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Phylogenetic Relationships of the Canary Islands Endemic Lizard Genus *Gallotia* (Sauria: Lacertidae), Inferred from Mitochondrial DNA Sequences

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Phylogenetic relationships among species and subspecies of the Canary Island endemic lizard genus *Gallotia* are inferred based on nucleotide sequences of fragments of 12S ribosomal RNA and cytochrome *b* mitochondrial genes. The four morphologically established species have also been recognized at the molecular level. Relative affinities among species follow an eastern–western geographic transect. The nearly extinct species *Gallotia simonyi* from the most western island of El Hierro is closely related to the common western species *Gallotia galloti*, the nearest branch to this pair is *Gallotia stehlini* from the central island of Gran Canaria, and finally, *Gallotia atlantica* from the two eastern and geologically oldest islands appears as the most distantly related species of the group. At the statistical level, four subspecies can be recognized in *G. galloti*, but only two in *G. atlantica*.

INTRODUCTION

Lacertids are represented in the Canary Islands by the endemic genus *Gallotia* (Arnold, 1973). As all the Canary Islands are of independent volcanic origin the speciation process of the genus is of great taxonomic and evolutionary interest and has been the object of numerous studies since the beginning of the last century (for a review see Machado *et al.,* 1985). Different morphological analyses agree in recognizing four *Gallotia* species with different distributions in the seven main islands of the Archipelago (Klemmer, 1976; Arnold, 1989): *Gallotia atlantica* inhabiting the eastern islands of Lanzarote and Fuerteventura and their coastal islets, *Gallotia stehlini* endemic to the central island of Gran Canaria, *Gallotia galloti* present in the western islands of Tenerife, La Gomera, La Palma, and El Hierro, and *Gallotia simonyi,* a species in danger of extinction, with a small known population on an almost inaccessible cliff in Hierro (Böhme and Bings, 1975). Reports of the presence of small populations of *G. atlantica* on Gran Canaria (Barquín and Martín, 1982) and of *G. stehlini* on Fuerteventura (Naranjo *et al.,* 1991) pose the question of the time of these colonizations and their degree of differentiation. Alternative phylogenetic relationships among the species have been proposed, with *G. stehlini* and *G. simonyi* being the closest related species (Arnold, 1989), *G. galloti* and *G. stehlini* as sister taxa, and *G. atlantica* less closely related (Thorpe *et al.,* 1985), *G. stehlini* being the most divergent species and *G. galloti* and *G. simonyi* the most related ones (Mayer and Bishoff, 1991), *G. galloti* and *G. atlantica* being sister species, and *G. stehlini* more divergent (Thorpe *et al.,* 1993a, 1994). Even more confusion exists at the subspecies level. For *G. atlantica* some authors recognize two subspecies, *G. a. atlantica* in Lanzarote and its islets and *G. a. mahoratae* in Fuerteventura and Lobos islet (Bischoff, 1985). Others claim four subspecies, *G. a. atlantica* inhabiting the main Lanzarote and Fuerteventura islands and Lobos, Graciosa, and Montaña Clara islets, *G. a. delibesi* for the small southeast Gran Canaria population, *G. a. laurae* only found in a volcanic badland named Malpaís de la Corona in the northeast of Lanzarote, and *G. a. ibagnezi* endemic to the Alegranza islet (Castroviejo *et al.,* 1985). Similar discrepancies exist to explain the radiation process of the species *G. galloti.* Morphological studies have recognized up to five subspecies, one for each main western island, El Hierro, La Gomera, La Palma, and two for Tenerife (Boettger and Müller, 1914; Bischoff, 1982). Recently, another subspecies, *G. g. insulanagae,* has been proposed for the Tenerife Roque de Fuera islet population (Martín, 1985). However, at the molecular level Thorpe *et al.* (1993b) suggested only two subspecific lineages in this species, one named Northern made up of La Palma, north Tenerife, and south Tenerife populations, and a Southern one including those of Gomera and Hierro.

