Specimens SANT herbarium / Collector
Erica kingaensis Engl. *subsp. Bequaertii* (De Wild.) R.Ross	Rwenzori, Congo/3000 m/1997	Pulgar
Erica rossii L. J. Dorr	Rwenzori, Congo/3000 m/1997	Pulgar
Erica trimera subsp. trimera (Engl.) H.J.Beentje	Rwenzori, Congo/3500 m/1997	Pulgar

Table 2-4. Genera used as outgroup for *Erica*.

Species	Collection Place / Altitude [m] / Year	Voucher Specimens SANT herbarium / Collector
Calluna Vulgaris (L.) Hull	Pontevedra, Spain/900 m/ 2010	3223 J. Fagúndez
Daboecia cantabrica (Huds.) K. Koch	Pontevedra, Spain/900 m/ 2010	3224 J. Fagúndez

Table 2-5. Species used for parental inheritance analysis of chloroplast and mitochondria organelles.

Species	Collection Place
Erica arborea L. (mother plant)	K. Kramer´s private garden, Germany
Erica carnea L. (father plant)	K. Kramer´s private garden, Germany
E. ×*oldenburgensis* (hybrid)	K. Kramer´s private garden, Germany

2.2 Preparation of plant material for molecular analysis

Herbarium specimen samples as well as fresh plant material were stored in Silica Gel Orange (Roth, Karlsruhe, Germany) for long term preservation at room temperature. 20 mg of silica gel dried leaves were harvested and put in a 2 ml tube. The tubes were then frozen at -80°C for 24 h. Subsequently the frozen leaf samples were fine grinded to a powder using a Qiagen Tissuelyser (Retsch GmbH, Haan, Germany) with 3 mm stainless steel grinding balls for 4 min at a frequency of 30/s.

2.3 Plant DNA extraction

Extraction of nuclear and chloroplast DNA from plant leaves was performed using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following its manufacturer´s protocol. The extracted plant genomic DNA of each sample was then dissolved in 200 µl TE buffer and stored at -20°C.

2.4 Polymerase chain reaction

The nuclear and chloroplast genome regions were amplified from the same accession for each species using a standard PCR method. Amplification reactions for all PCR assays presented a final volume of 50 µl with 1 µl of plant DNA. The amplification solution contained: 1x Go Taq® reaction buffer green (Promega GmbH, Mannheim, Germany), 200 µM of each dATP, dCTP, dGTP and dTTP (Promega GmbH, Mannheim, Germany), 0.40 µM of each

oligonucleotide primer forward and reverse and 1.5 U/µl Go Taq® DNA polymerase (Promega GmbH, Mannheim, Germany). Control tubes without DNA samples were performed as negative controls.

All PCR oligonucleotide primers used in this study were synthesized by biomers.net GmbH, Ulm, Germany, dissolved at 100 µM in sterilized double distilled water, and stored at -20°C.

2.4.1 Chloroplast marker region *trnL-trnL-trnF*

Universal primers trnTc and trnTf developed by Taberlet et al. (1991) were implemented (Table 2-5) for PCR amplification and sequencing of the two chloroplast DNA regions *trnL* intron and *trnL-trnF* spacer together. Location of the *trnL* intron and *trnL-trnF* spacer regions sequenced and position of the primers are shown in Figure 2-2.

Amplification of the assay was performed in a thermal cycler Tgradient thermoblock (Biometra, Göttingen, Germany). The conditions used consisted of a lid temperature of 99°C and a first step at 94°C for 2 min for denaturation followed by 35 cycles consisting of 1 min denaturation at 94°C, annealing at 52°C for 30sec and 2 min at 72°C. At the end a final extension at 72°C for 5 min. Storage at 10°C ran until the samples were collected.

Table 2-6. Primer sequences for detection of *trnL* intron and trnL- *trnF* spacer

Primer	Primer sequence (5´- 3´)	Size of PCR product (bp)	Reference
trnLc	cgaaatcggtagacgctacg	800-900	(Taberlet et al. 1991)
trnFf	atttgaactggtgacacgag		

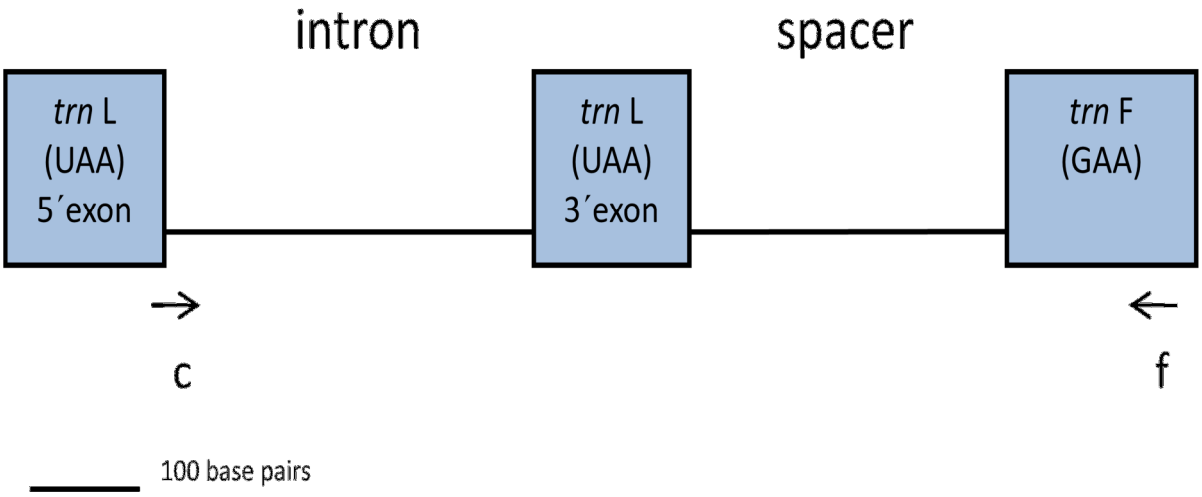


Figure 2-1. cpDNA *trnL* intron and trnL- *trnF* spacer regions. Positions and directions of primers used for amplification and sequencing are shown by arrows. Coding regions are represented as boxes; dark thin lines represent intron and spacer regions.

2.4.2 Chloroplast marker regions *5´trnK-matK*-intron and *matK* gene

The chloroplast regions *5´trnK-matK* intron and part of the *matK* gene were amplified and sequenced with two primer pairs. The forward primer matK6 (Shaw et al. 2005), the new reverse primer matK79 designed in this work for *Erica* species (Table 2-6), and the primers matK1F and matK1600R (McGuire and Kron 2005) (Table 2-7) respectively. Reverse primer matK79 amplified an overlapping region between the gene and the intron which was used to align both region parts as one. Location of *5´trnK-matK* intron and *matK* gene regions sequenced and position of the primers are shown in Figure 2-3.

Amplification of the two assays were performed in a thermal cycler Tgradient thermoblock (Biometra, Göttingen, Germany). The conditions used consisted of a lid temperature of 99°C and a first step at 94°C for 2 min for denaturation followed by 35 cycles consisting of 1 min denaturation at 94°C, annealing at 53°C for 30sec and 2 min at 72°C. At the end a final extension at 72°C for 5 min. Storage at 10°C ran until the samples were collected.

Table 2-7. Primer sequences for detection of *5´trnK-matK*-intron

Primer	Primer sequence (5´- 3´)	Size of PCR product (bp)	Reference
matK6	tgggttgctaactcaatgg	800-900	(Shaw et al. 2005)
matK79R	actcctgaaagataagcga		Designed in this work

Table 2-8. Primer sequences for detection of *matK* gene

Primer	Primer sequence (5´- 3´)	Size of PCR product (bp)	Reference
matK1F	acgaattcatggtccggtgaagtgttcg	800-900	(McGuire and Kron 2005)
matK1600R	tagaattccccggttcgctcgccgttac		

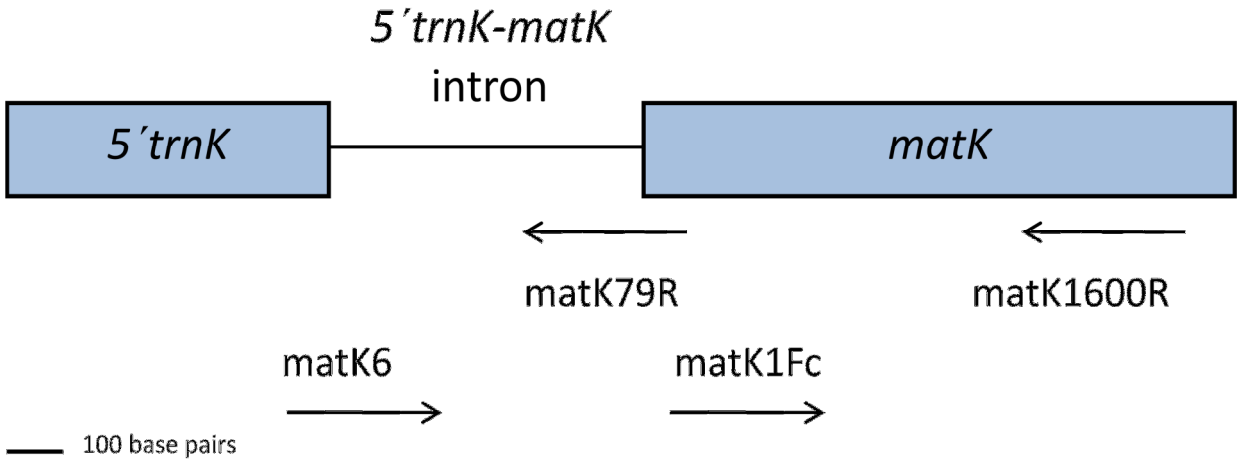


Figure 2-2. cpDNA *5´trnK-matK* intron and *matK* gene regions. Positions and directions of primers used for amplification and sequencing are shown by arrows. Coding regions are represented as boxes; dark thin line represent intron region.

2.4.3 Nuclear ribosomal DNA region

Molecular detection of the ITS region of the plant samples was carried out with a single primer set for the standard PCR as well as for the sequencing process. Primers ITS17se and ITS26se developed by Sun et al. (1994) were used (Table 2-4). Location of the ITS region sequenced and position of the primers are shown in Figure 2-1.

Amplification of the assay was performed in a thermal cycler Tgradient thermoblock (Biometra, Göttingen, Germany). The conditions used consisted of a lid temperature of 99°C and a first step at 94°C for 2 min for denaturation followed by 35 cycles consisting of 1 min denaturation at 94°C, annealing at 68°C for 30sec and 2 min at 72°C. At the end a final extension at 72°C for 5 min. Storage at 10°C ran until the samples were collected.

Table 2-9. Primer sequences for detection of ITS

Primer	Primer sequence (5´- 3´)	Size of PCR product (bp)	Reference
ITS17se	acgaattcatggtccggtgaagtgttcg	900	(Sun et al. 1994)
ITS26se	tagaattccccggttcgctcgccgttac		

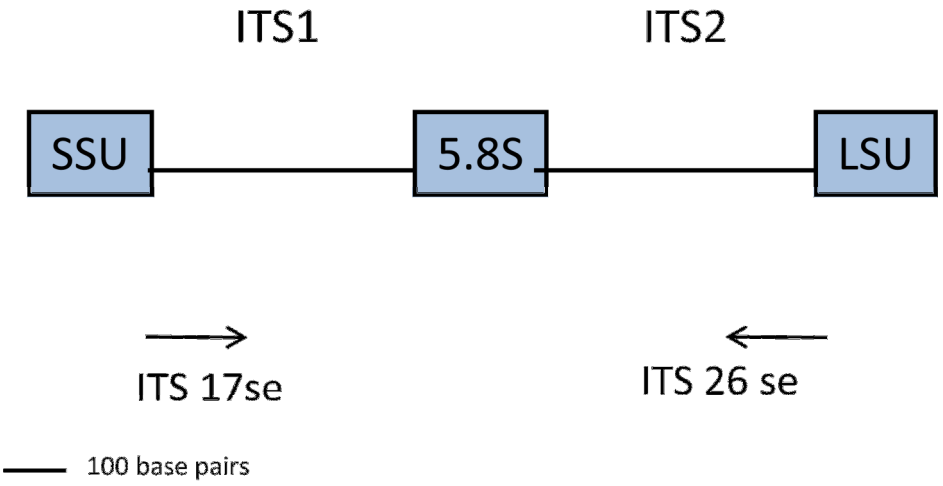


Figure 2-3. nrITS (internal transcribed spacer) region. Positions and directions of primers used for amplification and sequencing are shown by arrows. Coding regions of the ribosomal subunits are represented as boxes; dark thin lines represent ITS spacer regions.

2.4.4 Mitochondrial marker region *cox3*

The mitochondrial marker region cox3 was analysed via standard PCR. Amplification and sequencing was achieved using a single primer set developed by Duminil et al. (2002).

2.5 Gel electrophoresis

All amplified PCR products obtained in this work were separated by electrophoresis on 1% agarose gel (Bio Budget technologies GmbH, Krefeld, Germany) with 1x TAE buffer. TAE buffer mixed with agarose was heated in the microwave for 5 min until the agarose particles completely dissolved, and cooled down to a temperature of about 35 – 40°C. Once polymerized, the gel was placed in the electrophoresis apparatus (Bio-Rad, CA, USA) with the wells near the cathode, so that the negative DNA samples towards the positive anode ran. Per well an aliquot of 15 µl of PCR product was given. To estimate the size of the sample 8 µl of a 1000 bp DNA ladder (GeneRuler™, Fermentas, St. Leon-Rot, Germany) was loaded. The gels ran for about 50 min by 100 V.

