

GENETIC EVALUATION OF SIRENS IN TEXAS

by

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ABSTRACT

Sirens have always been a taxonomically controversial group. Historically, morphological characters were used to delineate taxonomic groups. However, the neotenic characters seen in sirens complicate taxonomic assignment. While it has been traditionally thought that only lesser sirens (*Siren intermedia*) resided in Texas, it has been well known that sirens in southern Texas are morphologically divergent from typical lesser sirens. The taxonomic classification of these sirens in southern Texas has been long debated and currently remains in question. The sirens in eastern Texas, however, are not morphologically divergent from that of typical lesser sirens, leaving us to wonder where in Texas these two groups meet and how their evolutionary relationship may help define the taxonomic identity of southern Texas sirens. It is important that we answer this question since sirens in some parts of southern Texas have been classified as state-threatened and protection of an undefined taxon is problematic. Since morphological identification has been challenging, I approached this issue using modern molecular phylogenetics. I sequenced 4 mitochondrial genes (CO1, ND2, 16S, and ND5) from samples of both southern Texas and eastern Texas sirens, as well as sirens from out of state. Using those sequences and other siren sequences published online, I created phylogenetic trees, using maximum likelihood and Bayesian analyses, and haplotype networks. These analyses revealed that, of the 4 genes sequenced, there were conflicting results, with CO1 and ND2 representing one relationship and 16S and ND5 representing

another. When concatenated, the maximum likelihood and Bayesian analyses of the sequences sided with the latter 2 genes, suggesting that eastern Texas sirens belong to a clade nested within the southern Texas sirens. Despite the conflicts, there was a consensus among the results in that the sirens of southern Texas seem to relate closer to lesser sirens than greater sirens or reticulated sirens. As such, there does not seem to be a significant difference between sirens within southern Texas, but sirens in eastern and southern Texas were somewhat divergent from each other. While the scope of this project is too small to definitively determine the exact relationship between these Texas sirens, my data strongly supports the sirens of southern Texas are lesser sirens.

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GENETIC EVALUATION OF SIRENS IN TEXAS

INTRODUCTION

Taxonomic debate

The genus *Siren* represents a morphologically unique group of amphibians whose taxonomic placement has been debated. Currently, sirens belong to the order Caudata (the extant salamanders), the suborder Sirenoidea, and the family sirenidae (the sirens), though this placement was not always readily accepted. Upon their initial discovery, Linnaeus (using morphological characters) placed sirens (the first known adult salamander with external gills) into its own order: Meantes (Jamieson and Sever 2003). This group eventually became current day Sirenoidea. Over time, naturalists became unsure of this placement, as they believed sirens to be the “imperfect” larval forms of other salamanders (Jamieson and Sever 2003).

This confusion was caused by the fact that sirens are paedomorphic, containing both larval and adult morphological characters at maturity (Jamieson and Sever 2003). Larval characters such as absent posterior limbs, external gills, a fin-like tail, and lidless eyes are all present in sirens and retained through adulthood (Noble 1931). Paedomorphic species like these present a problem because they not only share general larval characters that are many times homoplastic, but also often fail to develop clade-specific adult traits (Wiens et al. 2005). Historically, taxonomic assignments were made

using phenotypic characteristics and this neoteny creates a morphology that is very misleading for classification (Jamieson and Sever 2003, Wiens et al. 2005).

This neoteny proved to be a persistent problem and our current classification of sirens was not reached until molecular techniques became available to use in conjunction with morphology (Jamieson and Sever 2003). Some of the molecular studies that have been conducted have suggested that sirens were the earliest diverging salamander or that Sirenidae is sister to Proteidae (Frost et al. 2006). However, the currently accepted classifications in Caudata recognize Sirenidae as sister to all salamanders, exclusive of Cryptobranchidae and Hynobiidae (Pyron and Wiens 2011). While the current classification of Sirenidae has largely been accepted, classification of lineages within the genus *Siren* remain much more in question.

Siren is one of the 2 genera found in the family Sirenidae, with the other being *Pseudobranchius* (the dwarf sirens; Petranks 1998). As a whole, the genus *Siren* can be described as long eel-shaped salamanders with external gills, each with 3 gill slits, and only forelimbs present, each with 4 toes (Petranks 1998). Within *Siren* there are 3 currently described species: the greater siren (*Siren lacertina*), the lesser siren (*Siren intermedia*), and the reticulated siren (*Siren reticulata*; Graham et al. 2018).

The greater siren (*S. lacertina*) was the first species to be described (in 1766) and, as it was recognized to be unique from all other salamanders, placed in its own genus (Jamieson and Sever 2003). This species' range extends across the U.S. coastal plains from the District of Columbia through Florida and southern Alabama (International Union for Conservation of Nature and Natural Resources [IUCN] 2022; Figure 1). In

addition to the morphological features characteristic of the genus, *S. lacertina* is described as being dark olive green to light or dark gray in color, with the dorsum being darker than the venter (Bishop 1943, Petranka 1998). There may be small dark spots on the back, and/or light flecks and blotches on the sides and belly, often green or yellow in tint. They range from 500-980 mm in length, and have between 36-39 costal grooves (Bishop 1943, Petranka 1998).

The lesser siren was originally described in 1826 and is the most widespread of the sirens (Lannoo 2005). This species ranges from Virginia to central Florida, west into Texas and adjacent Mexico, as well as north in the Mississippi Valley to southern Michigan (IUCN 2022; Figure 2). In combination with the general morphological characters of the genus, *S. intermedia* is described as being anywhere from a dark brown or green to a light gray, often with tiny black spots scattered on the dorsum and sometimes light flecks on the venter (Martof 1973, Petranka 1998, Dixon 2000, Tipton et al. 2012). They vary in length from 180-690 mm and have between 31-38 costal grooves (Martof 1973, Petranka 1998, Dixon 2000, Tipton et al. 2012).

The reticulated siren is the most recently defined species of *Siren*, having only been described in 2018 (Graham et al. 2018). The species has a range that is currently limited to the Panhandle of Florida and a neighboring portion of lower Alabama (Graham et al. 2018; Figure 3). On top of the general characteristics of the genus, *S. reticulata* is described as being olive green to gray in color, with a prominent reticulated pattern of dark blotches on the back and, in some cases, continuing onto the lighter yellowish venter

(Graham et al. 2018). Thus far, known specimens of *S. reticulata* provide a range of 265-415 mm for total length and 38-42 for number of costal grooves (Graham et al. 2018).

Of these 3 species, only one, the lesser siren, has defined subspecies. While disputed, most authorities recognize at least 2 subspecies of *S. intermedia*: the eastern lesser siren (*S. intermedia intermedia*) and the western lesser siren (*S. intermedia nettingi*; Petranksa 1998). Adequately named, the western lesser siren makes up the western portion of the lesser siren's range, and the eastern lesser siren the eastern portion (Petranksa 1998; Figure 2). These subspecies are thought to meet, and perhaps overlap, in Mississippi and Alabama, where some believe intergrades may actually exist (Martof 1973, Petranksa 1998). Outside of geographic range, these subspecies are separated by a couple of morphological distinctions. *Siren intermedia intermedia* is said to be dark in color with small black dots, 31-35 costal grooves, and a maximum length of 380 mm (Bishop 1943, Martof 1973, Petranksa 1998). *Siren intermedia nettingi* is said to be dark to light gray with small dark spots on the dorsum and light flecks on the venter, with 33-37 costal grooves and a maximum length of 502 mm (Bishop 1943, Martof 1973, Petranksa 1998).

In addition to the forementioned species and subspecies within *Siren*, another taxon may exist in southern Texas. Goin (1942) noted that sirens found in southern Texas were much larger in size than *S. intermedia nettingi* which, at the time, was thought to be the only siren taxa in Texas. Because of a lack of quality specimens, he was unable to give this group a separate designation, and tentatively included them in *S. intermedia nettingi*.

Brown (1950) recognized this reported size difference between sirens in southern Texas and eastern Texas and decided to recognize this southern group as *S. lacertina* and the eastern group as *S. intermedia*, thus claiming two species of siren in Texas. He did, however, note that this designation of *S. lacertina* was tentative and also decided to give them the common name of Rio Grande siren.

After the collection of more material, Goin (1957) determined that the sirens of southern Texas had a higher costal groove count (36-38) than any *S. intermedia* specimens and had coloration differences (namely smaller spots) from *S. lacertina* specimens. As a result, he designated this southern Texas group as the subspecies *Siren intermedia texana* with the common name Rio Grande siren.

Martof (1973) accepted this subspecies designation and recognized *S. intermedia texana*. Villela and Brandon (1992) conducted a study that concluded there were 2 species of siren in Texas, *S. lacertina* and *S. intermedia nettingi*, based on the length measurements of 8 siren specimens, 5 of which were from Texas. The *S. lacertina* designation applied to the larger sirens and the *S. intermedia nettingi* designation to the smaller sirens in southern Texas, where they were thought to occur in sympatry. Petranka (1998) did not recognize Villela and Brandon's *S. lacertina* designation and only considered the subspecies *S. intermedia texana* to reside in southern Texas, with a noted zone of integration between them and the *S. intermedia nettingi* of eastern Texas.

Dixon (2000) decided to recognize the sirens of southern Texas as a separate species known as *Siren texana* based on personal communications of molecular data that supported a southern Texas siren specimen and a Florida *S. lacertina* specimen as

representing different species. Lannoo (2005) recognized the controversy that had been created around these sirens in southern Texas but having decided that Dixon did not present enough evidence, continued to consider the sirens in southern Texas to be *S. lacertina*.

Tipton (2012) decided that there was enough consensus on the distinctness of the southern Texas sirens that they deserved their own species status, but in recognition of the lack of evidence for this designation, did so under the name “*Siren* species ‘Rio Grande’”. Dixon (2013) ended up retracting his prior species designation *Siren texana* after more molecular data was communicated to him regarding the lack of evidence that there is more than one species in Texas, prompting him to then recognize the South Texas group under the subspecies *S. intermedia texana*. Most recently, LaFortune (2015) argued that these southern Texas sirens represent the separate species *Siren texana*, based on the comparison of mtDNA sequences of 8 siren specimens from Texas, 1 *S. lacertina* specimen, and 1 *S. intermedia* specimen.

Currently, the controversy over the taxonomy of sirens in southern Texas remains and has left authorities to recognize this group under various names and taxonomic designations (Lannoo 2005, Tipton et al. 2012, Dixon 2013, Kline and Carreon 2013). It is largely agreed that we need more information in order to solve this issue, as is reflected by AmphibiaWeb (2022), IUCN (2022), and NatureServe (2022a) who all recognize this southern Texas group as having the potential to represent *S. lacertina* and/or a subspecies of *S. intermedia*, but note that more data is necessary.

Despite the debate regarding taxonomy, it is widely accepted that this group of sirens in southern Texas have been deemed distinct from other siren taxa and many agree on a common description. These sirens are characterized as having 36-38 costal grooves, reaching a maximum length of 690 mm, and typically being light gray or brown in color, usually with tiny black spots on the dorsum (Martof 1973, Petranka 1998, Dixon 2000, Tipton et al. 2012).

The range of these sirens, like that of their taxonomic identity, lacks consistency across sources. Their ranges can vary from being secluded to a handful of counties in the Lower Rio Grande Valley, to extending up the coastal bend, south into Tamaulipas of Mexico, and even into parts of eastern Texas (Martof 1973, Petranka 1998, Lannoo 2005, LaFortune 2015; Figure 4). Though, many agree that Maverick County and Refugio County likely represent the northern boundaries, and somewhere in Mexico the southern boundary (Lannoo 2005, Tipton et al. 2012, Dixon 2013). The uncertainties surrounding range are complicated by the idea that there may also be zones of hybridization between these southern sirens and *S. intermedia nettingi* (Goin 1957, Petranka 1998).

The primary importance of investigating this issue of *Siren* taxonomy comes from the inability to protect a species that's classification remains uncertain. An alarming number of amphibians around the world are currently experiencing more rapid populations declines than ever historically documented (Catenazzi 2015). While population trends in sirens from southern Texas have never been formally investigated, it is assumed they are just as vulnerable to the same enigmatic factors that are affecting other amphibians.

In recognition of this, Texas Parks and Wildlife Department decided to list the southern Texas sirens as threatened under the taxonomic designation “South Texas siren (large form *Siren* sp. 1)” in 1987 (Dixon 2000, Texas Parks and Wildlife Department 2021). They have also been given the global rank of GNRQ which translates to “global rank not yet assessed for taxon with informal taxonomic status” and a state rank of S2 which translates to the species being considered “imperiled” at the state level (NatureServe 2022b). Since they still have no proper taxonomic designation, there is no clear and definitive range or identifying characteristics associated with these sirens. As a result, there have been no management or conservation efforts implemented yet for these sirens.

Environmental Factors

Within Texas, sirens are not only found in the south, but also in the eastern part of the state. While the range of this potentially unique southern Texas siren is still unclear, it is widely accepted that the sirens found in eastern Texas express characters more like that of typical lesser sirens and are generally considered to be *S. intermedia nettingi* (Dixon 2013). This leaves us to wonder where in Texas these 2 groups of sirens might meet, and how the differences between them may assist in our taxonomic investigation.

One major difference we see between these southern Texas and eastern Texas sirens is environment. The environmental conditions of the southern and eastern parts of the state differ greatly today, but they have differed even greater in past epochs. While the first fossil specimen belonging to the genus *Siren* was dated back to the middle Eocene, the first fossil of *Siren* found in Texas was dated to middle Miocene (Holman

2006). From fossil evidence, much of which is from the last glacial event in the late Pleistocene (Holman 2006), we can determine what the environment was probable to have looked like in the past and how that could have potentially affected the evolutionary relationships of sirens that we see today.

In present day, Texas is made up of 10 ecoregions and the typical proposed range for sirens in Texas falls within 5 of those ecoregions: the Gulf Coast Prairies and Marshes, the South Texas or Rio Grande Plains, the Piney Woods, the Post Oak Savannah, and the Blackland Prairies (Figure 5).

The Gulf Coast Prairies and Marshes are suspected to have represented a forested zone around 30,000 years ago, during the most recent full-glacial period, though this region is not well known in this period (Bryant and Holloway 1985). The post-glacial period (10,000 years ago – present) of this region is suggested to have been a relatively stable mosaic of grassland savannas mixed with pockets of oak and hickory woodlands, with a gradual increase in aridity over the years (Bryant and Holloway 1985). Today, the Gulf Coast Prairies and Marshes are characterized as a relatively level and slow draining plain with many creeks, rivers, sloughs, and bayous (Correll and Johnston 1979). They receive an evenly dispersed annual rainfall of between 75-125 cm, increasing towards the east (Correll and Johnston 1979). Many of the floodplains dry out in the summer months, leaving grassy prairies (Schmidly 2002). Among these gently rolling plains, there is little brush aside from the scattered pockets of live oak, acacia, and mesquite (Schmidly 2002).

