AN UNEXPECTED JOURNEY: ANURAN DECLINE RESEARCH AND THE INCIDENTAL ELUCIDATION OF A NEW CRYPTIC SPECIES ENDEMIC TO THE URBAN NORTHEAST AND MID-ATLANTIC US.

By

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ABSTRACT OF THE DISSERTATION

An unexpected journey: Anuran decline research and the incidental elucidation of a new cryptic species endemic to the urban Northeast and Mid-Atlantic US.

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In recent decades, anuran amphibians have suffered unprecedented declines throughout many parts of the world. In the eastern United States, one example of an enigmatic extirpation has emerged over recent decades in which leopard frogs disappeared from parts of New York City, Long Island, and surrounding mainland areas in New York and Connecticut. I conducted research into the causes of this extirpation and focused specifically on Long Island where the southern leopard frog, *Rana* (*Lithobates*) *sphenocephala*, was recognized to occur. This included work at two regionally extant populations outside the extirpation zone. Over time, I observed unusual differences between those two populations; at one, in southern New Jersey, frogs appeared typical for *R. sphenocephala*, at the other, on Staten Island, New York, frogs exhibited several atypical characteristics. Further research was needed to explore the reasons behind these differences.

Here, I present results from that research. The first stage (Chapter 1) was a molecular examination that focused on leopard frogs from Staten Island and three other regional populations later found to exhibit similar atypical characteristics. The results, supported by strong nuclear and mitochondrial phylogenetic evidence, revealed that all four populations were part of a cryptic genetic lineage that was distinct from *R. sphenocephala* and two other regionally similar congeners, the northern leopard frog, *R. pipiens*, and pickerel frog, *R. palustris*.

The discovery of this novel genetic lineage was followed by a subsequent study (Chapter 2) comparing bioacoustic and morphological characters between the same four congeners from chapter 1. The results revealed additional separation between the new species and its congeners and allowed us to taxonomically diagnose and describe the new species and name it formally as the Atlantic Coast leopard frog, *R. kauffeldi*. The new species is visually similar to *R. sphenocephala* and bioacoustically similar to the wood frog, *R. sylvatica*, which was also included in the bioacoustic analysis.

In the midst of this discovery, in October 2012, Hurricane Sandy made landfall across the same area where many known *R. kauffeldi* populations were located. This created cause for concern and prompted a study of the hurricane's impact on several of the most vulnerable populations in the New York City area (Chapter 3). *Rana kauffeldi* survived at all study locations, suggesting that this species is capable of withstanding large-scale coastal-flooding events and rapid salinity increases.

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INTRODUCTION

As species decline, they can eventually disappear either from the entirely of their ranges (through the process of extinction) or from certain local or regional parts of their ranges (through the process of extirpation). The reasons for such disappearances can vary by circumstance and the life history of the species involved. In some cases, the underlying factors may be clear and obvious, in others, the causes may be enigmatic and difficult to pinpoint. Enigmatic disappearances can be problematic when trying to protect and conserve the species involved in such declines, especially in areas of rapid urban development. With extinctions, this may be largely academic once a species has vanished completely and no individuals remain for recovery. In the case of extirpations, however, understanding the factors behind a disappearance in one region may be critical to the conservation of that species in another region where it still occurs.

Amphibians, and anurans in particular, have suffered widespread declines, extirpations, and extinctions in recent decades (Buck et al. 2015). These impacts have affected hundreds of species (Wake and Vredenburg 2008; Zhou et al. 2015) across many parts of the world (Stuart et al. 2004). Most examples of anuran declines in the United States (US) have come from areas to the west (Wake and Vredenburg 2008; Muths et al. 2012). However, in one recent decline, leopard frogs disappeared from the largest island in the continental US, Long Island, New York (NY), as well as adjacent coastal parts of mainland NY and Connecticut (CT).

My original dissertation research objective was to examine the causes of this leopard frog extirpation, and to focus specifically on Long Island, where *Rana sphenocephala*, a species common to the southeastern US, was recognized to occur and

reach its northern range limit. Historically, Long Island was considered a regional stronghold for leopard frogs (Schlauch 1978) before their decline over the past century and eventual putative disappearance from the last remnant populations on the east end of the island around the year 2000 (Kiviat 2011; Feinberg unpublished data). My approach was to perform a monitoring experiment where I collected eggs from populations outside the extirpation zone and raised the subsequent tadpoles in wetlands within the extirpation zone. I collected eggs from two extant areas where *R. sphenocephala* was considered the species of occurrence, in southern New Jersey (NJ) and Staten Island, NY, and reared the developing tadpoles in wetland enclosures on Long Island to examine the impacts of several potential extirpation drivers (e.g., disease, contaminants, invasive vegetation).

During the course of this work, I began to notice distinct differences between frogs at the two egg-source populations. The New Jersey population showed characteristics typical of *R. sphenocephala*, but the Staten Island population displayed unusual attributes, especially in terms of mating call and breeding phenology. The differences between these two populations were unusual and unexpected given that both locales were considered to harbor the same species. This suggested the possibility that two different species might be involved, including one that was a cryptic “look-alike” species to the other.

In light of the observed differences in populations, the overall objective of my research shifted to an investigation of genetic, bioacoustic, ecological, and morphological characters among the typical and atypical leopard frogs I had identified. Through examination and comparison of these attributes, my goal was to determine if the atypical

frogs actually constituted a unique and previously undocumented species that had remained undetected within the heart of the well-studied urban northeastern US.

Chapter 1 of my dissertation represents a published paper from 2012 on which I was second author (Newman et al. 2012). It includes a molecular investigation of leopard frogs from my Staten Island field site and three other areas in northern NJ and southeastern mainland NY where I helped identify similarly atypical leopard frog populations. Nuclear and mitochondrial data from these four populations were compared to control populations from other parts of the mid-Atlantic and northeastern US, where leopard frog species composition was not in question. Control populations included *R. sphenocephala* from my southern NJ field site, and northern leopard frogs, *R. pipiens*, and pickerel frogs, *R. palustris*, from populations to the north and east of the four atypical populations. I played an integral role in the development and coordination of this project and identifying the key problems and questions investigated therein. Further, I provided sizeable contributions to the collection of samples and natural history information, interpretation of results, and development of parts of the Introduction and Discussion sections along with first author Catherine E. Newman.

Chapter 2 was published in 2014, and follows the molecular work from chapter 1 with a comprehensive examination of bioacoustic and morphological characters to further investigate differences between leopard frogs of the mid-Atlantic and northeastern US (Feinberg et al. 2014). This chapter measures and compares attributes of mating calls and museum-specimen morphology between the atypical leopard frog group, recognized as a formally undescribed cryptic species, *R.* sp. nov., after chapter 1 (Newman et al. 2012), and several recognized congeners that again include *R. sphenocephala*, *R. pipiens*, and *R.*

palustris. A fifth congener, the wood frog, *R. sylvatica*, was also included within the bioacoustic analysis because of its similarity of call to *R.* sp. nov. This chapter also examines considerable long-standing confusion within the taxonomic history of eastern leopard frogs and seeks to explain how the existence of a hidden species might have contributed to some of that confusion. Lastly, this chapter considers additional ecological and morphological characteristics of the new species and explores conservation concerns and potential regulatory impacts and considerations.

Chapter 3 focuses on a major climate event, Hurricane Sandy, and its impacts on populations of the new species, which became formally recognized as the Atlantic Coast leopard frog, *R. kauffeldi*, after the publication of chapter 2 (Feinberg et al. 2014). On 29 October 2012, Hurricane Sandy made landfall across the same area where *R. kauffeldi* had first been identified only several months earlier (Newman et al. 2012). At the time of the storm, little was known about the new species or its ecology and overall geographic distribution outside the New York-New Jersey-Connecticut Tri-State area. Thus, given this tenuous early state of understanding, and the fact that the majority of known populations (at the time) fell within the tidal-storm surge floodplain of the storm, I initiated a study to assess impacts to the species. This work focused on several of the most vulnerable Tri-State area populations near the coast and within the urban landscapes of the New York City area. At the time of this study, the number of known *R. kauffeldi* populations in the Tri-State area had expanded from the initial four populations (from chapter 1) to 18 confirmed and 4 unconfirmed populations between central CT and extreme southern NJ. Several additional populations had also been identified farther to the south, based on bioacoustic evidence, but little information on the status or

distribution of *R. kauffeldi* was available from those areas (in the southern Mid-Atlantic region), and they were not included in this study.

All of the chapters in this dissertation have been written as stand-alone manuscripts. As such, they are formatted for publication, and in the case of chapters 1 and 2, follow the specific formatting of the journals they were already published in. Chapter 1 was written with Catherine E. Newman, Leslie J. Rissler, Joanna Burger, and H. Bradley Shaffer, and was published in *Molecular Phylogenetics and Evolution*. Chapter 2 was written with Catherine E. Newman, Gregory J. Watkins-Colwell, Matthew D. Schlesinger, Brian Zarate, Brian Curry, H. Bradley Shaffer, and Joanna Burger, and was published in *PLOS One*. Chapter 3 was written with Erik Kiviat, Matthew D. Schlesinger, and Joanna Burger, and is formatted and intended for publication in *Urban Ecosystems*.

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CHAPTER 1

A new species of leopard frog (Anura: Ranidae) from the urban northeastern US

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Abstract

Past confusion about leopard frog (genus Rana) species composition in the Tri-State area of the US that includes New York (NY), New Jersey (NJ), and Connecticut (CT) has hindered conservation and management efforts, especially where populations are declining or imperiled. We use nuclear and mitochondrial genetic data to clarify the identification and distribution of leopard frog species in this region. We focus on four problematic frog populations of uncertain species affiliation in northern NJ, southeastern mainland NY, and Staten Island to test the following hypotheses: (1) they are conspecific with Rana sphenocephala or R. pipiens, (2) they are hybrids between R. sphenocephala and R. pipiens, or (3) they represent one or more previously undescribed cryptic taxa. Bayesian phylogenetic and cluster analyses revealed that the four unknown populations collectively form a novel genetic lineage, which represents a previously undescribed cryptic leopard frog species, Rana sp. nov. Statistical support for R. sp. nov. was strong in both the Bayesian (pp = 1.0) and maximum-likelihood (bootstrap = 99) phylogenetic analyses as well as the Structure cluster analyses. While our data support recognition of R. sp. nov. as a novel species, we recommend further study including fine-scaled sampling and ecological, behavioral, call, and morphological analyses before it is formally described.

Keywords: *Rana pipiens*, *Rana sphenocephala*, *Lithobates*, Urban ecology, Amphibian decline, Species delimitation

1. Introduction

Leopard frogs of the *Rana pipiens* (=*Lithobates pipiens*) complex are widespread and common throughout much of the United States, but species delimitation and the associated taxonomy of the group have been challenging and contentious (Brown, 1973; Pace, 1974; Moore, 1975; Brown et al., 1977, 1990; Zug et al., 1982; Hillis, 1988; Frost et al., 2006, 2008, 2009; Pauly et al., 2009). While studies of range-wide phylogeography and systematics at the genus and species level are common (e.g., Pace, 1974; Hillis et al., 1983; Pytel, 1986; Hoffman and Blouin, 2004; Hillis and Wilcox, 2005; Oláh-Hemmings et al., 2010; Newman and Rissler, 2011), relatively little attention has been focused on taxonomic status and conservation needs of local or regional populations or subspecies (but see Di Candia and Routman, 2007; Hekkala et al., 2011). As is true for any group, appropriate conservation measures cannot be identified and implemented in the face of uncertain taxonomy (Köhler et al., 2005).

The species composition of leopard frogs in parts of the mid-Atlantic and northeastern US—hereafter the Tri-State area, including New Jersey (NJ), New York (NY), and Connecticut (CT)—has been questioned by biologists over the past several decades (Kauffeld, 1937; Yeaton, 1968; Schlauch, 1971; Pace, 1974; Klemens et al., 1987; Klemens, 1993). Currently, two species are recognized in the region (Conant and Collins, 1998). *Rana pipiens*, the northern leopard frog, is widely distributed across New England and the Great Lakes region, including the western two-thirds of CT and central and northern NY. From NJ, Long Island (NY), and southern mainland NY to the south, it is replaced by *R. sphenocephala* (=*L. sphenocephalus*), the southern leopard frog. While natural history collection data suggest the two species have a narrow zone of overlap in

southern NY (Fig. 1), no area of sympatry has been directly identified. Some earlier studies based on morphological data suggested the possibility of intergradation (Schlauch, 1971), whereas others speculatively discussed a putative third species in this region (Kauffeld, 1937; Klemens, 1993).

Although widespread and often common at the continental scale (Fig. 1), leopard frog populations have been severely declining in certain regions, resulting in extirpation from some portions of their historical range (Lannoo, 2005), including coastal regions and islands north and east of Long Island, NY (Ditmars, n.d.; Latham, 1971; Klemens, 1993; Feinberg, et al., unpublished data). Leopard frogs are also believed to be extirpated from highly developed areas including Long Island, NY (Kiviat, 2010; Feinberg et al., unpublished data); New Haven, CT; and Providence, Rhode Island (Klemens, 1993). While the exact causes of these declines are unclear, environmental pesticides and endocrine disruptors (Hayes et al., 2003; Lannoo, 2008), disease (Carey et al., 1999; Greer et al., 2005; Davis et al., 2007; Searle et al., 2011), habitat loss and alteration (Lannoo, 2005), and over-harvesting for use as laboratory specimens (Hillis, 1988; Klemens, 1993; Lannoo, 2005) have all been identified as contributing factors, particularly regarding *R. pipiens*. *Rana sphenocephala*, in contrast, remains relatively abundant throughout most of its range to the south, including coastal islands south of Long Island. However, near its northern range limit, it is listed as a Species of Special Concern in NY (NY Department of Environmental Conservation) and as endangered in Pennsylvania (PA) (Pennsylvania Fish and Boat Commission).

To gain a better understanding of the status and distributions of leopard frog populations in the Tri-State area, we analyzed mitochondrial and nuclear gene sequences

from four focal populations of unknown leopard frog species composition in northern NJ, southeastern mainland NY (two populations), and Staten Island, NY (one of the five boroughs of New York City). Direct observations by one of us (JAF) showed that these four populations exhibited several unique characteristics, including an advertisement call distinct from both *R. pipiens* and *R. sphenocephala*. We also analyzed three CT populations from localities within the traditionally accepted geographic range of R. pipiens. We evaluated three possible interpretations of the status of leopard frogs in the Tri-State area: (1) the four focal populations are conspecific with either *R. pipiens* or *R. sphenocephala*, (2) the populations are hybrids between *R. pipiens* and *R. sphenocephala*, or (3) the populations represent a previously undescribed leopard frog lineage distinct from *R. pipiens* and *R. sphenocephala*.

2. Materials and methods

2.1. Study area and sample collection

Our study region was focused on the Tri-State area of the northeastern US, including NY, NJ, and CT—a total area of roughly 40,000 km^2 (Fig. 1). The region includes an area of putative range overlap between *R. sphenocephala* and *R. pipiens* according to range maps downloaded from the IUCN [IUCN Red List of Threatened Species 2011.1 (http://www.iucnredlist.org)]. Our study included four focal populations of unknown leopard frog species composition: Great Swamp (NJ), Staten Island (NY), Putnam County (NY), and Orange County (NY) (Fig. 1). The Great Swamp and Staten Island sites fall within the geographic range of *R. sphenocephala* and outside the range of

R. pipiens, whereas the Putnam and Orange sites fall in the overlap zone of the two species' ranges. Leopard frog species composition in CT has also been questioned (Klemens, 1993), so we collected samples from three sites across CT to include in the analyses (Fig. 1).

Toe clips were taken from 3 to 10 individual frogs at each of the four focal sites and three populations in CT, as well as control sites for R. sphenocephala in southern NJ and R. pipiens in northeastern mainland NY (Fig. 1; Table 1). In addition, three morphologically ambiguous specimens from Long Island were included to determine if they represented an isolated relict population of leopard frogs, or if they were instead the pickerel frog *R. palustris* (=*L. palustris*) (Table 1). Three CT *R. palustris* specimens from the Yale Peabody Museum were also included as reference samples. Tissues were stored in 98% ethanol, and source frogs were measured, photographed, and subsequently released, or collected as vouchers to be deposited in either the Yale Peabody Museum or the University of Alabama Herpetological Collection.

2.2. DNA extraction and gene amplification

Genomic DNA was extracted at the University of California, Davis, using a standard salt extraction protocol. We amplified the ND2 and 12S–16S regions of the mitochondrial genome, including the intervening tRNA-Valine and partial flanking tRNA-Tryptophan sequences, for a total of 1444 bp. We also amplified the neurotrophin-3 (NTF3, 599 bp), tyrosinase (Tyr, 557–585 bp), Rag-1 (647–683 bp), seven-in-absentia (SIA, 362–393 bp), and chemokine receptor 4 (CXCR4, 550 bp) regions of the nuclear genome. All primer references and sequences are provided in Supplementary Table S1.

PCR amplification was performed in 18 µL reactions, consisting of 1.5 µL PCR Buffer II (10X, Applied Biosystems), 2.4 µL $MgCl_2$ (25 mM), 0.6 µL each primer (5 mM), 2.4 µL dNTP solution (5 mM), 1U AmpliTaq (Applied Biosystems), and 10–30 ng genomic DNA. All gene regions except 12S–16S were amplified using the following PCR protocol: initial denaturation at 95° for 1 min.; 38 cycles of 94° for 30 s, 63–65° (see Supplementary Table S1) for 45 s, 72° for 1 min.; and a final extension at 72° for 10 min. The amplification protocol for the 12S–16S region was as follows: initial denaturation at 94° for 30 s; 35 cycles of 94° for 45 s, 52° for 30 s, 72° for 1 min. and a final extension at 72° for 7 min. PCR products were sequenced in the forward and reverse directions at Beckman Coulter Genomics (Danvers, MA, USA). Contigs were assembled in Geneious v.5.3.6 (Drummond et al., 2011). Sequence fragments were trimmed to minimize missing data.

2.3. Mitochondrial sequence analysis

The 12S–16S and ND2 sequence fragments with associated tRNA fragments were concatenated and aligned using ClustalW in Geneious and manually adjusted. All sequences were uploaded to GenBank (see Supplementary Table S2 for accession numbers). Bayesian analyses were conducted in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with five partitions: 12S plus tRNA-Val, 16S, and each of the three ND2 codon positions. Based on output from jModelTest v.0.1.1 (Guindon and Gascuel, 2003; Posada, 2008) and convergence analyses of trial runs (data not shown), the 12S and ND2 partitions were assigned a GTR model of evolution, and the 16S partition was assigned an HKY model of evolution. The 12S

partition allowed across-site rate variation under a gamma distribution, and rates were allowed to vary among partitions. Bayesian analyses were run with random starting trees, two simultaneous runs of 10 million generations, and sampling from the posterior distribution of trees every 5000 generations. Tracer v.1.4.1 (Rambaut and Drummond, 2007) was used to assess convergence and to determine appropriate burn-in. The first 25% of samples were omitted as burn-in. Nodal support was further assessed with a maximum-likelihood (ML) analysis in RaxML v.7.0.3 (Stamatakis, 2006; Stamatakis et al., 2008) with 1000 bootstraps. *Rana clamitans* sequences (DQ347036, 12S–16S; AY206480, ND2) were downloaded from GenBank and used as an outgroup. Tajima's *D* and Fu's F_S were calculated in Arlequin v.3.5 (Excoffier and Lischer, 2010) to test for selection.

2.4. Nuclear sequence analysis

For each locus, sequences were aligned using ClustalW in Geneious and manually adjusted, and sequences were uploaded to Gen-Bank (Supplementary Table S2). Phylogenies were reconstructed for each locus individually and for the concatenated data set using unphased sequences (see below) in MrBayes. For the individual gene trees, models of evolution, based on jModelTest output and preliminary runs (data not shown), were as follows: HKY for CXCR4; HKY + G for NTF3, Rag-1, and Tyr; and JC for SIA. The concatenated data set was partitioned by locus, but a consistent lack of convergence suggested that this model was inappropriate for our data (results not shown). Bayesian analyses were thus run on the entire, unpartitioned nuclear data set, with an HKY model of evolution [based on jModelTest and trial runs (data not shown)]. All analyses were run

for 10 million generations and sampled every 5000 generations. Convergence was assessed in Tracer, and the first 25% of samples were omitted as burn-in. Nodal support was further assessed with 1000 ML bootstraps in RaxML. Tests for selection were done in Arlequin, using Tajima's *D* and Fu's F_S statistics.

