

POPULATION DYNAMICS IN A MID-ATLANTIC BREEDING POPULATION OF KING RAILS  
(*Rallus elegans*) AND COMPARATIVE GENOMIC DIVERSITY ACROSS THEIR RANGE

by

Carol A. Gause

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Directors of Thesis: Dr. Susan B. McRae and Dr. Christopher N. Balakrishnan

Major Department: Biology

King rail (*Rallus elegans*) populations have experienced severe declines throughout their range over the past half a century. As an obligate freshwater marsh bird, the king rail relies upon extensive, high-quality wetlands for survival and reproduction. Due to anthropogenic activities such as urban and agricultural development, the extent of suitable freshwater emergent marsh habitat has dramatically decreased— most notably in inland regions of the eastern United States. Additionally, sea level rise and an increase in frequency and intensity of storms due to climate change have led to more instances of saltwater inundation in coastal freshwater marshes, which can render these habitats unusable for king rails. The king rail remains relatively understudied compared to other more conspicuous threatened species, and it is becoming increasingly urgent to better understand this cryptic bird. This thesis presents findings from a regional survey of a mid-Atlantic source population of king rails and a comparison of population-level genetic diversity and structure across four distinct geographic populations using whole-genome sequencing.

In Chapter 1, I discuss the findings of a regional census encompassing a suspected mid-Atlantic source population at Mackay Island National Wildlife Refuge (NWR) that was last surveyed over 10 years ago. During the breeding season within this region, I conducted surveys of king rails and dominant wetland vegetation cover types in both managed and unmanaged coastal marshes. I compared occupancy and abundance between managed and unmanaged sites and employed model selection techniques to identify predictive factors. The analysis

revealed positive correlations of king rail occupancy and abundance with land management practices. Occupancy and abundance were negatively correlated with the percentage of survey plot cover by *Schoenoplectus* sp. and by woody vegetation (trees and shrubs). The effect size of *Schoenoplectus* sp. as a model covariate was driven by three specific survey points. Temporarily removing these points as an exploratory measure resulted in *Schoenoplectus* sp. no longer being a covariate in the top models. Using a Royle-Nichols Abundance-Induced Heterogeneity Model, I estimated the number of breeding individuals within the surveyed area to be  $437 \pm 218$ .

In Chapter 2, I compare genetic variation among migrant and resident king rail populations across the species range: two from the Atlantic flyway (a breeding population in coastal North Carolina and a population from Florida), and two breeding populations from the Mississippi flyway (northern Ohio and southeastern Arkansas). Samples from inland populations were provided by collaborators, and those from Florida were archived museum specimens. Whole genomes of 56 individual king rails were sequenced using the Illumina NovaSeq 6000 platform. Population-level genetic diversity was investigated using pairwise fixation index ( $F_{ST}$ ) comparison, nucleotide diversity ( $\pi$ ), and Watterson's estimator ( $\theta$ ). Population structure was analyzed using principal component analysis (PCA), discriminant analysis of principal components (DAPC), and population admixture.

The results uncover substantial gene flow and minimal population structure among king rail populations, with consistent genetic diversity estimates falling within the intermediate range. While these analyses show no clear geographic relevance in range-wide population structure, the DAPC reveals evidence of weak structure with geographic correlation. Caution is advised in interpreting these findings, as the sampled populations represent strongholds for the species in the face of significant loss of freshwater wetland habitat. These results emphasize the critical role of remaining king rail population strongholds within managed freshwater wetlands and underscore the importance of ongoing king rail conservation research.



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Carol A. Gause

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Directors of Thesis:

Dr. Susan B. McRae, Ph.D. and Dr. Christopher N. Balakrishnan, Ph.D.

Thesis Committee Members:

April M. H. Blakeslee, Ph.D.

Michael S. Brewer, Ph.D.

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by  
Carol A. Gause

APPROVED BY:

DIRECTOR

OF THESIS: \_\_\_\_\_

Susan B. McRae, Ph.D.

DIRECTOR

OF THESIS: \_\_\_\_\_

Christopher N. Balakrishnan, Ph.D.

COMMITTEE MEMBER: \_\_\_\_\_

April M. H. Blakeslee, Ph.D.

COMMITTEE MEMBER: \_\_\_\_\_

Michael S. Brewer, Ph.D.

CHAIR OF THE

DEPARTMENT OF BIOLOGY: \_\_\_\_\_

David R. Chalcraft, Ph.D.

DEAN OF THE

GRADUATE SCHOOL: \_\_\_\_\_

Kathleen T. Cox, Ph.D.

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# CHAPTER 1: HABITAT ASSOCIATIONS AND DISPERSAL OF KING RAILS IN A MID-ATLANTIC BREEDING POPULATION

## Abstract

Microhabitat selection and movement of secretive birds is critical for understanding their habitat, foraging, and nesting requirements. The king rail (*Rallus elegans*) is an obligate freshwater marsh bird that is difficult to study due to its secretive nature. King rail populations have been declining across the species' range for over a century, heightening concern for their persistence and leading to increases in conservation management within many state and federal lands. This chapter investigates the effect of land management on king rail occupancy and abundance at Mackay Island NWR and in the surrounding region. This project builds on previous studies at this site by providing estimates of the breeding population density of king rails in surveyed areas of the region. I began by surveying freshwater marshes within a 20-mile radius of Mackay Island NWR at sites surrounding Back Bay, in the tidewater region of Virginia, and the northern Currituck Sound in North Carolina. These sites were last surveyed systematically for king rails in 2010. Using species-specific, standardized call-broadcast surveys to detect king rails, and vegetation surveys to estimate wetland cover types, I compared occupancy and abundance at managed and unmanaged sites. I conducted model selection to identify factors that predict occupancy and abundance. I found that king rail occupancy and abundance was related to land management and to percent cover of dominant vegetation types (*Schoenoplectus* sp. and woody vegetation such as trees and shrubs). The effect size of *Schoenoplectus* sp. was substantially influenced by three specific survey points, and temporarily removing these points as an exploratory measure led to *Schoenoplectus* sp. no longer being a covariate in the top models. Based on a Royle-Nichols model, I estimated the number of breeding individuals to be  $437 \pm 218$  within the surveyed area.

## Introduction

The king rail is a secretive freshwater marsh bird whose populations have been declining throughout North America for over 50 years at a rate of 6.7% per year (Cooper 2008). The range of the king rail extends from the Atlantic coast of North America throughout the Midwest. As an obligate freshwater marsh bird, the king rail depends upon large tracts of high-quality wetland habitat with low levels of salinity for survival and reproduction (Taylor and Van Perlo 1988, Glisson et al. 2015). However, the extent of suitable freshwater emergent marsh habitat has dramatically decreased due to anthropogenic activities such as urban and agricultural development (Eddleman et al. 1988, Dahl 2011), particularly in inland regions of the eastern United States (Bolenbaugh et al. 2012). One report, prepared for the Mississippi Flyway Council, estimated as few as 137-443 king rail pairs within the entirety of the North American Midwest, including sections of Canada (Russell 2004). Globally, the king rail population is estimated to consist of 69,000 individuals (BirdLife International 2023).

Due to the drastic decrease in inland marsh habitat, king rail populations have become concentrated along the southeastern coasts of North America where many are year-round residents (Bolenbaugh et al. 2012, Kolts and McRae 2017). In these coastal freshwater marshes, sea level rise and an increase in frequency and intensity of storms due to climate change have led to more instances of saltwater inundation (IPCC 2023). This can render these marsh habitats unsuitable for king rails by changing vegetation composition and prey abundance (Romañach et al. 2019) and can lead to displacement or ecological selection against king rails (Maley 2012, Brinker et al. 2002, Coster et al. 2018). The king rail is listed as federally endangered in Canada and as threatened or endangered by 12 states in the United States (Kraus 2016, BirdLife International 2023).

Since population declines are attributed mainly to human-caused habitat loss, many of the current recommendations for king rail conservation involve increasing extents of contiguous

marsh habitat and climate change mitigation (Cooper 2008, Bolenbaugh et al. 2012, Brewer et al. 2023). Other suggestions have focused on preserving and improving freshwater marsh quality. Artificial land leveling, for example, is strongly advised against because king rails prefer heterogeneous landscapes at a large scale, and particularly areas that promote diversity in plants, soil, and hydrology (Bolenbaugh et al. 2012). A plan created by the U.S. Fish and Wildlife Service recommended splitting conservation planning into different regions depending upon geography and habitat (Cooper 2008). These regions would focus on improving habitat in targeted ways specific to the threats faced by king rails in that area. Across all managed sites, occasional disturbances such as flooding and prescribed burning are suggested as a means of improving freshwater marsh habitat quality for the benefit of king rail populations (Bolenbaugh et al. 2012).

At this study's focal site, Mackay Island National Wildlife Refuge (NWR), these techniques are used to keep marsh habitat at an early successional state, control invasive *Phragmites australis*, and maintain freshwater tidal marshes and impoundments that support a large breeding population of king rails. Specific management strategies include prescribed fire (a 3- to 5-year rotational burn strategy), herbicide application, and raising or drawing down water levels in impoundments at strategic times in the year. The breeding population of king rails at the refuge has been extensively studied and is suspected to serve as a source population for the broader region (Sullivan et al. 2009, iNaturalist 2023, S. McRae unpubl.) More than a decade of research conducted at this refuge has not only yielded valuable insights about this breeding population but has also yielded a wealth of information about the ecology, behavior, and genetics of the species (Brackett et al. 2013, Clauser and McRae 2017, Schroeder and McRae 2018, Johnson and McRae 2022). This research has also led to adjustments in refuge wetland management practices, such as modifications to impoundment

drainage schedules and the implementation of patchy prescribed burn strategies (Kolts and McRae 2017).

The goals of this study were to 1) relate king rail occupancy and abundance to habitat features and wetland management, and 2) to complete an updated census of the region. The surveyed areas included coastal marsh sites along the intra-coastal waterway and Back Bay within 20 miles of Mackay Island NWR (including Back Bay NWR, False Cape State Park, and North Landing River in Virginia in addition to sites in Currituck, North Carolina). Many of these locations were last surveyed for king rails in 2010 by Rogers et al. (2013).

We used standardized call-broadcast survey protocols and vegetation surveys during the peak king rail breeding season in the region. Call-broadcast surveys were adapted from the Standardized North American Marsh Bird Monitoring Protocol (Conway 2011) and adhered to the methods outlined in this protocol. Vegetation surveys were used to gather data on the dominant species with the goal of improving our understanding of the relationship between cover type and king rail occupancy. These surveys were conducted towards the end of the king rail breeding season when the vegetation had fully matured. We visually estimated the dominant vegetation cover types within each survey plot. Following survey data collection, I used occupancy modelling to better understand how management and habitat variables impact king rail occupancy and abundance. Finally, I estimated breeder density and population size for the areas that were surveyed. My results will inform wetland management at regional refuges in support of king rail conservation.



## Methods

### *Study site characteristics and management*

This study was conducted in freshwater marshes within, and around, Mackay Island NWR (36° 31' N, 75° 58' W), located by North Carolina's eastern-most border with Virginia. Mackay Island NWR consists of natural tidal marshes and four impounded wetlands. The water level within impoundments is raised in the winter for waterfowl use and drawn down in the spring to promote the growth of early successional vegetation. The USFWS enacts additional wetland management techniques at this site: herbicide application (a mixture of glyphosate and imazapyr, A. Bennitch, pers. comm.), and rotational prescribed burning (Rogers 2013). Both strategies are used to control the spread of invasive *Phragmites australis* that covers large tracts of land throughout the region. Prescribed burning also reduces encroachment of woody vegetation, thins emergent vegetation, and keeps the marsh at an early successional state. We surveyed a privately owned wetland on Knotts Island, located 3 km to the northeast of Mackay Island NWR, that is managed with similar objectives. This tract includes one impoundment and surrounding natural marsh that is managed for overwintering waterfowl. The landowner mechanically removes invasive *Phragmites australis* from the dike surrounding an impoundment using mowing equipment (T. Yarborough, pers. comm.).

Mackay Island NWR is surrounded by Currituck Sound and Back Bay, connected shallow oligohaline bodies of water. These water bodies are completely separated from the Atlantic Ocean by barrier islands. They form the northern extremity of North Carolina's intracoastal waterway and are fed by freshwater rivers. Due to being subject to wind tides, the water level rises in the natural marshes surrounding the sound whenever there is a strong south wind.

We surveyed two additional managed sites north of Mackay Island NWR: False Cape State Park and Back Bay NWR. These sites are adjacent to one another on the barrier island

north of the Virginia border. Due to their proximity, management at the state park and refuge is often coordinated. Invasive plant species, such as *Phragmites australis*, are controlled with herbicide (imazapyr and glyphosate, L. Mowbray, pers. comm.) as well as prescribed fire. Back Bay NWR consists of natural marsh subject to wind tides, and 13 impoundments that are managed for bird populations throughout the year. Similarly, False Cape State Park consists of natural wind-tide-influenced marshes and two impoundments managed with the goal of supporting bird populations.

In addition to these sites, we also selected survey plots in publicly accessible areas within 20 mi. of Mackay Island NWR, natural wetlands along North Landing River and the western edge of the Currituck Sound. Specific areas were selected for surveying based on visual assessment of habitat suitability. These survey plots were in areas that were not actively managed. A total of 58 points were surveyed among all sites (Figure 1.1).



