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RESEARCH ARTICLE

Combining genetic structure and demographic analyses to estimate persistence in endangered Key deer (*Odocoileus virginianus clavium*)

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Abstract Recent improvements in genetic analyses have paved the way in using molecular data to answer questions regarding evolutionary history, genetic structure, and demography. Key deer are a federally endangered subspecies assumed to be genetically unique, homogeneous, and have a female-biased population of approximately 900 deer. We used 985 bp of the mitochondrial cytochrome *b* gene and 12 microsatellite loci to test two hypotheses: (1) that Key deer are isolated and have reduced diversity compared to mainland deer and (2) that isolation of the Florida Keys has led to a small population size and a high risk of extinction. Our results indicate that Key deer are indeed genetically isolated from mainland white-tailed deer and that there is a lack of genetic substructure between islands. While Key deer exhibit reduced levels of genetic diversity compared to their mainland counterparts, they contain enough diversity to uniquely identify individual deer. Based on genetic identification, we estimated a census size of around 1000 individuals with a heavily skewed female-biased adult sex ratio. Furthermore, our genetic and contemporary demographic data were used to generate a species persistence model of the Key deer. Sensitivity tests within the population viability analysis brought to light the importance of fetal sex ratio and female survival as the primary factors at risk of driving the subspecies to extinction. This study serves as a prime example of how persistence models can be used to evaluate population viability in natural populations of endangered organisms.

Keywords Noninvasive · Fecal · Deer · Endangered

DNA sequences: Genbank accessions KT877248-KT877344.

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Introduction

In the past, scientists have utilized both demographics and genetics as a means to address the conservation of species of concern (e.g. Petit et al. 1998; Degner et al. 2007; Bristol et al. 2013; Grayson et al. 2014; Robert et al. 2015). Despite that both demographics and genetics have utility in devising plans for long-term conservation planning, researchers have argued about which data type provides the best evidence for short-term planning (Lande 1988; Caughley 1994; Hedrick et al. 1996). Demographically, when there are too few individuals in a population, random factors such as demographic stochasticity, environmental variation, and rare catastrophic events may drive a population to extinction (Gilpin and Soule 1986; van Noordwijk 1994). For example, in 1980 the endangered dusky seaside sparrow experienced a severe bottleneck, due to habitat alteration, in which the population declined to only six individuals. Consequently, those six individuals were all male, condemning the population to eventual extinction (Avise and Nelson 1989). On the other hand, genetic drift can also put populations at risk of extinction by loss of genetic diversity (e.g. Miller and Lambert 2004; Cheng et al.

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2012). For small populations, genetic drift can increase the probability that deleterious alleles will rise in frequency and that rare alleles will be lost from the population (Soule 1973). In the 1990s, the Florida panther (*Puma concolor coryi*) was exhibiting kinked tails, cowlicks, and sperm and heart defects as a result of inbreeding depression (Roelke et al. 1993). To battle the possible extinction of the subspecies, management brought Texas cougars (*P. c. stanleyana*), the closest geographical population, to Florida (Pimm et al. 2006). The introduction of genetic diversity reduced the effects of inbreeding depression and encouraged the recovery of the Florida panther (Johnson et al. 2010). Collectively, demographic instability and reduced genetic diversity can increase the chance of populations being caught in an extinction vortex (van Noordwijk 1994; Tanaka 2000), where small populations succumb to inbreeding depression and genetic drift, leading to a further reduction in genetic diversity over time, and hence, reduces population size further. Ultimately, the understanding of both demographics and genetics are crucial in small populations.

Historically, dissimilarity in sample collection between genetics and demographics limited researchers to choose which type of data to use for the investigation of how best to conserve a species. Genetic data have proven to be powerful given their ability to assess loss of genetic diversity relative to non-endangered species (e.g. O'Leary et al. 2014), amount of gene-flow among populations (e.g. Robinson et al. 2012), and species delimitations (e.g. Brown et al. 2014). Moreover, genetic techniques have facilitated the evaluation of historical demography though the estimation of population expansion (e.g. Hoffman and Blouin 2004) and admixture (e.g. Zachos et al. 2008) via mismatch distribution analysis and historical effective population size via coalescent techniques such as Bayesian skyline plots (e.g. Grazziotin et al. 2006). Contemporary demographic techniques have addressed a separate but equally important set of questions with regard to conservation including age structure (e.g. Martins et al. 2006), survival (e.g. Pradel et al. 1997), and census size (e.g. Rice and Harder 1977; Cantor et al. 2012).

Recent advances in analyses have enabled genetic techniques to evaluate questions of contemporary demography, opening the door for studies that combine the investigation of genetic diversity, structure and evolutionary history with estimates of contemporary demographic parameters. In combination, these techniques can provide an improved view of conservation for a particular species. A major step enabling combined genetic/demographic analyses was the acquisition of noninvasive genetic material facilitating researchers to incorporate genetic data as a means to estimates census size (e.g. Mowat and Paetkau 2002; Boersen et al. 2003; Coster et al. 2011; Morán-Luis et al. 2014) and sex ratio (e.g. Lindsay and Belant 2008; Brinkman and Hundertmark 2009; Morán-Luis et al. 2014). However, few studies have used genetic data to simultaneously answer questions regarding genetic diversity, structure and evolutionary history as well as contemporary demographic questions (e.g. Sugimoto et al. 2014). Moreover, genetic and demographic data can be combined in predictive models to evaluate the long-term survivability of the species of interest. These models [i.e. population viability analysis (PVAs)] incorporate genetic information, life history data and estimates of population parameters, alongside probabilistic functions of stochastic events, to determine the probability of persistence of a species.

Florida contains a large density of near-shore islands which facilitate examining how insular systems impact population demography, genetic diversity, and species persistence. During the last glacial maximum, about 18,000 years ago, the landmass of Florida was much greater in area and extended beyond the Dry Tortugas (Lazell 1989). Eight thousand years later, with rising sea level associated with glacial retreat, the land south of modern day Florida became disjoined, with the intervening ocean establishing a geographic barrier between the mainland and the Florida Keys (Lazell 1989). The contemporary Florida Keys are categorized by three groups of islands: Upper, Middle, and Lower Keys. The Lower Keys are the farthest group of islands from the mainland and are separated from the Middle Keys by the 11-km wide Moser Channel. The Lower Keys contain numerous subspecies which were historically described based on geographic isolation and morphological distinction of mainland sister taxa. The largest of these taxa, Key deer, have been found to be genetically unique relative to their mainland sister taxon (Ellsworth et al. 1994). In addition to their genetic differentiation, Key deer have numerous physical (Hardin et al. 1976; Klimstra et al. 1991; Klimstra 1992) and behavioral characteristics (Hardin et al. 1976) which set them apart from their mainland counterparts. Additionally, anthropogenic influences have further impacted the natural history of Key deer. Most importantly, the subspecies was hunted to near extinction in the early 1950s and has been listed as federally endangered since 1967 (USFWS 1999).