To test our hypotheses concerning the status of the four unknown populations, we used a Bayesian approach implemented in Structure v.2.3.3 (Pritchard et al., 2000; Falush et al., 2003) with an allelic data set (6% missing data) generated from our nuclear sequence data. We used the software Phase v.2.1 (Stephens et al., 2001; Stephens and Donnelly, 2003) to infer haplotypes for each locus in the five-locus sequence data set using a Bayesian algorithm. Each allele represented a single haplotype. Input files for Phase were generated from alignment nexus files using a Perl script (RC Thomson, unpublished).

Structure was used to determine the number of genetically distinct clusters (*K*) of samples. We implemented the admixture model (Pritchard et al., 2000), assumed correlation of allele frequencies among clusters (Falush et al., 2003), and assumed no other *a priori* population information. We tested values of *K* from 1 to 10. For each *K*, 20 iterations were run, each consisting of 100,000 generations after a burn-in of 10,000 generations. The appropriate value of *K* was determined by assessing the posterior probabilities (Pritchard et al., 2000) and ΔK values following Evanno et al. (2005). An individual was considered of mixed ancestry if its cluster membership probability *q* was between 0.10 and 0.90 (Vähä and Primmer, 2005).

For both mitochondrial and nuclear loci, measures of sequence divergence (uncorrected p), nucleotide diversity (π) and haplotype diversity (Hd) were determined at the species level using either DnaSP v.5.10.01 (Librado and Rozas, 2009) or Arlequin. Pairwise F_{ST} values were calculated in Arlequin from the concatenated, phased nuclear sequence data set.

3. Results

3.1. mtDNA phylogenetic analyses

The concatenated mtDNA data set consisted of 1461 bp and 15 unique haplotypes. Bayesian analyses of mtDNA revealed four distinct clades, three of which correspond to the known species *R. sphenocephala*, *R. pipiens,* and *R. palustris* (Fig. 2). The three samples from Long Island fell out with the *R. palustris* reference samples, rejecting the hypothesis that those frogs represented a relict population of leopard frogs on Long Island. All specimens from the four focal populations and three of five specimens from Middlesex, CT, formed a clade (hereafter *Rana* sp. nov.) distinct from *R. sphenocephala*, *R. pipiens*, and *R. palustris*. All other CT specimens grouped with *R. pipiens*. All four clades were strongly supported with Bayesian posterior probabilities (all 1.0) and ML bootstraps (all $\geq$99). Importantly, the sister group to the *R*. sp. nov. clade, with reasonably strong statistical support (Bayesian posterior probability = 1.0, ML bootstrap support = 0.78) is the pickerel frog *R. palustris* rather than *R. pipiens* or *R. sphenocephala*. Species-level π and Hd values are listed in Table 2. Pairwise sequence divergence between *R*. sp. nov. and the three recognized species were 6.79% (*R.*

palustris), 11.0% (*R. sphenocephala*), and 12.5% (*R. pipiens*), and pairwise divergence between the latter three described species ranged from 11.1% to 13.4% (Table 3). These data indicate that a differentiated lineage, distinct from *R. sphenocephala*, *R. pipiens*, and *R. palustris*, occurs in the region and may represent a previously unrecognized species if additional data confirm these mtDNA results.

The three outlier specimens from Middlesex, CT, are more difficult to interpret. Phylogenetically, they fell in the *R.* sp. nov. clade, but their geographic location substantially farther east than all other *R.* sp. nov. samples and, more importantly, their sympatry with *R. pipiens* at the same site made us question whether they represent a natural population of *R.* sp. nov. farther east than expected or human-mediated introductions. Given this uncertainty, we omitted these individuals from population genetic calculations, pending future sampling in CT, particularly the region between Middlesex County and the closest known *R.* sp. nov. population roughly 95 km due west in Putnam County, NY.

3.2. Nuclear phylogenetic analyses

Aligned sequence lengths for nuclear loci were 550 bp (CXCR4), 599 bp (NTF3), 683 bp (Rag-1), 393 bp (SIA), and 585 bp (Tyr). The concatenated data set consisted of 2810 bp of aligned, trimmed sequence. The number of variable sites for each locus ranged from 10 to 30 (Table 2). Species-level π and Hd values are listed in Table 2, and pairwise F_{ST} in Table 4. Tajima's *D* and Fu's F_S tests for selection were non-significant for all loci (Table 2), indicating that all sampled loci were selectively neutral.

Analyses of individual nuclear loci (Fig. 3) revealed varying degrees of support for the four species recovered in the mtDNA analysis (Fig. 2). Monophyly of *R. palustris* was strongly supported by four loci, *R. pipiens* by two loci, and *Rana* sp. nov. by one locus. None of the loci supported a monophyletic *R. sphenocephala*. Importantly, none of the loci recovered strong clade support for non-monophyly of any of the species. In other words, no strongly supported clade contained individuals of multiple species.

Bayesian analysis of the concatenated data set recovered three strongly supported clades corresponding to the three known species (*R. sphenocephala*, *R. pipiens*, *R. palustris*) (Fig. 4), although their interrelationships were unresolved. The remainder of the samples—those that formed the *R*. sp. nov. mtDNA clade—constituted an unresolved collection of samples that were excluded from all three currently recognized species. While we acknowledge the problems associated with phylogenetic analyses of concatenated nuclear data sets (e.g., Kubatko and Degnan, 2007), we emphasize the concordance among the delimitations in our mitochondrial (Fig. 2) and concatenated nuclear (Fig. 4) phylogenies, as well as the Structure analysis (Fig. 5, see below).

The number of inferred haplotypes per locus ranged from 10 to 19. Bayesian cluster analyses in Structure recovered four clusters ($\ln L = -504.0$, $\Delta K = 224.13$) consistent with the phylogenetic analyses (Fig. 5). As in the mtDNA analyses, *R. sphenocephala* grouped together in one cluster, *R. palustris* reference samples grouped with the Long Island specimens in a second cluster, all specimens from CT except three from Middlesex grouped with *R. pipiens* controls, and all specimens from the four focal populations grouped with three from Middlesex, CT, in a fourth cluster (*R*. sp. nov.). The three specimens from Middlesex, CT, that clustered with *R*. sp. nov. are the same three

that clustered with this group in the mtDNA sequence analyses. Cluster membership values of samples, *q*, ranged from 0.922 to 0.992. None of the samples were of admixed ancestry.

4. Discussion

4.1. Taxonomic status and geographic distribution of Rana sp. nov

Our data strongly support the recognition of three evolutionary lineages of leopard frogs in the Tri-State area, with the four focal populations collectively forming a new, previously undescribed leopard frog species (*R.* sp. nov.). Phylogenetic and cluster analyses revealed the four unknown populations to be a distinct group from all locally occurring, recognized leopard frog species, rejecting the hypotheses that those populations are conspecific with one or more of the known species or that they are admixed, intergrade populations. Mitochondrial pairwise sequence divergences between *R.* sp. nov. and the currently recognized species ranged from 6.79% to 12.9%, consistent with or greater than divergence estimates among other ranid species (Jaeger et al., 2001; Shaffer et al., 2004; Di Candia and Routman, 2007; Funk et al., 2008; Oláh-Hemmings et al., 2010). These high levels of divergence strongly suggest a lack of gene flow between *R.* sp. nov. populations and other leopard frog species, and cluster analysis indicated that none of the samples were of admixed ancestry.

Empirical methods for species delimitation (Sites and Marshall, 2004) could potentially add support to our conclusions. In addition, new methods have recently become available that use Bayesian analyses of multilocus sequence data to concurrently

estimate the species tree and delimit species (O'Meara, 2010; Niemiller et al., 2011). We argue that such analyses are not necessary in our case, however, because species delimitation is relatively straightforward given the data herein. The older species are, the more time they have had to accumulate various evidences of lineage divergence, such as diagnosable morphological characters, reproductive isolation, or reciprocal monophyly (de Queiroz, 2007; Shaffer and Thomson, 2007). In our study, genetic data suggest monophyly of each of the four species, and the sympatry of *R. pipiens* and *Rana* sp. nov. in Middlesex, CT, suggests some extent of reproductive isolation between the two. Together, reciprocal monophyly and reproductive isolation strongly indicate the reality of independently evolving lineages, which we designate as distinct species.

Based on our current, relatively sparse sampling, *R.* sp. nov. is restricted to northern NJ, extreme southeastern mainland NY, and Staten Island (Fig. 1), although range limits may extend as far as CT and northeastern PA (Pace, 1974). Three samples from Middlesex County, CT, suggest that the range potentially extends into the western half of CT, where *R.* sp. nov. is currently sympatric with *R. pipiens*. Additional sampling in western CT should help to clarify the range extent of *R.* sp. nov. However, we reiterate that our results show no evidence of hybridization between *R.* sp. nov. and either of the other two leopard frog species in the region, including central CT where *R.* sp. nov. and *R. pipiens* occur in sympatry, suggesting some level of reproductive isolation.

4.2. Conservation implications and recommendations

The geographic extent of *R.* sp. nov. is limited to a small portion of NJ, NY, and possibly CT and PA (Fig. 1). This northeastern endemic distribution is concordant with

few other amphibian taxa (but see *Pseudacris kalmi*; Lemmon et al., 2007) and presents a unique situation compared to more ‘‘standard’’ amphibian phylogeographic patterns (Rissler and Smith, 2010). Pending additional field sampling, the recognition of a distinct, geographically-restricted species suggests that conservation needs may be high, particularly in light of the tremendous human population density in this region and epidemic declines and extirpations from mainland and coastal regions of the Tri-State area, including Long Island (Feinberg et al., unpublished data), an area once considered a regional stronghold for leopard frogs (Schlauch, 1978).

Rana sphenocephala is currently (as of 2011) listed as a Species of Special Concern in NY; it is not listed in NJ. *Rana pipiens* is not a listed species in NY and is not known to be present in NJ. Our genetic data demonstrate that all of the leopard frogs collected in southern mainland NY for this study were *R.* sp. nov., rather than *R. sphenocephala*. Staten Island and the two populations in southern mainland NY (Orange, Putnam) are the only known extant putative *R. sphenocephala* populations in NY, suggesting that southern leopard frogs do not occur in NY, although information gaps remain regarding Long Island. Furthermore, *R. sphenocephala* is currently believed to be present throughout the entire state of NJ, but all of the samples collected in northern NJ were *R.* sp. nov. Our findings therefore have important implications for conservation and geographic range delimitation for not only *R.* sp. nov., but also *R. sphenocephala*, which until now has likely been erroneously considered to be part of the fauna of NY and northern NJ.

We strongly suspect that *R.* sp. nov. also occurred on Long Island based on historic descriptive literature and photographs (Overton, 1914a, 1914b; Villani, 1997).

Leopard frogs were once abundant on Long Island (Latham, 1971) but are now presumed extirpated (Kiviat, 2010; Feinberg et al., unpublished data). The samples that we analyzed from our field collections on Long Island came from recently metamorphosed tadpoles that our genetic data indicated are *R. palustris*. *Rana palustris* is still common in many central and eastern Long Island localities, and tadpoles and recent metamorphs of this species can be morphologically very similar to leopard frogs. The most recent verified photograph of a live leopard frog on Long Island was taken between 1994 and 1995 (Villani, 1997; Villani, pers. comm.). The historical and current status of leopard frogs on Long Island reflects a distressing trend throughout this region of rapid decline of leopard frog populations (Lannoo, 2005).

The geographic range of *R.* sp. nov. is very small and likely contains only a relatively small number of individual frogs. Amphibians are sensitive to small changes in their environment, and geographically restricted species with few individuals have a reduced chance for survival in the face of rapid climate change, pesticides, and disease (Lande, 1988). *Rana* sp. nov. potentially faces all of these threats, as the pesticide atrazine (Hayes et al., 2002, 2003, 2010), the fungus *Batrachochytrium dendrobatidis* (Morell, 1999; Bradley et al., 2002; Stuart et al., 2004; Greer et al., 2005; Searle et al., 2011), and *Ranavirus* outbreaks (Granoff et al., 1965) have been shown to have adverse effects on leopard frog populations in this and other regions (but see Voordouw et al., 2010).

Future studies should focus on the ecology and population genetics of *R.* sp. nov., including breeding phenology and call structure, and incorporate more fine-scaled sampling to gain a better understanding of the distribution of, and gene flow among,

existing populations. Ongoing additional work (Feinberg et al., unpublished) will address these issues and describe *R*. sp. nov. as a novel species, furthering our understanding of the *R. pipiens* species complex in this region. In light of this new systematic knowledge, the ‘‘precautionary principle’’ (Raffensperger et al., 1999; Georges et al., 2011) suggests that appropriate conservation measures should be considered for immediate implementation at the state and possibly federal levels. The northeastern US is generally viewed as a glacially-impacted region of low diversity compared to the southeastern US (Rissler and Smith, 2010) or California (Rissler et al., 2006), and thus this region has received relatively less scrutiny and study in recent decades compared to regions that are believed to harbor higher overall diversity (but see *Pseudacris kalmi* Lemmon et al., 2007). However, urban environments such as the northeastern US have been shown to be detrimental to anuran populations, primarily due to habitat fragmentation and isolation, road mortality, and contamination (Findlay and Houlahan, 1997; Hitchings and Beebee, 1997; Knutson et al., 1999; Gibbs et al., 2005). It is therefore likely that species endemic to the Northeast require swift management attention to preserve what biodiversity still remains in the region. Our study revealed a new leopard frog species in the midst of this highly developed region of the US, suggesting that the densely populated Northeast still harbors cryptic biodiversity that remains to be discovered.

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Table 1. Specimens used in genetic analyses. GSNWR = great swamp national wildlife refuge, BRSP = bass river state park. More specific locality information is available from authors. Sample IDs are listed in Supplementary Table S1.

Population	Sample size	Species (*a priori*)	Map code	County	State
Focal unknown populations					
Staten Island	6	Unknown	1	Richmond	New York
GSNWR	5	Unknown	2	Morris	New Jersey
Putnam	5	Unknown	3	Putnam	New York
Orange	3	Unknown	4	Orange	New York
Unknown populations in connecticut					
Hartford	5	Unknown	5	Hartford	Connecticut
Middlesex	5	Unknown	6	Middlesex	Connecticut
Litchfield	10	Unknown	7	Litchfield	Connecticut
Unknown populations on long Island					
Eastport	3	Unknown	10	Suffolk	New York
Control populations (known species)					
BRSP	5	*R. sphenocephala*	8	Burlington	New Jersey
Saratoga	6	*R. pipiens*	9	Saratoga	New York
Litchfield	1	*R. palustris*	7	Litchfield	Connecticut
New London	1	*R. palustris*	11	New London	Connecticut
Fairfield	1	*R. palustris*	12	Fairfield	Connecticut

Table 2. Species-level general descriptive statistics. Length = aligned sequence length (bp), #VS = number of variable sites, Hd = haplotype diversity, π = nucleotide diversity, θπ = number of pairwise nucleotide differences, θS = Watterson's estimator of genetic diversity.

	Descriptive statistics						Tests of neutrality			
	Length	#VS	Hd	π	θ_π	θ_S	Tajima's *D*	*P*-value	Fu's F_S	*P*-value
mtDNA	1444	309								
R. sphenocephala			0.833	0.0013	1.833	1.636	1.09	0.854	0.006	0.292
R. palustris			0.6	0.0048	1.2	0.876	1.753	0.988	1.938	0.798
R. pipiens			0.786	0.0008	7.029	5.003	1.505	0.943	3.215	0.9
Rana sp. nov.			0.582	0.0004	0.619	2.018	0.262	0.688	0.235	0.62
CXCR4	550	13								
R. sphenocephala			0.429	0.0016	1.333	1.414	−0.219	0.401	1.056	0.748
R. palustris			0.378	0.0021	0.956	1.414	−1.245	0.112	0.39	0.494
R. pipiens			0.633	0.0017	1.228	1.138	0.196	0.62	0.381	0.613
Rana sp. nov.			0.508	0.0012	0.617	0.94	−0.822	0.237	0.688	0.605
NTF3	599	10								
R. sphenocephala			0.533	0.0009	0.533	0.353	1.302	0.95	1.029	0.635
R. palustris			0.485	0.0012	0.485	0.331	1.066	0.886	1.003	0.569
R. pipiens			0.518	0.0008	1	1.593	−1.001	0.169	−1.128	0.284
Rana sp. nov.			0.514	0.0009	0.511	0.232	1.688	0.978	1.886	0.771
Rag-1	683	20								
R. sphenocephala			0.778	0.0025	1.711	1.414	0.807	0.785	0.251	0.538
R. palustris			0.8	0.001	1.848	1.656	0.429	0.696	0.737	0.677
R. pipiens			0.273	0.0043	0.594	0.683	−0.273	0.459	2.289	0.847
Rana sp. nov.			0.605	0.0018	1.533	1.394	0.263	0.653	0.94	0.716
SIA	393	19								
R. sphenocephala			0.429	0.0065	2.8	2.121	1.325	0.907	5.13	0.983
R. palustris			0.439	0.0004	0.47	0.662	−0.85	0.246	−0.725	0.097
R. pipiens			0.165	0.0012	0.168	0.455	−1.148	0.089	−1.722	0.025
Rana sp. nov.			0.163	0.0019	1.232	1.609	−0.637	0.284	1.349	0.793
Tyrosinase	585	30								
R. sphenocephala			0.929	0.0085	4.689	4.595	0.093	0.551	−0.892	0.262
R. palustris			0	0.0053	0	0	0	1	0	–
R. pipiens			0.803	0	3.065	2.048	1.412	0.926	2.73	0.886
Rana sp. nov.			0.767	0.0075	4.093	2.759	1.464	0.94	4.154	0.949

Table 3. Intraspecific and pairwise percent sequence divergence (uncorrected p), %SD, for mtDNA.

		Pairwise		
	Intraspecific (%)	*R. sphenocephala* (%)	*R. palustris* (%)	*R. pipiens* (%)
R. sphenocephala	0.11	–		
R. palustris	0.08	11.1	–	
R. pipiens	0.43	13.4	12.8	–
Rana sp. nov.	0.04	11.0	6.8	12.5

Table 4. Species-level pairwise F_{ST}, based on phased nuDNA.

	R. sphenocephala	*R. palustris*	*R. pipiens*
R. sphenocephala	–		
R. palustris	0.661	–	
R. pipiens	0.463	0.627	–
Rana sp. nov.	0.423	0.695	0.536

Table S1. Primer names, reference citations, sequences, and annealing temperatures used in genetic analyses.

Primer Name	Direction	Reference	Sequence	Annealing Temperature (°C)
ND2		New primer, designed by		64.5
ND2-F	Forward	PQ Spinks	5'- CCA CCC ACG AGC MAT TGA AGC -3'	
ND2-R	Reverse		5' GGG ATC RAG GCC CGY CTT TC -3'	
12S-16S		New primer, designed by JJ		52
12S-16SF	Forward	Apodaca	5'- AAA AAG CTT CAA AGA TAC CCC ACT AT -3'	
12S-16SR	Reverse		5'- GAC CAT GAT GCA AAA GGT ACG AGG -3'	
NTF3		Modified from Townsend et		65
NTF3-F	Forward	al.	5'- TCT TCC TTA TCT TTG TGG CAT CCA CGC TA -3'	
NTF3-R	Reverse		5'- ACA TTG RGA ATT CCA GTG TTT GTC GTC A -3'	
Tyrosinase		Modified from Bossuyt and		65
Tyr1bRana	Forward	Milinkovitch 2000, by GB	5'- AGG TCC TCT TRA GCA AGG AAT G -3'	
Tyr1gBufo	Reverse	Pauly to make more specific	5'- TGC TGG GCA TCT CTC CAG TCC CA -3'	
Rag-1		Modified from Chiari et al.		64.5
MartFL1	Forward	2004, by GB Pauly to make	5'- AGC TGC AGY CAG TAC CAC AAA ATG -3'	
AMPR1rana	Reverse	more specific to ranids	5'- AAT TCA GCT GCA TTT CCA ATG TC -3'	
SIA		Frost *et al*. 2006		65
SIA1	Forward		5'- TCG AGT GCC CCG TGT GYT TYG AYT A -3'	
SIA2	Reverse		5'- GAA GTG GAA GCC GAA GCA GSW YTG CAT CAT -3'	
CXCR4		Modified from Biju and		63-65
CXCR4_Rana.F	Forward	Bossuyt 2003	5'- TTC ACC CTT CCA TTC TGG TC -3'	
CXCR4_Rana.R	Reverse		5'- GCC ACG GCT TCT GTG ATA G -3'	

Table S2. GenBank accession numbers for specimens used in genetic analyses. Sample ID corresponds to Table 1.

