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Evolutionary Age of the Galápagos Iguanas Predates the Age of the Present Galápagos Islands

Kornelia Rassmann[1]

School of Biological Sciences, University of Wales at Bangor, Bangor, Gwynedd LL57 2UW, Great Britain and Zoologisches Institut, der Universität München, Luisenstr. 14, 80333 München, Germany

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New geological findings suggest that volcanoes existed over the Galápagos hotspot long before today's islands emerged less than 5 million years ago. The evolution of some of Galápagos' biota might have taken place on these former islands. This study investigates the evolutionary history of two of the archipelagos' older vertebrate taxa, the endemic Galápagos marine and land iguana (genera *Amblyrhynchus* and *Conolophus*). Mitochondrial rDNA sequences (in total about one kilobase of the 12S and 16S genes) were obtained from all extant genera of the family Iguanidae and the outgroup *Oplurus*. The phylogenetic analyses suggest that the Galápagos iguanas are sister taxa. Rate comparisons between the iguanid sequences and a corresponding set of sequences from ungulates with known fossil ages date their separation time at 10 million years, or more. The results strengthen the hypothesis that extended speciation times in the Galápagos are possible and provide an estimate of the minimum time inhabited islands of the archipelago may have existed.

INTRODUCTION

The Galápagos archipelago is famous for the rapid adaptive radiation of some of its vertebrate taxa. Enzyme electrophoretic analyses show that the divergence of the 13 Darwin finch species (Emberizinae) occurred within 5 million years (MY), or less (Grant, 1994; Polans, 1986), and thus within the age range of the present islands (White *et al.*, 1993). However, the biota of the Galápagos Islands also comprises taxa with high levels of genetic differentiation. The divergence times among the seven endemic Galápagos lava lizard species (*Tropidurus*), as well as among the six native geckos (*Phyllodactylus*), have been estimated as 9 MY, or more, on the basis of enzyme-electrophoretical and immunological data (Wright, 1983; Lopez *et al.*, 1992). Old evolutionary ages were also reported for the Galápagos land iguana (*Conolophus*) and the marine iguana (*Amblyrhynchus*), which, according to immunological data, diverged about 15 to 20 MY ago (Wyles and Sarich, 1983).

The separation times of these taxa predate the age of today's Galápagos islands. The archipelago is located nearly 1000 kilometers west of the nearest mainland and, as the product of a stationary mantle hotspot (Morgan, 1971), has never been in contact with any continental land mass. The ages of the individual islands increase in line with the eastward movement of the Nazca plate on which the Galápagos are situated. From the velocity of the plate motion it can be estimated that none of today's islands is older than 5 MY, and radiometric data predict a younger origin of the present archipelago (White *et al.*, 1993). However, Christie *et al.* (1992) analyzed material from submarine sea mounts to the southeast of the archipelago and proposed that they represent older, now sunken, islands of the archipelago. If this is true, then taxa such as the iguanas may have speciated on the former Galápagos islands and thus may have experienced much longer evolutionary times *in situ* than previously thought.

Despite the old age of the Galápagos iguanas, there is little apparent evolutionary divergence within the lineages. The land iguana comprises two species (*Conolophus subcristatus* and *C. pallidus*) and the marine iguana a single species (*Amblyrhynchus cristatus*). Immunological studies show low levels of genetic differentiation within both genera (Higgins and Rand, 1974, 1975; Higgins, 1977). Wyles and Sarich (1983) suggested two alternative models to explain this pattern of evolution. Divergence of the two taxa from a common ancestor might have taken place within the archipelago a long time ago, with little subsequent radiation. Alternatively, two separate ancestors from different iguana stocks might have colonized the Galápagos recently, not leaving enough time for further morphologi-

[1] Address correspondence and reprint requests to the author at School of Biological Sciences, University of Wales at Bangor, Bangor, Gwynedd LL57 2UW, Great Britain. Fax: +44-1248-37-1644. E-mail: k.rassmann@bangor.ac.uk.

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cal and genetic divergence on the islands. Unfortunately, the iguanid paleontological record is sparse (Estes, 1983; de Queiroz, 1987) and does not help to clarify the question. Steadman *et al.* (1991) report findings of iguana fossils on the Galápagos, but they appear to be Holocene representatives of the extant genus *Conolophus*. Etheridge (1964) detected a late Pleistocene fragmentary braincase and a body vertebra in Barbuda, which resembled several iguanids including *Amblyrhynchus* and *Conolophus*. However, reinterpreting the data from this fossil, de Queiroz (1987) concluded that it could not be assigned unambiguously to either of the two.

An alternative way to address the question is to reconstruct the phylogenetic history of the Galápagos iguanas. If the phylogeny indicates that the Galápagos iguanas do not share a direct common ancestor, then their separation within the archipelago can be rejected. More ambiguous to interpret would be the opposite outcome—that the two Galápagos taxa are their closest living relatives. The sister taxa status of the endemic Galápagos iguanas cannot prove their speciation within the archipelago; however, this would be the most parsimonious explanation of their occurrence in the Galápagos. The land and marine iguana represent two genera of the large iguanids (family Iguanidae *sensu* Frost and Etheridge, 1989), which also comprise *Dipsosaurus* (desert iguana, southern USA), *Brachylophus* (banded iguana, Fijian islands), *Sauromalus* (chuckwalla, southern USA), *Cyclura* (ground iguana, West Indies), *Iguana* (green iguana, Mexico, Central and South America, West Indies), and *Ctenosaura* (spiny-tailed iguana, Central America). An additional genus, *Enyaliosaurus* (Gicca, 1983), appears to form a subgroup of the taxa included in *Ctenosaura* (de Queiroz, 1987) and is presently not universally recognized. Much of the iguanid phylogeny is not yet understood, for example the relationship between the Galápagos iguanas and the *Ctenosaura*/*Enyaliosaurus* group, and a representative of *Enyaliosaurus* is therefore included in the molecular phylogenetic analysis presented here.

The lack of resolution of the iguanid phylogenetic relationships is surprising given the multitude of morphological and biochemical studies of this group (Avery and Tanner, 1971; Higgins and Rand, 1975; Wyles and Sarich, 1983; Etheridge and de Queiroz, 1988). A cladistic analysis of morphological and osteological data suggested that *Dipsosaurus* and *Brachylophus* were the evolutionary oldest Iguanidae (de Queiroz, 1987), with either of the two being the sister taxon to the remaining seven genera, a group named Iguanini (*sensu* de Queiroz, 1987). Within the Iguanini, de Queiroz (1987) distinguished four lineages: (i) the Galápagos marine and land iguana; (ii) *Ctenosaura* and *Enyaliosaurus;* (iii) *Cyclura* and *Iguana;* and (iv) *Sauromalus*. The branching order among these groups, however, was unresolved, leaving the phylogenetic events directly preceding the speciation of the two Galápagos iguanas unclear (de Queiroz 1987). In a later study, Norell and de Queiroz (1991) included two fossil iguanids in the morphological analyses, *Pumilia novaceki* and *Armandisaurus explorator,* and were able to place *Brachylophus* as the sister taxon to the Iguanini, but the cladogenesis among the four lineages within the Iguanini remained dubious.

