Jour. Iowa Acad. Sci. 108(3):79–83, 2001

Identification and Distribution of the Treefrogs *Hyla versicolor* and *Hyla chrysoscelis* in Iowa

CATHERINE E. C. OBERFOELL and JAMES L. CHRISTIANSEN

Department of Biology, Drake University, 25th and University Avenue, Des Moines, Iowa 50311

Historically, the tetraploid and diploid species pair of gray treefrogs, *Hyla versicolor* and *Hyla chrysoscelis*, were mapped collectively in Iowa because no macroscopic morphological characteristics useful in recognizing the two species had been found. The present study reports identification and separation of the species by counts of nucleoli in the palpebral membrane of the eye and by measurement of scanning electron micrographs of toepad epithelial projections. Subsequent morphometric data confirm that the two species are morphologically distinct in Iowa but that overlap in characters continues to make macroscopic morphology unreliable for identifying individuals. *H. chrysoscelis* is distributed sporadically though all but the northeastern quarter of Iowa, while *H. versicolor* is found roughly southeast of a diagonal line extending from the northeastern to the southwestern corner of the state. A genetically different population appears to reside in Cherokee Co. Iowa.

INDEX DESCRIPTORS: gray treefrogs, *Hyla versicolor, Hyla chrysoscelis,* Iowa frogs.

The *Hyla versicolor-Hyla chrysoscelis* complex is the only known diploid-tetraploid species pair of frogs in North America (Wasserman 1970, Dalrymple 1993). Chromosomal analysis shows *H. versicolor* Le conte to be tetraploid (4n = 48), and *H. chrysoscelis* Cope to be diploid (2n = 24) (Wasserman 1970). Due to morphological and ecological similarities, the two species cannot be distinguished by gross morphological characteristics (McAlpine et al. 1991). The inability to differentiate these species morphologically has resulted in clumping of their distributions (Christiansen and Bailey 1991, Conant and Collins 1991). Most of the current information on the ranges of the two species is based on call pulse rate analysis of the males. When calling sympatrically, the two species can be distinguished by the pitch and duration of their call (Jaslow and Vogt 1977, Ralin and Selander 1979, McAlpine et al. 1991, Dalrymple 1993). This is complicated by increased pulse rate with increased temperature and by intermediate pulse rates with hybrids (Gerhardt et al 1994). This study examines three methods of separating the frogs: gross morphology, measurement of toepad projections, and counts of nucleoli, and uses calls of only a few frogs as supporting evidence.

Johnson (1966) first recognized that *H. versicolor* and *H. chrysoscelis* were distinct species. Reliable methods of identifying individuals were developed by Cash and Bogart (1978) and Fitzgerald et al. (1981) who found that certain cells of the tetraploid form are larger than those of the diploid form and can be used to distinguish the species. Treefrogs have hexagonal epithelial toepad projections (Green 1979, Green and Carson 1988, Hanna and Barnes 1991), and Green (1980) found these toepad projections in *H. versicolor* to be 1.81 times greater in mean relative volume than those of *H. chrysoscelis*. This difference in size was assumed to be the result of the increased amount of genetic material in the cells of the tetraploid species (Green 1980).

The difference in the amount of genetic material can also be seen in the size of the nucleus and in the number of nucleoli present. Cash and Bogart (1978) found the nuclei of *H. versicolor* to be 2.1 times the size of *H. chrysoscelis* nuclei. Similarly, a greater number of nucleoli were found in the cells of *H. versicolor* than in *H. chrysoscelis*, reflecting the increased amount of genetic material within the cells of the tetraploid species (Cash and Bogart 1978). It was therefore concluded that examination at the cellular level was necessary to correctly identify the two species (Cash and Bogart 1978, Ralin and Rogers 1979, Fitzgerald et al. 1981, Hillis et al. 1987, Dalrymple 1993). The present study was conducted to verify the consistency of counts of nucleoli with measurements of toepads and to search for morphological features useful in distinguishing the species in Iowa. The resulting identifications were used to map the distributions of *H. versicolor* and *H. chrysoscelis* in Iowa.

