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E-Article

Polyploids with Different Origins and Ancestors Form a Single Sexual Polyploid Species

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abstract: Polyploidization is one of the few mechanisms that can produce instantaneous speciation. Multiple origins of tetraploid lineages from the same two diploid progenitors are common, but here we report the first known instance of a single tetraploid species that originated repeatedly from at least three diploid ancestors. Parallel evolution of advertisement calls in tetraploid lineages of gray tree frogs has allowed these lineages to interbreed, resulting in a single sexually interacting polyploid species despite the separate origins of polyploids from different diploids. Speciation by polyploidization in these frogs has been the source of considerable debate, but the various published hypotheses have assumed that polyploids arose through either autopolyploidy or allopolyploidy of extant diploid species. We utilized molecular markers and advertisement calls to infer the origins of tetraploid gray tree frogs. Previous hypotheses did not sufficiently account for the observed data. Instead, we found that tetraploids originated multiple times from extant diploid gray tree frogs and two other, apparently extinct, lineages of tree frogs. Tetraploid lineages then merged through interbreeding to result in a single species. Thus, polyploid species may have complex origins, especially in systems in which isolating mechanisms (such as advertisement calls) are affected directly through hybridization and polyploidy.

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Speciation by polyploidy has played an important role in diversification of major lineages (Ohno 1970), with some groups experiencing extensive bouts of polyploidization (e.g., bony fishes and ferns [Otto and Whitton 2000]). Although recent polyploidization events are rare in some groups such as vertebrates, polyploidization continues to be an important evolutionary force in many plant groups (White 1973; Otto and Whitton 2000). Polyploid species are formed either through genome duplication of a single diploid species (autopolyploidy) or through the fusion of two or more diploid genomes (allopolyploidy). Polyploid species are often formed recurrently. However, all known instances of allotetraploid species with multiple origins have been formed repeatedly from the same pair of diploid species (Soltis and Soltis 1999, 2000; Otto and Whitton 2000). Based on all known examples, allopolyploidization events from different pairs of progenitors are expected to result in new, isolated polyploid species (e.g., ferns [Grant 1981]; *Tragopogon* [Soltis and Soltis 1993]; salmonids [Turner 1984]; *Xenopus* [Evans et al. 2004]). This expectation was not realized in the taxa that are the focus of this study.

Gray tree frogs (*Hyla versicolor* complex) have been a central model system for studying polyploid speciation in animals (Maxson et al. 1977; Ralin and Selander 1979; Ralin et al. 1983; Romano et al. 1987; Ptacek et al. 1994). Two reproductively isolated advertisement call types differ in ploidy level (Wasserman 1970). The name *Hyla versicolor* has been assigned to the slow-trilling tetraploids and the name *Hyla chrysoscelis* to the fast-trilling diploids. Pulse rates of advertisement calls are completely nonoverlapping, with mean tetraploid pulse rates at 22.9 ($\pm$1.5 SD) pulses/s at 20°C in populations with the fastest rate and mean diploid pulse rates at 35.7 ($\pm$1.4 SD) pulses/s at 20°C in populations with the slowest rate (Gerhardt 2005; this study). Allozymes (Ralin and Selander 1979; Ralin et al. 1983; Romano et al. 1987), chromosomal location of ribosomal RNA genes (Wiley and Little 2000), immuno-

logical analysis (Maxson et al. 1977), and mitochondrial DNA sequences (Ptacek et al. 1994) have been used to support a wide variety of hypotheses concerning both the number and the nature of tetraploid origins in this group. Interpretations of the data conflict as to whether the diploid *H. chrysoscelis* represents a single or multiple species, whether tetraploids originated once or multiple times, and whether tetraploids are autopolyploid or allopolyploid. Here we show for the first time that a single interbreeding species of polyploid gray tree frogs has resulted from multiple origins of allotetraploids from three different diploid ancestors. Our results highlight the complex nature of polyploid origins and evolution.

Methods

Source Material

We studied the origins of tetraploids in the gray tree frog complex by sequencing three nuclear markers and the mitochondrial gene cytochrome *b* (cyt *b*) in 39 *Hyla chrysoscelis* and 32 tetraploid frogs from throughout their ranges (fig. 1*A*). We also sampled from seven *Hyla avivoca*, which has a restricted range in the south central United States and is the closest relative to gray tree frogs. Data from the nuclear genes rhodopsin I and recombination activation gene 1 support *H. avivoca* as the closest extant relative of gray tree frogs among all Nearctic *Hyla* (A. K. Holloway, D. M. Hillis, and D. C. Cannatella, unpublished manuscript). Other Nearctic *Hyla* (*H. femoralis, H. andersonii*, and *H. arenicolor*) were used as outgroups. Localities of samples used in analyses are provided in table A1.

Species Identification

Diploid frogs (*H. chrysoscelis*) and tetraploid frogs (*Hyla versicolor*) were distinguished in the field by mating calls. Advertisement-call pulse rate, analyzed with software designed by G. M. Klump, D. Polete, and J. Brown, was corrected for temperature to a mean of 20°C, which is near the middle of the range of breeding temperatures. The corrected pulse rates are completely nonoverlapping, allowing for unequivocal distinction between *H. chrysoscelis* and tetraploids (see mean values above).

Molecular Methods

If tetraploid gray tree frogs were formed by allopolyploidization, their nuclear genomes should contain information from both progenitors. Therefore, we used cloning techniques to recover all alleles present in polymerase chain reaction (PCR) amplifications of nuclear markers. Because there are few appropriate nuclear markers for examining relationships of closely related anurans, a cDNA library was created from a diploid gray tree frog (specimen noted in table A1). Two markers from this library, 65T and 11T, as well as the internal transcribed spacer 1 (ITS1) of the nuclear ribosomal repeat unit, were used in the following analyses. Detailed methods of library construction and the methods of DNA extraction, PCR, sequencing, and alignment of sequences are provided in the appendix. Briefly, nuclear genes were PCR amplified, and products were cloned into vectors. We directly sequenced PCR products of nuclear and mitochondrial markers and sequenced at least four colonies from cloning reactions for each nuclear marker in each individual. The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY830951–AY831027, AY831028–AY831189, AY831190–AY831384, AY833135–AY833261; table A3).

