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Copeia

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Comprehensive Analysis of Salamander Hybridization Suggests a Consistent Relationship between Genetic Distance and Reproductive Isolation across Tetrapods

Scott Lucas Melander[1] and Rachel Lockridge Mueller[1]

Hybridization between populations along the path to complete reproductive isolation can provide snapshots of speciation in action. Here, we present a comprehensive list of salamander hybrids and estimate genetic distances between the parental hybridizing species using one mitochondrial and one 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 groups 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.

SPECIES formation is typically a temporally extended process, occurring over countless generations as one population-level lineage diverges into two independent population-level lineages (de Queiroz, 1998). Because of the long timescales, studying this process is challenging; however, diverging lineages that retain the ability to reproduce 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 and Hewitt, 1985). If the admixture of alleles is intrinsically harmful, such as the hybrid being sterile, 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, 2008; Sloan et al., 2017; Hill et al., 2019). When the nuclear genome includes contributions from two species, but the mitochondrial genome comes from only one of the two, the gene products encoded by the two organelles cannot 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; Barreto and Burton, 2013; Borowiec et al., 2016; McFarlane et al., 2016; Prokić et al., 2018). We hypothesize that if an organism has a low metabolic rate to begin with, this change in OXPHOS functionality might have a smaller effect on overall hybrid fitness. This, in turn, could allow hybridization between species with more severely mismatched mitochondrial and nuclear genomes. 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 el al., 2006; Anderson and Gillooly, 2018). Literature reviews summarizing 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, we predict that salamander hybrids can tolerate greater levels of genetic divergence—a proxy for mitonuclear mismatch—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 disproportionate share of hybrid incompatibilities is found on either the X or Z chromosome (Presgraves, 2008; Lavretsky et al., 2015; Janoušek et al., 2019). In hybrid zones, mutations in sex chromosomes have been shown to limit introgression (Cortés-Ortiz et al., 2019) or even cause complete reproductive isolation between lineages (Johnson and Lachance, 2012; Hooper et al., 2019). Compared to other tetrapod clades, salamanders have variable genetic sex-determining mechanisms with either homomorphic sex chromosomes or heteromorphic sex chromosomes with either ZW or XY systems (Eggert, 2004). ZW and XY systems

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Table 1. Hybridization in salamanders.

Parental species				
Species 1	Sex chromosome type	Species 2	Sex chromosome type	Resources
Ambystomatidae				
Ambystoma dumerilii		*Ambystoma mexicanum*		Brandon, 1977
Ambystoma dumerilii		*Ambystoma rivulare*		Brandon, 1977
Ambystoma macrodactylum North Central		*Ambystoma macrodactylum* Rocky Mountains		Lee-Yaw et al., 2014
Ambystoma macrodactylum	ZW*	*Ambystoma mexicanum*	ZW$^{1.2.3}$	Brandon, 1977
Ambystoma maculatum Eastern		*Ambystoma maculatum* Western		Johnson et al., 2015
Ambystoma mexicanum	ZW$^{1.2.3}$	*Ambystoma tigrinum*	ZW$^{1.2.3}$	Woodcock et al., 2017
Ambystoma mexicanum	ZW$^{1.2.3}$	*Ambystoma opacum*	ZW*	Brandon, 1977
Ambystoma talpoideum		*Ambystoma texanum*		Brandon, 1977
Ambystoma texanum	ZW*	*Ambystoma tigrinum*	ZW$^{1.2.3}$	Brandon, 1977
Ambystoma tigrinum californiense	ZW$^{1.2.3}$	*Ambystoma mavortium*	ZW*	Fitzpatrick et al., 2009 Fitzpatrick and Shaffer, 2004 Riley et al., 2003
Cryptobranchidae				
Andrias davidianus		*Andrias japonicus*		Fukumoto et al., 2015
Dicamptodontidae				
Dicamptodon ensatus	XX^{PC}	*Dicamptodon tenebrosus*	XX^{PC}	Good, 1989
Hynobiidae				
Hynobius nebulosus		*Hynobius nigrescens*		Kawamura, 1953
Onychodactylus japonicus S-Tohoku		*Onychodactylus japonicus* SW-Honshu		Yoshikawa et al., 2012
Salamandrella keyserlingii	XX^{PC}	*Salamandrella tridactyla*	XX^{PC}	Malyarchuk et al., 2015
Plethodontidae				
Aneides klamathensis		*Aneides flavipunctatus*		Reilly and Wake, 2019
Batrachoseps gavilanensis		*Batrachoseps luciae*		Jockusch and Wake, 2002
Bolitoglossa franklini	XX^{PC}	*Bolitoglossa lincolni*	XX^{PC}	Wake et al., 1980
Desmognathus carolinensis		*Desmognathus orestes*		Mead and Tilley, 2000
Desmognathus conanti	XX*	*Desmognathus fuscus*	XX^{PC}	Bonett, 2002
Desmognathus fuscus	XX^{PC}	*Desmognathus ochrophaeus*	XX^{PC}	Sharbel et al., 1995
Desmognathus fuscus	XX^{PC}	*Desmognathus santeetlah*	XX*	Tilley, 1988
Ensatina eschscholtzii croceater	XX^{PC}	*Ensatina eschscholtzii platensis*	XX^{PC}	Pereira and Wake, 2009
Ensatina eschscholtzii eschscholtzii	XX^{PC}	*Ensatina eschscholtzii klauberi*	XX^{PC}	Devitt et al., 2011
Ensatina eschscholtzii oregonensis	XX^{PC}	*Ensatina eschscholtzii picta*	XX^{PC}	Pereira and Wake, 2009
Ensatina eschscholtzii oregonensis	XX^{PC}	*Ensatina eschscholtzii xanthoptica*	XX^{PC}	Pereira and Wake, 2009
Ensatina eschscholtzii platensis	XX^{PC}	*Ensatina eschscholtzii xanthoptica*	XX^{PC}	Alexandrino et al., 2005 Sweet, 1984
Eurycea bislineata	XX^{PC}	*Eurycea cirrigera*	XX^{PC}	Guttman and Karlin, 1986
Eurycea cirrigera	XX^{PC}	*Eurycea wilderae*	XX*	Kozak, 2003
Eurycea neotenes		*Eurycea tridentifera*		Kozak and Montanucci, 2001
Hydromantes ambrosii	XY$^{1.2.3}$	*Hydromantes italicus*	XY$^{1.2.3}$	Lunghi et al., 2018 Ficetola et al., 2019
Plethodon aureolus	XX*	*Plethodon shermani*	XX^{PC}	Highton and Peabody, 2000
Plethodon chattahoochee		*Plethodon chlorobryonis*		Highton and Peabody, 2000
Plethodon chattahoochee	XX*	*Plethodon shermani*	XX^{PC}	Highton and Peabody, 2000
Plethodon chattahoochee		*Plethodon teyahalee*		Highton and Peabody, 2000
Plethodon cheoah		*Plethodon teyahalee*		Highton and Peabody, 2000
Plethodon chlorobryonis		*Plethodon cylindraceus*		Highton and Peabody, 2000
Plethodon chlorobryonis	XX*	*Plethodon metcalfi*	XX^{PC}	Highton and Peabody, 2000
Plethodon chlorobryonis		*Plethodon teyahalee*		Highton and Peabody, 2000
Plethodon cinereus	XX^{PC}	*Plethodon electromorphus*	XX*	Lehtinen et al., 2016
Plethodon cylindraceus	XX*	*Plethodon glutinosus*	XX^{PC}	Highton and Peabody, 2000
Plethodon dorsalis	XX^{PC}	*Plethodon ventralis*	XX*	Highton, 1997 Duncan and Highton, 1979
Plethodon electromorphus	XX*	*Plethodon richmondi*	XX^{PC}	Highton, 1999
Plethodon fourchensis	XX*	*Plethodon ouachitae*	XX^{PC}	Shepard et al., 2011

Table 1. Continued.

