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Evolutionary basis of mitonuclear discordance between sister species of mole salamanders (*Ambystoma* sp.)

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Abstract

Distinct genetic markers should show similar patterns of differentiation between species reflecting their common evolutionary histories, yet there are increasing examples of differences in the biogeographic distribution of species-specific nuclear (nuDNA) and mitochondrial DNA (mtDNA) variants within and between species. Identifying the evolutionary processes that underlie these anomalous patterns of genetic differentiation is an important goal. Here, we analyse the putative mitonuclear discordance observed between sister species of mole salamanders (*Ambystoma barbouri* and *A. texanum*) in which *A. barbouri*-specific mtDNA is found in animals located within the range of *A. texanum*. We test three hypotheses for this discordance (undetected range expansion, mtDNA introgression, and hybridization) using nuDNA and mtDNA data analysed with methods that varied in the parameters estimated and the timescales measured. Results from a Bayesian clustering technique (STRUCTURE), bidirectional estimates of gene flow (MIGRATE-N and IMa2) and phylogeny-based methods (*BEAST, BUCKY) all support the conclusion that the discordance is due to geographically restricted mtDNA introgression from *A. barbouri* into *A. texanum*. Limited data on species-specific tooth morphology match this conclusion. Significant differences in environmental conditions exist between sites where *A. texanum* with and without *A. barbouri*-like mtDNA occur, suggesting a possible role for selection in the process of introgression. Overall, our study provides a general example of the value of using complimentary analyses to make inferences of the directionality, timescale, and source of mtDNA introgression in animals.

Keywords: *Ambystoma*, introgression, mitochondrial DNA, mitonuclear discordance, mole salamanders, nuclear DNA

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Introduction

Conceptually, different classes of genetic markers should show similar patterns of differentiation both within and between species as a result of their shared evolutionary history. This assumption allows for the inference of phylogenetic relationships among taxa. In practice, various markers often show different patterns of differentiation due to a variety of evolutionary processes (Avise 1994). For instance, genetic comparisons of closely related animal taxa show discordance between nuclear genes (nuDNA) and mitochondrial DNA (mtDNA). This lack of congruence (termed mitonuclear discordance) results from the introgression of mitochondrial genes from one population or species to another combined with low levels of nuclear introgression (Avise 1994). Studies that report mitonuclear discordance have become more common as researchers increasingly use mtDNA and nuDNA loci concertedly for phylogeographic and phylogenetic analyses in a range of taxa (Funk & Omland 2003; Chan & Levin

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2005; Gompert *et al.* 2008; Parham *et al.* 2013; Zieliński *et al.* 2013).

Identifying cases of mitonuclear discordance and understanding the underlying mechanisms is an important step in understanding evolutionary and ecological relationships between species or populations (Toews & Brelsford 2012). For example, different types of genetic introgression can alter ecological relationships among organisms (Ryan *et al.* 2009), create independent lineages (Robertson *et al.* 2006), or result in taxonomic misidentifications (reviewed in Funk & Omland 2003). Mitonuclear discordance can also results in a loss of genetic distinctiveness between species that results in uncertainty in specifying species' ranges, leading to the potential misidentification of cryptic species (Rohwer *et al.* 2001; Zieliński *et al.* 2013). Mitonuclear discordance has been explained by a variety of evolutionary mechanisms, including adaptive sweeps of mtDNA haplotypes, sex-biased hybridization, or demographic influences such as genetic drift (reviewed in Toews & Brelsford 2012). Although it is a well-recognized phenomenon, the methods used to detect and explain mitonuclear discordance vary in their approaches and assumptions. Thus, there is an active focus on evaluating the current methods used to detect cases of mitonuclear discordance and to identify the evolutionary mechanisms responsible (Funk & Omland 2003; Toews *et al.* 2013).

Here, we investigate a putative case of mitonuclear discordance within sister species of mole salamanders in Ohio, *Ambystoma barbouri* (Streamside Salamander) and *A. texanum* (Smallmouth Salamander). Amphibians display diverse patterns of mitonuclear discordance that result from multiple processes, including asymmetrical mtDNA introgression (reviewed in Toews & Brelsford 2012), asymmetrical nuDNA introgression (Di Candia & Routman 2007; Johanet *et al.* 2011), and the introgression of both mtDNA and nuDNA (Chatfield *et al.* 2010; Veith *et al.* 2012). *Ambystoma barbouri* and *A. texanum* are two morphologically similar salamander species but can be identified using species-specific patterns of tooth morphology and inhabit different breeding environments (Kraus & Petranka 1989). Despite this distinctiveness, these animals are known to interbreed in several sympatric areas of their ranges, likely leading to locations where mitonuclear discordance exists (Fig. 1; Niedzwiecki 2005). For example, based on three mtDNA loci and morphological characters, Niedzwiecki (2005) identified a single population of *A. texanum* in southwestern Ohio (Greene County) with a mtDNA haplotype most similar to that of *A. barbouri*. Eastman *et al.* (2009) identified two individuals that were potential hybrids based on mismatched mtDNA haplotypes. Greenwald & Gibbs (2012) subsequently discovered several individuals in central Ohio with *A. barbouri* mtDNA haplotypes that are >100 km from the nearest sample within the established range of *A. barbouri* ('unknown' individuals; Figs 1 and 2, Table S1, Supporting information). However, all examples involved a small number of samples, lack of information on nuDNA or morphological variation, and a limited set of methods to analyse the data. Additionally, *A. barbouri* displays multiple satellite populations (USGS 2012), leaving the possibility that mtDNA haplotypes have identified previously unknown populations of *A. barbouri*. Thus, understanding the extent of mitonuclear discordance and its possible causes requires detailed sampling and more comprehensive analyses of both mtDNA and nuDNA markers.

