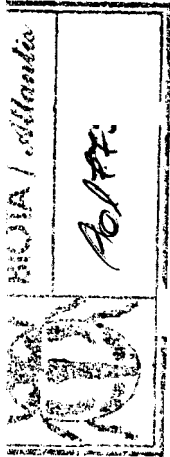


Tracking colonization and diversification of insect lineages on islands: mitochondrial DNA phylogeography of *Tarphius canariensis* (Coleoptera: Colydiidae) on the Canary Islands

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The genus *Tarphius* Erichson (Coleoptera: Colydiidae) is represented by 29 species on the Canary Islands. The majority are rare, single-island endemics intimately associated with the monteverde (laurel forest and fayal-brezal). The *Tarphius canariensis* complex is by far the most abundant and geographically widespread, occurring on Gran Canaria, Tenerife and La Palma. Eighty-seven individuals from the *T. canariensis* complex were sequenced for 411 bp of the mitochondrial DSA cytochrome oxidase I gene (COI), 597 bp of the COII gene and the intervening tRNA_{Leu} gene. A neighbour-joining analysis of maximum-likelihood distances put La Palma as a single monophyletic clade of haplotypes occurring within a larger clade comprising all Tenerife haplotypes. Gran Canarian haplotypes were also monophyletic occurring on a separate lineage. Using a combination of the phylogeographic pattern for *T. canariensis*, geological data, biogeography of the remaining species and estimated divergence times, we proposed a Tenerifean origin in the old Teno massif and independent colonizations from here to north-eastern Tenerife (Anaga), Gran Canaria and La Palma. New methods of estimating diversification rates using branching times were applied to each island fauna. All islands exhibited a gradually decreasing rate of genetic diversification similar to that seen for *Brachyderes rugatus* (Coleoptera: Curculionidae) from the Canary Islands.

Keywords: colonization; diversification; phylogeography; mitochondria; DS.4; Canary Islands; Coleoptera

1. INTRODUCTION

Oceanic archipelagos such as the Galápagos and Hawaiian Islands have provided model substrates for studying the processes of divergence and speciation. The faunal and floral richness of the Canary Islands has stimulated an increasing number of phylogeographic studies for this archipelago (for a review, see Juan *et al.* 2000). While many speciose groups exist on the archipelago, the most striking cases of diversification are among invertebrates, in particular the Coleoptera. Several recent studies have sought to use a molecular phylogenetic approach in reconstructing interspecific relationships within species-rich coleopteran genera (Juan *et al.* 1995, 1996a; Emerson *et al.* 1999, 2000a). While these studies have offered insight into the pattern of evolution of species assemblages, it is also of interest to understand what patterns and processes exist below the species level. This can best be achieved by examining the pattern of genetic diversity within species that have a broad geographical distribution both within single islands (e.g. Juan *et al.* 1996b, 1998) and across multiple islands (e.g. Emerson *et al.* 2000b).

The genus *Tarphius* Erichson (Coleoptera: Colydiidae) is represented by 29 species on five of the Canary Islands, i.e. Gran Canaria (four), Tenerife (13), La Gomera (eight), La Palma (five) and El Hierro (two). *Tarphius* are absent

on the eastern islands of Fuerteventura and Lanzarote which lack suitable habitat for this hygrophilic fungivorous beetle. The majority of *Tarphius* species are rare and intimately associated with the monteverde forest which, along with the pine woods, is viewed as a relict flora (Ciferri 1962). Only two species occur on more than one island: *Tarphius setosus* occurs on La Gomera and El Hierro and *Tarphius canariensis* occurs on Gran Canaria, Tenerife and La Palma. As well as being the most widespread species, *T. canariensis* is by far the most abundant *Tarphius* species on the archipelago. In this study, we examined the phylogeography and diversification of *T. canariensis* by sampling across its distribution on each of the islands of Gran Canaria, Tenerife and La Palma. Single, widespread species within island radiations such as *T. canariensis* may well represent complexes of species (Roderick & Gillespie 1996, or even paraphyletic assemblages such as is the case for *Drosophila grimshawi* on the Hawaiian Islands (Piano *et al.* 1997). Franz (1990) noted the confusion surrounding the taxonomy of *T. canariensis* and several other Canarian species. As part of a continuing study of the phylogeography of *Tarphius* across the entire archipelago we have identified four other species which are not phylogenetically distinct from *T. canariensis* and we include these in this present study. Figure 1 shows the collection sites for *T. canariensis*, *Tarphius simplex*, *Tarphius erosus*, *Tarphius elongatus* and *Tarphius supranubius*.