			GenBank Accession Numbers						
Sample ID	**Species (a priori)**	**Map Code**	**12S-16S**	**ND2**	**CXCR4**	**NTF3**	**RAG**	**Tyr**	**SIA**
Unknown Population #1									
CEN 10-11	Unknown	1	JN227366	JN227421	JN227091	JN227144	JN227198	JN227310	JN227254
CEN 10-21	Unknown	1	JN227375	JN227430	JN227100	JN227153	JN227207	JN227319	JN227263
CEN 10-22	Unknown	1	JN227376	JN227431	JN227101	JN227154	JN227208	JN227320	JN227264
CEN 10-23	Unknown	1	JN227377	JN227432	JN227102	JN227155	JN227209	JN227321	JN227265
CEN 10-24	Unknown	1	JN227378	JN227433	-	-	JN227210	JN227322	JN227266
CEN 10-30	Unknown	1	-	-	JN227108	JN227161	JN227216	JN227328	JN227272
Unknown Population #2									
CEN 10-25	Unknown	2	JN227379	JN227434	JN227103	JN227156	JN227211	JN227323	JN227267
CEN 10-26	Unknown	2	JN227380	JN227435	JN227104	JN227157	JN227212	JN227324	JN227268
CEN 10-27	Unknown	2	JN227381	JN227436	JN227105	JN227158	JN227213	JN227325	JN227269
CEN 10-28	Unknown	2	JN227382	JN227437	JN227106	JN227159	JN227214	JN227326	JN227270
CEN 10-29	Unknown	2	JN227383	JN227438	JN227107	JN227160	JN227215	JN227327	JN227271
Unknown Population #3									
CEN 10-31	Unknown	3	JN227384	JN227439	-	JN227162	JN227217	JN227329	JN227273
CEN 10-32	Unknown	3	JN227385	JN227440	JN227109	JN227163	JN227218	JN227330	JN227274
CEN 10-33	Unknown	3	JN227386	JN227441	JN227110	JN227164	JN227219	JN227331	JN227275
CEN 10-34	Unknown	3	JN227387	JN227442	JN227111	JN227165	JN227220	JN227332	JN227276
CEN 10-35	Unknown	3	JN227388	JN227443	JN227112	JN227166	JN227221	JN227333	JN227277
Unknown Population #4									
CEN 10-36	Unknown	4	JN227389	JN227444	JN227113	JN227167	JN227222	JN227334	JN227278
CEN 10-37	Unknown	4	JN227390	JN227445	JN227114	JN227168	JN227223	JN227335	JN227279
CEN 10-38	Unknown	4	JN227391	JN227446	JN227115	JN227169	JN227224	JN227336	JN227280
Unknown Populations in Connecticut									
CEN 10-45	Unknown	5	JN227398	JN227453	JN227122	JN227175	JN227231	JN227343	JN227287
CEN 10-46	Unknown	5	JN227399	JN227454	JN227123	JN227176	JN227232	JN227344	JN227288
CEN 10-47	Unknown	5	JN227400	JN227455	JN227124	JN227177	JN227233	JN227345	JN227289
CEN 10-48	Unknown	5	JN227401	JN227456	JN227125	JN227178	JN227234	JN227346	JN227290
CEN 10-49	Unknown	5	JN227402	JN227457	JN227126	JN227179	JN227235	JN227347	JN227291
CEN 10-50	Unknown	6	JN227403	JN227458	JN227127	JN227180	JN227236	JN227348	JN227292
CEN 10-51	Unknown	6	JN227404	JN227459	JN227128	JN227181	JN227237	JN227349	JN227293
CEN 10-52	Unknown	6	JN227405	JN227460	JN227129	JN227182	JN227238	JN227350	JN227294
CEN 10-53	Unknown	6	JN227406	JN227461	JN227130	JN227183	JN227239	JN227351	JN227295
CEN 10-54	Unknown	6	JN227407	JN227462	JN227131	JN227184	JN227240	JN227352	JN227296
CEN 10-55	Unknown	7	JN227408	JN227463	JN227132	JN227185	JN227241	JN227353	JN227297
CEN 10-56	Unknown	7	JN227409	JN227464	JN227133	JN227186	JN227242	JN227354	JN227298
CEN 10-57	Unknown	7	JN227410	JN227465	JN227134	JN227187	JN227243	JN227355	JN227299
CEN 10-58	Unknown	7	JN227411	JN227466	JN227135	JN227188	JN227244	JN227356	JN227300
CEN 10-59	Unknown	7	JN227412	JN227467	JN227136	JN227189	JN227245	JN227357	JN227301
CEN 10-60	Unknown	7	JN227413	JN227468	JN227137	JN227190	JN227246	JN227358	JN227302
CEN 10-61	Unknown	7	JN227414	JN227469	JN227138	JN227191	JN227247	JN227359	JN227303
CEN 10-62	Unknown	7	JN227415	JN227470	JN227139	JN227192	JN227248	JN227360	JN227304
CEN 10-63	Unknown	7	JN227416	JN227471	JN227140	JN227193	JN227249	JN227361	JN227305
CEN 10-64	Unknown	7	JN227417	JN227472	JN227141	JN227194	JN227250	JN227362	JN227306
Unknown Populations on Long Island									
CEN 10-17	Unknown	10	JN227372	JN227427	JN227097	JN227150	JN227204	JN227316	JN227260
CEN 10-18	Unknown	10	JN227373	JN227428	JN227098	JN227151	JN227205	JN227317	JN227261
CEN 10-20	Unknown	10	JN227374	JN227429	JN227099	JN227152	JN227206	JN227318	JN227262
Control Populations (Known Species)									
CEN 10-12	*R. sphenocephala*	8	JN227367	JN227422	JN227092	JN227145	JN227199	JN227311	JN227255
CEN 10-13	*R. sphenocephala*	8	JN227368	JN227423	JN227093	JN227146	JN227200	JN227312	JN227256
CEN 10-14	*R. sphenocephala*	8	JN227369	JN227424	JN227094	JN227147	JN227201	JN227313	JN227257
CEN 10-15	*R. sphenocephala*	8	JN227370	JN227425	JN227095	JN227148	JN227202	JN227314	JN227258
CEN 10-16	*R. sphenocephala*	8	JN227371	JN227426	JN227096	JN227149	JN227203	JN227315	JN227259
CEN 10-39	*R. pipiens*	9	JN227392	JN227447	JN227116	-	JN227225	JN227337	JN227281
CEN 10-40	*R. pipiens*	9	JN227393	JN227448	JN227117	JN227170	JN227226	JN227338	JN227282
CEN 10-41	*R. pipiens*	9	JN227394	JN227449	JN227118	JN227171	JN227227	JN227339	JN227283
CEN 10-42	*R. pipiens*	9	JN227395	JN227450	JN227119	JN227172	JN227228	JN227340	JN227284
CEN 10-43	*R. pipiens*	9	JN227396	JN227451	JN227120	JN227173	JN227229	JN227341	JN227285
CEN 10-44	*R. pipiens*	9	JN227397	JN227452	JN227121	JN227174	JN227230	JN227342	JN227286
YPM A9110	*R. palustris*	7	JN227418	JN227473	JN227142	JN227195	JN227251	JN227363	JN227307
YPM A9399	*R. palustris*	11	JN227420	JN227475	-	JN227197	JN227253	JN227365	JN227309
YPM A9389	*R. palustris*	12	JN227419	JN227474	JN227143	JN227196	JN227252	JN227364	JN227308

Figure Legend

Fig. 1. Range maps for *Rana pipiens* (light gray shading) and *R. sphenocephala* (dark gray shading) in the US. Black indicates range overlap. Inset: sampling localities for genetic analyses. Numbers correspond to Table 1. Green: *R. sphenocephala* range, blue: *R. pipiens* range, dark gray: range overlap. Red oval contains the four focal populations in this study. NY: New York, PA: Pennsylvania, NJ: New Jersey, CT: Connecticut, MA: Massachusetts, SI: Staten Island, LI: Long Island. Range maps were downloaded as ESRI shapefiles from the IUCN Red List spatial data collection (2011).

Fig. 2. Bayesian phylogeny for concatenated mtDNA (12S–16S and ND2). Nodal support: Bayesian posterior probabilities/maximum-likelihood bootstrap values. Tip labels correspond to Supplementary Table S2. Clade symbols correspond to Fig. 1.

Fig. 3. Bayesian phylogenies for individual nuDNA loci: (a) CXCR4, (b) NTF3, (c) Rag-1, (d) SIA, (e) Tyr. Nodal support: Bayesian posterior probabilities/maximum-likelihood bootstrap values. Outgroup root (*R. catesbeiana*) was removed for diagram simplicity. Tip labels correspond to Supplementary Table S2. Clade symbols correspond to Fig. 1. Colors correspond to inferred species: *R. sphenocephala* (green), *R. pipiens* (blue), *R. palustris* (orange), *Rana* sp. nov. (red).

Fig. 4. Bayesian phylogeny for concatenated nuDNA (CXCR4, NTF3, Rag-1, SIA, Tyr). Nodal support: Bayesian posterior probabilities. Tip labels correspond to Supplementary Table S2.

Fig. 5. Structure bar plot based on nuDNA. Population numbers are in parentheses under the text label and correspond to Table 1 and Fig. 1. Focal populations are marked with asterisks.

Figure 1

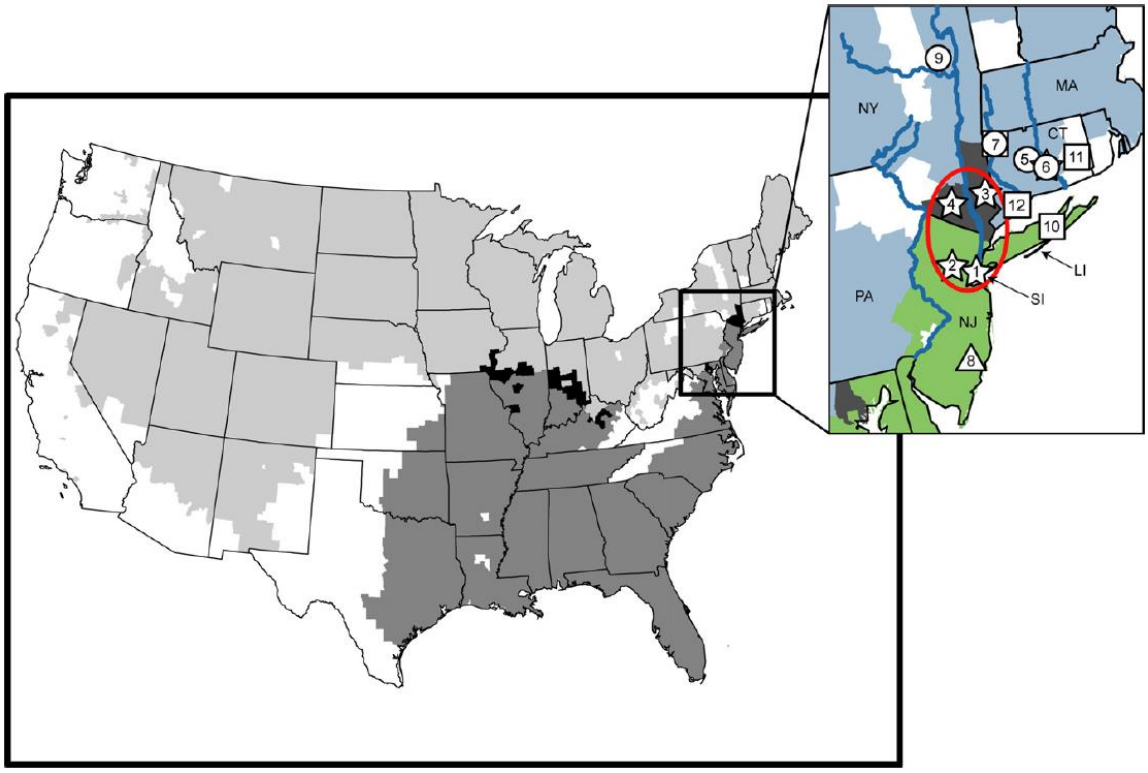

Figure 2

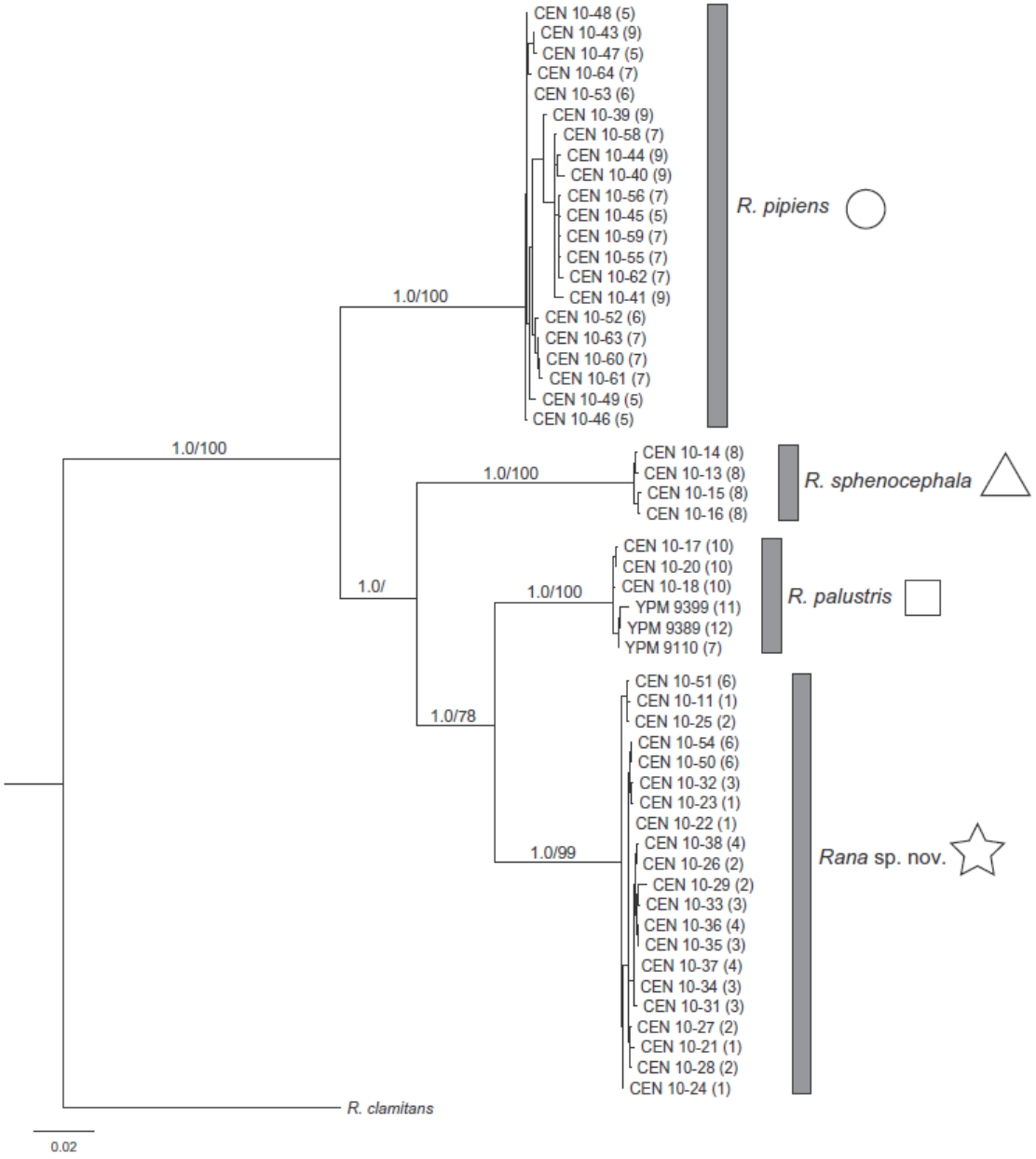
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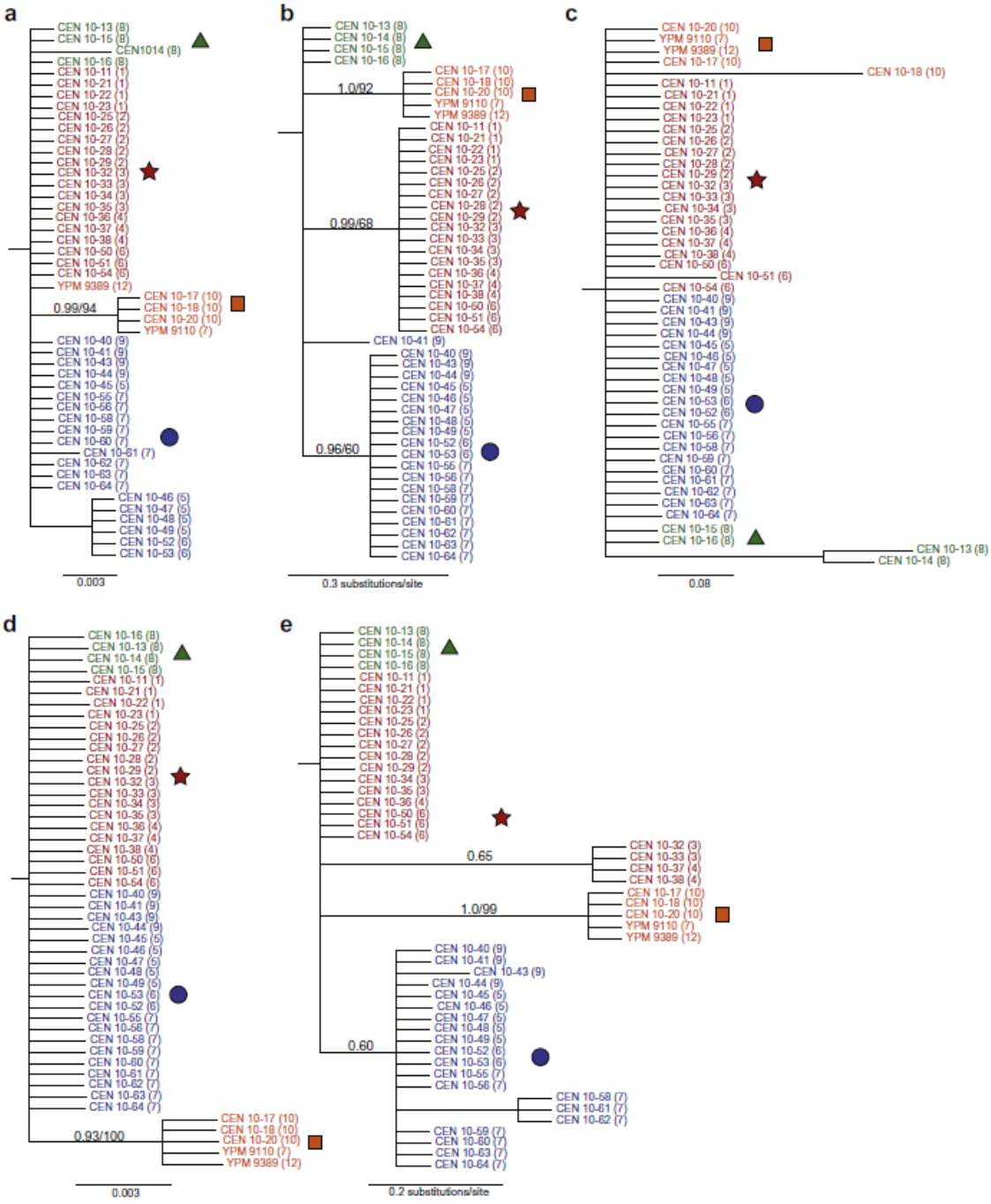
Figure 3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CEN 10-52 (6)
CEN 10-55 (7)
CEN 10-56 (7)
CEN 10-58 (7)
CEN 10-59 (7)
CEN 10-60 (7)
CEN 10-61 (7)
CEN 10-62 (7)
CEN 10-63 (7)
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0.93/100
CEN 10-17 (10)
CEN 10-18 (10)
CEN 10-20 (10)
YPM 9110 (7)
YPM 9389 (12)
0.003
e
CEN 10-13 (8)
CEN 10-14 (8)
CEN 10-15 (8)
CEN 10-16 (8)
CEN 10-11 (1)
CEN 10-21 (1)
CEN 10-22 (1)
CEN 10-23 (1)
CEN 10-25 (2)
CEN 10-26 (2)
CEN 10-27 (2)
CEN 10-28 (2)
CEN 10-29 (2)
CEN 10-34 (3)
CEN 10-35 (3)
CEN 10-36 (4)
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CEN 10-54 (6)
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CEN 10-33 (3)
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CEN 10-38 (4)
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CEN 10-18 (10)
CEN 10-20 (10)
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YPM 9389 (12)
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CEN 10-41 (9)
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CEN 10-46 (5)
CEN 10-47 (5)
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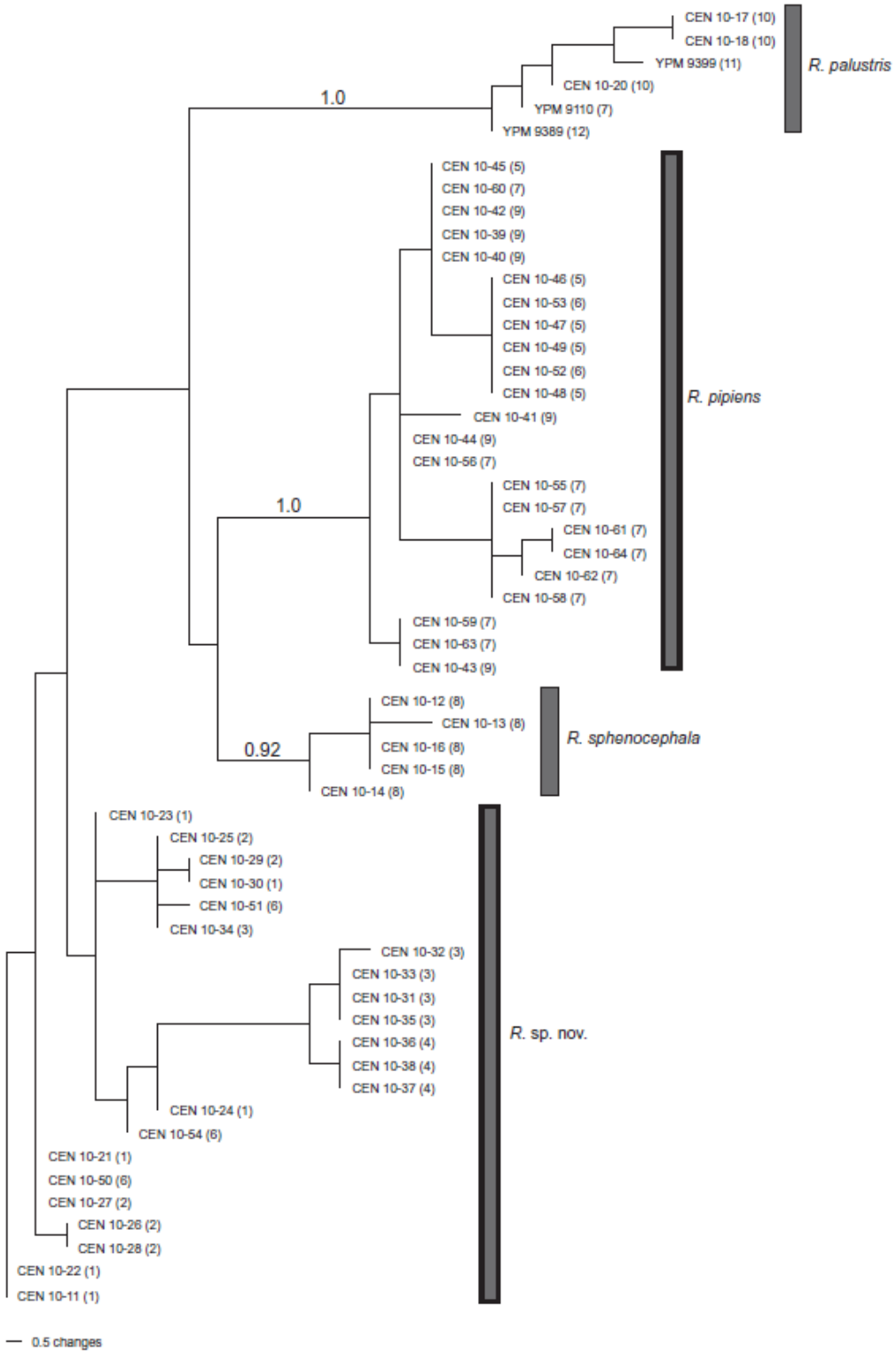
Figure 4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CEN 10-20 (10)
YPM 9110 (7)
YPM 9389 (12)
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CEN 10-42 (9)
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CEN 10-15 (8)
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CEN 10-35 (3)
CEN 10-36 (4)
CEN 10-38 (4)
CEN 10-37 (4)
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CEN 10-27 (2)
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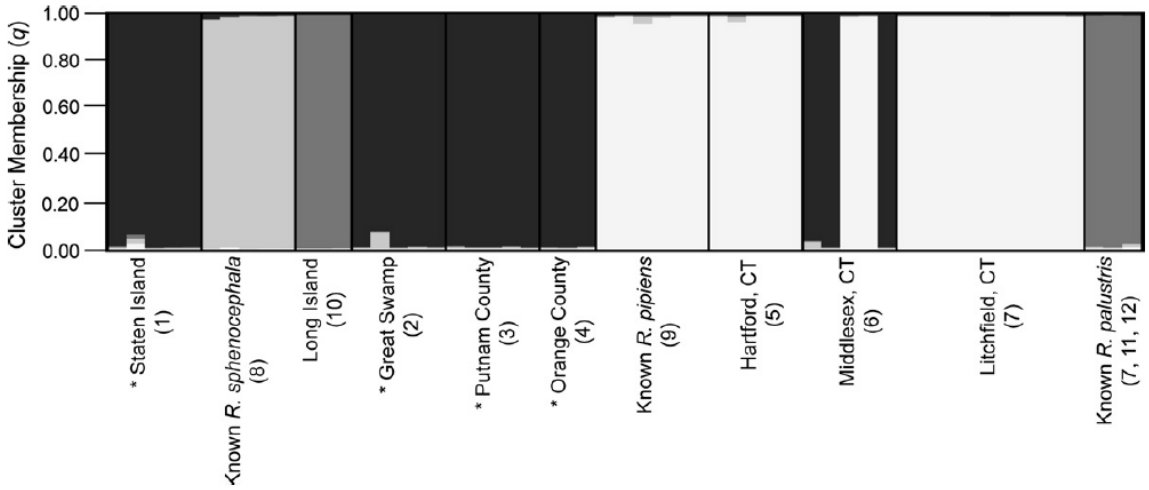
CEN 10-28 (2)
CEN 10-22 (1)
CEN 10-11 (1)
R. sp. nov.
0.5 changes

