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

Tyler B. DeVos[1] | **Dan G. Bock**[2] | **Jason J. Kolbe**[1]

[1]Department of Biological Sciences, University of Rhode Island, Kingston, Rhode Island, USA

[2]Department of Biology, Washington University, St. Louis, Missouri, USA

Correspondence
Tyler B. DeVos, Florida Natural Areas Inventory, Florida State University, Tallahassee, FL 32303, USA.
Email: tylerbdevos@gmail.com

Present address
Tyler B. DeVos, Florida Natural Areas Inventory, Florida State University, Tallahassee, Florida, USA
Dan G. Bock, Department of Botany and Biodiversity Research Centre, University of British Columbia, Vancouver, British Columbia, Canada

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Abstract

Invasive species can impact native populations through competition, predation, habitat alteration, and disease transmission, 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. porcatus*) in south Florida provides an ideal opportunity to study interspecific admixture across a heterogeneous landscape. We used reduced-representation sequencing to describe introgression in this hybrid system and to test for a relationship between urbanization and non-native ancestry. Our findings indicate that hybridization between green anole lineages was probably a limited, historic event, producing a hybrid population characterized by a diverse continuum of ancestry proportions. Genomic cline analyses revealed rapid introgression and disproportionate representation of non-native alleles at many loci and no evidence for reproductive isolation between parental species. Three loci were associated with urban habitat characteristics; urbanization and non-native ancestry were positively correlated, although this relationship did not remain significant when accounting for spatial nonindependence. Ultimately, our study demonstrates the persistence of non-native genetic material even in the absence of ongoing immigration, indicating that selection favouring non-native alleles can override the demographic limitation of low propagule pressure. We also note that not all outcomes of admixture between native and non-native 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.

KEYWORDS
admixture, green anole, hybridization, introgression, non-native, urbanization

1 | INTRODUCTION

Invasive species are widely known for their negative ecological impacts on native organisms, which often involve predation, competition, habitat alteration, and disease transmission (Case & Bolger, 1991; Gibbons et al., 2000; Gordon, 1998; Kraus, 2015; Vilcinskas, 2015). It is also well-known that invaders can interact with native species genetically through hybridization (e.g., Ellstrand & Schierenbeck, 2000; Fitzpatrick & Shaffer, 2007a, 2007b; Huxel, 1999; Vuillaume et al., 2015), with numerous examples documented in birds, mammals, fishes, and plants (Largiader, 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 indirectly exacerbated by scenarios in which an 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 and unmanaged.

Possible outcomes of 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 important (Bennett 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 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 may require 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 urbanization-induced habitat heterogeneity.

An ideal opportunity to investigate outcomes of hybridization between native and non-native species in an urban setting is provided by *Anolis* lizards in south Florida. Here, introduced Cuban green anoles (*Anolis porcatus*) have hybridized with native green anoles (*A. carolinensis*) across a patchwork of urban and remnant natural forest habitats. Microsatellite-based analyses suggest this secondary contact has resulted in a population of hybrid origin with a genetic makeup distinct from that of either parental species (Wegener et al., 2019). While the hybrid population is thought to have originated in the city of South Miami during or prior to 1987 (Kraus, 2009; Meshaka et al., 1997), 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 distinguished from parental species.

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). No contemporary route of introduction of additional *A. porcatus* to Florida is known 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 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., 2011). If tropical *A. porcatus* is better adapted to the warmer temperatures that dominate urban settings than *A. carolinensis*, non-native 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 favour the persistence of hybrid genotypes in a region with little to no ongoing *A. porcatus* immigration.

We capitalize on the unique nature of the *A. porcatus* × *A. carolinensis* hybrid system to understand the interplay between urbanization and introduction-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, the relationship between urbanization and invasion success, and the erosion of reproductive barriers by anthropogenic habitat disturbance.

2 | MATERIALS AND METHODS

2.1 | 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 (Largiader, 2008). *Anolis porcatus* is introduced in Florida and is morphologically indistinct from *A. carolinensis* (Camposano, 2011; Kolbe et al., 2007; Wegener et al., 2019). Although *A. porcatus* is native to Cuba and the two species have had allopatric distributions since before the Pliocene (the split between the two lineages is estimated to have occurred at least 6 million years ago; 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. porcatus* introduction (Wegener et al., 2019).

Anolis porcatus was first observed in south Florida in 1987 (Kraus, 2009; Meshaka et al., 1997), although the pathway by which founding individuals arrived remains unknown. Assuming a generation time of 1 year, this record suggests that hybridization has been occurring for a minimum of 34 generations. Kraus (2009) reports that *A. porcatus* arrived as a cargo stowaway—this explanation is very plausible but 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. porcatus* import or export. Moreover, trade and travel between south Florida and Cuba have been heavily regulated for over 60 years.

