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E-Article

Introgression across Hybrid Zones Is Not Mediated by Large X-Effects in Green Toads with Undifferentiated Sex Chromosomes

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q1 ABSTRACT: Divergence between incipient species remains an incompletely understood process. Hybrid zones provide great research potential, reflecting natural organismal genomic interactions and gene evolution in a variety of recombinants over generations. While sex chromosomes are known evolutionary drivers of reproductive isolation, empirical population genetics has mostly examined species with heteromorphic sex chromosomes. We recently reported restricted introgression at sex-linked markers in an amphibian system with homomorphic sex chromosomes (*Hyla*), consistent with a large X-effect, designating a greater role of sex chromosomes in driving hybrid incompatibilities. Here, using a similar approach, we examined two hybrid zones of Palearctic green toads (*Bufo viridis* subgroup), involving several lineages that arose at different times and form secondary contacts. We find no evidence for differential introgression of sex-linked versus autosomal markers across both zones. This absence of large X-effects in *Bufo* indicates that, unlike in *Hyla*, hybrid incompatibilities may not result from the faster-heterogametic sex and faster-male aspects of Haldane's rule. The recent suppression of XY recombination in *Hyla* but not in *Bufo* may have driven greater divergence between *Hyla* sex chromosomes, causing stronger reproductive isolation. Alternatively, stronger linkage among *Hyla*'s sex-linked markers could restrict introgression. We hypothesize that the degree of sex-specific recombination may condition the importance of homomorphic sex chromosomes in speciation.

Keywords: hybridization, hybrid zones, Haldane's rule, large X-effect, reproductive isolation, speciation.

Introduction

The initial stages of speciation remain incompletely understood. Especially at the onset of reproductive isolation of diverging lineages, the identification of barriers to gene flow in genomes presents an important research challenge that can be particularly well addressed in hybrid zones (Harrison 1990; Maroja et al. 2015; Payseur and Rieseberg 2016; Harrison and Larson 2016). Because of the diversity of recombinants over many generations, hybrid zones are excellent systems to study natural organismal genomic interactions as a result of the evolutionary forces acting on certain genes (Abbott et al. 2013) and mirroring the dispersal behavior of the organisms (Barton and Hewitt 1985). In such potentially "semipermeable" or "porous" genomes and gene pools, it is expected that neutral or advantageous alleles will cross the semipermeable species boundaries (Wu 2001), while genomic regions under divergent selection or causing reproductive isolation will not (Barton and Hewitt 1981; Harrison 1990; Payseur et al. 2010; Maroja et al. 2015; Harrison and Larson 2016), with recent evidence that locally adapted linked loci may be captured by young inversions (Lee et al. 2017).

Theoretical (e.g., Charlesworth et al. 1987) and empirical (e.g., Masly and Presgraves 2007; Sæther et al. 2007; Presgraves 2008) work has shown that sex chromosomes play a special role in the evolution of reproductive isolation between incipient species. This role is well illustrated by the two empirical rules of speciation. Haldane's (1922) rule established that in interspecies crosses the heterogametic sex (XY males, ZW females) is more affected, and evidence comes from the vast majority of animals (Schilthuizen et al. 2011), including humans (Mendez et al. 2016), and plants with heteromorphic sex chromosomes (Brothers and Delph 2010). The "large X-effect" refers to a disproportionally high contribution of X chromosomes to hybrid sterility (Turelli and Moyle 2007; Masly and Presgraves 2007), and beyond animals it has also been described in plants (Hu and Filatov 2016). These empirical patterns all involve

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a role of sex chromosomes in driving incompatibilities due to hemizygosity (dominance theory; Muller 1940; Turelli and Orr 1995), faster evolution (faster-X theory; Charlesworth et al. 1987), epistatic X-Y or Z-W interactions (faster-heterogametic sex theory; Tao and Hartl 2003; McDermott and Noor 2010), and the propensity of carrying faster-evolving male genes (faster-male theory; Parsch and Ellegren 2013).

However, evidence for Haldane's rule and large X-effects comes mainly from systems with heteromorphic XY or ZW chromosomes. In contrast, Haldane's rule-like patterns have scarcely been found in species with homomorphic sex chromosomes (Schilthuizen et al. 2011; Beukeboom and Perrin 2014), although there are exceptions (e.g., from F_1 crosses in fish; Bolnick et al. 2008). The vast majority of animals with genetic sex determination exhibit homomorphic (i.e., microscopically indistinguishable) sex chromosomes, with small nonrecombining sex-determining regions. Many homomorphic sex chromosomes are considered too young to have evolved large-scale structural differentiation. Do they already play a role in speciation? A metastudy of laboratory F_1 crosses suggested that taxa with heteromorphic sex chromosomes show a higher degree of reproductive isolation than taxa without sex chromosomes; taxa with homomorphic sex chromosomes appeared to be in an intermediate position (Lima 2014). Thus, the relative contribution of sex chromosomes to postzygotic isolation may depend on the level of degeneracy: strong dominance and faster-X effects should apply only to species with heteromorphic sex chromosomes but not to species with undifferentiated ones. Accordingly, F_1 postzygotic incompatibilities seem generally more severe in interspecies crosses with heteromorphic chromosomes than in those with homomorphic chromosomes (Presgraves and Orr 1998) or no sex chromosomes (Lima 2014). Yet the proximate role of homomorphic sex chromosomes on reproductive isolation remains widely unclear, especially under natural conditions like in hybrid zones (cf. Harrison and Larson 2016). Genetic incompatibilities may be expressed in later generations than the F_1, as recombination breaks down functional combinations that evolved independently in the diverging species.

Recently, we have reported restricted introgression at sex-linked compared with autosomal markers in an anuran hybrid zone between two incipient species of European tree frogs (*Hyla arborea*, *Hyla orientalis*), providing the first empirical evidence for a large X-effect in a system with homomorphic sex chromosomes (Dufresnes et al. 2016). Multilevel analyses (individual, population-wide, phylogenetics) support that the sex chromosomes of tree frogs are fully homomorphic due to recurrent XY recombination over macro- and microevolutionary times (Dufresnes et al. 2015*a* and references therein). Importantly, this large X-effect cannot result from the dominance or faster-X aspects of Haldane's rule, which are specific to degenerated sex chromosomes, but rather supports a role for faster-heterogametic sex or faster-male evolutionary processes. This first case study raises the question, Are homomorphic sex chromosomes generally important for speciation and, if not, what evolutionary features condition this importance? Data from additional fish and amphibian hybrid systems appear required to potentially draw more general conclusions.

In this article, we tested for a large X-effect across two hybrid zones between diploid lineages of Palearctic green toads (*Bufo viridis* subgroup), where sex is determined by XY homomorphic sex chromosomes (Stöck et al. 2013*a*; Tamschick et al. 2015). Using sequence markers spread over the sex chromosomes (flanking regions of two microsatellites and intronic regions of the master sex-determining candidate gene *dmrt1*; Stöck et al. 2013*a*; Tamschick et al. 2015), we have previously shown that there is no XY differentiation at the phylogenetic level, suggesting frequent XY recombination. We even measure low XY recombination directly by genotyping genetic green toad families, derived from natural mating pairs, collected in the field (Stöck et al. 2013*a*; Betto-Colliard et al. 2015). Here we focus on the genetic interactions between three taxa from the Palearctic green toad radiation: *Bufo viridis* sensu stricto (hereafter, *B. viridis*), *Bufo balearicus*, and *Bufo siculus*. Their phylogeny and phylogeography is well resolved, involving reciprocally monophyletic signals for all three taxa at both the nuclear level and the mitochondrial level (Stöck et al. 2006, 2008; Dufresnes et al. 2014*b*). The younger lineages (1.9 million years diverged), *B. viridis* and *B. balearicus* in northern Italy, exhibit introgression over ~120 km using mitochondrial markers and over ~50 km at nuclear levels, and their hybrid zone appears mediated by geographic barriers to dispersal rather than postzygotic isolation (Dufresnes et al. 2014*b*). The older lineages (2.6 million years diverged), *B. balearicus* and *B. siculus*, form a much narrower contact zone in northeastern Sicily with scarce mitochondrial introgression and nuclear admixture reaching only ~20 km, characteristic of nearly complete reproductive isolation (Colliard et al. 2010). In both systems, we measured differential introgression between sex-linked and autosomal loci either by fitting genomic clines to allele frequency data or by comparing the number of introgressed alleles between both marker types and compared our results with the *Hyla* system.

Material and Methods

DNA Sampling and Amplification of Autosomal and Sex-Linked Microsatellite Loci

Population genetic analyses were performed for two independent data sets. We used the sampling previously obtained from two hybrid zones, one between *Bufo viridis* and *Bufo balearicus* from the Po Plain of northeastern Italy (fig. 1;

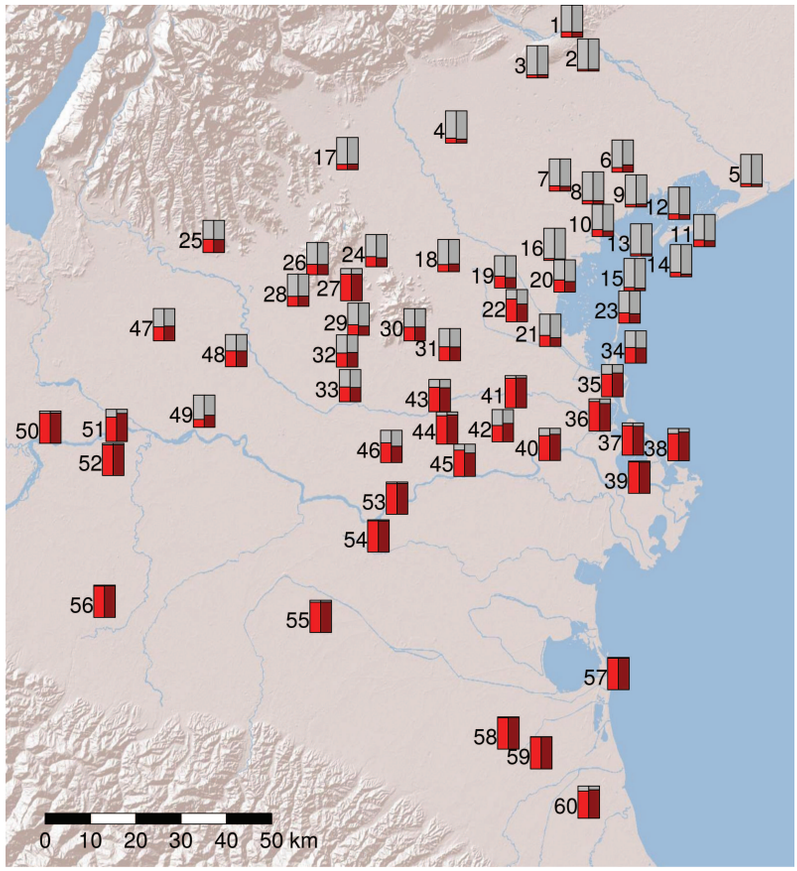

Figure 1: Map of the North Italian hybrid zone. Bars represent average Bayesian assignment probabilities to either *Bufo balearicus* (red) or *Bufo viridis* (gray) for each population. The left and lighter of each pair of bars represents assignment using autosomal markers, and the right and darker bar represents assignment with sex-linked markers.

