NRC Research Press

ARTICLE

Revisiting the evolution of the North American tetraploid treefrog (*Hyla versicolor*)

James P. Bogart, Patrick Burgess, and Jinzhong Fu

Abstract: *Hyla chrysoscelis* and *H. versicolor* are common treefrogs in eastern North America and are a cryptic diploid–tetraploid species pair. They are morphologically identical but *H. versicolor* is a tetraploid. They can be identified acoustically by the male's advertisement mating call, which has a pulse repetition rate that has twice as many pulses per second in the diploid species, *H. chrysoscelis*. We used isozymes, microsatellite DNA alleles, and mitochondrial cytochrome *b* sequences to test the hypothesis that gene exchange occurs between the diploid and tetraploid species in sympatric populations. Each method provided results that are best explained by occasional hybridization of female *H. versicolor* and male *H. chrysoscelis*. We propose that *H. versicolor* first arose from an autotriploid *H. chrysoscelis* female that produced unreduced triploid eggs. After *H. versicolor* became established, genes could be passed from *H. chrysoscelis* to *H. versicolor* in sympatric populations when these species hybridize. Their F_1 female progeny produce unreduced triploid eggs that are fertilized by haploid *H. chrysoscelis* sperm to reconstitute *H. versicolor*. Genes can be passed from diploid *H. chrysoscelis* to tetraploid *H. versicolor* in sympatric populations.

Key words: Amphibia, Anura, *Hyla versicolor* complex, polyploid, mitochondrial DNA, isozymes, microsatellites, treefrogs.

Résumé : *Hyla chrysoscelis* et *H. versicolor* sont des rainettes répandues dans l'Est de l'Amérique du Nord et forment une paire d'espèces cryptiques diploïde–tétraploïde. Elles sont identiques sur le plan morphologique, mais *H. versicolor* est tétraploïde. Elles sont identifiées de manière acoustique du fait que, chez l'espèce diploïde, *H. chrysoscelis*, l'appel d'accouplement du mâle compte deux fois plus de pulsations par seconde. Les auteurs ont employé des isozymes, des microsatellites et des séquences de la cytochrome *b* mitochondriale pour tester si des échanges géniques se produisent entre les espèces diploïdes et tétraploïdes chez des populations sympatriques. Chaque méthode a produit des résultats qui s'expliquent le mieux par une hybridation occasionnelle entre une femelle *H. versicolor* et un mâle *H. chrysoscelis*. Les auteurs proposent que *H. versicolor* serait issu d'une femelle autotriploïde *H. chrysoscelis* qui aurait produit des œufs triploïdes non-réduits. Une fois *H. versicolor* établi, des gènes pourraient passer du *H. chrysoscelis* au *H. versicolor* lorsque ces espèces s'hybrident. La progéniture femelle F_1 produit des œufs triploïdes non-réduits qui sont fécondés par du sperme haploïde du *H. chrysoscelis* pour reconstituer le *H. versicolor*. Des gènes peuvent ainsi passer du *H. chrysoscelis* diploïde au *H. versicolor* tétraploïde au sein de populations sympatriques. [Traduit par la Rédaction]

Mots-clés : Amphibia, Anura, complexe *Hyla versicolor*, polyploïde, ADN mitochondrial, isozymes, microsatellites, rainettes.

Introduction

Although less common than found in plants, polyploid species have been documented to occur in a wide range of animal taxa (Gregory and Mable 2005). Polyploid vertebrate species can be unisexual (e.g., parthenogenetic lizards and salamanders in the genus *Ambystoma*) or bisexual (e.g., several species of frogs). Forty-six bisexually reproducing polyploid species have independently arisen across 12 families of anuran amphibians (Bogart 1980; Mable et al. 2011; Schmid et al. 2015). Much of the research on naturally occurring polyploid vertebrates has focused on understanding the origin of the polyploid species and whether the polyploid is an allopolyploid, derived from hybridization event(s), or an autopolyploid, derived from a single ancestral species. Unisexual lizards in the genera *Aspidocelis* and *Darevskia* are allopolyploid (Moritz et al. 1992; Fu et al. 2000) and allopolyploid speciation resulted in five to seven allotriploid and allotetraploid bisexual species of Palearctic green toads (*Bufo viridis* subgroup) (Betto-Colliard et al. 2018). The diploid

Received 5 March 2020. Accepted 30 July 2020.

J.P. Bogart, P. Burgess, and J. Fu. Department of Integrative Biology, University of Guelph, Guelph, ON N1G 2W1, Canada.
Corresponding author: James P. Bogart (email: jbogart@uoguelph.ca).

species that hybridized and gave rise to these allopolyploid lizards and toads are genetically divergent, which may be a prerequisite for allopolyploid speciation (Betto-Colliard et al. 2018). A polyploid species that lives with, or close to, a morphologically similar diploid species and demonstrates quadrivalent chromosome associations during prophase in meiosis is suspected to be an autopolyploid species. The North American Grey Tree Frogs are diploid (*Hyla chrysoscelis*) and tetraploid (*Hyla versicolor*) cryptic species that are morphologically identical and *H. versicolor* does form quadrivalents (Bogart and Wasserman 1972). They are common frogs in central and eastern North America and can be found in sympatry in several areas across their extensive range. Acoustics was used to distinguish these species (Johnson 1959, 1963) prior to their recognition as a diploid–tetraploid species pair (Wasserman 1970; Bogart and Wasserman 1972). Males can be distinguished by mating call because diploid *H. chrysoscelis* has a pulse repetition rate that is twice as fast as tetraploid *H. versicolor*. The different mating calls serve as a premating isolating mechanism for mate choice in both species (Gerhardt 2005).

Even though *H. versicolor* complies with expected criteria for an autopolyploid species, it is possible that *H. versicolor* is an allopolyploid derived from hybridization of genetically distinct individuals from eastern and western populations of *H. chrysoscelis* based on immunological (Maxson et al. 1977), acoustic (Gerhardt 1974) and chromosomal (Wiley 1983) differences observed between "eastern" and "western" populations of *H. chrysoscelis*. Ptacek et al. (1994) sequenced mitochondrial cytochrome *b* (cyt*b*) from several populations of *H. versicolor* and *H. chrysoscelis*. They found that some *H. versicolor* individuals clustered within different *H. chrysoscelis* lineages. From their phylogenetic analysis of the sequence data, Ptacek et al. (1994) concluded that *H. versicolor* arose at least three times, twice from *H. chrysoscelis* and once from an unknown maternal ancestor. From cyt*b* and three nuclear markers, Holloway et al. (2006) constructed phylogenies that suggested that *H. versicolor* arose multiple times from *H. chrysoscelis* and from two other unknown lineages. But, as pointed out by Mable et al. (2011), polyploid taxa could potentially be problematic for phylogenetic analyses because they do not arise by cladogenesis.

The mechanism of polyploid formation has not been adequately studied for *H. versicolor* or for other naturally occurring cryptic anuran polyploids, but an artificially produced congeneric autotetraploid frog provides relevant information on the process. Nishioka and Ueda (1983) were able to produce bisexually reproducing autotetraploid *H. japonica* in the laboratory. Unreduced (diploid) eggs, resulting from a temperature shock that prohibited extrusion of the second polar body during female meiosis, when fertilized with normal (haploid) sperm produced viable triploid males and females. The triploid females produced unreduced (triploid) eggs that were fertilized with haploid sperm to produce fertile male and female tetraploids. No tetraploids were produced when triploid males were mated with diploid females. This "triploid bridge" hypothesis was proposed for the evolution of *H. versicolor* from *H. chrysoscelis* by Bogart and Wasserman (1972). Based on isozyme analyses, the genetic similarity of these frogs is greatest where the two species occur together (Ralin and Selander 1979; Ralin et al. 1983; Romano et al. 1987). All these studies considered the possibility that introgression is occurring in some areas by hybridization. A triploid hybrid female that produced unreduced (triploid ova) could mate with a male *H. chrysoscelis* to reconstitute *H. versicolor* so the hybrid would serve as a one-way bridge for gene flow from the diploid to the tetraploid. They all dismissed that possibility because females were consistently able to discriminate the different mating calls (Littlejohn et al. 1960), laboratory crosses (Johnson 1959; Ralin 1976) demonstrated reduced viability of F_1 hybrids and complete mortality of backcross progeny (Johnson 1963), and triploids had never been found in nature. The isozymes that have documented genetic similarity between *H. versicolor* and *H. chrysoscelis* by Ralin and Selander (1979) and Romano et al. (1987) all belonged to a slowly evolving group of proteins as opposed to plasma proteins that accumulate amino acid substitutions some 10 times more rapidly (Sarich 1977). A better test for more contemporary genetic similarity may be obtained using microsatellite DNA loci that have a rapid mutation rate (Ellegren 2004) and would be "fast" evolving. Espinoza and Noor (2002) used three microsatellites from SSR loci documented for *H. chrysoscelis* by Krenz et al. (1999) and a nuclear marker from a *H. versicolor* genomic DNA library to test the hypothesis that different lineages of *H. versicolor*, as documented by Ptacek et al. (1994), interbreed. Microsatellites have not been used to test the hypothesis that *H. versicolor* and *H. chrysocelis* exchange genes in sympatric populations.

Triploid hybrid males have been discovered in sympatric populations in West Virginia (Wiley et al. 1992; Gerhardt et al. 1994), and Oklahoma (Bogart and Bi 2013). Further evidence that female discrimination is not perfect was provided by Gerhardt et al. (1994) who found mis-mated pairs of *H. versicolor* and *H. chrysoscelis* in amplexus in a Missouri pond where the two species were breeding. Male hybrids (*H. chrysoscelis* × *H. femoralis* and *H. chrysoscelis* × *H. avivoca*) have previously been found in natural ponds (Mecham 1960; Pyburn 1960; Gerhardt 1974). Autotriploid *H. chrysoscelis* males and females were artificially produced by Keller and Gerhardt (2001). The triploid males produced mating calls with pulse rates that were intermediate between those produced by tetraploid *H. versicolor* and diploid *H. chrysoscelis*. These data, and similar findings on acoustic analyses of diploid, autotriploid, and autotetraploid *H. japonica* (Ueda 1993) as well as diploid and polyploid toads in the *Bufo viridis*

subgroup (Guignard et al. 2012), and diploid *Odontophrynus cordobae* and tetraploid *O. americanus* cryptic species in Argentina (Bogart and Wasserman 1972; Martino and Sinsch 2002), confirm a direct relationship that pulse repetition rate declines with ploidy elevation. Mating calls of new tetraploids would be expected to attract tetraploid females.

