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# Cryptic lineages and standing genetic variation across independent cane toad introductions

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## Abstract

Widespread introduced species can be leveraged to investigate the genetic, ecological and adaptive processes underlying rapid evolution and range expansion, particularly the contributions of genetic diversity to adaptation. *Rhinella marina*, the cane toad, has been a focus of invasion biology for decades in Australia. However, their introduction history in North America is less clear. Here, we investigated the roles of introduction history and genetic diversity in establishment success of cane toads across their introduced range. We used reduced representation sequencing (ddRAD) to obtain 34,000 SNPs from 247 toads in native (French Guiana, Guyana, Ecuador, Panama, Texas) and introduced (Bermuda, southern Florida, northern Florida, Hawai'i, Puerto Rico) populations. Unlike all other cane toad introductions, we found that Florida populations were more closely related to native Central American lineages (*R. horribilis*), than to native Southern American lineages (*R. marina*). Furthermore, we found high levels of diversity and population structure in the native range, corroborating suggestions that *R. marina* is a species complex. We also found that introduced populations exhibit only slightly lower genetic diversity than native populations. Together with demographic analyses, this indicates founding populations of toads in Florida were larger than previously reported. Lastly, within *R. marina*, only one of 245 putatively adaptive SNPs showed fixed differences between native and introduced ranges, suggesting that putative selection in these introduced populations is based upon existing genetic variation. Our findings highlight the importance of genetic sequencing in

understanding biological introductions and hint at the role of standing genetic variation in range expansion.

KEYWORDS
adaptation, amphibians, invasive species, phylogeography, population genetics – empirical

## 1 | INTRODUCTION

Invasive species are widely used as model systems in evolutionary biology. Many successful invasive species were introduced to multiple areas, creating replicated natural experiments in which to test a central question in evolutionary biology: how does genetic variation contribute to adaptation? Invasive species are useful in addressing this question because distinct introductions with differing founding population sizes can be compared and contrasted. If source populations are known, we can make additional inferences about historical genetic differentiation and even preadaptation (as in, adaptation to similar conditions in the source environment) to the novel environments they encounter in the introduced range (Hufbauer et al., 2011). Populations resulting from different introductions can also be compared to ask if evolution acts on the same genetic variants when similar stressors are experienced by introduced individuals (Fang et al., 2020; McGoey et al., 2020).

The invasive species paradox describes the common scenario in which introduced species undergoing severe bottlenecks are often able to adapt to their novel environments, despite low genetic diversity (Dlugosch & Parker, 2008; Schrieber & Lachmuth, 2017). Causes of this success could include plasticity, high mutation rate, or the presence of adaptive variation from the source population. Alternatively, these introduced populations may not actually be as genetically homogeneous as assumed. For instance the presence of multiple introductions from different population sources can result in high levels of genetic diversity, thus rejecting the paradox entirely (Kolbe et al., 2004). To parse these possibilities, it is essential to know the relationships between source populations in the native range and interpret population structure amongst introduced populations within this context. Comparing introduced populations to their sources can then shed light on the role of genetic variation and demographic history in establishment success.

The cane toad complex, *Rhinella* spp., is an excellent system for investigating changes in genetic diversity during successful establishment in introduced ranges. *Rhinella* species were introduced world-wide in the early to mid 1900s. However, until recently, research largely centered on introduced populations in Australia, Hawai'i, and Puerto Rico. Recent morphological work in the native range identified two species within *R. marina*, with *R. horribilis* occurring in northwestern South America, Central America and the southern United States and *R. marina* occurring exclusively in South America (Acevedo et al., 2016; Bessa-Silva, 2020), and this has been largely confirmed by genetic data (Rivera et al., 2021). Given this finding, gaps remain in our understanding of the broader context of invasion in *Rhinella*. In particular, our knowledge of source populations for introductions to Bermuda and Florida, USA is based largely on anecdotal evidence, thus it is unknown whether *R. horribilis* contributed to these introductions.

According to available records, Bermuda populations were introduced from Guyana in the early 1900s, and possibly even earlier (Lever, 2001). In Florida, there are records of toads being introduced from Puerto Rico in the 1940s, but these were considered unsuccessful (Easteal, 1981; King & Krakauer, 1966). A newspaper article cited in King and Krakauer (1966) reported that the source of the Florida introduction was an unintentional release of 100 toads from Colombia (probably *R. horribilis*) in 1955, followed by smaller releases from Suriname (*R. marina*). It has also been suggested, based on the seemingly sudden appearance of cane toads in northern Florida, that there were multiple introductions to Florida. Previous studies surveyed native and introduced populations of *Rhinella* spp. using microsatellite and mitochondrial DNA (Acevedo et al., 2016; Estoup et al., 2001; Slade & Moritz, 1998; Vallinoto et al., 2010), but these did not include Florida and lacked the resolution to estimate the demographic consequences of invasion (Estoup et al., 2001). These lower-resolution methods may miss more subtle population structure, and fail to detect past introduction events (Le Cam et al., 2020).

In contrast, the demographic and evolutionary consequences of introduction is well studied in Australia. Cane toads in Australia were introduced in a stepping stone fashion: toads from Guyana were introduced to Puerto Rico, whose descendants were introduced to Hawaii in the 1930s. Toads from Hawai'i were then introduced to Australia in the 1940s (Easteal, 1981; Lever, 2001). Australian cane toads exhibit decreases in genome wide average genetic diversity, but genetic diversity in putatively adaptive loci has increased across their introduced and rapidly expanding range (Selechnik, 2019). Additionally, studies on Australian toads have found evolution in dispersal ability, environmental niche occupancy, immune function, and cold-tolerance which may be linked to the maintenance of adaptive variation, or increased mutation rates creating variation (Rollins et al., 2015; Sales et al., 2021; Selechnik, 2019; Shine et al., 2011). Elucidating the phylogenetic history and evolutionary trajectories of separate introductions to Florida and Bermuda provide an opportunity to test the generalizability of these molecular processes.

Our goal was to perform the first genome-wide survey of the *Rhinella marina* species complex with a focus on native populations in both Central and South America and introduced populations in the Mid-Atlantic and North America. We used a reduced representation genomics approach (ddRAD) to address the following aims and questions: (i) Establish the phylogenic context of introductions by inferring introduction routes with genetic data. In particular, are all introduced populations *R. marina*, or was *R. horribilis* also introduced?; (ii) Evaluate evidence for the first condition of the "genetic paradox" in

Bermuda and Florida. Specifically, do introduced populations exhibit signatures of historical bottlenecks and decreased genetic variation? Alternatively, does cryptic admixture between distinct source populations result in increased genetic variation in introduced relative to native populations? and (iii) investigate patterns of selection in introduced populations by identifying loci putatively under selection within and across introduced populations. Are these variants new mutations or do they represent selection upon standing genetic variation from the native range? Do putatively adaptive loci exhibit higher genetic diversity than putatively neutral loci, thus maintaining genetic variation despite historical bottlenecks, as seen in other *R. marina* introductions?

Elucidating the history of invasions, source populations, and the distribution of genetic variability in invasive species can help us predict future establishment success and adaptive potential (Acevedo-Limón et al., 2020). Knowing the environmental conditions of the source lineages, as well as the amount of genetic variation can inform models of further range expansion (Razgour et al., 2019; Waldvogel et al., 2020). Finally, cane toads have become a model species for understanding the genomics of invasive species, and rapid evolution to novel environmental conditions more generally (see review: Rollins et al., 2015). Adding a genomic perspective on the presence of cryptic lineages, levels of genetic diversity, and phylogenetic history will motivate future studies on the molecular underpinnings of rapid adaptation in species encountering novel environmental conditions.

## 2 | MATERIALS AND METHODS

### 2.1 | Collection

We sampled *Rhinella marina* and *R. horribilis* across several localities (Figure 1). Sampling for introduced *R. marina* included Florida, USA in Miami ($n = 28$, hereafter, the "Southern" Florida population), Tampa ($n = 24$), and Lakeland ($n = 5$). As both Lakeland and Tampa populations occur near the northwestern range edge (Meshaka Jr., 2011), are closer to each other than to the southern sampling site, and Lakeland individuals were paraphyletic within the Tampa clade in the phylogenetic analysis (Figure S1), we grouped them together and refer to them as the "Northern" Florida population ($n = 29$). We included three additional introduced populations: Puerto Rico ($n = 10$), Bermuda ($n = 32$), and O'ahu, Hawai'i ($n = 29$).