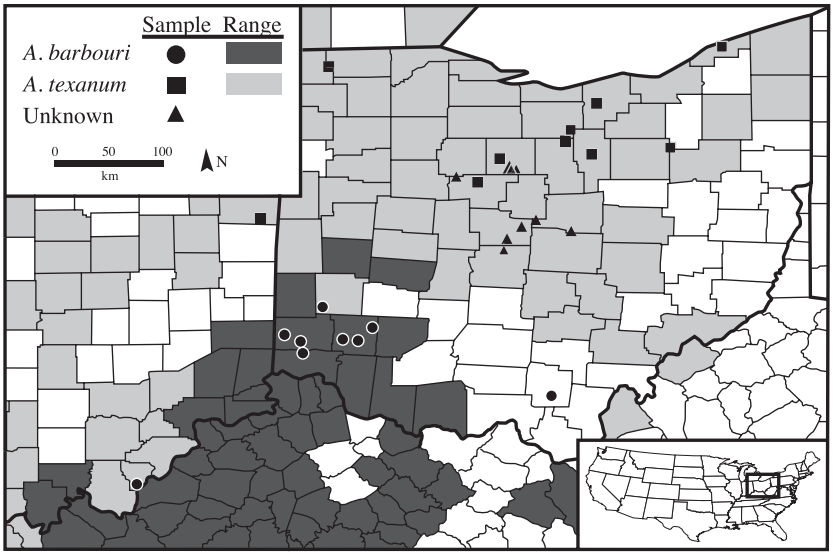

Fig. 1 Sampling locations and partial range maps for *Ambystoma barbouri*, *A. texanum*, and unknown individuals identified with mismatched mtDNA haplotypes. Range data are taken from the USGS National Amphibian Atlas (2012).

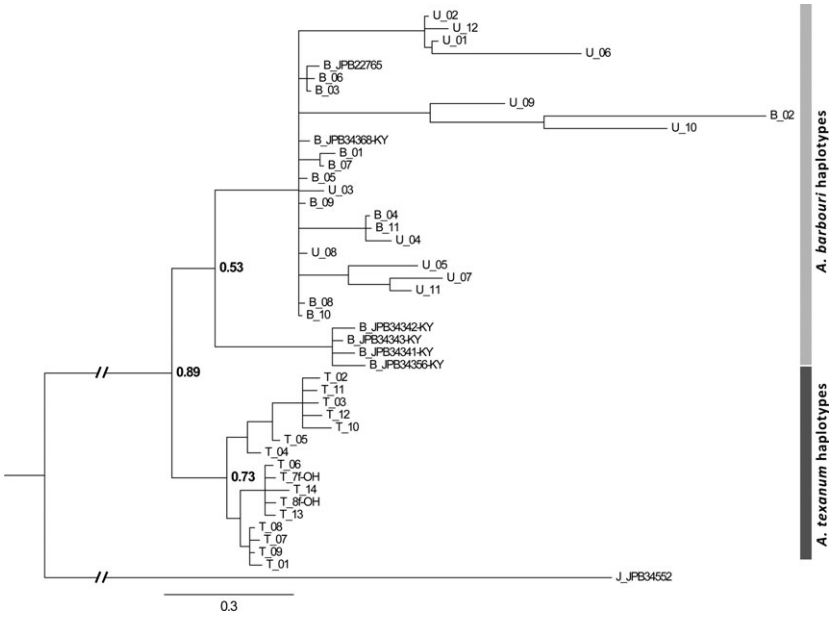

Fig. 2 Phylogenetic tree with posterior probabilities based on a 346 bp section of control region mtDNA (Primers F-THR, R-651; Shaffer & McKnight 1996; Bogart *et al*. 2007). The 39 individuals and reference samples are identified by letter (U = unknown, T = *Ambystoma texanum*, B = *A. barbouri* and J = *A. jeffersonianum*) and reference samples are included that have had species identity confirmed by morphology. See Table S1 (Supporting information) for more information about samples.

For this purpose, we collected samples from a broad range of sites (Fig. 1) and analysed them using a diagnostic mtDNA marker and 10 nuDNA sequence-based markers (c.f. Greenwald & Gibbs 2012). Initially, we sought to confirm that mitonuclear discordance was present in these salamanders and identify its geographical extent. Next, we tested three hypotheses that provide demographic or potentially adaptive explanations for the observed genetic pattern (Table 1). First, the presence of 'mismatched' mtDNA haplotypes could be explained as a consequence of *A. barbouri* having a larger range than previously recognized, and it has gone undocumented due to the difficulty in identifying the two species ('misidentification' hypothesis). Second, the 'unknown' individuals may be *A. texanum* with introgressed *A. barbouri* mtDNA from a historical hybridization event ('introgression' hypothesis; Toews & Brelsford 2012). Finally, the mitonuclear discordance could be due to ongoing but geographically restricted hybridization between *A. barbouri* and *A. texanum*, with the result that the 'unknown' individuals in central Ohio with mismatched genomes are hybrids ('hybridization' hypothesis).

Each of these hypotheses can be tested by comparing patterns of variation in nuDNA markers in the two parental species and in mismatched individuals

Table 1 Proposed hypotheses to explain the presence of salamander individuals with 'mismatched' mtDNA haplotypes in central Ohio. Each hypothesis is presented along with supporting predictions for each of four analysis types

	Analysis type and example program/procedure			
Hypothesis	Bayesian clustering (STRUCTURE)	Gene flow estimation (MIGRATE-N, IMA2)	Phylogeny estimation (*BEAST, BUCKY)	Morphology (Maxillary teeth)
A. barbouri misidentification	Unknown individuals group with *A. barbouri*	Symmetrical gene flow between *A. barbouri* and unknown group	Unknown individuals group with *A. barbouri*	Unknown individuals display rounded cusps (*A. barbouri* phenotype) on maxillary teeth
mtDNA introgression into *A. texanum*	Unknown individuals group with *A. texanum*	Symmetrical gene flow between *A. texanum* and unknown group	Unknown individuals group with *A. texanum*	Unknown individuals display pointed cusps (*A. texanum* phenotype) on maxillary teeth
Hybridization	Admixture within unknown group	Gene flow between unknown group and both parental groups	Admixture within unknown group	Unknown individuals potentially display intermediate phenotype

(Table 1). We used three types of methods for such tests: Bayesian clustering methods (Beaumont *et al.* 2001; Susnik *et al.* 2004; Grant *et al.* 2007; Pastorini *et al.* 2009; Bohling *et al.* 2012), Isolation-Migration (IM) methods (Barrowclough *et al.* 2005; Ackermann & Bishop 2010; Austin *et al.* 2011; Nevado *et al.* 2011) and species tree-based phylogenetic techniques (Sequeira *et al.* 2011; Melo-Ferreira *et al.* 2012; Parham *et al.* 2013). Each method varies in terms of assumptions made, the parameters estimated, and the timescale over which the estimation occurs. For example, genetic clustering techniques identify sets of genetically similar samples under minimal assumptions but provide no estimates of gene flow or effective population sizes. In contrast, many IM methods provide estimates of the direction and magnitude of gene flow but assume that populations are at genetic equilibrium and that retained ancestral polymorphism does not impact estimates of variation shared between populations. Finally, species tree-based phylogenetic techniques can account for retained ancestral polymorphism in estimates of polymorphism between species but assume that gene flow between species is limited. Utilizing a range of techniques allows for a more comprehensive assessment of the pattern of genetic discordance and its possible causes in these salamanders than is usually completed in past studies (Barrowclough *et al.* 2005; Parham *et al.* 2013). Overall, this study provides a general example of the value of using complimentary analyses to make inferences of the directionality, timescale, and source of mtDNA introgression in animals.

