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Δ^6 -Desaturase sequence evidence for explosive Pliocene radiations within the adaptive radiation of Macaronesian *Echium* (Boraginaceae)

Federico García-Maroto^{a,*}, Aurora Mañas-Fernández^a, José A. Garrido-Cárdenas^a, Diego López Alonso^b, José L. Guil-Guerrero^c, Beatriz Guzmán^d, Pablo Vargas^d

^aArea de Bioquímica, Universidad de Almería, Campus de la Cañada, 04120 Almería, Spain

^bDepartamento de Biología Aplicada, Universidad de Almería, Campus de la Cañada, 04120 Almería, Spain

^cDepartamento de Ingeniería Química, Facultad de Ciencias Experimentales, Universidad de Almería, Campus de la Cañada, 04120 Almería, Spain

^dReal Jardín Botánico, CSIC, Plaza de Murillo 2, 28014 Madrid, Spain

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ABSTRACT

The oceanic islands of Macaronesia provide an ideal temporal and spatial context to test hypotheses of plant evolution using a novel set of phylogenetic markers, Δ^6 -desaturase sequences. In contrast to the limited resolution of standard molecular markers (nrDNA and plastid sequences), the Δ^6 -desaturase sequence phylogeny of *Echium* unequivocally reconstructs its active colonization across islands and archipelagos (Madeira, the Canary Islands, and Cape Verde), as well as its subsequent geographical and ecological speciation. Molecular-clock estimates using penalized likelihood and Bayesian inference reveal two radiation processes coincident with two dramatic climatic changes recorded in the region: the advent of the cold Canarian sea current (ca. 4 Ma) and the establishment of a strong seasonality in the Pleistocene (1.8 Ma). Though *Echium* had available all the diversity of present-day Macaronesian environments (xeric and mesic scrub, laurisilva, pine forest, and subalpine habitats) in the Miocene, evolutionary divergence appears to have been triggered by an extension of fluctuating xeric and mesic habitats with the advent of Pliocene conditions. These *Echium* radiations not only fulfill traditional predictions of adaptive radiation (i.e., common ancestry, rapid speciation, and phenotype–environment correlation), but also, uniquely among Macaronesian species, trait utility of woodiness. A Pliocene transition from annuality to a bush or tree-like condition occurred in early *Echium* lineages. Maintenance of woodiness in major lineages, and reversal to an herbaceous condition by three independent events, is reported for the first time in plants of oceanic islands.

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1. Introduction

Oceanic islands are considered to be living laboratories for evolution because they furnish clear-cut spatial and temporal limits (Carlquist, 1974). Evolution on islands is so simplified that Humboldt (1817) and Darwin (1859) developed theories of vegetation and organic evolution on the basis of cursory observations in the Canary and Galapagos archipelagos. In particular, questions related to species origin, given the unique morphological differentiation that occurs on oceanic islands, were helpful for Darwin (1859) to formulate his theory of evolutionary change. Island radiations are associated with particular evolutionary processes conducive to adaptive radiation: genetic bottlenecks as populations initiate from

a small number of colonists, rapid rate of phenotypic evolution by character release from previous ecological constraints, and novel adaptations as populations evolve into a range of new niches (Bromham and Woolfit, 2004). Lamarck (1809), Darwin (1859), and Haeckel (1866) used the concept of adaptive radiation to link environment and organismic evolution, although that term was formally proposed by Osborn (1902). Later on, Huxley (1942) defined adaptive radiation as ‘an invasion of different regions of the environment by different lines within a group and secondarily their exploitation of different modes of life’. Simpson (1953) added a new twist to the definition of adaptive radiation by introducing a tempo scale. The most recent descriptions of adaptive radiation include analyses not only of common ancestry, phenotype–environment correlation and rapid speciation, but also trait utility in the course of evolution (Schluter, 2000). Although spectacular adaptive radiations in continental plants (Hughes and Eastwood, 2006) and animals (McCune, 1997) have been described, the characteristics of oceanic islands make them a compelling environment for testing instances of adaptive radiation.

* Corresponding author. Fax: +34 950 015476.

E-mail addresses: fgmaroto@ual.es (F. García-Maroto), amf331@ual.es (A. Mañas-Fernández), jcardena@ual.es (J.A. Garrido-Cárdenas), dlopez@ual.es (D.L. Alonso), jlguil@ual.es (J.L. Guil-Guerrero), bguzman@rjb.csic.es (B. Guzmán), vargas@rjb.csic.es (P. Vargas).

The Macaronesian archipelagos (the Azores, Madeira, the Canary Islands, and Cape Verde) were subjects of numerous early molecular studies aimed at interpreting patterns of dispersal and colonization, as well as the acquisition of particular characteristics (see Carine et al., 2004; Vargas, 2007, for revision). The *Sonchus* alliance (Kim et al., 1996), the *Aeonium* alliance (Mort et al., 2002), *Argyranthemum* (Francisco-Ortega et al., 1996), and the *Echium* group (Böhle et al., 1996), with over 25 species each, provide opportunities to investigate multiple patterns of evolution. They are ideal plant groups to test hypotheses of adaptive radiation and the adaptive significance ('utility') of some typical characters developed on islands such as the acquisition of woodiness. Macaronesia parallels oceanic archipelagos of the Pacific (Hawaii, Galápagos, Juan Fernández) in that tree-like forms occur in unrelated genera, and closely related relatives to woody species display herbaceous forms (Baldwin et al., 1998). A traditional hypothesis is that woodiness originated in the continent and was maintained exclusively in the Canary Islands after climatic changes in the Tethyan-Tertiary (Lems, 1960; Bramwell, 1972). Many phylogenetic studies, however, reveal that remarkable shifts from herbaceous to bush and tree-like species across different plant groups (taxonomic families) is the predominant pattern (see revisions in Baldwin et al., 1998; Carine et al., 2004; Vargas, 2007). Among the 1062 genera of Macaronesian vascular plants, secondary acquisition of woodiness has been documented by means of molecular phylogenetics in *Sonchus* (Kim et al., 1996), *Pericallis* (Panero et al., 1999), the *Aeonium* alliance (Mort et al., 2002), *Sideritis* (Barber et al., 2002), *Tolpis* (Moore et al., 2002), and *Convolvulus* (Carine et al., 2004). Remarkably, significant morphological diversity (as exemplified by growth habits), is found in 28 Macaronesian *Echium* species (Böhle et al., 1996), surpassing that of the remaining 40 continental relatives. Given that the majority of continental species of *Echium* are annuals, elucidating *Echium*'s evolutionary patterns, with particular regard to the trait of woodiness, is a compelling undertaking. In fact, *Echium* is the Macaronesian genus that displays the highest number of woody perennials (24), including 21 candelabra shrubs and three monocarpic-rosette-shrubs.

In addition to woodiness, the remarkable morphological diversity of *Echium* in Madeira, the Canary Islands, and the Cape Verde archipelagos has been repeatedly interpreted to be a remarkable case of adaptive radiation (Carlquist, 1974), although no explicit test has been implemented. A high number of taxonomic entities exemplify phenotype variation in Macaronesian *Echium*, where 28 species (see Appendix A) are classified into seven endemic taxonomic sections, two of which are monotypic (Bramwell, 1972). In addition to this spectacular species radiation, the lure of endemism has historically led to insightful studies of trichome micro-morphology (Lems and Holzapfel, 1968), wood anatomy (Carlquist, 1970), flavonoid chemosystematics (Bramwell, 1973), and molecular phylogenetics (Böhle et al., 1996) with the goal of linking particular attributes to habitat conditions. In fact, *Echium* occupies a wide range of ecological habitats, ranging from coastal xeric areas to humid laurel forest and volcanic subalpine zones (see Appendix A).

Comparative morphology was once an essential methodology for the investigation of radiations of insular oceanic floras. Molecular data can provide an escape from the potential circularity of using morphological characters to reconstruct patterns of evolution with the same characters, particularly in cases of adaptive radiation (Givnish and Sytsma, 1997). In this context, genetic markers have become powerful tools for inferring speciation, as long as levels of genetic differentiation parallel those of morphological differentiation into species. Among all molecular markers, non-coding cpDNA and nrDNA ITS sequences have been the most widely used in the phylogenetics of island plants (Baldwin et al., 1998; Vargas, 2007). A pioneer investigation using nrITS-1 and plastid spacer sequences concluded that the spectacular diversity of Macaronesian *Echium* is the result of colonization from a single

continental-herbaceous ancestor (Böhle et al., 1996). However, evolutionary patterns of inter-island colonization, plant habitat, and ecological shifts remained elusive due to limited sequence variation. Given the intrinsic limitations of these markers (i.e., the low levels of molecular variation across species), investigation of alternative fast-evolving genome regions has become essential to the study of insular evolution. Thus, in spite of technical difficulties involving primer design and paralogy (Fulton et al., 2002; Howarth and Baum, 2005), single-copy genes are increasingly gaining interest because of biparental inheritance, the rarity of cases of concerted evolution, limited alignment ambiguity, and low levels of homoplasy (Alvarez-Fernández and Wendel, 2003). In this study, we have used the 5'-regulatory region of the Δ^6 -desaturase gene as a molecular marker to study the evolution of *Echium*. This gene encodes an acyl-desaturase enzyme catalyzing the synthesis of polyunsaturated fatty acids (Alonso and Maroto, 2000). The functional Δ^6 -desaturase gene (*D6DES*) has been cloned from different Macaronesian *Echium* species (García-Maroto et al., 2002, 2006), and Southern blotting analysis revealed a single-copy in their genomes (García-Maroto et al., 2002). The inverse PCR technique allowed us to isolate the *D6DES* promoter region (*D6DES*-p) even from distant continental *Echium* species. Despite the significant nucleotide variability observed, feasibility of sequence alignment prompted us to investigate the use of the *D6DES*-p.