Due to export restrictions, we were unable to obtain specimens from Surinam (*R. marina*) or Colombia (*R. horribilis*), the proposed sources for the Florida introduction. However, we were able to obtain *R. marina* samples from Guyana ($n = 32$) and French Guiana ($n = 26$), which border Suriname. We also obtained tissues samples of specimens identified as *R. marina* in existing collections from Ecuador ($n = 13$; Museo de Zoología, Universidad Tecnológica Indoamérica; permit MAE-DNB-CM-2015-0017). We collected *R. horribilis* from Panama ($n = 32$) and Texas, USA ($n = 18$), which, like Colombian *Rhinella*, were assigned to *R. horribilis* by Acevedo et al. (2016). These specimens allow us to place *R. horribilis* in a phylogenic context with *R. marina*, and to determine if the *R. horribilis* lineage has contributed to invasive populations in the Mid-Atlantic, Caribbean and North America. The collection protocol is summarized in Mittan and Zamudio (2019). Briefly, toads collected for this study were hand captured, and euthanized using an MS-222 bath or Clove oil. Muscle, liver, and/or toe clips were taken and whole specimens deposited at the Cornell University Museum of Vertebrates (catalogue numbers: Florida a-0016126–41; Puerto Rico a-0016247–55; Texas a-0016256–75). Data on all samples, including locality, museum numbers, and species are listed in Table S1.

### 2.2 | DNA extraction

Muscle, liver, or toe clips were used for DNA extraction (see Table S1). For liver and toe clips we used the DNeasy Blood and

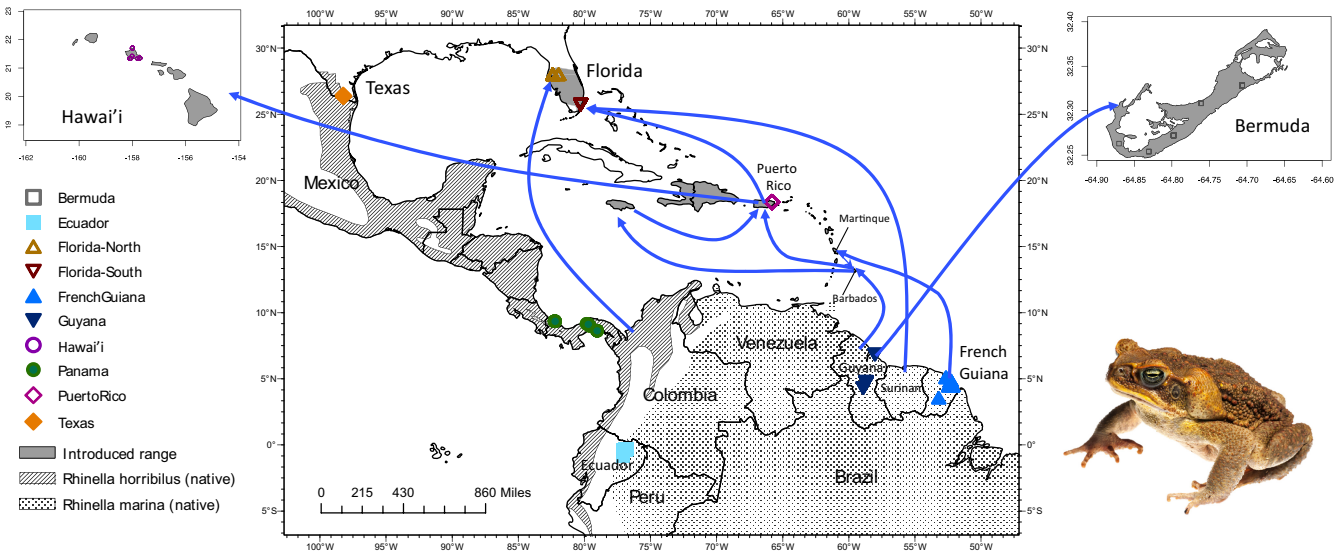


FIGURE 1 Map of sampled localities (points) and hypothesized introduction routes (arrows) from Easteal (1981).

Tissue Kit (Qiagen). For muscle tissue, which has less DNA content than liver or toe clip tissues, we used the QIAamp DNA Microkit (Qiagen). Following extraction, DNA concentrations were measured using QuantiFluor (Promega) according to the manufacturer's instructions. Samples with DNA concentrations below 20 ng/μl were concentrated by drying samples on a SpeedVac (ThermoFisher Scientific), until the DNA concentration reached at least 20 ng/μl.

## 2.3 | Sequencing

We prepared the ddRAD libraries following the protocol detailed in Polato et al. (2018). Briefly, purified DNA samples were cut using Msp1, and SbfI-HF enzymes. Each sample was then individually indexed and barcoded, with 48 individuals per library and 11 total libraries. Barcoded fragments were PCR amplified, and then size-selected to retain fragments between 200 and 700 bp. Final libraries were visualized on gels to ensure correct fragment size. Libraries were sequenced at the Cornell Biotechnology Research Centre on the Illumina HI-SEQ 2500 with 100 bp single-end sequencing with one lane per library.

## 2.4 | Data processing and SNP calling

Following sequencing, libraries were checked for quality using FastQC (Andrews, 2017). Reads were demultiplexed using process_radtags in the Stacks (version 2.3) pipeline with the following settings: removed reads with uncalled bases, rescue barcodes, eliminate reads failing Illumina's quality filter (Catchen et al., 2013). We then aligned our reads to the *R. marina* genome (Edwards et al., 2018) using bowtie2 using the "sensitive" option, and default settings (Langmead & Salzberg, 2012). We then used samtools (Li, 2011) to convert aligned reads to BAM format, filter reads with a quality score below 42, and sort bam files for further analyses. We called SNPs using the Stacks (version 2.3) program gstacks for referenced-based alignment using default settings.

We used an iterative approach to filter our assembled loci. First, we preformed baseline filtering in Stacks using populations: reads were required to be in at least 50% of individuals across all populations (- r 0.5), and present in at least seven of the 10 populations (-p 7), and we filtered SNPs exhibiting more than 50% heterozygosity (max_obs_het 0.5), as these may represent paralogous loci.

Second, we analysed the retained SNPs and loci in R (R Core Team, 2020) using plyr (Wickham, 2011) and pegas (Paradis, 2010), to determine: (i) Which SNPs to eliminate based on position within the loci, as the average number of SNPs may increase towards the ends of reads, indicating quality drop off; and (ii) to eliminate loci that fell in the top 5% of most variable (>8 SNPs per locus). We eliminated highly variable loci as SNP density should follow a Poisson distribution, with very few loci with more than seven SNPs (DaCosta & Sorenson, 2014). We used these assessments to create a list of loci that passed all filters, which we then used to filter loci in populations.

Third, we used plink to iteratively filter both individuals and loci for missingness across all populations (Purcell et al., 2007). This strategy is recommended because it filters loci and individuals with missing data, while maximizing the number of loci and individuals retained, compared to using a single filtering call (O'Leary et al., 2018). Lastly, using the retained loci and individuals, we applied a minor allele count (-mac 3) of 3, corresponding to a minor allele frequency of 0.01. Filtering by MAC balances the filtering of rare variants in the dataset due to sequencing error and retaining true low-frequency variants by requiring the allele to appear in three individuals (O'Leary et al., 2018). This final data set was used across all analyses except where otherwise noted. After filtering, we also generated a data set containing only one randomly selected SNP per locus, for analyses sensitive to linkage disequilibrium. Lastly, we used the option -fstats to obtain pairwise $F_{ST}$ values between populations (assigned by collection locality) and individuals.

## 2.5 | Population statistics

We used populations to calculate observed and expected heterozygosity ($H_o$ and $H_e$), and inbreeding ($F_{is}$) within populations (Stacks version 2.3; Catchen et al., 2013). We used HP-rare to quantify allelic richness (Ar), while controlling for differences in sample size between populations (Kalinowski, 2005).

## 2.6 | Phylogeny

We constructed a maximum likelihood phylogeny using IQtree version 2.1.1 (Minh et al., 2020) on the CIPRES science gateway (Miller et al., 2010), implementing the ascertainment bias correction (ASC) for SNP data without variable sites. The phylogenetic analysis was then performed using the best substitution model (tvm + F + ASC + R5) as determined by ModelFinder (Kalyaanamoorthy et al., 2017), with 1000 Ultrafast bootstrap replicates (Hoang et al., 2018), and the -bnni option to reduce overestimating bootstrap support.

## 2.7 | Population structure

To visualize amongst and between population variation, we executed a principal component analysis (PCA) on the 1 SNP per locus data set using the R packages adegenet (Jombart & Ahmed, 2011) and SNPRelate (Zheng et al., 2012) with all samples and with *Rhinella marina* samples alone. To detect subpopulation structure, we used fineRADstructure with all filtered SNPs per locus to generate a coancestry matrix (Malinsky et al., 2018). Heatmaps were generated using the R script provided with the fineRADstructure distribution.

We used ADMIXTURE to determine the number of populations present in our sampling, using the 1 SNP per locus data set. We tested population numbers (K) of 1–15, using 5-fold cross-validation to select the best K value (Alexander & Lange, 2011).

