The genetic value of *volans*: Assessing phylogenetics and population genetics of southern flying squirrels *(Glaucomys volans*) across their range and at the Kansas range edge

by

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Abstract

Southern flying squirrels (*Glaucomys volans*) are both charismatic and understudied. A species largely dependent on hardwood forest ecosystems for survival due to diet, locomotion, and nesting preferences, *G. volans* are widespread throughout the eastern United States. Given the strong associations of the species with forests, understanding the genetic health, history, and connectivity of regional populations can inform broader understandings of forest health, species conservation, and wildlife management priorities. Despite *G. volans*' wide range, most previous genetic work has focused along the northern edge of the species range, and large gaps in scientific knowledge have persisted to present. Among other considerations, morphologic assessments suggest the presence of four *G. volans* subspecies within the U.S., but no range wide phylogenetic assessments have been done. Further, species populations at range edges often experience environmental conditions that alter population connectivity, natural selection, and ultimately survival. My research includes both a range wide assessment and a regional focus on the western range edge of *G. volans*. In Kansas, *G. volans* is listed as a species of conservation concern largely due to a lack of data and knowledge as opposed to known population decline or genetic attributes.

My master's thesis focuses on addressing two critical knowledge gaps: (1) range-wide phylogenetic history and diversity and (2) the evolutionary legacy of western range edge populations in Kansas to understand conservation needs. First, I analyzed mitochondrial DNA to understand the maternal history of *G. volans* and population diversity. I employed third generation Nanopore MinION sequencing to generate complete mitogenomes, allowing for results to provide greater insight and greater confidence than is possible with only a single gene region. Based on mitochondrial DNA, I observed fewer genetic lineages of *G. volans* across the range than has been suggested through morphometric-based sub-species designations. In addition, I found high regional differentiation, and extremely low levels of genetic diversity at the species' western range edge. Evidence also supports anthropogenic influences on range-edge inter-population diversity. Second, I expanded the genetic assessment to include thousands of independent loci from across the nuclear genome to assess phylogenetic and population structure, meaningful evolutionary units, including adaptative and management units, and regional diversity and differentiation. This dataset supported greater regional population

structure, range-edge genetic fitness concerns, and indicated increased range-wide gene flow that suggests potential for human movement of this species through the pet trade. Ultimately, my research shows that *G. volans* contains two intra-specific lineages consistent with regional differentiation with overall relatively high genomic diversity, although there remain regional concerns and conservation needs for populations along the western range edge, particularly in Kansas.

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Dedication

To my mom and dad. For nurturing me in every way young woman and scientist could dream. I never would have made it this far without my biggest cheerleaders. Making you both proud is one of the greatest joys of my life.

And to Ada Lovelace, Rosalind Franklin, and Jane Goodall. For showing me what it means to be a woman in STEM. I may not be the mother of computing, DNA structure, or primates, but I will carve my own niche.

Chapter 1 - An Introduction to *G. volans* and Range Edge Genetics

Research Need and Overview

Very little scientific research has been conducted on the southern flying squirrel (*Glaucomys volan*s) in Kansas in recent times (but see Bernhardt et al. 2026). Due to this data deficiency and uncertainty regarding species management needs, the Kansas Department of Wildlife and Parks (KDWP) listed *G. volans* as a Species in Need of Conservation (SINC) in 1987. By 2020, the need for and merits of scientific research into the ecology and evolutionary biology of *G. volans* were apparent, leading to the KDWP sponsoring a multidisciplinary, multi-year research program into the ecology and evolution of *G. volans*. Genetic research on local and regional *G. volans* populations is necessary due to the complex life history of *G. volans*, the variable genetic trends at range edges, and the apparent shifts in suitable *G. volans* habitat in Kansas. While related work spans from home range analyses (Bernhardt et al. 2026) to distributional assessments (Robbins unpublished data), my thesis focused on evaluating *G. volans* genetic history and population genetics.

Though the need to assess Kansas *G. volans* drove this work, my thesis aimed to better contextualize these peripheral populations by conducting the first comprehensive multi-locus and range-wide genetic analysis of the species. In addition, this first chapter provides a detailed summary of the biology of *G. volans* based on a review of modern literature, given the existing mammalian species account is dated (Dolan and Carter 1977). This chapter also explores the known trends of species genetics at ranges edges and applies those trends to shape the research directions of Chapters 2 and 3. In my second chapter, I use novel nanopore-based MinION sequencing to enable analyses of full mitogenomes to evaluate *G. volans*' complex lineage history. In the third chapter, I apply additional phylogenetic and population genetic analyses to

nuclear DNA to further elucidate the genetic history patterns and modern population health of Kansas individuals against specimens from across the *G. volans* range. Ultimately, this body of work comprises not only the first Kansas-specific *G. volans* genetic analysis, but also the first range-wide multi-locus analysis, making this research valuable towards systematic reassessment, enhanced knowledge of life history, and improved conservation and management.

Species Account

Introduction - The southern flying squirrel (*Glaucomys volans*) is one of only three glissant rodent species (including *G. oregonensis* and *G. sabrinus*) native to North America (Arbogast 2007). All three species derive their common name “flying squirrel" (although a misnomer) from their propensity to glide by use of a patagium: an expanse of skin connecting the individual’s front and hind legs that acts like a parachute (Dolan and Carter 1977). Gliding is critical to these species’ arboreal life history (Dolan and Carter 1977), allowing for highly efficient movement within closed forest habitat (Flaherty et al. 2010). Despite a broad distribution across eastern North America and Central America (Figure 1.1A, Figure 1.1B), *G. volans* are relatively unstudied compared to other North American squirrel species. Although a full mammalian species account for *G. volans* is available, it is dated and does not consider the literature from recent decades (Dolan and Carter 1977). This species account will compile past research into the ecology and evolutionary history of *G. volans*.

Distribution, Taxonomic History, and Diversity- Glaucomys volans occur across the eastern half of North America as far north as southeastern Canada (Cassola 2016) and persist in isolated montane forests of Central America (Kerhoulas and Arbogast 2010). Given the strong forest associations of *G. volans*, the species’ range in the United States and Canada is limited on its western range edge by the Great Plains grasslands (Figure 1.1A). Along the northern edge, which

is sympatric with sister species *G. sabrinus*, the range of *G. volans* is primarily limited by winter climate (Weigl 1978). Historically, cold winters at the northern range edge limited survival of *G. volans* due to physiological constraints, allowing *G. sabrinus* a competitive advantage (Weigl 1978). However, as recent regional warming has made winters milder, *G. volans* have been observed expanding northwards and outcompeting local *G. sabrinus* populations (Wood et al. 2016).

Long before formal description, *G. volans* were known to indigenous populations (Mooney 1992). The first scientific documentation of *G. volans* in western science was Carl Linnaeus' 10th edition of *Systema Naturae* in 1758 where the populations in Virginia and Mexico were listed under the name *Mus volans* (von Linné and Salvius 1758). The genus nomenclature changed to *Glaucomys volans* in 1908 (Thomas 1908). Morphometric analyses further resolved the species into ten regionally distinct subspecies (Howell 1918; Hall and Kelson 1959; Goodwin 1961; Dolan and Carter 1977). More recently, genetic analyses have allowed for exploration of evolutionary relationships within the genus *Glaucomys*. The use of mitochondrial and microsatellite data has even identified a third *Glaucomys* species (*G. oregonensis*) that was previously considered part of *G. sabrinus* (Arbogast et al. 2017).

Within *G. volans*, several regional genetic studies have been conducted but critically, from both ecological and evolutionary perspectives, the existing literature for *G. volans* is geographically or otherwise limited. Most research has occurred on the northern range-edge or else does not have sufficient sampling to account for dynamics across the complete species' range. In the northeastern United States, microsatellites were used to detect three genetic clusters that were attributed to isolation driven by historical logging decreasing connectivity and leading

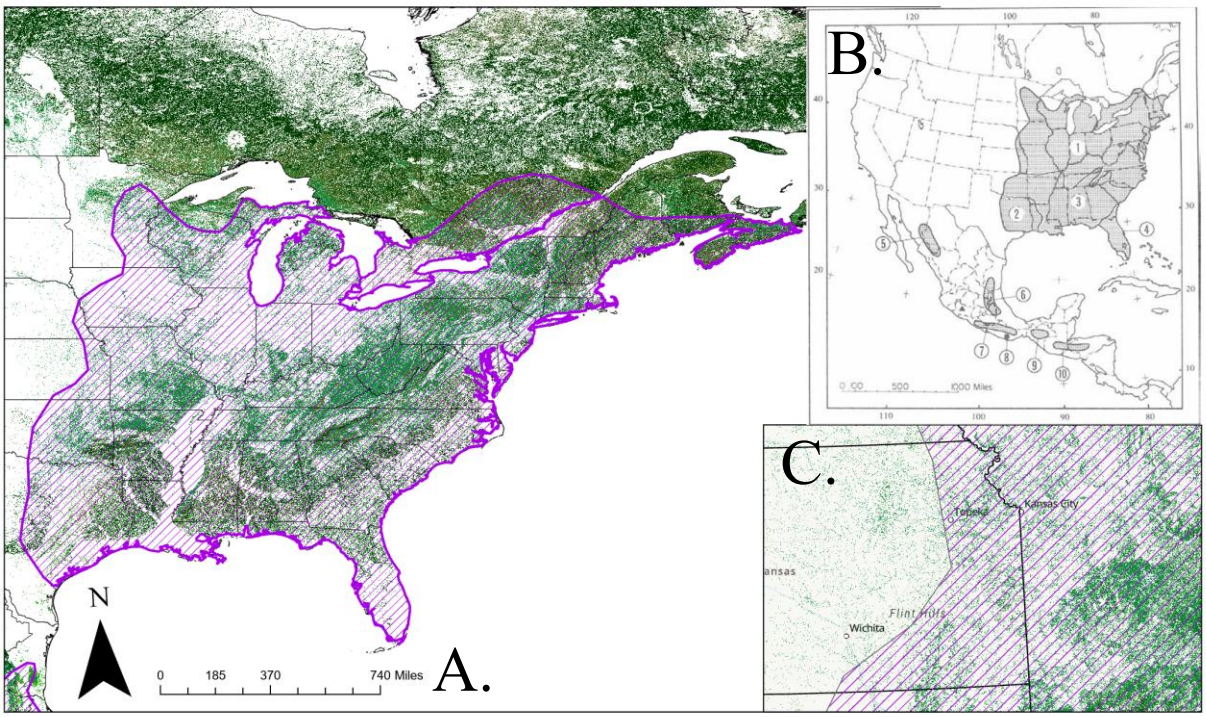

Figure 1.1 (A) Distribution of *G. volans* (purple outline) across North America with associated forest cover. (B) Figure 4 from Dolan and Carter 1977 reflecting subspecies distributions. (C) Visual of forested areas of Kansas and neighboring states showing possible forested dispersal corridors.

to group differentiation (Vivas-Toro et al. 2023). Along the northern sympatric zone, the first *G. volans* long-read genome was assembled and used to detect declines in effective population size 40-80 thousand years ago (Wolf et al. 2022). The study with the broadest range used mitochondrial Cytochrome-b to contextualize samples from the eastern half of the range within the broader species (Arbogast 2007). This work identified a single clade that is sister to *G. sabrinus* (Arbogast 2007).

Habitat and Nest-Site Selection- Rather than excavating new nest cavities, *G. volans* preferentially make use of existing cavities of various origin (Taulman 1999; Steinhoff et al. 2012). Often, these secondary nest cavities have been created by woodpeckers (Loeb 1993). Across the species' range, *G. volans* prefer nesting in dead standing trees ("snags") and living hardwood trees such as oak (*Quercus* sp.), maple (*Acer* sp.), and hickory (*Carya* sp.) (Taulman 1999; Holloway and Malcolm 2007; Steinhoff et al. 2012; Zweep et al. 2018). External nesting on pine (*Pinus* sp.) has been observed in pine-dominate forests (Taulman 1999; Steinhoff et al. 2012). Studies into tree selection across *G. volans*' range support plasticity to tree species selection based on forest diversity (Muul 1974). Use of man-made cavities in the form of nest boxes is also common, particularly during the winter months (Althoff and Althoff 2001; Reynolds et al. 2009). However, squirrels are neophobic (Bernhardt et al. 2026) and require extended periods averaging 11 months to begin using the boxes for nesting or caching (Althoff and Althoff 2001). Interestingly, the percentage of nest boxes utilized in some capacity by squirrels per study was typically high, but occupancy at any given time ranged significantly from 4.4% (Reynolds et al. 2009) to 60% (Althoff and Althoff 2001).

In addition to tree and nest type associations, nesting behavior of *G. volans* appears to be biased in favor of unmanaged forests, likely due to the increased cavity availability (snags not

being removed) and decreased environmental degradation (Taulman 1999). However, flying squirrels can exhibit behavioral shifts that allow them to persist in moderately disturbed habitats (Taulman et al. 1998). Furthermore, proximity to water can increase nesting likelihood, given that baited (foraging) traps had no strong correlation with distance to water, but sheltering trap capture rates declined with distance to water (Sonenshine et al. 1979). Generally, distances from water between 20 and 40 meters provide a critical threshold at which *G. volans* detections decline (Muul 1965; Taulman 1999).

Social Behaviors- The most prevalent social behavior of *G. volans* is aggregate nesting. Often, *G. volans* utilize wintering nests in aggregations of up to 25 individuals, depending on location (Layne and Raymond 1994; Althoff and Althoff 2001; Reynolds et al. 2009). These aggregations are particularly prevalent in the winter months to support thermoregulation (Dolan and Carter 1977; Merritt et al. 2001). In sympatric zones, heterospecific aggregations are sometimes observed, indicating the necessity of aggregations in thermoregulation (Olson et al. 2018). However, given the broad latitudinal range of *G. volans*, many areas of the species range remain warm enough to not necessitate aggregations for thermoregulation. Despite this, aggregations still occur, indicating that social elements may also help to explain this behavior (Layne and Raymond 1994).

Notably, nest aggregations appear to be influenced by kinship, with aggregations of closely related individuals being more prevalent than if aggregating was random (Thorington et al. 2010). Relatedness is associated with the ages of the aggregate individuals (Winterrowd et al. 2005). Mixed-age (adults and young) and subadult aggregations (which occur consistently throughout the year) are more genetically related than adult-only aggregations that primarily occur during breeding seasons (Winterrowd et al. 2005). Aggregates do accept unrelated

individuals, particularly if they are known to aggregate members, indicating the presence of aggregate social dynamics (Thorington et al. 2010; Thorington and Weigel 2011).

Diet- The diet of *G. volans* is heavily linked to their arboreal life history. Tree nuts and seeds comprise much of the diet, with hickory nuts and acorns consistently among the most cached and consumed across the species' range (Muul 1965; Helmick et al. 2014). The preference for nuts may be driven by their high caloric content providing energy for thermoregulation (Weigl 1978). As such, *G. volans* appear to cache nuts for winter consumption (Muul 1965).

However, while the *G. volans*' diet is dependent on nuts, the species is omnivorous. Other vegetation such as buds, bark, and various fruits are consumed when encountered (Muul 1965). Bird eggs, birds, insects, and small mammals are also consumed (Muul 1965). Based on stomach contents from 71 squirrels, acorns accounted for 74% of the diet, with pine seeds, holly and blueberry fruits, mosses, and other plants making up 25% and insects comprising only 1% (Harlow and Doyle 1990). The lack of vertebrate remains was possibly due to differential rates of tissue digestion, resulting in underrepresentation of vertebrate tissues (Harlow and Doyle 1990). Throughout the course of the year, diet contents fluctuate based on food availability driven by time of year and crop success (Muul 1965). The relatively flexible diet of *G. volans* likely influences survival as, when certain foods become scarcer, the species can shift its diet to other available sources (Karmacharya et al. 2013).

Reproduction- Reproduction of *G. volans* occurs twice a year (once in late spring, once in late summer) (Dolan and Carter 1977) and may be driven by changes in photoperiod (Muul 1965). However, whereas males under a natural photoperiod experienced higher numbers of reproductively active (scrotal) individuals as the photoperiod was increasing in spring, photoperiod alone did not explain the two "spikes" in birthing (Muul 1969). However, females

experience two periods of estrous, potentially facilitating two periods of breeding and birthing (Lee and Zucker 1990). Typically, *G. volans* give birth to litters of 2-6 young that differ in rate of maturation based on birthing period (Sollberger 1943; Linzey and Linzey 1979; Lee and Zucker 1990). While spring litters rapidly matured (averaging 2.5 months), summer litter required 6 to 8 months to mature (Lee and Zucker 1990). It may be the case that females born in the previous fall wait until later in the season to breed, resulting in breeding cycles associated with differential female ages (Sollberger 1943; Dolan and Carter 1977).

Dispersal and Home Ranges- Following maturation, *G. volans* disperse from their maternal home ranges, though knowledge of the extent to which young disperse is limited. In *G. sabrinus*, maximum natal dispersal of individuals was roughly 810 meters (Trapp et al. 2019). In contrast, Siberian flying squirrels (*Pteromys volans*) had more variable average natal dispersal distances which range from approximately 250 meters (second litter males) to >3,150 meters (first litter females) (Selonen et al. 2012), indicating that dispersal distance variation for a given species may depend on sex, timing, and spatial availability. In both comparison species, once home ranges were established, individuals appeared to remain in that region unless extreme disturbance or out-competition occurred.

Home range sizes for *G. volans* depend on regional locality, individual age and sex, and seasonality. In Maryland, minimum area-defined home ranges for adults averaged at 2.45 hectares (ha) for males and 1.95 ha for females, while juveniles averaged just 0.61 hectares (Bendel and Gates 1987). However, nearby New Hampshire contour mean ranges were much larger than those in Maryland and differed significantly by sex, with males averaging 16 ha while females only 7.2 ha (Fridell and Litvaitis 1990). While sex-based home range size differences were significant in Maryland, other studies indicate no statistical support for sex-based home

range sizes and indicate that defined home range size is highly variable depending on area analysis approach used. In Ontario, Arkansas, Illinois, and Kansas, sex was not significantly associated with differences in home size (Persad et al. 2025; Stone et al. 1997; Howard 2019; Bernhardt et al. 2026). Interestingly, when Kansas *G. volans* range sizes were measured using Minimum Convex Polygons, measures indicate an average range of roughly 1.55 hectares; but Kernal Density Estimates predict average ranges of 6.44 hectares for males and 3.94 for females (Bernhardt et al. 2026). Furthermore, Illinois *G. volans* home range sizes averaged 10.38ha (Jacques et al. 2017), but multiple studies indicated that this complete home range was substantially larger than the core range of 1.25 ha (Fridell and Litvaitis 1990). Ultimately, this indicates a level of range definition that needs greater clarification across the range, as what is core range versus exploratory range is still poorly understood. Yet another confounding variable is planar range versus topographic range, as in Arkansas some individual topographic home ranges were 20% larger than planimetric home ranges (Stone et al. 1997).

Seasonality may also be associated with home range. Particularly during winter with increased nesting aggregation, home ranges may differ in size and overlap more with other individuals compared with other seasons (Fridell and Litvaitis 1990). Overall, research suggests that *G*. volans contract their ranges and move less during colder temperatures and shorter day photo periods (Nelson and Sagot 2018). While home ranges are among the most-studied aspects of *G. volans*' ecology, more work is necessary across the species' range.

Notable Biotic Associations- The secondary cavity preferences of *G. volans* often results in a strong preference for woodpecker nests. *Glaucomys volans* are one of the top users of red-cockaded woodpecker (RCW) nests with up to 21-25% of monitored RCW nests containing *G. volans* (Loeb 1993; Laves and Loeb 1999). This interspecies dynamic is heavily parasitic as *G.*

volans disrupts active RCW nests. When management practices include mechanical removal of squirrels from red-cockaded woodpecker nests, more eggs were laid and the proportion of nests that produced at least one fledgling increased (Laves and Loeb 1999). Likewise, when nest boxes were introduced as an alternative for *G. volans* nesting, RCW nest success increased (Borgo et al. 2006). While trophic isotope analyses fail to detect signs of egg consumption by *G. volans* (Meyer and Rush 2024), camera detections in a similar geographic region indicate that *G. volans* are among the top predators of RCW from bird nest boxes (DeGregorio et al. 2019). Given that the IUCN reports red-cockaded woodpeckers as near threatened due to extirpation from their historicial habitat, the effects of *G. volans* as an RCW kleptoparasite are potentially extremely harmful to RCW species persistence (Birdlife International 2020).

Conversely, *G. volans* are also observed as hosts for various parasites and related diseases. Louse-borne *Rickettsia prowazekii* has resulted in transfer of typhus from *G. volans* to humans (McDade 1987; Chapman et al. 2009; Prusinski et al. 2014). Interestingly, where *G. sabrinus* and *G. oregonensis* were tested zones where no contact with *G. volans* was likely, no discernable presence of *Rickettsia prowazekii* was detected (Foley et al. 2007). Unfortunately, no scientific research has explored the prevalence of this disease in sympatric zones with southern flying squirrels, where it is possible that this disease may be assisting with the out-competition of *G. volans* against *G. sabrinus*. Another parasite of *G. volans* is the intestinal nematode *Strongyloides robustus* which has been explored in the *G. sabrinus* sympatric zone. While *G. volans* appears to have a much higher rate of *S. robustus* infection than *G. sabrinus,* some research indicates increased deleterious associations on *G. sabrinus* (Pauli et al. 2004), though other work does not observe correlations with body condition in either *Glaucomys* species (Krichbaum et al. 2009).

Regardless of whether disease is a contributing factor in the sympatric dynamics of *G. volans* and *G. sabrinus*, many other dynamic factors have been well-documented. As noted previously, the zone of sympatry between *G. volans* and *G. sabrinus* has been detected along the Canadian/United States boundary east of central Minnesota, with notable extensions south along the Appalachian Mountains (Weigl 1978; Arbogast et al. 2005). In sympatric zones, nest tree partitioning has been observed, with *G. volans* preferentially selecting trees with larger DBH values, as well as utilizing snags and deciduous hardwoods more frequently than *G. sabrinus* (O'Brien et al. 2021; Persad et al. 2025). The thicker trees by *G. volans* has been attributed in many studies to the lower cold tolerance of the species compared to *G. sabrinus*, thus requiring nest sites that are better at containing heat and excluding cold (O'Brien et al. 2021; Weigl 1978). This nest preference in *G. volans* is also likely casual to the higher rates of nest aggression and defense by *G. volans* against *G. sabrinus* (Weigl 1978). Much like their nest partitioning, *Glaucomys* diets also appear to partition with *G. volans* requiring nuts and seeds for winter survival, while *G. sabrinus* consumes mosses and lichens more abundantly (Weigl 1978; Persad et al. 2025). Despite this partitioning, *Glaucomys* species have been observed sharing nests (Olson et al. 2018) and there is increasing evidence that the two species hybridize (Garroway et al. 2010; Wolf et al. 2022), particularly as climate change has permitted *G. volans* to expand further northward (Rogic et al. 2016).

Human-Specific Associations- While *G. volans*' capacity as a host of parasites and pathogens makes the species relevant to human well-being, they are also sought after as pets and are commonly kept in captivity. Though many states have legislation making it illegal to own flying squirrels, the internet hosts multiple sites where "flying squirrels" (often without a species-specific title or locality information) are sold. Although captive breeding appears popular,

poaching is also common. In one recent case, over 3,000 Florida flying squirrels were poached over three years (Wong 2020). Human transport of flying squirrels has repercussions for their evolutionary legacies. Recent genetic analysis in Nebraska indicates that some local populations have genetic signatures dissimilar to known native populations, indicating possible pet trade to Nebraska followed by purposeful or accidental release of individuals into the wild (Wettschreck 2024). Subsequent breeding between transported squirrels and local resident populations may erode locally unique diversity and adaptations (particularly in mammals), with consequences for management of distinct populations (Uller and Leimu 2011). Human transportation of other forest products such as lumber may also facilitate unnatural dispersal of southern flying squirrels.

In contrast, humans also create barriers to the natural movement of flying squirrels. Principally, deforestation and urban development disrupt natural corridors of movement and dispersal for flying squirrels. In the same way that a dam may prevent water-obligate fish from moving upstream, creating gaps in the forest will curtail natural dispersal of *G. volans*. The size of these gaps may play a large role in *G. volans'* ability to maintain interpopulation connectivity. Gliding distances of up to 140 meters have been calculated based on tree heights and wind direction (Howard and Essner 2020) and gap crossing on foot has been observed up to 1,000 meters (Rizkalla and Swihart 2007). However, the energic costs of running and associated risks of predation are significantly greater than when gliding, likely limiting dispersal in the presence of gaps (Flaherty et al. 2010). Accounting for the influences of human-squirrel interactions likely will play a critical role in future management of *G. volans*.

Range Edges and Genetic Effects

Range Edge Fundamentals- The edges of a species range can be defined as the furthest geographic extent that a species can occupy. This definition hints at multiple processes and

features that limit species ranges and the effects of these edges on the genetics of range edge populations. Most generally, range edges are dictated by the limits of environmental suitability for a species. An obvious example is that of a coastline that limits the distribution of a terrestrial species. But in the absence of physical barriers, creation of edges follow shifts from suitable environmental conditions that facilitate species survival to conditions that are unable to sustain a species (Caughley et al. 1988). These environmental conditions are complex including biotic (i.e. preferred habitat, food availability, predator abundance) and abiotic (i.e. temperature and precipitation) factors (Cahill et al. 2014). Range edges may be abrupt or gradual shifts in environmental suitability (Caughley et al. 1988; Chuang and Peterson 2016). Often, range limits are strongly associated with the limits of the species' niche. The niche is the set (and scope) of environmental conditions that are suitable for a species to survive and a species' fitness declines when placed outside of its niche (Hargreaves et al. 2014). However, when niche limits do not fully explain the change in species fitness at the range edge, other factors such as species dispersal capacity are strongly correlated with range boundaries (Hargreaves et al. 2014; Chuang and Peterson 2016).

When range edges are linked with niche limits, it is often concluded that range edges are static but, by including a temporal perspective, range edges are more appropriately considered in flux. Traditional assessment of range shifts focused heavily on adaptation of populations along range edges which enabled species to expand into previously inhospitable areas (Angert et al. 2020). However, range shifts can also result from the expansion of suitable habitat because of climatic shifts, in addition to species adaptation. Across deep time (e.g., glacial cycling of the Pleistocene), global climate cycles have strongly influenced species distributional change (Hewitt 2004; Hampe and Petit 2005; Hope 2020). More recently during the Great Acceleration

(the epoch spanning the last century characterized by rapid expansion of human activity) (Steffen 2022), anthropogenic climate change has driven environmental abiotic and biotic changes leading to rapid directional range shifts with expanding (leading) and contracting (trailing) range edges (Hampe and Petit 2005; Cahill et al. 2013; Hope et al. 2013; Williams and Blois 2018; Hargreaves and Eckert 2019). While climate change may assist in the creation of newly suitable habitat, it may also result in previously suitable habitat becoming inhospitable as previous local adaptations to range-edge conditions lose their advantage, particularly if changes occur rapidly (Hargreaves and Eckert 2019). Critically, vertebrate species typically do not respond to abiotic environmental change directly, making knowledge of the biogeographic history of plant species important for understanding shifts in animal species with strong vegetation associations (Hargreaves and Eckert 2019; Slaton 2015).

Founding Effects and Dispersal- Dispersal of individuals from interior populations can greatly influence peripheral population genetic diversity (Chuang and Peterson 2016). Many factors contribute to a species' capacity to disperse such as kin competition, habitat quality, and physical capacity (Chuang and Peterson 2016). Dispersal can either establish new populations or introduce new individuals to an existing population. When dispersal occurs into novel locations, new populations can experience "founder effects" (Chuang and Peterson 2016). In contrast, dispersal of individuals into existing populations facilitates gene flow to the range edge (Ellstrand and Rieseberg 2016; Chuang and Peterson 2016). In both circumstances, the resulting gene pool can play a critical role in a population's capacity for adaptation and survival (Ellstrand and Rieseberg 2016; Chuang and Peterson 2016).

