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Molecular Systematics of the Frog Genus *Leptodactylus* (Amphibia: Leptodactylidae)

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A Contribution in Celebration of the Distinguished Scholarship of Robert F. Inger on the Occasion of His Sixty-Fifth Birthday

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Molecular Systematics of the Frog Genus *Leptodactylus* (Amphibia: Leptodactylidae)

Abstract

More than three-quarters of the described species of the frog genus *Leptodactylus* were sampled and analyzed using the quantitative immunological technique of micro-complement fixation. Eleven albumin antisera to representatives of the four described species groups in this genus were compared to one another and to albumins of all available species of *Leptodactylus*.

The results of this analysis indicated enormous albumin differentiation within the genus, suggesting that most species of *Leptodactylus* have been established since the Paleocene with modest speciation occurring throughout the Eocene, Oligocene, and Miocene. No evidence for Pleistocene speciation has been found.

The four species groups of *Leptodactylus* defined on morphological and behavioral criteria are not as clearly defined by this biochemical analysis. Albumins of species within each of the *L. pentadactylus, L. melanonotus,* and *L. ocellatus* groups are more similar to one another than to members of other groups. However, there is little evidence of close relationships among those members of the *L. fuscus* group available for study. *Leptodactylus riveroi* is not genetically close to any of the reference species and appears to represent yet another lineage in this genus. *Leptodactylus silvinambus* has its closest relatives among members of the *L. pentadactylus* species group.

There is considerable intraspecific albumin differentiation in *Leptodactylus bolivianus, L. fuscus, L. pentadactylus,* and *L. podicipinus.* Populations sometimes referred to as *L. ocellatus* from northeastern Brazil and Amazonia are specifically distinct from *L. ocellatus* from southeastern Brazil and Uruguay.

Introduction

The Neotropical frog genus *Leptodactylus* consists of over 45 species. Comparative morphological and behavioral data (Heyer, 1969, 1979, and other revisions cited therein) indicate that these species divide into four lineages. To test this hypothesis we have been gathering micro-complement fixation (MC'F) data on albumin evolution among *Leptodactylus* species since 1974. We initially anticipated that a few representative species samples would establish a molecular framework to determine the relationships among the major lineages within the genus. Our early results indicated that the problem of defining relationships within *Leptodactylus* was more complex than anticipated. It is only now that we have sufficient data from MC'F analyses to evaluate the utility of this approach for delineating relationships within the genus. Because of Robert F. Inger's interest in frog systematics, we offer this summary, warts and all, as a token of our appreciation for his influence on herpetology.

Our initial interest was to determine if MC'F analysis would indicate genetic groups that would correlate with the species groupings determined from other data sources. These species groups and their key diagnostic features are:

1. The *Leptodactylus melanonotus* species group (5 species)
   a. Toes fringed
   b. Males with thumb spines, no chest spines
   c. No dorsolateral folds
   d. Eggs laid in foamy mass on top of water
   e. Larvae uniformly dark, labial toothrow anterior to beak entire
2. The *Leptodactylus ocellatus* species group (4–6 species)
   a. Toes fringed
   b. Males with thumb spines, no chest spines
   c. Dorsolateral folds present
   d. Eggs laid in foamy mass on top of water
   e. Larvae uniformly dark, labial toothrow anterior to beak entire
3. The *Leptodactylus pentadactylus* species group (11 species)
   a. Toes ridged in juveniles, free in adults
   b. Males usually with thumb and chest spines
   c. Dorsolateral folds usually present
   d. Eggs laid in foamy mass on top of water
   e. Larvae mottled, labial toothrow anterior to beak divided
4. The *Leptodactylus fuscus* species group (at least 23 species)
   a. No fringes on toes
   b. Males without thumb or chest spines
   c. Dorsolateral folds usually present
   d. Eggs laid in foamy mass in an underground terrestrial incubating chamber
   e. Larvae mottled, labial toothrow anterior to beak divided.

Since these groups were initially defined (Heyer, 1969), two species have been described that are intermediate. *Leptodactylus riveroi* is very similar in overall appearance to *L. wagneri,* a *L. melanonotus* group member, but has a pair of dorsolateral folds, as found in the *L. ocellatus* group. The call of *L. riveroi* is distinctive and unlike that of any other *Leptodactylus* species (Heyer & Pyburn, 1983). *Leptodactylus silvinambus,* when described (McCranie et al., 1980), was associated with the *L. pentadactylus* group by default, as it was clearly distinct from members of the other three species groups. However, *L. silvinambus* is morphologically distinct from all other members of the *pentadactylus* group. In addition to determining whether MC'F analysis of *Leptodactylus* albumins would (1) substantiate the species groups summarized above, and (2) elucidate relationships among the species groups, we were interested in determining if albumin data would (3) shed light on the relationships of *L. riveroi* and *silvinambus* to other members of the genus.