In both the phylogenetic tree and ADMIXTURE analysis using all populations, we found that several individuals from coastal Guyana (Georgetown, Table S1) appeared to be more similar to Bermuda

than to samples from inland Guyana (Iwokrama, Table S1). To further investigate this pattern, we performed a "supervised" analysis in ADMIXTURE. We first created a reference panel with all individuals from Guyana and French Guiana, setting K = 2. This allowed us to train the ADMIXTURE model using the native populations. We then used the trained model to assign ancestry to all individuals from Puerto Rico, Hawai'i and Bermuda. We also performed this analysis with K = 3, to allow for population structure (coastal and inland populations) within Guyana. This analysis allowed us to assess to which of the potential source lineages the introduced populations in Bermuda, Puerto Rico, and Hawai'i were most similar.

### 2.8 | Demography

Past demography was inferred using StairWayPlot version 2.1 (Liu & Fu, 2020). We conducted analyses for each population except for Ecuador, due to small sample numbers and the presence of two sampled clades. We used StairwayPlot as it is better at detecting recent changes to demography than other methods, such as FastSimCoal (Liu & Fu, 2020). To generate the site frequency spectrum per each population, we used the data set without a minor allele count filter, so as not to exclude rare variants appearing in only one or two individuals. We then created a VCF file for each population using vcftools (Danecek et al., 2011). We used the python script easySFS to convert the VCF files to SFS, which also reduced the data set to one SNP per locus, as recommended (Overcast, 2020). SNP mutation rate in amphibians is not well known, so we used 1.9e-8, as has been used in similar studies of anurans, and is close to the general vertebrate mutation rate (Chan et al., 2017; Crawford, 2003). We used the 1-year generation rate across all populations to compare, however, we note there may be some variation in generation time across years and populations (Lever, 2001). All other settings were set to default values.

### 2.9 | Selection scans

We detected $F_{ST}$ outliers in PCAdapt (Privé et al., 2020), which identifies outliers while controlling for population structure. This method is also robust to the presence of admixture between individuals. As we were most interested in outliers that differentiated population pairs, we ran PCAdapt for selected pairwise comparisons. First, we compared each introduced population to its source population to detect selection in the introduced range. We did not compare Florida populations to the native range, as we could not identify the exact source populations(s) for the Florida introduction. However, since populations in southern Florida were established before populations in northern Florida, we compared these two populations to each other. As northern populations experience colder temperatures and may be differentially adapted to cold (Mittan & Zamudio, 2019), we wanted to examine genetic variation that may be under differential selection. Second, we compared Puerto Rico and Hawai'i to each other, to assess selection following introduction to Hawai'i as Hawaiian populations were sourced from Puerto Rico (Lever, 2001). Lastly, we compared Bermuda to Puerto Rico and Hawai'i to assess whether the three introductions show differential evolutionary trajectories.

We selected the number of PCA axes to retain using Cattell's rule (Cattell, 1966). We used a conservative false discovery rate of 0.001 to account for multiple testing. We then used SNPeff to identify outliers which fell within annotated genes (Cingolani et al., 2012; Edwards et al., 2018). We submitted the list of annotated genes to the PANTHER database to test for gene ontology enrichment, using the Benjamini-Hochberg false-discovery rate correction to account for multiple testing (Mi et al., 2010; Thomas, 2003).

Finally, we used the list of putatively adapted loci to investigate whether these loci exhibited higher genetic variation than putatively neutral loci in the same populations. Using the filtered dataset with all SNPs per locus, we calculated average genetic diversity and average haplotype diversity for putatively adaptive loci in Bermuda, Hawai'i, and Puerto Rico from per-locus haplotype statistics generated in Stacks (version 2.3; Catchen et al., 2013). The measure of genetic diversity assesses the number of distinct haplotypes without considering how different haplotypes are from each other, while haplotype diversity considers both the number of, and similarity between, observed haplotypes (Catchen et al., 2013). For each list of adaptive loci, we also calculated average genetic diversity and average haplotype diversity in the closest related native population, Guyana. Finally, we calculated average genetic diversity and average haplotype diversity across putatively neutral loci for each population, including Guyana.

## 3 | RESULTS

### 3.1 | SNP filtering

Following the initial round of filtering (no more than 50% missing data, present in seven of 10 populations, no more than 50% heterozygous loci), we retained 28,882 of 314,098 loci sequenced. After eliminating SNPs found at the end of loci, and excessively variable loci, we retained 19,896 loci. After iteratively filtering for individuals and loci for high degrees of missingness, we retained 247 of 255 individuals, and 16,780 loci. Our final data set, including the minor allele count filter, contained 34,827 SNPs across 13,690 loci. Our data set with one SNP per locus contained 13,690 SNPs.

### 3.2 | Phylogeny

To assess the evolutionary history of the introduced populations, we constructed a phylogenetic tree. Two major clades emerged (Figure 2); the first clade included samples collected in South America (French Guiana and Guyana), Puerto Rico, Bermuda, and Hawai'i; and the second included samples from North and Central America (Panama, Texas, Southern Florida, and Northern Florida). Samples from Ecuador grouped with South America, as an earlier divergence relative to the other South American populations (Figure 2).

### 3.3 | Population structure

The best number of groups in our ADMIXTURE analysis (based on lowest CV-value) were K = 8 (CV-value = 0.16515) and K = 10 (CV-value = 0.16383). For K = 8, French Guiana and Guyana comprised a single group, as did Puerto Rico and Hawai'i. Each of the remaining sampling locations were largely distinct but with some admixture between Northern and Southern Florida, as well as between Bermuda and Guyana (Figure 3a). For K = 10, northern Florida and southern Florida comprised a single group, Hawai'i contained two groups, one very similar to Puerto Rico, and the other more distinct, and Panama also contained two groups, corresponding to Western and Central sampling localities (Figure 3c). K = 9 had a higher CV-value than either K = 8 or K = 10, and contained the same groups as K = 8, except northern Florida was divided into two groups (Figure 3b).

In both the phylogenetic and ADMIXTURE analyses, a subset of Guyanese samples from coastal Guyana (Georgetown; Table S1) clustered more closely with Bermuda than with other Guyanese samples. To further investigate this pattern, we trained the ADMIXTURE algorithm with native *R. marina* (French Guiana and Guyana), and then used that model to evaluate ancestry of introduced *R. marina* (Bermuda, Puerto Rico and Hawai'i) (Figure 4). The best value for the native range was K = 2, splitting French Guiana and Guyana into two groups with admixture present in two individuals from French Guiana sampled in a more forested, inland region (Saul; Table S1) (Figure 4a). At K = 2, introduced populations are indistinguishable: all individuals show majority (~90%) Guyanese ancestry (Figure 4c). K = 3 identifies the Bermuda-clustering individuals as a distinct group (Figure 4b), and all introduced individuals show admixture from all three populations, with most of their ancestry from the Bermuda-clustering subgroup (~0.6) within Guyana (Figure 4d). Bermuda shows more ancestry from the smaller Guyana group than do Hawai'i and Puerto Rico.

We used the coalescent model in fineRADstructure to investigate pairwise ancestry between samples (Figure S2). As in ADMIXTURE analyses, we recovered two large, highly supported clades. Again, we found that Florida populations were most closely related to native populations in Central America. Individuals within the Ecuadorian clade were highly similar to each other.

Principal component analyses were also consistent with two major clades. We found that approximately 20% of variation was

