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ORIGINAL RESEARCH

Surveys that prioritize site number over time per site will result in better gastropod status assessments: a case study on the rediscovery of Big Black Rocksnail

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Abstract
Freshwater gastropods are among the most imperiled organisms on Earth. Yet, they are among the most understudied freshwater taxa. Numerous freshwater gastropod species have gone extinct in the last 100 years, but recent rediscoveries indicate that some species were prematurely declared extinct. Such premature extinction declarations remove legal protections, which could facilitate actual extinction. Thus, research and policy recommendations are needed so surveys provide the best information possible for conservation. Here, we examined the case of *Lithasia hubrichti*, a freshwater gastropod endemic to the Big Black River in Mississippi that was last seen in 1965. In 2022, a freshwater mollusk survey resulted in finding *L. hubrichti* alive. An additional survey effort in 2023 that prioritized sampling as many sites as possible in a single day clarified the current range of *L. hubrichti*. Genomic analyses indicated that the species has persisted with a large population size for thousands of years, rather than ever falling below a survey detection limit. When considering the case of *L. hubrichti* and other recent freshwater gastropod rediscoveries, we conclude that freshwater gastropod surveys should emphasize sampling as many sites as possible under favorable sampling conditions when targeting rare species, rather than expending high sampling effort at a small number of sites or when stream conditions may impact ability to detect target species. We also advocate for policies that encourage partnerships with landowners, which was required to rediscover *L. hubrichti*.

Keywords Conservation genomics · Demographic modeling · Extinction · Survey design · Pleuroceridae · RAD-seq

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Introduction

Conservation status assessments, conservation priority setting, and decisions about whether a species will receive special protections under state and federal laws rely upon foundational survey data (Jones et al. 2013; Smith et al. 2018). Survey data are also critical to understanding historical trends and, in the most extreme case, for declaring a species extinct. However, poorly-designed surveys and monitoring programs can cause more harm than good (Yoccoz et al. 2001; Lindenmayer and Likens 2009). For example, inadequate surveys could lead a governmental agency to prematurely declare a species extinct, which would then cause a species in need of conservation to be overlooked. Although recommendations for effective monitoring exist, they rely on conservation practitioners being able to make informed study designs suitable for the taxon or ecosystem of interest (Lindenmayer and Likens 2009). Data to inform such study designs are often lacking for understudied invertebrates, which are usually those most in need of conservation attention (Johnson et al. 2013; Haag and Williams 2014). Thus, information is required for understudied taxa about whether the best approach for surveys will be those that emphasize sampling as many sites as possible or those that emphasize expending a high sampling effort at a small number of sites. For species that have been rediscovered after being considered extinct, understanding why and how surveys failed to detect those species should also ensure better overall status assessments. Moreover, to influence policy makers, conservation-related success stories are needed to emphasize the real-world benefits of well-designed surveys for understudied taxa.

Freshwater gastropods are among the most imperiled taxa in the world, but they are also among the least researched (Johnson et al. 2013; Böhm et al. 2021). Freshwater gastropods persist ubiquitously throughout a variety of aquatic habitats and drive ecosystem function by playing a pivotal role in aquatic food webs and nutrient cycling (Strong et al. 2008; Atkinson et al. 2023). The southeastern United States is a hotspot of freshwater gastropod biodiversity where many species are restricted to single drainages or isolated within small springs (Lydeard and Mayden 1995; Johnson et al. 2013). In the last 35 years, at least eight freshwater gastropods that were once considered extinct have been rediscovered (e.g., Adams and Gerberich 1988; Hershler et al. 1990; Minton et al. 2003; Ó Foighil et al. 2011; Whelan et al. 2012; and see Johnson et al. 2013; Whelan et al. 2022). However, past research has been equivocal as to why those species were overlooked to the point of being declared extinct. For example, one reason a species could be prematurely declared extinct is that no reasonable amount of survey work would have allowed for past detection if a given species underwent drastic, below detection limit population size declines across its range and then expanded in response to habitat improvements. Another reason would be that survey work was inadequate if a species persisted at some locations that were simply not surveyed prior to an extinction declaration. Determining why an understudied species was overlooked should reveal whether premature declaration of extinctions can be avoided.

No other freshwater gastropod family has seen as many putative extinctions as Pleuroceridae (Johnson et al. 2013; IUCN 2022). In the most extreme instance, the entire genus *Gyrotoma* went extinct after numerous impoundments were installed on the Coosa River in Alabama, USA during the 20th century (Bogan and Pierson 1993). However, recent work offers encouraging results for pleurocerids, including the rediscovery of the narrow-range endemic *Leptoxis compacta* (Whelan et al. 2012). Furthermore, some species were recently determined to be more widespread than previously thought (e.g., *Leptoxis ampla* and *Pleu-*

rocera foremani) (Whelan et al. 2019; Redak et al. 2021). Yet, a question remains as to why these species and populations were overlooked for many years.

