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A MOLECULAR PHYLOGENETIC PERSPECTIVE ON THE ORIGINS OF MORPHOLOGICAL NOVELTIES IN THE SALAMANDERS OF THE TRIBE PLETHODONTINI (AMPHIBIA, PLETHODONTIDAE)

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A major goal of evolutionary research is the elucidation of the process by which large morphological and adaptive discontinuities arise in evolving lineages. There is much disagreement as to the validity of explaining macroevolutionary change by microevolutionary mechanisms. Bock (1979), for example, concluded that macroevolution is simply the consequence of additive microevolutionary changes; whereas Gould (1980) envisaged a potential saltational origin for the essential features of key adaptations. In a recent review of paleontological discoveries, Stanley (1979) argued that macroevolution cannot be adequately explained by microevolutionary mechanisms.

Bock (1979) stated that if a macroevolutionary modification can be shown to be a summation of a sequence of small steps, each of which can be explained by mechanisms of microevolution, then the macroevolutionary explanation can be reduced to a microevolutionary one and no additional mechanisms are required. He suggested a "sequential species analysis" of species-rich genera or groups of related genera, in which the end forms demonstrate a large difference in morphology or other features and which contain enough species to illustrate intermediate stages, in order to determine whether such a summation of small steps provides a plausible explanation of macroevolution. Such an analysis must include detailed comparisons of the descriptive, functional and

ecological morphology of the features involved in the evolutionary change.

The North American salamanders of the tribe Plethodontini (genera *Aneides, Ensatina* and *Plethodon*) provide an opportunity to study the morphological changes associated with a shift from a terrestrial to an arboreal adaptive zone. These salamanders comprise one of two plethodontid lineages that lack an aquatic larval stage and thus have evolved true terrestriality. *Ensatina* and *Plethodon* are terrestrial. *Plethodon* is believed to resemble the ancestor of this group closely in ecology and morphology (Wake, 1960); *Ensatina* is a morphologically distinct derivative of the ancestral stock (Wake, 1963, 1966). Compared to *Plethodon,* the species of *Aneides* show a progression of morphological and ecological specializations for arboreal and scansorial locomotion that are paralleled by changes that strengthen the jaws (Wake, 1960, 1963). We present a sequential species analysis of these morphological changes in the framework of phylogenetic reconstructions from electrophoretic and immunological protein comparisons to evaluate the evolutionary origin of this adaptive and morphological transition.

Materials and Methods

Cladistic Analysis

The five species of *Aneides, Ensatina* (the single species *eschscholtzii*) and *Plethodon* (all species) are treated as separate units for morphological analysis. Data were presented by Lowe (1950), Wake (1960, 1963, 1966), Highton (1962), Wake and Dresner (1967) and Lombard and Wake (1977). We analyze these data to examine concordance or lack thereof between independently derived data sets (for discussion of this approach, see Nelson, 1979). We selected for a cladistic analysis 17 morphological characters that show low variability and are known to distinguish groups. Our criterion for the determination of ancestral and derived states is outgroup comparison (Hennig, 1966; Eldredge and Cracraft, 1980), using other tribes and subfamilies of plethodontid salamanders, and related groups such as ambystomatids. A description of the characters and their alternative states follows; ancestral states are denoted by upper case letters and derived states by the corresponding lower case letter:

1. *Tail base region.* Three general conditions were recognized in plethodontids by Wake and Dresner (1967). *A.* The wound-healing specialization, considered to be a derived state in the Plethodontidae, is associated with specializations for tail loss, and is found in *Aneides* and *Plethodon. a.* The constricted-base tail, a further derivation of this state, is associated with true tail autotomy in *Ensatina.*
2. *Tongue. B. Aneides* and *Plethodon* have relatively generalized tongues (Mode II; Lombard and Wake, 1976, 1977), with broad pads that are seldom used for projection beyond the mouth. *b.* The tongue of *Ensatina* is also relatively generalized (Mode III; Lombard and Wake, 1976, 1977) but is smaller, more projectile and has a number of structural differences in relation to *Aneides* and *Plethodon.*
3. *Legs and their use. C. Aneides* and *Plethodon* use a sprawling locomotion, with the legs held out from the body as in many other groups of salamanders. The hind legs are longer than the front legs, and the femur exceeds the humerus in length. There are no plantar tubercules. *c. Ensatina* has long legs that can be held rigid, directly under the body. The humerus is as long or longer than the femur (Wake, 1963) and distinct plantar tubercules are present.
4. *Jaws and the extent of tooth-bearing surfaces. D.* Relative to *Aneides* and *Plethodon, Ensatina* has weak jaws (slender dentaries and maxillaries and low, small prearticulars), and long tooth rows. The tooth-bearing preorbital process of the vomer is relatively very long. All teeth are smaller and

more numerous than those of *Aneides* and *Plethodon*. The condition in *Ensatina* resembles that of ambystomatids. *d*. In *Aneides* and *Plethodon,* the maxillary, premaxillary, dentary and prearticular bones are stouter than in *Ensatina* but the vomer is smaller. The tooth-bearing areas are less extensive and the teeth are larger.

