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# Hybridization in the Diploid-Tetraploid Treefrogs *Hyla chrysoscelis* and *Hyla versicolor*

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**The first cytologically confirmed natural hybrids between the diploid-tetraploid gray treefrogs, *Hyla chrysoscelis* and *H. versicolor*, were two triploid males initially detected by their distinctive advertisement calls. Analyses of the pulse-repetition rate and shape of the advertisement calls of these triploid hybrids suggest that one of the two previously reported putative hybrids was a hybrid and the other male was a *H. chrysoscelis*. The calls of the field-recorded hybrids were intermediate in pulse rate in comparison to the parental species and significantly different in structure than were the calls of laboratory-reared hybrids described by Mable and Bogart (1991). Mated pairs were collected in six syntopic populations in south-central Missouri. Flow cytometric identifications revealed that five (about 6.7%) of the 75 pairs in two of these ponds were mismated; these five matings represented about 3% of all 181 pairs examined from all Missouri populations. The rarity of sexually mature hybrids, despite a fairly high frequency of matings in at least two localities, indicates severe selection against hybrids. In four of the five mismated pairs, the female was *H. versicolor*. Analyses of mtDNA sequences showed that the female parent of the two cytologically confirmed natural hybrids was also *H. versicolor*.**

NATURAL hybridization among North American treefrogs has been frequently reported; most hybrids were males that were detected by their distinctive advertisement calls (Gerhardt, 1974; Gerhardt et al., 1980). The cryptic species of gray treefrogs, *Hyla chrysoscelis* and *H. versicolor* are a diploid-tetraploid complex that was first recognized by differences in the pulse-repetition rate (pulse rate) of the advertisement calls of males (Johnson, 1966; Wasserman, 1970). Laboratory crosses between the two species resulted in high mortality during the first week of the larval period, but survivorship to two weeks after fertilization was often as high as about 40% (Johnson, 1963; Ralin, 1976). Triploid hybrids that reached sexual maturity in the laboratory had greatly reduced fertility (Johnson, 1963).

Although the two species often form mixed breeding aggregations in ponds in the mid-Atlantic and central United States, no cytologically confirmed natural hybrid has been reported. Zweifel (1970) suggested that a male from Cape May County, New Jersey, was a hybrid because of the intermediate nature of the pulse rate of its advertisement calls. Ralin (1968, 1977), Jaslow and Vogt (1977), Bogart and Jaslow (1979) and Gerhardt (1982) provided quantitative data on the pulse rates of about 250 gray treefrogs from areas of sympatry. On the basis of temperature-corrected pulse rate, only one frog from Laclede County, Missouri, was considered to be a putative hybrid (Gerhardt, 1982). However, Mable and Bogart (1991) reported that the calls of laboratory-produced hybrids had pulse rates ("note repetition rate" in their terminology) that were not significantly different from those of *H. versicolor*. Thus, they speculated that hybrids may be "acoustically hidden" in sympatric populations.

Here we report the discovery of two cytologically verified, triploid hybrids in a mixed-species chorus in West Virginia. Our acoustic analyses of their calls, which were quite different from those of the laboratory-reared hybrids described by Mable and Bogart (1991), indicate that the male recorded in Laclede County, but not the individual recorded by Zweifel (1970), was probably a hybrid. We argue that triploid hybrids are not acoustically hidden in nature and that adult hybrids are exceedingly rare.

Females of both species show well-developed phonotactic selectivity for synthetic calls with pulse rates or shapes typical of conspecific males in the same populations (Gerhardt, 1982; Gerhardt and Doherty, 1988). Is female phonotactic selectivity alone sufficient to explain the rarity of adult hybrids? Here we provide an estimate of the frequency of mismating and a qualitative assessment of its direction based on a four-year period of sampling of mated pairs in syntopic populations in Missouri. We describe a method for unequivocal determination of ploidy that requires neither killing nor karyotyping the an-

imal. Determining ploidy is necessary because individuals of the two species cannot be distinguished reliably by external morphology. Schlefer et al. (1986) provide the only other direct estimate of the frequency and direction of mismating in a hylid species based on identifying males and females in mated pairs. We also determined the species of the female parent(s) of the two hybrids from West Virginia by sequencing a region of the cytochrome *b* gene from mitochondrial DNA of these individuals and individuals of the parental species from the same locality. Lamb and Avise (1986) also used data from mitochondrial DNA to determine the species of females that were involved in hybridization between *H. cinerea* and *H. gratiosa.*