2.2 | Sample selection and processing

Samples of muscle and liver tissue were obtained from the Kolbe Laboratory tissue library at the University of Rhode Island and were strategically selected to construct a data set with balanced sex ratios, diverse temporal sampling, and putative hybrid representatives from a variety of locations in and around South Miami, FL. Samples were selected based on known geographic distribution patterns of hybrids and parental species (Campbell-Staton et al., 2012; Wegener et al., 2019). In addition to 63 hybrid samples, 15 *A. porcatus* from Western Cuba and two individuals of unknown ancestry from Parkland, FL were also selected. Tissue samples from 14 *A. carolinensis* from Hobe Sound, FL—a location 185 km 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 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. Three of the replicate samples were from *A. porcatus*, while all other supplemental samples were from hybrids. 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).

2.3 | Data trimming and filtering

A quality control check for all raw sequence data was conducted using FastQC version 0.11.8 (https://www.bioinformatics.babra ham.ac.uk/projects/fastqc/). Reads were then trimmed using Trimmomatic version 0.39 (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 version 2.7.8 (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 data set 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 four reads were marked as missing data. Additional filters to exclude markers with minor allele frequencies less than or equal to 3% (i.e., with fewer than seven unique allele copies among all samples) 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 et al., 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 data set. 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 version 3.5.1 (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.

2.4 | 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 data set. Principal components and their corresponding eigenvalues were calculated using the R package ADEGENET version 2.1.1 (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 for a randomly selected subset of hybrid samples using the admixture ancestry model of STRUCTURE version 2.3.4 (Pritchard et al., 2000) with a maximum of eight possible genetic groups ($K = 8$). The number of hybrid samples included in this analysis was limited to 16 to satisfy the recommendation of even sampling across groups (Puechmaille, 2016). 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 burnin 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 version 2.3.1 (Francis, 2017).

Ancestry informative markers (AIMs) displaying fixed differences between *A. carolinensis* and *A. porcatus* were identified using a custom script in conjunction with BEDTOOLS version 2.27.1 (Quinlan & Hall, 2010). AIM genotypes for each individual were then converted to allele counts and used to calculate hybrid index (HI) values with the R package INTROGRESS version 1.2.3 (Gompert & Buerkle, 2010). These values were visualized in a triangle plot displaying individual hybrid class membership as a function of the relationship between HI and interspecific heterozygosity (IH). We also used INTROGRESS to visualize the ancestry of each individual at each AIM. For both analyses performed with INTROGRESS, the "fixed" parameter was conservatively set to "FALSE" due to low parental sample size.

2.5 | Population-based analyses

Patterns of introgression at individual loci were examined via Bayesian estimation of genomic clines using BGC version 1.03 with default settings and following manual recommendations (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 data set. The genomic cline model was run for 100,000 MCMC steps (the first 50,000 of which were discarded as burnin) 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 version 0.0.0.9000 (Martin & Chafin, 2021) and mapped to an ideogram of the *A. carolinensis* reference genome using a custom script.

2.6 | Environmental association analyses

To investigate the relationship between anthropogenic habitat disturbance and the genotypes of hybrid green anoles, canopy cover and impervious surface area (i.e., surfaces impermeable to water) 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 × 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^2 to a maximum of 1538 m^2 in size (average = 260 m^2 for males and 410 m^2 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 a *K* value (i.e., predefined number of genetic groups) determined by results from STRUCTURE, DAPC, and the broken stick stopping rule. To maximize the number of loci tested, the 70% call rate data set was used as input for these analyses. We chose not to filter LFMM input markers for linkage to allow for observation and interpretation of disequilibrium-related clustering patterns along the genome. 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 (Rellstab et al., 2015). Both models used a least-squares estimation method with ridge penalties and were run in R with the package LFMM version 2 (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 version 2.12.0 (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 tested for correlations between habitat characteristics and HI as estimated using AIMs. Spatial autocorrelation was assessed via Moran's *I* test and accounted for using spatial lag models run in R with the package SPDEP version 1.1-3 (Bivand & Wong, 2018).

3 | RESULTS

Following trimming, mapping, and filtering of the raw sequence data, 147,594 SNPs from 101 individuals (70 hybrids, 16 *A. carolinensis* [native parental species], and 15 *A. porcatus* [non-native parental species]; Tables S1 and S2) were retained in the 95% call rate data set, while 222,567 SNPs from 100 individuals were retained in the 70% call rate data set. 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 data set. 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. porcatus* 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. PC1 and PC2 each explained similar proportions of variation, with PC1 differentiating parental species from one another and PC2 differentiating parental species from hybrids. 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 subsequent analyses.

