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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.

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Introduction

Biogeographic scenarios are created by evolutionary biologists when they repeatedly see similar patterns between taxonomic groupings and geographic distributions. This study was

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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.

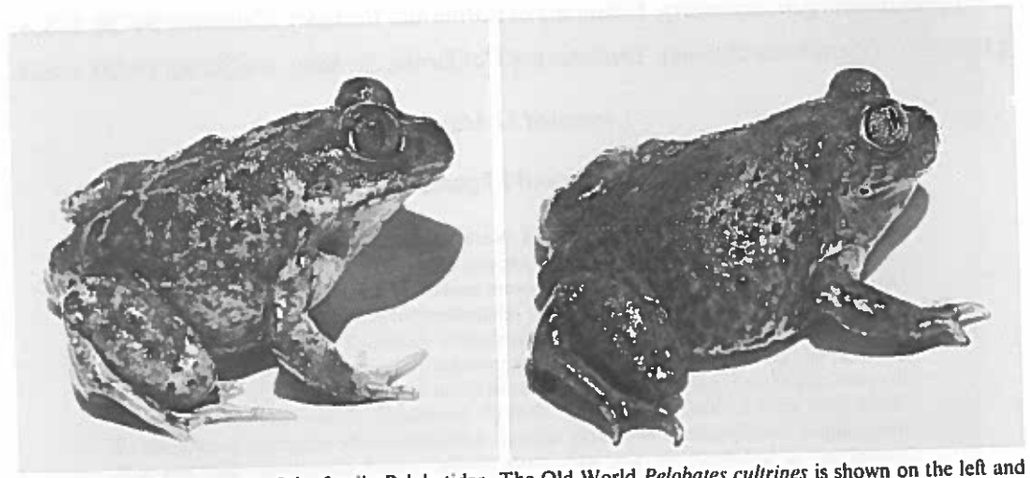

PLATE I. Spadefoot toads of the family Pelobatidae. The Old World *Pelobates cultripes* is shown on the left and the North American *Scaphiopus hammondi* on the right. The spade on the right hind foot of *S. hammondi* appears particularly prominent. Other readily apparent similarities of these two genera include the vertical pupils and the overall body shape. The snout-to-vent length of *P. cultripes* runs between 5 and 7·5 cm and that of *S. hammondi* between 4 and 6·5 cm.

Highly specialized salamanders of the genus *Hydromantes* are found in western North America and in a restricted part of Europe. The great distance separating similar animals of these two regions has been regarded as a major zoogeographical enigma. A biochemical study of the genetic relationships in this genus (Wake, Maxson *et al.*, 1978) showed that this was a natural assemblage of species, more closely related to one another than to any other plethodontid genus. This result eliminates from further consideration any hypotheses that the morphological similarity of the animals on the two continents is due to evolutionary convergence. Furthermore, interpretation of the protein data suggested that the European and American units last shared a common ancestor at the end of the Oligocene, about 28 million years ago.

"Spadefoot" frogs of the family Pelobatidae are found in North America and in Europe and fringing areas of northern Africa. They share a characteristic keratinous tubercle on the hind foot which aids them in digging into sandy soils and is the basis for their common name. Three species of spadefoot toads live in Europe and north Africa (genus *Pelobates*)* and six

*Pasteur & Bons (1959) describe a fourth species, *Pelobates varaldii* of Morocco. A consensus is lacking as to whether *P. varaldii* is really a distinct species. Some (*e.g.* Roček, 1982) recognize it as such, while others (*e.g.*, Sanchíz, 1977; Arnold & Burton, 1978) consider *P. varaldii* synonymous with or a subspecies of *P. cultripes*. Our designation of the Moroccan *Pelobates* sample used here as *P. cultripes* (Table I) reflects this latter view.

species occur in North America (genus *Scaphiopus*). The American species are placed in two subgenera: *Scaphiopus* and *Spea*. Although the Old and New World species are placed in different genera, the animals show many external similarities besides the distinctive spades (Plate I and illustrations in Stebbins, 1966; Conant, 1975; Arnold & Burton, 1978). Savage (1973) found that the restricted European-North American distribution of pelobatid genera was a unique case during a comprehensive analysis of the biogeography of frogs. He proposed that *Pelobates* was derived from a *Scaphiopus*-like ancestor that migrated to Eurasia during the Oligocene, the same time of separation as suggested for the salamanders. We performed an immunological study of all of the North American spadefoots against one species from Europe. The test was whether spadefoot albumin molecules would give comparable results to *Hydromantes* and support the hypothesis of parallel zoogeographical histories between similar cognate species in diverse groups of amphibians living on different continents.

