A Cretaceous divergence time between pelobatid frogs (Pelobates and Scaphiopus): immunological studies of serum albumin

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Serum albumin was purified from the Old World spadefoot toad Pelobates cultripes and from the New World spadefoot toads Scaphiopus hammondi and Scaphiopus couchi and injected into rabbits. The resulting antisera were used in the quantitative micro-complement fixation test to assess the degree of genetic relatedness between Pelobates and Scaphiopus as well as among six different species of Scaphiopus. Although Pelobates and Scaphiopus are morphologically and ecologically similar, and are considered to be close relatives, our immunological data suggest a divergence time of about 110 million years B.P. The albumins of the four species within the subgenus Spea (S. hammondi, S. intermontanus, S. bombifrons, and S. multiplicatus) were very similar. Albumins of the subgenus Scaphiopus (S. couchi and S. holbrooki), in turn, were more similar to each other than either was to S. hammondi. The intra-Scaphiopus results are consistent with previously reported electrophoretic studies, and a high correlation is observed between albumin immunological and electrophoretic genetic distances. These data and results from other biochemical studies provide no support for a single biogeographic model to explain the Holarctic distributions of amphibians.

Contents

Introduction ........................................ 481
Pelobatid evolutionary relationships and fossil history ............... 483
Immunological comparisons of frogs .................................. 484
Materials and methods ......................................... 484
Specimens and albumin purification .................................. 484
Immunological techniques .......................................... 485
Calculations .................................................. 486
Results .......................................................... 486
Discussion ...................................................... 488
Pelobates-Scaphiopus divergence .................................... 488
Intra-Scaphiopus relationships ..................................... 490
Cladogenesis and biogeographic models .............................. 491
Summary ....................................................... 492
References ..................................................... 492

Introduction

Biogeographic scenarios are created by evolutionary biologists when they repeatedly see similar patterns between taxonomic groupings and geographic distributions. This study was...
undertaken to determine whether a peculiar distributional pattern noted among anatomically similar salamanders had the same temporal sequence in a group of morphologically similar frogs. That we found the two cases quite different from one another provides us with a cautionary lesson concerning the assumption that morphological similarity provides much information about either the zoogeographical history or the degree of genetic relatedness in taxonomic groupings.


PELOBATID EVOLUTIONARY RELATIONSHIPS AND FOSSIL HISTORY

The family Pelobatidae is a morphologically well-defined group and, among frogs, it has a relatively good fossil record that extends back to the Cretaceous (Estes, 1970; Spinat, Boublik et al., 1971). Duellman (1975) recognized a superfamily Pelobatoidea which contained two families. The Pelodictyidae (genus Pelodytes) has living representatives only in Europe, but the group was present in the Oligocene of North America (Thilen, 1974). Pelodytes is more aquatic than Pelobates and Scaphiopus. Its morphological distinctiveness has been noted by many workers, and while Pelodytes is retained in the Pelobatidae by some workers (Savage, 1973; Arnold & Burton, 1978), a growing trend is to place this genus in its own family (Lynch, 1973; Duellman, 1975; Sokol, 1981 a,b).

The Pelobatidae includes two subfamilies, the Megophryinae and the Pelobatinae. The living megophryines are restricted to southeastern Asia, but the group has a long fossil record that extends outside this region. Estes (1970) considers the fossil genus Eopelobates of North America and Europe to be a megophrynine. There are six recognized species of Eopelobates, and the genus is known from the late Cretaceous to the middle Miocene. Estes (1970) argued that Eopelobates gave rise to the common ancestor of Scaphiopus and Pelobates.

The Pelobatinae includes the so-called spadefoots, Scaphiopus of North America and Pelobates of Europe and extreme western North Africa. The earliest pelobatine in Europe is from latest Oligocene to earliest Miocene, 25 to 26 million years B.P. (R. Estes, pers. commun.), and the earliest North American pelobatine (Scaphiopus skinneri) is from early Oligocene, 32 to 36 million years B.P. (Holman, 1968; R. Estes, pers. commun.). Zweifel (1956) and Estes (1970) argued in favor of a common ancestor for the two genera. While the putative ancestor, Eopelobates, occurred in both the Old and New Worlds, Estes (1970) favored a North American origin of the spadefoots. The Holarctic spread of the spadefoots was thought by Estes (1970) to have occurred during the early Eocene. While Estes (1970) discussed Holarctic distributions without being specific about dispersal routes, Savage (1973) was explicit concerning migrion on of spadefoots to Eurasia via the Bering land bridge in the Oligocene.

