doi:10.1111/j.1558-5646.2009.00617.x

ISLAND BIOGEOGRAPHY OF GALÁPAGOS LAVA LIZARDS (TROPIDURIDAE: *MICROLOPHUS*): SPECIES DIVERSITY AND COLONIZATION OF THE ARCHIPELAGO

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Received September 12, 2008
Accepted November 23, 2008

The "lava lizards" (*Microlophus*) are distributed throughout the Galápagos Archipelago, and consist of radiations derived from two independent colonizations. The "Eastern Radiation" includes *M. bivittatus* and *M. habeli* endemic to San Cristobal and Marchena Islands. The "Western Radiation" includes five to seven historically recognized species distributed across almost the entire Archipelago. We combine dense geographic sampling and multilocus sequence data to estimate a phylogenetic hypothesis for the Western Radiation, to delimit species boundaries in this radiation, and to estimate a time frame for colonization events. Our phylogenetic hypothesis rejects two earlier topologies for the Western Radiation and paraphyly of *M. albemarlensis*, while providing strong support for single colonizations on each island. The colonization history implied by our phylogeny is consistent with general expectations of an east-to-west route predicted by the putative age of island groups, and prevailing ocean currents in the Archipelago. Additionally, combined evidence suggests that *M. indefatigabilis* from Santa Fe should be recognized as a full species. Finally, molecular divergence estimates suggest that the two colonization events likely occurred on the oldest existing islands, and the Western Radiation represents a recent radiation that, in most cases, has produced species that are considerably younger than the islands they inhabit.

KEY WORDS: **Galápagos, lizards, mitochondrial DNA, molecular timing of colonization, nuclear DNA, oceanic islands, phylogeny.**

Oceanic islands have been model systems in evolutionary studies for well over a century (Emerson 2002; Whittaker et al. 2008), and the Galápagos Archipelago, located about 960 km west from the coast of Ecuador, has figured prominently among them. Galápagos is one of the most recent oceanic island formations (Christie et al. 1992), and a consensus biogeographic history (reviewed in Grehan 2001) has favored an over-water colonization model for the origin of its many endemic radiations. Most of these studies have emphasized early models of Galápagos colonization events that were initially constrained to a 4 to 5 million year time frame set by the estimated ages of the oldest current islands (Cox 1983). The subsequent discovery of underwater seamounts representing former Galápagos islands to the east of the current archipelago on the east-shifting Nazca Plate, extended the

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temporal window for colonization to at least 17 million years (Werner et al. 1999; Werner and Hoernle 2003). This extension still does not preclude even earlier landmasses given the 80 to 90 million-year existence of the ocean-floor hotspot (Christie et al. 1992). An extended window of time may have allowed for overwater colonization by founders of a group onto emerged volcanic islands with a series of subsequent dispersals in a westward direction onto younger islands, in the interim, older islands were transported east and eventually eroded below sea level. The "conveyer belt" mechanism was proposed by Axelrod (1972) as a general evolutionary scenario for many Pacific island biotas, and has been invoked to explain the evolution of some of the Galápagos taxa with molecular divergence estimates that exceeded the age of existing islands (Wright 1983; Wyles and Sarich 1983; Rassmann 1997; Sequeira et al. 2008).

Knowledge of both geographic sources and approximate arrival times of the ancestors of endemic radiations is a key component to better understand the evolution of the Galápagos biota. This requires well-corroborated phylogenetic hypotheses of clades that include all Galápagos endemic species of a given radiation, and reliable molecular estimates of divergence times for these clades. Available divergence time estimates for Galápagos radiations are questionable due to either imprecise external reference points, or the use of nonspecific extrinsic calibrations derived from unrelated groups (but see Schmitz et al. 2007 for an exception). More recently, advances in molecular methods for estimating divergence times, especially with multigene datasets, reduce the uncertainties associated with simplistic assumptions made in earlier studies (Thorne and Kishino 2002). We use this approach to estimate colonization times of the Galápagos Archipelago by lizards of the genus *Microlophus*.

THE GALÁPAGOS LAVA LIZARDS

The seven to nine Galápagos species of *Microlophus* are hypothesized to have radiated asymmetrically after two independent colonization events from the mainland; nonmonophyly of the insular group is supported by multiple lines of evidence including allozyme polymorphisms (Wright 1983), immunological distances (Lopez et al. 1992), and both mtDNA (Heise 1998; Kizirian et al. 2004) and nuclear sequence data (Benavides et al. 2007). The two island clades include a small "Eastern Radiation" consisting of two species endemic to San Cristobal (*M. bivittatus*) and Marchena (*M. habeli*) islands, and a larger "Western Radiation" of five to seven species (see Baur 1892; Van Denburgh and Slevin 1913; Kizirian et al. 2004) that inhabit most of the southern and western islands (Fig. 1). Both radiations appear to have been established in the oldest islands of the archipelago (i.e., San Cristobal [Eastern] and Española [Western]), with subsequent divergence hypothesized via the westward colonization of younger islands.

To date, the lava lizards are only one of two endemic Galápagos terrestrial groups for which two separate origins are proposed; Wright (1983) hypothesized two separate colonization events from mainland South America for geckos of the genus *Phyllodactylus* (fig. 10, p. 149) and lava lizards (the genus was *Tropidurus* in the 1983 paper; see fig. 11, p. 150), on the basis of allozyme data. The *Phyllodactylus* radiation has not been rigorously tested, and all other studies of endemic Galápagos radiations suggest single colonization events (Parent and Crespi 2006; but see general caveats summarized by Emerson 2002). Available divergence estimates suggest that origins of some groups, including lava lizards (Lopez et al. 1992), marine and land iguanas (genera *Amblyrhynchus* and *Conolophus*, respectively; Rassmann 1997), and *Galapaganus* weevils (Sequeira et al. 2000) may predate the ages of the oldest current islands. For *Microlophus* the available estimates place the arrival times from 2.45 (Wright 1983; allozyme distance data) to 34 million years ago (Lopez et al. 1992; albumin immunological distance data). In contrast, estimates for the origin of other Galápagos endemics are more recent, and match the estimated geological age of the existing islands (see below).

OBJECTIVES OF THIS STUDY

We have collected an extensive molecular dataset for the Galápagos lava lizards, and here we extend the study of Benavides et al. (2007) by focusing on species boundaries and relationships within the Western Radiation to address several questions relevant to the evolution of this group. First, we evaluate the recent proposal by Kizirian et al. (2004) and Kizirian and Donnelly (2004) for the Western Radiation, in which *M. albemarlensis* is interpreted as a single entity that these authors recognized as a complex due to its paraphyly with respect to several other diagnosable species (see table 3 in Kizirian and Donnelly 2004). Two other insular groups were characterized as weakly divergent and morphologically nondiagnosable and remained unnamed. Kizirian et al. (2004) follow the earlier taxonomy of Van Denburgh and Slevin (1913), and applied the name *M. albemarlensis* to populations from four large islands and numerous "satellite islets" to each of these. The *albemarlensis* complex as recognized in these papers is distributed throughout the islands of Isabela, Fernandina, Santa Cruz–Santa Fe, and Santiago (and the associated satellite islets of all of these). More recently, however, Benavides et al. (2007) showed strong support for reciprocal monophyly of three mtDNA haploclades for the Isabela–Fernandina, Santa Cruz–Santa Fe, and Santiago Island complexes; some of these clades were also supported by nuclear gene regions, and all were strongly supported by the combined mtDNA–nuclear datasets (Benavides et al. 2007, fig. 8). Ignoring the summation of names by Van Denburgh and Slevin (1913) and Kizirian and Donnelly (2004), Benavides et al. (2007) restricted the name *M. albemarlensis* to the Isabela–Fernandina islands, and

