

# Rapid introgression of invasive alleles following hybridization between a native *Anolis* lizard species and a cryptic invader across an urban landscape

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## Abstract

Invasive species can impact native populations through competition, predation, and habitat alteration, but also genetically through hybridization. Potential outcomes of hybridization span the continuum from extinction to hybrid speciation and can be further complicated by anthropogenic habitat disturbance. Hybridization between the native green anole lizard (*Anolis carolinensis*) and a morphologically similar invader (*A. porcatum*) in south Florida provides an ideal opportunity to study interspecific admixture across a heterogeneous landscape of urban and forested habitats. We used reduced-representation sequencing to describe introgression in this hybrid system and to test for a relationship between urbanization and invasive ancestry. Our findings indicate that hybridization between green anole species was likely a limited, historic event, and that patterns of backcrossing have produced two distinct genetic clusters within the hybrid population. Genomic cline analyses revealed rapid introgression and disproportionate representation of invasive alleles at many loci, and no evidence for reproductive isolation between the two species. We also found a positive relationship between urbanization and invasive ancestry, although the mechanism driving this association remains unclear. Ultimately, our findings demonstrate the persistence of non-native genetic material even in the absence of ongoing immigration, indicating that selection favoring invasive alleles can override the demographic limitation of low propagule pressure. However, we also note that not all outcomes of admixture between native and invasive species should be considered intrinsically negative. Hybridization with ecologically robust invaders can lead to adaptive introgression, which may facilitate the long-term survival of native populations otherwise unable to adapt to anthropogenically mediated global change.

## INTRODUCTION

Invasive species are widely known for their negative ecological impacts on native organisms, which often involve predation, competition, and habitat alteration (Case & Bolger, 1991; Gibbons et al., 2000; Gordon, 1998; Kraus, 2015). It is also well-known that invaders can interact with native species genetically through hybridization (e.g., Ellstrand & Schierenbeck, 2000; Fitzpatrick and Shaffer, 2007a; Huxel, 1999; Vuillaume et al., 2015), with numerous examples documented in birds, mammals, fishes, and plants (Largiadere, 2008). Such events can threaten the genetic integrity of native populations and in some cases may even result in the extinction of rare native species (Rhymer & Simberloff, 1996; Riley et al., 2003; Todesco et al., 2016). The dangers of genetic swamping and loss of native genotypes are exacerbated by invasion scenarios in which the invader is morphologically indistinguishable from its native congener (Morais & Reichard, 2018; Riley et al., 2003; Wegener et al., 2019; Wielstra et al., 2016). These cryptic invaders cannot be visually identified, and as a result, can spread and impact native species while remaining undetected.

Possible outcomes of interspecific hybridization vary on a situational basis. When hybridizing species are parapatric, a stable tension zone may form and persist (Barton & Hewitt, 1989; Gay et al., 2008; Pinto et al.,

2019). Though the majority of hybridization research has been conducted in tension zone systems (Gompert et al., 2017), prolonged admixture may also lead to adaptive introgression (Hedrick, 2013; Whitney et al., 2010), the formation of a new, hybrid species (Rieseberg, 1997; Salazar et al., 2010; Schumer et al., 2014), or the extinction of one parental species via genetic swamping (Todesco et al., 2016; Wolf et al., 2001). These less commonly studied outcomes may be more likely in invasion scenarios, since invaders usually spread from a point of introduction within the novel range, rather than from a range boundary. The outcome ultimately realized is determined by a suite of ecological and genomic factors not yet fully understood, but among which spatial and environmental characteristics, relative population sizes, and propagule pressure are thought to be included (Bennet et al., 2010; Gompert et al., 2017).

Hybridization during biological invasions can be further complicated when occurring across landscapes disrupted by anthropogenic activity (Grabenstein & Taylor, 2018; Riley et al., 2003). For example, Riley et al. (2003) found that hybridization between a native tiger salamander and a cryptic invader occurred at much higher levels in artificial water bodies than in natural breeding pools. These differences were better explained by non-random patterns of mating and survival than by the relative proportions of hybrid individuals in each pool, suggesting that invaders may be more successful and hybridize more frequently in habitats which have been disturbed from their natural state. For organisms that live and breed in habitats occupied by humans, urbanization can be one such major source of disturbance.

Urban habitats are characterized by major environmental alterations relative to undisturbed habitats, including reduced canopy cover and an increase in impervious surface area. Together, these features facilitate rapid surface heating and lead to diurnal temperatures up to 10°C warmer than those observed in corresponding natural habitats (Kim, 1992). This “urban heat island” effect requires organisms living in cities to adapt to warmer conditions, and is especially critical for ectothermic species, which rely on external conditions and thermoregulatory behaviors to maintain an operational body temperature (Ackley et al., 2015; Battles & Kolbe, 2019; Hall & Warner, 2018). Urban heat island conditions have also been linked to an increase in pollutant concentration (Sarrat et al., 2006), as well as changes in patterns of wind, humidity, and precipitation (Taha, 1997). Since anthropogenic habitat disturbance has been shown to play a role in reducing reproductive barriers and promoting hybridization between both naturally co-occurring species (Grabenstein & Taylor, 2018) and native-invasive species pairs (Beninde et al., 2018; Riley et al., 2003; Walters et al., 2008), it is essential that studies of hybrid populations in urban areas consider the possible implications of habitat heterogeneity.

An ideal opportunity to investigate outcomes of hybridization between native and invasive species in an urban setting is provided by *Anolis* lizards in south Florida. Here, invasive Cuban green anoles (*Anolis porcatius*) have hybridized with native green anoles (*Anolis carolinensis*) across a patchwork of urban and remnant natural forest habitats, resulting in a population of hybrid origin with a genetic makeup distinct from that of either parent species (Wegener et al., 2019). While the hybrid population is thought to have originated in the city of South Miami, the limits of its distribution remain unknown. This is because the parental species are not morphologically distinct (Camposano, 2011; Wegener et al., 2019) and thus hybrids cannot be easily identified.

Aside from its occurrence across a mosaic of habitat types, this hybridization event is notable for two other reasons. First, while a few previous instances of hybridization among anole species have been recorded (Gabot-Rodriguez et al., 2020; Gorman & Atkins, 1968; Jenssen, 1977; Jezkova et al., 2013; Kohler et al., 2010; MacGuigan et al., 2017), such events are rare within the nearly 400-species *Anolis* clade (Losos, 2009). Second, the genetically distinct nature of the hybrid population is indicative of historic admixture followed by in situ population differentiation (Wegener et al., 2019). There is no known contemporary route of introduction through which additional *A. porcatius* could continue to arrive in Florida, and the founding propagule is thought to have been relatively small (Wegener et al., 2019). Therefore, this hybrid population presents an ideal opportunity to study the effect of unusually low propagule pressure on hybridization outcomes and to assess the consistency of introgression across a heterogenous landscape.