After the electrophoresis was finished, the gels were submerged in a 1.5:5 1xTAE - ethidium bromide (Serva Electrophoresis GmbH, Heidelberg, Germany) solution for 20 min. Ethidium bromide has the capacity to intercalate nucleic acids and to fluoresce orange when exposed to ultraviolet light, allowing the amplified DNA samples to be detected and analyzed.

At the end the gels were viewed under ultraviolet light to analyze the results. Black and white pictures were done with a digital camera to record the data.

2.6 DNA sequencing

All PCR amplified products were cleaned up with ExoSAP-IT® (USB/Affymetrix Inc., High Wycombe, United Kingdom) previously diluted in distilled water until reaching a concentration of 1:1. Afterwards the clean-up protocol was completed as described by the manufacturer. The already cleaned up PCR products were then sent for sequencing to the companies Macrogen Inc. (Seoul, Korea), Eurofins MWG GmbH (Ebersberg, Germany), and Sequiserve GmbH (Vaterstetten, Germany) which also sequenced and edited the sequences of the hybrid *Erica x Stuartii*, allowing the detection of both paternal sequence information.

2.7 DNA sequence alignment

Raw DNA sequences obtained from the nuclear and chloroplast genome of the analyzed plants were edited and aligned using the software Geneious 5.0.4 (2013) and manually corrected to create the corresponding contiguous sequences (contigs) of each plant species. Contigs sequences were trimmed at the beginning and at the end, when presenting not readable parts. Only unambiguous sequences were aligned, so that cloning was not necessary to be conducted. Subsequently the compiled alignments of the different DNA regions were generated for the further calculation of the phylogenetic trees.

2.8 Phylogenetic analysis

Matrices of the aligned DNA sequences of the different examined regions were implemented for the calculation of the phylogenetic trees. The chloroplast DNA regions data sets: *trnL-trnL-trnF* and *5´trnK-matK* intron and *matK* gene were first treated individually and then combined in a concatenated alignment as one data set to create the different phylogenetic trees. Data matrix of nuclear ribosomal region ITS was treated separately, to create its own phylogenetic tree.

Phylogeny was inferred using maximum parsimony analysis performed with the software PAUP* 4.0b10 (Swofford 2002). The strict consensus tree was calculated performing parsimony Ratchet algorithm (Nixon 1999) generated by Prap2 (Müller 2004) employing 10 random addition cycles of 200 replicates with 25% upweighting to 2 of the characters in the iterations, with command file for PAUP* 4.0b10. Heuristic searches were conducted for most parsimonious analysis with the following setting options: with unordered and unweighted characters, that is all positions were stated to be equally likely to change and all characters changes were assumed to be equally likely to occur, 100 random replicates, TBR (tree-bisection-reconnection) branch swapping, ignoring uninformative characters, and alignment gaps treated as missing data. Internal support of branches was assessed by bootstrapping (Felsenstein 1985) with 1000 random replicates. Decay values for further approach of support of the tree topology were calculated using PRAP2 and PAUP* 4.0b10 with setting options implemented in the ratchet. Phylogenetic trees were outgrouped with the Ericoideae genera

Daboecia cantabrica and *Calluna vulgaris*. Generated trees were arranged and plotted with the software TreeGraph2 (Stöver and Müller 2010).

2.9 Test for data congruence

Congruence of chloroplast and nuclear DNA data matrices was tested manually for each topological node as well as with the incongruence length difference test (ILD) (Faris et al. 1995). The probability threshold (*p* value) to reject the null hypothesis was set to $p > 0.05$. The ILD test was calculated in Paup* (Swofford 2002).

2.10 Parental inheritance of mitochondria and chloroplast organelles

Resulted DNA sequence alignments of mitochondrial region *cox3* and chloroplast regions *trnL-trnL-trnF* and *matK* gene were manually compared base by base with each other. This enabled the determination of sequence array similarity of the hybrid with the corresponding sequence array of the parent.

2.11 High-resolution flow cytometry

Flow cytometry was implemented as a method for relative quantification of total plant nuclear genome of selected South African and European *Erica* plant species. Procedure was performed as described below.

2.11.1 Nuclei isolation and staining

Cell nuclei were extracted from new sprout shoots of ca. 1.5 cm long using CyStain UV precise P kit (Partec, Germany). Plant shoots were cut into small pieces with a scalpel in Petri dishes containing 800 µl of nuclei extraction buffer, and incubated at room temperature for 1 hour. The samples were then filtrated through a 50 µm CellTrics disposable filter (Partec,

Germany), stain with 1.6 ml staining solution and kept in the dark approximately 1 hour until their subsequently measurement.

2.11.2 Nuclei analysis

Measurement was performed at the facilities of the Institute for molecular medicine and experimental immunology (Univeritätsklinikum Bonn, Germany) in a BD FACSCanto II flow cytometer (BD Biosciences, Germany). 405 nm violet Laser was used for excitation and a 450/40 nm band pass filter was required for recording the DAPI fluorescence. The relative total genome amount was represented by the mean peak position in a DAPI fluorescence intensity histogram. The measurement of the analysed plant species was done in two times due to irregular acquisition of samples in the wild. Each time a sample species used for the previous test was used as a reference for comparison. Data was featured using Flowjo analysis software version 9.3.1 (http://www.flowjo.com).

2.12 Pollen storage and vitality test

Pollen vitality of *Erica* species was tested after a total time storage period of six months. This analysis was achieved by a fluorescence microscopy technique describe below.

2.12.1 Pollen material and storage

Pollen was obtained from flowers cultivated in the greenhouse that were full developed, when corolla and internal organs reach their normal adult morphology (Schumann et al. 1995). Anthers were separated from the flowers and dried in open 2 ml single tubes (Eppendorf, Hamburg, Germany) in an exsicator filled with silica gel (Roth, Karlsruhe, Germany) at 5°C for 1 week. Afterwards the tubes were sealed and stored at -20°C for a total period of time of approximately six months.

2.12.2 Stereo microscope techniques

Pollen grains were observed under a stereo microscope Leica MZ16F (Wetzlar, Germany) with a digital camera JVC 3CCD ky-F75U (Japan) before the store procedure started. This enabled there storage and documentation in pictures of their morphological aspects such as shape and size. Pictures were viewed with the software Diskus v. 4.80.6346 (Königswinter, Germany).

2.12.3 Fluorescence microscope techniques

Pollen vitality was tested under the microscope Leica Leitz DMRB (Wetzlar, Germany) with a digital camera Hitachi HV-20A (Japan), applying fluorescence methods. Fluorescein diacetate (FDA) (Sigma-Aldrich, USA) at an excitation wavelength of 450 to 490 nm was used to test the quality of the pollen grains. FDA was prepared before use in a stock solution in acetone at 2 $mgml^{-1}$ (Heslop-Harrison et al. 1984). The working solution for this assay had a concentration of 6 x 10^{-5} M FDA in 0.5 sucrose (Pinillos and Cuevas 2008). Pictures were viewed with the software Diskus v. 4.80.6346 Fluorescence HV20 (Königswinter, Germany).

3 Results

The main purpose of this study is to clarify the phylogenetic relationship at a species level of the genus *Erica* principally using European taxa as well as including a representative group of species with an African origin, that is from Central and South Africa. To reach this objective, molecular phylogenetic assays based on nuclear and chloroplast DNA regions with specific primer pairs and subsequently sequencing were carried out, enabling the calculation of the respective trees based on the created data matrices. Chloroplast and mitochondria inheritance in *Erica* was molecular analysed, elucidating their parental origin for the given species. Eighteen *Erica* species were selected and their relative total DNA was measured applying flow cytometry. At the end two different *Erica* plant species were chosen to test their pollen vitality through fluorescence microscopic methods.

3.1 Analysis of combined chloroplast data

Combined sequences of total aligned and concatenated chloroplast regions data set: *trnL-trnL-trnF*, *5´trnK-matK*-intron and *matK* gene of 244 taxa samples, resulted in a matrix length of 2738 total characters, with 1898 characters being constant, 351 variable characters parsimony uninformative, and 489 characters parsimony informative. Displayed in Figure 3-1 is the strict consensus tree of 1723 equally parsimonious trees found.

The tree was highly resolved with strong support in the nodes with a bootstrap proportion of 100%, and decay value of 3 indicating the monophyly of the clade of the European and African *Ericas* compared to the outgroup genera. The hybrid *Erica x Stuartii* was grouped together with the *Erica tetralix* clade with a strong support confirmed by a bootstrap proportion of 100% and decay value of 6 for the clade node.

The concatenated alignment had 980 (35.8%) identical sites, with a pairwise identity of 85.9%. Ungapped lengths of total sequences showed a mean of 2191.7 bases long. Insertions/deletions (indels) were 1-20 bp large. Most indels were in the non-conservative DNA of the respective chloroplast regions studied. GC content for the analysed chloroplast region was 25.9% for the total taxa. Polymorphism search under the parameters of a minimum

coverage of 1 read and minimum variant frequency of 0.25 per read resulted in a transition/transversion ratio of 1.

3.2 Analysis of nuclear data

Aligned ITS sequences of 242 taxa samples resulted in a matrix length of 945 total characters, with 522 characters being constant, 142 variable characters parsimony uninformative, and 281 characters parsimony informative. Displayed in Figure 3-2 is the strict consensus tree of 86 equally parsimonious trees found after the search.

The tree was highly resolved with strong support in the nodes with a bootstrap proportion of 99%, and decay value of 14 indicating the monophyly of the clade of the European and African *Ericas* compared to the outgroup genera. From the hybrid *Erica x Stuartii* two ITS sequences were obtained from the same individual, meaning that not different samples were used for this purpose. One sequence of *Erica x Stuartii* was grouped together with the *Erica mackayana* clade and the other one clustered together with the *Erica tetralix* clade showing a strong support confirmed by bootstrap proportions of 90% and 100% and decay values of 3 and 7 for the clade nodes respectively.

The alignment presented 351 (37.1%) identical sites, with a pairwise identity of 94.9%. Ungapped lengths of total sequences showed a mean of 836.6 bases long. Insertions/deletions (indels) were 1-7 bp large. Most indels were in the regions between 130-400 bp and 600-880 bp; in between conserved regions were observed. GC content of the ITS region was 45.5% for the total taxa. Polymorphism search under the parameters of a minimum coverage of 1 read and minimum variant frequency of 0.25 per read resulted in a transition/transversion ratio of 5.75.

3.3 Incongruence length difference test

ILD test resulted in a low probability ($p=0.01$) accepting the null hypothesis, that is the overall topology of both nuclear and chloroplast trees is significantly incongruent, indicating that the observed data cannot be merged together in a single tree. As well incongruence patterns were tested manually by comparing both trees, leading to the decision of not merging

them, due to important differences located in the node topologies which can be seen in Figures 3-1 and 3-2 and discussed in the next chapter.

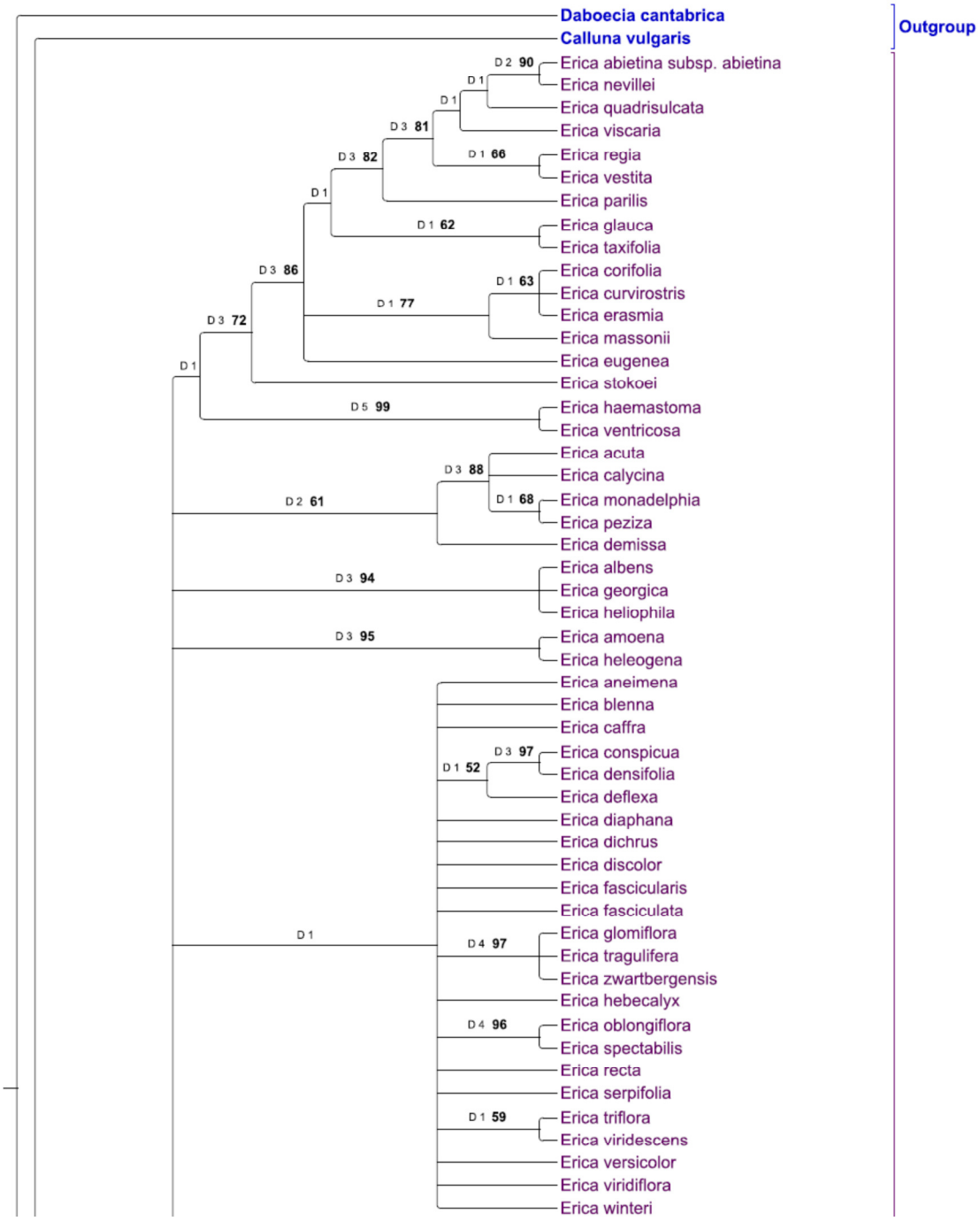


Figure 3-1. Strict consensus of 1723 most parsimonious trees of chloroplast data (Length=1446, CI=0.704, RI=0.871) with decay (D) and bootstrap (bold) values.

