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SPECIATION BY POLYPLOIDY IN TREEFROGS: MULTIPLE ORIGINS OF THE TETRAPLOID, *HYLA VERSICOLOR*

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Abstract.—Speciation by polyploidy is rare in animals, yet, in vertebrates, there is a disproportionate concentration of polyploid species in anuran amphibians. Sequences from the cytochrome *b* gene of the mitochondrial DNA (mtDNA) were used to determine phylogenetic relationships among 37 populations of the diploid-tetraploid species pair of gray treefrogs, *Hyla chrysoscelis* and *Hyla versicolor*. The diploid species, *H. chrysoscelis*, consists of an eastern and a western lineage that have 2.3% sequence divergence between them. The tetraploid species, *H. versicolor*, had at least three separate, independent origins. Two of the tetraploid lineages are more closely related to one or the other of the diploid lineages (0.18%–1.4% sequence divergence) than they are to each other (1.9%–3.4% sequence divergence). The maternal ancestor of the third tetraploid lineage is unknown. The phylogenetic relationships between the two species and among lineages within each species support the hypothesis of multiple origins of the tetraploid lineages.

Key words.—Amphibian, Anura, *Hyla versicolor* complex, mitochondrial DNA, polyploidy, speciation, tree frogs.

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Polyploidy can represent a nearly instantaneous form of speciation because polyploid individuals are reproductively isolated from their nonpolyploid ancestors (Orr 1990). Although common in plants, speciation by polyploidy is relatively rare in animals (Müller 1925; White 1978; Orr 1990), probably because it disrupts sex determination and dosage compensation in those animals with chromosomal sex determination (Müller 1925; Dobzhansky 1951, 1970; White 1954). Among vertebrate species that have arisen via polyploidy, there is a disproportionate concentration in anuran amphibians. Polyploid taxa have been documented in 12 genera in nine families of frogs and toads (Kuramoto 1990; Tymowska 1991).

The diploid-tetraploid cryptic species pair of gray treefrogs, *Hyla chrysoscelis* and *Hyla versicolor*, present an intriguing example of polyploid speciation. Wasserman (1970) first hypothesized that the tetraploid ($2n = 48$) gray treefrog, *H. versicolor*, arose by polyploidy from the diploid *H. chrysoscelis* ($2n = 24$). Two lineages of the diploid species have been described differing in advertisement calls, allozymes, and chromosome polymorphisms (Gerhardt 1974; Ralin 1977; Wiley 1983; Wiley et al. 1989). The existence of more than one diploid lineage has raised the question of whether the tetraploid *H. versicolor* arose once or multiple times from one or both of these diploid lineages.

The primary goal of this research was to determine the number of origins of the tetraploids using a phylogenetic analysis of mitochondrial DNA (mtDNA) from frogs throughout the ranges of both species. A phylogenetic analysis of mtDNA molecules is the best method for reconstructing the evolutionary origin of a taxon. Unlike allozymes or nuclear genes, the mitochondrial genome is generally inherited maternally in higher animals (Avise and Lansman 1983) and does not recombine. Low levels of paternally inherited mtDNA have been detected in hybrid strains of mice (Gyllensten et al. 1991), but the absolute leakage of paternal molecules appears small. Thus, the phylogeny produced from mtDNA is an unambiguous tree of the mutational history of the mtDNA genome through time, a "gene tree" as outlined by Nei (1987), Pamilo and Nei (1988), and Avise (1989). Moreover, the more rapid evolution of the mtDNA genome enhances the chance that new lineages will appear shortly after speciation and develop diagnostic mutations for each phylogenetic branch. This tracing of matriarchical lineages allows one to test monophyly versus polyphyly for a particular mtDNA lineage (Avise 1986). Mitochondrial DNA analyses have been used to trace the maternal ancestor in polyploid frogs

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TABLE 1. Localities, number of Individuals, and mitochondrial DNA (mtDNA) haplotypes for each frog species sequenced.

