

Molecular phylogeny and dating of an insular endemic moth radiation inferred from mitochondrial and nuclear genes: The genus *Galagete* (Lepidoptera: Autostichidae) of the Galapagos Islands

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Abstract

Galagete is a genus of microlepidoptera including 12 nominate species endemic to the Galapagos Islands. In order to better understand the diversification of this endemic insular radiation, to unravel relationships among species and populations, and to get insight into the early stages of speciation, we developed a phylogenetic reconstruction based on the combined mitochondrial cytochrome oxidase I (555 bp) and II (453 bp), and the nuclear elongation factor-1 α (711 bp) and wingless (351 bp) genes. Monophyly of the genus is strongly supported in the Bayesian and maximum likelihood analyses suggesting a single colonization event by a common ancestor. Two cases of paraphyly observed between species are hypothesized to represent imperfect species limits for *G. espanolaensis* nested within the *G. turritella* clade, and introgressive hybridization or lineage sorting in the case of the population of *G. protozona* from Santa Fe nested within the *G. gnathodoxa* clade. A geologically calibrated, relaxed molecular clock model was used for the first time to unravel the chronological sequence of an insular radiation. The first split occurring within the *Galagete* lineage on the archipelago is estimated at 3.3 ± 0.4 million years ago. The genus radiated relatively quickly in about 1.8 million years, and gives an estimated speciation rate of 0.8 species per million years. Although the colonization scenario shows a stochastic dispersal pattern, the arrival of the ancestor and the diversification of the radiation coincide with the chronological emergence of the major islands.

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1. Introduction

Remote volcanic archipelagos, like the Canary, Hawaiian, and Galapagos Islands, have played a significant role in studies of speciation processes that frequently focus on the outcome of endemic insular radiations, the process by which one species evolves into multiple species over a relatively short time scale (i.e. Emerson and Oromi, 2005;

Gillespie, 2004; Grant, 1999; Jordan et al., 2003; Shaw, 2002).

True oceanic islands have been recognized as natural laboratories for studying evolution (Whittaker, 2002) and phylogenetic approaches have recently opened new insights into these systems (Emerson, 2002), particularly with regard to molecular time calibration in the Canary Islands (Emerson and Oromi, 2005), the Hawaiian Islands (Baldwin and Sanderson, 1998; Fleischer et al., 1998; Hormiga et al., 2003; Mendelson and Shaw, 2005), and the Galapagos Islands (Bollmer et al., 2006; Caccone et al., 1999; Rassmann, 1997; Sato et al., 2001; Sequeira et al., 2000).

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Molecular dating analyses have been criticized for a variety of reasons including calibrations using suspect fossil dates, violation of the molecular clock assumption, absence of confidence intervals, and use of inappropriate taxa (Graur and Martin, 2004). The reliability of the modern molecular clock has also been questioned in the case of explosive radiations (Bromham, 2003; Bromham and Penny, 2003), in particular in the case of island endemic radiations because they combine a number of factors, such as reduced population size, elevated speciation rate, adaptation to new niches and release from previous ecological constraints, which could influence rate evolution and make the molecular clock run faster. But Bromham and Woolfit (2004) found no support for a consistent increase in rates in island taxa compared to their mainland relatives. More recently, new methods enabling the incorporation of variable rates into molecular dating have become available (for a review, see Renner (2005)). Such techniques offer greater potential for insight into the history of lineages with poor or non-existent fossil records, and enable estimates of the time of origin of any biological lineage (Welch and Bromham, 2005).

The Galapagos provide the best opportunity to examine the molecular dating of an endemic radiation because of their discrete geographical nature, the absence of historical connection between the archipelago and the mainland, and the known geological age of their component islands. Within the Galapagos archipelago, an increase in the age of the islands from west to east is attributed to the eastward displacement of the Nazca plate over a mantle hotspot present beneath the youngest island, Fernandina (Bailey, 1976; Cox, 1983; White et al., 1993). Although the emerged islands are young (3.3 million years old at the most), evidence of drowned seamounts east of San Cristobal, which might have been islands, extend the temporal window available for evolution in the Galapagos to about 9 million years (Christie et al., 1992).

The microlepidoptera of the genus *Galagete* are a distinctive element of the moth fauna of the Galapagos because they represent the largest endemic radiation of Lepidoptera in the archipelago and are present on all major islands at almost all altitudes (Landry, 2002). The genus comprises 12 nominate species with variable distributions encompassing the whole archipelago for some species to single-island endemics (Landry, 2002; Schmitz and Landry, 2005). In a cladistic analysis based on morphological characters (Landry, 2002), the monophyly of *Galagete* and that of three species groups were recovered: a clade formed by the three larger species (*G. gnathodoxa*, *G. protozona*, and *G. seymourensis*), the *G. espanolaensis* and *G. turritella* pair, and a clade composed of *G. cinerea*, *G. consimilis*, and *G. darwini*. Species like *G. consimilis*, *G. darwini*, and *G. espanolaensis* are superficially so similar that they can be identified reliably from their genitalia only (Landry, 2002). *Galagete* was placed by Landry (2002) in the Autostichidae, Symmocinae *sensu* Hodges (1998), which also includes the Autostichinae and Holcopogoninae. However

Kaila's (2004) morphological analysis showed that the Autostichidae *sensu* Hodges (1998) are paraphyletic with regard to the Glyphidoceridae and Lecithoceridae placed in an Autostichid assemblage.

Little is known about the ecology of *Galagete* apart from their scavenging feeding habits which are consistent with those of other Autostichidae (Hodges, 1998). For example, the larva and pupa of *G. protozona* were discovered recently in droppings of the Galapagos Land Iguana *Conolophus subcristatus* (Schmitz and Landry, 2007a).

Here, we present a phylogenetic analysis of all species and most known populations of *Galagete* based on genes from both mtDNA and nDNA in a combined analysis. We compare the resulting phylogeny to morphologically based hypotheses (Landry, 2002) and examine important factors in the diversification of this insular endemic radiation in the Galapagos. We apply a relaxed molecular clock approach developed in a Bayesian framework incorporating geological estimates for the age of emergence of the islands. This provides a chronological template of evolution to investigate the time of divergence and colonization of the radiation. We compare the phylogenetic pattern and the age estimates of molecular divergence obtained for *Galagete* with other groups to elucidate particular sequences of dispersal and colonization among the Galapagos Islands.

2. Material and methods

2.1. Specimen sampling

We sampled all of the 12 described species of the genus *Galagete* and analyzed 47 specimens collected in the Galapagos archipelago on 13 islands from 2003 to 2006 (Fig. 1). All specimens were collected alive and immediately stored in EtOH 100%, except for one dry and pinned specimen of *G. levequei* from Santa Cruz. Outgroup taxa were chosen from the Gelechioidea: one from the family Gelechiidae and seven from different lineages of the Autostichid assemblage of Kaila (2004) (Table 1).