5. *Premaxillary bones. E*. The presence of two premaxillary bones, found in *Ensatina* and *Plethodon,* is an ancestral state. *e*. A single premaxillary, found in *Aneides,* is derived (Wake, 1966).
6. *Number and structure of teeth. F*. Salamander teeth are primitively small and bicuspid, as in *Ensatina* and *Plethodon* (Lowe, 1950; Wake, 1963). The teeth of *Plethodon* are slightly larger than those of *Ensatina*. *f*. The teeth of *Aneides* are larger and less numerous than those of *Plethodon,* and at least the males of all species have some large, unicuspid teeth. Interspecific variation in the tooth structure of *Aneides* is great (Wake, 1963).
7. *Arrangement of superficial throat musculature. G. Ensatina* and *Plethodon* have a generalized arrangement of throat musculature and lack the gularis muscle. *g*. The throat muscles of *Aneides* are arranged differently than in *Ensatina* and *Plethodon* and the gularis muscle is present (Piatt, 1935; Hilton, 1952; Wake, 1960, 1966). The gularis muscle strengthens the throat-constricting musculature.
8. *Tarsal organization. H. Ensatina* and *Plethodon* have tarsal cartilages arranged as in all five-toed members of the plethodontid subfamily Desmognathinae and tribe Hemidactylini: the fifth tarsal is small and is excluded from an articulation with the centrale by the articulation of the relatively large fourth distal tarsal with the fibulare. *h*. In *Aneides,* the fifth distal tarsal is relatively large and it articulates with the centrale; the relatively small fourth distal tarsal is thereby excluded from articulating with the fibulare. As a result of this reorganization, the entire tarsus is narrower than in more generalized salamanders, and there is a more channeled distribution of force from the arm to the digits (Wake, 1960; 1963, Fig. 6; 1966).
9. *Carpus organization. I*. The carpus is relatively broad in *Ensatina* and *Plethodon,* and the centrale does not articulate with the ulnare because of a broad articulation of the intermedium and fourth distal carpal. This is the typical condition in generalized salamanders. *i*. In *Aneides* the carpus is narrowed and the four elements mentioned above meet, or nearly meet, in a four-way intersection (Wake, 1963, Fig. 6).
10. *Terminal phalanges. J*. The terminal phalanges of *Ensatina* and *Plethodon* are like those of most salamanders in being rounded at the tip. *j*. Those of *Aneides* are distally flattened, expanded and recurved, with a proximal, ventrally directed process for attachment of a large ligament (Lowe, 1950; Wake, 1963). These are climbing specializations, and the proximal portion of the phalanx has a pronounced ventral projection to which is attached a strong tendon (Wake, 1963).
11. *Maxillary-prefrontal articulation. K*. The facial process of the maxillary bone barely overlaps the prefrontal bone in *Ensatina* and *Plethodon*. *k*. In *A. aeneus* and *A. hardii* the maxillary overlaps the prefrontal extensively. *k'*. In the remaining *Aneides,* the two bones interlock in a strong, complex articulation (Wake, 1963); this condition is derived from state *k*.
12. *Skull shape. L*. The western *Aneides* have skulls that are shaped generally like those of *Ensatina* and *Plethodon* (cf. Figs. 1–4, Wake, 1963). The skulls are higher and narrower than

in *A. aeneus,* and the frontals often appear "pinched" between the eyes. *l. Aneides aeneus* has a broad, flat skull, and the facial region is rather short (Wake, 1963, Fig. 2). The nasals are about as broad as long and the frontal processes of the premaxillae are short and weak. This is part of a general flattening of the body in this species, which often inhabits granite crevices.

13. *Otic crests.* Dorsal crests in the otic region provide area for the origin of jaw and head raising muscles and are found in males of all species of *Aneides* (Wake, 1963). Low crests in different positions on the otic capsule occur in *Ensatina* and some species of *Plethodon. M.* In *A. aeneus* crests are clearly present but poorly developed. *m.* In the remaining species crests are well developed and conspicuous.
14. *Carpal fusion. N.* All species of *Aneides, Ensatina* and *Plethodon,* except *A. hardii,* have eight carpal elements. *n.* There are only seven carpal elements in *A. hardii* as a result of the fusion of the ulnare with the intermedium (Wake, 1963).
15. *Vomers. O. Ensatina, Plethodon, A. aeneus* and *A. hardii* have vomers bearing preorbital processes, as in generalized species. *o.* Vomerine processes are absent from the remaining species of *Aneides* (Wake, 1963).
16. *Limb and digit length. P.* The limbs and digits of *A. flavipunctatus* and *A. hardii* are relatively short and resemble those of *Plethodon* and other generalized plethodontids (Wake, 1963). *p.* The limbs and digits of *A. aeneus, A. ferreus* and *A. lugubris* are relatively long.
17. *Coossification. Q.* The absence of coossification is ancestral. *q.* The anterior cranial elements of the face of *A. lugubris* are extensively coossified with the overlying skin. This condition is unique in the tribe Plethodontini, and is found elsewhere among plethodontids only in *Phaeognathus* (Wake, 1963, 1966).

Protein Comparisons

Microcomplement fixation was used to compare the albumins of five species of *Aneides* to each other, to two eastern plethodons (*Plethodon glutinosus, P. richmondi*), to two western plethodons (*P. neomexicanus, P. vehiculum*) and to *Ensatina eschscholtzii.* The antisera to albumins of *Ensatina* and *Plethodon* were prepared and described in an earlier study (Maxson et al., 1979). Individual antisera were prepared to albumins of each of the five species of *Aneides* by published procedures (Maxson et al., 1979). A total of 1–1.5 mg of albumin was administered per rabbit over the 13-week immunization period. Three rabbits were used for each antiserum pool with the exception of *A. flavipunctatus* for which two rabbits produced sufficiently high titer antibodies for use. We pooled antisera that have been tested for purity (Champion et al., 1974) and used these pools to determine albumin immunological distances, which are roughly equivalent to the number of amino acid differences between the albumins compared (Maxson and Wilson, 1974).

The averaged reciprocal values of albumin immunological distance were used to construct phylogenetic trees according to the procedure of Fitch and Margoliash (1967). The relationships of five groups of species were evaluated by averaging the immunological distance comparisons for the species included in them and constructing trees corresponding to five hypothetical relationships of the groups. The five groups are as follows: eastern *Aneides* (*A. aeneus*), western *Aneides* (*A. ferreus, A. flavipunctatus, A. hardii, A. lugubris*), *Ensatina eschscholtzii,* eastern *Plethodon* (*P. glutinosus, P. richmondi*) and western *Plethodon* (*P. neomaxicanus, P. vehiculum*). The geographical units corresponding to eastern and western *Aneides* and *Plethodon* are shown in Figure 1. We also constructed phylogenetic trees from albumin immunological distances by treating

all species as distinct taxa. Alternative phylogenetic trees were evaluated by the "percent 'standard deviation'" criterion of Fitch and Margoliash (1967) and by the degree to which negative branch lengths are minimized. The degree of regularity of albumin evolution was evaluated by comparing the amounts of change occurring along the diverging branches of the tree showing the best fit to the data set (Wilson et al., 1977, and included references). We estimated times of divergence using the relationship that each unit of albumin immunological distance corresponds to approximately 0.583 Myr of separation (Maxson and Maxson, 1979).