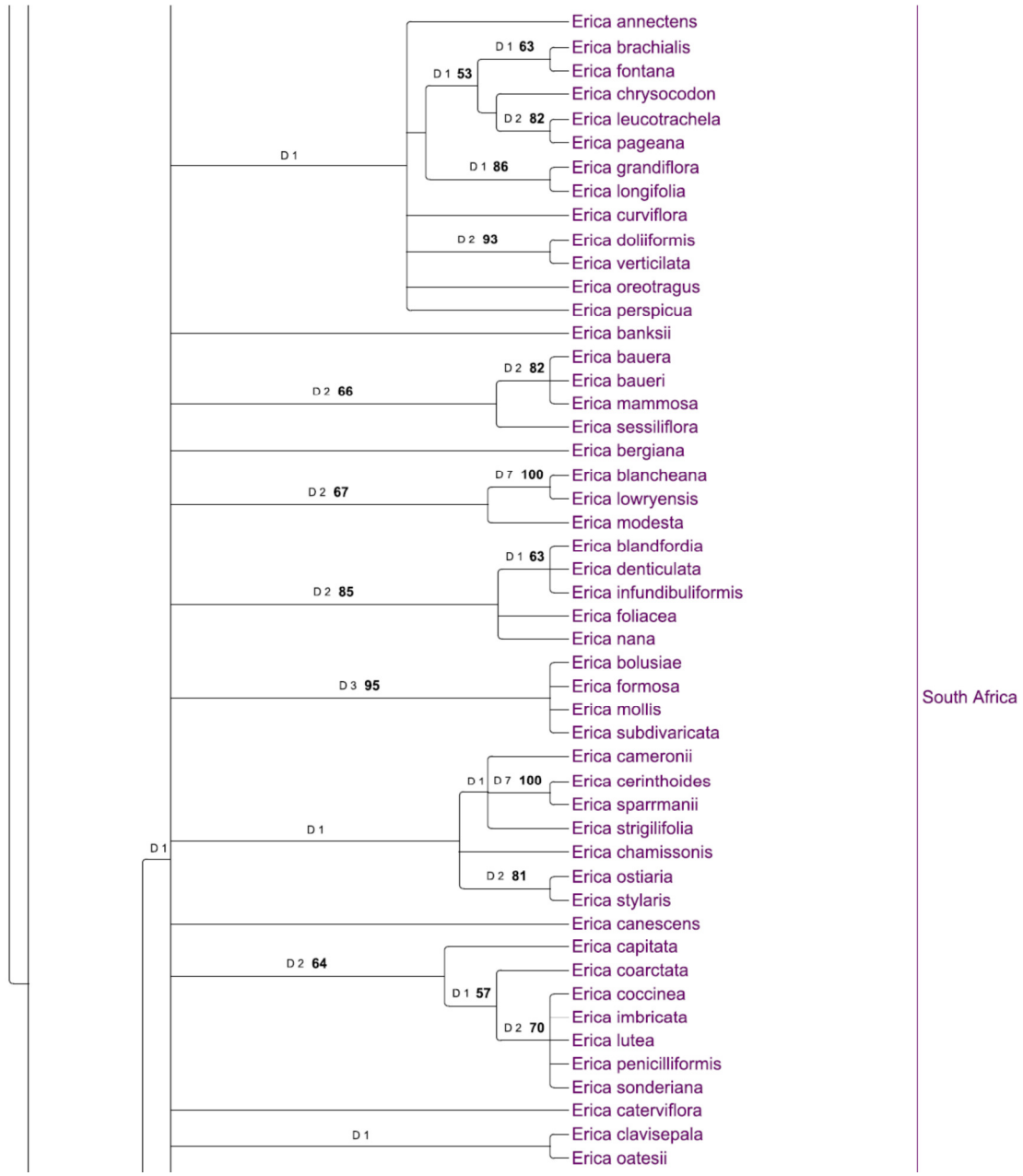


Figure 3-1. (Continued) Strict consensus of 1723 most parsimonious trees of chloroplast data (Length=1446, CI=0.704, RI=0.871) with decay (D) and bootstrap (bold) values.

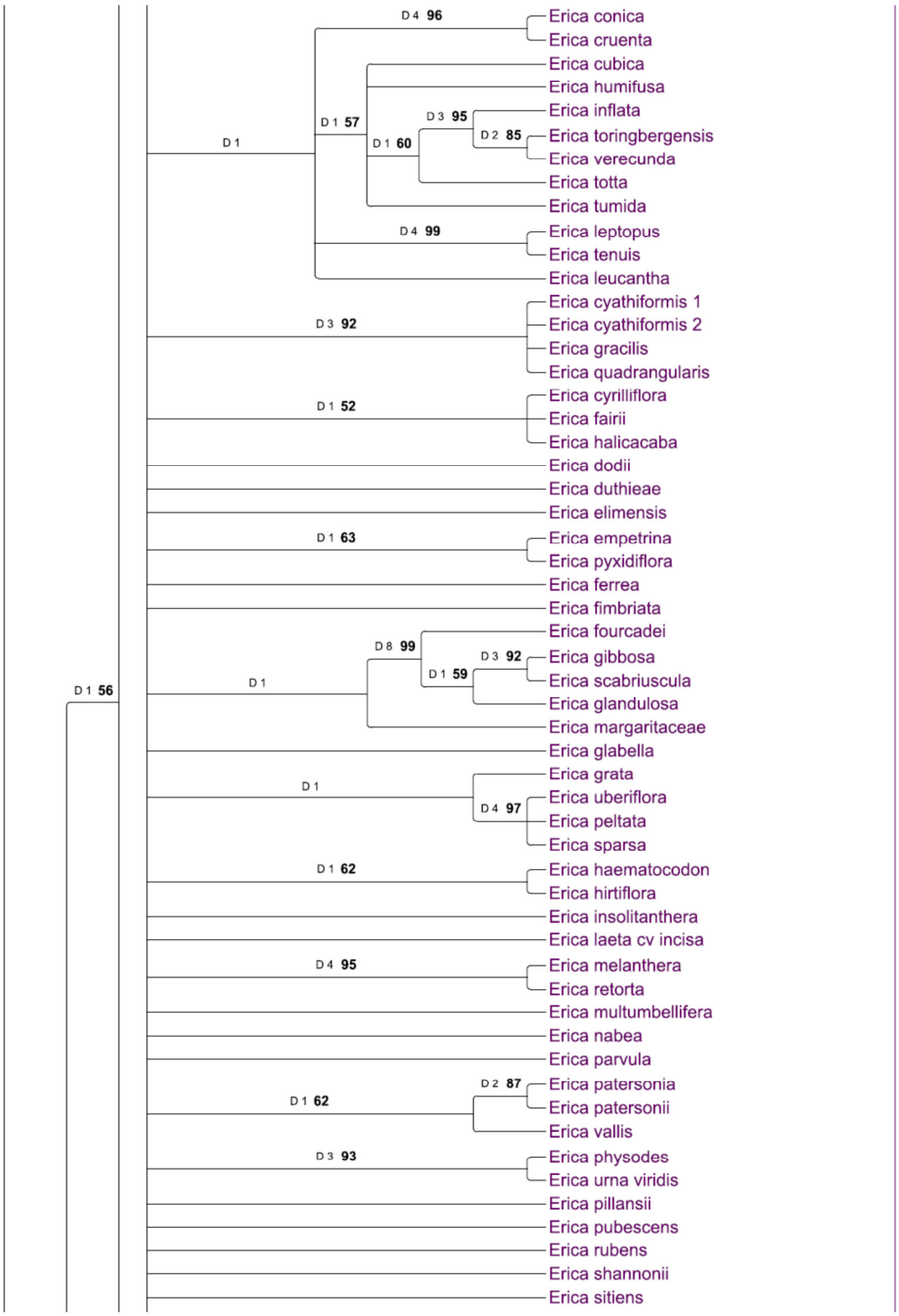


Figure 3-1. (Continued) Strict consensus of 1723 most parsimonious trees of chloroplast data (Length=1446, CI=0.704, RI=0.871) with decay (D) and bootstrap (bold) values.

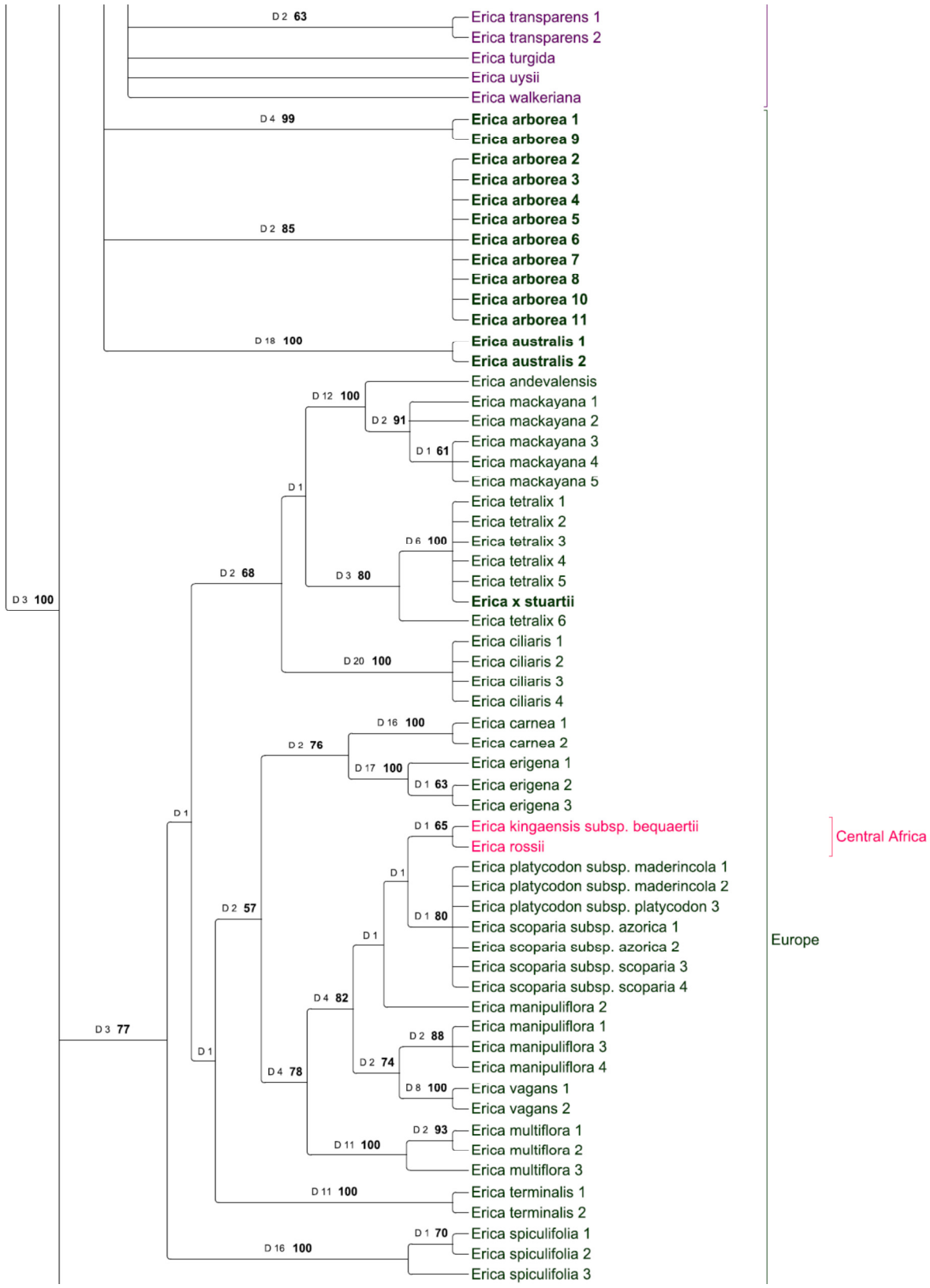


Figure 3-1. (Continued) Strict consensus of 1723 most parsimonious trees of chloroplast data (Length=1446, CI=0.704, RI=0.871) with decay (D) and bootstrap (bold) values.