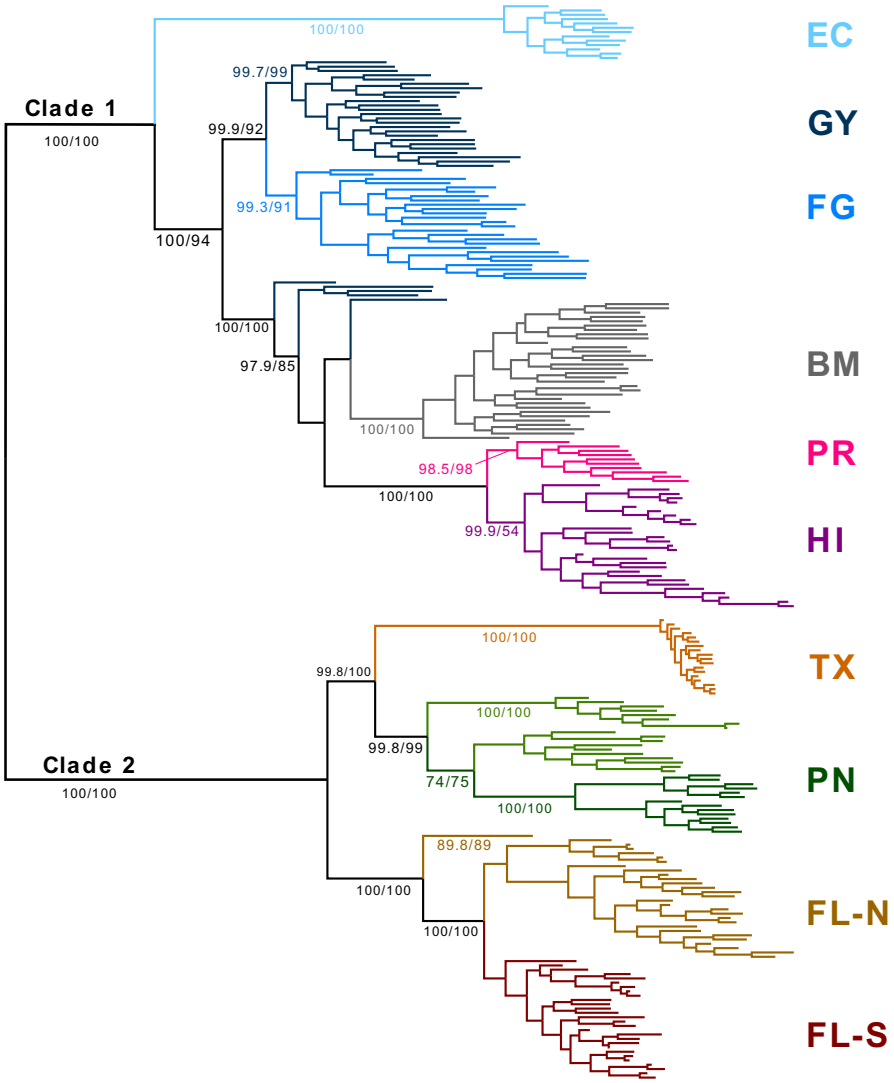


FIGURE 2 IQTree phylogeny, colours as in Figure 1. The two shades of green in the Panama clade correspond to the two groups recovered in the ADMIXTURE analysis. Tree is rooted at the midpoint with bootstrap support >0.75 listed above branches.

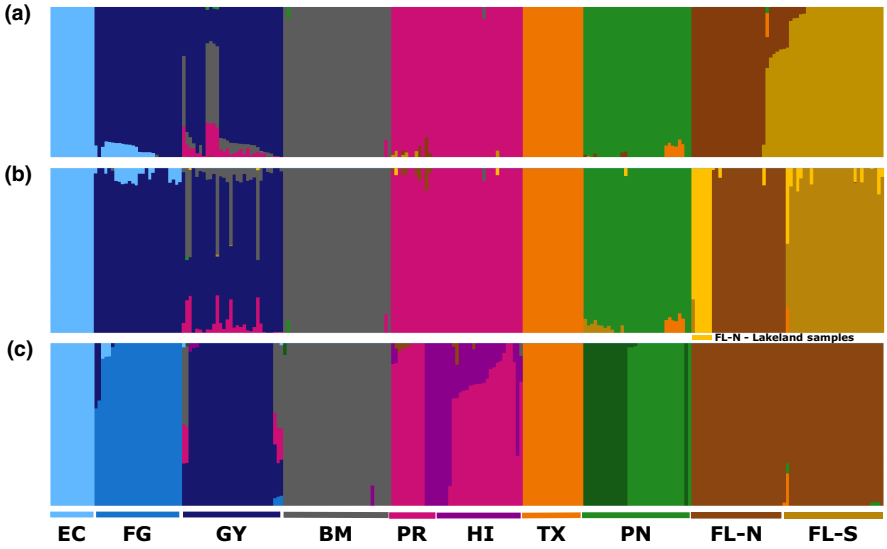


FIGURE 3 ADMIXTURE results for (a) K = 8, (b) K = 9, and (c) K = 10. Colours as in Figure 1.

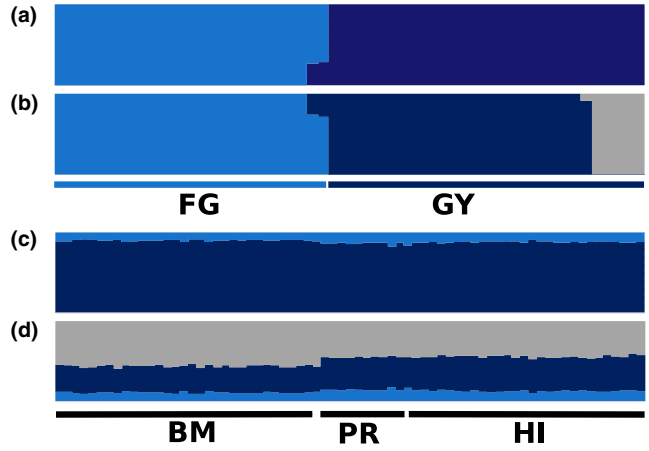


FIGURE 4 Supervised ADMIXTURE for French Guiana, Guyana, and Bermuda. Dark blue represents Guyana, light blue represents French Guiana, and grey represents Bermuda. (a) Reference panel with K = 2. (b) Population assignments for Bermuda, Hawai'i and Puerto Rico based on K = 2 reference panel. (c) Reference panel with K = 3. (d) Population assignments for Bermuda, Hawai'i and Puerto Rico based on K = 3 reference panel.

explained by the first two axes (Figure 5). The first axis separates the two major clades, the second resolves populations within each major clade. Introduced populations in the Caribbean cluster tightly with each other, and with Guyana and French Guiana (Figure 5b). Southern Florida and Northern Florida are also similar to each other, and distinct from both Panama and Texas along PC2.

## 3.4 | Population statistics

Levels of differentiation between Puerto Rico and Hawai'i are low, as is differentiation between Puerto Rico and Hawai'i and Guyana and French Guiana ($F_{ST}$ 0.03–0.10; Table 1). Northern and southern Florida also exhibit a relatively low level of differentiation ($F_{ST}$ =0.05), and together, show the least differentiation in comparison to Panama ($F_{ST}$ =0.13) than any other native population.

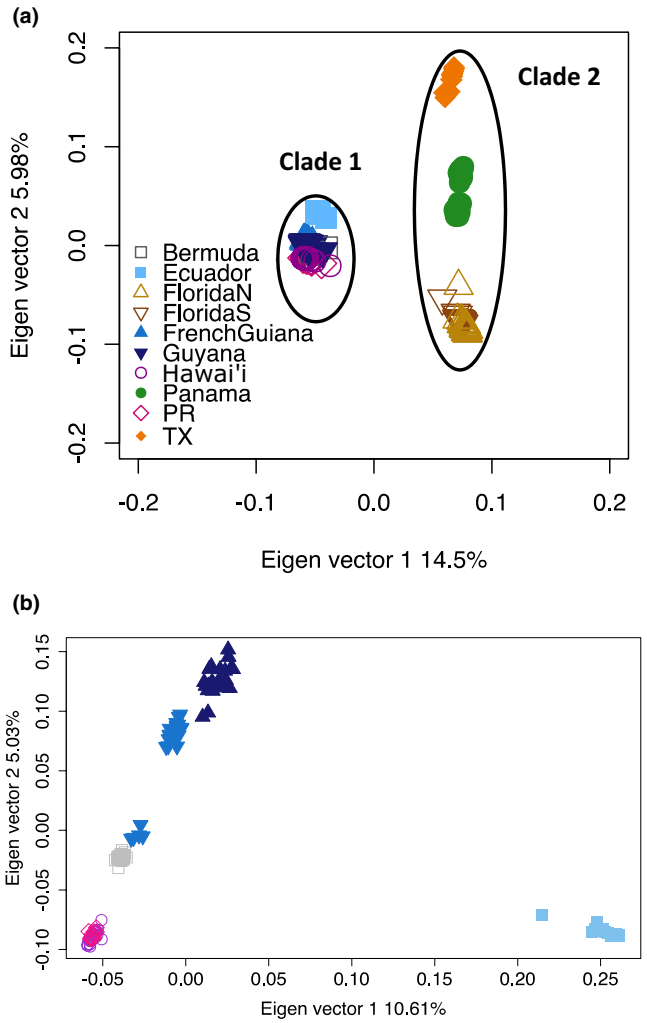


FIGURE 5 Principal component analysis. (a) all samples. (b) *Rhinella marina* (clade 1) only.

Number of private alleles, allelic richness, percent polymorphic loci, and Pi show that introduced populations are less genetically diverse than their closest native-range relatives (Table 2, Figure 6).

Most measures of genetic diversity were similar across the introduced populations (Table 2, Figure 6), except for the low number of private alleles in Puerto Rico (68), particularly in comparison to Hawai'i (575). This is probably driven primarily by differences in sample size (PR, $N = 10$; HI, $N = 29$), as the number of private alleles detected is strongly influenced by sample size (Allendorf et al., 2022). Further, allelic richness, which was corrected for sample size was similar between the populations (PR, Ar = 1.29; HI, Ar = 1.28), as was observed heterozygosity (Figure 6).

Both Texas and Ecuador (native populations), show less genetic diversity across some of these measurements than other native-range populations, and Texas had the lowest percentage of polymorphic loci of any population. Lastly, there is no clear pattern between introduced and source populations for observed heterozygosity or inbreeding, although inbreeding is quite low overall.

