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Molecular phylogeny of the Canary Island lacertids (*Gallotia*): mitochondrial DNA restriction fragment divergence in relation to sequence divergence and geological time

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Abstract

Restriction fragment length polymorphisms of 6 base pair recognising endonucleases are used to reconstruct the phylogeny of the endemic Canary Island lacertid, *Gallotia*. The division into conventional species is upheld by this molecular analysis and the western Canary Island lizard (*G. galloti*) and eastern Canary Island lizard (*G. atlantica*) are hypothesized to be sister species. A more comprehensive study of the intraspecific relationships of *G. galloti*, based on nineteen restriction enzymes, indicates that there are distinct southern and northern lineages within this species. The phylogenetic analysis does not uphold the conventional subspecies, but suggests an alternative arrangement with one northern (La Palma, Tenerife) and one southern (Gomera, Hierro) subspecies. The inferred timing of molecular divergence of populations of *G. galloti*, based on RFLP analysis, is compatible with the geological timing for island origin and fossil data. Mantel tests show that mitochondrial RFLP divergence is correlated with mitrochondrial 12s rRNA and cytochrome oxidase I sequence divergence and highly correlated with mitochondrial cytochrome b sequence divergence.

Introduction

Earlier morphological studies of the systematics of the three main species of the endemic Canary Island lacertids of the genus *Gallotia* (Thorpe, 1985a, b; Thorpe, Watt & Baez, 1985) provided the impetus for a wide range of nucleic acid studies

(Thorpe et al., 1993a) which have suggested a colonization sequence for the western species. A previous study of restriction fragment length polymorphisms (RFLP) of mitochondrial DNA, based on two 4-base recognition restriction endonucleases, provided preliminary evidence on the relationships within the western species (*G. galloti*), but no evidence on the interspecific relationships (Thorpe et al., 1993b). This study, based primarily on 6 base recognition endonucleases, aims to investigate the phylogenetic relationships within the genus *Gallotia* and species *G. galloti* and compare RFLP divergence with nucleotide sequence divergence and geological times.

Materials and methods

Ten specimens were collected from each sample locality. For *G. galloti* these were on La Palma, Hierro, Gomera, north Tenerife and south Tenerife; for *G. stehlini* the sample locality was from the only island it inhabits, Gran Canaria; for *G. atlantica* the sample localities were on Lanzarote and from Arinaga, Gran Canaria (which may be an introduced population) (Fig. 1). The outgroup was *Lacerta viridis* from southern France.

The following seventeen restriction endonucleases were used in this study with the recognised base sequence given in brackets:- *Hind*III (A/AGCTT), *Kpn*I (GGTAC/C), *Asp*700 (GAANN/NNTTC), *Bam*HI (G/GATCC), *Cla*I (AT/CGAT), *Dra*I (TTT/AAA), *Eco*RI (G/AGCTT), *Ksp*I (CCGC/GG), *Pst*I (CTGCA/G), *Sac*I

