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# Albumin Evolution and its Phylogenetic Implications in the Plethodontid Salamander Genera *Plethodon* and *Ensatina*

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**Comparative studies were made of albumin evolution in all species of the salamander genera *Plethodon* and *Ensatina*. The albumins of all species were compared by the quantitative micro-complement fixation technique. *Ensatina* was seen to be phyletically remote from all species of *Plethodon*. The eastern large and eastern small *Plethodon* were shown to be more similar to one another than to the species of western *Plethodon*. *P. neomexicanus* was closest to the western species of *Plethodon*. Two distinct subsets were disclosed within the eastern small assemblage of *Plethodon*. Within the eastern large assemblage of *Plethodon*, *P. wehrlei* and *P. punctatus* were found to constitute a separate lineage.**

**We suggest that *Ensatina* diverged from the lineage giving rise to *Plethodon* near the beginning of the Tertiary. A late Eocene divergence for the eastern and western *Plethodon* species is indicated. Overall these salamanders appear to have been experiencing considerable molecular evolution, without equivalent morphological change, throughout their Cenozoic duration.**

Lungless salamanders of the genus *Plethodon* are widely distributed in forested areas of North America, and they have been the subject of many taxonomic, ecological, genetic, and morphological studies. Currently, 26 species are recognized (Table 2): six occur west of the crest of the Cascade Mountains in the Pacific Northwest, two in the Rocky Mountains (one in northern Idaho and western Montana and one in northern New Mexico), and the remainder are found in the eastern half of North America. The monotypic genus *Ensatina* ranges from British Columbia to Baja California, in and west of the Cascade-Sierra Nevada system. For many years populations now placed in *Ensatina* were considered to be species of *Plethodon,* and, on

morphological grounds, the genera are thought to be close relatives (Dunn, 1926; Wake, 1966). *Plethodon, Ensatina* and another close relative, *Aneides,* constitute the tribe Plethodontini (Wake, 1966).

Extensive taxonomic investigations of *Plethodon* (Highton, 1962, 1972; Brodie, 1970) have produced hypotheses concerning the phylogeny of *Plethodon,* and morphological studies (Wake, 1963, 1966) have formed the basis for hypotheses relating to the relationship of *Ensatina* and *Plethodon* and the biogeographic history of the group. Currently three major assemblages of *Plethodon* are recognized: eastern small, eastern large and western (Table 2).

Wake (1966) suggested that *Ensatina* is a primitive genus, representing a lineage independent from all species of *Plethodon.* Furthermore, the eastern small and the eastern large *Plethodon* are thought to be phyletically closer to one another than either group is to members of the western assemblage of *Plethodon.* The relationships of the New Mexican species, *P. neomexicanus,* are not entirely clear. For example, Highton (1962) considered the geographically isolated *P. neomexicanus* to be closer to the eastern small *Plethodon* than to other groups. Wake (1966) suggested that the western *Plethodon* might be the closest relatives of *P. neomexicanus* on the basis of vertebral structure. Mizuno and Macgregor (1974) found that certain repetitive DNA fractions of western *Plethodon* were more similar to *P. neomexicanus* than to other *Plethodon* species.

Over the past several years the quantitative technique of micro-complement fixation (MC'F) has been used in analyzing phyletic relationships among amphibians (Maxson and Wilson, 1975; Maxson, 1977; Maxson et al., 1977; Wake et al., 1978). We therefore combined efforts to test alternative phylogenetic hypotheses in *Plethodon* using quantitative micro-complement fixation studies of serum albumins. This approach allows estimation of sequence differences between albumins of related species (Maxson and Wilson, 1974; Wilson et al., 1977). These differences can be allocated to different lineages to generate a phylogenetic hypothesis based on the most parsimonious arrangement of the molecular data. Assuming that amino acid differences between albumins of different taxa accumulate in a mainly time-dependent manner (Wilson et al., 1977), we predicted that 1) *Ensatina* should be equally distinct from all species of *Plethodon*; 2) the *Ensatina-Plethodon* difference should be larger than any differences measured between *Plethodon* species; 3) *Plethodon* of the same species group would have fewer differences than members of different species groups; and 4) the average differences between species groups would be concordant with prevailing hypotheses of phylogenetic branching (Highton, 1962, 1972).