2.2. DNA extraction, PCR amplification, and sequencing

Whole genomic DNA was extracted from the adult thorax and head using the Nucleospin kit (Macherey-Nagel), except for the specimen of *G. protozona* collected on Baltra, a larva found in Land Iguana droppings (Schmitz and Landry, 2007a). Abdomens and wings were conserved in gelatin capsules as vouchers and for confirmation of specimen identification by dissection of the genitalia. The vouchers are deposited in the Muséum d'histoire naturelle, Geneva, Switzerland (MHNG). Detailed data on collection localities and DNA GenBank Accession numbers of all sequences included in our analysis are given in Table 1.

Fragments of cytochrome oxidase I and II (COI and COII), elongation factor-1 α (EF1 α), and wingless (WG) were amplified using polymerase chain reaction (PCR) with the primer combinations listed in Table 2. The rationale for

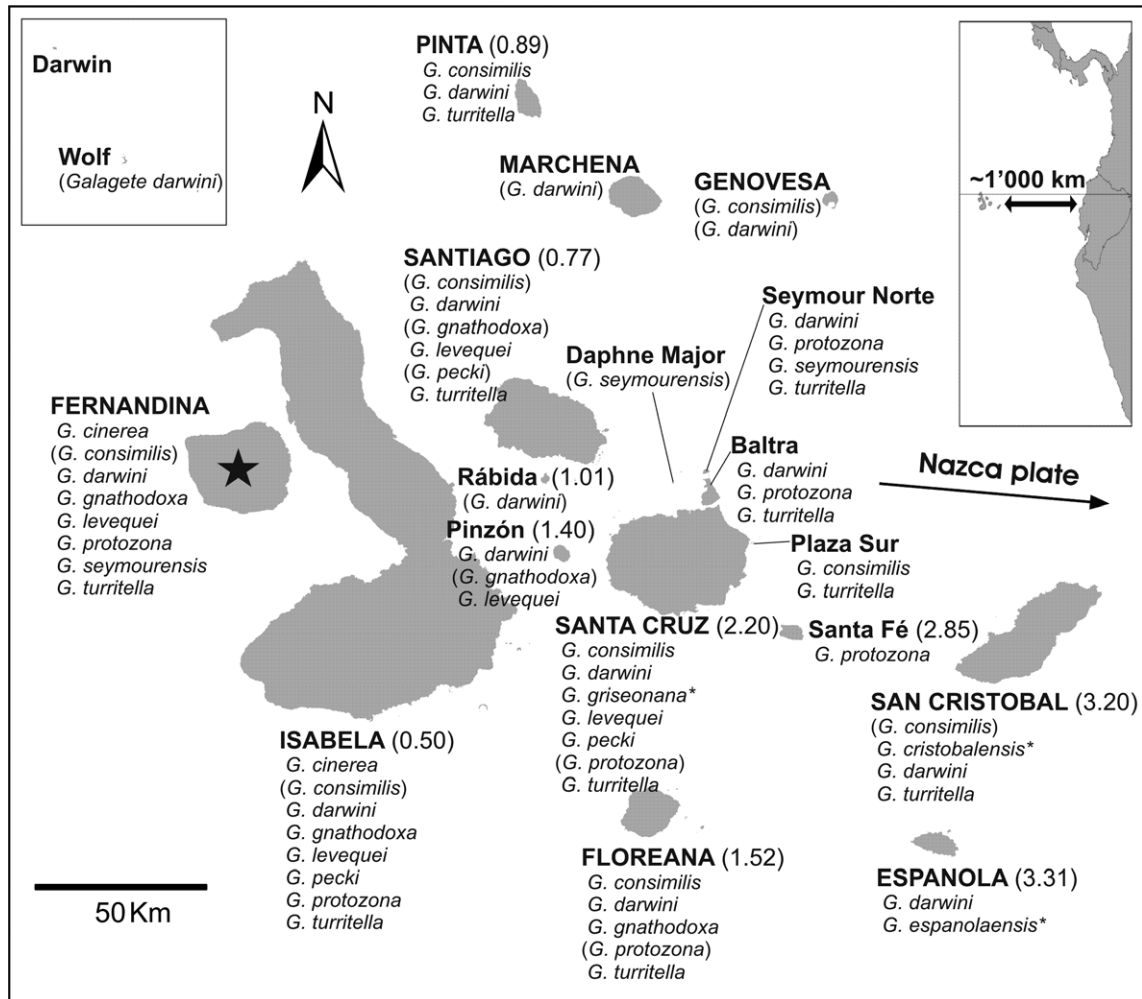


Fig. 1. Distribution of species of *Galagete* occurring in the Galapagos Islands. Species names in parentheses refer to populations for which samples could not be obtained for sequencing, but which are known from specimens in collections. Asterisks indicate the species considered as single-island endemics. Numbers in parentheses refer to the proposed maximum geological ages for each island (Bailey, 1976; Cox, 1983; Geist et al., 1994; White et al., 1993), estimated in millions of years. The star on Fernandina indicates the position of the archipelago's hotspot. Inset on the right is the geographic location of the Galapagos Islands relative to the equator and the South American continent.

the choice of these genes is that they have proven to be informative at different levels in several phylogenetic studies of Lepidoptera (Caterino et al., 2000).

The length of COI is ~1500 bp but we focused on its second half following the variability survey presented by Lunt et al. (1996) in other Insecta. Furthermore, because females are the heterogametic sex in Lepidoptera, the inviability of interspecific hybrid females expected under Haldane's Rule makes mtDNA less vulnerable to introgression (Sperling, 2003) that might lead to a disagreement between species trees and gene trees (Maddison, 1997; Pamilo and Nei, 1988; but see Funk and Omland (2003) and Rubinoff and Holland (2005) for different inheritance of mtDNA). As a contrast to the faster evolving sequence in COI and COII, we also sequenced part of the nuclear gene EF1 α and WG which are usually considered useful at higher taxonomic levels (Sperling, 2003).

The amplifications were performed in a total volume of 50 μ l. Thermal profiles for COI and COII (94 $^{\circ}$ C for 30 s;

47 $^{\circ}$ C for 30 s; 72 $^{\circ}$ C for 2 min), EF1 α (95 $^{\circ}$ C for 1 min; 55 $^{\circ}$ C for 1 min; 72 $^{\circ}$ C for 2 min), and WG (95 $^{\circ}$ C for 1 min; 50 $^{\circ}$ C for 1 min; 72 $^{\circ}$ C for 2 min), started with 5 min of denaturation at 95 $^{\circ}$ C, were repeated for 35 cycles, and were followed by a final step for 10 min at 72 $^{\circ}$ C.