**Figure 1.1** Field sites where call-broadcast surveys were conducted. Gray dots represent survey plots. Unmanaged, publicly accessible sites included North Landing River (A) and areas along the western edge of the Currituck Sound (C). Sites with land management activity included Back Bay NWR and False Cape State Park (B) and Mackay Island NWR and privately-owned wetlands (D).

### *Sampling Design*

During the peak of the king rail breeding season, from late April through early July of 2022, we conducted surveys for breeding king rails adapted from the Standardized Marsh Bird Monitoring Protocol (Conway 2011) at wetlands within a 20-mile radius of Mackay Island NWR. Surveys were conducted at 58 points along roads and waterways. Survey points were at least 400 m apart, to reduce the probability of sampling the same individuals. At Back Bay NWR, False Cape State Park, Mackay Island NWR, and the managed private land on Knotts Island, I selected survey points that were close to the random points sampled in 2009-10 (Rogers et al. 2013). These points were sampled from the road access points rather than walking into the marsh. This was, in part, to maximize the chances of detecting king rails and to expand the pool of survey points that could be effectively covered within a limited time window.

At other sites, points were selected in extents of open emergent vegetation of at least 40 hectares that were broadly similar in composition to occupied areas in the focal study area (Kolts and McRae 2017). The selection of survey plots was constrained by several factors, including the quality and availability of habitat, accessibility, and the specific timeframe for conducting surveys. Due to these limitations, there were no sites surveyed to the east of Mackay Island NWR. A lack of resources (e.g., boat training) and the limited availability of refuge staff prevented us from accessing eastern sides of the sound along the barrier island, such as Currituck NWR.

We visited each survey point three times at intervals of no fewer than ten days. Standardized call broadcasts included periods of passive listening followed by recordings of three king rail calls in the following order: *grunt*, *kek*, and *kek-burr*. There

was a period of 15 seconds of silence between calls. The *grunt* call preceded *kek* calls due to research that suggests *grunt* calls (used for contact and neighbor recognition) are more likely to elicit a call response compared to *keks* that are used in mating and territorial contexts (Schroeder and McRae 2019). Surveys occurred within the window of time when marsh birds were most active (Conway et al. 2004, Schroeder and McRae 2020). Morning surveys were conducted between 30 minutes prior to sunrise and 2 hours after. Evening surveys occurred from 2 hours prior to sunset until 30 minutes after. The sound pressure of the played calls was 80-90 dB measured at 1 m in front of the speaker using a sound level meter mobile application (National Institute for Occupational Safety and Health, 2023).

In addition to recording detections during surveys, we also collected information about distance and direction of individual responding king rails in addition to response call type. Distance was determined by visually estimating the distance to landmarks (*e.g.*, distinct vegetation or snags) where the call was thought to originate. There were 1-6 trained observers for each survey (3 observers per survey on average). In cases where observers provided varying distance estimates, an average distance was calculated. We surveyed during favorable weather conditions to increase detection likelihood. Five sample survey-specific covariates that could impact detection were measured during each survey: the number of observers (1-6), wind (Beaufort scale), temperature (°C, The Weather Company 2023), sky conditions (a standardized scale of 0-8 to consider general sky conditions), and background noise (on a scale of 0-4).

Two categories of site covariates were recorded at each plot: vegetation composition (land cover) and the presence of land management activity (Table 1.1). At the end of the breeding season, once vegetation was well-established (June 23<sup>rd</sup> through July 18<sup>th</sup>), we visually approximated vegetation based on dominant species composition as a percentage of plot coverage (200 m radius from a survey point). Additionally, the mean height of herbaceous vegetation was visually estimated. Land management was categorized as a binary variable (0 =

no management, 1 = management) due to the similar management practices employed across all managed sites.

**Table 1.1** Model covariates and their descriptions.

Covariate	Description
<i>Juncus</i>	Percent cover of black needlerush ( <i>Juncus</i> ). Co-occurs with few wetland plant species and can form monocultures over large areas (Hosier 2018); grows where the water table is close to the soil surface or in standing water. King rails are known to nest in stands of black needlerush, using it as nesting material (Meanley 1969).
<i>Typha</i>	Percent cover of a plot by cattail species ( <i>Typha</i> sp.) — primarily <i>Typha latifolia</i> and <i>Typha angustifolia</i> . Freshwater wetland species that prefer waterlogged soils or standing water (Hosier 2018). King rails are known to nest in stands of cattail as well as use it as nesting material (Meanley 1969).
<i>Phragmites</i>	Percent cover of a plot by invasive common reed ( <i>Phragmites australis</i> ). Grows up to 3.65 m tall and clonal growth results in nearly impenetrable stands (Hosier 2018). King rails have never been found to nest in common reed, though they have been observed using it for shelter (Kolts and McRae 2017).
<i>Schoenoplectus</i>	Percent cover of bulrush, most notably common three-square bulrush ( <i>Schoenoplectus pungens</i> ). Common three-square can be found in freshwater marshes and prefers to grow in standing water (Albert et al. 2013). There are some observations of king rail nests built in patches of common three-square at Mackay Island NWR (S. McRae, unpubl.).
Grasses and sedges	Percent cover of a plot by various genera of grasses and sedges (e.g., <i>Spartina</i> sp. and <i>Eleocharis</i> sp.) that did not individually constitute a large portion of the area (fewer than 5 plots had $\geq 10\%$ cover of grasses and sedges).
Woody	Percent cover of a plot by trees and shrubs. Some notable species include wax myrtle ( <i>Myrica cerifera</i> ), groundsel tree ( <i>Baccharis halimifolia</i> ), and pine trees ( <i>Pinus</i> sp.).

**Table 1.1** Continued.

Covariate	Description
Mixed emergents	Percent cover by various herbaceous emergents, often growing as mats. Notable species include water pennyworts ( <i>Hydrocotyle</i> sp.), alligator weed ( <i>Alternanthera philoxeroides</i> ), and American water plantain ( <i>Alisma subcordatum</i> ). This category consists of aquatic emergent species and did not constitute a large portion of the area (fewer than 5 plots had $\geq 10\%$ cover of that specific genus).
Management	A binary variable (0 = no management, 1 = management) due to the similar management practices employed across all managed sites.
Wind	Beaufort scale (0 = smoke rises vertically, 1 = wind direction shown by smoke drift, 2 = leaves rustle, 3 = leaves and small twigs in constant motion, 4 = raises dust and loose paper, 5 = small trees sway and crested wavelets appear on inland waters).
Noise	Background noise as a scale (0 = no noise, 1 = faint noise, 2 = moderate noise such that some birds cannot be heard beyond 100 m, 3 = loud noise such that some birds cannot be heard beyond 50 m, 4 = intense noise such that some birds cannot be heard beyond 25 m).
Temperature	Temperature recorded at the start of the callback survey from The Weather Channel (in °F, converted to °C).
Sky	Sky conditions scale (0 = clear or few clouds, 1 = partly cloudy, 2 = cloudy or overcast, 3 = fog or smoke, 4 = slight rain/ drizzle, 5 = snow, 8 = rain showers).
Observers	The number of trained observers participating in a survey (range: 1-6 individuals).



### *Occupancy and abundance models*

I used the software PRESENCE (version 2.13.47) in addition to RPresence (version 2.13.52) and the package unmarked (version 1.3.2) in R (version 4.3.1) to fit two models to the data: a simple, single-season, single-species model and a Royle-Nichols Abundance-Induced Heterogeneity Model (Hines 2006, Chandler et al. 2023, MacKenzie et al. 2002, Royle and Nichols 2003). These models were chosen to estimate occupancy probability ( $\psi$ ), mean site abundance ( $\hat{\lambda}$ ), and site population size ( $\hat{N}$ ). The assumptions for a simple occupancy model include:

- 1) Sites were closed to immigration or emigration.
- 2) There were no false detections.
- 3) Detections across sites were independent.

We addressed assumptions by conducting surveys during the peak breeding season when king rail pairs had established territories (assumption 1), training all observers in king rail identification (assumption 2), and separating survey points by at least 400 m (assumption 3). In addition, the Royle-Nichols model assumes that abundance follows a Poisson distribution such that inherent detection probability ( $\hat{p}$ ) increases when there are more individuals at a site ( $N_i$ ).

Data were organized into separate sets: detection histories, site covariates, and sample covariates. The data were re-formatted for analysis in PRESENCE and R. Detection histories were transformed into presence-absence (also known as detection/non-detection) format. A “0” indicates no detection at a survey plot for a given survey instance, and a “1” indicates that there was at least one positive detection. I used the scale function in R to standardize and z-transform all covariates prior to importing data into the software PRESENCE for modeling.

Results were subsequently validated in R using the packages unmarked and RPresence to model occupancy and abundance. MuMIn (version 1.47.5) was used to rank models and

AICcmodavg (version 2.3-2) was used for model selection and multimodal inference (Bartoń 2023, Mazerolle 2023). Model averaging was completed using packages RPresence and AICcmodavg. I used the dredge function to evaluate all possible combinations of models and rank the output by AICc. The simple occupancy and Royle-Nichols candidate models that could best explain occupancy probability were gathered into corresponding subsets.

To evaluate support for models and the strength of each covariate's effect on occupancy and abundance, I used a corrected Akaike's Information Criterion (AICc) due to sample size (defined here as the number of survey points multiplied by three visits) being small relative to the number of parameters being evaluated (Burnham and Anderson 2004, Hurvich and Tsai 1989). Models with  $\Delta AICc \leq 2$  from the top model were considered acceptable. Due to model selection uncertainty caused by a low-weighted top model, I used full-model averaging (Symonds & Moussalli 2011, Lukacs et al. 2009) to evaluate the effect sizes of covariates ( $\beta$ ) in the top models. Covariate effect sizes ( $\beta$ ) are reported with 85% and 95% confidence intervals, as recommended by Sutherland et al. (2023) for a more suitable representation of parameter estimate uncertainty. I used a Mackenzie-Bailey goodness-of-fit test to assess model fit. This test was chosen over a Pearson's chi-square test because the Pearson test can produce inflated and misleading results, particularly when assessing fit in occupancy models with covariates (MacKenzie and Bailey 2004).

I estimated the number of king rails ( $\hat{N}$ ) specifically within the surveyed area of each field site by multiplying the number of sites surveyed by the estimated mean site abundance ( $\hat{\lambda}$ ) derived from the Royle-Nichols model. King rail site densities were then estimated for each site by dividing  $\hat{N}$  by the total surveyed area. Total area was calculated by multiplying the area of a 200-m radius plot by the total number of survey points at a site. Occupancy probability ( $\psi$ ) was derived from the best Royle-Nichols model due to its better fit of the data. Given the shared wetland management activity of Back Bay National Wildlife Refuge (NWR) and False Cape

State Park, these two locations were combined for the purpose of this analysis. These estimates are reported with standard error ( $\pm$  SE) and should not be extrapolated beyond our surveyed area.

To further test the strength of the covariate, *Schoenoplectus* sp., all modeling steps were repeated after the removal of three survey points from the dataset that were outliers. These points had a relatively high estimated percentage of *Schoenoplectus* sp. (> 30%) relative to all other survey points in the region. One point was located at Back Bay NWR and two in False Cape State Park. Other survey points had no greater than 20% of this bulrush.

## Results

### *Occupancy and abundance*

The dredge function returned 4,096 simple occupancy candidate models, of which 5 models had  $\Delta AICc \leq 2$  from the top model (Table 1.3). Software PRESENCE, RPresence, and unmarked all ranked the same top model; the model including the variables land management, *Schoenoplectus* sp. cover, and woody vegetation cover best explained king rail occupancy ( $AICw=0.31$ ). King rail presence was positively correlated with land management and negatively related to *Schoenoplectus* sp. cover and woody vegetation cover. This model was chosen over the other candidates with strong support because model averaging resulted in lower effect sizes for some parameters. These included the grasses and sedges covariate and all sample covariates (Table 1.4). All parameters included in the top model retained effect sizes after model averaging, making the top model the most supported in addition to the most parsimonious. Despite this, model fit was assessed to be poor based on the Mackenzie-Bailey goodness-of-fit

test ( $\chi^2=18.35$ ,  $p=0.003$ ), suggesting that there is unmodeled heterogeneity in detection probability.

**Table 1.2** Model notation for candidate models of king rail site occupancy and abundance.

Model notation	Description
$\Psi(M + S + W)$	The probability that occupancy is influenced by land management, represented as a binary variable (0 denoting unmanaged land and 1 indicating managed land), along with the percentage of landcover consisting of <i>Schoenoplectus</i> sp. and woody vegetation ( <i>i.e.</i> , trees and shrubs).
$\Psi(M + S + W + G)$	The probability that occupancy is influenced by land management, represented as a binary variable (0 denoting unmanaged land and 1 indicating managed land), along with the percentage of landcover consisting of <i>Schoenoplectus</i> sp., woody vegetation ( <i>i.e.</i> , trees and shrubs), and diverse grasses and sedges.
$p(\cdot)$	Constant detection probability.
$p(\text{Wind})$	Detection probability as a function of wind speed (Beaufort scale).
$p(\text{Sky})$	Detection probability as a function of cloud cover (ordinal variable on a scale of 0-2).
$p(\text{Noise})$	Detection probability as a function of the level of background noise (ordinal variable on a scale of 0-2).
$\lambda(M + S + W)$	The probability that abundance is influenced by land management, represented as a binary variable (0 denoting unmanaged land and 1 indicating managed land), along with the percentage of landcover consisting of <i>Schoenoplectus</i> sp. and woody vegetation ( <i>i.e.</i> , trees and shrubs).