Methods

Samples and genotyping

We collected DNA samples from the tail tips of 39 individual salamanders from across Ohio that spanned the previously described ranges of *A. barbouri* and *A. texanum* (Fig. 1; Table S1, Supporting information). Within the putative ranges of each species, *A. barbouri* samples were collected from streams ($n = 11$), whereas *A. texanum* samples were collected in ponds ($n = 26$). DNA was extracted from tail tips using Qiagen DNeasy tissue kits (Qiagen, Valencia, CA). Each sample was then analysed in two ways. First, to identify species, each sample was sequenced for a 346 bp section of control region mtDNA from an amplicon generated using primers F-THR and R-651 (Shaffer & McKnight 1996; Bogart *et al.* 2007) that contains species-specific polymorphisms (Bogart *et al.* 2007; Greenwald & Gibbs 2012). Individuals were classified as having either *A. barbouri* or *A. texanum* mtDNA based on the presence/absence of specific mtDNA polymorphisms (Table S2, Supporting information) and whether they clustered with reference *A. texanum* or *A. barbouri* samples (Fig. 2). Samples collected in the putative range of *A. texanum* that were identified as having *A. barbouri* mtDNA were designated as 'unknown'. We also sequenced all samples at 10 nuDNA loci (see Table S3, Supporting information for primer sequences) consisting of seven anonymous DNA loci (Smith *et al.* 2005; Greenwald & Gibbs 2012) and partial sequences from three protein-coding loci (Vieites *et al.* 2007). We followed PCR protocols as described in Vieites *et al.* (2007) and Greenwald & Gibbs (2012). All sequences were aligned with MUSCLE v.3.8.31 (Edgar 2004), and nuDNA sequences were phased using Phase (Stephens *et al.* 2001; Stephens & Donnelly 2003) as implemented in DNASP v5.10.1 (Librado & Rozas 2009). Tests for Hardy-Weinberg equilibrium (HWE) across loci and groups were conducted with GENALEX 6.501 (Peakall & Smouse 2006, 2012), and linkage disequilibrium between pairs of loci was evaluated using GENEPOP 4.2 (Raymond & Rousset 1995; Rousset 2008).

Population and phylogenetic analyses

First, we used the Bayesian clustering program STRUCTURE (version 2.3.3; Pritchard *et al.* 2000) to evaluate the assignment of the unknown group of samples (putative *A. texanum* individuals with *A. barbouri* mtDNA) to either potential parental species. We followed the method of Gibbs *et al.* (2010) by first confirming that the *A. barbouri* and *A. texanum* groups are clearly detected as distinct clusters and then including the unknown group, while specifying $K = 2$ genetic clusters in the analysis. All STRUCTURE analyses were run using all 39 individuals in an admixture model with allele frequencies correlated and without sample location priors (Hubisz *et al.* 2009). Each run included a burn-in period of 5×10^5 repetitions followed by 7.5×10^5 MCMC repetitions. Convergence was determined based on the examination of ln likelihood graphs and the consistency of results across three separate runs. Lastly, we calculated *q*-values and associated 95% confidence intervals to interpret the proportion of unknown individuals' genomic assignment to the two parental species.

Second, we used the program MIGRATE-N (version 3.3.1; Beerli 2006) to estimate directional migration rates and effective population sizes using all 39 individuals and the 10 nuclear loci described above. An advantage of MIGRATE-N is that it provides a framework to evaluate the fit of different multiple migration models to the data by comparing Bayes factors. To test hypotheses about the evolutionary origin of the unknown samples, we compared seven *a priori* migration matrix models (Beerli & Palczewski 2010; Fig. S1, Supporting information). Three of the proposed models reflect gene flow

scenarios that would support the three main explanations for mitochondrial-range mismatch: misidentification, mtDNA introgression, and hybridization. Two additional models were included that added a one-way migration rate between the unknowns and *A. barbouri*. These models reflect the potential directionality of a historical hybridization event that would result in mtDNA introgression into *A. texanum*. Finally, two global models were included that reflect either symmetric gene flow or unidirectional gene flow between the three groups. For each MIGRATE-N analysis, initial theta and migration values were generated using the default F_{ST} calculation and the initial genealogies were sampled started from a random tree. As no previous information concerning migration rates or theta values for these species was available, we used uniform priors and slice sampling for parameter distributions. Static heating was used with temperatures of 1, 1.5, 3 and 1×10^6. Three long chains with 1×10^4 burn-in repetitions followed by 1×10^4 recorded steps for every 100 steps, resulting in a total of 1×10^6 sampled genealogies. Convergence was determined by investigating the smoothness of parameter histograms and the consistency of results across three separate runs. The probability of different models in fitting the data was then compared using Bayes factors as described in Beerli & Palczewski (2010).

Third, we used the program IMA2 (Hey & Nielsen 2004) to estimate parameters (e. g. divergence times) not estimated with MIGRATE-N. Unlike MIGRATE-N, IMA2 explicitly accounts for shared retained ancestral polymorphism between taxa and does not assume that the taxa under study are at genetic equilibrium (Hey & Nielsen 2004). Based on the results from STRUCTURE (see below), we assumed that the unknown samples contained true *A. texanum* nuclear genomes. Therefore, we pooled these samples with the designated *A. texanum* samples and compared this sample ($n = 28$ individuals) with the *A. barbouri* group ($n = 11$). We ran all sequences through the Perl script IMGC (Woerner *et al.* 2007) to confirm that the data represented single nonrecombining blocks of sequence which conformed to an infinite allele mutation model. We found deviations from this model for seven loci. Based on program recommendations, we deleted between 1–7 individual sequences (mean = 3.8) to satisfy the mutation and recombination assumptions and used this modified data set in subsequent IMA2 analyses.

