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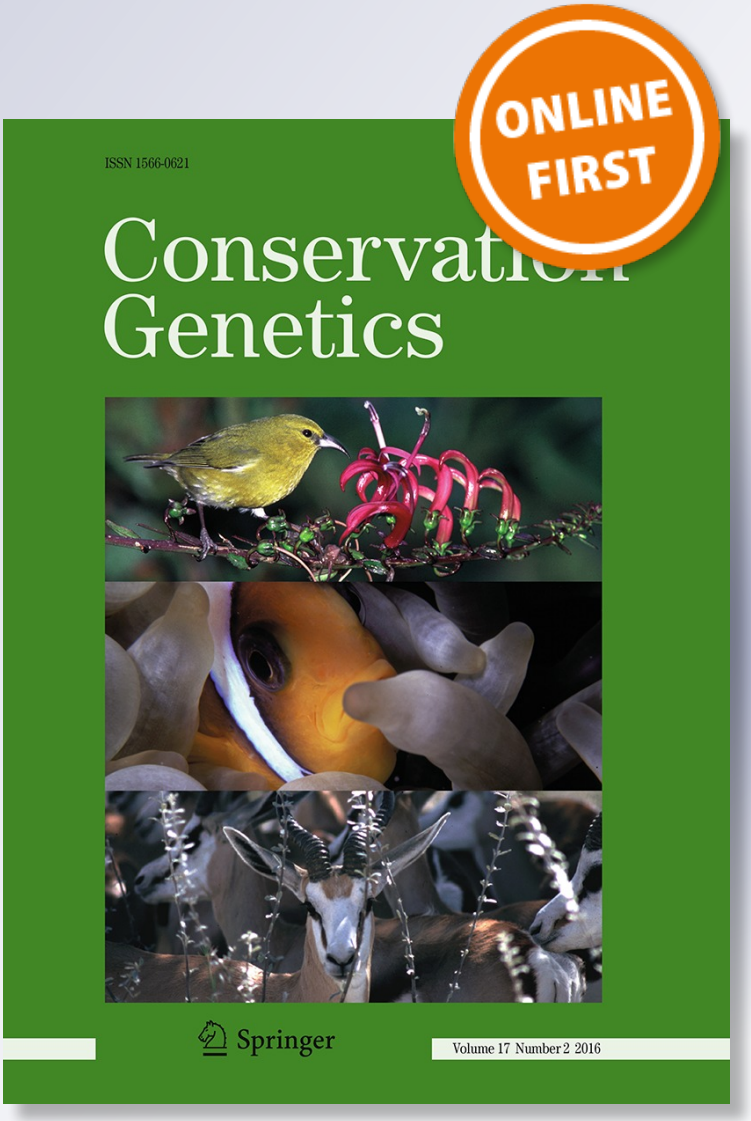

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RESEARCH ARTICLE

Phylogenetic and taxonomic assessment of the endangered Cumberland bean, *Villosa trabalis* and purple bean, *Villosa perpurpurea* (Bivalvia: Unionidae)

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Abstract Inadequate understanding of the phylogeography, taxonomy, and historical distribution of two critically imperiled freshwater mussels, Cumberland bean, *Villosa trabalis*, and purple bean, *Villosa perpurpurea*, has hindered management and recovery actions related to population restoration within their extant ranges. For more than 100 years, the purple-to-pink nacre of *V. perpurpurea* and white nacre of *V. trabalis* have been the only defining phenotypic characteristics used to distinguish each species. Genetic samples were analyzed from 140 individuals collected from 10 streams located in Virginia, Tennessee, and Kentucky, representing all known extant populations of each species. A 784-bp section of the mitochondrial DNA *ND1* region was sequenced to assess the phylogeography and taxonomic validity of these taxa. Results of our phylogenetic analyses showed 100 % Bayesian posterior support for two distinct clades, one occurring in the Cumberland River basin and the other in the Tennessee River basin, separated by a mean genetic distance of 4 %. Mean genetic distances between haplotypes within each clade was <1 %. Among individuals from the Cumberland River basin, the nacre of shells was white to bluish-white, but in the Tennessee River basin, nacre graded from white to pink to dark purple; thus, nacre color is a variable and inconsistent character in nominal *V. trabalis* and *V. perpurpurea* occurring in the Tennessee River basin. Our data suggest that these morphologically similar species do not co-occur, as was previously believed. Instead, we conclude that the two species most likely share a common ancestor, but became isolated within each basin and experienced allopatric speciation. Updates to nomenclature, taxonomic placement, and recovery plans for the investigated species are needed.

Keywords Freshwater mussels · Mitochondrial *ND1* · Nacre color · Mantle lure · Species recovery plan

Introduction

North America has the most diverse assemblage of freshwater mussels (Bivalvia: Unionoida) in the world, with at least 300 recognized taxa (Turgeon et al. 1998; Lydeard et al. 2004; Graf and Cummings 2007). The Tennessee-Cumberland River zoogeographic province is a primary center of this diversity, accounting for approximately 37 % of the total North American mussel fauna (Ortmann 1918, 1925; Haag 2012). Two species endemic to this region, Cumberland bean, *Villosa trabalis* (Conrad 1834) and purple bean, *V. perpurpurea* (Lea 1861), exist only in small fragmented populations relative to their historical distribution and abundance. Each of these species has been listed as federally endangered (in danger of extinction) under the United States Endangered Species Act, and the U.S. Fish and Wildlife Service (USFWS) considers remaining populations vulnerable to various anthropogenic impacts (USFWS 1984, 2004).

These species are difficult to distinguish from one another in the field and historically were thought to co-occur in the Tennessee River basin, but only *V. trabalis* is known from the Cumberland River basin (Fig. 1). Some

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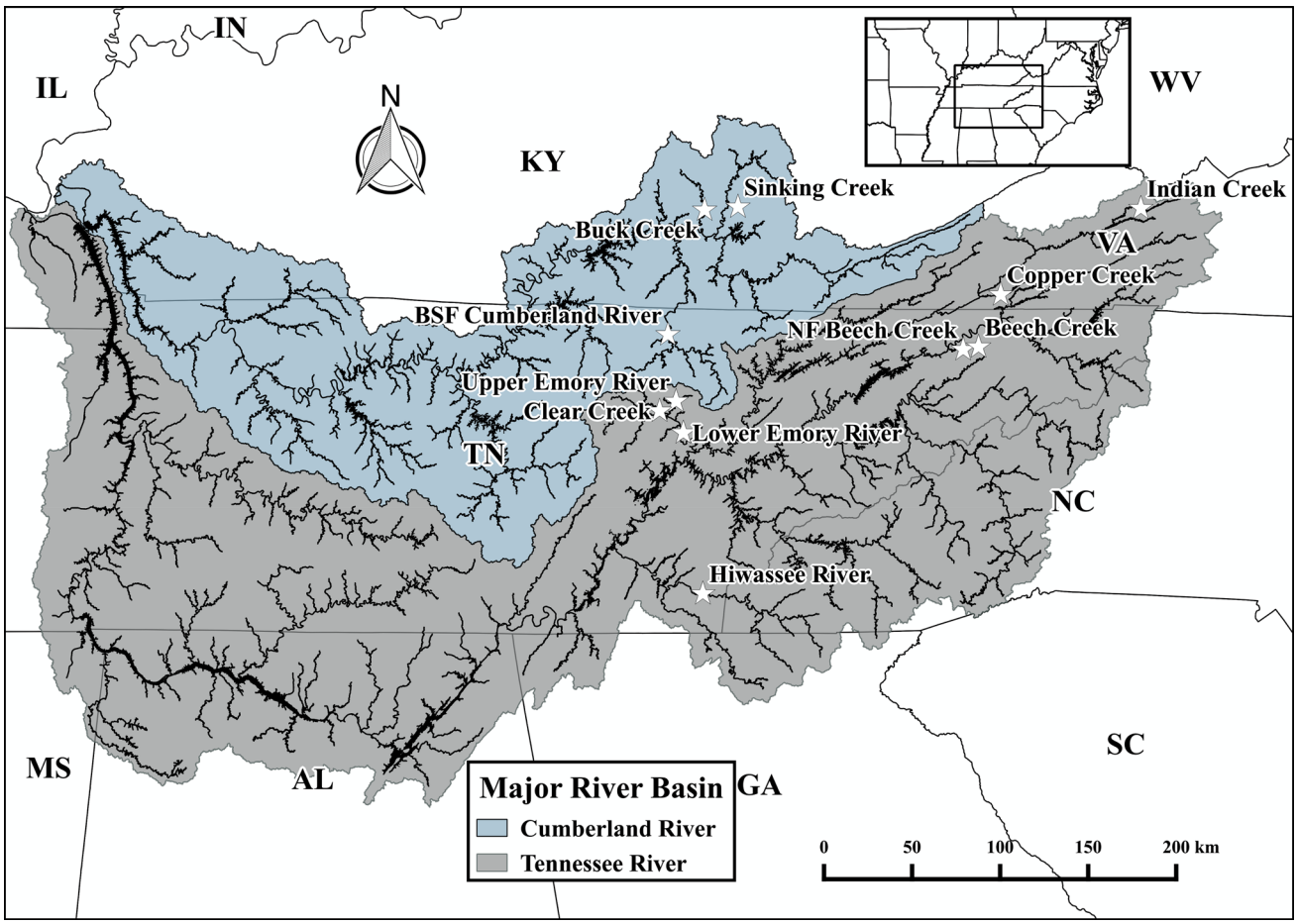