To test the hypothesis that gene exchange occurs between *H. versicolor* and *H. chrysoscelis*, we examined microsatellite DNA alleles from specimens of both species from five sympatric populations. If asymmetric gene flow has recently occurred in the sympatric populations, we would expect that the two species would share microsatellite alleles. Isozymes were also examined from the same individuals to compare with data obtained by Romano et al. (1987) from other sympatric populations. Mitochondrial cyt*b* sequences were examined to detect female lineages for the species in sympatry and to compare nuclear genes that were estimated by sampling isozymes and microsatellites with mitochondrial cyt*b*. This comparison could also provide information on possible interspecific gene flow to see if genes would be more likely to be passed from *H. chrysoscelis* to *H. versicolor* or from *H. versicolor* to *H. chrysoscelis*.

Materials and methods

Based on a paper by Duellman et al. (2016), the generic name *Dryophytes* was applied to hylid frogs in North America that include *H. versicolor* and *H. chrysoscelis* (Frost 2020). The generic name change has not been generally accepted and the justification for changing the name from *Hyla* to *Dryophytes* is tenuous (see Faivovich et al. 2018). We continue to use *Hyla* for these frogs that have a rich history in the scientific literature with that generic name.

Male specimens were collected during opportunistic field trips to sympatric populations in Manitoba, Maryland, Michigan, Minnesota, Virginia, and Wisconsin taken during the spring breeding season in 1982, 1988, 1989, and 1992. Sympatric populations were identified when both species were heard to be vocalizing in the same pond or in very close ponds. The "slow-calling" *H. versicolor* were collected and placed in separate containers from collections of "fast-calling" *H. chrysoscelis*. Frogs were transported to the laboratory in Guelph, toe-clipped for individual identification, maintained in captivity, and used for MSc research projects that investigated the genetics (Danzmann 1982; Mable 1989) and physiology (McLister 1992) of these frogs. Individuals were all karyotyped and their tissues (heart, portions of the liver and skeletal muscle, and spleen) were removed, placed in 1.5 mL microtubes, and frozen in an equal volume of deionized water in an ultra-low freezer (–80 °C) for isozyme analyses that were done from 1982 to 1994. In 2017, DNA was extracted from some of the frozen tissue (heart, skeletal muscle, and spleen) that had been used for the isozyme investigation. The extracted DNA was used for mitochondrial cyt*b* sequences and microsatellite analyses. The localities and the number of specimens used for each analysis are provided in Table 1 and more detailed information for each specimen is included in the supplemental data, Table S1[1].

Table 1. Localities for *Hyla versicolor* (vers) and *H. chrysoscelis* (chrys) and sample size of individuals that were used to analyze isozymes, microsatellites (Micros), and mitochondrial cytochrome *b* (cyt*b*) sequences.

		No. of Individuals		
	Locality	Isozymes	Micros	cyt*b*
1	Manitoba, Canada. Whiteshell Prov. Park			
	vers	24	20	1
	chrys	12	11	3
2	Maryland, Prince George Co.			
	vers	23	16	9
	chrys	33	21	14
3	Michigan, Jackson Co.			
	vers	21	11	5
	chrys	8	5	4
4	Minnesota, Mahnomen Co.			
	vers	48	35	12
	chrys	10	10	10
5	Wisconsin, Columbia Co.			
	vers	7	5	3
	chrys	14	17	4
6	Virginia, King George Co.			
	vers	7	—	1
	chrys	15	—	5
7	Illinois, Stephenson Co.			
	vers	14	9	3

Chromosomes

Chromosomes were obtained from corneal epithelial squashes according to the methods outlined by Bogart (1981). At least 2.5 h prior to sacrifice, the frog was injected with 0.5 cc of colchicine solution (1 mg/cc). When sacrificed, one eye was removed and placed in distilled water for 2 h, held over glacial acetic acid for 1 min, placed back in distilled water, and the cornea was picked off the eye with forceps and placed on a siliconized cover slip with a drop of 70% acetic acid that is picked up on a slide. The preparation is inverted and squashed using a band of bibulous paper and thumb pressure. Chromosomes were observed and photographed using phase contrast optics. Chromosome number was used to confirm the identification of diploid *H. chrysoscelis* ($2n = 24$) and tetraploid *H. versicolor* ($4n = 48$).

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2020-0031.

Table 2. Presumptive structural gene loci examined in *Hyla versicolor* and *H. chrysoscelis* populations.

Locus (abbreviation)	E.C. No.*	Tissue†	Gel‡
6-Phosphogluconate dehydrogenase (*6Pgd*)	1.1.1.44	L	2
Aconitase (*Acon-1*)	4.2.1.3	L	1
Aconitase (*Acon-2*)	4.2.1.3	L	1
Aspartate amino transaminase (*Aat-1*) (=*Got-1*)§	2.6.1.1	HMS	2
Aspartate amino transaminase (*Aat-2*) (=*Got-2*)	2.6.1.1	L	2
Isocitrate dehydrogenase (*Idh-1*)	1.1.1.42	HMS	2
Isocitrate dehydrogenase (*Idh-2*)	1.1.1.42	HMS	2
Lactate dehydrogenase (*Ldh-1*)	1.1.1.27	HMS	1
Lactate dehydrogenase (*Ldh-2*)	1.1.1.27	HMS	1
Malate dehydrogenase (*Mdh-1*)	1.1.1.37	HMS	1
Mannose 6-phosphate isomerase (*Mpi*)	5.3.1.8	L	1
Phosphoglucose isomerase (*Pgi*)	5.3.1.9	HMS	2
Phosphoglucomutase (*Pgm-1*)	2.7.5.1	L	1
Phosphoglucomutase (*Pgm-2*)	2.7.5.1	L	1
Superoxide dismutase (*Sod-1*)	1.15.1.1	L	1

*Standardized enzyme-numbering system established by the nomenclature committee of the International Union of Biochemistry (IUBC 1984).

†Tissues used to resolve the enzyme systems were liver (L) or a combination of heart, skeletal muscle, and spleen (HMS).

‡The electrophoretic conditions for the gels were (1) amine-citrate, gel, and tray buffer adjusted to pH 6.5 (Clayton and Tretiak 1972) run for 3 h at 250 V; (2) tris-citrate, gel buffer pH 6.7, and tray buffer pH 6.3 (Selander et al. 1971) run for 3–4 h at 150 V.

§*Got* (glutamate oxaloaceto-transaminase) is a synonym of aspartate amino transaminase (*Aat*). Previous studies on *H. versicolor* and *H. chrysoscelis* used the earlier enzyme designation (*Got*).

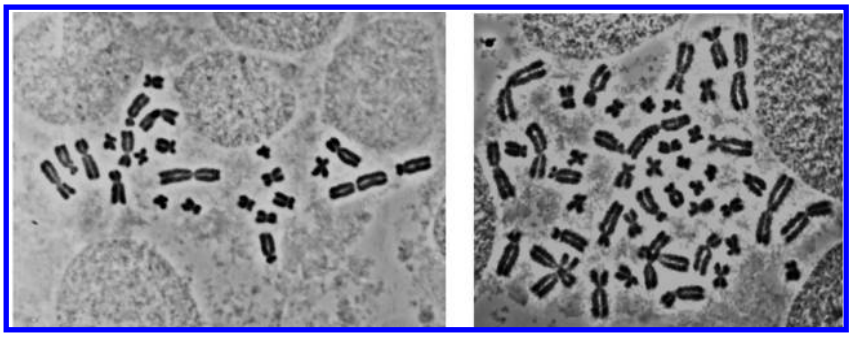
Isozymes

Tissues used for isozyme electrophoresis were liver (L) and a combination of heart, skeletal muscle, and spleen (HMS). Just prior to electrophoresis, the frozen tissues were ground in 1.5 mL microtubes using a sharp glass rod and centrifuged for 2 min in a microfuge. The supernatant was absorbed in small wicks of Whatman #3 filter paper and were air-dried prior to being inserted in the gel at the origin. Horizontal starch gel electrophoresis followed the procedures outlined by Selander et al. (1971). We used buffer systems described by Selander et al. (1971) and Clayton and Tretiak (1972) and staining recipes that were provided by Selander et al. (1971). Fifteen isozyme loci (Table 2) were resolved and analysed from individual *H. versicolor* and *H. chrysoscelis* collected from sympatric populations in Virginia, Maryland, Michigan, Wisconsin, and Manitoba as well as from a population of *H. versicolor* in Illinois. Electrophoretic allozymes for each enzyme locus were labelled on the gel from A (the most anodally migrating allozyme) to E. Allozyme allele frequencies were calculated for each species in each population. Tetraploid *H. versicolor* possesses twice as many alleles as *H. chrysoscelis* and, with only two alleles, heterozygotes can be symmetrical (AABB) or asymmetrical (ABBB, AAAB). Staining intensity (dosage) of the isozyme alleles was used to distinguish the number of alleles in heterozygotes (Danzmann and Bogart 1982) and to assist with gene frequency calculations. The calculated allele frequencies were analysed using POPTREE2 (Takezaki et al. 2010).

