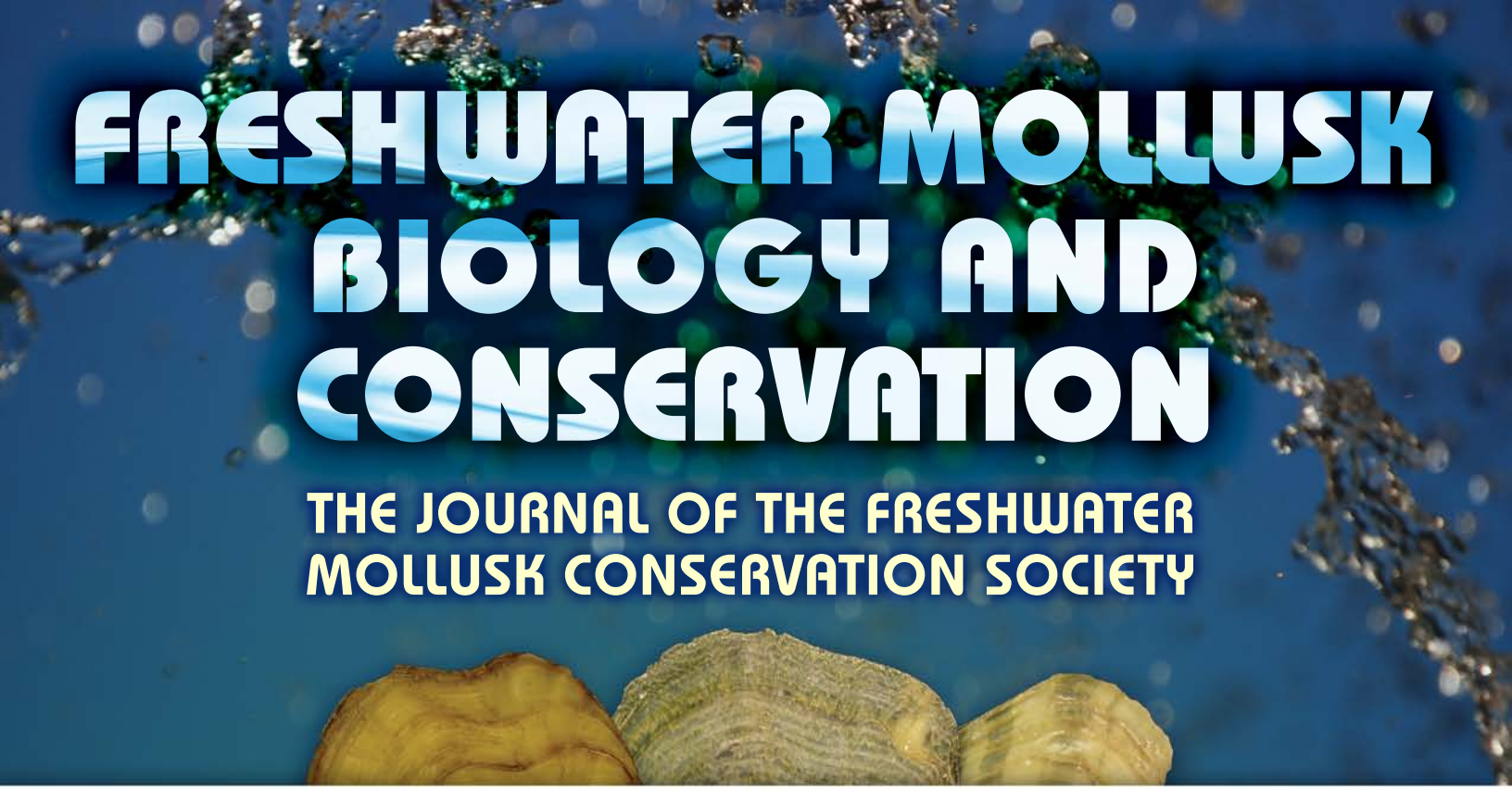

FRESHWATER MOLLUSK BIOLOGY AND CONSERVATION

THE JOURNAL OF THE FRESHWATER MOLLUSK CONSERVATION SOCIETY

VOLUME 24 NUMBER 2 SEPTEMBER 2021

Freshwater Mollusk Biology and Conservation 24:43–58, 2021

DOI:10.31931/fmbc-d-20-00002

REGULAR ARTICLE

RIVERSCAPE-SCALE MODELING OF FUNDAMENTALLY SUITABLE HABITAT FOR MUSSEL ASSEMBLAGES IN AN OZARK RIVER SYSTEM, MISSOURI

Kayla N. Key*[1,2], Amanda E. Rosenberger[3], Garth A. Lindner†[4], Kristen Bouska[5], and Stephen E. McMurray[6]

[1] *West Tennessee River Basin Authority, Tennessee Department of Environment and Conservation, Humboldt, TN 38343 USA*
[2] *Tennessee Cooperative Fishery Research Unit, Department of Biology, Tennessee Tech University, Cookeville, TN 38505 USA*
[3] *U.S. Geological Survey, Tennessee Cooperative Fishery Research Unit, Department of Biology, Tennessee Tech University, Cookeville, TN 38505 USA*
[4] *University of Missouri, School of Natural Resources, Columbia, MO 65211 USA*
[5] *U.S. Geological Survey, Upper Midwest Environmental Sciences Center, La Crosse, WI 54603 USA*
[6] *Missouri Department of Conservation, Science Branch, Columbia, MO 65201 USA*

ABSTRACT

Identifying the physical habitat characteristics associated with riverine freshwater mussel assemblages is challenging but crucial for understanding the causes of mussel declines. The occurrence of mussels in multispecies beds suggests that common physical factors influence or limit their occurrence. Fine-scale geomorphic and hydraulic factors (e.g., scour, bed stability) are predictive of mussel-bed occurrence, but they are computationally challenging to represent at intermediate or riverscape scales. We used maximum entropy (MaxEnt) modeling to evaluate associations between riverscape-scale hydrogeomorphic variables and mussel-bed presence along 530 river km of the Meramec River basin, USA, to identify river reaches that are fundamentally suitable for mussels as well as those that are not. We obtained the locations of mussel beds from an existing, multiyear dataset, and we derived river variables from high-resolution, open-source datasets of aerial imagery and topography. Mussel beds occurred almost exclusively in reaches identified by our model as suitable; these were characterized by laterally stable channels, absence of adjacent bluffs, proximity to gravel bars, higher stream power, and larger areas of contiguous water (a proxy for drought vulnerability). We validated our model findings based on model sensitivity using a set of mussel-bed locations not used in model development. These findings can inform how resource managers allocate survey, monitoring, and conservation efforts.

KEY WORDS: freshwater mussels, conservation planning, unionids, hydrogeomorphology, MaxEnt, riverscape

INTRODUCTION

Our knowledge of the physical habitat characteristics associated with mussel beds remains incomplete (Haag 2012). Understanding the habitat associations of mussels is foundational for further understanding the threats to mussel populations and enacting regional and strategic conservation efforts to improve the status of mussels nationwide (FMCS 2016). Mussel presence has been predicted most successfully at large spatial scales, using variables such as watershed geology, soils, land use, and topography (Strayer 1983;

†Current affiliation: Indiana Department of Natural Resources, Indianapolis, IN 46204 USA
*Corresponding Author: kaylakey@me.com

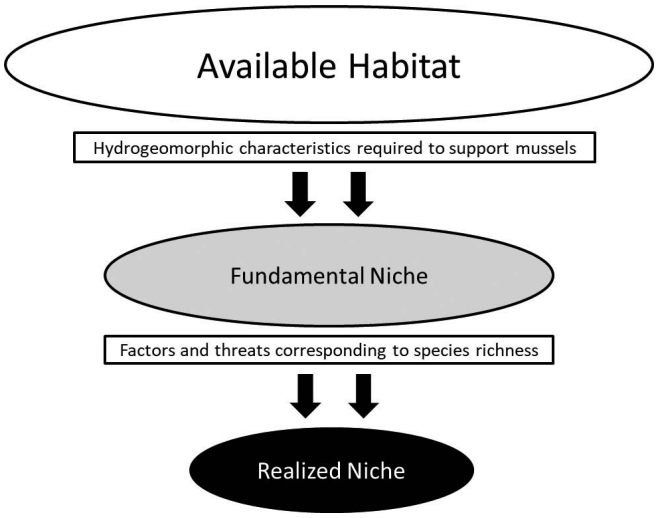

Figure 1. Conceptual model of the approach used to depict habitat requirements of freshwater mussels, starting with the identification of the fundamental habitat requirements through a description of hydrogeomorphic features of the river system. Based on this, one can then investigate other factors limiting mussel species, including anthropogenic threats.

Arbuckle and Downing 2002; McRae et al. 2004; Daniel and Brown 2014; Walters et al. 2017). However, large-scale factors such as geology and topography are not tractable for management actions.

Reach-level factors are influenced more easily by management, but efforts to predict mussel occurrence at this scale have been less successful (Strayer 2008). Hydrogeomorphic variables related to substrate stability (e.g., shear stress, channel stability) show promise for predicting where mussels occur (Hardison and Layzer 2001; Allen and Vaughn 2010; Drew et al. 2018). Unfortunately, generalizing these results to other streams can be difficult due to varying methods and differences in size and geomorphological processes among lotic systems (Layzer and Madison 1995; Steuer et al. 2008; Pandolfo et al. 2016). Furthermore, measurements of hydraulic variables at small scales are time-consuming, making it impractical to generate data needed for watershed-scale inference, which is most useful for resource managers (Fausch et al. 2002).

An investigation of mussel habitat associations at a riverscape scale (continuous spatial data across a longitudinal river gradient) may be most useful to prioritize management efforts within a watershed (Bouska et al. 2018). For such a framework, the first step is inferring what combinations of reach-scale habitat factors provide fundamentally suitable conditions for mussels. This is analogous to the single-species, fundamental niche concept, which describes "a state of environment which would permit the species to exist" (Hutchinson 1957; Fig. 1). The advantage of the riverscape scale is that we can use those data to spatially identify where these combinations of habitat factors do and do not occur in the river system of interest. However, at a riverscape scale, it is time-consuming and expensive to generate the hydrogeomorphic data needed to predict mussel occurrence (e.g., shear stress). Easily obtainable riverscape-scale data are needed that represent or are responsive to hydrogeomorphic processes and can predict mussel occurrence in similar ways. If the fundamental niche can be described or quantified, it can be contrasted with the realized niche, which describes where animals actually occur according to the influence of other factors, such as competition or anthropogenic impacts. Such riverscape-scale habitat models can allow inference about whether mussel declines or absence in a particular stream reach are due to habitat factors or other factors, such as human impacts on water quality (Bouska et al. 2018; Fig. 1).

Mussels often occur in multispecies beds, suggesting that common physical factors influence or limit the occurrence of multiple species (Vaughn 1997), and the probability of occurrence of species of management concern increases with increased assemblage richness (Zipkin et al. 2009; Lueckenhoff 2015). For these reasons, it is reasonable—and most informative to managers—to assess habitat relationships associated with entire, multispecies mussel beds, rather than using traditional species-specific habitat models. It also may be most useful to depict such associations along a continuous riverscape scale instead of imposing preconceived spatial scales (e.g., hydrologic units) of unknown relevance to the organisms; such an approach allows scale to arise from analytical results (Parsons et al. 2004) and better represents multiple levels of ecological organization.

The goal of this study was to delineate fundamentally suitable and unsuitable river reaches for mussels based on habitat variables (i.e., in the absence of other limiting factors, such as water quality or fish hosts) in the Meramec River basin of Missouri on a riverscape scale. We confined our study to midsized river reaches suitable for snorkel-based sampling, such as timed visual searches. We evaluated habitat features associated with mussel beds, with a focus on riverscape-scale hydrogeomorphic variables associated with water availability, channel stability, and stable gravel substrate. Our objectives were to (1) generate a dataset of spatial layers representing hydrogeomorphic stream characteristics relevant to mussel ecology derived from open-source, remotely sensed data, (2) develop a fundamental niche habitat model to evaluate associations between the presence of mussel beds and hydrogeomorphic stream characteristics, and (3) validate the model by testing its overall sensitivity and evaluate the occurrence of mussel beds outside of fundamentally suitable habitat as delineated by the model. Because the model is intended only to delineate, in general, fundamentally suitable and unsuitable habitat, rather than to predict actual bed location, we did not evaluate model specificity. We hypothesized that specific combinations of physical features would be associated with mussel beds and the absence of these features would characterize sizeable portions of the river as fundamentally unsuitable for mussel beds.

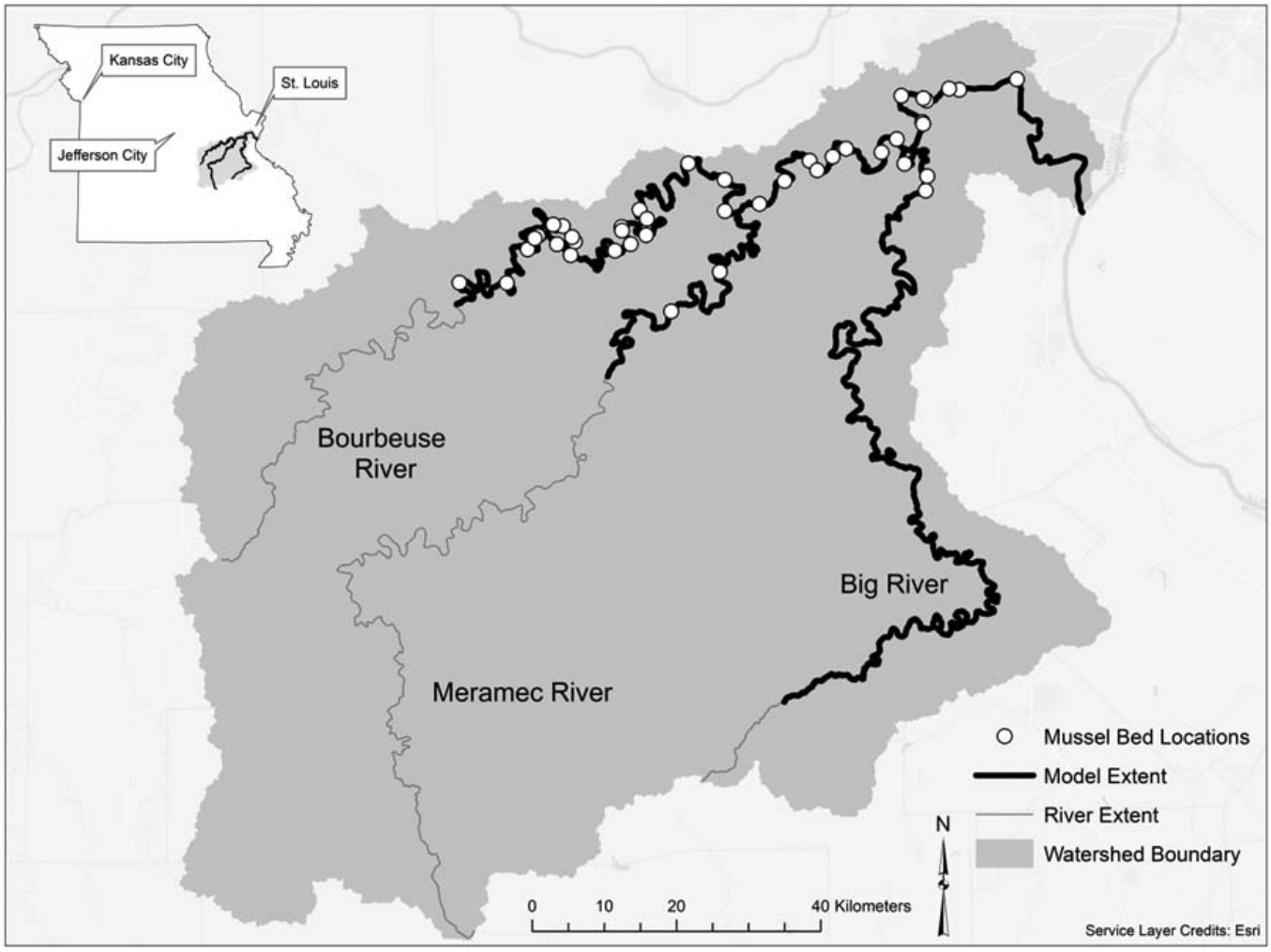

Figure 2. Map of the Meramec River basin showing the Meramec River and its two largest tributaries, the Big and Bourbeuse rivers. The inset map shows the location of the Meramec River basin in Missouri, USA. The study reach (model extent) is indicated by bolded streamlines and mussel-bed locations used in modeling and validation are shown in white circles.

METHODS

Study Area

The study was focused on the Meramec River basin (the Meramec, Big, and Bourbeuse rivers, "MBB" hereafter as an acronym for this basin; Fig. 2) in the northeastern Ozark Plateau in Missouri. The MBB is located within the Upper Mississippi mussel faunal province (Haag 2010), and it is a hotspot of mussel diversity in the midwestern United States (Roberts and Bruenderman 2000; Hinck et al. 2012). The Meramec River has two major tributaries, the Big River (2,473 km^2) and the Bourbeuse River (2,183 km^2). Stream substrates in the basin are dominated by gravel (Jacobson and Primm 1997). Mussel diversity has declined in the MBB, potentially due to altered floodplains, channel incision, invasive species, and water pollution (TNC 2014). The basin has a drainage area of 10,308 km^2 and is approximately 70% forested (MSDIS 2011). The remainder of the watershed is composed of row-crop agriculture, pasture, and low-density industry and urbanization, with the exception of the heavily urbanized St. Louis metropolitan region, near the point at which the Meramec River flows into the Mississippi River (Homer et al. 2012). Our study was located along 530 km of the main channels of the Meramec, Big, and Bourbeuse rivers (the MBB) upstream of the urbanized part of the watershed (Fig. 2).

Mussel Survey Dataset

We used the Missouri Department of Conservation (MDC) mussel database (data available upon request to and subject to the approval of the Missouri Department of Conservation, 3500 East Gans Road, Columbia, MO, 65201) to extract geographic locations from mussel surveys completed by the department. This dataset is a large, statewide, and long-term database managed by MDC biologists and includes survey information for specific mussel-bed locations across Missouri. Information on these mussel beds includes GPS points, survey method used, list of species found, and number of individuals found, but it

includes little or no information on survey design. To be consistent with the dates of hydrogeomorphic data used in this study (2012 and 2014), we extracted survey information for sites in the MBB sampled after 1993 by wading, snorkeling, or diving. We removed from the dataset sites where mussels were not reported, sites that were represented by haphazard or incidental collections, or sites that were sampled only by tactile search methods. Sites located within 180 m of another site were considered one collective bed. We chose this distance based on previous studies of average mussel-bed length in the MBB (Lueckenhoff 2015; Schrum 2017). The remaining dataset included 106 unique mussel-bed locations. To build our model, we then selected a subset of 42 mussel-bed locations representing the highest-richness sites; these beds individually contained 14–31 species and, collectively, the entire Meramec River mussel fauna (Key 2019). The remaining 64 mussel-bed locations individually contained 2–26 species and a total of 39 species. We used these beds to determine the model's sensitivity by assessing how many of these beds fell within areas that our model defined as suitable habitat. This validation is an additional step beyond the standard validation methods used regularly in model development, such as AUC values described subsequently (see "Model Building").

Generation of Hydrogeographic Variables

A critical step for setting up the workflow of generating the spatial hydrogeomorphic variables is to define the stream dimensions and the location of the stream channel. To do so, we used the definition of bankfull flow as the minimum width-to-depth ratio for any location along the stream channel (Wolman 1955). We used two readily available spatial data sources: light detection and ranging (LiDAR) and National Hydrography Dataset (NHD). LiDAR coverage was accessed through the Missouri Spatial Data Information Service (MSDIS 2011) and flown between 2012 and 2014. LiDAR tiles (1-m horizontal resolution) were mosaicked into a single, seamless digital elevation model (DEM) and resampled to 10-m resolution. LiDAR does not penetrate water, and bathymetric data for the entire watershed do not exist and would be extremely costly to generate. Therefore, we used available elevation data from DEMs and minimum stream-bottom elevation from the National Hydrography Dataset (USGS 2004) as a measure of channel depth.

Throughout the length of the MBB study reaches, we generated 200-m cross sections at 10-m intervals at right angles to the stream centerline. Using these cross sections, the elevations of the underlying DEM were assigned to points along each cross section at 3-m intervals. The width-to-depth ratio was determined by first identifying the minimum elevation along a cross section taken from the underlying LiDAR. We determined that the minimum elevations from LiDAR represented either an in-channel gravel bar or a value generated from the hydro-flattening; either way, the values provide the best available method for determining channel-bank heights over large spatial domains. Using MATLAB (MathWorks 2016), the horizontal and vertical distances of this minimum elevation point were then calculated relative to all other points in that same cross section. The minimum value of the ratios of the horizontal distances relative to the vertical distances identified the depth of water at which bankfull flows occur (Fig. S1). The resulting water depth was added to the minimum elevation taken from the LiDAR to define the bankfull elevation for each cross section. The bankfull elevations for the cross sections were interpolated into a continuous grid using natural neighbor interpolation. The DEM was then subtracted from the bankfull elevation grid to approximate the channel polygon, where positive values are the channel and negative values are outside the channel. Areas deemed outside the channel were removed through visual inspection (e.g., near-channel gravel mines). The channel polygon was then smoothed, and a stream centerline was derived. Cross sections for each point at 10-m intervals along the derived centerline were then created and clipped to the channel polygon to determine channel width at 10-m intervals (Fig. S1).

After we defined the stream dimensions and location of the channel, we developed four hydrogeomorphic variables generated solely from LiDAR coverage, including two bluff adjacency variables (bluff adjacency, binary, "ba"; bluff adjacency area, continuous, "baa") and two stream power index variables (stream power class, binary, "spc"; stream power index, continuous, "spi") (Table 1 and Fig. S2). We identified bluffs as steep cliffs, usually along stream meanders; bluffs are found throughout the MBB. The bluff adjacency variables were created by classifying each pixel in the DEM using a neighborhood search criterion for change in elevation from each pixel centroid. Here pixels were stratified by range criteria to classify those areas of the landscape with a slope equal to or greater than 100% or 45°. Pixels with a range, or change in elevation, from the focal pixel to one of the surrounding eight pixels greater than or equal to a 10-m change in elevation were classified as bluffs. Having classified areas of the landscape as high-slope or bluffs, we then used the stream centerline to search at 10-m intervals for adjacent bluffs within a buffer of 1.5 channel widths (one channel width from each bank). Each point along the centerline was classified as either adjacent or not adjacent to a bluff as the ba variable. The variable baa represented the total bluff area within 1.5 channel widths for each stream centerline point at 10-m intervals. The value or class for bluff adjacency was attributed to each cross section at 10-m intervals and then interpolated using natural neighbors into a continuous gridded variable (Fig. S2).

The stream power variables were created by defining stream power as an index, $spi = \ln(A_d) \times S_{500}$, where spi is the stream power index, A_d is the total drainage area upstream of the site, and S_{500} is the slope over 500 m (Moore et al. 1991). Drainage area was solved by burning the stream centerline into the DEM, which enforces correct drainage of the flow direction and accumulation grids, then attributing the drainage area for the centerline points. For each centerline point at 10-m intervals, slope was calculated over a 500-m interval, spanning

Table 1. List of hydrogeomorphic variables generated, including the abbreviated names, the type of layer (continuous/binary), ecological justification, methodological description, and hypotheses of where mussels are expected. * denotes layers used in our model.

Habitat Characteristic ("layer name"): type	Justification	Description	Hypothesis
*Bluff adjacency area ("baa"): continuous	Conversations with malacologists indicate that mussel beds are usually found in the vicinity of bluffs adjacent to the stream channel	Total bluff area (m^2) within one channel width of each bank	The probability of mussel presence increases with increasing bluff area adjacent to the channel
Bluff adjacency ("ba"): binary		Whether there is a bluff within one channel width of each bank	The probability of mussel presence increases in channels adjacent to bluffs
*Stream power index ("spi"): continuous	Stream power is a major control of slope toe erosion (Nefeslioglu et al. 2008), which can have negative effects on mussels (Hartfield 1993)	Index of potential energy of water in the channel, using $spi = \ln(A_d) \times S_{500}$	The probability of mussel presence increases in areas with moderate stream power
Stream power class ("spc"): binary		Potential energy of water in the stream channel, classed as either high or low, based on spi	The probability of mussel presence increases in areas with low stream power
*Lateral channel stability ("lcs"): binary	Lateral channel movement and bank erosion could disrupt substrate stability and mussel occurrence (Strayer 1999; Strayer et al. 2004)	Lateral channel movement of > 10 m in 17 years classed as unstable, all else classed as stable	The probability of mussel presence increases in stable channels
*Gravel/pool class ("gpc"): binary	(1) Conversations with malacologists indicate that mussels are frequently found near gravel bars, and (2) areas with persistent gravel bars indicate areas that have stable beds, a necessary condition for mussel persistence (Bates 1962; Peck 2005; Zigler et al. 2008)	Reaches dominated by gravel are classed at gravel, all else classed as pool reaches	The probability of mussel presence increases within gravel class reaches
Gravel bar proximity ("gbp"): binary		All areas within 100 m of a gravel bar are classed as adjacent to a gravel bar, all else classed as not adjacent to a gravel bar	The probability of mussel presence increases within 100 m of gravel reaches
*Distance to gravel bar ("dgb"): continuous		Euclidean distance (m) to nearest gravel bar	The probability of mussel presence increases in areas with close proximity to gravel reaches
*Low-flow surface availability index ("lwai"): continuous	Refuge during drought periods is necessary for mussel survival (Golladay et al. 2004)	Cross-sectional average of the area of water pixels surrounding each cell, normalized by stream width	The probability of mussel presence increases in areas with higher low-water availability index values
Low-flow surface water availability class ("lwac"): binary		Cross-sectional average of the area of water pixels surrounding each cell, normalized by stream width, classed as high or low	The probability of mussel presence increases in areas with high low-water availability classification

A_d is the total drainage area upstream of the site, and S500 is the slope over 500 m.

250 m upstream and downstream of every point. A moving average of 50 m was used to smooth the estimates of slope. The value of spi for each point was attributed to the corresponding cross section at 10-m intervals and then interpolated using natural neighbors into a continuous gridded variable. The gridded variable was classified into a binary variable of high and low stream power classes using the mean value as the break between the two classes (spc; Fig. S2).

Aerial imagery was used as the primary source for deriving six additional hydrogeomorphic variables (Table 1). Three variables characterized the channel based on the properties of gravel bars in the imagery (gravel/pool class, binary, "gpc"; gravel bar proximity, binary, "gbp"; distance to gravel bar, continuous, "dgb") (Fig. S3). Because bathymetry data were not available, we created two variables as proxies for water availability during low flows (low-flow surface water

availability index, continuous, "lwai"; low-flow surface water availability class, binary, "lwac") (Fig. S4). The final variable represented lateral channel stability ("lcs") (Fig. S5). To derive the gravel variables, unsupervised classifications were performed on images from the National Agriculture Imagery Program (NAIP; 1-m horizontal resolution) (MSDIS 2011). This is leaf-on imagery gathered over six days in June 2012 and seven days in July 2014 at low water conditions, which were conducive for identifying exposed gravel bars. The raster grid was converted to a polygon vector layer and polygons were classified as either gravel, water, or other by using the ISO Cluster Unsupervised Classification in the Spatial Analyst Toolbox in ArcMap 10.7. The classification results were processed by filtering, smoothing, and cleaning boundaries using respective tools in the Spatial Analyst Toolbox. The postprocessed classification was visually inspected for misclassified regions by comparing to the original aerial imagery. We identified stable gravel bars by using only pixels that were identified as gravel in both 2012 and 2014 imagery; all others were identified as pool. These data worked well for our study because a near-record flood occurred in 2013; therefore, those gravel bars that remained after that flood were considered stable. The cross sections derived from LiDAR were then used to classify the reaches as either a gravel bar or pool. If a cross section was intercepted by a gravel polygon, then that entire cross section was classified as gravel. Due to the nature of the error in data collection for in-channel habitat characteristics from left bank to right bank and the scale of our environmental data, we did not distinguish between suitable and unsuitable habitat laterally within the channel. Instead, we focused on delineating whole reaches longitudinally along the river as suitable or unsuitable habitat.

The variable gpc was derived by interpolating using natural neighbors to create a continuous, longitudinal representation of gravel and pool classes (Fig. S3). The variable gbp was derived by classifying each centerline point as a distance either greater than or less than 100 m to a gravel reach (Fig. S3). The dgb variable was the continuous representation of the Euclidean distance to gravel bars for every centerline point (Fig. S3).

We derived low-water availability variables (lwai and lwac) by performing an eight-cell focal search window on each cell within the channel to assign the total area of water pixels surrounding each cell, including that cell's area. The cross sections were assigned the average value of the pixels intercepting each cross section, which was then divided by channel width to account for changes in channel size due to drainage-area scaling. The variable lwai was derived by interpolating cross sections to generate a continuous grid of low-flow surface water availability index. The continuous lwai grid was classified into a binary variable of high- and low-flow water surface availability (lwac) using the median value as the threshold between the classes (Fig. S4). We generated 100 random points within our channel to validate the unsupervised classifications. Each point was assigned the class code corresponding with the location of the classified layer, with 92% of the points correctly classified through visual inspection of aerial imagery. We acknowledge that this variable does not perfectly represent the vulnerability of any given point to dewatering during low flow because we do not have depth data. However, at the riverscape scale in which we are modeling, our low-flow water availability variables can help identify areas likely to contain water during low flow periods, making them potentially important target locations for managers.

We used aerial imagery to classify the channel into one of two lateral channel stability classes (lcs: laterally stable and laterally unstable), using Leaf-off Digital Orthophoto Quarter Quads (DOQQs) from 1990 (1-m horizontal resolution) and 2007 (0.6-m horizontal resolution) (MSDIS 2011). We used this set of imagery because the leaf-off aspect makes delineating the stream channel less challenging. The approximate bankfull lines for each year were digitized using visual clues, including shadows, breaks in vegetation and substrate, scour lines from high flows, and an overlain semitransparent hillshade from the DEM. Areas where the lines diverged by more than 10 m between the two sets of images were classified as laterally unstable, while areas where the lines did not diverge by more than 10 m were classified as laterally stable (Fig. S5).

Model Building

The maximum entropy modeling method known as MaxEnt (Phillips 2017) was used to generate habitat models for mussel beds in the MBB. This method uses presence-only data to find the probability distribution of maximum entropy (i.e., closest to uniform) given constraints of known locations and hydrogeomorphic variables relative to the spatial extent of the analysis (Raxworthy et al. 2007). Because absence points in our case could not be generated with certainty, MaxEnt generated 10,000 pseudo-absence points automatically. MaxEnt generated a map showing predicted habitat suitability for each area of the landscape (given the spatial grain size) with values ranging from 0 to 1, where 0 is the most unsuitable and 1 is the most suitable habitat. A portion of mussel-bed locations ($n = 42$) were used in the MaxEnt model. All continuous hydrogeomorphic variables used in the final model were not highly correlated. Most variables had a correlation coefficient of < 0.29; however, the variables dgb and gpc had a slightly higher correlation coefficient of 0.58. The relative contribution of hydrogeomorphic variables in the habitat suitability model was assessed in MaxEnt via Jackknife analysis. Run type was set to bootstrap to generate test data with 20% of the presence data, and random seed was chosen to randomize test data. Replicates were set to 50 and iterations set to 5,000. All other settings in MaxEnt were set to default. The models were built using 80% of the total amount of presence data used in the model ($n = 42$), referred to as "training data," to generate the algorithms relating the hydrogeomorphic variables to the habitat suitability of every parcel on the landscape. The remaining 20% of mussel-bed locations that were withheld from MaxEnt were used to test the model. The

Table 2. Summary of values for each of the two classes of the six binary hydrogeomorphic variables and the values of the sample mussel beds for the same six layers. Included for both the six layers and the sample points are the percentages for each class, the minimum and maximum lengths (m) of each reach class, and the mean and standard deviation of each reach class.

Habitat characteristic	Class	Percent of layers	Percent of samples	Reach length minimum	Reach length maximum	Mean reach length	Reach length standard deviation
Bluff adjacency ("ba")	Adjacent	40.6	40.6	30	3,885	615	523
	Not adjacent	59.4	59.4	10	9,977	886	1,159
Gravel/pool class ("gpc")	Gravel	51.3	59.4	37	9,943	603	874
	Pool	48.7	40.6	50	4,392	571	498
Gravel bar proximity ("gbp")	<100 m	67.3	71.0	61	1,1761	845	1,028
	>100 m	32.7	29.0	10	4,187	412	502
Lateral channel stability ("lcs")	Stable	85.4	88.4	148	47,643	4,464	6,395
	Unstable	14.6	11.6	73	4,545	784	869
Low-flow surface water availability class ("lwac")	High	58.1	55.1	95	7,216	1,392	1,304
	Low	41.9	44.9	113	11,819	989	1,352
Stream power class ("spc")	High	43.1	50.7	47	6,661	1,014	900
	Low	56.9	49.3	142	7,427	1,365	1,334

area under the receiver operating curve (AUC) measures the probability that presence locations have a higher habitat suitability score than randomly chosen pseudo-absence points (Phillips and Dudik 2008). Models with average AUC values greater than 0.5 are considered sufficiently better than a random model at distinguishing among habitat suitability of presence locations from pseudo-absence points (Elith 2002). We used the test gain value to assess which environmental variables were the most important for model fit (Phillips 2017). Gain is a likelihood (deviance) statistic that maximizes the probability of the presence in relation to the background (pseudo-absence) data. Taking the exponent of the final gain gives the (mean) probability of the presence sample(s) compared to the pseudo-absences. We developed response curves to investigate the relationships between specific values within hydrogeomorphic variables and suitable/unsuitable reaches. In the absence of information-theoretic approaches available for MaxEnt model selection, we selected the best-fit model as our final model using our 10 variables (Table 1) and a stepwise model selection approach, preferentially selecting variables in a stepwise manner leading to high total model AUC values and containing only those variables with sizeable individual effects on model results when other variables are removed (following Elith 2002).

The raw model results were converted to a binary map of suitable and unsuitable reaches using the equal test sensitivity and specificity logistic threshold of 0.45. This commonly used threshold sets sensitivity equal to specificity (Phillips 2017; Cao et al. 2013). Once reaches were delineated, a buffer of 40 m was used to separate suitable and unsuitable habitats to account for areas of transition. The buffer size selected represents the average length of transitional values of habitat suitability seen across the watershed between long, continuous reaches of high habitat suitability values versus low suitability values. The portions of river highlighted as suitable habitat by this approach are measurable reaches of suitable habitat. These reaches vary in length, with the length depending on the continuity of stream characteristics and the relationship of mussel presence to those characteristics.

All spatial analyses were performed in ArcGIS and projected to NAD 1983 UTM Zone 15N (ESRI 2011). Due to computational limitations, we were not able to perform the image classification analysis on aerial imagery at 1 m. The finest resolution we were able to classify and process was 10 m. Therefore, all other layers were resampled to a 10-m resolution using majority setting on the resample tool in ArcMap, and the final product has a 10-m resolution.

Model Validation

The remaining mussel-bed locations that were not used in model development ($n = 64$) were used to validate the model. The location within the mussel bed at which these GPS points were taken is unknown in this dataset. To reduce the potential GPS errors on our results, we considered points that were located within 180 m (average mussel-bed length in the MBB; see above) of a suitable reach to be within suitable habitat. We reported the percentage of validation mussel-bed locations that were within a suitable reach (predicted by the MaxEnt model). We used a Pearson's chi-squared test to determine if validation mussel-bed locations were found disproportionally in suitable versus unsuitable reaches.

RESULTS

Hydrogeomorphic Variables

LiDAR derived variables.—Bluff adjacency area (baa) ranged from 0 to 28,173 m^2, with a mean of 1,393 m^2. For bluff adjacency (ba), 41% of total channel length was adjacent to a bluff, and 59% was not (Table 2). The baa for the entire

Table 3. Minimum, maximum, mean, and standard deviation for the four continuous habitat layers that we generated and the values of the layers at mussel-bed locations.

Habitat characteristic	Layer/Location	Minimum	Maximum	Mean	Standard deviation
Bluff adjacency area ("baa)	Layer	0	28,173	1,393	2,769
	Mussel location	0	17,678	1,129	2,672
Distance to gravel bar ("dgb")	Layer	0	1,820	121	222
	Mussel location	0	371	67	105
Low-flow surface water availability index ("lwai")	Layer	0	13.30	4.53	1.98
	Mussel location	0.78	8.55	4.34	1.73
Stream power index ("spi")	Layer	−0.0146	0.0243	0.0035	0.0031
	Mussel location	−0.0073	0.0120	0.0037	0.0029

model extent and mussel-bed locations had a 21% difference between the means (Table 3). For ba, the percentages representing the length of each class comprising the entire model extent and mussel-bed locations were identical. Stream power index (spi) values ranged from –0.0146 to 0.0243, with a mean of 0.0035. The spi for the entire model extent and mussel-bed locations had a 6% difference between the means (Table 3). For stream power class, (spc), 43% of total channel length had high stream power, and 57% had low stream power (Table 2). The spc had a 7.6% difference for the percentage of each class comprising the entire model extent and mussel-bed locations.

