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RESEARCH

Genetic support for discrete conservation units of the fossorial rodent *Geomys pinetis*

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Abstract

Knowledge of the population genetic structure and diversity of at-risk species is essential to accurately evaluate population viability and define units for conservation and management. The southeastern pocket gopher (*Geomys pinetis)* is a fossorial rodent native to the imperiled longleaf pine savannas of the southeastern United States. Its recent decline has made it a species of 'high conservation concern' by state agencies. Previous phylogenetic analyses suggested two distinct lineages within the species occurring east (*G. p. pinetis*) and west (*G. p. mobilensis*) of the Apalachicola-Chattahoochee-Flint River (ACF) Basin, a phylogeographic break for many species. However, little is known about the genetic substructure within each region. We examined neutral and putatively adaptive variation in 9373 single nucleotide polymorphisms (SNPs) to assess the extent of genetic structure across the species' geographic range. We confirmed significant genetic divergence of populations east–west of the ACF Basin, predating the Last Glacial Maximum, supporting the presence of two evolutionary independent lineages. Our results indicate additional strong genetic substructuring within each lineage and possible non-neutral variation across latitudes. Given the high degree of genetic differentiation and lack of evidence for secondary contact among populations within the ACF Basin, we recommend that *G. pinetis* be managed as two conservation units corresponding to distinct lineages representing *G. pinetis* and *G. mobiliensis*.

Keywords Cryptic species · Genetic structure and diversity · Single nucleotide polymorphisms · Southeastern pocket gopher

Introduction

A prerequisite for guiding population restoration and preserving biodiversity is accurately delineating evolutionary lineages, their genetic composition, and population substructure (Frankham 2010). However, uncertainty about the precise delimitation of species and identifying distinct populations within species remain significant challenges (Moritz 1994; Coates et al. 2018). Polytypic species are often divided into lineages that are not genetically uniform and may exhibit a high level of genetic structure due to different selection pressures and historical isolation (Coates et al. 2018). Populations may also show variations in phenotypic traits, demography, gene flow, and genetic diversity, which can affect the adaptive potential of the population (Frankham 2005). Therefore, robust conservation strategies include defining conservation units (CUs) that delineate distinct groups of populations (Crandall et al. 2000; Reed and Frankham 2003; Funk et al. 2012) to help guide management

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decisions. CUs typically have been defined as either evolutionary significant units (ESUs) or management units (MUs) (Ryder 1986; Moritz 1994; Funk et al. 2012). However, CUs can also be identified from cryptic (i.e., undiagnosed) sibling species (Bickford et al. 2007), warranting legal protection and separate management considerations.

Identifying CUs requires a phylogeographic approach where geographic sampling is broad enough to minimize the chance of under-representing genetic variation while increasing the phylogenetic resolution. A phylogeographic approach can be essential for cryptic species where the lack of morphological variation belies evolutionary distinction. However, phylogenetic studies often suffer from limited geographic- and genomic-scale sampling (Robinson et al. 2014; Garrick et al. 2015). This is an issue that may be exacerbated when involving cryptic species because of the lack of geographic resolution to delineate taxonomic or conservation units. In contrast, phylogeographic studies limited to single genes (particularly organellar markers) may provide a picture of broader geographic landscape patterns but may poorly resolve evolutionary history in many instances (Coates et al. 2018).

One such potentially cryptic species is the southeastern pocket gopher, *Geomys pinetis*, found in the current and former range of imperiled longleaf pine savannas (Noss 1989; Noss et al. 1995; Frost 2007) in the southeastern United States (Golley 1962; Pembleton and Williams 1978; Wilkins 1987). The species has been designated a species of conservation concern in the states in which they occur (Alabama, Georgia, Florida: Alabama Department of Conservation and Natural Resources 2015; Georgia Department of Natural Resources 2015; Florida Fish and Wildlife Conservation Commission 2019) due to lack of biological knowledge about the species, apparent population declines (Scott 2008), and ecological importance (Huntley and Inouye 1988; Reichman and Seabloom 2002; Clark et al. 2018). Although recent studies have provided more knowledge about the ecology and movements of *G. pinetis* (Warren et al. 2017a, b; Clark et al. 2018; Pynne et al. 2019a, b; Bennett et al. 2020; Duncan et al. 2020; Parsons et al. 2022), there remains limited information about the genetic structure and diversity of the species (Soto-Centeno et al. 2013). Additionally, the southeastern U.S. has a prominent phylogeographic area of discontinuity between the Atlantic and Gulf Coastal sister taxa (Soltis et al. 2006). A significant boundary for many vertebrate, invertebrate, and plant species in this region is the Apalachicola-Chattahoochee-Flint (ACF) River Basin (Fig. 1). In addition to this major phylogeographic break, other major rivers often contribute to the genetic structuring of terrestrial species (Soltis et al. 2006).

There has been regular debate over the validity of southeastern pocket gopher taxonomy (Pembleton and Williams 1978; Hall 1981; Williams and Genoways 1980; Laerm et al. 1982). Early taxonomic work recognized four pocket gopher species in the southeastern U.S.: the widespread polytypic *G. pinetis* and three geographically restricted species: *G. colonus, G. fontanelus,* and *G. cumberlandius*, each respectively restricted to the Coastal Plain of Camden County, Chatham County, and Cumberland Island, GA (Hall 1981). After a statistical analysis of the morphological variation, the genus was reduced to a single species consisting of two subspecies, the widespread *G. p. pinetis* and range-restricted *G. p. fontanelus,* the latter distinguished by the presence of a fontanel (Williams and Genoways 1980).

Early allozyme and mitochondrial DNA revealed substantial genetic divergence east and west of the ACF that did not reflect the taxonomy at the time (Avise et al. 1979; Laerm et al. 1982; Sudman et al. 2006; Chambers et al. 2009; Soto-Centeno et al. 2013). Subsequently, *G. p. pinetis* and *G. p. mobilensis* were recognized as morphologically indistinguishable subspecies, with *G. p. fontanelus* assumed to be extirpated from its narrow distribution (Chambers et al. 2009). Molecular phylogenetics based on mitochondrial and nuclear DNA (Sudman et al. 2006; Chambers et al. 2009) have led to calls for reconsidering the taxonomic status of *G. p. mobilensis*. Unfortunately, these studies only examined one or two specimens from each subspecies, making more detailed inferences about lineage isolation impossible. Despite the interest in characterizing genetic variation, *G. pinetis* remains data deficient due to a lack of information on the extent of range-wide population structure and diversity.