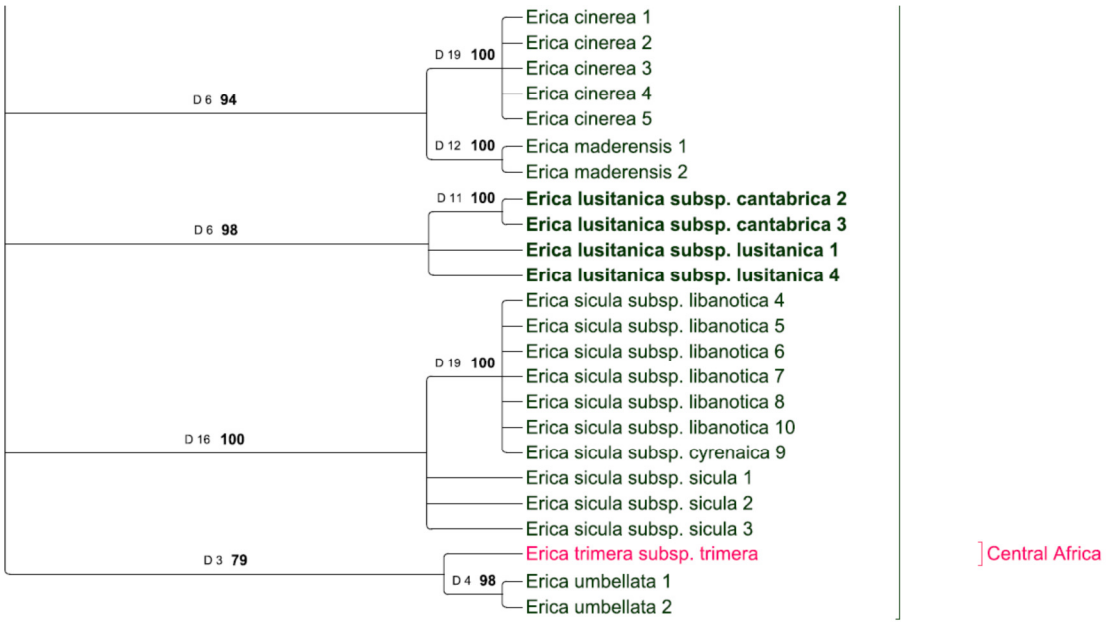


Figure 3-1. (Continued) Strict consensus of 1723 most parsimonious trees of chloroplast data (Length=1446, CI=0.704, RI=0.871) with decay (D) and bootstrap (bold) values.

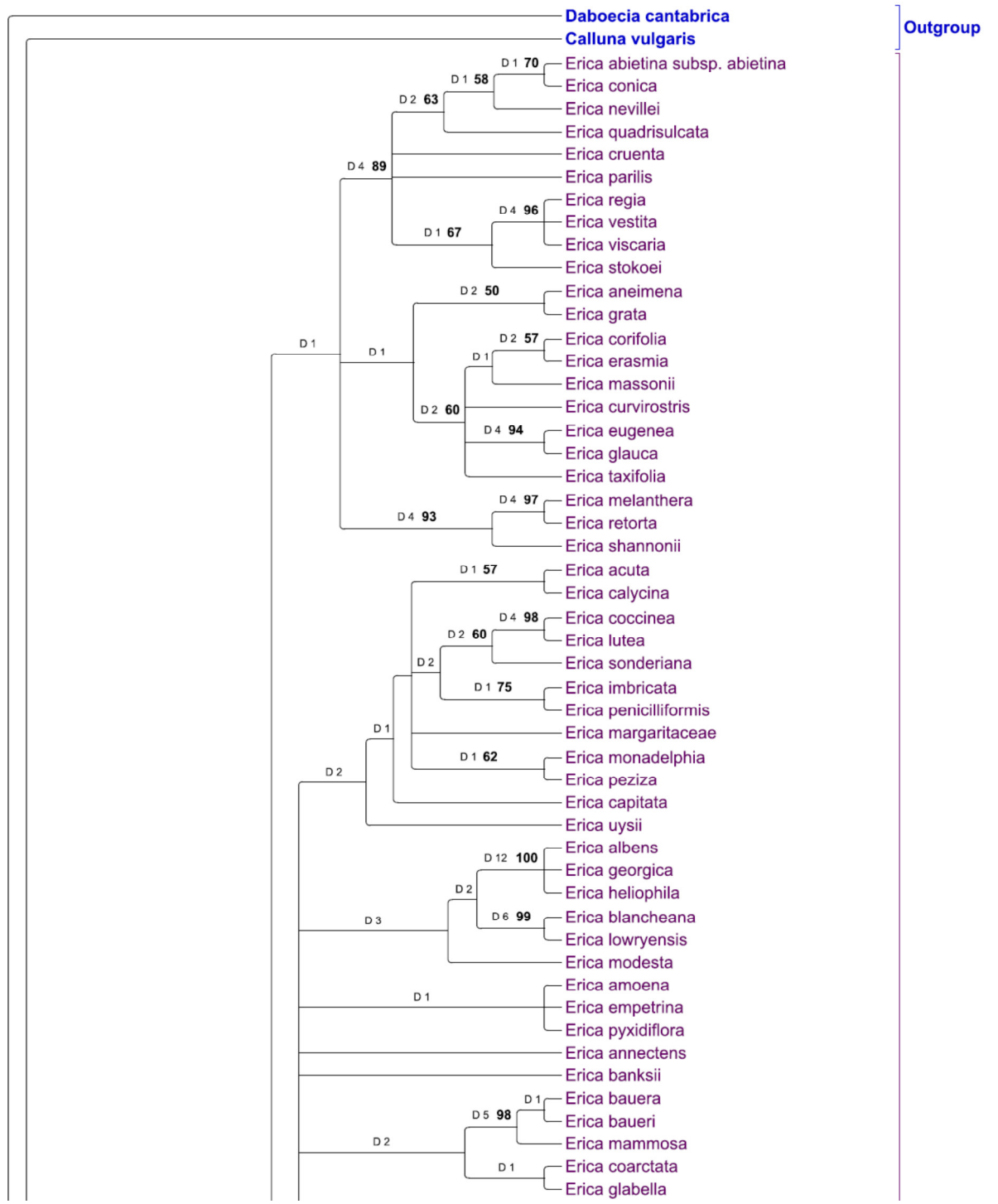


Figure 3-2. Strict consensus of 86 most parsimonious trees of nuclear data (Length=1278, CI=0.479, RI=0.846 with decay (D) and bootstrap (bold) values.

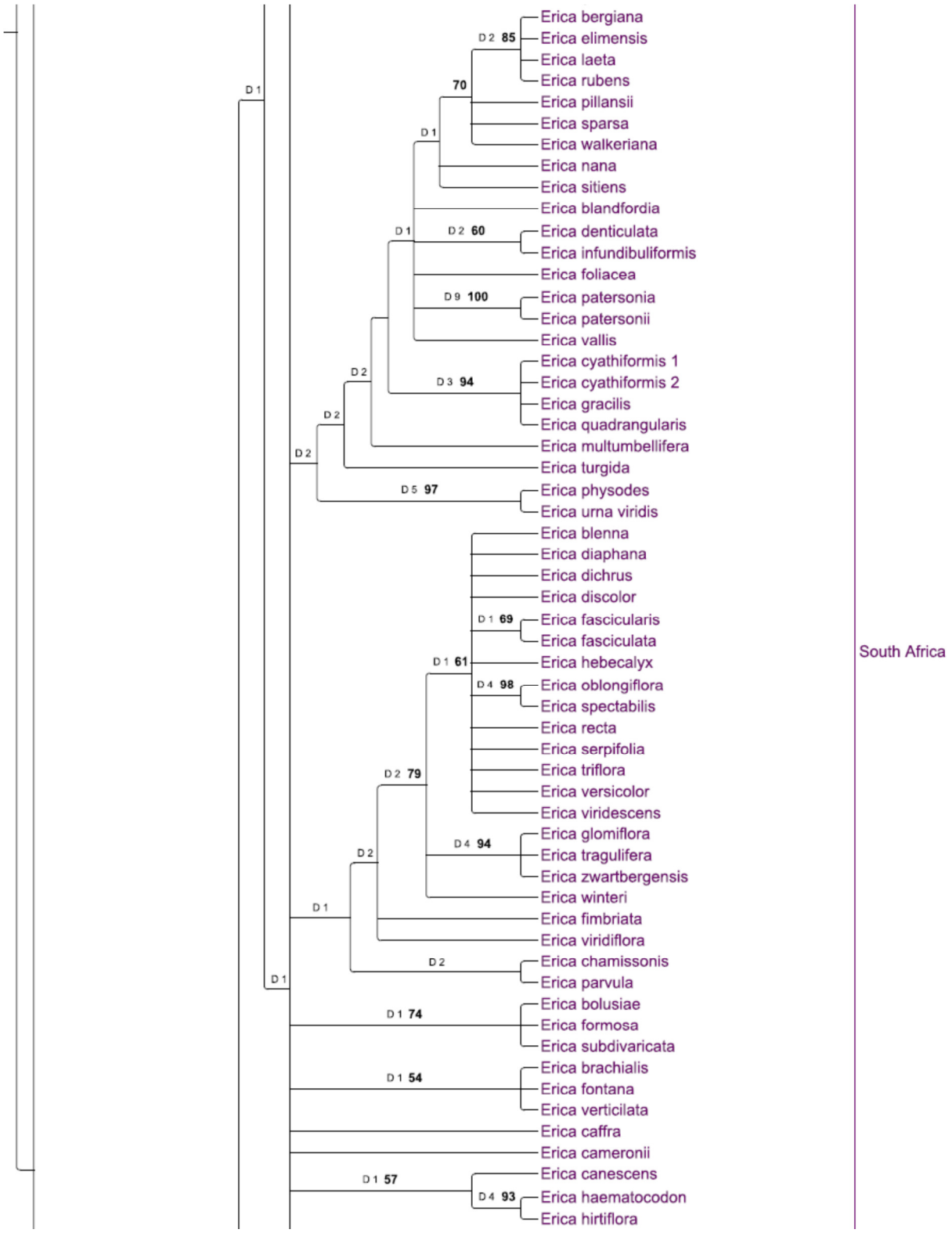


Figure 3-2. (Continued) Strict consensus of 86 most parsimonious trees of nuclear data (Length=1278, CI=0.479, RI=0.846 with decay (D) and bootstrap (bold) values.

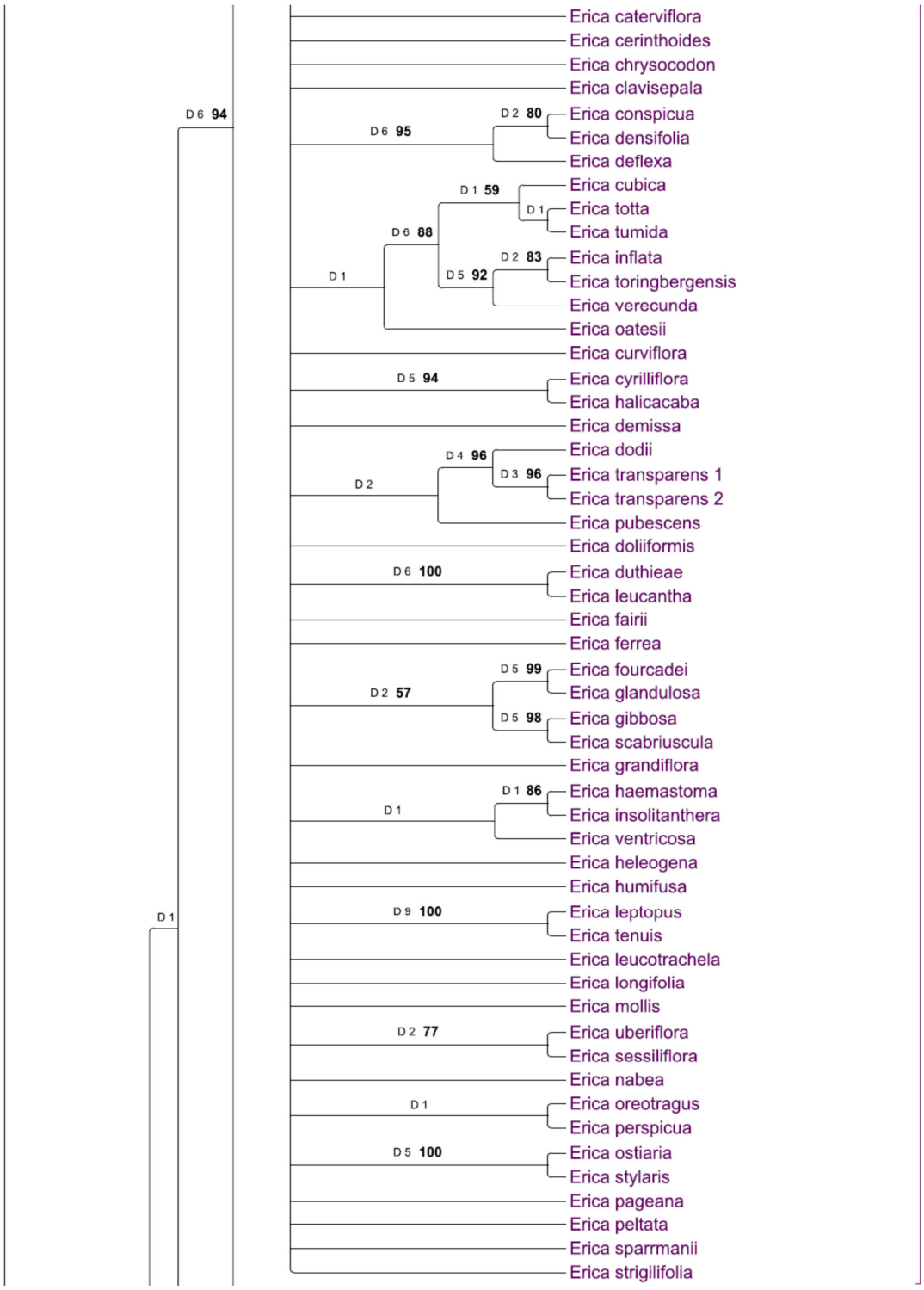


Figure 3-2. (Continued) Strict consensus of 86 most parsimonious trees of nuclear data (Length=1278, CI=0.479, RI=0.846 with decay (D) and bootstrap (bold) values.