In accounting for the role that anthropogenic habitat disturbance may play in facilitating hybridization

between green anoles in South Miami, it is necessary to consider how the habitat associated with the niche of the hybridizing species differs between urban and natural forest settings. From a lizard’s perspective, urban habitats are characterized by smoother substrates, greater amounts of impervious surface area, sparser canopy cover, and higher temperatures relative to corresponding natural habitats (Battles & Kolbe, 2019; Kolbe et al., 2016; Winchell et al., 2016). Among these factors, the relative increase in average temperature suggests a mechanism by which urban habitats could promote increased hybridization. Studies of anoles and other ectotherms have found that thermal tolerance limits are correlated with the natural thermal conditions existing across a species’ geographic range (Grigg & Buckley, 2013; Gunderson et al., 2018; Hertz et al., 1979; Sunday et al., 2010). If tropical *A. porcatius* is better adapted to the warmer temperatures that dominate urban settings than *A. carolinensis*, invasive individuals and hybrids may outcompete pure *A. carolinensis*—but only in the warmer urban habitats where non-native genotypes are adaptive. An ability to withstand higher temperatures may become increasingly advantageous as current climate change and urbanization trends contribute to an increase in environmental temperatures (Battles & Kolbe, 2019; Frishkoff et al., 2019; Huey et al., 2009) and could favor the persistence of hybrid genotypes in a region with little to no ongoing *A. porcatius* immigration.

We capitalize on the unique nature of the *A. porcatius* x *A. carolinensis* hybrid system to understand the interplay between urbanization and invasion-mediated hybridization. Using genome-wide SNP data from strategically sampled hybrid individuals and parental species, we combine population genetics, genomic cline analyses, and environmental association analyses to fill existing knowledge gaps regarding the influence of both demographic and environmental factors on hybridization outcomes. Our analyses aim to shed light on the dynamics of genetic swamping resulting from a cryptic invasion, on the relationship between urbanization and invasion success, and on the erosion of reproductive barriers by anthropogenic habitat disturbance.

## MATERIALS AND METHODS

### Study species

*Anolis carolinensis* is an arboreal (trunk-crown) lizard found throughout much of the southeastern USA, and is the only *Anolis* species native to the United States (Campbell-Staton et al., 2012; Losos, 2009). It is a popular model organism for which an abundance of genetic and natural history data are available (Losos & Schneider, 2009) but belongs to a taxonomic group currently underrepresented in studies of hybridization (Largiadere, 2008). *Anolis porcatius* is invasive in Florida and is morphologically indistinct from *A. carolinensis* (Camposano, 2011; Kolbe et al., 2007; Wegener et al., 2019). Although *A. porcatius* is native to Cuba and the two species have had allopatric distributions since before the Pliocene (the split between the two lineages is thought to have occurred at least 6 mya; Campbell-Staton et al., 2012), they are known to hybridize. Hybrid green anoles have been identified in the South Miami area, which is thought to be the original location of the *A. porcatius* invasion (Wegener et al., 2019).

The Cuban green anole was first observed in south Florida in 1987 (Kraus, 2009; Meshaka et al., 1997), although the mechanism by which founding individuals arrived remains uncertain. Kraus (2009) reports that *A. porcatius* arrived as a cargo stowaway—this explanation is very plausible but entirely speculative. Release or escape of individuals transported intentionally for commercial pet trade is also a common introduction pathway for lizards (Romagosa et al., 2009) but is less likely in this scenario since neither the CITES Trade Database nor the U.S. Fish and Wildlife Service’s Law Enforcement Management Information System contain any record of *A. porcatius* import or export.

### Sample Selection and Processing

Samples of muscle and liver tissue were obtained from the Kolbe Lab tissue library at the University of Rhode Island and were strategically selected to construct a dataset with balanced sex ratios, diverse temporal sampling, and hybrid representatives from a variety of locations near South Miami, FL. In addition to 63 hybrid samples, 15 *A. porcatius* from Western Cuba and two green anoles of unknown ancestry from Parkland, FL were also selected. Tissue samples from 14 *A. carolinensis* from Hobe Sound, FL—a location 185 kilometers north of the known, hybrid population in South Miami—were obtained from the cryogenic

collection of the Harvard Museum of Comparative Zoology. Genomic DNA was extracted from all 94 samples using a commercially available Bioline DNA extraction kit and extract concentration and purity were assessed via nanodrop. The DNA samples were then sent to Admera Health (NJ, USA) for double digest restriction-site associated DNA (ddRAD) sequencing. Libraries were prepared using the restriction enzymes *SphI* and *EcoRI*. Following a size selection step that retained fragments of 450 - 650 bp, the libraries underwent 150-bp paired-end sequencing on the Illumina HiSeq 4000 platform. These data were supplemented with ddRAD data for 14 additional individuals (including five replicate individuals sequenced in both groups). Libraries for these 14 supplemental samples were constructed using the same restriction enzymes as all other samples, but underwent a slightly different size selection procedure retaining fragments of 550 - 710 bp (Bock et al., 2021).

### Data Trimming and Filtering

A quality control check for all raw sequence data was conducted using FastQC v0.11.8 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were then trimmed using Trimmomatic v0.39 (Bolger et al., 2014) to exclude Illumina-specific sequences and to remove bases below a quality score of 20 from the start and end of each read, cut any windows (width = 3 bp) within which the average quality score fell below 15, and remove individual reads with a total length of less than 36 bp. Trimmed reads were mapped against the *Anolis carolinensis* reference genome (Alfoldi et al., 2011) using dDocent v2.7.8 (Puritz et al., 2014) with a mismatch penalty of 4 and a gap opening penalty of 6. SNPs were then called from this assembly and filtered in dDocent under both 70% and 95% call rates to create versions of the dataset suitable for population (70% call rate) and individual-based (95% call rate) analyses. For both call rates, only biallelic markers were retained, complex variants (non-SNPs) were excluded, markers with mapping quality scores less than or equal to 20 were removed, and genotypes with fewer than 4 reads were marked as missing data. Additional filters to exclude markers with minor allele frequencies less than or equal to 3% and to remove individual samples with more than 30% missing data were applied prior to downstream analyses as appropriate. To reduce potential effects of linkage disequilibrium (LD), loci were also pruned using the `snp_autoSVD` function of the R package `bigsnpr` (Prive, 2018) prior to use in genetic clustering analyses.

To check for anomalies indicative of sample contamination, we used a custom script and `vcflib` (Garrison et al., 2021) to calculate allele balance ratios (O’Leary et al., 2018) for all heterozygote calls supported by 15 or more reads within the fully filtered, 70% call rate dataset. Any samples for which more than 20% of calls displayed ratios less than 0.2 or greater than 0.8 were removed. An identity by state (IBS) analysis was then performed in R v3.5.1 (R Core Team, 2018) using `SNPRelate` (Zheng et al., 2012) to check for library effects. Specifically, we compared pairwise IBS values of replicate samples sequenced in both ddRAD sequencing groups to pairwise values for all other sample combinations, after which the version of each pair with the lower number of reads was removed.