Electrophoretic comparisons of proteins were used to provide independent evaluations of the relationships of the *Aneides* species using *P. neomexicanus* as an outside comparison. Samples consist of five individuals collected from the same localities as those of the animals used for the immunological comparisons except for *A. ferreus,* for which a nearby locality was used. The use of small samples for electrophoretic protein comparisons of different species is justified by the results of Sarich (1977), Nei (1978) and Gorman and Renzi (1979); this is a special case of the exemplar method of Sokal and Sneath (1963, p. 161). Voucher specimens of the animals used for protein comparisons are deposited in the Museum of Vertebrate Zoology, University of California, Berkeley and in the collection of Richard Highton, University of Maryland. The localities, which consisted of no more than a few hectares of continuous habitat, are as follows: *A. aeneus* (Audra State Park, Barbour Co., West Virginia), *A. ferreus* (electrophoretic comparisons: Mary's Peak, Benton Co., Oregon; immunological comparisons: West Bank Rd. 7.2 km N. Smith River Rd., Douglas Co., Oregon), *A. flavipunctatus* (Uvas Canyon, Croy Creek, 0.8 km below Sveadal, Santa Clara Co., California), *A. hardii* (near Sunspot, Otero Co., New Mexico), *A. lugubris* (Potter Valley Rd., 0.3 km N. jct. Hwy. 20, Mendocino Co., California), *Ensatina eschscholtzii* (Pinehurst Rd. N. jct. Canyon Rd., Moraga, Contra Costa Co., California). The localities for *P. glutinosus, P. neomexicanus, P. richmondi* and *P. vehiculum* are the same as those given by Highton and Larson (1979).

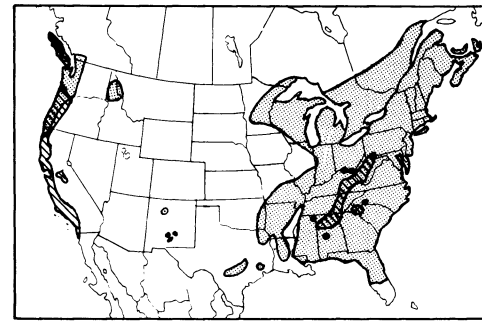

FIG. 1. Geographical distributions of the genera *Aneides* and *Plethodon* in North America. Regions marked with diagonals and solids represent *Aneides*; stipples denote *Plethodon*. Eastern populations of *Aneides* comprise the species *A. aeneus*; populations in New Mexico comprise *A. hardii*; *A. ferreus, A. flavipunctatus,* and *A. lugubris* inhabit the west coast.

Tissue homogenates from each animal were centrifuged to obtain an aqueous extract of proteins for electrophoretic analysis. Samples were stored at −70 to −80 C. Protein extracts were analyzed by starch gel electrophoresis using Sigma starch. Buffers were prepared according to Selander et al. (1971); protein assays were according to Harris and Hopkinson (1976), Selander et al. (1971) and Shaw and Prasad (1970). Buffer system-assay combinations are listed in Table 1.

Electrophoretic protein comparisons are summarized by the genetic distance measurements of Nei (1972, 1978) and Rogers (1972). Phylogenetic trees were constructed from the Rogers distances using the method of Fitch and Margoliash (1967) which was recommended for use with electrophoretic data by Prager and Wilson (1978). Alternative phylogenetic trees were evaluated by the "percent 'standard deviation' " criterion of Fitch and Margoliash (1967) and by the minimization of negative branch lengths. The degree of

TABLE 1. *Starch gel electrophoresis buffer system-assay combinations. All buffers are according to Selander et al. (1971) and assays are according to Harris and Hopkinson (1976), Selander et al. (1971) and Shaw and Prasad (1970). General proteins were stained with amido black.*

Protein assay	Buffers*					
	LiOH	T.C. 6.7	T.C. 8.0	T. HCl	T.M.	T.V B.
Aconitase (Acon)			X			
Adenylate kinase (Ak)		X				
Creatine kinase (Ck)		X				
Diaphorase (Dia)	X					
Esterase (Est)	X					
Glucose-6-phosphate dehydrogenase (G-6-pd)						X
Glucosephosphate isomerase (Gpi)						X
Glutamate dehydrogenase (Gdh)					X	
Glutamic oxaloacetic transaminase (Got)						X
Glyceraldehyde phosphate dehydrogenase (Gapdh)			X			
α Glycerophosphate dehydrogenase (αGpd)			X			
Indophenol oxidase (Ipo)	X					
Isocitrate dehydrogenase (Idh)			X			
Lactate dehydrogenase (Ldh)	X					
Leucine aminopeptidase (Lap)			X			
Malate dehydrogenase (Mdh)		X				
Malic enzyme (Me)					X	
Mannose-6-phosphate isomerase (Mpi)	X					
Peptidase (Pep)	X					
6-Phosphogluconate dehydrogenase (6-Pgd)						X
Proteins A, B (pt A, B)				X		

* Buffer abbreviations: T.C. 6.7 (Tris citrate pH 6.7), T.C. 8 0 (Tris citrate pH 8.0), T.M. (Tris maleic EDTA), T HCl (Tris HCl), T V.B. (Tris verzene borate).

regularity of the average rate of protein evolution was evaluated by comparing the amounts of change occurring along the diverging branches of the phylogenetic tree showing the best fit to the data (Wilson et al., 1977, and included references). Divergence time estimates were derived from the Nei (1972, 1978) genetic distances using the relationship that each genetic distance unit corresponds to approximately 14 Myr of divergence (Maxson and Maxson, 1979). The proportion of rapidly and slowly evolving proteins selected for this analysis is approximately equal to those of the studies from which the time calibration of genetic distance was determined.

RESULTS

Cladistic Analysis

A cladogram constructed from the alternative states of 17 morphological characters is presented in Figure 2. For these characters, 17 states are uniquely derived in the tribe Plethodontini. One character state (*p.* elongated limbs and digits) arose in parallel in the *A. aeneus* lineage and in the common ancestor of *A. ferreus* and *A. lugubris*. Three taxa are not characterized by any uniquely derived states; these taxa are the genus *Plethodon* and the species *A. ferreus* and *A. flavipunctatus*. The single largest concentration of morphological change is observed in the common ancestor of the *Aneides* species.

Protein Data

The titers of the five *Aneides* antisera ranged from 1,300 (*A. flavipunctatus*) to 6,800 (*A. aeneus*) with an average titer of 3,500 and a typical slope of 380. These values are essentially the same as those reported for antisera to the albumins of *Ensatina* and *Plethodon,* for which the titer ranged from 1,000 to 6,200, $\bar{x}$ = 3,900, and the slope averaged 380 (Maxson et al., 1979).