In this paper, we first evaluate the utility of *D6DES*-p by contrasting its phylogenetic signal with those derived from cpDNA and ITS-1 sequence data (Böhle et al., 1996). We next investigate sister-group relationships and the role of inter-island vs. intra-island colonization and speciation. Lastly, we test the adaptive radiation hypothesis that has been historically proposed on the basis of morphology and habitat exploitation (Lems and Holzapfel, 1968; Bramwell, 1972; Carlquist, 1974), but not fully confirmed because of the limitations of phylogenetically standard DNA sequences (Böhle et al., 1996). Sequence data were accordingly used to estimate divergence times and to elucidate evolutionary processes of species characteristics, particularly habitat shifts and woodiness, in the subtropical habitats of Macaronesia.

2. Materials and methods

2.1. Plant materials

All 27 *Echium* taxa described by Bramwell (1972) as endemic to the Macaronesian islands were sampled. Additionally, a subsequently described endemic, *Echium bethencourtii* (Santos, 1983) was also included (Appendix A). Seeds were collected from plants at their natural locations in successive campaigns carried out from 1998 to 2000 (Guil-Guerrero et al., 2000a,b, 2001). Seed pools were obtained from a variable number of plant individuals in the field (typically >10). They were deposited in the germplasm bank at the Royal Botanic Garden of Madrid (Appendix A). Two continental *Echium* species, *E. sabulicola* and *E. tenue*, were used as outgroup taxa based on a previous ITS phylogenetic analysis, which placed them as sister to the Macaronesian clade (*E. sabulicola*, from Böhle et al. (1996); *E. tenue* from our unpublished results). Genomic DNA was extracted following a CTAB-based procedure (Taylor and Powel, 1982), either from seedling pools containing 7–11 plantlets germinated in growth chambers from the seeds collected in Macaronesia, or from silica-dry leaves from at least four individuals collected from the two continental taxa.

2.2. Cloning and sequencing procedures

The 5'-regulatory region of the Δ^6 -desaturase gene (*D6DES*) of *Echium* (García-Maroto et al., 2002) was used in this study. About

1.3 kb of the sequence upstream of the initiator ATG were initially obtained for the *D6DES* gene of *Echium gentianoides* and *Echium pitardii* by genomic walking using the inverse-PCR (iPCR) technique (Ochman et al., 1988). Briefly, genomic DNA was digested with different restriction enzymes and the resulting fragments were circularized with T4 DNA-ligase under low DNA concentration. PCR using specific primers for the known coding region of *D6DES* allowed the amplification of suitable iPCR fragments encompassing the 5'-region, which were then cloned into a T-vector and fully sequenced. This region was shown to be sufficient to direct the heterologous expression of the *GUS* reporter gene with the same pattern observed for the *D6DES* gene in *Echium* (García-Maroto et al., unpublished results). We will therefore refer to this sequence as the Δ^6 -desaturase promoter (*D6DES*-p).

D6DES-p sequences used for further analysis were generated by direct sequencing of DNA fragments after PCR amplification of total genomic DNA extracted from seedling pools of each taxon (see above). This strategy required an iterative approach using different primer combinations against conserved nucleotide stretches, as well as optimization of the PCR conditions, to obtain a sufficient amount of each product. In the first step, the upstream primer EG-UP1 (5'-CGTTCCAATACTATCGTACAAACATGC-3'), located at the -1212 position relative to the ATG in the *E. gentianoides* sequence, was used in combination with the downstream primer EG-DW1 (5'-AATGTACTTCTTGATTGCATTAGCCAT-3'), which corresponds to the first nine residues of the protein encoded by the *D6DES* gene. Re-amplification, using EG-UP1 and the nested primer EG-DW2 (5'-GATACGCAAGAGTGTGAATACTTC-3') located at -52, was performed when a low amplicon amount was obtained in the first PCR reaction. Amplification products for a considerable number of taxa (20 out of 30) were obtained at this stage. For the remaining 10 taxa, new inner primers were designed based on conserved stretches found by alignment of the already available sequences. One of the three overlapping upstream primers, EG-UP2 (-1214): 5'-GTCGTTCCAATACTATCGTACAAACAT-3', EG-UP3 (-1216): 5'-TTGTCGTTCCAATACTATCGTACAAAC-3' or EG-UP4 (-1220): 5'-GTTTTGTCGTTCCAATACTATCGTAC-3', was alternatively tried in combination with the downstream primer EG-DW3 (-162): 5'-GAGATGTCAATGTTTCAGAAGTTATG-3'.

PCR were conducted essentially as described (García-Maroto et al., 2006) and the amplicon products were purified from agarose gels (QIAEX-II agarose gel extraction kit, QIAGEN). PCR optimization was required for a number of taxa and it was accomplished by modification of the annealing temperature and/or the Mg^{2+} concentration. Sequencing was performed directly on the purified DNA fragments using an ABI-310 genetic analyzer (Applied Biosystems) and BigDye v.3 chemistry. The sequences corresponding to the 5'-end of the *D6DES*-p were used in the study, and they have been deposited in the GenBank database under their respective accession numbers (Appendix A).

Pooling of multiple individuals for each species allowed us to obtain a consensus sequence for each sampled population including the alleles contained in the population. In order to retrieve as much information as possible, informative sites were rechecked on the sequence chromatograms to look for overlapping peaks that would be indicative of allelic variation. Four positions were identified in four different species that contained two different bases (overlapping chromatographic peaks) in one position. They were appropriately encoded with IUPAC symbols in the matrix and analyzed.

2.3. Phylogenetic analyses

Nucleotide sequences (Appendix A) were easily aligned using the program Clustal X 1.62b (Thompson et al., 1997), with further manual adjustments. Phylogenetic analyses were performed using maximum parsimony (MP), maximum likelihood (ML), and

Bayesian inference (BI). Parsimony-based and ML analyses were implemented in PAUP* 4.0b10 (Swofford, 2003) with equal weighting of all characters and of transitions:transversions. Heuristic searches were replicated 1000 times with random taxon-addition sequences, tree bisection-reconnection (TBR), branch swapping and with the options MULPARS, STEEPEST DESCENT in effect and holding 10 trees per replicate in the ML analyses. Relative support of clades identified by parsimony was assessed by full bootstrapping (1000 replicates) using the heuristic search strategy as indicated above. In addition, to determine the simplest model of sequence evolution that best fits the sequence data, hierarchical likelihood-ratio test (hLRT) and Akaike information criterion (AIC) were implemented by using MrModeltest 1.1b (Nylander, 2002), which is a simplified version of Modeltest 3.06 (Posada and Crandall, 1998). Bayesian inference was conducted using MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001) under the general-time reversible model (GTR) of DNA substitution selected by MrModeltest. Two identical searches with eight million generations each (four MCM, chain temperature = 0.2; sample frequency = 100) were performed. In both runs, probabilities converged on the same stable value after approximately 100,000 generations. A 50% majority-rule consensus tree was calculated for each matrix from the pooled sample using the *sumt* command in MrBayes to yield the final Bayesian estimate of phylogeny. Though bootstrap and posterior probability values do not measure the same type of robustness estimates, we used both methods to provide branch support in MP and BI analyses, respectively. Insertions/deletions (indels) were coded as appended characters following the logic of Kelchner (2000) and Simmons and Ochoterena (2000).