Fig. 1 Sampling locations for extant populations of *Villosa perpurpurea* and *V. trabalis* in streams of the Cumberland and Tennessee river basins, where tissue was collected from live individuals in 2013–2015 and previously preserved specimens. GPS coordinates of sampling locations are available in Table 1

authors (Ortmann 1918; Frierson 1927) have argued that these two taxa are actually phenotypic variants of the same species, and that only nominally are they separate species. Morphometric analysis of larvae (glochidia) by Hoggarth (1988) and molecular analysis of DNA sequences by Kuehnl (2009) concluded that the two species were, indeed, valid. Further phylogenetic analyses by Kuehnl (2009) have suggested that the two species do not belong in the genus *Villosa* but rather in the genus *Venustaconcha*, along with the genetically and morphologically similar species *Venustaconcha ellipsiformis* and *V. pleasii*; their analysis supports the nomenclature proposed by Frierson (1927) based on similarities in shell characteristics. Still, past studies lacked large sample sizes encompassing all population segments, and additional work was needed to test these inferences. In terms of shell characters, the two species are nearly indistinguishable except for the color of the nacre, where *V. perpurpurea* typically has purple nacre, which may grade to pink or almost white (hereafter referred to as generally purple to white), and *V. trabalis* has bluish-white to white nacre (hereafter referred to as generally white) (Parmalee and Bogan 1998). To facilitate attachment of glochidia to their host fishes, both species have evolved highly modified mantle tissues to serve as lures, termed "mantle-lures", which closely resemble and mimic prey items, such as insect larvae and pupae. Mantle lures can be important characters for species taxonomy in mussels (Jones et al. 2006; Zanatta and Murphy 2006).

Historically, specimens of *V. trabalis* have been reported from throughout the Cumberland River basin in Kentucky (KY) and Tennessee (TN), and in the Tennessee River basin from the headwaters in Virginia (VA) downstream to Muscle Shoals, Alabama (AL) (Fig. 1). The species is considered extirpated from AL, Georgia (GA), and from the headwaters of the upper Tennessee River basin in VA. Both *Villosa perpurpurea* and *V. trabalis* have been reported historically as co-occurring in the Clinch River in Scott Co. and Russell Co., VA; Beech Creek in Hawkins Co., TN; and the Obed River in Cumberland Co., TN, which has supported speculation that they may be the

same species. Further, the two species share similar life-history traits and habitat preferences. Layzer and Madison (1995) noted that detectability of gravid females was greatest for *V. trabalis* during December to February, similar to when *V. perpurpurea* are gravid and more detectable. Also, sculpin (*Cottus* spp.), greenside darter (*Etheostoma blennioides*), and fantail darter (*Etheostoma flabellare*) are confirmed suitable hosts for both mussel species (Layzer and Madison 1995; Watson 1999).

The population recognized as *Villosa perpurpurea* in the Emory River, TN, has been reassessed since 2011 and is larger than previously thought (Dinkins et al. 2012; Hubbs 2012). However, both species have declined to the point where captive propagation and reintroduction are likely necessary for their continued survival. The Freshwater Mollusk Conservation Center (FMCC) at Virginia Tech University and the Aquatic Wildlife Conservation Center (AWCC) operated by the Virginia Department of Game and Inland Fisheries (VDGIF) have been successfully propagating and rearing *V. perpurpurea* for reintroduction efforts in VA since 2002. Similarly, other mussel propagation facilities in AL, KY and TN have been propagating these species using broodstock from additional populations. Nevertheless, before further recovery activities can go forward, the USFWS and state agencies need a better understanding of the taxonomic and genetic variability within and among populations of the two species. Until their taxonomic relationship can be substantiated, propagation for augmentation and reintroduction will be hindered by inadequate understanding of justifiable stocking locations in which to place reared cohorts within each species' currently recognized range.

Recovery of both species is a high priority for the USFWS and state agencies, which are involved in complementary conservation projects. Thus, the purpose of this study was to assess the phylogeography and taxonomic validity of *Villosa perpurpurea* and *V. trabalis* using mitochondrial DNA sequences and phenotypic traits of both species in order to guide future recovery efforts.

Materials and methods

Tissue collection and preparation

Tissue samples from live and preserved individuals were collected from 2013 to 2015 from all drainages known to contain extant populations of *Villosa perpurpurea* and *V. trabalis*: (1) *V. perpurpurea* (live individuals), Copper Creek, Scott Co., VA; Indian Creek, Tazewell Co., VA; Beech Creek, Hawkins Co., TN; North Fork Beech Creek, Hawkins Co., TN; Clear Creek, Morgan Co., TN; Lower Emory River, Morgan Co., TN; and Upper Emory River, Morgan Co., TN; (2) *V. perpurpurea* (preserved specimens), Upper Emory River, Morgan Co., TN; (3) *V. trabalis* (live individuals), Hiwassee River, Polk Co., TN; and Sinking Creek, Laurel Co., KY; and (4) *V. trabalis* (preserved specimens), Sinking Creek, Laurel Co., KY; Buck Creek, Pulaski Co., KY; and Big South Fork Cumberland River, McCreary Co., KY (Fig. 1). Sample sizes and GPS coordinates for all individuals sampled are reported in Table 1. GPS coordinates were delineated using QGIS (2009) software (Open Source Geospatial Foundation Project).

Live individuals were gently opened to a width of 6–8 mm to non-lethally access the foot and mantle, which were swabbed to obtain a tissue sample (Henley et al. 2006) using an Isohelix (Harrietsham, UK) SK-2 buccal swab (Moyer and Díaz-Ferguson 2012). The sample then was transported on ice to the Virginia Tech Integrated Life Sciences Building (VT-ILSB), where it was chemically stabilized in lysis buffer. DNA extraction was performed using the Isohelix DDK Isolation Kit.

All preserved specimens were held at −20 °C at the Center for Mollusk Conservation (CMC) operated by the Kentucky Department of Fish and Wildlife Resources (KDFWR) in Frankfort. Following a 30-min thawing period, small pieces of mantle tissue (10–20 mg) were clipped from each specimen (Naimo et al. 1998) and preserved in 95 % ethanol. All samples were transported on ice to VT-ILSB, where they were stored at −20 °C prior to DNA extraction. Total genomic DNA was isolated using a DNeasy DNA extraction kit (Qiagen). The concentration of DNA was determined using a Hoefer TKO 1000 fluorometer to provide a standardized quantity for use in the polymerase chain reaction (PCR).

