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Unraveling *Siren* (Caudata: Sirenidae) systematics and description of a small, seepage specialist

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Abstract

For approximately four decades, scientists have known of the existence of several undescribed species of *Siren* in the southeastern United States Coastal Plain. One of these species, *S. reticulata*, was recently described, but a small, seepage-dwelling species has remained undescribed until now. To resolve outstanding questions concerning the phylogeny of *Siren*, we collected sequence and morphometric data from specimens across the range of *Siren*. We found *S. lacertina* and *S. reticulata* to represent strongly supported monophyletic groups, with *S. reticulata* having a sister relationship to all other *Siren*. Additionally, we found five distinct mtDNA lineages within what has been recognized as *S. intermedia*. *Siren lacertina* and type-locality *S. intermedia* (lineage A) are sister mtDNA lineages, whereas *S. intermedia* lineages B and C show a high level of mitogenomic divergence from type-locality *S. intermedia*. Analyses of two scnDNA loci revealed that *S. lacertina* is monophyletic but nested with low positional support in a clade including the three *S. intermedia* mtDNA lineages. Further study is needed to determine whether *S. intermedia* lineages A, B, and C represent distinct species or incompletely sorted lineages. We restrict the range of *S. intermedia* to the region from the Escambia and Perdido river drainages of Florida and Alabama eastward through Virginia (the combined ranges of lineages A, B, and C). We also elevate *S. i. nettingi* (lineage E) to species status and include the larger *S. i. texana* form in that taxon, generating a species that occurs from the Mobile Bay drainages westward through the Mississippi Basin and southwest into northeastern Mexico. Lastly, we describe a new miniature species, *S. sphagnicola*, that ranges from the Florida Parishes of Louisiana eastward to the westernmost tributary creeks of Choctawhatchee Bay in the western Florida panhandle.

Key words: Sirenidae, phylogeny, *Siren sphagnicola*, new species, Gulf Coastal Plain

Introduction

The family Sirenidae belongs to the order Caudata (salamanders) and currently comprises two genera, *Siren* Linnaeus and *Pseudobranchus* Gray. All extant members possess external gills with comb-like filaments, lidless eyes, and laterally compressed tails with fin blades; additionally, they lack pelvic girdles and associated hindlimbs, a trait unique within salamanders (Martof 1974). Despite the abundance of *Siren* in some aquatic habitats (Gehlbach & Kennedy 1978; Godley 1980; Sorensen 2004), relatively little research has been conducted on interspecific differences in natural history and phylogeography of the group.

Three *Siren* species are currently recognized. The Greater Siren (*S. lacertina* Linnaeus & Österdam [see Wahlgren 2011]) has been reported in the southeastern U. S. Coastal Plain from the vicinity of Chesapeake Bay, Virginia, south through peninsular Florida, and west to southwestern Alabama (Petranka 1998). The Lesser Siren (*S. intermedia* Barnes) ranges from northern Mexico along the Coastal Plain to Virginia and north throughout the Mississippi River drainage (Powell *et al.* 2016). The recently described Reticulated or Leopard Siren (*S. reticulata* Graham, Kline, Steen & Kelehear) has been reported to occur from the Fish River in Baldwin Co., Alabama, east to Okaloosa Co., Florida, and Covington Co., Alabama (Graham *et al.* 2018).

Relatively little taxonomic work on sirens has been conducted since Petranka (1998) suggested that multiple additional species could likely be described using genetic analyses. Additionally, confusion has existed regarding the validity of the description of *S. intermedia* due to a lack of diagnostic features (Noble & Marshall 1932). Noble

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and Marshall (1932) were unable to verify that the specimens Le Conte examined while describing *S. intermedia* were not actually small *S. lacertina*, because the specimen series was lost in the fire that destroyed the Lyceum of Natural History of New York in 1866, and the superficial differences used by Le Conte had no diagnostic value. Ultimately, Noble and Marshall (1932) provided a narrowed description of *S. intermedia* and suggested that the name be applied to the smaller *Siren* species found in the vicinity of the purported type locality. Three subspecies of *S. intermedia* have been described: *intermedia* Goin, 1942; *nettingi* Goin, 1942; and *texana* Goin, 1957. Prior to the use of genetic techniques, taxonomic assignments of *Siren* from the Mississippi River drainage southwest to the Rio Grande Valley were debated (Noble & Marshall 1932; McDaniel, 1969). Flores-Villela and Brandon (1992) attempted to assign sirens found in the Rio Grande Valley to either *S. intermedia* or *S. lacertina* using historical measurements of specimens and their egg capsules. Zhang and Wake (2009) sequenced mitochondrial genomes, and Bonett *et al.* (2013) sequenced nuclear genes from *Siren* and *Pseudobranchus* while investigating different aspects of caudatans. LaFortune (2015) examined *Siren* from Texas and failed to find genetic support for recognition of "texana" as a taxon distinct from *S. i. nettingi* and rejected the assignment by Flores-Villela and Brandon (1992) of "texana" specimens to *S. lacertina*. Although LaFortune (2015) contributed insights into the breadth of *Siren* diversity, she did not revise the taxonomy. Highton *et al.* (2017) thought the taxonomic statuses of the three *S. intermedia* subspecies remained unclear and deserved careful evaluation. Graham *et al.* (2018) used molecular data to describe *S. reticulata* from the western Florida panhandle and southern Alabama, but they indicated additional sampling and sequencing would be required to refine its placement and explain the variation among specimens that were identified as *S. intermedia*. Powell *et al.* (2019) provided a key distinguishing the three *Siren* species from each other using tail as a proportion of total length, presence or absence of gold spotting, style and presence of black spotting, and tail tip shape.

Herein, we use morphological and molecular data to evaluate *Siren*, focusing on specimens attributed to *S. intermedia* in Florida, Georgia, and Alabama. For approximately four decades, scientists have been aware of the existence of a small, seepage-dwelling siren in the Blackwater, East Bay, and Yellow river drainages of the western Florida panhandle that did not conform to descriptions of any recognized species. This miniature siren was investigated alongside what is now *S. reticulata* in the 1990s using allozyme, mitochondrial, and karyotype data, but results were not published. Licht and Lowcock (1991) determined that the karyotype of the miniature siren was 2n = 48, which differed from their *S. i. intermedia* karyotypes in the Florida panhandle (2n = 62) and peninsula (2n = 52) and from León and Kezer's (1974) *S. i. nettingi* from northern Texas (2n = 46). Enge (2005) trapped this undescribed siren species in five steephead streams in the Blackwater and Yellow river drainages and referred to it as the "Least Siren" (*S.* cf. *intermedia*). Moler (2019a) further mentioned this undescribed species. We describe this small siren species that inhabits seepage areas in the eastern Gulf Coastal Plain. We also evaluate the validity and assignment of *S. intermedia* subspecies based on genetic and morphological variation, including the large form from southern Texas and northeastern Mexico referred to as "texana."

Material and methods

Specimen acquisition. We collected specimens from most Florida drainages using traps, dipnets, and hand-raking. We focused primarily on collecting specimens in Florida because our suspected new species had only been documented from the East Bay, Blackwater, and Yellow river basins based upon previous herpetological surveys and specimens accessioned in the Florida Museum of Natural History. We used satellite imagery to select accessible sampling sites for the miniature siren in areas with seepage bogs and creeks in the Yellow, Blackwater, and Escambia river drainages. We collected larger species by dipnetting or trapping floodplains, creeks, and freshwater marshes accessible from roadsides or campgrounds. Because this project was primarily funded by the Florida Fish and Wildlife Conservation Commission, most Florida fieldwork was conducted by agency staff and volunteers. We obtained non-Florida specimens from private individuals and museums to supplement our sampling efforts and to ascertain the ranges of newly described species and potentially undescribed species from previous studies. We used ArcGIS (ESRI 2015) to plot the distribution of specimens we analyzed and determined to belong to recovered *Siren* species.

Molecular analyses. We derived sequence data from liver, muscle, or tail-tip tissues preserved in 95% ethanol or RNALater. We extracted genomic DNA from tissues using the QIAGEN DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) mixes contained 9.5 µl H_2O, 12.5 µl Go*Taq*® Master Mix (Promega Corp, Madison, Wisconsin, USA), 1.0 µl each primer (10 µM), and 1.0 µl genomic DNA template. We amplified two mitochondrial DNA (mtDNA) regions: 16S ribosomal RNA (472 bp) and nicotinamide adenine

dinucleotide dehydrogenase subunit 4 (ND4, 718 bp). We also amplified two single-copy nuclear DNA (scnDNA) regions: recombination activating gene 1 (RAG1, 714 bp) and Sodium/Calcium Exchanger 1 (NCX1, 541 bp). Primers used for PCR and sequencing appear in Table 1. We conducted PCRs by denaturing at 95°C for 5 min, then 35 cycles of amplification (denaturing at 95°C for 1 min, annealing at primer-specific temperatures (Table 1) for 1 min, and extension at 72°C for 1 min), followed by a final extension at 72°C for 5 min. For each PCR product, 4.0 µl were electrophoresed on a 2% agarose gel, visualized with GelRed™ staining (Biotium Inc., Hayward, California, USA), and compared with a DNA standard. PCR products with a distinct band were sent to Genewiz, Inc (South Plainfield, New Jersey, USA) for sequencing. We assembled, verified, and aligned sequence files with Geneious 9.1.8 (https://www.geneious.com), after which we trimmed sequences to the aforementioned lengths for each region. We submitted genetic sequence data to GenBank (OP440447–OP440530, OP442970–OP443313) for public access.

TABLE 1. Primers used for PCR and sequencing gene fragments.

Gene region	DNA marker	Primer name	Primer sequence	Annealing temp. (°C)	Source
16S	mtDNA	16Sar	CGCCTGTTTATCAAAAACAT	51°C	Kessing *et al.* 1989
16S	mtDNA	16Sbr	CCGGTCTGAACTCAGATCACGT	51°C	Kessing *et al.* 1989
NADH-4	mtDNA	DW1641T	TGACTACCAAAAGCTATGTAGAAGC	52°C	
NADH-4	mtDNA	ND4-Leu Croc	CATTACTTTTACTTGGATTTGCAC	52°C	
RAG1	scnDNA	SirenidaeRAG1bF	ACG CCT TGA AGC ATC CCA GAA CAT	58°C	Bonett *et al.* 2013
RAG1	scnDNA	SirenidaeRAG1bR	CCA ATG GTG CTT CAG AAC ATC CTC	58°C	Bonett *et al.* 2013
NCX1	scnDNA	NCX1 Siren F	ATC GCG ATT TGC GTC TAC GTG	58°C	Bonett *et al.* 2013
NCX1	scnDNA	NCX1 Siren R	ACA CCA CTG TTC CTT CGG TGA ACT	58°C	Bonett *et al.* 2013

Phylogenetic analysis. We ran Bayesian Inference (BI) using BEAST v2.5.0 (Drummond *et al.* 2006; Bouckaert *et al.* 2014) to estimate the phylogenetic relationships of our samples. The corrected Akaike Information Criterion (AICc) in jModelTest (ver. 2.1.7; Guindon & Gascuel 2003; Darriba *et al.* 2012) determined that the best-fit nucleotide substitution model was General Time Reversible (GTR) + gamma (Γ) for 16S and ND4 regions. We produced the mtDNA phylogeny using the 'BEAUti2: standard' template (Heled & Drummond 2010). We generated parameters for the mtDNA tree using a relaxed lognormal clock, estimated base frequencies, a coalescent constant population size, and randomly generated starting trees. We set the Markov chain Monte Carlo runs to 20 million generations with 2 million generations of burn-in and retained every 5,000th sample. Median joining networks (Bandelt 1999) of haplotypes were generated for RAG1 and NCX1 using PopART (Leigh & Bryant 2015). We used ASAP (Puillandre *et al.* 2021) to estimate species delimitations. We calculated minimum between lineage p-distances and maximum p-distances within lineages using TaxI2 (Steinke *et al.* 2005) and the average number of nucleotide substitutions per site (Dxy) between lineages and fixed genetic differences of mitochondrial data using DnaSP (ver. 6.12.01; Rozas *et al.* 2017).

Morphological analyses. A recent dichotomous key by Powell *et al.* (2019) used body measurements and proportions to distinguish among *Siren* species. We measured snout-vent length (SVL) from tip of the snout to the anterior of the cloacal sphincter (following Noble & Marshall 1932). For specimens with apparently undamaged and non-regenerated tails, we measured tail length (TaL) as the distance to the tail tip from the anterior margin of the cloacal sphincter. We also measured head width, head length, interorbital distance, eye snout distance, and chest width of specimens that were well preserved and intact (not bloated, desiccated, or damaged by dissection) following diagrams provided by Bingham *et al.* (2018). We standardized these size data by taking the log of each of five measurements and then subtracting each transformed measurement from its respective transformed SVL (Mosimann & James 1979). We determined sex by examining gonads after dissection. We tentatively assigned life stage based on a specimen's size relative to the smallest reproductive female of the lineage (i.e., smallest specimen with ova or

eggs present in ovaries or oviducts), but we did not use life stage in any analysis. We classified some specimens as "undeterminable" for sex or life stage because of small size or previously removed reproductive organs.