### Individual-based Analyses

To investigate admixture at the individual level, a subset of 10,000 SNPs was selected randomly from the fully-filtered, LD-pruned, 95% call rate dataset. Principal components and their corresponding eigenvalues were calculated using the R package `adegenet` v2.1.1 (Jombart & Ahmed, 2011) and visualized using `ggplot2` (Wickham, 2016). Discriminant analysis of principal components (DAPC) was also performed using `adegenet`, which employs the  $k$ -means algorithm to identify the number of genetic clusters for which BIC is minimized and between-group variation is maximized.

Individual admixture proportions were estimated using the admixture ancestry model of `STRUCTURE` v2.3.4 (Pritchard et al., 2000) with a maximum of 8 possible genetic groups ( $K = 8$ ). Twenty independent runs were performed for each value of  $K = 1-8$ . Each `STRUCTURE` simulation was run for a total of 150,000 Markov Chain Monte Carlo (MCMC) steps preceded by a burn-in of equal length. The most likely value of  $K$  was inferred via the Evanno method (Evanno et al., 2005) and the associated individual admixture proportions were plotted using the R package `pophelper` v2.3.1 (Francis, 2017).

Ancestry informative markers (AIMs) displaying fixed differences between *A. carolinensis* and *A. porcatius*

were identified using a custom script in conjunction with BEDTools v2.27.1 (Quinlan & Hall, 2010). AIM genotypes for each individual were then converted to allele counts and used to calculate hybrid index values with the R package *introgress* v1.2.3 (Gompert & Buerkle, 2010). These values were visualized in a triangle plot displaying individual hybrid class membership as a function of the relationship between hybrid index and interspecific heterozygosity. We also used *introgress* to visualize the ancestry of each individual at each AIM.

## Population-based Analyses

To describe the extent of genetic differentiation between the hybrid anoles and parental species, we used *vcftools* (Danecek et al., 2011) to calculate weighted  $F_{ST}$  in non-overlapping windows (width = 50,000 bp) across the genome. We considered all pairwise combinations of hybrid, *A. carolinensis*, and *A. porcutus* sample groups. Prior to this analysis, missing data in the 70% call rate dataset (not filtered for percent missing data per individual) were imputed with BEAGLE v5.2 (Browning et al., 2018).

Patterns of introgression at individual loci were examined via Bayesian estimation of genomic clines using *bgc* v1.03 (Gompert & Buerkle, 2012). For this analysis, only AIMs were tested and any AIMs missing allele depth values (an effect of the decomposition of multiple nucleotide polymorphisms during the AIM identification process) were excluded from the dataset. The genomic cline model was run for 100,000 MCMC steps (the first 50,000 of which were discarded as burn-in) and included both a genotype-uncertainty component with a sequence error probability of 0.001 and an ICARrho component accounting for linked loci with a maximum free recombination distance of 0.5. Loci categorized as alpha and/or beta outliers were identified and plotted using the R package *ClineHelpR* v0.0.0.9000 (Martin & Chafin, 2021) and mapped to an ideogram of the *A. carolinensis* reference genome using a custom script.

## Environmental Association Analysis

To investigate the relationship between anthropogenic habitat disturbance and the genotypes of hybrid green anoles, canopy cover and impervious surface area were selected as quantitative proxies of urbanization. These variables are commonly used as metrics of urbanization (Borden et al., 2022), are expected to be negatively correlated (i.e., canopy cover will be low and impervious surface area will be high in distinctly urban habitats), and are related to environmental temperatures relevant for lizards (Ackley et al., 2015; Battles & Kolbe, 2019; Kolbe et al., 2016). Using GPS coordinates for the site of capture of each hybrid individual, values for both variables were extracted from the 2016 US Forest Service Tree Canopy Cover and 2016 Percent Developed Imperviousness GIS layers produced by the National Land Cover Database (Yang et al., 2018). These layers provide habitat data at a spatial resolution of 30 x 30 m, which should be interpreted as representing the average condition across the home range of an individual anole rather than the specific microhabitat in which that individual was observed. Arboreal anoles (*A. carolinensis* included) have been found to utilize larger quantities of two-dimensional space than their more terrestrial counterparts (Schoener & Schoener, 1982), with a recent study of *A. carolinensis* habitat use in an urban setting (Weber et al., 2021) reporting individual home ranges as varying from a minimum of 16 m<sup>2</sup> to a maximum of 1,538 m<sup>2</sup> in size (average = 260 m<sup>2</sup> for males and 410 m<sup>2</sup> for females).

Associations between the selected environmental variables and SNP genotypes observed in hybrid green anoles were assessed using two separate latent factor mixed models (LFMMs) with K values determined by results from STRUCTURE, DAPC, and the broken stick stopping rule. To maximize the number of loci tested, the 70% call rate dataset was used as input for these analyses. LFMM was selected over other environmental association methods because it accounts for neutral and population genetic structure as a random factor and has been shown to be a suitable modelling choice for scattered, individual-based sampling designs as in this study (Reilstab et al., 2015). Both models used a least-squares estimation method with ridge penalties and were run in R with the package LFMM v2 (Caye et al., 2019). Following initial model estimation, the genomic inflation factor (GIF) for each model was manually adjusted to achieve an appropriate p-value distribution. Correction for multiple comparisons was implemented by converting p-values to q-values with the package *qvalue* v2.12.0 (Storey et al., 2015) and assessing significance at a false discovery rate (FDR)

threshold of 0.05. The NCBI *Anolis carolinensis* Annotation Release 102 (O’Leary et al., 2016) was then used to determine the location of significant loci relative to known genomic features.

To further investigate the relationship between urbanization and hybridization in this system, we implemented Wilcoxon rank-sum, Mantel, and partial Mantel tests in R. These analyses aimed to compare the distributions of habitat characteristics associated with groups of hybrid individuals displaying differing proportions of *A. porcatius* ancestry. These hybrid groups were assigned based on results of the DAPC analysis described above.

## RESULTS

Following trimming, mapping, and filtering of the raw sequence data, 147,594 SNPs from 101 individuals (70 hybrids, 16 *A. carolinensis*, and 15 *A. porcatius*; Tables S1, S2) were retained in the 95% call rate dataset, while 222,567 SNPs from 100 individuals were retained in the 70% call rate dataset. The read intervals from which SNPs were called covered 11.6% of the *A. carolinensis* reference genome. Assessment of per-sample allele balance revealed abnormal values indicative of contamination for two hybrid samples, which were excluded from all versions of the dataset. IBS analysis confirmed that differences between the five technical replicates were minimal (IBS [?] 98.7%; Figure S1), allowing data from nine quality-filtered hybrid samples sequenced in a separate study to be included in all analyses.

The principal component analysis produced well-defined clusters for both parental species, while hybrid individuals were distributed along a diagonal line (Figure 1). This line spanned the intermediate space between *A. carolinensis* and *A. porcatius* along the first principal component (PC1) but was extreme relative to both parental clusters along PC2. Collectively, PC1 and PC2 described 25.6% of the observed genetic variation, with no other PC axis describing more than 2.2% of the variation individually. Two samples of unknown ancestry from Parkland, FL fell within the *A. carolinensis* cluster rather than along the hybrid continuum and so were classified as pure *A. carolinensis* for all downstream analyses.