Exclusion of Recombinant Sequences

During the initial PCR amplification before cloning, partially extended products may prime another allele in the next round of amplification. To minimize the number of partial amplicons, we designed primers that amplified less than 1 kb of each marker. Chimeric sequences were identified by comparing the sequences of multiple copies of alleles from each individual; these sequences were excluded from analyses. We tested for recombination in the remaining sequences using the following methods: pairwise scanning in Recombination Detection Program (RDP), version 2b.08 (Martin and Rybicki 2000), and the Bayesian multiple-change-point model in OhBrother (Suchard et al. 2002). Both programs take multiple tests into account. The only marker with evidence for recombination was ITS1. Parsimony-informative characters were also examined by eye on the most parsimonious ITS1 tree in MacClade 4.06 (Maddison and Maddison 2000). Putative recombinant sequences (sequences with homoplasies in >15% of sites and/or sequences that were identified in RDP) were excluded. Tests for recombination on the remaining sequences using RDP indicated that no other sequences were recombinants (Martin and Rybicki 2000). Analyses using OhBrother indicated that there was reasonably strong evidence against recombination in the final data set (Bayes factor, −1.884; Suchard et al. 2002).

Phylogenetic Analyses

Phylogenetic relationships among aligned sequences were examined with Bayesian methods of inference. Before analysis, sequences with less than 1% pairwise sequence divergence were pruned from each data set to allow better visualization of the data and increased computational ef-

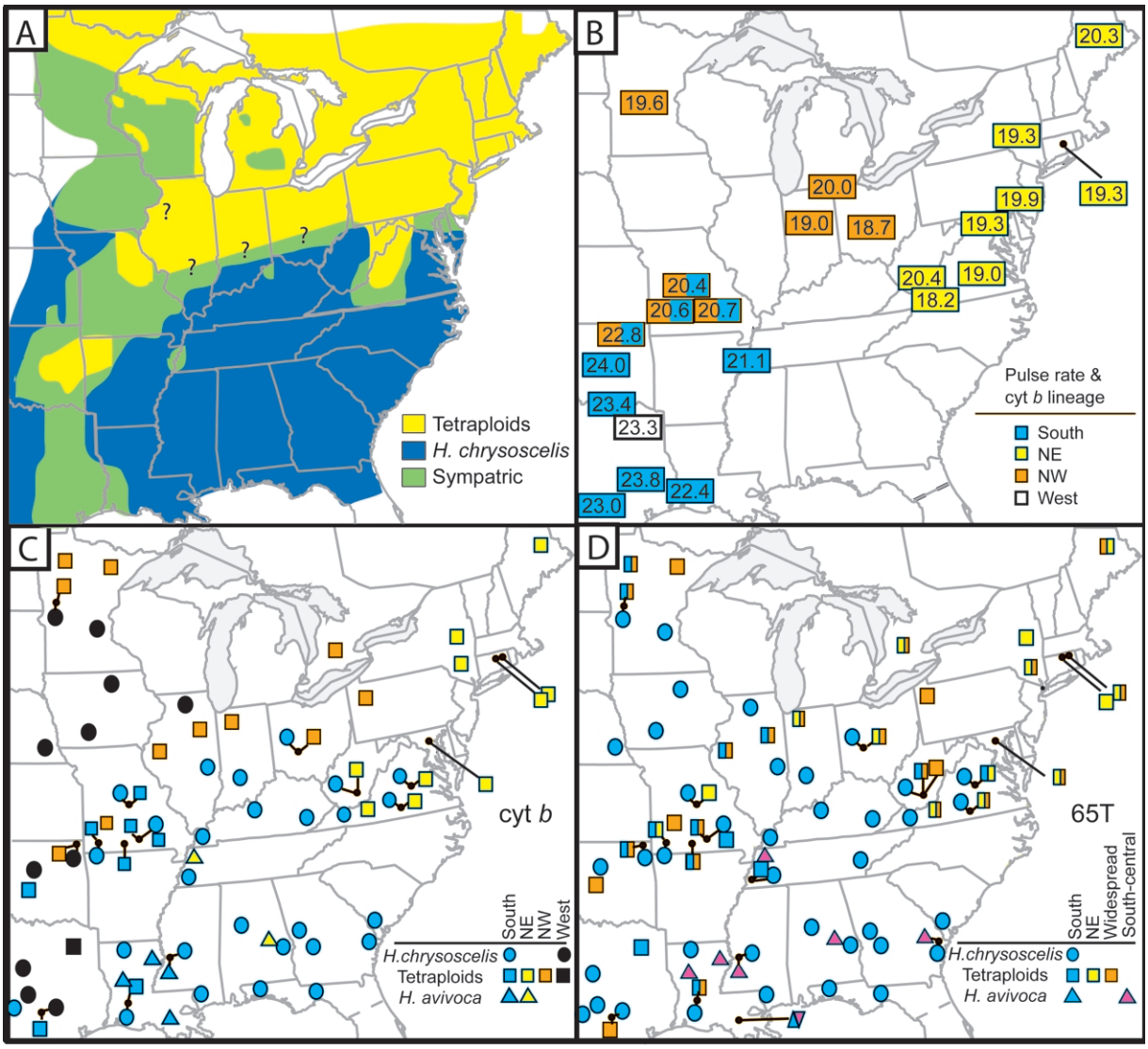

Figure 1: Geographic distribution and pulse rates of gray tree frogs and distribution of allele lineages for cyt *b* and 65T. *A*, Distribution of *Hyla chrysoscelis* and tetraploid gray tree frogs, modified from articles by Blackburn et al. (2002) and Gerhardt (1999). Exact details of the distribution are unknown in some areas of the Midwest and are denoted with a question mark. *B*, Population means of tetraploid pulse rates with colors representing cyt *b* haplotype lineages. Distribution of cyt *b* haplotype lineages (*C*) and nuclear marker 65T allele lineages (*D*) for gray tree frogs and *Hyla avivoca*. Squares with two colors represent heterozygous individuals.