Dufresnes et al. 2014*b*) and one between *Bufo balearicus* and *Bufo siculus* from northeastern Sicily (fig. 2; Colliard et al. 2010). These samples were analyzed with anonymous microsatellites (12 loci in the North Italian hybrid zone and 10 loci in the Sicilian hybrid zone developed from a genomic library, enriched for repetitive sequences), most of them autosomal as tested in genetic families (cf. Stöck et al. 2013*a*). We reused the sampling schemes and DNA specimens and added 15 newly developed transcriptome-based markers in the North Italian hybrid zone as well as 16 newly developed transcriptome-based markers in the Sicilian hybrid zone (Gerchen et al. 2016). Importantly, sex linkage or autosomal locations of these markers have previously been demonstrated using genetic families of these taxa as well as interspecies crosses (Gerchen et al. 2016). In total, we used 10 sex-linked and 17 autosomal markers in the North Italian hybrid zone and 12 sex-linked and 14 autosomal markers in the Sicilian hybrid zone; the underlying data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.sk5sv1q (Gerchen et al. 2018). Tests for sex linkage corroborated evidence for conserved synteny across the anuran *LG1* for Palearctic green toads. Mapping transcriptome-based green toad markers to the *Xenopus tropicalis* linkage groups suggested that toad markers spanned at least half of the entire length of *LG1* (i.e., ~100 Mbp; see fig. 1 in Gerchen et al. 2016). Similarly, linkage maps obtained with some of the classical microsatellites suggested distances of 50–145 cM on the female maps (Stöck et al. 2013*a*; Tamschick et al. 2015).

The sampling of the North Italian hybrid zone included 312 green toads from 60 localities (table S1; tables S1–S3 are available online); the Sicilian hybrid zone analysis comprised 192 green toads from 12 locations (table S2). As described elsewhere (Gerchen et al. 2016), toads were genotyped using 10-μL multiplex polymerase chain reactions (PCRs) with markers assigned to three separate master mixes (table S3). The PCR protocol comprised the following: initial denaturation at 95°C for 3 min; 35 cycles of denaturation

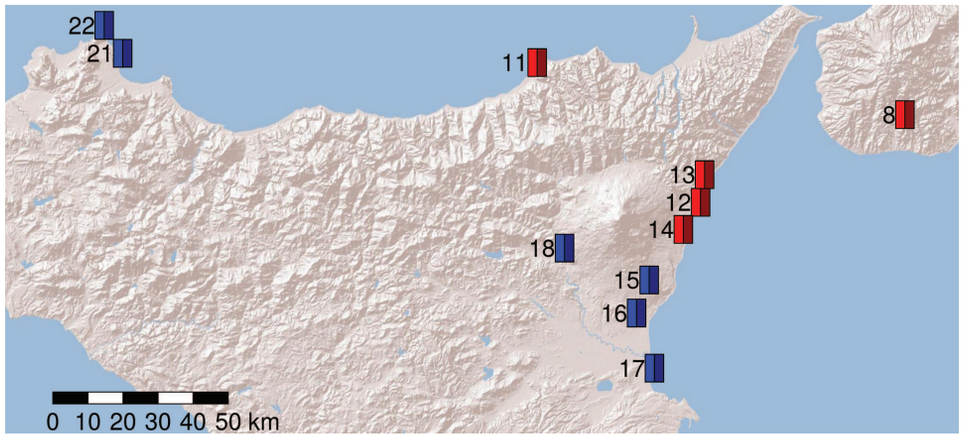

Figure 2: Map of the Sicilian hybrid zone. Bars represent average Bayesian assignment probabilities to either *Bufo balearicus* (red) or *Bufo siculus* (blue) for each population. The left and lighter of each pair of bars shows assignment using autosomal markers, and the right and darker bar shows assignment with sex-linked markers.

at 94°C for 30 s, annealing at optimal annealing temperature (table S2) for 60 s, and elongation at 72°C for 40 s; and final elongation at 72°C for 10 min. PCR products were diluted in RNAse-free water (master mix I, 1 : 15; master mix II, 1 : 20; master mix III, 1 : 5). Genotyping was performed on an ABI 3500xL capillary sequencer (Applied Biosystems) at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) with 0.5 μL of PCR product diluted in 9.25 μL of Hi-Di formamide (Applied Biosystems) and 0.25 μL of GeneScan 500 ROX size standard (Life Technologies). Genotypes were scored using Genemapper (ver. 4.1; Applied Biosystems).

Population Genetic Analyses

Genotypes were analyzed using the Bayesian clustering software Structure (ver. 2.3.3; Pritchard et al. 2000), which computes probabilities of assignments of individuals into a predefined number (K) of groups by maximizing Hardy-Weinberg equilibrium in each group. The analyses involved a burn-in value of 10,000 generations followed by 100,000 Markov chain Monte Carlo iterations, using an admixture model and correlated allele frequencies among populations. To confirm that each hybrid zone was the result of contact between two main genetic groups ($K = 2$), we first determined the most likely number of genetic clusters by comparing the ΔK statistic (Evanno et al. 2005) computed by Structure Harvester (ver. 0.6.94; Earl and von Holdt 2012). For each value of K from 1 to 10, we performed 10 replicate Structure analyses. To verify that $K \neq 1$ (which the ΔK statistics cannot test), we also explored population differentiation by conducting principal component analyses (PCAs) on individual genotypes (R packages ade4 and adegenet; Jombart 2008; R Development Core Team 2016). In the North Italian hybrid zone, we considered as "pure" populations those having assignment probabilities <0.1 or >0.9 in Structure runs with $K = 2$ and without cytonuclear discordance.

To infer the hybrid status, we collected the distribution of ancestry coefficients (Q) for every individual. Individuals were considered admixed if this distribution excludes 0 and 1. This approach allows us to disentangle true hybrids from individuals with uninformative genotypes (e.g., Sá-Pinto et al. 2010; Dufresnes et al. 2014*b*). We used the linkage model in Structure (Falush et al. 2003) to get a side-by-side output for individual markers. This allowed us to compare posterior assignment probabilities for different markers independently.

Given the difference in polymorphism between anonymous versus transcriptomic-based microsatellites (Dufresnes et al. 2014*c*), we tested whether our newly developed transcriptome-based markers were comparable in assignment performance with other microsatellite markers developed from enriched libraries. We compared the number of alleles found among transcriptome-based and classical microsatellite markers in the North Italian and Sicilian hybrid zones using nonparametric paired Wilcoxon tests in R. In addition, we tested whether transcriptome-based markers were able to differentiate between supposedly pure populations in both hybrid zones by performing PCAs based on only the genotypes of transcriptome-based markers using ade4 and adegenet (Jombart 2008).

Since the few available green toad–specific linkage maps cover only some of the analyzed genetic markers (Stöck et al. 2013*a*; Betto-Colliard et al. 2015) and no assembled genome is available, we could not incorporate reliable linkage information into the analyses. Therefore, we performed two separate runs with different linkage assumptions for the data set: one with all markers unlinked and

one with an estimation based on map distances of orthologous genes (if available) from the linkage map of the western clawed frog *X. tropicalis* (Wells et al. 2011) that shows some amount of structural conservation (synteny) with green toads (cf. Brelsford et al. 2013).

Analyses of Differential Introgression in the North Italian Hybrid Zone

To compare sex-linked and autosomal levels of introgression, we computed an admixture index ranging from 0 (pure parental of either species) to 0.5 (50:50 hybrid) based on the Structure probabilities of assignment (cf. Dufresnes et al. 2016). We tested whether differences were significant in the North Italian hybrid zone using nonparametric paired Wilcoxon tests in R, including only confidently assigned hybrids (see above).

An alternative way of testing differential introgression is to fit clines to the distribution of assignment probabilities for the two classes of loci (sex linked or autosomal), a method known as genomic clines (Fitzpatrick 2013). Basically, this approach compares the amount of introgression of a specific group of markers to the overall genomic introgression rate. A neutral marker class will behave according to the average genome background (with a linear relationship), whereas a marker class involved in reproductive isolation will feature less admixture than the genome background (clinal relationship, where cline width is proportional to the amount of selection).

For this analysis, we computed the individual hybrid index as the probability of assignment to the *B. balearicus* cluster for all markers, averaged over sex-linked markers and over autosomal markers, available from the side-by-side output of Structure. Then we fitted two-parameter maximum likelihood genomic clines (Fitzpatrick 2013) to these hybrid indices. Specifically, we used the native optim function in R to optimize the parameters u (relative cline position) and v (relative slope of the cline). We made three independent fits: (*a*) sex-linked markers against genomic hybrid indices, (*b*) autosomal markers against genomic hybrid indices, and (*c*) all markers against genomic hybrid indices (the latter to obtain the result of the likelihood function for calculating the probability of the cline fit). To account for potential noise, which might have caused imperfectly pure assignments of pure individuals (due to lack of informativeness of some genotypes), we also performed these analyses without those individuals whose hybrid indices were <0.1 and >0.9. We then tested whether our data are significantly better explained by two independent sex-linked and autosomal clines than by a single cline combining both marker types. Specifically, we tested whether the likelihood increase in the two-cline model justified the addition of two additional free parameters compared with the one-cline model.

Analyses of Differential Introgression in the Sicilian Hybrid Zone

As previously shown (Colliard et al. 2010), *B. siculus* and *B. balearicus* show advanced reproductive isolation and very limited introgression across their hybrid zone in Sicily (see "Results"), which is thus little informative for the population genetics approaches applied above. Therefore, we chose an alternative and simply compared the total number of introgressed alleles between autosomal and sex-linked markers present in populations. We defined an allele as introgressed to the other species' gene pool if it was otherwise found exclusively in the putatively pure populations of either *B. balearicus* (populations 8 and 11) or *B. siculus* (populations 18, 21, and 22), which are spatially separated from the hybrid zone and also do not provide evidence of mitochondrial introgression (Colliard et al. 2010). Then we compared the number of markers showing signs of introgression and the number of alleles obtained from relating introgression between sex-linked versus autosomal markers using χ^2 tests in R (R Development Core Team 2016). We used the R package pwr (Champely 2017) to compute the effect size for both χ^2 tests, assuming a type II error probability of 10% or 30%. Because the number of introgressed alleles might depend on basal polymorphism and the informativeness of loci, we also fitted generalized linear mixed models (GLMMs) to predict them from chromosomal status (sex linked or autosomal), accounting for the species diagnosticity of loci (as their contribution to axis 1 of the PCA, which distinguishes between the two species) and the total number of alleles. GLMMs were performed in R (lme4 package) following a backward selection procedure, and significance was tested using 1,000 bootstrap replicates (Faraway 2006).

Results

A total of 218 green toads could be confidently assigned as hybrids in the North Italian hybrid zone of *Bufo balearicus* and *Bufo viridis*. Based on these individuals, we found no significant difference between the admixture indices of sex-linked and autosomal markers (paired Wilcoxon test: $V = 12{,}488$, $P = .5542$; fig. 3). Genetic clines provided no evidence for differential introgression between sex-linked and autosomal loci. First, analyses assuming no linkage between markers yield linear clines (u close to 0 and v close to 1; Fitzpatrick 2013) for both autosomal and sex-linked markers (fig. 3; table 1). The increase in likelihood does not justify adding two additional parameters for individual autosomal and sex-linked clines ($P = .819$). Second, adding additional linkage information does not result in relevant differences in cline shape, nor does it justify a model with two separate clines for autosomal and sex-linked marker sets (table 1; fig. S1*A*; figs. S1–S8 are available online). Third,

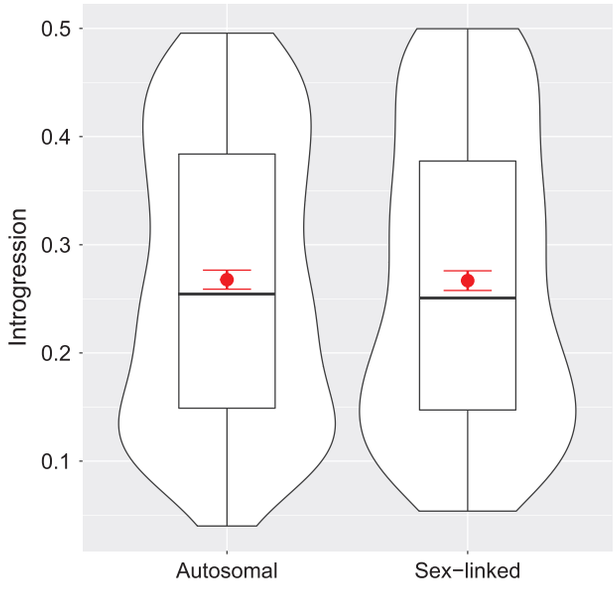

Figure 3: Combined violin plots and boxplots of the average introgression between autosomal and sex-linked markers in the North Italian hybrid zone (*Bufo balearicus*, *Bufo viridis*). The red circle shows the mean, and the red whiskers show the SE. The bar in the center of the box represents the median, and the lower and upper edges of the box represent the first and third quartiles, respectively. The black whiskers depict the highest or lowest value within 1.5 interquartile ranges (no outlier values beyond this range occur).

reanalyses considering only individuals with genomic hybrid indices >0.1 and <0.9 also show similar cline shapes, and the difference between single-cline and two-cline models always remain nonsignificant (table 1; fig. S1*B*, S1*C*).

