

THESIS

NATURAL CASES OF SALAMANDER HYBRIDIZATION SUGGEST A CONSISTENT
RELATIONSHIP BETWEEN GENETIC DISTANCE AND REPRODUCTIVE ISOLATION
ACROSS TETRAPODS

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ABSTRACT

NATURAL CASES OF SALAMANDER HYBRIDIZATION SUGGEST A CONSISTENT RELATIONSHIP BETWEEN GENETIC DISTANCE AND REPRODUCTIVE ISOLATION ACROSS TETRAPODS

Hybridization between populations along the path to complete reproductive isolation can provide snapshots of speciation in action. Here, we present the first comprehensive list of natural salamander hybrids and estimate genetic distances between the parental hybridizing species using a mitochondrial and nuclear gene (MT-CYB and RAG1). Salamanders are outliers among tetrapod vertebrates in having low metabolic rates and highly variable sex chromosomes. Both of these features might be expected to impact speciation; mismatches between the mitochondrial and nuclear genomes that encode the proteins for oxidative metabolism, as well as mismatches in heteromorphic sex chromosomes, can lead to reproductive isolation. We compared the genetic distances between hybridizing parental species across four main tetrapod clades that differ in metabolic rates and sex chromosome diversity: salamanders, lizards, mammals, and birds. Our results reveal no significant differences, suggesting that variation in these traits across vertebrates does not translate into predictable patterns of genetic divergence and incompatible loci in hybrids.

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INTRODUCTION

As lineages diverge (i.e. species form), the separating populations accumulate genetic distance, and eventually the distance becomes great enough to prevent reproduction, keeping the species on completely independent trajectories (de Queiroz 1998). Studying this process is challenging because it typically occurs over long timescales, but populations along the path to complete reproductive isolation can provide snapshots of speciation in action (Harrison and Larson 2016; Soltani et al., 2017). Classic examples include ring species such as *Ensatina eschscholtzii*, where different steps of speciation can be examined in a single taxon by looking at how the populations distributed around the geographic “ring” interbreed or hybridize (Pereira and Wake, 2009; Devitt et al., 2011). Specifically, hybrids provide an opportunity to identify the key genetic factors that become reproductive barriers when divergent genomes mix (Arnold 1997; Toro et al., 2002; Harrison and Larson, 2016; Qvarnström et al., 2016). Typically, populations that come into contact and interbreed after isolation will have alleles interact in new, untested ways (Barton 1985). If this mixture of alleles is harmful, such as the hybrid being sterile or having low fitness, these negative interactions are called Dobzhansky–Muller or Bateson–Dobzhansky–Muller incompatibilities (BDMIs) and are a source of reproductive isolation (Bateson, 1909; Dobzhansky 1937; Muller 1942; Orr 1996).

One special case of BDMIs is when the mitochondria and the nucleus are mismatched after hybridization — i.e. mitonuclear discordance (Ellison and Burton 2007; Sloan et al., 2017; Hill et al., 2019). When the nucleus comes from one species and the mitochondria comes from a second, the gene products encoded by the two organelles can’t always functionally interact, causing reduced fitness and contributing to reproductive isolation. More specifically, this

reduced fitness can be reflected in the metabolic costs of being a hybrid, which include increased respiration rates, increased levels of reactive oxygen species (ROS), and increased metabolic rate (Olson et al., 2010; Gvoždík 2012; Borowiec et al., 2016; McFarlane et al., 2016; Prokić et al., 2018). However, if an organism has a low metabolic rate to begin with, this drop in OXPHOS productivity might have a smaller effect on overall hybrid fitness. This could allow hybridization between species with more genetically distant mitochondrial and nuclear genomes before mismatches between the two parental species cause reproductive isolation. Tetrapods are a good model system in which to test this hypothesis because of the wide range of metabolic rates that exists in the clade (Pough 1980; White et al., 2006; Anderson and Gillooly 2018). Literature reviews summarizing natural hybrids in birds, mammals, and lizards have been published previously (Fitzpatrick 2004; Jančúchová-Lásková et al., 2015); birds have the highest metabolic rates, followed by mammals, and then lizards. Salamanders (Order Caudata) are an important clade to incorporate into a comparative analysis of tetrapods because they have the lowest metabolic rates (Pough 1980; Glatten et al., 1992), and there are many reported cases of salamander hybrids. With their low metabolic requirements, salamander hybrids might be able to tolerate greater levels of genetic divergence between parental species before complete reproductive isolation occurs.

Sex chromosomes are also important during speciation and the emergence of reproductive isolation or hybrid incompatibility (Lima 2014) because of Haldane's rule, where the heterogametic sex is more likely to be infertile by a variety of potential mechanisms, or the related large X/Z effect, where a disproportionately high amount of hybrid incompatibilities are found on either the X or Z chromosome (Presgraves 2008; Lavretsky et al., 2015; Janoušek et al., 2018). In hybrid zones, mutations in sex chromosomes have been shown to limit introgression

(Cortés-Ortiz et al., 2018) or even cause complete reproductive isolation between lineages (Johnson and Lachance, 2012; Hooper et al., 2018). Compared to other tetrapod clades, salamanders have variable genetic sex determining mechanisms with either homomorphic sex chromosomes or heteromorphic sex chromosomes and either ZW or XY systems (Eggert 2005). This makes the salamander clade a good system for studying the effects of heteromorphic sex chromosomes on reproductive isolation (Evans et al., 2012), as this diversity yields a snapshot into heteromorphic sex chromosome evolution (Charlesworth et al 2005; Evans et al., 2012).

There are numerous studies published on hybrids in salamanders, both from long-term stable hybrid zones and conservation efforts (Fitzpatrick and Shaffer, 2004; Fukumoto et al., 2015), but to date, there is no published review of natural salamander hybrids. With a few notable exceptions (e.g. Twitty 1963; Gvoždík 2012; Prokić et al, 2018), there have not been extensive published experimental crosses in salamanders like those done in other vertebrate clades (e.g. toads, Malone and Fontenot, 2008). Here, we summarize the known cases of natural hybridization in salamanders. We then use this dataset to compare the genetic distances across which viable hybrids can form in different tetrapod clades and test whether differences in metabolic rate and sex chromosomes impact hybridization.

MATERIALS AND METHODS

Compilation of Salamander Hybrids

The first goal was the establishment of a comprehensive list of published salamander hybrids (Table 1). Taxonomy is continually revised to provide scientific names that convey accurate information about species boundaries as well as the evolutionary relationships among species. Making these species designations is a rich discipline, with disagreements caused by taxonomists requiring different levels of divergence or isolation between populations before they are formally named as species (Highton 1998; Kuchta and Wake 2014). For this review, we used a general lineage concept of species where a species equates to a population-level evolutionary segment (de Queiroz 1998). We looked for populations far enough along their own evolutionary trajectories that when secondary contact occurred, the populations did not completely admix into a single population. By doing this, we were able to include several instances of hybridization in which the hybridizing populations have not been formally named as species, which allowed us to compile the maximum amount of information about hybrid salamanders.

Database searches were performed using the terms salamander, newt, hybrid, and contact zone. Databases used were JSTOR, Web of Science, and Wildlife & Ecology Studies Worldwide. Using the same terms, additional sources were found with the search engine Google Scholar. The searches were undertaken from January 2018 to June 2019 and included research published between 1979-2019. Many taxonomic changes occurred during this 40-year period; whenever there was a conflict in species name, the current listing on AmphibiaWeb (amphibiaweb.org) was used to resolve the issue.

Intensity of Research on Different Salamander Families

To check whether the reported number of hybrids within each salamander family was a function of the intensity of publication, a general review was also conducted on how well each family of salamanders is represented in literature. Using the Web of Science database, searches were performed using terms based on variations on the salamander family names with two to three search terms used per family. Terms used were: Ambystomatidae (Ambystomatid and *Ambystoma*), Amphiumidae (Amphiumid and *Amphiuma*), Cryptobranchidae (Cryptobranchid and *Cryptobranchus*), Dicamptodontidae (Dicamptodontid and *Dicamptodon*), Hynobiidae (Hynobiid and *Hynobius*), Plethodontidae (Plethodontid and *Plethodon*), Proteidae (Proteid), Rhyacotritonidae (Rhyacotritonid and *Rhyacotriton*), Salamandridae (Salamandrid and *Salamandra*), and Sirenidae (Sirenid). The genus names *Proteus* and *Siren* were excluded due to their use in Greek mythology; because the corresponding salamander families are small, missing papers is unlikely. The number of articles per family was recorded divided by the number of species in each family based off AmphibiaWeb as of June 2019 (amphibiaweb.org). To test whether the reported number of hybrids within each salamander was related to the intensity of publication, a Kendall correlation coefficient was calculated comparing papers per species and hybrids per species for each family.