Microsatellite DNA

Tissues previously used for isozymes (above) were thawed and small pieces of heart and skeletal muscle were chosen for DNA extraction using a Promega Wizard Genomic DNA Purification Kit. We tested the 21 primer sequences developed for *H. andersonii* microsatellites (Warwick and Lemmon 2014) using DNA extractions from *H. versicolor* and *H. chrysoscelis*. Six primer sequences consistently amplified microsatellite DNA alleles with tetranucleotide repeat motifs from the same individuals that were used for isozymes (Table 1). Forward primers for each locus were fluorescently labelled with tetramethyl rhodamine. DNA was amplified with standard PCRs for microsatellite DNA using an annealing temperature of 57 °C for *Ha*1092, *Ha*1168, *Ha*1518, and *Ha*8862 and 60 °C for *Ha*2876. PCR reactions using primers for *Ha*502 used annealing temperatures of 57 °C and 60 °C. PCR products were electrophoresed on vertical, 6% denaturing polyacrylamide gels alongside a Genescan™-350 TAMRA size standard ladder. Gels were scanned with Hitachi FMBioII® or FMBioIII® fluorescent image scanning units and were scored relative to the ladder using ImageAnalysis® imaging software version 3 (MiraiBio Inc.). Scoring was verified visually to ensure accuracy. Dosage of microsatellites can not be determined, and we have taken a conservative approach to estimate microsatellite allele frequency in *H. versicolor*. The number of sampled chromosomes is a necessary parameter in POPTREE2. All four chromosomes would be counted for a homozygote and for heterozygotes that demonstrate four microsatellite

Fig. 1. Mitotic metaphase chromosomes of a diploid male *Hyla chrysoscelis* (2*n* = 24) and a tetraploid male *H. versicolor* (4*n* = 48) derived from corneal epithelial squash preparations. The unstained chromosomes were photographed using phase contrast optics with a 63× oil objective. Other than number, the chromosomes of the two species are identical using this method (Bogart 1980). The largest chromosomes are about 15 μm in length.



alleles. Three chromosomes would be counted for an individual that demonstrates three microsatellite alleles and, for two alleles, the individual would be treated as a diploid heterozygote. Microsatellite allele frequencies were calculated using the adjusted number of chromosomes for *H. versicolor* and analysed using POPTREE2 (Takezaki et al. 2010).

Mitochondrial cytochrome *b* sequences

DNA from many of the same samples used for isozymes and for microsatellites was amplified using standard PCR methods and the same primers that were used by Ptacek et al. (1994) with the annealing temperature optimized at 46 °C. The PCR products were purified using a Qiagen QIAquick PCR Purification Kit, and directly sequenced using Big Dye sequencing protocols (ABI) with an ABI 3730 automatic sequencer. The same primers were used for both PCR and sequencing. Sequences were edited using BioEdit 7.2.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using CLUSTALX (Thompson et al. 1997). A haplotype gene tree was constructed from the aligned sequences using the maximum-likelihood method implemented in PAUP* (version 4.0a, build 167). A best-fit substitution model was first selected based on BIC in PAUP*, and a heuristic search was conducted with 100 random addition replicates. A bootstrap analysis was also conducted with 100 replicates in PAUP*.

Results

Chromosomes

Mitotic metaphase chromosome spreads of field-collected fast-calling frogs (*H. chrysoscelis*) all had 24 mitotic chromosomes and slow-calling frogs (*H. versicolor*) consistently demonstrated 48 mitotic chromosomes (Fig. 1). Karyotypes of these species have previously been published (Bogart and Wasserman 1972; Bogart 1980; Schmid et al. 2018). In the present study, chromosome number was used to confirm species identification for all individuals.

Isozymes

Isozyme alleles were resolved from individuals of *H. versicolor* and *H. chrysoscelis* collected from sympatric populations in Virginia, Maryland, Michigan, Wisconsin, and Manitoba as well as from a population of *H. versicolor* in Illinois. Allozyme frequencies for 15 isozyme loci are provided in Table 3. Many of the same isozyme loci were investigated by Ralin and Selander (1979) and Romano et al. (1987). Allozyme frequencies for *Ldh-1* and (or) *Ldh-2* were not adequately resolved for Maryland, Wisconsin, and Manitoba individuals and are not included in Table 3. In general, both species share the same allozymes and have similar frequencies of the most common allozymes in the two species in sympatric populations. Some rare allozymes were only found in *H. versicolor* (*Acon-1* C, *Ldh-1* A, *Ldh-2* C, *Mpi* D, *Pgm-1* C, *Sod-1* D, *Sod-1* E). *Idh-1* C was only found in *H. chrysoscelis*. The tree derived from the allozyme frequencies show no basic dichotomy of the two species (Fig. 2). Sympatric populations of *H. chrysoscelis* and *H. versicolor* from Manitoba, Michigan, and Virginia are each grouped together in their own clades.

Microsatellites

Primer sequences designed for *H. andersonii* amplified six polymorphic microsatellite loci in both *H. versicolor* and *H. chrysoscelis*. As many as 13 microsatellite alleles were observed from each of three loci (*Ha*502, *Ha*2876, and *Ha*8862), and 12 alleles from one other locus (*Ha*1092). Only two alleles were resolved for *Ha*1518 and four alleles for *Ha*1168. The microsatellite allele frequencies for *H. versicolor* and *H. chrysoscelis* from sympatric populations in Maryland, Michigan, Wisconsin, Minnesota, and Manitoba as well as *H. versicolor* from Illinois are provided in Table 4. Tissue samples from Virginia, that were used for isozymes, did not provide adequate DNA for microsatellite amplification. Although it is evident that these species share many microsatellite alleles, the frequencies and the number of alleles varied considerably within and between populations. The number of observed alleles is directly related to sample size in the highly polymorphic loci. The tree derived from microsatellite allele frequencies (Fig. 3) only groups sympatric populations in Maryland and Minnesota.

Table 3. Isozyme allele frequencies at 10 loci in populations of *Hyla versicolor* (vers) and *H. chrysoscelis* (chrys).

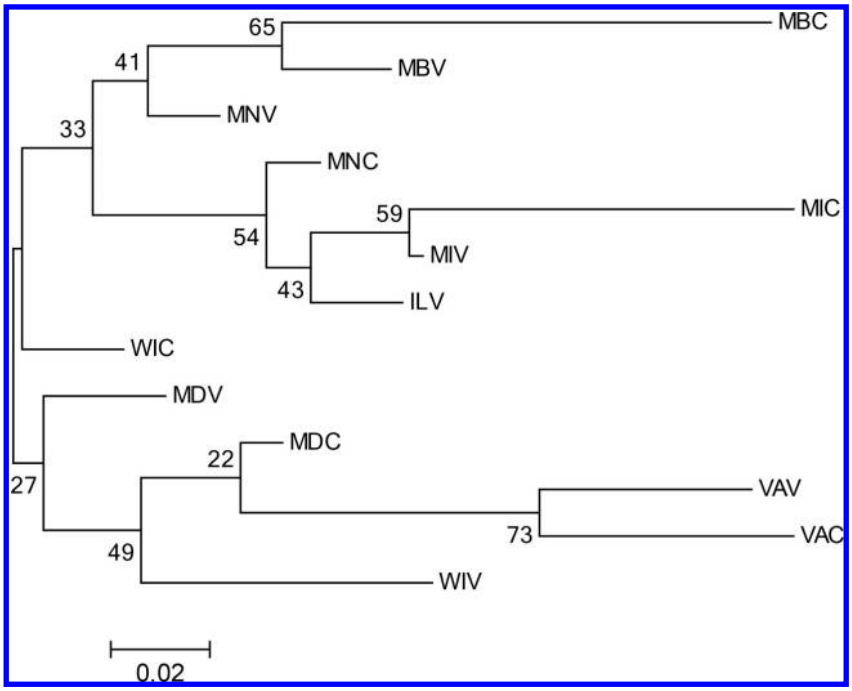
	Localities and Species										
	Virginia		Maryland		Michigan		Illinois	Wisconsin		Manitoba	
Locus	vers	chrys	vers	chrys	vers	chrys	vers	vers	chrys	vers	chrys
6Pgd (N)*	(28)	(22)	(60)	(42)	(72)	(6)	(52)	(24)	(26)	(80)	(20)
A	0.464	0.5	0.55	0.595	0.514	1.0	0.25	0.792	0.462	0.85	0.8
B	0.536	0.5	0.45	0.405	0.486	—	0.75	0.208	0.538	0.15	0.2
Acon-1 (N)	(20)	(24)	(92)	(54)	(52)	(16)	(48)	(28)	(24)	(92)	(22)
A	0.4	0.042	0.098	0.056	0.962	1.0	0.812	0.857	0.25	0.261	0.182
B	0.55	0.958	0.848	0.944	0.038	—	0.188	0.143	0.75	0.739	0.818
C	0.05	—	0.054	—	—	—	—	—	—	—	—
Acon-2 (N)	(16)	(10)	(44)	(18)	(12)	(16)	(28)	(20)	(12)	(59)	(8)
A	0.5	—	0.091	0.167	0.167	0.188	0.286	0.45	0.167	0.458	0.125
B	0.5	1.0	0.818	0.833	0.833	0.812	0.714	0.35	0.667	0.288	0.875
C	—	—	0.091	—	—	—	—	0.2	0.167	0.254	—
GOT-1 (N)	(28)	(22)	(64)	(64)	(76)	(16)	(52)	(28)	(26)	(96)	(24)
A	—	—	—	—	0.026	—	—	—	—	—	0.125
B	0.642	0.227	0.062	0.068	0.303	0.625	0.558	0.214	0.115	0.906	0.875
C	0.357	0.773	0.938	0.932	0.671	0.375	0.442	0.786	0.885	0.094	—
GOT-2 (N)	(28)	(22)	(64)	(34)	(8)	(10)	(52)	(28)	(20)	(92)	(22)
A	1.0	1.0	0.859	0.853	1.0	1.0	1.0	0.75	0.9	0.891	1.0
B	—	—	0.047	0.147	—	—	—	0.143	—	0.076	—
C	—	—	0.094	—	—	—	—	0.107	0.1	0.033	—
IDH-1 (N)	(20)	(30)	(92)	(52)	(76)	(16)	(56)	(28)	(24)	(92)	(24)
A	1.0	0.467	0.663	0.712	0.882	1.0	0.857	0.714	1.0	0.467	0.916
B	—	0.066	0.337	0.25	0.118	—	0.143	0.286	—	0.533	0.083
C	—	0.467	—	0.038	—	—	—	—	—	—	—
IDH-2 (N)	(20)	(4)	(56)	(12)	(8)	(10)	(56)	(20)	(14)	(84)	(18)
A	—	—	0.071	0.5	—	—	—	—	0.143	0.143	—
B	1.0	1.0	0.929	0.5	1.0	1.0	1.0	1.0	0.857	0.857	1.0
LDH-1 (N)	(16)	(22)	—	(10)	(52)	(6)	(44)	(4)	(4)	—	—
A	—	—	—	—	0.019	—	0.114	—	—	—	—
B	0.062	0.182	—	0.2	0.346	—	0.204	1.0	—	—	—
C	0.938	0.818	—	0.8	0.635	1.0	0.682	—	1.0	—	—
LDH-2 (N)	(12)	(8)	—	—	(44)	(6)	(44)	(4)	—	—	—
A	1.0	1.0	—	—	0.636	0.833	0.386	0.25	—	—	—
B	—	—	—	—	0.341	0.167	0.309	0.75	—	—	—
C	—	—	—	—	0.023	—	0.204	—	—	—	—
MDH-1 (N)	(28)	(30)	(92)	(42)	(80)	(16)	(56)	(28)	(28)	(96)	(24)
A	1.0	1.0	0.989	1.0	0.725	0.5	0.946	0.929	0.786	0.698	0.583
B	—	—	0.011	—	0.262	0.5	0.054	0.071	0.214	0.281	0.375
C	—	—	—	—	0.013	—	—	—	—	0.021	0.042
MPI (N)	(4)	(22)	(64)	(44)	(20)	(10)	(40)	(20)	(22)	(32)	(22)
A	1.0	0.864	0.594	0.25	0.25	—	0.625	—	0.045	0.094	0.227
B	—	0.045	0.375	0.727	0.65	1.0	0.325	0.75	0.864	0.25	0.409
C	—	0.091	0.031	0.023	0.05	—	0.05	0.25	0.091	0.656	0.364
D	—	—	—	—	0.05	—	—	—	—	—	—
PGI (N)	(4)	(8)	(40)	(8)	(20)	(10)	(40)	(24)	(24)	(84)	(24)
A	—	—	—	—	0.05	—	—	—	0.042	0.381	0.417
B	1.0	1.0	1.0	1.0	0.95	1.0	1.0	1.0	0.958	0.619	0.583
PGM-1 (N)	(20)	(22)	(36)	(16)	(64)	(6)	(44)	(24)	(22)	(20)	(20)
A	0.2	0.227	0.111	0.188	0.094	—	0.091	0.042	—	0.141	0.05
B	0.8	0.773	0.889	0.812	0.734	1.0	0.864	0.958	1.0	0.859	0.95
C	—	—	—	—	0.172	—	0.045	—	—	—	—
PGM-2 (N)	(20)	(22)	(40)	(18)	(68)	(16)	(52)	(28)	(26)	(88)	(22)
A	0.05	0.136	0.85	0.5	0.191	—	0.231	0.893	0.615	0.864	0.91
B	0.85	0.727	0.15	0.278	0.809	1.0	0.769	0.071	0.308	0.136	0.09
C	0.1	0.136	—	0.222	—	—	—	0.036	0.077	—	—

