

Albumin evolution in West Indian frogs of the genus *Eleutherodactylus* (Leptodactylidae): Caribbean biogeography and a calibration of the albumin immunological clock

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(With 3 figures in the text)

Antisera to serum albumins from five West Indian species of the frog genus *Eleutherodactylus* were prepared, and the reciprocal immunological distances (IDs) obtained were used to provide a time frame for the evolution of this group in the West Indies. One-way IDs were obtained to 25 additional species within the genus, with emphasis on those from the West Indies. These immunological data support both a recent classification of *Eleutherodactylus* based on an analysis of slow-evolving allozyme loci, and the monophyly of the 17 native Jamaican species as indicated by a more comprehensive electrophoretic study. This is in contrast to the results of morphological studies supporting multiple invasions of Jamaica by *Eleutherodactylus*. Within the subgenus *Euhyas*, IDs ranged from 6–27 between Jamaican species, whereas between species on different islands the range was 29–67. The subgenus *Syrrhophus* in southern North America was found to be the sister group to the subgenus *Euhyas*, a western Caribbean clade. *Pelorius*, a subgenus restricted to Hispaniola, was found to be the sister group of the subgenus *Eleutherodactylus* in the West Indies. The largest IDs obtained for West Indian species were those between the two major groups, the subgenera *Eleutherodactylus* and *Euhyas*.

The albumin immunological clock for *Eleutherodactylus* was calibrated with three events in the geologic history of the Caribbean: the breakup of the proto-Antilles (65–75 million years before present [mybp]), the emergence of Jamaica (20–30 mybp), and the uplift of the Blue Mountains in Jamaica (5–10 mybp). Immunological distances corresponding to those events yield a calibration of 1 ID = 0.60 million years (my), the same as that previously obtained for other groups of amphibians and thus supports the use of albumin immunological distance as a molecular chronometer in the genus *Eleutherodactylus*.

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Introduction

Time is an important dimension of evolutionary history, yet for most species there are no fossils to provide this information. However, some molecules evolve at a relatively constant rate and can be used as an evolutionary chronometer that, once calibrated, can be used to date phylogenetic divergence events. This is particularly useful in groups that have a poor fossil record, such as amphibians. A time frame is essential in biogeographic analysis because a major objective is to relate evolutionary history to earth history. Over the past two decades, molecular clocks have provided that time frame and enhanced our understanding of biogeography in many groups (Sarich & Wilson, 1967; Wilson, Carlson & White, 1977; Thorpe, 1982; Maxson & Roberts, 1984).

Albumin is one of the most widely used molecules for dating evolutionary divergences (e.g. Wilson *et al.*, 1977; Collier & O'Brien, 1985; Cadle, 1988; Maxson & Maxson, 1990). The number of amino acid differences that have accumulated in this molecule since the divergence of two species is estimated indirectly by the immunological technique of micro-complement fixation (MC'F) (Champion *et al.*, 1974; Maxson & Maxson, 1986). The albumin clock initially was calibrated using mammalian fossil data (Wilson *et al.*, 1977; Carlson, Wilson & Maxson, 1978), and this calibration (1 ID=0.55-0.60 my) has been supported in other vertebrate groups, including amphibians (Maxson, Sarich & Wilson, 1975; Maxson, 1984, 1991).

In this study, we use albumin immunological data in the frog genus *Eleutherodactylus* to address current issues in Caribbean biogeography. The relatively poor overwater dispersal ability of this group makes it a potential indicator of plate tectonic movement (Hedges, 1989a). This feature, coupled with the timing of several events in the geologic history of the Caribbean, offers an opportunity to obtain an independent calibration of the albumin clock.

Caribbean biogeography

Currently, two competing theories of Caribbean biogeography are being debated. The dispersal theory suggests that the West Indian biota is the product of overwater transport from the mainland throughout the Cenozoic (Matthew, 1918; Simpson, 1956). The vicariance theory, on the other hand, argues that the relationships of the West Indian biota are the reflection of the geological relationships (plate tectonic history) of the islands (Rosen, 1976, 1985). Positions in which both of these mechanisms are seen to be important also have been taken (MacFadden, 1980, 1981; Hedges, 1982, 1989a; Buskirk, 1985).

Recent hypotheses for the complex geologic history of the Caribbean suggest considerable movement of the islands during the Cenozoic, although the degree of movement, relative positions, and conditions of these islands are still points of contention (Burke, 1988; Donnelly, 1988; Perfit & Williams, 1989). Amber fossils of insects (Wilson, 1985) and vertebrates (Rieppel, 1980; Böhme, 1984; Poinar, 1987; Poinar & Cannatella, 1987) from Hispaniola establish the presence of these groups at a relatively early date (perhaps as far back as 40 mybp; Lambert, Frye & Poinar, 1985), although terrestrial fossils are virtually unknown from other time periods in the Tertiary. In addition to the lack of fossils for dating divergence times, accurate phylogenetic reconstructions are not available for most West Indian plant and animal groups. Therefore, the relative role of these two mechanisms, dispersal and vicariance, remains unresolved.