Here, we describe survey work that resulted in rediscovering *Lithasia hubrichti*, a species thought to be extinct for nearly 60 years (Hartfield 1993; Johnson et al. 2013). In doing so, we examine the real-world benefits of improved surveys for freshwater gastropods. *Lithasia hubrichti* had not been seen since its description in 1965, and the species was previously known from only one location in Mississippi, USA (Table S1; Clench 1965; Hartfield 1993). However, the aforementioned discoveries of putatively extinct or extirpated pleurocerids motivated us to look for *L. hubrichti*. We compared the conchological and radular morphology of collected specimens with historical museum material to ensure that putative *L. hubrichti* individuals were not another species. We also generated genomic data to model demographic history of the species, which can provide insights as to whether *L. hubrichti* was overlooked as result of low abundance at some point since its description or if it was overlooked because it persisted within an unsurveyed stretch of river. This study also explains why other rediscovered freshwater gastropods were overlooked in the recent past and should influence future status assessments of understudied invertebrates.

Methods

Survey and initial species identification

During the fall of 2021, 89 sites were surveyed for freshwater mussels in the mainstem Big Black River (Ellwanger et al. 2021). While sampling, a single relic shell that resembled *L. hubrichti* was found. This finding spurred a targeted survey for *L. hubrichti* during infrequently-low water levels (2.1 m river gauge height; Bovina, Mississippi) on October 21st, 2022 and August 30th, 2023. To access survey sites, private land access was requested and granted by local landowners. In addition to re-sampling the relic shell location, we surveyed 15 other mainstem localities across 25 river km that were selected through aerial imagery and the presence of gravel. Each site was searched by a 5–6 person survey team for approximately 15 min. At locations where *L. hubrichti* occurred, a rapid density estimate was made by counting all *L. hubrichti* individuals within five randomly-placed quadrats (0.50×0.50 m). At each quadrat, we recorded substrate (%), water depth (m), and benthic stream velocity (m/s). Following habitat assessments, all gastropod species captured were counted and identified in the field. Species identification followed Clench (1965), Burch and Tottenham (1980), and Johnson et al. (2013). We tentatively identified shells with a globose body whorl, at least two rows of axial tubercules, indented sutures, a subovate aperture, and a columella callous as *Lithasia hubrichti*. Most individuals were returned to the site; however, representative specimens of each species were retained at every location. Collected specimens were preserved following Fukuda et al. (2008), placed in 95% ETOH, and vouchered at the Mississippi Museum of Natural Science (MMNS) Malacological Collection in Jackson, Mississippi.

Morphological documentation

Twelve individuals were used for morphological documentation and genetic analyses. Shells were photographed and qualitative comparisons of shell features were made to type material and other museum records (Fig. 1). Foot and head tissue clips were taken separately and digested overnight in 180 µL of Buffer ATL (Qiagen) and 10 µL of Proteinase K (New England Biolabs) at 56 °C. Following tissue digestions of head clips, three relatively intact radulae were removed from buffer, mounted, and visualized via scanning electron microscopy (SEM) following Whelan (2016).

Genomic analyses

Genomic data for 12 individuals from the rediscovery site were generated to measure baseline genetic diversity, check for presence of subpopulation structure, and infer demographic history. DNA was extracted from aforementioned foot tissue digestions with the Qiagen DNeasy Plant Kit following manufacturer's instructions, except a small modification was made to incorporate a proteinase K digestion step. A plant kit was used because it removes mucus polysaccharides that are prevalent in pleurocerid tissues better than standard animal tissue kits (Whelan et al. 2019). DNA was quantified with a Qubit fluorometer and normalized to 20 ng/µL.

A genome-wide single nucleotide polymorphism (SNP) dataset was generated using a 3-enzyme restriction site associated DNA sequencing approach (3RAD-seq; Bayona-Vásquez et al. 2019). Normalized DNA (20 ng/µL) was digested with XbaI, EcoRI, and NheI restriction enzymes. Illumina adaptors with in-line barcodes were attached via a ligation reaction. A random i5 index and an i7 barcode index were attached via limited-cycle PCR. A Blue Pippen (Sage Science) was used to size select for fragments of 440–608 bp in length. The full library prep protocol is available at https://github.com/NathanWhelan/3RAD_pro-