Materials and Methods

The quantitative immunological technique of MC'F was used to compare albumins of the frogs of the genus *Leptodactylus.* Albumins were obtained from blood and phenoxyethanol extracts of muscle from most described species of *Leptodactylus.* Antisera were prepared to purified albumins of 11 species, representing the four major described species groups: *melanonotus* group—*L. podicipinus*; *ocellatus* group—*L. ocellatus, L. bolivianus*; *pentadactylus* group—*L. pentadactylus, L. fallax, L. flavopictus, L. labyrinthicus, L. laticeps*; *fuscus* group—*L. fuscus, L. labrosus, L. notoaktites.* Collection and voucher information on all specimens used in this study are indicated in the Appendix. Some of the antisera used in this study have been described earlier (Heyer & Maxson, 1982a,b; Maxson & Heyer, 1982).

All antisera were prepared and all MC'F analyses were carried out according to established procedures (Maxson et al., 1979; Champion et al., 1974). Data are reported as immunological distance (ID) units (IDU) which, for albumin, represent amino acid differences in the two albumins being compared (Wilson et al., 1977; Benjamin et al., 1984). The mean rate of albumin evolution is such that 100 IDU accumulate for every 55–60 million years that two lineages have been reproductively isolated from one another (Wilson et al., 1977).

Results and Discussion

The average titer (and slope) of the 11 antisera is 3600 (and 390). Although the averages are typical of previous MC'F studies of albumin evolution in vertebrates, three antisera had exceptionally low titers of 1100 (*L. fallax*) and 1300 (*L. laticeps, L. pentadactylus*). These low titers make it technically difficult to use these antisera, particularly at IDs greater than 50 units. The remaining eight antisera had an average titer of 4500 and an average slope of 390.

TABLE 1. Matrix of reciprocal immunological distances (ID) among 10 species of *Leptodactylus*.

Antigens	ID measured with antisera to albumins of:									
	FA	FL	LB	PT	BO	OC	FU	NO	LR	PO
fallax (FA)	0	62	44	36	153	135	≥170	>127	≥115	>160
flavopictus (FL)	79	0	49	56	>120	132	≥176	>125	≥111	>113
labyrinthicus (LB)	84	60	0	33	162	···	≥174	>126	≥110	>110
pentadactylus (PT)	64	49	37	0	160	>160	>180	>127	~112	>113
bolivianus (BO)	110	105	>120	112	0	52	>185	>78	87	>110
ocellatus (OC)	>113	140	>147	~118	77	0	≥178	>125	~135	>160
fuscus (FU)	>109	108	≥150	~115	~184	162	0	>150	>150	>110
notoaktites (NO)	···	112	≥171	≥119	≥175	···	120	0	98	>160
labrosus (LR)	>110	113	132	118	···	···	>185	>90	0	>85
podicipinus (PO)	···	131	~150	>140	···	···	~190	>140	>144	0

··· = Comparison not carried out.

Reciprocal Reactions

If MC'F analysis were a perfect measure of amino acid sequence difference, rather than an *estimate* of such sequence differentiation, it would be possible to determine the ID between two species using an antiserum to either species. For example, the ID measured between two species, X and Y, should be the same whether using antiserum to X or antiserum to Y. In practice, a deviation from perfect reciprocity (Maxson & Wilson, 1975) which averages 10%–15% is usually encountered in amphibian albumin studies (Heyer & Maxson, 1983; Maxson, 1984). This deviation is, in part, attributable to experimental technique and considerations of protein structure. The lower the deviation from perfect reciprocity, the greater is the confidence that actual amino acid substitutions in the albumin protein are being measured. Reciprocal tests are important in order to (1) draw phylogenetic conclusions, (2) determine the confidence level that the experiments are actually measuring amino acid substitutions, and, as a consequence, (3) provide a framework for interpreting one-way tests.

The reciprocal test data for 10 of the 11 species of *Leptodactylus* are presented in Table 1. The deviation from reciprocity of this matrix is very high and most IDs are very large, with several of the values approaching or exceeding the resolution of the technique (Maxson & Maxson, 1986). Initially, we were very surprised by these large values. Most of the values indicate that the species studied had a common ancestor a very long time ago. Because of the generality of large values, we have not completed the data matrix for those tests that we confidently predict would result in large values but add no information regarding degree of similarity.

A second observation is that among the 10 species tested only two closely related clusters of species are identified. All the other species are distantly related to each other at about the same level of distance. The first cluster includes *Leptodactylus fallax-flavopictus-labyrinthicus-pentadactylus*; the second cluster is comprised of *L. bolivianus-ocellatus*. The first cluster species are all members of the *L. pentadactylus* species group; the second cluster pair are members of the *L. ocellatus* species group.

A third observation, for those cases where both reciprocal values are less than 100 IDU, is that considerable variation exists in the similarity of reciprocal values. At one extreme are the reciprocal values for *Leptodactylus fallax* (84) and *labyrinthicus* (44), where the difference in values, 40 IDU, is almost as large as one of the values itself, 44 IDU. In this instance, both antisera exhibit significant nonreciprocity in estimating ID. The *L. labyrinthicus* antiserum underestimates values and the *L. fallax* antiserum gives overestimates. When the Sarich-Cronin (1976) correction for such nonrandomness in reciprocity is applied, the estimates become 49 and 52 IDU, respectively. At another extreme is the pair of *L. labyrinthicus* and *pentadactylus*, where the values (37 and 33 IDU) fall within experimental error of ±2 IDU per test (e.g., experiments from same sample sources run on different days). However, when correcting for the *L. labyrinthicus* antiserum, the two estimates are 41 and 33 IDU. This is no longer as ideal as the uncorrected values, but still not unreasonable for typical albumin studies.