DNA sequences

Mitochondrial DNA (mtDNA) sequences from the first subunit of NADH dehydrogenase (*ND1*) were amplified by polymerase chain reaction (PCR) in a BioRad MyCycler thermal cycler using primers reported in Campbell et al. (2005). The *ND1* gene was selected because Jones et al. (2006) showed that it contained a greater number of variable nucleotide sites among *Epioblasma* spp. than other commonly analyzed mtDNA gene regions for unionids, including cytochrome oxidase I, cytochrome-*b*, and *16S*. The PCR amplification solutions for *ND1* consisted of 20–50 ng of genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 µM of each primer, 0.4 mg/mL BSA, and 1.5 U GoTaq DNA polymerase (Promega Corporation) in a total volume of 20 µL. Touchdown PCR thermal cycling conditions (Don et al. 1991) were: 95 °C for 30 s; followed by 10 cycles of 95 °C for 30 s, 0.5 °C temperature step-downs every cycle from 62.0 to 57.5 °C for 45 s, and 72 °C for 1 min; 25 cycles of 95 °C for 30 s,

Table 1 Mussel sampling locations and sample sizes for the mitochondrial NADH subunit-1 (*ND1*) sequences for investigated populations of *Villosa perpurpurea* and *V. trabalis* in 2013–2015

Nominal species	Sampling location	GPS coordinates	River drainage	*ND1* (*N*)	Accession numbers for sequences unique to sampling location*
V. perpurpurea	Copper Creek, VA	36.66629N, 82.61506 W	Clinch	4	KT964368–KT964370
V. perpurpurea	Indian Creek, VA	37.08750N, 81.75775W	Clinch	14	KT964368–KT964369; KT964371–KT964372
V. perpurpurea	Clear Creek, TN	36.09287N, 84.70286W	Obed-Emory	3	KT964375–KT964376
V. perpurpurea	Emory River, TN	35.98539N, 84.55786W	Obed-Emory	1	KT964374
V. perpurpurea	Upper Emory River, TN	36.14092N, 84.60201W	Obed-Emory	21	KT964368; KT964373
V. perpurpurea	Beech Creek, TN	36.40939N, 82.75224W	Holston	23	KT964377–KT964378
V. perpurpurea	North Fork Beech Creek, TN	36.39693N, 82.84475W	Holston	11	KT964377
V. trabalis	Hiwassee River, TN	35.18587N, 84.43745W	Hiwassee	30	KT964379–KT964386
V. trabalis	BSF Cumberland River, KY	36.62368N, 84.57365W	Cumberland	2	KT964387–KT964389
V. trabalis	BSF Cumberland River, TN	36.54763N, 84.66566W	Cumberland	3	KT964390
V. trabalis	Buck Creek, KY	37.07958N, 84.42729W	Cumberland	4	KT964387–KT964388
V. trabalis	Sinking Creek, KY	37.09437N, 84.22317W	Cumberland	24	KT964387
Total				140	

Additional tissue sampling and location information are available in the methods section. Approximate site coordinates are reported in decimal degrees. Sample size *N* includes additional DNA sequences obtained from NIH GenBank Sequence database and reported in Appendix 1

* Accession numbers for mitochondrial *ND1* sequences published in the NIH GenBank Sequence database (http://www.ncbi.nlm.nih.gov/genbank/)

0.3 °C temperature step-downs every cycle from 56.0 to 49.3 °C for 45 s, and 72 °C for 1 min; a final extension at 72 °C for 5 min; and a final hold at 4 °C. PCR-amplified DNA sequences were purified using a Qiagen PCR Purification Kit before being checked by electrophoresis, and then sequenced using an ABI 3130 × 1 automated DNA sequencer at the Virginia Biocomplexity Institute (VBI).

The DNA sequences were aligned and edited using the program SEQUENCHER, version 3.0 (Gene Codes Corporation). Additional *ND1* sequences for *Villosa perpurpurea*, *V. trabalis*, *Venustaconcha ellipsiformis*, *Venustaconcha pleasii*, were obtained from the NIH GenBank Sequence database (http://www.ncbi.nlm.nih.gov/genbank/) and aligned within the DNA sequence dataset to increase our sample size (Appendix 1). Two additional *ND1* sequences, one from *Villosa fabalis* and another from *Epioblasma capsaeformis* also were obtained from GenBank and used as outgroup taxa to root phylogenetic trees.

Phylogenetic analysis was conducted primarily to assess the genetic distinctiveness of DNA sequence haplotypes of *Villosa perpurpurea* and *V. trabalis*. Analysis of variable nucleotide sites was used to infer ancestral genealogical relationships among haplotypes and to provide statistical support for any inferred taxonomic groups. Following the Phylogenetic Species Concept (Cracraft 1983), taxa forming a monophyletic clade were considered a single species. The model of sequence evolution used to calculate mean genetic distance (*D*) was determined by the program MEGA, v6.0 (Tamura et al. 2013), which was the T92 model (Tamura 1992). Intraspecific genetic variation among haplotypes was estimated using uncorrected *p*-distance in DnaSP, v5.10.1 (Rozas and Rozas 1995). A phylogenetic reconstruction was estimated using Bayesian inference in MrBayes, v3.2.5 (Huelsenbeck and Ronquist 2001). MrBayes was run for 1,000,000 generations and 8 chains, sampling trees every 1000 generations. Posterior probabilities were calculated using the tree topologies that remained after the burn-in trees from 200,000 generations were excluded (i.e., after the tree score likelihood values had stabilized). Stabilization of likelihood scores was confirmed visually by plotting scores in Microsoft Excel to determine when scores stabilized asymptotically. The final tree figure was created in FigTree, v1.4.2 (Rambaut 2012), using the consensus tree from these runs with nodes labeled indicating support from posterior probabilities.

Assessment of mantle lure and shell nacre characteristics

Mantle lures of gravid females were photographed during early spring 2014, including single individuals from the following locations: Hiwassee River, Polk Co., TN, 19 February 2014; Indian Creek, Tazewell Co., VA, 2 March 2014; and Beech Creek, Hawkins Co., TN, 14 March 2014. Photographs were taken underwater using a waterproof Pentax Optio WG-3 16.0 MP (Pentax Ricoh Imaging Company, Ltd.) digital camera set to its super-macro function, and then processed in iPhoto for OS X (Apple,

Inc.). The same camera was used to photograph shells of selected specimens held at the FMCC shell collection and the North Carolina Museum of Natural Science, Raleigh, NC, between 26 February and 18 March 2015, to document nacre color in study populations of each species. For live individuals, nacre color was determined visually by TWL by inspecting the external umbo region of the shell.

Results

Haplotypic and phylogenetic analysis of mtDNA sequences

The mtDNA *ND1* gene region was sequenced for 77 *Villosa perpurpurea* and 63 *V. trabalis*, representing the 7 and 4 remaining extant populations of these two species, respectively (Table 1). The matrix of aligned *ND1* mtDNA sequences contained 784 base-pairs (bp), of which 45 were variable (Table 2). Of these variable sites, 21 were fixed (contained only one nucleotide type) within our dataset between all individuals within the Tennessee River basin for *V. perpurpurea* and all individuals within the Cumberland River basin for *V. trabalis* (Table 2). Excluding DNA sequences for outgroups obtained from GenBank, observed nucleotide site variation defined a total of 23 haplotypes within and among the two clades; 5 haplotypes were observed in the Clinch River (CL) pooled population of *V. perpurpurea*, 6 haplotypes in the Obed-Emory River (OE) pooled population of *V. perpurpurea*, 2 haplotypes in the Holston River (HO) pooled population of *V. perpurpurea*, 8 haplotypes in the Hiwassee River (HW) population of *V. trabalis,* and 4 haplotypes in the Cumberland River (CU) pooled population of *V. trabalis* (Tables 2, 3). Nucleotide diversity (π) was highest within HW ($\pi = 0.003$) and lowest within HO ($\pi = 0.0001$); intraspecific genetic variation within each population is summarized in Table 3. Within populations, haplotypes were characterized by low divergence (<0.01 %) and nucleotide diversity, typically exhibiting only a few

Table 2 Haplotypes and variable sites of mtDNA *ND1* sequences of *Villosa perpurpurea* (*Vper*) sampled in the Clinch River drainage, VA, Obed-Emory River drainage, TN, and Holston River drainage, TN; and *V. trabalis* (*Vtra*) sampled in the Hiwassee River drainage, TN, and the Cumberland River basin, KY/TN in 2013–2015