Aerial-imagery-derived variables.—The gravel class constituted 51% of the 530 river km, while pool class was 49% of the total channel length. The gpc had the largest discrepancy between entire model extent and mussel-bed locations with an 8.1% difference between the two. The gbp variable had a 3.7% difference for the percentage of each class comprising the entire model extent and mussel-bed locations. The percent difference between the means of the entire model extent and mussel-bed locations was 58% for the dgb, which was the greatest difference between the variables and bed locations among the continuous variables (Table 3).

The continuous representation of the low-flow surface water availability index values ranged from 0 to 13.3 and had an average index value of 4.5 in the lwai variable. The lwai and mussel-bed locations had a 4% difference between the means, which was the lowest among the continuous variables (Table 3). For the binary lwac variable, average reach length was 1,392 m. The high class in the lwac was 58% of the total reach length, while the low class was 42% (Table 2). The lcs variable was classified as either laterally stable or unstable, where 85% of the 530 river km were classified as stable and 15% as unstable. The average reach length for the stable class was 4,464 m and for the unstable class was 784 m (Table 2). The lwai and lcs both had a 3% difference for the percentage of each class comprising the entire model extent and mussel-bed locations.

Model Results

Model generation.—The training and test AUC values of the top model were 0.75 and 0.62, respectively. Six hydrogeomorphic variables were used in the best model: lcs, dgb, gpc, spi, baa, and lwai (Table 1). The model results show separation of habitat into suitable and unsuitable habitat (Fig. 3). Jackknife analysis indicated that lcs, dgb, lwai, and spi contributed significantly to the final model, while baa and gpc did not (Fig. 4). Response curves indicated that suitable reaches differed from unsuitable reaches in values of baa, lcs, dgb, spi, and lwai (Fig. 5). However, all available spi values in the response curve were above the 0.5 probability of presence; therefore, we cannot report definitively relationship of mussel presence with this variable. More specifically, areas predicted as suitable were in reaches with laterally stable channels, with zero or very low bluff area, near gravel bars, with slightly higher stream power, and with greater areas of contiguous water (Fig. 5).

Model validation.—Eighty-three percent (53 of 64) of the validation mussel beds fell within a suitable reach in the binary suitability model, and mussel beds were found disproportionally within suitable reaches ($\chi^2 = 18.77$, 1 df, $P < 0.05$). Eleven beds fell within reaches that the model predicted as unsuitable. Seven of these beds were within a river reach with a split channel (see Discussion) and one was in a relatively short, unsuitable reach between two long, suitable reaches. The remaining three beds were centrally located within an unsuitable habitat reach and had no evident characteristics that could help inform future modeling efforts.

DISCUSSION

Our study successfully delineated suitable habitat for mussel presence in the MBB. Our model results suggest that mussel beds are dependent on multiple channel features, particularly the presence of laterally stable channels, proximity to gravel bars, and greater contiguous expanses of water. One potential source of bias in our dataset is that mussel biologists typically focus their sampling effort on gravel bars and avoid less-accessible, deeper areas of rivers, and our dataset suffers from this source of bias. Sampling a randomly selected set of stream reaches identified by our models as unsuitable is necessary to ground truth our model results. Nevertheless, our approach shows that readily available aerial imagery and topography data can provide useful description of stream-

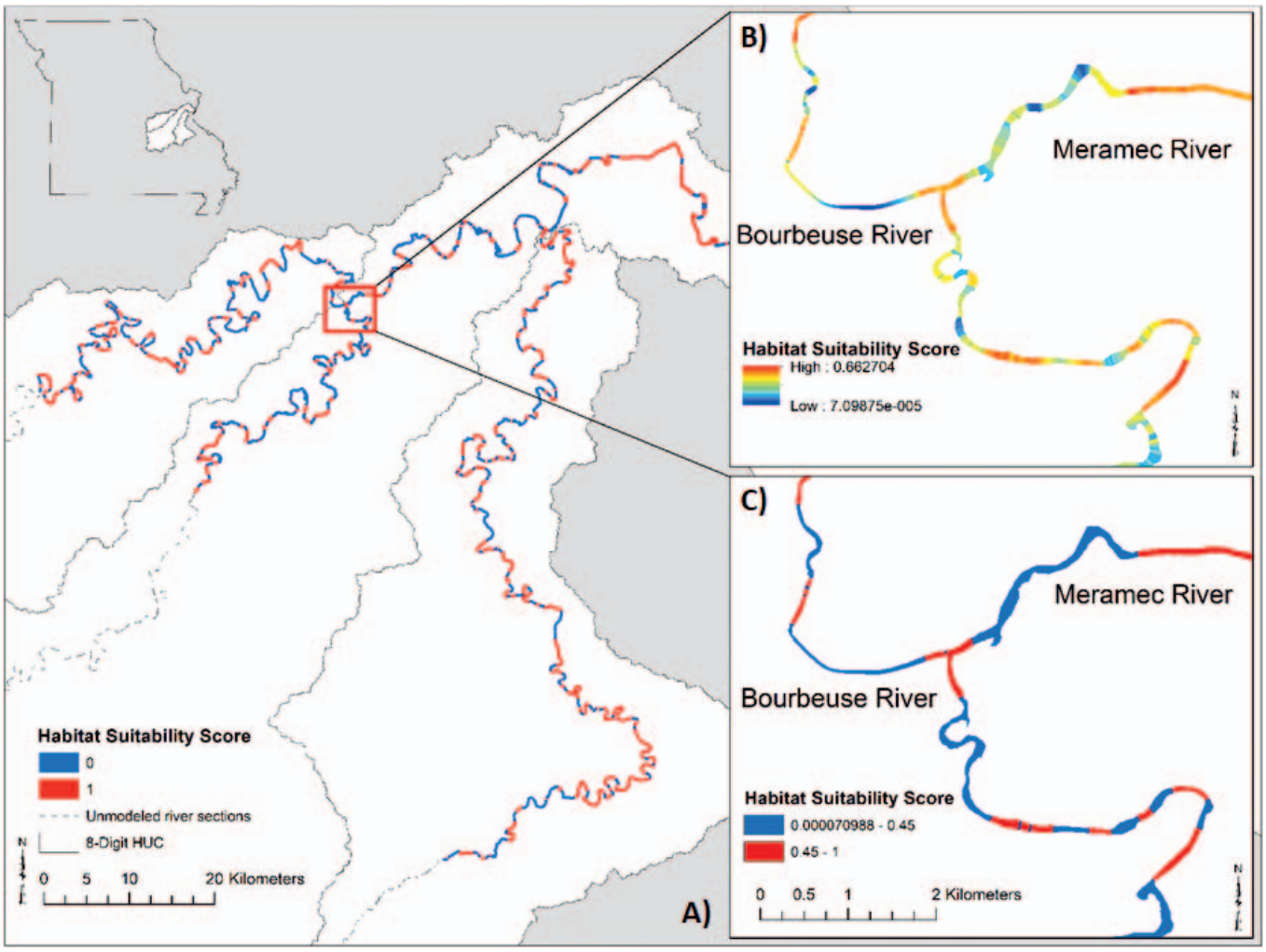

Figure 3. Model results for classifying stream reaches in the Meramec River basin with regard to their suitability for mussel beds. (A) Map of the entire study area showing a binary classification (suitable or unsuitable). (B) Detailed map of an example section of the watershed showing continuous suitability scores. (C) Detailed map of an example section of the watershed showing binary scores.

reach characteristics associated with the occurrence of mussel beds at a riverscape scale.

Other aspects of our model findings require ground-truthing due to the nature of available large-scale hydro-geomorphic data. For example, we used aerial imagery to define low-flow surface water availability as a proxy for identifying potential drought refugia. Ground-truthing based on bathymetric data is necessary to evaluate the extent to which low-flow surface water availability represents vulnerability to emersion during drought. Similarly, lateral bank stability may or may not reflect the type of substrate stability that other papers have associated with mussel occurrence on a smaller spatial scale. Evaluating these factors can help refine approaches for utilizing large-scale watershed data. Other challenges associated with using riverscape-scale hydrogeomorphic data include lack of availability of such data in some areas, extensive canopy shading of smaller streams, and the computational power required to run models with detailed spatial information.

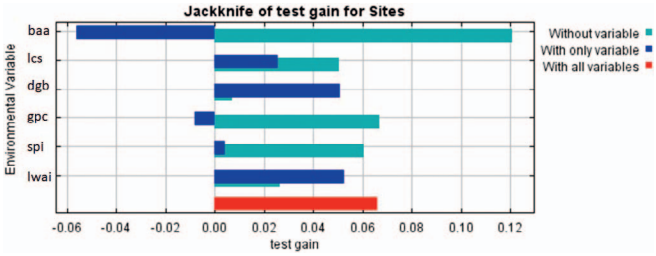

Figure 4. Results of jackknife analysis of the contribution of individual habitat variables to model performance. Final model variables include bluff area adjacency (baa), lateral channel stability (lcs), distance to gravel bar (dgb), gravel/pool class (gpc), stream power index (spi), and low water availability (lwai)

Many factors that influence mussel presence were not included in our model, such as fish-host relationships, species-specific differences in habitat requirements, and anthropogenic influences. However, identifying minimum characteristics of habitats necessary to support mussel presence provides a

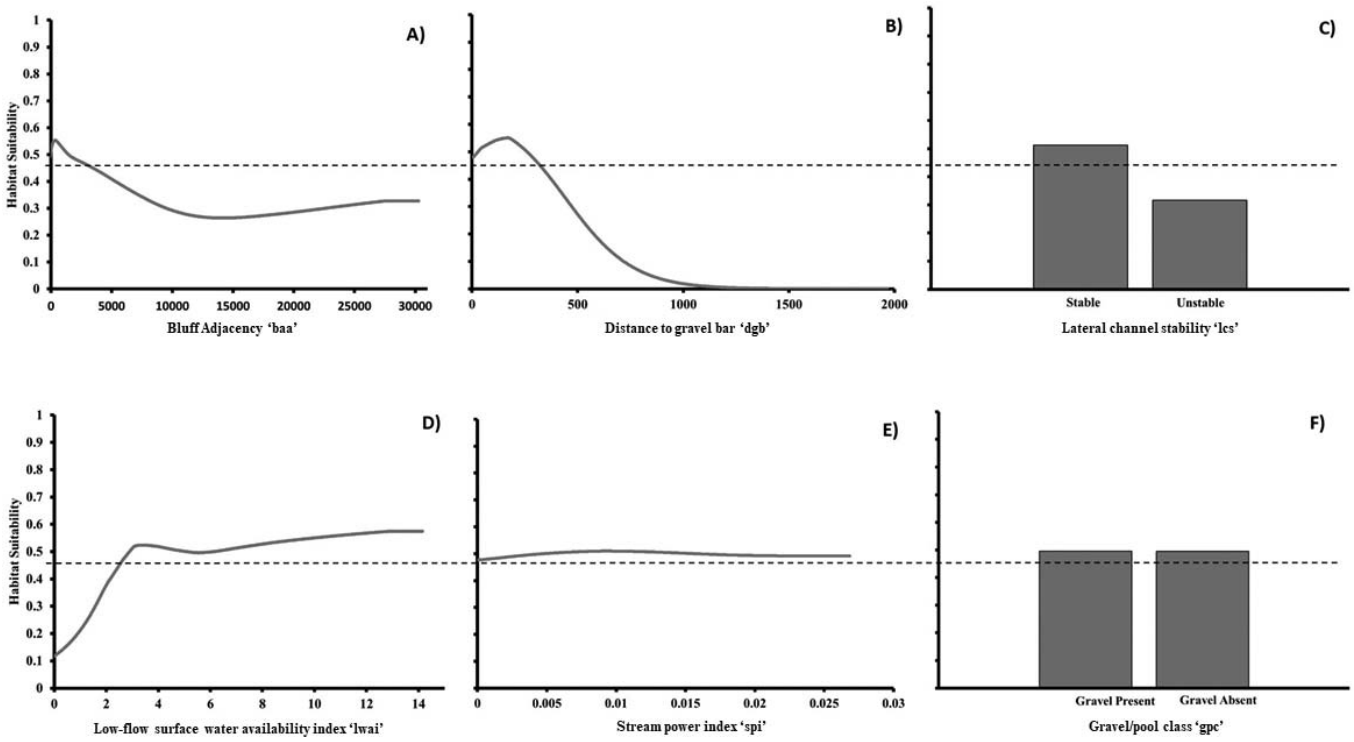

Figure 5. Response curves for hydrogeomorphic variables used in the habitat model. (A) bluff area adjacency (baa), (B) distance to gravel bar (dgb), (C) lateral channel stability (lcs), (D) low water availability (lwai), (E) stream power index (spi), and (F) gravel/pool class (gpc). The dashed line represents the equal sensitivity and specificity logistic threshold used to delineate habitats predicted as suitable ($\geq$0.45) and unsuitable ($<$0.45).

baseline that can allow the effects of other factors to be evaluated (Bouska et al. 2018; Fig. 1). Although the datasets we used are specific to the Meramec River basin, our approach is amenable to other river systems where researchers have access to comprehensive mussel-survey data. LiDAR is quickly becoming ubiquitous in the contiguous United States, and high-resolution aerial imagery for some areas often can be found dating back decades, making this approach a reasonable option in some areas where data are available. Our methods also may be applicable for other benthic organisms, such as snails, insects, or crayfish.

We developed an approach that uses readily available, continuous, longitudinal data to describe associations of hydrogeomorphic features and the presence of mussel beds to identify suitable mussel habitat at a large scale. We specifically address a recognized knowledge gap for understanding mussel-habitat distributions (FMCS 2016) by using hydrogeomorphic variables and scaling them to the channel in a continuous, longitudinal manner. By using riverscape-scale, fundamental niche habitat modeling, managers can target specific river reaches for mussel surveys, reintroduction efforts, and other management activities.

ACKNOWLEDGMENTS

We thank the following individuals for contributions throughout various stages of the project: Scott Faiman (MDC), Ivan Vining (MDC), Rob Pulliam (MDC), Andy Roberts and Joshua Hundley (US Fish and Wildlife Service), Craig Scroggins (MDC), and Jodi Whittier (MU). We also would like to thank the reviewers of this manuscript for their comments and suggestions. This work was funded by the Missouri Department of Conservation. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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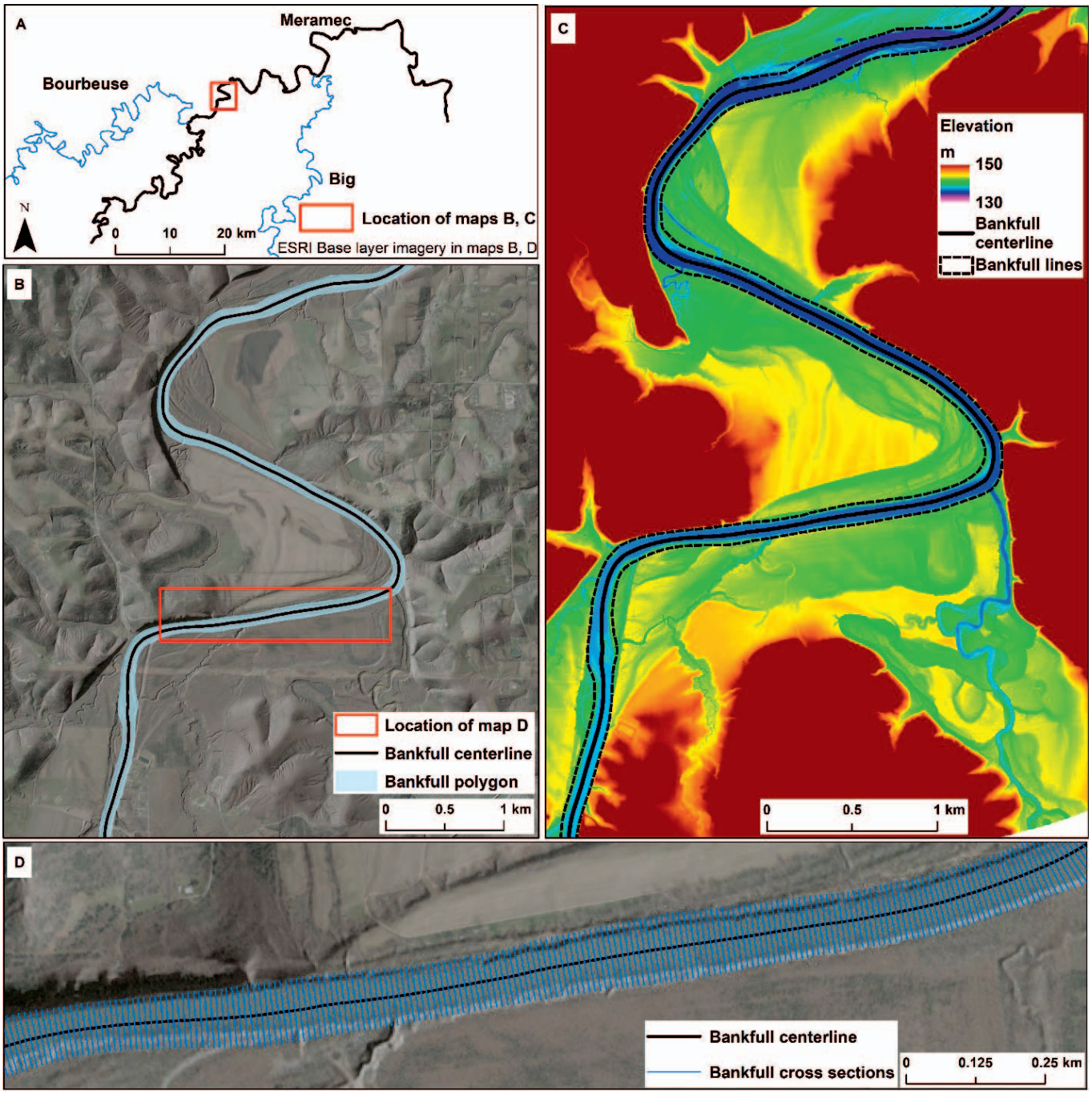

Figure S1. Examples of the stream channel delineation from LiDAR: A) main channel network of the Meramec River watershed, B) the stream centerline and the bankfull polygon superimposed over aerial imagery and a semi-transparent hillshade of the LiDAR digital elevation model, C) the stream centerline and the bank lines superimposed over the DEM used for the channel delineation, and D) the stream centerline and bankfull cross sections superimposed over aerial imagery and a semi-transparent hillshade of the LiDAR digital elevation model.

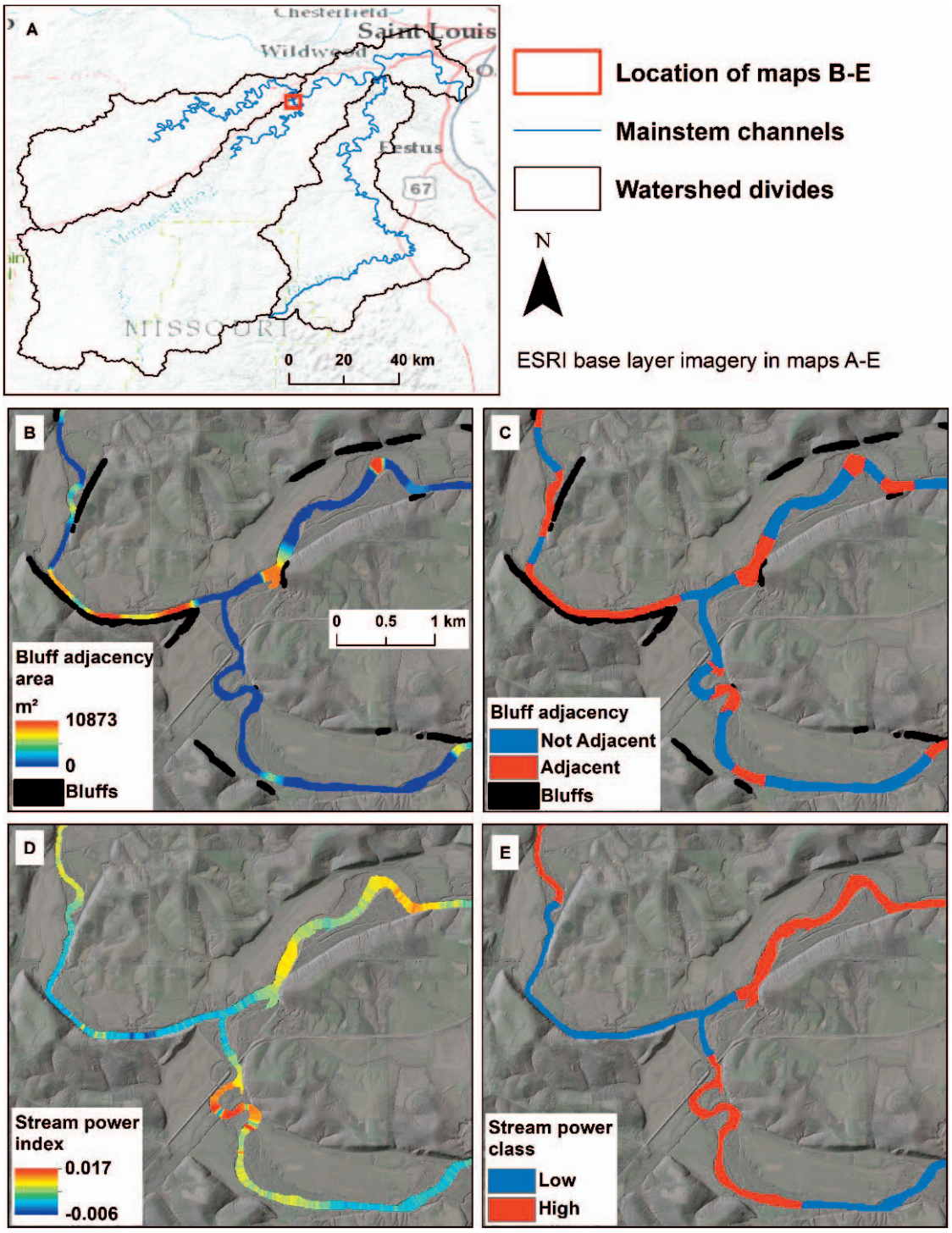

Figure S2. Example maps of bluff adjacency and stream power layers: A) map of the Meramec River watershed with locations of the subsequent detailed maps, B) bluff adjacency area (baa) continuous layer with classified bluffs, C) bluff adjacency (ba) binary layer with classified bluffs, D) stream power index (spi) continuous layer, and E) stream power class (spc) binary layer. The base layers for maps B-E are composed of aerial imagery overlaid with a semi-transparent hillshade derived from the LiDAR digital elevation model.

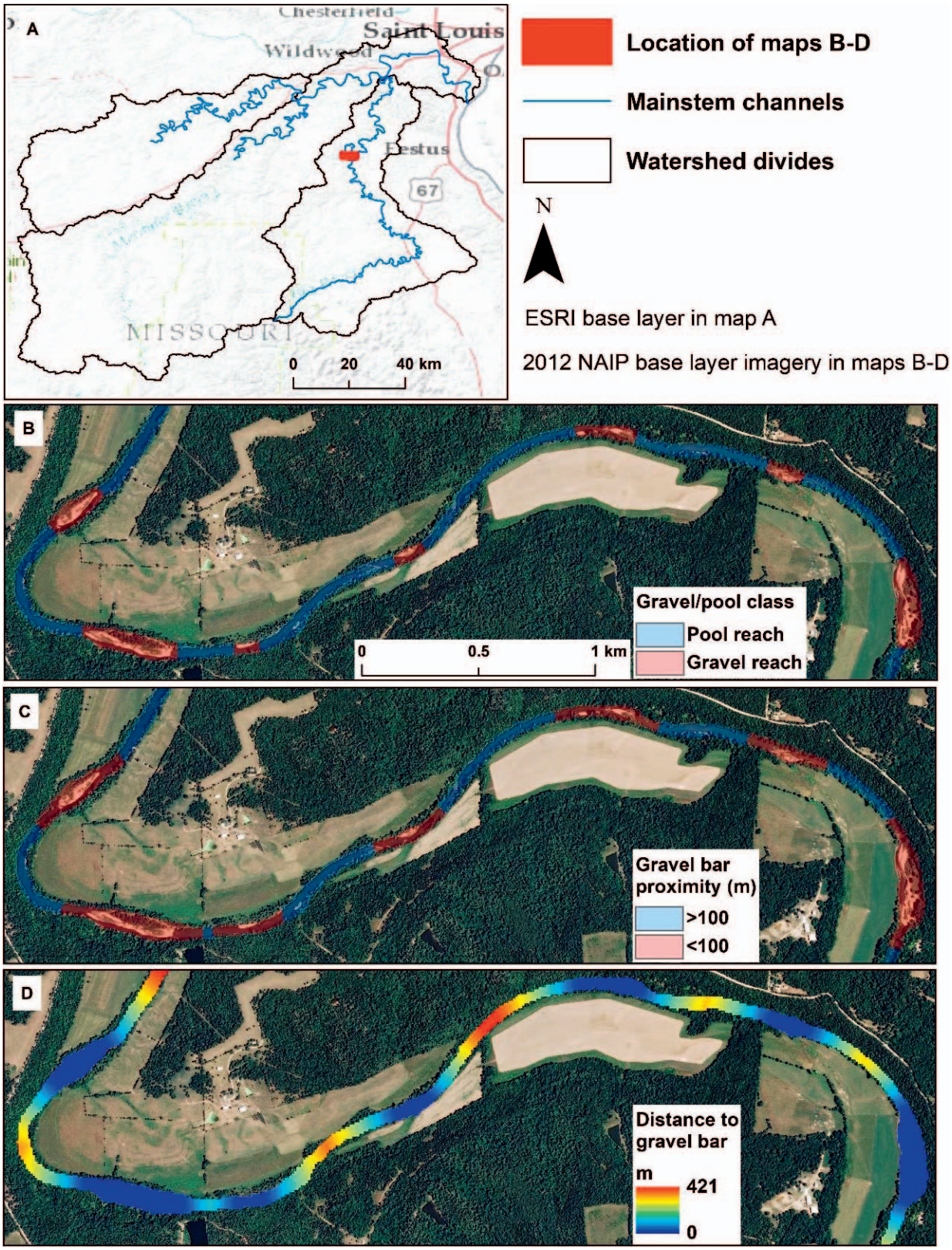

Figure S3. Example maps of gravel layers: A) map of the Meramec River watershed with locations of the subsequent detailed maps, B) semi-transparent gravel/pool class (gpc) binary layer superimposed over aerial imagery to show underlying stream channel, C) semi-transparent gravel bar proximity (gbp) binary layer, and D) distance to gravel bar (dgb) continuous layer.

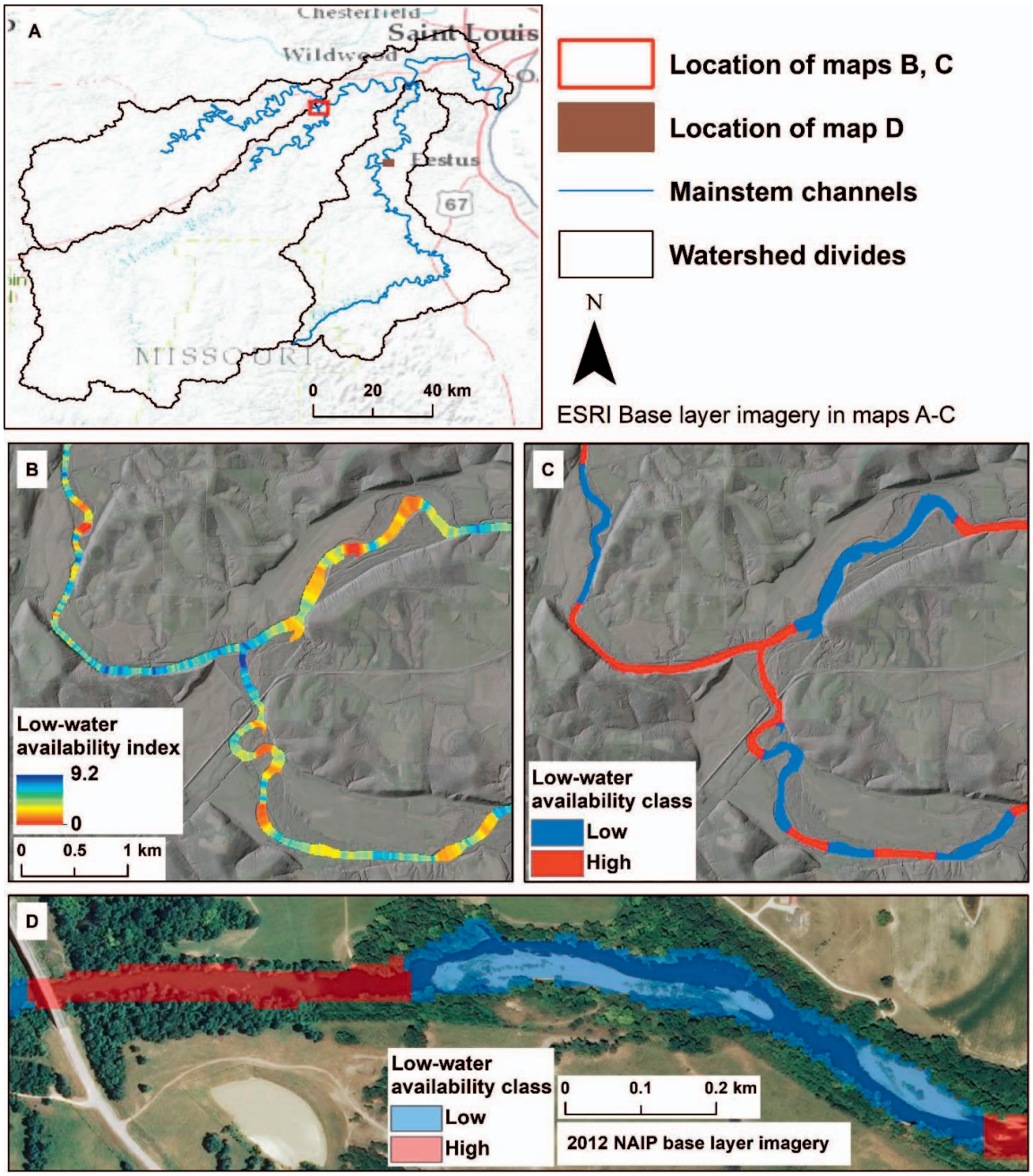

Figure S4. Example maps of low-flow surface water availability layers: A) map of the Meramec River watershed with locations of the subsequent detailed maps, B) low-flow surface water availability index (lwai) continuous layer, C) low-flow water availability class (lwac) binary layer, and D) large-scale example of semi-transparent low-flow surface water availability class binary layer showing the underlying stream channel classified as either low low-water surface availability or high low-flow surface water availability. The base layers for maps B and C are composed of aerial imagery overlaid with a semi-transparent hillshade derived from the LiDAR digital elevation model.

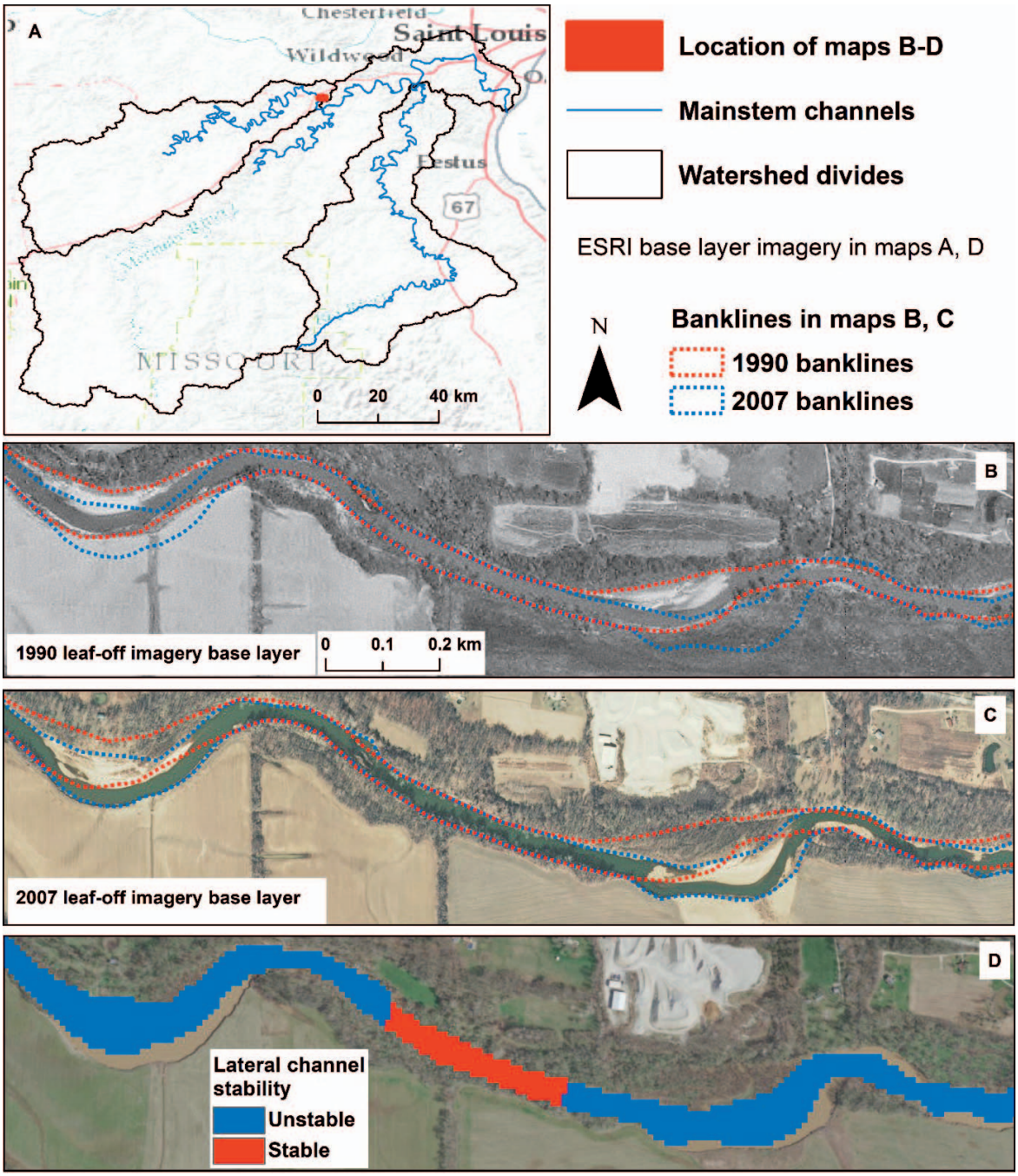

Figure S5. Example maps of lateral channel stability layer: A) map of the Meramec River watershed with locations of the subsequent detailed maps, B) digitized bank lines from 1990 and 2007 superimposed over 1990 leaf-off aerial imagery, C) digitized bank lines from 1990 and 2007 superimposed over 2007 leaf-off imagery, and D) lateral channel stability binary layer from the digitized bank lines.

Freshwater Mollusk Biology and Conservation 24:59–86, 2021

DOI:10.31931/fmbc-d-19-00040

REGULAR ARTICLE

HIDING IN PLAIN SIGHT: GENETIC CONFIRMATION OF PUTATIVE LOUISIANA FATMUCKET *LAMPSILIS HYDIANA* (MOLLUSCA: UNIONIDAE) IN ILLINOIS

Alison P. Stodola[1]*, Charles Lydeard[2], James T. Lamer[3], Sarah A. Douglass[1], Kevin S. Cummings[1], and David Campbell[4]

[1] *Illinois Natural History Survey, Prairie Research Institute, University of Illinois at Urbana-Champaign, Champaign, IL 61820 USA*
[2] *Department of Biology and Chemistry, Morehead State University, 150 University Blvd, Lappin Hall Room 103, Morehead, KY 40351 USA*
[3] *Illinois River Biological Station, Illinois Natural History Survey, Prairie Research Institute, Havana, IL 62644 USA*
[4] *Department of Natural Sciences, Gardner-Webb University, 110 S Main St, no. 7270, Boiling Springs, NC 28017 USA*

ABSTRACT

Understanding the status and distribution of species is fundamental for conservation. However, recent genetic work has challenged the known distributions of some unionid taxa. The recognized range of the Louisiana Fatmucket *Lampsilis hydiana* spans watersheds from east Texas northward to southern Arkansas and eastward to western Mississippi. Specimens with morphological similarities to *L. hydiana* have been observed in Illinois and were presumed to be Fatmucket *Lampsilis siliquoidea* based on known distributions of *Lampsilis* species in Illinois. We examined specimens from Illinois and completed comparative genetic analyses using the mitochondrial genes *cox1* and *nad1* for species resembling *L. siliquoidea*. Our results show two morphologically similar, yet genetically distinct, species in Illinois. One of these species was genetically similar to *L. siliquoidea*, and one of these species showed little-to-no genetic difference from topotypic *L. hydiana*. The confirmation of *L. hydiana* populations within Illinois is significant for documenting the faunal diversity of the state. The varying degree of phenotypic separation confirms the need for further morphological research within *Lampsilis*, as well as genetic research throughout the updated known range of *L. hydiana*.