Because most *Siren* lose color after preservation and some species have similar coloration and patterns in life, costal grooves have become the primary character used to distinguish among taxa. Costal grooves correspond to single vertebrae and their associated trunk muscles (Highton 1957; Litvinchuk & Borkin 2003). However, methods for counting costal grooves in salamanders are inconsistent (Litvinchuk & Borkin 2003) and need to be standardized for comparative purposes. Researchers often neglected to mention their methodology (Noble & Marshall 1932; Goin 1942; Flores-Villela & Brandon 1992; Parmley & Gaddis 1999), causing variations of up to three counted grooves. One counting method included the axillary groove that touched the posterior of the forelimb and the inguinal groove that touched the anterior of the hindlimb (Cope 1889; Dunn 1923). Another method excluded the axillary and inguinal grooves and counted only grooves between but not touching the limbs (Misawa 1989). We chose to use the latter method because the axillary groove is usually obfuscated by the flexible arm pit (and sometimes gills), making the first post axillary groove the first distinct "groove" on the body. Because *Siren* lacks hindlimbs, an alternative termination point for counting grooves needs to be specified. McDaniel (1969) counted "all complete grooves between axilla and vent," whereas Graham *et al.* (2018) counted grooves "from the axial region to the posterior vent." We chose to terminate our count at the last groove before the anterior edge of the cloacal sphincter both to align this count with our SVL measurement and to avoid counting additional costal grooves when the cloaca expands posteriorly in the cloacal groove, which is not visible in every specimen.

We produced graphs comparing costal grooves and TaL-to-TL proportions of the different mtDNA lineages recovered. We conducted principal component analysis and linear discrimination analysis using R 4.2.0 (R Core Team 2022) on our size-corrected morphological data to test whether variation existed among *Siren* lineages.

We modeled the effects of lineage on the number of costal groves using a Conway-Maxwell Poisson regression (Huang 2017) instead of a conventional Poisson regression because goodness of fit assessments based on simulations of model residuals, which were implemented in the DHARMa package (Hartig 2021), indicated evidence of significant under-dispersion (dispersion ratio = 0.024, p-value < 0.001). Lineage was the only predictor variable in this model and was included as a categorical variable. Following model-fitting, we tested for significant pairwise differences in the expected number of costal groves among the lineages (emmeans package; Length 2021).

For all other measured structures, we tested for differences among lineages using Kruskal-Wallis tests. For all measurements except SVL and TaL, we used the difference between the log of SVL and the log of the other measurement as the response variable. We first assessed if the data met the assumptions of ANOVA. We tested for equality of variance among lineages using a Bartlett's test, and we tested for normality using a Shapiro-Wilk test. If a measurement had either unequal variance among lineages or lacked a normal distribution, we tested for significant differences among groups using a Kruskal-Wallis test. If a Kruskal-Wallis test indicated that significant differences existed, we followed up with a post-hoc Dunn's test. We adjusted p-values of post-hoc Dunn's tests using the methods of Benjamini & Hochberg (1995). All tests were compared to $\alpha = 0.05$ and were performed in R.

Data for the 294 specimens are provided here: 10.6084/m9.figshare.22043294.

Results

Phylogenetic analysis. Our mitochondrial tree of *Siren* returned five lineages of *S. intermedia*, a monophyletic *S. lacertina* nested within *S. intermedia*, and divergent *S. reticulata* that rests sister to all other *Siren*. *Siren lacertina*, *S. reticulata*, and all except one *S. intermedia* lineage (C) are well supported (posterior probability [PP] > 0.95).

Herein, we refer to the *S. intermedia* mtDNA clade consisting of specimens collected east of the Apalachicola drainage in Florida and Georgia (including the purported type locality) as *S. intermedia* lineage A. This clade has a sister relationship to *S. lacertina* in the mtDNA tree (Fig. 1) and shares haplotypes for RAG1, but *S. lacertina* is monophyletic on the NCX1 gene (Fig. 2). *Siren intermedia* collected from the Apalachicola river drainage west to the Escambia river drainage form a separate mtDNA clade (Fig. 1), *S. intermedia* lineage B. Specimens from North and South Carolina form a third mtDNA clade, *S. intermedia* lineage C. The few specimens of lineage C in our dataset exhibited relatively high genetic variability. Because this lineage is the only one with a PP < 1 (PP = 0.77), we believe that it should be treated as a catch-all for the under sampled Atlantic drainages from the Savannah River north to Chesapeake Bay, and we suspect that future studies may show that it comprises several lineages. Lineage D consists of the miniature siren form collected from the western Florida panhandle along with a single larva (genetic

sample only) purportedly from Talisheek Creek in St. Tammany Parish, Louisiana. The final *S. intermedia* lineage (E) belongs to the western subspecies *S. i. nettingi* that ranges from the Mobile Bay drainages west and north through the Mississippi River drainage and south through the Gulf coast drainages to the State of Veracruz, Mexico. Both nuclear and mitochondrial data show that the eastern subspecies, *intermedia,* and the western subspecies, *nettingi* (including "texana"), are paraphyletic and share no haplotypes on either of the nuclear loci. Additionally, *S. lacertina* is nested within the five *S. intermedia* mitochondrial clades with a sister relationship to *S. intermedia* lineage A. Statistics for minimum pairwise identity, number of fixed differences, maximum pairwise identity, and average number of nucleotide substitutions per site (Dxy) for both mtDNA loci are presented in Tables 2 and 3.

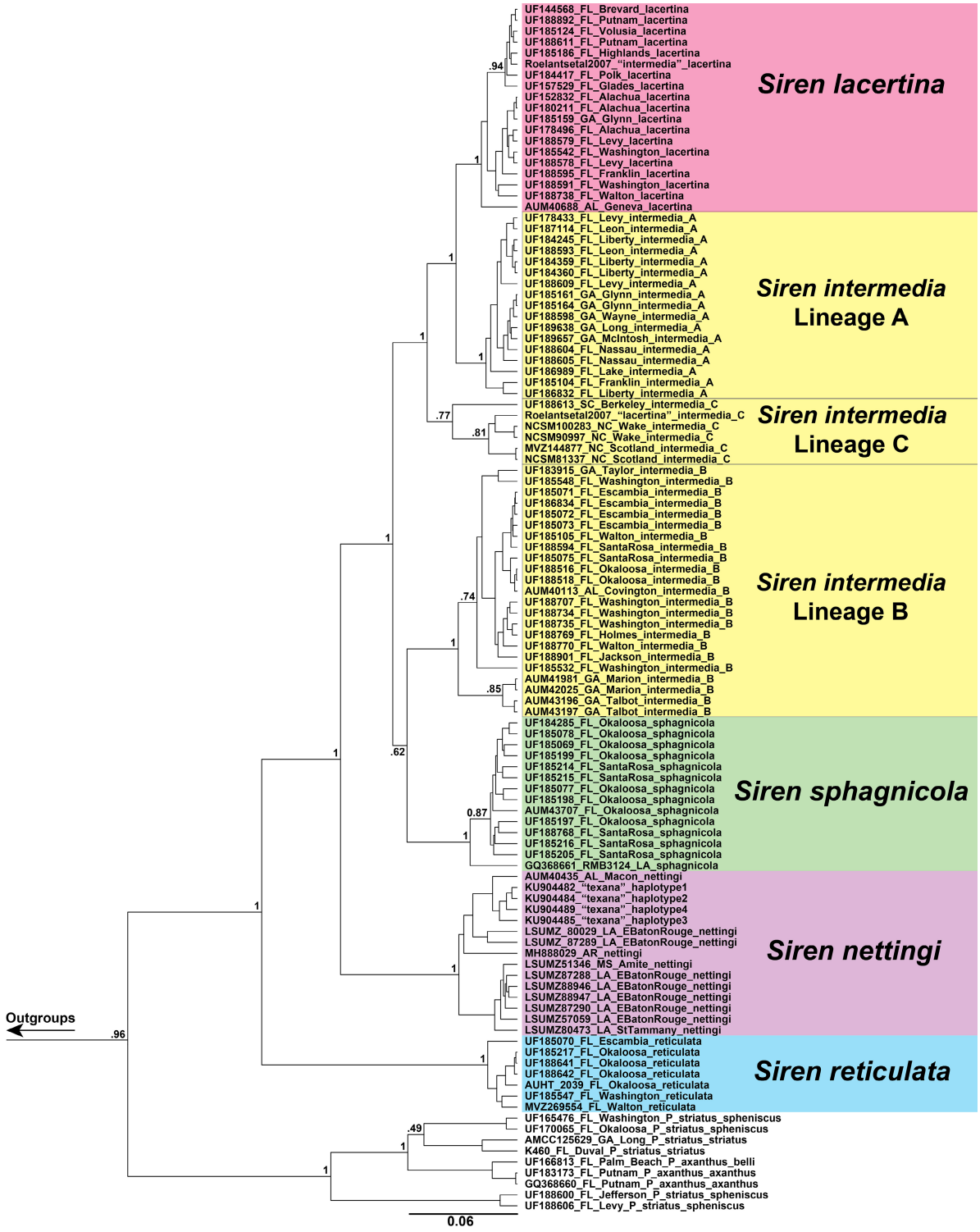

FIGURE 1. Bayesian probability tree of concatenated 16S and ND4 regions inferred using BEAST2. Individual specimen labels include voucher ID or GenBank number if no voucher, state, county, and lineage.

TABLE 2. Minimum pairwise identity and (number of fixed differences) are below the diagonal, maximum pairwise identity on the gray diagonal, and average number of nucleotide substitutions per site (Dxy) above the diagonal within and between each lineage of *Siren* and the sister genus *Pseudobranchus* for 16S sequence data (496 base pairs).

	lacertina	***intermedia* lineage A**	***intermedia* lineage B**	***intermedia* lineage C**	***cf. intermedia* lineage D "*sphagnicola*"**	***cf. intermedia* lineage E *nettingi***	***reticulata***	***Pseudobranchus***
lacertina	0.01066	0.01457	0.02112	0.01643	0.01536	0.05721	0.04988	0.10393
***intermedia* lineage A**	0.008529 (3)	0.01279	0.02747	0.02193	0.02496	0.06389	0.05676	0.10503
***Intermedia* lineage B**	0.006397 (1)	0.01706 (4)	0.01919	0.02175	0.02068	0.06425	0.05871	0.09666
***Intermedia* lineage C**	0.008529 (2)	0.01493 (4)	0.006397 (1)	0.01279	0.01599	0.05906	0.05615	0.10597
***cf. intermedia* lineage D "*sphagnicola*"**	0.01066 (4)	0.02132 (8)	0.008529 (3)	0.01066 (4)	0.01279	0.06103	0.05721	0.10792
cf. intermedia* lineage E *nettingi	0.05117 (19)	0.05757 (22)	0.05117 (19)	0.0533 (20)	0.05544 (22)	0.02132	0.07013	0.11161
Reticulata	0.04691 (21)	0.0533 (23)	0.04904 (21)	0.05117 (23)	0.05544 (26)	0.0661 (27)	0.0043	0.09367
Pseudobranchus	0.0917 (26)	0.09168 (25)	0.08316 (22)	0.09168 (25)	0.09808 (29)	0.1002 (24)	0.08955 (22)	0.08529

TABLE 3. Minimum pairwise identity and (number of fixed differences) are below the diagonal, maximum pairwise identity on the gray diagonal, and average number of nucleotide substitutions per site (Dxy) above the diagonal within and between each lineage of *Siren* and the sister genus *Pseudobranchus* for ND4+tRNA's sequence data (717 base pairs).

	lacertina	***intermedia* lineage A**	***intermedia* lineage B**	***intermedia* lineage C**	***cf. intermedia* lineage D "*sphagnicola*"**	***cf. intermedia* lineage E *nettingi***	***reticulata***	***Pseudobranchus***
Lacertina	0.04463	0.05143	0.09277	0.08379	0.10325	0.09593	0.13621	0.14960
***Intermedia* lineage A**	0.03944 (12)	0.03068	0.08576	0.07211	0.09878	0.08935	0.13508	0.15040
***Intermedia* lineage B**	0.0779 (35)	0.08062 (33)	0.04742	0.07806	0.07086	0.07729	0.12068	0.14048
***Intermedia* lineage C**	0.0618 (22)	0.05485 (23)	0.06206 (25)	0.05866	0.09088	0.09634	0.13617	0.14654
***cf. intermedia* lineage D "*sphagnicola*"**	0.08696 (39)	0.09129 (42)	0.05775 (22)	0.07123 (26)	0.03905	0.09111	0.13338	0.14574
cf. intermedia* lineage E *nettingi	0.07801 (29)	0.06818 (29)	0.06215 (16)	0.06497 (22)	0.06713 (24)	0.07273	0.10340	0.12883
Reticulata	0.1152 (72)	0.1195 (76)	0.1072 (57)	0.1133 (68)	0.1103 (65)	0.08333 (39)	0.03077	0.13291
Pseudobranchus	0.133 (41)	0.1401 (44)	0.1164 (28)	0.1325 (38)	0.1221 (39)	0.1082 (29)	0.1077 (36)	0.1409

Haplotype networks of RAG1 and NCX1 do not fully reflect the relationships inferred from the mitochondrial data, but taken together, their signal supports that gene flow among the mitochondrial lineages is also at least partially restricted from a nuclear genomic perspective. The RAG1 haplotype network shows several shared haplotypes of *S. intermedia* lineages A–C and *S. lacertina* and high diversity among *S. intermedia* lineages A–C (Fig. 2). We also found that *S. intermedia* lineage D forms a phylogroup with only three RAG1 haplotypes present, lineage E is similarly a separate phylogroup with 15 haplotypes, and *S. reticulata* has only one haplotype unique from other *Siren* (Fig. 2). The NCX1 haplotype network shows *S. lacertina*, *S. reticulata*, and *S. intermedia* lineage E haplotypes each forming one phylogroup (Fig. 2). *Siren intermedia* lineages A and B share several haplotypes, whereas lineage C forms a phylogroup but is only one mutation away from lineage A (Fig. 2). *Siren intermedia* lineage D has two sets of non-sister haplotypes with one set sister to lineage E (*nettingi*) and the other sister to the lineage A+ B complex (Fig. 2).