**Table 1.2** Continued.

Model notation	Description
$\lambda(M + S + W + G)$	The probability that abundance is influenced by land management, represented as a binary variable (0 denoting unmanaged land and 1 indicating managed land), along with the percentage of landcover consisting of <i>Schoenoplectus</i> sp., woody vegetation ( <i>i.e.</i> , trees and shrubs), and diverse grasses and sedges.
$r(.)$	Constant inherent detection probability.
$r(\text{Wind})$	Inherent detection probability as a function of wind speed (Beaufort scale, range=0-4).
$r(\text{Sky})$	Inherent detection probability as a function of cloud cover (ordinal variable, range=0-2).
$r(\text{Noise})$	Inherent detection probability as a function of the level of background noise (ordinal variable, range=0-2).
$r(\text{Temp})$	Inherent detection probability as a function of the temperature (°C).

**Table 1.3** Candidate models for a simple, single-season occupancy analysis ranked by AICc value. Site covariates include vegetation type percentages and management as a binary variable. Sample covariates include sky (range = 0-2), noise (range = 0-2), and wind (Beaufort scale, range = 0-4). AICw is a measure of relative model likelihood. For model notations, see Table 1.2.

Model	AICc	$\Delta$ AICc	AICw	-2*log-likelihood
$\Psi(M + S + W), p(.)$	202.79	0.00	0.31	192.43
$\Psi(M + S + W + G), p(.)$	203.42	0.63	0.23	190.43
$\Psi(M + S + W + G), p(\text{Sky})$	203.91	1.12	0.18	191.41
$\Psi(M + S + W), p(\text{Wind})$	204.15	1.36	0.16	191.65
$\Psi(M + S + W), p(\text{Noise})$	204.53	1.74	0.13	189.86

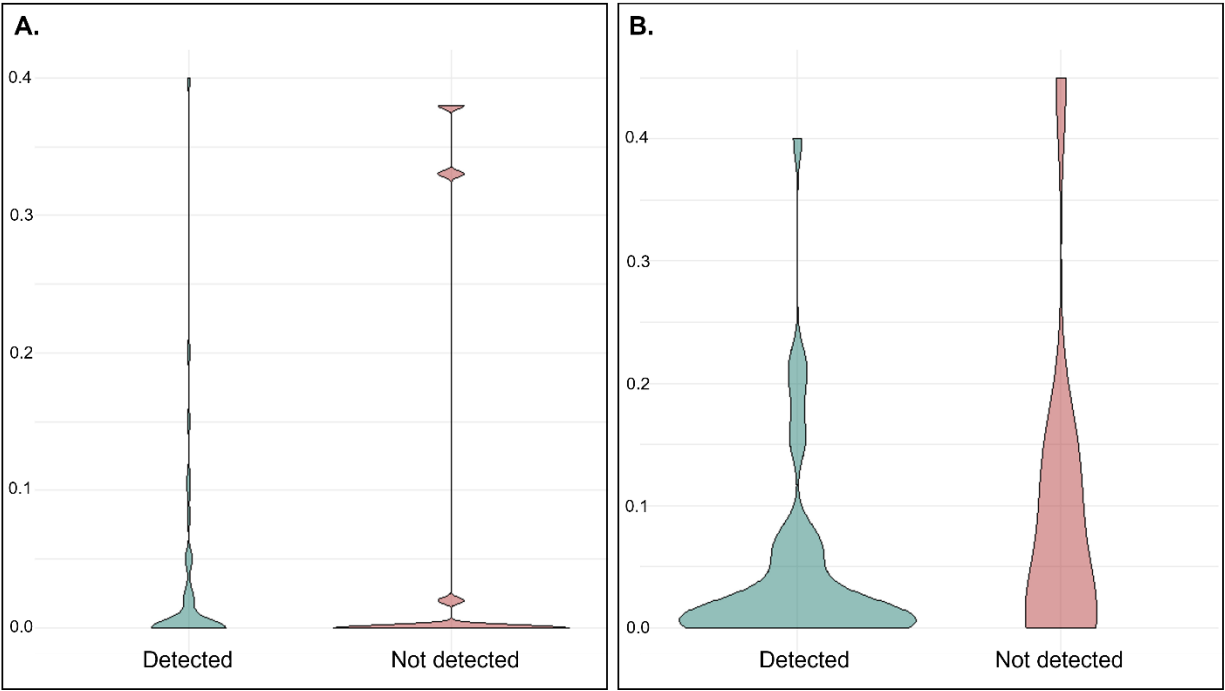
**Table 1.4** Results of full model averaging for covariate coefficients ( $\beta$ ) for the top simple occupancy model. “True  $\beta$ ” refers to the model-averaged estimates that account for model uncertainty, and “Subset  $\beta$ ” refers to estimates from the top five models.

	$\Psi(M)$	$\Psi(S)$	$\Psi(W)$	$\Psi(G)$	$p(\text{Sky})$	$p(\text{Wind})$	$p(\text{Noise})$
True $\beta$	5.78	-2.75	-2.85	-0.11	0.05	0.02	-0.02
Subset $\beta$	5.78	-2.75	-2.85	-0.55	0.23	0.17	-0.16



To account for heterogeneity in detection probability, I used a Royle-Nichols model to estimate mean king rail site abundance ( $\hat{\lambda}$ ). The model that accounted for the most variation among surveys and sites included management, *Schoenoplectus* sp., and woody vegetation as site covariates (AIC $\omega$ =0.19; Table 1.5). According to model coefficients, king rail abundance is positively associated with land management activity and negatively associated with *Schoenoplectus* sp. and woody vegetation. The effect size ( $\beta$ ) of the covariate management is  $\beta=0.92 \pm 0.17$  (95% CI=0.58- 1.24; 85% CI=0.67- 1.16), *Schoenoplectus* sp.  $\beta=-0.25 \pm 0.13$  (95% CI=-0.51 - 0.02; 85% CI=-0.45 - -0.06), and woody vegetation  $\beta=-0.37 \pm 0.14$  (95% CI=-0.63 - -0.10; 85% CI=-0.56 - -0.17). Of survey points within managed sites, 38 out of 41 sites (93%) had at least one king rail detection across three survey visits. However, only 8 of 17 survey points (47%) within unmanaged sites had at least one king rail detection. There were higher percentages of *Schoenoplectus* sp. and woody vegetation within a few plots that had no king rail detections (Figure 1.2). Following the recommendations of Sutherland et al. (2023), the *Schoenoplectus* sp. and woody covariates were kept in the top model due to the 85% CI not overlapping zero. A MacKenzie-Bailey goodness-of-fit test showed that model assumptions were met ( $\chi^2= 9.09$ ,  $p= 0.13$ ).

The calculated occupancy probability ( $\psi$ ) adjusts for imperfect detectability during surveys. Occupancy probability for all survey plots within Back Bay National Wildlife Refuge (NWR) and False Cape State Park was estimated to be  $0.99 \pm 0.02$ . Similarly, the estimated occupancy probability for all surveyed sites within Mackay Island NWR was  $0.99 \pm 0.01$ . The privately owned wetland was estimated to have a near perfect estimated occupancy probability of  $1.00 \pm 0.001$ , but it represented only four points surveyed in a small area (~50 ha). Collectively, the unmanaged public access sites had a lower estimated occupancy probability of  $0.85 \pm 0.27$ . The survey sites that used wetland management techniques (Back Bay NWR, False Cape State Park, Mackay Island NWR, and the privately owned wetlands) had greater



**Figure 1.2** Distribution of plot coverage percentage by **A.** *Schoenoplectus* sp. or **B.** woody vegetation. The “Detected” category includes survey points with at least one positive king rail detection from among three survey visits, whereas “Not detected” denotes survey points with no detections.

**Table 1.5** Royle-Nichols Abundance-Induced Heterogeneity candidate models ranked by AICc value. Site covariates include vegetation type percentages and management as a binary variable. Sample covariates include sky (range =0-2), noise (range =0-2), and wind (Beaufort scale, range =0-2). For model notation, see Table 1.2.

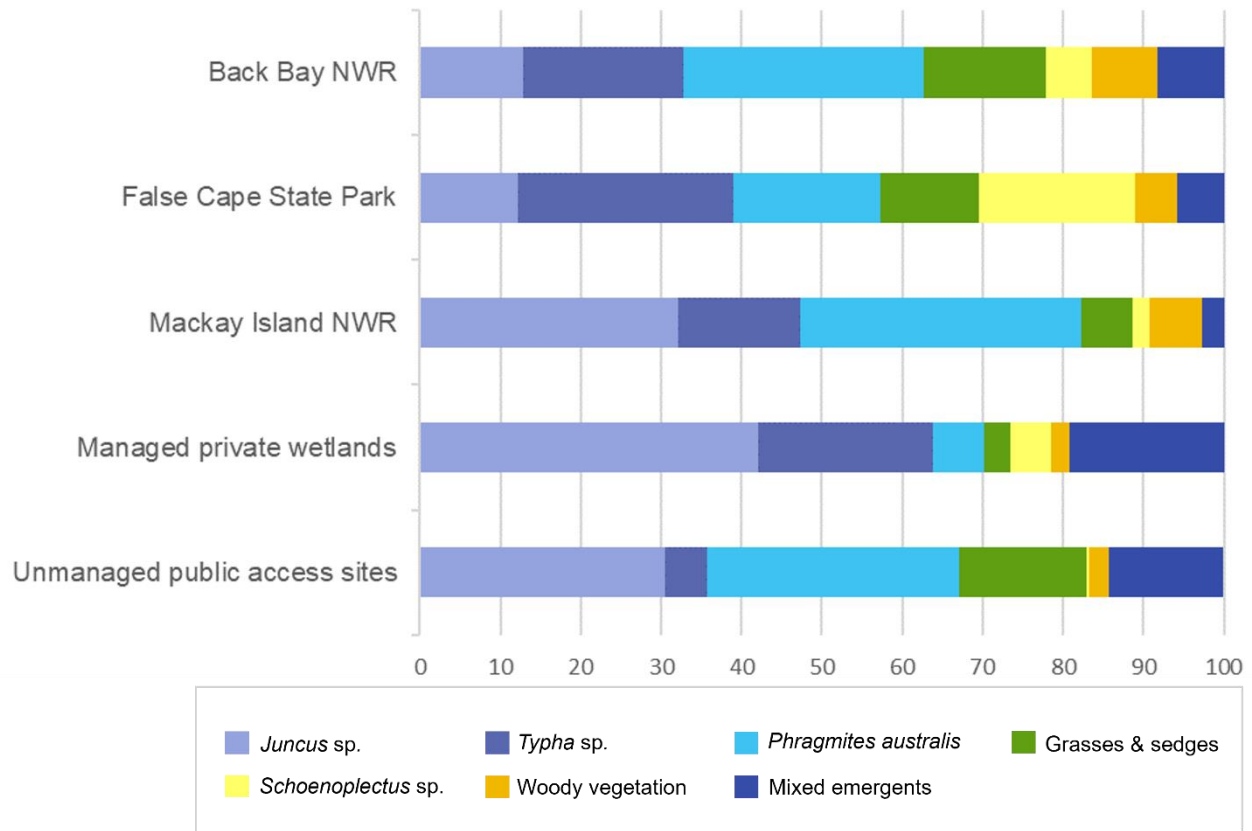
Model	AICc	$\Delta$ AICc	AICw	-2*log-likelihood
$\lambda(M + S + W), r(.)$	189.26	0.00	0.19	178.90
$\lambda(M + S + W + G), r(.)$	189.33	0.07	0.18	176.83
$\lambda(M + S + W), r(\text{Sky})$	189.36	0.10	0.18	176.86
$\lambda(M + S + W + G), r(\text{Sky})$	189.37	0.11	0.18	174.70
$\lambda(M + S + W), r(\text{Noise})$	189.82	0.56	0.14	177.32
$\lambda(M + S + W), r(\text{Wind})$	191.06	1.80	0.08	178.56

king rail abundances and densities despite having higher average levels of *Schoenoplectus* sp. and woody vegetation, in contrast to the unmanaged public access sites (Figure 1.3; Table 1.6).

The exploratory removal of three survey points that had >30% plot coverage of *Schoenoplectus* sp. resulted in a dataset of plots with an average of 2% *Schoenoplectus* sp. (range: 0-20%). The top Royle-Nichols models ( $\Delta AICc \leq 2$ ) no longer included *Schoenoplectus* sp. as a covariate. According to the model coefficients, king rail abundance remains positively associated with land management activity and negatively associated with woody vegetation. The covariate effect sizes remained the same in the updated top model. The effect size of the covariate management is  $\beta=0.92 \pm 0.17$  (95% CI=0.58- 1.24; 85% CI=0.67- 1.16) and woody vegetation is  $\beta=-0.37 \pm 0.14$  (95% CI=-0.63 - -0.10; 85% CI=-0.56 - -0.17). A MacKenzie-Bailey goodness-of-fit test showed that model assumptions were met ( $\chi^2= 10.17$ ,  $p= 0.08$ ).

**Table 1.6** Estimates of mean site abundance, population size, and density of king rails within the surveyed area of each site. Total area was calculated by multiplying the area of a 200-m radius plot by the total number of survey points at a site. Site abundance ( $\hat{\lambda}$ ) was estimated by the Royle-Nichols model. Population size ( $\hat{N}$ ) was calculated by multiplying  $\hat{\lambda}$  by the number of survey points at a site. Density was estimated by dividing  $\hat{N}$  by the total area surveyed at a site.

