



Incipient radiation versus multiple origins of the Galápagos *Croton scouleri* (Euphorbiaceae)

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ABSTRACT

Aim Island radiations imply the emergence of numerous species in a short period of time. Downscaling at the infraspecific level, considerable differentiation among populations can be a sign of ‘incipient radiation’. However, this process remains largely unexplored. We focus on one of the most outstanding cases of infraspecific morphological variation in the Galápagos flora. Our hypothesis is that the phenotypic variation of *Croton scouleri* is a sign of incipient radiation, in which a single colonization has generated new lineages with considerable morphological differentiation.

Location The Galápagos Islands and Neotropics.

Methods One hundred and forty-four nuclear ribosomal DNA (ITS) and plastid *trnL-F* sequences of *Croton* sect. *Adenophylli* were used to test the hypothesis of a single ancestry (monophyly) of *C. scouleri* using a phylogenetic approach. Sequence data were analysed using Bayesian inference (BI) and maximum likelihood (ML). A complementary phylogeographical analysis of *C. scouleri* and phylogenetically related species was also performed using 123 plastid sequences (*trnL-F*, *petL-psbE*, *trnH-psbA*) in search for common ancestry of Galápagos lineages.

Results The phylogenetic approach revealed that the closest relatives of *C. scouleri* were *C. alnifolius*, *C. pavonis* and *C. rivinifolius*. However, we failed to support monophyly of *C. scouleri* populations. Despite finding numerous haplotypes (14 polymorphic sequences/9 substitution-based sequences), their distribution across *Croton* species prevented us from inferring common ancestry for *C. scouleri*. The phylogeographical reconstruction revealed multiple lineages related to the origin of *C. scouleri*.

Main conclusions Lack of monophyly likely indicates that an incipient radiation from a single ancestor does not account for the striking infraspecific phenotypic variation in *C. scouleri*. This morphological diversity could be explained by recurrent biogeographical connections between Galápagos and the mainland, involving multiple colonizations to the islands from the continent rather than back colonizations from the islands to the mainland. Morphological, reproductive, geographical and ecological evidence better support the scenario of recurrent colonizations from the continent in different periods of time.

Keywords

Euphorbiaceae, ITS, leaf morphotypes, oceanic islands, *petL-psbE*, phylogeny, phylogeography, plastid DNA, *trnH-psbA*, *trnL-F*

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INTRODUCTION

Oceanic islands harbour a great deal of evolutionary radiations. Characteristics such as isolation, niche availability, topographic complexity or habitat heterogeneity of many archipelagos favour diversification and adaptive evolution (Paulay, 1994). Therefore, some of the most striking plant and animal radiations documented to date have been found on islands. Among the most significant examples are the Hawaiian honeycreepers (Freed *et al.*, 1987) and lobeliads (Givnish *et al.*, 2009), the Macaronesian *Echium* L. (García-Maroto *et al.*, 2009) and *Aeonium* Webb & Berthel. (Jørgensen & Olesen, 2001), and the Galápagos *Scalesia* Arn. (Blaschke & Sanders, 2009) and finches (Grant & Grant, 2008).

The process of evolutionary radiation involves the emergence of numerous lineages and species from a common ancestor in a short period of time (Schluter, 2000). However, at the infraspecific level, considerable morphological differentiation can be a sign of 'incipient radiation', where populations with derived characters represent 'infant' lineages over the course of radiation (Coyne & Orr, 2004; Balao *et al.*, 2010). Incipient or not, the first step for the proposal of any radiation is to establish that the current diversification is a consequence of a single origin (Schluter, 2000). In other words, rapid differentiation associated with radiation needs to be preceded by colonization of a single lineage. Knowledge of the number of colonizations helps determine whether the range of morphological and ecological differentiation among species arose from single or multiple lineages. The same is true at a lower taxonomic level. Incipient radiations have been seldom documented and there is a lack of molecular studies aimed at gaining a deeper insight into significant divergence of populations on islands.

Croton scouleri Hook. f. (Euphorbiaceae) is the only species on the Galápagos Islands belonging to the genus *Croton* L., one of the largest monophyletic genera within angiosperms (over 1200 spp., van Ee *et al.*, 2011). This species is endemic to the archipelago (Wiggins & Porter, 1971) and thus is interpreted as the result of a single colonization from the Americas (Svenson, 1946b). Most *Croton* fruits are explosive capsules with no adaptations for long-distance dispersal. Nevertheless, *C. scouleri* is an abundant arborescent shrub widely distributed across the Galápagos archipelago (Fig. 1). Morphological variation within this species offers one of the most outstanding cases of the Galápagos flora as revealed by its nine taxonomic varieties, of which four had been originally described at the species level (see Wiggins & Porter, 1971). Indeed, variation in plant habit and especially leaf shape of *C. scouleri*, led Hamann (1979) to speculate about an 'early' speciation within the archipelago. For instance, *C. scouleri* displays strong extremes in leaf shape, such as broad-leaved forms (see Appendix S1a in Supporting Information) and narrow-leaved forms (see Appendix S1b). A wide span of intermediate or elliptic-leaved forms between these two morphotypes are also frequent (Fig. 2), but show little

geographical correlation (Wiggins & Porter, 1971). These characteristics make *C. scouleri* a good candidate to test the hypothesis of incipient radiation on oceanic islands.

