1978 JOURNAL OF HERPETOLOGY 12(4):555-558

# Cytological Differentiation of the Diploid-Tetraploid Species Pair of North American Treefrogs (Amphibia, Anura, Hylidae)

Marion N. Cash and James P. Bogart[1]

LSU School of Medicine, Shreveport, Louisiana 71103, USA and
Department of Zoology, University of Guelph, Guelph, Ontario, N1G 2W1

*ABSTRACT*—Current differentation of tetraploid *Hyla versicolor* LeConte and diploid *H. chrysoscelis* Cope relies upon acoustical or chromosomal analyses requiring live specimens. The tetraploid cells have larger nuclei and more nucleoli so it is possible to identify both preserved and living specimens simply and reliably. The possibility of identifying preserved museum specimens should ensure a more accurate and complete range for this interesting species pair.

* * *

*Hyla chrysoscelis* and *H. versicolor* represent a diploid-tetraploid species pair (Bogart and Wasserman, 1972). So far, no morphometric or ecological factors have been found to distinguish between these biological species over their combined recognized range (see Conant, 1975). Our current knowledge of the geographical ranges of these two species has been roughly outlined by pulse rate analyses of calls from males of each species (Brown and Brown, 1972; Jaslow and Vogt, 1977; Johnson, 1966; Ralin, 1968; 1977; Zweifel, 1970). The identification of females is conjectural.

This study was undertaken to determine new methods of identification applicable to preserved specimens which would allow ranges to be accurately mapped with greater ease and rapidity than that possible with methods using live specimens. Certain cells of tetraploid *H. versicolor* are larger than those of diploid *H. chrysoscelis* (Bogart and Wasserman, 1972), and the nuclei of *H. versicolor* contain almost twice the amount of DNA found in the nuclei of *H. chrysoscelis* (Bachmann and Bogart, 1975). Thus, the physical dimensions of the tetraploid nuclei should be greater than those of their diploid counterparts. Also, the number of nucleoli should be increased in the tetraploid species. Nucleoli are formed by nucleolus organizer regions (NORs) which have been associated with secondary constrictions of particular chromosomes in several species (Barr, 1966; Dearing, 1934; Kaufmann, 1938). Secondary constrictions have been identified in both species (Bogart and Wasserman, 1972), and an increased number of "nucleolar chromosomes" probably exists in the duplicated set of chromosomes in the tetraploid nuclei. A study was made to determine if differences in nuclear size and nucleolar number could be demonstrated and used as criteria to identify tadpoles and adults of the two species.

## MATERIALS AND METHODS

Adults of *Hyla versicolor* and *H. chrysoscelis* were obtained from several localities in Louisiana and Texas. The frogs were artificially crossed (see Mecham, 1965) to produce tadpoles of both species as well as triploid hybrids from both combinations of parents. Standard paraffin sections were prepared from tadpoles of comparable developmental stages and stained with hematoxylin and eosin. Areas of the sections were projected with a camera lucida microscopic assembly (Murad, 1966). The same magnification was used for all measurements. Nuclei of the mantle cell layer of the spinal cord were selected for this study because of their nearly spherical shape. Fifty nuclei were measured from each of 5 *H. chrysoscelis,* 5 *H.*

[1]Reprint requests to JPB.

*versicolor,* and 8 hybrid tadpoles (four from each parental combination). Ploidy was confirmed prior to fixation by chromosome counts following Bogart (1968).

Nucleoli were counted in cells by squashing tadpole tail tips and staining by the procedure outlined by Fernandez-Gomez et al. (1969). The technique was altered by substituting 50% lactic acid for acetic acid. Three hundred nucleolar counts were obtained from each of five *H. chrysoscelis* tadpoles, five *H. versicolor* tadpoles, and six hybrid tadpoles (three from each parental combination). Cells for nucleolar count were obtained from adult frogs by squashing small pieces of nictitating membrane using the same staining technique. This tissue was specifically chosen in order to minimize the alteration of museum specimens. The adult specimens used had been preserved for as long as 20 years and were maintained in 40% isopropanol. These specimens were identified as *H. versicolor* or *H. chrysoscelis* (20 of each) according to collecting locality using the range map of Ralin (1968). These localities currently contain allopatric populations of these species which was later confirmed by call and chromosome analyses.