With the aim of constrasting the mainly morphologi-

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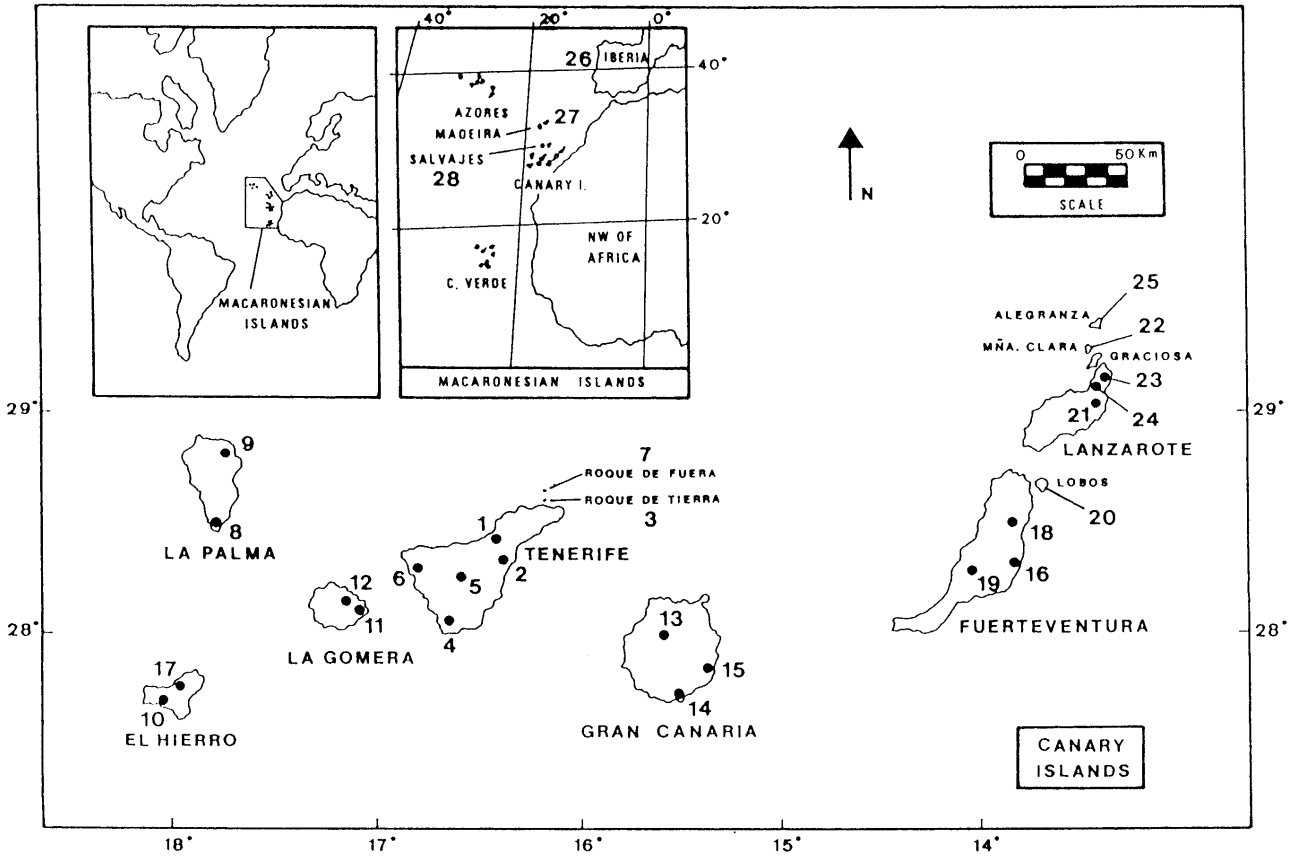

FIG. 1. Distribution of the endemic lizards (genus *Gallotia*) of the Canary Islands and the outgroup lizards (genera *Lacerta* and *Podarcis*) analyzed in the present study. Numbers of the localities correspond with numbers in Table 1.

cal phylogenies proposed for *Gallotia* with one based on molecular characters, we have studied representatives of each recognized taxon at the mitochondrial DNA level. Sequences with different evolving rates and taxonomic utility (Kocher *et al.*, 1989) have been used to detect inter- and intraspecific differentiation without the strong influence of adaptive convergence often encountered with morphological data.

MATERIALS AND METHODS

Specimens Analyzed

The geographic origin of the studied specimens is shown in Fig. 1. Specimens were captured in the field (w) or obtained from collection material kept in 70% ethanol (c). In both cases a piece of the tail approximately 0.5 g in weight was clipped off; after that live animals were released in the same capture place. Table 1 shows the number of samples and locality of capture for each *Gallotia* species and their putative subspecies. *Lacerta lepida* and *Podarcis dugesii* were included as outgroup genera. Both subspecies proposed for *P. dugesii* (Bischoff *et al.*, 1989) were also examined.