Information about the frequency and direction of hybridization bear on an important issue concerning the evolution of these two species. Because the two species are genetically incompatible and form mixed-species breeding aggregations in many parts of their ranges, the gray treefrog complex is an excellent system for studying reproductive character displacement (Butlin, 1987). Indeed, Ralin (1977) claimed to have documented character displacement in the pulse rate of the advertisement calls of both species in Texas and Oklahoma. However, Bogart and Wasserman (1972) and even Ralin (1977) also speculated that the polyploidization phenomenon itself may result in pulse rate differences and female phonotactic selectivity sufficient to isolate the two incipient species in the absence of natural selection. Documenting mating mistakes would confirm the role of selection in the evolution of courtship signals and responses.

## Materials and Methods

*Species identification by flow cytometry.*—Early in this study we attempted to differentiate between the two species on the basis of differences in red blood cell size (Bogart and Wasserman, 1972; Ralin, 1977). However, we found a great deal of within-species variation and were unsatisfied with the reliability of this technique. Certainly it would be difficult, if not impossible, to detect triploid individuals using red blood cell size. Hence, we developed the following procedures to determine the ploidy of individuals by flow cytometry.

We anesthetized frogs by immersion in a 0.2% aqueous solution of 3-aminobenzoic ethyl ester (MS 222; Sigma, Inc.), buffered with sodium bicarbonate to achieve a pH of about 7 and collected about 20 μl of blood in a capillary tube by clipping the wall of a vein under the tongue with small dissecting scissors. Frogs recovered quickly from this procedure and began feeding within a day. The blood sample was placed in 2 ml of PBS/EDTA solution [1.37 M NaCl, 0.015 M $KH_2PO_4$ (monobasic), 0.08 M $Na_2HPO_4$ (dibasic), 0.027 M KCl and 5 mM EDTA]. We washed the blood cell suspension twice with PBS/EDTA and centrifuged the sample at 2800 rpm for five minutes between washings. After the second wash, the cells were resuspended in 2.5 ml of cold 100% ethanol, in which they were fixed for 24 h at 4 C. We then removed the ethanol by centrifugation. After another wash with PBS/EDTA, followed by centrifugation, we resuspended the cells in 1 ml of PBS/EDTA and performed cell counts using a hemocytometer. The final concentration was adjusted to 1 $\times$ $10^6$ cells per ml. We centrifuged the samples, decanted the supernatant, and added 1 ml of Krishan stain (Krishan, 1975). Krishan stain contains propodium iodine, which intercalates into the DNA and thereby allows for the determination of cell ploidy by quantification of fluorescence intensity. We filtered (nylon mesh size: 70 μm) stained samples immediately prior to analysis in the flow cytometer to remove clumped cells and other debris.

We used an EPICS 753 flow cytometer (Coulter Cytometry, Hialeah, Florida) with a 5-watt laser tuned at 488 nm using a 150-mW output. We optically aligned the instrument using 10-μm, full-bright, fluorescent polystyrene microspheres (Coulter Immunology) with coefficients of variation kept at 2% or less. We collected approximately 10,000 nuclei and used a dual parameter histogram of forward angle light scatter (FALS) versus log 90 degree light scatter (L90LS) to gate out debris. We displayed red fluorescence emission (610 low-pass filter) of Krishan stained cells as a single parameter histogram of linear integral red fluorescence (IRFL) on a scale of 256 channels, with fluorescence intensity corresponding to DNA content. We performed the analysis using Cytologic Software (Coulter Cytometry). We froze blood collected from known individuals of *H. versicolor* and *H. chrysoscelis* and analyzed an aliquot of each as a control with every batch of test individuals. The IRFL peak channel of samples from the tetraploid *H. versicolor* was approximately twice that of the diploid *H. chrysoscelis.*

For our studies of interspecific matings, we collected mated pairs of gray treefrogs at breeding sites where males of both species were calling in Laclede (2 ponds), Phelps (2 ponds), Webster (1 pond), and Crawford (1 pond) counties, Missouri. We placed each pair in a separate con-

tainer until we reached a site where we could separate each pair briefly with little risk of losing them. A male usually called within five minutes of being separated from the female, and we thus identified its species. We then returned each pair to the same container for transport to the laboratory. At the laboratory, frogs were toe clipped for subsequent identification and pairs maintained in the same container. Both males and females of each pair were identified using flow cytometry.