Microcomplement fixation tests were

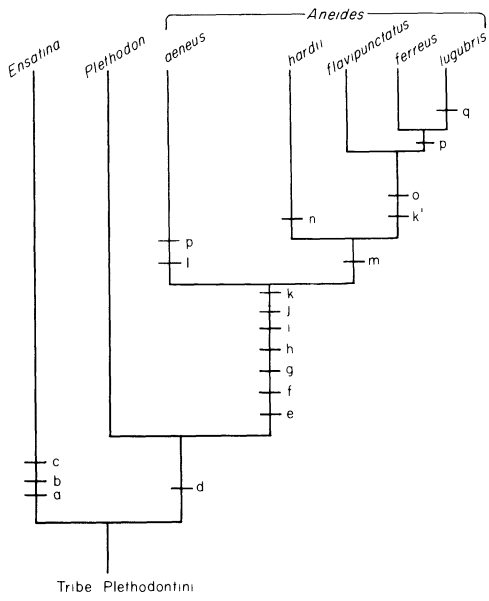

FIG. 2. A cladogram of the tribe Plethodontini based on 17 morphological characteristics. Ancestral states of all characters characterize the root of the tree; lower case letters denote derived characteristics. There is only one parallelism; character *p* changes on the *A. aeneus* lineage and in the common ancestor of *A. ferreus* and *A. lugubris*. See Materials and Methods for accounts of these characters.

carried out on all 45 species pairs (Table 2). For the 10 *Aneides* comparisons, percent standard deviation from reciprocity (Maxson and Wilson, 1975) was a surprisingly high 26%. When comparisons to *A. lugubris* are omitted, the standard deviation drops to a more typical value of 13%. In the four comparisons involving *A. lugubris*, the values obtained with this antiserum were an average of 29 units low relative to the reciprocal measurement. When all nine reciprocal comparisons of *A. lugubris* to *Plethodon, Ensatina* and the other *Aneides* are considered, the average deficit is 23 units. The percent standard deviation from reciprocity for the 45 comparisons involved in this work was 15.8%, however, which is more typical of earlier amphibian studies (Maxson and Wilson, 1975).

Phylogenetic trees corresponding to five hypotheses of the relationship of *Aneides* to *Plethodon* are shown in Figure 3. A tree showing the relationships of all species compared immunologically is given in Figure 4. The branching pattern of this tree corresponds to that of Figure 3a. The %SD statistic generally increases with increasing numbers of taxa included in a tree; therefore, the tree in Figure 4 has a higher %SD than the one in Figure 3a, although their branching patterns do not differ. Comparisons of the amounts of change occurring along the diverging branches of this tree suggest that rates of albumin evolution have been somewhat variable. For example, *A. hardii* has undergone roughly one half and *A. ferreus* has undergone roughly one tenth of the amount of albumin change seen in the *A. aeneus, A. flavipunctatus* and *A. lugubris* lineages. Estimates of divergence times from these data must have a higher vari-

TABLE 2. *Matrix of immunological distances among albumins of 10 species of plethodontid salamanders.*

	Antisera									
	A	F	T	L	H	V	N	G	R	E
A. aeneus (A)	0	63	103	65	51	79	68	101	94	107
A. ferreus (F)	53	0	23	11	28	64	41	101	72	105
A. flavipunctatus (T)	59	28	0	34	51	55	54	89	74	100
A. lugubris (L)	81	46	74	0	56	95	69	101	102	115
A. hardii (H)	46	29	59	33	0	45	35	86	68	122
P. vehiculum (V)	48	64	65	58	60	0	33	88	70	90
P. neomexicanus (N)	70	53	84	59	53	29	0	79	69	118
P. glutinosus (G)	81	98	117	101	83	79	74	0	44	105
P. richmondi (R)	72	90	95	65	65	65	65	44	0	97
Ensatina (E)	124	119	113	105	126	120	117	106	102	0

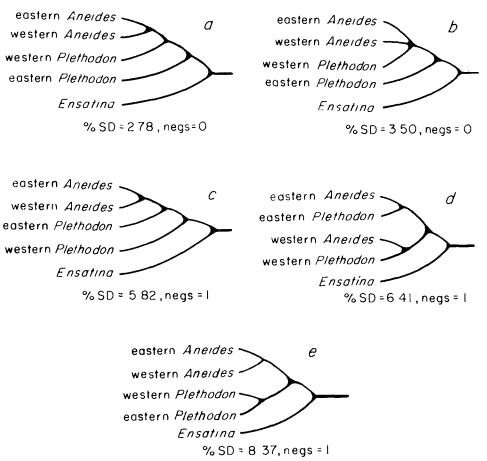

FIG. 3. Five hypotheses of the relationship of *Aneides* and *Plethodon*. Phylogenetic trees corresponding to five hypotheses are constructed from albumin immunological distances according to the procedure of Fitch and Margoliash (1967). Alternative trees are evaluated using a measure of the deviation of the tree from the data set (%SD, Fitch and Margoliash, 1967) and by counting the number of negative branches (negs.); these values should be minimized by the best tree. By both criteria, hypothesis *a* is favored.

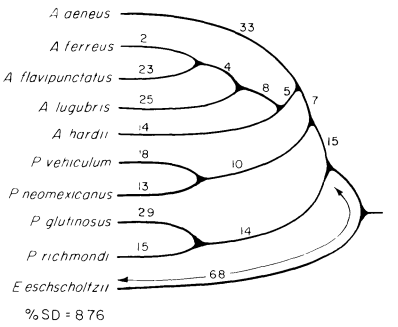

FIG. 4. Phylogenetic tree constructed from albumin immunological distance according to the method of Fitch and Margoliash (1967) relating five species of *Aneides*, four species of *Plethodon* and one species of *Ensatina*. %SD = 8.76.

ance (Nei, 1977) than estimates from other molecular data for which more strictly time dependent evolution is observed.

Electrophoretic protein comparisons are presented in Table 3 and summarized by genetic distance measurements in Table 4. Divergence time estimates for the major cladogenetic events of the tribe Plethodontini are derived from these data and from the immunological data and are presented in Table 5.

A phylogenetic tree constructed from the genetic distances (Rogers, 1972) is shown in Figure 5. This is judged to be the best of several alternatives according to the criteria given above. The amounts of change occurring along the diverging branches of the tree are approximately equal, suggesting a reasonably constant average rate of protein evolution.

DISCUSSION

Phylogenetic Relationships

The cladistic analysis of morphological variation (Fig. 2) establishes monophyly of the genus *Aneides* and shows that *Aneides* and *Plethodon* constitute a lineage distinct from *Ensatina*. Protein comparisons indicate that the *Ensatina* and *Aneides/Plethodon* lineages split during the Paleocene epoch (Maxson et al., 1979, Table 5). The exact relationship of *Aneides* to *Plethodon* is not resolved on the basis of morphological variation, because no derived morphological features distinguish the genus *Plethodon*. Circumstantial biogeographical evidence considered in the light of previous protein comparisons suggests that *Aneides* is an offshoot of the western *Plethodon* lineage. Protein comparisons indicate that the eastern and western lineages of *Plethodon* split late in the Eocene epoch (Highton and Larson, 1979; Maxson et al., 1979). Wake (1966) suggested that *Aneides* probably derived from a *Plethodon*-like ancestor in the more recent Oligocene or Miocene epochs on the basis of biogeographical and paleobotanical observations. The preponderance of *Aneides* species in the west and the fact that the most generalized species (*A. hardii*) occurs there caused Wake (1966) to propose a western origin for the genus *Aneides*.

