Efficacy of Non-lethal Molecular Methods in Elucidating Distribution of Gray Treefrog Complex (*Hyla chrysoscelis/versicolor*) in Kansas

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ABSTRACT

Globally, amphibians are the most imperiled vertebrate taxa in part because they rely on both aquatic and terrestrial ecosystems. Specifically, their permeable skin makes them uniquely susceptible to habitat degradation and alteration. Cope's Gray Treefrog (*Hyla chrysoscelis*) and the Gray Treefrog (*Hyla versicolor*) are a diploid-tetraploid, morphologically indistinguishable sister pair of cryptic anurans native to Kansas. Since 1987, the distribution of gray treefrogs in Kanas has extended west but the status of each species in the complex in Kansas is not known beyond its documented combined western expansion. Currently, species identification cannot be determined by nonlethal techniques. Consequently, which species or if species remain in sympatry across the expanding range has not been determined. Therefore, the objectives of this research were to determine 1) the updated range distribution for both species that comprise the gray treefrog complex in Kansas, 2) to determine if mitochondrial DNA can be used to distinguish the two species in the complex, and 3) establish a non-invasive sampling technique that can be useful in future studies of amphibian populations. Results of this study indicate cytochrome b was not a useful molecular marker to distinguish between the two species in the gray treefrog complex. Consequently, the status of individual species distributions remains unknown. Buccal swabs were effective for collection of mtDNA even when stored at room temperature for up to a week.

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# INTRODUCTION

All organisms rely on the biodiversity and healthy structure and functioning of natural systems. Biodiversity has direct, positive effects on ecosystem services such as nutrient cycling, carbon storage, climate regulation, water quality, and pollination (Balvanera et al., 2006). Ecosystem services support local and global economies and are conservatively estimated to be worth $145 trillion per year (Costanza et al., 2014). Biodiversity at local, regional, and global scales provides food security, protection from pathogens and infectious disease, and pest reduction (Chivian, 2002). Furthermore, healthy ecosystems have been foundational to the innovative history of modern medicine (Chivian, 2002).

The current rates of extinction across all taxonomic groups is worrisome given that humans and other life rely on biodiversity and the associated structure and functioning of ecosystems. Current extinction rates are above background extinction rates, and the magnitude of the biodiversity crisis is extensive (Singh, 2002). Of all terrestrial vertebrate taxa, amphibians are the most imperiled. Forty-one percent of known amphibian species are threatened (IUCN, 2020). Amphibians are vital to the proper functioning of ecosystems globally and contribute to trophic interactions in both terrestrial and aquatic ecosystems (Regester et al., 2006). Additionally, amphibians are consumers of arthropods and serve as a food source for predators in higher trophic levels (Hopkins 2007). Accordingly, the unprecedented loss of amphibians are a great risk to biodiversity, the health of ecosystems, and human communities globally (Stuart et al., 2004).

The threats that amphibians face and their declines are context dependent (Blaustein & Kiesecker, 2002), complex, and remain misunderstood (Green et al., 2020). Amphibians are highly susceptible to environmental changes due to the permeability of their skin and their

reliance on both aquatic and terrestrial ecosystems (Quaranta et al., 2009). Documented threats to amphibians include chytridiomycosis (Skerratt et al., 2007), climate change, pollution, habitat loss and degradation, invasive species (Gibbons et al., 2000), and UV-B radiation (Blaustein et al., 1994). These threats to amphibian populations seldom occur in isolation (Blaustein & Kiesecker, 2002), and compound projected losses in amphibian diversity (Hof et al., 2011).

To conserve amphibians, a better understanding of habitat associations and their role in broader ecosystems is essential. Barriers to successful amphibian conservation include gaps in our understanding of species distributions at local, regional, and global scales, as well as population dynamics and species interactions (Hortal et al., 2015). There is a need for more research on local population demographics (Grant et al., 2020), as well as information on spatial distribution of amphibians (Hof et al., 2011).

Additional barriers to amphibian conservation include outdated assessments of conservation status for many species and insufficient data to determine conservation status (IUCN, 2020). This underscores two important points: first, demographic information must be determined while there are ample population sizes, and second, non-lethal or minimally invasive sampling techniques need to be employed to fill the gaps in our understanding, particularly for use in vulnerable populations. Rare and endangered species frequently are the focus of conservation efforts because of the need for urgent conservation action. However, there is merit in studying common species, because they generally comprise a higher proportion of individuals in assemblages (Gaston, 2008), and provide for a baseline of comparison to future population conditions. Abundant species might influence an ecosystem disproportionately more than rare species, and loss of an abundant species can have severe consequences (Gaston, 2008).

