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"Resolution" of Diploid-Tetraploid Tree Frogs

In their report on the albumin immunology of the diploid-tetraploid species complex of *Hyla chrysoscelis* and *Hyla versicolor* (*1*), Maxson *et al.* state that all *H. chrysoscelis* individuals tested against both antiserums could be unequivocally assigned to either the eastern or western population group. However, this statement is then contradicted by a note (*2*). The two diploid heterozygous individuals from the Angelina National Forest in extreme eastern Texas are explained away as "occasional hybrids" between eastern and western *H. chrysoscelis*. Examination of a range map of the complex (*3*) would have revealed that, less than 40 km to the west of this locality, the tetraploid *H. versicolor* completely divides the range of *H. chrysoscelis* westward to central Texas and northward from the Texas and Louisiana coasts to southwestern Missouri. "Occasional" hybridization in southwestern Missouri could scarcely account for the appearance of two of two heterozygous individuals in extreme eastern Texas. If Maxson *et al.* are proposing that two distinct forms of *H. chrysoscelis* that rarely hybridize coexist in eastern Texas (that is, are distinct species), then the probability that two of two randomly selected individuals would both be hybrids is infinitesimally small. Moreover, the data they present are not relevant to the question of specific status. Species distinctness of sympatric forms is determined by fieldwork, not by immunological distances. In addition, moderate frequencies of the eastern allele in western populations, and vice versa, could very well have gone undetected simply because sample sizes are only three to five individuals (six to ten genes) per population (*1*). Therefore the data at worst contradict, and at best provide no support for, the statement (*1*) that eastern and western *H. chrysoscelis* have ever maintained separate gene pools.

Nor do the data support the statement (*1*) that gene exchanges between the two forms of *H. chrysoscelis* have not been evolutionarily significant in the last 4 million years. The genetically intermediate tetraploid *H. versicolor* (*1*, *4*) must have arisen from either a continuously existing intermediate population of *H. chrysoscelis*, or from one or more heterozygous individuals produced as a result of secondary contact of the two forms. Either case represents evolutionarily significant gene exchange between the two forms subsequent to their acquisition of different major albumin alleles.

To demonstrate that the two forms of *H. chrysoscelis* are genetically different enough to have produced an allotetraploid, Maxson *et al.* state (*1*) that the formation of quadrivalents during meiosis, as occurs in *H. versicolor*, is often taken as an indication of allopolyploidy in plants. This is a complete reversal of the generally accepted view (*5*).

The average distance between the albumins of eastern and western *H. chrysoscelis* is approximately 7 immunological distance units (IDU) (*1*). Consistent with a number of assumptions about IDU's and amino acid substitutions in frog albumins, and the rate of evolution of frog albumins (*6*), the genetic difference inferred from this distance is thought to be too large to be a simple geographic allele frequency difference within a single, wide-ranging species. Despite claims to the contrary (*1*), sample procedures, sample sizes, and statistical analyses of results in prior albumin immunological studies (*7*) have not been suitable for detecting albumin immunoalleles in populations. This possibility has been ignored in interpretations of results in these studies (*6*, *7*).

The average distance of about 7 IDU (a calculated divergence time of approximately 4 million years) is used in a fashion unwarranted by the statistical situation. First, tests of eastern albumin with western antiserum and western albumin with eastern antiserum differ from a perfect reciprocity by 100 percent (an average immunological distance of 4.5 in the former test and of 9.3 in the latter). The difference in the reciprocal tests is highly significant ($P < .001$). Combining them to obtain an average of 7 IDU is not statistically valid. Furthermore, since the eastern and western albumins are tested directly against each other and not against that of a third form, it is illogical to attribute the deviation from perfect reciprocity to "a different number of amino acid substitutions in the albumins of the eastern and western lineages since they last shared a common ancestor" [reference 13 in (*1*)]. Therefore the actual distance could be as small as 4.5 ± 1.7 IDU. Second, a figure of ± 2 IDU, which is characterized as "expected from the normal polymorphic structure of evolving populations" is elsewhere given as the maximum standard deviation observed "on repeated runs of a single sample" [reference 12 in (*1*)]. Finally, interindividual standard deviations range from ± 0.7 IDU to ± 1.7 IDU in the sample groups. Given this variation, one can seriously question whether the average immunological distance of 7 between the eastern and western albumins represents a reasonable measure of genetic distance between the two forms of *H. chrysoscelis*. The actual immunological distance could be small enough to be accounted for by only one or two amino acid substitutions.