ficiency due to the reduction of equally optimal topologies. Analyses using pruned and unpruned data sets did not differ in branching patterns for the deepest divergences, and computational time was drastically increased. Initial parsimony trees were used to evaluate appropriate models of evolution. Heuristic searches for the most parsimonious trees using unweighted data were conducted using 100 random taxon-addition replicates with PAUP*, version 4.0b10 (Swofford 2002). Models of evolution were evaluated with likelihood ratio tests of tree scores from parsimony trees in PAUP*, version 4.0b10 (Swofford 2002). Scripts within Nexus files were used to automate all processes in PAUP*. Phylogenetic analyses were performed in MrBayes, version 3.04b (Huelsenbeck and Ronquist 2001). We used four Markov chains at default temperature settings for each run in order to traverse a larger area of tree space and avoid entrapment in local topological optima. The chain was sampled every 100 generations for 10 million generations for ITS1 and five million generations for 65T, 11T, and cyt *b*. Bayesian posterior probabilities were

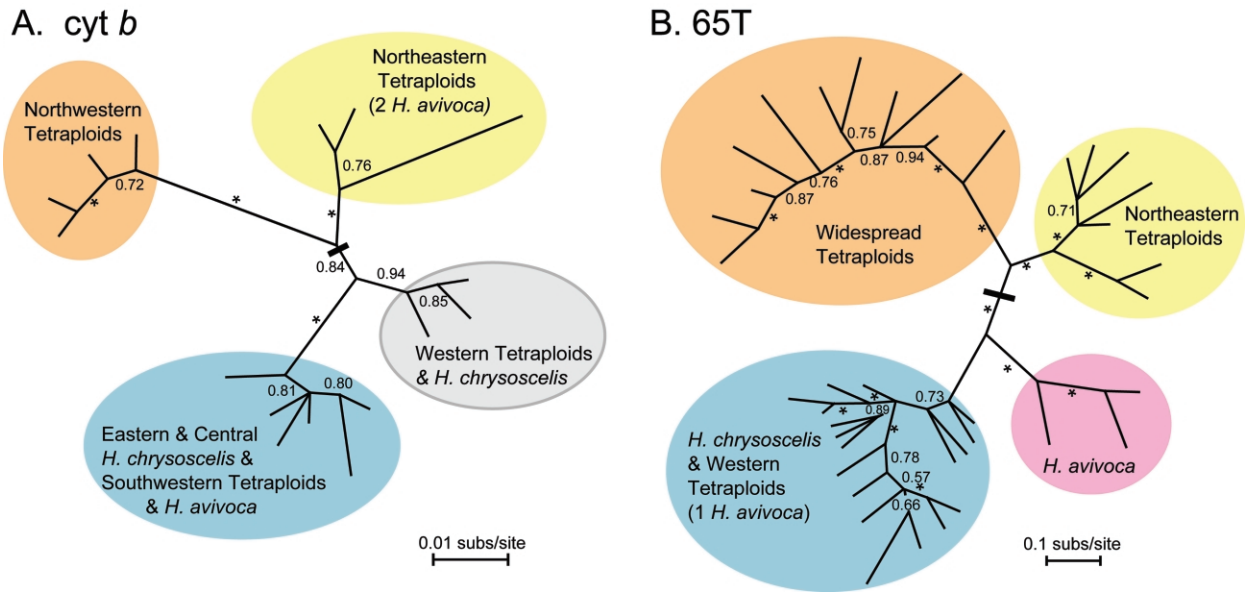

Figure 2: Bayesian inference of phylogenetic relationships of gray tree frogs and *Hyla avivoca*. *A*, cyt *b* gene; *B*, nuclear marker 65T. Posterior probabilities next to branches (asterisk represents >95% posterior probability); colors correspond to those in figure 1*C*, 1*D*. Two clades in each tree contain tetraploids but no *Hyla chrysoscelis* (orange and yellow clades). Taxa with <1% sequence divergence were excluded from analyses, but genotypes are reflected in figure 1. Black bars represent the position of the root.

estimated as the proportion of trees sampled after burn-in that contained each of the observed bipartitions (Larget and Simon 1999; Huelsenbeck and Ronquist 2001). Four separate analyses for each gene were run to examine convergence of bipartition posterior probabilities. Bipartition posterior probabilities differed by less than 5% between runs.

Expected Phylogenetic Relationships and Genotypes

Within populations, diploid frogs formed single lineages with low levels of divergence (<0.1% for 900 bp of 12S–16S mitochondrial DNA; A. K. Holloway, D. M. Hillis, and D. C. Cannatella, unpublished manuscript). Alleles of tetraploids are expected to cluster with their diploid progenitors. Therefore, if tetraploids were formed from a single autopolyploidy event, all tetraploids would form a single clade in gene trees. In contrast, multiple autopolyploidy events would produce a pattern with tetraploid clades nested within two or more diploid lineages. In any case, all nuclear alleles from an individual tetraploid frog would group together under autopolyploidy, whereas under allopolyploidy, the nuclear alleles of an individual would form two clusters, each united to a different diploid clade.

Linkage disequilibrium between marker loci would indicate autopolyploid origins because polyploids would have inherited sets of alleles from a single progenitor. Conversely, we would expect no linkage disequilibrium with allopolyploid origins. Linkage disequilibrium between marker loci was calculated as

$$D = p(A_1B_1) - [p(A_1) \times p(B_1)],$$

where A_1 is allele 1 of locus A and B_1 is allele 1 of locus B (Hartl and Clark 1997).

Results

Four well-supported and geographically distinct mitochondrial (cyt *b*) haplotype lineages (figs. 1*C*, 2*A*) were found, supporting previous findings of multiple tetraploid origins (Ptacek et al. 1994). The geographic groupings indicate the provenance of a majority of individuals from a particular lineage. Eastern and central *Hyla chrysoscelis*, southwestern tetraploids, and the majority of *Hyla avivoca* formed one haplotype lineage. Western *H. chrysoscelis* and one tetraploid formed a second haplotype lineage. A third lineage was represented only in only northwestern tetraploids and not in any *H. chrysoscelis* or *H. avivoca*. Sampling from the northeastern part of the tetraploid range revealed a fourth haplotype lineage shared with *H. avivoca* but not found in any *H. chrysoscelis*.

We recovered four allele lineages of the nuclear fragment 65T (fig. 2*B*), with one geographical group (northeast)

roughly concordant with the cyt *b* tree (fig. 1*C*, 1*D*). One allele lineage was present in western tetraploids, all *H. chrysoscelis*, and a single *H. avivoca* from Louisiana. Two additional allele lineages were present in tetraploids but were absent in *H. chrysoscelis* and *H. avivoca*; one was primarily northeastern, and the other was widespread throughout the range of tetraploids. A fourth allele was present in all *H. avivoca* but not in any gray tree frogs.