Figure 5


Cluster Membership (q)
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0.60
0.40
0.20
0.00
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Known R. sphenocephala (8)
Long Island (10)
* Great Swamp (2)
* Putnam County (3)
* Orange County (4)
Known R. pipiens (9)
Hartford, CT (5)
Middlesex, CT (6)
Litchfield, CT (7)
Known R. palustris (7, 11, 12)

CHAPTER 2

Cryptic Diversity in Metropolis: Confirmation of a New Leopard Frog Species (Anura: Ranidae) from New York City and Surrounding Atlantic Coast Regions

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Abstract

We describe a new cryptic species of leopard frog from the New York City metropolitan area and surrounding coastal regions. This species is morphologically similar to two largely parapatric eastern congeners, *Rana sphenocephala* and *R. pipiens*. We primarily use bioacoustic and molecular data to characterize the new species, but also examine other lines of evidence. This discovery is unexpected in one of the largest and most densely populated urban parts of the world. It also demonstrates that new vertebrate species can still be found periodically even in well-studied locales rarely associated with undocumented biodiversity. The new species typically occurs in expansive open-canopied wetlands interspersed with upland patches, but centuries of loss and impact to these habitats give some cause for conservation concern. Other concerns include regional extirpations, fragmented extant populations, and a restricted overall geographic distribution. We assign a type locality within New York City and report a narrow and largely coastal lowland distribution from central Connecticut to northern New Jersey (based on genetic data) and south to North Carolina (based on call data).

Introduction

In order to develop clear understandings of species and their ecologies, distributions, and conservation needs, they must first be properly identified and accurately delimited [1]. Such efforts can be complicated, however, by the presence of cryptic species – species that, due to morphological similarity, have been incorrectly included with one or more other species under a single species classification [2]. Identifying cryptic species can be difficult though, which presents taxonomic and conservation challenges. These challenges can be further exacerbated in heavily altered environments and areas where extirpations and habitat loss have led to insufficient numbers of individuals or populations for sampling. Nonetheless, a cryptic species discovery can have important implications for multiple species, including the new species itself and its cryptic congeners [1]. Further, cryptic species can be found in unexpected locales [3], and in some regions, reflect surprisingly high levels of diversity [4]. Left undetected, however, cryptic species can remain concealed among other species, which can be problematic if seemingly common or widespread nominal species actually contain hidden component species that are range-restricted, rare, or even extinct [1,2].

Considerable effort has been given to identifying and cataloging new species, cryptic and otherwise, over the past few decades. In the case of amphibians, these efforts carry added urgency in the face of severe global declines and extinctions and also reveal strongholds of undocumented species, often in areas of tropical species richness or poorly known composition [4,5]. In contrast, far less attention or discovery has been associated with urban areas and other highly developed or well-documented regions, especially those outside the tropics. Among anurans, for example, only two truly novel species (that

is, taxa that were not previously recognized as subspecies) have been reported from the continental United States (US) and Canada since 1986 [3,6,7]. In this paper we describe the most recent of these, a cryptic leopard frog lineage that was first identified from the New York City region in 2012 [3]. Few examples of undescribed vertebrate diversity exist in the recent literature from highly urbanized regions and areas with well-established taxonomic infrastructures.

The species we describe here was first identified by Newman *et al.* [3] via molecular data. It constitutes the newest member of the *Rana pipiens* complex and occupies parts of the lower Northeast and mid-Atlantic US within the densely populated and heavily industrialized Interstate-95 (I-95) corridor. This is one of the largest human population centers on earth [8] and a region where endemic vertebrate species are rare. The long-term concealment and recent discovery of a novel anuran here is both surprising and biogeographically significant, and illustrates how new species can occur almost anywhere. It also raises potentially important conservation concerns: amphibians can be sensitive to disease, contaminants, and environmental perturbations, and their low vagility can be particularly problematic in fragmented and urban landscapes [9]. Also worrisome are enigmatic declines that have led to disappearances of leopard frogs from parts of the Northeast and mid-Atlantic US [10-13]; this includes some relatively non-urbanized coastal, suburban, and agricultural regions in southeastern New York (NY) [3,14], southern Connecticut (CT) [11], and presumably parts of northeastern Pennsylvania (PA) where they were reported historically, but not in recent decades [15-20].

Here, we expand upon the initial genetic results presented by Newman *et al.* [3] to name, diagnose, and describe the new species. We present several lines of supporting evidence, but focus on bioacoustic signals and molecular data. We also provide a brief history of relevant taxonomic confusion within the *R. pipiens* complex, comparisons to similar species, and information on distribution, ecology, and conservation status.

Taxonomic Overview

Although one of the most well-known and best-studied amphibian groups on earth, the *R. pipiens* complex has long been a source of taxonomic uncertainty and nomenclatural debate in eastern North America [21-27]. Our work resolves some of this confusion. In this section we review relevant background information to provide appropriate context for our discovery.

The unsettled taxonomic history of the *R. pipiens* complex spans several centuries and has been fueled largely by a lack of scientific consensus and changing species concepts across those years. This has led to numerous synonyms and conflicting species frameworks over time [28]. Ultimately, however, only two species, *R. sphenocephala* and *R. pipiens*, received lasting consideration and taxonomic recognition in the east [26,29]. *Rana sphenocephala*, the southern leopard frog, has a reported range from extreme southeastern NY to Florida (FL) and west from Texas to Iowa [30]. *Rana pipiens*, the northern leopard frog, ranges from eastern Canada, New England, and the northern mid-Atlantic, west to the Pacific Coast states and British Columbia [30]. These two species are generally parapatric along the US East Coast [29,30], although Pace [26] reported one possible example of sympatry from Bronx County, NY (but see Klemens *et al.* [31]).

Much of the historical discord and confusion surrounding the *R. pipiens* complex can be traced to the Northeast and mid-Atlantic US [26,27,32], especially the greater New York City metropolitan area [11,33,34] (referred to hereafter as the NY/NJ-metro area and defined to include southwestern CT, southeastern NY, New Jersey [NJ], and extreme eastern PA). This relatively small region has been associated with longstanding ambiguity regarding leopard frogs, including the type locality of *R. pipiens* itself [7,34,35] and as many as five different species names over the past 250 years [7,33].

In 1936, Kauffeld [35] attempted to reconcile some of this confusion. He did so by noting the possibility of a third, centrally occurring and unnamed "form" of leopard frog in the NY/NJ-metro area, between the recognized East Coast ranges of *R. sphenocephala* and *R. pipiens* at that time. Kauffeld [33] later combined his own examinations with subspecies descriptions by Cope [36] and putative type localities for *R. pipiens* to conclude that three distinct species did occur across the Northeast and mid-Atlantic US. He classified the northernmost species as *R. brachycephala* and reassigned *R. pipiens* – the binomial typically associated with the northernmost species – to his proposed central species (occupying much of the NY/NJ-metro area and mid-Atlantic region with extensions south along the coastal plain and west to Texas); *R. sphenocephala* was maintained as the southernmost species. Despite acknowledging the potential taxonomic confusion and backlash this could cause, Kauffeld [33] proposed these changes to reflect his conclusion that the type locality for *R. pipiens* fell within southeastern New York, where his reported central species occurred, not the northernmost species.

Kauffeld's three-species framework and taxonomic changes received some initial recognition [37-39] but did indeed face considerable scrutiny over time and failed to garner lasting support [23-25]. His proposals also provided the impetus for several studies that led to more conservative taxonomic frameworks, including the predominant mid-20th Century single-species interpretation that classified all North American leopard frogs as *R. pipiens* [24,40,41]. This determination was based on inconsistent differences among purported species and successful cross-breeding experiments with frogs from distant geographies [28,42]. Several decades later, relying primarily on morphology and bioacoustics, Pace [26] presented a detailed treatment of the *R. pipiens* complex that returned to a two-species arrangement in the eastern US, echoing arrangements prior to Kauffeld's work [43-45]. This included *R. sphenocephala* (referred to as *R. utricularia* by Pace) to the south, and *R. pipiens* to the north, with a species boundary centered in the NY/NJ-metro area. Pace's arrangement remained largely intact over subsequent decades, particularly across the eastern US.

Occasional discussion of distinct populations, potential intergradation, and cryptic species in the NY/NJ-metro area continued after Kauffeld [33], but remained largely speculative [11,46,47]. More recently, however, advances in molecular methods utilizing nuclear and mitochondrial markers have allowed for increasingly sophisticated species delimitations and analyses of phylogenetic and population genetic relationships. Initial molecular work by Newman *et al.* [3] demonstrated this, suggesting that an undescribed cryptic leopard frog lineage, termed *R.* sp. nov., does indeed occur between populations of *R. sphenocephala* and *R. pipiens* in the NY/NJ-metro area. They also reported mitochondrial data showing this species to be most closely related to the pickerel frog, *R.*

palustris, a morphologically distinct and readily identifiable species [29], rather than to *R. sphenocephala*, the species to which it had been included based on morphological similarity; nuclear data regarding interspecific relationships were inconclusive.

In retrospect, the long history of taxonomic and nomenclatural confusion in the NY/NJ-metro area was likely due to the unrecognized presence of a cryptic species occurring in close proximity to several similar congeners. For example, in the Philadelphia region – an area replete with historical confusion and variation reported among leopard frogs [26,27,48] – all four regional spotted congeners are now known to occur; *R. pipiens*, *R. palustris*, *R.* sp. nov., and *R. sphenocephala* each occur in succession along a narrow 90-km west-to-east transect between Berks County, PA and Burlington County, NJ [20,49].

Materials and Methods

Ethics Statement

The species described here was discovered during research activities conducted under an Institutional Animal Care and Use Committee Protocol (IACUC) from Rutgers University (#07-024). Additional field work and collection of the holotype specimen occurred under New York State Collect or Possess permit #969 (to MDS) in compliance with Yale University IACUC protocol #2012-10681.

Taxonomic Note

We briefly point to an area of unresolved taxonomic debate within the herpetological community. This debate centers on use of the historical genus name *Rana* versus a recently proposed replacement name, *Lithobates*, which has been applied to a number of North American ranid frog species [50]. Given that this issue still remains largely unsettled, we have followed the conservative taxonomic practice of continuing to use *Rana* for all North American ranid frogs, including the *R. pipiens* complex.

Morphology

Fieldwork to collect an adult male holotype was conducted in Richmond County, NY. The specimen was preserved in 10% neutral-buffered formalin, transferred to 70% ethanol and deposited at the Yale Peabody Museum of Natural History (YPM). We collected morphometric measurement data from 283 specimens, including the holotype (YPM 13217) and 282 other museum specimens across four species (*R.* sp. nov., *R. sphenocephala, R. pipiens,* and *R. palustris*), 30 US counties, seven eastern states, and Quebec, Canada (Table S1). When genetic data were not available to confirm species identification, we used a combination of morphology and location to classify preserved specimens based on our knowledge of species habitat preferences and distributions (Fig. 1). Straight-line measurements were taken to the nearest 0.01 mm with Mitutoyo Digimatic calipers. We measured 13 characters, 11 of which follow Napoli [51]: snout-vent length (SVL; anterior end of snout to posterior end of urostyle), head length (HL; anterior end of snout to occiput), head width (HW; at widest part of the head), eye diameter (ED; at widest point of eye), tympanum diameter (TD; at widest point of tympanum), foot length (FOL; tip of fourth toe to heel), eye to naris distance (END;

anterior eye to naris), naris to snout distance (NSD; naris to anterior end of snout), thigh length (THL; anterior knee to posterior urostyle), internarial distance (IND; closest distance between nares), and interorbital distance (IOD, closest distance between the eyes). We also include shank length (SL; knee to heel) following Heyer *et al.* [52] and dorsal snout angle (DSA; [arcsine ((HW/2)/HL) x 2) following Lemmon *et al.* [6].

We looked for univariate differences in species morphology using boxplots and one-way ANOVAs followed by Tukey HSD post-hoc pairwise comparisons. We used discriminant function analysis (DFA) to examine variation in multivariate space and determine which variables best discriminated among species. This was followed by a MANOVA to look for multivariate differences among species, and then Tukey HSD post-hoc pairwise comparisons. Because body size varied substantially among specimens, we removed this effect in our statistical analyses by using the residuals of a regression of snout-vent length on each morphometric variable. Foot length was not available for some specimens (n = 19), reducing the number of frogs with complete measurements to 264. Thus, we omitted these specimens from our DFA. All analyses were conducted in R, v. 2.15.2 and v. 3.0.2 [53], including package MASS.

We also examined color and patterning differences between leopard frog species. We compared dorsal spots (number of spots and percent dorsal area coverage) between the new species and its closest morphological congener, *R. sphenocephala*, following Platz [54]. For spot coverage, we imported images of both species (*R.* sp. nov., n = 22; *R. sphenocephala*, n = 18) into ArcMap 10.0 [55] and digitized polygons representing the dorsum and each spot as viewed from directly above in order to calculate the proportion of the dorsal surface covered by spots. We examined both variables using boxplots and t-

tests ($\alpha = 0.05$) to look for species differences. We also conducted several categorical comparisons between *R.* sp. nov. and *R. sphenocephala*, including 1) dorsal spot shape (round or elongate), 2) snout spot (present or absent), and 3) skin color (three color categories). We categorized a dorsal spot as 'elongate' if it was at least 2.5 times longer than wide at its widest point, but excluded eyelid spots from this analysis because the curvature of the eye made them difficult to assess. Lastly, we compared pigmentation on the posterior dorsal surface of the femur (thigh) among specimens of *R.* sp. nov., *R. sphenocephala*, and *R. pipiens*. This character was previously used to distinguish leopard frogs in regions where *R.* sp. nov. occurs [24,32]. We follow Moore [24] in referring to it as the "reticulum" and recognize two alternate states: light (light ground color with dark spots) or dark (dark ground color with light spots). All specimens used in spot and color comparisons are listed in Table S1. All photo vouchers were deposited at YPM.

Genetic Analysis

Following the methods described in Newman *et al.* [3], we extracted genomic DNA from a liver sample obtained from the holotype. We sequenced the ND2 and 12S-16S regions of the mitochondrial genome, including intervening and flanking tRNAs (1444 bp), and the nuclear genes neurotrophin-3 (NTF3, 599 bp), tyrosinase (Tyr, 557–585 bp), Rag-1 (647–683 bp), seven-in-absentia (SIA, 362–393 bp), and chemokine receptor 4 (CXCR4, 550 bp). PCR products were sequenced at Beckman Coulter Genomics (Danvers, MA, USA). All sequences generated in this study were uploaded to GenBank (accession number of hologenetypes: JX867559-JX867563). Data from the present study were added to the Newman *et al.* [3] data set, and Bayesian phylogenetic

analyses were run in MrBayes 3.1 [56,57] for each locus following the analyses described in Newman *et al.* [3] to verify the species identity of the holotype.

