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IDENTIFICATION OF GRAY TREEFROG POPULATIONS IN MIDDLE TENNESSEE: CHROMOSOME COUNTS FROM SQUASHED TADPOLE TAIL TIPS

GARY R. PRITTS AND BRIAN T. MILLER

Department of Biology, Middle Tennessee State University, Murfreesboro, TN 37132

ABSTRACT—The gray treefrogs *Hyla chrysoscelis* (2n = 24) and *Hyla versicolor* (4n = 48) are morphologically indistinguishable from each other. As a consequence, maps depicting the ranges of gray treefrogs usually represent composite distributions. We collected gray treefrog tadpoles from twenty localities and examined chromosome preparations to determine the occurrence of gray treefrog species in middle Tennessee. Chromosome squashes were prepared from the excised tail tips of ten tadpoles collected from each locality. All tadpoles sampled were identified as *H. chrysoscelis* (2n = 24); no *H. versicolor* (2n = 48) or hybrids (3n = 36) were found. A growing body of evidence suggests that *H. chrysoscelis* is the most common, if not the only, member of the gray treefrog complex in middle Tennessee.

The gray treefrog species complex consists of at least two sibling species, *Hyla chrysoscelis* and *Hyla versicolor.* Morphologically, these two species are indistinguishable from one another and historically were regarded as a single species. Two distinct vocalizations (a fast-trill and a slow-trill) were reported for gray treefrogs (Noble and Hassler, 1936; Hoffman, 1946; Wiley, 1983). Subsequently, populations with different vocalizations were found to be reproductively isolated (Johnson, 1959; 1963; Littlejohn et al., 1960). Reproductive isolation among populations of gray treefrogs with distinct vocalizations prompted Johnson (1966) to suggest that two species were being recognized as one. He designated the slow-trill species as *H. versicolor* and the fast-trill species as *H. chrysoscelis.* Wasserman (1970) karyotyped the two species and reported that *H. chrysoscelis* is a diploid species (2n = 24) and that *H. versicolor* is a tetraploid species (4n = 48).

Because of the difficulty in distinguishing between the sibling species, maps depicting the ranges of gray treefrogs often are composite distributions of both species. However, the tetraploid nucleus of *H. versicolor* is larger than the diploid nucleus of *H. chrysoscelis* and several laboratory techniques have been developed that facilitate identification of "gray treefrogs". Most of these techniques measure one or more physical parameters of the cell and are, consequently, indirect measures of ploidy (toe pad cell size, Green, 1979; Chaffin and Trauth, 1987; erythrocyte cell size, Bogart and Wasserman, 1972; Matson, 1990; nuclear diameter, Cash and Bogart, 1978; microcomplement fixation, Maxson et al., 1977; diameter of eyelid cells, Chaffin and Trauth, 1987; and nucleoli number per eyelid cell, Burkett, 1989). While data obtained from studies using these techniques often have been useful in determining more exact distributions for members of the gray treefrog complex, results occasionally have been inconclusive. Consequently, direct counts of chromosomes remain the most reliable method to differentiate between *H. chrysoscelis* and *H. versicolor.*

The gray treefrog complex occurs throughout Tennessee (Redmond and Scott, 1996), but few studies have attempted to identify populations to species. A notable exception is the work of Burkett (1989). He used nucleoli counts in preserved specimens and call rates in the field to identify gray treefrogs throughout much of Tennessee. His data suggest that *H. chrysoscelis* occurs statewide and that *H. versicolor* is limited in distribution to extreme southwestern and northeastern Tennessee. Because the number of nucleoli found within the cell thought to be characteristic to *H. chrysoscelis* has been found in specimens of *H. versicolor* (Little et al., 1989), the possibility exists that populations of *H. versicolor* could be misidentified in studies relying on nucleoli counts to identify species of gray treefrogs. The purpose of this study was to use counts of chromosomes, as obtained from squashed tadpole tail tips, to identify the species of gray treefrog that occurs in middle Tennessee.