Wyles and Sarich (1983) employed immunological comparisons of serum albumins (antisera to the albumins of *Amblyrhynchus* and *Conolophus*) and electrophoresis of plasma proteins to place the Galápagos iguanids phylogenetically within the Iguanidae. In agreement with the morphological studies, the biochemical data proposed a basal position for *Dipsosaurus* and *Brachylophus*, but the genetic relationships within the Iguanini were mostly unresolved. Because the immunological distance between the two Galápagos iguanas was slightly less than the distances between either of the two and the other Iguanini, Wyles and Sarich (1983) concluded that the land and marine iguana were sister taxa, but had shared only a brief period of common ancestry after their divergence from other iguanids. The most recent study on the phylogeny of the Iguanidae compared the results from morphological analyses and mitochondrial (mt) DNA sequence analyses of 959 nucleotides (nts) of the ND4 gene and the tRNA genes (Sites *et al.*, 1996). The molecular tree suggested a sister taxon relationship of the Galápagos iguanas, but the clade was not supported in the statistical analyses.

In this study, the mitochondrial 12S and 16S genes were chosen to reevaluate the molecular phylogeny of the Iguanidae, focusing particularly on the phylogenetic relationship between the two Galápagos iguanas and the cladogenesis preceding their speciation. Additionally, the DNA sequence data was utilized to approximate the evolutionary ages of some of the iguanid lineages. In view of the new geological data from the Galápagos area, such an analysis appeared particularly interesting (Carson, 1992). The oldest sea mount studied by Christie *et al.* (1992) was about 11 MY old, but the authors suggested that islands might have appeared and disappeared throughout the entire 80 to 90 MY history of the Galápagos hotspot activity. If the Galápagos iguanas are sister taxa, then they possibly speciated within the islands. In this case, their separation time would reflect the minimum time iguanas inhabited the archipelago, and thus the minimum time surfaced Galápagos islands must have existed. Hence, independent from the geological data, the molecular estimate may provide an approximation of the time available for speciation in the Galápagos. The two main questions addressed in this study are, therefore: (i) are the Galápagos iguanas sister taxa; and (ii) how old are the Galápagos iguanas?

MATERIALS AND METHODS

Tissue or blood samples of the large iguanids were obtained from museum, zoo, or personal collections and included *Amblyrhynchus cristatus, Conolophus pallidus, Ctenosaura similis, Enyaliosaurus quinquecarinata, Iguana iguana, Cyclura cychlura, Sauromalus obesus, Brachylophus fasciatus,* and *Dipsosaurus dorsalis. Oplurus* has been used frequently as an outgroup in the phylogenetic analyses of the Iguanidae (e.g., Wyles and Sarich, 1983; de Queiroz, 1987; Sites *et al.,* 1996), and *Oplurus cyclurus* (Opluridae *sensu* Frost and Etheridge, 1989; Madagascar) was chosen as an outgroup taxon in this study.

Total DNA was extracted from blood in a salt extraction (Bruford *et al.,* 1992) and from tissue using the Chelex extraction method (Walsh *et al.,* 1991). Regions of the mt 12S gene and 16S gene were amplified in PCR reactions using "universal" primers to obtain the 16S fragment (L02510 5′-C G C C T G T T T A T C A A A A A C A T-3′, H03063 5′-C T C C G G T T T G A A C T C A G A T C-3′) and the 3′ segment of the 12S gene (L01091, H01478 (Kocher *et al.,* 1989)) and primers designed for this study to amplify the 5′ region of the 12S gene (L00903 5′-T C T C G T G C C A G C C A C C G C G G T-3′, H01214 5′-T T A T A G G A C A G G C T C C T C T A-3′). In a pilot study on the variability of other mitochondrial genes, fragments of the cytochrome b gene were obtained with "universal" primers (L14724; H15149 (Irwin *et al.,* 1991)) and specifically designed primers (L14961 5′-C A G T A G C C C A C A T C T G C C G A G A-3′, H15394 5′-G T A T G G G T G G A A T G G A A T T T T-3′). Primer numbers refer to their position in the human mtDNA sequence (Anderson *et al.,* 1981). The same primers were used for generating single-stranded DNA and for direct sequencing. Homologous rDNA sequences from six ungulate species were obtained from the GenBank data base and represented two cervids: *Cervus unicolor* (GeneBank Accession No. M35875) and *Muntiacus reevesi* (M35877), which separated about 7 MY ago (Kraus and Miyamoto, 1991; Solounias, 1981), and four bovids: *Gazella thomsoni* (M86501) and *Madoqua kirki* (M86495), on average 16.5 MY apart (Allard *et al.,* 1992; Gentry, 1990), and *Bos taurus* (J01394) and *Capra hircus* (M55541), approximately 20 MY apart (Kraus and Miyamoto, 1991; Hamilton, 1973).

The iguanid and ungulate sequence sets were aligned separately using the overall multiple alignment algorithm provided in the program Clustal V (Higgins and Sharp, 1989). In regions with high numbers of indels, which were mostly found in the loop segments and in combination with mononucleotide repeats, the resulting alignment was ambiguous and adjustments by hand were necessary. Indel positions and regions with uncertain alignment were excluded in the phylogenetic analyses and rate estimates (125 nts in the iguanid and 46 nts in the ungulate sequences). Mononucleotide repeats were discarded, when they were at least 4 nucleotides in length, and when gaps had to be inserted to maximize the alignment among the nine Iguanidae or the six ungulates, respectively. An exception to this rule was the loop between the 16S stem sequences 35 and 35′, which was excluded in its entirety from the first indel position in this region to the last because unambiguous alignment seemed impossible (Fig. 1).

In total 838 nts of the iguanid rDNA sequences were used in the phylogenetic analyses (916 nts in the ungulates). Following the suggestion of Kim (1993), Kumar *et al.* (1993, p. 47), and Avise (1994, p. 124), multiple methods of tree reconstruction procedures were employed to evaluate the consistency of the phylogenetic estimations. Two of the methods, namely the maximum parsimony (MP) and the neighbor joining (NJ) method, did not assume equal rates of sequence evolution, but the maximum likelihood (ML) trees were generated under the constraint of a molecular clock, as implemented in the DNAMLK program of the PHYLIP package, version 3.55 (Felsenstein, 1993). The NJ tree and branch lengths were estimated with MEGA version 1.01 (Kumar *et al.,* 1993) under the assumptions of the Kimura-2-parameter model of nucleotide substitution. Unweighted MP analyses were performed using the exhaustive search routine in PAUP 3.1.1 (Swofford, 1993), which also served to evaluate the amount of phylogenetic signal in the rDNA sequences with the g1 test of Hillis (1991) and Hillis and Huelsenbeck (1992). Weighted MP tests, assuming a TS:TV ratio of 2:1, were run using the heuristic routine of PAUP 3.1.1. Unless stated otherwise, the default settings of these programs were applied. The statistical properties of the NJ tree were evaluated with the standard error test included in MEGA (Rzetsky and Nei 1992, 1993), and the reliabilities of all tree reconstructions were tested and compared using bootstrap resampling analyses performed in PHYLIP (or PAUP in the case of the weighted MP analysis). NJ and MP tree reconstructions were repeated 1000 times, the more time consuming ML and weighted MP procedures 100 times.

Rate heterogeneity was tested following the Wu and Li relative rates test (Wu and Li, 1985). The nucleotide substitution rates among the nine members of Iguanidae were compared with respect to the outgroup *Oplurus* (36 comparisons, critical value $z \approx 3.2$ for $P = 0.05$). Since the choice of a closely related outgroup is critical for analyzing relative rates in a given clade, the evolutionary rates within the members of the Iguanini were also tested separately using *Brachylophus* as an outgroup (21 comparisons, $z \approx 3.0$ for $P = 0.05$). *Bos taurus* served as an outgroup in the rate comparison between the two cervid taxa (1 comparison, $z \approx 1.96$ for $P = 0.05$) and *Cervus unicolor* when comparing the

rates among the four bovids (6 comparisons, $z \approx 2.6$ for $P = 0.05$).