We analysed these data under a two population isolation-migration model in which we estimate effective population size of the ancestor to both salamander species, time at which this ancestor diverged into the two present-day taxa, the effective population sizes of these contemporary taxa, and levels of ongoing gene flow between *A. barbouri* and *A. texanum* since their origin. We set up MCMC runs of IMA2 using a variety of starting random seeds, heating schemes and run lengths until we obtained repeatable estimates of all parameters. Our final run consisted of an MCMC run of 3×10^7 generations with a moderate level of chains (30) run with an aggressive heating scheme. We report parameter estimates based on the high point for the parameter distribution, along with error estimates based on the high and low values that define 95% of the distribution for each parameter estimated. To translate model estimates into demographic estimates, we followed the IMA2 manual using an estimated mutation rate of 8.5×10^{-9} per base per year derived from available estimates of mutation rates in single copy nuclear DNA in vertebrates (see Kubatko *et al.* 2011) and a generation time of 2.5 years based on observed age of first reproduction in *Ambystoma* (Petranka 1998).

Finally, to incorporate a long-term evolutionary perspective with phylogenetic analyses, we used two multispecies coalescent species tree estimators, *BEAST (version 1.7; Heled & Drummond 2010; Drummond *et al.* 2012a,b) and BUCKy (Larget *et al.* 2010) to estimate relationships among 'taxa' defined as the two groups of samples designated *a priori* as *Ambystoma* sp. and the unknown samples. The *BEAST analysis uses information from multiple gene trees to estimate a species tree. The BUCKy analysis similarly combines information from multiple gene trees, but within the framework of Bayesian concordance analysis (Larget *et al.* 2010). As outlined in Table 1, each hypothesis predicts different phylogenetic relationships among samples. Under the misidentification hypothesis, unknowns should group with *A. barbouri* samples, while the introgression hypothesis predicts the opposite pattern (unknowns with *A. texanum* samples). Finally, if ongoing hybridization is present the unknowns should show no clear grouping of unknowns with either *A. barbouri* or *A. texanum* samples.

For both phylogenetic analyses, we generated gene trees for each of the 10 loci with the MRBAYES plugin (version 3.2.1; Huelsenbeck & Ronquist 2001) implemented within the program GENEIOUS (version 5.6, Drummond *et al.* 2012a) using a generalized time-reversible model, unconstrained branch lengths, 1×10^6 chain length, and a 1×10^5 burn-in period (Fig. S2, Supporting information). Locus-specific nucleotide substitution models were chosen using JMODELTEST (version 2.1.2; Guindon & Gascuel 2003; Darriba *et al.* 2012) and implemented in *BEAST using BEAUTi version 1.6.1. Molecular clocks were imposed on all loci, the species tree prior was set to the Yule process, and the population size model was set to piecewise constant. We ran three independent runs with a chain length of 5×10^8, and parameters were logged every 5×10^4 generations.

The log files from these three runs were combined using LOGCOMBINER (version 1.6.1). We used TRACER (version 1.5) to examine effective sample size values and posterior estimate graphs. The resulting species tree was visualized using FIGTREE (version 1.4.0; http://tree.bio.ed.ac.uk/software/figtree/). For BUCKy analyses, gene trees were summarized with MBSUM by randomly selecting 1001 trees from each locus, and the summary trees were implemented using default parameters. We completed three independent BUCKy analyses at alpha values of 0, 1, and infinity. The alpha value indicates the level of discordance among gene tree topologies, with 0 and infinity representing no discordance and complete independence, respectively.

Morphological and environmental analyses

To supplement the genetic analyses, we also conducted a limited comparison of morphology, mtDNA assignment, and geographical location. The purpose of examining morphology was to potentially confirm the presence of individuals in central Ohio with a mismatch between mtDNA haplotype and species-specific morphology as suggested by the genetic analyses (see below). For this, we collected five individual salamanders from the same Crawford County (Ohio) sites as above during May 2012. Animals were sacrificed using an overdose of Tricaine (MS-222, 5 grams/litre) according to Institutional Animal Care and Use Committee (Protocol #2012A00000039) protocols. The maxillary tooth morphology was then examined and classified as either *A. barbouri*- or *A. texanum*-specific based on criteria described in Kraus & Petranka (1989).

Second, we were interested in whether there were differences in environmental conditions between sites occupied by unknowns and pure *A. texanum*. To limit the effects of spatial autocorrelation, we only included the samples from the *A. texanum* and unknowns that were sympatric, resulting in the inclusion of 22 individuals across 11 contiguous counties in central Ohio. For each of these sites, we extracted site-specific worldclim variables (Hijmans *et al.* 2005) with DIVA-GIS v. 4.0 (Hijmans *et al.* 2001) from locations containing each type of sample and used a principal component analyses (PCA) with SPSS v. 17 (IBM Corporation, Somers, NY, USA) to summarize the variation for each site. These factor scores for PCA 1–3 were then used as dependent variables for a MANOVA comparing the environmental characteristics of between sites with the *A. texanum* and unknown groups.

Results

mtDNA and nuDNA genotyping

Mitochondrial genotyping identified 15 unique haplotypes across the 39 samples from 31 sites across Ohio and Indiana. The phylogenetic tree produced by MRBAYES with all of the samples, including six reference *A. barbouri* individuals and two reference *A. texanum* individuals, clearly separated into two well-supported clades (Fig. 2). Fourteen individuals grouped with the *A. texanum* reference samples, whereas all other samples clustered with the reference *A. barbouri* individuals. Twelve of the samples with *A. barbouri*-like mtDNA haplotypes were well outside of the known range of *A. barbouri*, similar to the 'unknown' samples identified by Greenwald & Gibbs (2012). Following the mtDNA haplotype classification of the samples into *A. barbouri*, *A. texanum* and unknowns, all 39 individuals were successfully sequenced and aligned at the ten nuclear loci (Tables S1–S3, Supporting information) and these data were used for subsequent analyses. Six of the ten loci significantly differed from HWE after using a Bonferroni-corrected significance level ($P < 0.002$). Departures from HWE were group-specific, as only a single locus (E13E02) was in violation within all three groups (Table S4, Supporting information). No loci were found to be in linkage disequilibrium.