Four allele lineages of ITS1 were found (fig. 3*A*, 3*C*). One allele lineage was present in all *H. chrysoscelis*, *H. avivoca*, and some tetraploids. A second allele lineage was represented by a single *H. chrysoscelis*. A third allele lineage was composed of a single tetraploid and one *H. chrysoscelis*. A fourth allele lineage was composed entirely of tetraploids. There was no geographical concordance with other markers.

The vast majority of diploids and tetraploids, as well as one *H. avivoca*, shared a similar genotype for gene fragment 11T (fig. 3*B*, 3*D*). A different genotype was present in all other *H. avivoca*, one diploid, and one tetraploid. One *H. avivoca* was heterozygous at this locus.

The results from cyt *b*, 65T, and ITS1 support the hypothesis that diploid lineages besides *H. chrysoscelis* and *H. avivoca* contributed to tetraploid formation. The data for markers 65T and 11T indicate that gene flow between *H. avivoca* and gray tree frogs is restricted. However, we did find evidence for either occasional hybridization or persistent ancestral allele lineages in *H. avivoca*. We maintain that *H. avivoca* was probably not a major player in the formation of tetraploids, based on the genetic data and advertisement call structure. All gray tree frog advertisement calls are pulsed, 1-s-duration calls that have a bimodal frequency spectrum. Conversely, *H. avivoca* has a much longer call with a single emphasized frequency band. Natural hybrids between *H. avivoca* and *H. chrysoscelis* had much longer calls than any of the diploid or tetraploid gray tree frogs (Gerhardt 1974*b*), so tetraploids with a *H. avivoca* parent would be expected to have distinctly longer calls if call structure in contemporary frogs has been conserved.

Discussion

Our analyses support multiple events of allopolyploidization with subsequent reticulation of tetraploid lineages. One line of evidence comes from the geographic distribution of alleles. If tetraploids were autopolyploids, we would expect large geographic areas of homozygous genotypes among the tetraploids, reflecting origins from diploid parents of the same genotype. As illustrated in figure 1*D*, this is not the case; most tetraploid frogs are heterozygous, reflecting parentage from different allele lineages. Alternatively, subsequent mixing of autopolyploid lineages could result in heterozygous tetraploids. Under this hypothesis, we would also expect geographic concordance of allele lineages in tetraploids. However, this pattern is not observed; although the cyt *b* haplotype lineages demonstrate geographic distinction (fig. 1*C*), only one 65T allele lineage (northeast) roughly corresponds to the distribution of a cyt *b* haplotype lineage (fig. 1*C*, 1*D*). For ITS1, the two predominant allele lineages in tetraploids show geographic distinction. An autopolyploid origin seems unlikely given the lack of geographic concordance and lack of evidence of linkage disequilibrium (*D*) between marker alleles in tetraploids (cyt *b*–65T, average $D = 5.4 \times 10^{-18}$).

Evidence for three distinct genomes within tetraploids indicates that *Hyla chrysoscelis* and at least two other diploid species contributed to forming the tetraploid species. In three markers (cyt *b*, 65T, and ITS1), tetraploid gray tree frogs have allele lineages not represented by any *H. chrysoscelis* or *Hyla avivoca* (fig. 2). These allelic lineages represent a deeper level of divergence than observed among the sampled diploid frogs, which may indicate that these allelic lineages descended from separate diploid species. Geographic distribution of the cyt *b* and 65T markers strongly suggests the ancestral presence of three diploid species. Taken together, these lines of evidence suggest that extinct lineages may have contributed to the origins of the tetraploids.

A model of hybridization among all possible pairs of diploids is presented in figure 4. We hypothesize that two unknown or extinct diploid lineages occupied the northeastern and northwestern parts of the range (where no diploid gray tree frogs currently exist), participated in the allopolyploid origin of at least one lineage of tetraploids, and were subsequently replaced by them. The high level of sequence divergence among tetraploid haplotype lineages relative to divergence within other hylid species supports the species status of the two unknown lineages. Pairwise distances for cyt *b* haplotype lineages were 2%–3.5% between tetraploid haplotype lineages, which is higher than the divergence within other species of Nearctic hylids (e.g., pairwise distance for cyt *b* was <2% within species for both *Hyla andersonii* and *Hyla femoralis*; A. K. Holloway, D. M. Hillis, and D. C. Cannatella, unpublished manuscript).

Despite an allopolyploid origin from distinct and divergent diploid lineages, the extant tetraploids are not reproductively isolated from one another (Espinoza and Noor 2002). Moreover, different combinations of the alleles from the various diploids are present in extant tetraploid populations (fig. 1*C*, 1*D*). Thus, we interpret the reproductive continuity within the extant tetraploids as indicative of one species, which should continue to bear the name *Hyla versicolor*.