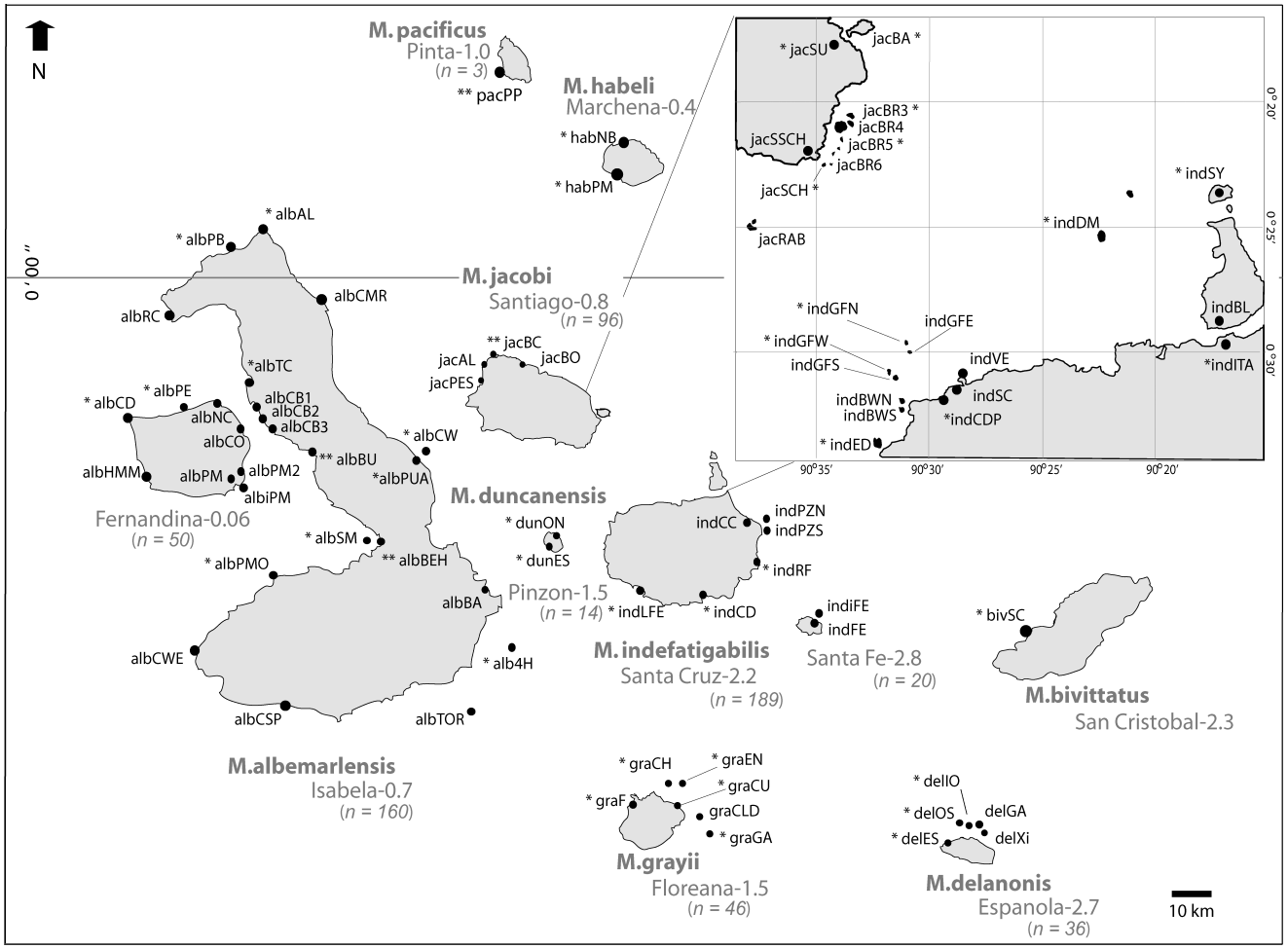

Figure 1. **Distribution of the nine species of *Microlophus* (Baur 1892) endemic to the Galápagos Archipelago; the "Eastern Radiation" includes only *M. bivittatus* (biv) and *M. habeli* (hab) endemic to San Cristobal and Marchena Islands, respectively. The "Western Radiation" includes the seven species for which sampling sites are plotted here (sampling points and type locality details are given in Appendix S1). The inset shows sampling points from small islets adjacent to the islands of Santa Cruz and Santiago. Abbreviations for the Western Radiation species names are (east-to-west): del, *M. delanonis* (Española Island); gra, *M. grayii* (Floreana); ind, *M. indefatigabilis* (Santa Cruz, Santa Fe); dun, *M. duncanensis* (Pinzón); jac, *M. jacobi* (Santiago); alb, *M. albemarlensis* (Isabela, Fernandina); and pac, *M. pacificus* (Pinta). Asterisks next to names identify 40 localities from which we chose 44 unique cyt *b* haplotypes within the Western Radiation. In some cases, two divergent haplotypes were sampled from the same locality and these are identified by two asterisks (see text for detailed explanation). The DNA from these lizards/haplotypes was also used to sequence all gene regions used in the phylogenetic analyses. The numbers following island names are consensus estimates of subaerial age for islands of the Galápagos Archipelago (taken from Vicenzi et al. [1990], Parent and Crespi [2006], and Arbogast et al. [2006]). The numbers in parentheses beneath island names refer to the total number of specimens sampled by island in this study.**

recognized the Santiago and Santa Cruz–Santa Fe populations as *M. jacobi* and *M. indefatigabilis*, respectively (both names are original to Baur [1892]). In Figure 1, we show the distribution of the seven species recognized by Benavides et al. (2007).

The seven species comprising the Western Radiation are allospecies (i.e., they are endemic to single islands or island complexes; type localities are given in Appendix S1); none show sympatry. Because geographic sampling was limited in both the Kizirian et al. (2004) and the Benavides et al. (2007) studies, and the latter was not focused on species delimitation, our sampling effort here was designed to provide a robust test of monophyly versus paraphyly of the entities included in the *M. albemarlensis* complex. We use the mtDNA locus as a "first pass" estimator of population history and species limits in this clade (Zink and Barrowclough 2008), but we are fully cognizant of the limitations of this approach, especially for recent divergence events (Hudson and Coyne 2002). In this study, we interpret strong support for mtDNA monophyly of these island populations as evidence that they are "candidate species" (Morando et al. 2003), and corroboration by nuclear genes suggests that these groups comprise

genealogical species (de Queiroz 1998, 2005b). A subset of the total number of lizards sequenced for cyt *b* was then used for phylogenetic analyses of multiple mitochondrial and nuclear gene regions, to obtain a best estimate of the species tree for the Western Radiation.

We test our best-supported combined-data topology for the Western Radiation against alternatives presented in earlier studies that show varying degrees of discordance regarding the direction and sequence of inter-island colonization events (Wright 1983; Lopez et al. 1992; Heise 1998; Kizirian et al. 2004; Benavides et al. 2007). From this result, we present a refined hypothesis for colonization routes and the sequence of derivation for the seven species we recognize in this radiation. Finally, we address the temporal issue by estimating the timing of the two *Microlophus* colonization events from continental South America. More specifically, did one or both colonization events of the Archipelago predate the ages of the oldest current islands, or are both radiations derived within the time frame of the ages of the existing islands?

Materials and Methods

TAXON AND GEOGRAPHIC SAMPLING DESIGN

We collected tissues from 614 lizards from 78 localities representing the Western Galapagos radiation (as defined in Benavides et al. 2007), which significantly increases the area covered by previous studies (Heise 1998; Kizirian et al. 2004). All sampled localities are plotted in Figure 1, and details of each (tissue voucher numbers, name of locations, sample sizes, and geographical coordinates) are presented in Appendix S1. In the field, samples were taken nondestructively (tail tips or toe clips were stored in silica or ethanol) and all lizards were released at their capture points.

We used information on cyt *b* haplotype relationships (because it provides strong phylogenetic signal for recent splits within the genus; Benavides et al. 2007) to guide a subsampling design for collection of additional sequence data. We constructed haplotype networks using the statistical parsimony algorithm of Templeton et al. (1992) implemented in the TCS program version 1.16 (Clement et al. 2000; http//:inbio.byu.edu/Faculty/kac/crandall_lab/Computer.html), and used relationships defined by the 95% parsimony limit to infer ancestral and derived haplotypes. Ambiguous network connections (loops, which represent homoplasy) were resolved using predictions from coalescent theory, as validated with empirical datasets (Crandall and Templeton 1993; Pfenninger and Posada 2002).

The subset of cyt *b* nonredundant haplotypes selected from haplotype networks was subsequently screened for three additional mitochondrial and 10 nuclear gene regions (Table 1). This subset included all Galápagos terminals used by Benavides et al. (2007; $n = 20$), in addition to 25 terminals selected here to encompass ancestral and derived haplotypes within each network. The Galápagos Western Radiation samples were complemented with 11 additional terminals for coverage of: (1) two species of the Galápagos Eastern Radiation (two terminals from Marchena and one from San Cristobal Islands); (2) three continental species of the *Occipitalis* group (two terminals for *M. koepckeorum*, *M. stolzmanni*, and *M. occipitalis*); (3) three species of the *Peruvianus* group (*M. peruvianus*, *M. theresiae*, and *M. thoracicus*); and (4) two outgroup taxa (*Tropidurus oreadicus* and *T. insulanus*). The larger cyt *b* dataset ($n = 614$) is further used to describe patterns of population structure and to statistically evaluate island features are most strongly associated with the genetic diversity on each island (E. Benavides, H. L. Snell, H. M. Snell, J. B. Johnson, and J. W. Sites, unpubl. ms.).

GENE SAMPLING AND LABORATORY PROCEDURES

Both nuclear and mitochondrial genes used here have been previously evaluated for their informativeness at different levels of divergence within *Microlophus* (Benavides et al. 2007; Table 1). In this study, we used the four mtDNA and eight of the nine nuclear gene regions used by Benavides et al. (2007), and two protein-coding nuclear genes recently made available through the Squamate Tree of Life project (Dyneinaxonemal heavy chain 3 [DNAH3], and Natural killer-triggering receptor [NKTR]; Townsend et al. 2008). Total genomic DNA was extracted using the QIAGEN DNeasy kit (Qiagen, Valencia, CA) according to the standard protocol. All gene regions were amplified via PCR in a cocktail containing 2.0 μl of template DNA (approximate concentration estimated on a 2% agarose gel), 8 μl dNTPs (1.25 mM), 4 μl 10× *Taq* buffer, 4 μl each primer (10 μl), 4 μl MgCl (25 mM), 22 μl distilled water, and 0.25 μl *Taq* DNA polymerase (5 U/μl; Promega Corp., Madison, WI). Primers and PCR profiles are given in Benavides et al. (2007), and for the new genes include: DNAH3_F1 5′- ggtaaaatgatagaagaytactg-3′, DNAH3_R6 5′-ctkgagttrgahacaatkatgccat-3′, and NKTR_F1 5′-agtaaatgggaytckgartcaaa-3′, NKTR_R3 5′-kcgtgcygtctty ctwacttca-3′. Double-stranded PCR amplified products were checked by electrophoresis on a 1% agarose gel (the size of the target region was estimated using a molecular weight marker), purified using a GeneClean III kit (BIO101, Inc, Vista, CA), and directly sequenced in both directions on a Perkin Elmer ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA). Excess of Dye Terminator was removed with CentriSep spin columns (Princeton Separations, Inc., Adelphia, NJ), and sequences were generated on an ABI Prism 3730 capillary autosequencer at the DNA Sequencing Center at Brigham Young University. All sequences are deposited in GenBank (accession numbers given in Appendixes S1 and S2).

Table 1. Genetic variability for mitochondrial and nuclear gene regions across three nested levels of clade depth in the genus *Microlophus*, with models of substitution selected for each partition. Asterisks identify nuclear loci with indels for which length adjustments made by PRANK alignments (see text for details).

Genes	Aligned bp	Number of variable sites			Number of parsimony informative sites			Model selected
		All taxa	*Occipitalis* group	Western Radiation	All taxa	*Occipitalis* group	Western Radiation	
mtDNA:								
Cyt *b*	1037	430	409	274	385	361	238	TrN+I+G
ND4	661	330	283	160	268	221	143	TrN+I+G
16S	510	135	102	47	105	83	45	GTR+I+G
12S	844	264	173	74	184	121	60	GTR+I+G
nucDNA:								
Anon*	400	104	55	18	50	31	12	K80+G
Atrp*	255	58	41	8	35	25	4	TrN+G
Ck*	362	90	47	8	60	24	4	K80
Cmos	524	58	20	3	19	10	3	K80
Cryba*	811	205	98	38	135	53	28	TVMef+G
DNAH3	660	58	26	11	37	19	5	HKY+I
Enol*	273	42	18	7	30	9	5	K80
Gapdh*	342	69	28	10	29	18	8	K80+G
NKTR*	831	102	37	12	76	24	7	HKY+I
RP40*	798	123	83	13	60	49	11	TrNef+G
Indels	111	63	40	5	63	20	3	Variable+G
Totals	8419	2130	1460	688	1530	1068	576	

ALIGNMENT AND PHYLOGENETIC ANALYSES

Forward and reverse sequences for each individual were edited and manually aligned using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI); protein-coding genes (cyt *b*, ND4, Cmos, DNAH3, and NKTR) were translated to insure that reading frames were intact across sequence lengths, ribosomal regions were first aligned with the program MUSCLE (Edgar 2004) and then proofread by eye in accord to secondary structure models. We used PRANK (Loytynoja and Goldman 2005) to align nuclear introns to maximize base-pair identity in conserved indel-flanking sequence blocks, and to identify indel events as phylogenetic characters (Benavides et al. 2007). A decision theory approach implemented in DT-ModSel (Minin et al. 2003) was used to select substitution models for all phylogenetic reconstructions (see also Sullivan and Joyce 2005). Phylogenetic analyses were performed for two different datasets: (1) nonredundant cyt *b* haplotypes summarizing genetic variability of the Western Radiation′s initial pool of 614 specimens (obtained with the COLLAPSE program [http://bioag.byu.edu/zoology/crandall_lab/programs.htm]); and (2) the subset of 56 terminals described above, for which 13 additional gene regions were sequenced to recover both shallow and deeper nodes of the tree (8308 bp total). We also considered a modification of the original 56 terminals dataset, in which indels from nuclear introns were coded as binary characters (Simmons and Ochoterena 2000; $n = 111$ indels; 8419 characters total; Table 1). Indels from loop regions of the 12S and 16S mitochondrial genes were uninformative and were not coded.

