Copeia, 2000(3), pp. 860–862

Validity of Nucleolar Number for Identification of the Diploid-Tetraploid Gray Treefrogs, *Hyla chrysoscelis* and *Hyla versicolor*

MICHAEL J. KELLER

The identification of the cryptic diploid-tetraploid Gray Treefrogs, *Hyla chrysoscelis* and *H. versicolor*, from preserved specimens is problematic using conventional methods, such as karyotyping. Nucleolar number has been used as an index of ploidy for this purpose, based on the assumption of a single nucleolus per chromosome set. However, cytogenetic studies of nucleolar organizer regions (NORs) have shown that putative secondary NORs occur in some populations of the diploid, *H. chrysoscelis*. This study demonstrates that secondary NORs in the diploid genome are associated with additional nucleoli in interphase cell nuclei. The number of nucleoli is proportional to the total number of primary and secondary NORs, not ploidy level, and, therefore, is not a valid character for identification of specimens from populations having secondary NORs. Because little is known about the frequency of secondary NORs in most *H. chrysoscelis* populations, nucleolar number should be avoided as a primary means of species identification.

THE Gray Treefrogs *Hyla chrysoscelis* and *Hyla versicolor* make up a diploid-tetraploid species complex with an extensive range that encompasses much of eastern North America between southern Canada and northern Florida, from the Atlantic seaboard westward to central Texas and Manitoba (Blair, 1958). The distributional patterns of each species, however, are complex and poorly understood, as illustrated by conflicting accounts published over the years (Ralin, 1968, 1977; Romano et al., 1987). The tetraploid, *H. versicolor*, is a cryptic species with no distinct morphological features to distinguish it from the diploid, *H. chrysoscelis* (Ralin, 1968; Ralin and Rogers, 1979). Live specimens can be identified accurately by differences in advertisement call structure (Johnson, 1966), chromosome number (Wasserman, 1970), relative DNA content per cell (Bachman and Bogart, 1975; Gerhardt et al., 1994), or cell size (Bogart and Wasserman, 1972; Matson, 1990); however, these methods are difficult or impossible to use with preserved animals. Cash and Bogart (1978) proposed distinguishing between diploid and tetraploid gray treefrogs on the basis of nucleolar number, which had previously been shown to accurately indicate ploidy level in salamanders (Fankhauser and Humphrey, 1943). Unlike cell size, nucleolar number is not altered in preserved tissues and is often used for the identification of museum specimens (e.g. Hillis et al., 1987; Bogart and Jaslow, 1979; Little et al., 1989).

Nucleoli are formed in the nucleus by the chromosomal nucleolar organizer regions (NORs), which mark the locations of large ribosomal DNA repeats. The nucleolar count method explicitly assumes that there is a single NOR per set of chromosomes, such that diploids never have more than two nucleoli, whereas tetraploids will have up to four. However, Wiley et al. (1989) reported the occurrence of a variety of secondary NOR sites in *H. chrysoscelis*, as well as a loss of primary NORs in *H. versicolor*. It is not known whether secondary NORs produce nucleoli independent of the primary nucleoli during the cell cycle. If the presence of secondary NORs increases the number of nucleoli, the nucleolar count method may overestimate the ploidy of *H. chrysoscelis* specimens resulting in their misidentification as *H. versicolor*. Here, I present results from a cytogenetic study of a highly polymorphic population of *H. chrysoscelis* that confirms the nucleolus-forming capability of secondary NORs.

MATERIALS AND METHODS

Ten *H. chrysoscelis* (four males and six females) were collected from a breeding assemblage in Phelps County, Missouri, in June 1998. Samples of whole blood were collected from each individual by nonlethal cardiocentesis, and lymphocytes were cultured following the methods of Wiley and Meisner (1984) and Wiley et al. (1989). Five drops of 10 μg/ml Colcemid (Gibco) were added to the culture media for six hours to synchronize actively dividing cells at metaphase. Blood cells were treated with a hypotonic solution of 0.05 M KCl prior to fixation in 3:1 methanol:acetic acid. Three drops of concentrated cell suspension were applied to cold, wet slides, dried over steam for 30–90 sec, and air-dried for 1–2 h. Nucleoli and potential

TABLE 1. CHROMOSOMAL LOCATIONS OF PRIMARY AND SECONDARY NORS AND FREQUENCY OF CELLS WITH DIFFERENT NUCLEOLAR COUNTS FOR *Hyla chrysoscelis*.

