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Albumin Phylogeny for Clawed Frogs (*Xenopus*)

Abstract. *Comparisons of albumin indicate that the frogs commonly used by North American molecular and developmental biologists under the name of* Xenopus muelleri *belong to another species,* X. borealis. *Phylogenetic analysis of the albumin data reveals two major groups of* Xenopus *species, one containing only* X. tropicalis *and the other, called the* X. laevis *group, containing the remaining species of the genus. The phylogenetic tree, in conjunction with evidence from chromosomes and DNA content, leads to the hypothesis that total genome duplication occurred in the common ancestor of the* X. laevis *group.*

Clawed frogs (genus *Xenopus*) are often used for research in molecular and developmental biology (*1*). Interpretation of the results of such research is sometimes dependent on accurate identification of the specimens used. Species of frogs which are very alike morphologically can differ greatly at the gene level (*2–4*) so that misidentification can be a serious problem for biologists working with them. Large genetic differences between morphologically similar frogs arise because frog anatomy has undergone slow evolutionary change while their genes and proteins have evolved at standard rates. By contrast, mammalian species that differ conspicuously in morphology can be extremely similar at the gene level (*5*).

The genus *Xenopus*, like several other frog genera, is old and morphologically conservative. This genus probably arose more than 90 million years ago at the same time as the common ancestor of all living placental mammals (*6*). As an aid to identifying and classifying the members of this genus, we have compared the serum albumins of a variety of species and subspecies by electrophoretic and immunological techniques. Serum albumin has already proved useful for distinguishing among other frog species and for elucidating their phylogenetic relationships (*2–4*).

Albumin, the major acidic protein in *Xenopus* plasma (*7*), was partially purified from plasma of six species by Sephadex G-200 gel filtration (*8, 9*) in Geneva and then shipped to Berkeley at ambient temperature in the presence of 1.5 percent phenoxyethanol as a preservative (*10*). The albumin was further purified by ammonium sulfate fractionation and polyacrylamide gel electrophoresis in Berkeley. Each purified protein was homogeneous by immunological criteria. Each was identified as albumin by several criteria, including solubility, electrophoretic mobility in the native state, strong fluorescence in the presence of 8-anilino-1-naphthalene sulfonate (*11*) and, in four cases, amino acid composition. The albumins analyzed showed significant similarity in amino acid composition to albumins of frogs of the genus *Rana* (*12, 13*). The average value of Metzger's index, a convenient measure of compositional difference (*14*), was 9 for the *Rana-Xenopus* comparison. This is indicative of significant homology in amino acid sequence between *Rana* and *Xenopus* albumins (*15*).

Antiserums were made by injecting each purified albumin into a group of three Dutch-Belted rabbits. After a 3-month period of immunization (*4*), antiserums were collected and pooled in inverse proportion to their titers in the microcomplement fixation test (*16*) and then tested for purity by several of the methods summarized elsewhere (*17*). Each antiserum pool was then reacted with the immunizing albumin as well as with the albumins of other frog species. The results of these comparisons are expressed as units of immunological distance. For albumin, there is indirect evidence for a correlation between immunological distance and the number of amino acid substitutions by which two albumins differ in sequence, one unit of immunological distance being roughly equivalent to one amino acid substitution (*3*). There is also a correlation between albumin immunological distance and genetic distance measured by the electrophoretic comparison of many enzymes of frogs (*18*).

The results obtained by testing the antiserum pool made against albumin from *X. laevis laevis* with albumin from other frogs are shown in Table 1. Each of these taxa was distinguishable from *X. l. laevis* on the basis of albumin reactivity in the microcomplement fixation test. The immunological distances of most taxa fell in the range from 5 to 19 units. The albumin of *X. tropicalis* was exceptional in differing by 57 units from that of *X. l. laevis* (*19*). *Hymenochirus* and *Pipa*, which belong with *Xenopus* in the family Pipidae, were extremely different from *Xenopus* with regard to the antigenic properties of albumin.

Many of the subspecies and species of *Xenopus* were distinguishable from one another on the basis of the electrophoretic mobility of their albumins, as in-

dicated in Table 1. Although most species appeared to have a single albumin, three species had two albumins. *Xenopus ruwenzoriensis*, which is hexaploid with respect to *X. laevis* (*20*), has three albumins according to our electrophoretic criteria.