These datasets were analyzed by Bayesian inference (BI; MR BAYES; Ronquist and Huelsenbeck 2003) and maximum-likelihood (ML; PHYML; Guindon and Gascuel 2003) methods. Bayesian analyses consisted of two independent runs of four chains sampling every 100 generations for 20 million generations. Each gene region was considered a separate partition because simpler or more complex partitions do not appear to enhance support (Benavides et al. 2007). Output parameters from Bayesian analyses were visualized using the program TRACER (ver. 1.4; Rambaut and Drummond 2003) to ascertain stationarity and whether the duplicated runs had converged on the same mean likelihood. Equilibrium samples were used to generate 50% majority rule consensus trees. The percentage of samples that recover any particular clade represents the posterior probability (*PP*) for that clade, and normally a value of $P \geq 95\%$ is taken as significant support for a clade (Huelsenbeck and Ronquist 2001). Because Bayesian methods may resolve bifurcations with strong support when relationships are really unresolved (a polytomy is not considered as a possible outcome, see Lewis et al. 2005), we used the program PHYML (Guindon and Gascuel 2003) to generate a maximum-likelihood (ML) phylogenetic hypothesis based on a single general-time-reversible model of sequence evolution with six substitution rates, a proportion of invariable sites, and

unequal rates among variable sites (GTR + I + G). Bootstrap values were compiled after 1000 replicates, and taken as evidence for significantly supported clades if ≥ 70 (Hillis and Bull 1993; with caveats). We considered nodes as strongly supported only if both the *PP* and bootstrap estimates exceeded the above values. All trees were rooted with outgroup taxa *Tropidurus oreadicus* and *T. insulanus* (the genera *Tropidurus* and *Microlophus* are sister taxa with a cis–trans Andean distribution; see justification in Benavides et al. [2007]).

We evaluate the proposal by Kizirian et al. (2004) that interprets *M. albemarlensis* as a complex due to its paraphyly with respect to several other species, and applies this name to populations from four large islands and numerous "satellite islets" to each of these. The *albemarlensis* complex as recognized by Kizirian et al. is distributed throughout the islands of Isabela, Fernandina, Santa Cruz–Santa Fe, and Santiago (and the associated satellite islets of all of these). Strong support for reciprocal monophyly of each of these island groups, especially for mtDNA and nuclear gene regions combined, and based on dense sampling, would falsify the Kizirian et al. hypothesis, and suggest that the three haploclades for the Isabela–Fernandina, Santa Cruz–Santa Fe, and Santiago Island complexes represent distinct species.

TESTING ALTERNATIVE COLONIZATION HYPOTHESES FOR THE WESTERN RADIATION

By considering estimated island ages and prevailing ocean currents, the direction of colonization events in the Galápagos Archipelago by passive transport from South America is expected to occur in an approximately east-to-west (older to younger islands) direction (Cox 1983; White et al. 1993). In a more general sense, this "progression rule" hypothesis postulates that the ancestor of an endemic island radiation colonized what is now the oldest extant island when it was young, and as each new volcano became available for colonization, a dispersal event associated with speciation occurred from the older to the younger volcano (Funk and Wagner 1995). For endemic species in an archipelago characterized by linearly aligned islands, a single species per island, no extinctions, and no back colonizations, the area cladogram would have a pectinate topology. In this ideal case, the oldest island and its endemic species would be the first branch at the base of the tree, and the youngest species and islands would occupy the most nested level of the tree (Funk and Wagner 1995, fig. 17.1; but see Emerson 2002 for possible exceptions).

Although a general east-to-west colonization pattern is expected for Galapagos, the islands are not linearly arranged by age, and colonization histories of endemic species may be more complex (e.g., Parent and Crespi 2006; Sequeira et al. 2008). Previously published studies suggest slightly different colonization routes within the Western Galápagos Radiation of *Microlophus*. Figure 2 summarizes alternative colonization scenarios hypothesized in three studies that sampled all relevant taxa. For example, Wright (1983) used an allozyme-based distance phenogram (fig. 4, p. 135) and geological evidence to construct a colonization hypothesis for the genus (fig. 11, p. 150). More recently, Heise (1998) and Kizirian et al. (2004) used mtDNA sequence data to derive different hypotheses of interspecific relationships within the Western Radiation, and proposed different island colonization scenarios. All three studies coincide in showing Española (*M. delanonis*) as the first island to be colonized (and it is the oldest or among the oldest extant islands inhabited by this clade), but differ in the sequence of subsequent colonization events (Fig. 2). In Heise (1998), *M. grayii* is the second species derived at the start of the colonization sequence of the Western Radiation (fig. 3.4, p. 65), but it is the last with *M. albemarlensis* in Wright's (1983) hypothesis. The Kizirian et al. hypothesis differs from the Wright and Heise proposals in that independent events led to the colonization of the western-most islands of Pinta, Isabela, and Fernandina via Floreana, and the islands of Santiago and Pinzón via Santa Cruz. We compared our topology to the Heise (1998) hypothesis by first constraining our tree to conform to the (*duncanensis* + (*indefatigabilis* + *jacobi*)) topology, and then constraining *M. grayii* (highly nested in our tree) to the second derived species in the radiation, as proposed by Heise (*M. delanonis* + (*M. grayii* + (five species)). We also forced our tree to Wright's (*pacificus* + (*albemarlensis* + *grayii*)) topology, and then again to his comb topology for the earliest four derived species (*delanonis* + (*indefatigabilis* + (*duncanensis* + (*jacobi* + (all other species))))). The topology of Kizirian et al. (2004) is similar to ours, but differs in the resolution of species boundaries. We ignore the Lopez et al. (1992) colonization scenario because their taxon sampling does not include all relevant species and therefore is too limited to be useful here (Shaw 2002).

We used the Shimodaira and Hasegawa (SH; 1999) likelihood comparison test as implemented in PAUP*. Ten thousand replicates were performed for every paired test resampling the partial likelihoods for each site (RELL model). The one-tailed SH test compares the fit of an a priori Western Radiation hypothesis to the fit of the data for our best-supported topology. Because there are sampling differences among all of these studies (with regard to the number of terminals sampled), alternative topologies were constructed in MacClade version 4.03 by rearranging only the branches representing island lineages in conflict based on the simplified species trees of Figure 2. For example, we tested our topology against Heise (1998) alternative topology one by forcing our tree to unite *M. jacobi* with *M. indefatigabilis* as sister species, while leaving the rest of our topology as it was recovered in our best estimate of the species tree. We then repeated this test by repositioning *M. grayii* in our tree to match Heise alternative topology two, and so on.

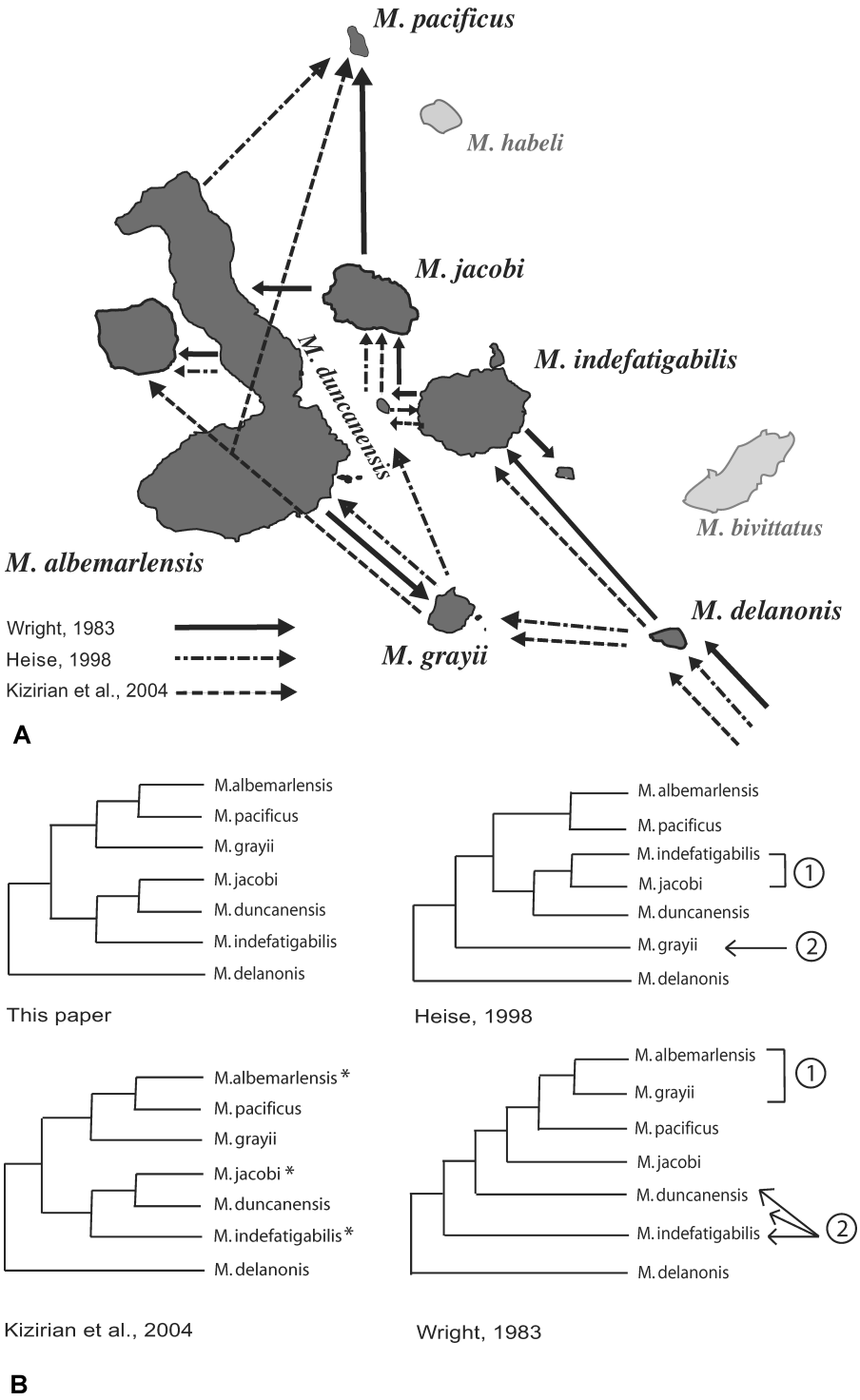

Figure 2. **(A) Colonization routes within the Western Radiation of *Microlophus* (dark islands, bold species names) reconstructed from inferred phylogenetic hypotheses, and (B) schematic representation of (simplified) phylogenetic hypotheses based upon mtDNA sequences (Heise 1998; Kizirian et al. 2004), allozyme distances (Wright 1983), and mitochondrial and nuclear sequences (this article). Brackets and arrows identify alternative topological constraints used in paired tests of Heise (1998) and Wright (1983) hypotheses, against that reported in this study (see text for details); the hypothesis of Benavides et al. (2007, fig. 8) is identical to that reported in this article (Fig. 5). Asterisks identify species recognized by Baur (1892), but considered as unnamed components of the *M. albemarlensis* complex by Kizirian et al. (2004).**

ESTIMATING COLONIZATION TIMES OF THE ARCHIPELAGO

We performed divergence time estimates for the multigene dataset on the basis of the best-supported topology based on nonbinary characters (56 terminals; 14 gene regions, 8308 bp). Large sequence datasets derived from genes with variable substitution rates should narrow confidence ranges for divergence estimates (Renner 2005; Rannala and Yang 2007), whereas the combination of mitochondrial and nuclear markers should yield robust estimates at shallow and deeper nodes (see Springer et al. 2003; Van Tuinen and Hardly 2004). Compared to earlier methods, the recent development of Bayesian MCMC methods has further refined divergence estimates by the use of models that incorporate rate heterogeneity through a lognormal model of rate variation (Thorne et al. 1998) and the extension of this approach to multilocus data appears to represent a significant advance (Thorne and Kishino 2002; but see Pulquério and Nichols 2006). This method applies divergence time calibrations that can be input as upper and lower bounds on nodes of a well-supported topology, thus allowing the MCMC algorithm to generate posterior distributions of rates and times at these nodes (Yang and Yoder 2003). Maximum-likelihood (ML) estimates for model parameters were obtained for each gene region with BASEML from the PAML (ver. 3.14) suite of options on individual Jukes–Cantor input trees (Yang 1997). ML estimates of tree branch lengths and their corresponding variance–covariance matrices were then obtained under each model using ESTBRANCHES from the MULTIDIVTIME package (Thorne and Kishino 2002). This was done for each gene partition, and MULTIDIVTIME was then used to run a MCMC chain (10^6 cycles sampled every 100 cycles) to estimate posterior distributions of times and substitution rates, based on all partitions (details are described by Renner and Zhang 2004).