Eleutherodactylus

With over 450 described species, *Eleutherodactylus* has more members than any other vertebrate

genus. They are direct-developing neotropical frogs that lay eggs on land (one species is ovoviviparous). In an effort to establish a phylogenetic framework for the genus, several large clades recently have been defined on the basis of morphology and allozymes (Lynch, 1986; Hedges, 1989a). These five groups, recognized as subgenera by Hedges, are: *Craugastor* (68 spp. in Middle America), *Eleutherodactylus* (275 spp., primarily in South America but with a subclade [*auriculatus* section] in the West Indies), *Euhyas* (78 spp. on islands in the West Indies), *Pelorius* (6 spp. in Hispaniola), and *Syrrhophus* (24 spp. in southern North America). The phylogeny of the South American species is poorly known and thus the subgenus *Eleutherodactylus* is likely a paraphyletic taxon. The remaining four subgenera are believed to be monophyletic (Lynch, 1986; Hedges, 1989a).

There are 17 native species of Jamaican *Eleutherodactylus* placed in five species groups (Hedges, 1989a): *cundalli* (3 spp.), *gossei* (5 spp.), *jamaicensis* (1 sp.), *luteolus* (3 spp.), and *nubicola* (5 spp.). One additional species (*johnstonei*) is known to have been introduced, and another (*planirostris*) is believed to have been introduced to Jamaica. These five native groups are supported by allozyme and chromosome data (Hedges, 1989a, b). Although previous classifications based on morphology have suggested a multiple origin for the native Jamaican species, the allozyme data support a single radiation.

Materials and methods

The species and localities sampled are listed in the Appendix. Voucher specimens for most species are in the United States National Museum, Smithsonian Institution (USNM). Animals were killed by cryothermy (Kennedy & Brockman, 1965). This method quickly anaesthetizes tropical amphibians and the tissues are not exposed to chemicals (e.g. ethyl ether or tricaine methanesulfonate). Blood was taken from the aorta with heparinized capillary tubes. If the animals were processed in the field, blood was mixed with an approximately equal volume of the tissue preservative phenoxyethanol (Nakanishi *et al.*, 1969) prepared as PPS (Gorman, Wilson & Nakanishi, 1971). Upon return to the laboratory, this was refrigerated until the sample was centrifuged to separate the plasma from the red blood cells (RBCs). If the frogs were processed in the laboratory, the fresh blood sample (no PPS) was immediately centrifuged. In both cases, the plasma and RBCs were separated and stored at -70°C . For most species, plasma samples of more than one individual from the same population were pooled to use in the production of antibodies or as antigens.

Albumin from 5 species of *Eleutherodactylus* (*gossei*, *inoptatus*, *montanus*, *nubicola* and *planirostris* II [from Jamaica]) was purified from plasma samples using polyacrylamide disk gel electrophoresis (7% gels) modified from the method of Davis (1964) and the albumin band visualized by fluorescence (Hartman & Udenfriend, 1969). In all cases, only a single band fluoresced. This band was cut from the gels and placed in isotris buffer (Champion *et al.*, 1974) to elute the albumin. Each sample purified gave a single precipitation arc when tested against an antiserum to *E. coqui* albumin (obtained from Linda R. Maxson, Penn State University, University Park, Pennsylvania) in a Ouchterlony double diffusion test. Antisera to these purified albumins were prepared in female New Zealand white rabbits following the method of Maxson, Highton & Wake (1979) except that the first marginal ear vein injection was given 4 weeks (rather than 3) after the second intradermal injection. The antisera to *montanus*, *nubicola* and *planirostris* II each were prepared in 2 rabbits, while those to *gossei* and *inoptatus* were prepared in 3 rabbits. Individual rabbit antisera each were pooled in inverse proportion to their MC_F titres and all experiments were done with these pooled antisera. The results of MC_F tests using purified albumin eluent as the antigen gave the same results as those in which whole plasma was used.

Experiments were performed as in Maxson & Maxson (1990) and run for 20 h at 2°C . Most antigen sources were plasma in isotris buffer; *planirostris* I (from Cuba), *unistrigatus* and *marnockii* antigens were muscle in PPS, diluted with isotris. At least 2 measures (repeatable within 2 ID units) were obtained for each ID, except

as noted in Table II. For the albumin molecule, one ID unit is equal to approximately one amino acid difference between the 2 albumins compared (Wilson *et al.*, 1977; Benjamin *et al.*, 1984; Maxson & Maxson, 1986).