KEY WORDS: Fatmucket, Louisiana Fatmucket, Illinois, *Lampsilis hydiana*, *Lampsilis siliquoidea*

INTRODUCTION

Accurate knowledge of the status and distribution of biota is fundamental for proper conservation of natural resources. Diversity is significant within unionid mollusks in the Mississippi basin (van der Schalie and van der Schalie 1950; Johnson 1980; Turner et al. 2000), yet an incomplete understanding of the genetic structure of many taxa (e.g., Campbell et al. 2005, Graf and Cummings 2007) leads to uncertainty regarding species distributions. Illinois has a diverse, well-documented freshwater mussel fauna that historically consisted of more than 80 species of Unionidae and one species of Margaritiferidae (Baker 1906, 1912; Parmalee 1967; Cummings and Mayer 1997; Tiemann et al. 2007). Range updates, such as discovering Bankclimber *Plectomerus dombeyanus* (Valenciennes, 1827) in Illinois in 2012, have been documented through sporadic or systematic surveys (Tiemann et al. 2007, 2013). Publication of such findings is valuable to regional conservation efforts, because federal and state agency conservation plans can apply only to species that are known to be present.

*Corresponding Author: alprice@illinois.edu

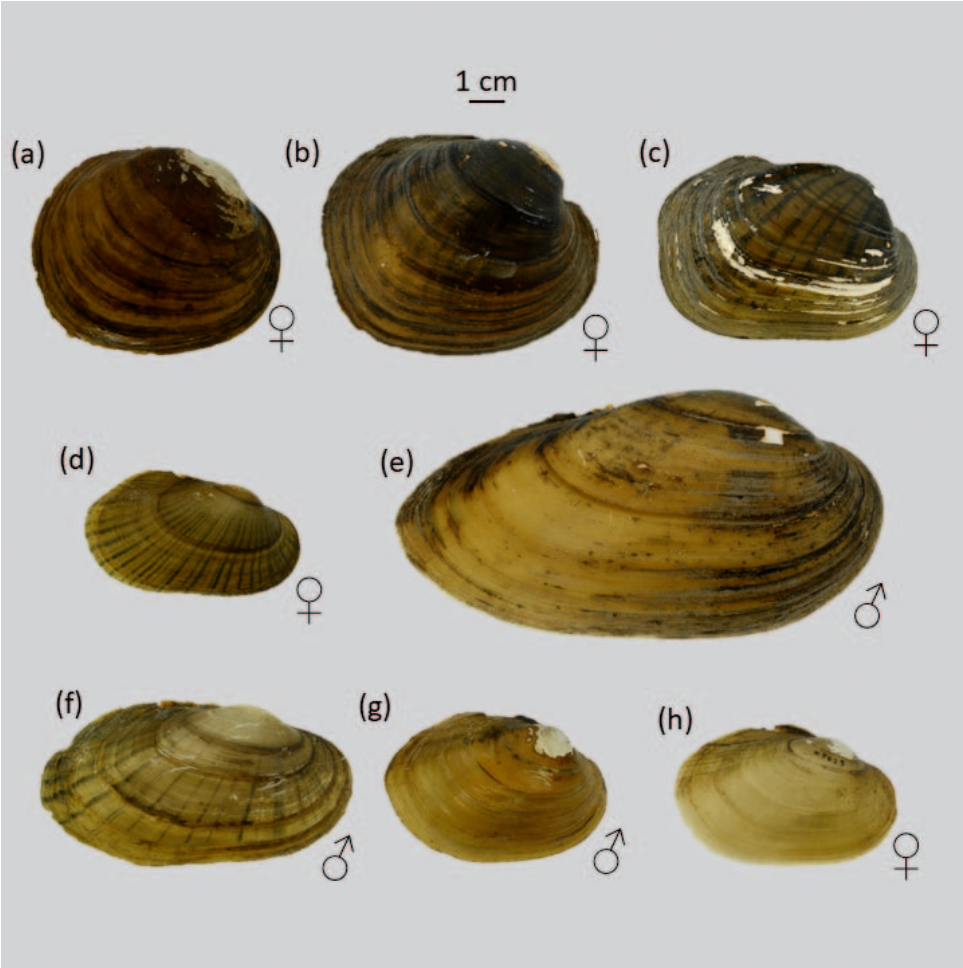

Figure 1. Example of variation in morphology of *Lampsilis* species included in our analyses: INHS Mollusk Collection Catalog Number, locality details, state. Sex noted by ♀ (female) or ♂ (male) and was determined by external shell morphology. (a) *Lampsilis abrupta* INHS 21521, Ohio; (b) *Lampsilis higginsii* INHS 30606, Mississippi River, Dubuque County, Iowa; (c) *Lampsilis hydiana* INHS 87783 Boeuf River, Richland Parish, Louisiana; (d) *Lampsilis radiata* INHS 38141, Yates County, New York; (e) *Lampsilis sietmani* INHS 32502, Illinois River, Pike County, Illinois; (f) *Lampsilis siliquoidea* INHS 41996, Mackinaw River, McLean County, Illinois; (g) *Lampsilis straminea* INHS 22926, Black Warrior River, Jefferson County, Alabama; (h) *Lampsilis virescens* INHS 21586, Paint Rock River, Alabama.

More than 20 species of *Lampsilis* are currently recognized in North America (Williams et al. 2017; FMCS 2019), and seven of those have been documented in Illinois by live material or shell (Tiemann et al. 2007). This diverse genus ranges across eastern and central North America and has shell morphology that varies from ovate—like Pink Mucket *Lampsilis abrupta* (Say, 1831)—to elongate and terete—like the newly described Canary Kingshell *Lampsilis sietmani* Keogh and Simons 2019 (Keogh and Simons 2019; Fig. 1). Fatmucket *Lampsilis siliquoidea* (Barnes, 1823) is one of the most widespread unionids in the world and has stable populations across most of its range. It occurs widely in the Mississippi and Great Lakes basins and is commonly encountered in Illinois rivers (Tiemann et al. 2007; Watters et al. 2009). Louisiana Fatmucket *Lampsilis hydiana* (Lea, 1838) (Fig. 1c)—a species previously reported from eastern Texas, Oklahoma, and Arkansas and east to Alabama (Burch 1975; Howells et al. 1996)—has a similar morphology to *L. siliquoidea* (Fig. 1f), but *L. hydiana* has never been genetically confirmed to exist in Illinois. Neither *L. hydiana* nor *L. siliquoidea* is of conservation concern in Illinois or at the federal level.

Lampsilis hydiana is described as having an elliptical, rayed, somewhat inflated shell and is distinguished from *L. siliquoidea* by a pearlier nacre, an umbo that is anterior, and an overall smaller average total length (Lea 1838). However, these two species have been considered indistinguishable at times (Vaughn et al. 1996) or as synonyms (Call 1895), which has led to uncertainty regarding their distributions. Based on literature reports and museum shell records, these species presumably co-occur in several drainages, such as the Big Black and Yazoo rivers in Mississippi (Jones et al. 2005). Additionally, specimens from Arkansas initially identified as *L. hydiana* included three genetically distinct groups that represented *L. hydiana* and two additional undescribed species (Harris et al. 2009). These divisions were supported by a shape

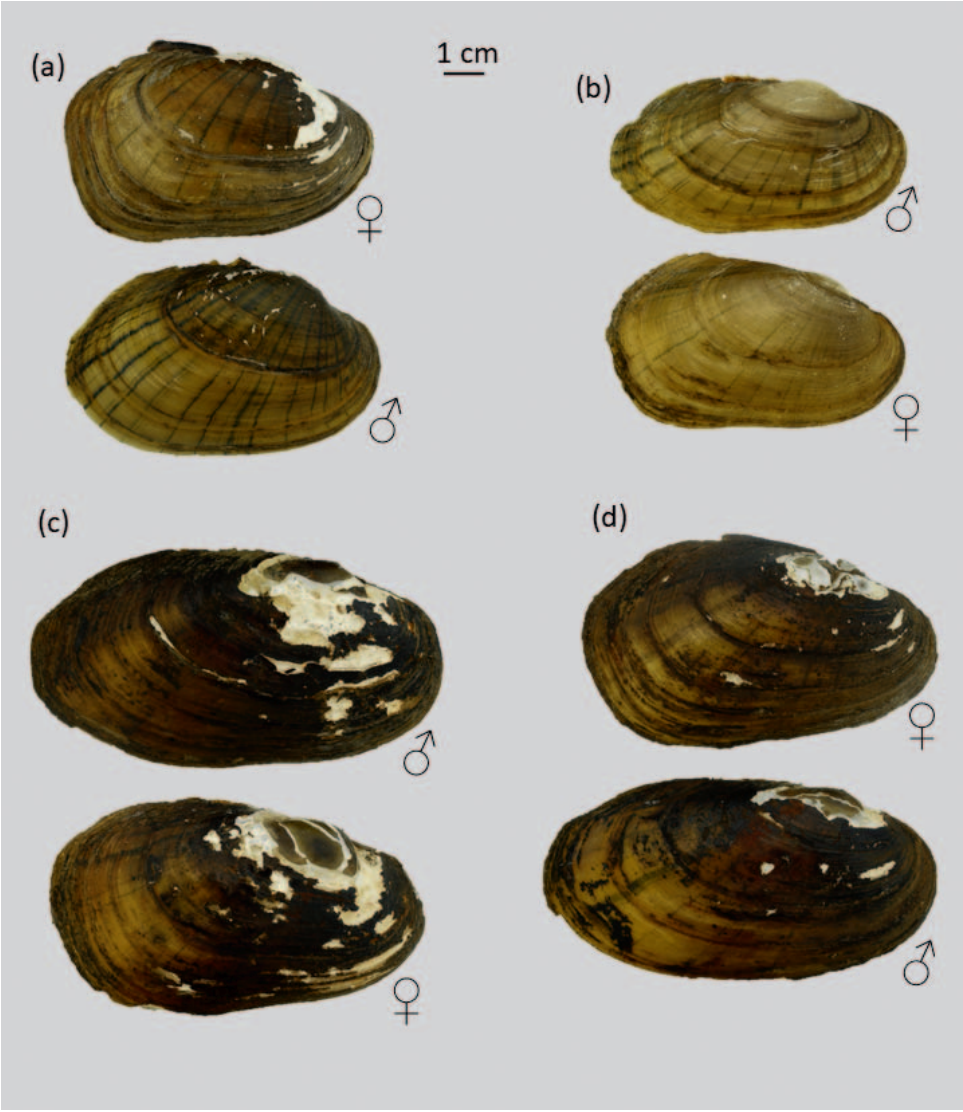

Figure 2. Representative images of some individuals of Illinois-collected *Lampsilis* included in our analyses (other images at https://doi.org/10.13012/B2IDB-5609050_V1): INHS Mollusk Collection Catalog Number (lower specimen number is arranged on top of each pair of images), locality details, and predetermined phenotype and confirmed genotype. Sex noted by ♀ (female) or ♂ (male) and was determined by external shell morphology. (a) INHS 45463-2 and 45463-3, Skillet Fork, Wayne County *Lampsilis hydiana* phenotype and *L. hydiana* genotype; (b) INHS 41996-1 and 41996-2, Mackinaw River, McLean County, Illinois, *Lampsilis siliquoidea* phenotype and *L. siliquoidea* genotype; (c) INHS 86787-5 and 86787-6 Lusk Creek, Pope County, *L. siliquoidea* phenotype and *L. hydiana* genotype; (d) INHS 45615-2 and 45615-9 Lusk Creek, Pope County, *L. siliquoidea* phenotype and *L. siliquoidea* genotype.

analysis, though there was some overlap in morphology (Harris et al. 2004; Harris et al. 2009). Thus, the range extent of *L. hydiana* remains unknown, and morphological characteristics to distinguish among *L. siliquoidea*, *L. hydiana*, and other similarly shaped *Lampsilis* species are lacking.

Certain specimens in several southern Illinois watersheds morphologically resemble *L. hydiana* (Fig. 2), though collection localities are well outside the published range of this species (Fig. 3). These specimens were typically identified as *L. siliquoidea*, despite morphologic resemblance to *L. hydiana*. The objective of our study was to determine taxonomic placement of the specimens that morphologically resemble *L. hydiana* to gain a better understanding of the distribution of *L. siliquoidea* and related species in Illinois.

METHODS

Mantle tissues of putative *L. hydiana* and *L. siliquoidea* from Illinois ($n = 83$ specimens from 25 sites) were collected from fresh, frozen, or ethanol-preserved individuals, used for DNA extraction, and catalogued in the Illinois Natural History Survey (INHS) Mollusk Collection, Champaign, Illinois (Appendix 1). Specimens came from the Big Muddy, Cache, Embarras, Kaskaskia, Little Wabash, Little Vermilion, Mackinaw, Sangamon, and Skillet Fork drainages and direct

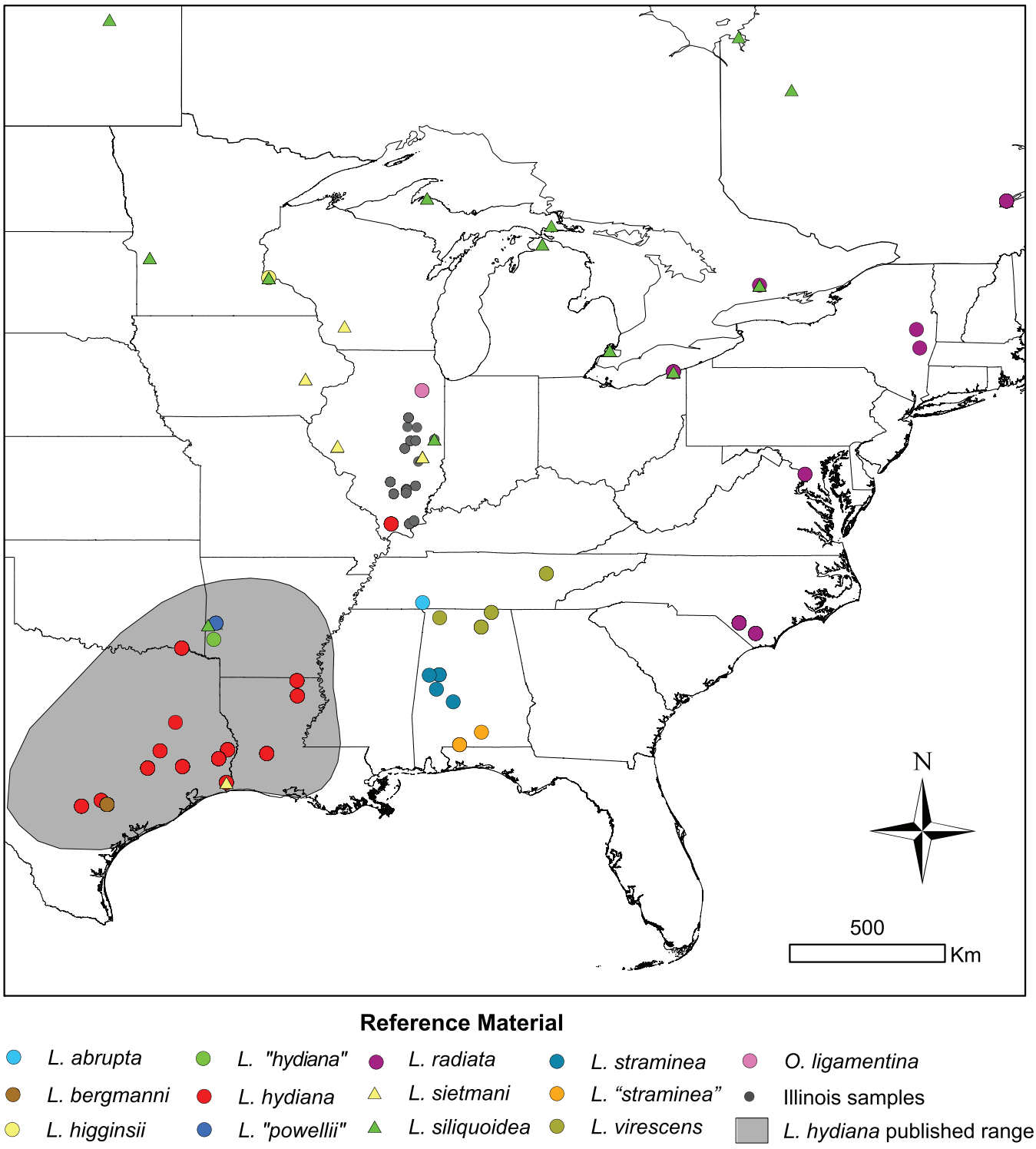

Figure 3. Approximate locations of reference materials used for this study. The previously published range for *Lampsilis hydiana* was adapted from Burch (1975) and Howells et al. (1996).

tributaries to the Ohio River (Big Grande Pierre, Lusk, and Rose creeks); images of external and internal valves of each specimen were made available via the Illinois Data Bank (https://doi.org/10.13012/B2IDB-5609050_V1). Initial species identifications were made from external shell morphology of each specimen prior to genetic analysis. Those that were more inflated, had a pearlier nacre, and had a shorter average total length in mature individuals were identified as putative *L. hydiana* ($n = 46$; Fig. 2a), while specimens that were more compressed, had a duller nacre, and had a longer average total

length in mature individuals were identified as *L. siliquoidea* ($n = 37$; Fig. 2b). Most of the putative *L. hydiana* were from specimens collected from the southern half of Illinois. Specimens used in this study were collected as part of other research projects, primarily during a statewide mussel survey for Illinois from 2009 to 2012. Funding constraints or curated tissue quality prevented us from using all available tissue samples from putative *L. hydiana* or *L. siliquoidea* in Illinois. Four *L. hydiana* specimens were collected from the Boeuf River, Louisiana, to provide comparative material (INHS 87783). In addition, comparative sequences were obtained from GenBank (Appendix 2).

DNA was extracted from approximately 2 mm × 2 mm mantle and muscle biopsies using the MagMAX–96 DNA Multi-Sample Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions, except samples were eluted in 40 µl of elution buffer 1 and 2 instead of 100 µl. Polymerase chain reactions (PCR) and primers for *cox1* and *nad1* DNA amplification followed Campbell and Lydeard (2012). PCR products were sequenced on a Life Technologies 3730xl DNA Analyzer (Applied Biosystems, University of Illinois Chicago Genome Research Core). The *cox1* region was 660 base pairs long, and the *nad1* region was 834 bases long (including 30 bases of *tRNA-Leu*). Not all reads clearly resolved all bases, however, and unreadable bases were entered as unknowns. Sequences were aligned using BioEdit (Hall 1999). The sequence alignments are available at the Illinois Data Bank (https://doi.org/10.13012/B2IDB-5609050_V1).

The relationships between species currently assigned to *Lampsilis* are not well resolved (Keogh and Simons 2019). To determine appropriate comparison taxa for our specimens, we performed preliminary phylogenetic analyses (details below) of all available *cox1* and *nad1* sequences for species currently assigned to *Lampsilis* (based on Williams et al. 2017), along with representatives of other genera in the tribe Lampsilini. These supported a clade of morphologically similar taxa that included *L. siliquoidea* and *L. hydiana*, along with Guadalupe Fatmucket *Lampsilis bergmanni* Inoue & Randklev, 2020, Arkansas Fatmucket *Lampsilis powellii* (Lea, 1852), Eastern Lampmussel *Lampsilis radiata* (Gmelin, 1791), *L. sietmani*, Rough Fatmucket *Lampsilis straminea* (Conrad, 1834), and Alabama Lampmussel *Lampsilis virescens* (Lea, 1858). In turn, this *siliquoidea* clade was most closely related to a clade that included Mucket *Ortmanniana ligamentina* (Lamarck, 1819), *L. abrupta*, and Higgins Eye *Lampsilis higginsii* (Lea, 1857), consistent with previous findings (Porto-Hannes et al. 2019; Inoue et al. 2020). Nomenclature follows Williams et al. (2017), with updates from recent works for *O. ligamentina* (Pfeiffer et al. 2019; Graf and Cummings 2021). As noted by Keogh and Simons (2019), confident assessment of the phylogenetic relationships of *Lampsilis* species within Lampsilini will require extensive sampling. Our goal was to find appropriate taxa for comparison with our *L. siliquoidea*–like and *L. hydiana*–like populations from Illinois, and we did not pursue the general phylogeny further. Based on these preliminary results, we included all available *cox1* and *nad1* sequences from the *siliquoidea* clade in our detailed analyses and used the *ligamentina* clade as the outgroup. The sequence identified as *L. powellii* in GenBank was treated as *L. hydiana* in our analyses (MF326971). Walters et al. (2021) also found this sequence to be *L. hydiana*, whereas true *L. powellii* was nearest to *L. siliquoidea*. A few sequences currently listed as *L. radiata* in GenBank (*cox1*: HQ153601, HQ153602, HQ153605; *nad1*: HQ153683, HQ153684, HQ153687, and HQ153691) were found to represent the "Cryptic *Lampsilis* sp." of McCartney et al. (2016). Those sequences did not place in the *siliquoidea* clade based on McCartney et al. (2016) and our preliminary analyses, thus we excluded them from the present analyses. Percent differences and number of base-pair differences were calculated for all sequences from the *siliquoidea* clade using PAUP*4.0a167 (Swofford 2002). Because many individuals had only one gene or the other sequenced, *cox1* and *nad1* were compared separately in these analyses. These calculations omit bases with uncertainty (e.g., A versus N is not counted as a difference, nor is that position counted in the total number of bases for calculating percentage). We used the program ABGD (Puillandre et al. 2012) to test the differentiation between species in the *siliquoidea* clade. To test the cutoff for different divisions, the number of steps was increased to 20 and relative gap width decreased to one; other settings used the default values.

For phylogenetic analyses, we used both parsimony and Bayesian approaches and included all individuals with data for both *nad1* and *cox1*. We concatenated the two genes, omitting the *tRNA-Leu* region. *Lampsilis sietmani* and *L. abrupta* had no *nad1* data available, but we included representative *cox1* sequences. In the ABGD analysis, one published *cox1* sequence identified as *L. hydiana* (EF033270, from the Cossatot River in Arkansas), the Escambia River *L. straminea* (four sequences), and the Neches River sequence of *L. sietmani* (two individuals with identical sequences) were somewhat divergent from the other sampled individuals, so they were also included despite having only *cox1* data available. If two individuals had the same haplotype for both *cox1* and *nad1*, that combined haplotype was included only once in the phylogenetic analyses. Maximum parsimony and "Group present/Contradicted" (GC) bootstrap analysis (Goloboff et al. 2003) in the computer program TNT 1.5 (Goloboff and Catalano 2016) used all the "new technology" search options. Parsimony analysis used 500 random addition replicates, and the bootstrap analysis used 500 bootstrap replicates, each with 10 random addition replicates. Bayesian analyses used 10,000,000 generations with 10 runs, each with eight chains. We used PAUP* to test data partitions, setting the codon positions as data blocks. Using likelihood criteria and the "greedy" heuristic, the AICc criterion supported a GTR+I model for *cox1* positions 1 and 3 and *nad1* position 2, GTR for *cox1* position 2 and *nad1* position 3, and GTR+G for *nad1* position 1. MrBayes 3.2.7 was used for Bayesian analyses (Ronquist et al. 2012). Each codon position was treated as a separate partition. The parameters revmat, shape,

pinvar, and statefreq were all unlinked. Convergence was determined by examining the standard deviation of split frequencies and confirming that they were under 0.01 (Ronquist et al. 2011), as well as by examination of the ESS values and trace plot in Tracer 1.7.1 (Rambaut et al. 2018). Tracer showed all ESS values well over 200, and the trace plot did not show any anomalies, so the standard 25% burn-in was used. We used PAUP* to calculate a majority-rule consensus of the Bayesian trees to obtain posterior probabilities, which facilitated outputting the tree as a graphic. Additionally, haplotype networks were constructed for *L. siliquoidea* and *L. hydiana* using median joining in PopART (Leigh and Bryant 2015).

RESULTS

The genetic results indicate that the *L. siliquoidea* and putative *L. hydiana* specimens from Illinois represent two distinct but closely related *Lampsilis* species. Sequences obtained for this study are available in GenBank (accession numbers MH560712-MH560762, MH560764-MH560777, MH588322-MH588394, MT537705-MT537725; Appendices 1, 2). Parsimony and Bayesian methods produced nearly identical results, with no differences in the affinities of the Illinois specimens. Parsimony analyses produced 319 trees of length 545 (as counted by TNT, which collapses polytomies, making a much smaller number of trees than PAUP*). In the Bayesian analysis, the standard deviation of split frequencies reached 0.01 after 1,735,000 generations. *Lampsilis hydiana* and *L. siliquoidea* were not sister taxa, but instead placed on different branches within the larger *siliquoidea* clade (Fig. 4). *Lampsilis siliquoidea* and *L. radiata* are sister taxa with relatively low genetic divergence, while *L. hydiana* is sister to *L. bergmanni*.

Sequences for *L. hydiana* versus *L. siliquoidea* had an average of 5.67% difference between them in *cox1* and 7.43% in *nad1*, similar to most other interspecies differences within the clade (Table 1). In contrast, the average differences within *L. hydiana* and within *L. siliquoidea* for both genes were under 0.5%, with some Illinois specimens sharing haplotypes with specimens from elsewhere. In particular, identical haplotypes were found in some Illinois specimens and some of the topotypic *L. hydiana* specimens sampled in this study (Appendix 2, Figs. 5, 6). Likewise, the haplotype networks show much larger differences between *L. hydiana* and *L. siliquoidea* than within them. In ABGD for *cox1*, all partitions with gap priors between 0.0183 and 0.00162 separated the Illinois specimens (along with many from other states) into two groups, corresponding to *L. hydiana* and *L. siliquoidea*. No partitions supported any further division of *L. hydiana* or *L. siliquoidea*, except for recognizing the Cossatot River, Arkansas "*L. hydiana*" as distinct for priors of 0.00886 or less in the initial partition and 0.0144 or less in the recursive partition. The species most difficult to distinguish from *L. hydiana* were *L. bergmanni* and Mobile Basin *L. straminea*, which separated only at gap priors of 0.00428 or less, whereas *L. siliquoidea* and *L. radiata* were separated at gap priors of 0.0546 or less. Gap priors of 0.00127 or less split up individual variation, which produced 116 groups. For *nad1*, partitions with gap priors between 0.0183 and 0.00264 separated *L. hydiana* and *L. siliquoidea* without dividing either one. Again, separation between *L. hydiana* and *L. bergmanni* or *L. straminea* was less clear, requiring gap priors of 0.00207 or less, which also began to split off divergent sequences within *L. hydiana*. Separation of *L. siliquoidea* from *L. radiata* was supported with the recursive partition at a gap prior of 0.0183 or less and the initial partition at a gap prior of 0.0144 or less. Intermediate gap priors generally agreed with currently recognized species, though some currently recognized species were divided into more than one group, especially if there was a geographic gap in the sampling (such as *L. sietmani* from Texas versus the upper Mississippi drainage).

Our results support the presence of *L. hydiana* in the Big Muddy, Cache, Embarras, Kaskaskia, Sangamon, Ohio, and Little Wabash drainages of Illinois (Fig. 7). Most drainages that we examined contained only *L. siliquoidea* or *L. hydiana*; however, both *L. siliquoidea* and *L. hydiana* were confirmed in the Sangamon River basin and in Horse, Big Grande Pierre, and Lusk creeks. Our morphological identifications matched the genetic confirmation in most cases (72 of 83 individuals were identified correctly; Appendix 1). Ten specimens that were determined morphologically to be *L. siliquoidea* were genetically confirmed as *L. hydiana*, and one specimen that was determined morphologically to be *L. hydiana* was genetically confirmed as *L. siliquoidea*. Three of four sites where these mismatches occurred had both *L. hydiana* and *L. siliquoidea* genotypes present (Big Grande Pierre Creek, Lusk Creek, and Horse Creek; Fig. 2c, 2d). The only individual sequenced from Salt Creek (of two total specimens from the Sangamon River drainage) was genetically confirmed as *L. hydiana* but was determined morphologically to be *L. siliquoidea*.

DISCUSSION

We used genetic analyses to confirm the presence of *L. hydiana* in Illinois. This genetic confirmation supports the species determinations by Anson A. Hinkley and Frank C. Baker more than a century ago (Illinois Natural History Survey, Prairie Research Institute 2021 [INHS Collections Data], referenced via previous identification field), that were made prior to the availability of genetic tools. It is unclear why *L. hydiana* was never included on Illinois species lists even though shells were deposited in the INHS Mollusk Collection bearing this identification. Regardless, we now have genetic support that the range of *L. hydiana* extends to latitude 40.1° N in the Sangamon River drainage, which is well north of the previously published range limit of latitude 34.6° N (Burch 1975; Howells et al. 1996; Inoue et al. 2020). While historical literature proclaimed the morphological differences between *L. hydiana* and *L. siliquoidea* to be "very clear cut" (Isley 1924),

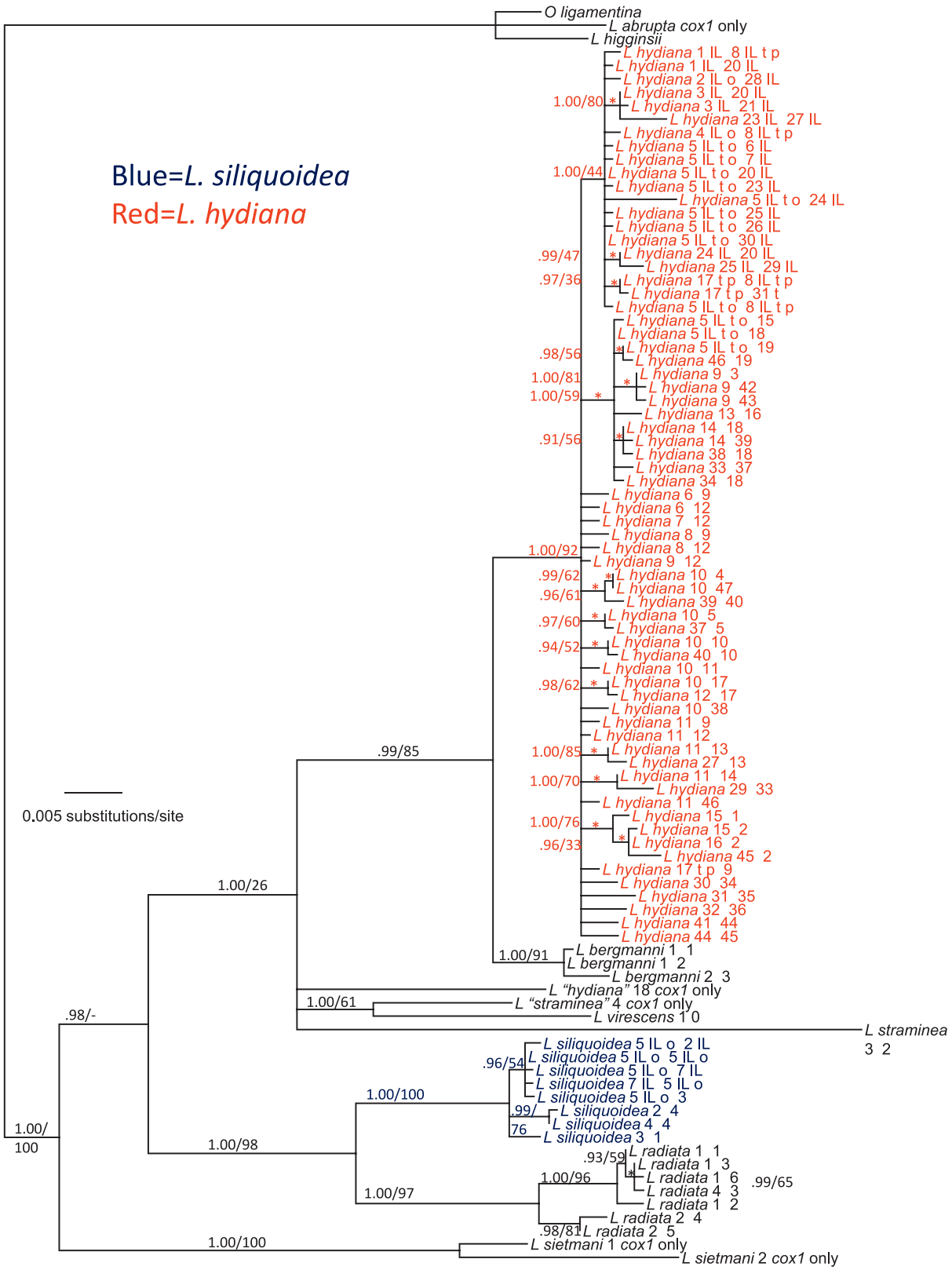

Figure 4. Phylogram of the Bayesian majority-rule consensus tree. Numbers on branches are Bayesian posterior probability/bootstrap GC percentage, - indicates under 50% bootstrap support; * denotes branches that did not have room for labeling the probabilities directly on the branch. Numbers after a name indicate *cox1* haplotype, followed by *nad1* haplotype (see Appendix 2). Letters after a name indicate a new sequence from Illinois, topotype *Lampsilis hydiana* (t), the sequence identified as *Lampsilis powellii* (p), or haplotypes found in other published sequences and in new ones from Illinois (o). *L.* "*hydiana*" indicates the divergent Cossatot River sequence, and *L.* "*straminea*" indicates the Escambia River population.

Table 1. Average, minimum, and maximum percent difference and number of base-pair differences in *cox1* and *nad1*.