Maxson *et al.* demonstrate that there are two albumin immunoalleles in this complex (*1*). They also show that one is present in major frequency in eastern *H. chrysoscelis* populations as far west as Mississippi, the other present in major frequency in central Texas (western) *H. chrysoscelis* populations, and that both are present in Texas populations of the

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tetraploid. Although their sample sizes are much smaller, these data appear to be consistent with the allele distributions at 1 of 12 electrophoretic loci that have been examined (*4*, *8*). Their data also show that both alleles are present in an eastern *H. chrysoscelis* population in extreme eastern Texas, nearly sympatric with *H. versicolor*.

It is regrettable that the exciting discoveries of the first case of albumin immunoalleles, and the first intermediate diploid population in this complex, were overlooked in favor of unwarranted speculation and erroneous interpretation.

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We have presented data demonstrating evolutionarily effective gene pool isolation of eastern and western populations of *Hyla chrysoscelis* (*1*). Ralin (*2*) misinterprets our phylogenetic argument. The data indicate that an estimated seven amino acid substitutions have been confined to either the eastern or western lineages since their original divergence. Protein sequence divergence within rapidly evolving proteins is a very sensitive indication of common or separate gene pools (*3*). The magnitude of albumin differentiation that we report has never been seen within a species (*4*), and is greater than the differences reported between albumins of some taxonomically distinct and reproductively isolated species of hylid and pipid frogs (*5*). This degree of divergence is measured by single individuals. Therefore the necessary existence of a barrier to gene flow is inferred without an exhaustive population survey, and Ralin's comments concerning sampling statistics are not relevant.

The presence of two variants in the eastern Texas population (3 IDU to eastern and 6 IDU to western *H. chrysoscelis*) invites speculation as to their origin. We agree with Ralin that it is unlikely that these eastern variants are hybrids. Whatever the case, it does not alter the measured albumin divergence between the eastern and western populations and their inferred historical isolation.

As to the significance of immunological nonreciprocity, a third species, the antibody-producing rabbit, is always implicitly involved because antibodies can only be made to immunogenic sites identifiable as "non-self." The albumin within the rabbit can influence the measurement if by chance it is "closer" to one or the other of the compared species. Nonreciprocal measurements are repeatably stable even when different rabbits are used (*6*). They are not, therefore, two samples of a single random variable, the "statistical situation" that Ralin criticizes (*2*). Nonreciprocity appears to reflect the stochastic nature of molecular evolution in the two independent lineages.

The proposed recent origin of the tetraploid species *H. versicolor* is not evidence of successful gene flow between the parental "forms" as Ralin states (*2*). There is no evidence of triploid frogs, and no other indication that alleles pass from the tetraploid back into the diploid populations.

Nowhere do we argue that quadrivalent formation in *H. versicolor* implies an allotetraploid origin. The existence of both eastern and western albumin alleles within *H. versicolor* is demonstrated directly by our data. The significance of quadrivalent formation is that it provides a genetic mechanism for generating the observed immunologically intermediate tetraploids. Because of the conservative nature of chromosomal evolution in frogs (*7*), quadrivalent formation would be expected whether the tetraploid arose through auto- or allotetraploidy.

We believe our original presentation is not "unwarranted speculation and erroneous interpretation" (*2*), but follows logically from the molecular evidence. Our argument crucially hinges only on the degree of divergence of the eastern and western albumins. While it is Ralin's prejudice that only fieldwork can determine "species distinctness" (*2*), it would appear that the phylogenetic dimension holds considerable promise of revealing important subtleties that might otherwise be overlooked. Further "resolution" will require additional data.

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