We employed genome-wide single nucleotide polymorphism (SNP) data to delineate CUs in *G. pinetis*. Specifically, our first objective was to assess the support for, and extent of, genomic divergence predicted based on previous mtDNA studies between populations demarcated at the ACF. We sought to determine whether the genomic SNP data would reflect deep divergence at the highest hierarchical level (i.e., previous phylogenetic evidence for an east–west split). Second, we predicted that the habitat specificity and a presumed low vagility of *G. pinetis* (Hickman and Brown 1973; Warren et al. 2017b; Pynne et al. 2019a), combined with increased fragmentation of longleaf pine savannas (Noss et al. 1995; Frost 2007) would result in a signature of reflecting a prominent role for genetic drift between local populations, as has been found in other pocket gopher species (Penney and Zimmerman 1976; Patton and Yang 1977; Patton and Feder 1981; Welborn and Light 2014). Demographic factors such as limited population connectivity and small home ranges will be reflected in neutral signatures of genetic variation, whereas outlier SNP loci may reflect environmental variation or gradients.

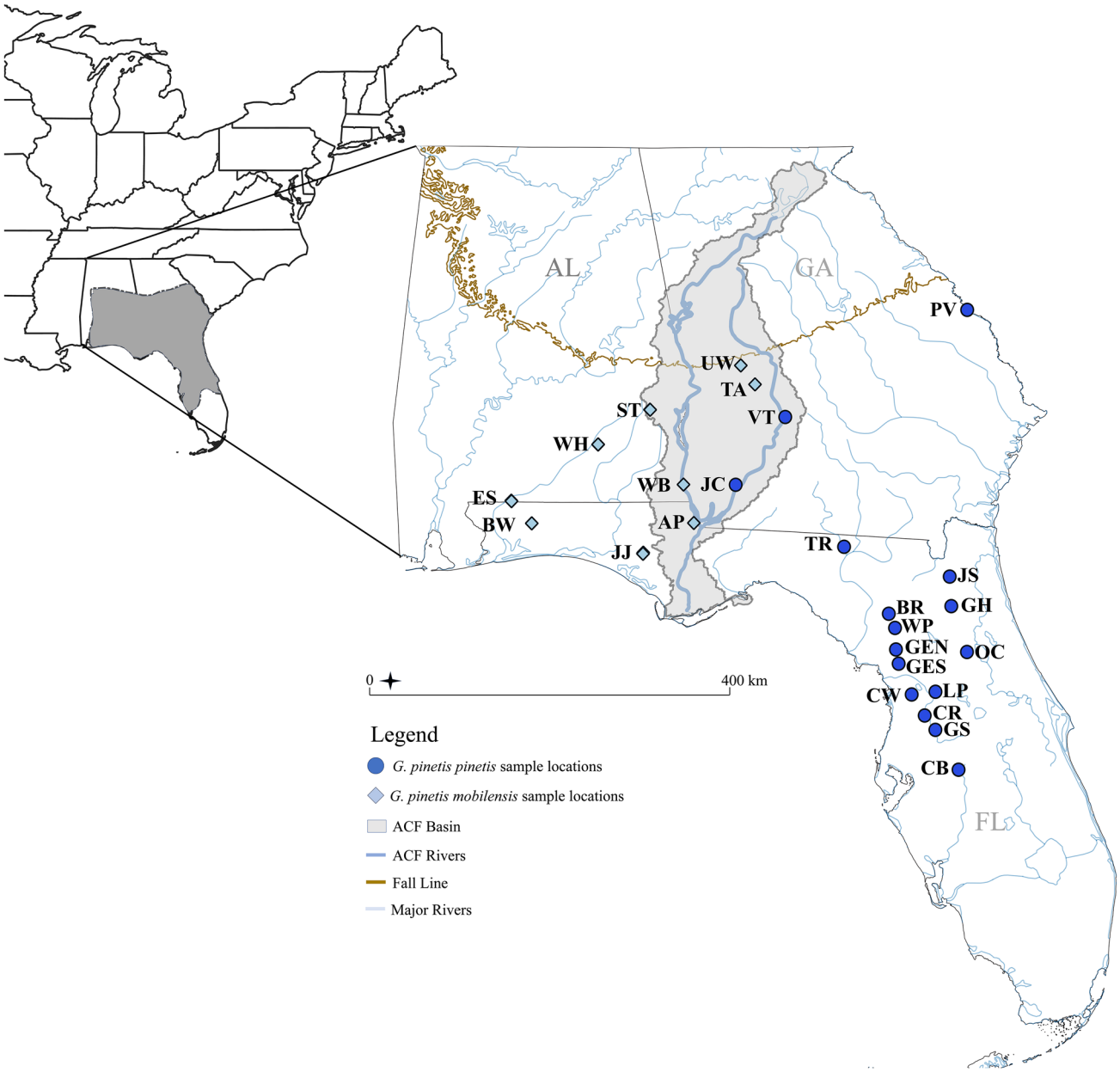

Fig. 1 Map of the United States (upper left) with the historical range of *Geomys pinetis* shaded in grey. The inset depicts the states of Georgia (GA), Alabama (AL), and Florida (FL), United States, with sampling locations labeled with site ID (Table 1: N = 25 sites, N = 121 individuals). Sampling location symbols and colors represent putative subspecies designations for *G. pinetis mobilensis* (light blue diamonds) and *G. pinetis pinetis* (dark blue circles). The ACF Basin is shaded in gray, and the ACF Rivers are represented by dark blue lines. Additional major rivers are drawn in light blue. The Fall Line represents the transition between the Piedmont and Coastal Plain physiographic provinces and delineates the northern range extent of *Geomys pinetis*)

Methods

Sampling, genotype-by-sequencing, and bioinformatics

We sampled 149 *G. pinetis* (1–26 individuals per sampling location (N = 30 locations)) from across their distribution, including Florida, Georgia, and Alabama (United States). Genetic sampling was conducted in a broader study examining habitat features associated with *G. pinetis* at local and broad spatial scales (Duncan et al. 2020; Parsons et al. 2022). Animals were live-trapped using Hart (Hart 1973), modified pitfall traps (Moore et al. 2019), and Sherman traps (LFAHD traps, H. B. Sherman Traps, Inc., Florida, USA). A tissue biopsy was taken from the tail, and the pocket gopher was released (IACUC #201509101). Genomic DNA was

extracted and purified using Solid Phase Reverse Immobilization (SPRI; Beckman Coulter Life Sciences). Due to low DNA yield, we performed Sygnis TruePrime Whole Genome Amplification (Sygnis TruPrime WGA) on 18 samples representing 12 locations.

Reduced representation libraries were constructed using a high-fidelity PstI restriction enzyme (New England BioLabs) and a modified genotype by sequencing (GBS) protocol (Wallace and Mitchell 2017) of Elshire et al. (2011). We generated two libraries, with samples from different locations distributed across each plate, and sequenced each plate on one of two NextSeq500 Illumina lanes (1 × 75 bp). Raw sequence data (range = 1–17 million reads per sample) were demultiplexed and quality filtered using the *process_radtags* program with parameters -e PstI, -t 65, -r, -c, -q in STACKS version 2.1 (Catchen et al. 2013).