The amplified PCR products were purified using the PCR Purification kit (Qiagen) and run on an ABI Prism 377 automated DNA sequencer, or an ABI Prism 3130xl genetic analyzer. The amplification primers were used for direct sequencing of the PCR fragments, and an additional internal primer, "Jerry", was used to replace "k698" to sequence the terminal region of COI. The same individual was sequenced for each gene, and sequences were translated into amino acids to check for reading frame. To avoid contamination, two or more individuals for some populations were sequenced days apart for the end of COI only to test for concordance between them (not shown). The resulting sequences encompass a 555 bp fragment in COI,

Table 1
Species of *Galagete* and outgroups included in the phylogenetic analyses

Species	Sample localities	COI GenBank No.	COII GenBank No.	EF1 α GenBank No.	Wingless GenBank No.
Outgroup:					
Autostichidae:					
Autostichinae					
<i>Autosticha modicella</i>	JAP: Hokkaido, Sapporo (K. Sugisima)	EF680572	EF680625	EF680680	EF680519
Autostichidae:					
Holcopogoninae					
<i>Holcopogon bubulcellus</i>	CR: Island of Krk, Kampelje (G. Baldizzone)	EF680568	EF680621	EF680676	EF680515
Autostichidae:					
Symmocinae					
<i>Taygete sphecochila</i>	EC: Galápagos, Santa Cruz	Landry et al., 2006	EF680619	EF680674	EF680513
<i>Oegoconia novimundi</i>	CH: Zurich, Steinmaur (R. Fritsch)	EF680569	EF680622	EF680677	EF680516
Lecithoceridae					
<i>Odites leucostola</i>	JAP: Hokkaido, Sapporo (K. Sugisima)	EF680570	EF680623	EF680678	EF680517
<i>Rhizosthenes falciformis</i>	JAP: Honsyū, Isikawa (T. Sarto)	EF680573	EF680626	EF680681	EF680520
Glyphidoceridae					
<i>Pseudodoxia achlyphans</i>	JAP: Hokkaido, Sapporo (K. Sugisima)	EF680571	EF680624	EF680679	EF680518
Gelechiidae					
<i>Chionodes Stefaniae</i>	EC: Galápagos, Pinzón (L. Roque)	Schmitz and Landry (2007b)	EF680620	EF680675	EF680514
Ingroup:					
Autostichidae:					
Symmocinae					
<i>Galagete cinerea</i> (1)	EC: Galápagos, Fernandina	EF680591	EF680645	EF680700	EF680539
<i>G. cinerea</i> (2)	EC: Galápagos, Isabela, Puerto Villamil	EF680580	EF680634	EF680689	EF680528
<i>G. consimilis</i> (1)	EC: Galápagos, Floreana	EF680605	EF680659	EF680714	EF680553
<i>G. consimilis</i> (2)	EC: Galápagos, Sta Cruz	EF680614	EF680669	EF680724	EF680563
<i>G. consimilis</i> (3)	EC: Galápagos, Pinta	EF680616	EF680671	EF680726	EF680565
<i>G. consimilis</i> (4)	EC: Galápagos, Plaza Sur	EF680617	EF680672	EF680727	EF680566
<i>G. cristobalensis</i> *	EC: Galápagos, San Cristóbal	EF680582	EF680636	EF680691	EF680530
<i>G. darwini</i> (1)	EC: Galápagos, Baltra	EF680612	EF680666	EF680721	EF680560
<i>G. darwini</i> (2)	EC: Galápagos, Española	EF680602	EF680656	EF680711	EF680550
<i>G. darwini</i> (3)	EC: Galápagos, Fernandina	EF680600	EF680654	EF680709	EF680548
<i>G. darwini</i> (4)	EC: Galápagos, Floreana	EF680615	EF680670	EF680725	EF680564
<i>G. darwini</i> (5)	EC: Galápagos, Isabela	EF680606	EF680660	EF680715	EF680554
<i>G. darwini</i> (6)	EC: Galápagos, Pinta	EF680607	EF680661	EF680716	EF680555
<i>G. darwini</i> (7)	EC: Galápagos, Pinzón	EF680609	EF680663	EF680718	EF680557
<i>G. darwini</i> (8)	EC: Galápagos, San Cristóbal	EF680590	EF680644	EF680699	EF680538
<i>G. darwini</i> (9)	EC: Galápagos, Santa Cruz	EF680618	EF680673	EF680728	EF680567
<i>G. darwini</i> (10)	EC: Galápagos, Santiago	EF680588	EF680642	EF680697	EF680536
<i>G. darwini</i> (11)	EC: Galápagos, Seymour Norte	EF680589	EF680643	EF680698	EF680537
<i>G. espanolaensis</i> *	EC: Galápagos, Española	EF680604	EF680658	EF680713	EF680552
<i>G. gnathodoxa</i> (1)	EC: Galápagos, Fernandina	EF680587	EF680641	EF680696	EF680535
<i>G. gnathodoxa</i> (2)	EC: Galápagos, Floreana	EF680576	EF680629	EF680684	EF680523
<i>G. gnathodoxa</i> (3)	EC: Galápagos, Isabela, Puerto Villamil	EF680585	EF680639	EF680694	EF680533
<i>G. griseonana</i> *	EC: Galápagos, Santa Cruz	EF680583	EF680637	EF680692	EF680531
<i>G. levequei</i> (1)	EC: Galápagos, Fernandina	EF680592	EF680646	EF680701	EF680540
<i>G. levequei</i> (2)	EC: Galápagos, Isabela, Volcano Alcedo	EF680595	EF680649	EF680704	EF680543
<i>G. levequei</i> (3)	EC: Galápagos, Santiago	EF680594	EF680648	EF680703	EF680542
<i>G. levequei</i> (4)	EC: Galápagos, Santa Cruz (L. Roque)	EF680574	EF680627	EF680682	EF680521
<i>G. levequei</i> (5)	EC: Galápagos, Pinzón	EF680610	EF680664	EF680719	EF680558
<i>G. pecki</i> (1)	EC: Galápagos, Isabela, Volcano Alcedo	EF680579	EF680633	EF680688	EF680527
<i>G. pecki</i> (2)	EC: Galápagos, Santa Cruz	EF680581	EF680635	EF680690	EF680529

(continued on next page)

Table 1 (continued)