Estes (pers. commun.) has outlined for us his current ideas concerning pelobatine origins and historical biogeography. Based on the presence of Eopelobates leptocladus in the late Cretaceous of Mongolia (Borukh-Białyńńska, 1978), which has clear pelobatine resem-
The immunological techniques employed here, quantitative micro-complement fixation, has been used for extensive comparisons of albumins from diverse groups of vertebrates. These studies indicate that micro-complement fixation can assess point mutational differences in albumin, that albumin may be used as an evolutionary clock, and that for many vertebrates—including mammals, several groups of reptiles, and frogs—the rate constant for albumin evolution is 1-7 units of immunological distance per million years per pair of lineages (Masson, Sarich et al., 1975; Prager & Wilson, 1975; Wilson, Carlson et al., 1977; Carlson et al., 1978). Because change at the protein level is related to time rather than to organismal change, the albumin clock can be used to provide a temporal framework despite great differences in rates of evolutionary change at supramolecular levels (such as anatomy, potential for interspecific hybridization, and chromosome structure) and despite the absence of a fossil record (Wilson, Masson et al., 1974; Wilson, Sarich et al., 1974; Masson, Sarich et al., 1975; Wilson, Carlson et al., 1977; Carlson, Wilson et al., 1978).

Most decades have seen a veritable explosion in the use of micro-complement fixation for comparisons of frog albumins. The resulting immunological distances have been used most extensively for constructing evolutionary trees, for estimating divergence times at various taxonomic levels, and for detecting cases of convergent morphological evolution. These investigations, particularly by Masson and her co-workers, have involved a majority of the extant frog families. Though citation of all the previous micro-complement fixation studies of frog albumins is beyond the scope of the present report, we list the families examined and representative works: Ranidae (Wallace, Masson et al., 1971; Wallace, King et al., 1973; Case, 1978; Post & Uzzell, 1981), Rhacophoridae (Wallace, Masson et al., 1971; Wallace, King et al., 1973), Hylidae (Masson & Wilson, 1975; Masson, 1976, 1977), Bufonidae (Masson, 1981a,b), Leptodactyliidae (Masson & Heyer, 1982), Myobatrachidae (Daugherty & Masson, In press), Discoglossidae (Masson & Szmyrta, 1979), Laeopelmatidae (Daugherty, Masson et al., In press), Asaphidae (L. Masson, pers. comm.), Pipidae (Bisbee, Baker et al., 1977), and Rhinophrynidae (Masson & Daugherty, 1980). With this report we add the Pelobatidae.

Materials and methods

Specimens and albumin purification

Serum samples were obtained from the nine different spadefoot toads listed in Table I. The preserved carcasses are deposited in the collection of the Museum of Vertebrate Zoology, University of California, Berkeley. Additional aliquots of serum, as well as tissues, are in the frozen tissue collection of the same institution.

* It has, however, recently been suggested by Roeluk (1982) and Spinath (cf. Ees & Sanchis, In press) that *Pelobates leptocephalus* may be a discoglossid rather than a pelobatid.
Results

Table II presents the results of micro-complement fixation tests in which all nine spadefoot toads were tested with the three different antisera. The two most salient features of these data are (1) the great difference between Pelobates and Scaphiopus and (2) the great similarity of the first four species, members of the subgenus Spea.

The average Pelobates Scaphiopus distance is 188 units if we consider only the reciprocal tests.* This value is close to three times as great as any intra-Scaphiopus value and suggests that these two genera separated long before any divergence occurred among the North American spadefoot toads.

The albumins of the members of Spea are seen as very similar by all three antisera. If the rates of evolution within Spea are relatively constant, it is to be expected that the outside reference points (i.e., S. couchii and Pelobates) would see them all as virtually equidistant. Indeed, S. intermontanus is indistinguishable from S. hammondii, and the most different representative of Spea, S. multiplicatus, differs from S. hammondii by only nine units.