Table 3 (*concluded*).

	Localities and Species										
	Virginia		Maryland		Michigan		Illinois	Wisconsin		Manitoba	
Locus	vers	chrys	vers	chrys	vers	chrys	vers	vers	chrys	vers	chrys
SOD-1 (N)	(16)	(16)	(28)	(10)	(52)	(6)	(36)	(12)	(6)	(52)	(4)
A	—	0.125	0.071	—	0.058	—	0.028	—	—	0.019	—
B	—	0.375	0.107	—	0.173	—	0.361	0.333	—	—	—
C	1.0	0.5	0.821	1.0	0.731	1.0	0.611	0.5	1.0	0.981	1.0
D	—	—	—	—	0.019	—	—	0.167	—	—	—
E	—	—	—	—	0.019	—	—	—	—	—	—

*(N) is the number of chromosomes examined to obtain microsatellite fragment frequencies.

Fig. 2. Neighbour-joining tree ("Distance/phylogeny") of *Hyla versicolor* (V) and *H. chrysoscelis* (C) from sympatric populations in Virginia (VA), Maryland (MD), Michigan (MI), Wisconsin (WI), Manitoba (MB), and from a population of *H. versicolor* in Illinois (IL) based on allozyme frequencies using the data in Table 3. The analysis was performed using POPTREE2 (Takezaki et al. 2010) with 1000 bootstraps.



Mitochondrial cyt*b*

Twenty-six haplotypes were recovered from 36 *H. versicolor* and 40 *H. chrysoscelis* that were sequenced for a total of 1004 bp after alignment and trimming the sequence ends. No gaps were added, and all sequences could be translated into amino acids. The ML analysis with best-fit model of HKY+G resulted in one best tree (Fig. 4) that included many of the same frogs that were also used for isozymes and microsatellites. Bootstrap values greater than 70 are mapped on the tree. A clear dichotomy exists between specimens of *H. versicolor* and *H. chrysoscelis*. The phylogeny was rooted with a specimen of *H. avivoca* from Kentucky and shows that *H. chrysoscelis* is basal to *H. versicolor*. *Hyla chrysoscelis* from Minnestoa, Wisconsin, and Manitoba form a separate cluster to *H. chrysoscelis* from Michigan, Maryland, and Virginia. The main cluster of *H. versicolor* is divided into two sister groups of frogs from eastern (Virginia and Maryland) and western (Michigan, Manitoba, Wisconsin, and Minnesota) populations.

Discussion

We sampled tetraploid *H. versicolor* and diploid *H. chrysoscelis* individuals that were collected from sympatric populations to test the hypothesis that there is genetic interaction between these two species. Our hypothesis would be rejected if synapomorphic alleles were not shared by the two species but were confined to one or to the other species. The data that were obtained using isozymes, microsatellites, and mitochondrial DNA sequences to examine possible gene exchange appear to be inconsistent. Isozymes show the most genetic similarity of these two species in sympatry (Fig. 2). Microsatellites group the two species in some sympatric populations such as Maryland, but the two species in Manitoba that were

Table 4. Microsatellite DNA allele frequencies at six loci in sympatric populations of *Hyla versicolor* (vers) and *H. chrysoscelis* (chrys) as well as a population of *H. versicolor* from Illinois.

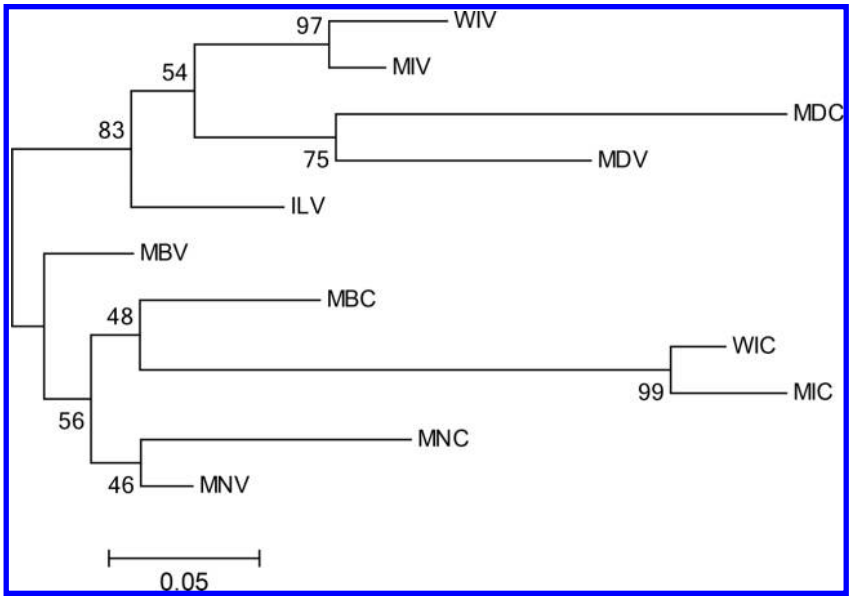
	Localities and species										
	Maryland		Michigan		Illinois	Wisconsin		Minnesota		Manitoba	
Locus	vers	chrys	vers	chrys	vers	vers	chrys	vers	chrys	vers	chrys
Ha502 (N)*	(35)	(42)	(20)	(8)	(30)	(15)	(26)	(79)	(20)	(39)	(18)
239	—	0.02	—	—	—	—	—	—	—	—	—
243	0.03	—	0.05	—	—	—	0.04	0.02	—	—	—
247	0.11	0.14	0.05	—	—	—	0.04	0.01	—	—	0.06
251	0.08	—	0.25	—	0.1	0.53	—	0.06	0.15	0.03	—
255	0.68	0.78	0.3	0.38	0.27	0.33	0.27	0.3	—	0.41	0.5
259	0.03	0.05	0.1	—	0.17	0.07	0.12	0.35	0.6	0.41	0.06
263	—	—	0.15	0.5	0.33	—	0.23	0.08	0.05	0.03	0.22
267	—	—	—	—	—	—	0.19	0.08	—	0.09	—
271	—	—	—	—	—	—	—	0.04	0.15	—	0.17
275	—	—	—	—	0.1	0.07	—	0.04	0.05	0.03	—
279	0.06	—	—	—	0.03	—	—	0.01	—	—	—
283	—	—	0.1	—	—	—	0.12	0.01	—	0.03	—
287	—	—	—	0.12	—	—	—	—	—	—	—
Ha1092 (N)	(44)	(44)	(29)	(10)	(17)	(11)	(32)	(79)	(20)	(58)	(22)
194	—	0.2	—	—	—	—	—	—	—	—	—
198	0.86	0.54	0.34	—	0.47	0.45	0.03	0.11	—	0.09	—
206	0.11	0.14	0.52	0.8	0.41	0.45	0.84	0.66	0.65	0.78	0.91
210	—	—	0.03	0.1	0.12	0.09	0.12	—	—	—	—
214	—	—	0.07	—	—	—	—	0.05	—	0.03	—
218	—	—	—	—	—	—	—	0.1	0.25	0.03	0.04
222	0.02	—	—	—	—	—	—	0.05	—	—	0.04
226	—	0.09	—	—	—	—	—	0.01	0.1	—	—
230	—	—	0.03	—	—	—	—	0.01	—	0.05	—
234	—	—	—	—	—	—	—	—	—	0.02	—
246	—	—	—	0.1	—	—	—	—	—	—	—
250	—	0.02	—	—	—	—	—	—	—	—	—
Ha1168 (N)	(54)	(46)	(30)	(10)	(34)	(20)	(34)	(144)	(20)	(80)	(22)
126	0.09	—	0.2	—	0.03	—	—	—	—	—	—
138	—	0.02	—	—	—	—	—	—	—	—	—
142	—	—	0.03	—	—	—	—	—	—	—	—
154	0.91	0.98	0.77	1.0	0.97	1.0	1.0	1.0	1.0	1.0	1.0
Ha1518 (N)	(34)	(32)	(28)	(8)	(16)	(10)	(30)	(54)	(20)	(20)	(16)
334	0.85	0.03	0.64	1.0	0.5	.7	0.97	0.61	0.75	0.7	0.62
346	0.15	0.97	0.36	—	0.5	0.3	0.03	0.39	0.25	0.3	0.38
Ha2876 (N)	(47)	(46)	(18)	(10)	(26)	(18)	(18)	(69)	(20)	(52)	(20)
122	0.02	—	0.22	1.0	0.2	0.22	0.05	0.49	0.55	0.29	0.35
126	0.3	—	0.06	—	—	—	—	0.04	—	0.06	—
130	—	—	—	—	0.2	0.11	0.11	—	—	0.04	0.05
134	0.02	0.41	0.33	—	0.1	0.11	0.06	0.01	—	0.12	—
138	—	0.11	—	—	0.04	0.11	0.11	—	—	—	—
142	0.08	0.02	0.11	—	—	0.11	0.11	0.03	—	—	—
146	0.21	0.09	0.06	—	—	0.17	—	—	—	—	—
150	0.3	0.35	—	—	0.3	0.11	—	0.36	0.35	0.27	0.25
154	0.06	0.02	0.11	—	0.1	0.06	0.06	0.04	0.05	0.21	0.35
158	—	—	0.06	—	0.1	—	0.06	—	—	—	—
162	—	—	—	—	—	—	—	—	0.05	—	—
166	—	—	—	—	—	—	—	0.01	—	—	—
170	—	—	0.06	—	—	—	—	—	—	0.02	—
Ha8862 (N)	(29)	(36)	(20)	(6)	(17)	(12)	(24)	(76)	(20)	(43)	(18)
198	—	—	0.05	—	—	—	—	—	—	—	—
206	—	0.06	—	—	—	—	—	—	—	—	—
210	—	0.08	0.1	—	0.06	—	0.04	0.06	—	0.09	—
214	0.52	0.17	0.25	0.67	0.12	0.25	0.29	0.1	0.2	0.16	0.28

