FINAL PERFORMANCE REPORT

As Required by

THE ENDANGERED SPECIES PROGRAM

TEXAS

Grant No. TX E-164-R

(F14AP00825)

Endangered and Threatened Species Conservation

Assessing the Conservation Status of Native Freshwater Mussels (Family: Unionidae) in the Trinity River basin

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31 August 2017

FINAL REPORT

STATE: ____Texas_____ GRANT NUMBER: ___TX E-164-R-1__

GRANT TITLE: Assessing the Conservation Status of Native Freshwater Mussels (Family: Unionidae) in the Trinity River basin.

REPORTING PERIOD: ____1 September 2014 to 31 August 2017_

OBJECTIVE(S). The primary objective of this study is to comprehensively survey the Trinity River, between Athens and Crockett, TX, to determine the location, density, and species composition of existing unionid mussel populations.

Segment objectives:

Task 1: site selection for the Trinity River basin (Sept – Oct 2014, year 1)

Task 2: comprehensive survey of Trinity River, Athens to Palestine, TX (Oct 2014– Sept 2015, yr 1)

Task 3: comprehensive survey of Trinity River, Palestine to Crockett, TX (Oct 2015 – May 2016, yr 2)

Task 4: assess the genetic diversity and begin to validate the taxonomic identity of *Fusconaia* species in the Trinity River basin (Oct 2014 – Sept 2016, years 1 &2).

Task 5: presence/absence survey for zebra mussels (Oct 2014 – Sept 2016, years 1 & 2)

Task 6: create distribution maps for Unionid and zebra mussels, evaluate the conservation status of state threatened mussel species in the Trinity River, and submit final report (June 2015– September 2016, yr 2)
Significant Deviations:

None.

Summary Of Progress:

Please see Attachment A.

Location: Trinity River in Anderson, Ellis, Freestone, Henderson, Houston, Kaufman, Madison, and Navarro counties, Texas.

Cost: ___Costs were not available at time of this report, they will be available upon completion of the Final Report and conclusion of the project.__

Prepared by: _Craig Farquhar_____ Date: _31 August 2017

Approved by: _____ Date:___31 August 2017_
C. Craig Farquhar

ATTACHMENT A

Assessing the Conservation Status of Native Freshwater Mussels (Family: Unionidae) in the Trinity River basin.

Final Report

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August 2017

Executive Summary

The Trinity River drainage was surveyed in 2014 – 2017 to examine the distribution and habitat associations of common and threatened species of freshwater mussels. We also examined species boundaries and genetic diversity of *Fusconaia* within this basin and the presence and prevalence of *Dreissena polymorpha* larvae (veligers) in the Trinity River between Lewisville Lake (SH121 Bridge in Dallas, TX) and SH84 Bridge near Palestine, TX (Figure 1). In total, 2,445 individuals from 18 species were observed across 61 sites in the 5 study reaches surveyed, which is low compared to other rivers in East and Central Texas. We observed two of the four threatened species known to occur in this basin: *Fusconaia chunii* and *Potamilus amphichaenus*. We did not find live or recent shell of *Lampsilis satura* or *Pleurobema riddellii*, the other two threatened species, despite both being recently reported by private contractors. We were able to evaluate photovouchers for several of those specimens and all were misidentifications. In general, species richness and abundance was greatest in the middle Trinity near Oakwood, TX, and in the Elm Fork of the Trinity River. Immediately downstream of the Dallas-Fort Worth Metroplex and Lake Livingston mussel species richness and abundance was reduced relative to the other reaches. Indicator species analysis revealed associations for all species observed, though only a few were significant. Conservation maps for threatened species from this drainage show range curtailment but also areas where populations continue to persist, except for *L. satura* and *P. riddellii*, and thus should be targeted for further conservation activities.

Phylogeny of three species (*F. askewi*, *F. chunii*, and *F. flava*), were analyzed from drainages in the Neches, Sabine, Trinity, San Jacinto, and six major rivers in Arkansas, Louisiana, and Texas. In total, we examined ~658 base pairs (bp) of the *cox1* gene from 270 individuals, ~608 bp of the *nad1* gene from 160 individuals, and ~533 bp of the *ITS1* gene from 104 individuals. Our results indicate that all three species are valid, which contradicts previous phylogenetic studies of this group. Our results show that there are two distinct evolutionary lineages within *F. askewi* and *F. chunii* as each formed a reciprocally monophyletic lineage, indicating no gene flow between the species. Therefore, we conclude that *F. chunii* is a valid species, which is endemic to the Trinity River drainage. Based on the reciprocal monophyly and allopatric distributions, it is likely *F. askewi* and *F. chunii* have undergone recent speciation during the early Pleistocene.

We observed veligers at two bridge crossings downstream of Lake Lewisville, which indicates that *D. polymorpha* are passively moving downriver from infested reservoirs. Densities at these two locations ranged from 0.83–3.33 veligers/m^3, which is orders of magnitude less than reported densities in the Illinois and Mississippi rivers where *D. polymorpha* has become established. Collected veligers were in the D-stage (≤112μm), which confirms that adult mussels are reproducing within Lake Lewisville. We did not find any *D. polymorpha* veligers that were larger than D-stage, nor did we ever observe any adults near our sampling locations, which suggests that *D. polymorpha* is not becoming established within the mainstem of the Trinity River. We hypothesize that although veligers are being released from Lake Lewisville, and other reservoirs in the

DFW Metroplex, unknown environmental factors are preventing their establishment, except immediately downstream of infested reservoirs. High turbidity and frequent flood pulses are likely explanations, though neither has been specifically evaluated.

Introduction

Freshwater mussels are in decline worldwide (Lydeard et al., 2004), and in North America, where mussels reach their highest diversity, more than two-thirds of the approximately 300 species require some degree of conservation protection (Williams et al., 1993; Strayer et al., 2004). In response to these declines, there has been a rapid increase of conservation efforts and prioritization of research targeting the ecology of freshwater mussels (NNMCC, 1998; Haag & Williams, 2014). Distribution surveys have been the cornerstone of these efforts because knowledge of past and present distribution and abundance is needed in order to understand the current status and threats to remaining populations. However, distribution data for many species of mussel occurring in the United States are limited (Lydeard et al., 2004), leaving status assessments and conservation decisions based on incomplete data.

Texas is faced with an impending conservation crisis regarding the plight of freshwater mussels (Howells et al., 1996, 1997), and unlike other regions in the United States, establishment of conservation priorities have begun only recently. In particular, 15 of 52 species of Texas mussel were listed as state-threatened in 2009 (TPWD, 2010), and shortly thereafter, 12 of those species were petitioned for protection under the U.S. Endangered Species Act (ESA) (USFWS, 2011). Included among those 12 species, 1 has been proposed as endangered and 5 have already advanced to candidacy and are pending review by U.S. Fish and Wildlife Service. As a result, there is an urgent need to collect information on the distribution and evolutionary history of these species to ensure listing decisions and conservation efforts are based on sound scientific information.

The Texas Wildlife Action Plan identifies several mussel species in the Trinity River basin as critically imperiled and calls for more information on their status and geographic location (TPWD, 2012). For this drainage, early reports indicate a diverse and productive fauna (Singley, 1893; Strecker, 1931). However, studies within the past 20 years, mainly near Dallas, TX, and nearby reservoirs, by private collectors and TPWD personnel, have described the fauna as being nearly extirpated (Randklev et al. 2010). Recent surveys on the Elm Fork of the Trinity River and the mainstem of the Trinity River indicate that this may not be the case, as several high-density mussel beds, including three state threatened species, were found near several bridge crossings undergoing construction (R.G. Howells pers. comm.). Recently, investigators have discovered populations of *Fusconaia askewi* (Texas pigtoe) and *Potamilus amphichaenus* (Texas heelsplitter) in the middle and upper Trinity River, indicating that research regarding the distribution of endemic and common species in this river is needed.

In an effort to address knowledge gaps regarding the geographic distribution, and conservation status of mussels and potential threats in the Trinity River basin, the objectives of this project were to (1) conduct a longitudinal survey of the Trinity River for rare and common mussel species; (2) assess the genetic diversity within the genus *Fusconaia;* and (3) conduct presence/absence surveys for zebra mussels in the middle and upper Trinity River drainage.

Objective 1: Comprehensive survey of unionids along the entire length of the Trinity River

Introduction

Freshwater mussels (Family: Unionidae) play an important role in freshwater ecosystems through nutrient cycling, increasing habitat heterogeneity, and as a food source for fishes, mammals, and birds (Haag & Williams, 2014). Due to their sensitivity to various environmental stressors, inability to move far from human-mediated perturbations, and reliance on certain fish species to complete their reproductive life cycle, they are one of the most imperiled taxonomic groups in North America (Williams et al., 1993). In Texas, 15 of 52 described species (29%) are listed as state threatened, of which 12 are being reviewed by U.S. Fish and Wildlife Service (USFWS) for listing under the Endangered Species Act (ESA) (TPWD, 2010; USFWS, 2001; 2011). For these species, detailed information on distribution and threats is lacking.

The Trinity River drainage located in central Texas historically supported a diverse mussel fauna (Singley, 1893; Strecker, 1931), including four endemics presently considered imperiled: *Fusconaia chunii*, *Lampsilis satura*, *Pleurobema riddellii*, and *Potamilus amphichaenus* (TPWD, 2010; USFWS, 2011). More recent studies have described the fauna as being nearly extirpated (Randklev et al., 2010), presumably because of intense urbanization within this drainage (Randklev et al., 2010; Burlakova et al., 2012). The Trinity River has a long-history of degraded water quality, particularly between Dallas to Lake Livingston due to contaminant loading from industrial and municipal wastewater treatment plants, raw sewage bypassing, urban runoff and in later years resuspension of contaminates during storm events (Davis, 1997; Land et al., 1998). These impacts resulted in episodic fish kills, which culminated (between 1970-1985) with 13 major fish kills, totaling 1.04 million fish (Land et al., 1998). Improvements in waste-water treatment practices brought about by state and federal legislation eventually led to improved water quality within the Trinity River (Davis, 1997; Land et al., 1998; Perkin and Bonner, 2016), which led to recovery of the mainstem fish assemblage and benthic macroinvertebrate community (Davis, 1997; Perkin and Bonner, 2016). However, little attention has been given to evaluating the status and current condition of the mussel fauna within the mainstem of the Trinity River.

In an effort to support conservation efforts for mussels in the Trinity River basin the objectives of this study were to: 1) examine the status of freshwater mussels along the entire length of the Trinity and the lower portion of the East Fork of the Trinity River, particularly for those species being reviewed for listing under the Endangered Species Act (ESA); 2) use the resulting data to examine habitat associations and spatial patterns in mussel occupancy; and 3) assess the conservation status of threatened species in this basin by creating Conservation Status Maps.

Methods

Study area

The Trinity River basin is located in Southwestern United States (central Texas– see Figure 1) and has an overall length of 579 km and encompasses approximately ~ 46,539 square km making it one of the larger river basins in Texas. The human population in the basin was ~ 6.9 million people in 2010, a majority of which (~ 5.3 million) reside in the Dallas-Fort Worth Metroplex (Perkin & Bonner 2016), located in the headwaters of the Trinity River. The Trinity River is formed by the Clear, West, Elm and East Forks, which then flows from just west of Dallas, TX, to ultimately the Gulf of Mexico (Kleinsasser & Linam, 1990).

Mussel surveys

Survey sites on the mainstem and East Fork of the Trinity River were selected by identifying reaches approximately 10 km in length on each river that could be accessed safely using a motorized boat or canoe. Within each reach, sites were selected using a random sampling design with 2 strata: river left or river right and 2) mesohabitat: banks, backwater (only for reaches in the East Fork of the Trinity) and riffles. All sites were 150 m^2 in area and were searched for 4 person-hours (p-h) visually and tactilely either by snorkel or SCUBA.

We used timed searches in each randomly selected mesohabitat type to locate mussels. The timed search method was chosen because it provides a more effective means of detecting rare species than quantitative sampling methodologies (Vaughn et al., 1997). At each site (i.e., mesohabitat type), we confined the search boundaries to the specific habitat type, ensuring that the search area did not exceed 150 m^2. Each site was then surveyed tactilely and visually for a total of 4 p-h. Surveyors were spread out in the search area and every effort was made to search all available microhabitats. At the end of each search interval, surveyors combined all live specimens into a mesh bag, which was kept submerged in water until completion of the survey. Following completion of the survey, all live mussels from each search period were identified to species, counted, measured and then returned back to the river into the appropriate habitat.

Data analysis

Several techniques were used to analyze characteristics of mussel assemblages and mesohabitat associations in the Trinity River drainage. For all analyses we used species complexes for *Fusconaia chunii/flava* (see Objective 2) and *Truncilla donaciformis/macrodon* because of uncertainties with their taxonomy. We generated sample-based species accumulation curves to estimate average pooled species richness among study reaches and habitat types (Kindt and Coe, 2005). Species accumulation curves were used in two ways because sampling effort (i.e., number of sites) varied among reaches: to (1) examine for significant differences among groups based on the non-overlapping variances (standard deviation) plotted with curves, and (2) determine if sampling effort was adequate based on whether a curve reached its asymptote (Gotelli and Colwell, 2001).

To estimate species richness for the entire study area, we used a first-order (nonparametric) Jackknife species richness estimator, which uses a resampling technique (Palmer, 1990; Kindt & Coe, 2005). We plotted mean CPUE of mussels by reach and habitat and tested for differences among groups using the nonparametric Kruskal-Wallace rank sum test. Each grouping variable (i.e., reach and habitat) was analyzed separately, and if significant differences were observed, a pairwise Wilcoxon rank sum test was implemented to identify differences between group levels.

We analyzed habitat associations of mussels in two ways. First, we calculated proportional abundance by habitat for each species; and Second, using indicator species analysis to test for significant mesohabitat associations (Dufrene & Legendre, 1997; De Cáceres & Legendre, 2009). This analysis calculates an indicator value (IndVal) index based on differences among proportional occurrence and relative abundance of species within groups (e.g., mesohabitat types) and tests for significant differences among groups using a permutation test (Dufrene & Legendre, 1997). The IndVal statistic represents two parameters, including the probability a site belongs to a specific habitat based on the presence of a species (A), and the probability of finding a species at a site belonging to its associated habitat (B). To ease the interpretation of the results, we restricted the analyses such that each species was only allowed to be associated with 1 habitat type.

To assess the conservation status of threatened and endemic species occurring in the Trinity River drainage, we developed range maps for *F. chunii/flava*, *L. satua*, *P.amphichaenus*, *P. riddellii, and T. donaciformis/macrodon*. Conservation status assessment maps are a way to efficiently determine the status of a given species and have been used in conservation assessments by U.S. Fish & Wildlife Service for rare aquatic species. Generally, conservation maps are suitable for coarse-level assessments and are generated using occurrence data mapped at a watershed scale using GIS. The resulting map can then be used to identify range size, survey needs, and high priority areas for conservation. To develop these maps, we followed methods presented in the Conservation Status Map package by the Georgia Conservation Status Map package provided by the Georgia Department of Natural Resources (http://www.georgiawildlife.com/conservation_status_assessment_maps). Occurrence was obtained from the current survey, state agencies (e.g., Texas Parks and Wildlife Department [TPWD], Texas Department of Transportation [TxDOT], Texas Commission on Environmental Quality [TCEQ], Texas Water Development Board [TWDB]), universities (e.g., University of Texas at Tyler, Texas A&M), museums (in state and out-of-state), published literature, and other known sources.