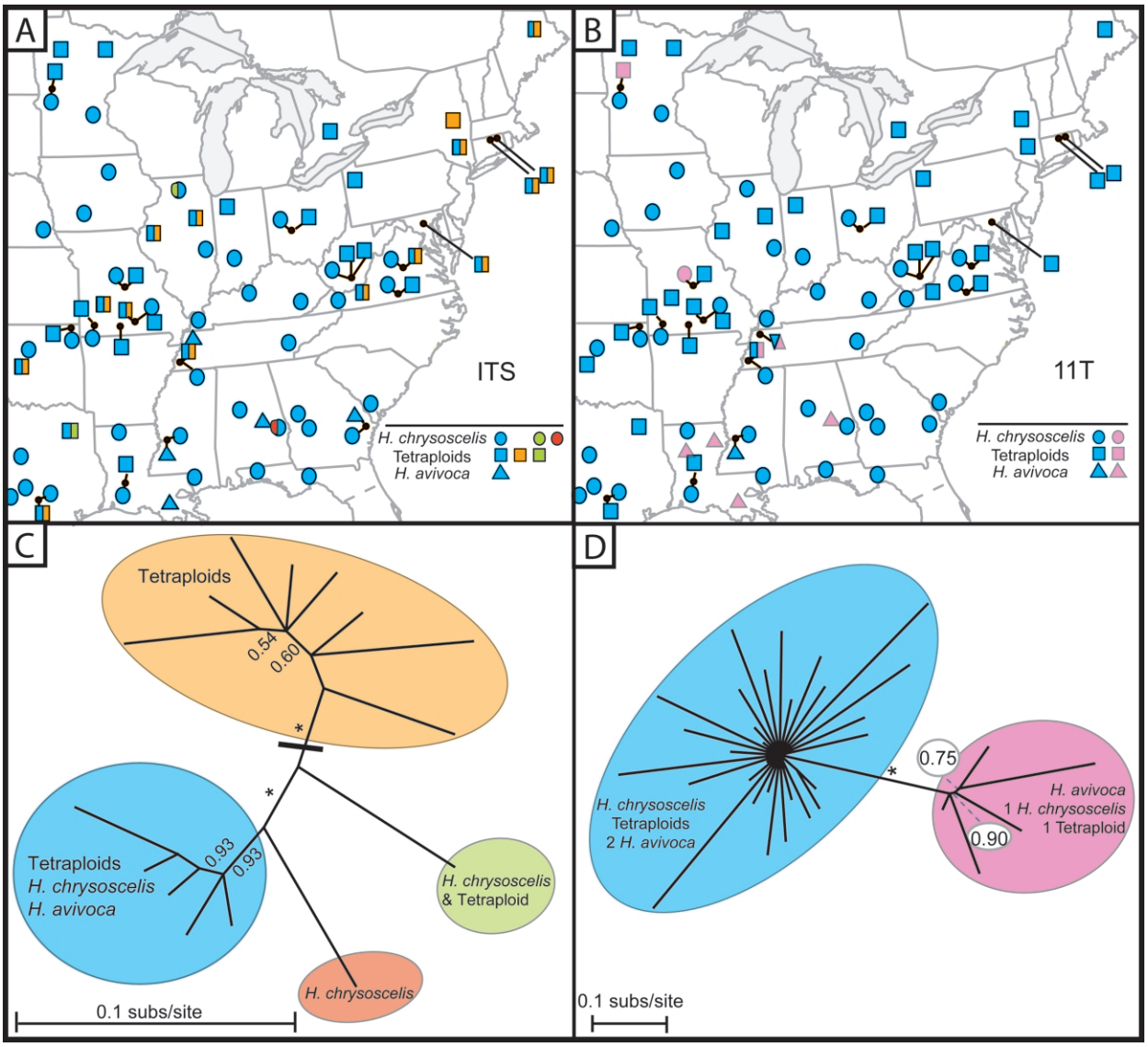

Figure 3: Distribution of ITS1 allele lineages and nuclear marker 11T allele lineages. Distribution of ITS1 allele lineages (*A*) and nuclear marker 11T allele lineages (*B*) for gray tree frogs and *Hyla avivoca*. Bayesian inference of phylogenetic relationships of gray tree frogs and *Hyla avivoca*: ITS1 (*C*), nuclear marker 11T (*D*). Posterior probabilities next to branches (asterisk represents >95% posterior probability). Black bar represents the position of the root for ITS1.

Geographic structure in tetraploid advertisement call pulse rates supports our hypothesis of multiple origins from different closely related diploid lineages with structurally similar advertisement calls. Tetraploids with southern cyt *b* haplotype lineages show a small but statistically significant difference in temperature-corrected pulse rate from the northwestern and northeastern lineages (fig. 1*B*; ANOVA results, overall $F = 836.29$, df $= 5$, $P < .0001$; NE vs. S, $F = 26.02$, $P < .0001$; NW vs. S, $F = 55.67$, $P < .0001$; NE vs. NW, $F = 2.47$, $P = .1168$; pulse rates were temperature corrected for each lineage separately). This disparity may reflect differences in the pulse rates of the diploid parental species, selection, drift, or all of these factors. Nevertheless, tetraploid females do not discriminate among these small differences, and the differences do not prevent interbreeding (Gerhardt 2005). Some tetraploid populations with intermediate pulse rates were found in southwestern Missouri and western Tennessee (fig. 1*B*). The geographic structure of pulse rates supports the ge-

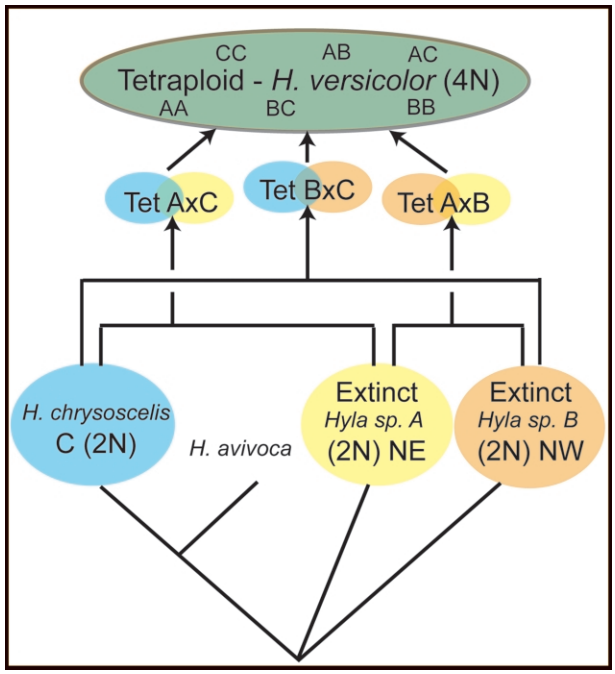

Figure 4: Model of tetraploid origins from diploid ancestors. The relationships of diploid gray tree frogs and *Hyla avivoca* are depicted in the tree at the bottom. Extinct diploids (2N), *H. sp. A* and *H. sp. B*, were inferred from tetraploid allele lineages. Tetraploid gray tree frogs, *Hyla versicolor* (4N), were formed multiple times from extinct diploid ancestors (A × B, A × C, and B × C). Tetraploid gray tree frogs now share this gene pool.

netic analysis, which suggests gene flow among lineages of tetraploids.