If we examine comparisons only involving the

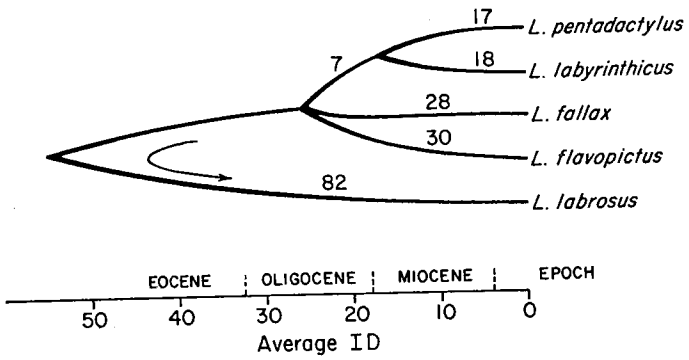

FIG. 1. Phylogenetic relationships among members of the *Leptodactylus pentadactylus* group using *L. labrosus* as an outgroup. The scale indicates the average immunological distance (ID) between species and the geological epochs.

four antisera in the *pentadactylus* group (*fallax, flavopictus, labyrinthicus, pentadactylus*) as presented in Table 1, the percentage of deviation from reciprocity is 18.6. However, after correcting for nonrandomness, this value drops to a more typical 7.6%. Generally, the reciprocal values do not correlate as well as desired, indicating that the degree of noise is such that all results must be interpreted very cautiously.

Production of antiserum to albumin from *Leptodactylus laticeps* led to such disparate reciprocal values for several tests that we think it inappropriate to discuss the relationships based on the available values for *laticeps*. For example, *laticeps* Ab tested against *pentadactylus* Ag, *bolivianus* Ag, and *ocellatus* Ag had values of 54, 70, and 75 IDU, respectively; whereas the tests of *pentadactylus* Ab, *bolivianus* Ab, and *ocellatus* Ab to *laticeps* Ag had IDU values of 105, 120, and 140, respectively. This kind of systematically asymmetrical reciprocity has been found in other studies as well (e.g., Sarich & Cronin, 1976; Maxson et al., 1985; Uzzell, 1982), although the precise reasons for the results are unknown at present.

Phylogenetic Considerations Based on Reciprocal Data

Examination of the reciprocal reactions in Table 1 reveals that only the members of the *pentadactylus* group (*fallax, flavopictus, labyrinthicus, pentadactylus*) and *Leptodactylus labrosus* have albumin distances close enough to be used in estimating a phylogeny. All of the other species have either overall larger distances or, in some cases, distances that could only be estimated as being greater than some large distance. Hence, we have estimated a tree only for the four members of the *pentadactylus* group and *L. labrosus*, a member of the *fuscus* group. Some of these data have been interpreted in an earlier study (Heyer & Maxson, 1982a). The additional antisera have not changed the conclusions of that earlier work, but have added some lineages not included at that time.

The data used in constructing the phylogeny in Figure 1 are those in Table 1. The standard deviation from reciprocity for the raw data is 15.6%; when the correction for nonrandomness was applied, this dropped to 11.0%. Both the raw data and the corrected data were used to build phylogenies according to the Maxson modification (Maxson, 1984) of the Wagner network described by Farris (1972). For both data sets, *pentadactylus* and *labyrinthicus* were each other's closest genetic relatives. The uncorrected data yielded a tree topology with *flavopictus* being the next lineage, but with a short branch length of only 3 IDU. The corrected data placed *fallax* closest to the first cluster, but the branch length was even less significant at 2.5 IDU. Thus we have presented in Figure 1 a topology that we think best interprets our data. There are three major lineages branching about the mid-Oligocene. The lineage leading to *pentadactylus* and *labyrinthicus* branches about early Miocene and the lineage leading to *labrosus* separates from that, leading to the *pentadactylus* group in the Paleocene. The standard deviations (Fitch & Margoliash, 1967) for the tree presented, comparing the raw and corrected input data, are 9.6% and 9.7%, respectively.

One-Way Reactions

Most of the data gathered in this study involve only one-way reactions of Ag samples against the antibodies raised to albumins of the 10 species shown in Table 1. A complete data matrix was

TABLE 2. One-way immunological distances (ID) in the *Leptodactylus melanonotus* group.

Antigens	ID to *L. podicipinus*
melanonotus	
Costa Rica	>80
El Salvador	>109
podicipinus	
Ybycuí	0
Cordillera	1
El Tirol	4
wagneri	
Tapajós	35

deemed prohibitive, in terms of time invested, versus results anticipated in view of the large distances. We chose to run all those reactions we predicted might show close values, together with a few reactions we did not predict would demonstrate close values. For any unpredicted results, further tests were performed in order to verify and/or understand the results. By and large, the tests corroborated the four previously defined species groupings. We therefore present the results by species groupings, followed by the results involving *Leptodactylus riveroi* and *silvinambus,* then conclude with those tests which did not corroborate the previously identified species groupings and/or which we view as problematical.