The Gray Treefrog (*Hyla versicolor*; Le Conte, 1825) and Cope's Gray Treefrog (*Hyla chrysoscelis*; Cope, 1880) [*Dryophytes = Hyla;* Duellman et al., 2016] are a diploid-tetraploid species complex native to the eastern United States and portions of southern Canada. *Hyla chrysoscelis* is diploid and possesses 12 pairs of chromosomes, and *Hyla versicolor* is tetraploid with 24 pairs (Wasserman, 1970). Commonly known as the gray treefrog complex, *H. versicolor* and *H. chrysoscelis* are morphologically indistinguishable. They are both 3.2 - 5.7 cm snout vent length (SVL) with gray to green mottled skin (Ralin, 1968), but these measurements are not useful for field identification. Both *H. versicolor* and *H. chrysoscelis* are cryptic and change their skin color to match their environment. Like other treefrogs, they have adhesive toe pads and feed on small insects. The two species are distinguishable by nucleus size (Cash & Bogart, 1978) and call (Johnson, 1959). In the past, nucleolar number was used to distinguish the two species (Cash & Bogart, 1978) but has since been discredited as a principal means of identification (Keller, 2000).

Similarities between the gray treefrogs also include many natural history characteristics. The two species share identical physiological responses to freezing across multiple populations from broad geographic areas (Irwin, 2003). Additionally, the gray treefrogs appear to share the same habitat preferences for forested areas near wetlands that provide adequate overwintering sites, foraging habitat, and refugia (Johnson et al., 2008; Pittman et al., 2008). Site fidelity is high in *H. chrysoscelis* during the non-breeding period (Pittman et al., 2008), but *H. versicolor* tends not to have strict breeding-pond fidelity (Johnson & Semlitsch, 2003).

*Hyla versicolor* and *H. chrysoscelis* call during the breeding season, which extends from March through August. *Hyla chrysoscelis* has a higher call pulse rate than *H. versicolor* (Gerhardt & Doherty, 1988), and the trill rates for both species are dependent on both ambient

temperature and body temperature (Mitchell & Pague, 2011). There has been debate over whether there is any overlap of call properties for these two species, with Gerhardt (1982) suggesting that the pulse rate of *H. versicolor* at higher temperatures would be comparable to the pulse rate of *H. chrysoscelis* at a lower temperature. Mitchell & Pague (2011) determined that there is no overlap in call characteristics based on body size or ambient temperature. Given these contrasting views, call characteristics between *H. versicolor* and *H. chrysoscelis* should not be the sole means for identification between the two species. Furthermore, only male frogs call during the breeding season at the appropriate time of day. Females, juveniles, and males during the non-breeding season do not call.

In the gray treefrog complex, mate choice is made solely by advertisement call for both *H. chrysoscelis* and *H. versicolor* (Gerhardt, 2001), and is evolutionarily and functionally complex (Schul & Bush, 2002). The possibility of heterospecific mate choice is most likely when a warm *H. versicolor* is in the same pond as a cool *H. chrysoscelis*, and it has been suggested that differences in call structure and female preference are likely not strong enough to completely eliminate mismatched pairs (Gerhardt, 2005). Gerhardt et al. (1994) documented a single naturally occurring individual with an intermediate call frequency and suggested it was evidence of hybridization. Historically it was thought that hybridization did not result in viable offspring (Johnson 1959), and there was evidence for selection against *H. versicolor* × *H. chrysoscelis* hybrids (Gerhardt et al., 1994). However, recent studies indicate that hybridization may occur rarely in sympatric populations (Bogart et al., 2020).

The origin of polyploidy in the gray treefrog complex has been the subject of study for decades and has been contentious (Ptacek et al., 1994; Holloway et al., 2006; Bogart et al., 2020). Holloway et al. (2006) concluded that the tetraploid originated from allopolyploidy

through limited interbreeding among extant and extinct treefrog species and subsequent occasional gene flow between these groups. Bogart et al. (2020) provided an alternative explanation and evidence to support tetraploid formation via autopolyploidy. Evidence that supports these alternative explanations of tetraploid origin included combinations of advertisement calls, mitochondrial and nuclear genes, and isozymes (Holloway et al., 2006; Bogart et al., 2020)

Sequence variation in the mitochondrial cytochrome b (cyt b) gene across the range of the gray treefrog complex has been documented (Ptacek et al., 1994; Holloway et al., 2006; Bogart et al., 2020). Bogart et al. (2020) found that mitochondrial DNA (mtDNA) appeared to separate *H. versicolor* and *H. chrysoscelis* into respective clades, and sympatric populations of *H. chrysoscelis* and *H. versicolor* did not share mtDNA haplotypes. These results suggested that differences in cyt b sequences might be sufficient to distinguish the two species. However currently, determining chromosome number via karyotype is the only definitive means of identification within this complex (Wasserman, 1970), a technique requiring the animals be euthanized.