DNA Extraction

Before treatment, alcohol samples were washed 30 min in 50 ml sterile distilled water. Total DNA was isolated from 0.1 g of tail tissue, homogenized in 500 μl of lysis buffer (50 mM KCl, 2.5 mM Cl_2Mg, 0.45% Nonidet P-40, 0.45% Tween 20, 10 mM Tris–HCl, pH 8.0). To this homogenate, 10 μl of 20% SDS and 10 μl Proteinase K (20 mg/ml) were added, and the mixture was incubated 4 h at 55°C with occasional agitation. Proteins were removed by consecutive phenol, phenol–chloroform, chloroform, and ether extractions. The aqueous phase containing total DNA was boiled 5 min and 5 μl was used as a template for the polymerase chain reaction (PCR) amplification.

DNA Amplification and Sequencing

Two segments of the mitochondrial genome were amplified: a 307-bp region of the cytochrome *b* (cyt*b*) gene, corresponding to sites 14842 to 15148 in humans (Anderson *et al.*, 1981), and an approximately 400-bp region of the 12S rRNA gene, corresponding to sites 1092 to 1477 in humans. Primers were the same as in Kocher *et al.* (1989). Amplifications were carried out in 100-μl volumes, and *Taq* polymerase and amplification buffer

TABLE 1

List of Specimens Examined in the Present Study

Species/subspecies Abreviations	Source[a]	Locality[b]
Gallotia g. eisentrauti		
Ggae-1	w	El Sauzal (Tenerife) [1]
Ggae-2	w	Arafo (Tenerife) [2]
Ggae-3	c	Roque de Tierra (Tenerife) [3]
Gallotia g. galloti		
Ggag-1	w	El Palmar (Tenerife) [4]
Ggag-2	c	Las Cañadas (Tenerife) [5]
Ggag-3	w	Masca (Tenerife) [6]
Gallotia g. insulanagae		
Ggai-1	c	Roque de Fuera (Tenerife) [7]
Gallotia g. palmae		
Ggap-1	c	Fuencaliente (La Palma) [8]
Ggap-2	c	Los Sauces (La Palma) [9]
Ggap-3	w	Los Sauces (La Palma) [9]
Gallotia g. caesaris		
Ggac-1	c	El Júlan (El Hierro) [10]
Gallotia g. gomerae		
Ggago-1	w	San Sebastián (La Gomera) [11]
Ggago-2	c	Hermigua (La Gomera) [12]
Gallotia stehlini		
Gst-1	w	Juncalillo (Gran Canaria) [13]
Gst-2	w	Maspalomas (Gran Canaria) [14]
Gst-3	c	Arinaga (Gran Canaria) [15]
Gst-4	w	B la Torre (Fuerteventura) [16]
Gallotia simonyi		
Gsis-1	c	Fuga de Gorreta (El Hierro) [17]
Gsis-2	c	Fuga de Gorreta (El Hierro) [17]
Gsis-3	c	Fuga de Gorreta (El Hierro) [17]
Gallotia a. atlantica		
Gata-1	w	La Asomada (Fuerteventura) [18]
Gata-2	w	La Asomada (Fuerteventura) [18]
Gata-3	c	Pájara (Fuerteventura) [19]
Gata-4	w	Lobos Islet [20]
Gata-5	w	Lobos Islet [20]
Gata-6	c	Guatiza (Lanzarote) [21]
Gata-7	c	Montaña Clara (Lanzarote) [22]
Gata-8	c	Montaña Clara (Lanzarote) [22]
Gallotia a. laurae		
Gatl-1	c	Los Jameos (Lanzarote) [23]
Gatl-2	c	Haría (Lanzarote) [24]
Gallotia a. ibagnezi		
Gati-1	c	Alegranza (Lanzarote) [25]
Gallotia a. delibesi		
Gatd-1	c	Arinaga (Gran Canaria) [15]
Lacerta lepida		
Lle-1	c	Badajoz [26]
Podarcis d. dugesii		
Pdud-1	w	Madeira [27]
Podarcis d. selvagensis		
Pdus-1	c	Salvaje Grande [28]

[a] w, wild; c, collection.
[b] Numbers in brackets correspond with numbers in Fig. 1.

were the standard ones from Promega. Cycle profile for the cyt*b* region was: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, for 35 cycles, and for 12S rRNA: 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, for 35 cycles. After precipitation with 0.6 vol polyethylene glycol/NaCl (20% wt/vol PEG 6000, 2.5 M NaCl) the amplified products were directly cycle sequenced using the fmol DNA sequencing system (Promega). In each case, both complementary strands were sequenced with the same primers as in the amplification process but in separate reactions. In all cases PCR specifications were: 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C, for 35 cycles.