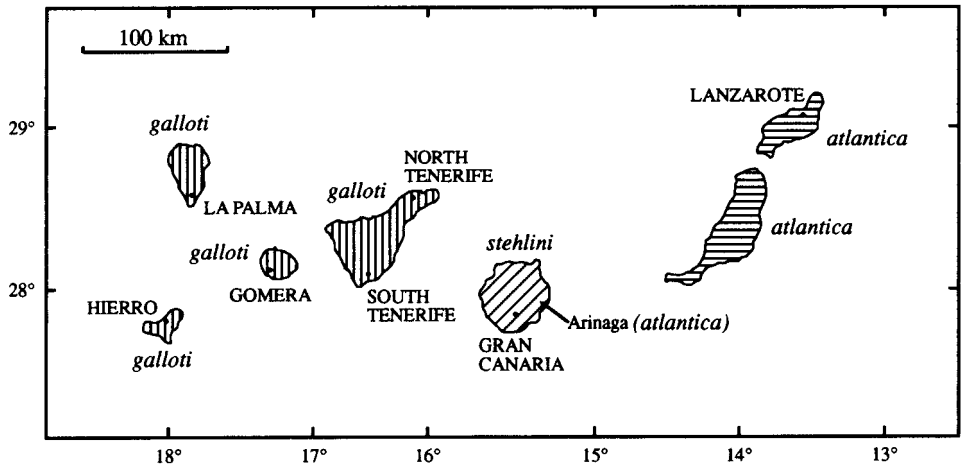

Fig. 1. Canary Island sample sites for *Gallotia*. *G. galloti* was sampled from La Palma, Hierro, Gomera and north and south Tenerife, *G. stehlini* was sampled from Gran Canaria and *G. atlantica* was sampled from Lanzarote and from a small (possibly introduced) population near Arinaga, in Gran Canaria. The distributions of these three main species are indicated by vertical hatching for *G. galloti*, horizontal hatching for *G. atlantica* and oblique hatching for *G. stehlini*.

(GAGCT/C), *Sal*I (G/TCGAC), *Sma*I (CCC/GGG), *Xho*I (C/TCGAG), *Apa*I (GGGCC/C), *Pvu*II (CAG/CTG), *Sty*I (C/CWWGG) and *Xba*I (T/CTAGA). The latter four enzymes were not used in the outgroup-rooted interspecific study. Mitochondrial DNA fragments were detected by either the hybridization technique described in Thorpe et al. (1993b), or directly by an antibody detection procedure given in the appendix. In addition, the '4-base' restriction enzymes listed in Thorpe et al. (1993b) were also used in some analyses.

The RFLP data was used to construct Fitch-Margoliash phylogenetic trees (see discussion in Swofford and Olsen, 1990) using the Phylip 3.3 package (Felsenstein, 1990) and bootstrapped Wagner trees. These trees gave the most consistent results across various sources of mtDNA data (McGregor, 1992). Outgroup rooted phylogenetic relationships within the genus were reconstructed using restriction fragment data from the first 13 endonucleases listed above. Phylogenetic relationships within the genus (without an outgroup) were reconstructed using restriction fragment data from all 17 endonucleases listed above. The data was also used to compute molecular divergence times and used in comparison with the nucleotide sequence divergence.

An intraspecific phylogeny for *G. galloti* was reconstructed, with a congener as an outgroup, using restriction fragment data from all 17 endonucleases listed above together with data from two previously studied 4-cut endonucleases (Thorpe et al., 1993b), and the inter-relationships within the northern lineage of *G. galloti* were reconstructed with data from these 19 enzymes together with a further four 4-cut enzymes listed in Thorpe et al. (1993b).

The genetic distances among populations within the genus obtained from RFLPs were correlated with those calculated from cytochrome b sequence divergence, cytochrome oxidase sequence divergence and 12s rRNA sequence divergence (Thorpe et al., 1993a) and the probability obtained using a Mantel test (Mantel, 1967; Manly, 1991; Thorpe, 1991) based on 10,000 randomizations.

Results

Restriction fragment information was available for eight specimens of *G. atlantica*, eight specimens of *G. stehlini* and twenty five specimens of *G. galloti* (between five and seven per locality). There was no heteroplasmy found and only one haplotype per locality.

The 13 endonucleases used to reconstruct the phylogeny of *Gallotia* (outgroup, *Lacerta viridis*) yielded 63 restriction fragments. The phylogeny (Fig. 2a) reaffirms the conventional species (Baez, 1987; Klemmer, 1976) and also indicates that *G. galloti* and *G. atlantica* are sister species, while geographically intermediate *G. stehlini* is less closely related. A mid-point rooted tree based on 79 restriction fragments from all 17 endonucleases also indicates *G. galloti* and *G. atlantica* as sister species (Fig. 2b). Both trees (Fig. 2a, b) show southern (Gomera, Hierro) and northern (north Tenerife, south Tenerife, La Palma) lineages within *G. galloti*, although some closely related populations are undifferentiated in the outgroup rooted tree (Fig. 2a).