The large regional differences in substitution rates in the iguanid, as well as in the ungulate sequences, required a secondary structure analysis, and separation time estimates were obtained from both the total sequence information and from the loop regions separately. Stem and loop regions in the 12S and 16S rDNA fragments were established following the models proposed for *Bos taurus* and for human mt rDNA (Gutell and Fox, 1988; Neefs *et al.*, 1993) (Fig. 1). Positions which were clearly not part of a stem in any of the taxa in the iguanid or, respectively, the ungulate data set were assigned to loop regions. These characters, with the exception of the positions already excluded in the phylogenetic analysis, were used in the estimate of divergence times when analyzing the loop regions separately (iguanids, 410 nts; ungulates, 466 nts). Because of the low number of TV substitutions among the seven Iguanini and among the six ungulate stem segments (in both on average 1.4 TV), an estimate based on the stem segments alone was impossible, at least for the TV rate estimation (see below).

The separation times of the iguanid lineages were calculated using two approaches. First, the ML branch length estimates resulting from the ML tree reconstructions were used, which incorporated TS and TV substitutions. Second, estimates were based on pairwise sequence comparisons, counting only the TV substitutions between the sequences. The estimates obtained with the TV method reflect the pairwise TV divergence between two sequences when sister taxa are compared, and the average TV divergence of multiple pairwise comparisons when taxa from different clades are compared. For the ML estimates, trees for the nine Iguanidae and *Oplurus* as outgroup were generated with the DNAMLK program from either the total sequences or from the loop regions only. They were also assessed with and without correcting for substitution rate variation among sites. When adjusting the settings in the DNAMLK program of the PHYLIP package, two categories of positions were defined with substitution rates of 0 and 2, respectively (C-option). The probabilities in which unvaried sites (rate 0) occurred were specified according to their percentage in the iguanid and ungulate rDNA sequences. The value of the average patch length (R-option) was defined as 2, which gave the best log-likelihood values for both iguanid and ungulate ML trees. The nucleotide substitution rates for the ungulate sequences were estimated accordingly. Three pairwise comparisons of ungulate sequences were used for these estimates (see above), and the mean was taken to calculate the average separation times between the iguanid lineages. Minimum age estimates were assessed by adding two times the sample standard diviation (SD) to the mean ungulate substitution rate.

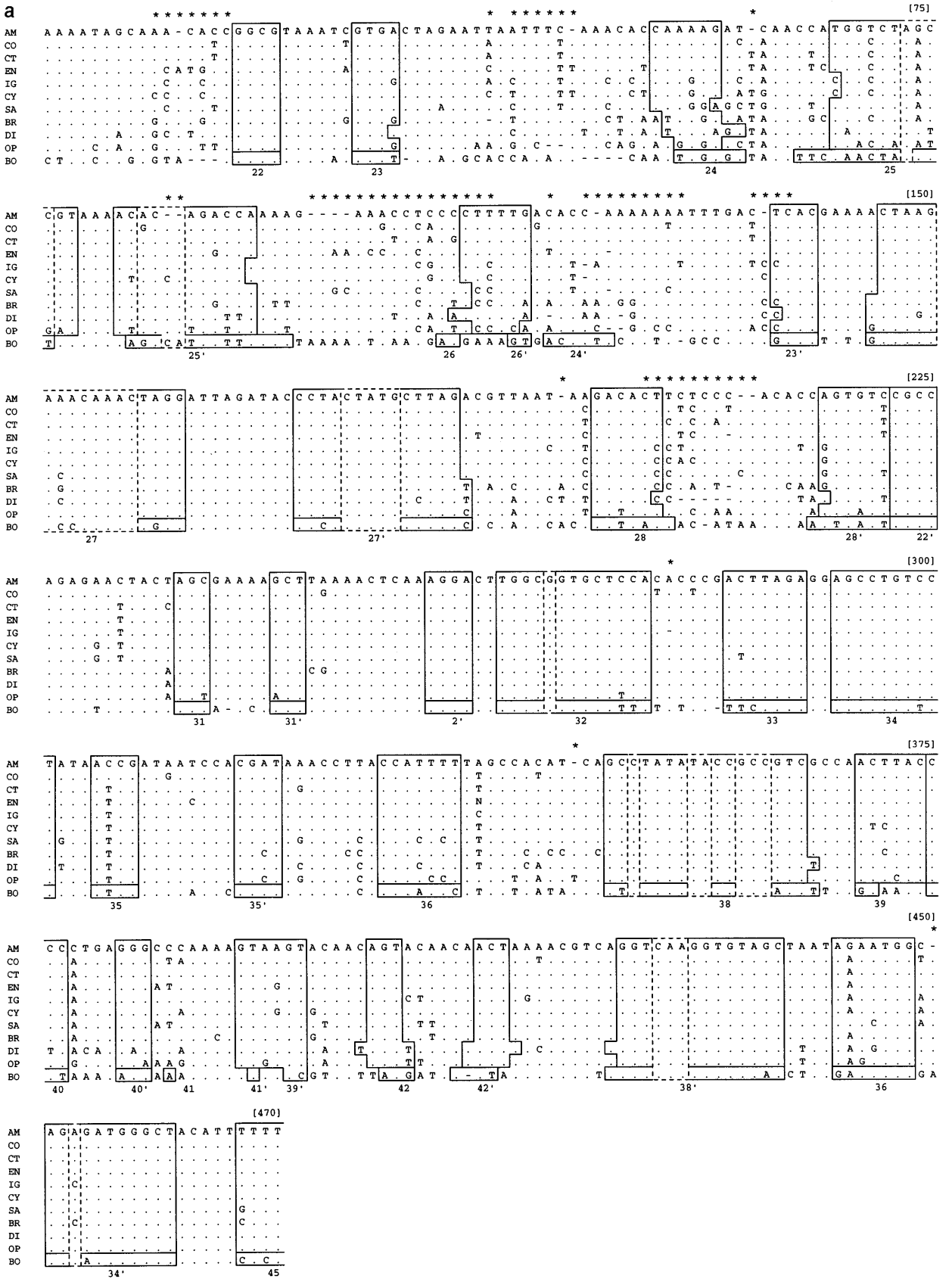
RESULTS

Iguanid Phylogeny

The quantity and quality of variation detected in the 12S and 16S rDNA segments appeared to be adequate for the molecular phylogenetic analysis of the iguanids. Among the 10 sequences, 196 of 838 positions used in the tree estimation were variable (17% of the stem positions, 27% in the loops), and 117 positions among the seven Iguanini (8% in the stems, 18% in the loops). The distribution of tree lengths obtained in the unweighted MP analysis of the nine Iguanidae and *Oplurus* was highly left skewed, and the g1 statistic suggested the presence of a strong phylognetic signal in the sequences ($g1 = -0.870$; $P < 0.01$) (Hillis, 1991; Hillis and Huelsenbeck, 1992).