Parental species				
Species 1	Sex chromosome type	Species 2	Sex chromosome type	Resources
Plethodon glutinosus	XX^{PC}	*Plethodon jordani*	XX^{PC}	Hairston et al., 1992
Plethodon glutinosus	XX^{PC}	*Plethodon kentucki*	XX*	Kuchta et al., 2016
Plethodon hoffmani		*Plethodon virginia*		Hairston et al., 1992 Highton, 2009 Dawley, 1987
Plethodon jordani	XX^{PC}	*Plethodon metcalfi*	XX^{PC}	Chatfield et al., 2010
Plethodon jordani	XX^{PC}	*Plethodon teyahalee*	XX*	Chatfield et al., 2010
Plethodon metcalfi	XX^{PC}	*Plethodon teyahalee*	XX*	Chatfield et al., 2010
Plethodon shermani	XX^{PC}	*Plethodon teyahalee*	XX*	Highton and Peabody, 2000
Proteidae				
Necturus maculosus	$XY^{1.2.3}$	*Necturus aff. lewisi*	$XY^{1.2.3}$	Nelson et al., 2017
Salamandridae				
Chioglossa lusitanica Northern		*Chioglossa lusitanica* Southern		Sequeira et al., 2005
Cynops pyrrhogaster Central		*Cynops pyrrhogaster* Western		Tominaga et al., 2018
Lissotriton helveticus	$XY^{2.3}$	*Lissotriton vulgaris*	$XY^{2.3}$	Johanet et al., 2011
Lissotriton montandoni	XY*	*Lissotriton vulgaris*	$XY^{2.3}$	Babik et al., 2005 Zieliński et al., 2013
Lissotriton vulgaris kosswigi	$XY^{2.3}$	*Lissotriton vulgaris vulgaris*	$XY^{2.3}$	Nadachowska and Babik, 2009
Lyciasalamandra antalyana		*Lyciasalamandra billae*		Johannesen et al., 2006
Notophthalmus viridescens		*Notophthalmus viridescens dorsalis*		Takahashi et al., 2011
Ommatotriton ophryticus		*Ommatotriton nesterovi*		van Riemsdijk et al., 2018
Ommatotriton ophryticus		*Ommatotriton vittatus*		Yoshikawa et al., 2010 van Riemsdijk et al., 2018
Pleurodeles nebulosus	ZW*	*Pleurodeles poireti*	$ZW^{2.3}$	Escoriza et al., 2016
Salamandra salamandra gallaica	XX^{PC}	*Salamandra salamandra bernardezi*	XX^{PC}	García-París et al., 2003
Salamandra fastuosa	XX^{PC}	*Salamandra terrestris*	XX^{PC}	Ventura et al., 2015
Salamandrina perspicillata	XX*	*Salamandrina terdigitata*	XX^{PC}	Canestrelli et al., 2014 García-París et al., 2003 Hauswaldt et al., 2011 Mattoccia et al., 2011 Arntzen et al., 2009
Taricha rivularis	XX*	*Taricha sierrae*	XX^{PC}	Twitty, 1963
Taricha rivularis	XX*	*Taricha torosa*	XX^{PC}	Twitty, 1963
Taricha torosa	XX^{PC}	*Taricha sierrae*	XX^{PC}	Kuchta, 2007
Triturus carnifex	$XY^{2.3}$	*Triturus cristatus*	$XY^{1.2.3}$	Arntzen et al., 2014
Triturus carnifex	$XY^{2.3}$	*Triturus dobrogicus*	XY*	Arntzen et al., 2014
Triturus carnifex	$XY^{2.3}$	*Triturus ivanbureschi*	XY*	Arntzen et al., 2014
Triturus carnifex	$XY^{2.3}$	*Triturus macedonicus*	XY*	Arntzen et al., 2014
Triturus cristatus	$XY^{1.2.3}$	*Triturus dobrogicus*	XY*	Arntzen et al., 2014
Triturus cristatus	$XY^{1.2.3}$	*Triturus ivanbureschi*	XY*	Arntzen et al., 2014
Triturus cristatus	$XY^{1.2.3}$	*Triturus macedonicus*	XY*	Arntzen et al., 2014
Triturus cristatus	$XY^{1.2.3}$	*Triturus marmoratus*	$XY^{1.2.3}$	Visser et al., 2017
Triturus ivanbureschi		*Triturus macedonicus*		Vučić et al., 2018

[1] Evans et al., 2012
[2] Hillis and Green, 1990
[3] Perkins et al., 2019
* Predicted sex chromosome type
[PC] Personal correspondence, Stan Sessions, 2019

are both found within multiple families, indicating that sex chromosomes are evolutionarily dynamic in salamanders; this makes the clade a good system for studying the effects of heteromorphic sex chromosome evolution on reproductive isolation (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). With a few notable exceptions (e.g., Twitty, 1963; Brandon, 1977; 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, Blair, 1972; Malone and Fontenot, 2008). Here, we summarize the known cases of hybridization in salamanders. We then use this dataset to compare the genetic distances across which viable hybrids can form in different tetrapod groups and test whether differences in metabolic rate and sex chromosomes impact hybridization.