Location	Total area surveyed (ha)	Site abundance ( $\hat{\lambda}$ )	Population size ( $\hat{N}$ )	Density (king rails/ ha)
Back Bay NWR & False Cape State Park	163.36	9 ± 4	111	0.68
Mackay Island NWR	301.59	11 ± 5	253	0.84
Managed private wetlands	50.27	11 ± 5	44	0.87
Unmanaged public access sites	213.63	2 ± 1	28	0.13



**Figure 1.3** The proportion of dominant wetland vegetation types at each survey site. The percentages for each vegetation type were visually estimated and averaged across all survey points following the conclusion of the final call-broadcast surveys.

## Discussion

This study accomplished an updated census of a mid-Atlantic source population of breeding King Rails. Initial analysis revealed that the data do not conform to a simple occupancy model, and accounting for detection heterogeneity (variation in detection probability) resulted in a model better suited for the data. The discrepancy in model fit may be attributed to variation in king rail abundance among sites. These site-specific differences in abundance can lead to heterogeneity in detection probabilities. This is a common and important source of heterogeneity to accommodate for in modeling (Royle and Nichols 2003).

Within areas of high-quality king rail habitat, management practices, less *Schoenoplectus* sp. (common three-square bulrush)-dominated cover and less woody vegetation were key influencers of occupancy and abundance. Apart from the negative correlation between king rail abundance and common three-square bulrush, our findings are consistent with previous king rail studies (Darrah and Krementz 2009, Pierluissi 2006, Bolenbaugh et al. 2012). It is worth noting that despite woody vegetation having a negative correlation with king rail abundance during the breeding season, this does not necessarily imply that king rails always avoid wooded areas. In fact, king rails have been observed using flooded forest habitat in the non-breeding season (Kolts and McRae 2017).

The negative relationship between abundance and *Schoenoplectus* sp. cover was unexpected. *Schoenoplectus pungens* is common in the region (Meanley 1969), and at Mackay Island NWR, 35 of 299 king rail nests found between 2011- 2017 were in patches of three-square bulrush as well as intermixed with *Juncus*, *Typha*, or other emergent plant species (S. McRae, unpubl.). Using the terminology of Sutherland et al. (2023), the *Schoenoplectus* sp. covariate is a “confusing” variable due to its p-value falling within the range of 0.05 - 0.175 ( $p=0.06$ ). This result was strongly influenced by two survey plots with no king rail detections in which *Schoenoplectus* sp. accounted for 33-38% of habitat cover. An additional point was

excluded from the exploratory analysis as it exceeded the 30% cut-off with *Schoenoplectus* sp. comprising 40% of habitat cover, and there were king rail detections at each survey. Removing these three survey points from the dataset resulted in six top models, none of which featured *Schoenoplectus* sp. as a covariate of interest, calling into question the validity of its negative influence on king rail occupancy and abundance.

*Schoenoplectus pungens*, common three-square bulrush, is known to tolerate water depths exceeding 1 m (Albert et al. 2013), so a potential explanation for the negative association could be that it is found in deeper water that king rails avoid (Kolts 2014, Gawlik and Crozier 2007, Brewer et al. 2023). Further investigation is needed to clarify this interaction and should include hydrological measurements in areas of marsh dominated by different plant species.

A previous census of the region by Rogers et al. (2013) involved surveying 41 plots three times at Back Bay NWR, False Cape State Park, Mackay Island NWR, and privately owned wetlands on Knotts Island. For analysis, Rogers et. al (2013) combined Back Bay NWR and False Cape State Park, and additionally combined Mackay Island NWR and the privately owned wetlands. This resulted in two distinct geographic areas for comparison. Results from this study are not directly comparable to ours due to differences in survey design and due to differences in model fit. However, it is interesting to note that our mean site abundance ( $\hat{\lambda}$ ) and occupancy probability ( $\psi$ ) estimates are considerably higher for Back Bay NWR and Mackay Island NWR. Rogers et al. (2013) estimated king rail abundance at Back Bay NWR to be  $0.66 \pm 0.22$  and estimated abundance at Mackay Island NWR to be  $1.45 \pm 0.38$ . My estimates are 92% and 86% higher for these sites, respectively.

Occupancy probability for Back Bay NWR and Mackay Island NWR were calculated separately by Rogers et al. (2013) based on plot burn status. They estimated occupancy probability at Mackay Island NWR to be  $0.95 \pm 0.06$  for plots burned within the previous 2 years



and  $0.68 \pm 0.13$  for non-recently burned plots. They estimated occupancy probability for recently burned plots at Back Bay NWR to be  $0.77 \pm 0.20$  and  $0.28 \pm 0.13$  for non-recently burned plots. My derived occupancy estimates for Mackay Island NWR and Back Bay NWR greatly surpass the values reported by Rogers et al. (2013). This difference is notable, particularly considering the limited prescribed burning conducted in 2020 and 2021 due to pandemic-related constraints, resulting in the surveyed areas being unburned in the previous 2 years.

The larger estimates reported here are likely due in part to differences in survey methodology. In the study by Rogers et al. (2013), the research design included a random selection of survey points and surveyors waded into the marsh to conduct the call-broadcast surveys. Surveys were conducted at the pre-selected points irrespective of whether the habitat was suitable for king rails. Moreover, birds may have been displaced during the process of surveyors traveling through the survey plot, possibly limiting their detections. By contrast, my survey points were on roads and along waterways surrounded by high quality king rail habitat. This allowed me to complete survey routes efficiently without compromising detection probability. The likelihood of detection could have further been enhanced by the fact that king rails at Mackay Island NWR appear to be more densely distributed in proximity to refuge roads as opposed to the central marsh areas during the breeding season. The reason is not clear, but their abundance along roads that are open to the public daily suggests an effect of lower predation pressure (S. McRae, pers. comm.). In addition to targeting emergent marsh that appeared to be suitable king rail habitat, we conducted surveys only under favorable weather conditions (e.g., on average, wind was scored on the Beaufort scale as a 2 and sky was scored as a 1) which increased the probability of detection.

The aim of this research is to inform land-management decisions for a suspected critical source population of breeding king rails at Mackay Island NWR and the surrounding region. Specific goals were to relate aspects of high-quality freshwater marsh habitat to king rail

occupancy and abundance on managed and unmanaged lands. Since the publication of the king rail status report by Cooper (2008), there has been over a decade of marsh management by refuge managers to keep habitat at an early successional state. This study validates the effectiveness of these strategies employed by refuge, state park, and private land managers.

It is imperative that marsh conservation efforts are validated and supported to encourage continued efforts and to elucidate the specifics of king rail habitat preferences to allow for a more nuanced approach to land management. With the progression of climate change, emerging threats should be of serious concern to those interested in king rail conservation. These include more frequent and intense storms, as well as rising sea levels, which pose a severe risk to freshwater marsh ecosystems due to increased salination resulting from storm surges and saltwater intrusion. Although king rails exhibit adaptability in their nest-building behavior, they may not be able to respond rapidly enough to sudden water level rises during storm events (Clauser and McRae 2016). Additionally, king rails adapting for water fluctuations with taller nests could face elevated risks of predation and nest failure due to reduced concealment by vegetation (Mainwaring et al. 2014).

Our findings underscore the effectiveness of regional land management efforts and shed light on a potential new habitat variable to investigate: the percent cover of *Schoenoplectus* species. The negative correlation of *Schoenoplectus* sp. with king rail abundance should be interpreted with caution due to the effect size indicating somewhat weak evidence for the relationship. I recommend that future studies consider gathering supplementary data, such as hydrological data, and conducting further analyses to clarify this interaction that is suggested by the model.

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## CHAPTER 2: INVESTIGATING KING RAIL POPULATION STRUCTURE AND GENETIC DIVERSITY USING WHOLE GENOME SEQUENCING

### Abstract

Species that migrate can be at higher risk of experiencing environmental stressors related to climate change because they rely on many stopover points and resources along their migratory routes. Anthropogenic land conversion leads to the loss of staging areas and breeding habitat. King rail populations are believed to have declined largely due to habitat loss and fragmentation. King rails vary in their migratory behavior, and though migrant populations may overlap with residents in the non-breeding season, breeding populations are separated by migratory status as well as by geography. The objective of this chapter is to investigate if separate breeding populations show variation in genetic structure. I will then explore whether genetic variation corresponds to differences in migratory status. Here, I compare genetic diversity among inland populations of king rails (a fully migratory Ohio population and a southeastern Arkansas population with a mixed migration status) and southeastern coastal populations of king rails (a resident North Carolina population and a Florida population consisting largely of king rails of unknown migration status) using whole genome sequencing (WGS). My efforts provide novel information about the genetic diversity and structure of these populations.

My results indicate substantial gene flow among king rail populations, suggesting minimal population structure across the king rail range. Genetic diversity estimates are consistent across all sampled populations and are in the intermediate range. Admixture analysis and principal component analysis produced no evidence of geographically correlated population structure. However, a discriminant analysis of principal components provided evidence of weak population structure with geographic correlation. While these findings initially appear positive,

caution is warranted in their interpretation. The sampled populations represent relict strongholds for the species, as most freshwater wetlands within the species range support few, if any, king rails. The dynamics may shift with additional habitat loss exacerbated by climate change. Therefore, I encourage the continuation of king rail conservation efforts and research.

## **Introduction**

Freshwater marshes in the United States have become increasingly fragmented (Dahl 2011). Fragmented habitats can result in decreased genetic connectivity, genetic introgression, and reduced adaptation potential for species that depend upon them (Barr et al. 2015, Coster et al. 2018). This is especially applicable for species that migrate. King rails exhibit among-population variation in migratory behavior: some individuals remain at breeding sites year-round and others migrate to northern breeding sites in the spring (Meanley 1969, Taylor and Van Perlo 1988). Additionally, some king rails migrate through the Midwest, while others use the Atlantic Flyway (Pickens and King 2013, Kolts and McRae 2017). These differences may have led to distinct breeding populations that are geographically separated by the Appalachian Mountains and expanses of habitat devoid of wetlands.

Wetlands have been lost due to anthropogenic land conversion and climate change (Bolenbaugh et al. 2012, Eddleman et al. 1988, IPCC 2023). Remaining freshwater marshes of sufficient size to support king rails lack habitat connectivity and tend to be concentrated in coastal areas (Bolenbaugh et al. 2012, Kolts and McRae 2017), where king rails are at risk of being displaced by salt-tolerant species (Maley 2012, Coster et al. 2018). The clapper rail (*Rallus crepitans*), sister taxon to the king rail, is a saltmarsh specialist and can also be found in brackish water (Eddleman and Conway 1998). The species can overlap within these intermediate salinity zones. In some cases, this has resulted in reproductively viable king and



clapper rail hybrids (Maley 2012, Coster et al. 2018). In other instances, when levels of salinity have surpassed the tolerance levels of king rails and shifts occur in the emergent vegetation structure, king rails are displaced entirely (Brinker et al. 2002).

To better understand the genetic diversity and structure within king rail populations, I used whole genome sequencing (WGS) to describe genetic variation in four geographically distinct populations of king rails: a coastal resident breeding population at Mackay Island NWR (Kolts and McRae 2017), suspected resident king rails from various locations across the state of Florida, migratory and resident individuals from Freddie Black Choctaw WMA in Arkansas, and one inland migrant population located at Winous Point Marsh in Ohio. Conservation genomic projects allow us to discern the genetic diversity and degree of genomic divergence among populations, which can be applied to conservation and management decisions (Andrews et al. 2018, Hohenlohe et al. 2021).

King rails are notoriously difficult to capture, which can result in studies that must rely on relatively few samples (Kane et al. 2019, Brewer et al. 2023, Kolts and McRae 2017). This makes acquiring king rail genetic samples particularly difficult. Despite this, crucial insights can still be obtained from a few samples if the dataset contains a large number of loci (Nei 1978), such as those produced from whole genomes. This is further supported by empirical studies that affirm that datasets generated through whole genome sequencing possess the capability to accurately delineate population structure, even with limited individual samples per population (Iannucci et al. 2021; Gaughran et al. 2017; Attard et al. 2018). This method is therefore particularly advantageous for threatened and elusive species such as the king rail. Using the whole genomes of 54 king rails, I characterized genetic variation and population structure for four populations from across the range.