Before de novo assembly of loci, we used a subset of N = 8 individuals selected to represent the geographic range to assess the performance of three main parameters (m = 3–7, M = 1–8, and n = M and M − 1 and M + 1) on detecting polymorphic loci. These variables control STACKS's locus formation and polymorphism identification (Paris et al. 2017). Based on our evaluation, we selected parameter combinations m = 2, M = 4, n = M = 4, and an r = 0.8 for de novo SNP discovery (Fig. S1). In populations, we retained a single SNP per locus and filtered out loci with a maximum heterozygosity > 0.5 and a minimum minor allele frequency ≤ 0.05. We conducted additional filtering in VCFtools (Danecek et al. 2011). We excluded sites with a minimum mean read depth across individuals of < 15 and sites with > 25% missing data. We included only sites with a minor allele count ≥ 3 and removed individuals with > 50% missing data (Table S1). Unless otherwise stated, all subsequent analyses were performed in R version 3.6.2 (R Core Team 2019).

To define non-outlier and outlier data sets, we screened for outlier loci using the pcadapt (Privé et al. 2020) package, which uses a principal components analysis (PCA) to detect population structure and distinguish outlier loci based on Mahalanobis distances (Luu et al. 2017). We assessed a range of *K* values from 1 to 20 to select the optimal choice for *K*. Outlier SNPs were identified using Mahalanobis distances, which were transformed into adjusted p-values (Benjamini and Hochberg 1995) at an alpha of 0.01. Following this, we retained a neutral dataset (non-outlier loci), an outlier dataset, and a total dataset (neutral and outlier loci) for downstream analyses.

Phylogeny

To examine phylogenetic structure within the neutral SNP dataset, we constructed a maximum likelihood phylogenetic tree on the total dataset with IQ-TREE using the web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al. 2016). We created a matrix for phylogenetic analysis using vcf2phylip v2.0 (Ortiz 2019). The ultrafast bootstrap assessed branch support with 1000 replicates (Hoang et al. 2018) and the SH-aLRT test (Guindon et al. 2010). The best-fit substitution model (TUM + F + G4) was determined using the auto function, which implements ModelFinder (Kalyaanamoorthy et al. 2017).

Genetic structure

To determine the extent of hierarchical structuring among locations, we followed the workflow of Funk et al. (2012) to assess the extent of genome structuring of evolutionary units. We first examined all loci to delineate the overall population structure that may be shaped by neutral and adaptive divergence (total dataset). We then examined neutral loci to detect differences shaped primarily by gene flow and drift. Lastly, we examined outlier loci separately to examine genetic divergence potentially shaped by environmental or other putatively adaptive variation.

We performed PCA on the three data partitions described above using the R package pcadapt (Privé et al. 2020) and visualized results with ggplot2 (Wickham 2016). The number of principal components retained in the PCA was determined by examining a scree plot in which PCs retained corresponded to eigenvalues to the left of where the curve on the plot leveled out (Cattell 1966). We assessed the optimal number of principal components from a K value from 1 to 20.

We then estimated recently shared coancestry among individuals at neutral SNPs using the program fineRADstructure (Malinsky et al. 2018). First, we re-ran STACKS, omitting the—write-single-snp option to include all SNPs in each locus to improve estimates of nearest-neighbor relationships. Because our SNPs are not sorted based on a reference genome, we ordered loci based on linkage disequilibrium (LD) using the sampleLD.R script provided with the program. This preliminary step reduces the effect of LD on diagnosed clusters (Malinsky et al. 2018). We calculated the nearest neighbor haplotype relationships and generated a heatmap to visualize recently shared coancestry.

Because spatial proximity can substantially affect the structure of genetic variation, we evaluated spatial hierarchical population structure using spatial PCA (sPCA; Jombart and Ahmed 2018) and a model-based clustering approach implemented in tess3r (Caye et al. 2016; Caye and Francois 2016). A centered, scaled PCA was calculated in adegenet (Jombart and Ahmed 2018) with the *spca* function and applying Delaunay triangulation to model the connectivity network between spatially referenced trap locations. We examined scree plots to determine whether global and local spatial components were present. Global scores (largest positive PC) indicate the presence of spatial structure, whereas

local scores (smallest negative PC) may reflect greater than expected differentiation between neighboring groups. The significance of the largest global (global.rtest) and local (local.rtest) scores were tested using 9999 permutations. For the spatial Bayesian clustering method implemented in tess3r, we used the projected least squares algorithm and a maximum K = 25 with 10 replicates of each K. We examined the cross-validation score for each value of K to identify the number of clusters. The best choice of *K* was based on when the curve exhibited a plateau or started to increase (Caye et al. 2016). Individual ancestry coefficient maps were visualized with kriging spatial interpolation.

Population statistics

We calculated genetic diversity estimates of *G. pinetis* sampling locations with GENODIVE version 3.05 (Meirmans 2020) with non-outlier (neutral) loci. Estimates include observed heterozygosity (H_O), expected heterozygosity (H_E), and inbreeding coefficient (G_{IS}; analogous to F_{IS}) for each sample locality with a sample size greater than one.

To assess levels of genetic differentiation among populations, we used the *gl.fst.pop* function from StAMPP (Pembleton et al. 2013) in dartR (Gruber et al. 2018) to calculate unbiased pairwise F_{ST} (Weir and Cockerham 1984) between sampling locations with a sample size greater than one for the full range and eastern region (values were not estimated for the western region separately due to small sample sizes). We calculated the adjusted p-value from the Benjamini-Hochberg (Benjamini and Hochberg 1995) procedure and 95% confidence intervals with 10,000 bootstrap replicates.

Demographic analyses

We estimated the timing of divergence between the two clades distributed on either side of the ACF using DIYABC-RF (Collin et al. 2021), which implements approximate Bayesian computation combined with supervised machine learning. We assumed a single historical split from a common ancestral population of size N_A into two independent populations, *mobilensis* (N1) and *pinetis* (N2), at some past time (t). Because parameter estimation is sensitive to population substructure (i.e., particularly within the *pinetis* clade), we restricted our analysis to the 29 samples from JC (Fig. 1; Table 1) and the combined 16 *mobilensis* samples (both sets of samples had high inferred internal coancestry, see results). We excluded SNPs missing in all individuals in one population, which reduced the data set to 5782 neutral SNPs across the 45 individuals. Final training set simulations (5,000,000) applied uniform priors between 1000–10,000,000 for NA and 1000–1,000,000 for N1 and N2, and 1000–5,000,000 for t. We used five noise variables and ran 10,000 training set simulations, and the number of sampled trees was 1000. Training set simulations were run five times to assess the similarity in results. For parameter estimation (t), we examined the out-of-bag computation of global and local accuracy measures, reporting the NMAE (i.e., the normalized mean absolute error, representing the average absolute difference between the point estimate and the true simulated value divided by the true simulated value) with the mean presented as a point estimate. We also report the 90% coverage of true simulated values.