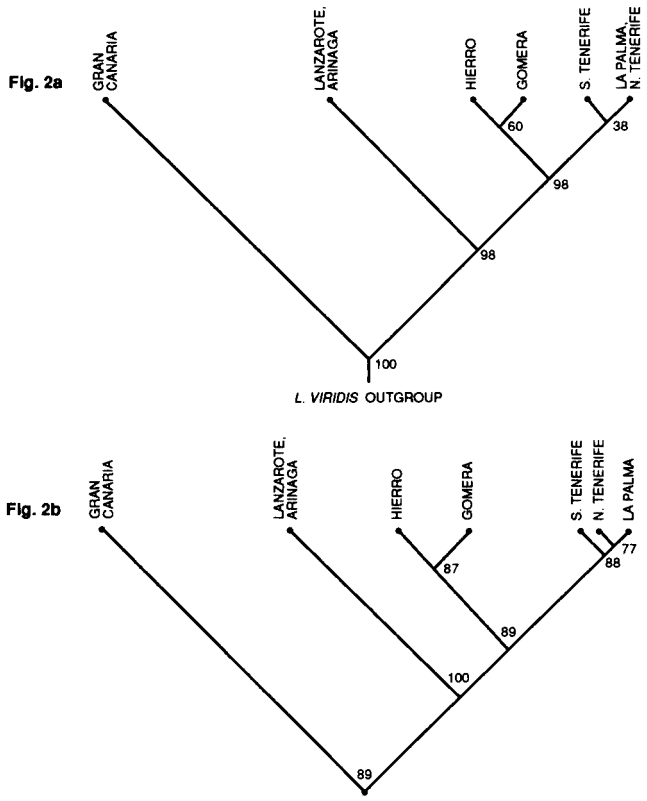

Fig. 2. Fitch-Margoliash phylogenetic trees of *Gallotia* samples. The bootstrapped Wagner tree had an identical topology so the bootstrap values are indicated at each node. Fig. 2a is based on restriction fragments from thirteen 6-cut endonucleases, outgroup rooted with *Lacerta viridis*, while Fig. 2b is based on seventeen 6-cut endonucleases. The main lineages represent the conventional species, *G. stehlini* (Gran Canaria), *G. atlantica* (Lanzarote, Arinaga) and *G. galloti* (Hierro, Gomera, La Palma, north and south Tenerife).

The evolutionary distances among populations, computed from this restriction fragment data for the 17 enzymes (Nei & Li, 1979; Nei, 1987) are given in Table 1. When these evolutionary distances among populations of *Gallotia* are interpreted in terms of divergence times, assuming the standard rate of 2% per million years (Wilson et al., 1985), then *stehlini* diverges from other lineages at ca 5.6 m.y.a., *atlantica* diverges from *galloti* at ca 3.4 m.y.a., the northern and southern intraspecific lineages of *galloti* diverge at ca 1.7 m.y.a., Hierro and Gomera diverge at ca 0.4 m.y.a. and La Palma diverges from north Tenerife at ca 0.3 m.y.a.

Restriction fragment data from all seventeen 6-cut endonucleases together with two (*Mva*I, *Hae*III) 4-cut endonucleases were used to construct an intraspecific phylogeny (Fig. 3a) for *G. galloti*, rooted with *G. stehlini* (with 156 fragments). The data differentiates closely related taxa and the tree indicates that *G. galloti* has

Table 1. Evolutionary distance among populations based on seventeen 6-base endonucleases. Sample codes are GC, *G. stehlini* from Gran Canaria; NT, ST, LP, GM, HI, *G. galloti* from north Tenerife, south Tenerife, La Palma, Gomera, Hierro; LA, AR, *G. atlantica* from Lanzarote and Arinaga.

	LA	AR	GC	LP	NT	ST	GM
AR	0.000						
GC	0.109	0.109					
LP	0.064	0.064	0.111				
NT	0.074	0.074	0.115	0.006			
ST	0.064	0.064	0.111	0.008	0.015		
GM	0.070	0.070	0.108	0.024	0.031	0.028	
HI	0.070	0.070	0.108	0.033	0.041	0.038	0.008