Genetic Distances Between Hybridizing Species of Salamanders

The genetic distances across the parental species for each hybridizing salamander pair were estimated using the mitochondrial gene cytochrome *b* (MT-CYB) (Figure 2a). MT-CYB has long been used as a phylogenetic and phylogeographic marker for salamanders, so there is wide coverage for many different species (Johns and Avise, 1998). MT-CYB has also been used as a proxy for overall genetic distance in summaries of hybridizing pairs of species in other

vertebrate taxa (Fitzpatrick 2004; Jančúchová-Lásková et al., 2015). For each parental species, the longest sequence without ambiguous nucleotides was downloaded from NCBI GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). In some cases, this involved extracting the MT-CYB sequence from a complete mitochondrial genome sequence. Of the 68 salamander species pairs known to hybridize, MT-CYB sequence data were available for 52 (Table 2). For each hybridizing pair, a pairwise alignment was calculated with default ClustalW settings implemented in MEGA X (Kumar et al., 2018). Each alignment was then trimmed to the first and last overlapping nucleotide position. After trimming, the alignments ranged from 345 to 1141 base pairs. The genetic distances were estimated with PAUP * Version 4.0a (Swofford 2002). The nucleotide substitution model used was HKY85 + Γ (Swofford 1998). In order to maximize the amount of sequence data used, the Γ distribution shape parameter α was estimated using the MEGA X maximum likelihood model selector based on a ClustalW alignment of all 73 salamander MT-CYB sequences used in this study (Kumar et al., 2018). To test whether differences between the two largest salamander families (Plethodontidae and Salamandridae) drive overall patterns of genetic distances across which salamander hybrids form, a non-parametric Mann-Whitney test was used.

In order to test how representative MT-CYB genetic distances are in describing the overall genetic divergence between species, the substitution rate of mitochondrial genes versus nuclear genes was compared. This was accomplished by dividing the genetic distance of the mitochondrial gene MT-CYB by the genetic distance of the nuclear gene recombination activating 1 (RAG1) for the parental species that hybridize (Table 2). RAG1 was selected since the gene has widespread use in phylogenetic studies and was available for the most taxa (Chiari et al., 2009). For every parental species available, the longest RAG1 sequence without

ambiguous nucleotides was downloaded from GenBank. RAG1 genetic distances between parental species that hybridize were then estimated using the same methods as for MT-CYB genetic distances.

Genetic Distances Between Hybridizing Species of Salamanders Compared with Hybrids in Other Vertebrate Clades

The MT-CYB genetic distances between hybridizing parental species of salamanders were compared to those of birds, mammals, and lizards. These clades were selected because they span part of the range of vertebrate metabolic rates (Pough 1980; White et al., 2006; Anderson and Gillooly 2018) and because of the existence of published summaries of known, naturally occurring hybrids (Fitzpatrick 2004; Jančúchová-Lásková et al., 2015). A hybrid toad summary was excluded because it focused primarily on experimental crosses (Malone and Fontenot, 2008). In some vertebrate classes, many closely related species hybridize, or a singular species hybridizes several times. This creates a potential bias when looking at many pairwise comparisons as a single taxon becomes overrepresented, and the resulting data becomes dependent on a few select taxa (Fitzpatrick 2004). Previously published work in mammals and birds resolved this issue by removing repeated taxa to remove nonindependence (Fitzpatrick 2004), so the list of hybrids for salamanders and lizards was also pared down to make comparable results. For salamanders, the order was pared down to a single pair that can hybridize per genus. When a genus had multiple hybridizing species pairs, the pair with the greatest MT-CYB genetic distance was used. The list of hybrids for lizards was also pared down to the largest MT-CYB genetic distance per genus based on previously published work (Jančúchová-Lásková et al., 2015). Using this pared down list for all four vertebrate classes, MT-CYB genetic distances were estimated between the parental species for each hybridizing species

pair (Table 2). For every species included, the longest MT-CYB sequence without ambiguous nucleotides was downloaded from NCBI GenBank. A pairwise alignment was then calculated for each hybridizing species pair with default ClustalW settings implemented in MEGA X (Kumar et al., 2018). Each alignment was then trimmed to the first and last overlapping nucleotide position. As was done for the salamanders, genetic distance was estimated using PAUP * Version 4.0a with an HKY85 + Γ nucleotide substitution model (Swofford 2002). For each of the vertebrate classes, the shape of the Γ distribution shape parameter α was estimated using the MEGA X maximum likelihood model selector based on a ClustalW alignment of all MT-CYB sequences included in this study (46 birds, 50 mammals, and 40 lizards) (Kumar et al., 2018). A Kruskal-Wallis test was then used to determine if the genetic distance across parental species that hybridize differs among the vertebrate classes.

In order to test how representative MT-CYB genetic distances are in describing the overall genetic divergence between species, the substitution rate of mitochondrial genes relative to the substitution rate of nuclear genes was compared across the four vertebrate clades (Figure 4). This was accomplished by dividing the genetic distance of the mitochondrial gene MT-CYB by the genetic distance of the nuclear gene RAG1 for the parental species that hybridize (Table 2). For every parental species available, the longest RAG1 sequence without ambiguous nucleotides was downloaded from GenBank. RAG1 genetic distances between parental species that hybridize were then estimated using the same methods as for MT-CYB genetic distances. A Kruskal-Wallis test was used to determine if the ratio of MT-CYB/RAG1 genetic distances differed between the four vertebrate clades.

Relationship Between Sex Chromosomes and Hybridization in Salamanders

A list of which salamanders have heteromorphic sex chromosomes was generated to see if the presence of heteromorphic sex chromosomes impacted hybridization. Salamanders with heteromorphic sex chromosomes were identified by using two published reviews and a recently published amphibian karyotype database (Hillis and Green, 1990; Evans et al., 2012; Perkins et al., 2019). In the available literature, it is not always apparent whether a salamander species is known to have homomorphic sex chromosomes or if the species was merely never confirmed as having heteromorphic sex chromosomes (Perkins et al., 2019). As such, additional unpublished information on salamanders that are known to have homomorphic sex chromosomes was obtained (S. Sessions, Personal Communication, June 19th, 2019). MT-CYB genetic distances between parental species with heteromorphic sex chromosomes that hybridize were compared to the genetic distances between parental species with homomorphic sex chromosomes that hybridize using a non-parametric Mann-Whitney test. A comparison was also made between the overall percentage of salamanders known to hybridize and the percentage of salamanders with heteromorphic sex chromosomes known to hybridize using a one-sided exact test of goodness-of-fit. The overall results were similar whether the analyses were performed using only the species confirmed to have homomorphic sex chromosomes (based on personal communication) or using the larger dataset that assumed that all salamanders that have not been reported to have heteromorphic sex chromosomes have homomorphic sex chromosomes.

RESULTS

Natural Hybrids in Salamanders

This review found 68 pairs of genetically distinct salamander lineages that hybridize in nature (Table 1). These lineages represent 10.5% of named salamander species and 7 out of 10 salamander families (Figure 1). The seven families represented include Plethodontidae (38 species), Salamandridae (25 species), Ambystomatidae (4 species), Hynobiidae (4 species), Cryptobranchidae (2 species), Dicamptodontidae (2 species), and Proteidae (2 species) (Figure 1). The families of Amphiumidae, Rhyacotritonidae, and Sirenidae have no recorded cases of hybrids. Many pairs that hybridize come from Plethodontidae and Salamandridae, consistent with these being the two largest families of salamanders (475 and 120 species, respectively) (amphibiaweb.org). The third largest salamander family (Hynobiidae with 79 species) contains proportionally fewer hybrids with just 2 recorded pairs that can hybridize (Figure 1). There is no case of hybridization occurring between species of different genera.

Intensity of Research on Different Salamander Families

There were a total of 32,499 papers published using the salamander family related terms. Out of the large salamander families, the family with the highest number of papers per species, Ambystomatidae, did not have the highest number of reported hybrids (Figure 1). However, *Ambystoma mexicanum* is a contributor to the high paper-per-species count because it is a model lab animal and makes up a quarter (2694) of the Web of Science results for Ambystomatidae (Figure 1) (Voss et al., 2010). The two families with the highest numbers of species have the lowest intensity of publication: Hynobiidae (22 papers per species) and Plethodontidae (13 papers per species). There are proportionally more papers published for families with fewer

species: Cryptobranchidae (336 papers per species), Amphiumidae (270 papers per species), Proteidae (110 papers per species), Dicamptodontidae (107 papers per species), Sirenidae (79 papers per species), and Rhyacotritonidae (44 papers per species). There was no significant correlation between the number of papers per species and the number of salamanders found to hybridize per family (Kendall's rank correlation $P = 0.146$).