Bioacoustic Analysis

We recorded calls of the new species with an Olympus DS-40 digital voice recorder and Sennheiser MKE 400 directional microphone at a sampling rate of 44.1 kHz and 16-bit sampling size. We converted files to .wav format using Roxio Sound Editor (Sonic Solutions, Novato, CA, USA) and analyzed calls with RAVEN Pro v. 1.4 [58] using the following settings: spectrogram FFT length 2048, Hanning window size 1024, amount of overlap between FFT samples 90, and power spectrum FFT length 2048. We analyzed calls from three populations (two in Richmond County, NY; one in Bergen County, NJ). For comparison, we also recorded and analyzed calls from four congeners using these same methods unless otherwise stated (Table S2); these included *R. sphenocephala, R. pipiens, R. palustris,* and an acoustically similar species outside the leopard frog complex, *R. sylvatica*. We examined two populations of *R. sphenocephala* (Middlesex Co., NJ and Burlington Co., NJ), one population of *R. pipiens* (Columbia Co., NY), one population of *R. palustris* (Suffolk Co., NY), and three populations of *R. sylvatica* (Queens Co., NY, Suffolk Co., NY, and Larimer Co., Colorado). We did not collect frogs used in our call analysis, but deposited call vouchers at YPM (Table S2).

We measured seven variables: call length (CL; time from beginning to end of a single call), call rate (CR; based on time between starts of successive calls), call rise time (CRT; time from call start to maximum amplitude), call duty cycle (CDC; call length / [call length + time to next call start]), pulse number (PN; number of pulses in a call),

pulse rate (PR; based on time between start of first and last pulse), and dominant frequency (DF; frequency of highest energy in a call). We mostly follow parameters and terminology from Cocroft and Ryan [59] but follow Lemmon *et al.* [6] for CDC and PN. We derived trait averages from four consecutive calls per individual unless otherwise noted (Table S2). For the purposes of this study, we examined only the primary mating call of each species, defined as the advertisement call by Heyer *et al.* [52]. This approach provided a clear means for comparing species and minimized confusion presented by secondary call signals. Thus, all secondary repertoires were considered to fall outside the scope of our objectives and were not analyzed here. We compared call differences between species using the same univariate and multivariate statistical procedures described for our morphological analyses. Call rate and call length are frequently correlated with water temperature, so we adjusted these two parameters to a common water temperature of 14°C for our statistical analyses following Lemmon *et al.* [6]. We used regression equations from *R.* sp. nov. in place of *R. pipiens* and *R. palustris* because both species were recorded at only one site each under a single temperature regime, and thus lacked sufficient variation for us to generate their own species-specific regression equations.

Nomenclatural Acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank,

the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub:2E7F07A6-19B1-4352-B5B7-A227A93A37CD. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central and LOCKSS.

Results

Diagnosis and Description

***Rana kauffeldi* sp. nov.** urn:lsid:zoobank.org:act:149ED690-FA7D-4216-A6A1-AA48CC39B292.

Holotype. YPM 13217, adult male (Fig. 2, Table 1), collected from Bloomfield region, Richmond County (Staten Island), NY, United States, on 15 November 2011, by B. R. Curry.

Paratypes. YPM 13559, subadult male (paragenetypes: GenBank accession numbers JN227403, JN227458, JN227127, JN227180, JN227236, JN227348, JN227292) and YPM 13560, adult male (paragenetypes: GenBank accession numbers JN227404, JN227459, JN227128, JN227181, JN227237, JN227349, JN227293); both collected from Wangunk Meadows in Portland, CT by T. Mahard and M. Blumstein on 15 September 2010; genetically confirmed within the same clade as the holotype [3].

Referred material. YPM 13920, juvenile (GenBank accession numbers JN227377, JN227432, JN227102, JN227155, JN227209, JN227321, JN227265); collected as an egg by J. A. Feinberg from the type locality on 27 March 2009 (hatched in captivity and raised *in situ* within a field enclosure on Long Island, NY, for a separate research project); genetically confirmed within the same clade as the holotype [3]. AMNH 121857–121858, juveniles; collected from type locality on 3 August 1984 by P. R. Warny and E. Johnson.

Etymology. The specific epithet is a patronym in recognition of Carl F. Kauffeld who studied the *R. pipiens* complex in the NY/NJ-metro area and concluded that three distinct species, including an undocumented central species, occurred there.

Common Name. We propose the common name 'Atlantic Coast Leopard Frog' for this species.

Synonymy. Given the complex nomenclatural history of leopard frogs in the NY/NJ-metro area, we searched for potential synonyms within the range of *R. kauffeldi* before assigning a binomial and identified five candidates: *R. pipiens* Schreber [60], *R. halecina* Daudin [61], *R. utricularius* Harlan [48], *R. virescens virescens* Cope [36], and *R. brachycephala* Cope [36] as elevated to species rank by Kauffeld [33]. Based on our review and commentary by Lavilla *et al.* [62] and Frost [7], we determined that none of these candidates has clear unequivocal support or the precise locality information or type specimens necessary to warrant assignment to the new species. Most recently, Frost *et al.* [50] proposed *Lithobates pipiens* as a systematic replacement for *Rana pipiens*, but the type locality was not changed, and, as noted earlier, disagreements in the herpetological

community as to the utility and appropriateness of *Lithobates* remain largely unsettled at this time.

We include *R. pipiens* as a synonym because its type locality has been restricted to various parts of the NY/NJ-metro area where *R. kauffeldi* occurs [7,34,35,63,64]. However, given the lack of precision, geographic consensus, or a physical type specimen, Pace [26] designated a neotype from Tompkins County in central NY (UMMZ 71365). We follow Pace, and thus consider *R. pipiens* to be removed from further geographic consideration, and also agree with Smith [65] and Pace [26] that the frog illustrated by Schreber [60] most resembles the northernmost species, not the species described here. Thus recircumscription of the geographic range of *R. pipiens* is unwarranted and, despite the confusion and numerous synonymies from the NY/NJ-metro area, no other synonym conclusively warrants resurrection. We also refer briefly to Lavilla *et al.* [62] and point out that *R. halecina* was introduced to translate a Swedish name but was not intended as a scientific name. Further, it comes only from an observation and lacks an explicit type locality or type specimen.

Diagnosis. *Rana kauffeldi* is morphologically similar to *R. sphenocephala* and *R. pipiens*, but distinguishable by 1) advertisement call (Fig. 3, Table 2; Figs. S1 and S2), 2) genetics [3], 3) habitat (see *Distribution*), 4) geographic distribution (Fig. 1), and 5) a combination of morphological characters (Table 1; Figs. S3 and S4).

The advertisement call is a single-noted unpulsed ‘chuck’ that is distinct from the pulsed ‘ak-ak-ak’ of *R. sphenocephala* and the snore-like calls of *R. pipiens* and *R. palustris*. The quivering ‘quack’ of *R. sylvatica* is superficially similar but consists of discrete bouts of 2-4 rapidly pulsed notes that are never accompanied by secondary

‘groans’ as occasionally emitted by *R. kauffeldi*. Although sympatric with *R. kauffeldi*, *R. sylvatica* is morphologically and genetically distinct and typically calls from smaller canopied wetlands and forested pools whereas *R. kauffeldi* usually calls from larger, open-canopied wetlands.

Adult male *R. kauffeldi* possess very large, laterally paired external vocal sacs that distinguish them from all similar congeners except *R. sphenocephala*. Additionally, *R. kauffeldi* has a dark femoral reticulum (Fig. 4*a*) whereas northeastern populations of *R. sphenocephala* and *R. pipiens* typically have a light reticulum (Fig. 4*b*). This diagnostic was 100% consistent in *R. kauffeldi* from NY and NJ (n = 27) and *R. pipiens* from the northeastern US and Canada (n = 46), and was 88.6% consistent in *R. sphenocephala* from NJ (n = 35). The diagnostic value of this character may be limited to northern regions, however, as Moore [24] noted that leopard frogs predominantly exhibit a dark reticulum across portions of the Southeast where *R. sphenocephala* is broadly distributed.

Rana kauffeldi may be further distinguished from *R. sphenocephala* by a tympanic spot that is typically duller, less well-defined, and rarely pure white (as in *R. sphenocephala*); from *R. pipiens* by a light spot in the center of the tympanum that is often small and faint (but occasionally absent); and from *R. palustris* by pale inner thighs without deep yellow coloration and round, unaligned dorsal spots.

Description. Body moderate and robust; head longer than wide. Dorsal outline of snout acuminate; lateral snout profile round. Nares dorsolaterally oriented, slightly protuberant, around two-thirds closer to tip of snout than anterior corner of eye. Canthus rostralis distinct and angular; loreal region steep and slightly concave. Eyes large and protuberant; diameter slightly less than combined eye-to-naris and naris-to-snout

distances. Internarial distance nearly equal to eye-to-naris distance. Tympanum distinct and relatively large (> 65% diameter of the eye); bordered dorsally and posteriorly by faint supratympanic fold. Distinct dorsolateral fold runs uninterrupted from posterior eye to pelvic insertion of femur. Forearms relatively short and robust; unwebbed fingers; relative length III>I>II>IV. Fingers lack fringes; tips rounded without expansion; subarticular tubercles small, round, and moderately prominent. No palmer tubercles appear present. First finger slightly swollen at base with faint nuptial pad; all other fingers slender. Hindlimbs relatively long, moderately robust; thigh and shank length nearly equal. Relative toe lengths IV>V>III>II>I; toes have rounded tips without expansion; subarticular tubercles small, round, and prominent. Inner tarsal fold connects tarsus to large, distinct, elliptical, elevated inner metatarsal tubercle. Indistinct, small outer metatarsal tubercle faintly evident. Toe IV very long and slender; toe V slightly fringed; webbing present between all toes; webbing formula **I**1 – 2**II**1^{+} – 2⅓**III**1^{+} – 3^{+}**IV**3 – 1**V** following Savage [66]. Skin on dorsum smooth with several raised folds running between and parallel to dorsolateral folds. Flanks, thighs, and shanks smooth. Ventral surface mostly smooth with papillae-like granulation on groin and thighs. Large, distinct, paired lateral external vocal sacs.

Color in life. In photographs taken before preservation, dorsal ground color of holotype varies from mint-gray in bright lighting (Fig. 2*a*) to light olive green in darker conditions (Fig. 2*b*). Medium to dark brown spots irregularly distributed across dorsum and lateral body; more elongate or barred on the limbs. Distinct black postorbital patch encompasses dorsal and posterior tympanum along the supratympanic ridge. Labial margins slate gray with light mottling and distinct ivory stripe above the upper margin;

terminates under the tympanum (continues to anterior forearm in females). Dark canthal band runs from snout tip through the nare and iris, along outer edge of dorsolateral fold; terminates above the arm. On snout, inner edge of canthal band is paralleled by light brown band that continues through the eyelid to merge with a dorsolateral fold that varies from gold (Fig. 2*a*) to bronze (Fig. 2*b*) in different lighting. Iris gold with dark intrusions at corners. Vocal sac slightly darker than surrounding skin. Lower flank of holotype pale with light yellowish-green hues and smaller, lighter spots and mottles; these intrude onto ventral margins, throat, or body in some individuals. Tympanum finely granulated brown color with black flecks; central spot creamy and subtly defined in holotype; bright and well defined or entirely absent in some individuals. Reticulum and anterior ventral margin of thigh dark with distinct light flecks or mottles; off-white in holotype, occasionally bone-white (Fig. 4*a*), light yellow (Fig. 4*c*) or green (Fig. 4*d*) in some individuals. Ventral limbs of holotype pinkish-gray with scattered mottles; body pale white. Inner tarsal fold and outer metatarsal tubercle are bright white against a dark brown tarsal background; webbing pale gray.

Color in preservative. Generally similar to that in life with several notable distinctions. Ground color dark olive green in holotype (Fig. 2*c*) but can range from tan to dark brown in other specimens (as in paratypes YPM 13559 and 13560). Colored flecks and mottles in life appear white in preservative. Ventral body and limbs of holotype cream, light mottling behind knees (Fig. 2*d*). Dorsolateral fold of holotype rust brown (Fig. 2*c*); off-white to brown in other individuals. Tympanic spot, when present as in the holotype, typically subtle and grayish white.

Genetics

Holotype (YPM 13217) falls within the *R. kauffeldi* clade (*R.* sp. nov. in Newman *et al.* [3]) in the mitochondrial phylogeny (results not shown). Mitochondrial and nuclear haplotypes are identical to other *R. kauffeldi* samples. As reported by Newman *et al.* [3], *R. kauffeldi* is genetically distinct from all other regionally occurring spotted ranid frogs (*R. sphenocephala, R. pipiens, and R. palustris*). The mitochondrial phylogeny suggests that *R. kauffeldi* is most closely related to *R. palustris*. Average pairwise mitochondrial sequence divergence (uncorrected p) is similar to genetic divergences between other closely related species in the *R. pipiens* complex (Newman *et al.* [3]).

Distribution

Rana kauffeldi is known from three states (CT, NY, NJ) based on genetic samples [3] and seven states (NY, NJ, PA, Delaware [DE], Maryland [MD], Virginia [VA], and North Carolina [NC]) based on bioacoustic sampling reported here. The estimated range from these samples is approximately 780 km, north-to-south, from central CT to northeastern NC (Fig. 1). The range is narrow, however, east-to-west, occurs almost entirely within the densely populated I-95 corridor, and is smaller than most if not all other ranid frogs along the eastern North American seaboard. Within the presented range, we depict a core sampling area (Fig. 1, purple shading) where gaps in genetic and bioacoustic information were filled by other lines of evidence (e.g., specimens, photographs, geology, or historical literature). *Rana kauffeldi* appears to occur parapatrically in this core area. Beyond the core area, we depict an extended area of potential occurrence (Fig. 1, yellow shading) based on habitat features and proximity to

known bioacoustic confirmations in DE, MD, VA, and NC. Within the yellow shading we also note the potential for sympatry with *R. sphenocephala* (in the south) and *R. pipiens* (in the north) based on genetic, bioacoustic, and specimen sampling (see *Discussion*).

Rana kauffeldi has a mesic distribution that is wider in the north and narrows from Trenton, NJ, to the Delmarva Peninsula. This part of the range essentially follows the Delaware River floodplain and the Atlantic Fall Line – the geologic interface between the relatively xeric Atlantic coastal plain where *R. sphenocephala* occurs, and more interior and upland regions to the west – where *R. pipiens* occurs. This species is usually abundant where it occurs, but populations in the NY/NJ-metro area tend to be disjunct and isolated from one another and often occur in highly fragmented landscapes with limited connectivity or dispersal opportunities. *Rana kauffeldi* was generally included within the range of *R. sphenocephala* prior to its discovery, but northern mainland populations from northeastern PA to central CT may have been included within *R. pipiens* instead (Fig. 1, yellow shading).

We also consider *R. kauffeldi* to have previously occurred within parts of an apparent extirpation zone that includes most of coastal NY and southern CT (Fig. 1). We used multiple lines of evidence to inform this conclusion, including historical locality information [11,33], photographs [67-69], call descriptions [68,70], personal communications (A. Sabin and F. C. Schlauch), and museum specimens (Table S1). Our assessment of museum specimen and photographs included frogs from Long Island (n = 27) and Bronx County, NY (n = 7). Based on our examination, 29 of these 34 frogs were *R. kauffeldi*. Two other individuals, from xeric parts of Long Island, NY (Suffolk

County), appeared to be *R. sphenocephala* (AMNH 125956, 176153). The remaining three frogs were *R. pipiens*, two of which (AMNH 106549, 106550) came from the Bronx County site previously noted by Pace [26] and Klemens *et al.* [31], where specimens of *R. kauffeldi* (AMNH 52342, 106551-10654) were also collected historically. The third was a lone individual from western Long Island, in Queens County, NY (AMNH 36651). We also examined specimens (n = 9) from two presumably extirpated sites in southeastern CT (New Haven County) (Table S1). All were *R. pipiens*, but neither site is coastal or located within a bottomland riparian floodplain where *R. kauffeldi* would be expected to occur.

Morphological Evidence

Univariate analysis recovered significant differences among 11 of 12 size-corrected characters between *R. kauffeldi* and *R. sphenocephala*, *R. pipiens*, and *R. palustris* (Fig. S1). *Rana kauffeldi* had 1) the shortest eye-to-naris distance ($F_{3,279}$ = 28.41, $p < 0.0001$), 2) shortest thigh length ($F_{3,279}$ = 22.63, $p < 0.0001$), and 3) shortest shank length ($F_{3,279}$ = 27.95, $p < 0.0001$) of the four species examined. *Rana kauffeldi* had 4) narrower eyes ($F_{3,279}$ = 41.61, $p < 0.0001$), 5) a wider head ($F_{3,279}$ = 14.59, $p < 0.0001$), 6) and longer interorbital distance ($F_{3,279}$ = 35.02, $p < 0.0001$) than *R. sphenocephala* and *R. pipiens*. *Rana kauffeldi* also had 7) a shorter head than *R. sphenocephala* and a longer head than *R. pipiens*, ($F_{3,279}$ = 16.00, $p < 0.0001$), 8) a longer internarial distance than *R. sphenocephala* and a shorter internarial distance than *R. pipiens* ($F_{3,279}$ = 8.48, $p < 0.0001$), 9) a larger tympanum diameter than *R. pipiens* and *R. palustris* ($F_{3,279}$ = 14.42, $p < 0.0001$), 10) a shorter naris-to-snout distance ($F_{3,279}$ =

19.92, $p < 0.0001$) than *R. pipiens*, and 11) a wider snout angle than *R. sphenocephala* ($F_{3,279} = 32.04$, $p < 0.0001$). The unadjusted summary data for all 13 morphometric characters are also presented (Table 1).

In multivariate space using DFA, we found considerable morphological overlap among all four species examined (Fig. S2), but some significant differences were detected ($F_{3,260} = 120.0$, $p < 0.0001$). The DFA correctly classified 78.0% of specimens (Table S3). Post-hoc Tukey's HSD tests showed all pairwise comparisons to be significantly different from one another ($p < 0.0001$) except for *R. sphenocephala* and *R. palustris* ($p = 0.9966$). The first discriminant function accounted for 58.4% of the variation in the data with tympanum diameter loading most heavily, while the second function accounted for 31.4% of the variation with eye-to-naris distance having the greatest load (Table S4).

Previous studies report fewer and smaller dorsal spots among leopard frogs from areas where *R. kauffeldi* occurs [24,32], and we found that *R. kauffeldi* indeed has fewer dorsal spots than *R. sphenocephala* (mean = 13.18±3.22 SD vs. 20.44±4.10 SD, respectively) ($t = -4.32$, two-tailed $p < 0.001$) and less dorsal surface covered by spots (mean = 13.56%±3.29 vs. mean = 22.13%±7.76, respectively) ($t = -6.12$, two-tailed $p < 0.0001$) (Fig. S3). Dorsal spot shape also differed; only 35.71% ($n = 42$) of *R. kauffeldi* had one or more elongated spot compared to 61.16% ($n = 67$) of *R. sphenocephala* examined. Further, snout spots were present in 32.86% ($n = 70$) of *R. kauffeldi* versus 16.88% ($n = 77$) of *R. sphenocephala*. Lastly, we found considerable categorical color differences between *R. kauffeldi* ($n = 75$) (74.7% = dark olive to mint-gray, 24.0% = green to light brown, and 1.3% = bright green) and *R. sphenocephala* ($n = 94$) (46.8% =

dark olive to mint-gray, 39.4% = green to light brown, and 13.8% = bright green). Multi-colored frogs were categorized by their lightest color.

Bioacoustic Evidence

The unpulsed advertisement call of *R. kauffeldi* is typically emitted in evenly spaced, repeated series that can include up to 27 ‘chucks’ over 22 s. Calls were recorded at multiple locations within the type locality. Five males (YPM 14137-14140; Table S2) were recorded at the specific location where the holotype itself was heard calling and collected (but not recorded). These frogs were recorded between 2028 and 2042 h on 15 March 2012 (11°C air, 10°C water) and had the following mean characteristics: call length 60.55 ms (54.00-71.25±6.74 SD), call rate 1.10 calls/s (0.90-1.33±0.15), call rise time 33.55 ms (29.00-39.75±4.55), call duty cycle 0.07 (0.05-0.10±0.02), pulse number 1.00 (1.00±0.00), pulse rate 0, and dominant frequency 1296.30 Hz (1211.23-1421.20±85.50). Recordings from one of these frogs (YPM 14137 and 14172) were used to represent temporal and spectral features for *R. kauffeldi* in comparison to *R. sphenocephala*, *R. pipiens*, *R. palustris*, and *R. sylvatica* in Fig. 3.

We compared summary data for all *R. kauffeldi* to the four other species (Table 2). Frogs were recorded opportunistically with water temperatures ranging from 8 to 25.6°C (Table S2), reflecting the different geographies and phenologies among species. The temperature range was less variable, however, when grouped and averaged by species; *R. kauffeldi* (12.56°C±2.87 SD), *R. sphenocephala* (18.30°C ±7.80), *R. pipiens* (18.00°C ±0), *R. palustris* (15.00°C ±0), and *R. sylvatica* (9.68°C ±0.94).