Sequence Analysis

Nucleotide sequences were aligned using the CLUSTAL V program (Higgins *et al.*, 1992). Following Hedges *et al.* (1991) every length difference of one or more bases was scored as a single mutational event. To accomplish this, the difference matrix based on the aligned sequences was modified in such a way that in all pairs of comparisons each indel was considered as a single transitional difference. Sequence divergence between taxa for cyt*b* and 12S rRNA were calculated by the Kimura's (1980) two-parameter method using the MEGA 1.01 program (Kumar *et al.*, 1993). A combined analysis of cyt*b* and 12S rRNA was not carried out because not all the specimens had been analyzed for both sequences. Phylogenies were constructed assuming constant (UPGMA; Sneath and Sokal, 1973) and variable (NJ; Saitou and Nei, 1987) evolutionary rates, also using the MEGA 1.01 program. Confidence in the tree topologies was evaluated by computing the standard errors of branching points in the UPGMA tree (Nei *et al.*, 1985) using the Jin and Ferguson (1990) program and by the bootstrap method with 2000 iterations in the NJ tree (Felsenstein, 1985) using the MEGA 1.01 program.

RESULTS

Amplified DNA from fresh and collection material gave sequences in agreement with taxonomic assignments irrespective of their origin. In spite of several attempts under different conditions we were unable to amplify collection specimens Ggago-2 and Gatl-1 for 12S rRNA and Ggag-2, Ggai-1, Ggae-3, Gata-3, and Gatl-2 for cyt*b*.

For cyt*b* sequence no indels were observed, and 118 of the 307 positions were variable among 30 different sequences (Fig. 2). Most (73.7%) substitutions occurred in third base codon positions. Only 30 substitutions gave amino acid replacements (Fig. 3) and 15 of them were not conservative. The two histidines involved in heme ligation (Howell and Gilbert, 1988) are among the invariant amino acids.

The size of the 12S rRNA fragment ranges from 389 bp for some *G. atlantica* specimens to 393 bp for the

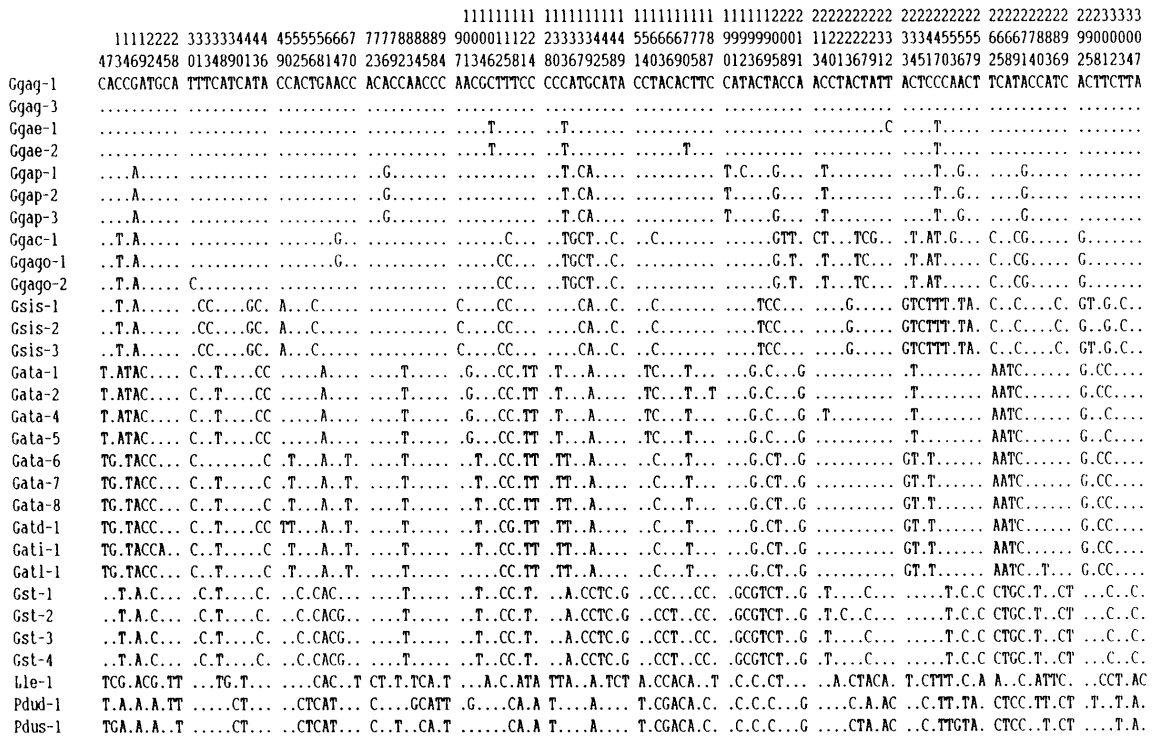

FIG. 2. Cyt*b* nucleotide differences among the 30 specimens studied. Site 4 corresponds with position 14,845 in the published human reference sequence (Anderson *et al.*, 1991).

species *G. galloti* and *P. dugesii* due to small indels of 1–3 bp. Intraspecific size polymorphism was only detected in *G. atlantica* and *P. dugesii* (Fig. 4). In addition, 97 of 396 positions were variable among 33 different sequences (Fig. 4).