Our immunological comparisons support the conclusion that *Aneides* derived from a western *Plethodon* lineage; the tree corresponding to this hypothesis is more consistent with the data than any of the others considered (%SD = 2.78; see Fig.

TABLE 3. *Protein variation within and among five species of* Aneides *and* Plethodon neomexicanus.

Protein	Samples					
	aeneus	*ferreus*	*flavipunctatus*	*hardii*	*lugubris*	*P. neomexicanus*
*N**	5	5	5	5	5	5
Acon	e (0.6) f (0.4)	b	a	c	d	e
Ak	b	c	d	b	c	a
Ck	b	b (0.1) c (0.9)	d	a	b	d
Dia	d	b (0.4) d (0.6)	c (0.1) d (0.9)	d	b (0.4) d (0.6)	a
Est	e	g	a	c (0.5) e (0.5)	b (0.8) d (0.2)	f (0.9) h (0.1)
G-6-pd	b	a	a	a	a (0.5) b (0.5)	a
Gpi	a (0.6) b (0.4)	b (0.3) d (0.7)	a	e	d	c
Gdh	c	c	c	a (0.2) c (0.8)	c	b
Got-1	b	d	d	a (0.7) c (0.3)	d	b
Got-2	b	c	c	a	c	d
Gapdh	a	a (0.1) c (0.9)	b	f	e	d
αGpd	e	a (0.4) d (0.6)	d	a	c	b
Ipo	a	e	e	d	c	b
Idh-1	a	c	e (0.2) f (0.8)	c (0.1) d (0.9)	b	b
Idh-2	d	b (0.8) e (0.2)	b (0.9) d (0.1)	b (0.5) c (0.5)	a	a
Ldh-1	a	b	d	e	c	f
Ldh-2	a	a	b	d	a	c
Lap	b	b	c	c	c	a
Mdh-1	b	c	c	c	c	a
Mdh-2	b	d	c	a	e	a
Me-1	c	a	a	d	d	b
Me-2	b	d	b	c	d	a
Mpi	c	b	d (0.4) e (0.6)	b	a	f
Pep-1	c (0.5) f (0.5)	f	e	a (0.1) b (0.9)	d	g
Pep-2	b	c	a (0.2) b (0.8)	b	b	b
6-Pgd	a (0.8) c (0.2)	c	c	d (0.3) e (0.7)	e (0.9) f (0.1)	b
pt A	a	c	e	b	d	b
pt B	f	d	c	a	b (0.8) c (0.2)	e

* The number of animals sampled per protein

TABLE 4. *Genetic distance measures of Rogers (1972, above diagonal) and Nei (1978, below diagonal) for electrophoretic protein comparisons of five species of* Aneides *and* Plethodon neomexicanus.

Samples	*aeneus*	*ferreus*	*flavipunctatus*	*hardii*	*lugubris*	*P. neomexicanus*
aeneus	0	.815	.819	.813	.793	.893
ferreus	1.723	0	.623	.783	.651	.943
flavipunctatus	1.739	.971	0	.760	.731	.888
hardii	1.784	1.569	1.466	0	.740	.831
lugubris	1.629	1.075	1.313	1.366	0	.861
P. neomexicanus	2.336	3.286	2.269	1.890	2.039	0

3a), indicating that *Plethodon* is paraphyletic with respect to *Aneides*. An alternative hypothesis, that the eastern *Aneides,* western *Aneides* and western *Plethodon* lineages diverged from a common ancestor simultaneously following their separation from the eastern *Plethodon* lineage, is less consistent with the protein data (%SD = 3.50; Fig. 3b) than the hypothesis represented by Figure 3a; it also requires paraphyly and extensive parallelism of morphological evolution. The hypothesis that *Aneides* arose in the east (Fig. 3c; %SD = 5.82) and the hypothesis that it arose independently in the east and west (Fig. 3d; %SD = 6.41) are less compatible with the protein data than are either of the preceding hypotheses. The hypotheses represented by Figure 3c and 3d both require paraphyly of *Plethodon*; the latter also requires polyphyly for *Aneides*. The tree corresponding to a morphologically completely monophyletic assemblage (Fig. 3e; %SD = 8.37) is less compatible with the protein data than are any of the hypotheses presented above.

Time estimates (Table 5) from the immunological and electrophoretic data place the separation of *Aneides* from the western plethodons in the Oligocene epoch as suggested by Wake (1966) on the basis of biogeographical and paleobotanical data. The recent discovery of fossil vertebrae assignable to western *Plethodon* and *Aneides* from lower Miocene rocks in eastern Montana documents the early separation of the genera (Tihen and Wake, 1981). The electrophoretic data indicate that the species of *Aneides* separated from each other over a period of approximately 10 Myr in the Miocene epoch (Fig. 5; Table 5). The immunological data suggest that the separation of *A. aeneus* from the western *Aneides* was considerably earlier than the electrophoretic data suggest; immunological divergence time estimates within the western lineage, however, are comparable to the electrophoretic

TABLE 5. *Time estimates of major cladogenetic events of the tribe Plethodontini based on Nei's (1978) genetic distance and on albumin immunological distance using the relationship that a genetic distance of 1 corresponds to an immunological distance of 24 which represents approximately 14 Myr of divergence (Maxson and Maxson, 1979).*

Comparison			Divergence time Myr		
			(Nei *D*)	(AID)	Epoch
Ensatina	—	*Aneides/Plethodon*	—	64.5	Paleocene
Aneides	—	eastern *Plethodon*	—	48.3	Eocene
Aneides	—	western *Plethodon*	33.1	38.1	Oligocene/Eocene
A. aeneus	—	western *Aneides*	24.1	35.8	Miocene/Oligocene
A. hardii	—	*A. lugubris* group	20.5	23.5	Miocene
A. lugubris	—	*A. ferreus/A. flavipunctatus*	16.7	22.7	Miocene
A. ferreus	—	*A. flavipunctatus*	13.6	14.0	Miocene

estimates. These estimates are consistent with the suggestion of Lowe (1950) and Wake (1966) that there has been no post-Miocene movement of plethodontid salamanders between eastern and western North America, and that the eastern and western *Aneides* share only ancient common ancestry. Our estimated times of divergence within *Aneides* are slightly earlier than Lowe's (1950); he suggested that *A. aeneus* separated from the western *Aneides* in the late Miocene but our data suggest an early Miocene divergence. Lowe (1950) thought that *A. hardii* separated from the other western *Aneides* no more recently than the Pliocene, while our data suggest a mid-Miocene divergence.