The growing season for the flora in the Gulf Coast Prairies and Marshes is very long, at around 300 days, because of the warm temperatures and high humidity of the

area (Correll and Johnston 1979). Most recent changes to this area include increased urbanization, which has led to habitat destruction through increased traffic at the ports and the creation of reservoirs and flood controls (Schmidly 2002). The increase in anthropogenic change has been caused by an increase in agricultural practices, of which irrigation and pesticide use greatly impact local wildlife (Schmidly 2002). There has also been a noted rise in sea level, which is 3-4 times the global rate along the eastern part of the Texas coast (Davis 2011). This is a result of many factors including erosion from hurricanes, fluid withdraw by the petroleum industry, and soil compaction. The central and southern Texas coast is also experiencing a rise in sea level, but at a rate below that of the eastern coast, and only slightly above the global rate. This area experiences some erosion, but less than the eastern coast, likely because many rivers feed into this part of the gulf and the sediment deposits have helped stabilize this portion of the coastline. This area is, however, experiencing noticeable effects of climate change, with many rivers and streams, that used to flow continuously, drying up (Davis 2011).

The South Texas or Rio Grande Plains, during the last glacial period, likely consisted of a mix of grasslands, oak scrublands, and a few humid woodlands (Bryant and Holloway 1985). Towards the end of this period, the climate began to shift and become drier, prompting the landscape to transition toward more grasslands and scrublands than woodlands. This trend continued in the post-glacial period, where the vegetation consisted of some limited wooded areas dispersed within larger grasslands and scrublands. It is suspected that around 6,000 years ago the climate became quite stable, with only slight changes in temperature and rainfall patterns, as there is not much evidence of changes in vegetational structure (Bryant and Holloway 1985). Present day,

the South Texas Plains are dominated by flat to gently rolling, arid, open prairies and shrublands, sparsely broken up by streams flowing into the Gulf of Mexico and the Rio Grande River (Correll and Johnston 1979, Schmidly 2002). The soil varies from sandy to rocky and is often covered with various densities of mesquite and other brush (Schmidly 2002). These plains receive an annual rainfall of between 40-75 cm, again increasing towards the east, with peaks in the early and late summer season, though the high summer temperatures of the area result in high evaporative loss (Correll and Johnston 1979). Most recent changes to this ecoregion mainly consist of effects from agricultural practices, namely ranching, of which overgrazing has had a significant impact (Schmidly 2002). This area also has the issue of urbanization, but to a much lesser extent, and mostly around the border shared with Mexico (Schmidly 2002).

The Piney Woods is theorized to have represented a deciduous forest in the last glacial period, though this area is also not well known during this period (Bryant and Holloway 1985). As this period came to an end, there is evidence that the deciduous forest remained and likely only changed in terms of composition, fluctuating in percentages of certain taxa. The post-glacial period for this area continued this trend, as we see evidence of a migration of pine that mixed with the remnants of the late-glacial deciduous woodlands (Bryant and Holloway 1985). Today, the Piney Woods ecoregion contains hilly pine forests with numerous streams that feed into larger rivers (Schmidly 2002). This forest varies in density, with the thickest parts becoming impenetrable with vines and brush, to the thinner parts containing strips of open grassland (Schmidly 2002). The area, which generally has little drainage, receives an average annual rainfall of 90-130 cm, evenly distributed throughout the year (Correll and Johnston 1979). Most recent

changes in this area include habitat destruction which, while contributed to by cultivation of farmland, is primarily the result of timber and oil industries (Schmidly 2002). Another significant impact in this area was historic hog farming, as overstocked hogs became free range and also contributed to habitat destruction (Schmidly 2002).

The Post Oak Savannah area was heavily forested during the full-glacial period and assumed to be cold in temperature (Bryant and Holloway 1985). As this period progressed, there was a steady warming and drying trend. This transitioned the fragile balance of open deciduous forests and grassland regions towards a grassland dominant region. These climatic trends continued in the post-glacial period, replacing the previous oak-woodlands with the present oak-savannah (Bryant and Holloway 1985). Today, the Post Oak Savannah is characterized by hilly deciduous forest with a grassland understory, receiving an annual rainfall of 90-115 cm, primarily in early summer (Correll and Johnston 1979). Most recent changes to the ecoregion include an increase in urbanization which has caused a great deal of habitat destruction and fragmentation, since this area's fertile soils have triggered an over cultivation of the land (Schmidly 2002). As a result, much of the remaining woodlands are concentrated around the few streams and springs that still flow (Schmidly 2002).

The Blackland Prairies ecoregion, similar to the Post Oak Savannah, was heavily forested during the full-glacial period and cold in temperature with a trend of warming and drying as time went on (Bryant and Holloway 1985). The change in climate triggered a movement away from woodland and toward grassland habitats, resulting in the current landscape (Bryant and Holloway 1985). Present day, the Blackland Prairies

ecoregion consists of a true tallgrass prairie with relatively recent woody encroachment (Schmidly 2002). This area has a mixture of rocky and rich waxy soil with an annual rainfall of around 75-100 cm, increasing to the east, with rapid surface drainage (Correll and Johnston 1979). Most recent changes to this habitat include large amounts of cultivation and, in turn, urbanization as a result of the rich soils of the lowlands (Schmidly 2002). This has caused habitat destruction, fragmentation, and a drastic change in the flora of the area (Schmidly 2002).

One factor that seems to separate the ecoregions where we see these morphologically distinct southern sirens, typical *Siren intermedia*, and no sirens at all, is water. Being fully aquatic, sirens are, at least in part, limited by their water resources, and when these resources are unavailable, their ability to estivate (Luhring and Holdo 2015). The ecoregions where sirens are not typically documented (Cross Timbers and Prairies, Edward's Plateau, Rolling Plains, High Plains, and Trans-Pecos Mountains and Basins), have precipitation levels, historic and recent, that are more sporadic and/or lower than areas where sirens are usually found (Correll and Johnston 1979, Bryant and Holloway 1985), resulting in few, if any, permanent or semi-permanent wetlands suitable for sirens. This brings up the most noticeable distinction between areas where the two morphologically different siren groups reside: precipitation predictability.

Sirens in eastern Texas (typical of *Siren intermedia*) reside in the Blackland Prairies, the Post Oak Savannah, and the Piney Woods. All of these ecoregions not only have relatively high levels of rainfall, but they also have much more predictable rainfall (Correll and Johnston 1979, Bryant and Holloway 1985). The ecoregions that contain the

seemingly distinct population of sirens, the South Texas Plains and the Gulf Coast Prairies (namely the central and southern portions), often receive less precipitation, and it is much less predictable (Correll and Johnston 1979, Bryant and Holloway 1985).

This unpredictability is largely contributed to by hurricanes in the area (Davis 2011, Walls et al. 2013). Hurricane inundation has a large effect on these habitats, as the flooding represents a significant source of water for some areas and can allow for movement between normally isolated bodies of water (Small et al. 2009, Walls et al. 2013). These periodic hurricane inundations may actually be what allows sirens to persist in some more isolated and drier areas.

In these more arid environments, animals will have to survive through longer dry bouts between wet periods. This could explain the difference in siren body size, since there is evidence that size directly correlates with estivation length (Luhring and Holdo 2015). It has been shown that drought survival of sirens can be correlated with body size, because the amount of fat reserves determines how long a siren can survive through estivation (Luhring and Holdo 2015). This could easily present a selective pressure towards larger body sizes in these populations with more unreliable water resources.

Additionally, since the Miocene, there have been significant rises and drops in the sea level of the Gulf of Mexico (Davis 2011), enough for sirens to feasibly go through repeated cycles of dispersion and isolation. This could facilitate divergence through vicariance. One or more of these factors may help explain the morphological differences, but still begs the question: how do the sirens of Texas relate taxonomically?

Utility of Genetics

Various authorities have proposed several hypotheses to answer how sirens in southern Texas relate to sirens elsewhere: (1) sirens in southern Texas are the same subspecies as sirens in eastern Texas (*Siren intermedia nettingi*), (2) sirens in southern Texas represent a third subspecies of *S. intermedia* (“*Siren intermedia texana*”), (3) sirens in southern Texas represent a separate species of *Siren* (“*Siren texana*”), (4) sirens in southern Texas are actually a very disjunct population of *Siren lacertina*, (5) there are actually multiple species of siren in southern Texas (Goin 1957, Villela and Brandon 1992, Lannoo 2005, Dixon 2013, LaFortune 2015).

As exemplified by the prior descriptions of *Siren* taxa, many of their differentiating factors are unreliable for identification. Range, in many cases, is not well known, the exact boundaries vary between authorities, and there are overlaps between some taxa (Petranka 1998). For example, the IUCN’s range of *S. intermedia* excludes many specimen records, especially in the eastern U.S., and depicts a perceived hiatus in southern Texas that is not supported by specimen records (IUCN 2022; Figure 3). Similarly, the IUCN recognizes an isolated population of *S. lacertina* in northeastern Mexico despite a lack of evidence, but excludes several specimen records between this population and their perceived eastern range, making it unclear if the range is incorrect or the identification of the excluded specimens is incorrect (IUCN 2022; Figure 1). As for morphology, the primary criteria used to separate the different taxa are number of costal grooves, length, and color/pattern. Costal groove counts tend to vary between sources, have overlapping ranges between species, and can significantly vary from siren to siren

(Martof 1973). Length presents an obvious problem with juveniles, and can also be problematic with adults, as size is hypothesized to vary with environmental conditions (Bonett et al. 2013). Siren coloration contains a very large amount of variation in most species, bringing into question its utility in species identification (Petranka 1998).

Because of this difficulty with morphological identification within *Siren*, I decided to investigate the taxonomic identity of the sirens in southern Texas using genetics. Genetics can provide more clarity on evolutionary relationships since it lacks vulnerability to morphological homoplasy, which has a clear presence in salamanders (Mueller et al. 2004). I specifically chose to analyze mitochondrial genes, which mutate at a more rapid rate than nuclear genes and thus have the potential to offer more information on the evolutionary history of the species (Olsen and Woese 1993, Zardoya and Meyer 1996). Previous studies have been successful in using genetics to describe new species, including that which described the reticulated siren (Graham et al. 2018). That is why my objective was to use genetic analyses to explore the taxonomic relationships of sirens in Texas in hopes to clarify their classification.

METHODS

Sample Collection

All tissue samples utilized in this study came from the West Texas A&M University tissue collection or were loaned from the Wichita State University collection and Oklahoma Collection of Genomic Resources.

The samples from the WTAMU tissue collection were collected from sirens captured between the years 2005-2021 using a variety of active and passive trapping

techniques. The active trapping techniques included using a bag seine of either 9.1 m length and 2.5 mm square mesh or 18.3 m length and 12.7 mm square mesh. Passive trapping techniques included using large and small modified fyke nets and a variety of minnow traps. The large modified fyke nets had a lead measuring 14.5 m by 88 cm with the body of the trap measuring 4.5 m in length and being comprised of 2 rectangular frames in front that measure 88 cm in height by 120 cm in width and contained a vertical slit-shaped funnel the full height of the frame followed by 5 circular hoops that supported 3 regular-shaped funnels, all with square mesh of 10 mm. The small modified fyke nets are similar to the large ones but with a lead measuring 7.4 m by 67 cm, the body of the trap measuring 3.3 m in length, the rectangular frames measuring 67 cm tall by 95 cm wide, and only 4 rings toward the back that supported 2 funnels. The minnow traps used include pyramid traps, Gee traps, and Minnie traps (Memphis Net & Twine Co. Inc., Memphis, TN). The pyramid traps were constructed according to Shirley and Lutz (2009) using vinyl-coated 6.35 mm square mesh wire. All passive traps were baited with canned cat food and/or a light source. Three types of light sources were used: Cyalume sticks, battery operated fishing light lures, and water activated fishing light lures. Traps were set in any suitable freshwater with vegetation, such as ditches, small ponds, or resacas. They were placed in relatively shallow water with one side of the trap parallel with structure or vegetation in the pond and secured in place with a piece of rebar threaded through the mesh into the substrate. Once a siren was captured, a scalpel or scissors were used to take approximately 0.5-2 cm of tissue from the tail, which was then immediately preserved in 95% ethanol.

These samples from the WTAMU tissue collection originated from different locations in Florida and Texas. The Florida locations include River Styx (Alachua County) and the Rodman Reservoir (Putnam County), both of which are located in central Florida. The Texas locations are much more far spread and include Guadalupe Delta Wildlife Management Area (WMA), The Nature Conservancy's Southmost Preserve, Powderhorn WMA, Gus Engeling WMA, Laguna Atascosa National Wildlife Refuge (NWR), and Carrizo Springs, TX (Dimmit County).

Guadalupe Delta WMA is an estuarine property in Refugio County, Texas. The property is characterized by many interconnected bodies of water with a diverse community as a result of the varying salinities across the site (Sullivan et al. 2020). The salinity of different areas is highly correlated with seasonality and proximity to freshwater inflow, which in turn has a direct effect on the diversity of that area (Sullivan et al. 2020). Sirens were collected in an upland resaca with relatively high water permanence. The resaca generally ranged from 0.5-1.5 m in water depth, was surrounded by a dense thornscrub canopy dominated by retama (*Parkinsonia aculeata*) and lime prickly ash (*Zanthoxylum fagara*), and contained a large quantity of downed woody vegetation that made it structurally complex.

Southmost Preserve is located in Cameron County and represents a unique property that was originally an orchard before it was acquired by The Nature Conservancy (Vasquez 2020). This property is typical of the Lower Rio Grande Valley with a semi-arid and subtropical climate, and management of the property primarily entails restoration of the native thornscrub habitat and conservation of the remaining

Mexican sabal palm forests (Vasquez 2020). Sirens from here were collected in an old, concrete-lined, warehouse loading dock that had been converted to a water garden by staff of the preserve. The area ranged to about 1 m deep, was highly vegetated, and was relatively stable in water level. Although this water garden was artificial, sirens were not stocked into it and were presumed to have naturally colonized the area from nearby natural resacas adjacent to the Rio Grande River.