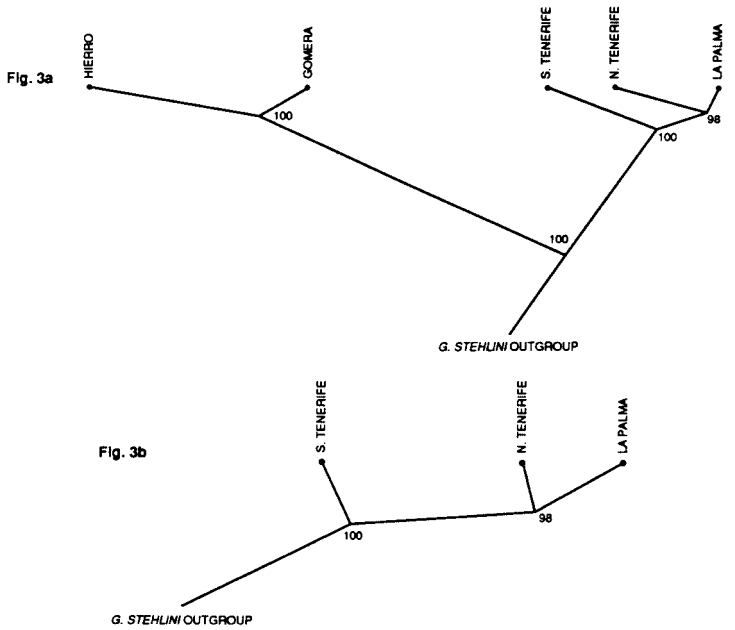

Fig. 3. (a) Fitch-Margoliash phylogenetic tree (without the assumption of equal branch lengths) of *G. galloti* samples outgroup rooted by *G. stehlini*, based on restriction fragments from seventeen 6-cut and two 4-cut endonucleases, showing southern and northern lineages. (b) Northern lineage based on restriction fragments from all seventeen 6-cut and all six 4-cut endonucleases giving the best estimate available from RFLPs of topography and branch length. The bootstrap values at nodes are derived as in Fig. 2.

southern (Hierro, Gomera) and northern (Tenerife, La Palma) lineages with north Tenerife and La Palma as sister groups. When the evolutionary distance among populations, based on these 19 endonucleases, are interpreted in terms of divergence times then the northern and southern lineages of *galloti* diverge at ca 1.7 m.y.a., Hierro and Gomera diverge at 0.6 m.y.a. and La Palma diverges from north Tenerife at 0.3 m.y.a.

The relationships within the northern lineage of *G. galloti* (Fig. 3b), are confirmed by a separate analysis based on the fragment data from these seventeen 6-cut enzymes pooled with the six 4-cut enzymes previously studied (Thorpe et al., 1993b) which surveys 206 fragments. The analysis shows the La Palma population to have diverged more from their common ancestor than the current north Tenerife population and indicates a divergence time as low as 0.2 m.y.a.

Discussion

While mtDNA is only maternally inherited, and may not be congruent with nuclear genome, the phylogenetic analyses of the mtDNA in this study support the conventional arrangement of the populations into three species, *G. galloti* (western), *G. stehlini* (central) and *G. atlantica* (eastern) (*G. simonyi* is not represented). The western *G. galloti* and eastern *G. atlantica* are sister species even though they are geographically separated by *G. stehlini* occupying the central island of Gran Canaria.

The existence of northern and southern lineages for *G. galloti* is indicated in all analyses in this study. This supports the view, initiated by the study of 4-cut endonucleases, that the six conventional subspecies (Baez, 1987; Klemmer, 1976; Martin, 1985) should be replaced by two subspecies reflecting these two intraspecific lineages (Thorpe et al., 1993b). That is, *Gallotia galloti galloti* for the northern lineage (Tenerife, its islets and La Palma) and *Gallotia galloti caesaris* for the southern lineage (Hierro and Gomera).

The analyses which differentiate among all samples of *G. galloti* (Figs. 2a, 3a) produce a topography that is congruent with those based solely on 4 cut endonucleases (Thorpe et al., 1993b) and other sources of nucleic acid data, e.g., mtDNA cytochrome b nucleotide sequences, cytochrome oxidase I nucleotide sequences, 12s rRNA nucleotide sequences and RAPD analysis of the nuclear genome (Thorpe et al., 1993a). This congruence argues for the sensitivity and reliability of RFLP data in phylogenetic analysis when a sufficient number of restriction enzymes are employed. Moreover, the reliability and utility of RFLPs is further illustrated by comparing the genetic divergence among populations in the genus based on seventeen restriction enzymes (Tab. 1) with those based on mtDNA nucleotide sequences (sequences in Thorpe et al., 1993a). There is a correlation of $r = 0.95$ between RFLP genetic distances and cytochrome b sequence genetic distances (Mantel test $P = 0.0002$), a correlation of $r = 0.86$ between RFLP genetic distances and cytochrome oxidase I sequence genetic distances (Mantel test $P = 0.0036$), and a correlation of $r = 0.89$ between RFLP genetic distances and 12s rRNA sequence genetic distances (Mantel test $P = 0.0047$). Consequently, the RFLPs, based on seventeen "6 cut" restriction enzymes, give comparable information to sequence data, particularly that of cytochrome b sequence.