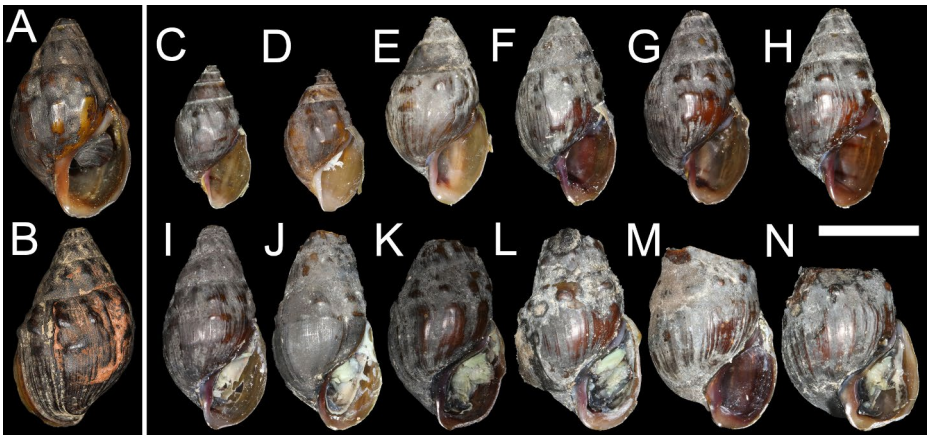

Fig. 1 Holotype and sequenced individuals of *L. hubrichti*. (**A**) Holotype: MCZ 210,956, apertural view. (**B**) Holotype: MCZ 210,956, subapertural view, (**C**) MMNS 18,232, (**D**) MMNS 18,233, (**E**) MMNS 18,234, (**F**) MMNS 18,235, (**G**) MMNS 18,236, (**H**) MMNS 18,237, (**I**) MMNS 18,238, (**J**) MMNS 18,239, (**K**) MMNS 18,240, (**L**) MMNS 18,241, (**M**) MMNS 18,242, (**N**) MMNS 18,243. Scale bar = 1 cm.

tocols. 3RAD libraries for *Lithasia hubrichti* were pooled together with libraries from other projects and were sequenced on an Illumina NovaSeq 6000 SP flow cell using paired end, 2×150 bp chemistry.

Raw Illumina data were demultiplexed with the STACKS 2.61 program *process_radtags* (Rochette et al. 2019) following Whelan et al. (2023). Reads were demultiplexed in two steps: (1) first by the Illumina i7 index that distinguished between 96-well plates used in library prep and (2) then by inline barcodes on both reads that distinguished among individuals on each 96-well plate. Barcodes were allowed to have up to one mismatch during demultiplexing. During the second demultiplexing step, reads without the XbaI and EcoRI enzyme cut sites in the forward and reverse reads, respectively, were discarded.

After demultiplexing, data were assembled with the STACKS pipeline denono_map.pl. Parameters for assembly were determined following Paris et al. (2017), which suggested 5 as the minimum coverage per stack (-m), 3 as the maximum distance allowed between stacks (-M), and 3 as the maximum distance allowed between catalog loci (-n). After assembly, two datasets were created with the STACKS program *populations* by discarding loci that were not present in 80% of individuals, that had a minimum minor allele frequency of less than 2.5%, and that had a maximum observed heterozygosity of 0.5; one dataset was allowed to have multiple SNPs per locus and the other only included one SNP per locus for analyses that assumed SNPs were unlinked. A third dataset for demographic analyses that used the allele frequency spectrum was created with *populations*, as above, but the minimum minor allele frequency was set to 0.01, loci had to be present in every individual, and multiple SNPs per locus were permitted. The vcf file from this third dataset (Table S2) was converted to an allele frequency spectrum using the script vcf2dadi.py with an easySFS projection value of 12 to maximize the number of segregating sites (Gutenkunst et al. 2009). Final datasets were named based on filtering parameters (Table S2).

Average observed heterozygosity, expected heterozygosity, and nucleotide diversity were calculated with STACKS using dataset R80_maf025_multi (Table S2). Rarefied allelic richness was calculated with the R (R Core Team 2022) package PopGenReport (Adamack and Gruber 2014) and dataset R80_maf025_multi. Genomic background and the best-fit number of genetic clusters (K) was assessed with ADMIXTURE (Shringarpure et al. 2016) and the AdmixPipeline (Mussmann et al. 2020) using dataset R80_maf025_single because ADMIXTURE assumes that loci are unlinked; the number of genetic clusters was assessed via 20% cross-validation with 10 replicates for K = 1–3. Genetic structure was further examined with discriminant analysis of principle components (DAPC) and fineRADstructure with dataset R80_maf025_multi because both methods allow for linked SNPs. The best-fit K for DAPC was assessed with the find.clusters command of the R package adegent (Jombart and Ahmed 2011), allowing a maximum of 4 clusters. The best-fit K was determined to be 1, so further DAPC steps were not performed. fineRADstructure was run by first calculating the coancestry matrix with RADpainter and then by assigning individuals to populations with finestructure (Lawson et al. 2012), running the Markov chain Monte Carlo for 2,000,000 generations, sampling every 1,000 generations and using 1,000,000 generations as burn-in.