### 3.5 | Demography

Introduced populations founded by only a few individuals often exhibit signatures of genetic bottlenecks, a sharp drop in historical population size, which can persist over multiple generations. Bermuda and Hawai'i each show evidence of an extreme bottleneck, a relatively rapid and drastic reduction in effective population size (Figure 7). For Bermuda, the population size declined steeply between 200–400 years ago, with the lowest point reached around 200 years ago and persisting for several generations (indicated by low population size across several years). Hawai'i also shows evidence for a severe bottleneck at this time, followed by a rapid increase in population size. We find evidence of a less rapid decline in effective population size in northern Florida in comparison to Bermuda and Hawai'i. Further, the population did not grow rapidly post-bottleneck, and the contemporary effective size is still smaller than historical levels. Texas was the only native population showing a relatively recent increase in effective population size, occurring approximately 100 years ago.

We note that $N_e$ is sensitive to life history, sex ratio, generation time and mutation rate, and a number of these factors are not controlled for in our comparisons. Generation time is dependent on climate, sex ratio may fluctuate between populations, and the true mutation rate of bufonid toads is unknown, thus the estimated timing of historical demography events should be considered approximate.

### 3.6 | Selection

We detected significant outliers in all pairwise comparisons within the *Rhinella marina* lineage (Table 3). Of the 245 outliers, 27 occurred within protein coding regions, with 20 variants predicted to have a "low" or "moderate" effect on protein function. Of all $F_{ST}$ outliers 76% were annotated with known functions (Table S2).

We identified four SNPs that were outliers across all source versus introduced comparisons, 28 outliers shared across two such comparisons, and 150 in at least one comparison (see Table 4 for shared variants). We identified seven missense mutations (*spr*, *GPAM*, *Mpdul*, *RNF123*, *LLGL1*, and two unknown proteins, Table S2). Each of these mutations occurred in introduced populations, with four occurring in multiple comparisons (Table 4). All but one mutation (*Mpdul*) was observed in Hawai'i. We did not find evidence for overrepresentation of any gene ontology category within annotated variants (Table 5).

In comparisons between introduced populations, we identified five outliers in the Puerto Rico/Hawai'i comparison, of which three were shared with the Bermuda/Hawai'i comparison. We detected 90 in the Bermuda/Hawai'i comparison, and 75 in Bermuda/Puerto Rico, with 20 outliers shared between the two comparisons (see Table S2 for annotations).

All 12 outliers we identified in the comparison between southern and northern Florida were unique to that comparison. We did not find evidence for gene ontology enrichment; however, two of the eight annotated genes have been implicated in bone development disorders (*P4HA3*, *LGR4*) (The UniProt Consortium et al., 2021).

In Bermuda and Hawai'i, putatively adaptive loci were less genetically diverse than putatively neutral loci (Table 6). In contrast, outlier loci in Puerto Rico were on average more diverse than neutral loci. In all three introduced populations, genetic and haplotype diversity were lower than for the same loci in Guyana. Lastly, loci identified as putatively adaptive in one or more introduced population were more diverse in Guyana than they were in the corresponding introduced population (Table 6).

## 4 | DISCUSSION

Cane toads are an excellent model for studying evolution because multiple introductions yield repeated natural "experiments". We leveraged this system to investigate the role of introduction history and genetic variation on successful introduction with the following goals: (i) infer routes of introduction; (ii) evaluate evidence for the first condition of the "genetic paradox", specifically, signatures of population bottlenecks and decreased genetic variation in introduced populations; and (iii) investigate patterns of genetic variation across introductions at putatively adaptive and neutral loci. Population structure and introduction routes supported by our findings are summarized in Figure 8. We confirmed anecdotal shipping records and found that introduced populations in Florida probably came from a different source population, and thus different species (*R. horribilis)* than the other introduced populations in Bermuda, Puerto Rico, and Hawai'i (*R. marina*), although we were unable to identify the original source with our current sampling. We found reductions in genetic diversity associated with invasion in all cases, suggesting singular introductions or multiple introductions from genetically similar source populations to each studied locality. Lastly, we found many shared outliers across population comparisons, consistent with selection or genetic drift acting upon standing genetic variation, rather than de novo mutation.

TABLE 1 Pairwise $F_{ST}$

| Population | FG | GY | BM | PR | HI | TX | PN | FL-S | FL-N |
|---|---|---|---|---|---|---|---|---|---|
| Ecuador | 0.14 | 0.13 | 0.21 | 0.28 | 0.25 | 0.49 | 0.26 | 0.32 | 0.32 |
| French Guiana | | 0.04 | 0.09 | 0.09 | 0.1 | 0.24 | 0.17 | 0.19 | 0.19 |
| Guyana | | | 0.06 | 0.07 | 0.08 | 0.22 | 0.16 | 0.17 | 0.18 |
| Bermuda | | | | 0.1 | 0.11 | 0.34 | 0.21 | 0.24 | 0.24 |
| Puerto Rico | | | | | 0.04 | 0.46 | 0.23 | 0.28 | 0.29 |
| Hawai'i | | | | | | 0.4 | 0.24 | 0.28 | 0.28 |
| Texas | | | | | | | 0.18 | 0.28 | 0.29 |
| Panama | | | | | | | | 0.13 | 0.13 |
| Florida S. | | | | | | | | | 0.06 |

TABLE 2 Population statistics

| | EC | FG | GY | BM | PR | HI | TX | PN | FS | FN |
|---|---|---|---|---|---|---|---|---|---|---|
| N | 13 | 26 | 30 | 32 | 10 | 29 | 18 | 32 | 28 | 29 |
| Ar | 1.66 | 1.42 | 1.39 | 1.34 | 1.29 | 1.28 | 1.46 | 1.45 | 1.35 | 1.33 |
| Priv | 2776 | 5147 | 4810 | 891 | 68 | 575 | 1302 | 5548 | 1073 | 970 |
| % Poly | 0.38 | 1.10 | 1.19 | 0.61 | 0.42 | 0.52 | 0.15 | 0.75 | 0.50 | 0.51 |

Abbreviations: %Poly, percent of polymorphic loci; Ar, allelic richness; *N*, number of individuals; Priv, number of private alleles.

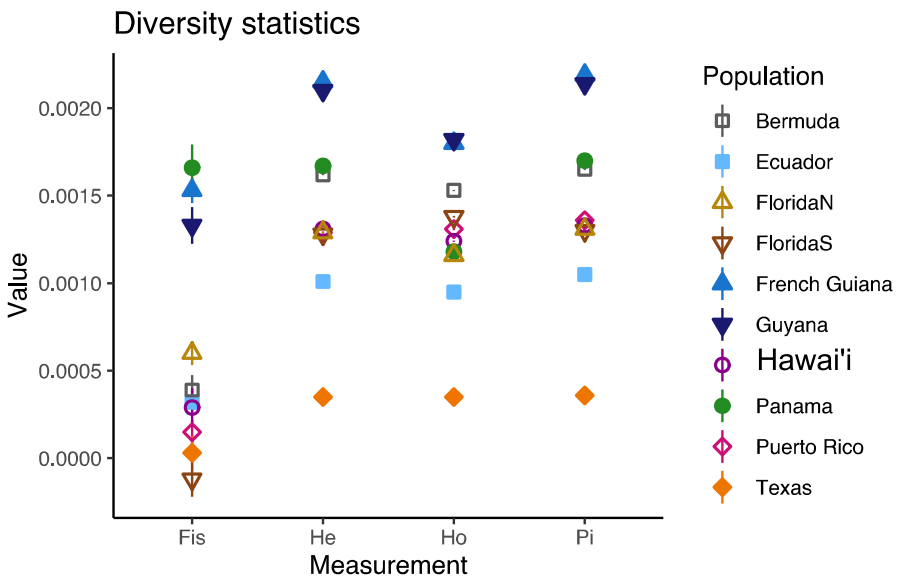


FIGURE 6 Population statistics. $F_{IS}$, inbreeding; $H_o$, observed heterozygosity; $H_e$, expected heterozygosity; pi, nucleotide diversity.

## 4.1 | Phylogenetics and introduction history

We found consistent support for two lineages of *Rhinella* in our sampling, namely *R. marina* and *R. horribilis*. Clade 1 contained native populations from South America, as well as introduced populations from Puerto Rico, Hawai'i, and Bermuda. The close relationship between these introduced populations and native populations from Guyana and French Guiana is consistent with introduction routes already established for Australia, namely that *R. marina* from the Guiana shield were introduced to Bermuda, Puerto Rico, and then Hawai'i (via Puerto Rico) (Lever, 2001) (Figure 8). Clade 2 contained native populations from Central (Panama) and North (Texas, USA) America, as well as introduced populations from southern and northern Florida, USA. Thus, the invasive toads found in Florida are more closely related to *R. horribilis* than to *R. marina*. Our analyses also recovered patterns of population bottlenecks in introduced populations that were not seen in the native populations. Although the precise mutation rate for *Rhinella* is unknown, our results nonetheless roughly correlate with known introduction dates (Lever, 2001).

We found no strong evidence for elevated genetic diversity from multiple introductions to any of our sampled populations: all introduced populations we sampled have lower allelic richness and nucleotide diversity than native populations, and we do not see excess heterozygosity in introduced individuals, as we might expect in admixed individuals. The introduction size to Florida seems to be larger than the reported 100 individuals, with $N_e$ estimates around 800.