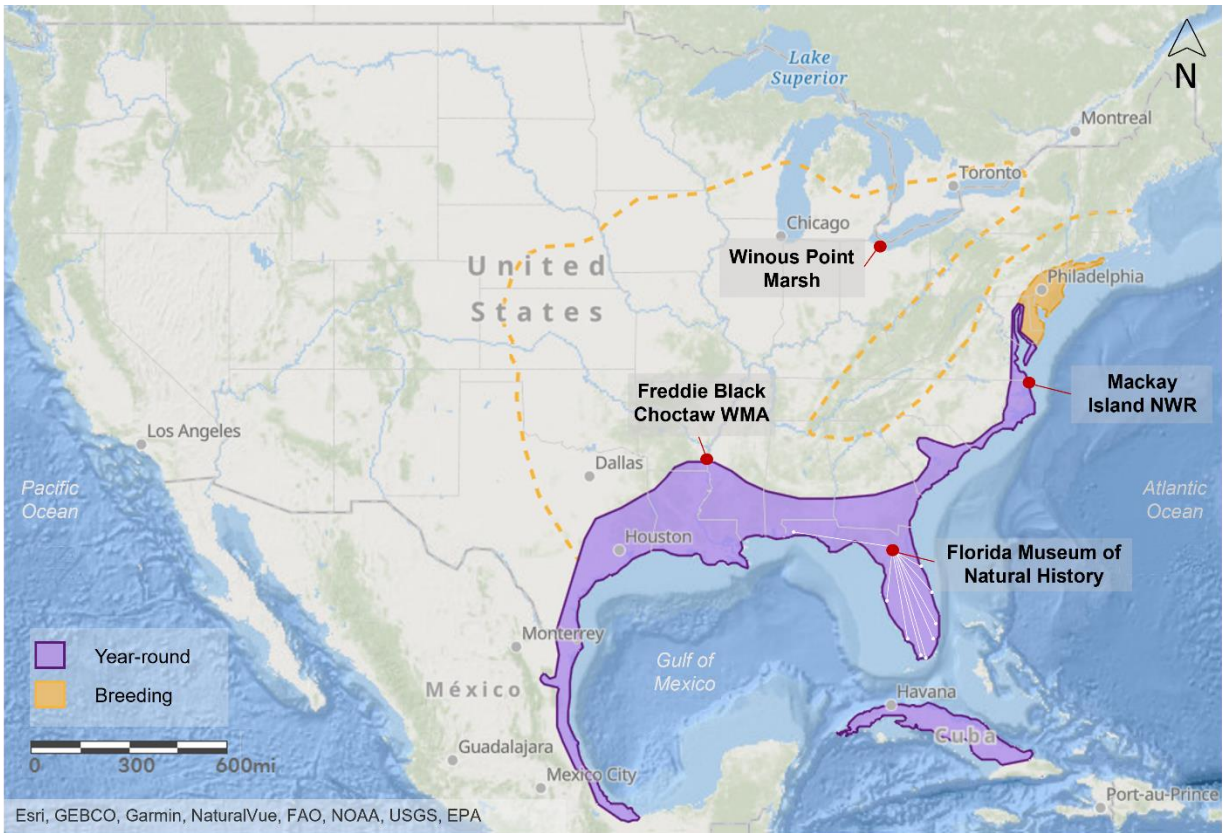
## Methods

### *Descriptions of study populations*

I compared four geographically distinct king rail populations to assess genetic diversity and gene flow across the species' range. Three of these were breeding populations: Mackay Island NWR, North Carolina, Freddie Black Choctaw WMA, Arkansas, and Winous Point Marsh, Ohio. Mackay Island NWR is located near the resident and migrant range transition, but the breeding population of king rails at this site is known to consist largely of non-migratory residents (Kolts and McRae 2017). For further details about this site, please see the methods section of chapter 1. The fourth population consisted of samples of unknown breeding status collected during various seasons within the state of Florida during the years 2002-2018. These samples were generously provided by the Florida Museum of Natural History and were collected from across the state of Florida (Figure 2.1, Appendix Table A1).

Dr. Dustin Brewer of the University of Central Michigan contributed 10 samples from migrant king rails caught during the 2020 and 2021 breeding seasons at Winous Point Marsh in Ohio. Winous Point Marsh is a large freshwater wetland complex along the southwestern shore of Lake Erie. King rails have been captured and studied for research purposes at this site for almost 10 years (Shirkey et al. 2017). Most of the wetlands at this study site are impounded and managed by the Winous Point Marsh Conservancy, a non-profit organization (Kane et al. 2019).

Jessica Schmit of the University of Arkansas contributed 16 king rail samples from Freddie Black Choctaw Wildlife Management Area (WMA) in Arkansas during the 2022 breeding season. The migratory status of some king rails at this site was ascertained using GPS tracking. This revealed that the breeding population consisted of migratory and resident individuals. Freddie Black Choctaw West WMA contains emergent freshwater wetlands located along the Mississippi River that are managed by the Arkansas Game and Fish Commission.



**Figure 2.1** Sites of sampled populations superimposed on the king rail range map. Year-round (purple) and breeding-only (orange) distributions are shaded. The dotted orange line indicates the extent of the breeding range where king rails are expected but uncommon. Red dots represent sample sites. White lines connecting to the Florida Museum of Natural History (red dot, Florida) represent the localities of the museum specimen samples. Range estimates were sourced from the National Audubon Society.

### *Capturing, Marking, and Sampling Adult King Rails*

The necessary permits to capture, band, and collect blood samples from king rails were obtained (Federal Banding Sub-permit 23728-H, Federal Research & Monitoring Special Use Permit BKB-S.McRae-2022, East Carolina University Institutional Animal Care and Use Committee approval, Appendix). During the beginning of the breeding season (approximately April 20th through May 31st) at Mackay Island NWR, we captured adult king rails using nets, audio lures, and a king rail taxidermic decoy. Males are especially responsive to *kek* calls during this time which are used to attract a mate and for territorial defense (Zembal and Massey 1985, Schroeder and McRae 2019). Until the end of May, king rails could be captured opportunistically on the refuge if they were heard calling next to a non-public refuge road during evening surveys. To capture adults, a whoosh net and decoy were deployed near the calling bird. Once set, an audio lure was used to attract the bird to the target area. I recorded the time and location of each capture, the capture method, and standard morphometrics were taken (e.g., bill height, bill width, bill length, tarsus length, flattened wing chord, and weight). A USGS numbered band and a unique combination of color-bands were applied to each adult for individual identification upon resighting. I collected 50- $\mu$ l blood samples drawn from the brachial vein and preserved the samples in 1.0 mL of 100% ethanol.

### *Sample selection*

Due to a restricted pool of available samples, we opted to sequence all available samples from Ohio. Selection criteria for samples from North Carolina and Florida included balanced sex ratios, high DNA quality, and complete background records. North Carolina samples were chosen from the entire pool of adult samples that had been collected during the period of 2011 to 2022. When possible, samples from king rails at Mackay Island NWR were selected from known resident breeders that had been observed at the site in both the breeding and non-breeding seasons or were individuals that had been tracked for up to one year at the

site (Kolts and McRae 2017). After the available pool of such samples diminished, subsequent selections were based on the time of capture and the completeness of records. Samples were selected such that all three populations were represented as evenly as possible (a minimum of 10 samples per site, Table 2.1). Sequencing costs limited the total number of samples, and sample numbers per population were constrained by the number of king rails captured. Given limited knowledge of migration status in the Arkansas and Florida populations, when possible, I selected samples collected during the breeding season and that had relatively complete records. Within the Arkansas population, priority was given to individuals that had been fitted with transmitters to improve our chances of definitively ascertaining the migratory status of as many birds as possible.

**Table 2.1** Sample sites, population migration status, season of sample collection, and total number of samples per population. Samples from Florida were generously provided by the Florida Museum of Natural History and are sourced from throughout the state. Additional information about the Florida king rail specimens can be found in Appendix A Table A1.

State	Site	Central site coordinates	Status	Collection season	Samples
Ohio	Winous Point Marsh	41°47' N, -83°15' W	Migratory	Breeding	10
North Carolina	Mackay Island NWR	36°31' N, 75°58' W	Resident	Breeding	18
Arkansas	Freddie Black Choctaw WMA	33°35' N, -91°9' W	Resident & Migratory	Breeding	16
Florida	State-wide	See Appendix A	Unknown	Breeding and non-breeding	12

### *DNA extraction, library preparation, and whole genome sequencing*

I extracted DNA from blood samples using a DNeasy Blood and Tissue Kit (Qiagen). DNA concentration and purity were assessed using a NanoDrop spectrophotometer (2000 series). Sample library preparation and whole genome sequencing were outsourced to The North Carolina State University Genomic Sciences Laboratory. Whole genomes were sequenced paired-end (PE) using the Illumina NovaSeq 6000 platform with a target of 10X coverage. This target depth was chosen because lower coverage is sufficient to discover polymorphisms that segregate at a high frequency while remaining cost-effective (Nielsen et al. 2011). Genomes were sequenced paired-end because the alignment of sequences as read pairs increases the accuracy of alignment (Nakazato et al. 2013).

### *Pre-processing, mapping, sorting, and down-sampling*

The North Carolina State University Genomic Science Laboratory completed the demultiplexing process before providing us with the raw FASTQ files containing 150-bp PE reads for 45 king rails. I received an additional 10 king rail whole genomes from Dr. Elisa Elizondo of Dr. Greg Shriver's lab at the University of Delaware. Five of these individuals were breeding adults collected from Freddie Black Choctaw WMA and the remaining five were breeding adults sourced from Mackay Island NWR. Raw forward and reverse sequencing reads were filtered and trimmed using fastp (version 0.23.3), and quality reports were generated for all files (Chen et al. 2018). The default filters were enabled for all files (phred quality score  $\geq$  Q15 and  $\leq$  40% of bases are allowed below the quality threshold).

I used the Burrows Wheeler Aligner (version 0.7.17) for short read alignment to a reference genome (Li and Durbin 2009). Reads were aligned to a high-quality clapper rail genome (GenBank accession no. JAQOTC000000000.1; Elizondo et al. 2023). Clapper rails

and king rails are sister taxa, making clapper rails an ideal reference for alignment. I ordered the scaffolds within the clapper rail reference genome prior to alignment using the scaffold utility within the RagTag toolkit (version 2.1.0; Alonge et al. 2022). Scaffolds were put into chromosome order using an annotated chicken (*Gallus gallus*) reference genome (GenBank accession no. JAENSK000000000.1; Warren et al. unpubl.). Aligned king rail genomes were converted into binary alignment and map (BAM) file format and sorted using SAMtools (version 1.12; Danecek et al. 2021). To normalize depth of coverage to approximately 10x as much as possible, some files were reduced to retain only a random subset of the reads using DownsampleSam from Picard Tools (version 3.1.1; Broad Institute 2019). Average depth and mapping statistics were generated using the depth and flagstat functions within SAMtools.

### *Genomic diversity and population structure*

I estimated and compared genomic diversity of four king rail populations by estimating nucleotide diversity ( $\pi$ ; Nei and Li 1979), Watterson's estimator ( $\theta$ ; Watterson 1975), and pairwise  $F_{ST}$  values (Weir and Cockerham 1984). I used the Analysis of Next Generation Sequencing Data (ANGSD) software suite (version 0.941-17) to estimate population genetic diversity and differentiation because this program suite is particularly advantageous for analyzing low-coverage NGS data in non-human organisms, particularly in cases where a reference population is unavailable (Korneliussen et al. 2014). Prior to analysis, I identified autosomal chromosomes and corresponding genomic coordinates. These sites were then used as a filter in downstream analyses with ANGSd using the sites (-sites) flag. I reduced to a purely autosomal dataset because our sample group is comprised of male and female individuals, and datasets with pooled sexes have the potential to polarize some results by sex (Benestan et al. 2017, Thia 2023). During preliminary analyses, disparities emerged between the genomic data and the species identification record for two individuals. The initial PCA and admixture analyses



suggested that two individuals within the Florida population were likely clapper rails and had been misidentified as king rails (Figure A, Appendix). Consequently, I excluded these two probable clapper rails from the dataset.

Population-level genetic diversity statistics,  $\theta$  and  $\pi$ , were calculated using the realSFS tool within ANGSD. For each population, I estimated site allele frequencies (SAF) based on individual genotype likelihoods using the SAMtools model, and I included filtering parameters (-minMapQ 30 -minQ 20 -minInd 8) for refining which bases to include and a SNP calling filter parameter (-baq 1) to improve accuracy by considering alignment uncertainties (Li 2011; command: `angsd -b BamFileList -ref Ordered.CLRA.fasta -minMapQ 30 -minQ 20 -GL 1 -minInd 8 -doSaf 1 -baq 1 -sites sites.list -out SAF`). Following this, I generated the folded site frequency spectrum (SFS) using the chromosome-ordered clapper rail reference genome as the ancestral filter (-anc), calculated per-site theta values, and calculated  $\pi$  and  $\theta$  with the ANGSD ThetaStat program following the methodology explained by Nielsen et al. (2011). Folded SFS was used since the reference genome was used for the ancestor filter, and folded SFS does not require ancestral state information (Nielsen et al. 2012). Instead, it assumes that a given reference allele serves as the ancestral state. Population-level  $\pi$  was calculated by extracting the nucleotide diversity column (tP) from the output file, and then dividing that value by the number of sites (nSites). Population-level  $\theta$  was calculated by extracting the corresponding column (tW) from the output file, and then dividing that value by the number of sites (nSites).

I used pairwise fixation index ( $F_{ST}$ ) comparisons to investigate genetic variation within populations compared to that between populations. I used the population SAF files previously generated to calculate 2D SFS for each pair of populations (command: `realSFS Population1.saf.idx Population2.saf.idx > Pop1_Pop2.ml`; `realSFS fst index Population1.saf.idx Population2.saf.idx -sfs Pop1_Pop2.ml -fstout FST.Out.Pop1_Pop2`). To extract the  $F_{ST}$  values

from the binary output files, I used the realSFS command again (Arkansas and North Carolina populations: realSFS fst stats FstOut.AR\_NC.fst.idx > FstOut.AR\_NC.fst).

I investigated population structure via principal components analyses (PCA), discriminant analysis of principal components (DAPC), and admixture analysis. I used PCAngsd and NGSadmix within program suite ANGSD which uses genotype likelihoods from variable sites as inputs to compose a covariance matrix and population admixture proportions (Meisner and Albrechtsen 2018, Skotte et al. 2013). I used the angsd function to estimate genotype likelihoods (the probability of 0, 1, or 2 minor alleles at a given site) for all individuals using the SAMtools model. I used a single nucleotide polymorphism (SNP) filter to limit the resulting SNP dataset to significant polymorphic sites (command: angsd -b BamFileList -ref Ordered.CLRA.fasta -GL 1 -doMajorMinor 1 -doMaf 1 -SNP\_pval 1e-6 -doGlf 2 -sites sites.list -out BeagleInputFile). The resulting SNP genotype likelihood dataset was stored in beagle file format. I used NGSadmix to estimate admixture proportions because it was found to be more accurate for this task than PCAngsd (Meisner and Albrechtsen 2018). NGSadmix lacks the capacity to identify the most likely number of populations (K). To address this, I ran NGSadmix 3 times for K=1-10, and then I used the  $\Delta K$  method to identify the optimal value of K (Evanno et al. 2005).