Results/Discussion

Distribution and abundance

We surveyed mussels at 61 sites (5 study reaches) in the mainstem of the Trinity River and the East Fork of the Trinity River (Figure 1). A sixth reach on the Elm Fork is included for comparison and data for this reach comes from environmental impact assessments associated with construction projects on this river (Halff 2013a,b; ACI

2015). In total, 2,445 live individuals from 18 species were found during this survey (Table 1). The three most abundant species (i.e., proportional abundance ≥ 0.15) were *Lampsilis teres*, *Quadrula nobilis*, and *Quadrula mortoni*, which proportionally comprised of 0.21, 0.18 and 0.15 of total individuals collected, respectively (Table 1). The dominant mussel species by reach also varied such that *L. teres* was the most abundant species in Reaches 1, 2, and 5, while *Q. nobilis* was dominant in Reach 3 and *Quadrula apiculata* in Reaches 4 and 6 (Table 1). Overall proportional occurrence by species (i.e., proportion of sites a species was observed) varied from 0.03 to 0.41 (Table 1) and the following species were considered prevalent (i.e., proportional occurrence ≥ 0.25 or 15/61 sites) across the study area: *L. teres* (0.41), *Leptodea fragilis* (0.25), and *T. donaciformis*/*macrodon* (0.25).

Species richness varied by reach and appeared to be related to proximity to Dallas-Fort Worth and Lake Livingston (Figure 2A). Reaches 3 and 6 had the highest species richness, Reaches 1, 2 and 5 had intermediate levels of species richness and Reach 4 had the lowest level of species richness (Figure 2A). Sampling effort was generally sufficient; however, species accumulation curves for Reaches 4 and 5 failed to asymptote (Figure 2A), indicating richness is expected to be higher if more sites were sampled. Species richness estimated for the entire study area (JACK1 = 20.0 ± 0.00) was higher than observed richness (n = 18), suggesting additional species might still be present within the mainstem of the Trinity, Elm Fork of the Trinity, and East Fork of the Trinity. Species not detected here but are known to occur in this drainage (historically or presently) include: *Anodonta suborbiculata* (flat floater), *Cyrtonaias tampicoensis* (Tampico pearlymussel), *Glebula rotundata* (round pearlshell), *Lasmigona complanata* (white heelsplitter), *Ligumia subrostrata* (pondmussel), *Pleurobema riddellii* (Louisiana pigtoe), *Strophitus undulatus* (creeper), *Uniomerus declivis* (tapered pondhorn), *Uniomerus tetralasmus* (pondhorn), *Utterbackia imbecillis* (paper pondshell), and *Villosa lienosa* (little spectaclecase)(Strecker, 1931; Howells et al., 1996). Though, most of these species (e.g., *L. subrostrata* and *U. imbecillis*) occur primarily in slack water habitats such as oxbows and sloughs, which were not surveyed during this study.

Mean CPUE ranged from 1.40 ± 0.60 (SE) mussels/p-h (Reach 1) to 27.51 ± 10.00 mussels/p-h (Reach 3) and averaged 8.30 ± 4.91 mussels/p-h across reaches (Figure 2B). Kruskal-Wallis rank sum test revealed significant differences in CPUE among study reaches ($\chi^2 = 17.55$, df = 5, p = 0.004), and pairwise comparisons using Wilcoxon rank sum test indicated the significant differences between Reaches 3 & 4 (p = 0.047) and Reaches 4 & 6 (p = 0.033); Reaches 1 & 6 (p = 0.074) were marginally significant. These results indicate mussel abundance was highest in Reach 3, located ~ 275 rkm downstream from Dallas-Fort Worth, and in Reach 6, located in the Elm Fork of the Trinity River upstream of Dallas-Fort Worth (Figures 1 & 2B). Comparing our overall abundance results to other rivers (mean ± SE; middle Brazos – 63.69 ± 14.69, lower Sabine – 21.68 ± 4.93, and lower Guadalupe – 63.30 ± 10.5) where similar sampling methodology was used indicates mussel abundance on average is low (Randklev et al., 2014a, b; Tsakiris & Randklev, 2016), though similar to other impaired waterbodies (mean ± SE; Lower Brazos – 8.69 ± 1.94; Rio Grande – 3.75 ± 10.66) (Randklev et al., 2014c; Randklev et al. 2017).

Mussel-habitat associations

Mussel species occupancy varied by mesohabitat type (Table 1) and as a result so did species richness, though not significantly (Figure 3A). Species accumulation curves for riffle and bank habitats reached an asymptote, suggesting our sampling effort was sufficient. However, the species accumulation curve for backwater habitat did not asymptote, which suggests additional species might be present within this habitat type. CPUE ranged from 8.24 mussels/p-h in backwater to 16.14 mussels/p-h in banks (Figure 3B). Kruskal-Wallis test revealed no significant differences in CPUE ($\chi^2 = 0.49$, df = 2, p = 0.78) across habitat types.

Results from the Indicator species analysis show that 6 of the 20 species observed in the Trinity River basin were associated significantly with one specific mesohabtiat type. *Potamilus amphichaenus* was associated with banks, *P. grandis*, *Q. apiculata*, *T. parvum*, and *T. texasense* with backwater, and *Q. nobilis* with riffles (Table 2). The remaining species also show mussel-mesohabitat associations, but they were not significant.

Status of threatened species

Abundance by reach and habitat

During the course of our surveys we found live individuals of *P. amphichaenus*, presently listed as state-threatened and pending review for federal protection by U.S. Fish and Wildlife Service (USFWS, 2011). We also found *F. chunii/flava* and *T. donaciformis/macrodon,* which do not have a state or federal conservation status due, in part, to uncertainties with their taxonomy. CPUE of threatened species generally followed a similar trend as overall abundance estimates of mussels throughout the study reaches (Figure 4). For *F. chunii/flava*, overall mean CPUE was 1.57 ± 0.90 and ranged from 0.05 ± 0.05 mussels/p-h (Reach 5) to 5.34 ± 0.13 mussels/p-h (Reach 6); this species was moderately abundant in Reach 3 (3.15 ± 0.25 mussels/p-h) and was not observed, live or shell, in Reach 4 (Figure 4A). CPUE by habitat for *F. chunii/flava* ranged from 0.75 ± 0.55 mussels/p-h in backwater to 1.83 ± 1.83 mussels/p-h in banks; mean abundance in riffles were 1.64 ± 0.88 mussels/p-h (Figure 4D). Thus, abundance for *F. chunii/flava* was highest in Reaches 3 and 4 and backwater and riffle habitats were most productive for this species.

For *P. amphichaenus* overall mean CPUE was 0.07 ± 0.04 mussels/p-h, which is low relative to *F. chunii/flava*. Mean CPUE was consistent across the study reaches where it occurred (Reach 2 – 0.21 ± 0.21 mussels/p-h; Reach 3 – 0.23 ± 0.23 mussels/p-h) (Figure 4B). We did not find live or shell of this species from Reaches 1, 4, 5 and 6. *Potamilus amphichaenus* was only observed in bank habitats, which had a mean CPUE of 0.18 ± 0.11 mussels/p-h for this species (Figure 4E). Taken together, these results indicate that Reaches 2 and 3 are most productive for this species and banks are its optimal habitat.

Overall mean CPUE for *T. donaciformis/macrodon* was 0.12 ± 0.07 mussels/p-h, which is higher than *P. amphichaenus* but lower than *F. chunii/flava*. Mean CPUE across the study reaches ranged from 0.02 ± 0.02 mussels/p-h (Reach 4) to 0.42 ± 0.15 mussels/p-h (Reach 3) (Figure 4C). We did not find live or shell for this species from Reaches 1 and 6. CPUE by habitat varied with bank (0.12 ± 0.06 mussels/p-h) and riffles (0.15 ± 0.09 mussels/p-h) having similar abundance, while CPUE was on average lowest in backwater habitats (0.02 ± 0.02 mussels/p-h) (Figure 4F). Thus, *T. donaciformis/macrodon* appears to be most abundant in Reach 3 and bank and riffles are its optimal habitat.

Conservation Status maps

For *F. chunii/flava* our Conservation Maps show it occurring throughout the middle and upper Trinity River drainage, though there appear to be only three significant populations: Elm Fork of the Trinity (Reach 6), East Fork of the Trinity River (Reach 5) and the mainstem near Oakwood, TX (Reach 3) (Figure 5). Of these, the Elm Fork of the Trinity and Oakwood populations appear to be the most robust in terms of prevalence (i.e., number of sites where this species occurs) and overall abundance. Our maps also indicate a potential range decline as this species currently occupies only ~11% of the HUC10s within its presumptive range (Figure 5).

The distribution of *L. satura* within the Trinity River drainage is largely unknown. Archaeological specimens of this species from the late Holocene have been collected from the upper Trinity River drainage (Wolverton & Randklev 2016), but this species has yet to be found, live or shell, during contemporary surveys (Figure 6). *Lampsilis satura* currently occurs in the San Jacinto, Neches-Angelina and Sabine basins and so its absence from the Trinity is enigmatic (Strecker, 1931; Howells et al., 1996). Recent reports of live individuals from the Elm Fork and mainstem near Dallas by private contractors are questionable and several have proven to be misidentifications (Neck 1990; McDermid et al., 2013) and so any report of this species from this drainage should be confirmed using genetics.

Potamilus amphichaenus historically occurred throughout the Trinity River drainage (Howells et al. 1996) but now appears to be restricted to the mainstem and nearby tributaries between Dallas-Fort Worth and Lake Livingston (Figure 7). Similar to *F. chunii/flava*, stronghold populations (i.e., high prevalence and abundance) for this species occur primarily between Trinidad (Reach 2) and Oakwood, TX (Reach 3). Our evaluation of current occupancy of *P. amphichaenus* relative to its presumptive range indicates a potential range decline as this species currently occupies ~7% of the HUC10s within its range (Figure 7).

Pleurobema riddellii was described from Dallas, Texas, but water quality issues in the late 19th and early 20th centuries are thought to have locally extirpated this species (Strecker, 1931; Read & Oliver, 1953; Read, 1954). To date, no live or recent shell has been found during contemporary surveys of this river (Figure 8). Recent reports of live individuals from the Elm Fork or mainstem near Dallas by private contractors appear to be misidentifications (Halff 2013a,b; ACI 2105 Figure 8). We examined photovouchers

of several specimens from these collections purported to be this species and all appear to be misidentified *Q. mortoni* (western pimpleback), *F. chunii/flava*, or *T. truncata*.

Truncilla donaciformis/macrodon historically occurred throughout the Trinity drainage (Howells et al. 1996). Our Conservation Status maps show this species primarily occurring within the middle Trinity, though there appears to be a disjunct population in the lower Reaches of the East Fork of the Trinity River (Figure 9). Stronghold populations for this species occur mainly near Oakwood, TX (Reach 3). Finally, this species appears to have undergone a range decline as it currently occupies only ~10% of the HUC10s within its presumptive range (Figure 9).

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Table 1. Species of mussel and their respective abundance, proportional abundance (prop), occurrence (number of times a species occurred at a site) and proportional occurrence. In addition, proportional abundance of species is presented by reach and habitat type. Habitat types are as follows: BH = bank, BW = backwater, and R = riffle.

Species	Abundance		Occurrence		Proportional abundance by reach						Proportional abundance by habtat		
	n	prop	n	prop	1	2	3	4	5	6*	BH	BW	R
Amblema plicata	24	0.01	2	0.03	0.00	0.00	0.01	0.00	0.00	0.02	0.01	0.00	0.01
Arcidens confragosus	14	0.01	4	0.07	0.00	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01
Fusconaia chunii/flava	231	0.09	10	0.16	0.13	0.01	0.11	0.00	0.03	0.03	0.12	0.00	0.09
Lampsilis hydiana	0	0.00	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lampsilis teres	505	0.21	25	0.41	0.58	0.36	0.13	0.00	0.68	0.07	0.27	0.61	0.08
Leptodea fragilis	108	0.04	15	0.25	0.02	0.07	0.04	0.01	0.05	0.03	0.07	0.04	0.02
Megalonaias nervosa	174	0.07	4	0.07	0.00	0.00	0.09	0.01	0.00	0.01	0.08	0.00	0.08
Obliquaria reflexa	59	0.02	11	0.18	0.05	0.02	0.02	0.00	0.03	0.24	0.02	0.01	0.03
Plectomerus dombeyanus	78	0.03	3	0.05	0.00	0.00	0.04	0.00	0.00	0.00	0.02	0.00	0.05
Potamilus amphichaenus	30	0.01	7	0.11	0.00	0.06	0.01	0.00	0.00	0.00	0.03	0.00	0.00
Potamilus purpuratus	93	0.04	7	0.11	0.01	0.01	0.05	0.00	0.00	0.03	0.04	0.00	0.05
Pyganodon grandis	4	0.00	3	0.05	0.00	0.00	0.00	0.04	0.00	0.03	0.00	0.01	0.00
Quadrula apiculata	84	0.03	9	0.15	0.03	0.00	0.00	0.93	0.02	0.29	0.01	0.23	0.02
Quadrula mortoni	364	0.15	12	0.20	0.10	0.12	0.17	0.00	0.05	0.05	0.13	0.01	0.19
Quadrula nobilis	450	0.18	7	0.11	0.00	0.18	0.22	0.00	0.00	0.00	0.13	0.00	0.26
Quadrula verrucosa	172	0.07	9	0.15	0.07	0.13	0.07	0.00	0.04	0.14	0.06	0.01	0.09

*Data comes from Halff (2013a, b) and ACI (2015).

Table 1. Continued.

Species	Abundance		Occurrence		Proportional abundance by reach						Proportional abundance by habtat		
	n	prop	n	prop	1	2	3	4	5	6*	BH	BW	R
Truncilla donaciformis/macrodon	40	0.02	15	0.25	0.00	0.04	0.01	0.01	0.01	0.00	0.02	0.00	0.02
Toxolasma parvum	13	0.00	4	0.07	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.05	0.00
Toxolasma texasense	2	0.00	2	0.03	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.00
Truncilla truncata	0	0.00	0	0.00	0.01	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00

Table 2. Species mesohabitat associations based on indicator species analysis. IndVal represents the test statistic, *A* is the probability a site belongs to a specific habitat based on the presence of a species, *B* is the probability of finding a species at a site belonging to its associated habitat and p is the level of significant ($\alpha = 0.10$). Habitat types are as follows: BH = bank, BW = backwater, and R = riffle.