In this study, we sought to evaluate how putative isolation of a wide-ranging species impacts genetic diversity, structure and evolutionary history as well as contemporary demography of Key deer (*Odocoileus virginianus clavium*), a subspecies of white-tailed deer (*O. virginianus*). Moreover, we used these data to generate a species persistence model (i.e. PVA) to evaluate the extinction probability of this island subspecies. In general, we evaluated how genetic data can be used to assess genetic and contemporary demographic questions regarding the conservation status of Key deer. Specifically, we used the mitochondrial cytochrome *b* gene and variation present in 12 microsatellite loci to

address two hypotheses. First, we hypothesized that Key deer are isolated and hence should be differentiated and have reduced genetic diversity compared to mainland deer. This hypothesis is based on previous work conducted by Ellsworth et al. (1994), who identified a single, unique haplotype in Key deer using restriction enzymes. This hypothesis is further supported by two sources of evidence: (a) the geographic distance between the Keys and mainland Florida, which would suggest that Key deer are unable to disperse from the Keys to the mainland; and (b) research of other insular species which has shown that island populations typically contain reduced levels of genetic diversity compared to their mainland counterparts (Frankham 1997). Second, we hypothesized that isolation to the Florida Keys has led to a small population size, a female-biased sex ratio, and a high probability of extinction. This hypothesis is based on previous estimates of Key deer census size [a 5% annual increase in Key deer census size (Lopez et al. 2004) starting with an estimate of 587 deer (Roberts 2005) would lead to a prediction of 900–1000 deer], sex ratio [studies on Key deer which have also shown a female-biased adult sex ratio (Lopez et al. 2003b)], and studies that have shown that insular species exhibit higher risk of extinction, which are typically exacerbated by low genetic diversity, population size, and suboptimal habitat (Alcover et al. 1998; Frankham 1998; Manne et al. 1999; Ricklefs 2009). These data serve as a prime example of how persistence models can be used to evaluate population viability in natural populations of endangered organisms.

Materials and methods

Sampling collection

In order to evaluate broad-scale differentiation of white-tailed deer throughout Florida, we collected tissue samples from 4 counties in Florida, USA (Fig. 1): Collier (n = 30), Palm Beach (n = 8), Monroe (n = 10), and Orange (n = 30). Additionally, we collected samples from Ohio (n = 2) and West Virginia (n = 2) to compare with the Florida samples. All white-tailed deer samples were donated by individuals as a result of legal hunting, road kill, or by Florida Fish and Wildlife Conservation Commission.

To compare genetic differentiation between mainland white-tailed deer and Key deer and to evaluate genetic structure and demography within Key deer, we additionally collected fecal (n = 350) and tissue (n = 21) samples from

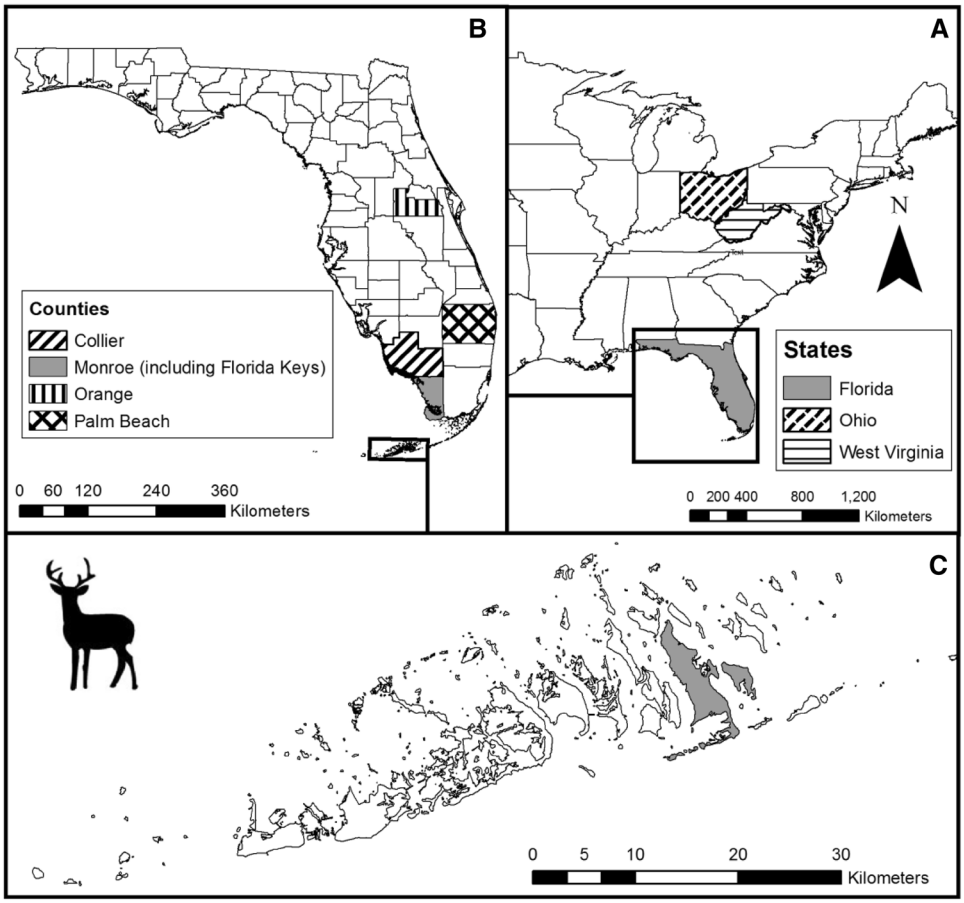

Fig. 1 Map of sampling locations. **a** Ohio and West Virginia samples were collected as outgroup samples. **b** Samples were collected from four counties within Florida to represent the Florida mainland population. **c** Key deer samples were collected from Big Pine Key and No Name Key

Key deer originating from No Name Key (NNK) and Big Pine Key (BPK) during two sampling sessions (Fig. 2). These two islands represent the core of the Key deer population and contain approximately 75% of the global Key deer population (Lopez 2001). The initial sampling occurred from April 2013 through May 2013; the second session occurred from July 2013 until March 2014. To ensure that collections were sampled uniformly throughout NNK and BPK, we established 29 1-km grids covering these islands using ArcMap10. The size of grids was based on the monthly home range size of male Key deer (USFWS 1999) and the amount of effort needed to collect fecal samples across the two islands. Using a random number generator, we assigned a direction and distance along the edge of each grid to mark the starting point of each transect. We then walked each transect in an approximate straight line to a point 1-km away on the opposite side of the grid. Along each transect, we continuously searched for piles of fecal pellets. Pellet groups which were scattered or contained an abnormally high amount of pellets were not collected in order to reduce the risk of a sample being from multiple individuals. Additionally, only pellet groups which appeared to be shiny with a mucus sheen were sampled to ensure the highest probability of successful DNA extraction (Brinkman et al. 2010). Moreover, we did not collect samples within 24 h of rainfall to maximize collection of pellets with high DNA quality. Due to environmental conditions in the Keys, we could not estimate the number of days pellets were exposed to weather conditions. For each pellet group which met our criteria, we collected 6 pellets and georeferenced the sample site using a Garmin GPSmap 60CSx. We used fresh gloves for each pellet group and

