

Genetic diversity of three endangered species of *Echium* L. (Boraginaceae) endemic to Cape Verde Islands

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Abstract. *Echium hypertropicum*, *E. stenosphon* and *E. vulcanorum* are the three endemics representative of the genus *Echium* (Boraginaceae) in Cape Verde archipelago. The aim of this study is to provide a first attempt at estimating genetic diversity among natural populations of these endangered *Echium* species based on RAPD, so as to provide data available for future appropriate strategies for their conservation. PCO and UPGMA of RAPD analysis suggests a close genetic proximity between the Southern endemic species (*E. hypertropicum* and *E. vulcanorum*) and shows that the levels of polymorphism strongly differ between these two *Echium* species (27 and 29% respectively) and *E. stenosphon* (74%), the Northern endemic species. Mantel test also corroborates a close genetic proximity between genetic and geographic data. Population genetic analysis of *E. stenosphon* revealed low levels of gene flow between islands ($N_m = 0.32$) being S. Nicolau the most isolated as evident in PCO. Furthermore the differentiation between groups of individuals belonging to putative subspecies was tested by AMOVA. According to our results there is no genetic basis to consider the two subspecies of *E. stenosphon* namely ssp. *stenosphon* and ssp. *lindbergii*. Our results enable us to suggest that *E. stenosphon* must be ranked as a Threatened species. Measures aiming at conservation of *E. hypertropicum* and *E. vulcanorum* must be implemented at short-term taking into account the small number of existing plants and its low genetic variability.

Introduction

Echium is a genus of the Boraginaceae with irregular flowers, predominantly insect pollinated, that is distributed in Macaronesia, Europe, West Asia, North and South Africa (Retief and Wyk 1998). 27 species of the genus are endemic to the Canary, Madeira and Cape Verde archipelagos of the Macaronesian islands, whereas the remaining congeners (~30) are found in circummediterranean-west Asian Floras (Böhle et al. 1996). *Echium hypertropicum* Webb, *E. stenosphon* Webb and *E. vulcanorum* A. Chev. are the three endemic representatives of the genus in the Cape Verde archipelago.

The archipelago comprises ten islands of volcanic origin divided into the Northern (windward) and Southern (leeward) groups, situated in the Atlantic Ocean outside the westernmost cape of the African mainland, about 500 km West of Senegal and 1500 km South of the Canary Islands (Figure 1). The topography of Cape Verde ranges from dry plains to high, active volcanoes. While all the archipelago is generally dry and has suffered from severe draughts in the 20th century, the mountainous areas receive significantly more rainfall than the lowlands. According to the first Cape Verde red list (Leyens and Lobin 1996), *Echium hypertropicum* as well as *E. vulcanorum* are Endangered and *E. stenosiphon* is considered Vulnerable. Nowadays, these endemic species are limited to a small number of individuals per population, which are being progressively overwhelmed by invasive species, and are also subjected to a number of negative human factors, such as agriculture and pasture or use as firewood. Insular ecosystems such as the Macaronesian archipelagos greatly contribute to the floristic richness and vegetation diversity (Sunding 1973). Nevertheless, the biodiversity of islands is naturally restricted to narrow geographical limits and is extremely vulnerable, as evidenced by the high number of endangered species. The decrease in endemic species populations is a serious problem in Cape Verde, where more than half of the flora consists of exotic species (Brochmann et al. 1997).

The intraspecific variation of these *Echium* species has been considered important, and their taxonomy has been controversial and based only on morphological characters (Bramwell 1972; Martins 1995; Brochmann et al. 1997). Genetic diversity is a critical feature because it contributes directly to the

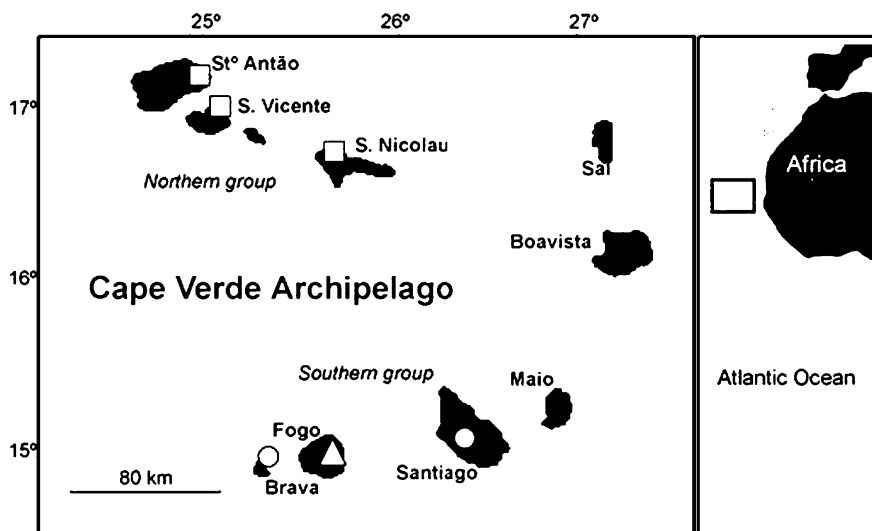


Figure 1. Location of Cape Verde Islands and geographic distribution of *Echium* species: *E. hypertropicum* (●) from Santiago and Brava Islands; *E. vulcanorum* (▲) from Fogo Island; and *E. stenosiphon* (■) from St. Antão, S. Nicolau and S. Vicente.

likelihood of persistence and ecological success of a species (Huenneke 1991; Menges 1991). The preservation of diversity is a fundamental goal in conservation biology, and the study of population genetics has been identified as one of its main priorities (Holsinger and Gottlieb 1991).

Molecular genetic analyses have proven helpful to detect genetic variation and establish species circumscription on a complementing many morphological studies. Random Amplified Polymorphic DNA (RAPD) is a commonly used PCR (polymerase chain reaction) technique for detection of genetic variability due to its simplicity, low cost and unnecessary previous knowledge of the genome (Williams et al. 1990). The RAPD technique is particularly useful for population studies (Parker et al. 1998), as it surveys the entire genome, rather than selected fragments, as with microsatellites and allozyme markers (Kingston et al. 2004). It is appropriate for rare and endangered species, because it requires only small amounts of tissue (Esselman et al. 2000; Fischer et al. 2000).