We have previously studied the phylogeography and diversification of a species of weevil (*Brachyderes rugatus*) occurring on several of the Canary Islands (Emerson

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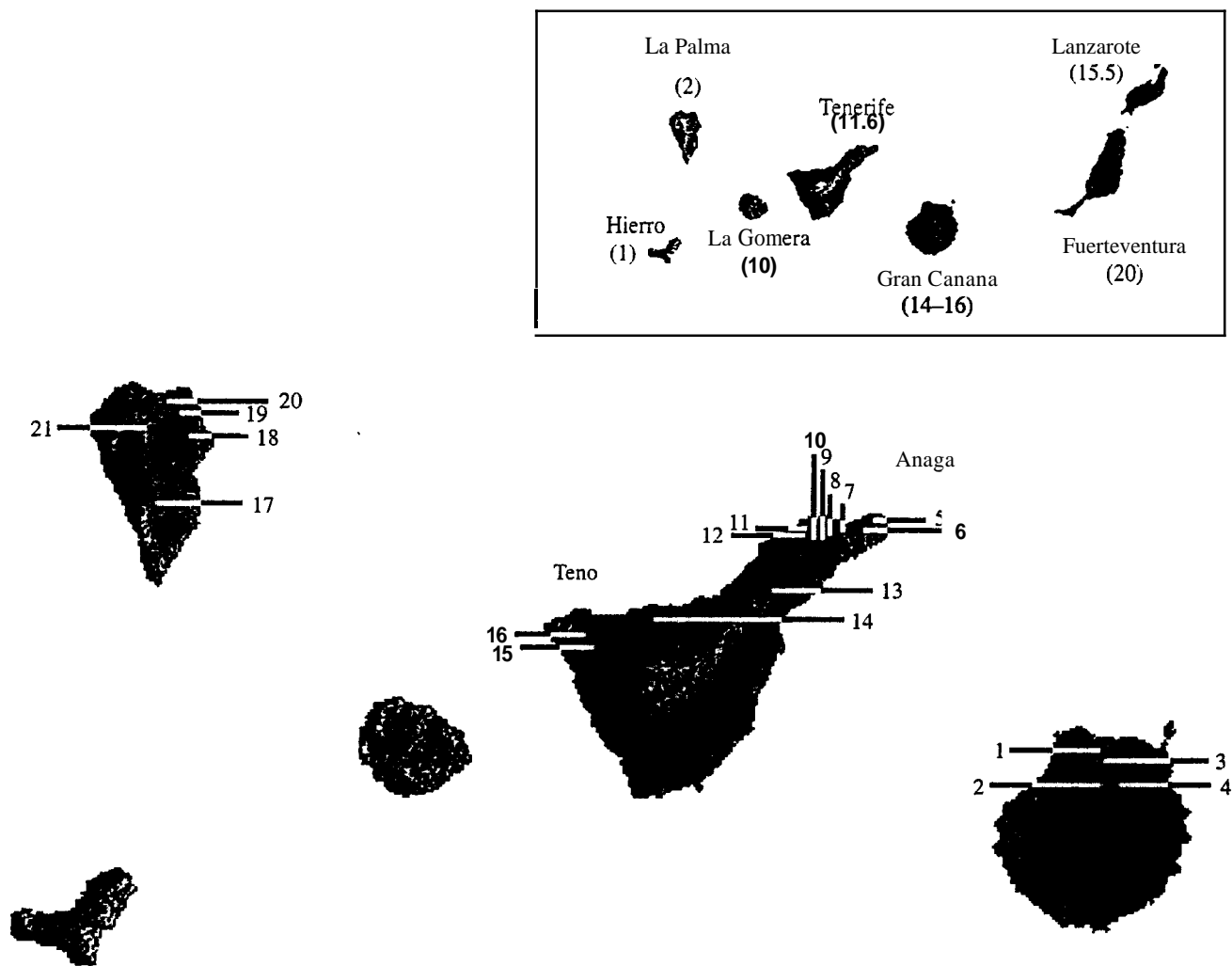