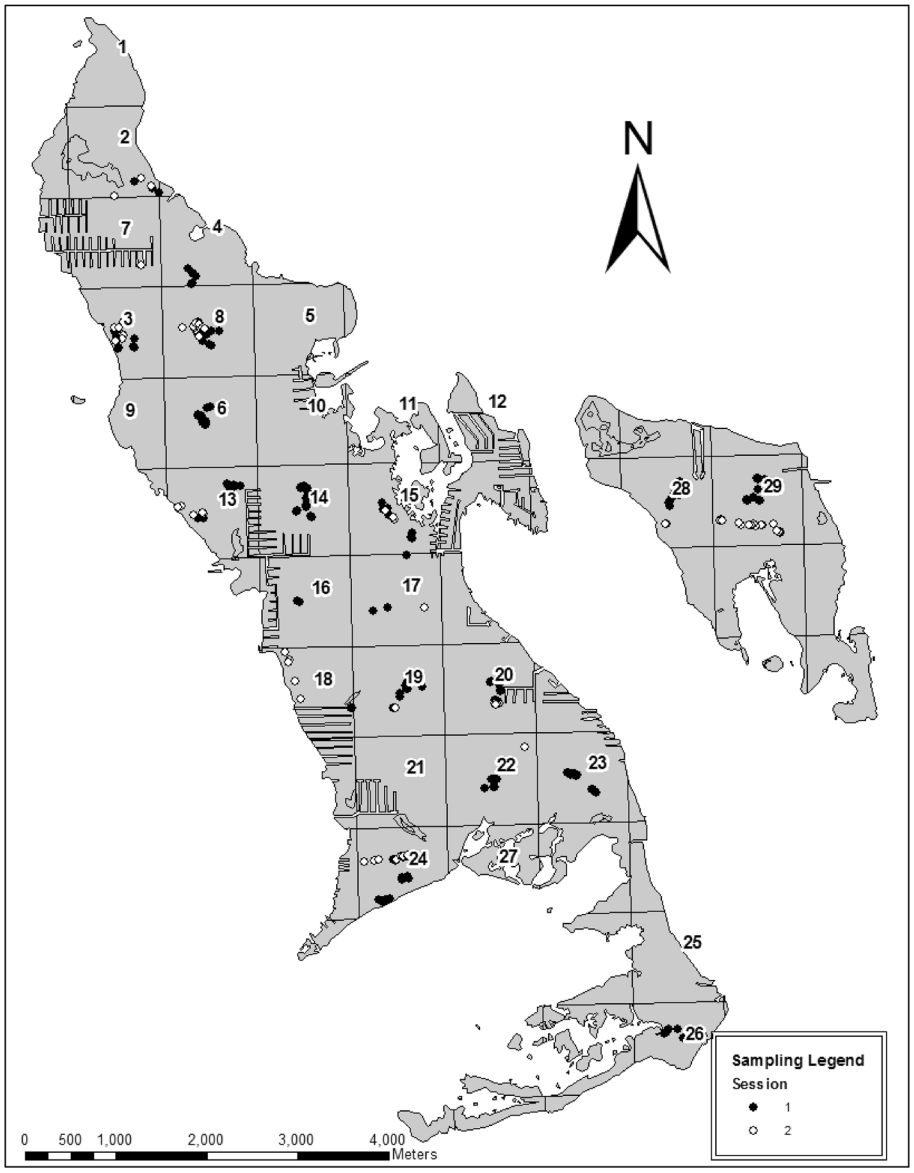

Fig. 2 Sampling locations of Key deer. BPK and NNK are the easternmost islands in the Lower Keys of Florida. Sampling session one is denoted by *closed circles*; second session by *open circles*

stored samples in Drierite desiccant (W. A. Hammond Drierite Co., Xenia, OH). Fecal samples were collected during the parturition season; however, fawn pellets are easily distinguished in size from adult and yearling pellets and were not collected in this study. In addition to the collection of fecal pellets, tissue samples were taken from Key deer using biopsy darts (PneuDart, Inc.) in grids with high human population density due to the difficulty in locating fecal pellets and the inability to walk a transect through private property.

For sex identification, we used the same fecal samples as above. Additionally, for methodological control of sex identification, we collected fresh fecal samples from three Key deer males and three Key deer females. One control sample male was collected from BPK, the remaining five control samples were obtained from the captive Key deer population at the Ellie Schiller Homosassa Springs Wildlife State Park, Florida. These six fecal samples were only used to validate the methodology for sex identification and were not used in any other analyses.

DNA extraction

We extracted DNA from tissue using serapure beads following the protocol of Rohland and Reich (2012). Fecal DNA was extracted using two pellets following the QIamp Stool Kit (QIAGEN) manufacturer's instructions with two modifications. First, to account for absorption of the lysis buffer and maximize DNA yield, we added the minimum amount of lysis buffer to each sample to obtain a final amount of 1.4 mL lysis buffer. The amount of lysis buffer varied by sample depending on how much of the liquid was absorbed by the pellets. Second, we used 100 µL of water heated to 70 °C for the elution step, following recommendations by Tursi et al. (2013).

DNA amplification

For the broad scale phylogenetic analyses, we sequenced 985 base pairs of the mtDNA cytochrome *b* gene. Amplification of the cyt*b* gene was conducted in 40 µL reactions using the following concentrations: 4 µL 10× PCR Buffer, 4 µL 25 mM $MgCl_2$, 4 µL 10 mM dNTP, 0.4 µL DMSO, 1.8 µL each 10 µM forward and reverse primer, 0.8 µL *Taq* polymerase, and 4 µL DNA (50 ng/µL). Primers were developed based on published mitochondrial genomes of *O. virginianus* within GenBank (accession numbers: HQ332445.1 and NC_015247; Forward 5′-GTCATTCAA CTACAAGAACACYA-3′; Reverse 5′-TATTGAATGTAC TACAAAGACTTA-3′). Amplification conditions were as follows: 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 30 s at 54 °C, 1 min at 72 °C, followed by a final extension for 15 min at 72 °C. Subsequent PCR product was sequenced at Eurofins Genomics and University of Arizona Genetics Core (UAGC).

To assess fine scale genetic structure and demographic parameters, we genotyped all samples using 12 previously published polymorphic microsatellite loci which had been optimized for *O. virginianus* (Table 1). Microsatellite PCR products were genotyped at UAGC. PCRs for fecal DNA were conducted in 15 µL reactions using the following concentrations 0.3 µL 40 mM dNTP, 1.5 µL GeneAmp® 10× PCR Gold Buffer, 0.15 µL DMSO, 0.1875 µL 10 µM forward primer, 0.75 µL 10 µM 6-fam dye, 0.75 µL 10 µM reverse primer, 0.15 µL AmpliTaq Gold® DNA Polymerase, and 1.5 µL DNA (specific concentration of $MgCl_2$ are shown in Table 1). Amplification conditions were based

Table 1 Primers and PCR conditions for microsatellite data generated for Key deer

Locus	Citation	[$MgCl_2$] (mM)	T_A	Error rate (%)	PIC
BL25	Bishop et al. (1994)	2.0	52	N/A	0
ILSTS011	Brezinsky et al. (1993)	2.0	52	6.25	0.506
OarFCB193	Talbot et al. (1996)	2.0	52	0	0.480
INRA011	Vaiman et al. (1992)	2.0	52	0	0.528
Cervid1	DeWoody et al. (1995)	2.5	52	25	0.562
P	Jones et al. (2000)	2.0	52	0	0.433
R	Jones et al. (2000)	3.5	52	12.5	0.513
IGF1	Kirkpatrick (1992)	3.0	52	6.25	0.681
N	Jones et al. (2000)	2.0	52	18.75	0.768
Rt9	Wilson et al. (1997)	3.0	54	12.5	0.641
BM4107	Talbot et al. (1996)	2.5	52	12.5	0.732
Q	Jones et al. (2000)	2.0	52	0	0.744

All PCR reactions were run using a standard protocol (see "DNA amplification" section). Concentration of $MgCl_2$ and primer annealing temperatures varied by locus (TA). Error rates within noninvasive samples are calculated from the re-amplification of 16 samples. PIC values range from 0.433 to 0.768 (excluding BL25) with a mean value of 0.599

on the protocol of Anderson et al. (2002). The following modifications were made for fecal DNA: initial denaturation of 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at T_A (specific annealing temperatures are shown in Table 2), and extension for 1 min at 95 °C, followed by a final extension of 10 min at 72 °C. For tissue DNA, the protocol was the same as for fecal DNA, but the initial denaturation was conducted for 4 min and the amplification was run for 35 cycles. All fecal samples were initially amplified across 12 loci under the optimal conditions. Samples which failed at >50% of the loci were discarded from the study. The remaining samples were rerun under the same conditions if