We found few alleles showing a signal of introgression across the *Bufo balearicus*/*Bufo siculus* Sicilian hybrid zone (figs. 4, 5): 4 of the 14 autosomal markers show introgression, with only 11 introgressed alleles in populations 12–17 (of 2,932 alleles, excluding missing data). Five of 12 sex-linked markers have 19 introgressed alleles among 2,520 alleles in total. The proportion of markers with introgressed alleles ($\chi^2 = 0.0819$, $P = .7747$) is not significantly different between autosomal and sex-linked markers. Power analyses revealed that the number of markers ($n = 26$) would allow detecting only very large differences of introgression (63.6% assuming a type II error probability of 10%; 48.7% assuming a type II error probability of 30%). However, there is also no significant difference in the relative number of introgressed alleles ($\chi^2 = 2.8591$, $P = .091$) between sex-linked and autosomal markers. The high total number of alleles ($n = 5{,}452$) would be sufficient to detect moderately small differences of introgression rates (4.4% assuming a type II error probability of 10%; 3.4% assuming a type II error probability of 30%). Accordingly, chromosomal status was not a significant predictor of these variables in the GLMMs (number of introgressed loci, $P = .49$; number of introgressed alleles, $P = 1.0$). The diagnosticity of loci was a sig-

Table 1: Results of cline fitting ~~for cline fits~~ based either on all individuals or on individuals with intermediate hybrid indices and Structure runs using different linkage parameters

Fit	*u*	*v*	ln	*P*
	Cline fits based on all individuals			
No linkage				.819
Autosomal	.011759	1.017073	−2,223.83	
Sex linked	−.019467	.971569	−1,335.28	
Genomic	0	1	−3,559.30	
Linkage estimates based on *X. tropicalis* linkage map				.852
Autosomal	−.001947	1.008475	−2,252.50	
Sex linked	.003767	.986134	−1,345.82	
Genomic	0	1	−3,598.47	
	Cline fits based on individuals with genomic hybrid indices >.1 and <.9			
No linkage				.937
Autosomal	−.001202	1.006738	−1,884.19	
Sex linked	.002952	.989264	−1,114.63	
Genomic	0	1	−2,998.88	
Linkage estimates based on *X. tropicalis* linkage map				.843
Autosomal	−.012971	1.000846	−1,910.76	
Sex linked	.023558	1.000013	−1,129.94	
Genomic	0	1	−3,040.87	

Note: The terms *u* and *v* are parameters of the cline fits, ln is the likelihood of the fit, and *P* is the result of testing whether the data are better explained fitting two individual clines instead of one. *X. tropicalis* = *Xenopus tropicalis*.

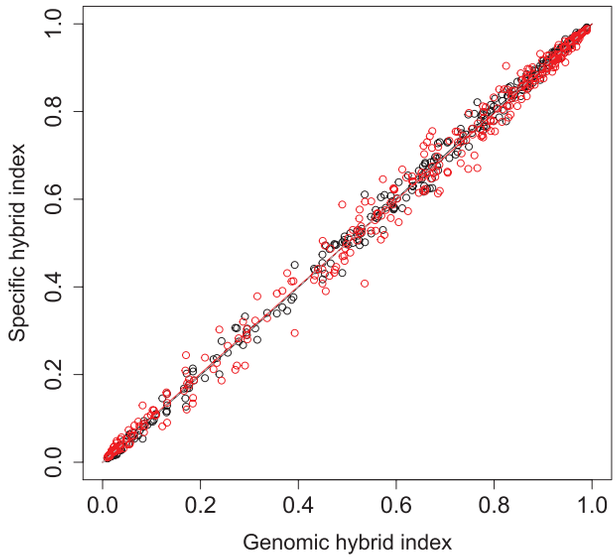

Figure 4: Genomic clines in the North Italian hybrid zone. Specific hybrid indices of autosomal (black) and sex-linked (red) markers are plotted against genomic hybrid indices (*Bufo balearicus*, *Bufo viridis*). The analysis was performed assuming no linkage between markers (for other linkage scenarios, see the supplemental material).

nificant random variable in both analyses ($P < .001$), but ~~their~~ polymorphism was not ($P = .44$ and $P = 1.0$, respectively), and ~~it~~ was discarded from the final analyses.

Discussion

We did not find any significant differences between the introgression levels of sex-linked and autosomal loci in either of the *Bufo* hybrid zones using several complementary approaches and different linkage assumptions. A large X-effect would have resulted in lower sex-linked than autosomal introgression between the species' gene pools. To the contrary, our data support no such effects in Palearctic green toads, which possess homomorphic sex chromosomes; this situation strongly contrasts with the similarly surveyed *Hyla* system (*H. arborea*/*H. orientalis*; Dufresnes et al. 2016). While some homomorphic sex chromosomes disproportionally bear hybrid incompatibilities compared with autosomes (Dufresnes et al. 2016), we here present an amphibian system in which this is not suggested. Rather, our findings indicate that the role played by homomorphic sex chromosomes in the evolution of reproductive isolation is not ubiquitous and might be system dependent, implying that different mechanisms of genetic incompatibilities are involved.

Unlike the very informative *Bufo viridis*/*Bufo balearicus* hybrid zone, the narrow contact and restricted gene flow reduced our power to detect differential introgression between *Bufo siculus* and *Bufo balearicus* but should have been sufficient to detect differences as low as 4%, considering the number of alleles. However, we acknowledge the limitations and constraints associated with natural systems (e.g., hybrid zone dynamics, especially demographic

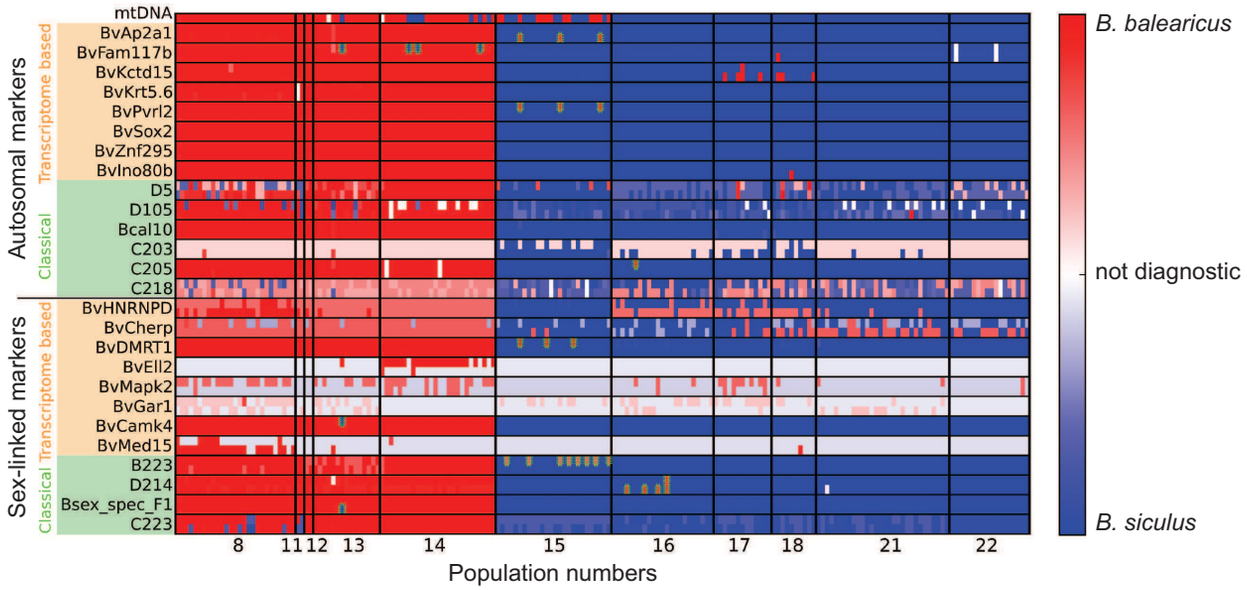

Figure 5: Mitochondrial haplotypes and specificity of individual ~~a~~lleles in the Sicilian hybrid zone (*Bufo balearicus*, *Bufo siculus*) for autosomal and sex-linked markers. Markers are either transcriptome-based or classical microsatellites. ~~A~~lleles with a dashed green margin are introgressed~~;~~ mtDNA = mitochondrial DNA~~;~~

effects like genetic drift, and potential selection in insular populations as Sicily) and the to-date still-limited genetic resources (namely, the relatively low number of available cross-amplifying markers and the lack of a closely related reference genome for mapping these markers).

Nevertheless, the question remains: the available data support the notion that the large X-effect is not shaping patterns of admixture in Palearctic green toads, contrasting with the *Hyla* system (*H. arborea*/*H. orientalis*; Dufresnes et al. 2016). Our finding appears highly relevant to speciation research, as it suggests that the importance of homomorphic sex chromosomes in the evolution of reproductive isolation is not ubiquitous and might be system dependent.

Why do homomorphic sex chromosomes appear important for reproductive isolation in some systems (e.g., *Hyla*) but not in others (e.g., *Bufo*)? We discuss several potential explanations that may condition the evolution and expression of sex-linked hybrid incompatibilities in *Hyla* but not in *Bufo*.

First, these systems may not have reached the same levels of reproductive isolation. The transition of *H. arborea* and *H. orientalis* alleles appears clearly mediated by selection against hybrids at their secondary contacts (Dufresnes et al. 2015*b*, 2016). In contrast, selection against hybrids may be more relaxed between *B. viridis* and *B. balearicus*, as their wide range of introgression and numerous backcrossed individuals testify (Dufresnes et al. 2014*b*). Stronger reproductive isolation may thus likely explain why sex chromosomes play a greater role in *Hyla* than in *Bufo*. However, under this hypothesis we would expect less introgression at sex-linked markers between the Sicilian taxa *B. siculus* and *B. balearicus*, which are clearly reproductively isolated (Colliard et al. 2010). Yet we cannot rule out that the absence of such a signal in Sicily stems from the limited informativeness of this hybrid zone due to limited (if any) contemporary gene flow.