2.4. Molecular-clock analyses

Rates of diversification were obtained by using penalized likelihood (PL) and Bayesian estimators. We used Felsenstein's (1988) tree-wide likelihood-ratio (LR) test for rate-constancy of molecular evolution across lineages of the *D6DES*-p trees. Most parsimonious trees were described in PAUP* 4.0b10 with and without a molecular-clock constraint. Trees were then described with gamma-distributed rate variation and four rate categories were assumed. Differences in the log-likelihoods of clock-constrained and clock-unconstrained trees were assessed for statistical significance following the logic of Baldwin and Sanderson (1998). To infer divergence times, we used tree topology and branch lengths obtained from the ML results. The tree most congruent with the Bayesian consensus tree was accepted for analysis. Each unconstrained and constrained ML phylogram was tested for rate-constancy using the χ^2 test of Langley and Fitch (Sanderson, 2003) implemented in r8s v1.60 for Unix. We rejected the null hypothesis of constant rate ($\chi^2 = 116.117$; df = 28; $p < 0.001$) and then, divergence times were estimated with a penalized likelihood (PL) approach implemented with the truncated Newton (TN) algorithm. Initial results were obtained under the following parameters: cvstart = 0.5, cvinc = 0.5, cvnum = 5 with cross-validation enforced. The rate smoothing with the lowest cross-validation scores was selected and the dating procedure was repeated with the following parameters: collapse, num_time_guesses = 5, and num_resarts = 5. Cross-validation suggested 3.2 as the best smoothing parameter. Estimating the length of branches derived from the root node of a tree is problematic because of the uncertain placement of the root along the branch linking the outgroup to the ingroup. An empirical solution is to exclude from divergence time reconstruction the outgroup used during branch-length estimation. In this way, the root node is shifted to the next node within the original phylogram and the lengths of the branches derived from it are accurate (Sanderson, 2002). In this study, *E. sabulicola*, the sister to the remaining taxonomic sample during branch-length

estimation, was excluded from the molecular-clock tests and divergence time reconstructions. The divergence between *E. tenue* and all other *Echium* species became the new root of the tree. This node is also the calibration point. In the absence of fossil records, a conservative approach to calibrate the rate-constant *D6DES*-p trees was performed to obtain a maximum age estimate for the most recent common ancestor (i.e., the initial island colonizer) of the Macaronesian *Echium*. Based on the assumption that diversification of the genus would not have preceded the occurrence of the extant islands, we used 20.6 Ma, a conservative maximum age for the oldest island (Fuerteventura, Canary Islands) (Fig. 1), as our calibration date for the divergence of Macaronesian and continental lineages (Carracedo et al., 2002).

Resulting ages of internal nodes were then contrasted with maximum ages of subaerial island formation and island endemics. The implementation of r8s and node time estimates are depicted in a chronogram. To obtain confidence intervals, 1000 replicates of a full heuristic bootstrap (random taxon-addition sequence, Multrees, Steepest Descent, TBR branch swapping, holding one tree at each step) were obtained enforcing a topologically constrained ML tree (previously used) using the simplest sequence evolution model (GTR).

A likelihood-ratio-based relative rate test was performed in r8s (rrlike command) to test whether the lineages have evolved from the most recent ancestor at the same constant rate. Additionally, a Bayesian dating method was used to infer divergence time estimates and associated confidence intervals (Kishino et al., 2001). The procedure outlined in Rutschmann (2005) was followed, which involves the use of three programs: *Baseml* (PAML3.15; Yang, 1997), *Estbranches* (Thorne et al., 1998), and *Multidivtime* (Thorne and Kishino, 2002). Model parameters (base frequencies,

transition/transversion ratio, and alpha shape parameter of the gamma distribution accounting for among-site rate heterogeneity) were estimated using the most complex substitution model implemented in *Baseml*, F84+G. Maximum likelihood estimates of the branch lengths and their variance-covariance matrix were then estimated by the program *Estbranches*. The program *Multidivtime* determines the posterior distributions of substitution rates and divergence times and uses a MCMC procedure. The following settings for the prior distributions were used in *Multidivtime* (in 10 Ma units): *rttm* and *rtmsd* set at 2.1 (mean and standard deviation of the prior distribution for the time separating the ingroup root and the present based on maximum age of subaerial island formation), *rtrate* and *rtratesd* set at 0.08 (mean and standard deviation of the prior distribution for the rate of molecular evolution at the ingroup node; median distance between ingroup root and tips determined using results obtained by *Estbranches*), and *brownmean* and *brownsd* set at 0.4 (mean and standard deviation of the prior distribution of the Brownian motion parameter ν determining the change for the logarithm of rate of molecular evolution over time; see Rutschmann, 2005). The *bigtime* value (value surpassing age of any nodes in the tree) was set at 100 Ma. All other parameters were left at their default values (see *Multidivtime* manual readme file; <http://statgen.ncsu.edu/thorne/multidivtime.html>). The Markov chain was run for 500,000 generations and sampled every 100 generations following an initial burn-in of 100,000 generations (not sampled).

2.5. Inferring patterns of character evolution

Woody growth traits, habitat, and geographical distributions related to island ages were determined using taxonomic accounts

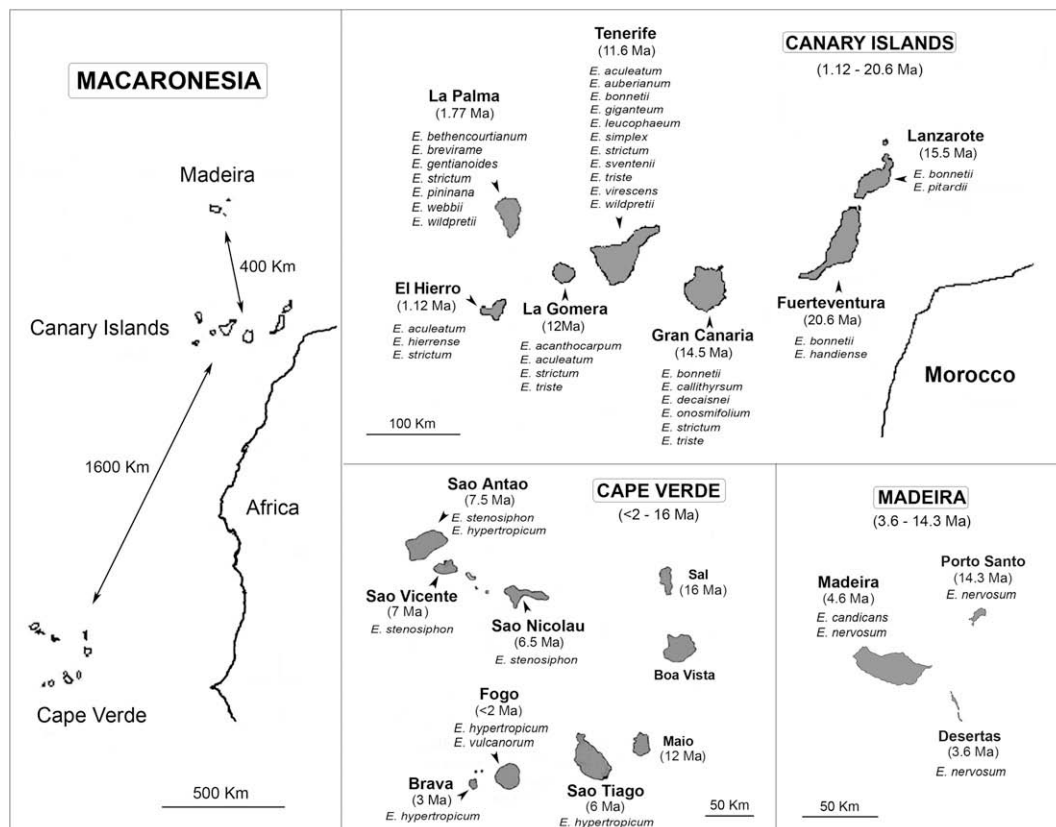


Fig. 1. Geographical distribution of *Echium* species endemic to Macaronesia. Available island ages, estimated from oldest subaerial volcanism, are indicated within brackets (Geldmacher et al., 2000; Carracedo et al., 2002; Holm et al., 2006). Approximate distances between Madeira, Canary Islands and Cape Verde archipelagos are indicated by double arrows.

(Bramwell, 1972, 1973) and personal observations. Character reconstructions were performed using the character-state optimization function of MacClade 3.01 (Maddison and Maddison, 1992), and calculations of ancestral state probabilities were performed using a Bayesian model-averaging approach (Pagel and Meade, 2007). Character-state reconstruction in MacClade assumed Fitch parsimony. The MP tree that displayed the most branch agreement with the BI tree was chosen for character tracing. Given the complex assignment of parallelism and/or reversals, the less restrictive “all most parsimonious trees” option from MacClade was illustrated and discussed (Fig. 3; Supplementary Fig. 1). Additionally, probabilities of ancestral states for the habitat and habit were estimated using the BayesMultiState program (contained in the BayesTraits 1.0 package) (Pagel and Meade, 2007), using the MCMC method and allowing transitions between character states in both directions. To reduce the autocorrelation of successive samples, 2000 Bayesian trees (1000 independent runs) were drawn from the distribution of 158×10^3 trees at stationarity, which equates to sampling every 7900th generation of the chains used in the phylogenetic analysis. As suggested in the BayesMultiState manual, to reduce some of the uncertainty and arbitrariness of prior choosing in MCMC studies, we used the hyperprior approach, specifically the reversible-jump (RJ) hyperprior with a gamma prior (mean and variance seeded from uniform distributions on the interval 0 to 10). Preliminary analyses were run to adjust the *ratedev* parameter until the acceptance rates of proposed changes was around 20–40%. Using a *ratedev* of 10, we ran the RJ MCMC analyses for each trait three times independently for 1.0×10^7 iterations, sampling every 100th iteration (to produce 90,000 sampled points) and discarding the first 1,000,000 iterations. We used the “Addnode” command to find the proportion of the likelihood associated with each of the possible states at each node.