Table 2 Final parameters input into program Vortex

Parameter	Value	Source
Inbreeding depression	**Inbreeding depression = 0, 3, 6.29*,[a], 12**	*Standard value given by Vortex
	Percent due to recessive lethal alleles = 25, 50*,[a]	*Standard value given by Vortex
Reproductive system	Polygynous	Based on white-tailed deer (Clutton-Brock 1989)
	Age of first offspring females = 1	USFWS (1999)
	Age of first offspring males = 2	USFWS (1999)
	Maximum lifespan = 7	Lopez et al. (2003b)
	Maximum number of broods per year = 1	USFWS (1999)
	Maximum number of progeny per brood = 3	USFWS (1999)
	Sex ratio at birth—in % males = 59, 66, 74	See "Materials and methods" section
	Maximum age of female reproduction = 7	Lopez et al. (2003b)
	Maximum age of male reproduction = 3*,[a], 7	*Klimstra (1992)
Reproductive rates	% Adult females breeding = 82	Folk and Klimstra (1991)
	SD in % breeding due to EV = 10*	Standard value given by Vortex
	Distribution of number of offspring per female per brood (4 combinations tested: 1, 2, 3 offspring respectively)	*Folk and Klimstra (1991); USFWS (1999)
	Combination 1 = 83, 17, 0	
	Combination 2 = 60, 40, 0	
	Combination 3 = 20, 50, 30	
	Combination 4*,[a] = 82, 17, 1	
Mortality rates	***Females = see "Materials and methods" section***	Lopez et al. (2003b)
	SD due to EV = 0.1	Standard value given by Vortex
	Males—age 0–1 = 28, 32*,[a]	*Lopez et al. (2003b)
	Males—age 1–2 = 18, 39*,[a], 50	*Lopez et al. (2003b)
	Males—age after age 2 = 18, 39*,[a], 50	*Lopez et al. (2003b)
	SD due to EV = 0.1	Standard value given by Vortex
Parameter	Value	Source
Catastrophes	Number of catastrophes = 2	Standard value given by Vortex
	Frequency = 1[a], 50	Modeled range from 1 to 50% and showed no change
	Severity – reproduction = 1	Lopez et al. (2003a)
	Survival = 1	Lopez et al. (2003a)
Mate monopolization	**Males in breeding pool = 25, 100[a]**	Modeled range from 25 to 100% and showed no change
Initial population size	Population size = 1006	This study
	Stable age distribution with no males surviving after age 3	Klimstra (1992)
Carrying capacity	**250, 607, 1500*,[a], 2000**	*This value represents a 50% increase over estimated census size
Harvest	None	Phillip Hughes (pers. comm)
Supplementation	None	Phillip Hughes (pers. comm)
Genetics	Additional loci only and 11 neutral loci to be modeled	This study

All data input into the persistence model were based on results from this study, previously published Key deer literature, or standard Vortex values. During model examination, parameters in bold were evaluated for impacts on species persistence

[a]Illustrates values used in the standard model when multiple values were evaluated, yet variation did not influence likelihood of extinction. Parameters italicized were used in sensitivity analyses due to their importance in species persistence

loci failed to amplify during the initial screening. Samples which failed to amplify a second time under the initial conditions were rerun at decreasing annealing temperature in 2° increments until an annealing temperature of 46 °C was reached (Fig. S1). Samples which failed to display clear peaks went through the amplification temperature-cycle twice. To estimate the genotype error rates in the fecal samples, we re-amplified 11 loci across 16 samples which had been shown to work successfully.

Sex identification was determined using intron 7 of the zinc-finger locus (Lindsay and Belant 2008). Amplification of intron 7 was conducted in 10 µL reactions following the protocol of Lindsay and Belant (2008). The X-linked allele (displayed for males and females) is visualized as a smaller band on a 2% agarose gel, while the Y-linked allele (males only) is double the size of the X-linked allele caused by an insertion in the Y-linked allele of intron 7. The larger, Y-linked, allele is at greater risk of allelic dropout in degraded samples such as feces. To monitor allelic dropout (i.e. to monitor the risk and rate of dropout of the larger Y-linked alleles in males), each PCR reaction was run with two positive controls: one male and one female. As previously stated, these positive controls were fecal pellets collected from Key deer with known sex.

Analyses

To address the genetic isolation of Key deer in hypothesis one, we edited cyt*b* sequences in Sequencher v5.1 (Gene Codes Inc., Ann Arbor, MI, USA) and aligned the data in MEGA6 (Tamura et al. 2013) using ClustalW. We created a TCS network (Clement et al. 2000) using popART (http://popart.otago.ac.nz) to find unique haplotypes and identify the relationship among haplotypes. To evaluate mitochondrial diversity in terms of nucleotide and haplotype diversity, we used the program DnaSP v5.0 (Librado and Rozas 2009).

To assess nuclear genetic diversity and structure between the Keys and mainland and within the Keys, we first determined allele sizes using the program GENEMARKER (SoftGenetics, LLC) and used GenAlEx6 (Peakall and Smouse 2006) to assess if loci were in Hardy–Weinberg equilibrium (HWE) for each population and to calculate observed heterozygosity (H_O). Next, we used FSTAT (Goudet 2001) to estimate expected heterozygosity (H_E) and levels of allelic richness. We tested for significance in genetic diversity between Key deer and mainland deer via Welch's *t* test in R (R Core Team 2013). Finally, to test for structure within and between the mainland and the Keys we ran the program STRUCTURE (Pritchard et al. 2000) with ten independent runs for each value of *K* (1–6), 100,000 burn-in, and 500,000 iterations. We used the Evanno method (Evanno et al. 2005) to estimate ΔK as implemented in the program STRUCTURE HARVESTER (Earl and VonHoldt 2011). To complement the findings in STRUCTURE, we estimated F_{ST} between the mainland and the Keys using the program GenePop v4.0 (Rousset 2008).

We calculated the probability of identity (P_{ID}) and probability of sibling identity (P_{sib}) using GenAlEx6 as a means to uniquely identify individual Key deer, as well as use the program CERVUS (Kalinowski et al. 2007) to calculate the polymorphic information content (PIC) as a second method to assess diversity across loci [PIC values greater than 0.5 are considered highly informative for distinguishing individuals and loci with PIC values between 0.25 and 0.5 are considered reasonably informative (Botstein et al. 1980)]. The ability to distinguish between individuals and siblings is crucial to calculating a census size and is based on the amount of genetic diversity within the population. We identified unique individuals and possible recaptures of the same individual utilizing two programs, which use different methods to correct for error. The first program, COLONY (Jones and Wang 2010), was used to determine full sibs under an assumption of a 20% error rate. The inclusion of an error rate corrects for known issues involving non-invasive genetic sampling such as allelic dropout and false alleles (Waits and Paetkau 2005). By including error into the analyses, we were able to account for inconsistencies in identifying recaptured individuals which may not be exact matches due to allelic dropout. The second program, CERVUS, identified unique individuals under the conditions of 4 mismatching loci and 6 matching loci. Although both of these programs allow individuals to potentially be assigned as identical, even if there were differences within a locus, assigned matches from COLONY and CERVUS were further scrutinized by eye to confirm matching individuals. Any single allele that was identified as different between samples disqualified the classification of samples as matches. However, we allowed four instances of allelic dropout between possible matches. In many cases, four cases of allelic dropout were not required to have a match between individuals. Additionally, the majority of possible matches had missing data for at least one locus (e.g. matched at 10 loci with the eleventh locus missing entirely). To account for missing data, we recalculated P_{ID} and P_{sib} for all matches such that the recalculated value only included loci in which data were present. If the P_{ID} and P_{sib} did not exceed the threshold values of 0.001 and 0.05 (Schwartz and Monfort 2008), respectively, they were recorded as the same individual.