DAPC indicated a K value of 4 (Figure S2), correctly identifying *A. carolinensis* and *A. porcatius* as distinct genetic groups and subdividing the 70 hybrid individuals between two separate clusters of  $n = 31$  (hereafter referred to as hybrid group 1) and  $n = 39$  (hybrid group 2; Figures 2A, 2B). While discriminant function 1 placed both hybrid groups close to *A. carolinensis* and far from *A. porcatius*, discriminant function 2 situated the hybrid clusters closest to *A. porcatius*, with group 2 located further from the *A. carolinensis* cluster than group 1 (Figures 2C, 2D). Geographically, individuals assigned to hybrid group 1 were associated primarily with the northernmost, southernmost, and westernmost sampling sites, whereas group 2 individuals were associated almost exclusively with the central and easternmost sites (i.e., primarily in and around South Miami; Figure 3). When DAPC was repeated with a dataset containing only the hybrid individuals,  $K = 2$  was the most likely number of groups; individual group assignments remained consistent with results for the full dataset.

Unlike DAPC, STRUCTURE supported a K value of 3 (Figure S3), assigning the *A. carolinensis* and *A. porcatius* samples to their own groups while representing the hybrid samples as an admixed population composed of a mixture of genetic material from *A. carolinensis*, *A. porcatius*, and a third genetic group (Figure S4). When forced to divide all individuals among  $K = 2$  populations, STRUCTURE combined *A. carolinensis* and *A. porcatius* into a single group, while still displaying hybrids as admixed. An identical version of the analysis run with only the hybrid samples supported a K value of 2, assigning admixture proportions generally consistent with the hybrid groups identified by DAPC.

Of 222,567 SNPs in the 70% call rate dataset, 10,269 were identified as AIMs. These markers were used to produce a triangle plot (Figure 4), which integrates measures of interspecific heterozygosity (IH) and hybrid index (HI). Most hybrid individuals were positioned in the center of this bi-dimensional plot, as expected for advanced-generation backcrosses. The distribution of datapoints along the right side of the triangle (indicative of *A. carolinensis* ancestry) suggests that the more geographically widespread group 1 hybrids are the result of more recent backcrosses with *A. carolinensis*. A marker ancestry plot produced with the AIM data (Figure 5) also illustrates the high level of introgression occurring across the hybrid genome,

although a small number of fixed loci and genomic regions of low admixture are also evident.

For hybrid group 1, weighted  $F_{ST}$  values were lower when compared to *A. carolinensis* (mean = 0.207) than to *A. porcatius* (mean = 0.349), indicating that group 1 hybrids are less genetically differentiated from native *A. carolinensis* (Figure 6A). Conversely, group 2 hybrids displayed lower weighted  $F_{ST}$  values when compared to *A. porcatius* (mean = 0.325, vs. 0.385 for comparison with *A. carolinensis*) throughout all of the genome except for a portion of chromosome 2 where  $F_{ST}$  values converged (Figure 6B). For all genomic windows within both groups, hybrid comparisons with parental species always yielded  $F_{ST}$  values lower than those corresponding to genetic differentiation between the parental species themselves (mean = 0.601).

A total of 8,551 AIMs were examined via Bayesian estimation of genomic clines. Of these loci, 53.5% were identified as alpha outliers, beta outliers, or both (Table 1; Figure S5). All loci identified as beta outliers displayed negative beta scores, suggesting that no ancestry-informative allele from either parental species is being selected against. Of the loci identified as alpha outliers, 91.8% were negative, revealing that alleles indicative of *A. porcatius* ancestry are more than nine times more likely to be overrepresented in the hybrid genome than those unique to *A. carolinensis*. Finally, among loci identified as both alpha and beta outliers, 98.7% displayed negative values for both alpha and beta—a combination representing rapid introgression of *A. porcatius* alleles. When mapped to the *A. carolinensis* genome, loci identified as alpha outliers were distributed across all six macrochromosomes (Figure S6A), while those identified as beta outliers were concentrated nearly exclusively on chromosome 3 (Figures S6B, S6C).

A wide range of canopy cover (0-80%) and impervious surface area (0-70%) conditions were represented among the 68 hybrid individuals for which location data were available. As expected, these variables were negatively correlated ( $r = -0.759$ ,  $p < 0.0001$ ; Figure S7). The LFMM testing the association between canopy cover and genotype (manually adjusted GIF = 0.96; Figure S8A) identified a single significant SNP at position 19,549,307 on chromosome 1 ( $q = 0.0176$ ). A Manhattan plot of the model (Figure S9A) also revealed a series of closely-positioned, near-significant loci on an unassigned scaffold (NW\_003338792.1), the clustering of which suggest that a more liberal FDR threshold would also have identified the SNP at position 1,663,486 as significantly correlated with canopy cover. The LFMM testing the association between impervious surface area and hybrid genotype (manually adjusted GIF = 1.00; Figure S8B) identified two loci in close proximity on chromosome 2 (positions 135,319,503 and 135,321,966, Figure S9B) as significant ( $q = 0.0037$  for both loci). Of the three significant loci identified by the two models, all are located in currently unannotated regions of the *A. carolinensis* genome. All of these loci also display the same homozygous genotype across both parental species, while an alternate allele occurs at low frequencies (7.5 – 8.0%) among hybrids.

We found that both canopy cover (mean<sub>1</sub> = 37.4%, mean<sub>2</sub> = 17.8%,  $p = 0.0021$ ) and impervious surface area (mean<sub>1</sub> = 14.9%, mean<sub>2</sub> = 24.8%,  $p = 0.0132$ ) differed significantly between hybrid groups, with group 1 individuals occurring in more natural habitats (higher canopy cover and lower impervious surface area) than group 2 individuals. However, we also documented significant spatial autocorrelation among observations for both canopy cover (Mantel  $r = 0.14$ ,  $p = 0.005$ ) and impervious surface area (Mantel  $r = 0.14$ ,  $p = 0.019$ ), suggesting spatial non-independence for these variables. With the confounding effect of geographic location held constant, we confirmed a significant correlation of hybrid group membership with canopy cover (Mantel  $r = 0.10$ ,  $p = 0.005$ ) but not with impervious surface area (Mantel  $r = 0.02$ ,  $p = 0.175$ ).

## DISCUSSION

In this study, we used high-resolution SNP data to describe the nature and extent of hybridization between native *A. carolinensis* and invasive *A. porcatius* in South Miami, and to investigate the link between urbanization and hybrid ancestry. Our results support a description of the hybrid population as genetically distinct relative to both parental species (Wegener et al., 2019) and reveal two genetic subgroups within the hybrid population. We report rapid introgression of *A. porcatius* alleles at a large number of individual loci, demonstrating that hybrids have retained non-native alleles even in the absence of ongoing immigration of this invasive species. We also provide preliminary evidence for a positive association of urban habitats with

increased *A. porcatius* ancestry, although our hypothesis regarding the role of thermal conditions in facilitating hybridization requires further investigation. Ultimately, our findings reveal an unusual case of adaptive introgression resulting from a limited (possibly isolated) introduction event, and contribute to an increased understanding of the genetic, demographic, and environmental determinants of hybridization outcomes.