Bayesian clustering

Samples classified *a priori* as *Ambystoma barbouri* and *A. texanum* were perfectly segregated under a $K = 2$ model in STRUCTURE with all individual assignment probabilities >0.99 (results not shown). When the analyses were repeated under a $K = 2$ model with the unknowns included, all unknown individuals were assigned to the *A. texanum* cluster (Fig. 3). The mean assignment coefficient to cluster two ($q2 \pm 95\%$ confidence interval)

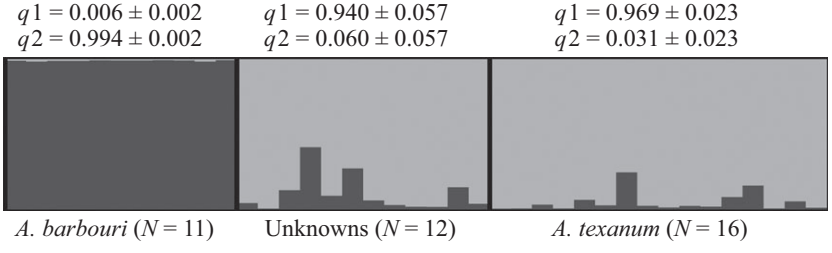

Fig. 3 STRUCTURE bar plot for $K = 2$ with q-values (SE) that represent proportion of ancestry to each group. Plot was created with distruct (version 1.1; Rosenberg 2003).

for *A. barbouri* was 0.994 ± 0.002. The mean assignment coefficients to cluster one ($q1$ ± 95% confidence interval) for *A. texanum* and the unknowns were 0.969 ± 0.023 and 0.940 ± 0.057, respectively. The log likelihood plots showed convergence in all runs, and three repeated runs generated the same results. To confirm that our findings were robust to the assumption made by STRUCTURE of HWE within populations, we reanalysed our data using an alternative method which does not assume HWE (k-sample clustering implemented in the R package *adegenet* Jombart 2008; R Development Core Team 2011) and obtained the same result (not shown).

Estimates of population size and gene flow

The 'from *A. barbouri*' MIGRATE-N model had the highest probability among the models considered (Bezier lmL = −6077.44, model probability = 1.0, Fig. 4). This model includes symmetric gene flow between the *A. texanum* and unknown groups and one-way gene flow from *A. barbouri* to the unknown group. Theta values for all three groups were similar in magnitude, with *A. barbouri* having a slightly lower value (Θ = 0.00139) compared with either *A. texanum* (Θ = 0.00168) or the unknown group (Θ = 0.00162). The migration statistic *M* (immigration rate/mutation rate), which measures the relative importance of immigration over mutation as a source of novel variation in a population, was more than an order of magnitude higher for the symmetric gene flow parameter between the unknowns and *A. texanum* (*M* = 9534.1) than the one-way gene flow parameter from the *A. barbouri* to the unknown group (*M* = 347.0). These results indicate that *A. texanum* and unknown samples form a single panmictic unit with the addition of a relatively small amount of gene flow from *A. barbouri* to the unknown group. This supports the hypothesis that the mitonuclear divergence found in the unknowns is due to mtDNA introgression from *A. barbouri* into central Ohio populations of *A. texanum*.

IMA2 analyses identify a small ancestral population of ~4000 individuals which split into existing populations of *A. texanum* and *A. barbouri* approximately 400 000 years before present (ybp), although error estimates of this value are large (95% of estimated values: 261 538–3750 000 ybp). Contemporary populations of both species have effective population sizes that are 3–8 times larger than the ancestral population [point estimate of N_e for *A. texanum*: 12 462 individuals (95% value range: 6808–21 432); N_e for *A. barbouri*: 32 885 (95% range: 20 500–50 846)]. In contrast with population size estimates produced by MIGRATE-N, the more northerly species (*A. texanum*) has a smaller effective population size. Additionally, there are low levels of ongoing gene flow between these species after considering shared similarity due to retained ancestral polymorphism. Coalescent-based estimates of the number of effective migrants moving from *A. texanum* to *A. barbouri* is 0.164 (95% range: 0.026–0.673), while the same value for gene flow in the opposite direction is slightly higher (0.285 [95% range: 0.018–1.26]) although the 95% ranges of values for each point estimate substantially overlap. These results show that although *A. barbouri* and *A. texanum* are a recently evolved pair of sister species, they have been isolated for

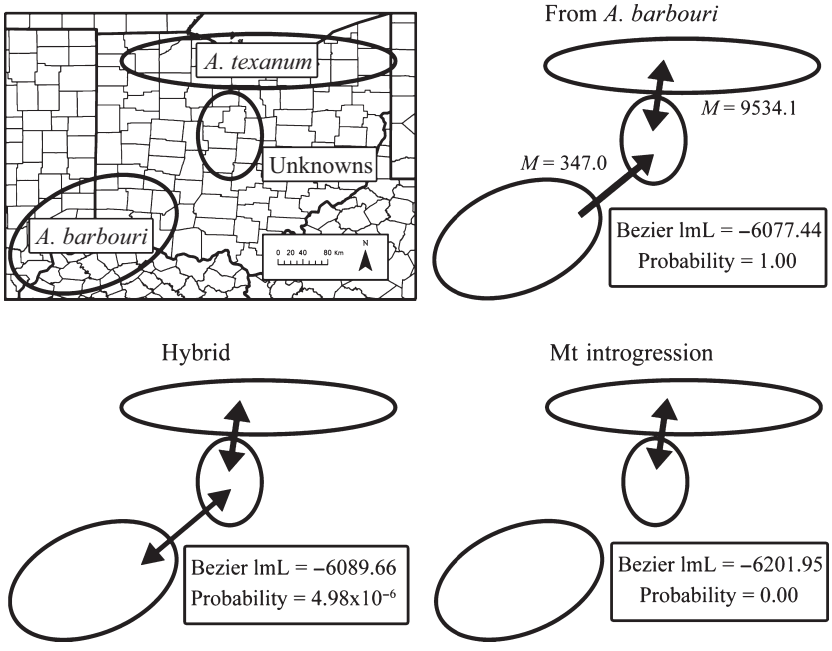

Fig. 4 Probabilities of three primary hypotheses regarding the identification of mismatched mtDNA haplotypes in central Ohio *Ambystoma*. Migration rates (*M*) and Bezier log likelihood values were produced with MIGRATE-N and probabilities were calculated as in Beerli & Palczewski (2010).