We considered a maximum of seven calibration points to place hard bounds on the ages of selected nodes within the *Occipitalis* group (Fig. 3). Prior information on clade ages is based on the subaerial maximum-age estimates for selected islands given in Hickman and Lipps (1984), Vicenzi et al. (1990), White et al. (1993) and Geist (1996), and we used consensus island ages to calibrate appropriate nodes (see Fig. 1). For each calibration we make use of the age of the younger island between each pair of sister taxa because this gives the maximum age for that split (Fleisher et al. 1998; Magallon 2004). For example, the Eastern Radiation includes only the sister pair *M. habeli* and *M. bivittatus* endemic to Marchena and San Cristobal islands, respectively. The ages of these islands are estimated to be 0.4 million years (Marchena) and 2.3 million years (San Cristobal), thus we calibrate the node joining these species as 0.4 million years. Our set of calibration points excluded the split between populations of Fernandina and Western Isabela (< 0.03 million years); the two islands still share cyt *b* haplotypes (Fig. 4) evidence of recent migration thus rendering our phylogenetic approach nonsuitable for this clade (Ho et al. 2007). We used the following prior distributions (in units of 10 million years): RTTM = 1.0, RTTMSD = 2.0 RTRATE and RTATESD = 0.013; BROWNMEAN and BROWNSD = 0.036. The first two numbers define the mean and standard deviation for the prior distribution of the age of the root, and were chosen in the light of the approximate minimum age of arid conditions in the modern Peru–Chile Desert (Hartley and Chong 2002). The value of BIGTIME (= 23) was chosen to reflect geological evidence for Andean uplift to an elevation of 2000 m (Gregory-Wodzicki 2002; Pirie et al. 2006), which is the approximate upper elevational limit of the distribution of the basal species *M. koepckeorum* and *M. stolzmanni* on the western slopes of this divide.

The estimation of divergence times based on inferred ages of volcanic islands could be confounded by several factors. Fossils are virtually nonexistent on most oceanic islands, and sources of temporal information like potassium–argon (K–Ar) calibrations used to extrapolate island ages on the basis of exposed strata can be biased if these are particularly poor in potassium or not the oldest subaerial stratum for any given island (Geist 1996). On the other hand, geological hotspots can have a history of island formation and disappearance (White et al. 1993; Werner and Hoernle 2003; Heads 2005; Whittaker et al. 2008). Island submergence may lead to lineage extinction, which in turn can be a source of error when estimating divergence times because it introduces the possibility that one or more nodes could be older than the time constraint (calibration) used for extant islands (Emerson et al. 2000; Emerson 2002).

The Galápagos Archipelago has also been affected by cyclical changes in the sea level as a result of glacial advance every 10,0000 years for the past 1.0 million years (Jordan and Snell 2008). The direct consequence of these cycles is the periodic contact of satellite islets to major islands, but not the submergence of major islands. At longer time spans, the evidence for emergence/submergence cycles in the oldest islands is inconclusive. For example, lava fields in San Cristobal ($\sim$ 2.3 million years) are virtually unmodified and do not show evidence of erosion or soil formation that a submerged period would produce (Geist 1996). These issues are not unique to Galápagos, however, and in fact they are typical of all oceanic archipelagos (Whittaker et al. 2008). Because of the intrinsic problems with the calibration of K–Ar geological timescales, we preferred not to run divergence analyses under one set of assumptions. Thus, in addition to the use of fixed calibration points, we performed a jack-knife analysis of reciprocal compatibility of the constraint nodes; this involved repeating the dating calculations after removal and replacement of each one of the calibration constraints in turn. These calibration sets cross-check the sensitivity of our estimates for the two colonization times of interest, and for each of the internal calibration points (Rutschmann et al. 2007).

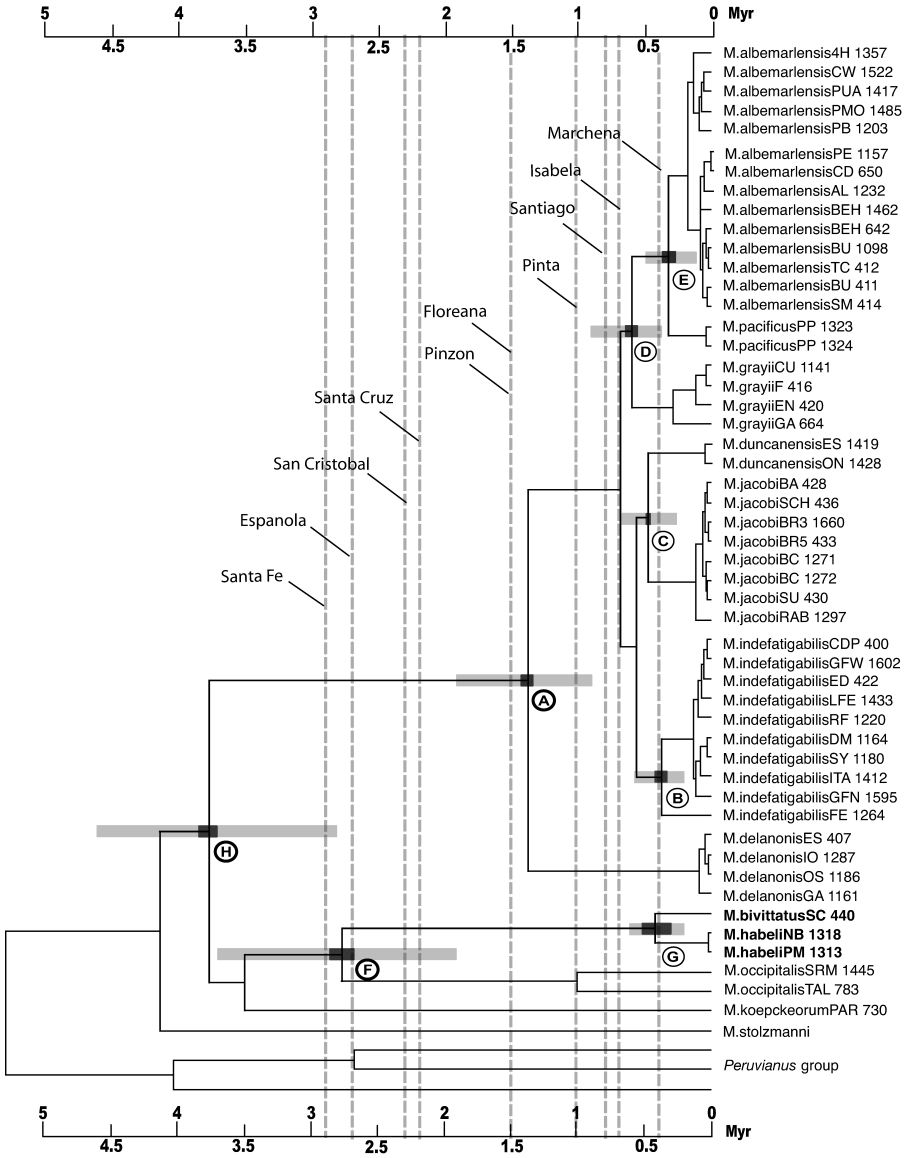

Figure 3. **Phylogenetic chronogram showing calibration points to estimate colonization times for the Eastern and Western Galápagos Radiations of *Microlophus* (A to F). Circles given in bold (A, H, and F) are nodes of interest to date the colonization events that lead to the Western and Eastern radiations, respectively. All nodes except node H were alternatively used as calibration points and these were inferred using the consensus subaerial ages for the islands shown here by the dotted lines. The following constrains were used: (A) the split between *M. delanonis* and the remaining six species of the Western Radiation (2.2 million years); (B) the split between *M. indefatigabilis* populations of Santa Cruz and Santa Fe Islands (2.2 million years); (C) the split between *M. duncanensis* and *M. jacobi* of Pinzón and Santiago islands, respectively (0.8 million years); (D) the split between *M. grayii* and the *albemarlensis-pacificus* clade (1.0 million years); (E) the split between *M. albemarlensis* and *M. pacificus* (0.7 million years); (F) the split between *M occipitalis* and the two species of the Eastern Radiation (2.3 million years); and (G) the split between *M. habeli* and *M. bivittatus* (0.4 million years). We used scalars of 10 and 23 for the prior age of the root node (RTTM) and the maximum possible age of this divergence (BIGTIME), respectively. Point estimates, standard deviation (dark boxes) and 95% confidence intervals (lighter boxes) shown in this graphic correspond to runs with no reference calibration points for these nodes. Names in bold font depict the two species of the Eastern Galápagos Radiation.**

Results

CYTOCHROME-B HAPLOTYPE NETWORKS

We recovered a total of 11 independent haplotype networks by applying the statistical parsimony algorithm implemented in the TCS program (Fig. 4). In all cases, haplotypes separated by up to 14 mutational steps had greater than 95% probability of being parsimoniously connected (i.e., no superimposed mutations). Single networks describe genealogical relationships among populations from Española (*M. delanonis*), Pinta (*M. pacificus*), Pinzón (*M. duncanensis*), and Santiago (*M. jacobi*). Two networks describe