Phylogenetic trees were constructed using the neighbour-joining method of Saitou & Nei (1987) and a modification of the distance Wagner procedure (Maxson *et al.*, 1979). The Cronin & Sarich (1975) method of correction for non-reciprocity was employed to investigate the relative contribution of individual antisera to the non-reciprocity found in the data set; however, trees were constructed from uncorrected data.

Results

The MC'F titres (at 75% complement fixation) of the five antisera produced were: *gossei*, 7900 (slope 350); *inoptatus*, 3200 (slope 300); *montanus*, 3400 (slope 350); *nubicola*, 4100 (slope 250); and *planirostris*, 8500 (slope 300). Data for reciprocal tests and unidirectional tests for all five antibodies are shown in Table I. The percentage standard deviation from reciprocity (Maxson & Wilson, 1975) is 8.6, a value typical of anuran studies (Maxson, 1984). Examining the Cronin & Sarich (1975) correction factors (Table I), no individual antibody appears to contribute the majority of deviation from reciprocity, although the *nubicola* and *inoptatus* antisera seem to underestimate values, while the *montanus* antiserum overestimates IDs. The data are highly metric, with only four of 80 possible three-way comparisons (5%) violating the triangle inequality (Farris, 1981).

The phylogenetic trees constructed from reciprocal data by the neighbour-joining and the modified distance Wagner methods have the same branching pattern and nearly identical branch lengths, therefore only the modified distance Wagner tree is shown (Fig. 1). The three representatives of the subgenus *Euhyas* form a group, with the two Jamaican taxa as sister species. The representative of the subgenus *Pelorius*, *inoptatus*, clusters with *montanus* (subgenus *Eleutherodactylus*) and both have a similar mean ID (108.5 and 116.7, respectively) from the *Euhyas* species. The branch lengths indicate that albumin evolution in the subgenus *Euhyas* may be slightly slowed relative to *Eleutherodactylus* and *Pelorius*. A relative rate test (see Baverstock *et al.*, 1989) was not employed because an antibody to the only appropriate outgroup, a representative of

TABLE I
Matrix of reciprocal albumin immunological distances between the five antisera to West Indian *Eleutherodactylus* used in this study, and one-way distances to a mainland species *E. marnockii*. The correction factor (CF) for each antibody is also shown

	Antibodies				
	GOS	NUB	PLN	MON	INP
CF	0.98	1.13	0.92	0.89	1.12
Antigens					
<i>E. gossei</i> (GOS)	0	22	45	128	106
<i>E. nubicola</i> (NUB)	25	0	45	133	102
<i>E. planirostris</i> II (PLN)	42	32	0	105	104
<i>E. montanus</i> (MON)	119	101	114	0	71
<i>E. inoptatus</i> (INP)	121	115	103	88	0
<i>E. marnockii</i>	62	55	68	87	91

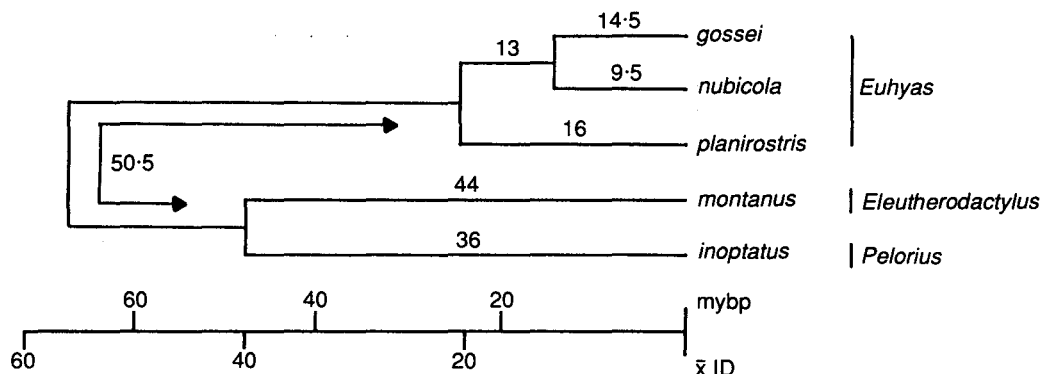


FIG. 1. Phylogenetic tree of five species (representing three subgenera) of *Eleutherodactylus* constructed using a modified distance Wagner method. Branch lengths are indicated above each branch. Approximate times of divergence are indicated based upon the standard calibration of 100 ID = 60 my. The percentage standard deviation (Fitch & Margoliash, 1967) of tree values from input values is 0.98; the percentage standard error (Prager & Wilson, 1976) is 0.64.

the subgenus *Craugastor*, was not available. However, rate tests at the distances involved (approximately 150 ID) are of dubious value due to the technical limitations of MC'F at distances that large (Maxson & Maxson, 1986).