*Mitochondrial DNA sequence analysis.*—We killed frogs with an overdose of MS222, removed liver tissue and stored it at −80 C. We later extracted total DNA from homogenized liver tissue by digestion for 20 h at 37 C in 100 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 0.1% SDS, and 50 nM dithiotheritol/proteinase K (4 μg). DNA was extracted twice with phenol, once with a mixture of equal volumes of phenol and chloroform/isoamyl alcohol (24:1), and then once with chloroform/isoamyl. The sample was concentrated by ethanol precipitation.

We used the polymerase chain reaction (PCR; Saiki et al., 1985) to amplify a 600 base-pair segment of the cytochrome *b* mitochondrial 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 5′ to 3′ was TGAGGACAAATATCATTCTGAGGGGC-TGCAG. The H-strand primer was designed from the published sequence of the bullfrog, *Rana catesbeiana* (Yoneyama, 1987), and its sequence 5′ to 3′ was TCTTCTACTGGTTGT-CCTCCGATTCA. The 5′ base of this primer corresponds to position 214 in the published sequence. The amplification occurred in 100 μl of a solution containing 67 mM Tris (pH 8.8), 6.7 mM $MgSO_4$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, with each dNTP at 1 mM, each primer at 1 μM, genomic DNA (4 μg), and eight units of *Thermus aguaticus* polymerase (Perkins-Elmer/Cetus, Oakland, California). Each cycle of the PCR consisted of 1 min denaturation at 94 C, 1.5 min annealing at 50 C, and 1.5 min extension at 72 C for 30 cycles. We removed the top layer of mineral oil and precipitated the amplification product with 1 ml of cold 100% ethanol overnight at −20 C. After centrifugation, we removed the ethanol and allowed the remaining pellet to dry. Following Sambrook et al. (1989), we resuspended the DNA pellet in 10 μl of sterile water and added 20 μl of formamide dye. We then further purified the sample by using gel fractionation (3.5% acrylamide gel at 200 v for about 1 h). We stained the gel with ethidium bromide and visualized the DNA with longwave UV light. We then used a razor blade to excise the gel fragment containing the amplified product of the appropriate size and placed it in 300 μl of DNA elution buffer (Sambrook et al., 1989; A. R. Templeton and K. Shaw, pers. comm.).

We eluted the DNA from the acrylamide slice for 16–24 h in a 37 C waterbath, removed it by pipetting, and precipitated it in 1 ml of 100% ethanol for 30 min at −20 C. After centrifugation for 20 min, we collected the pellet and washed it with 70% ethanol and allowed it to dry. Finally, we resuspended the DNA in 30 μl of sterile water. This DNA solution was used as template in sequencing reactions with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, California). These reactions were run on the Applied Biosystems Model 373A Sequencing System at the DNA Core Facility, University of Missouri, Columbia.

*Acoustic recordings and analysis.*—We tape recorded both species of gray treefrogs and hybrids in Summers County, West Virginia, with a Sony Pro-Walkman cassette recorder and a Sennheiser directional microphone (ME80). The calls of the putative hybrid recorded by Zweifel (1970) and those of *H. chrysoscelis* recorded on the same date and locality were provided as a cassette copy of the original recording (American Museum of Natural History, Tape No. 150; equipment unspecified). The second putative hybrid and males of both species were recorded in May 1980, in Laclede County, Missouri, by M. Rapp, using a Nagra IV recorder (19 cm/sec) and Sennheiser 415 microphone. The body temperature of each frog was measured with a quick-reading thermometer immediately after each recording.

We used a Kay 5500 DSP sonagraph and custom-designed software to determine pulse rate, pulse shape, and dominant frequencies. Pulse rate is the reciprocal of the pulse period, the time from the beginning of one pulse within a pulse train to the beginning of the next pulse. Our software computed a mean pulse period for each of five or more advertisement calls, computed a grand mean, and converted to pulse rate.