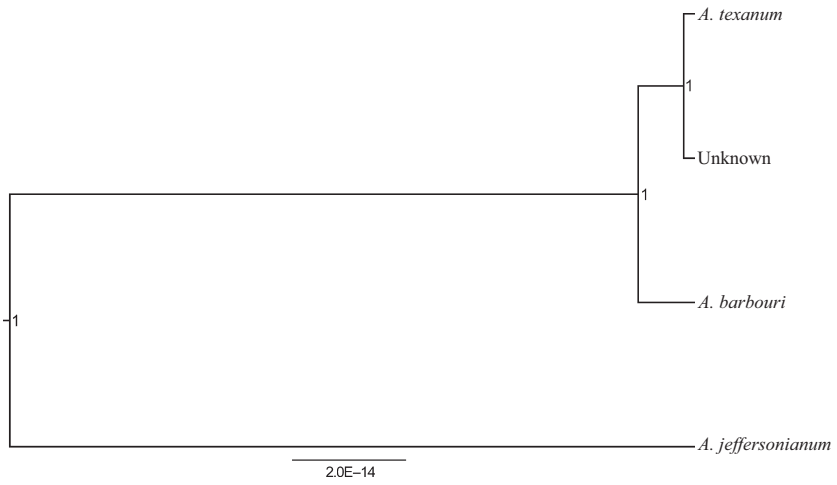

Fig. 5 Result of maximum clade credibility species tree analysis with *BEAST including 10 nuclear loci from all unknown, *Ambystoma texanum* and *A. barbouri* individuals. Numbers on branches indicate posterior probabilities.

a substantial period of time (>100 000 generations) but also continue to experience limited (but nonzero) amounts of gene flow.

Phylogenetic analyses

The 10 gene trees produces by MRBAYES provided varying levels of resolution for the three groups (Fig. S2, Supporting information). The *BEAST analysis including all 10 nuclear loci produced a species tree topology with a highly supported clade ($P = 1.0$) consisting of *A. texanum* and the unknowns with *A. barbouri* as a sister group (Fig. 5). This result supports the conclusion of mtDNA introgression from *A. barbouri* into *A. texanum* (Table 1). In contrast, the BUCKy analysis produced a single poorly resolved tree with low concordance factors (Fig. S3, Supporting information) and hence is uninformative in discriminating among the hypotheses in Table 1.

Morphological and environmental analyses

The mtDNA analysis of five adult salamanders collected for morphological analyses showed they contained a mix of *A. barbouri* haplotypes ($n = 3$) and *A. texanum* haplotypes ($n = 2$). However, all five individuals displayed maxillary teeth with pointed cusps that are diagnostic of *A. texanum* (see Fig. 2 in Kraus & Petranka 1989; data not shown).

The PCA procedure generated three components that together explained 94.47% of the total variation. Principal component 1 (43.28% of total variation explained) had high loadings (>0.093) from five bioclim variables related to rainfall (annual precipitation, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter and precipitation of coldest quarter). Therefore, PC 1 was interpreted as capturing the variation in precipitation. The second component (33.75% of total variation explained) captured variation in temperate (bioclim variables with loadings >0.92: annual mean temperature, maximum temperature of warmest month, mean temperature of wettest quarter, mean temperature of warmest quarter and mean temperature of coldest quarter). Principal component 3 (17.44% of total variation explained) reflects annual variation in temperature and precipitation (variables with high (> 0.80) loadings: temperature seasonality, precipitation seasonality, and annual temperature range).

Overall, PCA components were significantly different between the sympatric *A. texanum* and unknowns (MANOVA, $F = 9.507$, hypothesis d.f. = 3, $P < 0.001$). However, a component-by-component analysis shows that only PC 1 scores were significantly different between the two groups ($t = -3.457$, 95% confidence interval = −(1.924–0.476), $P = 0.002$). Therefore, unknown individuals that were sympatric with *A. texanum* individuals were associated with sites that had higher amounts of annual precipitation. However, the differences in mean annual precipitation between the *A. texanum* (942.5 mm ± 4.24 SE) and unknown samples (973.5 mm ± 8.27 SE) are relatively small.

Discussion

Our genetic analyses show that the unknown salamanders in central Ohio are *A. texanum* individuals with introgressed *A. barbouri* mtDNA. Our work has both methodological and evolutionary implications for studying mitonuclear discordance in nature. Below, we discuss issues to do with using multiple analyses of genetic data to investigate mitonuclear discordance, their relationship to past observations of introgression in *Ambystoma*, and what mechanisms may have led to the observed patterns in these salamanders and the general implications of our work for understanding the causes of mitonuclear discord in vertebrates.