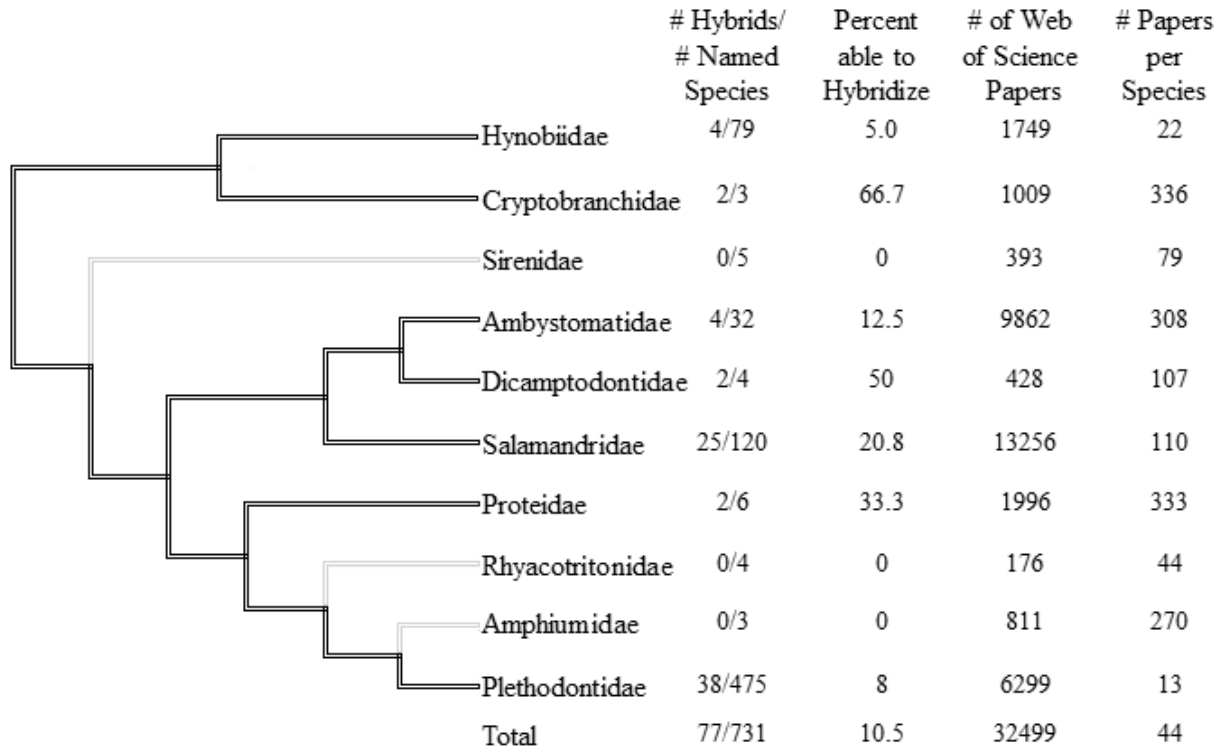


Figure 1. Salamander hybrids are found in most families (cf. Weins et al., 2011). About 10.5% of salamanders are known to hybridize with over half belonging to Plethodontidae. There was no significant correlation between the number of papers per species and the proportion of salamanders found to hybridize (Kendall's rank correlation $P = 0.146$)

Salamander Hybrid Genetic Distances

For the 52 parental pairs of salamanders that have published MT-CYB sequence data, the mean genetic distance was 0.107 (Table 2). *Lissotriton vulgaris* X *Lissotriton helveticus* had the largest genetic distance of 0.376 while *Plethodon teyahalee* X *Plethodon shermani* had the smallest distance of 0.003 (Table 2). The two families with the most pairs that can hybridize have slightly differing distributions of genetic distance (Plethodontidae, mean = 0.114;

Salamandridae, mean = 0.0916). However, a non-parametric Mann-Whitney test showed no significant difference in genetic distance between the two families, ($n_{\text{Plethodontidae}} = 35$, $n_{\text{Salamandridae}} = 15$, $W = 336$, $P = 0.1231$). For the 29 parental pairs of salamanders that have published RAG1 data, the mean genetic distance was 0.00569 (range 0.000682 to 0.0199) (Table 2). The genetic distance ratio of the mitochondrial gene MT-CYB over the nuclear gene RAG1 between salamanders that hybridize was highly variable (Figure 2b). The ratio ranged from 0.6x to 112x with most species pairs having a higher mitochondrial genetic distance (median ratio = 21.5).

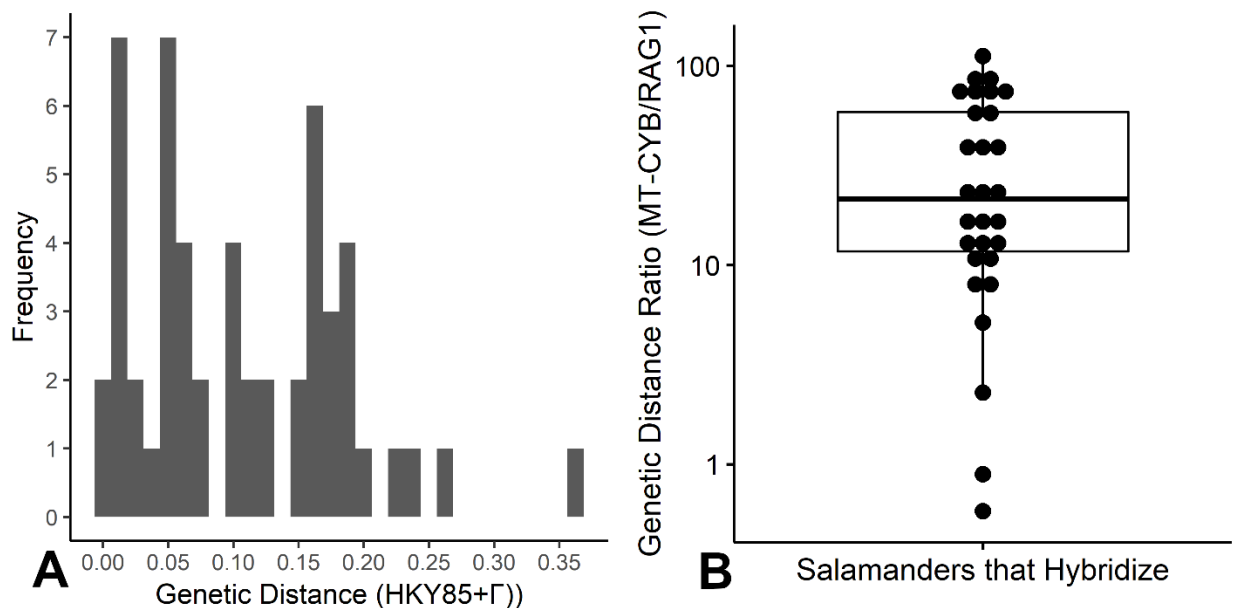


Figure 2. A) Histogram of cytochrome *b* (MT-CYB) genetic distance estimates (HKY85 + Γ) between salamanders that hybridize. Graph bin width is 0.0125. B) Ratio of mitochondrial MT-CYB genetic distance to nuclear recombination activating 1 (RAG1) genetic distance between parental species of salamanders that hybridize on a log scale. The ratio of genetic distance ranged from 0.6 to 112 with the majority of species pairs having a higher mitochondrial genetic distance (median = 21.5).

Genetic Distances Between Parental Species that Hybridize in Different Clades

After paring down to the pair that can hybridize with the highest genetic distance per genus, there were 23 pairs of hybrids for birds, 20 pairs for lizards, 25 pairs for mammals, and 16 pairs for salamanders (Table 2). The median MT-CYB genetic distances for the groups were: lizards (0.181), birds (0.167), mammals (0.152), and salamanders (0.125) (Figure 3). There were

no significant differences in distributions of genetic distance among the four groups ($\chi^2 = 2.8024$, $P = 0.4231$). Across all vertebrates, birds had both the lowest and highest genetic distances with *Regulus calendula* X *Regulus satrapa* (0.002) and *Gallus gallus* X *Alectura lathami* (0.366). Using the pared down list of hybrids, RAG1 sequences were available for 12 pairs of hybrids for birds, nine pairs for lizards, nine pairs for mammals, and nine pairs for salamanders (Table 2). The median MT-CYB/RAG1 genetic distance ratio for the four groups were: lizards (25.8), salamanders (21.0), mammals (14.0), and birds (10.6) (Figure 4). There were no significant differences among the four vertebrate classes ($P = 0.0915$).