Powderhorn WMA is located in Calhoun County in the coastal plains of Texas. This property is characterized by shallow waters with ranging salinities, including a diversity of habitats such as seagrass beds and oyster reefs (Matich et al. 2016). Sirens were collected from naturally occurring, shallow, upland pans. Although these pans lacked woody vegetation, they tended to be <0.7 m deep, were rather ephemeral (often drying down annually), and had extensive flooded mats of Bermuda grass (*Cynodon dactylon*) that provided abundant cover.

Gus Engeling WMA is located in Anderson County, which falls within the Trinity River Basin. This area is characterized by Post Oak Savannah and Bottomland Hardwood Forest that is scattered with lakes, ponds, creeks, marshes, and swamps (Riedle et al. 2015). Sirens from Gus Engeling WMA were collected from a shallow (<0.5 m water depth) swampy area below the dam of an artificial lake (= Berry Lake). The swamp was open-canopied, extremely highly vegetated with emergent and submergent aquatic vegetation (particularly pennywort [*Hydrocotyle* sp.] and bulrush [*Scirpus* spp.]) and had a bottom consisting of decomposing organic detritus approaching the depth of the water.

Laguna Atascosa NWR is located in Cameron County in the Lower Rio Grande Valley. This property is within the coastal plains of Texas and is characterized by its various lakes, estuaries, and marshes, which are broken up by sand and clay ridges and contain varying salinities (Fernandez 1999). Sirens were collected from a number of different wetlands at Laguna Atascosa NWR. These included natural, Bermuda grass dominated pans, roadside ditches, and small ponds that were either natural or man-made. Ponds included those surrounded by dense, thornscrub canopies as well open-canopied sites. Other than the Bermuda grass pans, wetlands where sirens were collected at Laguna Atascosa tended to lack or have very little emergent vegetation. All sampled wetlands on the site were somewhat ephemeral, with Bermuda grass pans and ditches tending to dry annually while ponds often retained water for several years between drying events.

Carrizo Springs is a town in Dimmit County that falls in the Rio Grande Plains ecoregion, also referred to by the more descriptive name of the Tamaulipan Thornscrub ecoregion (Folks et al. 2014). This area is also characterized by warm temperatures and unpredictable rainfall (Folks et al. 2014). The sole collection site on this area consisted of a man-made, earthen stock tank that tended to hold water for several years between drying events. The surrounding area is currently quite arid and dominated by mesquite (*Prosopis glandulosa*) and acacia (particularly *Vachellia farnesiana* and *Vachellia rigidula*). Although most currently existing wetlands in the general area are man-made, historically a large number of natural, nearly permanent, artesian well-fed wetlands were found in the region. These had largely disappeared by the early to mid-1900's because of declining water tables from agricultural drawdown (Mason 1960).

The samples loaned from Wichita State University came from the US Department of Energy's Savannah River Site, located in western South Carolina, and samples loaned from the Oklahoma Collection of Genomic Resources came from Red Slough WMA, located in McCurtain County in southeastern Oklahoma.

In addition to the samples which I was able to obtain tissue from, I also utilized samples that had sequences publicly available on the genetic database GenBank. From this database, sequences were chosen based on their utility to this study and the availability of information on the sample.

Genetic Analysis

For all samples, I analyzed the mitochondrial genes CO1, ND2, ND5, and 16S. These genes were chosen as a result of what siren sequences were available on GenBank (to use for comparisons) and what genes would provide the most information. All of the genes that were chosen are known for informing evolutionary relationships in amphibians and therefore are commonly used for phylogenetic work and the most available in the form of published sequences (Olsen and Woese 1993, Zardoya and Meyer 1996). Primer sequences for genes of interest were generated using the National Center for Biotechnology Information's primer-blast tool and the mitochondrial genome of a southern Texas siren as a template (GenBank KU904487.1).

Genomic DNA was extracted from tissues using the Qiagen DNeasy Blood and Tissue Kit (QIAGEN Sciences, Germantown, MD) using the recommended protocol. Polymerase Chain Reaction (PCR) was used to amplify each target gene with the following 25 µl reaction conditions: 1 µl of genomic DNA, 1µl of 10mM forward primer,

1 µl of 10mM reverse primer, 12.5 µl of (GoTaq MasterMix, Promega, Madison, WI, USA), and 9.5 µl of water. Amplification conditions for the CO1 and ND2 genes were 35 cycles with an annealing temperature of 53 °C and a 2-minute elongation period. Amplification conditions for the 16S and ND5 genes were 35 cycles with an annealing temperature of 55 °C and a 2-minute elongation period. The PCR products were visualized using gel electrophoresis and the bands of target genes were cut from the gel and extracted using a QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD). Purified PCR products were sequenced on an ABI Prism 3700 DNA Analyzer (Eurofins Genomics, Louisville, KY).

Sequence results were processed using Sequencher (Gene Codes Corporation, Ann Arbor, MI) and aligned in Mesquite (Mesquite v.3.7, www.mesquiteproject.org, accessed 10 March 2022) using opal package (Wheeler and Kececioglu 2007). Aligned sequenced data included both the sequences I produced here, and those siren sequences published on GenBank, which were used to construct phylogenies using the CIPRES science gateway (Miller et al. 2010).

Maximum likelihood phylogenetic trees were created for each gene and a concatenated tree was created with all 4 genes using RAxML-HPC v8.2.12 (Stamatakis 2014). Within RAxML, I employed the GTR+ Γ substitution model for each gene which was partitioned by codon position where appropriate, and bootstrapping was halted automatically using the autoMRE criteria. These maximum likelihood models provide node support values (also referred to as bootstrap values) that indicate the proportion of times a specific relationship was found in phylogenies that were generated by resampling,

with replacement, the original data matrix. This value is often interpreted as a confidence percentage and values of 70 and higher are typically considered significant (Hillis and Bull 1993), however, just as with alpha values, numbers below this may still hold some level of significance (Baum and Smith 2013).

I also conducted a Bayesian analysis on my concatenated data using Mr. Bayes v.3.2.7 (Ronquist and Huelsenbeck 2003). Mr. Bayes was run for 10,000,000 generations, with sampling every 1000 generations, discarding 25% of the generations as burn-in, and using 4 Markov chains with default parameters. Bayesian analyses provide posterior probability values which indicate the probability of that branch belonging in that spot given the probabilities of the alternatives. Similar to bootstrap values, posterior probabilities are often interpreted as confidence percentages, with values of 0.95 and above (on a scale of 0-1) typically being considered significant (Baum and Smith 2013).

TCS haplotype networks were also created, with just Texas samples, for each individual gene using PopART (Clement et al. 2002).

RESULTS

Sample Collection

Thirty-seven sirens were successfully sequenced across the 4 target genes. Of these individuals, 2 were from Oklahoma, 3 were from South Carolina, 3 were from Florida, and 29 were from Texas (Table 1, Figure 6). Within Texas, 1 was from Carrizo Springs, 2 were from TNC Southmost Preserve, 2 were from Powderhorn WMA, 3 were from Guadalupe Delta WMA, 6 were from Gus Engeling WMA, and 15 were from

Laguna Atascosa NWR. Of these sites, Powderhorn WMA (Figure 7) and Laguna Atascosa NWR (Figure 8) were the only ones to have multiple sampling ponds.

I also utilized GenBank sequences from 18 sirens across the 4 genes. These included 1 individual from Louisiana, 3 individuals from Illinois, 5 individuals from Florida, and 9 individuals from Texas (Table 2, Figure 6).

Genetic Analysis

Of the 37 individuals I processed and sequenced, 31 individuals had successful sequences for the CO1 gene, 29 for the ND2 gene, 27 for the 16S gene, and 19 for the ND5 gene (Table 1).

Of the 18 individuals whose sequences were obtained from GenBank, 14 had sequences for CO1, 11 for ND2, 17 for 16S, and 9 for ND5 (Table 2).

For simplicity, in the following paragraphs I refer to all Texas samples from south of Washington County as “southern Texas sirens” and all Texas samples east of Washington County as “eastern Texas sirens” (Figure 6).

The CO1 maximum likelihood tree revealed a clear distinction between sirens identified as *S. lacertina* and *S. reticulata*, vs. sirens identified as *S. intermedia*, with a bootstrap value of 100 for the *S. intermedia* clade (Figure 9). Within this *S. intermedia* clade, there were 2 distinct clades, one of eastern Texas and Oklahoma sirens (bootstrap = 95) which was sister to one of southern Texas sirens and a Louisiana siren (bootstrap = 71), with a single GenBank siren from eastern Texas that is excluded from both of these groups.

The ND2 maximum likelihood tree depicted a similar pattern of 3 clades (Figure 10). The *S. intermedia* clade had a bootstrap value of 96, with the eastern Texas-Oklahoma clade (bootstrap = 81) sister to the southern Texas clade (bootstrap = 66). Unfortunately, this tree did not include a Louisiana siren sample as there was not one available on GenBank.

The 16S maximum likelihood tree found that *S. intermedia* formed one monophyletic clade as well (bootstrap = 72), this time containing sirens from Texas, Louisiana, Oklahoma, and Illinois (Figure 11). Specimens from Illinois (putatively our only exemplars of *S. intermedia intermedia*) were early diverging and sister to a clade of remaining *S. intermedia* (bootstrap = 65). Similar to the previous trees, the singular GenBank eastern Texas siren separated itself from the rest of the Texas sirens, however, unlike the previous trees, there is a clade of eastern Texas-Oklahoma-Louisiana sirens (bootstrap = 71) nested within the southern Texas sirens.

The ND5 maximum likelihood tree depicted a clear clade of Texas-Oklahoma sirens (bootstrap = 99; Figure 12). The only *S. intermedia* siren not included in this clade was the eastern Texas GenBank siren, which was found to be more closely related to *S. lacertina* and *S. reticulata* sirens. Within the Texas-Oklahoma clade, there was once again an eastern Texas-Oklahoma clade (bootstrap = 100) nested in the southern Texas sirens.

The concatenated maximum likelihood tree revealed relationships like that seen in 16S, but with stronger support (Figure 13). *Siren intermedia* was found to be monophyletic and distinct from the *S. lacertina* and *S. reticulata* sirens on a branch with a

bootstrap value of 99. In this *S. intermedia* clade, there was a large clade of Texas-Oklahoma-Louisiana sirens (bootstrap = 89), only excluding sirens from Illinois. The eastern Texas GenBank siren, again, was sister to a clade (bootstrap = 89) that contained a group of eastern Texas-Oklahoma-Louisiana sirens nested within the southern Texas sirens. In this final subclade the Louisiana siren sample fell out first, leaving a strongly supported clade (bootstrap = 99) of eastern Texas-Oklahoma sirens.

The Bayesian analysis of the concatenated results demonstrated the same trends seen in the maximum likelihood analysis of the concatenated data with most major clades being strongly supported (posterior probability = 1.0; Figure 14). This analysis gave a posterior probability (PP) of 0.99 for the *S. intermedia* clade, a PP of 1.0 for the Texas-Oklahoma-Louisiana clade, a PP of 1.0 for that clade excluding the eastern Texas GenBank siren, a PP of 1.0 for the eastern Texas-Oklahoma-Louisiana clade nested in the southern Texas sirens, and a PP of 1.0 for the eastern Texas-Oklahoma clade.

The CO1 haplotype network illustrated a shared haplotype between several samples from Southmost Preserve, Laguna Atascosa, and southern Texas GenBank, as well as another shared haplotype between some of the Powderhorn and Guadalupe Delta samples (Figure 15). The remaining southern Texas samples all represented a haplotype that differed by only 1 or 2 nucleotides from the closely related wide-spread haplotypes, indicating very close relationships between all of the southern Texas individuals. Collectively, Southern Texas sirens are shown to be more divergent from the eastern Texas sirens (10+ nucleotide substitutions). It should also be noted that within the eastern Texas sirens, the eastern Texas GenBank sample and the Gus Engeling samples

were relatively divergent from each other, and both approximately equally divergent from haplotypes found in south Texas. As a result, the Texas samples have essentially split themselves into 3 genetically distinct and equally divergent populations: the southern Texas sirens, the Gus Engeling sirens, and the eastern Texas GenBank siren.

The ND2 haplotype network depicted a similar pattern to the CO1 network, with the same close relationships between the southern Texas samples, a more distant relationship between the southern Texas sirens and eastern Texas sirens, and a distant relationship between the 2 eastern Texas groups (Figure 16). This once again displays 3 groups (the southern Texas sirens, the Gus Engeling sirens, and the eastern Texas GenBank siren) that are all equally distinct from each other.

The ND5 haplotype network, like that of the CO1 and ND2 networks, depicted the same close relationships within the southern Texas sirens, with a shared haplotype between samples from Laguna Atascosa, Powderhorn, and Guadalupe Delta, and a more distant relationship between the southern Texas sirens and the eastern Texas sirens (Figure 17). Additionally, the 2 eastern Texas groups are seen to have a distant relationship between themselves, yet again dividing the samples into 3 distinct groups of southern Texas sirens, Gus Engeling sirens, and the eastern Texas GenBank siren.

The 16S haplotype network revealed close genetic relationships, not only between the southern Texas sirens, but between all Texas sirens (Figure 18). The eastern Texas GenBank siren shared a haplotype with samples from Southmost Preserve, Laguna Atascosa, Guadalupe Delta, and southern Texas GenBank, and the Gus Engeling sirens, which all shared a haplotype, had a very close relationship to the southern Texas sirens.

These close relationships and lack of diversity in haplotypes indicate a scarcity of mutations in this gene, limiting the amount of information it can provide on the evolutionary relationships of sirens in Texas.

DISCUSSION

My Analyses

Across all of the phylogenetic trees, it was clear that there is a distinct separation between sirens identified as *S. intermedia* and *S. lacertina*. The Illinois sirens identified as *S. intermedia*, for which genes they were available, were divergent, but related to, all other sirens identified as *S. intermedia*, potentially representing the genetic distinction between *S. intermedia intermedia* (Illinois) and *S. intermedia nettingi* (the remaining samples). Within this group of *S. intermedia nettingi*, the samples from Oklahoma were consistently found to be sister to samples from eastern Texas, but there were conflicting placements of the single Louisiana sample, with its closest relationship being to the southern Texas sirens in the CO1 tree and to the eastern Texas sirens in the 16S tree. I found the phylogenetic trees of the different genes to also reveal conflicting stories on the siren relationships within Texas.