No.	Locality	Species	Haplotype	*N*
1	Meade Co., Kentucky	*H. chrysoscelis*	A	1
2	Stoddard Co., Missouri	*H. chrysoscelis*	A	1
3	Russell, Co., Alabama	*H. chrysoscelis*	B	1
4	Summers Co., West Virginia	*H. chrysoscelis*	B	1
5	Smythe Co., Virginia	*H. chrysoscelis*	B	1
6	Jackson Co., Florida	*H. chrysoscelis*	C	1
7	Okaloosa Co., Florida	*H. chrysoscelis*	C	1
8	Muscogee Co., Georgia	*H. chrysoscelis*	C	1
9	Monroe Co., Tennessee	*H. chrysoscelis*	C	1
10	Hinds Co., Mississippi	*H. chrysoscelis*	C	1
11	Goochland Co., Virginia	*H. chrysoscelis*	D	2
12	Houston Co., Georgia	*H. chrysoscelis*	D	1
13	Shelby Co., Tennessee	*H. chrysoscelis*	E	1
14	Phelps Co., Missouri	*H. chrysoscelis*	E	3
15	Mecklenburg Co., Virginia	*H. chrysoscelis*	F	1
16	Nassau Co., Florida	*H. chrysoscelis*	F	1
17	Shelby Co., Tennessee	*H. versicolor*	G	1
18	Cleveland Co., Oklahoma	*H. versicolor*	H	1
19	Bastrop Co., Texas	*H. versicolor*	H	1
20	Allen Parrish, Louisiana	*H. versicolor*	I	1
21	Allen Parrish, Louisiana	*H. chrysoscelis*	J	1
22	Tillman Co., South Carolina	*H. chrysoscelis*	K	1
23	Travis Co., Texas	*H. chrysoscelis*	L	1
24	Hennepin Co., Minnesota	*H. chrysoscelis*	M	1
25	Saline Co., Missouri	*H. chrysoscelis*	N	1
26	Kendall Co., Texas	*H. chrysoscelis*	O	1
27	Eastland Co., Texas	*H. chrysoscelis*	P	1
28	Payne Co., Oklahoma	*H. chrysoscelis*	Q	1
29	Penobscot Co., Maine	*H. versicolor*	R	1
30	Goochland Co., Virginia	*H. versicolor*	S	1
31	Summers Co., West Virginia	*H. versicolor*	S	2
32	Giles Co., Virginia	*H. versicolor*	S	1
33	Campbell Co., Virginia	*H. versicolor*	T	1
34	Phelps Co., Missouri	*H. versicolor*	U	1
35	Guelph, Ontario, Canada	*H. versicolor*	V	2
36	Clearwater, Minnesota	*H. versicolor*	W	2
37	Saline Co., Missouri	*H. versicolor*	X	1
38	Chatham Co., Georgia	*H. femoralis*	Y	1
39	Gila Co., Arizona	*H. arenicolor*	Z	1

(Spolsky and Uzzell 1984; Carr et al. 1987) and lizards (Brown and Wright 1983; Wright et al. 1983) and to test for the existence and direction of introgression in other species (Ferris et al. 1983; Avise and Saunders 1984; Lamb and Avise 1986).

We used a mtDNA phylogeny to address the specific question: Did the tetraploid species, *H. versicolor,* have a single origin from one lineage of the diploid or multiple independent origins from two or more lineages? The single-origin hypothesis would be supported if geographically disparate representatives of *H. versicolor* form a monophyletic lineage with just one lineage of *H. chrysoscelis.* The hypothesis of multiple origins would be supported if there were several distinct phyletic lines, each composed of frogs of both species.

MATERIALS AND METHODS

Collection and Identification of Gray Treefrogs

Sequences of 27 individuals from 24 populations of *Hyla chrysoscelis* and 17 individuals from 13 populations of *Hyla versicolor* were used in phylogenetic analysis (table 1). Locations of the collecting sites are shown in figure 1. *Hyla femoralis* and *H. arenicolor* were used as outgroup species.