### Hybrid population structure

The genetic divergence between hybrids and parental genotypes that we report here agrees with the microsatellite-based results of Wegener et al. (2019) and supports the hypothesis of an historic hybridization event with little to no ongoing *A. porcatius* immigration. Differentiation of the hybrid population along the second PC axis likely corresponds to mutations and/or changes in allele frequencies (e.g., founder effects) that occurred following the original hybridization event. Such allele frequency changes may occur randomly due to genetic drift or can result from natural selection (Barton, 2000; Buffalo & Coop, 2020).

The positioning of hybrids in PCA space along a diagonal line angling toward the *A. carolinensis* cluster can also be explained by the historic admixture hypothesis. If indeed immigration of *A. porcatius* was restricted and is no longer occurring, contemporary hybrid anoles must breed either with conspecific hybrids or backcross with native *A. carolinensis*. Backcrossing of hybrid individuals to the native parent will erode differentiation relative to *A. carolinensis*, while crosses of hybrid individuals will maintain or increase the divergence of offspring from parental species. A competing (though seemingly less likely) explanation is that the hybrid line angles directly toward a theoretical cluster representing the original parental *A. porcatius* propagule, which was in some way genetically distinct from the *A. porcatius* sampled in our study. While our samples were collected from the western *A. porcatius* clade in Cuba, which displays mitochondrial haplotypes most similar to those observed in the South Miami hybrid population (Kolbe et al., 2007; Wegener et al., 2019), the possibilities of substructure within the western clade or founder effects resulting from a small immigrant propagule remain.

Substructure of the hybrid population resulted in the assignment of hybrid individuals to two groups, with discriminant function 2 placing hybrid group 1 in closer proximity to *A. carolinensis* than group 2. These assignments were further supported by STRUCTURE, which estimated higher *A. carolinensis* ancestry proportions for hybrids belonging to group 1. Although the hybrid range boundary is currently unknown, the spatial distribution of group 1 individuals, which occurred primarily along the outermost sites sampled in our study, suggests that these individuals are closer to the range boundary and thus more likely to encounter and backcross with pure *A. carolinensis* than group 2 individuals located closer to the (putative) center of the hybrid swarm. Indeed, patterns of hybrid ancestry and heterozygosity at loci displaying fixed differences between parental species confirm that the difference between hybrid groups is related to patterns of backcrossing with *A. carolinensis*. These results support our conclusion that hybridization was a limited, historic event and that contemporary *A. porcatius* immigration is low or nonexistent.

Although both plant and animal hybrids resulting from crosses among three parental species have been reported (Bi & Bogart, 2006; Floate et al., 2016; McDonald et al., 2008), we consider contribution of genetic material from a third species to be an unlikely explanation for the observed genetic differentiation of the hybrid anoles from their parents. Since our analyses clearly identified substructure within the hybrid population in addition to differentiating between *A. carolinensis* and *A. porcatius*, any higher-tier genetic structure resulting from a third parental species would also have been revealed had it been present. Furthermore, hybridization among *Anolis* species is rare (Jezkova et al., 2013; Losos, 2009) and—beyond *A. porcatius*—no anole species known to occur in South Miami is thought to be capable of producing fertile hybrids with *A. carolinensis* (Morris et al., 2021). Indeed, of the 26 mitochondrial haplotypes observed among 86 hybrid green anoles sampled by Wegener et al. (2019), all indicated maternal ancestry from either *A. carolinensis* or *A. porcatius*. The divergent features of the hybrid population are thus best interpreted as resulting from genetic differentiation of *A. carolinensis* x *A. porcatius* hybrids following historic admixture, rather than hybridization with an unknown third parental species.

### Introgressive hybridization

Although hybrid ancestry averages for AIM loci showed a much higher genetic contribution from *A. carolinensis* overall, a large proportion (40.3%) of individual markers displayed higher than expected *A. porcatius* ancestry in comparison to the average across all AIM loci. Furthermore, a quarter of these loci (10% of all AIMS) displayed both excess *A. porcatius* ancestry and unusually rapid rates of introgression (i.e., negative alpha + negative beta outliers), while only 0.1% of AIMS departed from null expectations regarding increased introgression of *A. carolinensis* alleles. Fixed AIM loci can show drastically larger proportions of significant alpha and beta outliers than markers that do not display fixed differences between parental species (McFarlane et al., 2021), so the proportions reported here should not be extrapolated as representative of introgression rates across the entire hybrid genome. However, the notable inequality in counts of positive and negative outliers for both the alpha and beta parameters demonstrates a clear trend of rapid introgression, which has resulted in the accumulation and retention of *A. porcatius* alleles rather than those of the more abundant, native *A. carolinensis*.

Caution is warranted when interpreting the evolutionary drivers of observed genomic cline patterns, as significant outliers may indicate directional selective pressure within a population but can also result from genetic drift (Fitzpatrick, 2013; Gompert & Buerkle, 2011). Indeed, considering the large number of significant loci identified by most genomic cline analyses, it is inevitable that at least some outliers will be linked to drift rather than selection (Gompert & Buerkle, 2012; McFarlane et al., 2021). Because genetic drift is a random process and natural selection is not, comparison of clines measured in replicate populations can be used to distinguish between these causes when fitness values of individual alleles are unknown (Jeffery et al., 2017; Teeter et al., 2009). However, additional *A. carolinensis* x *A. porcatius* hybrid populations resulting from replicate introductions are not known to exist, so from this perspective selection and drift remain confounded in our study system.

Nonetheless, we note that two aspects of our genomic cline results are decidedly non-random, and therefore consistent with the action of natural selection. First, the majority of alpha outliers in the hybrid genome are negative and correspond to excess introgression of alleles from the rarer invader (*A. porcatius*). This finding deviates from both modeled and observed results for neutral alleles, which predict the opposite—that the direction of introgression will be from the native species into the invader regardless of relative population size (Currat et al., 2008). Indeed, while genomic cline analyses conducted in several other hybrid systems have identified similar imbalances in positive and negative alpha outliers, few report equally large differences in beta outliers (Haines et al., 2019; Oswald et al., 2019; Souissi et al., 2018; Sung et al., 2018). McFarlane et al. (2021) report AIM-based genomic cline patterns for hybrids of native red deer (*Cervus elaphus*) and introduced sika (*C. nippon*) in Scotland that do meet both criteria, although their findings are ultimately opposite to ours in that alleles from the native deer were shown to be rapidly introgressing into the population of the invader, rather than vice versa. Second, most of the beta outliers in our study were clustered on chromosome 3 (Figure S6B,C), indicating rapid introgression of *A. porcatius* alleles at this genomic region. Here as well, results depart from expectations under neutral genetic drift, which should affect polymorphism genome-wide. The reason behind the over-representation of outliers on chromosome 3 remains to be clarified, although one possibility is positive selection at adaptive loci on this chromosome followed by fixation of adjacent regions due to linkage drag. Future studies focused on genetic mapping of adaptive traits in this system will be well-positioned to investigate this hypothesis. If indeed adaptive in nature, alleles of *A. porcatius* origin can be expected to continue to spread outward from South Miami, as has been documented in other systems (e.g., Fitzpatrick et al., 2010).