The unweighted MP analysis of the 10 sequences resulted in four most parsimonious trees (tree length (TL) = 356, consistency index (CI) = 0.694, rescaled consistency index (RC) = 0.276) (Figs. 2a–2d). The weighted MP analysis yielded two topologies (Figs. 2a and 2d), and the NJ and ML tree estimations both recovered topology 2a. All trees suggested *Dipsosaurus* to be the oldest evolutionary lineage among the Iguanidae, and *Brachylophus* the next oldest and the closest living relative to the Iguanini. Within the Iguanini, a group joining *Sauromalus* with *Iguana* and *Cyclura* and a sister taxon relationship of the latter two were consistently found (this clade of three taxa is here called "*Sauromalus* group"). The topologies differed from each other in the proposed branching patterns

FIG. 1. Alignment and secondary structure of the partial mitochondrial 12S and 16S genes of the 9 Iguanidae, *Amblyrhynchus cristatus* (AM), *Conolophus pallidus* (CO), *Ctenosaurus similis* (CT), *Enyaliosaurus quinquecarinata* (EN), *Iguana iguana* (IG), *Cyclura cychlura* (CY), *Sauromalus obesus* (SA), *Brachylophus fasciatus* (BR), *Dipsosaurus dorsalis* (DI), and the outgroup taxon *Oplurus cyclurus* (OP), aligned to the sequence of the ungulate representative *Bos taurus* (BO). Dashes refer to gaps in the alignment. Positions excluded in the data analysis are marked by asterisks. Stem regions are enclosed in boxes, with some of the nonpairing nucleotides enclosed in dashed lines. (a) 12S rDNA fragments: Position 1 in the iguanid alignment corresponds to position 00921 in the human mt 12s gene, position 470 to the human position 01373 (Anderson *et al.*, 1981). The numbers of the stems relate to those published in Neefs *et al.* (1993). Stems 26 and 41 are omitted in the iguanid rDNA. (b) 16S rDNA fragments: Position 1 corresponds to position 02533 and position 514 to position 03033 in the human mtDNA. The numbering of stems is arbitrary. A secondary structure analysis using the computer program DNASIS (Pharmacia) suggested an additional stem (38a) in the iguanid 16S rDNA, which is not found in the ungulate sequences.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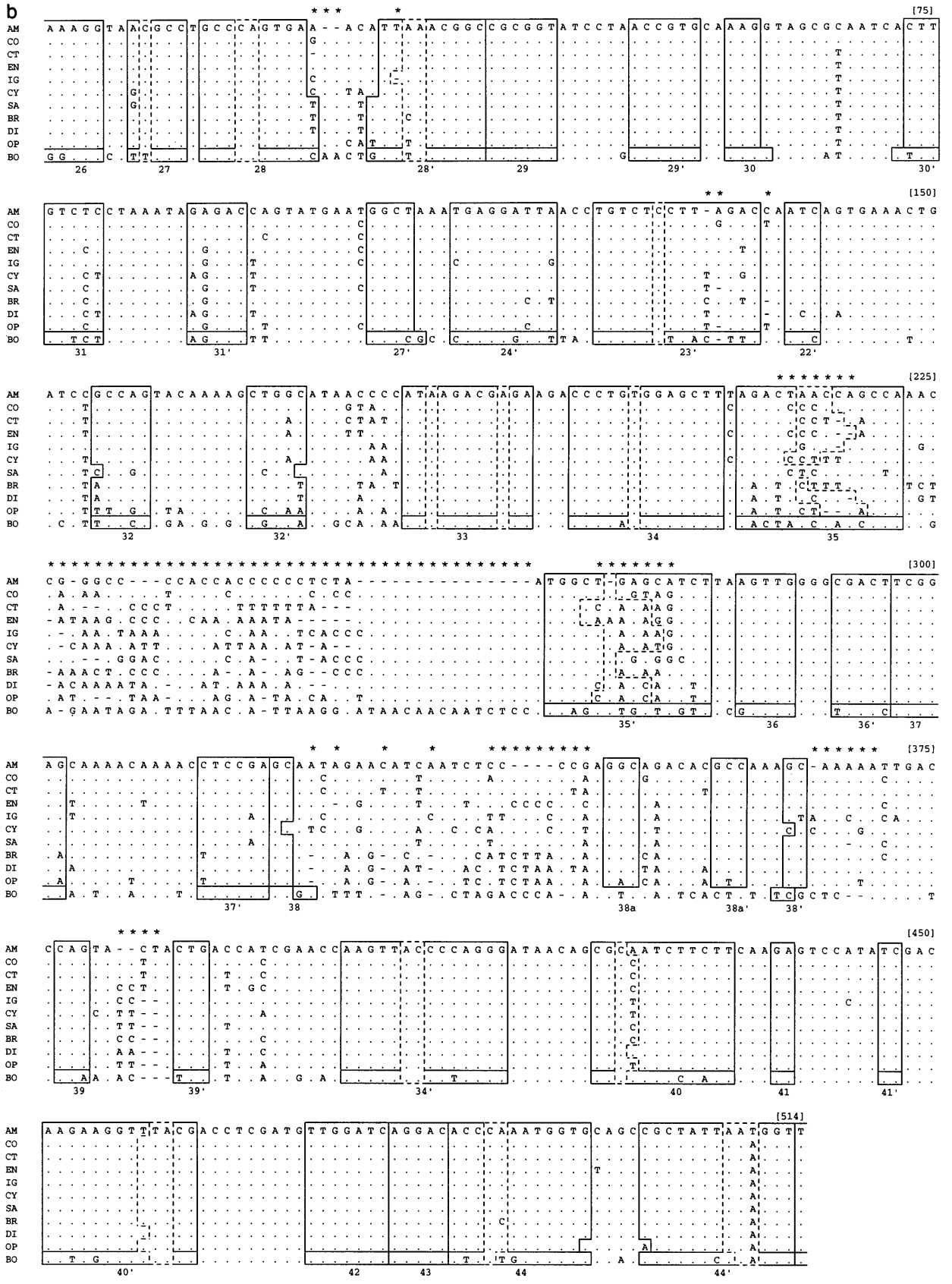

FIG. 1—*Continued*

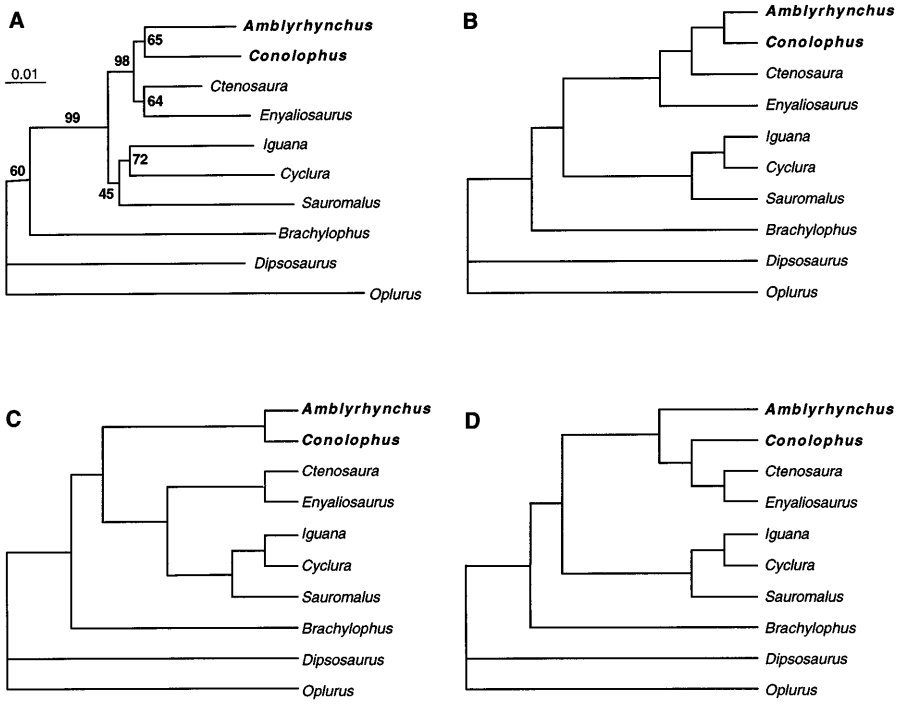

FIG. 2. (A) Tree topology suggested in the NJ, ML, and MP analyses of the 9 Iguanidae and the outgroup *Oplurus*. The branch lengths reflect those obtained in the NJ analysis, and confidence probabilities for the NJ tree topology are given above the branches. The unweighted MP analysis predicted 3 additional trees with equal lengths (B–D), and the weighted MP analyses yielded 1 alternative tree (D). The two Galápagos iguana genera are indicated in bold face type.

among the *Sauromalus* group and the remaining four Iguanini. Three of the four trees indicated a sister taxon relationship of the two Galápagos iguana genera, *Amblyrhynchus* and *Conolophus*.