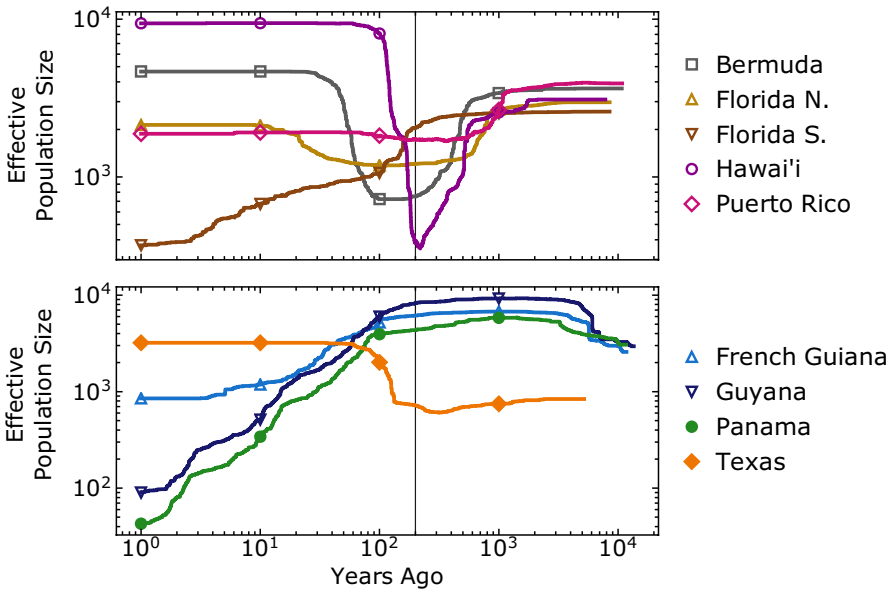


**FIGURE 7** Demographic analyses performed in stairway plot. Y-axis denotes effective population size x-axis denotes time (years ago.) the black vertical line in each plot marks 200 years ago.

**TABLE 3** $F_{ST}$ outlier summary

| | Total | Exon mis/syn | Intron | Up | Down | Intergenic | Effects low/mod/hi |
|---|---|---|---|---|---|---|---|
| Guyana × Bermuda | 29 | 1<br>0/1 | 19 | 0 | 2 | 11 | 1/0/0 |
| Guyana × Puerto Rico | 82 | 4<br>0/2 | 40 | 7 | 0 | 38 | 5/0/0 |
| Guyana × Hawai'i | 38 | 2<br>1/1 | 22 | 5 | 5 | 15 | 2/1/0 |
| Bermuda × Puerto Rico | 75 | 6<br>1/1 | 40 | 16 | 8 | 31 | 5/1/0 |
| Bermuda × Hawai'i | 91 | 11<br>7/2 | 49 | 13 | 11 | 32 | 4/7/0 |
| Hawai'i × Puerto Rico | 5 | 3<br>3/0 | 1 | 0 | 0 | 1 | 0/3/0 |
| Florida N. × Florida S. | 12 | 0<br>0/0 | 8 | 2 | 0 | 4 | 0/0/0 |

*Note*: "Total SNPs" indicates the total number of outliers detected in each comparison. "Exon" contains the number of SNPs located within exons, and mis/syn indicates the number of missense and synonymous SNPs, respectively. "Intron" indicates the number of SNPs located within intronic regions. "Up" and "down" indicate the number of SNPs found within 2 kb upstream or downstream of a gene. "Intergenic" SNPs are those found in intergenic regions, farther than 2 kb from a gene. Row totals are higher than the number of SNPs, as individual SNPs may fall under more than one category. "Effects" lists the predicted impact of mutations based on SNPeff, that is, a missense mutation would probably have a moderate effect on gene function.

Thus, the bottleneck associated with the Florida introduction was probably less extreme than previously reported anecdotally.

Introduced populations of *Rhinella* in Florida are more closely related to those from Panama than those from French Guiana or Guyana (Figure 2). However, the Florida populations were still fairly differentiated from Panama and Texas (Table 1). Thus, the sampled populations are either not the exact source populations, or the individuals introduced to Florida were not a representative sample of the genetic variation in these native populations. Genetic drift following introduction could increase $F_{ST}$ between the source and introduced populations; however, genetic diversity in Florida, while lower than Panama, is still relatively high compared to the other introductions. Thus, such high levels of differentiation between Florida and Panama do not appear to be fully attributable to drift. Of previously proposed introduction routes, our findings are most compatible with an introduction from Colombia (King & Krakauer, 1966). While we were unable to obtain samples from Colombia, previous mitochondrial work suggests that Panamanian and Venezuelan lineages bordering Colombia are distinct from Guyana and French Guiana (Acevedo et al., 2016). Given the reciprocal monophyly of southern and northern Florida populations, it is possible that they resulted from two introductions. Nonetheless, the low $F_{ST}$ between the two populations suggests that if they do represent separate introductions, the source localities were closely related.

Within Clade 1 (=*Rhinella marina*), our results indicate that introduced populations receive the majority of their ancestry from

**TABLE 4** Outliers detected in multiple source (Guyana) versus introduction comparisons

| Contig | Pos | BM | PR | HI | Type | Nearest gene | Annotation |
|---|---|---|---|---|---|---|---|
| 1349 | 119822 | X | X | X | intergenic | RMA_00009427 | LINE-1 (Homo sapiens) |
| 1427 | 349433 | X | X | X | intron | RMA_00001449 | NFAT5 (Homo sapiens) |
| 3167 | 475399 | X | X | X | intron | RMA_00000328 | Protein of unknown function |
| 3703 | 90260 | X | X | X | intron | RMA_00028737 | mat2b (Xenopus tropicalis) |
| 6295 | 265664 | X | X | | intron | RMA_00009562 | ncoa2 (Xenopus laevis) |
| 3984 | 58942 | X | X | | intron | RMA_00022098 | gata6-a (Xenopus laevis) |
| 13,068 | 50059 | | X | X | synonymous | RMA_00035866 | Trnt1 (Mus musculus) |
| 5303 | 168690 | | X | X | synonymous | RMA_00022418 | XKR5 (Homo sapiens) |
| 5303 | 168716 | | X | X | intron | RMA_00022418 | XKR5 (Homo sapiens) |
| 10,541 | 189809 | | X | X | intron | RMA_00010703 | Aftph (Mus musculus) |
| 10,644 | 263733 | | X | X | intron | RMA_00014659 | Chchd1 (Mus musculus) |
| 1157 | 178921 | | X | X | intron | RMA_00011384 | Kiaa1109 (Mus musculus) |
| 1308 | 594774 | | X | X | intron | RMA_00002901 | Tbck (Mus musculus) |
| 15,793 | 47685 | | X | X | downstream | RMA_00035638 | pol (Drosophila melanogaster) |
| 16,457 | 86794 | | X | X | downstream | RMA_00031877 | Protein of unknown function |
| 19,978 | 16118 | | X | X | intergenic | RMA_00030974 | Protein of unknown function |
| 23,472 | 111595 | | X | X | intergenic | N/A | N/A |
| 4849 | 38139 | | X | X | upstream | RMA_00022342 | OPA1 (Gallus gallus) |
| 608 | 223156 | | X | X | intron | RMA_00019530 | KIF27 (Homo sapiens) |
| 6170 | 25127 | | X | X | intron | RMA_00007220 | Lmf1 (Mus musculus) |
| 6757 | 6305 | | X | X | intergenic | RMA_00027538 | pou3f2-b (Xenopus laevis) |
| 7679 | 259490 | | X | X | intron | RMA_00005192 | PPARGC1B (Homo sapiens) |
| 7712 | 55520 | | X | X | intron | RMA_00023760 | Protein of unknown function |
| 8050 | 143616 | | X | X | intron | RMA_00022278 | KCNMB4 (Homo sapiens) |
| 938 | 724683 | | X | X | upstream | RMA_00002032 | PKIG (Bos taurus) |
| 343 | 185350 | | X | X | intron | RMA_00019958 | Cystatin (Pseudechis porphyriacus) |
| 3536 | 21595 | | X | X | downstream | RMA_00023497 | cuta (Xenopus laevis) |
| 3849 | 61419 | | X | X | intron | RMA_00016053 | NCK1 (Homo sapiens) |

*Note*: The contig number: "Contig" and basepair within the contig: "Pos" identify the genomic coordinates of each SNP. "Type" and "effect" as in Table 3. Nearest gene indicates the gene name assigned in the *Rhinella marina* reference genome. For intergenic SNPS, genes within 2 kb are listed.

Guyana (Figure 4). A recent study in French Guiana described an inland "rainforest" and a "coastal" morph of *Rhinella marina* (DeVore et al., 2021). In Guyana, our sampling was largely inland, with five coastal individuals from Georgetown, Guyana, while in French Guiana, our sampling was primarily coastal, with two inland individuals from Saul, French Guiana. DeVore et al. (2021) did not include genetic data for these two morphs; however, in our study, introduced populations appear to be more genetically similar to the coastal morph. Indeed, Guyanese samples from the coast were more closely associated with Bermuda in our phylogenetic analyses than with Guyanese samples collected further inland (Figure 1, Figure 2).

