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Co-occurrence and Hybridization between *Necturus maculosus* and a Heretofore Unknown *Necturus* in the Southern Appalachians

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Abstract.—**The only mudpuppy (Caudata: Proteidae) known to occur in the Tennessee Valley of the Interior Highlands and Southern Appalachians is the Common Mudpuppy (*Necturus maculosus*). *Necturus maculosus* is not known to co-occur with any other congeners. Here, we report evidence that an additional *Necturus* occurs in the Hiwassee River, a tributary of the Tennessee River, in eastern Tennessee. Some specimens from the Hiwassee River are clearly identified as *N. maculosus*, but others resemble the Neuse River Waterdog (*N. lewisi*), known from only the Tar-Neuse river system draining to the Atlantic Ocean on the opposite side of the Appalachian Mountains. Concordance between color pattern, mitochondrial DNA, and four nuclear loci demonstrate that these two co-occurring forms represent distinct lineages rather than color variants within a single, panmictic population. A few mismatched genotypes (7 of 32 individuals in total) suggest rare hybridization and backcrossing. Phylogenetic analyses indicate that the new form (hereafter *N. aff. lewisi*) is related to *N. lewisi* and *N. punctatus* (both species from the Atlantic Coastal Plain and Piedmont), but whether this population was introduced, is a naturally disjunct population of *N. lewisi*, or a heretofore unknown species is yet unclear. Regardless, its existence raises new questions about the evolutionary and ecological dynamics of riverine salamander communities in southern Appalachia and for conservation and management.**

In well-studied regions of the world, discovering diversity previously unknown to science is becoming increasingly uncommon (Ceballos and Ehrlich, 2009; ISSE, 2011). The Southern Appalachians in eastern North America is a global hotspot of salamander biodiversity and has been subjected to intense systematic study for more than a century (Hairston, 1987; Petranka, 1998; Highton and Peabody, 2000). New salamander diversity often is described after phylogenetic analyses of molecular data reveal substantial divergence among morphologically similar lineages (i.e., cryptic species; Highton and Peabody, 2000; Crespi et al., 2010; Tilley et al., 2013). In such cases, a described taxon is "split" into multiple species or subspecies (Isaac et al., 2004; Zachos et al., 2013). Rarely have distinctly new species been discovered in the past several decades from the Southern Appalachians (but see Wynn et al., 1988; Camp et al., 2009).

The genus *Necturus* (Proteidae) includes five species of large neotenic salamanders commonly referred to as mudpuppies and waterdogs. All are endemic to eastern North America (Petranka, 1998). *Necturus maculosus* (Common Mudpuppy) has the largest range in the genus (Fig. 1), occurring from southern Canada southward into northern Mississippi, Alabama, and Georgia in the Interior Plateau, and into northern Louisiana west of the Mississippi River (Petranka, 1998; Pasachnik and Niemiller, 2011). It is the only *Necturus* known from the Tennessee Valley, including the Hiwassee River and other major tributaries of the Tennessee River (Pasachnik and Niemiller, 2011). All other described *Necturus* species inhabit streams and rivers within the Piedmont, Atlantic Coastal Plain, and Gulf Coast Plain that ultimately drain into the Atlantic Ocean or Gulf of Mexico independently of the Mississippi (Petranka, 1998).

Many *Necturus* in the Hiwassee River of eastern Tennessee resemble *N. maculosus* (Fig. 2). They have typical coloration and patterning, including irregular black spots on the dorsum, a grayish-to-whitish venter with some spotting, and a distinct facial stripe running from the canthus through the eye and extending to the gills (Viosca, 1937; Petranka, 1998; Pasachnik and Niemiller, 2011). In contrast, some individuals have larger, more distinct but less numerous spots on both dorsum and venter, mottled brown dorsal ground coloration, and lack a well-defined facial stripe (Fig. 3). These individuals resemble the Neuse River Waterdog (*N. lewisi*), known from the Neuse and Tar rivers in the Piedmont and Atlantic Coastal Plain regions of North Carolina (Fig. 1). These rivers are on the opposite side of the Eastern Continental Divide from the Hiwassee River and drain into the Atlantic Ocean via Pamlico Sound, whereas the Hiwassee River joins the Tennessee River and then the Mississippi River to drain into the Gulf of Mexico (Viosca, 1937; Ashton, 1990; Petranka, 1998).

Here, we use molecular genetics to examine whether individuals morphologically resembling *N. lewisi* are distinct from co-occurring individuals identified as *N. maculosus* within the Hiwassee River of eastern Tennessee. We incorporate phylogenetic data from all currently recognized *Necturus* species to assess the evolutionary relationships of Hiwassee River *Necturus* to the other members of the genus. Hereafter, we refer to *N. lewisi*–like salamanders from the Hiwassee River as *N. aff. lewisi* to indicate that they are closely related to *N. lewisi* but might represent an undescribed taxon (Sigovini et al., 2016).