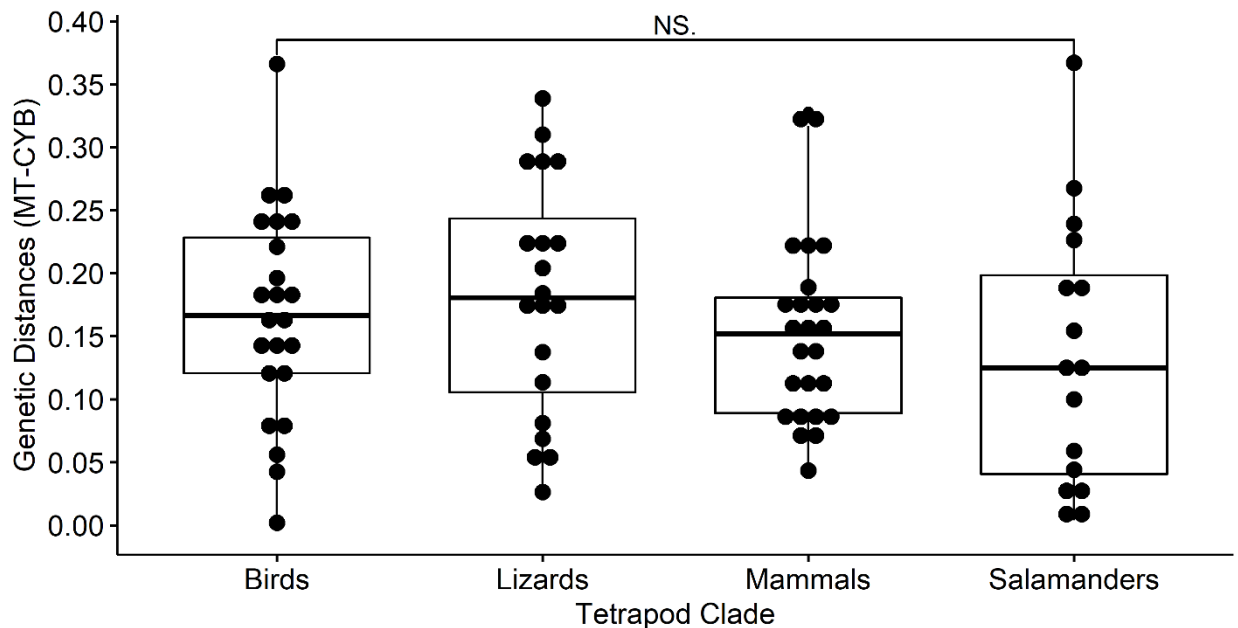


Figure 3. Cytochrome *b* (MT-CYB) genetic distances between parental species that hybridize in different tetrapod groups. Kruskal–Wallis H test showed no significant differences across the groups ($p = 0.423$). Salamanders are not hybridizing across greater genetic distances.

Relationship Between Sex Chromosomes and Hybridization in Salamanders

Forty-eight species of salamanders in seven families have been discovered with heteromorphic sex chromosomes (Ambystomatidae, Hynobiidae, Plethodontidae, Proteidae, Salamandridae, Sirenidae, and Proteidae) (Table 3). (Continued next page).

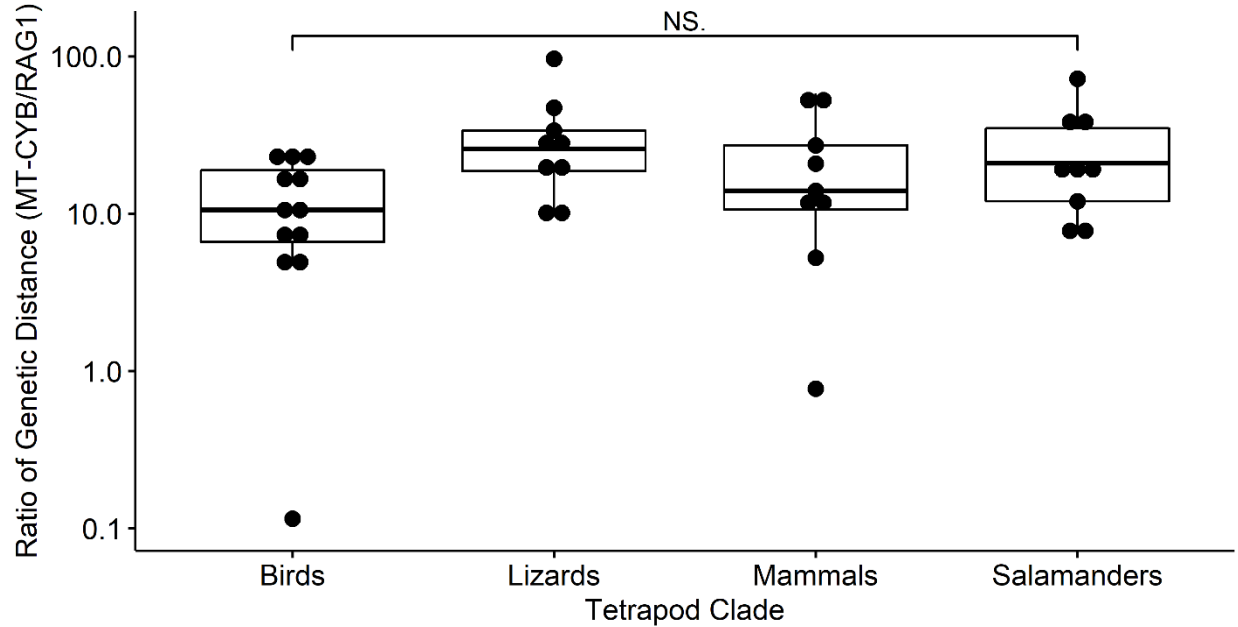


Figure 4. Genetic distance ratio of mitochondrial cytochrome b (MT-CYB) to nuclear recombination activating 1 (RAG1) for pairs of species that hybridize in major tetrapod clade on a log scale. Kruskal–Wallis H test showed no strong significant differences across the four groups ($p = 0.0915$).

Salamander families are not characterized by a single type of sex chromosome with different members of Salamandridae and Plethodontidae having both ZZ/ZW and XX/XY systems. Out of the salamanders with sex chromosomes, 13/48 are known to hybridize with a different species (Table 3). There is no difference between percentage of salamanders that hybridize with known sex chromosomes and the known overall salamander hybridization rate ($P = 0.999$). Also, the genetic distances between parental salamanders that hybridize with sex chromosomes (median = 0.104) and parental salamanders that hybridize without sex chromosomes (median = 0.139) are not significantly different ($p=0.639$) (Figure 5). Based on these analyses, there is no indication that the presence of sex chromosomes hinders hybridization in salamanders.

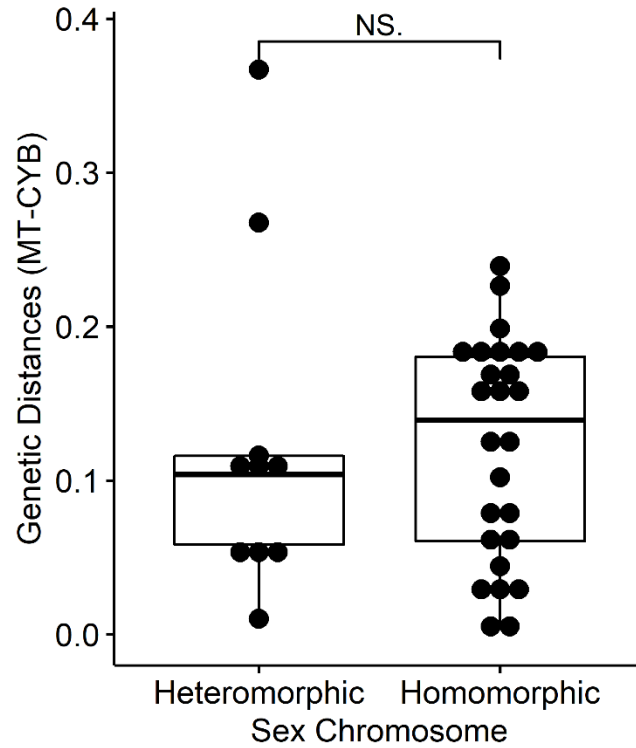


Figure 5. Genetic distances between salamanders that hybridize with heteromorphic sex chromosomes (median = 0.104) and salamanders that hybridize without sex chromosomes (median = 0.139). There is no significant difference ($p=0.639$) between salamanders with sex chromosomes and salamanders without sex chromosomes