Biases in the data treatment prior to the phylogenetic analyses can affect the outcome of a molecular tree reconstruction. In the iguanid study, a high number of indels and regions with ambiguous alignment were excluded from the data set. To test whether this led to distortions in the tree estimation, an unweighted MP analysis was conducted including all positions and treating deletions as a fifth base. The single resulting tree (TL = 676, CI = 0.678, RC = 0.292) matched topology 2a, inferring a sister taxon status of the Galápagos iguanas.

The statistical significance of particular clusters in the NJ tree estimation was tested with the standard error test of Rzetsky and Nei (1992, 1993). Two nodes were highly supported, suggesting the monophyly of the seven Iguanini (confidence probability (CP) = 99%) and the grouping of the Galápagos iguanas with *Ctenosaura* and *Enyaliosaurus* (here named '*Amblyrhynchus* group') (CP = 98%) (Fig. 2a). Bootstrap resampling analyses were performed to compare the reliabilities of the NJ, ML, and MP tree reconstructions. The 50% consensus trees obtained in the bootstrap analyses matched the topology given in Fig. 2a, regardless of the method used. However, the bootstrap values for a particular node in the tree varied considerably for the different methods (Fig. 3a). The values for the node suggesting *Brachylophus* as a sister taxon to the Iguanini ranged between 68 and 87%. The monophyly of the Iguanini, on the other hand, was equally strong supported with all methods (96–100%). Within this group the values differed again, in particular for the node joining the taxa of the *Sauromalus* group and the node joining the *Amblyrhynchus* group taxa. The *Amblyrhynchus* group was moderately supported only by the NJ bootstrap value (77%), whereas the ML bootstrap value for this group was 59% and the MP value only 23% (Fig. 3a). Conversely, the *Sauromalus* group was better supported with the MP method (70 and 73%) and the ML method (82%), than with the NJ method (47%). In summary, when the 10 taxa were analyzed jointly only the monophyly of the Iguanini was significantly supported in the bootstrap analyses.

The sequences of the monophyletic Iguanini were then analyzed on their own. The unweighted MP tree reconstruction resulted in a single tree (TL = 171, CI = 0.772, RC = 0.329) (Fig. 3b). The grouping of the seven taxa into the *Amblyrhynchus* and the *Sauromalus* clade was more consistently found with all methods than in the previous analyses (75–91%) (Fig. 3b). Similarly, testing the four taxa of the *Amblyrhynchus* group alone gave a single MP tree (TL = 72, CI = 0.931, RC = 0.543), and all bootstrap analyses supported the

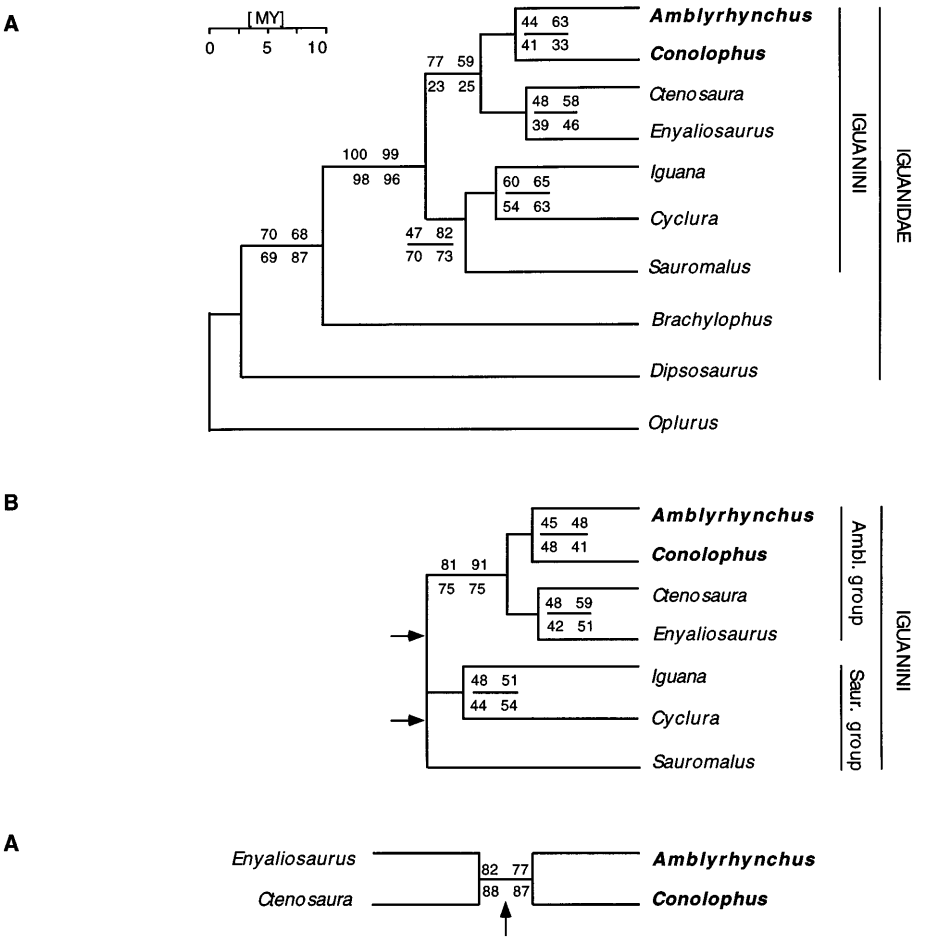

FIG. 3. The 50% majority rule consensus trees obtained in the ML, NJ, and MP bootstrap analyses including (A) the 9 Iguanidae and the outgroup *Oplurus*, (B) the 7 Iguanini, and (C) the 4 *Amblyrhynchus* group taxa. The bootstrap values are given above the branches for the NJ and the ML method, and below for the unweighted and weighted MP methods. Arrows indicate the possible positions of roots in the trees. (A) The branch lengths in the tree reflect those obtained in the ML tree reconstruction when using the total sequence information (stems and loops) and without adjusting for substitution variation. The scale reflects the age estimates obtained from this tree. The same tree topology was obtained when using only the loop regions or when adjusting the settings in the DNAMLK program to the high number of unvaried sites in the iguanid DNA.

separation of the Galápagos iguana lineage and the *Ctenosaura*/*Enyaliosaurus* lineage (77 to 88%) (Fig. 2c). Sixty three positions were variable among the sequences of the four taxa, 12 were parsimoniously informative, and 7 of the informative sites were synapomorphies for *Amblyrhynchus* and *Conolophus* (3 for *Amblyrhynchus* and *Ctenosaura;* 2 for *Amblyrhynchus* and *Enyaliosaurus*). Outgroups could not be defined *a priori* in the tree reconstructions of the iguanid subgroups, and roots had to be determined *a posteriori* to establish the branching order among the taxa. Roots were indicated in the trees obtained with the DNAMLK program and could be determined through midpoint rooting for the NJ and MP trees. The resulting tree topologies suggested that the *Amblyrhynchus* group was monophyletic (Fig. 3b), and that the Galápagos iguanas were sister taxa (Fig. 3c).