The endemic status of *C. scouleri* in the Galápagos involves the assumption that all its morphological variation results from a single ancestor that evolved within the archipelago. However, this assumption has not been tested by any molecular phylogenetic study, and thus the origin of the species remains unclear. Our working hypothesis is that the enormous phenotypic variation in *C. scouleri* reflects an incipient radiation from a single ancestor. The considerable isolation of the Galápagos archipelago (*c.* 1000-km west of South America) and the absence of diaspore specialization in *Croton* for long-distance dispersal also points towards the hypothesis of a single colonization event (see Vargas *et al.*, 2012). Studies on phylogenetic relationships have helped identify single versus multiple origins of plants on oceanic islands based on monophyletic versus polyphyletic groups (see Silvertown, 2004). In this study, we test the monophyly of the Galápagos lineages of *C. scouleri* to establish single origin from the continent. To this end, a large sample of *Croton* species from section *Adenophylli* from the Neotropics was used to perform phylogenetic analyses. In addition, a complementary phylogeographical approach was explored by reconstructing ancestral haplotypes and lineage relationships within the Galápagos Islands. Genealogical relationships help interpret incipient differentiation of insular and continental morphotypes.

MATERIALS AND METHODS

Study species

Croton scouleri has not been included in any molecular phylogeny; however, it was classified in the Neotropical *Croton* sect. *Adenophylli* on basis of morphological characters (van Ee *et al.*, 2011). The distribution of *C. scouleri* in the volcanic archipelago of Galápagos encompasses 12 of the 13 main islands (> 10 km²), being only absent from the island of Baltra (Fig. 1). It is an abundant arborescent shrub with a wide habitat range, occurring both in the arid lowlands and moist uplands. Within sect. *Adenophylli* (223 species), the narrow-leaved shape is not common and it is only found in a few species (e.g. *C. sagraeanus* Müll.Arg. from Cuba, *C. linearis* Jacq. from Bahamas, Florida, Cuba and Jamaica), including the *C. scouleri* on the Galápagos Islands [*C. scouleri* f. *macraei* (Hook. f.) G. L. Webster, see Appendix S1b]. *Croton scouleri* was considered to be exclusively dioecious (Wiggins & Porter, 1971), despite the observation that most of the species of the genus *Croton* are monoecious (van Ee *et al.*, 2011). However, monoecy has also been recorded in few individuals of *C. scouleri* on the islands of Genovesa, Isabela and Española, with a frequency of 8–15% of monoecious individuals in the populations studied (Mauchamp, 1997) (see also Fig. 2d).