Table 1. Natural Hybridization in Salamanders

Parental Species		Resources
Species 1	Species 2	
Ambystomatidae		
<i>Ambystoma macrodactylum</i> North Central	<i>Ambystoma macrodactylum</i> Rocky Mountains	Lee-Yaw et al., 2014
<i>Ambystoma maculatum</i> Eastern	<i>Ambystoma maculatum</i> Western	Johnson et al., 2015
<i>Ambystoma tigrinum californiense</i>	<i>Ambystoma mavortium</i>	Fitzpatrick et al., 2009 Fitzpatrick and Shaffer, 2004 Riley et al., 2003
Cryptobranchidae		
<i>Andrias davidianus</i>	<i>Andrias japonicus</i>	Fukumoto et al., 2015
Dicamptodontidae		
<i>Dicamptodon ensatus</i>	<i>Dicamptodon tenebrosus</i>	Good, 1989
Hynobiidae		
<i>Onychodactylus japonicus</i> S-Tohoku	<i>Onychodactylus japonicus</i> SW-Honshu	Yoshikawa et al., 2012
<i>Salamandrella keyserlingii</i>	<i>Salamandrella tridactyla</i>	Malyarchuk et al., 2014
Plethodontidae		
<i>Batrachoseps gavilanensis</i>	<i>Batrachoseps luciae</i>	Jockusch and Wake, 2002
<i>Bolitoglossa franklini</i>	<i>Bolitoglossa lincolni</i>	Wake et al., 1980
<i>Desmognathus carolinensis</i>	<i>Desmognathus orestes</i>	Mead and Tilley, 2000
<i>Desmognathus conanti</i>	<i>Desmognathus fuscus</i>	Bonett, 2002
<i>Desmognathus fuscus</i>	<i>Desmognathus ochrophaeus</i>	Sharbel et al., 1995
<i>Desmognathus fuscus</i>	<i>Desmognathus santeetlah</i>	Tilley, 1988
<i>Ensatina eschscholtzii croceater</i>	<i>Ensatina eschscholtzii platensis</i>	Pereira and Wake, 2009
<i>Ensatina eschscholtzii</i>	<i>Ensatina eschscholtzii klauberi</i>	Devit et al., 2011
<i>Ensatina eschscholtzii oregonensis</i>	<i>Ensatina eschscholtzii picta</i>	Pereira and Wake, 2009
<i>Ensatina eschscholtzii oregonensis</i>	<i>Ensatina eschscholtzii xanthoptica</i>	Pereira and Wake, 2009
<i>Ensatina eschscholtzii platensis</i>	<i>Ensatina eschscholtzii xanthoptica</i>	Alexandrino et al., 2005 Sweet, 1984
<i>Eurycea bislineata</i>	<i>Eurycea cirrigera</i>	Guttman and Karlin, 1986

Table 1 (continued).

<i>Eurycea cirrigera</i>	<i>Eurycea wilderae</i>	Kozak, 2003
<i>Eurycea neotenes</i>	<i>Eurycea tridentifera</i>	Kozak and Montanucci, 2001
<i>Hydromantes ambrosii</i>	<i>Hydromantes italicus</i>	Lunghi et al., 2017 Ficetola et al., 2019
<i>Plethodon aureolus</i>	<i>Plethodon shermani</i>	Highton and Peabody, 2000
<i>Plethodon chattahoochee</i>	<i>Plethodon chlorobryonis</i>	Highton and Peabody, 2000
<i>Plethodon chattahoochee</i>	<i>Plethodon shermani</i>	Highton and Peabody, 2000
<i>Plethodon chattahoochee</i>	<i>Plethodon teyahalee</i>	Highton and Peabody, 2000
<i>Plethodon cheoah</i>	<i>Plethodon teyahalee</i>	Highton and Peabody, 2000
<i>Plethodon chlorobryonis</i>	<i>Plethodon cylindraceus</i>	Highton and Peabody, 2000
<i>Plethodon chlorobryonis</i>	<i>Plethodon metcalfi</i>	Highton and Peabody, 2000
<i>Plethodon chlorobryonis</i>	<i>Plethodon teyahalee</i>	Highton and Peabody, 2000
<i>Plethodon cinereus</i>	<i>Plethodon electromorphus</i>	Lehtinen et al., 2016
<i>Plethodon cylindraceus</i>	<i>Plethodon glutinosus</i>	Highton and Peabody, 2000
<i>Plethodon dorsalis</i>	<i>Plethodon ventralis</i>	Highton, 1997 Duncan and Highton, 1979 Highton, 1999
<i>Plethodon electromorphus</i>	<i>Plethodon richmondi</i>	Highton, 1999
<i>Plethodon fourchensis</i>	<i>Plethodon ouachiteae</i>	Shepard et al., 2011
<i>Plethodon glutinosus</i>	<i>Plethodon jordani</i>	Hairston et al., 1992
<i>Plethodon glutinosus</i>	<i>Plethodon kentucki</i>	Kuchta et al., 2016 Hairston et al., 1992 Highton, 2009 Dawley, 1987
<i>Plethodon hoffmani</i>	<i>Plethodon virginia</i>	Chatfield et al., 2010
<i>Plethodon jordani</i>	<i>Plethodon metcalfi</i>	Chatfield et al., 2010
<i>Plethodon jordani</i>	<i>Plethodon teyahalee</i>	Chatfield et al., 2010
<i>Plethodon metcalfi</i>	<i>Plethodon teyahalee</i>	Chatfield et al., 2010
<i>Plethodon shermani</i>	<i>Plethodon teyahalee</i>	Highton and Peabody, 2000
Proteidae		
<i>Necturus maculosus</i>	<i>Necturus aff. lewisi</i>	Nelson et al., 2017

Table 1 (continued).

Salamandridae		
<i>Chioglossa lusitanica</i> Northern	<i>Chioglossa lusitanica</i> Southern	Sequeira et al., 2005
<i>Cynops pyrrhogaster</i> Central	<i>Cynops pyrrhogaster</i> Western	Tominaga et al., 2017
<i>Lissotriton helveticus</i>	<i>Lissotriton vulgaris</i>	Johanet et al., 2011
<i>Lissotriton montandoni</i>	<i>Lissotriton vulgaris</i>	Babik et al., 2005
		Zieliński et al., 2013
<i>Lissotriton vulgaris kosswigi</i>	<i>Lissotriton vulgaris</i>	Nadachowska and Babik, 2009
		Johannesen et al., 2006
<i>Lyciasalamandra antalyana</i>	<i>Lyciasalamandra billae</i>	Takahashi et al., 2011
<i>Notophthalmus viridescens</i>	<i>Notophthalmus viridescens dorsalis</i>	Riemsdijk et al., 2018
<i>Ommatotriton ophryticus</i>	<i>Ommatotriton nesterovi</i>	Yoshikawa et al., 2010
<i>Ommatotriton ophryticus</i>	<i>Ommatotriton vittatus</i>	Riemsdijk et al., 2018
		Escoriza et al., 2016
<i>Pleurodeles nebulosus</i>	<i>Pleurodeles poireti</i>	García-París et al., 2003
<i>Salamandra salamandra gallaica</i>	<i>Salamandra bernardezi</i>	Ventura et al., 2015
<i>Salamandra fastuosa</i>	<i>Salamandra terrestris</i>	Canestrelli et al., 2014
		García-París et al., 2003
<i>Salamandrina perspicillata</i>	<i>Salamandrina terdigitata</i>	Hauswaldt et al., 2011
		Mattocchia et al., 2011
		Arntzen et al., 2009
<i>Taricha torosa</i>	<i>Taricha sierrae</i>	Kuchta, 2007
<i>Triturus carnifex</i>	<i>Triturus cristatus</i>	Arntzen et al., 2014
<i>Triturus carnifex</i>	<i>Triturus dobrogicus</i>	Arntzen et al., 2014
<i>Triturus carnifex</i>	<i>Triturus ivanbureschi</i>	Arntzen et al., 2014
<i>Triturus carnifex</i>	<i>Triturus macedonicus</i>	Arntzen et al., 2014
<i>Triturus cristatus</i>	<i>Triturus dobrogicus</i>	Arntzen et al., 2014
<i>Triturus cristatus</i>	<i>Triturus ivanbureschi</i>	Arntzen et al., 2014
<i>Triturus cristatus</i>	<i>Triturus macedonicus</i>	Arntzen et al., 2014
<i>Triturus cristatus</i>	<i>Triturus marmoratus</i>	Visser et al., 2016

Table 2. Hybrid pairs and accession numbers for MT-CYB and RAG1. Lists of bird hybrids and mammal hybrids modified from Fitzpatrick 2004. Lizards modified from Jančúchová-Lásková et al., 2015. Only the highest distance per genus was used in the salamander (*) for the cross-clade comparison (Figure 3).

Species 1	Accession Number		Species 2	Accession Number		Genetic Distance (HKY85 + Γ)		
	MT-CYB	RAG1		MT-CYB	RAG1	MT-CYB	RAG1	MT-CYB/RAG1
Birds								
<i>Acrocephalus scirpaceus</i>	LT671508.1		<i>A. palustris</i>	AJ004774.1		0.121		
<i>Aegypius monachus</i>	AY987266.1	EF078711.1	<i>Gyps fulvus</i>	AY987261.1	EU496474.1	0.120	0.005	22.0
<i>Agapornis nigrigenis</i>	AF001328.1	GQ505193.1	<i>Melopsittacus undulatus</i>	DQ467903.1	DQ143354.1	0.265	0.036	7.3
<i>Alectura lathami</i>	KF833611.1	AF294687.2	<i>Gallus</i>	L08376.1	NM001031188.1	0.366	0.076	4.8
<i>Branta canadensis</i>	EU585629.1		<i>Cairina moschata</i>	L08385.1		0.235		
<i>Bugeranus carunculatus</i>	U27556.1		<i>Grus canadensis</i>	EU166997.1		0.074		
<i>Buteo buteo</i>	X86741.1	EU345528.1	<i>Pernis apivorus</i>	X86758.1	EF078753.1	0.185	0.018	10.2
<i>Carduelis chloris</i>	AY495384.1		<i>Emberiza citrinella</i>	AY495392.1		0.221		
<i>Ciconia ciconia</i>	KJ456229.1		<i>C. nigra</i>	U72771.1		0.184		
<i>Cathartes aura</i>	EU166984.1	EF078766.1	<i>Coragyps atratus</i>	KX534417.1	KM876315.1	0.167	0.008	21.8
<i>Columba livia</i>	KC675192.1	AY228768.1	<i>Leucosarcia melanoleuca</i>	AF483327.1	EF373512.1	0.239	0.032	7.4
<i>Diphylloides magnificus</i>	X74255.1		<i>Paradisaea minor</i>	U25737.1		0.159		
<i>Egretta garzetta</i>	MH645659.1		<i>Nycticorax nycticorax</i>	AF193829.1		0.143		
<i>Falco columbarius</i>	EU233049.1	EU233167.1	<i>F. tinnunculus</i>	EU233121.1	EU233241.1	0.140	0.006	24.4
<i>Ficedula albicollis</i>	DQ674491.1	XM005046928.1	<i>F. hypoleuca</i>	KJ930552.1	DQ466798.1	0.042	0.004	11.0

Table 2 (continued).