Males were identified in the field by their calls,

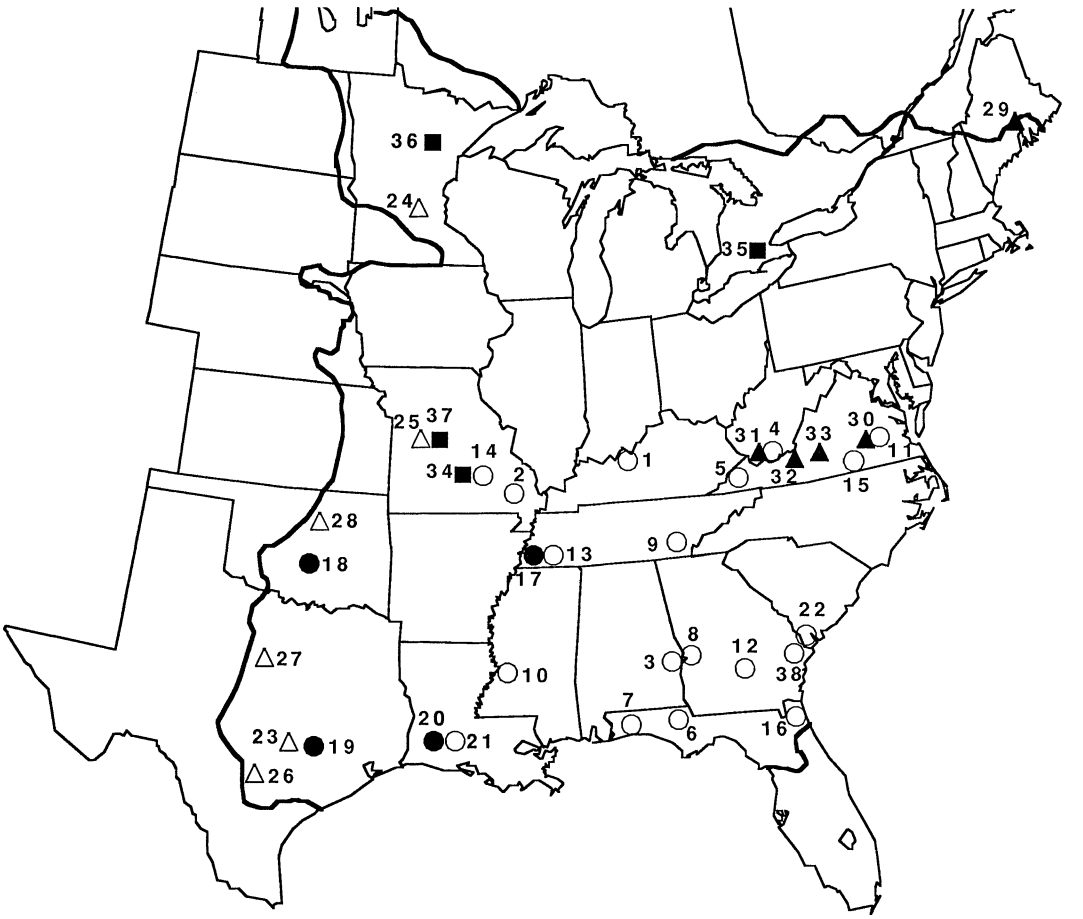

FIG. 1. Map showing the collecting sites of individuals of *Hyla versicolor* (solid symbols) and *Hyla chrysoscelis* (open symbols). The solid lines indicate the approximate boundaries of the distribution of the gray treefrog complex. See table 1 for locality information, haplotype, and number of individuals sequenced. Individuals that were in the same clade in the tree of figure 3 have the same type of symbol. Key to symbols: solid symbols—*H. versicolor*; ■, northwestern lineage; ●, southwestern lineage; ▲, eastern lineage; open symbols—*H. chrysoscelis*; ○, eastern lineage; △, western lineage.

and most were tape recorded for later acoustic analysis in the laboratory. Females and noncalling males were identified by flow cytometry (Krishan 1975; Gerhardt et al. 1994), which measures the total DNA content of small blood samples. Treefrogs have nucleated red blood cells, and thus, flow cytometry is an effective method of species identification.

Extraction, Amplification, and Sequencing of mtDNA

Frogs were euthanized by immersion in a 0.2% aqueous solution of 3-aminobenzoic ethyl ester (MS 222, Sigma, Inc.). Genomic DNA was extracted from liver tissue using standard phenol/chloroform extraction procedures outlined by Hillis et al. (1990). The sample was then concentrated by ethanol precipitation.

We used the polymerase chain reaction (Saiki et al. 1985) to amplify a 600 base-pair segment from the mitochondrial cytochrome *b* gene using primers synthesized by the DNA core facility at the University of Missouri, Columbia. The first primer, located on the L-strand, was the reverse complement to H15149 designed by Kocher et al. (1989); its sequence is 5′-TGAGGACAAATATCATTCTGAGGGGCTGCAG-3′. The H-strand primer was designed from the pub-

lished sequence of the bullfrog, *Rana catesbeiana* (Yoneyama 1987), and its sequence is 5′-TCTTCTACTGGTTGTCCTCCGATTCA-3′. The 5′ base of this primer corresponds to position 214 in the bullfrog sequence. Amplification was performed under standard conditions following the protocol outlined by Kocher et al. (1989). The amplification product was concentrated by ethanol precipitation and further purified by gel fractionation on a 3.5% acrylamide gel (Sambrook et al. 1989; for use of this protocol for purifying PCR products, A. R. Templeton and K. Shaw pers. comm. 1990).

This purified, amplified DNA was used as template in the sequencing reactions with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, California). The fluorescently dye-labeled template DNA was run on the Applied Biosystems Model 373A DNA Sequencing System at the DNA Core Facility, University of Missouri, Columbia.

Phylogenetic Analyses

The DNASTAR software program (Release 63, DNASTAR, Inc., Madison, Wisconsin) was used to align sequences and to detect base-pair differences. The number of transition and transversion substitutions and the number of "base positions shared" (Miyamoto et al. 1989) were determined and used to compute pairwise sequence divergence estimates expressed as percentages. Parsimony analysis (PAUP, Version 3.0s, Swofford 1989) was used to construct a phylogenetic tree for the cytochrome *b* segment. Sequences for *H. femoralis* and *H. arenicolor* were used to root the tree. These two species were chosen based upon their phylogenetic relationships to *H. chrysoscelis* and *H. versicolor* determined by allozymes (Hedges 1986). Both species are outside of the monophyletic clade that contains *H. versicolor* and *H. chrysoscelis* but are found in the next closely related clade. The two species are closely related within this clade but are not sister taxa. The strict consensus tree was constructed using the heuristic search option in PAUP. The shortest tree found by the heuristic search served as the initial limit in a branch-and-bound search.

Polyphyletic groups of each species were forced into monophyly using the constraints option in PAUP. A heuristic search was then conducted to find the most parsimonious constrained tree. Following Templeton (1983), a Wilcoxon signed ranks test compared the character state changes between the constrained tree and the most parsimonious tree by testing for equal medians in the distribution of nucleotide changes in the two trees. For the Cladistic Permutation test (Faith 1991; Faith and Cranston 1991), we used our original data set to produce 100 randomly generated data sets in which transversions were assigned a weight five times that of transitions (the observed transition/transversion ratio was 4.5). The random data sets were created by randomizing only the data among the ingroup taxa; outgroup taxa were included in the parsimony analyses to root the trees. Randomizations were achieved using software provided by D. Hillis and J. Huelsenbeck; data from four populations that differed by just one step from the most similar population were omitted to make the computations more manageable. To test specifically for polyphyly, heuristic searches were then conducted with either *H. versicolor* or *H. chrysoscelis* constrained into monophyly, and the magnitudes of differences between constrained and nonconstrained trees were noted. Polyphyly for a taxon is supported if no more than 5% of the 100 comparisons have a tree length difference equal to or greater than the difference between the nonconstrained and constrained trees from the original data. Finally, a bootstrap analysis (Felsenstein 1985) with 1000 replications was also performed to test the statistical confidence of each monophyletic group in the topology.