The electrophoretic and immunological protein comparisons indicate that *A. ferreus* and *A. flavipunctatus* are the most closely related of the three west coast species (Figs. 4, 5; Table 5). This is unexpected in light of the observation that *A. ferreus* and another west coast species, *A. lugubris,* share a derived feature (elongated limbs and digits) that is not shared with *A. flavipunctatus* (Fig. 2). The cladistic analysis (Fig. 2) already requires that this feature have evolved in parallel in eastern and west coast *Aneides,* suggesting that this character may have greater than expected evolutionary plasticity, so that a second parallel event is not surprising.

Our protein comparisons suggest that *Aneides* and *Plethodon* are old compared to other salamander genera. The plethodontid genus *Hydromantes* is also known to be very old (Wake et al., 1978). Protein comparisons suggest that the salamandrid genera *Notophthalmus* and *Taricha* are more closely related to each other than are members of any pair of *Aneides* species other than *A. ferreus* and *A. flavipunctatus* (Ayala, 1975). Bush et al. (1977) estimate on the basis of fossils that the average age of salamander genera is 23.4 Myr. Our data suggest that *Aneides* is between 24 and 38 Myr old and that *Plethodon* is between 48 and 64 Myr old. Estimates of the ages of these genera cannot be made with greater precision from the biochemical data because we can only date the separation of different lineages and can only say that the morphological changes resulting in the recognition of different genera arose before the first intrageneric divergence. The time ranges for *Aneides* and *Plethodon* include the divergence of each lineage from its nearest outside relative and the date of the first cladogenetic event within each genus. Of the vertebrate groups considered by Bush et al. (1977), only frogs, crocodiles and turtles are composed of genera with average ages exceeding that of salamander genera.

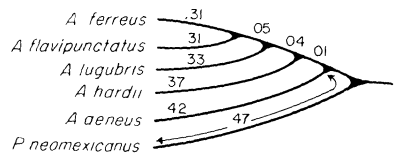

FIG. 5. Phylogenetic tree constructed from Rogers (1972) genetic distances according to the method of Fitch and Margoliash (1967) relating five species of *Aneides* and one species of *Plethodon*. %SD = 2.93.

Modes and Rates of Morphological Evolution

In his consideration of the evolution of higher categories, Miller (1949) suggested that this process depends upon the appearance of "key innovations" or inventions that permit a fairly rapid departure of a group of organisms from a preceding ecological sphere. He proposed that these innovations arise as preadaptations and promote the appearance of supportive adaptations during the ecological transition. Miller (1949) stated that the key innovation has two important results. It permits adaptive radiation by release from former ecological competitors and tends to develop gaps between the new group and the ancestral group because intermediates that may have existed suffer competition from occupants of both the old and the new ecological planes. Subsequent studies supported the importance of key innovations and preadapted structures in evolutionary change (Bock, 1965; Liem, 1974; Russell, 1979). The ecological and mor-

phological transition from *Plethodon* to *Aneides* can be characterized as having two key innovations from which the other changes followed. The rearrangement of carpals and tarsals provided for a redistribution of forces to the fingers and toe tips that facilitated climbing, and the fusion of the premaxillary bones provided a foundation for the evolution of a strengthened jaw (Wake, 1966).

Transitional stages of the two key innovative features of *Aneides* do not exist today. We suspect that these features were produced in single steps, and that no intermediate conditions are possible. The two key innovations occur elsewhere in the family Plethodontidae (Wake, 1966). Premaxillary bones of plethodontids are either fused or separated in adult animals; no intermediate degrees of fusion have been observed. Premaxillary bones are united in all plethodontid embryos and can be united in adults either by developmental failure or by secondary fusion following developmental separation. It may be impossible to determine which route has been followed in *Aneides,* but either could theoretically be achieved by a single developmental alteration. The carpal and tarsal arrangements of *Aneides* are found elsewhere only in *Chiropterotriton* group α (Wake and Lynch, 1976), a tropical group with many arboreal species. As with the premaxillary bones, there are no known examples of intermediates between the conditions found in *Plethodon* and other generalized salamanders, and in *Aneides* and *Chiropterotriton* α. Furthermore, there are developmental reasons to suspect that either one or the other arrangement will occur (especially in the tarsus) and that no intermediate condition is possible (Wake, 1980).

Although intermediate stages of the two key innovations of *Aneides* apparently do not exist, intermediate stages of the ecological and morphological changes or "supportive adaptations" initiated by the key innovations are observed. Phylogenetic trends were analyzed by Wake (1960) using Maslin's principles for phylogenetic inference from morphology (Maslin, 1952). Morphological features of extant forms are arranged in a continuum of variation, called a morphocline. Maslin's principle of identity of morphoclines and chronoclines states that morphoclines are partially or entirely identical to the gradual changes in a character vertically in time (chronoclines) from which they have evolved; if one extreme of a morphocline resembles a condition found in the less modified members of related groups of the same rank, then that extreme is primitive.

Wake (1960) identified 10 morphoclines in *Aneides* for which the primitive extreme can be determined (Table 6). Nine of these morphoclines represent modifications of the teeth or jaws. Relative to *Aneides,* the maxillary and mandibular teeth in *Plethodon* are greater in number, short and conical in shape, and fill a greater proportion of the maxillary and mandibular rami, respectively. The morphoclines illustrate a gradual transformation of tooth morphology in *Aneides*. The primitive case is observed in each instance in *A. hardii,* and for some features there is a sexual dimorphism in which the female is closer to the primitive extreme than the male. Female *A. hardii* have a relatively large number of maxillary and mandibular teeth (averages are 15.2 and 18.5, respectively, for adults); the maxilla is almost completely toothed and unexpanded posteriorly, and the bicuspid teeth are very short and conical. Male *A. hardii* have somewhat longer and distally recurved unicuspid teeth. The maxillary and mandibular teeth in male *A. hardii* are also less numerous, averaging 6.9 and 10.0 teeth, respectively. Male *A. aeneus* have still longer teeth that are distally recurved and spinelike. There is further reduction in tooth number (6.4 maxillary, 7.4 mandibular teeth on the average) and the portion of the maxilla that is toothed is correspondingly less. The reduction of the number of maxillary and mandibular teeth is paralleled by a reduction in the number of anterior vomerine teeth, associated with reduction in size of the preorbital process of the vomer. Dorsoventral expansion of the posterior por-

