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9. Included in this list are bovine serum albumin, methylated bovine albumin, bovine serum globulin, egg albumin, egg globulin, egg phosvitin, α-casein, gelatin, fibrinogen, calf thymus histone, protamine chloride, fetuin (GIBCO), and poly-L-aspartic acid. In each instance, the final concentration of the protein tested was 1.2 mg per milliliter of medium.
10. The standard molecules for the interpolation of the molecular weight were thyroglobulin (molecular weight, 670,000), β-galactosidase (molecular weight, 500,000), and catalase (molecular weight, 250,000).
11. Nerve growth factor (Wellcome Research Laboratories) was used at 0.1 and 1 unit/ml; phytohemagglutinin (Wellcome), at 1 : 100 and 1 : 1000 dilutions of the purchased material as recommended by the manufacturer; concanavalin A, at 10 and 25 μg/ml; wheat germ agglutinin (gift of M. M. Burger), at 10, 100, and 200 μg/ml; pokeweed mitogen (GIBCO), at 25, 50, and 100 μg/ml.
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Convergent Morphological Evolution Detected by Studying Proteins of Tree Frogs in the Hyla eximia Species Group

Abstract. *Protein studies have uncovered an apparent case of convergent evolution among North American tree frogs. The species* Hyla eximia *and* Hyla regilla *are so similar in external morphology that the* "wrightorum" *subspecies is assigned by some authorities to* H. eximia *and by others to* H. regilla. *Yet microcomplement fixation experiments show that* "wrightorum" *albumin, though virtually indistinguishable from authentic* H. eximia *albumin, differs as much from* H. regilla *albumin as from albumins of species outside the genus* Hyla, *such as* Acris crepitans. *The morphological resemblance of* "wrightorum" *to* H. regilla *is thus probably due to convergence.*

Protein studies can uncover cases of convergent anatomical evolution because protein evolution and anatomical evolution proceed independently (*1–4*). We describe here a case of convergent evolution in frogs.

Although frog fossils are known from rocks 150 million years old (*5*), living frogs (order Anura) are exceedingly uniform in anatomy and way of life. Despite the many speciation events responsible for the existence today of thousands of frog species, frogs have retained much the same way of life for 150 million years (*6*). Frogs are so alike in anatomy and way of life that it is very difficult for zoologists to detect convergent evolution within the order. Nevertheless, some notable cases of convergence are established. Tree frogs, for example, have evolved several times independently from ground-dwelling frogs (*5*). This happened both in the New World, where a bufonoid stock gave rise to the tree frog family Hylidae, and in the Old World, where ranoid frogs gave rise to the tree frog family Rhacophoridae (*5*). The anatomical similarities among convergent tree frogs are so great, however, that when a distinguished zoologist was confronted not long ago with a rhacophorid, mislabeled as coming from Brazil, he misclassified it as a new hylid (*7*).

We now present evidence for a case of convergent evolution among North American tree frogs of the genus *Hyla*. These animals have been classified into several species groups (*8*) on the basis of external morphology, skin color and pattern, osteology, and mating calls of adults, as well as larval morphology and, in some cases, potential for interspecific hybridization. This report deals with the *eximia* species group. As defined by Blair (*9*) and Duellman (*10*), this group includes seven or eight species, of which we have studied the following four: *H. eximia, H. euphorbiacea, H. regilla,* and *H. cadaverina.* Our interest in this species group arose from a disagreement which has developed over the taxonomic placement of an Arizona population, referred to here as the *"wrightorum"* subspecies. Duellman (*10*) assigned *"wrightorum"* to *H. eximia,* although a quantitative study of external morphology led Jameson *et al.* (*11*) to assign it to *H. regilla.* Our protein studies indicate that the morphological resemblance of *"wrightorum"* to some subspecies of *H. regilla* is due to convergence.

We compared the serum albumins of these species as well as other North American hylids by a quantitative immunological approach. Serum or plasma samples were obtained from representatives of the frog species listed in Table 1. Albumin was purified from two of them (*"wrightorum,"* Apache County, Arizona and *H. regilla hypochondriaca,* Contra Costa County, California) by preparative polyacrylamide gel electrophoresis (*12*) and injected into groups of three or four rabbits. After a 3-month period of immunization by a published method (*2*), antiserums were collected, pooled in inverse proportion to their microcomplement fixation titers, and tested for purity by several of the methods outlined by Arnheim and Wilson (*13*). Each antiserum pool was then tested for reactivity with the unpurified albumin present in serum from each of the species listed in Table 1. Reactivity was measured by the quantitative microcomplement fixation method (*14*). The results are given in immunological distance units, which are defined elsewhere (*14–16*). Immunological distance (y) is generally related to percentage difference in amino acid sequence (x) by the equation $y \cong 5x$ (*14, 16, 17*). For the particular case of albumin, there is direct empirical evidence that each unit of immunological distance is roughly equivalent to one amino acid substitution (*18*).