RESULTS

DNA Sequence Variation

The 565 base-pair segment from the cytochrome *b* gene has been deposited in GenBank for *Hyla chrysoscelis* from Meade County, Kentucky (accession no. L22017) and *Hyla versicolor* from Cleveland County, Oklahoma (accession no. L22018). For three populations of *H. versicolor* (Goochland County, Virginia; Phelps County, Missouri; and Allen Parish, Louisiana), two individuals per locality were sequenced. Individuals within a population had identical sequences. Sequences from three individuals from one population of *Hyla chrysoscelis* (Phelps County, Missouri) and two individuals from another (Jasper County, South Carolina) were also identical within populations. Thus, we assumed that a sequence from one individual represented the predominant mitochondrial DNA (mtDNA) haplotype for that population. Twenty-four hap-

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                    POSITION NUMBER
   0 0 0 1 1 1 1 1 2 2 2 2 2 2 2 3 3 3 3 3 3 3 4 4 4 4 4 5 5 5
   6 7 9 2 3 5 6 9 0 0 1 5 7 7 7 1 5 5 6 7 7 7 1 3 6 6 8 1 2 4
   6 8 4 1 3 7 6 0 2 5 7 4 1 2 3 1 3 7 0 0 6 9 5 4 0 9 1 7 8 5

A  A T T C A C G A T T C G C A C A T - C A A A T A T C C T C A
B  . . . . . . . . . . . . . . . G . . . . . . . . . . . . . .
C  . . . . . . . . . . . C . . . G . . . . . . . . . . . . . .
D  . . . . . . . . . . . C T T . . . . . . . . . . . . . . . .
E  . . . . . . . . . . . C . . . G . . . . . . . . . . . . . .
F  . . . . . . . . . . . C . T . . . . . . . . . . . . . . . .
G  . . . . . . . G . . . C . . . . . . . . . . . . . T . . .
H  . . . . . . . . . . . C . . . . . . . . . . . . . T . . .
I  . . . . . . . . . . . . . . . . . . . . . . . . . T . . .
J  . . . . . . . . . . . C . . . . . . . . . . . . . . . . .
K  . . . . . . . . . . . C . T . . . . . . . . . . . . . . .
L  . . C . . . A . A . . C . C . . . . . G G G . . . T . . . .
M  . C . . G T A . . A T C . C . . . . . G G . . . T . . . G
N  . . C . . . A . . A T C . C . . . . . G G G . . . T . . . .
O  . . C . . . A . . A T C . C . . . . . G G G . . . T . . G .
P  . . C . . . A . . A T C . C . . . . . G G . . . T . . G .
Q  . . C . . . A . . A T C . C . . . . . G G . . . . T . . . .
R  . . C T . . A . . A T C . . . . . A G G . C . . . . . . .
S  . . C T . . A . . A T C . C . . . . A G G . C . . . . . . .
T  . . C T . . A . . A T C . C . . . . A G G G C . . . . . . .
U  G C . . G T A G . A T C . T T . - T . G G . . G C . . . . .
V  G C . . G T A G C A T C . T T . - T . G G . . G C . . . . .
W  G C . . G T A G . A T C . T T . - T . G G . . C C . . C . .
X  G C . . G T A G . A T C . T T . - T . G G . . G C . . . . G
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FIG. 2. Aligned DNA sequences showing variable nucleotide positions among the 24 haplotypes of the two species of gray treefrogs. The nucleotide at each variable position is given for haplotype A, *Hyla chrysoscelis* from Meade County, Kentucky. In the other sequences, the nucleotide is defined when different from haplotype A; identity with haplotype A is indicated by a dot. Dashes indicate deletions.