I used R package ggplot2 (version 3.4.2) to visualize results, and adegenet (version 2.1.10) to conduct a DAPC (Wickham 2016, Jombart 2008). I used DAPC to further investigate population structure because it can be used to identify more fine-scale population differences when there is admixture in ancestry (Jombart et al. 2010, Jombart and Ahmed 2011). Like PCA, DAPC can effectively handle extensive SNP datasets, making it ideal for studies that use whole genomes. To choose the appropriate number of principal components (PCs) to keep, I used the xvalDapc function within the package adegenet to perform stratified cross-validation with a range of principal component values. After performing cross validation with the default 30

replications, I selected the range of PCs with the highest proportion of successful outcome prediction (5-30 PCs) for cross validation using 1000 replications. I chose 1000 replications to increase the stringency of replication without making the process overly computationally intensive. I extracted the number of PCs with the lowest root mean squared error from the output and used this as the optimal number of PCs in the DAPC.

## Results

After sequencing, the estimated average coverage was approximately 31X (with a range of 10-66X). Post-filtering and trimming, an average of 98% of all reads successfully passed fastp filters for all files. The average proportion of reads that mapped to the reference genome was 98%, and the average percentage of properly paired reads was 94%. The proportion of reads properly mapped was comparable but slightly higher than other studies that used reference-based read mapping (Díez-del-Molino et al. 2020, Foote 2023). The average depth across merged forward and reverse reads spanned from 5.03-31.6X. Subsequent down-sampling resulted in an estimated average coverage of 9.47X (range 5.03-14.2X).

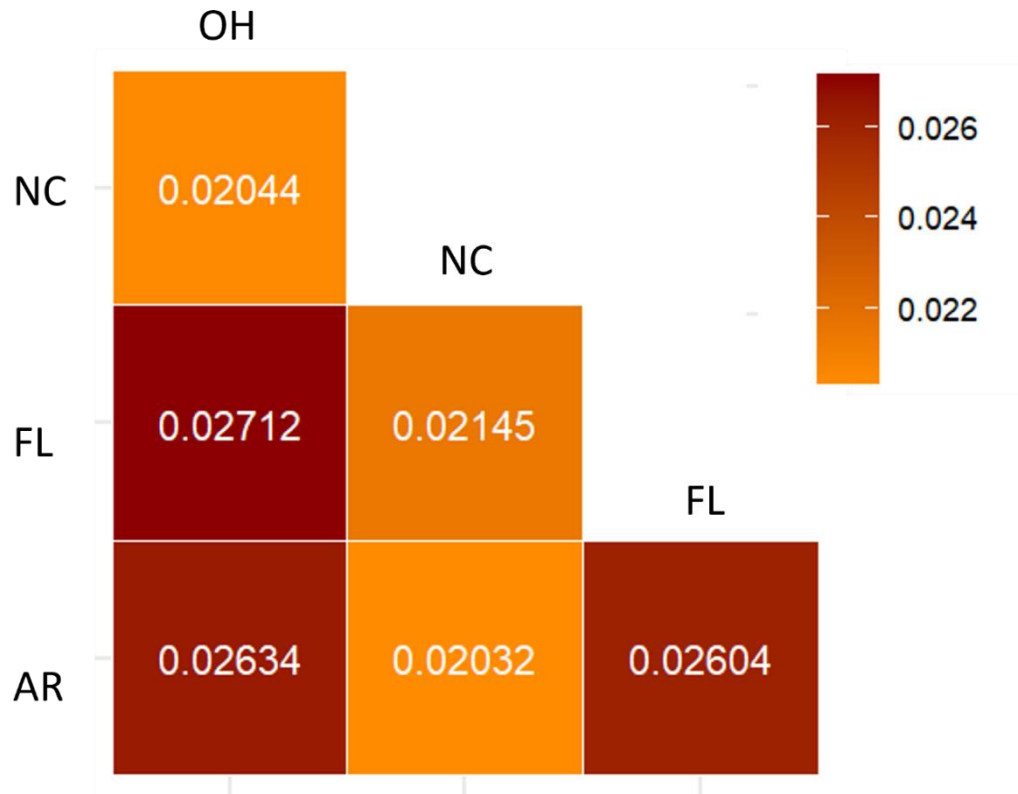
### *King Rail Population Comparison*

Pairwise  $F_{ST}$  values among all four populations were below 0.03 with a range from 0.02032 to 0.02712. Ohio and Florida had the largest pairwise  $F_{ST}$  value, but it was only marginally greater than the next highest value by approximately 0.0008 (Figure 2.2). All four populations had similar estimates of nucleotide diversity ( $\pi$ ), and Watterson's estimator ( $\theta$ ) values were larger and varied slightly more (Table 2.2). Watterson's  $\theta$  values ranged from 0.0035 to 0.0041, and  $\pi$  values ranged from 0.0032 to 0.0035.

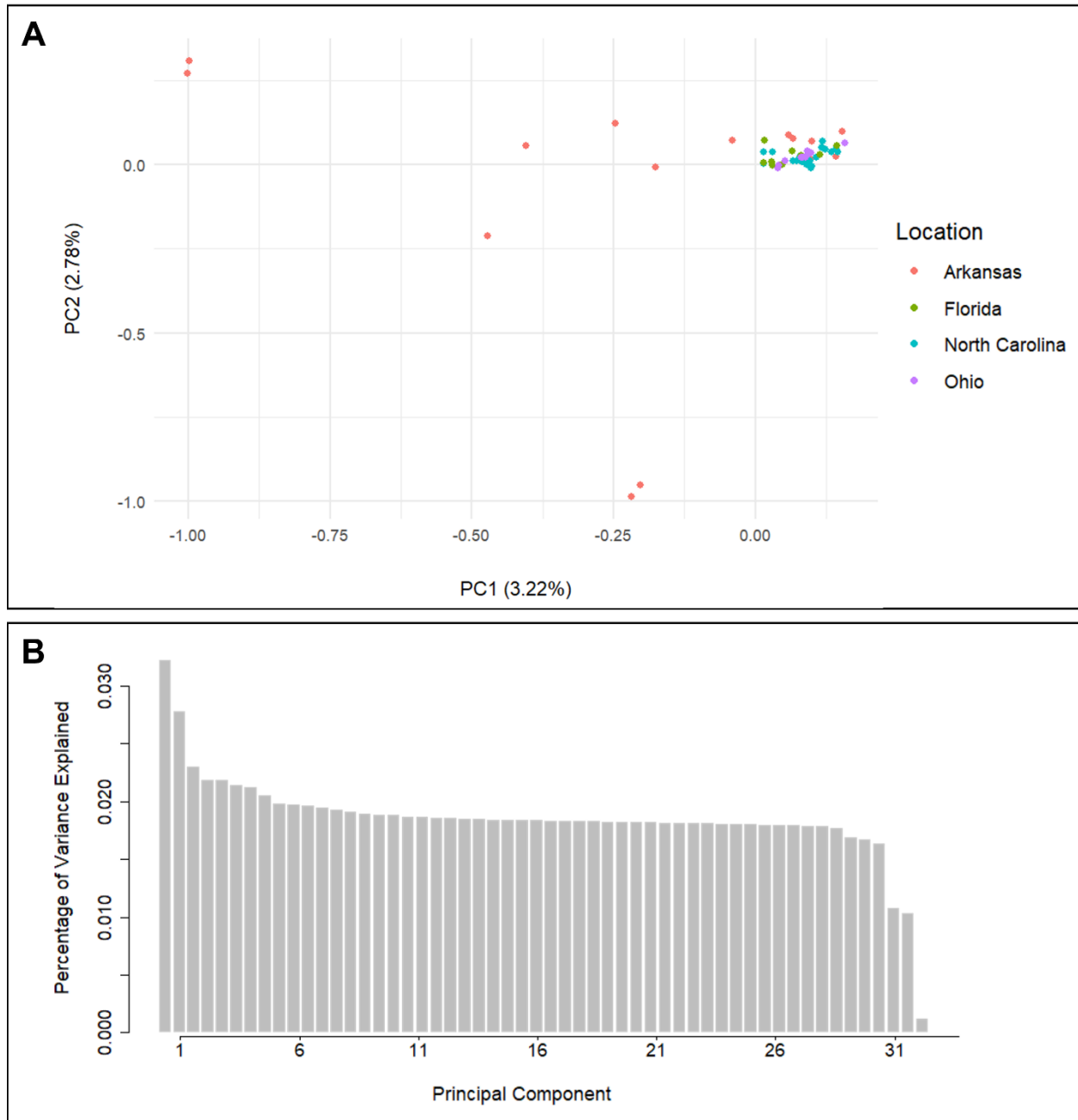
Plotted PCA results for whole-autosome SNP genotype likelihoods display a single distinct cluster that consists of king rails from all sample locations (Figure 2.3). With the exception of eight samples from Arkansas, the remaining samples formed a relatively homogeneous group without clear separation along PC1 or PC2. PC1 and PC2 cumulatively accounted for 6% of the dataset's variation. Eight Arkansas samples have more distinct separation along PC1 and PC2 compared to other samples.

**Table 2.2** Estimates of nucleotide diversity ( $\pi$ ) and Watterson's estimator ( $\theta$ ) for 54 king rails across four sample locations.

State	Site	Nucleotide diversity ( $\pi$ )	Watterson's $\theta$
Ohio	Winous Point Marsh	0.0032	0.0035
North Carolina	Mackay Island NWR	0.0035	0.0041
Arkansas	Freddie Black Choctaw WMA	0.0035	0.0040
Florida	State-wide	0.0033	0.0037



**Figure 2.2** Pairwise fixation index ( $F_{ST}$ ) values for four populations of king rails. Populations are represented by the abbreviation of the state in which they originated (AR = Arkansas, FL = Florida, NC = North Carolina, OH = Ohio).



**Figure 2.3** Principal component analysis results (A) for 54 king rail samples sourced from four sampling locations (16 samples from Arkansas, 10 samples from Florida, 18 samples from North Carolina, and 10 samples from Ohio). Scree plot (B) represents principal components identified by PCA and their corresponding percentage of explained variance.

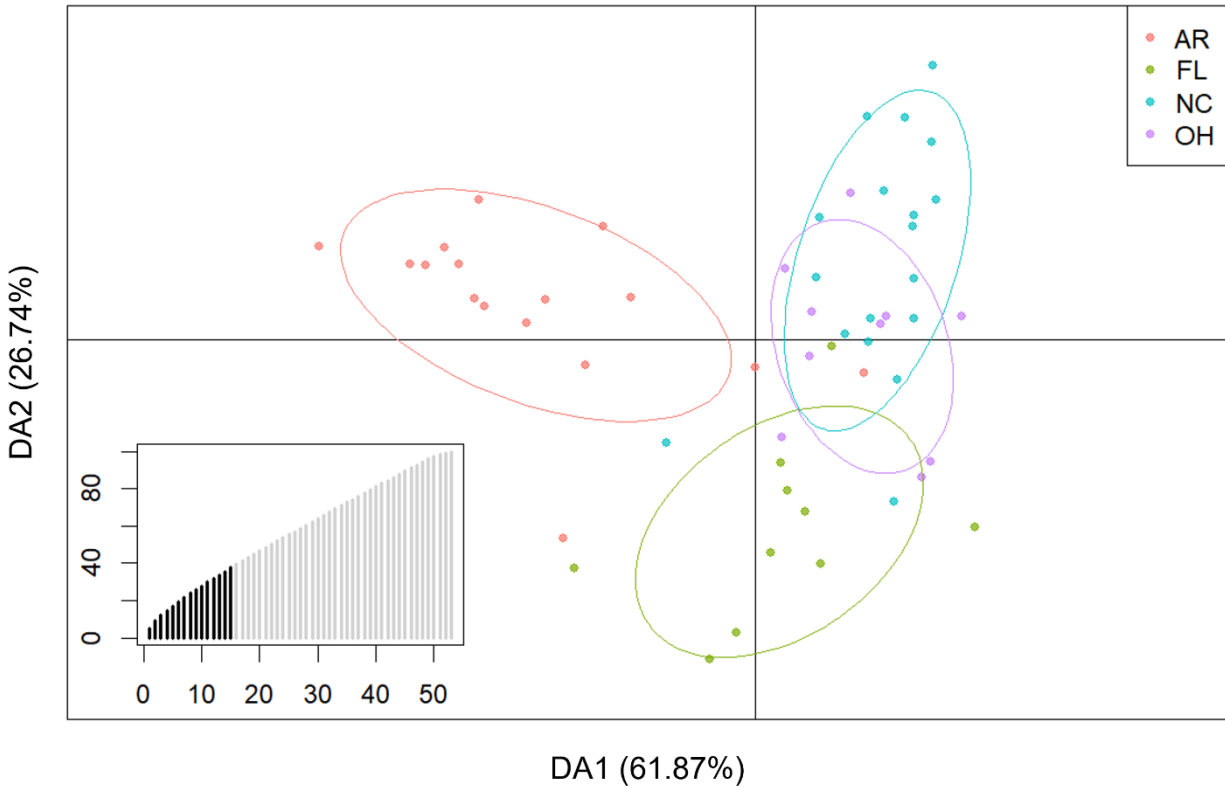
When conducting the DAPC, I identified the optimal number of principal components to be 15 using cross validation. Retaining the first 15 PCA eigenvalues (37.6% of total variance in the original PCA dataset), the DAPC explained 88.61% of the variance in the data (Figure 2.4). DAPC resulted in a different grouping pattern than that of the PCA. King rails from Florida and Arkansas separated out from North Carolina and Ohio along DA1 while Florida also separated out along DA2. Similar to the PCA result, Ohio and North Carolina king rails group closely together at the center. Populations are surrounded by 95% confidence ellipses (Friendly et al. 2013). On average, the model predicted 44 out of 54 (81.48%) king rails to belong to their *a priori* assigned cluster (*i.e.*, geographic population). The proportions of assignments to each *a priori* defined cluster are as follows:

- 13 out of 16 Arkansas samples (81.25%)
- 8 out of 10 Florida samples (80%)
- 13 out of 18 North Carolina samples (72.22%)
- 10 out of 10 Ohio samples (100%)

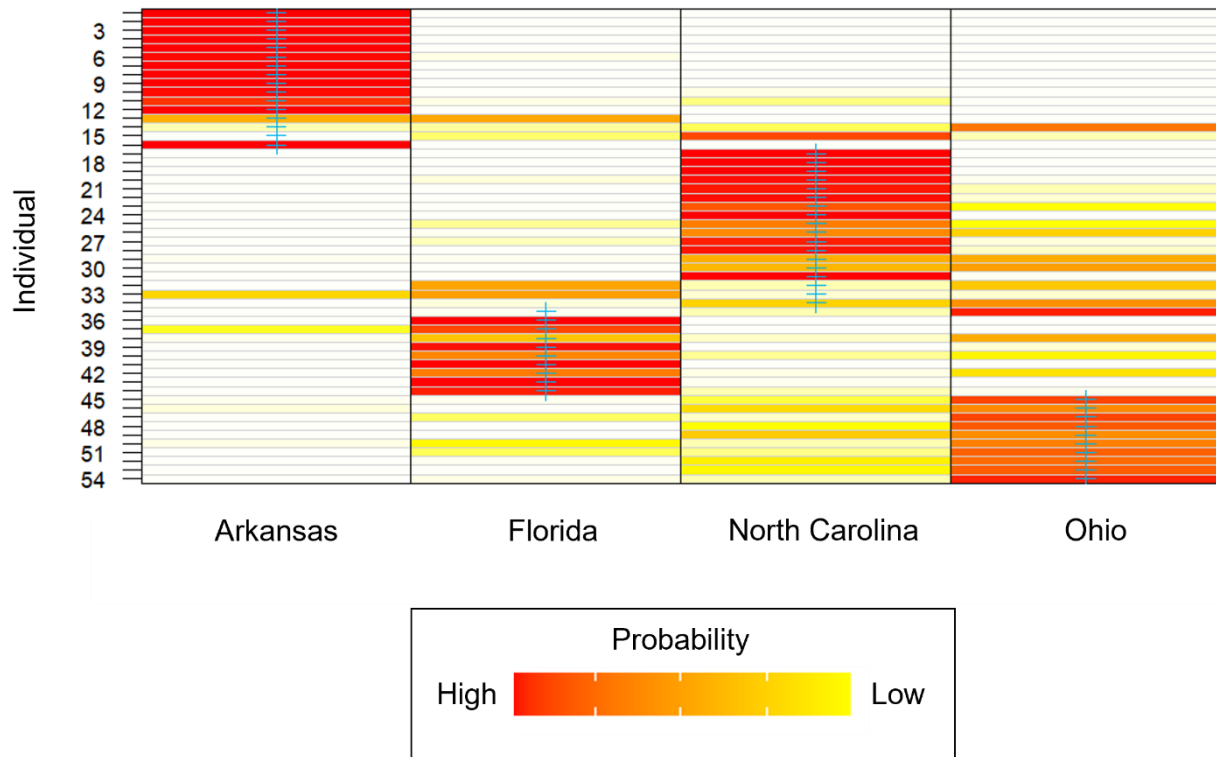
In most cases, DAPC classification was consistent with the *a priori* assignments (Figure 2.5).

After conducting population structure analysis with NGSadmix, 10 candidate models for population assignments (K) were examined. The Delta K ( $\Delta K$ ) method indicated that K=2 was the optimal model ( $\Delta K=21.54$ ), and K=3 was the next best value ( $\Delta K=7.87$ ).  $\Delta K$  values ranged from 1.74 to 21.54. Structure plots and admixture proportions did not reveal clear population groupings (Figure 2.6). Additionally, each NGSadmix run of a given K value resulted in different patterns of admixture. This instability was especially apparent for Arkansas samples across different runs of the K=2 model.

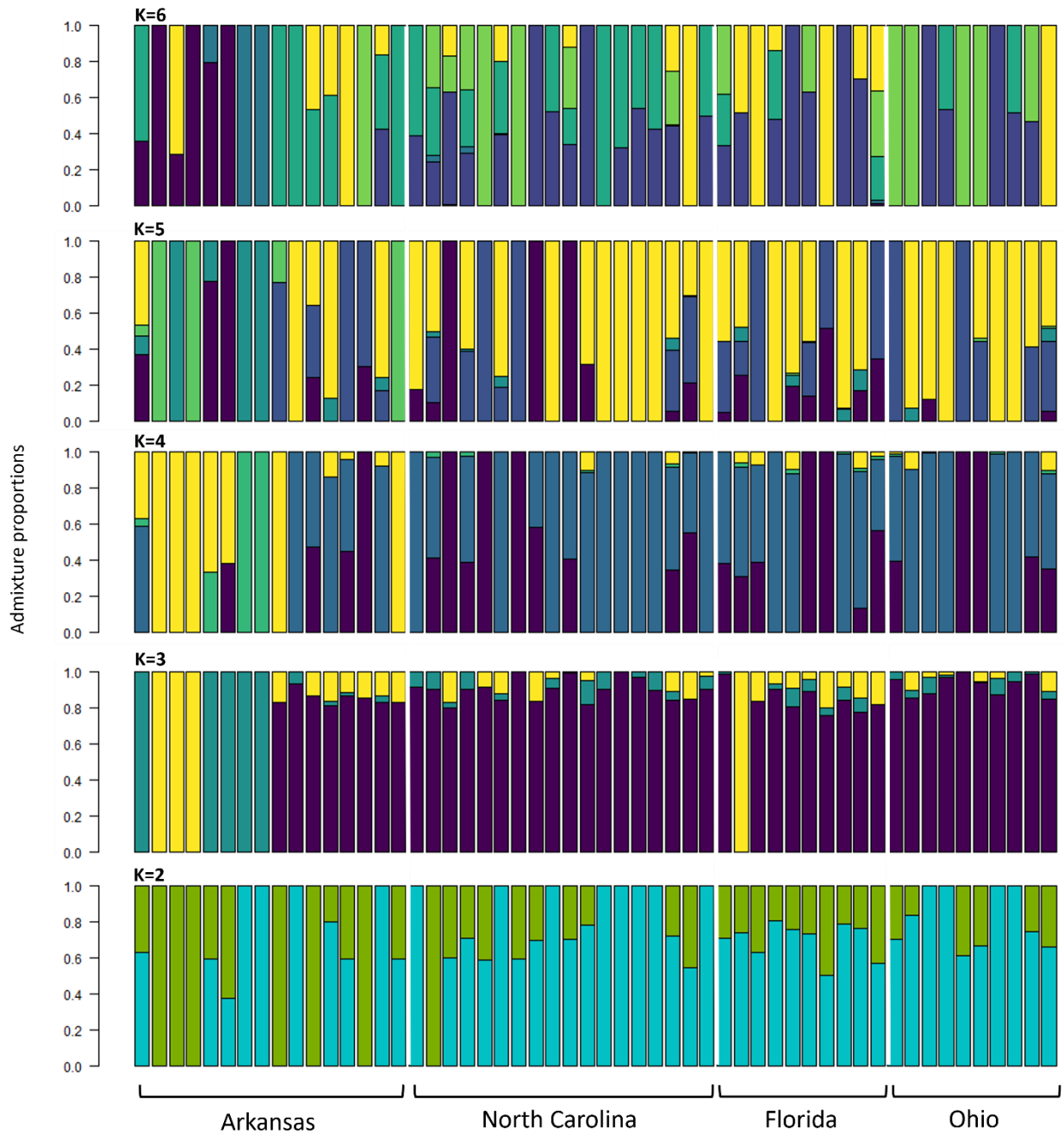




**Figure 2.4** DAPC plot with the first 15 PCA eigenvalues retained (37.6% of total PCA variation) and 95% confidence ellipses for 54 king rails sourced from four sampling locations. The inset plot in the bottom-left displays PCA eigenvalues retained during dimension-reduction in black. The y-axis of the inset plot represents the percent of cumulative variance in the original data that is captured by the PCA eigenvalues retained.



**Figure 2.5** Membership probabilities assigned by the Discriminant Analysis of Principal Components (DAPC). Rows represent individual king rails. Blue crosses indicate which cluster individuals were assigned to *a priori* (*i.e.*, geographic populations), and bar colors indicate the probability of belonging to each geographical cluster column (red = high probability, orange = intermediate probability, yellow = low probability).



**Figure 2.6** Population structure determined by NGSadmix for king rails sourced from four geographic populations. Each admixture plot represents a number of population assignments (K, defined *a priori*) for K= 2-6. The x-axis represents admixture proportions.

## Discussion

My analyses support the hypothesis of population admixture in king rails. Low pairwise  $F_{ST}$  values and consistent genetic diversity across populations suggest a predominantly homogeneous breeding population. PCA also suggests that there is gene flow between all populations. It is not clear why eight Arkansas king rails separated out within the PCA more than all other samples. Initial exploratory assessments for hybridization with clapper rails suggest none of the individuals from Arkansas were hybrids (Figure A1, Appendix). Additionally, it is important to note that the limited explanatory power of PC1 and PC2 warrants caution in interpretation.

DAPC results provide contradictory evidence of weak population structure. To maximize variation between king rail sample populations, I used DAPC retaining the first 10 PCA eigenvalues. Large proportions of correctly assigned individuals in the DAPC support defined clusters of king rails rather than admixed groups (Jombart et al. 2010). The larger proportions of successful reassignment to *a priori* defined groups suggest that there is some level of structure correlating to geographic population.

Interestingly, the Ohio samples cluster tightly at the center of the DAPC. However, these samples did not appear visually distinct from other groups within the admixture plots. Further exploration of population structure through admixture analysis did not reveal distinct separation by sample populations. Each successive run of a given K value resulted in slightly different patterns of population admixture, suggesting low model confidence and a lack of stability in identifying structure. Furthermore, it is important to acknowledge that although the  $\Delta K$  method supported K=2 as the optimal model, it has been proven that in cases where K is actually more appropriately defined as 1, the  $\Delta K$  method may exhibit a bias toward K=2 over alternative k-values (Cunningham et al. 2020). Therefore, it is plausible that the optimal population assignment is K=1, and so this admixture analysis should be interpreted carefully. Alternatively, the optimal

model could be explained by the occurrence of introgression. However, it must be noted that during the preliminary analysis, the inclusion of a clapper rail did not result in a more distinct separation— with the exception of two potentially misclassified samples from Florida (Appendix Figure A1) that were removed from subsequent analyses. Additionally, eight Arkansas samples that visually separated in the PCA plot did not separate out as clapper rails in either the preliminary admixture analysis or PCA (Appendix Figure A2).

My findings closely align with one of the outcomes of a recent dissertation (Elizondo 2023) that investigated the population demography and population genomics of clapper rails. This study included two populations of king rails for comparison purposes. Population connectivity was investigated using ddRAD sequencing for 162 clapper rail samples. Elizondo (2023) reported low pairwise  $F_{ST}$  values for all clapper rail population comparisons, ranging from less than 0.01 to 0.012 for six geographically separated clapper rail populations, and reported a pairwise  $F_{ST}$  value of 0.01 for two king rail populations sourced from North Carolina and Louisiana. The study concluded that clapper rail populations along the U.S. Atlantic and Gulf coasts are panmictic.

Similarly, my results support king rail populations having a high level of population connectivity despite differences in migratory status and geographic barriers between migratory flyways. In spite of differences in spatial distributions of these species – with king rails extending into the Midwest and clapper rails being confined to coastal regions – the similarity in low pairwise  $F_{ST}$  values is notable. The low values reported for linearly-distributed saltmarsh-inhabiting clapper rails are somewhat intuitive. In contrast, a similar result is somewhat surprising for king rails considering the presence of resident populations distributed over a vast geographic area in my analyses. One plausible explanation for this is significant movement between populations, potentially involving individuals displaying plasticity in migratory behavior.

Alternatively, gene flow may be facilitated through the dispersal of individuals across all populations.

Prior to this project, it was unknown how contiguous king rail populations were or if there was gene flow across migratory flyways. To date, there have been few published studies that involve tracking king rail movements outside of the breeding season (Kolts and McRae 2017), and only one has tracked large-scale migratory movements (Kane et al. 2019). In the study, four king rails captured at Winous Point Marsh, Ohio by Kane et al. (2019) were tracked over autumn migration to southern overwintering sites. Remarkably, one individual not only traveled eastward before heading south, but its trajectory to the Atlantic migratory flyway crossed over the Appalachian Mountains, a significant geographic feature separating the Mississippi Flyway and the Atlantic Flyway. Although the breeding location of this individual in subsequent seasons remains unknown, these observations hinted at the likelihood of gene flow between king rail populations. This hypothesis is supported by the results of my study.