MATERIALS AND METHODS

Twenty breeding sites of gray treefrogs were located throughout middle Tennessee by the presence of calling males (Fig. 1). Twenty tadpoles were collected from each site and transported to the laboratory. Tadpoles were identified as "gray treefrogs" by use of key characters (Altig, 1970) and by identification of froglets that metamorphosed in the laboratory.

Chromosomes were counted from squashes obtained from the excised tail tips of ten tadpoles selected randomly from each site. The chromosome preparations were obtained by modifying a method described by Bogart (1968). The tadpoles were submersed in a 0.006% colchicine solution for 12 h; colchicine arrests cell division at metaphase and consequently facilitates chromosomal preparations. Following 12 h submersion in colchicine, the tadpoles were anesthetized with tricaine methylsulfonate and their tail tips were excised. The excised tail tip was fixed to a slide by placing it over glactic acid fumes for one min. Orecin was applied to the tissue for two min to stain and darken the chromosomes. The tissue preparation was then placed into 70% glactic acid for five min to decolorize the rest of the tissue. A glass cover slip was placed over the tissue and a standard thumb

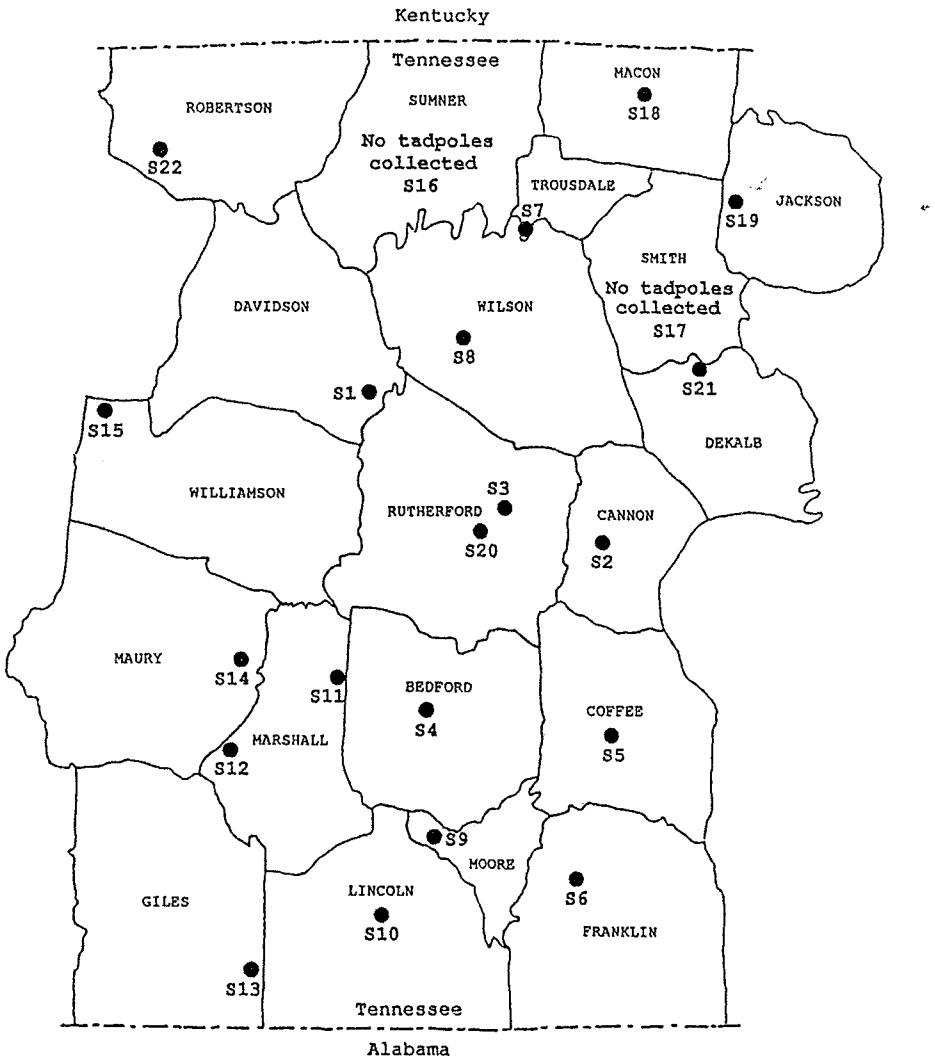

FIG. 1. Map of middle Tennessee. Collection sites dotted and numbered.

squash applied. The chromosome squash was sealed by applying clear fingernail polish to the edges of the cover slip, and an identifying label was applied to each slide. Two hundred tail tip squashes were prepared.

All preparations were viewed with a Nikon microscope equipped with a Nikon AFX attachment (Preiser Scientific, Durham, North Carolina). The chromosomes were photographed at 1000× on Ilford Pan F 50 film. However, the chromosomes often stacked up on one another, and seldom spread-out in a single plane. Consequently, chromosomes were counted from the slides rather than from the photographs. Most chromosomes could readily be seen and counted by adjusting the depth of field on the microscope while viewing the squashed preparation. No attempt was made to karyotype the samples as we were interested in determining only the number of chromosomes the tail tip cells contained (24 chromosomes = *H. chrysoscelis,* 36 = hybrid, 48 = *H. versicolor*).

RESULTS