DAPC indicated a *K* value of 4 (Figure S2), although the difference in BIC between *K* = 3 and *K* = 4 was small. The analysis correctly identified *A. carolinensis* and *A. porcatus* as distinct genetic groups and subdivided the 70 hybrid individuals between two separate clusters of *n* = 31 and *n* = 39 (Figure 2a,b). While discriminant function 1 placed both hybrid groups close to *A. carolinensis* and far from *A. porcatus* (Figure 2c), discriminant function two situated the hybrid clusters to either side of *A. porcatus*, with both hybrid groups displaying highly platykurtic distributions suggestive of a genetic continuum rather than distinct clustering (Figure 2d). Subgroup assignments did not correspond to sampling year, sex, or sequencing batch. When DAPC was repeated with a data set containing only the

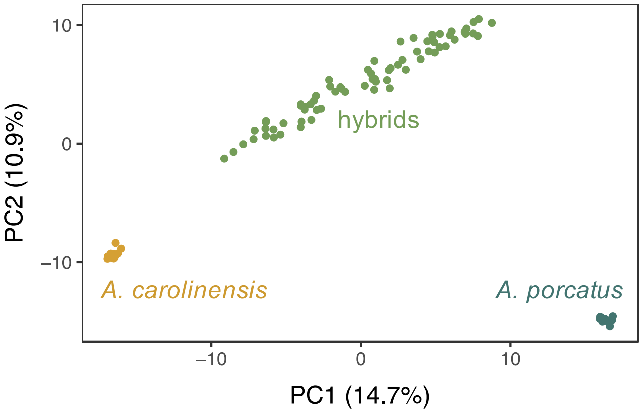

FIGURE 1 Principal component plot displaying genetic differentiation between *Anolis carolinensis* (*n* = 16), *A. porcatus* (*n* = 15), and their hybrids (*n* = 70). Hybrid individuals collected from South Miami are intermediate to the parental species along PC1 and extreme relative to both parental species along PC2. [Colour figure can be viewed at wileyonlinelibrary.com]

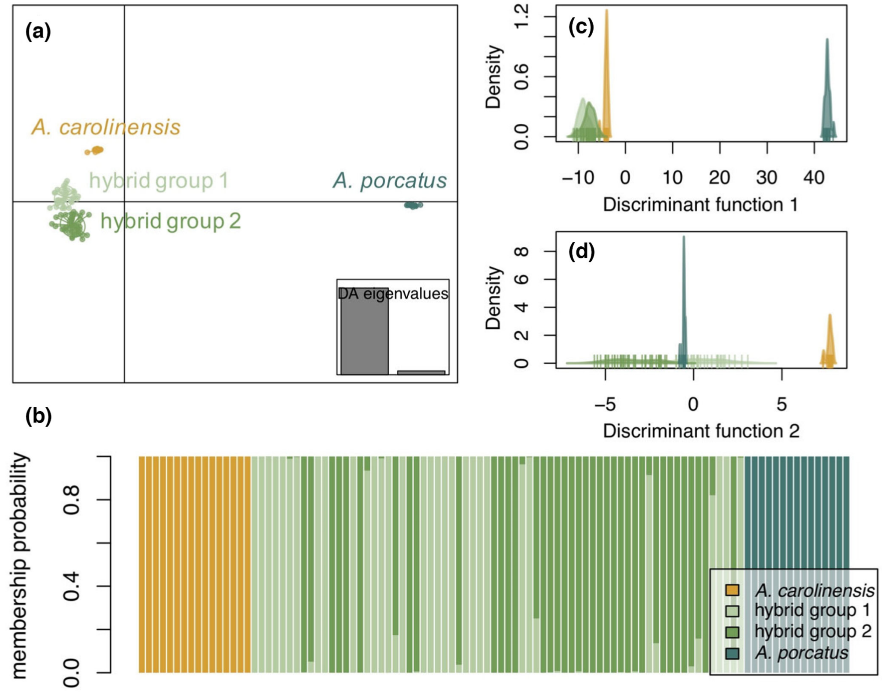

FIGURE 2 Results of DAPC performed on hybrid anoles and parental species. DAPC identified $K = 4$ groups: the two parental species and a subdivision of the hybrids into two separate genetic clusters (a, b). The first discriminant function groups both hybrid clusters with *A. carolinensis* (c), while the second discriminant function differentiates among *A. carolinensis*, hybrid group 1, and hybrid group 2 (d). [Colour figure can be viewed at wileyonlinelibrary.com]