If dispersing individuals establish new populations, founder effects may result in genetic diversity of founded populations that differs greatly from the genetic diversity of populations in

the species' core range (Chuang and Peterson 2016; Slatkin and Excoffier 2012). Founding populations are normally very small as few individuals successfully disperse to the same location. This means that after only a few generations, the alleles possessed by the founders can rapidly become fixed and highly differentiated from other populations through genetic drift (Slatkin and Excoffier 2012). Often the founding effects are negative with founded populations having disproportionately high rates of deleterious, recessive alleles that become abnormally common through inbreeding and drift (Kivisild 2013). However, founding can be advantageous if the establishing individuals are abnormally "fit" which allowed them to be more successful at dispersing (Chuang and Peterson 2016; Shine et al. 2011). These advantageous alleles can then drift to fixation creating an "Olympic Village" of individuals with abnormally high rates of advantageous dispersal-associated alleles (Chuang and Peterson 2016; Shine et al. 2011; Schmid et al. 2019).

Like dispersal that establishes novel populations, when dispersing individuals introduce genes into an existing range edge population (which are often still relatively small) can be advantageous or deleterious (Chuang and Peterson 2016). Introduced alleles may help to mask the negative impacts of inbreeding through genetic rescue (Brown and Kodric-Brown 1977; Bell et al. 2019) and may help a population to adapt to a wider breadth of environmental conditions (Usui et al. 2023). Alternatively, introduced alleles may swamp-out locally adapted genotypes (combinations of alleles), resulting in loss of local genetic advantages and result in the loss of range-edge populations (Phillips 2012; Kirkpatrick and Barton 1997). The complex and semi-random dynamics that govern dispersal, founding, and gene flow at species range edges play critical roles in developing local gene pools that ultimately dictate survival.

Genetics at Stable and Contracting Edges- At geographically stable range edges, natural selection is often the dominant evolutionary process acting on populations (Angert et al. 2020). Where habitat suitability of range edges is lower compared to the core range, decreased fitness provides a feature on which selection may act (Angert et al. 2020; Bontrager et al. 2021). However, the specific mode of selection (balancing, diversifying, or purifying) depends on the gene pool present in the population and the strength of the environmental stressor (Angert et al. 2020). Stable population boundaries can be valuable as long-term reservoirs of highly adapted genotypes that are not found elsewhere within a species range that may be capable of facilitating future successful range expansions into regions with unfavorable conditions.

Genetics at Expanding Edges- Expanding edges expose a species to novel conditions that facilitate shifts in genetic diversity because of drift and selection (Miller et al. 2020). For instance, founder effects may allow otherwise-uncommon mutations to persist along the expanding range in a model dubbed "allelic surfing" (Edmonds et al. 2004). While most surfing alleles are neutral, advantageous or deleterious alleles may surf, potentially leading to range-edge fitness consequences (Klopfstein et al. 2006; Lehe et al. 2012; Peischl et al. 2015). Simulations indicate that mutations that would normally be lost due to drift or selection can rapidly fix if the mutation is at the front of the range expansion (Klopfstein et al. 2006; Burton and Travis 2008). This capacity for mutations to bypass selective pressure leads to the possibility of abnormal allelic diversity and genotypes in expanding range edge populations. While this process can lead to the fixation of deleterious alleles, the fixation of these alleles typically slows expansion, in turn allowing selection to begin acting as a driving mechanism in these populations, preventing deleterious alleles from becoming excessively harmful to successful and prolonged range expansion (Peischl et al. 2015).

Beyond allelic surfing, "spatial sorting" can drive distinctive trends in edge population diversity and fitness (Shine et al. 2011; Phillips and Perkins 2019). As discussed previously, range expansion may lead to "Olympic Villages" with higher rates of alleles that improve dispersal capacity (Shine et al. 2011; Chuang and Peterson 2016; Miller et al. 2020). Spatially, this means that certain alleles are preferentially "sorted" to the range edges (Phillips and Perkins 2019). These advantageous alleles that mediate dispersal success often favor increased fecundity to increase the likelihood of successful species dispersal (Miller et al. 2020). However, increased fecundity often induces a trade-off for individual viability (Phillips et al. 2010; Phillips and Perkin 2019). While this mechanism is advantageous while populations are expanding, once a population is established this decreased viability may incur its own survival tradeoffs, potentially leading to the loss of range-edge populations as those populations possess a greater genetic capacity to disperse than persist in a location.

Critical Trends and Conservation Importance of Range Edge Populations- Given the combined theory outlined, populations at range edges often have significantly smaller effective population sizes compared to populations in the interior of the species range, making drift a stronger force than natural selection (Vucetich and Waite 2003). This is likely compounded by relatively high population isolation at the periphery which results in decreased gene flow and potentially creates genetically distinct populations along the range edge (Vucetich and Waite 2003; Eckert et al. 2008; Hardie and Hutchings 2010). Given the distinctive adaptations of range edge populations, these populations are critical for conservation as they may be able to persist in conditions that are lethal to core populations (Hampe and Petit 2005; Rehm et al. 2015). This is both theoretically possible and observable. Among those mammals that were thought extinct but subsequently rediscovered, roughly 60% were found in the outer half of their historicial range and 15% were

found in entirely different biomes than their historicial populations (Fisher 2011). Evidently, range edge populations are often those that persist during climate changes, making them important to consider both in active research, as well as conservation and management.

Genetic Predictions for Kansas *G. volans*

While *G. volans* possesses a wide geographic range, its abrupt range edge across the latitudinal extent of the Great Plains allows me to develop existing evolutionary theory about range edge populations into predictions for investigating their genetic diversity, phylogenetic history, and conservation needs. Across the Great Plains distributional edge for *G. volans*, Kansas is uniquely situated. Based on morphometric accounts of *G. volans* in North America, Kansas sits at the range intersection of two of the four subspecies (*G. v. volans*, *G. v. saturatus*) with possibly a third (*G. v. texensis*; found in Texas) being connected to Kansas via forest corridors (Dolan and Carter 1977). Being both a subspecies contact zone and range edge, research focused on *G. volans* in Kansas has the potential to explore both systematics and population genetics of the species under a unique set of conditions in an area with an extensive recent history of intense forestry management.

While Kansas is frequently dubbed a “prairie state” as it is predominantly prairie and agricultural lands, many riparian gallery forests exist in the eastern third of the state (Chase and Strickler 1968). Data from the 1960s reports over 1.2 million acres of predominately oak forest centered in the southeastern corner of the state (Chase and Strickler 1968). Historically, commercial logging was an important industry in Kansas with roughly 8 million cubic feet of lumber being extracted from Kansas in 1965 alone (Chase and Strickler 1968). Through multiple mechanisms, the extent of gallery forests have gradually expanded westward across Kansas since the mid-1800s (Abrams 1986; Knight et al.1994). By 2015, Kansas forest cover surpassed 2.5

million acres with the top five tree groups all being capable of reaching the large diameters favored by *G. volans* (Meneguzzo 2016).

Though the earliest museum record of *G. volans* from Kansas (KU:2301) was collected in 1892, an extensive ecological and evolutionary history likely developed for the species in Kansas across previous centuries. The history of logging and forest expansion in the state has likely provided fluctuating connectivity through time. Given that in Kansas, hardwood forests that typify preferred habitats for *G. volans* are not continuous either now or historically, connectivity of populations is likely more circuitous and associated with forested corridors following major watershed drainages (Figure 1.1). Based on the range-edge distribution of all Kansas populations and direction of these drainages as they extend into neighboring states, connectivity between drainages is possibly much lower than connectivity within drainages (Figure 1.1) (Knight et al.1994).

Synthesis and Predictions- Considering the context of range-edge genetics and Kansas *G. volans*, several key predictions arise. First, given the high degree of watershed isolation between forest corridors in Kansas, populations are expected to be highly divergent across watersheds while maintaining low genetic diversity within these isolated corridors. In a similar vein, it is likely that lineages of *G. volans* in Kansas will be more closely related to those from neighboring regions with corridor connectivity, than between regions in Kansas. When these populations' genetic diversity and fitness are compared to populations with greater regional connectivity, Kansas populations will likely exhibit lower diversity which may translate to reduced fitness. Furthermore, given the history of logging within Kansas, populations are likely to exhibit signals of decreased population size in the last two centuries. Finally, due to distinct range-edge environmental conditions compared to core range populations, if range-edge selective pressures

have had a significant impact on Kansas populations, signals of selection may be evident in parts of the genome that improve range-edge fitness such as mitochondrial metabolic genes. Ultimately, Kansas populations of *G. volans* are likely to be distinct both compared to other regions across *G. volans*' range, as well as between populations in the state. Given the complex evolutionary history of range-edge populations, my thesis tests these predictions with multiple lines of genomic evidence, from which I relate my findings to the ecology of this forest specialist, and synthesize my results towards positive future conservation outcomes.

Chapter 2 - Mitochondrial Analysis of *G. volans*

Introduction

Given the proposed complexity of *Glaucomys volans* (southern flying squirrel) genetics at their range-edge in Kansas (as reviewed in Chapter 1), evaluating multiple genetic perspectives of *G. volans* is necessary to best inform species assessments and conservation. This thesis chapter will assess *G. volans* phylogenetics, population histories, and functional selection based on mitogenomes (the complete ~16,500 base pair genomes housed in mitochondria). Given the complex history of mitogenomes and their applications in research, I will first outline the background of both, followed by my chapter-specific goals and hypotheses.

Mitochondrial Fundamentals- Though nuclear DNA codes for most of the traits that define species that arise through classic evolutionary processes including mitosis, the DNA housed in mitochondria is distinctive and has been widely used in research of biodiversity and cellular processes. Arising from endosymbiosis of free-living bacteria within eukaryotic cells over 1.5 billion years ago (Gray and Doolittle 1982), mitochondria retained their ancestral DNA which has become functionally associated with eukaryotic nuclear DNA and performs critical life functions, especially related to metabolism (Wallin 1922). Mitochondrial DNA is passed from parent to offspring during sexual reproduction but, in mammals and other orders, mitochondrial DNA is maternally inherited whereas nuclear DNA is bi-parentally inherited (Hutchinson et al. 1974). Despite mitochondrial DNA having little to no recombination across generations (Hagström et al. 2014), mitogenomes have an estimated mutation rate roughly ten times higher than that of the nuclear genome (Brown et al. 1979). The research benefits of rapidly-mutating, nonrecombinant DNA that is strictly maternally inherited are numerous, from informing phylogenetic systematic relationships and population histories, to an understanding of selection

regimes on mitochondrial protein coding genes. The latter is especially important in helping understand species adaptations and survival across temperature and precipitation gradients as many of the protein coding genes in the mitogenome play critical roles in metabolic processes that are strongly impacted by extreme environments.

History of Mitochondrial Sequencing- Since early genetic sequencing in the middle to late 20th century, sequencing technology has progressed at an ever-increasing rate. From early Sanger sequencing (Sanger et al. 1977) to "next generation" Roche 454 and Illumina sequencing, dramatic improvements to sequencing depth, length, and accuracy occurred (Ye et al. 2014; Forsythe et al. 2021). These advances drove a shift from sequencing single loci or specific genes (primarily Cytochrome-b) (Castresana 2001) to complete mitogenomes (Smith et al. 2016). More recently, sequencing technology has advanced to a "third generation" spearheaded by Oxford Nanopore and Pacific Biosciences (Athanasopoulou et al. 2021). The major innovation of third generation approaches is that they can generate long read sequences ranging from thousands to millions of base pairs (Athanasopoulou et al. 2021). These technologies also employ adaptive sampling which provides researchers the ability to "weed out" non-target DNA and sequence only DNA that matches target genome regions, in turn refining workflows and precision without the need for primer-specified DNA amplification through polymerase chain reaction (Yang et al. 2025). As such, we can now target and sequence whole mitogenomes from non-model wildlife, although the use of those sequences to inform broader phylogenetic or population genetic work is still in its infancy (Franco-Sierra et al. 2020; Nguyen et al. 2025).

Chapter Goals and Hypotheses- Given that the technology for rapid and relatively cheap sequencing of full mitogenomes is still in its early application, several multifaceted goals guided this project. They included (1) Generating the first whole mitogenomes for samples of *G. volans*

from across their sampled range. Considering tissue samples from loans are sometimes not of the best quality, I anticipated moderate mitogenome coverage across the sampling range and higher coverage in Kansas where tissues were recently acquired and fresh. (2) Estimating a U.S. range phylogeny of *G. volans* with an outgroup species to assess systematic inter and intra-specific relationships. I hypothesized fewer clades would be present across the North American range than morphometric work predicted for recognized subspecies, as morphological differences between subspecies are often qualitive and not discrete. Furthermore, given the isolated watersheds in Kansas with connectivity to disparate localities, I expected to observe Kansas individuals being included in multiple phylogenetic clades. (3) Compare phylogenies based on each of the 13 protein-coding regions within the mitogenome. I anticipated similar phylogenetic topologies across all protein-coding regions given that all genes within mitogenomes are linked, but with potential differences in relationships or posterior support values as well as divergence time estimates due to different rates of evolution within genes compared to the complete mitogenome. (4) Assess population and clade genetic diversity and differentiation. I hypothesized that substantial differentiation and variation in diversity would be present between intra-specific clades, but that inferring these would be challenging in some instances due to small sample sizes. (5) Evaluate the demographic history of the species, major clades, and localized populations. I expect that a large population increase will be observed roughly 20 thousand years ago, coincident with the end of the Last Glacial Maximum (LGM) when the retreat of glaciers from much of *G. volans'* current range would have opened suitable habitat for colonization, allowing rapid population growth. (6) Assess signals of selection across mitochondrial coding regions. Given that all mitochondrial genes are involved with metabolic function, it is challenging to hypothesize which genes will experience the greatest selection, though I expect

purifying selection to be far greater across the genome than diversifying selection, given that these genes code for important functions and so should experience selection for stability as opposed to (potentially deleterious) change. Ultimately, the directive of this work is to assess the genetic history of *G. volans* and the functional implications of genomic variation for population health at their range edge using novel sequencing and comprehensive analyses.

Methods

Ethical Considerations- All *G.volans* were captured and handled under the approval of the Kansas State University IACUC (permit #4801).

Specimen Collection- We conducted field-based sample collection of *G. volans* within Kansas from May 2024 through February of 2025. Two types of trapping were used to maximize the likelihood of *G. volans* collection, including (1) live trapping using Sherman traps (H.B. Sherman Traps, Inc., Tallahassee, FL) from May-July 2024, and (2) nest box surveys through the 2024-2025 winter season.

Sherman trapping was conducted through two independent field trapping efforts (hereafter "short-term" and "long-term" trapping) that differed primarily in their duration. For both sampling groups, trapping sites were selected for areas with large hard-mast producing trees. At each site, four to ten pairs of Sherman traps were placed five to twenty meters apart (Slade et al. 1993; Engel et al.1992). Each pair of traps consisted of one horizontal tree trap placed two to three meters above the ground on a wooden platform, and one ground trap below the tree trap (Bendel and Gates 1987; Engel et al. 1992). Traps were baited with an oatmeal and peanut butter mixture, sweet horse feed, or sunflower seeds (Kuykendall and Keller 2011; Nelson and Sagot 2018; Howard et al. 2020). Traps were checked daily to minimize capture

stress and were rebaited whenever bait was depleted or when wet or molding (Powell and Proulx 2003).

The short-term trapping occurred at Mined Lands Wildlife Area, Fall River Wildlife Area, and Quivira Boy Scout Ranch (Figure 2.1A). Each trapping event lasted four nights with four to six trap arrays set each night. To cover more of each regional site in a short period of time, some trap arrays were rotated to new locations mid-week. Sites that had very low trap success of any small mammals were preferentially moved. The "long-term" trapping established 15 trap arrays at Grand Osage Wildlife Area (Figure 2.1A) that were maintained from mid-May to mid-July (Bernhardt et al. 2026). Traps were baited and set five to seven nights each week, with closures due to inclement weather. In total, only three individual flying squirrels were trapped from the long-term sites.

As an alternative sampling method, 84 nest boxes were built and deployed from July through October 2024 at 23 forested locations (Figure 2.1B). *Glaucomys volans* have been observed nesting in large groups during winter months; thus, boxes were deployed to attract such aggregations (Thorington and Weigl 2011). Boxes were built to the specifications outlined in Althoff and Althoff (2001). At each forest, we deployed boxes in sets of three with at least 30 meters between boxes and with boxes positioned three to six meters above ground level (Althoff and Althoff 2001). Boxes were unbaited to avoid the possibility of bait molding and deterring nesting. At Grand Osage Wildlife Area (Kansas Department of Wildlife and Parks; KDWP) and Perry Lake (U.S. Army Corps of Engineers), we deployed an additional 12 and 6 boxes respectively, as these were focal areas of collaborative work with state biologists.

Nest boxes were checked at least twice from November 2024 through February 2025. We checked an additional 15 boxes at Grand Osage Wildlife Area that had been placed three years

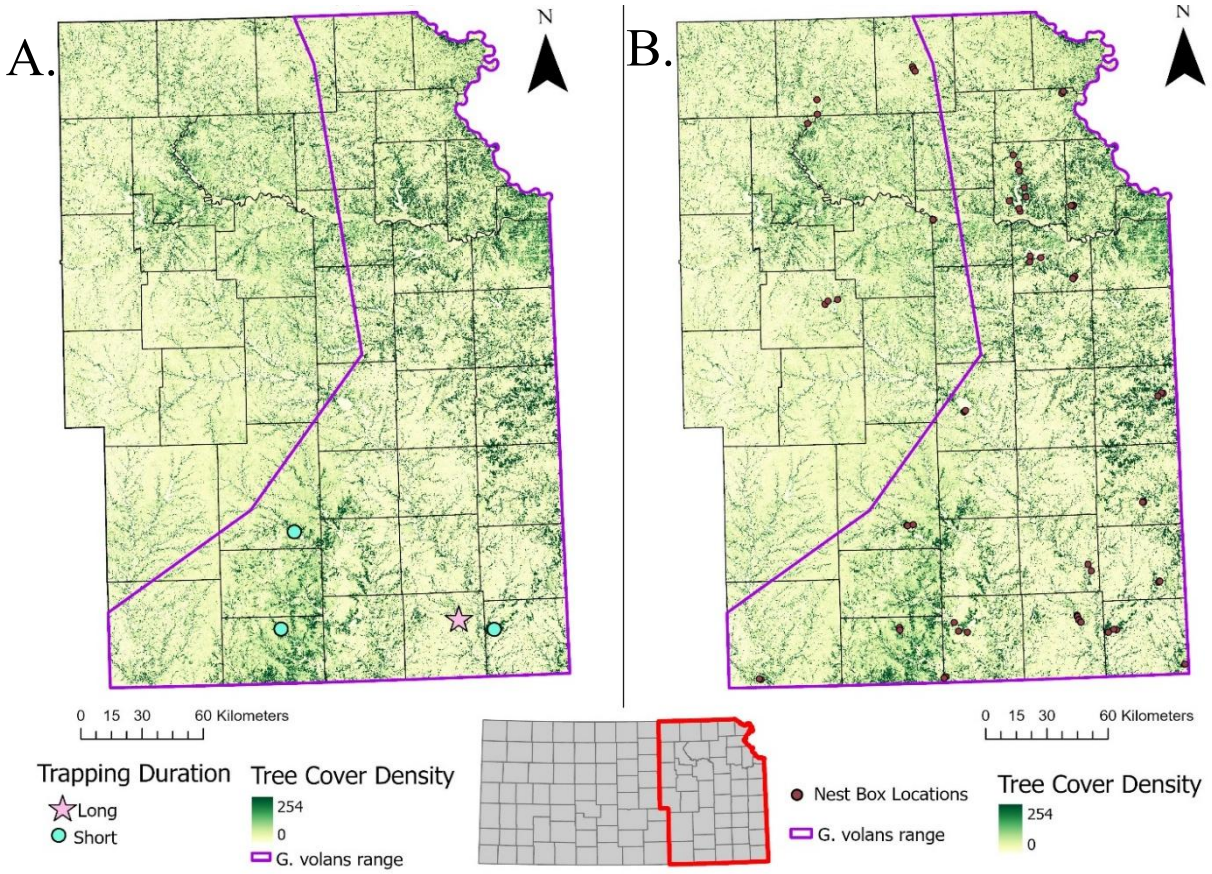

Figure 2.1 Maps of Kansas *G. volans* sampling efforts based on *G. volans* range and tree canopy density. (A) Long term (star) and short term (circle) Sherman trapping sites. (B) Nest box locations across range.

prior by KDWP land managers. These boxes were checked weekly between November 29th and December 20th, 2024, and captured *G. volans* were handled and processed using protocols developed by Bernhardt et al. (2026). For all trapped *G. volans*, ear biopsies were sampled from both ears and placed in 100% ethanol for preservation (Cinelli et al. 2007). Fifteen individuals were processed from nest boxes with one individual being identified as having been trapped previously during long-term Sherman trapping. Only the three-year-old nest boxes at Grand Osage Wildlife Area yielded squirrels within the project timeframe, suggesting squirrels may be neophobic towards new boxes. Between Sherman and nest box trapping efforts, 17 individuals were sampled within Kansas between June 2024 and June 2025, all from Grand Osage.

Additional *Glaucomys* samples were acquired via loans from natural history collections. Specimens were requested to maximize geographic coverage across the range of *G. volans* given available specimens, with individuals for sister species *G. oregonensis* and *G. sabrinus* being included as outgroups. In total, 103 *Glaucomys* samples (17 from field work and 86 from museum loans) were collected for mitochondrial analyses (Appendix Figure A.1). Of these, 34 (32 *G. volans* and 2 *G. oregonensis*) were successfully sequenced for complete mitogenomes (Appendix Figure A.1). Sanger sequencing of Cyt-b was successful on an additional 50 samples (44 *G. volans*, 2 *G. oregonensis*, 4 *G. sabrinus*) (Appendix Figure A.2).

DNA Extraction and Quantification- Tissues from both field and museum samples were extracted using standard salt extraction methods (Fleming and Cook 2002; Miller et al. 1988). We quantified DNA extractions using the Qubit 4 Fluorometer (ThermoFischer Scientific) with Broad Range dsDNA chemistry. Extraction volumes used for quantification were between 1µl and 10µl. Extractions with concentrations below 20 ng/µl were reextracted and quantified as available tissues allowed.

Mitochondrial Genome Library Preparation and Sequencing- Full mitochondrial genome sequencing was conducted for a subset of the total samples available. Samples for mitogenome sequencing were selected based on high DNA concentration (ranging from 47.2 to 490 ng/µl) and to maximize species range coverage. Three sequencing libraries containing 16, 12, and 16 samples were prepared using the Oxford Nanopore Technology (ONT) Native Barcoding Kit SQK-NBD114.24 V14. Library preparation followed manufacturer protocols for R10.4.1 (FLO-MIN114) MinION chemistry and flowcells. Total DNA input by sample ranged from 14.52 to 600 ng, with all but four geographically significant samples using 400 ng in 12 µl input volume, adjusting final volume with milliQ water depending on DNA concentration. Prepared libraries were stored at -10°C for 1 month, 3 months, and 2 days respectively. Libraries were loaded onto new flow cells following manufacturer protocols, with the exception of the use of a lower concentration of Bovine Serum Albumin (BSA, 10 mg/ml) resulting in 25µl BSA and 1,150 µl Flow Cell Flush. Library Beads were used instead of Library Solution given the low viscosity of libraries.

For all MinION sequencing runs, run time was constrained to 24 hours or until complete flow cell pore depletion, whichever occurred first. Sequencing control was conducted using MinKNOW software (ONT) and the MinION Mk1D (versions 25.03.9, 25.05.14, and 25.05.12, respectively). For the first two libraries, sequencing was done on a Windows desktop computer (Intel(R) Core(TM) i7-1065G7 CPU @ 1.30GHz, 1498 Mhz, 4 Cores, 8 Logical Processors, 1Tb local storage). While adaptive sampling was enabled for both runs, insufficient processing power prevented adaptive sampling, resulting in shotgun runs. The third library was run on a MacBook Pro with M4 processor, with effective adaptive sampling of DNA matching a reference library

consisting of all mammalian mitogenomes sourced from the NCBI Organelle database as of the 8th of May 2025.

Mitogenome Post Sequencing Analysis- Raw sequencing results (were basecalled on the MacBook using ONT MinION's MinKNOW software (v25.05.12, chemistry DNA 5 kHz, super-accurate, minimum Q-score 7). I then used MinKNOW to process barcodes (trimming barcodes and barcoding both ends) on all sequences that passed base calling. MinIONQC (Lanfear et al. 2019) was used to determine reasonable cross-library thresholds for quality filtering. Resulting sequences were then filtered and trimmed using chopper (De Coster and Rademakers 2023). Sequences were filtered for average quality scores of 10 or greater and minimum sequence lengths of 100 base pairs and then trimmed until to the first base pair of at least a quality of 10. A final aligned file for each sample was then generated using MiniMap2 (Li 2021). Alignments were analyzed for completeness in IGV (Robinson et al. 2011) to determine level of depth across the mitogenome. Ten samples were dropped from analysis (nine lacked any significant region of alignment over 500 bp, one was sequenced twice to check sequencing consistency) resulting in a dataset of 34 individuals (Appendix Figure A.1A). To account for interspecies variation, multiple sequence alignment was performed via MUSCLE (Edgar 2022) in MEGA (Kumar et al. 2024). Mitogenomes were annotated and all protein coding regions were extracted using MitoZ (Meng et al. 2019). Extracted protein coding regions were realigned via MUSCLE in MEGA.

Cytochrome-b Sequencing-The Cytochrome-b (Cyt-b) protein-coding gene was sequenced for 75 samples but only 50 were successful and unique from mitogenome sequences (Appendix Figure A.1B). The Cyt-b gene was amplified using polymerase chain reaction using primers L14724 and H15915 (Irwin et al. 1991). Reaction conditions were consistent with those from previous work (Galfano et al. 2025). Products were cleaned using ExoSap-It (Bell 2008), diluted 1:5 and

then Sanger sequenced at Eurofins Genomics. In Geneious Prime (v. 2026.0.1, https://www.geneious.com), sequences were aligned to a *G. volans* reference Cyt-b sequence (GenBank AJ389531.1) and manually cleaned and trimmed. Final sequence lengths ranged from 509 to 1,112 base pairs.

Phylogenetic Trees- Bayesian phylogenetic trees for aligned mitogenomes and protein coding regions were generated in BEAST2 (version 2.7.7) (Bouckaert et al. 2014). For mitogenome trees and all protein coding regions except Cyt-b, the dataset consisted of the 34 sequences generated on the MinION sequencer. For the Cyt-b tree, the 50 additional sequences generated from Sanger sequencing were aligned to the 34 Cyt-b sequences extracted from the mitogenomes in MEGA.

Parameters for all trees were set in BEAUti2 (v. 2.7.8). The tree parameters were set up using the BEAST Model Test (Bouckaert and Drummond 2017) with both transitionTransversionSplit and empirical frequencies selected. The Relaxed Clock Log Normal clock model and tree prior Coalescent Constant Population Size were used. Given estimates of both most recent common ancestor (MRCA) and mitogenome mutation rate exist, mitogenome trees were run with each as a prior to avoid confounding or overfitting data. A mean clock rate of 0.02 mutations per base pair per million years was assumed and birthrate was set to a gamma distribution (Brown et al. 1979). Estimates place *Glaucomys* species divergence roughly 700,000 to 1.3 million years ago (Arbogast 1999); thus, an MRCA prior of 1 million years with a normal distribution, monophyletic structure, and sigma value of 0.15 (giving an age range of 0.7 to 1.3 million years) was used. For each set of priors, a preliminary tree with an MCMC chain length of 1 million was run in BEAST2 to confirm reasonable parameters, followed by a final run with a

chain length of 10 million. For only the Sanger sequence-inclusive Cyt-b dataset, I conducted down sampling with LogCombiner (v. 2.7.7; Drummond and Rambaut 2007) for every ten trees.