We treated pulse shape as follows. Dividing the time to reach 50% of peak amplitude of a pulse by the time to reach peak (100%) amplitude allowed us to categorize the form of the rise (rate of increase in amplitude). The ratio was about 0.5 for a linear rise, greater than 0.5

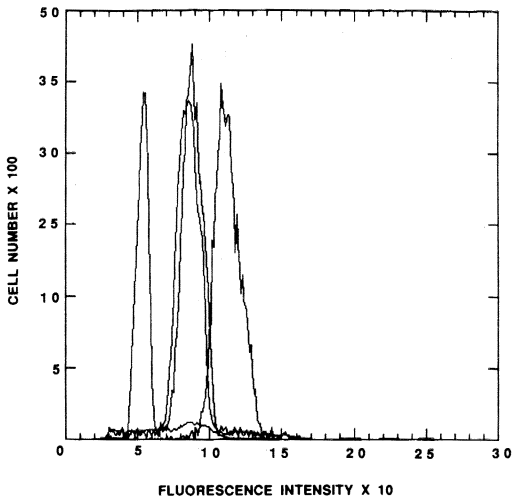


Fig. 1. Flow cytometric displays used to determine the ploidy of gray treefrogs (*Hyla chrysoscelis, H. versicolor*) and hybrids collected at Pipestem, Summers County, West Virginia. The peak in fluorescence intensity at 52 corresponds to an individual of *H. chrysoscelis*, the peak at 120, to a *H. versicolor*, and the peaks at 85 and 88, to the hybrids.

for an exponential rise, and less than 0.5 for a logarithmic (or inverse exponential) rise. We expressed the time to reach peak amplitude as a percentage of pulse duration and defined beginnings and endings of pulses at points that were about 5% of maximum amplitude. Expressing the time to reach full amplitude as a percentage of pulse duration permits comparisons over a broad range of temperature because pulse duration is inversely related to temperature. Frogs of both species tend to keep pulse duty cycle (ratio of pulse duration to pulse period) constant over the temperature range at which nearly all breeding occurs (about 15–25 C; Gerhardt and Doherty, 1988). Using a 5% criterion for defining the beginnings and endings of pulses makes it possible to compare more confidently pulse durations measured from recordings with different signal-to-background noise ratios. Our software displayed oscillograms of pulses and provided horizontal and vertical cursors that were superimposed to determine the values of each of the parameters described above. Dominant frequencies were determined from power spectra generated by averaging over the entire duration of one to five calls. The spectra of the calls of gray treefrogs and hybrids have bimodal spectra. The greatest amplitude was in the high-frequency band (at about 2 kHz); we also determined the maximum amplitude in the low frequency band and expressed its amplitude relative to that in the high-frequency band in decibels.

## Results

*Identification of species and confirmation of the ploidy of putative hybrids by flow cytometry.*—We identified 62 males of *H. versicolor* and 80 males of *H. chrysoscelis* from Missouri and West Virginia on the basis of their advertisment calls. Without exception, flow cytometry of the same frogs unequivocally confirmed these identifications.

The two interspecific hybrids recorded at Pipestem, West Virginia, were detected by the intermediate nature of their calls. The two putative hybrids and two representatives of each of the parental species were toe-clipped for individual identification and sent back to the laboratory in Missouri. Flow cytometry was conducted by technicians who did not know the sources of the blood samples. The results for four of the animals are shown in Figure 1. The peak to the left, centered at a fluorescence intensity of about 52, corresponds to an individual of *H. chrysoscelis*; the peak to the far right, centered at about 120, to an individual of *H. versicolor*. The two intermediate peaks, with a fluorescent intensity of about 85–88, correspond to the hybrids. Subsequently, we sent these frogs to J. E. Wiley, who confirmed the triploid nature of the two hybrids by determination of the metaphase karyotypes. Wiley et al. (1992) shows the karyotype of one of the triploids. After their return to Missouri, we euthanized the frogs and removed their livers for mtDNA extraction. The specimens are deposited in the Museum of Natural History at the University of Kansas.

*Parentage of hybrids.*—Because mtDNA is maternally inherited, and the sequences of both hybrids and one individual of *H. versicolor* from the same population were identical, the female parent of the hybrids was a *H. versicolor*. Two additional individuals of *H. versicolor* from two different localities in Virginia also had haplotypes identical to those of the hybrids and *H. versicolor* from West Virginia. Two other individuals of *H. versicolor*, one from a third locality in Virginia and one from a locality in Maine, differed by a single base pair from the Virginia and West Virginia specimens. The individual of *H. chrysoscelis* had a sequence that differed at 12 nucleotide positions from that of the hybrids and *H. versicolor* from the same locality.