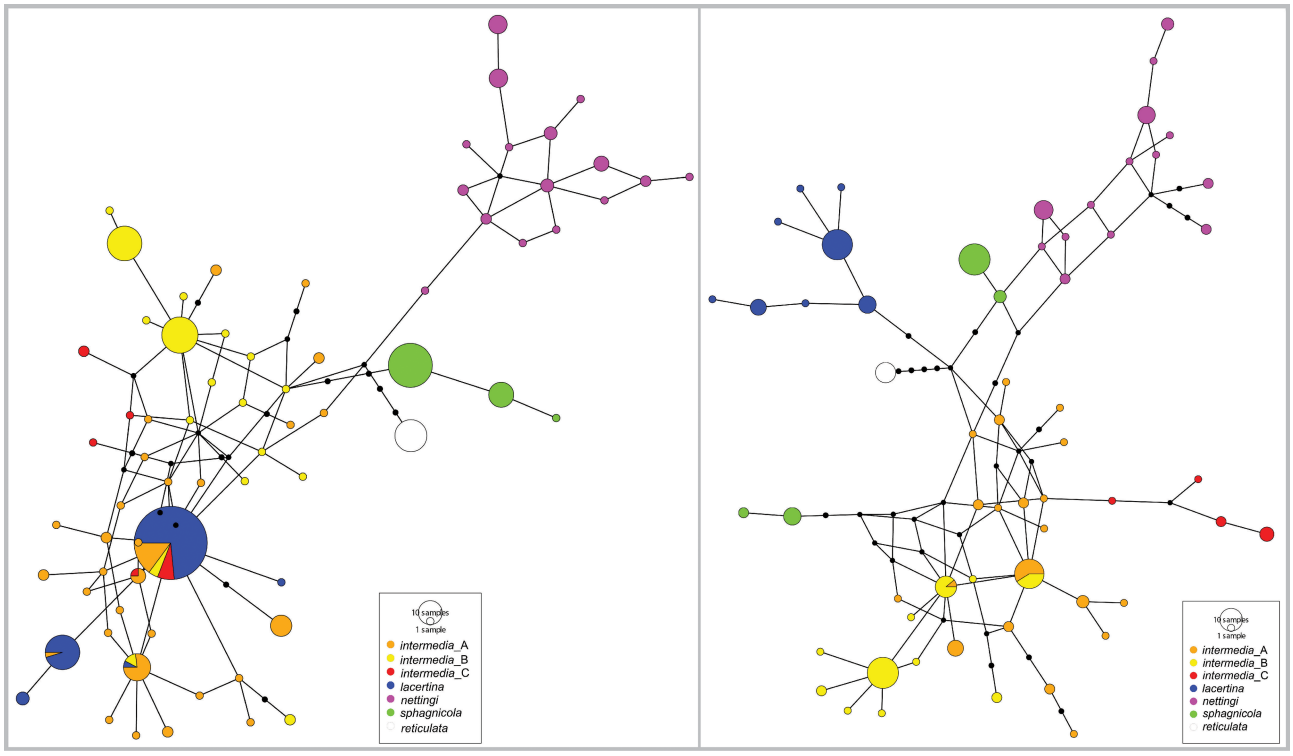

FIGURE 2. Median joining networks of *Siren* haplotypes for RAG1 (left) and NCX1 (right) regions made using PopART.

The ASAP species estimates based on genetic distance identified most lineages as candidate species, but it separated the *S. intermedia* lineage D sample from Louisiana from the Florida lineage D samples, and samples of *S. intermedia* lineage C from each another. We believe that these splits are likely due to natural genetic variation by geographic distance and to missing samples from intervening drainages.

Morphological analyses. Ranges in the proportion of TaL to total length (TL) for each *Siren* species overlap substantially (Fig. 3). Tails of most *Siren* species comprise up to 32–36% of their TL. Some *S. intermedia* from various lineages exhibit more extreme proportions and have tails >40% of their TL, but the range in tail proportions still overlaps substantially with other *Siren*.

Comparisons of costal groove count by mtDNA lineages are presented in Fig. 4. Costal groove counts of *S. intermedia* (lineage A = 29–33, lineage B = 29–32, lineage C = 30–33) and *S. lacertina* (35–39) do not overlap. Costal groove counts of *S. intermedia* lineage E "*nettingi*" (31–36) overlap those of *S. intermedia* and slightly overlap those of *S. lacertina* (Fig. 4), but identification is simplified because lineage E is apparently not sympatric with *S intermedia* lineages A–C or *S. lacertina*. *Siren reticulata* has the highest costal groove counts (39–41), and only one of 56 *S. lacertina* specimens examined had 39 costal grooves. Costal groove counts of our *S. intermedia* lineage D *"sp. nov."* (30–33) overlap those of *S. intermedia* lineages B and E (Fig. 4). In areas of sympatry between *S. intermedia* lineages B and D, lineage B specimens have a reduced costal groove range of 29 (n =12), 30 (n = 23), or 31 (n = 3). Most specimens of each species cluster in a range of one or two costal grooves with few outliers.

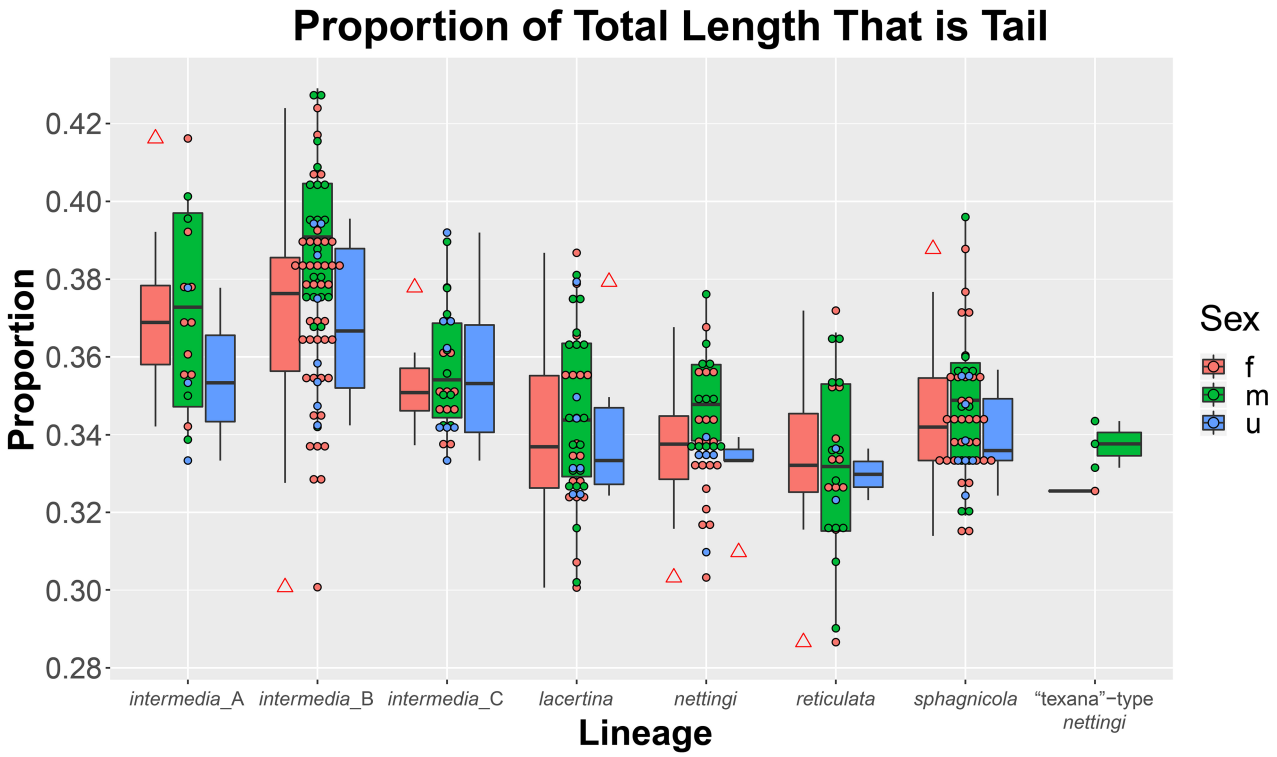

FIGURE 3. Box plots with points colored by sex showing distribution of tail proportions of *Siren* lineages. Outliers are noted with red triangles on the associated box plot.

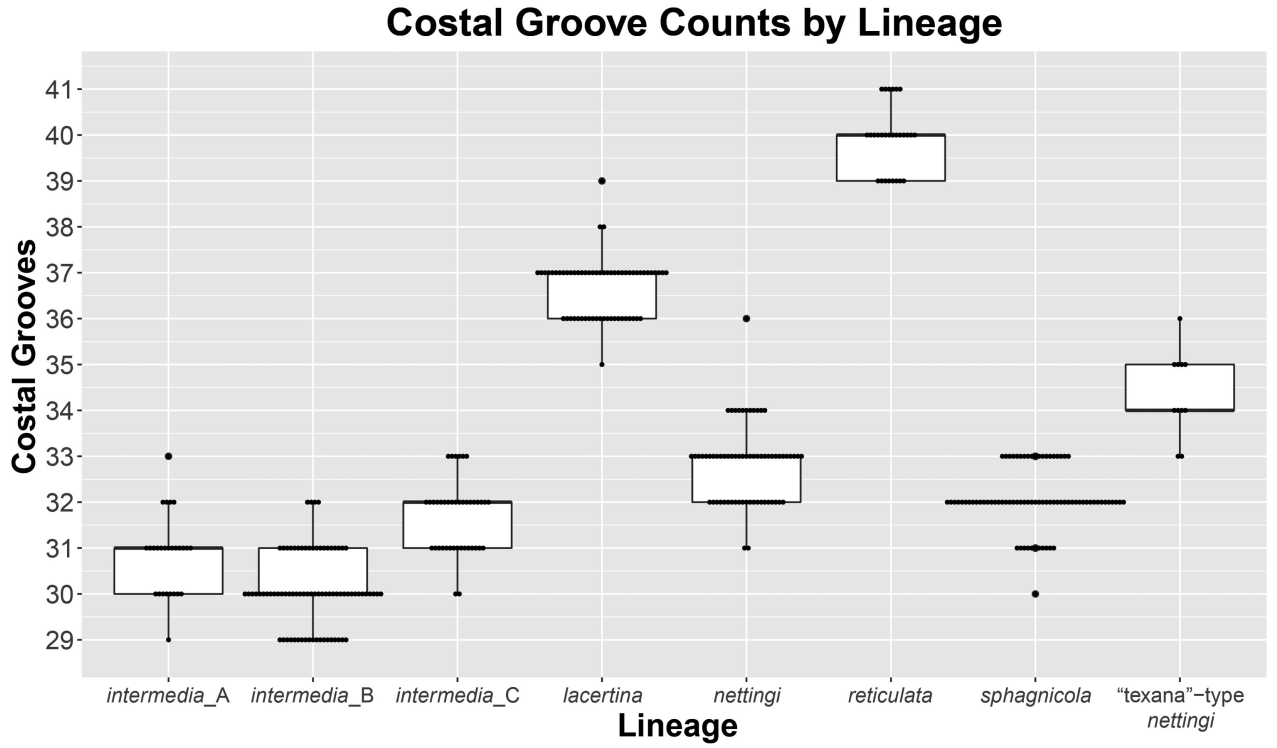

FIGURE 4. Box plots with points representing specimens per showing distribution of costal groove counts of *Siren* lineages.

Our statistical analyses of costal grooves by lineage using Conway-Maxwell Poisson regression and of each linear measurement by lineage using Dunn's tests showed that significant differences existed in the distributions of most species (Table 4). Distributions of all standardized linear measurements except tail length are plotted in Fig. 5. All *Siren* lineages had statistically distinct distributions of costal grooves except *intermedia* C and *intermedia*

D *sp. nov.* Head length was the most distinct measurement and had the least number of nonsignificant differences among lineages. Comparisons of morphological measurements of lineages A and B using the Kruskal-Wallis test and subsequent Dunn's test found that six of seven measured features did not differ significantly. These were our best-sampled *intermedia* lineages and included juveniles and adults, whereas lineages C and E contained mostly adults. Interestingly, lineage C did not differ significantly from lineages A and B in only two of seven measurements, whereas lineage E did not differ significantly from lineage A in four measurements, lineage B in three measurements, and lineage C in five measurements.