Following tree generation, Tracer (version 1.7.2; Rambaut et al. 2018) was used to assess Effective Sample Size estimates (ESS). For the 10 milllion MCMC chain length, all ESS values were above 200 (Dellicour et al. 2021). A burn-in of 10% was then applied to the tree file in TreeAnnotator (v. 2.7.7; part of the BEAST2 package). Tree visualization and editing were done in FigTree (v. 1.4.4; Rambaut 2009). Individual phylogenetic trees were also generated for each of the 13 protein-coding regions with the same MRCA prior defined as for the whole genome. Given the variability in coding region mutation rates, this approach was both more empirical and allowed estimations of relative mutation rates across protein-coding regions.

Genetic Diversity and Population Pairwise-Differentiation- To assess species, clade, and population-level diversity and differentiation, DnaSP (v. 6.12.03; Rozas et al. 2017) was used to estimate nucleotide diversity and population pairwise divergence from the mitogenome dataset. To best account for inter- and intra- specific trends, three iterations of population partitioning were conducted: (1) species-level (*G. volans* against *G. oregonensis*), (2) clade-level (*G. volans* clades as determined by the mitogenome phylogenetic tree), (3) population-level (*G. volans* geographic populations). While large sample sizes are beneficial for increasing resolution and confidence, inclusion of a disproportionate number of individuals from a given site may bias results. As such, only one sample from Grand Osage (KT 2314) and two from Ouachita Forest (KT 2624 and KT 2658) were included for species- and clade-level analyses. Two samples from Ouachita Forest were used as, in preliminary trees, they were present in different clades, representing different patterns of genetic diversity. For population-level analysis, all individuals in both populations (Grand Osage Wildlife Area, KS; Ouachita National Forest, AR) were

included. For each partition, multi-sequence alignment files using MEGA's MUSCLE were generated for only those relevant individuals. In DnaSP, coding regions were set based on positions defined by MitoZ on the multi-sequence alignment, and the genetic code was set to mtDNA Mammals. For each "population" within each of the three partitions, GC content, haplotype diversity (Hd), nucleotide diversity (π), and Tajima's D (D) with p-values (Tajima 1989). Pairwise population divergence (average number of nucleotide substitutions per site between populations, D_{xy}) was calculated for all relevant pairwise population sets, also in DnaSP.

This same process and population partitioning was repeated for the comprehensive Cyt-b (Sanger and mitogenome subsampled) dataset. Population partitions again included (1) species-level (*G. volans*, *G. oregonensis*, and *G. sabrinus*), (2) clade-level (*G. volans* phylogenetic clades), (3) population-level (Grand Osage Wildlife Area, KS; Ouachita National Forest, AR). All populations had larger samples sizes due to the inclusion of the 50 Cyt-b sequences. Sample removal was once again conducted for Grand Osage and Ouachita populations at species and clade level analyses with the same three individuals kept to reflect the range of local diversity.

Demographic History- Bayesian skyline plots of demographic history (effective population size over time) were generated in BEAST2 using the BEAUti2 BDSKY package (Drummond et al. 2005). In all iterations, BEAST and BEAUti were used to generate trees using the same parameters as the complete mitogenome trees, with the key prior being the mutation rate calculated from the mitogenome MRCA-prior tree. The tree prior was set to Coalescent Bayesian Skyline. Following confirmation of reasonable parameters in Tracer, each Bayesian Skyline-specific tree generated during early phylogenetic tree development was tested. For both mitochondrial and Cyt-b datasets, groups were parameterized as (1) all *G. volans* and (2)

independent groups based on clades from the respective phylogenetic trees. As skyline plots require genetic variation to inform predictions, skyline plotting of the Ouachita Forest population was conducted using the larger Cyt-b dataset.

Signals of Selection-To evaluate selection across the mitogenome dataset, two programs were used: FUBAR (Murrell et al. 2013) and MEME (Murrell et al. 2012). Three iterations of FUBAR and MEME analyses were conducted through the HyPhy package (v. 2.5.64; Pond et al. 2020) with the gene-specific NEXUS alignment of each protein coding region and phylogenetic tree (built using the subset of mitogenomes and the prior of the mitogenome mutation rate) for (1) all *G. volans* mitogenomes and (2) each clade group identified in the mitogenome phylogeny. For all datasets, the same subsampling of Grand Osage and Ouachita Forest individuals was conducted to avoid biasing results. Protein coding regions were cleaned of stop codons in the interactive HyPhy interface with the ascribed vertebrate mitochondrial DNA as the nucleotide format. Multi-sequence alignment was conducted with MUSCLE in MEGA. FUBAR results were significant if the posterior probability was at least 0.9. MEME results were significant if the p-value was less than 0.1. Levels of selection across genes was then manually evaluated and graphed using custom R scripts.

Results

Mitogenome Post Sequencing Analysis- All 34 successful mitogenomes had complete coverage across the reference genome. Given that the reference was from an individual in Pennsylvania, those alignments of southern *G. volans* and *G. oregonensis* individuals exhibited greater variation from the reference. Locus depth ranged from 4-1,763x but most samples averaged 30-100x depth.

Mitogenome Phylogenetic Trees- Regardless of MRCA or mutation rate prior, the mitogenome phylogeny supports two clades of *G. volans* (Figure 2.2). The "Southwest" clade comprises individuals from Texas, Arkansas, Oklahoma, and the southeastern corner of Kansas (Figure 2.3A). The "Widespread" clade comprises individuals from the remainder of the range and includes some individuals from Arkansas and Kansas (Figure 2.3A). Of the four sites in Kansas, two (Quivira and the Southeastern Corner) were contained within the Southwest clade, while the Widespread clade contained Grand Osage and Northern Kansas samples (Figure 2.2, Figure 2.3B). Furthermore, Ouachita National Forest ("Ouachita Forest") contained samples that belonged to both clades (Figure 2.2, Figure 2.3C). When the most recent common ancestor (MRCA) of all *Glaucomys* individuals was provided, a divergence time of roughly 0.98 mya (95% confident interval (95 CI) = 0.698-1.288) and a mutation rate of 0.045 mutations per site per million years were produced (Figure 2.2A). When a mutation rate of 0.02 was provided, the MRCA of all individuals was roughly 2.13 mya (95 CI = 1.878-2.380), roughly double that of the MRCA-prior tree (Figure 2.2B). Based on the MRCA-prior tree, the split between the two *G. volans* clades occurred 0.183 mya (95% CI = 0.123-0.246), which is more recent than coalescence among *G. oregonensis* individuals.

Cyt-b Phylogenetic Tree- The Sanger-inclusive Cyt-b phylogeny presents a topology like the complete mitogenome phylogeny, although each of the two major clades has a single sample that falls into a basal position with lower nodal support (Figure 2.4). One of these is the sample from northern Kansas. In addition, there is support for Grand Osage individuals existing within the Widespread clade. Both the southeastern corner (Cherokee Co.) and Quivira populations are strongly supported as within the Southwest clade. When given the MRCA prior used for the

mitogenome tree, the mutation rate of the large Cyt-b dataset was estimated to be 0.048 with MRCA values of 0.982 (95 CI 0.677-1.261).

Mitogenome-Derived Phylogenetic Trees Based on Protein-Coding Genes- Mitogenome protein-coding gene phylogenies show relatively consistent structure, with generally low nodal support (Appendix A Figures 2-8). Of these coding regions, expected MRCA of all individuals ranged from 0.973 (COX3 and ND4L) to 0.979 (mitogenome-only Cyt-b) mya. The estimated mutation rate ranged from 0.0318 (COX3) to 0.0581 (ND6), though no rates were significantly different given confidence around estimates (Appendix Figure A.9). Notably, only the COX2 and ND4 gene trees provided strong nodal support for all individuals within two clades as was shown for the whole mitogenome dataset, though most returned a similar overall topology (except for ND4L). The ND4L phylogeny did not become more like the mitogenome phylogeny, even when using the reverse complement sequence to reflect the gene's placement on the Light Strand (Appendix Figure A.7B).

Genetic Diversity and Population Pairwise-Differentiation- The GC content across mitogenomes exhibited very little variation but was slightly higher in protein-coding regions (Table 2.1). *G. volans* had over double the variable sites (S) as the two *G. oregonensis* samples and each sample was a distinct haplotype (due in part to the removal of closely related individuals). The Widespread clade and Ouachita Forest had higher numbers of variable sites than the Southwest clade and Grand Osage populations respectively. Notably, Grand Osage had only two haplotypes across six individuals, and only six total variable sites across the entire mitogenome.

When looking at the Cyt-b dataset, the GC content was higher than that of the mitogenome coding region average (Table 2.1). *G. sabrinus* had very few variable sites in

contrast to the other two species. As in the mitogenome data, the Widespread clade and Ouachita Forest populations had more variable sites than the Southwest clade and Grand Osage respectively. The 14 Grand Osage Cyt-b sequences were invariant.

Nucleotide diversity (π) within each "population" was highly variable depending on group. At the species level using mitogenome data, *G. volans* had four times the nucleotide diversity (0.0086) as *G. oregonensis* (0.0023) (Table 2.2). However, when using Cyt-b data which included a broader geographic sampling of *G. oregonensis* (Figure 2.3), *G. volans* had three times the diversity of *G. sabrinus* (0.0037), but only half the diversity (0.0098) of *G. oregonensis* (0.02) (Table 2.2). When comparing *G. volans* clades, the Southwest clade had greater diversity based on both mitogenome and Cyt-b data (0.007 and 0.0082, respectively) than the Widespread clade (0.0048 and 0.0025) (Table 2.2). At local scales, as expected due to the lack of variable sites at Grand Osage, Ouachita Forest had 100 times the diversity (0.011) as Grand Osage (0.0001) based on mitogenome data (Table 2.2). The Cyt-b data reflects a similarly high level of diversity at Ouachita (0.00816), whereas the Cyt-b data from Grand Osage exhibited only a single haplotype (Table 2.2).

For values of average pairwise sequence divergence (D_{xy}), highest differentiation was between *G. sabrinus* and *G. oregonensis*, with *G. volans* having values of 0.066 and 0.069 against each of those species, based on Cyt-b data (Table 2.3). Mitogenome D_{xy} species data indicates a lower level of differentiation (0.055) between *G. volans* and *G. oregonensis* (Table 2.3). Based on mitogenome and Cyt-b data, the Southwest and Widespread are differentiated by about one fifth of that between species (0.014 to 0.019), with local populations in Kansas and Arkansas being even less differentiated (0.008 to 0.011) (Table 2.3). The significantly higher diversity of *G. oregonensis* based on Cyt-b data is likely a function of the broader species range

covered by the Cyt-b dataset, whereas the low *G. sabrinus* value is likely the result of a small sampling region (Figure 2.3).

Demographic History- Across both mitogenome and Cyt-b Bayesian Skyline Plots, there was significant growth in the effective population size between 15 and 35 thousand years ago for the species as a whole and for the Widespread clade (Figure 2.5). Estimated growth was also observed for the Southwest clade, though insignificant (Figure 2.5). Significance assessments are based on a demographic shift that exceeds the 95% confidence interval of the plot. For the species at large and mitogenome version of the Widespread clade, these increases coincide with the timeframe of global warming following the last glacial maximum (LGM). Mitogenome-based models for the Widespread clade and full species indicate a possible population size decline in the last several centuries, though these declines are not significant (Figure 2.5). For local populations, the Grand Osage population in Kansas did not exhibit any variability and as such, I could not infer any genetic demographic change. However, sufficient variation was present in the Ouachita Forest Cyt-b data. The latter model reflects a moderate but significant population growth around the last glacial maximum (Figure 2.6.).

When considering demographic changes, Tajima's D is informative. Of all Tajima's D values, the Widespread clade (mitogenome and Cyt-b datasets) exhibited significantly negative D values (-1.99 and -2.45 respectively) (Table 2.2). Negative Tajima's D values reflect an excess of low frequency polymorphisms. This can result from either recent demographic expansion or purifying (negative) selection but is most often used to infer population expansion within phylogeographic studies.

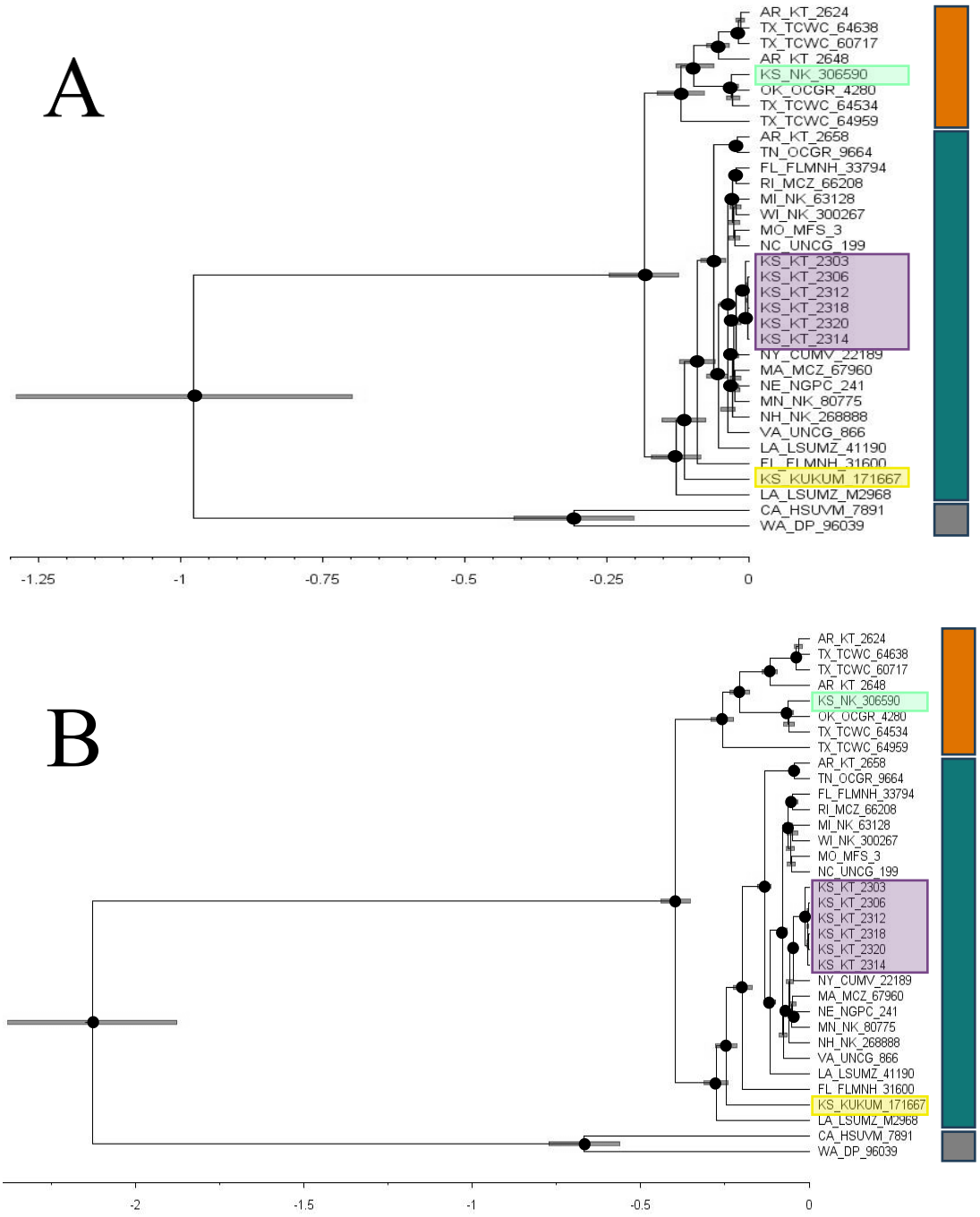

Figure 2.2 Bayesian phylogenetic trees based on full mitogenomes of *Glaucomys volans* and sister species *G. oregonensis*. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age. Parameterization differed with (A) prior set to most recent common ancestor of 1 mya and (B) prior set to a mutation rate of 0.02 mutations per site per million years.

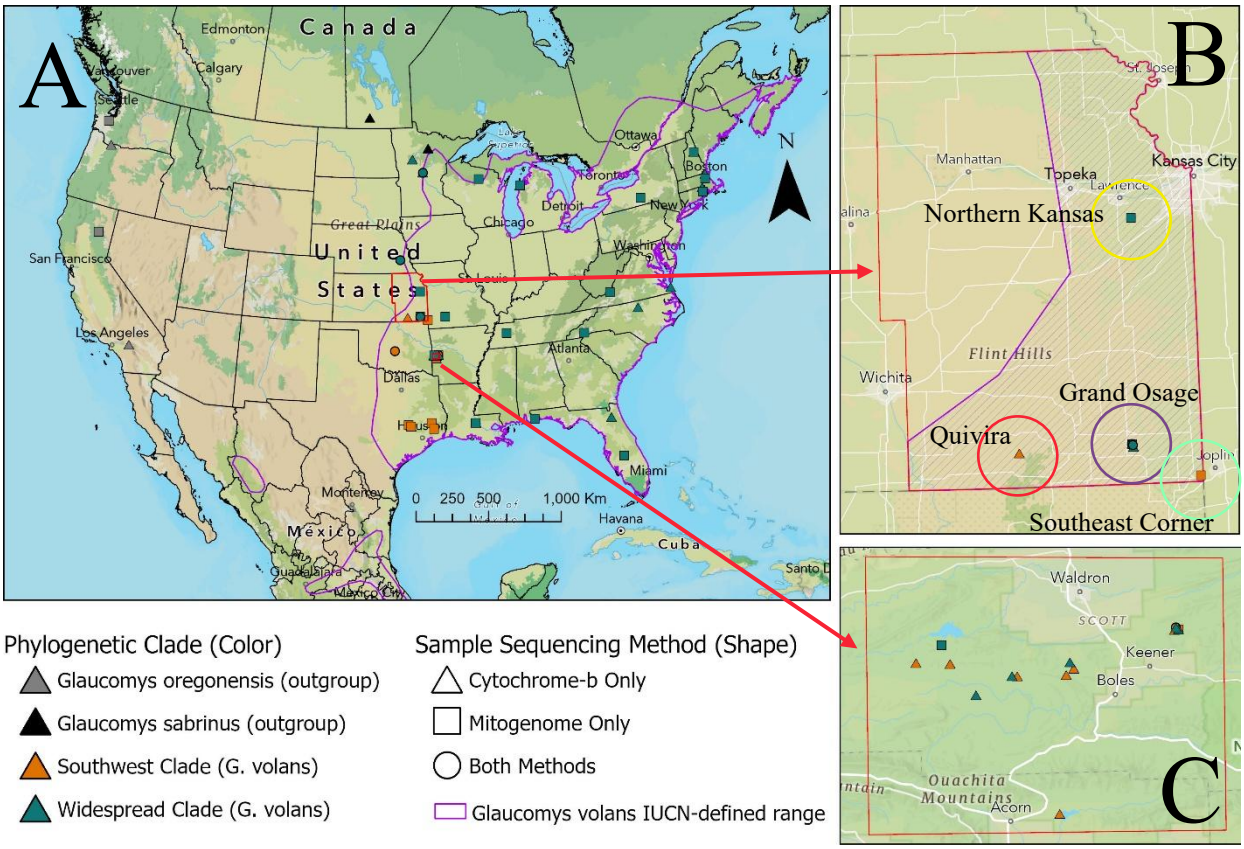

Figure 2.3 Distribution of mitochondrial samples based on sequencing type and resulting phylogenetic clade. Includes maps of complete range (A), Kansas (B), and Ouachita Forest, Arkansas (C).

Signals of Selection- Based on FUBAR (which tests for both diversifying and purifying selection), only two codon loci were deemed under significant diversifying selection (in CYTB and ND5) across the *G. volans* species, while 235 codons were identified as purifying selection (Table 2.4; Appendix Figure A.10A). Of the diversifying codons, the CYTB codon was not significant for either clade but the ND5 codon (the 475th codon in the protein coding region) was significant in the Widespread clade (Table 2.4, Table 2.5, Appendix Figure A.11A). In both clades, one position in the ND6 gene was significant, though the codons were different (Table 2.5, Appendix Figure A.11A, Appendix Figure A.12A).

In MEME results (which only tests for diversifying selection), four sites were strongly supported as experiencing diversifying selection for *G. volans* species (Table 2.4; Appendix Figure A.10B). Of these, three were in ND5 and one was in ND2 (Table 2.2). One site (the 475th codon in ND5) was also significant in the FUBAR analysis (Table 2.5), though it was not significant for either clade in further MEME tests. The ND2 codon was significant in the Widespread clade (Table 2.5, Appendix Figure A.11B). Of the remaining two ND5 codons, each clade test was significant at one of these codons (Table 2.5, Appendix Figure A.11B, Appendix Figure A.12B).

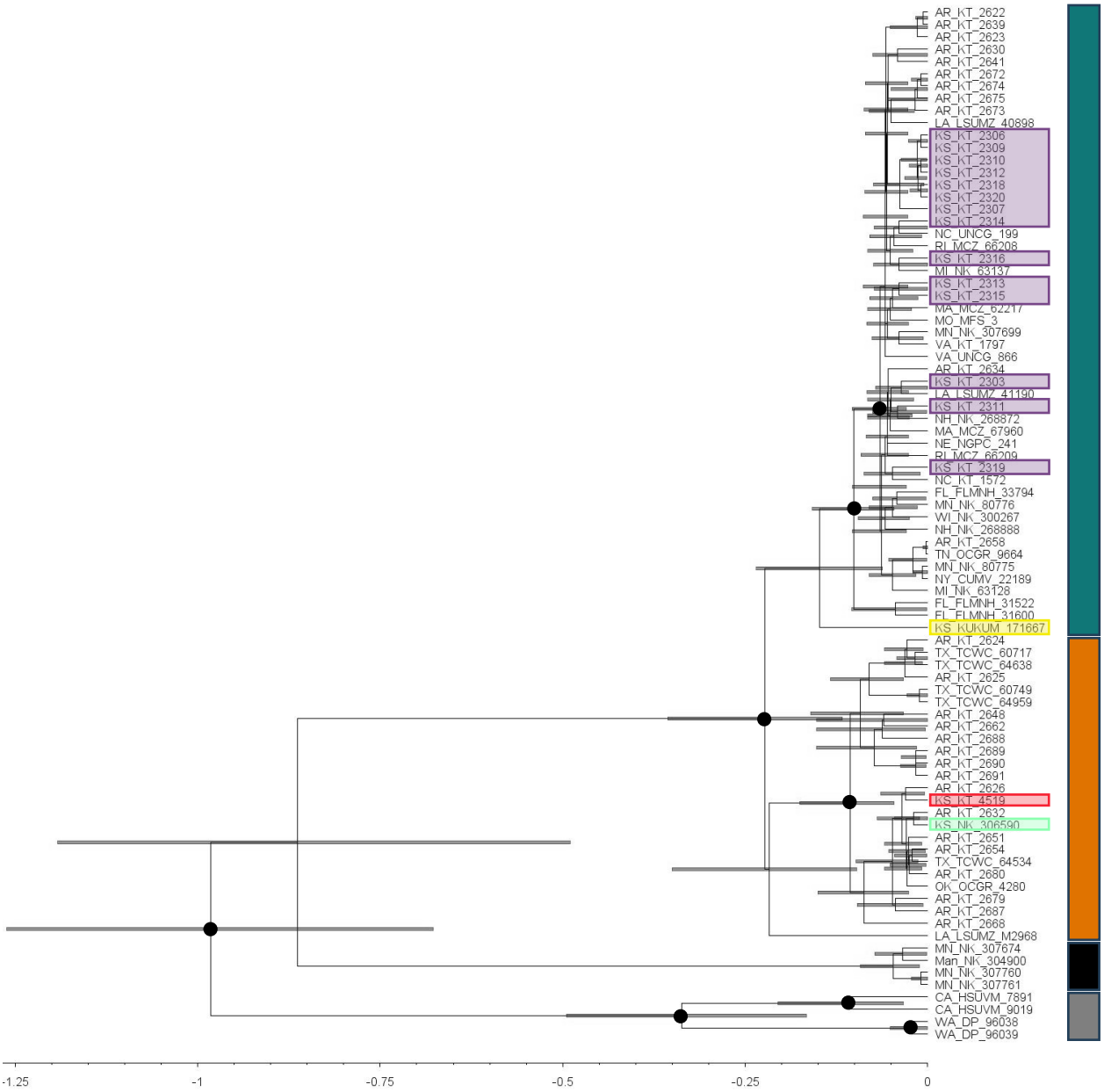

Figure 2.4 Bayesian phylogenetic trees based on Cyt-b sequences from mitogenome and Sanger sequencing. Vertical bars indicate clades of the outgroups of *G. sabrinus* (black) and *G. oregonensis* (grey), Widespread (blue), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), Grand Osage (purple), and westernmost site (red). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age.

Table 2.1 Basic sequence and population statistics for both mitogenome and Cytochrome-b datasets categorized into sub-datasets used for population genetic and selection analyses.

		# Samples	GC Content (Complete)	GC Content (Non-Coding)	GC Content (Coding)	# Variable Sites (S)	# Haplotypes	Haplotype Diversity (Hd)
Mitogenomes	**Species**							
	Glaucomys volans	26	0.38	0.37	0.38	799	26	1
	Glaucomys oregonensis	2	0.38	0.37	0.38	372	2	1
	Clades							
	Southwest	7	0.38	0.37	0.38	287	7	1
	Widespread	19	0.38	0.37	0.38	513	19	1
	***G. volans* Local Populations**							
	Grand Osage, Kansas	6	0.38	0.37	0.38	6	2	0.33
	Ouachita Forest, Arkansas	3	0.38	0.37	0.38	263	3	1
Cytochrome-b	**Species**							
	Glaucomys volans	38	0.41	NA	NA	39	23	0.89
	Glaucomys oregonensis	4	0.41	NA	NA	34	4	1
	Glaucomys sabrinus	4	0.40	NA	NA	4	3	0.83
	***G. volans* Clades**							
	Southwest	10	0.41	NA	NA	18	9	0.98
	Widespread	28	0.41	NA	NA	22	16	0.65
	***G. volans* Local Populations**							
	Grand Osage, Kansas	14	0.42	NA	NA	0	1	0
	Ouachita Forest, Arkansas	27	0.42	NA	NA	10	9	0.81

Table 2.2 Nucleotide diversity and Tajima's D values for mitogenome and Cytochrome-b datasets.

		# Individuals	Nucleotide Diversity (Pi)	Tajima's D	Tajima's D P-value
Mitogenomes	**Species**				
	Glaucomys volans	26	0.0086	-1.31	>0.10
	Glaucomys oregonensis	2	0.0023	NA	NA
	***G. volans* Clades**				
	Southwest	7	0.0070	-0.14	>0.10
	Widespread	19	0.0048	-1.99	<0.05
	***G. volans* Local Populations**				
	Grand Osage, Kansas	6	0.0001	-1.37	>0.10
	Ouachita Forest, Arkansas	3	0.0106	NA	NA
Cytochrome-b	**Species**				
	Glaucomys volans	38	0.0098	-1.33	>0.10
	Glaucomys oregonensis	4	0.0204	1.09	>0.10
	Glaucomys sabrinus	4	0.0037	0.65	>0.10
	***G. volans* Clades**				
	Southwest	10	0.0082	-0.79	>0.10
	Widespread	28	0.0025	-2.45	<0.01
	***G. volans* Local Populations**				
	Grand Osage, Kansas	14	0	NA	NA
	Ouachita Forest, Arkansas	27	0.0082	-0.89	>0.10

Table 2.3 Pairwise population sequence divergence (D_{xy}) between reduced-sample populations based on species, *G. volans* clade, and *G. volans* local populations based on both complete mitogenome sequences and Cytochrome-b data.

