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Cytonuclear equilibrium following interspecific introgression in a turtle lacking sex chromosomes

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When reproductive barriers break down, interspecific hybridization can lead to gene flow between evolutionarily distinct species. Studying the fate of these introgressing elements can offer valuable insights into the factors contributing to reproductive isolation. We have identified a population of false map turtles (*Graptemys pseudogeographica*) that hybridized historically with the common map turtle (*Graptemys geographica*), but were subsequently isolated from interbreeding for several generations by unique geological events. Although many studies conclude that genic interactions involving sex chromosomes impact the introgression of mitochondrial or nuclear genomes, *Graptemys* turtles have environmental sex determination, and thus introgression can be explored while controlling for the effects of sex-specific heterogameity. We identified and sequenced a species-specific mitochondrial control region marker, as well as two nuclear markers (ODC and HNFAL), in turtles from across the ranges of these species. We found both nuclear and mitochondrial introgression in our study population, and present evidence consistent with the proposed time range of reproductive contact and isolation. We also report an absence of cytonuclear or linkage disequilibrium among markers, indicating that some important pre- and postzygotic barriers to gene flow that characterize other systems are absent in *Graptemys*. Finally, we show that *Graptemys* turtles have a complex molecular evolutionary history, and that leaks in reproductive barriers probably occur frequently. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, **106**, 405–417.

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Despite molecular and morphological divergence, hybridization between animal species is a well-documented phenomenon (Wilson, Maxson & Sarich, 1974; Schwenk, Brede & Streit, 2008). Although many hybrid offspring are sterile, fertile hybrid offspring may backcross into one or both parental species, leading to the introgression of DNA from one species into the genomic background of another (Dowling & Secor, 1997; Gay *et al*., 2007; Bee & Close, 2009). Introgression is of particular interest to evolutionary biologists because it can reveal how novel genetic elements spread in a population. In particular, investigating the fate of different genomic elements following hybridization can shed important light on the forces contributing to reproductive isolation.

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Species that have been reproductively isolated for an extended time often evolve mechanisms that prevent interbreeding (prezygotic isolation; Wade *et al*., 1994; Capy *et al*., 2000) or suffer from reduced fitness of hybrids (postzygotic isolation; Sasa, Chippindale & Johnson, 1998; Behrmann-Godel & Gerlach, 2008). In the absence of these barriers to gene flow, hybridization is predicted to result in the loss of genetic distinctness between the hybridizing species and the ultimate extinction of one or both parental species (Wolf, Takebayashi & Rieseberg, 2001). In the last two decades, cases of interspecific hybridization have been documented in a wide range of turtle species (Fritz & Baur, 1994; Fritz, 1995; Karl, Bowen & Avise, 1995; Stuart & Parham, 2007). These cases are noteworthy, given the long time since divergence and the extensive morphological differentiation of the species involved. For example, although

they diverged 6–8 Mya (Lamb *et al*., 1994), hybrids between the emydid turtles *Graptemys geographica* and *Graptemys pseudogeographica* have been suspected based on phenotypic evidence (Vogt, 1978 cited in Fritz, 1995; R. C. Vogt, pers. comm.). By comparison, grey wolves (*Canis lupus*) and coyotes (*Canis latrans*), the natural hybridization and introgression of which have received considerable attention over the last two decades, diverged only two million years ago (Lehman *et al*., 1991; Roy *et al*., 1994).

Several studies have investigated nuclear and mitochondrial introgression in naturally hybridizing populations (Crochet *et al*., 2003; Sullivan *et al*., 2004). Often, substantial mitochondrial introgression occurs, whereas nuclear introgression is completely absent (reviewed in Chan & Levin, 2005). One explanation for mitochondrial introgression in the absence of nuclear introgression is that there are many more functional chromosomal than mitochondrial genes, and thus natural selection is much more likely to filter these out from the foreign background (Powell, 1983; reviewed in Rognon & Guyomard, 2003). A second explanation for limited nuclear introgression is that interactions involving the sex chromosomes cause hybrid males to suffer reduced fitness (Haldane's rule), allowing hybrid females to transmit the introgressing mitochondria at a disproportionately high rate. Because they make similar empirical predictions, it is difficult to disentangle the relative impact of these two factors on introgression. Importantly, turtles of the genus *Graptemys* exhibit environmental sex determination and lack sex chromosomes (Bull & Vogt, 1979; Ewert, Jackson & Nelson, 1994), and thus allow for an investigation of gene introgression in the two genomes without being influenced by genic interactions involving the sex chromosomes.

When multiple genetic elements introgress across species lines, they often remain in close statistical association. In particular, if the hybridization event was recent or is ongoing, introgressed mitochondrial and nuclear genes are predicted to exhibit cytonuclear disequilibrium, whereby they co-occur more often than is expected by chance (Ballard & Whitlock, 2004). In addition, cytonuclear disequilibrium will occur as a result of assortative mating among hybrids or selection against the disruption of co-adapted gene complexes (Arnold, 1993). These factors leading to cytonuclear disequilibrium (mate choice and genetic divergence) are predicted to increase with time since separation (Coyne & Orr, 1998). In systems where both mitochondrial and nuclear introgression between distinct species have been quantified, cytonuclear disequilibrium is common (Harrison & Bogdanowicz, 1997; Latta, Linhart & Mitton, 2001; Won *et al*., 2003).

Reelfoot Lake (TN, USA) was formed as a result of coseismic uplift from the New Madrid earthquakes of 1811–1812, which caused the Mississippi river to rapidly fill in the newly formed lake basin (Johnston & Schweig, 1996). Following this uplift, the lake was separated from the Mississippi River by several kilometers of land, and it is therefore likely that the turtles currently residing in Reelfoot Lake are descendants of the migrants associated with the initial uplift, in 1812. Whereas the presence of *G. pseudogeographica* is well documented in this lake, *G. geographica* has never been observed there, despite decades of intensive sampling (Collins, Benz & Deck. 1997; M. Ewert, unpubl. data).