Table 1 Site location ID, state, and number of samples (N) of *Geomys pinetis* for each sampling location

Location ID	State, USA	N	H_O	H_E	G_{IS}
ES	AL	2	0.020	0.036	0.450
ST	AL	4	0.042	0.061	0.314
WH	AL	2	0.030	0.052	0.418
PV	GA	6	0.015	0.030	0.489
UW	GA	1	–	–	–
TA	GA	1	–	–	–
VT	GA	5	0.100	0.168	0.406
JC	GA	24	0.055	0.081	0.316
WB	GA	1	–	–	–
BW	FL	1	–	–	–
AP	FL	1	–	–	–
JJ	FL	3	0.049	0.091	0.461
JSF	FL	3	0.034	0.068	0.494
GH	FL	3	0.046	0.089	0.488
BR	FL	6	0.086	0.122	0.293
WP	FL	5	0.091	0.124	0.263
TR	FL	5	0.084	0.119	0.300
GEN	FL	7	0.102	0.138	0.262
GES	FL	6	0.095	0.127	0.247
OC	FL	6	0.040	0.057	0.301
CW	FL	6	0.074	0.104	0.286
LP	FL	7	0.079	0.105	0.248
CR	FL	6	0.074	0.100	0.260
GS	FL	5	0.056	0.078	0.281
CB	FL	5	0.036	0.067	0.464

Population genetic statistics are included for each *Geomys pinetis* sample location with N > 1: observed heterozygosity (H_O), expected heterozygosity (H_E), and the inbreeding coefficient (G_{IS})

Results

Sampling, genotype-by-sequencing, and bioinformatics

After applying additional quality control filters (Table S1), we retained 121 individuals from 25 sampling locations and 9373 loci (Table 1; Fig. 1). We selected K = 3 as the optimal choice for pcadapt outlier analysis, resulting in 1794 outlier

loci being identified (Fig. S2). We retained 9373 loci for the total dataset, 7579 SNPs for neutral, and 1794 SNPs for the outlier dataset.

Phylogeny

Two well-supported major clades emerged in our phylogenetic analysis (Fig. 2). The *mobilensis* clade, west (Fig. 2), included all pocket gophers collected from Alabama and the panhandle of Florida, west of the Apalachicola and Chattahoochee rivers and all pocket gophers collected at three locations east of the Chattahoochee River in Georgia (UW, TA, and WB, Fig. 1). The *pinetis*, east (Fig. 2), clade is further subdivided into three clades: one consists of three locations (JC, VT, and TR, Fig. 1) in south and central Georgia and north Florida. Of the latter, JC was located west of the Flint River, within the ACF basin. This clade diverged early from the rest of the *pinetis* clade (Fig. 2). A second clade consists of JS, PV, and GH (Fig. 2). This clade represents populations distributed in northeast Florida and the furthest northeast population in Georgia. The third and largest clade consists of the remaining Florida peninsula populations (Fig. 2). There is considerable geographic structuring within each clade.

Genetic structure

Principal component analysis was consistent with the phylogenetic analysis, with a large separation between *mobilensis* and *pinetis* samples for all three data partitions along the first axis (Fig. 3A–C). The first two axes explained approximately

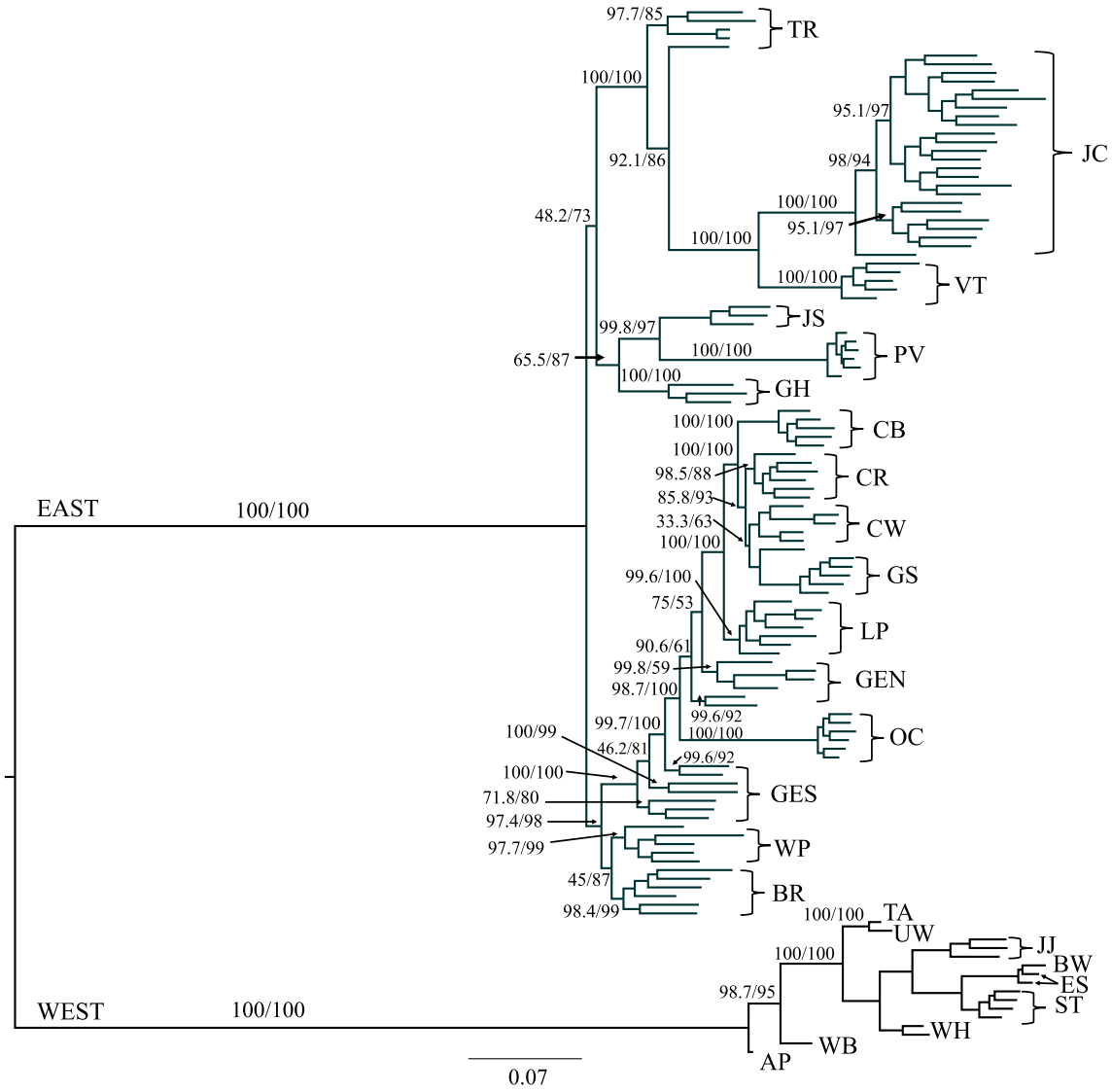

Fig. 2 IQTree phylogeny of *Geomys pinetis* individuals (N = 121; 9373 loci). The tree is rooted at the midpoint with UF bootstrap support/SH-aLRT listed near nodes (tips are labeled with site ID; Table 1)