*Frequency of mismating in south-central Missouri.*—In Table 1, we summarize our identifications of frogs found in amplexus in ponds where males

TABLE 1. FREQUENCY OF MISMATING BETWEEN *Hyla versicolor* AND *H. chrysoscelis* IN SYNTOPIC POPULATIONS IN MISSOURI. All frogs were identified by flow cytometry, and most males were also identified by their advertisement calls.

| Locality | Pairs *H. versicolor* | Pairs *H. chrysoscelis* | Mismated pairs |
|---|---|---|---|
| Laclede County | | | |
| 1989 | 7 | 4 | 0 |
| 1990 | 17 | 16 | 1 |
| 1991 | 7 | 10 | 2 |
| 1992 | 7 | 2 | 2 |
| Phelps County | | | |
| 1989 | 5 | 12 | 0 |
| 1990 | 8 | 5 | 0 |
| 1991 | 3 | 13 | 0 |
| 1992 | 5 | 35 | 0 |
| Webster County | | | |
| 1989 | 11 | 3 | 0 |
| Crawford County | | | |
| 1992 | 4 | 2 | 0 |
| Totals | 74 | 102 | 5 |

of both species called at the same time and place. Mismated pairs were found in both of the ponds in Laclede County. In four of the five mismated pairs, the female was a *H. versicolor*. During most years, we estimate that there were approximately equal numbers of males of both species at one of the Laclede County ponds; *H. chrysoscelis* was somewhat (about 60–70%) more prevalent at the other pond than was *H. versicolor*. There were approximately equal numbers of males of both species in the ponds in Phelps County during 1990 and 1991, but males of *H. chrysoscelis* were more common in 1992. The three mismated pairs represent 2.8% of the pairs that we identified from all six syntopic populations in Missouri. At the two ponds in Laclede County, mismated pairs constituted about 6.6% of the 75 pairs. The putative hybrid (see below) recorded by M. Rapp and reported by Gerhardt (1982) was also found in Laclede County at a locality about 14 km east of the two ponds at which we found mismated pairs.

*Acoustic analyses.*—We show oscillograms of the calls of the confirmed interspecific hybrids and parental individuals recorded at the same time and place in West Virginia in Figure 2. In Table 2, we present a summary of our measurements of some temporal properties of these vocalizations, those of putative hybrids reported by Zweifel (1970) and Gerhardt (1982), and those of males of the parental species recorded at the same times and places. The pulse rates of the confirmed and putative hybrids were intermediate with respect to those of the parental species. Notice that even in the calls of the hybrids the variability of pulse rate is low (see Gerhardt, 1991, for extensive data demonstrating the low variability of pulse rate within and between individuals of *H. versicolor*). We plot pulse rate as a function of temperature for all of our recordings from West Virginia in Figure 3. Analyses of the calls of more than 100 frogs from Laclede County (see below) confirm that the values reported for the representative males of the parental species are typical for the temperatures at which the calls were recorded. Our estimate (Table 2) of the pulse rate of the putative hybrid recorded by Zweifel (1970) is within 2% of the value he reported.

The analyses of pulse shape and rise time sug-

TABLE 2. TEMPORAL AND SPECTRAL PROPERTIES OF THE ADVERTISEMENT CALLS OF CONFIRMED AND PUTATIVE HYBRIDS BETWEEN *Hyla versicolor* AND *H. chrysoscelis* COMPARED WITH THOSE OF REPRESENTATIVES OF THE PARENTAL SPECIES.

| Locality | Species | Temperature (C) | Mean (SD) pulse rate (pulses/sec) | Mean (SD) pulse shape (time: 50%/100% peak) | Mean (SD) rise time (time to peak/dur.) | Dominant frequency (Hz) | Secondary peak (Hz, dB) |
|---|---|---|---|---|---|---|---|
| West Virginia | *H. chrysoscelis* | 20.8 | 46.2/sec (0.4) | 0.30 (0.07) | 0.29 (0.07) | 2212 | 1190 (−3) |
| | Hybrid No. 1 | 20.8 | 30.0/sec (1.1) | 0.50 (0.10) | 0.72 (0.08) | 1888 | 930 (−1) |
| | Hybrid No. 2 | 19.6 | 30.7/sec (0.3) | 0.54 (0.09) | 0.62 (0.04) | 1988 | 1020 (−10) |
| | *H. versicolor* | 19.7 | 19.8/sec (0.2) | 0.50 (0.05) | 0.67 (0.03) | 1850 | 990 (−14) |
| Missouri | *H. chrysoscelis* | 21.4 | 51.7/sec (0.1) | 0.30 (0.03) | 0.36 (0.02) | 2460 | 1330 (−10) |
| | Putative hybrid | 21.2 | 32.2/sec (0.7) | 0.55 (0.06) | 0.55 (0.02) | 2360 | 1250 (−15) |
| | *H. versicolor* | 21.0 | 22.3/sec (0.3) | 0.53 (0.03) | 0.63 (0.02) | 2450 | 1220 (−7) |
| New Jersey | *H. chrysoscelis* | 23.7 | 45.3/sec (0.2) | 0.45 (0.04) | 0.38 (0.04) | 2360 | 1220 (−7) |
| | Putative hybrid | 24.6* | 37.9/sec (0.1) | 0.32 (0.04) | 0.49 (0.04) | 2640 | 1390 (−11) |