The aims of this study, based on RAPD analysis, were: (1) to provide a first attempt at estimating genetic diversity among natural populations of the *Echium* species endemic to Cape Verde Islands; (2) determine the infraspecific diversity within *E. stenosiphon* and evaluate the distinctiveness of subspecies in a molecular basis.

Studied species and taxonomy

Echium hypertropicum and *E. vulcanorum* are endemic to the Southern group of Cape Verde Islands (Figure 1). These species are perennial woody candelabra shrubs up to 2 m high with strong compact branches. They bear large inflorescences, which are often pyramidal in shape with white flowers with blue stripes. *Echium hypertropicum* differs from *E. vulcanorum* by its subglabrous branches and greyish, finely hispid leaves (Brochmann et al. 1997). According to previous records *Echium hypertropicum* occurs at Santiago and Brava whereas *E. vulcanorum* is endemic to Fogo Island.

Echium stenosiphon is endemic to the Northern islands of S. Nicolau, S. Vicente and St.º Antão (Figure 1). *Echium stenosiphon* is a moderately branched, sub-shrub up to 1.5 m high. The inflorescence is lax with violet-blue to pink flowers. This species shows considerable variation, especially in indumentum characters, leaf-shape and dimensions of the corolla tube (Bramwell 1972; Brochmann et al. 1997). Petterson (1960) described deviating specimens from mountain areas in St.º Antão as *Echium lindbergii* B. Petters., which was later reduced to *E. stenosiphon* ssp. *lindbergii* (Pett.) Bramw. According to Bramwell (1972), the taxon occurs in mountain areas of St.º Antão, whereas *Echium stenosiphon* ssp. *stenosiphon* is confined to coastal slopes up to 400–500 m in the three Northern islands. The infraspecific taxa were not considered in the Cape Verde Flora (Martins 1995) although the intraspecific morphological variation was noted.

Table 1. Geographic origin of the 57 individuals of *Echium* from Cape Verde Islands included in the study.

Taxon	Island/population size*	Population	Accession	Altitude (m)	Latitude (north)	Longitude (west)		
<i>Echium hypertropicum</i>	Santiago <400 individuals		<i>Ey 1</i>	760	15°11'46"	23°41'35"		
		Serra da	<i>Ey 3</i>	760	15°11'46"	23° 41'35"		
		Malagueta	<i>Ey 5</i>	760	15°11'46"	23°41'35"		
			<i>Ey 26</i>	900	15°03'00"	23°38'30"		
		Pico da Antónia	<i>Ey 27</i>	900	15°03'00"	23°38'30"		
			<i>Ey 28</i>	1000	15°03'00"	23°38'29"		
		Rui Vaz	<i>Ey 29</i>	820	15°02'15"	23°35'57"		
			<i>Ey 30</i>	700	15°03'01"	23°38'32"		
			<i>Ey 35</i>	780	15°10'36"	23°41'14"		
		Serra da	<i>Ey 37</i>	790	15°11'02"	23°41'23"		
		Malagueta	<i>Ey 38</i>	780	15°11'02"	23°41'23"		
			<i>Ey 39</i>	800	15°11'14"	23°41'26"		
		<i>Echium stenosisiphon</i>	S. Nicolau <1000 individuals		<i>Esj 31**</i>	–	–	–
					<i>Esj 32**</i>	–	–	–
					<i>Esn 40</i>	640	16°38'12"	24°21'05"
Ribeira Fajã	<i>Esn 41</i>			640	16°38'05"	24°21'00"		
	<i>Esn 42</i>			1100	16°37'40"	24°21'03"		
Monte Gordo	<i>Esn 43</i>			1050	16°37'43"	24° 20'59"		
	<i>Esn 44</i>			1100	16°37'36"	24°20'59"		
	<i>Esn 45</i>			1050	16°37'20"	24°20'46"		
Ribeira Brava	<i>Esn 46</i>			800	16°37'08"	24°19'58"		
Chamiço	<i>Esn 47</i>			600	16°36'27"	24°20'05"		
Cabeceira da	<i>Esn 48</i>			650	16°36'31"	24°19'11"		
Ribeira Brava	<i>Esn 49</i>			750	16°36'51"	24°19'35"		
R ^a Queimada	<i>Esn 50</i>			70	16°39'06"	24°18'26"		
	<i>Esn 51</i>			200	16°36'32"	24°08'23"		
Ribeira	<i>Esn 52</i>			300	16°36'26"	24°08'20"		
Juncalinho	<i>Esn 53</i>	30	16°36'43"	24°08'24"				
	<i>Esn 54</i>	90	16°36'47"	24°09'42"				

S. Vicente <50 individuals		<i>Esv 55</i>	760	16°52'22"	24°55'59"		
		<i>Esv 56</i>	760	16°52'15"	24°56'00"		
Monte Verde		<i>Esv 57</i>	760	16°52'13"	24°56'01"		
		<i>Esv 58</i>	600	16°52'28"	24°55'50"		
		<i>Esv 59</i>	550	16°52'33"	24°56'00"		
		<i>Esa 60***</i>	960	17°03'44"	25°12'19"		
St. Antão >1000 individuals	Chã de Morte	<i>Esa 61***</i>	1100	17°05'36"	25°13'24"		
		<i>Esa 62***</i>	940	17°04'20"	25°04'56"		
		<i>Esa 63***</i>	1400	17°06'44"	25°03'50"		
		<i>Esa 64***</i>	1450	17°06'47"	25°03'44"		
		<i>Esa 65***</i>	1350	17°05'18"	25°09'16"		
		<i>Esa 66***</i>	1450	17°05'36"	25°08'59"		
		<i>Esa 67***</i>	1460	17°05'36"	25°08'59"		
		<i>Esa 68***</i>	700	17°09'11"	25°05'01"		
		<i>Esa 69</i>	400	17°08'00"	25°02'31"		
		<i>Esa 70</i>	500	17°07'07"	25°03'11"		
		<i>Esa 71</i>	200	17°10'40"	25°02'34"		
		<i>Esa 72***</i>	1500	17°06'04"	25°02'12"		
		<i>Esa 73***</i>	1400	17°06'56"	25°03'38"		
		<i>Esa 74***</i>	1400	17°06'56"	25°03'38"		
		<i>Echium vulcanorum</i>	Fogo	Monte Losna	<i>Ev 6</i>	1780	14°55'23"
<i>Ev 7</i>	1780				14°55'22"	24°20'09"	
<i>Ev 8</i>	1790				14°55'22"	24°20'11"	
<i>Ev 9</i>	1790				14°55'21"	24°19'58"	
<i>Ev 12</i>	1750				14°55'31"	24°19'56"	
Bordeira	<i>Ev 23</i>			2300	14°55'01"	24°21'09"	
	<i>Ev 24</i>			2200	14°54'59"	24°21'07"	
	<i>Ev 25</i>			2000	14°54'56"	24°20'57"	