	Intraspecific	To *hydiana*	To *siliquoidea*
cox1			
L. bergmanni	0.08% (0.00–0.33)	1.74% (1.18–2.74)	5.21% (4.19–6.12)
	0.55 bp (0–2)	11.18 bp (7–18)	32.86 bp (22–37)
L. hydiana	0.32% (0.00–1.98)	0.32% (0.00–1.98)	5.67% (4.38–7.29)
	2.02 bp (0–13)	2.02 bp (0–13)	35.63 bp (20–43)
"*L. hydiana*"	n/a	3.48% (3.09–4.45)	5.35% (4.80–6.00)
	n/a	20.19 bp (14–26)	31.00 bp (25–34)
L. radiata	0.63% (0.00–1.53)	5.37% (4.04–7.07)	2.23% (1.39–3.03)
	3.42 bp (0–8)	31.64 bp (16–42)	13.11 bp (8–19)
L. sietmani	0.66% (0.00–2.60)	6.46% (5.88–8.16)	7.17% (6.46–8.45)
	4.23 bp (0–16)	41.20 bp (26–53)	45.43 bp (34–52)
L. siliquoidea	0.46% (0.00–1.74)	5.67% (4.38–7.29)	0.46% (0.00–1.74)
	2.89 bp (0–11)	35.63 bp (20–43)	2.89 bp (0–11)
L. straminea	0.46% (0.16–0.69)	2.08% (1.55–3.53)	5.60% (4.44–6.24)
	2.67 bp (1–4)	12.64 bp (8–23)	33.74 bp (23–39)
"*L. straminea*"	0.39% (0.16–0.63)	3.08% (2.50–4.10)	5.72% (4.83–6.50)
	2.50 bp (1–4)	19.56 bp (16–27)	35.64 bp (25–40)
L. virescens	0.87% (0.00–2.01)	4.66% (3.35–6.89)	7.42% (5.91–9.17)
	3.52 bp (0–8)	22.64 bp (16–31)	36.05 bp (29–44)
	Illinois intraspecific	Topotype *hydiana*	Other conspecific specimens
L. hydiana Illinois	0.16% (0.00–1.52)	0.20% (0.00–1.23)	0.30% (0.00–1.98)
	1.06 bp (0–10)	1.30 bp (0–8)	1.91 bp (0–13)
L. siliquoidea Illinois	0.01% (0.00–0.15)	n/a	0.41% (0.00–1.41)
	0.42 bp (0–4)	n/a	2.66 bp (0–9)
	Intraspecific	To *hydiana*	To *siliquoidea*
nad1			
L. bergmanni	0.21% (0.00–0.70)	1.70% (0.67–3.10)	7.50% (6.19–8.39)
	1.22 bp (0–4)	9.92 bp (2–17)	43.16 bp (23–49)
L. hydiana	0.63% (0.00–2.36)	0.63% (0.00–2.36)	7.43% (5.34–8.86)
	3.73 bp (0–15)	3.73 bp (0–15)	47.68 bp (16–61)
L. radiata	0.60% (0.00–1.66)	6.76% (5.37–8.66)	4.91% (3.43–5.71)
	4.30 bp (0–12)	41.49 bp (21–61)	32.52 bp (13–39)
L. siliquoidea	0.31% (0.00–0.96)	7.43% (5.34–8.86)	0.31% (0.00–0.96)
	2.06 bp (0–7)	47.68 bp (16–61)	2.06 bp (0–7)
L. straminea	11.70% (11.70–11.70)	6.95% (1.26–12.30)	10.12% (7.13–12.39)
	57 bp (57 bp)	38.17 bp (3–66)	56.12 bp (25–72 bp)
L. virescens	n/a	5.62% (4.17–6.76)	5.55% (5.10–6.24)
	n/a	37.42 bp (16–42)	40.43 bp (22–44)
	Illinois intraspecific	Topotype *hydiana*	Other conspecific specimens
L. hydiana Illinois	0.12% (0.00–1.45)	0.20% (0.00–1.45)	0.87% (0.00–2.36)
	0.98 bp (0–11)	1.63 bp (0–11)	5.14 bp (0–15 bp)
L. siliquoidea Illinois	0.20% (0.00–0.84)	n/a	0.43% (0.00–0.96)
	1.65 bp (0–7)	n/a	2.65 bp (0–7)

n/a = not applicable (either only a single sequence was available or irrelevant [*Lampsilis siliquoidea* were not compared with topotypic *Lampsilis hydiana* separately from other *hydiana*]). Number of base-pair differences is affected by including short published sequences.

we obviously did not find that to be the case for all the individuals analyzed. At sites where both *L. hydiana* and *L. siliquoidea* genotypes were present, we were unable to separate these individuals using only shell morphology (Fig. 2c, 2d). A more detailed morphological analysis may reveal additional characters that we did not consider, such as quantifying height to length ratio or measuring shell thickness (Keogh and Simons 2019). We recognize that our study's

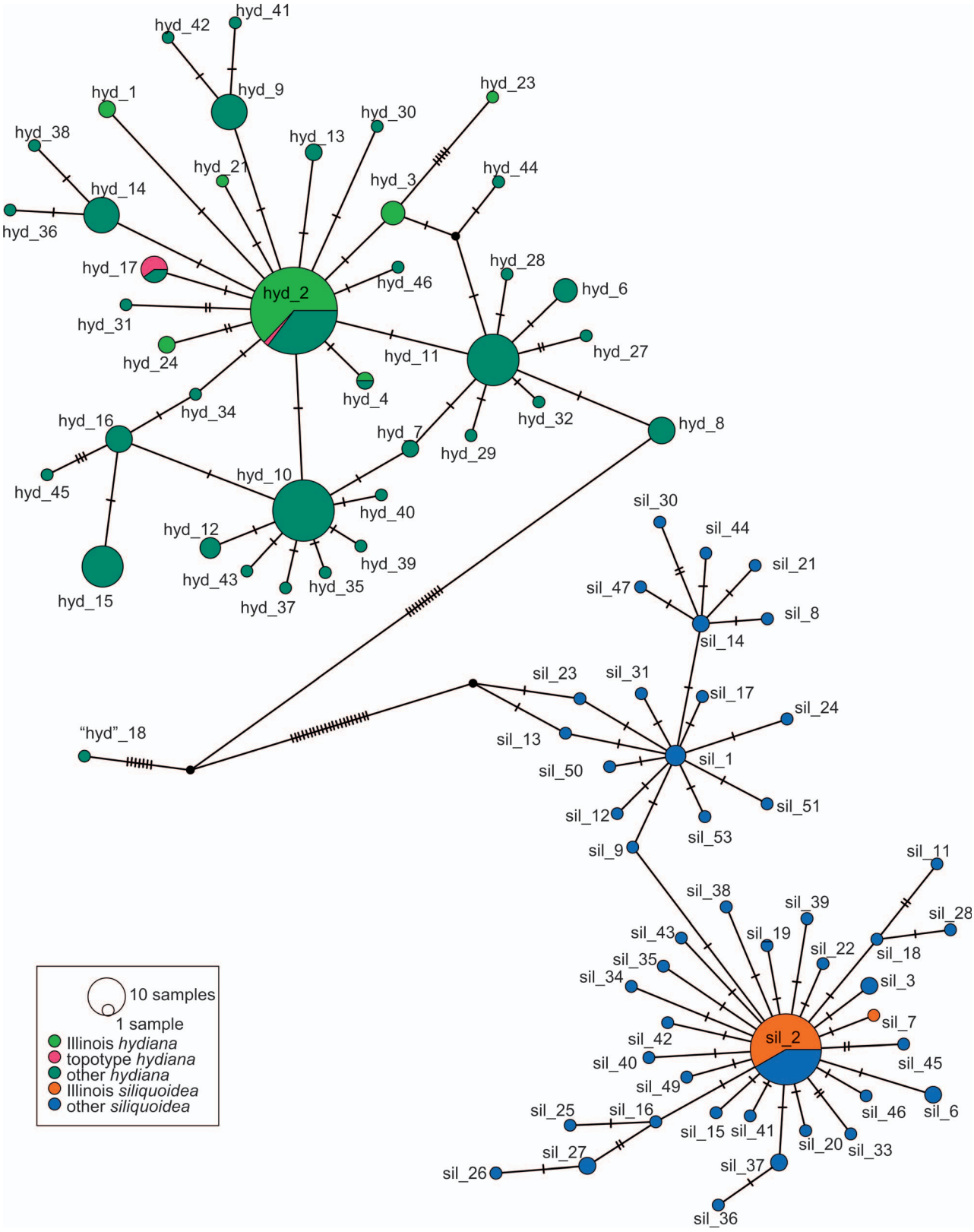

Figure 5. Haplotype network for *cox1* data. Numbers are the haplotype number (Appendices 1, 2). Bars on connecting lines indicate the number of base-pair differences between specimens; size of circles indicates the number of individuals with that haplotype.

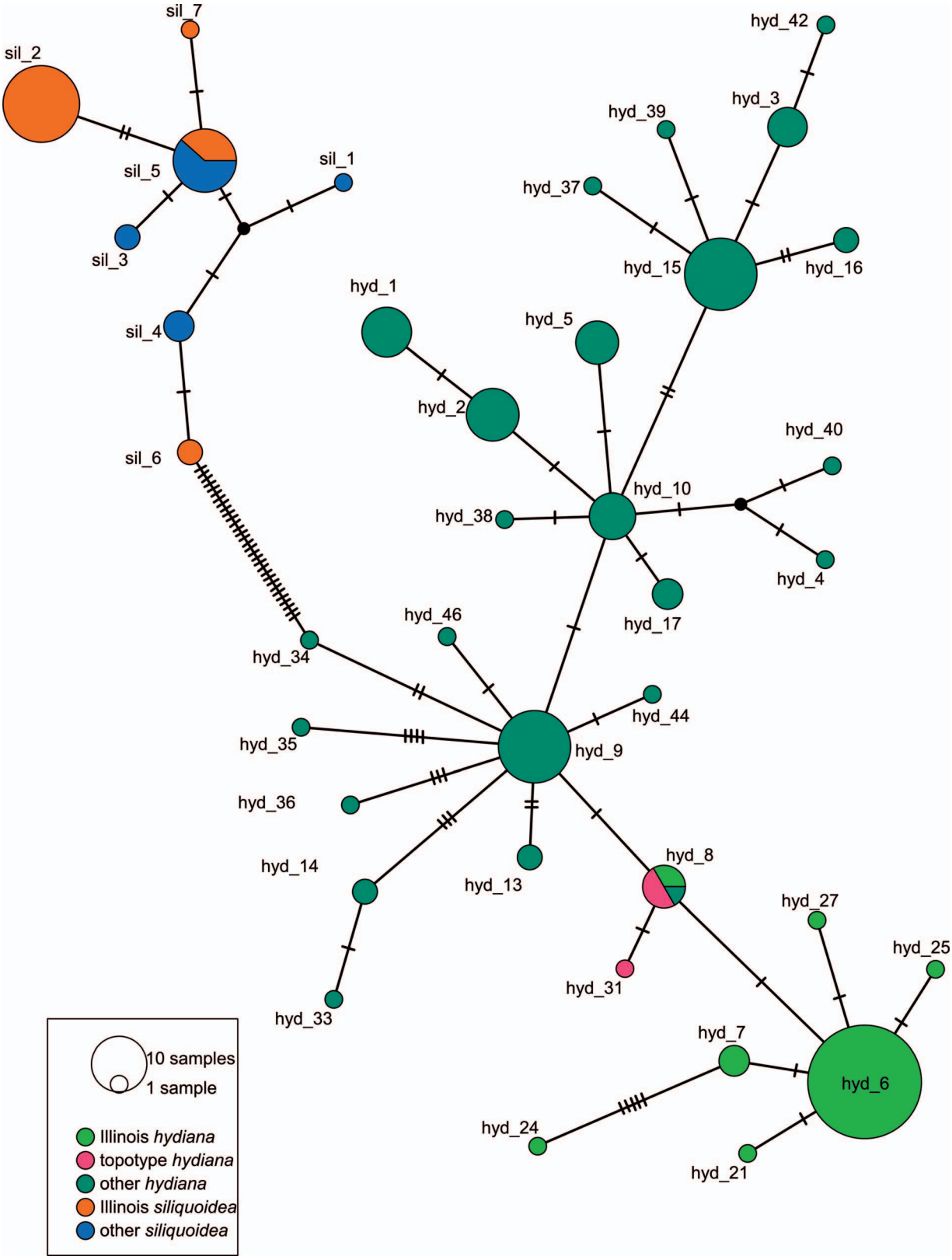

Figure 6. Haplotype network for *nad1* data. Numbers are the haplotype number (Appendices 1, 2). Bars on connecting lines indicate the number of base-pair differences between specimens; size of circles indicates the number of individuals with that haplotype.

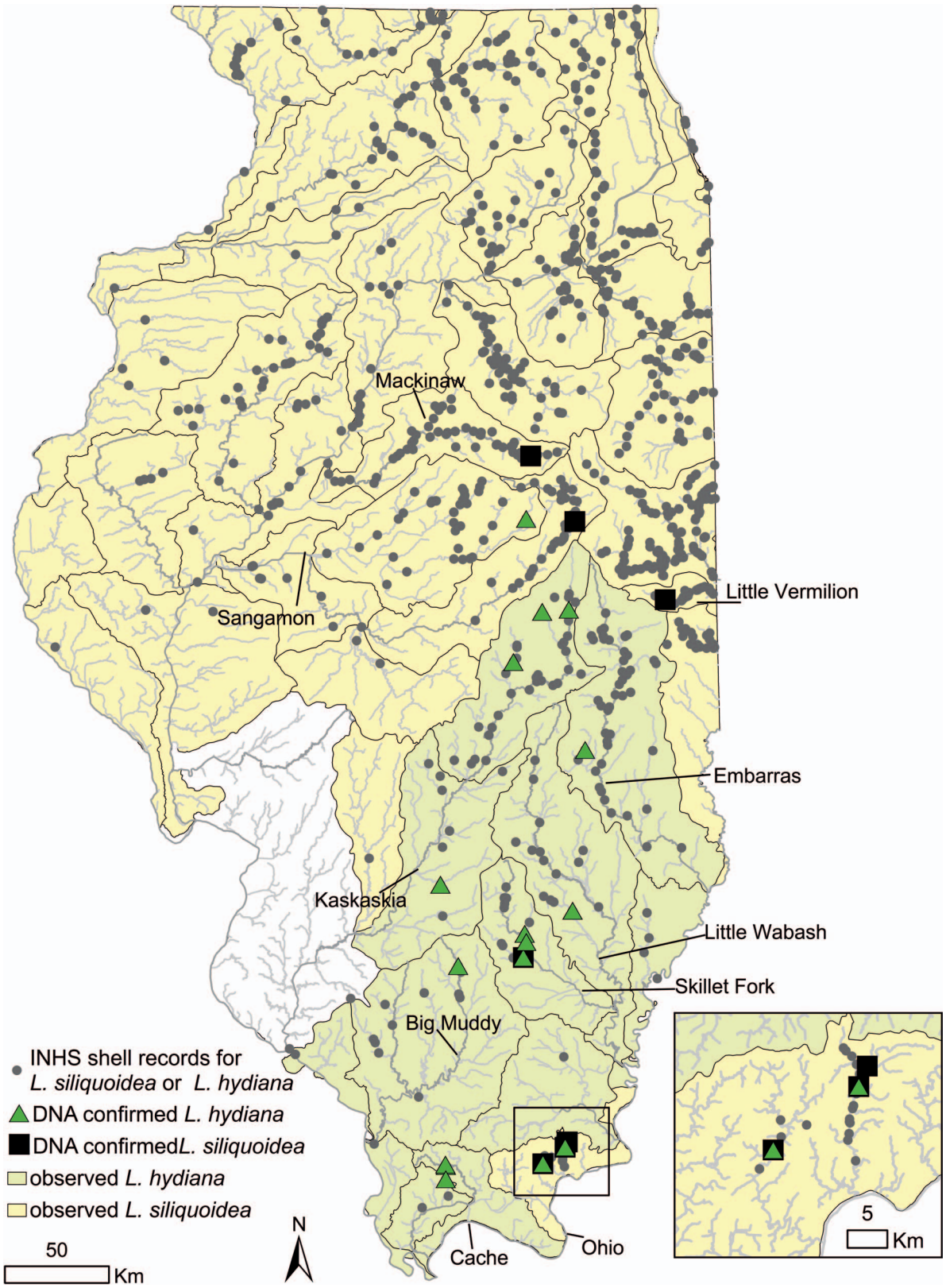

Figure 7. Locations of all shell records observed for *Lampsilis hydiana* and *Lampsilis siliquoidea* from Illinois in the INHS Mollusk Collection, Champaign, Illinois (gray closed circles), with genetic confirmation of *L. hydiana* (green triangles) and *L. siliquoidea* (black squares) plotted within each watershed, with pertinent rivers labeled. Watershed shading indicates species assignments based on observed external shell morphology prior to genetic analysis; green shading = shell characters match *L. hydiana* and yellow shading = shell characters match *L. siliquoidea*.

small sample size limits our understanding of the overall extent of *L. hydiana* in Illinois. Likewise, mitochondrial introgression, selective pressures, or incomplete lineage sorting (Doucet-Beaupré et al. 2012; Chong et al. 2016) could have produced anomalous genetic patterns. Additional nuclear molecular markers and a more detailed morphometric analysis of these populations may provide a clearer picture of relationships of *Lampsilis* populations in Illinois (Graf and Cummings 2006; Bogan and Roe 2008; Chong et al. 2016).

Our results suggest that *L. siliquoidea* and *L. hydiana* are closely related to each other but are not sister taxa. The sister taxon relationship between *L. siliquoidea* and *L. radiata* fits with previous classifications, as *L. siliquoidea* has been treated as a subspecies of *L. radiata* (Watters et al. 2009). Relationships between other members of the *siliquoidea* clade have not been discussed in detail, particularly as *L. sietmani* and *L. bergmanni* were described very recently. However, a relationship between *L. straminea*, *L. bergmanni*, and *L. hydiana* would not be surprising on biogeographic grounds, as their ranges adjoin each other.

Our discovery of both *L. hydiana* and *L. siliquoidea* in Illinois highlights the possibility of overlooked diversity elsewhere. Previous studies found some specimens identified as *L. hydiana* from the Arkansas and Red River systems in Arkansas were genetically distinct from topotypic *L. hydiana* (Turner et al. 2000; Lewter et al. 2003; Harris et al. 2004). A *cox1* sequence from one of those populations (Chapman et al. 2008; GenBank accession number EF033270) was divergent from true *L. hydiana* (Keogh and Simons 2019 and present analyses). Similarly, sequences in GenBank identified as *L. powellii* (from Breton et al. 2011 and Robicheau et al. 2018; GenBank accession numbers HM849075 and HM849218) matched topotypic *L. hydiana* (Walters et al. 2021 and present analyses). However, Harris et al. (2004) and Walters et al. (2021) found their sequences for *L. powellii* were closest to *L. siliquoidea*. *Lampsilis straminea* is reported to range from eastern Louisiana to central Florida, but data for *cox1* separated specimens from the Escambia drainage versus those from the Mobile basin; no other populations have been analyzed genetically. Thus, further analyses of the *siliquoidea* clade are likely to reveal additional new records. The recent descriptions of *L. sietmani* and *L. bergmanni* highlight the possibility of additional undescribed or incorrectly synonymized species in this group (Inoue et al. 2020; Keogh and Simons 2020).

Our analysis provides additional support showing that the *siliquoidea* clade is one of several distinct groups currently assigned to the genus *Lampsilis*, even though species in this clade are morphologically and genetically distinct from the type of the genus, Pocketbook *Lampsilis ovata* (Say, 1817). Other species seem to be genetically divergent from both the *siliquoidea* clade and from type *Lampsilis*, including Texas Fatmucket *Lampsilis bracteata* (Gould, 1855) (Harris et al. 2004; Porto-Hannes et al. 2019; Inoue et al. 2020), the cryptic *Lampsilis* sp. of McCartney et al. (2016), and the clade of Northern Brokenray *Lampsilis brittsi* Simpson, 1900, Arkansas Brokenray *Lampsilis reeveiana* (Lea, 1852), and Speckled Pocketbook *Lampsilis streckeri* Frierson, 1927 (Harris et al. 2004). One other species recognized in *Lampsilis*, Neosho Mucket *Lampsilis rafinesqueana* Frierson, 1927, has not yet been analyzed genetically but has an unusual combination of anatomical and shell features (Harris et al. 2004). As Keogh and Simons (2019) pointed out, a thorough analysis of Lampsilini will be necessary to determine the correct placement of these taxa.

Accurate species delineation is critical to developing sound conservation strategies for freshwater mussels, particularly because many species of conservation concern are managed or closely monitored at the state level. At press time, three *Lampsilis* species are endangered in Illinois: *L. abrupta* and *L. higginsii* are federally protected, while Wavyrayed Lampmussel *Lampsilis fasciola* Rafinesque, 1820 is listed only at the state level. Other common, widespread *Lampsilis* species, such as Plain Pocketbook *Lampsilis cardium* (Rafinesque, 1820) and *L. siliquoidea*, are often used by local and state authorities for propagation and augmentation following habitat restoration efforts. Our analysis emphasizes the need for managers to follow best practices during augmentation and reintroduction activities to avoid cross-basin contamination, as hidden diversity may be present even in common, presumably well-understood species (McMurray and Roe 2017; Inoue et al. 2020).

ACKNOWLEDGMENTS

Funding was provided by the US Fish and Wildlife Service–State Wildlife Grant program to the Illinois Department of Natural Resources and the Illinois Natural History Survey. A special thanks to Diane Shasteen, Ann Marie Holtrop, and Robert Szafoni for project support, Rachel Vinsel for collection and curatorial support, Jen Mui for graphic assistance, and many field assistants. Thanks also to Dr. Andrea Porras-Alfaro and Phil Scheibel for help with bioinformatics. Todd Slack (USACOE ERDC) collected the topotypic *L. hydiana* specimens. TNT is made available with the sponsorship of the Willi Hennig Society.

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Appendix 1. Illinois specimens and sequences used in analysis, haplotype number for reference to Figures 4–6, GenBank accession number, INHS catalog number, approximate waterbody location, and our preliminary putative identification based on external shell characteristics.

Species	Gene	Haplotype	GenBank accession no.	INHS catalog no.	Waterbody	Putative species
L. hydiana	*nad1*	22	*MT537714	INHS 35065-1	Cache River	*L. hydiana*
L. hydiana	*cox1*	5	*MT537719	INHS 35065-3	Cache River	*L. hydiana*
L. hydiana	*nad1*	23	*MT537715	INHS 35065-3	Cache River	*L. hydiana*
L. hydiana	*nad1*	24	*MT537716	INHS 35065-4	Cache River	*L. hydiana*
L. hydiana	*cox1*	5	*MT537720	INHS 35065-4	Cache River	*L. hydiana*
L. hydiana	*cox1*	21	*MT537721	INHS 39742-1	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	3	*MT537717	INHS 39742-4	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MT537705	INHS 39742-4	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	3	*MT537718	INHS 39742-5	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	21	*MT537706	INHS 39742-5	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560721	INHS 86789-1	Big Grande Pierre Creek	*L. siliquoidea*
L. hydiana	*nad1*	25	*MH588328	INHS 86789-1	Big Grande Pierre Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560723	INHS 86789-2	Big Grande Pierre Creek	*L. siliquoidea*
L. hydiana	*nad1*	20	*MH588329	INHS 86789-2	Big Grande Pierre Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560732	INHS 45495-1	Big Muddy River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588336	INHS 45495-1	Big Muddy River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560715	INHS 45495-2	Big Muddy River	*L. hydiana*
L. hydiana	*nad1*	6	*MH588324	INHS 45495-2	Big Muddy River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560716	INHS 45495-3	Big Muddy River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588325	INHS 45495-3	Big Muddy River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588326	INHS 45495-4	Big Muddy River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560717	INHS 45495-5	Big Muddy River	*L. hydiana*
L. hydiana	*nad1*	6	*MH588327	INHS 45495-5	Big Muddy River	*L. hydiana*
L. hydiana	*cox1*	4	*MH560739	INHS 45455-1	Bradshaw Creek	*L. hydiana*
L. hydiana	*nad1*	8	*MH588344	INHS 45455-1	Bradshaw Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588345	INHS 45455-2	Bradshaw Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588346	INHS 45455-3	Bradshaw Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560740	INHS 45460-1	Brush Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588347	INHS 45460-1	Brush Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560741	INHS 45460-2	Brush Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588348	INHS 45460-2	Brush Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560742	INHS 45460-3	Brush Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588349	INHS 45460-3	Brush Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560743	INHS 45460-4	Brush Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588350	INHS 45460-4	Brush Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560744	INHS 45460-5	Brush Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588351	INHS 45460-5	Brush Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560751	INHS 45482-1	Cypress Creek	*L. hydiana*
L. hydiana	*nad1*	7	*MH588361	INHS 45482-1	Cypress Creek	*L. hydiana*
L. hydiana	*cox1*	25	*MH560752	INHS 45482-2	Cypress Creek	*L. hydiana*
L. hydiana	*nad1*	29	*MH588362	INHS 45482-2	Cypress Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560753	INHS 45490-1	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588363	INHS 45490-1	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	3	*MH560754	INHS 45490-2	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588364	INHS 45490-2	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560755	INHS 45490-3	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588365	INHS 45490-3	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588366	INHS 45490-4	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588367	INHS 45490-5	East Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560713	INHS 45491-1	Elm River	*L. hydiana*

Appendix 1, continued.

Species	Gene	Haplotype	GenBank accession no.	INHS catalog no.	Waterbody	Putative species
L. hydiana	*nad1*	20	*MH588322	INHS 45491-1	Elm River	*L. hydiana*
L. hydiana	*cox1*	1	*MH560714	INHS 45491-2	Elm River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588323	INHS 45491-2	Elm River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588352	INHS 45462-1	Horse Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560745	INHS 45462-2	Horse Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588353	INHS 45462-2	Horse Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560746	INHS 45462-3	Horse Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588354	INHS 45462-3	Horse Creek	*L. hydiana*
L. hydiana	*cox1*	24	*MH560747	INHS 45462-4	Horse Creek	*L. hydiana*
L. hydiana	*nad1*	20	*MH588355	INHS 45462-4	Horse Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560735	INHS 45449-1	Lake Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588339	INHS 45449-1	Lake Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	1	*MH560736	INHS 45449-3	Lake Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	8	*MH588340	INHS 45449-3	Lake Fork Kaskaskia River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588341	INHS 45449-4	Lake Fork Kaskaskia River	*L. hydiana*
L. hydiana	*cox1*	5	*MH560724	INHS 86787-1	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	30	*MH588376	INHS 86787-1	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560725	INHS 86787-2	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	20	*MH588330	INHS 86787-2	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560727	INHS 86787-3	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	7	*MH588331	INHS 86787-3	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560728	INHS 86787-4	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	26	*MH588332	INHS 86787-4	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560729	INHS 86787-5	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	7	*MH588333	INHS 86787-5	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560730	INHS 86787-6	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	20	*MH588334	INHS 86787-6	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560731	INHS 86787-7	Lusk Creek	*L. siliquoidea*
L. hydiana	*nad1*	20	*MH588335	INHS 86787-7	Lusk Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560718	INHS 22361	Muddy Creek	*L. hydiana*
L. hydiana	*cox1*	5	*MH560719	INHS 35459	Salt Creek	*L. siliquoidea*
L. hydiana	*cox1*	5	*MH560748	INHS 45463-1	Skillet Fork	*L. hydiana*
L. hydiana	*nad1*	20	*MH588356	INHS 45463-1	Skillet Fork	*L. hydiana*
L. hydiana	*nad1*	20	*MH588357	INHS 45463-2	Skillet Fork	*L. hydiana*
L. hydiana	*cox1*	5	*MH560749	INHS 45463-3	Skillet Fork	*L. hydiana*
L. hydiana	*nad1*	20	*MH588358	INHS 45463-3	Skillet Fork	*L. hydiana*
L. hydiana	*nad1*	20	*MH588359	INHS 45463-4	Skillet Fork	*L. hydiana*
L. hydiana	*cox1*	2	*MH560750	INHS 45463-5	Skillet Fork	*L. hydiana*
L. hydiana	*nad1*	28	*MH588360	INHS 45463-5	Skillet Fork	*L. hydiana*
L. hydiana	*cox1*	5	*MH560737	INHS 45453-3	Twomile Slough	*L. hydiana*
L. hydiana	*nad1*	20	*MH588342	INHS 45453-3	Twomile Slough	*L. hydiana*
L. hydiana	*cox1*	5	*MH560738	INHS 45453-4	Twomile Slough	*L. hydiana*
L. hydiana	*nad1*	20	*MH588343	INHS 45453-4	Twomile Slough	*L. hydiana*
L. hydiana	*cox1*	23	*MH560734	INHS 45443	West Okaw River	*L. hydiana*
L. hydiana	*nad1*	27	*MH588338	INHS 45443	West Okaw River	*L. hydiana*
L. hydiana	*cox1*	3	*MH560733	INHS 45447	West Okaw River	*L. hydiana*
L. hydiana	*nad1*	20	*MH588337	INHS 45447	West Okaw River	*L. hydiana*
L. siliquoidea	*nad1*	6	*MT537712	INHS 35786-1	Little Vermilion River	*L. siliquoidea*
L. siliquoidea	*nad1*	6	*MT537713	INHS 35786-4	Little Vermilion River	*L. siliquoidea*
L. siliquoidea	*nad1*	5	*MT537707	INHS 41996-1	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MT537722	INHS 41996-2	Mackinaw River	*L. siliquoidea*

Appendix 1, continued.

Species	Gene	Haplotype	GenBank accession no.	INHS catalog no.	Waterbody	Putative species
L. siliquoidea	*nad1*	5	*MT537708	INHS 41996-2	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MT537723	INHS 41996-3	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*nad1*	5	*MT537709	INHS 41996-3	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MT537724	INHS 41996-4	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*nad1*	7	*MT537710	INHS 41996-4	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*cox1*	7	*MT537725	INHS 41996-5	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*nad1*	5	*MT537711	INHS 41996-5	Mackinaw River	*L. siliquoidea*
L. siliquoidea	*nad1*	5	*MH588388	INHS 35558	Big Ditch	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560756	INHS 45613-1	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588368	INHS 45613-1	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588375	INHS 45613-10	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560757	INHS 45613-3	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588369	INHS 45613-3	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560758	INHS 45613-4	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588370	INHS 45613-4	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560759	INHS 45613-5	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588371	INHS 45613-5	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560760	INHS 45613-6	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588372	INHS 45613-6	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560761	INHS 45613-8	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588373	INHS 45613-8	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560762	INHS 45613-9	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588374	INHS 45613-9	Big Grande Pierre Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588386	INHS 86788	Horse Creek	*L. hydiana*
L. siliquoidea	*cox1*	5	*MH560769	INHS 45615-10	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588382	INHS 45615-10	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560770	INHS 45615-12	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588383	INHS 45615-12	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560771	INHS 45615-13	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588384	INHS 45615-13	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560772	INHS 45615-18	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588385	INHS 45615-18	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560773	INHS 45615-19	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560764	INHS 45615-2	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588377	INHS 45615-2	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560765	INHS 45615-3	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588378	INHS 45615-3	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560766	INHS 45615-4	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588379	INHS 45615-4	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560767	INHS 45615-6	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588380	INHS 45615-6	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560768	INHS 45615-9	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588381	INHS 45615-9	Lusk Creek	*L. siliquoidea*
L. siliquoidea	*cox1*	5	*MH560774	INHS 45471-1	Rose Creek	*L. siliquoidea*
L. siliquoidea	*nad1*	2	*MH588387	INHS 45471-1	Rose Creek	*L. siliquoidea*

*Newly generated sequences.

Appendix 2. Additional sequences used in analysis, with haplotype reference number (see Figs. 4–6), GenBank accession number, specimen identification, and approximate waterbody location.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. abrupta	*cox1*		*MH560776	UAUC3531	Tennessee River, Diamond Island	New
L. bergmanni	*nad1*	2	MK672463	UGUA01	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672464	UGUA02	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672465	UGUA03	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672466	UGUA04	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672467	UGUA05	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672468	UGUA07	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672469	UGUA08	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672470	UGUA09	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672471	UGUA10	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672472	UGUA11	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672473	UGUA12	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672474	UGUA13	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672475	UGUA14	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672476	UGUA15	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672477	UGUA16	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672478	UGUA17	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672479	UGUA18	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672480	UGUA19	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672481	UGUA20	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	3	MK672482	UGUA21	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672483	UGUA22	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672484	UGUA23	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672485	UGUA24	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672486	UGUA25	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672487	UGUA26	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	3	MK672488	UGUA27	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672489	UGUA28	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672490	UGUA29	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	3	MK672491	UGUA30	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672492	UGUA31	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672493	UGUA32	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672494	UGUA33	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	3	MK672495	UGUA34	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672496	UGUA35	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	2	MK672497	UGUA36	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	1	MK672498	UGUA37	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	3	MK672499	UGUA39	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*nad1*	3	MK672500	UGUA40	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672718	UGUA01	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672719	UGUA02	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672720	UGUA03	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672721	UGUA04	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672722	UGUA05	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672723	UGUA07	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672724	UGUA08	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672725	UGUA09	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672726	UGUA10	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672727	UGUA11	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672728	UGUA12	Guadalupe River drainage	Inoue et al. 2020

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. bergmanni	*cox1*	1	MK672729	UGUA13	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672730	UGUA14	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672731	UGUA15	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672732	UGUA16	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672733	UGUA17	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672734	UGUA18	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672735	UGUA19	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672736	UGUA20	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	2	MK672737	UGUA21	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672738	UGUA22	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672739	UGUA23	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672740	UGUA24	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672741	UGUA25	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672742	UGUA26	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	2	MK672743	UGUA27	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672744	UGUA28	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672745	UGUA29	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	2	MK672746	UGUA30	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672747	UGUA31	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672748	UGUA32	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672749	UGUA33	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	2	MK672750	UGUA34	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672751	UGUA35	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672752	UGUA36	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	1	MK672753	UGUA37	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	2	MK672754	UGUA39	Guadalupe River drainage	Inoue et al. 2020
L. bergmanni	*cox1*	2	MK672755	UGUA40	Guadalupe River drainage	Inoue et al. 2020
L. higginsii	*nad1*		EF213061		Upper Mississippi drainage	Zanatta and Murphy 2006 Unpublished
L. higginsii	*cox1*		GU085287	1	Upper Mississippi drainage	Boyer et al. 2011
L. "*hydiana*"	*cox1*	18	EF033270	H1230	Cossatot River, Red River, Arkansas	Chapman et al. 2008
L. hydiana	*cox1*	17	*MH560720	INHS 87783-2	Boeuf River	New
L. hydiana	*nad1*	31	*MH588389	INHS 87783-1	Boeuf River	New
L. hydiana	*nad1*	8	*MH588390	INHS 87783-2	Boeuf River	New
L. hydiana	*nad1*	8	*MH588391	INHS 87783-3	Boeuf River	New
L. hydiana	*nad1*	8	*MH588392	INHS 87783-4	Boeuf River	New
L. hydiana	*nad1*	12	MK672437	BRA01	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	9	MK672438	BRA02	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672439	BRA03	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	9	MK672440	BRA04	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672441	BRA05	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672442	BRA06	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672443	BRA07	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	9	MK672444	BRA08	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672445	BRA09	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	46	MK672446	BRA10	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	9	MK672447	BRA11	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672448	BRA12	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672449	BRA13	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672450	BRA14	Brazos River drainage	Inoue et al. 2020

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. hydiana	*nad1*	12	MK672451	BRA15	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672685	BRA01	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	8	MK672686	BRA02	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	6	MK672687	BRA03	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672688	BRA04	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	7	MK672689	BRA05	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	8	MK672690	BRA06	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672691	BRA07	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	6	MK672692	BRA08	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	8	MK672693	BRA09	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672694	BRA10	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672695	BRA11	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	6	MK672696	BRA12	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	8	MK672697	BRA13	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	8	MK672698	BRA14	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	6	MK672699	BRA15	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672700	BRA16	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672701	BRA17	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672702	BRA18	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672703	BRA19	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672704	BRA20	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672705	BRA21	Brazos River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672706	BRA22	Brazos River drainage	Inoue et al. 2020
L. hydiana	*nad1*	37	MK672388	CAL01	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	11	MK672389	CAL02	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	38	MK672390	CAL08	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	10	MK672391	CAL09	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	11	MK672392	CAL15	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	11	MK672393	CAL16	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	11	MK672394	CAL19	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*nad1*	11	MK672395	CAL20	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	33	MK672611	CAL01	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672612	CAL02	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672613	CAL08	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672614	CAL09	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672615	CAL15	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672616	CAL16	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672617	CAL19	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672618	CAL20	Calcasieu River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK226685	012TS	Guadalupe River drainage	Porto-Hannes et al. 2019
L. hydiana	*cox1*	16	MK226686	013TS	Guadalupe River drainage	Porto-Hannes et al. 2019
L. hydiana	*cox1*	16	MK226687	016TS	Guadalupe River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	2	MK226704	016TS	Guadalupe River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	1	MK226709	012TS	Guadalupe River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	1	MK672452	GUA06	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*nad1*	1	MK672453	GUA16	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*nad1*	1	MK672454	GUA17	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*nad1*	1	MK672455	GUA18	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*nad1*	1	MK672456	GUA19	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672707	GUA06	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672708	GUA16	Guadalupe River drainage	Inoue et al. 2020

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. hydiana	*cox1*	15	MK672709	GUA17	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672710	GUA18	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672711	GUA19	Guadalupe River drainage	Inoue et al. 2020
L. hydiana	*nad1*	3	MG030352		Neches River drainage	Marshall et al. 2018
L. hydiana	*cox1*	9	MK226688	138TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*cox1*	10	MK226689	159TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*cox1*	10	MK226690	200TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*cox1*	10	MK226691	214TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	5	MK226705	214TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	47	MK226706	159TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	4	MK226707	200TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	3	MK226708	138TS	Neches River drainage	Porto-Hannes et al. 2019
L. hydiana	*nad1*	3	MK672411	NEC10	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	5	MK672412	NEC11	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672413	NEC12	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	17	MK672414	NEC13	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	40	MK672415	NEC14	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	10	MK672416	NEC15	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	5	MK672417	NEC16	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	12	MK672418	NEC17	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	5	MK672419	NEC18	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	5	MK672420	NEC19	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	42	MK672421	NEC20	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	5	MK672422	NEC21	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	43	MK672423	NEC22	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	44	MK672424	NEC23	Neches River drainage	Inoue et al. 2020
L. hydiana	*nad1*	3	MK672425	NEC24	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	12	MK672641	NEC01	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672642	NEC03	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672643	NEC06	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672644	NEC07	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672645	NEC09	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672646	NEC10	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	37	MK672647	NEC11	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	38	MK672648	NEC12	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	12	MK672649	NEC13	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	39	MK672650	NEC14	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	40	MK672651	NEC15	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672652	NEC16	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672653	NEC17	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672654	NEC18	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672655	NEC19	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672656	NEC20	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672657	NEC21	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672658	NEC22	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	41	MK672659	NEC23	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672660	NEC24	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	42	MK672661	NEC25	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672662	NEC26	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672663	NEC27	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672664	NEC29	Neches River drainage	Inoue et al. 2020

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. hydiana	*cox1*	43	MK672665	NEC30	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672666	NEC31	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672667	NEC32	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672668	NEC33	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672669	NEC34	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672670	NEC35	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672671	NEC37	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672672	NEC38	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	9	MK672673	NEC40	Neches River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK391871	JFBM22432 1	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*cox1*	5	MK391872	JFBM22432 2	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*cox1*	5	MK391873	JFBM22432 3	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*cox1*	5	MK391874	JFBM22432 4	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*cox1*	4	MK391875	JFBM22432 5	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*cox1*	5	MK391876	JFBM22432 6	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*cox1*	5	MK391877	JFBM22432 7	Ohio River drainage	Keogh and Simons 2019
L. hydiana	*nad1*	9	MK672379	OUA01	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*nad1*	13	MK672380	OUA02	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*nad1*	13	MK672381	OUA03	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	17	MK672596	OUA01	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672597	OUA02	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	27	MK672598	OUA03	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672599	OUA04 IF01	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672600	OUA05 IF02	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	7	MK672601	OUA06 VL01	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672602	OUA07 VL02	Ouachita River drainage	Inoue et al. 2020
L. hydiana	*nad1*	33	MK672382	RED04	Red River drainage	Inoue et al. 2020
L. hydiana	*nad1*	34	MK672383	RED06	Red River drainage	Inoue et al. 2020
L. hydiana	*nad1*	35	MK672384	RED07	Red River drainage	Inoue et al. 2020
L. hydiana	*nad1*	36	MK672385	RED08	Red River drainage	Inoue et al. 2020
L. hydiana	*nad1*	14	MK672386	RED09	Red River drainage	Inoue et al. 2020
L. hydiana	*nad1*	14	MK672387	RED10	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	28	MK672603	RED02	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672604	RED03	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	29	MK672605	RED04	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	30	MK672606	RED06	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	31	MK672607	RED07	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	32	MK672608	RED08	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672609	RED09	Red River drainage	Inoue et al. 2020
L. hydiana	*cox1*	11	MK672610	RED10	Red River drainage	Inoue et al. 2020
L. hydiana	*nad1*	17	MK672396	SAB01	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672397	SAB02	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	15	MK672398	SAB03	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672399	SAB04	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672400	SAB05	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	16	MK672401	SAB06	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	16	MK672402	SAB07	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672403	SAB08	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	39	MK672404	SAB09	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672405	SAB10	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	17	MK672406	SAB11	Sabine River drainage	Inoue et al. 2020