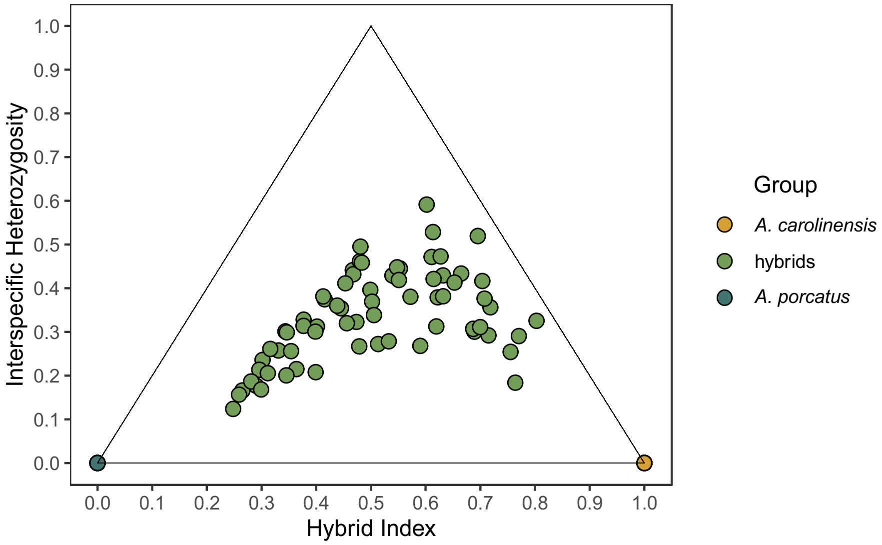

FIGURE 3 Triangle plot of hybrid class as determined by the relationship between HI and IH at the individual level. These values were calculated using only loci displaying fixed differences between parental *Anolis* species ($n = 10{,}269$). [Colour figure can be viewed at wileyonlinelibrary.com]

hybrid individuals, $K = 2$ was the most likely number of groups; individual hybrid group assignments remained consistent with results for the full data set.

Unlike DAPC, STRUCTURE supported a K value of 2 (Figure S3), assigning the *A. carolinensis* and *A. porcatus* samples to their own groups. The ancestry of hybrid samples included in this analysis was assigned, in various proportions, to both parental species (Figure S4). At $K = 3$, STRUCTURE again assigned *A. carolinensis* and *A. porcatus* to their own groups but characterized the hybrid samples as belonging to an admixed population composed of a mixture of genetic material from *A. carolinensis*, *A. porcatus*, and a third genetic group. A similar analysis run with the full group of hybrid samples and no parental individuals supported a K value of 2 (Figure S5), assigning a wide variety of admixture proportions that again suggested a genetic continuum.

Of 222,567 SNPs in the 70% call rate data set, 10,269 were identified as AIMs. These markers were used to produce a triangle plot (Figure 3), which integrates measures of IH and HI. Most individuals were positioned in the centre of this bidimensional plot, as expected for advanced-generation hybrids. The distribution of datapoints along the right side of the triangle (indicative of *A. carolinensis* ancestry) suggests that hybrids with higher native ancestry proportions are the result of more recent backcrosses with *A. carolinensis*. Conversely, the lack of datapoints along the left side of the triangle reveals that hybrids with lower native ancestry proportions result from sustained breeding within the hybrid population rather than recent backcrosses with *A. porcatus*. Geographically, individuals with the highest native ancestry proportions were associated primarily with the northernmost, southernmost, and westernmost (i.e., peripheral) sampling sites, whereas advanced generation hybrids were associated almost exclusively with the central and easternmost sites (i.e., primarily in and around South Miami; Figure 4). Marker ancestry plots produced with the AIM data (Figure 5) further illustrate 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.