We worked with albumin, not only because of considerable experience in our laboratory with the study of species

differences in this protein (*1, 2, 15, 19–21*), but also because albumin evolves faster than most other proteins. Whereas the average rate of protein evolution is one amino acid substitution per 100 residues per 10^7 years (*22*), that of albumin appears to be twice as fast (*23*). Albumin is also nearly twice as large as the average protein, having about 580 amino acids in a single polypeptide chain (*24*). For these reasons, it is a useful protein for detecting sequence differences among closely related species.

Although we studied primarily albumin in these tree frogs, protein evolution proceeds with sufficient regularity (*25*) to make us confident that species whose albumins differ greatly will also differ substantially at other loci as well (*26*). There is evidence for a strong correlation ($r \geq .8$) between albumin immunological distance and other measures of genetic distance (*3, 27*). Hence, we believe that the immunological distance results given below are indicative of the genetic distances among the species compared.

The immunological results in Table 1 show that antiserum to the albumin of *"wrightorum"* reacted very strongly with the albumins of authentic *H. eximia* and *H. euphorbiacea* but far less strongly with the albumins of various *H. regilla* subspecies and *H. cadaverina*. Conversely, antiserum to the albumin of one subspecies of *H. regilla* reacted very strongly with the albumins of all other subspecies of *H. regilla* tested and rather strongly with *H. cadaverina* albumin, but rather weakly with the albumins of *H. eximia, "wrightorum,"* and *H. euphorbiacea*.

It is inferred that the albumins of *"wrightorum," H. eximia,* and *H. euphorbiacea* differ from each other by a very small number of amino acid substitutions. Likewise, the various subspecies of *H. regilla* probably differ from the reference subspecies (*H. regilla hypochondriaca*) in albumin sequence by an average of only about two substitutions. However, roughly 75 amino acid differences may exist between the albumins of *H. regilla* and *"wrightorum."*

The large antigenic difference between the albumins of *H. regilla* and *"wrightorum"* is comparable in magnitude to that between these two species and species belonging to other species groups or even to other genera (see Table 1). *Acris crepitans*, for example, is representative of a North American hylid lineage that abandoned the tree frog niche and diverged considerably in anatomy and way of life from other North American hylids (*10*). Yet *Acris* albumin is as similar to that of *"wrightorum"* as is that of *H. regilla* (see Table 1).

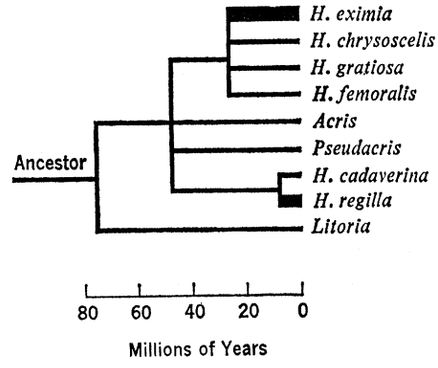

Fig. 1. Phylogenetic tree for the albumins of some North American hylids. The tree depicts the probable order of branching of lineages and their possible divergence times. Distance on the vertical axis has no evolutionary significance. The method of tree construction, devised by R. D. Maxson and L. R. Maxson (*12*), is based on those of Fitch and Margoliash (*30*) and Farris (*31*). As a reference albumin, from a species that is phylogenetically outside this group of North American hylids, we used that of the Australian hylid *Litoria aurea*. The conjectural time scale is based on the assumption, discussed elsewhere (*1, 2, 15, 21*), that differences between the albumins of two lineages have accumulated at the average rate of 100 immunological distance units per 60 million years. The heavy lines indicate the lineages along which convergent morphological evolution may have taken place (*29*).