In addition to the patterns described above, the total lack of positive beta outliers in the hybrid genome warrants additional discussion. Positive beta values correspond to reduced rates of introgression, and loci involved in reproductive isolation between species will deviate from genome-wide averages in this way (Gompert & Buerkle, 2010; McFarlane et al., 2021; Taylor et al., 2014). Wegener et al. (2019) suggested revision of the currently paraphyletic *A. porcatius* group such that the western clade of *A. porcatius* is subsumed into *A. carolinensis*, citing morphological similarity (Camposano, 2011) and violation of the biological species concept (Mayr, 1982) as justifications for this taxonomic alteration. Hybridization scenarios often blur species boundaries, and an increased understanding of the genetic details of admixture and introgression has led

many to critique the biological species concept as insufficient for delineating species status (Gompert et al., 2014, 2017; Mallet, 2007, 2020). To ameliorate the difficulties of assigning hybridizing organisms to the discreet “different species” and “same species” categories set forth by the biological species concept, hybrid systems should be viewed as representing various intermediate stages of the speciation process, with early stages characterized by abundant gene flow, many hybrid individuals, and little to no reproductive isolation, while later stages display opposite characteristics (Gompert et al., 2017; Payseur, 2010). The total absence of positive beta outliers among the fixed AIM loci in our study demonstrates that, despite an estimated 6–12 million-year period of geographic isolation (Campbell-Staton et al., 2012; Wegener et al., 2019), none of the loci we sequenced and analyzed are contributing to reproductive isolation between *A. carolinensis* and *A. porcatius*. This indicates that speciation differentiating the two anole lineages is still in its earliest stages and considerably strengthens the argument that *A. carolinensis* and western *A. porcatius* have not yet diverged enough to constitute different species. Future studies of anole diversification and speciation should assess locus-specific cline width for other known hybrid populations produced by putatively distinct parental species. If no evidence of reproductive isolation between parental species (i.e., positive beta outliers) is present, species status may need to be reassessed.

### Relationship between urbanization and hybridization

Only three loci were found to be significantly associated with environmental variables indicative of urbanization (i.e., canopy cover or impervious surface area)—a surprisingly small proportion of the 222,567 loci tested. While most studies with similar methods identify hundreds to thousands of significant SNPs (Bekkevold et al., 2020; Dudaniec et al., 2018; Frichot et al., 2013; Guerrero et al., 2018), lower proportions are not entirely unprecedented. For example, Caye et al. (2019) tested their LFMM package on a set of 345,067 CpG sites collected from patients with the autoimmune inflammatory disease rheumatoid arthritis (RA) and found only nine of the sites to be significantly correlated with tobacco consumption. These nine included genes with clear ties to RA, demonstrating that analyses returning low proportions of significant loci can still yield meaningful and accurate results.

Of the three loci we identified as significant, all were located in currently unannotated regions of the *A. carolinensis* genome, preventing characterization of their potential functions. No locus was significantly associated with both canopy cover and impervious surface area, and none of the significant loci displayed fixed differences between parental species (preventing comparison with AIMs identified as genomic cline outliers). Allele frequency patterns displayed by the three significant loci suggest that these markers correspond to mutations that arose in the hybrid population after the initial hybridization event, and thus that alleles unique to hybrid anoles may play a greater role in environmental adaptation than those associated with either parental species. However, this conclusion should be viewed as preliminary for two reasons. First, rapid adaptation to novel environments is more likely to result from standing genetic variation than from new mutations (Barrett & Schluter 2008). Second, each of the hybrid-specific minor alleles occur at low frequencies, so parental populations would need to be sampled in greater numbers to confirm that these alleles are indeed unique to the hybrid population. The fact that the three loci associated with environment had minor alleles of low frequency also leaves open the possibility that a small number of individuals occurring in unusual habitats could have driven the association signal.

While these results do not disprove our hypothesis that increased temperatures in urban habitats have facilitated the spread of *A. porcatius* alleles, they also provide little support for such a conclusion. Next steps in addressing this hypothesis will require the collection of live anoles from both hybrid groups, for which measures of thermal tolerance, such as critical thermal maximum ( $CT_{max}$ ), can be compared both between discrete groups and across the continuous hybrid index spectrum.  $CT_{max}$  values should also be incorporated into a genome-wide association study to identify specific loci that may be involved in the genetic regulation of thermal tolerance. A significant association between  $CT_{max}$  and *A. porcatius* allele frequency at any such locus would provide strong support for the thermal facilitation hypothesis.

The association of hybrid genotypes with environmental factors

indicative of urbanization was further investigated by our exploration of the relationship between hybrid group assignment and habitat type. Group 2 individuals, which have higher proportions of *A. porcat* ancestry, were found in habitats with 20% less canopy cover and 10% more impervious surface area than habitats associated with group 1 individuals. These differences may result in relatively warmer microhabitats for group 2 hybrids (Battles & Kolbe, 2019). After accounting for spatial autocorrelation, this relationship held true for canopy cover, but not for impervious surface area. The association of group 2 hybrids with low canopy cover values supports the hypothesis that invasive genotypes are more abundant in anthropogenically disturbed habitats, although the lack of a significant association with impervious surface area indicates some uncertainty in the mechanism driving this pattern. Ultimately, we provide preliminary evidence for a relationship between urbanization and hybridization in the *A. carolinensis* x *A. porcat* system, although both the ecological cause and underlying genomic basis of this pattern remain to be elucidated.

## Conservation implications and conclusions

Native *A. carolinensis* is both abundant and widely distributed across the southeastern United States (Campbell-Staton et al., 2012; Losos, 2009), so the isolated hybridization scenario reported here is not a conservation concern in and of itself. However, our observations could be informative in systems where hybridization does constitute a genuine concern. We demonstrate that genetic material from an invasive species introgresses rapidly and persists in native populations even after (1) pure parental individuals of the invasive species are no longer present and (2) immigration of additional invasive individuals has ceased. Such introgression may occur randomly as an unpredictable outcome of genetic drift (McFarlane et al., 2021), but in this system is much more likely the result of positive selection on non-native alleles. These findings suggest that, in situations where a rare native species with a restricted range is threatened by hybridization with an invader, management should focus on identifying pure native individuals and preventing these individuals from breeding with hybrids—rather than simply trying to eliminate the non-native species.