Figure 1. Sampling locations and number of individuals sequenced (in parentheses) for *Tarphius* (ca, *canariensis*; si, *simplex*; su, *supranubius*; er, *erosus*; el, *elongatus*) on the Canary Islands (maximum age of islands in millions of years shown in inset). 1, Brezal de Moya (five ca); 2, Barranco Oscuro (five ca); 3, Los Tilos de Moya (five ca); 4, Zamora (four ca); 5, Cabezo del Tejo (six si); 6, Barranco de Iguete (two si); 7, Vueltas de Taganana (two ca and two si); 8, Casas de la Cumbre (two ca); 9, Mirador Pico del Inglés (two si); 10, Mirador Cruz del Carmen (four ca); 11, El Batán (six ca and two si); 12, Barranco de Pedro Alvarez (four ca); 13, Las Lagunetas (four ca); 14, El Lagar (one el); 15, Monte del Agua 1 (four ca); 16, Monte del Agua 2 (two ca and two er); 17, Refugio El Pilar (eight ca); 18, Cubo de la Galga (one ca); 19, Los Tilos (four ca); 20, Gallegos (eight ca); 21, Roque de los Muchachos (two su).

et al. 2006). That study showed that, although the phylogeographic structure for *B. rugatus* mitochondrial DS.4 (mtDNA) sequences differed between islands, their diversification rates were not dissimilar. Each of the four islands exhibited a decreasing rate of mtDNA diversification over time, sometimes marked by sudden, periodic changes.

In their purest form, phylogeographic appraisals deal with the spatial distributions of alleles, the phylogenetic relationships of which are known or can be estimated (Avice 2000). In this study, we sequenced 87 individuals from the *T. canariensis* complex for 444 bp of the mtDNA cytochrome oxidase 1 gene (COI), 597 bp of the COII gene and the intervening tRNA_{Leu} gene. We tested the monophyly of the *T. canariensis* complex presented in this paper thoroughly by individually analysing a further 20 Canary Island *Tarphius* as outgroups. Bootstrap support for their monophyly was 100% for a series of neighbour-

joining analyses and the phylogenetically closest species, *Tarphius congestus* from Tenerife, was included in this study as an outgroup.

2. METHODS

(a) DNA extraction, polymerase chain reaction amplification and sequencing

Tarphius were collected from the Canary Islands from 1996 to 1998 (figure 1). DNA was extracted and purified by the methods described in Emerson *et al.* (1999; with the exception of, due to the small size of individuals (2.5–4 mm), whole beetles being used for extraction. Parallel specimens for each species and locality have been deposited in the collection of Pedro Oromí. The primers used for mtDNA amplification were Cl-J-2513 (designed by B.E.) (5'-CTATCGGAGGCTTACTGGAGTAGT-3') and TK-N-3782 (Eva, Harrison Laboratory, Cornell University, Ithaca, NY, USA) (5'-GAGACCATTACTTGCTTTTCAGT