A total of 8551 AIMs were examined via Bayesian estimation of genomic clines. Of these loci, 53.5% were identified as alpha outliers (i.e., loci associated with directional introgression), beta outliers (i.e., loci introgressing at unusually rapid or reduced rates), or both (Table 1; Figure S6). All loci identified as beta outliers displayed negative beta scores, suggesting that no ancestry-informative allele from either parental species is being strongly selected against. Of the 3760 loci identified as alpha outliers, 91.8% were negative, revealing that alleles indicative of *A. porcatus* 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

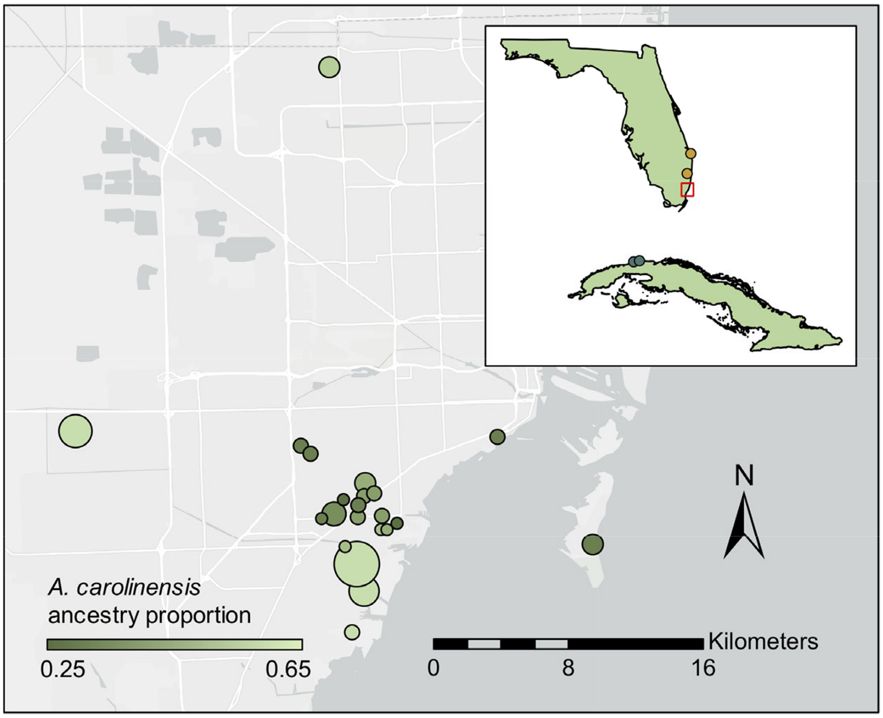

FIGURE 4 Geographic distribution of *Anolis* hybrids in South Miami, FL. Symbol size corresponds to the number of individuals sampled at each site, ranging from n = 1–12. Symbol colour indicates the average *A. carolinensis* ancestry proportion (based on INTROGRESS HI values) of hybrids sampled at each site. Within the inset map, gold and teal circles indicate the locations from which parental *A. carolinensis* in Florida and *A. porcatus* in Cuba were sampled. [Colour figure can be viewed at wileyonlinelibrary.com]

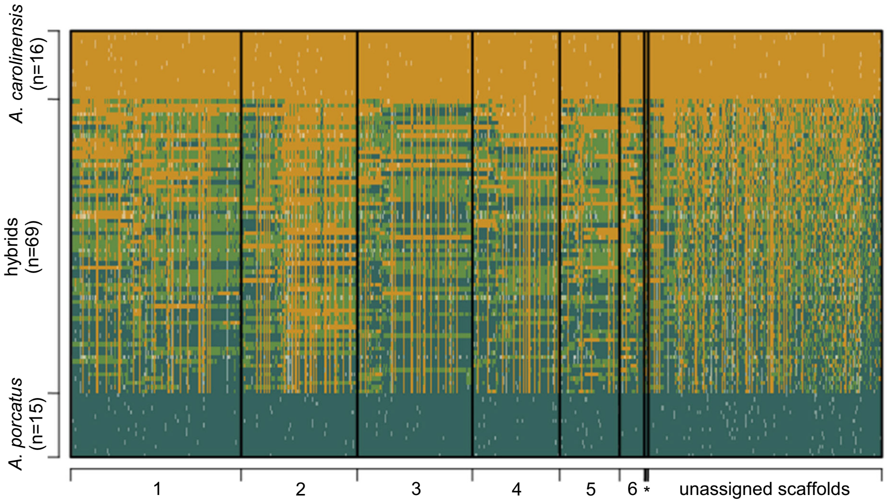

FIGURE 5 Ancestry plot of observed hybrid genotypes at n = 10,269 loci displaying fixed differences between parental species. Columns correspond to loci (ordered by chromosome), while rows correspond to individual lizards. Gold indicates a homozygous *Anolis carolinensis* genotype, teal indicates a homozygous *A. porcatus* genotype, green indicates a heterozygous genotype, and white indicates missing data. The asterisk (*) indicates loci mapped to any of the seven *A. carolinensis* microchromosomes. Loci mapped to the "unassigned scaffolds" bin are currently unplaced within the *A. carolinensis* reference genome and thus are not meaningfully ordered. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Outlier status of 8551 fixed, ancestry-informative loci, as assigned by Bayesian estimation of genomic clines.