<i>Garrulus glandarius</i>	AB242559.1		<i>Perisoreus infaustus</i>	U86042.1		0.247		
<i>Hirundo rustica</i>	DQ119526.1	AY443290.1	<i>H. pyrrhonota</i>	AF074591.1	AY056997.1	0.181	0.010	18.0
<i>Larus canus</i>	AB208756.1		<i>L. ridibundus</i>	FM209923.1		0.056		
<i>Phylloscopus collybita</i>	HQ608821.1		<i>P. trochilus</i>	MH079362.1		0.146		
<i>Pterocnemia pennata</i>	U76054.1		<i>Rhea americana</i>	L78808.1		0.084		
<i>Regulus calendula</i>	AY329472.1	AY057028.1	<i>R. satrapa</i>	AJ004329.1	AY443327.1	0.002	0.018	0.1
<i>Scolopax rusticola</i>	KM434134.1	AY228802.1	<i>Vanellus vanellus</i>	KM577158.1: 13657-14802	AY339126.1	0.260	0.052	5.0
<i>Turdus migratorius</i>	AF197835.1	KC789829.1	<i>T. philomelos</i>	AY495411.1	AY307214.1	0.196	0.013	15.4
Lizards								
<i>Amblyrhynchus cristatus</i>	AY948118.1	KR350710.1	<i>Conolophus subcristatus</i>	AY948122.1	KR350708.1	0.219	0.008	25.8
<i>Anolis aeneus</i>	EU557103.1	JN112592.1	<i>A. trinitatis</i>	AF493592.1	JN112645.1	0.286	0.009	30.7
<i>Carlia rubrigularis</i> NORTH	AF181042.1		<i>C. rubrigularis</i> South	AF181056.1		0.229		
<i>Crotaphytus bicinctores</i>	EU037682.1		<i>C. collaris</i>	EU037482.1		0.137		
<i>Ctenosaura bakeri</i>	GU331976.1		<i>C. similis</i>	GU331975.1		0.184		
<i>Gambelia sila</i>	EU037370.1		<i>G. wislizenii</i>	EU037415.1		0.026		
<i>Iberolacerta galani</i>	HQ234901.1	KY762187.1	<i>I. monticola</i>	HQ234897.1	EF632220.1	0.050	0.001	33.8
<i>Iguana delicatissima</i>	KX610607.1		<i>I. iguana</i>	AF020251.1		0.172		
<i>Kentropyx calcarata</i>	JQ639739.1		<i>K. striata</i>	JQ639672.1		0.223		
<i>Lacerta agilis</i>	AF373032.1	EF632222.1	<i>L. schreiberi</i>	AF372103.1	KY762190.1	0.310	0.017	18.7

Table 2 (continued).

<i>Lacerta pater</i>	AF378964.1		<i>Timon lepidus</i>	JX626302.1		0.204		
<i>Leiolepis guttata</i>	NC014179.1		<i>L. reevesii</i>	EU305052.1		0.339		
<i>Liolaemus bibronii</i>	JN410531.1		<i>L. gracilis</i>	JN410538.1		0.177		
<i>Nactus multicaarinatus</i>	KC581486.1	HM997172.1	<i>N. pelagicus</i>	KC581545.1	EU054275.1	0.081	0.004	20.8
<i>Oligosoma otagense</i>	JN999970.1	EU568093.1	<i>O. waimatense</i>	JN999978.1	EU568094.1	0.114	0.001	96.4
<i>Phrynocephalus putjatai</i>	KF691634.1	KC551413.1	<i>P. vlangualii</i>	KF691642.1	KJ195972.1	0.058	0.005	10.6
<i>Phrynosoma cornutum</i>	AY141087.1	DQ385423.1	<i>P. coronatum</i>	AY141097.1	FJ356738.1	0.291	0.030	9.7
<i>Plestiodon japonicus</i>	EU203134.1	HM161196.1	<i>P. laticutatus</i>	EU203035.1	HM161203.1	0.175	0.004	47.2
<i>Podarcis melisellensis</i>	AY185036.1		<i>P. sicula</i>	AY770890.1		0.292		
<i>Zootoca vivipara carniolice</i>	AY714929.1		<i>Z. vivipara vivipara</i>	AY714913.1		0.069		
Mammals								
<i>Alcelaphus buselaphus</i>	AJ222681.1		<i>Damaliscus lunatus</i>	AF016635.1		0.161		
<i>Arctocephalus pusillus</i>	AM181018.1		<i>Zalophus californianus</i>	AM422164.1		0.088		
<i>Bos bison</i>	AF036273.1		<i>B. indicus</i>	AF419237.2		0.089		
<i>Camelus bactrianus</i>	JX177500.1	XM010964664.1	<i>C. dromedarius</i>	KU509220.1	XM011000597.1	0.170	0.004	47.9
<i>Capra caucasica</i>	AF034738.1		<i>C. sibirica</i>	KF990328.1		0.110		
<i>Cervus elaphus</i>	AB001612.1		<i>Odocoileus hemionus</i>	AF091630.1		0.226		
<i>Chinchilla brevicaudata</i>	AF464756.1		<i>C. lanigera</i>	AF122820.1		0.069		
<i>Equus caballus</i>	DQ223535.1	NM001256901.1	<i>E. grevyi</i>	X56282.1	AY239184.1	0.118	0.009	13.0

Table 2 (continued).

<i>Eulemur macaco</i>	AF081049.1	HM759153.1	<i>E. mongoz</i>	AF081051.1	EU342315.1	0.140	0.005	27.1
<i>Halichoerus grypus</i>	GU167293.1		<i>Phoca hispida</i>	X82304.1		0.043		
<i>Hylobates agilis</i>	AJ010583.1		<i>H. hoolock</i>	Y13304.1		0.178		
<i>Kobus kob</i>	AF052939.1		<i>K. megaceros</i>	AJ222686.1		0.136		
<i>Lama guanicoe</i>	U06428.1		<i>Vicugna vicugna</i>	U06430.1		0.083		
<i>Lepus europaeus</i>	AY745112.1		<i>L. timidus</i>	AJ279424.1		0.152		
<i>Macaca fascicularis</i>	AF295584.1		<i>Mandrillus sphinx</i>	JQ068151.1		0.328		
<i>Martes martes</i>	AF154975.1		<i>M. putorius</i>	X94925.1		0.218		
<i>Mastomys natalensis</i>	JX292865.1	DQ023475.1	<i>Mus musculus</i>	AY057804.1	NM009019.2	0.317	0.060	5.2
<i>Microtus californicus</i>	AF163891.1	KC953523.1	<i>M. montanus</i>	AF119280.1	KC953524.1	0.189	0.018	10.6
<i>Ovis aries</i>	AF034730.1	XM012134165.2	<i>O. canadensis</i>	EU365985.1	AY239177.1	0.089	0.006	14.0
<i>Panthera leo</i>	X82300.1	AB109364.1	<i>P. pardus</i>	JF720058.1	XM019470137.1	0.107	0.002	58.0
<i>Pecari tajacu</i>	DQ179055.1		<i>Tayassu pecari</i>	AY534303.1		0.073		
<i>Peromyscus difficilis</i>	AF155394.1		<i>P. truei</i>	FJ800579.1		0.221		
<i>Spermophilus major</i>	AF157903.1		<i>S. pygmaeus</i>	AF157907.1		0.158		
<i>Ursus americanus</i>	U23556.1		<i>U. arctos</i>	U18870.1		0.172		
<i>Vulpes lagopus</i>	LT559489.1		<i>V. vulpes</i>	AY928669.1		0.181		
Salamanders								
<i>Andrias davidianus</i>	KU131042.1	MH106790.1	<i>A. japonicus</i>	AB208679.1	AY583346.1	0.100*	0.0047	21.5

Table 2 (continued).