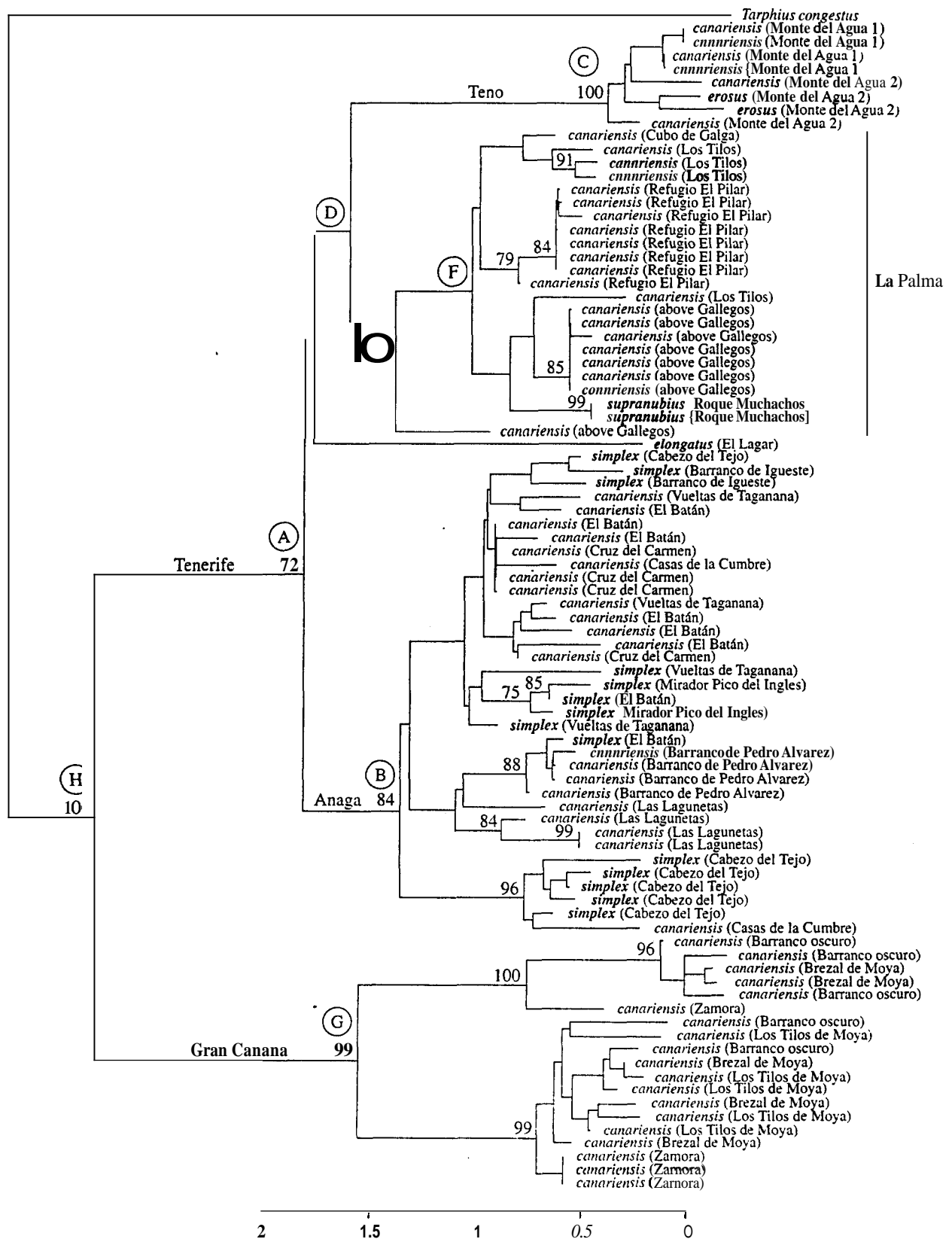


Figure 2. Neighbour-joining tree of maximum likelihood distances for species of the *T. canariensis* complex using mitochondrial COI and COII sequence data. Bootstrap values are indicated for nodes gaining more than 70% support (1000 replicates). Letters indicate nodes for which ages were estimated using the method of Sanderson (1997).

CATCT-3'). These primers amplify a fragment of 1286 bp of the COI, *tRNA_{Leu}* and COII gene regions. Polymerase chain reactions (PCRs) were carried out in 100 μ l volumes containing 2.5 μ l of each primer (10 μ M) and $MgCl_2$ (2 mM). One microlitre of

extract was used for amplification. Each of 40 PCR cycles comprised denaturation at 94 °C for 1 min, annealing at 35 °C for 30 s and extension at 72 °C for 1 min. The PCR products were purified with Promega 'Wizard PCR Clean Up System'

minicolumns (Promega, Southampton, UK) following the manufacturer's recommendations. Sequencing reactions were performed using Perkin-Elmer BigDye terminator reaction mix with PCR primer Cl-J-2513 and several internal primers (sequences can be provided upon request) and run on a Perkin-Elmer ABI automated sequencer (Perkin-Elmer, Cheshire, UK).