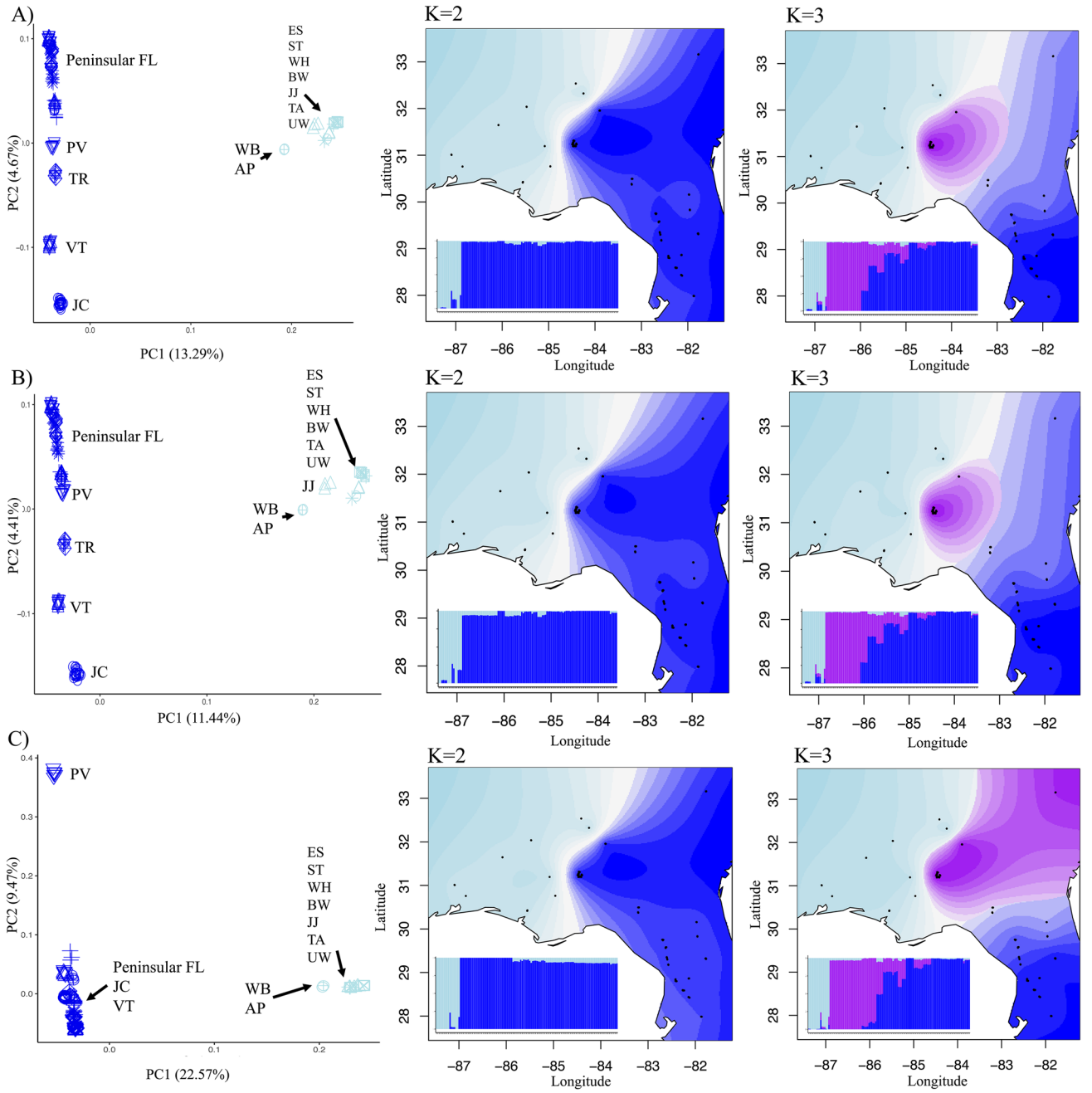

Fig. 3 Principal Component Analysis (PCA: column 1) and range-wide population structure estimated with TESS3 (for K = 2 and K = 3: columns 2 and 3) conducted for *Geomys pinetis* (N = 121) with the **A** total dataset (neutral and outlier (N = 9373)), **B** neutral (N = 7579), and **C** outlier (1794) single nucleotide polymorphisms (SNPs). Sampling locations included in the PCAs (left) are denoted by symbols and labeled within the plot. Geographic maps of ancestry coefficients are presented at $K = 2$ and 3 ancestral populations from the TESS3 analysis (right). Individual sampling locations are represented by black dots, and colors on the map indicate ancestry coefficients with spatial interpolation. Individual ancestry coefficients are presented as bar plots (bottom left) on the map. Colors indicate putative *G. pinetis mobilensis* (light blue) and *G. pinetis pinetis* (dark blue) sites

16–18% of the variation when examining all or neutral loci (Fig. 3A, B). For the same data partitions, the second axis describes the variance among *pinetis* populations, with JC, VT, and TR being more differentiated (Fig. 3A, B), reflecting the phylogenetic results (Fig. 2). The first two PCs from the PCA performed on the outlier loci explained approximately 30% of the variance (Fig. 3C). Here, *mobilensis* and *pinetis* again separated on the first axis (Fig. 3C). However, the second axis separated PV (eastern Georgia) from the rest of the *pinetis* populations. In contrast, PV was intermediate

and closely allied with peninsular Florida based on the total and neutral dataset PCAs (Fig. 3A, B). PCAs conducted on the *mobilensis* and *pinetis* regions separately generally reflected spatial gradient (i.e., northwest to southeast) with similar levels of variance explained compared to the total dataset PCA (Fig. S3). Notably, the eastern outlier PCA separated PV from all other locations along the first axis (16% variance explained), whereas the second axis reflected the northwest-to-southeast gradient (Fig. S3C).