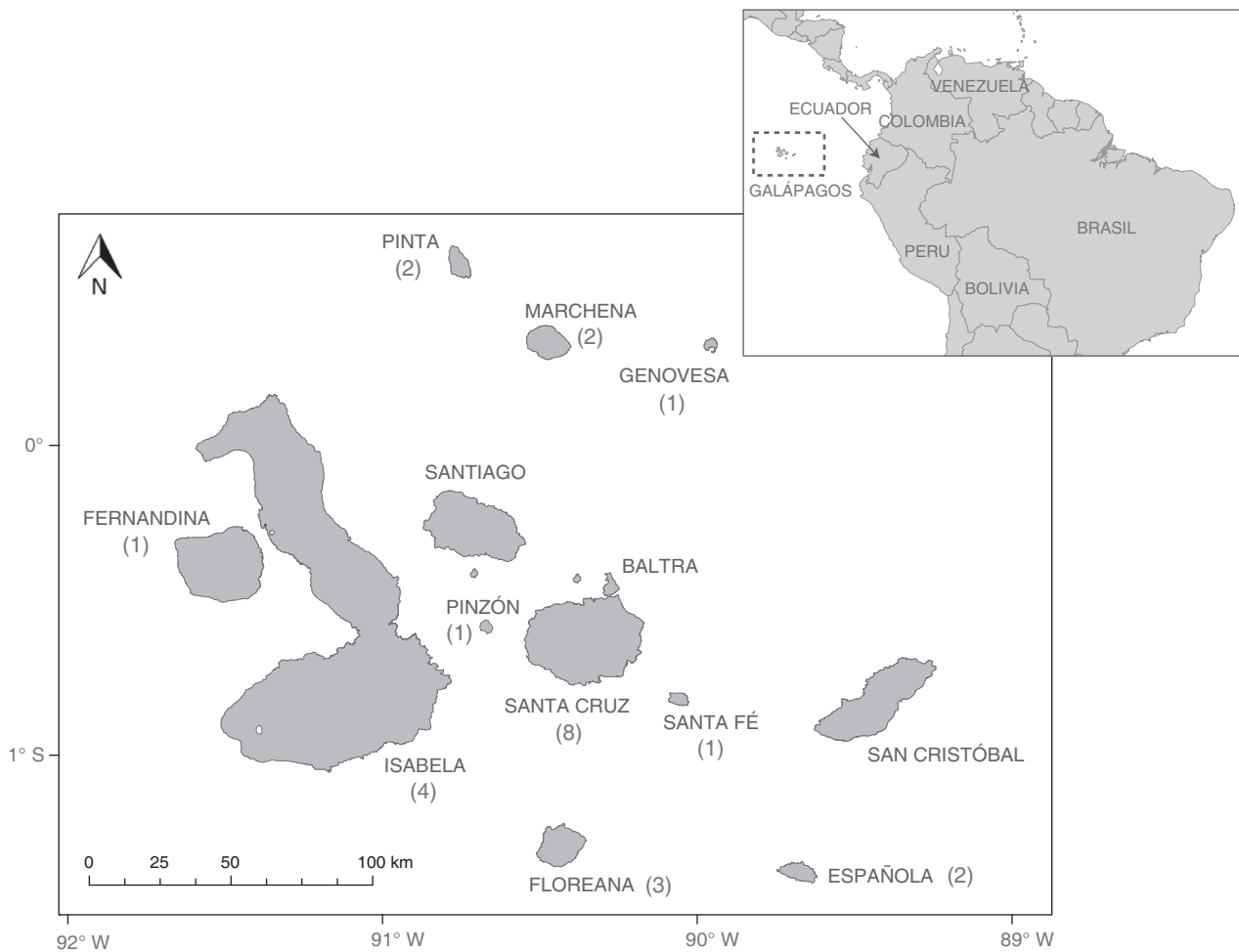


Figure 1 Map of the Galápagos archipelago, showing its global location (top-right) and the main islands (> 10 km²). *Croton scouleri* is distributed in all the main islands except from Baltra. The number of samples sequenced per island is indicated between brackets.

Sampling and sequencing

Plant material was collected in the field and from herbarium specimens [C, HUEFS, LPB, MA, MICH, MO, MU, QCNE, U, USM, WIS; herbarium acronyms follow Thiers (2015)]. Several sequences were also obtained from Riina *et al.* (2009) (see Appendix S2 for details of the sample origins). Within our study group we selected representatives of different islands and leaf morphotypes. We included 25 samples of *C. scouleri* covering much of its morphological variation and distribution in the Galápagos Islands (10 out of the 12 islands of occurrence), and 47 samples of *Croton* sect. *Adenophylli* representing most of the morphological variation and geographical distribution in the Neotropics (Mexico-Central America, Caribbean, eastern Brazil, southern South America, Andes). Our sample selection benefited from an ongoing phylogenetic analysis based on DNA sequences of sect. *Adenophylli* (Riina *et al.*, unpubl. data), which helped to infer closely-related taxa. From these 47 samples, 12 were of *C. rivinifolius* Kunth, an endemic species from Ecuador to which *C. scouleri* was originally ascribed (Svenson, 1946a). In

addition, *C. caracasanus* Pittier (sect. *Corylocroton*) and *C. sampatik* Müll. Arg. (sect. *Sampatik*) were used as the out-group in the phylogeny. Total genomic DNA was isolated using the DNeasy Plant Mini-Kit (Qiagen Inc., Carlsbad, CA, USA) following the manufacturer's recommended protocols.

The internal transcribed spacer region (ITS) of the nuclear ribosomal DNA and the plastid spacer *trnL-F* have proven to be useful markers to infer phylogenetic relationships within the genus *Croton* (Berry *et al.*, 2005; Riina *et al.*, 2009). Therefore, we chose these two DNA regions in our phylogenetic approach. The ITS region was amplified using the primers ITS-1 (Urbatsch *et al.*, 2000) and ITS-4 (White *et al.*, 1990). The plastid *trnL* intron and *trnL-F* intergenic spacer were amplified using primers 'c' and 'd', and 'e' and 'f' respectively (Taberlet *et al.*, 1991). After 2 min pre-treatment at 94–95 °C, polymerase chain reaction (PCR) conditions were: 30 cycles of 1 min at 95 °C, 2 min at 55–60 °C and 1–2 min at 72 °C; cycles were followed by a final extension at 72 °C for 10 min. A volume of 1 µL of bovine serum albumin at 1 mg mL⁻¹ was included in each 25 µL reaction to improve the efficiency of the amplification. PCR products



Figure 2 Morphological variation of *Croton scouleri* in leaf shape and inflorescence. Scale bar, 1 cm. (a) Broad-leaved morphotype; (b) Elliptic-leaved morphotype; (c) Narrow-leaved morphotype; (d) Monoecious inflorescence at Punta Espejo, Marchena Island. Female flowers are located at the base of the inflorescence axis, whereas male flowers are at more distal positions. Photo (d): P. Vargas.

were sequenced using an ABI Prism H 3730xi DNA sequencer at the Macrogen Institute (Macrogen Co., Seoul, Korea). Sequences were aligned, and manually adjusted, using MAFFT 6.814b (Kato *et al.*, 2002) implemented in the GENEIOUS 6.1.7 software (Drummond *et al.*, 2011).

To further explore the origin of *C. scouleri* variation, we performed a phylogeographical analysis in search for common ancestry. First, a pilot study based on three to eight individuals from different islands and 19 plastid DNA regions was done (Taberlet *et al.*, 1991; Hamilton, 1999; Shaw *et al.*, 2005, 2007). Secondly, we chose the following three DNA regions as a result of the variability and quality of the amplifications obtained from herbarium samples: *trnL-F* (Taberlet *et al.*, 1991), *trnH-psbA* (Hamilton, 1999) and *petL-psbE* (Shaw *et al.*, 2007). For the *petL-psbE* region, one internal primer (5'-CATCGAATATTCTTTACTACTGG-3') was herein designed to facilitate the amplification of a short fragment because of failure in the amplification of the complete region. For this phylogeographical approach, we

assembled a matrix including samples of *C. scouleri* and their closest mainland relatives based on the phylogenetic analysis. PCR protocols and sequencing followed the above description. All new sequences were deposited in GenBank (see Appendix S2 for accession numbers).