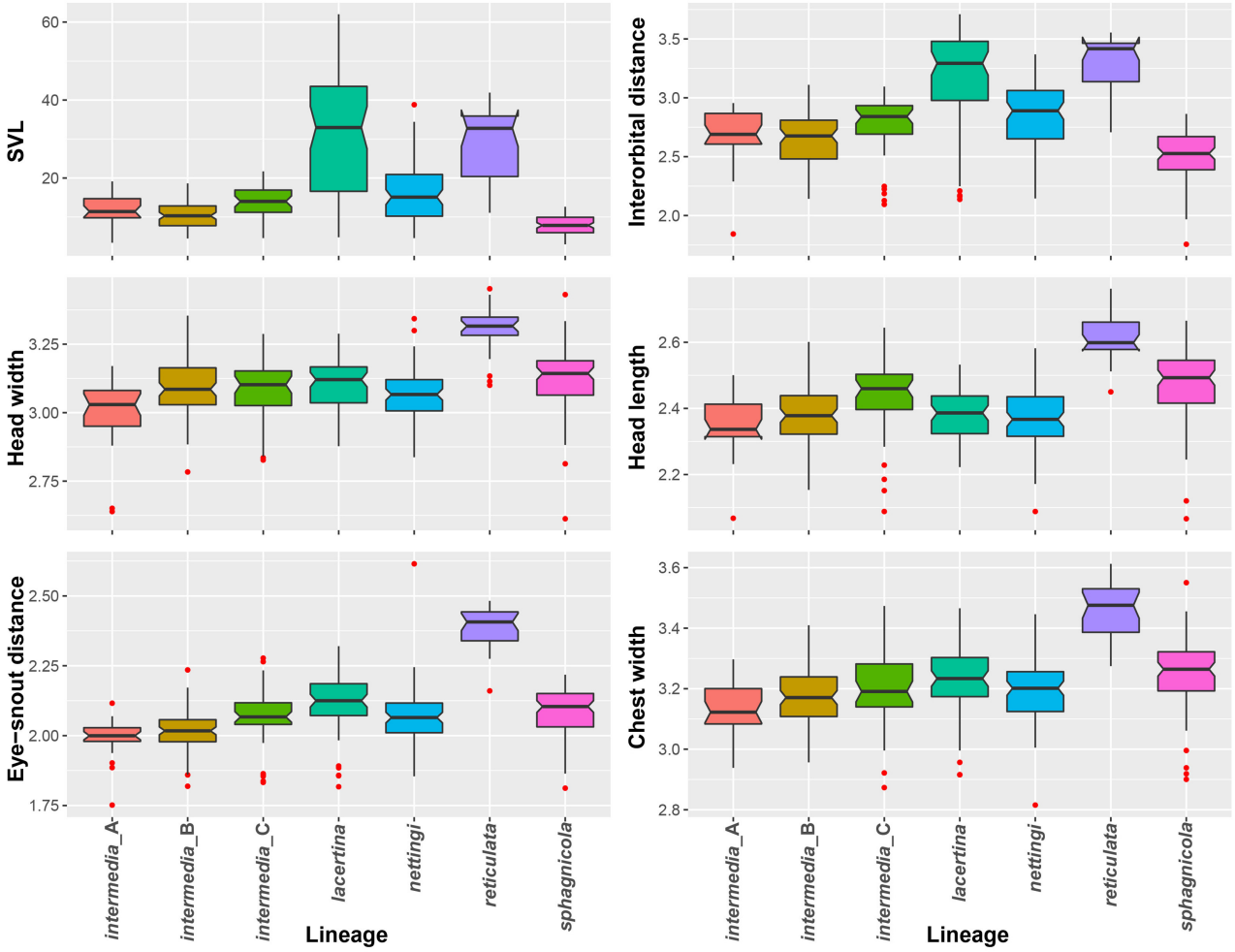

FIGURE 5. Box plots of the distributions of snout-vent length and the five size-corrected linear measurements compared using linear discriminant analysis.

The results of our linear discriminant function analysis of size-corrected measurements are shown in Fig. 6, with LD1 representing 54.86% of variation and LD2 representing 40.66%. Both LD1 and LD2 are primarily influenced by eye-snout distance, then head length, and then interorbital distance. This analysis indicates that *S. reticulata* is readily distinguishable from other *Siren* species using these measurements, whereas *S. lacertina* and *S. intermedia* lineage D have confidence ellipses that separate them from the mostly overlapped *S. intermedia* lineages A, B, C, and E.

Species and lineage determination. We attributed specimens to a species or mitochondrial lineage based initially on whether it was strongly supported by sequence data (PP > 0.95). These clades correspond to the thresholds identified using ASAP except for *S. intermedia* lineages C, D, and E. Lineages C and D lack specimens spanning several drainages. When a specimen was not sequenced, we assigned it to a lineage based on costal groove count (*S. lacertina* and *reticulata*), geography (based on the ranges of *S. intermedia* lineages A, B, C, and E), and (rarely) phenotypic traits that we found separated lineages with overlapping ranges (*S. intermedia* lineages B and E versus lineage D).

TABLE 4. Comparisons of morphometric comparisons between recovered mtDNA lineages of *Siren*. Non-distinct distributions ($p > 0.05$) are highlighted on light gray.

Comparison	Costal grooves	SVL	Tail length	Interorbital	Head width	Head length	Eye snout	Chest width
intermedia A–*intermedia* B	<0.001	0.119	0.198	0.002	0.221	0.158	0.052	0.103
intermedia A–*intermedia* C	<0.001	0.161	0.317	0.005	0.001	0.001	0.012	0.002
intermedia B–*intermedia* C	<0.001	0.003	0.043	0.495	0.001	0.002	0.156	0.013
intermedia A–*lacertina*	<0.001	<0.001	0.001	<0.001	0.218	<0.001	<0.001	0.003
intermedia B–*lacertina*	<0.001	<0.001	<0.001	0.164	0.499	<0.001	0.002	<0.001
intermedia C–*lacertina*	<0.001	<0.001	0.001	0.209	0.002	0.007	0.062	<0.001
intermedia A–*nettingi*	<0.001	0.058	0.120	0.046	0.290	0.001	0.006	0.469
intermedia B–*nettingi*	<0.001	<0.001	0.002	0.051	0.385	0.002	0.101	0.027
intermedia C–*nettingi*	<0.001	0.279	0.209	0.086	0.001	0.301	0.478	<0.001
lacertina–*nettingi*	<0.001	<0.001	0.007	0.006	0.375	<0.001	0.042	<0.001
intermedia A–*reticulata*	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.157
intermedia B–*reticulata*	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.479
intermedia C–*reticulata*	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.036
lacertina–*reticulata*	<0.001	0.185	0.376	<0.001	<0.001	<0.001	<0.001	<0.001
nettingi–*reticulata*	<0.001	<0.001	0.007	<0.001	<0.001	<0.001	<0.001	0.091
intermedia A–*sphagnicola*	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
intermedia B–*sphagnicola*	<0.001	0.001	<0.001	0.007	<0.001	<0.001	<0.001	<0.001
intermedia C–*sphagnicola*	0.128	<0.001	<0.001	0.022	0.028	0.148	0.013	0.011
lacertina–*sphagnicola*	<0.001	<0.001	<0.001	0.092	<0.001	0.042	0.227	<0.001
nettingi–*sphagnicola*	<0.001	<0.001	<0.001	<0.001	<0.001	0.030	0.004	<0.001
reticulata–*sphagnicola*	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

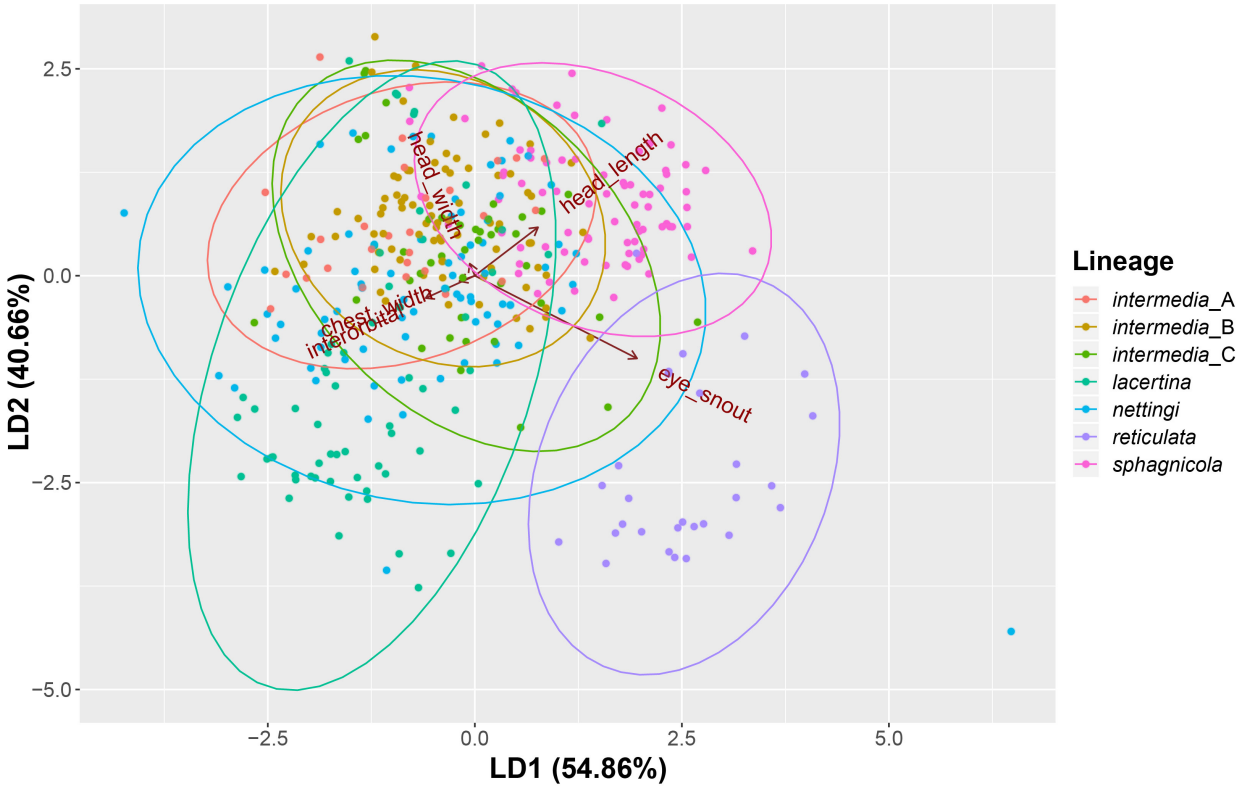

FIGURE 6. Results from our linear discriminant function analysis based on the five size-corrected linear measurements taken from 396 *Siren* specimens. Except for *S. reticulata*, each lineage failed to create meaningful separation.

GenBank sequences with complementing datasets (gene sequences not used in this paper) for comparison are presented with the matching *Siren* lineage in Table 5. Because specimens are lacking, we were unable to include costal groove counts or phenotypic traits to compare with our ranges for each lineage.

TABLE 5. Referenced GenBank and SRA files with identifications based on sequence data generated during this project. Publications referenced are the original use of the sequence data. Genbank sequences for genes not included in this paper are included for reference only. Duplicates of some GenBank entries are not included and should be referenced using the sequences mentioned here. Specimens with only genes that we did not sequence are also not included.

GenBank (NCBI) voucher(s)	Publication(s)	Genes respective to the specimen sequenced.	Species ID in publication	Our species ID	Specimen voucher
JX145019, JX145008, JX145036, JX144996	Bonett *et al.* (2013)	RAG1, POMC, SLC8a3, NCX1	*Siren lacertina*	*Siren intermedia* (Lineage B)	None
GQ368661, AY650140, JX145005, JX145033, JX144993	Zhang and Wake (2009); Bonett *et al.* (2013)	mtDNA genome, RAG1, POMC, SLC8a3, NCX1	*Siren intermedia*	*Siren sphagnicola*	None
SRX2382491 (SRA database)	Irisarri *et al.* (2017)	Transcriptome mapped mtDNA reads	*Siren lacertina*	*Siren nettingi*	None
EF107184, EF107471, EF107247, EF107307, EF107405	Roelants *et al.* (2007)	16S rRNA, CXCR4, NCX1, RAG1, SLC8A3	*Siren lacertina*	*Siren intermedia* (Lineage C)	None
EF107186, EF107473, EF107249, EF107309, EF107407	Roelants *et al.* (2007)	16S rRNA, CXCR4, NCX1, RAG1, SLC8A3	*Siren intermedia*	*Siren lacertina* mtDNA	None

Discussion

Species identification and lack of types. Sirens possesses few physical attributes to distinguish among species. Coloration and pattern can vary widely even within the same species from the same locality and cannot reliably be used to diagnose species. For example, adult *S. lacertina* that were displaced en masse from a wetland in Gainesville, Alachua Co., Florida, by flooding from Hurricane Irma exhibited several phenotypes. Patterns ranged from heavily spotted to spotless, and background coloration was blue-gray, black-brown, or forest green. Yellow labial stripes and nose spots were solid, faint, or absent. Gold flecking was present or absent on the body. Identification of preserved specimens is particularly difficult because the pattern may disappear, the color often turns grayish or brown, and although yellow pigmentation sometimes appears as an absence of the background color, fine yellowish markings are often lost.

Confusion exists regarding validity of the description of *S. intermedia* (Noble & Marshall 1932). The only physical feature that currently accurately distinguishes *S. intermedia* from *S. lacertina* is costal groove count, but this character was not used in the descriptions by Barnes (1826) and Le Conte (1828). The specimens that Le Conte collected and examined to describe *S. intermedia* were destroyed and cannot be examined. Therefore, we designate a neotype from near the purported type locality of Riceboro, Georgia (Harper 1935). Some of the syntypes may have been young *S. lacertina* instead of *S. intermedia*, because Le Conte failed to examine an oviparous female (Noble & Marshall 1932). Le Conte described *S. intermedia* as being intermediate in size between *S. lacertina* and *P. striatus*, and he mentioned that *S. intermedia* had smaller gills than *S. lacertina.* Noble (1924) noted that reduced gill size is not a distinguishing feature of *S. intermedia*, because gills of *S. lacertina* would be similarly reduced in size if specimens were placed in "irritating fluids." Le Conte also did not mention the enlarged (hypertrophic) cranial abductor muscles of large males, which is a feature we have found useful in distinguishing adult males near their maximum size.

