Parallel changes in mate-attracting calls and female preferences in autotriploid tree frogs

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For polyploid species to persist, they must be reproductively isolated from their diploid parental species, which coexist at the same time and place at least initially. In a complex of biparentally reproducing tetraploid and diploid tree frogs in North America, selective phonotaxis—mediated by differences in the pulse-repetition (pulse rate) of their mate-attracting vocalizations—ensures assortative mating. We show that artificially produced autotriploid females of the diploid species (*Hyla chrysoscelis*) show a shift in pulse-rate preference in the direction of the pulse rate produced by males of the tetraploid species (*Hyla versicolor*). The estimated preference function is centred near the mean pulse rate of the calls of artificially produced male autotriploids. Such a parallel shift, which is caused by polyploidy *per se* and whose magnitude is expected to be greater in autotetraploids, may have facilitated sympatric speciation by promoting reproductive isolation of the initially formed polyploids from their diploid parental forms. This process also helps to explain why tetraploid lineages with different origins have similar advertisement calls and freely interbreed.

Keywords: sympatric speciation by polyploidy; reproductive isolation; acoustic signal preference;
tree frog; *Hyla chrysoscelis*; *Hyla versicolor*

1. INTRODUCTION

Speciation by polyploidy has generated enormous organismal diversity in plants [1] and also played a significant role in the early evolution of vertebrates [2]. Among modern vertebrates, polyploid speciation has occurred relatively recently in diverse groups of sexually reproducing fish and anuran (frogs and toads) amphibians [3]. As in plants, there is evidence that some polyploid species are autopolyploids (products of whole genome duplication), but most polyploids arise through hybridization (allopolyploidy) [3]. Either process can occur multiple times [1,3–5]. Regardless of the mode or mechanism, newly formed polyploid lineages must initially coexist with their diploid parental forms, which are likely to be present in much larger numbers. To become established, it is thus crucial that polyploid individuals remain reproductively isolated from parental individuals. Assortative mating by ploidy, which occurs in both plants and animals [6,7], could provide such an isolating mechanism. Could a shift to a higher ploidy level automatically result in changes in individuals of both genders that contribute to this function?

In frogs, cryptic species pairs with different ploidy levels often cannot be distinguished by external morphology but differ in the fine-scale temporal properties of the male advertisement (mate-attracting) call [8,9]. Differences in such calls first led to the discovery of a diploid–tetraploid complex of North American gray tree frogs [8,10], and playback experiments showed that females of both ploidy types show strong (intensity-independent) preferences for conspecific signals based on such differences [7].

Moreover, a previous study of artificially produced auto-triploids of the diploid species in this complex (*Hyla chrysoscelis*; $2n = 24$) found that the pulse rate of their calls shifted in the direction of the lower values of the tetraploid species (*Hyla versicolor*; $4n = 48$) [11]. This result and a comparable study [12] of a Japanese tree frog (*Hyla japonica*) provide unequivocal evidence that polyploidy *per se* can affect behaviourally significant call properties. The potential for automatic prezygotic isolation would be greatly enhanced if female preferences were to show a corresponding shift [8]. Here, we report the results of playback experiments with autotriploid females of *H. chrysoscelis* that demonstrate such a parallel shift in preference. These immediate effects of polyploidy *per se* (most likely the increase in cell size) on the communication system almost certainly contributed to the origin of reproductive isolation of wild-type (WT) individuals of the two species. Such effects may also help to explain the fact that multiple lineages of naturally occurring tetraploids with different, independent origins have calls with similar pulse rates and interbreed extensively [5,13]. The genetic incompatibility between *H. chrysoscelis* and *H. versicolor* further serves to select against mismatings in areas of current overlap [14].