3. Results

3.1. Analysis of D6DES vs. cpDNA and ITS-1 sequences

A consensus sequence from 7 to 11 plantlet DNAs of the D6DES-p was obtained for each of the 30 *Echium* species. Despite significant sequence length variation, the aligned matrix unequivocally resulted in a total of 695 sites, of which 149 were variable

(Table 1). The resulting alignment generated 14 informative indels (Table 1). Pairwise comparisons (using the commonly used Kimura-2-parameters distance) allowed comparisons with previous ITS and cpDNA sequence data (Böhle et al., 1996). The average sequence divergence among Macaronesian species was 3.8%, which is almost twice that observed in ITS-1 sequences (2.0%), and 20 times higher than sequences from the plastid *trnL-F* spacer (Table 1).

3.2. Phylogenetic analyses

Bayesian inference (BI), maximum parsimony (MP), and maximum likelihood (ML) analyses rendered largely congruent results. Of the 24 models of sequence evolution tested, the general-time reversible model (GTR), which accounts for variable base frequencies in a symmetrical substitution matrix, produced the best fit for the data. Our D6DES phylogenetic hypothesis suggests monophyly for the 28 Macaronesian sequences (100% pp, 100% ML, and MP bs), which is in agreement with previous results (Böhle et al., 1996), and an analysis performed with a more extended sample of ITS-1 sequences (our unpublished data). Within this group, the tree topology depicted three major, well-supported groups (Figs. 2 and 4). Group 1 contains four species (three bushes and one annual) primarily from the easternmost Canarian islands (Fig. 2, Supplementary Fig. 1). Group 2 is formed by the three bush-like endemics of Cape Verde plus two annual Canarian species. In group 3, the remaining 19 species from Madeira and the Canary Islands join together. Herbaceous species are therefore found not only in continental outgroup species, (*E. tenue*, *E. sabulicola*) but also in the three major Macaronesian groups, ranging from annual-biannual (*Echium bonnetii*, *E. pitardii*, *Echium triste*) to perennial (*Echium auberianum*) herbs. Multiple placements across our D6DES-p phylogeny is observed for species occurring on different Canarian Islands, while the *Echium* endemics of the Cape Verde and Madeira archipelagos form part of the most limited subclades in groups 2 and 3, respectively (Fig. 2; Supplementary Fig. 1).

Our parsimony (MacClade) (Fig. 3) and Bayesian (BayesTraits) (Table 3) reconstructions mostly agree and reveal recent shifts in morphological characters and reversions from woodiness to herbaceousness. The parsimony reconstruction unequivocally

Table 1

Summary of sequence characteristics for D6DES-p, ITS-1 and *trnL-F* spacer regions from *Echium* (Boraginaceae) species. For comparative purposes, the same data set, comprising all 28 Macaronesian taxa and the continental *E. tenue* and *E. sabulicola*, was employed for D6DES-p (this work) and GenBank ITS-1 sequences (15), while for plastid *trnL-F* sequences, available sequences for 21 Macaronesian plus two chosen continental species, *E. vulgare* and *E. creticum*, were used (15). Best-fitting model estimates were performed using Mrmodeltest v2.2.

Feature	D6DES-p	ITS-1	trnL-F spacer
Informative indels (bp length) ^a	14 (1–96)	3 (1)	2 (7–35)
Length range, bp (average)	394–638 (462)	229–232 (231)	595–638 (628)
Aligned length (bp)	695	234	666
Number of variable/informative sites (%)	149 (32%)/82 (18%)	38 (16%)/29 (12%)	23 (4%)/19 (3%)
CI ¹ (CI)	0.82 (0.89)	0.92 (0.93)	0.95 (0.96)
RI	0.90	0.96	0.97
Mean G + C content (%)	20.7	53.3	26.2
Average sequence divergence K-2-p (MSE)			
Outgroup vs. islands	16.5% (2.1)	10.3% (2.1)	3.1% (0.7)
Islands vs. islands	3.8% (0.4)	2.0% (0.5)	0.2% (0.1)
Canary Islands	3.9% (0.4)	2.1% (0.5)	0.2% (0.1)
Madeira	2.0% (0.6)	0%	n.a.
Cape Verde	0.4% (0.2)	0.3% (0.3)	n.a.
Best-fitting model estimate	GTR	SYM + G	GTR + I
Proportion of invariable sites (I)	0	0	0.847
Gamma shape parameter (G)	Equal rates	0.407	Equal rates
Transitions average (range)	13/9.21 (0–26)	4/3.03 (0–13)	1/0.4 (0–2)
Transversions average (range)	8/5.76 (0–26)	3/3.00 (0–13)	3/2.92 (0–13)
Transitions/transversions average	1.5/2.08	1.1/1.11	0.3/0.09

^a Abbreviations: bp, base pairs; CI, consistency index; RI, retention index; MSE, mean standard deviation; n.a., not available.

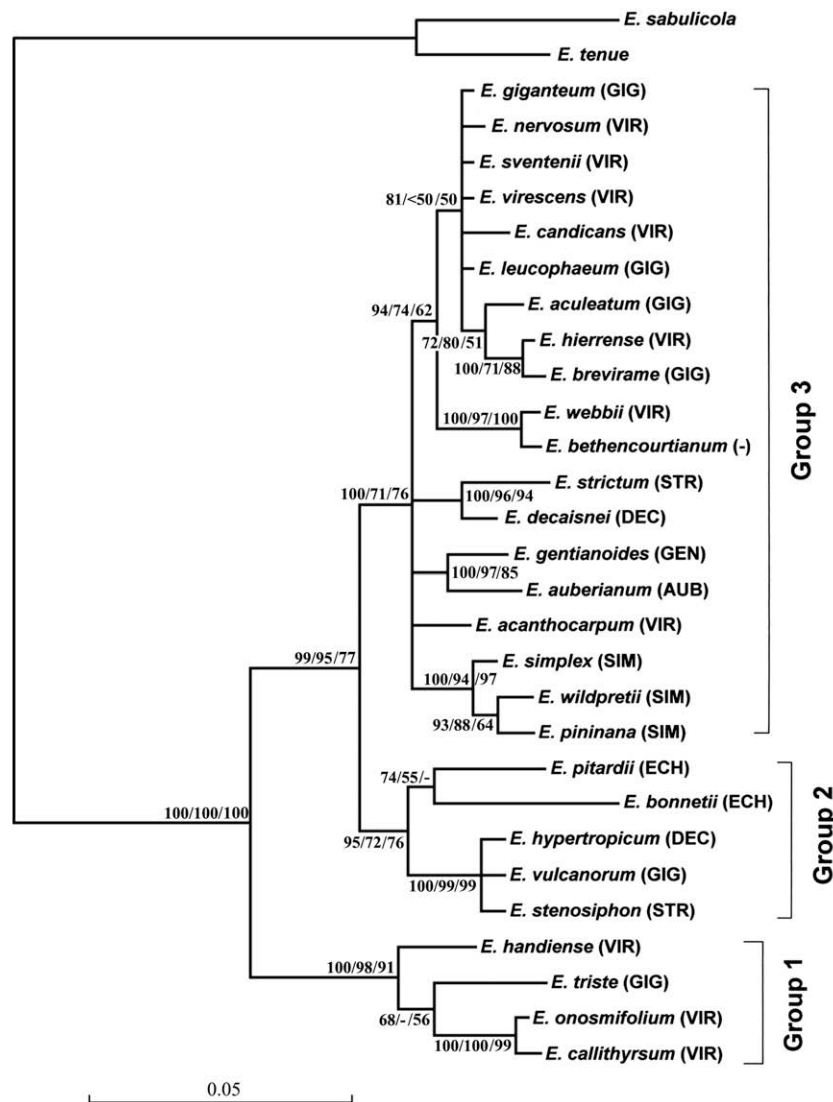


Fig. 2. D6DES-p phylogeny of the 28 *Echium* species endemic to Macaronesia. The figure shows the majority-rule consensus tree generated by Bayesian Inference performed under the GTR model of DNA substitution selected by MrModeltest 1.1b (Posada and Crandall, 1998). Posterior probability percentages are indicated on nodes before slash (/). Maximum Parsimony analysis of D6DES-p sequences, recoding insertions/deletions (indels) as additional characters yielded a strict consensus tree of 198 most parsimonious trees of 217 steps (CI = 0.825, excluding uninformative characters; RI = 0.900). Numbers on nodes after slash (/) indicate bootstrap values. BI branch incongruences relating to MP clades are indicated with a hyphen (-). In the analyses, *Echium sabulicola* and *E. tenue* served as outgroup taxa. Species circumscription in eight *Echium* sections considered by Bramwell (1972) is indicated by the following initials: GIG, Gigantea; VIR, Virescentia; STR, Stricta; DEC, Decaisnea; GEN, Gentianoidea; AUB, Auberiana; SIM, Simplicia; ECH, *Echium*. The three major groups are indicated.