* This temperature measurement could have been an error. See the text.

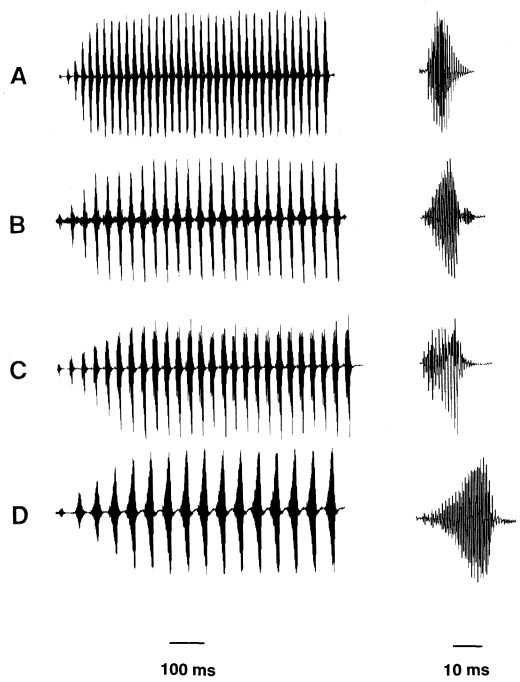


Fig. 2. Oscillograms of advertisement calls of gray treefrogs and hybrids recorded 8 June 1990 at Pipestem, Summers County, West Virginia. (A) *Hyla chrysoscelis,* recorded at 20.8 C; (B and C) two confirmed triploid hybrids, recorded at 20.2 C and 20.8 C, respectively; (D) *H. versicolor,* recorded at 21.0 C. In the first column, we show one complete call. Notice that the interpulse interval (reciprocal of pulse rate) of the hybrid calls is intermediate in comparison with that of the parental species. In the second column, we show, with an expanded time base, one typical pulse from the call. Note that the pulses of *H. versicolor* and the hybrids have a nearly linear form of rise that reaches peak amplitude after the pulse is more than half completed. The pulse of *H. chrysoscelis* has a logarithmic form of rise, and peak amplitude occurs before the pulse is half completed (see also Table 2).

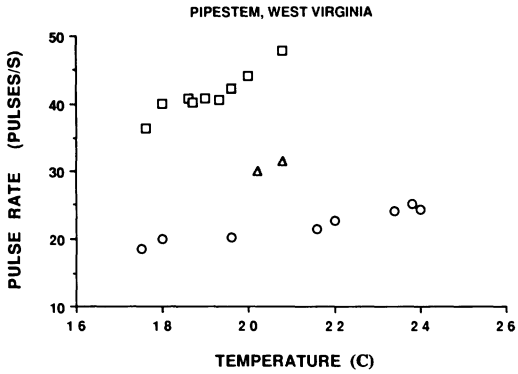


Fig. 3. Pulse rate of advertisement calls of nine males of *Hyla chrysoscelis* (squares), two confirmed hybrids (triangles), and eight males of *H. versicolor* (circles) plotted against temperature. Frogs were recorded in Summers County, West Virginia on 8–9 June 1990. No error bars for the pulse rate estimates of each frog are shown because the variability from call to call is very low (see Table 2; Gerhardt, 1991).