Materials and Methods

Study Area and Sampling.—We sampled for *Necturus* periodically from May through September in 2012–2015 in a 4-km reach of the Hiwassee River in Polk County, Tennessee, located between the Appalachia Powerhouse and the confluence with the Tennessee River. We also sampled in neighboring tributaries of the Tennessee River, including the Little River, Tellico River, and Citico Creek (Fig. 1; Table S1). We captured *Necturus* under rocks and logs during snorkel surveys. In March 2015, we also used baited minnow traps to sample *Necturus* in deeper pools

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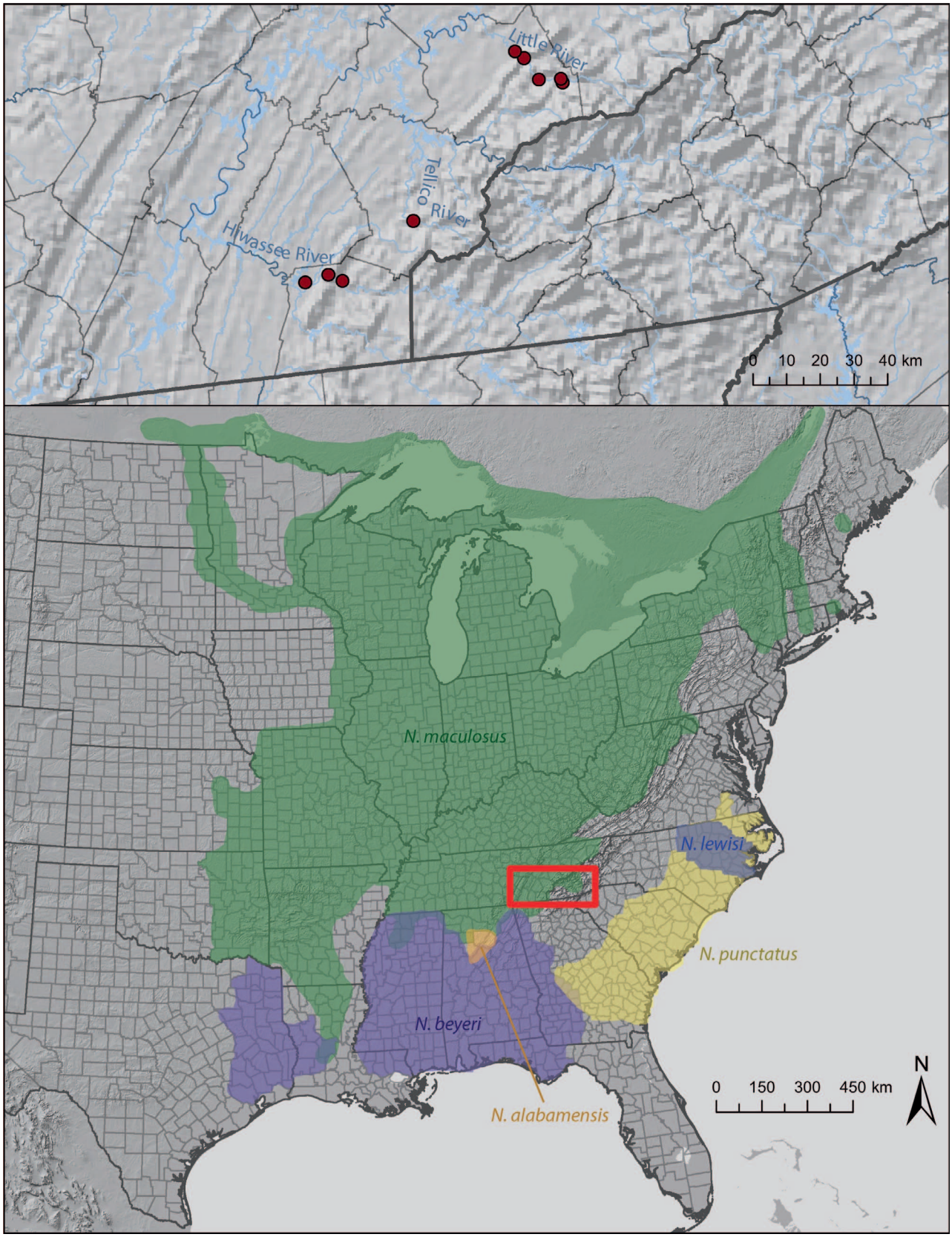

FIG. 1. Sampling locations (top) and geographic ranges of known *Necturus* species (bottom) based on county records and most recently published distribution maps (Petranka, 1998; Beane et al., 2010; Pasachnik and Niemiller, 2011).