METHODS

Our study used a total of 156 frogs, either freshly collected or preserved specimens in the Drake University Research Collection (DURC). Some of the latter had been in storage for as many as 26 years. All specimens were eventually fixed in 13% formalin for at least 24 hours and stored in 5% formalin. Thirteen morphological measurements were made with dial calipers as outlined by Ralin and Rogers (1979) and Mason (1988). Measurements of bilateral structures were made on the animal's right side. Eight specimens were initially identified by call when several were calling in sympatric populations where they could easily be distinguished. These specimens served as reference points for comparisons with toepad and cellular characteristics. Morphological data for adult frogs were analyzed by group comparison test (t-test) for two samples with equal variance and reanalyzed with ANOVA. For this study, individuals less than 35mm snout vent length (SVL) were considered juveniles, and those 35mm SVL or greater were considered adults.

The third and fourth toepads on the left front foot of 17 specimens were excised and prepared by standard procedure for examination on the scanning electron microscope (SEM) (Postek et al. 1980). Fixation with glutaraldehyde was omitted due to prior fixation with formalin. Measurements of the toepad epithelial projections were made on micrographs at a magnification of 2,590X. Width mea-

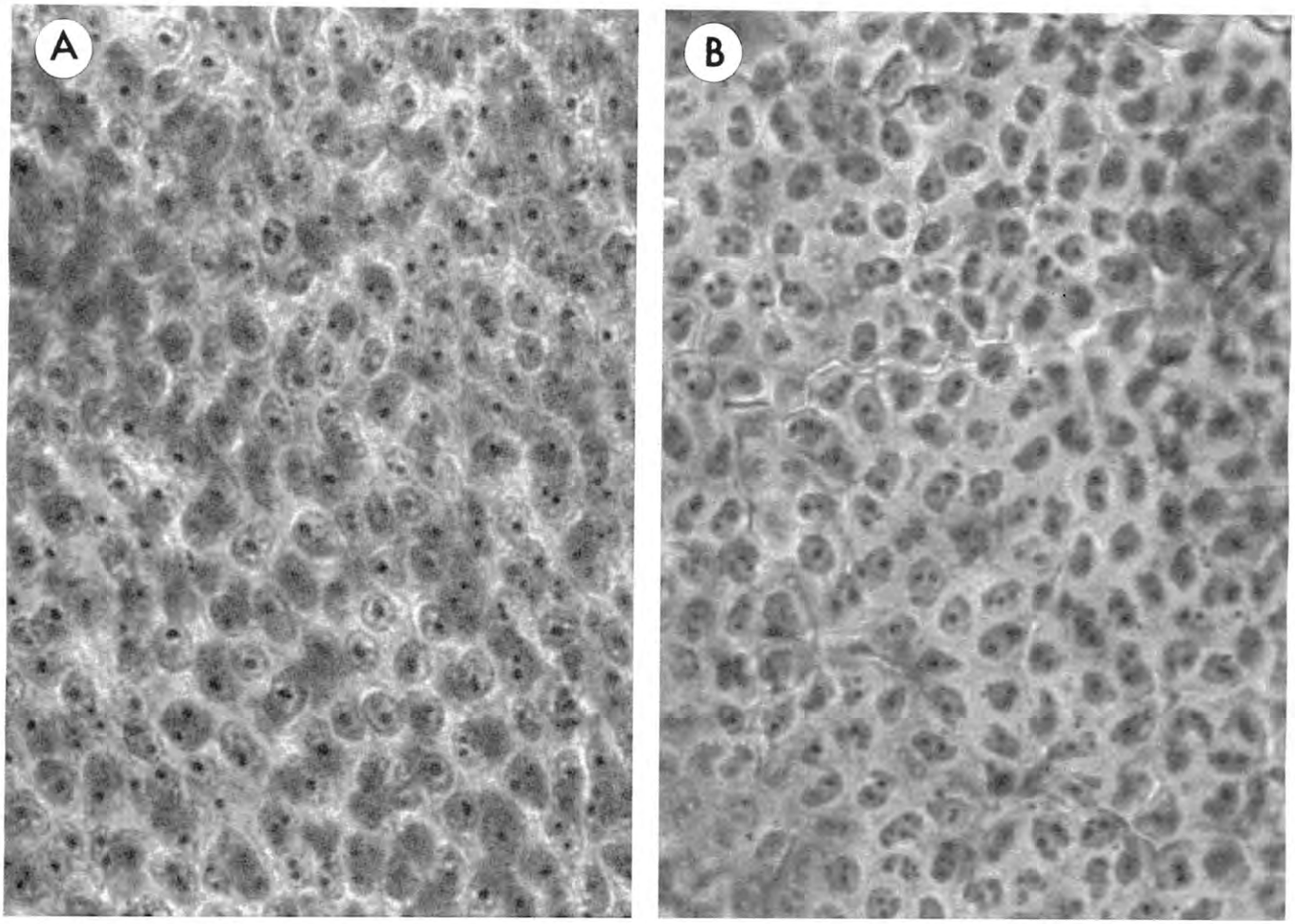

Fig. 1. Micrographs of silver stained palpebral spectacles. Cells of *H. chrysoscelis* (A) have one or two nucleoli per cell, never more. Cells of *H. versicolor* (B) often have 3–4 nuclei per nucleus. Many of those showing only one or two have a third outside of the focal plain. (1000×).

surements were made on the micrographs at the widest point on the cell to the nearest millimeter, averaged, and converted to micrometers.

Counts of nucleoli were made on silver stained slides of flattened palpebral spectacles, the transparent membrane covering the cornea (Fitzgerald et al. 1981). The palpebral spectacles were excised, and slides were prepared by the technique of Fernandez-Gomez et al. (1969). Their technique was modified by extending the initial fixation with 1:1 1% hydroquinone: 10% formalin to 4–6 hours and by reducing the silver staining incubation time to 8–12 hours. Because this staining technique employed the same concepts as photographic development, after excision no metal utensils were used, and procedures were performed under darkroom conditions. These tissues were examined and photographed under oil emersion at 1,000X. Specimens whose nuclei frequently contained three or four nucleoli were categorized as *H. versicolor.