Phylogeny reconstruction

Phylogenetic relationships among *C. scouleri* and its sect. *Adenophylli* relatives were explored using maximum likelihood (ML) and Bayesian inference (BI) analyses. Prior to these analyses, the simplest model of sequence evolution that best fits each sequence dataset was determined using the Akaike information criterion in jMODELTEST 2.1.3 (Durraba *et al.*, 2012). For BI, MRBAYES 3.2.2 (Ronquist & Huelsenbeck, 2003) was used to conduct an exploratory analysis on the ITS, *trnL-F* and combined ITS+*trnL-F* matrices. For each DNA region, four chains were run for five million generations fitting the optimal models of substitution to each

molecular partition (SYM+G for ITS, and GTR+G for *trnL-F*). The resulting ITS and *trnL-F* trees were visually inspected to check for congruence. There was no incongruence between the ITS and *trnL-F* phylogenies, so the concatenated dataset was analysed. The final BI analysis of the combined dataset was conducted with each model substitution fitted to each molecular partition. Four chains were run twice for 15 million generations with a sample frequency of 1000, and discarding the first 25% generations as burn-in. Chain convergence was assessed with TRACER 1.5 (Rambaut & Drummond, 2009), and a 50% majority rule consensus tree was calculated to estimate the BI phylogeny.

The ML analysis was performed using RAXML (Stamatakis, 2006) as implemented in RAXMLGUI 1.31 (Silvestro & Michalak, 2012) with the alignment divided into two partitions (ITS+*trnL-F*) and a unique GTR*GAMMA model of evolution assigned to each partition. Branch support was assessed by performing a thorough bootstrap analysis (1000 bootstrap replicates) with 100 runs.

Phylogeographical approach

Plastid markers are known to be one of the most reliable for inferring plant colonization by seed dispersal (e.g. Guzmán & Vargas, 2009; Fernández-Mazuecos & Vargas, 2011). The ptDNA genome is structurally stable, haploid, non-recombinant and maternally inherited in most angiosperms (Birky, 2001; Avise, 2009), including species within Euphorbiaceae (Corriveau & Coleman, 1988). The three ptDNA regions (*trnL-F*, *trnH-psbA* and *petL-psbE*) were concatenated in order to detect polymorphism and reconstruct the ancestral genotype (haplotype) of *C. scouleri* (Birky, 2001). The resulting dataset was analysed through a statistical parsimony algorithm (Templeton *et al.*, 1992), as implemented in tcs 1.21 (Clement *et al.*, 2000), to infer genealogical relationships among haplotypes. The maximum number of differences resulting from single substitutions among haplotypes was calculated with 95% confidence limits, and initially treating gaps as missing data. In addition, two more analyses of haplotypic diversity were performed: (1) by treating gaps as the fifth state, and (2) using ribotypes from the ITS region as a separate dataset.

Distribution of leaf morphotypes

Croton scouleri has two main types of leaf shape: broad-leaved (Fig. 2a & see Appendix S1a) and narrow-leaved (Fig. 2c & see Appendix S1b) forms. To infer the frequency of each leaf morphotype in *C. scouleri*, we inspected a total of 107 herbarium specimens representing populations of the 12 islands. However, because some herbarium specimens displayed intermediate leaf forms (elliptic), we established three categories for the character leaf morphotype (Fig. 2a–c) and all specimens were visually classified accordingly. The frequency of each leaf morphotype has not been previously studied for *C. scouleri* and this study is considered exploratory.

RESULTS

Sequence variation and phylogeny

The aligned length of the combined ITS and *trnL-F* sequences was 1710 bp (621 bp for ITS; 1089 for *trnL-F*). There were 163 variable sites for the ITS and 123 for the *trnL-F* datasets. The ITS alignment had no missing characters, whereas the *trnL-F* alignment had 3.5% missing characters.

The two phylogenetic analyses (BI and ML) of *Croton* sect. *Adenophylli* revealed that the closest relatives of *C. scouleri* were *C. alnifolius* Lam. (Ecuador, Peru), *C. pavonis* Müll.Arg. (Ecuador, Peru) and *C. rivinifolius* (Ecuador) (Fig. 3). The four species formed a well-supported clade (Bayesian posterior probability = 1; maximum likelihood bootstrap value = 100). However, this clade had low internal resolution because the two DNA regions used did not provide enough variation (two substitutions in ITS; three substitutions in *trnL-F*) to infer species relationships and monophyly of *C. scouleri* populations.

Haplotype analysis of *C. scouleri* and closest relatives

Molecular variation of the ITS sequences in *C. scouleri* and its closest mainland relatives was defined only by three ribotypes as a result of four substitutions (see Appendix S3). The most common ribotype (R2) was shared by *C. scouleri*, *C. alnifolius*, *C. pavonis* and *C. rivinifolius*. Ribotypes R1 and R3 were displayed by one sample each of *C. alnifolius* and *C. scouleri* respectively.