Alpha		Beta		Both			
+	−	+	−	$+\alpha -\beta$	$-\alpha -\beta$	Neither	Total
298/3	2595/2501	0/0	811/431	11/0	856/854	3980/2445	8551/6234

Note: Alpha outliers display a greater-than-expected contribution of genetic material from one parental species (positive = excess *A. carolinensis* ancestry, negative = excess *A. porcatus* 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.

as both alpha and beta outliers, 98.7% displayed negative values for both alpha and beta—a combination representing rapid introgression of *A. porcatus* alleles. When mapped to the *A. carolinensis* genome, loci identified as alpha outliers were distributed across all six macrochromosomes (Figure S7a), while those identified as beta outliers were concentrated nearly exclusively on chromosome 3 (Figure S7b,c).

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 ($r = -.759$, $p < .0001$; Figure S8). The LFMM testing the association between canopy cover and genotype (manually adjusted GIF = 0.96; Figure S9a) identified a single significant SNP at position 19,549,307 on chromosome 1 ($q = 0.0176$). A Manhattan plot of the model (Figure S10a) also revealed a series of closely-positioned, near-significant loci on an unassigned scaffold (NW_003338792.1), the clustering of which suggests 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 S9b) identified two loci in close proximity on chromosome 2 (positions 135,319,503 and 135,321,966, Figure S10b) 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 percent canopy cover ($r = -.299$, $p = .013$; Figure 6a) and percent impervious surface area ($r = .267$, $p = .028$; Figure 6b) were significantly correlated with non-native ancestry, with hybrids having lower *A. porcatus* ancestry proportions occurring in more natural habitats (i.e., higher canopy cover and lower impervious surface area). For both predictor variables, each 10% shift in habitat condition was associated with a 2% shift in non-native ancestry. However, we also documented significant spatial autocorrelation among observations for both canopy cover (Moran's $I = 0.610$, $p < .001$) and impervious surface area (Moran's $I = 0.594$, $p < .001$), suggesting spatial nonindependence. After accounting for the confounding effect of geographic location, both environmental predictors were reduced to near-significance ($p = .051$ and $p = .097$; Table 2).

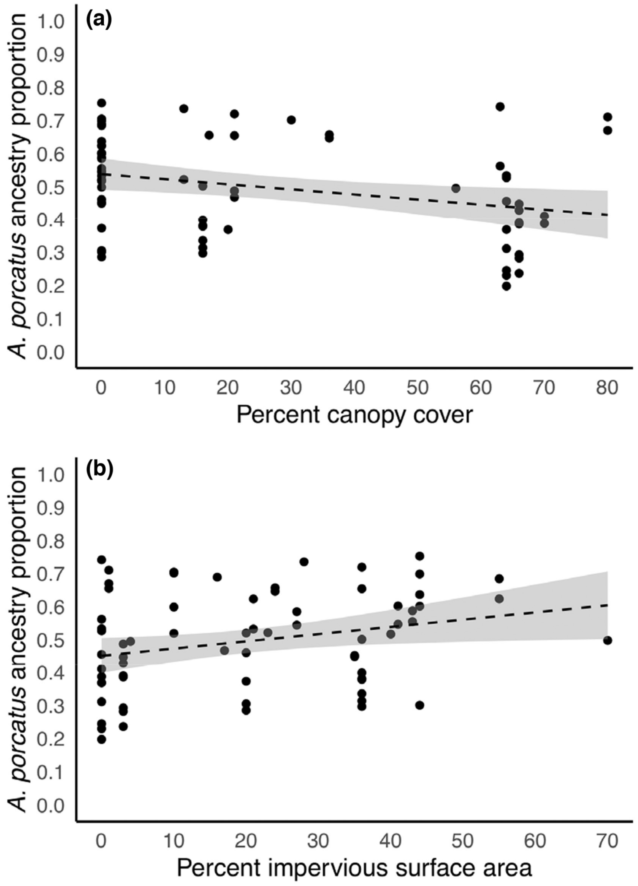

FIGURE 6 Scatterplots, regression lines, and 95% confidence intervals demonstrating correlations (prior to correction for spatial nonindependence) of *A. porcatus* ancestry with canopy cover ($r = -.299$, $p = .013$; (a)) and impervious surface area ($r = .267$, $p = .028$; (b)) for hybrid anoles ($n = 68$). For both environmental predictors, each 10% shift in habitat condition is associated with a 2% shift in non-native ancestry.