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; Špinar, Boubelík *et al.*, 1971). Duellman (1975) recognized a superfamily Pelobatoidea which contained two families. The Pelodytidae (genus *Pelodytes*) has living representatives only in Europe, but the group was present in the Oligocene of North America (Tihen, 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 megophryine. 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 only the so-called spadefoots, *Scaphiopus* of North America and *Pelobates* of Europe and extreme northwestern Africa. The earliest pelobatine in Europe is from latest Oligocene to earliest Miocene, 25 to 26 million years B.P. (R. Estes, pers. comm.), and the earliest North American pelobatine (*Scaphiopus skinneri*) is from early Oligocene, 32 to 36 million years B.P. (Holman, 1968; R. Estes, pers. comm.). Zweifel (1956) and Estes (1970) argued in favour of a common ancestor for the two genera. While the putative ancestor, *Eopelobates*, occurred in both the Old and New Worlds, Estes (1970) favoured 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 migration of spadefoots to Eurasia via the Bering land bridge in the Oligocene.

Estes (pers. comm.) has outlined for us his current ideas concerning pelobatine origins and historical biogeography. Based on the presence of *Eopelobates leptocolaptus* in the late Cretaceous of Mongolia (Borsuk-Białynicka, 1978), which has clear pelobatine resem-

blances,* as well as the presence in Mongolia in the Oligocene of *Macropelobates* (which he thinks is probably more like *Pelobates* than like *Scaphiopus* but more primitive than either extant genus), Estes now suggests an Asian origin of pelobatines. He believes the latest Eocene-earliest Oligocene was an important evolutionary time, and that it may have been during this period that pelobatines spread.

Immunological comparisons of frogs

The immunological technique employed here, quantitative micro-complement fixation, has been used for extensive comparisons of albumins from diverse groups of vertebrates. These studies indicated that micro-complement fixation can assess point mutational difference 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 (Maxson, Sarich *et al.*, 1975; Prager & Wilson, 1975; Wilson, Carlson *et al.*, 1977; Carlson, Wilson *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, Maxson *et al.*, 1974; Wilson, Sarich *et al.*, 1974; Maxson, Sarich *et al.*, 1975; Wilson, Carlson *et al.*, 1977; Carlson, Wilson *et al.*, 1978).

The past decade has 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 Maxson 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, Maxson *et al.*, 1971; Wallace, King *et al.*, 1973; Case, 1978; Post & Uzzell, 1981), Rhacophoridae (Wallace, Maxson *et al.*, 1971; Wallace, King *et al.*, 1973), Hylidae (Maxson & Wilson, 1975; Maxson, 1976, 1977), Bufonidae (Maxson, 1981*a*,*b*), Leptodactylidae (Maxson & Heyer, 1982), Myobatrachidae (Daugherty & Maxson, In press), Discoglossidae (Maxson & Szymura, 1979), Leiopelmatidae (Daugherty, Maxson *et al.*, In press), Ascaphidae (L. Maxson, pers. comm.), Pipidae (Bisbee, Baker *et al.*, 1977), and Rhinophrynidae (Maxson & 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 Roček (1982) and Špinar (cf. Estes & Sanchíz, In press) that *Eopelobates leptocolaptus* may be a discoglossid rather than a pelobatid.

TABLE I
Species and localities of spadefoot toads

Species	Locality
Scaphiopus hammondi	Boundary of Alameda and San Joaquin Cos., California
S. intermontanus	Mono Co., California
S. bombifrons 1	Payne Co., Oklahoma
S. bombifrons 2	Cochise Co., Arizona
S. multiplicatus	Lubbock Co., Texas
S. holbrooki holbrooki	Beaufort Co., North Carolina
S. holbrooki hurteri	Payne Co., Oklahoma
S. couchi	Cochise Co., Arizona
Pelobates cultripes	Rabat-Salé Prefecture, Morocco

Albumin was purified from the serum of *Pelobates cultripes*, *Scaphiopus hammondi*, and *S. couchi* by a single preparative electrophoresis step on 7% polyacrylamide slab gels at pH 8·9, essentially as described (Prager & Wilson, 1975; Prager, Fowler *et al.*, 1976).

Immunological techniques

Each purified albumin was injected into two or three Dutch Belted rabbits according to a schedule and protocol similar to those described (Prager & Wilson, 1975; Prager, Fowler *et al.*, 1976): initial immunization in Freund's supplemented complete adjuvant, Freund's incomplete adjuvant at seven weeks, intravenous injections at 10 and 11 weeks, bleeding by cardiac puncture at 12 weeks. Each rabbit received approximately 100–300 μg of pure albumin per injection.