MATERIALS AND METHODS

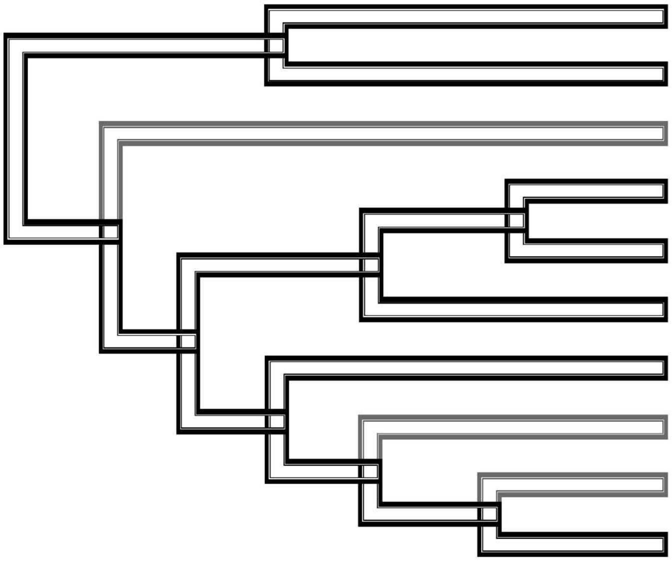
Compilation of salamander hybrids.—The first goal was the establishment of a comprehensive list of published salamander hybrids, which did not exist when we began this research (Table 1). Taxonomy is continually revised to provide scientific names that convey accurate information about species boundaries as well as the evolutionary relationships among species. Making these species designations is a rich discipline, with disagreements among taxonomists requiring different levels of divergence or isolation between populations before they are formally named as species (Highton, 1998; Kuchta and Wake, 2016). For our literature review, we used a general lineage concept of species where a species equates to a segment of a population-level evolutionary lineage (de Queiroz, 1998). We chose to include 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. We included cases where secondary contact occurred because of human-mediated introductions, and we also included laboratory crosses. Hybridization that resulted in polyploidy was excluded to eliminate the confounding variable of increased ploidy levels. Species with evidence of historical, but not ongoing, hybridization were also excluded. This compilation of hybridizing species represents extant salamanders that are known to have had the opportunity to hybridize and done so successfully; there are undoubtedly pairs of lineages that would be able to hybridize, given the opportunity (either with or without human mediation), as well as undetected instances of natural hybridization. The criteria we chose allowed us to compile the maximum amount of information about hybrid salamanders from the literature, as well as make comparisons with existing literature surveys on birds, mammals, and lizards that applied similar criteria (Fitzpatrick, 2004; Jančúchová-Lásková et al., 2015).

Database searches were performed using the terms salamander, newt, hybrid, and contact zone. Databases used were JSTOR, Web of Science, and Wildlife and 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 February 2020 and included research published between 1979–2020. Many taxonomic changes occurred during this 40-year period; whenever there was a conflict in species name, the current listing on AmphibiaWeb (https://amphibiaweb.org) was used to resolve the issue.

Intensity of research on different salamander families.—To check whether or not 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 the literature (Fig. 1). 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 standardized by dividing by the number of species in each family based on AmphibiaWeb as of July 2020 (https://amphibiaweb.org). To test whether the reported number of hybrids within each salamander family 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; Fig. 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 groups (Fitzpatrick, 2004; Jančúchová-Lásková et al., 2015). For each parental species, the longest high-quality sequence was downloaded from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). In some cases, this involved extracting the MT-CYB sequence from a complete mitochondrial genome sequence. When multiple equally long, high-quality sequences were available, one was selected at random. Of the 76 salamander species pairs known to hybridize, MT-CYB sequence data were available for 62 (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 1,141 base pairs. The genetic distances were estimated with PAUP * Version 4.0a (Swofford, 2002) using the HKY85 + Γ nucleotide substitution model (Hasegawa et al., 1985). 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 81 salamander MT-CYB sequences used in this study (Kumar et al., 2018).

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 (Fig. 2B). 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



	# Hybrids/ # Named Species	Percent able to Hybridize	# of Web of Science Papers	# Papers per Species
Hynobiidae	5/82	6.1%	1749	21
Cryptobranchidae	2/4	50%	1009	252
Sirenidae	0/5	0%	393	79
Ambystomatidae	9/32	28.1%	9862	308
Dicamptodontidae	2/4	50%	428	107
Salamandridae	29/125	23.2%	13256	106
Proteidae	2/8	25%	1996	250
Rhyacotritonidae	0/4	0%	176	44
Amphiumidae	0/3	0%	811	270
Plethodontidae	42/486	8.6%	6299	13
Total	91/753	12.1%	32499	43

Fig. 1. Salamander hybrids are found in most families (Pyron and Wiens, 2011). About 12.1% 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 $P = 0.236$).

and was available for the most taxa (Chiari et al., 2009). For every parental species available, the longest high-quality RAG1 sequence was downloaded from GenBank. When multiple equally long, high-quality sequences were available, one was selected at random. 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 tetrapod groups.—The MT-CYB genetic distances between hybridizing parental species of salamanders were compared to those of birds, mammals, and lizards (i.e., squamate reptiles excluding snakes). These tetrapod groups were selected because they span part of the range of vertebrate metabolic rates (Pough, 1980; White el al., 2006; Anderson and Gillooly, 2018) and because of the existence of published summaries of known hybrids that include natural, human-introduced, and lab-crossed hybridization events (Fitzpatrick, 2004; Jančúchová-Lásková et al., 2015). A hybrid toad summary was excluded because it focused only on experimental crosses (Blair, 1972; Malone and Fontenot, 2008). In some tetrapod groups, many closely related species hybridize, or a single species hybridizes several times. This creates a potential bias when looking at many pairwise comparisons as a single taxon becomes overrepresented, and the results become 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

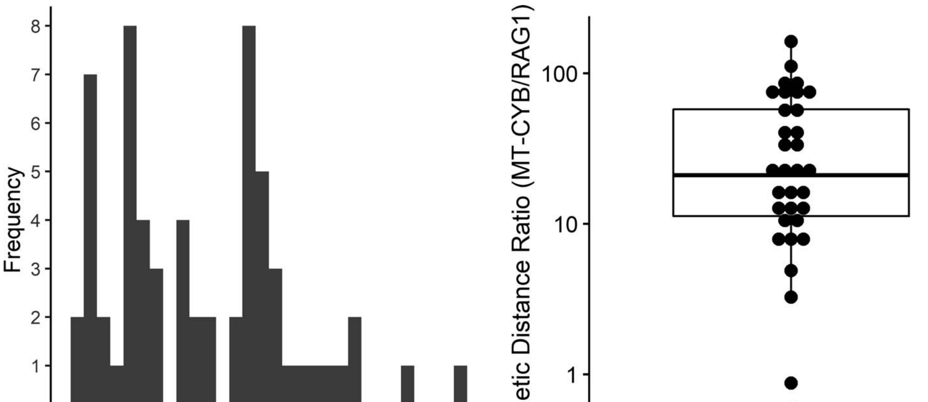


Fig. 2. (A) Histogram of cytochrome *b* (MT-CYB) genetic distance estimates (HKY85 + Γ) between salamanders that hybridize. Graph bin width is 0.0125. (B) Ratio of mitochondrial MT-CYB genetic distance to nuclear recombination activating 1 (RAG1) genetic distance between parental species of salamanders that hybridize on a log scale. The ratio of genetic distance ranged from 0.6 to 162 with the majority of species pairs having a higher mitochondrial genetic distance (median = 21.1).

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 salamanders (*) for the cross-clade comparison (Fig. 3).