Counting costal grooves is especially important to distinguish between similar-sized *S. intermedia* and *S. lacertina*. For example, adult and subadult *S. intermedia* (Figs. 7A & 7E) and subadult *S. lacertina* (Fig. 8A) from the Altamaha drainage in Georgia appear similar in coloration and pattern. However, differentiating *Siren* species based on costal groove counts may be difficult, because some overlap exists in counts and researchers often start and end their counts on different costal grooves. We recommend counting only grooves that are posterior to, but not touching, the forelimbs and are anterior to the cloaca (Petranka 1998). Powell *et al.* (2019) suggested that tail proportions could be used to distinguish among *Siren* species and that TaL is greater than, less than, or equal to one-fourth the TL (one-third the SVL). We found that all *Siren* lineages have overlapping ranges of tail proportions, with most species having tails that comprise approximately one-third of their TL (half of their SVL). *Siren intermedia* has a much larger range in tail-length proportions than other species, and it is the only species with specimens in our dataset that had tails over 40% of their TL (Fig. 3).

Our analyses of external morphological features likely leave much to be desired in comparing the different lineages. Because the distributions of measurements were not normal, we had to compare the distributions themselves using Kruskal-Wallis and Dunn's tests. We suspect that although the distribution of SVL-standardized measurements of many species proved to be statistically significant, most of these data may not be useful for specimen identification except for *S. reticulata*, as can be seen in our linear discrimination analysis (Fig. 6). Our linear discrimination analysis also indicated that *S. lacertina* and *S. intermedia* lineage D produced diverging confidence ellipses, though the exclusive areas were still less than 50% over the ellipse. Because we did not curate our measured sirens nor discriminate by size class and sexual maturity, we suspect a sampling bias likely existed among species that we could readily collect in our focus area in the western Florida panhandle and those we obtained on loan from museums. The samples we collected had a more diverse representation of age groups than did museum specimens. This particularly affected specimens of *S. intermedia* lineage C from the Carolinas and Virginia and lineage E (*nettingi* + "texana"), which consisted mostly of adults.

Species ranges. Our genetic analyses of mtDNA and scnDNA revealed that *S. intermedia* and *S. nettingi* are allopatric, non-sister species. However, *S. intermedia* and *S. lacertina* are close sister species. Additionally, *S. intermedia* includes three widespread, distinct mtDNA lineages. *Siren intermedia* lineage A is found from eastern Georgia south through the Florida peninsula to Lake Okeechobee and west to the Apalachicola River. Lineage B has been found from the Apalachicola drainage west to the Escambia drainage. Allozyme and karyotype data suggest this lineage may extend into the Big Bend region of northwestern peninsular Florida (Paul Moler pers. comm.),

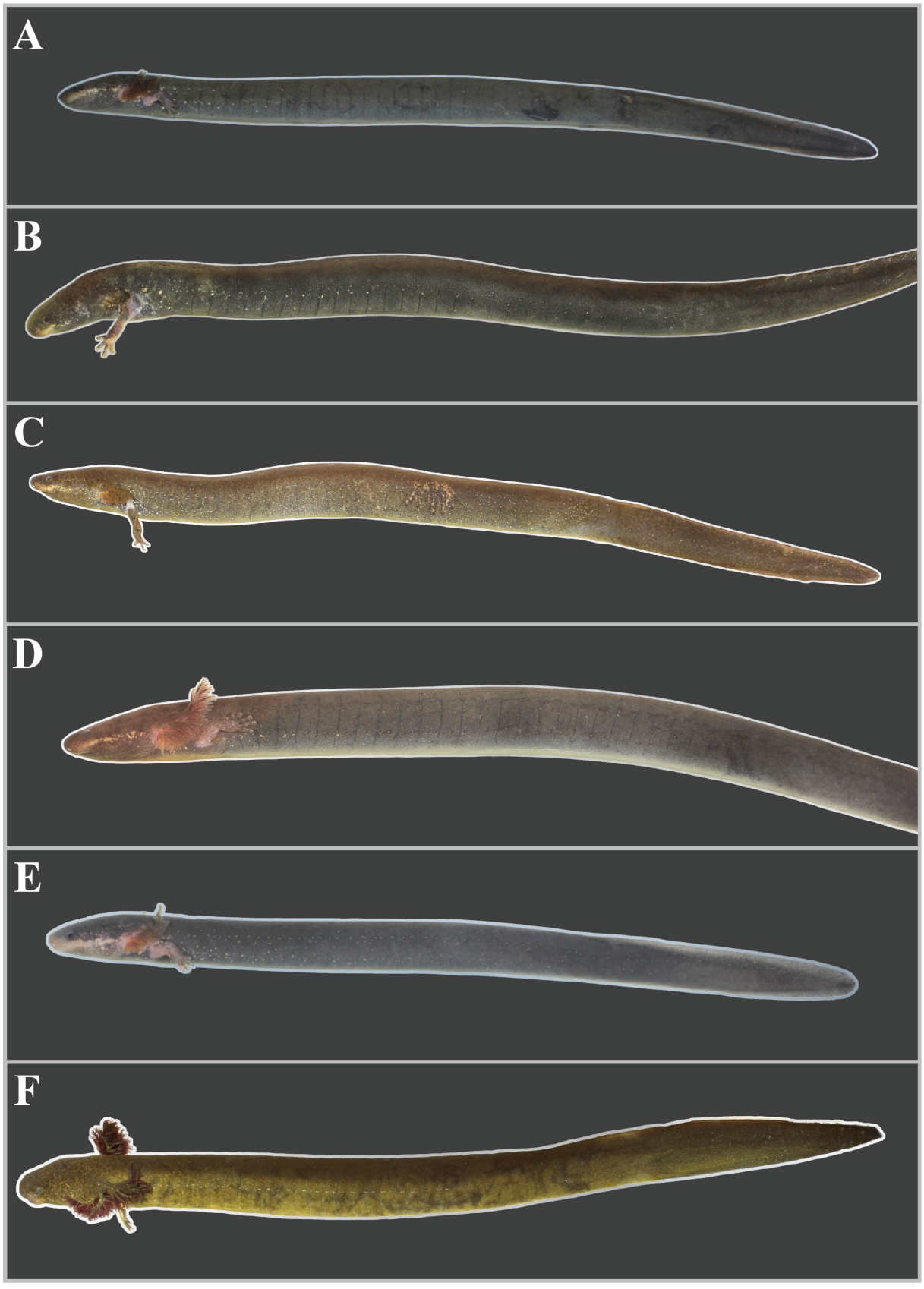

FIGURE 7. Live *Siren intermedia* type specimens belonging to mtDNA lineage A. A Neotype from Wayne Co., Georgia (UF Herp 190369). B Paratype from McIntosh Co., Georgia (UF Herp 189657). C Paratype from Nassau Co., Florida (UF Herp 188604). D Paratype from Nassau Co., Florida (UF Herp 188605). E Paratype from Wayne Co., Georgia (UF Herp 190370). F Paratype from Lake Co., Florida (UF Herp 186989).

but sequence data from samples we collected did not indicate this. *Siren intermedia* lineage C ranges from the Savannah River drainage in Georgia north through Virginia. Although we did not sequence any samples from the Savannah River, our assumption of this southern boundary is supported by unpublished cytochrome B sequence data and allozyme data from samples sequenced by Paul Moler and collaborators in the 1990s. Lineage C is the most genetically distinct *S. intermedia* lineage among the eastern lineages, and we recommend that it continue to be recognized as *S. intermedia* until further investigations determine if all or parts of the lineage warrant taxonomic recognition. *Siren intermedia* lineage D "*sp. nov.*" ranges from the westernmost creeks feeding into Choctawhatchee Bay (Garnier Creek) in the western Florida panhandle west to the Pearl River in Louisiana, where the Talisheek Creek sample was found. It is likely absent from portions of this broad geographic area, as we were unable to find samples in the Escambia drainage in Florida, but several older AUM specimens and recently collected specimens from Alabama and Mississippi resemble Florida specimens. The range of *S. lacertina* extends from the vicinity of Chesapeake Bay south through the Florida peninsula and westward only as far as Basin Bayou on the northern side of Choctawhatchee Bay. Its range was thought to extend farther west, likely because of the inclusion of specimens now attributed to the recently described *S. reticulata* (Graham *et al.* 2018). *Siren reticulata* is the most genetically and morphologically distinct *Siren* and placed sister to other *Siren* in our mtDNA tree while still nested within *Siren* as one of three clades in our scnDNA tree. A high costal groove count differentiates *S. reticulata* and *S. lacertina* from all sympatric *Siren* species; the costal groove count rarely overlaps in these two species.

FIGURE 8. A Subadult *Siren lacertina* from McIntosh Co., Georgia (UF Herp 190368). B Subadult *S. lacertina* from Alachua Co., Florida. C Large juvenile *S. lacertina* from Alachua Co., Florida. D Adult *S. lacertina* from Levy Co., Florida.

We describe a new siren species, whose maximum size is smaller than other *Siren* species. This new species uses shallow, seepage habitats not used by sympatric siren species. It is most closely related to *S. nettingi* and has a similar number of costal grooves. Most specimens were collected from partially submerged sphagnum moss, root mats of grasses, or muddy accumulations on wetland margins in < 20 cm of water. The shallow creeks and pools inhabited by this species are typically nutrient-poor and have abundant carnivorous plants such as sundews (*Drosera*), pitcher plants (*Sarracenia*), bladderworts (*Utricularia*), and butterworts (*Pinguicula*).

As part of our genetic analyses of *Siren*, we downloaded all available sequence data from GenBank and the only publicly available Sequence Read Archive (SRA) files. After inputting the sequences (or corresponding aligned NGS reads) into our dataset, we found that five specimen identifications did not match the corresponding species in our phylogeny, one of which was our new species (lineage D). Most of these apparently misidentified sequences lacked specimens that could be examined, but we obtained collection localities for specimens used by Bonett *et al.* (2013). We found that purported *S. lacertina* specimens matched our sequence data for the *S. intermedia* lineage B specimen from Georgia, which had a spotted and gold-flecked pattern similar to *S. lacertina* of comparable size. This lack of *S. lacertina* in analyses conducted by Bonett *et al.* (2013) explains why their relationships among *Siren* species differed from those found by Graham *et al.* (2018). However, we suspect that higher-level relationships between Sirenidae and the other caudate families found by Bonett *et al.* (2013) will remain unchanged.

Our genetic analyses leave unresolved the status and exact distribution of *S. intermedia* lineages A and C. Additional study is needed to determine if these *S. intermedia* clades represent distinct taxonomic groups or intergrading populations that were once isolated. To develop a range-wide phylogeny of *Siren*, additional specimens need to be analyzed genetically and morphologically from key river drainages, notably the Atlantic coastal drainages north of the Altamaha River/Blackbeard Creek drainages, drainages between the Mobile Basin and the Mississippi River, and drainages in Texas and northeastern Mexico. By focusing on these areas, the geographic distribution and genetic variance within lineage D and *S. reticulata* will become clearer, and the relationships of *S. intermedia* lineages A, B, and C will be resolved sufficiently to make judgments on other potential taxonomic changes. The relationship and variation in size and other traits between populations of *S. nettingi* and the "texana" form require additional scrutiny to fully understand the relationship between the giant and medium-sized individuals that make up this complex.

Taxonomic revisions and recommendations

***Siren sphagnicola* sp. nov.**
(Figs. 1–6, 11–14)
Common name. Seepage Siren

Holotype. UF Herp 185209 (Fig. 11A), adult female from Junior Walton Pond in Okaloosa Co., Florida, USA (30.69270°N, 86.47250°W, datum WGS84, elev. 30 m) (Fig. 12 A & 12B). Collected on 18 January 2019 by Matthew Fedler, Paul Moler, and Pierson Hill.

Paratypes. UF Herp 161516, 162498, 162568, 163271, 164240, 164241, 164242, 164243, 184285, 185195, 185200, 185201, 185208, 185209, 185214, 185215, 188766, 188767, 190036, 190037, 185205, 185216, 185197, 185198, 185210. Locality information for the various paratype localities is available via FLMNH's UF Herpetology database (http://specifyportal.flmnh.ufl.edu/herps/).

Description of holotype. The holotype has 32 costal grooves and faint black dorsal spots extending from the head to the forelimbs. It lacks approximately 3 mm of its tail tip. In life, it was mouse gray on its venter and sides with a grayish brown dorsum. Sensory pits on the head are well defined and beige in color. Measurements are 97 mm SVL, 49 mm TaL, 3.9 mm interorbital distance, 7.1 mm head width, 11.1 mm head length, 3.6 mm eye-snout distance, and 4.4 mm chest width.