Hillis et al. (1987) conducted the most recent study of distributions of the gray treefrog complex in Kansas. Karyotypes were used to identify 108 gray frogs; eighty-five of the frogs were diploid (*H. chrysoscelis*) and 23 were tetraploid (*H. versicolor*). *Hyla chrysoscelis* occurred throughout a majority of the eastern third of the state and occupied areas farther west, ranging to the Flint Hills region (Figure 2, reconstructed from Hillis et al., 1987). *Hyla versicolor* occurred in the southeastern-most corner of the state, along the Kansas-Missouri border and in a small area within the Chautauqua Hills. No individuals of either species were documented in the southern Flint Hills. Since1987, gray treefrogs have extended their range westward (Collins et

al., 2010), possibly along riparian corridors. Documentation of range expansion includes voucher specimens deposited in the Herpetology Collection at the Sternberg Museum of Natural History in Hays, Kansas. Though not identified by karyotype, collections included traditional whole individuals, as well as liver and muscle tissue stored in 95% ethanol.

Previous studies on *H. chrysoscelis* and *H. versicolor* in Kansas have used lethal sampling methods to karyotype each individual (Collins & Hillis, 1985; Hillis et al. 1987). Considering the global amphibian decline, as well as ethical concerns of whole individual sampling, moving away from lethal and highly invasive sampling techniques should be a priority (Perry et al., 2011). Furthermore, establishing reliable, efficient, noninvasive sampling methods for a variety of amphibian families allows populations to be sampled more frequently, and standardized methods can be applied to a broader range of species including those of conservation interest. Clipping toes of frogs is a non-lethal method of collecting genetic material but can cause infection, reduce balance and mobility, and lower survival, particularly for treefrogs that use toe pads and disks to climb (McCarthy & Parris, 2004). Although toe clipping is considered ethical and necessary for certain studies (Perry et al., 2011), in terms of individual pain, stress, and likelihood of future mortality, alternatives to toe clipping should be considered in scientific studies involving amphibians (Parris et al., 2010).

Swabbing portions of an amphibian to collect cells is an alternative to whole tissue collection for DNA testing. Skin swabbing is a technique that reduces handling time and individual stress, but results vary based on anatomical differences among amphibian species, DNA contamination resulting from different modes of amplexus, or inconsistent sample processing and storage (Prunier et al., 2012; Müller et al., 2013; Pichlmüller et al., 2013; Ringler, 2018). Buccal swabs have been used to collect DNA samples in turtles, salamanders, true frogs,

and toads (Poschadel & Möller, 2004), newts, and European tree frogs (Broquet et al., 2007). Swabbing protocols should aim to reduce injury and stress to the frog, while also collecting sufficient cell density (Pidancier et al., 2003).

The distribution of the gray treefrog complex in Kansas is unknown beyond its documented western expansion. Species identification across seasons cannot be determined by nonlethal techniques. Consequently, it is unknown if one or both species in the gray treefrog complex have expanded their range or if the species remain in sympatry in just a portion of the expanding range. Therefore, the objectives of this research were 1) to determine the updated range distribution for both species in the gray treefrog complex in Kansas, 2) to determine if cytochrome b can be used to distinguish the two species, and 3) to establish a viable, noninvasive sampling technique for genetic material that can be useful in future studies of amphibian populations. Establishing molecular methods with common species, like gray treefrogs that are of least conservation concern, reduces risk of testing methods on threatened or endangered populations. Non-lethal molecular methods allow for flexibility in sample type, as both fresh tissues and museum vouchers can be used when the latter are preserved in ethanol. The same protocol for DNA collection can be applied to tissues and buccal swabs as an alternative to whole individual or tissue collection. It is hypothesized that cyt b will be effective in differentiating between the two species in the complex, and will help determine ranges for the two species in Kansas.

# METHODS

## *Field Collection*

Sample areas were defined by the distribution of documented vouchers of the gray treefrog complex (Taggart, 2022), and the voucher localities tissue collections at the Sternberg Museum of Natural History, Hays Kansas. Sample sites were selected to address the apparent gaps in the distribution of vouchers and with emphasis on the western edge of the expanding range (Figure 1). Cryptic frog species are most easily detected during the breeding season, when males call. Searching and sampling gray treefrogs spanned May – August 2021 to coincide with the gray treefrog breeding season. Sampling efforts included opportunistic searches in eastern and east-central Kansas using visual surveys and calls. To overcome some of the challenges of obtaining access to private land, I recruited citizen scientists via social and professional networks in regions where gray treefrogs were documented or historically present.

Voucher specimens were collected at locations outside the documented range to verify presence at specific time and place (Clemann et al., 2014). Individuals were euthanized and preserved according to IACUC (#21-013), and in compliance with state scientific collection permits. Toe clips were collected after voucher specimens were processed for museum deposition.