The geology of the Canary Islands is such that each western island arose volcanically from the sea floor without being in contact with one another, or the African mainland (Anguita & Hernan, 1975; Carrecedo, 1979), and are separated

by deep sea channels. Consequently, one may interpret the intraspecific phylogeny of the western lizard in terms of colonization sequence (Thorpe et al., 1993a). When the intraspecific *G. galloti* tree (Fig. 3a) is interpreted in terms of colonization sequence using the topography and geographic distance procedure in Thorpe et al. (1993a), then one gets on origin in Tenerife and two separate westward expansions to the younger island to the west. In the north, La Palma is colonized from north Tenerife and in the south Hierro is colonized, via Gomera, from south Tenerife. The alternative procedure for reconstructing colonization sequence suggested by Thorpe et al. (1993a), which is based on branch lengths and topography, indicates the same colonization sequence when the northern lineage relationships are based on the maximum available data (i.e., fragments from seventeen 6-cut enzymes plus 6 4-cut enzymes giving the tree in Fig. 3b).

The geological times of island origin may vary according to author (Ancochea et al., 1990; Abdel-Monem, Watkins & Gast, 1971, 1972; Anguita & Hernan, 1975, 1986; Carrecedo, 1979). Anguita & Hernan (1975) give times for all the pertinent islands and therefore their dates are followed here, but alternative times by other authors do not contradict the following conclusions.

Angutia and Hernan (1975) suggest that Tenerife arose 15.7 m.y.a.; Gomera arose 12 m.y.a.; La Palma arose 1.6 m.y.a.; and Hierro arose 0.75 m.y.a. The molecular (DNA) clock times are compatible with these geological times in that the 'DNA clock' does not suggest that an island is colonized before its time of geological origin (Thorpe et al., 1993a). If the molecular clock times were greater than the times for geological origin the clock concept would have to be rejected (assuming the geological dates are accurate). The RFLP data suggests an origin on Tenerife ca 3.4 m.y.a., a colonization of Gomera at 1.7 m.y.a., a colonization of Hierro at 0.4–0.6 m.y.a., and a colonization of La Palma at ca 0.3–0.2 m.y.a. Moreover, times for early fossils of a range of species suggest these volcanic islands were habitable at, or before, the colonization dates suggested by the molecular clock (McGregor, 1992).

Nevertheless, a comparison of relative rates (Beverley and Wilson, 1984; Desalle and Templeton, 1988) within various lineages (Tab. 2) indicates that the "clock" is not entirely constant and that founder effects are implicated, particularly in the colonization of Hierro and to some extent in the divergence of *G. stehlini*. Also, the geological times only give an upper bound to the possible colonization time, and not a lower bound. Consequently, they cannot be used to give a narrow time zone against which to precisely, and independently, judge the rate of this RFLP DNA divergence. The existence of any fossil will give an indication of when the island was habitable, but one needs species specific fossils to give a lower bound. Unfortunately, when pertinent species specific fossils are available, as in *G. galloti* on Gomera, the dates are not precise enough to be useful. For example, fossil finds on Gomera are only listed as Pleistocene. If fossils, with precise dates, were available for the younger islands of La Palma and Hierro, this would be particularly useful in narrowing the possible 'window' for colonization and thereby giving an independent time with which to calibrate the molecular clock.

Table 2. Relative rate test on RFLP genetic distances taken from McGregor (1992). Sample codes are as for Table 1.