Haplotype and polymorphic nucleotide sites		Populations[a,b]											
		CL		OE			HO		HW	CU			
	11112222333333333444445555555666677777	CC	IC	CLC	ER	UER	BC	NBC	HW	BSF	BUC	SC	Total
	124556844553578012346899234790112266044600146												
	522270447067292067363403324721041514389356448												
Vper1	GACCGGATTCAGCCAAACTGTCACCTCCGACATCTGATGGTTACA	1	8			18							27
Vper2	T·······························C············	2	2										4
Vper3	················G····························	1											1
Vper4	········C····································		2										2
Vper5	·······C·································C···		2										2
Vper6	·········A···································					3							3
Vper7	····························AG·····A·········				1								1
Vper8	·····························G·····A·········			1									1
Vper9	··········C················T·····T···········			1									1
Vper10	·················T·········T·····T···········						20	11					31
Vper11	·················T·········T·····T··G········						3						3
Vtra1	···························T··T··T···········								4				4
Vtra2	···TA····A·················T·····T······C····								1				1
Vtra3	··················C········T·····T···········								10				10
Vtra4	···TA······················T·····T······C····								5				5
Vtra5	···························T·····T···········			1					4				5
Vtra6	····A······················T·····T······C····								2				2
Vtra7	····T······················T·····T···········								3				3
Vtra8	·························C·T·····T········G··								1				1
Vtra9	·**GT**TA**CG**····**ATTTG**···**ACTGTT**·**T**TA··**G**·T**C**····**A**···**TG**									1	2	24	27
Vtra10	·**GT**T·**CG**····**ATTTG**···**ACTGTT**·**T**TA··**G**·T**C**····**A**···**TG**									1	2		3
Vtra11	·**GT**TA**CG**····**ATTTG**···**ACTGTT**·**T**TA··**G**·T**C**··C·**A**···**TG**									1			1
Vtra12	·**GT**T·**CG**····**ATTTG**···**ACTGTT**·**T**TA··**G**·T**C**···A**A**···**TG**									2			2
Pooled Population Total (*N*)			18		25		34		30			33	140

[a] CL = Clinch River drainage; OE = Obed-Emory River drainage; HO = Holston River drainage; HW = Hiwassee River drainage; CU = Cumberland River Basin

[b] CC = Copper Creek, VA; IC = Indian Creek, VA; CLC = Clear Creek, TN; ER = Mainstem Emory River, TN; UER = Upper Emory River, TN; BC = Beech Creek, TN; NBC = North Fork Beech Creek, TN; HW = Mainstem Hiwassee River, TN; BUC = Buck Creek, KY; SC = Sinking Creek, KY; BSF = Big South Fork Cumberland River, KY/TN.

Underlined nucleotides in bold font represent presumably diagnostic sites observed in samples from pooled populations in the Cumberland River basin

nucleotide differences among haplotypes. A maximum of eight nucleotide differences between haplotypes was observed in HW. Mean genetic distance (D) was lowest between CL and OE and highest between CL and CU; among population mean genetic distance is summarized in Table 4. Mean genetic distance among haplotypes within the four upper Tennessee River basin (UTRB) populations (Fig. 1) was $D = 0.0043$, while mean genetic distance among haplotypes within CU was $D = 0.0005$. Among population comparisons within CU revealed a range of divergence from the four UTRB populations ranging from $D = 0.0387$–0.0434. Mean genetic distance between the two pooled river basins of CU and UTRB was $D = 0.0409$.

Comparison of CL and OE *ND1* haplotypes revealed the lowest divergence ($D = 0.0016$) between populations, with the *Vper1* haplotype shared between these two populations (Table 4). Nominal *V. perpurpurea* populations collectively showed diverse mtDNA lineages, with 11 total haplotypes present, ranging from 1 to 4 mutational steps from the most common haplotype (*Vper1*) (Fig. 2). The HW *ND1* haplotypes showed low divergence from the CL ($D = 0.0030$), OE ($D = 0.0027$), and HO ($D = 0.0039$) populations. One haplotype observed in HW, *Vtra5*, was the same as in an individual from Clear Creek within the OE population (Table 2; Fig. 2). The HO samples contained low nucleotide diversity, with only 2 haplotypes observed and each containing a single unique polymorphic site. One of these haplotypes, *Vper10,* accounted for 31 of the 34 samples analyzed from the HO population. The HO sequences were only 3–4 mutational steps removed from the CL and OE haplotypes, indicating low divergence ($D = 0.0060$–0.0065) across these three populations (Table 4). The CU samples exhibited four unique haplotypes, each of which with 21 fixed nucleotide differences relative to all samples observed in the UTRB (Table 2; Fig. 2).

Table 4 Pairwise comparisons of mean genetic distance (D) observed among pooled populations of *Villosa perpurpurea* and *V. trabalis* in 2013–2015

Population	CL	OE	HO	HW	CU
CL	–				
OE	0.0019	–			
HO	0.0066	0.0060	–		
HW	0.0068	0.0062	0.0039	–	
CU	0.0434	0.0426	0.0403	0.0387	–

The T92 model of nucleotide substitution (Tamura 1992) was used to estimate genetic distance among pooled populations and basins. Population segments are abbreviated as follows: *CL* Clinch River drainage, VA/TN; *OE* Obed-Emory River drainage, TN; *HO* Holston River drainage, TN; *HW* Hiwassee River drainage, TN; *CU* Cumberland River basin, KY/TN

Phylogenetic analysis of mtDNA sequence haplotypes showed nominal *Villosa perpurpurea* and the HW population of *V. trabalis* to comprise together a genetically distinct, monophyletic lineage (Fig. 3). Only minor differences, typically 1–5 bp, were observed among haplotypes between the CL, EO, HW, and HO populations, and further, only two presumably fixed nucleotide differences were observed between CL and the other three populations within the clade (Table 3). Similarly, sampled individuals of nominal *V. trabalis* from Cumberland River tributaries grouped together into their own monophyletic lineage; all recovered clades were well supported statistically by high posterior probability values (Fig. 3). The tree topology placed the Tennessee River basin clade, the Cumberland River basin clade, *Venustaconcha ellipsiformis*, and *V. pleasii* all together as sister taxa, while indicating similar levels of divergence for these four clades from outgroup taxa (Fig. 3).

Table 3 Summary of intraspecific sequence variation observed at the mitochondrial *ND1* gene among pooled populations of *Villosa perpurpurea* and *V. trabalis* in 2013–2015

Nominal Species	Population	N	No. unique haplotypes	Mean nucleotide substitutions, K (range)	Pairwise divergence*	Haplotype diversity (h)	Nucleotide diversity (π)
V. perpurpurea	Clinch (CL)	18	5	1.3 (0–6)	0.000–0.008	0.71242	0.00224
V. perpurpurea	Obed-Emory (OE)	25	6	1.0 (0–7)	0.000–0.009	0.48000	0.00176
V. perpurpurea	Holston (HO)	34	2	0.1 (0–1)	0.000-0.001	0.17045	0.00013
V. trabalis	Hiwassee (HW)	30	8	1.7 (0–8)	0.000–0.009	0.83678	0.00301
V. trabalis	Cumberland (CU)	33	4	0.5 (0–4)	0.000–0.005	0.53024	0.00068

* Estimated using uncorrected p-distance

Shell and mantle lure characters

All HW mussels had white nacre, while CL and OE specimens had nacre grading from purple to pink, and HO specimens had either purple or white nacre with no apparent intergradation (Fig. 4). Live individuals in the CL, OE and HO populations exhibited the most variability in nacre color, whereas individuals in the HW and CU populations only exhibited white nacre (Table 5). Females of each UTRB population had similar mantle lures (Fig. 5a–c), with undulating mantles drawing attention to large protruding gill ovisacs. These gill ovisacs appeared partitioned and were typically off-white in color and marked with a thin honeycomb-pattern overlapped by numerous minute black dots. When fully charged and extended, the most posterior partition of the gill ovisacs appeared less globular and dark in color, perhaps mimicking a tipulid larva (Fig. 5b). Mantle flap tissue was purple on the outer edge and completely white on the inner edge, which created noticeable color contrast when undulated and focused attention toward the protruding gill (Fig. 5c). The mantle lures of mussels in CU were structurally similar to those observed in UTRB, with undulating mantles and protruding gill ovisacs; however, mussels in CU had gills that were black in color with no visible honeycomb-pattern (Fig. 5d). Mantle flap tissue contrasted from dark black on the outer edge and completely white on the inner edge.