	Population Group 1	Population Group 2	Pairwise D_{xy}
Mitogenomes	*G. volans* (n = 26)	*G. oregonensis* (n= 2)	0.055
	Southwest Clade (n = 7)	Widespread Clade (n = 19)	0.014
	Grand Osage, KS (n = 6)	Ouachita Forest, AR (n = 3)	0.011
Cytochrome-b	*G. volans* (n=38)	*G. oregonensis* (n= 4)	0.069
	G. volans (n=38)	*G. sabrinus* (n= 4)	0.066
	G. oregonensis (n=4)	*G. sabrinus* (n= 4)	0.080
	Southwest Clade (n=10)	Widespread Clade (n=28)	0.019
	Grand Osage, KS (n=14)	Ouachita Forest, AR (n=27)	0.008

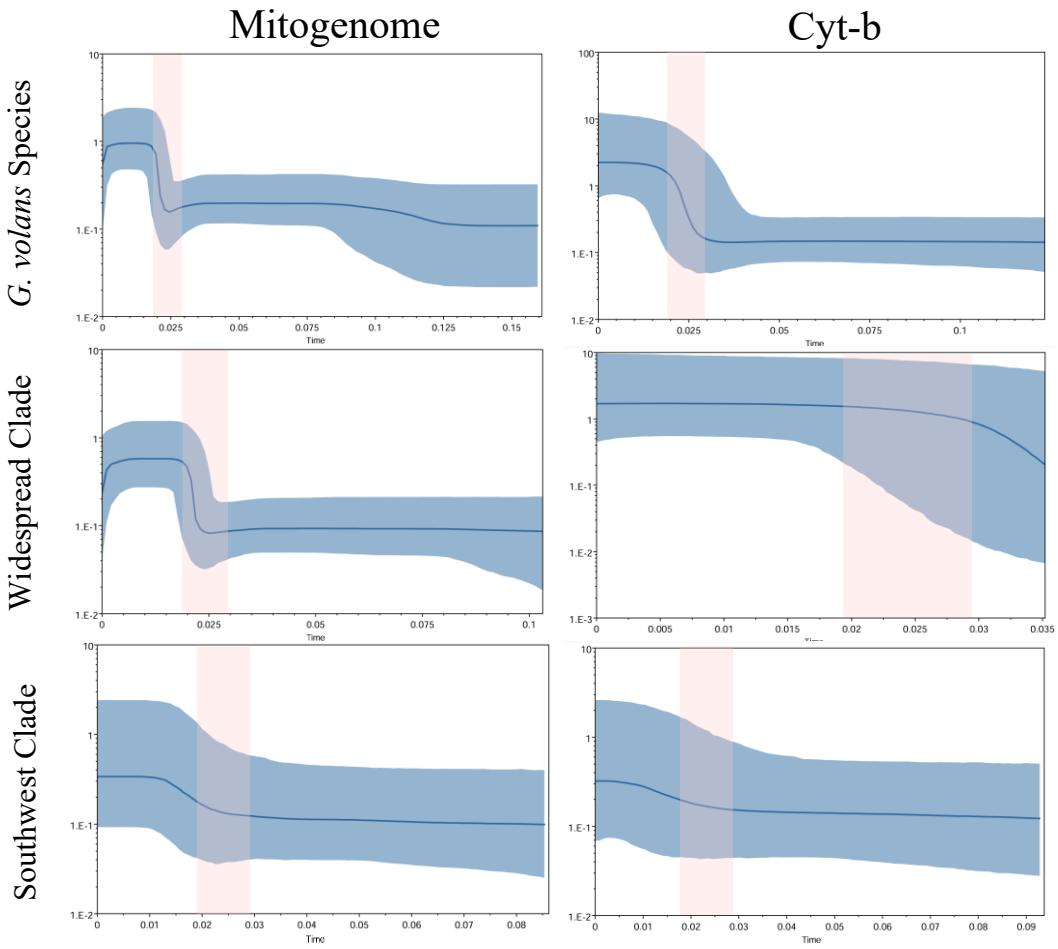

Figure 2.5 Bayesian skyline plots of effective population size over time based on mitogenome (left column) and Cyt-b (right column) data for the full *G. volans* species and both clades. The lighter blue band represents the 95% confidence interval of values. Pink bands represent the period of the last glacial maximum 19-29 million years ago.

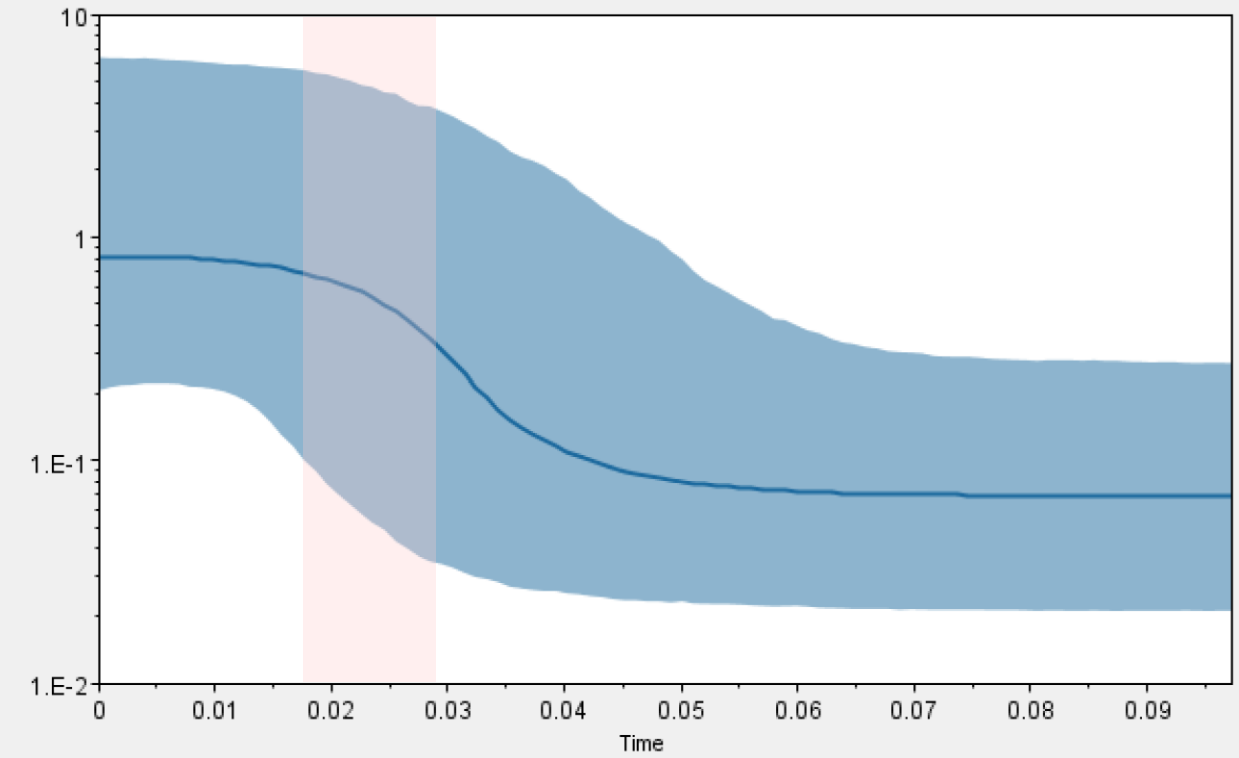

Figure 2.6 Bayesian skyline plots of effective population size over time based on Cyt-b data for Ouachita Forest, AR. The lighter blue band represents the 95% confidence interval of values. Pink bands represent the period of the last glacial maximum 19-29 million years ago.

Table 2.4 Number of codons within each mitogenome protein-coding region undergoing significant purifying or diversifying selection based on FUBAR and MEME analyses.

			ATP6	ATP8	COX1	COX2	COX3	CYTB	ND1	ND2	ND3	ND4	ND4L	ND5	ND6	Total Codons
FUBAR	Diversifying	Complete G. volans	0	0	0	0	0	1	0	0	0	0	0	1	0	2
		Widespread Clade	0	0	0	0	0	0	0	0	0	0	0	1	1	2
		Southwest Clade	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	Purifying	Complete G. volans	23	2	56	21	11	7	42	23	2	15	11	17	5	235
		Widespread Clade	23	2	38	15	23	7	29	24	1	16	7	13	1	199
		Southwest Clade	7	0	21	6	18	13	14	19	4	16	6	10	4	138
MEME	Diversifying	Complete G. volans	0	0	0	0	0	0	0	1	0	0	0	3	0	4
		Widespread Clade	0	0	0	0	0	0	0	1	0	0	0	1	0	2
		Southwest Clade	0	0	0	0	0	0	0	0	0	0	0	1	0	1

Table 2.5 Trends in identification of specific codons under diversifying selection as identified by FUBAR or MEME applications to each *G. volans* dataset. Green "Y" values indicate the associated codon was identified by the specific program as experiencing significant diversifying selection within the corresponding dataset, while red "N" values indicate the opposite.

		Detected in Species?		Detected in Widespread Clade?		Detected in Southwest Clade?	
Protein Coding Region	Intra-Region Codon	FUBAR	MEME	FUBAR	MEME	FUBAR	MEME
CYTB	184	Y	N	N	N	N	N
ND2	36	N	Y	N	Y	N	N
ND5	211	N	Y	N	N	N	Y
	465	N	Y	N	Y	N	N
	475	Y	Y	Y	N	N	N
ND6	10	N	N	Y	N	N	N
	109	N	N	N	N	Y	N

Discussion

My findings merit a reassessment of the *G. volans* subspecies classifications, give insights into this species' history and present trends, and suggest that future continued efforts towards conservation of Kansas populations are warranted.

Species Phylogenetics and Systematics- The mitogenome and Cyt-b trees have strong posterior support for the division of *G. volans* into two clades that coalesced roughly 183,000 years ago, coincident with the penultimate Illinoian glacial phase. This aligns with previous mitochondrial phylogenetic work that indicated two monophyletic groups within *G. volans* (Kerhoulas and Arbogast 2010). Of these two clades, the Southwest clade has a slightly broader range than that of the previously described *G. v. texensis* (Dolan and Carter 1977). However, the Widespread clade appears to cover the range of the other three subspecies (*G. v. volans*, *G. v. saturatus*, and *G. v. querceti*) (Dolan and Carter 1977) with no strongly supported, regionally consistent clades within the broad clade. Even with more robust sampling in the Cyt-b dataset, finer clade structuring with posterior support was not apparent. Given the moderate time since divergence, and only low pairwise sequence divergence (~1.4%) these clades represent intra-specific units of analysis (de Jong et al. 2025; Chan et al. 2026). This supports my goal four hypothesis that clear intra-specific clades with strong support would be present. The clear differentiation between two lineages occurring within *G. volans* warrants recognition as units of analysis for conservation. However, these units are not coincident with current subspecies classifications. While there is some debate over the conservation merits of subspecies classifications (Mayr 1982; Haig et al. 2006; Reydon and Kunz 2021), sub-species often remain diagnosable, and if so, do merit legal and conservation status (Hope and Frey 2022). Given that genetic data represent valid criteria towards diagnosing subspecies, my mitochondrial analyses recognize diversity consistent with

two subspecies. The samples analyzed from Texas, Arkansas, and southeast Kansas are consistent with the sub-species *G. v. texensis*, although the historical range of this sub-species needs updated geographic scope (Dolan and Carter 1977). The remainder of the samples are consistent with the combined distribution of the *G. v. querceti*, *G. v. saturatus*, and *G. v. volans*, where the latter name takes precedence as senior synonym (Dolan and Carter 1977). This reclassification supports my prediction under my second goal (generation of a mitogenome-based species phylogeny), which was that fewer clades would be supported than the number of regional morphologically classified subspecies. However, formal rearrangement of taxonomy should remain pending until a nuclear perspective can either support or refute the mitochondrial evidence.

Species Demographic and Evolutionary History- Beyond phylogenetics, this work made headway in exploring the genetic history of *G. volans*. From an effective population size perspective, as I predicted in goal five which sought to assess demographic change over time, the most significant demographic trend was one of expansion, especially in the Widespread clade. The estimated timing of expansion was closely coincident with the timeframe of the last glacial maximum (LGM) (Clark et al. 2009). Given that effective population size remained high following this period of growth, it would suggest that post-LGM warming was the driver of expansion. At the LGM, the preferred habitat (hardwood forests) of *G. volans* were restricted to southern range limits, limiting *G. volans*' range (Bemmels and Dick 2018). However, as the glaciers retreated, extant hardwood forests in the southeast U.S. expanded, subsequently creating suitable range for *G. volans* north to the Canadian border (Bemmels and Dick 2018). This trend has also been observed in genetic studies at the species' modern northern range limit (Petersen and Stewart 2006). While this trend persists in the Widespread clade, the Southwestern clade

experienced comparatively moderate population growth, likely representing a glacial phase refugium in Texas, followed by more localized range shifts, as has also been seen for a refugial population of preferred hard-mast trees such as *Carya* (Bemmels and Dick 2018).

The Tajima's D statistic results further support these lineage-specific demographic trends. The significantly negative value for the Widespread clade indicates one of several factors: (1) a selective sweep has occurred or (2) there has been dramatic population expansion following a bottleneck or founding event. Based on the demographic shifts following the last glacial maximum, the latter is more supported in this system. The lack of detected diversifying selection fails to support the possibility of a selective sweep being the cause of the negative Tajima's D value. The insignificant Tajima's D value of the Southwest clade further supports that this clade did not experience rapid population expansion, as it existed in its own refugia without rapid expansion as the glaciers retreated.

Despite the constricted region of the Southwest clade, it exhibited higher genetic diversity compared to the Widespread clade. While genetic diversity is sometimes a function of area (as regional drift and selection drive geographically distant populations to distinct genetic structure), this is not always the case. Namely, the regional restriction prior to rapid expansion of the Widespread clade following the LGM may have acted as a bottleneck or founding event followed by further loss of diversity through rapid leading-edge expansion. While sufficient time has occurred for mutation to begin to reintroduce genetic diversity, signals of decreased diversity may still be present in that clade.

Kansas G. volans- The inclusion of Kansas samples across both subspecies clades highlights Kansas as an important region for assessing *G. volans* diversity. The Grand Osage population, while monophyletic, was most closely related to an individual from New York. Given the

distance between New York and Kansas, combined with the small dispersal range of the species, it seems that these individuals may have been transplanted by humans, such as via pet trade and subsequent release, or through movement of freight or lumber into the area. One explanation for transplantation is that Grand Osage was previously a military installation, where personnel likely experienced high turnover. The individuals from the southeastern corner of Kansas and Quivira (the most western Kansas individual) are both closely related to individuals from Arkansas and Texas. These make geographic sense, as there are corridors of gallery forests that connect their sampling locations to the regions occupied by their closest phylogenetic relations. This indicates natural colonization. Finally, the northern individual is basal to the Widespread clade (on par with individuals from Florida and Louisiana), lending further support for a south to north post-glacial colonization that extended as far west as Kansas. As such, Kansas represents an important region for contemporary genetic diversity of these squirrels.

In addition to unique genetic diversity in Kansas, the evidence supports different levels of conservation concern among these populations. In particular, the Grand Osage population in southern Kansas has exceptionally low nucleotide diversity. All 14 individuals with sequenced Cyt-b were represented by a single haplotype. Similarly, for the six mitogenomes sequenced from Grand Osage, there were only six variable sites across the complete 16,550 base pair sequence (0.036% of the mitogenome). This exceptionally low genetic variation across the mitogenomes suggests the potential for similar "flatlining" across the nuclear genome. This genetic signal could be indicative of a founder event accompanied by subsequent inbreeding. Given anecdotes but unconfirmed evidence of recent extirpation of other Kansas populations, it is possible that the signal detected at Grand Osage is a consequence of a system in relaxation (extinction driven isolates) where future local extirpations such as at Grand Osage may be

predicted. Given the combined evidence from mitochondrial DNA, the Grand Osage population in Kansas could be considered both high risk, but also potentially a result of human introduction, lending conflicting evidence towards future conservation priorities. Additional evidence from the nuclear genome is necessary prior to formal recommendations. Further work should also be conducted to sample and evaluate other Kansas populations to determine the extent to which these trends are present.

Arkansas G. volans- While not originally a focal group in this project, the Ouachita National Forest population in Arkansas provides a distinctive foil to Kansas and Grand Osage. While previous work has detected the dual maternal lineages at Ouachita (Cook 1999), this work expands this support. Likewise, the exceptionally high diversity at Ouachita is likely informed by this dual lineage history, as well as the evident habitat-island structure in the region which may be leading to independent drift events across isolated populations (Cook 1999). Of all sampled sites and published work, this appears to be the only documented site of direct contact between the two clades. This region is also of high importance for ongoing wildlife management as these squirrels, due to their pervasive, parasitic effects on red-cocked woodpeckers, are often purposefully removed from the landscape by managers. While demographic trends and current diversity suggest no harm to populations from periodic removal, the evolutionary complexity evident in this region may warrant management consideration.

Selection and Protein-Coding Regions- Looking at the rare instances of diversifying selection in the *G. volans* mitogenome, the only coding gene that markedly stood out was ND5 based on its consistent presence in both FUBAR and MEME results. One of the subunits in the mitochondrial NADH dehydrogenase complex I, ND5 is critical in the regulation of cellular respiration and meeting energy demands (Bai et al. 2000; Chomyn 2001). Given that *G. volans* have high energy

demands due to their lack of hibernation, energetic lifestyle including gliding locomotion, and small size, the presence of diversifying selection (and moderate purifying selection as well) is logical as this gene is critical to this species' functional survival. These findings achieve my sixth goal of assessing selection and support my hypothesis that purifying selection would be more prevalent. Furthermore, the clades demonstrated lower internal values of both diversifying and purifying selection than the population at large, supporting differentiation between the clades.

Beyond *G. volans*, this study's third goal sought to explore how the mitogenome provided critical insights not possible from single genes, such as Cty-b. First, different genes returned differences in phylogenetic topology, and as compared to the entire mitogenome with different levels of nodal support. Given that many phylogenetic studies are conducted using just the Cyt-b or Cytochrome c oxidase subunit I (COX1) (neither of which fully aligned with the mitogenome phylogeny), it is possible that a wealth of previous literature using these loci may have failed to reflect true matrilineal phylogenetic relationships. Second, all protein-coding gene regions exhibited different average mutation rates. Given that evolutionary rates are a critical component towards understanding the timeframe of diversification within phylogeographic studies, this dataset now offers a set of comparative rates above a single Cyt-b estimate for future work. Also, given the relatively well-established evidence for timing of divergence between species of *Glaucomys*, the mitochondrial rates estimated based on this calibration were notably higher than the "standard" 0.02 per site per million years for mitochondrial DNA which is often invoked (Brown et al. 1979). Rate estimates for *G. volans* were like other rate calibrations among mammals, including shrews (Hope et al. 2010) and other rodents (Hope et al. 2014).

Finally, generally mitochondrial genes are considered to evolve in a putatively neutral manner, an assumption which is critical for assessing rates of change and timing of evolution,

and by establishing if and where selection is occurring across mitogenomes, we might avoid violating this assumption when assessing mutation rates. Ultimately, given that full mitogenomes are now both easy to obtain and relatively cheap, they provide much more insight to evolutionary relationships and processes than any single gene, as reflected by differences in basic measures of genetic diversity between my Cyt-b and full mitogenome dataset.

Conclusion- Ultimately, beyond the importance of this study as a litmus test on the value of mitogenomes and the functional capacity to utilize them, this study reshapes the understanding of *G. volans*. Not only does this research begin to explain how *G. volans* diverged into two distinct subspecies, but how those subspecies shifted overtime and have differentiated themselves from each other. More consequentially, the Kansas population (Grand Osage) at the center of this research indicates that substantial concern over long-term genetic health and population persistence is warranted for Kansas populations, particularly as Kansas appears to be a vestige of old colonization events intermixing with anthropogenic-mediated diversity. While mitogenomes provide only a maternal lens through which to view a species, this chapter facilitates the first understanding of the species that encompasses the extremes of the U.S. range. Beyond that, it highlights the importance of, and hazards faced by, western range edge populations. The next important step will be to combine evidence from the mitochondrial genome with that of the nuclear genome in Chapter 3.

Chapter 3 - Nuclear Analysis of *G. volans*

Introduction

In Chapter 2, I demonstrated multiple benefits of using mitochondrial DNA (mtDNA) to inform our understanding of the evolutionary ecology of *Glaucomys volans*. However, mtDNA has limits for inferring evolutionary legacies. The exploration of nuclear DNA provides insights into these trends and more that mtDNA cannot. In my third chapter, I use data from across the *G. volans* nuclear genome and with range-wide geographic sampling to further refine my assessments of population structure, genetic differentiation, and evolutionary processes through time. I will begin by outlining the value and history of nuclear DNA research, then explain what the nuclear genome can tell researchers and managers that mitochondrial DNA cannot. I will conclude the introduction by outlining the specific value of nuclear DNA research to the *G. volans* species and outline the goals and hypotheses that guided this chapter.

The History of Nuclear DNA- Housed in the nucleus of cells, nuclear DNA is bi-parentally inherited (i.e. Mendelian inheritance). Across Eukaryotes, there is more than 200,000-fold variation in genome size (Gregory 2001), though this does not appear to correlate with organismal complexity (Thomas 1971). Unlike mitochondrial DNA, nuclear DNA experiences recombination (events where genetic content is exchanged between maternally and paternally inherited chromosomes) (Meselson and Radding 1975). This can advantageously create beneficial allele combinations or be harmful when it results in the separation of advantageous alleles. Following the discovery of the nuclear DNA structure in 1953 (Watson and Crick 1953), it took over 20 years for the first bacteriophage genome to be sequenced (Sanger et al. 1977). This sequencing technology, dubbed “Sanger Sequencing”, reflected the foundation of the “first generation” of sequencing (Kchouk et al. 2017). In the following decades, sequencing advanced

into the “second generation” (Ronaghi et al. 1998; Bentley et al. 2008) which enabled greater data output volume, increased sequencing parallelization, and decreased costs per base pair (Shendure and Ji 2008). During this transitionary period, first generation sequencing remained important, even helping sequence the first complete human genome (International Human Genome Sequencing Consortium 2004). Sequencing has since progressed into the “third generation” (though first- and second-generation sequencing remain staples in modern research) which has its own distinct advantages that were discussed in Chapter 2. This progression of nuclear DNA sequencing provides many sequencing options, depending on the research in question.

The Importance of Nuclear DNA to Population Genetics and Phylogenetics- The most obvious benefit of nuclear DNA to genetic research lies in the magnitude of data that can be used to infer variation and evolution. In the case of humans, which have an estimated 0.1% variation in the genome between two individuals (Li and Sadler 2001), this equates to roughly three million pairwise differences (Zhang and Hewitt 2003). While the degree of variation differs between species (Zhang and Hewitt 2003), a similar level of variation is expected and observed in other mammals. These variable sites (also known as single nucleotide polymorphisms or “SNPs”) may provide more powerful insights into genetic trends than the single locus of the mitogenome. Likewise, as nuclear DNA is bi-parentally inherited, it provides insights into the influence of both parents on genetic diversity through measures such as admixture and interpopulation gene flow that mitochondrial data cannot fully represent. As such, nuclear DNA is a meaningful supplement to traditional mitochondrial approaches and critical to assessing certain genetic trends not observable in mitochondrial DNA.

Furthermore, nuclear SNPs can be used to infer groups of individuals with signals of locally adaptative advantage or that are on unique neutral evolutionary trajectories (demographically independent) that may be considered of disproportionate importance for conservation and management. For instance, using a large SNP dataset, Evolutionarily Significant Units (ESUs) can be detected, which are critical for identifying distinct populations of conservation importance based on ecological and genetic distinction (Moritz 1994; Funk et al. 2012). However, the SNP datasets can be broken down further into punitively neutral and nonneutral loci. From these two sub-datasets, two more sets of units can be identified: Management Units (MUs) based on neutral loci and Adaptive Units (AUs) based on nonneutral loci. MUs identify populations based on connectivity while AUs identify populations based on adaptive variation (Barbosa et al. 2018). These units and the differences between them can help inform meaningful species conservation but can be much more rigorously detected using nuclear DNA.

Potential Nuclear DNA Pitfalls - Nuclear DNA does have some research limitations. Due to the high (and variable) recombination rates across the genome, clear phylogenetic relationships can be challenging to identify (Posada and Crandall 2002). As such, inferring the true phylogenetic topology of a species with significant recombination (especially in cases of admixture between populations or species) can be challenging, if not impossible. Furthermore, like mitochondrial DNA research, the results of analyses can likely be influenced by regional sample sizes. Logically, larger sample sizes are typically preferrable, as they provide greater insights into the true genetic dynamics and structure of the study system. However, as discussed in Chapter 2, when the sampling is uneven such that the sample set does not reflect an accurate spread of diversity, analyses can be biased in the direction of the sub-dataset that is overrepresented. While

this is not a true pitfall of nuclear DNA, as this trend can happen with any genetic dataset, it is important to consider when conducting analyses on a potentially skewed dataset. Ultimately, while nuclear DNA is a critical piece to understanding the population and phylogenetics of species, it is important to account and address the potential pitfalls of these analyses as well.

The Value of G. volans *Nuclear DNA*- While many *G. volans* genetic studies have used nuclear DNA to evaluate the species, most of these studies have limited geographic scope (Rogic et al. 2016; Wolf et al. 2022; Vivas-Toro et al. 2023). While some mitochondrial studies explored somewhat broader ranges (Kerhoulas and Arbogast 2010), the only "range-wide" nuclear study (Wettschreck 2024) had a sample distribution that still lacked northeastern individuals, making a true range-wide assessment impossible. The major impediment to a comprehensive range-wide study of *G. volans* is the lack of availability of tissue samples from sky-island populations occurring through Mexico. However, previous studies have demonstrated the clear evolutionary distinction of these populations from those further north, whereas the inter-population relationships of *G. volans* north of Mexico remain poorly resolved (Kerhoulas and Arbogast 2010). This work provides the first contiguous range-wide (i.e. the range of *G. volans* excluding Central America) genetic account of the species using both mitochondrial and nuclear data that can assess phylogenetic history, local and regional diversity, and identify units significant for conservation.

Chapter Goals and Hypotheses- To develop the nuclear genomic perspective of *G. volans*, a series of nuclear-specific tests were performed and then compared with the mitochondrial results from Chapter 2 to address the following goals and hypotheses. (1) Generate outgroup-inclusive Maximum-Likelihood (ML) and Neighbor-Net phylogenetic trees. The latter method better accounts for ongoing gene flow and reticulation compared with the presumption of bifurcating

evolution based on ML. These topologies will be used to further assess genetic variation and the validity of subspecies designations. I hypothesize that both nuclear trees will reflect a topology indicating two clades as from the mitochondrial results, but that the network-based analysis will best represent the history of these squirrels, as the contiguous range of *G. volans* provides ample opportunities for gene flow that the ML tree is unable to represent. (2) Assess levels of discordance between the nuclear ML tree and mitogenomic Bayesian tree. Given my previous prediction of widespread gene flow, the potential also for sex-biased dispersal, and the cultural evidence of pet trade and human movement of individuals, it seems likely that mitonuclear discordance will be observed and that it will be most evident in range edge populations where dispersal may more greatly impact population diversity. (3) Explore levels admixture and population structuring, following by determining SNP loci under selection and conducting ESU, MU, and AU assessment through PCA plots of structured clustering using parsed locus-sets. As phylogenetic clades are expected to experience differentiation and potential geographic isolation, I expect the admixture model to reflect the same ideal number of populations as there are strongly supported phylogenetic clades. However, larger tested population numbers are likely to reflect regional diversification because of differential selective pressure across the broad range of *G. volans.* In this same vein, given this variable selective pressure, I hypothesize that regional clustering of individuals will be present in all PCA plots. However, clusters greater regional connectivity will cluster in the MU plot while groups under similar environmental conditions will be more clustered in the AU plot. (4) Assess divergence at species, phylogenetic clade, PCA cluster, and local population partitions. I hypothesize that as the populations become more geographically constrained, especially towards the range periphery, decreased population diversity will be apparent and interpopulation differentiation will be a function of geographic

distance. Finally, (5) analyze how nuclear and mitogenome diversity indices differ. In a null scenario, I expect similar metrics to reflect similar trends despite their different modes of inheritance and mutation rates. To address each goal, methods were developed based on tools and pipelines that are widely used in modern population genetic studies.