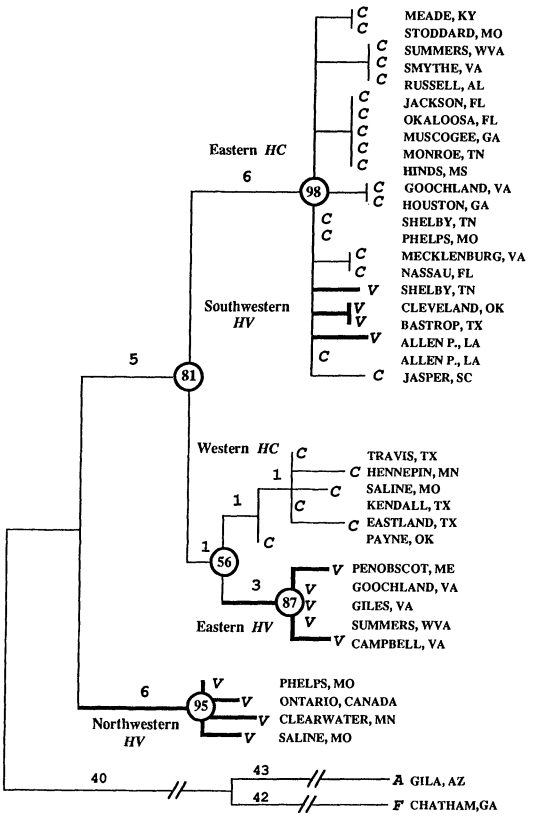

FIG. 3. The strict consensus tree for the cytochrome *b* gene. Numbers above the branches correspond to branch lengths and circled numbers are bootstrap values from 1000 bootstrap replications. See figure 1 for an overview of the distribution of the lineages identified in the tree.

lotypes were distinguished among the 37 gray tree frogs sampled.

There were 30 variable nucleotide positions between the two gray treefrog species (fig. 2). The first 22 and the last 54 nucleotide sites were identical among populations. Base-pair variation in the remainder of the sequence occurred as 28 single nucleotide substitutions, and a paired, one-nucleotide insertion and deletion. Twenty-three of the substitutions were transitions, four were transversions, and one site exhibited both kinds of change. The transition/transversion ratio was 4.5.

There were 27 (84%) silent mutations and three missense mutations causing changes in the amino acid sequence. The silent substitutions occurred with equal frequency in regions of the protein that are considered to be both highly conserved and nonconserved between species (Howell 1989). The high proportion of silent mutations observed is to be expected because this segment of cytochrome *b* comprises part of the coding region of an essential mitochondrial protein whose structure and function must be maintained (see also Anderson et al. 1982; Brown et al. 1982). Only three mutations led to amino acid substitutions, and all resulted in fairly conservative changes in terms of side-chain alterations.

We found 105 additional variable nucleotide positions in the comparisons between the gray treefrogs and the two outgroup species, *Hyla femoralis* and *Hyla arenicolor*. Between the *H. chrysoscelis* haplotype A and *H. femoralis* and *H. arenicolor* the sequence divergence was 16.1% and 14.7%, respectively. Percent sequence di-

vergence between the two outgroup taxa was 14.3%. Pairwise comparisons of percent sequence divergence among the 26 haplotypes are shown in table 2.

Phylogenetic Relationships

Maximum parsimony analysis yielded the strict consensus tree for the cytochrome *b* gene shown in figure 3. A heuristic search showed that the most parsimonious tree had 168 steps. A branch-and-bound search with 168 set as the initial limit found no shorter trees. The randomization technique of Faith and Cranston (1991) confirmed that the original data set was phylogenetically informative. The shortest tree generated from heuristic searches of each of 100 randomly generated data sets based on our data was 209 steps, which is 41 steps longer than the most parsimonious tree.

Populations of *H. chrysoscelis* were polyphyletic showing two distinct mtDNA lineages. These lineages corresponded well with the geographic origins of the populations. The western lineage was composed of populations from the extreme western edge of the species distribution, including Texas, Oklahoma, west-central Missouri, and Minnesota. The remaining populations of *H. chrysoscelis* clustered within the eastern mtDNA lineage. Percent sequence divergence between eastern and western lineages of *H. chrysoscelis* was 1.4% to 2.3%.

Cytochrome *b* sequences from *H. versicolor* were assignable to at least three distinct mtDNA lineages, two of which were closely related to one or the other of the diploid lineages. Southwestern populations of *H. versicolor* from western Tennessee, Louisiana, Texas, and Oklahoma, were most closely related to diploid populations from the eastern lineage (0.18% to 1.1% sequence divergence from eastern *H. chrysoscelis* populations; 0.18% to 0.35% sequence divergence among the four populations of *H. versicolor*). Eastern populations of *H. versicolor* from Maine, West Virginia, and Virginia were most closely related to the western diploid lineage (0.71% to 1.4% sequence divergence from the western populations of *H. chrysoscelis*; 0.18% to 0.35% sequence divergence among the five populations of *H. versicolor*). The third mtDNA lineage for the tetraploid was composed of populations from the northwestern portion of the species range, that is, Missouri; Minnesota; and Ontario, Canada. Percent sequence divergence among the four populations in this lineage ranged from 0.18% to 0.53%. No diploid populations were found in this clade. Percent sequence divergences among the three lineages of *H. versicolor* were from 1.9% to 2.3% between the eastern and southwestern lineages, 2.8% to 3.4% between the northwestern and southwestern lineages and 2.7% to 3.0% between the eastern and northwestern lineages.

Results of the Wilcoxon signed ranks test supported polyphyly of lineages of both species; the most parsimonious tree constraining *H. chrysoscelis* into monophyly required eight additional steps ($T = -2.04$, $P < 0.02$), and the most parsimonious tree constraining *H. versicolor* into monophyly was seven steps longer ($T = -2.07$, $P < 0.02$). The Cladistic Permutation Probability test also supported polyphyly for both species. In none of the pairwise comparisons of the randomized data sets were the differences between the constrained and nonconstrained trees as great as the differences of 19 and 20 steps observed in the original analyses of *H. chrysoscelis* and *H. versicolor,* respectively. Finally, bootstrap analysis supported the separation of the three clades of *H. versicolor* (fig. 3).