* Specimens with only one or two nucleoli in each nucleus were categorized as *H. chrysoscelis* (Cash and Bogart 1978). After completion of this portion of the study, specimens were collected from three counties on the northern Iowa border: Emmet, Winnebago, and Worth. These frogs were initially identified by call and the identification was confirmed by counts of nucleoli in hepatocyte and duodenal nuclei. The slides were cut at seven μm and stained with hematoxylin and eosin.

Specimens identified by counts of palpebral spectacle nucleoli were analyzed for consistency of toepad projection measurements by group comparison tests with pooled variances. All frogs collected were preserved and catalogued into the DURC where they remain available to researchers. Prepared slides and photographs are stored as well and are identified by the specimen catalog number.

RESULTS

The counts of nucleoli in silver-stained palpebral spectacles showed an apparent natural break among the 156 specimens examined. A block of typically small frogs had one, and often two, nucleoli per nucleus in each specimen (Fig. 1A). Frogs that were somewhat larger had two to four nucleoli (Fig. 1B). These differences were expected because the larger frogs, being tetraploid, would have more DNA than would smaller diploid frogs. Observations from stained hepatocytes and intestinal epithelial cells were consistent with those of the palpebral spectacles.

Typical specimens of each species identified on the basis of number of nucleoli were compared on the basis of size of toepad projections to verify the value of counts of nucleoli as a primary species-identifying character in Iowa. Toepads were larger in *H. versicolor* than in *H. chrysoscelis* (Table 1). *H. chrysoscelis* from Cherokee Co. had larger toepad projections than most individuals from other locations (Table 1). These individuals also had a more *H. versicolor*-like call, although

Table 1. Counts of nucleoli and measurements of toepad projections for *Hyla versicolor* and *H. chrysoscelis* collected in Iowa. t-test P = 0.016