ID	NORs		Cells w/ #nucleoli					Mean #nuc.	Max. #nuc.	%Cells >2 nuc.
			1	2	3	4	5			
Hc203	6p−		100	—	—	—	—	1.00	1	0
Hc010	6p−	2p	60	40	—	—	—	1.40	2	0
Hc008	6p−	4q	63	37	—	—	—	1.37	2	0
Hc006	6p6p		79	21	—	—	—	1.21	2	0
Hc148	6p6p	4q	36	47	17	—	—	1.81	3	17
Hc149	6p6p	7q	55	31	14	—	—	1.59	3	14
Hc146	6p6p	$1p_{dist}1p_{dist}$	10	45	34	11	—	2.46	4	45
Hc011	6p6p	$1p_{cent}7q$	16	47	31	6	—	2.27	4	37
Hc007	6p6p	3p7q	20	31	34	15	—	2.44	4	49
Hc009	6p6p	$2p4q_{ter}7q$	20	40	29	8	3	2.34	5	40

NORs on chromosomes were silver-stained using the colloidal developer method of Howell and Black (1980). Slides were dried overnight, and coverslips were applied with Permount mounting medium. For each individual, at least 10 sets of metaphase chromosomes were observed at 1000× magnification to determine chromosomal locations of NORs.

Red blood cell nuclei were used for the nucleolar counts because they do not divide and would not be affected by culture conditions or colcemid treatment. Counts made from white blood cell nuclei gave similar results. The number of nucleoli per cell was counted for 100 cells from each individual, and the mean and maximum number of nucleoli per cell and the proportion of cells with three or more nucleoli were calculated.

RESULTS

The Phelps County, Missouri, population of *H. chrysoscelis* exhibited an exceptionally high degree of polymorphism, with seven different secondary NORs. Only one individual of the 10 examined had the expected NOR complement with only primary NORs on the short arms of both homologues of chromosome 6 (6p6p), whereas three individuals possessed the 6p-NOR on only a single homologue. Of the seven secondary NOR sites, two were located on the short arm of chromosome 1 at distal and centromeric positions ($1p_{dist}$ and $1p_{cent}$), two were on the long arm of chromosome 4 at terminal and medial positions ($4q_{ter}$ and 4q), and the remaining three occupied medial positions on the short arms of chromosomes 2 and 3 (2p, 3p) and the long arm of chromosome 7 (7q). The sample NOR frequencies suggest that secondary NORs are common in this population.

Six of the 10 individuals examined had nuclei containing more than two nucleoli, and four of those individuals had nuclei with more than three nucleoli. The maximum number of nucleoli per cell was equal to the total number of primary and secondary NORs, independent of specific complement, and the mean number of nucleoli per cell increased with total NOR number (Table 1).

DISCUSSION

The direct relationship between the maximum number of nucleoli per cell and the total number of primary and secondary chromosomal NORs confirms that the secondary NOR sites actively contribute to nucleolus formation during the cell cycle. The occurrence of secondary NOR sites in the *H. chrysoscelis* genome violates the assumption of one NOR per chromosome set underlying the use of nucleolar counts to determine ploidy level in gray treefrogs. Using the maximum number of nucleoli per cell as an indicator of ploidy level would correctly identify only three of the 10 individuals in this study as diploid. Cash and Bogart (1978) suggested that individuals could be correctly classified as tetraploids, as opposed to diploids or triploid hybrids, if more than 40% of nuclei contained three or more nucleoli. Even with that relatively high threshold criterion, at least three of the diploids in this study would be misidentified as tetraploids (Hc146, Hc007, and Hc009; Table 1).

The number and frequency of secondary NOR sites in the study population were surprisingly high, especially considering the small number of individuals examined. An extensive cytological survey by Wiley et al. (1989) of 34 *H. chrysoscelis* populations sampled throughout the species range found a total of eight secondary NOR sites, in addition to five alternative pri-

mary NOR sites. In that study, only five populations possessed secondary NORs, and the most polymorphic of those only had three secondary sites.