***Leptodactylus melanonotus* Group**—Antiserum is available for *L. podicipinus*; the sample used for antibody production was obtained from frogs from Ybycuí, Paraguay. Samples of other populations of *L. podicipinus* from Paraguay range from 1–4 IDU when tested by MC'F (table 2). *Leptodactylus podicipinus* is more closely related to *L. wagneri,* with which it is marginally sympatric, than it is to *L. melanonotus* (table 2). *Leptodactylus podicipinus* occurs in southern South America; *L. wagneri* occurs broadly throughout Amazonia and the lowlands of South America east of the Andes; and *L. melanonotus* occurs in southernmost United States and from Mexico through Central America to Ecuador west of the Andes. Thus, the closest relative of *podicipinus* is its geographically closest form, *wagneri.* Based on morphology and distributions, we predict that when *L. dantasi* and *pustulatus* are tested (the remaining two species of the *L. melanonotus* group), they will show closer relationships with *L. podicipinus* and *wagneri,* with which they are mostly parapatric, than with the geographically distant *L. melanonotus.*

***Leptodactylus ocellatus* Group**—Several geographic samples of *L. bolivianus* and *ocellatus* were tested against antisera to *bolivianus* and *ocellatus.* From all of the tests run (tables 1, 3, 6; LRM, pers. obs.), *bolivianus* and *ocellatus* are each other's closest relatives, but the relationship is not especially close, averaging about 60 IDU. There appears to be some population differentiation among the samples of *bolivianus* tested, but again the data should not be overinterpreted (table 3). Of particular concern is the value of 8 IDU for the second sample from Bolívar State, Venezuela. This value exceeds experimental error and is twice that found for two other geographic samples from the state of Bolívar. With the exception of this one high value, all the remaining values suggest a relatively undifferentiated population structure for *L. bolivianus* in South America. The geographic samples for *L. ocellatus* show a different pattern, with relatively small IDs among samples of *L. ocellatus* from Uruguay, southern and southeastern Brazil, and significantly higher distances for a series of samples from Amazonia and northeastern Brazil. The magnitude of these differences is consistent with specific differentiation of the two groups of populations, which are hereby so considered.

TABLE 3. One-way tests in the *Leptodactylus ocellatus* group.

Antigens	ID measured with antisera to albumins of: *bolivianus*	*ocellatus*
bolivianus		
Venezuela	0	52
Venezuela, Bolívar (1)	3	49
Venezuela, Bolívar (2)	8	50
Venezuela, Bolívar (3)	4	47
Brazil, Acre	2	66
Brazil, Madeira	3	64
Peru	3	...
ocellatus		
Brazil, São Paulo	77	0
Brazil, Minas Gerais	...	0
Brazil, Santa Catarina	...	6
Uruguay	...	7
Brazil, Madeira	...	26
Brazil, Purus	...	25
Brazil, Santarém	...	27
Brazil, Ceará (1)	...	26
Brazil, Ceará (2)	...	30

Cei (1970) used Libby's photronreflectometric technique to demonstrate that the serum of *Leptodactylus ocellatus* from Argentina (Córdoba) differed significantly from the serum of *L. chaquensis* (Tucumán, Argentina) and from a sample of another *L. ocellatus* group population from around São Paulo, Brazil, which Cei identified as *L. mac-*

TABLE 4. One-way tests in the *Leptodactylus pentadactylus* group.

Antigens	ID measured with antisera to albumins of:			
	fallax	***flavopictus***	***labyrinthicus***	***pentadactylus***
fallax	0	62	44	36
flavopictus				
Brazil, São Paulo (1)	79	0	49	56
Brazil, São Paulo (2)	...	0	...	43
knudseni				
Venezuela, Amazonas	12	...	18	...
Venezuela, Bolívar	16	...	20	...
Brazil, Madeira	13	54	11	32
Brazil, Tapajós	≤17	56	18	38
Peru	...	47	11	29
labyrinthicus				
Brazil, São Paulo	...	...	4	35
Brazil, Ceará	84	60	0	33
pentadactylus				
Panama	64	49	37	0
Ecuador, Coastal (1)	...	...	...	5
Ecuador, Coastal (2)	...	...	...	8
Peru, Amazon	...	...	...	9
rugosus	24	≤43	57	67
stenodema				
Brazil, Madeira	~11	61	38	51
Peru	5	...	38	48
syphax	87	46	61	61

rosternum. Based on Gallardo's (1964) revision, we would consider that our sample from Boracéia represented *L. ocellatus* and the samples from Amazonia and northeastern Brazil *L. macrosternum.* Thus, we are not certain whether Cei's and our interpretations of *L. macrosternum* are the same. What is clear is that considerable biochemical evolution has taken place in this complex with very little accompanying morphological change. We would expect *L. chaquensis* to show closer relationships to our concept of *L. ocellatus* than to the Amazonian and northeastern Brazilian member of this species group.