To estimate the census size of Key deer, we used two methodologies: mark-recapture in the program MARK (White and Burnham 1999) and spatially-explicit capture recapture (SECR) in the package *secr* (Efford 2014) in R. In MARK, we used the standard closed capture model (Otis et al. 1978) which assumes that, for the duration of

the experiment, the population does not change through immigration, emigration, births, or deaths. For the purpose of the study, our data approximate a closed capture model based on features of our population: (1) that movement into and out of NNK and BPK are negligible given that the majority of the Key deer individuals inhabit these two islands (Lopez et al. 2004; Barrett and Stiling 2006); and (2) that we can distinguish yearling from adults owing to pellet size differences. Therefore, we tested two biologically plausible closed capture models based on Otis et al. (1978). The first model assumed that the probability of capture and recapture remained equal and constant between first and second captures. The second model allowed time (sampling occasion) to remain constant within captures, but vary between first and second captures. We used Akaike's Information Criterion (AIC_c, adjusted for sample size) to determine which model best explained the data. The second method to estimate census size utilized SECR analysis. SECR differs from traditional mark-recapture in that it includes individuals found in the same sampling session ("occasion") and uses the coordinates of each sample to inform the density estimate. We generated a mask area based on the ArcMap shapefile of BPK and NNK using the *maptools* package in R. For the SECR analysis, we tested two models, following the same models we used in MARK, and assessed the best model using AIC_c. From the calculation of density estimated in *secr*, we multiplied this value by the area of BPK and NNK to estimate the census size ($N = DA$). Additionally, we estimated effective population size using Colony. Effective population size was used to compare the amount of diversity present in the Keys population to the estimated census and calculate a census size/effective size ratio.

To estimate the sex ratio within the Key deer population as part of the second hypothesis, we counted the number of males and females identified in the Key deer population using gel electrophoresis. The total number of males and females found in the Key deer population was then divided by the total number of samples which successfully amplified for sex identification.

Finally, we combined the genetic and demographic data into a species persistence model using the program Vortex v10.0.8.0. Life history traits and genetic data were based on results from this study, previously published literature for Key deer, or standard Vortex values (Table 2). Specifically, the model included the following information: a polygynous mating system (Clutton-Brock 1989), age of reproduction is 1 year for females and 2 years for males (USFWS 1999), maximum brood of one each year (USFWS 1999), and average lifespan of 7 years (Lopez et al. (2003b). Additionally, female Key deer may reproduce until death, therefore maximum age of female reproduction was input as average lifespan, whereas male maximum age of reproduction is 3 years (Klimstra 1992). Folk and Klimstra (1991) estimated the frequency of breeding females to be 82%. For the distribution of number of offspring per female in a brood, we tested multiple distributions including 83% of the pregnant females carried one fetus and 17% carried twins (Folk and Klimstra 1991), as well as a distributions that included triplets as documented by USFWS (1999). Mortality rates were based off the model-averaged survival estimates for NNK, South BPK, and North BPK from Lopez et al. (2003b). Within the Florida Keys, the main contributor to catastrophes is hurricanes, of which Key deer remain relatively unaffected (Lopez et al. 2003a), therefore we retained high levels of survival and reproduction during catastrophes. The model included a stable age distribution with no males surviving after age three Klimstra (1992). Finally, Key deer are not harvested given their protection status, nor are translocations being used as a current management plan for BPK and NNK.

To determine aspects of the model that impacted species persistence, we ran 20 iterations for each model while changing individual model parameters, these included: catastrophes, mate monopolization, maximum age of male reproduction, levels of inbreeding depression, fecundity, carrying capacity, fetal sex ratio, male survival, and female survival. Not all model parameters impacted probability of extinction (see Results); however, for those that did, we ran sensitivity analyses to determine how varying parameters related to persistence. Specifically, we varied percent males born (59, 66, and 74%) and female mortality in the sensitivity analyses. The values for fetal sex ratio represent the two published extremes (Hardin 1974; Folk and Klimstra 1991) and an intermediate value. Adult female mortality was modeled using two methods: constant mortality rate and a function to account for negative density-dependent survival:

$$= 18 \times (\mathrm{EXP}(N/K)/\mathrm{EXP}(K/K))$$

with N = census size and K = carrying capacity. The constant in the equation for female mortality represents the published value for adult mortality (Lopez et al. 2003b). In the sensitivity analyses, female mortality was evaluated under three different levels: decreased mortality (10% fawns, 10% adults), baseline mortality (28% fawns, 18% adults), and increased mortality (38% fawns, 28% adults). We ran the sensitivity analyses for 100 samples to model changes due to stochasticity. We simulated the data to look 50 years into the future and evaluate likelihood of persistence.

Results

Cytb sequencing

A total of 985 bp of the cyt*b* gene were successfully amplified from 94 white-tailed deer and Key deer samples. Of those samples, we identified 16 unique haplotypes with the most distinct group (two haplotypes found in the Keys and Collier County) being separated by 23 base pairs from the next most closely related haplotype (Orange County; Fig. 3). Of non-Florida sequences, there were three haplotypes: two in Ohio and one West Virginia. One Ohio haplotype was shared with Collier County and was closely related to the samples from West Virginia. The second haplotype found in Ohio grouped more closely to samples from Florida. Within the Keys, all samples exhibited a single unique cyt*b* haplotype. The Keys haplotype differs from its closest related haplotype, found in Collier County, by one base pair.

Population genetics

We were able to successfully genotype 164/350 samples collected (47% success rate) with 37% yielding complete genotypes. We found all 12 microsatellite loci were polymorphic within and among the mainland deer samples, whereas only 11 of the loci were polymorphic within the Key deer population. In the mainland we tested for Hardy–Weinberg equilibrium (HWE) in Collier, Orange, Monroe, and Palm Beach counties. After conducting a sequential Bonferroni correction (Rice 1989), only three loci were out of HWE (loci R and IGF1 in Collier County and locus R in Orange County). We saw no patterns of loci or populations that were consistently out of HWE; therefore, all populations and loci were used in downstream analyses, despite the possibility for low frequency null alleles in some populations. Ohio and West Virginia were not tested for HWE due to small sample sizes. Moreover, samples from these populations were not evaluated for within-population levels of genetic diversity or among-population genetic differentiation. In contrast to the mainland populations tested, the Keys population deviated from HWE expected values in 11 out of 12 loci. However, this result was not surprising given known issues associated with noninvasive genetic sampling (Waits and Paetkau 2005). The average error rate across all loci was 8.52% (Table 1), which according to Smith and Wang (2014), is below the suggested threshold (50% for estimating structure and 20% for genetic diversity) for conducting genetic analyses. Additionally Smith and Wang (2014) described samples sizes above 100 reduced the influence of allelic dropout and false alleles prevalent in noninvasive studies. The one locus that did not deviate from HWE was locus BL25, the monomorphic locus in the Keys. Average allelic richness ranged from 3.37 in the Keys to 5.51 in Orange County (Table 3). Key deer were found to contain significantly reduced levels of allelic richness compared to the mainland population (Welch's t test; $t=-2.771$, $df=20.501$, $P=0.012$). Based