Evolutionary Rates

Divergence times between taxa can be estimated, provided that the evolutionary rates across lineages are similar. The Wu and Li relative rates test (Wu and Li, 1985) applied in this paper did not detect unequal rates among the iguanid rDNA sequences (nine Iguanidae, total sequences: $z_{max} = 1.12$, $P > 0.05$ for all pairwise comparisons; loops: $z_{max} = 2.22$, $P > 0.05$; seven Iguanini, total sequences: $z_{max} = 0.88$, $P > 0.05$; loops: $z_{max} = 0.67$, $P > 0.05$). Therefore, equal rates were assumed in the calculations. Minor rate heterogeneities among some ungulate rDNA sequences were found in previous studies (Kraus and Miyamoto, 1991; Allard *et al.*, 1992), but the subfragments used for the rate comparisons in this study did not show significant rate differences (Wu and Li test: cervids, total sequences:

TABLE 1a

Percentage Pairwise Divergences among Iguanid mt rDNA Sequences

Genera compared	AM	CO	CT	EN	IG	CY	SA	BR	DI	OP
Total sequences										Transversions
Amblyrhynchus		1.1	1.1	1.3	1.8	2.2	1.8	4.3	3.5	4.9
Conolophus	3.0		0.5	1.1	1.2	1.3	1.4	3.7	2.9	4.5
Ctenosaura	2.5	3.1		0.8	1.2	1.6	1.4	3.7	3.3	4.5
Enyaliosaurus	3.8	3.7	2.8		1.8	2.2	1.6	3.6	3.7	4.8
Iguana	3.3	4.9	4.1	4.8		1.6	1.4	3.9	3.8	4.8
Cyclura	3.8	5.1	4.2	4.1	4.2		2.0	4.5	4.2	4.7
Sauromalus	4.8	5.9	4.8	5.1	5.0	5.1		4.2	3.8	4.5
Brachylophus	5.0	5.7	5.5	5.6	5.9	5.6	6.4		4.9	5.9
Dipsosaurus	5.6	7.3	5.7	6.5	6.9	6.7	6.4	5.6		4.3
Oplurus	6.9	8.4	6.6	7.4	7.8	8.4	7.9	6.7	7.9	
	Transitions									
Loop regions										Transversions
Amblyrhynchus		2.0	2.0	2.2	3.2	3.7	2.7	6.8	6.1	7.3
Conolophus	5.1		1.0	2.0	2.2	2.2	2.2	5.9	4.6	6.8
Ctenosaura	3.7	5.4		1.5	2.2	2.7	2.2	5.9	5.6	6.8
Enyaliosaurus	5.4	5.6	4.7		3.2	3.7	2.2	5.9	6.1	7.1
Iguana	3.7	7.1	4.9	5.9		2.4	2.0	6.1	6.8	7.6
Cyclura	3.7	6.3	4.6	5.1	4.9		2.9	7.6	6.8	6.6
Sauromalus	5.1	7.3	4.6	5.4	5.1	4.6		6.1	5.9	6.6
Brachylophus	5.9	7.6	6.8	7.6	7.6	6.3	7.3		8.1	9.3
Dipsosaurus	5.4	8.8	5.6	7.1	6.1	6.3	6.3	4.9		6.1
Oplurus	4.6	7.8	4.6	6.4	5.9	7.1	5.4	5.9	4.6	
	Transitions									

$z_{max} = 0.44$, $P > 0.05$; cervids, loops: $z_{max} = 1.21$, $P > 0.05$; bovids, total sequences: $z_{max} = 0.89$, $P > 0.05$; bovids, loops: $z_{max} = 0.69$, $P > 0.05$). Adopting the evolutionary rate from these fragments was therefore possible, but probably only for taxa with similar levels of evolutionary divergence. Comparisons of the total (TS and TV) sequence divergences between the 3 ungulate pairs suggested a similar range of genetic distances as among the Iguanini (Table 1), and separation times were estimated only for the taxa of this group. While the overall rates of sequence evolution appeared to be constant, the segments within the analyzed rDNA sequences differed largely in their nucleotide substitution rates, as well as their base compositions. Similar base frequencies were found in the iguanid stems and ungulate stems and the iguanid and ungulate loops, respectively (Table 2). Thus, the secondary structure analysis made it possible to compare DNA regions with similar structure and potentially similar functional constraints in the rate estimation.

In a first approach, the divergence times of the Iguanini were calculated from the branch lengths in the ML trees. The same ML tree topology was obtained

TABLE 1b

Percentage Pairwise Divergences among Ungulate mt rDNA Sequences

Genera compared	CE	MU	BO	CA	MA	GA	CE	MU	BO	CA	MA	GA
	Total sequences				Transversions		Loops				Transversions	
Cervus		0.4	1.6	1.8	2.6	2.7		0.9	2.6	3.2	4.1	4.5
Muntiacus	3.4		1.6	1.8	2.6	2.5	4.7		2.6	3.2	4.1	4.1
Bos	7.6	6.9		1.2	1.9	2.0	7.9	6.0		1.9	2.4	2.8
Capra	6.0	5.6	6.3		1.5	1.6	6.7	5.2	7.5		2.2	2.6
Madoqua	5.6	5.9	6.0	5.0		0.8	6.7	6.9	7.3	7.3		1.3
Gazella	5.9	6.1	5.6	5.8	4.9	6.7	7.1	5.4	8.2	5.6		
	Transitions						Transitions					

TABLE 2

Base Composition (in Percentage + SD) in the Iguanid and Ungulate rDNA Sequences

	Stem regions		Loop regions	
	Iguanids	Ungulates	Iguanids	Ungulates
Guanine	30.6 ± 0.7	28.5 ± 0.4	11.0 ± 0.5	11.5 ± 0.4
Adenine	21.3 ± 0.8	21.6 ± 0.4	47.1 ± 1.3	46.8 ± 0.8
Uracil	24.0 ± 0.4	27.4 ± 0.4	18.0 ± 0.8	20.3 ± 0.3
Cytosine	24.1 ± 0.5	22.4 ± 0.4	24.0 ± 1.5	21.4 ± 0.5

Note. Base frequencies are estimated among the 9 Iguanidae and *Oplurus* and among the 6 ungulate sequences, based on the positions included in the phylogeny and separation time estimation.

when using the total sequence information or when using only the loop regions and with or without adjustment of the settings in the DNAMLK program to substitution rate variation (Fig. 3a). The average separation time between the two Galápagos iguanas was estimated as 10.5 MY, and the entire Iguanini clade appeared not to be older than 17.6 MY, with slightly higher values resulting for the analysis of the loop regions alone (Table 3, left columns). When the settings of the program were adjusted to the high number of unvaried sites in the iguanid and ungulate rDNA sequences, the separation times increased, suggesting an age of 17.2 MY for the Galápagos marine and land iguana, when only the loop regions were analyzed (Table 3, middle columns). The second approach, counting only TV substitutions, resulted in higher estimates than the ML method. The average TV divergence rate (per nucleotide and MY) between the ungulate total sequences analyzed in this study was $5.6 \times 10^{-4} \pm 8.7 \times 10^{-5}$ and $1.0 \times 10^{-3} \pm 2.0 \times 10^{-4}$ for the loop regions. Using these rates, the evolutionary age of the Galápagos marine and land iguana was estimated as about 19 MY and that of the Iguanini as about 29 MY (Table 3, right columns).

DISCUSSION

The purpose of this study was to test the sister taxon relationship of the Galápagos iguanas and to estimate their separation times. When analyzing the nine Iguanidae and *Oplurus*, a direct common ancestry of the Galápagos marine and land iguana was proposed in most, but not all tree reconstructions, and the node joining the two was not sufficiently confirmed in the bootstrap analyses. The standard error test in the NJ tree estimation suggested a high probability for a grouping of the Galápagos iguanas with *Ctenosaura* and *Enyaliosaurus* (Fig. 2a), but the NJ, ML, and MP bootstrap support for this node was low. Thus, the branching order among the seven Iguanini was generally unsupported.