(b) Phylogenetic analysis

Sequence data were analysed using the program Modeltest (Posada & Crandall 1998, (http://bioag.byu.edu/zoology/crandall_lab/modeltest.htm). This analysis enables a comparison of different models of DNA substitution in a hierarchical hypothesis-testing framework. The models tested so far are the Jukes-Cantor, Kimura two-parameter, Tamura-Nei with equal base frequencies, Kimura three-parameter, SYM, F81, HKY85, Tamura-Nei, Kimura three-parameter with unequal base frequencies and general time-reversible models (for details of the models, see Posada & Crandall (1998)). Each model was tested with four rate heterogeneity models: (i) equal rates, (ii) estimating the number of invariant sites, (iii) estimating the gamma shape parameter across all sites, and (iv) estimating both the number of invariant sites and the gamma shape parameter for variable sites. Likelihood values were obtained for each of the 40 models using PAUP* (Swofford 1998) and then analysed with Modeltest in order to determine which of the models best described the data (log-likelihood ratio tests for nested hypotheses or Akaike information criterion (Akaike 1973) for non-nested hypotheses). The optimal model defined by Modeltest was then used to estimate maximum-likelihood distances for a neighbour-joining analysis using PAUP*.

(c) Estimation of divergence times

Several features of intraspecific gene genealogies complicate the direct application of a molecular clock in estimating divergence times (Emerson *et al.* 2000a, Sanderson (1997) developed algorithms for estimating divergence times from non-ultrametric trees. His method of non-parametric rate smoothing estimates a local rate of molecular evolution for each branch and then seeks to minimize the differences between these. The program r8s (anonymous ftp from pub director) at loco.ucdavis.edu) also allows for one or more nodes to be assigned ages. This then allows absolute ages for other nodes to be inferred. Final solutions for all analyses using r8s were perturbed using a factor of 0.05 and searches restarted from ten different initial times.

(d) Analysis of clade diversification

We used the method of Paradis (1997, 1998a, b) in order to estimate diversification from a phylogenetic tree with statistical models derived from survival analysis (Cox & Oakes 1984). Using the ages of the divergences it is possible to estimate the diversification rate (δ) with a maximum-likelihood approach and (i) test for temporal variations in the diversification rate within a clade, and (ii) test for variation in the diversification rate between clades. The fit of the data to three types of model can be tested with a maximum-likelihood approach using Diversi v.0.1 (anonymous ftp from pub directory at evol.isem.univ-montp2.fr). Model A assumes constant diversification through time, model B assumes a diversification rate that is either increasing or decreasing through time and model C assumes a break-point in time with different diversification rates before and after. The fit of the data to each model is assessed with either (i) likelihood ratio tests for nested models (in this case model A is nested within both models B and

C), or (ii) Akaike information criterion when there is no nesting relationship (as is the case between models B and C). Specifically defined models of diversification can be constructed for analysis of between-clade variation, ranging from the null model where all clades have the same δ to a model where all clades have a different δ . All likelihood ratio tests were performed at the 5% probability level. This framework provided a robust means of investigating variation in the diversification rate.

3. RESULTS

Totals of 444 and 597 bp were sequenced for the COI and COII genes, respectively (the 3'-end of the COII gene was not sequenced) and the intervening tRNA_{leu} gene was 65 bp in length. Their sequences have been deposited in the EMBL nucleotide sequence database under accession numbers AJ403037-AJ403104. Variability within the tRNA_{leu} gene was low with a total of six haplotypes, and data for this gene were excluded from subsequent analyses. COI and COII exhibited similar levels and patterns of variability and both log-likelihood ratio tests and Akaike information criterion from Modeltest (including other *Tarphius* species) supported the general time-reversible model as the best-fit substitution model for the combined COI and COII sequence data. The estimates of the substitution rates were 0.720 for A-C, 14.049 for A-G, 0.714 for A-T, 1.518 for C-G, 7.254 for C-T and 1.0 for G-T and the proportions of invariant sites and gamma shape parameters were estimated to be 0.587 and 1.283, respectively. Figure 2 is a neighbour-joining tree of the maximum-likelihood distances incorporating these parameters and using empirical nucleotide frequencies.