## *DNA Sampling*

Individually packaged sterile cotton swabs and Eppendorf tubes were used to collect and store buccal samples and toe tissues. A sterilized blunt metal spatula was used to open the mouth of a frog, a cotton swab was inserted in the buccal cavity, and gently rotated until the swab was saturated. The buccal swabs were placed into dry, sterile tubes and labeled with a unique

identification number. A toe clip from the same individual was placed into a sterile tube filled with 95% ethanol and corresponding identification number. After each individual was sampled, all metal instruments were flame sanitized. Attempts were made to minimize handling time to reduce stress on the frogs.

Metadata collected for each sample included the date, ambient temperature, and GPS location. Samples were transported or shipped at ambient temperature until arrival on campus. Upon arrival, samples were frozen at -18°C until they were processed. This protocol was used because historically keeping dry samples (not stored in ethanol) at ambient temperature has caused no adverse effect on DNA extraction success; however, previous studies only kept swabs at ambient temperature for one to eight hours (Broquet et al., 2007). Some swabs in this study were maintained at ambient temperature for multiple days and up to a week due to logistic constraints in the field or as the result of shipping times. Buccal swabs were stored long term (~ 6 months) at -18°C prior to extraction and toe clips were stored at room temperature in the same ethanol solution used at the time of collection.

*DNA Extraction, Amplification, and Sequencing*

The mitochondrial cytochrome b (cyt b) gene was targeted to distinguish between *H. versicolor* and *H. chrysoscelis.* Cyt b was selected because it was used successfully in previous investigations of the evolutionary history of the gray treefrog complex (Bogart et al., 2020; Holloway et al., 2006; Ptacek et al., 1994), and with other amphibians (Pidancier et al., 2003). These studies indicated that there was variability in cyt b between species, and that different populations had different haplotypes (Ptacek et al., 1994; Bogart et al., 2020).

DNA was extracted from toe clips and museum tissues by using a DNeasy Tissue Kit (QIAgen) following the manufacturer's protocol. DNA was extracted from buccal swabs by using the same kit with initial overnight incubation in 280 µl ATL (instead of 180µl ATL) followed by use of a QIAShredder (QIAgen) after incubation according to manufacturer's protocol. DNA from buccal swabs was eluted in 50 µl buffer AE to concentrate the DNA from these potentially degraded samples.

The 565 base-pair segment of cyt b was amplified following standard polymerase chain reaction (PCR) protocol and using PuRe Taq Ready-To-Go PCR Beads (Cytiva). PCRs contained 25µl total volume with 200 µM concentration of each dNTP, 50 mM KCl, and 1.5 mM $MgCl_2$ in the reconstituted solution. Primers used were those established by Ptacek et al. (1994) and used by Bogart et al. (2020). Thermal cycling conditions for PCR reactions were initial denaturing for 3 minutes at 93°C; followed by 35 cycles consisting of one minute at 93°C, one minute at 52°C, then one minute at 72°C; followed by a final elongation for 5 minutes at 72°C. PCR products were isolated via electrophoresis in 2% E-Gels (Invitrogen) using standard loading buffers, and extracted using the QIAgen Gel Extraction Kit. The manufacturer's protocol was modified to included incubating samples with PE for four minutes, and in EB buffer for four minutes. The purified PCR products were Sanger sequenced by Azenta (New Jersey).

*Genetic Analysis*

Cyt b sequences were cleaned and analyzed using Geneious Prime software (version 2022.0.2). Reference sequences of *H. chrysoscelis* and *H. versicolor* from Minnesota, Michigan, and Wisconsin, and identified by karyotype (Bogart et al., 2020), were downloaded from GenBank to Geneious. The sequences generated from the present study were aligned with the

reference sequences using the CLUSTAL algorithm in Geneious Prime. Phylogenetic inference was made using the GTR + F + G4 substitution model (IQTree ModelFinder, Minh et al., 2020) to infer a maximum likelihood tree using IQTree (Nguyen et al., 2015) that was visualized in FigTree. *Hyla femoralis* was selected as the outgroup to root the trees, because it is a closely related species but is not a sister taxon. One-thousand bootstrap replicates were generated using the ultrafast algorithm (Minh et al., 2013) to infer confidence in the topology. Samples were spatially represented using ArcGIS and compared to historical occurrences in Kansas.

## RESULTS

I obtained toe clips and buccal swabs from 27 gray treefrogs during the 2021 field season, representing populations from seven counties across eastern and central Kansas (Figure 2). The sample documented in Harvey County represents a county record along the western edge of the distribution (Figure 2).

Buccal swabs were collected from 23 of 27 individuals sampled in the field. Buccal swabs were not collected from recently metamorphosed frogs and smaller juvenile frogs, because the buccal cavities were too small. For these small size restricted frogs, toe clips alone were collected for genetic analysis.