The CO1 and ND2 maximum likelihood trees indicated that the southern Texas sirens and eastern Texas sirens represent two separate groups that have a common ancestor from which they each diverged. The 16S, ND5, and concatenated maximum likelihood trees and the Bayesian concatenated tree all indicated that the eastern Texas sirens are a distinct group nested within the southern Texas sirens, revealing that they diverged from the southern Texas group. However, this does exclude the eastern Texas

sample that was acquired from GenBank, as it placed itself in various positions across the different trees but was consistent in the fact that it was always significantly differentiated from the other siren samples of eastern Texas. I would like to apply some caution to this sample since it has displayed some unusual relationships and, having originated from GenBank, I cannot attest to its validity. Despite these conflicts, all of the trees agreed that the sirens of southern Texas do have a slight genetic distinction from the sirens of eastern Texas.

Likewise, most of the haplotype networks indicated that there is a separation of the southern Texas sirens, the Gus Engeling WMA eastern Texas sirens, and the GenBank eastern Texas siren. While this was supported by the CO1, ND2, and ND5 networks, the 16S haplotype network differed in its close relationships between all Texas sirens. As previously mentioned, this likely reflects a lack of genetic mutations and therefore a lack of information on their evolutionary relationships. Overall, the haplotype networks depicted a nearly equidistant relationship between the 3 groups, which could likely represent isolation by distance. As seen in Figure 6, these groups have a significant geographical separation and only small divergences from each other. This further emphasized the point that the sirens of southern Texas are only somewhat distinct from sirens in eastern Texas.

While all of the analyses agreed that the sirens of southern Texas have some level of differentiation from the sirens of eastern Texas, the conflicts regarding their exact relationship prevent me from making a definitive conclusion about the taxonomic status of sirens in southern Texas or how they relate to those in eastern Texas. That being said,

my results can still provide some insight on the likelihood of the various hypotheses that have been proposed various authorities to explain the sirens in this region.

Comparisons with Historic Hypotheses

Hypothesis 1.— Sirens in southern Texas are the same subspecies as sirens in eastern Texas (*Siren intermedia nettingi*; Villela and Brandon 1992). While my results support the idea that sirens of eastern and southern Texas have some level of differentiation, that differentiation is slight and could simply represent isolation by distance. The geographic distance between the sirens in southern Texas and the sirens in eastern Texas (as well as between the two eastern Texas groups) is significant and the small amount of genetic difference seen in the haplotype networks could be representative of this separation. This would also explain the separation and differentiation with the Louisiana sample and Oklahoma samples with respect to the Texas samples in my phylogenetic trees. This hypothesis is usually argued along with the idea that sirens in southern and eastern Texas look different because of the different growing conditions. While there is sufficient published evidence for this, I cannot attest to it, as my data only speaks to genetics, not morphology. Given the gaps between the Texas sample locations in my data, I cannot currently determine if this genetic differentiation is simply isolation by distance. This leaves me to say that my results have the potential to support the idea that sirens in southern Texas and eastern Texas represent the same subspecies, but I lack sufficient evidence to make any stronger claims regarding this hypothesis.

Hypothesis 2.– Sirens in southern Texas represent a third subspecies of *S. intermedia* (“*Siren intermedia texana*”; Goin 1957). Subspecies is typically a designation that is strictly based on morphology, not genetics (Hillis 2020). Because there is a clear difference in morphology between sirens in southern Texas and eastern Texas, a subspecies designation could be the most appropriate classification for this southern group. However, since subspecies are simply morphological, we do not expect a reproductive barrier or therefore any genetic differences. While the genetic differentiation between my southern Texas and eastern Texas samples was slight, genetic continuity would be expected. This does not necessarily negate the possibility of southern Texas sirens representing a separate subspecies, but again leaves me to say that my results only have the potential to support this hypothesis, as I lack sufficient evidence to claim anything further.

Hypothesis 3.– Sirens in southern Texas represent a separate species of *Siren* (“*Siren texana*”; LaFortune 2015). LaFortune came to the conclusion that there was a significant enough difference between the genetics of southern Texas sirens and *Siren intermedia* and *Siren lacertina*, for the southern Texas sirens to be considered their own species. However, the results of my study continuously placed all southern Texas sirens in a strongly supported clade of *S. intermedia*. This means, according to my data, that if sirens in southern Texas are to be considered a species separate from *S. intermedia*, so should sirens in eastern Texas, and every other clade nested in the *S. intermedia* clade. Because of the separation seen between the 2 groups of eastern Texas sirens, this would actually insinuate that there are 3 species of sirens in Texas. Since sirens in eastern Texas are typically accepted to be *S. intermedia*, there is very little support that they are

deserving of a separate species designation. Considering that my data included some of the same samples as the LaFortune (2015) study, I believe their conclusions were misled by the small sample size. This leads me to conclude that my results lend no support to the hypothesis that sirens in southern Texas belong to a species separate from *S. intermedia*.

Hypothesis 4.— Sirens in southern Texas are actually a very disjunct population of *Siren lacertina* (Villela and Brandon 1992). Prior to our current knowledge on siren relationships, it was a valid hypothesis that these larger than average sirens found in southern Texas may belong to the greater siren species (Strecker 1915, Stejneger and Barbour 1923). However, the relationships represented in all of my analyses revealed a strongly supported separation of Texas sirens from those identified as *S. lacertina*. It was also seen that the southern Texas samples consistently formed a clade with, and were nested within, other available sequences of *S. intermedia*. This leads me to conclude that my results provide no evidence that any sirens in Texas are *S. lacertina*, and are in fact *S. intermedia*.

Hypothesis 5.— There are actually multiple species of siren in southern Texas (Villela and Brandon 1992). It was seen in my analyses that all the sirens from various locations in the southern portion of the state (south of Washington County) were not highly differentiated from one another, including sirens with around 400 km between their collection locations. That being said, the relatively small number of samples I analyzed may have simply not detected a second taxa if it was significantly less common than the one I sampled. However, my samples from southern Texas did come from a

diversity of wetland types which may have improved my chances of detecting multiple taxa if they occur. Overall, I can say that my current results do not provide any support for the hypothesis that there is more than one species of siren in Texas.

This brings up the range of these sirens. As previously touched on, the range of the sirens inhabiting southern Texas varies across authorities, but is often accepted as the lower Rio Grande valley, extending north towards Maverick County in the west, somewhere around Refugio and Aransas Counties in the east, and south into Mexico (Petranka 1998, Lannoo 2005, Tipton et al. 2012, Dixon 2013). The results from this study support this range, and perhaps even extend its eastward boundary. Two of the siren samples I processed were from Powderhorn WMA located in Calhoun County, which would extend the range of the “Rio Grande siren”, but only by one county. I also included in my analyses a GenBank siren from the Attwater Prairie Chicken NWR in Colorado County, TX, which would further extend the eastern boundary of the siren’s range, but I say this with caution, as I cannot attest to the quality or validity of a sample I did not personally process.

To conclude, my results support that the sirens of southern Texas represent a group with a slight differentiation from sirens of eastern Texas, though it is still uncertain how this genetic differentiation translates taxonomically. At this point, I conclude that all sirens in Texas, according to my data, belong to *S. intermedia*, but what subspecies each geographic group represents is still in question.

Future directions

While this study has positively contributed to our knowledge on Texas sirens, in order to come to more concrete conclusions regarding their taxonomic status, further research is vital. Sequencing more individuals as well as sequencing more genes, would greatly clarify the phylogenetic relationships within the siren populations of Texas. This sample expansion would be most productive if concentrated in the gaps my study left between the southern and eastern sample locations. Filling these gaps can help determine which subspecies designation is most appropriate. A gradient in haplotypes between the southern and eastern locations would likely represent the same subspecies across Texas, while a distinct separation between the phenotypes of these areas would be more likely to represent a distinction between two separate subspecies. Additionally, in order to address the source of the noted morphological differences between sirens in Texas, a common garden experiment using geographically separated specimens would prove helpful.

MANAGEMENT IMPLICATIONS

Our understanding of the taxonomic relationships between the siren populations in Texas is not just pertinent to the furtherment of science itself, but particularly to the management and conservation of the disappearing organisms. As it stands right now, the sirens of southern Texas cannot be adequately protected under such a vague designation as “South Texas siren (large form *Siren* sp. 1)”. Once these populations are given an officially recognized taxonomic designation, regardless of what it may be, we can then sufficiently identify what populations need protection and implement management plans for their conservation. The results of my study currently provide no evidence that sirens

of the Lower Rio Grande Valley are significantly different or should be treated any different from siren populations along the central coast. This, in turn, suggests that the threatened status of the “South Texas siren” could be reevaluated, as they no longer seem to be as unique or isolated as originally assumed.

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Table 1. The sample ID, location of origin, parent collection, and genes successfully sequenced for all sirens processed and utilized in this study.

Sample	Location	Collection	Successful Sequences
WSU5199	Savannah River Site, SC	Wichita State University	CO1, ND2, 16S, ND5
WSU6687	Savannah River Site, SC	Wichita State University	CO1, ND2, 16S, ND5
WSU717	Savannah River Site, SC	Wichita State University	CO1, ND2, 16S, ND5
OMNH9190	Red Slough WMA, OK	Oklahoma Collection of Genomic Resources	CO1, ND2, 16S, ND5
OMNH9195	Red Slough WMA, OK	Oklahoma Collection of Genomic Resources	CO1, ND2, 16S, ND5
PAS13050	River Styx, FL	West Texas A&M University	ND2, 16S, ND5
WT1373	Rodman Reservoir, FL	West Texas A&M University	CO1, 16S
WT1374	Rodman Reservoir, FL	West Texas A&M University	16S
RTK1079	TNC Southmost Preserve, TX	West Texas A&M University	CO1, ND2, 16S
RTK1080	TNC Southmost Preserve, TX	West Texas A&M University	CO1, ND2, 16S
RTK1167	Carrizo Springs, TX	West Texas A&M University	CO1, ND2, 16S, ND5
RTK2614	Powderhorn WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
RTK2615	Guadalupe Delta WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
RTK2616	Guadalupe Delta WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
RTK2617	Guadalupe Delta WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
RTK2618	Powderhorn WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
SITE007	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2
SITE008	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2
SITE009	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2, 16S, ND5

Table 1. Cont. The sample ID, location of origin, parent collection, and genes successfully sequenced for all sirens processed and utilized in this study.

Sample	Location	Collection	Successful Sequences
SITE011	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2, 16S, ND5
SITE012	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2, ND5
SITE100	Laguna Atascosa NWR, TX	West Texas A&M University	16S
SITE103	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2
SITE104	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2, 16S
SITE105	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2
SITE111	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2
SITE112	Laguna Atascosa NWR, TX	West Texas A&M University	ND2
SITE113	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, ND2, 16S, ND5
SITE116	Laguna Atascosa NWR, TX	West Texas A&M University	CO1, 16S
GSIN005	Gus Engeling WMA, TX	West Texas A&M University	16S
GSIN006	Gus Engeling WMA, TX	West Texas A&M University	16S
GSIN200	Gus Engeling WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
GSIN201	Gus Engeling WMA, TX	West Texas A&M University	CO1, ND2, 16S, ND5
GSIN202	Gus Engeling WMA, TX	West Texas A&M University	CO1, ND2
GSIN203	Gus Engeling WMA, TX	West Texas A&M University	CO1, 16S, ND5

Table 2. The GenBank ID, location of origin, and genes with available sequences for each GenBank sample utilized in my analyses. *These ID codes represent different genes for the same individual; in the concatenated data these individuals are referred to as USNM574579 and Sreticulata.

GenBank ID	Location	Genes Available
KU871392.1	South Texas	CO1, ND2, 16S, ND5
KU904482.1	South Texas	CO1, ND2, 16S, ND5
KU904483.1	South Texas	CO1, ND2, 16S, ND5
KU904484.1	South Texas	CO1, ND2, 16S
KU904485.1	South Texas	CO1, ND2, 16S
KU904486.1	South Texas	CO1, ND2, 16S, ND5
KU904487.1	South Texas	CO1, ND2, 16S, ND5
KU904488.1	Florida	CO1, ND2, 16S, ND5
KU904489.1	Attwater Prairie Chicken NWR, TX	CO1, ND2, 16S
KU985926.1	Florida	CO1
MN135479.1*	Plaucheville, LA	CO1
MN135550.1*	Plaucheville, LA	16S
MH888024.1	Jasper County, TX	CO1, ND2, 16S, ND5
NC036927.1	Florida	CO1, ND2, 16S, ND5
DQ283181.1	Florida	16S
X86287.2	Alexander County, IL	16S
X86321.1	Alexander County, IL	16S
Y10946.1	Illinois	16S
MH806873.1*	Florida	ND5
MH806874.1*	Florida	16S
MH808031.1*	Florida	CO1

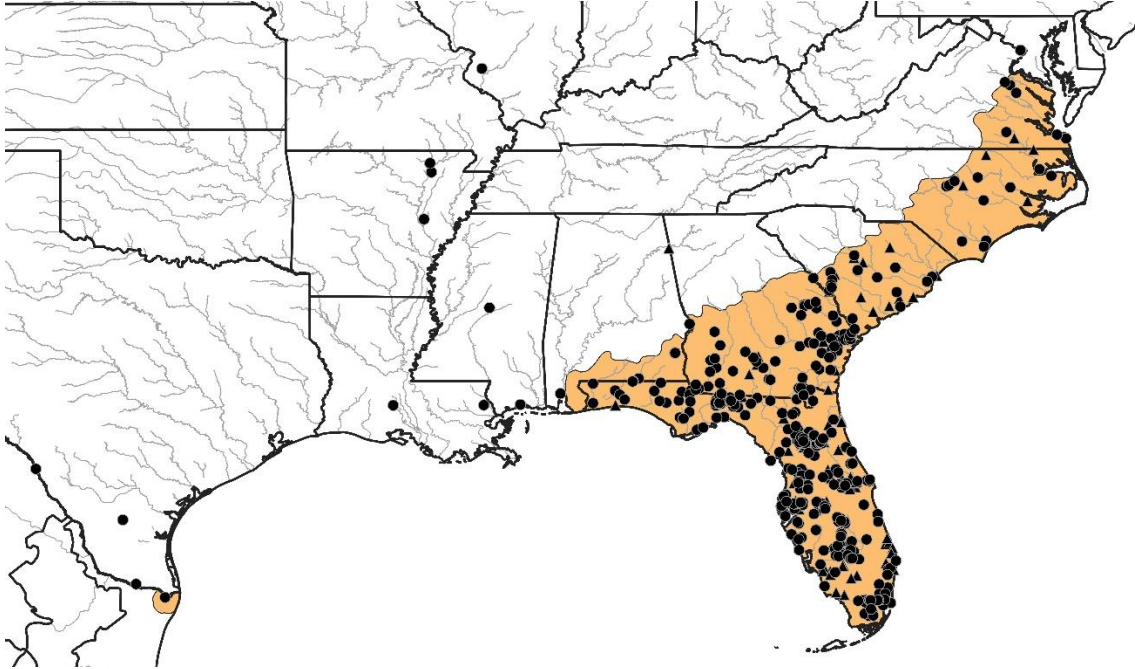


Figure 1. A map of specimen records for *Siren lacertina*. Specimen locations acquired from VertNet are represented by a circle (n = 1290) and those acquired from iNaturalist are represented by a triangle (n = 178). The overlaying polygon indicates one accepted range of *Siren lacertina* (IUCN), highlighting the inconsistencies between records and range.