gest that the putative hybrid from Missouri was an interspecific hybrid. As in the calls of confirmed hybrids, the pulses of this individual had a nearly linear form and reached full amplitude only after the pulse was more than 50% completed. The individual identified as a possible hybrid by Zweifel (1970) was almost certainly a *H. chrysoscelis.* The form of its pulses was distinctly logarithmic (Fig. 4); and, although the time to reach full amplitude was longer than that of the reference specimen of *H. chrysoscelis,* the value was well within that reported elsewhere for this species (Gerhardt and Doherty, 1988; Note: the species labels in the fig. 1 of that paper were mistakenly reversed). We speculate that Zweifel made an error in measuring the temperature of the frog. The air temperature was recorded as 23.7 C for another gray treefrog recorded earlier (8:40 pm) in the evening; the water temperature was measured as 23 C for a leopard frog (*Rana pipiens*) recorded immediately before the putative hybrid (recorded at 9:20 pm) and as 19 C for a cricket frog (*Acris crepitans*) recorded immediately afterward (9:30 pm). It seems unlikely that the body temperature of the putative hybrid (reported as 24.6 C) would be significantly higher than either the air or water temperature. Finally, Zweifel (1970) found only individuals of *H. chrysoscelis* at the Cold Spring locality.

## Discussion

We have established for the first time that natural hybridization occurs between the cryptic diploid-tetraploid pair of gray treefrogs. Both hybrids were triploids and had a mtDNA sequence that was identical to that of a *H. versicolor* and differed from that of a *H. chrysoscelis* from the same population. Hence, these individuals could not have been triploid *H. chrysoscelis* which, as hypothesized by Bogart and Wasserman (1972), may occur as an intermediate stage in the polyploid speciation of *H. versicolor.*

Our surveys of mated pairs in breeding sites in sympatry provide only the second estimate of the frequency of mismating between a pair of treefrog species. Schlefer et al. (1986) found that five (11%) of 45 pairs of *H. cinerea* and *H.*

*gratiosa* were mismated in disturbed sites in Alabama. All females in the mismated pairs between individuals of the two species were *H. gratiosa*. In five other pairs, one of the male frogs was identified as a hybrid or backcross product; three of the females in these pairs were *H. gratiosa* and one was *H. cinerea*. Whereas these authors studied a site where introgressive hybridization had been documented for many years, our samples were from ponds at which not even a single putative hybrid has been found.

Only two putative and two confirmed hybrids between the two gray treefrog species have been detected by their advertisement calls, even though there has been extensive sampling of calls conducted by several research groups (see above). We have analyzed additional recordings (246 males of *H. chrysoscelis* and 162 of *H. versicolor*) of both species from syntopic populations throughout the eastern United States since Gerhardt's (1982) report. The current total count is 658 males of both species; the three adult hybrids thus represent about 0.45% of the total. This percentage is almost certainly at least an order of magnitude higher than the true percentage, given that the number of frogs recorded in syntopic populations was a small fraction of the numbers that were unequivocally identified by ear as either *H. chrysoscelis* or *H. versicolor* by ourselves and other researchers who can confidently distinguish between the calls of the two species. As discussed below, hybrids produce distinctive calls that are unlikely to be mistaken with those of either parental species.

We identified 62 males of *H. chrysoscelis* and 48 of *H. versicolor* from Laclede County, Missouri, from recordings. Combining these totals with those from flow cytometry, there were 99 adults of *H. chrysoscelis* and 90 of *H. versicolor*. The single adult (putative) hybrid represents about 0.5% of the 189 males. Again this is certainly a highly exaggerated percentage.

In view of the relatively high incidence (6.6%) of mismating in the two ponds in Laclede County, we suggest that most of the offspring of mispaired frogs fail to reach sexual maturity. Much of the mortality is expected because of the reduced viability of hybrids that Johnson (1959, 1963) and Ralin (1976) documented in laboratory crosses; these authors found that only 16.8–43.5% of the hybrid tadpoles survived more than two weeks. However, we cannot rule out the possibility that environmental selection in later larval stages and after metamorphosis also increases mortality among the hybrids that develop normally. Schlefer et al. (1986) concluded that environmental selection increased the mortality of hybrids between *H. cinerea* and *H. gratiosa*, which are genetically compatible.