Table 4 (*concluded*).

	Localities and species										
	Maryland		Michigan		Illinois	Wisconsin		Minnesota		Manitoba	
Locus	vers	chrys	vers	chrys	vers	vers	chrys	vers	chrys	vers	chrys
218	0.24	0.3	0.1	—	0.41	0.27	0.04	0.16	0.1	0.05	0.06
222	0.03	0.08	0.1	—	0.06	0.25	0.08	0.13	0.1	0.19	0.22
226	—	0.11	0.05	—	—	—	0.13	0.16	0.2	0.16	0.06
230	0.07	0.06	0.1	0.33	0.18	—	0.21	0.09	0.05	0.12	—
234	0.03	0.11	0.15	—	0.12	0.08	0.13	0.14	0.15	0.02	0.17
238	0.07	0.03	0.05	—	0.06	0.17	0.08	0.09	0.1	0.14	0.17
242	—	—	—	—	—	0.08	—	0.05	0.05	0.07	0.06
246	0.03	—	0.05	—	—	—	—	—	—	—	—
330	—	—	—	—	—	—	—	—	0.05	—	—

*(N) is the number of chromosomes examined to obtain microsatellite fragment frequencies.

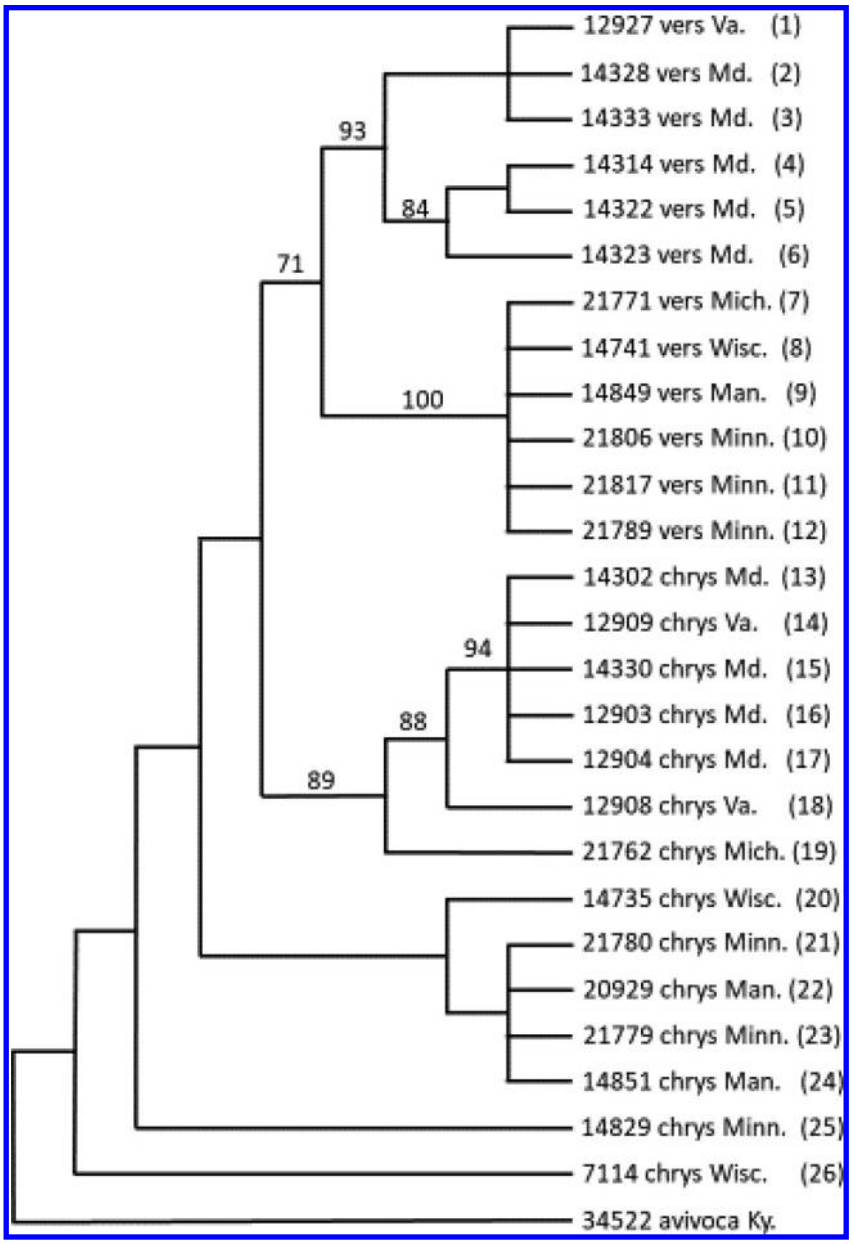
Fig. 3. Neighbour-joining tree ("Distance/phylogeny") of *Hyla versicolor* (V) and *H. chrysoscelis* (C) from sympatric populations in Maryland (MD), Michigan (MI), Wisconsin (WI), Minnesota (MN), and Manitoba (MB), and from a population of *H. versicolor* in Illinois (IL) based on microsatellite DNA allele frequencies using the data in Table 4. The analysis was performed using POPTREE2 (Takezaki et al. 2010) with 1000 bootstraps.



closely grouped with isozymes appear distantly related from each other by microsatellites (Fig. 3). Mitochondrial DNA sequences partition *H. versicolor* and *H. chrysoselis* into major clades of the two species (Fig. 4), and no sympatric population was found to have *mt*DNA that was shared by both *H. versicolor* and *H. chrysoscelis*. These data do, however, provide information that helps to improve our understanding of the possible mating and embryological factors that lead to the evolution of *H. versicolor* and temporal information that do demonstrate that gene flow occurred from *H. chrysoscelis* to *H. versicolor* rarely over time. Isozymes and microsatellites sample nuclear genomes and the mitochondrial cyt*b* sequences provide information on female lineages. Microsatellites are highly variable, have a rapid mutation rate (Ellegren 2004), and are most useful for quantifying recent gene flow in contemporary populations. Isozymes are very conservative (Sarich 1977; Ralin and Selander 1979) and provide a measure of possible historical gene flow. Our cyt*b* sequence data (Fig. 4) show that all our *H. versicolor* samples could be derived from a single common ancestor that arose from *H. chrysoscelis* that shared a common ancestor with Michigan *H. chrysoscelis*. Our samples do not include specimens over the complete range of these two species as were sampled in much larger range coverage of these frogs in North America by Ptacek et al. (1994) and Holloway et al. (2006) who both provided sequence evidence for multiple origins of *H. versicolor*.