Whereas nucleolar counts can overestimate ploidy level in *H. chrysoscelis*, they may also underestimate ploidy level in the tetraploid, *H. versicolor*. Wiley et al. (1989) reported the absence of NORs on homologues of chromosome 6 in individuals from 15 populations of *H. versicolor* sampled from widespread areas throughout the range of the species. Individuals with one to four NORs were observed, and although no nucleolar counts were performed, by definition the maximum number of nucleoli must be proportional to NOR number. Thus individuals with only one or two NORs would be misidentified as diploids. Therefore, loss of NORs also may contribute significantly the misidentification of gray treefrogs.

It is clear that nucleolar counts can be misleading when used to determine the ploidy level of gray treefrogs. Therefore, nucleolar counts should be avoided as a primary method of determining ploidy level and species identity in gray treefrogs. Several studies have indicated that cell dimensions are significantly larger in *H. versicolor* than in *H. chrysoscelis* (e.g., Bogart and Wasserman, 1972; Cash and Bogart, 1978; Matson, 1990). The direct measurement of cell or nuclear size, although more tedious and subject to increased variance from osmotic damage to preserved tissues, is perhaps the best alternative to nucleolar counts for identifying preserved specimens.

Acknowledgments

This project would not have possible without the support of H. C. Gerhardt and J. David. I am also grateful for the critical review of this manuscript by M. Bee and C. van der Weele. All collections were made under Missouri permit 4510, and work was carried out under University of Missouri Animal Care Protocol 1910.

Literature Cited

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

Blair, W. F. 1958. Mating call in speciation of anuran amphibians. Am. Nat. 92:27–51.

Bogart, J. P., and A. P. Jaslow. 1979. Distribution and call parameters of *Hyla chrysoscelis* and *Hyla versicolor* in Michigan. Royal Ontario Museum, Life Science Contribution 1173. Toronto, ON, Canada

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

Cash, M. N., and J. P. Bogart. 1978. Cytological differentiation of the diploid-tetraploid species pair of North American treefrogs (Amphibia, Anura, Hylidae). J. Herpetol. 12:555–558.

Fankhauser G., and R. R. Humphrey. 1943. The relation between number of nucleoli and number of chromosome sets in animal cells. Proc. Nat. Acad. Sci. (USA) 29:344–350.

Gerhardt, H. C., M. B. Ptacek, L. Barnett, and K. G. Torke. 1994. Hybridization in the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *Hyla versicolor*. Copeia 1994:51–59.

Hillis, D. M., J. T. Collins, and J. P. Bogart. 1987. Distribution of diploid and tetraploid species of gray treefrogs (*Hyla chrysoscelis* and *Hyla versicolor*) in Kansas. Am. Midl. Nat. 117:214–217.

Howell, W. M., and D. A. Black. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36:1014.

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

Little, M. L., B. L. Monroe Jr., and J. E. Wiley. 1989. The distribution of the *Hyla versicolor* complex in the northern Appalachian highlands. J. Herpetol. 23:299–303.

Matson, T. O. 1990. Erythrocyte size as a taxonomic character in the identification of Ohio *Hyla chrysoscelis* and *H. versicolor*. Herpetologica 46:457–462.

Ralin, D. B. 1968. Ecological and reproductive differentiation in the cryptic species of the *Hyla versicolor* complex (Hylidae). Southwest. Nat. 13:283–299.

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

———, and J. S. Rogers. 1979. A morphological analysis of a North American diploid-tetraploid complex of treefrogs (Amphibia, Anura, Hylidae). J. Herpetol. 13:261–269.

Romano, M. A., D. B. Ralin, S. I. Guttman, and J. H. Skillings. 1987. Parallel electromorph variation in the diploid-tetraploid gray treefrog complex. Am. Nat. 130:864–878.

Wasserman, A. O. 1970. Polyploidy in the common tree toad *Hyla versicolor* Le Conte. Science 167:385–386.

Wiley, J. E., and L. F. Meisner. 1984. Synergistic effect of TPA and T-cell mitogens in nonmammalian vertebrates. In Vitro 20:932–936.

———, M. L. Little, M. A. Romano, D. A. Blount, and G. R. Cline. 1989. Polymorphism in the location of the 18S and 28S rRNA genes on the chromosomes of the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *Hyla versicolor*. Chromosoma 97:481–487.

Division of Biological Sciences, 105 Tucker Hall, University of Missouri, Columbia, Missouri 65211. E-mail: c626284@showme.missouri.edu. Submitted: 7 June 1999. Accepted: 19 Jan. 2000. Section editor: J. D. McEachran.