Many of the frogs identified as *X. muelleri* in North America came from the vicinity of Nairobi in the central highlands of Kenya (*21*). According to the personal experience of one of us (M.F.) and to Alex Duff-MacKay, curator of amphibians at the National Museum in Nairobi, however, *X. muelleri* does not occur in the central highlands. Using antiserums to albumin from *X. borealis*, we find that the albumins of *X. borealis* and Nairobi specimens labeled "*X. muelleri*" (*22*) are immunologically identical. Antiserums to *X. muelleri* albumin readily distinguish *X. muelleri* albumin from the albumins of *X. borealis* and Nairobi specimens labeled "*X. muelleri*." We think that the designation of specimens coming from Nairobi and now widely used in American laboratories as *X. muelleri* was in error; these specimens belong to the species *X. borealis* (*23*).

From the results obtained by testing the six antiserums with each of the six albumins (Table 2), we constructed a phylogenetic tree for these albumins. This tree, shown in Fig. 1, depicts the order of branching of the lineages leading to the living species. The albumins fall into two phylogenetic groups: one containing *X. tropicalis*, and the other containing the remaining five species. On the basis of nomenclatorial priority, we refer to the latter group as the *X. laevis* species group. The divergences among species within the latter group occurred much more recently than the divergence between this group and the *X. tropicalis* group. This inference is consistent with fossil evidence indicating that both species groups were in existence 15 million years ago and that the genus itself originated about 90 million years ago (*6*). Included in the tree is the lineage leading to *Hymenochirus*, another genus of African pipid frogs. The albumin data imply that this lineage separated from the one leading to *Xenopus* long before the separation of the two species groups within *Xenopus*.

The phylogenetic tree facilitates interpretation of recent evidence regarding the chromosomes and DNA content of various species of *Xenopus* (*20, 24*). Whereas *X. tropicalis* has only 20 two-armed chromosomes and 3.6 picograms of DNA per nucleus, species of the *X. laevis* group have 36 or more two-armed

Table 1. Comparison of albumins from several species with albumin of *Xenopus l. laevis* by microcomplement fixation and electrophoresis. The *X. laevis* and *X. tropicalis* species groups are defined on the basis of the present albumin study.

Species*	Source	Immunological distance	Electrophoretic mobility†
	X. laevis *group*		
X. l. laevis	Fishoek, South Africa	0	126 (121)
X. l. petersi	Zambia	5	109
X. l. victorianus	Kampala, Uganda	9	124
X. fraseri	South Cameroon	9	127, 133
X. ruwenzoriensis	Ruwenzori Mountains, Uganda	12	123, 126, 129
X. vestitus	Kigesi, Uganda	13	120, 129
X. muelleri	Ifakara, Tanzania	13	123
X. clivii	Addis Ababa, Ethiopia	16	130
X. borealis	Kiambu and Marsabit, Kenya	19	124
	X. tropicalis *group*		
X. tropicalis	Ivory Coast	57	118
	Other genera		
Hymenochirus sp.	Africa‡	180	
Pipa pipa	South America‡	> 200	

*Voucher specimens deposited at Museum of Vertebrate Zoology, University of California, Berkeley. †Relative to human albumin which was assigned a mobility of 100; the value in parentheses refers to a minor protein component. Electrophoresis was done in a gel slab containing 7 percent polyacrylamide at *p*H 8.8. ‡Purchased from Hermosa Reptile Farm, Hermosa Beach, California.

Table 2. Average immunological distances among the albumins of six *Xenopus* species. Each distance is the average of the two values obtained in reciprocal tests. For this reason, the unidirectional distances in Table 1 are not necessarily identical with those given here.

Albumin	Immunological distance for *Xenopus*					
	clivii	*fraseri*	*muelleri*	*borealis*	*l. laevis*	*tropicalis*
X. clivii	0					
X. fraseri	7	0				
X. muelleri	4	3	0			
X. borealis	12	12	10	0		
X. l. laevis	13	9	16	17	0	
X. tropicalis	51	55	58	54	61	0