Species	Sample localities	COI GenBank No.	COII GenBank No.	EF1 α GenBank No.	Wingless GenBank No.
<i>G. protozona</i> (1)	EC: Galápagos, Baltra	Schmitz and Landry (2007a)	EF680668	EF680723	EF680562
<i>G. protozona</i> (2)	EC: Galápagos, Fernandina	EF680586	EF680640	EF680695	EF680534
<i>G. protozona</i> (3)	EC: Galápagos, Isabela, Volcano Alcedo	EF680584	EF680638	EF680693	EF680532
<i>G. protozona</i> (4)	EC: Galápagos, Seymour Norte	Schmitz and Landry (2007a)	EF680630	EF680685	EF680524
<i>G. protozona</i> (5)	EC: Galápagos, Santa Fé (L. Roque)	EF680575	EF680628	EF680683	EF680522
<i>G. seymourensis</i> (1)	EC: Galápagos, Fernandina	EF680599	EF680653	EF680708	EF680547
<i>G. seymourensis</i> (2)	EC: Galápagos, Seymour Norte	EF680577	EF680631	EF680686	EF680525
<i>G. turritella</i> (1)	EC: Galápagos, Baltra	EF680613	EF680667	EF680722	EF680561
<i>G. turritella</i> (2)	EC: Galápagos, Fernandina	EF680603	EF680657	EF680712	EF680551
<i>G. turritella</i> (3)	EC: Galápagos, Floreana	EF680598	EF680652	EF680707	EF680546
<i>G. turritella</i> (4)	EC: Galápagos, Isabela, Volcano Alcedo	EF680578	EF680632	EF680687	EF680526
<i>G. turritella</i> (5)	EC: Galápagos, Pinta	EF680608	EF680662	EF680717	EF680556
<i>G. turritella</i> (6)	EC: Galápagos, Plaza Sur	EF680611	EF680665	EF680720	EF680559
<i>G. turritella</i> (7)	EC: Galápagos, Santiago	EF680593	EF680647	EF680702	EF680541
<i>G. turritella</i> (8)	EC: Galápagos, Seymour Norte	EF680597	EF680651	EF680706	EF680545
<i>G. turritella</i> (9)	EC: Galápagos, Santa Cruz	EF680596	EF680650	EF680705	EF680544
<i>G. turritella</i> (10)	EC: Galápagos, San Cristóbal	EF680601	EF680655	EF680710	EF680549

Samples not collected by the first or third author are indicated by collector's name in parentheses. Asterisks indicate the species considered as single-island endemics.

Table 2
Primers used in the amplification of COI, COII, EF1 α , and WG

Gene	Primer (forward or reverse)	Sequence of primer (5'–3')	Reference
COI	k698 (f)	TAC AAT TTA TCG CCT AAA CTT CAG CC	Caterino and Sperling (1999)
	Pat2 (r)	TCC ATT ACA TAT AAT CTG CCA TAT TAG	Caterino and Sperling (1999)
	Jerry (f)	CAA CAT TTA TTT TGA TTT TTT GG	Caterino and Sperling (1999)
COII	Stefi (f)	ATA CCT CGT CGT TAT TCT GAT TAT CC	Present study
	Eva (r)	GAG ACC ATT ACT TGC TTT CAG TCA TCT	Caterino and Sperling (1999)
EF1 α	EF44 (f)	GCY GAR CGY GAR CGT GGT ATY AC	Monteiro and Pierce (2000)
	EFrcM4 (r)	ACA GCV ACK GTY TGY CTC ATR TC	Monteiro and Pierce (2000)
WG	LepWG1 (f)	GAR TGY AAR TGY CAY GGY ATG TCT GG	Brower and DeSalle (1998)
	LepWG2a (r)	ACT NCG CAR CAC CAR TGG AAT GTR CA	Brower and DeSalle (1998)

453 bp in COII, 711 bp in EF1 α , and 351 bp in WG, representing 2070 bp altogether.

2.3. Phylogenetic analysis

Sequences were easily assembled manually, edited, and aligned with the software BIOEDIT 7.0.5 (Hall, 1999). Alignments were unambiguous. Homogeneity tests of base frequencies were conducted on all alignments by using the base frequencies χ^2 test as implemented in PAUP* 4.0b10 (Swofford, 2003) to estimate if the sequences have evolved with the same pattern of nucleotide substitution. A partition homogeneity test, using a heuristic search of 1000 replicates, was applied on the alignments of mtDNA and nDNA to verify if the two partitions could be analyzed in concatenation. Both data sets were first analyzed inde-

pendently to look for potential conflicts. The concatenated data sets were used then for further phylogenetic analyses.

Hierarchical-likelihood ratio tests performed with MODELTEST 3.04 (Posada and Crandall, 1998) determined that a General Time Reversible model with rate variation among sites and a proportion of invariable sites (GTR + Γ_8 + I) represent the best fit model of nucleotide substitution for the combined datasets. The Maximum Likelihood (ML) method was then performed with a heuristic search and random addition of sequences as implemented in PAUP* 4.0b10, with the starting tree obtained via stepwise addition of taxa, and then swapped using the tree-bisection-reconnection (TBR) algorithm. Outgroup taxa (Table 1) used were chosen from each subfamily inside the Autostichid assemblage (Kaila, 2004). The reliability of internal branches was assessed using the bootstrap method

(Felsenstein, 1985) with 1000 replicates by using the program PHYML 2.4.4 using the same model (Guindon and Gascuel, 2003). Bayesian posterior probabilities were calculated using a Metropolis-coupled, Markov Chain, Monte Carlo (MCMCMC) sampling approach, as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2003), using a $GTR + \Gamma_8 + I$ model. Four simultaneous Markov Chains were run in parallel twice for 1 million generations with trees sampled every 10 generations. Stationarity was evaluated graphically and the first 250,000 trees were discarded as burn-in. MrBayes was set to estimate model parameters independently and simultaneously for each gene partition. All above-mentioned analyses were run through the Bioportal web-based service platform for phylogenomic analysis at the University of Oslo (www.biportal.uio.no). The combined data provided robust phylogenetic reconstructions that we considered the departure point for further examination of the *Galagete* radiation.

2.4. Molecular dating

The Bayesian relaxed molecular clock approach was applied using the program package MULTIDIVTIME (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002). This program allows the incorporation of multiple time constraints, and takes into account both molecular and geological uncertainties to estimate the variance of divergence times. Calculations were performed on the ML tree obtained from the combined dataset. The module ESTBRANCHES was first used to estimate branch lengths of the constrained topology and the corresponding variance–covariance matrices. *Taygete sphecephila* was used as the outgroup because it was found to be the closest Galapagos relative based on morphological characters (Landry, 2002; Landry et al., 2006). The $F84 + \Gamma_8$ model (model complexity is limited by the dating program) was used with maximum likelihood parameters previously estimated by PAML 3.14 (Yang et al., 1997). The output from ESTBRANCHES was then used as the input file for the module MULTIDIVTIME to run a Markov Chain Monte Carlo (MCMC) for estimating the mean posterior divergence times on nodes, with associated standard deviation, and 95% credibility interval. MCMC was sampled every 100 generation over 1 million generations, after a burn-in period of 100,000 cycles. The following priors were used for the data set: 9.1 million years ago (Mya) for the expected time units between root and tips, corresponding to the age of the oldest drowned island east of the Galapagos hotspot (Christie et al., 1992), and 17 Mya (Werner and Hoernle, 2003) for the highest possible number of time units between root and tips. Other priors for gamma distribution of the rate at root node and the Brownian motion constant describing the rate variation (i.e. the degree of rate autocorrelation along the descending branches of the tree), were derived from the median branch length for the data set as advised by Thorne et al. (1998). Two internal calibration dates corresponding to the upper estimates for the age