The maximum ingroup genetic divergence observed was 6.9% (maximum likelihood). Within islands, the maximum genetic divergences were 1.8% for La Palma, 4.2% for Tenerife and 5.6% for Gran Canaria. The divergences of ingroup taxa from the outgroup (*T. congestus*) ranged from 5.5 to 8.2%. The key features of the tree are as follows.

- (i) No haplotypes are shared between islands.
- (ii) Gran Canaria is composed of a single monophyletic clade of haplotypes.
- (iii) La Palma is composed of a single monophyletic clade including *T. supranubius* haplotypes.
- (iv) The La Palma clade occurs within a larger clade comprising all Tenerife haplotypes.
- (v) There are two well-supported clades within Tenerife corresponding to Anaga and Teno (figures 1 and 2). Both *T. simplex* and *T. erosus* haplotypes occur within these clades, respectively, but *T. elongatus* falls outside these two clades.

(a) Diversification times

An analysis of the phylogenetic pattern for the three islands was performed using the calibrated option of r8s (Sanderson 1997). Although Tenerife has a maximum age estimate of 11.6 million years (Myr), much of the island is geologically younger with volcanic activity over the last 2 Myr that connected the more ancient separate islets of Anaga and Teno (Ancochea *et al.* 1990). Several features

suggest that the distribution of *T. canariensis* on Tenerife postdates this connection (as opposed to a chance dispersal prior to the connection) with an origin in Teno.

- (i) The maximum uncorrected genetic divergence of 3% between Anaga and Teno is consistent with an upper age of ca. 1.5 Myr based on the rate estimates of DeSalle *et al.* (1987) and Brower (1994). However, it must be recognized that the generality of these rate estimates across invertebrate taxa is unknown.
- (ii) The similar phylogenetic divergence of the geographically intermediate *T. elongatus* (which occurs on geologically young terrain) suggests that this lineage evolved as part of a dispersive colonization process along the north-west coast of Tenerife.
- (iii) The closest related species to the *T. canariensis* complex, i.e. *T. congestus*, is the unique species restricted to Teno.

The maximum possible age for node A (figure 2), which separates Anaga from Teno, is 2 Myr, the beginning of the volcanic period connecting Teno and Anaga. By using this calibration we can thus generate maximum age estimates for nodes B–H. The nodal age estimates were 1.67 Myr for B, 0.89 Myr for C, 1.75 Myr for D, 1.51 Myr for E, 1.16 Myr for F, 1.80 Myr for G and 2.42 Myr for H. These age assignments are maximum estimates as it is likely that *T. canariensis* colonization from Teno to Anaga was more recent than 2 Myr ago.

(b) Analysis of diversification

Mitochondrial DSA clades for each island were analysed separately by calibrating the roots to an arbitrary value of 1 in order to generate relative divergence times on a scale of 0 to 1 and carry out analyses of temporal variation within a clade and between clades. Model B (gradual change in diversification) was always better supported than model A (constant diversification) for all three islands by both likelihood ratio tests and Akaike information criterion values. The β -values were > 1 suggesting that diversification rates are decreasing ($\beta = 1.98, 1.99$ and 2.58 for Gran Canaria, Tenerife and La Palma, respectively). A series of analyses of temporal variation were run for each island fauna with sequential break-points (T_c) for model C (which assumes a T_c with different diversification rates before and after) along the relative time-scale of 0 to 1. The results of these analyses are given in figure 3 along with the Akaike information criterion values for the fit of the data for each island to model B. The model with the lower Akaike information criterion is the one that best describes the data. Diversification on all islands was best described by a gradual decrease in rate (model B), but there were break-points for all islands when model C approached favour for describing the data. These break-points suggest that shifts in the diversification rate occurred late rather than early in the period of diversification.

We tested homogeneity in the diversification rate between islands in an analysis of between-clade variation in the diversification rate by comparing a model with different diversification rates for each island and a model with a similar diversification rate between islands. Likelihood ratio tests could not reject the hypothesis of a homogenous diversification rate between islands.