2. MATERIAL AND METHODS

(a) *Generation of autotriploids*

Amplectic *H. chryscoscelis* pairs were collected from southern Missouri, USA during the summers of 2006–2008. Pairs were allowed to oviposit and fertilize eggs naturally. About 5–10 min after fertilization, clutches of eggs were removed and submerged in 0–3°C water and ice to prevent exclusion of the second polar body [15]. After cold-shocking, clutches of eggs were then transferred to individual containers until

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they hatched into tadpoles; dead embryos (approx. 50%) were removed daily. Tail tip clips from small haphazardly sampled numbers of tadpoles from each treatment were karyotyped (method 1 below) to ensure that a large proportion of individuals were triploids. Tadpoles were transferred to outdoor 800 l cattle tanks, where they metamorphosed at about 28–45 days after fertilization. Metamorphs were transferred to the laboratory, where they were maintained individually at 20–30°C and ambient humidity, first in 16 oz containers where they were fed fruit flies and crickets 4–7 days per week (less than 1 year old) and then in 77 oz containers where they were fed crickets 2–4 days per week (more than 1 year old). Females attained sexual maturity (indicated by size and visibility of eggs through the semi-transparent skin in the inguinal area) at about 22 months after fertilization.

(b) *Karyotyping*

Method 1: tail tip. A subset of tadpoles was karyotyped to verify that the cold-shocking procedure produced both triploids and diploids. A 4-mm section of the tail tip was removed and placed in a colchicine solution (31 μg ml^{-1}) for 1 h, and then placed in distilled water for 1 h. The tail tip was squashed in a drop of 70 per cent acetic acid and chromosomes counted under phase contrast on a compound microscope at 600× magnification.

Method 2: cell culture. On day 1, frogs were subcutaneously injected with 0.05 ml phytohaemagglutinin (PHA) and fasted. On day 3, whole blood was sampled by cardiocentesis, cultured in supplemented 50 per cent Liebovitz L-15 medium for 6–10 h, and subsequent cell-fixation and slides made following Wiley & Little [16], except no mitogens were added to the culture medium, as PHA was provided *in vivo*.

Method 3: flow cytometry. Whole blood was sampled as above, fixed in 95 per cent ethanol and analysed via flow cytometry on a BD FACScan following procedures described by Ptacek *et al.* [4].

(c) *Synthetic stimuli synthesis*

Each signal had a spectrum consisting of two spectral peaks (1.2 and 2.4 kHz; the amplitude of the 1.2 kHz component was 6 dB less than that of the 2.4 kHz component). The amplitude–time envelope of pulses was shaped to resemble that of natural advertisement calls; such signals have been found to be as attractive as pre-recorded natural calls [7]. Pulse duration was adjusted to maintain the pulse duty cycle at 50 per cent, and pulse number was adjusted so that the total call duration of alternative stimuli was as close as possible to being equal (figure 1). Two alternatives were combined in stereo files so that each stimulus occupied a separate channel, and there were equal periods of silence between the end of one alternative and the beginning of the other alternative. Additional details are provided by Gerhardt [7].

(d) *Playback experiments*

Females were treated with progesterone and prostaglandin to induce phonotactic behaviour (details in Gordon & Gerhardt [17], where it was demonstrated that there were no differences in selectivity between hormone-induced phonotaxis and that of reproductively active, non-treated females), and subsequently tested individually in two-speaker playback experiments at 85 dB SPL (sound pressure level in (dB) re 20 μPa) at 20 ± 2°C. The same acoustic chamber, equipment and testing methods were used to assess the pulse-rate preferences of females of both WT species [7]. Briefly, females were released remotely from an acoustically
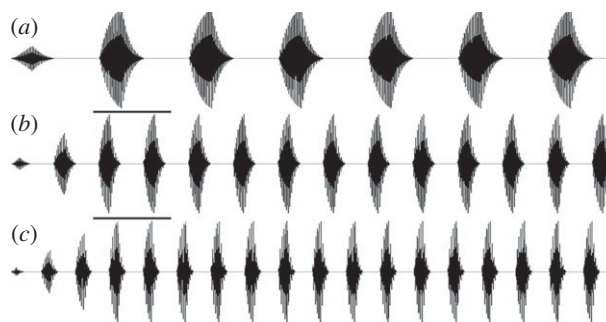