Demographic history for *L. hubrichti* was estimated from the allele frequency spectrum using fastsimcoal2 v2.7 (Excoffier et al. 2013). We modeled eight single-population scenarios (Fig. 2). Parameters were estimated from 1,000,000 simulations and 40 expectation/conditional maximization optimization cycles with 50 replicates for each model. Mutation

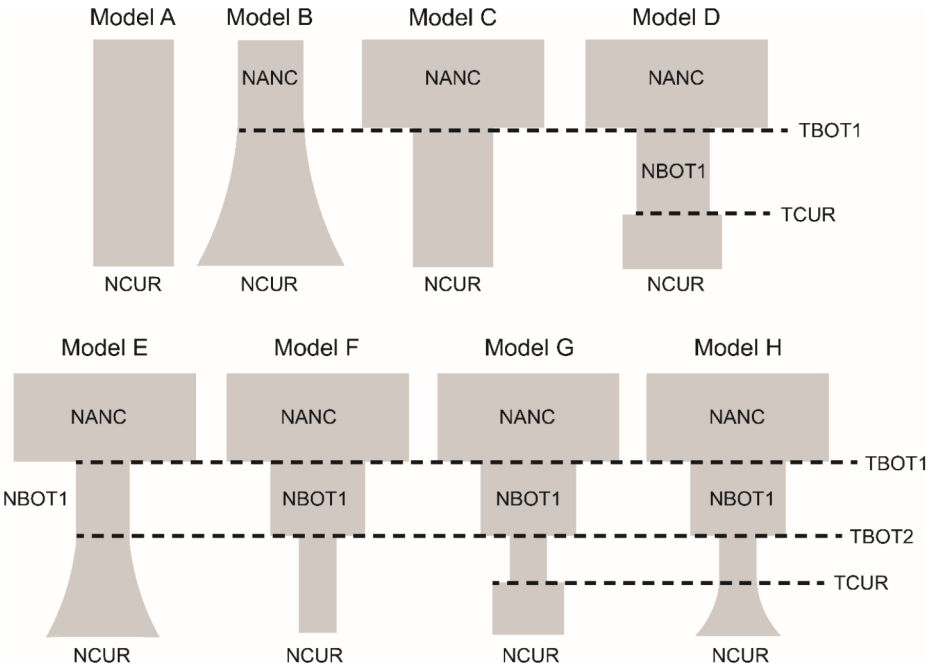

Fig. 2 Eight demographic scenarios modelled for *L. hubrichti* in fastsimcoal2. Model E is the best-fit model according to analyses with fastsimcoal2. Parameter estimates include current (NCUR) and ancestral (NANC, NBOT1, NBOT2) effective population sizes, exponential growth rate (GRATE in models B, E, and H) and timing of demographic events in generations (TCUR, TBOT1, TBOT2).

rates were set to 2.5×10^{-8} per site per generation following recommendations from Excoffier et al. (2013) given unknown mutation rates for pleurocerid species. The best-fit model was identified using Akaike's Information Criterion (AIC). 95% confidence intervals (CI) were estimated by first creating 100 SFS datasets via non-parametric bootstrapping in fastsimcoal2 and then running each simulated dataset with 50 replicates as above.

Results

Survey

Lithasia hubrichti was rediscovered in the mainstem Big Black River on October 22, 2022. During the subsequent survey on August 30th, 2023, we found the species at five of 16 sampling locations, including one site downstream of the type locality (Fig. 3). We predominantly captured *L. hubrichti* nearshore along shoals at shallow depths (<0.25 m) in slow to moderate current (0.07–0.67 m/s). However, the species was also encountered in lower abundance at shoal adjacent habitats (e.g., shallow backwaters, downstream pools). *Lithasia hubrichti* was exclusively found in unconsolidated substrates comprised of pebble and gravel (8–72 mm) with minor amounts of siltation. We failed to capture *L. hubrichti* in