Our univariate analysis revealed significant differences among species in 6 of 7 call parameters (Fig. S4). *Rana kauffeldi* had 1) a lower pulse rate ($F_{4,40} = 293.0$, $p < 0.0001$) and 2) shorter call duration than all other species ($F_{4,40} = 171.0$, $p < 0.0001$), and 3) a lower pulse number ($F_{4,40} = 280.9$, $p < 0.0001$) and 4) a lower call rise time than all species except *R. sylvatica* ($F_{4,40} = 85.3$, $p < 0.0001$). *Rana kauffeldi* also had 5) a lower call duty cycle than all species except *R. pipiens* ($F_{4,40} = 37.8$, $p < 0.0001$), and 6) a call rate that was higher than *R. pipiens* and *R. palustris* and lower than *R. sylvatica* ($F_{4,40} = 44.8$, $p < 0.0001$). Dominant frequency did not differ significantly among the five species ($F_{4,40} = 2.3$, $p = 0.0744$).

In multivariate space using DFA, we found clear separation in call parameters among all species (Fig. S2). The DFA correctly classified 95.6% of calls ($F_{4,40} = 323.7$, $p < 0.0001$). The only classification errors were two *R. sylvatica* classified as *R. kauffeldi* (Table S5). Post-hoc Tukey's HSD tests showed all pairwise comparisons to be significantly different from one another ($p < 0.001$) except for *R. kauffeldi* and *R. sylvatica* ($p = 0.9991$). Pulse rate was excluded from the DFA because *R. kauffeldi* has only one pulse per call. The first discriminant function accounted for 61.0% of the variation in the data with call rise time loading most heavily, while the second function accounted for 24.3% of the variation with call length contributing the greatest load (Table S6).

Ecology, Behavior, and Natural History

Rana kauffeldi inhabits a restricted range of mesic lowland habitats that primarily includes coastal freshwater wetlands, tidally influenced backwaters, and interior riparian

valley floodplains. This species is typically associated with large wetland complexes composed of open-canopied marshes, wet meadows, and slow-flowing systems with ample open upland and early-successional habitats. Aquatic conditions are usually clear, shallow, and sometimes ephemeral, with emergent shrubs or stands such as cattail, *Typha* spp., or the invasive common reed, *Phragmites australis*.

Rana kauffeldi begins breeding around the same time as *R. sylvatica* and *R. sphenocephala* and slightly in advance of *R. pipiens* and *R. palustris*. In NY, we have observed migratory activity on rainy nights with above-average temperatures in early February, and have documented the onset of chorusing after several days of above-average temperatures in early-to-mid March. Choruses are most consistent nocturnally, with air temperatures ranging from 10-18°C, but sustained diurnal and nocturnal chorusing is common early in the season and through the initial 2-3 week peak breeding period (late March and early April in NY), especially on warmer days. Thereafter, chorusing tapers to a more episodic nocturnal and precipitation-based regime from mid-April through early June (in NY). We have not observed opportunistic mid-summer chorusing as we and others [26,71] have for *R. sphenocephala,* but we have observed occasional second breeding periods with the onset of cooler autumn temperatures and precipitation (late August through November).

Individuals may exhibit a limited degree of color change around a general base color that can vary widely between frogs, from light green to dark brown. Holmes [72] noted that leopard frogs (*sensu lato*) tend towards darker nocturnal shading and brighter, more vivid diurnal colors (as a putative mode of camouflage). Some degree of seasonal color change also appears to exist in *R. kauffeldi*; we often observed frogs with darker,

drabber color and fainter tympanic spots in the early spring, and more vivid and varied overall color and brighter, more defined tympanic spots later in the season.

During breeding, males congregate in concentrated groups, or possible leks [26], that typically include five or more frogs, with as few as 30 cm between individuals. Males call while floating in shallows with emergent vegetation and as little as 20 cm of water. As stated by Mathewson [73], their calls are low-pitched and do not carry far. This is especially apparent in the presence of louder, higher pitched sympatric species like spring peepers (*Pseudacris crucifer*). Thus dense aggregations may have compensatory value, especially when faced with noisy conditions [74] or acoustic competition from other anurans [9,75,76]. Egg masses are often clustered in groups or deposited near one another. Porter [32] and Moore [77] discussed eggs and embryonic development among specimens (referred to as *R. pipiens*) from Philadelphia and NJ, respectively, that we consider *R. kauffeldi*.

Little is known about non-breeding activity or dispersal in *R. kauffeldi*, but leopard frogs have been described as being fairly terrestrial on Staten Island [73]. In our work, we observed individuals on land later in the season, but also noted periods, typically in summer and early fall, when few if any individuals could be found. Diet is not specifically known, but presumably similar to those reported for other regional leopard frog species.

Discussion

Hidden Diversity in a Well-Documented Urban Region

The description of *R. kauffeldi* brings the current number of New World leopard frogs to 19 (excluding *R. palustris*) and the total number of native ranid frog species from the US mainland and Canada to 30 [7]. Despite the vast size of this area, new frog discoveries north of Mexico are infrequent, and thus geographically significant. For example, *R. kauffeldi* and the Cajun chorus frog, *P. fouquettei*, [6] are the only newly described anurans (not former subspecies) north of Mexico in nearly three decades (since 1986) [7], and *R. kauffeldi* is the first anuran from the US Atlantic coast since the New Jersey chorus frog, *P. kalmi*, was originally recognized (as a subspecies) in 1955 [7].

The specific region where *R. kauffeldi* was first identified, the New York City metropolitan area (with a type locality less than 15 km from the Statue of Liberty) is also significant. It provides an example of new species discovery, not from a tropical biodiversity hotspot or poorly studied region, but rather the glacially impacted urban Northeast; one of the most developed, heavily settled, and well-inventoried places on earth. Novel and undescribed vertebrate species are unexpected here (particularly amphibians) and thus carry considerable interest and value. The last amphibian described from NY or New England was the Fowler's toad, *Bufo fowleri*, in 1882 [78], and *R. kauffeldi* follows the northern cricket frog, *Acris crepitans*, in 1854 [79], as the seventh amphibian described from NY [7]. Several other points warrant consideration. For one, this discovery clearly demonstrates that human knowledge of the natural world remains incomplete even in the best-known locales. Second, although new frog discoveries are generally uncommon north of Mexico, they do still occur periodically. Third, the two most recent examples (*R. kauffeldi* and *P. fouquettei* [6]) are both cryptic species. Taken together, these points suggest that occasional future discoveries from well-cataloged

areas may continue, but probably in the form of additional cryptic species rather than morphologically distinct taxa (which are likely already cataloged).

Although *R. kauffeldi* is a cryptic species, it is a relatively large, conspicuous, non-fossorial species nonetheless, and acoustically distinct. That it remained ill-defined and poorly documented within one of the largest population centers on earth [8] spanning eight eastern US states and several major North American cities, is rather remarkable. As a point of comparison, we consider another cryptic species group from the eastern US, the gray treefrogs *Hyla versicolor* and *H. chrysoscelis*. Despite being arboreal, smaller, and less conspicuous than leopard frogs, these two congeners were recognized as separate and distinct species nearly 50 years earlier (in 1966) by call their differences [7,80].

In part, the sustained concealment of *R. kauffeldi* may have been due to its narrow and fragmented range, short and cold-season calling regime, and low frequency (less audible) call. Repeated acoustic misidentification may have also played a concealing role; many colleagues with whom we communicated recalled unusual calls from frog populations now known to be *R. kauffeldi*. Some attributed these calls to *R. sylvatica* in unusual habitats; others presumed call variation within *R. sphenocephala*. Given these examples and the generally stereotyped and species-specific nature of frog calls [4,9] and the nuanced-but-critical role they can play in identifying species, we encourage greater scrutiny and examination of aberrant calls elsewhere, especially when encountered and heard consistently across entire populations or regions. Such efforts may reveal additional diversity, especially in areas of systematic uncertainty or contact zones where opportunities for hybridization and speciation are most likely.

Biogeography and Distributional Relationships with Close Congeners

New species can have important biogeographic implications, particularly when they occur within intricate species groups and complex geographic regions. In the case of *R. kauffeldi*, its discovery from the Northeast and mid-Atlantic US has direct consequences for three species across eight states (Fig. 1). Its range draws entirely from two cryptic congeners, *R. sphenocephala* and *R. pipiens*. Thus, the recognized distributions of both congeners will decrease correspondingly where *R. kauffeldi* occurs alone. These changes will refine certain ecological understandings and distributional patterns too. For example, contrary to a previously defined statewide distribution in NJ, *R. sphenocephala* is now exclusively restricted to xeric habitats such as the Pine Barrens. This constitutes a considerable departure from a previous range over a wide variety of habitats and geologies to a newly defined range that conforms to the coastal distributions of many southern herpetofaunal species.

Distributional relationships vary between *R. kauffeldi* and its close congeners. The general distributions of *R. kauffeldi* and its sister species *R. palustris* (as reported in Newman *et al*. [3]) overlap broadly [29,30], though we did not find them together in the field and noted different general habitat preferences that may keep the two species ecologically isolated. Conversely, the distribution of *R. kauffeldi* is generally parapatric with *R. sphenocephala* and *R. pipiens*, but examples of sympatry do exist with both species. Newman *et al.* [3] provided genetic evidence of sympatry without hybridization with *R. pipiens* in CT, and we viewed museum specimens noted by both Pace [26] and Klemens *et al.* [31] that suggest additional potential sympatry in northwestern NJ (*R. kauffeldi:* AMNH 35138; *R. pipiens:* AMNH 13114, 35139). We also identified areas of

sympatry between *R. kauffeldi* and *R. sphenocephala* in southeastern VA from bioacoustic evidence (North American Amphibian Monitoring Program), and suspect additional overlap in southern locales. Lastly, based on museum specimens from areas where leopard frogs are now extirpated, we note several isolated examples of possible *R. sphenocephala* from xeric eastern Long Island, NY, and *R. pipiens* from Queens and Bronx Counties, NY (Table S1). Historical species composition in these areas remains unclear, however. These sparse samples may reflect natural historical populations (and potential areas of overlap with *R. kauffeldi*) or possible human introductions; isolated geographic records can suggest captive releases [3,31], particularly in urban areas. Thus, we excluded both urban *R. pipiens* occurrences from Fig. 1.

Delineating Complicated Historical Ranges in Heavily Modified Landscapes

Determining the distribution of new species is essential to the process of identifying and interpreting their broader biogeographic implications. In the case of cryptic species, identifying regional compositions and reassigning museum specimens can be challenging but important, especially in heavily impacted landscapes with extirpations or species overlap. In our work, leopard frogs were simply unavailable across vast landscapes due to habitat loss and extirpations. Where individuals were available, differentiating similar-looking congeners was difficult. To overcome such challenges, several strategies can provide pathways forward, including 1) using genetic and bioacoustic methods at sites where new species and their cryptic congeners still occur to delineate species and study habitats, interactions, and hybridization; 2) using genetics and morphology to identify subtle physical differences, if any, between species; and 3)

applying these insights to museum specimens and extirpated locales to help assess historical compositions and distribution where populations no longer exist. These pathways (along with genetic examination of archival specimens when possible) can link genetic and bioacoustic tools with museum specimens and morphology and can also help inform future conservation strategies and range map development.

Management and Conservation

The addition of *R. kauffeldi* to the North American faunal record and species lists of at least eight US states will have implications at various regulatory and management levels. This will include possible threatened or endangered species considerations in certain areas, and may require further assessment of the status of *R. kauffeldi* and its cryptic congeners in some of these impacted areas. It may also provide further opportunity to investigate and verify species composition and boundaries throughout different parts of the range. This may be challenging, however, especially in states where leopard frogs (*sensu lato*) already receive legal protections and in areas where multiple species are found to co-occur. Thus, reliable, field-ready characters that distinguish similar taxa, and research on potential hybridization, are key priorities. We also leave open the possibility that *R. kauffeldi* may extend farther south.

The discovery of *R. kauffeldi* has several broad conservation implications. For one, it reaffirms that refined taxonomic information is essential for implementing proper conservation measures [2,3]. It also reinforces the critical role that basic natural history and alternative methods, such as bioacoustic techniques, can have in distinguishing potentially rare cryptic species. Lastly, it demonstrates that undocumented species can

still reside in some of the most urbanized and densely inhabited parts of the world; these areas can harbor significant biodiversity and, with proper management, simultaneously protect that diversity and provide valuable educational opportunities to urban communities. The United Nations Environment Programme and US Fish and Wildlife Service's Urban Wildlife Refuge Initiative have both focused recent efforts on protecting urban biodiversity and enhancing the value and scope of urban wildlife refuges. The discovery of *R. kauffeldi* adds another important observation to the growing consensus that we must protect sensitive species where they occur, not just in pristine environments. Findings such as this also provide invaluable opportunities to highlight and enhance access for increasingly urban societies to experience new species discoveries and taxa of high conservation concern firsthand.

The overall conservation status of *R. kauffeldi* awaits further definition of distribution and habitat use and should be considered data deficient in the IUCN classification system. On-the-ground assessments, coupled with genetic and bioacoustic data, will be critical to this and allow for more complete mapping of boundaries and overlap with related taxa. If the distribution is indeed narrow and fragmented (as reported here), it may pose some cause for concern as geographically restricted species are often at risk of extinction due to demographic stochasticity [81]. Several other conservation considerations warrant mention. First, survival prospects of *R. kauffeldi* populations in the NY/NJ-metro area vary from tenuous to stable, with the most vulnerable populations being those that are small and isolated and threatened by succeeding canopy closure and development. Second, dense breeding groups and strong metapopulation structure may be essential features of *R. kauffeldi* demography, but may also represent key vulnerabilities

in the face of habitat impacts. Rorabaugh [82] expressed similar concerns in noting metapopulation susceptibility, habitat impacts, and canopy closure as potential threats for *R. pipiens*. Lastly, on a broader scale, climatic events (e.g., rising sea levels, increased storm frequencies and intensities) have the ability to alter coastlines and threaten proximate low-lying freshwater wetlands and any amphibian populations therein with potentially harmful saline inundation.

Leopard frogs (*sensu lato*) have already vanished from some parts of North America [30] including several areas specifically within the northern range of *R. kauffeld* [10,11,13]. Some of these disappearances were likely caused by direct habitat loss or alteration, especially in urban landscapes [10,31]. Others, however, occurred enigmatically within less-developed coastal, suburban, and semi-rural areas (Fig. 1); this includes Long Island [3,13], the largest island in the continental US and a former leopard frog stronghold [10] where potential causes of extirpations (e.g., disease, invasive species, and contaminants) are being assessed [83] (J. A. Feinberg and J. Burger, unpublished data). Counterintuitively, *R. kauffeldi* persists in several locales within New York City (Staten Island) and the adjacent NJ Meadowlands. These sites are heavily industrialized and have endured severe long-term anthropogenic impacts and invasion by the common reed, *Phragmites australis*. Most offer large habitat areas, however, which may provide an important clue to survival. The surprising persistence of populations within these urban landscapes, while not completely understood, is encouraging and may have implications for management and restoration possibilities elsewhere, in the future.

We finish with a cautionary note regarding reintroductions, repatriations, and translocations. Moving species to restore extirpated populations is a common

conservation and management practice, but one that can have unintended risks and consequences. For example, had a leopard frog restoration been implemented on Long Island before the 2007 discovery of extant populations on nearby Staten Island (that were later found to be *R. kauffeldi*), the incorrect species (*R. sphenocephala*) would have been moved from known populations further to the south that harbor *R. sphenocephala*, not *R. kauffeldi*. Thus, careful consideration of systematics and population genetics at both donor and recipient site ends is critical to responsibly conducting any such endeavors.

Conclusions

In diagnosing, describing, and defining the Atlantic Coast leopard frog, *R. kauffeldi*, we add a new and potentially at-risk cryptic vertebrate species to the northeastern and mid-Atlantic US fauna. *Rana kauffeldi* can be characterized as 1) potentially vulnerable with highly specialized and restrictive habitat needs; 2) locally abundant where present, but often only occurring in isolated and scattered locales; 3) having a restricted distribution across heavily populated, urbanized regions; and 4) having suffered extirpations from certain areas. Concerns over habitat loss and degradation continue today, along with a suite of other threats (e.g., disease, contaminants) that may pose additional future challenges.

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Table 1. Mean morphological parameters for four species of *Rana*.

		R. kauffeldi	*R. sphenocephala*	*R. pipiens*	*R. palustris*
Variable	**Holotype**	**(*n* = 160)**	**(*n* = 46)**	**(*n* = 47)**	**(*n* = 30)**
SVL	50.03	57.16±9.81	57.92±10.03	58.24±9.76	51.73±7.80
range		20.34–85.07	42.47–84.1	42.25–83.23	31.53–66.24
HL	18.87	18.59±2.81	19.92±2.98	18.26±2.79	17.42±2.25
range		11.53–27.49	14.77–28.02	13.30–25.75	11.06–21.05
HW	15.73	18.87±3.40	18.09±3.25	18.66±3.11	17.41±2.46
range		9.95–26.60	12.65–25.83	13.75–25.75	10.69–22.23
ED	6.29	4.69±1.01	5.65±1.49	6.29±1.04	4.21±1.21
range		1.19–7.80	2.82–9.51	3.74–8.52	2.72–7.43
TD	4.18	4.81±0.91	4.68±0.84	4.43±0.85	3.92±0.55
range		1.77–7.15	3.15–6.54	3.00–6.92	2.65–5.00
FOL	43.52	48.35±8.12	49.73±7.96	50.65±7.51	44.28±5.75
range		17.79–65.35	36.57–69.84	38.67–66.82	28.97–56.62
END	3.81	3.98±0.66	4.74±0.97	4.38±0.64	4.00±0.63
range		2.25–5.97	3.40–7.44	3.27–6.08	2.62–5.30
NSD	3.19	3.78±0.78	4.02±0.84	4.59±0.91	3.52±0.52
range		1.20–6.31	2.69–7.04	3.11–7.11	2.55–4.74
THL	29.09	27.24±4.90	30.26±6.49	30.42±5.98	27.07±4.18
range		15.61–41.81	20.12–48.22	20.87–45.27	17.75–35.69
IND	3.53	3.95±0.80	3.75±0.74	4.29±0.80	3.79±0.77
range		1.18–6.05	2.15–5.60	2.87–6.11	2.71–5.38
IOD	3.55	4.19±0.84	3.68±0.72	3.40±0.68	3.63±0.76
range		1.88–6.72	2.57–5.24	2.26–4.67	2.41–5.32
SL	28.65	31.98±5.32	33.60±6.36	34.89±5.68	30.91±4.66
range		18.65–46.96	20.91–49.27	25.89–48.46	19.76–40.79
DSA	0.86	1.06±0.10	0.94±0.08	1.07±0.08	1.05±0.07
range		0.76–1.32	0.79–1.12	0.93–1.22	0.94–1.20

All measurements in mm, unless otherwise noted. Mean includes ± standard deviation (SD). Thirteen characters are listed as follows: snout-vent length (SVL), head length (HL), head width (HW), eye diameter (ED), tympanum diameter (TD), foot length (FOL), eye-to-naris distance (END), naris-to-snout distance (NSD), thigh length (THL), internarial distance (IND), interorbital distance (IOD), shank length (SL), and dorsal snout angle (DSA, radians). Nineteen frogs were omitted from FOL measurements (see Table S1). Note: the above values come from unadjusted (raw) data whereas size-corrected residual values were used in all other morphometric analyses.

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Table 2. Mean primary (advertisement) call parameters for five species of *Rana*.

	R. kauffeldi	*R. sphenocephala*	*R. pipiens*	*R. palustris*	*R. sylvatica*
Variable	**(*n* = 13)**	**(*n* = 8)**	**(*n* = 4)**	**(*n* = 11)**	**(*n* = 9)**
CL (ms)	55.81±10.86	534.45±158.69	1905.42±352.45	1429.90±237.24	205.89±86.27
range	33.25–71.25	364.50–796.00	1604.50–2409.33	1130.00–1825.00	85.25–330.25
CR (calls/s)	1.34±0.46	1.38±0.39	0.07±0.01	0.19±0.09	1.72±0.77
range	0.70–2.35	0.96–1.90	0.06–0.08	0.09–0.33	0.68–2.85
CRT (ms)	31.52±7.66	422.64±159.81	1299.65±223.73	856.40±218.27	169.85±80.75
range	18.00–47.25	212.33–636.00	1001.5–1519.67	595.33–1267.67	57.50–289.75
CDC	0.07±0.02	0.71±0.05	0.14±0.03	0.28±0.10	0.39±0.24
range	0.05–0.10	0.62–0.79	0.10–0.16	0.12–0.41	0.06–0.66
PN	1.00	7.85±1.05	38.83±7.76	61.15±9.10	2.51±0.67
range	1.00	6.25–9.50	29.50–48.33	47.50–78.67	1.50–3.33
PR (pulses/s)	0	13.57±3.53	19.79±1.92	42.52±5.41	7.79±1.17
range	0	9.77–17.82	17.75–22.38	30.26–47.96	6.19–9.23
DF (Hz)	1383.11±116.41	1214.86±226.09	1174.91±103.91	1264.43±251.86	1426.79±214.89
range	1211.23–1593.48	785.98–1476.58	1098.20–1327.90	947.50–1937.97	947.47–1679.60

Seven bioacoustic characters are listed as follows: call length (CL), call rate (CR), call rise time (CRT), call duty cycle (CDC), pulse number (PN), pulse rate (PR), and dominant frequency (DF). Mean includes ± standard deviation (SD). Note: the above values come from unadjusted (raw) data; in all other bioacoustic analyses CL and CR were corrected to a common temperature of 14°C, following Lemmon *et al.* [6].