While data from unidirectional tests may be less reliable as estimators of amino acid sequence difference than the averages of reciprocal values, they can be used to classify species into groups (Maxson & Wilson, 1975; Shochat & Dessauer, 1981). The mean ID from the three *Euhyas* antisera to *marnockii*, a member of the subgenus *Syrrhophus*, is 61.7 ± 3.8 ($\bar{x} \pm S.E.$), while the IDs measured from *montanus* and *inoptatus* are higher (Table I). These data place *marnockii* within the range of IDs found in the genus *Eleutherodactylus* and support the classification proposed by Hedges (1989a) in which *Syrrhophus* was lowered to subgeneric status within *Eleutherodactylus*, as well as strongly suggesting that *Syrrhophus* is the sister group to *Euhyas*.

The results of unidirectional tests of the three *Euhyas* antibodies to 25 additional species are shown in Table II. All of the native Jamaican species show the lowest IDs to the Jamaican antisera (*gosei* and *nubicola*). The average ID between the *gosei* and *nubicola* species groups is 21.8 ± 1.0 . In contrast, the average ID of the *planirostris* antiserum to all native Jamaican species (the *luteolus* series) is 36.7 ± 1.6 . The ID between the antibody against *planirostris* II (a population in Jamaica, believed to be introduced from Cuba) and *planirostris* I (a native Cuban population) is two, which is within the level of variation found within a single species (Highton, Maha & Maxson, 1989) and is not significantly different from zero when experimental error (± 2 ID; Maxson & Maxson, 1979) is considered. The average ID of Hispaniolan species of the subgenus *Euhyas* (*bakeri*, *pictissimus* and *jugans*) from the *luteolus* series (*gosei* and *nubicola*) antisera (46 ± 4.8) is approximately the same as from the *planirostris* antiserum (49.7 ± 6.4).

Within the subgenus *Eleutherodactylus*, all members of the *auriculatus* section have IDs to the *Euhyas* antisera of greater than 73 (91.8 ± 3.6). One mainland species examined, *unistrigatus* from Ecuador, falls within the range of values obtained for *auriculatus* section species with an average ID of 91.5 ± 0.5 to *Euhyas*. However, the other mainland species examined, *binotatus* from Brasil, shows the largest ID in this study (162 to the *nubicola* antiserum). Because only one ID was obtained for this species (due to the small amount of the sample) and because it is so much higher

TABLE II

Immunological distances of 28 species of *Eleutherodactylus* to antisera from three species representing the subgenus *Euhyas* (indicated by an asterisk). *Eleutherodactylus planirostris* II is from a population in Jamaica (believed to be introduced from Cuba); *planirostris* I is from La Habana, Cuba. Classification follows Hedges (1989a)

	Antisera		
	(Jamaica)		(Cuba)
	GOS	NUB	PLN
Antigens			
Subgenus <i>Euhyas</i>			
<i>luteolus</i> series (Jamaica)			
<i>luteolus</i> group			
<i>grabhami</i>	22	16	32
<i>luteolus</i>	23	19	38
<i>sisyphodemus</i>	— ^a	(18) ^b	—
<i>gossei</i> group			
<i>fuscus</i>	11	23	31
* <i>gossei</i>	0	22	45
<i>junori</i>	6	19	48
<i>pantoni</i>	12	23	32
<i>pentasyringos</i>	11	27	34
<i>cundalli</i> group			
<i>cavernicola</i>	13	12	31
<i>cundalli</i>	13	14	29
<i>glaucoreius</i>	11	13	34
<i>jamaicensis</i> group			
<i>jamaicensis</i>	14	8	31
<i>nubicola</i> group			
<i>alticola</i>	18	10	38
<i>andrewsi</i>	20	15	42
<i>griphus</i>	19	12	41
* <i>nubicola</i>	25	0	45
<i>orcutti</i>	—	13	—
<i>bakeri</i> series (Hispaniola)			
<i>bakeri</i>	51	40	54
<i>pictissimus</i> series (Hispaniola)			
<i>pictissimus</i>	33	42	37
unassigned to series			
<i>jugans</i> (Hispaniola)	67	43	58
* <i>planirostris</i> II (Cuba)	42	32	0
<i>planirostris</i> I (Cuba)	—	—	2
Subgenus <i>Eleutherodactylus</i>			
<i>auriculatus</i> section (West Indies)			
<i>martinicensis</i> series (PR and LA)			
<i>cooki</i>	103	80	102
<i>coqui</i>	119	88	99
<i>johnstonei</i>	80	74	89
<i>richmondi</i>	93	91	84
unassigned to section			
<i>binotatus</i> group			
<i>binotatus</i> (Brasil)	—	(162)	—
<i>unistrigatus</i> group			
<i>unistrigatus</i> (Ecuador)	—	91	92
Subgenus <i>Craugastor</i> (Middle America)			
<i>bransfordii</i>	—	> 150	—