Second, since the dominance or faster-X aspects of Haldane's rule are specific to degenerated sex chromosomes, a large X-effect involving homomorphic sex chromosomes should be caused only by the faster-male and faster-heterogametic sex aspects of Haldane's rule (Dufresnes et al. 2016). Under the fair assumption that both anuran systems (*Hyla*, *Bufo*) face some postzygotic isolation (for bufonid toads and green toads, see "Supplementary Text 11" in the supplemental material, available online), our results could thus indicate that these mechanisms do not act uniformly in *Bufo* and *Hyla*. In fact, the role played by sex-linked genes in male-expressed traits has received little empirical support. Most studies have suggested that these traits are autosomal, and so the faster-male hypothesis is not driven by sex chromosomes (e.g., in *Xenopus*; Malone and Michalak 2008). Hence, the large X-effect of *Hyla* may rather result from faster-heterogametic sex effects only. These are caused by incompatibilities between X- and Y-linked genes, for which epistatic interactions are required for proper meiosis and sexual differentiation (Tao and Hartl 2003; McDermott and Noor 2010). Given that *Hyla* and *Bufo* share homologous sex-determining systems—namely, XY male heterogamety at linkage group 1 (Brelsford et al. 2013; Stöck et al. 2013*a*, 2013*b*; Tamschick et al. 2015; Dufresnes et al. 2015*a*)—we should thus expect similar incompatibilities in both systems. Yet these incompatibilities were not detected from the empirical green toad hybrid zone data. However, one major intrinsic difference between the sex chromosome dynamics of *Bufo* and *Hyla* may well reconcile this discrepancy: the degree of XY recombination differs remarkably between these amphibians. In *Hyla*, XY recombination has presumably stopped in males during the postglacial expansion (<15,000 years; Dufresnes et al. 2014*a*) and was never detected among hundreds of controlled crosses from western and northern Europe (Berset-Brändli et al. 2008; Stöck et al. 2011; Dufresnes et al. 2014*a*, 2015*a*). Accordingly, Y chromosomes have evolved some genetic differentiation from X chromosomes in these regions, which encompass the area of contact between *H. arborea* and *H. orientalis* (Dufresnes et al. 2014*a*). In contrast, all green toads examined to date exhibit a low but nonzero rate of XY recombination in males (Stöck et al. 2013*a*, 2013*b*; Betto-Colliard et al. 2015; Tamschick et al. 2015), and preliminary data suggest a possibly narrow Y nonrecombining region (manuscript in preparation).

Therefore, the lack of XY recombination over thousands of generations may have accelerated the rate of evolution of *Hyla* sex chromosomes (through enhanced drift and purifying selection acting on entire haplotypes), which in turn triggered more X-Y incompatibilities in interspecific hybrids (e.g., the X alleles of *H. arborea* are no longer compatible with the Y alleles of *H. orientalis*) and the evolution of a detectable large X-effect. In contrast, these processes might not apply in *Bufo*, where sex chromosomes may rather evolve at a similar rate as autosomes, and speciation progresses without disproportionally more sex-linked than autosomal incompatibilities.

Although X and Y chromosomes are homomorphic in *Hyla*, it is also conceivable that their recent divergence in northern Europe could have initiated the early degeneration of some Y-linked sequences through accumulation of deleterious mutation and gene loss. Under this hypothesis, the large X-effect of tree frogs might also result, in part, from the dominance or faster-X aspects of Haldane's rule, which are normally specific to degenerated sex chromosomes (e.g., Balakrishnan et al. 2013; Sharbrough et al. 2017). Following the rationale presented above, these effects clearly do not apply in *Bufo* either, where Y degeneration is prevented by frequent XY recombination.

Third, recombination rates in hybrid populations may also play a major role in the differences observed. As sug-

gested by theoretical work on hybrid zone modeling, gene flow is expected to be more restricted at X-linked loci than autosomes because full linkage at sex chromosomes prevents neutral alleles to escape hemizygous selection of disadvantageous alleles in XY hybrids (Muirhead and Presgraves 2016). In the absence of XY recombination, these selective sweeps will thus result in what we interpret as a large X-effect (i.e., restricted introgression at sex-linked genes), even if the same amount of incompatibilities are involved on sex chromosomes as autosomes. For instance, Hu and Filatov (2016) have recently shown a large X-effect in two dioecious plants (*Silene*), where the majority of X-linked genes have Y-linked homologs but recombination between ~~XY~~ $_1$ has been suppressed, a situation similar to *Hyla*. In contrast, introgression at homomorphic sex chromosomes with smaller nonrecombining regions is thus expected to mirror autosomal ones, as we here documented in *Bufo*. However, this interpretation assumes that the recombination rates of hybrid individuals are similar to parental ones, an assumption refuted by empirical and theoretical literature. Indeed, hybrid recombination can be influenced by multiple factors, such as incompatibilities in genes involved in the recombination machinery (Balcova et al. 2016), chromosomal inversions (Kirkpatrick and Barton 2006), and fine-tuning of recombination patterns to favor linkage of genetic combinations promoting reinforcement (Hall and Kirkpatrick 2006). Knowledge of hybrid recombination rates would thus nicely complement recent advances in our understanding of the role played by sex-linked genes in reproductive isolation.

In ~~sum~~, our study shows that homomorphic sex chromosomes are not ubiquitous drivers of reproductive isolation and thus stresses the need for additional studies to draw general conclusions. In particular, we emphasize that future work, while testing for a large X-effect in additional systems, should account for the amount of recombination rates in sex chromosomes in comparison with autosomes.

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Literature Cited

Abbott, R., D. Albach, S. Ansell, J. W. Arntzen, S. J. E. Baird, N. Bierne, J. Boughman, et al. 2013. Hybridization and speciation. Journal of Evolutionary Biology 26:229–246.

Balakrishnan, C. N., C. Chapus, M. S. Brewer, and D. F. Clayton. 2013. Brain transcriptome of the violet-eared waxbill *Uraeginthus granatina* and recent evolution in the songbird genome. Open Biology 39:130063.

Balcova, M., B. Faltusova, V. Gergelits, T. Bhattacharyya, O. Mihola, Z. Trachtulec, C. Knopf, et al. 2016. Hybrid sterility locus on chromosome X controls meiotic recombination rate in mouse. PLoS Genetics 22:e1005906.

Barton, N. H., and G. M. Hewitt. 1981. Hybrid zones and speciation. Pages 109–145 *in* W. D. Atchley and D. S. Woodruf, eds. Evolution and speciation. Cambridge University Press, Cambridge.

———. 1985. Analysis of hybrid zones. Annual Reviews in Ecology and Systematics 16:113–148.

Berset-Brändli, L., J. Jaquiéry, T. Broquet, Y. Ulrich, and N. Perrin. 2008. Extreme heterochiasmy and nascent sex chromosomes in European tree frogs. Proceedings of the Royal Society B 275:1577–1585.

Betto-Colliard, C., R. Sermier, S. Litvinchuk, N. Perrin, and M. Stöck 2015. Origin and genome evolution of polyploid green toads in Central Asia: evidence from microsatellite markers. Heredity 114:300–308.

Beukeboom, L., and N. Perrin. 2014. The evolution of sex determination. Oxford University Press, Oxford.

Bolnick, D. I., M. Turelli, H. Lopez-Fernandez, P. C. Wainwright, and T. J. Near. 2008. Accelerated mitochondrial evolution and "Darwin's Corollary": asymmetric viability of reciprocal F_1 hybrids in centrarchid fishes. Genetics 178:1037–1048.

Brelsford, A., M. Stöck, C. Betto-Colliard, S. Dubey, C. Dufresnes, H. Jourdan-Pineau, N. Rodrigues, R. Savary, R. Sermier, and N. Perrin. 2013. Homologous sex chromosomes in three deeply divergent anuran species. Evolution 67:2434–2440.

Brothers, A. N., and L. F. Delph. 2010. Haldane's rule is extended to plants with sex chromosomes. Evolution 64:3643–3648.

Champely, S. 2017. pwr: basic functions for power analysis. R package version 1.2-1. https://CRAN.R-project.org/package=pwr.

Charlesworth, B., J. Coyne, and N. Barton. 1987. The relative rates of evolution of sex chromosomes and autosomes. American Naturalist 130:113–146.

Colliard, C., A. Sicilia, G. F. Turrisi, M. Arculeo, N. Perrin, and M. Stöck. 2010. Strong reproductive barriers in a narrow hybrid zone of West-Mediterranean green toads *Bufo viridis* subgroup with Plio-Pleistocene divergence. BMC Evolutionary Biology 10:232.

Dufresnes, C., Y. Bertholet, J. Wassef, K. Ghali, R. Savary, B. Pasteur, A. Brelsford, et al. 2014*a*. Sex-chromosome differentiation parallels postglacial range expansion in European tree frogs *Hyla arborea*. Evolution 68:3445–3456.

Dufresnes, C., L. Bonato, N. Novarini, C. Betto-Colliard, N. Perrin, and M. Stöck. 2014*b*. Inferring the degree of incipient speciation in secondary contact zones of closely related lineages of Palearctic green toads *Bufo viridis* subgroup. Heredity 113:9–20.

Dufresnes, C., A. Borzée, A. Horn, M. Stöck, M. Ostini, R. Sermier, J. Wassef, et al. 2015*a*. Sex-chromosome homomorphy in Palearctic tree frogs results from both turnovers and X-Y recombination. Molecular Biology and Evolution 32:2328–2337.

Dufresnes, C., A. Brelsford, P. Béziers, and N. Perrin. 2014*c*. Stronger transferability but lower variability in transcriptomic- than in anonymous microsatellites: evidence from hylid frogs. Molecular Ecology Resources 14:716–725.

Dufresnes, C., A. Brelsford, J. Crnobrnja-Isailovic, N. Tzankov, P. Lymberakis, and N. Perrin. 2015*b*. Timeframe of speciation inferred from secondary contact zones in the European tree frog radiation *Hyla arborea* group. BMC Evolutionary Biology 15:155.

Dufresnes, C., T. Majtyka, S. J. E. Baird, J. F. Gerchen, A. Borzée, R. Savary, M. Ogielska, N. Perrin, and M. Stöck. 2016. Empirical evidence for large X-effects in animals with undifferentiated sex chromosomes. Scientific Reports 6:21029.

Earl, D. A., and B. M. von Holdt. 2012. Structure Harvester: a website and program for visualizing Structure output and implementing the Evanno method. Conservation Genetics Resources 4:359–361.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software Structure: a simulation study. Molecular Ecology 14:2611–2620.

Falush, D., M. Stephens, and J. K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Faraway, J. J. 2006. Extending the linear model with R: generalized linear, mixed effects and nonparametric regression models. Chapman & Hall/CRC, Boca Raton, FL.

Fitzpatrick, B. M. 2013. Alternative forms of genomic clines. Ecology and Evolution 3:1951–1966.

Gerchen, J. F., C. Dufresnes, and M. Stöck. 2018. Data from: Introgression across hybrid zones is not mediated by large X-effects in green toads with undifferentiated sex chromosomes. American Naturalist, Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.sk5sv1q.

Gerchen, J. F., S. J. Reichert, J. T. Röhr, C. Dieterich, W. Kloas, and M. Stöck. 2016. A single transcriptome of a green toad (*Bufo viridis*) yields candidate genes for sex determination and -differentiation and non-anonymous population genetic markers. PLoS ONE 11: e0156419.

Haldane, J. B. S. 1922. Sex ratio and unisexual sterility in hybrid animals. Journal of Genetics 12:101–109.

Hall, D. W., and M. Kirkpatrick. 2006. Reinforcement and sex linkage. Evolution 60:908–921.

Harrison, R. G. Hybrid zones: windows on evolutionary process. 1990. Pages 69–128 *in* D. Futuyma and J. Antonovics, eds. Oxford surveys in evolutionary biology. Oxford University Press, New York.

Harrison, R. G., and E. L. Larson. 2016. Heterogeneous genome divergence, differential introgression, and the origin and structure of hybrid zones. Molecular Ecology 25:2454–2466.

Hu, X.-S., and D. A. Filatov. 2016. The large-X effect in plants: increased species divergence and reduced gene flow on the *Silene* X-chromosome. Molecular Ecololgy 25:2609–2619.

Jombart, T. 2008. Adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405.

Kirkpatrick, M., and N. Barton. 2006. Chromosome inversions, local adaptation and speciation. Genetics 173:419–434.