***Leptodactylus pentadactylus* Group**—The results of reciprocal and one-way tests (tables 1, 4) suggest that the MC'F data corroborate a cluster of species previously defined on morphological bases (*fallax, flavopictus, knudseni, labyrinthicus, pentadactylus*; Heyer, 1979). There appears to be some variation within samples of *L. pentadactylus.* An additional sample run from coastal Ecuador since our previously published data (Heyer & Maxson, 1982b) does not corroborate our previous zoogeographic statement regarding *L. pentadactylus.* We had previously stated that the Amazonian populations of *L. pentadactylus* were differentiated from the Middle American-coastal South American populations. However, the current data do not suggest such a clear-cut pattern. Until further samples are evaluated, we conservatively interpret the samples to represent the same species, with no interpretation of patterns of differentiation among samples.

The available data do not allow an unambiguous hypothesis of relationships among the members of this cluster. If only the reciprocal data are used, it is quite clear that *Leptodactylus pentadactylus* and *labyrinthicus* are each other's closest relatives. However, it is not clear whether *L. knudseni* is a closer relative of *labyrinthicus* or *pentadactylus,* as one-way tests to *knudseni* give low and similar values to both *fallax* and *labyrinthicus.* An attempt to produce antibodies to *knudseni* to resolve this problem was unsuccessful. Based on larval and adult morphology, *L. fallax* is much more similar to *flavopictus, knudseni, labyrinthicus,* and *pentadactylus* than to *stenodema.* The immunological data conflict with the morphological data; they show a closer relationship between *fallax* and *stenodema* than between *fallax* and any other taxa in the *pentadactylus* group (table 4). Resolution of this conflict will require additional data.

***Leptodactylus fuscus* Group**—Most of the one-way tests run against *fuscus, labrosus,* and *notoaktites* antisera give large ID values and do not demonstrate any particularly close relationships (table 5). Three examples that do suggest close relationships are therefore of interest. First, the geographic samples of *fuscus* tested against *fuscus* antisera prepared from individuals from Boracéia, São Paulo, Brazil, demonstrate close relationships relative to other tests against *fuscus*; however, the intraspecific tests do not make complete geographic sense. The identical value of zero of samples from Boracéia and Paraguay is understandable, but the distances of 13–16 IDU to samples from Argentina (Tucumán), northeastern Brazil, and central Amazonia (Manaus) are greater than one would anticipate for intraspecific variation. Previously reported values of *fuscus* from Argentina-Boracéia, Brazil (14) and Manaus-Boracéia, Brazil (0) (reported in Heyer & Maxson, 1982a) are incorrect in part. The Manaus-Boracéia test had in fact not been run; Heyer thought it had and concluded that the absence of a value was a zero. Tests repeated from additional aliquots from the Argentine antigen yield a value of 16 IDU (within experimental error), but the Manaus-Boracéia distance is also 16 as shown in Table 5. *Leptodactylus fuscus* as currently defined would be an excellent candidate for detailed electrophoretic analysis.

The second suggested close relationship is *Leptodactylus labrosus* and *ventrimaculatus.* The significance of this relationship has been discussed elsewhere (Heyer & Maxson, 1982a). The third set of close relationships is that between *L. notoaktites* and *elenae* (35) and *L. notoaktites* and *mystaceus* (11–20). These three species were, until recently (Heyer, 1978), considered conspecific; they are morphologically very similar and their distributions are essentially parapatric.

Based on overall morphological similarity, one of us (WRH) predicted that *fuscus* would show close relationships to *camaquara, cunicularius, gracilis,* and *longirostris,* of the species tested. The extremely large ID values argue against close relationships for these pairings, however.

TABLE 5. One-way tests in the *Leptodactylus fuscus* group.

Antigens	ID measured with antisera to albumins of:		
	fuscus	***labrosus***	***notoaktites***
albilabris	76	66	<60
bufonius	≥196	>80	>74
camaquara	147	114	59
cunicularius	144	109	57
elenae	99	~116	35
fragilis	>100	≥122	55
fuscus			
Boracéia	0	>150	>150
Paraguay	0	...	...
Argentina	16	...	...
NE Brazil	13	...	...
Manaus	16	...	...
gracilis	100	≥122	68
latinasus	90	...	~73
longirostris	≥144	...	92
mystaceus			
Madeira	>100	...	20
Tapajós	...	...	17
Trombetas	...	...	11
mystacinus	>100	...	~67
troglodytes	103	≥84	~94
ventrimaculatus	≥153	6	~94

Leptodactylus riveroi* and *silvinambus*—*Leptodactylus riveroi antigen was tested against the antisera of *bolivianus, fuscus, ocellatus, pentadactylus,* and *podicipinus.* All of the tests resulted in very large ID values (table 6), indicating that *L. riveroi* is not closely related to any of the species tested. As the species tested represent the morphological diversity within the genus, it is likely that *L. riveroi* has no close relative within the genus.

Leptodactylus silvinambus antigen was tested against the antibodies of *bolivianus, fallax, flavopictus, fuscus, labyrinthicus, pentadactylus,* and *podicipinus.* In this case, relatively low IDU values were found between *silvinambus* and *fallax,* and *silvinambus* and *flavopictus* (table 6). These results indicate that *silvinambus* is related to some members of the *L. pentadactylus* group.

Unusual/Problematical Data—Some of the re-

TABLE 6. One-way tests comparing *Leptodactylus riveroi* and *L. silvinambus* to other species of *Leptodactylus.*

Antigens	ID measured with antisera to albumins of:							
	FA	**FL**	**LB**	**PT**	**BO**	**OC**	**FU**	**PO**
riveroi	...	...	...	≥104	≥180	~90	~169	>130
silvinambus	17	25	62	76	80	...	≥151	130

Abbreviations are the same as in Table 1.