Gray treefrog tissues from 27 museum specimens, representing 15 counties in Kansas, were included in this investigation in order to increase sample size (Figure 2). Two additional museum specimens of *H. chrysoscelis* from Florida were added in an attempt to improve confidence in taxonomic identification. DNA was extracted from a total of 46 gray treefrogs for this project and are compared to locations from Hillis et al. (1987) on Figure 2.

Due to logistic supply-chain restrictions, only a portion of the collected toe clips and buccal swabs were sequenced. However, a majority (16 of 27) of the collected toe clips were sequenced and included in the taxonomic analysis. DNA was extracted from 10 of 23 buccal swabs and all the swabs that were processed yielded sufficient DNA to be sequenced. Seven of 10 buccal swabs were successfully sequenced but three samples failed to provide adequate sequence information due to priming errors. Ultimately, buccal swabs were as effective as tissues for amplifying mtDNA, regardless of short-term storage temperature after collection.

Using *H. femoralis* as the outgroup, the sequences of cyt b from Kansas and three samples of *H chrysocelis* from Florida, and the geographically nearest samples identified by karyotype and sequenced by Bogart et al. (2020) were joined to form a maximum-likelihood tree (Figure 3). There was little variation in the cyt b sequences among individuals from disparate localities. The maximum-likelihood tree did not depict sufficient differences to distinguish among samples (Figure 3). The samples with verified species identifications did not form distinctive clades from each other or samples from Kansas based on sequences from cyt b (Figure 3). Accordingly, cyt b was not an effective means of differentiating between *H. chrysoscelis* and *H. versicolor* in Kansas.

## DISCUSSION

Genes within the mitochondrial genome, such as cyt b, have been used routinely to identify species across taxa in a variety of conservation applications (Parson et al., 2000, Hsieh et al., 2001), including species authentication of animal products (Yan et al., 2005, Kappel et al., 2017) and to evaluate genetic structure within populations (Bradley & Baker 2001). Specifically, genetic variability of cyt b within the gray treefrogs has been used in attempts to elucidate

species patterns and postulate the origins of the diploid-tetraploid complex for over two decades (Ptacek et al., 1994, Holloway et al., 2006, Bogart et al., 2020). Accordingly, a main objective of this research was to determine if cyt b was an effective genetic marker to indicate species level differences between gray treefrogs in Kansas.

The results of this study indicate that cyt b was not effective in differentiating between species in Kansas and that the hypothesis about its utility in this context was not supported. Based on research available after the conclusion of this study, results produced by this investigation are not unexpected (Booker et al., 2022). Booker et al. (2022) completed a large-scale, range-wide study on the gray treefrog complex that included analysis of the entire mitochondrial genome, 244 nuclear loci, and used various phylogenetic models to infer relationships between the two species; but included only one specimen from Kansas. Booker et al. (2022) concluded that *H. versicolor* formed via autopolyploidy and cited evidence of intermediate allele frequencies, close pairwise genetic differences, and model coalescence on autopolyploidy. The models supported a geographic origin in the northeastern United States and stepwise migration through the Midwest and south to eastern Texas. The analysis also indicated that geneflow continues from *H. chrysoscelis* to *H. versicolor* where they occur in sympatry (Booker et al., 2022). Consistent with results from this study, single genetic markers did not distinguish species, but rather distinctive clades were derived from the broader scope of genetic material (Booker et al., 2022). Based on Booker et al. (2022), it would not be unexpected to see few differences between cyt b sequences among the members of the species complex, therefore my inability to make species-level distinctions from my Kansas samples is unsurprising.

Across their ranges there is documented hybridization and unidirectional gene flow from *H. chrysoscelis* to *H. versicolor* in areas of sympatry (Bogart et al., 2020, Booker et al., 2022).

The impacts of hybridization and continuing unidirectional gene flow on population genetic structure is relatively unknown for the complex (Booker et al., 2022). However, polyploids are generally more adaptive to hybridization relative to related diploids, given that interploid reproductive interactions largely result in increased genetic diversity for the polyploid (Bogart & Bi, 2013). It would be important to better understand the relationship between *H. versicolor* and *H. chrysoscelis* in Kansas to make conclusions about conservation implications of hybridization in the complex for the state. However, Booker et al. (2022) suggest that hybridization is a regularly occurring interaction where species co-occur.

An additional objective of this research was to determine which species occupied the western edge of the observed range of gray treefrogs. In Kansas, it remains unknown which species occupies the western extent of the state given that species identification cannot be determined with confidence using non-invasive methods. It also remains unknown if the frogs that lived in sympatry in 1987 remain sympatric now, or if areas of sympatry are common. To understand species relationships and potential conservation implications in the state, it would be important to answer these questions. It is useful to consider what options remain for differentiating the species with absolute certainty. Other than male breeding calls that are subjective, karyotyping remains the only way to identify treefrogs of all sexes and ages. However, karyotyping is time intensive and requires lethal sampling. Monitoring and management of gray treefrog populations in the state will be challenging given the limitations of these methods.