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. hydiana	*nad1*	18	MK672407	SAB12	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672408	SAB13	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672409	SAB14	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	18	MK672410	SAB15	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	12	MK672619	SAB01	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672620	SAB02	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672621	SAB03	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672622	SAB04	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	34	MK672623	SAB05	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	13	MK672624	SAB06	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	13	MK672625	SAB07	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672626	SAB08	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672627	SAB09	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672628	SAB10	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672629	SAB11	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672630	SAB12	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672631	SAB13	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672632	SAB14	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672633	SAB15	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	2	MK672634	SAB16	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	10	MK672635	SAB17	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	35	MK672636	SAB18	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	36	MK672637	SAB19	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672638	SAB23	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	14	MK672639	SAB24	Sabine River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672640	SAB25	Sabine River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672457	SAN01	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*nad1*	1	MK672458	SAN02	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672459	SAN03	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672460	SAN04	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*nad1*	1	MK672461	SAN05	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672462	SAN06	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672712	SAN01	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672713	SAN02	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672714	SAN03	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672715	SAN04	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672716	SAN05	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*cox1*	15	MK672717	SAN06	San Antonio River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672428	SJC01	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	19	MK672429	SJC05	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	19	MK672430	SJC06	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672431	SJC07	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	19	MK672432	SJC08	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	19	MK672433	SJC09	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	19	MK672434	SJC10	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	15	MK672435	SJC11	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672436	SJC12	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	16	MK672676	SJC01	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672677	SJC05	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672678	SJC06	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	16	MK672679	SJC07	San Jacinto River drainage	Inoue et al. 2020

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Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. hydiana	*cox1*	46	MK672680	SJC08	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672681	SJC09	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672682	SJC10	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	5	MK672683	SJC11	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*cox1*	16	MK672684	SJC12	San Jacinto River drainage	Inoue et al. 2020
L. hydiana	*nad1*	45	MK672426	TRI05	Trinity River drainage	Inoue et al. 2020
L. hydiana	*nad1*	2	MK672427	TRI06	Trinity River drainage	Inoue et al. 2020
L. hydiana	*cox1*	44	MK672674	TRI05	Trinity River drainage	Inoue et al. 2020
L. hydiana	*cox1*	45	MK672675	TRI06	Trinity River drainage	Inoue et al. 2020
L. hydiana	*cox1*	17	*MH560712	INHS 87783-1	Boeuf River	New
L. hydiana	*cox1*	17	*MH560722	INHS 87783-3	Boeuf River	New
L. hydiana	*cox1*	5	*MH560726	INHS 87783-4	Boeuf River	New
L. hydiana	*cox1*	14	MH161354	UAUC3508	Neches River	Burlakova et al. 2019
L. "*powellii*"	*cox1*	17	MF326971	H2610	Ouachita River drainage	Robicheau et al. 2018
L. "*powellii*"	*nad1*	8	MF326971	H2610	Ouachita River drainage	Robicheau et al. 2018
L. "*powellii*"	*cox1*	17	HM849075	H2610	Ouachita River drainage	Breton et al. 2011
L. "*powellii*"	*nad1*	8	HM849218	H2610	Ouachita River drainage	Breton et al. 2011
L. radiata	*cox1*	3	MK226692	2	Hudson River	Porto-Hannes et al. 2019
L. radiata	*cox1*	3	MN432619	mH34	Hudson River drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	6	MN432616	mH31	Lake Ontario drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	1	HQ153594	COX67	Lake Waccamaw	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153595	COX68	Lake Waccamaw	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153596	COX69	Lake Waccamaw	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153597	COX70	Lake Waccamaw	McCartney et al. 2016
L. radiata	*cox1*	4	HQ153598	COX71	Lake Waccamaw	McCartney et al. 2016
L. radiata	*nad1*	2	HQ153676	NAD55	Lake Waccamaw	McCartney et al. 2016
L. radiata	*nad1*	3	HQ153677	NAD56	Lake Waccamaw	McCartney et al. 2016
L. radiata	*nad1*	3	HQ153678	NAD57	Lake Waccamaw	McCartney et al. 2016
L. radiata	*nad1*	3	HQ153679	NAD58	Lake Waccamaw	McCartney et al. 2016
L. radiata	*nad1*	3	HQ153680	NAD59	Lake Waccamaw	McCartney et al. 2016
L. radiata	*cox1*	19	MN432650	mH65	Potomac drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	20	MN432651	mH66	Potomac drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	7	MN432620	mH35	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	8	MN432621	mH36	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	9	MN432623	mH38	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	10	MN432624	mH39	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	11	MN432629	mH44	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	12	MN432631	mH46	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	13	MN432633	mH48	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	14	MN432634	mH49	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	15	MN432642	mH57	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	16	MN432644	mH59	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	17	MN432645	mH60	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*cox1*	18	MN432646	mH61	St. Lawrence drainage	Porto-Hannes et al. 2021
L. radiata	*nad1*	4	EF446098		Lake Erie drainage	Kneeland and Rhymer 2007
L. radiata	*cox1*	2	KC408769	H18	Lake Erie drainage	Krebs et al. 2013
L. radiata	*cox1*	21	KC408770	H19	Lake Erie drainage	Krebs et al. 2013
L. radiata	*cox1*	5	KC408771	H20	Lake Erie drainage	Krebs et al. 2013
L. radiata	*cox1*	1	HQ153599	COX72	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016

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Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. radiata	*cox1*	2	HQ153600	COX73	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153603	COX76	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153604	COX77	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153606	COX79	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153607	COX80	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153608	COX81	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153609	COX82	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*cox1*	1	HQ153610	COX83	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	1	HQ153681	NAD60	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	5	HQ153682	NAD61	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	3	HQ153685	NAD64	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	2	HQ153686	NAD65	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	1	HQ153688	NAD67	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	1	HQ153689	NAD68	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	2	HQ153690	NAD69	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	6	HQ153692	NAD71	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. radiata	*nad1*	3	HQ153693	NAD72	Waccamaw, Yadkin/Pee Dee, Lumber rivers	McCartney et al. 2016
L. sietmani	*cox1*	2	MK391838	TAMUNRI8052 2	Neches River	Keogh and Simons 2019
L. sietmani	*cox1*	2	MK391839	TAMUNRI8052 3	Neches River	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391843	JFBM22438 1	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391844	JFBM22438 2	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391845	JFBM22438 3	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391846	JFBM22438 4	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391847	JFBM22438 5	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391848	JFBM22438 6	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391849	JFBM22438 7	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391850	JFBM22433	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391851	INHS 27760	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391853	INHS 32502	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	3	MK391856	JFBM22439	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	4	MK391857	JFBM22439 photo	Upper Mississippi drainage	Keogh and Simons 2019
L. sietmani	*cox1*	1	MK391858	WI River photo	Upper Mississippi drainage	Keogh and Simons 2019
L. siliquoidea	*cox1*	49	MN432647	mH62	Great Lakes drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	50	MN432648	mH63	Great Lakes drainage	Porto-Hannes et al. 2021

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. siliquoidea	*cox1*	1	MH012239	Fatmucket1	Lake Erie drainage	Metzger et al. 2018
L. siliquoidea	*cox1*	38	MN432628	mH43	Lake Huron drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	6	MN432617	mH32	Lake Michigan drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	31	MN432614	mH29	Lake Ontario drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	35	MN432625	mH40	Lake Ontario drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	42	MN432636	mH51	Lake Ontario drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	44	MN432638	mH53	Lake Ontario drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	45	MN432639	mH54	Lake St. Clair	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	51	MN432649	mH64	Lake St. Clair drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	43	MN432637	mH52	Lake Winnipeg drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	52	MN432652	mH67	Little Vermilion River, Illinois	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	53	MN432653	mH68	Little Vermilion River, Illinois	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	32	MN432615	mH30	Meramec drainage	Porto-Hannes et al. 2021
L. siliquoidea	*nad1*	5	MK672508	MS01	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	5	MK672509	MS02	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	5	MK672510	MS03	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	5	MK672511	MS04	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	3	MK672512	MS05	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	5	MK672513	MS06	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	3	MK672514	MS07	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*nad1*	5	MK672515	MS08	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672774	MS01	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672775	MS02	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672776	MS03	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672777	MS04	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672778	MS05	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672779	MS06	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672780	MS07	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	5	MK672781	MS08	Mississippi drainage	Inoue et al. 2020
L. siliquoidea	*cox1*	36	MN432626	mH41	Mississippi drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	37	MN432627	mH42	Mississippi drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	34	MN432622	mH37	Nottaway drainage, Hudson Bay, Canada	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	3	MF326973	H2655	Red River drainage	Robicheau et al. 2018
L. siliquoidea	*nad1*	1	MF326973	H2655	Red River drainage	Robicheau et al. 2018
L. siliquoidea	*cox1*	41	MN432635	mH50	Rupert drainage, Hudson Bay, Canada	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	4	MK226693		St. Lawrence drainage	Porto-Hannes et al. 2019
L. siliquoidea	*nad1*	4	MK226710		St. Lawrence drainage	Porto-Hannes et al. 2019
L. siliquoidea	*cox1*	33	MN432618	mH33	St. Lawrence drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	39	MN432630	mH45	St. Lawrence drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	40	MN432632	mH47	St. Lawrence drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	46	MN432640	mH55	St. Lawrence drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	47	MN432641	mH56	St. Lawrence drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	48	MN432643	mH58	St. Lawrence drainage	Porto-Hannes et al. 2021
L. siliquoidea	*cox1*	2	MK391878	JFBM22440 1	Upper Mississippi drainage	Keogh and Simons 2019
L. siliquoidea	*cox1*	6	MK391879	JFBM22440 2	Upper Mississippi drainage	Keogh and Simons 2019
L. siliquoidea	*nad1*	4	AY094386	UAUC 882	Douglas Lake, Cheboygan County, Michigan	Buhay et al. 2002
L. siliquoidea	*cox1*	2	DQ494752	UAUC882	Douglas Lake, Cheboygan County, Michigan	Serb 2006

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. siliquoidea	*cox1*	1	AF156521	UMMZ 265709a	Huron River, Michigan	Graf and Ó Foighil 2000
L. siliquoidea	*cox1*	5	AF156522	UMMZ 265709b	Huron River, Michigan	Graf and Ó Foighil 2000
L. siliquoidea	*nad1*	4	AY158747	LSILIQ	Lake Erie drainage	Serb et al. 2003
L. siliquoidea	*cox1*	8	KC408744	H1	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	4	KC408745	H2	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	9	KC408746	H3	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	10	KC408747	H4	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	11	KC408748	H5	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	12	KC408749	H6	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	13	KC408750	H7	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	14	KC408751	H8	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	15	KC408752	H9	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	16	KC408753	H10	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	2	KC408756	H13	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	19	KC408757	H14	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	20	KC408758	H15	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	21	KC408759	H16	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	22	KC408760	H17	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	23	KC408761	H21	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	24	KC408762	H22	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	25	KC408763	H23	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	26	KC408764	H24	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	27	KC408765	H25	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	28	KC408766	H26	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	29	KC408767	H27	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	30	KC408768	H28	Lake Erie drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	3	HM849076	H2655	Red River drainage	Breton et al. 2011
L. siliquoidea	*nad1*	5	HM852926	BM20297	Upper Mississippi drainage	Boyer et al. 2011
L. siliquoidea	*nad1*	5	HM852927	BM19848	Upper Mississippi drainage	Boyer et al. 2011
L. siliquoidea	*cox1*	17	KC408754	H11	Upper Mississippi drainage	Krebs et al. 2013
L. siliquoidea	*cox1*	18	KC408755	H12	Upper Mississippi drainage	Krebs et al. 2013
L. "*straminea*"	*cox1*	4	MK391881	JFBM22424	Escambia River drainage	Keogh and Simons 2019
L. "*straminea*"	*cox1*	5	MK672782	ESC04	Escambia River drainage	Inoue et al. 2020
L. "*straminea*"	*cox1*	6	MK672783	ESC05	Escambia River drainage	Inoue et al. 2020
L. "*straminea*"	*cox1*	7	MK672784	ESC06	Escambia River drainage	Inoue et al. 2020
L. straminea	*cox1*	1	MK391880	JFBM22423	Alabama River drainage	Keogh and Simons 2019
L. straminea	*cox1*	1	MK391882	JFBM:22426	Tombigbee River drainage	Keogh and Simons 2019
L. straminea	*nad1*	2	*MH588393	UAM3543	32.674–87.765, Black Warrior River drainage	New
L. straminea	*cox1*	3	MH161355	UAUC 3543	Black Warrior River drainage	Burlakova et al. 2019
L. straminea	*nad1*	1	DQ445163	UAUC694	Black Warrior River, near Fosters, Alabama	Unpublished
L. straminea	*cox1*	2	EF033271	H1369	Not stated	Chapman et al. 2008
L. virescens	*cox1*	1	MK672787	Lvir TEN01	Tennessee River drainage	Inoue et al. 2020
L. virescens	*cox1*	1	*MH560775	AABC	Paint Rock River	Alabama Aquatic Biodiversity Center
L. virescens	*cox1*	1	JF326433		Tennessee River drainage	Campbell and Lydeard 2012
L. virescens	*nad1*		JF326443		Tennessee River drainage	Campbell and Lydeard 2012
L. virescens	*cox1*	1	JQ437390	PR 7106	Tennessee River drainage	Moyer and Díaz-Ferguson 2012
L. virescens	*cox1*	2	JQ437391	PR 7108	Tennessee River drainage	Moyer and Díaz-Ferguson 2012
L. virescens	*cox1*	3	JQ437392	1	Tennessee River drainage	Moyer and Díaz-Ferguson 2012

Appendix 2, continued.

Species	Gene	Haplotype	GenBank accession no.	Specimen ID	Waterbody	Reference
L. virescens	*cox1*	4	JQ437393	2	Tennessee River drainage	Moyer and Díaz-Ferguson 2012
Ortmanniana ligamentina	*cox1*		*MH560777	UAM241	Kankakee County, Illinois, Kankakee River	New
O. ligamentina	*nad1*		*MH588394	UAM241	Kankakee County, Illinois, Kankakee River	New

*Newly generated sequences.

Freshwater Mollusk Biology and Conservation 24:87–103, 2021

DOI:10.31931/fmbc-d-20-00015

REGULAR ARTICLE

PHENOTYPIC PLASTICITY AND THE ENDLESS FORMS OF FRESHWATER GASTROPOD SHELLS

Nathan V. Whelan[1,2]*

[1] *Southeast Conservation Genetics Lab, Warm Springs Fish Technology Center, U.S. Fish and Wildlife Service, 203 Swingle Hall, Auburn, AL 36849 USA*
[2] *School of Fisheries, Aquaculture, and Aquatic Sciences, College of Agriculture, Auburn University, 203 Swingle Hall, Auburn, AL 36849 USA*

ABSTRACT

Freshwater gastropod shells display a striking amount of variation. Shell characters are the foundation of most freshwater gastropod taxonomy and the basis for identifying most species. However, intraspecific shell variation is common, and the mechanisms that give rise to this variation are often unclear. One source of shell variation is phenotypic plasticity, in which one genotype gives rise to multiple phenotypes as a response to environmental cues. This phenomenon is often invoked as an explanation for intraspecific shell variation in gastropods, but its existence has not been confirmed experimentally or otherwise in many gastropod lineages. I review the evidence for phenotypic plasticity in freshwater gastropods, and I discuss areas of research needed for a better understanding of intraspecific shell variation. Phenotypic plasticity is well documented in the superorder Hygrophila, but evidence in other freshwater gastropod groups is limited or nonexistent because of the scarcity of common garden experiments for those groups. Despite statements to the contrary, studies that show correlations of shell traits with environmental factors, population genetic analyses, and phylogenetic inference fail to provide evidence of phenotypic plasticity. Researchers must be careful not to postulate about phenotypic plasticity without evidence. I argue that phenotypic plasticity should not be the default hypothesis for explaining intraspecific shell variation in freshwater gastropods and that more common garden experiments are needed to test its existence. Genomic studies of mantle gene expression and transgenerational epigenetic studies also will increase our understanding of gastropod shell variation.

KEY WORDS: morphology, ecomorph, common garden, biomineralization, review

INTRODUCTION

Shells are the most prominent feature of freshwater gastropods (snails and limpets). They protect the animals, form the basis of most taxonomy and species identification, and are linked inextricably to many aspects of gastropod biology (Brusca and Brusca 2003). Gastropods have arguably the greatest diversity of shell forms of any shell-bearing molluscan group. Adult shells of freshwater species range in size from less than 3 mm to more than 16 cm (Thompson 1977; Burch and Tottenham 1980; Hayes et al. 2012), and they can be dome shaped, coiled, extremely ornamented, or without distinguishing features (Figs. 1–3). Intraspecific shell variation is also common and extensive. For example, shell coiling can be dextral or sinistral (Figs. 2, 3), and both forms can be present in the same population (Fig. 3A, B; Freeman and Lundelius 1982; Asami et al. 2008; Tiemann and Cummings 2008; Abe and Kuroda 2019). Many other types of shell variation occur among and within populations of the same species (e.g., Whelan et al. 2012; Zuykov et al. 2012).

For most freshwater gastropod groups, our current knowledge of shell variation does not extend past superficial documentation of shell forms, and the genetic and environmental mechanisms that influence shell shape are largely unknown. Gastropod shells are hypothesized to be under strong selection from predators (Vermeij 1974, 1982; Vermeij and Covich 1978), an idea that has been corroborated

*Corresponding Author: nathan_whelan@fws.gov

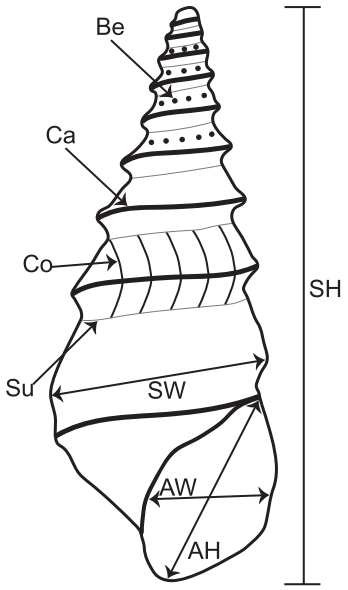

Figure 1. Artist rendering of a dextral freshwater snail shell with example shell features and measurements. Abbreviations: Be, beads (small, round protrusions); Ca, carinae (horizontal ribs); Co, costae (vertical ribs); Su, suture (connection point between two whorls); SH, shell or spire height; SW, shell width (usually measured at widest points across body whorl); AW, aperture width (usually measured across widest points); AH, aperture height (usually measured from contact point of the top aperture lip with shell body across to the widest point on anterior aperture lip). This figure does not include spines (see Fig. 3F), tubercles, lirae, or other uncommon types of ornamentation.

experimentally for many marine gastropods (Palmer 1979; Vermeij 2015). However, less evidence is available for freshwater groups. Stream flow also is hypothesized to influence shell shape (Statzner 2008), but the advantages conferred by different shapes to slower or faster flows are unclear. Genomic and proteomic tools have been used to study the genetic basis of shell shape in only a few lineages (reviewed by Kocot et al. 2016; Song et al. 2019), and difficulties associated with captive rearing of many lineages hinder experimental studies on shell variation. Our poor knowledge of the causes of shell variation may have led to overdescription of gastropod taxa (Burch 1982; Graf 2001), and species hypotheses in most groups have yet to be tested with molecular data.

One source of shell variation is phenotypic plasticity, when a single genotype gives rise to multiple phenotypes through developmental responses to biotic or abiotic environmental factors such as presence of predators, stream flow, and Ca^{2+} limitation (Bradshaw 1965; West-Eberhard 1989). This phenomenon is often invoked to explain shell variation in freshwater gastropods. The term also has been used in the gastropod literature simply to describe intraspecific shell variation, but it must be distinguished from heritable genetic variation that causes variation (Table 1). The extent to which phenotypic plasticity contributes to shell shape is well studied in a few lineages such as *Potamopyrgus antipodarum* and *Ampullaceana balthica* (see Patterns and Causes of Shell Variation). However, the extent to which shell variation can be attributed to phenotypic plasticity is poorly understood in most freshwater groups.

Understanding the basis of shell variation in gastropods is important to many research areas, including ecological interactions, systematics, and conservation. For example, misinterpreting phenotypic shell morphs as distinct species could alter fundamentally how we interpret ecological interactions between those morphs. Uncritically dismissing shell variation between two distinct species as phenotypic plasticity would lead to erroneous taxonomic conclusions and underestimation of biodiversity. Conversely, describing ecomorphs that result from phenotypic plasticity as distinct species would lead to overestimation of biodiversity. Such erroneous taxonomic conclusions could lead to inappropriate conservation actions because species-level taxonomy typically informs delineation of management units (Margules and Pressey 2000).

I review the evidence for phenotypic plasticity in freshwater gastropods. I discuss the types of evidence needed to attribute shell variation to phenotypic plasticity, and I assess whether the evidence supports phenotypic plasticity as a common cause of shell variation across freshwater gastropods. I focus on freshwater gastropods because phenotypic plasticity may be more common in freshwater lineages than in marine lineages, even though the reasons for this pattern are unclear (Bourdeau et al. 2015). My goals are to review what is known about phenotypic plasticity, clarify confusion about the evidence for phenotypic plasticity that has permeated some freshwater gastropod literature, and identify research that is needed to better understand the basis of shell variation in freshwater gastropods.

WHAT KIND OF EVIDENCE IS NECESSARY TO ATTRIBUTE SHELL VARIATION TO PHENOTYPIC PLASTICITY?

Testing for phenotypic plasticity requires careful experimental design (Table 1). The most powerful approach for testing the cause of intraspecific shell variation is a common garden experiment. Common garden experiments are designed specifically to evaluate phenotypic plasticity by growing individuals from different populations in a common environment and measuring the expression of traits of interest (de Villemereuil et al. 2016). Examples of shell traits measured in common garden experiments include size (e.g., Krist 2002; Hoverman et al. 2005), shape (e.g., Kistner and Dybdahl 2013), shell thickness (e.g., Hoverman et al. 2005), crush resistance (e.g., Lakowitz et al. 2008), and presence–absence of discrete characters (e.g., Whelan et al. 2012; see Fig. 1). Shell variation observed in the absence of environmental variation can then be attributed to specific stimuli or genetic variation. A disadvantage of common garden experiments is that they require the ability to breed and grow offspring of the study species to a size at which shell traits of interest are expressed and measurable. In part for this reason, common garden studies are far less common than claims about

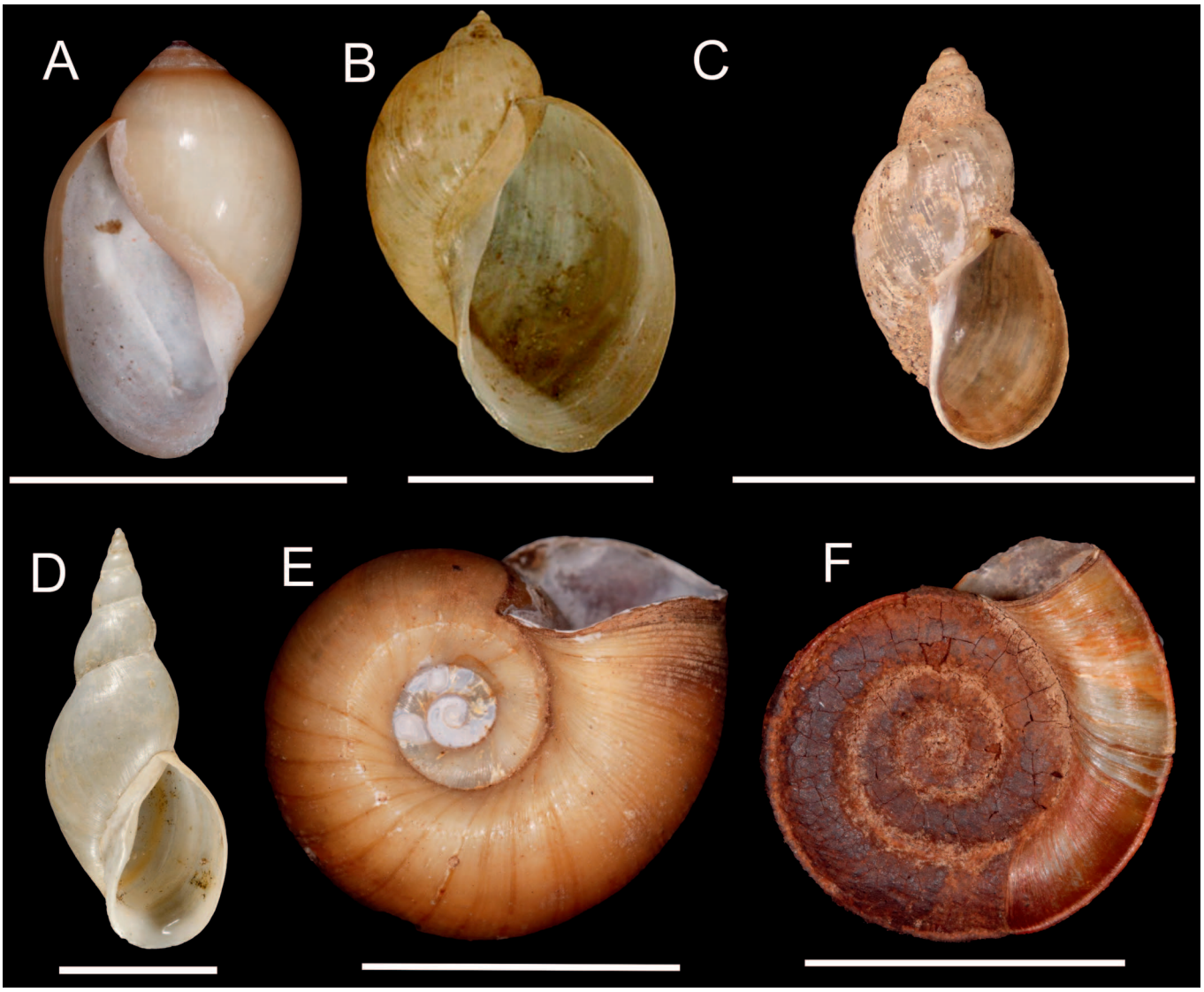

Figure 2. A sample of Hygrophila shell morphologies. Shells are from the Auburn Museum of Natural History (AUMNH) unless otherwise noted. (A) *Physella* sp. (AUMNH 905). (B) *Ampullaceana balthica* (photo by J. Trausel and F. Slieker; Langeveld et al. 2020; licensed under http://creativecommons.org/licenses/by/4.0/). (C) *Galba humilis* (University of Michigan Museum of Natural History, UMMNH 75881). (D) *Ladislavella humilis* (Chicago Academy of Sciences, CHAS MAL23622). (E) *Helisoma anceps* (AUMNH 8010). F) *Anisus complanatus* (AUMNH 5412). Scale bars, 1 cm.

phenotypic plasticity causing shell variation (see subsequent; Table 2).

Reciprocal transplant experiments also can be used to make inferences about phenotypic plasticity. In this approach, individuals from two or more populations or environments are transplanted into the other environment, and shell traits are measured in the new environment. A disadvantage of this approach is that transplanted individuals may have lower survival than native individuals, which would make possible inferences about adaptation but potentially obscure patterns of phenotypic plasticity (de Villemereuil et al. 2016). Transplant experiments also run the risk of inadvertent release of nonnative individuals, which must be avoided. Transplant experiments are uncommon for freshwater gastropods (Tables 1 and 2).

Genomic approaches such as sequencing genes involved in shell shape or other traits have been used in other organisms to determine whether intraspecific variation is genetically controlled or caused by phenotypic plasticity (e.g., McCairns and Bernatchez 2010; Flamarique et al. 2013; Chang and Yan 2019). This approach is difficult, particularly for polygenic traits, and I am aware of no such studies in freshwater snails.

Other approaches for examining intraspecific shell variation cannot provide solid evidence about phenotypic plasticity. Many studies on freshwater gastropods show correlations or clinal variation between shell traits and environmental factors or geography (Table 2). These patterns can appear to provide compelling evidence for phenotypic plasticity. However, clinal variation can have an underlying genetic basis (e.g., Ma et al. 2010; McKechnie et al. 2010; Paaby et al. 2010; Machado et

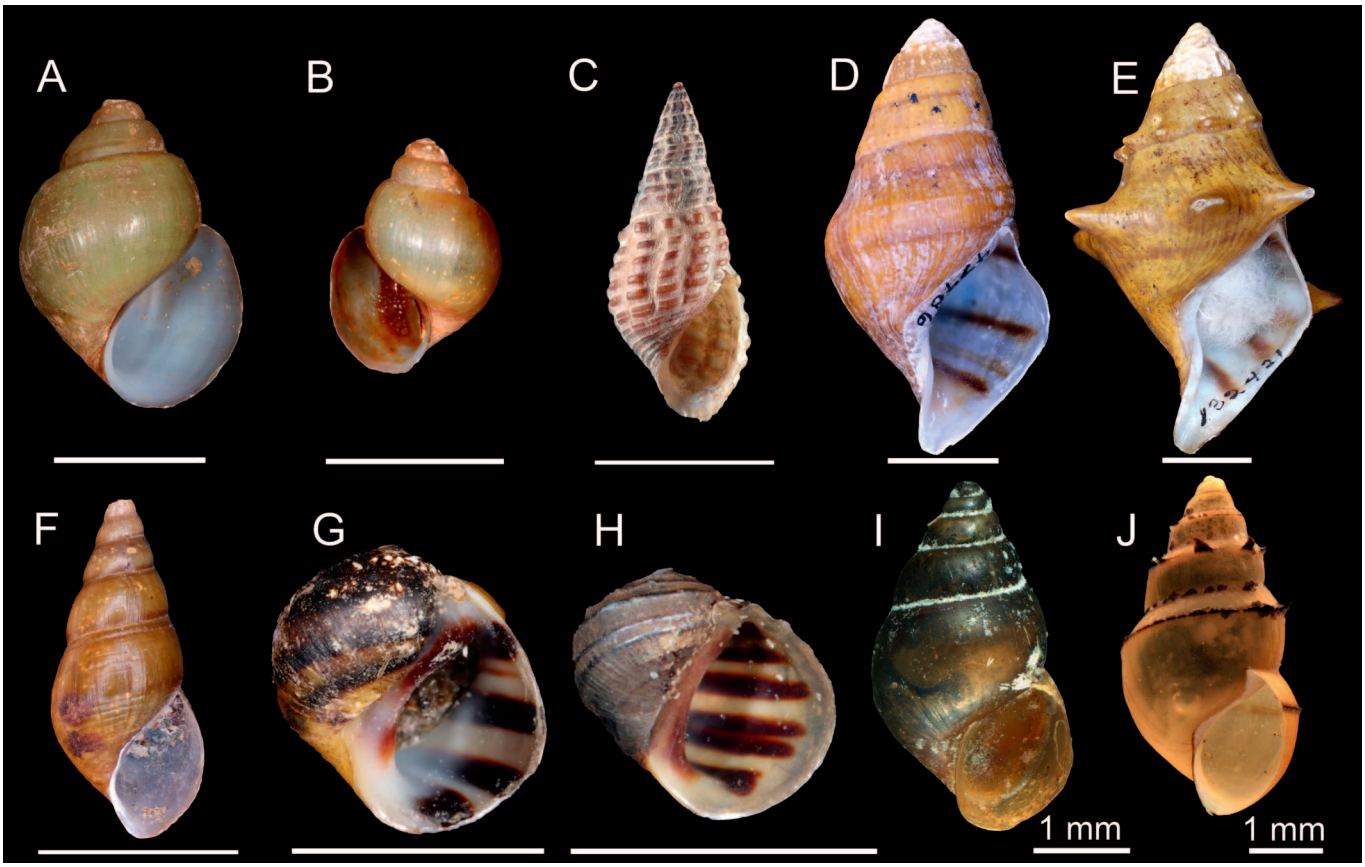

Figure 3. A sample of Caenogastropoda shell morphologies. Shells are from University of Michigan Museum of Natural History (UMMNH) unless otherwise noted. (A) Dextral *Campeloma regulare* (photo by N. Whelan). (B) Sinistral *Campeloma regulare* (photo by N. Whelan). (C) *Tarebia granifera* (iBOL 2016). (D) Smooth *Io fluvialis* (UMMNH 49486). (E) Spined *Io fluvialis* (UMMNH 132421). (F) *Semisulcospira libertina* (UMMNH 153930). (G) Smooth *Leptoxis ampla* (photo by N. Whelan). (H) Carinate *Leptoxis ampla* (photo by N. Whelan). (I) Smooth *Potamopyrgus anitopodarum* (photo by K. Mahlfeld, D. Roscoe, F. Climo; Ueda 2020). (J) Spined *P. antipodarum* (photo by M. Bowie; Ueda 2020). (I, J) Licensed under http://creativecommons.org/licenses/by-nc/4.0/. Scale bars, 1 cm unless otherwise noted.

al. 2016; Koch et al. 2021). Consequently, correlations or clinal variation by themselves cannot provide unequivocal evidence about whether genetic differences, phenotypic plasticity, or both, contribute to shell variation.

Population genetic analyses in combination with morphological analyses are a powerful tool for understanding spatial genetic patterns, gene flow, and conservation needs of freshwater gastropods (Table 2). However, they cannot provide evidence for phenotypic plasticity because such studies use noncoding loci (e.g., microsatellites) or loci not

Table 1. Study types used to examine shell variation in freshwater gastropods.

Type of Study	Can Provide Evidence of Phenotypic Plasticity?	Example Studies
Common garden experiments	Yes	Krist 2002; Hoverman and Relyea 2007; Whelan et al. 2012; Kistner and Dybdahl 2013; Goeppner et al. 2020
Reciprocal transplant experiments	Yes	Negovetic and Jokela 2001
Sequencing and analyzing genes controlling shell traits	Yes	No studies to date for freshwater gastropods
Correlations or clinal variation between shell traits and environmental factors or geography	No	Dupoy et al. 1993; Minton et al. 2008; Cazenave and Zanatta 2016
Population genetic analyses in combination with morphological analyses	No, but could provide some evidence against plasticity	Dillon 2011, 2014; Dillon et al. 2013; Verhaegen et al. 2018b; Whelan et al. 2019
Phylogenetic analyses without sequencing genes controlling shell morphology	No	Ó Foighil et al. 2011; Becker et al. 2016; Hirano et al. 2019; Strong and Whelan 2019

Table 2. Summary of studies evaluating phenotypic plasticity in freshwater gastropod shells. All studies refer to intraspecific variation unless otherwise noted.

Taxon	Type of Variation	Proposed Cause of Variation	Cause Confirmed?[a]	Proposed Cue[a]	Cue Confirmed?[a]	Evidence Type	Source(s)
Superorder Hygrophila							
Lymnaeidae							
Ampullaceana balthica	Crush resistance, shape	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Brönmark et al. 2011, 2012; Lakowitz et al. 2008
Ampullaceana balthica	Crush resistance	Phenotypic plasticity	Yes	Predators	Yes	Common garden	
Ampullaceana balthica	Mantle pigmentation, seen through shell	Phenotypic plasticity	Yes	Predators, UV light	Yes	Common garden	Ahlgren et al. 2013
Ampullaceana balthica	Shape	Phenotypic plasticity	Yes	Flow rate	Yes	Common garden	Lam and Calow 1988; Wullschleger and Jokela 2002
Ladislavella elodes, Galba humilis	Shape	Acknowledged uncertainty	—[a]	—	—	Correlation with habitat factors	Ross et al. 2013
Multiple lymnaeids	Chirality dimorphisms	Genetic	Yes	—	—	Crossing experiments, gene editing	Freeman and Lundelius 1982; Asami et al. 2008; Abe and Kuroda 2019
Multiple lymnaeids	Shell abnormalities	Acknowledged uncertainty	—	—	—	Analysis of museum specimens	Zuykov et al. 2012
Planorbidae							
Planorbella trivolvis	Shape, thickness	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Hoverman et al. 2005; Hoverman and Relyea 2007; Hoverman et al. 2014
Planorbella campanulata	Shape, thickness	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Hoverman et al. 2014
Helisoma anceps	Shape, thickness	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Hoverman et al. 2014
Ferrissia rivularis	Shape	Phenotypic plasticity	Yes, but not interspecific as originally proposed	Flow	Yes	Common garden	Dillon and Herman 2009
Gyraulus spp.	Spire coiling	Phenotypic plasticity	No	None proposed	—	Phylogenetic analyses	Clewing et al. 2015
Anisus leucostoma	Spire coiling	Genetic	Yes	—	—	Multigeneration breeding in common garden	Boettger 1949
Biomphalaria glabrata	Spire coiling	Genetic	Yes	—	—	Multigeneration breeding in common garden	Richards 1971

Table 2, continued.