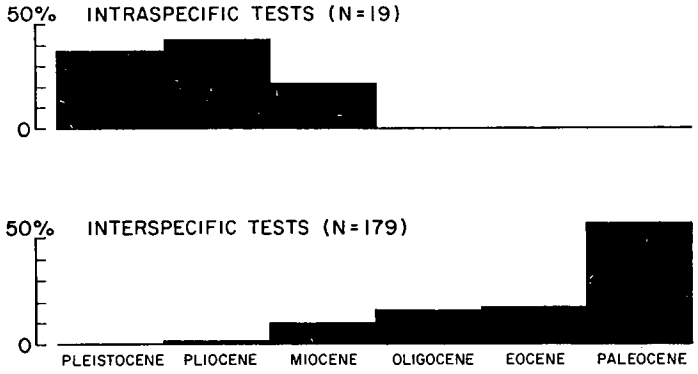

FIG. 2. Histograms showing frequency of pairwise immunological distance (ID) comparisons indicative of lineages diverging in the indicated geological epochs. **Top,** intraspecific comparisons; **bottom,** interspecific comparisons. The Paleocene label includes presumed Paleocene divergences plus all older divergence estimates.

sults that were unexpected or that indicated a problem with the antisera in estimating amino acid differences in the albumin proteins being compared have already been discussed. These include the conflicting morphological and immunological data on the relationships between *Leptodactylus fallax* and *stenodema.* The failure to produce an albumin antiserum to *L. knudseni* that yields consistent results may be due to a duplicated albumin locus in this lineage.

Finally, the following results of tests run between previously defined species groups are unusual. *Leptodactylus pentadactylus* is clearly most closely related to other members of the *pentadactylus* group (table 4). However, *pentadactylus* antiserum, when tested against antigens of *fragilis, mystaceus* (two geographic samples), and *mystacinus,* gave values of 38, 47, 53, and 58 IDU, respectively. These values are consistent with the values of *pentadactylus* with other *pentadactylus* group members, such as *rugosus, stenodema,* and *syphax.* We would have predicted that these one-way ID values should have been in the same range as the tests between *pentadactylus* and *fuscus* (115 IDU) and *pentadactylus* and *labrosus* (118 IDU). However, *L. pentadactylus* is the other low titer antiserum; it has consistently given somewhat lower ID values (see table 1), although these values are still lower than can be accounted for solely on the basis of reciprocals of *pentadactylus* to other species. The same kind of problem is evident in the antigen sample on *melanonotus* from El Salvador which was tested against *L. pentadactylus* antiserum; the test result was 36 IDU. The antiserum of *L. fallax* gave very low ID values when tested against two different antigen samples of *L. albilabris.* Both tests gave values of about 12 IDU. This, together with the apparently unusually low IDU values observed between *fallax* and *stenodema,* indicates that, at best, the *fallax* values should be used cautiously. If it proves that the *fallax* antiserum is not interacting in a uniform and predictable manner with other *Leptodactylus* antigens, then all test results involving *fallax* antiserum are suspect.

Divergence Times in *Leptodactylus*

Extensive studies of albumin evolution in diverse vertebrates have indicated that albumin accepts amino acid substitutions throughout the molecule at a stochastically regular rate (Maxson & Wilson, 1975; Maxson et al., 1975; Wilson et al., 1977). This rate for amphibians was estimated to be an average of 1.7 IDU per million years of divergence (Maxson et al., 1975). A calibration based on a more restricted but better dated fossil record for mammals corresponds to 1.8 IDU per million years of divergence (Wilson et al., 1977). Using these calibrations for all of the tests run with *Leptodactylus,* we arrive at some rather surprising conclusions (fig. 2).

For intraspecific comparisons we see two different patterns. Either populations of the same species are genetically indistinguishable, or allopatric populations assigned to the same species have been reproductively isolated since as long ago as the Miocene. Examining the interspecific comparisons, we find only 1% of the species comparisons indicative of a Pliocene divergence. The remainder of the comparisons show moderate genetic divergence occurring from the Miocene through the Eocene, and most of the divergence dating to the Paleocene or earlier (the limits of resolution of MC'F comparisons of albumin do not allow comparisons between albumins which differ by over 35% in their sequence; this amount of change ac-

cumulates by roughly 100–120 million years of separation [Wilson et al., 1977]). In view of the large number of tests run with *Leptodactylus,* the trends are not likely to change with more comparisons of additional species. Even if some of the values are incorrect, the noise is likewise negligible in terms of the summary presented in Figure 2.

At least two explanations can be proposed for these data. First, albumin may be evolving at a much faster rate in *Leptodactylus* than in most other vertebrate lineages. Alternatively, the genus *Leptodactylus,* as presently constituted, is a very old lineage with most species established since the Paleocene and modest speciation occurring throughout the Eocene, Oligocene, and Miocene. A little additional speciation occurred in the Pliocene, and essentially no major speciation events date to the Pleistocene. Two kinds of independent data—fossil and biochemical—can be brought to bear to distinguish between these two alternative explanations.