FIG. 2. Example of *N. maculosus* from the Hiwassee River, Tennessee. Three photographs of the same specimen (N59) were adjusted to the same total length and merged.

that could not normally be targeted during snorkel surveys (McDaniel et al., 2009; Craig et al., 2015).

We photographed each specimen, measured total length (TL) and snout–vent length (SVL), and took 1 cm of tail tissue for molecular analyses. We collected voucher specimens of both forms (two *N. aff. lewisi* and nine *N. maculosus*), currently held in the University of Tennessee's Department of Ecology and Evolutionary Biology collection (Table S1).

To ensure complete taxon sampling for each gene, we obtained tissue from two *N. maculosus* from Lincoln Lake, Michigan; two *N. punctatus* from Drowning Creek, North Carolina; two *N. lewisi* from Contentnea Creek, North Carolina; and one *N. alabamensis* from Sipsey Fork, Alabama (Table S1). We also downloaded from GenBank all available *Necturus* sequence data corresponding to loci used in this study: AY141897, AY650136–AY650137, AY916042–AY916043, DQ517763, EF107245, EF107279, EF107305, EF107338, EF107442, GQ368658, JX144985–JX144990, JX144997–JX145002, JX145009–JX145014, JX145025–JX145030, and KC165593.

FIG. 3. Example of *N. aff. lewisi* from the Hiwassee River, Tennessee. Three photographs of the same specimen (N61) were adjusted to the same total length and merged.

DNA Sequencing.—We extracted DNA from tail tips by using DNeasy kits (QIAGEN Inc., Valencia, California, USA). We used polymerase chain reaction (PCR) to amplify fragments of one mitochondrial and four nuclear loci following previously published primers and protocols (Table S2; Weisrock et al., 2005; Bonett et al., 2013). The five loci included 823 base pairs (bp) of mitochondrial NADH dehydrogenase 2 (ND2), 541 bp of sodium-calcium exchanger 1 (NCX1), 481 bp of pro-opimelanocortin (POMC), 1402 bp of recombination activating protein-1 (RAG1), and 393 bp of solute carrier family 8 member 3 (SLC8a3). We developed a new internal forward primer for the ND2 locus due to poor amplification in some samples (ND2fi: 5′-GCAACAGAAGCCACTACTAAATA-3′). We found that a touchdown protocol (Palumbi, 1996) yielded the best products for sequencing, with minimal nonspecific amplification. Detailed PCR conditions are given in Table S2 and primer sequences are listed in Table S3.

We purified PCR products using exonuclease I and shrimp alkaline phosphatase (ExoSap-IT, Santa Clara, California, USA) and had them sequenced in both directions by using the PCR primers at the University of Tennessee's Molecular Biology Resource Facility on an ABI 3730 sequencer (Life Technologies, Carlsbad, California, USA). We used Sequencher 5.0.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) to edit and align sequence reads into contigs. We inspected all nuclear sequence chromatograms to identify heterozygous sites. Heterozygotes were rare and their alleles easily reconstructed as alleles observed elsewhere in homozygous state (Clark, 1990). There was no evidence of recombination according to the four-gamete test (Hudson, 1985). Therefore, the resolution of heterozygotes as carrying known alleles is the haplotype reconstruction with highest likelihood (Stephens et al., 2001). Unique alleles were aligned with published *Necturus* sequences from GenBank by using the align-to-reference algorithm in Sequencher. Unique sequences of each locus have been accessioned to GenBank (KX842531–KX842547, KY225843–KY225861).

Gene Tree Estimation.—We estimated gene trees independently for each locus by using the Bayesian Markov chain Monte Carlo (MCMC) algorithm of mrBayes v3.2 (Ronquist et al., 2012). We used jModelTest v2.1.7 to choose the best fit nucleotide substitution models (Darriba et al., 2012). Because all loci were protein coding sequences, we partitioned each by codon position. For each locus, we ran four replicate MCMC searches, each with four chains (three hot, one cold), for 10 million generations and sampled parameters and trees every 1000 generations after a 2.5 million generation burnin. We assessed convergence qualitatively by agreement among independent runs and quantitatively by the standard deviation of split frequencies (SDSF < 0.01), effective sample sizes (ESS $> 1{,}000$), and potential scale reduction factors (PSRF ~ 1.000). We generated a 50% majority-rule consensus tree for each locus by using trees sampled from the stationary distributions. Each gene tree was midpoint rooted in FigTree v1.4.3.

Combined analysis: To present a summary tree, we used *BEAST v2.4.5 (Heled and Drummond, 2010) to simultaneously estimate the five gene trees and a single containing tree (species tree) under the assumption that *N. aff. lewisi* should be treated as a distinct taxonomic unit from *N. lewisi*. Data were entered as alleles (two per individual except for mtDNA) and assigned to taxa based on the separate gene trees from mrBayes (Figs. S1–S5). We used the same codon partitions and substitution models as in the mrBayes analysis. We ran four independent MCMC

chains for 2×10^8 generations each by using a lognormal relaxed clock model, a Yule-process speciation prior, and sampling every 10,000 generations. We used Tracer v1.6.0 and TreeAnnotator (part of the BEAST package) to assess stationarity and establish an adequate burnin. We assessed posterior support for each node using TreeAnnotator and visualized the posterior distribution of containing trees with Densitree v2.0 (part of the BEAST package).