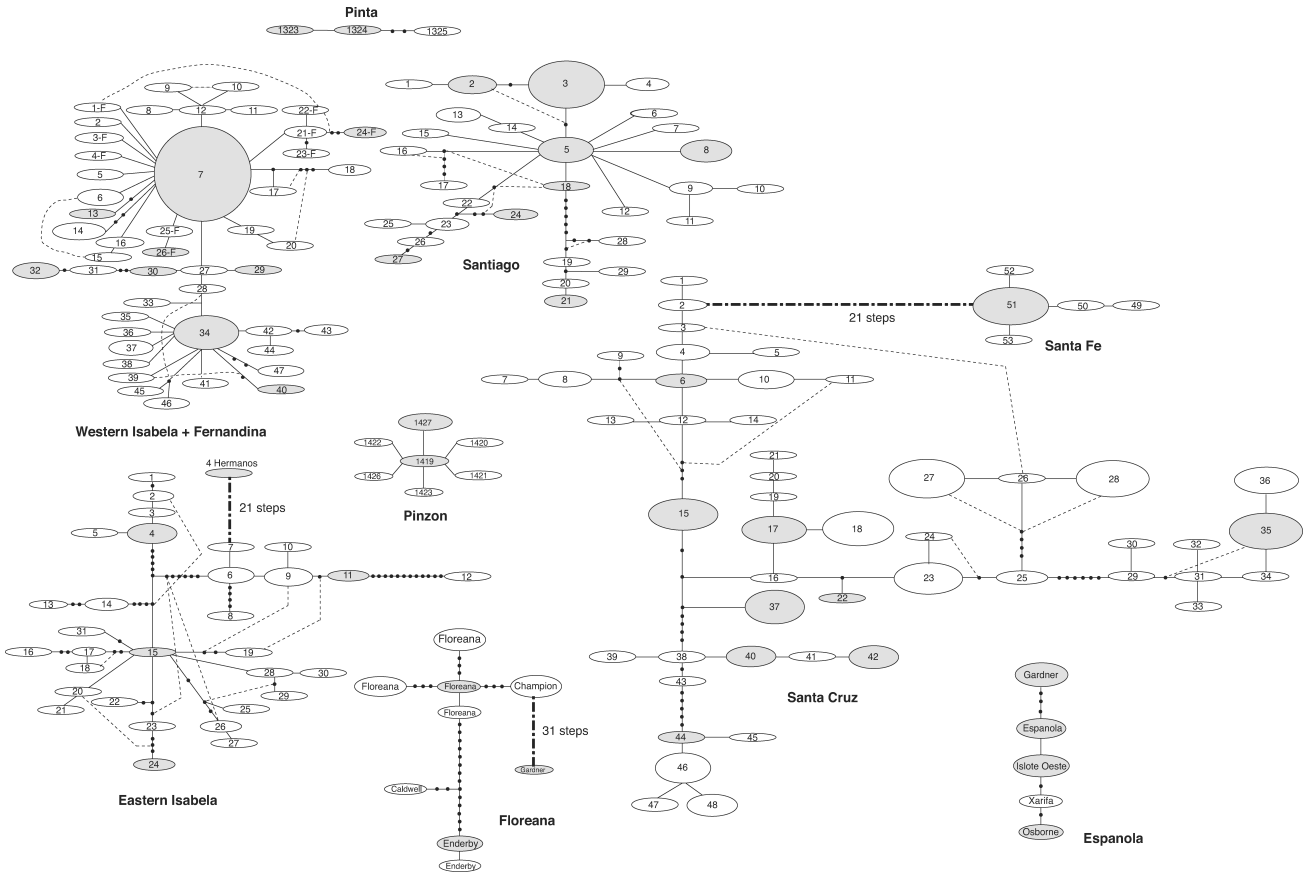

Figure 4. Cytochrome *b* haplotype networks describing genealogical relationships among 614 individuals collected from 78 localities across nine islands representing the Western Radiation. Networks were constructed using the statistical parsimony algorithm of Templeton et al. (1992), under a 95% limit of 14 steps. The size of each oval is proportional to the frequency of each haplotype and haplotypes shaded in gray were sequenced for 13 additional gene regions for phylogenetic analyses (see methods section). The letter "F" after some haplotypes in the Western Isabela–Fernandina network identifies haplotypes unique to Fernandina Island. Islet names replace haplotype numbers in Floreana and Española networks. Solid dots represent unsampled haplotypes and gray dashed lines indicate discarded network loops.

the relationships among populations of Santa Cruz (*M. indefatigabilis*) and Floreana (*M grayii*), and in both cases the additional network corresponds to satellite islet populations isolated from the closest main islands (Gardner from Floreana [31 mutational steps], and Santa Fe from Santa Cruz [21 mutational steps]). Three separate networks describe genealogical relationships of Isabela and Fernandina. The "Western network" shows genealogical relationships that include haplotypes exclusive to Fernandina ($n = 6$) and haplotypes exclusive to, or shared with Isabela ($n = 41$). The "Eastern network" groups haplotypes from the Eastern coast of Isabela ($n = 31$) and it is separated by 21 inferred mutation steps from a third single haplotype from the Cuatro Hermanos islets (Fig. 4). These network genealogies guided our subsampling strategy for phylogenetic analyses of a concatenated dataset with 13 additional gene regions. Within each network, we generally selected either ancestral haplotypes that were connected to most others by one or a few steps (as in the Isabela–Fernandina network), or high-frequency haplotypes recovered at different points within a network, and then haplotypes most distant from these (most others in Fig. 4).

PATTERNS OF VARIATION

Table 1 summarizes patterns of variation in all loci used in this study across three nested levels of taxon sampling for the 54 ingroup terminals used to recover the phylogenetic history of the Western Galápagos Radiation within the *Occipitalis* group of *Microlophus*. The majority of nuclear genes are informative at the deeper levels within the genus whereas the reverse is true for the mtDNA locus, but in the aggregate, the nuclear loci collectively provided 282 and 90 parsimony informative sites in the *Occipitalis* group and Western Radiation clades, respectively. Parsimony informative sites in several nuclear genes (e.g., Cryba) include indels as well as base changes, and in the nuclear NKTR region a complete codon deletion was found in all terminals except the two outgroups and *M. occipitalis* and *M. thoracicus* from the mainland.

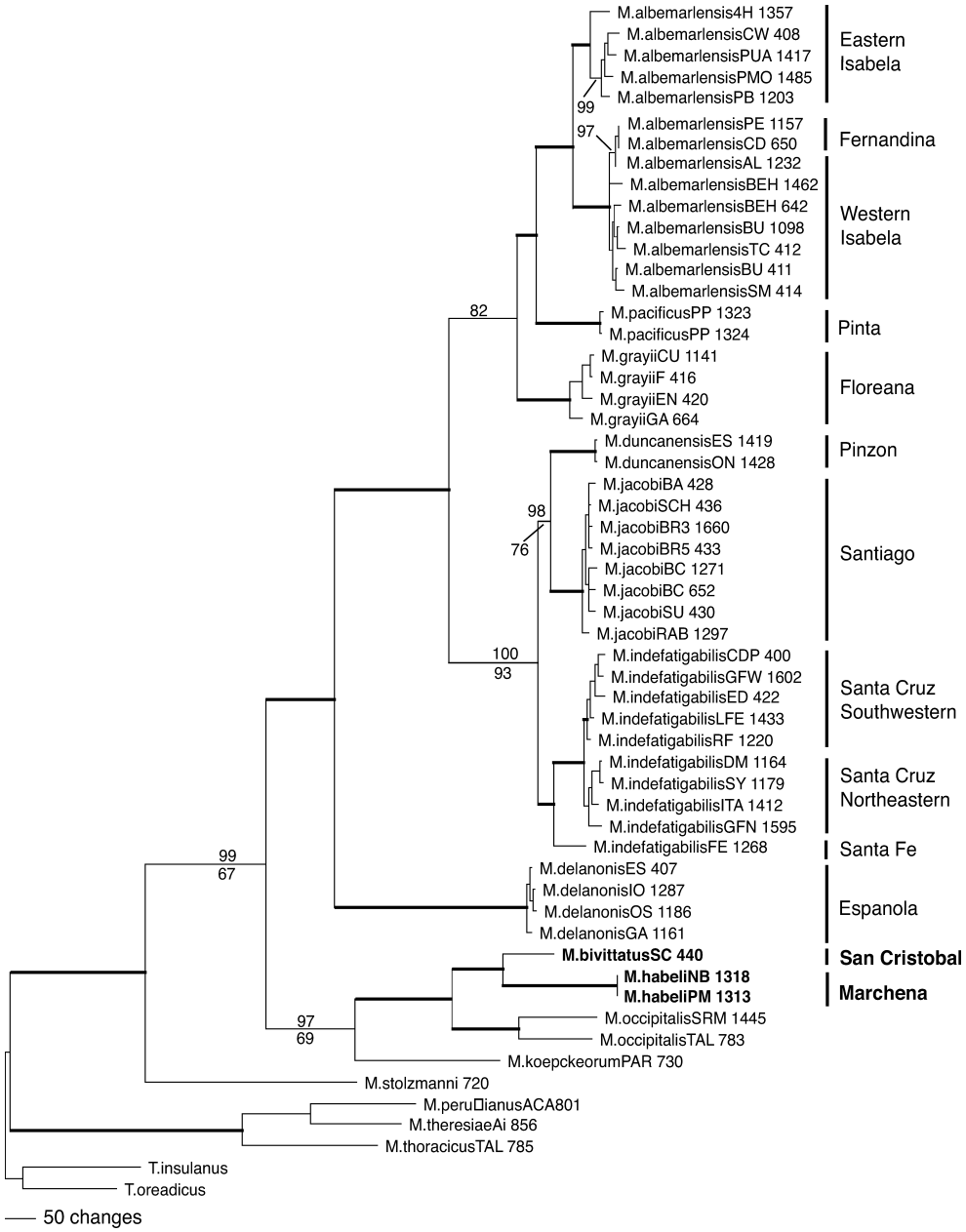

Figure 5. Maximum-likelihood phylogram of 54 ingroup terminals of *Microlophus*. Numbers above branches represent Bayesian posterior probabilities (ln $L = -35300.283$), and those below are ML bootstrap values (ln $L = -36591.34152$; Bayesian and ML trees are nearly indistinguishable). Branches with ML bootstrap support values > 100% and *PP* > 1.0 are identified by a thick black line. Island and species names given in bold identify taxa representing the Eastern Radiation. Subclades showing within-island population structure recovered for the islands of Santa Cruz and Isabela–Fernandina. The postscripts after the species name identify localities in Figure 1 and Appendix S1.

PHYLOGENETIC ANALYSES

The cyt *b* gene tree (not shown) based on 188 nonredundant Western Galápagos haplotypes recovered individual island (including satellite islets) and species haploclades (following the Baur [1892] taxonomy) with the 100/100 levels of Bayesian/bootstrap support for: Española (*M. delanonis*), Floreana (*M. grayii*), Santa Cruz (*M. indefatigabilis*), Santa Fe (*M. indefatigabilis*), Santiago (*M. jacobi*), and Pinzón (*M. duncanensis*). Support was as strong (100/96 and 100/89, respectively) for the Pinta (*M. pacificus*) and Isabela + Fernandina (*M. albemarlensis*) haploclades (these are all Baur [1892] names recognized in Benavides et al. 2007). Monophyly for all islands/species is thus strongly supported, and on average long branches separate island clades and lead to comparatively short branches for all within-island

Table 2. Results of the paired Shimodaira–Hasegawa topological constraints tests of our best tree compared to two alternative hypotheses each proposed by Heise (1998) and Wright (1983) (see Fig. 2).

Tree	–ln *L*	Diff–ln *L*	*P*	Topology compared
This article, Figure 5	43930.65917	(Best)	—	This article, Figure 2B
	50616.52170	6685.86253	0.000	Heise (1998) alternative 1, Figure 2B
	52614.06928	8683.41011	0.000	Heise (1998) alternative 2, Figure 2B
	50703.11280	6772.45363	0.000	Wright (1983) alternative 1, Figure 2B
	50692.98024	6762.32107	0.000	Wright (1983) alternative 2, Figure 2B

terminals. These island haploclades correspond to the separate networks described above, including the three islands (Santa Cruz–Santa Fe, Floreana–Gardner, and Isabela–Fernandina) for which separate networks correspond to strongly supported regional subclades in the cyt *b* tree.