Lee, C.-R., B. Wang, J. P. Mojica, T. Mandáková, K. V. S. K. Prasad, J. L. Goicoechea, N. Perera, et al. 2017. Young inversion with multiple linked QTLs under selection in a hybrid zone. Nature Ecology and Evolution 1:0119.

Lima, T. G. 2014. Higher levels of sex chromosome heteromorphism are associated with markedly stronger reproductive isolation. Nature Communications 5:4743.

Malone, C., and P. Michalack. 2008. Physiological sex predicts hybrid sterility regardless of genotype. Science 319:59.

Maroja, L. S., E. L. Larson, S. M. Bogdanowicz, and R. G. Harrison. 2015. Genes with restricted introgression in a field cricket *Gryllus firmus*/*Gryllus pennsylvanicus* hybrid zone are concentrated on the X chromosome and a single autosome. G3: Genes, Genomes, Genetics 5:2219–2227.

Masly, J. P., and D. C. Presgraves. 2007. High-resolution genome-wide dissection of the two rules of speciation in *Drosophila*. PLoS Biology 5:e243.

McDermott, S. R., and M. A. F. Noor. 2010. The role of meiotic drive in hybrid male sterility. Philosophical Transactions of the Royal Society B 365:1265–1272.

Mendez, F. L., G. D. Poznik, S. Castellano, and C. D. Bustamante. 2016. The divergence of Neandertal and modern human Y chromosomes. American Journal of Human Genetics 98:728–734.

Muirhead, C. A., and D. C. Presgraves. 2016. Hybrid incompatibilities, local adaptation, and the genomic distribution of natural introgression between species. American Naturalist 187:249–261.

Muller, H. J. 1940. Bearing of the *Drosophila* work on systematics. Pages 185–268 *in* J. S. Huxley, ed. The new systematics. Clarendon, Oxford.

Parsch, J., and H. Ellegren. 2013. The evolutionary causes and consequences of sex-biased gene expression. Nature Reviews Genetics 14:83–87.

Payseur, B. A. 2010. Using differential introgression in hybrid zones to identify genomic regions involved in speciation. Molecular Ecology Resources 10:806–820.

Payseur, B. A., and L. A. Rieseberg. 2016. A genomic perspective on hybridization and speciation. Molecular Ecology 25:2337–2360.

Presgraves, D. C. 2008. Sex chromosomes and speciation in *Drosophila*. Trends in Genetics 24:336–343.

Presgraves, D. C., and H. A. Orr. 1998. Haldane's rule in taxa lacking a hemizygous X. Science 30:952–954.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

R Development Core Team. 2016. R: a language and environment for statistical computing. Vienna. http://www.R-project.org.

Sæther, S. A., G.-P. Sætre, T. Borge, C. Wiley, N. Svedin, G. Andersson, T. Veen, et al. 2007. Sex chromosome–linked species recognition and evolution of reproductive isolation in flycatchers. Science 318:95–97.

Sá-Pinto, A., S. J. E. Baird, C. Pinho, P. Alexandrino, and M. Branco. 2010. A three-way contact zone between forms of *Patella rustica* (Mollusca: Patellidae) in the central Mediterranean Sea. Biological Journal of the Linnean Society 100:154–169.

Schilthuizen, M., M. C. Giesbers, and L. W. Beukeboom. 2011. Haldane's rule in the 21st century. Heredity 107:95–102.

Sharbrough, J., J. C. Havird, G. R. Noe, J. W. Warren, and D. B. Sloan. 2017. The mitonuclear dimension of Neanderthal and Denisovan ancestry in modern human genomes. Genome Biology and Evolution 96:1567–1581.

Stöck, M., A. Horn, C. Grossen, D. Lindtke, R. Sermier, C. Betto-Colliard, C. Dufresnes, et al. 2011. Ever-young sex-chromosomes in European tree frogs. PLoS Biology 9:e1001062.

Stöck, M., C. Moritz, M. Hickerson, D. Frynta, T. Dujsebayeva, V. Eremchenko, J. R. Macey, T. J. Papenfuss, and D. B. Wake. 2006. Evolution of mitochondrial relationships and biogeography of Palearctic green toads (*Bufo viridis* subgroup) with insights in their genomic plasticity. Molecular Phylogenetics and Evolution 41:663–689.

Stöck, M., R. Savary, C. Betto-Colliard, S. Biollay, H. Jourdan-Pineau, and N. Perrin. 2013*a*. Low rates of X-Y recombination, not turnovers, account for homomorphic sex chromosomes in several diploid species of Palearctic green toads *Bufo viridis* subgroup. Journal of Evolutionary Biology 3:674–682.

Stöck, M., R. Savary, A. Zaborowska, G. Górecki, A. Brelsford, B. Rozenblut-Kościsty, M. Ogielska, et al. 2013*b*. Maintenance of ancestral sex chromosomes in Palearctic tree frogs: direct evidence from *Hyla orientalis*. Sexual Development 7:261–266.

Stöck, M., A. Sicilia, N. Belfiore, D. Buckley, S. Lo Brutto, and M. Arculeo. 2008. Post-Messinian evolutionary relationships across the Sicilian channel: mitochondrial and nuclear markers link a new green toad from Sicily to African relatives. BMC Evolutionary Biology 8:56.

Tamschick, S., B. Rozenblut-Kościsty, L. Bonato, C. Dufresnes, P. Lymberakis, W. Kloas, M. Ogielska, and M. Stöck. 2015. Sex chromosome conservation, *DMRT1*-phylogeny and gonad morphology in diploid Palearctic green toads *Bufo viridis* subgroup. Cytogenetic and Genome Research 144:315–324.

Tao, Y., and D. L. Hartl. 2003. Genetic dissection of hybrid incompatibilities between *Drosophila simulans* and *D. mauritania*. III. Heterogeneous accumulation of hybrid incompatibilities, degree of dominance, and implications for Haldane's rule. Evolution 57: 2580–2598.

Turelli, M., and L. C. Moyle. 2007. Asymmetric postmating isolation: Darwin's corollary to Haldane's rule. Genetics 176:1059–1088.

Turelli, M., and H. A. Orr. 1995. The dominance theory of Haldane' rule. Genetics 140:389–402.

Wells, D. E., L. Gutierrez, Z. Xu, V. Krylov, J. Macha, K. P. Blankenburg, M. Hitchens, et al. 2011. A genetic map of *Xenopus tropicalis*. Developmental Biology 354:1–8.

Wu, C. I. 2001. The genic view of the process of speciation. Journal of Evolutionary Biology 14:851–865.

References Cited Only in the Supplemental Material

Blair, W. F. 1972. Evolution in the genus *Bufo*. University of Texas Press, Austin.

Kawamura, T., M. Nishioka, M. Sumida, and M. Ryuzaki. 1982. Viability of hybrids among Japanese, Chinese, Taiwan, European and American toads. Japanese Journal of Genetics 57:677–678. [In Japanese.]

Kawamura, T., M. Nishioka, and H. Ueda. 1980. Inter- and intraspecific hybrids among Japanese, European and American toads. Scientific Reports of the Laboratory of Amphibian Biology, Hiroshima University 4:1–125.

Malone, J. H., and B. E. Fontenot. 2008. Patterns of reproductive isolation in toads. PLoS ONE 3:e3900. doi:10.1371/journal.pone.0003900.

Nishioka, M., M. Sumida, H. Ueda, and Z. Wu. 1990. Genetic relationships among 13 *Bufo* species and subspecies elucidated by the method of electrophoretic analyses. Scientific Reports of the Laboratory of Amphibian Biology, Hiroshima University 10:53–92.

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Q1. Au: Please be sure to check the math carefully to ensure that nothing has gone awry during translation from Word or LaTeX, editing, or typesetting and advise if anything seems amiss. Note that journal style is to set roman labels attached to variables, and edits were made as such; please ensure that they are okay or advise.
Q2. Au: Okay to edit to read "has mostly"?
Q3. Au: Is "homomorphic (i.e., microscopically indistinguishable) sex chromosomes" okay as edited?
Q4. Au: Correct that LG1 is a gene symbol? Okay to make it italic?
Q5. Au: Is "without those individuals whose hybrid indices were <0.1 and >0.9" okay as edited?
Q6. Au: Okay to edit to read "hybrid zone" (not "contact zone") here?
Q7. Au: Is "but their polymorphism was not . . . , and it was discarded from the final analyses" okay as edited?
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Supplementary information on:

Introgression across hybrid zones is not mediated by Large X-effects in green toads with undifferentiated sex chromosomes

The American Naturalist

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Key words: Hybridization, Hybrid zones, Haldane's rule, Large X-effect, reproductive isolation, speciation.

Table S1: ID, locality, size and proportion of *B. balearicus* / *B. viridis* mitochondrial haplotypes (not available for all individuals) for the sampled populations.

Population ID	Longitude	Latitude	Number of Individuals	Mitochondrial haplotypes (*B. balearicus* / *B. viridis*)
1	12.149	45.849	7	7 / 0
2	12.194	45.783	18	17 / 0
3	12.052	45.758	18	4 / 14
4	11.826	45.631	8	3 / 5
5	12.649	45.546	1	1 / 0
6	12.290	45.524	2	2 / 0
7	12.133	45.531	7	3 / 3
8	12.261	45.499	6	1 / 5
9	12.301	45.481	1	1 / 0
10	12.235	45.448	2	0 / 2
11	12.455	45.466	3	3 / 0
12	12.421	45.460	6	6 / 0
13	12.360	45.429	14	14 / 0
14	12.388	45.426	2	2 / 0
15	12.324	45.342	4	4 / 0
16	12.100	45.401	1	1 / 0
17	11.523	45.579	17	14 / 2
18	11.805	45.379	3	3 / 0
19	11.963	45.341	2	1 / 1
20	12.100	45.289	4	1 / 3
21	12.066	45.271	1	1 / 0
22	11.995	45.281	1	1 / 0
23	12.309	45.297	8	8 / 0
24	11.604	45.361	4	3 / 1
25	11.150	45.417	1	1 / 0
26	11.439	45.358	9	8 / 1
27	11.534	45.322	1	1 / 0
28	11.386	45.311	10	1 / 7
29	11.581	45.254	3	3 / 0
30	11.746	45.244	23	6 / 12

Table S1 continued.

31	11.772	45.223	5	2 / 0
32	11.522	45.224	13	10 / 3
33	11.530	45.124	8	3 / 4
34	12.326	45.168	2	2 / 0
35	12.261	45.121	4	0 / 4
36	12.226	45.066	1	0 / 1
37	12.274	45.056	5	0 / 5
38	12.392	45.014	1	0 / 1
39	12.336	44.959	1	0 / 1
40	12.087	45.024	5	0 / 2
41	11.992	45.061	1	0 / 1
42	11.932	45.045	1	0 / 1
43	11.780	45.079	19	2 / 12
44	11.823	45.040	1	0 / 1
45	11.850	44.999	1	0 / 1
46	11.645	45.004	1	0 / 1
47	11.012	45.244	7	1 / 6
48	11.213	45.193	3	0 / 3
49	11.123	45.073	1	0 / 1
50	10.694	45.042	14	0 / 11
51	10.879	45.036	1	0 / 1
52	10.870	44.988	5	0 / 3
53	11.661	44.902	4	1 / 3
54	11.609	44.827	6	0 / 4
55	11.448	44.668	4	1 / 3
56	10.846	44.698	4	1 / 3
57	12.278	44.554	1	0 / 1
58	11.971	44.429	3	0 / 3
59	12.032	44.412	1	0 / 1
60	12.196	44.298	1	0 / 1

Table S2: Population ID, locality, size and proportion of *B. balearicus* / *B. siculus* mitochondrial haplotypes (not available for all individuals) for the sampled populations.