Population genetics: To evaluate the extent of interbreeding between forms within the Hiwassee River, we estimated deviations from Hardy–Weinberg and linkage equilibrium by using the R package 'genetics' v1.3.8.1 (Warnes et al., 2013). We assume the contiguous 4-km sampling area represents a single undivided population. Based on the gene trees (Figs. S1–S5), we classified each unique allele in the Hiwassee sample as belonging to the *N. lewisi/punctatus* clade or the *N. maculosus/alabamensis/beyeri* clade. We then classified each individual's genotype as homozygous for *N. lewisi*–like alleles, heterozygous, or homozygous for *N. maculosus*–like alleles. We used the exact test (Engels, 2009) and randomization to test for deviations from single-locus Hardy–Weinberg expectations. To assess linkage disequilibrium (co-occurrence of *N. aff. lewisi* alleles within individuals) for each pair of loci, we calculated correlation coefficients and used randomization to test the null hypothesis of independent assortment within the Hiwassee sample.

To provide an overall summary of the population structure within the Hiwassee River, we used STRUCTURE v2.3.2.1 (Pritchard et al., 2000; Falush et al., 2003) to estimate ancestry proportions for each individual. We assumed an admixture model with two ancestral populations ($K = 2$), correlated allele frequencies, and standard uninformative priors on frequencies and individual ancestries. We ran 10 independent chains with 10^6 generation burnin and 10^6 post burnin generations.

Morphology.—We estimated means and 95% confidence intervals for total length, tail length, and relative tail length (tail length/total length) to compare adults captured for this study with data for *N. lewisi* reported by Viosca (1937). Although Viosca (1937) did not report variances or standard deviations, we estimated standard errors and 95% confidence intervals using his reported means and sample sizes for *N. lewisi* and assuming equal variances between *N. lewisi* and *N. aff. lewisi*. We make this working assumption for the purposes of heuristic comparison. In the same manner, we computed two-sample *t*-statistics to test the null hypothesis of no difference between the measurements of Viosca (1937) of *N. lewisi* and our measurements of *N. aff. lewisi*.

Results

Gene Trees.—Gene trees for each locus (from 30,000 samples from the posterior distributions) are presented in Figs. S1–S5. All four independent chains resulted in identical tree topologies for each gene. The maximum standard deviation of split frequencies across all genes and parameters was 0.009264, the minimum ESS was 3404.9, and all PSRFs were unity to three significant digits (Table S4); therefore, we are confident that the resulting gene trees shown in Figures S1–S5 represent the stationary distribution of each Bayesian MCMC analysis.

Combined analysis: Species tree analysis assuming *N. aff. lewisi* is a distinct taxonomic unit indicated strong support for a close sister relationship between *N. aff. lewisi* and *N. lewisi* (Fig. 4, posterior probability 0.9999). All four independent chains yielded the same result even with no burnin. With 25% burnin the posterior ESS ranged from 1,101 to 1,221 and stationarity of the Markov chains was indicated by absence of any trends in trace plots.

Gene trees for all five loci and the combined tree recovered the same phylogenetic pattern described by Bonett et al. (2013). There were two well-supported clades, one including *N. lewisi* and *N. punctatus* and the other including *N. alabamensis*, *N. beyeri*, and *N. maculosus*. Sequences from the Hiwassee River (from specimens N01 through N32) group unambiguously in either one or the other of these two clades. Sequences generated from the same individual almost always grouped in the same clade across gene trees; however, there were seven individuals (22% of the Tennessee sample) with discordant genotypes. Within the Hiwassee sample, 6 of the 22 individuals (27% of the sample) were identified morphologically as *N. aff. lewisi* but found to have one or two *maculosus*-like alleles (Fig. 5). We found one additional individual from the Little River (identified in the field as *N. maculosus*) to have one *lewisi*-like allele, resulting in an estimated 8.3% individual admixture estimate from STRUCTURE (Fig. 5). Individuals morphologically identified as *N. aff. lewisi* always had all or most alleles group with the *N. lewisi/punctatus* clade, and individuals morphologically identified as *N. maculosus* had all or most alleles group with the *N. maculosus* clade (Fig. 5).

Quantitatively, divergences between *N. aff. lewisi* and *N. lewisi* sequences were slightly greater, on average, than divergences between the recognized species *N. alabamensis* and *N. beyeri* (Table S5). For example, mitochondrial ND2 sequences averaged 3.48% (±0.44 SD) divergence between *N. aff. lewisi* and *N. lewisi*, in comparison to average 3.35% (±0.08) divergence between *N. alabamensis* and *N. beyeri*.