	Accession number			Accession number		Genetic distance (HKY85 + Γ)		
Species 1	MT-CYB	RAG1	Species 2	MT-CYB	RAG1	MT-CYB	RAG1	MT-CYB/RAG1
Birds								
Acrocephalus scirpaceus	LT671508.1		*A. palustris*	AJ004774.1		0.121		
Aegypius monachus	AY987266.1	EF078711.1	*Gyps fulvus*	AY987261.1	EU496474.1	0.120	0.005	22.0
Agapornis nigrigenis	AF001328.1	GQ505193.1	*Melopsittacus undulatus*	DQ467903.1	DQ143354.1	0.265	0.036	7.3
Alectura lathami	KF833611.1	AF294687.2	*Gallus gallus*	L08376.1	NM001031188.1	0.366	0.075	4.9
Branta canadensis	EU585629.1		*Cairina moschata*	L08385.1		0.235		
Bugeranus carunculatus	U27556.1		*Grus canadensis*	EU166997.1		0.074		
Buteo buteo	X86741.1	EU345528.1	*Pernis apivorus*	X86758.1	EF078753.1	0.185	0.018	10.2
Carduelis chloris	AY495384.1		*Emberiza citrinella*	AY495392.1		0.221		
Ciconia ciconia	KJ456229.1		*C. nigra*	U72771.1		0.184		
Cathartes aura	EU166984.1	EF078766.1	*Coragyps atratus*	KX534417.1	KM876315.1	0.167	0.008	21.8
Columba livia	KC675192.1	AY228768.1	*Leucosarcia melanoleuca*	AF483327.1	EF373512.1	0.239	0.036	7.4
Diphyllodes magnificus	X74255.1		*Paradisaea minor*	U25737.1		0.159		
Egretta garzetta	MH645659.1		*Nycticorax nycticorax*	AF193829.1		0.143		
Falco columbarius	EU233049.1	EU233167.1	*F. tinnunculus*	EU233121.1	EU233241.1	0.140	0.006	24.4
Ficedula albicollis	DQ674491.1	XM005046928.1	*F. hypoleuca*	KJ930552.1	DQ466798.1	0.042	0.004	11.0
Garrulus glandarius	AB242559.1		*Perisoreus infaustus*	U86042.1		0.247		
Hirundo rustica	DQ119526.1	AY443290.1	*H. pyrrhonota*	AF074591.1	AY056997.1	0.181	0.010	18.0
Larus canus	AB208756.1		*L. ridibundus*	FM209923.1		0.056		
Phylloscopus collybita	HQ608821.1		*P. trochilus*	MH079362.1		0.146		
Pterocnemia pennata	U76054.1		*Rhea americana*	L78808.1		0.084		
Regulus calendula	AY329472.1	AY057028.1	*R. satrapa*	AJ004329.1	AY443327.1	0.002	0.017	0.1
Scolopax rusticola	KM434134.1	AY228802.1	*Vanellus vanellus*	KM577158.1: 13657–14802	AY339126.1	0.260	0.052	5.0
Turdus migratorius	AF197835.1	KC789829.1	*T. philomelos*	AY495411.1	AY307214.1	0.196	0.013	15.4
Lizards								
Amblyrhynchus cristatus	AY948118.1	KR350710.1	*Conolophus subcristatus*	AY948122.1	KR350708.1	0.219	0.008	25.8
Anolis aeneus	EU557103.1	JN112592.1	*A. trinitatis*	AF493592.1	JN112645.1	0.286	0.009	30.8
Carlia rubrigularis NORTH	AF181042.1		*C. rubrigularis* South	AF181056.1		0.229		
Crotaphytus bicinctores	EU037682.1	MK780621.1	*C. collaris*	EU037482.1	FJ356749.1	0.137	0.007	21.1
Ctenosaura bakeri	GU331976.1		*C. similis*	GU331975.1		0.184		
Gambelia sila	EU037370.1		*G. wislizenii*	EU037415.1		0.026		
Iberolacerta galani	HQ234901.1	KY762187.1	*I. monticola*	HQ234897.1	EF632220.1	0.050	0.001	33.8
Iguana delicatissima	KX610607.1		*I. iguana*	AF020251.1		0.172		
Kentropyx calcarata	JQ639739.1		*K. striata*	JQ639672.1		0.223		
Lacerta agilis	AF373032.1	EF632222.1	*L. schreiberi*	AF372103.1	KY762190.1	0.310	0.017	18.7
Lacerta pater	AF378964.1		*Timon lepidus*	JX626302.1		0.204		
Leiolepis guttata	NC014179.1		*L. reevesii*	EU305052.1		0.339		
Liolaemus bibronii	JN410531.1		*L. gracilis*	JN410538.1		0.177		
Nactus multicarinatus	KC581486.1	HM997172.1	*N. pelagicus*	KC581545.1	EU054275.1	0.081	0.004	20.9
Oligosoma otagense	JN999970.1	EU568093.1	*O. waimatense*	JN999978.1	EU568094.1	0.114	0.001	96.4
Phrynocephalus putjatai	KF691634.1	KC551413.1	*P. vlangalii*	KF691642.1	KJ195972.1	0.058	0.005	10.6

Table 2. Continued.