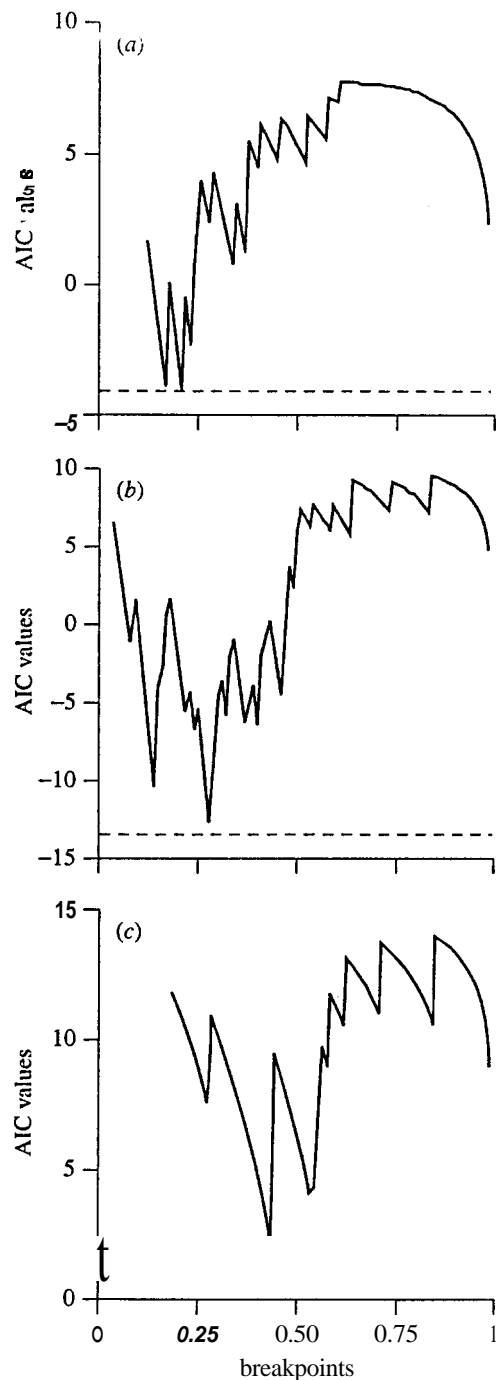


Figure 3. Distribution of Akaike information criterion values for models B (gradually increasing or decreasing diversification rate) (broken line) and C (a break-point in time with different diversification rates before and after) (solid line) for (a) Gran Canaria, (b) Tenerife and (c) La Palma. The x-axis represents break-points for model C and the y-axis represents Akaike information criterion values. Model C was evaluated against model B for 100 break-points. The model with the lowest Akaike information criterion was selected as the one that best described the data.

4. DISCUSSION

Tarphius canariensis is clearly not a monophyletic group. Less clear is the extent to which a revision of the taxonomy of this group is warranted without further data from independent genetic loci (see Avise 2000, pp. 285–308). The

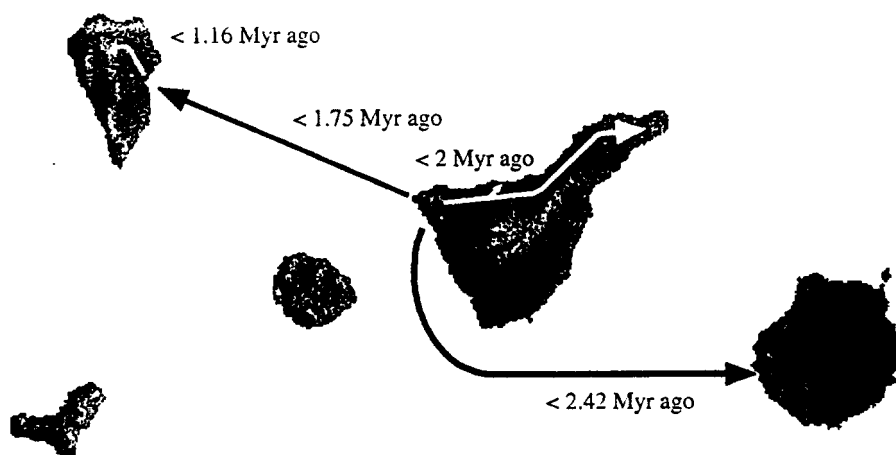


Figure 4. Proposed colonization sequence and approximate timing of colonization events (Myr ago) for the *T. canariensis* complex on the Canary Islands.