Discussion

Phylogenetic assessment

We conducted a phylogenetic assessment of all extant populations of two federally listed endangered species, *Villosa perpurpurea* and *V. trabalis* to resolve their taxonomic status. Our main findings were: (1) DNA sequence variation at the mitochondrial *ND1* gene revealed that

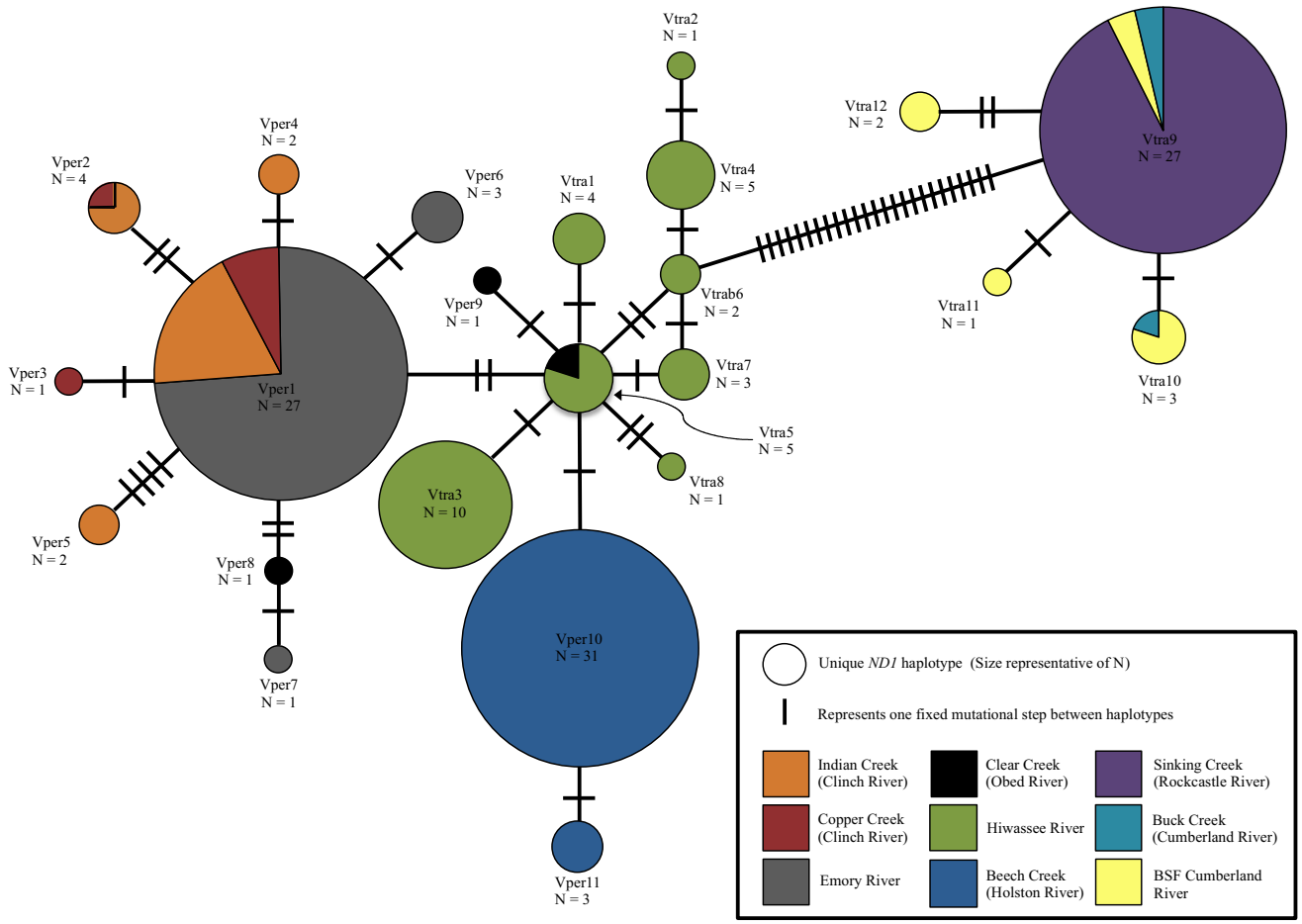
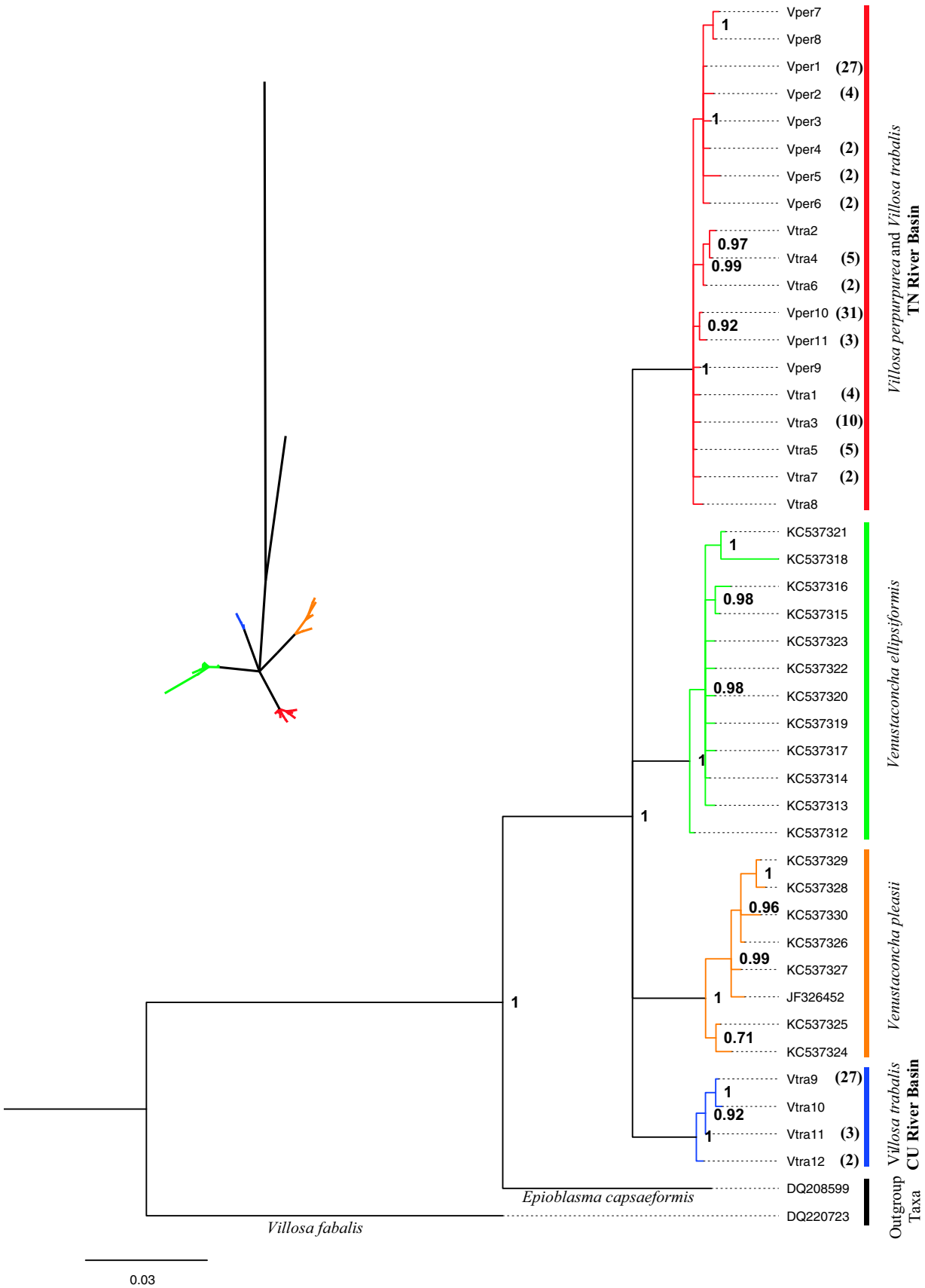


Fig. 2 Spanning network for a 784-bp region of the mitochondrial *ND1* gene of *Villosa perpurpurea* and *V. trabalis* haplotypes sampled in the Cumberland and Tennessee basins in 2013–2015. The network was produced in TCS, version 1.2.1 (Clement et al. 2000). The figure includes DNA sequences from NIH GenBank Sequence database (http://www.ncbi.nlm.nih.gov/genbank/). Accession numbers of all DNA sequences used in this study are available in Appendix 1