Population genetics: Within the Hiwassee River, 6 of 22 individuals possessed alleles from both clades (Fig. 5; Table S1). Nuclear alleles differed by a small number of nucleotides and none failed the four-gamete test, consistent with little to no recombination among homologous alleles within the sample (Hudson 1985). Homozygous individuals always were homozygous for an allele grouping unambiguously with either the *N. lewisi/punctatus* clade or the *N. maculosus* clade (Fig. 5). The few heterozygous samples could always be resolved, assuming no recombination, as pairs of alleles observed elsewhere in homozygous state.

All nuclear loci were significantly deviant from Hardy–Weinberg expectations within the Hiwassee River sample, and all pairwise linkage disequilibria were positive and statistically significant (Tables 1 and 2). Multilocus ancestry estimates (Q) from STRUCTURE also illustrate the co-occurrence of two distinct genotypic clusters with the most admixed individual (specimen N24) estimated to have 83% *N. aff. lewisi* ancestry (Fig. 5). Therefore, we reject the null hypothesis that *Necturus* within the Hiwassee River constitute a single, randomly mating population. Instead, our sample consists predominantly of individuals sharing genetic affinity with *N. lewisi*, a smaller number of *N. maculosus*, and a few individuals with evidence of some mixed ancestry.

Morphology.—Our measurements of total length and tail length for *N. aff. lewisi* were substantially smaller than those reported for *N. lewisi* (Table 3). Although there might be several confounding variables, Viosca's means are upward of 50% larger than ours, suggesting a real biological difference between *N. aff. lewisi* from the Hiwassee River and the original *N. lewisi* from the Neuse and Tar rivers. Our estimates of relative tail length tend to be slightly

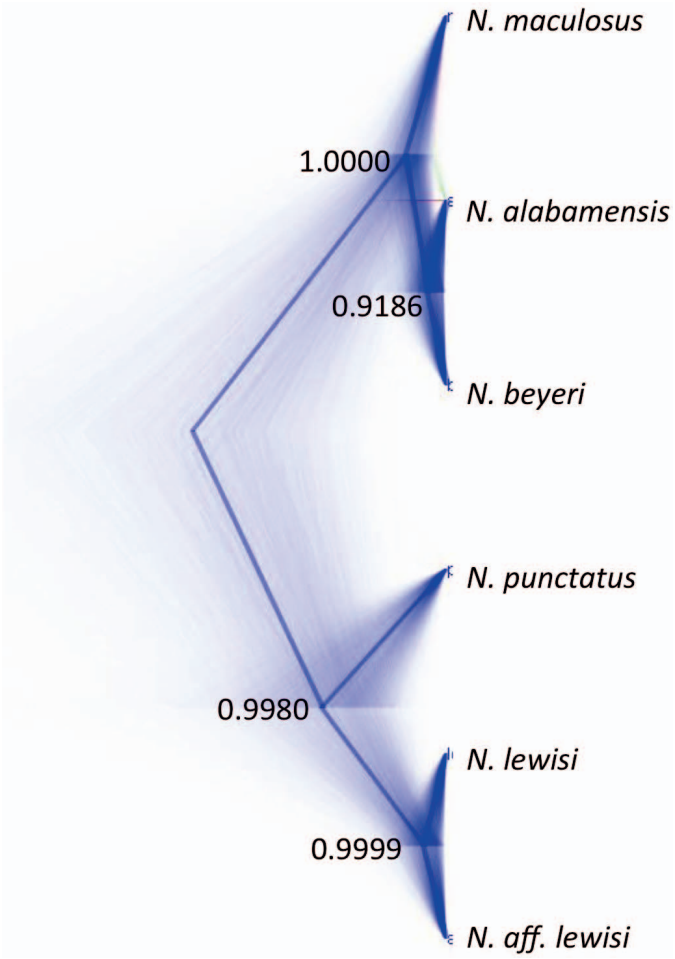

FIG. 4. Summary "species tree" (treating *N. aff. lewisi* as an operational taxonomic unit) from *BEAST visualized with Densitree. Thick lines illustrate the consensus tree and thinner lines show 10,000 samples from the posterior distribution of species trees. Posterior support at each node was estimated as the fraction of the posterior sample including the clade.

smaller than the estimate based on Viosca (1937), but the estimated 95% confidence intervals overlap (Table 3). Therefore, we have no strong evidence of a difference in tail/body proportion, but reasonable evidence that *N. aff. lewisi* in the Hiwassee River tend to be smaller than *N. lewisi* from North Carolina. Differences in measurement are confounded by differences in preservation and observer, but the magnitudes of differences in means are substantial. Obviously, the biological significance of differences in measurements cannot be inferred without common garden experiments.