Variation in the combined ptDNA dataset (*trnL-F*, *petL-psbE*, *trnH-psbA*) for *C. scouleri* and relatives was the result of eight substitutions and four gaps, distributed as follows: in *trnL-F*, three substitutions and one gap; in *petL-psbE*, three substitutions and one gap; in *trnH-psbA*, two substitutions and two gaps. The analysis of the three combined ptDNA regions treating gaps as missing data yielded nine haplotypes of *C. scouleri* and the three closest mainland relatives. Haplotype relationships depicted a single network with no loops (Fig. 4a). Eight of the nine haplotypes in Fig. 4(a) were found in *C. scouleri*, and seven were exclusive of this species (1, 3, 4, 5, 6, 7, 8). In contrast, the other three species in the clade harboured only two haplotypes (2, 9).

Five more haplotypes were detected when considering gaps as a fifth state (Fig. 4b). In this case, four missing haplotypes and one loop were also inferred. *Croton scouleri* harboured 11 haplotypes, nine being private (1, 3, 4, 5, 6, 9, 11, 12, 13), whereas *C. alnifolius*, *C. pavonis* and *C. rivinifolius* displayed five haplotypes (2, 7, 8, 10, 14). From these, two haplotypes (7, 14) were exclusive of *C. rivinifolius*.

In both haplotype networks (Fig. 4a,b), haplotype 2 was the most frequent and widely distributed, and it was shared by the four species of the ingroup clade. These genealogical reconstructions did not provide sufficient resolution to precisely infer the origin of all the lineages of *C. scouleri*.

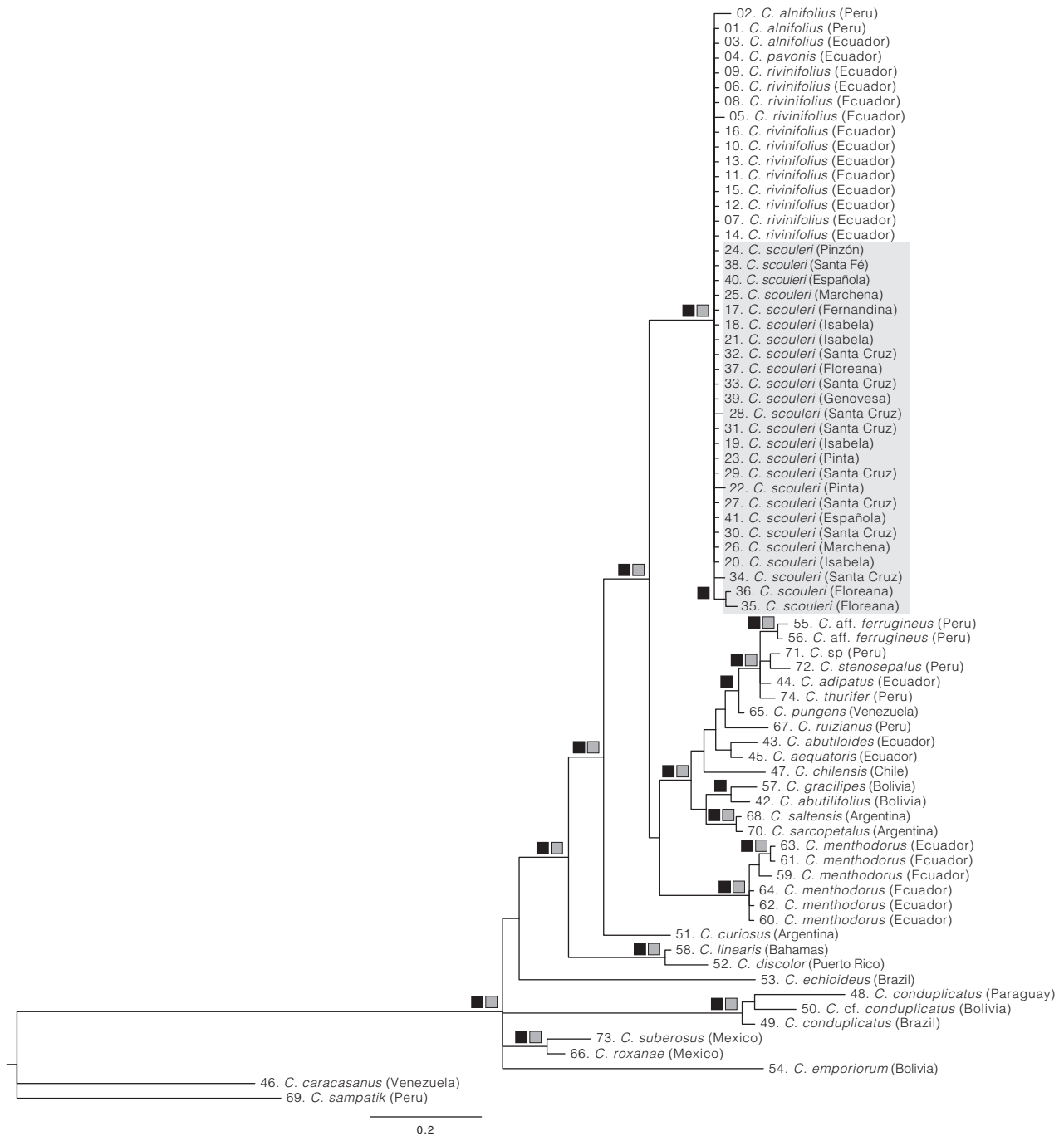


Figure 3 Fifty per cent majority-rule consensus tree obtained from the Bayesian phylogenetic analysis of ITS and *trnL-F* sequences of *Croton* sect. *Adenophylli*. Black and grey squares above the nodes represent Bayesian posterior probabilities $\geq 95\%$ and maximum likelihood bootstrap values $\geq 75\%$, respectively. Sample provenance (country) is indicated within brackets after taxa names, except for *C. scouleri* (grey shaded), for which each Galápagos island is indicated.