	Accession number			Accession number		Genetic distance (HKY85 + Γ)		
Species 1	MT-CYB	RAG1	Species 2	MT-CYB	RAG1	MT-CYB	RAG1	MT-CYB/RAG1
Phrynosoma cornutum	AY141087.1	DQ385423.1	*P. coronatum*	AY141097.1	FJ356738.1	0.291	0.030	9.8
Plestiodon japonicus	EU203134.1	HM161196.1	*P. latiscutatus*	EU203035.1	HM161203.1	0.175	0.004	47.2
Podarcis melisellensis	AY185036.1		*P. sicula*	AY770890.1		0.292		
Zootoca vivipara carniolice	AY714929.1		*Z. vivipara vivipara*	AY714913.1		0.069		
Mammals								
Alcelaphus buselaphus	AJ222681.1		*Damaliscus lunatus*	AF016635.1		0.161		
Arctocephalus pusillus	AM181018.1		*Zalophus californianus*	AM422164.1		0.088		
Bos bison	AF036273.1		*B. indicus*	AF419237.2		0.089		
Camelus bactrianus	JX177500.1	XM010964664.1	*C. dromedarius*	KU509220.1	XM011000597.1	0.170	0.004	48.2
Capra caucasica	AF034738.1		*C. sibirica*	KF990328.1		0.110		
Cervus elaphus	AB001612.1		*Odocoileus hemionus*	AF091630.1		0.226		
Chinchilla brevicaudata	AF464756.1		*C. lanigera*	AF122820.1		0.069		
Equus caballus	DQ223535.1	NM001256901.1	*E. grevyi*	X56282.1	AY239184.1	0.118	0.009	13.1
Eulemur macaco	AF081049.1	HM759153.1	*E. mongoz*	AF081051.1	EU342315.1	0.140	0.005	27.4
Halichoerus grypus	GU167293.1		*Phoca hispida*	X82304.1		0.043		
Hylobates agilis	AJ010583.1		*H. hoolock*	Y13304.1		0.178		
Kobus kob	AF052939.1		*K. megaceros*	AJ222686.1		0.136		
Lama guanicoe	U06428.1		*Vicugna vicugna*	U06430.1		0.083		
Lepus europaeus	AY745112.1		*L. timidus*	AJ279424.1		0.152		
Macaca fascicularis	AF295584.1	XM005578172.2	*Mandrillus sphinx*	JQ068151.1	HM759046.1	0.328	0.001	353.2
Martes martes	AF154975.1		*M. putorius*	X94925.1		0.218		
Mastomys natalensis	JX292865.1	DQ023475.1	*Mus musculus*	AY057804.1	NM009019.2	0.317	0.055	5.7
Microtus californicus	AF163891.1	KC953523.1	*M. montanus*	AF119280.1	KC953524.1	0.189	0.0173	10.9
Ovis aries	AF034730.1	XM012134165.2	*O. canadensis*	EU365985.1	AY239177.1	0.089	0.006	14.1
Panthera leo	X82300.1	AB109364.1	*P. pardus*	JF720058.1	XM019470137.1	0.107	0.002	58.2
Pecari tajacu	DQ179055.1		*Tayassu pecari*	AY534303.1		0.073		
Peromyscus difficilis	AF155394.1		*P. truei*	FJ800579.1		0.221		
Spermophilus major	AF157903.1		*S. pygmaeus*	AF157907.1		0.158		
Ursus americanus	U23556.1	DQ240717.1	*U. arctos*	U18870.1	XM026511863.1	0.172	0.008	21.0
Vulpes lagopus	LT559489.1	GU167551.1	*V. vulpes*	AY928669.1	XM026012934.1	0.181	0.179	1.0
Salamanders								
Ambystoma dumerilii	AY659994.1		*A. mexicanum*	AY659991.1		0.053		
Ambystoma macrodactylum	EF036633.1		*A. mexicanum*	AY659991.1		0.208		
Ambystoma mexicanum	AY659991.1		*A. tigrinum*	AY659992.1		0.075		
Ambystoma mexicanum	AY659991.1	AY323752.1	*A. opacum*	AY691730.1	AY650130.1	0.256*	0.030*	8.4*
Ambystoma talpoideum	MG822788.1		*A. texanum*	GU078471.1		0.263		
Ambystoma texanum	GU078471.1		*A. tigrinum*	AY659992.1		0.172		
Andrias davidianus	KU131042.1	MH106790.1	*A. japonicus*	AB208679.1	AY583346.1	0.099*	0.004*	21.1*
Batrachoseps gavilanensis	KM203055.1	KM202898.1	*B. luciae*	KM203053.1	KM202878.1	0.187*	0.011*	17.1*
Bolitoglossa franklini	MK165231.1	KC614439.1	*B. lincolni*	GU725464.1	KC614440.1	0.030*	0.003*	12.0*
Desmognathus carolinensis	EU314306.1	KR732369.1	*D. orestes*	EU314288.1	KR827015.1	0.161	0.003	57.6
Desmognathus conanti	KY659020.1	KR732370.1	*D. fuscus*	AY728227.1	KR732372.1	0.236*	0.011*	20.7*
Desmognathus fuscus	AY728227.1	KR732372.1	*D. ochrophaeus*	EU314289.1	KR732377.1	0.165	0.013	12.9

Table 2. Continued.

Species 1	Accession number		Species 2	Accession number		Genetic distance (HKY85 + Γ)		
	MT-CYB	RAG1		MT-CYB	RAG1	MT-CYB	RAG1	MT-CYB/RAG1
Desmognathus fuscus	AY728227.1	KR732372.1	*D. santeetlah*	EU314270.1	KR732384.1	0.196	0.012	16.3
Dicamptodon ensatus	AY734600.1	EF107335.1	*D. tenebrosus*	AAW70411.1	AY650132.1	0.044*	0.005*	8.0*
Ensatina eschscholtzii croceater	L75796.1		*E. eschscholtzii platensis*	FJ151995.1		0.063		
Ensatina eschscholtzii eschscholtzii	FJ151951.1		*E. eschscholtzii klauberi*	L75801.1		0.181		
Ensatina eschscholtzii oregonensis	FJ151696.1		*E. eschscholtzii picta*	FJ151670.1		0.059		
Ensatina eschscholtzii oregonensis	FJ151696.1		*E. eschscholtzii xanthoptica*	FJ151887.1		0.223		
Ensatina eschscholtzii platensis	FJ151995.1		*E. eschscholtzii xanthoptica*	FJ151887.1		0.177		
Eurycea bislineata	AY728217.1	AY691706.1	*E. cirrigera*	NC035494.1	FJ750236.1	0.124*	0.017*	7.5*
Eurycea cirrigera	NC035494.1	FJ750236.1	*E. wilderae*	JQ920621.1	JQ920766.1	0.101	0.021	4.9
Eurycea neotenes	AY528400.1	AY650122.1	*E. tridentifera*	AY014848.1	KF562669.1	0.009	0.010	0.9
Hydromantes ambrosii	FJ602258.1	FJ602321.1	*H. italicus*	FJ602299.1	EU275791.1	0.059*	0.0008*	71.9*
Hynobius nebulosus	HM036356.1	AY650144.1	*H. nigrescens*	JQ929922.1	KJ715356.1	0.309*	0.010*	32.0*
Lissotriton helveticus	DQ821238.1		*L. vulgaris*	EU880339.1		0.360		
Lissotriton montandoni	DQ821254.1		*L. vulgaris*	EU880339.1		0.010		
Ommatotriton nesterovi	KX682106.1		*O. ophryticus*	DQ821267.1		0.011		
Ommatotriton ophryticus	DQ821267.1		*O. vittatus*	EU880338.1		0.014		
Plethodon aureolus	DQ994914.1	DQ995012.1	*P. shermani*	DQ994985.1	DQ995063.1	0.166	0.002	73.9
Plethodon chattahoochee	DQ994919.1	DQ995014.1	*P. teyahalee*	DQ994990.1	DQ995068.1	0.047	0.003	13.5
Plethodon chattahoochee	DQ994919.1	DQ995014.1	*P. chlorobryonis*	DQ994923.1	DQ995016.1	0.051	0.003	15.3
Plethodon chattahoochee	DQ994919.1	DQ995014.1	*P. shermani*	DQ994985.1	DQ995063.1	0.051	0.002	24.7
Plethodon cheoah	DQ994921.1		*P. teyahalee*	DQ994990.1		0.046		
Plethodon chlorobryonis	DQ994923.1		*P. cylindraceus*	DQ994928.1		0.016		
Plethodon chlorobryonis	DQ994923.1		*P. teyahalee*	DQ994990.1		0.011		
Plethodon chlorobryonis	DQ994923.1		*P. metcalfi*	DQ994956.1		0.160		
Plethodon cinereus	AY378042.1	AY691703.1	*P. electromorphus*	AY378060.1	DQ995024.1	0.151	0.013	11.3
Plethodon cylindraceus	DQ994928.1	DQ995022.1	*P. glutinosus*	DQ994937.1	DQ995027.1	0.169	0.003	56.1
Plethodon dorsalis	GQ464404.1	DQ995023.1	*P. ventralis*	DQ994993.1	DQ995071.1	0.034	0.003	9..83
Plethodon electromorphus	AY378060.1	DQ995024.1	*P. richmondi*	AY378072.1	DQ995051.1	0.081	0.002	39.2
Plethodon fourchensis	FJ611481.1	DQ995026.1	*P. ouachitae*	FJ266744.1	AY691704.1	0.179	0.002	86.9
Plethodon glutinosus	DQ994937.1	DQ995027.1	*P. jordani*	DQ994947.1	DQ995032.1	0.184	0.002	82.0
Plethodon glutinosus	DQ994937.1	DQ995027.1	*P. kentucki*	DQ994948.1	DQ995033.1	0.186*	0.005*	34.9*
Plethodon hoffmani	AY378047.1	DQ995029.1	*P. virginia*	AY378049.1	DQ995072.1	0.047	0.0007	68.4
Plethodon jordani	DQ994947.1	DQ995032.1	*P. metcalfi*	DQ994956.1	DQ995040.1	0.076	0.0007	111.2
Plethodon jordani	DQ994947.1	DQ995032.1	*P. teyahalee*	DQ994990.1	DQ995068.1	0.162	0.002	84.6
Plethodon metcalfi	DQ994956.1		*P. teyahalee*	DQ994990.1		0.156		
Plethodon shermani	DQ994985.1	DQ995063.1	*P. teyahalee*	DQ994990.1	DQ995068.1	0.003	0.001	3.3
Salamandra salamandra bernardezi	DQ092219.1		*S. salamandra gallaica*	KX094979.1		0.024		
Salamandra salamandra fastuosa	DQ221234.1		*S. salamandra terrestris*	AY222503.1		0.006		
Salamandrella keyserlingii	AY593141.1	AY650145.1	*S. schrenckii*	AB363608.1	KJ855096.1	0.153*	0.004*	41.6*
Salamandrina perspicillata	DQ821207.1	HQ915345.1	*S. terdigitata*	EU880332.1	HQ915218.1	0.004*	0.007*	0.6*
Taricha rivularis	EU880334.1	AY650133.1	*T. torosa*	DQ196247.1	EF107340.1	0.161*	0.007*	21.9*
Taricha rivularis	EU880334.1		*T. sierrae*	DQ196282.1		0.167		
Taricha sierrae	DQ196282.1		*T. torosa*	DQ196247.1		0.123		