Buccal swabbing as a non-lethal sampling technique for gray treefrogs in Kansas was useful in producing adequate mtDNA for sequencing. Although mtDNA is not useful for elucidating differences in the gray treefrog complex, buccal swabbing and DNA extraction may

be useful for obtaining population genetics information for other threatened or vulnerable amphibian species in the state. Mitochondrial DNA can be useful for understanding population genetic structure, which may be increasingly important in the conservation of endangered or at-risk populations (Shaffer et al., 2000; Najibzadeh et al., 2018; Ramírez et al., 2020). Where research objectives allow, ethical use of species should dictate that these minimally invasive techniques supplant traditional collections. This research demonstrates the efficacy of minimally invasive DNA sampling techniques that do not require whole specimen collection.

Broquet et al. (2007) extracted DNA from swabs that were maintained at ambient temperature for one to eight hours prior to storage at -20°C or -80°C. In my study, logistical restrictions, such as volunteers mailing samples, precluded immediate freezing of the swabs. Pidancier et al. (2003) successfully amplified and sequenced the cyt b gene from six species of amphibians, including three frog species, two salamander species, and one newt species, from buccal swabs stored immediately at -18°C. However, DNA was successfully extracted from only three of six species provided from swabs stored at room temperature for nine weeks. Pidancier et al. (2003) suggested immediately freezing buccal swabs to preserve DNA quality; however freezing samples in the field presents many challenges. My findings suggest that mtDNA can be amplified from swabs kept at ambient temperature for up to a week without reduced efficacy of mtDNA amplification. This study is valuable because it adds to our understanding of the limits of buccal swabbing. Furthermore, adult treefrogs are indeed large enough to obtain adequate swab saturation with minimal handling time. It would be worthwhile to investigate future questions such as determining how long swabs can be maintained at ambient temperature and still provide quality DNA for sequencing.

Non-lethal techniques established for smaller taxa in this study should be applied to other species of conservation concern. Buccal swabbing might be useful in the investigation of population dynamics for small anurans, such as Red-spotted Toad (*Anaxyrus punctatus*), Chihuahuan Green Toad (*A. debilis*), or Spring Peeper (*Pseudacris crucifer*), for which whole individual sampling may have negative effects on populations. Managers can implement buccal swabbing at relatively low cost with no harm to individuals to understand population genetic structure.

In conclusion, gray treefrog conservation in Kansas is complex due to difficulty in distinguishing the two species and the recent documentation of gene flow and hybridization between species in sympatric areas (Booker et al., 2022). Species designations are essential elements of traditional conservation management and planning, and the basis of most legal protections. In the case of the gray treefrogs in Kansas, it may be more useful to consider the two species as one conservation unit until more information on the specific impacts of hybridization in sympatric populations is available.