The morphological similarity between *"wrightorum"* and some subspecies of *H. regilla* probably arose by convergent evolution. Alternatively, the similarity could be due to retention of morphological features present in their common ancestor; in other words, these frogs could be "living fossils." To distinguish between these two possibilities, we constructed a phylogenetic tree for some of the species represented in Table 1. The tree shown in Fig. 1 was constructed from the albumin data in Table 1 as well as from additional albumin data obtained with antiserums to albumins of numerous hylid species. The additional data, the method of tree construction, and the justification for the time scale will be published elsewhere (*28*). As illustrated in Fig. 1, the *H. eximia* and *H. regilla* lineages are only remotely related phylogenetically (*29*). Thus, their morphological simi-

Table 1. Immunological comparison of the albumins of *"wrightorum"* and *Hyla regilla* with those of various North American hylids. Antiserums to the albumins of *"wrightorum"* or *H. regilla hypochondriaca* were tested by the microcomplement fixation method (*14*) for reactivity with the albumins present in serum from the species listed. A few results obtained with the second antiserum have been published (*1*). Values in parentheses were obtained in a reciprocal test with antiserum to albumin from the species on the left against albumin from *"wrightorum"* or *H. regilla hypochondriaca*. Voucher specimens of all these species and subspecies are deposited in the Museum of Vertebrate Zoology, University of California, Berkeley, and the Museum of Natural History, University of Kansas, Lawrence.

Source of albumin tested		Immunological distance from reference species	
Taxonomic group	Locality	*"Wrightorum"*	*H. regilla hypochondriaca*
	Eximia *species group*		
"Wrightorum"	Apache County, Arizona	0	78
H. eximia	South Tepic, Nayarit, Mexico	1	81
H. euphorbiacea	Vera Cruz, Mexico	3	81
H. cadaverina	San Diego County, California	70	13
H. regilla hypochondriaca	Contra Costa County, California	71	0
H. regilla deserticola	Inyo County, California	72	1
H. regilla sierrae	Desolation Wilderness, California	75	4
H. regilla regilla	Linn County, Oregon	71	3
H. regilla pacifica	Benton County, Oregon	72	2
H. regilla cascadae	Jefferson County, Oregon	72	0
H. regilla curta	San Ignacio, Baja California Sur	70	0
	Other species groups		
H. chrysoscelis	Lincoln Parish, Louisiana	31	84
H. gratiosa	Scotland County, North Carolina	29 (48)	82 (87)
H. femoralis	Florida (dealer)	45 (56)	89 (100)
	Other genera		
Pseudacris triseriata	Licking County, Ohio	82 (81)	75 (64)
Acris crepitans	Douglas County, Kansas	83 (76)	62 (66)

larities probably arose independently.

Note added in proof: After this report went to press, P. G. Haneline and S. M. Case undertook an electrophoretic comparison of enzymes in the specimens whose albumins we had compared immunologically. There is approximate agreement between the electrophoretic and immunological results. In particular, the comparison of enzymes encoded by ten genes indicates that whereas the major alleles at most loci in "*wrightorum*" are identical electrophoretically with those of *H. eximia*, the major alleles at most loci in *H. regilla* are different. We are grateful to Haneline and Case for permission to refer to the electrophoretic evidence, which they intend to publish in full later. These workers are affiliated with the California Academy of Sciences, San Francisco, and the Department of Zoology, University of California, Berkeley, repectively. Their electrophoretic studies are being carried out in S. Y. Yang's laboratory, Museum of Vertebrate Zoology, University of California, Berkeley.

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Malignant Melanoma: Specific Immunity Induced by Bacillus Calmette-Guérin in BALB/c Mice

Abstract. *Previous treatment of BALB/c mice with bacillus Calmette-Guérin (BCG) will significantly protect them against intramuscular challenge of S-91 melanoma but not against a mammary carcinoma or a methylcholanthrene-induced sarcoma. Lymphocyte-mediated cytotoxicity studies correlate with in vivo data in showing that BCG-immune lymphocytes are specifically cytotoxic to S-91 melanoma target cells but not to the carcinoma or sarcoma target cells.*

Injection of bacillus Calmette-Guérin (BCG) into animals produces not only specific immunity to tubercle bacilli but also leads to development of nonspecific immunity to a variety of foreign antigens (*1*), among which are a number of malignant tumors. In humans, a nonspecific imune stimulation by BCG has been employed in the treatment of sarcomas (*2*), leukemia (*3*), and, most notably, malignant melanoma (*4*). Although animal investigations have shown nonspecific BCG-induced regressions of sarcomas (*5*), mammary carcinoma (*6*), and leukemia (*7*), there is an absence of animal studies demonstrating the effectiveness of BCG in the prevention and treatment of melanoma. We now document BCG-induced protection against a murine malignant melanoma and demonstrate preliminary evidence that suggests the possibility of a specific BCG-induced immunity to this malignant melanoma.

Three different malignant tumors—a melanoma (S-91), a mammary carcinoma, and a methylcholanthrene-induced sacroma—were used in syngeneic BALB/c mice. Test animals received 0.1 ml of BCG (Research Foundation,