Figure 5 presents our best-supported species tree for 56 terminals sequenced for all 14-gene regions, of which 45 were subsampled from the 11 eleven cyt *b* haplotype networks of the Western Radiation (Fig. 4). The topology of Figure 5 is identical to that reported in Benavides et al. (2007) when all terminals are collapsed to the named taxa, and Bayesian probabilities and ML bootstrap values of 100/100 support the majority of species/island groups, plus a number of shallower and deeper nodes. The inclusion of a binary indel partition did not alter either tree topology or branch support for any of the analyses, and is not considered further. Within the Western Radiation, the species–island relationships implicit in this tree agree with previous topologies in which *M. delanonis* (Española) is basal to all other species. Our topology also recovers *M. grayii* (Floreana) as the sister taxon of the two westernmost species; *M. albemarlensis* (Isabela–Fernandina) and *M. pacificus* (Pinta), albeit with weak support ($PP = 0.82$). We also recover a "central islands" subclade with strong support, in which *M. indefatigabilis* from Santa Cruz–Santa Fe are placed as a sister taxon to the clade of *M. jacobi* (Santiago) + *M. duncanensis* (Pinzón).

Table 2 shows the results of the SH tests comparing our best topology (Figs. 2 and 5) to alternative relationships presented by Heise (1998) and Wright (1983). All of these topologies represented significantly worse alternatives to our best tree. Pairwise tests rejected the Heise placement of *M. jacobi* + *M. indefatigabilis* as sister taxa (alternative 1), and the position of *M. grayii* as sister to all species except *M. delanonis* (alternative 2). Similarly, none of Wright's species relationships, for example, *M. albemarlensis* and *M. grayii* as sister taxa (alternative 1), or the derivation sequence that assumes *M. indefatigabilis* as basal but just internal to *M. delanonis* (alternative 2) is acceptable. We therefore consider the topology shown in Figure 5 to be the best working hypothesis of relationships among the seven species that comprise the Western Radiation.

ESTIMATED ARRIVALS OF THE GALÁPAGOS COLONISTS

The divergence time estimates based on alternate calibration points are summarized in Table 3, and Figure 3 shows a chronogram to which these calibrations have been added to facilitate comparison in a graphical context. Our results generally give consistent divergence time estimates; in five of the seven calibrated nodes these estimates show no large difference when that particular node was not calibrated (nodes A, B, C, D, E), whereas the absence of a calibration reference for nodes F and G increases the divergence time estimates for these two nodes (Table 3). The point estimates for divergence of the Eastern Radiation from its mainland sister species (*M. occipitalis*; node F in Fig. 3 and Table 3) range from ~2.09 to 2.8 million years, and the highest posterior density (HPD) interval for this node (2.794 ± 0.474) corresponds to the run that excluded this calibration point. This is the only estimate that does not overlap the point estimated age of San Cristobal Island (2.3 million years), but the standard deviation and credibility intervals for this and all other estimates do overlap this island's estimated age.

Because there is no extant mainland sister species to the Western Radiation (Figs. 3 and 5), we cannot date its initial colonization in the same manner as for the Eastern Radiation. However, we can estimate the age of the first split within this clade, and then the earliest split between the continent and the Galápagos (nodes A and H, respectively, in Fig. 3). The point estimates for divergence between *M. delanonis* and the remaining taxa (node A) range from 1.39 to 1.69 million years and are not particularly sensitive to iterative deletion and replacement of other nodes (Table 3). Although these estimates postdate the age of the oldest extant island (Española; 2.7 million years), the highest HPD interval for this split (1.397 ± 0.250; Table 3) corresponds to the run that excluded this calibration point, but still includes the putative age of Española in its 95% confidence interval (Fig. 3). At the next deep node (H), our point estimates range from 3.69 to 4.54 million years, with the highest number sensitive to the iteration without node F. Other than this "outlier" value, all others range between 3.69 and 3.73 million years, and the 95% confidence values for the lower of these estimates approach but do not overlap the

Table 3. Divergence date estimations for selected nodes of the Western and Eastern Radiations of *Microlophus*. We conducted seven iterative runs with $n - 1$ node calibrations to gauge sensitivity of divergence time estimates based on slightly different prior information; numbers given in bold on the diagonal are estimates obtained when that node was not calibrated, and underlining identifies highest-probability density values. All nodes represent original calibration points identified by letters in Figure 3, and three estimates of interest (A, F, and H) are given in bold. Numbers indicate point estimates of island ages, ± SD, with 95% credibility intervals given below each estimate in parentheses.

Node description	Alternative calibrations							
	All calibrations	2nd-node G	3rd-node F	4th-node B	5th-node C	6th-node E	7th-node D	8th-node A
G: *M. habeli* vs. *M. bivittatus* (0.4 million years)	0.315±0.063 (0.167–0.397)	**0.424±0.122 (0.201–0.677)**	0.332±0.057 (0.185–0.398)	0.314±0.064 (0.161–0.397)	0.315±0.064 (0.163–0.397)	0.314±0.064 (0.161–0.397)	0.315±0.063 (0.172–0.397)	0.316±0.063 (0.166–0.397)
F: *M. occipitalis* vs. the Eastern Radiation (2.3 million years)	2.095±0.167 (1.685–2.294)	2.134±0.146 (1.763–2.295)	**2.794±0.474 (1.948–3.780)**	2.096±0.166 (1.684–2.294)	2.096±0.167 (1.683–2.293)	2.096±0.166 (1.684–2.294)	2.096±0.166 (1.684–2.293)	2.095±0.167 (1.676–2.293)
B: *M. indefatigabilis*—Santa Cruz vs. Santa Fe (2.2 million years)	0.367±0.088 (0.214–0.553)	0.374±0.088 (0.222–0.564)	0.441±0.103 (0.256–0.655)	**0.368±0.087 (0.216–0.559)**	0.365±0.087 (0.212–0.551)	0.368±0.087 (0.216–0.559)	0.369±0.087 (0.215–0.558)	0.367±0.086 (0.214–0.554)
C: *M. duncanensis* vs. *M. jacobi* (0.8 million years)	0.479±0.101 (0.299–0.697)	0.488±0.100 (0.310–0.703)	0.570±0.111 (0.352–0.774)	0.479±0.101 (0.297–0.691)	**0.478±0.101 (0.303–0.698)**	0.479±0.101 (0.297–0.691)	0.479±0.102 (0.293–0.697)	0.479±0.102 (0.299–0.697)
E: *M. albemarlensis* vs. *M. pacificus* (0.7 million years)	0.313±0.080 (0.178–0.492)	0.318±0.081 (0.181–0.499)	0.376±0.092 (0.213–0.572)	0.314±0.079 (0.177–0.485)	0.309±0.078 (0.175–0.480)	**0.314±0.079 (0.177–0.485)**	0.313±0.080 (0.174–0.486)	0.311±0.078 (0.178–0.484)
D: *M. grayii* vs. *albemarlensis–pacificus* (1.0 million years)	0.584±0.122 (0.369–0.852)	0.594±0.121 (0.378–0.855)	0.697±0.135 (0.439–0.956)	0.582±0.120 (0.366–0.838)	0.577±0.120 (0.367–0.835)	0.582±0.120 (0.366–0.838)	**0.582±0.123 (0.362–0.843)**	0.581±0.122 (0.365–0.846)
A: *M. delanonis* vs. remaining species (2.2 million years)	1.393±0.243 (0.959–1.917)	1.416±0.240 (0.980–1.921)	1.688±0.278 (1.122–2.158)	1.396±0.243 (0.955–1.910)	1.390±0.242 (0.954–1.905)	1.396±0.243 (0.955–1.910)	1.399±0.245 (0.956–1.913)	**1.397±0.250 (0.958–1.940)**
H–Earliest split between the continent & Galápagos	3.695±0.455 (2.851–4.645)	3.743±0.445 (2.921–4.667)	4.541±0.681 (3.264–5.905)	3.703±0.458 (2.847–4.658)	3.697±0.459 (2.853–4.655)	3.703±0.458 (2.847–4.658)	3.709±0.458 (2.831–4.650)	3.698±0.457 2.853–4.662)

point-estimated age for Española (2.7 million years; Fig. 3). This places the oldest split between continental and the Galápagos Western Radiation as predating one of the oldest of the subaerial islands, but the earliest split within the Western Radiation as postdating the age of Española. From these results, we infer that the ancestor of *M. delanonis* probably colonized Española Island sometime between 3.7 and 1.4 million years ago, and that the subsequent evolution of the Western Radiation occurred less than ~1.4 million years; it is thus considerably younger than the initial founding of the Eastern Radiation.

Discussion

SPECIES DIVERSITY IN THE WESTERN RADIATION

Van Denburgh and Slevin (1913) originally recognized the following five species and distributions in the Western Radiation of *Microlophus*: *M. delanonis* (Española), *M. grayii* (Floreana); *M. albemarlensis* (Fernandina, Isabela, Santiago, and Santa Cruz–Santa Fe), *M. duncanensis* (Pinzón), and *M. pacificus* (Pinta). In a phylogenetic classification (de Queiroz and Gauthier 1990), Kizirian et al. (2004) recognized four of these same species, but they did not use the unappended binomial "*M. albemarlensis*" because it was recovered as paraphyletic in their mtDNA trees. These authors instead recognized a "*M. albemarlensis* complex" distributed across the "Western Galápagos," and including *M. duncanensis*, *M. grayii*, and *M. pacificus* (Table 3). Importantly, samples were limited both with respect to numbers of individuals and localities representing each species or island group (Table 1), and Kizirian et al. recognized the provisional nature of their classification.

Our results provide strong support for the monophyly of all island groups, which are coincident with the seven "Baur species" recognized by Benavides et al. (2007). Similarly, the cyt *b* gene tree of 188 nonredundant haplotypes from 78 populations of the Western Radiation recovers all seven of the recognized Baur species as reciprocally monophyletic (data not shown). This monophyly is retained in all seven species with very strong nodal support in the multilocus phylogeny (Fig. 5), even though the number of synapomorphic base changes that support these clades among nuclear sequences ($n = 5256$ bp) is small to modest in some taxa, that is, *M. albemarlensis* (0), *M. pacificus* (10) *M. grayii* (8), *M. indefatigabilis* (1), *M. duncanensis* (5) *M. jacobi* (3), *M. delanonis* (18). We therefore reject the Kizirian et al. (2004) hypothesis of mtDNA paraphyly for the "*M. albemarlensis* complex"; populations assigned to this name are restricted to the Fernandina–Isabela island complex, and recognized here as a "candidate species" by our previously stated criteria. The "weakly differentiated" populations that remained unnamed by Kizirian et al. included those we recognize here as *M. indefatigabilis* (also as a "candidate species" restricted to the Santa Cruz–Santa Fe Islands complex) and *M. jacobi* (restricted to the Santiago Island complex). We suggest that these names now be provisionally applied to populations from these island complexes, even though morphological differences between them may be hard to discern (Van Denburgh and Slevin 1913, p. 188), and they will require confirmation from nuclear markers. We suggest this because we consider the mtDNA locus to be the ideal marker for a "first pass" investigation, the mtDNA haploclades are concordant with geographic distributions (Wiens and Penkrot 2002), and this marker in general identifies "candidate species" that are usually not incompatible with expectations of multilocus coalescence (Zink and Barrowclough 2008). The Kizirian et al. study was based on very small number of localities of *M. albemarlensis* from Fernandina and Isabela (two and three samples, respectively), and we suspect that their recovery of *M. albemarlensis* as paraphyletic (fig. 3) is an undersampling artifact (Zwickl and Hillis 2002; DeBry 2005).