Conversely, we also report an association of hybrid genotypes with environmental variables indicative of urbanization, suggesting that hybridization may lead to adaptive introgression when occurring in an anthropogenically disturbed habitat. Although the genetic swamping that occurs in a hybrid swarm is generally considered undesirable (Fitzpatrick & Shaffer, 2007b; Huxel, 1999; Vuillaume et al., 2015), adaptive introgression is increasingly being viewed as a positive hybridization outcome (Hamilton & Miller, 2016; Pardo-Diaz et al., 2012). Adaptive alleles inherited from a resilient invader have the potential to facilitate the persistence of native species in the face of ever-accelerating global change (Fitzpatrick & Shaffer, 2007a; Oziolor et al., 2019) and thus may constitute a valuable conservation tool. Rather than dismissing all forms of native-invasive hybridization as undesirable, we should carefully consider both the benefits and risks of admixture from an ecological perspective—particularly in terms of long-term persistence when facing multiple dimensions of global environmental change.

Through detailed examination of the unique *A. carolinensis* x *A. porcat* hybrid system, our study reveals that the demographic factors of low population size and negligible immigration rate are alone insufficient to prevent rapid, directional introgression of invasive alleles into a large native population. Rather, the limitation of low propagule pressure may be overcome by selection favoring the persistence of invasive alleles in urban habitats. While our findings enhance current understanding of the roles these factors play in influencing hybridization outcomes, further study will be necessary to fully describe the demographic and environmental determinants of introgression in the *A. carolinensis* x *A. porcat* hybrid system. Next steps should prioritize identifying hybrid range boundaries, which will require broad, systematic sampling of green anole genetic material ranging from the southeast coast of Florida into the Everglades and north along the heavily developed east coast of Florida. Such a sampling design will also accommodate the application of additional environmental association analyses and will permit the collection of microhabitat and thermal data that can be used to more rigorously test our preliminary findings regarding the relationship between non-native alleles and urbanization. While various studies have reported similar associations between anthropogenic disturbance and the spread of invasive genotypes via hybridization, the underlying causes of such associations are rarely discussed beyond general statements that invasive species are typically resilient,

and thus better able to withstand habitat disturbance (Beninde et al., 2018; Riley et al., 2003; Walters et al., 2008; but see Fitzpatrick & Shaffer, 2004). We suggest that the identification of specific mechanisms underlying these patterns should be a primary goal of future research both in this system and in the field of invasion genetics as a whole.

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## DATA ACCESSIBILITY AND BENEFIT-SHARING SECTION

### Data Accessibility Statement

Contingent on acceptance, raw sequence data will be deposited in the Sequence Read Archive (SRA). All script used for analyses will be archived in a publicly accessible GitHub repository.

### AUTHOR CONTRIBUTIONS

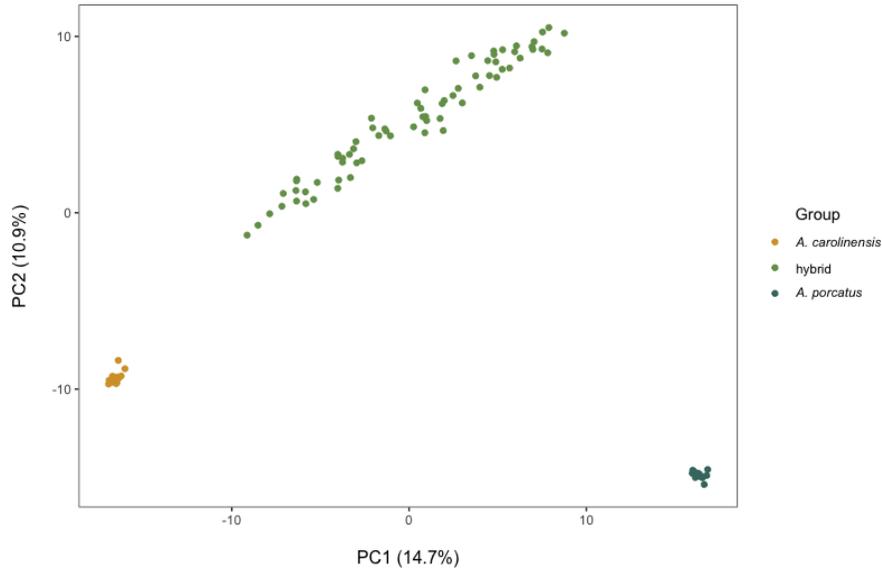
T.B.D., D.G.B., and J.J.K. contributed to study design. Lab work and analyses were performed by T.B.D. with instruction from D.G.B. The paper was written by T.B.D. with input from D.G.B. and J.J.K.

### TABLES AND FIGURES

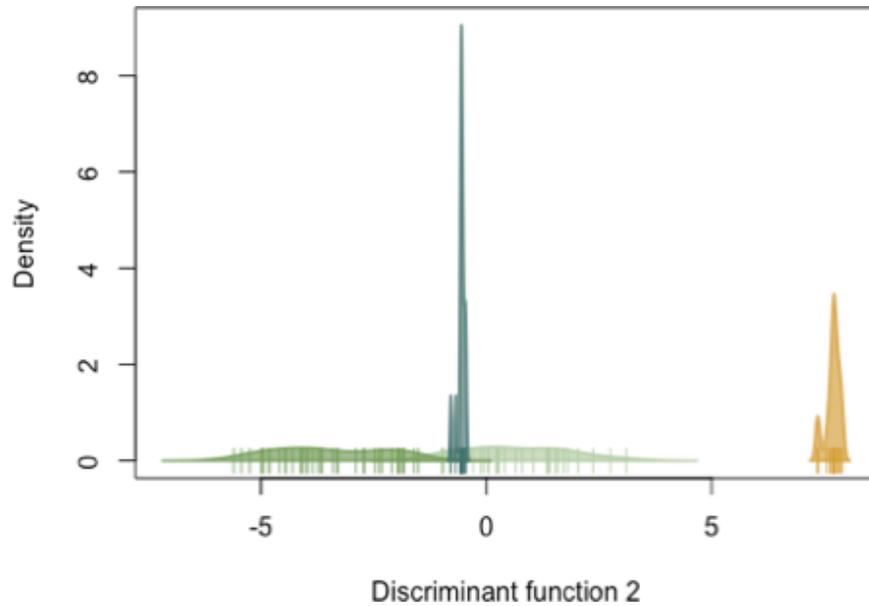
**Table 1.** Outlier status of 8,551 fixed, ancestry-informative loci, as assigned by Bayesian estimation of genomic clines. Alpha outliers display a greater-than-expected contribution of genetic material from one parental species (positive = excess *A. carolinensis* ancestry, negative = excess *A. porcatius* ancestry), while beta outliers display unusual rates of introgression (positive = reduced introgression, negative = rapid introgression). The first value within each cell represents the total number of loci assigned to the corresponding

outlier status, while the second represents the subset of those loci currently mappable to a known chromosomal location.