TABLE II

<table>
<thead>
<tr>
<th>Amigen</th>
<th>Antiserum</th>
<th>S. hammondii</th>
<th>S. couchii</th>
<th>P. cultripes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scaphiopus (Spea)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. hammondii</td>
<td>0</td>
<td>68</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>S. intermontanus</td>
<td>0</td>
<td>69</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>S. bombifrons 1</td>
<td>5</td>
<td>68</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>S. bombifrons 2</td>
<td>5</td>
<td>70</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>S. multiplicatus</td>
<td>9</td>
<td>64</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Pelobates cultripes</td>
<td></td>
<td>125</td>
<td>187</td>
<td>0</td>
</tr>
</tbody>
</table>

*The values presented are derived from Table II. The averages of reciprocal values are shown in italics. The entries for S. holbrooki are averages for the subspecies S. h. holbrooki and S. h. hurteri.

The entry of 222 for the Pelobates-S. holbrooki comparison was derived as follows: it was noted (Table II) that the values determined with the antiserum to Pelobates tested against S. hammondii and S. couchii exceeded the reciprocal values by 58 and 68 units, respectively. It was further noted (Table II) that the anti-Pelobates versus S. holbrooki and S. couchii distances were virtually identical (256 and 255, respectively). It was therefore assumed that an antiserum to S. holbrooki would probably see Pelobates as about 68 units less distant than the antiserum to Pelobates saw S. holbrooki, giving a value of 236 minus 68, or 188 units. This computed value of 188 for anti-S. holbrooki versus Pelobates was then averaged with the measured 256 for anti-Pelobates versus S. holbrooki to yield 222.

Fig 1: Spadefoot toad evolutionary tree based on albumin immunological distances in Table III and constructed according to the Fitch-Margoliash method. The F value is 3.7% and the percentage standard deviation 7.9%. Two additional trees were constructed, in which the two alternative arrangements among the Scaphiopus lineages were explored. For these latter trees the F value was 9.9-13.4%, the percentage standard deviation 31-37%, and the sum of the negative branch lengths 12 or 30. The branching order and the relative branch lengths of the main lineages were the same for the UPGMA tree as shown here for the Fitch-Margoliash tree.
grouped most closely to one another, and *S. hammondii* represents the third main lineage within the genus. There appears to be considerable heterogeneity of rates of albumin evolution: the amount of change on the *S. hammondii* lineage is markedly less than that along the *S. holbrooki-couchi* lineage. Even within this latter clade there appears to be a two-fold difference in rates. In this regard it is worth remembering that the albumin clock is a probabilistic, not metronomic, clock (Carlson, Wilson et al., 1978).

In Fig. 2 albumin immunological and Nei electrophoretic measures of genetic distance are compared. Similar relative degrees of difference are measured by the two approaches and there is a good correlation between these two measures of genetic distance, as previously reported (Sarich, 1977; Maxson & Maxson, 1979). The least-squares line calculated from the points in Fig. 2 is rather different from the regression line and does not pass through the origin, but instead intersects the abscissa at $D=0.032$, corresponding to $I=0.73$. The correlation coefficient for the least-squares line is nevertheless high ($r=0.95$).

![Fig. 2. Albumin immunological distance versus electrophoretic distance for members of the genus *Scaphiopus*.](image)

**Discussion**

**Pelobates-Scaphiopus divergence**

Though the Old World spadefoot toads (*Pelobates*) bear a considerable morphological resemblance to the North American *Scaphiopus*, no evaluation of their relatedness at the molecular level has previously been made. The present albumin immunological comparison indicates that the divergence between these genera is ancient. Our estimates for the initiation of divergence of *Scaphiopus* and *Pelobates* range from 75 to 150 million years B.P., with a value of 110 million years calculated from the average intergeneric distance of 188 units. The minimum estimate predates the beginnings of the Cenozoic (65 million years ago). We thus argue in favour of a Cretaceous separation for the two genera. These results do not support our initial hypothesis of similar zoogeographic histories between spadefoot toads and *Hydromantes* salamanders.