◀**Fig. 3** Unrooted and rooted trees representing phylogenetic relationships of unique haplotypes observed in *Villosa perpurpurea* and *V. trabalis* populations across the Cumberland and Tennessee River basins in 2013–2015 and hypothesized sister taxa *Venustaconcha ellipsiformis* and *V. pleasii* (sequences from Zanatta and Harris 2013), inferred from the mitochondrial *ND1* region (784 bp) using Bayesian consensus trees. *Numbers* on branches are calculated posterior probabilities for nodes. *Numbers* in parentheses signify total observed samples matching haplotype if greater than 1. Final average standard deviation of split frequencies was 0.008683, with the most likely tree possessing a -ln likelihood of −2948.570, with burn-in set to 20,000 and mean -ln likelihood of −2984.77. Outgroup taxa sequences for *Venustaconcha ellipsiformis*, *V. pleasii*, *Villosa fabalis*, and *Epioblasma capsaeformis* were acquired from the NIH GenBank Sequence database (http://www.ncbi.nlm.nih.gov/genbank/). Accession numbers of all DNA sequences used in this study are available in Appendix 1

investigated populations of *V. perpurpurea* and *V. trabalis* in the Tennessee River basin are the same species and genetically distinct from populations of *V. trabalis* in the Cumberland River basin, (2) mitochondrial DNA sequence haplotypes of *V. perpurpurea* and *V. trabalis* in the Tennessee River basin differed from haplotypes of *V. trabalis* in the Cumberland River basin by 21 fixed and diagnostic nucleotide sites, and (3) mitochondrial DNA sequence haplotypes of *V. perpurpurea* and *V. trabalis* in both river basins grouped together into a larger monophyletic clade that included DNA sequence haplotypes of *Venustaconcha ellipsiformis* and *V. pleasii*, further supporting the finding of Kuehnl (2009) that *V. perpurpurea* and *V. trabalis* belong in the genus *Venustaconcha*.

The 21 fixed nucleotide differences observed between mussels from the Cumberland River basin versus those from the Tennessee River basin convincingly demonstrates that distinct genetic lineages occur in each basin and have been diverging genetically for millennia. These taxa most likely evolved from a single common ancestor, whose populations following historical glacial expansion and retreat (or other natural historical processes) became geographically isolated from one another in the headwaters of these two major basins, driving allopatric speciation (Inoue et al. 2014; Jones et al. 2015). A very similar finding by Jones and Neves (2010) showed that two populations of tan

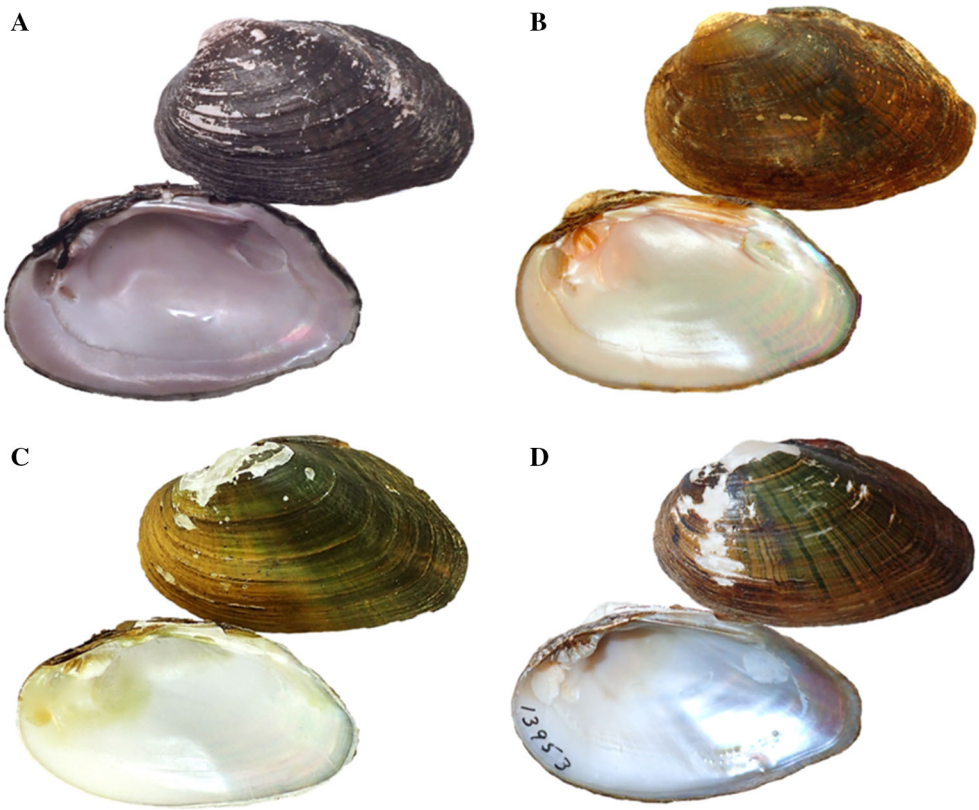


Fig. 4 Nacre *color* variation among shells of *Villosa perpurpurea* and *V. trabalis*: **a** female *V. perpurpurea* (specimen collected by Lane 2014) from Beech Creek, Hawkins Co., TN; **b** female *V. perpurpurea* (Neves 1991) from Copper Creek, Scott Co., VA; **c** male *V. trabalis* (Ahlstedt 1992) from Hiwassee River, Polk Co., TN; and **d** male *V. trabalis* (Athearn 1966) from Rockcastle River, Jackson Co., KY. Specimens **a–c** are at the shell collection located at the Freshwater Mollusk Conservation Center, Blacksburg, VA. Specimen **d** is at the North Carolina Museum of Natural Sciences, Raleigh, NC (Athearn Collection, 13953), courtesy A.E. Bogan. Photographs were taken by T.W. Lane, March–April 2015

Table 5 Nacre color and respective sample sizes of live *Villosa perpurpurea* and *V. trabalis* sampled at stream locations in upper Tennessee River and Cumberland River basins in 2014

Population	Nacre color observed on umbo				
	White	Pink	Purple	No data*	Total (*N*)
Clinch River (CL)	1	2	13	–	16
Obed-Emory (OE)	6	16	3	–	25
Holston River (HO)	8	–	8	15	31
Hiwassee River (HW)	30	–	–	–	30
Cumberland River (CU)	25	–	–	–	25
Total (*N*)	70	18	24	15	127

Nacre color was visually determined by inspecting the external umbo region of the shell

* No data means that shells were not visually inspected

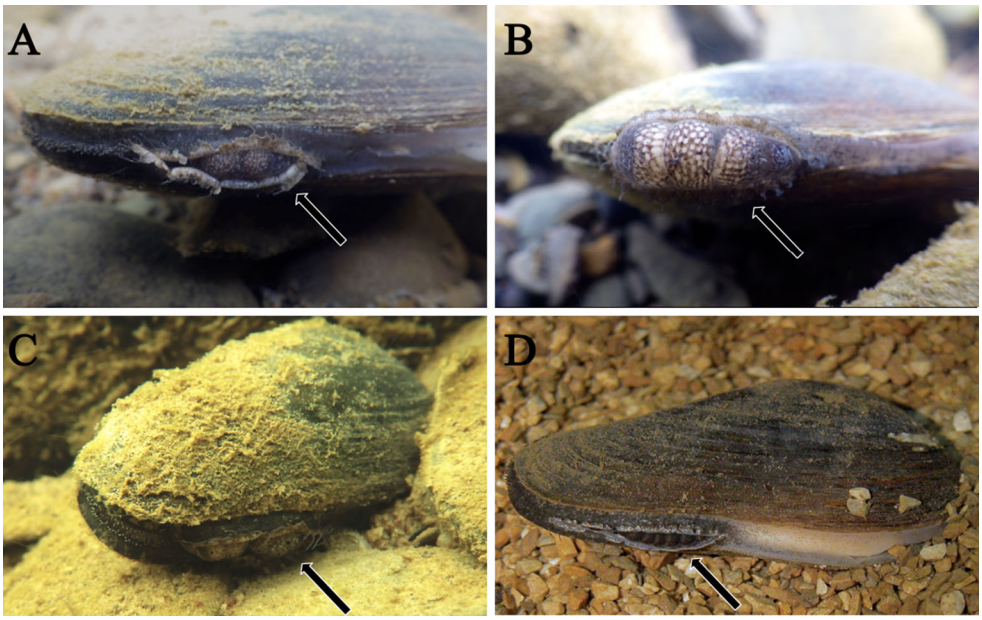


Fig. 5 Mantle-lure displays of selected female mussels: **a** *Villosa perpurpurea*, Indian Creek, Tazewell Co., VA; **b** *V. perpurpurea*, Beech Creek, Hawkins Co., TN; **c** *V. trabalis*, Hiwassee River, Polk Co., TN; **d** and nominal *V. trabalis*, Buck Creek, Laurel Co., KY. Photographs **a**–**c** were taken in native streams by Lane in Spring 2014. Photograph **d** (courtesy, M. McGregor) was taken of an individual held at the Center for Mollusk Conservation, Kentucky Department of Fish and Wildlife Resources, Frankfort, KY