^a Reaction was not performed

^b Only one ID estimate was obtained

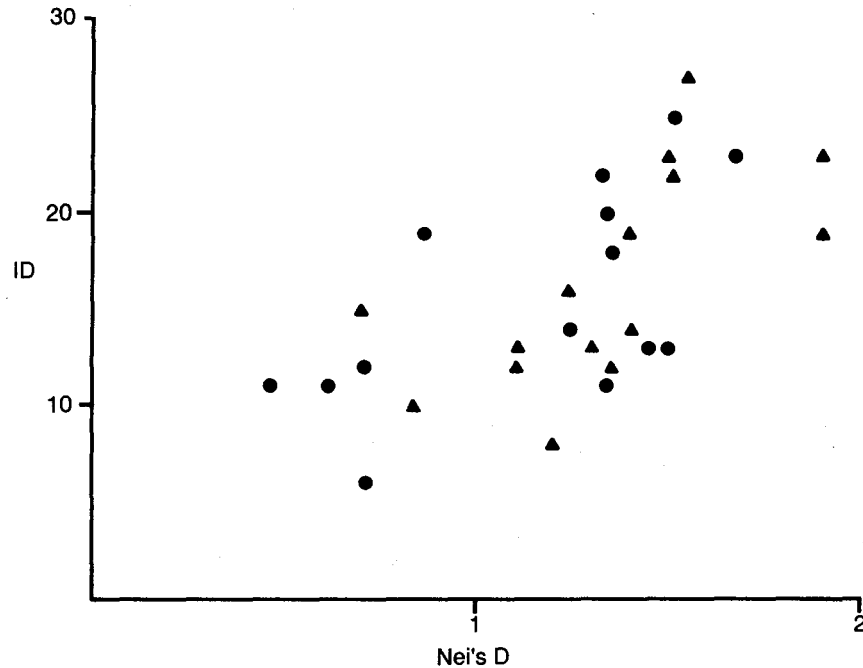


FIG. 2. Relationship between albumin immunological distance (this study) and Nei's genetic distance (Hedges, 1989b). Circles (●) = *E. gossei* antiserum ($r=0.62$, slope = 11); triangles (▲) = *E. nubicola* antiserum ($r=0.65$, slope = 12).

than those for the other *Eleutherodactylus*, this species will not be considered further in the analyses involving the subgenus *Eleutherodactylus*. The ID for *Eleutherodactylus bransfordii*, representing the subgenus *Craugastor* (Lynch, 1986; Hedges, 1989a) falls outside (ID > 150) all values obtained, except for *binotatus*.

The relationship between ID and Nei's (1978) genetic distance (D) calculated from allozyme data (Hedges, 1989b) is shown in Fig. 2. Product-moment correlation coefficients (r) for these data ($r=0.62$, $n=14$, for distances between *gossei* and other native Jamaican species; $r=0.65$, $n=15$, for *nubicola*) are slightly lower than those reported for other amphibian groups (Wyles & Gorman, 1980; Hedges, 1986). Only genetic distances below two are used in these calculations because above this level there is little correlation (Maxson & Maxson, 1979; Hedges, 1986). The statistical significance of these correlations could not be assessed because reciprocal distances were not available for all possible species pairs, a requirement of the Mantel test (Mantel, 1967; as applied by Schnell, Watt & Douglas, 1985).

The slopes of the regression lines, adjusted to pass through the origin, were lower ($m=11$ for the *gossei* distances, $m=12$ for *nubicola*) than any that have been reported for vertebrates (Wyles & Gorman, 1980). This is at least partly due to the fact that the Nei's D values were calculated from data collected by the method of sequential electrophoresis (Coyne, 1982), which uses additional electrophoretic conditions to detect more differences between species than are found with conventional electrophoresis, thus increasing the genetic distance between them. This relationship was predicted by Maxson & Maxson (1979).