The resulting antisera to each immunogen were heated, stored, and pooled in inverse proportion to their micro-complement fixation titres as described (Champion, Prager *et al.*, 1974). The titres (for 75% peak fixation with the homologous antigen, the immunogen) of the pools of the three antisera directed towards *P. cultripes* and *S. hammondi* albumin were, respectively, 4600 and 3200; the pool titre of the two antisera elicited by *S. couchi* albumin was 5200. The slopes of the antiserum pools, which are a measure of the variation in peak complement fixation as a function of antiserum concentration (Champion, Prager *et al.*, 1974), ranged from 332 to 374. Both the titres and slopes are typical of rabbit antisera to frog albumins (cf. Wallace, Maxson *et al.*, 1971; Champion, Prager *et al.*, 1974).

That the antibodies were directed overwhelmingly if not solely towards albumin was demonstrated by the fact that the same micro-complement fixation curves were obtained whether purified albumin or whole serum was used as the antigen. In the Ouchterlony double diffusion test (done according to Prager, Brush *et al.* (1974)), a single line of immunoprecipitation was observed when the antiserum pools were tested against whole serum.

Quantitative micro-complement fixation was carried out as described (Champion, Prager *et al.*, 1974) except that all buffers contained 2·5 μg of merthiolate per ml. Whole sera were used as the antigen sources, except in the case of *Pelobates*, where the purified albumin was generally used.

Immunological distance, which is the measure of the degree of antigenic difference in the micro-complement fixation test, is equal to 100 times the log of the factor by which the antiserum concentration must be raised for a heterologous antigen to produce a complement fixation curve whose peak height is equal to that produced by the homologous antigen (Champion, Prager *et al.*, 1974). There is a strong correlation between immunological distance and amino acid sequence difference among related proteins, such that for several proteins of known sequence a 1% sequence difference results in 5–7 units of immunological distance (Prager, Welling *et al.*, 1978).

Calculations

Evolutionary trees were constructed according to the method of Fitch & Margoliash (1967) and evaluated as described by Prager & Wilson (1978). The UPGMA (unweighted pair-group) method (Sneath & Sokal, 1973) was also used for tree construction.

For comparison of the immunological distances presented here with electrophoretic measures of genetic distance among members of the genus *Scaphiopus*, we used the Nei identity (I) values given in Table II of Sattler (1980). We converted I to genetic distance, D, with the equation $D = -\ln I$ (Nei, 1975).

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. couchi* and *Pelobates*) would see them all as virtually equidistant. Indeed, *S. intermontanus* is indistinguishable from *S. hammondi*, and the most different representative of *Spea*, *S. multiplicatus*, differs from *S. hammondi* by only nine units.

TABLE II

Immunological distances among spadefoot toad albumins

Antigen	Antiserum		
	S. hammondi	*S. couchi*	*P. cultripes*
Scaphiopus (Spea)			
S. hammondi	0	68	183
S. intermontanus	0	69	183
S. bombifrons 1	5	68	178
S. bombifrons 2	5	70	177
S. multiplicatus	9	64	183
Scaphiopus (Scaphiopus)			
S. holbrooki holbrooki	44	35	254
S. holbrooki hurteri	38	35	257
S. couchi	59	0	255
Pelobates cultripes	125	187	0

*The percentage standard deviation from perfect reciprocity (Champion, Soderberg *et al.*, 1975) for the three comparisons in which antisera to both species were available is 14·6%. This value is in agreement with those obtained from data sets for many different proteins (*e.g.*, Champion, Soderberg *et al.*, 1975; Maxson & Wilson, 1975; Prager, Welling *et al.*, 1978).

Results of the semi-quantitative Ouchterlony double diffusion test (Prager, Fowler *et al.*, 1976) among *S. hammondi*, *S. h. holbrooki*, *S. couchi*, and *Pelobates* with the three antisera were entirely consistent with the results shown in Table II.

Table III summarizes the immunological distances used to construct evolutionary trees among the four main lineages studied here: *Spea* (represented by *S. hammondi*), *S. holbrooki*, *S. couchi*, and *Pelobates*. Figure 1 shows the most likely relationships among these principal lineages based on the values in the table. As was apparent from Tables II and III, the relationship of *Pelobates* to *Scaphiopus* is a distant one. *Scaphiopus holbrooki* and *S. couchi* are

TABLE III

*Average immunological distances among spadefoot toad albumins**

Species compared	*S. hammondi*	*S. holbrooki*	*S. couchi*	*P. cultripes*
Scaphiopus hammondi	–	41	*64*	*154*
S. holbrooki		–	35	222†
S. couchi			–	*221*
Pelobates cultripes				–

*The values presented are derived from Table II. The averages of reciprocal values are shown in italics. The entries for *S. holbrooki* are averages of the values 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. hammondi* and *S. couchi* 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. couchi* 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 256 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.