4 | DISCUSSION

In this study, we used high-resolution SNP data to describe the nature and extent of hybridization between native *A. carolinensis* and non-native *A. porcatus* 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

TABLE 2 Results from spatial lag models comparing environmental predictors to non-native ancestry proportions of *A. carolinensis* × *A. porcatus* hybrids ($n = 68$).

							Predictor impact					
	Predictor estimate		Rho		Residual autocorrelation		Direct		Indirect		Total	
Ancestry predictor	Value	*p*	Value	*p*	Value	*p*	Value	*p*	Value	*p*	Value	*p*
Canopy cover	−0.001	.051	0.419	<.001	0.209	.648	−0.001	.022	<0.001	.052	−0.002	.027
Impervious surface area	0.001	.097	0.423	<.001	0.303	.582	0.001	.127	<0.001	.156	0.002	.131

both parental species (Wegener et al., 2019). We report rapid introgression of *A. porcatus* alleles at many individual loci, demonstrating that hybrids have retained non-native alleles even in the absence of ongoing immigration of *A. porcatus*. Ultimately, our findings reveal a 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.

4.1 | Hybrid population structure

Both the observed genetic divergence between hybrids and parental genotypes and the total lack of pure *A. porcatus*, F1 hybrids, and recent *A. porcatus* backcrosses among the 70 green anoles sampled support the hypothesis of an historic hybridization event with little to no ongoing *A. porcatus* immigration. Differentiation of the hybrid population along the second PC axis probably corresponds to mutations and/or changes in allele frequencies (e.g., founder effects) that occurred following the original hybridization event at least 34 generations ago. 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. porcatus* was restricted and is no longer occurring, contemporary hybrid anoles must breed either with other hybrids or backcross with native *A. carolinensis*. Backcrossing of hybrid individuals to the native parental species will erode differentiation relative to *A. carolinensis*, while crosses among 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. porcatus* propagule, which was in some way genetically distinct from the *A. porcatus* sampled in our study. While our samples were collected from the western *A. porcatus* 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.

While DAPC split the hybrid individuals between two groups, the observed PCA and DAPC clustering patterns as well as the ancestry proportion estimates generated by STRUCTURE and INTROGRESS all support the conclusion that variation in ancestry within the hybrid population occurs across a continuous gradient rather than in distinct groups. Although the hybrid range boundary is currently unknown, the concentrated occurrence of individuals with higher native ancestry proportions at the peripheral sites sampled in our study suggests that these hybrids are closer to the range boundary and thus more likely to encounter and backcross with pure *A. carolinensis* than individuals located closer to the (putative) centre of the hybrid swarm.

4.2 | 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. porcatus* ancestry in comparison to the average across all AIM loci. Furthermore, a quarter of these loci (10% of all AIMs) displayed both excess *A. porcatus* ancestry and unusually rapid rates of introgression (i.e., negative alpha and 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. porcatus* alleles rather than those of the more abundant, native *A. carolinensis*. Though our sample size was limited, probably contributing to high among-locus variation in cline parameters (Gompert & Buerkle, 2011), we note that the number of admixed individuals we analysed is on par with those reported in recent genomic cline analyses (e.g., Jahner et al., 2021; Zhang et al., 2022). Other characteristics of our study system should facilitate the study of introgression via genomic clines, including the strong allele frequency differences between parental species, the fact that we could easily separate hybrids from parental genotypes (advanced generation hybrids are unlikely to have been erroneously included in the parental groups), and the wide distribution of individual admixture proportions among the hybrids.

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, 2012; McFarlane et al., 2021). We note that two aspects of our genomic cline results are decidedly nonrandom, and therefore consistent with the action of natural selection. First, most alpha outliers in the hybrid genome are negative and correspond to excess introgression of alleles from the rarer species (*A. porcatus*). This finding deviates from both modelled 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 deer (*C. nippon*) in Scotland that do meet both criteria, although 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. porcatus* 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. porcatus* 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).

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 (Gompert et al., 2014, 2017; Mallet, 2007, 2020; Mayr, 1982). To ameliorate the difficulties of assigning hybridizing organisms to discreet "different species" and "same species" categories, hybrid systems should be viewed as representing various intermediate stages of the speciation process (Gompert et al., 2017; Payseur, 2010). Wegener et al. (2019) have suggested revision of the currently paraphyletic *A. porcatus* group such that the western clade of *A. porcatus* is subsumed into *A. carolinensis*. The 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 analysed are contributing to reproductive isolation between *A. carolinensis* and *A. porcatus*. 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. porcatus* 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, particularly when hybridization occurs apparently unimpeded in undisturbed habitats, species status may need to be reassessed.