Diagnosis. *Siren sphagnicola* has typical *Siren* characteristics: external gills with three fimbriate gill stalks, three associated gill slits, four toes on the forelimbs, lack of pelvic girdle and hindlimbs, and a thin, pigment-bearing mucus layer that overlies the keratinized skin. A combination of traits distinguishes it from other members of the genus. It has 30–33 costal grooves (Fig. 3) and a mouse gray base color with occasionally a light, grayish brown sheen on the dorsum (Fig. 11). Small juveniles in the post-macrocephalic larval stage, which is >2 months of age

according to diagrams of growth/transition rates of *S. nettingi* provided by Noble & Marshall (1932), have the same gray coloration as adults (Fig. 11) and lack the orange, red, or yellow highlights present on other *Siren* juveniles of similar age (Fig. 13). A few adult specimens examined have small, well-defined black spotting on the head and occasionally on the dorsum (Fig. 11). Sensory pits on the head are more visible than those on heads of other *Siren* species and are typically ivory to beige colored, which may denote an absence of gray pigment rather than the presence of chromatophores (Fig. 11). This species lacks the yellow labial stripe present in young *S. lacertina* (Figs. 13B & 13F), *S. intermedia* (Figs. 13A, 13C, 13G, & 13I), and *S. nettingi* examined from the Mobile Bay drainage. Some juvenile *S. intermedia* in eastern populations also lack the light labial stripe. A few small juvenile *S. sphagnicola* have yellow spots or a short, broken stripe where a labial stripe is present in other species (Figs. 13E & 13H). *Siren sphagnicola* also lacks the post-cranial yellow or gold flecking found in many *S. lacertina* (Fig. 8), *S. intermedia* (Fig. 10), and *S. nettingi*. Gill stalk coloration is typically rosy pink to red in recently captured specimens but fades to grayish pink in captivity, likely due to changes in acidity or oxygenation of water. Intact tail tips are rounded, whereas partially regenerated tails (frequently observed) often taper to an abrupt point after the tail fin blade (Fig. 11). Regenerated portions of the tail seem to lack the density of gray pigment found in non-regenerated portions; thus, the regenerated portion is easily distinguished by its pinkish gray hue. Regenerated portions of the tail of other *Siren* species examined match the normal body coloration or have a brownish hue.

Size. *Siren sphagnicola* is the smallest known species in the genus *Siren*. Additionally, all specimens examined are shorter than the maximum length given for both species of *Pseudobranchus* (Moler 2019b, c), making *S. sphagnicola* the smallest member of Sirenidae based on our current understanding. The largest specimen examined (AUM 27973) had an SVL of 126 mm, but it lacked a complete tail. The largest specimen with a complete tail (AUM 8960) had an SVL of 120 mm and a TaL of 76 mm (196 mm TL). We attributed these AUM specimens to *S. sphagnicola* based upon costal groove count, lack of labial striping and gold flecking found on sympatric *S. nettingi* and *S. intermedia*, and presence of beige-colored facial pores. Reproductive females have been found as small as 71 mm SVL (111 mm TL). When comparing measurement distributions of *Siren* lineages, *S. sphagnicola* was not distinct from any other single lineage in, at most, two of seven measurements (Table 4).

Natural history and distribution. Based on our surveys in Florida, populations appear to be robust and widely distributed in suitable microhabitats in the Blackwater and Yellow river drainages and the western two-thirds of Eglin Air Force Base, including several streams that flow into the western side of Choctawhatchee Bay (Fig. 14). This suspected microhabitat specialist has been found in headwater seepage areas of steephead streams, mucky seeps farther downstream, muddy and/or densely vegetated seepage bogs, shallow-water depressions lined with dense sphagnum moss or filled with leaves along seepage-fed streamside terraces, and other types of shallow streams with mucky, detrital, or sandy bottoms (Enge 2005) (Fig. 12). In contrast, *S. intermedia* collected at localities near (<200 m) *S. sphagnicola* were found in leaf packs not associated with seeps and adjacent to deeper water. Incised (gully-eroded) first- and second-order streams (Strahler 1964) lack the microhabitats used by *S. sphagnicola* (and many other salamander species), because accumulations of leaf litter and other detritus are constantly flushed from streams and scoured from surface pools by heavy rainfall events. Common, syntopic amphibian species are the Southern Cricket Frog (*Acris gryllus* [Le Conte]), Bronze Frog (*Lithobates clamitans* Latreille), Southern Two-lined Salamander (*Eurycea cirrigera* [Green]), and Southern Red Salamander (*Pseudotriton ruber vioscai* Bishop). One-toed Amphiuma (*Amphiuma pholeter* Neill) and Two-toed Amphiuma (*A. means* Garden) may be present but are less abundant than the aforementioned species (Enge 2005).

Deep, steephead ravine systems (Means 1981, 2000) and more shallow-gradient, seepage bogs in upland habitats near the Gulf of Mexico may have served as "evolutionary engines" during periods of elevated sea levels, producing the Florida Bog Frog (*Lithobates okaloosae* Moler, 1985), Bog Dwarf Salamander (*Eurycea sphagnicola* Wray, Means, & Steppan, 2017), and *S. sphagnicola*. A sea level rise of only 2–5 m would have led to saltwater inundation of the mouths of these deep steephead valleys, thus isolating ancestral populations of freshwater species (Means 2000). We suspect the range of *S. sphagnicola* is similar to that of *E. sphagnicola*, which also inhabits the sphagnum-lined margins of streams and associated seepage habitats (Wray *et al.* 2017).

Siren sphagnicola has a smaller geographic distribution than other *Siren* species. Most specimens have been found in the Blackwater, Yellow, and Escambia/Conecuh river drainages of Florida and Alabama (Fig. 14). Elsewhere, its range is poorly known, but we believe it is restricted to the environs of sandy, seepage-fed creeks in the lower Gulf Coastal Plain as far west as the Florida Parishes of Louisiana (Fig. 14). Locality information from outside Florida is entirely based on preserved AUM specimens and sequence data from a GenBank specimen that match

both mtDNA and scnDNA markers. Few *Siren* museum vouchers with genetic material exist from Mississippi (42 total specimens via Vertnet search and only one with available tissue, which we sequenced) and the Florida Parishes of Louisiana (63 total via Vertnet search; one of two tissues requested yielded DNA), and we did not examine most museum specimens from this area that lacked tissue samples.

Etymology and common name. The specific epithet is derived from *Sphagnum*, the generic name for sphagnum moss, and the Latin suffix -cola, meaning inhabitant or dweller. The species epithet is used as noun in apposition. This siren is frequently found in and under mats of *Sphagnum* in and along streams and the margins of other bodies of water. Because of its affinity for seepage-fed streams and wetlands, we suggest Seepage Siren as the common name.

Specimens examined. See Supplemental Table 1.

***Siren nettingi* (Goin, 1942)**
(Figs. 1–6 & 9)
Common name. Western Siren

Holotype. Carnegie Museum 7580. Adult female collected in May 1928 from Imboden, Lawrence Co., Arkansas, USA (Goin 1942).

Paratypes. See Goin (1942).

Diagnosis. *Siren nettingi* has typical *Siren* characteristics: external gills with three fimbriate gill stalks, three associated gill slits, four toes on the forelimbs, lack of pelvic girdle and hindlimbs, and a thin, pigment-bearing mucus layer that overlies the keratinized skin. Goin (1942) distinguished *S. nettingi* from *S. intermedia* by the presence of well-defined light spots on the sides and venter and also specifying that these were not the bar-like streaks found in *S. lacertina*. Additionally, Goin (1942, p. 212) stated that *S. nettingi* has "about two more costal grooves (usually 33 in *intermedia*, 35 in *nettingi*)," a value slightly overlapping but less than the average for *S. lacertina*. See comments below for possible reasons for variation in data.

In areas of sympatry, the costal groove range of *S. nettingi* overlaps that of *S. sphagnicola. Siren nettingi* has 32–34 costal grooves, with two outliers of 31 and 36 (Fig. 4). Goin (1942) gave a slightly higher range of 33–37 costal grooves, but we infer that he included the axial groove or terminated counting grooves at a point posterior to our stopping point. *Siren nettingi* specimens similar in size to *S. sphagnicola* can be distinguished by the presence of a solid yellow labial stripe and rostral patch, whereas *S. sphagnicola* has, at most, several beige spots where the labial stripe occurs in other species. In addition, bodies of small *S. nettingi* often have a green or yellowish hue and gold flecking, both of with are lacking in *S. sphagnicola*.

Comments. This species occupies a large geographic area, and we analyzed relatively few specimens, mainly from Alabama and Louisiana (Fig. 9). Our information may not represent the full extent of variation found in this species. Reinhard *et al.* (2013) provided photographs of larvae and mature adults of this species.

After examining the holotype, we concluded that the well-defined, light spots on the venter referenced by Goin (1942) represent sensory pores rather than chromatophores. Chromatophores on the holotype are neither defined nor extensive in coverage. Regardless of this distinction, neither the distinct, light-colored sensory pores nor the yellow to yellow-green flecking of chromatophores is unique to *S. nettingi*. These pores are frequently obfuscated by the slime layer but become more visible when this is removed. This slime layer frequently sloughs off of poorly preserved or frozen specimens.

Validity of the Rio Grande Siren (*S. intermedia texana* Goin, 1957) as a taxonomic unit distinct from *S. nettingi* has been questioned in recent decades. Our analyses suggest that *S. nettingi* and "texana" are either closely related sister taxa or ecomorphs of the same species adapted to habitats present in different regions. Further investigation of this topic is needed, but we treated "texana" as a distinct unit for morphological analyses. Furthermore, *S. i.* "texana" is no longer recognized by Highton *et al.* (2017), who accepted the erroneous designation by Flores-Villela and Brandon (1992) that the large siren from southern Texas and Mexico is *S. lacertina* (LaFortune 2015).

Size. *Siren nettingi* has a reported maximum TL of 686 mm for the larger "texana" form found in southern Texas and Mexico, whereas the smaller form found in the rest of its range has a reported maximum TL of 502 mm (Martof 1973a). Flores-Villela and Brandon (1992) cited Goin (1957) for total lengths given by Martof (1973a and 1973b) and incorrectly reported the TL as the SVL for both the "texana" form of *S. nettingi* (then *S. intermedia texana*) and *S. lacertina*.

Distribution. Based on our examination of museum specimens and genetic analyses, *S. nettingi* occurs from the Mobile Bay drainage westward, and native populations do not occur in any Florida drainage (Fig. 9). A single specimen that sequenced as *S. nettingi* was collected from Tates's Hell State Forest, Franklin Co., Florida, but we suspect this specimen was translocated as live fish bait. Based on phenotypes and costal groove counts, Goin (1942) suggested that Florida Parishes of Louisiana represented the break between the then-recognized subspecies *intermedia* and *nettingi*. This boundary was later considered to be a hybridization zone that stretched into Mississippi (Boyd & Vickers 1963) and then to the Mobile Bay drainage (Caldwell & Howell 1966). We suspect part of this confusion resulted from examining *S. sphagnicola* and attributing them to *S. i. intermedia* because of their resemblance to grayish *S. intermedia* that can be found farther east. Furthermore, *S. nettingi* is currently thought to be the only *Siren* species that occurs from the Mississippi River west and south to Veracruz, Mexico.

Common Name. We suggest the common name Western Siren. Except for the Mobile Bay drainage system, where *S. reticulata* is present, *S. nettingi* represents the largest or only siren (from the Mississippi River westward) present throughout its range; thus, the "Lesser" moniker is misleading.

Specimens examined. See Supplemental Table 1.

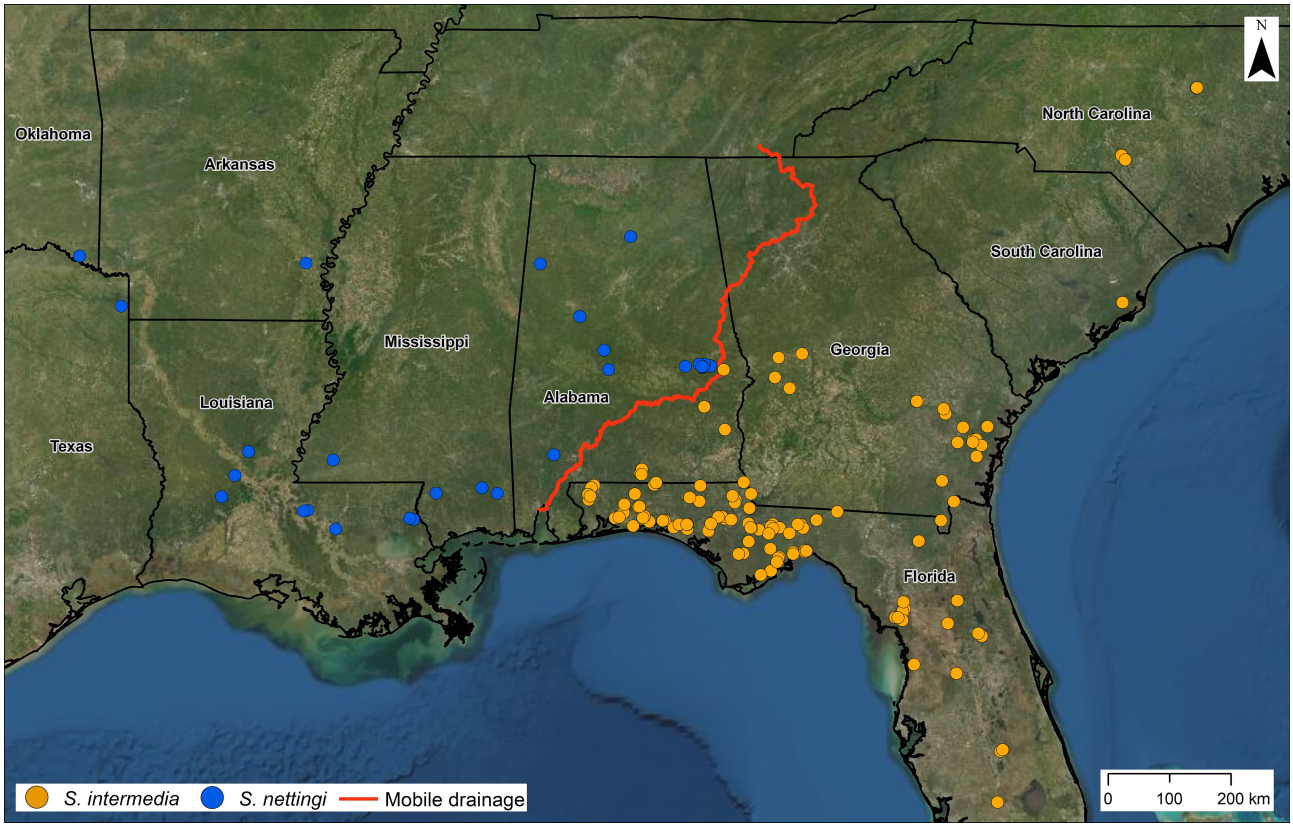

FIGURE 9. Distribution map of specimens assigned to *Siren intermedia* (orange circles) and *S. nettingi* (blue circles). The red line is the eastern boundary of the Mobile drainage from EDNA Derived Watersheds for Major Named Rivers (https://edna.usgs.gov/watersheds/kml_index.htm). The Mobile drainage lacks *S. intermedia* records and is the easternmost drainage where *S. nettingi* occurs. Our westernmost records of S. *intermedia* are from the Escambia drainage.