Discussion

Higher-level relationships

Representatives from all five subgenera of *Eleutherodactylus* were examined in this study and thus it is possible to obtain an initial estimate of their relationships. The five antisera used represent the three subgenera that occur in the West Indies, although three are against species in the subgenus *Euhyas*. In addition, important information can be gained from one-way distances, particularly when they are to multiple species in a group, and when some phylogenetic structure already is known (e.g. Hedges, 1989a). The finding that the subgenera *Euhyas* and *Syrrhophus* apparently are sister groups has at least some support from morphology. Both groups contain a relatively high proportion of species with distinct glandular areas. Also, they are predominantly ground-dwelling species that occur in geographically adjacent regions: southern North America (*Syrrhophus*) and the western Caribbean (*Euhyas*). Hedges (1989a) suggested that *Syrrhophus* was derived by dispersal from Cuban *Euhyas*.

The subgenus *Pelorius* consists of six species of large frogs found exclusively on Hispaniola (Hedges & Thomas, 1987; Hedges, 1989a). The albumin data indicate that this group is more closely related to the members of the subgenus *Eleutherodactylus* than to *Euhyas*, and may have been derived from the *Eleutherodactylus* lineage on Hispaniola.

The subgenus *Craugastor* appears to be the most divergent of the five subgenera within the genus *Eleutherodactylus*. However, this is based upon a distance to only one representative without benefit of a reciprocal distance so confirmation of this must await additional data.

Jamaican Eleutherodactylus

The albumin immunological data provide further support for the monophyly of native Jamaican *Eleutherodactylus*. This is in agreement with allozyme studies (Hedges, 1989a, b) but is in contrast with all classifications based on morphology (Dunn, 1926; Goin, 1954; Schwartz & Fowler, 1973; Crombie, 1977; Schwartz, 1985; Joglar, 1989). Those studies have placed the Jamaican species in two or more groups and have suggested a multiple origin for these species. The allozyme and immunological data, however, offer a simpler biogeographic hypothesis: one colonization event with subsequent adaptive radiation. The morphological similarity of some Jamaican species with species on other islands thus appears to be morphological convergence as a result of similar ecologies (see Hedges, 1989a, b).

Details of relationships of the 17 native Jamaican *Eleutherodactylus* could not be addressed with antisera to only two of the species. Allozyme and chromosome data have been used to address that question elsewhere (Hedges, 1989b). Instead, the one-way IDs (Table II) between the *gossei* and *nubicola* antisera allow species groups defined by those other data to be tested. Low IDs between *gossei* and the four other *gossei* group species (6–12) compared with distances between *E. gossei* and *nubicola* group species (18–25) support the recognition of the *gossei* group. Likewise, distances between *nubicola* and the four other *nubicola* group species (10–15) compared with distances between *nubicola* and the *gossei* group species (19–27) provide support for the monophyly of the *nubicola* group. The three *luteolus* group species had relatively high (16–23) distances to both antisera which is in agreement with (but does not necessarily support) their placement in a separate group. The single species in the *jamaicensis* group and the three members of the *cundalli* group all had relatively low IDs (8–14) to both antisera. Little information regarding the relationships of the

species groups can be gleaned from the albumin data alone. A detailed discussion of the relationships of Jamaican *Eleutherodactylus*, based on a synthesis of allozyme, chromosome and albumin data, is given in Hedges (1989b).

Caribbean biogeography and the albumin immunological clock

The phylogenetic framework for this study is based primarily on an analysis of slow-evolving allozyme loci in West Indian *Eleutherodactylus* using sequential electrophoresis (Hedges, 1989a). In that study, two major West Indian groups were defined. The subgenus *Euhyas* is a western Caribbean clade that includes 27 of the 33 Cuban species, all 17 native Jamaican species, and 33 of the 55 Hispaniolan species (mostly South Island = areas south of the Cul de Sac/Valle de Neiba). The other major group is the *auriculatus* section of the subgenus *Eleutherodactylus*, an eastern Caribbean clade which includes all five Lesser Antillean species, all 17 from the Puerto Rico Bank, 16 from Hispaniola (mostly North Island), and six from Cuba.

The strong correlation between phylogeny and geography in West Indian *Eleutherodactylus* suggests that the tectonic history of the Caribbean has played a role in the evolutionary history of this group. Hedges (1989a) proposed that the two major West Indian groups of *Eleutherodactylus* diverged in the early Tertiary as a result of the breakup of the proto-Antilles. The subgenus *Euhyas* likely occupied Cuba, dispersing to Jamaica and the South Island of Hispaniola after those areas emerged in the early Miocene. The subgenus *Eleutherodactylus* (*auriculatus* section) probably occupied the North Island of Hispaniola and/or Puerto Rico, and dispersed relatively recently to Cuba and the Lesser Antilles. After the North and South Islands collided (between 10–20 mybp), there was limited overland dispersal of the two groups. The existence of the Eocene *Eleutherodactylus* (subgenus unknown) in amber from the North Island (Poinar & Cannatella, 1987) is in agreement with that hypothesis, but otherwise does not contribute crucial information on dates of divergence. Using the above hypothesis, an independent calibration of the albumin immunological clock can be made for West Indian *Eleutherodactylus*.