In many cases of natural hybridization, secondary contact occurs between previously separated species, and frequent reproduction between the species is possible (Esa, Waters & Wallis, 2000; Payseur, Krenz & Nachman, 2004). In such cases, the direction and extent of hybridization may be quantified, but the long-term fate of introgressing genetic elements is obscured by ongoing gene flow between the species. In cases of ancient hybridization, the introgression of genetic material into a population is often either complete or absent, because the time since hybridization exceeds the coalescent time of the gene(s) involved (Berthier, Excoffier & Ruedi, 2006; Good *et al*., 2008). Reelfoot Lake represents a unique and important evolutionary laboratory. Specifically, if the lake was first populated by *G. pseudogeographica* that had previously had the opportunity to hybridize with *G. geographica*, but have since been restricted from contact, then genetic elements from the two species have had several generations to interact without further contact between the species. Although such an environment has been explored in the laboratory in short-lived organisms (Aubert & Solignac, 1990; Edmands *et al*., 2005), this turtle population offers an opportunity to investigate this scenario in a long-lived species in nature.

MATERIAL AND METHODS

Study system

The genus *Graptemys* consists of 12 aquatic species distributed throughout east central North America (Ernst, Barbour & Lovich, 1994). *Graptemys geographica* is the most divergent of the *Graptemys* turtles, and is readily distinguished from *G. pseudogeographica* by a number of morphological and molecular features (Vogt, 1980; Ernst *et al*., 1994; Lamb *et al*., 1994; Stephens & Wiens, 2003; Myers, 2008). Vogt (1993) described *Graptemys ouachitensis* and *G. pseudogeographica* as sister species, assigning species status to *G. ouachitensis* primarily on

phenotypic traits that are now known to overlap in parts of their range (Myers, 2008). Janzen, Ast & Paukstis (1995) report significant physiological differences between sympatric populations of the species, arguing that they confirm the species status. Although Lamb *et al*. (1994) found monophyly in the *G. pseudogeographica* and *G. ouachitensis* groups, Walker & Avise (1998) note that the mtDNA divergence between *G. ouachitensis* and *G. pseudogeographica* is less than the within-species variation observed in most of the turtle species in their range, and argue that the genus has been oversplit at the species level. Subsequent phylogenetic analysis has shown low divergence and limited support for monophyly between *G. pseudogeographica* and *G. ouachitensis* (Myers, 2008; Smith, 2008; Spinks *et al*., 2009). Regardless of whether separate species designations are assigned to *G. pseudogeographica* and *G. ouachitensis*, the low molecular and phenotypic divergence suggests a recently shared evolutionary history of these lineages.

Identifying species-specific markers

In order to identify species-specific genetic markers, tissue samples from 28 representative samples were acquired from across the species' ranges, including 14 *G. geographica*, seven *G. pseudogeographica* and seven *G. ouachitensis* (Fig. 1A,B). Two samples from *Chrysemys picta* were collected for use as an outgroup. Fossil data suggest that the *Chrysemys*/*Graptemys* split occurred in the early Miocene (~15 Mya; Lamb *et al*., 1994; Near, Meylan & Shaffer, 2005). Samples were collected by us, donated by M. Ewert, or were acquired from specimens in the UC Davis or University of Minnesota Bell Museum collections. Tissue samples were preserved in 95% ethanol and refrigerated at approximately 4 °C until DNA extraction. DNA was extracted with a Puregene DNA extraction kit for cells and tissue (Gentra Corporation, Minneapolis, MN, USA). Extracted DNA was stored in hydration buffer at –20 °C until genetic analysis.

A 387-bp fragment of the mitochondrial control region was amplified using primers developed by M. Sorenson at the University of Massachusetts (5′-CAAGGGTGGATCGGGCATAAC-3 and 5′-GTGC CTGAAAAAACAACCACAGG-3′; Freedberg *et al*., 2005) corresponding to base pairs 15 780–16 288 of the *Chrysemys picta* mitochondrial genome (AF069423). Polymerase chain reaction (PCR) amplification was performed in 10-μL reactions consisting of 10 mM Tris buffer, pH 8.4, 0.2 mM of each primer, 1.5 mM $MgCl_2$, 0.15 mM dNTP, 0.5 U *Taq* DNA polymerase, and ~50 ng of DNA template. An initial denaturation (4 min at 95 °C and 5 min at 72 °C) was