## Materials and Methods

Serum albumin was purified from plasma of five species of plethodontid salamanders by single step polyacrylamide gel electrophoresis (Maxson, 1973). Albumin was identified by its fluorescence in the presence of 8-anilino-l-naphthalene sulfonate (Hartman and Udenfriend, 1969). Antisera to pure albumins were prepared in male New Zealand White or Dutch Belted rabbits by the following procedure, modified from Sarich (1969). Rabbits received an initial intradermal injection of Freund's Complete Adjuvant with albumin solution (1.2:1), followed at seven weeks by a second intradermal injection using Freund's Incomplete Adjuvant and albumin. Both injections were distributed among two sites on the rabbits' haunches. Three weeks later one ml of albumin solution was given intravenously in the marginal ear vein. This was repeated one week later. The rabbit was bled from the marginal ear vein or by heart puncture one week following the second intravenous injection. A total of 1–3 mg of albumin was administered per rabbit over the thirteen-week immunization period. Three rabbits were used for each immunogen and individual antisera were tested for purity by the criteria described by Wallace et al. (1973). Individual antisera were pooled in inverse proportion to their micro-complement fixation (MC'F) titers and all reported results were obtained with these pooled antisera. Reactivity was measured by the quantitative MC'F technique. Protocol for titering and MC'F experiments is given in Champion et al. (1974). Data are reported in immunological distance units, which are a measure of the sequence difference between the proteins compared (Champion et al., 1974). For albumin it has been determined that one unit of immunological distance is roughly equivalent to one amino acid difference between the albumins compared (Maxson and Wilson, 1974).

Antisera were produced to albumin from several members of the same population for the

TABLE 1. MATRIX OF IMMUNOLOGICAL DISTANCES AMONG THE ALBUMINS OF 5 SPECIES OF PLETHODONTID SALAMANDERS.

| Species tested | Antisera | | | | |
|---|---|---|---|---|---|
| | G | R | N | V | E |
| *Plethodon glutinosus* (G) | 0 | 44 | 74 | 79 | 105 |
| *Plethodon richmondi* (R) | 44 | 0 | 65 | 65 | 97 |
| *Plethodon neomexicanus* (N) | 79 | 69 | 0 | 29 | 118 |
| *Plethodon vehiculum* (V) | 88 | 70 | 33 | 0 | 90 |
| *Ensatina eschscholtzii* (E) | 106 | 102 | 117 | 120 | 0 |

TABLE 2. ALBUMIN COMPARISONS AMONG SALAMANDERS OF THE GENERA *Plethodon* AND *Ensatina*.

| Species | Immunological Distance | | | | |
|---|---|---|---|---|---|
| | Anti-G* | Anti-R | Anti-N | Anti-V | Anti-E |
| Eastern large *Plethodon* | | | | | |
| *P. glutinosus* | 0 | 44 | 74 | 79 | 105 |
| *P. jordani* | 10 | 50 | 78 | 89 | —† |
| *P. ouachitae* | 9 | 48 | 63 | 81 | 106 |
| *P. caddoensis* | 11 | 44 | 69 | 84 | 114 |
| *P. yonahlosse* | 15 | 46 | 69 | 81 | 101 |
| *P. fourchensis* | 10 | 39 | 63 | 74 | — |
| *P. punctatus* | 47 | 39 | 52 | 69 | — |
| *P. wehrlei* | 49 | 39 | 50 | 72 | 113 |
| Eastern small *Plethodon* | | | | | |
| *P. richmondi* | 44 | 0 | 65 | 65 | 97 |
| *P. hoffmani* | 42 | 1 | 53 | 66 | 99 |
| *P. cinereus* | 43 | 4 | 67 | 70 | 96 |
| *P. serratus* | 44 | 4 | 59 | 69 | 101 |
| *P. shenandoah* | 44 | 3 | 54 | 69 | — |
| *P. nettingi* | 49 | 3 | 58 | 64 | 99 |
| *P. hubrichti* | 45 | 2 | 53 | 70 | 95 |
| *P. dorsalis* | 25 | 42 | 68 | 82 | — |
| *P. welleri* | 27 | 41 | 57 | 79 | — |
| *P. websteri* | 25 | 38 | 63 | 78 | — |
| *P. neomexicanus* | 79 | 69 | 0 | 29 | 118 |
| Western *Plethodon* | | | | | |
| *P. vehiculum* | 88 | 70 | 33 | 0 | 90 |
| *P. dunni* | 71 | 76 | 32 | 13 | 81 |
| *P. elongatus* | 72 | 60 | 18 | 29 | 93 |
| *P. stormi* | 88 | 59 | 20 | 19 | 102 |
| *P. larselli* | 81 | 71 | 22 | 30 | 106 |
| *P. vandykei* | 84 | 75 | 29 | 34 | — |
| *P. idahoensis* | 84 | — | 29 | 37 | — |
| *Ensatina eschscholtzii* | 106 | 102 | 117 | 120 | 0 |