DISCUSSION

Our study revealed that two distinct *Necturus* coexist in the Hiwassee River in eastern Tennessee. *Necturus maculosus* was previously known to occur in the region, but it was not known to coexist or overlap geographically with any other *Necturus* (Petranka, 1998; Pasachnik and Niemiller, 2011). The newly discovered form is most similar to *N. lewisi*, but it might be a new undescribed taxon. Although sampling has been limited, the known geographic distribution of *N. aff. lewisi* currently is restricted to the Hiwassee River watershed. Given that *N. lewisi* is found only in the Tar and Neuse rivers, which both drain to

			Loci					
ID	Taxon	River	m	R	N	S	P	Q
N01	*aff. lewisi*	Hiw	L4	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N02	*aff. lewisi*	Hiw	L4	--	L5	--	L5	91.4%
				--	L5	--	m2	
N03	*aff. lewisi*	Hiw	L5	L4	L5	L4	L1	99.4%
				L4	L5	L4	L1	
N04	*aff. lewisi*	Hiw	L5	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N05	*aff. lewisi*	Hiw	L6	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N06	*aff. lewisi*	Hiw	L4	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N07	*aff. lewisi*	Hiw	L7	L3	L5	L4	L4	96.0%
				L3	L5	L4	m2	
N10	*aff. lewisi*	Hiw	L8	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N11	*aff. lewisi*	Hiw	L9	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N22	*aff. lewisi*	Hiw	L2	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N23	*aff. lewisi*	Hiw	L4	L5	L4	L4	L5	99.4%
				L5	L4	L4	L5	
N24	*aff. lewisi*	Hiw	L4	L5	m1	L4	L5	83.0%
				L5	m1	L4	L5	
N25	*aff. lewisi*	Hiw	L4	L5	L5	L4	L5	99.4%
				L5	L5	L4	L5	
N27	*aff. lewisi*	Hiw	L3	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N28	*aff. lewisi*	Hiw	L10	L6	L5	L4	L5	99.4%
				L6	L5	L4	L5	
N29	*aff. lewisi*	Hiw	m4	L6	L5	L4	L5	98.8%
				L6	L5	L4	L5	
N30	*aff. lewisi*	Hiw	m4	L6	L5	L4	L5	98.8%
				L6	L5	L4	L5	
N31	*aff. lewisi*	Hiw	m4	L4	L5	L4	L5	98.8%
				L4	L5	L4	L5	
N08	*maculosus*	Hiw	m2	m5	m1	m2	m1	0.6%
				m5	m1	m2	m1	
N09	*maculosus*	Hiw	m3	m5	m1	m1	m1	0.6%
				m5	m1	m2	m1	
N26	*maculosus*	Hiw	m3	m7	m1	m1	m1	0.6%
				m7	m1	m1	m1	
N32	*maculosus*	Hiw	m8	m7	m1	m1	m1	0.6%
				m7	m1	m1	m1	
N20	*maculosus*	Cit	m6	m6	m3	m1	m1	0.6%
				m6	m3	m2	m1	
N21	*maculosus*	Cit	m7	m6	m3	m1	m1	0.6%
				m6	m3	m1	m1	
N12	*maculosus*	Tel	m4	m5	m1	m2	m1	0.6%
				m5	m1	m2	m1	
N13	*maculosus*	Lit	m4	m6	m1	m1	m1	0.6%
				m6	m1	m2	m1	
N14	*maculosus*	Lit	m4	m3	m1	m2	m1	0.6%
				m3	m1	m2	m1	
N15	*maculosus*	Lit	m5	m6	m1	m2	m1	0.6%
				m6	m1	m2	m1	
N16	*maculosus*	Lit	m4	m6	m1	m1	m1	0.6%
				m6	m1	m2	m1	
N17	*maculosus*	Lit	m4	m4	m1	m2	m1	0.6%
				m4	m1	m2	m1	
N18	*maculosus*	Lit	--	--	--	m1	m1	1.3%
				--	--	m2	m1	
N19	*maculosus*	Lit	m4	m5	m1	m2	L5	8.3%
				m5	m1	m2	m2	

FIG. 5. Genotypes of *Necturus* sampled in Tennessee. Taxon was assigned in the field based on morphological differences between *N. maculosus* (Fig. 2) and *N. aff. lewisi* (Fig. 3). River abbreviations are a follows: Hiw, Hiwassee River; Cit, Citico Creek; Tel, and Tellico River; Lit, Little River. Alleles are color coded based on their clade affinities in the gene trees (Figs. S1–S5): *N. lewisi*–like alleles are light gray and *maculosus*-like alleles are dark gray. Genotypes are represented by two lines per individual except for the mitochondrial ND2 haplotype (m). Nuclear loci are R, RAG1; N, NCX1; S, SLC8a3; and P, POMC. Specific allele names correspond to Table S1. *Q* is the estimated ancestry proportion from STRUCTURE. Dashes indicate missing data.