As customary in studies of animal mtDNA, there is a pronounced transition bias in both sequences, particularly within subspecies. Transition–transversion ratios decline with increasing sequence divergence (Table 2).

Significant differences in sequence divergence were found between the two regions studied here. The cyt*b* is 3 times more variable than 12S rRNA at the subspecies level, 2-fold at species level, but only 1.2 when genera are compared (Table 3).

The UPGMA and NJ analyses of the cyt*b* data produced essentially identical topologies (Figs. 5A and 5B), as did those for the 12S rRNA data (Figs. 5C and 5D). The possible effects that rRNA secondary structure could have in these analyses have not been taken into account (see also Hedges *et al.*, 1991). As a consequence of their different variability, each fragment provides information for taxa at different levels. Subspecies branches were only significantly detected in the cyt*b* analyses, whereas the species relationships were better inferred from the 12S rRNA, likely as the result of greater saturation evident in the cyt*b* sequences.

DISCUSSION

The average sequence divergence among the Lacertidae genera studied here for cyt*b* (0.245 ± 0.025) and 12S rRNA (0.195 ± 0.010) are within the ranges observed for other lizards (Hedges *et al.*, 1991) and fish genera (Zhu *et al.*, 1994). From UPGMA and NJ analyses the Canary Islands endemic genus *Gallotia* appears as a monophyletic group (Fig. 5) as was already proposed based on morphological (Arnold, 1973) and karyological analyses (Cano *et al.*, 1984; López-Jurado *et al.*, 1986).

Affinities among species follow an eastern–western geographic transect. The nearly extinct species *G. simonyi*, from the most western island of El Hierro, appears as a sister species of *G. galloti* with a genetic distance for cyt*b* of only 0.122 ± 0.008. Furthermore, in the 12S rRNA topologies *G. simonyi* clusters within the *G. galloti* complex, being more closely related to the Tenerife and La Palma subspecies than to its sympatric subspecies from El Hierro (Fig. 5). These molecular data do not support the oldest taxonomic classification that considered *G. simonyi simonyi* and *G. simonyi stehlini* as races of a single polytypic species (Steindachner, 1889; Schenkel, 1901). Neither do they support the more recent idea (Arnold, 1989) that has considered *G. simonyi* and *G. stehlini* as the most closely related species of the genus. Our data are, however, in

	Sites																				
																					1
	1	1	1	1	2	2	3	3	3	4	5	5	6	7	7	7	7	7	8	0
	8	0	1	3	4	4	8	4	5	8	3	0	7	4	1	4	6	7	8	6	1
Ggag-1	C	I	I	I	I	N	N	I	A	H	H	I	L	M	Y	S	L	F	T	L	L
Ggap-1	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.
Ggac-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	I	.	.
Ggago-1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
Ggago-2	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.	.
Gsis-1	.	.	T	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	V	I	P
Gsis-2	.	.	T	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	V	I	P
Gsis-3	.	.	T	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	V	I	P
Gata-1	.	T	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	I	.	.
Gata-2	.	T	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	I	.	.
Gata-4	.	T	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	I	.	.
Gata-5	.	T	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	I	.	.
Gata-6	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
Gata-7	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
Gata-8	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
Gatd-1	.	T	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	V	.	.
Gati-1	Y	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
Gatl-1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V	.	.
Gst-1	.	.	.	.	.	.	.	.	.	.	V	.	A	.	.	.	.	.	.	.	.
Gst-2	.	.	.	.	.	.	.	.	.	.	V	.	A	.	P	.	.	.	.	.	.
Gst-3	.	.	.	.	.	.	.	.	.	.	V	.	A	.	.	.	.	.	.	.	.
Gst-4	.	.	.	.	.	.	.	.	.	.	V	.	A	.	.	.	.	.	.	.	.
Pdud-1	.	.	.	T	.	T	A	V	.	.	Y	.	M	L	.	.	M	Y	.	I	.
Pdus-1	.	.	.	T	.	T	T	.	.	.	Y	.	M	L	.	.	M	Y	.	I	.
Lle-1	.	.	.	V	.	T	S	.	T	.	Y	L	M	L	.	.	I	Y	S	.	.