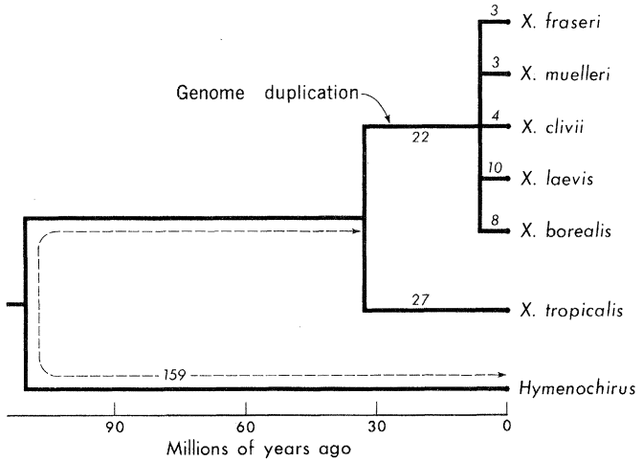

Fig. 1. A phylogenetic tree for the albumins of six species of *Xenopus* and a *Hymenochirus* species. This tree is based on the immunological distances among *Xenopus* species given in Table 2 and on immunological distances to *Hymenochirus* obtained with antiserums to the following albumins: *X. l. laevis* (180), *X. borealis* (196), and *X. tropicalis* (188). Several alternative trees were constructed by use of the Fitch and Margoliash and Farris methods (*28*). The results disagreed only with respect to the order of branching of lineages within the *X. laevis* species group. We therefore regard the branching order within this group as uncertain; this results in part from the postulated genome duplication which would have produced duplicate loci for albumin. The number by each line shows the amount of albumin change (in immunological distance units) allocated to that lineage. The time scale is based on the assumption that albumin has evolved in pipid frogs at a fairly steady rate of about 1.7 immunological distance units per million years per pair of species compared, as it does in nonpipid frogs and mammals (*2–4*). This time scale is consistent with the fossil evidence cited in the text as well as with other fossil evidence (*29*) indicating that the family Pipidae arose at least 130 million years ago.

chromosomes and 6.4 pg or more DNA per nucleus. The simplest interpretation of these findings, in view of the albumin phylogeny, is that there was a duplication of the genome (accompanied by a small number of chromosomal rearrangements) in the common ancestor of the *X. laevis* group, as indicated in Fig. 1 (*25*). Thus the *Xenopus* species commonly used for molecular studies of development and genome organization probably are tetraploid, that is, have two pairs of genes for every function. Although this finding may surprise those who study genome organization in *Xenopus*, it will not surprise herpetologists, who are beginning to recognize that polyploidy is rather common in frogs of several families (*26*).

The duplication event referred to in Fig. 1 evidently occurred so long ago that evolutionary divergence has taken place between the duplicated genes. The complex electrophoretic patterns observed for the enzymes lactate dehydrogenase, malate dehydrogenase, and superoxide dismutase in *X. laevis* and *X. borealis* (*27*) are consistent with this possibility. While the existence of duplicate genes may complicate attempts to study the molecular basis of development in *Xenopus*, biologists now have a good opportunity for the molecular study of evolutionary aspects of gene duplication in a laboratory animal.

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Ribavirin: Efficacy in the Treatment of Murine Autoimmune Disease

Abstract. *Ribavirin, a drug with known antiviral activity, was given to mice with established lupus nephritis. Ribavirin was effective in prolonging survival, reducing the titer of antibodies to DNA, and reversing proteinuria. Other antiviral agents were not effective in the dosages used.*

Female New Zealand black by New Zealand white F_1 (NZB/W) mice develop an autoimmune disease characterized by immune complex glomerulonephritis, antibodies to DNA and RNA, lupus erythematosus (LE) cells, and shortened life expectancy (*1*). These findings are also seen in human systemic lupus erythematosus (SLE). Although genetic, immunologic, and hormonal factors have been implicated in the pathogenesis of this disorder, recent studies suggest that viruses may be causative agents of autoimmune disease in both animals and man (*2*). These studies indicate that a new approach to the therapy of immune complex disease might be directed against viral infection. We therefore studied the effect of several antiviral drugs and an immunostimulatory drug on the natural history of NZB/W lupus.

NZB/W female mice (152, aged 20 weeks), already manifesting autoimmunity were randomly selected to receive intraperitoneal injections of one of the following drugs: ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) at a dose of 250 mg/kg twice weekly; ara-A (9-β-D-arabinofuranosyladenosine) at a dose of 600 mg/kg twice weekly; phosphonoacetic acid at a dose of 750 mg/kg five times weekly; and levamisole (L-2,3,5,6-tetrahydro-6-phenylimidazo-[2,1*b*]-thiazole·HCl) at a dose of 10 mg/kg twice weekly, or normal saline twice weekly.

Drug dosages were based on previous short-term studies that suggested that the above schedule would be relatively nontoxic while still demonstrating antiviral activity (*3*). Levamisole was included in the therapy groups because of its ability to stimulate immune responses and the suggestion of usefulness in rheumatological disorders (*4*). Treatment was started at 20 weeks of age when demonstrable immune-complex deposition in the kidneys and circulating antibodies to nucleic acid are present, thus making the experimental system analogous to clinical situations.

All mice were followed for the development of antibodies to DNA, proteinuria, and for survival. The protein con-