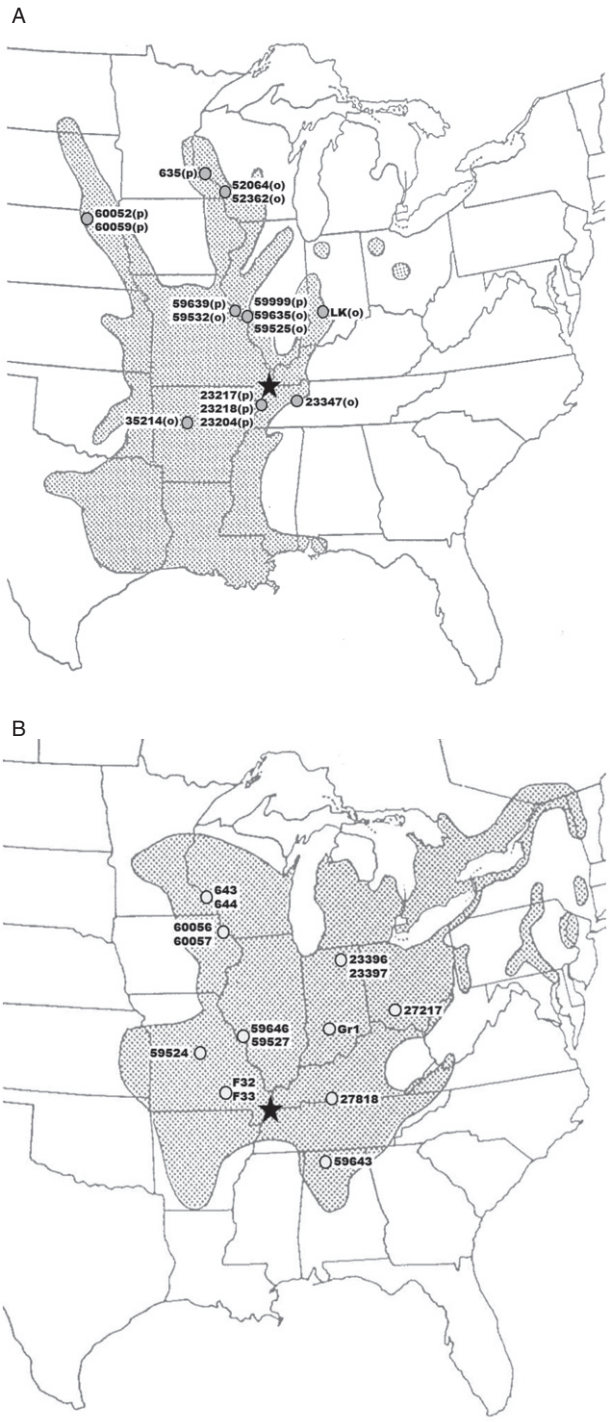

Figure 1. Distribution map and locations of representative samples for: A, *Graptemys pseudogeographica* (p) and *Graptemys ouachitensis* (o); and B, *Graptemys geographica*. Reelfoot Lake is shown with a star. One *G. pseudogeographica* sample (11150) had no locality data. Range maps are modified with permission from Ernst, Barbour & Lovich 1994.

followed by 40 cycles of 40 s at 95 °C, 1 min at 55 °C, and 2 min at 72 °C, and a final 8-min extension at 72 °C. PCR products were purified with QIAquick PCR purification kits (Qiagen, Valencia, CA, USA) using a microcentrifuge extraction protocol or with EXO-SAP enzymatic incubation. The purified PCR product was amplified at 1/24th scale using the ABI Big Dye v3.1. (2 min at 96 °C, 15 s at 50 °C, and 4 min at 60 °C, followed by 25 cycles of 30 s at 96 °C, 15 s at 50 °C, and 4 min at 60 °C). Sequencing clean-up was performed with ABI big-dye Xterminator kit. Sequence analysis was performed on an ABI 377 gel sequencer and/or on an ABI 3730 capillary sequencer. Sequences were processed, aligned, and analysed with GENEIOUS 4.5.5.

We identified two sets of nuclear markers capable of distinguishing museum specimens of *G. pseudogeographica* and *G. geographica* at multiple nucleotide positions. Markers were developed from internal primers for an intron of the ornithine decarboxylase antizyme (*ODC*; P. Spinks, pers. comm.) and an intron from the hepatocyte nuclear factor (*HNFAL*) from sequences downloaded from GenBank. No markers were found at either nuclear locus that were capable of distinguishing *G. pseudogeographica* from *G. ouachitensis* samples.

Sequencing of the ODC intron was performed with two sets of internal primers: ODC4 (5′-GGGTTTCTTTCAATTGCTGTAGTAA-3′ and 5′-CAG AGCACCGCTGGGAAT-3′) amplified a 464-bp region and ODC5 (5′-GGCTGAACGTAACAGAGGAACTA-3′ and 5′-TGTCATCTCTGTTCTTGTGGAAG-3′) amplified a 908-bp region. After removing the region of overlap and stretches of unclear sequence, 939 bp of sequence was used. A PCR amplification was performed in 10-μl volumes consisting of the same quantities of reagents listed above. For ODC, an initial amplification cycle (4 min at 95 °C) was followed by 40 cycles of 40 s at 95 °C, 1 min at 58 °C, and a 1-min elongation at 72 °C. Samples were purified and were amplified with the same PCR protocol as described above, using both forward and reverse primers.

Sequencing of the HNFAL region was performed with one set of internal primers (5′-CAGCAAT GATAGAACCCAGGA-3′ and 5′-GATGACAGCCACA TTCGTTC-3′), amplifying a 220-bp region. The PCR protocol was the same as for ODC, but the annealing temperature was lowered to 58 °C. Samples were purified and were amplified with the reverse primer.

Sample collection

In June of 2000–2003, 298 nesting *G. pseudogeographica* were collected on land at Reelfoot Lake, TN, USA. Reelfoot Lake is located outside of the known range of *G. geographica*, and no *G. geographica* individuals have ever been found in three decades of sampling (M. Ewert, unpubl. data) or in an intensive 3-year survey of 3500+ freshwater turtles at this lake (Collins *et al*., 1997). All turtles exhibited the unambiguous post-orbital patterning and eye pigmentation that is characteristic of *G. pseudogeographica kohnii* (Vogt, 1993). Tissue samples were collected and the turtles were released in the manner described in Freedberg *et al*. (2005).

Molecular analysis of Reelfoot Lake samples

Most samples produced clear reads throughout the entire control region and nuclear markers. For all three markers sequenced, only sequences that produced unambiguous reads at multiple informative sites were included. Sequences that were unclear for longer stretches were excluded from the analysis. In total, 269 samples produced clear reads for all three markers, 19 samples produced clear reads at two markers, and three samples produced clear reads for one marker.

Control region sequences were aligned in CLUSTALW. Phylogenetic analysis was performed using MEGA 5.0 (Tamura *et al*., 2011). Maximum-likelihood trees were constructed and branch clustering was bootstrap tested (1000 replicates). After a best-fitting nucleotide substitution model was identified, evolutionary distances were computed using the Tamura three-parameter method with discrete gamma-distributed evolutionary rates. Phylogenetic analysis was not performed on the ODC or HNFAL data because of the limited number of informative sites at these loci. Haplotype frequencies were calculated by dividing the total number of copies of each haplotype by the total number of copies of each genome (n for mtDNA, $2n$ for nuclear DNA). Nuclear loci were tested for departures from the Hardy–Weinberg equilibrium in ARLEQUIN 3.5. Haplotypes were reconstructed from unphased genotypes using fastPHASE 1.2, with default settings (Scheet & Stephens, 2006). The presence of recombination among reconstructed ODC haplotypes was tested with RecombiTEST (Piganeau, Gardner & Eyre-Walker, 2004) using the MAXIMUM CHISQUARE detection algorithm (Maynard Smith, 1992). There were too few polymorphisms to test for recombination at the HNFAL locus.