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Table S1. List of *Rana* specimens examined. Fluid specimens were examined from the following museums: Yale Peabody Museum (YPM), American Museum of Natural History (AMNH), Academy of Natural Sciences of Drexel University (ANSP), Carnegie Museum of Natural History (CM), and Sam Noble Oklahoma Museum (OMNH). Analysis type codes are as follows: morphometric analysis (M), dorsal spot analyses (number of spots and percent coverage) (D), dorsal spot shape analysis (SH), snout spot analysis (SN), color analysis (C), and femur reticulum analysis (F). Two additional codes indicate 'examination-only', for femur reticulum (R) and extirpation zone composition (E); these were not used in any specific analysis. * = Foot length unavailable, not included in morphometric analysis. † = Photo voucher only, deposited at YPM. †† = Photo voucher with tissue, deposited at YPM. § = Single photo voucher of amplexed pair. §§ = Holotype.

Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. kauffeldi	YPM	13559		CT	Middlesex	M	*
R. kauffeldi	YPM	13560		CT	Middlesex	M	*
R. kauffeldi	YPM	13561		CT	Middlesex	M	*
R. kauffeldi	AMNH	35503	see Moore (1944) Pl. 62, #5	NJ	Bergen	D,M,SN	
R. kauffeldi	AMNH	35512	see Moore (1944) Pl. 62, #6	NJ	Bergen	D,M,SN	
R. kauffeldi	AMNH	84220		NJ	Bergen	F	
R. kauffeldi	AMNH	121324		NJ	Bergen	F	
R. kauffeldi	AMNH	121340		NJ	Bergen	F	
R. kauffeldi	AMNH	121341		NJ	Bergen	F	
R. kauffeldi	AMNH	1720		NJ	Bergen	M	
R. kauffeldi	AMNH	1721		NJ	Bergen	M	
R. kauffeldi	AMNH	12855		NJ	Bergen	M	
R. kauffeldi	AMNH	35504		NJ	Bergen	M	
R. kauffeldi	AMNH	35505		NJ	Bergen	M	
R. kauffeldi	AMNH	35509		NJ	Bergen	M	
R. kauffeldi	AMNH	35510		NJ	Bergen	M	
R. kauffeldi	AMNH	35511		NJ	Bergen	M	
R. kauffeldi	AMNH	52344		NJ	Bergen	M	
R. kauffeldi	AMNH	63848		NJ	Bergen	M	
R. kauffeldi	AMNH	63849		NJ	Bergen	M	
R. kauffeldi	AMNH	63850		NJ	Bergen	M	
R. kauffeldi	AMNH	67491		NJ	Bergen	M	
R. kauffeldi	AMNH	81337		NJ	Bergen	M	
R. kauffeldi	AMNH	81338		NJ	Bergen	M	
R. kauffeldi	AMNH	81339		NJ	Bergen	M	
R. kauffeldi	AMNH	81840		NJ	Bergen	M	
R. kauffeldi	AMNH	121124		NJ	Bergen	M	

Table S1.	**Continued**					
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type
R. kauffeldi	AMNH	121190		NJ	Essex	M
R. kauffeldi	AMNH	121191		NJ	Essex	M
R. kauffeldi	AMNH	121192		NJ	Essex	M
R. kauffeldi	AMNH	121193		NJ	Essex	M
R. kauffeldi	AMNH	121194		NJ	Essex	M
R. kauffeldi	AMNH	121195		NJ	Essex	M
R. kauffeldi	AMNH	121198		NJ	Essex	M
R. kauffeldi	AMNH	121199		NJ	Essex	M
R. kauffeldi	AMNH	121200		NJ	Essex	M
R. kauffeldi	AMNH	121203		NJ	Essex	M
R. kauffeldi	AMNH	121204		NJ	Essex	M
R. kauffeldi	AMNH	121205		NJ	Essex	M
R. kauffeldi	AMNH	121206		NJ	Essex	M
R. kauffeldi	AMNH	121208		NJ	Essex	M
R. kauffeldi	AMNH	121209		NJ	Essex	M
R. kauffeldi	AMNH	121210		NJ	Essex	M
R. kauffeldi	AMNH	121212		NJ	Essex	M
R. kauffeldi	AMNH	121213		NJ	Essex	M
R. kauffeldi	AMNH	121214		NJ	Essex	M
R. kauffeldi	AMNH	121215		NJ	Essex	M
R. kauffeldi	AMNH	121216		NJ	Essex	M
R. kauffeldi	AMNH	121217		NJ	Essex	M
R. kauffeldi	AMNH	121218		NJ	Essex	M
R. kauffeldi	AMNH	121219		NJ	Essex	M
R. kauffeldi	AMNH	121220		NJ	Essex	M
R. kauffeldi	AMNH	121221		NJ	Essex	M
R. kauffeldi	AMNH	121222		NJ	Essex	M
R. kauffeldi	AMNH	121223		NJ	Essex	M
R. kauffeldi	AMNH	121224		NJ	Essex	M
R. kauffeldi	AMNH	121225		NJ	Essex	M
R. kauffeldi	AMNH	121226		NJ	Essex	M
R. kauffeldi	AMNH	121227		NJ	Essex	M
R. kauffeldi	AMNH	121228		NJ	Essex	M

Table S1.	**Continued**					
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type
R. kauffeldi	AMNH	121229		NJ	Essex	M
R. kauffeldi	AMNH	121230		NJ	Essex	M
R. kauffeldi	AMNH	121231		NJ	Essex	M
R. kauffeldi	AMNH	121232		NJ	Essex	M
R. kauffeldi	AMNH	121233		NJ	Essex	M
R. kauffeldi	AMNH	121234		NJ	Essex	M
R. kauffeldi	AMNH	121235		NJ	Essex	M
R. kauffeldi	AMNH	121236		NJ	Essex	M
R. kauffeldi	AMNH	121237		NJ	Essex	M
R. kauffeldi	AMNH	121238		NJ	Essex	M
R. kauffeldi	AMNH	121240		NJ	Essex	M
R. kauffeldi	AMNH	121241		NJ	Essex	M
R. kauffeldi	AMNH	121243		NJ	Essex	M
R. kauffeldi	AMNH	121244		NJ	Essex	M
R. kauffeldi	AMNH	121245		NJ	Essex	M
R. kauffeldi	AMNH	51018		NJ	Morris	M
R. kauffeldi	AMNH	51019		NJ	Morris	M
R. kauffeldi	AMNH	51020		NJ	Morris	M
R. kauffeldi	AMNH	51021		NJ	Morris	M
R. kauffeldi	AMNH	51022		NJ	Morris	M
R. kauffeldi	AMNH	64499		NJ	Morris	M
R. kauffeldi	AMNH	79579		NJ	Morris	M
R. kauffeldi	AMNH	79580		NJ	Morris	M
R. kauffeldi	AMNH	79581		NJ	Morris	M
R. kauffeldi	AMNH	84181		NJ	Morris	M
R. kauffeldi	AMNH	121321		NJ	Morris	M
R. kauffeldi	AMNH	121322		NJ	Morris	M
R. kauffeldi	AMNH	121323		NJ	Morris	M
R. kauffeldi	AMNH	121325		NJ	Morris	M
R. kauffeldi	AMNH	121326		NJ	Morris	M
R. kauffeldi	AMNH	121327		NJ	Morris	M
R. kauffeldi	AMNH	121328		NJ	Morris	M
R. kauffeldi	AMNH	121329		NJ	Morris	M

Table S1.	**Continued**					
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type
R. kauffeldi	AMNH	121330		NJ	Morris	M
R. kauffeldi	AMNH	121331		NJ	Morris	M
R. kauffeldi	AMNH	121332		NJ	Morris	M
R. kauffeldi	AMNH	121333		NJ	Morris	M
R. kauffeldi	AMNH	121334		NJ	Morris	M
R. kauffeldi	AMNH	121335		NJ	Morris	M
R. kauffeldi	AMNH	121336		NJ	Morris	M
R. kauffeldi	AMNH	121337		NJ	Morris	M
R. kauffeldi	AMNH	121338		NJ	Morris	M
R. kauffeldi	AMNH	121339		NJ	Morris	M
R. kauffeldi	AMNH	121342		NJ	Morris	M
R. kauffeldi	AMNH	121343		NJ	Morris	M
R. kauffeldi	AMNH	121344		NJ	Morris	M
R. kauffeldi	AMNH	18730		NJ	Passaic	M
R. kauffeldi	AMNH	70296		NJ	Passaic	M
R. kauffeldi	AMNH	70366		NJ	Passaic	M
R. kauffeldi	AMNH	70367		NJ	Passaic	M
R. kauffeldi	AMNH	77415		NJ	Passaic	M
R. kauffeldi	AMNH	79391		NJ	Passaic	M
R. kauffeldi	AMNH	121103		NJ	Passaic	M
R. kauffeldi	AMNH	121104		NJ	Passaic	M
R. kauffeldi	AMNH	121105		NJ	Passaic	M
R. kauffeldi	AMNH	121106		NJ	Passaic	M
R. kauffeldi	AMNH	121107		NJ	Passaic	M
R. kauffeldi	AMNH	121113		NJ	Somerset	M
R. kauffeldi	AMNH	121114		NJ	Somerset	M
R. kauffeldi	AMNH	121116		NJ	Somerset	M
R. kauffeldi	AMNH	121117		NJ	Somerset	M
R. kauffeldi	AMNH	121118		NJ	Somerset	M
R. kauffeldi	AMNH	121119		NJ	Somerset	M
R. kauffeldi	AMNH	121120		NJ	Somerset	M
R. kauffeldi	AMNH	121121		NJ	Somerset	M
R. kauffeldi	AMNH	121316		NJ	Union	M

Table S1.	**Continued**					
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type
R. kauffeldi	AMNH	121317		NJ	Union	M
R. kauffeldi	AMNH	121318		NJ	Union	M
R. kauffeldi	AMNH	121319		NJ	Union	M
R. kauffeldi	AMNH	121320		NJ	Union	M
R. kauffeldi	AMNH	52342		NY	Bronx	E,M
R. kauffeldi	AMNH	106551		NY	Bronx	E,M
R. kauffeldi	AMNH	106552		NY	Bronx	E,M
R. kauffeldi	AMNH	106553		NY	Bronx	E,M
R. kauffeldi	AMNH	106554		NY	Bronx	E,M
R. kauffeldi	AMNH	41292	Long Island	NY	Nassau	E
R. kauffeldi	AMNH	21016		NY	Orange	M
R. kauffeldi	AMNH	103134		NY	Orange	M
R. kauffeldi	AMNH	103135		NY	Orange	M
R. kauffeldi	AMNH	164382		NY	Orange	M
R. kauffeldi	AMNH	14386	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14515	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14516	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14517	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14518	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14519	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14520	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14521	Long Island	NY	Queens	E
R. kauffeldi	AMNH	14522	Long Island	NY	Queens	E
R. kauffeldi	AMNH	38193	Long Island	NY	Queens	E
R. kauffeldi	AMNH	38194	Long Island	NY	Queens	E
R. kauffeldi	AMNH	38195	Long Island	NY	Queens	E
R. kauffeldi	AMNH	23030	see Moore (1944) Pl. 62, #4	NY	Richmond	D,M,SN
R. kauffeldi	AMNH	121857		NY	Richmond	F,M
R. kauffeldi	AMNH	121858		NY	Richmond	F,M
R. kauffeldi	AMNH	581		NY	Richmond	M
R. kauffeldi	AMNH	636		NY	Richmond	M
R. kauffeldi	AMNH	3542		NY	Richmond	M
R. kauffeldi	AMNH	3543		NY	Richmond	M

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. kauffeldi	AMNH	3699		NY	Richmond	M	
R. kauffeldi	AMNH	3700		NY	Richmond	M	
R. kauffeldi	AMNH	23029		NY	Richmond	M	
R. kauffeldi	AMNH	23031		NY	Richmond	M	
R. kauffeldi	AMNH	23032		NY	Richmond	M	
R. kauffeldi	AMNH	125959		NY	Richmond	M	
R. kauffeldi	AMNH	125960		NY	Richmond	M	
R. kauffeldi	AMNH	125961		NY	Richmond	M	
R. kauffeldi	AMNH	125962		NY	Richmond	M	
R. kauffeldi	YPM	13791		NY	Richmond	C	†
R. kauffeldi	YPM	13822		NY	Richmond	C	†
R. kauffeldi	YPM	13823		NY	Richmond	C	†
R. kauffeldi	YPM	13828		NY	Richmond	C	†
R. kauffeldi	YPM	13832		NY	Richmond	C	†
R. kauffeldi	YPM	13833		NY	Richmond	C	†
R. kauffeldi	YPM	13837		NY	Richmond	C	†
R. kauffeldi	YPM	13843		NY	Richmond	C	†
R. kauffeldi	YPM	13844		NY	Richmond	C	†
R. kauffeldi	YPM	13845		NY	Richmond	C	††
R. kauffeldi	YPM	14018		NY	Richmond	C	†
R. kauffeldi	YPM	13217		NY	Richmond	C,D,F,M,SH,SN	§§
R. kauffeldi	YPM	13788		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	13789		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	13847		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	13851	Top frog	NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	13853		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	13854		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	14020		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	14021		NY	Richmond	C,D,F,SH,SN	†
R. kauffeldi	YPM	14107		NY	Richmond	C,D,F,SH,SN	††
R. kauffeldi	YPM	13820		NY	Richmond	C,D,SH,SN	†
R. kauffeldi	YPM	13821		NY	Richmond	C,D,SH,SN	†
R. kauffeldi	YPM	14022		NY	Richmond	C,D,SH,SN	†

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. kauffeldi	YPM	14027		NY	Richmond	C,D,SH,SN	†
R. kauffeldi	YPM	14029		NY	Richmond	C,D,SH,SN	†
R. kauffeldi	YPM	14108		NY	Richmond	C,D,SH,SN	††
R. kauffeldi	YPM	13866		NY	Richmond	C,D,SN	†
R. kauffeldi	YPM	13862		NY	Richmond	C,F,SH,SN	†
R. kauffeldi	YPM	13863		NY	Richmond	C,F,SH,SN	†
R. kauffeldi	YPM	13865		NY	Richmond	C,F,SH,SN	†
R. kauffeldi	YPM	13920		NY	Richmond	C,F,SH,SN	
R. kauffeldi	YPM	13921		NY	Richmond	C,F,SH,SN	
R. kauffeldi	YPM	14024		NY	Richmond	C,F,SH,SN	†
R. kauffeldi	YPM	14025		NY	Richmond	C,F,SH,SN	†
R. kauffeldi	YPM	14111		NY	Richmond	C,F,SN	†
R. kauffeldi	YPM	13790		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	13827		NY	Richmond	C,SH,SN	††
R. kauffeldi	YPM	13836		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	13838		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	13840		NY	Richmond	C,SH,SN	††
R. kauffeldi	YPM	13842		NY	Richmond	C,SH,SN	††
R. kauffeldi	YPM	13848		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	13849		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	13864	Amplexed male	NY	Richmond	C,SH,SN	§
R. kauffeldi	YPM	13872		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14019		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14023		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14026		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14028		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14048		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14086		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	14110		NY	Richmond	C,SH,SN	†
R. kauffeldi	YPM	13768	Amplexed female	NY	Richmond	C,SN	§
R. kauffeldi	YPM	13768	Amplexed male	NY	Richmond	C,SN	§
R. kauffeldi	YPM	13792		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13793	Bottom frog	NY	Richmond	C,SN	†

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. kauffeldi	YPM	13824		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13825		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13826		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13829		NY	Richmond	C,SN	††
R. kauffeldi	YPM	13830		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13831		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13834		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13835		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13841		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13864	Amplexed female	NY	Richmond	C,SN	§
R. kauffeldi	YPM	13867		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13868		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13869		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13870		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13871		NY	Richmond	C,SN	†
R. kauffeldi	YPM	14017		NY	Richmond	C,SN	†
R. kauffeldi	YPM	14032		NY	Richmond	C,SN	†
R. kauffeldi	YPM	14033		NY	Richmond	C,SN	†
R. kauffeldi	YPM	13850		NY	Richmond	D,F,SH,SN	†
R. kauffeldi	YPM	13767		NY	Richmond	F	†
R. kauffeldi	YPM	14112		NY	Richmond	F,SH,SN	
R. kauffeldi	AMNH	5369	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	18674	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	121856	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	125957	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	125958	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	130217	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	130218	Long Island	NY	Suffolk	E	
R. kauffeldi	AMNH	132936	Long Island	NY	Suffolk	E	
R. kauffeldi	Overton (1941a)	Figure 34	Long Island	NY	Suffolk	E	
R. kauffeldi	Overton (1941a)	Figure 38	Long Island	NY	Suffolk	E	
R. kauffeldi	Overton (1941b)	Page 34 (right)	Long Island	NY	Suffolk	E	
R. kauffeldi	ANSP	17675		PA	Bucks	M	

Table S1.	**Continued**					
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type
R. kauffeldi	ANSP	32684		PA	Bucks	M
R. kauffeldi	ANSP	2787		PA	Philadelphia	M
R. kauffeldi	ANSP	2788		PA	Philadelphia	M
R. kauffeldi	ANSP	2804		PA	Philadelphia	M
R. kauffeldi	ANSP	2879		PA	Philadelphia	M
R. kauffeldi	ANSP	2892		PA	Philadelphia	M
R. kauffeldi	ANSP	19222		PA	Philadelphia	M
R. kauffeldi	Porter (1941)	Figure 1	Right photo	PA	Philadelphia	D,SN
R. palustris	YPM	6103		CT	Fairfield	M
R. palustris	YPM	7142		CT	Fairfield	M
R. palustris	YPM	10631		CT	Fairfield	M
R. palustris	YPM	10303		CT	Hartford	M
R. palustris	YPM	10304		CT	Hartford	M
R. palustris	YPM	10307		CT	Hartford	M
R. palustris	YPM	10308		CT	Hartford	M
R. palustris	YPM	11757		CT	Hartford	M
R. palustris	YPM	9018		CT	Litchfield	M
R. palustris	YPM	9110		CT	Litchfield	M
R. palustris	YPM	1070		CT	New Haven	M
R. palustris	YPM	1074		CT	New Haven	M
R. palustris	YPM	2974		CT	New Haven	M
R. palustris	YPM	3067		CT	New Haven	M
R. palustris	YPM	3129		CT	New Haven	M
R. palustris	YPM	3133		CT	New Haven	M
R. palustris	YPM	3134		CT	New Haven	M
R. palustris	YPM	3190		CT	New Haven	M
R. palustris	YPM	4907		CT	New Haven	M
R. palustris	YPM	4908		CT	New Haven	M
R. palustris	YPM	4909		CT	New Haven	M
R. palustris	YPM	10225		CT	New Haven	M
R. palustris	YPM	10684		CT	New Haven	M
R. palustris	YPM	6673		CT	New London	M
R. palustris	YPM	6647		CT	Tolland	M