DURC Number[a]	Nucleoli Counts	Projection Width
H. versicolor		
1574	3 and 4	16 μm
1570	3 and 4	16 μm
1569	3	18 μm
1568	3	18 μm
Mean/Std. Deviation		17.0 μm/1.5470
H. chrysoscelis		
1611	1 and 2	12 μm
2648	1 and 2	12 μm
1599	1 and 2	14 μm
2668	1 and 2	14 μm
1593	1 and 2	14 μm
1594	1 and 2	15 μm
1598	1 and 2	15 μm
2671	1 and 2	15 μm
1595	1 and 2	16 μm
2670[b]	1 and 2	16 μm
1596	1 and 2	16 μm
2669[b]	1 and 2	17 μm
Mean/Std. Deviation		14.67 μm/1.5569

[a]Des Moines University Research Collection
[b]Cherokee County Specimens

Table 2. Morphological features of *H. versicolor* (h.v.) and *H. chrysoscelis* (h.c.) already separated on the basis of counts of nucleoli. Numbers in t-test and F-test columns are calculated probabilities that the means for the two species are different. All measurements except head width are lengths (maximum distance) or distance between the two mentioned points (mm).

	Mean H.v.	Mean H.c.	Variance H.v.	Variance H.c.	t-test	F-test
SVL mm	44.91	41.92	20.56	16.10	0.0001	0.1647
femur	22.14	21.10	5.83	3.64	0.0068	0.0305
shank	20.38	19.49	4.09	3.06	0.0090	0.1226
foot	28.32	26.62	10.77	6.85	0.0013	0.0359
forearm	10.32	9.85	1.35	1.02	0.0150	0.1309
upperarm	8.34	7.74	1.17	0.97	0.0013	0.2262
hand	11.45	10.56	2.03	1.14	0.0001	0.0106
toepad	2.61	2.44	0.39	0.25	0.0902	0.0393
head (L)	13.88	13.03	2.18	1.21	0.0003	0.0097
eye-nostril	3.54	3.37	0.40	0.29	0.1108	0.1067
nostril-lip	3.73	3.36	0.31	0.26	0.0001	0.2465
tympanum	3.30	3.11	0.22	0.13	0.0091	0.0202
head (W)	16.07	14.88	4.72	2.17	0.0003	0.0010

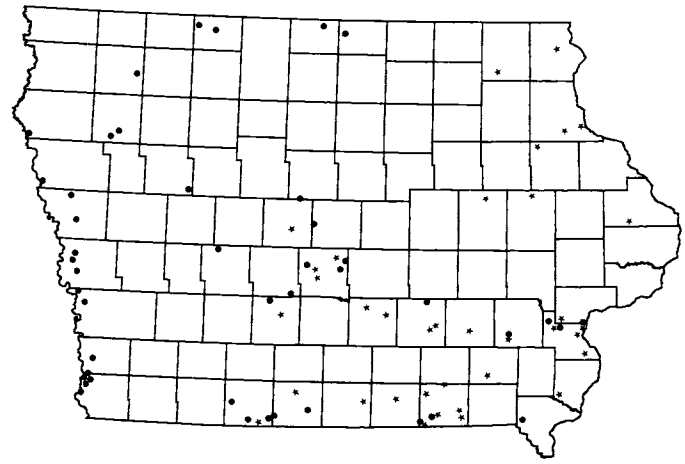

Fig. 2. Distribution of *H. chrysoscelis* (Solid Circles) and *Hyla versicolor* (Stars) in Iowa. All specimens were identified by counts of nucleoli in palpebral spectacles except those in the central counties on the northern border. Those were initially identified by call and confirmed by counts of nucleoli in hematoxylin and eosin stained hepatocytes and duodenum.

no calls were recorded. Even including this population, no *H. chrysoscelis* were found with toepad projections greater than 17μm. The overlap of toepad projection sizes in Table 1 is consistent with the overlap seen in other size-related characters examined in this study.

When studies of nucleoli and toepads were complete, it was evident that two species of gray treefrogs were present in Iowa. To determine whether these two populations in Iowa represented morphologically different animals, gross measurements of all frogs greater than 35mm SVL were compared (Table 2). While exhibiting great overlap, *H. versicolor* was significantly larger than *H. chrysoscelis* (P = <.05) in 11 of 13 characters (t-test) and in 7 of 13 with ANOVA. Although the means of these measurements were significantly different, measurement of all characters overlapped.