of emergence of two islands were used as constraints in the analysis under the assumption that the *Galagete* taxa colonized the islands shortly after their emergence: these dates correspond to the oldest volcano on Isabela (Alcedo, 0.5 Mya; Geist et al., 1994), and the emergence of Floreana (1.52 Mya; White et al., 1993). These dates reflect our choice to use lower internal calibration points scattered through the topology to avoid discarding potentially useful information at the base of the tree. To estimate rates of speciation, we used the Yule estimator as implemented in Mendelson and Shaw (2005). The speciation rate (SR) for young monophyletic clade is estimated as $SR_{in} = ([\ln N]/t)$ where N is the number of extant species and t is the divergence time.

3. Results

3.1. Sequence data

The final concatenated alignment, excluding outgroups, was 2070-bp long, including 1008 mtDNA (COI and COII) characters, of which 317 were variable (31.4%), 62 of which were first codon position, 12 second codon position, and 243 third codon position, and 1062 nDNA (EF1 α and WG) characters of which 197 were variable (18.5%), 21 of which were first codon position, 7 second codon position, and 169 third codon position. The data showed an A/T ratio of 74% for mtDNA, and 44% for nDNA. The χ^2 homogeneity test of base frequencies indicated no significant deviations between taxa for both sets (mtDNA: $\chi^2 = 12$; $df = 138$; $P = 1.00$, and nDNA: $\chi^2 = 15$; $df = 138$; $P = 1.00$). The partition homogeneity test on the concatenated alignments was performed and showed no significant incongruence between the two data sets ($P = 0.13$). Therefore, the combined mitochondrial and nuclear dataset was used in further phylogenetic analyses.

The ingroup genetic distance (corrected for multiple hits with $GTR + \Gamma_8 + I$) ranged up to 18.5% for the mtDNA, and 6.6% for the nDNA. The combined alignment was unambiguous for both sets and without indels, as expected for protein-coding genes, except for a 3 bp insertion observed in COII for the whole ingroup.

Several facts indicated that the PCR mtDNA products were mitochondrial, rather than nuclear pseudogenes (Bensasson et al., 2001; Zhang and Hewitt, 1996). First, no unexpected stop codons or frameshift mutations were present in the coding sequences. Second, several long PCR were performed in one specimen for each species. Third, low guanine content (26%) suggests a mitochondrial origin.

3.2. Phylogenetic analyses

The ML tree of the combined mitochondrial and nuclear dataset is shown in Fig. 2, with the supports of nodes estimated with the two methods (MrBayes, ML). The concatenated dataset shows increasing Bayesian posterior probabilities (PP) and ML bootstrap supports (BS) than

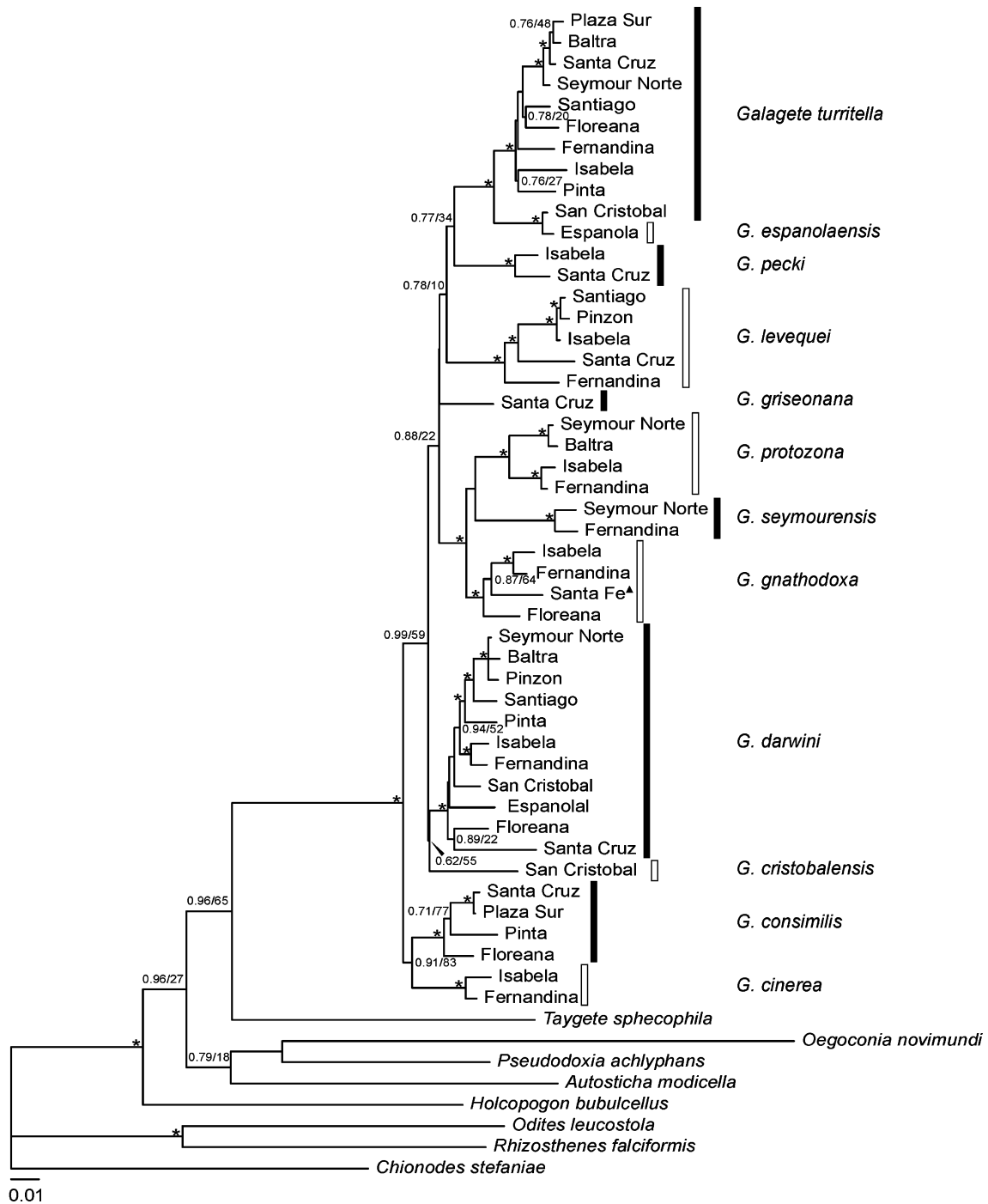


Fig. 2. ML phylogram of *Galagete* species and population relationships based on a combined molecular analysis of 1008 bp mtDNA and 1062 bp nDNA sequence data, with nodal support indicated (Bayesian analysis, ML). A node is shown as fully resolved (*) if its posterior probability is $\geq 95\%$ and its bootstrap value is ≥ 75 , respectively. Nodal support is indicated if its posterior probability is $\geq 75\%$ or its bootstrap value is ≥ 55 . The \blacktriangle symbol indicates the *G. protozona* population on Santa Fe.