The first calibration point is the breakup of the proto-Antilles. The proto-Antilles formed in the Cretaceous as an island arc or isthmus connecting North and South America. Although the extent of land emergence (islands vs. isthmus) is poorly known (Perfit & Williams, 1989), the breakup of that land mass probably occurred 65–75 mybp (Burke, 1988; Ross & Scotese, 1988). The mean ID between the subgenera *Euhyas* and *Eleutherodactylus*, the groups that presumably diverged at this time (Hedges, 1989a), is 116.7 ± 5.1 (based upon reciprocal distances of *Euhyas* and *montanus* antibodies).

The next possible calibration point is the emergence of Jamaica, which occurred in the late Oligocene, 20–30 mybp (Robinson, Lewis & Cant, 1970; Horsfield & Roobol, 1974; Comer, 1974; Arden, 1975; Kashfi, 1983; Buskirk, 1985). That is the earliest time that the subgenus *Euhyas* could have colonized the island and maintained a continuous lineage. Unfortunately, the upper time limit for the dispersal of the subgenus *Euhyas* to Jamaica is less clear. The morphological diversity encompassed by the 17 native species suggests a relatively long occupation of the island, and thus the time interval for the emergence (20–30 mybp) is used, assuming that dispersal occurred soon after emergence. Other members of this subgenus are found on Cuba and the South Island of Hispaniola. However, it is unlikely that the South Island was a source area for the colonization of Jamaica, because it, too, was submerged or only recently emergent at that time. Cuba was the most likely source, but the Cuban sister species or sister group to the Jamaican radiation is presently unknown. The Cuban species used in this study, *planirostris*, closely resembles some Jamaican

species (*cundalli* group) morphologically, although perhaps convergently, and also is an excellent disperser. Thus, the Cuban lineage that includes *planirostris* is not an unlikely candidate, and the mean of reciprocal IDs between *planirostris* and the Jamaican species *gossei* and *nubicola* is 41 ± 3.1 .

Finally, a third calibration point is the uplift of the Blue Mountains in eastern Jamaica, which began 5–10 mybp (Horsfield, 1973; Comer, 1974; Wadge & Dixon, 1984). The evolution of the *nubicola* species group appears to be associated with this uplift because four out of the five species are restricted to this region of Jamaica (Hedges, 1989b). The sister groups to the *nubicola* group are the *jamaicensis* and *cundalli* groups (Hedges, 1989a). The mean ID between *nubicola* and the four species in those two groups is 11.8 ± 1.3 , with the assumption being that the divergence of the *nubicola* group occurred at the onset of the Blue Mountain uplift.

These three calibration points and associated intervals are shown in Fig. 3. The slope of the resulting line, adjusted to pass through zero, is 1.67. This gives a calibration of 1 ID = 0.60 my, which agrees with that obtained in other vertebrate groups (Maxson *et al.*, 1975; Maxson, 1991). This provides further support for the use of albumin immunological distance as a molecular clock to date times of divergence. Such independent calibrations are important to establish the robustness of the albumin clock.

As our knowledge of evolutionary history increases with the more widespread use of molecular techniques, and the refinement of methods of analysis, there will be a greater need for methods of dating divergence events. It is unlikely that fossils will provide that information, at least for the majority of species. In many studies, the protein albumin has been shown to be useful in dating