According to previous accounts, Bermuda represents a distinct introduction event from Puerto Rico and Hawai'i. Indeed, Bermuda shows different ancestry proportions than Puerto Rico and Hawai'i, and a higher average $F_{ST}$ (Table 1, ~0.10) with Puerto Rico and Hawai'i than Puerto Rico and Hawai'i have with each other (Table 1, 0.05). It is possible that the Puerto Rico and subsequent Hawaiian introductions were founded by a "rainforest" morph, while the source population in Bermuda was more similar to the "coastal" morph. However, since we only sampled two localities in Guyana, it is possible that the strong affinity between the coastal Guyana samples and the Bermuda samples is due to ascertainment bias. In this case, more complete sampling across the coast could better resolve the source localities for the Bermuda introduction.

Further, our analyses showed that Hawai'i, Puerto Rico and Bermuda derive approximately 10% of their ancestry from French Guiana. This may be due to incomplete lineage sorting between inland French Guiana and Guyana populations, a common pattern in amphibians, and within the *R. marina* and *R. horribilis* species complex in particular (Firneno et al., 2020; Rivera et al., 2021). This pattern could also result from admixture between French Guiana and Guyana at some point in the introduction history. According to

historical documents, toads from both Guyana and French Guiana were introduced to Barbados in the 1830s and 1840s. Toads from Barbados were then shipped to several other islands, including Puerto Rico, thus, admixture within Puerto Rico and Hawai'i may have occurred along this route (Lever, 2001).

Lastly, selection may have shaped population structure in a way not captured by ADMIXTURE. Bermuda is more temperate than Puerto Rico and Hawai'i, with more seasonal fluctuation in temperature and cooler winters. Bermuda also experiences more consistent rainfall throughout the year, whereas Puerto Rico and Hawai'i have wet and dry seasons. Selection analyses show many outliers in Bermuda that are not shared by Puerto Rico and Hawai'i. Thus, the differentiation may be due to selection in a different environment than what is experienced by populations in Puerto Rico or Hawai'i. Alternatively, this differentiation could be due to genetic drift in Bermuda, which differentiated the allele frequencies in relation to Puerto Rico and Hawai'i. Further study of the fitness consequences of $F_{ST}$ outliers in Bermuda in the Bermuda versus Puerto Rican environments would be needed to robustly test these alternatives.

We find that Ecuador diverges earlier than other populations in South America. Recent work in the native range of *Rhinella* suggests cryptic diversity in this area (Rivera et al., 2021), nonetheless, the Ecuadorian specimens are morphologically consistent with *Rhinella marina*. High differentiation between Guiana Shield populations, Ecuador, Panama, and Texas are suggestive of distinct lineages. Both *Rhinella marina* and *R. horribilis* are described from our sampling range, with Panamanian and Texan toads formally assigned to *R. horribilis* (Acevedo et al., 2016; Figures 1 and 8). However, reciprocal monophyly between the clades and high $F_{ST}$ values suggest than they may be part of a larger species complex, as has been postulated elsewhere (Acevedo et al., 2016; Bessa-Silva, 2020; Rivera et al., 2021; Vallinoto et al., 2010). Although beyond the scope of our current sampling and study, further investigation into the native range of the *R. marina* complex may uncover additional species, or at least highly divergent lineages.

Within Clade 1, our findings correspond with previous studies and historical records: Bermuda, Puerto Rico, and Hawai'i are closely related to populations from the Guiana Shield, and Puerto Rico and Hawai'i are nearly identical to each other, consistent with an introduction from Puerto Rico to Hawai'i. In contrast, the Florida introduction appears to derive from a distinct lineage, possibly in Colombia or Panama. Thus, drawing comparisons between Florida and well-studied introductions in Hawai'i, Puerto Rico and Australia (which originates from Puerto Rico), must be done with caution, as Florida does not appear to be a parallel introduction from the same lineage/species as other *Rhinella* introductions.

TABLE 5 GO categories of annotated genes

| Process | % all | % outliers |
|---|---|---|
| Developmental process (GO:0032502) | 4.42 | 10.53 |
| Multicellular organismal process (GO:0032501) | 3.92 | 10.53 |
| Cellular process (GO:0009987) | 31.11 | 73.68 |
| Reproduction (GO:0000003) | 0.40 | 5.26 |
| Localization (GO:0051179) | 7.47 | 10.53 |
| Reproductive process (GO:0022414) | 0.40 | 5.26 |
| Multiorganism process (GO:0051704) | 0.03 | 0.00 |
| Biological adhesion (GO:0022610) | 1.21 | 10.53 |
| Immune system process (GO:0002376) | 0.59 | 0.00 |
| Biological regulation (GO:0065007) | 16.51 | 47.37 |
| Growth (GO:0040007) | 0.09 | 0.00 |
| Signalling (GO:0023052) | 5.89 | 5.26 |
| Metabolic process (GO:0008152) | 18.90 | 57.89 |
| interspecies interaction between organisms (GO:0044419) | 0.19 | 0.00 |
| Pigmentation (GO:0043473) | 0.06 | 0.00 |
| Response to stimulus (GO:0050896) | 7.23 | 10.53 |
| Biological phase (GO:0044848) | 0.16 | 0.00 |
| Rhythmic process (GO:0048511) | 0.16 | 0.00 |
| Locomotion (GO:0040011) | 1.25 | 5.26 |

*Note*: The "all" column indicates the percentage of all sequenced SNPs associated with each biological process. The "outliers" column indicates the percentage of outlier SNPs associated with each process. Note that a gene can be annotated with more than one biological process, so totals are greater than 100%.

## 4.2 | Recent population declines, and native range demography

While both Bermuda and Hawai'i exhibit rapid decreases in population size followed by rapid population increases, most native range

TABLE 6 Genetic diversity (g) and haplotype diversity (h) at putatively adaptive versus putatively neutral loci in comparisons between source (Guyana) and introduced populations of *R. marina*

| | Introduced | | Native source (Guyana) | |
|---|---|---|---|---|
| Population | Adaptive (g/h) | Neutral (g/h) | Adaptive (g/h) | Neutral (g/h) |
| Bermuda | 0.136/0.155 | 0.169/0.201 | 0.359/0.431 | 0.112/0.135 |
| Hawai'i | 0.084/0.09 | 0.136/0.162 | 0.277/0.312 | |
| Puerto Rico | 0.154/0.171 | 0.110/0.130 | 0.259/0.294 | |

*Note*: Adaptive loci in the "source (Guyana)" column are the same loci in the adaptive column for "introduced" populations to allow comparisons between the same loci in the introduced versus source populations.

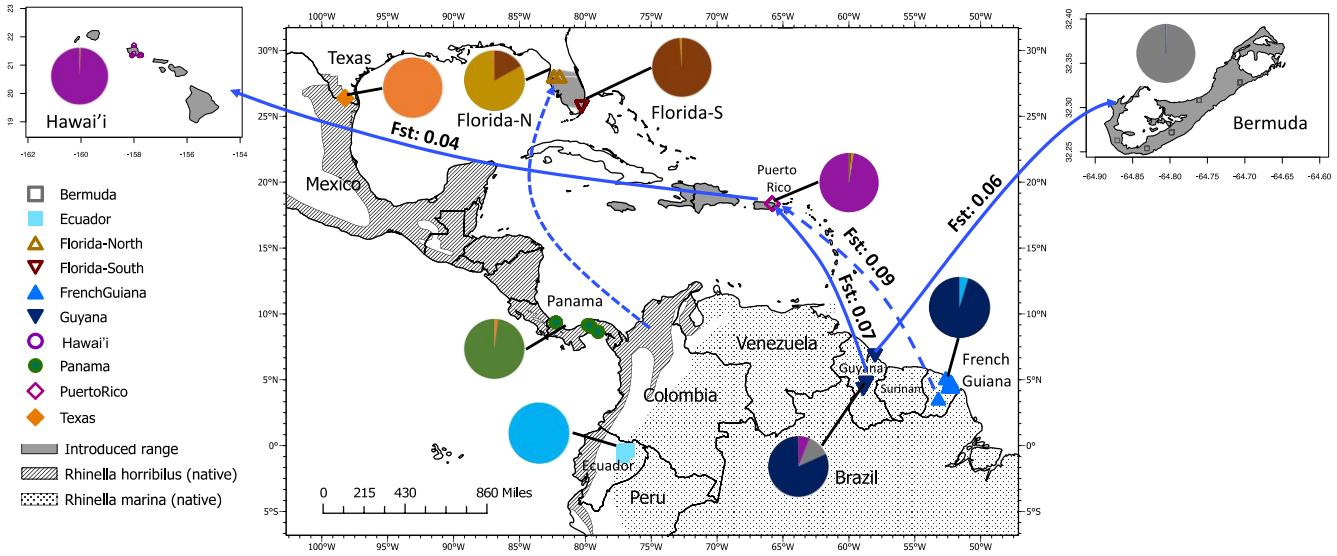


FIGURE 8 Summary of findings showing introduction routes consistent with analyses. Circles show ADMIXTURE results for K = 8, arrows indicate introduction routes, numbers above arrows indicate $F_{ST}$ between connected populations. Dotted lines indicate uncertainty in exact source population.

populations, as well as southern Florida exhibited a slow decrease in population size over the past 200 years. The steady decrease in the southern Florida toads is consistent with anecdotal reports of "marked declines" in Miami-Dade county reported in the 1980s, although the cause of this decline is unknown (Lever, 2001). Population decreases in the native range are consistent with global reports of amphibian declines associated with habitat loss, disease, and climate change (Campbell Grant et al., 2020; Lips et al., 2005). In contrast, the population in Texas shows a modest increase in population size. Texas is the northern range limit of native *Rhinella horribilis*, and anecdotal records have suggested that this population is expanding at this range edge (Guadiana et al., 2020; Mayra Overyides, personal communication).