Methods

Specimen Collection- As noted in chapter 2, extensive Kansas-based field collections and museum loans were gathered for associated mitochondrial work. The same samples were utilized for nuclear sequencing and analysis, but with the inclusion of additional loaned samples (refer to Appendix Figure B.1).

Nuclear Sequencing- To obtain nuclear data, we conducted double digest restriction-site associated DNA sequencing (ddRADseq). Samples were selected based on ability to cover range-wide distributions and to increase population-level coverage for localities with multi-sample abundance (Appendix Figure B.1). Methodology of digestion, size selection, amplification, purification, and pooling followed standard procedures as described by DaCosta and Sorenson (2014) and used restriction enzymes SbfI and EcoRI. DNA quantification prior to pooling was performed using the Qubit Flex with Broad Range dsDNA chemistry to assess 1µl of library. Sequencing was performed on the Illumina NovaSeq X Plus (2x150 base pair) platform at Admera Health. A total of 134 samples were successfully sequenced including 96 exclusively for this project and 38 using identical methods shared from a separate project (Wettschreck 2024). Of these samples, 130 were *Glaucomys volans* and 4 were *Glaucomys oregonensis* (Appendix Figure B.1). No sequencing of the third species, *G. sabrinus*, was performed although future inclusion of this outgroup species is in progress.

Nuclear ddRADseq Post Sequencing Cleaning- Sequences were first trimmed of padding sequences and filtered for length greater than 50 base pairs using the package gbstrim.pl (https://bitbucket.org/jgarbe/gbstrim). Stacks process_radtags (Catchen et al. 2013) was utilized to demultiplex sequences and filter out sequences with uncalled bases or with sliding window regions (15% sequence length) below a quality score of 10. The Burrows-Wheeler Alignment index tool (Li 2013) was applied to a *G. volans* reference genome (GenBank CA_020662805.1) to define a reference library. Alignment of sequences to the reference was done with BWA-MEM (Li 2013). Sequences were then sorted and indexed with Samtools using functions sort and index respectively (Danecek et al. 2021). Stacks SNP calling was conducted on four sub-datasets: (1) all *G. volans* samples and outgroup individuals (for phylogenetic tree generation and species-level population genetics), (2) all *G. volans* samples without outgroup individuals (for phylogenetic tree generation, selection identification, and PCA plots), (3) a reduced set of *G. volans* (to avoid biasing analyses towards large-sampled populations) with outgroups (for phylogenetic clade-level population genetics), (4) reduced set of *G. volans* without outgroups (for PopClust and local and PCA-based population genetics). In order, these are the "complete interspecies", "complete *G. volans*", "reduced interspecies", and "reduced *G. volans*" datasets. For the reduced datasets, populations with more than three individuals were reduced to three individuals that covered the greatest range of positions in preliminary phylogenetic trees. These individuals were consistent between the reduced interspecies and *G. volans* datasets to maximize comparative potential.

For each dataset, aligned sequences were analyzed using the ref_map.pl pipeline in Stacks for loci and single nucleotide polymorphisms (SNPs) (Catchen et al. 2013). SNPs were reduced to one per locus in the populations module in Stacks (--write-single-snp) (Catchen et al.

2013). Following this reduction to a maximum of one SNP per locus, many retained loci did not have any SNPs. The post-reduction interspecies dataset consisted of 232,932 loci and 69,012 SNPs while the reduced interspecies dataset consisted of 190,109 loci and 53,171 SNPs. The complete *G. volans* dataset contained 226,810 loci and 64,727 SNPs and the reduced *G. volans* dataset contained 181,755 loci and 48,727 SNPs.

Variant sites were filtered using R packages *SNPfiltR* (DeRaad 2022) and *vcfR* (Knaus and Grunwald 2017). Reasonable filtering thresholds were selected by iterating through various threshold parameter sets and determining the effects on resulting PCA plots. For all datasets, reasonable thresholds were determined to be a minimum depth of 10, minimum quality score of 30, and a maximum depth of 100. Due to differences in the sample diversity (between reduced and complete datasets) and given that the inclusion of outgroups normally results in higher missing data (given that greater genetic distances result in more fixed differences across loci), SNP missingness by sample and SNP completeness by SNP were different for each dataset (Table 3.1). Note that the SNP missingness threshold was iteratively reduced to maximize sample retention and minimize missingness.

Table 3.1 Variable SNPfiltR thresholds and resulting dataset statistics.

Dataset	Missingness by Sample	Completeness by SNP	# Retained Individuals	# Variants	% Missing Data
Complete Interspecies	0.82	0.90	118	2,475	5.185
Complete *G. volans*	0.72	0.95	112	1,660	2.144
Reduced Interspecies	0.75	0.90	60	2,277	4.091
Reduced *G. volans*	0.70	0.95	54	1,651	1.733

The *G. volans* datasets were then analyzed to identify and remove SNPs deviating significantly from Hardy-Weinberg equilibrium (HWE) using the R *pegas* package (v. 1.4; Paradis 2010) where populations were defined as individuals clustering together in *adegenet* (v. 2.1.11; Jombart 2008) PCA plots generated automatically by *SNPfiltR* (DeRaad 2022). SNPs were removed if they were out of HWE across all populations (the conservative "out all" approach), as the populations were somewhat ambiguous to define and we did not want to falsely remove true instances of structure (Pearman et al. 2022; Hsu 2026). The resulting datasets comprised 1,659 SNPs and 1,651 SNPs respectively for the complete and reduced *G. volans* datasets. When considering filtering for HWE loci in the complete and reduced interspecies datasets, concerns were present about the outgroup's ability to inform HWE values, as either the outgroup was considered one population to increase sampling strength, or else was separated into distinct, small populations which lacked strong statistical support. While the former option was considered, concerns were introduced regarding the Wahlund Effect (Wahlund 1928) due to the known structure between the two outgroup populations (Yuan et al. 2022). Given these concerns, the limited capacity in which both outgroup datasets were used, and the already minute number of observed loci out of HWE in *G. volans*, these two datasets were not filtered based on HWE values.

ddRADseq Phylogenetics- A rooted Maximum-Likelihood phylogeny of the complete interspecies dataset was generated in RAxML Next Generation (RAxML-NG, v. 1.2.2; Kozlov et al. 2019). To determine the ideal model for RAxML-NG, ModelTest-NG (v. 0.1.7; Darriba et al. 2020) was applied to the SNP datafiles with candidate models of heterogeneity limited to uniform and discrete Gamma rate categories. To account for ascertainment bias, the Lewis model was applied but given RAxML-NG identifies ambiguous sites as invariant, these sites were

removed using the ascbias.py script (https://github.com/btmartin721/raxml_ascbias) with modifications recommended on GitHub. RAxML-NG was run with the optimum model produced across BIC, AIC, and AICc test results, as well as Lewis' method for ascertainment bias correction. Rather than setting a discrete number of bootstrap replicates, up to 1000 replicates were permitted with "bootstopping" preformed every 50 trees with a cutoff of 0.03. Trees were visualized and annotated in FigTree (v. 1.4.4; Rambaut 2009). To assess the phylogenetic, distance-based network, that accounts for potential gene flow between populations, a Neighbor-Net analysis was run on the complete *G. volans* dataset in SplitsTree (v. 6.7.4; Huson and Bryant 2024). The Neighbor-Net was calculated using uncorrected p-distances. The Neighbor-Net tree was visualized and annotated in the SplitsTree GUI.

Assessment of Mitonuclear Discordance- To determine differences in phylogenetic history between individual mitochondrial and nuclear DNA, a tanglegram was generated and annotated in Dendroscope (v. 3.8.10; Huson and Scornavacca 2012). Two iterations of tanglegram, both with the intra-species nuclear RAxML-NG tree, were generated: one using the more strongly supported but reduced-sample mitogenome tree and one using the less supported but more sample-rich Cytochrome-b tree. To reduce noise in the tanglegram, the nuclear was reduced to only those samples that were also present in the mitochondrial comparison dataset.

Detection of Neutral and Nonneutral Loci Under Selection- Following file conversion in PGDSpider (v. 2.1.1.3; Lischer and Excoffier 2012), filtered SNPs were analyzed for neutrality in Bayescan (v. 2.1; Foll and Gaggiotti 2008). In Bayescan, iterations were run with prior odds of 10, 100, and 1000. All iterations had 5,000 MCMC output iterations, a thinning interval of 10, 20 pilot runs with 5000 iterations each, and a burn-in of 50,000 iterations. Across the three prior

odds tests, the number of identified loci decreased from 12 to 4 to 3. Given these small values, all loci were considered nonneutral if they appeared in any iteration.

An alternative approach to nonneutral allele identification using *pcadapt* (v. 4.4.1; Luu et al. 2017) was conducted to avoid the chance of Bayescan failing to identify all nonneutral loci. This program required file conversion using Plink (v. 1.90-b6.21; Chang et al. 2015). Prior to accounting for false discovery, 49 SNPs were significant at a p-value threshold of 0.05. Then the Benjamini-Hochberg Procedure was applied to account for false discovery with a threshold of 0.1, resulting in a significant dataset of only 23 SNPs at a threshold of 0.1. Of these, only four aligned with those identified by Bayescan. Given the small sample size of nonneutral alleles, the derived nonneutral allele set consisted of all SNPs identified as nonneutral using either method, as only four were common between the two. To account for bias, the neutral allele set consisted of all alleles that were determined to be neutral by both methods.

Population Clustering and Variance- To evaluate population structure, PopCluster (v. 1.7.0; Gurinovich et al. 2019) was applied to the reduced *G. volans* dataset and was run for K values 2 through 20 with 10 iterations each. An optimal K value was determined by evaluating DLK2 and F_{ST}/F_{IS} estimators. Plotting results was done in the PopCluster GUI with additional manual modification.

Evaluation of variation and clustering of ESUs, AUs, and MUs, used *dartR* (v. 2.9.9.5; Mijangos et al. 2022) with independent runs on each of the SNP datasets (complete, neutral, nonneutral). Prior populations were defined by phylogenetic groups and PopCluster results, though population-absent simulations were also conducted for all datasets. Pairwise plots of all PCA axes accounting for at least 4% of variation were plotted with ellipses representing 95% of points within each identified population for each dataset.

Population Diversity and Divergence Estimations- Measures of observed heterozygosity, expected heterozygosity, nucleotide diversity (π), and the inbreeding coefficient (F_{IS}) were calculated in the Stacks' populations script (Catchen et al. 2013). To account for diversity at multiple levels within the species, four iterations using the most suitable and balanced SNP set were conducted. These iterations were (1) species-level (the reduced interspecies dataset), (2) phylogenetic clade-level (the reduced *G. volans* dataset based on Neighbor-Net groups) , (3) region-level (regional genetic clusters based on PCA results using the complete *G. volans* dataset), and (4) local-level (individuals found at the same locality for those with robust sample sizes, using individuals from the complete *G. volans* dataset). Pairwise population fixation (F_{st}) values were calculated in R package *hierfstat* (v. 0.5-11; Goudet and Jombart 2024), using the equation proposed by Weir and Cockerham (1984), for each of the same four population sets used in the Stacks populations script.

Results

Phylogenies- For the RAxML-NG tree, the ideal model was determined to be TVM+G4 and bootstrapping converged between 600 and 650 replicates. Despite this bootstrap convergence, bootstrap values for most RAxML-NG tree branches was low (below 95 and the majority below 40) (Figure 3.1; Appendix Figure B.2). However, high bootstrap values support the two species (*G. volans* and *G. oregonensis*) as well as some inter-sample relationships (Figure 3.1). The only regional monophyletic group are the samples from Grand Osage, Kansas, with strong bootstrap support (Figure 3.1). Additionally, the northern Kansas sample (KS-KUKUM171667) is strongly supported in a group with one of the Lincoln, Nebraska individuals (Figure 3.1).

Though lacking rigorous support (Appendix Figure B.2), four monophyletic groups are identifiable in the "consensus" topology: (1) two basal Florida individuals, (2) the remaining

Florida individuals and one Louisiana sample, (3) all Texas, Arkansas, Oklahoma, Nebraska, and Kansas samples combined, and (4) all remaining samples (Figure 3.1). Hereafter, these will be the "Basal Florida", "Southeast", "Southwest", and "Northeast" RAxML groups respectively.

The structure of the Neighbor-Net SplitsTree shows a topology that reflects 99.2% of identified distance. Two major distinctive groups are apparent: a Southwest group consisting of the same individuals as the RAxML tree, and a "Widespread" group that included all remaining *G. volans* individuals (Figure 3.2). Within the Widespread group, the individuals from Florida, Louisiana, and Tennessee from a cluster (except for one Louisiana individual) (Figure 3.2). Four distinct subgroups are present within the Southwest clade: (1) all Grand Osage, Kansas individuals, (2) the northern Kansas sample and one Nebraska individual (UNO004, the same as clustered strongly with the same Kansas sample in the RAxML tree), (3) all remaining Nebraska individuals, and (4) both remaining Kansas samples and all Texas, Arkansas, and Oklahoma samples (Figure 3.2). It is worth highlighting that in both trees, all Kansas individuals are contained within the Southwest group. However, the net-like structure between the groups and subgroups indicates reticulation through gene flow between these clusters, reflecting incomplete genetic isolation.

Mitonuclear Discordance- High mitonuclear discordance is present within both the mitogenome- and Cyt-b-aligned tanglegrams (Figure 3.3). Except for Grand Osage group (Figure 3.3A) and some Ouachita Forest family groups (Figure 3.3B), all groups identified in RAxML and Neighbor-Net analyses have variable topology. When direct phylogenetic assessments of the mitochondrial phylogeny and nuclear Neighbor-Net are conducted, most samples remain in the same group, but a series of individuals at the western range edge are part of the Southwest nuclear cluster and the mitochondrial Widespread (Figure 3.4).

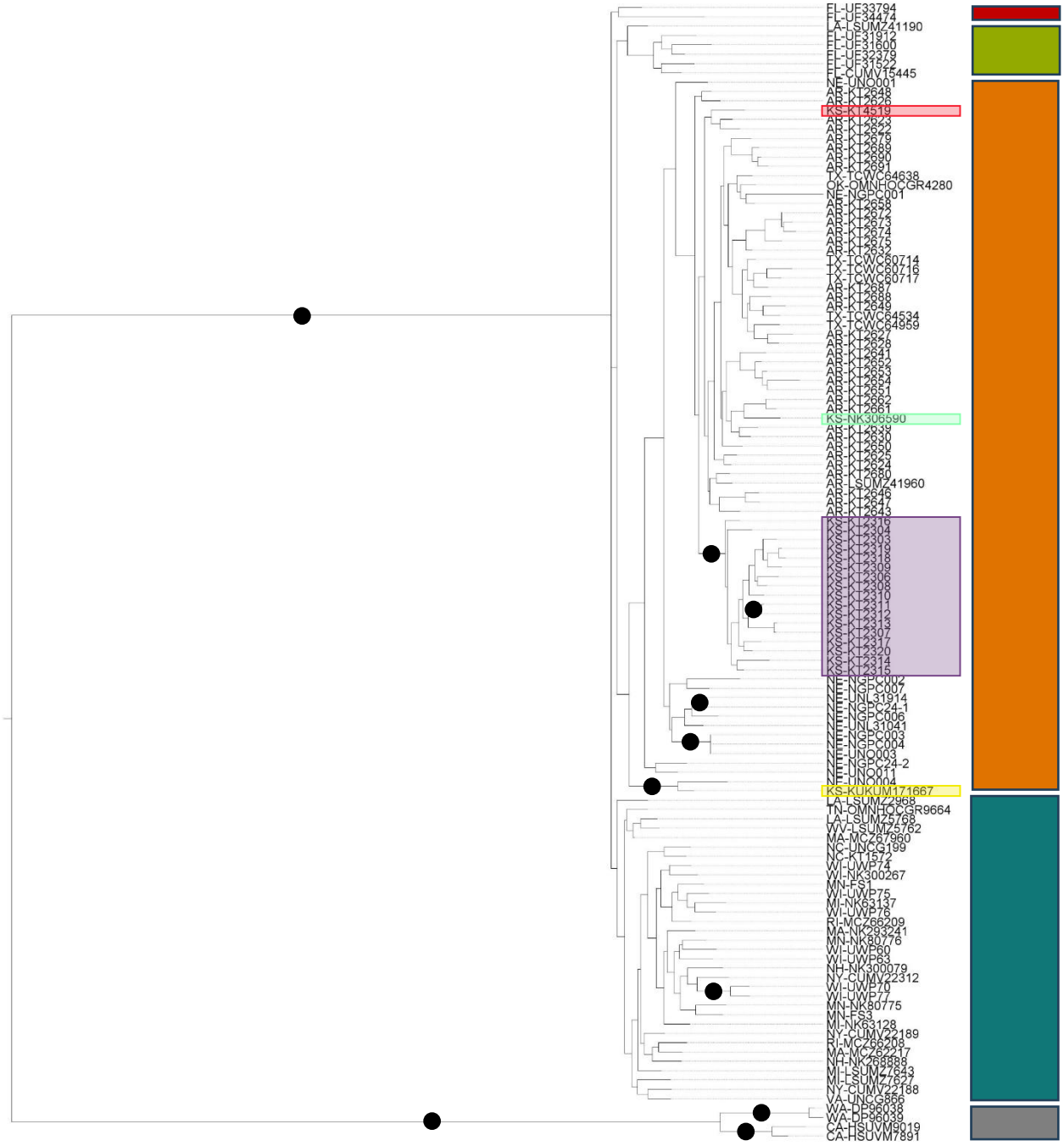

Figure 3.1 RAxML phylogeny of the complete *Glaucomys* (*G. volans* and outgroup *G. oregonensis*) dataset. Black dots indicate branches with bootstrap support ≥ 95. Vertical bars represent proposed clades as Basal Florida (dark red), Southeast (green), Southwest (orange), Hodgepodge (teal), and *G. oregonensis* outgroup (grey). Kansas samples are highlighted base on locality at western Quivira Boy Scout Ranch (red), the southeastern state corner (green), Grand Osage Wildlife Area (purple), and northern Kansas (yellow).

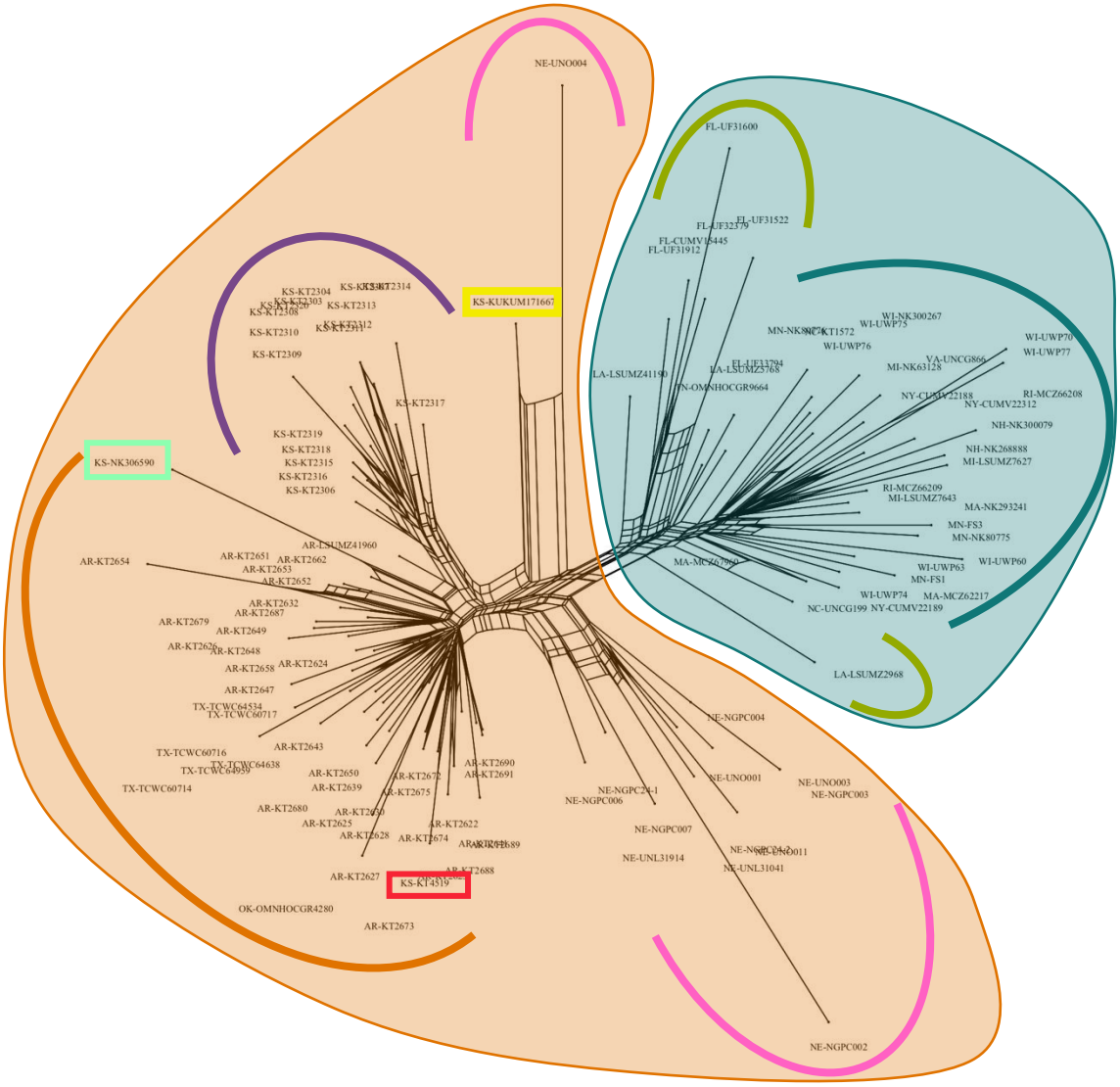

Figure 3.2 Neighbor-Net phylogeny of the complete dataset. Shaded regions indicate Southwest (orange) and Widespread (teal) groups. Colored arcs indicate clusters apparent in the network and subsequent PCA plots. These clusters are Majority Southwest (orange), Grand Osage (purple), Nebraska and Northern Kansas (pink), North (teal), and Southeast (green). Boxed samples are Kansas samples from Northern Kansas (yellow), the Southeastern corner (pale green), and Quivira (red).

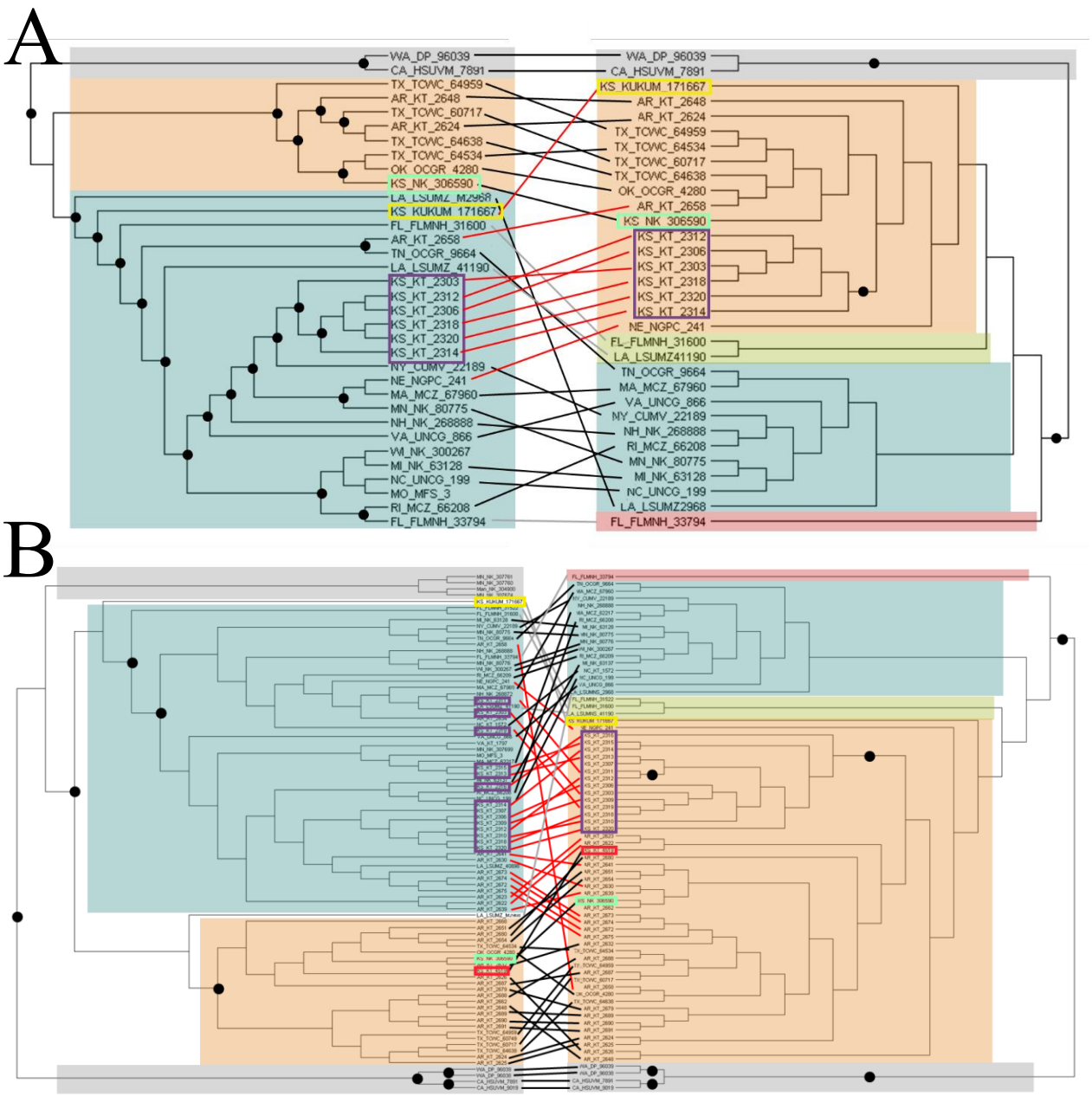

Figure 3.3 Tanglegrams of mitochondrial topology (left) against nuclear topology (right) for mitogenome (A) and Cyt-b (B) trees. Lines connect samples present in both trees. Black indicates consistent clade between trees, red indicates discordance between the Southwest and Widespread clades, and grey indicates different clades but not Southwest/Widespread. Dots indicate posterior support of at least 0.95 for mitochondrial trees and bootstraps of at least 95 for nuclear trees. Colors are based on monophyletic groups using the standard Southwest (orange), Widespread (teal), and outgroup (grey), as well as Basal Florida (red) and Florida/Louisiana (green). Kansas samples have clear-centered boxes where colors correspond to localities at Grand Osage (purple), Quivira Scout Ranch (red), the southeast corner of Kansas (bright green), and northern Kansas (yellow);

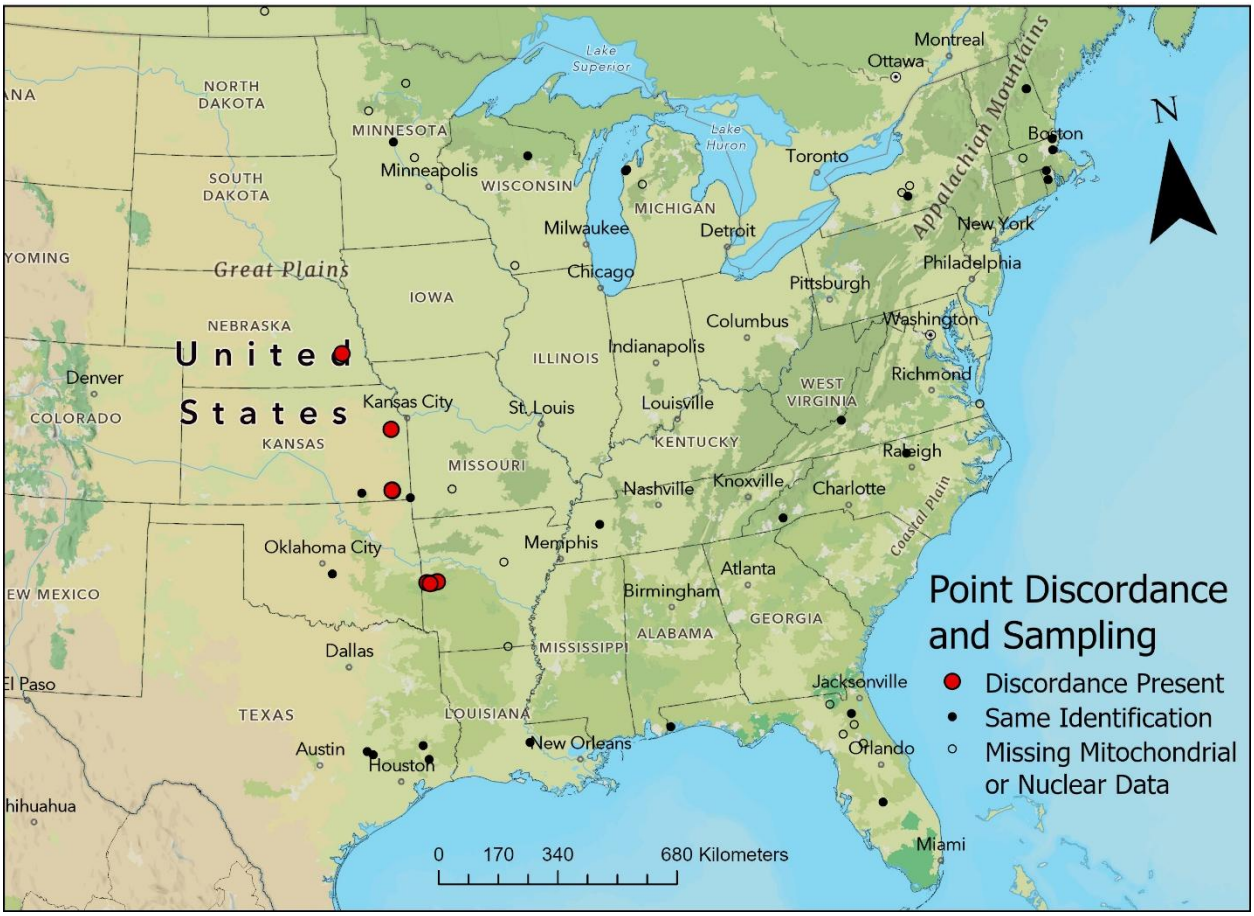

Figure 3.4 Mapping of *G. volans* sampling localities and samples that differed in their phylogenetic group classification between mitochondrial and nuclear phylogenies, but only those between the two consistent clades (Southwest and Widespread) (red dots).

