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Immunological Resolution of a Diploid-Tetraploid Species Complex of Tree Frogs

Abstract. *Micro–complement fixation studies of eastern and western populations of the North American tree frog* Hyla chrysoscelis *reveal they have been genetically isolated for about 4 million years. Immunological comparisons of populations of the cryptic tetraploid* Hyla versicolor *indicate a recent origin, from hybridization between eastern and western* H. chrysoscelis.

The North American cryptic species pair of hylid frogs, *Hyla chrysoscelis* and *H. versicolor*, are respectively diploid and tetraploid (*1, 2*). Despite their remarkable morphological resemblance, these two species can be distinguished by karyotypic analysis, the nonoverlapping pulse rates of the male mating calls, and their incompatibility in hybridization tests (*3, 4*).

Electrophoretic studies have demonstrated that populations of the diploid *H. chrysoscelis* from South Carolina, Georgia, Ohio, and Mississippi are genetically different from diploid populations of central Texas (*5*). The two population groups, "eastern" and "western" *H. chrysoscelis*, can be readily distinguished because they are monomorphic for different alleles at the *LDH-B* [heart LDH (lactate dehydrogenase)] locus. The two groups of populations also have significantly different, but overlapping, mating call pulse rates and durations (*5*).

We were interested in determining the time of separation of *H. chrysoscelis* into eastern and western gene pools, and estimating the age of origin of the tetraploid, *H. versicolor*. To approach these questions, we used the quantitative immunological technique of micro–complement fixation. This approach is useful in estimating divergence times of related species (*6*), in reconstructing phylogenetic histories (*7*), and in detecting cases of convergent evolution (*8*). At the time of collection, the species of all frogs used in our study were identified by mating call (*9*). Subsequent karyotypic analyses of samples from each population confirmed the specific designations (*2, 10*).

Antiserums were produced to purified serum albumin of *H. chrysoscelis* from Oktibbeha County, Mississippi, and of *H. chrysoscelis* from an allopatric locality in Bastrop County, central Texas, by techniques described in Champion *et al.* (*11*). The two antiserums were tested for cross reaction with plasma samples of *H. chrysoscelis* from the same populations, from other eastern and western localities, and with plasma samples of *H. versicolor* from Texas and northeastern populations. Genetic distances between sample pairs are reported in immunological distance units (IDU). For hylid albumins, we have shown that a single IDU corresponds roughly to a single amino acid substitution (*8*), and the mean evolutionary rate of albumin is 1 IDU per 0.6 million years (*6*).

Thirty-three *H. chrysoscelis* were tested against both the eastern and western antiserums (Table 1). Serum albumins of 15 *H. chrysoscelis*, from four eastern localities, were identical to the Mississippi *H. chrysoscelis* antiserum (IDU = 0.4 ± 0.7), but an average distance of 4.5 ± 1.7 IDU from the central Texas, or western, *H. chrysoscelis* antiserum (*12*). Conversely, 18 frogs from five western populations were essentially identical to the antiserum against central Texas *H. chrysoscelis* (IDU=0.9 ± 1.3), but an average distance of 9.3 ± 1.5 IDU from the eastern *H. chrysoscelis* antiserum (*13*). The populations tested include one population sympatric with *H. versicolor*, three allopatric populations within 50 km to the west of sympatry and one population at the western extremity of the range in central Texas. In all of these cases, *H. chrysoscelis* individuals tested against both antiserums could be unequivocally assigned to either the eastern or western population group (*14*). Such magnitudes of immunological distance are unknown among populations of other species of hylid frogs. For example, immunological studies of geographically widely separated populations of *H. crucifer* show no albumin differentiation (*15*), and extensive studies of geographically separated and morphologically diverse *H. regilla* populations, many considered subspecies (*16*), differ

Table 1. Albumin comparisons involving populations of eastern and western *Hyla chrysoscelis*. The numbers in parentheses indicate the number of individuals for which immunological distance was measured.

Individual plasma from	Immunological distance to *Hyla chrysoscelis*	
	Eastern*	Western†
Eastern populations:		
Mississippi (homologous)	0.0	5.0
Mississippi (4)	0.5	5.2
Ohio (4)	0.5	6.5
Georgia (3)	0.3	4.0
South Carolina (4)	0.2	2.2
Average (15)	0.4 ± 0.7	4.5 ± 1.7
Western (Central Texas) populations:		
Bastrop County (homologous)	9.0	0.0
Bastrop County (4) (Alum Creek, sympatric)	8.5	1.0
Bastrop County (3) (Colorado River, allopatric)	8.0	0.0
Bastrop County (3) (Elgin, allopatric)	9.7	0.0
Gonzales County (5) (Highway 90, allopatric)	10.8	2.2
Gillespie County (3) (Fredriksburg, allopatric)	9.0	0.3
Average (18)	9.3 ± 1.5	0.9 ± 0.9

*Antiserum produced to *H. chrysoscelis* from Oktibbeha County, Mississippi. †Antiserum produced to *H. chrysoscelis* from Bastrop County, Texas.