Notes: (*) approximately population size per island. In situ conservation action at Bordeira region limited the estimating population sizes in Fogo Island. (**) Plants collect from Cape Verde Botanical Garden at Santiago (Esj 31 and 32), which have been propagated by cuttings from S. Nicolau material. (***) *E. stenosphon* Webb ssp. *lindbergii* (Pett.) Bramw.

Material and methods

Sampling

The fieldwork was carried out in the Cape Verde Islands during June 2000 and February 2001. The survey took place in the islands of Santiago, Fogo, S. Nicolau, S. Vicente and St.º Antão. Young leaves for DNA analysis were collected in the field and rapidly dried in ziplock bags containing silica gel, as recommended by Chase and Hills (1991). A total of 57 *Echium* samples representing all surveyed populations of each of the three species were collected (Table 1). In cases where no previous herbarium record was known for the species at that locality, a voucher was collected and deposited at the Herbarium of the Centro de Botânica (LISC) – Instituto de Investigação Científica Tropical of Lisbon – Portugal.

RAPD analysis

DNA was extracted from 0.3 to 0.5 g silica-dried leaves using the CTAB (cetyltrimethylammonium bromide) based method (Doyle and Doyle 1987). RAPD reactions were performed using 20–30 ng of genomic DNA in a Biometra UNO II with a 3-h program of 45 cycles (Yu and Pauls 1992). 80 random primers from Operon Technologies (Alameda, California, USA) kits OPA, OPC, OPD and OPH, were tested. Seventeen primers that revealed a reproducible pattern over several independent PCR reactions were used: OPA03, OPA04, OPC05, OPC08, OPD02, OPD08, OPD11, OPD13, OPD15, OPD20, OPH01, OPH 03, OPH04, OPH05, OPH08, OPH15, OPH20. Amplification products were visualized on agarose gels (1.5% w/v). Sizes of all RAPD fragments were estimated using size standard marker (marker XVI, Boehringer Mannheim) and compared among all gels for the same primer using image analyzer software (Molecular Analyst and Molecular Fingerprinting; BioRad, Inc.). Gel data were summarized in a binary matrix that was edited by comparison with gel images, scoring only fragments that showed a consistent amplification pattern between repeated runs. This procedure greatly reduced the potential number of bands that could have been scored, but avoided mis-scoring of bands for which co-migration could not be sure.

Statistics and multivariate analyses

The edited matrix including all scored RAPD fragments of the 57 individuals of the three species was used to calculate the percentage of polymorphic bands for each of the three *Echium* species. To illustrate the relatedness among these three species, the same matrix was subjected to principal coordinate analyses (PCO) using ADE-4 ver. 1.04 (Thioulouse et al. 1997) and cluster analysis

using un-weighted pair-group method of arithmetic averages (UPGMA) with the Jaccard's index of similarity (NTSYS-pc ver. 2.0, Rohlf 1998). The Mantel test between genetic matrix (Jaccard's index of similarity) and geographic data was performed using NTSYS-pc ver. 2.0 (Rohlf 1998).

To test whether or not populations of *Echium stenosiphon* were isolated by island and to examine hierarchical population structure we estimated the degree of differentiation among populations of this species (Φ) and performed analysis of molecular variance (AMOVA). Phi-statistics (Φ) summarizes the degree of differentiation between population divisions and is analogous to *F*-statistics (Excoffier et al. 1992; Excoffier 2001). For the AMOVA, variation was partitioned at three levels, among regions (islands of S. Nicolau, S. Vicente and St^o Antão), among populations within islands and within populations. We also tested for the genetic differentiation between the two subspecies (*E. stenosiphon* ssp. *stenosiphon* and *E. stenosiphon* ssp. *lindbergii*). AMOVA was partitioned at three different levels: among subspecies, among populations within subspecies and within populations. All calculations were performed with Arlequin ver. 2.0 (Schneider et al. 2000) and Genalex ver. 5.1 (Peakall and Smouse 2001).

Results

Population surveys

Echium hypertropicum: populations of this species appear at Serra da Malagueta, where it can be found between 350 and 800 m of altitude, forming small populations or as isolated individuals mainly at SW or NE exposure. Other populations were found at Rui Vaz (N exposure, altitudes 600–800 m) and few scattered individuals appeared up to 1000 m at Pico da Antónia (Table 1). Despite the fact that this species has been recorded from Brava Island, twice in the last 130 years (Gomes et al. 1995), no plant was found on that island during the fieldwork.

Echium stenosiphon: populations of this species were found at St^o. Antão, S. Vicente and S. Nicolau (Table 1). Two main populations (Ribeira do Juncalinho and Monte Gordo) and other small dispersed nuclei were recorded at S. Nicolau, one at S. Vicente (Monte Verde), and six main populations at St^o. Antão plus some dispersed individuals. This species subsist from zones of low altitude (e.g., 30 m in S. Nicolau) up to 1500 m (St^o. Antão). Populations grow in areas exposed to trade winds from N/NE.

Echium vulcanorum: only two populations of this species were found at Bordeira (the largest) and Monte Losna in Fogo Island. The plants occupy habitats between 1700 and 2300 m altitude, generally with SW/SE exposure (Table 1).