## 4.3 | Maintenance of genetic variation during introduction

Cane toads are an example of the "genetic paradox", in which we see many examples of rapid adaptation despite records of small founding populations (Mittan & Zamudio, 2019; Rollins et al., 2015). Studies of successful introduced species have sometimes found evidence of cryptic introductions, resulting in increased genetic diversity upon which selection could act (Acevedo-Limón et al., 2020; Kolbe et al., 2004; Sheldon et al., 2018). We did not recover evidence of increased genetic diversity in introduced ranges due to admixture between distinct source localities. Nonetheless, we found only modest decreases in genetic diversity in introduced populations (Table 2, Figure 6).

Some studies find that high mutation rates may explain rapid adaptation in introduced species (Cobben et al., 2017). A recent study of *Rhinella marina* in Australia found surprisingly high levels of adaptive genetic diversity despite small introduction sizes, and one proposed explanation was an unusually high mutation rate in *R. marina* (Selechnik, 2019). Two of the four outliers shared across all three native range to introduced range populations are involved with genome stability and epigenetic modifications (*LINE-1*, *mat2b*, Table 4) (Yuan et al., 2019; Zhang et al., 2020). In addition, one of the two outliers shared by Bermuda and Puerto Rico in comparison with their native source was *ncoa2*, a coactivator also involved in histone modification, and the regulation of gene expression, particularly in early development (Tanizaki et al., 2021). The possibility that retrotransposon activity increases mutation rate during introduction has been observed in both birds and introduced ants (Cobben et al., 2017; Schrader et al., 2014). However, while increased mutation rates could potentiate adaptive changes, our demographic analyses using a standard mutation rate for amphibians align well with historical records of *R. marina* species complex introductions to the Caribbean and thus do not suggest an unusually high mutation rate amongst our sampled lineages. Further, putatively adaptive loci are not more genetically diverse than putatively neutral loci within introduced populations (Table 6). Loci identified as outliers in introduced populations were on average more diverse in the native range population (Guyana), suggesting that putative selection in introduced populations is occurring in parts of the genome with high standing genetic variation in the native range.

Alternatively, life history strategy can contribute to maintenance of genetic variation. The fate of genetic variation following a founding event is largely influenced by the number of individuals that survive and contribute to the gene pool following introduction (Simberloff, 2009). Meta-analyses showed that propagule pressure (the number of individuals introduced to a new location) was a key factor in establishment success (Cassey et al., 2018). Reproductive strategy, in particular, has been found to contribute to establishment

success in mammals (Capellini et al., 2015), as well as in reptiles and amphibians (Allen et al., 2017). In line with these predictions, cane toads are r-strategy reproducers, producing many offspring, as many as 30,000 eggs per clutch, with little maternal investment (Lever, 2001; Meshaka Jr., 2011). The introduction in southern Florida does exhibit a decrease in population size around the time of introduction (Figure 7), nonetheless, this population size is around 800 individuals, which is higher than anecdotal reports suggesting an accidental release of just 100 individuals (Easteal, 1981). Although Hawai'i's population bottleneck was severe (Figure 7), the population size rebounded quickly which may have prevented further loss of genetic diversity, and provided more opportunity for de novo mutations (reflected in the number of private alleles, Table 2) as the population size increased. Similarly, Bermuda shows a rapid rebound in population size following a severe population bottleneck. Thus, exponential population growth following expansion is one potential mechanism by which introduced species may both maintain, and potentially increase genetic diversity (Nei et al., 1975).

In addition to reproductive strategy, cane toads possess other life-history traits that may predispose them to establishment success. Records of intentional cane toad introductions record high survival rates (Lever, 2001). In fact, compared to other amphibians, toads (Bufonidae) may be particularly suited to range expansions. A phylogenetic analysis of Bufonidae found seven life-history characteristics associated with large range sizes and dispersal amongst toads: terrestrial adults, large body size, parotoid glands, presence of fat bodies, aquatic oviposition sites, large clutch sizes, and larvae that did not depend upon maternal food supplementation (Van Bocxlaer et al., 2010). While these characteristics are not present in all Bufonidae, they are true of *R. marina* and *R. horribilis*. We also detected several outliers involved with reproductive processes, discussed below. Thus, over both long and short evolutionary timescales, life history appears to play a role in establishment success and range expansion of Bufonidae.

Maintenance of genetic variation during establishment and range expansion may allow introduced species to adapt to novel conditions (Briski et al., 2018). In combination with only modest decreases in genetic variation, we also found evidence of selection in all introduced populations. Amongst putatively selective variants, we found that nearly 20% of outliers in native versus introduced populations were found in more than one comparison. This may be due to pre-existing differences prior to introductions or parallel selection across independent invasions. We did not detect fixed differences between Guyana and Bermuda or between Guyana and Hawai'i, and only one fixed difference between Guyana and Puerto Rico; however, Bermuda and Puerto Rico and Bermuda and Hawai'i showed nine and three fixed differences, respectively. This suggests selection for different alleles or drift acting upon standing variation present in Guyana, suggesting that adaption could be largely due to existing variation in the native range. Further, outlier loci in introduced populations also tended to be more genetically diverse that neutral loci within the source population, providing further support for the role of standing genetic diversity in selection in introduced ranges (Table 6).

Amongst annotated outliers, we found several interesting candidates for future study. In particular, we found outliers associated with reproduction, metabolism, and locomotion (Table 5). Evolution of reproductive traits are consistent with the role of life history and propagule pressure on invasion success, and the evolution of locomotor traits have also played a role in the cane toad expansion in Australia (Hudson et al., 2020; Kosmala et al., 2017; Shine et al., 2011). We also found missense mutations within genes associated with metabolic activity (*spr*, *GPAM*, *Mpdul*, *RNF123*, *LLGL1*). As both energy storage and adaptation to novel temperature regimens are implicated in physiological and transcriptomic studies of the range expansion in Australian toads (McCann et al., 2014; Rollins et al., 2015; Selechnik, 2019), and physiological studies in Floridian toads (Gardner et al., 2020; Mittan & Zamudio, 2019), the role of metabolic adaptation may also be important to ultimate establishment success. Future studies investigating the fitness effects of these variants could elucidate their potential role in adaptation to these environments.

Within the Florida introduction, we previously documented evidence of incipient local adaptation to cold in the north versus south of Florida (Mittan & Zamudio, 2019). We cannot identify the underlying genetic architecture of cold-tolerance with genomic data alone. Nonetheless, the lack of fixed differences and low $F_{ST}$ values between the two Florida populations suggests that adaptation is due to plasticity and/or selection on shared variation, rather than preadapted genetic variants from an environmentally distinct source population in northern Florida.

Our findings emphasize the importance of genomic analyses in determining introduction history. The toads introduced to Florida represent a distinct lineage, probably belonging to *Rhinella horribilis*, thus a distinct species from that in other *Rhinella* introductions. This phylogenetic information will be instrumental in testing new hypotheses about the impact of introduction history on adaptation to novel environments. Furthermore, it presents an opportunity to study *Rhinella* introductions not only as natural experiments replicated within a species, but also replicated across lineages. Already, our results suggest that maintenance of high levels of genetic diversity following introduction may potentiate selection for alternative alleles across introductions, and we expect this study system to continue yielding insight into adaptation to novel environments.

## AUTHOR CONTRIBUTIONS

Cinnamon S. Mittan-Moreau, Kelly R. Zamudio and Luís Felipe Toledo conceived the study. Cinnamon S. Mittan-Moreau, Crystal Kelehear, Jamie Bacon, Juan M. Guayasamin and Andrew Snyder contributed samples and performed laboratory work. Cinnamon S. Mittan-Moreau analysed the data. Cinnamon S. Mittan-Moreau led the writing of the manuscript, and all authors contributed to editing and finalizing the study.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Raw sequence data and metadata will be made available on the Sequence Read Archive at NCBI (accession PRJNA880569) upon publication.

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## SUPPORTING INFORMATION

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