Outgroup	Ingroup		Rates		
	A	B	A	B	A/B
LA	GC	LP	0.079	0.033	2.4
		NT	0.076	0.039	1.9
		ST	0.079	0.033	2.4
		GM	0.075	0.034	2.2
		HI	0.075	0.034	2.2
GC	LA	LP	0.031	0.033	1.0
		NT	0.035	0.039	1.1
		ST	0.031	0.033	1.0
		GM	0.035	0.034	1.0
		HI	0.035	0.034	1.0
GC/LA	LP	NT	0.000	0.013	–
		ST	0.008	0.008	1.0
		GM	0.023	0.025	1.1
		HI	0.032	0.034	1.1
GC/LA/LP	NT	ST	0.028	0.030	1.1
		GM	0.044	0.050	1.1
		HI	0.054	0.069	1.3
GC/LA/LP/NT	ST	GM	0.039	0.010	2.1
		HI	0.049	0.103	2.1
GC/LA/LP/NT/ST	GM	HI	0.002	0.044	22.0

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Appendix

Antibody detection of mitochondrial DNA

The method was adapted from a kit which was originally designed as a probing system. It involved sulphonating the cytosine residues in the probe DNA and, after

hybridization, detection of probe with an anti-sulphonated cytosine monoclonal antibody and a secondary antibody conjugated with alkaline phosphatase. Altering the protocol by sulphonating digested mtDNA which had been blotted onto a membrane, and then detecting the sulphonated fragments with the antibodies improved results. The technique works well for Southern blotted DNA i.e. for six-base recognition fragments enzymes, but with electroblotted DNA, it has less utility.

The procedure was further improved by substituting the alkaline phosphatase conjugate with a horseradish peroxidase conjugate and detecting this with the luminol/iodophenol/hydrogen peroxide light emitting system now sold by Amersham as part of their ECL hybridisation kit. In this system Luminol is broken down to 3-aminophalate by the action of peroxidase and hydrogen peroxide with Iodophenol acting as an enhancer of light production. The system has since been superseded in sensitivity by alkaline phosphatase/dioxetane based light emission systems e.g. Tropix's AMPPD.

The peroxidase luminescence system has a high sensitivity and produces a permanent record on film. The procedure is as follows.

1. Membrane Sulphonation

This is carried out by bathing the membrane overnight in a solution of two chemicals – O-methoxylamine (1–13 dilution of a 2 M stock) and sodium metabisulphite (1–3 dilution of a 2 M stock). Following this the membrane is washed in 2 × SSC (Saline Sodium Citrate) for 10 minutes.

2. Primary antibody

After washing, the membrane is blocked for 1 hour in Tris-buffered saline (50 mM Tris pH 7.5, 200 mM NaCl (TBS)), 0.05% Tween-20, 1% BSA. Antibody is added at 1 : 5000 dilution in TBST with 3.5 mg/ml Heparin to reduce background. The membrane is incubated, with shaking, for 1 hour. The membrane is washed in TBS/0.05% Tween-20 (TBST) for 3 × 15 minutes.

3. Secondary antibody

The conjugate (horseradish peroxidase (HRP) or alkaline phosphatase (AP)) is added to the membrane in TBST at 1 : 1000 dilution and incubated, with shaking, for 1 hour. For HRP washing is for 3 × 15 minutes in TBS. For AP washing is 2 × 15 minutes in TBS followed by 15 minutes in 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl and 50 mm MgCl2 (substrate buffer).

4. Luminescent development (HRP)

Tris-buffered Luminol and Tris-buffered Iodophenol/Hydrogen peroxide from the ECL kit (Amersham) are mixed 1 : 1 and poured over the membrane. After 1 minute the membrane is blotted gently on filter paper, placed in a bag and exposed to film for 1 minute. The film is developed and another put in its place for up to 1 hour.

5. Colour development (AP)

With an alkaline phosphatase conjugated secondary antibody the procedure was identical to the HRP protocol up until the final wash prior to development. The development chemicals 0.33 mg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) in 100% Dimethylformamide and 0.16 mg/ml nitroblue tetrazolium (NBT) in 70% Dimethylformamide are made up in substrate buffer. This is then poured over the membrane which is then incubated at 37° C in the dark for about 30 minutes. BCIP/NBT are precipitated as blue/purple dye at the site of the alkaline phosphatase. The reaction is stopped by washing the membrane with T.E. buffer to remove the enzyme substrates.