Figure 1. Oscillograms of three of the seven test stimuli used in two-choice experiments, which varied in pulse rate. (*a*) Synthetic stimuli of 20 pulses per second (pps); (*b*) stimuli of 40 pps; (*c*) stimuli of 60 pps. The pulse duty cycle (ratio of pulse duration to the pulse period), which changes little in natural calls produced at different temperatures, was held constant between stimuli at 50%. Details are presented in Gerhardt [7]. Scale bar, 100 ms.

transparent cage located midway between two speakers that were 2 m apart, and monitored using an infrared-sensitive video system. A response was scored when the female moved to within 10 cm of one of the speakers. Acoustic stimuli consisted of three synthetic calls that had been used in the previous study [7], as well as four additional synthetic calls with other pulse-rate values that allowed us to estimate a preference function. Oscillograms of three of the test stimuli are shown in figure 1.

Females were karyotyped only after all testing sessions were completed. Because not all females responded in every test within a given session, responses were tabulated over as many as six sessions separated by at least 14 days. For additional direct comparisons of phonotactic selectivity, WT diploids from the same populations as the parents of autotriploids were tested with several of the same pairs of alternatives. Because temperature affects pulse-rate preferences [7], no choice was recorded if the test subject's body temperature departed by more than 2°C from the 20°C target. Only one response per female was recorded in any particular test; at least a 6 min elapsed between a subsequent trial of the same female using a different stimulus call. No carry-over effects have been found in such multiple tests of WT gray tree frogs (*H. versicolor*) [18].

3. RESULTS

Sexually mature females resulting from cold-shock treatments were tested in two-stimulus, forced-choice playback experiments using synthetic calls that differed in pulse rate. Subsequent karyotyping of 52 individuals that responded in at least one test confirmed that 49 individuals were autotriploids. The three females that developed from cold-shocked eggs but did not become triploid served as controls. We estimated a pulse-rate preference function from the choices of the autotriploids in tests of six pairs of alternative stimuli (figure 2a). For comparison, we show the pulse-rate preference functions of WT tetraploids and diploids in figure 2b (see electronic supplementary material, table S1 for details). Female preference functions based on pulse rate in WT frogs from Missouri are unimodal, with peaks at about 20 pulses per second (pps) and 55 pps, for tetraploids and diploids, respectively (figure 2b) at 20°C [7]. The pulse-rate
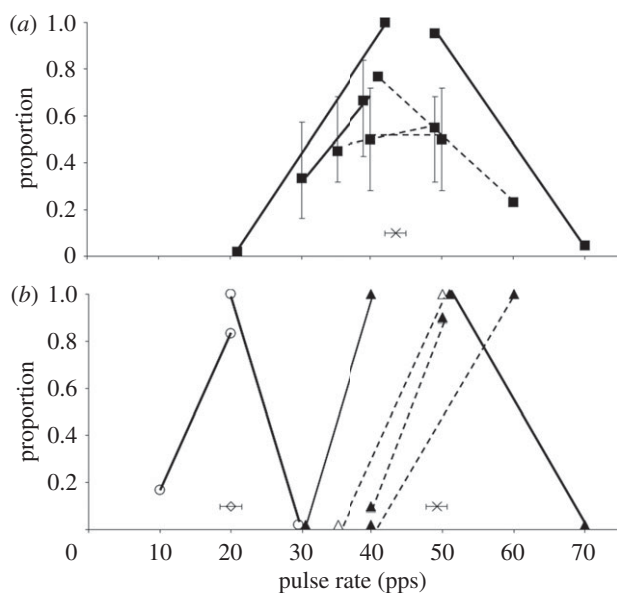
Figure 2. Shift in preference function caused by autotriploidy *per se*. (*a*) Pulse-rate preference function estimated for auto-triploid *Hyla chrysoscelis*. (*b*) Preference function estimates for wild-type *H. chrysoscelis* (filled triangles, new data; open triangles, data from Gerhardt [7]) and *Hyla versicolor* (open circles, data from Gerhardt & Doherty [18]). Each line connects points showing the proportion of females choosing each alternative; dashed lines in both panels highlight significantly different responses between (*a*) autotriploids and (*b*) diploids (filled triangles and open triangles). Error bars are 95% credible intervals (numerically equal to confidence limits because we assumed a uniform prior), for tests in which the responses were not significantly different than random. In all other tests, preferences were statistically significant ($p < 0.05$, two-tailed binomial; table 1). Below the preference functions are symbols indicating the mean pulse rate of the calls of autotriploid and wild-type *H. chrysoscelis* (X in (*a*) and (*b*), respectively), and *H. versicolor* (open diamonds, *b*) [11,18] at 20°C; error bars are s.d.