FIG. 3. Cyt*b* amino acid differences among the 30 specimens studied. Sites correspond with the published human reference peptide sequence (Anderson *et al.*, 1991).

agreement with previous molecular results based on albumin immunological analyses (Mayer and Bischoff, 1991).

Less clear is the relative proximity of the three well-defined species, *G. atlantica*, *G. stehlini*, and *G. galloti*. Each mtDNA region supports a different clustering of these species. The cyt*b* data set presents *G. atlantica* and *G. galloti* as the more related species with 67% of bootstrap support on the NJ tree (Fig. 5B). The same relative positions hold for the UPGMA tree, but standard errors on nodes overlap (Fig. 5A). Conversely, in the 12S rRNA analyses *G. atlantica* significantly branches off before the *G. galloti–G. stehlini* pair in both trees (Figs. 5C and 5D). In order to avoid saturation problems due to the faster divergence rate of cyt*b* compared to 12S rRNA, we reanalyzed cyt*b* at the amino acid level, and also taking into account only first and second codon positions (data not shown). The topology in these trees was similar to that obtained with 12S rRNA sequences but without statistical significance. Therefore *G. galloti* and *G. stehlini* appear to be sister taxa, with *G. atlantica* more distantly related to them. This is not in agreement with previous molecular results based on albumin immunological analyses (Mayer and Bischoff, 1991), mtDNA restriction fragment polymorphism (Thorpe *et al.*, 1993a), mtDNA sequences, and nuclear RAPD analyses (Thorpe *et al.*, 1994) but does agree with the interspecific relationships found in earlier morphological numerical analyses (Thorpe *et al.*, 1985; Arnold, 1989).

Intraspecific differentiation is only detected with the cyt*b* data set. For the species *G. galloti*, up to four of the six morphologically recognized subspecies can be also distinguished at the molecular level; *G. g. gomerae* in La Gomera, *G. g. caesaris* in El Hierro, *G. g. palmae* in La Palma, and *G. g. galloti* in Tenerife (Figs. 5A and 5B). In accordance with the radiation of the species proposed in previous molecular studies by Thorpe *et al.* (1993ab), *G. galloti* specimens from Tenerife cluster first with those of La Palma and are well differentiated from those of La Gomera and El Hierro (Figs. 5A and 5B). The subspeciation within Tenerife proposed by Bischoff (1982), with *G. g. galloti* from southern and central parts of the Island and *G. g. eisentrauti* from the Anaga peninsula and the majority of the North coast, is not statistically supported in the UPGMA tree (Fig. 5A). However, it deserves mention that specimens morphologically classified as *G. g. galloti* (Ggag-1 and Ggag-3) cluster together in 89% of the cases in the bootstrap analyses, and those recognized as *G. g. eisentrauti* (Ggae-1 and Ggae-2) did so in 85% (Fig. 5B). Apparently, analyses including larger numbers of specimens and/or the most variable mtDNA regions will be necessary in order to distinguish these morphological levels of differentiation.

Morphologically, the intraspecific variation of *G. atlantica* is not as obvious as with *G. galloti* (Thorpe, 1985). This fact is corroborated, at the molecular level, by the average values of their intraspecific distances (3.06 and 5.57%, respectively). On the phylogenetic trees only two clusters of *G. atlantica* specimens can be distinguished, those belonging to the Lanzarote island and surrounding islets and those from Fuerteventura and Lobos (Figs. 5A and 5B). These results are fully consistent with the classification proposed by Bischoff (1982) based on the variability of several biometric characters: one subspecies, *G. a. atlantica*, for the Lanzarote cluster and one subspecies, *G. a. mahoratae*, for Fuerteventura. This classification is in disagreement with that of Castroviejo *et al.* (1985). Although Gran Canaria has a greater geographic distance from Lanzarote than from Fuerteventura, the representative specimen of the *G. atlantica* population found on the Gran Canaria island (Gatd-1) belongs to the Lanzarote subspecies *G. g. atlantica* (Figs. 5A and 5B),