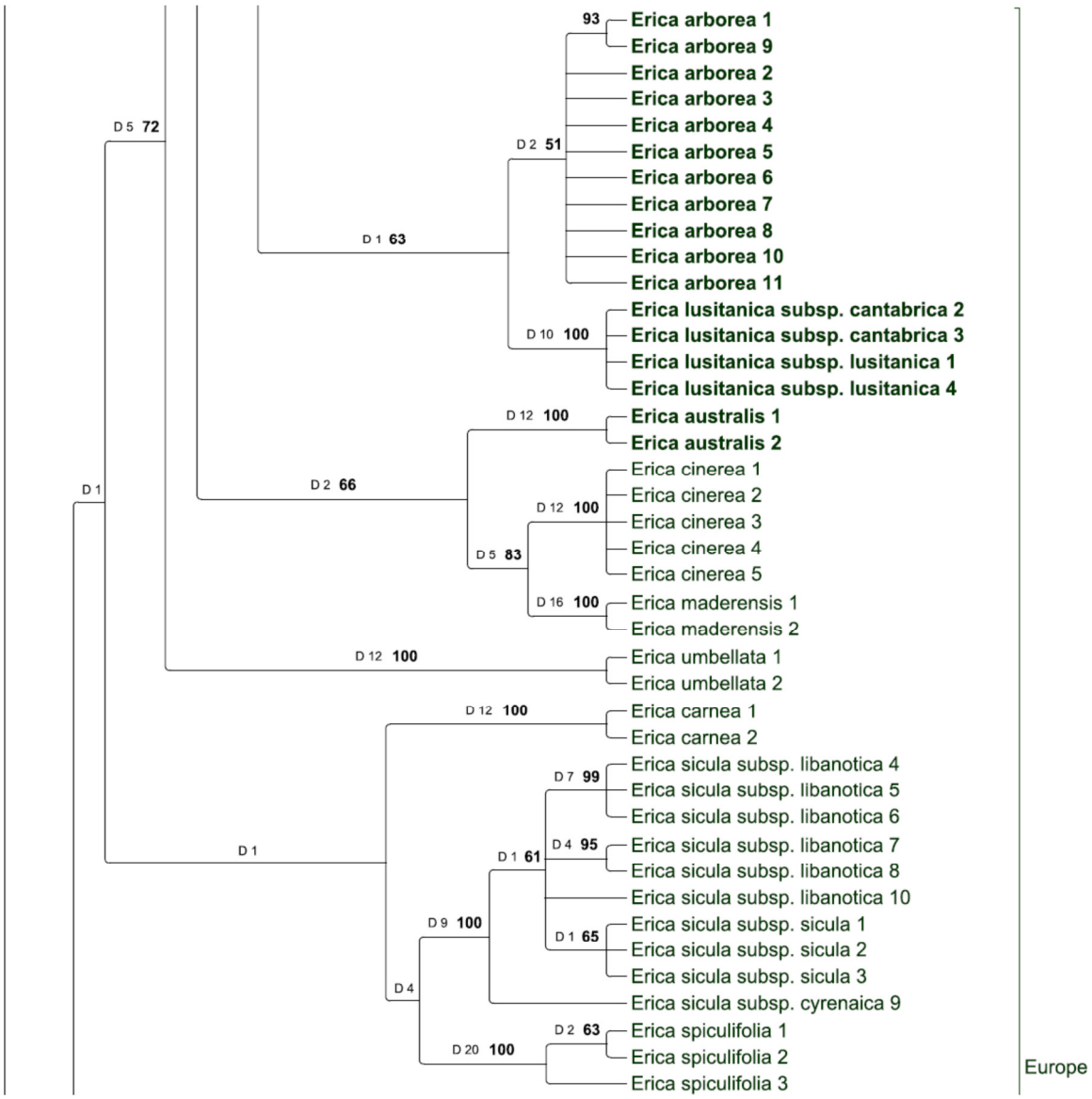


Figure 3-2. (Continued) Strict consensus of 86 most parsimonious trees of nuclear data (Length=1278, CI=0.479, RI=0.846 with decay (D) and bootstrap (bold) values.

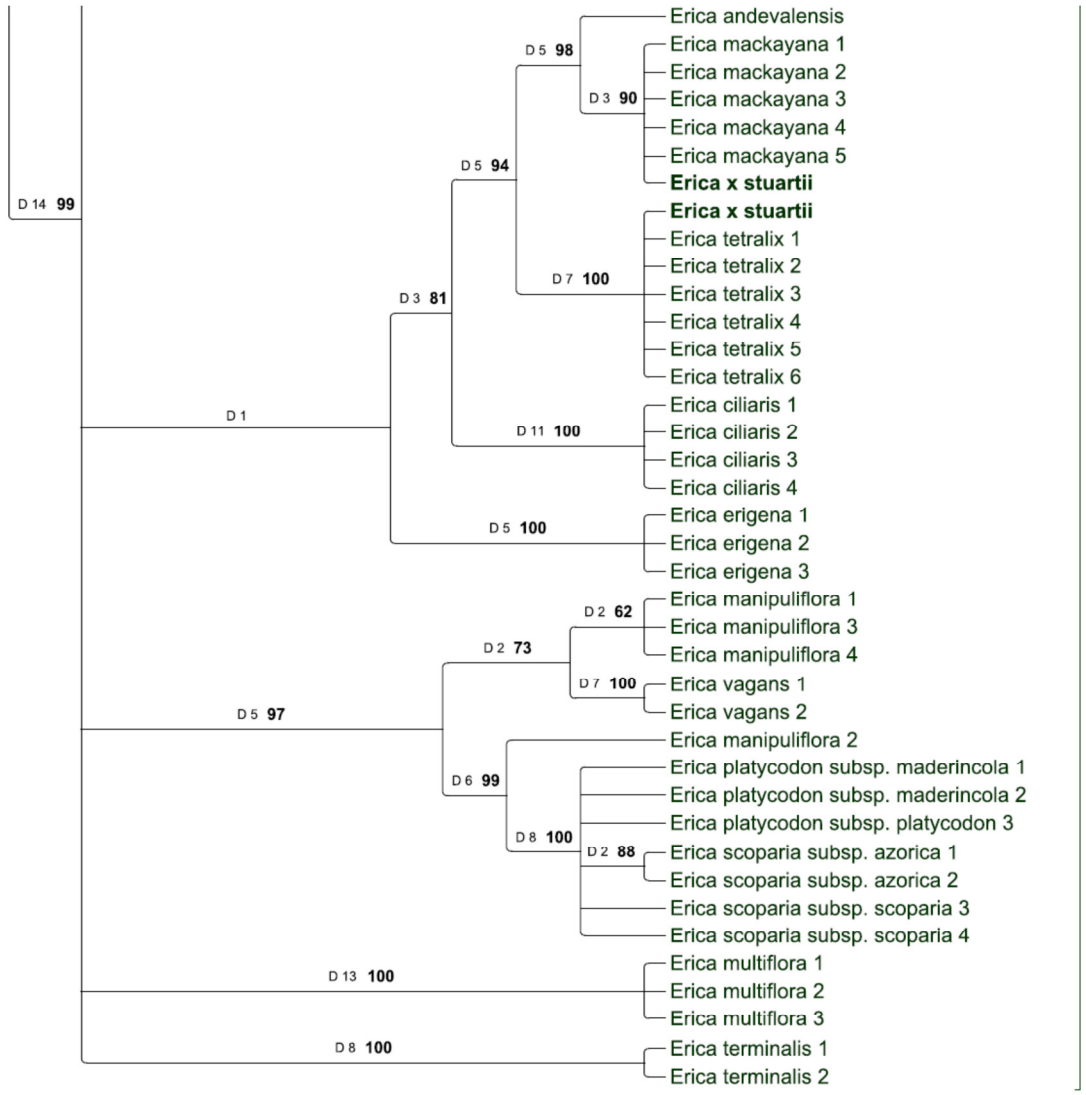


Figure 3-2. (Continued) Strict consensus of 86 most parsimonious trees of nuclear data (Length=1278, CI=0.479, RI=0.846 with decay (D) and bootstrap (bold) values.

3.4 Parental inheritance of cell organelles

The analysis of the artificial hybrid *E.* × *oldenburgensis* and its parent plants (*Erica arborea* as the mother plant and *Erica carnea* as the father plant) resulted in a clear pattern of maternal inheritance for both organelles. Chloroplast and mitochondrial sequences were identical to its mother plant *Erica arborea*, but clear punctual differences were found when the sequences were compared with its father plant *Erica carnea.*

3.5 Relative quantification of total plant nuclear genome

Flow cytometry analyses results of the relative total cell genome size from plant leaves of 18 different plant samples from the genus *Erica* in two terms is shown in Figures 3-3 A and B. *Erica laeta* was rated twice to be used as a possible reference standard control for both measurements. Relative fluorescence units (RFU) represent the estimation of absolute nuclear DNA amount. Comparing the peaks in the G_0/G_1 phase of the cell cycle *Erica cinerea* presents the smallest relative nuclear DNA content with approximately 30 RFU and *Erica bauera* presents the largest relative nuclear DNA content with approximately 180 RFU. All samples reveal to have the same ploidy level, except for the *Erica bauera* sample that apparently resulted to have a higher one, as it can be deduced from the first two peaks of the graphic together with the resulted RFU values.

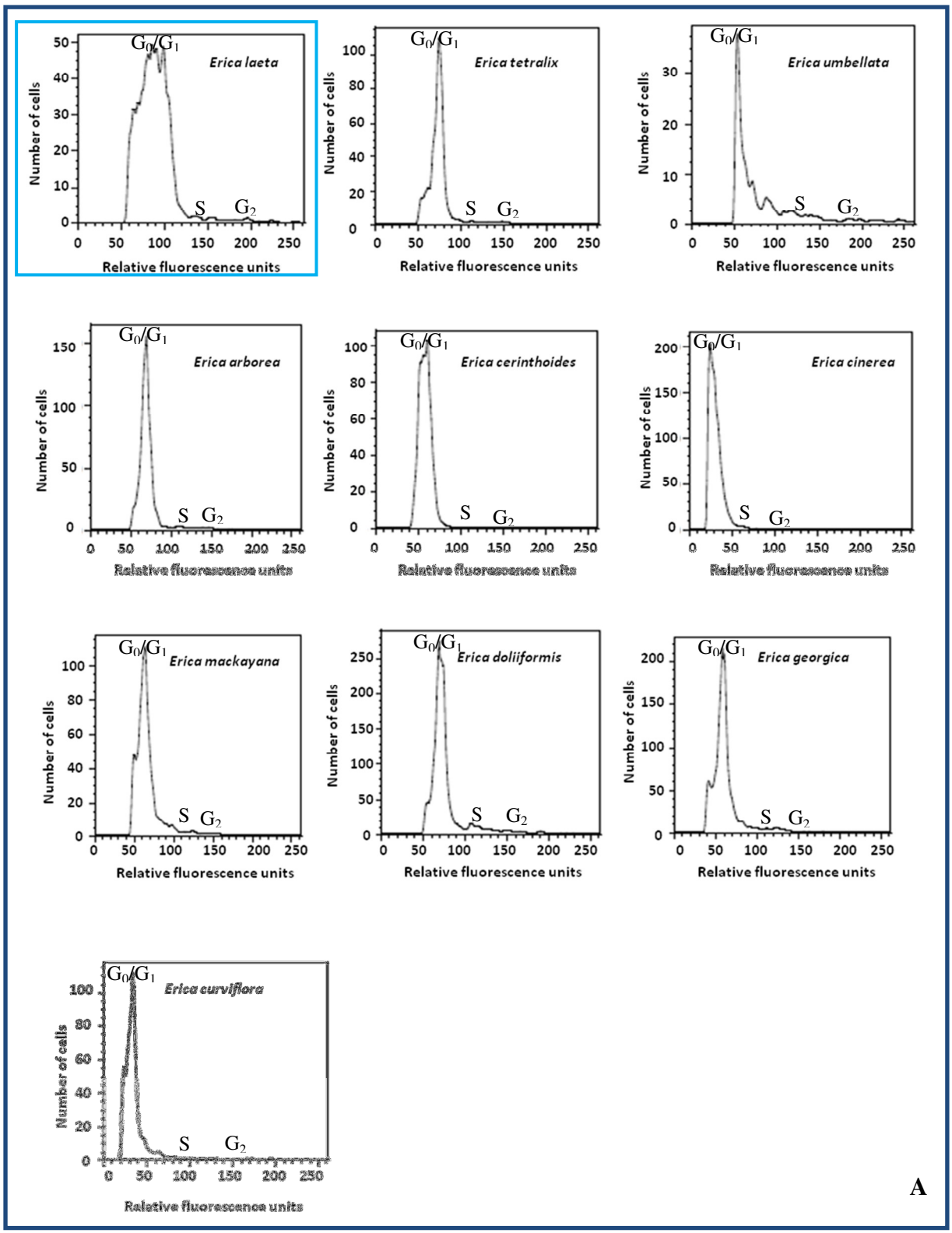


Figure 3-3. (Continued)