* The five antisera used are the same as in Table 1.
† Not done.

following species: *Plethodon neomexicanus* (New Mexico), *P. glutinosus* (New York), *P. richmondi* (Virginia), *P. vehiculum* (Oregon), and *Ensatina eschscholtzii* (California). Plasma was also obtained from 22 additional species of *Plethodon*, which are listed in Table 2. Voucher specimens are deposited in the collection of R. Highton in the Department of Zoology, University of Maryland, and in the Museum of Vertebrate Zoology, University of California, Berkeley (see appendix for locality data).

## Results and Discussion

Antisera titers ranged from a low of 1000 (*Ensatina*) to a high of 6200 (*P. richmondi*). The average titer of 3900 and average slope of 380 is typical of that found in small anurans (Maxson, 1973). MC'F tests were carried out on all ten species pairs and the averages of these reciprocal tests (Table 1) were used for phylogenetic reconstruction. The percent standard deviation from reciprocity as defined in Maxson and Wilson (1975) for these plethodontid salamanders is 5.6%, considerably lower than that reported in any anuran studies (Maxson and Wilson, 1975) or in unpublished studies of tropical plethodontid salamanders where for five antisera the average percent standard deviation from reciprocity was 12.7%.

The phylogenetic tree in Fig. 1 was constructed from the data matrix of Table 1 by a method described by Farris (1972), but modified so that each monophyletic group of the tree utilizes only the cladistically closest outside lineage in assigning its relative limb length. This procedure minimizes inference errors (Maxson and Wilson, 1975). The percent "standard deviation" as defined by Fitch and Margoliash (1967) as a measure of "goodness of fit" of the tree to the data was only 1.8%, considerably lower than that reported for any immunological studies of proteins of amphibians, birds, conifers or most carnivores (Prager and Wilson, 1978). The percent error (Prager and Wilson, 1976) of our tree is 2.5%, also considerably lower than usually seen in immunological studies of a wide variety of organisms. A Fitch-Margoliash tree was calculated for comparison. This tree had the same branching pattern as the tree shown in Fig. 1, the only difference being in the amounts of change assigned along each lineage—the maximum difference being 4.5 IDU's. The percent "standard deviation" of this tree was greater, 2.2%, and the percent error was also greater, 3.4%.

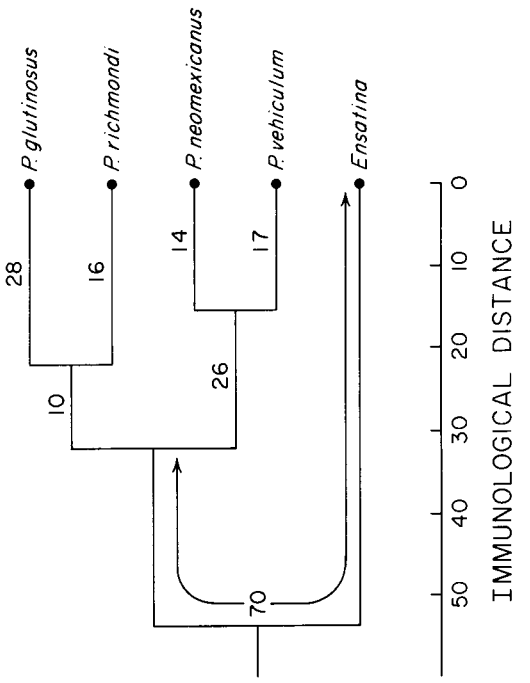


Fig. 1. Phylogenetic relationships among the albumins of the lungless salamanders, *Plethodon* and *Ensatina*. This phylogeny gives the branching order of the lineages leading to the modern species. Methods of construction are described in the text. The numbers on the branches are the amounts of albumin change (expressed in immunological distance units) estimated to have occurred along each branch. The immunological distance scale indicates the average amount of albumin change that has occurred since each branch point in the tree.