preference function of autotriploids is also unimodal, but flatter than in either WT species (figure 2*a*). Whereas WT females of *H. chrysoscelis* strongly preferred a call with a pulse rate of 50 pps (close to the mean population value of 49.2 pps) to alternatives of 35 and 40 pps (figure 2*b*), female autotriploids failed to show a preference in these two tests (figure 2*a* and table 1). More significantly, autotriploids preferred an alternative of 40 pps to an alternative of 60 pps, whereas WT *H. chrysoscelis* showed the opposite preference (table 1). The three diploid controls made the same choices as naturally occurring diploids (electronic supplementary material, table S1). A previous study found that the mean pulse rate of the calls of autotriploid males at 20°C was about 42 pps [11].'

4. DISCUSSION

How might our results concerning the effects of polypoidy *per se* bear on polyploid speciation in the gray tree frog complex and other polyploid speciation events? Two general mechanisms are responsible for speciation by polyploidy. Autopolyploids arise when alterations in meiosis result in unreduced gametes. Allopolyploids are hybrids that retain the diploid complements of both parental forms. Most polyploid species have an even number

of chromosome sets (tetraploids or octaploids, but see Stöck *et al.* [19]). On the one hand, some authorities suggest that there is a 'triploid bridge' to even-numbered chromosome sets [20], and Japanese researchers created autotetraploids of several species by crossing autotriploids created through cold-shock to WT diploids [12,21]. Rapid drops in temperature ('cold-shocks') occur frequently during the early breeding season within the range of gray tree frogs [3], and this vulnerability of large numbers of externally fertilized eggs laid in fresh water has been noted as support for this mechanism in fishes and anuran amphibians [3]. Our demonstration that autotriploid females fail to reject some calls with pulse rates within the range of variation of WT diploids adds to the plausibility of this scenario. On the other hand, recent evidence based on the analysis of mitochondrial and nuclear DNA indicates that the tetraploid lineages of *H. versicolor* arose as allopolyploids involving ancestors of *H. chrysoscelis* and two extinct taxa [5]. Further support for this mechanism stems from the fact that hybridization in fishes and anuran amphibians is common and often increases the frequency of unreduced gametes [3].

Regardless of the mechanism of speciation, the fact that calls and preferences in artificially produced autopolyploid frogs both shift in the direction of WT polyploids shows that changes associated with polyploidy *per se* can contribute automatically to species isolation. The most likely proximate (general) cause is the well-documented direct relationship between ploidy level and cell size, which occurs in both autopolyploids and allopolyploids [3,8,11,22]. Indeed, a telling result from a previous study was the fact that pulse rate did not shift from that of diploid controls in three male autotriploids of *H. chrysoscelis* whose cell size also failed to increase [11]. Nevertheless, because different tissues and systems control vocalization and auditory pattern recognition, respectively, we had no *a priori* reason to expect the parallel change documented here. Our results should thus inspire studies of the specific proximate mechanisms affected by changes in cellular dimensions caused by polyploidy.