Linkage disequilibrium between the *geographica* alleles at the two nuclear loci was calculated by first predicting haplotype frequencies for each multilocus genotype and then applying the formula: $D = p_{oh} - p_o p_h$, where o represents the *geographica* allele at the ODC locus and h represents the *geographica* allele at the HNFAL locus. Cytonuclear disequilibrium between the mitochondrial locus and

each of the nuclear loci was calculated using the formula: $D = p_{mn} - p_m p_n$, where *m* represents the *geographica* alleles at the mt locus and *n* represents the *geographica* allele at each of the nuclear loci. Departures from the null expectation of $D = 0$ were tested with a likelihood-ratio test in ARLEQUIN with 100 000 permutations.

Statistical analysis of hybridization and lineage sorting

Incomplete lineage sorting and hybridization make different predictions about the gene trees underlying the species phylogeny. Because incomplete lineage sorting results from the failure of haplotypes to sort during speciation events, any haplotypes present in both lineages during the speciation event will have accumulated substantial divergence in this rapidly evolving region over the long time since divergence, and thus would exhibit substantial divergence today (Holder, Anderson & Holloway, 2001; Joly, McLenachan & Lockhart, 2009). To distinguish between the contrasting hypotheses of incomplete lineage sorting and hybridization, we followed the method of Joly *et al*. (2009). In brief, sequence differences for the species under consideration are compared with a null distribution of sequence distance expected under incomplete lineage sorting. First, we generated estimates of population size ($\theta = 4N_E\mu$) and divergence times ($\tau = T\mu$) using the reference samples and *Chrysemys picta* out-group samples for the mitochondrial control region in MCMCcoal 1.2 (Rannala & Yang, 2003; Burgess & Yang, 2008). Using coalescent simulations in MCcoal, 999 gene trees were simulated, selecting different parameters θ_i and τ_i from the species tree posterior distribution. Sequences of the same length as the original reference samples were then simulated independently using the simulated gene trees in SEQ-GEN 1.3.2 (Rambaut & Grassly, 1997) and the best-fitting nucleotide substitution model identified by JMODELTEST (HKY + Γ; Posada, 2008). Finally, the shortest Hamming distance (the number of nucleotide differences between sequences) between *G. geographica* and *G. pseudogeographica* was calculated for all replicates. The sequence distance between the Reelfoot Lake *G. pseudogeographica* and the reference *G. geographica* samples could then be calculated and compared with the null distribution.

In addition, we used molecular sequence data from Myers (2008) to estimate divergence time estimates for the splits between *Chrysemys picta* and the *Graptemys*, and within *Graptemys* between *G. geographica* and *G. pseudogeographica*/*G. ouachitensis*, and finally between *G. pseudogeographica* and *G. ouachitensis* in MCMCcoal. For each species, we included two sequences at six loci for a total of 4071 bp (GenBank accession numbers JN993967–JN993987, L28776, L28781, U81345, and AF069423). The analysis incorporated rate differences between loci and a heredity scalar to enhance divergence time estimates.

RESULTS

Identification of species-specific markers

The control region data from the representative samples collected from across the species ranges formed two well-defined clades separated by 5.7% sequence divergence (30 nucleotides): one composed of all of the *G. pseudogeographica*/*G. ouachitensis* samples and the other composed of all of the *G. geographica* samples (Fig. 2). The two separate branches were supported by a bootstrap value of 96. Within the *G. pseudogeographica*/*G. ouachitensis* clade there was low overall divergence (< 1.2%) and no clear support for a *G. ouachitensis*/*G. pseudogeographica* separation. There was extremely low divergence within the *G. geographica* clade (< 0.2%), with only one variable nucleotide position. There was an average of 7.7% divergence between *Graptemys* and the out-group *Chrysemys*, and an average of 30-bp divergence between *G. geographica* and *G. pseudogeographica*. Using the multilocus data set we estimated divergence between the out-group and *Graptemys* at 9.8–10.8 Mya, and the split between *G. geographica* and *G. pseudogeographica* at 4.6–5.5 Mya. Thus, for the control region data, with an estimated 5 million years of divergence and 30-bp difference between these lineages, we can crudely calculate a molecular clock of 6 bp Myr^{-1} (base pairs/million years) for this region. The low level of sequence divergence suggests that this estimate is not significantly influenced by saturation.

For the ODC locus, 27 representative samples produced clear, unambiguous reads, and were used in subsequent analyses. Examination of the representative samples and Reelfoot Lake specimens revealed four distinct haplotypes, each separated from the other three by at least six nucleotide positions (Table 1). Single nucleotide polymorphisms at other positions were rare, and all parental haplotypes reconstructed from unphased genotypes aligned unambiguously with one of these four haplotypes. Recombination analysis revealed no evidence for recombination among the four haplotypes (max $\chi^2 = 3.35$, $P = 0.95$). Five of the seven representative specimens from *G. pseudogeographica* were homozygous for haplotype A, and the other two specimens were heterozygous for haplotypes A and C (Table 2). Three of the six *G. ouachitensis* samples were