Genetic diversity of *Echium hypertropicum*, *E. stenosisiphon* and *E. vulcanorum*

The use of seventeen primers for the 57 plants of these three species produced a matrix of 144 characters. The primers that revealed the highest level of polymorphism overall were OPH01, OPD15 and OPC08 (Table 2). *Echium stenosisiphon* (Es), the species with the largest distribution in Cape Verde Islands, showed the highest level of polymorphism (74%). *Echium hypertropicum* (Ey) and *E. vulcanorum* (Ev) occur in a reduced number of populations and showed a low level of polymorphism, 27 and 29% respectively (Table 2). *Echium stenosisiphon* displayed the greatest number of loci (115) when compared to *E. hypertropicum* and *E. vulcanorum* (83 and 84 respectively). Four bands obtained with primers OPA3, OPD13, OPD20, and OPH8 were specific and constant to all *Echium stenosisiphon* samples. Three species-specific markers were also obtained with primers OPD13, OPD20 and OPH5 for *Echium vulcanorum* and two (primers OPD2 and OPH5) for *E. hypertropicum*. These two species also shared six markers (primers OPA3, OPD2, OPD8, OPC5, OPH5 and OPH20) that were absent from all *E. stenosisiphon* samples.

Principal coordinate analyses (PCO) – Analysis of the three species showed a clear separation between *Echium stenosisiphon* and the other two *Echium* species: *E. hypertropicum* and *E. vulcanorum* (Figure 2). PCO 1 and PCO 2 represent 70.2% of the total species variation. The species are clearly separated in opposite sides of PCO 1 axis (56.5%) that splits *Echium stenosisiphon* from

Table 2. Primers and percentage of polymorphism detected in 57 plants of *Echium hypertropicum*, *E. stenosisiphon* and *E. vulcanorum*.

Primer	<i>Echium hypertropicum</i>			<i>Echium stenosisiphon</i>			<i>Echium vulcanorum</i>		
	Loci	Poly	% Pp	Loci	Poly	% Pp	Loci	Poly	% Pp
OPA 03	5	0	0	7	3	43	5	0	0
OPA 04	7	1	14	9	6	67	6	1	17
OPC 05	5	0	0	6	4	67	4	0	0
OPC 08	4	1	25	5	5	100	3	2	67
OPD 02	6	1	17	5	3	60	5	1	20
OPD 08	5	1	20	5	3	60	6	1	17
OPD 11	7	3	43	8	6	75	7	2	29
OPD 13	3	0	0	7	4	57	4	0	0
OPD 15	4	2	50	6	6	100	5	2	40
OPD 20	4	1	25	6	4	67	5	2	40
OPH 01	3	2	67	5	5	100	3	2	67
OPH 03	4	1	25	6	4	67	6	2	33
OPH 04	8	4	50	8	5	63	7	4	57
OPH 05	6	2	33	7	6	86	6	2	33
OPH 08	6	3	50	10	8	80	5	2	40
OPH 15	3	0	0	8	7	88	4	1	25
OPH 20	3	0	0	7	6	86	3	0	0
Total	83	22	27	115	85	74	84	24	29

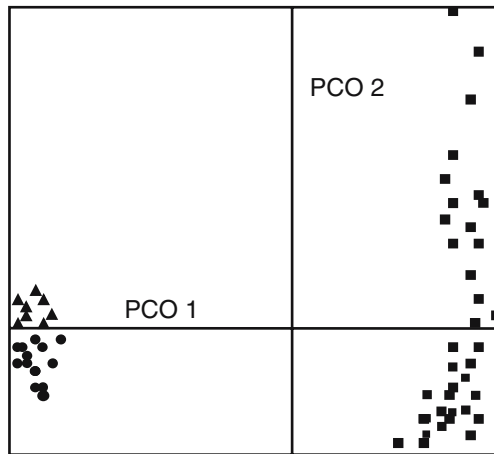


Figure 2. PCO of 57 individual plants of *Echium hypertropicum* (●), *E. vulcanorum* (▲) and *E. stenosphon* (■) [S. Nicolau (Esn), S. Vicente (Esv) and St.º Antão (Esa)] (cf. Table 1). PCO 1 and PCO 2 represent 56.5 and 13.7%, respectively of the variance.

E. hypertropicum and *E. vulcanorum*. In a second PCO analysis including only samples of *Echium hypertropicum* and *E. vulcanorum*, individuals of these two species appear as well separated along PCO 1 axis which accounted for 66.5% of the variation (Figure 3).

UPGMA analysis – UPGMA analysis using Jaccard index of similarity for the 57 individuals of the three species produced a dendrogram that revealed a

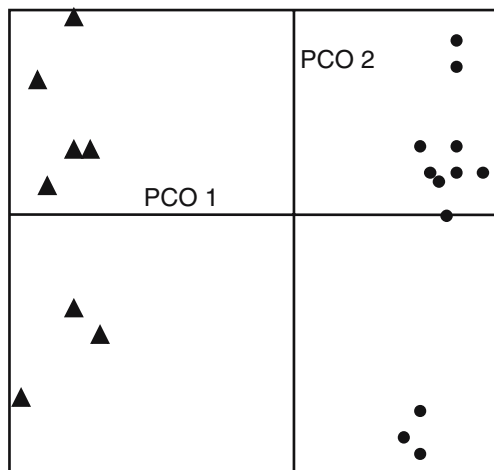


Figure 3. PCO of 20 individual plants of *Echium hypertropicum* (●) and *E. vulcanorum* (▲). PCO 1 and PCO 2 represent 66.5 and 11.4%, respectively of the variance.