Discussion

Evidence for Multiple Origins of Tetraploids

Our analyses of mitochondrial DNA (mtDNA) sequences show a polyphyletic arrangement of the tetraploid frogs, with at least three separate origins. Southwestern populations of the tetraploid (Tennessee, Louisiana, Texas, and Oklahoma) are located within the eastern clade of *Hyla chrysoscelis.* The exact number of origins and their phylogenetic relationships to populations of *H. chrysoscelis* within this clade cannot be resolved. This entire clade is shown as a polytomy; the levels of sequence divergence are comparable both between populations within species (0.18%–0.53%) and between populations of the two species (0.18%–1.1%). Analysis of a more variable region of the mtDNA genome, such as D-loop sequences, might further resolve the evolutionary history of this clade. A second distinct monophyletic group is composed of eastern populations of the tetraploid (Maine, West Virginia, and Virginia). The diploid ancestors of this tetraploid lineage come from the western clade of *H. chrysoscelis.* A third tetraploid lineage is composed of populations from the northwestern portion of the species range (Missouri; Minnesota; and Ontario, Canada) and is not closely related to any of the diploid populations included in the

TABLE 2. Pairwise comparisons among haplotypes. Numbers above the diagonal are estimates of percent sequence divergence computed as *S/BPS*, where *S* is the number of substitutions and *BPS* is the number of base positions shared (Miyamoto et al. 1989). Numbers below the diagonal are the number of base-pair substitutions.

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	—	.18	.35	.53	.18	.35	.53	.35	.18	.35	.88	1.8	1.8
B	1	—	.18	.71	.35	.53	.71	.53	.35	.35	1.1	1.9	1.9
C	2	1	—	.53	.18	.35	.53	.35	.53	.35	.88	1.8	1.8
D	3	4	3	—	.35	.18	.71	.53	.71	.53	.71	1.8	1.8
E	1	2	1	2	—	.18	.35	.18	.35	.18	.71	1.6	1.6
F	2	3	2	1	1	—	.53	.35	.53	.35	.53	1.6	1.6
G	3	4	3	4	2	3	—	.18	.35	.53	1.1	1.9	1.9
H	2	3	2	3	1	2	1	—	.18	.35	.88	1.8	1.8
I	1	2	3	4	2	3	2	1	—	.53	1.1	1.9	1.9
J	2	3	2	3	1	2	3	2	3	—	.88	1.8	1.8
K	5	6	5	4	4	3	6	5	6	5	—	2.1	2.1
L	10	11	10	10	9	9	11	10	11	10	12	—	.35
M	10	11	10	10	9	9	11	10	11	10	12	2	—
N	11	12	11	11	10	10	12	11	12	11	13	1	3
O	11	12	11	11	10	10	12	11	12	11	13	1	3
P	10	11	10	10	9	9	11	10	11	10	12	2	2
Q	9	10	9	9	8	8	10	9	10	9	11	1	3
R	10	11	10	11	9	10	11	10	11	10	13	6	8
S	11	12	11	11	10	10	12	11	12	11	13	5	7
T	12	13	12	12	11	11	13	12	13	12	14	4	6
U	17	18	17	16	16	15	16	17	18	17	18	14	16
V	18	19	18	17	17	16	17	18	19	18	19	15	17
W	18	19	18	17	17	16	17	18	19	18	19	15	16
X	18	19	18	17	17	16	17	18	19	18	19	15	15
Y	91	92	92	92	91	91	92	91	91	91	92	87	89
Z	83	84	83	83	82	82	84	83	84	83	82	81	81

	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
A	1.8	1.8	1.8	1.9	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
B	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
C	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
D	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
E	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
F	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
G	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
H	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
I	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
J	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
K	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1	2.1
L	.35	.35	.35	.35	.35	.35	.35	.35	.35	.35	.35	.35	.35
M	—	—	—	—	—	—	—	—	—	—	—	—	—
N	—	1	1	1	2	2	2	2	2	2	2	2	2
O	1	—	1	1	2	2	2	2	2	2	2	2	2
P	1	1	—	1	2	2	2	2	2	2	2	2	2
Q	1	1	1	—	2	2	2	2	2	2	2	2	2
R	2	2	2	2	—	1	1	2	2	2	2	2	2
S	2	2	2	2	1	—	1	2	2	2	2	2	2
T	2	2	2	2	1	1	—	2	2	2	2	2	2
U	14	14	14	14	14	14	14	—	1	1	1	2	2
V	15	15	15	15	15	15	15	1	—	1	1	2	2
W	15	15	15	15	15	15	15	1	1	—	1	2	2
X	15	15	15	15	15	15	15	1	1	1	—	2	2
Y	87	87	87	87	87	87	87	88	89	89	89	—	1
Z	81	81	81	81	81	81	81	82	83	83	83	1	—

phylogeny. Two possible explanations exist. First, we sampled only a few populations of *H. chrysoscelis* from the northwestern and north central parts of the range and perhaps missed collecting individuals of a third diploid lineage. Second, the third mtDNA diploid lineage may be extinct (Avise et al. 1984). Indeed, this would be the situation if the third tetraploid lineage arose from ancestral *H. chrysoscelis,* that is, individuals that existed before the present day eastern and western lineages of *H. chrysoscelis* diverged. Such ancient mtDNA lineages have been observed in hybrid polyploid salamanders (Hedges et al. 1992).