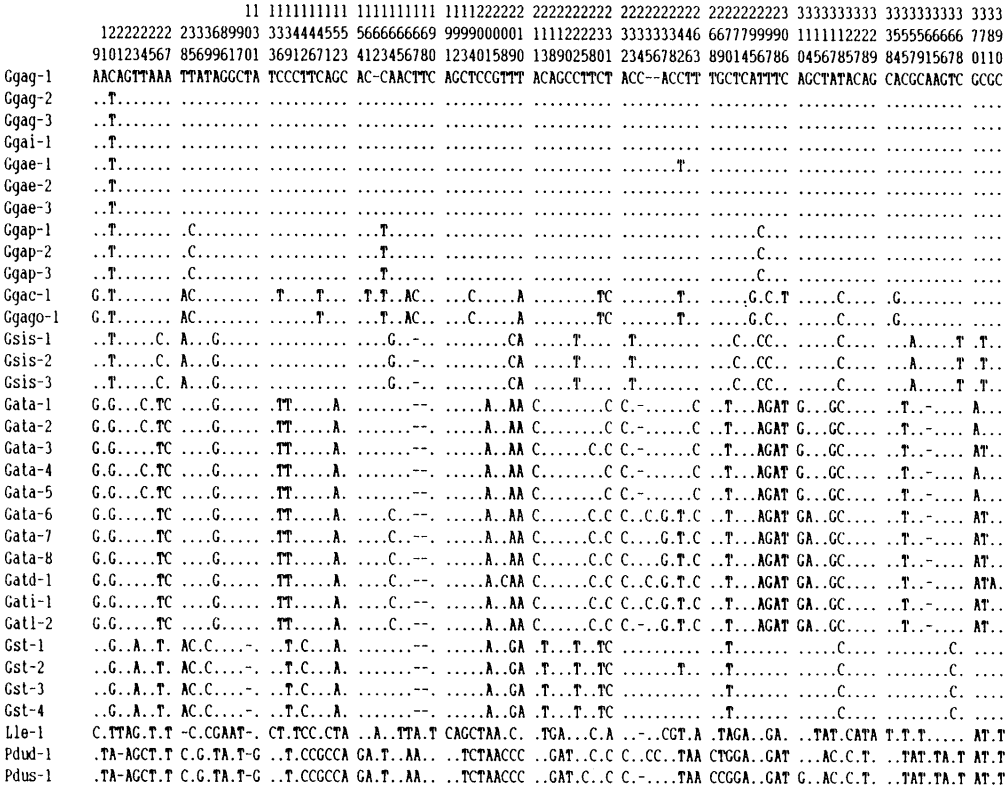

FIG. 4. 12S rRNA nucleotide differences among the 33 specimens studied. Site 9 corresponds with position 1100 in the published human reference sequence (Anderson *et al.,* 1991).

TABLE 2

Transitions (Ts), Transversions (Tv), and Ts/Tv Ratios within Subspecies and between Subspecies, Species, and Genera

	cyt*b*		
	Total sequence	Third position	12S rRNA
Within subspecies			
N	40	40	58
Ts ± SE	1.98 ± 0.95	1.48 ± 1.24	0.83 ± 0.99
Tv ± SE	0.13 ± 0.33	0.13 ± 0.33	0.00
Ts/Tv	15.23	11.38	
Between subspecies			
N	60	60	73
Ts ± SE	14.37 ± 4.20	10.92 ± 2.94	7.64 ± 4.48
Tv ± SE	1.72 ± 1.01	1.57 ± 0.95	1.23 ± 1.17
Ts/Tv	8.35	6.96	6.21
Between species			
N	252	252	305
Ts ± SE	33.76 ± 4.64	25.44 ± 4.74	20.18 ± 4.15
Tv ± SE	8.60 ± 2.52	8.25 ± 2.51	9.13 ± 2.94
Ts/Tv	3.93	3.08	2.21
Between genera			
N	83	83	92
Ts ± SE	35.33 ± 3.56	27.29 ± 3.36	38.15 ± 1.82
Tv ± SE	27.78 ± 5.94	20.20 ± 4.85	28.28 ± 2.10
Ts/Tv	1.27	1.35	1.35

with a mean divergence from these specimens of only 0.5%, whereas the mean distance from those of Fuerteventura is 4.6%. The colonization of Fuerteventura by *G. stehlini* seems to be more recent as the Fuerteventura representative (Gst-4) is identical to the Gst-3 specimen from Gran Canaria. Finally, the subspeciation proposed by Bischoff *et al.* (1989) for the outgroup species of this study, *P. dugesii,* for the populations from Madeira (*P. dugesii dugesii*) and Salvajes islands (*P. dugesii selvagensis*) is sound at the molecular level (Figs. 5A and 5B).