4.3 | 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 (e.g., Caye et al., 2019). However, we also note that the small number of significant SNPs identified in our study may be linked to low statistical power resulting from use of a hybrid sample set ($n = 68$) smaller than is typical for genome-wide association type analyses. While both the high levels of admixture in our study system and application of ridge penalties in the LFMM analysis help to compensate for low sample size, false negatives resulting from suboptimal power should still be considered a possibility.

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 at the three significant loci suggest that alleles unique to hybrid anoles may play a greater role in environmental adaptation than those associated with either parental species, although parental populations would need to be sampled in greater numbers to confirm that these low-frequency minor alleles are indeed unique to the hybrid population. We also note that rapid adaptation to novel environments is more likely to result from standing genetic variation than from new mutations (Barrett & Schluter, 2008), and that the observed association signal could have been driven by a small number of individuals occurring in unusual habitats. While these results do not disprove our hypothesis that increased temperatures in urban habitats have facilitated the spread of *A. porcatus* alleles, they also provide little support for such a conclusion. Next steps in addressing this hypothesis will require the collection of live anoles for which measures of thermal tolerance, such as critical thermal maximum (CT_{max}), can be compared across the HI spectrum. CT_{max} values should be incorporated into a genome-wide association study to identify loci that may be involved in the genetic regulation of thermal tolerance. The extent to which urban populations of *A. carolinensis* have already adapted to warmer conditions encountered in cities should also be investigated by comparing thermal tolerance and associated genetic polymorphisms between pairs of urban and natural populations in areas where hybridization with *A. porcatus* has not yet occurred.

The association of hybrid genotypes with environmental factors indicative of urbanization was further investigated by a broader exploration of the relationship between genetic ancestry and habitat type. On average, hybrids with higher proportions of *A. porcatus* ancestry were found in habitats with less canopy cover and more impervious surface area than were individuals with less *A. porcatus* ancestry. These differences may result in relatively warmer microhabitats for hybrids with more non-native alleles (Battles & Kolbe, 2019), which seem to be most abundant in anthropogenically disturbed habitats. However, neither relationship remained significant after accounting for spatial autocorrelation. The near-significance of both predictor variables suggests that significant correlations may be revealed by increased sample sizes or sampling of more variation in the predictor variables. It also remains possible that any existing relationship between environment and ancestry may be driven by dispersal patterns of hybrids from one or more points of introduction rather than adaptation to thermal extremes; indeed, this scenario could account for the observed influence of spatial autocorrelation on model results.

4.4 | 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 major conservation concern in and of itself. However, our observations could be informative in systems where hybridization is of concern. We demonstrate that genetic material from a non-native species introgresses rapidly and persists in native populations even after (1) pure parental individuals of the introduced species are no longer present and (2) immigration of additional non-native individuals has ceased. Such introgression may occur randomly as an unpredictable outcome of genetic drift (McFarlane et al., 2021), but in this system is 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, the potential ecological benefits of admixture should also be considered on a case-by-case basis; in certain existing hybrid zones, adaptive alleles inherited from a resilient invader may facilitate the persistence of native species that would otherwise succumb to the pressures of ever-accelerating global environmental change (Fitzpatrick & Shaffer, 2007a; Hamilton & Miller, 2016; Oziolor et al., 2019; Pardo-Diaz et al., 2012).

Through detailed examination of the unique *A. carolinensis* × *A. porcatus* 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 non-native alleles into a large native population. Rather, the limitation of low propagule pressure may be overcome by selection favouring the persistence of non-native alleles. While our findings enhance current understanding of the roles that demographic 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* × *A. porcatus* hybrid system. While various studies have reported associations between anthropogenic disturbance and the spread of non-native 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.

AUTHOR CONTRIBUTIONS

Tyler B. DeVos, Dan G. Bock, and Jason J. Kolbe contributed to study design. Laboratory work and analyses were performed by Tyler B. DeVos with instruction from Dan G. Bock. The manuscript was written by Tyler B. DeVos with input from Dan G. Bock and Jason J. Kolbe.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw sequence data used in this study are accessible in the NCBI Sequence Read Archive (SRA) under BioProject ID: PRJNA931787 (Accession nos SAMN33099350–SAMN33099450). All code used for analyses has been made available in a publicly accessible GitHub repository located at https://github.com/tylerdevos/green_anole_hybridization_2023.

ORCID

Tyler B. DeVos https://orcid.org/0000-0001-8302-2560
Dan G. Bock https://orcid.org/0000-0001-7788-1705

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