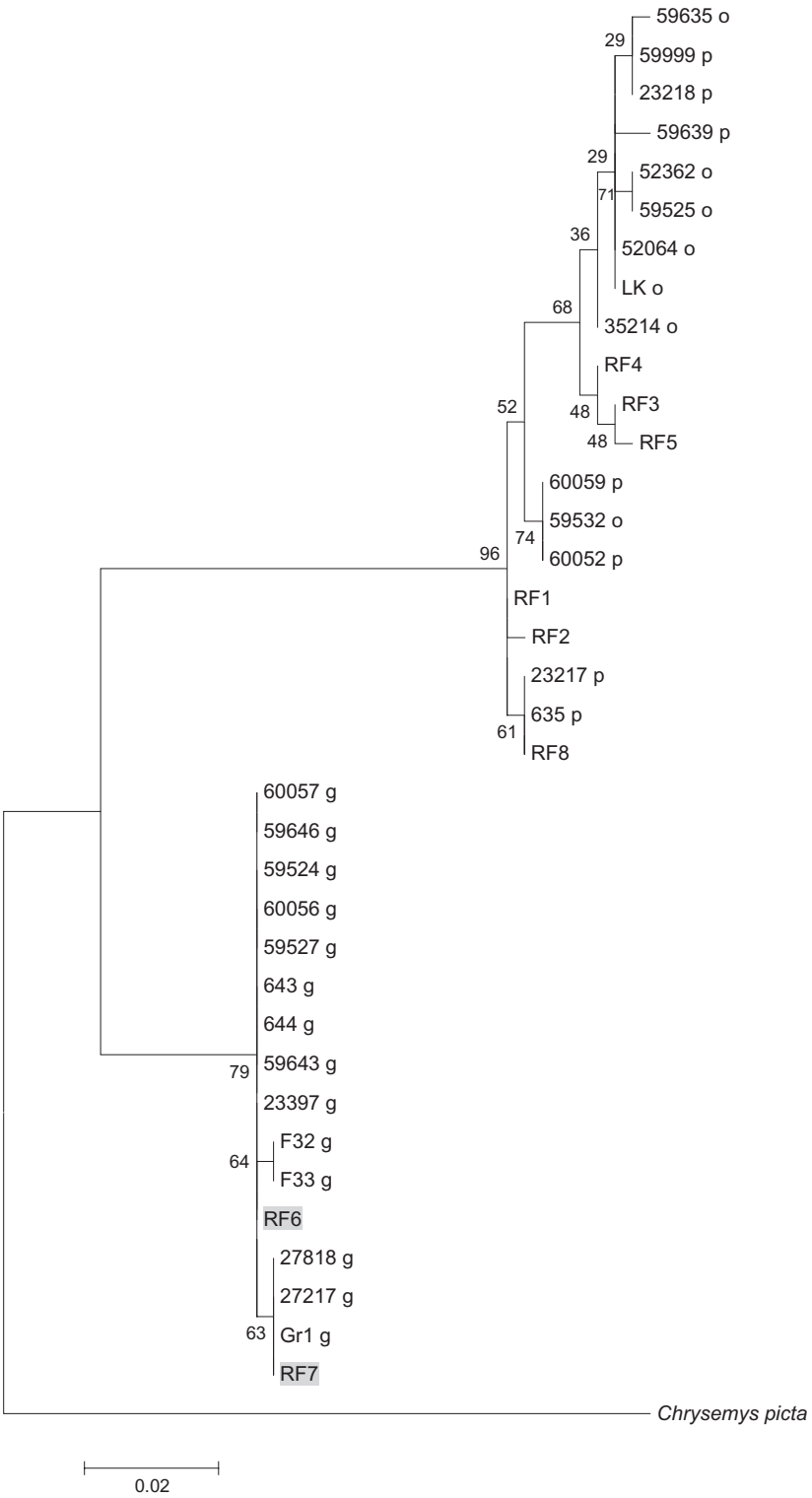

Figure 2. Phylogeny of the mitochondrial control region inferred using the maximum-likelihood method based on the Tamura three-parameter model with discrete gamma-distributed evolutionary rates. Two haplotypes from *Graptemys pseudogeographica* from Reelfoot Lake (RF6 and RF7, highlighted) aligned unambiguously with the *Graptemys geographica* clade. The tree with the highest log likelihood (–869.1721) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. There were a total of 389 positions in the final data set.

Table 1. Variable positions and haplotype assignments for the nuclear ODC locus. For haplotype $R^{C/B}$, the underlined region indicates where recombination between haplotypes C and B occurred

Position Haplotype	*110*	*146*	*148*	*370*	*396*	*434*	*491*	*550*	*641*	*787*	*808*	*820*	*1099*	*1204*	*1224*
A	T	C	C	–	C	T	C	G	G	T	A	A	A	A	A
B	C	T	C	–	T	G	T	A	G	T	A	C	G	A	C
C	T	C	C	AA	T	T	T	A	G	T	C	A	A	G	A
D	T	C	T	AA	T	T	T	A	A	C	A	C	A	A	A
$R^{C/B}$	T	C	C	AA	T	T	T	A	G	T	A	C	G	A	C

Table 2. Diploid genotypes for the nuclear ODC and HNFAL loci in representative samples of *Graptemys pseudogeographica* (P), *Graptemys ouachitensis* (O), and *Graptemys geographica* (G)

ID	Species	ODC genotype	HNFAL genotype
60059	P	AA	11
59999	P	AA	11
635	P	AA	11
23217	P	AA	11
23218	P	AA	11
59639	P	AC	11
60052	P	AC	11
23204*	P	–	11
11150*	P	–	11
LK	O	AA	11
52362	O	AA	11
59635	O	AA	11
59525	O	AC	11
52064	O	AC	11
35214	O	AC	11
23347*	O	–	12
F33	G	BB	22
59527	G	BB	22
59646	G	BB	22
60056	G	BB	22
59524	G	BB	22
59643	G	BB	22
Gr1	G	BB	22
23397	G	BB	22
27217	G	BB	22
27818	G	BB	22
643	G	BB	22
644	G	BB	22
F32	G	$BR^{B/C}$	22
60057	G	BA	12
23396*	G	–	22

*Denotes HNFAL sequence downloaded from GenBank.

homozygous for haplotype A, and the other three were heterozygous for haplotypes A and C. Twelve out of 14 representative specimens from *G. geographica* were homozygous for haplotype B, one sample was heterozygous for B and A, and the other sample was heterozygous for B and another allele, $R^{B/C}$, that apparently resulted from recombination of haplotypes B and C. This recombinant allele was characterized by the haplotype-C nucleotide at the first three divergent positions, and by the haplotype-B nucleotide at the last three divergent positions. A fourth haplotype (D) was found in several Reelfoot Lake samples, but was not seen in any of the 27 representative samples. No consistent differences were observed between the *G. pseudogeographica* and *G. ouachitensis* samples at the ODC locus. One representative sample from *G. ouachitensis* yielded a clear control region haplotype, but repeated attempts at sequencing the nuclear loci in this turtle were unreadable.