Taxon	Type of Variation	Proposed Cause of Variation	Cause Confirmed?[a]	Proposed Cue[a]	Cue Confirmed?[a]	Evidence Type	Source(s)
Physidae							
Physella virgata	Thickness	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Langerhans and DeWitt 2002
Physella virgata	Growth rate	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Crowl and Covich 1990
Physella heterostropha	Shape	Phenotypic plasticity, genetic variation	Yes, combination	Predators	Yes	Common garden	DeWitt 1998
Physella virgata	Shape	Phenotypic plasticity, genetic variation	Yes, combination	Temperature	Yes	Common garden	Britton and McMahon 2004
Physa acuta	Shape, crush resistance	Phenotypic plasticity, genetic variation	Yes, combination	Predators	Yes	Common garden	Goeppner et al. 2020; Tariel et al. 2020
Physa acuta	Shape	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Ross et al. 2014
Physa acuta, Physella carolinae	Shape	Interspecific genetic variation	Yes	—	—	Common garden	Dillon and Jacquemin 2015
Subclass Caenogastropoda							
Tateidae							
Potamopyrgus antipodarum	Shape, size	Phenotypic plasticity, genetic variation	Yes, mixture of both	Lake depth	Yes	Reciprocal transplant	Negovetic and Jokela 2001
Potamopyrgus antipodarum	Shape, size	Phenotypic plasticity, genetic variation	Yes, mixture of both	Flow	Yes	Common garden	Kistner and Dybdahl 2013
Potamopyrgus antipodarum	Shape, size	Phenotypic plasticity, genetic variation	Genetic cause confirmed, plasticity equivocal	Flow	No	Correlation with habitat factors, genetics	Verhaegen et al. 2018a
Potamopyrgus antipodarum	Presence of spines	Phenotypic plasticity, genetic variation	Genetic cause confirmed, plasticity equivocal	Predators, parasites	No	Correlation with habitat factors and genetics	Holomuzki and Biggs 2006; Verhaegen et al. 2018b; Vergara et al. 2016; Levri et al. 2005
Potamopyrgus antipodarum	Shape, size	Phenotypic plasticity, but acknowledged uncertainty	No	Flow, stream size, nutrient availability	No	Correlation with habitat factors	Haase 2003
Potamopyrgus antipodarum	Shape, size	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Kistner and Dybdahl 2014; Vergara et al. 2016
Hydrobiidae							
Pyrgophorus coronatus	Shape	Phenotypic plasticity	Yes	Temperature	Yes	Common garden	Albarrán-Melzér et al. 2020

Table 2, continued.

Taxon	Type of Variation	Proposed Cause of Variation	Cause Confirmed?[a]	Proposed Cue[a]	Cue Confirmed?[a]	Evidence Type	Source(s)
Pyrgulopsis robusta	Shape	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Kistner and Dybdahl 2014
Ampullariidae							
Pomacea canaliculata	Size, thickness	Phenotypic plasticity	Yes	Temperature	Yes	Common garden	Tamburi et al. 2018
Pomacea canaliculata	Size, shape, crush resistance	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Guo et al. 2009
Pomacea canaliculata	Shape	Genetic variation	Yes	—	—	Common garden	Estebenet and Martín 2003
Viviparidae							
Cipangopaludina chinensis	Size, organic content	Phenotypic plasticity	Yes	Predators	Yes	Common garden	Prezant et al. 2006
Cipangopaludina japonica and Heterogen longspira	Shape	Acknowledged uncertainty	—	—	—	Mitochondrial phylogenetics and observed shell variation	Hirano et al. 2015
Campeloma dicisum	Shape	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Ross et al. 2014
Campeloma spp.	Chirality	Acknowledged uncertainty	—	—	—	Observed shell variation	Figure 1; Tiemann and Cummings 2008, and references therein
Superfamily Cerithioidea							
Thiaridae							
Tarebia granifera	Shape	Phenotypic plasticity	Yes	Temperature	Yes	Common garden	Albarrán-Melzér et al. 2020
Melanoides tuberculata	Shape, color	Phenotypic plasticity	Yes	None proposed	—	Common garden	Van Bocxlaer et al. 2015
Semisulcospiridae							
Semisulcospira reiniana	Shape	Phenotypic plasticity, genetic variation	Yes, combination	Flow	Yes, but effect possibly overstated	Common garden	Urabe 1998, 2000
Pleuroceridae							
Elimia livescens	Shape	Phenotypic plasticity	Yes, in one of three populations analyzed	Predator	Yes, for one population	Common garden	Krist 2002
Elimia comalensis	Growth rate	None proposed	—	—	—	Growth rates of two populations based on single-collection observations	Minton et al. 2007

Table 2, continued.

Taxon	Type of Variation	Proposed Cause of Variation	Cause Confirmed?[a]	Proposed Cue[a]	Cue Confirmed?[a]	Evidence Type	Source(s)
Elimia potosiensis	Shape	Phenotypic plasticity	No	Stream position	—	Correlation with habitat factors	Minton et al. 2011
Io fluvialis	Shape, presence of ornamentation	None proposed	—	—	—	Correlation with habitat factors	Adams 1915
Lithasia geniculata	Shape, presence of ornamentation	Phenotypic plasticity	No	Flow, stream position	No	Correlation with habitat factors	Minton et al. 2008
Lithasia geniculata	Shape, crush resistance	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Minton et al. 2018
Elimia livescens	Shape	Phenotypic plasticity	No	Many environmental variables	No	Correlation with habitat factors	Dunithan et al. 2012
Elimia livescens	Shape	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Cazenave and Zanatta 2016
Elimia livescens	Shape	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Ross et al. 2014
Pleurocera acuta	Shape	Acknowledged uncertainty	—	—	—	Correlation with habitat factors	Ross et al. 2014
Pleurocera acuta, P. canaliculata, P. pyrenella	Interspecific shape	Phenotypic plasticity	No	Stream size	No	Correlation with habitat factors, allozyme variation	Dillon et al. 2013
Elimia clavaeformis, Pleurocera unciale	Interspecific shape, presence of carinae	Phenotypic plasticity	No	Stream size and other unknown environmental variables	No	Correlation with habitat factors, allozyme variation	Dillon 2011
Elimia livescens, Elimia semicarinata, Lithasia obovata	Interspecific shape	Phenotypic plasticity	No	Predation, substrate, flow	No	Allozyme variation	Dillon 2014
Elimia spp. from Georgia and Florida, USA	Interspecific shape, presence of sculpture	Acknowledged uncertainty	—	—	—	Allozyme variation	Dillon and Robinson 2011
Leptoxis ampla	Presence of carinae	Genetic variation	Yes	—	—	Common garden, genetics	Whelan et al. 2012; 2019
Leptoxis spp.	Shape, discrete characters like striae	Interspecific genetic variation	Yes	—	—	Common garden	Whelan et al. 2015

Table 2, continued.

Taxon	Type of Variation	Proposed Cause of Variation	Cause Confirmed?[a]	Proposed Cue[a]	Cue Confirmed?[a]	Evidence Type	Source(s)
Subclass Neritimorpha							
Neritidae							
Theodoxus fluviatilis	Color, shape	Phenotypic plasticity	No	Substrate	No	Correlation with habitat factors	Zettler et al. 2004
Theodoxus spp.	Color, shape	Phenotypic plasticity, but possibly meant intraspecific variation of any cause	No	—	—	Observed shell variation within species	Sands et al. 2020; Glöer and Pešić 2015
Theodoxus jordani	Color	Phenotypic plasticity	No	UV light	No	Geographical distribution of color morphs	Heller 1979
Subclass Heterobranchia							
Valvatidae							
Valvata lewisi	Unattached spire in some individuals	None proposed	—	—	—	Documentation of variation	Clarke 1973; Burch and Tottenham 1980
Valvata lewisi	Unattached spire in some individuals	None proposed	—	—	—	Documentation of sympatric variation, lack of variation at COI mitochondrial and ITS2 nuclear genes	Hinchliffe et al. 2019

COI = cytochrome c oxidase subunit I; ITS2 = internal transcribed spacer 2; UV = ultraviolet.

[a] —, indicates not applicable.

involved with shell shape (e.g., allozymes, genome-wide single-nucleotide polymorphisms). Population genetic analyses could provide evidence that shell variation is not the result of phenotypic plasticity if shell shape and genetic variation are highly correlated (e.g., Whelan et al. 2019), but other lines of evidence are needed to be conclusive because genome-wide genetic variation may not indicate differences in genes involved with shell variation.

Examining shell traits in a phylogenetic context is also common for freshwater gastropods (Tables 1 and 2), but phylogenetic studies without sequencing genes involved with shell morphology cannot provide information about whether phenotypic plasticity causes shell variation. That is, closely related individuals could have differences in the genes controlling shell traits, but be identical with respect to genes used to infer a phylogeny (e.g., mitochondrial genes). The one exception would be if genes involved in shell variation are used for phylogenetic tree inference, but this has not been done for freshwater snails. By contrast, phylogenetic results showing that two entities are distinct species could be used as evidence that observed shell variation is not caused by phenotypic plasticity because variation in genes controlling shell shape can be assumed to have accumulated since the species diverged.

Finally, evidence of plasticity in one group of freshwater snails is not suitable evidence that phenotypic plasticity controls shell traits in other groups. "Freshwater gastropods" is a polyphyletic group. Freshwater habitats have likely been invaded by gastropods at least 30 times (Strong et al. 2008), and all freshwater gastropods have not shared an ancestor for at least 350 million yr (Zapata et al. 2014). This means that factors that control shell traits likely vary widely among disparate lineages. Even within a lineage (e.g., family or genus), the existence of phenotypic plasticity in one species does not necessarily support its existence in other members of the lineage.

PATTERNS AND CAUSES OF SHELL VARIATION

Superorder Hygrophila

Phenotypic plasticity is unusually well studied in the superorder Hygrophila (Table 2). Hygrophila gastropods lack an operculum, and many have a thin and transparent shell (Fig. 2). Historically, these gastropods were considered pulmonates, but molecular phylogenetic analyses determined Pulmonata to be polyphyletic (Jörger et al. 2010). Nevertheless, the term Pulmonata, or pulmonate, is still in use (e.g., Goeppner et al. 2020). Many Hygrophila are reared easily in captivity, which makes common garden experiments and other experimental approaches feasible.

Lymnaeidae.—Phenotypic plasticity is perhaps better documented for *Ampullaceana balthica* (=*Radix balthica*; Fig. 2B) than for any other freshwater snail (Table 2). In common garden experiments, *A. balthica* shell shape was strongly influenced by the presence of predators (Brönmark et al. 2011, 2012), and shell shape varied such that crush resistance to specific predators was maximized in the presence of the specific predator (e.g., crayfish vs. fish; Lakowitz et al. 2008). Brönmark et al. (2012) showed that production of predator-resistant shells carried a fitness trade-off in which higher crush resistance was associated with reduced growth and fecundity. Mantle pigmentation in *A. balthica*, which can be seen through the shell, is also influenced by predatory fish and UV light (Ahlgren et al. 2013). Shell shape of *A. balthica* also displays phenotypic plasticity in response to flow, with individuals having proportionally larger apertures in the presence of higher flow (Lam and Calow 1988; Wullschleger and Jokela 2002). Furthermore, some responses to flow by *A. balthica* appear to be epigenetic because shell shape of subsequent generations can be influenced by stimuli experienced by parents before egg laying (Wullschleger and Jokela 2002).

Both *Galba humilis* (Fig. 2C) and *Ladislavella elodes* (Fig. 2D) display shell-shape variation that is associated with abiotic environmental factors, such as substrate composition, watershed drainage area, and pH (Ross et al. 2014). This variation was revealed only by geometric morphometrics, and the variation is subtle and likely not readily perceptible to the human eye. Ross et al. (2014) acknowledged that the causes of variation in these two species are unclear because shape variation is correlated only with environmental factors.

Some lymnaeid species display rare shell abnormalities such as detachment between whorls and bulges on the external shell surface. The causes of such abnormalities are unknown, but they are unlikely to be caused by phenotypic plasticity in every case (Zuykov et al. 2012). The growth of abnormal spires may be similar to intraspecific chirality dimorphisms (i.e., dextral or sinistral) where a small percentage of individuals will have shell chirality opposite of what is common for the species (Freeman and Lundelius 1982; Asami et al. 2008; Abe and Kuroda 2019). Chirality dimorphisms are not well studied in most species, but multiple studies on *Peregriana peregra* (=*Lymnaea peregra*) and *Ampullaceana balthica* indicate that chirality is heritable (Freeman and Lundelius 1982; Asami et al. 2008) and probably controlled by a single gene (Abe and Kuroda 2019).

Planorbidae.—Juvenile *Planorbella trivolvis* (=*Helisoma trivolvis*) grew thicker or wider shells depending on whether individuals were exposed to predatory crayfish or waterbugs, respectively (Hoverman et al. 2005; Hoverman and Relyea 2007; Tamburi et al. 2018). Sexually mature *P. trivolvis* can initiate production of thicker shells when exposed to predators, but previously deposited shell is not modified, emphasizing the developmental aspect of phenotypic plasticity (Hoverman and Relyea 2007). In one of the few common garden experiments that included multiple freshwater snail species, Hoverman et al. (2014) showed that *P. trivolvis*, *P. campanulata*, and *Helisoma anceps* (Fig. 1F) all expressed predator-induced phenotypic plasticity in shell shape or thickness, but phenotypic responses varied by species: *H. anceps* developed a lower spire and thicker shell in the

presence of crayfish and water bugs; *P. campanulata* developed a lower spire and wider shells in the presence of water bugs, but was unresponsive to crayfish; and *P. trivolvis* responded differently to each predator, developing a wider shell in response to water bugs but a thicker shell in response to crayfish.

Intraspecific variation in shell spire shape of planorbids is at least sometimes genetically controlled. Some planorbid lineages comprise individuals that grow abnormal, corkscrew-like spires (Zuykov et al. 2012; Clewing et al. 2015), which is heritable and genetically controlled in at least two species, *Anisus leucostoma* (Fig. 2F; Boettger 1949) and *Biomphalaria glabrata* (Richards 1971). Clewing et al. (2015) hypothesized that corkscrew-like spires in *Gyraulus* were ecomorphs caused by phenotypic plasticity, but no experiments were done that could corroborate their hypothesis.

Phenotypic plasticity was invoked to explain interspecific shell-shape variation between the limpets *Ferrissia californica* (=*Ferressia fragilis*) and *Ferressia rivularis* (Dillon and Herman 2009). This conclusion was based on shell shape differences between wild populations that were not present when offspring of each population were raised in a common garden, and the result was the basis for synonymization of *F. californica* and *F. rivularis* (Dillon and Herman 2009). However, Walther et al. (2010) demonstrated that *F. californica* is a valid species and that Dillon and Herman (2009) examined only *F. rivularis* sensu stricto. Thus, the shell variation documented by Dillon and Herman (2009) was a result of intraspecific phenotypic plasticity within *F. rivularis*. This example emphasizes the importance of accurate taxonomy and species identification for studying shell variation.

Physidae.—Common garden experiments showed that both genetic differences and phenotypic plasticity affect intraspecific shell variation in at least some physids (Fig. 2A). The genealogy of *Physella heterostropha* (=*Physa heterostropha*) individuals influences the degree to which phenotypic plasticity modifies shell shape (DeWitt 1998), and genetic variation in *P. virgata* influences shell shape more than thermal environment (Britton and McMahon 2004). Furthermore, *P. virgata* has faster growth rates in the presence of predators, but faster growth has the cost of delayed reproduction (Crowl and Covich 1990). *Physella virgata* also responds similarly to molluscivorous and nonmolluscivorous fish: it grows thicker shells even in the presence of a nonmolluscivorous fish, which results in decreased fecundity (Langerhans and DeWitt 2002). This demonstrates that phenotypic plasticity can sometimes result in reduced fitness, but provides no offsetting benefit if environmental cues are too general (i.e., any fish vs. a fish predator).

Physella acuta shell shape can vary with abiotic environmental factors, such as pH and substrate composition, but it is unclear to what extent this variation is genetically controlled or a result of phenotypic plasticity (Ross et al. 2014). Both plasticity and genetic background affected shell shape and crush resistance of *P. acuta* in response to predators (Goeppner et al. 2020; Tariel et al. 2020). Interestingly, common garden experiments showed that the presence of predators also can have transgenerational effects as shell shape appeared to be partly influenced by predator cues experienced by parents and grandparents (Goeppner et al. 2020; Tariel et al. 2020). Dillon and Jacquemin (2015) showed that shell variation between *P. acuta* and *P. carolinae* was genetically controlled, indicating that the two species should not be synonymized despite their ability to hybridize.

Subclass Caenogastropoda

Tateidae.—Environmental correlates of shell variation are better demonstrated for the minute and highly invasive *Potamopyrgus antipodarum* (Fig. 3I, J) than for any other non-Hygrophila freshwater gastropod. *Potamopyrgus antipodarum* has larger and more slender shells and larger apertures in riverine environments compared with individuals in lakes, both within and beyond its native range (Verhaegen et al. 2018a). Invasive *P. antipodarum* and native *Pyrgulopsis robusta* in the Snake River drainage, USA, both have larger apertures in riverine environments compared with individuals in lakes, suggesting convergent environmental adaptation (Kistner and Dybdahl 2014). In its native range, *P. antipodarum* shells are larger at more downstream-riverine locations, and shell size increases with depth in lakes (Haase 2003; Vergara et al. 2016). The selective advantages of such shell variation are unclear because larger apertures do not convey resistance to dislodgement (Verhaegen et al. 2019), and environmental correlations do not inform the causes of shell variation.

Spines on *P. antipodarum* shells are more common in lakes than in rivers (Fig. 1J; Holomuzki and Biggs 2006; Verhaegen et al. 2018b), and spine prevalence appears to increase with lake depth (Vergara et al. 2016). The presence of parasites also was associated with larger shells (Levri et al. 2005), but whether parasites induce larger size or simply infect larger individuals has not been determined. Individuals with parasites also are less likely to have spines, but again, whether parasites influenced shell morphology is unclear (Levri et al. 2005). Spines may provide predator defense, but they incur the cost of increased drag (Holomuzki and Biggs 2006). These relationships suggest that flow and predators influence spine development in opposite ways, but spines also are associated with genetic variation (Verhaegen et al. 2018b), casting doubt that phenotypic plasticity alone determines spine development in *P. antipodarum*.

I am aware of only two studies on *P. antipodarum* that were common garden or transplant experiments. Negovetic and Jokela (2001) demonstrated through transplantation to different wild habitats that shell shape was influenced by both genetics and environmentally induced plasticity, but shell size was influenced only by phenotypic plasticity. Their findings were corroborated by the common garden experiments of Kistner and Dybdahl (2013). No study has examined spine development in a common garden experiment. Clearly, shell

morphology of *P. antipodarum* is correlated with environmental factors, but data on the mechanisms underlying shell variation are either equivocal (Haase 2003; Levri et al. 2005; Holomuzki and Biggs 2006; Kistner and Dybdahl 2014; Vergara et al. 2016) or suggest an interplay between genetics and phenotypic plasticity (Negovetic and Jokela 2001; Kistner and Dybdahl 2013; Verhaegen et al. 2018a, 2018b).

Hydrobiidae.—Using geometric morphometrics, Albarrán-Melzér et al. (2020) showed that *Pyrgophorus coronatus* grew slightly wider at lower temperatures, but the differences were exceedingly small and probably not readily perceptible to the human eye. However, small differences may be meaningful to snails, and shell width may affect thermoregulation (Albarrán-Mélzer et al. 2020). Shell variation in *Pyrgulopsis robusta* is correlated with environmental factors, but the cause of this variation is unknown (Kistner and Dybdahl 2014).

Ampullariidae.—A common garden experiment with *Pomacea canaliculata* showed that interpopulation shell-shape variation was genetically controlled (Estebenet and Martín 2003). By contrast, another common garden experiment confirmed phenotypic plasticity in *P. canaliculata*; shells grew larger, but were thinner, at higher temperatures, likely due to reduced shell deposition per unit area as the active edge of the mantle moved forward faster (Tamburi et al. 2018). Another common garden experiment revealed sex-specific phenotypic plasticity in *P. canaliculata* in which shell height was reduced in females in the presence of a turtle predator, but not in males (Guo et al. 2009). However, both male and female *P. canaliculata* grew shells with greater crush resistance and smaller opercula when exposed to a turtle predator (Guo et al. 2009).

Viviparidae.—In the only common garden study conducted with Viviparidae, *Cipangopaludina chinensis* (=*Bellamya chinensis*) produced offspring with greater shell organic content and slightly larger shells in the presence of a crayfish predator (Prezant et al. 2006). Studies of more obvious traits provide no unequivocal evidence for phenotypic plasticity in the family. *Cipangopaludina japonica* and *Heterogen longispira* are indistinguishable on mitochondrial gene trees, but are distinguished easily by shell-suture depth, size of the body whorl, and aperture shape (Hirano et al. 2015). This variation was hypothesized to be the result of phenotypic plasticity within a single species, but the possibility of undiscovered genetic variation sufficient to support the existence of two species was acknowledged (Hirano et al. 2015). Like some Hygrophila, *Campeloma* spp. can display intraspecific differences in chirality (Fig. 3A, B; Tiemann and Cummings 2008, and references therein), but the underlying causes of this variation are unknown. Furthermore, *Campeloma decisum* shell shape varies with abiotic environmental factors, but the cause of this variation is also unknown (Ross et al. 2014).

Superfamily Cerithioidea

Thiaridae and Semisulcospiridae.—Common garden experiments have shown that temperature induced small shell-shape differences in the thiarid *Tarebia granifera* (Fig. 3C) in its invasive range, but the degree of shell-shape variation was lower than in a sympatric, native snail *Pyrgophorus coronatus* (see *Tateidae*; Albarrán-Mélzer et al. 2020). Common garden experiments also showed that phenotypic plasticity results in coloration and small shape differences in the thiarid *Melanoides tuberculata* (Van Bocxlaer et al. 2015). Studies on the semisulcospirid *Semisulcospira reiniana* suggested a larger environmental effect than genetic effect on shell shape, but estimates of heritability were confounded by an experimental design that failed to account for paternal shell shape (Urabe 1998, 2000). Thus, environmental influence on the shell shape of *S. reiniana* may be overstated.

Pleuroceridae.—The Pleuroceridae exhibits extensive shell variation within recognized species. For example, the seminal study of Adams (1915) documented striking clinal variation in the genus *Io* (Fig. 3D, E). Many studies have invoked phenotypic plasticity as a cause of shell variation in Pleuroceridae (e.g., Minton et al. 2008; Dillon 2011, 2014; Minton et al. 2011; Dunithan et al. 2012; Dillon et al. 2013). However, the evidence presented in these studies is limited to correlations with environmental factors or examination of allozyme variation unrelated to shell traits, and none provide unequivocal evidence of phenotypic plasticity. Other studies have documented shell variation in *Lithasia geniculata*, *Elimia* spp., and *Pleurocera acuta* that is correlated with environmental factors or stream position, but have not proposed a cause for this variation (Minton et al. 2007, 2018; Dillon and Robinson 2011; Ross et al. 2014; Cazenave and Zanatta 2016).

Only three common garden experiments have been done on the family, and only one showed evidence of phenotypic plasticity. In the latter study, *Elimia livescens* grew a slightly narrower ($<$1.0 mm difference) shell in the presence of a predator cue, but this effect was observed in individuals from only one of three populations studied (Krist 2002). Two studies on *Leptoxis ampla*, including a common garden experiment, showed that the presence of carinae and other intraspecific shell variation are genetically controlled (Fig. 3G, H; Whelan et al. 2012, 2019). Common garden rearing of all currently recognized, extant *Leptoxis* species indicated that interspecific shell variation is under genetic control and not a result of phenotypic plasticity (Whelan et al. 2015), supporting the validity of each species.

Despite claims to the contrary, evidence does not support the existence of widespread phenotypic plasticity in pleurocerids. Considering the attention devoted to describing shell variation in the Pleuroceridae, it is curious that little research has attempted to examine the causes or adaptive significance of that variation. For example, no studies have examined the potential adaptive significance or cause of the extensive variation documented by Adams (1915) in *Io*, and *Io fluvialis* is currently the only species recognized in the genus (Johnson et al. 2013; MolluscaBase 2021). An impediment to study of the Pleuroceridae is that its taxonomy is in need of revision (Graf 2001; Johnson et al. 2013). This issue complicates the

study of shell variation because uncertainty persists about whether shell differences are intra- or interspecific.

Other Freshwater Gastropod Groups

No conclusive evidence exists that shell variation in other gastropod groups is a result of phenotypic plasticity. Freshwater Neritidae in the genus *Theodoxus* have variable shell coloration and shape (Zettler et al. 2004; Sands et al. 2020), and Heller (1979) suggested that the different shell coloration provides differential protection from predators and UV radiation in specific environments. However, these studies were descriptive or designed to test selective advantages of shell coloration, not underlying causes of variation. Thus, unsubstantiated claims by some authors that morphological variation in *Theodoxus* is a result of phenotypic plasticity (Zettler et al. 2004; Glöer and Pešić 2015; Sands et al. 2020) should be approached with caution. Morphological variation in Valvatidae is understudied, but some individuals of *Valvata lewisi* grow abnormal, corkscrew-like shells (Baker 1931; Clarke 1973; Burch and Tottenham 1980; Hinchliffe et al. 2019). The cause and distribution of corkscrew-like individuals of *V. lewisi* need more research as no common garden experiments have been done, and genetic data are equivocal (Hinchliffe et al. 2019).

GENERAL PATTERNS OF PHENOTYPIC PLASTICITY AND SOURCES OF CONFUSION

Most documented examples of phenotypic plasticity in freshwater gastropods are for the Hygrophila, and phenotypic plasticity appears to be widespread in this group. Most other groups have not been studied well enough to determine the extent of phenotypic plasticity and whether this cause of shell variation is rare or merely poorly documented. Yet, phenotypic plasticity is often stated, or implied, to be common throughout freshwater gastropods (e.g., Urabe 2000; Glaubrecht and Köhler 2004; Minton et al. 2008, 2011; Dillon 2011, 2014; Dunithan et al. 2012; Dillon et al. 2013; Clewing et al. 2015). Such statements appear to stem from untested assumptions and confusion about the types of studies that can confirm phenotypic plasticity. Confusion about the causes of shell variation also appears to stem from using the term "phenotypic plasticity" to mean any type of intraspecific shell variation (e.g., Glaubrecht and Köhler 2004; Glöer and Pešić 2015; Marković et al. 2019; Sands et al. 2020). Researchers must consider what types of evidence are necessary to confirm phenotypic plasticity (Table 1) before invoking it uncritically to explain shell variation.

Importantly, none of the studies discussed in the previous section provided evidence that phenotypic plasticity is the cause of shell variation used to diagnose two putative species. At least two studies on freshwater gastropods showed that interspecific variation is genetically controlled and heritable, thus supporting the distinctiveness of those taxa (Dillon and Jacquemin 2015; Whelan et al. 2015). When phenotypic plasticity is clearly documented as a cause of shell variation between two putative species, synonymy may be warranted, especially if coupled with supporting evidence such as phylogenetic analyses. However, several studies have proposed taxonomic revisions based on unproven claims of phenotypic plasticity as a cause of shell variation (e.g., Dillon and Herman 2009; Dillon 2011, 2014; Dillon et al. 2013), which should be avoided.

Confusion in the literature also exists about the degree to which shells vary due to phenotypic plasticity. In many cases, phenotypic plasticity has a small effect on shell shape (e.g., a slightly wider aperture or thicker shell). This variation may not be perceptible to the human eye, but in some cases, it has demonstrated fitness benefits (e.g., Lakowitz et al. 2008; Hoverman et al. 2014; Albarrán-Melzér et al. 2020). Variation in larger, more conspicuous shell traits such as spines, carinae, etc., also have been attributed to phenotypic plasticity, but almost always without evidence and often based only on speculation about fitness benefits (e.g., Minton et al. 2008; Dillon 2011; Dunithan et al. 2012). Currently, there is no evidence that phenotypic plasticity is the cause of shell ornamentation and the potential fitness benefits of ornamentation are mostly unknown, but few studies have examined these traits.

Much of the confusion about phenotypic plasticity in freshwater gastropods seems to come down to expectations and generalizations. That is, to what extent should we expect phenotypic plasticity to cause shell variation? In Planorbidae, where phenotypic plasticity is well documented in multiple species, the prevalence of phenotypic plasticity may be high. In *P. antipodarum*, an interplay between genetics and plasticity appears to influence shell shape (Verhaegen et al. 2018a). Clearly, some authors expect phenotypic plasticity to be common in pleurocerids, but phenotypic plasticity has been documented in only one pleurocerid species. Thus, data do not support broad generalizations, and phenotypic plasticity must be evaluated on a case-by-case basis to better understand its prevalence in freshwater gastropods.

FUTURE DIRECTIONS

The greatest research needs for advancing our understanding of phenotypic plasticity in freshwater gastropods are studies with broader taxonomic focus. Although model systems are useful, what makes a system easy to study (e.g., ease of raising in captivity) does not necessarily make the system general enough to explain a phenomenon in disparate lineages. In freshwater gastropods, generalizations are not possible currently because most studies have been conducted with easily studied systems (e.g., Hygrophila). We lack common garden experiments for most groups, and difficulties with raising many groups in captivity are an obstacle. For example, at least some species of pleurocerids can be raised in captivity, but their captive culture needs are more exacting than those of Hygrophila (e.g., larger tanks with flow), and it may take 3–6 mo or longer after hatching before traits of

interest are expressed and measurable (Whelan et al. 2012, 2015). Research is needed to develop cost-effective captive-propagation methods for many freshwater gastropods. Such research would improve our ability to study morphological variation and also would be useful for conservation efforts.

Comparative studies among freshwater gastropod lineages also should be pursued, particularly those that examine the potential adaptive value of shell variation. Predation is an important factor in gastropod evolution, and research examining the value of shell traits in predator defense would be fruitful for better understanding phenotypic plasticity. Experimental studies also are needed to examine how shell traits influence fitness relative to flow and stream size (e.g., rivers vs. lakes or tributaries vs. mainstem) and other abiotic variables. Trade-offs between traits such as shell thickness, size, and ornamentation also are of interest. Such studies have the potential to reveal broad patterns and processes that contribute to evolution of shell traits. Phylogenetic comparative analyses should be a major component of comparative studies because they would reveal broad evolutionary patterns of phenotypic plasticity in freshwater gastropods.

Genomic tools also should be used to advance understanding of phenotypic plasticity. By examining mantle tissue, the tissue responsible for shell growth, RNA-sequencing experiments could identify genes involved in biomineralization and reveal how differential expression contributes to phenotypic plasticity. Coupling common garden experiments with gene expression studies will be fruitful. Genomic tools also could be used in a comparative framework to examine sequence differences of biomineralization genes among closely related species or populations. Such studies would allow researchers to determine whether morphological variation is caused by genetic differences without having to do common garden experiments. Thus, genomic data may make studying phenotypic plasticity in difficult-to-propagate species more cost-effective. Evidence of transgenerational effects of predators on shell morphology in *Physella acuta* (Goeppner et al. 2020; Tariel et al. 2020) suggests epigenetic studies also are needed.

Finally, future studies must be precise in how they use the term phenotypic plasticity. The term should not be used to describe morphological variation when the underlying cause is unknown. Authors also should be precise when referring to shell traits. Terms such as "robustly shelled" and "fusiform" are subjective and vague; traits such as these require quantification by geometric morphometrics or other methods so that they are repeatable by other researchers.

CONCLUSION

Even in the genomic era, shells will continue to be a focus of malacologists. Given the limited number of lineages that have been studied with genetic or common garden experiments, phenotypic plasticity cannot be invoked based on its documentation in other groups. In other words, phenotypic plasticity should not be a default hypothesis for explaining difference in shell morphology. I argue that a high bar should be set when concluding that morphological variation is a result of phenotypic plasticity, and that bar requires common garden experiments or direct studies of genes that control shell shape. By adopting this standard, researchers can avoid past mistakes and clarify misconceptions about the causes of shell variation in freshwater gastropods.

ACKNOWLEDGMENTS

I thank Nicholas Gladstone (Auburn University) and Ashantye' Williams (U.S. Fish and Wildlife Service) for feedback on this article and help with Figure 1. Discussions with Paul Johnson, Jeff Garner (Alabama Department of Conservation and Natural Resources), and Ellen Strong (National Museum of Natural History) also contributed to my interest in this topic. Paul Johnson and Michael Buntin (Alabama Department of Conservation and Natural Resources) kindly shared *Campeloma* shells for photographing. Jonathan Armbruster (Auburn University) and Melissa Callahan (Auburn University) provided access to Auburn University Museum of Natural History. Two anonymous reviewers and editor Wendell Haag provided feedback that improved this article. The findings and conclusions in this article are those of the author and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

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Freshwater Mollusk Biology and Conservation 24:104–113, 2021

DOI:10.31931/fmbc-d-20-00008

REGULAR ARTICLE

DIVERSITY AND PREDICTED FUNCTION OF GUT MICROBES FROM TWO SPECIES OF VIVIPARID SNAILS

Ernest North[1,†] and Russell L. Minton[2]*

[1] *Department of Biological and Environmental Sciences, University of Houston Clear Lake, Houston, TX 77058 USA*

[2] *Department of Biology, Gannon University, 109 University Square, Erie, PA 16541 USA*

ABSTRACT

Animal gut bacteria are involved in numerous critical functions. In snails, gut bacteria play crucial roles in organic material digestion and nutrient production and have been implicated in aspects of reproduction. Snail gut microbes are known to differ between species and even between anatomical compartments of the digestive tract; dietary changes are also known to alter snail gut flora. In an effort to better understand their diversity and function, we studied the gut microbial communities from two viviparid snails, *Campeloma decisum* and *Cipangopaludina japonica*. We were interested in whether significant differences in bacterial community composition existed between the two species, and whether differences in microbial diversity corresponded to differences in community function. Using next-generation sequencing of the bacterial 16S V4 region, we found no significant differences in alpha and beta diversity between *Ca. decisum* and *Ci. japonica*. Firmicutes and Proteobacteria were the most abundant bacterial phyla in both species, while Bacteroidetes had a higher mean abundance in *Ci. japonica*. Nine taxonomic groups were present in significantly different mean abundances between the snail species. Pseudomonads and Enterobacteriaceae were notably more abundant in *Ca. decisum*, while Proteobacteria and Chitinophagaceae were more abundant in *Ci. japonica*. Peptidoglycan synthesis, pyruvate fermentation, and aerobic respiration by cytochrome *c* were the three most abundant microbial pathways represented in the viviparid gut. Fourteen functional pathways differed significantly between *Ca. decisum* and *Ci. japonica*, potentially correlated with differences in bacterial community composition and snail life history. Our data fill in data gaps regarding gut microbes in Viviparidae and highlight future research paths examining the prevalence of Firmicutes and unidentified diversity in both snail species.

KEY WORDS: bacteria, microbial communities, *Campeloma*, *Cipangopaludina*, next-generation sequencing

INTRODUCTION

Animal gut bacteria are critical for the health of their hosts; they affect nutrition, behavior, immune responses, and development (Uzbay 2019). This has been demonstrated in snails as well, where gut microbes are ubiquitous contributors to many physiological processes. Snail gut bacteria play crucial roles in digesting organic material and producing nutrients (Hu et al. 2018). They break down structural carbohydrates such as cellulose, chitin, and lignin, and they provide nitrogen and organic precursors for the production of nucleic acids and the metabolism of energy (Nicolai et al. 2015; Pinheiro et al. 2015; Aronson et al. 2017). Up to 80% of plant-derived carbohydrates are broken down by bacterial enzymes that augment the snail's own digestive enzymes (Charrier et al. 2006). Gut bacteria also have been shown to differ between sexual and asexual populations of the same snail species, suggesting a microbial aspect to reproduction (Takacs-Vesbach et al. 2016).

Multiple factors are known to influence the composition and function of the animal gut microbiome. Bacterial communities can vary significantly between individuals and between species. Diet, geography, season, and disease have been shown to cause variation in—and potential disruption of—a host's gut flora (Colman et al. 2012; King et al. 2012;

[†]Current address: AveXis, Inc., 2275 Half Day Road Suite 300, Bannockburn, IL 60015 USA

*Corresponding Author: minton001@gannon.edu

Tang et al. 2019). Gut microbes also are subject to spatial and temporal differences throughout the host lifecycle (Llewellyn et al. 2016; Triplett et al. 2020). Three planorbid snail species exhibited significant differences in gut microbial diversity and abundance at both the individual and species levels (Van Horn et al. 2012). Different compartments of the digestive system of *Achatina fulica* possessed different microbial communities; these same communities differ further between active and estivating individuals (Pawar et al. 2012). The addition of sugarcane to the diet of *A. fulica* changed the taxonomic composition of the gut microbiome (Cardoso et al. 2012).