The coancestry matrix generated by *fineRADstructure* (Fig. 4) reflected strong recent coancestry among all individuals from either side of ACF, with all *mobilensis* having high overall recent shared coancestry reflected evenly across geographic locations. Many sampled locations within *pinetis* also had high within-location coancestry (i.e., VT, JC, PV, OC, CB, GS). Other locations, particularly on the Florida peninsula, had high levels of genetic mixing as indicated by the more uniform pairwise coancestry among locations (CW and CR, GEN and GES, WP, and BR; Fig. 4). Any indication of recent genetic mixing between *mobilensis* and *pinetis* locations was negligible, including among geographically proximate locations in the ACF.

Spatial PCA conducted on the neutral dataset revealed a strong global spatial signal when all pocket gophers were assessed as a single dataset. The first eigenvalue was 285.9 ($P < 0.0001$), and the smallest negative was −3.44 ($P = ns$), the latter suggesting that local structure was absent. The significant first eigenvalue could reflect isolation by distance in continuously distributed species with limited spatial dispersal ability. However, in this case, the spatial pattern reflected in the first PC characterized the deep divergence between the *pinetis* samples separated at the ACF basin (Figs. 2 and S4). Significant global spatial structures and an absence of local structure were also detected when *mobilensis* and *pinetis* were evaluated separately, likely reflecting actual isolation by distance within each lineage.

For tess3r the cross-entropy curves in each case exhibited a change in curvature beginning at $K = 2$ and included $K = 3$ (Fig. S5). The two identified lineages clustered separately for the total and neutral datasets (Fig. 3; Figs. S6, S7). For $K = 3$, the *pinetis* lineage consisted of a western Georgia and an eastern Georgia + Florida cluster (Fig. 3A–B; Figs. S6S, S7). When the outlier loci were assessed, the results for $K = 2$ were the same as previous, but the $K = 3$ resulted in a north–south split within the *pinetis* lineage (Fig. 3C; Fig. S8).

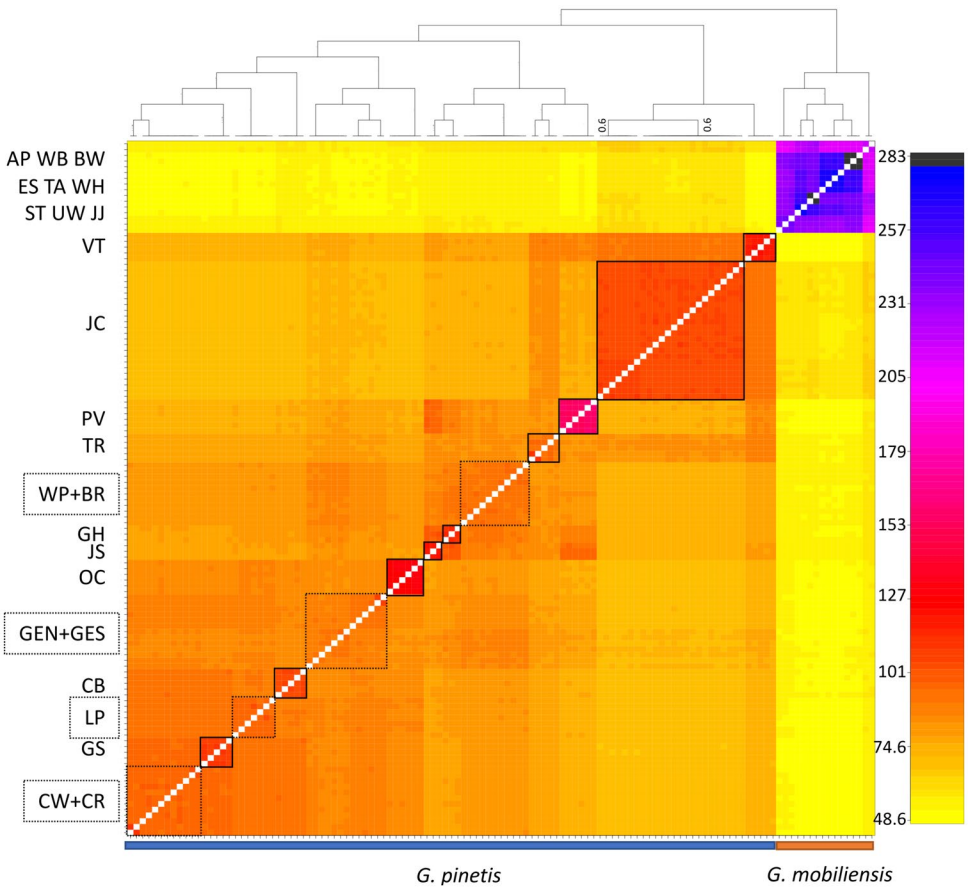

Fig. 4 Clustered fineRADstructure coancestry matrix. The color gradient (scale to right of figure) ranges from low (yellow) to high (black) coancestry, with a corresponding numeric scale. Each cell represents the coancestry value between pairs of individuals. Individuals within recognized subspecies cluster together, with high coancestry among the nine locations representing *mobilensis*. Substructure is evident within the eastern lineage (*pinetis*). Support for clustered branch nodes (top) are all equal to 1 unless otherwise noted. Box outlines delineate sample locations for *pinetis*, with dotted outlines reflecting geographically proximate locations

Population statistics

Levels of H_E ranged from 0.030 (PV in east GA) to 0.168 (VT in GA), with lower H_O than expected for all sampling locations (Table 1). G_{IS} estimates ranged from 0.247 (GES in peninsular FL) to 0.494 (JSF in north FL) (Table 1). Pairwise population differentiation estimates for the total dataset ranged from a minimum of $F_{ST}=0.044$ to as high as $F_{ST}=0.938$ (Table S2). Generally, the highest differentiation estimates ($F_{ST}>0.7$) were between *mobilensis* and *pinetis* locations (Table S2). The lowest F_{ST} values were calculated between sampling locations within peninsular FL. In contrast, the largest ($F_{ST}>0.9$) were between PV and *mobilensis* locations (Table S2). Pairwise F_{ST} estimates were broadly consistent but lower when calculated with neutral loci (Table 2). Pairwise differentiation based on outlier loci (Table S3) was considerably greater between PV and the rest of the *pinetis* sample locations than neutral loci (Table 2).

Demographic analyses

Under our simple two-population divergence model, *mobilensis* and *pinetis* split from a common ancestor approximately 1.3×10^6 generations ago (averaged 90% CI 3.3×105–3.0×106). Assuming two generations per year corresponds to a divergence time that pre-dated the Last Glacial Period (LGP, Table S4, Fig. S9). Although the point estimates have relatively high posterior error values (Local error ~ 0.398), the 90% credibility estimates from each replicate predate LGP and are bounded by the start of the Pleistocene Era.