Population ID	Longitude	Latitude	Number of Individuals	Mitochondrial haplotypes (*B. balearicus* / *B. siculus*)
8	15.894	37.985	27	27 / 0
11	14.699	38.118	2	2 / 0
12	15.230	37.789	2	2 / 0
13	15.243	37.769	15	13 / 1
14	15.174	37.691	26	18 / 7
15	15.063	37.561	26	13 / 13
16	15.022	37.476	23	0 / 23
17	15.080	37.334	13	0 / 13
18	14.788	37.643	10	0 / 10
21	13.352	38.170	30	0 / 30
22	13.292	38.213	18	0 / 18

Table S3: Optimal annealing temperature (T_A) and composition of mastermixes for microsatellite PCRs. Fwd. and rev. are forward and reverse primers with a concentration of 10 pmol/µl each. The total reaction volume for each PCR including DNA was 10 µl.

Mastermix I	Mastermix II	Mastermix III
T_A: 61°C	T_A: 59.5°C	T_A: 63°C
1 µl DNA (10 ng/µl)		
1 µl 10x TopTaq PCR Buffer (Qiagen, with 15 mg $MgCl_2$)		
1 µl dNTP-Mix (2,5 mM of each dNTP)		
0.1 µl TopTaq Polymerase (Qiagen)		
0.25 µl BvHNRNPD fwd. and rev.	0.5 µl BvMed15unig fwd. and rev.	0.25 µl BvKctd15 fwd. and rev.
0.1 µl BvEll2 fwd. and rev.	0.25 µl BvGar1 fwd. and rev.	0.5 µl BvAp2a1 fwd. and rev.
0.25 µl BvDMRT1 fwd. and rev.	0.5 µl BvMed15 fwd. and rev.	0.35 µl BvSox2 fwd. and rev.
0.1 µl BvCherp fwd. and rev.	0.15 µl BvCamk4 fwd. and rev.	0.25 µl BvZnf295 fwd. and rev.
0.1 µl BvMapk2 fwd. and rev.	0.25 µl BvIno80b fwd. and rev.	0.25 µl BvFam117b fwd. And rev.
-	-	0.5 µl BvDyrk1a fwd. and rev.
-	-	0.35 µl BvKrt5.6 fwd. and rev.
-	-	0.5 µl BvPvrl2 fwd. and rev.
5.3 µl RNAse free water	3.4 µl RNAse free water	1 µl RNAse free water

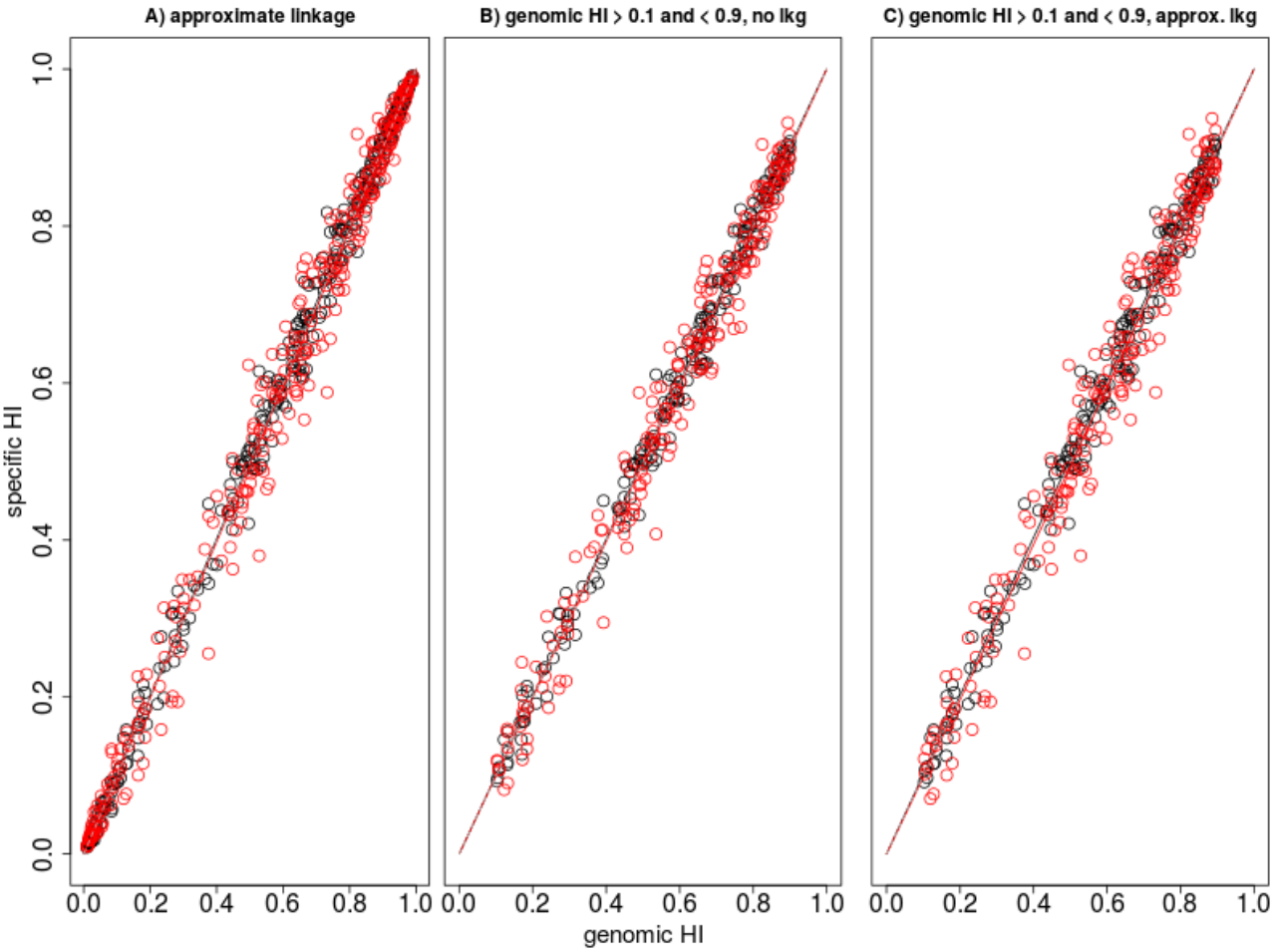

Figure S1: Genomic clines in the North-Italian hybrid zone (*B. balearicus* / *B. viridis*). Specific hybrid indexes of autosomal (black) and sex-linked (red) markers plotted against genomic hybrid indexes. Analysis was performed with three different datasets:
A) with linkage information estimated from the *X. tropicalis* linkage map,
B) assuming no linkage (lkg) between markers and with clines based on individuals with genomic hybrid indexes > 0.1 and < 0.9,
C) with linkage (lkg) information estimated from the *X. tropicalis* linkage map and with clines based on individuals with genomic hybrid indexes > 0.1 and < 0.9.

Supplemental Material for: Jorn F. Gerchen, Christophe Dufresnes, Matthias Stock. 2018. "Introgression across Hybrid Zones Is Not Mediated by Large X-Effects in Green Toads with Undifferentiated Sex Chromosomes." The American Naturalist 192(5). DOI: 10.1086/699162.

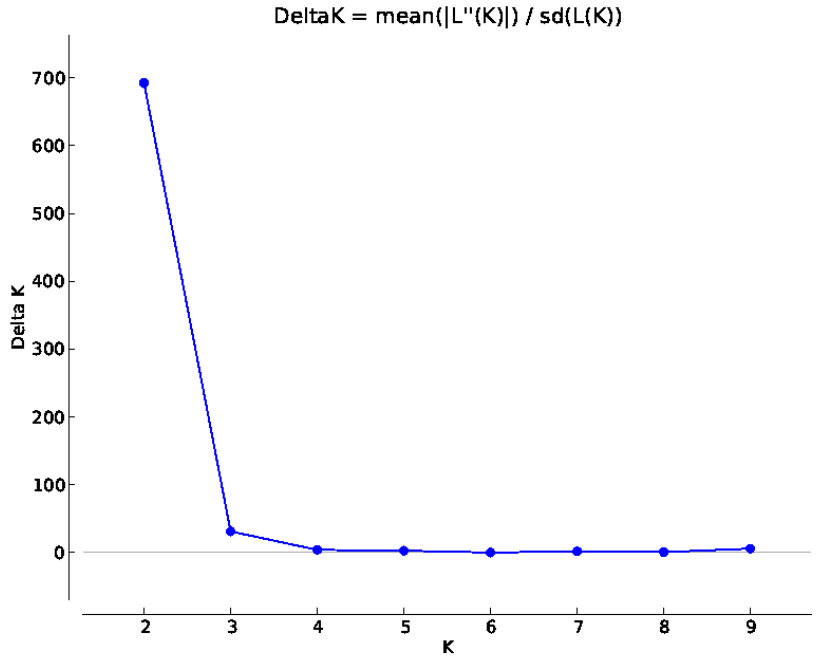

Figure S2: Delta K in the North-Italian hybrid (*B. balearicus* / *B. viridis*) zone resulting from 10 Structure runs for each K value from K = 1 to K = 10.

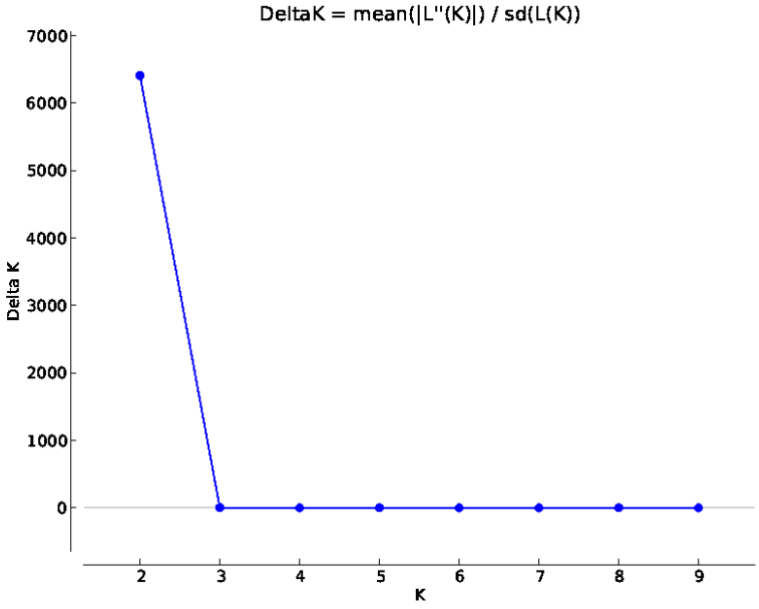

Figure S3: Delta K in the Sicilian hybrid zone (*B. balearicus* / *B. siculus*) resulting from 10 Structure runs for each K value from K = 1 to K = 10