Siren intermedia Barnes, 1826

(Figs. 1–7, 9, 10, & 13)

Common name. Intermediate Siren

Neotype: UF Herp 190369 (Fig. 7A), adult female (gravid) from Wayne Co., Georgia, USA (31.50109°N, 81.91324°W, datum WGS84, elev. 13 m). Collected on 30 January 2020 by Dirk Stevenson, Arik Hartmann, and Matthew Fedler.

Description of neotype: The neotype has 32 costal grooves and faint black spots on the dorsum extending from the head to the fourth costal groove behind the forelimbs. In life, it had a bluish gray venter and sides and a

dark grayish brown dorsum. The slightly darker tail tip may indicate old regeneration. A broken chartreuse labial stripe runs from a few millimeters posterior of the nares to just anterior of the gills. Light yellow spots are present ventrolaterally on the head, often surrounding sensory pores. Sparse and more randomly placed small (<3 mm) yellow spots are posterior to the gills on the forelimbs and lateral/ventrolateral portions of the body, terminating roughly one-third of the distance between forelimbs and cloaca. The gills have sparse, minute, chartreuse yellow spots. Minor abrasions are present near the cloaca. Measurements are 146 mm SVL, 85 mm TaL, 6.3 mm interorbital distance, 12.9 mm head width, 18.5 mm head length, 5.8 mm eye-snout distance, and 8.4 mm chest width.

Paratypes: UF Herp 188598, 189657 (Fig. 7B), 188604 (Fig. 7C), 188605 (Fig. 7D), 186989 (Fig. 7F), 190370 (Fig. 7E), 190371, 190374, 190375. Locality information for these specimens can be found via the Florida Museum of Natural History's web database (http://specifyportal.flmnh.ufl.edu/herps/).

Diagnosis. *Siren intermedia* has typical *Siren* characteristics: external gills with three fimbriate gill stalks, three associated gill slits, four toes on the forelimbs, lack of pelvic girdle and hindlimbs, and a thin, pigment-bearing mucus layer that overlies the keratinized skin. This species is best distinguished from other sympatric *Siren* lineages by costal groove count, which varies geographically. We restrict this species to the recovered *S. intermedia* lineages A, B and C. All *S. intermedia* lineages combined that we examined have 29–33 costal grooves (Fig. 4). Lineage B has a more restricted costal groove range of 29 (n =12), 30 (n = 23), or 31 (n = 3) in areas of sympatry with *S. sphagnicola*., which has 31–33 costal grooves in the East Bay, Yellow, Blackwater, and Escambia river drainages. Specimens of lineage B that are comparable in size to *S. sphagnicola* typically have yellow labial stripes, rostral patches, and ventrolateral flecking (Fig. 10).

Siren intermedia lineages A (type clade) (Fig. 7) and C (Fig. 10) can be distinguished from sympatric *Siren* species by having fewer than 34 costal grooves. These two lineages exhibit hypervariability in pattern and coloration. Yellowish labial stripes and rostral patches may be present or absent. Numerous distinct black spots may be present, absent, or occur in patches on the dorsal and lateral surfaces of the head and body, and some individuals possess reticulated patterns (UF Herp 186989, 192033, and an unvouchered specimen from the same locality as 192033). These traits are also present in other *Siren* species and may be variable within populations of any of these species, emphasizing the need for accurate costal groove counts to distinguish from other species in the vicinity.

Original description, first redescription, and comments. Le Conte (1828) provided the following description: "Its greatest length is twelve inches: colour uniform dusky, very slightly paler beneath, sometimes faintly speckled with darker above." Barnes (1826) and Harlan (1827) announced that the species was going to be described and provided the same information when summarizing Le Conte's forthcoming manuscript. According to Harlan (1827): "Colour resembling that of the Lacertina; branchiae resembling those of the Striata [i.e., *Pseudobranchus striatus* Le Conte]. Length about one foot."

We designated a neotype from near the type locality for the express purpose of clarifying the taxonomic status of a nominal taxon whose specimens (syntypes) were destroyed. Smith *et al.* (1975) clarified that the name should be attributed to Barnes (1826). Le Conte's (1828) description provided additional traits that are generic among *Siren* and failed to identify any features that distinguish it from other *Siren* given our current understanding of the genus. *Siren intermedia* is extremely variable in both color and pattern, and many phenotypes overlap those of *S. lacertina* and *S. nettingi*.

The original type locality was purportedly Le Conte's Woodmanston Plantation, Riceboro, Georgia (Harper 1935). We chose the neotype locality, which is approximately 45 km SW of Woodmanston Plantation, because all sequenced specimens from the surrounding region and Altamaha River drainage belong to the same mtDNA lineage as our single Woodmanston Plantation specimen. The neotype locality is easily accessible and yielded many *S. intermedia* compared to other localities in the area. Additionally, the locality has stereotypical habitat for *S. intermedia* consisting of a cypress swamp with many small creeks and large accumulations of muck, leaf packs, and submerged Creeping Rush (*Juncus repens* [Michaux]).

Noble and Marshall (1932) provided a redescription of *S. intermedia* but failed to state where the specimens they examined came from, and they likely included specimens from populations later split into the *nettingi* and *texana* subspecies of *S. intermedia*. This would explain their higher costal groove range for *S. intermedia* (up to 36 grooves). Additionally, they stated that the outer capsule of the egg was wider for *S. lacertina* than *S. intermedia*. This may ultimately be proven true by future studies, but their data are contradictory in light of the revised *Siren* taxonomy. Noble and Marshall (1932) compared specimens of what they considered to be *S. intermedia* and *S. lacertina* from the same locality (Maverick Co., Texas) to each other, as well as *S. intermedia* and *S. lacertina* from

Oakley, Berkeley Co., South Carolina. A recent genetic study (LaFortune 2015) found that the Rio Grande drainage has only one genetic group of *Siren*; thus, Noble and Marshall (1932) and Flores-Villela and Brandon (1992) were comparing large and smaller individuals of the same species (now *S. nettingi*) using different names. Their findings indicate that different-sized individuals of the same species may have different-sized eggs, or they were comparing specimens with eggs at various stages of development.

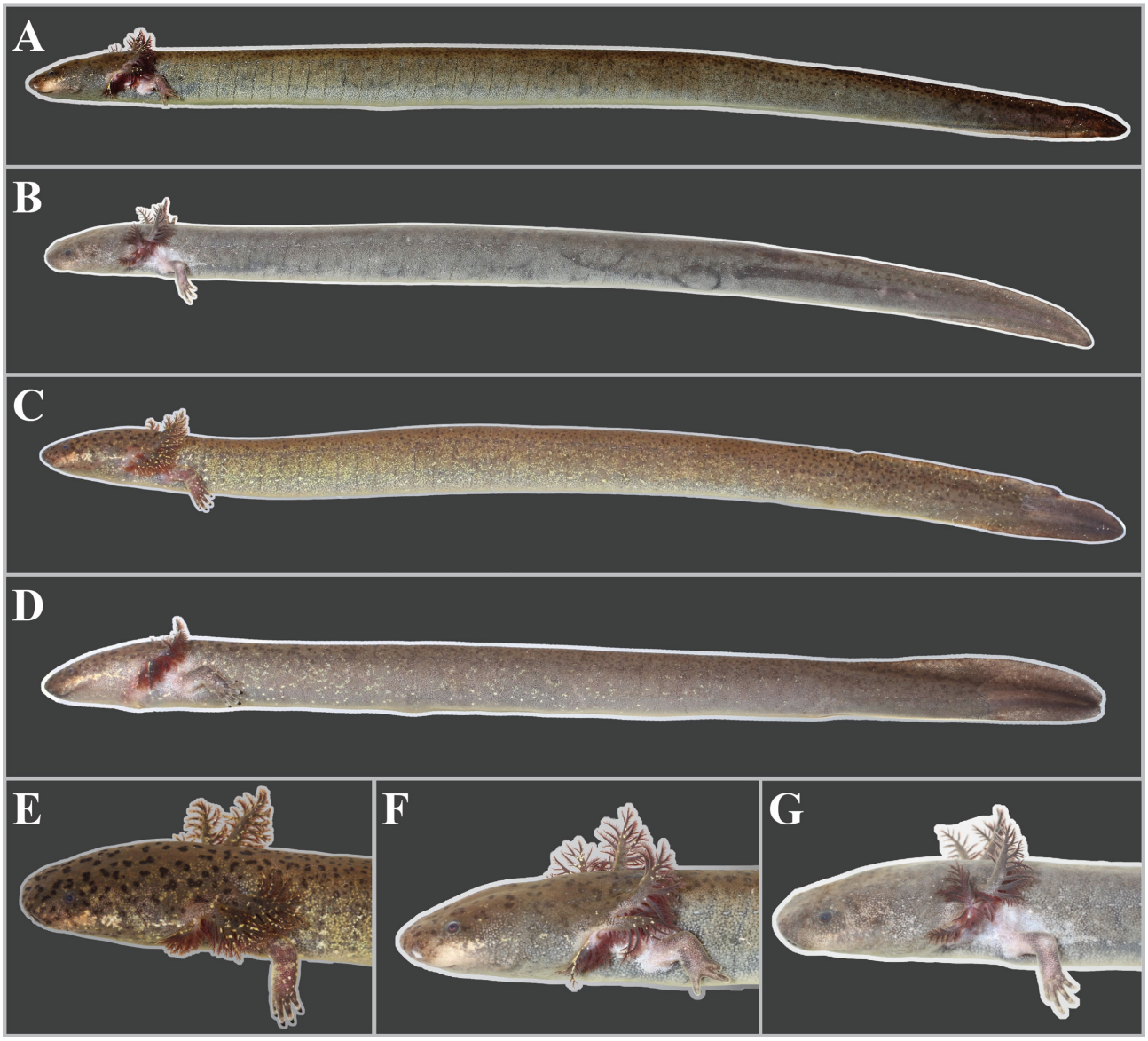

FIGURE 10. Live *Siren intermedia* specimens belonging to mtDNA lineages B and C. A Lineage C from Berkeley Co., South Carolina (UF Herp 188612). B Lineage C from Berkeley Co., South Carolina (UF Herp 188613). C Lineage B from Holmes Co., Florida (UF Herp 188769). D Lineage B from Jackson Co., Florida (UF Herp 188901). E Head of lineage B specimen from Holmes Co., Florida (UF Herp 188769). F Head of lineage C specimen from Berkeley Co., South Carolina (UF Herp 188612) (lineage C). G Head of lineage C specimen from Berkeley Co., South Carolina (UF Herp 188613). The latter two specimens are from the same locality but differ dramatically in pattern and coloration.