Gene exchange between these species in our sympatric populations can be explained by the "triploid bridge" hypothesis (Bogart and Wasserman 1972; Ralin and Selander 1979; Nishioka and Ueda 1983). *Hyla versicolor* first arose when an autotriploid *H. chrysoscelis* (3*n*) produced unreduced triploid ova that were fertilized by haploid *H. chrysoscelis* sperm (Fig. 5, *1*). A female *H. versicolor* mates with a male *H. chrysoscelis* and produces triploid

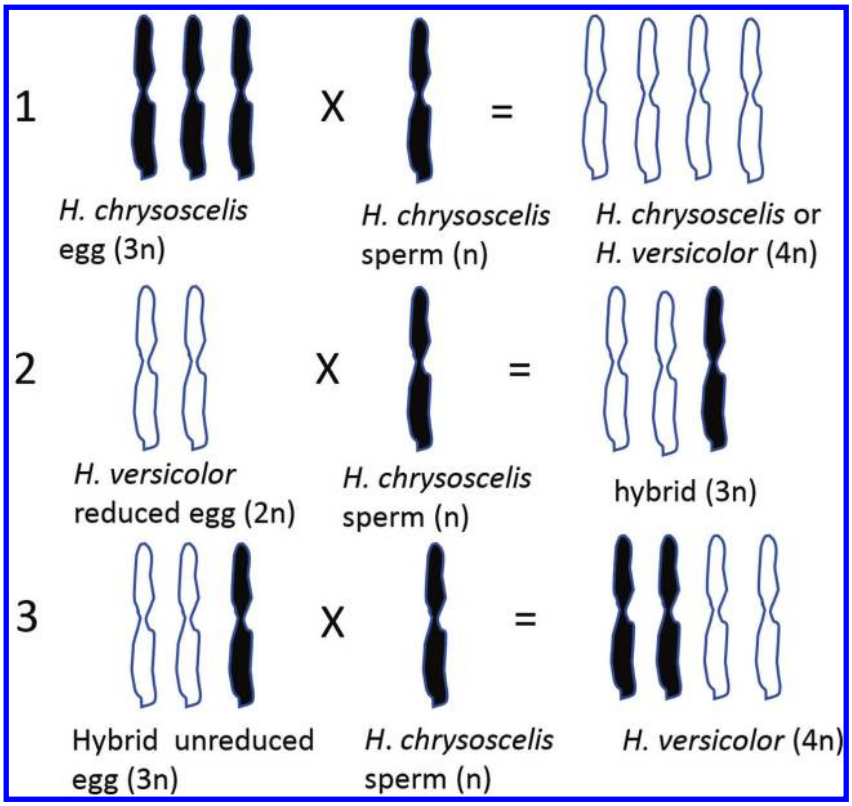
Fig. 4. A maximum-likelihood tree derived from the cytochorome *b* sequence data that were rooted with a sequence from a male specimen of *Hyla avivoca* from Kentucky. Twenty-six haplotypes were recovered from 74 specimens of *H. versicolor* and *H. chrysoscelis*. Percent bootstrap values greater than 70 are included at the nodes on the tree. Specimen numbers are J.P. Bogart catalogue numbers. Only unique haplotypes are included in the tree. Many individuals from the same population were found to share the same haplotype. Some haplotypes were shared in individuals from different populations: haplotype 7 is shared with *H. versicolor* in Michigan, Illinois, and Wisconsin; haplotype 15 is shared with *H. chrysoscelis* specimens in Maryland and Virginia; haplotype 20 is shared with Wisconsin and Minnesota *H. chrysoscelis*; haplotype 22 is shared in *H. chrysoscelis* from Minnesota and Manitoba; and haplotype 24 is shared in *H. chrysoscelis* from Wisconsin and Manitoba. Haplotypes for all specimens sequenced are provided in the supplementary data, Table S1[1].



hybrids (Fig. 5, *2*). Female hybrids produce unreduced triploid eggs that are fertilized by *H. chrysoscelis* sperm and result in tetraploid *H. versicolor* (Fig. 5, *3*). Genes from *H. chrysoscelis* are included in the "new" *H. versicolor* from the initial hybridization and from sperm incorporation. The newly incorporated genes are then included in the *H. versicolor* population through subsequent random mating of the tetraploids.

Results from artificial breeding experiments (Johnson 1959, 1963; Ralin 1976) have been used as evidence that these two species are incompatible and are genetically isolated (Ralin and Selander 1979; Romano et al. 1987). F_1 hybrids are deemed to have reduced viability, and backcross progeny are not viable. But Johnson (1959) did produce viable F_1 males and females, and he only used F_1 males for his backcross experiments (Johnson 1963). His

Fig. 5. Proposed origin and subsequent triploid bridge hypothesis that could explain gene sharing between diploid *Hyla chrysoscelis* and tetraploid *H. versicolor*. (*1*) Unreduced (3*n*) ova from an autotriploid *H. chrysoscelis* are fertilized with *H. chrysoscelis* sperm to produce the original(?) male and female *H. versicolor* tetraploids. (*2*) Diploids and tetraploids hybridize and produce triploids. (*3*) Triploid hybrid females produce unreduced triploid eggs that are fertilized by haploid *H. chrysoscelis* sperm to produce new tetraploids. Genes can pass from diploid *H. chrysoscelis* to tetraploid *H. versicolor* in sympatric populations.



female F_1 hybrids were not used in backcross experiments because they failed to develop mature ova. It is difficult to raise hybrids or even frogs from control crosses in the laboratory. Ralin (1976) used survival of individuals two weeks after hatching as a measure of genetic competency as further development was compromised by environmental factors. Tucker and Gerhardt (2012) were able to raise autotriploid *H. chrysoscelis* to sexual maturity in outdoor cattle tanks and found that female triploids took 22 months to attain sexual maturity. The triploid males were used in an acoustic investigation that demonstrated that ploidy is related to pulse rate (Keller and Gerhardt 2001), and the triploid females were used in acoustic discrimination experiments (Tucker and Gerhardt 2012). The F_1 crosses performed by Johnson (1959) used specimens collected between March and May in 1958, and his backcrosses were done using F_1 males in the spring breeding season of 1959 (Johnson 1963). The age of first reproduction for females of both *H. chrysoscelis* and *H. versicolor* is two years (Duellman and Trueb 1986). It is possible that Johnson's F_1 females did not have time to develop mature ova. Although triploid hybrid males do produce sperm that can activate development when used in crosses (Johnson 1963; Nishioka and Ueda 1983; Mable 1989), embryonic mortality is high and tetraploids have not been produced. Unreduced ova are commonly produced by interspecific hybrids and in crosses that involve individuals with different ploidy levels (Bogart 1972; Mable et al. 2011; Mason and Pires 2015), so it is reasonable to assume that triploid *H. versicolor* × *H. chrysoscelis* females could produce triploid ova (VVC) that would result in tetraploids (VVCC) when fertilized with haploid *H. chrysoscelis* sperm. Backcrosses using F_1 triploid females (VVC) and *H. versicolor* males (with diploid sperm) would result in pentaploids (VVVVC), which, if viable, would be expected to produce aneuploid gametes.

Hybridization could involve female *H. versicolor* and male *H. chrysoscelis* or female *H. chrysoscelis* and male *H. versicolor*. Mitochondrial sequences from field-collected male hybrids in West Virginia (Gerhardt et al. 1994) and Oklahoma (Bogart and Bi 2013) all align with sympatric *H. versicolor* that was also the identified female in four of five mis-mated pairs found in Missouri ponds (Gerhardt et al. 1994). All *H. versicolor* in our sympatric populations had *H. versicolor* female ancestors (Fig. 4). If the original F_1 cross had a *H. chrysoscelis* female, *H. versicolor* would be included in a clade of *H. chrysoscelis* as documented by Ptacek et al. (1994) for southwestern *H. versicolor* from Tennessee, Oklahoma, Texas, and Louisiana. Hybridization is likely a rare event because it has been well documented that females of both species do discriminate and

are acoustically attracted to their own males (Gerhardt 2005). It is not known if *H. versicolor* arose more than one time from autotriploid *H. chrysoscelis*. If so, *H. versicolor* might be found in some populations within the range of *H. chrysoscelis* that are geographically distant from other populations of *H. versicolor*. Hybridization to produce triploids is a more likely event but can only occur in sympatric populations and gene flow can only occur from diploid *H. chrysoscelis* into tetraploid *H. versicolor*. If other, perhaps extinct, species have contributed to the *H. versicolor* gene pool, as suggested by Holloway et al. (2006), an unknown sympatric diploid male (XX) might have mated with a triploid *H. versicolor* × *H. chrysoscelis* hybrid (VVC) female to produce tetraploid hybrid (VVCX) males and females that could interbreed with *H. versicolor* (VVCC) and incorporate genes from the unknown male. It is more difficult to explain how two unknown species could hybridize to produce a new tetraploid that would interbreed with extant *H. versicolor* as was proposed by Holloway et al. (2006).

Sex determination

Sex chromosomes are homomorphic in both *H. versicolor* and *H. chrysoscelis*, but heteromorphic XY chromosomes are found in *H. femoralis* and WZ chromosomes are found in *H. squirella* (Schmid et al 2018). *Hyla japonica* is heterogametic (XY) (Schmid et al. 2018). Experiments that produced tetraploids from autotriploid *H. japonica* found equal numbers of male and female triploids and tetraploids (Nishioka and Ueda 1983). Autotriploid *H. chrysoscelis* are both males and females (Tucker and Gerhardt 2012) as are hybrids of *H. versicolor* and *H. chrysoscelis* derived from crosses in either direction (Johnson 1963). In an XY system, triploid females would be XXX and, when fertilized with X or Y sperm, would produce XXXX female and XXXY male tetraploids. If the system was WZ, triploids would all be WZZ and the tetraploids would all be WZZZ and likely all females. Male *H. versicolor* could produce equal numbers of XX and XY sperm using a tetrasomic mode of chromosomal segregation during meiosis (Danzmann and Bogart 1983). How polyploid frogs avoid problems such as sexual imbalance (Muller 1925) or dosage compensation (Orr 1990) is currently not known and may involve various chromosomes and several genes (Bewick et al. 2010; Evans et al. 2012; Furman and Evans 2016; Miura 2017).

Other polyploid anurans

Our proposed evolution of tetraploid *H. versicolor* from diploid *H. chrysoscelis*, and occasional gene flow from *H. chrysoscelis* into *H. versicolor* via a triploid bridge, is probably not a unique phenomenon in the Anura. In South America, diploid *Odontophrynus cordobae* and tetraploid *O. americanus* are morphologically indistinguishable, have different mating calls (Martino and Sinsch 2002), and triploid hybrids have been found (Grenat et al. 2018). *Phyllomedusa tetraploidea* is an autotetraploid that hybridizes with diploid *P. distincta* (Haddad et al. 1994). The tetraploid arose from *P. distincta* or from an unknown diploid lineage that shared a common ancestor with *P. distincta* (Brunes et al. 2010). The four tetraploid species of *Neobatrachus* in Australia are all believed to be autotetraploids, have advertisement calls with lower pulse rates from their diploid ancestral species, triploid hybrids have been found in sympatric diploid/tetraploid populations, and they have asymmetric gene flow from diploids to the tetraploids as well as interspecific gene flow between the autotetraploid species (Novikova et al. 2020). Triploid and tetraploid species in the Eurasian *Bufo viridis* subgroup are allopolyploids (Betto-Colliard et al. 2018) as are the African diploid, tetraploid, octoploid, and dodecaploid species of *Xenopus* (Evans et al. 2015). It is very likely that allopolyploidy is initiated by reduced eggs in F_1 hybrid females (Stöck et al. 2010).