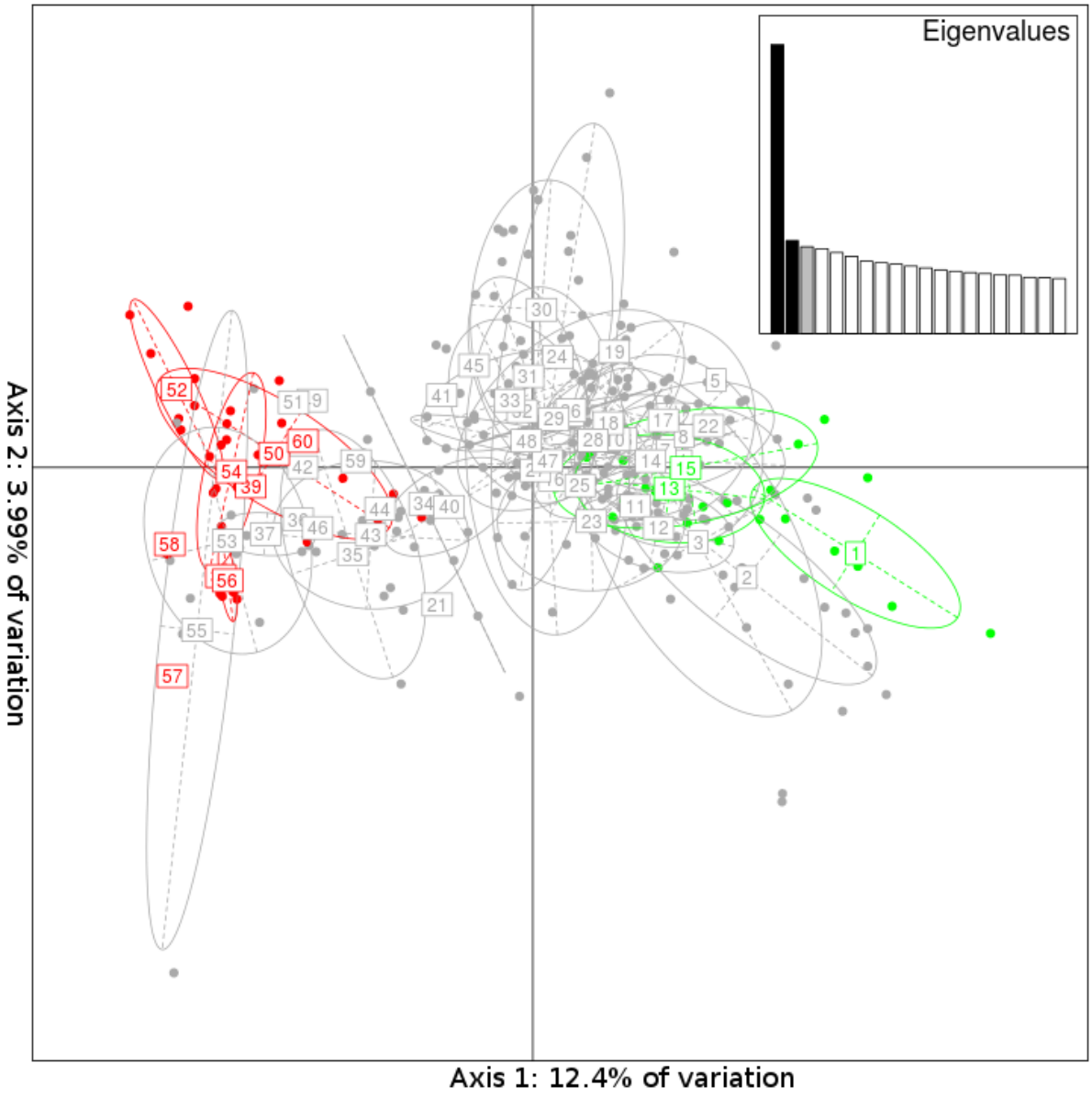

Figure S4: Principal component analyses based on genotypes in the North-Italian hybrid zone (*B. balearicus* / *B. viridis*). Probably pure *B. balearicus* populations with average Structure assignment probabilities < 0.1 and having exclusively *B. balearicus* mitochondrial haplotypes are marked in red and supposed pure *B. viridis* populations with Structure assignment probabilities >0.9 and having exclusively *B. viridis* mitochondrial haplotypes are marked in green. The first axis separating both clusters accounts for 12.4 % of the variation.

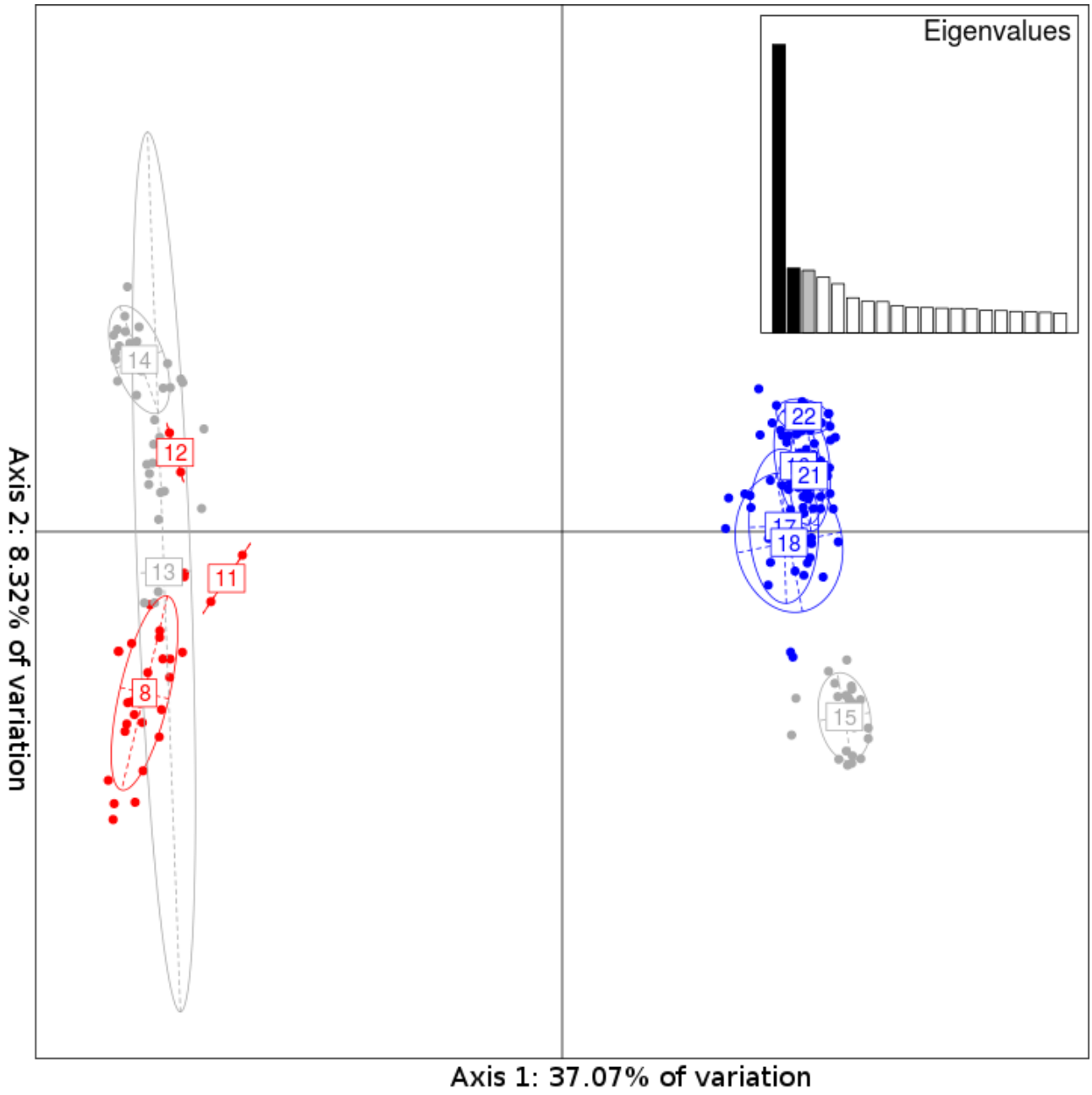

Figure S5: Principal component analyses based on genotypes in the Sicilian hybrid zone (*B. balearicus* / *B. siculus*). Probably pure *B. balearicus* populations with average Structure assignment probabilities < 0.1 and having exclusively *B. balearicus* mitochondrial haplotypes are marked in red and supposed pure *B. siculus* populations with Structure assignment probabilities >0.9 and having exclusively *B. siculus* mitochondrial haplotypes are marked in blue. The first axis separating both clusters accounts for 37.07 % of the variation.

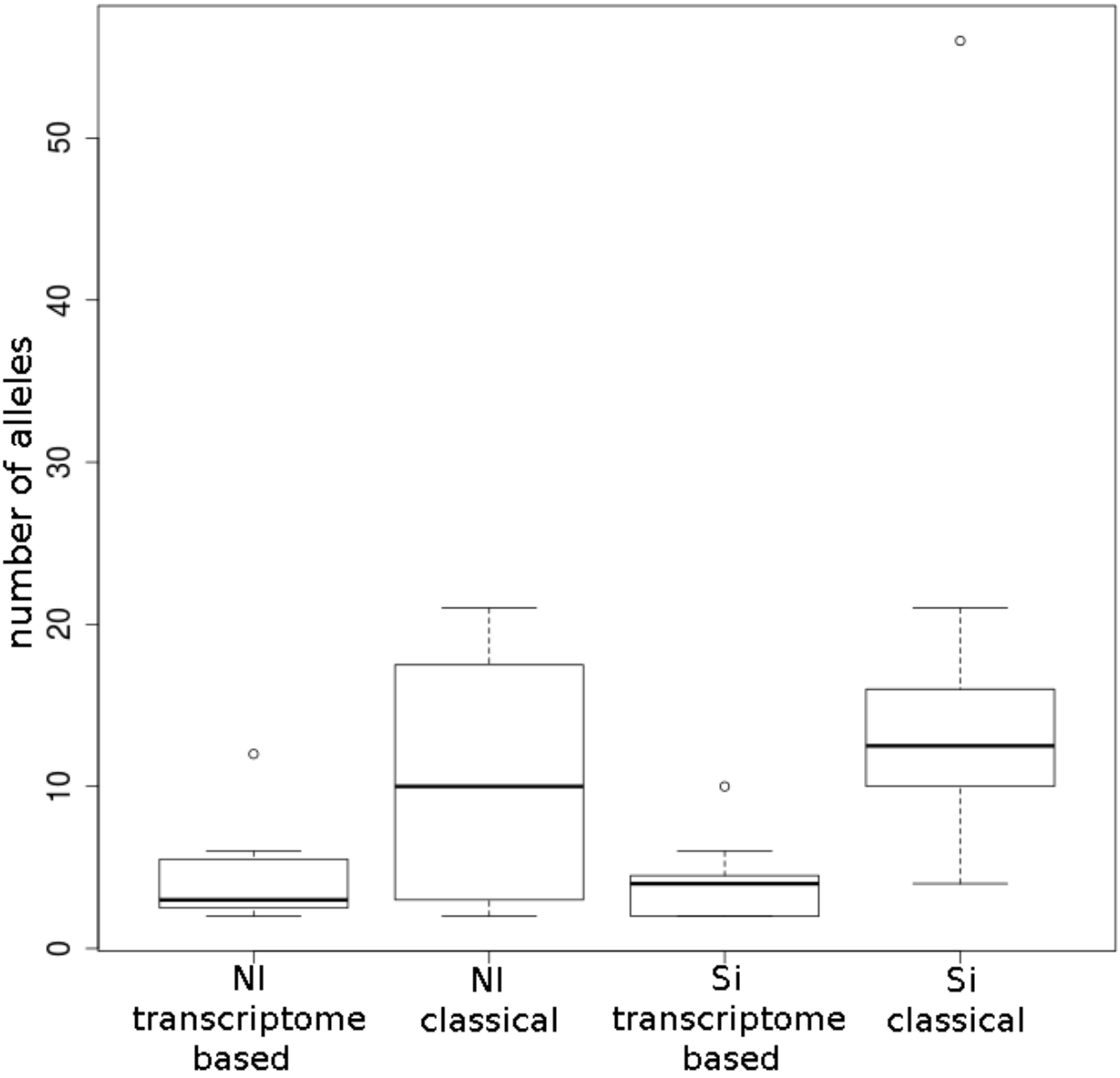

Figure S6: Number of alleles of newly developed transcriptome-based and classical microsatellite markers in the North-Italian (NI; *B. balearicus* / *B. viridis*) and the Sicilian (Si; *B. balearicus* / *B. siculus*) hybrid zone.