shows new acquisition of an herbaceous habit three times from woody Canarian ancestors (Fig. 3A), and the Bayesian analysis largely confirms this (Table 3). In contrast, the three species displaying monocarpic-rosette-tree habits (node 5; posterior probability = 1.00) form a well-supported monophyletic group (100% pp, 94% bs, Fig. 2) distributed exclusively in the central-west Canarian islands of Tenerife and La Palma (Fig. 3A). Strict xerophyllous species are widespread on every island and account for 14 of the 28 endemics. The Macaronesian group appears to have initially maintained lowland xeric preferences in the three subclades, and subsequently acquired characteristics permitting colonization of higher elevation subalpine zones (*Echium vulcanorum*, group 2) (Fig. 3B), although this preference is not resolved by the Bayesian inference. Broader ecological shifts from a xerophyllous ancestor have occurred in the course of evolution in particular species-rich lineages of group 3. Equivocal ancestry is inferred at mid-term levels (node 10) of the parsimony and the Bayesian reconstructions (Fig. 3B and Table 3) involving 9 species of a large lineage of group

3, which may reflect fluctuating xeric and humid habitat preferences for ancestors and new species in the long period of the Pliocene (see below).

3.3. Estimates of divergence times

Table 2 summarizes the results of the relative rate test implemented in r8s and time estimates of D6DES-p sequences based on penalized likelihood and Bayesian estimators. The two methods rendered congruent results and are remarkably compatible with the maximum age of subaerial island formation and endemics.

Calibration of the Macaronesian *Echium* lineage at the maximum age conceivable for this neoendemic plant radiation (i.e., at 20.6 million years ago (Ma), the oldest subaerial rocks in Macaronesia, see Carracedo et al., 2002) yields the following maximum ages affected by their respective standard deviations using both ML and BI approaches (Table 2): (i) <11 Ma (mean values of 6.56 and 6.95 in the ML and BI analysis, respectively) for the first

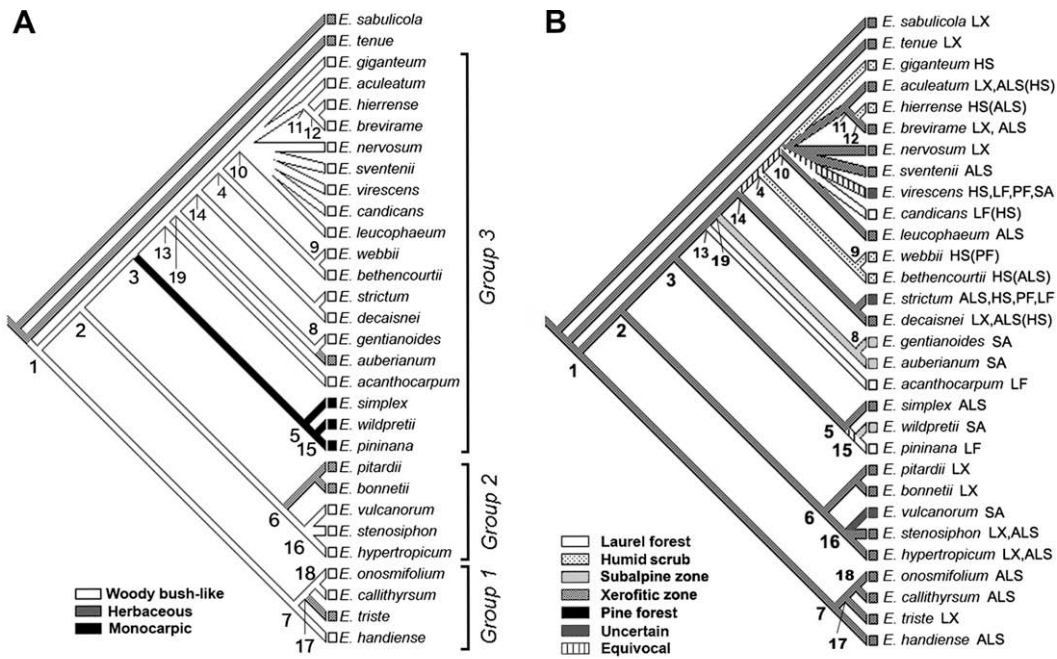


Fig. 3. Hypothesis of character evolution for woodiness (A) and habitat (B) based on one of the 198 most-parsimonious trees obtained in the *D6DES*-p analysis. This tree is congruent with the Bayesian inference (see text). The character reconstruction was obtained by implementing “all most parsimonious states” optimization in MacClade (Maddison and Maddison, 1992). Numbers beside nodes indicate node number (see Table 3). Particular abbreviations are: LX, Lowland xerophytic to coastal desert; ALS, arid lowland scrub; HS, humid scrub; PF, pine forest; LF, laurel forest; SA, subalpine zone.

lineage divergence of extant species (node 1); (ii) <8.2 Ma (mean values of 4.18 and 5.15) for the divergence of the second lineage split giving rise to Cape Verde species (node 2); (iii) <6.4 Ma (mean values of 2.74 and 3.91) for the first evidence of dramatic phylogenetic radiation into five sublineages of 19 species (node 3); and (iv) <3.4 Ma (mean values of 1.31 and 0.51) for the second radiation of 9 species (node 10). Time estimates for the other divergence times are given in Table 2 and depicted in Fig. 4. We hereafter discuss mean timing values provided by the ML approach for the sake of brevity and to facilitate comparison with most angiosperm studies.

4. Discussion

4.1. Δ^6 -Desaturase sequences in plant phylogenetics

The Δ^6 -desaturase promoter (*D6DES*-p) shows great promise as a new molecular marker in plant phylogenetics for several reasons: (i) a high number of variable (149) and informative (82) nucleotide substitutions in a fragment encompassing less than 400 shared

positions; (ii) ease of sequence alignment within the same genus; (iii) low homoplasy values (CI = 0.82; RI = 0.90) given the number of accessions (30) and variable sites; (iv) low ti:tv ratio, unbiased base pair composition, and lack of saturation preventing problems of long branch attraction and statistical inconsistency associated with these issues; and (v) single-copy condition, while alternative nuclear markers are usually taken from multiple to low-copy genes. Previous Southern blotting experiments (García-Maroto et al., 2002) revealed a single-copy locus for *D6DES*-p thus avoiding the problems associated with paralogy. Our results indicate that this desaturase promoter has the potential to be used as a phylogenetic marker in plants containing the Δ^6 -acyl-desaturase enzyme involved in the synthesis of polyunsaturated fatty acids.

4.2. The colonization history of Macaronesian *Echium*

Together, the volcanic history, geographic isolation, and disarmonic biota of the Macaronesia archipelagos all suggest that plant life arrived by long-distance dispersal and undertook complex

Table 2

Divergence times (\pm standard deviations) of *D6DES*-p sequences based on maximum likelihood and Bayesian estimators (see text for description of different analyses) and calculations for the relative rate test implemented in r8s.

Node number ^a	Maximum likelihood clock method	Bayesian relaxed-clock method	Relative rate test				
			Likelihood clock model	Likelihood non-clock	LRStat	df ^b	p
1	6.564 \pm 1.398	6.950 \pm 3.91	–	–	–	–	–
2	4.185 \pm 1.366	5.150 \pm 3.04	–72.83	–72.38	0.91	1	0.35
3	2.745 \pm 1.115	3.913 \pm 2.44	–55.42	–53.95	2.93	4	0.6
4	2.225 \pm 1.077	1.959 \pm 1.44	–30.77	–29.15	3.24	1	0.1
5	1.183 \pm 0.794	1.747 \pm 1.38	1.99	–5.61	–15.20	1	<0.001
6	3.304 \pm 1.143	4.001 \pm 2.49	–13.59	–12.34	2.50	1	0.15
7	2.856 \pm 0.788	3.560 \pm 2.40	–11.75	–10.59	2.32	1	0.15
8	1.904 \pm 1.035	2.150 \pm 1.54	–3.94	–3.80	0.29	1	0.6
9	0.186 \pm 0.224	0.499 \pm 0.589	–2.69	–1.31	2.77	1	0.1
10	1.310 \pm 1.516	0.509 \pm 0.463	–23.12	–10.12	26.02	6	<0.001

^a Node numbers as in Fig. 4.

^b df, degrees of freedom.

Table 3

Means \pm confidence intervals (95%) of posterior probabilities of Bayesian inference character-state evolution after successive iterations (9,000,000) by reversible-jump (RJ) Markov chain Monte Carlo (MCMC). Ancestral character state traced in MacClade (Maddison and Maddison, 1992) is marked in bold (see Fig. 3). Values in the table reflect estimates based on averaging over 2000 Bayesian trees. The 95% confidence intervals of the posterior probabilities were all less than ± 0.28 . Habitat character states, herbaceous/woody bush-like/monocarpic. Habitat character states, laurel forest/xerophytic zone humid scrub/subalpine zone/pine forest.