All populations of gray treefrogs sampled were identified as *Hyla chrysoscelis* (2n = 24); no *Hyla versicolor* (4n = 48) or hybrids (3n = 36) were identified within the sampled populations. Complete complements of chromosomes were found in nearly all preparations. However, only twenty-three chromosomes were counted in several preparations and twenty-five chromosomes were counted in one preparation. We assumed that the anomalous counts were artifacts resulting from improper cell squashes.

DISCUSSION

Techniques that measure physical parameters of cells as an indirect count of ploidy are often used to identify museum specimens of the gray treefrog complex, but these techniques have had varying degrees of success. For instance, Chaffin and Trauth (1987) found two size classes (*H. chrysoscelis* and *H. versicolor*) of eyelid cells in the specimens they examined from Arkansas, but in some populations they also found cells of an intermediate size. Matson (1990) found that cell size differed between populations of the same species located in different geographic areas. Also, Green (1979) matched toe pad cell size and the species of treefrog less than 80% of the time. The use of call rates also has had a varying degree of success in the identification of gray

treefrog species; Gerhardt (1974) has shown that the pulse rate of the call can vary among conspecifics.

To further complicate interpretation of results obtained from techniques avoiding direct counts of ploidy, natural hybridization has been reported between *H. versicolor* and *H. chrysoscelis* (Gerhardt et al., 1994). Indeed, several authors have suggested the possibility of multiple origins of polyploidy in the gray treefrog complex (Chaffin and Trauth, 1987; Hillis et al., 1987; Ptacek et al., 1994). We suggest that chromosome counts of cells from the tips of tadpole tails provide a definitive method of identifying members of the gray treefrog complex.

Burkett (1989) used an indirect measure of ploidy (nucleoli counts) to determine the distribution of *H. chrysoscelis* and *H. versicolor* in Tennessee. The range map he constructed suggests that *H. chrysoscelis,* but not *H. versicolor,* occurs in middle Tennessee. Our data support Burkett's conclusions, as we found no evidence of polyploidy in any of our samples. Wiley (1982) and Miller (1991), using direct chromosome counts, also identified populations in middle Tennessee (Wilson County and Warren County, respectively) as *H. chrysoscelis.* Therefore, a growing body of evidence suggests that *H. chrysoscelis* is the only member of the gray treefrog complex that occurs in middle Tennessee.

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