However, it is notable that in one analysis haplotype 9 appears unrelated to the other 10 haplotypes of *C. scouleri* (see Fig. 4b), needing a minimum of four steps to be connected to haplotype 2. The absence of loops in the clade including these haplotypes suggests lack of homoplasy, two different lineages in the Galápagos and thus, rejecting the hypothesis of a single origin of *C. scouleri*.

Leaf and inflorescence variation

Visual inspection of 107 herbarium specimens yielded different frequencies of occurrence of the three leaf morphotypes. The most abundant morphotype was broad-leaved (62.6%), followed by the narrow-leaved (23.4%) and the intermediate elliptic-leaved (14.0%).

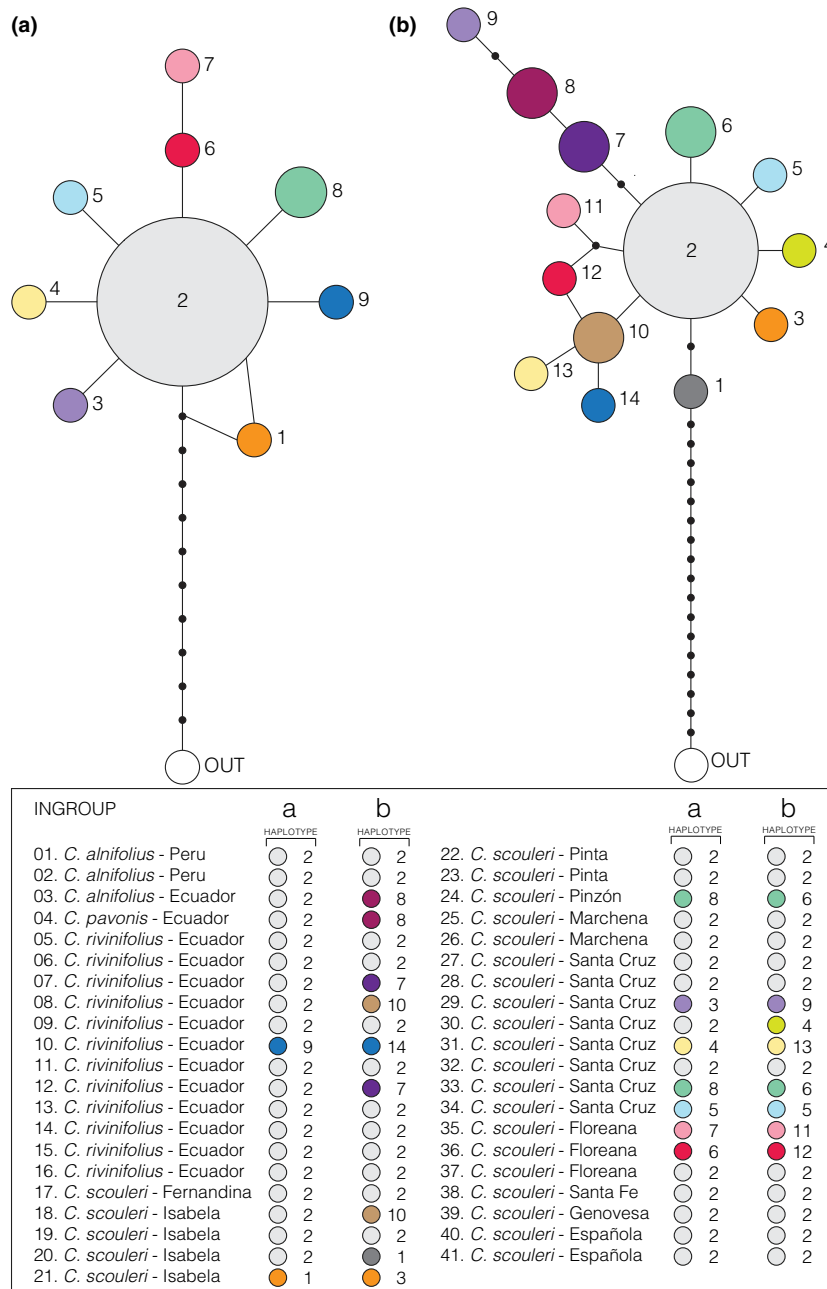


Figure 4 Statistical parsimony networks of *Croton scouleri* and its closest relatives (ingroup, Fig. 3) based on ptDNA haplotypes (*trnL-F*, *petL-psbE* and *trnH-psbA*). (a) Substitution-based haplotype network (treating gaps as missing data). (b) Polymorphic haplotype network (treating gaps as fifth state). In both networks, each haplotype is represented by a colour and a number. Lines represent single nucleotide substitutions; dots indicate absent haplotypes (extinct or not found). Haplotype circle size is proportional to frequency. The two haplotypes (a, b) for each sample are indicated in the inset. Sample provenance (country) is indicated after taxa names, except for *C. scouleri*, for which each Galápagos island is shown. See Appendix S2 for specific information of each sample.