TABLE 1. Hardy–Weinberg tests for each nuclear locus within the Hiwassee River sample of *Necturus*. Genotypes are summarized as LL when both alleles were from the *lewisi*/*punctatus* clade, MM when both alleles were from the *maculosus* clade, and LM when an individual was inferred to have one allele from each clade. F_{IS} is Wright's standardized measure of deviation from expected heterozygote frequencies. *P* values are given for the randomization test (10,000 replicates) and the exact test (Warnes et al., 2013).

	Genotype count					
Locus	LL	LM	MM	F_{IS}	*P*(10,000 replicates)	*P*(exact)
NCX1	17	0	5	1.00	<0.0001	1.06×10^{-5}
POMC	16	2	4	0.75	0.0014	0.001815
RAG1	17	0	4	1.00	<0.0001	5.07×10^{-5}
SLC8a3	17	0	4	1.00	<0.0001	5.07×10^{-5}

the Atlantic Ocean via Pamlico Sound (Fig. 1), occurrence of a close relative on the other side of the Eastern Continental Divide raises questions regarding the biogeographic, hydrologic, and ecological history of the region. One possibility is that *N. lewisi* were recently introduced into the Hiwassee River by humans. In contrast, if the Hiwassee form is native, the primary questions are (1) what explains its geographic distribution and (2) should it be classified as a new species?

Translocations and deliberate introductions of aquatic salamanders have been linked to the live bait industry (Martof, 1953; Fitzpatrick and Shaffer, 2007; Picco and Collins, 2008), and *N. maculosus* are reportedly used as live bait in many parts of their range (Petranka, 1998; Miesen and Hauge, 2005). There are at least three reasons, however, to question translocation by fishermen and the live bait industry as the origin of *N. aff. lewisi* in the Hiwassee River. First, the practice is not particularly common in the region (Copeland et al., 2009). Second, most salamanders used as live bait are *Desmognathus* or *Ambystoma*, both of which are more desirable and more easily obtained in large numbers than the relatively obscure and narrowly distributed *N. lewisi* (Braswell and Ashton, 1985; Miesen and Hauge, 2005; Picco and Collins, 2008; Copeland et al., 2009). Finally, *N. lewisi* might be unpalatable to many fish, making them unlikely commodities for anglers (Brandon and Huheey, 1985). Currently, there is no evidence for or against human translocation as the possible origin of the Hiwassee population. Range wide genetic analysis of *N. lewisi* and *N. aff. lewisi* will be necessary to test for a recent anthropogenic origin for the Hiwassee River population versus an ancient biogeographic connection to the Tar and Neuse (Johnson et al., 2011).

If *N. aff. lewisi* is native to the Hiwassee River (and possibly other tributaries of the Tennessee River system), it represents a biogeographic anomaly. Previously, *Necturus* was distinctly divided into an Atlantic clade (*N. lewisi* and *N. punctatus*) and a Gulf of Mexico clade (*N. alabamensis*, *N. beyeri*, and *N. maculosus*), with one member having spread northward, mostly within the greater Mississippi River watershed (Bonett et al., 2013). Better description of its geographic range and likely timescale of separation from its common ancestor with the Atlantic *lewisi*/*punctatus* clade would greatly enrich our understanding of the historical biogeography of aquatic life across the Eastern Continental Divide. Many terrestrial and semiaquatic salamanders are found on both sides of the Appalachians, but this pattern is unusual for fully aquatic vertebrates (Page et al., 2011). Using the raw data from Matamoros et al. (2015), we identified 10 freshwater fish species that naturally occur in both watersheds (Table S6). All of those species are currently more widely distributed than *N. lewisi*, but they illustrate the plausibility of a natural, recent geographic distribution including the Hiwassee, Tar, and Neuse rivers.

TABLE 2. Linkage disequilibria (*D*′, standardized measure of deviation from independent assortment) between alleles from the *N. lewisi* vs. *N. maculosus* clades within the Hiwassee River sample. All *P* values < 0.001.

Locus	POMC	RAG1	SLC8a3	mtDNA
NCX1	0.7412	0.9997	0.9997	0.7066
POMC		0.9997	0.9997	0.7066
RAG1			0.9997	0.9996
SLC8a3				0.9996

TABLE 3. Morphological comparisons between *N. lewisi* and *N. aff. lewisi*. Means for *N. lewisi* were reported by Viosca (1937) for 12 adults from North Carolina. Original data for *N. aff. lewisi* ($N = 18$) are given in Table S1 and include six putatively introgressed individuals. Statistical comparisons assume equal variances (estimated from the *N. aff. lewisi* data).