<i>Batrachoseps gavalanensis</i>	KM203055.1	KM202898.1	<i>B. luciae</i>	KM203053.1	KM202878.1	0.189*	0.0107	17.7
<i>Bolitoglossa franklini</i>	MK165231.1	KC614439.1	<i>B. lincolni</i>	GU725464.1	KC614440.1	0.030*	0.0025	12.1
<i>Desmognathus carolinensis</i>	EU314306.1	KR732369.1	<i>D. orestes</i>	EU314288.1	KR827015.1	0.163	0.0028	58.7
<i>Desmognathus conanti</i>	KY659020.1	KR732370.1	<i>D. fuscus</i>	AY728227.1	KR732372.1	0.239*	0.0111	21.6
<i>Desmognathus fuscus</i>	AY728227.1	KR732372.1	<i>D. ochrophaeus</i>	EU314289.1	KR732377.1	0.167	0.0124	13.4
<i>Desmognathus fuscus</i>	AY728227.1	KR732372.1	<i>D. santeetlah</i>	EU314270.1	KR732384.1	0.199	0.0117	16.9
<i>Dicamptodon ensatus</i>	AY734600.1	EF107335.1	<i>D. tenebrosus</i>	AAW70411.1	AY650132.1	0.044*	0.0054	8.1
<i>Ensatina eschscholtzii croceater</i>	L75796.1		<i>E. eschscholtzii platensis</i>	FJ151995.1		0.064		
<i>Ensatina eschscholtzii eschscholtzii</i>	FJ151951.1		<i>E. eschscholtzii klauberi</i>	L75801.1		0.183		
<i>Ensatina eschscholtzii oregonensis</i>	FJ151696.1		<i>E. eschscholtzii picta</i>	FJ151670.1		0.059		
<i>Ensatina eschscholtzii oregonensis</i>	FJ151696.1		<i>E. eschscholtzii xanthoptica</i>	FJ151887.1		0.226*		
<i>Ensatina eschscholtzii platensis</i>	FJ151995.1		<i>E. eschscholtzii xanthoptica</i>	FJ151887.1		0.179		
<i>Eurycea bislineata</i>	AY728217.1	AY691706.1	<i>E. cirrigera</i>	NC035494.1	FJ750236.1	0.125*	0.0160	7.8
<i>Eurycea cirrigera</i>	NC035494.1	FJ750236.1	<i>E. wilderae</i>	JQ920621.1	JQ920766.1	0.102	0.0198	5.1
<i>Eurycea neotenes</i>	AY528400.1	AY650122.1	<i>E. tridentifera</i>	AY014848.1	KF562669.1	0.009	0.0102	0.9
<i>Hydromantes ambrosii</i>	FJ602258.1	FJ602321.1	<i>H. italicus</i>	FJ602299.1	EU275791.1	0.059*	0.0008	72.2
<i>Lissotriton helveticus</i>	DQ821238.1		<i>L. vulgaris</i>	EU880339.1		0.367*		
<i>Lissotriton montandoni</i>	DQ821254.1		<i>L. vulgaris</i>	EU880339.1		0.010		
<i>Ommatotriton nesterovi</i>	KX682106.1		<i>O. ophryticus</i>	DQ821267.1		0.012		

Table 2 (continued).

<i>Ommatotriton ophryticus</i>	DQ821267.1		<i>O. vittatus</i>	EU880338.1		0.014*		
<i>Plethodon aureolus</i>	DQ994914.1	DQ995012.1	<i>P. shermani</i>	DQ994985.1	DQ995063.1	0.168	0.0022	75.1
<i>Plethodon chattahoochee</i>	DQ994919.1	DQ995014.1	<i>P. teyahalee</i>	DQ994990.1	DQ995068.1	0.047	0.0034	13.7
<i>Plethodon chattahoochee</i>	DQ994919.1	DQ995014.1	<i>P. chlorobryonis</i>	DQ994923.1	DQ995016.1	0.051	0.0033	15.5
<i>Plethodon chattahoochee</i>	DQ994919.1	DQ995014.1	<i>P. shermani</i>	DQ994985.1	DQ995063.1	0.051	0.0021	25.0
<i>Plethodon cheoah</i>	DQ994921.1		<i>P. teyahalee</i>	DQ994990.1		0.047		
<i>Plethodon chlorobryonis</i>	DQ994923.1		<i>P. cylindraceus</i>	DQ994928.1		0.016		
<i>Plethodon chlorobryonis</i>	DQ994923.1		<i>P. teyahalee</i>	DQ994990.1		0.011		
<i>Plethodon chlorobryonis</i>	DQ994923.1		<i>P. metcalfi</i>	DQ994956.1		0.163		
<i>Plethodon cinereus</i>	AY378042.1	AY691703.1	<i>P. electromorphus</i>	AY378060.1	DQ995024.1	0.153	0.0131	11.7
<i>Plethodon cylindraceus</i>	DQ994928.1	DQ995022.1	<i>P. glutinosus</i>	DQ994937.1	DQ995027.1	0.171	0.0030	57.2
<i>Plethodon dorsalis</i>	GQ464404.1	DQ995023.1	<i>P. ventralis</i>	DQ994993.1	DQ995071.1	0.034	0.0034	9.9
<i>Plethodon electromorphus</i>	AY378060.1	DQ995024.1	<i>P. richmondi</i>	AY378072.1	DQ995051.1	0.081	0.0020	39.6
<i>Plethodon fourchensis</i>	FJ611481.1	DQ995026.1	<i>P. ouachitae</i>	FJ266744.1	AY691704.1	0.181	0.0020	88.3
<i>Plethodon glutinosus</i>	DQ994937.1	DQ995027.1	<i>P. jordani</i>	DQ994947.1	DQ995032.1	0.187	0.0022	83.4
<i>Plethodon glutinosus</i>	DQ994937.1	DQ995027.1	<i>P. kentucki</i>	DQ994948.1	DQ995033.1	0.188*	0.0052	35.8
<i>Plethodon hoffmani</i>	AY378047.1	DQ995029.1	<i>P. virginia</i>	AY378049.1	DQ995072.1	0.047	0.0007	68.8
<i>Plethodon jordani</i>	DQ994947.1	DQ995032.1	<i>P. metcalfi</i>	DQ994956.1	DQ995040.1	0.076	0.0007	112.0
<i>Plethodon jordani</i>	DQ994947.1	DQ995032.1	<i>P. teyahalee</i>	DQ994990.1	DQ995068.1	0.164	0.0020	80.2

Table 2 (continued).

<i>Plethodon metcalfi</i>	DQ994956.1		<i>P. teyahalee</i>	DQ994990.1		0.158		
<i>Plethodon shermani</i>	DQ994985.1	DQ995063.1	<i>P. teyahalee</i>	DQ994990.1	DQ995068.1	0.003	0.0014	2.3
<i>Salamandra salamandra bernardezi</i>	DQ092219.1		<i>S. salamandra gallaica</i>	KX094979.1		0.025*		
<i>Salamandra salamandra fastuosa</i>	DQ221234.1		<i>S. Salamandra terrestris</i>	AY222503.1		0.006		
<i>Salamandrella keyserlingii</i>	AY593141.1	AY650145.1	<i>S. schrenckii</i>	AB363608.1	KJ855096.1	0.154*	0.0036	42.3
<i>Salamandrina perspicillata</i>	DQ821207.1	HQ915345.1	<i>S. terdigitata</i>	EU880332.1	HQ915218.1	0.004*	0.0065	0.6
<i>Taricha sierrae</i>	DQ196282.1		<i>T. torosa</i>	DQ196247.1		0.125*		
<i>Triturus carnifex</i>	NC 015788.1		<i>T. cristatus</i>	HQ697273.1		0.103		
<i>Triturus carnifex</i>	NC 015788.1		<i>T. dobrogicus</i>	HQ697274.1		0.104		
<i>Triturus carnifex</i>	NC 015788.1		<i>T. macedonicus</i>	HQ697278.1		0.058		
<i>Triturus cristatus</i>	HQ697273.1		<i>T. dobrogicus</i>	HQ697274.1		0.115		
<i>Triturus cristatus</i>	HQ697273.1		<i>T. marmoratus</i>	HQ697279.1		0.267*		
<i>Triturus cristatus</i>	HQ697273.1		<i>T. macedonicus</i>	HQ697278.1		0.116		
<i>Triturus marmoratus</i>	HQ697279.1		<i>T. pygmaeus</i>	HQ697280.1		0.048		

Table 3. Heteromorphic Sex Chromosomes in Salamanders. Of the 48 species with sex chromosomes, 13 have been shown to hybridize with a different species. A one-sided exact test of goodness-of-fit for the lower bounds showed no significance ($P = 0.999$). The presence of sex chromosomes is not an inherent barrier to hybridization in salamanders.