	Habitat	Habitat
Root	0.61 /0.26/0.13	0.18/ 0.27 /0.19/0.18/0.18
Node 1 ^a	0.14/ 0.78 /0.08	0.19/ 0.25 /0.19/0.19/0.18
Node 2	0.14/ 0.81 /0.05	0.20/ 0.25 /0.18/0.21/0.16
Node 3	0.04/ 0.89 /0.07	0.27/ 0.17 /0.19/0.23/0.14
Node 4	0.01/ 0.98 /0.01	0.14/0.26/0.37/0.11/0.12
Node 5	0.00/0.00/ 1.00	0.22/ 0.36 /0.11/0.21/0.10
Node 6	0.45/ 0.49 /0.06	0.15/ 0.36 /0.16/0.18/0.15
Node 7	0.13/ 0.83 /0.04	0.13/ 0.48 /0.14/0.13/0.12
Node 8	0.36/ 0.59 /0.05	0.13/0.09/0.11/ 0.55 /0.12
Node 9	0.00/ 1.00 /0.00	0.02/0.01/ 0.93 /0.02/0.02
Node 10	0.00/ 1.00 /0.00	0.13/0.37/0.34/0.08/0.08
Node 11	0.01/ 0.98 /0.01	0.10/ 0.47 /0.26/0.01/0.09
Node 12	0.00/ 1.00 /0.00	0.06/ 0.22 /0.62/0.05/0.05
Node 13	0.02/ 0.97 /0.01	0.26/ 0.16 /0.20/0.23/0.14
Node 14	0.02/ 0.97 /0.01	0.16/ 0.17 /0.24/0.27/0.15
Node 15	0.01/0.00/ 0.99	0.38/0.05/0.10/0.36/0.10
Node 16	0.00/ 1.00 /0.00	0.07/ 0.54 /0.07/0.25/0.06
Node 17	0.33 /0.60/0.07	0.16/ 0.18 /0.24/0.27/0.15
Node 18	0.01/ 0.99 /0.00	0.02/ 0.92 /0.02/0.02/0.02
Node 19	0.03/ 0.95 /0.01	0.15/ 0.42 /0.15/0.14/0.14

^a Node coding as in Fig. 3.

evolutionary processes in the last 21 Ma (Carracedo et al., 2002). The spectacular differentiation of *Echium* attracted the attention

of island biologists in the last century (Carlquist, 1965; Bramwell, 1975), but accurate hypotheses of colonization and evolutionary patterns of Macaronesian species remained elusive (but see Böhle et al., 1996). The reconstruction of major *D6DES*-p lineages suggest a sequential colonization in Macaronesia resulting in early differentiation of two currently species-poor lineages (groups 1 and 2), followed by the emergence of a species-rich lineage (group 3) containing a large group of 19 species (Fig. 4, Supplementary Fig. 1). High molecular *D6DES* variation, broad across-lineage species distribution, and sister-group relationships of Canarian species are congruent with the previous hypothesis of an initial colonization and diversification on the Canary Islands, followed by secondary colonization of Madeira and the Cape Verde group (Böhle et al., 1996). Once again, the Canary Islands appear to have harbored the ancestor responsible for spawning new lines of evolution to distant archipelagos (Baldwin et al., 1998). In our historical reconstruction (Supplementary Fig. 1), a single colonization from the Canarian islands to the Cape Verde islands is suggested, whereas limited resolution in clade 3 prevented us from concluding whether high levels of *D6DES* sequence divergence (2% K-2-p) between the two Madeiran species is the result of a single, ancient introduction or multiple colonizations. Irrespective of the actual agents responsible for Macaronesian dispersal to other oceanic islands, tree-based reconstructions of dispersal-trait ancestry for the Canarian native flora shed light on the ability of successful colonizers with endozoochorous (34%) and epizoochorous (8%) syndromes, as well as a considerable number of successful unassisted diaspora (24%) (Vargas, 2007). Although no specific long-distance mechanism has been described (except possibly adhesive potential in Ridley (1930)), the recurrent success of *Echium*

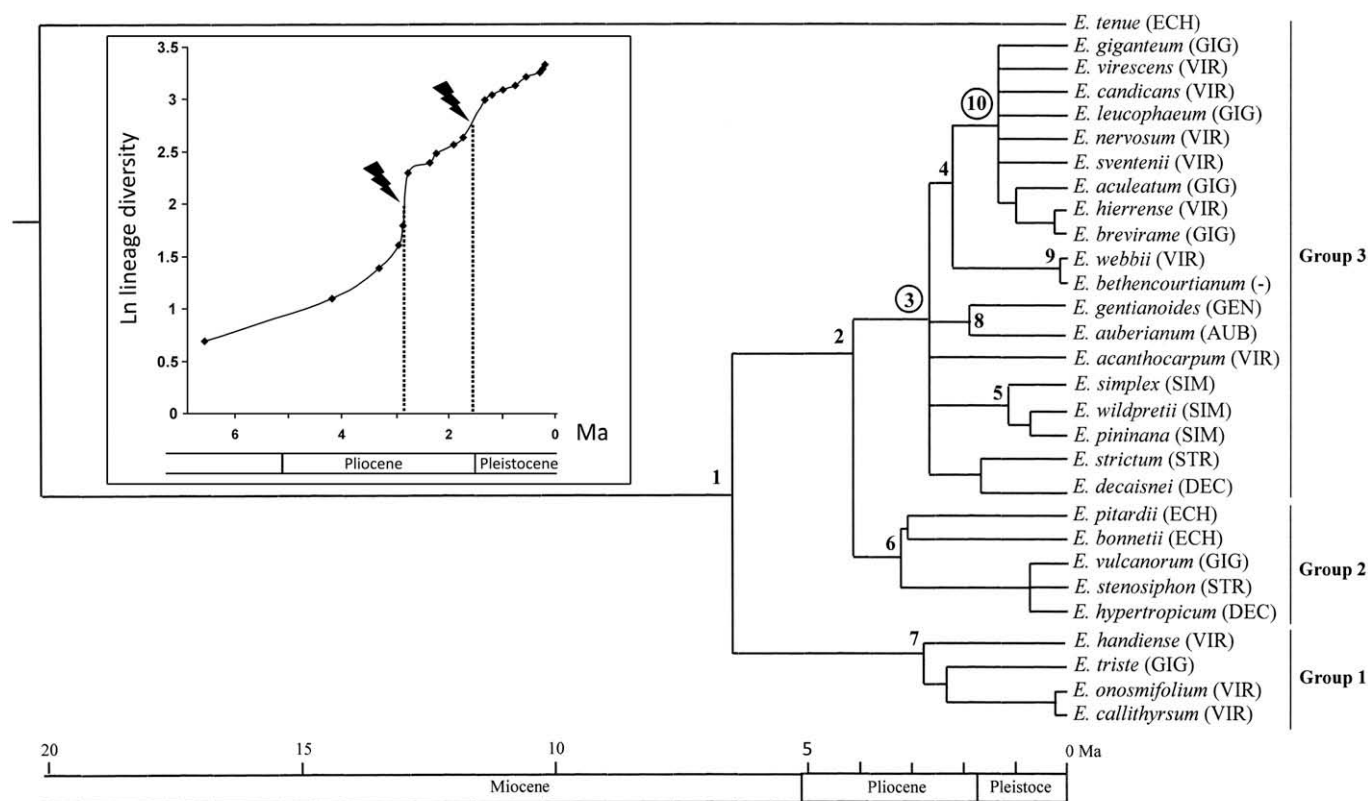


Fig. 4. Chronogram of 1000 maximum likelihood (ML) trees from the penalized likelihood analysis as implemented in r8s (Sanderson, 2003). Ages are based on a 20.6-Ma calibration point for the continental-insular divergence at the maximum age conceivable for the oldest subaerial rocks in Macaronesia (Fuerteventura) (Carracedo et al., 2002). Confidence limits for relevant nodes, represented by numbers above nodes, are also provided in Table 2. The two radiation events at about 3 and 1 Ma are marked by circles enclosing the node numbers 3 and 10. The inset graph depicts the natural logarithm (Ln) trace of lineage accumulation through time (i.e., lineage-through-time plot). Arrows indicate two significant increments in lineage numbers postdating the two strong aridity events of the Pliocene (ca. 4 Ma) and the Pleistocene (<1.8 Ma).

dispersal is attested to not only by inter-archipelago colonization from the Canary Islands to Cape Verde (a distance of 1600 km) and to Madeira (a distance of 400 km) (Fig. 1), but also by colonization within the Canary Islands. Prompt dispersal events between the youngest islands, such as La Palma and El Hierro (<2 Ma) (Supplementary Fig. 1), reflect an ongoing process of colonization of new, uplifting islands since the arrival of the first *Echium* colonizer.