Discussion

In agreement with previous genetic work (Avise et al. 1979; Sudman et al. 2006; Chambers et al. 2009; Soto-Centeno et al. 2013), our results provide strong evidence that southeastern pocket gophers consist of two distinct lineages that have been reproductively isolated for an extended period (i.e., likely since the Pleistocene) and may have undergone some degree of adaptive divergence. We also found support for our prediction that the habitat specificity and a presumed low vagility of *G. pinetis* (Hickman and Brown 1973; Warren et al. 2017b; Pynne et al. 2019a), combined with increased fragmentation of longleaf pine savannas (Noss et al. 1995; Frost 2007), resulted in a signature of strong genetic drift, as has been found in other pocket gopher species (Penney and Zimmerman 1976; Patton and Yang 1977; Patton and Feder 1981; Welborn and Light 2014). Factors such as limited population connectivity and small home ranges will be reflected in neutral signatures of genetic variation, whereas outlier SNP loci may reflect environmental variation or gradients (Soto-Centeno et al. 2013).

Geomys is restricted to south of the Fall Line, where the Piedmont changes to the Coastal Plain physiographic province (Fig. 1). The portion of the ACF below the Fall Line underwent significant geological and environmental changes during the Pleistocene (11,700 to ~ 2.6 mya). Multiple glacial cycles lead to fluctuating sea levels, greatly expanding the Coastal Plain during glacial periods and leaving marine dunes during inter-glacial high stands (Torak and McDowell 1995). At the ACF, these changes included expanded floodplain development and increased stream sinuosity (Couch et al. 1997), making the area less suitable for subterranean mammals. Further north, away from the coast, the rivers of the ACF flow through areas characterized by ridges and bluffs, lending itself to the structuring of *Geomys* populations. Before European settlement, the ACF Coastal Plain was partially vegetated by wiregrass and pine savannah, though the majority was riparian forest and swamp (Wharton 1978). These environmental features and the limited vagility of pocket gophers likely explain the occurrence of divergent monophyletic nuclear and mitochondrial lineages that appear to meet at the ACF.

At regional and local scales (within eastern and western lineages), all pairwise comparisons of neutral markers reflect a substantial degree of demographic isolation. The lowest pairwise F_{ST} exceeded 0.07 and was between Florida peninsular populations separated by only ~ 26 km. Most measures of divergence within *pinetis* and *mobilensis* lineages far exceed that value, suggesting that local populations separated by 10 s of km are likely demographically independent and should thus be considered management units. Although some of our pairwise F_{ST} estimates involved small sample sizes, this is potentially less of a concern for larger SNP datasets due to the bi-allelic nature of these markers (see Nazareno et al. 2017 for discussion).

Previous applications of genetic variation to infer MUs have relied on tests to reject panmixia or, more recently, on approaches that measure whether genetic divergence exceeds a predefined threshold (Palsbøll et al. 2007). Our data supports the lack of panmixia among pairwise comparisons even between locations separated as little as 13 km (e.g., GEN and GES), as all F_{ST} values were highly significant. Although not quantified directly here, our values of F_{ST} (Table 2) indeed suggest that migration rates are far lower than 10% per generation (Palsbøll et al. 2007; Waples and Gaggioti 2006).

At the range-wide level, patterns in outlier loci reflected the same ACF partition that was detected by neutral loci (Fig. 3). Clearly, demographic isolation has led to significant genetic differences reflected in the exceptionally high F_{ST} scores (> 0.8) differentiating the two lineages. This

Table 2 Pairwise population differentiation estimates (F_{ST}) for the full range of *Geomys* sampling locations with a sample size > 1 (N = 20) examined with neutral loci (N = 7579)

	BR	CB	CR	CW	GEN	GES	GS	LP	OC	TR	WP	JS	JJ	GH	JC	VT	ES	ST	WH
BR																			
CB	0.371																		
CR	0.305	0.215																	
CW	0.308	0.223	0.072																
GEN	0.096	0.255	0.162	0.161															
GES	0.182	0.271	0.158	0.149	0.068														
GS	0.399	0.341	0.190	0.201	0.263	0.262													
LP	0.280	0.258	0.133	0.121	0.155	0.139	0.235												
OC	0.451	0.586	0.497	0.504	0.407	0.442	0.590	0.467											
TR	0.198	0.462	0.383	0.387	0.231	0.278	0.462	0.365	0.524										
WP	0.045	0.377	0.299	0.300	0.087	0.172	0.394	0.275	0.473	0.210									
JS	0.246	0.553	0.449	0.445	0.258	0.332	0.553	0.414	0.613	0.320	0.261								
JJ	**0.700**	**0.797**	**0.751**	**0.746**	**0.683**	**0.704**	**0.787**	**0.744**	**0.822**	**0.711**	**0.706**	**0.779**							
GH	0.164	0.498	0.415	0.408	0.199	0.277	0.514	0.372	0.569	0.295	0.187	0.292	0.755						
JC	0.331	0.437	0.423	0.426	0.352	0.376	0.457	0.416	0.484	0.245	0.329	0.360	0.600	0.356					
VT	0.432	0.618	0.554	0.552	0.426	0.472	0.624	0.533	0.674	0.345	0.442	0.552	0.789	0.522	0.273				
ES	**0.755**	**0.856**	**0.803**	**0.796**	**0.730**	**0.753**	**0.845**	**0.791**	**0.879**	**0.765**	**0.759**	**0.854**	0.409	**0.829**	**0.641**	**0.842**			
ST	**0.760**	**0.841**	**0.799**	**0.795**	**0.739**	**0.757**	**0.835**	**0.789**	**0.860**	**0.770**	**0.765**	**0.830**	0.372	**0.813**	**0.648**	**0.831**	0.383		
WH	**0.746**	**0.846**	**0.792**	**0.788**	**0.722**	**0.747**	**0.837**	**0.781**	**0.869**	**0.755**	**0.747**	**0.836**	0.341	**0.808**	**0.632**	**0.833**	0.604	0.388	
PV	0.347	0.595	0.478	0.479	0.308	0.377	0.602	0.449	0.671	0.401	0.376	0.557	**0.855**	0.551	0.369	0.608	**0.918**	**0.888**	**0.909**

Pairwise values between *mobilensis* and *pinetis* are bold. Significance was assessed by an adjusted p-value from the Benjamini–Hochberg procedure and 95% confidence intervals with 10,000 bootstrap replicates. All pairwise comparisons were significant

similarity in genetic differentiation between neutral and outlier loci could belie underlying novel combinations of alleles and unique evolutionary features (Palumbi et al. 2001). For example, ecological niche modeling further supports the possibility of adaptive divergence east and west of the ACF (Soto-Centeno et al. 2013). The evolutionary divergence between *pinetis* and *mobilensis* also includes differences in chromosomal fundamental number (Laerm et al. 1982) and each lineage being host to a distinct sister species of parasitic lice (Nadler and Hafner 1993; Sudman et al. 2006). Thus, our consistent east–west split between neutral and outlier SNPs may reflect neutral and putatively adaptive divergence. Although there is no direct confirmation that the detected outlier loci represent adaptively divergent portions of the genome (e.g., using an annotated genome), differences between neutral and outlier loci found within the *pinetis* portion of the range (Fig. 3B, C) do suggest that outlier loci are not simply reflecting historical demographic processes. Minimally, an annotated reference genome and denser SNP sampling will be necessary for elucidating the extent of adaptive divergence.