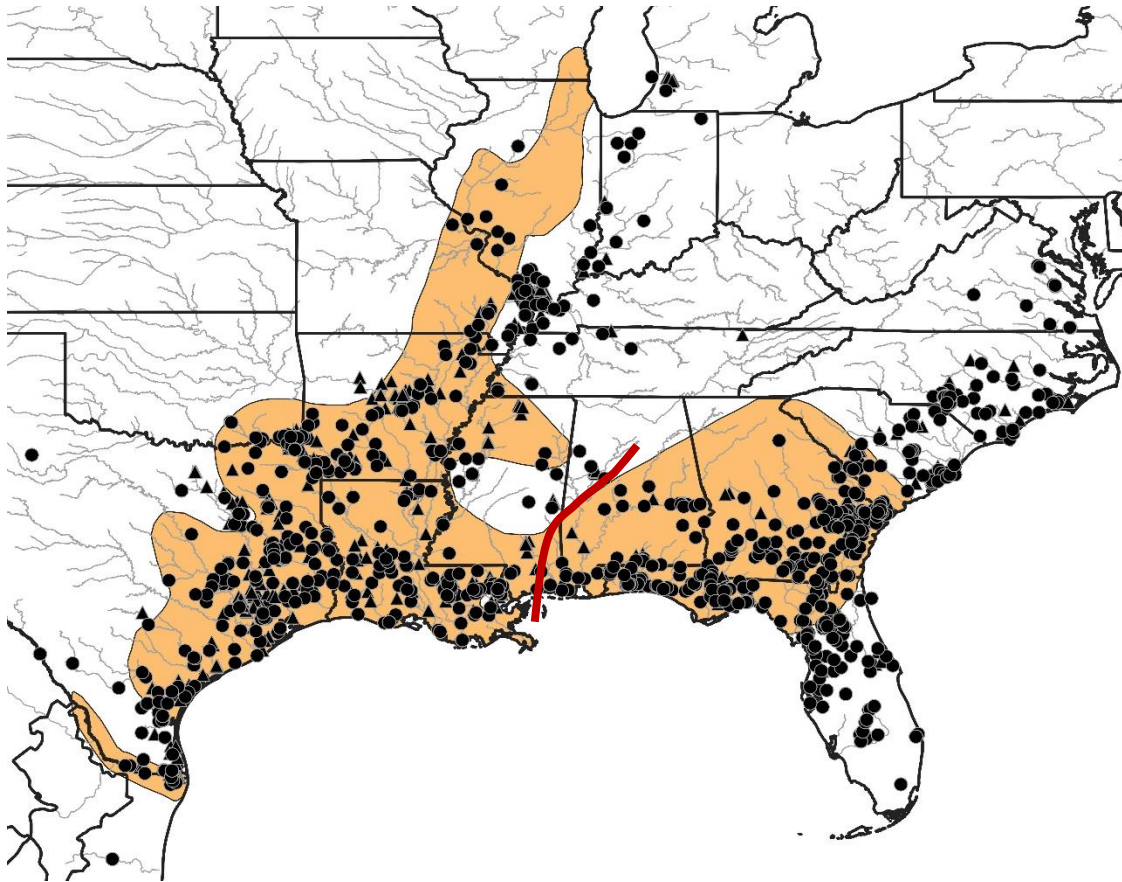


Figure 2. A map of specimen records for *Siren intermedia*. Specimen locations acquired from VertNet are represented by a circle (n = 4209) and those acquired from iNaturalist are represented by a triangle (n = 452). The overlaying polygon indicates one accepted range of *Siren intermedia* (IUCN), highlighting the inconsistencies between records and range. The red line dividing the specimen locations of Alabama and Mississippi indicates the assumed boundary between the western and eastern subspecies.

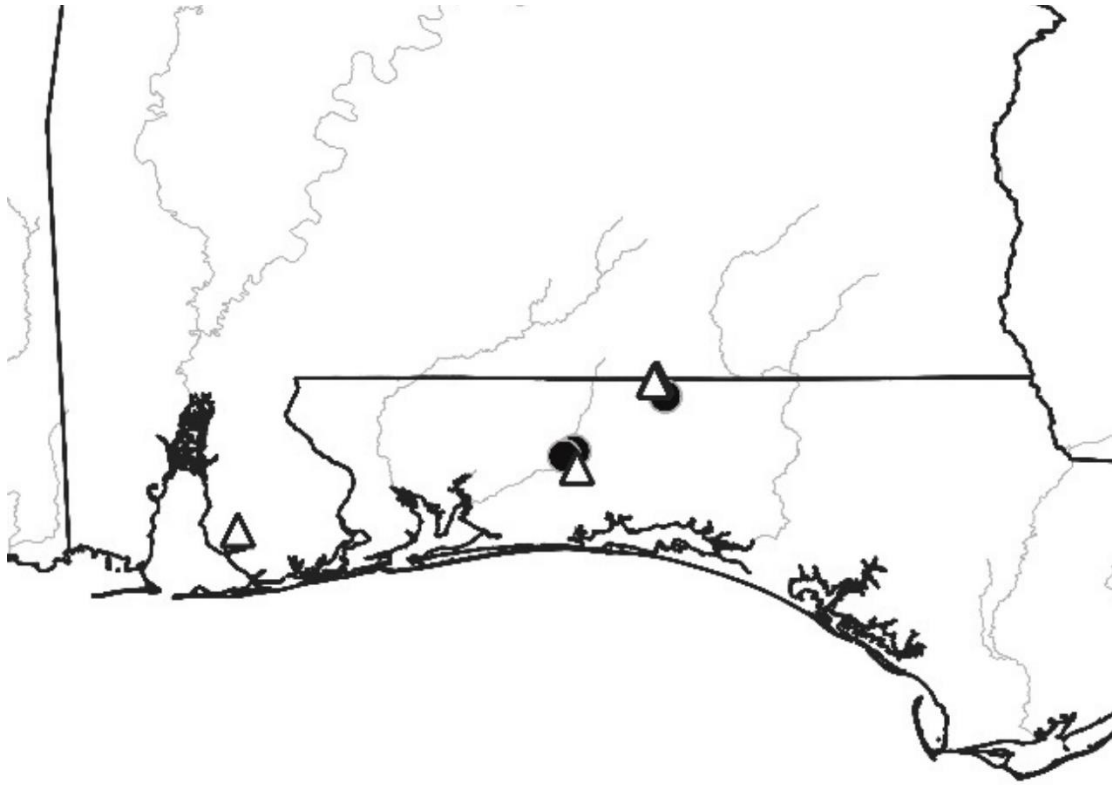


Figure 3. A map of specimen records for *Siren reticulata*. Specimen locations acquired from VertNet are represented by a circle ($n = 8$) and those acquired from iNaturalist are represented by a triangle ($n = 3$).

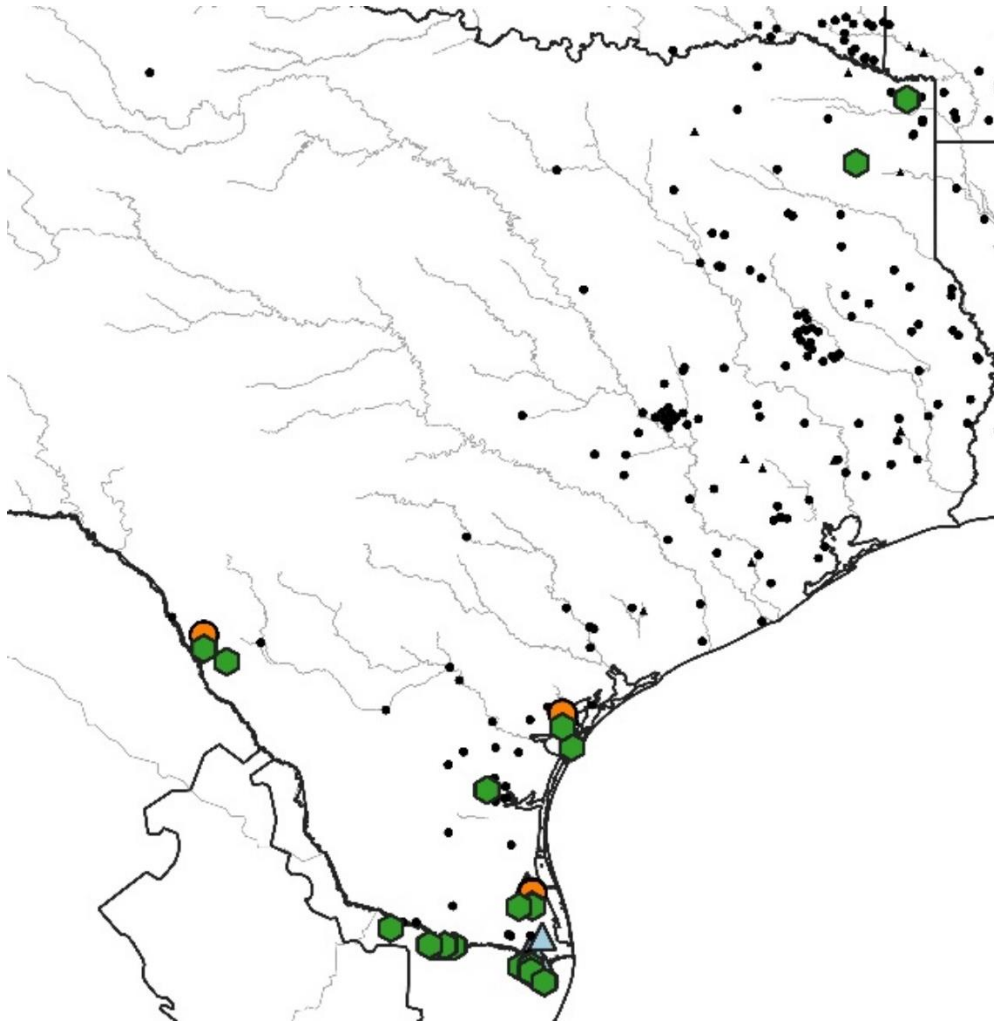


Figure 4. A map of specimen records for sirens in Texas. Large points indicate specimens identified as “*texana*”, with circles representing VertNet specimens identified as *Siren intermedia texana* (n = 4), hexagons representing VertNet specimens identified as *Siren texana* (n = 38), and triangles representing iNaturalist specimens identified as *Siren intermedia texana* (n = 6). Small points indicate specimens identified as *S. intermedia* (other than *S. intermedia texana*), with circles representing locations acquired from VertNet and triangles representing locations acquired from iNaturalist.

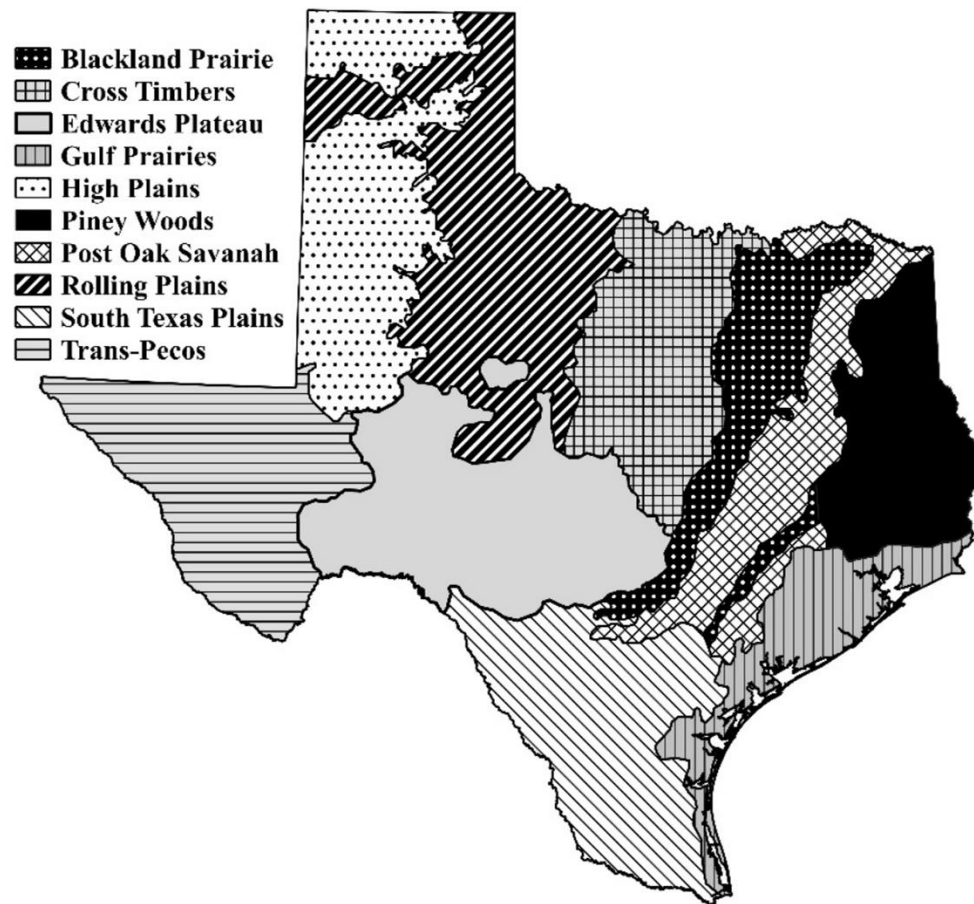


Figure 5. A map of the ecoregions of Texas.