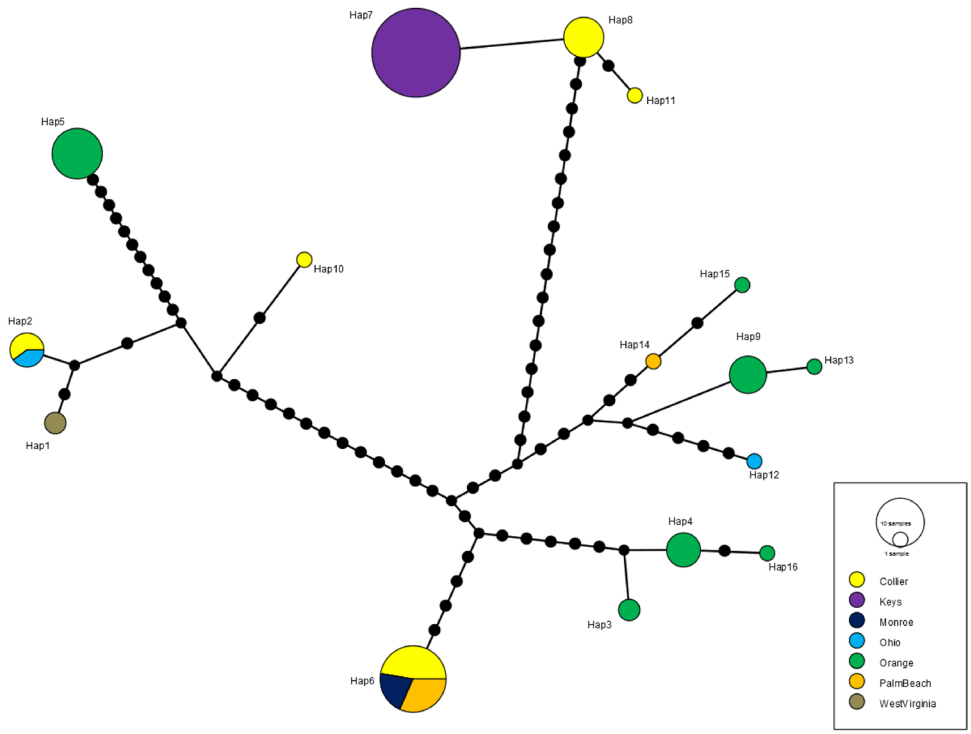

Fig. 3 Haplotype network showing the relationship of haplotypes from multiple populations sampled in this study. As shown, the haplogroup containing the Keys and Collier County are 22 basepairs away from the next related haplotypes. Additionally, the Keys contain a unique haplotype, one basepair away from samples taken from Collier County

Table 3 Genetic diversity for five populations of mainland Florida white-tailed deer

Population	Mitochondrial diversity					Microsatellite diversity			
	n	No. of haplotypes	No. of segregating sites	Π	*h*	n	H_O	H_E	Allelic richness
Keys	34	1	0	0	0	185	0.299 ± 0.036	0.592 ± 0.060	3.747 ± 0.354
Collier	20	4	42	0.018 ± 0.000	0.684 ± 0.014	30	0.651 ± 0.054	0.726 ± 0.065	5.349 ± 0.551
Orange	25	5	45	0.019 ± 0.000	0.769 ± 0.011	30	0.677 ± 0.063	0.732 ± 0.062	5.509 ± 0.586
Monroe	4	1	0	0	0	10	0.732 ± 0.047	0.752 ± 0.042	5.076 ± 0.435
Palm Beach	7	1	0	0	0	8	0.650 ± 0.076	0.780 ± 0.042	5.266 ± 0.452

Genetic diversity shows number of individuals used (n), number of haplotypes, number of segregating sites, nucleotide diversity (π), haplotype diversity (*h*), average observed and expected heterozygosity (H_O and H_E, respectively) and allelic richness. Nucleotide diversity, haplotype diversity, average heterozygosity estimates and allelic richness are reported as mean ± standard error

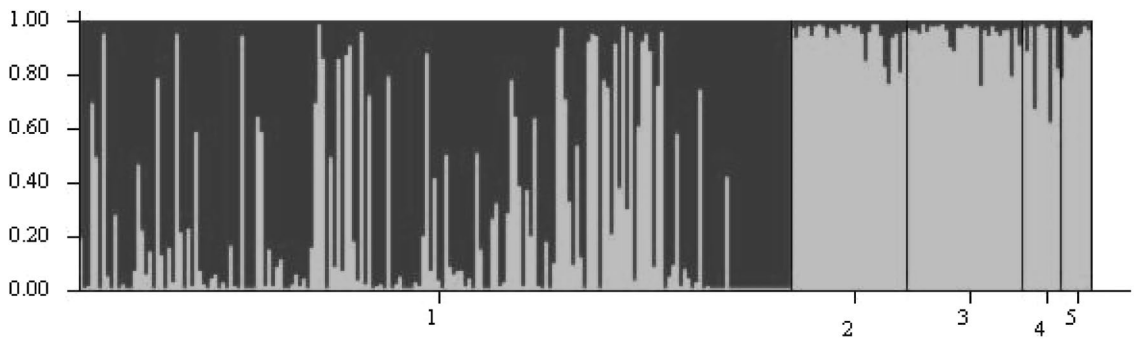

Fig. 4 Output of the Structure analysis when K = 2. The output shows structure between the Keys (Keys = 1) and mainland (Collier = 2, Orange = 3, Monroe = 4, Palm Beach = 5) with no structure within the Keys or mainland

Table 4 Pairwise F_{ST} values between populations

Population	Keys	Collier	Orange	Monroe	Palm Beach
Keys	–	–	–	–	–
Collier	**0.204**	–	–	–	–
Orange	**0.202**	**0.041**	–	–	–
Monroe	**0.207**	0.022	**0.074**	–	–
Palm Beach	**0.155**	**0.052**	**0.040**	**0.057**	–

The Keys have the highest amount of differentiation when compared to other populations. Within the mainland, there is little differentiation between populations. Numbers in bold are significantly greater than zero based on Welch's *t* test

on the genetic diversity estimate, the P_{ID} (2.4×10^{-9}) and P_{sib} (2.4×10^{-4}) were calculated to be less than the threshold (Schwartz and Monfort 2008) which allowed for the genetic tagging of individuals. Similarly, the average PIC value across loci was 0.599 (Table 1), indicating a sufficient level of diversity across loci. One locus (BL25) had a PIC value of zero due to the lack of allelic variation in Key deer.

Based on Structure, we identified K = 2 clusters as the best fit for the data using the Evanno et al. (2005) method: the Keys and mainland Florida (Fig. 4). Although all pairwise F_{ST} values were significant except for the comparison between Collier and Monroe Counties, the numerical values were much greater between the Keys and the mainland (0.155–0.207) than among mainland populations (0.022–0.074; Table 4), further supporting that these populations fall into two clusters (i.e. mainland versus Keys). One caveat of the high F_{ST} value is that the Keys population is out of HWE leading to a possible inaccurate estimate of F_{ST}. However, Smith & Wang (2014) determined that when error rates are less than 20%, estimates of F_{ST} and genetic diversity are able to be appropriately evaluated. Hence, these data should reflect accurate estimates of differentiation between Keys and mainland populations.