Value of multiple methods of analysis

Our study illustrates the power of using multiple analyses to investigate presumed cases of mitonuclear discordance and offers more comprehensive approach than many recent studies based on mtDNA and nuDNA variation (Nevado *et al.* 2011; Sequeira *et al.* 2011; Melo-Ferreira *et al.* 2012). The methods we used represent novel approaches to investigating discordance and were chosen to complement each another in light of the strengths and limitations of each technique. For example, our use of model selection within MIGRATE-N was novel in that it allowed us to statistically compare the likelihood of different models accounting for patterns of discordance as described in Table 1. These results also provide information concerning the directionality of the mtDNA introgression, as there was well-supported unidirectional gene flow from *A. barbouri* to the unknown group. This suggests the mtDNA introgression resulted from *A. barbouri* individuals invading populations of *A. texanum*. While model selection has not been commonly used in investigations of mitonuclear discordance, it is increasingly becoming an important tool for evaluating demographic hypotheses about phylogeography (Carstens *et al.* 2013), hybridization (Kubatko 2009) and species delimitation (Csilléry *et al.* 2010; Camargo *et al.* 2012), and we see an important role for it in future investigations of genetic discordance in natural populations. A weakness of MIGRATE is that it assumes a migration-drift equilibrium in each population and does not account for the potential impact of incomplete lineage sorting (ILS) on levels of genetic similarity between populations. We feel this is not a significant issue for two reasons: first, the estimated average time for lineage sorting to occur after divergence between populations is 2–3 Ne generations (Neigel & Avise 1986). Based on our estimates of Ne (<40 000) and generation time (2.5 years–see above), there has been sufficient time for lineage sorting to have taken place in these species as they diverged. Second, the results of the IMA2 program, which takes into account ILS, match the best supported model from MIGRATE-N in terms of directions and magnitude of migration. When investigations of mitonuclear discordance have relied solely on observing conflicting patterns of mtDNA- and nuDNA-based phylogenetic trees (Di Candia & Routman 2007; Bossu & Near 2009; Chen *et al.* 2009; Spinks & Shaffer 2009), the effects of ILS have been difficult to address. The same can be said for analyses that make interpretations based on a clustering method such as STRUCTURE (Gompert *et al.* 2008; Veith *et al.* 2012). Methods such as STRUCTURE are valuable in that they have few assumptions but are limited in their ability to produce specific parameter estimates. However, recent work has shown that both IMA2 (Strasburg & Rieseberg 2010) and species tree analyses (Knowles & Carstens 2007) are relatively robust to violation of assumptions and do account for ILS. Here, we show that leveraging analyses that do and do not account for ILS can provide a thorough and complete evaluation of the timing and direction of introgression.

Mitonuclear discordance in Ambystoma

Amphibians are one of the most common groups in which mitonuclear discordance has been identified, although there are examples from other animals (reviewed in Toews & Brelsford 2012). However, the majority of identified cases of mitonuclear discordance have been recognized in frogs, with many fewer cases in salamanders (Chan & Levin 2005). *Ambystoma* salamanders provide many examples of genetic introgression, including adaptive introgression from invasive into native species (Ryan *et al.* 2009) and extensive introgression between multiple species within the unisexual *Ambystoma* complex (Bi & Bogart 2006; Bogart *et al.* 2007; Bi *et al.* 2009). Specifically, mitochondrial haplotypes originally derived from *A. barbouri*, similar to those described here in *A. texanum*, are found within the entire unisexual *Ambystoma* complex (Robertson *et al.* 2006; Bogart *et al.* 2007). Unisexuals are hypothesized to be the result of an ancient hybridization involving a common ancestor most similar to *A. barbouri*. The persistence of this independent mitochondrial lineage, given the cytonuclear interactions of up to five genomes from other *Ambystoma* species, suggests that there is some property of *A. barbouri*-like haplotypes or *Ambystoma* nuclear genomes that allow for reduced cytonuclear conflict after introgression (Bogart *et al.* 2007, 2009).

This study is not the first to discover *A. barbouri*-like mtDNA haplotypes within *A. texanum* populations, yet it is the first to characterize discordance and evaluate putative causes. A range-wide genetic survey by Niedzwiecki (2005) revealed a single *A. texanum* individual from Greene County Ohio that contained an *A. barbouri* mtDNA haplotype, and a single individual from southern Indiana was identified by Eastman *et al.* (2009). Greene County is one county north of Warren County, where we identified all specimens sampled as pure *A. barbouri*. The individual identified by Niedzwiecki suggests that the sampling gap between central and southwestern Ohio in our study likely contains populations of *A. texanum* with *A. barbouri*-like mtDNA. Because the range of *A. texanum* is many times larger than that of *A. barbouri*, it is surprising that no other mtDNA mismatches have been found, especially near the other recognized zone of introgression between the

two species in western Kentucky. This geographical pattern suggests that *A. texanum* may have carried mtDNA northward from previous introgression events during glacial maxima. This scenario is supported from other studies which show that species with expanding ranges are more likely to carry introgressed mitochondria from other sympatric species with more stable distributions (Petit & Excoffier 2009; Keck & Near 2010). Finally, our results from MIGRATE-N and IMA2 do support low levels of historical gene flow from *A. barbouri* into *A. texanum* which is counter to reports of these species being strongly isolated from each other even when in close proximity (Kraus & Petranka 1989). While having separate breeding habitats could limit the chances of hybridization between these species, antipredator adaptations against fish predation on *A. barbouri* may reinforce the reproductive barrier between *A. texanum* and *A. barbouri* (Storfer & Sih 1998).

A unidirectional pattern of mtDNA introgression from *A. barbouri* into *A. texanum* is biologically likely for two reasons. First, even though these species breed in different habitats, there are more observations of *A. barbouri* using *A. texanum* habitat than the opposite. While *A. barbouri* primarily breed in headwater streams, there are multiple accounts of *A. barbouri* breeding in ponds (Kraus & Petranka 1989; Venesky & Parris 2009). In contrast, there are fewer reports of *A. texanum* breeding in streams (Petranka 1984), supporting a higher likelihood that the original source of genetic introgression was from *A. barbouri* individuals invading *A. texanum* populations. Secondly, the dispersal of *A. barbouri* into the range of *A. texanum* could be explained by a combination of habitat connectivity and reduced landscape resistance. The northeastern extent of the *A. barbouri* range lies within the same major river drainage (The Scioto River) of the unknown samples which may have provided a likely corridor for the movement of *A. barbouri* individuals.

Mechanisms of mitochondrial introgression

Multiple processes have been hypothesized to be responsible for cases where mtDNA has introgressed from one species into another, and this work takes a novel approach to testing these hypotheses. Most mechanisms fall into the categories of adaptive introgression, demographic differences and sex-biased asymmetries (Toews & Brelsford 2012). While patterns of mitonuclear discordance have been identified across many taxa, few studies have explicitly linked a pattern of discordance to a particular process. Instead, many authors have proposed mechanisms as determined by the geographical patterns of discordance (extent of mtDNA introgression and frequency of introgressed haplotype) and characteristics of the focal species (sex determination, mating strategies and relative abundances). In this light, the mitochondrial introgression in *A. barbouri* and *A. texanum* is unusual in the distance within the range of *A. texanum* that the *A. barbouri*-like haplotypes have spread. When foreign mtDNA haplotypes appear at a distance >50% of the total range, these foreign haplotypes tend to be at fixation, suggesting an adaptive introgression of mtDNA (Quesada *et al*. 1999; McGuire *et al*. 2007; Melo-Ferreira *et al*. 2009; Brelsford *et al*. 2011). In the case of these two *Ambystoma* species, *A. barbouri*-like mtDNA haplotypes have been detected in far <50% of the range of *A. texanum*, but the distance from the nearest area of sympatry that these introgressed haplotypes are found is relatively large (~150 km). While the geographical extent of discordance suggests that the mtDNA haplotypes may provide an adaptive advantage, the frequency of introgressed haplotypes is not near fixation as one would predict. Recent preliminary sampling within the transitional gradient of mtDNA haplotypes (Crawford County) identifies wetlands with ~50–75% introgressed haplotypes <2 km from wetlands with 100% *A. texanum* haplotypes (Denton, unpublished data).