Fossil Data—The fossil record for the genus could provide evidence on the age of some taxa and provide an independent calibration of albumin evolution in this group. Unfortunately, as is true for most amphibians, the published fossil record of *Leptodactylus* is not sufficient to allow an independent assessment for this taxon.

Biochemical Data—Cei and his colleagues have investigated relations among varied species of *Leptodactylus,* using comparisons of skin amines and polypeptides as well as qualitative comparisons of serum proteins by means of precipitin analyses. These approaches give some insight into associations of groups of species based on relative similarities of small skin polypeptides (Cei & Erspamer, 1966; Cei et al., 1967) and patterns of behavior with antiserum raised to whole serum (Cei, 1970). However, skin amines and polypeptides are small molecules which appear to evolve rather rapidly and are not useful as general probes of relationships among relatively old taxa such as anurans. The MC'F analyses of albumin evolution have the advantage over precipitin analyses, in that the latter provide only a qualitative estimate describing some overall averaging of general similarities of an unknown mixture of serum proteins. The MC'F analyses, on the other hand, have been demonstrated to be an efficient estimator of amino acid differences between the species compared (Maxson & Maxson, 1986), and it is this capability that permits us to extrapolate time estimates from the ID measurements.

At present the only relevant molecular study on *Leptodactylus* (besides MC'F analyses) is an analysis of four species of Costa Rican *Leptodactylus* by starch gel electrophoresis (Miyamoto, 1981). While Miyamoto agreed with the species group assignments as used in this paper, it is difficult to use his data to support or refute the great age implied by the albumin studies. Because gel electrophoresis can only detect the first amino acid substitution causing a change in mobility, only relatively recently separated populations can be accurately diagnosed with gel electrophoresis due to the problem of multiple substitutions (Maxson & Maxson, 1979). Thus, if the species are as old as the albumin data imply, electrophoretic data cannot refute the albumin results! However, even Miyamoto's results show that over 80% of the loci scored between *pentadactylus* and *melanonotus* and between *pentadactylus* and *bolivianus* have fixed allelic differences, suggesting a long independence of lineages, such as the albumin data indicate. Thus, we suggest that the albumin data are the best available estimates of divergence time presently available for *Leptodactylus.* We propose these data be given serious consideration until falsified.

Conclusions

One of our original research aspirations with molecules and *Leptodactylus* was to provide an exemplary showcase of molecular and morphological evolution for a vertebrate genus. The MC'F albumin data do not allow the depth of interpretation we had anticipated at the outset. Most of the MC'F albumin data corroborate the species groupings determined on the basis of other criteria (mostly morphological in nature). However, some MC'F tests suggest close relationships that cross the previously defined species groupings. The rather poor reciprocity seen with results of reciprocal tests indicates that there may be some problems with *Leptodactylus* albumins resulting in poor antisera or there may be multiple paralogous albumins in this genus, confounding the results. Although only single proteins were identified and purified, all albumins may not be homologous in this genus. Because of that possibility, we are hesitant to accept blindly all of the ID values. This noise in the *Leptodactylus* data also prevents us from unambiguously resolving divergence events among closely related species (with moderate ID values), such as members of the *Leptodactylus pentadactylus* species cluster. The members of this cluster

(*fallax, flavopictus, knudseni, labyrinthicus, pentadactylus*) have a uniquely derived tadpole (Heyer, 1979), defining this cluster as monophyletic, yet the branching sequences among its members cannot be unambiguously proposed due to the noise in our MC'F albumin data.

The possible great age of most *Leptodactylus* species, together with the noise in the MC'F albumin data, lead us to the conclusion that MC'F albumin analysis is not the ideal choice for evaluating genetic relationships among most *Leptodactylus* species. Our studies have shown that there are some very interesting problems that should be pursued. Intraspecific variation in the widespread species *L. fuscus* and *ocellatus* should be studied in detail, probably with mitochondrial DNA analyses and/or electrophoretic techniques. Overall genetic relationships among *Leptodactylus* species should be examined using a more slowly evolving molecule than albumin, or by the direct sequencing of ribosomal genes.

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Appendix

Specimens used for MC'F analysis are listed. An LM (Linda Maxson) number ties into any additional data not listed here. Other abbreviations are for museum collections where specimen vouchers are deposited (museum number given) or will be deposited (no museum number given). Museum abbreviations are per standardized list given in *Copeia* (1985: 802–832). Ab = antibody produced.