Demographics

Combined with 21 tissue samples, we identified 173 unique deer to be used in downstream demographic analyses. Within sampling session one we found six matches (i.e. samples that were identical within the first sampling session), sampling session two had two matches. Comparisons between sampling sessions revealed eight recaptures of sampling session one deer in sampling session two. In

MARK and *secr*, the most supported model stated that the probability of capture remains constant within sampling sessions, but varied between sessions (Table 5). Both programs gave similar results: MARK estimated a census size of 986.69 (SE = 316.81) individuals and *secr* estimated 1,006.93 (SE = 242.30) individuals (Table 5). These numbers are surprisingly higher than the genetic effective population size, which was estimated to be 11 individuals (95% CI 6–28). Therefore, the ratio of effective/census population size is approximately 0.011. Finally, we were able to successfully amplify intron 7 of the zinc-finger locus in 70 samples. In all 70 of PCRs in which these samples amplified, positive controls were also amplified. Out of the 70 samples, we were able to identify 65 females and five males showing a heavily female-biased adult population. This result may be influenced by allelic dropout of the larger Y-linked allele; however, we did not see indication of allelic dropout within our controls or of the initial validation samples which consisted of three known males and three known females. Indeed, our control males always amplified both alleles (or failed to amplify completely) whereas our female controls never exhibited a false male pattern.

Population viability assessment

The genetic analyses directly informed three characteristics necessary in building the PVA: lack of structure of Keys samples, estimates of census size, and estimates of adult sex ratio. Indirectly, the model also enabled us to predict the rate of genetic loss over time. By altering individual parameters in the PVA analysis, we found that only two variables (female survival and fetal sex ratio) impacted long-term census size and species persistence of the Key deer. When all remaining variables were substituted with alternative values (Table 2), the model of species persistence was minimally impacted. Specifically for Fig. 5a, d, a decrease in overall mortality allowed the population to persist without variation in fetal sex ratio or mortality. Similar to Fig. 5c, f, in which an increase in overall mortality caused the population to decline over time, fetal sex ratio and difference in mortality models did influence rate of extinction, however given more time, all simulations become extinct. In contrast, when female survival is increased or decreased beyond the value estimated from field data (Fig. 5b, e; Lopez et al. 2003b), we found that female survival itself is the primary factor impacting persistence. Whereas when female survival is at the value estimated from the field, persistence is dependent upon fetal sex ratio. Fetal sex ratio impacted the rate of extinction such that at higher male-biased fetal sex ratios (e.g. 74% males), extinction was reached more quickly (Fig. 5). Under all scenarios, when the species is extinction bound, density-dependent mortality slows the rate of extinction.

Discussion

This study highlights the utility in using genetic techniques to answer questions related to both genetics and contemporary demography. Our results indicate that Key deer are genetically isolated from mainland white-tailed deer and that there is a lack of genetic substructure between BPK and NNK. Moreover, Key deer exhibit reduced levels of genetic diversity compared to their mainland counterparts; however, they contain enough diversity of which to uniquely identify individual deer. Based on genetic identification, we estimated a census size of around 1000 individuals with a heavily skewed female-biased adult sex ratio. Moreover, we were able to combine genetic and contemporary demographic data to generate a species persistence model for Key deer. Sensitivity tests within the PVA brought to light the importance of fetal sex ratio and female survival as the primary factors at risk of driving the subspecies to extinction. Below, we discuss the evolutionary history of Key deer, contemporary demographic estimates of Key deer and how each of these factors contributes to species persistence.

Evolutionary history

White-tailed deer are the most widespread ungulate in the Americas, ranging from the United States to Peru. Throughout their range, they are believed to consist of around 40 subspecies. The southeastern United States is home to eight of the subspecies. Previous research has sought to address differentiation in the southeastern US deer populations (Ellsworth et al. 1994), including five islands where deer

Table 5 Models tested and AICc scores and weight for census size

Program	Model	AIC_c	AIC_c weight	Census estimate
MARK	{N, p(constant) = c(constant)}	−1139.65	0.18	1012.97 ± 326
MARK	**{N, p(time) = c(time)}**	**−1142.74**	**0.82**	**986.69 ± 317**
secr	g0	883.98	0.17	1006.93 ± 242
secr	**g0t**	**880.86**	**0.83**	**1006.93 ± 242**

The two best models for each method are in bold. See text for contents of these models

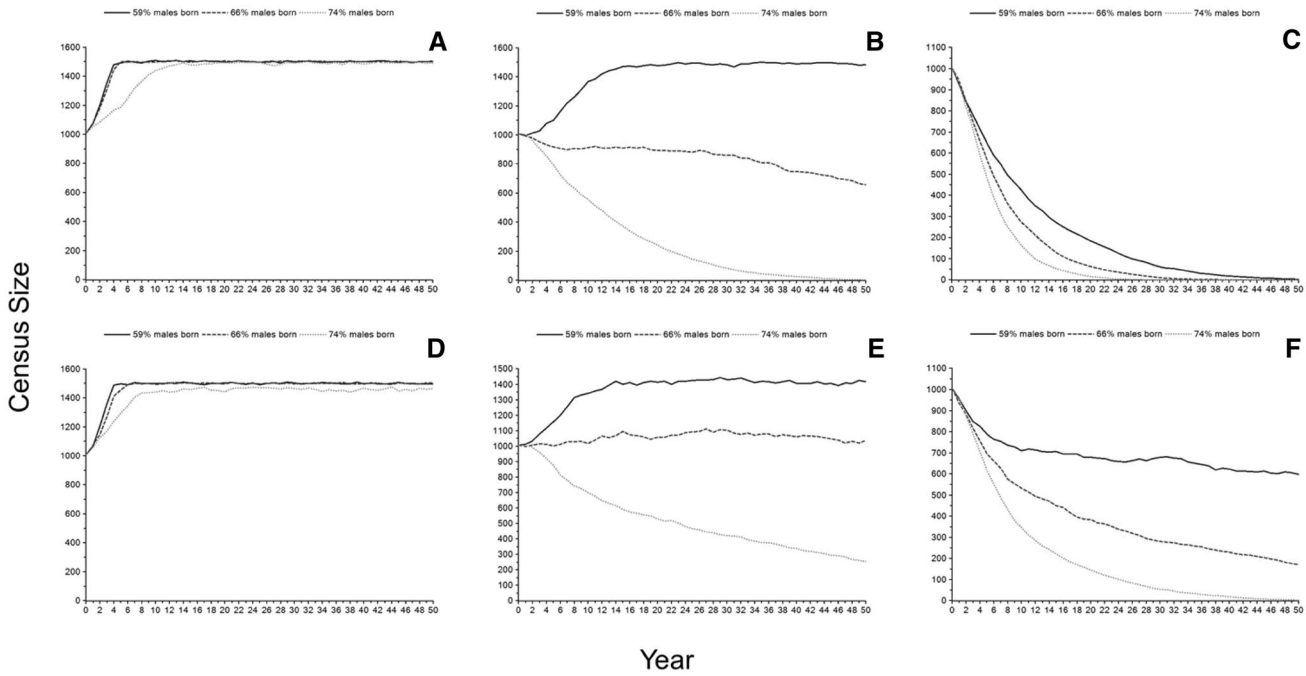

Fig. 5 Vortex simulations for persistence of Key deer. All six graphs are shown with variable fetal sex ratios, but each plot differs according to female mortality. **a** Decreased and constant mortality, **b** baseline and constant mortality, **c** increased and constant mortality, **d** decreased density-dependent mortality, **e** baseline and density-dependent mortality, and **f** increased and density-dependent mortality

are known to inhabit: Pinckney Island (South Carolina), Wassaw Island (Georgia), St. Vincent Island (Florida), Blackbeard Island (Georgia), and the Lower Florida Keys. The latter two are the only islands to have been described to contain a unique haplotype (Ellsworth et al. 1994). However, Blackbeard Island contains a second haplotype which is found on the mainland, while the Key deer is the only insular white-tailed deer population known to contain a haplotype not shared with the mainland.