Species	**Habitat type**			**Statistics**			
	BH	**BW**	**R**	***A***	***B***	***IndVal***	***p***
Amblema plicata		x		0.75	0.15	0.34	*0.353*
Arcidens confragosus			x	0.45	0.23	0.32	0.371
Fusconaia chunii/flava			x	0.47	0.50	0.49	0.162
Lampsilis hydiana		x		0.46	0.07	0.19	0.685
Lampsilis teres	x			0.32	0.58	0.43	0.969
Leptodea fragilis			x	0.32	0.50	0.40	0.796
Megalonaias nervosa			x	0.57	0.27	0.39	0.149
Obliquaria reflexa			x	0.52	0.45	0.49	0.310
Plectomerus dombeyanus			x	0.79	0.14	0.33	0.277
Potamilus amphichaenus	x			1.00	0.29	0.54	**0.009**
Potamilus purpuratus			x	0.35	0.27	0.31	0.950
Pyganodon grandis		x		0.76	0.31	0.48	**0.023**
Quadrula apiculata		x		0.68	0.46	0.56	**0.044**
Quadrula mortoni			x	0.54	0.41	0.47	0.274
Quadrula nobilis			x	0.76	0.27	0.46	**0.065**
Quadrula verrucosa			x	0.57	0.41	0.48	0.178
Truncilla donaciformis/macrodon			x	0.57	0.32	0.43	0.288
Toxolasma parvum		x		0.87	0.15	0.37	**0.086**
Toxolasma texasense		x		1.00	0.15	0.39	**0.048**
Truncilla truncata		x		0.64	0.15	0.31	0.330

Figure 1. Map of the sample sites and study reaches in the Trinity River basin

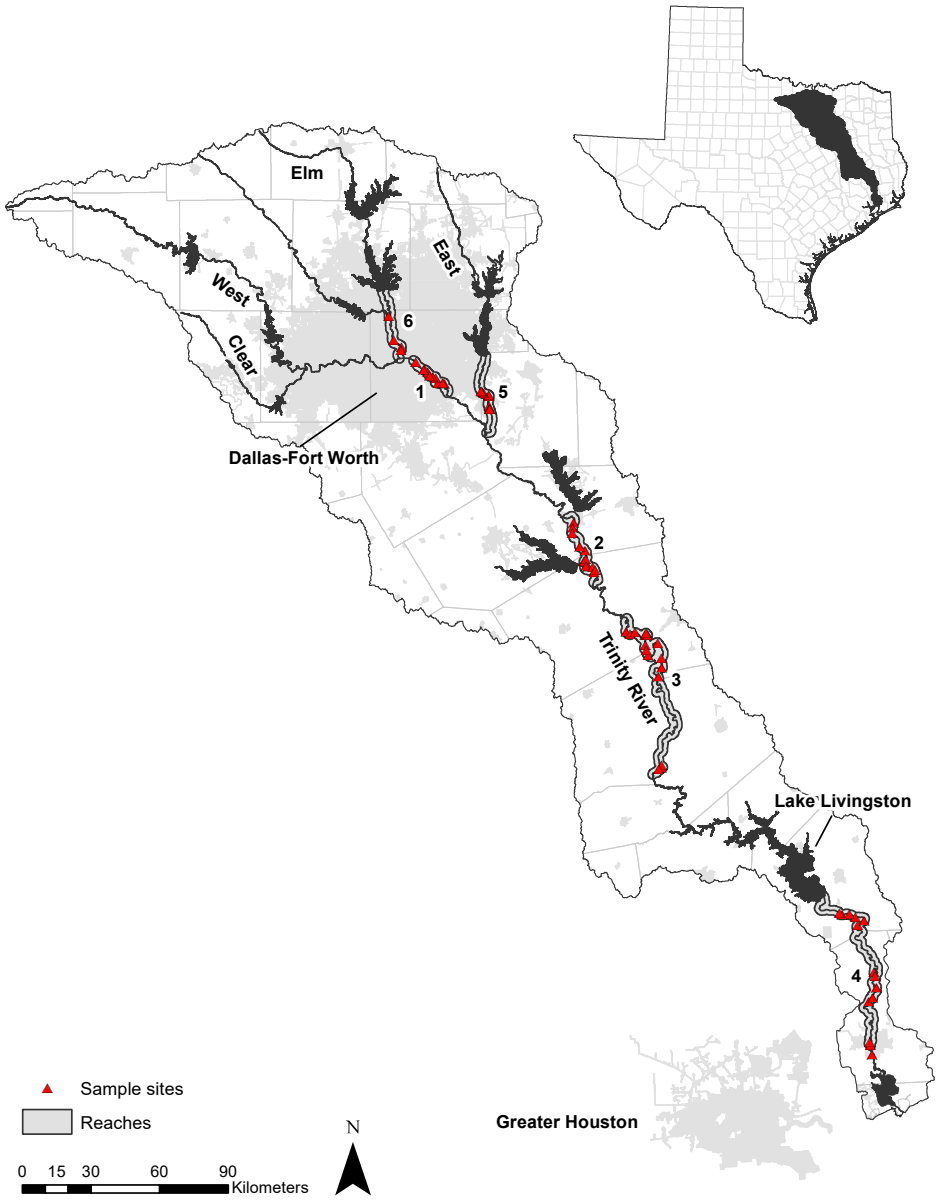


Figure 2. (A) Species accumulation curves and (B) catch-per-unit effort (CPUE) of mussels by reach in the Trinity River basin, Texas. Error bars are ± 1 SE.

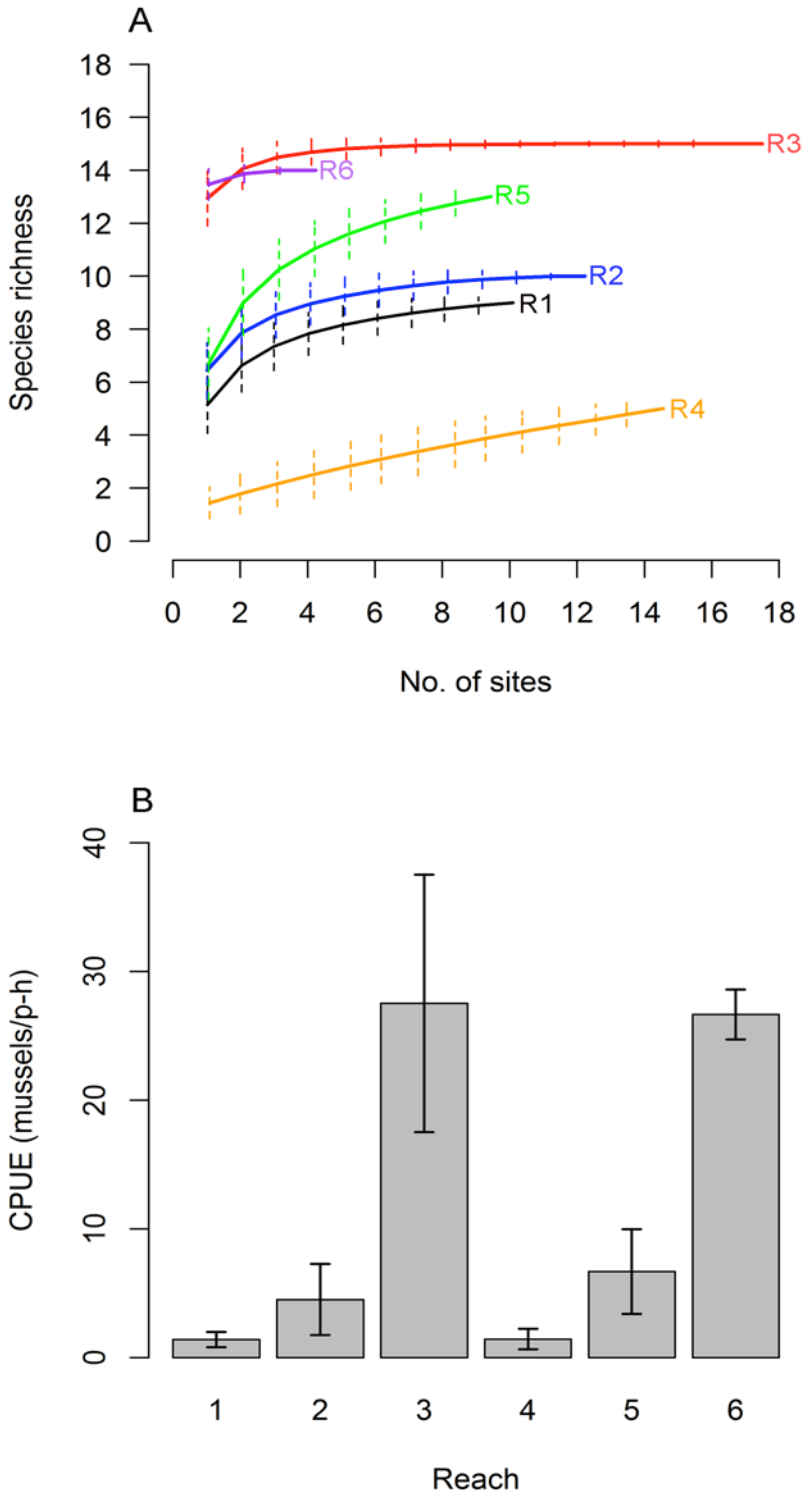


Figure 3. (A) Species accumulation curves and (B) catch-per-unit effort of mussels by habitat type in the Trinity River basin, Texas. Error bars are ± 1 SE. Habitat types are as follows: BH = bank, BW = backwater, and R = riffle.

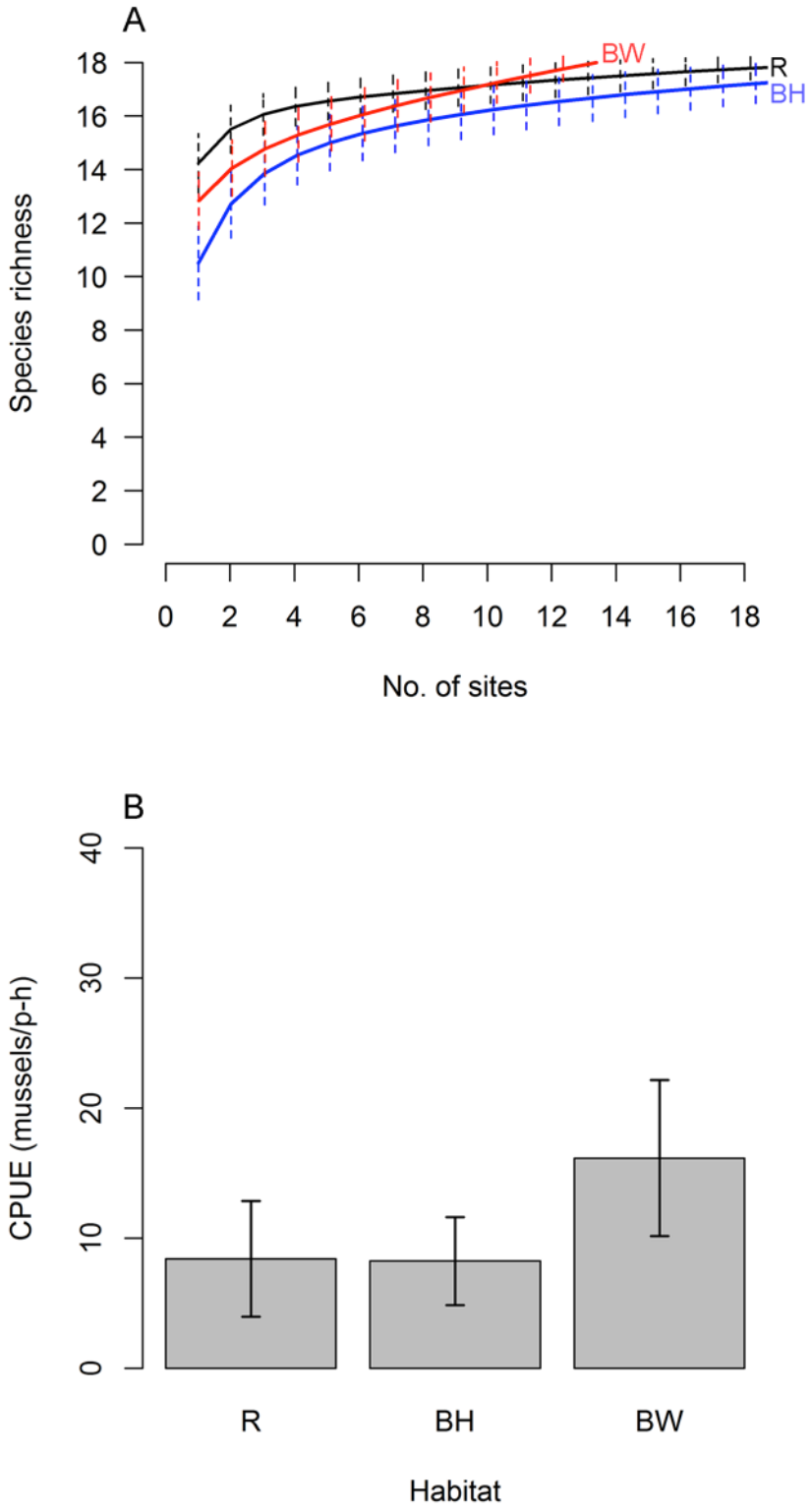


Figure 4. CPUE by reach and habitat type of *Fusconaia chunii/flava*, *Potamilus amphichaenus*, and *Truncilla donaciformis/macrodon* in the Trinity River basin, Texas. Error bars are ± 1 SE. Habitat types are as follows: BH = bank, BW = backwater, and R = riffle.

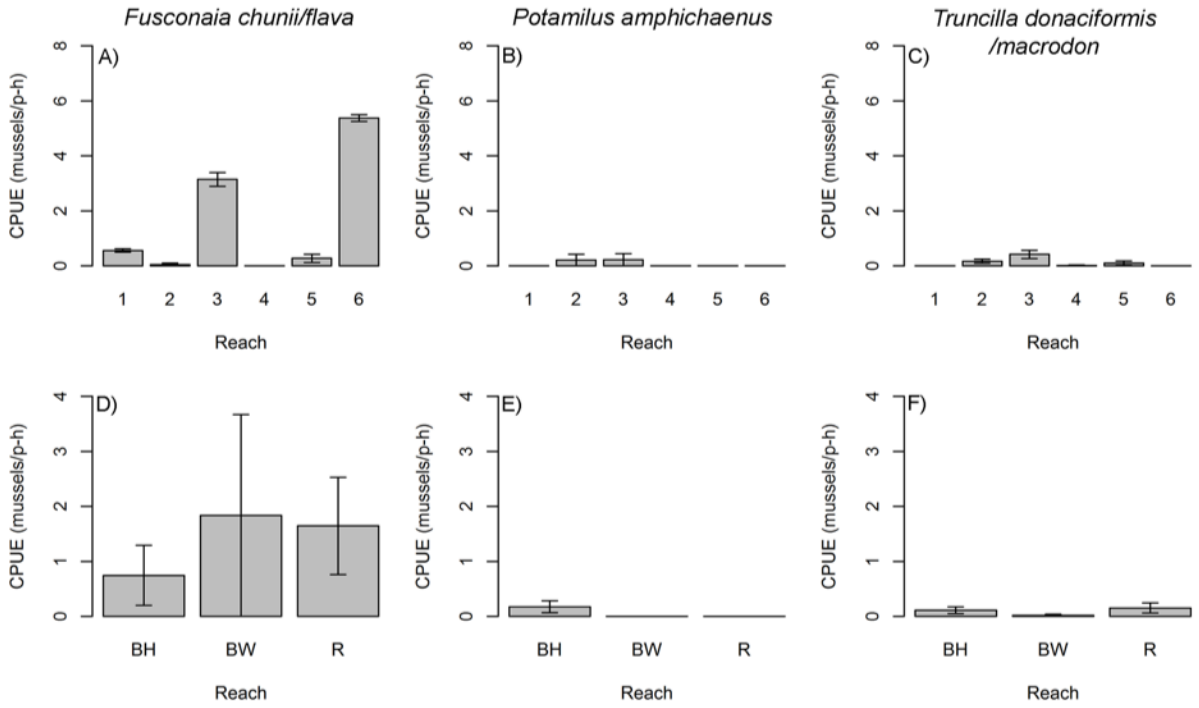


Figure 5. Conservation assessment map for *Fusconaia chunii*/*flava*. These two species are grouped because they are morphologically indistinguishable and co-occur (see results of Objective 2 for more details). Survey data used to construct this map are taken from the present study plus those obtained from museums, academic, state, and federal agencies and include both live and shell. HUC10s are colored based on date of sampling. Dashed black line denotes presumptive range.