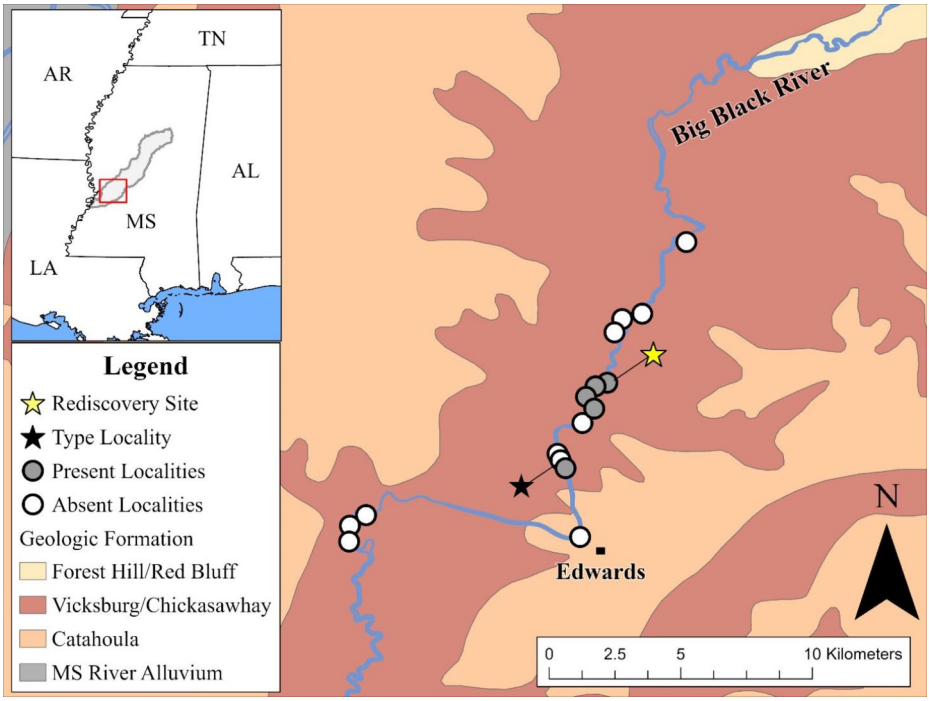

Fig. 3 Map of the known range of *L. hubrichti*, in the Big Black River with a surface geology overlay.

habitats with fast current (>1 m/s), at depths greater than one meter, or in substrates dominated by sand or silt.

The rediscovery site, also the most upstream location of occurrence, contained a large shoal that stretched across the width of the channel. The gravel there was coated in a dark, iron oxide patina and was noticeably different compared to unstained gravel at sites where *L. hubrichti* did not occur. Among all sites surveyed, the rediscovery site had the highest observed average density of *L. hubrichti* at 434.4 individuals/m^2 (±97.9 SE) with densities ranging between 116 and 668 individuals/m^2. In comparison, the second most abundant site only had an average density of 43.2 individuals/m^2 (±14.2 SE). *Lithasia hubrichti* densities were lowest at the site located 5 river km downstream from the rediscovery site, with an average density of 9.6 individuals/m^2 (±2.7 SE).

During our survey, we encountered a diverse mollusk community. Among the 16 sites, we found three other species of freshwater gastropods including another species of Pleuroceridae, *Pleurocera acuta*, and two species of Viviparidae, *Callinina georgiana* and *C. subpurpura*. We note that the taxonomy of Pleuroceridae needs revision and that *P. acuta* likely consists of multiple unrecognized species (Whelan et al. 2022). Nevertheless, no other *Pleurocera* is known to occur in the eastern Mississippi River tributaries of Mississippi, and the *Pleurocera* encountered in the Big Black River is classified as *P. acuta* under current taxonomy (Johnson et al. 2013; Whelan et al. 2022).

Conchological and radular morphology comparison

Shells of newly-collected individuals were similar in appearance to historical collections and type material (Fig. 1, S1), but a large amount of conchological variation is present in the species (Fig. 1). Based on size, sequenced individuals likely include ages ranging from subadult, or early adult (Fig. 1C, D), to adult, with the range in adult size indicating multiple year classes (Fig. 1E-N). Younger individuals have two rows of tubercules on the body whorl and a mostly intact spire of at least 3 whorls (Fig. 1C, D). In contrast, older individuals retain only the top row of tubercules and often have an extremely eroded spire (Fig. 1K-N). The aperture of *L. hubrichti* is subovate, becoming more ovate as individuals age (Fig. 1). The columella callous, which is the defining shell character for *Lithasia*, is present, but not prominent in most individuals (Fig. 1).

Radular morphology further confirmed that *L. hubrichti* was accurately identified because radulae visualized here match radulae visualized from a specimen collected from the type locality in 1964 (North Carolina Museum of Natural Sciecnes 59,904; Table S1; Whelan 2016). The rachidian has a dagger-like central cusp that is flanked on both sides by 4–5 pointed denticles (Fig. S2). The lateral teeth have a wide, rectangular cusp with 1–2 small, but pointed, outer denticles. There are three inner denticles on the lateral teeth, with the innermost denticle being comparatively blunt. The inner marginal teeth have six wide denticles, whereas the outer marginal teeth have 12–13 narrow denticles. The greater variation in denticle number on some teeth seen here, compared to Whelan (2016), appears to be the result of more than one individual being analyzed. The observed level of radula morphological variation is similar, or less than, what has been documented within other pleurocerid species (Whelan and Strong 2016).