For the HNFAL locus, 27 representative samples produced clear, unambiguous reads. Four additional sequences were downloaded from GenBank and were included in the analysis. Two haplotypes separated at two nucleotide positions were found among the representative samples. All haplotypes reconstructed from unphased genotypes aligned with one of these two haplotypes. The two haplotypes were strongly associated with species identities among the representative samples: 13 of 14 representative *G. geographica* samples that were successfully sequenced were homozygous for allele 2, and one was heterozygous for alleles 1 and 2. This individual was also heterozygous for species-specific markers at the ODC locus, and may signify a recent history of hybridization in this lineage. Fifteen out of 16 representative *G. pseudogeographica*/*G. ouachitensis* samples were homozygous for allele 1, whereas one *G. ouachitensis* sample was heterozygous for alleles 1 and 2.

Quantitative analysis of Reelfoot Lake samples

For the control region locus, 270 out of 284 Reelfoot *G. pseudogeographica* samples aligned with the *G. pseudogeographica* branch. Fourteen samples aligned with the *G. geographica* branch. These 14 samples were characterized by two separate haplotypes, each of which aligned perfectly with some of the representative *G. geographica* samples, suggesting that the divergence between these two haplotypes occurred prior to hybridization.

The deep divergence of these lineages and high mutation rate of the control region marker suggest that the perfect alignment of the *G. pseudogeographica* samples with *G. geographica* haplotypes cannot result from incomplete lineage sorting. The null distribution of minimum sequence distance between *G. geographica* and *G. pseudogeographica* under incomplete lineage sorting ranged from 9 to 45 nucleotide differences, with the mean at 26.89. Comparing the empirical sequence distances for the Reelfoot *G. pseudogeographica* samples with *G. geographica* and the null distribution, RF1–RF5 and RF8 haplotypes were well within this distribution (values ranged from 26 to 33). In contrast, incomplete lineage sorting was strongly rejected ($P = 0.001$) for all 14 samples from both the RF6 and RF7 haplotypes, as these haplotypes had minimum distances of 0, well outside the null distribution.

For the ODC locus, A was the most commonly observed allele, followed by D, B, and C (Table 3). The locus showed no significant departure from the Hardy–Weinberg (HW) equilibrium ($Het_{obs} = 0.482$, $Het_{exp} = 0.476$, $P = 0.19$). Although failure to detect departure from HW equilibrium may be caused by rare alleles, only one allele (C) was found in fewer than 20 turtles, and all three turtles possessing this allele were heterozygous for this allele and the most common allele. Given its near-perfect association with the *G. geographica* representative samples, we can assign haplotype B to *G. geographica* and examine the level of *G. geographica* introgression at the ODC locus. Although haplotype D did not appear in any of the representative samples, it aligned with haplotypes A and C at five of the six positions where haplotypes A and C shared a nucleotide that differed from haplotype B, and thus there is no justification for assigning haplotype D to *G. geographica*. The frequency of *G. geographica* haplotype B in Reelfoot Lake was 0.099. The HNFAL locus similarly showed no departure from HW equilibrium ($Het_{obs} = 0.058$, $Het_{exp} = 0.056$, $P = 1.0$). The allele frequency of the *G. geographica* allele (allele 2) for HNFAL in Reelfoot Lake was 0.029.

The gene frequency of the *G. geographica* ODC allele was significantly greater than that of the *G. geographica* mt or HNFAL allele (ODC versus mt, $\chi^2 = 6.36$, d.f. = 1, $P < 0.02$; ODC versus HNFAL, $\chi^2 = 22.6$, d.f. = 1, $P < 0.0001$). There was no difference in the gene frequencies of the HNFAL and mt markers ($\chi^2 = 2.117$, d.f. = 1, $P = 0.146$). There was no difference in the gene frequency of the mt marker and the combined gene frequencies of the two nuclear markers ($\chi^2 = 0.981$, d.f. = 1, $P = 0.322$). No combinations of markers showed deviations from linkage equilibrium for the *G. geographica* alleles (ODC-mt, distance, $D = -0.0031$; HNFAL-mt, $D = -0.0013$; ODC-HNFAL, $D = 0.0009$; d.f. = 1, $\chi^2 = 0.16$, $P = 0.69$).

Table 3. Frequency of nuclear and mitochondrial haplotypes from *Graptemys pseudogeographica* found in Reelfoot Lake

ODC		
Genotype	Frequency	%
AA	130	45.8
AB	37	13.0
AC	3	1.1
AD	90	31.7
BB	6	2.1
BD	7	2.5
DD	11	3.9
HNFAL		
Genotype	Frequency	%
11	260	93.8
12	16	6.9
Mitochondrial		
Haplotype	Frequency	%
RF1	162	57.0
RF2	93	32.7
RF3	3	1.1
RF4	2	0.7
RF5	9	3.2
RF6	7	2.5
RF7	7	2.5
RF8	1	0.4

DISCUSSION

We examined interspecific genetic introgression between two species of *Graptemys* turtles that have been naturally restricted from gene flow for ~200 years. We found strong evidence for hybridization and gene introgression from *G. geographica* into

a population of *G. pseudogeographica*, two taxa that diverged approximately 5 Mya. We isolated both mitochondrial and nuclear markers that were capable of distinguishing between these two species, and found several *G. pseudogeographica* that aligned unambiguously with *G. geographica* mitochondrial or nuclear genotypes. In addition, coalescent simulations allowed us to reject incomplete lineage sorting as an explanation for this trend, indicating that the discordance in the phylogeny resulted from hybridization and subsequent introgression.