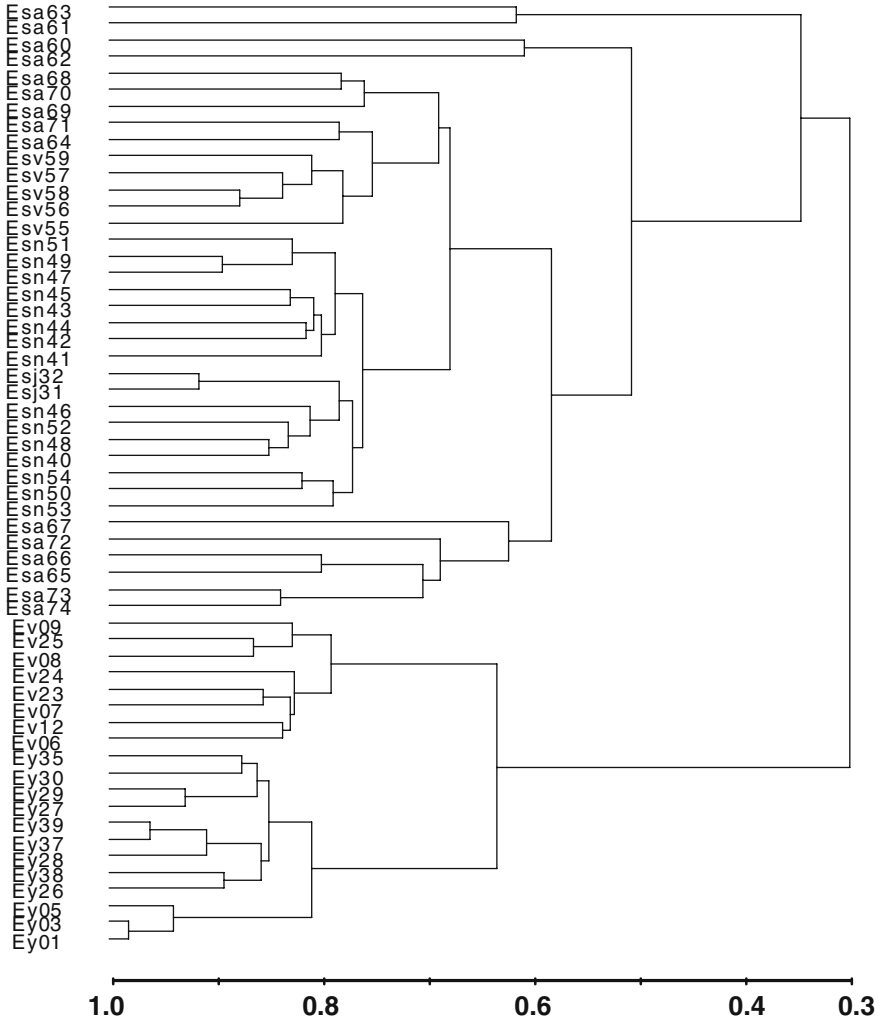


Figure 4. UPGMA analysis of 57 individual plants based on Jaccard's similarity index. *Echium hypertropicum* (Ey), *E. vulcanorum* (Ev) and *E. stenosiphon* (Es).

clear separation of all species (Figure 4). *Echium hypertropicum* (Ey) and *E. vulcanorum* (Ev) form two clusters well separated from *E. stenosiphon* (0.3 similarity). *Echium stenosiphon* appear as a separate entity where it is possible to recognise some clearly well delimited populations (Esn, S. Nicolau).

Mantel Test – The Mantel test with 5000 permutations indicated a *p*-value = 0.0004 for the positive correlation ($r = 0.59$) found between matrices of RAPD data and of geographic distance expressed as UTM co-ordinates, thus evidencing a strong geographic pattern between genotypes.

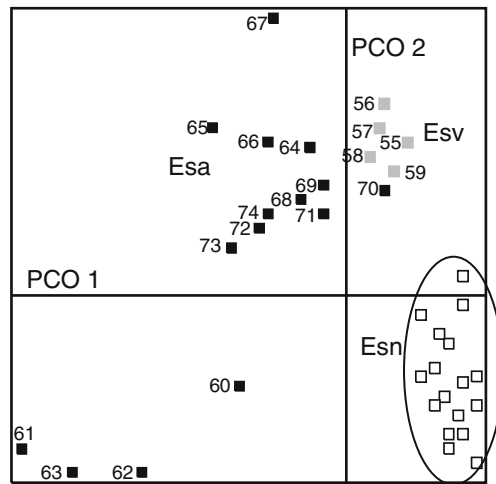


Figure 5. PCO of 37 individual plants of *Echium stenosphon*: S. Nicolau (Esn: □), S. Vicente (Esv:■) and St. Antão (Esa: ■). PCO 1 and PCO 2 represent 41.5 and 11.7%, respectively of the variance.

Echium stenosphon population genetic analysis

The genetic differentiation of *Echium stenosphon* populations was independently analyzed due to its wider range of distribution in three islands of Northern group and the high levels of polymorphism obtained in the overall analysis.

Principal coordinate analyses (PCO) and UPGMA analysis – A partial analysis of *Echium stenosphon* plants reveals a congruent grouping among individuals of the S. Nicolau Island (Figures 5 and 6). Individuals from St. Antão displayed more diversity in terms of RAPD patterns. Furthermore, the individuals Esa 61, 62 and 63 are scattered along the left side of the first axis suggesting that they correspond to the most divergent genotypes when compared with Esn genotypes in the other extreme of the axis.

AMOVA – Analysis of molecular variance within and among *Echium stenosphon* populations from S. Nicolau, S. Vicente and St. Antão revealed that most variance could be attributed to genetic variation within populations (60.6%; Table 3). This analysis showed that levels of genetic diversity detected between islands were proportionally much higher than those within different islands (29.7 and 9.8%). Phi-statistics (Φ) which summarizes the degree of differentiation among population divisions indicated that the degree of differentiation among populations within islands ($\Phi_{sc} = 0.139$) is around half of the differentiation among islands ($\Phi_{ct} = 0.297$). The greatest degree of differentiation being shown by the variation among individuals within populations ($\Phi_{st} = 0.394$).