riffleshell (*Epioblasma florentina walkeri*), one in the Cumberland River basin and another in the Tennessee River basin, warranted recognition as separate subspecies. Outside of the Tennessee-Cumberland River province, Zanatta and Harris (2013) provided evidence for allopatric speciation in populations of the sister species *Venustaconcha ellipsiformis* and *V. pleasii* in the Great Lakes, upper Ohio River, and Mississippi River basins. In our study, all haplotypes from the Tennessee River basin formed a distinct monophyletic clade with 100 % posterior probability support, as did all haplotypes from the Cumberland River basin (Fig. 3). There was no support for these two genetically distinct clades overlapping geographically in either basin. Thus, the strong phylogeographic concordance between the geographic and mtDNA data suggests that these two lineages are separate species. Since the mtDNA genome is small, haploid and not recombinant, analysis of another mtDNA gene region likely would only strengthen our current findings. Although not presented in this study, analysis of ten DNA microsatellite loci showed the same phylogeographic concordance as the mtDNA, supporting our conclusion of species status for each lineage (Lane et al. 2015).

Morphological assessment and observations

Our phylogenetic analyses indicated that the current taxonomic status of *V. perpurpurea* and *V. trabalis* is not valid. The taxonomic confusion can be attributed to the two species being distinguished by shell nacre color, purple

versus white, respectively. Our data clearly show that nacre color is not a character appropriate for distinguishing these two taxa. Our results for UTRB mussels showed that all HW specimens had white nacre, while CL and OE specimens had individuals grading from white to pink to purple nacre, and HO specimens had either purple or white nacre with no apparent intergradation (Fig. 4; Table 5).

We note that in streams with more alkaline pH and karst geology, e.g., those in the Valley and Ridge physiographic province, we have observed mostly purple-nacred individuals. In contrast, in streams with more neutral pH, nacre was predominantly white. This is the case for each of the sampled streams within CU, which are located in the Cumberland Plateau physiographic province, as well as for the sampled streams in OE and HW, whose headwaters originate in predominantly non-karst geology in the Appalachian Plateau and Blue Ridge physiographic provinces, respectively. The only stream in our study where this observation was not consistent was in Beech Creek, where nacre color was highly variable. Nacre color has been shown to be a variable trait across multiple unionid species and our findings may warrant further investigation into species complexes that are separated taxonomically using this character, e.g., lilliput *Toxolasma parvum,* pale lilliput *T. cylindrellus* and purple lilliput *T. lividum* (Williams et al. 2008). We further note that non-intergradation and continuous variation of nacre color occurs not only within and among the focal species of this study, but both within and among populations of other unionid mussel species, e.g., Alabama spike *Elliptio arca* and threehorn wartyback *Obliquaria reflexa* (see Haag 2012, Plate 11), and the white and purple nacre forms of spike *Elliptio dilatata*, among others.

Our knowledge of genetic, physiological and environmental determinants of color in mollusk nacre remains incomplete. Haag (2012) surmised that the continuous distribution of color variation in particular unionid species and its variability among populations would imply a connection to locally fixed alleles or quantitative genetic traits strongly influenced by environmental factors. In marine bivalves, the inner nacreous layer is created by a specific region of the epithelial cells of the mantle, involving expression of a number of genes and transcription factors (Jackson et al. 2006, 2010). Gene expression profiling among the respective mantle tissues that secrete red or white nacre in individual moon scallop *Amusium pleuronectes* (Huang et al. 2015) showed differential expression of genes involved in organic pigment assembly (notably vitellogenins) and biomineralization processes. Comparing gene expression among white, golden, black, and partially colored variants of Pacific oyster *Crassostrea gigas*, Feng et al. (2015) showed differential expression of ATP-binding cassette transporters, tyrosinase, and *notch* genes. While greater progress has been achieved in understanding processes of nacre coloration in marine mollusks, some progress has recently been realized for freshwater mussels, where recent work has investigated the effects of metal ions, organic pigments, and structural colors (Karampelas et al. 2009; Ji et al. 2013). Carotenoid pigments affect nacre coloration in freshwater pearl mussel *Hyriopsis cumingi*; levels are higher in purple lines than in white lines (Li et al. 2104a) and shell color is a partially heritable trait (Zhu 2011). Apolipoproteins (Apo) mediate the intracellular uptake of not only lipids, but also carotenoids; the level of Apo expression is correlated to carotenoid content and purple nacre coloration in *H. cumingi* (Li et al. 2014b). Using next-generation sequencing, Bai et al. (2013) identified 33 genes differentially expressed in white or purple individuals of *H. cumingi*, notably including biomineralization genes. Such genes included cobalamin, which has been associated with purple coloration of freshwater pearls (Yang et al. 2004). Much more work is needed to understand the genetic, physiological, and environmental bases of coloration of nacre in unionid species. Expression of this phenotypic trait may be partially influenced by environmental conditions, including water or substrate chemistry.

Additional morphological observations also support our genetically-based inference that populations in each basin represent distinct species. For example, glochidia of *Villosa perpurpurea* from the upper Clinch River in the UTRB are smaller than those of *V. trabalis* from the Rockcastle River, Cumberland River basin (Hoggarth 1988). Also, Simpson (1914) described pronounced shell dissimilarities between male and female specimens of both *V. perpurpurea* and *V. trabalis*, noting these features are typically less exaggerated in the former than in the latter. In addition, we have observed that young *V. trabalis* from the upper Cumberland River basin have bright green rays and yellow periostracum, whereas young *V. perpurpurea* from the upper Clinch River and *V. trabalis* from the Hiwassee River have thin dark green to black rays and light brown periostracum. As noted in the results, the mantle lures of mussels in the Cumberland River basin appear much darker than those of mussels in the Tennessee River basin, the latter typically having a honeycomb-pattern gill marked with numerous black dots (Fig. 5). While our sample size of photographed individual mantle-lures per population is small—one photographed female from each population—our field observations over the last 10 years are greater in number. For example, both TWL and JWJ collectively have observed >30 females of *V. perpurpurea* displaying their mantle-lures in Indian Creek, VA, >10 females of *V. perpurpurea* in Beach Creek, TN, and >20 females of *V. trabalis* in the Hiwassee River, TN. Due to current velocity, in-stream obstacles (e.g., rocks and wood debris),

specific orientation and position of the female mussel in situ, light conditions, cold water temperatures, photographing the mantle-lure display is extremely challenging. However, we have observed minimal variation in the color and morphology of the gill ovisac and mantle-lure of female mussels in these populations. Hence, it is likely these soft-anatomy traits are conserved and diagnostic between the Cumberland and Tennessee River basin populations. Increased sample sizes are needed to reach definitive conclusions and can be achieved in a future study by collecting live individuals from native streams and photographing them in a laboratory setting.

Taxonomic considerations

Our data support recognition of two genetically distinct species, which warrant placement in the genus *Venustaconcha* (see Frierson 1927; Thiele 1934) with *V. ellipsiformis* and *V. pleasii*, a finding reached previously by Kuehnl (2009). These four species are similar in shell characteristics, e.g., relatively thick-shelled, with numerous fine rays on the posterior portions of the valves. Females of each species have structurally similar mantle lures but with distinguishing features. In addition to morphology, each species shows similarities in habitat selection, life history characters, fish hosts, and heightened reproductive activity in winter and early spring months (Parmalee and Bogan 1998; Watson 1999; Williams et al. 2008; Watters et al. 2009; Zanatta and Harris 2013), suggesting that they evolved from a common ancestor and share a similar ecological niche.