Genomic analyses

After demultiplexing and decloning, an average of 1,269,062 paired-end reads per sample (range: 329,395-3,017,774) were used for assembly. Depending on the threshold of allowed missing data for each dataset, the number of loci (i.e., RAD-tags or assembled contigs) ranged from 4,115 to 6,161. Of those loci, the number with at least one SNP ranged from 1,381 to 2,102 (Table S2). The number of SNPs in each dataset, depending on missing data, minimum minor allele frequency, and whether multiple SNPs per locus were allowed, ranged from 2,102 to 4,762 (Table S2).

Genetic diversity, as measured by summary statistics, was high. Rarefied allelic richness was 2.042 (SE = 0.0089), average nucleotide diversity was 0.2975 (SE = 0.0021), and average observed heterozygosity was 0.2600 (SE = 0.0021). Average expected heterozygosity was 0.2846 (SE = 0.0020). The inbreeding coefficient was 0.0918 (SE = 0.0108). Genetic structure analyses revealed a lack of subpopulation structure as all analyses indicated that only one genetic cluster was present in the data (Figs. S3, S4).

Fastsimcoal2 demographic analysis identified Model E, a single bottleneck followed by gradual exponential growth, as the best-fit model for the sampled *L. hubrichti* population (Table S3, Fig. 2). Parameter estimates for the best-fit model indicate a historical population of *L. hubrichti* that underwent a minor bottleneck before gradually expanding over approximately four million generations that resulted in a slow, but continuous, population increase over time (Table 1). The exact generation time for *L. hubrichti* is unknown, but pleurocerids

Table 1 Parameter estimates and 95% confidence intervals (95% CI) for the best-fit demographic model (i.e., Model E: a single bottleneck followed by exponential growth) inferred by fastsimcoal2.

Parameter Name	Parameter Estimate	95% CI
Current Ne	8,905,797	1,068,200–6,640,154
Bottleneck Ne	2,300,835	489,756–2,837,413
Ancestral Ne	2,501,615	2,343,242–3,403,160
Timing of bottleneck in generations	3,984,700	4,541–3,363,202
Start of exponential growth in generations	3,934,603	1,448–3,295,065
Exponential growth rate	-1.19E-09	(-7.49E-05) - (-1.01E-09)

can live 2–9 years and usually become sexually mature in the first year (Whelan et al. 2015). Therefore, using a generation time of 1 year, the inferred demographic model suggests that the historical bottleneck and subsequent growth occurred during the Pliocene. Since there is no additional population expansion or decline following the initial bottleneck and growth during the Pliocene, demographic modeling also suggests that *L. hubrichti* has maintained a robust population size for at least four million years.

Discussion

We rediscovered *Lithasia hubrichti* after it was overlooked for nearly 60 years, Despite being considered extinct, population genomic data indicated that *L. hubrichti* has persisted at high abundance in some stretches of the Big Black River before and since the species was formally described. Thus, the species was overlooked because not enough sites were surveyed, not because the species had suffered a range-wide decrease in abundance. Rapid surveys, like the ones done here, also led to previous gastropod rediscoveries (Ó Foighil et al. 2011; Whelan et al. 2012) or discovery of larger ranges (Whelan et al. 2019; Redak et al. 2021). Since the description of *L. hubrichti* there have been multiple attempts to detect the species at its type locality by United States Fish and Wildlife Service and other researchers (P. Hartfield, *pers. observation*; F. Thompson, *pers. communication*; L. Hubricht, *pers. communication*), but because the species distribution also coincides with a publicly-inaccessible reach of the river, seldom effort was made to find the species at novel localities. Furthermore, low water conditions may have been important in the rediscovery of *L. hubrichti* due to the naturally-high turbidity levels within the Big Black River at base flow. Thus, our results and those of past studies clearly demonstrate that sampling as many sites as possible under low water levels, rather than expending high sampling effort at one or a few sites, is the best strategy for uncovering overlooked populations of understudied gastropods. That is, sampling more sites will give the best odds at encountering habitats where narrow-range endemics persist. In contrast, sampling a small number of sites (e.g., those at road crossings or only the type locality) with a high amount of effort or when sampling conditions are not ideal (e.g., high water levels) can result in failing to capture any given species and result in premature extinction declarations.