Multiple independent origins of reproductively compatible tetraploids from more than two diploid ancestors have not been reported, even in groups such as plants in which speciation by polyploidy is common (Grant 1981; Otto and Whitton 2000). Given that the tetraploid and diploid frogs are reproductively isolated (Littlejohn et al. 1960; Johnson 1963; Gerhardt et al. 1994), recognition and subsequent mating among the different tetraploid lineages seems counterintuitive. Premating reproductive isolation between diploids and tetraploids occurs because tetraploid pulse rates are approximately half those of the diploids, and females strongly discriminate against calls that differ by this order of magnitude from those of conspecific calls (Littlejohn et al. 1960; Johnson 1966; Gerhardt 1974*a*, 1994).

All tetraploid gray tree frogs (regardless of origins) have a slower pulse rate than *H. chrysoscelis*, suggesting that a reduction in pulse rate may be a direct consequence of ploidy level (Keller and Gerhardt 2001), a phenomenon observed in some other tetraploid frog species (Martino and Sinsch 2002). Tetraploid cells have approximately twice the volume of diploid cells, even though the adult body size is indistinguishable (Cash and Bogart 1978). This suggests a possible mechanism for mate recognition among different tetraploid lineages: polyploidization may lead to a predictable and consistent change in the advertisement call, perhaps as a direct result of increased cell size (Keller and Gerhardt 2001), which would result in automatic isolation of the tetraploids from the diploids but identification of other tetraploids as possible mates. The idea that cell size alters pulse rates is supported by two empirical studies. Autotriploid gray tree frogs and autotetraploid *Hyla japonica* had lower pulse rates than diploid controls (Ueda 1993; Keller and Gerhardt 2001). However, changes in cell size appear to be insufficient to account for all of the difference in pulse rate between *H. chrysoscelis* and *H. versicolor*. Changes in cell size coupled with reinforcement against mating with frogs of a different ploidy level may have driven the differences between *H. chrysoscelis* and *H. versicolor* to their current levels.

Our hypothesis for the origins of tetraploids resolves the apparent conflicts among previous data sets on gray tree frogs. Although allozyme data have been used to support a single origin hypothesis of the tetraploids, many tetraploid populations contained allozyme alleles that were not found in any of the extant *H. chrysoscelis* populations (Ralin and Selander 1979; Ralin et al. 1983; Romano et al. 1987), which supports our hypothesis of extinct diploid species. Studies of both immunological and mitochondrial gene data also found evidence for tetraploid populations

that were not closely related to any *H. chrysoscelis* sampled (Maxson et al. 1977; Ptacek et al. 1994). Our study of both mitochondrial and nuclear loci from a comprehensive geographic sample demonstrates that tetraploids originated from at least three diploid species, including the extant *H. chrysoscelis* and two other apparently extinct species. It is possible that extant populations of these "missing" diploid species will be discovered, but to date only tetraploid populations of gray tree frogs have been reported from northern regions where we would expect these species to have occurred (based on the present distribution of the allelic lineages).

An alternative to our hypothesis of three diploid progenitor species is that a single ancestral diploid species was present and contained all extant tetraploid alleles. Various populations of this widespread diploid gave rise to independent lineages of tetraploids. However, over time, extinction of some diploid populations reduced variability to the levels observed today. However, if this were true, the probability of finding multiple tetraploid-only alleles at every locus would be extremely low; *H. chrysoscelis* would have contained unprecedented genetic variation (compared with any other Nearctic hylid), and there would be no explanation for the strong geographic distribution of the distinct allelic lineages among the tetraploids. Therefore, our hypothesis that all pairwise combinations of three diploid lineages generated a single species of tetraploid gray tree frogs, *H. versicolor*, is the most straightforward and consistent with the data.

The multiple origins of a single, reproductively compatible tetraploid species from different combinations of three diploid ancestors is unexpected (and previously unreported). Our hypothesis is consistent with the view of a species as a reproductively connected lineage (e.g., Frost and Hillis 1990), although in this case, the sexual polyploid lineage has polyphyletic origins. The tetraploids are now interacting reproductively and share a common mate-recognition system despite their independent origins from hybridization events among different diploid species. Other examples exist of reproductively interacting lineages that have arisen in parallel, even in the absence of polyploidy (Boughman et al. 2005). Cases such as these are problematic for conceptual views of species as entities that are indistinct from clades (e.g., Mishler 1999). If only monophyletic entities were recognized as species of gray tree frogs, then either all gray tree frogs (plus *H. avivoca*) would have to be considered a single species or each of the different polyploid combinations would have to be recognized as distinct species. We view both of these options as unacceptable: the first option would not recognize sympatric, reproductively isolated populations of diploids and tetraploids as distinct species, and the second would not recognize the reproductive continuity among the different polyploid combinations.

Many tetraploids show evidence of recurrent formation, but these instances of multiple origins have always resulted from crossing of the same two diploid species, although sometimes from different populations (Bell 1982; Soltis and Soltis 1999, 2000; Otto and Whitton 2000). However, if there is a causal relationship between polyploidy and critical aspects of the advertisement call (or other features important for recognition and reproductive isolation), recognition between different allotetraploid lineages would be expected. Organisms such as ferns (Grant 1981) and African clawed frogs (Evans et al. 2004, 2005) have long, complicated histories of polyploid speciation. Resolution of the gray tree frog story adds another facet to the complex relationships between polyploids and their progenitors as well as between closely related polyploid lineages.

Acknowledgments

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APPENDIX

Detailed Methods

Library Construction

A cDNA library was created from testes tissue of gray tree frogs (PT3577-1; Clontech, Palo Alto, CA). Messenger RNA was extracted (K3064-1; Clontech, Palo Alto, CA) and reverse transcribed to produce double-stranded cDNA that was then ligated into plasmids. Plasmids were transformed into bacteria to produce colonies containing single genes. Single-gene copies were amplified and sequenced using plasmid primers (see Sequencing Protocol below). These sequences were compared to gene sequences in GenBank (Altschul et al. 1990; Boguski et al. 1993). Genes that were either very conserved or from the mitochondrial genome were discarded. For genes that either had no match or were divergent from other sequences in the database, gene-specific primers were designed and two individuals from each of four populations were sequenced

to examine variation. Two markers developed from this library, 65T and 11T, were used.