The name *Unio perpurpurea* (Lea 1861) should no longer be considered valid. Instead, as Ortmann (1925) correctly suggested, the name *U. perpurpurea* should be considered a synonym of *V. trabalis*, and its associated color morphs should be considered phenotypic variants. The species name *Unio trabalis* (Conrad 1834), whose type locality is Flint Creek, AL, in the lower Tennessee River basin (Ortmann 1925; Williams et al. 2008), should receive priority as the species name for the genetic lineage occurring in the Tennessee River and its tributaries, historically within AL, GA, TN, VA. With no evidence that this lineage occurs or occurred in the Cumberland River basin, we propose changing the common and scientific names of this species to Tennessee bean *Venustaconcha trabalis* (Conrad 1834).

Unio troostensis (Lea 1834), whose type locality is Stones River, TN, in the Cumberland River basin, is the oldest available name (Parmalee and Bogan 1998) and should receive priority for the genetic lineage extant in the Cumberland River basin within KY and TN. With no evidence that this lineage occurs or occurred in the Tennessee River drainage, we propose the common and scientific names Cumberland bean *Venustaconcha troostensis* (Lea 1834).

The genetic and morphological data presented in this study have clarified long-standing confusion over the systematics and taxonomy of these species. It is now evident that the original taxonomy of *Unio trabalis* (Conrad 1834) and *U. troostensis* (Lea 1834), respectively—though based on shell traits alone—was valid, and these names were maintained from 1834 to 1861. This taxonomy then was confounded by description of the *U. trabalis* synonym, *U. perpurpureus* (Lea 1861), followed by Simpson (1900) incorrectly synonymizing *U. troostensis* with *U. trabalis* and concurrently upholding the validity of *U. perpurpurea*.

Implications for conservation and management

The USFWS has written recovery plans for *Villosa perpurpurea* and *V. trabalis* based on an outdated concept of their taxonomic status and distribution in the Tennessee–Cumberland province (USFWS 1984, 2004). Recovery plans for each species will need to be revised based on our taxonomic revision, reassessments of their historical and current distribution, conservation status, life-history data, and threats analyses in their respective river basins. Shell morphology, and sexually dimorphic characters, as well as information ascertained since the recovery plans were written (e.g., glochidia size, mantle-lure characteristics, suitable fish hosts), also will need to be thoroughly reviewed and revised for each taxon. For example, many of the previous shell and soft tissue observations and descriptions of *V. trabalis*, e.g., Simpson (1914), were made using collections combining specimens from the Cumberland and Tennessee river basins. Specifically, sample sizes of soft-anatomy and shell traits will need to be increased to provide a more robust analyses beyond the data and observations presented in our study. More generally, it is critical that phylogenetic studies of mussels include large sample sizes across all known populations for focal taxa, without which we would not have been able to reach defensible inferences on the phylogenetic and taxonomic status of *V. perpurpurea* and *V. trabalis*.

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and laboratory analyses. We thank Dr. Monte McGregor (Kentucky Department of Fish and Wildlife Resources) and Todd Fobian (Alabama Department of Conservation and Natural Resources) for collaboration with mantle lure photography and access to tissue samples from preserved specimens. We thank Dr. Arthur Bogan and colleagues (North Carolina Museum of Natural Sciences) for access to preserved specimens. We are grateful to Bob Butler, USFWS for providing useful comments on a previous draft. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the Commonwealth of Virginia or U.S. Government. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the USFWS.

Appendix 1

Table 6.

Table 6 Locations and accession numbers for additional DNA sequences included in phylogenetic analyses of *Villosa trabalis* and *V. perpurpurea*; sequences were obtained from the NIH GenBank database

Species	Author(s)	Collector(s)	Location(s)	Drainage/basin	Accession number
Villosa perpurpurea	Buhay et al. (2002)	S. Ahlstedt	Beech Creek, TN	Tennessee	DQ445190
	Kuehnl (2009)	J. Jones	Beech Creek, TN	Tennessee	GQ921297
			Indian Creek, VA	Tennessee	GQ921298
			Indian Creek, VA	Tennessee	GQ921299
			Beech Creek, TN	Tennessee	GQ921300
	Present study	T. Lane	Copper Creek, VA	Tennessee	KT964368
			Indian Creek, VA		
			Upper Emory River, TN		
			Copper Creek, VA	Tennessee	KT964369
			Indian Creek, VA		
			Copper Creek, VA	Tennessee	KT964370
			Indian Creek, VA	Tennessee	KT964371
			Indian Creek, VA	Tennessee	KT964372
			Upper Emory River, TN	Tennessee	KT964373
			Emory River, TN	Tennessee	KT964374
			Clear Creek, TN	Tennessee	KT964375
			Clear Creek, TN	Tennessee	KT964376
			Beech Creek, TN	Tennessee	KT964377
			North Fork Beech Creek, TN		
			Beech Creek, TN	Tennessee	KT964378
Villosa trabalis	Buhay et al. (2002)	S. Ahlstedt	BSF Cumberland River, TN	Cumberland	DQ445195
	Kuehnl (2009)	M. McGregor	Buck Creek, KY	Cumberland	GQ921256
			Sinking Creek, KY	Cumberland	GQ921257
			Buck Creek, KY	Cumberland	GQ921258
			Sinking Creek, KY	Cumberland	GQ921259
			Sinking Creek, KY	Cumberland	GQ921260
		S. Ahlstedt	BSF Cumberland River, TN	Cumberland	GQ921301
			BSF Cumberland River, TN	Cumberland	GQ921302
	Present study	T. Lane	Hiwassee River, TN	Tennessee	KT964379
			Hiwassee River, TN	Tennessee	KT964380
			Hiwassee River, TN	Tennessee	KT964381
			Hiwassee River, TN	Tennessee	KT964382
			Hiwassee River, TN	Tennessee	KT964383
			Hiwassee River, TN	Tennessee	KT964384
			Hiwassee River, TN	Tennessee	KT964385

Table 6 continued

Species	Author(s)	Collector(s)	Location(s)	Drainage/basin	Accession number
			Hiwassee River, TN	Tennessee	KT964386
		M. McGregor	BSF Cumberland River, KY	Cumberland	KT964387
			Buck Creek, KY		
			Sinking Creek, KY		
			BSF Cumberland River, KY	Cumberland	KT964388
			Buck Creek, KY		
			BSF Cumberland River, KY	Cumberland	KT964389
		S. Ahlstedt	BSF Cumberland River, TN	Cumberland	KT964390
*Venustaconcha ellipsiformis**	Zanatta and Harris (2013)	Authors	Boubeuse River, MO	White	KC537312
			Multiple Locations	Great Lakes	KC537313
				Illinois	
				Upper Mississippi	
				White	
			Ferson Creek, IL	Illinois	KC537314
			Ferson Creek, IL	Illinois	KC537315
			Ferson Creek, IL	Illinois	KC537316
			Mackinaw River, IL		
			Gasconade River, MO	White	KC537317
			Gasconade River, MO	White	KC537318
			Horse Creek, IL	Illinois	KC537319
			Mackinaw River, IL	Illinois	KC537320
			Zumbo River, MI	Upper Mississippi	KC537321
			Zumbo River, MI	Upper Mississippi	KC537322
			Zumbo River, MI	Upper Mississippi	KC537323
*Venustaconcha pleasii**	Campbell and Lydeard (2012)	C. Barnhardt	Beaver Creek	White	JF326452
	Zanatta and Harris (2013)	Authors	Flat Creek, MO	White	KC537324
			Flat Creek, MO	White	KC537325
			Flat Creek, MO	White	KC537326
			Flat Creek, MO	White	KC537327
			James River, MO	White	KC537328
			James River, MO	White	KC537329
			James River, MO	White	KC537330
*Epioblasma capsaeformis**	Jones et al. (2006)	J. Jones	Clinch River, TN	Upper Tennessee	DQ208599
*Villosa fabalis**	Zanatta and Murphy (2006)	D. Zanatta	Allegheny River, PA	Upper Ohio	DQ220723

* Outgroup taxa

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