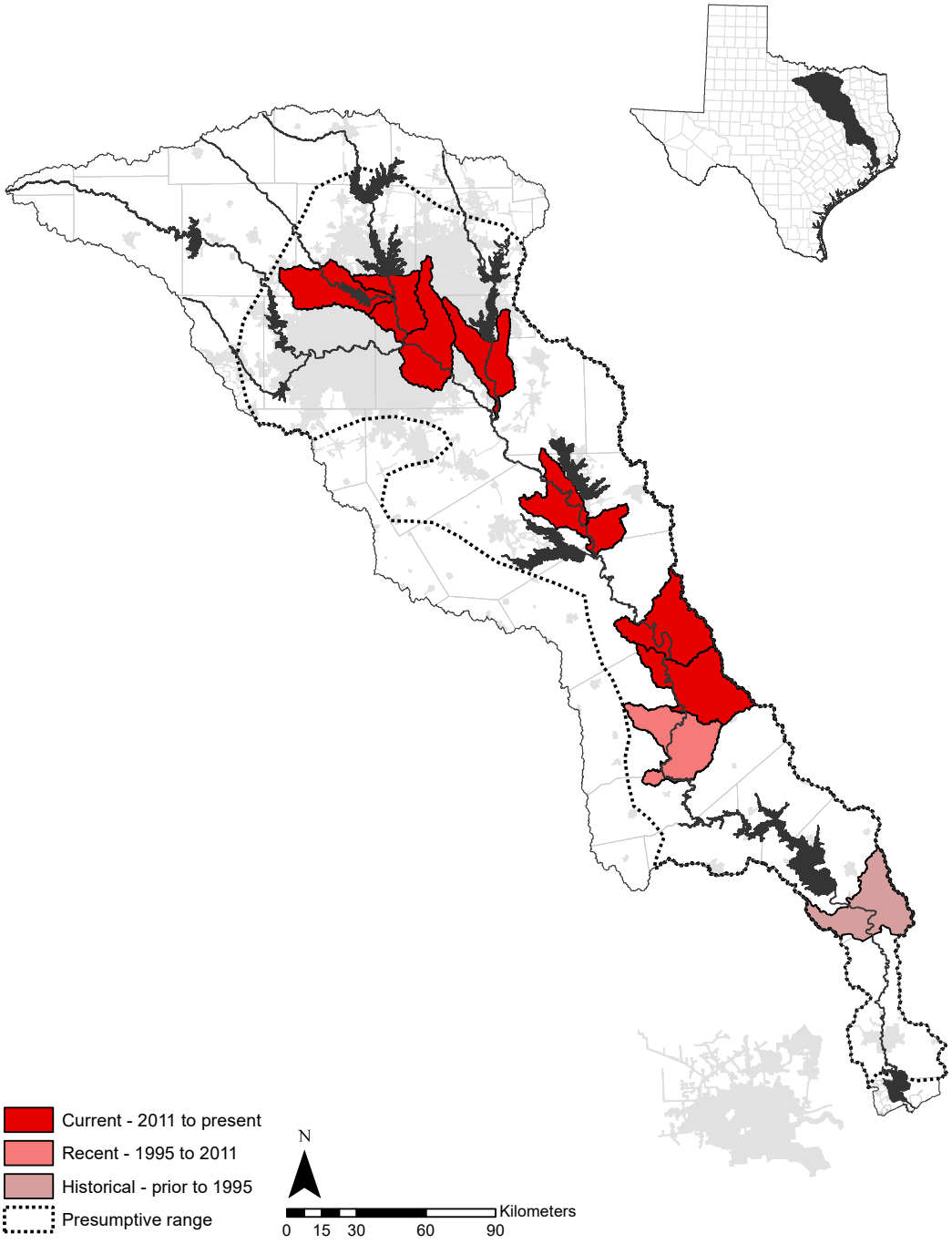


Figure 6. Conservation assessment map for *Lampsilis satura* (sandbank pocketbook). Survey data used to construct this map are taken from the present study plus those obtained from museums, academic, state, and federal agencies and include both live and shell. HUC10s are colored based on date of sampling. Dashed black line denotes presumptive range.

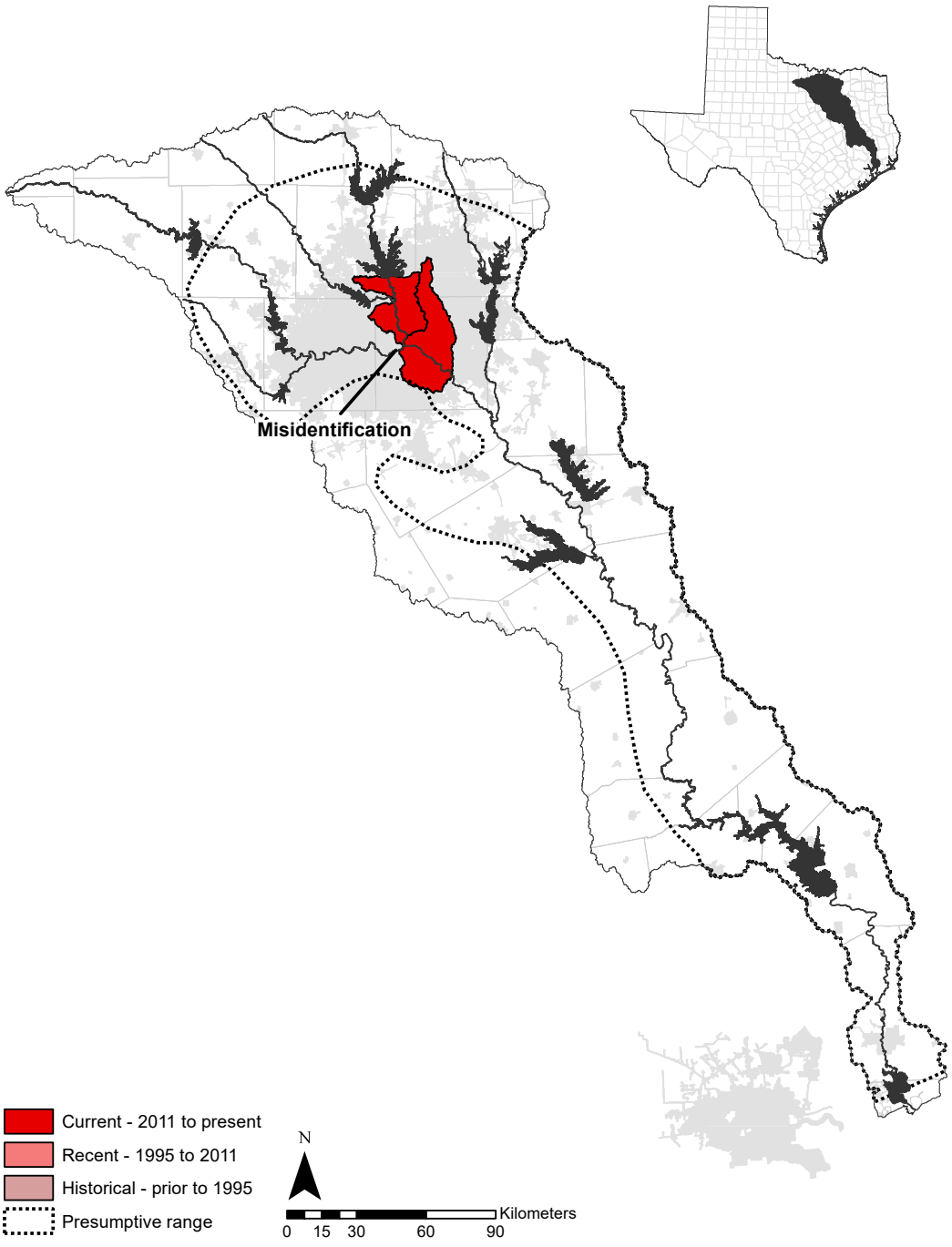


Figure 7. Conservation assessment map for *Potamilus amphichaenus* (Texas heelsplitter). Survey data used to construct this map are taken from the present study plus those obtained from museums, academic, state, and federal agencies and include both live and shell. HUC10s are colored based on date of sampling. Dashed black line denotes presumptive range.

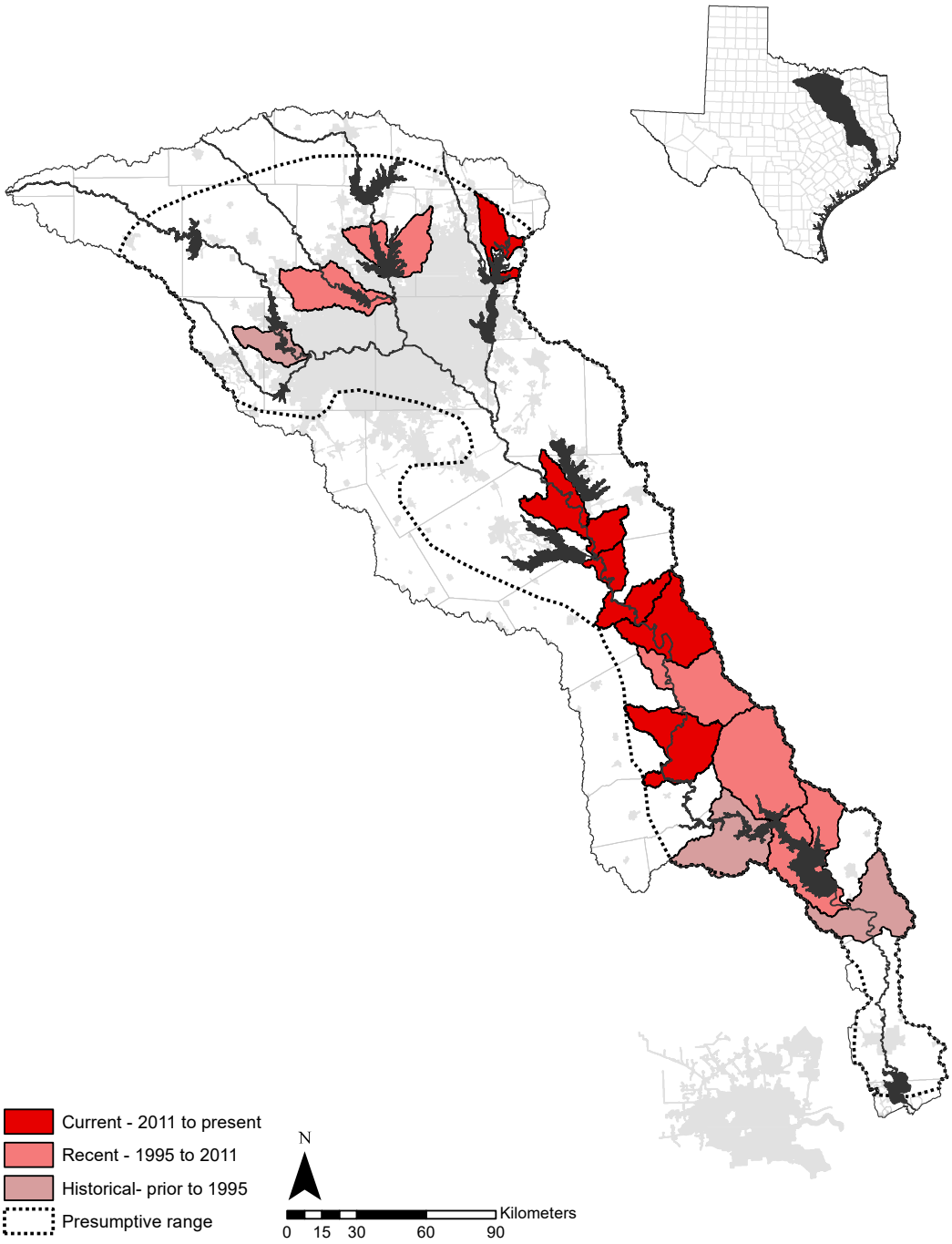


Figure 8. Conservation assessment map for *Pleurobema riddellii* (Louisiana pigtoe). Survey data used to construct this map are taken from the present study plus those obtained from museums, academic, state, and federal agencies and include both live and shell. HUC10s are colored based on date of sampling. Dashed black line denotes presumptive range.

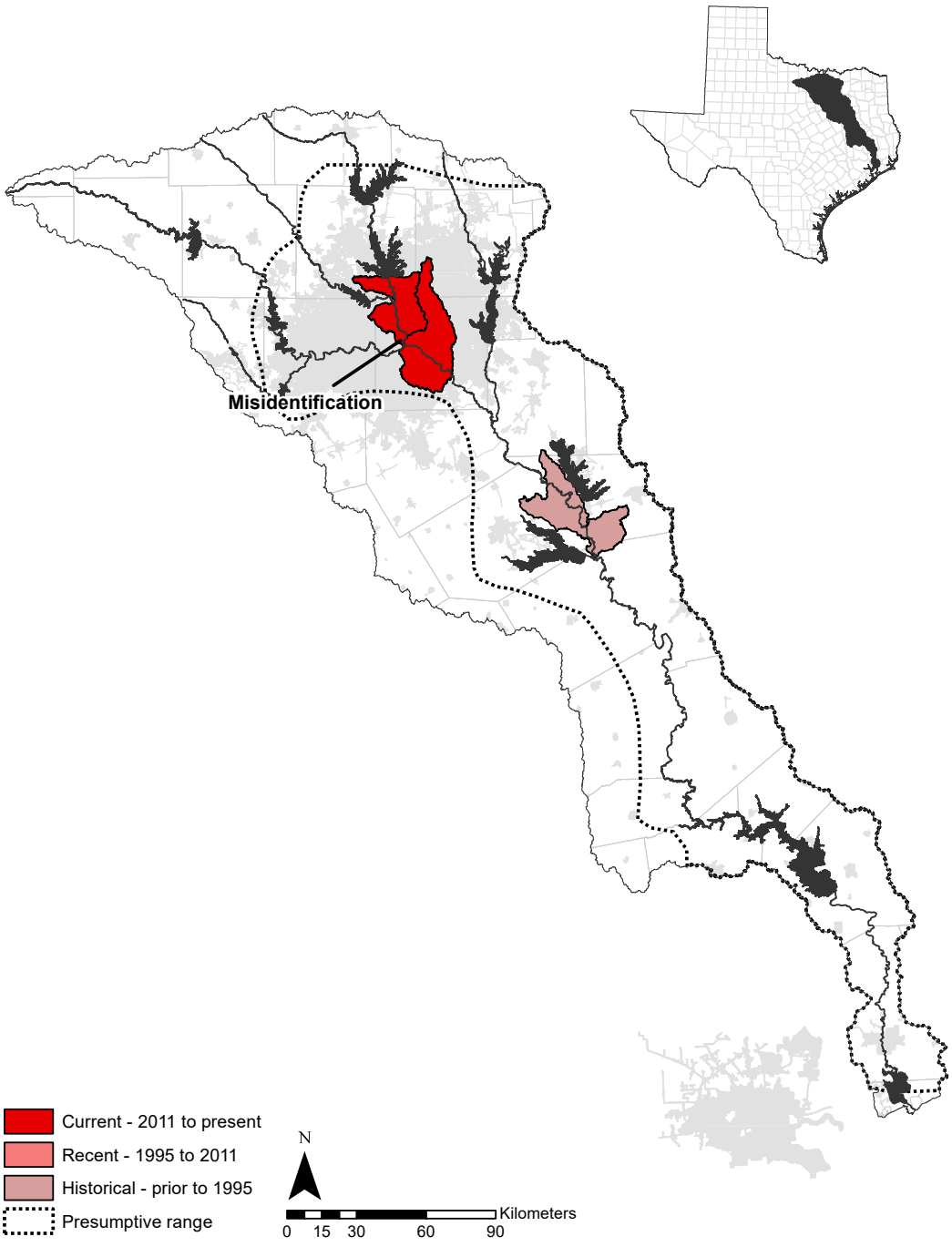
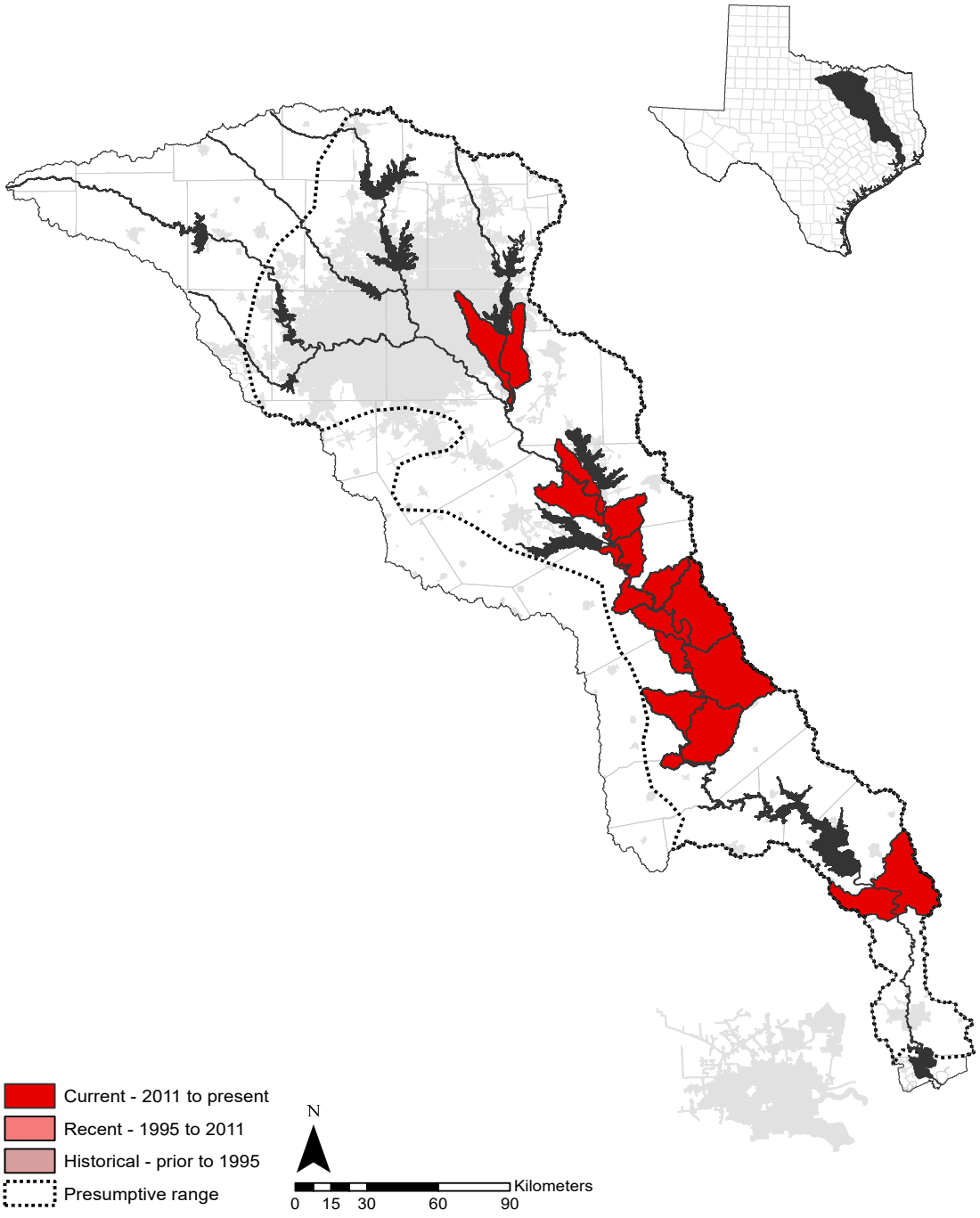