found for the separate genes alone and no incongruence was observed between the resulting reconstructions of both data sets (not shown). The tree topologies obtained with MrBayes and ML for the combined analysis were identical. All sequenced species of *Galagete* are grouped in a strong monophyletic clade in both reconstructions (1.00 PP; 100% BS). This clade is further subdivided in 11 fully resolved clades (all with 1.00 PP; 100% BS; except the

G. darwini clade with 1.00 PP and 85% BS) which represent all the known species of *Galagete* including nearly all extant populations from the various islands, with two exceptions: *G. espanolaensis* which is nested inside the *G. turritella* clade (1.00 PP; 100% BS) and the population of *G. protozona* from Santa Fe which is nested inside the *G. gnathodoxa* clade (1.00 PP; 100% BS). Other interesting features appearing in the combined data tree is the mono-

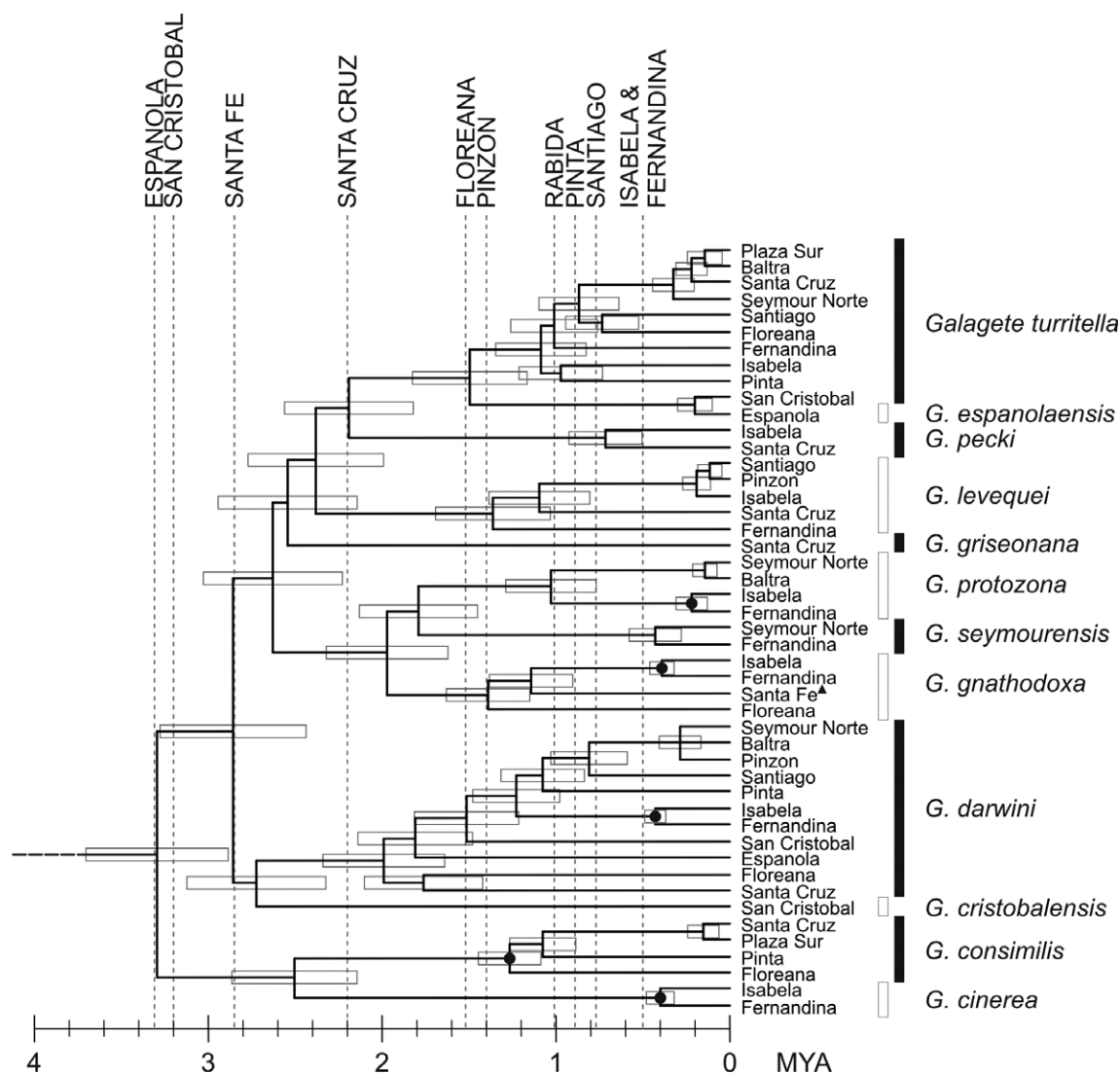


Fig. 3. Chronogram obtained from the combined mtDNA and nDNA data sets for the *Galagete* ingroup. Ages are inferred with the Bayesian rate autocorrelation method using five nodes under geological constraints (black dots). Horizontal boxes stand for \pm one standard deviation around divergence ages. Vertical dashed lines indicate the estimated age of emergence of the islands. The \blacktriangle symbol indicates the *G. protozona* population on Santa Fe.

phyletic grouping of the three larger species, *G. gnathodoxa*, *G. protozona*, and *G. seymourensis*, (1.00 PP; 99% BS) recovered from the cladistic analysis based on morphology (Landry, 2002), and the basal placement of *G. cinerea* and *G. consimilis* (0.91 PP; 83% BS).