Species	Family	Sex Chromosome Type	Hybrid (Yes/No)
<i>Ambystoma jeffersonianum</i> ³	Ambystomatidae	ZW	No
<i>Ambystoma laterale</i> ¹²³	Ambystomatidae	ZW	Yes
<i>Ambystoma mexicanum</i> ¹²³	Ambystomatidae	ZW	Yes
<i>Ambystoma tigrinum</i> ¹²³	Ambystomatidae	ZW	Yes
<i>Hynobius hidamontanus</i> ¹³	Hynobiidae	ZW	No
<i>Hynobius quelpaertensis</i> ¹³	Hynobiidae	ZW	No
<i>Hynobius tokyoensis</i> ³	Hynobiidae	ZW	No
<i>Aneides ferreus</i> ¹²³	Plethodontidae	ZW	No
<i>Bolitoglossa subpalmata</i> ¹²³	Plethodontidae	XY	No
<i>Chiropetrotriton dimidiatus</i> ¹²³	Plethodontidae	ZW	No
<i>Cryptotriton veraepacis</i> ¹²³	Plethodontidae	XY	No
<i>Dendrotriton bromeliacius</i> ²³	Plethodontidae	XY	No
<i>Dendrotriton cuchumatanus</i> ²	Plethodontidae	XY	No
<i>Dendrotriton rabbi</i> ¹²³	Plethodontidae	XY	No
<i>Dendrotriton xolocalcae</i> ¹²³	Plethodontidae	XY	No
<i>Hydromantes ambrosii</i> ¹²³	Plethodontidae	XY	Yes
<i>Hydromantes flavus</i> ¹²³	Plethodontidae	XY	No
<i>Hydromantes imperialis</i> ¹²³	Plethodontidae	XY	No
<i>Hydromantes italicus</i> ¹²³	Plethodontidae	XY	Yes
<i>Hydromantes genei</i> ³	Plethodontidae	XY	No
<i>Hydromantes supramontis</i> ¹³	Plethodontidae	XY	No
<i>Nototriton abscondens</i> ¹³	Plethodontidae	XY	No

Table 3 (continued).

<i>Nototriton picadoi</i> ¹²³	Plethodontidae	XY	No
<i>Nototriton richardi</i> ¹	Plethodontidae	XY	No
<i>Oedipina cyclocauda</i> ¹	Plethodontidae	XY	No
<i>Oedipina parvipes</i> ¹²	Plethodontidae	XY	No
<i>Oedipina poelzi</i> ¹²³	Plethodontidae	XY	No
<i>Oedipina pseudouniformis</i> ¹	Plethodontidae	XY	No
<i>Oedipina uniformis</i> ²³	Plethodontidae	XY	No
<i>Thorius dubitus</i> ¹²³	Plethodontidae	XY	No
<i>Thorius pennatulus</i> ²³	Plethodontidae	XY	No
<i>Necturus alabamensis</i> ³	Proteidae	XY	No
<i>Necturus beyeri</i> ¹²³	Proteidae	XY	No
<i>Necturus lewisi</i> ¹²³	Proteidae	XY	Yes
<i>Necturus maculosus</i> ¹²³	Proteidae	XY	Yes
<i>Necturus punctatus</i> ¹²³	Proteidae	XY	No
<i>Ichthyosaura alpestris</i> ³	Salamandridae	XY	No
<i>Lissotriton boscai</i> ³	Salamandridae	XY	No
<i>Lissotriton helveticus</i> ²³	Salamandridae	XY	Yes
<i>Lissotriton italicus</i> ²³	Salamandridae	XY	No
<i>Lissotriton vulgaris</i> ²³	Salamandridae	XY	Yes
<i>Pleurodeles poireti</i> ¹²³	Salamandridae	ZW	Yes
<i>Pleurodeles waltl</i> ¹²³	Salamandridae	ZW	No
<i>Triturus carnifex</i> ²³	Salamandridae	XY	Yes
<i>Triturus cristatus</i> ¹²³	Salamandridae	XY	Yes
<i>Triturus karelinii</i> ¹³	Salamandridae	XY	No
<i>Triturus marmoratus</i> ¹²³	Salamandridae	XY	Yes
<i>Siren intermedia</i> ¹²	Sirenidae	ZW	No

¹Evans et al., 2012 ²Hillis and Green, 1990 ³Perkins et al., 2019

DISSCUSSION

Overall, we found that hybridization is widespread throughout the salamander order occurring in both the biggest and smallest families (Figure 1). The largest four families (Ambystomatidae, Hynobiidae, Plethodontidae, and Salamandridae) account for nearly 97% of all salamander species and account for over 90% of the hybrids (Figure 1). With respect to geographic location, hybrids are widespread in the Northern Hemisphere, occurring in North America, Europe, and Asia. In contrast, there is only one reported case of salamanders hybridizing in the tropics, occurring between *Bolitoglossa franklini* and *Bolitoglossa lincolni* (Table 1). *Bolitoglossa* is the largest genus of salamanders (132 species) and it occurs exclusively in the tropics. More generally, 18% of all salamander diversity occurs in the tropics, suggesting that the low number of tropical hybrids is not a result of low tropical species diversity (amphibiaweb.org), but may reflect general differences in mechanisms underlying the origin and maintenance of species in temperate versus tropical areas worthy of further investigation (Kozak and Wiens 2010).

The mitochondrial genetic distances (estimated with MT-CYTB) between parental species of salamanders that hybridize spanned two orders of magnitude (0.003 to 0.376) while the nuclear genetic distances (estimated with RAG1) spanned a single order (0.001 to 0.020). The ratio of genetic distances between these two genes provides an estimate of the difference in substitution rates of the two genomes. In vertebrates, the mitochondrial genome typically has a higher substitution rate than the nuclear genome (Brown et al., 1979). However, this ratio is variable across genes and species in all of the major vertebrate clades including amphibians, where the majority of the estimates are from frogs (Allio et al., 2017). Our salamander estimates

(0.6 to 112, median = 21.5) are consistent with existing estimates for other vertebrate taxa, suggesting similar relative evolutionary rates between the two genomes. Because the MT-CYTB and RAG1 sequences used are short (345–1141 bp and 556–1510 bp, respectively) and represent single loci, some of the variation in genetic distance estimates reflects sampling error.

The ratio between mitochondrial and nuclear genetic distances can also be used to identify candidate examples where loci have introgressed from one parental species to another; in these cases, the genetic distance would be low at the introgressed locus relative to the non-introgressed locus. Based on our genetic distance ratios, potential examples of salamander mitochondrial introgression include *Salamandrina perspicillata* X *Salamandrina terdigitata* (0.6), *Eurycea neotenes* X *Eurycea tridentifera* (0.9), and *Plethodon shermani* X *Plethodon teyahalee* (2.3) (Table 2). Additional cline-based analyses of mitochondrial and nuclear introgression across the hybrid zone between these species would be informative.

With each tetrapod clade, the genetic distance between parental species that hybridize is highly variable, but we found no significant differences in genetic distances among the clades. In addition, there were no significant differences in the ratio of mitochondrial to nuclear genetic distances among the clades. Taken together, these results suggest that speciating lineages of salamanders retain the ability to hybridize at similar overall genetic divergence levels as speciating lineages in other vertebrate clades. Thus, the differences in metabolic rates across vertebrates do not appear to translate into predictable patterns of overall genetic divergence and incompatible loci in hybrids. This pattern may reflect a similar evolved match between metabolic “supply and demand” (i.e. ATP use and ATP synthesis) (Darveau et al., 2002) in all tetrapod clades, irrespective of overall metabolic rates, such that increased metabolic costs in hybrids have equal detrimental effects on fitness. Cline analyses of OXPHOS-specific loci, combined

with OXPPOS functional data in parental species and hybrids, would allow more rigorous testing of this hypothesis.

The presence of heteromorphic sex chromosomes does not appear to deter salamanders from hybridizing (Figure 3). This pattern suggests that, despite evolutionary lability in amphibian sex chromosomes (Hillis and Green, 1990; Evans et al., 2012; Sessions et al., 2016), the underlying genetic sex-determining factors might be sufficiently conserved to not be a barrier to reproduction. Consistent with this explanation, for some salamanders, the difference between the heteromorphic sex chromosomes is extremely small (Schartl et al., 2016; Keinath et al., 2018), showing that the selective pressures might be similar on each chromosome or that the region of suppressed recombination on the X or Z chromosomes is small (unlike in birds or mammals) (Schartl et al., 2016). Additionally, evidence that cryptobranchid salamander homomorphic sex chromosomes have been conserved for nearly 60 million years (Hime et al., 2019) suggests that the underlying genes for determining sex may be highly conserved. Finally, we note that the relationship between sex chromosome morphology and reproductive isolation is complex; in some cases, even homomorphic sex chromosomes show a large X/Z effect (e.g. *Hyla arborea* and *H. orientalis*, Dufresnes et al., 2016).

In conclusion, this first comprehensive review of natural salamander hybrids revealed hybrids throughout the order with a wide range of genetic divergences between parental species. Despite being outliers among tetrapods in metabolic rate and sex chromosome diversity, both of which were predicted to impact the relationship between genetic divergence and reproductive isolation, our analyses suggest that salamanders hybridize across similar levels of genetic divergence to the other examined tetrapod clades. This study further demonstrates the power of comparative studies of hybridization as an approach to understanding the process of speciation.

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