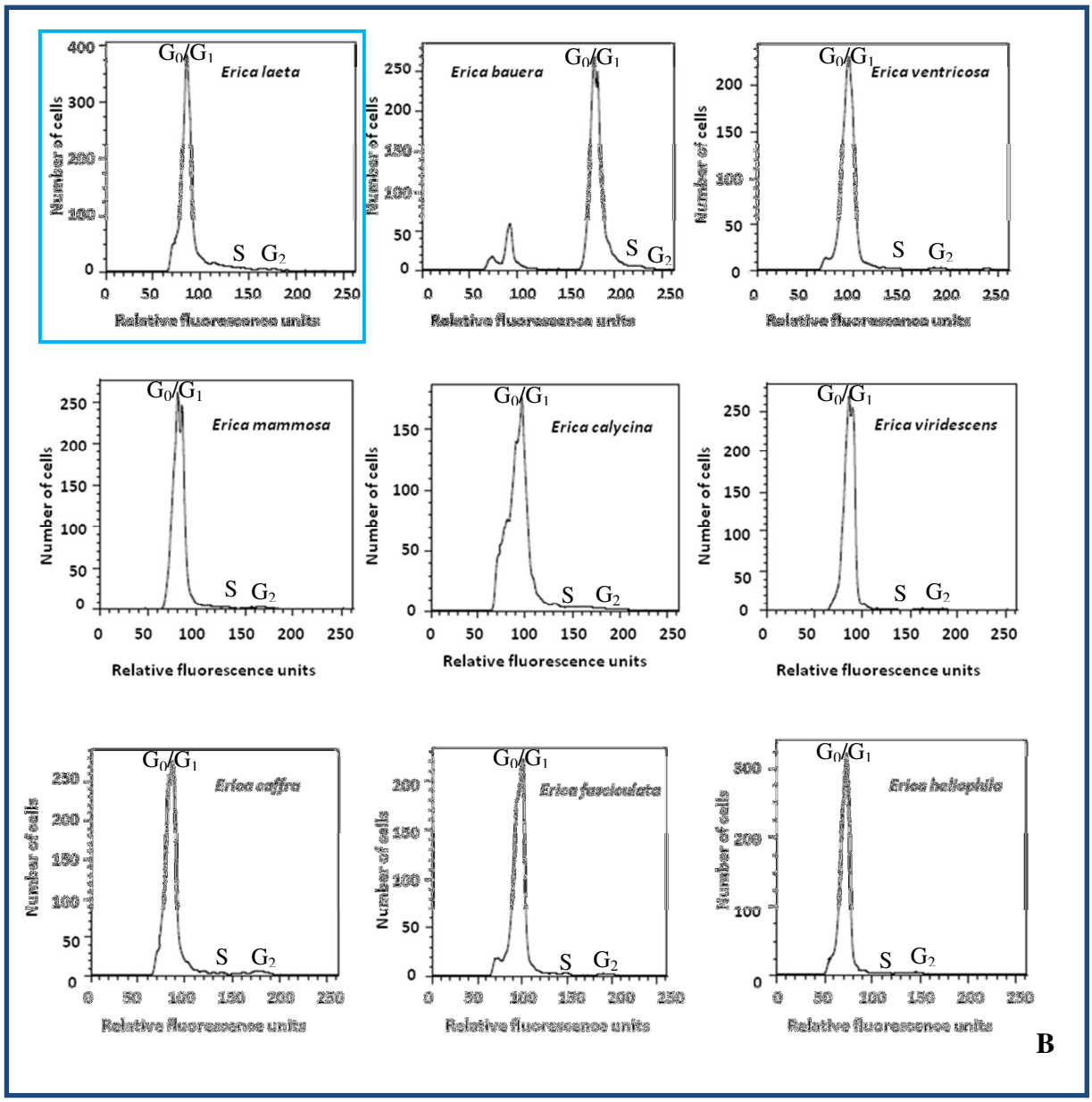


Figure 3-3 (Continued) Flow cytometric histograms of genome analysis made in two terms (**A** and **B**) of 10 and 9 *Erica* species respectively. Cell nuclei were stained with DAPI. Peaks show the relative quantification of total nuclear genome in relative fluorescence units (RFU) from the analysed plant leave cells in the different cell cycle stages G_0/G_1, S and G_2. *Erica laeta* was measured each time to be used as a possible control standard of the procedure.

3.6 Stereomicroscopic structure of flowers and pollen grains

Plant samples were viewed under the stereomicroscope before pollen grains were collected and stored. Structures of hermaphrodite flowers of *Erica ventricosa* THUNBERG 1785 and *Erica bauera* ANDREWS 1812 with their pale pink petals, stamens with anthers, stigma and pollen grains can be seen in Figures 3-4 and 3-5. As already described by Schumann et al. (1995) the first one is commonly known as 'Franschhoek heath' growing to 900 mm high with tubular corolla flowers of 12 to 16 mm long with lobes curling backwards and minutely crested anthers, exists in cultivation in England since the nineteenth century and flowers from October until January. The second one is also ordinary called 'bridal heath' or 'Albertinia heath' is one of the most grown *Ericas* characterized to be an up to 1.5 m high shrub when grown in cultivation, with typical grey-green leaves and white pinkish tubular corollas of 16 to 20 mm long and anthers with long awns, flowering in spring and autumn.

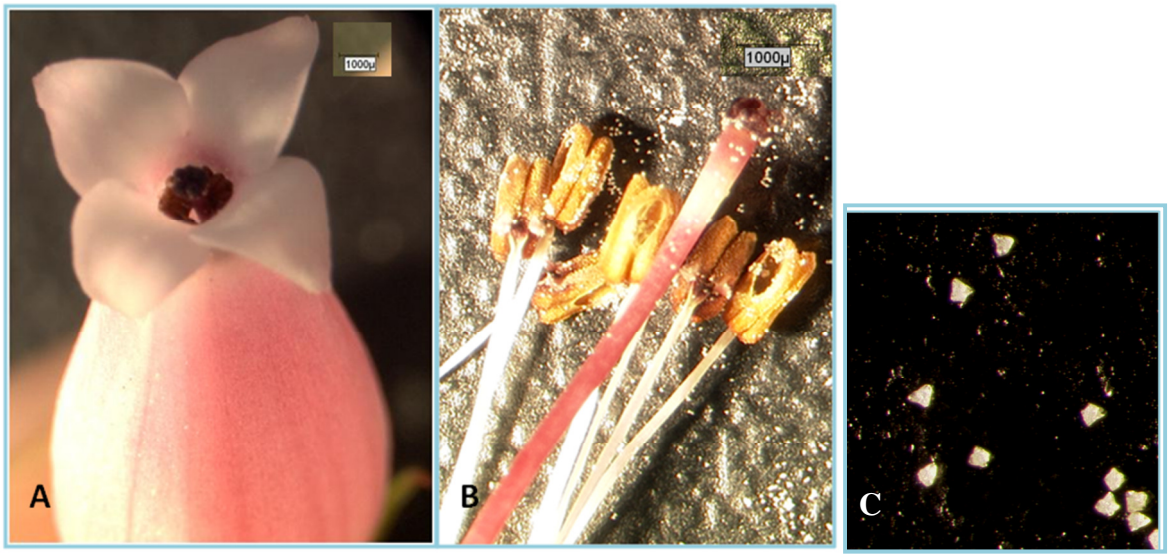


Figure 3-4. *Erica ventricosa* **A**: tubular corolla flower with pale pink petals; **B**: stigma and stamens with minutely crested anthers and pollen grains; **C**: pollen grain tetrads.

Figure 3-5. *Erica bauera* **A**: tubular corolla flower with pale pink petals; **B**: stigma and stamens with anthers presenting long awns; **C**: stigma, anther and pollen grains; **D**: pollen grain tetrads.

3.7 Fluorescence microscopic structure of pollen tetrads

Random pollen samples were viewed under the fluorescence microscope before and after the storage period to score their vitality after the fluorochromatic reaction test. Figures 3-6 and 3-7 show some examples of living as well as dead cells within a pollen tetrad for the studied species *Erica ventricosa* and *Erica bauera* respectively.

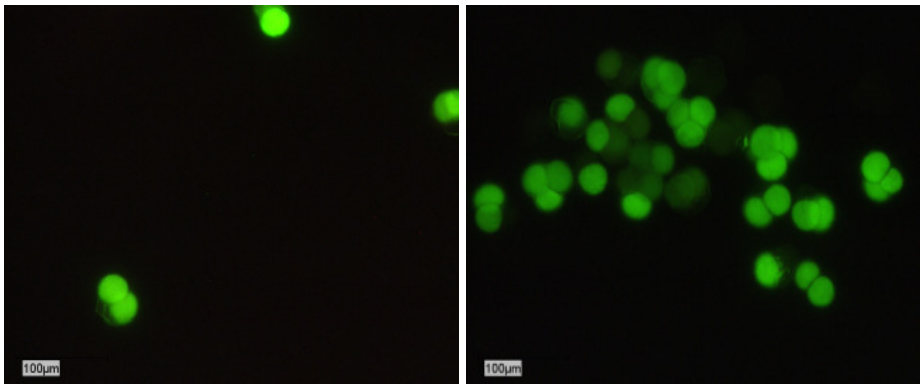


Figure 3-6. Pollen tetrads of *Erica ventricosa* at day=0 detected under the fluorescence microscope. Living cells are bright green after the fluorochromatic reaction with fluorescein diacetate (FDA) seen under the fluorescence microscope with UV-light.

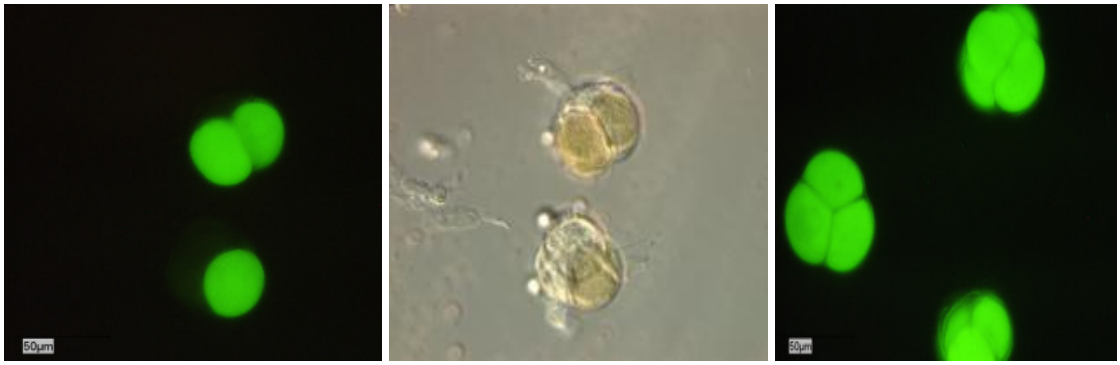
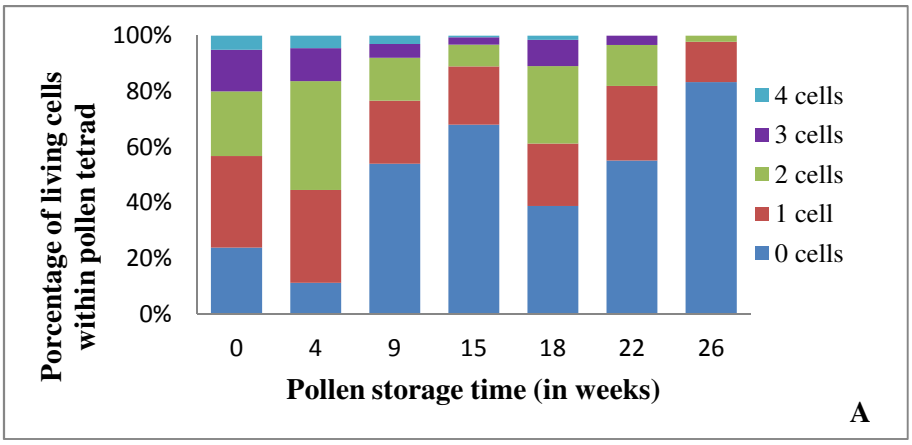


Figure 3-7. Pollen tetrads of *Erica bauera* at day=0 detected under the fluorescence microscope. Living cells are bright green after the fluorochromatic reaction with fluorescein diacetate (FDA) seen under the fluorescence microscope with UV-light.