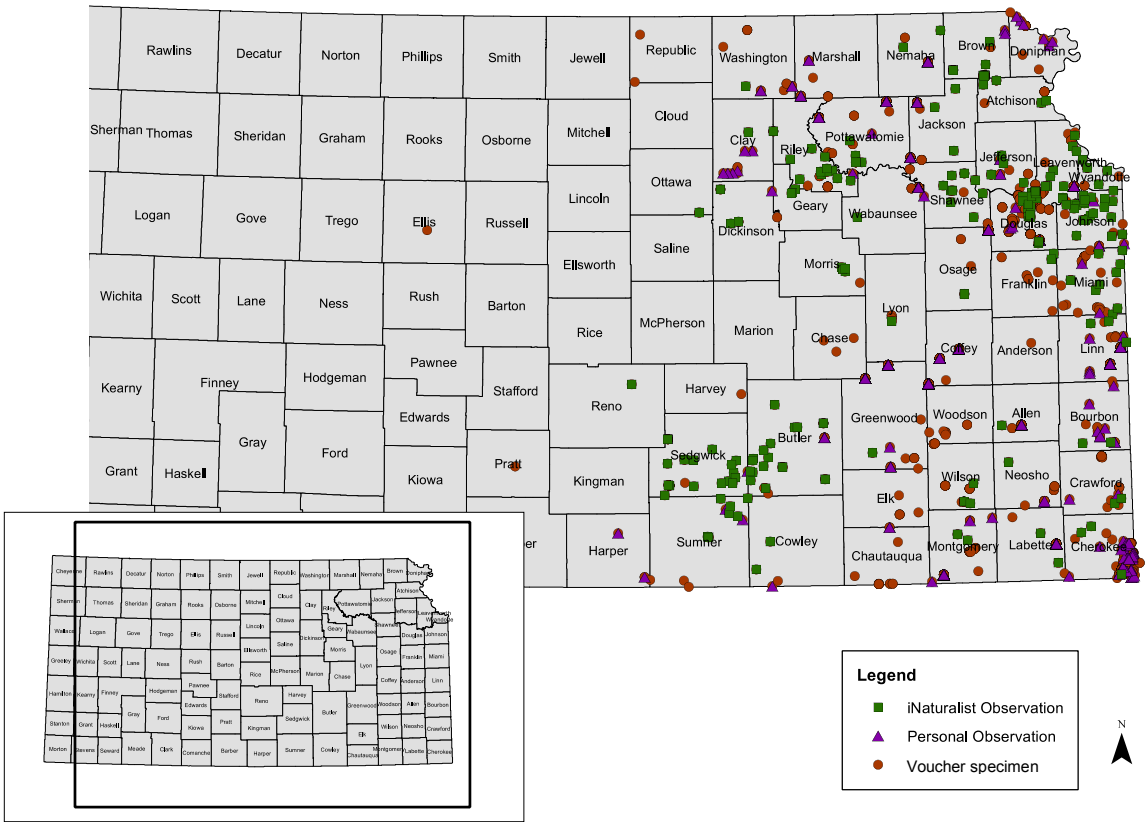


Figure 1: Map of all modern gray treefrog (*Hyla versicolor/chrysoscelis*) records in Kansas including museum voucher specimens, iNaturalist observations, and personal observations. Different record types are distinguished by color and shape. Data courtesy of Kansas Herp Atlas, https://webapps.fhsu.edu/ksherp/account.aspx?o=30&t=7.

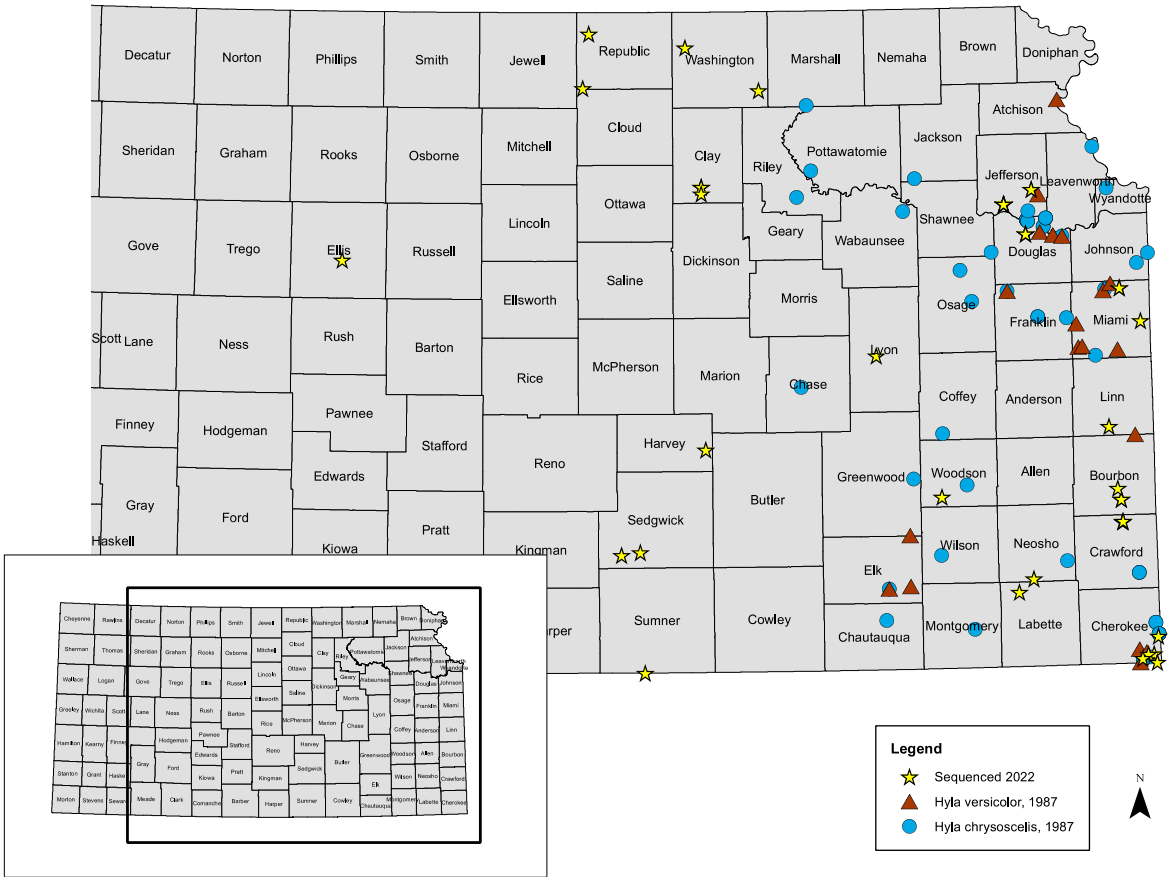


Figure 2: Map of gray treefrog records based on karyotype from Hillis et al. (1987). Stars represent locations of both samples collected during the field season and museum samples sequenced for this study.