Figure 9. Conservation assessment map for *Truncilla donaciformis/macrodon*. These two species are grouped because the identify of *T. donaciformis* in this basin remains unresolved. Survey data used to construct this map are taken from the present study plus those obtained from museums, academic, state, and federal agencies and include both live and shell. HUC10s are colored based on date of sampling. Dashed black line denotes presumptive range.



Objective 2: Assess the genetic diversity and taxonomic identity of *Fusconaia* species in the Trinity River basin.

Introduction

Rivers in East Texas harbor high species diversity and endemism of freshwater mussels (Burlakova et al., 2011). In the Sabine-Trinity Province including the Calcasieu, Neches, Sabine, and Trinity rivers (*sensu* Haag 2010), currently four nominal species in the genus *Fusconaia* are recognized, of which three are considered endemic to this province: *Fusconaia askewi*, *Fusconaia chunii*, and *Fusconaia lananensis*. Recent molecular analyses of this group have concluded that: (1) *F. lananensis* is not a valid species and likely a junior synonym of *F. askewi* (Burlakova et al., 2012; Campbell & Lydeard, 2012); (2) *F. chunii* is likely a senior synonym of *F. askewi* (Burlakova et al., 2012) or a junior synonym of *F. flava* (Vidrine, 1993; Howells et al., 1996), although a more recent phylogenetic study of the tribe Pleurobemini (which includes the genus *Fusconaia*) indicated that *F. chunii* is genetically distinct from *F. askewi* or *F. flava* (Inoue et al., unpublished); and (3) *F. flava* likely occurs in the Mississippi Embayment Province of Texas (i.e., the Red River drainages; Howells et al., 1996). However, these conclusions have not been explicitly tested using a molecular approach that includes multiple markers, topotypic specimens and a large sample size, in terms of numbers per site and geographic coverage. This is problematic as several of these species are of conservation concern. Thus, genetic studies are needed for *F. askewi*, *F. chunii*, *F. lananensis*, and *F. flava* to resolve their taxonomic status and evolutionary history.

Significant knowledge gaps exist regarding species boundaries and genetic diversity of *Fusconaia* in east Texas, which has the potential to hinder conservation activities for species in this group. Thus, the goal of this project was to delineate species boundaries and assess phylogenetic relationships of *Fusconaia* species, specifically in the Trinity River basin. Our specific objectives were to 1) use two mitochondrial DNA (mtDNA) genes and one nuclear DNA fragment to delineate species boundaries, and 2) assess intra- and inter-specific morphological variations using Fourier shape morphometrics to see if differences in genetic lineages are reflected in external morphology.

Methods

Sample Collection

We collected 132 individuals of *F. askewi* from the Neches and Sabine River drainages, 74 individuals of *F. chunii* from the Trinity River drainage (including the East Fork of the San Jacinto River), and 83 individuals of *F. flava* from six major river drainages in Arkansas, Louisiana, and Texas for molecular and morphometric analyses (Figure 1). Initial species identification was based on collected localities. Live specimens were euthanized with ethanol and then separated into soft tissue and shell. Soft tissue was preserved in absolute ethanol until DNA extraction and shells were scrubbed inside and out to remove any remaining tissue in preparation for the morphometric analyses.

Sample Preparation, DNA Sequencing, and Phylogenetic Analysis

We extracted genomic DNA from mantle tissue using standard CTAB/chloroform extraction followed by ethanol precipitation (Saghai-Maroof et al., 1984). We amplified two mitochondrial (mtDNA) genes, cytochrome *c* oxidase subunit I (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*), and one nuclear DNA segment, internal transcribed spacer 1 (*ITS1*). We used the *cox1* primers described by Folmer et al*.,* (1994), *nad1* primers described by Campbell et al*.* (2005), and the *ITS1* primers described by *King et al.* (1999). Because of difficulty amplifying *cox1*, we used two alternative *cox1* primer sets described by Campbell et al. (2005) and Inoue et al. (2015). PCR products were visualized using 1% agarose gel electrophoresis and purified with ExoSAP-IT (Affymetrix) or GenCatch Gel Extraction Kit (Epoch Life Science). We employed Eurofins Genomics (Louisville, KY) for DNA sequencing. Sequences were assembled and aligned using SeqMan Pro v14.0 (DNASTAR. Madison, WI). We used MAFFT v7 (Katoh & Standley 2013) to perform multiple sequence alignment.

We generated haplotype networks (Clement et al*.,* 2000) for mtDNA (i.e., concatenated two mtDNA genes) and nuclear *ITS1* segment separately in POPART (http://popart.otago.ac.nz/). Phylogenetic trees were reconstructed using Bayesian inference (BI) for mtDNA. We did not reconstruct *ITS1* phylogenetic tree due to low variation within the segment (see Results). We included 14 species in the tribe *Pleurobemini* (10 *Fusconaia* species, three *Pleurobema* species, *Eurynia dilatata*) to evaluate phylogenetic relationships. We also included *Quadrula pustulosa* and *Arcidens confragosus* as outgroups. Prior to the phylogenetic analysis, we used METAPIGA v3.1 (Helaers & Milinkovitch, 2010) to identify unique haplotypes and evaluate substitution saturation in the dataset. We only used unique haplotypes for the phylogenetic analysis and used KAKUSAN4 (Tanabe, 2011) to estimate best-fit model of nucleotide substitution for each codon partition of *cox1* and *nad1*. Based on Bayesian information criterion (BIC), the best substitution model for the *cox1* dataset was GTR+Γ for the first codon, F81 for the second codon, and HKY+Γ for the third codon. For the *nad1* dataset, the best substitution model was K80+Γ for the first codon, HKY for the second codon, and HKY+Γ for the third codon. Phylogenetic analysis was performed with MRBAYES v3.2.6 (Ronquist et al*.,* 2012). Two simultaneous Markov chain Monte Carlo runs (MCMC; each chain containing three heated chains and one cold chain) were executed for 8×10^6 generations, with trees sampled every 1000 generations for a total of 8001 trees in the initial samples. We used TRACER v1.5 (Rambaut & Drummond, 2009) to assess the convergence of MCMC by plotting the log-likelihood scores for each sampled point. When the likelihood values reached a plateau with sufficient effective sample sizes (ESS > 200), we considered the Markov chains stationary. Accordingly, we discarded the first 25% of trees (2000 trees) as burn-in, and the remaining trees were retained and evaluated using the 50% majority rule for a consensus tree.

We used DNASP v5.10 (Librado & Rozas, 2009) to estimate number of haplotypes (H), mean number of nucleotide differences (K), and mean nucleotide diversity (π) from each gene for five groups. The groups were assigned to individuals by species identified by the phylogeny and collected drainages (i.e., *F. askewi* from the

Neches River, *F. askewi* from the Sabine River, *F. chunii* from the Trinity River, *F. flava* from the Trinity River, and *F. flava* from elsewhere; see Results). We used MEGA v7.0.16 (Kumar et al., 2016) to estimate pairwise genetic divergence between groups under maximum composite likelihood method for the concatenated mtDNA and *ITS1* datasets separately.

Divergence Time Estimate and Species Delineation

We used a molecular clock method implemented in BEAST v2.4.5 (Bouckaert et al., 2014) to estimate divergence time among *Fusconaia* species and delineate species boundaries. We used only the mtDNA dataset. A random starting tree was estimated under the HKY+Γ model for each *cox1* and *nad1* dataset with estimated base frequencies. A relaxed lognormal clock model and Yule model were used. We used a substitution rates available for Unionoida (Froufe et al*.,* 2016), where the substitution rate was $2.56 \times 10^{-9} \pm 0.6 \times 10^{-9}$ substitutions site^{-1} year^{-1} estimated from two *Unio* species currently separated by the Strait of Gibraltar (Froufe et al., 2016). Analysis was run for 1×10^{7} generations with sampling every 1000 generations and a burn-in of 25% of the total saved trees, and the remaining trees were retained and evaluated using the maximum clade credibility method for a consensus tree.

Fourier Morphometric Analysis

We used Fourier shape morphometrics to compare the outlines of shell shapes among populations and species. We used a total of 236 individuals for the analysis and tested five groups: *F. askewi* (Neches; n = 64), *F. askewi* (Sabine; n = 40), *F. chunii* (Trinity; n = 41), *F. flava* (Trinity; n = 29), and *F. flava* (widespread; n = 62). The widespread *F. flava* included those collected from the Arkansas River drainage (West Fork Point Remove Creek, AR), Calcasieu River drainage (Bundick Creek, LA), Ouachita River drainage (the mainstem, Little Missouri River, and North Fork of the Saline River, AR), and Red River drainage (Cossatot River, AR) obtained from the Arkansas State University Museum of Zoology. We took digital photographs of the right valve of each specimen with a Canon EOS7D SLR camera. We first extracted the outline of the shell by cropping the image using Adobe® Photoshop® CC v2015.0.0 (Adobe Systems). Using cropped shell images, shell outline was described by 20 Fourier coefficients using SHAPE v1.3 (Iwata & Ukai, 2002). We analyzed morphological variation within and between species through principal component analysis (PCA) and used multivariate analysis of variance (MANOVA) and discriminant function analysis (DFA) to determine how frequently principal component (PC) scores correctly distinguished between groups. We used the first 10 PC axes for MANOVA and DFA.

Results

We examined ~658 base pairs (bp) of the *cox1* gene from 270 individuals, ~608 bp of the *nad1* gene from 160 individuals, ~533 bp of the *ITS1* gene from 104 individuals. We did not find any indication of substitution saturation in the concatenated mtDNA dataset (data not shown). Haplotype networks revealed two distinct clusters in the *cox1* and *nad1* datasets (Figure 2A and B). In both mtDNA networks, one cluster comprised of *F. askewi* from the Neches and Sabine rivers and individuals initially

identified as *F. chunii* from the mainstem above Lake Livingston and the East Fork of the Trinity River. The other cluster consisted of individuals initially identified as *F. chunii* from the East Fork of the San Jacinto River and the mainstem of the Trinity River (hereafter, *F. flava* from the Trinity River) and *F. flava* collected from elsewhere (hereafter, widespread *F. flava*). For the first cluster, *F. askewi* shared the same haplotypes between drainages and comprised of large number of haplotypes (14 and 16 haplotypes for *cox1* and *nad1*, respectively; Figure 2A and B). *Fusconaia chunii* had unique haplotypes, which were distinct from those of *F. askewi* by >2 bp. In the second cluster, *F. flava* from the Trinity River shared the same haplotype with widespread *F. flava* in the *cox1* network (Figure 2A), while *F. flava* from the Trinity River had unique haplotypes in the *nad1* network (Figure 2B). The haplotype network of *ITS1* showed no genetic structure among species (Figure 2C). All three species (*F. askewi*, *F. chunii*, and *F. flava*—including ones from the Trinity River and elsewhere) shared a single haplotype.

Similarly, the BI phylogeny based on the mtDNA dataset showed that individuals collected from the Trinity River between Lake Livingston and East Fork of the Trinity River spread into two lineages: *F. chunii* clade and *F. flava* clade (Figure 3). *Fusconaia chunii* formed a monophyletic clade, which is sister to a monophyletic clade of *F. askewi* with a shallow branch separation, and none of *F. askewi* or *F. chunii* individuals placed in both clades. While *F. chunii* showed high posterior probability, the clade of *F. askewi* had low support. The clade of *F. askewi* and *F. chunii* formed a sister clade with *F. mitchelli* with a high posterior probability. *Fusconaia flava* did not show phylogeographic patterns and formed a sister clade with *F. masoni*.
Genetic diversity was relatively similar among groups and between the mtDNA genes, except for widespread *F. flava* and *ITS1* (Table 1). For the mtDNA dataset, the number of haplotypes within groups ranged from four to 12 for *F. askewi*, *F. chunii*, and *F. flava* from the Trinity River. Widespread *F. flava* had high genetic diversity (i.e., number of haplotypes and mean nucleotide diversity). Genetic diversity for *ITS1*, on the other hand, was similarly low among groups (Table 1). Number of haplotypes ranged from one to five and mean nucleotide diversity ranged from 0 to 0.002 for *ITS1*. Pairwise genetic divergence among species ranged from 0.01 (*F. askewi* from the Sabine vs. *F. chunii*) to 0.0387 (*F. chunii* from the Trinity River vs. *F. flava* from the Trinity River), whereas genetic divergence within species was relatively small (ranged from 0.0034 to 0.006) for the concatenated mtDNA dataset (Table 2). For the *ITS1* dataset, genetic divergence among populations and species was relatively low (ranged from 0.0001 to 0.002).

Estimate of divergence time between the lineage of *F. askewi* and *F. chunii* and the lineage of *F. flava* was 7.82 million years ago (Ma) (95% credible interval: 4.39–11.84 Ma) during the late Miocene Epoch. Estimate of divergence time between *F. askewi* and *F. chunii* was recent around 1.82 Ma (0.64–3.13 Ma) during the early Pleistocene Epoch.