3.3. Molecular dating

The first radiation event on the archipelago within the *Galagete* lineage obtained with the Bayesian relaxed molecular clock on the combined dataset and ML tree (Fig. 3) occurred around 3.3 ± 0.4 Mya. The genus radiated relatively quickly in about 1.8 million years, and the analysis gives an estimated speciation rate of 0.8 species per million years. The mini-radiation of the three larger species happened at 2.0 ± 0.3 Mya. *G. cristobalensis* and *G. griseonana*, two single-island endemics, split off at 2.7 ± 0.4 Mya and 2.5 ± 0.4 Mya respectively, and repre-

sent the oldest extant lineages of the radiation. The first split in the radiation coincides with the emergence of the oldest islands of Espanola and San Cristobal, the second with the emergence of Santa Fe. The radiation of 11 species of *Galagete* took place on the islands of Espanola, San Cristobal, Santa Fe, Santa Cruz, and Floreana, and was completed at 1.5 ± 0.3 Mya before the emergence of the other islands (dates indicated in Fig. 3).

4. Discussion

4.1. Outgroups

It is worth emphasizing three interesting features observed within the relationships to the outgroup taxa. First, the Autostichid assemblage *sensu* Kaila (2004) is strongly supported by both methods (MrBayes, ML), excluding the Lecithoceridae *Odites leucostola* and

Rhizosthenes falciformis. Second, the Bayesian analysis supports the paraphyly of the Autostichidae *sensu* Hodges (1998) and the basal position of *Holcopogon bubulcellus* (Holcopogonidae: Holcopogoninae), which is consistent with the results obtained by Kaila (2004). Third, regarding the Symmocinae *sensu* Hodges (1998), in which *Galagete* and *Taygete* were placed (Landry, 2002) with *Oegoconia*, the posterior probabilities support the close relationship of *T. sphecochila* with *Galagete*, but also the paraphyly of the subfamily because of the position of *Oegoconia novimundi*.

Taygete sphecochila was originally described from specimens reared from a nest of *Polistes* wasps on Trinidad and was probably introduced to the Galapagos by anthropological means, jointly with *Polistes* nests (Causton et al., 2006; Landry et al., 2006). It is now identified as the closest relative of *Galagete* in morphological (Landry, 2002; Landry et al., 2006) and molecular analyses (this study). Interestingly, Darwin's finches diverged also from a likely common ancestor occurring within the Caribbean islands (Burns et al., 2002). However, Central and South America are basically terra incognita for Gelechioidea and *T. sphecochila* may be more widespread than currently known.

4.2. *Galagete* phylogenetic relationships

Our analysis of mtDNA and nDNA sequence data strongly supports the monophyly of *Galagete* and thus, the hypothesis of a single colonization event.

Although some clades have unstable positions probably because of the very short internal branches in the likelihood analyses, all main lineages are supported. The lack of support in the internal structure of the trees could reflect the signature of the isolation and divergence of many populations in a short time frame, and thus, the tree topology probably is an accurate representation of historical relationships between taxa. The large number of genes used in this study and the extensive sampling reinforce this point of view. Therefore, it should be considered as a valid phylogenetic hypothesis because it might represent a recent simultaneous speciation event (Hoelzer and Melnick, 1993). *Galagete* does not yet represent an adaptive radiation according to Schluter (2000) because it does not satisfy the evolution of ecological diversity within a rapidly multiplying lineage, but only the evolution of its phenotypic diversity. However, the life history of most *Galagete* species is poorly known and further studies will be necessary to assess the ecological diversity of the genus.

The rapid speciation mechanism within *Galagete* may have happened twice in the *Galagete* colonization scenario, resulting in two major polytomies in the phylogeny (Fig. 3). The first event occurred at the base of the radiation just after the early colonizers arrived on the archipelago at around 3.3 Mya, and the second event is situated inside the radiation, with the simultaneous appearance of the three larger *Galagete* species between 1.5 and 2.0 Mya.

The occurrence of more than one species within a clade could be explained by problems in recognizing species boundaries due to morphological variation, interspecific introgressive hybridization, or incomplete lineage sorting of ancestral polymorphism (Funk and Omland, 2003). Two examples can be found in the phylogeny of *Galagete*.

First, *Galagete espanolaensis*, nested inside the *G. turritella* clade, may represent a geographical variant of the second species present on the oldest island of the archipelago, but it may also represent a valid taxon distinct from *G. turritella*, occurring on Espanola and San Cristobal. Interestingly, the three superficially indistinguishable species, *G. consimilis*, *G. darwini*, and *G. espanolaensis*, are genetically distinct and evolved separately. Two other cases of cryptic speciation were observed in the giant Galapagos tortoise population on Santa Cruz (Russello et al., 2005) and the Galapagos warbler finch in the archipelago (Petren et al., 1999; Tonniss et al., 2005), with one species occurring on the larger central islands while the other species are dispersed on smaller islands.

Second, the peripheral population of *G. protozona* on Santa Fe, nested inside the *G. gnathodoxa* clade, is also of interest. Hybridization cannot be ruled out between the morphologically similar *G. gnathodoxa* and *G. protozona* as they often occur in sympatry or are present on adjacent islands. The peripheral population on Santa Fe may have been differentially and profoundly affected by past introgression. On the other hand, the isolation of this population may be more easily explained by incomplete lineage sorting. This hypothesis is supported by the budding speciation concept which produces patterns of paraphyly in both nuclear and mitochondrial loci (Funk and Omland, 2003), as shown with this *G. protozona* peripheral isolate. But since distinguishing between introgression and incomplete sorting is often difficult (Funk and Omland, 2003), it is tentative yet to decide between the two causes for this paraphyletic relationship.

One factor that can accelerate lineage sorting is the relatively smaller population size due to bottlenecks presumably occurring on numerous occasions during the colonization of new islands in the Galapagos archipelago: this theory, first developed by Mayr (1954), is based on the influence of the founder effect in reducing levels of heterozygosity and leads, through random sampling error, to rapid differentiation between source and founder populations (Chakraborty and Nei, 1976; Hartl and Clark, 1989). During this study we found two examples of intra-archipelago diversification, *G. levequei* and *G. pecki*, which show both remarkable variation of the wing pattern between different island populations and well defined intraspecific relationships (see Fig. 2). Specimens of *G. levequei* encountered on Fernandina are almost completely dark-brown compared to those found on the other islands, which are predominantly white, but there are intermediate forms. This great phenotypic variation encountered within *G. levequei* illustrates an interesting case of linear morphological gradation through the archipelago, with distinct

genetic differentiation. The same may apply to the *G. pecki* populations in which variation is so striking that the population on Santa Cruz was thought to be a new species until it was described as a new subspecies based on morphological and molecular characters (Schmitz and Landry, 2005). Further studies will be necessary to estimate the role of founder effect in the differentiation of these taxa.

Ricklefs and Bermingham (1998) have analyzed phylogenies of the West Indian avifauna and suggested that as populations get older, they become more restricted in their distribution and suffer an increased probability of extinction. Subsequent extinction among local populations may lead to global island extinction, or a new phase of range expansion leading to a renewal of the cycle (the “taxon cycle” theory, for a review see Emerson (2002), Ricklefs and Bermingham (2002)). Within the Galapagos archipelago, according to this theory one can assume that the ranges of two species, *G. cristobalensis* and *G. griseonana*, which occur now only on older islands, may be due to local extinctions of once widespread ancestors.