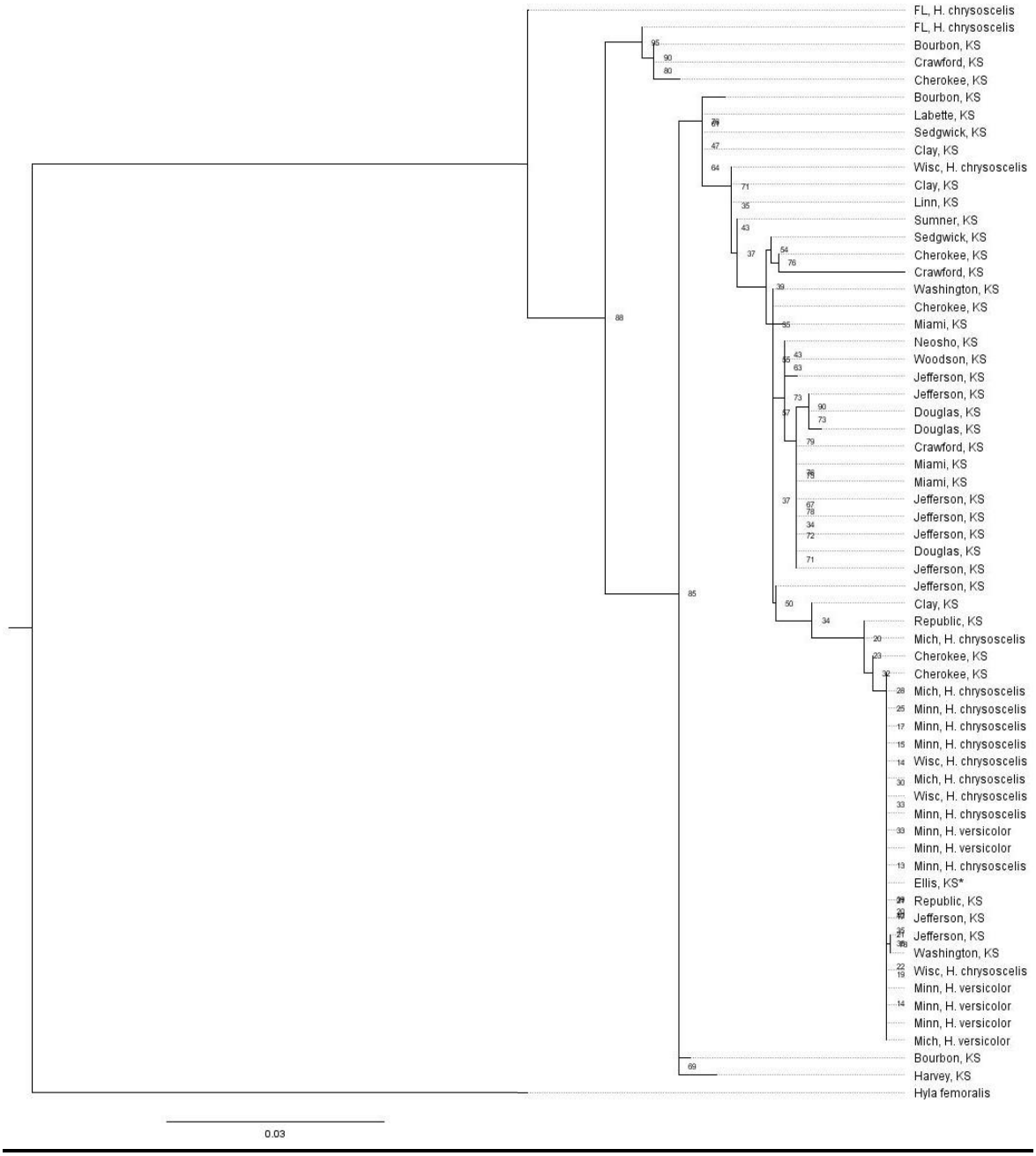


Figure 3: Maximum likelihood tree of identified cyt b sequences from Bogart et al. (2020), and putative *Hyla versicolor* and *H. chrysoscelis* sequences from this study, with *Hyla femoralis* as the outgroup. Locations from Kansas are identified by county. Bootstrap values generated from 1000 replicates. [Mich = Michigan, Minn = Minnisota, Wisc = Wisconsin, and FL = Florida].

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