4.3. Timing of differentiation

Substitution rates of ITS sequences (Böhle et al., 1996) and geological timing of Macaronesian island formation are consistent with our *D6DES* estimate of species and lineage divergence as calibrated at 20.6 Ma (Fig. 4). Despite the fact that our calibration point is not based on fossil records, the molecular-clock approaches are consistent with the sharing of most recent common ancestors, island ages, and present-day species distributions. Based on these results, we can infer that the introduction of *Echium* in Macaronesia may have taken place shortly after the first Canary Island was formed. This lineage dating implies that early divergence occurred in the Upper Miocene for Canarian *Echium*, and that a high number of extant species (19) are the result of an explosive lineage divergence (group 3) in the middle Pliocene, as previously calculated based simply on levels of sequence divergence (Böhle et al., 1996).

Climatic changes have been recorded in that area, coincident with the establishment of the cold Canarian sea current ca. 4 Ma (Meco et al., 2005). The constant warm climate of the region was initially replaced by alternating periods of strong aridity and heavy rains (Meco et al., 2005). This situation culminated in the first glaciation in the northern-hemisphere followed by an intense aridity crisis preceding the Pleistocene (ca. 1.8 Ma) (Meco et al., 2006). Irrespective of the molecular-clock method used, two radiations likely occurred after the onset of these two dryness events, as suggested by the two divergence estimates at nodes 3 and 10 (see Table 2). However, dry environments similar to those found in the Mediterranean basin and all the Canarian islands may have been in existence since island formation. MP optimization of character evolution consistently reveals an ancestral xerophyllous state for the *Echium* founder colonizing Macaronesia (Fig. 3B). In fact, the Macaronesian flora has been linked to that spreading from the Mediterranean basin and adjacent deserts in the late Miocene and Pliocene when the first northern-hemisphere glaciation and the desertification of most of northern Africa led to massive extinction and migration of plants (Fauquette et al., 1999). Xerophyllous ancestry at multiple nodes in the McClade optimization of the phylogenetic reconstruction (Fig. 3B), coupled with the predominant xerophyllous of continental species and the large extent of xeric habitats in Macaronesia, lead us to hypothesize that speciation from early colonizers has been continuously occurring in dry environments (Jorgensen and Olesen, 2001). In summary, although ongoing xeric habitats have been in existence since the Canary Islands' origin, we propose that the advent of strong aridity in two events of the Pliocene (ca. 4 Ma) and the Pleistocene (<1.8 Ma) may have accelerated two radiation processes (inset in Fig. 4).

4.4. Testing adaptive radiation in *Echium*

Adaptive radiation is defined as the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage, and it is characterized by four distinctive features: (1) common ancestry, (2) rapid speciation, (3) phenotype–environment correlation; and (4) trait utility (Schluter, 2000). Morphological differentiation from a single ancestor was interpreted as adaptive radiation *per se* through most of the 20th century (Givnish and Sytsma, 1997). In

light of its considerable degree of morphological diversity, Canarian *Echium* was one of the first genera used to support morphological radiation of plants on oceanic islands (Carlquist, 1965; Lems and Holzapfel, 1968). The inferred phylogenetic histories (Fig. 2) further support profound morphological radiation in Macaronesian lineages of *Echium* displaying disparate morphologies acquired in the course of evolution from a single colonizer, as exemplified by the recognition of seven taxonomic sections (Bramwell, 1972).

Interdependence of environments, instead of morphology *per se*, and lines of evolution were explicitly defended by Lems and Holzapfel (1968) and Bramwell (1975) for Canarian *Echium*. The character change of leaf-trichome types (Lems and Holzapfel, 1968) and the phylogenetic relationships of species (Böhle et al., 1996; this paper) exemplify a dynamic evolution. Leaves with spineless and deep veins on the upper leaf surface canalizing water to dripping points at the apex were associated with laurisilva species (*Echium acanthocarpum*, *Echium candicans*, *Echium pininana*), whereas spiny and silky leaves prevailed in species of xeric habitats (e.g., *Echium aculeatum*, *Echium breviflorum*) (Bramwell, 1973). The *D6DES* inference for this set of characters provides strong evidence for the maintenance of xerophyllous in all lineages coupled with the independent exploitation of laurel forests by *Echium* (Fig. 3B). Our analysis demonstrates that the circularity of using morphological characters subject to selection can lead to misinterpretations of sectional taxonomy and species relationships (Bramwell, 1972, 1973, 1975) (Fig. 2), particularly in the accelerated change occurring on oceanic islands (Givnish and Sytsma, 1997; Baldwin and Sanderson, 1998).

Simpson (1953) added a new twist to the definition of adaptive radiation by introducing a temporal scale. This refinement takes on a deeper meaning in oceanic islands a few million years old, which is a relatively short span of time to investigate island speciation from continental relatives. Instances of radiation on Hawaii (<6 Ma), the Galapagos (<5 Ma), and Macaronesia (<21 Ma) were considered adaptive radiations because profound morphological and ecological shifts occurred in a relatively short period of time (Carlquist, 1974). Polytomies and short internal *D6DES* branches occurring exclusively in Madeiran–Canarian species (group 3 in Fig. 4) lead us to infer rapid radiation within the radiation historically considered for *Echium*. Our time-calibrated phylogeny (Fig. 4) suggests that two periods of explosive radiation resulted in a high number of lineages and species (19), most likely in the last five million years (Pliocene–Quaternary). Fundamental causes of rapid differentiation are poorly understood and have been related to divergence selection in different habitats (Schluter, 2000). Major ecological conditions were already in existence long before these two extremely rapid radiations occurred in Macaronesia (Meco et al., 2005, 2006). Accordingly, this cladogenetic pattern of evolution may simply reflect survival of previous explosive radiations of *Echium* widely extinct since island formation (21 Ma). Alternatively, dramatic shifts in paleoclimatic conditions in the Pliocene may have favored the emergence of new microhabitats within existing biotic zones that were successfully exploited (Fauquette et al., 1999). It is interesting to note a correlation between the two accelerated speciation events and two dramatic climatic changes recorded in Macaronesia: the Pliocene establishment of a strong seasonality and aridity coincident with the advent of the cold Canarian sea current (ca. 4 Ma), and the onset of the climatic changes of the Pleistocene (1.8 Ma) (Meco et al., 2005, 2006). Chronograms of additional plant groups will furnish time-calibrated phylogenies to elucidate whether a recurrent pattern of explosive radiation occurred in the majority of large genera of the Macaronesian flora as a result of these two climatic events. Therefore, *Echium* radiations not only fulfill traditional predictions of adaptive radiation (i.e., common ancestry, rapid speciation, and phenotype–environment correlation), but also, uniquely among

Macaronesian species, utility of the woodiness trait (Schluter, 2000).

4.5. Evolution of woodiness

Non-exclusive hypotheses have traditionally been proposed to account for spectacular woodiness examples in the neo-flora of oceanic islands (Wallace, 1878; Carlquist, 1965; Baldwin et al., 1998). Selection for successful pollination with large, long-lasting inflorescences, niche competition among initial colonizers, and promotion of the outbreeding ratio to overcome inbreeding depression may be related to *Echium* longevity and woodiness (Böhle et al., 1996). Irrespective of the causes generating woodiness, the trait utility of this character is manifested by the large number of woody plant groups that rapidly evolved from herbaceous ancestors not only in Macaronesia (*Sonchus*, *Isoplexis*, *Aeonium* group, *Pericallis*), but also in the Hawaiian (silversword alliance, *Schieda*), Galápagos (*Scallesia*), and Juan Fernández (*Dendroseris*) archipelagos.

The secondary growth responsible for woody habit is an ancient evolutionary process predating the divergence of gymnosperms and angiosperms. Recent molecular data indicate that genes and basic mechanisms regulating woody growth have also been maintained in herbaceous species, as exemplified by the model plant *Arabidopsis* (Groover, 2005). These sources of evidence provide an interesting framework to understand the rapid acquisition of woodiness in island radiations, since not only the genes but also the basic genetic architecture appear to have already been present in herbaceous ancestors. Although we sampled only a few mainland species (2), the facts that many *Echium* species from the continent are annuals, and that a basal-most position of woody species is inferred in our character-evolution reconstruction using *D6DES*-p sequences, suggest an early transition to woodiness of Macaronesian *Echium*. Then, an early acquisition of woody forms appears to have been maintained and spawned into the three prime lineages (Fig. 3A). Trait utility of plant habit in Macaronesia is additionally manifested by further shifts. Reversal to an herbaceous condition had not, however, been documented in Macaronesian plant groups after acquisition of

bush-like forms once (*Sonchus*, *Isoplexis*, *Aeonium* group) or multiple times (*Pericallis*) (Kim et al., 1996; Panero et al., 1999; Mort et al., 2002; Bräuchler et al., 2004). We herein confirm reversal to an herbaceous condition in woody lineages of *Echium* (Böhle et al., 1996) and report for the first time three independent formations of annual species (*E. bonnetii*, *E. pitardii*, *E. triste*) in dry habitats of Macaronesia (Fig. 3A). *Echium* lineages undergoing selection for loss of woodiness may have independently met similar conditions. A dynamic evolution of woodiness and herbaceousness supports the view that recycling of genetic mechanisms related to secondary growth might play an active role in flowering plants of oceanic islands (Carlquist, 1974), once given enough diversification time (predating the Pliocene in *Echium*). We argue that strong selective pressure in dry habitats resembling those of African deserts, where over 56% of the flora is annual (Braun-Blanquet, 1964), may be responsible for herbaceous return in the three xerophyllous annuals. Whether future co-option of genes and regulatory mechanisms will drive herbaceousness from woody Macaronesian plants besides *Echium* is a matter of uncertainty. However, one might expect them to do so, considering the recurrent shifts of vascular plants on the continent where differential growth of the shoot-apical meristem gave rise to multiple woody and herbaceous forms during the evolution of cambium (Groover, 2005).