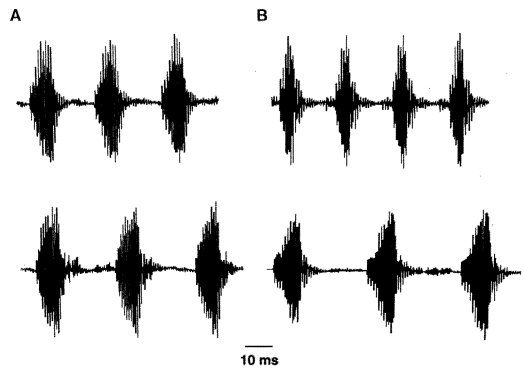


Fig. 4. Oscillograms of representative pulses from the advertisement calls of a (A) *Hyla chrysoscelis* (upper trace) recorded at 23.7 C and a putative hybrid (lower trace) recorded at 24.6 C. Both frogs were recorded on 20 May 1967, at the same locality in Cape May County, New Jersey (Zweifel, 1970; American Museum of Natural History Tape No. 150, cuts 3 and 5, respectively); (B) *H. chrysoscelis* (upper trace) recorded at 21.4 C and a putative hybrid (lower trace), recorded at 21.2 C. Both frogs were recorded on 29 May 1980, at the same pond in Laclede County, Missouri. Notice that the pulses from the calls of *H. chrysoscelis* and the putative hybrid from New Jersey have a distinctly logarithmic form of onset, whereas those of the putative hybrid from Missouri have a nearly linear or slightly exponential form of onset. See also Table 2.

Phylogenetic analyses of mtDNA sequences of cytochrome *b* from throughout the distribution of the gray treefrog complex indicate that the tetraploid *H. versicolor* has arisen independently at least three times from *H. chrysoscelis* (Ptacek, 1991; Ptacek et al., in press). The *H. versicolor* from Missouri and West Virginia belong to different lineages. However, both of these areas involve secondary contact (sympatry) with *H. chrysoscelis*. That is, the *H. chrysoscelis* with which the tetraploids are currently sympatric is not the same lineage of *H. chrysoscelis* from which they arose. Future studies should compare the frequency and direction of mismating in other contact zones, where individuals of *H. versicolor* occur with diploids of the same lineage from which they were derived.

Our results indicate that studies of reproductive character displacement in *H. chrysoscelis* and *H. versicolor* will be worthwhile. That is, the ploidy change that results in speciation may affect call structure and female selectivity as suggested by Bogart and Wasserman (1972) and Ralin (1977), but the change does not ensure perfect species discrimination. Mating mistakes must,

therefore, constitute a rather important selective force on female selectivity, male calls or both in some areas, and such selection is likely to have been a significant factor in the past. Indeed, Ptacek (1992) provides evidence for character displacement in the choice of calling positions in the two species in south-central Missouri; she also speculates that the higher incidence of mismating in the two ponds in Laclede County could reflect her observations that males of both species tended to call from less elevated sites in those ponds than in other ponds.

The advertisement calls of the confirmed and putative hybrids recorded in the field differed significantly from those recorded by Mable and Bogart (1991). These authors raised an unspecified number of triploid hybrids from laboratory crosses and reported summary statistics based on acoustic analyses of an unspecified number of calls. Hybrids had a mean pulse rate of 23.73 pulses/sec at an air temperature of 22 C, compared with 22.02 pulses/sec for field-recorded *H. versicolor* and 37.77 pulses/sec for field-recorded *H. chrysoscelis*. The mean duration of the calls of the hybrids was significantly shorter than those of either parental species, and their spectral structure differed in that the dominant frequency of the lower band was intermediate with respect to those in the calls of the parental types. The frequency of the upper band was similar to that in *H. chrysoscelis*. Finally, a sonagram of one of the calls of a hybrid showed highly irregular timing in the pulses making up the call.

As shown above, the calls of the natural hybrids we recorded were distinctly intermediate in pulse rate, and the pulses were regularly spaced as in the calls of the parental forms. The duration and the spectral structure of the calls of the parental species are influenced by chorus density and body size, respectively (Gerhardt, 1991). Values of these two properties overlap broadly between the two species (Gerhardt, 1982; Ralin, 1977), and the calls of the confirmed hybrids that we analyzed had values that were well within the same ranges of variation. Whereas we recorded all frogs in the field, Mable and Bogart (1991) recorded hybrids in aquaria and parental types in the field. We have observed that gray treefrogs confined in highly reflective acoustic environments produce abnormally short calls. Moreover, gray treefrogs raised in the laboratory, or even recorded in the laboratory after being maintained there for a few months, produced calls with pulse rates that were somewhat lower than those of males calling in the field at the same temperature (e.g., Burger, 1980; Doherty and Gerhardt, 1984). Proper comparisons of the calls of hybrids and parental individuals require that all recordings be made under the same conditions, i.e., in the laboratory (preferably in semianechoic conditions) or in the field.

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