Our suggestion of a Cretaceous divergence time within a family or subfamily is by no means unprecedented among frogs, but instead may be added to a long list of frog families in which immunological measurements indicate divergence at the familial or subfamilial levels during the Cretaceous. These groups include the Ranidae (Wallace, Maxson et al., 1971; Wallace, King et al., 1973), Hylidae (Wallace, Maxson et al., 1971; Maxson & Wilson, 1976, 1977), Bufonidae (Maxson, 1981a; Leptodactylidae (Maxson & Heyer, 1982), Myobatrachidae (Daugherty & Maxson, in press), and Pipidae (Bisbee, Baker et al., 1977). Indeed, a Cretaceous divergence time within genera has been reported for *Rana* (Wallace, Maxson et al., 1971; Wallace, King et al., 1973), *Hyla* (Wallace, Maxson et al., 1971), and *Bufo* (Maxson, 1981a). The earliest reported intrafamilial (subfamilial) divergence time and the comparison most similar to the *Pelobates-Scaphiopus* case may be that of *Xenopus* and *Hymenochirus*, both members of the subfamily *Xenopodinae* of the family Pipidae. The average intergeneric immunological distance of 188 units suggested an early Cretaceous divergence (Bisbee, Baker et al., 1977), a time which was consistent with fossil data indicating that *Xenopus* arose 90 million years ago and the Pipidae 130 million years or more ago (cf. references in Bisbee, Baker et al., 1977).

The Cretaceous estimate for the time of separation of *Pelobates* and *Scaphiopus* is not contradicted by any fossil evidence. Furthermore, it does produce specific predictions about the molecular similarity of other genera within the family Pelobatidae and about the occurrences of fossil types in the geological record. If the megophrynine genus *Epelobates* is ancestral to both *Pelobates* and *Scaphiopus*, then it is to be expected that all living members of this subfamily (*Megophrys*, *Leptobrachium*, *Nesobia*, *Oreolax*, *Scutiger*, *Vibrissaphora*) will express very low levels of cross-reaction in immunological comparisons when tested against these two pelobatine genera. Successful immunological tests, moreover, will probably have to be carried out using a more slowly evolving molecule than albumin. The fossil record predicts little about the relative similarity of *Pelodytes* to either the pelobatine or megophryine genera. The oldest relatives of *Pelodytes* come from Oligocene deposits in North America (Tihen, 1974). If the current trend to recognize *Pelodytes* as a member of a family different from Pelobatidae (Lynch, 1973; Duellman, 1973, Sokol, 1981a,b) represents the taxonomic recognition of important phyletic changes in this lineage, and not recently derived characters related to an aquatic lifestyle, then it is expected that *Pelodytes* will also show very little immunological resemblance to either *Pelobates* or *Scaphiopus*. A final prediction arising from our interpretation of the molecular data is that pelobatine fossil remains can be expected from throughout Cenozoic strata, and even back into Cretaceous rocks.

If the assumption of a clock-like rate of change of the albumin molecule is correct, and the divergence of the pelobatine frog genera occurred within the Cretaceous, these results indicate an extreme age for the “spadefoot toad” morphotype. Ample documentation shows that evolution at the molecular and organismal levels does not proceed at the same rate (e.g., Wilson, Maxson et al., 1974; Wilson, Sarich et al., 1974; King & Wilson, 1975; Maxson & Wilson, 1975; Wilson, Carlson et al., 1977; Wake, 1981). Thus, while at the protein
level frogs have evolved at the standard vertebrate rate, they have changed little at the anatomical and chromosomal levels in comparison with placental mammals (Wilson, Maxson et al., 1974; Wilson, Sarich et al., 1974). Unless future molecular studies report a closer relationship of Pelobates to either pelobatine genus, and thereby raise a question about the relationship of Pelobates to either pelobatine genus, the most reasonable explanation of the ancestral phenotype of such a combined lineage, the most reasonable explanation of the morphological similarity between Pelobates and Scaphiopus is evolutionary stasis since the time of their separation in the Cretaceous.