For the Fourier morphometrics, the PCA yielded eight distinct eigenvalues and described >90% of the total variation among individuals (Figure 5). The PC1 axis described 42% and the PC2 axis described 25% of the total variation. The PCA plot with

assigned groups by species showed that morphological variation was similar across all three species, although *F. flava* showed more morphological variation across its geographic distribution (Figure 5). The MANOVA revealed that shell morphologies were significantly different between all pairs of groups (Wilk's $\Lambda = 0.1613$; $F_{40,843.7} = 13.03$; $P < 0.001$), except between *F. chunii* and *F. flava* from the Trinity River ($P = 0.736$). The DFA on average correctly assigned 57% of individuals to the correct group but this varied by species and drainage. For *F. askewi,* DFA was able to correctly classify 97% and 76% of the individuals from the Neches and Sabine rivers, although it was less accurate in correctly assigning individuals to their respective drainages (78% Neches and 53% Sabine). In contrast, the DFA was less accurate in correctly assigning individuals to *F. chunii* (59%) or *F. flava* (28%) because it was unable to differentiate these two species; 48% of the *F. flava* from the Trinity were incorrectly assigned as *F. chunii.* Interestingly, correct classification of *F. flava* from outside of Trinity River was high, 69%.

Discussion

We confirmed the presence of three *Fusconaia* species in the Sabine-Trinity Province: *F. askewi*, *F. chunii*, and *F. flava*, which contradicts previous phylogenetic studies of this group (Burlakova et al*.,* 2012). Our results indicate that there are two distinct evolutionary lineages within *F. askewi* and *F. chunii* as each formed a reciprocally monophyletic lineage, indicating no gene flow between the species. Furthermore, our results show that *F. chunii* is a valid species and genetically distinct from *F. askewi*, which is not unexpected given that the Trinity River is separated from the Neches and Sabine rivers by the Gulf of Mexico. The distribution of *F. askewi* likely includes only the Neches and Sabine rivers, which represents a reduction in the presumptive range for this species. Based on the reciprocal monophyly and allopatric distributions, it is likely *F. askewi* and *F. chunii* have undergone recent speciation during the early Pleistocene.

In addition to *F. chunii*, the Trinity River has syntopic *F. flava* whose morphologies are indistinguishable from those of *F. chunii*. In the case of *F. flava*, their wide geographic distribution has likely shaped the morphological variation observed within this species, which is presumably in response to local environmental conditions (Graf, 1998). The lack of phylogeographic structuring and genetic divergence among drainages for *F. flava* suggests that this species may have high dispersal capability via the movement of its fish hosts, which may have allowed rapid range expansion to formally glaciated areas (Inoue et al., 2014).

Currently, *F. askewi* is listed as threatened by the state of Texas (TPWD, 2010) and USFWS has yet to consider it for ESA listing. Additionally, *F. lananensis* is listed as stated-threatened and has been proposed by USFWS for listing under the ESA (USFWS, 2009). Previous studies of molecular systematics and the findings of the current study show that this species is a junior synonym of *F. askewi* and so its proposed listing by USFWS is likely not warranted. Additionally, our results show that *F. chunii* is a valid species and based on recent surveys in the Trinity River drainage (see Task 1) appears to

have a restricted distribution, occurring primarily in the mainstem of the Trinity River above lake Livingston to Dallas-Fort Worth (see Figure 5 for Objective 1). Given this species limited distribution and endemism to the Trinity River drainage, state and federal agencies should consider evaluating its conservation status to determine whether listing at the state and federal level is appropriate. However, during these assessments managers should be mindful that this species can co-occur with *F. flava*, which is morphologically indistinguishable from *F. chunii*.

The current study clarified the taxonomic status of *F. chunii* and found cryptic diversity in the Trinity River. Future molecular research for threatened species in this basin should focus on understanding the population genetic structure, genetically effective population size, and rate of gene flow among populations. The results of the current study also indicate that cryptic diversity may be prevalent in other mussel species, which has been documented for several rare species in Central Texas (Pfeiffer et al. 2016; N. Johnson unpublished data), and so additional study of molecular systematics and population genetics of rare and common species is warranted and should be a top research priority for state and federal agencies.

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Table 1. Summary statistics of *cox1*, *nad1*, and *ITS1* for *Fusconaia askewi* from the Neches and Sabine rivers, *Fusconaia chunii* from the Trinity River, and *Fusconaia flava* from the Trinity River (including the San Jacinto River) and elsewhere.

		cox1				*nad1*				*ITS1*			
Species	Drainage	*n*	*H*	*K*	π	*n*	*H*	*K*	π	*n*	*H*	*K*	π
Fusconaia askewi	Neches	45	7	1.2	0.0028	24	7	1.8	0.0032	13	1	0	0
Fusconaia askewi	Sabine	62	10	1.0	0.0022	39	12	2.4	0.0047	26	5	0.4	0.0008
Fusconaia chunii	Trinity	44	6	1.3	0.0021	42	5	0.6	0.0012	32	3	0.1	0.0003
Fusconaia flava	Trinity	28	4	0.9	0.0015	28	5	0.7	0.0013	19	2	0.1	0.0006
Fusconaia flava	Widespread	91	30	2.6	0.0075	27	15	5.6	0.0095	14	4	1.0	0.0020

H, number of haplotypes; *K*, mean number of base pair differences; *n*, number of samples, π, nucleotide diversity

Table 2. Pairwise genetic divergence for the concatenated mtDNA (below diagonal) and *ITS1* (above diagonal) from *Fusconaia askewi*, *Fusconaia chunii*, and *Fusconaia flava*.

	Fusconaia askewi (Neches)	*Fusconaia askewi* (Sabine)	*Fusconaia chunii* (Trinity)	*Fusconaia flava* (Trinity)	*Fusconaia flava* (Widespread)
Fusconaia askewi (Neches)	–	0.0007	0.0001	0.0002	0.0014
Fusconaia askewi (Sabine)	0.0034	–	0.0008	0.0009	0.0020
Fusconaia chunii (Trinity)	0.0105	0.0100	–	0.0003	0.0015
Fusconaia flava (Trinity)	0.0383	0.0379	0.0387	–	0.0016
Fusconaia flava (widespread)	0.0384	0.0382	0.0403	0.0060	–

Table 3. Confusion matrix for *Fusconaia askewi* from the Neches and Sabine rivers, *F. chunii* from the Trinity River, and *F. flava* from the Trinity River (including the San Jacinto River) and elsewhere based on the Fourier morphometric analysis. Values are in proportion. Rows correspond to *a priori* assignment to the groups and columns correspond to predicted groups.

	Fusconaia askewi (Neches)	*Fusconaia askewi* (Sabine)	*Fusconaia chunii* (Trinity)	*Fusconaia flava* (Trinity)	*Fusconaia flava* (widespread)
Fusconaia askewi (Neches)	0.78	0.19	0	0.02	0.02
Fusconaia askewi (Sabine)	0.23	0.53	0.05	0.18	0.03
Fusconaia chunii (Trinity)	0.05	0.07	0.59	0.29	0
Fusconaia flava (Trinity)	0.07	0.10	0.48	0.28	0.07
Fusconaia flava (widespread)	0.27	0.03	0	0	0.69

Figure 1. Map showing the collection sites in the United States. Colors represent collected species (red = *Fusconaia askewi*; yellow = *Fusconaia chunii*; blue = *Fusconaia flava*). Shapes correspond to genetic-only specimens (squares), morphometrics-only specimens (triangles), and specimens used for both genetics and morphometric analyses (circles). Species identification was based on the phylogenetic analyses (Fig. 3).

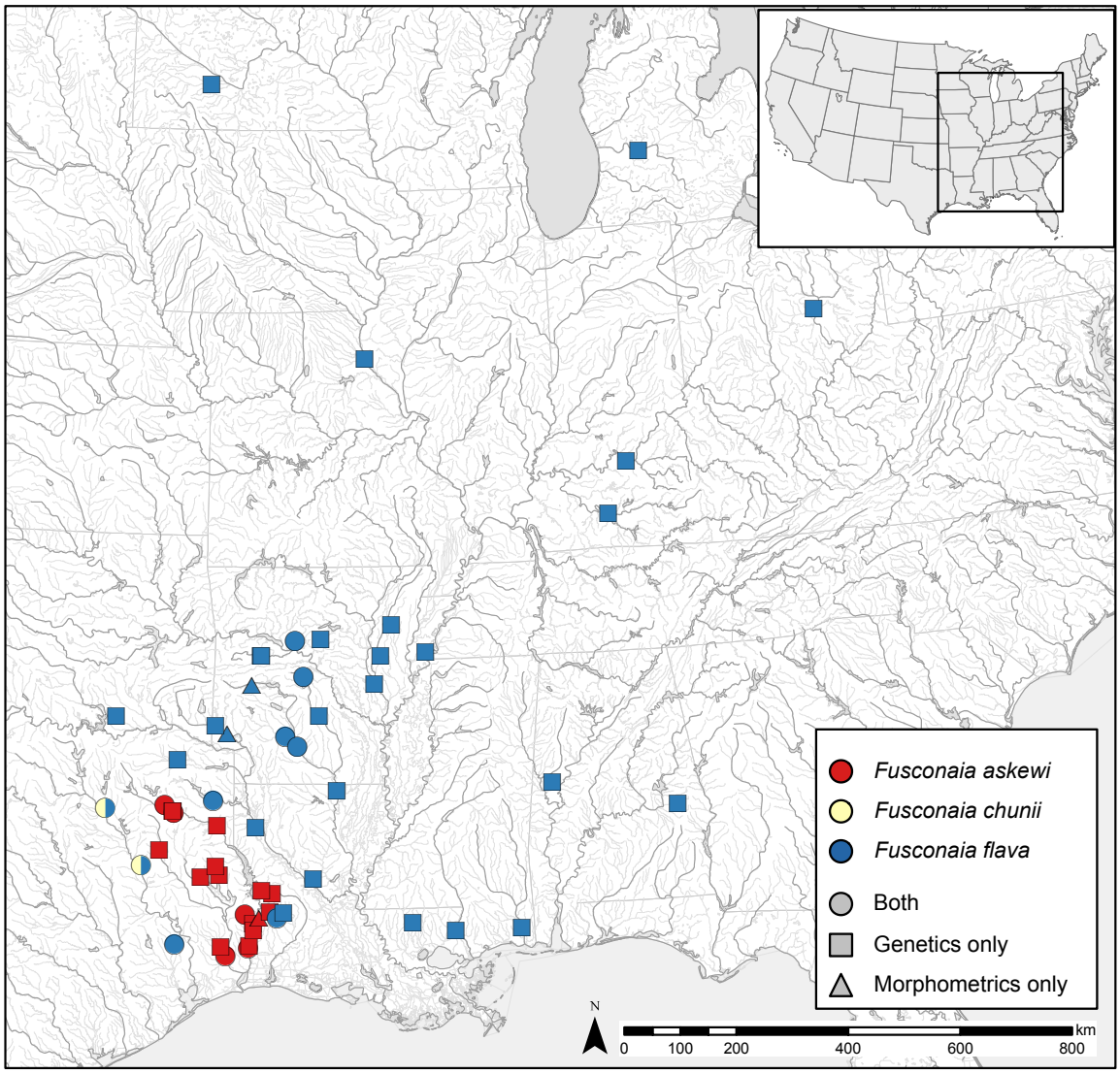


Figure 2. Haplotype networks of *Fusconaia* species for (A) *cox1*, (B) *nad1*, and (C) *ITS1*. Colors correspond to species and collected drainages (red = *Fusconaia askewi* from the Neches River; orange = *F. askewi* from the Sabine River; yellow = *Fusconaia chunii* from the Trinity River; light blue = *Fusconaia flava* from the Trinity River; dark blue = widespread *F. flava*). Each line represents one base pair difference between haplotypes, black dots are inferred missing haplotypes, and haplotype frequency is relative to the size of the circles.

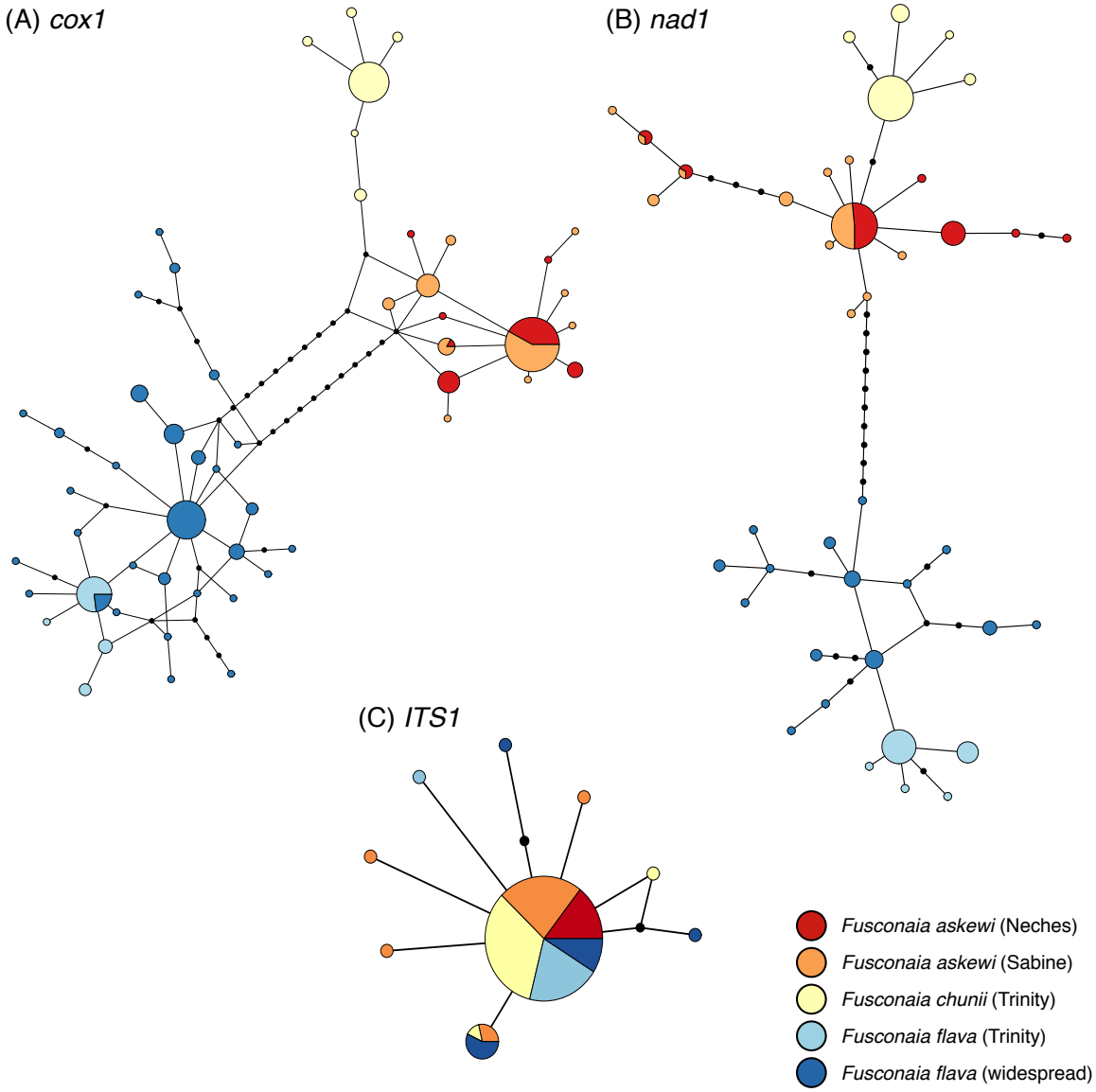
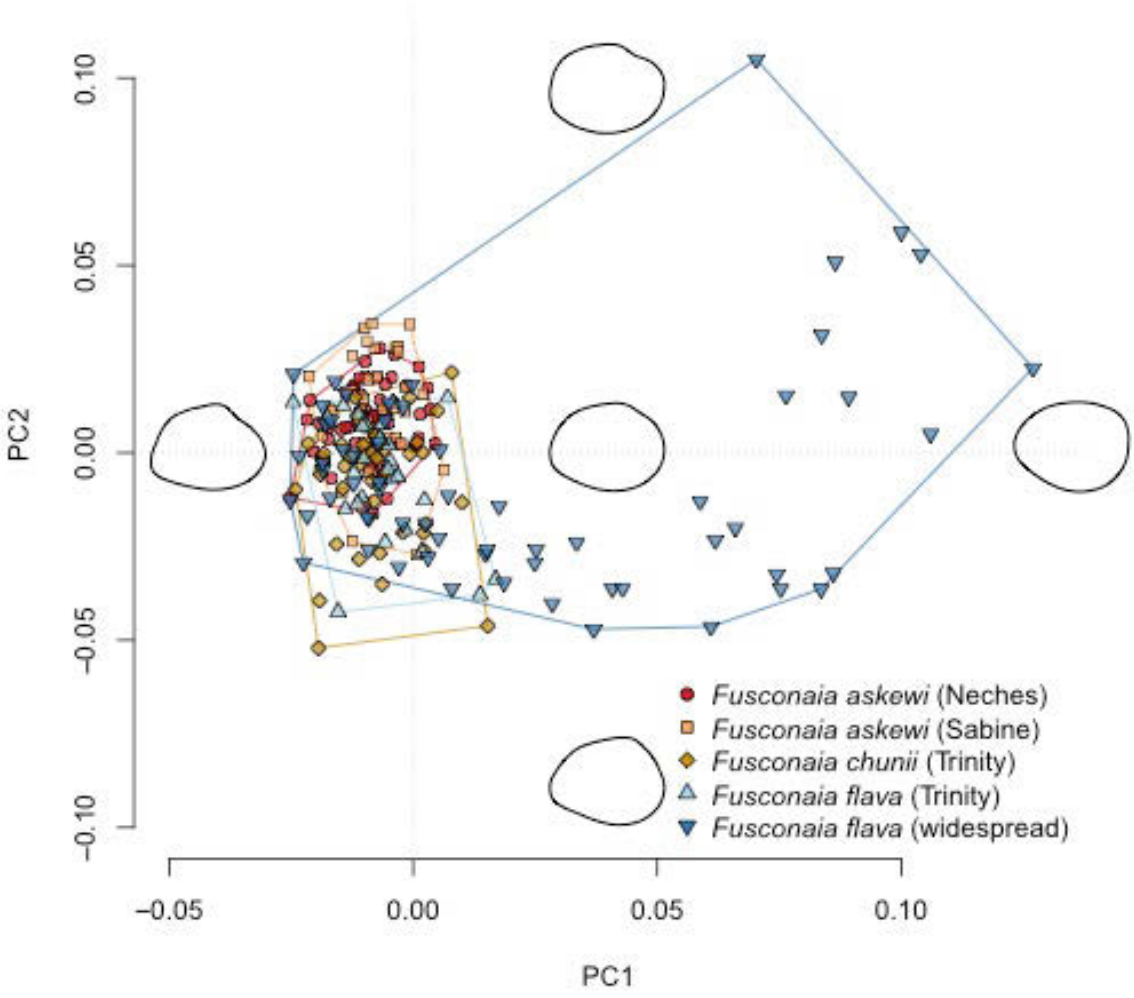