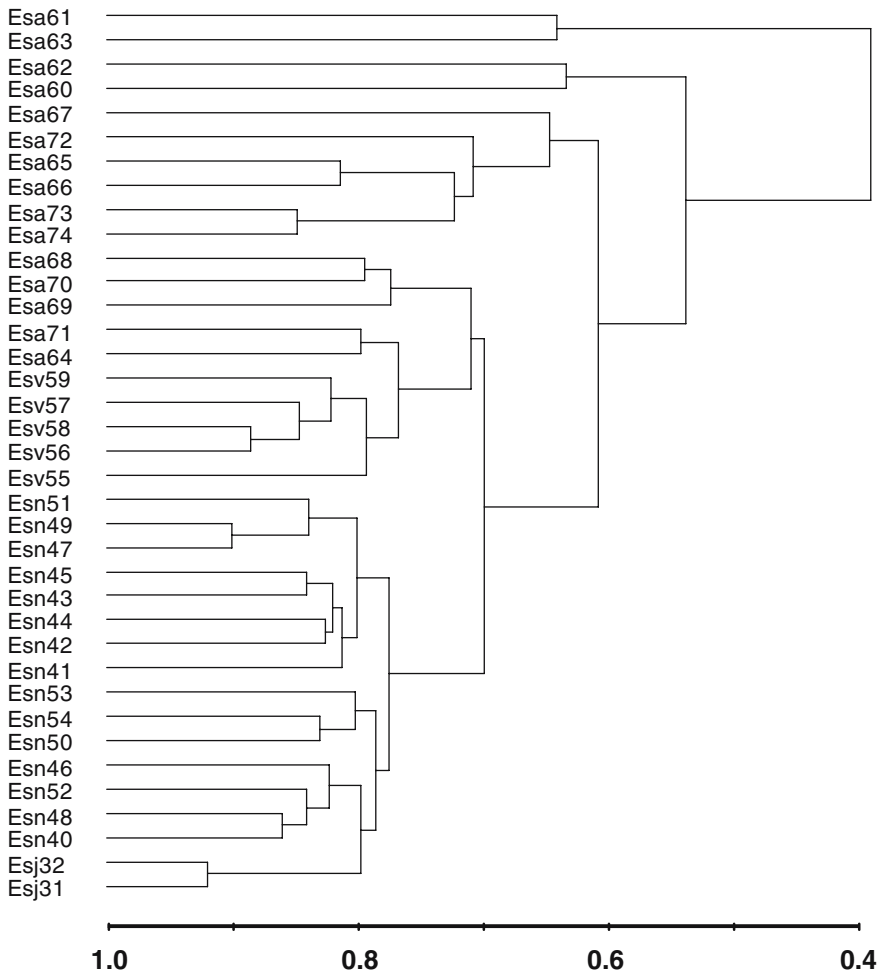


Figure 6. UPGMA of 37 individual plants of *Echium stenosphon* [S. Nicolau (Esn), S. Vicente (Esv) and St. Antão (Esa)] based on Jaccard's similarity index (cf. Table 1).

In a second analysis, the differentiation between groups of individuals putatively belonging to *Echium stenosphon* ssp. *stenosphon* and *E. stenosphon* ssp. *lindbergii* was evaluated (Table 4). One of the groups corresponded to *Echium stenosphon* ssp. *stenosphon* and included all populations from S. Nicolau and S. Vicente Islands, and three individuals (Esa 69; 70; 71; Table 1) that were collected on coastal slopes up to 400–500 m on St. Antão Island. The other group corresponding to *Echium stenosphon* ssp. *lindbergii* comprised all populations from mountain areas of St. Antão, two of them (Esa 73; 74; Table 1) were collected at the same area as that of the type specimen (St. Antão, Cova, 31.12.1953. *H. Lindberg*; holotype: H). The variation among

Table 3. Test of genetic subdivision for populations of *Echium stenosisiphon*.

Level of population hierarchy	d.f.	SSD	Variance component	% of total variation	p-value	Φ-statistics
Variation among islands	2	116.743	4.253	29.657	< 0.001	Φ _{ct} = 0.297
Variation among populations within islands	9	114.567	1.399	9.755	< 0.006	Φ _{sc} = 0.139
Variation within populations	23	199.833	8.688	60.588	< 0.001	Φ _{st} = 0.394

Notes: The AMOVA was partitioned at three different levels: among islands (S. Nicolau, S. Vicente and St^o. Antão), among populations within islands and within populations. The data show the degrees of freedom (d.f), sum of squares deviation (SSD), variance component estimate, percentage of the total variation, phi-statistics (Φ) analogous to *F*-statistics.

Table 4. Test of genetic subdivision for *Echium stenosisiphon* ssp. *stenosisiphon* and *E. stenosisiphon* ssp. *lindbergii*.

Level of population hierarchy	d.f.	SSD	Variance component	% of total variation	p-value	Φ-statistics
Variation among subspecies	1	86.603	4.562	29.947	< 0.001	Φ _{ct} = 0.299
Variation among populations within subspecies	8	111.840	1.364	8.954	< 0.004	Φ _{sc} = 0.129
Variation within populations	25	232.700	9.308	61.098	< 0.001	Φ _{st} = 0.389

Notes: The AMOVA was partitioned at three different levels: among subspecies, among populations within subspecies and within populations. Under *E. stenosisiphon* ssp. *lindbergii* are included all individuals but three (Esa 69, 70, 71) from St^o. Antão, all the remaining individuals from S. Nicolau; S. Vicente and low land individuals from St^o. Antão (up to 500 m, Esa 69, 70, 71) are included under *E. stenosisiphon* ssp. *stenosisiphon*. The data show de degrees of freedom (d.f), sum of squares deviation (SSD), variance component estimate, percentage of the total variation, phi-statistics (Φ) analogous to *F*-statistics.

subspecies (29.9%) was lower than the observed variation within populations (61.0%).

Mantel Test – The Mantel test with 1000 permutations (*p*-value = 0.001) revealed a positive correlation (*r* = 0.42) between *E. stenosisiphon* RAPD data and geographic distance expressed as UTM co-ordinates, thus evidencing a strong geographic pattern between genotypes.

Discussion

Genetic diversity of Echium hypertropicum, E. stenosisiphon and E. vulcanorum

RAPD analysis detected strikingly different levels of genetic variation for the three species. *Echium hypertropicum* and *E. vulcanorum* showed very low levels of genetic polymorphism (27 and 29% respectively) and *E. stenosisiphon*, a species with a wider distribution displayed 74% of polymorphism. According to traditional views, rare species exhibit lower levels of variation than

widespread ones. Although some rare plant species have little or no detectable variation [e.g., *Gentianella germanica* (Fischer and Matthies 1998), *Neolitsaea sericea* (Wang et al. 2005), *Saxifraga cernua* (Bauert 1998)] other rare species maintain high levels of genetic diversity [e.g., *Changium smyrnioides* (Fu et al. 2003); *Leucopogon obtectus* (Zawko et al. 2001), *Silene rothmaleri* (Cotrim et al. 2003)]. According to Karron (1991) comparisons between a given species and a mean for all rare species neglect the evolutionary history of the species under consideration. A more meaningful comparison, Karron (1991) argued, is that between a rare species and a more widely distributed congener. It is apparent that several rare species are as polymorphic as or more polymorphic than their widespread congeners. According to Gitzendanner and Soltis (2000) while there may be a slight reduction in genetic variation in rare species relative to their widespread congeners, it is not the case that rare species are confined to low levels of diversity. Finding values above or below that found in a widespread congener offers a useful point of reference from which to begin interpreting the biology of a rare species.