Acknowledgments

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Appendix A

See Appendix A.

Appendix A

Material list and characteristics of the *Echium* taxa including ecology, plant habit, geographical distribution, collection site, voucher, and GenBank Accession No. of the *D6DES*-p sequences. Ecological ranges are abbreviated as follows. LX: Lowland xerophytic to coastal desert; ALS, arid lowland scrub; HS, humid scrub; PF, pine forest; LF, laurel forest; SA, subalpine zone. Species distribution is indicated for individual Canarian islands. PA, La Palma; TE, Tenerife; FU, Fuerteventura; LA, Lanzarote; GO, La Gomera; HI, El Hierro, GC, Gran Canaria. CVE and MAD stand for the Cape Verde and Madeira archipelagos, respectively.

Taxa	Ecology	Habit	Distribution	Collection site	Voucher	GenBank Accession No.
<i>E. acanthocarpum</i> Svent.	LF	Candelabra tree	GO	Roque de Agando (La Gomera), Canaries	RJBM16-PV	DQ315582
<i>E. aculeatum</i> Poirret	LX, ALS (HS)	Candelabra tree	TE, GO, HI	Cumbres de Masca (Tenerife), Canaries	RJBM04-PV	DQ315568
<i>E. auberianum</i> Webb & Berth.	SA	Perennial herb	TE	Los Azulejos (Tenerife), Canaries	RJBM24-PV	DQ315596
<i>E. bethercourtii</i> Santos	HS (ALS)	Candelabra tree	PA	Gallegos (La Palma), Canaries	RJBM21-PV	DQ315595
<i>E. bonnetii</i> Coynei var. <i>bonnetii</i>	LX	Annual–biannual herb	TE, GC, FU, LA	Volcán de Guimar (Tenerife), Canaries	RJBM02-PV	DQ315578
<i>E. breviflorum</i> Spr. & Hutch.	LX, ALS	Candelabra tree	PA	Barranco del Río (La Palma), Canaries	RJBM07-PV	DQ315594
<i>E. callithyrsum</i> Webb	ALS	Candelabra tree	GC	Tenteniguada (Gran Canaria), Canaries	RJBM18-PV	DQ315573
<i>E. candicans</i> DC	LF (HS)	Candelabra tree	MAD	Malhada Valha (Madeira) Madeira	RJBM15-PV	DQ315587
<i>E. decaisnei</i> Webb & Berth. subsp. <i>decaisnei</i>	LX, ALS (HS)	Candelabra tree	GC	Araucas (Gran Canaria), Canaries	RJBM25-PV	DQ315593
<i>E. gentianoides</i> Webb ex Coynei	SA	Candelabra tree	PA	Cumbre de Garafia (La Palma), Canaries	RJBM23-PV	DQ315575
<i>E. giganteum</i> L. fil.	HS	Candelabra tree	TE	La Florida (Tenerife), Canaries	RJBM03-PV	DQ315567
<i>E. handiense</i> Svent.	ALS	Candelabra tree	FU	Pico de la Zarza (Fuerteventura), Canaries	RJBM22-PV	DQ315577
<i>E. hierrense</i> Webb ex Bolle	HS (ALS)	Candelabra tree	HI	El Golfo (Hierro), Canaries	RJBM19-PV	DQ315581
<i>E. hypertropicum</i> Webb	LX, ALS	Candelabra tree	CVE	Serra Malagueta (Sao Tiago), Cape Verde	RJBM26-PV	DQ315592
<i>E. leucophaeum</i> Webb ex Spr. & Hutch.	ALS	Candelabra tree	TE	Las Mercedes (Tenerife), Canaries	RJBM06-PV	DQ315588
<i>E. nervosum</i> Dryand. In Aiton	LX	Candelabra tree	MAD	Funchal (Madeira) Madeira	RJBM14-PV	DQ315574
<i>E. onosmifolium</i> Webb & Berth	ALS	Candelabra tree	GC	Paso de la Plata (Gran Canaria), Canaries	RJBM17-PV	DQ315572

Appendix A (continued)

Taxa	Ecology	Habit	Distribution	Collection site	Voucher	GenBank Accession No.
<i>E. pininana</i> Webb & Berth.	LF	Monocarpic–rosette–tree	PA	Cubo de la Galga (La Palma), Canaries	RJBM11-PV	DQ315586
<i>E. pitardii</i> A. Chev. var. <i>pitardii</i>	LX	Annual–biannual herb	LA	Famara (Lanzarote), Canaries	RJBM01-PV	DQ315569
<i>E. sabulicola</i> Pomel	LX	Annual–biannual herb	Mediterr./Morocco	Moulouya valley (Oujda), Morocco	HUAL-3218	DQ315570
<i>E. simplex</i> DC	ALS	Monocarpic–rosette–tree	TE	Taganana (Tenerife), Canaries	RJBM10-PV	DQ315584
<i>E. stenosphon</i> Webb	LX, ALS	Candelabra tree	CVE	Monte Verde (Sao Vicente), Cape Verde	RJBM28-PV	DQ315591
<i>E. strictum</i> L. fil.	ALS, HS, PF, LF	Candelabra tree	TE, GC, PA, GO, HI	San Antonio (Tenerife), Canaries	RJBM27-PV	DQ315590
<i>E. sventenii</i> Bramw.	ALS	Candelabra tree	TE	Barranco Seco de Adeje (Tenerife), Canaries	RJBM13-PV	DQ315579
<i>E. tenue</i> Lam.	LX	Annual–biannual herb	Morocco	Casablanca, Morocco	HUAL-3220	DQ315571
<i>E. triste</i> Svent.	LX	Annual to perennial herb	GC, TE, GO	Barranco Manchitas (Tenerife), Canaries	RJBM05-PV	DQ315576
<i>E. virescens</i> DC var. <i>angustissimum</i> Bolle	HS, LF, PF, SA	Candelabra tree	TE	Barranco de Tamadaya (Tenerife), Canaries	RJBM12-PV	DQ315583
<i>E. vulcanorum</i> A. Chev.	SA	Candelabra tree	CVE	Cha das Caldeiras (Ilha do Fogo), Cape Verde	RJBM08-PV	DQ315589
<i>E. webbii</i> Coynccii	HS (PF)	Candelabra tree	PA	La Cumbrecita (La Palma), Canaries	RJBM20-PV	DQ315580
<i>E. wildpretii</i> Pearson ex Hook fil. subsp. <i>wildpretii</i>	SA	Monocarpic–rosette–tree	TE, PA	Los Azulejos (Tenerife), Canaries	RJBM09-PV	DQ315585

Appendix B

See Appendix B.

Appendix B

Bayesian inference of trait evolution after successive iterations of the chain (9,000,000) by reversible-jump Markov chain Monte Carlo. Means \pm confidence intervals (95%) of the log-likelihoods (Lh) and rate coefficients are shown.

Trait	Log-likelihood (Lh)	qAB	qAC	qAD	qAE	qBA	qBC	qBD	qBE	qCA	qCB	qCD	qCE
Habitat	−33.08 \pm 0.05	25.02 \pm 0.13	3.60 \pm 0.77	16.49 \pm 0.43	26.27 \pm 0.18	56.59 \pm 0.21	24.87 \pm 0.22	41.63 \pm 0.77	39.48 \pm 0.37	47.97 \pm 0.07	42.33 \pm 0.36	43.74 \pm 0.45	43.90 \pm 0.02
Habit	−15.87 \pm 0.008	13.31 \pm 0.07	10.71 \pm 0.07	–	–	13.35 \pm 0.06	5.68 \pm 0.04	–	–	9.70 \pm 0.07	10.35 \pm 0.07	–	–
qDA	–	qDB	–	qDC	–	qDE	–	qEA	–	qEB	–	qEC	–
50.89 \pm 0.33	–	41.86 \pm 0.13	–	28.61 \pm 0.01	–	45.32 \pm 0.017	–	51.80 \pm 0.46	–	38.40 \pm 0.88	–	25.02 \pm 0.41	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2009.04.009](https://doi.org/10.1016/j.ympev.2009.04.009).

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