Data for the five reciprocal tests and for unidirectional tests with 22 additional species of *Plethodon* are reported in Tables 1 and 2. Only results of reciprocal tests were used in constructing Fig. 1, but the data from unidirectional tests have value in making approximate assignment of species to the branches of that figure on the basis of albumin similarities.

As predicted, *Ensatina* has the greatest immunological distance from all species in our data set, 103 ± 2 units ($\bar{x}$ ± s.e.). The data for *Plethodon* generally conform to expectations. The eastern large *Plethodon* (represented by *P. glutinosus*) and the eastern small *Plethodon* (represented by *P. richmondi*) are more similar to each other than either is to the western *Plethodon* (represented by *P. vehiculum*). The average

TABLE 3. ALBUMIN IMMUNOLOGICAL DISTANCES AND DIVERGENCE TIMES IN *Plethodon* AND *Ensatina*.[a]

| | Eastern | | Western | |
|---|---|---|---|---|
| | *P. glutinosus* | *P. richmondi* | *P. neomexicanus* | *P. vehiculum* |
| Eastern Large | | | | |
| I. | 9–15 (5.4–9 m.y.) | 45.2 ± 1.6 (27.1 m.y.) | | |
| II.* | 48.0 ± 1.0 (28.8 m.y.) | 39.0 (23.4 m.y.) | | |
| Eastern Small | | | 61.9 ± 1.9 (37.1 m.y.) | 74.5 ± 1.7 (44.7 m.y.) |
| I. | 44.4 ± 0.8 (26.6 m.y.) | 1–4 (0.6–2.4 m.y.) | | |
| II.† | 25.7 ± 0.7 (15.4 m.y.) | 40.3 ± 1.2 (24.2 m.y.) | | |
| Western | 75.1 ± 2.3 (45.1 m.y.) | | 18–33 (10.8–19.8 m.y.) | 13–37 (7.8–22.2 m.y.) |
| *Ensatina*[b] | 102.6 ± 1.7 (61.6 m.y.) | | 103.4 ± 5.1 (62.0 m.y.) | |

[a] Average immunological distances are calculated where appropriate, elsewhere ranges are indicated. Approximate time in millions of years is calculated using 100 units of immunological distance accumulating every 60 million years (Maxson and Wilson, 1975).
* *P. punctatus* and *P. werhlei*.
† *P. dorsalis*, *P. welleri* and *P. websteri*.
[b] These values include all possible reciprocal measurements involving *Ensatina*.

distance between 13 comparisons of the large (excluding *punctatus* and *wehrlei*, see below) and small (excluding *dorsalis*, *welleri* and *websteri*, see below) eastern *Plethodon* is 44.8 ± 0.8 units. The average distance for 51 comparisons of eastern and western (including *P. neomexicanus*) species of *Plethodon* is 70 ± 1.4 units. Excluding *P. neomexicanus*, 31 comparisons remain with an average immunological distance of 74.8 ± 1.5 units.

Although Highton (1962) assigned *P. neomexicanus* to the eastern small *Plethodon*, Wake (1963) thought that the vertebral structure of *P. neomexicanus* was more similar to that of western than eastern *Plethodon*. *P. neomexicanus* seemed to be more logically associated with the western than eastern species on biogeographical grounds as well (Wake, 1966). Recently studies of chromosomes and DNA sequence homologies among species of *Plethodon* by Mizuno and Macgregor (1974) led these authors to suggest that *P. neomexicanus* had closer affinities to western *Plethodon* than to eastern *Plethodon*. They noted that *P. neomexicanus* "was exceptional in showing little homology with other species." The albumins of *P. neomexicanus* and the western *Plethodon* are far more similar than are those of *P. neomexicanus* and eastern *Plethodon* (Table 3). These data are consistent with the view that *P. neomexicanus* is closer to the western than to the eastern *Plethodon*.

The albumins of several species of western *Plethodon* (notably *P. elongatus* and *P. larselli*) are more similar to the albumin of *P. neomexicanus* than they are to the western *P. vehiculum*. These data reinforce Mizuno and Macgregor's finding that among the western *Plethodon*, *P. elongatus* is distinct from *P. vehiculum* and *P. dunni*.