Sequence and Sample Availability

The nucleotide sequences in this paper are available from the GenBank/EMBL databases and are as fol-

TABLE 3

Mean Distances within and between Subspecies and between Species and Genera

	Cyt*b*		12S rRNA	
	N	*d* ± SE	*N*	*d* ± SE
Within subspecies	40	.006 ± .004	58	.002 ± .003
Between subspecies	60	.055 ± .018	73	.023 ± .015
Between species	252	.157 ± .024	305	.079 ± .018
Between genera	83	.244 ± .025	92	.193 ± .010

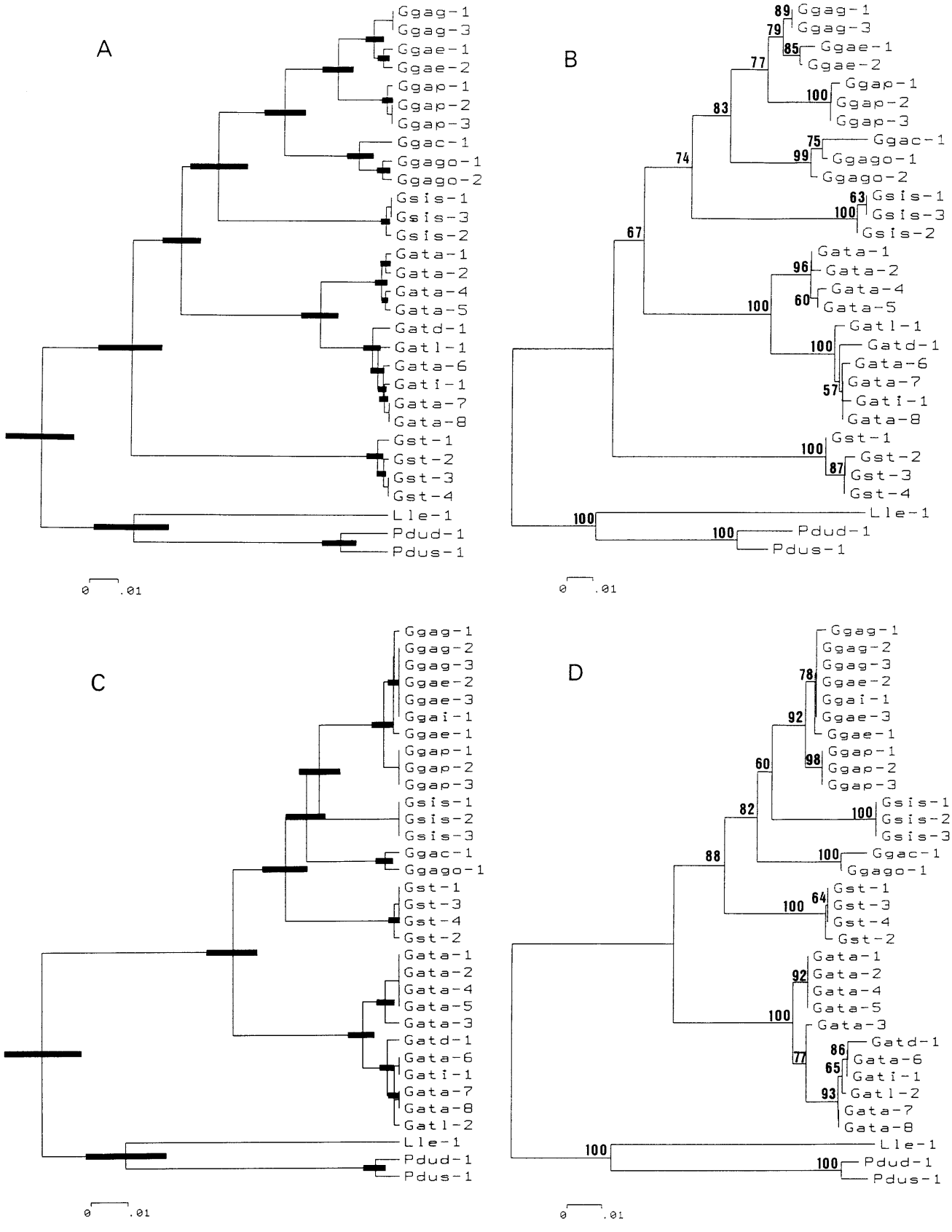

FIG. 5. Relationships among species and subspecies of lizard genus *Gallotia*, obtained by UPGMA (A, C) and neighbor joining methods (B, D), based on cyt*b* (A, B) and 12S rRNA (C, D) sequences. Numbers on trees indicate the percentage of bootstrapped trees supporting each node.

lows: *G. galloti* Z48034 and Z48038; *G. atlantica* Z48035 and Z48040; *G. stehlini* Z48036 and Z48039; *G. simonyi* Z49751 and Z49752; *P. dugesii* Z48037 and Z48041; and *L. lepida* Z48049 and Z48050 for cyt*b* and 12S rRNA, respectively. The collection material analyzed in this paper is at the Zoology Department of La Laguna University.

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