Table 2. Continued.

Species 1	Accession number		Species 2	Accession number		Genetic distance (HKY85 + Γ)		
	MT-CYB	RAG1		MT-CYB	RAG1	MT-CYB	RAG1	MT-CYB/RAG1
Triturus carnifex	NC 015788.1		*T. cristatus*	HQ697273.1		0.102		
Triturus carnifex	NC 015788.1		*T. dobrogicus*	HQ697274.1		0.104		
Triturus carnifex	NC 015788.1		*T. macedonicus*	HQ697278.1		0.058		
Triturus cristatus	HQ697273.1		*T. dobrogicus*	HQ697274.1		0.114		
Triturus cristatus	HQ697273.1		*T. marmoratus*	HQ697279.1		0.263		
Triturus cristatus	HQ697273.1		*T. macedonicus*	HQ697278.1		0.115		
Triturus marmoratus	HQ697279.1		*T. pygmaeus*	HQ697280.1		0.048		

list of hybrids for salamanders and lizards was also pared down to make results comparable. For salamanders, the dataset was pared down to a single pair that can hybridize per genus; all genera are monophyletic. 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). MT-CYB genetic distances between the parental species for each hybridizing species pair were calculated for birds, mammals, and lizards using the same methods as for salamanders (Fig. 3, Table 2). For each of the tetrapod groups, a different Γ distribution shape parameter α was estimated using 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 tetrapod groups. Effect size was calculated and reported in terms of η^2.

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 (Fig. 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). RAG1 genetic distances between the parental species for each hybridizing species pair were calculated for birds, mammals, and lizards using the same methods as for salamanders. A Kruskal-Wallis test was used to determine if the ratio of MT-CYB/RAG1 genetic distances differed among the four tetrapod groups. A Kruskal-Wallis test was also used to determine if RAG1 genetic distance differed among the four tetrapod groups. Effect size was calculated for both tests and reported in terms of η^2.

Relationship between sex chromosomes and hybridization in salamanders.—A list of the salamanders that 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). To further clarify, additional unpublished information on salamanders that are known to have homomorphic sex chromosomes was obtained (S. Sessions, pers. comm., 19 June 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 t-test (Fig. 5). 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

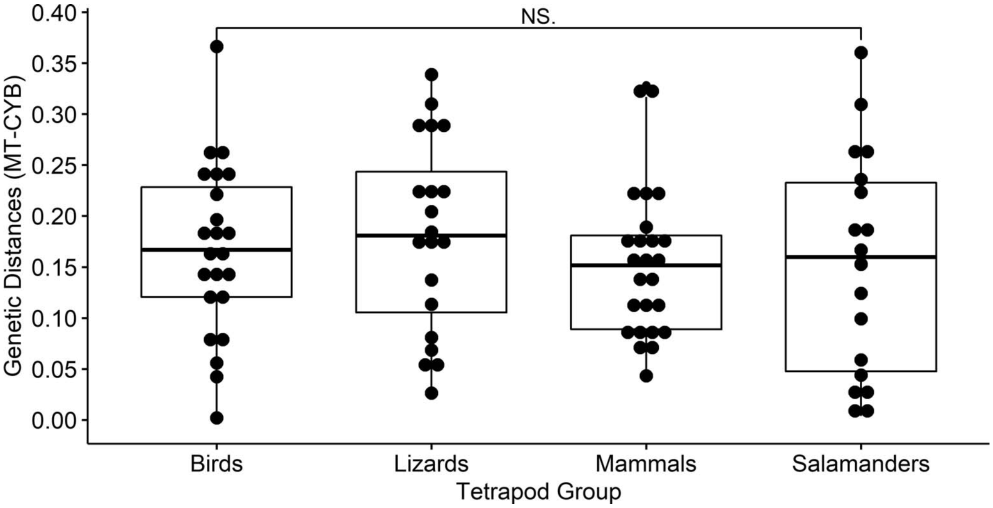


Fig. 3. Cytochrome *b* (MT-CYB) genetic distances between parental species that hybridize in different tetrapod groups. Kruskal-Wallis H test showed no significant differences across the groups ($P = 0.661$). Salamanders are not hybridizing across greater genetic distances.

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

Hybrids in salamanders.—Our literature review found 81 pairs of genetically distinct salamander lineages that hybridize in nature (Table 1). These lineages represent 12.1% of named salamander species and seven out of ten salamander families (Fig. 1). The seven families represented include Plethondontidae (42 species), Salamandridae (29 species), Ambystomatidae (9 species), Hynobiidae (5 species), Cryptobranchidae (2 species), Dicamptodontidae (2 species), and Proteidae (2 species; Fig. 1). Many pairs that hybridize come from Plethondontidae and Salamandridae, consistent with these being the two largest families of salamanders (486 and 125 species, respectively; https://amphibiaweb.org). The third largest salamander family (Hynobiidae with 82 species) contains proportionally fewer hybrids with only three recorded pairs that can hybridize (Fig. 1). The families Amphiumidae, Rhyacotritonidae, and Sirenidae have no recorded cases of hybrids. These families of salamanders contain very few species with the largest of the three, Sirenidae, only containing five species. There is no case of hybridization occurring between species of different genera. Overall, these results are consistent with those of another recently published study (Patton et al., 2020).