tion of the maxilla is greater in *A. aeneus* than *A. hardii*. The extreme reduction of tooth number and length of tooth row is found in the west coast species *A. lugubris* (average 6.4 maxillary, 7.4 mandibular teeth), *A. ferreus* (average 3.9 maxillary, 5.3 mandibular teeth) and *A. flavipunctatus* (average 5.7 maxillary, 5.0 mandibular teeth). The teeth are longest and most distally recurved in the west coast species and the teeth are slightly compressed laterally in *A. flavipunctatus* and greatly laterally compressed in *A. ferreus* and *A. lugubris*. The portion of the maxilla that bears teeth is smallest in the western species, particularly *A. lugubris,* and the posterior portion of the maxilla is dorsoventrally expanded and extended gradually in the sequence *A. flavipunctatus, A. ferreus* and *A. lugubris*. Compared to *Plethodon,* the maxillary-prefrontal articulation is strengthened in *Aneides*. *Aneides hardii* and *A. aeneus* are closest to the *Plethodon* condition; *A. flavipunctatus* and *A. lugubris* are intermediate but complex, and *A. ferreus* represents an extreme condition.

The spinelike teeth and strengthened jaws of the more derived *Aneides* do not appear to be associated with any fundamental change of food items taken by these species. Relatively large and small arthropods and molluscs constitute the primary food items of some *Plethodon* (Whitaker and Rubin, 1971; Jaeger, 1972), *A. hardii* (Johnston and Schad, 1959), *A. ferreus* (Bury and Martin, 1973; Lynch, 1974), *A. flavipunctatus* (Lynch, 1974), and *A. lugubris* (Bury and Martin, 1973; Lynch, 1974; Maiorana, 1978). *Aneides ferreus* and *A. lugubris* also eat salamanders of the genus *Batrachoseps* (Wake, 1960) but the studies cited above indicate that this is an infrequent event.

Wake (1964) proposed that the enlarged dentition and increased skull strength of *Aneides* are related to the manner of feeding in arboreal as contrasted with terrestrial habitats. In capturing and ingesting relatively large food items, terrestrial salamanders seize the prey and quickly turn their heads to restrain the prey against the ground. This practice is cumbersome and difficult in arboreal situations in which the maintenance of perch takes precedence over other maneuvers. Wake (1964) suggested that the jaw specializations of *Aneides* increase the efficiency of seizing and holding prey, and that this increased efficiency is of greater significance to scansorial than to terrestrial organisms. The ability to feed in an arboreal situation may give *Aneides* advantages in competitive encounters important for its success. Wake (1964) noted that the only *Aneides* which lacks salamander competitors, *A. hardii,* is the least arboreal species; it also lacks the jaw specialization observed in the other species. An alternative interpretation is that the jaw and tooth specializations of some species may faciliate aggressive encounters between territorial males (see Cupp, 1980).

The morphoclines (Table 6) are consistent with an interpretation that the jaw and skull modifications were produced gradually by the separate transformations of many small morphological features. The morphocline for limb length indicates that this modification was also gradual. Although *A. aeneus* is usually close to *A. hardii* at the ancestral extremes of the morphoclines and the west coast species are closer to the derived extreme, the variation of the position of these species on different morphoclines suggests that morphological variants may be under separate genetical and developmental control, even though they are functionally related.

The pattern of morphological evolution in *Aneides* corresponds well with that observed at the generic level by Miller (1949) and Bock (1979) in birds, Liem (1974) in fishes, and Russell (1979) in lizards. The apparently sudden appearance of a key innovation followed by a gradual adjustment of functionally associated features is observed in *Aneides*. The mechanisms of macroevolutionary change in these relatively slowly evolving salamanders must therefore indicate either a lengthening of this process of morphological transformation relative to other groups, or a lower rate of initiation of changes that can func-

TABLE 6. *Morphoclines observed in interspecific comparisons of* Aneides *(from Wake, 1960).*

Feature	Ancestral extreme	Derived extreme	Clinal series (Ancestral–Derived)
1. *Number of maxillary and mandibular teeth	High (max = 13.1, mand = 16.1)	Low (max = 3.9, mand = 5.0)	*hardii-aeneus-lugubris ferreus-flavipunctatus*
2. Compression of maxillary and mandibular teeth	conical	extremely compressed	(*hardii, aeneus*)-*flavipunctatus-ferreus-lugubris*
3. Length of mandibular tooth row	long	short	*hardii-aeneus-lugubris-ferreus-flavipunctatus*
4. Mandibular tooth length	short	long	*hardii-aeneus-(ferreus, flavipunctatus)-lugubris*
5. Maxillar-prefrontal articulation	simple	complex	(*hardii, aeneus*)-(*flavipunctatus, lugubris*)-*ferreus*
6. Toothlessness and dorsoventral expansion of posterior portion of maxilla	fully toothed, unexpanded	partially toothed, greatly expanded	*hardii-aeneus-ferreus-flavipunctatus-lugubris*
7. Preorbital process of vomer	well developed	absent	*hardii-aeneus-(ferreus, flavipunctatus-lugubris)*
8. Number of anterior vomer teeth	high	low	*hardii-aeneus-lugubris-(ferreus, flavipunctatus)*
9. Development of occipito-otic crest	poorly developed	well developed	(*hardii, aeneus*)-(*ferreus, flavipunctatus*)-*lugubris*
10. Limb length	very short	long	*hardii-flavipunctatus-(aeneus, ferreus, lugubris)*

* Numbers of teeth given here and in the discussion are from Wake (1960) and include only mature, functional teeth For estimates of the total numbers of tooth loci in the species of *Aneides,* see Wake (1963).

tion as key innovations to precipitate a morphological transformation. These alternatives are evaluated by analysis of the morphological changes in the perspective of divergence time estimates derived from the protein comparisons.

Seven morphological changes occurred along the lineage separating all *Aneides* from the common ancestor of *Aneides* and the western plethodons. These are all qualitative changes in limb and jaw morphology and cannot be considered to be strictly quantitative changes in ancestral features. Both changes judged to be key innovations occurred on this lineage. The immunological and electrophoretic data suggest that the duration of this lineage was 2.3 Myr or 9.0 Myr, respectively. The major morphological changes observed in the tribe Plethodontini took place during a period of time that was small compared to the duration of the genera. Two major morphological transformations, those of skull morphology and locomotion, occurred nearly simultaneously with respect to the age of the tribe. The morphological changes associated with the evolution of *Aneides* occurred rapidly enough that the ages of these genera would be comparable to those of the more rapidly evolving mammals and teleosts (Bush et al., 1977) if morphological transitions of this nature were more frequently initiated. Because the individual morphological changes associated with the origin of *Aneides* do not appear to be genetically or developmentally coupled, the concentration of these changes in time cannot be explained as the result of a single genetic change. The rate of evolution of these salamanders is prob-

ably limited by the appearance of morphological changes that can function as key innovations and promote changes in way of life. Once these changes are initiated, independent changes may be rapidly promoted by the requirements of the new adaptive zone.