Despite recent efforts, relatively little is known about freshwater snail gut microbiomes (Hu et al. 2018; Lyra et al. 2018; Li et al. 2019; Huot et al. 2020). Viviparidae comprises operculate live-bearing snails whose females brood their young in a pouch formed from the palatal oviduct. The Pointed Campeloma, *Campeloma decisum*, is native to the USA, ranging from the Great Lakes and Mississippi River drainages east to the Atlantic slope (Clench 1962). *Cipangopaludina japonica*, the Japanese Mysterysnail, likely was introduced to North America from Asia in the late 19th century by food vendors and spread through intentional and accidental means (Wood 1892; Rothlisberger et al. 2010). In the USA, the species is widespread, reaching its highest density in the Great Lakes and northeastern states (Perez et al. 2016). *Campeloma decisum* and *Ci. japonica* occupy similar habitats and ecological niches: individuals are frequently found on soft sediments in rivers and lakes, and both species are presumed to filter feed from the water column as well as ingest organic material present in the substrate (Allison 1942; Chamberlain 1958; Bocxlaer and Strong 2016).

To explore the diversity and function of these important systems, we studied the gut microbial communities of two viviparid snails. We were interested in testing three central predictions regarding the gut microbes in *Ca. decisum* and *Ci. japonica*. First, we predicted that estimates of alpha and beta diversity would not differ significantly between the snail species given their similar environmental and ecological niches. Second, we predicted that those similarities in diversity would correspond to nonsignificant differences in the estimated bacterial group abundances between the two species. Finally, we predicted that similar *Ca. decisum* and *Ci. japonica* bacterial communities would possess similar estimated microbiome functions. Our ultimate goal was to determine how knowledge of their gut microbial communities affects our understanding regarding the biology and life history of these two species.

METHODS

We acquired 14 *Cameploma decisum* and 13 *Cipangopaludina japonica* from a single collection event at a single site on the Flat River in Lowell, Michigan (42.934° N, 85.339° W) in August 2017. Snails were frozen live at –80°C after collection. After removing the bodies from the shells, we rinsed the animals in deionized water and dissected the intestines and posterior portion of the stomachs. Following the manufacturer's directions, we used the DNeasy PowerSoil (Qiagen) kit to extract microbial genomic DNA from the tissues. We sent the DNA samples to MrDNALab (Shallowater, TX), where the bacterial 16S V4 region was amplified by PCR using the 515F-806R primer pair (Caporaso et al. 2011) and sequenced on an Ion Torrent PGM (Thermo Fisher). Ion Torrent methods produce unidirectional reads of approximately 250 bp by using forward and reverse sequencing primers that are subsequently assembled. MrDNALab performed all quality control on the sequencing output using their proprietary pipeline. Briefly, sequences were depleted of primers; short sequences ($<$ 150 bp) and sequences with ambiguous base calls were removed. Sequences were quality-filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated sequences were denoised; unique sequences identified by sequencing or PCR point errors were removed, as were chimeras; and ends were trimmed.

We used QIIME2 (Bolyen et al. 2019) to analyze the assembled reads. We de-replicated our sequences (vsearch option) and removed any amplified sequence variants (ASVs) present in fewer than two snail samples and/or with abundances below 10 reads summed across all samples (McDonald et al. 2012; Bokulich et al. 2013; Rognes et al. 2016; Takacs-Vesbach et al. 2016). We used the align-to-tree-mafft-fasttree pipeline to perform multiple sequence alignment of our ASVs, mask ambiguously aligned regions, and build maximum likelihood trees (Price et al. 2010; Katoh and Standley 2013). We compared three measures of ASV alpha diversity between *Ca. decisum* and *Ci. japonica*: Shannon diversity as a quantitative estimate of community richness (Shannon 1948), Faith's PD as a qualitative estimate of community richness incorporating phylogenetic relatedness (Faith 1992), and Pielou's index as a measure of community evenness (Pielou 1966). For beta diversity comparison between the snail species, we utilized a permutational multivariate analysis of variance (PERMANOVA) based on generalized UniFrac distances (Anderson 2001; Lozupone and Knight 2005; Chen et al. 2012). Generalized distances combine presence-absence and abundance data with phylogenetic distances between ASVs in the computations, while adjusting the weighting on the branches (Chang et al. 2011). Beta diversity was also assessed at a sampling depth of 5,600 ASVs. Significance of all between-species measures was determined using Kruskal-Wallis tests at $P < 0.05$. We additionally visualized relationships between individual snails using UPGMA based on generalized UniFrac distances in QIIME2.

We hierarchically classified our ASVs using QIIME2 and a pretrained naïve Bayesian classifier based on the 99% OTU Greengenes 13_8 database (DeSantis et al. 2006; Pedregosa et al. 2011; Bokulich et al. 2018). Each ASV was classified to the lowest phylum, family, and genus assigned by the classifier, and abundances were compared between snail species. For unclassified groups that differed significantly between snail species, we selected 50 random ASVs from each group and used the NCBI blastn tool (Altschul et al. 1990) with default

Table 1. Alpha diversity statistics for individual viviparid snails. Cd, *Campeloma decisum*; Cj, *Cipangopaludina japonica*; ASVs, amplified sequence variants.

Snail	Raw reads	ASVs	Shannon diversity	Faith's PD	Pielou's evenness
Cd1	63,884	29,325	4.18	5.13	0.45
Cd2	82,596	61,399	5.58	3.77	0.57
Cd3	75,356	53,078	5.83	7.75	0.58
Cd4	48,528	30,732	6.46	7.27	0.64
Cd5	64,572	38,370	6.96	7.03	0.68
Cd6	63,046	28,933	6.62	9.89	0.67
Cd7	67,309	45,817	6.26	11.75	0.62
Cd8	70,799	41,512	5.41	6.21	0.57
Cd9	38,322	19,989	6.29	13.50	0.66
Cd10	62,095	38,213	6.60	8.24	0.65
Cd11	59,945	38,340	6.35	8.30	0.63
Cd12	16,784	5,642	6.68	7.75	0.70
Cd13	72,397	39,562	3.32	5.65	0.42
Cd14	52,784	25,131	5.71	4.19	0.61
Cj1	58,969	36,240	4.66	8.12	0.50
Cj2	59,771	39,162	4.99	6.82	0.54
Cj3	78,576	52,858	5.63	8.79	0.58
Cj4	75,647	44,210	5.09	5.25	0.53
Cj5	81,376	61,783	5.49	4.42	0.56
Cj6	27,526	10,008	6.43	13.15	0.72
Cj7	80,157	45,606	7.15	5.40	0.71
Cj8	59,666	38,299	5.74	11.20	0.59
Cj9	65,165	38,069	6.07	4.90	0.64
Cj10	35,717	20,102	5.40	10.48	0.58
Cj11	66,937	39,112	6.67	5.21	0.67
Cj12	38,265	19,800	4.67	11.55	0.53
Cj13	64,135	35,494	6.78	15.28	0.67

settings to assess similarity compared to sequences accessioned in GenBank. Finally, we predicted the functional composition of each snail species' gut microbial metagenome using PICRUSt2 (Douglas et al. 2020). PICRUSt2 reconstructs a simulated metagenome from the samples provided, then predicts the function of the metagenome through comparison to the prokaryotic portion of the MetaCyc database (Caspi et al. 2018). ASVs aligning with less than 80% similarity to the reference sequences were excluded, as were those exceeding a nearest sequenced taxon index of 2.0, based on the default settings in PICRUSt2. Significant taxonomic and functional differences between species were identified in ALDEx2 (Fernandes et al. 2014). ALDEx2 uses centered log ratios to convert absolute feature counts to relative abundances normalized for sequencing effort modeled from a Dirichlet process (Holmes et al. 2012; Rosa et al. 2012). Significant differences were assessed by ALDEx2 using estimated *P*-values from Welch's *t*-tests controlled for Benajmini-Hochberg false-discovery rates (FDR) less than 0.1 (Welch 1947; Benjamini and Hochberg 1995).

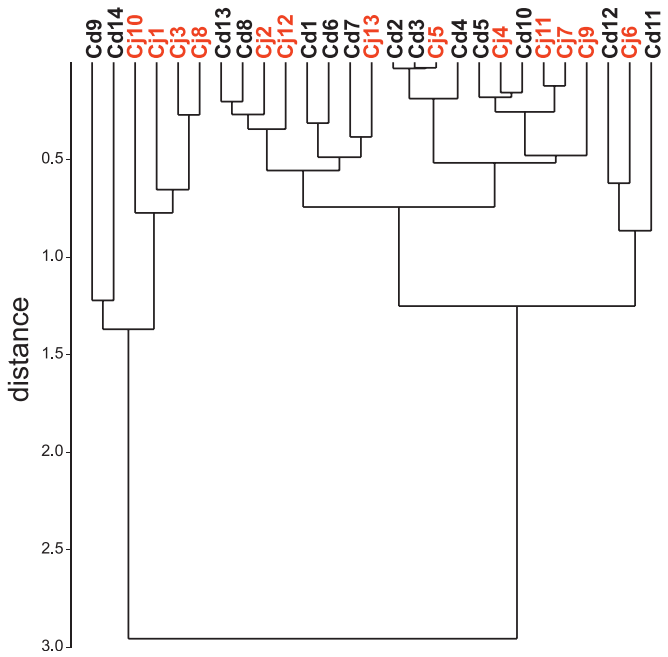

Figure 1. UPGMA dendrogram of pairwise generalized UniFrac distances between individual snails. Black labels, *Campeloma decisum*; red labels, *Cipangopaludina japonica*.

RESULTS

A total of 1,630,324 raw reads were generated for all 27 snails examined, yielding 976,786 total ASVs after dereplicating and filtering. Mean raw reads and ASVs for *Ca. decisum* were 59,887 and 35,432, respectively, and 60,916 and 36,980 for *Ci. japonica*. There was no significant difference in mean ASV number recovered between species (*t*-test, $t = 0.29$, $P = 0.77$). Neither Shannon diversity, nor Pielou's evenness, nor Faith's PD were significantly different between species (Shannon, $H = 0.53$, $P = 0.47$; Pielou, $H = 0.19$, $P = 0.66$; Faith, $H = 0.24$, $P = 0.63$; Table 1). Beta diversity assessed through PERMANOVA of generalized UniFrac distances also did not differ between species (999 permutations, $F = 1.78$, $Q = 0.14$). A UPGMA based on generalized UniFrac distances showed that samples from each snail species did not cluster together (Fig. 1).

Firmicutes and Proteobacteria were the most abundant bacterial phyla in both *Ca. decisum* and *Ci. japonica*, while Bacteroidetes had a mean abundance 100× higher in *Ci. japonica*. Nine families were identified as occurring at $\geq 1\%$ relative abundance in at least one snail species, with Bacillaceae being most abundant in both. Enterobacteriaceae was recovered from only *Ca. decisum*. *Bacillus* was the most abundant genus identified in both snails, with five genera occurring at $\geq 1\%$ relative abundance in at least one snail species. Gut-microbe mean relative abundances are summarized in Figure 2. At the family level, five classifications were significantly more abundant in *Ca. decisum*: families Pseudomonadaceae, Enterobacteriaceae, Mycobacteriaceae, and Staphylococcaceae and order Rhizobiales unclassified to

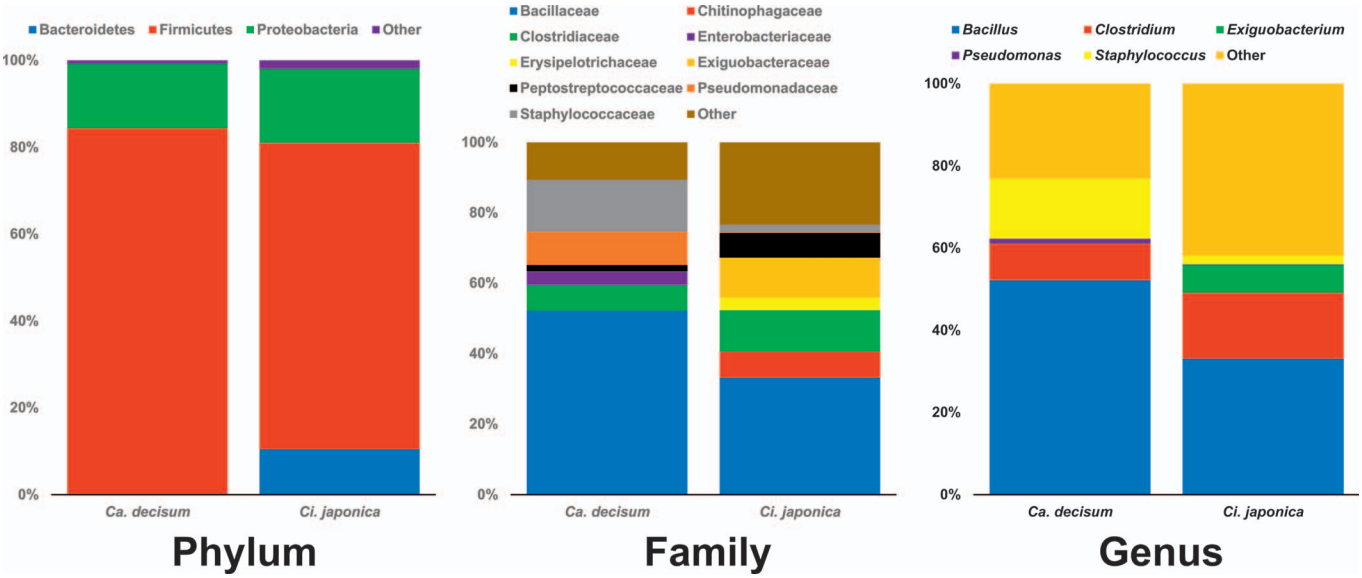

Figure 2. Mean gut-microbe relative abundances between *Campeloma decisum* and *Cipangopaludina japonica*. Values are mean relative percent abundance by snail species. Classification is the lowest hierarchical level that ASVs were assigned to and had relative abundances $\geq 1\%$ in at least one species; the remaining groups are placed in "Other."

family. In *Ci. japonica*, two classifications were significantly more abundant: bacteria unclassified beyond kingdom Bacteria and the phylum Proteobacteria. Analysis of genus-level classifications indicated similar differences compared to those seen at the family level, with the addition of bacteria unclassified beyond family Chitinophagaceae being more abundant in *Ci. japonica*. Comparisons between the snail species with *P*-values, FDR values, and effect sizes are shown in Table 2.

From the unclassified past kingdom and past family Proteobacteria in *Ci. japonica*, 50 ASVs each were compared against GenBank using blastn. All ASVs from the unclassified kingdom group had the same best match, a *Mycoplasma* sp. isolated from *Biomphalaria glabrata* (GenBank accession

Table 2. Significant gut-microbe differences between *Campeloma decisum* and *Cipangopaludina japonica*. Analysis indicates at which taxonomic level the two species were compared. Classification is the lowest hierarchical level that ASVs were assigned in the comparison. Species column reflects in which species the bacterial group was detected at the significantly higher relative abundance. Statistics are the estimated *P*-values derived from Welch's *t*-tests, Benjamini-Hochberg false discovery rates (FDR), and effect sizes. Significance was measured at FDR $\alpha < 0.1$.

Analysis	Classification	Species	*P*	FDR	Effect size
Phylum	Kingdom Bacteria	*Ci. japonica*	0.0130	0.076	0.836
Family	Family Pseudomonadaceae	*Ca. decisum*	0.0009	0.024	0.891
	Family Enterobacteriaceae	*Ca. decisum*	0.0097	0.071	0.755
	Family Mycobacteriaceae	*Ca. decisum*	0.0076	0.049	0.972
	Family Staphylococcaceae	*Ca. decisum*	0.0018	0.032	0.894
	Order Rhizobiales	*Ca. decisum*	0.0080	0.064	0.812
	Kingdom Bacteria	*Ci. japonica*	0.0118	0.073	0.835
	Phylum Proteobacteria	*Ci. japonica*	0.0001	0.005	1.371
Genus	Family Pseudomonadaceae	*Ca. decisum*	0.0005	0.017	1.089
	Family Enterobacteriaceae	*Ca. decisum*	0.0109	0.087	0.773
	Genus *Mycobacterium*	*Ca. decisum*	0.0088	0.067	0.931
	Genus *Staphylococcus*	*Ca. decisum*	0.0034	0.060	0.801
	Order Rhizobiales	*Ca. decisum*	0.0093	0.086	0.800
	Genus *Pseudomonas*	*Ca. decisum*	0.0091	0.096	0.691
	Kingdom Bacteria	*Ci. japonica*	0.0123	0.091	0.812
	Family Chitinophagaceae	*Ci. japonica*	0.0051	0.056	0.953
	Phylum Proteobacteria	*Ci. japonica*	0.0001	0.008	1.371

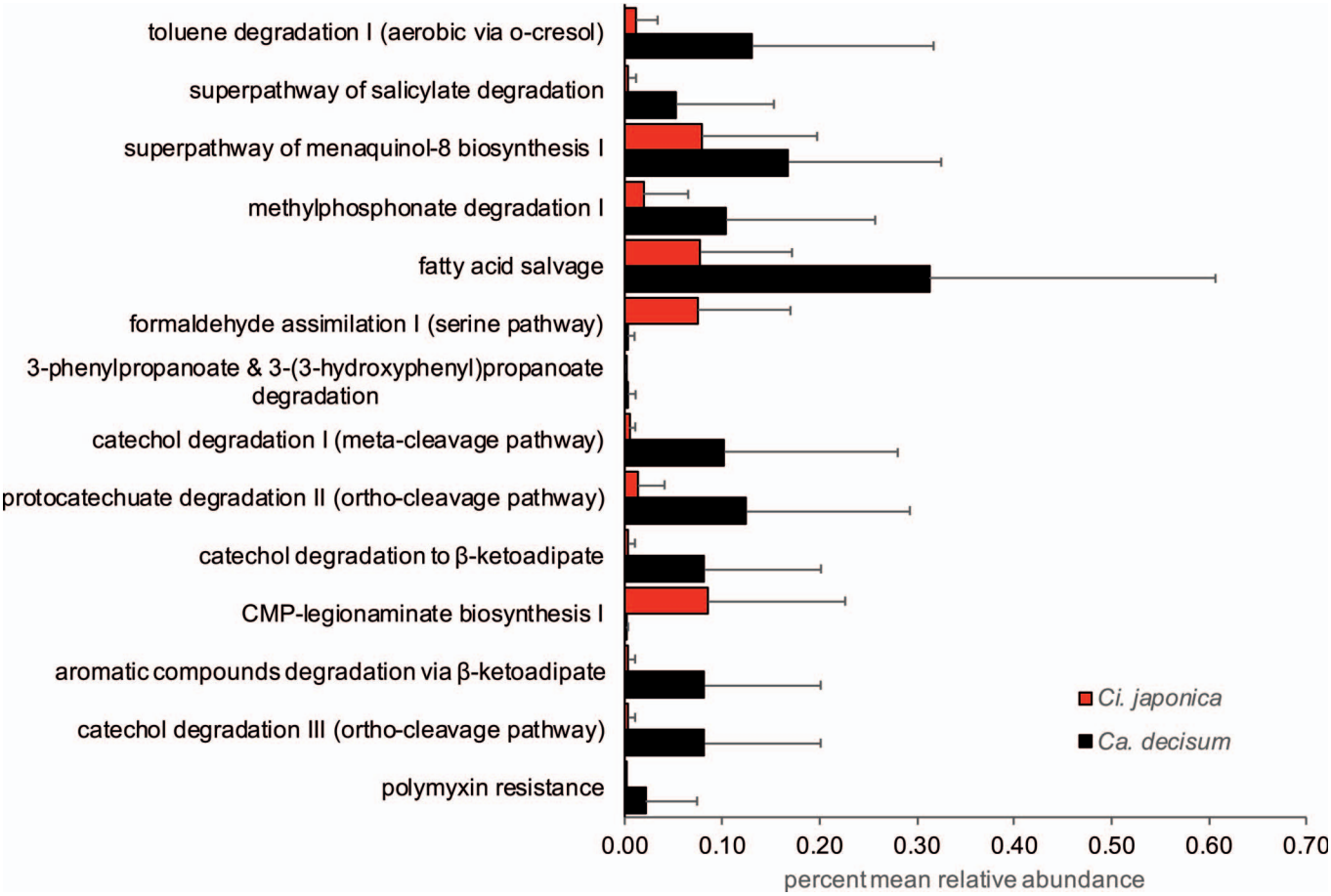

Figure 3. Functional pathway abundances for *Campeloma decisum* and *Cipangopaludina japonica*. Only those pathways that differed significantly (FDR < 0.1) between snail species are shown. Error bars represent one standard deviation.

number CP013128). All ASVs from unclassified Proteobacteria matched best to an unclassified Gammaproteobacteria isolated from the gut of *Achatina fulica* (JN211207).

PICRUSt2 analysis of predicted metagenomes identified peptidoglycan synthesis, pyruvate fermentation, and aerobic respiration by cytochrome *c* as the three most abundant microbial pathways represented in the viviparid gut; they were the only pathways to occur at greater than 1% relative abundance in each snail species. Significant differences in predicted function between *Ca. decisum* and *Ci. japonica* were detected in 14 pathways (Fig. 3). CMP-legionaminate biosynthesis and formaldehyde assimilation pathways were more abundant in *Ci. japonica*. In *Ca. decisum*, degradation pathways (aromatic compounds, catechol, methylphosphonate, proponoate, protocatechuate, salicylate, toluene), fatty acid salvage, menaquinol-8 biosynthesis, and polymyxin resistance pathways were more abundant.

DISCUSSION

Given the importance of gut microbes in an animal's life history and the paucity of knowledge regarding the gut flora of freshwater snails, we aimed to better understand the gut microbial communities in *Ca. decisum* and *Ci. japonica*. Our results represent the first efforts to estimate the diversity and predicted function of gut bacteria in Viviparidae and the second examination of gut flora from a freshwater snail species native to North America (Van Horn et al. 2012). Our data supported our first prediction that *Ca. decisum* and *Ci. japonica* would possess significantly different alpha and beta diversities. Microbiome estimates from both species were not significantly different in terms of ASV richness, evenness, or phylogenetic diversity. Additionally, beta diversity measured by generalized UniFrac distances did not significantly differ between samples from the two species. In general, gut microbiomes arise from two main sources: vertical parental transmission and horizontal environmental acquisition (Rothschild et al. 2018). Research has consistently shown that the environment plays the largest role in shaping gut microbial communities (Preheim et al. 2011; Schmidt et al. 2019). Since our snail samples were taken from the same locality during the same collection event—and given their similar known ecologies—we were unsurprised to find no significant differences between diversity measures in *Ca. decisum* and *Ci. japonica*.

In both *Ca. decisum* (84.24%) and *Ci. japonica* (70.58%),

bacteria in Firmicutes showed the highest relative abundance, with Proteobacteria being the next most abundant (14.75% and 17.02%, respectively). Proteobacteria have been observed to be the most abundant microbial phylum in the gut of terrestrial, marine, and freshwater snails (Pawar et al. 2012; Lyra et al. 2018; Ito et al. 2019). Of the snail species whose gut flora have been studied, the majority are either herbivores or periphyton grazers/scrapers (e.g., *Achatina fulica*, *Biomphalaria glabrata*, *Radix auricularia*, and *Batillus cornutus*). Rare exceptions exist, including the deep sea, bone-eating *Rubyspira osteovora* and the generalist *Pomacea canaliculata* (Johnson et al. 2010; Oosterom et al. 2016). In our viviparid samples, Firmicutes were most abundant. Members of this group have been found in low abundance among snail gut microbes and are slightly more abundant in freshwater species (Takacs-Vesbach et al. 2016; Lyra et al. 2018; Huot et al. 2020). Firmicutes do, however, compromise a major component (up to 64%) of the gut flora associated with soil-dwelling invertebrates, including earthworms, isopods, springtails, and millipedes (König 2006). Given that *Ca. decisum* and *Ci. japonica* filter feed and process detritus from sediment, their diets may include food sources more similar to those found in soil habitats than to herbivores or periphyton ingesters. Within Firmicutes, Bacillaceae and *Bacillus* were the most abundant in the two viviparid species. This suggests that the diets of *Ca. decisum* and *Ci. japonica* contain many organic plant polymers, including cellulose and hemicellulose. *Bacillus* are capable of digesting available carbohydrates and recalcitrant biological materials, such as chitin and lignocellulose (König et al. 2006). *Clostridium* (Clostridiaceae) were also abundant in both snails' digestive tracts, further reflecting a diet heavy in plant polysaccharides (Boutard et al. 2014).

Significant differences in relative microbial abundance were observed for several bacterial groups in *Ca. decisum* and *Ci. japonica* and, thus, did not support our second prediction. In *Ca. decisum*, Pseudomonadaceae and *Pseudomonas* were two of the more abundant groups. Pseudomonads are better known from the gut flora of terrestrial snails than freshwater snails (Nicolai et al. 2015; Takacs-Vesbach et al. 2016; Hu et al. 2018) and may participate in the anaerobic hydrolysis of plant carbohydrates (Buettner et al. 2019). Enterobacteriaceae, Staphylococcaecae, and *Staphylococcus* were also more abundant in *Ca. decisum*. Enterobacteriaceae are commonly found in animal gut microbiota, where they ferment sugars to lactic acid and other products; in addition, most can reduce nitrate to nitrite (Octavia and Lan 2014). Staphylococci are known from the digestive systems of freshwater fish and mussels but are rarely represented in snails (Jami et al. 2015; Weingarten et al. 2019). The role of these bacteria in the animal gut is poorly known, though they may provide mechanisms for hydrocarbon breakdown (Kayath et al. 2019).

In studies of animal gut microbes, many bacteria remain unclassified by the methods employed or are able to be classified only at higher taxonomic levels (Thomas and Segata 2019). This was the case for *Ci. japonica*, where bacteria that could not be classified past kingdom and those that could not be classified past Proteobacteria were significantly more abundant relative to *Ca. decisum*. Using blastn, we were able to match subsets of each group to microbes isolated from other snail taxa. All 50 ASVs from the unclassified bacterial kingdom group matched to a *Mycoplasma* sp. isolated from the freshwater planorbid *Biomphalaria glabrata*. We found it interesting that *Mycoplasma* was detected in *Ci. japonica* but not in *Ca. decisum. Mycoplasma* species are well-characterized intracellular animal parasites (Razin et al. 1998). Invasive species may harbor bacteria from their native range, but they also develop novel associations with microbes found where they are introduced (Bankers et al. 2020). While present in *Ci. japonica*, these unclassified, *Mycoplasma*-like bacteria were present in low numbers (0.34% mean abundance). We hypothesize that *Ci. japonica* may be more susceptible to *Mycoplasma* infection in its introduced range than the indigenous *Ca. decisum*. All 50 ASVs from unclassified Proteobacteria were most similar to an unclassified Gammaproteobacteria isolated from the gut of the land snail *Achatina fulica* (Pawar et al. 2012). Gammaproteobacteria are common animal gut microbes, and the unclassified ASVs suggest the presence of novel bacterial taxa from the family in *Ci. japonica*.

Gut bacteria comprise both those microbes that live in the animal host symbiotically and those that are ingested as food or ingested nonselectively through feeding. We found groups that were likely ingested by the snails, given that they are considered environmental taxa and not present in animal digestive systems. *Cipangopaludina japonica* had a high, but not significantly different, relative abundance of *Exiguobacterium* (Exiguobacteraceae). These bacteria are ubiquitous in soil and freshwater and have been shown to break down a variety of plant carbohydrates (Kasana and Pandey 2018). Chitinophagaceae were significantly more abundant in *Ci. japonica*. As their name implies, these bacteria can hydrolyze chitin from the environment and are found primarily in soils and aquatic sediments (Lim et al. 2009; Madhaiyan et al. 2015). They are often poor hydrolyzers of plant carbohydrates such as cellulose and starch, but they can ferment sugars into organic acids (Sangkhobol and Skerman 1981). Their abundance in *Ci. japonica* may be a result of untested dietary or microhabitat differences between snail species. Bacteria from the order Rhizobiales were significantly more abundant in *Ca. decisum*. Rhizobiales are frequently associated with plants; some taxa are nitrogen-fixing bacteria associated with the rhizosphere, while others are intracellular pathogens (Delmotte et al. 2009). Mycobacteriaceae and *Mycobacterium* were also significantly more abundant in *Ca. decisum*. These are ubiquitous soil bacteria and not gut flora (Pontiroli et al. 2013). The relative abundance differences of these in *Ca. decisum* versus *Ci. japonica* suggest diet or microhabitat differences between the two snails, as the snails are ingesting and processing different materials.

Our data also failed to support our third prediction, since significant differences were observed in the predicted microbial community functions of *Ca. decisum* and *Ci.*

japonica, although in pathways of low abundance (1.65% of total abundance summed across functions). In *Ca. decisum*, degradation pathways for aromatic compounds, catechol, protocatechuate, and salicylate are all associated with *Pseudomonas* species that were significantly more abundant (e.g., Chan et al. 1979; Harayama and Rekik 1990; Díaz 2004). These degradation pathways are interconnected mechanisms for bacteria to break down environmental pollutants such as phthalates, hydroxybenzoates, and toluene (Parales and Harwood 1993; Przybylińska and Wyszkowski 2016). The fatty acid salvage pathway also appears to be an additional means for *Pseudomonas* to produce long-chain fatty acids (Yuan et al. 2012). Phenylpropanoate degradation, a pathway for the breakdown of aromatics in Proteobacteria (Burlingame and Chapman 1983), was also more abundant in *Ca. decisum*. We hypothesize that the significant abundance of *Pseudomonas* and degradation pathways were the result of snail hosts adapting to environmental contamination. While the Flat River is considered a relatively "healthy" river, agricultural runoff, septic systems, and other human activities are thought to be pollution sources (Michigan Department of Environmental Quality 2006). These activities generate significant amounts of aromatic contaminants that can end up in freshwater sediments (Malaj et al. 2014). By harboring more pseudomonads and Proteobacteria that can degrade and metabolize the contaminants, *Ca. decisum* may increase their survivorship in polluted fresh waters. *Cipangopaludina japonica* may possess other pathways to deal with contamination or may not be able to host native pseudomonads as readily as *Ca. decisum.*

Other functional pathways that lacked clear correlations to the environment were more abundant in *Ca. decisum*. Menaquinol-8 biosynthesis, methylphosphonate degradation, and polymyxin resistance all showed higher abundance in *Ca. decisum*. Menaquinols function in the bacterial electron chain; they also participate in the production of vitamin K2 for their animal hosts (Meganathan 2001). Phosphonate degradation provides bacteria with an alternate source of phosphorous in addition to the breaking of phosphoester bonds of phosphates (Huang et al. 2005). Polymixins are polypeptides produced by Gram-positive Bacilliaceae that disrupt the outer membrane of Gram-negative bacteria. These results may suggest that *Ca. decisum* has a different requirement for vitamin K2 than *Ci. japonica* and possibly needs alternate sources of phosphorous. The presence of polymyxin resistance may be tied to the increased abundance in *Pseudomonas*, which are known to acquire resistance and may require it to persist in the same microbiome with polymyxin-producing Bacilliaceae (Tam et al. 2005).

Two bacterial pathways were more abundant in the predicted metagenomes from *Ci. japonica*. The formaldehyde assimilation pathway is used by methanotrophic bacteria to oxidize methane into formaldehyde, which can be used to form intermediates needed for other metabolic pathways (Quayle and Ferenci 1978). The increased presence of methane oxidation implies that *Ci. japonica* is directly or indirectly ingesting more material from anaerobic sediment than *Ca. decisum*. While both species burrow into the sediment, this finding may suggest that *Ci. japonica* spends more time burrowed or burrowed deeper than *Ca. decisum* (Szal and Gruca-Rokosz 2020). CMP-legionaminate biosynthesis was also more abundant in *Ci. japonica*. This pathway is involved in sialic acid metabolism, one means by which pathogenic bacteria can avoid the host immune system (Schoenhofen et al. 2009). Abundance of the pathway may correlate with the potential presence of *Mycoplasma* in *Ci. japonica* and not *Ca. decisum*. The binding of *Mycoplasma* to host cells is modulated by sialic acid residues (Nishikawa et al. 2019) and may explain why the pathway is significantly more abundant in *Ci. japonica*.

Our results highlight limitations and paths for future research on freshwater snail gut microbes. Next-generation, high-throughput sequencing methods have become the standard for exploring microbial diversity using 16S sequences (Poretsky et al. 2014). Our data were generated using Ion Torrent chemistry, an older method that generates fewer sequence reads per sample with higher error rates. Illumina technology is seen as superior, generating higher numbers of more accurate reads, though each method biases its results differently (Salipante et al. 2014). While both methods generate statistically consistent taxonomic and functional microbial profiles when read numbers are similar (Onywera and Meiring 2020), the increase in read number from Illumina methods may provide more adequate sampling of the gut flora. Also, our use of the intestine and partial posterior stomach for analysis may have been suboptimal, since separate digestive compartments possess their own set of microbes (Pawar et al. 2012). We were able to minimize some variation in our data by using snails collected from the same location at the same time but did not tightly control for how much stomach was used. We also did not assess the microbial diversity of the water and sediment at the collection site, so our determinations of enteric versus environmental taxa may be skewed or incorrect. Although they were from the same site, it is unknown whether the two snail species occupied identical microhabitats. Subtle differences in microhabitats may be reflected in the significant differences in low-abundance groups and functions (Fiore et al. 2020). Finally, our results indicated that Firmicutes were the major component of viviparid gut flora. This finding is in sharp contrast to all other mollusks whose gut microbes have been assessed. Determining why Firmicutes were the dominant phylum and not others, namely Proteobacteria, would allow a better integrative approach to understanding viviparid diet, metabolism, habitat usage, and life history. More complete pathway analysis and biochemical testing would also test hypotheses of taxon and pathway abundances that differ between species.

ACKNOWLEDGMENTS

We thank David Zanatta for specimen collection. Heath Mills, Michael LaMontagne, and Clinton Creech offered microbiology advice throughout the project. Three anonymous

reviewers provided constructive comments and suggestions. Funding was provided in part by a Texas Academy of Science undergraduate research grant to EN and by Gannon University departmental funds to RLM.

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Freshwater Mollusk Biology and Conservation 24:114–123, 2021

DOI:10.31931/fmbc-d-20-00011

REGULAR ARTICLE

INVASIVE BLACK CARP AS A RESERVOIR HOST FOR THE FRESHWATER MOLLUSK PARASITE *ASPIDOGASTER CONCHICOLA*: FURTHER EVIDENCE OF MOLLUSK CONSUMPTION AND IMPLICATIONS FOR PARASITE DISPERSAL

Barry C. Poulton[1]*, Jennifer Bailey[2], Patrick T. Kroboth[1], Amy E. George[1], and Duane C. Chapman[1]

[1] *U.S. Geological Survey, Columbia Environmental Research Center, 4200 E. New Haven Road, Columbia, MO 65201 USA*

[2] *U.S. Fish and Wildlife Service, La Crosse Fish Health Center, 555 Lester Avenue, Onalaska, WI 54650 USA*

ABSTRACT

Black Carp (*Mylopharyngodon piceus*) has invaded the Mississippi River and is a potential threat to native mollusks. During prior diet research, we discovered that the fluke *Aspidogaster conchicola*, a mollusk parasite, occurs regularly in the gastrointestinal tract of Black Carp. The fluke remains in fish intestines for extended periods after the fish has consumed its host. Flukes were found in 33% of the wild Black Carp examined, and numbers ranged from 1 to 802, with no pattern evident across seasons of fish capture. Treating the flukes as indicators of prior mollusk consumption, we adjusted the percent occurrence of mollusks from 26.6% to 54.1%, indicating that the previously reported incidences for bivalves (22.8%) and gastropods (16.5%) in the diet of wild Black Carp are likely to be underestimated. Based on percent occurrences in Black Carp, larger fish (>791 mm) had significantly higher fluke occurrence (42.6%) and fish captured from lentic habitats had significantly greater fluke-adjusted mollusk occurrence (87.5%). These diet-occurrence estimates, coupled with the presence of gravid *A. conchicola* and evidence of their continued viability in Black Carp intestines, indicate that these fish retain evidence of mollusk consumption for extended periods after evacuation of the gastrointestinal tract. Consequently, Black Carp has the potential to disperse this parasite to other mollusks.