	Mean	Upper 95% CI[a]	Lower 95% CI	*t* (df = 28)	*P*
Total length (mm)					
N. lewisi	202.0	222.1	181.9	5.36	5.2×10^{-6}
N. aff. lewisi	130.9	147.4	114.5		
Tail length (mm)					
N. lewisi	77.2	84.9	69.5	6.17	5.7×10^{-7}
N. aff. lewisi	45.8	52.1	39.4		
Relative tail length					
N. lewisi	0.382	0.411	0.354	1.61	0.059
N. aff. lewisi	0.352	0.375	0.329		

[a] CI, confidence interval.

The question of how to classify *N. aff. lewisi* (if it is native) is not straightforward. Taxonomists favoring different species concepts and criteria are likely to disagree over the interpretation of molecular and morphological data alone (De Queiroz, 2007; Carstens et al., 2013). Complete reproductive isolation would satisfy any species definition. Ideally, we would like to perform statistically powerful tests of reproductive isolation between individuals from the Hiwassee River and *N. lewisi* from North Carolina. Such an analysis is extremely impractical given restrictions on collecting and the difficulty of setting up natural breeding physiology and behavior in captivity (Stoops et al., 2014). Moreover, given the evidence presented here for hybridization between *N. aff. lewisi* and *N. maculosus*, complete reproductive isolation between any named species in the clade is unlikely. In such cases, taxonomic delimitations often are based on a more general concept of species as genetically distinct lineages (De Queiroz, 2007; Shaffer and Thomson, 2007). Species criteria are the practical standards for deciding whether a group of organisms should be classified as a species (De Queiroz, 1998). Taxonomists with different philosophies argue for different criteria; those debates have gone on for decades and will not be resolved here. In our view, a pragmatic definition of "species" for conservation is "a distinct group of organisms meriting independent legal status because extinction of such a group would constitute a substantial loss of biological diversity" (Pasachnik et al., 2010; Fitzpatrick et al., 2015). Therefore, future research will focus on determining which, if any, species criteria are met by *N. aff. lewisi* relative to *N. lewisi*.

In our view, the most urgent question is whether *N. aff. lewisi* is native or introduced. If it is native, it would be an important target of conservation management and research regardless of its assigned taxonomic rank. The U.S. Endangered Species Act is regularly applied to subspecies and distinct population segments in addition to taxonomic species (USFWS and NMFS, 1996), and the state of Tennessee independently determines conservation status of species within its borders (Tennessee State Wildlife Action Plan Team, 2015). At present, *N. aff. lewisi* has been documented from the Hiwassee River only despite survey efforts in other river systems by us and others (e.g., Nickerson et al., 2002). *Necturus aff. lewisi* might occur in other tributaries of the Tennessee River in eastern Tennessee, but additional survey work is needed to ascertain its distribution and abundance. Potential impacts to these aquatic salamanders include hydroelectric management, siltation from agriculture and forestry practices, and the amphibian chytrid and ranavirus pathogens that are known to be present in some Eastern Hellbender (*Cryptobranchus alleganiensis*) populations within the same watersheds (Souza et al., 2012). Chytrid has been detected in *Necturus alabamensis* and *Necturus beyeri* previously (Chatfield et al., 2012) and could pose a threat given known carriers in the same waterways. Ranavirus has not yet been detected in mudpuppies, but this may reflect a lack of sampling.

Necturus lewisi is classified as Near Threatened by the International Union for Conservation of Nature (Braswell and Hammerson, 2004), but it has been petitioned by the Center for Biological Diversity to be listed under the U.S. Endangered Species Act (Center for Biological Diversity, 2010). The North Carolina Wildlife Action Plan states that aquatic species from the Neuse Basin are threatened due to increasing impoundments, forestry, agriculture, and development (North Carolina Wildlife Resources Commission, 2015). If *N. aff. lewisi* should be classified as a disjunct population of *N. lewisi*, it might reduce the perceived need for listing of the species. Alternatively, expanding the known species range to include the Hiwassee River would not represent a dramatic increase in the number of known populations, and a listing decision might still be based largely on the rate of habitat destruction and local population extirpation.

We also provide evidence of limited hybridization between *N. aff. lewisi* and *N. maculosus* in the Hiwassee River and possible introgression in *N. maculosus* in the Little River (specimen N19). If *N. aff. lewisi* is in fact an introduced population of *N. lewisi*, then hybridization could constitute a threat to native *N. maculosus* in addition to potential threats owing to ecological interactions (Fitzpatrick et al., 2015). If *N. aff. lewisi* is native, then understanding the ecological and genetic factors facilitating coexistence of the two species, despite hybridization and likely competition, will be critical for assessing conservation status.

Future studies on *N. aff. lewisi* should focus on its genetic relationship to *N. lewisi* (with particular attention to the question of whether it is native), its geographic distribution, and its interactions with *N. maculosus* (both ecological and genetic).

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Supplementary Data

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