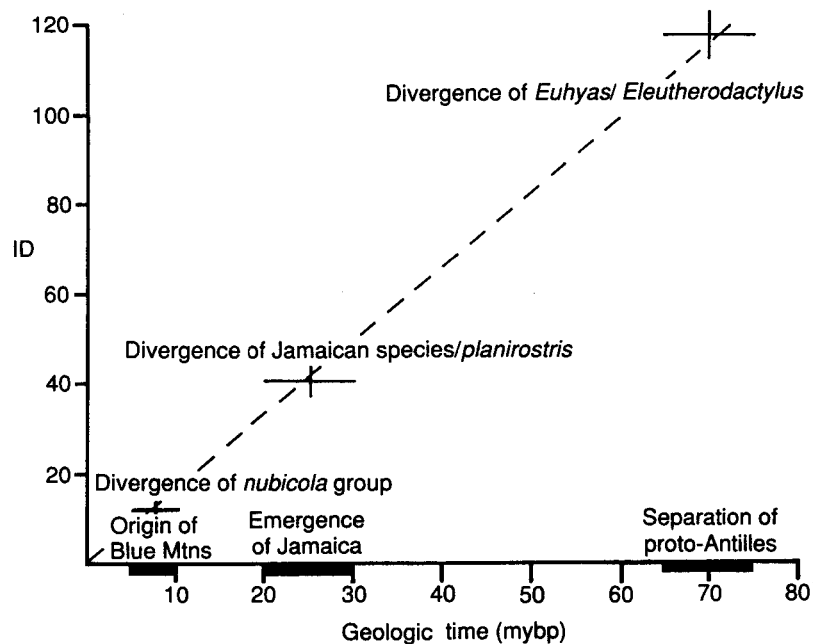


FIG. 3. Calibration of the albumin immunological clock for *Eleutherodactylus*. The calibration points (crosses) are mean immunological distances (\pm S.E.) corresponding to three events in the geologic history of the Caribbean. The dashed line corresponds to a calibration of 100 ID = 60 my.

evolutionary divergences through the indirect estimation of amino acid substitutions by MCF. However, improvements in estimates of both time and phylogeny likely will be seen in future studies when new methods of DNA amplification and sequencing (Mullis *et al.*, 1986; Saiki *et al.*, 1988) are applied to the albumin molecule (in progress).

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Appendix

Localities and voucher specimens

Numbers refer to preserved specimens in the United States National Museum of Natural History, unless otherwise noted (JPB = James P. Bogart, private collection; LM = Linda Maxson, private collection; SBH = S. Blair Hedges, frozen tissue collection). Species for which an antiserum was made are indicated by an asterisk. Localities indicated by a dagger are those used in Hedges (1989a).

Cuba: *planirostris* I, La Habana, Ciudad de La Habana, (JPB 4335, JPB 4347); and *planirostris* II (introduced), †Jamaica, 2.9 km NW Port Maria (305411–305413, SBH 161159–185, SBH 161187–195).

Jamaica: *alticola*, †St. Thomas, Blue Mountain Peak (266337, 266339–340); *andrewsi*, †St. Andrew, 1.3 km W Hardwar Gap (269235); *cavernicola*, †Clarendon, Jackson's Bay Caves, c. 1.6 km ESE of Jackson's Bay (266353–354, 266356); *cundalli*, Trelawny, 10.1 km NW Troy (305362–363), and Trelawny, 0.8 km N Burnt Hill (305364); *fuscus*, †St. James, 3.2 km W Mocho (266376–377, 266379–380); *glaucoreius*, Portland, 1.6 km N Section (305365–367); **gossei*, †St. James, 3.2 km W Mocho (269236, 266383–387); *grabhami*, †Trelawny, c. 11 km NNW Quick Step at Matta Stick (Marta Tick) Cave (266391, 266393–394, 269237), and St. James, 2.4 km W Mocho (305368–369); *griphus*, †Trelawny, c. 11 km NNW Quick Step at Matta Stick (Marta Tick) Cave (266401, 266404–405, 269238); *jamaicensis*, †St. Andrew, c. 2.4 km NW Hardwar Gap (SBH 101509); *johnstonei*, Trelawny, 7.7 km WNW Troy (305414–419); *junori*, †Trelawny, 7.7 km WNW Troy (269239–240); *luteolus*, †St. James, 2.4 km W Mocho (269246); **nubicola*, St. Andrew, 1.3 km W Hardwar Gap (305370–405); *orcutti*, Portland, 4.2 km N Hardwar Gap (266436–440); *pantoni*, Trelawny, c. 11 km NNW Quick Step at Matta Stick (Marta Tick) Cave (305406–407); *pentasyringos*, †Portland, 2.3 km S Fellowship (266453–454), and Portland, 5.8 km W Ecclesdown (305408–410); *sisyphodemus*, †Trelawny, c. 11 km NNW Quick Step at Matta Stick (Marta Tick) Cave (266466–467).

Hispaniola: *bakeri*, Haiti, Grande Anse, 11.2 km S, 1.9 km E Marche Leon (airline distance) (305423–426); *inoptatus*, Dominican Republic, Pedernales, 1.8 km N Los Arroyos, (257757–758); *jugans*, †Haiti, SE, 8.0 km NW Seguin (266315–317, 269278); *montanus*, Dominican Republic, 18 km SE La Vega (via the old road) (266306); *pictissimus*, †Dominican Republic, Barahona, Los Patos (266313–314).

Puerto Rico: *cooki*, c. 3.0 km SW Yabucoa (305420); *çoqui*, 1.3 km S, 1.1 km E El Yunque Peak (airline distance) (305421–422); *richmondi*, El Yunque, vic. of the peak (266328).

North America: *marnockii*, Texas, Austin (LM 2921).

Central America: *bransfordii*, Panama (LM 1142).

South America: *binotatus*, Brasil, São Paulo, Boracéia (LM 1491); *unistrigatus*, Ecuador (LM 2920).