Despite the fact that the different, independently derived lineages of *H. versicolor* involved hybridization, the calls of all three lineages have the same basic structure: trains of pulses with the same shape (slow, linear rise time) and duration, and two spectral peaks of similar frequency and relative amplitude [5,18]. Pulse rate, a key quantitative call trait, varies geographically but the largest difference between different tetraploid lineages is about 15 per cent [5]; by comparison, the maximum pulse-rate variation among populations of *H. chrysoscelis* is about 30 per cent [23]. We therefore suggest that the parental taxa involved must have been closely related and that males would have produced similar advertisement calls or at least had calls with properties that resulted in polyploid offspring with similar calls in all three tetraploid lineages. Otherwise, their present similarity would have had to be a result of differential effects of polyploidy *per se*, selection for call convergence, or both. A strong possibility is that the parental forms were lineages of *H. chrysoscelis*, a species which shows considerable phenotypic and genetic differentiation throughout its extensive range of distribution. For example, differences in the chromosome bearing the nuclear organizing region (NOR) occur among

Table 1. Results of wild-type (WT) diploid and autotriploid *Hyla chrysoscelis* female two-choice tests. Stimuli pairs used to estimate the preference function of WT and autotriploid *H. chrysoscelis*. Proportion of females responding to alternative 1 (= Alt 1) are listed, along with 95% credible intervals. n, number of females responding; χ^2-values (d.f. = 1) for 4×4 χ^2-tests of the difference of the proportions choosing Alt 1 by ploidy level. LCL, lower confidence limit; UCL, upper confidence limit.

Alt 1 (pulses per second)	Alt 2	ploidy	n	alt 1 choices (%)	χ^2-value (p)
40	20	triploid	11	100 (LCL = 76)	—
40	30	triploid	24	67 (45–84)	8.96 (<0.01)
		diploid	10	100 (LCL = 74)	
40	50	triploid	22	50 (28–72)	15.57 (<0.01)
		diploid[a]	30	3 (UCL = 17)	
40	60	triploid	13	77 (46–95)	13.61 (<0.01)
		diploid	10	0 (UCL = 26)	
50	35	triploid	31	55 (36–73)	9.18 (<0.01)
		diploid[a]	14	100 (LCL = 80)	
50	70	triploid	21	95 (LCL = 76)	0.58 (>0.30)
		diploid	10	100 (LCL = 74)	

[a]Data from previously published study Gerhardt [7].

present-day populations of *H. chrysoscelis*, and some tetraploids (*H. versicolor*) have been found with two NORs located on the same chromosomes (6p6p8p8p) as those in diploid hybrids between wide-ranging 6p and 8p NOR lineages [16,24]. Note that hybridization does not inevitably result in allopolyploidy [3].

Although the difference in pulse rate between diploid controls and autotriploids was only about 13 per cent in *H. chrysoscelis* and *H. japonica* [11,12], pulse rate in autotetraploids of the latter species showed a decrease of about 24 per cent [12]. A difference of this magnitude would have made possible call discrimination by both diploids (ancestral *H. chrysoscelis* and extinct diploid lineages) and presumably by the newly formed allotetraploids (*H. versicolor*) [7]. As discussed by Coyne & Orr [25], differences in calls alone caused by a shift to a higher polyploid level would suffice to promote successful speciation assuming that the genetic incompatibility of diploids and tetraploids would constitute strong selection against mismatings and that sufficient variation in female preference existed. While the enhanced selectivity of females of *H. chrysoscelis* in areas of sympatry with *H. versicolor* supports this argument [14], discrimination by the tetraploids against the calls of the diploid parental forms would have been immediately facilitated by shifts in pulse-rate preference such as that demonstrated in this study. Studies of autotetraploids are required to reveal if indeed there is a further shift in preference to lower values that would parallel the further shift in pulse rate expected in male calls based on the shift observed in autotetraploids of *H. japonica* [12].

In summary, we have shown that parallel changes brought about by polyploidy could have instantly facilitated the reproductive isolation of newly formed polyploid frogs from their diploid parents. Such a mechanism, probably resulting from changes in cell size, represents another path to the coupling of senders and receivers in addition to recently documented genetic mechanisms [26]. These results should also inspire future research concerned with documenting changes in cellular dimensions in neuromuscular systems and the auditory system, and their effects on calls and preferences, respectively. For example, recent neurophysiological studies suggest that temporal selectivity in the midbrain is mediated by auditory-neuron resonance, which, in turn, would be expected to be affected by cell size [27]. Finally, because changes in cell dimensions can also be caused by environmental factors during development in diploids across diverse taxa, these results may also have important implications for phenotypic alterations of communication systems in general [28,29].

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