Leptodactylus albilabris
LM 29; Puerto Rico, Isla Vieques; USNM

Leptodactylus bolivianus
LM 1267–8; Brazil, Amazonas, Bôca do Acre; USNM 202444–5
LM 1269; Brazil, Amazonas, Borba; USNM 202451
LM 1102; Peru, Madre de Dios, Tambopata Reserve; USNM 247351, 247354
LM 524; Venezuela, Bolívar, S of Ciudad Bolívar
LM 360; Venezuela, Bolívar, Ciudad Guayana; USNM 229778
LM 18; Venezuela, Bolívar, 28 km E El Palmar
LM 1266; Ab; Venezuela, Sucre, Cumaná

Leptodactylus bufonius
LM 417; Paraguay, Central, Villeta; USNM

Leptodactylus camaquara
LM 1275; Brazil, Minas Gerais, Serra do Cipó; USNM 217647

Leptodactylus cunicularius
LM 1272–3; Brazil, Minas Gerais, Serra do Cipó; USNM

Leptodactylus elenae
LM 32–5, 62–3; Paraguay, El Tirol; USNM

Leptodactylus fallax
LM 10; Ab; Dominica, near Coulibistri; USNM 218253–4

Leptodactylus flavopictus
LM 1276; Ab; Brazil, São Paulo
LM 1277; Brazil, São Paulo, Boracéia; USNM 209215

Leptodactylus fragilis
LM 1289–90; Panama, Canal Zone, Gamboa; USNM 203650–1

Leptodactylus fuscus
LM 1280; Argentina, Tucumán
LM 1279; Brazil, Amazonas, Manaus; USNM 202506
LM 1283; Brazil, Ceará, Santana do Cariri; USNM 216071
LM 1281–2; Ab; Brazil, São Paulo, Boracéia; USNM 209221–2
LM 12; Paraguay, El Tirol; USNM

Leptodactylus gracilis
LM 1285; Argentina, Tucumán

Leptodactylus knudseni
LM 1288; Brazil, Pará, Parque Rio Tapajos; USNM
LM 1348; Brazil, Rondônia, Calama; USNM 202516
LM 1286–7; Peru, Madre de Dios, Tambopata Reserve; USNM
LM 523; Venezuela, Amazonas, Tama-Tama
LM 522; Venezuela, Bolívar, Ciudad Bolívar

Leptodactylus labrosus
LM 1295; Ab; Ecuador, Río Palenque Biological Station; UIMNH 94604

Leptodactylus labyrinthicus
LM 1351–2; Ab; Brazil, Ceará, Santana do Cairiri; USNM 216079
LM 1296; Brazil, São Paulo, Assis; USNM 207674

Leptodactylus laticeps
LM 1298; Ab; Argentina; UIMNH 94163

Leptodactylus latinasus
LM 550; Argentina, Tucumán

Leptodactylus longirostris
LM 1302; Brazil, Pará, Parque Rio Tapajos; USNM

Leptodactylus melanonotus
LM 1304; Costa Rica
LM 548; El Salvador, Cuscatlán, El Sitio de los Hidalgo

Leptodactylus mystaceus
LM 1264; Brazil, Pará, Parque Rio Tapajos; USNM

LM 1263; Brazil, Pará, Reserva Rio Trombetas; USNM
LM 1305; Brazil, Rondônia, Calama; MZUSP

Leptodactylus mystacinus
LM 1306; Brazil, São Paulo, Fazenda do Veado; USNM 208092

Leptodactylus notoaktites
LM 1316; Ab; Brazil, Paraná, nr São João da Graciosa; USNM 217791–3

Leptodactylus ocellatus (= *ocellatus*)
LM 1337; Brazil, Minas Gerais, Serra do Cipó; USNM
LM 413; Brazil, Santa Catarina. Rio dos Cedros; USNM 243753
LM 1317; Ab; Brazil, São Paulo, Boracéia; USNM 209230
LM 1323; Uruguay, Maldonado, Sierra de Animas; USNM 217801

Leptodactylus ocellatus (= *macrosternum*?)
LM 1321; Brazil, Amazonas, Rio Madeira, Manicoré; MZUSP
LM 1319; Brazil, Amazonas, Rio Purus, Beruri; USNM 202512
LM 1326; Brazil, Ceará, Santana do Cariri; MZUSP
LM 1327; Brazil, Ceará, Santana do Cariri; MZUSP
LM 11; Brazil, Pará, Santarem; USNM

Leptodactylus pentadactylus
LM 1332; Ecuador, Río Palenque Biological Station; USNM
LM 1333; Ecuador, Río Palenque Biological Station; USNM
LM 1328; Ab; Panama, Canal Zone; UIMNH 94165
LM 791; Peru, Amazonas, Río Cenepa, Río Huampami; USNM

Leptodactylus podicipinus
LM 25; Paraguay, Cordillera; USNM
LM 26–7; Paraguay, El Tirol; USNM
LM 61; Ab; Paraguay, Ybycuí; USNM

Leptodactylus riveroi
LM 528–9; Venezuela, Amazonas, nr Tama-Tama

Leptodactylus rugosus
LM 1334; Venezuela, Bolívar, La Escalera; KU 181028

Leptodactylus silvinambus
LM 19, 22–24; Honduras, Ocotepeque, Belen Gualcho and El Chagüitón

Leptodactylus stenodema
LM 1335; Brazil, Amazonas, Rio Madeira, Restauração; MZUSP
LM 788; Peru, Amazonas, Río Cenepa, Río Huampami; USNM

Leptodactylus syphax
LM 1366x; Brazil, Minas Gerais, Serra do Cipó; USNM 218156

Leptodactylus troglodytes
LM 1355; Brazil, Ceará, Santana do Cariri; USNM 216080

Leptodactylus ventrimaculatus
LM 1356; Ecuador, Río Palenque Biological Station; USNM

Leptodactylus wagneri
LM 1341; Brazil, Pará, Parque Rio Tapajos; USNM