Beyond the seven species we formally recognize, we suggest that additional cryptic species diversity may be present in the Western Radiation, particularly within *M. indefatigabilis*. A deep split separates populations of this species from Santa Cruz and Santa Fe islands; the two island groups are reciprocally monophyletic with 100/100 Bayesian/ML bootstrap support in the cyt *b* tree (not shown), and the two haploclades correspond to two distinct TCS networks separated by 21 substitutions (Fig. 4). By some criteria these differences are sufficient to recognize these groups as distinct species (Cardoso and Vogler 2005), and this divergence is strongly corroborated by nuclear data (11 microsatellite loci) recently reported by Jordan and Snell (2008). These authors sampled 17 populations of lizards from Santiago and some of its associated satellites (representing *M. jacobi*), Santa Cruz (with satellites), and Santa Fe (*M. indefatigabilis*); sample sizes were 32 individuals for all localities but one ($n = 14$). Multilocus nuclear genotypes show that three satellite islets of Santa Cruz (Daphne Major, North Guy Fawkes, and South Guy Fawkes) are strongly divergent from large island populations as a result of loss of genetic variation and/or retention of private alleles (Jordan and Snell 2008, table 2). All of these populations, however, are part of the Santa Cruz cyt *b* network and differentiated by at most six substitutions (well within the 14-step parsimony limit). Taking both lines of evidence together, these satellite populations have diverged in their nuclear genomes relatively recently by loss of some alleles, whereas the Santa Fe population is strongly differentiated in both mitochondrial and nuclear genomes. Sea depth contours of 60 and 130 m, indicating approximate island contours at 12,000 and 17,000 years (the LGM), reveal that Santa Fe remained fully isolated from Santa Cruz throughout Pleistocene sea level fluctuations (unlike most of the smaller islets; Jordan and Snell 2008, fig. 1). We thus recommend that the Santa Fe population of *M. indefatigabilis* be recognized as a valid species, *M. barringtonensis* (Baur 1892).

Other possible species may be represented by the two networks found in the Fernandina–Isabela complex, and the highly divergent haplotypes connected to the Eastern Isabela and Floreana networks (Fig. 4), but these require further study.

MONOPHYLY OF SPECIES/ISLAND COMPLEXES

The evidence for monophyly discussed above indicates that all island species have likely been derived from single founder events, yet it seems improbable that these lizards would fail to recolonize some of the larger and closely bunched central islands more than once (i.e., Isabela, Pinzón, Santa Cruz, and Santiago; Fig. 1). Lizards have well-developed long-distance dispersal capabilities (de Queiroz 2005a) and two primary colonization events of Galápagos have been demonstrated. Kizirian et al. (2004) suggested that inter-island gene flow might account for the weak differentiation among lizard populations inhabiting the central group of islands separated by relatively small distances (i.e., the unnamed *M. albemarlensis* complex in table 3). These authors suggested that this might occur by reversal of normally northwesterly flowing Humboldt Current during *El Niño* years, coupled with higher rainfall causing Galápagos freshwater systems to flood, and to occasionally wash vegetative mats downstream to the ocean. In such a scenario stowaway lizards (Censky et al. 1998) could be transported among the clumped islands in the center of the archipelago. Such intermittent movements could foster gene flow and maintain weak divergence within the *M. albemarlensis* clade (Kizirian et al. 2004; p. 768). However, the Jordan and Snell (2008) study of gene flow among populations of *M. indefatigabilis* (referred to as *M. albemarlensis* by Kizirian et al.) in the central region of the archipelago does not support this hypothesis. Overall, absence of evidence for multiple colonization events by lava lizards on any of the Galápagos Islands suggests either that: (1) predominant ocean currents within the archipelago are insufficient to carry passively dispersed taxa between islands with sufficient frequency to establish new founders after the initial colonization event; or (2) new colonists are occasionally founded but fail to establish after arrival. The first explanation does not seem likely given that all but the most extremely isolated islands in Galápagos were colonized by *Microlophus*, and other taxa equally dependent on passive transport have multiply colonized some islands, including other lizards (Wright 1983), the giant tortoises (*Geochelone*; Ciofi et al. 2002; Rusello et al. 2005), and land snails (*Bulimulus*; Parent and Crespi 2006).

Alternatively, islands in close proximity are more likely to exchange genes (MacArthur and Wilson 1967), and if there is occasional dispersal between islands, the high habitat diversity on the large and "middle aged" islands suggest that low habitat diversity or the lack of ecological opportunity would have not prevented successful founding of new lizard populations. We offer two mutually compatible explanations for recolonization failure; first, if *Microlophus* are ecological generalists, as suggested by the range of habitats they occupy on large topographically complex islands (Stone et al. 2002), then once established, resident populations would make colonization by a congener much harder. This suggestion is indirectly supported by two observations. First, numerous studies have shown that ecologically similar species exist together on islands less often than expected by chance (Diamond 1975; Lomolino 2000), suggesting that interspecific competition has a central role in the composition of island assemblages (Gotelli and McCabe 2002). Second, groups characterized by multiple within-island colonizations (finches, etc.) are those in which resource partitioning between sympatric species is pronounced (Grant and Grant 1998). Additionally, recolonizations may also be precluded by pronounced sexual selection, as evidenced by secondary sexual ornamentation, in the *Occipitalis* group (Werner 1978; Watkins 1996, 1997, 1998). For example, males of *M. duncanensis* (Pinzón) are dull colored whereas females are brightly colored in contrast to the neighboring islands of Santa Cruz, Isabela, or Santiago (E. Benavides, pers. obs.) Thus sexual selection may have accelerated the differentiation and retention of morphological differences among island species to the extent that new founders may be at a mating disadvantage.

PHYLOGENY AND COLONIZATION HISTORY OF THE WESTERN RADIATION

The Western Radiation of *Microlophus* represents a classic "non-adaptive" radiation in the sense that each major island is inhabited by a single species (with the possible exception of Isabela). Because lizards are capable long-distance dispersers by passive drift on ocean currents (Censky et al. 1998; de Queiroz 2005a), colonization of oceanic archipelagos should be heavily influenced by predominant currents. In the eastern Pacific, the prevailing Humboldt Current flows from the west coast of South America in a northwesterly direction past the Galápagos Archipelago at a speed of about seven knots (Wright 1983), whereas the islands themselves are shifting eastward on the Nazca Plate over a stationary volcanic plume (Cox 1983; Werner et al. 1999; Werner and Hoernle 2003). This "conveyor belt" mechanism appears to have been operating for at least 80 to 90 million years, based on the ages of submerged seamounts east of the ocean-floor hotspot (Christie et al. 1992). In an approximately linear volcanic system (Hawaii) sequential colonization/speciation in low-vagility passive drifters should follow from older to younger islands and result in a pectinate tree topology. This follows the "progression rule" of Funk and Wagner (1995), but it assumes no extinctions, that each island will be colonized from the nearest older island, and that there have not been any "back colonizations" from younger to older islands (see also Emerson 2002).

Although islands of the Galápagos Archipelago are clumped by age groups (Fig. 1) rather than linear, the characteristic "one

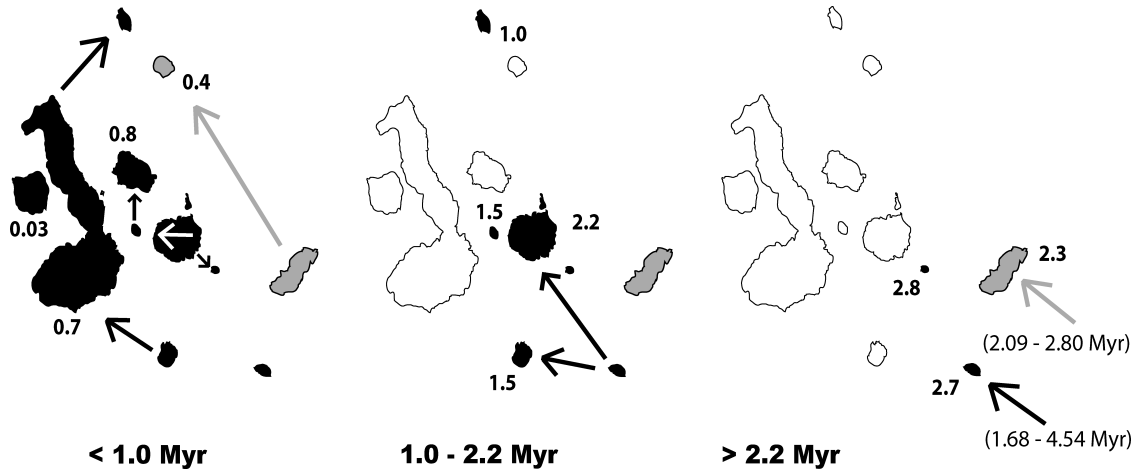

Figure 6. **Lava lizards island colonization events in the context of an approximate geological history of the Galápagos Archipelago summarized for three arbitrary time scales. The panel depicts the islands' emergence sequence from right to left (islands older than 2.2 million years, islands between 1.0 and 2.2 million years, and islands younger than 1.0 million years) and numbers given in bold indicate consensus island ages in million years. Submerged islands are shown by approximate outlines (which do not necessarily correspond to the shape of these islands when they emerged) and subaerial islands are depicted with solid shapes. The sequence and tempo of *Microlophus* colonization events on the emerged islands started with both the Western Galápagos Radiation (in black) and the Eastern Galápagos Radiation (in gray) are based on the topology shown in Figure 5, and divergence time estimates presented in Table 3 and Figure 3.**

species one island" distribution for *Microlophus*, the strong evidence for a single colonization of each island, and lack of evidence for extinctions (Stone et al. 2002) or back colonizations, lead to similar expectations for a general east-to-west sequence of speciation events. If true, then the earliest derived species should be basal clades endemic to the oldest islands, whereas more recently derived species should be restricted to younger islands, and presumably founded by ancestors rafted from older islands. Our phylogenetic hypothesis is in agreement with all previous studies in that Española was the first island to be colonized (the endemic *M. delanonis* is the sister species of a well-supported clade that contains all others in this group; Fig. 5). The sequence of derivation of the remaining six species in the western radiation shows that the initial colonization was followed by an overall southeast-to-northwest colonization of younger islands (with some exceptions, see below), in a pattern consistent with the prevailing ocean current that runs in a northwesterly direction for much of the year (Pak and Zaneveld 1973; Wyrtki et al. 1976).