Conclusion

To examine gene sharing in *H. versicolor* and *H. chrysoscelis*, we partitioned the two species in sympatric populations as two separate populations. Our genetic clustering analyses of isozyme and microsatellite alleles, derived using POPTREE2 (Takezaki et al. 2010), assumes random mating within populations. *Hyla versicolor* and *H. chysoscelis* are not randomly mating with each other but, in several populations, *H. versicolor* appears to be more closely related to *H. chrysoscelis* than to *H. versicolor* in other populations. Our data clearly show that *H. versicolor* and *H. chrysoscelis* do share several genes. Allozymes were also observed to be most similar in other sympatric populations of the two species by Romano et al. (1987), but they did not interpret those data as gene-sharing. The more rapidly mutating microsatellites provide a more contemporary picture of historical gene exchange, followed by subsequent microsatellite mutation, and intraspecific recombination. Microsatellite analyses show that hybridization is likely a rare event but has now been confirmed by finding a few triploid males in sympatric populations and probably occurred over time in more than one sympatric population. Cytochrome *b* sequences show that all the *H. versicolor* in the sympatric populations that we examined were derived from *H. versicolor* females, so *H. versicolor* must have been the female in F_1 *H. versicolor* × *H. chrysoscelis* hybridizations that could pass genes from *H. chrysoscelis* to *H. versicolor*. Female triploid hybrids have yet to be found in nature, but female treefrogs do not vocalize so diploid, triploid, and tetraploid females can not be identified without ploidy evaluation. Hybridization may have independently occurred in all our sympatric populations. Based on microsatellite analyses, a more recent hybridization may have happened in sympatric populations in Maryland and Minnesota. Genes passed from *H. chrysoscelis* into *H. versicolor* would likely be rapidly dispersed in the *H. versicolor* population and would be expected to elevate heterozygosity in *H. versicolor*.

If "new" *H. versicolor* can arise repeatedly from *H. chrysoscelis* and (or) *H. versicolor* × *H. chrysoscelis* hybrids, the taxonomic status of *H. versicolor* is questionable. *Hyla versicolor* is the only bisexually reproducing polyploid vertebrate species in North America and should prove to be a valuable species for several research initiatives. We prefer to maintain *H. versicolor* as a valid taxon and treat sympatric populations as possible hybrid zones.

Acknowledgements

J.P.B. thanks former graduate students, Roy Danzmann, David Green, Barbara Mable, Jim McLister, Leslie Rye, and Alison Taylor for collecting the frogs used in this investigation and for memorable frog collecting trips. Care and treatment of frogs followed protocols approved by the Animal Care Committee of the University of Guelph. This research was supported with NSERC (Canada) grants to J.P.B. and J.F. All sequences are deposited in GenBank (accession numbers MT759638–MT759713).

References

Betto-Colliard, C., Hofmann, S., Sermiar, R., Perrin, N., and Stöck, M. 2018. Profound genetic divergence and asymmetric parental genome contributions as hallmarks of hybrid speciation in polyploid toads. Proc. R. Soc. B Biol. Sci. **285**: 20172667. doi:10.1098/rspb.2017.2667. PMID:29436499.

Bewick, A.J., Anderson, D.W., and Evans, B.J. 2010. Evolution of the closely related, sex-related genes *DM-W* and *DMRT1* in African clawed frogs (*Xenopus*). Evolution, **63**(3): 698–712. doi:10.1111/j.1558-5646.2010.01163.x. PMID:21044062.

Bogart, J.P. 1972. Karyotypes. *In* Evolution in the genus *Bufo*. *Edited by* W.F. Blair. University of Texas Press, Austin. pp. 171–195.

Bogart, J.P. 1980. Evolutionary implications of polyploidy in amphibians and reptiles. *In* Polyploidy: biological relevance. *Edited by* W.H. Lewis. Plenum Press, New York and London. pp. 341–378.

Bogart, J.P. 1981. Chromosome studies in *Sminthillus* from Cuba and *Eleutherodactylus* from Cuba and Puerto Rico (Anura: Leptodactylidae). Life Sciences Contributions, Royal Ontario Museum.

Bogart, J.P., and Bi, K. 2013. Genetic and genomic interactions of animals with different ploidy levels. Cytogenet. Genome Res. **140**: 117–136. doi:10.1159/000351593. PMID:23751376.

Bogart, J.P., and Wasserman, A.O. 1972. Diploid-polyploid cryptic species pairs: a possible clue to evolution by polyploidization in anuran amphibians. Cytogenetics, **11**: 7–24. doi:10.1159/000130172. PMID:5020841.

Brunes, T.O., Sequiera, S., Haddad, C.F.B., and Alexandrino, J. 2010. Gene and species trees of a Neotropical group of treefrogs: genetic diversification in the Brazilian Atlantic Forest and the origin of a polyploid species. Mol. Phylogenet. Evol. **57**: 1120–1133. doi:10.1016/j.ympev.2010.08.026. PMID:20813192.

Clayton, J.W., and Tretiak, D.N. 1972. Amine-citrate buffers for *p*H control in starch gel electrophoresis. J. Fish. Res. Board Can. **29**(8): 1169–1172. doi:10.1139/f72-172.

Danzmann, R.G. 1982. An examination of gene expression in the tetraploid *Hyla versicolor*, and in interspecific hybrids with the diploid treefrog, *H. chrysoscelis*. M.Sc. thesis, University of Guelph, Guelph, Ont.

Danzmann, R.G., and Bogart, J.P. 1982. Gene dosage effects on MDH isozyme expression in diploid, triploid, and tetraploid treefrogs of the genus *Hyla*. J. Hered. **73**: 277–280. doi:10.1093/oxfordjournals.jhered.a109639. PMID:7108184.

Danzmann, R.G., and Bogart, J.P. 1983. Further evidence for a polymorphism in gametic segregation in the tetraploid treefrog *Hyla versicolor* using a glutamate oxaloacetic transaminase locus. Genetics, **103**: 753–769. PMID:6852524.

Duellman, W.E., and Trueb, L. 1986. Biology of amphibians. McGraw-Hill, New York.

Duellman, W.E., Marion, A.M., and Hedges, S.B. 2016. Phylogenetics, classification, and biogeography of the treefrogs (Amphibia: Anura: Arboranae). Zootaxa, **4104**: 1–109. doi:10.11646/zootaxa.4104.1.1. PMID:27394762.

Ellegren, H. 2004. Microsatellites: simple sequences with complex evolution. Nat. Rev. Genet. **5**: 435–445. doi:10.1038/nrg1348. PMID:15153996.

Espinoza, N.R., and Noor, M.A. 2002. Population genetics of a polyploid: is there hybridization between lineages of *Hyla versicolor*? J. Hered. **93**: 81–85. doi:10.1093/jhered/93.2.81. PMID:12140266.

Evans, B.J., Pyron, R.A., and Wiens, J.J. 2012. Polyploidization and sex chromosome evolution in Amphibians. *In* Polyploidy and genome evolution. *Edited by* P.S. Soltis and D.E. Soltis. Springer-Verlag, Berlin Heidelberg. pp. 385–410. doi:10.1007/978-3-642-31442-1_18.

Evans, B.J., Carter, T.F., Greenbaum, E., Gvoždík, V., Kelley, D.B., McLaughlin, P.J., et al. 2015. Genetics, morphology, advertisement calls, and historical records distinguish six new polyploid species of African Clawed Frog (*Xenopus*, Pipidae) from West and Central Africa. PLoS ONE, **10**(12): e0142823. doi:10.1371/journal.pone.0142823. PMID:26672747.

Faivovich, J., Pereyra, M.O., Luna, M.C., Hertz, A., Blotto, B.L., Vásquez-Almazán, C.R., et al. 2018. On the monophyly and relationships of several genera of Hylini (Anura: Hylidae: Hylinae), with comments on recent taxonomic changes in hylids. South Am. J. Herpetol. **13**: 1–32. doi:10.2994/SAJH-D-17-00115.1.

Frost, D.R. 2020. Amphibian species of the world: an online reference. Version 6.0. American Museum of Natural History, New York, USA. Electronic Database Available from http://research.amnh.org/herpetology/amphibia/index.html [accessed 2020-07-13]. doi:10.5531/db.vz.0001.

Fu, J., MacCulloch, R.D., Murphy, R.W., and Darevsky, I.S. 2000. Clonal variation in the Caucasian rock lizard *Lacerta armeniaca* and its origin. Amphib.-Reptilia, **21**: 83–89. doi:10.1163/156853800507291.

Furman, B.L.S., and Evans, B.J. 2016. Sequential turnovers of sex chromosomes in African clawed frogs (*Xenopus*) suggest some genomic regions are good at sex determination. G3, **6**: 3625–3633. doi:10.1534/g3.116.033423. PMID:27605520.

Gerhardt, H.C. 1974. Mating call differences between eastern and western populations of the treefrog *Hyla chrysoscelis*. Copeia, **1974**: 534–536. doi:10.2307/1442547.

Gerhardt, H.C. 2005. Advertisement-call preferences in diploid-tetraploid tree frogs (*Hyla chrysoscelis* and *Hyla versicolor*): implications for mate choice and the evolution of communication systems. Evolution, **59**: 395–408. PMID:15807424.

Gerhardt, H.C., Ptacek, M.B., Barnett, L., and Torke, K.G. 1994. Hybridization in the diploid-tetraploid treefrogs *Hyla chrysoscelis* and *Hyla versicolor*. Copeia, **1994**: 51–59. doi:10.2307/1446670.