While my results might imply a certain degree of population stability, it is imperative to recognize that this could be altered by the escalating impacts of climate change and habitat loss. Furthermore, my results indicate that king rails exhibit intermediate levels of genetic diversity. Despite the consistency in nucleotide diversity estimates across king rail populations, these values are at the lower end of intermediate compared to values reported by Leffler et al. (2012). In this study, which encompassed 167 species of animals across 14 phyla, most species displayed  $\pi$  values ranging from 0.0005 to 0.05, with a median of 0.0065. Comparatively,  $\pi$  values for four king rail populations are lower than the median value reported in the study, but still fall within an intermediate zone. While my genetic diversity estimates are higher than those of some vulnerable and endangered avian taxa (Chaves et al. 2020, Foote 2023), it is noteworthy that they are lower than some avian species of least concern (Dutoit et al. 2017, Smeds et al. 2015).

In a study by Ruan et al. (2018), Eurasian moorhens (*Gallinula chloropus*) were sampled from 19 sites across their range in China, and these groups were found to exhibit no population structure and experience significant gene flow. Key evidence for this discovery included intermediate genetic diversity ( $\pi$ ) levels for all populations ranging from 0.0035 to 0.0079. Like the king rail, this species in the Rallidae family has significant evidence of panmixia. However, unlike the king rail, the Eurasian moorhen is ranked as a species of least concern (BirdLife International 2019), and there are somewhat higher  $\pi$  estimates across their range.

Lower estimates of genetic diversity in king rails may be explained by population decline. A study by Evans and Sheldon (2008) examined 194 bird species to understand the relationship between genetic diversity, conservation status, and population size. Their results indicated that small population size is a key factor in the correlation between low levels of genetic diversity and an increased risk of extinction. The authors argue that conservation initiatives should prioritize preventing additional loss of genetic diversity to enhance the chances of saving declining species. Other studies also support this suggestion after linking lower levels of genetic diversity in populations to reduced population health and survival (Reed and Frankham 2003, Messaoudi et al. 2002). For these reasons, continued research efforts that delve deeper into the assessment of range-wide genetic diversity for the king rail are encouraged.

In addition to conservation genomics research, access to whole-genome data opens many possibilities for answering long-unknown questions about king rails. One idea of interest involves the identification of candidate genes, particularly those associated with migration. This could potentially uncover a genetic basis of migratory behaviors in king rails. An additional research direction could be conducting further comparative genomics between king and clapper rails as it holds potential for uncovering shared or distinct genomic features. Building from the methodology of previous studies, there are many opportunities to uncover novel information using whole genomes (Delmore et al. 2015, Montejo-Kovacevich et al. 2020, Iannucci et al.,

Jensen et al. 2021), something that is especially useful for a cryptic species such as the king rail. Other examples include investigating drivers of selection or employing genome-wide association studies (GWAS) to associate specific genomic regions with traits of interest in king rails.

Beyond genomic exploration, using whole genomes has practical implications for conservation efforts (Hohenlohe et al. 2021). Population genetics can significantly contribute to wildlife management and conservation through the identification of distinct populations (Moore et al. 2014, Allendorf et al. 2012, Andrews et al. 2018). Harnessing whole-genome datasets, future studies could aim to infer king rail movement patterns, estimate effective population sizes, or assess adaptive potential in the face of challenges associated with climate change and wetland loss. These future directions align with a broad conservation agenda for the king rail and would provide actionable insights for preserving and managing king rail populations in the years to come.



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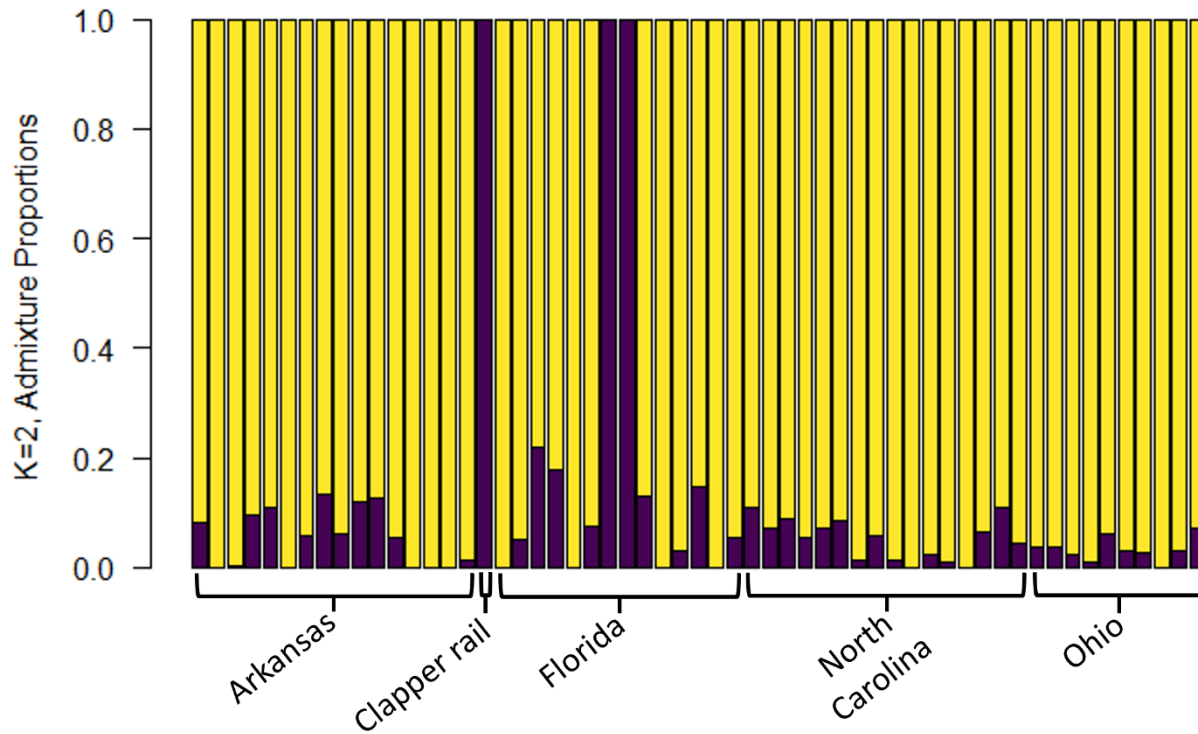
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## Appendix

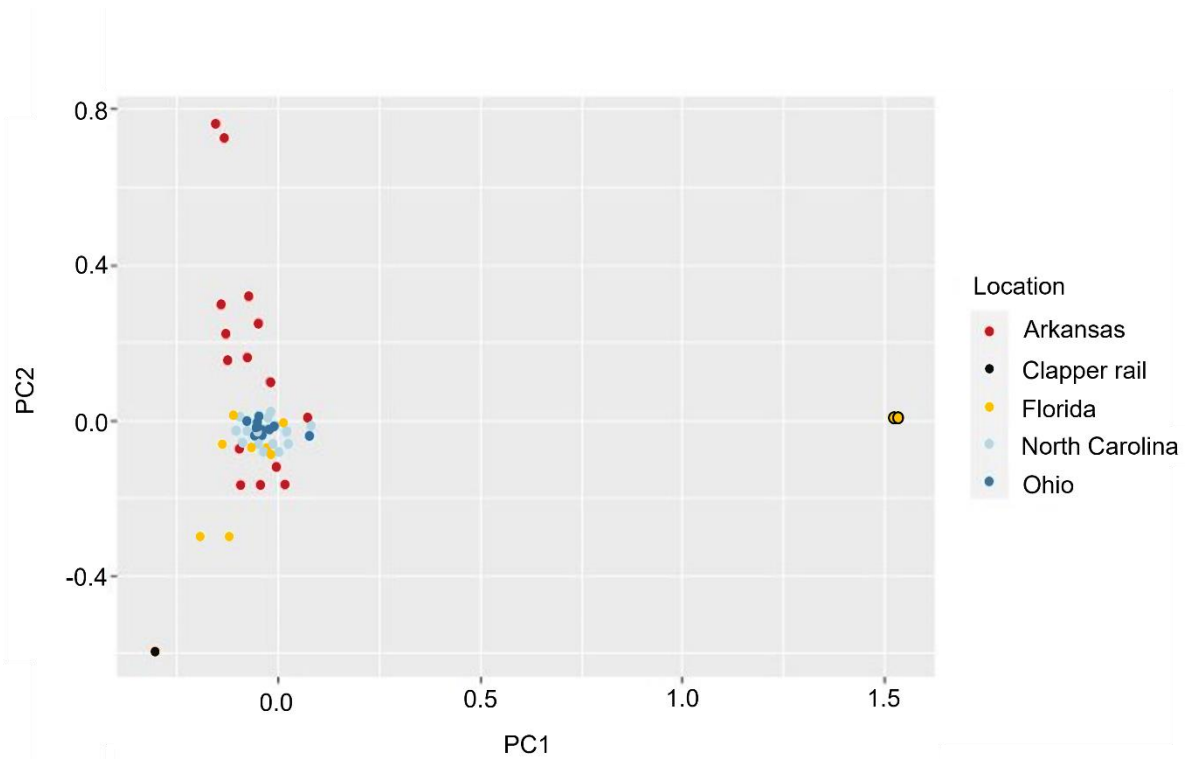
### Appendix A

**Table A1.** Selected samples and their corresponding records provided by the Florida Museum of Natural History. Two samples (in bold) are likely clapper rails.

Sample ID	Species ID	Sample	Geography	Locale	Date collected	Sex
43215	<i>Rallus elegans</i>	Tissue	Florida, Lee Co.	Ft. Myers	7/9/2002	M
43689	<i>Rallus elegans</i>	Tissue	Florida, Pinellas Co.	Vicinity St. Petersburg	12/6/2003	F
45049	<i>Rallus elegans</i>	Tissue	Florida, Escambia Co.	Pensacola	10/19/2005	M
45309	<i>Rallus elegans</i>	Tissue	Florida, Escambia Co.	Pensacola	3/30/2006	F
45655	<i>Rallus elegans</i>	Tissue	Florida, Lee Co.	Ft. Myers	9/14/2006	M
49804	<i>Rallus elegans</i>	Tissue	Florida, Collier Co.	Us 41, 0.25 Mi. E Sr 29	7/26/2012	M
<b>49805</b>	<b><i>Rallus elegans</i></b>	<b>Tissue</b>	<b>Florida, Collier Co.</b>	<b>Us 41, 1 Mi. W Faka-Union Canal</b>	<b>12/11/2010</b>	<b>M</b>
<b>49806</b>	<b><i>Rallus elegans</i></b>	<b>Tissue</b>	<b>Florida, Collier Co.</b>	<b>Us 41, West of S.R. 29</b>	<b>12/22/2005</b>	<b>F</b>
50753	<i>Rallus elegans</i>	Tissue	Florida, Brevard Co.	N/A	10/31/2014	M
50930	<i>Rallus elegans</i>	Tissue	Florida, Brevard Co.	Palm Bay	12/8/2014	F
51747	<i>Rallus elegans</i>	Tissue	Florida, Broward Co.	Davie, 11656 Sw 11Th Manor	4/11/2016	F
53078	<i>Rallus elegans</i>	Tissue	Florida, Miami-Dade Co.	Miami, Miami-Dade Medical Complex	9/10/2018	M



**Figure A1.** Population structure determined by NGSadmix for king rail whole genomes sourced from four geographic populations and one clapper rail whole genome.  $K=2$  was found to be the best model based on the  $\Delta K$  method. The individuals from Florida that grouped with the clapper rail were collected from Collier County in 2005 and 2010, respectively.



**Figure A2.** PCAngsd principal component analysis results for 56 king rail whole genomes sourced from four sampling locations and one clapper rail whole genome. The two Florida samples that were identified as likely clapper rails are outlined in black. These two individuals from Florida were collected from Collier County in 2005 and 2010, respectively.





May 6, 2022

Susan McRae, Ph.D.  
Department of Biology, ECU

Subject: Protocol D253c, original approval date 09/17/2019

Dear Dr. McRae:

The amendment#6 to your Animal Use Protocol entitled, "Ecology and genetics of the King Rail" (AUP#D253c) was reviewed by this institution's Animal Care and Use Committee on 05/04/2022. The following action was taken by the Committee:

"Approved as submitted"

**\*\*Please contact Aaron Hinkle prior to any hazard use\*\***

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

A handwritten signature in black ink, appearing to read "Jamie DeWitt".

Jamie DeWitt, Ph.D.  
Vice-Chair, Animal Care and Use Committee

JD/GD

enclosure



May 6, 2022

Susan McRae, Ph.D.  
Department of Biology, ECU

Subject: Protocol D339b, original approval date 02/24/2022

Dear Dr. McRae:

The amendment#2 to your Animal Use Protocol entitled, "Monitoring secretive marshbirds in coastal refuges and associated wetlands" (AUP#D339b) was reviewed by this institution's Animal Care and Use Committee on 05/04/2022. The following action was taken by the Committee:

"Approved as submitted"

**\*\*Please contact Aaron Hinkle prior to any hazard use\*\***

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.

Sincerely yours,

A handwritten signature in black ink, appearing to read "Jamie DeWitt", is written over a horizontal line.

Jamie DeWitt, Ph.D.  
Vice-Chair, Animal Care and Use Committee

JD/GD

enclosure