Despite the heterogeneity of sampling, our results indicate a set of two allopatric species, *Echium hypertropicum* and *E. vulcanorum*, with narrow distribution and reduced genetic diversity. The first is now only known at Santiago Island (with no more than four hundred individuals, mainly distributed in Serra da Malagueta and Pico da Antónia) whereas the second occupies zones of high altitude at Fogo Island, where it is represented by two main populations, Bordeira (the largest) and Monte Losna. According to old records, both species suffered considerable reduction of area being now restricted to mountains with less habitat destruction by human action. *Echium stenosisiphon* is present in three of the Northern islands of Cape Verde archipelago in small populations, especially in S. Vicente where less than a half of a hundred individuals exist. Occurring in different islands and in scattered populations, they have the potential for higher genetic diversity especially to St. Antão where we can find *Echium stenosisiphon* in a wide range of altitude and climate (arid and sub-humid areas). Moreover species classified as rare exhibit a diverse array of local population sizes, habitat specificities, and even geographic ranges (Rabinowitz 1981), which can also explain part of the different values, obtained for genetic polymorphism for these three congener species.

Regardless of all three species of *Echium* here studied being endemics, these species may display different forms of rarity influencing their genetic variability. Likewise the interaction of rarity and breeding systems can create different patterns of genetic variation. *Echium* species are known to be outcrossing (Bramwell 1973) although different levels of this breeding system may be present in each species. Heterostyly, defined as the reciprocal placement of stigma and anthers in two (dystily) or three (tristyly) floral morphs of one species (Lloyd and Webb 1992), is viewed as a way to promote outcrossing (Barret et al. 2000). Stigma-height dimorphism is reported from several genera of Boraginaceae [e.g., *Anchusa* (Philipp and Schou 1981) and *Lithodora* (Barret

et al. 2000)]. Our observations indicate the presence of dystily in all three species here studied Figure 7a–f).

Echium hypertropicum and *E. vulcanorum* display dense compound inflorescences bearing flowers with five stamens exserted from the corolla tube Figure 7a–d and g, h). *Echium stenosisiphon* has a lax inflorescence bearing flowers with only two exserted stamens Figure 7e, f and i). Flowers of the *Echium hypertropicum* and *E. vulcanorum* type present probably higher potential for self-pollination due to the higher number of anthers in the vicinity of stigma and thus source of pollen from the same genotype when compared to *E. stenosisiphon* flowers. The probability of self-pollination in the two Southern species is likely to be higher than in *Echium stenosisiphon*. Studies in another Boraginaceae (*Pulmonaria obscura*) indicated that despite the presence of heterostyly, floral dimorphism does not increase the number of inthermorph pollinations in each of the two flower types (Olesen 1979). According to the same author the presence of heterostyly does not increase the number of intermorph pollinations in each of the two flower types. This discrepancy may be caused by a considerable intrafloral flow of pollen, resulting from the shaking of the flowers, movement of pollinators, or small insects in the corolla tube. These movements of pollen inside the same flower reduce the potential

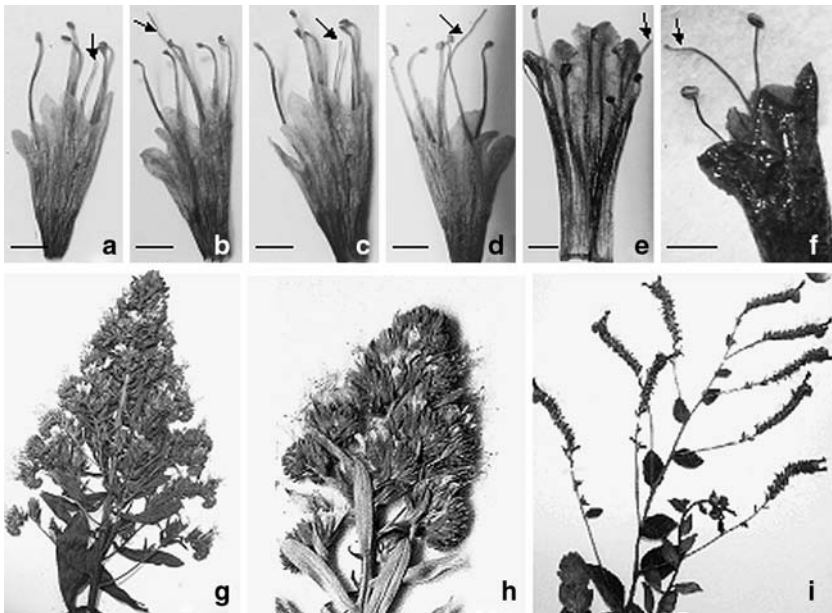


Figure 7. Heterostyly in flowers of *Echium hypertropicum* (a–b), *E. vulcanorum* (c–d) and *E. stenosisiphon* (e–f). Stamens 5, all exserted (a–d). Bars: 4 mm. Longitudinal section of corolla (e). Bar: 4 mm. Corolla lobes with two stamens exserted (f). Bar: 2 mm. Arrows indicate the styles. Inflorescences of *Echium hypertropicum* (g), *E. vulcanorum* (h) and *E. stenosisiphon* (i). Inflorescences diameters were 23–15, 4–3, and 18–15 cm, respectively.

for cross-pollination instead of enhancing it. Thus the striking differences in genetic polymorphism of the three species of *Echium* studied are probably also related to differences in breeding systems associated with different forms of dystily.

The high correlation of genetic variation patterns of these three species with the geographic distance between genotypes (Mantel test $r = 0.59$, p -value = 0.0004) together with UPGMA indicate The UPGMA clearly indicates a closely genetic proximity between the two Southern endemic species (*Echium hypertropicum* and *E. vulcanorum*). Furthermore our data indicates that *Echium stenosphon*, a Northern endemic species, has probably also a different life history and independent origins can be postulated. The results are reinforced by the morphological differences among these two groups of species, given that the first are candelabra shrubs with strong compact branches and flower rich inflorescences whereas the later is a moderately branched, sub-shrub with few flowered inflorescence.