DNA Extraction and PCR Conditions

Total genomic DNA was extracted from preserved liver, muscle, or toe clips using the DNeasy extraction kit (69506; Qiagen, Valencia, CA). Thermal conditions for 25-μL PCR reactions included initial denaturation at 95°C for 2 min followed by 35 30-s cycles of denaturation at 94°C, annealing at variable temperatures for 30–45 s (see table A2), and extension at 72°C for 1 min and finally one extension at 72°C for 10 min. The nuclear ribosomal subunit, internal transcribed spacer region 1, was amplified using the Failsafe PCR Buffer G (FSP995G; Epicentre, Madison, WI). All other genes were amplified using stock buffers supplied with Taq polymerase (M0267L; NEB, Beverly, MA). For nuclear genes, a small portion of PCR products was used in cloning reactions. The remainder of the PCR product of nuclear genes and the entire PCR product from cytochrome *b* was cleaned using the Qiaquick PCR purification kit (28104; Qiagen, Valencia, CA). Concentration of clean PCR products was determined by electrophoresis on a 1% agarose gel with 0.3μg/mL ethidium bromide.

Cloning of PCR Products

Four microliters of PCR product were used in each ligation reaction. Protocols for ligation and transformation followed those described in the cloning manual (K4500-40; Invitrogen, Carlsbad, CA). Ligations were carried out for the maximum suggested time (30 min), and transformations were carried out for the minimum suggested time (5 min). Transformed products were grown overnight at 37°C on LB plates containing X-Gal (64 μg/mL) for blue/white screening and kanamycin (50 μg/mL). White colonies were picked, added directly to PCR cocktails, and amplified using the plasmid primers M13F and M13R from Invitrogen with an annealing temperature of 55°C. PCR and cleaning protocols were as described above.

Sequencing and Alignment

Ten-microliter cycle sequencing reactions with BigDye V3.1 (ABI, Foster City, CA) as the dye terminator and gene-specific or plasmid primers were carried out for 25 cycles, each with denaturation at 94°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. Dye terminators were removed with Sephadex G-50 (S-6022; Sigma, St. Louis, MO) in Centrisep columns (CS-901; Princeton Separations, Adelphia, NJ). Sequencing was performed on an ABI3100 sequencer (Applied Biosystems, Foster City, CA). Sequences were compiled in Sequencher, version 4.0 (GeneCodes, Ann Arbor, MI), and consensus sequences were exported to ClustalX (Thompson et al. 1997) for preliminary alignment. Final alignment was done by eye using MacClade, version 4.06 (Maddison and Maddison 2000). Sequences were submitted to GenBank (table A3).

Table A1: Locality information

Species, field no.	State	County
Hyla avivoca:		
MP710	Alabama	Macon
DCC3857	Georgia	Chatham
HCG21	Louisiana	Grant
H146	Louisiana	Jefferson
HCG84	Louisiana	Madison
MP607	Mississippi	Hinds
HCG28	Tennessee	Obion
Hyla chrysoscelis:		
DCC3883	Alabama	Bibb
MP458	Alabama	Russell
MP732	Florida	Leon
MP670	Florida	Okaloosa
MP002	Georgia	Chatham
MP649	Georgia	Houston
MP859	Georgia	Upson
DCC3874	Iowa	Butler
MP706	Iowa	Clarke
INHS131T	Illinois	Edgar
INHS924T	Illinois	Ogle
DCC3751	Indiana	Monroe

Table A1 (*Continued*)

Species, field no.	State	County
MP753	Kentucky	Carlisle
MP630	Kentucky	Laurel
MP452	Kentucky	Meade
MP195	Louisiana	Allen
MP847	Louisiana	Lincoln
MP632	Minnesota	Hennepin
MP802	Minnesota	Ottertail
MP773	Missouri	Barry
MP386	Missouri	Howell
MP350	Missouri	Phelps
MP724	Mississippi	Hancock
MP249	Mississippi	Hinds
MP804	Nebraska	Otoe
DCC3829	Ohio	Ross
MP329	Oklahoma	Ottawa
MP327	Oklahoma	Payne
MP205	South Carolina	Jasper
MP692	Tennessee	Monroe
MP696	Tennessee	Shelby
MP723	Texas	Bastrop
MP686	Texas	Eastland
MP647	Texas	Gillespie
MP135	Texas	Travis
MP273	Virginia	Goochland
MP816	Virginia	Mecklenberg
MP701	Virginia	Smyth
MP651	West Virginia	Summers
MP625	Ontario, Canada	Guelph
DCC3807	Connecticut	Tolland
DCC3800	Connecticut	Windham
INHS950T	Illinois	Hancock
INHS399T	Illinois	Iroquois
DCC3768	Indiana	Porter
MP162	Louisiana	Allen
DCC3823	Maryland	Ann Arundel
MP576	Maine	Penobscot
MP702	Minnesota	Clearwater
MP795	Minnesota	Ottertail
DCC3864	Minnesota	St. Louis
MP759	Missouri	Barry
MP045	Missouri	Greene
MP409	Missouri	Howell
MP370	Missouri	Oregon
MP359	Missouri	Ozark
MP524	Missouri	Phelps
DCC3787	New York	Rensselaer
DCC3795	New York	Westchester
DCC3828	Ohio	Ross
MP099	Oklahoma	Cleveland
MP793	Oklahoma	Ottawa
DCC3832	Pennsylvania	Crawford
MP700	Tennessee	Shelby
MP019	Texas	Bastrop
MP717	Texas	Smith
MP020	Virginia	Giles

Table A1 (*Continued*)

Species, field no.	State	County
MP296	Virginia	Goochland
MP809	Virginia	Mecklenberg
MP676	West Virginia	Summers
MP678	West Virginia	Summers
Outgroups:		
Hyla andersonii:		
WED5445	New Jersey	Burlington
Hyla arenicolor:		
DCC3043	Texas	…
HCG2	Arizona	Coconino
Hyla femoralis:		
DCC3858	Georgia	Chatham

Note: Asterisk denotes specimen used for construction of cDNA library.

Table A2: Gene product and PCR primers

Gene	Fragment size (bp)	Forward primers	Reverse primers	Anneal temperature (°C)
cyt *b*	712	MVZ25-L[a] or MVZ15-L[a]	CytbAR-H[a]	52
ITS1	800	18b[b]	5.8c[b]	55
65T	650	65T_149F (5′—cccagggtaaattgtccgcagta—3′)	65T_526R (5′—gttgggaaacactggtg—3′)	58
11T	304	11T_84F (5′—tggagtacccctttaaatctgaat—3′)	11T_388R (5′—ataaagtgcataagtaaaagtgaa—3′)	60

[a] cyt *b* primers are from (Goebel et al. 1999).
[b] ITS1 primers are from (Hillis and Dixon 1991).