Intensity of research on different salamander families.—We identified 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 (Fig. 1). However, *Ambystoma mexicanum* is a contributor to the high papers-per-species count because it is a model lab animal and makes up a quarter (2,694) of the Web of Science results for Ambystomatidae (Fig. 1; Voss et al., 2010). Two of the families with the highest numbers of species have the lowest intensity of publication: Hynobiidae (21 papers per species) and Plethondontidae (13 papers per species). There are proportionally more papers published for families with fewer species: Amphiumidae (270 papers per species), Cryptobranchidae (252 papers per species), Proteidae (249.5 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 $P = 0.236$). We

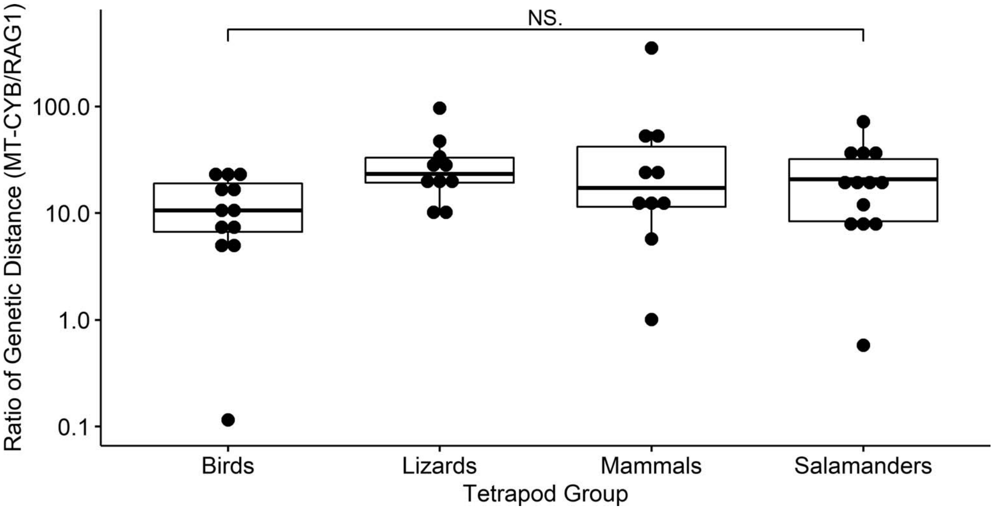


Fig. 4. Genetic distance ratio of mitochondrial cytochrome *b* (MT-CYB) to nuclear recombination activating 1 (RAG1) for pairs of species that hybridize in major tetrapod clade on a log scale. Kruskal-Wallis H test showed no strong significant differences across the four groups ($P = 0.108$).

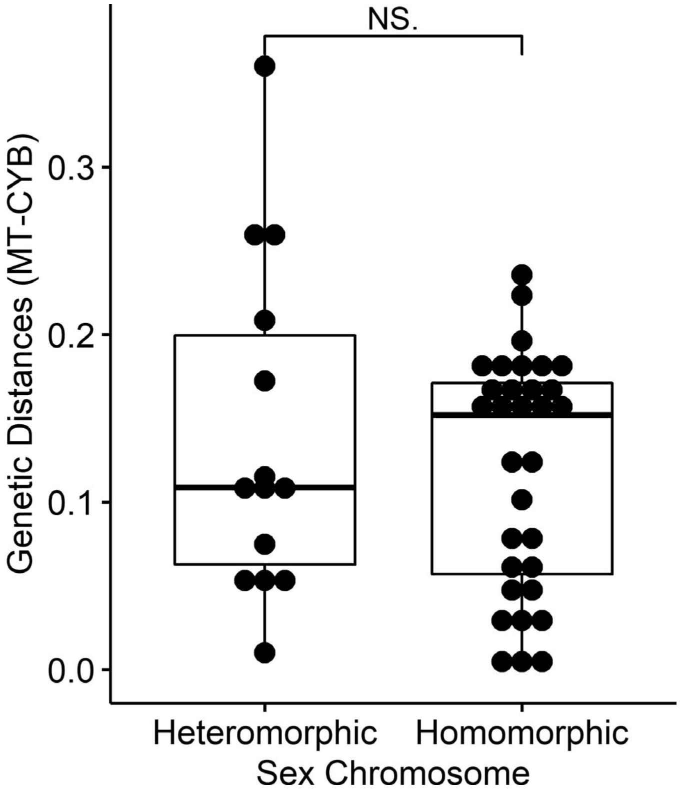


Fig. 5. Genetic distances between salamanders that hybridize with heteromorphic sex chromosomes (median = 0.109) and salamanders that hybridize without sex chromosomes (median = 0.152). There is no significant difference (P = 0.5293) between salamanders with sex chromosomes and salamanders without sex chromosomes.

acknowledge that studies of salamanders address far more biological questions than only those that are relevant to hybrid identification. Further analyses based on refined literature searches that target only the most pertinent disciplines (e.g., phylogeography, systematics) would provide a stronger test of the relationship between research effort and hybrid identification.

Salamander hybrid genetic distances.—For the 62 parental pairs of salamanders that have published MT-CYB sequence data, the mean genetic distance was 0.117 (Table 2). *Lissotriton vulgaris* X *Lissotriton helveticus* had the largest genetic distance of 0.360, while *Plethodon teyahalee* X *Plethodon shermani* had the smallest distance of 0.003 (Table 2). For the 33 parental pairs of salamanders that have published RAG1 data, the mean genetic distance was 0.00657 (range 0.000683 to 0.0306; Table 2). The genetic distance ratio of the mitochondrial gene MT-CYB over the nuclear gene RAG1 between salamanders that hybridize was highly variable (Fig. 2B). The ratio ranged from 0.6x to 162x with most species pairs having a higher mitochondrial genetic distance (median ratio = 21.1).

Genetic distances between parental species that hybridize in different tetrapod groups.—After paring down the salamanders and lizards to the pair that can hybridize with the highest genetic distance per genus, the across-tetrapod comparison included 23 pairs of hybrids for birds, 20 pairs for lizards, 25 pairs for mammals, and 18 pairs for salamanders (Table 2). The median MT-CYB genetic distances for the groups were: lizards (0.181), birds (0.167), salamanders (0.160), and mammals (0.152; Fig. 3). There were no significant differences in distributions of genetic distance among the four groups ($\chi^2 = 1.5922$, $P = 0.66$, $\eta^2 = -0.0172$). Across all tetrapods, 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, 10 pairs for lizards, 10 pairs for mammals, and 13 pairs for salamanders (Table 2). The median RAG1 genetic distances for the four groups were: birds (0.0151), mammals (0.00722), salamanders (0.00651), and lizards (0.00600). There were no significant differences among the four tetrapod groups ($\chi^2 = 5.8748$, $P = 0.12$; $\eta^2 = 0.0701$). The median MT-CYB/RAG1 genetic distance ratio for the four groups were: lizards (23.4), salamanders (20.7), mammals (17.6), and birds (10.6; Fig. 4). There were no significant differences among the four tetrapod groups ($\chi^2 = 6.0734$, $P = 0.11$; $\eta^2 = 0.075$).