KEY WORDS: invasive carp, reservoir host, fluke parasites, mollusks, diet

INTRODUCTION

Aspidogastrean trematodes, such as the common and widespread *Aspidogaster conchicola* Von Baer (Trematoda: Aspidogastridae), are regularly encountered parasites in freshwater unionid mussels and gastropods in the United States (Hendrix et al. 1985; Alves et al. 2015). This group of flukes can reside as a secondary occurrence in vertebrate species that feed on their hosts, including fish and turtles (Fulhage 1954; Rohde 1972, 2002). Currently, however, direct infection of vertebrate hosts by eggs or larval stages is unknown in this group of flukes. Compared with digenetic flukes with life cycles that include intermediate hosts, this fluke has a more ancestral life cycle (Olson et al. 2003) with direct development that requires only one host for maturation. Infection occurs when a mollusk ingests the egg stage during feeding on benthic substrates (i.e., grazing by snails) or—in unionid mussels—through their filtering apparatus (Huehner 1984). Autoinfection also has been suggested in this species, where the entire ontogenetic development may occur within one host individual (Williams 1942; Rohde 1973, 1994). Nonciliated fluke larvae (aspidocidia; Huehner and Etges

*Corresponding Author: bpoulton@usgs.gov

1977) hatch immediately after uptake and begin maturation, most often residing in the pericardial cavity, renal cavity, or viscera of the host mollusk (Huehner and Etges 1981); however, under certain conditions, the eggs or young larvae may become encapsulated or encysted in certain body tissues (Pauley and Becker 1968). This fluke possesses a ventral adhesive disk (opisthaptor) used as a holdfast, a sensory organ, and a source for secretion of digestive enzymes (Bakker and Diegenbach 1974; Fredricksen 1980). Adult flukes feed on the hemolymph and epithelial cells of the host by ingestion through the mouth (Bakker and Davids 1973; Huehner et al. 1989). Fish and other vertebrates consume infected mollusks and act as facultative hosts for *A. conchicola* (i.e., reservoir hosts), where adult flukes remain in the intestinal tract for extended periods after tissues of the mollusk host are digested (Rohde 1972; Evtushenko et al. 1994). The fluke *A. conchicola* has been reported in the intestinal tract of several freshwater fish species worldwide (Alves et al. 2015), including Grass Carp (*Ctenopharyngodon idella*) and Black Carp (*Mylopharyngodon piceus*) in Eurasia, and most recently, in wild-caught Black Carp of the Mississippi River Basin (Poulton et al. 2019). In Eurasia, among the parasites found in Zebra Mussels (*Dreissena* spp.), *A. conchicola* is the only fluke species that is also native to North America (Molloy et al. 1997).

The Black Carp is one of four invasive species that are commonly referred to as Asian carps; the others are Bighead Carp (*Hypophthalmichthys nobilis*), Silver Carp (*Hypophthalmichthys molitrix*), and Grass Carp (Kocovsky et al. 2018). These fish are receiving increasing attention in the United States because of their potential adverse effects on native species and aquatic ecosystems (Chapman and Hoff 2011; Nico and Neilson 2019; USFWS 2019). Black Carp was imported to the Unites States in the 1970s and 1980s to control digenetic trematodes in aquaculture by consuming their snail hosts; the intent was to improve the quality of aquaculture-produced food fishes (Venable et al. 2000; Ledford and Kelly 2006). Black Carp is characterized by molariform pharyngeal teeth adapted to crush mollusk shells during feeding (Liu et al. 1990; Shelton et al. 1995; He et al. 2013). The known molluscivorous habits of Black Carp—in combination with the expanding geographic range of wild fish within the Mississippi River Basin (Kroboth et al. 2019)—have led to concern that they may threaten populations of native and imperiled unionid mussel species in the United States (Nico et al. 2005; DeVaney et al. 2009; Nico and Jelks 2011; Hodgins et al. 2014). Most of the research available on Black Carp has been based on aquaculture studies. For example, Nico et al. (2005) summarized accounts for trematode parasites that reside in Black Carp muscle or liver tissue and use mollusks as intermediate hosts, but further noted the lack of information on parasites infecting wild populations. Diet, diseases, parasites, and ecological consequences of establishment have been identified as key components in the ongoing assessments of Black Carp invasion risk into the Great Lakes region (D.A.R. Drake, Fisheries and Oceans Canada, Burlington, Ontario, Canada, personal communication) and Mississippi River Basin (ACRCC 2019; USFWS 2019).

Understanding the frequency and extent of mollusk consumption in Black Carp is important for evaluating both diet composition and risk of invasion to native mollusks as well as for assessing effects on invasive mollusks. Bivalves and gastropods in the diet are most easily identified through their shells or shell fragments, but sometimes they cannot be identified or counted because these fish partially regurgitate mollusk shell material or expel fragments without swallowing them. During recent examination of wild Black Carp diets (Poulton et al. 2019), the fluke *A. conchicola* was commonly encountered in the gastrointestinal tract, even in fish that contained no other evidence of mollusk consumption. The ecological significance of this discovery was unknown at the time of that publication, but we recognized the need to further investigate the presence of these flukes in Black Carp and their importance to freshwater mollusks. As a reservoir host, Black Carp may share the same life stage of *A. conchicola* with the primary host mollusks, but also could serve as a source of infective organisms (Haydon et al. 2002) in addition to providing evidence that primary hosts were consumed in the diet some time before capture. Here, we report the specific interpretation of fluke incidence in the gastrointestinal tract from the original 109 wild Black Carp examined (Poulton et al. 2019), including the treatment of flukes as indicators of previous mollusk consumption and the potential role of Black Carp as a vector for dispersal of *A. conchicola*. Specifically, our study goals were to (1) report the abundance (infection rate) of *A. conchicola* flukes in wild Black Carp and any seasonal patterns in their numbers among fish examined, (2) provide an alternate method in estimating percent occurrence of mollusks in Black Carp diet based on the presence of flukes, and (3) test the significance of fluke and mollusk occurrence among fish of different sizes and the habitats where fish were captured. We also review the current ecological knowledge on *A. conchicola* flukes as related to freshwater mollusks, including longevity, viability, host pathways, potential damage to host tissues, and parasitic transmission. We also discuss the potential role of Black Carp as a vector of this mollusk parasite and summarize our study findings considering the risk to mollusks within the current range of Black Carp in the Mississippi River Basin. Our intent is to improve the understanding of linkages between wild Black Carp and mollusk hosts and to provide critical information for mollusk conservation efforts in riverine systems.

FIELD-SITE DESCRIPTION

Wild Black Carp, captured year-round from the Mississippi River Basin during 2009–2017, were acquired through collaborative research efforts involving commercial fisherman, federal and state agencies, and universities. They were used for multiple research projects, including distribution monitoring, life history, genetics, determination of origin, and age and growth. Black Carp were captured with hoop nets (51%; the

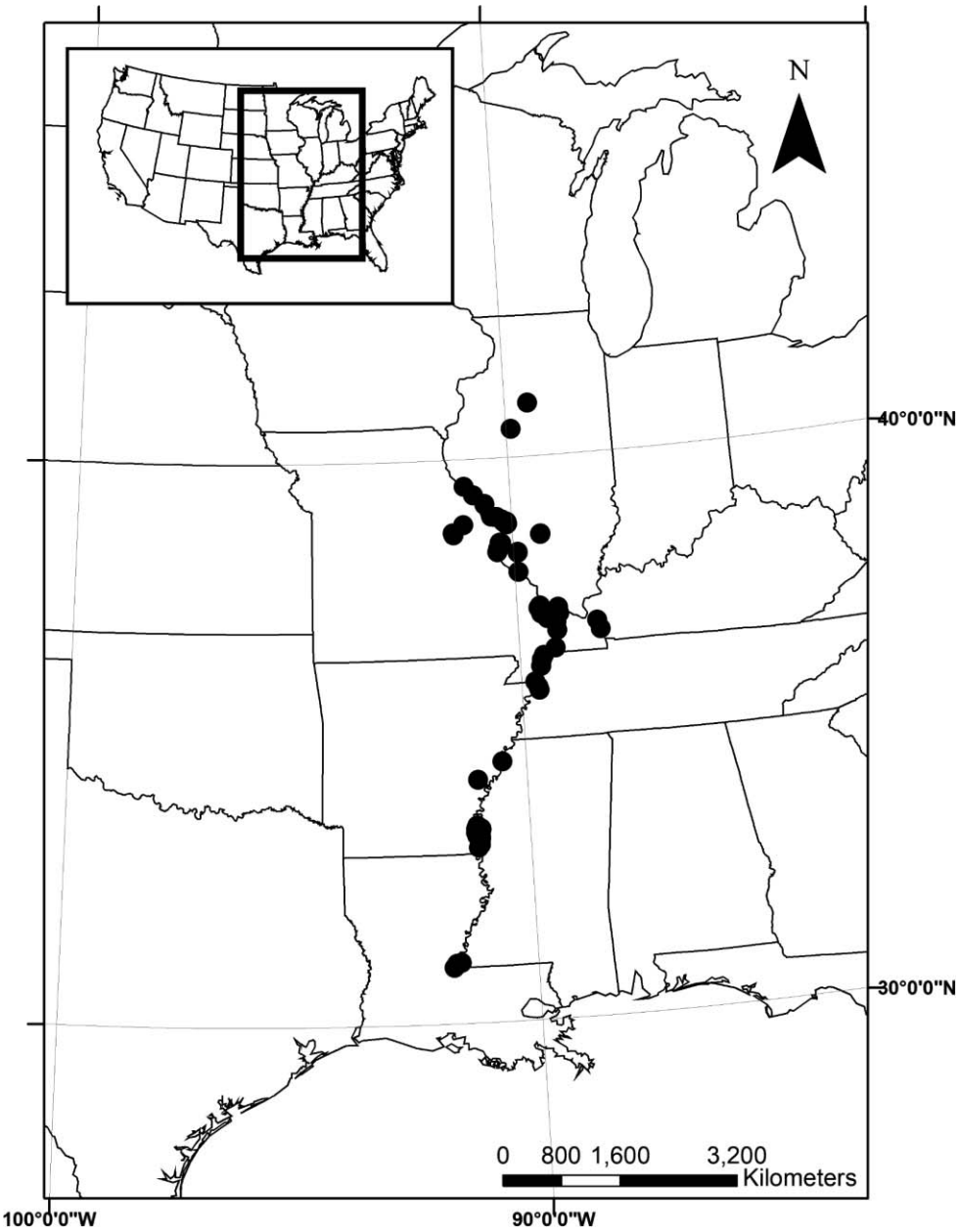

Figure 1. Capture locations of Black Carp (*Mylopharyngodon piceus*) examined in this study from the Mississippi River Basin in 2009–2017 ($n = 109$).

others were captured with various or unreported methods) by commercial fishing activities in riverine and backwater habitats. The geographic range of samples included the Mississippi River mainstem and the Atchafalaya, Cumberland, Illinois, Kaskaskia, Ohio, and White River basins (Fig. 1). Specific fish capture locations, methods, and dates are available at https://doi.org/10.5066/P9K88CWF.

METHODS

Gastrointestinal tract samples were dissected from 109 wild-caught Black Carp and preserved in formalin for diet analysis. Laboratory preparation methods, taxonomic identification of contents, and taxa-specific data analysis for these samples are detailed in the recently published diet study (Poulton et al. 2019). Flukes removed from these samples were stained and mounted on glass slides for species identification via electron microscopy, based on descriptions by Bailey and Tompkin (1971), Hathaway (1971), Halton and Lyness (1971), and Huehner and Etges (1977). To investigate any seasonal patterns in flukes, we compared fluke abundance (number of flukes found in individual fish) across seasons (astronomical winter, spring, summer, and fall). To provide additional comparisons of mollusks and flukes present in the gastrointestinal tract, we used frequency of occurrence based on diet data (Poulton et al. 2019). This occurrence is defined as

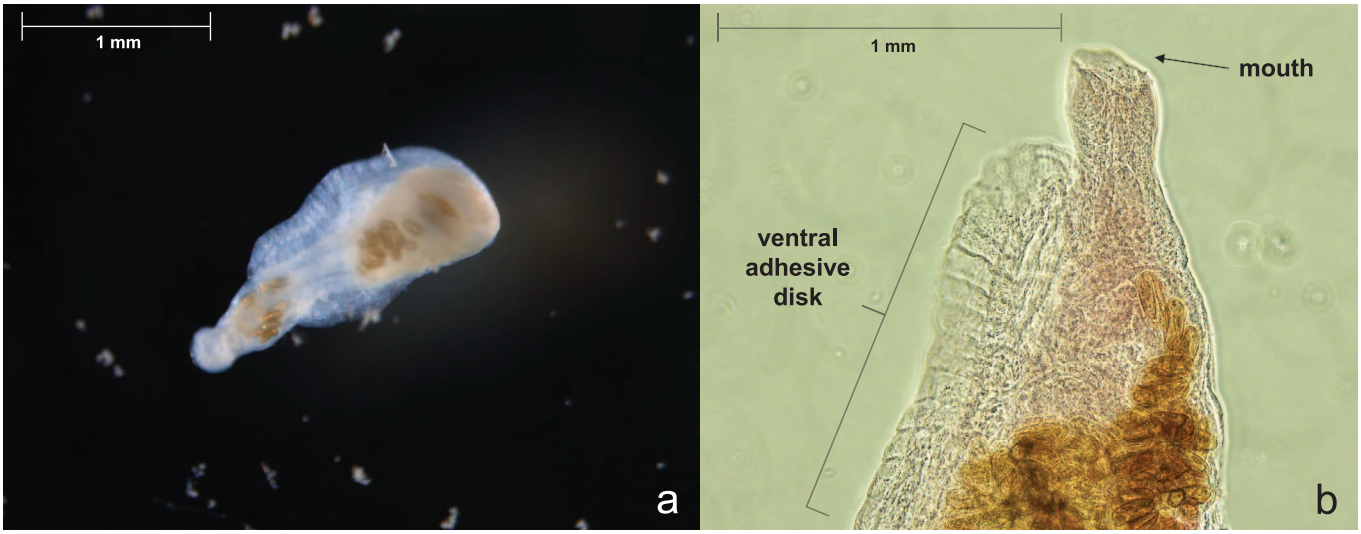

Figure 2. The fluke *Aspidogaster conchicola* found in the gastrointestinal tract of Black Carp (*Mylopharyngodon piceus*) captured from the Mississippi River Basin in 2009–2017. The images represent (a) dorsal view, showing gravid fluke with eggs, and (b) lateral view, showing buccal funnel (mouth) and opisthaptor (adhesive disk). Photograph© (a) Anne Herndon, (b) Jennifer Bailey.

the relative number of fish (percentage of total examined) that contained each specific diet group or taxa (percent incidence of Buckland et al. 2017). This diet measurement, as used for inventory of preserved gastrointestinal contents in this study, is based on presence–absence data only and does not take into account the abundance of diet items (i.e., in samples or the environment) or the timing of feeding, ingestion, or digestion (i.e., when items were ingested or gut evacuation rates).

For comparisons and statistical analyses, we recalculated percent occurrence for mollusks among diet samples, accounting for the presence of flukes as an indicator of prior mollusk consumption (=fluke-adjusted mollusks). We accomplished this by using the original mollusk occurrence estimate of 26.5% (Poulton et al. 2019) and adding percent occurrence estimates for the following: (1) fish containing *A. conchicola* flukes only and (2) fish containing flukes co-occurring with nonmollusk diet items. To investigate whether fluke occurrence differed among fish size classes or capture locations, we compared fluke classes (percent occurrence of flukes and fluke-adjusted mollusks) across two fish sizes (above and below the median total length, in millimeters) and two fish capture location habitats (lotic, mainstem flowing water habitats, including side channels and chutes; and lentic, off-channel areas, including backwater sloughs, oxbows, or reservoirs). Because of nonnormality of the data and bias associated with fish capture, we used nonparametric Kruskal–Wallis/Mann–Whitney U-tests ($\alpha = 0.05$) for these comparisons (Excel formatted for analysis; Microsoft, Redmond, WA, USA). Based on this information, we reinterpreted the contribution of mollusks to Black Carp diets and provide discussion of implications related to the risk of Black Carp invasion on native freshwater mollusks and the potential of *A. conchicola* dispersal.

RESULTS

The fluke *A. conchicola* (Fig. 2) was present in 36 (33%) of the 109 wild Black Carp examined. The abundance of flukes within individual fish (mean = 50.1, SE = 23.1) varied widely from 1 to 802, and seasonal means ranged from 3.4 to 122.4, with the highest mean in fish captured during spring (Fig. 3). All five fish with more than 100 flukes in their gastrointestinal tract were captured during May–September, but we found no significant differences in fluke abundances in Black Carp across seasons (Fig. 3). This fluke was the only diet item found in the gastrointestinal tracts of 18 fish and co-occurred with nonmollusk diet items in 12 additional fish (Fig. 4). Only 6 of the 36 fish containing flukes had shell remains of mollusks; the other 30 fish were added to the calculation of percent mollusk occurrence to account for prior ingestion of fluke-infected mollusk prey items, resulting in an estimated mollusk occurrence of 54.1%. Fluke percent occurrence did not differ significantly between capture habitats (Table 1), but was significantly higher in larger fish above the median of 791 mm total length (42.6%, $P = 0.04$). Fluke-adjusted mollusk occurrence did not differ significantly between the two Black Carp size classes (Table 1), but was significantly higher in fish captured from the lentic vs lotic habitats (87.5%, $P = 0.005$).

DISCUSSION

In general, the ecology and effects of intestinal flukes in freshwater fish are more poorly understood than those that infect other tissues or organs, carry human diseases, or require intermediate hosts for development. Because of the widespread distribution, more ancestral life cycle, and extensive list of host species, *A. conchicola* is relatively well known among fish trematodes, although it has no known medical importance to humans. Black Carp was reported as a reservoir host for *A.*

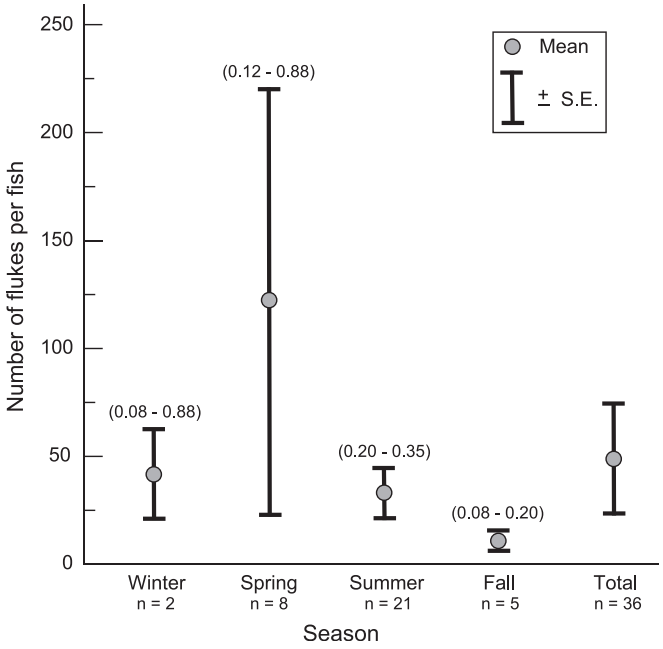

Figure 3. Fluke abundance by season of fish capture (mean number of *Aspidogaster conchicola* per fish examined, ±1 SE) for Black Carp (*Mylopharyngodon piceus*) captured from the Mississippi River Basin in 2009–2017. Only the 36 fish containing flukes are included, and range in *P* values for seasonal comparisons are given in parentheses (Kruskal–Wallis/Mann–Whitney U-tests, $\alpha = 0.05$ significance level).

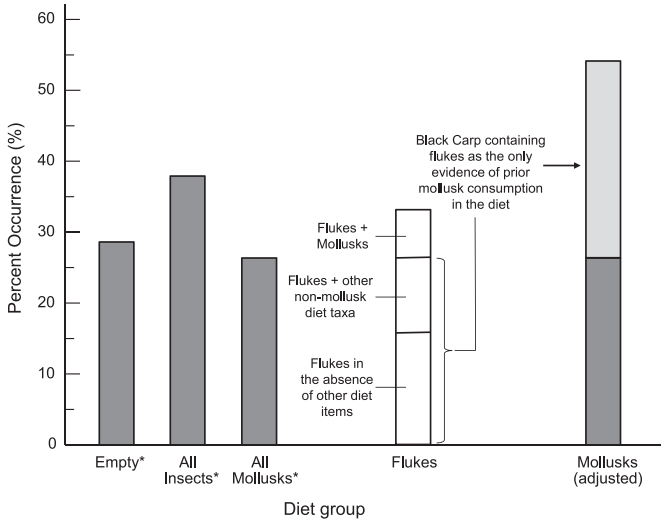

Figure 4. Percent occurrence ($n = 109$) of diet groups found in gastrointestinal tracts of Black Carp (*Mylopharyngodon piceus*) captured from the Mississippi River Basin in 2009–2017 (empty = no identifiable contents). Data with an asterisk (*) shown for comparison are from Poulton et al. (2019), and flukes (*Aspidogaster conchicola*) are further subdivided from data in that publication.

conchicola, resulting from consumption of infected unionid mussels (*Cristaria plicata*) in the Amur River, China (Evtushenko et al. 1994; Nico et al. 2005). According to the aspidogastrean summary provided by Alves et al. (2015), *A. conchicola* has the highest number of known host associations among this group of flukes, with freshwater bivalves and gastropods making up 78% of the total reported (122), although this number may be conservative because endosymbionts infecting imperiled species of mollusks are often overlooked (Brian and Aldridge 2019). Aspidogastrean flukes, including the closely related genus C*otylogaster*, are commonly found in the gastrointestinal tract of benthic-feeding freshwater fishes native to the Mississippi River Basin (Alves et al. 2015), including Blue Catfish (*Ictalurus furcatus*) and Freshwater Drum (*Aplodinotus grunniens*), that also occasionally consume mollusks. Specific U.S. records of *A. conchicola* in fish only included Shorthead Redhorse (*Moxostoma macrolepidotum*) and Common Carp (*Cyprinus carpio*; Alves et al. 2015); thus, our discovery of this fluke in wild Black Carp represents the first U.S. report for this fish species. The occurrence frequencies of *A. conchicola* across populations of other fish species are unknown and thus not available for comparisons. Because *A. conchicola* infects a wide variety of mollusk taxa, we were unable to determine which infected mollusk group(s) was consumed by individual fish.

The fluke *A. conchicola* is known to obtain a higher infection intensity (number of flukes per host individual) than other aspidogastrean species, with as many as 1,545 reported in one individual mollusk (Nelson et al. 1975). Some researchers have reported that flukes are more prevalent in mollusk hosts during winter (Bailey and Tompkin 1971; Halton and Lyness 1971). However, the variable infection intensity for host mollusk taxa and individuals (Huehner and

Table 1. Statistical comparisons of occurrence (percent incidence in the diet) for flukes (*Aspidogaster conchicola*) and mollusks (*, adjusted for fluke presence) based on fish size (total length [TL], median = 791 mm) and capture habitat (lotic, mainstem flowing water habitats, including side channels and chutes; and lentic, off-channel areas, including backwater sloughs, oxbows, or reservoirs) for 109 Black Carp (*Mylopharyngodon piceus*) from the Mississippi River Basin in 2009–2017 (Kruskal–Wallis/Mann–Whitney U-tests, $\alpha = 0.05$ significance level).

Parameter	Comparison	Fluke Occurrence	*P*	Mollusk Occurrence*	*P*
Fish size (TL; mm)	At or below median ($n = 55$)	23.6		47.3	
	vs		0.04		0.20
	Above median ($n = 54$)	42.6		59.3	
Fish capture habitat	Lentic ($n = 16$)	18.8		87.5	
	vs		0.32		0.005
	Lotic ($n = 93$)	35.5		47.3	

Etges 1977; Huehner 1984; Carney 2015) precludes us from relating fluke numbers in Black Carp samples to seasonality in mollusk ingestion rates, diet abundances among prey items, or specific mollusk taxa consumed in the diet. Therefore, we are reporting fluke abundances in Black Carp merely as a reference for comparison across seasons of fish capture and for any future studies that may examine abundance of *A. conchicola* in other vertebrate host species. There are no documented reports in the literature that this fluke species causes harm to fish, although trematodes in general are known to feed on the mucosal layers lining the intestinal tract (Roberts and Janovy 2000; Rohde 2005). A closely related fluke species, *Aspidogaster limacoides* Diesing, that is a common mollusk parasite in Europe is known to significantly damage gut tissues in host fish (Rahanandeh et al. 2016), and this observation may warrant further studies. Similarly, little is known about whether infections of *A. conchicola* can significantly reduce the fitness or viability of host mollusks or their populations. Some have reported changes in tissue histopathology (Michelson 1970) and variability of effects across host species and infection sites (Bakker and Davids 1973; Danford and Joy 1984). Pauley and Becker (1968) documented metaplasia in host tissues with high rates of fluke infection, and Benz and Curran (1997) concluded that *A. conchicola* infections would likely cause adverse effects in host unionid mussels. Pavluchenko and Yermoshyna (2017) also reported an increase in the cardiac index and reduced function in ciliated membranes of the filtering apparatus in unionids as infection rates increased. The ecological significance of *A. conchicola* infection in host mollusks and the extent to which infection might affect their populations, imperilment status, or vulnerability as a prey item are currently unknown. The potential of *A. conchicola* to adversely affect the integrity of host tissues and mollusk fitness in combination with our results suggests that further research on the sublethal effects of *A. conchicola* infections is needed, especially considering the widespread nature of this parasite among freshwater mollusks.

Among the 109 Black Carp examined for diet items (Poulton et al. 2019), the 30 fish containing flukes without other evidence of mollusk consumption (i.e., shell fragments or mollusk structures) likely fed upon mollusks sometime before capture, because mollusk ingestion is the only known pathway for *A. conchicola* infection in vertebrates. The time between mollusk ingestion and fish capture is unknown and cannot be easily approximated with our data, especially because gut evacuation rates are poorly known for Asian carps in general (Nico et al. 2005; Chapman and Hoff 2011). Based on the literature, we conclude that the presence of these flukes is a viable indicator of previous mollusk consumption in Black Carp. Although insects were reported at a higher incidence in diet samples (37.6%, Poulton et al. 2019), our fluke-adjusted mollusk occurrence of 54.1% is consistent with diet accounts for both cultured and wild fish given by Nico et al. (2005) and the common description of Black Carp as a molluscivore. It is likely that the previously reported percent occurrences for gastropods (16.5%), unionid mussels (13.7%), and all bivalve mollusks (22.8%) in the diet of wild Black Carp are lower than actual contributions to the diet because the presence of *A. conchicola* flukes was not accounted for in those estimates. Therefore, omitting these fish from the calculation of mollusk occurrence undervalues both the importance of these organisms in the diet and the risk of Black Carp invasion to freshwater mollusks.

Studies on the longevity, viability, and development of *A. conchicola* within and outside mollusk hosts were partially summarized by Huehner and Etges (1977). This fluke species can survive in water or saline solution for 2–5 wk in the laboratory (Van Cleave and Williams 1943; Rohde 1972), indicating that these flukes can remain viable outside the host for extended periods. However, these flukes are not motile in their adult (sexually mature) stage, with transmission and subsequent infection occurring through the egg, larval, or both forms (Rohde 1972; Bakker and Davids 1973). The *A. conchicola* flukes we found in Black Carp varied in size and growth phase, as described by Huehner and Etges (1977), but their development and maturation while residing within the intestinal tract of host fish have not been evaluated. Fredricksen (1980) suggested that some aspidogastrean fluke species probably continue their growth within the vertebrate hosts after the infected mollusks are consumed. Evtushenko et al. (1994) noted *A. conchicola* remained in the intestine of Black Carp after digestion of the host tissues, but did not specify fluke viability or a specific period. Our data showing 16.5% of fish examined contained this fluke in the absence of other diet items (Fig. 4) imply that the flukes remain in the gastrointestinal tract for extended periods after mollusk consumption, digestion of other diet items, and evacuation of mollusk shell fragments. This also implies that these fish may not have eaten for several hours or days before capture and that *A. conchicola* can withstand the enzyme activity associated with digestion. We recognize that commonly used methods for reporting gastrointestinal contents in fish, such as those outlined in Buckland et al. (2017) for qualitative presence–absence data such as ours, often assume a prey item has been recently consumed within the gut evacuation time period. Although our modified approach to calculating mollusk percent occurrence is nonstandard and beyond this definition, the extended presence of these flukes in a reservoir host such as Black Carp represents evidence of prior mollusk consumption regardless of time period or gut evacuation rate.

The habitats that Black Carp currently occupy within the invaded range of the Mississippi River Basin can be partially inferred by capture information and determination of prey habitats in combination with percent occurrence data for mollusks and *A. conchicola* flukes. Diet information associated with feeding zones and modes of prey capture (Poulton et al. 2019) indicate Black Carp feed on mollusks that are sediment dwelling or attached to hard substrates, both of which are well represented among the diet items consumed by these fish (Poulton et al. 2019) and known mollusk species host fish infected by *A. conchicola* (Alves et al. 2015). When

fluke presence in the gastrointestinal tract is accounted for in mollusk estimates, larger Black Carp (above the median total length for this study of 791 mm) and those captured in lentic environments have significantly higher occurrences (Table 1). Furthermore, of the 18 fish containing *A. conchicola* flukes as the only diet item present, all were collected in lotic environments, indicating that these fish had spent more time evacuating gut contents (i.e., while in hoop nets before retrieval or during transit between feeding locations). Conversely, fish containing mollusks, flukes, or both and captured in lentic habitats may have been actively feeding or had ingested mollusks more recently, as supported by our significantly higher adjusted mollusk occurrence estimates of 87.5% (Table 1) and the significantly greater diet taxa richness in fish collected from these environments (Poulton et al. 2019). However, many of the Black Carp that we examined were acquired from commercial fishers; therefore, the bias associated with season and gear preferences of these activities (including variation in time spent in capture gear such as hoop nets) warrants consideration when interpreting these results. Further investigations related to Black Carp movements and habitat use in riverine systems are underway and are needed to thoroughly assess the effects of Black Carp invasion on assemblages or specific taxa of mollusks.

The ecological importance of Black Carp as a potential carrier or transmitter of *A. conchicola* has not been investigated. Literature suggests that transmission pathways of aspidogastrean flukes among and between mollusk populations are associated with the environmental requirements of host unionids (such as current velocity and substrate factors) and that the dispersal of egg and nonciliated larval stages of *A. conchicola* may be passive (Huehner 1984; Carney 2015). These papers did not specify the mode of fluke transmission or the role of fish carriers, but Ferguson et al. (1999) recognized the passage of fluke eggs through the feces of mollusk-eating turtles as a component of aspidogastrean life cycles. Many of the flukes that we found in Black Carp were gravid (Fig. 2), and laboratory studies have shown that eggs of *A. conchicola* are immediately infective upon uptake by a mollusk host (Huehner and Etges 1972, 1977). If viable *A. conchicola* eggs or larvae pass through the digestive tract of Black Carp during waste evacuation, these fish may be dispersing the fluke to other feeding sites within riverine habitats suitable for colonization of host mollusks. Considering the presence of gravid flukes and their apparent extended longevity in the gastrointestinal tract, it seems likely that Black Carp may play a role in the dispersal of *A. conchicola*, both directly to mollusks and indirectly to other benthic dwelling fishes that feed on mollusks. To date, there are no literature reports that eggs or larvae of *A. conchicola* can infect fish directly. Viability of *A. conchicola* eggs passing through the digestive tract of fish have not been studied; thus far, dispersal by Black Carp can only be inferred by our observance of gravid flukes and the proximity of these fish to the mollusk habitat where they forage.

The invasion and expansion of Black Carp in the United States could deplete native unionid mollusks, a group with documented *A. conchicola* infections. Literature, including recent and ongoing efforts to evaluate Black Carp invasion risks (ACRCC 2019; USFWS 2019), has highlighted adverse effects on native freshwater mollusks as being the primary concern. The list of *A. conchicola* mollusk hosts in the United States (Alves et al. 2015) contains approximately 61 freshwater mussels and 3 gastropods within the currently known range of wild Black Carp in the Mississippi River Basin (Kroboth et al. 2019), including unionids classified as threatened (1), endangered (3), or of special concern (14) based on Williams et al. (1993). To date, Black Carp diet includes six taxa of unionids and two gastropod families from this list (Poulton et al. 2019), as well as *Corbicula* and *Dreissena*, both of which are documented *A. conchicola* hosts in Europe and Canada, respectively. Additional Black Carp are being examined for diet analysis, and we expect the number of mollusk taxa documented in the diet of wild fish (and listed as *A. conchicola* hosts by Alves et al. [2015]) to increase as they further expand their range in the United States. Among the eight freshwater fish hosts of aspidogastrean flukes listed by Alves et al. (2015) that are present in the United States, four are native species and three of them (Shorthead Redhorse, Blue Catfish, and Freshwater Drum) occasionally feed on mollusks. The other four are nonnative species and are known hosts of *A. conchicola*: Common Carp and the three invasive species Black Carp, Grass Carp, and Round Goby (*Neogobius melanostomus* (Pallus)) that occur in the same habitats of riverine ecosystems where mollusks are common. Kelly et al. (2009) theorized that parasitic spillback can occur when newly invasive hosts lead to increases in native parasites, which ultimately may cause higher infection burdens for native hosts. Given that the transmission of aspidogastrean flukes potentially may involve multiple pathways that include both invasive mollusk hosts (*Dreissena* and *Corbicula*) and invasive fish hosts that are currently expanding their ranges in the United States (Black Carp, Grass Carp, and Round Goby), a future increase in *A. conchicola* fluke infections in native unionid mussels and gastropods is theoretically possible, especially considering the high densities that these invasive species can attain (Graney et al. 1980; Schloesser et al. 1996; Johnson et al. 2005; Higgins and Vander Zanden 2010; Sass et al. 2014; Sullivan 2016). These relationships also add another dimension to the knowledge of mussel–fish host associations as described in papers related to unionid mussel life history (Schwalb et al. 2013) and shared parasitism among multiple hosts (Brian and Aldridge 2019). Although *A. conchicola* is widespread, Black Carp acting as a reservoir host could enhance their density or, ultimately, increase infection rates or occurrences within mollusk individuals or populations.

Reservoir–host pathways are important for controlling emerging diseases of domestic animal and wildlife populations (Daszak et al. 2000), but many of these pathways have not been well characterized for species that do not infect humans. Our assessment of *A. conchicola* flukes present in Black Carp

indicates that this fish species is a reservoir host that shares a reciprocal pathway with the freshwater mollusks that it consumes in its diet. Although the capture bias associated with our fish and the qualitative nature of presence–absence diet data preclude us from determining ingestion timing, feeding rates, or specific taxa consumed, the omission of these flukes from mollusk occurrence estimates would seriously undervalue their importance in the diet of wild Black Carp. Our data show that *A. conchicola* flukes remaining in the gastrointestinal tract provide an indication of mollusk consumption even after the digestion of host tissues and the fragments of their shells have been expelled or evacuated as waste. Recently published diet data for wild Black Carp in the United States (Poulton et al. 2019) suggest that this species has a more opportunistic and insectivorous diet than prior species descriptions (Nico et al. 2005) or that their application in aquaculture as a gastropod biological control would suggest (Venable et al. 2000; Ledford and Kelly 2006). Poulton et al. (2019) also noted the ability of Black Carp to expel mollusk shell fragments orally after ingestion and the resulting difficulty in identification of mollusk taxa in diet samples. This limits our ability to determine the potential effects of Black Carp on specific imperiled freshwater mollusk populations without further advances in collection methods and determination of habitats where Black Carp and potentially threatened mollusk taxa co-occur. Not only do mollusks provide a greater relative contribution to their diet than was previously determined (Poulton et al. 2019) but also wild Black Carp currently occupying the Mississippi River Basin are carrying gravid *A. conchicola* flukes with them as they expand their geographic distribution and could be a source of infections to mollusks while they move between habitats and forage in areas with suitable conditions for parasite transmission.

Collectively, literature and our additional interpretation of *A. conchicola* flukes in Black Carp indicate that the effects of this fish and that of fluke infections on native freshwater mollusks warrant further study in several areas of research. Although McElwain (2019) recently discussed the low likelihood that eukaryotic organisms such as flukes would be responsible for undiagnosed die-offs or other declines of unionid mussels, the fact that parasite infection rates are poorly known, especially for rare and imperiled mollusks, may lead to additional research and improvements in parasite detection for these taxa. Relatively few imperiled species are included among the mollusk hosts infected by *A. conchicola* (Alves et al. 2015), but as noted by Brian and Aldridge (2019), endosymbionts have not been adequately surveyed in rare taxa and dead specimens are not available for examination. We suspect that other rare mollusks not yet reported may also be hosts of this fluke, but development of nonlethal detection methods may be needed to accurately assess infection rate, occurrence, and population viability within mollusk assemblages. Furthermore, laboratory studies are needed to determine declines in mollusk fitness with high rates of infection and to quantify any negative cellular, tissue-, or organ-level effects this fluke might have on hosts. Additional knowledge on reservoir–host pathways associated with *A. conchicola* flukes, particularly the longevity and development of *A. conchicola* within reservoir hosts, would also be helpful in characterizing environmental and habitat conditions favorable for parasitic transmission between Black Carp and mollusks. Although Black Carp are not intentionally ingesting *A. conchicola* during their foraging behavior, they may facilitate dispersal of a parasite that readily infects the mollusks they frequently consume in the wild.

ACKNOWLEDGMENTS

We thank Shannon Amiot, Anne Herndon, and Lauren Mott for assistance with laboratory processing and data preparation and the commercial fishers and biologists who provided fish for this study. We also thank Bethany Kunz and two anonymous reviewers for their helpful suggestions regarding earlier versions of the article. This study was partially funded through the Great Lakes Restoration Initiative and the U.S. Geological Survey, Ecosystems Mission Area. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Freshwater Mollusk Biology and Conservation

ISSN 2472-2944