Table A3: GenBank accession numbers

Specimen	cyt *b*	G65T	ITS	G11T
MP002	AY830951	AY831028	AY831190–191	AY833135
MP019	AY830952	AY831029	AY831192–195	AY833136–37
MP020	AY830953	AY831030–31	AY831196–198	AY833138–41
MP045	AY830954	AY831032–33	AY831199–202	AY833142
MP099	AY830955	AY831034–37	AY831203–206	AY833143–44
MP135	AY830956	AY831038	AY831207–208	AY833145–46
MP162	AY830957	AY831039–41	AY831209–211	AY833147–48
MP195	AY830958	AY831042–43	AY831212–213	AY833149–50
MP205	AY830959	AY831044–45	AY831214–215	AY833151
MP249	AY830960	AY831046	AY831216–217	AY833152–53
MP273	AY830961	AY831047–48	AY831218–219	AY833154
MP296	AY830962	AY831049–51	AY831220–223	AY833155–56
MP327	AY830963	AY831052–53	AY831224–225	AY833157
MP329	AY830964	AY831054–55	AY831226–227	AY833158–59
MP350	AY830965	AY831056–57	AY831228–229	AY833160–61
MP359	AY830966	AY831058–60	AY831230–231	AY833162–63
MP370	AY830967	AY831061–62	AY831232–235	AY833164–65
MP386	AY830968	AY831063–64	AY831236	AY833166
MP409	AY830969	AY831065–66	AY831237–239	AY833167–68
MP452	AY830970	AY831067–68	AY831240–241	AY833169
MP458	AY830971	AY831069–70	AY831242–243	AY833170
MP524	AY830972	AY831071–72	AY831244–245	AY833171–73

Table A3 (*Continued*)

Specimen	cyt *b*	G65T	ITS	G11T
MP576	AY830973	AY831073–75	AY831246–249	AY833174
MP625	AY830974	AY831076–78	AY831250–253	AY833175–76
MP630	AY830975	AY831079–80	AY831254–255	AY833177
MP632	AY830976	AY831081	AY831256–257	AY833178–79
MP647	AY830977	AY831082–83	AY831258–259	AY833180
MP649	AY830978	AY831084–85	AY831260–261	AY833181
MP651	AY830979	AY831086–87	AY831262–263	AY833182
MP670	AY830980	AY831088–89	AY831264–265	AY833183
MP676		AY831090	AY831266–268	AY833184–85
MP678	AY830981	AY831091–94	AY831269–270	AY833186
MP686	AY830982	AY831095	AY831271	AY833187–88
MP692		AY831096	AY831272–273	AY833189–90
MP696	AY830983	AY831097–98	AY831274–275	AY833191
MP700		AY831099–102	AY831276–279	AY833192–93
MP701	AY830984	AY831103–104	AY831280–281	AY833194–95
MP702	AY830985	AY831105–107	AY831282–283	AY833196–98
MP706	AY830986	AY831108–109	AY831284–286	AY8331990
MP717	AY830987	AY831110	AY831287–289	AY833201–3
MP723	AY830988	AY831111–112	AY831290–291	AY833204–5
MP724	AY830989	AY831113–114	AY831292–293	AY833206–7
MP732	AY830990	AY831115	AY831294–295	AY833208
MP753	AY830991	AY831116–117	AY831296–297	AY833209
MP759	AY830992	AY831118–119	AY831298–300	AY833210
MP773	AY830993	AY831120–121	AY831301–302	AY833211–12
MP793	AY830994	AY831122–124	AY831303–305	AY833213
MP795	AY830995	AY831125–126	AY831306–307	AY833214–16
MP802	AY830996	AY831127–128	AY831308–309	AY833217
MP804	AY830997	AY831129	AY831310–311	AY833218–19
MP809	AY830998	AY831130–131	AY831312–315	AY833220–21
MP816	AY830999	AY831132–133	AY831316–317	AY833222–23
MP847	AY831000	AY831134–135	AY831318–319	AY833224
MP859	AY831001	AY831136–137	AY831320–321	AY833225
DCC3751*	AY831002	AY831138–139	AY831322–323	AY833226–27
DCC3768	AY831003	AY831140–141	AY831324–327	AY833228–29
DCC3787	AY831004	AY831142–143	AY831328–331	AY833230–31
DCC3795	AY831008	AY831144–146	AY831332–335	AY833232
DCC3800	AY831021	AY831147–149	AY831336–339	AY833233
DCC3807	AY831005	AY831150–151	AY831340–343	AY833234
DCC3823	AY831006	AY831152–153	AY831344–348	AY833235
DCC3828	AY831007	AY831154–157	AY831349–351	AY833236–37
DCC3829	AY831022	AY831158–159	AY831352–353	AY833238
DCC3832	AY831009	AY831160–163	AY831354–357	AY833239–40
DCC3857		AY831177–178	AY831377	
DCC3864	AY831010	AY831164	AY831358–360	AY833241–43
DCC3874	AY831011	AY831165–166	AY831361–362	AY833244–45
DCC3883	AY831012	AY831167	AY831363–364	AY833246
INHS131T	AY831013	AY831168–169	AY831365–366	AY833247
INHS399T	AY831014	AY831170–172	AY831367–369	AY833248–49
INHS924T	AY831015	AY831173–174	AY831370–371	AY833250–51
INHS950T	AY831016	AY831175–176	AY831372–376	AY833252
MP607	AY831017	AY831179–180	AY831378	AY833259
MP710	AY831020	AY831181	AY831379	AY833260–61
H146	AY831023	AY831182–183	AY831380	AY833253
HCG21	AY831018	AY831184		AY833254
HCG28	AY831024	AY831185–186	AY831381	AY833255–56

Table A3 (*Continued*)

Specimen	cyt *b*	G65T	ITS	G11T
HCG84	AY831019	AY831187–188		AY833257–58
DCC3858	AY831025		AY831384	
WED54451	AY831026	AY831189		
DCC3043	AY831027		AY831382	
HCG2			AY831383	

Note: Asterisk denotes specimen used for construction of cDNA library.

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