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). Salamander families are not characterized by a single type of sex chromosome; different members of Salamandridae and Plethodontidae have ZZ/ZW and XX/XY systems. Out of the salamanders with known heteromorphic sex chromosomes, 12/48 are known to hybridize with a different species (Table 3). There is no difference between the percentage of salamanders that hybridize with known heteromorphic sex chromosomes and the known overall salamander hybridization rate ($P = 0.99$). Also, the genetic distances between parental salamanders that hybridize with known heteromorphic sex chromosomes (median = 0.109) and parental salamanders that hybridize with homomorphic sex chromosomes (median = 0.152) are not significantly different ($P = 0.53$; Fig. 5). Based on these analyses, there is no indication that the presence of heteromorphic sex chromosomes hinders hybridization in salamanders.

DISCUSSION

Overall, we found that hybridization is widespread throughout the salamander clade, occurring in both the biggest and smallest families (Fig. 1). The largest four families (Ambystomatidae, Hynobiidae, Plethodontidae, and Salamandridae) account for nearly 96% of all salamander species and account for over 91% of the hybrids (Fig. 1). With respect to geographic location, known hybrids are widespread in the Northern temperate zone, 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 *B. lincolni* (Table 1). *Bolitoglossa* is the largest genus of salamanders (134 species), and it occurs exclusively in the tropics. More generally, close to half of all salamander diversity occurs in the tropics, suggesting that the low reported number of tropical hybrids is not a result of low tropical species diversity (https://amphibiaweb.org). If the low reported number of hybrids is an accurate reflection of the underlying biological reality, it

Table 3. Heteromorphic sex chromosomes in salamanders.

Species	Family	Sex chromosome type	Hybrid (Yes/No)
Ambystoma jeffersonianum [3]	Ambystomatidae	ZW	No
Ambystoma laterale [1,2,3]	Ambystomatidae	ZW	No
Ambystoma mexicanum [1,2,3]	Ambystomatidae	ZW	Yes
Ambystoma tigrinum [1,2,3]	Ambystomatidae	ZW	Yes
Hynobius hidamontanus [1,3]	Hynobiidae	ZW	No
Hynobius quelpaertensis [1,3]	Hynobiidae	ZW	No
Hynobius tokyoensis [3]	Hynobiidae	ZW	No
Aneides ferreus [1,2,3]	Plethodontidae	ZW	No
Bolitoglossa subpalmata [1,2,3]	Plethodontidae	XY	No
Chiropterotriton dimidiatus [1,2,3]	Plethodontidae	ZW	No
Cryptotriton veraepacis [1,2,3]	Plethodontidae	XY	No
Dendrotriton bromeliacius [2,3]	Plethodontidae	XY	No
Dendrotriton cuchumatanus [2]	Plethodontidae	XY	No
Dendrotriton rabbi [1,2,3]	Plethodontidae	XY	No
Dendrotriton xolocalcae [1,2,3]	Plethodontidae	XY	No
Hydromantes ambrosii [1,2,3]	Plethodontidae	XY	Yes
Hydromantes flavus [1,2,3]	Plethodontidae	XY	No
Hydromantes imperialis [1,2,3]	Plethodontidae	XY	No
Hydromantes italicus [1,2,3]	Plethodontidae	XY	Yes
Hydromantes genei [3]	Plethodontidae	XY	No
Hydromantes supramontis [1,3]	Plethodontidae	XY	No
Nototriton abscondens [1,3]	Plethodontidae	XY	No
Nototriton picadoi [1,2,3]	Plethodontidae	XY	No
Nototriton richardi [1]	Plethodontidae	XY	No
Oedipina cyclocauda [1]	Plethodontidae	XY	No
Oedipina parvipes [1,2]	Plethodontidae	XY	No
Oedipina poelzi [1,2,3]	Plethodontidae	XY	No
Oedipina pseudouniformis [1]	Plethodontidae	XY	No
Oedipina uniformis [2,3]	Plethodontidae	XY	No
Thorius dubitus [1,2,3]	Plethodontidae	XY	No
Thorius pennatulus [2,3]	Plethodontidae	XY	No
Necturus alabamensis [3]	Proteidae	XY	No
Necturus beyeri [1,2,3]	Proteidae	XY	No
Necturus lewisi [1,2,3]	Proteidae	XY	Yes
Necturus maculosus [1,2,3]	Proteidae	XY	Yes
Necturus punctatus [1,2,3]	Proteidae	XY	No
Ichthyosaura alpestris [3]	Salamandridae	XY	No
Lissotriton boscai [3]	Salamandridae	XY	No
Lissotriton helveticus [2,3]	Salamandridae	XY	Yes
Lissotriton italicus [2,3]	Salamandridae	XY	No
Lissotriton vulgaris [2,3]	Salamandridae	XY	Yes
Pleurodeles poireti [1,2,3]	Salamandridae	ZW	Yes
Pleurodeles waltl [1,2,3]	Salamandridae	ZW	No
Triturus carnifex [2,3]	Salamandridae	XY	Yes
Triturus cristatus [1,2,3]	Salamandridae	XY	Yes
Triturus karelinii [1,3]	Salamandridae	XY	No
Triturus marmoratus [1,2,3]	Salamandridae	XY	Yes
Siren intermedia [1,2]	Sirenidae	ZW	No

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

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). However, low reported numbers of hybrids could also mean that additional cases have yet to be discovered and described. Additional research will allow discrimination between these two possibilities.

Both the mitochondrial genetic distances (estimated with MT-CYB) and the nuclear genetic distances (estimated with RAG1) between parental species of salamanders that hybridize spanned two orders of magnitude (0.003 to 0.360 and 0.0007 to 0.020, respectively). 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 162, median = 21.1) are consistent with existing estimates for other vertebrate taxa, suggesting similar relative evolutionary rates between the two genomes. Because the MT-CYB and RAG1 sequences used are short (345–1,141 bp and 556–1,510 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 our dataset, potential cases of mitochondrial introgression would show a ratio much less than the median (i.e., <<21.1) These potential cases include *Salamandrina perspicillata* X *Salamandrina terdigitata* (MT-CYB /RAG1 ratio = 0.6) and *Eurycea neotenes* X *Eurycea tridentifera* (MT-CYB / RAG1 ratio = 0.9; Table 2). In phylogeographic studies, hybridization between *Salamandrina perspicillata* and *S. terdigitata* was identified to have limited mitochondrial introgression (Mattoccia et al., 2011). Additional cline-based analysis of mitochondrial and nuclear introgression across the hybrid zones of salamanders with extreme mitochondrial/nuclear genetic distance ratios would be informative.

Within each tetrapod group, the genetic distance between parental species that hybridize is highly variable, but we found no significant differences in genetic distances among the groups. In addition, there were no significant differences in the ratio of mitochondrial to nuclear genetic distances among the groups. 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 tetrapod groups. Thus, the differences in metabolic rates across tetrapods 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 OXPHOS 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 (Fig. 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* X *H. orientalis*, Dufresnes et al., 2016).

In conclusion, this comprehensive analysis of salamander hybrids revealed hybrids throughout the clade 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 groups. This study further demonstrates the power of comparative studies of hybridization as an approach to understanding the process of speciation.

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