Although hybridization probably occurred prior to the establishment of Reelfoot Lake, the polymorphism seen among the introgressed *G. geographica* haplotypes suggests that the introgression occurred evolutionarily recently. Specifically, neutral mitochondrial polymorphisms are expected to drift to fixation over time in small populations (Ballard & Whitlock, 2004). This conclusion is further supported by the complete lack of divergence between each of the introgressed *G. geographica* haplotypes and the representative *G. geographica* samples. Given our clock estimate of 6 bp Myr^{-1} for this region, one nucleotide substitution should separate distinct lineages, on average, every 170 000 years. Considering that neither of these Reelfoot lineages showed any divergence from *G. geographica* samples, it is highly likely that the hybridization events occurred within the last few hundred thousand years.

Although selection often restricts interspecific introgression of one or both genomes, *G. geographica* mitochondrial and nuclear DNA have persisted in this population for at least 200 years. By contrast, laboratory experiments often reveal strong selection against backcrossed hybrid lines soon after hybridization because of 'F_2 breakdown' resulting from genic interactions (Dobzhansky, 1937; Sawamura, Davis & Wu, 2000). In particular, barriers to nuclear introgression tend to evolve rapidly as a result of prezygotic isolating mechanisms (Chan & Levin, 2005). Studies reporting no nuclear introgression, despite mitochondrial introgression in other animal taxa, commonly involve species that diverged less than 5 Mya (*Drosophila*, 0.5–1 Mya, Bachtrog *et al*., 2006; chipmunks, 3 Mya, Good *et al*., 2008; hares, < 2.5 Mya, Alves *et al*., 2003; tilapia, 3.3 Mya, Rognon & Guyomard, 2003). Although precise estimates of genome-specific introgression rates require a large number of loci (Carling & Brumfield, 2008), we found nuclear introgression at two unlinked loci in the present study. Whereas genetic drift or selection on linked loci may allow some introgressed markers to remain in a population despite weak selection against hybrids (Seehausen, 2004), our finding of introgression at all three loci examined suggests that introgression is not generally restricted in this system.

A combination of genetic, geographic, and ecological forces can lead to the evolution of reproductive isolation between species (Coyne & Orr, 1998; Nosil, Harmon & Seehausen, 2009). Ecological factors are often associated with the incomplete evolution of reproductive isolation, particularly when fewer niche dimensions are involved in the speciation process. Specifically, divergent selection on one trait leads to minimal opportunity for correlated response on other loci that are important for genetic incompatibility (Nosil *et al*., 2009). Dietary preference appears to have played a critical role in the diversification of *Graptemys* (Myers, 2008), with *G. geographica* exhibiting substantially greater molluscivory than *G. pseudogeographica* (Ernst *et al*., 1994; Lindeman, 2000). If dietary specialization was the principal factor leading to reproductive isolation in this system, it may help to explain why complete reproductive isolation has not evolved.

Another factor that may facilitate introgression of both genomes in *Graptemys* is an absence of sex chromosomes. In systems with heteromorphic sex chromosomes, Haldane's rule predicts that hybrids of the heterogametic sex will be more likely to experience infertility or inviability, inhibiting the opportunity for introgression. This pattern is generally attributed to deleterious interactions between the X chromosome and 'foreign' autosomes that are expressed in F_1 heterogametic individuals, as homogametic individuals can hide any such harmful interactions by the presence of the second 'native' X chromosome. Empirical studies of hybrid zones support the predictions of Haldane's rule: in male heterogametic systems, nuclear introgression is limited relative to mitochondrial introgression (reviewed in Arntzen *et al*., 2009), whereas mitochondrial introgression is prevented in many taxa characterized by female heterogameity (Tegelström & Gelter, 1990; Sperling, 1993; Crochet *et al*., 2003).

In *Graptemys* and most other turtles, offspring sex is determined by incubation temperature, a system that greatly limits the opportunity for sex chromosome evolution (Bull, 1983). Moreover, because sex chromosomes have been absent from the lineage leading to *Graptemys* for over 200 Myr (Janzen & Phillips, 2006), reinforcing selection to avoid hybrid matings has probably not been as strong as in taxa characterized by sex chromosomes. The extensive reports of hybridization in other turtle taxa also involve species with non-chromosomal sex determination (Fritz & Baur, 1994; Fritz, 1995; Karl *et al*., 1995; Stuart & Parham, 2007).

Cytonuclear equilibrium

Contrary to many other studies of interspecific introgression, we found no evidence of cytonuclear

disequilibrium between the *G. geographica* nuclear and mitochondrial markers in our study population. Assuming there is no positive selection on our markers, cytonuclear equilibrium indicates that backcrossing has occurred for at least eight generations without any additional interspecific gene flow (Arnold, 1993). This estimate is consistent with the notion that *G. geographica* ceased hybridizing with *G. pseudogeographica* prior to or shortly after the inception of the lake, as the lake was formed 190 years before this study, and *Graptemys* turtle generations range from 8–50 years (Vogt, 1980). *Graptemys geographica* has not been reported within 70 km of Reelfoot Lake (http://emys.geo.orst.edu), and if the distribution of *G. geographica* has not shifted significantly in the last several hundred years, it is likely that the last opportunity for hybridization between the species occurred prior to the establishment of this population.