Use of nucleolar counts as a reliable way to distinguish *H. versicolor* from *H. chrysoscelis* in Iowa has made it possible to plot the distribution of the two species. These plots showed *H. versicolor* to be distributed across most of the southeastern half of Iowa (Fig. 2) and we found several areas in the southeastern quarter where the species were sympatric. In addition, populations were studied later in Emmet, Winnebago, and Worth counties that were initially identified as *H. chrysoscelis* on the basis of the call. The temperature was cool, app 15°C, but the frogs had a short, metallic call typical of what we had associated with this species elsewhere in Iowa. This identification was confirmed by counts of nucleoli in hematoxylin and eosin stained hepatocytes and duodenum. These frogs are also plotted on Fig. 2, filling in an hiatus in north-central Iowa that resulted from inadequate sampling of that area at the time of this study.

Extensive overlap existed in central and eastern Iowa with the two species collected sympatrically or nearly so in Muscatine, Louisa, and Davis Counties (Fig. 2). This could allow opportunity for hybridization as reported by Gerhardt et al (1994). However, we see no evidence for this with the possible exception of large *H. chrysoscelis* found in Cherokee Co. There, all specimens collected were larger than typical of *C. chrysoscelis* and had calls similar to *H. versicolor* but nucleoli counts were typical of *H. chrysoscelis*. A pure population of hybrids with the parental species unrepresented seems unlikely.

Color and spotting pattern for 17 freshly collected specimens of *H. versicolor* and nine *H. chrysoscelis* were examined. Both species were sometimes solid green with no evidence of spotting on the back and both were sometimes gray with dark gray lichen-like markings to variations of green with gray markings. It was suspected that only *H. versicolor* would have the lichen-like markings bordered with dark black, but this too was present in the unusual population of *H. chrysoscelis* from Cherokee Co. The color, large size, and call suggest that the *H. chrysoscelis* from Cherokee Co. may represent a genetically different population of the species. These characters could also sug-

gest that this Cherokee Co. population of *Hyla versicolor* has lost primary nucleolar organization regions, resulting in fewer nucleoli than normal. We are hesitant to suggest that it represents hybridization.

DISCUSSION

Counts of nucleoli have been considered definitive in differentiating *H. versicolor* and *H. chrysoscelis* in states south of Iowa (Cash and Bogart 1978, Fitzgerald et al. 1981). For this reason, counts of nucleoli were used as the primary character in separating the 156 specimens. Of the 156 specimens examined by nucleoli staining, 65 were identified as *H. versicolor* and 91 as *H. chrysoscelis.* Cash and Bogart (1978) found counts of three and four nucleoli in the cells of *H. versicolor* with some cells exhibiting one or two nucleoli. The cells of *H. chrysoscelis* never contained more than one or two, reflecting the doubling of genetic material for *H. versicolor.* This was consistent with our analysis of toepad projections, typically larger in *H. versicolor* than *H. chrysoscelis* ($P = 0.016$). This trend of larger toepads in *H. versicolor* supports the additional genetic material found in the tetraploid cells (Cash and Bogart 1978; Green 1980). Due to overlap of toepad projection measurements, they are somewhat less reliable in separating the species than are counts of nucleoli.

A serious question involving this method of separating these species was raised by Keller (2000). He found that the number of nucleolar organizer regions ranged as high as four in *H. chrysoscelis* from a population in Phelps Co. Missouri, suggesting that those frogs could have up to four nucleoli. Similarly, Wiley et al (1989) provided evidence that *H. versicolor* may develop fewer than the expected number of nucleolar organizer regions resulting than fewer than three or four nucleoli, a feature we observed in many cells. The frog population we studied in Cherokee County could therefore be *H. versicolor* with an unusually small number of nucleoli. It is apparent that calls and morphology should be used with nucleolar counts (Fitsgerald et al 1981) but that in combination with these factors, nucleolar counts are usually effective in separating populations of these species in Iowa. Color pattern is ineffective for separating these species in Iowa. Our examination of hepatocytes and duodenal nuclei in several specimens for which we had counts of palpebral spectacle nucleoli suggested that nucleoli could be counted in these tissues as well. We used these counts to verify field identification of *H. chrysoscelis* from three northern Iowa counties. While 7 μm sections could eliminate some nucleoli from many cells, three nucleoli need be found in only a few cells to confirm that the species is *H. versicolor.* We found this method faster and easier than the complex silver staining procedure used for palpebral spectacles.