Our search for sexual expression of *C. scouleri* flowers across the main islands (Española, Fernandina, Floreana, Genovesa, Isabela, Marchena, Pinta, Pinzón, San Cristóbal, Santa Cruz, Santa Fé and Santiago) supports dioecy as the predominant reproductive system. However, monoecy has been recently found on the island of Marchena, where inflorescences with male and female flowers were observed on five individuals (A. Traveset, pers. obs., Fig. 2d).

DISCUSSION

Island biota has provided some of the most informative cases of radiations worldwide, and early stages of radiations have been interpreted as incipient diversification within lineages (e.g. Vijverberg *et al.*, 2000; McGlaughlin & Friar, 2011; Stacy *et al.*, 2014). Nevertheless, in light of the phylogenetic and phylogeographical results, the pronounced morphological

variation in the Galápagos *C. scouleri* does not seem to be the result of an incipient radiation. Neither the ITS and *trnL-F* phylogeny nor the ptDNA network supported the monophyletic origin of the species and, consequently, our results suggest that alternative hypotheses to incipient radiation better explain the infraspecific phenotypic variation found in *C. scouleri*.

Continental relatives and morphological differentiation of *C. scouleri*

Croton scouleri has been the focus of taxonomic disagreement as a result of its striking morphological variation. Wiggins & Porter (1971) refer to its variation patterns as ‘nearly intractable to rational taxonomic treatment’. Our DNA sequence analyses support its phylogenetic relationship with *C. alnifolius*, *C. pavonis* and *C. rivinifolius* from Ecuador and Peru, with which *C. scouleri* forms a well-supported clade. Some morphological characters also link these four species into a natural group. They all share stellate trichomes, indumentum density and distribution, petiolar glands, and inflorescence and flower morphology. Regarding to the inflorescence, however, some differences arise among the four taxa. Dioecious individuals (dominant) coexisting with monoecious plants (less frequently) have been documented for *C. scouleri* (see Mauchamp, 1997 and Fig. 2d in this study). *Croton alnifolius* and *C. rivinifolius* are considered dioecious species, although a case of monoecy in *C. rivinifolius* has been detected in a herbarium specimen inspected by us (Riina, Real Jardín Botánico (CSIC-RJB), Madrid, pers. obs.). On the other hand, *C. pavonis* seems consistently monoecious (Riina, pers. obs.). The four species also show ecological similarities and differences. *Croton scouleri* is widely distributed in the archipelago of Galápagos, from arid lowlands (where it can be dominant) to moist uplands (0–1600 m a.s.l.) (Hamann, 1979). In the mainland, *C. pavonis* occurs mainly from 1500–2000 m a.s.l. in humid montane forests (Jørgensen & León-Yáñez, 1999). *Croton rivinifolius* forms part of the coastal vegetation (0–200 m a.s.l.), and *Croton alnifolius* occurs in the Peruvian coastal desert, where the vegetation is largely restricted to the fog-zone or *lomas* (small hills) between 200–800 m (Dillon *et al.*, 2011).

Taxonomic proximity of *C. scouleri* and *C. rivinifolius* had already been stressed by Svenson (1946a), who included the former in the latter based on their morphological similarities. However, Webster (1970) and Wiggins & Porter (1971) re-instated *C. scouleri* as a distinct and endemic species to the Galápagos archipelago arguing that the seeds of *C. rivinifolius* differ from those of *C. scouleri* in being ‘distinctly grooved or ridged instead of pitted’. Indeed, seed and leaf morphologies have been used to delimit morphological variation in *C. scouleri* (Fig. 2a–c & see Appendix S1) into nine taxa by numerous botanists (e.g. J. D. Hooker, N. J. Andersson, H. K. Svenson, A. N. Stewart).

Our inspection of herbarium specimens indicated that the broad-leaved form appears to be the most abundant (62.6%

of occurrence) in the Galápagos populations. This is also the most common morphotype of the three closest *Croton* relatives (*C. alnifolius*, *C. pavonis* and *C. rivinifolius*) in the continent. The fact that narrow-leaved plants are found only in Galápagos populations (c. 25% of the individuals inspected), together with the high number of haplotypes, indicate relatively ancient occurrence and differentiation of *Croton* on the islands.

The great morphological variation in *C. scouleri* paralleled the high diversity of plastid haplotypes. In particular, *C. scouleri* sequence variation, with eight of the nine substitution-based haplotypes (Fig. 4a) and 11 of 15 polymorphic haplotypes (Fig. 4b), outnumbered that of its closest mainland relatives (two substitution-based and five polymorphic haplotypes). In other words, most of these haplotypes were private to *C. scouleri*. Both narrow-leaved and broad-leaved individuals showed exclusive haplotypes (see Appendix S2). However, any relationships between haplotypes and morphotypes were not evident. A deeper phylogeographical research of *C. scouleri* within the Galápagos archipelago would be necessary to test any correlation between genetic diversity and morphotypes, especially regarding leaf and seed morphology.