Gregory, T.R., and Mable, B.K. 2005. Polyploidy in animals. *In* The evolution of the genome. *Edited by* T.R. Gregory. Elsevier Academic Press, London. pp. 428–517.

Grenat, P., Salas, N., Pollo, F., Otero, M., Baraquet, M., Sinsch, U., and Martino, A. 2018. Naturally occurring triploids in contact zones between diploid/tetraploid *Odontophrynus cordobae* and *O. americanus* (Anura, Odontophrynidae). Amphib.-Reptilia, **39**: 1–10. doi:10.1163/15685381-00003141.

Guignard, M., Büchi, L., Gétaz, M., Betto-Colliard, C., and Stöck, M. 2012. Genome size rather than content might affect call properties in toads of three ploidy levels (Anura: Bufonidae: *Bufo viridis* subgroup). Biol. J. Linn. Soc. **105**: 584–590. doi:10.1111/j.1095-8312.2011.01837.x.

Haddad, C.F.B., Pombal, J.P., Jr., and Batistic, R.F. 1994. Natural hybridization between diploid and tetraploid species of leaf-frogs, genus *Phyllomedusa* (Amphibia). J. Herpetol. **28**: 425–430. doi:10.2307/1564953.

Holloway, A.K., Cannatella, D.C., Gerhardt, H.C., and Hillis, D.M. 2006. Polyploids with different origins and ancestors form a single sexual polyploid species. Am. Nat. **167**: E88–E110. doi:10.1086/501079. PMID:16670990.

International Union of Biochemistry Nomenclature Committee (IUBC). 1984. Enzyme nomenclature. Academic Press, New York.

Johnson, C. 1959. Genetic incompatibility in the call races of *Hyla versicolor* Le Conte in Texas. Copeia, **1959**: 327–335. doi:10.2307/1439891.

Johnson, C. 1963. Additional evidence of sterility between call-types in the *Hyla versicolor* complex. Copeia, **1963**: 139–143. doi:10.2307/1441281.

Keller, M.J., and Gerhardt, H.C. 2001. Polyploidy alters advertisement call structure in gray treefrogs. Proc. R. Soc. B Biol. Sci. **268**: 341–345. doi:10.1098/rspb.2000.1391. PMID:11270429.

Krenz, J.D., Semlitsch, R.D., Gerhardt, H.C., and Mahoney, P.A. 1999. Isolation and characterization of simple sequence repeat loci in the gray tree frog, *Hyla chrysoscelis*. Genome, **42**(4): 676–680. doi:10.1139/g98-166. PMID:10464791.

Littlejohn, M.J., Fouquette, M.J., Jr., and Johnson, C. 1960. Call discrimination by female frogs of the *Hyla versicolor* complex. Copeia, **1960**: 47–49. doi:10.2307/1439846.

Mable, B.K. 1989. Genetic investigation of interspecific hybrids between tetraploid and diploid species of treefrogs (genus *Hyla*). M.Sc. thesis, University of Guelph, Guelph, Ont.

Mable, B.K., Alexandrou, M.A., and Taylor, M.I. 2011. Genome duplication in amphibians and fish: an extended synthesis. J. Zool. **284**: 151–182. doi:10.1111/j.1469-7998.2011.00829.x.

Martino, A., and Sinsch, U. 2002. Speciation by polyploidy in *Odontophrynus americanus*. J. Zool. **257**: 67–81. doi:10.1017/S0952836902000663.

Mason, A.S., and Pires, J.C. 2015. Unreduced gametes: meiotic mishap or evolutionary mechanism? Trends Genet. **31**: 5–10. doi:10.1016/j.tig.2014.09.011. PMID:25445549.

Maxson, L., Pepper, E., and Maxson, R.D. 1977. Immunological resolution of a diploid-tetraploid species complex of tree frogs. Science, **197**: 1012–1013. doi:10.1126/science.197.4307.1012. PMID:17784138.

McLister, J.D. 1992. Laryngeal morphology and musculature in the species of the gray treefrog complex, *Hyla chrysoscelis* and *Hyla versicolor* (family Hylidae). M.Sc. thesis, University of Guelph, Guelph, Ont.

Mecham, J.S. 1960. Introgressive hybridization between two southeastern treefrogs. Evolution, **14**: 445–457. doi:10.2307/2405994.

Miura, I. 2017. Sex determination and sex chromosomes in Amphibia. Sex Dev. **11**: 298–306. doi:10.1159/000485270. PMID:29241181.

Moritz, C.C., Uzzell, T., Spolsky, C., Hotz, H., Darevsky, I., Kupriyanova, I., and Danielyan, F. 1992. The material [sic] ancestry and approximate age of parthenogenetic species of Caucasian rock lizards (*Lacerta*: Lacertidae). Genetica, **87**: 53–62. doi:10.1007/BF00128773.

Muller, H.J. 1925. Why polyploidy is rarer in animals than in plants. Am. Nat. **59**: 346–353. doi:10.1086/280047.

Nishioka, M., and Ueda, H. 1983. Studies on polyploidy in Japanese treefrogs. Sci. Rep. Lab. Amphib. Biol. Hiroshima Univ. **6**: 207–252.

Novikova, P.Y., Brennan, I.G., Booker, W., Mahony, M., Doughty, P., Lemmon, A.R., et al. 2020. Polyploidy breaks speciation barriers in Australian burrowing frogs *Neobatrachus*. PLoS Genet, **16**(5): e1008769. doi:10.1371/journal.pgen.1008769. PMID:32392206.

Orr, H.A. 1990. 'Why polyploidy is rarer in animals than in plants' revisited. Am. Nat. **136**: 759–770. doi:10.1086/285130.

Ptacek, M.B., Gerhardt, H.C., and Sage, R.D. 1994. Speciation by polyploidy in tree frogs: multiple origins of the tetraploid, *Hyla versicolor*. Evolution, **48**: 898–908. doi:10.1111/j.1558-5646.1994.tb01370.x. PMID:28568266.

Pyburn, W. 1960. Hybridization between *Hyla versicolor* and *H. femoralis*. Copeia, **1960**: 55–56. doi:10.2307/1439852.

Ralin, D.B. 1976. Comparative hybridization of a diploid-tetraploid cryptic species pair of treefrogs. Copeia, **1976**: 191–196. doi:10.2307/1443792.

Ralin, D.B., and Selander, R.K. 1979. Evolutionary genetics of diploid-tetraploid species of tree frogs of the genus *Hyla*. Evolution, **33**: 595–608. doi:10.1111/j.1558-5646.1979.tb04713.x. PMID:28563941.

Ralin, D.B., Romano, M.A., and Kilpatrick, C.W. 1983. The tetraploid treefrog *Hyla versicolor*: evidence for a single origin from the diploid *H. chrysoscelis*. Herpetologica, **39**: 212–225.

Romano, M.A., Ralin, D.B., Guttman, S.I., and Skillings, J.H. 1987. Parallel electromorph variation in the diploid-tetraploid gray treefrog complex. Am. Nat. **130**: 864–878. doi:10.1086/284752.

Sarich, V.M. 1977. Rates, sample sizes, and the neutrality hypothesis for electrophoresis in evolutionary studies. Nature, **265**: 24–28. doi:10.1038/265024a0. PMID:64931.

Schmid, M., Evans, B.J., and Bogart, J.P. 2015. Polyploidy in Amphibia. Cytogenet. Genome Res. **145**: 315–330. doi:10.1159/000431388. PMID:26112701.

Schmid, M., Bogart, J.P., and Hedges, H.B. 2018. The Arboranan frogs: evolution, biology, and cytogenetics. Cytogenet. Genome Res. **155**: 1–324. doi:10.1159/000492098.

Selander, R.K., Smith, M.H., Yang, S.Y., Johnson, W.E., and Gentry, J.B. 1971. Biochemical polymorphism and systematics in the genus *Peromyscus*, I. Variation in the old-field mouse (*Peromyscus polionotus*). Stud. Genet. **7103**: 49–90.

Stöck, M., Ustinova, J., Lamatsch, D.K., Schartl, M., Perrin, N., and Moritz, C. 2010. A vertebrate reproductive system involving three ploidy levels: hybrid origin of triploids in a contact zone of diploid and tetraploid Palearctic green toads (*Bufo viridis* subgroup). Evolution, **64**: 944–959. doi:10.1111/j.1558-5646.2009.00876.x. PMID:19863582.

Takezaki, N., Nei, M., and Tamura, K. 2010. POPTREE2: software for constructing population trees from allele frequency data and computing other population statistics with windows interface. Mol. Biol. Evol. **27**: 747–752. doi:10.1093/molbev/msp312. PMID:20022889.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **24**: 4876–4882. doi:10.1093/nar/25.24.4876. PMID:9396791.

Tucker, M.A., and Gerhardt, H.C. 2012. Parallel changes in mate-attracting calls and female preferences in autotriploid tree frogs. Proc. R. Soc. B Biol. Sci. **279**: 1583–1587. doi:10.1098/rspb.2011.1968. PMID:22113033.

Ueda, H. 1993. Mating calls of autotriploid and autotetraploid males in *Hyla japonica*. Sci. Rep. Lab. Amphib. Biol. **12**: 177–189. doi:10.15027/311.

Warwick, A.R., and Lemmon, E.M. 2014. Development and characterization of 21 microsatellite loci for the Pine Barrens treefrog (*Hyla andersonii*). Conserv. Genet. Resour. **6**: 719–721. doi:10.1007/s12686-014-0197-5.

Wasserman, A.O. 1970. Polyploidy in the common tree toad *Hyla versicolor* Le Conte. Science, **167**: 385–386. doi:10.1126/science.167.3917.385. PMID:5409740.

Wiley, J.E. 1983. Chromosome polymorphisms in *Hyla chrysoscelis*. Copeia, **1983**: 273–275. doi:10.2307/1444732.

Wiley, J.E., Meyne, J., Little, M.L., and Stout, J.C. 1992. Interstitial hybridization sites of the $(TTAGGG)_n$ telomeric sequence on the chromosomes of some North American hylid frogs. Cytogenet. Cell Genet. **61**: 55–57. doi:10.1159/000133368. PMID:1505232.