Echium stenosphon population genetic analysis

PCO and cluster analyses of populations of *Echium stenosphon* revealed that S. Nicolau populations form a separate cluster from that of St. Antão and S. Vicente. However plants of St. Antão display higher genetic variability being scattered along the first axis of PCO (56.5% of the variation). AMOVA results showed that only 29.7% of the diversity could be attributed to variation among islands whereas 60.6% is attributed to individuals within populations. Phi-statistics (Φ) showed an intermediate value within populations ($\Phi_{st} = 0.394$), which is compatible with reduced levels of gene flow ($Nm = 0.32$). The closest geographic distance between St. Antão and S. Vicente Islands is about 20 km, whereas the distance between St. Antão and S. Nicolau is approximately 70 km. These facts together with the high positive correlation found in Mantel test ($r = 0.42$) corroborate the hypothesis that gene flow is less frequent between St. Antão and S. Nicolau than between St. Antão and S. Vicente, which may explain the genetic distinction of S. Nicolau genotypes. Assuming that the number of colonists decreased with altitude, high-altitude populations are expected to be more physically isolated due to more pronounced founder effects. In the mountainous islands such as St. Antão and S. Nicolau, sheer cliffs emerge abruptly from the ocean. St. Antão and S. Nicolau represent the most mountainous islands of the Northern group, although the first may be at a neighboring geographic distance with S. Vicente. Due to its plain lowland relief and exposure to the prevailing Trade winds, main direction S. Vicente may function as a genetic source for St. Antão. *Echium stenosphon* is found in St. Antão from 200 to 1500 m altitude showing high ecological amplitude, its habitats being frequently exposed to Trade winds from NE. The Northeastern trade winds may explain differences in genetic differentiation and diversity between islands. In

fact S. Nicolau plants display lower genetic diversity in the context of the species when compared with St. Antão, populations from which display the greatest diversity. As these islands are so distant (> 70 km) and given the significant Mantel correlation between the genetic data and geographic distance, it is possible that gene flow is more frequent within St. Antão than within S. Nicolau leading to a greater genetic diversity in the first and to genetic drift in the second.

Echium stenosisiphon ssp. stenosisiphon and *E. stenosisiphon ssp. lindbergii*

Petterson (1960) has considered the deviating specimens from mountain areas in St. Antão, as a separate species *Echium lindbergii*. According to Bramwell (1972) the specimens presenting extremes of range of some characters like leaf-shape, indumentum and corolla tube dimensions represent two subspecies, *Echium stenosisiphon ssp. stenosisiphon* and *E. stenosisiphon ssp. lindbergii*. Great morphological variation was also reported for the taxon by Romeiras (2005) although, this was not consistent with the subspecies types. According to Bramwell (1972) one of the taxa, *Echium stenosisiphon ssp. lindbergii*, occurs in mountain areas of St. Antão whereas the other, *E. stenosisiphon ssp. stenosisiphon*, is confined to coastal slopes up to 400–500 m in St. Antão, in S. Nicolau and S. Vicente Islands. Martins (1995) in the Cape Verde Flora noted the great morphological variability of *Echium stenosisiphon*, but disregarded the infra-specific taxa. Our analysis showed that populations of *Echium stenosisiphon* of St. Antão display higher genetic diversity (Figure 5) although the genetic variation found among putative subspecies (29.9%) was much lower than the observed variation within populations (61.0%). Thus we consider that there is no genetic basis to consider these entities as different subspecies, as proposed by Bramwell (1972).

Conservation actions for Cape Verde Echium species

The success of any conservation action can only be guaranteed if it is based on a thorough knowledge of the species to be protected, as well as a deep understanding of the reasons that led to the current situation. Our study strongly indicates the urgency of *in situ* and *ex situ* programs conservation for the *Echium* species of Cape Verde, especially concerning *E. hypertropicum* and *E. vulcanorum*, due to the low genetic diversity showed by RAPD analysis. *Echium hypertropicum*, considered as Endangered in the Red Data Book of Cape Verde (Leyens and Lobin 1996), is subjected to a number of negative human factors, such as agriculture and pasture. Also the exotic species *Lantana camara* and *Furcraea foetida* have invaded their natural habitats threatening *Echium hypertropicum* populations. *Echium vulcanorum* is also considered Endangered, but an *in situ* conservation action have been carried at the Bordeira region (in the Natural Park of Fogo Island) and a population with a

relatively high number of individuals exists, due to extensive work done on this island by Leyens (2002). The establishment of conservation programs like this in other islands is strongly suggested to guarantee *Echium* species survival in Cape Verde. Moreover local conservation programs should encourage the control of expansion of exotic species. Concerning *ex situ* conservation actions they may include the creation of island nurseries where stock plants could be grown for reinforcement of existing populations.

In what concerns *Echium stenosphon* the relatively large amount of within-island genetic variation suggests that this species does not show high levels of fragmentation as is common in other rare plant species (Schneller and Holderegger 1996). However the S. Nicolau populations are the less genetically diverse and in consequence they present a higher risk of inbreeding depression via reduction of gene flow. This species is classified as Indeterminate for St^o. Antão, Vulnerable for S. Vicente and at Lower Risk for S. Nicolau (Leyens and Lobin 1996). According to our data and considering the lower populations sizes the later category should be reviewed and S. Nicolau populations of *Echium stenosphon* should be classified as Vulnerable. Also population's surveys in St^o. Antão Island would be advisable in order to clarify its conservation status.

Considering the genetic differentiation of *Echium stenosphon* detected among islands, we propose that each island should be considerate as distinct component concerning conservation measures. Also, the existence of genetic differences among populations of these three islands has clear implications for germoplasm collection for *ex situ* conservations approaches, and ideally the full range of natural variation should be sampled. However, considering the situation of this species on S. Vicente Island with only two small populations, reintroduction of germoplasm from St^o. Antão (with a greater genetic diversity and presenting higher genetic proximity with S. Vicent populations) may be a viable way to guarantee the existence of *Echium stenosphon* in S. Vicente.

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