Population Clustering and Variance- Within the complete *G. volans* SNP dataset, four discrete population clusters are present: Grand Osage, the Southwest, Nebraska, and the "Widespread" clade (Figure 3.5A). As there is overlap between the proposed Florida/Louisiana population and Widespread population, these two populations cannot be considered as independent Evolutionarily Significant Units (Figure 3.5A). Of the three Kansas samples not from Grand Osage, two (Quivira and the northern site) are within the 95% CI thresholds of the Southwest and Nebraska populations respectively (Figure 3.5A). The southeastern corner sample appears to be intermediate between these two populations (Figure 3.5A). These clusters strongly mirror the two groups identified in the Neighbor-Net phylogeny, with greater structuring in the Southwest clade (Figure 3.6).

In the neutral SNP-only dataset, the PCA plot shows Grand Osage and the Southwest populations as distinctive Management Units (MUs) (Figure 3.5B). Nebraska individuals are also mostly distinct, although the ellipse slightly overlaps another (Figure 3.5B). The remaining populations (Florida/Louisiana and Widespread) do not significantly differ from each other (Figure 3.5B). In the non-neutral SNP PCA, only Grand Osage is a significantly variant population from the remaining individuals (Figure 3.5C). This indicates two Adaptive Units within *G. volans*.

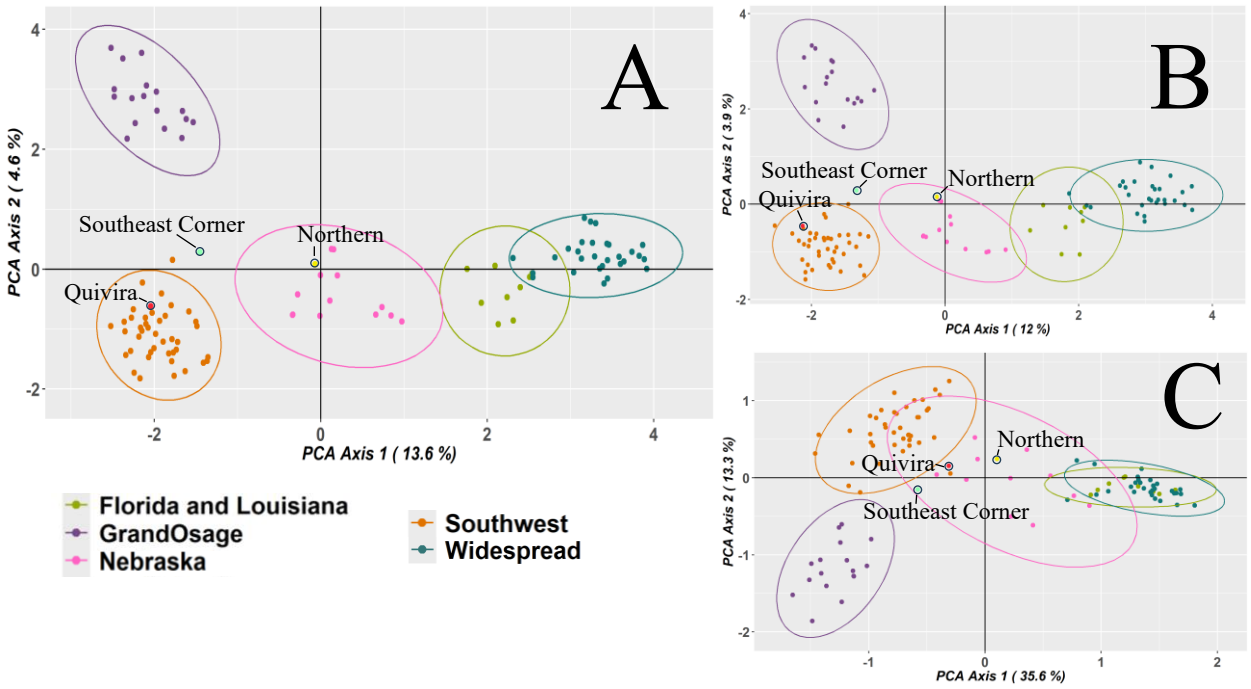

Figure 3.5 Principle Component Analyses of (A) all filtered SNPs from the complete *G. volans* dataset, (B) only neutral SNPs, and (C) nonneutral SNPs. Ovals represent 95% confidence intervals of identified populations. Non-Grand Osage Kansas samples are indicated with labels in each plot.

Two K values were deemed import in the PopCluster results. The F_{ST}/F_{IS} measure identified an optimal K of 4, while the DLK2 identified an optimal K of 15. Within the K-4 plot, clusters appear to align strongly with the clusters observed in PCA plots and phylogenic topologies (Figure 3.7A), however the Florida and Louisiana group is distinct while the Nebraska and Grand Osage individuals are predominantly included in the Southwest population. The Quivira (KS-KT4519) and southeastern Kansas (KS-NK306590) are members of a distinct "population", with one Texas individual sharing partial genetic similarity to this group (Figure 3.7A).

Within the K-15 plot, clustering appears to be primarily by geographic region (Figure 3.6B). Instances of this geographic clustering are Texas and Arkansas (Group 1), Minnesota and Wisconsin (Group 2), Florida (Group 3), Nebraska and northern Kansas (Group 6), various Northeast samples (Group 9), Texas and Oklahoma (Group 10), Grand Osage and Arkansas (Group 11), Florida and Louisiana (Group 13), and New York, North Carolina, and Tennessee (Group 14) (Figure 3.7B). Three populations consisted of single individuals: Group 4 (Quivira, KS), Group 7 (New Hampshire), and Group 15 (southeastern KS) (Figure 3.7B). The remaining Groups 5, 8, and 12 have some evidence of regional clustering, but contain individuals from geographically disparate populations. Group 5 is highly variable, with individuals from North Carolina, Michigan, and Louisiana (Figure 3.7B). Group 8 contains predominantly Northeast samples, but also a Michigan individual (Figure 3.7B). Last, Group 12 contains midwestern samples, as well as a Virginia sample (Figure 3.7B).

Population Diversity and Divergence Estimations- Between species, *G. volans* had a higher level of nucleotide diversity (0.086) compared to *G. oregonensis* (0.068) (Table 2). Each *G. volans* monophyletic group exhibited as much, if not more, genetic diversity (0.086-0.105) than the species as a whole (Table 2). Within both the PCA and local groups, Grand Osage had the lowest

level of nucleotide diversity (0.05) (Table 2). Universally, F_{IS} values were low, with the smallest local group measure being in the Platteville, WI population and the highest in the Ouachita Forest population (though Grand Osage was comparable) (Table 2).

The inter-species F_{ST} was 0.683 (Figure 3.8A). Between phylogenetic groups, the greatest differentiation was between the Southwest and Widespread groups (0.153) and the lowest was between the Florida/Louisiana and Widespread groups (0.06) (Figure 3.8B). Within the PCA and local groups, Grand Osage was most like the Southwest group (0.12) and Ouachita Forest (0.123) respectively (Figure 3.8C, Figure 8D). The most different populations from Grand Osage were the Widespread (0.203) and Wisconsin populations (0.281) (Figure 3.8C, Figure 8D). In both PCA and local F_{ST} measures, the Grand Osage population exhibited highest differentiation of any observed between any two populations.

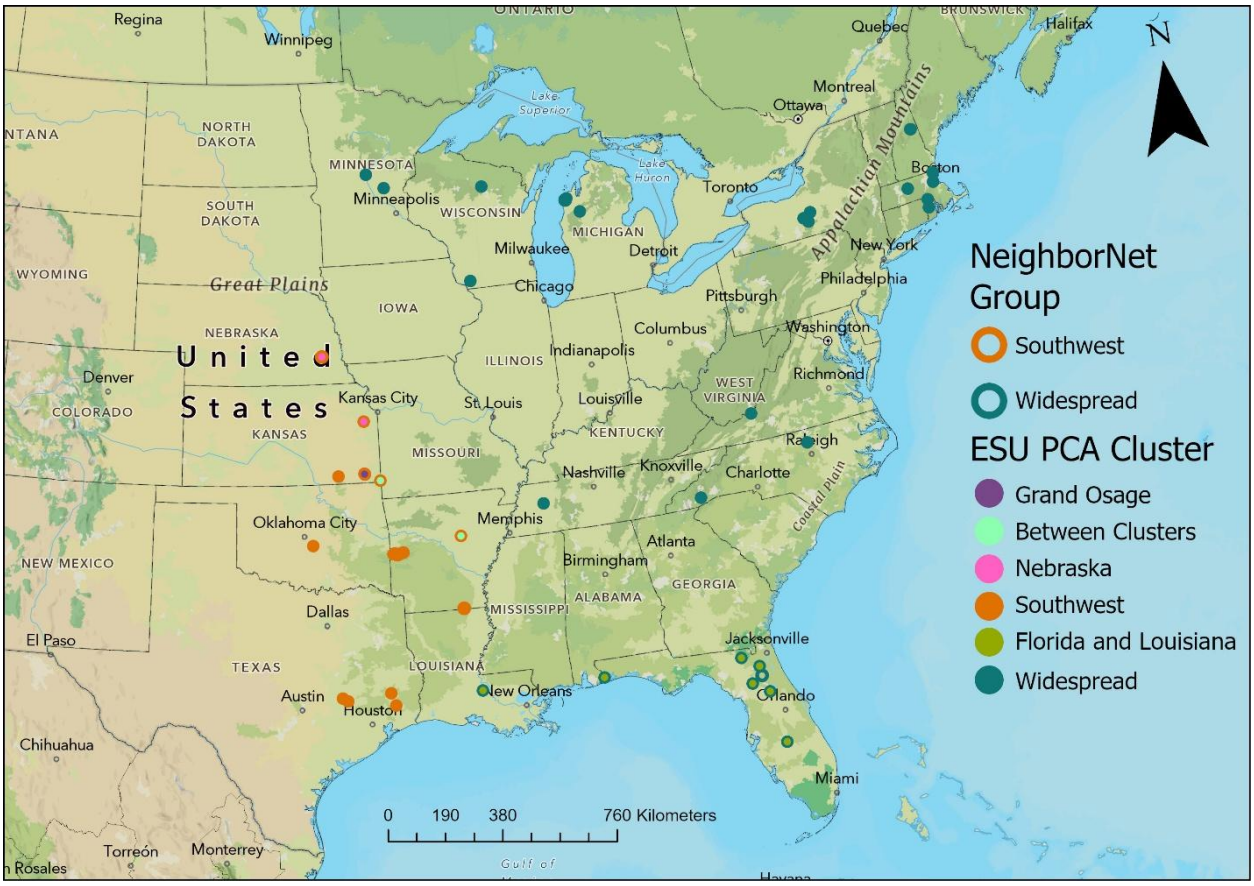

Figure 3.6 Sampling map of ddRADseq with coloring based on Neighbor-Net grouping (circle outlines) and PCA ESU clusters (internal colors).

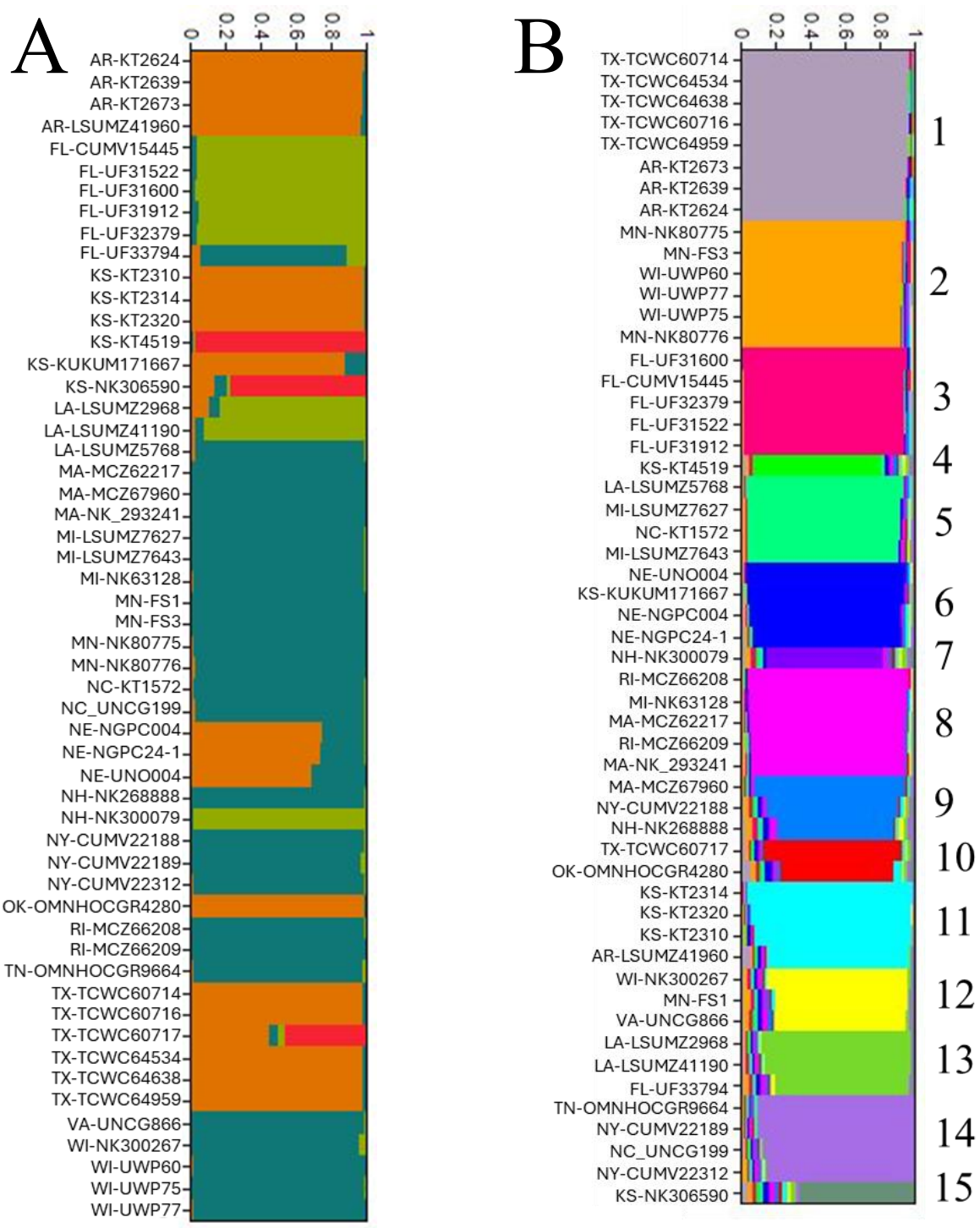

Figure 3.7 PopCluster admixture clustering analysis for K values of (A) four and (B) fifteen. (A) is organized by sample locality and colored based on detection of PCA clusters Florida and Louisiana (green), Southwest (orange), Widespread (teal), and Quivira, Kansas (red). (B) is organized by cluster which are labeled on the right. Color was randomly assigned for (B) clusters.

Table 3.2 Diversity and inbreeding statistics of population groups at the species, phylogenetic group, PCA cluster, and geographically local levels.

		Average Individuals Per SNP	Observed Heterozygosity	Expected Heterozygosity	Nucleotide Diversity (Pi)	Inbreeding Coefficient (Fis)	Fis Standard Error
Species	*G. volans*	53.720	0.068	0.085	0.086	0.094	0.034
	G. oregonensis	3.708	0.029	0.058	0.068	0.075	0.012
Phylogenetic	Southwest Group	19.590	0.069	0.084	0.086	0.070	0.014
	Florida and Louisiana Group	5.947	0.091	0.096	0.105	0.033	0.006
	Widespread Group	26.531	0.086	0.091	0.093	0.036	0.016
PCA	Southwestern	41.277	0.065	0.068	0.069	0.023	0.024
	Widespread	37.230	0.079	0.088	0.089	0.053	0.024
	Grand Osage, KS	16.600	0.046	0.049	0.050	0.011	0.017
	Nebraska and Northern KS	13.588	0.056	0.066	0.069	0.042	0.013
Local	Ouachita Forest, AR	31.600	0.065	0.067	0.068	0.014	0.018
	Grand Osage, KS	16.600	0.046	0.049	0.050	0.011	0.017
	Lincoln, NE	12.593	0.056	0.065	0.067	0.038	0.013
	Platteville, WI	6.976	0.074	0.077	0.083	0.021	0.004

A

	G. oregonensis (n=4)
G. volans (n=56)	0.683

B

	Florida and Louisiana	Widespread
Florida and Louisiana (n=6)		
Widespread (n=27)	0.06	
Southwest (n=20)	0.142	0.153

C

	Grand Osage, KS	Nebraska and Northern KS	Southwest
Grand Osage, KS (n=17)			
Nebraska and Northern KS (n=14)	0.197		
Southwest (n=42)	0.12	0.118	
Widespread (n=52)	0.203	0.113	0.165

D

	Grand Osage, KS	Lincoln, NE	Ouachita, AR
Grand Osage, KS (n=17)			
Lincoln, NE (n=13)	0.209		
Ouachita, AR (n=32)	0.123	0.127	
Platteville, WI (n=7)	0.281	0.154	0.218

Figure 3.8 Measures of pairwise F_{ST} between populations at the (A) species, (B) phylogenetic group, (C) PCA cluster, and (D) geographic locality levels. Only groups with at least4 individuals were considered, so some small groups at the phylogenetic and PCA cluster levels are not present.

Discussion

In this chapter, several valuable findings have come to light. Most obviously, the evolutionary history of *G. volans* north of Mexico appears both shallow (based on universally low intraspecies F_{ST} values), but also highly complex. Though two phylogenetic clades that are largely coincident with the identified mitogenome clades are present, evidence of discordance seemingly driven by a recent northward range shift of the Southwest clade has introduced gene flow that limits support within the tree topology. Of the identified populations seemingly impacted by the Southwest range shift, Grand Osage has a distinctive set of different mitochondrial and nuclear genomes. Additionally, Grand Osage shows signals of adaptive and neutral distinction from all other populations that potentially warrant unique management considerations. These findings help expand the understanding of the underlying genetic diversity and history of *G. volans* holistically, as well as regional populations, especially Kansas. This work also highlights the importance of incorporating multi-locus nuclear data into phylogeographic analysis, rather than assuming mitochondrial DNA can capture the complete genetic history and diversity of a species.

Phylogenetic Structure of G. volans - The nuclear population structure of *G. volans* is far less clearly defined than mitochondrial. Despite the bootstrap convergence of the RAxML phylogenetic analysis, there was a lack of support for major clades. While monophyletic groups were detected, the lack of support means that these cannot be used to infer species substructure on their own. However, the remaining analyses (Neighbor-Net, ESU and MU PCA plots, and PopCluster) all support the presence of at least two distinct clusters within *G. volans* that align with Southwest and Widespread mitochondrial clades, although multiple individuals exhibited discordant clade membership across locus-sets. Some additional sub-structure was also detected

across nuclear analyses. Collectively, this supports my hypothesis for my first goal which sought to evaluate the phylogenetic structure of *G. volans* and which anticipated two clades. Grand Osage and Nebraska (with the inclusion of the northern Kansas individual) distinctly clustered within all analyses, except for the PopCluster at a K of 4. Therein, Grand Osage was clustered within the Southwest population, and the Nebraska individuals appear to share genetic data from both the Widespread and Southwest populations. Interestingly, the PopCluster K=4 results identified Kansas populations and Florida populations that are not significantly distinct in the PCA plots, though the latter was considered in the figures. The Grand Osage, Nebraska, Florida, and southern Kansas groups that are variably present as differentiated groups from the two main clusters (Widespread and Southwest) depending on the analysis represent instances of localized differentiation. However, these groups are consistently present within the Neighbor-Net clades and possess much lower genetic differentiation (F_{ST}) with the Widespread and/or the Southwest group, compared to differentiation between the Widespread and Southwest groups. This lack of consensus on population structuring does not support my goal four hypotheses which predicted population structuring consistent across the tested methods, particularly the PopCluster analysis.

However, based on the consistent presence of the Widespread and Southwest groups in all analyses, higher rates of differentiation between these groups than any others, and consistency of groupings and localities with the majority of the mitochondrial phylogenetic findings from Chapter 2, I continue to support the reclassification of *G. volans* into two subspecies rather than the four described by Dolan and Carter (1977), and earlier work. Though these recommendations are supported, I also advise further work and sampling in Florida and the central *G. volans* range to assess if any other subspecies may exist but in highly isolated areas. The merit of these

subspecies' classifications may also be slightly undercut by the evidence of gene flow between these apparent subspecies.

Discordance and Gene Flow- Every analysis of the nuclear DNA in this chapter indicates gene flow across the *G. volans* range. The low bootstrap support of the RAxML tree, despite the rigorous filtering and strong outgroup support, is likely indicative of some form of gene flow resulting in incomplete lineage sorting (Eckert and Carstens 2008). The appearance of a net-like structure in the Neighbor-Net and high abundance of mitonuclear discordance further indicates that gene flow is occurring both within clades and among them. Along the western range boundary, it appears that while many individuals are maternally in the Widespread lineage, paternally inherited DNA is from the Southwestern clade. This indicates that there is a permeable subspecies boundary not far from the western range edge. Since the consistent trend is that individuals with Widespread haplotypes exhibit Southwestern nuclear genomes, there appears to be northward and eastward expansion of the Southwest subspecies into regions historically occupied by the Widespread subspecies. Furthermore, while the maternal genetic legacy indicates a phylogeographic history coincident with isolation in two distinct southern refugia during the LGM followed by post-glacial expansion, the nuclear history is much less well-defined and suggests substantial genomic mixing during the post-glacial phase of range expansion. Given the geographic scope and directionality of mitonuclear discordance, it appears that the Widespread clade historically occupied most of the western range edge (north of Oklahoma), but that recent expansion of the Southwest clade northward along the western range edge is introducing novel genetic diversity into the region. The intermediate nature of Nebraska between the two subspecies in the PCA plots and the admixed K-4 values indicate the presence of both subspecies in that region contributing genetic material to the sampled individuals.

Potentially, this implies the current or recent northern extent of the Southwest expansion, as Widespread individuals are still present.

Generally, decreased F_{ST} values were observed between populations that are more geographically proximate, as was anticipated in my fourth and fifth goals of identifying diversity and divergence. This implies that geographic distance may influence genetic differentiation (though formal tests of isolation by distance were not conducted). However, this trend does have exceptions. Consider again that the Grand Osage population expressed a mitochondrial most recent common ancestor with an individual from the Widespread clade (New York). It is then possible that either genotypes of the Widespread clade within this dataset are well mixed across eastern North America, or that some other mechanism such as anthropogenically mediated gene flow is acting to facilitate mixing of genetic legacies across this vast area. Although most groups identified in the K=15 PCA were geographically proximate (as also observed in previous studies) (Vivas-Toro et al. 2023), several groups consisted of individuals from disparate locations. Ultimately, this implies that these individuals may have been moved (or are the offspring of individuals moved) by people either directly or indirectly. The multiple idiosyncrasies within this dataset warrant further fine-scale investigation towards identifying movement of squirrels as pets or through other human-mediated activities. Ultimately, if the natural diversity of *G. volans* is being confounded by human activity, it will make management actions more challenging.

Grand Osage, Kansas- Across the nuclear analyses, *G. volans* at Grand Osage Wildlife Area stands out as a distinctive group. Not only was the Grand Osage group the only strongly supported clade in the RAxML tree, but the PCA plots used to determine Evolutionarily Significant Units, Management Units, and Adaptive Units all identified Grand Osage as a distinct entity. While the nucleotide diversity of the Grand Osage population was lower than

other localized populations, the species did not have a high inbreeding coefficient (F_{IS}), implying that sufficient gene flow has occurred which allowed the population to maintain genetic diversity. This is in stark contrast to the maternal genetic diversity which was essentially nonexistent (see Chapter 2), implying a role of male-biased dispersal into Grand Osage to supplement the genomic diversity without impacting the mtDNA signal. However little research supports tree squirrels exhibiting male-biased dispersal and Siberian squirrels instead show female-biased natal dispersal (Hanski and Selonen 2009). Based on PopCluster results and F_{ST} values, this dispersal appears to be from individuals aligned with Arkansas and Texas, indicating corridors of dispersal north for the Southwest subspecies. Grand Osage is the only Adaptive Unit distinct from the species at large implying that it is undergoing adaptive responses to environmental conditions at this range edge, supporting the assertion that these regions as important for unique diversity.