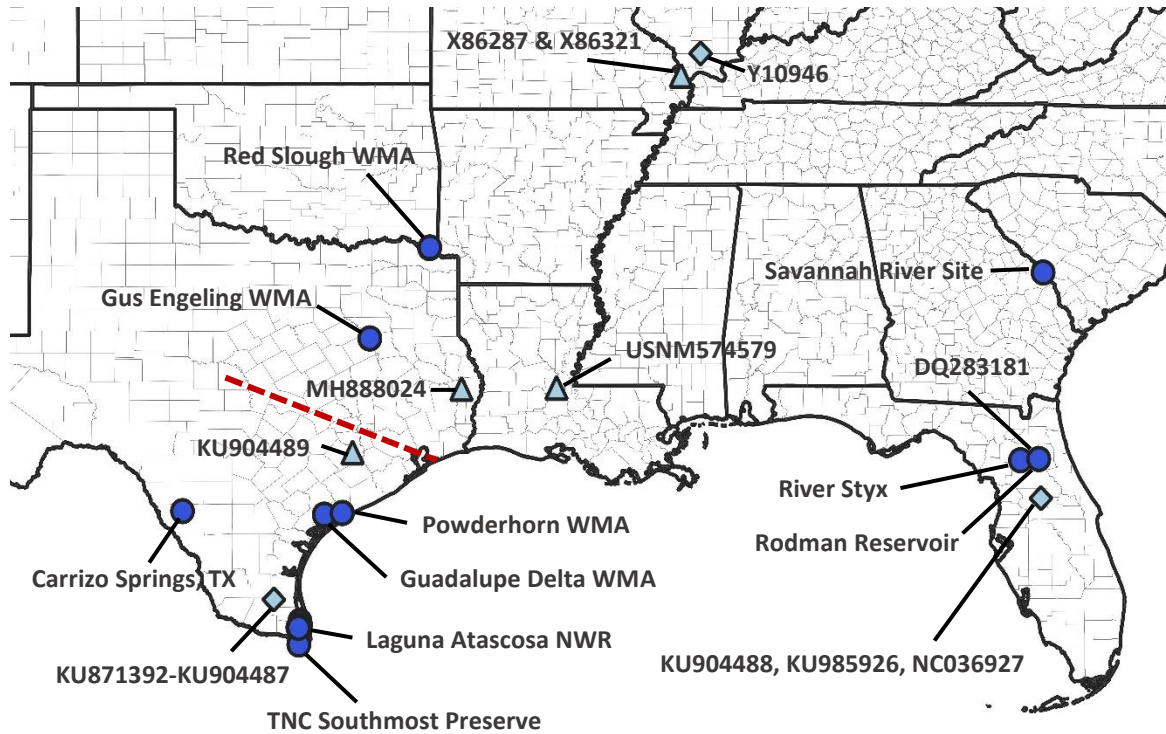


Figure 6. A map of all locations for sirens whose sequences were utilized in this study. GenBank siren locations are depicted in light blue and locations for sirens sequenced in this study are depicted in dark blue. The shape of the points represents quality of location information with circles representing a location where the exact body of water is known, triangles representing the city or county being known, and diamonds representing any location information more coarse than county. The dashed line indicates the separation between southern Texas samples and eastern Texas samples using Washington County as a reference.

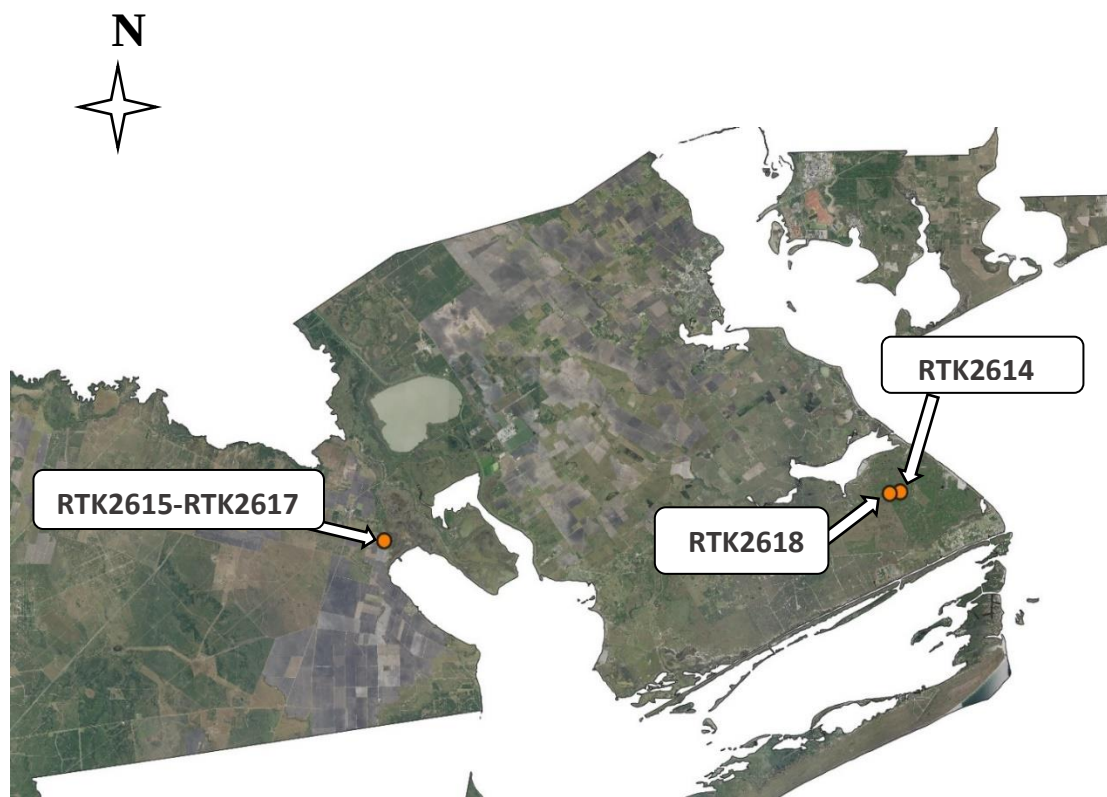


Figure 7. A map of Refugio and Calhoun Counties with location points for siren samples sequenced from Powderhorn Wildlife Management Area and Guadalupe Delta Wildlife Management Area.

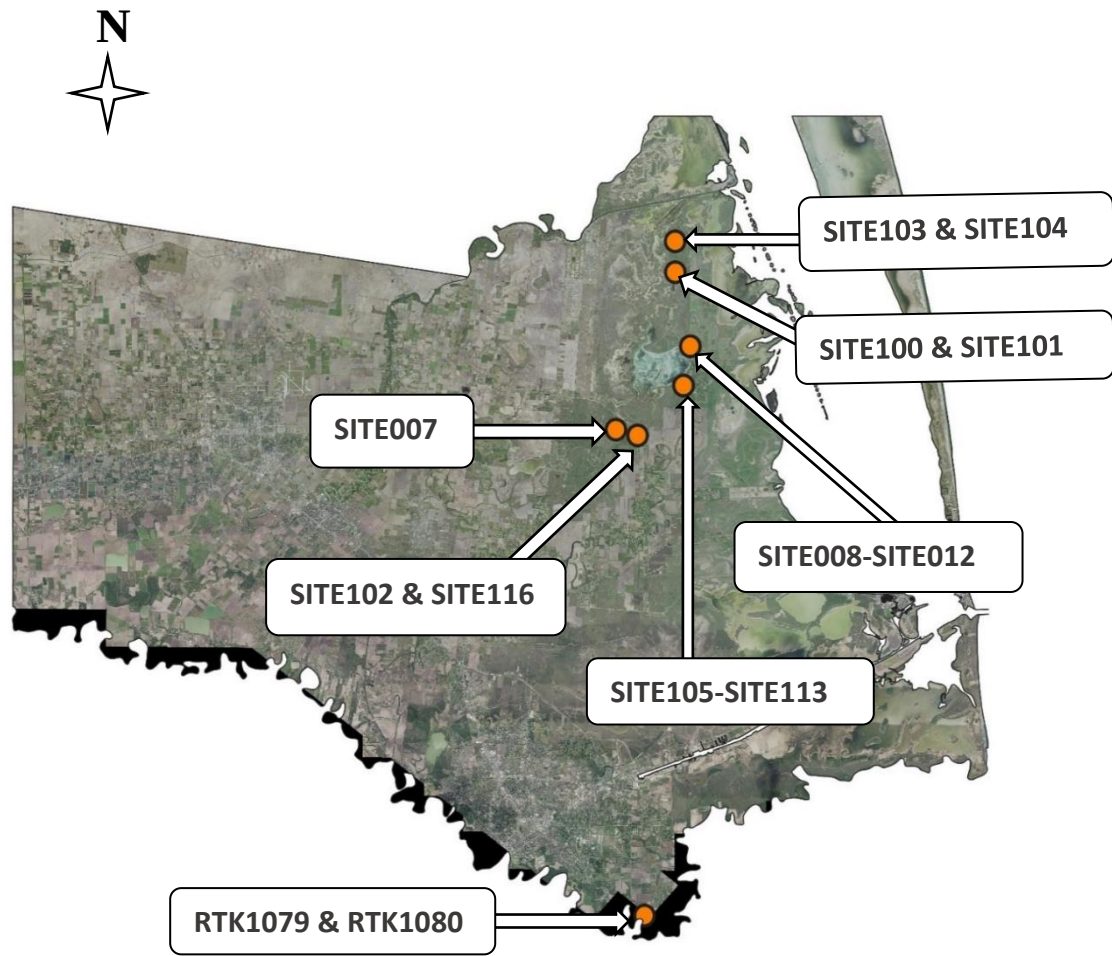


Figure 8. A map of Cameron County with location points for siren samples sequenced from Laguna Atascosa National Wildlife Refuge (SITE samples) and Southmost Preserve (RTK samples).

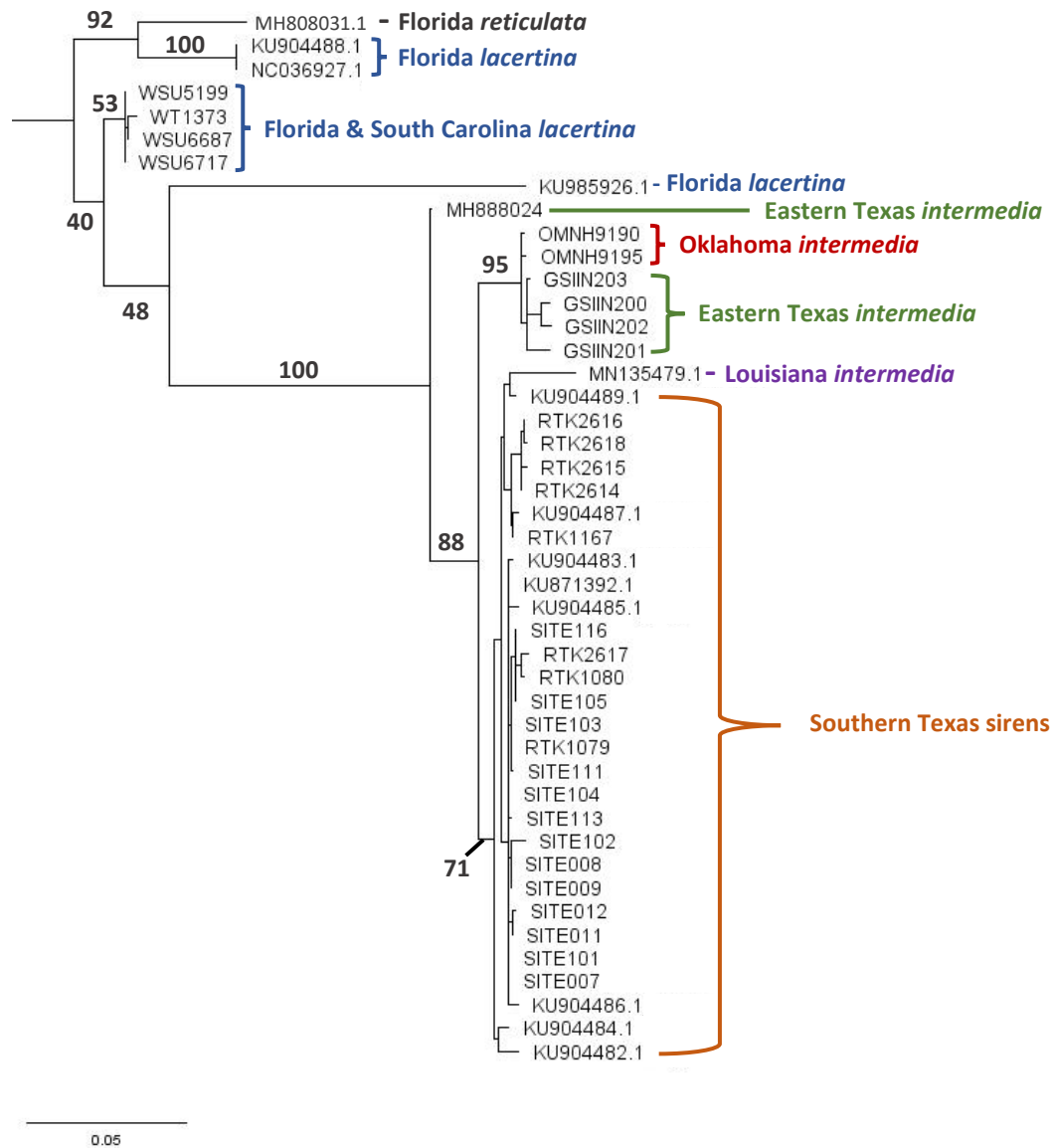


Figure 9. The CO1 maximum likelihood tree with each sample's location and identified species, if available. Bootstrap values are presented next to each node, excluding numbers deemed insignificant due to low value or short branch length. This tree was rooted with *Pseudobranchius*.