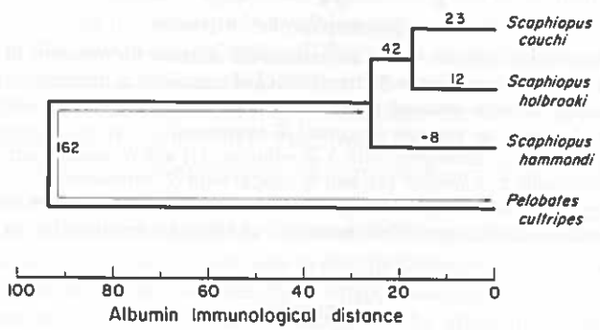

FIG. 1. Spadefoot toad evolutionary tree based on albumin immunological distances in Table III and constructed according to the Fitch-Margoliash method. The F value is 3·3% and the percentage standard deviation 7·5%. Two additional trees were constructed, in which the two alternative arrangements among 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. hammondi* 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. hammondi* 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 $D=0{\cdot}32$, corresponding to $I=0{\cdot}73$. The correlation coefficient for the least-squares line is nevertheless high ($r=0{\cdot}95$).

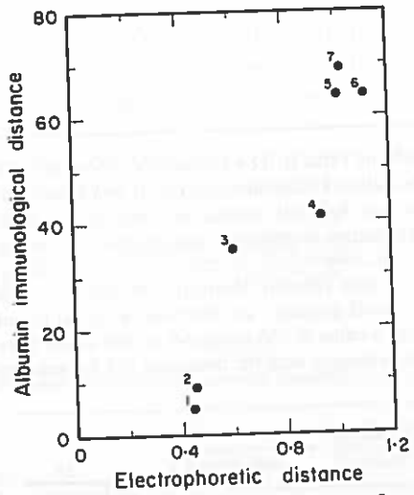

FIG. 2. Albumin immunological distance versus electrophoretic distance for members of the genus *Scaphiopus*. The electrophoretic distances, *D*, were based on Sattler (1980) and calculated as described (*Materials and methods*). The immunological distances, *y*, were obtained from Table II. Values for the two *S. bombifrons* and the two *S. holbrooki* specimens were averaged, as were the reciprocal *S. hammondi-S. couchi* comparisons. The seven points plotted represent comparisons of *S. hammondi* with *S. bombifrons* (1) and *S. multiplicatus* (2); *S. couchi* with *S. holbrooki* (3); *S. hammondi* with *S. holbrooki* (4); and *S. couchi* with *S. hammondi* (5), *S. multiplicatus* (6), and *S. bombifrons* (7). The regression line through the origin is described by $y=55D$. The line calculated by the method of least squares has a correlation coefficient *r* of 0·95 between *y* and *D* and is described by the equation $y=87D-28$.

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, 1975; Maxson, 1976, 1977), Bufonidae (Maxson, 1981*a*), 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, 1981*a*). 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 Xenopinae 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 megophryine genus *Eopelobates* is ancestral to both *Pelobates* and *Scaphiopus*, then it is to be expected that all living members of this subfamily (*Megophrys*, *Leptobatrachium*, *Nesobia*, *Oreolalax*, *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, 1975; Sokol, 1981*a*,*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 *Pelodytes* to either pelobatine genus, and thereby raise a question about 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 (Wasserman, 1957, 1964, 1970; Wasserman & Bogart, 1968), chromosomal analyses (Wasserman & Bogart, 1968; Wasserman, 1970), and electrophoretic results (Sattler & Mecham, 1979; Sattler, 1980). While the assemblages of species assigned to *Spea* and *Scaphiopus* are well differentiated, most recent authors favour 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 dendrogram derived 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. hammondi* and *S. couchi* (Table II) show that *S. hammondi* 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. couchi*. These results are consistent with the percentage of successful hybrid development (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 intermontanus*, in turn, we found to be indistinguishable from *S. hammondi* 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. Within the subgenus *Spea* divergence of the extant taxa may have begun as little as six million years ago. No living species has a fossil record that extends back earlier than the Pleistocene (Zweifel, 1956; Kluge, 1966; Estes, 1970).

Within the subgenus *Scaphiopus* divergence of the lineages leading to *S. couchi* 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. hammondi* than is *S. couchi*. At the same time, *S. holbrooki* and *S. couchi* 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 our 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, pterygoid process of maxilla present, palatine present, paratoid 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. couchi*, 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. Lanza & Vanni (1981) have recently asserted that the chronology proposed by Wake, Maxson *et al.* (1978) is wrong, based on their belief 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 *Hyla* separated from each other about 40 million years ago. Wallace, King *et al.* (1973) estimate a time of divergence 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 spadefoot 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 spadefoot 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 spadefoot toads was very different from that of congeneric plethodontid salamanders with a similar Holarctic distribution.

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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 the morphological differences observed in the adults (Lynch, 1973) and also in the larval chondrocrania and larval filter apparatuses (Sokol, 1981*a*,*b*).

It is of interest, furthermore, that although the spadefoots *Scaphiopus* and *Pelobates* are commonly considered to be morphologically similar in many respects, Sokol (1981*a*,*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.