Given the evidence presented above, I suggest that one of two events occurred at Grand Osage. One possible event is that the population was founded by members of the Southwest clade which began to diversify in relative isolation until female individuals of the Widespread clade were introduced. In theory, those females could have possessed a significant competitive advantage or higher birth rates than Southwest females, which eventually lead to the complete loss of the Southwest haplotype, though nuclear signals were persevered. Following this, differentiation persisted and any subsequent gene flow came from Southwest individuals. Though possible, the likelihood of such trends is unlikely. The more likely explanation is that through human introduction or natural colonization, a population of individuals in the Widespread clade was established at Grand Osage and was highly isolated from the surrounding region. After generations of adaptive divergence or drift driven by the previously discussed

trends of range edge populations (see Chapter 1), the Grand Osage population diverged from other populations and lost most of its genetic diversity (both mitochondrial and nuclear). Notably, it is rare to observe a population devoid of mitochondrial diversity but which maintains nuclear diversity, given that the higher rate of mitogenome mutation facilitates more rapid mitochondrial evolution (Brown et al. 1979). For the observed nuclear diversity to be present, it follows that infrequent immigration of Southwest males introduced nuclear genetic diversity to Grand Osage while not altering the mitochondrial diversity. The distinction of Grand Osage within the Southwest clade can then be explained by the set of loci that differentiated during past drift or adaptation. Ultimately, more research is necessary to further clarify the specific dynamics at play at Grand Osage, but it provides a distinctive and uncommon trend in population genetics.

The Rest of Kansas- Beyond Grand Osage, Kansas harbors interesting genetic diversity and specialization. The Quivira individual is highly similar to the Southwest subspecies while the northern individual is strongly associated with Nebraska individuals based on both PCA and phylogenetic topologies. Beyond these populations, the individual in the southeastern corner of Kansas appears to be more intermediate between these two clusters in the PCA plot, reflective of its intermediate geographic locality. While the exact cause of this differentiation is challenging to parse out (Lawson et al. 2018), the distinction is likely associated with genotypes distinct from the Southwest clade at large which is likely driven by local adaptive drivers. While diversity is present across the *G. volans* range, Kansas appears to be distinct from these in the extent and depth of this diversity. As such, Kansas exhibits arguably higher genomic complexity within *G. volans* than anywhere else in eastern North America, as is expected at a range edge, that warrants particular attention from managers and conservationists.

Conclusions- The proposed restructuring of subspecies based on mitochondrial lineages remains supported by the nuclear evidence. However, substantial gene flow (likely natural and human-mediated) provides confounding evidence across locus sets and may indicate that a more holistic approach to species management is needed in addition to regional management. At the western range edge of *G. volans*, previously unexplored genetic diversity, adaptive populations, and changes in regional subspecies ranges are apparent. Considering the changing landscape on the western edge, extra management attention should be paid to this region and ensuring regional connectivity is preserved. Ultimately, the history of *G. volans* coupled with recent demographic trends reflect a species under population flux. The species' strong associations with humans further confound evolutionary trajectories but also provides an avenue through which the public can be encouraged to engage and support expanded conservation of forests, both for *G. volans* and other flora and fauna that occupy those habitats.

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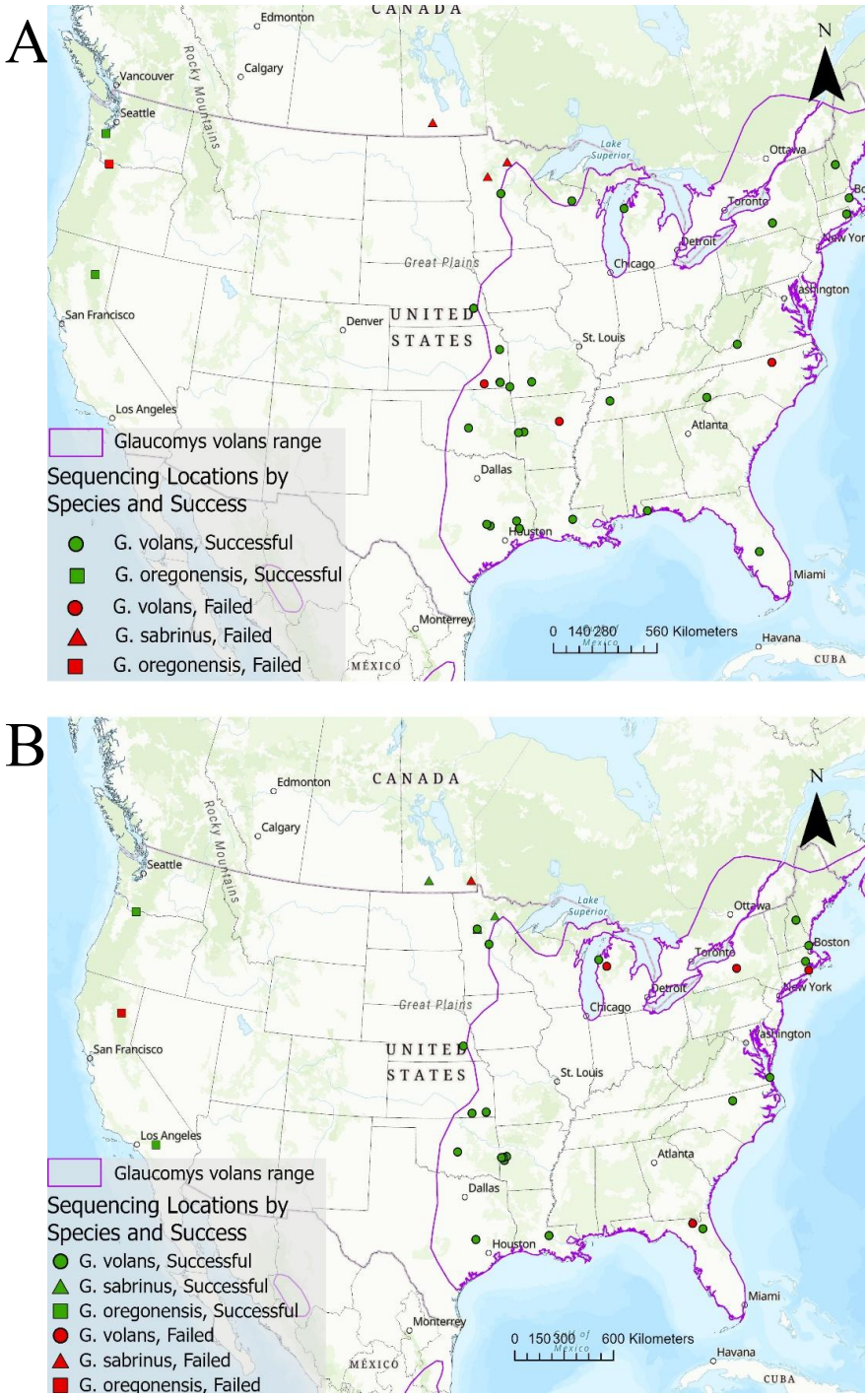
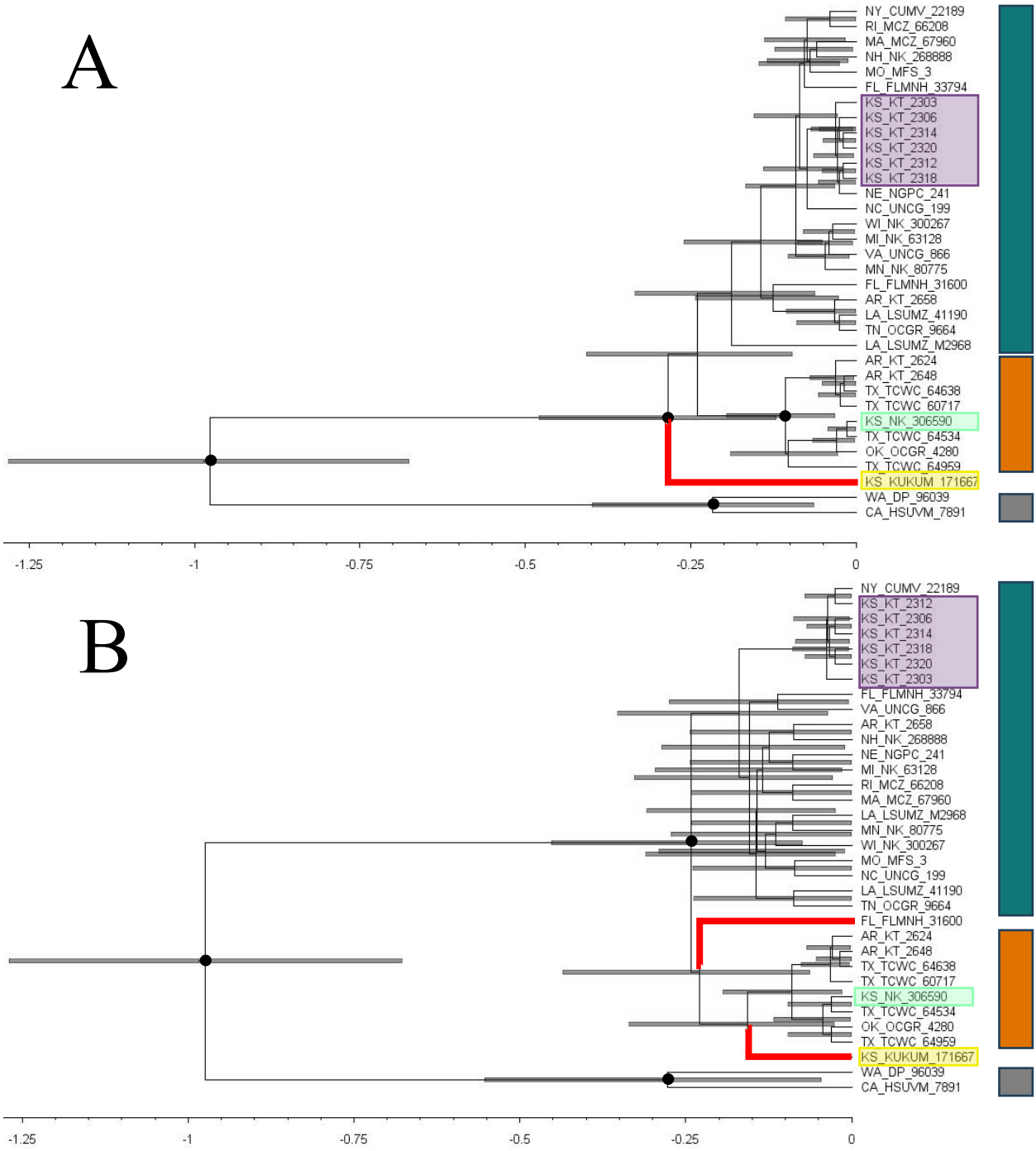
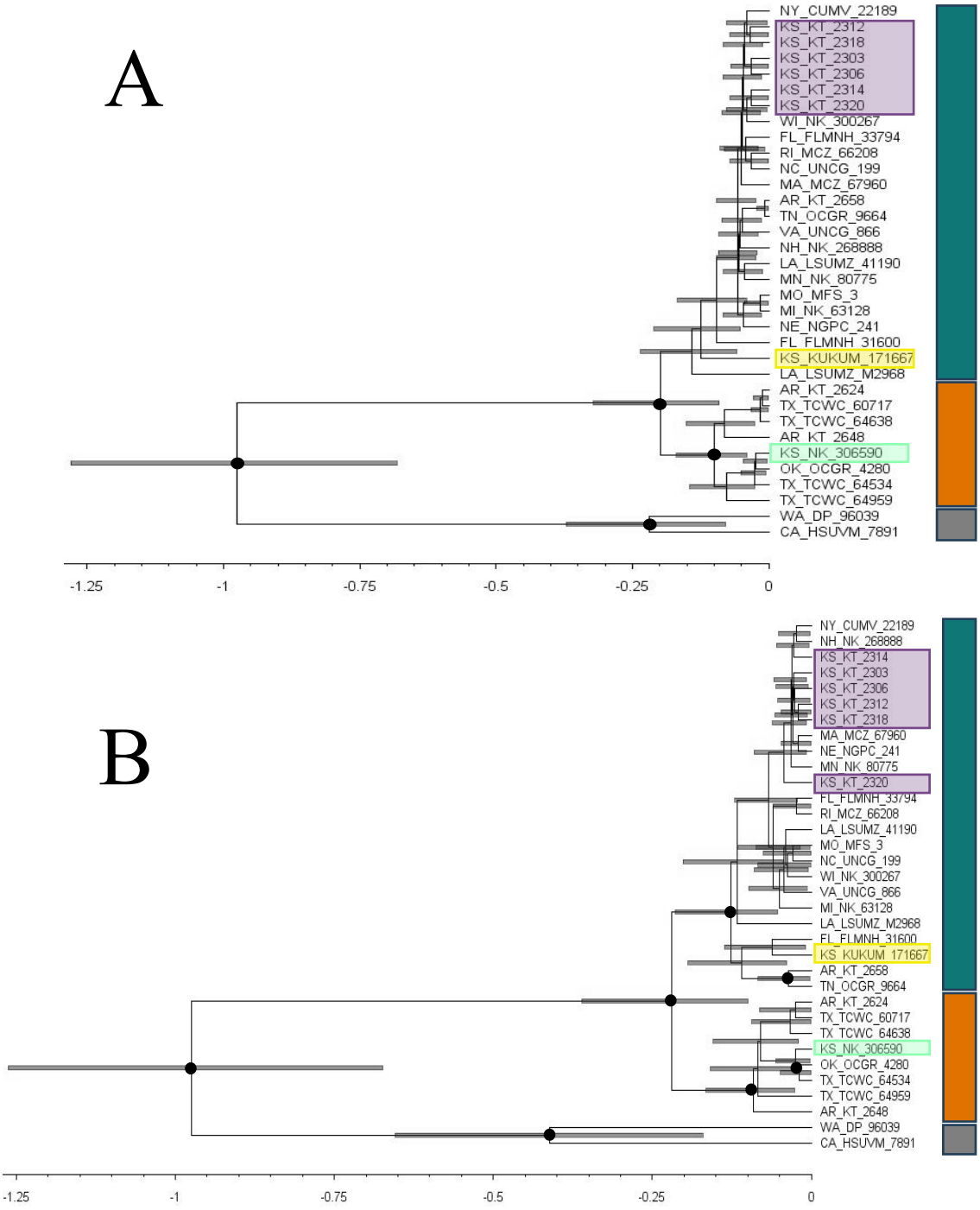
Appendix A - Chapter 2 Supplementary Figures



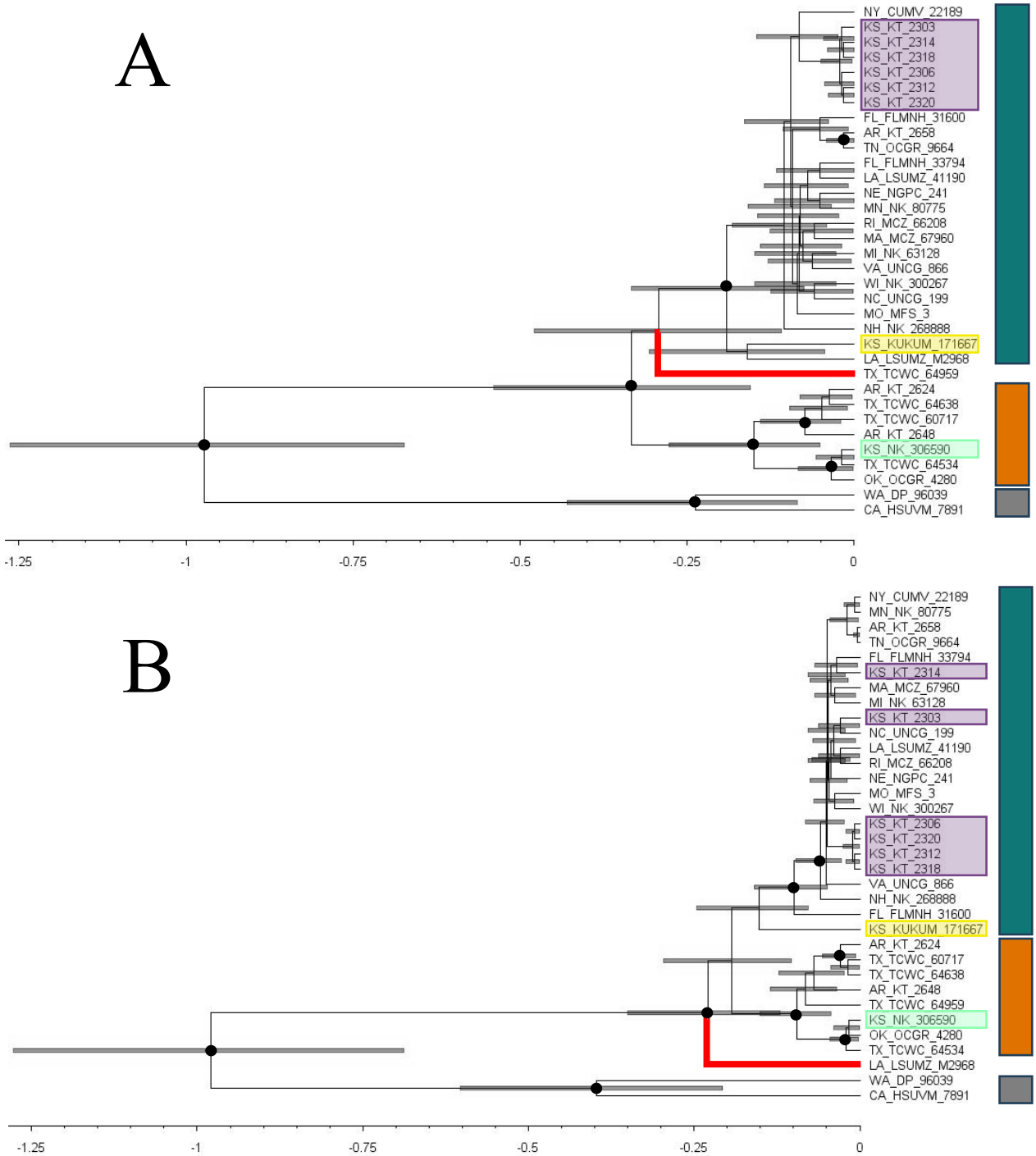
Appendix Figure A.1 Mitochondrial sequencing sample locations and sequencing results from A) Nanopore MinION mitogenome sampling and B) Sanger Cytochrome-b sampling.



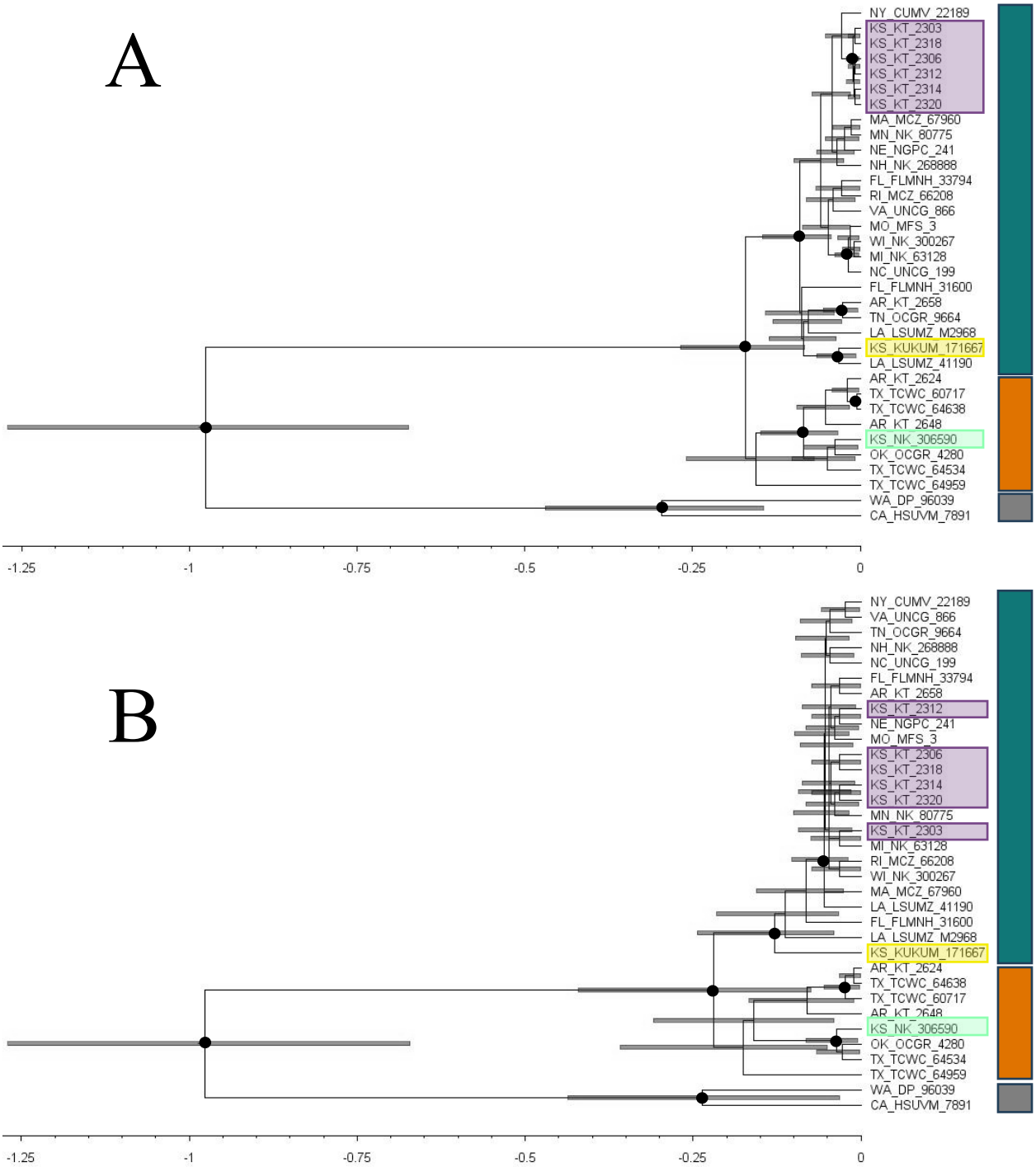
Appendix Figure A.2 Bayesian phylogenetic trees based on (A) ATP6 and (B) ATP8 protein-coding genes. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age. Red branches indicate samples that have a significantly different topology from the mitogenome tree.



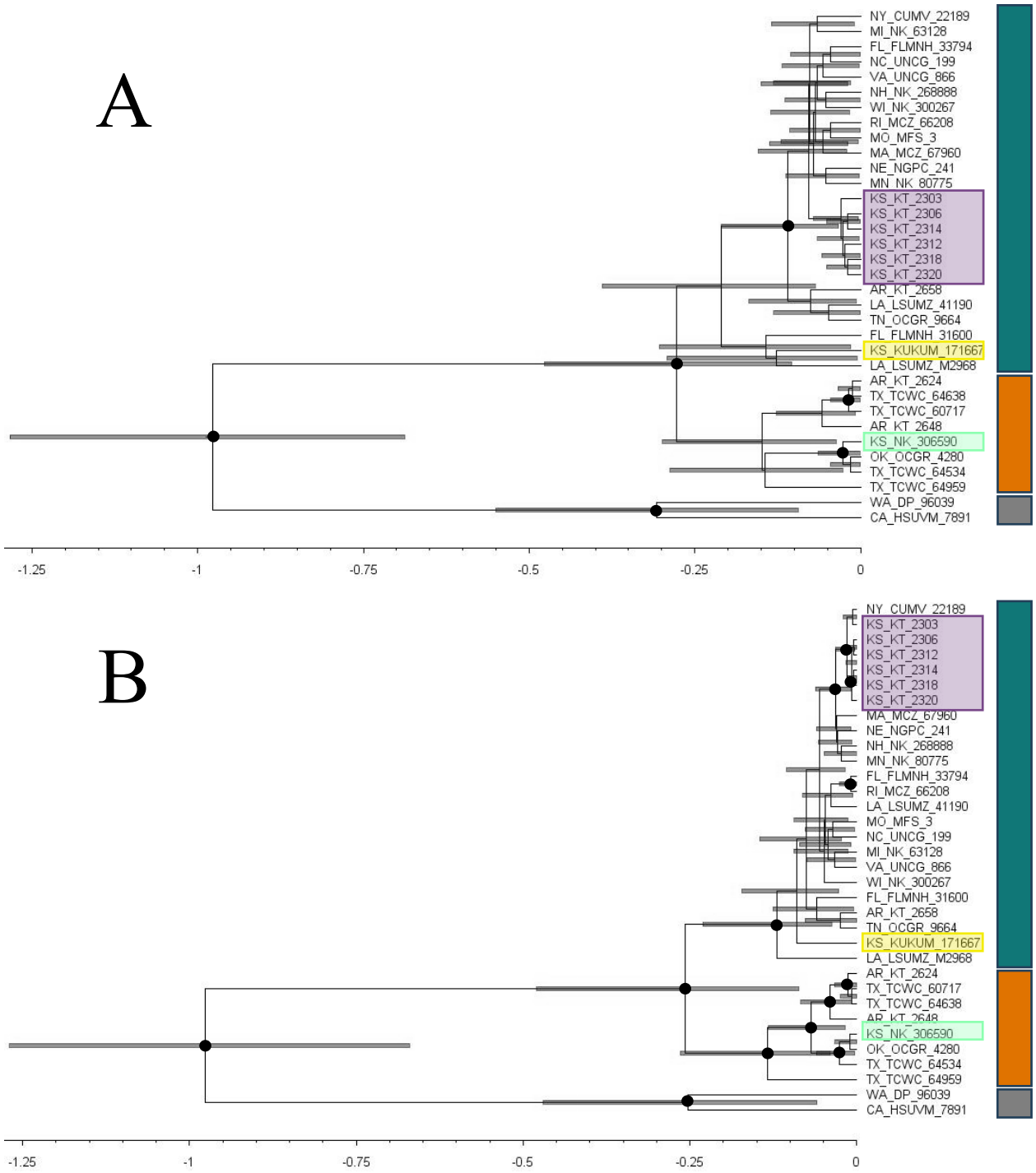
Appendix Figure A.3 Bayesian phylogenetic trees based on (A) COX1 and (B) COX2 protein-coding genes. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age.