Focusing on the relationship between the Key deer subspecies and mainland white-tailed deer, we found that there was high haplotype diversity among and within mainland populations, while genetic diversity was greatly reduced in the Keys. This result is not surprising, given that when a once widespread species becomes confined to a smaller area and limited resources, census size and genetic diversity are typically reduced. Indeed, the relationship between size of land occupied and genetic diversity have been found to be positively correlated in other species (e.g. White and Searle 2007). In contrast to the high genetic diversity found within mainland populations, Key deer all contained a single mtDNA haplotype. It is not surprising that Key deer exhibit such a reduction in genetic diversity relative to their mainland white-tailed deer ancestors. In addition to their isolation from the mainland, Key deer experienced an extreme bottleneck due to overexploitation and the population plummeted to about 25 individuals in the early 1950s (USFWS 1999). Additionally, Key deer mtDNA diversity indicates a lack of structure within Key deer. Although the lack of structure contrasts with studies of Key deer movement between BPK and NNK which suggest low levels of dispersal (Lopez 2001), it is paralleled by the nuclear markers, which supported a single panmictic population between the two islands. In addition, we identified a reduced level of allelic richness relative to mainland deer, supporting the lack of gene flow between Key deer and mainland deer.

Contemporary demography

Census estimates for models in MARK and *secr*, were similar and ranged from about 987–1012 individuals. Other studies have evaluated the census size estimates between *secr* and other mark-recapture programs (e.g. CAPTURE and MARK) and found similar estimates between programs (e.g. Gray and Prum 2012; Rayan et al. 2012). Moreover, estimates of standard error (of 200–300) suggest that even with only 16 recaptures (8 within and 8 between sampling sessions), we were able to estimate census size with relatively high confidence. A census size estimate of around 1,000 individuals suggests that the Key deer population is either continuing to increase from the estimated 25 individuals in the 1950s until present or that previous studies have underestimated census size. The last census count of Key deer on BPK and NNK estimated 555–619 deer in 2005

(Roberts 2005). In a previous study by Lopez et al. (2004), estimating a population size between 453 and 517 in 2001, they noted that the Key deer population on BPK and NNK is increasing at about 5% annually. Based on the 5% annual increase starting from the last census estimate in 2005, the contemporary estimate from Lopez et al. (2004) would be around 900 individuals, about 10% smaller than our contemporary estimate.

Historic population size of the Key deer population has been estimated to be between 600 and 700 individuals (Seal et al. 1990) with previous estimates of carrying capacity around 600 (Harveson et al. 2006). However, our current census size estimate exceeds historical size estimates and carrying capacities. These high numbers may be due to (1) the population recovering and not reaching carrying capacity (i.e. previous carrying capacity estimates were inaccurate) or (2) recent habitat changes have favored the Key deer. Humans may have artificially increased the Key deer carrying capacity by the addition of fresh water and food (Peterson et al. 2005). In fact, Key deer have increased their use of urban developments during the period from 1970 to 2000 (Harveson et al. 2007). Even with few houses providing additional sustenance, the extra resources can significantly influence population dynamics (Peterson et al. 2005).

Previous work on sex ratio in Key deer focused on both fetal and adult sex ratios and found contrasting results. In two different studies (Hardin 1974; Folk and Klimstra 1991), the fetal sex ratio was observed to be skewed towards males. In contrast to the fetal sex ratio, Lopez et al. (2003b) reported an adult female-biased (approximately 70% females) sex ratio during an observational study. Our results support a pattern similar to Lopez et al. (2003b); however, the results suggested a more striking sex ratio consisting of ~90% females. In natural populations, fetal sex ratio in mammals has been shown to be influenced by numerous variables including female health and rank (e.g. Verme 1969; McGinley 1984; Clutton-Brock and Iason 1986; Symington 1987). At the adult stage, the sex ratio is determined by physical factors ranging from natural competition among sexes to anthropogenic causes. Similar to our findings, female:male sex ratios of approximately 10:1 have been found in other cervids such as elk (Noyes et al. 1996) and mule deer (Scribner et al. 1991); however, these examples include species that have been directly impacted by preferential hunting of males. Another example, Île Longue feral sheep (*Ovis aries*), also exhibit a heavily male-skewed adult sex ratio due to female mortality caused by harassment from males during breeding season (Réale et al. 1996). In all of these cases, the genetic consequences of such a skewed sex ratio is low effective population size and high effective/census size ratios, leading to decreased genetic diversity over time (Harris et al. 2002). For Key deer, the explanation for the extreme female-biased sex ratio does not likely have natural causes. Rather, road mortality data likely play a role in skewing the sex ratio towards females. Specifically, studies have revealed a greater number of male deaths each year caused by deer-vehicle collisions (DVCs; Lopez et al. 2003b). DVCs have been shown to be a large factor in the survival of numerous species, particularly ungulates. In Utah, white-tailed deer bucks were killed at twice the rate of their density in the population, while doe survival was proportionate to their density in the population (Lehnert et al. 1998). Deer behavior is a likely culprit as to why DVCs favor male deer. As predicted by a polygynous mating system (Perrin and Mazalov 2000), white-tailed deer males have been shown to disperse significantly more than females. Etter et al. (2002) showed that male fawns had a dispersal rate of 50% while female fawns only dispersed at 7% in a suburban environment. Similar to mainland white-tailed deer, Key deer males are the primary dispersers (Lopez 2001), making them more likely to move across roads than female deer and collide with vehicles.

Persistence modeling

Population viability assessments have been used to evaluate reintroduction programs of species to their historical range and determine species persistence in light of threats to the population. In our study, we utilized both genetic and demographic data to evaluate the persistence of Key deer for the next 50 years. The genetic data informed the number of populations to be equal to one, consisting of all individuals found on BPK and NNK and provided the input of allele frequency data into the model to evaluate loss of genetic diversity. The demographic data informed the initial census size for all models and provided information with regard to the adult sex ratio. Surprisingly, only two variables (female survival and fetal sex ratio) were the main drivers of species persistence. The models illuminated the importance of females within the Key deer population. Variables that increased the number of females increased the likelihood of long-term persistence. On the other hand, the number of males does not influence time to extinction. Assuming that Key deer, similar to mainland white-tailed deer and other ungulates, are polygamous (Clutton-Brock 1989), we should expect that it would require few males to sustain the population. Similar to our results, it was identified in a released population of elk (*Cervus elaphus*) that female survival, sex ratio, and calf survival were found to have high elasticity in the PVA and greatly influence the population growth rate and success of the population (Murrow et al. 2009). With regard to long-term persistence of Key deer, the limiting factor is the number of females. When evaluating the combined role of fetal sex ratio and female survival, this study revealed that the tipping point

for species persistence in the Key deer is near 66% fetal sex ratio, 28% female fawn survival, and 18% adult female density-dependent survival (Fig. 5). Values less favorable lead to extinction while values more favorable lead to growth capped at carrying capacity.

In summary, Key deer provided a model system in which to use modern genetic techniques to evaluate questions related to traditional genetic data (e.g. genetic structure and diversity) and demography. We found that Key deer are genetically isolated from mainland white-tailed deer and contain reduced levels of genetic diversity. However, they contain enough genetic diversity to identify individual deer in which to estimate census size using genetic tagging. Through genetic mark-recapture, we found that the Key deer population has continued to increase from their historic population size of around 25 individuals. To evaluate population stability in Key deer, management should continue to monitor the census size of the population. Moreover, because females are critical for Key deer survival, future studies should focus on obtaining more accurate estimates of fetal sex ratio and methods to reduce female mortality. Ultimately, we provide evidence that Key deer are recovering and under continued management practices, we expect their continued persistence into the next 50 years.

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Author contributions VV, PH, EH., conceived the ideas; VV and PH acquired samples; VV collected the data; VV and EH analyzed the data; VV and EH led the writing.

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