Intra-Scaphiopus relationships

Relationships within the genus Scaphiopus have been considered on the basis of fossil and osteological data (Zweifel, 1956; Kluge, 1966), artificial hybridization experiments (Zweifel, 1957, 1964, 1970; Wasserman & Bogart, 1968), chromosomal analyses (Wasserman, 1957, 1964, 1970; Wasserman & Bogart, 1968; Wasserman, 1970), and electrophoretic results (Sattler & Wasserman, 1968; Wasserman & Bogart, 1970; Sattler, 1980). While the assemblages of species assigned to Spea and Scaphiopus are well differentiated, most recent authors favor treating these taxa as subgenera. The oldest Spea fossil is from the early Miocene (Kluge, 1966) and the oldest Scaphiopus fossil is from the early Oligocene (Holman, 1968).

The evolutionary tree proposed in Fig. 1 is identical in branching structure to the cladogram derivated by Sattler (1980) from electrophoretic evidence. We do not include S. bombifrons and S. multiplicatus in our Figure because we lacked antisera to these two species. However, our one-way comparisons of both these species to S. hammondii and S. couchii (Table II) show that S. hammondii is closer to S. bombifrons than it is to S. multiplicatus, and that these three species are much closer to each other than they are to S. couchii. These results are consistent with the percentage of successful hybrid development. S. hammondii (Wasserman, 1957, 1964, 1970) and the degree of karyotypic (Wasserman & Bogart, 1968; Wasserman, 1970) and electrophoretic (Sattler, 1980) similarity within Spea and between the two subgenera. Figure 2 depicts the agreement of relationships predicted from electrophoretic and immunological measurements. Scaphiopus intermedius, in turn, we found to be indistinguishable from S. hammondii at the albumin locus; neither Sattler (1980) nor Wasserman (1957, 1970) studied this species.

Our data support the recognition of at least two distinct groups within the genus Scaphiopus, as predicted by the osteological, developmental, and electrophoretic data. Scaphiopus, as defined by Zweifel (1956, 1966), Kluge (1966), Estes (1970), and holbrooki (Zweifel, 1956, 1966, 1970) is the subgenus of the lineages leading to S. couchii and S. holbrooki began about 21 million years ago, according to our data and interpretations. Neither species has a Tertiary fossil record, but the subgenus is known from the early Oligocene, over 30 million years ago. Thus all of our interpretations concerning time are consistent with known fossil occurrences.

Our data suggest that S. holbrooki is somewhat more closely allied to S. hammondii than is S. couchii. At the same time, S. holbrooki and S. couchii are closer to each other than to any other species examined. It is possible that the subgenus Scaphiopus is a paraphyletic taxon. Zweifel (1956) explicitly states that the subgenus Scaphiopus is more primitive than Spea. Kluge (1966) curiously avoided discussion of ancestral and derived states, but his interpretation of his Table 5 leads us to support Zweifel's (1956) contention. A duplicated isocitrate dehydrogenase locus in the species belonging to the subgenus Spea (Sattler & Mecham, 1979) is an additional derived (molecular) character which distinguishes these frogs from the subgenus Scaphiopus. If the subgenus Scaphiopus is founded mainly on ancestral traits (such as maxilla and squamosal in contact, dermal encrustation on skull, pterygodid process of maxilla present, patellae present, parotid glands present, etc.), it is possible that Spea (apparently a monophyletic taxon based on shared, derived traits) was derived from a lineage within Scaphiopus that had already diverged from the ancestors of S. couchii, but was ancestral to the stock that gave rise to S. holbrooki. The anti-Pelobates data argue against such an interpretation, for the distances between Pelobates and Spea (177–183, mean 181) are substantially less than those between Pelobates and Scaphiopus (254–257, mean 255). This result, however, is mitigated by the fact that we have more confidence in measurements in the 35–70 range than in the range between 170 and higher values. Our estimates of timing within the genus Scaphiopus must be interpreted cautiously, for the above data strongly suggest differences in the rates of change along various lineages. The Fitch-Margoliash tree in Fig. 1 implies that virtually all change between Spea and Scaphiopus occurred in the latter lineage.