Figure 3. Phylogenetic tree reconstructed by Bayesian analysis for mtDNA. Bayesian posterior probabilities are shown in shaded diamonds along the nodes (white < 0.91, gray = 0.91 – 0.99, black > 0.99). The tree was rooted with *Arcidens confragosus*. Bold bars along clades correspond to the focal species. Asterisks (*) represent individuals from the Trinity River initially identified as *Fusconaia chunii*.

Figure 4. Biplots from principal component analysis (PCA) of Fourier morphometrics. Colors and shapes of points correspond to species and collected drainages (red circle = *Fusconaia askewi* from the Neches River; orange squares = *F. askewi* from the Sabine River; dark yellow diamonds = *Fusconaia chunii* from the Trinity River; light blue triangles = *Fusconaia flava* from the Trinity River; dark blue downside triangles = widespread *F. flava*). Polygons enclose convex hulls of each group. Outlined shell shapes represent a mean shape (meddle) and ±2 × standard deviations on PC1 and PC2 axes.



Objective 3: Presence/Absence Survey for Zebra Mussels in the middle and upper Trinity.

Introduction

Zebra mussels, *Dreissena polymorpha* (Pallas, 1771), represent one of the most important biological invasions in North America (USGS, 2017). *Dreissena polymorpha* was likely first introduced to the United States in 1985 but was not discovered until 1988 in Lake St. Clair (Hebert et al., 1989; Griffiths et al., 1991). It is likely they were introduced via ballast water in ships coming from the Black Sea near Ukraine (Hebert et al., 1991; McMahon, 1996; Ram & Mcmahon, 1996; Mackie & Schloesser, 1996). Since 1988, *D. polymorpha* has spread to 28 states within the US (USGS, 2017) infesting both river drainages as well as reservoirs and lakes. Unfortunately, this spread will likely continue until all suitable habitat has been colonized (Strayer, 2009).

In Texas, *D. polymorpha* was first discovered by a concerned citizen in Lake Texoma on April 3 2009 (TPWD 2009) and within 8 years it has spread across the state. As of August 2017 it has infested nearly a quarter of the river basins in Texas, which include the Red, Trinity, Brazos, Colorado, and Guadalupe River basin. Within these basins, *D. polymorpha* has been primarily reported from reservoirs, though several river populations are known to occur (TPWD 2017). Because *D. polymorpha* has been found primarily in lakes demonstrates that boat traffic may be a significant source of dispersal for these animals and that it may not be able to colonize riverine systems in Texas.

Dreissena polymorpha can cause serious economic costs to municipal, hydroelectric, transportation, and industrial water infrastructure by fouling intake structures (MacIsaac, 1996; O'Neill, 1997). This can also impact ecosystem services of lakes and streams by negatively impacting the native fauna. Of concern, is the impact of *D. polymorpha* to native mussel species (Bivalvia: Unionoida; hereafter, unionids), which as a group are of high conservation concern (Texas Register 35, 2010). *Dreissena polymorpha* can use unionids as a substrate for attachment, particularly in areas with soft substrate, which impedes their ability to feed and respire (Hebert et al. 1991). *Dreissena polymorpha* can also outcompete native unionids for food resources (Haag et al., 1993; Hebert et al., 1991). The introduction of *D. polymorpha* has resulted in the extirpation of unionid populations within in the Mississippi River and Great Lakes Basins (Ricciardi, Neves, and Rasmussen 1998) and this trend is likely to continue.

We evaluated the presence and prevalence of *D. polymorpha* larvae (veligers) in the Trinity River between Lewisville Lake (SH121 Bridge in Dallas, TX) and SH84 Bridge near Palestine, TX (Figure 1). We chose Lewisville Lake as the uppermost extent of our sampling because this reservoir is currently infested with *D. polymorpha* and we were interested in examining how far veligers can travel downstream from source locations to assess the likelihood of *D. polymorpha* colonizing the mainstem of the Trinity River. Our specific objectives were to: (1) evaluate the presence and abundance of *D. polymorpha* at monitoring stations along the middle and upper Trinity; and (2) determine whether water quality parameters such as calcium hardness, alkalinity, pH,

temperature, transparency, and dissolved oxygen (DO) are suitable for veliger survival within this reach.

Methods

Study area

We conducted *D. polymorpha* veliger sampling on a monthly basis at the following six locations in the reach of the Trinity River between SH121 Bridge and SH84 Bridge from September 2016 to April 2017: SH121 Bridge crossing (below Lake Lewisville); SH12 Loop Bridge crossing; S. Beltline Rd. Bridge crossing; SH85 Bridge crossing; US287 Bridge; and SH84 Bridge crossing near Palestine, TX (Figure 1). These sites were chosen due to ease of access and several had boat launches, which could be points of introduction as *D. polymorpha* are known to be spread by boat traffic (Britton and McMahon 2005).

Veliger sampling

Veligers were sampled by using a plankton net with 63 µm mesh and a 30-cm diameter opening (Figure 2). Because we were sampling flowing water locations rather than reservoirs, we used the equation listed below to determine the amount of time needed to sample a standardized volume of 1000 L. We used an OTT MF pro flow meter to determine velocity (V) and the area was based on diameter of the net opening (A).

$$Q = V*A$$

Where Q = discharge (m^3/s)

V = Velocity (m/s)

A = Area of net opening (m^2)

$$1000L = 1m^3$$

$$T = 1m^3 / Q$$

At each site, samples were taken near midchannel from a depth where the water level was 0.5 m above and below the net. This depth generally corresponded with areas of the channel where settlement of *D. polymorpha* was likely to occur. In order to ensure constant flow into the plankton net, we kept the net opening perpendicular to the flow by attaching two number 4 dive weights to one side of the net opening with zip ties (Figure 3). The net was held in the flow for the time calculated with the above formulae. Upon completion, the net was quickly removed from the water and rinsed with filtered site water, or RO/DI into a Dolphin sampling bucket (Figure 4). The attached filter cups were then rinsed into a 250mL container with enough absolute ETOH to reach a final concentration of 90-95% ETOH. Two replicate samples were collected at each site for each sampling period.

Cross-polarized microscopy

Samples were assessed using cross-polarized microscopy under a dissecting microscope. This technique is useful for differentiating *D. polymorpha* veligers from detritus or other calcareous species (Figures 5 and 6; Johnson 1995). This technique

requires polarizing filters that are installed above and below the stage of a dissecting scope with a light source coming from the bottom up through the sample (Figure 7). Objects showing birefringence were investigated further by measuring and assessing shape and were subsequently identified following Nichols and Black (1994).

Sample workup

Preserved samples were filtered through a 55-µm mesh filter to separate the sample from ETOH and were then diluted in a specific amount of water (starting with 100 mL) depending on the amount of detritus contained in the sample. The diluted sample was mixed with a plunging type mixing rod to ensure homogenization of the sample. While mixing, a 6 mL subsample was removed and placed into a Bogorov counting chamber for cross-polarized microscopy. We adjusted the dilution volume depending on difficulty of microscopy process due to high concentration of detritus. These steps were necessary as the Trinity River is generally turbid.

We standardized the amount of effort among samples by taking multiple subsamples until 60% of the sample was assessed or until 100 veligers were detected. This method considers the variability of samples and detection effort (Jim Stoeckel pers. comm). However, because we never arrived at 100 veligers per sample, we took subsamples until 60% of the sample was assessed.

Adult zebra mussel observations

During the veliger sampling, we inspected hard structures (e.g., bridge pylons, rip-rap, boulders/cobble) for adult *D. polymorpha*.

Water quality assessment

We obtained water quality data on calcium hardness, pH, water temperature, dissolved oxygen, and transparency from the TCEQ Surface Water Quality Web Reporting Tool (https://www80.tceq.texas.gov/SwqmisPublic/public/default.htm). These data were then evaluated to determine if water conditions were suitable for zebra mussel survival within the sampled reach.

Results

During our survey from September 2016 until April 2016, veligers were detected at only two of the 6 sites sampled. Specifically, veligers were collected at the SH121 Bridge crossing and SH12 Loop Bridge crossing (Figure 1). At these locations mean density of veligers was low, ranging from 0.83 to 3.33 veligers/m^3. The detection limit based on our sampling design was 0.0016 veligers/ m^3. Veliger density was highest at the SH12 Loop Bridge in January 2017 ($3.33 \pm 2.36/m^3$) and at SH121 Bridge site in May 2017 ($1.67 \pm 0.0/m^3$) (Table 1). We did not detect any veligers downstream of these sites and no adult *D. polymorpha* were ever observed at these or the other sampling sites. Of the water quality parameters examined at our sampling locations all were suitable for *D. polymorpha* except for transparency (Cohen 2015; McMahon 2015).

Discussion

Our study showed that *D. polymorpha* veligers are moving downstream of Lake Lewisville, though in low densities. Collected veligers were in the D-stage (≤112μm), which confirms that adult mussels are present in Lake Lewisville and they are reproducing. These veligers are likely being released into the Trinity River as part of normal reservoir operations. However, veliger density in our reach was extremely low compared to other more heavily infested river systems. In our study reach, densities ranged from 0.83–3.33 veligers/m^3, which is orders of magnitude less than reported densities in the Illinois and Mississippi rivers where 10,000 veligers/m^3 is common (Stoeckel et al., 2004; Schneider et al., 2003).

We did not find any *D. polymorpha* veligers that were larger than D-stage, nor did we ever observe any adults near our sampling locations. We hypothesize that although veligers are being released from Lake Lewisville and traveling downstream, unknown environmental factors are preventing their establishment within the mainstem of the Trinity River. High turbidity and flashy flows, which characterizes much of the Trinity River, are likely the factors. High turbidity has been shown to be negatively correlated with *D. polymorpha* occupancy (Strayer, 1991) and it is well known that food quality can influence the survival of *D. polymorpha,* specifically the inorganic to organic ratio of the seston. Ratios of 0.5–1.71 of inorganic to organic seston are known to reduce individual growth rate of *D. polymorpha* to zero (Madon et al., 1998; Schneider et al., 1998). Given the high level of turbidity within the Trinity it is likely that veligers may be food limited, though this has not been demonstrated. Flood flows can also hinder *D. polymorpha* veliger dispersal by preventing settlement and attachment to hard surfaces due to increases in bottom shear stress (Horvath & Lamberti 1999; Rehmann et al., 2003), but as with seston ratios has not been specifically tested. Our results show that although the Trinity River appears suitable for *D. polymorpha* (McMahon, 2015), based on overall water quality, other factors such as high turbidity and flashy flows are likely restricting the colonization of the species downstream of Lewisville Lake, or other infested reservoirs. However, future studies are needed to specifically test whether this is the case and monitoring should continue in the upper Trinity until it is determined that downstream dispersal does not pose a threat to native riverine communities or water infrastructure.

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Table 1. Zebra mussel abundances from Sept 2016-May 2016. Abundances are reported in mean number of veligers/m^3 ± 1 standard deviation. NS = not sampled.

Date	TX-121	TX-12 Loop	S. Beltline Rd.	SH 85	USH-287	US-79/84
9/23/2016	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
10/19/2016	**0.83 ± 1.18**	**1.67 ± 2.36**	0 ± 0	0 ± 0	0 ± 0	0 ± 0
11/18/2016	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12/13/2016	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
1/27/2017	0 ± 0	**3.33 ± 2.36**	0 ± 0	0 ± 0	0 ± 0	0 ± 0
2/28/2017	0 ± 0	0 ± 0	NS	NS	NS	NS
3/21/2017	0 ± 0	0 ± 0	NS	NS	NS	NS
5/4/2017	**1.67 ± 0.0**	0 ± 0	NS	NS	NS	NS

Figure 1. Map of sampling locations.