No clear pattern emerges from the *G. darwini* and *G. turritella* lineages. Both are common and present on each island and islet in the archipelago, often found close to the shore. Although some island populations are obviously closely related, the lack of branch support inside these clades suggests that gene flow has occurred recently between the different populations.

4.3. Origin, dating, and biogeography

Changes in ocean currents were affected since 4.6 Mya due to the closure of the Isthmus of Panama, which was

completed 3.6 Mya (Haug and Tiedemann, 1998), and were probably accompanied by alterations in the direction of the prevailing winds. The source of the founder of the *Galagete* group is probably coastal South America, which lies 1000 km from the archipelago. Maybe one of the southeast trade winds carried the early colonizers of *Galagete* from the continent across the open waters of the Pacific Ocean to the Galapagos Islands by passive aerial transport, a mode of dispersal which is thought to account for the presence of most of the small-winged insects in the Galapagos (Peck, 2001). The oldest split within the *Galagete* lineage is estimated in this study at 3.3 ± 0.4 Mya, a result that seems reasonable compared to the earliest emergence date of the archipelago (3 Mya for the islands currently emerged but 9 Mya for the drowned seamounts). The rate of speciation of 0.8 species per million years corresponds to that calculated for the entire radiation of the Hawaiian *Laupala* crickets (Mendelson and Shaw, 2005). Compared to other native elements of the Galapagos fauna (see Table 3 for references), the *Galagete* colonization event happened after the arrival of the endemic iguanas (10.5–19.5 Mya) and *Galapaganus* weevils (10.7–12.1 Mya), which have diverged on the actual drowned islands, and before the giant Galapagos tortoises (1.5–2.0 Mya), the Darwin finches (1.2–2.3 Mya), and the Galapagos hawk (0.05–0.25 Mya), which represent recent arrivals on the extant islands. Although molecular estimates for Galapagos taxa are often plagued with problematic calibrations and emphasize previous use in the literature as justification for models (Grehan, 2001), new analytical methods, such as used here, enable the possibility to challenge recurrent questions on the chronological colonization scenario of oceanic islands.

Table 3

Molecular estimates (millions of years) determining the initial split within the lineage and the temporal window of divergence from the ancestor in different organism lineages of the Galapagos

Species	Initial split within lineage	Divergence with ancestor	Method and data	Reference
<i>Phyllodactylus</i> lizards	NA	8.9	Allozyme data calibrated with Nei and Sarich estimations for electrophoretic data	Wright (1983)
<i>Tropidurus</i> lava lizards	NA	10.2	Allozyme data calibrated with Nei and Sarich estimations for electrophoretic data	Wright (1983)
Galapagos iguanids	15.0–20.0	NA	Immunological distance based on albumin molecular clock	Wyles and Sarich (1983)
<i>Tropidurus</i> lava lizards	3.0–34.0	33.0–48.0	Immunological distance based on albumin molecular clock	Lopez et al. (1992)
Galapagos cotton <i>Gossypium</i>	NA	24.0–33.0	cpDNA sequence divergence based on comparison for plastid encoded rbc-L data	Wendel and Albert (1992)
Galapagos daisy <i>Scalasia</i>	NA	1.9–6.2	cpDNA sequence divergence based on comparison for plastid encoded rbc-L data	Schilling et al. (1994)
Galapagos iguanids	10.5–19.5	NA	mtDNA molecular clock for 12 S and 16 S rDNA calibrated with ungulate rates	Rassmann (1997)
Giant Galapagos tortoise	1.5–2.0	6.0–12.0	mtDNA molecular clock for Cytb and 16 S rDNA calibrated with ectotherms rates	Caccone et al. (2002, 1999)
Flightless <i>Galapaganus</i> weevils	10.7–12.1	NA	mtDNA molecular clock for COI calibrated with arthropods rates	Sequeira et al. (2000)
Darwin's finches	NA	1.2–2.3	mtDNA molecular clock for Cytb calibrated with geese rates	Sato et al. (2001)
Galapagos hawk	NA	0.05–0.254	mtDNA molecular clock for Cytb, COI, and ND2 calibrated with geese rates	Bollmer et al. (2006)
Galapagos <i>Galagete</i> microlepidoptera	2.9–3.7	NA	Bayesian relaxed molecular clock approach for mtDNA and nDNA	Present study

The common ancestor of all *Galagete* species, which may be phenotypically similar to *G. cinerea* and *G. consimilis*, could have arrived on the extant island of Espanola, or even before on the currently submerged islands like Iguanids and weevils (Table 3). During initial colonization, opportunities for allopatric differentiation were probably limited. The radiation may have accelerated later as the archipelago increased in number of islands, but was accomplished before the last extant islands emerged. The apparent lack of any simple evolutionary pattern from older to younger islands distinguishes the Galapagos from more linear examples of animal radiation on hotspot archipelagos such as Hawaii (Roderick and Gillespie, 1998) and the Marquesas (Cibois et al., 2004). Although the simple stepping-stone colonization model is predominant in the Canary Islands, deviations from such a pattern have also been observed on this archipelago (Juan et al., 2000). The complex geological history and settings of the Galapagos Islands whose volcanoes are not aligned in a chain (White et al., 1993) may be reflected by the complexity of the colonization patterns. In the Galapagos, the historical biogeographic pattern expected if speciation followed in tandem with island formation, which is recovered in reptile and Land snail phylogenies (Caccone et al., 2002; Kizirian et al., 2004; Parent and Crespi, 2006), is in contrast with that found for birds (Bollmer et al., 2006; Tonniss et al., 2005) and our findings for Lepidoptera. The main cause is certainly the high vagility of the winged species which obscures the evolutionary pathways within the archipelago with probable back-colonizations underlining a stochastic dispersal pattern (Cowie and Holland, 2006). Although the nature of colonization in *Galagete* is stochastic, the arrival of the ancestor and the diversification of the radiation coincide with the chronological emergence of the major islands.

It would be of interest to add microsatellites to the dataset as they have demonstrated use for reconstructing phylogenies of closely related taxa like Darwin's finches (Petren et al., 1999, 2005) or giant Galapagos tortoise populations (Ciofi et al., 2002, 2006; Russello et al., 2005). Such a detailed level of information would help to refine our understanding of this endemic radiation. A more comprehensive study on the ecology of the various *Galagete* species would also be worthwhile to confirm its adaptive nature and to elucidate the environmental causes underlying this insular radiation. Critical knowledge on *Galagete* is still missing and necessary for further inference regarding the conservation status of its species, ecological role, and importance. All these would be useful and contribute to strategies of arthropod conservation on the archipelago.

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