Cytonuclear equilibrium indicates that strong assortative mating between hybrid and parental lines is absent in this population. Although *G. geographic* and *G. pseudogeographica* are morphologically distinct and maintain species integrity over large regions of sympatry (Ernst *et al*., 1994), hybrids, once they occur, may be morphologically similar enough to *G. pseudogeographica* to allow them to freely backcross with pure individuals. Furthermore, males of both species court females through head bobbing (Vogt, 1980), suggesting that displays of hybrid males may be successful in enticing pure females, and vice versa. Finally, the rarity of encounters with genetically similar mates probably encourages hybrids to mate with more commonly encountered pure *G. pseudogeographica* individuals, a trend that has been documented in several animal systems (reviewed in Malmos, Sullivan & Lamb, 2001).

Our finding of cytonuclear equilibrium also suggests an absence of selection against disrupted cytonuclear pairings at loci linked to these markers. Because the function of many mitochondrial genes is dependent upon interactions with nuclear genes, hybridization and subsequent backcrossing often causes co-adapted mitochondrial/nuclear gene complexes to become disrupted, selecting against individuals with mismatched nuclear and mitochondrial DNA (Cruzan & Arnold, 1999). Hybrid lineages characterized by a mitochondrial haplotype in a foreign nuclear background suffer decreased fitness in laboratory studies of invertebrates (Edmands, 1999; Sackton, Haney & Rand, 2003), plants (Pollak, 1991), and yeast (Lee *et al*., 2008). Because individuals with incompatible mitochondrial and nuclear genomes are at a fitness disadvantage, this disruption can serve as a barrier to mitochondrial introgression. If *G. pseudogeographica* with foreign mitochondrial DNA suffer from decreased fitness, selection would reduce *G. geographica* mitochondrial introgression relative to *G. geographica* nuclear introgression.

The slow rate of mitochondrial evolution in turtles makes cytonuclear incompatibility among these two lineages unlikely. A comparison of mitochondrial clock rates in animals shows that turtles exhibit an 8- to 10-fold slowdown compared with the 2% per million year clock reported for a wide array of animal taxa (Avise *et al*., 1992). An examination of the divergence of 1020 bp of the protein-coding cytochrome *b* gene in *Graptemys* shows that although 22 nucleotide substitutions separate the *G. pseudogeographica* and *G. geographica* sequences, only one results in an amino acid substitution, from leucine to isoleucine (GenBank accession nos FJ770601 and FJ770598). In contrast, wolves (*Canis lupus*) and coyotes (*Canis latrans*), known to hybridize and introgress over large stretches of their range (Roy *et al*., 1994), are separated by 12 amino acid substitutions over the homologous region (GenBank accession nos NC008093 and NC011218). Although a chronological clock has been invoked to predict the rate of evolution of reproductive isolation in other taxa (Bolnick & Near, 2005), the accuracy of such a clock may be strongly dependent on mutation rate.

Reproductive Isolation in Graptemys

Both of the *G. geographica* haplotypes present in our *G. pseudogeographica* population were found in the representative *G. geographica* samples, indicating that at least two hybridization events between these species have occurred in this region. The *G. pseudogeographica* and *G. geographica* nuclear haplotypes in our population were in HW equilibrium, supporting the conclusion that strong assortative mating between hybrid and 'pure' *G. pseudogeographica* lineages is not occurring in this population. Furthermore, the presence of *G. pseudogeographica* nuclear alleles in two of our representative *G. geographica* samples, one from north-eastern Iowa and one from southern Missouri, and a *G. geographica* allele in a *G. ouachitensis* sample from central Tennessee, indicates that hybridization has occurred elsewhere. Coupled with reports of wild hybridization between *G. geographica* and *G. ouachitensis*, and between *G. geographica* and *G. pseudogeographica*, in Wisconsin (Vogt, 1978; R. Vogt, pers. comm.), it would appear that low levels of hybridization may occur over the large overlapping ranges of these species. Captive hybridization between *Graptemys oculifera* and *Graptemys barbouri* (reported in Fritz, 1995), which diverged 2.5–3.5 Mya (Lamb *et al*., 1994), and between *G. pseudogeographica* and *G. ouachitensis* (J. Harding, unpubl. data), which diverged

approximately 0.8 Mya, further suggest that the recent radiation in this genus may be associated with an incomplete evolution of reproductive isolation.

We found no evidence for monophyly separating *G. ouachitensis* and *G. pseudogeographica* in either the nuclear or mitochondrial markers. Previous attempts at resolving the phylogenetic relationships of these groups using control region data have found monophyly with weak statistical support and low sequence divergence (Lamb *et al*., 1994; Smith, 2008). Conversely, we found multiple instances of paraphyly with our control region marker. Although this pattern is consistent with hybridization between *G. ouachitensis* and *G. pseudogeographica*, the recent divergence times of these lineages means that incomplete lineage sorting cannot be ruled out as an explanation for the incongruent phylogenies. Under either scenario, the geographical variation and occasional overlap of the phenotypic traits used to distinguish these species, coupled with the intertwined phylogeny observed here, lends support to the notion that the two groups may maintain reproductive isolation in parts of their range while interbreeding in others.

The control region and HNFAL markers showed low levels of divergence within each of the two major branches, whereas the ODC locus revealed a more complex pattern of relatedness. In addition to the primary haplotypes that characterized the *G. geographica* (haplotype B) and *G. pseudogeographica* (haplotypes A and C) representative samples, we found a fourth haplotype, haplotype D, that showed nearly as much divergence from haplotype A (eight sites) as it did from *G. geographica* (nine sites). Notably, this is more than half of the divergence observed between haplotype A and *Chrysemys* (12 sites) over this region. The conspicuous absence of haplotype D from any of our representative *Graptemys* samples is surprising given its commonality in our population ($q = 0.212$). Although the high divergence between this haplotype and the other three would suggest that it could have come from another *Graptemys* species, no other *Graptemys* species are found within 300 km of Reelfoot Lake. Additional surveying of *Graptemys* from other populations may reveal if this anomalous genotype has resulted from interspecific hybridization or deep cryptic variation within *G. pseudogeographica*.

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