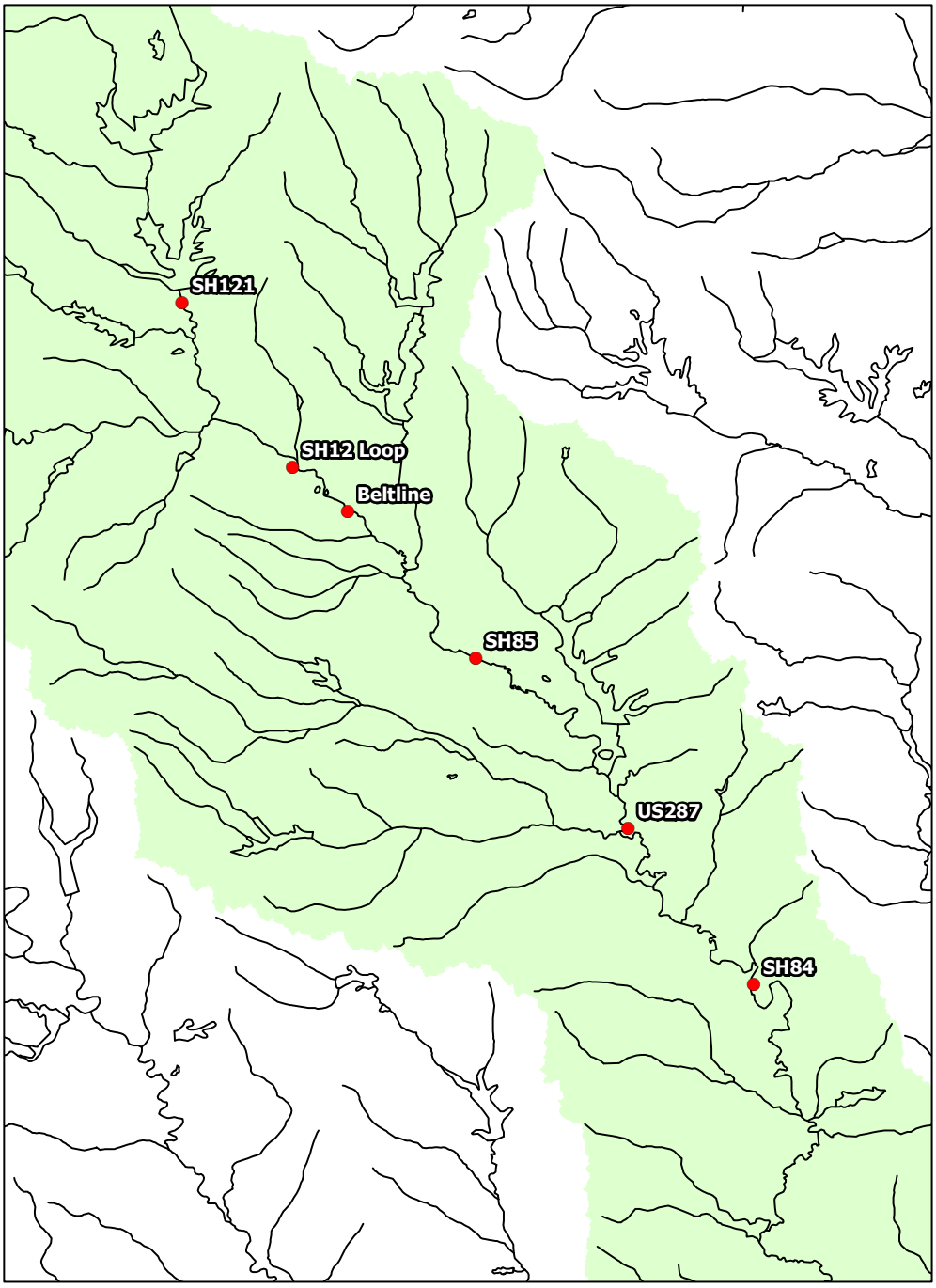

Figure 2. 63µm mesh plankton net with 0.3m opening diameter with cod end adapter installed.

Figure 3. Plankton net with weight added to one side of net opening.

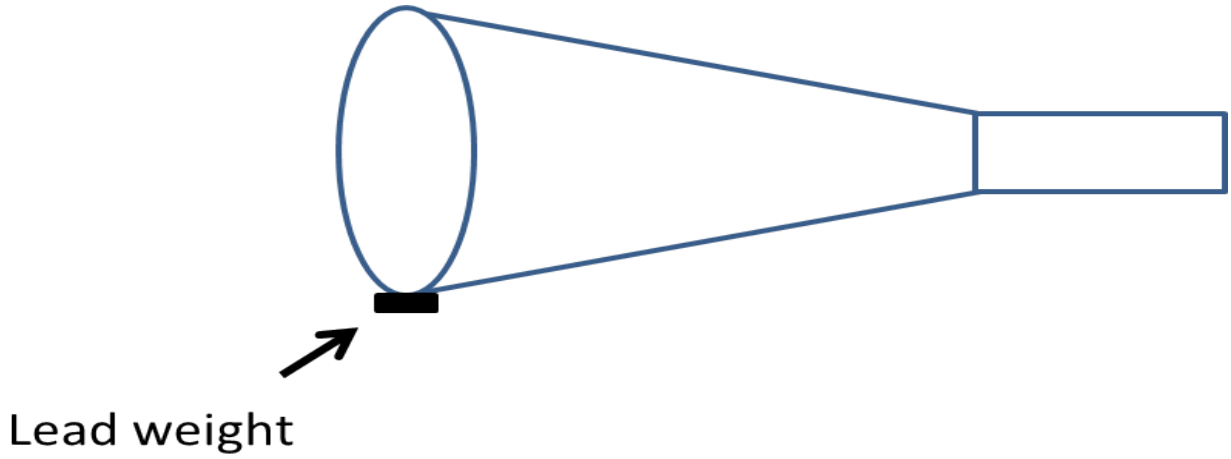

Figure 4. Dolphin sampling bucket attached to cod end adapter.

Figure 5. From Johnson 1995 showing zooplankton with calcareous shells exhibiting the "Maltese cross" or birefringence under cross polarized lighting. A, C, & E show zooplankton under normal lighting while B, D, & F show the same zooplankton under cross-polarized lighting.

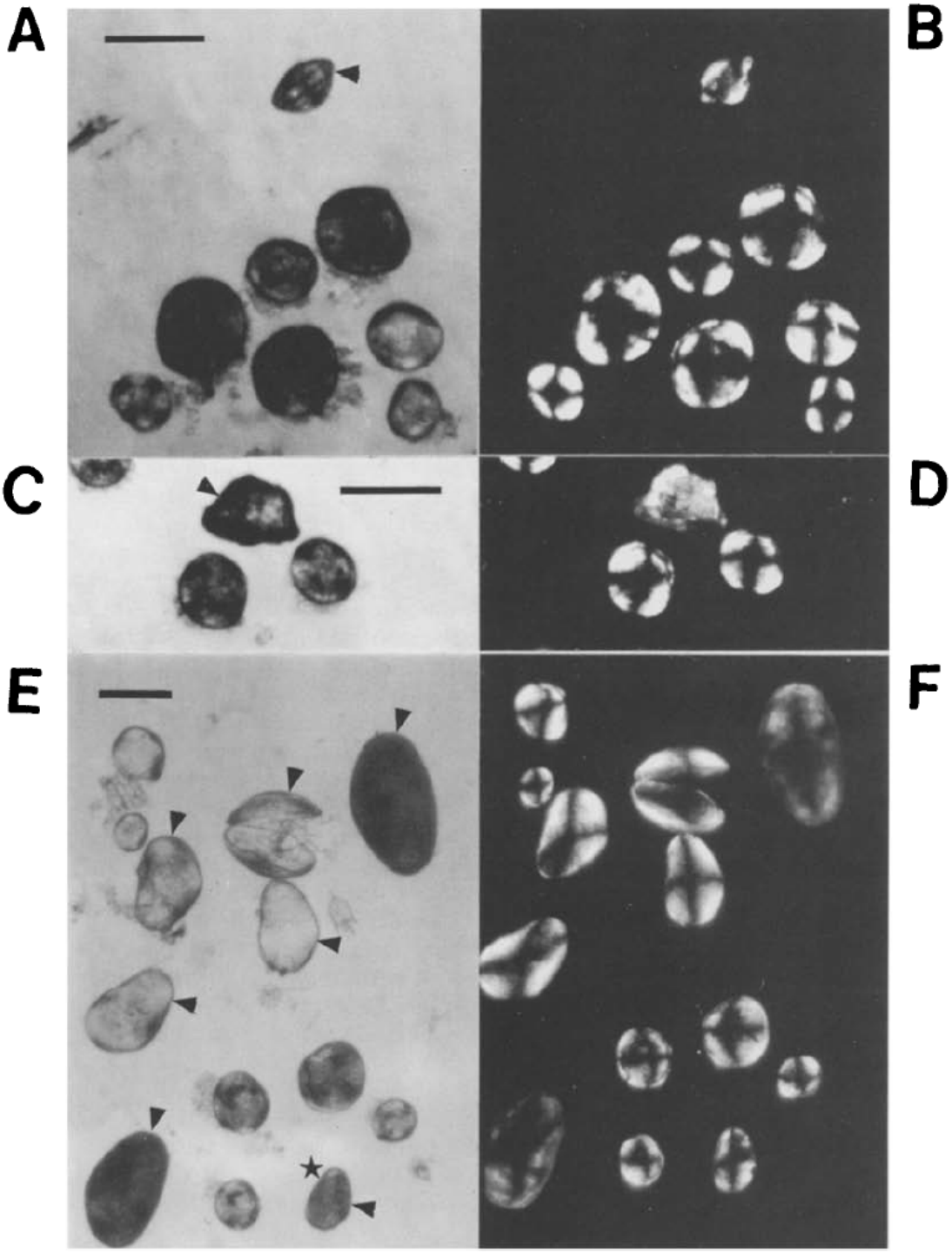

Figure 6. From Johnson 1995 showing how zooplankton with calcareous shells stand out among phytoplankton and other detritus in samples. A, & C show sample fields under normal lighting while B. & D show the same fields under cross-polarized lighting.

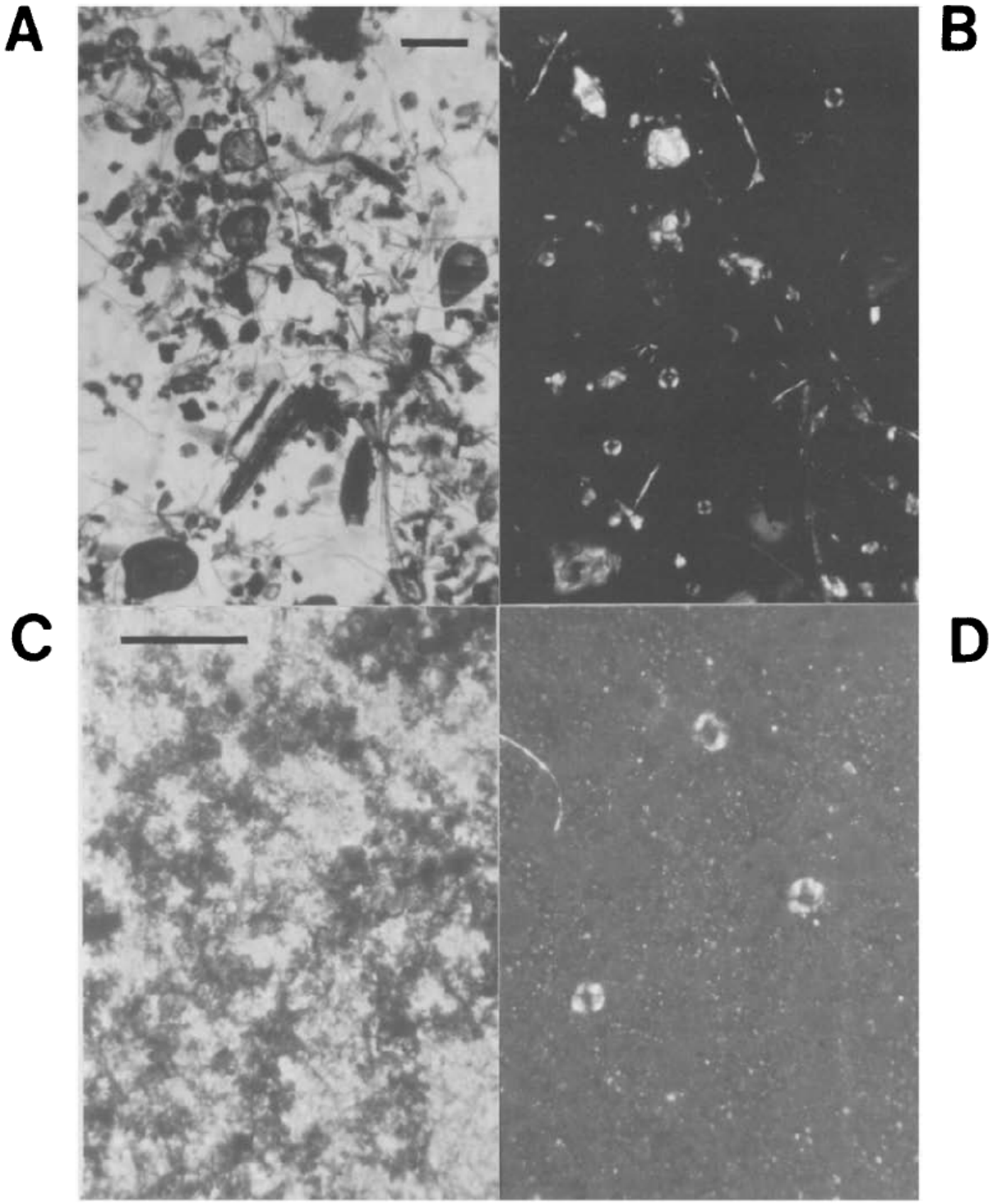

Figure 7. From Johnson 1995 illustrating how to set up a dissecting scope for cross polarized light microscopy showing the placement of polarizing filters and light source.

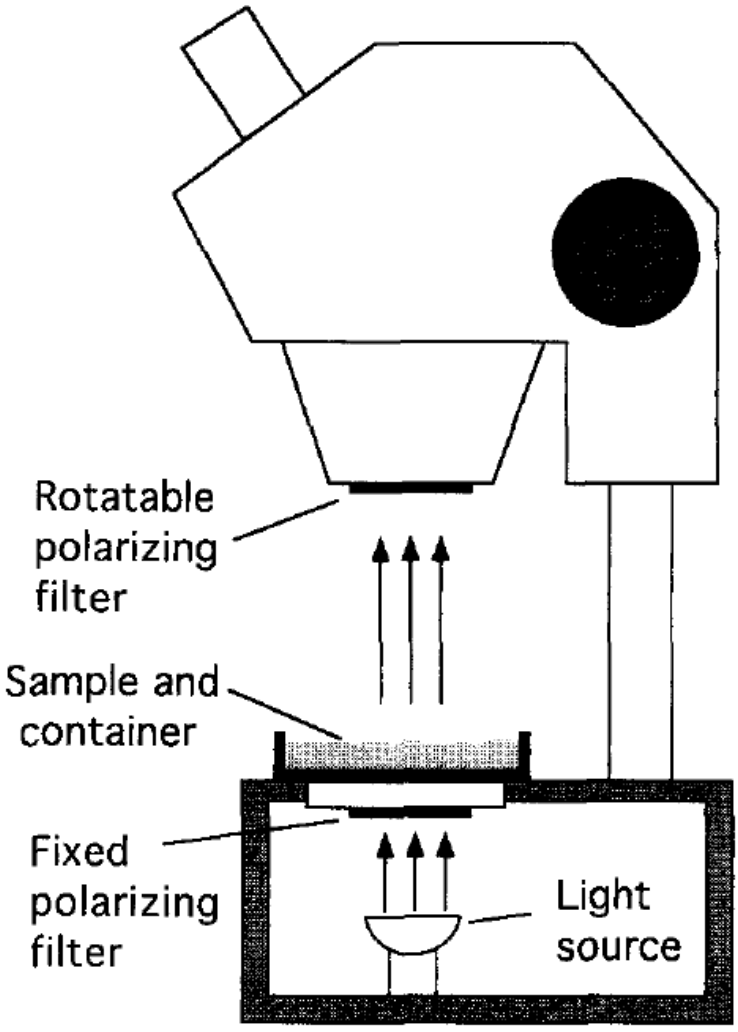

Fig. 1. Schematic diagram of microscope retrofitted with cross-polarizing filters.