Size. The largest specimen we examined (UF Herp 186989) measured 191 mm SVL and 125 mm TaL (316 mm TL). Noble & Marshall (1932) reported a male *S. intermedia* (MCZ Herp A-140) from Georgetown, South Carolina, that measured 212 mm SVL and 347 mm TL. This animal is likely a member of *S. intermedia* lineage C. Goin (1957) provided 240 mm SVL and 381 mm TL for the maximum size of "*S. i. intermedia*" specimens he examined. However, he failed to provide the collecting locality or attribute the measurements to a vouchered specimen; thus, we are unable to assign it to a lineage. Because Goin assumed that only *S. i. intermedia* was present in the coastal drainages of Mississippi, Alabama, and the Florida Parishes of Louisiana, he may have attributed the size to a specimen

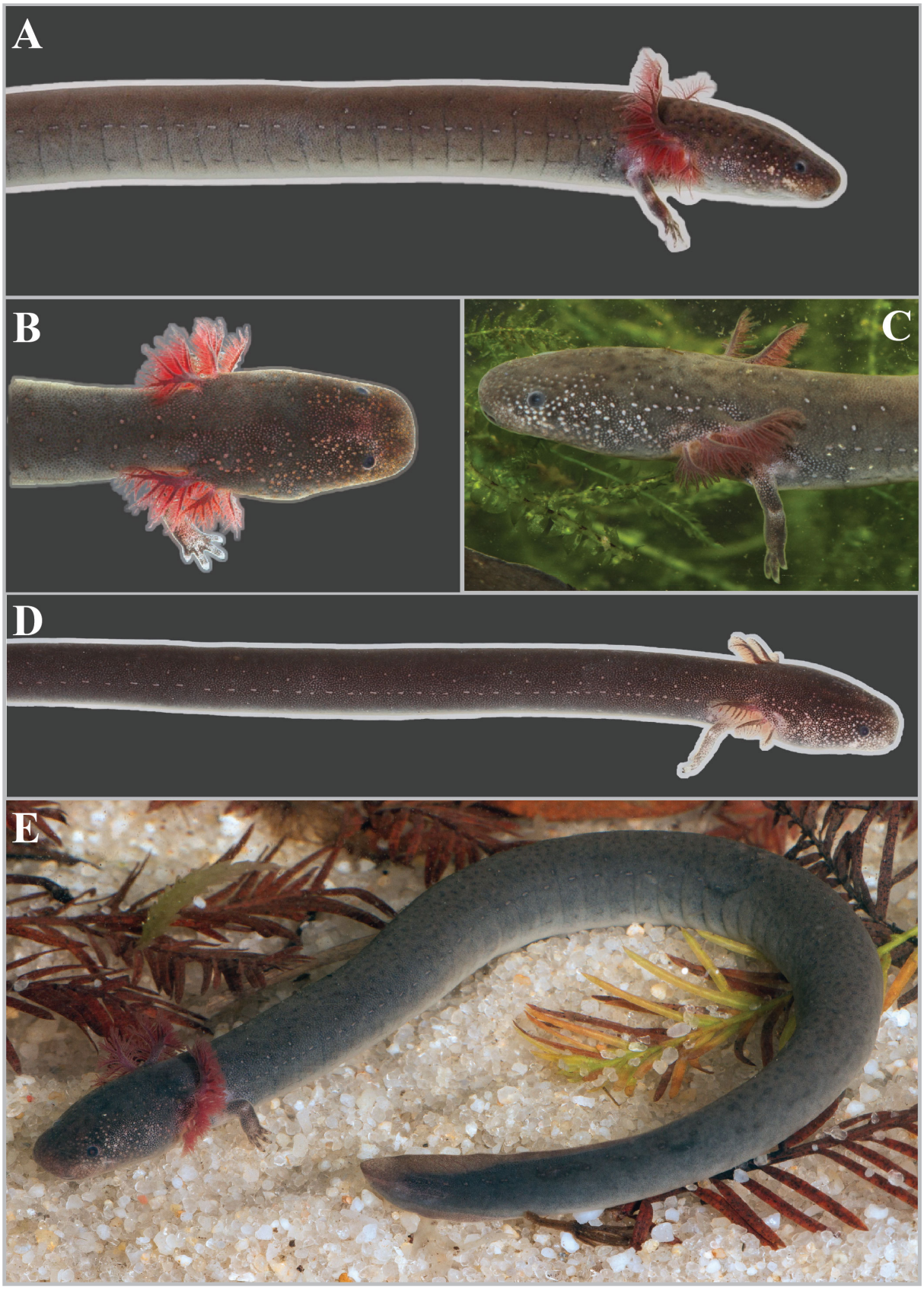

FIGURE 11. Live *Siren sphagnicola* specimens. A Holotype specimen from Okaloosa Co., Florida (UF Herp 185209). B Dorsal view of an adult found in shallow water where specimens were buried in the sandy bottom. C Dorsolateral view of an adult found in a deeper creek with deep muck deposits where specimens were found by digging through sphagnum root masses and fluid muck. D Juvenile from a shallow, sand-bottomed creek. E Adult hypertrophic male with a partially regenerated tail.

FIGURE 12. *Siren sphagnicola* habitats. A Type locality at Junior Walton Pond, Okaloosa Co., Florida, showing the feeding stream channel lined with thick muck and vegetation. B Type locality at Junior Walton Pond, Okaloosa Co., Florida, showing sphagnum mats and assorted vegetation covering a thick layer of muck and root masses. C Surface of a sphagnum bog on Bull Pen Branch, Okaloosa Co., Florida, where dense vegetation and 60 cm of muck provide ideal habitat for *S. sphagnicola*. D Seep at the head of Krul Lake, Santa Rosa Co., Florida, where shallow surface water flows over 15–90 cm of fluid muck covered by grassy vegetation and sphagnum mats. E & F Hillside seepage pools along Carr Spring Branch, Okaloosa Co., Florida, where intermittent pools are deceptively deep and filled with fluid muck, like many other localities inhabited by *S. sphagnicola*.

that we now recognize as *S. nettingi*. Furthermore, his measurements closely match the size of the largest *S. nettingi* specimen we sequenced (LSUMZ 87289; 270 mm SVL, 408 mm TL) and the largest *S. nettingi* in the AUM collection (AUM 40435; 242 mm SVL, 365 mm TL). The smallest sexually mature female (UF Herp 190904) measured 75 mm SVL and 119 mm TL.

Distribution. Our findings restrict *S. intermedia* to Atlantic and Gulf drainages from the vicinity of Chesapeake Bay south to central Florida and west throughout the Florida panhandle (Fig. 9). Specimens attributed to this species from the Mobile Bay drainage westward are likely either *S. nettingi* or *S. sphagnicola*.

Common name. The first common name assigned to *S. intermedia* was Intermediate Siren (Gray 1831), and we suggest using this name instead of Lesser Siren because there are three larger sirens (*S. lacertina, S. reticulata, S. nettingi*) and three smaller sirens (*S. sphagnicola*, *Pseudobranchus axanthus, P. striatus)*. This also aligns the epithet with the common name.

Specimens examined. See Supplemental Table 1.

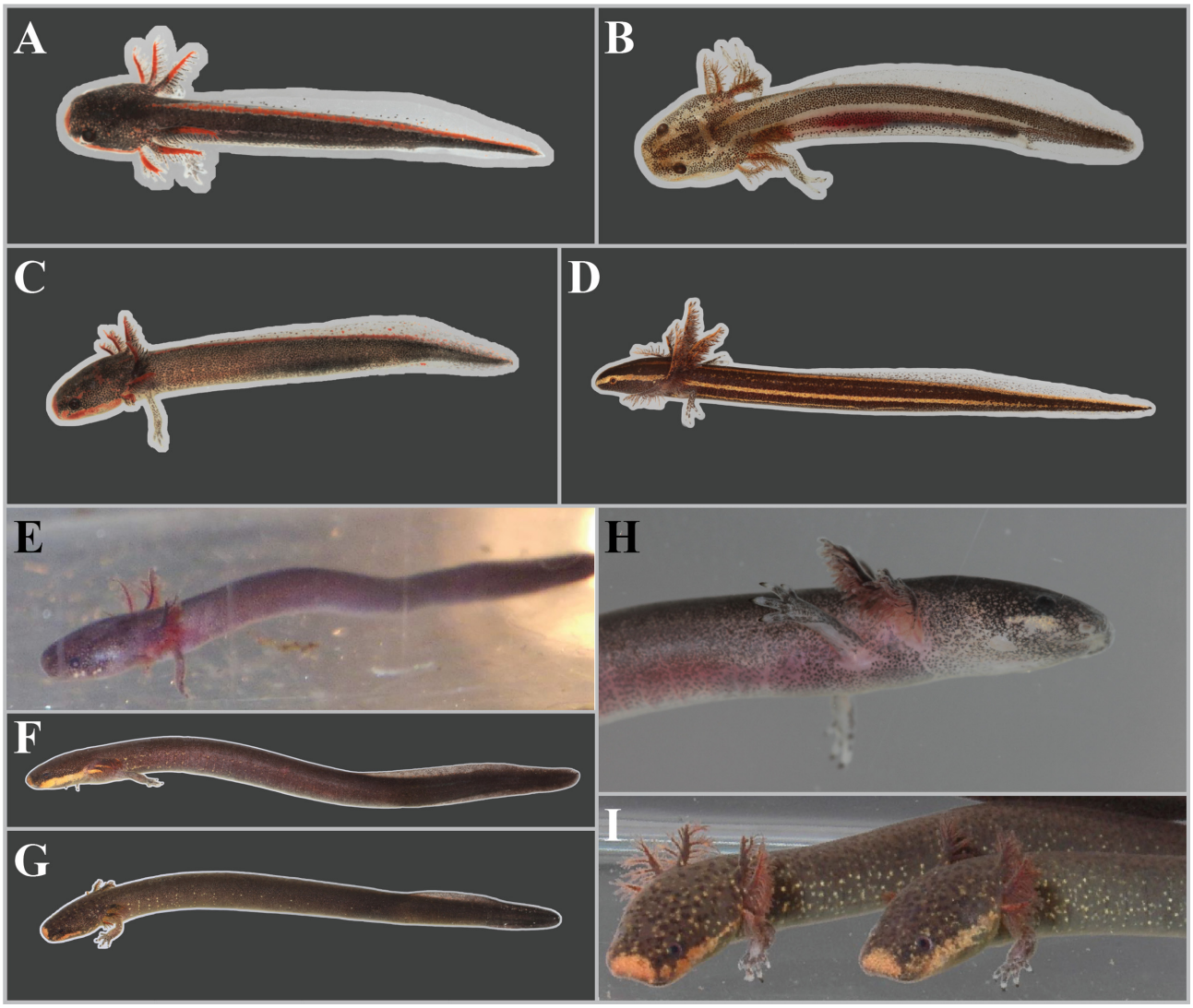

FIGURE 13. A comparison of larval and juvenile sirens. A Larval lineage B *Siren intermedia* from Escambia Co., Florida. B Larval *S. lacertina* from Jefferson Co., Florida (UF Herp 188601). C Larval lineage A *S. intermedia* from Liberty Co., Florida, that is slightly farther along in development than UF Herp 188601. D Larval *Pseudobranchus striatus spheniscus* from Jefferson Co., Florida (UF Herp 188600). E Juvenile *S. sphagnicola* (~3 cm TL) from Okaloosa Co., Florida, no voucher. F Juvenile *S. lacertina* from Walton Co., Florida (UF Herp 188739). G Juvenile lineage B *S. intermedia* from Washington Co., Florida (UF Herp 188735). H Head of a juvenile *S. sphagnicola* from Okaloosa Co., Florida (UF Herp 185068. I Juvenile lineage B *S. intermedia* from Escambia Co., Florida (UF Herp 185073 & UF Herp 185074).

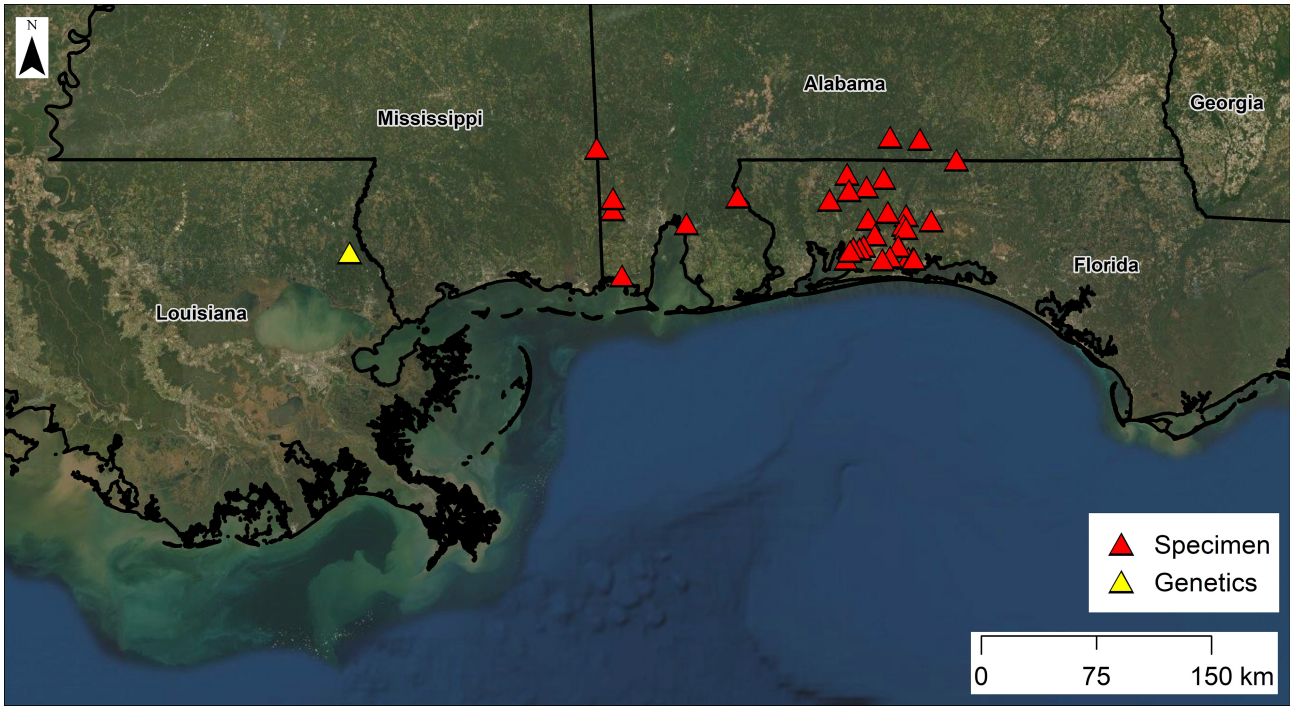

FIGURE 14. Distribution map of specimens assigned to *Siren sphagnicola*. Red triangles represent localities with museum voucher specimens. Species of these specimens were determined using sequence data if tissue samples were available or by comparing costal groove counts and patterns to data from sequenced specimens. The yellow triangle represents a locality with genetic data only.

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