Our conclusions about the need to sample as many sites as possible to understand whether a species is still extant and to understand the range of imperiled species likely hold across

Pleuroceridae and other freshwater gastropod families. As with *L. hubrichti*, other recent rediscoveries of freshwater gastropods in multiple families have occurred at locations where the putatively extinct species was locally abundant and easily sampled in a short time frame (Hershler et al. 1990; Ó Foighil et al. 2011; Whelan et al. 2012; Johnson et al. 2013). These rediscoveries resulted from increased freshwater mollusk surveys that emphasized unsampled, often hard to reach, sites. The ease of finding the species from the above examples, once the appropriate site was surveyed, supports the notion that our case study on *Lithasia hubrichti* is widely applicable.

To the best of our knowledge, demographic modeling has not been done on any other freshwater gastropod that was once thought to be extinct. However, genetic diversity estimates that were previously reported for another species that was once considered extinct, *Leptoxis compacta*, are also not consistent with what is expected after a bottleneck (Wright et al. 2020). Furthermore, no study has presented data that would indicate that previously-rediscovered gastropods were overlooked because they underwent a severe bottleneck followed by population expansion that facilitated their rediscovery. Therefore, genomic data examined here and from previous studies support our hypothesis that rediscovered species were overlooked because not enough sites were sampled, not because of a lack of survey effort at sampled sites. Thus, if tradeoffs between site number and survey effort per site are needed because of limited resources, our findings, and those of others, support policies and practices that favor sampling as many sites as possible to assess conservation status. Such surveys are particularly necessary before a species is declared extinct. Surveys that emphasize a higher number of sites over more time spent at any given site will also fill in gaps of our understanding of contemporary ranges for any under-surveyed species.

Policies and practice that emphasize surveys with many sites will also need to be coupled with policies that encourage collaborative relationships between landowners and conservation practitioners. However, positive engagement with landowners can be difficult, at least in some countries like the United States. For example, in the United States, Brook et al. (2003) found that over 50% of landowners in the range of the endangered Preble's Meadow Jumping Mouse either refused, or would refuse, to allow biologists to perform surveys on their land. In the case of *L. hubrichti*, sampling at the rediscovery site during low enough flow would have been extremely difficult (i.e., at least a 2+hour jet boat ride over shallow shoals), or impossible, without having been granted access to field sites by local private landowners. Without access through their property, *L. hubrichti* may still be considered extinct.

Our results also emphasize the benefits of considering multiple taxonomic groups when performing surveys. The discovery of one dead *L. hubrichti* shell was the impetus for our overall finding, but the shell was sampled during drainage-wide survey work focusing on freshwater mussels. Had the shell been discarded as a non-target taxon, *L. hubrichti* would likely still be considered extinct. Similarly, the case of *L. hubrichti* emphasizes the need for taxonomic expertise, which is limited for freshwater gastropods and other invertebrate groups. Without type comparisons and morphological comparisons of radulae, the identity of the snails collected in the Big Black River would be much more uncertain. Such uncertainty could have dissuaded agencies from pursuing conservation of *L. hubrichti*. We are certainly not the first to emphasize the importance of taxonomy in surveys and conservation, and we hope that this case study will aid in stimulating policies that encourage and fund taxonomic expertise and research.

Conservation status and recommendations for Lithasia hubrichti.

Prior to our survey, *L. hubrichti* was only known to historically occur at its type locality. Our findings extend the historical range of the species by approximately 5 river km (Fig. 3). Where found, *Lithasia hubrichti* is locally abundant, particularly at the site of rediscovery (Fig. 3). Even with an extended historical range, *L. hubrichti* has the smallest historical range of any non-spring-associated pleurocerid (Johnson et al. 2013; Whelan et al. 2022). *Lithasia hubrichti* also has a disjunct range from other species in the genus, which could have happened via historical dispersal of *Lithasia* from the Mississippi River, followed by isolation. Any future biogeographic study of *Lithasia* should include *L. hubrichti* and species from the Mississippi River.

In 1989, failure to find *L. hubrichti* at the type locality was attributed to an upstream chemical spill (*pers. communication*, Leslie Hubricht letter to USFWS, 1989). While we have no direct evidence to support claims of a chemical spill, we cannot rule it out. If a chemical spill was the reason *L. hubrichti* disappeared from the type locality, at some point *L. hubrichti* may have also disappeared at the lowermost site where we found the species present in 2023. If such an event occurred, it would mean that *L. hubrichti* likely migrated downstream since the pollution event because pleurocerids have extremely downstream-biased migration patterns (Whelan et al. 2019; Redak et al. 2021). Yet, that scenario seems unlikely given the distributional gap between the lowest two sites where we found *L. hubrichti* and given that the type locality is in-between those two sites. Instead, we think a more likely explanation is that the species was never extirpated from the lowest site, but instead not surveyed; notably the reported survey in 1989 only included the type locality (*pers. communication*, Leslie Hubricht letter to USFWS, 1989). Regardless of the cause for extirpation at the type locality, *L. hubrichti* remains susceptible to a single catastrophic event.