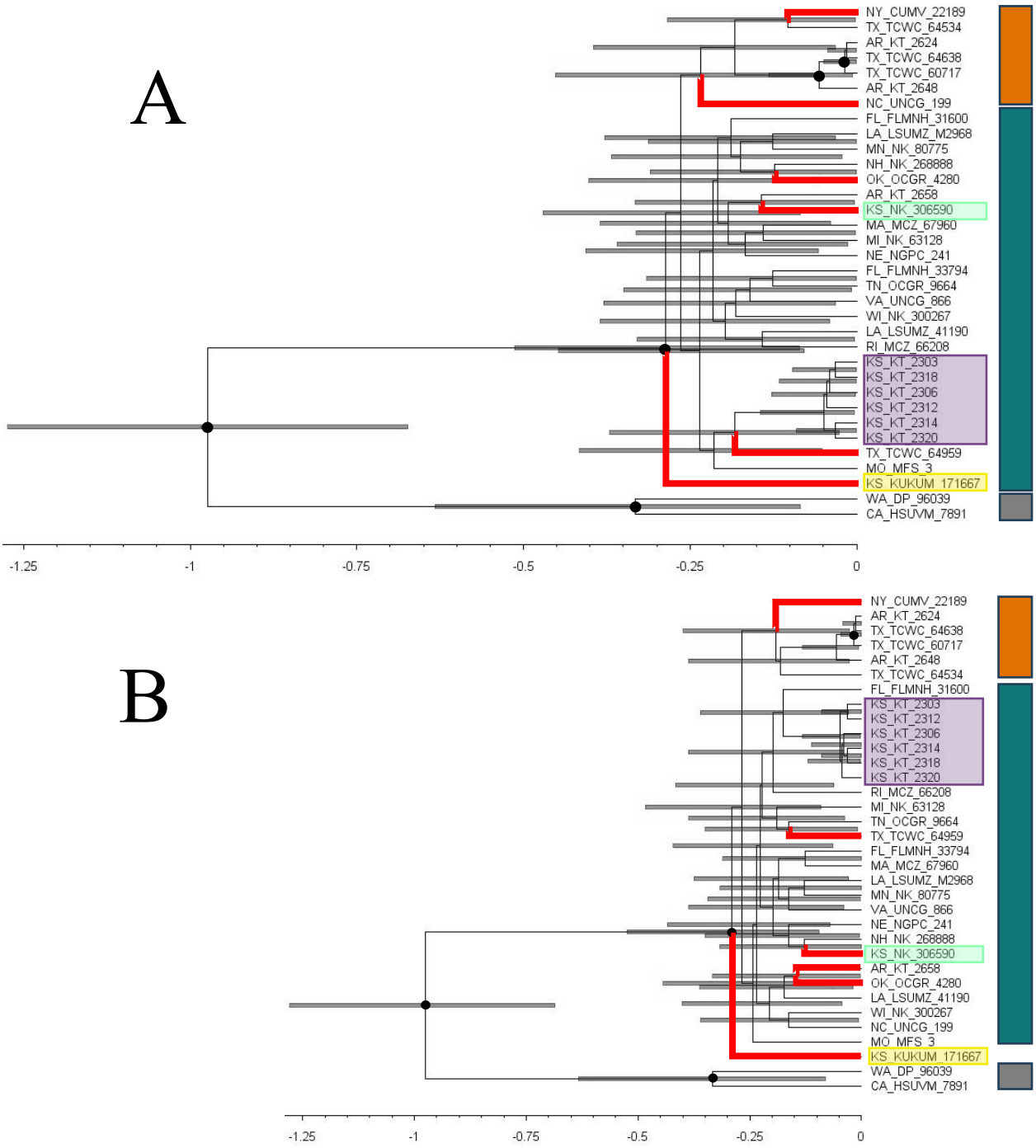
Appendix Figure A.4 Bayesian phylogenetic trees based on (A) COX3 and (B) Cyt-b protein-coding genes. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age. Red branches indicate samples that have a significantly different topology from the mitogenome tree.



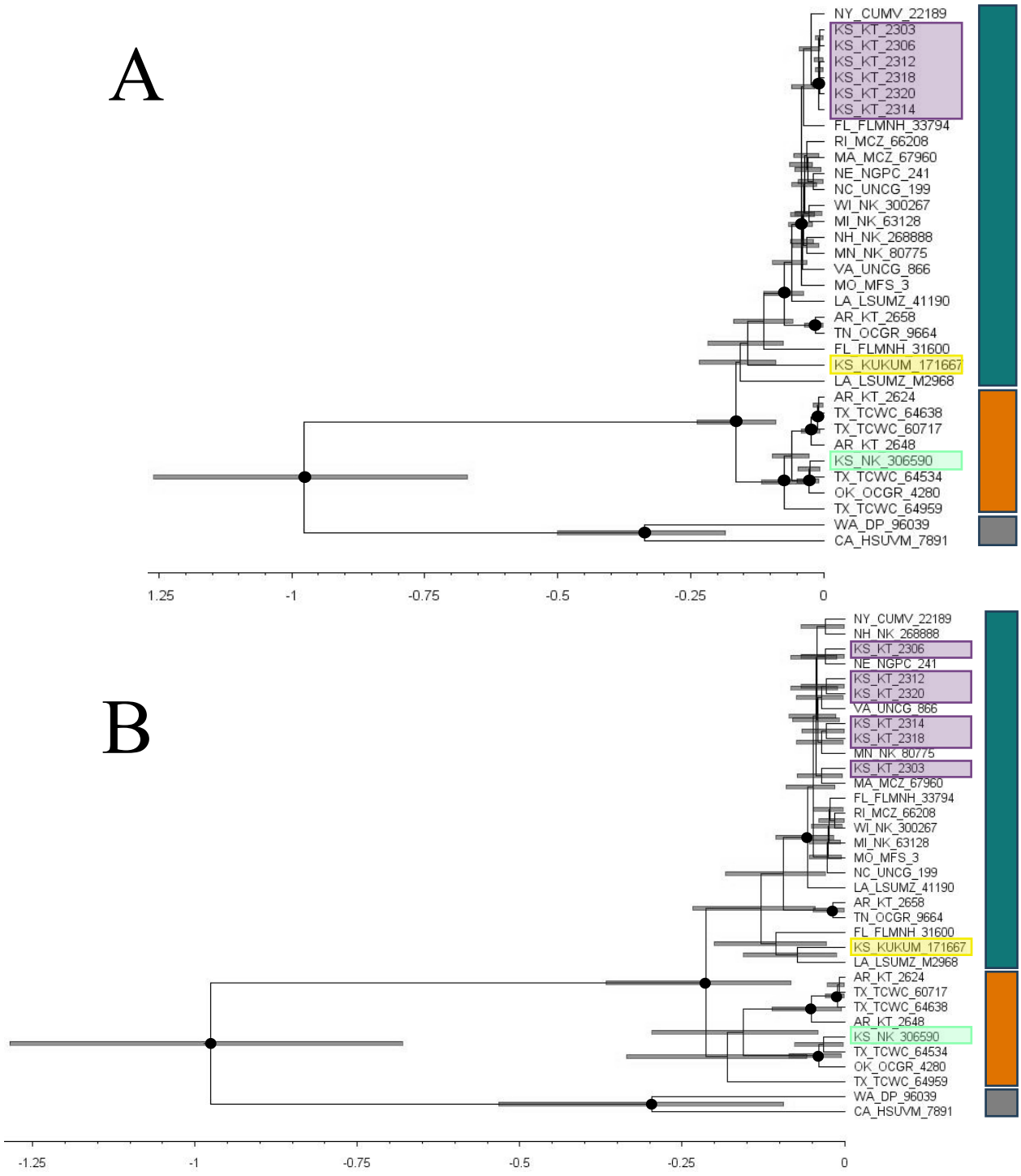
Appendix Figure A.5 Bayesian phylogenetic trees based on (A) ND1 and (B) ND2 protein-coding genes. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age.



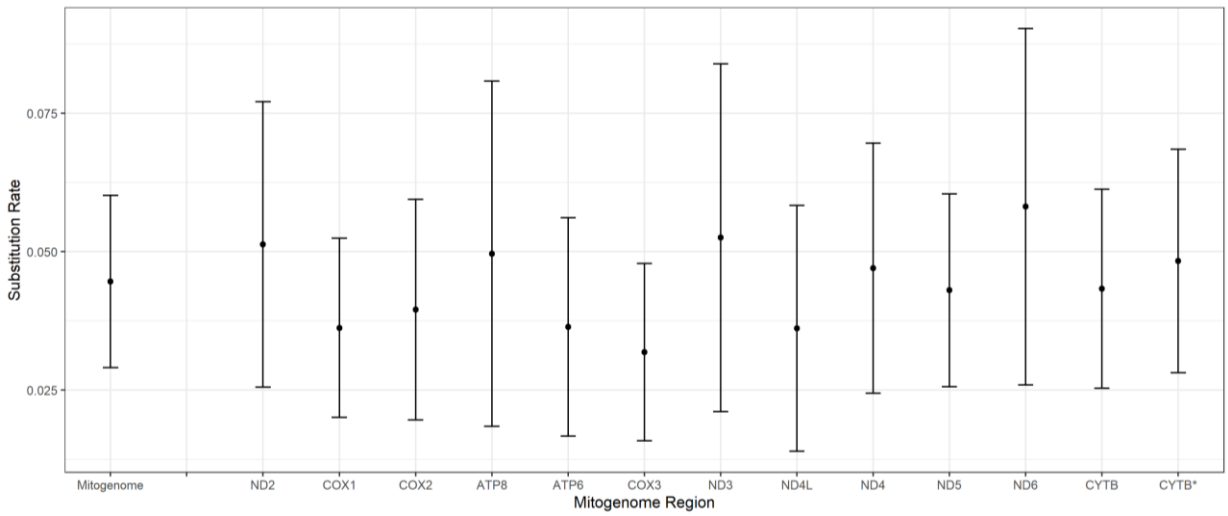
Appendix Figure A.6 Bayesian phylogenetic trees based on (A) ND3 and (B) ND4 protein-coding genes. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age.



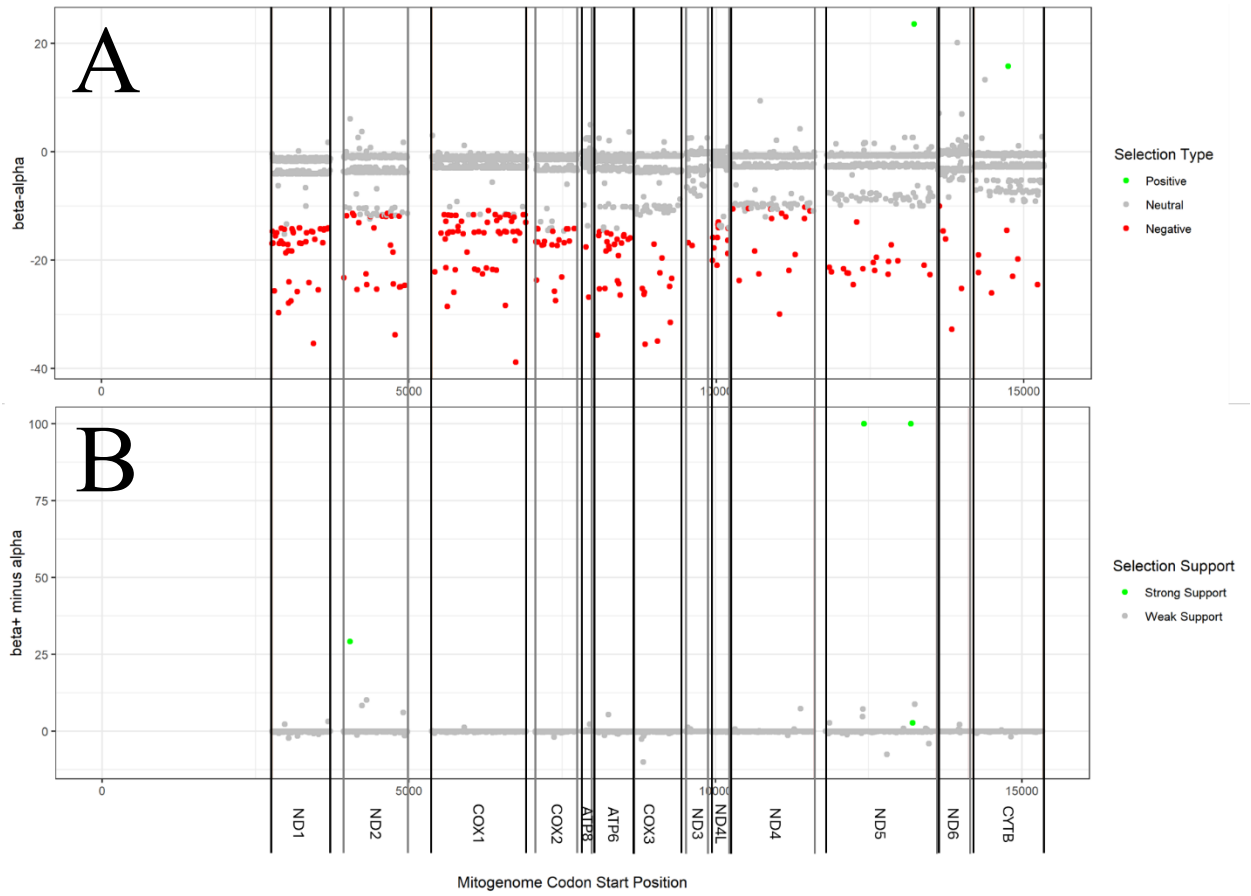
Appendix Figure A.7 Bayesian phylogenetic trees based on ND4L protein-coding genes using (A) original and (B) reverse complement sequences. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age. Red branches indicate samples that have a significantly different topology from the mitogenome tree.



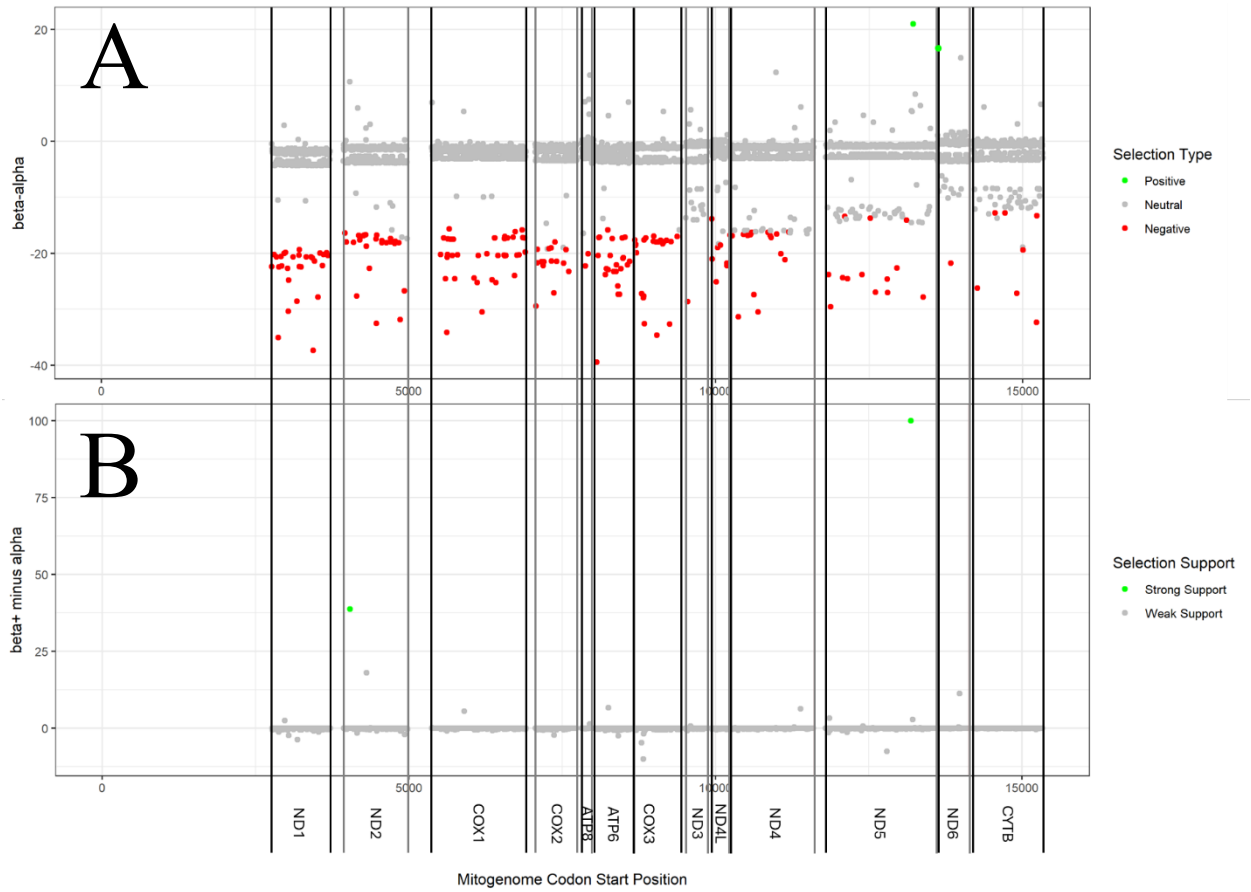
Appendix Figure A.8 Bayesian phylogenetic trees based on (A) ND5 and (B) ND6 protein-coding genes. Vertical bars indicate clades of the outgroup (grey), Widespread (teal), and Southwest (orange). Kansas individuals are highlighted based on locality as southeast corner (green), northernmost site (yellow), and Grand Osage (purple). Tree bifurcations with posterior probabilities of at least 0.95 are indicated with black dots at the node. Horizontal node bars indicate 95% confidence intervals of node age.



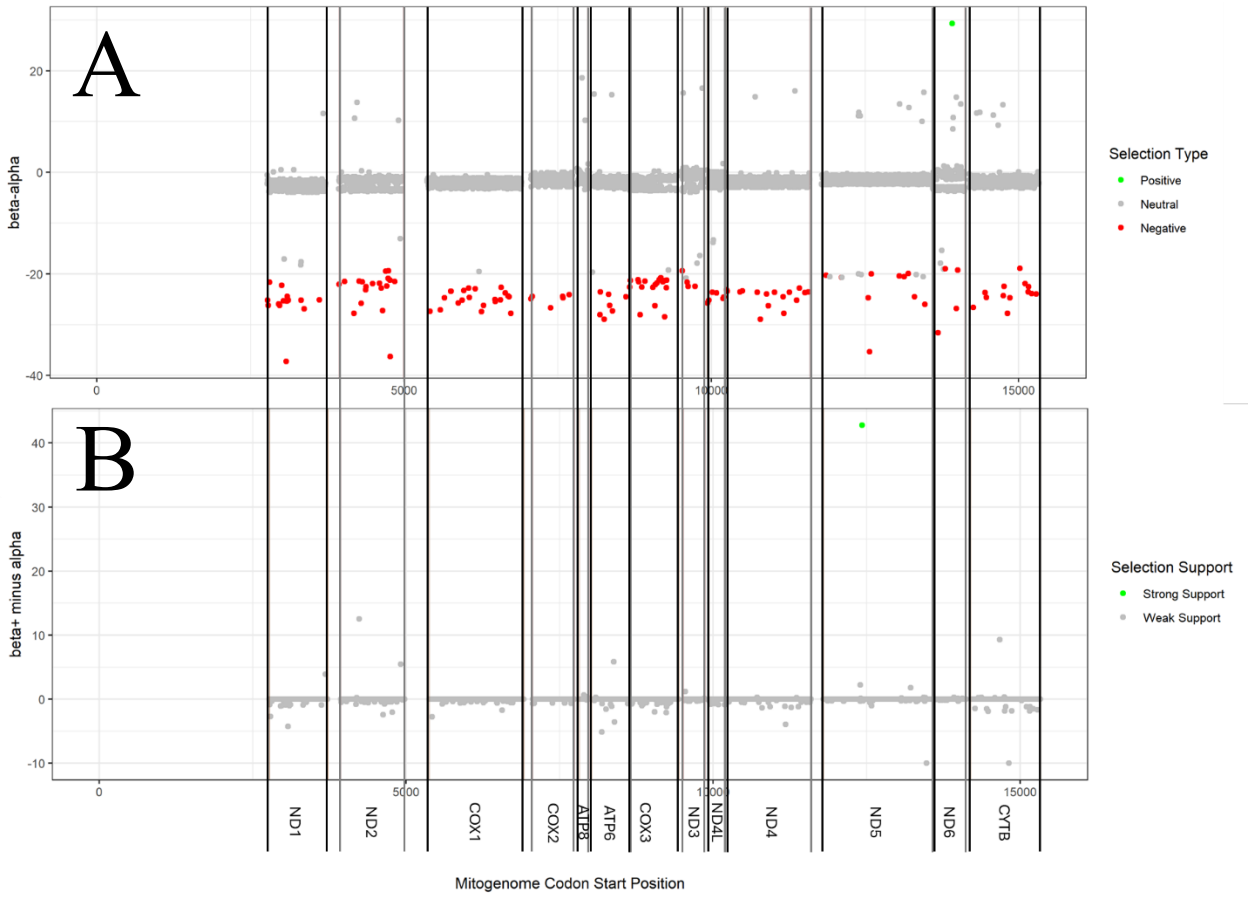
Appendix Figure A.9 Mutation rates (as number of mutations per site per million years) of the mitogenome (far left) and coding regions in order of position in mitogenome. CYTB* indicates the estimation with Sanger sequences included. Error bars indicate two standard deviations from the estimated mutation rate.



Appendix Figure A.10 Visual representation of selection along the mitogenome based on (A) FUBAR and (B) MEME analyses on a *G.volans* range-wide dataset. Y-axes are approximations of selection strength but are not precise.

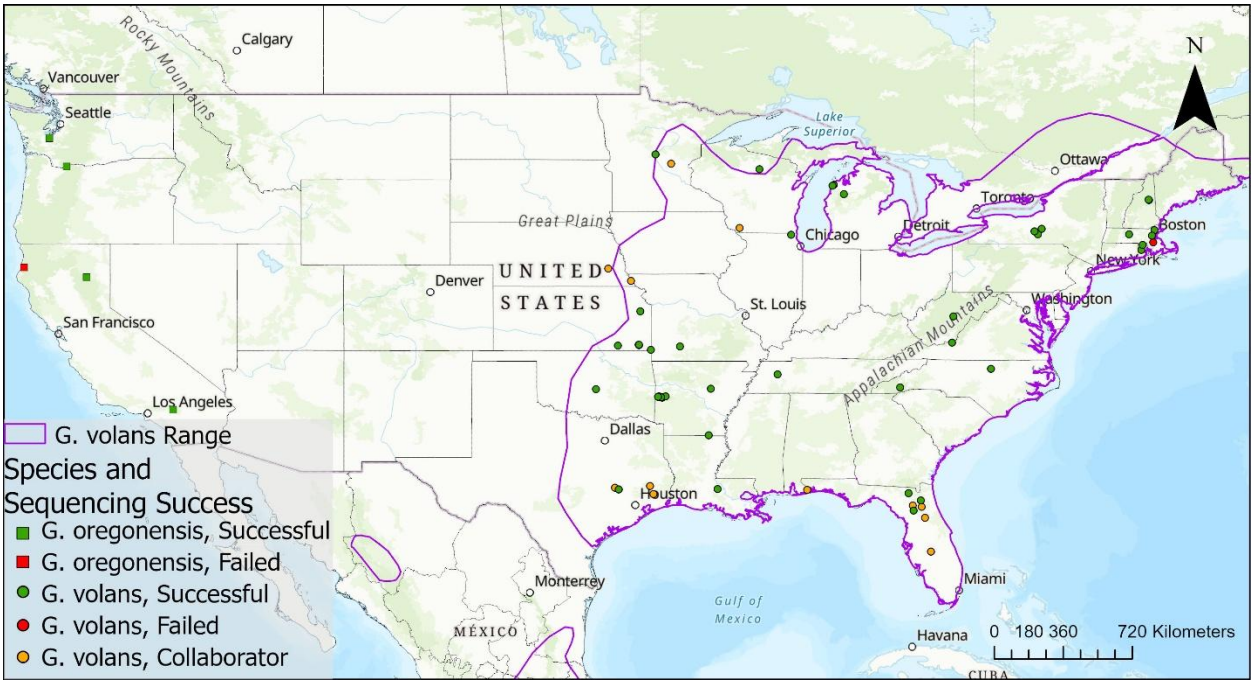


Appendix Figure A.11 Visual representation of selection along the mitogenome based on (A) FUBAR and (B) MEME analyses on a *G.volans* Widespread clade dataset. Y-axes are approximations of selection strength but are not precise.

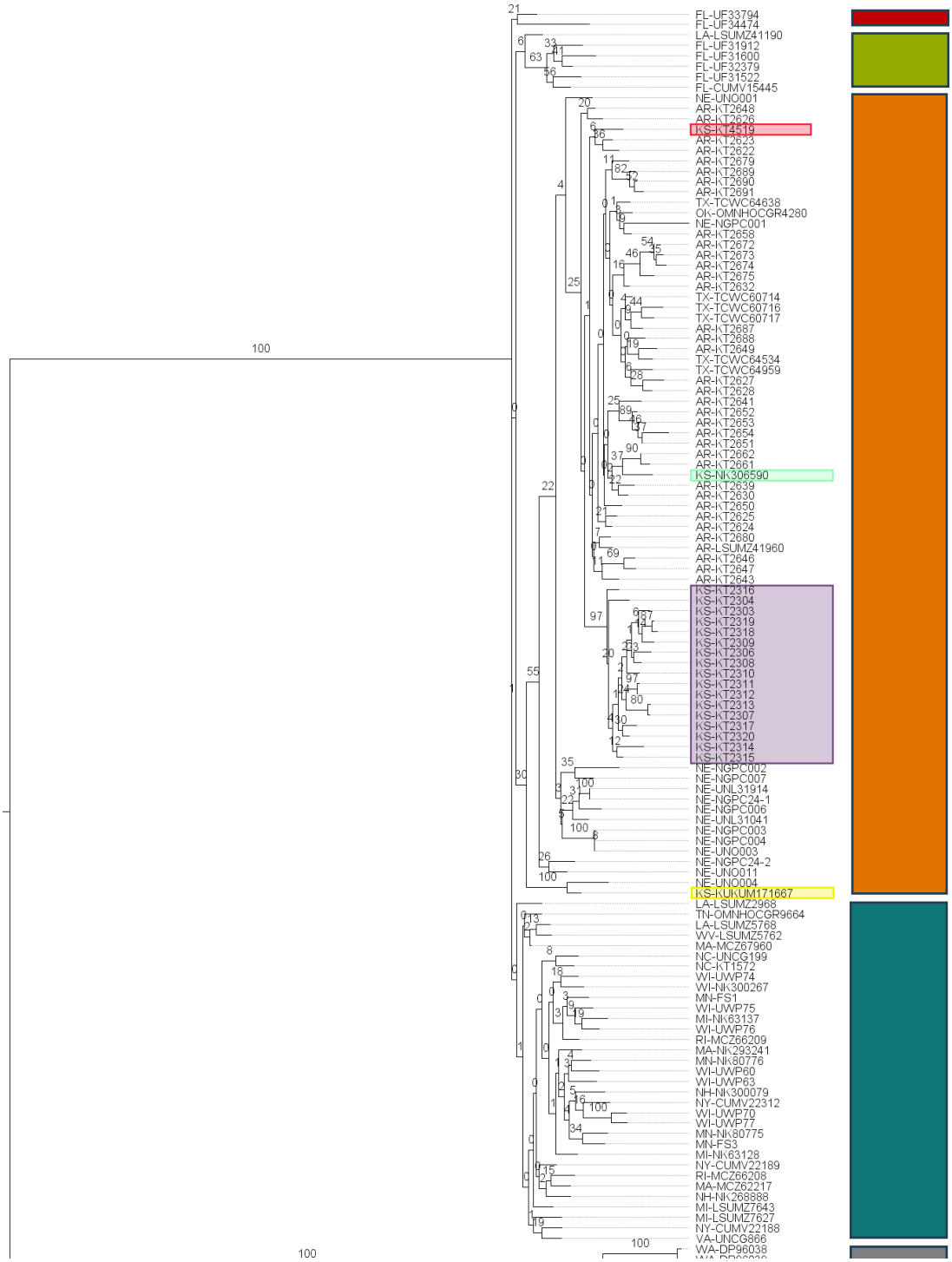


Appendix Figure A.12 Visual representation of selection along the mitogenome based on (A) FUBAR and (B) MEME analyses on a *G.volans* Southwest clade dataset. Y-axes are approximations of selection strength but are not precise.

Appendix B - Chapter 3 Supplementary Figures



Appendix Figure B.1 Map of *Glaucomys* sampling localities and ddRADseq sequencing success (green), failure (red), or third-party acquisition (orange).



Appendix Figure B.2 RAxML phylogeny of the complete *Glaucomys* (*G. volans* and outgroup *G. oregonensis*) dataset showing all bootstrap values (numbers along branches). Vertical bars represent proposed clades as Basal Florida (dark red), Southeast (green), Southwest (orange), Hodgepodge (teal), and *G. oregonensis* outgroup (grey). Kansas samples are highlighted base on locality at western Quivira Boy Scout Ranch (red), the southeastern state corner (green), Grand Osage Wildlife Area (purple), and northern Kansas (yellow).