Although adaptive introgression has been demonstrated in other amphibians (Pfennig 2007; Fitzpatrick *et al*. 2010), determining the adaptive value of introgressed mtDNA is difficult (Toews & Brelsford 2012; Toews *et al*. 2013). *Ambystoma texanum* individuals with *A. barbouri*-like mtDNA were significantly more likely to be present at localities with higher levels of precipitation. Even though the average difference in annual precipitation of sites with mitonuclear discordance was small (~3% of an average year's total), the statistical significance of this pattern within such a small geographical area lends support to this being a real biological phenomenon. Higher levels of precipitation at the sites were mitonuclear mismatch is present suggesting a role for differences in moisture in the environment as a driver of selection for the *A. barbouri* mtDNA haplotype populations of *A. texanum*. One potential explanation could involve the temporal components of each species' breeding strategies. *Ambystoma texanum* are explosive breeders that rely on the sudden filling of temporary wetlands in the spring, while *A. barbouri* breed during an overlapping period of 4–5 months from December–April (Petranka 1984). Because of the semipermanence of *A. barbouri* breeding streams, they may be more adapted to the wetter environment of stream sides and *A. texanum* with *A. barbouri*-like mtDNA are limited to wetland environments with higher precipitation. While this is not an adaptive advantage of having *A. barbouri*-like mtDNA, precipitation variables may predict the extent of introgression. This association with wetter

environments does not exclude the potential of some other beneficial property of the introgressed haplotypes for which the limitation of wetter environments is a trade-off. While more investigation is needed to determine the process behind the mtDNA introgression, recent studies have successfully uncovered the adaptive significance of introgressed mtDNA haplotypes, especially with more recent techniques to assay mitochondrial metabolism and efficiency (Ruiz-Pesini *et al.* 2004; Moyer *et al.* 2005; Grant *et al.* 2006; Toews *et al.* 2013).

Demographic differences and sex-biased asymmetries between *A. texanum* and *A. barbouri* provide less convincing explanations for the mitonuclear discordance between these species that is observed in central Ohio. A large shift in the range of *A. barbouri* that would leave behind a wake of mtDNA (Rohwer *et al.* 2001) is unlikely due to the environmental specificity of *A. barbouri*. Large discrepancies in relative abundance between species that have influenced introgression in other systems (Chan & Levin 2005; Linnen & Farrell 2007) are also unlikely due to the narrow range of sympatry and the separation of breeding habitats for each species. Another potential mechanism responsible for mitochondrial introgression would be an extension of Haldane's rule (Haldane 1922), which predicts that during hybridization, the heterogametic sex is most likely to suffer a fitness loss. In a XY sex determination system, this would predict a higher fitness for females. However, *Ambystoma* display a ZW sex determination system (reviewed in Hillis & Green 1990). This contradicts the observed pattern of introgressed mitochondria in *A. texanum*, but conclusions are difficult to make due to a lack of clarity concerning the sex determination system in *Ambystoma* and amphibians as a whole (see Robertson *et al.* 2006). Finally, female-biased dispersal could potentially initiate mitochondrial introgression, but there is no support for sex-biased dispersal in *Ambystoma* (Trenham *et al.* 2001). If any demographic differences have influenced the mitochondrial introgression between *A. texanum* and *A. barbouri*, it may be the asymmetrical behavioural reproductive isolation described above. Because breeding densities of *A. barbouri* would be predicted to be lower than *A. texanum* due to a longer breeding season, *A. barbouri* females may be less likely to discriminate against a male *A. texanum*.

Conclusions

Mitonuclear discordance is a widespread phenomenon that is likely an important force in the shaping of genetic diversity between species. Our work makes three general contributions to the study of this process in natural populations. First, it provides an example of a comprehensive methodological framework for investing this phenomenon that is based on a diverse set of approaches. In particular, Table 1 provides a model testing framework in which specific results from different analyses can be used to infer the processes underlying mitonuclear discord in any animal. Second, our results provide an example of the extent to which species boundaries are genetically permeable and a possible example how selection acting through environmental variation may constrain mitochondrial introgression between species (Ballard & Melvin 2010). Finally, our results provide yet another caution of the sole use of mtDNA for species identification (e.g. DNA barcoding) in taxa with poorly known geographical distributions (Rubinoff 2006).

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Data accessibility

DNA sequences: Genbank accessions KJ610094–KJ610523

DNA sequence assemblies used in analyses, input files and climate data: Dryad DOI:10.5061/dryad.n0r41

Sampling locations are uploaded online as supplemental material.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Identification information for 37 salamander individuals from Ohio and Indiana.

Table S2 Nucleotide identity at 20 polymorphic sites used to distinguish *Ambystoma texanum* and *A. barbouri* with a 346 bp section of mtDNA control region using primers F-THR and R-651 (Shaffer & McKnight 1996, Bogart *et al*. 2007).

Table S3 Details of 10 nuclear loci that were sequenced for 37 *Ambystoma* salamanders.

Table S4 Genetic summary information for each nuclear locus by group.

Fig. S1 Probabilities and descriptions of all tested models regarding the identification of mismatched mtDNA haplotypes in central Ohio *Ambystoma*.

Fig. S2 Gene trees produced for each nuclear locus across 37 *Ambystoma* individuals using the MRBAYES plugin (version 3.2.1; Huelsenbeck & Ronquist 2001) implemented within the program GENEIOUS (version 5.6, Drummond *et al*. 2012a).

Fig. S3 Consensus species tree for 37 *Ambystoma* salamanders using the program BUCKY (Larget, Kotha, Dewey, & Ané, 2010).