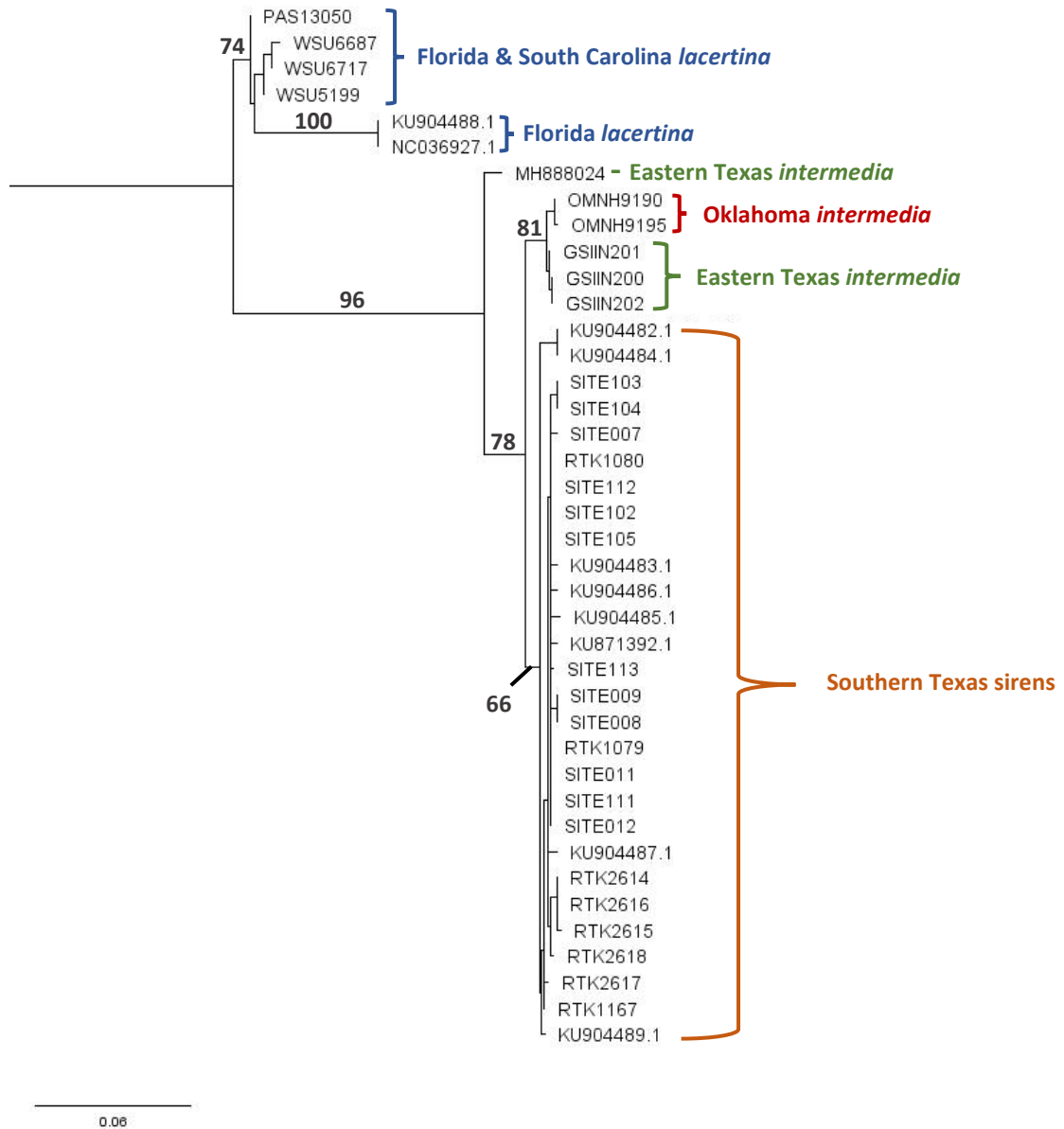


Figure 10. The ND2 maximum likelihood tree with each sample's location and identified species, if available. Bootstrap values are presented next to each node, excluding numbers deemed insignificant due to low value or short branch length. This tree was rooted with *Pseudobranchius*.

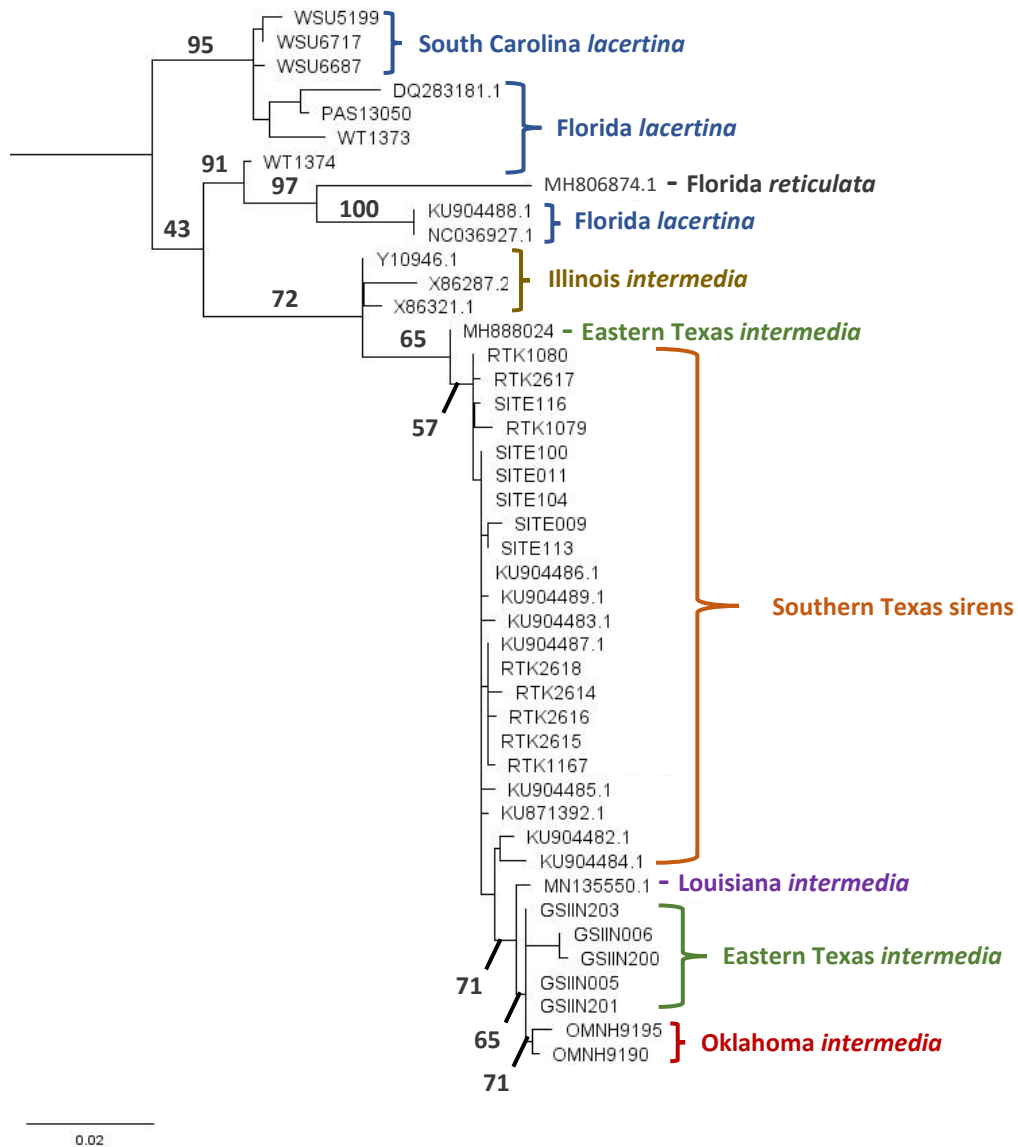


Figure 11. The 16S maximum likelihood tree with each sample's location and identified species, if available. Bootstrap values are presented next to each node, excluding numbers deemed insignificant due to low value or short branch length. This tree was rooted with *Pseudobranchius*.

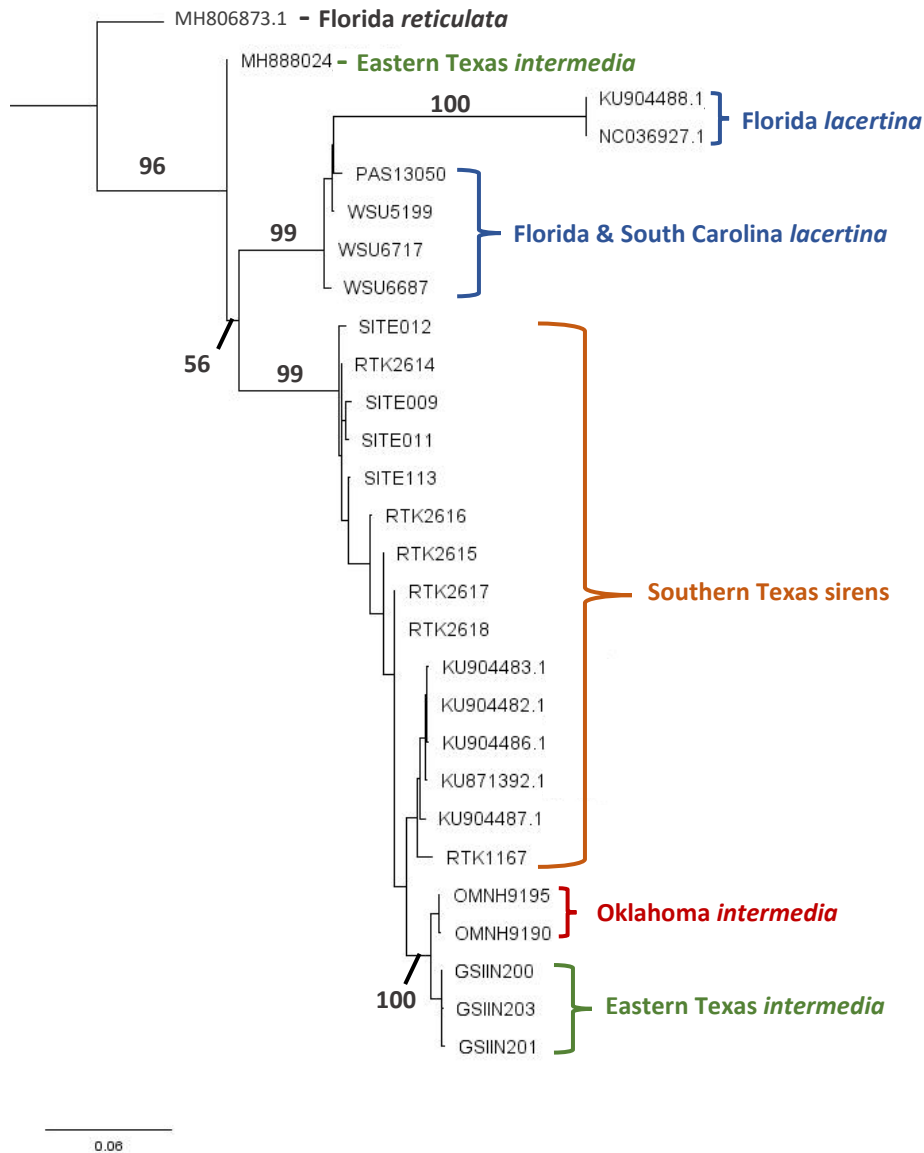


Figure 12. The ND5 maximum likelihood tree with each sample's location and identified species, if available. Bootstrap values are presented next to each node, excluding numbers deemed insignificant due to low value or short branch length. This tree was rooted with *Pseudobranchius*.

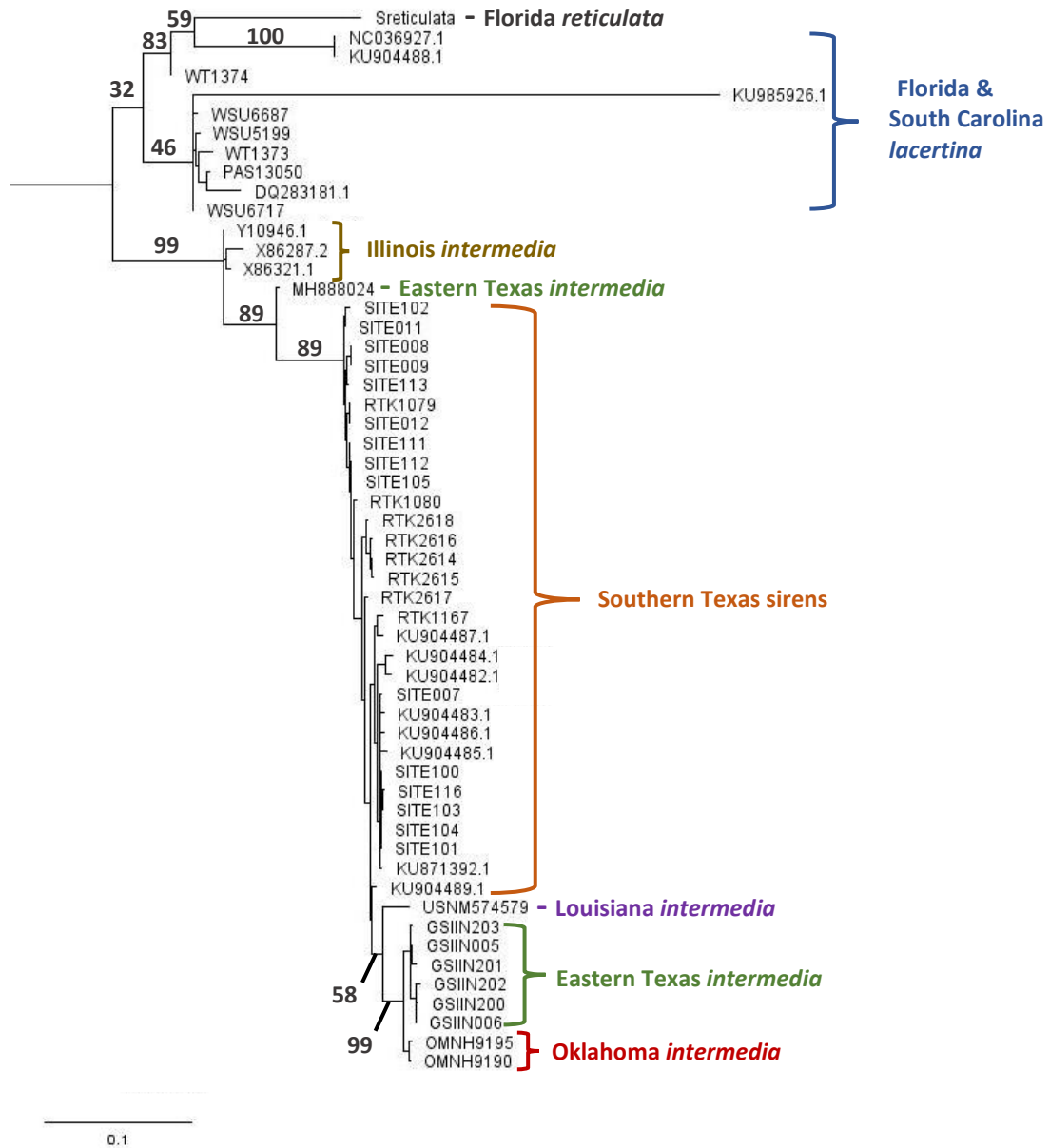


Figure 13. The concatenated maximum likelihood tree with each sample's location and identified species, if available. Bootstrap values are presented next to each node, excluding numbers deemed insignificant due to low value or short branch length. This tree was rooted with *Pseudobranchius*.