TABLE 1. Nuclear diameter and nucleolar number comparisons between tadpoles of *Hyla chrysoscelis, H. versicolor* and their hybrids.

| Tadpoles | Mean Nuclear Diameter in arbitrary units (S.D.) | Mean Nucleolar Number |
|---|---|---|
| *H. chrysoscelis* | 4.6 (.08) | 1.9 |
| *H. versicolor* | 6.0 (.30) | 2.6 |
| *H. chrysoscelis* X *H. versicolor* | 5.1 (.30) | 2.1 |
| *H. versicolor* X *H. chrysoscelis* | 5.2 (.30) | 2.4 |

## RESULTS

The results obtained from the nuclear measurements and nucleolar counts are presented in Table 1. Analysis of variance for nuclear diameter indicated that the observed differences between the groups of tadpoles were statistically significant ($P < 0.01$). A closer inspection of the diffferences between the four individual groups was accomplished by calculating the least significant differences. This statistical method also indicated that the difference between the two species was significant ($P < 0.01$). *H. versicolor* nuclei differed significantly from both groups of hybrid nuclei ($P < 0.01$), and a significant difference was also demonstrated between *H. chrysoscelis* nuclei and the hybrid nuclei ($P < 0.05$). There was no significant difference between the two groups of hybrid nuclei. A chi-square goodness of fit test involving nucleolar counts from all four groups (see Table) indicated statistically significant difference between the groups ($P < 0.001$). It became evident that the cells in tetraploid individuals possess a greater average number of nucleoli than do diploid cells. Therefore, the possibility of distinguishing with some accuracy between *H. chrysoscelis* and *H. versicolor* tadpoles on the basis of their nucleolar number did exist. We found that such classification was possible by imposing the criterion that any individual be classified as a member of the species *H. versicolor* if more than 40% of the examined cells contain three or more nucleoli. Using this method, all the diploid and tetraploid tadpoles in this study were correctly classified. The museum specimens which fell within the range of *H. versicolor* consistently possessed cells containing three or four nucleoli, while those specimens assigned by locality to *H. chrysoscelis* had one or two nucleoli per cell. The nuclei shown in Figures 1 and 2 are typical of the results obtained from the museum specimens and, using the above criterion established for the tadpoles, the species could easily be identified.

## DISCUSSION

The results from this study indicate that the measurement of nuclear diameter from paraffin sections is an accurate morphological method of species recognition in this cryptic species pair. When the mean diameters were converted to measurements of spherical volume, *H.*

*versicolor* nuclei were approximately 2.1 X the size of the *H. chrysoscelis* nuclei. This measurement is comparable to the expected doubling of the nuclear size resulting from the presence of twice the amount of chromatin in the tetraploid nuclei. When the data from both groups of hybrids were pooled, the triploid nuclei were found to be approximately 1.4 X the size of the diploid *H. chrysoscelis* nuclei. This degree of difference approaches the 50% increase expected for the increased amount of chromatin present. Because of possible mutilation of museum specimens and the time involved, nucleolar count is the method of choice for identification of preserved frogs.

Nucleolar number varied among individuals as well as among different cells from the same individual. Such variation has been noted by others working with different species of plants or animals. The nucleolar cycle in the human cell, which is known to contain 10 NORs was investigated by Anastassova-Kristeva (1977). The nucleolar kinetics is such that the ten primary nucleoli associate progressively through interphase and dissociate during prophase. The association results in fewer, larger, denser nucleoli which, according to Anastassova-Kristeva, corresponds to the association between the acrocentric SAT chromosome pairs which contain the NORs.

The technique of nucleolar count should be valuable in helping to rapidly outline a more complete geographical range for this interesting species pair or "complex" (see Ralin, 1977) which is a prerequisite to a better understanding of the polyploid phenomenon.

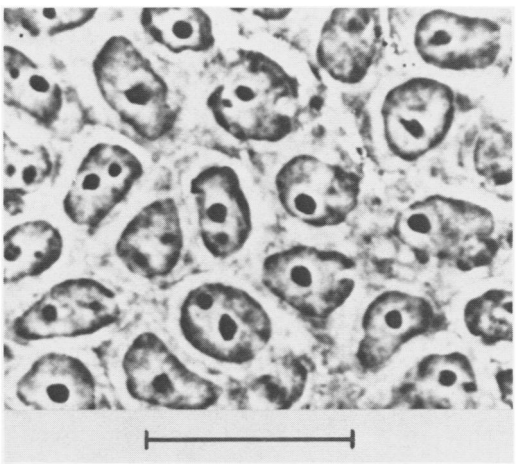

FIGURE 1. Photomicrograph to demonstrate the nucleoli found in a diploid frog from Angelina County in Texas. The specimen was preserved in May, 1968.