The lineage separating the three west coast species from their common ancestor with *A. hardii* was also of relatively short duration (0.8 or 3.8 Myr according to immunological and electrophoretic comparisons, respectively). The development of the complex maxillary-prefrontal articulation seen in the western species and loss of the vomerine preorbital process took place during this period. The lineage separating the western *Aneides* from their common ancestor with *A. aeneus* developed an otic crest. The duration of this lineage cannot be precisely determined because the electrophoretic and immunological estimates differ considerably (3.6 Myr and 12.5 Myr, respectively).

Compared to the age of the genus *Aneides,* the major morphological changes happened in relatively short periods of time, producing the appearance of rapid, punctuational change followed by somewhat slower, gradual adjustments. The rapid appearance of the major changes does not require, however, that their origin was a saltational rather than a gradual one. Bock (1979) suggested that microevolutionary changes can accumulate to produce a macroevolutionary change in less than 5,000 years and probably in less than 1,000 years, which is considerably less than the estimated duration times of the lineages along which these changes occurred. We believe that the rearrangements of carpals and tarsals and the fusion of the premaxillary bones represent two developmental changes that arose without adaptive transitional stages, probably as the results of small genetic alterations; the suite of changes that accompanied and followed these key innovative features, however, requires no mechanisms inconsistent with those of microevolutionary change. The only apparent example of a developmental alteration that simultaneously changed numerous aspects of morphology is the case of intraspecific variation of *A. flavipunctatus* (Lynch, 1974, 1981; Larson, 1980). Some populations of this species demonstrate a multiple-character juvenalization of adult morphology (Lynch, 1974, 1981). Larson (1980) presented evidence that this developmental alteration occurred independently at least twice, and argued that this multiple-character parallelism contradicts an interpretation that the intraspecific differentiation of *A. flavipunctatus* proceeded entirely by the accumulation of many smaller restricted changes in morphology. A single developmental origin for multiple-character changes need not be postulated, however, to explain the major features of the evolution of *Aneides*.

Taxonomic Implications

Aneides, Ensatina and *Plethodon* form a monophyletic assemblage within the Plethodontidae (Wake, 1966), and both *Ensatina* and *Aneides* display adaptively significant morphological specializations that distinguish them from *Plethodon.* These specializations are derived character states at least equivalent to the kinds of characters and magnitudes of difference upon which the generic category is established in salamander classification. The species of *Plethodon* show remarkable similarity in osteology; a plausible argument can be made, however, that *Plethodon* retains the ecology and morphology of the ancestral stock of the tribe Plethodontini (Wake, 1966). Our immunological and electrophoretic protein comparisons show that *Aneides* is more similar to the western plethodons than to the eastern plethodons, and that western plethodons are more similar to *Aneides* than to their eastern congeners. If our assumption that protein evolution is a divergent process is correct, *Plethodon* is a paraphyletic taxon, as defined by Farris (1974) and Platnick (1977). We make no recommendations for taxonomic changes, however, because only the present classification

clearly distinguishes adaptively and morphologically distinct forms.

These and other recently published results suggest that paraphyly of taxa may be widespread in plethodontids. The species *Plethodon dorsalis* and *P. websteri* are so similar morphologically that they were considered conspecific until Highton (1979) presented biochemical evidence of their genetic distinctness in sympatry. A third, morphologically distinct species, *P. welleri,* is more closely related to *P. dorsalis* than either of these is to *P. websteri* (Larson and Highton, 1978) suggesting that *P. dorsalis* and *P. websteri* retain ancestral morphology and that morphological evolution in *P. welleri* was comparatively rapid. The assemblage of species known as the eastern small plethodons was also recently shown to be paraphyletic (Highton and Larson, 1979; Maxson et al., 1979); the eastern small plethodons of the *P. welleri* group are more closely related to the eastern large plethodons than either of these is to the eastern small plethodons of the *P. cinereus* group. Again, it appears that along two lineages, an ancestral feature (small size) has been retained while a third lineage changed.

We suspect that in groups where morphological evolution may be extremely conservative, paraphyly of taxa may be very common. During phylogenesis in these groups, certain adaptive morphologies stabilize and exist for millions of years. A sublineage may evolve an evolutionary novelty that will form the basis for new adaptive morphologies while the old adaptive morphology continues in the remainder of the assemblage. The clade showing the morphological changes is recognized as a new taxon. This pattern is illustrated by the relationships of the anuran genera *Acris, Hyla* and *Pseudacris* (Maxson and Wilson, 1975). These situations can be detected only when we have combinations of molecular and morphological data as presented in this paper. We suspect that molecular and morphological analyses of widespread amphibian genera such as *Bufo* and *Rana* and their close relatives will show that paraphyly is a common phenomenon.

Summary

Immunological and electrophoretic protein comparisons are analyzed in conjunction with a cladistic analysis of morphological variation to evaluate the rates and modes of morphological evolution in the salamanders of the tribe Plethodontini. Phylogenetic inferences from different data sets are highly congruent. The protein comparisons indicate that the genera *Aneides* and *Plethodon* are relatively old and that the morphological and ecological transitions responsible for the differentiation of *Aneides* from a *Plethodon*-like ancestor occurred over a time interval that was small compared to the duration of these genera. The evolution of *Aneides* featured changes in locomotion and in jaws; for each change we can identify single specific morphological features that qualify as "key innovations" or preadaptations that facilitated the morphological and ecological transitions. The appearance of morphological changes that can function as key innovations may be the limiting step in the evolution of these salamanders; our data indicate that, once initiated, complex morphological changes may be achieved relatively rapidly. The two key innovations in *Aneides* may have been produced by single developmental alterations; however, a sequential morphological analysis of *Aneides* species suggests that associated morphological modifications occurred gradually by the accumulation of numerous, independent genetic changes. There is no need to invoke single, multiple-character alterations to explain the major features of morphological evolution in this group. Relatively rapid morphological transformations within groups that are otherwise slowly evolving may be expected to generate paraphyletic taxa, as observed here. This paraphyly can only be detected by a combined morphological and molecular analysis such as this one. We suggest that paraphyly is common among amphibian taxa.

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