Although no macroscopic morphological characteristics can be used to distinguish individual specimens of these species, they can be used to identify most pure populations in Iowa (Table 2). While t-tests showed means of 11 of 13 characters to be significantly larger in *H. versicolor,* ANOVA recognized only 7 of the 13 as distinguishing, reflecting the overlap and large variances involved. Ralin and Selander (1979) and Ptacek et al. (1994) suggested that evolutionary closeness could explain the overlap. We suspect that using 35mm snout-vent length as the criterion for defining adults may have excluded several adults of *H. chrysoscelis,* the smaller species. *H. chrysoscelis* was never larger than *H. versicolor* in mean value for any character examination and excluding small adults of this species may have made our morphometric analysis less distinguishing than it may actually be.

The mapping of the two species presented herein (Fig. 2), showing *H. chrysoscelis in the* southwestern half of Iowa and *H. versicolor* in most of the eastern half, follow the distribution pattern seen in Kansas by Hillis et al. (1987) with the more aridity-adapted *H. chrysoscelis* found farther west. Ralin (1968) observed that the species have different ecological requirements in that *H. chrysoscelis* can tolerate and may prefer lower humidity than *H. versicolor.* In Iowa, *H. chrysoscelis* tends to be present in more arid areas such as the loess hills, and *H. versicolor* tends to be limited to the more humid Mississippi River Valley. A study relating relative humidity to calling individuals shows a trend toward a greater number of *H. versicolor* calling at higher humidities and more of *H. chrysoscelis* calling at lower humidities (Ralin 1968). Ralin (1968) also reported a difference in calling position, and stomach contents indicated that *H. chrysoscelis* was more arboreal than *H. versicolor.* In forests, relative humidity decreases with height above ground. Where the species live sympatrically, different ecological preferences could explain how they reduce direct competition.

Even including the northern populations we have recently identified, comparison with historical maps of the complex reveals few populations in the northern two tiers of counties in Iowa. This could reflect habitat destruction due to agriculture, sampling error, or other factors. Sampling in the northern tier counties was intense during the course of a search for *Acris crepitans* in 1995 and 1996 by Van Gorp (unpublished records in Drake Research Collection). Much of northern Iowa is heavily farmed and lacks choice habitat. Historically, the area was tall grass prairie with habitat for treefrogs along streams and adjacent wetlands. This area is also on the Wisconsinan glacial deposit and is higher and cooler than the surrounding terrain (Prior 1991). Other studies verify the declining amphibian populations occurring in a north to south pattern which would affect this section of Iowa (Van Gorp and VanDeWalle 1995, Van Gorp 1996). Our discovery of *H. chrysoscelis* in three north-central counties may suggest that temperature extremes may be an important factor in selection for this seemingly more hardy species.

LITERATURE CITED

CASH, M. N. and J. P. BOGART. 1978. Cytological differentiation of the diploid-tetraploid species pair of North American treefrogs (Amphibia, Anura, Hylidae). Journal of Herpetology 12(4):555–558.

CHRISTIANSEN, J. L. and R. M. BAILEY. 1991. The salamanders and frogs of Iowa. Iowa Department of Natural Resources; Des Moines. Nongame Technical. Series. 3. 24pp.

CONANT, R. and J. T. COLLINS. 1991. A field guide to reptiles and amphibians, eastern and central North America. Houghton Mifflin, Boston 450pp.

DALRYMPLE, J. M. 1993. The gray treefrogs *H. versicolor* and *H. chrysoscelis:* The discovery of the only known diploid-tetraploid species pair of anurans in North America. Bulletin of the Chicago Herpetological Society 28(7):137–139.

FERNANDEZ-GOMEZ, M. E., J. C. STOCKERT, J. F. LOPEZ-SAEZ, and G. JIMMENEZ-MARTIN. 1969. Staining plant cell nucleoli with $AgNO_3$ after formalin-hydroquinone fixation. Stain Technology 44:48–49.