Cladogenesis and biogeographic models

Prior to the study of Wake, Maxson et al. (1978), various authors had provided evidence that the two European species and the three Californian species of Hydromantes were more closely related to congeners on the same continents than on different continents. The immunological study did not change this cladistic hypothesis, but it did provide a time estimate, 28 million years, for the separation of the two major lineages. Lana & Vanni (1981) have recently asserted that the chronology proposed by Wake, Maxson et al. (1978) is wrong, based on the idea that it is unlikely that the Bering land bridge was used as a dispersal route by Hydromantes. Their arguments are based solely on what they find credible about distributional patterns, rather than on any data, and thus cannot be considered further.

As in the case of Hydromantes, our analysis of relationships among pelobatids does not require any change in existing cladistic hypotheses, which group the American species relatively closely and separate them from Pelobates. If the salamanders and frogs had undergone a similar zoogeographic history, one would expect a common time estimate for initiation of divergence of the two major groups. In fact, albumin is much more differentiated among the frogs than the salamanders, and differentiation within Scaphiopus alone exceeds that within the entire genus Hydromantes.

Some other studies have examined the degree of albumin differentiation between European and North American amphibians. Maxson & Wilson (1975) argued that North American and Eurasian Hydros separated from each other about 40 million years ago. Wallace, King et al. (1973) estimate a time of emergence of North American and Eurasian Rana of 33 to 37 million years. Both of these estimates are substantially greater than the estimates for Hydromantes and much less than those for pelobatines. Accordingly, we conclude that Holarctic amphibians have gained their current distributions at different times, and no single vicariant event is likely to be responsible for current cladistic and distributional patterns.
Summary

In the present report we have obtained immunological information confirming published evolutionary relationships among the spindlefoot toads of the genus Scaphiopus, based on electrophoretic studies (Sattler, 1980). We have extended the comparison of the family Pelobatidae to include measurements of the Pelobates-Scaphiopus difference, which suggests a divergence time of around 110 million years and the ancient establishment of the spindlefoot toad morphology. Evolutionary stasis is the probable explanation for the present similarity in appearance of these two genera. The molecular data indicate that the biogeographic history of the spindlefoot toads was very different from that of congeneric plethodontid salamanders with a similar Holarctic distribution.

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REFERENCES


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**Note Added in Proof**

After this paper was accepted for publication, O. M. Sokol kindly provided us with specimens of *Pelodytes punctatus* (preserved carcasses on deposit in the Department of Anatomy, College of Medicine, University of South Alabama, Mobile; serum and tissues in the frozen collection of the Museum of Vertebrate Zoology, University of California, Berkeley). With the three antisera used in this work, *Pelodytes* albumin shows less resemblance to *Scaphiopus* and *Pelobates* than these genera do to one another, both in immunodiffusion tests, judged by line strengths and patterns of spur formation, and micro-complement fixation tests. The immunological distances (cf. Table II) measured with antisera to *S. hammondi*, *S. couchi*, and *Pelobates* are 181, 222, and 208, respectively. (*Rana pipiens*, *Bufo boreas*, and *Xenopus laevis* were used as controls and did not react at all in immunodiffusion tests with the three antisera.) These results are consistent with the scenario presented in the Discussion, recognizing a remote alliance of *Pelodytes* to the pelobatines and suggesting divergence off of the lineage leading to *Pelodytes* prior to the *Pelobates-Scaphiopus* split. The large immunological distances between *Pelodytes* and the pelobatine genera parallel and morphological differences observed in the adults (Lynch, 1973) and also in the larval chondrocrania and larval filter apparatuses (Sokol, 1981a,b).

It is of interest, furthermore, that although the spadefoots *Scaphiopus* and *Pelobates* are commonly considered to be morphologically similar in many respects, Sokol (1981a,b) finds their larval chondrocrania and filter apparatuses to be quite different. It should also be noted that Roček (1982) places these two genera in different families and suggests that they did not arise from a common *Eopelobates* ancestor. Roček's (1982) interpretation of the fossil evidence is at variance with that of Estes (cf. Introduction), but this paleontological controversy is beyond the scope of the present report.