3.8 Pollen vitality

The percentage of fluorescent pollen cells within the pollen tetrad samples incubated in FDA solution during the storage time is given in Figures 3-8 A and B. In the case of *E. Bauera* week = 0 (Fig. 3-8 B) it was not possible to distinguished the fourth cell because of its position and thus considered 3-4 cells together in the same category for this time period. The percentage of non-viable tetrads, that is the percentage of tetrads presenting cero living cells, is presented in Figures 3-9 A and B for *Erica ventricosa* and *Erica bauera* respectively. Pollen grains of both species present at the end of the selected storage period tetrads with at least one or two living cells, comprising around 20-50% of the total observed tetrads. The number of non-viable tetrads increases up to 50-80% and the ones with four living cells decreases up to 15-40% during the total storage period of the pollen grains.



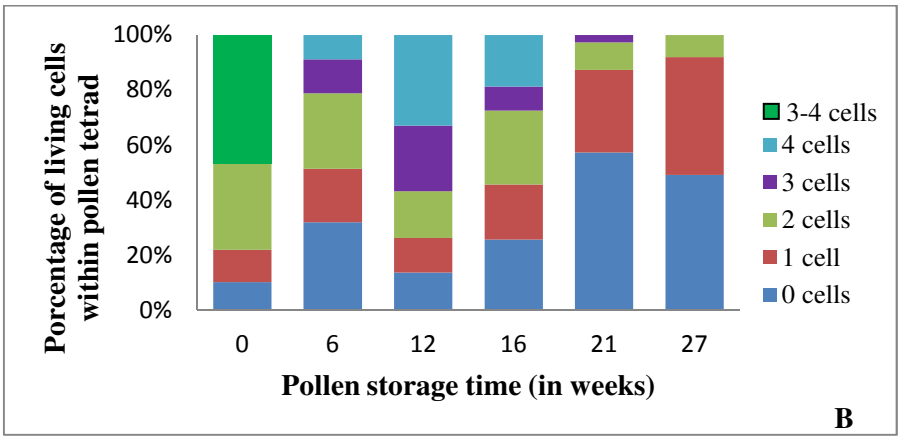


Figure 3-8. Pollen vitality of flowers (n=3) of **A** *Erica ventricosa* and **B** *Erica bauera* after different storage periods at -20°C. Average number of living cells within a pollen tetrad detected under the fluorescence microscope.

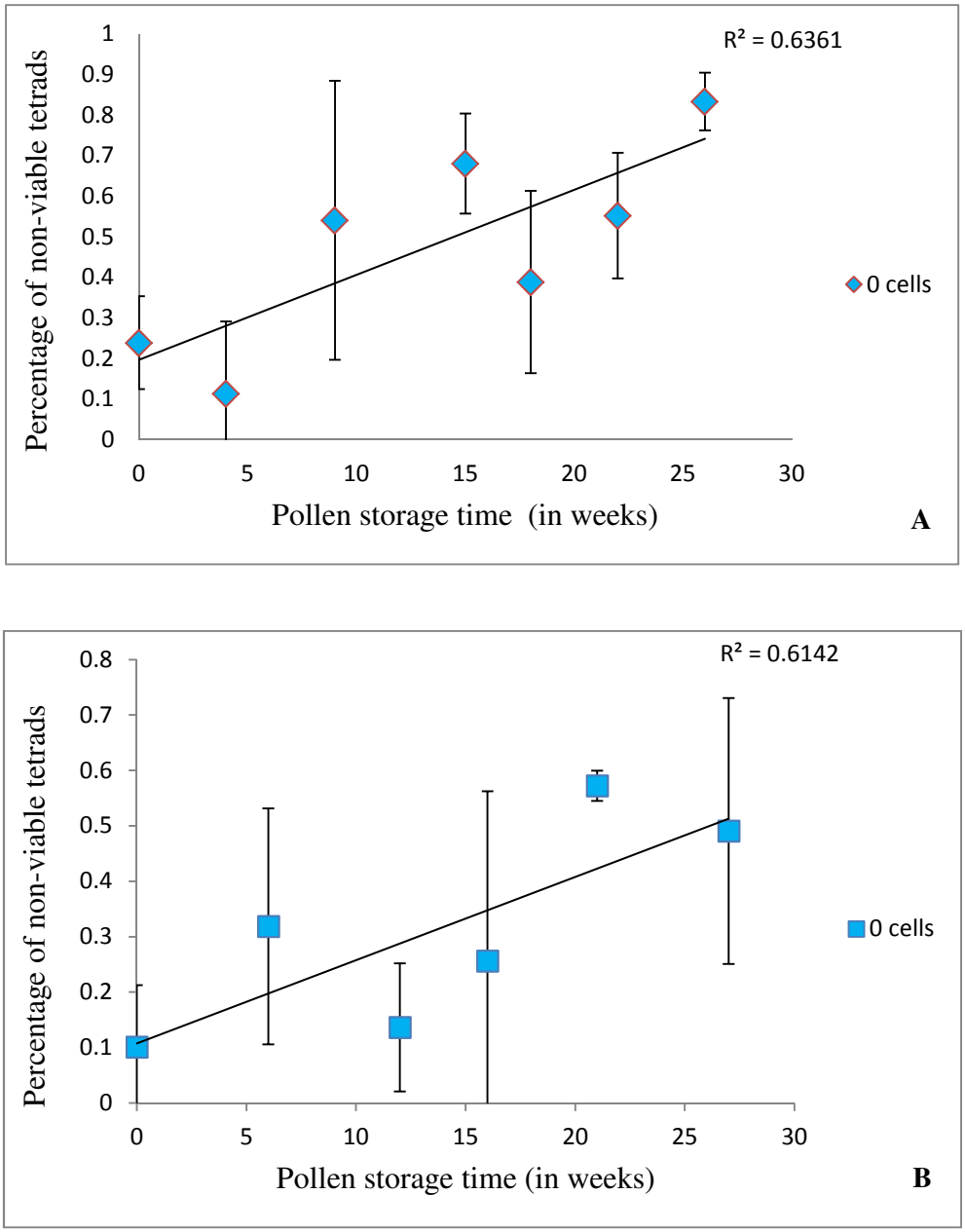


Figure 3-9. Pollen viability flowers (n=3) of **A** *Erica ventricosa* and **B** *Erica bauera* after storage at -20°C. Percentage of non-viable tetrads detected under the fluorescence microscope.

4 Discussion

Elucidating molecular phylogenetic of the studied *Erica* species in this work aims their arrangement in groups or clades according to their genetic relationship within and between the given northern and southern heather species. The parental inheritance of the cell organelles chloroplast and mitochondria in *Erica* was also molecularly tested enabling the understanding of the plastid inheritance as well as of the hybrid origin. Relative total genome DNA of eighteen selected *Erica* species was quantified via flow cytometry. Finally the pollen vitality of two different *Erica* plant species was assessed during a period of time of around six months by means of fluorescence microscopic procedures.

Using molecular characters such as nuclear or chloroplast DNA regions comprising genes, introns and spacers, is considered a suitable tool to explain evolutionary processes and phylogenetic structures among individuals (Álvarez and Wendel 2003). This is based on their sequence array itself compared among the taxa with emphasize on variation patterns, and on DNA-nucleotide mutations within the sequences, considering that non-genic DNA regions evolved rapidly. Chloroplasts have a genome range in size from 120 to 170 kbp for all land plants and are structured in four intercalated parts mainly: two inverted repeats (IR) disposed as mirror images of each other, a large single copy (LSC), and a small single copy (SSC). The small and large single copy regions have a substitution rate of up to two or three times higher than the two inverted repeats (Shaw et al. 2007). Nuclear ribosomal DNA region internal transcribed spacer (ITS) is positioned between ribosomal genes 18S and 26S, and comprises the sequences: ITS1, 5.8S and ITS2 (White et al. 1990). Because the ITS region is conservative throughout organisms but divergent in their nucleotide order, is considered adequate for phylogenetic studies as well (Baldwin et al. 1995; Sun et al. 1994).

McGuire and Kron (2005) performed the first phylogeographic analysis in *Erica* in which a small set of European and South African species were included, confirming the hypothesis of a European origin of the genus as well as the monophyly of the South African taxa. Recently Pirie et al. (2011) obtained a similar result, but from a much extent sample of approximately 45% of the total *Erica* species from European, South African and other African regions including Central Africa, Madagascar and the Mascarene Islands. They stated the monophyly for the whole African clade including *E. arborea* in a basal polytomy. Although here only

29% of the total *Ericas* has been assessed, the results in the current work are also principally in accordance with a European origin of the genus and the monophyly of the South African clade.

In this study three species from Central East Africa are also included in the cpDNA tree. *E. trimera* was positioned, together with the European species *E. umbellata*, as sister to the South African clade but *E. rossii* and *E. kingaensis* are merged within the *scoparia-vagans* European clade. Considering that no nuclear information was generated for these samples, it may be assumed with the available data that migration episodes could have occurred north-south in both directions in different moments at least between the European and Central East African nucleus as has already been demonstrated in *E. arborea* (Désamoré et al. 2011). Yet, in the present work, the hypothesis of a single episode of migration to the Cape region together with an intense radiation afterwards is also supported by the given results.

The monophyly of the studied northern and southern species is clearly supported by the resulting trees for both nuclear and chloroplast markers, with the exception of *E. arborea* and *E. manipuliflora*. Although populations of *E. arborea* are monophyletic in the nuclear data, they have two clades unresolved in the plastid data. There, a polytomy with other taxa in the chloroplast tree appears. Désamoré et al. (2011) explained the occurrence of different haplotypes within the *E. arborea* by different episodes of migration and the existence of refugia during the last glaciations, principally at the Iberian Peninsula. In their work the highest genetic variability, that is the highest number of haplotypes, was found in the Iberian Peninsula and in the East African populations, suggesting an African centre of origin and a reservoir on the western end of the Mediterranean. As for *E. manipuliflora*, one population is sister to the *E. scoparia* clade both for cpDNA and ITS trees (Figures 3-1 and 3-2). *E. manipuliflora* shows a high morphological variability (McClintock 1989; Fagúndez and Izco 2010), and in-depth studies on this taxon should be performed to clarify its taxonomic arrangement. Pirie et al. (2011) either found a strong support for analysed species with more than one sample, or they found non-supported para or polyphyly, just as obtained for *E. arborea* in this work.

Several basal branches of the cpDNA tree are not or only poorly supported by bootstrap (Figure 3-1). *E. lusitanica* species are positioned as sister to the rest of the *Erica* species and they are divided in two clades. One clade joins together *E. umbellata*, a distinct Iberian species, with the East African species *E. trimera*, sister to one more clade which includes *E.*

australis, the different populations of *E. arborea* and another clade with all the South African species. The second branch includes the core of the northern species and the other two eastern African species. *E. cinerea*, together with its close relative *E. maderensis*, and *E. sicula* are positioned in basal branches with low supporting values. *E. spiculifolia* is the sister species of the rest. This species has several exclusive characters within the northern species such as the absence of bracteoles, a characteristic raceme and a distinct chromosome number (Fagúndez and Izco 2008). But it should be considered that these mere morphological differences in cladistics may only describe in this case an autapomorphy of the given species, that is a singular attribute only found in a group within a clade, saying nothing about relativeness with the other ones, solely just showing differences from the rest. A basal position on the whole genus for *E. spiculifolia* was obtained by McGuire and Kron (2005) from their phylogenetic analysis with chloroplast and nuclear markers.

The position of *E. terminalis* is another case of an unresolved species. It has been related to *E. tetralix* and its relatives (Bayer 1993), but seed morphology clearly resembles it to *E. cinerea* and mostly to *E. maderensis* (Fagúndez and Izco 2009). *E. terminalis* also has some clear differential characters such as pollen in monads, a character only shared with *E. spiculifolia* among the northern heathers (Oldfield 1959; Nelson 2009).

Two main branches are supported by the bootstrap analysis within the northern clade. The first one corresponds to the Atlantic heathers, all glandular species that occur in humid habitats in temperate, oceanic climates. The clade includes *E. tetralix, E. ciliaris, E. mackayana* and *E. andevalensis*. This last species is a close relative of *E. mackayana* and has been considered a subspecies of the former one (McClintock 1989). The second clade includes a set of Mediterranean and Atlantic species and *E. carnea* from the high altitudes of the Alps, living up to 3000 m altitude. They share several characters such as the absence of anther appendages, mostly glabrous stems and a very light pink corolla with a broad mouth and exserted anthers. *E. carnea* and *E. erigena*, two very similar species included by Bentham in its own subgenus because of its basal anther insertion (Bentham 1839; Hansen 1950; Bayer 1993; Fagúndez and Izco 2003) are joined and are sister to a second clade where *E. multiflora, E. manipuliflora, E. vagans* and *E. scoparia*, including *E. platycodon*, are merged with the two eastern African species *E. rossii* and *E. kingaensis*. *E. scoparia* and its close Macaronesian relatives are distinguished by its clear anemophilous syndrome: small flowers, greenish corolla, exserted anthers, peltate style end and absence of nectariferous disk. It has

been suggested that these characters are highly correlated with the pollination type and have evolved independently in a wide number of plant lineages as an adaptation to ecological conditions and pollinators availability (Friedman and Barret 2009), and also that these dramatic changes can take place in a short time and only with a few genes being involved.