FITZGERALD, K. T., H. M. SMITH, and L. J. GUILETTE Jr. 1981. Nomenclature of the diploid species of the diploid-tetraploid *Hyla versicolor* complex. Journal of Herpetology 15(3):356–360.

GERHARDT, H. C., M. P. PTACEK, L. BARNETT, and K. G. TORKE. 1994. Hybridization in the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *Hyla versicolor.* Copeia 1994(1):51–59.

GREEN, D. M. 1979. Treefrog toe pad: comparative surface morphology using scanning electron microscopy. Canadian Journal of Zoology 57: 2033–2046.

GREEN, D. M. 1980. Size differences in adhesive toe-pad cells of treefrogs of the diploid-polyploid *Hyla versicolor* complex. Journal of Herpetology 14:15–19.

GREEN, D. M., and J. CARSON. 1988. The adhesion of treefrog toe-pads to glass: cryogenic examination of a capillary adhesion system. Journal of. Natural History 22:131–135.

HANNA, G. and W. J. P. BARNES. 1991. Adhesion and detachment of

the toe pads of tree frogs. Journal of Experimental Biology 155:103–125.

HILLIS, D. M., J. T. COLLINS, and J. P. BOGART. 1987. Distribution of diploid and tetraploid species of gray tree frogs (*Hyla versicolor* and *Hyla chrysoscelis*) in Kansas. American Midland Naturalist 171(1):214–217.

JASLOW, A. P., and R. C. VOGT. 1977. Identification and distribution of *Hyla versicolor* and *Hyla chrysoscelis* in Wisconsin. Herpetologica 33(2): 201–205.

JOHNSON, F. C. II. 1966. Species recognition in the *Hyla versicolor* complex. Texas Journal of Science 18:361–364.

KELLER, M. A. 2000. Validity of nucleolar number for identification of the diploid-tetraploid gray treefrogs, *Hyla chrysoscelis* and *Hyla versicolor.* Copeia 2000(3):860–862.

MASON, T. O. 1988. The *Hyla chrysoscelis—Hyla versicolor* complex in Ohio. Ph.D. dissertation, Kent State University, Kent, OH.

MCALPINE, D. F., T. J. FLETCHER, S. W. GORMAN, and I. T. GORMAN. 1991. Distribution and habitat of the tetraploid gray Treefrog, *Hyla versicolor,* in New Brunswick and Eastern Maine Canadian Field-Naturalist 105:526–529.

POSTEK, M. T., K. S. HOWARD, A. H. JOHNSON, and K. L. MCMICHAEL. 1980. Scanning electron microscopy: a student's handbook. Ladd Research Industries. xv, 305pp.

PRIOR, J. C. 1991. Landforms of Iowa. University of Iowa Press. Iowa City. 153pp.

PTACEK, M. B., H. C. GERHARDT, and R. D. SAGE. 1994. Speciation by polyploidy in treefrogs: multiple origins of the tetraploid, *Hyla versicolor.* Evolution 48(3):898–908.

RALIN, D. B. 1968. Ecological and reproductive differentiation in the cryptic species of the *Hyla versicolor* complex (Hylidae). Southwestern Naturalist 13(3):283–300.

RALIN, D. B., and J. S. ROGERS. 1979. A morphological analysis of a North American diploid-tetraploid complex of treefrogs (Amphibia, Anura, Hylidae). Journal of Herpetology 13(3):261–269.

RALIN, D. B., and R. K. SELANDER. 1979. Evolutionary genetics of diploid-tetraploid species of treefrogs of the genus *Hyla.* Evolution 33:595–608.

VANGORP, C. D., and T. J. VANDEWALLE. 1995. Survey of Blanchard's cricket frog in southeastern Minnesota. Report to the Minnesota DNR non-game division, St. Paul, MN.

VANGORP, C. D. 1996. Survey of Blanchard's cricket frog in southwestern Minnesota. Report to the Minnesota DNR non-game division, St. Paul, MN.

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

WILEY, J. E., 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.