Based upon their protein electrophoresis results, Ralin et al. (1983) hypothesized that the tetraploid frogs had a single origin from a genetically and geographically intermediate population of diploids. Romano et al. (1987) concluded that convergent similarities in allele frequencies observed between the diploid and tetraploid species in sympatry were caused by similar selective regimes in the common environment rather than multiple origins of the tetraploids. Ralin et al. (1983) concluded that the single-origin hypothesis was a more parsimonious explanation than multiple origins of the tetraploid because a multiple origins hypothesis must assume that (1) a complex series of parallel independent mutations occurred between geographically distant tetraploid populations; and (2) northeastern and southern tetraploids arose simultaneously from geographically distant diploid populations, followed by a third, much later, origin from *H. chrysoscelis* in the central part of the range. Although the exact location and order of origins for each tetraploid lineage cannot be determined by our mtDNA data, the geographic distribution of each tetraploid lineage (e.g., the eastern tetraploid lineage having its origin from the western diploid lineage), and the divergence of the northwestern lineage from the other two, fit a pattern of multiple origin events for *Hyla versicolor* that is consistent with the scenario that was rejected by Ralin et al. (1983).

Four independent lines of evidence have been offered to support the separation of *H. chrysoscelis* into genetically distinct "eastern" and "western" lineages. First, the advertisement calls of eastern and western populations differ in tem-

TABLE 2. Extended.

N	O	P	Q	R	S	T	U	V	W	X	Y	Z
1.9	1.9	1.8	1.6	1.8	1.9	2.1	3.0	3.2	3.2	3.2	16.1	14.7
2.1	2.1	1.9	1.8	1.9	2.1	2.3	3.2	3.4	3.4	3.4	16.3	14.9
1.9	1.9	1.8	1.6	1.8	1.9	2.1	3.0	3.2	3.2	3.2	16.3	14.7
1.9	1.9	1.8	1.6	1.9	1.9	2.1	2.8	3.0	3.0	3.0	16.3	14.7
1.8	1.8	1.6	1.4	1.6	1.8	1.9	2.8	3.0	3.0	3.0	16.1	14.5
1.8	1.8	1.6	1.4	1.8	1.8	1.9	2.7	2.8	2.8	2.8	16.1	14.5
2.1	2.1	1.9	1.8	1.9	2.1	2.3	2.8	3.0	3.0	3.0	16.3	14.9
1.9	1.9	1.8	1.6	1.8	1.9	2.1	3.0	3.2	3.2	3.2	16.1	14.7
2.1	2.1	1.9	1.8	1.9	2.1	2.3	3.2	3.4	3.4	3.4	16.1	14.9
1.9	1.9	1.8	1.6	1.8	1.9	2.1	3.0	3.2	3.2	3.2	16.1	14.7
2.3	2.3	2.1	1.9	2.3	2.3	2.5	3.2	3.4	3.4	3.4	16.3	14.5
.18	.18	.35	.18	1.1	.88	.71	2.5	2.7	2.7	2.7	15.4	14.3
.53	.53	.35	.53	1.4	1.2	1.1	2.8	3.0	3.0	2.7	15.8	14.3
—	.35	.53	.35	1.2	1.1	.88	2.7	2.8	2.8	2.8	15.2	14.2
2	—	.18	.35	1.2	1.1	.88	2.7	2.8	2.8	2.8	15.6	14.5
3	1	—	.53	1.4	1.2	1.1	2.8	3.0	3.0	3.0	15.8	14.3
2	2	3	—	.88	.71	.88	2.3	2.5	2.5	2.5	15.2	14.2
7	7	8	5	—	.18	.35	2.7	2.8	2.8	2.8	16.1	14.7
6	6	7	4	1	—	.18	2.7	2.8	2.8	2.8	15.9	14.5
5	5	6	5	2	1	—	2.8	3.0	3.0	3.0	16.1	14.7
15	15	16	13	15	15	16	—	.18	.35	.18	15.4	14.5
16	16	17	14	16	16	17	1	—	.53	.35	15.2	14.3
16	16	17	14	16	16	17	2	3	—	.53	15.8	14.9
16	16	17	14	16	16	17	1	2	3	—	15.6	14.7
86	88	89	86	91	90	91	87	86	89	88	—	14.3
80	82	81	80	83	82	83	82	81	84	83	81	—

perature-corrected pulse rate; males from the southeastern part of the range have lower pulse rates than those in the west (Gerhardt 1974; Ralin 1977). Second, electromorph differences in allozyme alleles between eastern and western *H. chrysoscelis* also exist (Ralin et al. 1983). Third, microcomplement fixation studies of albumin show eastern and western populations of *H. chrysoscelis* differ by an average of seven immunological distance units (Maxson et al. 1977). Finally, variation in the chromosomal location of ribosomal RNA (rRNA) genes in *H. chrysoscelis* (Wiley et al. 1989) also suggests "eastern" and "western" lineages. Opinions differ on whether these two diploid lineages constitute separate species and none of these studies have satisfactorily resolved whether *H. versicolor* arose from within a lineage or through hybridization between the two lineages of *H. chrysoscelis*.