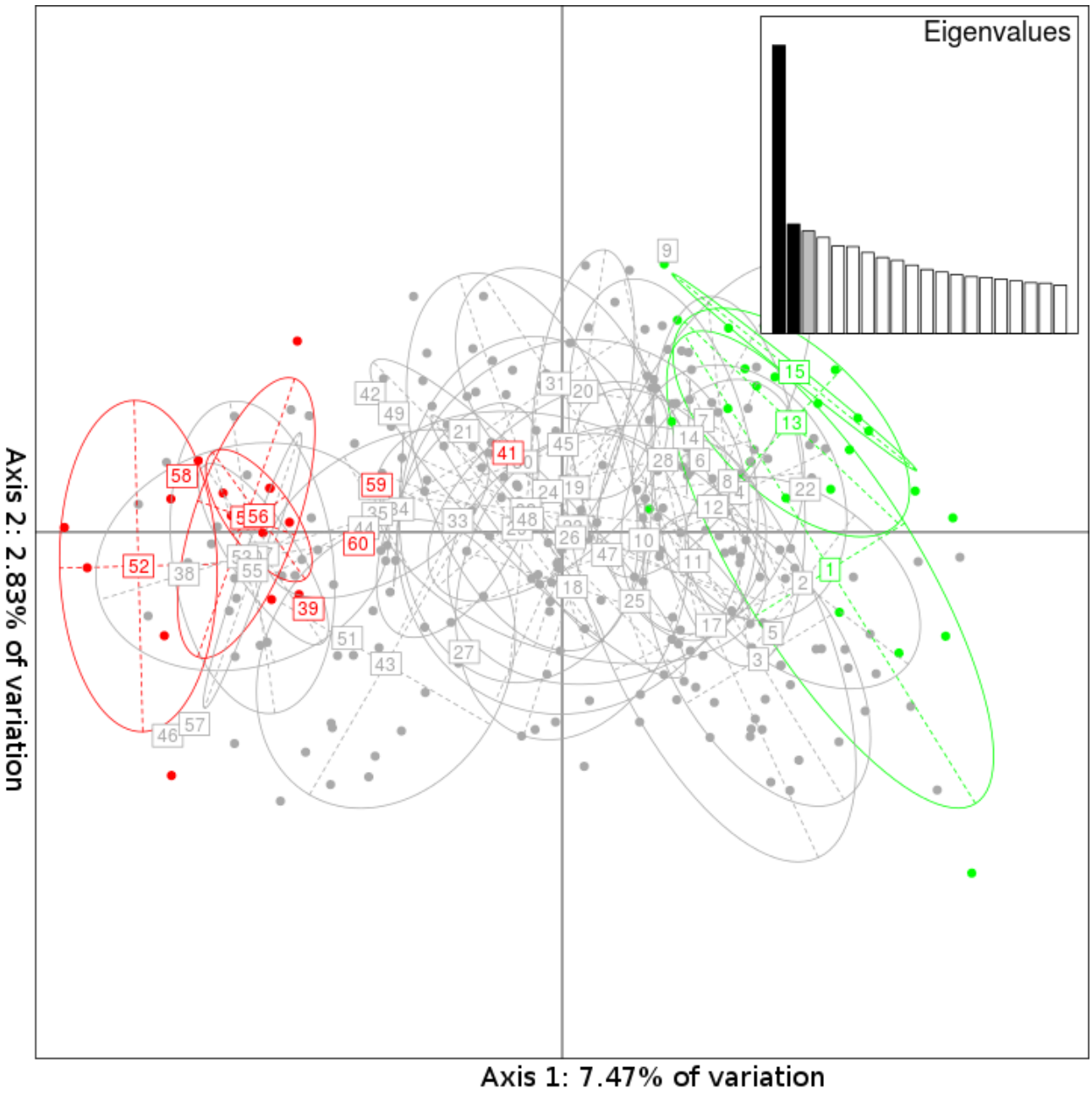

Figure S7: Principal component analyses based on genotypes of transcriptome-based markers in the North-Italian hybrid zone (*B. balearicus* / *B. viridis*). Supposed pure *B. balearicus* populations with average Structure assignment probabilities < 0.1 and having exclusively *B. balearicus* mitochondrial haplotypes are marked in red and supposed pure *B. viridis* populations with Structure assignment probabilities >0.9 and having exclusively *B. viridis* mitochondrial haplotypes are marked in green. The first axis separating both clusters accounts for 7.47 % of the variation.

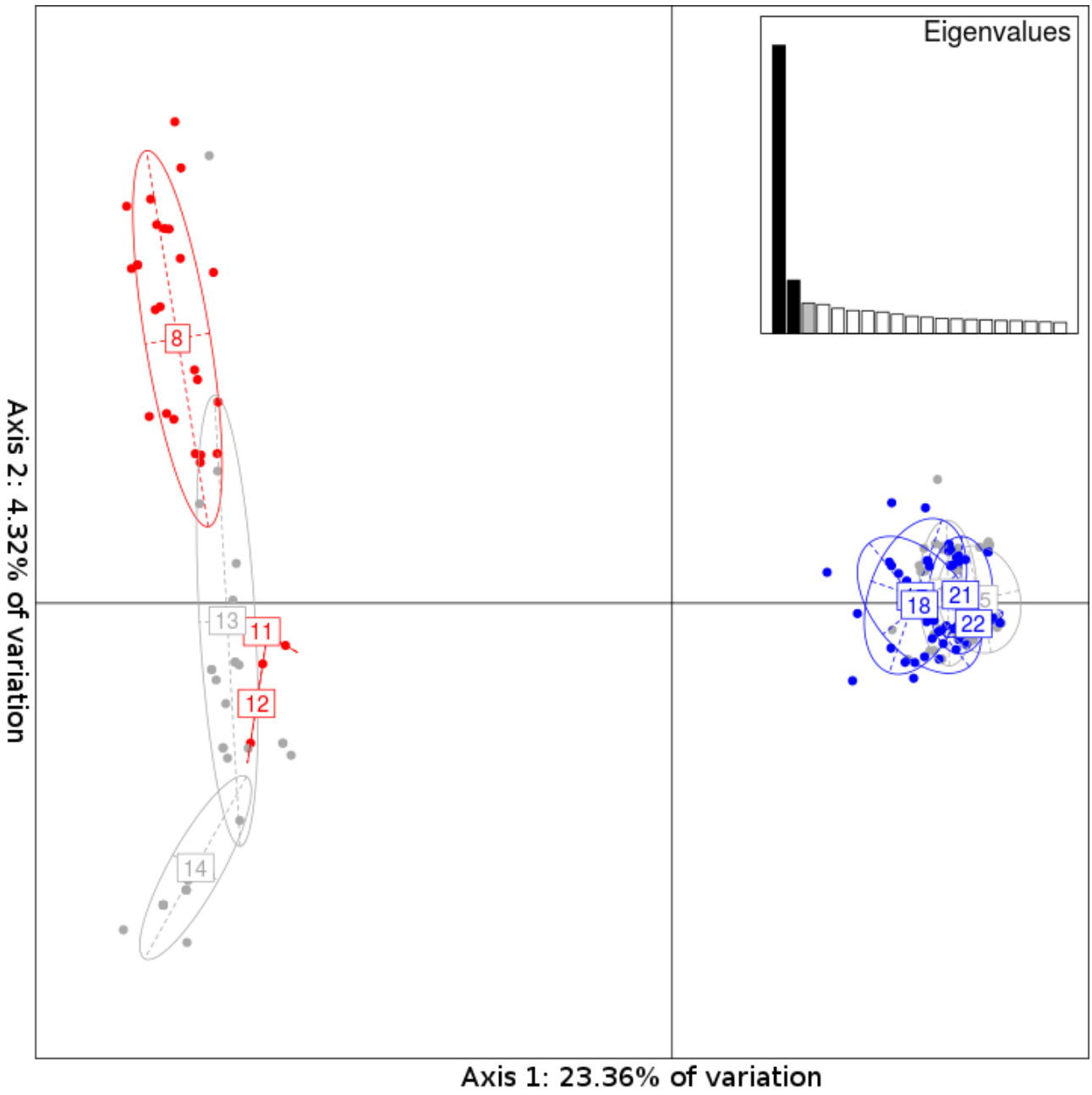

Figure S8: Principal component analyses based on genotypes of transcriptome-based markers in the Sicilian hybrid zone (*B. balearicus* / *B. siculus*). Supposed pure *B. balearicus* populations with average Structure assignment probabilities < 0.1 and having exclusively *B. balearicus* mitochondrial haplotypes are marked in red and supposed pure *B. siculus* populations with Structure assignment probabilities >0.9 and having exclusively *B. siculus* mitochondrial haplotypes are marked in blue. The first axis separating both clusters accounts for 23.36 % of the variation.

Supplementary Text 11

Hybridizability and sex-biased survival in crosses of bufonid toads and evidence from Palearctic green toads

Sex-biased survival has been documented in extensive laboratory crosses of bufonid toads (1900 crosses involving 92 species), where hatching success, the number of larvae produced, and the percentage of tadpoles reaching metamorphosis is inversely related with genetic divergence (Blair 1972, Malone and Fontenot 2008), resulting in 70% of male-biased and in 30% of female-biased offspring. Hybrid females from crosses between closely related species were completely fertile, while approximately half (53%) of the hybrid males were sterile, with sterility predicted by genetic divergence (Blair 1972, Malone and Fontenot 2008).

Diploid green toads of the *B. viridis* group have been involved in many crossing experiments (Blair, 1972; Kawamura et al., 1980, 1982; Nishioka et al., 1990), some resulting in asymmetric viability, depending on parental sex in reciprocal crosses. In accordance with a divergence-dependent F_1-fitness, more diverged green toad lineages (Colliard et al. 2010) showed asymmetric F_1-survival in reciprocal crosses of *B. balearicus* x *B. siculus*, but a complete hybrid breakdown in the F_2, whereas population genetics evidence showed advanced nuclear admixture ('F_2' and backcrosses) between *B. balearicus* and *B. viridis* and implied that at least some hybrids had become adult and fertile, as they successfully reproduced (Dufresnes et al. 2014).

References:

Blair WF: Evolution in the genus *Bufo*. Austin and London, 1972.

Colliard, C., A. Sicilia, G.F. Turrisi, M. Arculeo, N. Perrin, M. and M. Stöck 2010. Strong reproductive barriers in a narrow hybrid zone of West-Mediterranean green toads *Bufo viridis* subgroup with Plio-Pleistocene divergence. BMC Evolutionary Biology 10:232.

Dufresnes, C., L. Bonato, N. Novarini, C. Betto-Colliard, N. Perrin, and M. Stöck 2014. Inferring the degree of incipient speciation in secondary contact zones of closely related lineages of Palearctic green toads *Bufo viridis* subgroup. Heredity 113:9–20.

Kawamura T, Nishioka M, Ueda H 1980. Inter- and intraspecific hybrids among Japanese, European and American toads. Scientific Reports of the Laboratory of Amphibian Biology, Hiroshima University 4:1-125.

Kawamura T, M. Nishioka, M. Sumida, and M. Ryuzaki 1982. Viability of hybrids among Japanese, Chinese, Taiwan, European and American toads. Japanese Journal of Genetics. 57:677-678. (in Japanese).

Nishioka, M., M. Sumida, H. Ueda, and Z. Wu 1990. Genetic relationships among 13 *Bufo* species and subspecies elucidated by the method of electrophoretic analyses. Scientific Reports of the Laboratory of Amphibian Biology, Hiroshima University 10:53-92.

Malone J.H. and B.E. Fontenot 2008. Patterns of reproductive isolation in toads. PLoS ONE 3(12): e3900. doi:10.1371/journal.pone.0003900.