only by a maximum of 2 to 3 IDU (*7*). Furthermore, recent immunological studies of clawed toads (*Xenopus*) have revealed several different species of this genus which differ only by 3 to 9 IDU (*17*). The immunological distances observed between the eastern and western populations of *H. chrysoscelis* can be achieved only by independent sequential amino acid substitutions in the albumins of each population. Therefore, we may infer that these populations have maintained separate gene pools, and that they have done so, for a period of roughly 4 million years. While electrophoretic studies of these populations also indicate such genetic differentiation (*5*), it should be noted that the ability to measure cumulative differences at one locus also allows us to infer total independence of the gene pools and to estimate the time of divergence. There are significant differences between eastern and western *H. chrysoscelis* mating calls in pulse rate and call duration. Although eastern females prefer average eastern calls to average western calls, the overlap in these interpopulational responses is large enough to permit the conclusion that eastern females exhibit a secondary preference for a western call (*18*). Coupled with the fact that the eastern and western forms produce viable laboratory hybrids (*4, 14*), it seems likely that the two forms of *H. chrysoscelis* can exchange genes when they come in contact. Our albumin data indicate that such exchanges have not been evolutionarily significant in the past 4 million years. It has been shown (*19*) that protein evolution in anurans proceeds independently of morphological evolution and independently of ability to form viable hybrids. Thus it is not surprising to find that these populations have undergone genetic differentiation, despite their morphological similarity and apparent genetic compatability.

Individuals from a number of populations of the tetraploid *H. versicolor* were also tested against the antiserums formed against both diploid *H. chrysoscelis* populations (Table 2). Results were remarkably variable, particularly in the Texas populations, and therefore representative individuals are listed separately. Some of the frogs from the Texas populations, collected from the same pond on the same night, show completely reciprocal relationships. For example, frogs 72 and 74 from the Sam Houston National Forest (an allopatric *H. versicolor* locality) are immunologically indistinguishable, respectively, from western *H. chrysoscelis* and eastern *H. chrysoscelis*.

Table 2. Albumin comparisons between eastern and western *Hyla chrysoscelis* and representatives of *Hyla versicolor*. Numbers in parentheses indicate the number of individuals.

Hyla versicolor populations	Immunological distance to *Hyla chrysoscelis*	
	Eastern	Western
New Jersey (2)	0	7
New York (1)	0	10
Texas		
Sam Houston		
National Forest:		
No. 72*	6	0
No. 74	0	5
No. 75	5	4
No. 76	1	4
No. 78	2	2
No. 117	3	9
Bastrop State Park:		
No. 226	5	1
No. 227	5	9
No. 228	8	2
No. 239	4	5
No. 242	0	9
No. 253	6	6
No. 406	10	1
No. 407	3	10
No. 409	8	8
No. 410	6	7

*The numbers represent individual frogs.

The Texas *H. versicolor* fall into roughly three classes. One class is immunologically identical to eastern *H. chrysoscelis*, a second is identical to western *H. chrysoscelis*, and the third and largest class is immunologically heterogeneous. Never before has any immunological population study exhibited variation in excess of the ± 2 IDU expected from the normal polymorphic structure of evolving populations (*7, 8*). An interpretation consistent with the data in Table 2 is that the tetraploid *H. versicolor* arose as a result of allotetraploidy involving eastern and western *H. chrysoscelis*. Individual *H. versicolor* homozygous for either albumin will have an ID of 0 to the appropriate antibody, such as No. 72 and No. 74 above. Such data also indicate that the allotetraploidy was recent since the albumins are still immunologically indistinguishable from those of their respective diploid ancestors. Individuals of any of the three heterozygous genotypes will have unpredictable, but intermediate, immunological distances. Studies of meiotic chromosomes of *H. versicolor* show quadrivalent formation, which in plants is often an indication of allotetraploidy (*2*). Alternatively, apparent tetraploid homozygotes could be accounted for by multiple autopolyploid origins. Distinguishing between allo- and autopolyploidy is not possible at this time.

The conclusions to be drawn from the albumin data are consistent with all other genetic data available (*4, 5*). Eastern and western diploid populations have differentiated into two recognizable groups, this differentiation having started some 4 million years ago. The tetraploid, *H. versicolor*, appears to be of recent origin, as suggested by Blair (*20*) and Ralin (*5*), because of the allotetraploidy of the eastern and western *H. chrysoscelis* populations. A thorough study of albumins, key electrophoretic loci (*21*), mating calls, and karyotypes, in sympatric and allopatric populations from throughout the range of this species complex is now needed.

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13. If reciprocity were perfect, eastern antiserum with homologous western albumin (Bastrop County) and western antiserum with homologous eastern albumin (Mississippi) would give identical immunological distances. The deviation from perfect reciprocity, 9 and 5 IDU respectively, is attributable to a different number of amino acid substitutions in the albumins of the eastern and western lineages since they last shared a common ancestor.
14. Two individuals (Nos. 1029 and 1030) from the western extreme of the eastern population appear to be heterozygous diploids. The presence of occasional hybrids is to be expected because mating calls have not been perfected as a premating isolating mechanism between eastern and western *H. chrysoscelis* (*18*).
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