The chloroplast genome has largely been the first choice for phylogenetic analyses in plants (Chat et al. 2004; Shaw et al. 2007) mainly because of its conserved structure and the presence of the single copy regions with a high nucleotide substitution rate (Perry and Wolfe 2002). In turn, the nuclear genome has been used mostly as an aim to reinforce the phylogeny obtained from chloroplast markers and to prevent errors occurring due to hybridization and introgression events or lineage sorting (Doyle 1992). Results from the nuclear ITS markers were poorly supported for some samples and problems aroused because of high presence of double peaks or polymorphisms in the chromatograms after sequencing (Figure 3-2). As a consequence such samples were removed from this analysis.

Several clades were obtained equal to those of the cpDNA trees such as the *E. tetralix-ciliaris* clade or the *E. scoparia-vagans* clade with the exception of the absence of *E. multiflora* whose position was unresolved (Figure 3-2). *E. arborea* is again placed with the South African species in a strongly supported clade, but this time *E. lusitanica* is its closest relative. Several morphological characters are shared by these two species such as a whitish corolla; however other features such as seed morphology are strikingly different (Fagúndez and Izco 2010). Two other clades form a polytomy with the arborea-South African clade, one of *E. umbellata* and a second one with *E. australis* and *E. cinerea* including *E. maderensis*. Both *E. umbellata* and *E. australis* are endemic to the western half of the Iberian Peninsula and northern Morocco. This area has been known as a tertiary refugium in several groups (Postigo Mijarra et al. 2008) and probably for *E. arborea*, where the highest diversity of haplotypes was found (Désamoré et al. 2011). *E. australis* and *E. umbellata*, together with *E. arborea*, are the closest relatives to the South African species according to cpDNA and ITS trees, as found by McGuire and Kron (2005) and Pirie et al. (2011).

The southern heathers studied here represent only around 23% of the total South African *Ericas* species described so far. More species should be analysed before stating general information, but only small phylogenetic variation was present between the species studied, particularly when compared with the European *Erica* species. Here only few different clades comprising different species elucidate from the trees revealing a tight relationship and narrow

origin between the taxa. They all form a robust group in both trees. There could be several reasons to explain the higher number of *Erica* species in this region with such a close phylogenetic relationship. One is the fact that South African species are mostly seeders, in contrast with the European resprouter species. Seeders may show to have higher diversity forced by natural selection after a fire event, and considering that the cape floristic region has a favourable climate with mild temperatures and winter rainfall that may so induce the post fire germination of the plants (Segarra-Moragues and Ojeda 2010). Another point is the absence of a glaciation period in the south of Africa which was probably responsible for the extinction of many European species. A last argument could be the ability of *Erica* to interspecific cross pollinate, and so assuming a hybrid origin for the South African *Erica* species. Further clarification of the phylogenetic relationship and origin should include more South African *Erica* species.

Although several authors have claimed for the use of combined trees even if low values of IDL test have been obtained (Gillespie and Kron 2010) in this study the trees obtained from the combined cpDNA and the ITS were clearly incongruent and the topology of the trees were markedly different (test IDL), leading to the final decision of not merging them. Conflicts present among both trees may be due to different evolutionary history of markers, as a result of analytical artefacts such as paralogy or long-branch attraction, or as a result of biological phenomena such as incomplete linage sorting or hybridization, among others (Pirie et al. 2009; Blanco-Pastor et al. 2012; de Viliers et al. 2013). Then it is important to accept the given incongruences between trees and not to join them, otherwise relevant evolutionary information will be discarded while contradictory merged data will decrease the clades support or even lead to equivocal relationships (McDade 1992; Bull et al. 1993; Lecointre and Deleporte 2005; Pirie et al. 2009).

Angiosperms normally present a maternal-inherited plasmatic organelles DNA, but they may also follow a bi-parental or only paternal-inherited plastid and mitochondria DNA (Harris and Ingram 1993; Hagemann 2004). No specific analysis had been performed before in *Erica* or the *Ericeae* (see discussion for *Calluna* in Rendell and Ennos 2002). Although no confirmation was obtained by Kron et al. (1993) in *Rhododendron*, and by Rendell and Ennos (2002) in *Calluna*, these studies suggest the maternal path as the most probable case for the chloroplast inheritance in both groups. However, a strict paternal inheritance of the chloroplast has been documented in the related family Actinidiaceae (Ericales) for the

kiwifruit, but with a maternal inheritance of mitochondria (Chat et al. 2004). The detail analysis obtained from *E.* × *oldenburgensis*, an artificial hybrid from controlled parental specimens, clearly shows the mother inheritance of both organelles chloroplast and mitochondria at least for this particular cross. In the case of the hybrid *E.* × *stuartii* is clearly clustered with a different parental species when chloroplast or when both sequences obtained from nuclear DNA are analysed (Figures 3-1 and 3-2). Assuming also a maternal inheritance of the chloroplast for these species, *E. tetralix* is the maternal parent, while the paternal is *E. mackayana*. A strongly nuclear DNA inheritance is given by *E. mackayana*, since one of the sequences of the hybrid is clustered with its populations in a well-supported clade. The position of other species like *E. cinerea*, *E. umbellata*, *E. lusitanica* and *E. australis*, and other clades is also markedly different when chloroplast and nuclear DNA are used on the phylogenetic reconstruction. A possible explanation is a homoploid hybrid origin for these species. This could be the case of *E. lusitanica*, a tall shrub that shares several phenotypic characters with *E. arborea*. Both species were originally included in the same section Arsace (Hansen 1950; Bayer 1993). However, some features are strikingly different for the two species such as seed morphology (Fagúndez and Izco 2010). *E. lusitanica* is positioned within the *E. arborea* and the South African clade in the ITS tree, but its position on the chloroplast tree is unresolved and not related to the arborea-South African clade. The origin of *E. lusitanica* could be interpreted as homoploid hybridization between two lineages, with *E. arborea* as a probably parental species. Differences between plastid and nuclear markers trees could rely on the fact that the first ones are uniparentally inherited while the second ones have a biparentally DNA heredity pathway, meaning that lineage sorting of plastid happens faster, increasing species monophyly while lacking hybridization events compared to the nuclear-encoded DNA markers (Palumbi et al. 2001; Chan and Levin 2005; Hedrick 2007; de Viliers et al. 2013). Although it remains open to properly identify the causes of the tree incongruences by running a proper test to identify potential incomplete linage sorting versus hybridization, that is for example by coalescent simulations techniques, a reticulate evolution within these species can be suggested as a possible explanation for the incongruences found for the phylogeny of the group using different data sets.

Although genome quantification through flow cytometry is a well and rapid implemented method nowadays with the capacity of measuring large populations in a short time without the exert need of using tissue with dividing cells (Shapiro 2003; Doležel and Bartos 2005; Loureiro et al. 2006; Nowack et al. 2007), it is also a method that requires a precise handling

and can present some problems such as the need of fresh tissue material, the presence of by products in the cell disturbing the nuclei staining, and the lack of DNA reference standards for the measurement (Doležel and Bartos 2005; Clarindo and Carvalho 2011). *Erica* species are diploid with 2n=24, with the exception of *E. spiculifolia* with 2n=36 (Maude 1940; Nelson and Oliver 2005) and their C-value interpreted as the constancy of DNA per organism is of 2C DNA amount in the G_1 phase (Swift 1950), in this study the ploidy number of the individual analysed plants it was not corroborated but *Erica bauera* certainly showed a double peak. Because in the G_1 phase of the cell cycle only two copies of unreplicated genome can be found, this can be interpreted as higher ploidy level, that is as a higher number of chromosome copies for this karyotype, or as a higher DNA amount of this sample. According to the resulted measurements, between the other *Erica* species there was a very similar relative amount of DNA present. This can lead to the conclusion that if the ploidy level as well as the relative DNA amount of the samples is similar, than it can be thought of a possible crossing between plant species aiming the creation of new varieties.

A fluorochromatic reaction with fluorescein diacetate (FDA) is a reliable method to be implemented to assess pollen vitality (Pinillos and Cuevas 2008). In this study a simple protocol for the storage of pollen grains was performed. Stamens were stored for a period of time of around six months to achieve so a possible pool of living pollen tetrads capable of crossing *Erica* plant species that flower in different seasons of the year. It could be observed that even after the final storage period both South African *Erica* species chosen still showed a number of pollen tetrads with one or two living cells, although the number of dead ones increased linearly with the time. Although it is already well known that plants can dispose of pollen grain units as a single cell or in groups of two or more cells (Copenhaver 2005), nevertheless, it remains open to proof if this amount of living cells within the pollen unit would be suitable enough for the pollination event itself, that is if the pollen tube will germinate and fertilize the ovule, or if it is necessary that always four living cells within the pollen tetrad are present to achieve a positive pollination event of the plants.

Considering the factor that heathers as already described may present the capability of interspecific fertilization when parent plants coexist in one habitat or better when pollen grains achieve the stigma of another plant, meaning by this the faculty of positive interspecific fertilization through cross pollination ending in the generation of viable seeds that will so germinate into a F_1 hybrid, then a proper elucidation of the *Erica* phylogenetic tree, as well as

a complex study of their genome amount and pollen storage capacity, aiming a better understanding and clarification of the species relations will encourage and facilitate their plant breeding by promoting the interspecific crossing of *Erica* plants, and by this their diversity.

5 Summary

The angiosperm (flowering plant) genus *Erica* L. is taxonomically included in the large family their Ericaceae. It normally grows as a woody perennial shrub or subshrub and some species can reach up to tree heights of several meters. The allocation of the genus *Erica* is confined mainly in a north to south vertical dispersion line, comprising whole Europe with 20 species described and all the way across the African continent until the Cape of Good Hope, there, a high level of diversification with around 750 species can be found so far. A species bonding for the European and African region is presented mainly by the following species: *Erica arborea* L. and *Erica australis* L. *Erica scoparia* L., *Erica ciliaris* L., *Erica erigena* R. Ross., *Erica multiflora* L., *Erica umbellata* L., *Erica sicula* Gussone and *Erica terminalis* Salisb. Heathers are of considered importance as a useful crop for horticulture and other human commodities, as well as for the conservation of ecosystems and landscapes.

In the current work a total of 244 plants from Europe and South and Central East Africa were studied. Samples came from the collections of: Dr. Jaime Fagúndez from the University of Santiago de Compostela in Spain, the Bundesgarten-Belvedere Vienna in Austria, Kirstenbosch National Botanical Garden from Cape Town in South Africa, Botanic Gardens of the Rheinische Friedrich-Wilhelms-Universität Bonn in Germany, and from the Gartenbauzentrum Straelen in Germany. Plant samples were analysed using molecular phylogenetic methods applying specific primers for DNA marker regions: *trnL-trnL-trnF* and *5´trnK-matK* in the chloroplast, as well as the nuclear DNA marker ITS, for amplification and sequencing for the subsequently calculation of the matrix and phylogenetic trees. The parental inheritance of cell organelles: mitochondria and chloroplast was tested by analysing one artificial hybrid and its respective parents via mitochondrial marker region *cox3*, and the above implemented chloroplast markers.

Cell nuclei of leaves stained with DAPI were measured by means of flow cytometry methods to estimate the quantification of the relative total plant nuclear genome. In the last step pollen

vitality of two *Erica* species was tested after storage of pollen grains under cold conditions and subsequently staining of them with FDA solution for exposure under the fluorescence microscope, by this a possible viable storage time period of the pollen grains of around six months was attempted.

- Northern and Southern heathers relationship: results agree with a European origin of the genus *Erica* and the monophyly of the South African clade.
- Infraspecific variability of Northern and Southern heathers: monophyly of the studied northern and southern species is clearly supported. All species show to be monophyletic except for the widely spread *E. arborea*, and *E. manipuliflora*.
- Paraphyly of the northern species was also corroborated, although three taxa from Central East Africa were polyphyletic, underlying different episodes of colonization.
- Combined analysis: trees resulted from the combined cpDNA and the ITS analysis were clearly incongruent and the topology of the trees were markedly different (test IDL).
- Hybridization and evolution in *Erica*: a maternal inheritance of both cell organelles mitochondria and chloroplast is suggested.
- Relative genome: all tested samples revealed to have a similar genome size, with the exception of *E. bauera* that seemed to present a higher ploidy level.
- Vitality of pollen grains after storage proved to have a positive result of two living cells within a pollen tetrad after the storage period of six months.

After this work an even higher amount of *Erica* plant species should be further tested to be able to accomplish an even more extensive phylogenetic analysis of the *Erica* genus, in fact, a reticulated network study aiming a better comprehension of the relationships and origin between the existing species should be done.

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