Among other pleurocerid species, the conservation status of *Lithasia hubrichti* is most similar to *Leptoxis foremani* and *Leptoxis compacta*. *Leptoxis foremani* was once thought to be extinct (Lydeard and Mayden 1995) and had a historical range that encompassed much of the mainstem Coosa River and larger tributaries like the Oostanaula River. Now, the species is federally endangered under the U.S. Endangered Species Act and known from only two sites in the Oostanaula River (Powell and Hartfield 2014). Similarly, *L. compacta* is a Cahaba River drainage, Alabama, USA species that was once thought to be extinct (Whelan et al. 2012) and that was recently proposed for listing as endangered under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2023). All three species currently persist only in a small portion of the mainstem of a major river, making them susceptible to a single, catastrophic point source pollution event. However, *L. hubrichti* has a smaller current range than *Le. compacta* and *Le. foremani*, which occupy at least 9 and 19 km stretches of river, respectively. The known historical range of *L. hubrichti* is also much smaller than *Le. compacta* and *Le. foremani*, which could make standard conservation techniques for *L. hubrichti* more difficult. For example, locations exist within the historical range of *Le. compacta* that are potential sites for reintroduction that would protect the species from a single point source pollution event, but such sites (e.g., Buck Creek in Shelby County, Alabama, USA for *Le. compacta*) may require habitat improvements prior to reintroduction efforts. In contrast, *L. hubrichti* lacks this assurance because sites are linearly located to one another that even if habitat at the type locality was improved to a point where reintroduction could be successful, a reintroduced population would likely be susceptible to any event that

causes decline elsewhere within the known range. Given how little we know about the basic biology and ecology of *L. hubrichti*, reintroductions outside its historical range should be avoided (George et al. 2009; IUCN/SSC 2013; Strayer et al. 2019). Thus, the best conservation approach for *L. hubrichti* will likely be habitat protection and long-term monitoring to ensure stable population sizes. Sampling additional sites within the Big Black River drainage may also be required to fully understand its distribution.

Despite the restricted known range of *L. hubrichti*, we documented considerably higher genomic diversity, as estimated by summary statistics like heterozygosity and nucleotide diversity, than what was previously documented in other imperiled pleurocerids like *Leptoxis ampla* (Whelan et al. 2019), *Pleurocera foremani* (Redak et al. 2021), and *Le. compacta* (Wright et al. 2020). However, *L. hubrichti* does not appear to harbor subpopulation genomic structure like *Le. compacta* (Wright et al. 2020) and *Le. ampla* (Whelan et al. 2019). Wright et al. (2020) hypothesized that the subpopulation structure of *Le. compacta* was a result of extirpated ancestral populations contributing to the overall genomic diversity of the species, and the absence of subpopulation structure may be a result of *L. hubrichti* having a restricted historical range. Observed heterozygosity was lower than expected heterozygosity and F_{IS} was nearly 0.1 in *L. hubrichti*, possibly indicating that drift and inbreeding will result in a long-term decline of genetic diversity. Nevertheless, *L. hubrichti* has greater genetic diversity than other pleurocerids (see results; Whelan et al. 2019; Wright et al. 2020; Redak et al. 2021). Thus, genetic drift is unlikely to cause problems for *L. hubrichti* over the foreseeable future unless exacerbated by a human-mediated bottleneck.

A formal status assessment for *L. hubrichti* by state and federal agencies is immediately needed as the species exhibits low population redundancy and is susceptible to catastrophic events. However, comprehensive surveys in the Big Black River drainage for *L. hubrichti* will not be a simple task because most locations in the drainage are remote, and ideal conditions for snail surveys (i.e., low enough water for collection by hand) make boat travel difficult. For now, research, surveys, and habitat improvements should be the focus of *L. hubrichti* conservation efforts.

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Author contributions All authors contributed to study conception and design. Fieldwork was performed by C.R.R., R.J.E, P.D.H., A.S.R, D.S.R., and M.D.W. N.V.W. and S.A.D. performed molecular lab work. N.V.W. and S.A.D. performed molecular data analyses. S.A.D. performed SEM work. C.R.R. and N.V.W. wrote the first draft of the manuscript, and all authors edited previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Code used in genetic analyses are available from https://github.com/nathanwhelan/Lithasia_hubrichti_pop-gen. All raw sequence data has been uploaded to NCBI SRA under BioProject PRJNA1075138. Input data for various analyses and the 1989 letter from L. Hubricht to USFWS are available on FigShare DOI: 10.6084/m9.figshare.23780781.

Declarations

Competing interests The authors declare no competing interests.

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