The bootstrap values resulting from the different tree reconstruction methods for a particular node varied markedly. When the substitution rate variation is high among different sites, which is characteristic for rDNA genes, the ML method is supposed to be less efficient than the NJ method, and the weighted and unweighted parsimony methods are more affected than both the NJ and the ML methods (Tateno *et al.*, 1994). When the settings in the ML bootstrap analysis of the 10 sequences were adjusted to the high number of unvaried positions in the rDNA fragments, the resulting values were more similar to those of the NJ method. The bootstrap value for the node joining the *Amblyrhynchus* group taxa, for example, was 77% with the NJ method and 59% with the ML method when the settings were not corrected (Fig. 3a), but 71% with this method when the assumptions were changed (not indicated in Fig. 3a). Thus, the NJ, ML, and MP methods probably differed in their sensitivity to errors in the approxima-

TABLE 3

Separation Time Estimates [MY] for Several Clades within the Iguanini

	ML branch length estimate		ML branch length (corrected)		TV divergence estimate	
	Total	Loops	Total	Loops	Total	Loops
AM/CO	**10.5** (6.8)	**13.1** (7.7)	**11.8** (8.4)	**17.2** (11.0)	**19.2** (14.7)	**19.5** (13.9)
CT/EN	**9.5** (6.1)	**12.0** (7.0)	**10.4** (7.4)	**13.9** (8.9)	**14.9** (11.4)	**14.6** (10.5)
Ambl. group	**13.3** (8.6)	**14.8** (8.7)	**14.2** (10.1)	**19.1** (12.2)	**17.7** (13.6)	**17.8** (12.7)
IG/CY	**12.0** (7.8)	**11.1** (6.5)	**14.5** (10.3)	**13.9** (8.9)	**27.7** (21.3)	**24.4** (17.4)
Saur. group	**14.3** (9.3)	**12.8** (7.5)	**16.7** (11.8)	**15.5** (10.0)	**30.9** (23.7)	**24.4** (17.4)
Iguanini	**17.6** (11.4)	**17.8** (10.5)	**19.9** (14.1)	**20.9** (13.4)	**28.8** (22.1)	**26.8** (19.2)

Note. The average times of separation are estimated from ML tree branch lengths (with and without correcting for substitution rate variation at different positions) and from pairwise TV divergences; the results obtained when adopting the mean ungulate substitution rate for the calibration of the iguanid rate of sequence evolution are given in bold letters; a minimum separation time estimate (mean ungulate rate + 2 SD) is given in parentheses.

tion of the mode of sequence evolution, affecting their relative efficiencies in the tree reconstruction.

The lack of statistically significant resolution of most of the iguanid relationships is surprising, considering the relatively high number of variable positions (196 of 838 sites) and the strong phylogenetic signal present in the sequences ($g1 = -0.870$). A comparison of the sequences of the nine Iguanidae and *Oplurus* showed that 45% of all analyzed variable positions were uninformative in the sense of parsimony, and the figure increased to 60% when only the sequences of the Iguanini were compared. A pilot study based on partial sequences (300 nts) of the cytochrome b gene revealed that the ratio of uninformative to informative positions was equally high in a protein coding gene of the mitochondrion, with 52% (of 109 variable positions) uninformative among eight Iguanidae, and 63% (of 81 variable sites) among six Iguanini (excluding *Enyaliosaurus*). Thus, the large number of autapomorphies was not a peculiarity of the analyzed rDNA molecules. Instead, the findings may indicate a rapid cladogenesis among the Iguanini, followed by a long period of separate evolution. This was also evident from the NJ and ML tree estimations, which predicted much longer external than internal branches in the Iguanini and the *Amblyrhynchus* clade (Figs. 2a and 3a). Wollenberg *et al.* (1996) developed a statistical test for nonrandomness in the temporal pattern of lineage bifurcation. Using the ML branching times among the nine iguanids obtained with the DNAMLK program in this test suggested significant deviation of the observed mode of cladogenesis from a random pattern ($D = 0.57$, $P < 0.01$). Rapid separation of the Iguanini lineages also has been suggested by de Queiroz (1987) on the basis of osteological data, and is supported by the immunological data of Wyles and Sarich (1983), predicting similar genetic distances between either of the Galápagos iguanas and any of the mainland Iguanini.

Rapid cladogenesis can cause difficulties in the molecular tree reconstruction (Kraus and Miyamoto, 1991; Hay *et al.*, 1995). In particular, the MP method appears to be less efficient when short interior branches are combined with long exterior branches, with the power decreasing further, the more taxa are included (Takezaki and Nei, 1994; Tateno *et al.*, 1994). Alternatively, it could be argued that the outgroup included in this study (*Oplurus*) was too distant to efficiently polarize the character states within the Iguanidae in the MP analysis. If so, the taxa of the *Sauromalus* group should have represented more closely related, and therefore more appropriate, outgroups for the *Amblyrhynchus* group, or vice versa, and a separate analysis of the seven Iguanini should have led to a better supported topology within either group. This was not the case (Fig. 3b), and divergence of the *Amblyrhynchus* and the *Sauromalus* group taxa within a short period of time is the more likely explanation for the low statistical support of their relationships.

When analyzing the Iguanini together with outgroups, large amounts of sequence data may be needed to better resolve and support their phylogeny, especially when the MP method is applied. Here, the alternative approach was taken and the number of taxa reduced. Including only the monophyletic Iguanini in the analysis more reliably resolved the grouping of the Galápagos iguanas with *Ctenosaura* and *Enyaliosaurus*, regardless which tree estimation method was used (Fig. 3b). This result allowed a separate test of the *Amblyrhynchus* group. The sister taxon relationship of the Galápagos land and marine iguana was sufficiently supported in this analysis (77–88%), although most (81%) of the positions variable among the sequences were autapomorphies for one of the lineages, indicating their bush-like origin. However, *Amblyrhynchus* shared more than twice as many synapomorphies with *Conolophus* as with *Ctenosaura* or *Enyaliosaurus*. In summary, the findings of the molecular phylogenetic analyses justified the assumption that the Galápagos iguanas were their closest living relatives.

A recent study using sequence information from the mitochondrial ND4 and tRNA genes of the Iguanidae and several outgroups (Sites *et al.*, 1996) also proposed the monophyly of the *Amblyrhynchus* group taxa. The clade was sufficiently supported in the NJ bootstrap analysis (87%), but the MP analysis gave relatively weak support (66%). The sister taxon relationship of the marine and land iguana was not significantly resolved in this study (bootstrap values, MP < 50%, NJ = 64%). A promising future approach may be to combine all sequence information presently available, which, under certain circumstances, can lead to higher efficiencies in the tree reconstructions (Huelsenbeck *et al.*, 1996). Yet, an increase of sequence information may give more support to the tree proposed by the mitochondrial genes, but this tree may not reflect the species phylogeny (Wu, 1991). The likelihood of a contradiction between the gene and the species tree increases, when the time interval between two speciation events is short. Thus, the rapid cladogenesis among the Iguanini may have favored the occurrence of this phenomenon. Conversely, Sites *et al.* (1996) argue that mtDNA gene phylogenies have a high probability to resemble the species phylogeny, because the effective number of mitochondrial genes is lower than that of nuclear genes, and their coalescence times are shorter (Moore, 1995). The authors claim that the mtDNA coalescence times are especially low in iguanids because most species have polygynous mating systems and/or subdivided metapopulation structures, decreasing their effective population sizes (Sites *et al.*, 1996). The effective number of mitochondrial genes normally equals the female effective population size (N_e) and, in a panmictic population with sex ratio 1:1, is 4 times lower than the