two *T. erosus* haplotypes within Tenerife fall within the Teno clade of *T. canariensis* haplotypes. The taxonomic status of *Zerosus* has undergone several changes since its species description by Wollaston (1862). Wollaston (1865) changed his opinion by considering *Zerosus* to be only a race of *T. canariensis*. Uyttenboogaart (1937) considered *T. erosus* to be merely an extreme form of *T. canariensis* and Franz (1967) synonymized *Zerosus* with *Zcanariensis*, a suggestion which was subsequently supported by Dajoz (1977). However, more recently Israelson (1980) considered *Zerosus* to be a species in its own right. Our results do not resolve this taxonomic uncertainty. Similarly, *T. simplex* haplotypes are interspersed among *T. canariensis* haplotypes within the Anaga region of Tenerife. The species status of *T. simplex* has not been previously questioned, although a close relationship with *T. canariensis* has been recognized (Franz 1990). The phylogenetic relationships between haplotypes of *T. canariensis* and *T. erosus* and haplotypes of *T. canariensis* and *T. simplex* (figure 2) suggest the possibility of either incomplete lineage sorting between these pairs of closely related species or morphological variation within *T. canariensis*. Analysis of nuclear markers such as microsatellites, amplified fragment length polymorphism or randomly amplified polymorphic DNA may further clarify the systematics of *T. simplex* and *T. erosus*.

Dajoz (1971) described *T. elongatus* and Franz (1990) noted the affinity of this species with the *Zcanariensis* group. The *T. elongatus* lineage is phylogenetically (figure 2), geographically (figure 1) and morphologically (Dajoz 1971) distinct from the two remaining Tenerifean lineages of the Teno and Anaga haplotypes. Our results are not in conflict with the species status accorded to *T. elongatus*, but suggest it is derived from within the *T. canariensis* group.

Tarphius supranubius, as described by Franz (1984), is geographically, ecologically and morphologically distinct from *T. canariensis* on the island of La Palma. Franz (1990) did not recognize *T. supranubius* as belonging to this complex in his treatment of the *T. canariensis* group. *Tarphius supranubius* is unique within the *Tarphius* as it occurs at high altitude in subalpine regions, being found within the dead wood and bark of *Adenocarpus viscosus*

(Leguminosae). The phylogenetic position of *T. supranubius* haplotypes within the La Palma *T. canariensis* clade suggests a successful colonization and transition from the monteverde to the much harsher subalpine environment by a *Tcanariensis* ancestor.

We suggest a Teno origin for the *Zcanariensis* complex, as *Zcongestus*, the sister species to the complex, is a Teno endemic. Interestingly, the remaining Tenerifean *Tarphius* occur outside Teno, primarily in Anaga. Figure 4 summarizes the proposed route of colonization for this group with maximum age estimates for events calibrated from the upper age estimate of 2 Myr for the colonization along the northern coast of Tenerife.

Within-island maximum age estimates for existing haplotype diversity (nodes B, C, E and G) range from 0.89 Myr ago for the Teno clade to 1.8 Myr ago for Gran Canaria. Haplotype diversity is highest on Tenerife and Gran Canaria with 88 and 89% of sampled haplotypes, respectively, being unique, relative to only 52% for La Palma. However, despite this high level of diversity there is no obvious phylogeographic pattern within islands (excluding the Teno–Anaga disjunction on Tenerife), although some clustering of haplotypes from the same sampling location can be seen within Tenerife and La Palma. The pattern of haplotype diversification is better described by a decreasing rate of diversification for all three islands and a test of between-clade rate variation could not reject the null hypothesis of equivalent diversification rates across islands. This result is very similar to that observed for Canary Island populations of *B. rugatus* (Emerson *et al.* 2006). Although a decreasing rate of diversification may be a general treiid it is possible that, with further geographical sampling, the application of more refined models of diversification incorporating spatial information, such as those of Crandall & Templeton (1996, and references within), may be more discriminating.

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