Geomys pinetis is currently treated as a polytypic species. However, based on our study and previous evidence, we have adopted the null hypothesis that *G. pinetis* and *G. mobilensis* represent two distinct and reciprocally monophyletic sister species of pocket gophers that exhibit reproductive isolation, and would not freely interbreed if they were to occur in syntopy (sensu Gill 2014). This prediction places the burden of proof on rejecting our H_O by demonstrating the possibility of gene flow rather than explicitly assuming gene flow would be unfettered (Gill 2014). There is no evidence of secondary contact between *G. mobilensis* and *G. pinetis* occurring at the ACF. Secondary contact refers to the re-establishment of gene flow following (typically) allopatric divergence of sister populations and is often associated with the Quaternary glacial periods (Hewitt 1999). Secondary contact would be inferred by the presence of clinal patterns of genetic markers across the contact zone or evidence of recent admixture, neither of which was detected here nor in previously detailed mtDNA studies. Although we did not explore mtDNA variation in our samples, recent extensive mtDNA sequencing by Soto-Centeno et al. (2013) revealed a lack of syntopy of mtDNA lineages within the sampled ACF locations, suggesting that the nuclear and mitochondrial genomes are at equilibrium within eastern and western lineages. Finally, it should be noted that both lineages are close, centrally within the ACF, without major riverine barriers separating them (e.g., GC and WB; see Soto-Centeno et al. (2013)).

Our estimate of divergence timing is mainly consistent with that presented for mtDNA in Soto-Centeno et al. (2013). Our estimates span from just before the onset of the LGM (160,000 ybp) to very early in the Pleistocene (~ 1.5 mya; Table S4). However, mtDNA is expected to reach reciprocal monophyly faster than nDNA due to its smaller N_e (Birky et al. 1989). The divergence time of Soto-Centeno et al. (2013) is the estimate of Time to the Most Recent Common Ancestor (TMRCA), which is likely to be considerably earlier than the actual population divergence (*t*) (Edwards and Beerli 2000). Similarly, estimates of TMRCA based on single loci are expected to overestimate TMRCA, particularly if the two lineages have achieved reciprocal monophyly (Edwards and Beerli 2000). Thus, the strong degree of overlap between the multi-locus SNP dataset and the cyt b mtDNA results from Soto-Centeno et al. (2013) is at least partially accounted for by the uncertainty in estimating divergence times using single locus datasets and the lack of information on historical effective population sizes. Pocket gophers appear to have been isolated since before the last glacial maximum. Reciprocally monophyletic populations can be assumed to have developed novel combinations of alleles across many loci and may harbor unique features that should be considered by natural resource managers (Palumbi et al. 2001).

Given the possibility of substantial reproductive isolation (Coyne and Orr 2004), our recommendation accommodates the current and previous studies that reflect the large genetic (Sudman et al. 2006), chromosomal fundamental number differences (Laerm et al. 1982), in species interactions (Nadler and Hafner 1993), and environmental evidence (Soto-Centeno et al. 2013) supporting the absence of gene flow across short geographic distances at the ACF. Fine-scale transect studies spanning the ACF are needed to test for the presence of interbreeding and the extent of gene flow. However, ongoing gene flow appears unlikely given the patchy nature of suitable habitat in this region and the evidence of non-trivial genomic differentiation among populations within lineages. Thus, our null hypothesis of no gene flow seems to be the most appropriate hypothesis in lieu of any future evidence documenting substantive gene flow that would allow for its rejection.

Conservation and management implications

The recent decline of pocket gophers from across its historic range (Laerm et al. 1982; Ozier et al. 2005; Scott 2008) has led to it being considered a high-priority species in the state wildlife action plans (ADCNR 2015; FWC 2019; GA DNR 2015). Our recommendation of recognizing *G. mobilensis* and *G. pinetis* as distinct species will significantly affect their conservation and management. For example, conservation planning now must consider two species with relatively smaller distributions rather than one. Furthermore, these two species may require different management strategies. For instance, *G. mobilensis* is exceedingly rare (Parsons et al. 2022), requiring a greater need for species and habitat protection west of the ACF.

The small effective population size typical of pocket gopher colonies (Penney and Zimmerman 1976; Patton and Feder 1981; Zimmerman 1988; Visser et al. 2018; Steinberg and Patton 2000) and their limited ability for long-distance dispersal (Daily and Patton 1990; Hafner et al. 1998; Steinberg and Patton 2000) makes them vulnerable to demographic and environmental stochasticity. Recolonization following pocket gopher population extinction is unlikely due to the pocket gopher's low vagility. Management strategies, such as reintroductions or translocations (Pynne et al. 2023), may be necessary to mitigate population declines within lineages, but because of the large evolutionary divergence between *pinetis* and *mobilensis*, reintroductions and translocations should not be conducted between the two (Moritz 1999). Care should be taken within the ACF to avoid mixing the two evolutionary lineages—this will inevitably require genotyping representatives from each new colony to confirm their lineage. We recommend a more intensive, fine-scale genetic mapping of the two lineages across the ACF.

Additional research assessing pocket gopher dispersal patterns and landscape factors that affect individual movements is necessary to effectively decide how to facilitate gene flow in a fossorial species. Pocket gopher occurrence is high in areas with low levels of human disturbance (Duncan et al. 2020), so targeting these areas for conservation and management could support dispersal between protected areas. Reintroductions and translocations may be necessary to maintain genetic connectivity, increase genetic diversity, and reduce extinction risks within management units. However, selecting source and recipient sites should be carefully considered, and the relocation of individuals over long distances should be avoided due to the observed substantial genetic divergence and possible adaptive differences across latitudes.

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Author contributions JDA, RAM, LMC, RAG, and SBC conceptualized the study. SID, JTP and EIP conducted the field sampling. JDA, SID, generated data and JDA, SID, and CC performed data analysis. JDA and SID drafted the initial manuscript and all authors provided input and contributed to editing. All authors read and approved the final manuscript.

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Data availability The data for this study has been available in the Sequence Read Archive (SRA) of NCBI. Accession to cite for these SRA data: PRJNA1116731 Temporary Submission ID: SUB14478053 Release date: 2025-09-01.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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