effective number of nuclear genes (Birky *et al.*, 1983, 1989). A polygynous mating system leads to a high variance in male reproductive success, and thus to a lower total N_e, but should not reduce the female N_e which is relevant for mitochondrial genes. Also, in a subdivided population the coalescence times reflect the time until two ancestors in different subpopulations can be traced to the same subpopulation and thus depend primarily on the migration rate (Marjoram and Donnelly, 1994). Therefore, mtDNA coalescence times can be higher in a subdivided population than in a panmictic population of the same size. Bearing in mind the rapid cladogenesis of the Iguanini, this casts doubts on the assumption that the iguanid mtDNA phylogeny resembles the species phylogeny. The most convincing argument for the sister taxon relationship of the Galápagos iguanas thus comes from the comparison of the different data sets currently available. Molecular, morphological, and biochemical analyses consistently recover the marine and land iguana as the closest living relatives (Avery and Tanner, 1971; Wyles and Sarich, 1983; de Queiroz, 1987; Sites *et al.*, 1996). It must be concluded that the phylogeny of the Galápagos iguanas is consistent with the hypothesis that their direct common ancestor colonized the Galápagos, where they subsequently diverged *in situ*.

The evolutionary ages of the iguanids were estimated using an ungulate rate of sequence evolution. The rates of mitochondrial sequence evolution are likely to vary among taxon groups (Martin and Palumbi, 1993) and, perhaps, the accuracy of a separation time estimate is higher, when the rate is taken from a closely related taxon group. However, it is also crucial to adopt the rate from taxa with well-known phylogenetic relationships and divergence times (Marshall, 1990). Homologous rDNA sequences for reptile species with reliable molecular phylogenies and evolutionary ages were not available at the time of the study. The ungulate taxa used in this study were part of robust groupings in previous molecular analyses (Miyamoto *et al.*, 1990; Kraus and Miyamoto, 1991; Allard *et al.*, 1992). Their fossil record was relatively well-documented, providing good estimates of their separation times (Hamilton, 1973; Solounias, 1981; Gentry, 1990), which also covered the time range during which the Galápagos iguanas separated. Most importantly, the evolutionary rates of endotherm mtDNA are supposed to be faster than those of ectotherms (Avise *et al.*, 1992; Rand, 1994), and using an ungulate rate for the calibration of the iguanid rate of sequence evolution probably results in underestimates of their separation times. Therefore, adopting an ungulate rate provided a conservative test for the hypothesis that the divergence of the marine and land iguana predated the age of the present Galápagos Islands and was feasible for the purpose of this study.

The separation time estimates for the Galápagos iguanas ranged from 10.5 to 19.5 MY, with the lower values obtained with the ML method. The ML tree reconstruction is affected by substitution rate variation among different positions, which can lead to serious errors in the branch length estimation (Fukami-Kobayashi and Tateno, 1991; Tateno *et al.*, 1994). The choice of a suitable nucleotide substitution model, reflecting the characteristics of the molecular data, is therefore crucial. When the settings in the ML program were adjusted, better log-likelihood values were obtained for the resulting trees, and the age estimates increased (Table 3). Thus, deficiencies in the assumptions on the model of sequence evolution may have caused the low age estimates of the ML method. Interestingly, the ML estimates obtained for the total sequence information, including the stem regions, were lower than those for the loop regions only. It has been shown that the TS:TV substitution ratios in the stems of ungulate mitochondrial rDNA sequences are especially high (Gatesy *et al.*, 1994), and a similar bias was found in the iguanid stems. The TS:TV ratio can be specified in the ML program, but it reflects the overall assumptions for the analyzed sequences. Combining regions with different substitution patterns, such as the stem and loop segments, may therefore lead to incorrect branch length estimations. Compensatory mutations, which are frequent in the ungulate and the iguanid stem regions, may further influence the age estimation from the total sequences. The rates of compensation vary for different stem pairs (Gatesy *et al.*, 1994), and they may differ among ungulate and iguanid stem regions. It is therefore possible that the estimates based on the total sequences are less reliable than those from the loop regions alone. Considering the results of the TV method, it is conspicuous that the age of the *Amblyrhynchus* group is lower than that for the *Amblyrhynchus* and *Conolophus* lineages and, similarly, the age of the Iguanini lower than that of the *Sauromalus* group. Evidently, some pairwise TV divergences are lower among taxa from different clades than from the same clade (Table 1a). This is possible when the analyzed lineages separated rapidly, resulting in relatively similar TV divergences among all taxa. Additionally, the TV method gives only crude estimates, especially when the number of TV substitutions is low and a single sequence pair is compared.

In summary, the age estimates presented here are probably associated with large errors. However, even when the minimum time estimates are considered across all different calculations (using the mean ungulate rate + 2 SD), the separation between the Galápagos land and marine iguana must have occured before the oldest island of the present archipelago emerged (Table 3). The hypothesis is strengthened, considering that an endotherm rate was used in the calculation and their separation time underestimated. Also, due to ambiguous alignment, more variable positions were

excluded in the iguanid sequences than in the ungulate sequences, which may have decreased the age estimates further. Bearing this in mind, the estimates seem conservative and it is concluded that the speciation of the Galápagos iguanas took place between 10 and 20 MY ago. Hence, the DNA data are consistent with the findings of the immunological analyses, which predict an age of 15 to 20 MY for the two Galápagos iguana lineages (Wyles and Sarich, 1983).

CONCLUSIONS

The results of the phylogenetic analyses and the age estimations suggest that the land and marine iguana inhabited the former, now sunken, islands of the Galápagos. This conclusion is based on the assumption that they separated within the archipelago. The alternative hypothesis—that they diverged elsewhere and colonized the islands independently—has to be considered. This model would require two colonizations and, because the Galápagos iguanas appear to be sister taxa, the extinctions of both their ancestral stocks. Thus, the more parsimonious hypothesis is speciation *in situ*, requiring no extinctions and a single introduction of their common ancestor to the islands. Additional weight to the *in situ* speciation hypothesis may come from the rapid divergence among the Iguanini lineages. Judging from the ML time estimates, the divergence of *Amblyrhynchus* and *Conolophus*, as well as of *Iguana* and *Cyclura*, from their respective ancestors occured in 1.6 to 2.8 MY (Table 3). Perhaps the rapid cladogenesis of the Iguanini was triggered by the colonization of new habitat. In the case of *Iguana/Cyclura*, this would have been the colonization of the West Indies, and in the case of the Galápagos iguanas the colonization of the archipelago by their common ancestor.

Final proof for the *in situ* speciation of the Galápagos iguanas is hard to come by. According to current knowledge, however, this is the most parsimonious hypothesis. Under this assumption, the DNA data suggest that surfaced Galápagos islands have existed for 10 MY, or more, and Christie *et al.*'s (1992) proposition, that speciation times in the Galápagos can be extended, has gained support. Perhaps other Galápagos vertebrate taxa with old evolutionary ages, such as the Galapagos lava lizards (*Tropidurus*) and the geckos (*Phyllodactylus*), also inhabited the former islands. Again, it is possible that the high levels of genetic differentiation in these taxa originated from multiple introductions to the islands (Wright, 1983; Lopez *et al.*, 1992). Phylogenetic analyses, including mainland sister groups, promise more insight into the archipelago's colonization history, and thus a better understanding of the patterns and processes of evolution in the Galápagos.

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