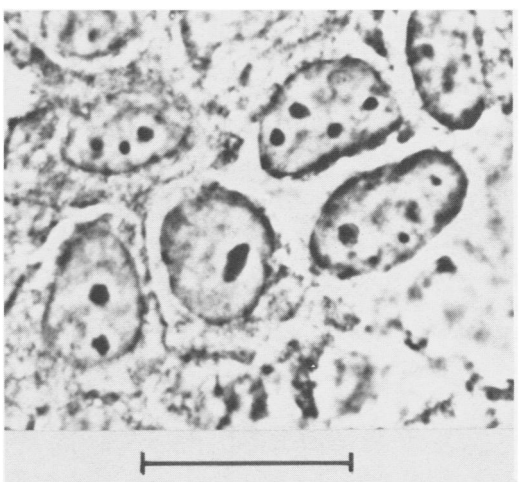

FIGURE 2. Photomicrograph of the nucleoli found in a tetraploid frog from Houston County in Texas. The specimen was preserved in April, 1966. Four nucleoli are evident in one of the nuclei. Both figures were taken at the same magnification under oil using phase contrast optics. The line represents 20 μm.

## ACKNOWLEDGMENT

We wish to thank J. L. Darling, Curator, The University of Texas at Arlington Vertebrate Collection, for the loan of preserved specimens, R. F. Walker for aid with statistical analysis, S. H. Cash for laboratory assistance, and D. L. DeSha and J. B. Mailhes for their helpful suggestions. This study was part of a MS thesis, partially supported by an in-house research grant from the College of Life Science Division of Research, Louisiana Tech University.

## LITERATURE CITED

Anastassova-Kristeva, M. 1977. The nucleolar cycle in man, J. Cell Sci. 25:103-110.

Bachmann, K. and J. P. Bogart. 1975. Comparative cytochemical measurements in the diploid-tetraploid species pair of hylid frogs, *Hyla chrysoscelis* and *H. versicolor*. Cytogenet. Cell Genet. 15:186-194.

Barr, H. J. 1966. Problems in the development and cytogenetics of nucleoli in *Xenopus*. Natn. Cancer Inst. Monogr. 23:411-424.

Bogart, J. P. 1968. Chromosome number difference in the amphibian genus *Bufo*: the *Bufo regularis* species group. Evolution 22:42-45.

———— and A. O. Wasserman. 1972. Diploid-polyploid cryptic species pairs: a possible clue to evolution by polyploidization in anuran amphibians. Cytogenetics 11:7-24.

Brown, L. E. and J. R. Brown. 1972. Mating calls and distributional records of treefrogs of the *Hyla versicolor* complex in Illinois. J. Herpetol. 6:233-234.

Conant, R. 1975. A field guide to reptiles and amphibians of eastern and central North America. 2nd ed.; Houghton Mifflin Co., Boston. 429 pp.

Dearing, N. H. 1934. The material continuity and individuality of the somatic chromosomes of *Amblystoma tigrinum* [sic], with special reference to the nucleolus as a chromosomal component. J. Morph. 56:157-159.

Fernandez-Gomez, M. E., J. C. Stockert, J. F. Lopez-Saez, and G. Jimenez-Martin. 1969. Staining plant cell nucleoli with $AgNO_3$ after formalin-hydroquinone fixation. Stain Tech. 44:48-49.

Jaslow, A. P. and C. Vogt. 1977. Identification and distribution of *Hyla versicolor* and *Hyla chrysoscelis* in Wisconsin. Herpetologica 33:201-205.

Johnson, C. 1966. Species recognition in the *Hyla versicolor* complex. Texas J. Sci. 18:361-364.

Kaufmann, B. P. 1938. Nucleolus-organizing regions in salivary gland chromosomes of *Drosophila melanogaster*. Z. Zellforsch. mikrosk. Anat. 28:1-11.

Mecham, J. S. 1965. Genetic relationships and reproductive isolation in southeastern frogs of the genera *Pseudacris* and *Hyla*. Amer. Midland Natur. 74:269-308.

Murad, J. L. 1966. A modified camera lucida assembly. Turtox News 44:20.

Ralin, D. B. 1968. Ecological and reproductive differentiation in the cryptic species of the *Hyla versicolor* complex (Hylidae). Southwest. Natur. 13:283-300.

————. 1977. Evolutionary aspects of mating call variation in a diploid-tetraploid species complex of treefrogs (Anura). Evolution 31:721-736.

Zweifel, R. G. 1970. Distribution and mating call of the treefrog, *Hyla chrysoscelis*, at the northeastern edge of its range. Chesapeake Sci. 11:94-97.

Accepted 26 May 1978