Multiple colonizations versus back colonization

Lack of strong phylogenetic distinctiveness of populations of *C. alnifolius*, *C. pavonis*, *C. rivinifolius* and *C. scouleri* was demonstrated by the absence of monophyletic groups within the well-supported clade that comprises these four species. Besides, no single common ancestry of the main Galápagos haplotype lineages is inferred from the haplotype network analysis. Therefore, the four species may have shared recent geographical and evolutionary histories. Once we failed to find support for single origin of *C. scouleri*, and thus incipient radiation, the question remains as to whether this pattern of morphological variation is exclusively the result of multiple biogeographical connections between Galápagos and the mainland. In other words, biogeographical relationships among *Croton* lineages in the Galápagos may be the result of two non-mutually exclusive origins: (1) multiple colonizations from the continent, or (2) back colonization from the islands to the mainland.

Oceanic islands are difficult to be colonized by most organisms. As a result, the majority of plant lineages on such islands are monophyletic, which indicates that they originated from a single colonization event (Gillespie & Clague, 2009). However, recent molecular phylogenetic analyses have provided valuable insights into the relationships of oceanic floras and their mainland relatives, showing that multiple colonizations of the islands have also occurred in a considerable number of genera (Carine *et al.*, 2004). In the Galápagos flora, phylogenetic evidence for multiple origins has already been demonstrated for *Darwiniothamnus* Harling (Andrus *et al.*, 2009), *Cordia* L. (Weeks *et al.*, 2010), *Cuscuta* L. (Stefanović *et al.*, 2007) and *Gossypium* L. (Wendel *et al.*, 2009).

Multiple colonizations have been also hypothesized for c. 25% of the genera of the Galápagos archipelago (Vargas *et al.*, 2012).

In contrast, back-colonization to the mainland, and thus islands serving as source area for continental taxa, appears to be a less frequent phenomenon. Nevertheless, the island-to-continent colonization hypothesis has been proposed in a few insular groups, such as *Aeonium* (Crassulaceae) (Mort *et al.*, 2002) and *Tolpis* Adans. (Park *et al.*, 2001; Moore *et al.*, 2002) from the Canary Islands (see Caujapé-Castells, 2011 for a review with emphasis on the Canary Islands). There is even the example of *Convolvulus* L., also from the Canary Islands, which shows phylogenetic patterns compatible with both multiple mainland-archipelago colonizations and one case of back colonization (Carine *et al.*, 2004). To the best of our knowledge, no case of back-colonization has been documented so far in the flora of the Galápagos Islands.

Hypothesis of multiple colonizations

The great morphological variation found in *C. scouleri* appears to be the result of differentiation on the Galápagos Islands and multiple colonizations. Our phylogeographical approach failed to support isolation of *Croton* in the Galápagos after a single colonization, which rules out exclusive in situ differentiation. Indeed, two leaf morphotypes (and intermediates) are widespread across the islands, of which one (broad-leaved) is most similar to that of the three continental species (*C. alnifolius*, *C. pavonis* and *C. riviniifolius*), while the other (narrow-leaved) appears to be the result of local differentiation from an earlier colonist. Further support for multiple continental *Croton* lineages coexisting in Galápagos is provided by seed morphology. The three continental closely related species to *C. scouleri* have distinctly grooved or ridged seeds and a size of c. 4-mm long. In Galápagos, *C. scouleri* individuals with broad leaves display larger (4–5-mm long, slightly ridged) seeds than those from narrow-leaved individuals that have smaller (3–4-mm long with pitted and mottled ornamentation) seeds (Riina *et al.*, Real Jardín Botánico (CSIC-RJB), Madrid, unpubl. data). In addition, we found molecular evidence for two Galápagos lineages directly connected to continental species (haplotypes 9 and 1; see Fig. 4b), indicating at least two independent colonization events from the continent. Considering that plants of any oceanic archipelago are the result of dispersal from the mainland, our morphological and molecular data are congruent with a hypothesis of at least two different lineages colonizing Galápagos followed by local differentiation. The fact that a higher number of haplotypes have been found in the Galápagos archipelago than in the three continental relatives also suggests a relatively long time of occurrence of *Croton* on the islands.

Therefore, based on our results and previous biogeographical patterns of Galápagos plants, a scenario of multiple colonizations seems more plausible than the back-colonization hypothesis. Future studies using more variable DNA

sequences (next generation sequencing data) correlated with individual phenotypes (biometric data) would help test the hypotheses of (1) two or more colonizations to the islands, (2) leaf morphological differentiation correlated with particular genotypes, and (3) whether two different leaf and seed morphotypes indicate differentiation at different times, and thus asynchronous colonization of Galápagos archipelago.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Holotypes of *Croton scouleri* illustrating extreme leaf-shapes.

Appendix S2 Voucher information.

Appendix S3 Statistical parsimony ribotype network of *Croton scouleri* and its closest relatives.

BIOSKETCHES

Beatriz Rumeu is a young researcher with a broad interest in biogeography, ecology and phylogeography, focusing on insular organisms. She has conducted research in the Canary Islands, Azores and Galápagos.

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Author contributions: P.V. and R.R. initiated the research and requested access to herbarium collections; B.R., P.V. and R.R. conceived the ideas; B.R. performed the lab work, conducted analyses and led the writing; B.R. and R.R. inspected herbarium specimens; all authors discussed the results and contributed to the writing and editing of the manuscript.

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