Our results from mtDNA sequences support the separation of *H. chrysoscelis* into eastern and western populations with those from the western edge of the species distribution forming a separate monophyletic clade. However, there is poor geographic congruence between eastern and western lineages defined by our mtDNA analysis and those defined by call differences, allozymes, and chromosome studies. The support for the separation of eastern and western *H. chrysoscelis* is the weakest portion of our tree topology with the western populations appearing as a separate group in only 56% of the bootstrap replications. However, the polyphyly of eastern and western lineages of *H. chrysoscelis* was supported by both the Cladistic Permutation Probability test and the Wilcoxon's signed ranks test suggesting strongly that the diploid occurs in at least two genetically definable lineages each giving rise to separate lineages of tetraploids.

The Mode of Polyploidy

Uncertainty remains about whether the tetraploid genomes in *H. versicolor* arose via autopolyploidy or allopolyploidy. In allopolyploidy, the chromosome sets in the derived species have originated from two or more ancestral species, whereas in autopolyploidy, all the chromosome sets have been derived from a single ancestral species (White 1978). The resolution of this question remains crucial to understanding

features of the distributional range of the tetraploid lineages and their ecological interactions with diploid *H. chrysoscelis*. Our study shows an unusual pattern of distribution between the geographical ranges of the three tetraploid lineages and the two diploid lineages. That is, tetraploids with a western *H. chrysoscelis* mtDNA genome coexist with the eastern *H. chrysoscelis*. This means that there is maximal differentiation between mtDNA genomes in some areas of sympatry. This pattern is continued in north central North America, where the third *H. versicolor* lineage, that has the most divergent mtDNA of all, lives with western *H. chrysoscelis* in many areas of sympatry. We cannot yet explain what evolutionary or ecological forces might have led to this pattern of maximal divergence of mtDNA genomes among coexisting sibling species. Additional questions, whose answers depend on knowing the mode of ploidy are (1) how genetic reorganization was achieved to permit the coexistence of a daughter with a parental species, and (2) how the derived taxa (*H. versicolor*) acquired the physiological capabilities to live in the northern regions of North America that are apparently uninhabitable to either of the parental populations? Does such increased ecological capacity arise simply from doubling the number of genetic elements that exist in a gene pool via autopolyploidy? Or, is the increased scope of the tetraploid a by-product of allopolyploidy and the genomic disruption that frequently occurs following hybridization (Sage et al. 1993)?

Although our mtDNA data speak convincingly in support of multiple origins of tetraploid gray treefrogs and to the existence of two evolutionary lineages of the diploid *H. chrysoscelis,* they are uninformative about the mode of polyploidy. However, our results now allow us to state precise criteria of what will define an auto- or allopolyploid mode of origin. The criteria for accepting one or the other definition depend on identifying the male and female *H. chrysoscelis* parents of the eggs that became polyploid. If the mating was between two individuals of the same mtDNA lineage (i.e., either both were eastern *H. chrysoscelis* or western *H. chrysoscelis*) then the tetraploids had an autopolyploid origin. If the tetraploids resulted from a hybridization mating between parents of different *H. chrysoscelis* mtDNA lineages this would indicate an allopolyploid origin.

We have no information on the identity of the male parent involved in the mating that preceded the polyploid event. At present, male-specific genetic or cytological markers have not been discovered in *Hyla* (Schmid et al. 1991). The most helpful, but still circumstantial, evidence about the identity of the male ancestor would come from identifying nuclear genomic markers in tetraploids that are diagnostic of the eastern and/or western *H. chrysoscelis* populations. But completely diagnostic alleles of electrophoretically detectable proteins have not been discovered to date (Ralin et al. 1983).

The strongest evidence for an autopolyploid mode of origin of *H. versicolor* may come from the behavior and cytological composition of their chromosomes. Cytological studies of *H. versicolor* showed that (1) multivalents are formed during meiosis (Bogart and Wasserman 1972), (2) morphological homogeneity exists within the homologous sets of chromosomes (Bogart and Wasserman 1972; Bogart 1980), and (3) the number of their nucleolar organizer regions corresponds to their level of ploidy (Cash and Bogart 1978; Bogart 1980). These three attributes correspond to the patterns seen in the other biparental, polyploid anurans that have arisen as autopolyploids (reviewed in Tymowska 1991). However, these cytological data still do not unequivocally answer whether the tetraploids arose solely from within one of the lineages of *H. chrysoscelis* or from a hybridization event between the two lineages. The low levels of genetic divergence between the two diploid lineages and the relatively recent origin of the tetraploid lineages could explain the morphological homogeneity among chromosome sets from both tetraploids and diploids regardless of their mode of origin. Further examination of nuclear molecular markers that are male specific, or at least lineage specific for the diploids, will be required before the mode of origin of the tetraploids and the genetic, distributional and ecological consequences of these speciation events can be fully understood.

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