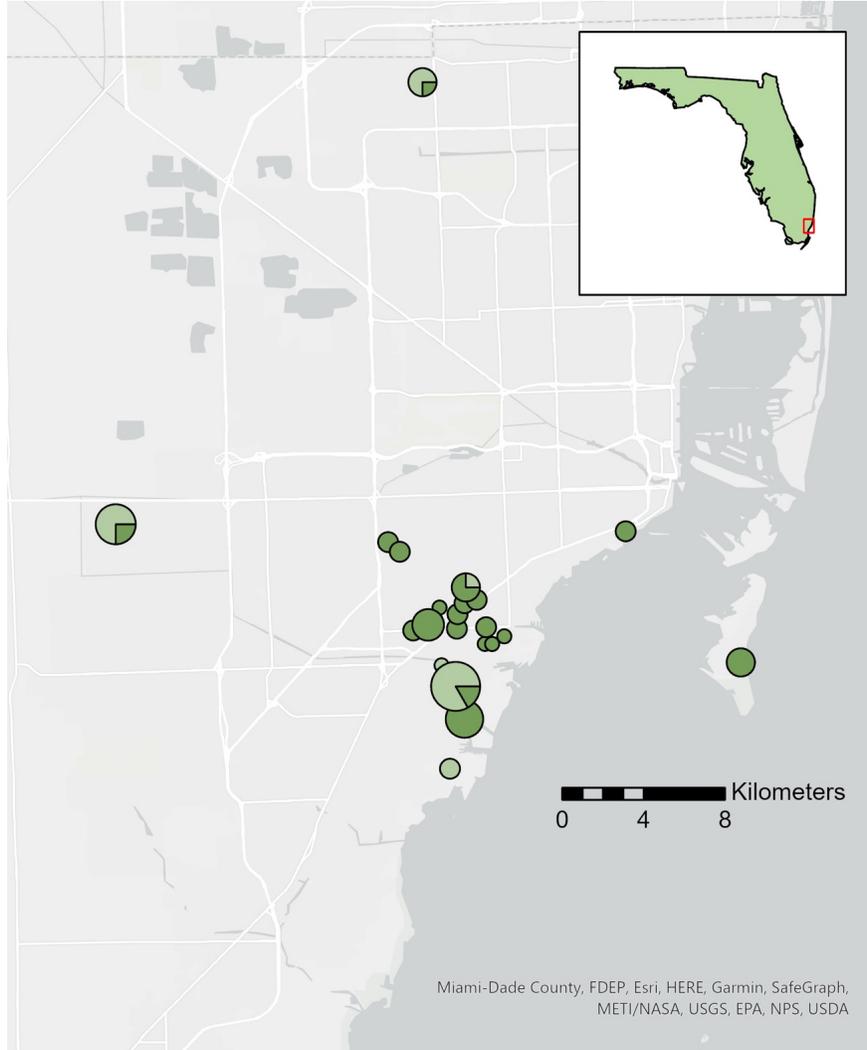
alpha	alpha	beta	beta	both	both	neither	total
+	-	+	-	+? -?	-? -?	3980 / 2445	8551 / 6234
298 / 3	2595 / 2501	0 / 0	811 / 431	11 / 0	856 / 854		



**Figure 1.** Principal component plot displaying genetic differentiation between *Anolis carolinensis* (n = 16), *A. porcatius* (n = 15), and their hybrids (n = 70). Hybrid individuals collected from South Miami are intermediate to the parental species along PC1 and extreme relative to both parental species along PC2.



**Figure 2.** Results of discriminant analysis of principal components (DAPC) performed on hybrid anoles and parental species. DAPC identified  $K = 4$  groups, subdividing the hybrids among two separate genetic clusters (A, B). The first discriminant function groups both hybrid clusters with *A. carolinensis* (C), while the second discriminant function differentiates among *A. carolinensis*, hybrid group 1, and hybrid group 2 (D).



**Figure 3.** Geographic distribution of hybrid groups in South Miami, FL. Pie chart size corresponds to the number of individuals sampled at each site, ranging from  $n = 1-12$ . Group 1 individuals are depicted in light green, while group 2 individuals are depicted in dark green.

#### Hosted file

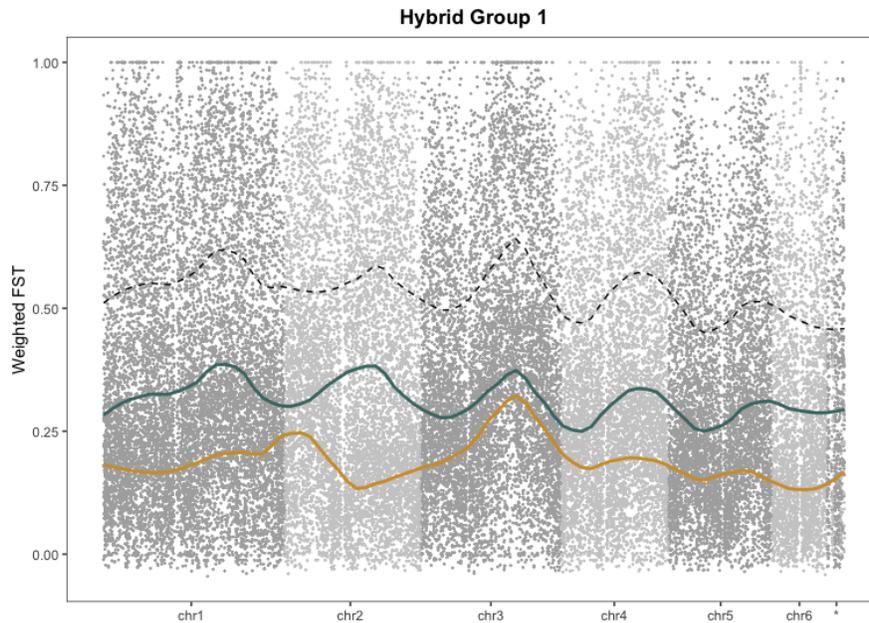
image11.emf available at <https://authorea.com/users/465039/articles/559769-rapid-introgression-of-invasive-alleles-following-hybridization-between-a-native-anolis-lizard-species-and-a-cryptic-invader-across-an-urban-landscape>

**Figure 4.** Triangle plot of hybrid class as determined by the relationship between hybrid index and inter-specific heterozygosity at the individual level. These values were calculated using only loci displaying fixed

differences between parental *Anolis* species ( $n = 10,269$ ).



**Figure 5.** Ancestry plot of observed hybrid genotypes at loci displaying fixed differences between parental species. Columns correspond to loci (ordered by chromosome), while rows correspond to individual lizards. Gold indicates a homozygous *Anolis carolinensis* genotype, teal indicates a homozygous *A. porcatius* genotype, green indicates a heterozygous genotype, and white indicates missing data. Loci mapped to the “unassigned scaffolds” bin are currently unplaced within the *A. carolinensis* reference genome and thus are not meaningfully ordered.



**Figure 6.** Weighted fixation index ( $F_{ST}$ ) values calculated for windows (width = 50,000 base pairs) across the genome. Pairwise  $F_{ST}$  values for hybrid-*Anolis carolinensis* are plotted in gold, hybrid-*A. porcatius* in teal, and *A. carolinensis* - *A. porcatius* in black (dashed). Calculations were performed separately for hybrid groups 1 (A) and 2 (B). The asterisk (\*) identifies loci mapped to any of the seven *A. carolinensis* microchromosomes (linkage groups a, b, c, d, f, g, and h).