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 reproducibly 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 autoploidoids (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 autotriploids 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. chrysoscelis 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...
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 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 cardiac puncture, 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 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 autotriplids 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 autotriplids. 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 autotriplids 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 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].
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 autoploid 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 autoploids 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 these differences in cellular 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].

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**Figure 2.** Shift in preference function caused by autotriploidy _per se_. (a) Pulse-rate preference function for autotriploid _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.

4. DISCUSSION

How might our results concerning the effects of polyploidy _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].

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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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