One way tests of all *Plethodon* to *P. richmondi* disclosed two immunologically distinct subsets in the eastern small assemblage. There are six species that are immunologically similar to *P. richmondi*: *P. hoffmani*, *P. cinereus*, *P. serratus*, *P. shenandoah*, *P. nettingi*, *P. hubrichti*. Immunological distances range from 1 to 4 (Table 3). Intrapopulation and intraspecific albumin variation is usually between 0–2 units (Maxson, 1973). This probably reflects the normal polymorphic structure of populations. Although distances measured between most amphibian species are usually greater than 2–4 units,

species pairs have been studied with very small distances such as those reported between *P. richmondi* and the group I eastern small species (Maxson and Wilson, 1974, 1975; Wilson et al., 1974; Wake et al., 1978).

The second subset (*P. welleri, P. dorsalis, P. websteri*) has immunological distances from *P. richmondi* of 38, 41 and 42. This distance approximates that obtained for comparisons of *P. richmondi* to the eastern large assemblage (Table 3). Although Highton (1962) placed *P. welleri* and *P. dorsalis* in the eastern small assemblage, he considered these species to form a distinct species group and, based on a phenetic analysis, he (Highton, 1972) considered these two species to be outliers to the main eastern small assemblage. The final species (*websteri*) is similar in morphology to *P. dorsalis*.

One way tests to *P. glutinosus* also reveal a dichotomy in the eastern small assemblage. Immunological distances of *P. glutinosus* to the seven species of the first subset (see above) range from 42–49 (Tables 2, 3), while distances for the three species in the second subset are 25 and 27. Another dichotomy occurs among the species previously associated with the eastern large assemblage. Immunological distances ranging from 9–15 (Tables 2, 3) were measured between *P. glutinosus* and one subset (*P. jordani, P. ouachitae, P. caddoensis, P. yonahlossee, P. fourchensis*). The remaining two species (*P. wehrlei, P. punctatus*) have immunological distances of 49 and 47 respectively relative to *P. glutinosus* but only 39 to *P. richmondi*. These data suggest that *P. wehrlei* and *P. punctatus* constitute a separate lineage.

Our data permit some speculation concerning the timing of subdivision of the *Ensatina-Plethodon* assemblage, if we use albumin as a molecular clock where 100 units of immunological distance accumulate in roughly 60 million years (Maxson and Wilson, 1975; Wilson et al., 1977). The average *Ensatina-Plethodon* distance (Table 3) suggests a divergence of the two lineages dating near the beginning of the Tertiary. Fifty-one comparisons of eastern and western *Plethodon* (Table 3) suggest a divergence during the late Eocene. This is earlier than previous estimates of divergence (Wake, 1966). The suggested Eocene date is consistent with the discovery of fossil vertebrae representing a western *Plethodon* in the lower Miocene of Montana (Tihen and Wake, in preparation).

In summary, our data suggest that *Plethodon* is an extremely ancient group which has remained very conservative in morphological differentiation during the entire period of its existence—the Cenozoic Era.

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*APPENDIX.* Exact locality data are available upon request of the authors.

| Species of Plethodon | State | County |
|---|---|---|
| *caddoensis* | Arkansas | Montgomery |
| *cinereus* | Indiana | Jackson |
| *dorsalis* | Illinois | Pope |
| *dunni* | Oregon | Benton |
| *elongatus* | Oregon | Curry |
| *fourchensis* | Arkansas | Polk |
| *glutinosus* | New York | Ulster |
| *hoffmani* | Pennsylvania | Somerset |
| *hubrichti* | Virginia | Bedford |
| *idahoensis* | Idaho | Kootenai |
| *jordani* | North Carolina | Macon |
| *larselli* | Oregon | Hood River |
| *neomexicanus* | New Mexico | Sandoval |
| *nettingi* | West Virginia | Tucker |
| *ouachitae* | Oklahoma | LeFlore |
| *punctatus* | Virginia | Rockingham |
| *richmondi* | Virginia | Wythe-Grayson |
| *serratus* | Arkansas | Polk |
| *shenandoah* | Virginia | Page |
| *stormi* | California | Siskiyou |
| *vandykei* | Washington | Pacific |
| *vehiculum* | Oregon | Benton |
| *websteri* | Alabama | Blount |
| *wehrlei* | New York | Cataraugus |
| *welleri* | North Carolina | Caldwell |
| *yonahlossee* | Tennessee-North Carolina | Unicoi-Mitchell |