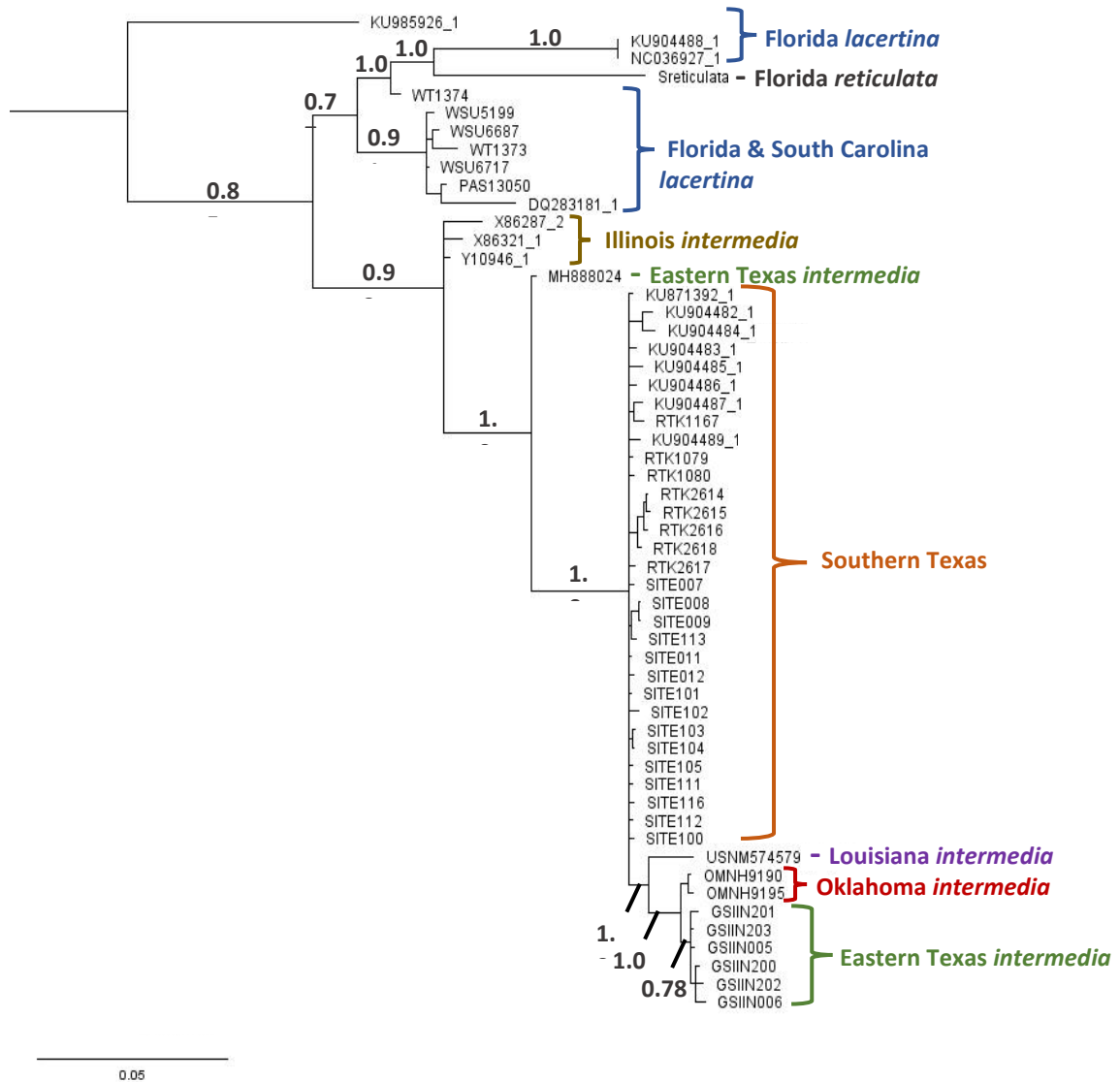


Figure 14. The concatenated Bayesian tree with each sample's location and identified species, if available. Posterior probabilities are presented next to each node, excluding numbers deemed insignificant due to low PP value or short branch length. This tree was rooted with *Pseudobranchius*.

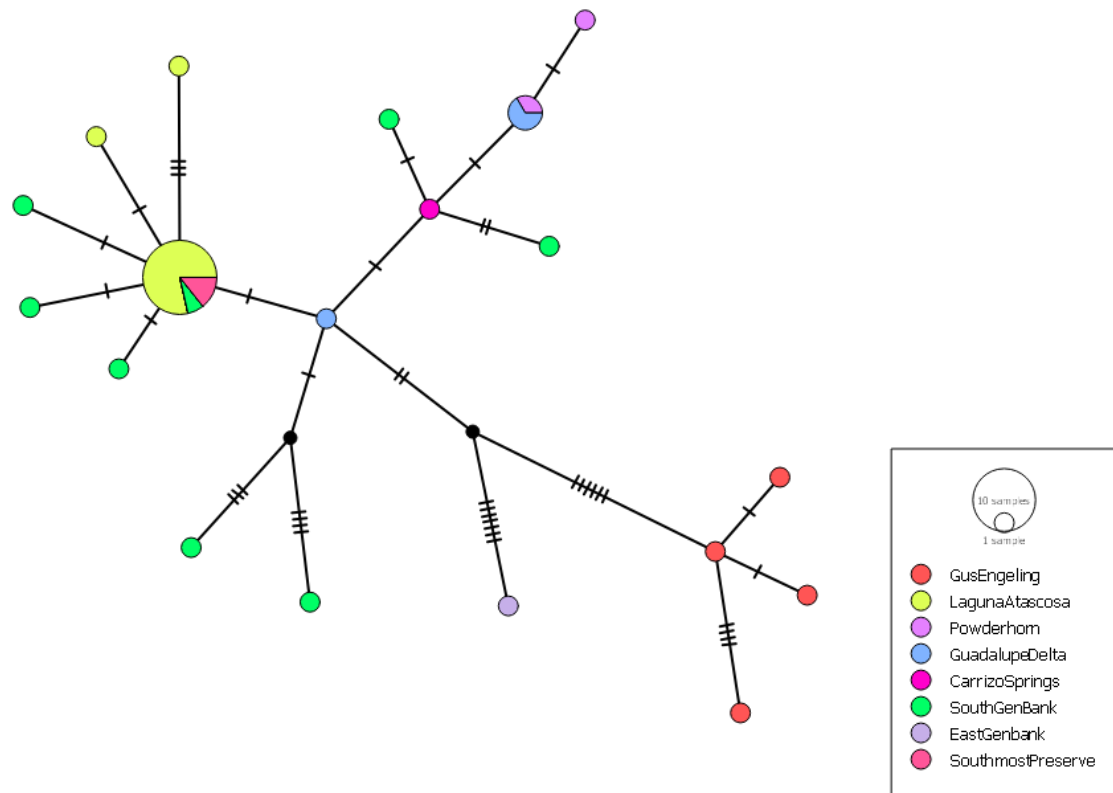


Figure 15. A Haplotype network of Texas sirens for the CO1 gene. The hatch marks represent the number of nucleotide differences, uncolored circles represent unsampled haplotypes, and the size of the circle is representative of the number of samples in that haplotype.

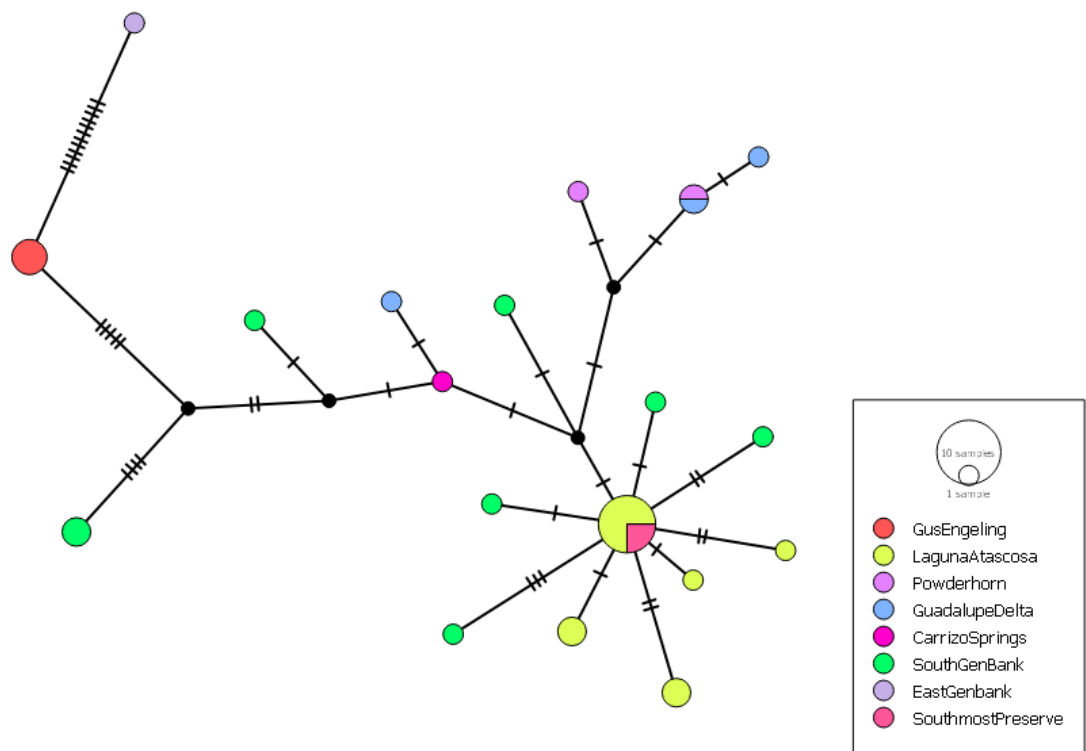


Figure 16. A Haplotype network of Texas sirens for the ND2 gene. The hatch marks represent the number of nucleotide differences, uncolored circles represent unsampled haplotypes, and the size of the circle is representative of the number of samples in that haplotype.

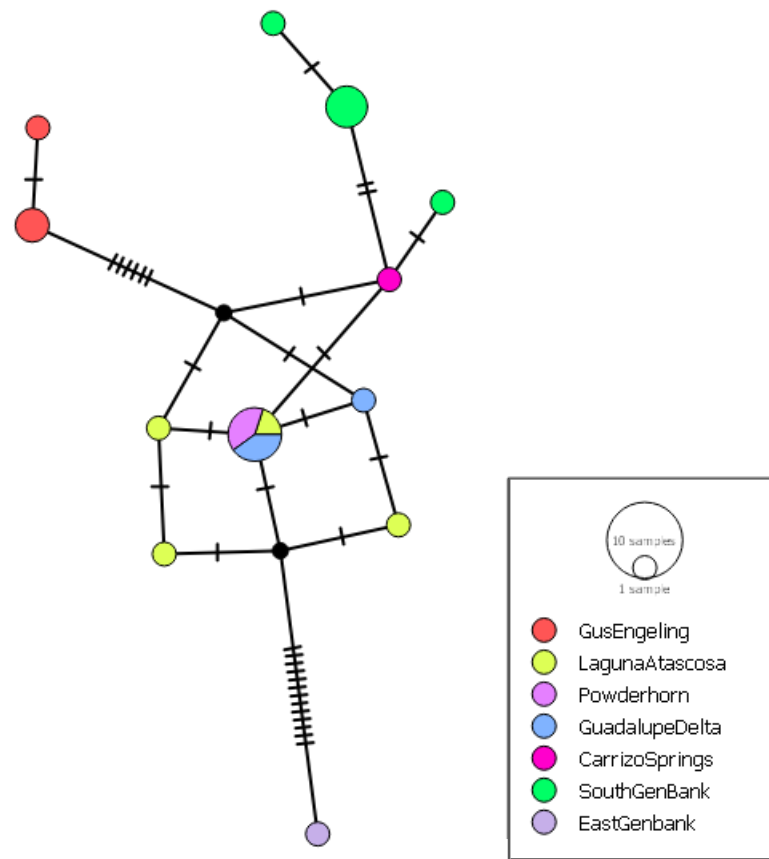


Figure 17. A Haplotype network of Texas sirens for the ND5 gene. The hatch marks represent the number of nucleotide differences, uncolored circles represent unsampled haplotypes, and the size of the circle is representative of the number of samples in that haplotype.

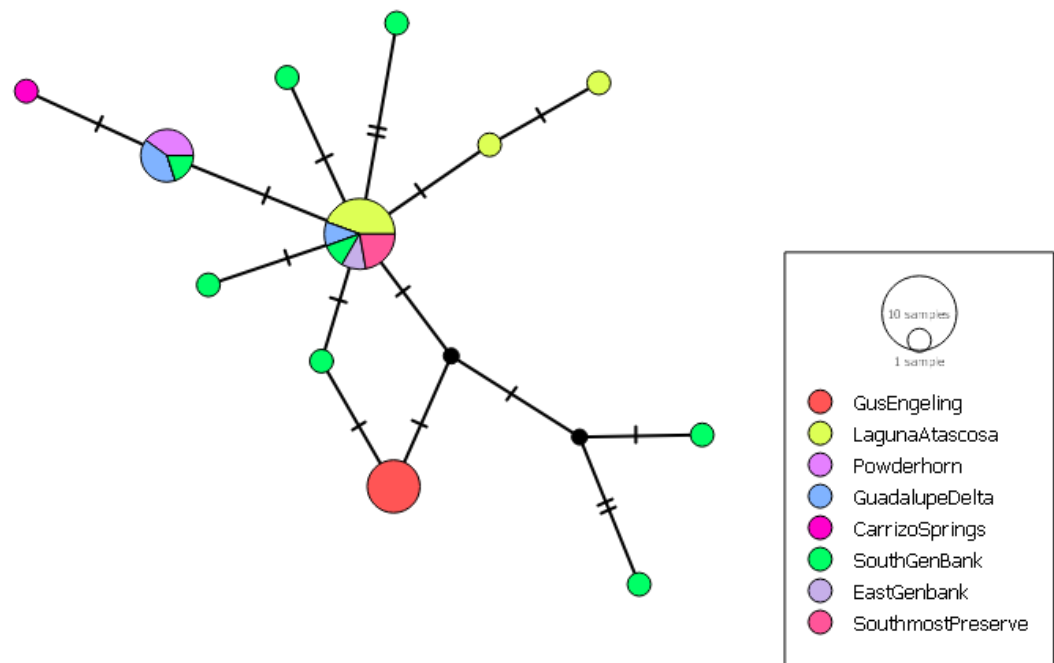


Figure 18. A Haplotype network of Texas sirens for the 16S gene. The hatch marks represent the number of nucleotide differences, uncolored circles represent unsampled haplotypes, and the size of the circle is representative of the number of samples in that haplotype.