In Figure 6, we graphically outline a working hypothesis for speciation within the Galápagos Archipelago, based on our best-supported species tree (Fig. 5), the above assumptions, and estimated colonization times of ancestral populations from the mainland (see below). The original colonization of Española could have pre- or postdated the colonization of San Cristobal (Fig. 6A), and these islands served as sources for subsequent divergence of the Western and Eastern Radiations, respectively. In the second phase of colonization, Española served as the source for two additional radiations; one of these colonized Santa Cruz and then Pinzón Islands of the central island group, whereas another founded the populations on Floreana (Fig. 6B). Note here that we invoke parsimony for interpretation of the sequence of colonization of the larger of two islands for sister species on the assumption that the larger island was colonized first (Santa Cruz is preferred over Santa Fe), or the closer island to the source rather than the more distant island (Pinzón is preferred over Santiago; see below).

In the last phases of colonization events, Isabela was colonized, and then followed by a relatively recent colonization to the "middle-aged" island of Pinta (Fig. 6C). Similarly, and after the colonization of Santa Cruz (Fig. 6B) the sequence of colonization events involved the subsequent radiations from Santa Cruz to Santa Fe (younger to older and against the prevailing surface currents in this case), and from Pinzón to Santiago (older to younger and in accord with surface currents). We hypothesize that lizards from Española colonized both Floreana and Santa Cruz almost simultaneously (HPD intervals overlap). Four islands were emergent at 1.5 million years (Fig. 6; 1.0–2.2 million years panel), and two of these were successfully colonized. This scenario can also explain the weakest point in our phylogeny which is the placement of *M. grayii* (Floreana). This species is poorly supported as the sister species of the *M. albemarlensis* + *M. pacificus* clade (Fig. 5; $PP = 0.82$, bootstrap < 50), and the mtDNA tree recovers *M. grayii* as the second derived species just internal to *M. delanonis* (not shown). However, we prefer the combined data tree (Fig. 5) as our working hypothesis, because it is based on multiple independent markers and we consider it the best estimate of the species tree. Second, the combined data tree shows relatively

long branches interspersed with short internal branches which indicate that some time splits were too short for the accumulation of sufficient synapomorphies to produce a robust phylogeny between species pairs (Weisrock et al. 2005). Newer coalescent approaches coupled with fast-evolving, unlinked nuclear sequences offer perhaps the best option for statistically rigorous resolution of the phylogenetic position of *M. grayii* (Jennings and Edwards 2005).

TIMING OF COLONIZATION OF THE TWO GALÁPAGOS RADIATIONS

Our molecular divergence estimates suggest that the Eastern Radiation does not predate the age of the oldest existing islands. This clade was likely founded at about 2.09–2.8 million years ago on the island of San Cristobal (which dates to approximately 2.3 million years), by the ancestor of *M. bivittatus* and *M. occipitalis*. Our estimate for the earliest split between the basal continental species (*M. stolzmanni*) and the ancestor of all Galápagos endemics are 3.69–4.54 million years, about a million years older than the consensus age for Española Island (2.7 million years), and in all cases the 95% HPD intervals of these estimates do not overlap with the putative age of this island (node H in Table 3). However, the Western Radiation was founded (by the ancestor of *M. delanonis*) on the island of Española some time before the split of *M. delanonis* and all other species in this clade (1.39–1.69 million years), and after the basal split noted above. Although we cannot infer a date for the colonization of Española, a midpoint between these two estimates would place the confidence interval of this event within the time frame of Española's emergence (between 1.69 and 3.69 million years). These results sharply contrast with those reported by Lopez et al. (1992), which estimated initial colonization events at 34 million years. The Lopez et al. estimate was based on pairwise distance coefficients of immunological cross-reactions of serum albumins, a method that assumes rate homogeneity along all branches and does not incorporate internal calibration points. In contrast, Wright's (1983) study based on allozyme distance coefficients and methods crude by today's standards, gave estimates of 2.45 million years for both *Microlophus* colonization events of Galápagos (table 5, p. 147). These are surprisingly close to our own estimates, and although we suggest that our estimates should be considered the best available hypotheses of the Galápagos colonization for this genus, we acknowledge that even sophisticated methods that incorporate more realistic assumptions have their limits (Pulquério and Nichols 2006).

Our estimates might be biased in at least two ways that are not mutually exclusive. First, despite advances in molecular clock divergence estimators, there is still much uncertainty about the quality of these estimates, and how much confidence we should place in them (Pulquério and Nichols 2006). Among other things, substitution rates for any gene in any lineage may be influenced by biological attributes such as body size, generation time, life history, metabolic rate, or population size, and any combination of these attributes may of course vary among lineages, and therefore influence rate heterogeneity among them. The issue of among-lineage rate variation has been addressed by methods such as that used here that remove the assumption of a constant substitution rate (Thorne and Kishino 2002), but this and related methods assume that rates are autocorrelated (nearby branches on the tree have similar substitution rates for the same gene). New methods have been proposed in which rates are not autocorrelated but are drawn from an underlying statistical distribution (Drummond et al. 2006); however, it is not yet clear which model best fits real data (Lepage et al. 2007). Other intrinsic issues that influence accuracy of divergence estimates, and for which there is yet no consensus about how to accommodate them, including the effects of selection, the discordance between substitution rates inferred from phylogenetic studies versus those observed in genealogies (Ho et al. 2005, 2007), and uncertainties inherent to calibration points (Heads 2005).

Addressing other possible biases to our divergence estimates is beyond the scope of this study, but we can comment on two relevant points. First, closely related species should share many of biological attributes, so within thoroughly sampled groups such as that studied here, substitutions rates may be relatively constant (Pulquério and Nichols 2006), making divergence time estimations based on relaxed clocks much more robust (Linder et al. 2005). Second, we used consensus island ages to calibrate internal nodes for estimating divergence times for the founders, and if these dates are seriously compromised, then of course our estimates will also be biased (Springer et al. 2003; Rutschmann et al. 2007). These ages were estimated by K–Ar dating of lava rock, and because these islands are formed by multiple eruptions, more than one age might be obtained for the same island if samples are taken from different lava strata. This last point is critical because island ages are oftentimes wrongly taken as errorless calibration points. We tried to overcome this pitfall by using alternate calibration references in our tree and we showed that the absence of a calibration reference adds a significant bias in only two nodes (G and F), and in only one (node F) does the estimate contradict geological information (Fig. 3). This protocol demonstrated that unless most island ages were biased in the same way, our estimates should be fairly conservative.

SYNTHESIS

If our colonization estimates are approximately correct, then the endemic *Microlophus* radiations are two of several that have colonized the Galápagos Archipelago within the time frame of the existing islands. These include tortoises, hawks, finches, mockingbirds, butterflies, warblers, beetles, and daisies; estimates for initial colonizations range from a low of 0.05 million years for the

Galápagos hawk to ranges of 1.6–5.5 million years for mockingbirds, and 1.9–6.2 million years for daisies, with a clumping of estimates between these (e.g., 2.0–3.0 million years for tortoises; 1.2–2.3 million years for finches; 2.5 million years for warblers; 3.7 and 4.7 million years for the two colonizations of geckos; 2.9–3.7 million years for butterflies, and multiple estimates for lava lizards; summarized in Appendix S3). In some cases, local extinctions may prevent strong inferences of the first island to be colonized from mainland (Bollmer et al. 2006), but most others show a pattern of colonization parallel to what we report in this article for the Western Radiation of *Microlophus*; older lineages usually inhabit older eastern islands whereas younger lineages occupy younger western islands (Rassmann 1997; Sequeira et al. 2000; Beheregaray et al. 2004; Bollmer et al. 2006). The similar initial colonization patterns, coupled with the range of initial colonization times and more idiosyncratic within-archipelago colonization routes (references above), imply that the assembly of the Galápagos biota took different routes and colonized at different times on the extant islands. Interestingly, within *Microlophus*, a number of relatively old islands have been colonized rather recently (Fig. 6); for instance, Santa Fe Island ($\sim$ 2.8 million years) was colonized by founders from Santa Cruz less than 0.441 million years; Pinta ($\sim$ 1.0 million years) was colonized by founders from the Isabela–Fernandina complex $<$ 0.378 million years ago), and Pinzón (1.5 million years) and Santiago (0.8 million years) were each colonized less than 0.5 million years ago (Table 3). The collective evidence suggests that the Western Radiation is less than 1.5 million years old, and that most islands have harbored *Microlophus* populations for only the last 0.5 million years (Fig. 3). This discordance between the rather old time of arrival to the Archipelago and the rather new within-island diversification times is striking and does not support the "progression rule" suggested for other Galápagos taxa (Beheregaray et al. 2004; Arbogast et al. 2006; Parent and Crespi 2006).

Over two decades ago, Wright (1983; p. 145) pointed out that there were "few, if any, areas on earth with better control, geologically speaking, over real or absolute time than that represented by the dataset for development of the Galapagos Archipelago." This prescient statement is now strongly validated by better resolved phylogenies and distributions for many endemic groups (Grehan 2001; Parent and Crespi 2006) and a unique biota that, although showing disturbing signs of human-caused stress, is still the most intact of all oceanic archipelagos on earth (Watkins and Cruz 2007). Refined geological studies should continue to reduce confidence intervals on island ages, whereas newer multilocus coalescent methods (e.g., Drummond et al. 2006; Knowles and Carstens 2007) now make it possible to investigate island colonization hypotheses with a level of precision not previously possible.

ACKNOWLEDGMENTS

We thank Susana Cardenas and the staff of the Charles Darwin Research Station and the Galápagos National Park Service for logistical support in the field, and the crew of the Queen Mabel. We thank Luis Coloma, Alessandro Catenazzi, Shaleyla Kelez, and Jesus Cordoba for tissue loans of mainland species, BYU students Travis Moss, John Wells, Paige Alsbury, and Ian Stehmeier for laboratory assistance, and Ray Grams for field assistance in Peru. EB thanks BYU for support, including the Department of Biology and the M. L. Bean Life Science Museum, BYU graduate mentoring and graduate research fellowships, a Larsen Scholarship, and a B. F. Harrison Scholarship. Additional support was provided by a graduate research award to EB from the Society of Systematic Biologists, and National Science Foundation awards DEB 0309111 (doctoral dissertation improvement award to JWS, Jr. and EB), and EF 0334966 ("Assembling the Tree of Life: The Deep Scaly Project: Resolving Higher Level Squamate Phylogeny Using Genomic and Morphological Approaches") to JWS, Jr.

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Associate Editor: M. Hellberg

Supporting Information

The following supporting information is available for this article:

Appendix A1: Species, type localities (in parentheses; from Van Denburgh and Slevin 1913), sampling points, geographical coordinates, and cyt *b* GenBank numbers for the seven taxa recognized here in the Western Galapagos radiation of *Microlophus;* abbreviations identify localities plotted in Figure 1, N = number of individuals sequenced per locality, and MSB numbers identify tissue vouchers catalogued in the Museum of Southwestern Biology (MSB), University of New Mexico.

Appendix A2: A. GenBank accession numbers for mitochondrial genes used in the phylogeny of 54 *Microlophus* terminals. B. GenBank accession numbers for 10 nuclear genes used in the phylogeny of 54 *Microlophus* terminals.

Appendix A3: Summary of recent molecular studies of endemic Galapagos species, with estimates of colonization times, the nature of the assumed molecular clock (intrinsic or extrinsic), the source of the calibrations used, and the inferred number of colonization events for each radiation (modified from Schmitz et al., 2007; and Bollmer et al., 2006).

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