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. palustris	YPM	10266		CT	Tolland	M	
R. palustris	YPM	12681		ME	Franklin	M	
R. palustris	YPM	6357		ME	Hancock	M	
R. palustris	YPM	12520		ME	Somerset	M	
R. palustris	YPM	12680		ME	Somerset	M	
R. pipiens	YPM	9315		CT	Litchfield	F,M	
R. pipiens	YPM	13052		CT	Litchfield	F,M	
R. pipiens	YPM	13564		CT	Litchfield	F,M	*
R. pipiens	YPM	13565		CT	Litchfield	F,M	*
R. pipiens	YPM	13566		CT	Litchfield	F,M	*
R. pipiens	YPM	13567		CT	Litchfield	F,M	*
R. pipiens	YPM	13568		CT	Litchfield	F,M	*
R. pipiens	YPM	13569		CT	Litchfield	F,M	
R. pipiens	YPM	13570		CT	Litchfield	F,M	*
R. pipiens	YPM	13571		CT	Litchfield	F,M	*
R. pipiens	YPM	13572		CT	Litchfield	F,M	*
R. pipiens	YPM	13573		CT	Litchfield	F,M	*
R. pipiens	YPM	13574		CT	Litchfield	F,M	*
R. pipiens	YPM	13575		CT	Litchfield	F,M	*
R. pipiens	YPM	13576		CT	Litchfield	F,M	*
R. pipiens	YPM	13577		CT	Litchfield	F,M	*
R. pipiens	YPM	13578		CT	Litchfield	F,M	*
R. pipiens	YPM	13612		CT	Litchfield	F,M	
R. pipiens	YPM	13562		CT	Middlesex	F,M	*
R. pipiens	YPM	13563		CT	Middlesex	M	*
R. pipiens	YPM	1022		CT	New Haven	E,F,M	
R. pipiens	YPM	1077		CT	New Haven	E,F,M	
R. pipiens	YPM	1143		CT	New Haven	E,F,M	
R. pipiens	YPM	2906		CT	New Haven	E,F,M	
R. pipiens	YPM	2907		CT	New Haven	E,F,M	
R. pipiens	YPM	2908		CT	New Haven	E,F,M	
R. pipiens	YPM	2909		CT	New Haven	E,F,M	
R. pipiens	YPM	3068		CT	New Haven	E,F,M	

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. pipiens	YPM	3069		CT	New Haven	E,F,M	
R. pipiens	AMNH	106549		NY	Bronx	E	
R. pipiens	AMNH	106550		NY	Bronx	E	
R. pipiens	AMNH	36651	Long Island	NY	Queens	E	
R. pipiens	YPM	1045		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	1048		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2965		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2966		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2967		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2968		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2969		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2970		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2971		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2972		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2973		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2976		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2977		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2978		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	2979		Quebec	Gaspe Peninsula	F,M	
R. pipiens	YPM	5808		RI	Aquidneck Island	F,M	
R. pipiens	YPM	5809		RI	Aquidneck Island	F,M	
R. pipiens	YPM	5810		RI	Aquidneck Island	F,M	
R. sphenocephala	YPM	10485		FL	Baker	M,R	
R. sphenocephala	YPM	1029		FL	Miami-Dade	M	
R. sphenocephala	YPM	2930		FL	Miami-Dade	R	
R. sphenocephala	YPM	4809		FL	Miami-Dade	R	
R. sphenocephala	YPM	3082		FL	Monroe	M,R	
R. sphenocephala	YPM	1023		FL	Palm Beach	M,R	
R. sphenocephala	YPM	7320		FL	Palm Beach	M,R	
R. sphenocephala	YPM	7321		FL	Palm Beach	M,R	
R. sphenocephala	YPM	13805		NC	Dare	C	†
R. sphenocephala	YPM	13803		NC	Dare	C,SN	†
R. sphenocephala	YPM	13804		NC	Dare	C,SN	†

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. sphenocephala	ANSP	3969		NJ	Atlantic	M	
R. sphenocephala	AMNH	121144		NJ	Burlington	F	
R. sphenocephala	AMNH	121145		NJ	Burlington	F	
R. sphenocephala	AMNH	121148		NJ	Burlington	F	
R. sphenocephala	AMNH	121149		NJ	Burlington	F	
R. sphenocephala	AMNH	121151		NJ	Burlington	F	
R. sphenocephala	AMNH	121153		NJ	Burlington	F	
R. sphenocephala	AMNH	121154		NJ	Burlington	F	
R. sphenocephala	AMNH	121155		NJ	Burlington	F	
R. sphenocephala	AMNH	121156		NJ	Burlington	F	
R. sphenocephala	ANSP	14939		NJ	Burlington	M	
R. sphenocephala	ANSP	14947		NJ	Burlington	M	
R. sphenocephala	ANSP	27110		NJ	Burlington	M	
R. sphenocephala	ANSP	28819		NJ	Burlington	M	
R. sphenocephala	ANSP	34478		NJ	Burlington	M	
R. sphenocephala	ANSP	36804		NJ	Burlington	M	
R. sphenocephala	CM	26238		NJ	Burlington	M	
R. sphenocephala	CM	26242		NJ	Burlington	M	
R. sphenocephala	CM	26243		NJ	Burlington	M	
R. sphenocephala	CM	26244		NJ	Burlington	M	
R. sphenocephala	CM	62005		NJ	Burlington	M	
R. sphenocephala	CM	140094		NJ	Burlington	M	
R. sphenocephala	OMNH	32414		NJ	Burlington	M	
R. sphenocephala	OMNH	32415		NJ	Burlington	M	
R. sphenocephala	OMNH	32416		NJ	Burlington	M	
R. sphenocephala	OMNH	32417		NJ	Burlington	M	
R. sphenocephala	OMNH	32418		NJ	Burlington	M	
R. sphenocephala	OMNH	32419		NJ	Burlington	M	
R. sphenocephala	OMNH	32420		NJ	Burlington	M	
R. sphenocephala	OMNH	32421		NJ	Burlington	M	
R. sphenocephala	OMNH	32422		NJ	Burlington	M	
R. sphenocephala	OMNH	32423		NJ	Burlington	M	
R. sphenocephala	YPM	13799		NJ	Burlington	C	†

Table S1.	Continued						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. sphenocephala	YPM	13856		NJ	Burlington	C	†
R. sphenocephala	YPM	14061		NJ	Burlington	C	†
R. sphenocephala	YPM	14076		NJ	Burlington	C	†
R. sphenocephala	YPM	14080		NJ	Burlington	C	†
R. sphenocephala	YPM	14082		NJ	Burlington	C	†
R. sphenocephala	YPM	14083		NJ	Burlington	C	†
R. sphenocephala	YPM	14072		NJ	Burlington	C,D,F,SH	†
R. sphenocephala	YPM	14085		NJ	Burlington	C,D,F,SH	†
R. sphenocephala	YPM	14035		NJ	Burlington	C,D,F,SH,SN	
R. sphenocephala	YPM	14039		NJ	Burlington	C,D,F,SH,SN	
R. sphenocephala	YPM	14097		NJ	Burlington	C,D,F,SH,SN	†
R. sphenocephala	YPM	13771		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	13794		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14058		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14059		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14064		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14069		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14070		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14084		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14089		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14096		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14102		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	14103		NJ	Burlington	C,D,SH,SN	†
R. sphenocephala	YPM	13769		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	13796		NJ	Burlington	C,F,SH,SN	†
R. sphenocephala	YPM	14034		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14036		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14037		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14038		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14040		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14041		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14042		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14043		NJ	Burlington	C,F,SH,SN	

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. sphenocephala	YPM	14044		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14045		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14046		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14047		NJ	Burlington	C,F,SH,SN	
R. sphenocephala	YPM	14088		NJ	Burlington	C,F,SH,SN	†
R. sphenocephala	YPM	14105		NJ	Burlington	C,F,SH,SN	†
R. sphenocephala	YPM	14063		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	14065		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	14068		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	14073		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	14077		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	14079		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	14106		NJ	Burlington	C,SH	†
R. sphenocephala	YPM	13772		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13795		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13797		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13800		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13857		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13859		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13860		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13861		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14053		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14054		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14055		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14056		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14060		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14066		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14067		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14071		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14081		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14090		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14092		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	14093		NJ	Burlington	C,SH,SN	†

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. sphenocephala	YPM	14100		NJ	Burlington	C,SH,SN	†
R. sphenocephala	YPM	13802		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	13858		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14049		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14057		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14062		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14074		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14075		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14078		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14087		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14091		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14094		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14095		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14098		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14099		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14101		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	14104		NJ	Burlington	C,SN	†
R. sphenocephala	YPM	13770		NJ	Burlington	SH,SN	††
R. sphenocephala	ANSP	19286		NJ	Camden	M	
R. sphenocephala	ANSP	15852		NJ	Cape May	M	
R. sphenocephala	ANSP	17606		NJ	Cape May	M	
R. sphenocephala	ANSP	17607		NJ	Cape May	M	
R. sphenocephala	ANSP	17608		NJ	Cape May	M	
R. sphenocephala	ANSP	17609		NJ	Cape May	M	
R. sphenocephala	ANSP	17610		NJ	Cape May	M	
R. sphenocephala	AMNH	116842		NJ	Middlesex	F	
R. sphenocephala	YPM	14030		NJ	Middlesex	C,F,SH,SN	†
R. sphenocephala	YPM	14031		NJ	Middlesex	C,SN	†
R. sphenocephala	ANSP	16394		NJ	Ocean	M	
R. sphenocephala	ANSP	16395		NJ	Ocean	M	
R. sphenocephala	CM	11593		NJ	Ocean	M	
R. sphenocephala	CM	11594		NJ	Ocean	M	
R. sphenocephala	CM	11595		NJ	Ocean	M	

Table S1.	**Continued**						
Taxon	Museum	Specimen No.	Notes	State/Province	County/Region	Analysis Type	
R. sphenocephala	CM	11596		NJ	Ocean	M	
R. sphenocephala	CM	28578		NJ	Ocean	M	
R. sphenocephala	CM	28579		NJ	Ocean	M	
R. sphenocephala	CM	39717		NJ	Ocean	M	
R. sphenocephala	OMNH	30537		NJ	Ocean	M	
R. sphenocephala	YPM	13801		NJ	Ocean	C,D,F,SH,SN	†
R. sphenocephala	YPM	14051		NJ	Ocean	C,F	†
R. sphenocephala	YPM	13798		NJ	Ocean	C,F,SH,SN	†
R. sphenocephala	YPM	14050		NJ	Ocean	C,SH,SN	†
R. sphenocephala	YPM	14052		NJ	Ocean	C,SH,SN	†
R. sphenocephala (tentative)	AMNH	125956	Long Island; further examination needed	NY	Suffolk	E	
R. sphenocephala (tentative)	AMNH	176153	Long Island; further examination needed	NY	Suffolk	E	

Table S2. List of *Rana* primary (advertisement) calls measured for bioacoustic data. All recordings are deposited at Yale Peabody Museum (YPM). Localities are listed by state, county, and city (or region, when more specific). Additional locality information is available from authors. Frog number 4 was omitted from analysis. Track time is provided for recordings with multiple analyzed frogs to differentiate individuals using the start time of their respective call series. * = Recorded with Olympus LS-1X series linear PCM recorder (at sample rate of 99.6 kHz and sample size of 24-bits). Each frog contributed call-attributes based on average values derived from four successive calls unless otherwise noted († = three successive calls, § = two successive calls). Frogs 15-18 recorded by E. Kiviat, frogs 23-26 by BRC, and frog 27 by S. Amburgey. All other frogs recorded by JAF.

Taxon	Frog No.	YPM Catalog No.	State	County	City or Region	Population	Water Temp. (°C)	Track Time (s)	
R. kauffeldi	1	14137, 14172	NY	Richmond	Bloomfield	1	10.0		
R. kauffeldi	2	14138	NY	Richmond	Bloomfield	1	10.0		
R. kauffeldi	3	14139	NY	Richmond	Bloomfield	1	10.0		
R. kauffeldi	5	14140	NY	Richmond	Bloomfield	1	10.0	7.35	
R. kauffeldi	6	14140	NY	Richmond	Bloomfield	1	10.0	7.73	
R. kauffeldi	7	14141	NY	Richmond	Bloomfield	1	11.9	7.14	
R. kauffeldi	8	14141	NY	Richmond	Bloomfield	1	11.9	16.31	
R. kauffeldi	9	14142	NY	Richmond	Bloomfield	1	11.9		
R. kauffeldi	10	14143	NJ	Bergen	Moonachie	2	17.2	7.84	
R. kauffeldi	11	14143	NJ	Bergen	Moonachie	2	17.2	23.36	
R. kauffeldi	12	14144	NJ	Bergen	Moonachie	2	17.2		
R. kauffeldi	13	14145	NY	Richmond	Bloomfield	3	13.0	0.31	
R. kauffeldi	14	14145	NY	Richmond	Bloomfield	3	13.0	10.30	
R. pipiens	15	14163	NY	Columbia	Hudson	1	18.0	8.37	*
R. pipiens	16	14163	NY	Columbia	Hudson	1	18.0	6.20	*†
R. pipiens	17	14163	NY	Columbia	Hudson	1	18.0	16.06	*
R. pipiens	18	14163	NY	Columbia	Hudson	1	18.0	53.44	*†
R. sphenocephala	19	14146	NJ	Middlesex	South Brunswick	1	11.0	9.31	
R. sphenocephala	20	14147	NJ	Middlesex	South Brunswick	1	11.0		
R. sphenocephala	21	14146	NJ	Middlesex	South Brunswick	1	11.0	4.03	
R. sphenocephala	22	14148	NJ	Middlesex	South Brunswick	1	11.0		
R. sphenocephala	23	14149	NJ	Burlington	New Gretna	2	25.6	32.79	*
R. sphenocephala	24	14150	NJ	Burlington	New Gretna	2	25.6	5.08	*
R. sphenocephala	25	14149	NJ	Burlington	New Gretna	2	25.6	2.27	*
R. sphenocephala	26	14150	NJ	Burlington	New Gretna	2	25.6	13.26	*†
R. sylvatica	27	14151	CO	Larimer	Cameron Pass	1	11.0		
R. sylvatica	28	14152	NY	Suffolk	Upton	2	10.1	13.90	

Table S2. Continued

Taxon	Frog No.	YPM Catalog No.	State	County	City or Region	Population	Water Temp. (°C)	Track Time (s)	
R. sylvatica	29	14152	NY	Suffolk	Upton	2	10.1	7.27	
R. sylvatica	30	14152	NY	Suffolk	Upton	2	10.1	4.53	†
R. sylvatica	31	14152	NY	Suffolk	Upton	2	10.1	18.35	
R. sylvatica	32	14152	NY	Suffolk	Upton	2	10.1	35.08	
R. sylvatica	33	14153	NY	Queens	Alley Park	3	8.0		
R. sylvatica	34	14154	NY	Queens	Alley Park	3	8.8	26.15	
R. sylvatica	35	14154	NY	Queens	Alley Park	3	8.8	2.83	
R. palustris	36	14155	NY	Suffolk	Calverton	1	15.0		§
R. palustris	37	14156	NY	Suffolk	Calverton	1	15.0		§
R. palustris	38	14157	NY	Suffolk	Calverton	1	15.0		
R. palustris	39	14158	NY	Suffolk	Calverton	1	15.0		
R. palustris	40	14159	NY	Suffolk	Calverton	1	15.0	2.42	†
R. palustris	41	14159	NY	Suffolk	Calverton	1	15.0	5.22	
R. palustris	42	14160	NY	Suffolk	Calverton	1	15.0		†
R. palustris	43	14161	NY	Suffolk	Calverton	1	15.0	1.13	†
R. palustris	44	14161	NY	Suffolk	Calverton	1	15.0	2.24	†
R. palustris	45	14162	NY	Suffolk	Calverton	1	15.0	11.86	†
R. palustris	46	14162	NY	Suffolk	Calverton	1	15.0	4.23	†

Table S3. Classification matrix for four *Rana* species using discriminant function analysis on morphometric variables.

	Pairwise			
Original	*kauffeldi*	*sphenocephala*	*pipiens*	*palustris*
kauffeldi	143	6	1	7
sphenocephala	15	31	0	0
pipiens	3	1	24	3
palustris	16	0	6	8

Table S4. Coefficients for three discriminant functions (from four species of Rana) for each of 12 morphological characters: head length (HL), head width (HW), eye diameter (ED), tympanum diameter (TD), foot length (FOL), eye-to-naris distance (END), naris-to-snout distance (NSD), thigh length (THL), internarial distance (IND), interorbital distance (IOD), shank length (SL), and dorsal snout angle (DSA).

	LD1	LD2	LD3
HL	-0.464	-1.098	-1.293
HW	-0.007	1.018	0.859
ED	0.512	-0.119	0.739
TD	-1.001	-0.315	0.65
FOL	-0.038	-0.051	0.103
END	0.035	-1.139	-0.123
NSD	0.528	0.191	0.597
THL	0.034	-0.028	-0.133
IND	-0.052	0.682	-0.441
IOD	-0.546	0.017	0.215
SL	0.375	0.238	-0.212
DSA	-2.02	-8.427	-17.234

Table S5. Classification matrix for five *Rana* species using discriminant function analysis on bioacoustic variables.

	Pairwise				
Original	*kauffeldi*	*sphenocephala*	*pipiens*	*palustris*	*sylvatica*
kauffeldi	13	0	0	0	0
sphenocephala	0	8	0	0	0
pipiens	0	0	4	0	0
palustris	0	0	0	11	0
sylvatica	2	0	0	0	7

Table S6. Coefficients for four discriminant functions (from five species of *Rana*) for each of six bioacoustic characters: call length (CL), call rate (CR), call rise time (CRT), call duty cycle (CDC), pulse number (PN), and dominant frequency (DF).

	LD1	LD2	LD3	LD4
CL	1.808	7.698	2.16	-1.97
CR	-0.605	-0.779	1.187	-1.039
CRT	-2.806	-1.751	0.964	4.409
CDC	1.263	4.855	-8.933	-3.346
PN	0.185	-0.199	-0.023	-0.057
DF	0	-0.003	0.001	-0.001

Figure Legend

Figure 1. Leopard frog distributions in the Northeast and mid-Atlantic US. Left: currently recognized IUCN (2012) range maps for *R. pipiens* (green) and *R. sphenocephala* (red) with areas of potential overlap (hatched). Right: newly interpreted distributions for all three leopard frog species including *R. kauffeldi*. Symbols indicate known *R. kauffeldi* populations and purple shading depicts areas where our field work has confirmed the occurrence of *R. kauffeldi*. Yellow shading indicates areas of less intensive examination and sampling; *R. kauffeldi* may occur in these areas based on habitat and proximity to known populations. Potential sympatry is also possible in the yellow shaded areas, with *R. sphenocephala* (from Long Island southward), or *R. pipiens* (north and west of Long Island). The type locality for *R. kauffeldi* is indicated by an arrow. doi:10.1371/journal.pone.0108213.g001.

Figure 2. Photographs of *Rana kauffeldi* sp. nov. holotype (YPM 13217). Male frog presented live: (a) whole body, dorsolateral view and (b) dorsal view; and preserved: (c) dorsal view and (d) ventral view. Photographs taken by BRC (a), BZ (b), and GWC (c–d). doi:10.1371/journal.pone.0108213.g002.

Figure 3. Primary (advertisement) calls of five *Rana* species from the study region. Species include *R. kauffeldi* (column 1), *R. sphenocephala* (column 2), *R. pipiens* (column 3), *R. palustris* (column 4), and *R. sylvatica* (column 5). Depicted individuals were recorded within 8 °C of each other at 10.0, 11.0, 18.0, 15.0, and 10.1 °C, respectively. Row 1 shows waveforms of primary call sequences (12 s scale) (note: *R. pipiens* contains secondary grunts). Rows 2 and 3 show single-call waveforms and spectrograms, respectively (750 ms scale). Row 4 shows power spectra for each single call. Numbers assigned to waveforms in row 1 indicate and identify different individuals. Format adapted from Lemmon et al. [6]. doi:10.1371/journal.pone.0108213.g003.

Figure 4. Reticulum shading patterns. Examples include (a) dark state, *Rana kauffeldi* (YPM 14143); (b) light state, *R. sphenocephala* (YPM 14097); (c) *R. kauffeldi* yellow variant (YPM 13767); (d) *R. kauffeldi* green variant (YPM 14025). Photographs taken by E. Kiviat (a), M. Cram (b), and BRC (c, d). doi:10.1371/journal.pone.0108213.g004.

Figure S1. Box and whisker plots comparing the size-corrected residuals of 12 morphological characters among four *Rana* species. Species include *R. kauffeldi* (kauf), *R. palustris* (palu), *R. pipiens* (pipi), and *R. sphenocephala* (sphe). For whisker plots, black bars = median, boxes = 25^{th}–75^{th} quartiles, whiskers = minimum and maximum values but exclude outliers (represented by open circles). For each character, species whose measurements differed significantly ($P<0.05$) in a one-way ANOVA are denoted with different letters atop the plot. Side notches in boxes indicate significantly different medians.

Figure S2. Discriminant function analyses (DFA). Left: DFA using 12 size-corrected morphological characters measured from 264 frogs examined across four *Rana* species. Right: DFA using six bioacoustic characters measured from 45 frogs examined across five *Rana* species. Species include *R. kauffeldi* (circles), *R. sphenocephala* (triangles), *R. pipiens* (plus signs), *R. palustris* (x-crosses), and *R. sylvatica* (red squares). Morphological characters include all variables from Figure S1. Bioacoustic characters include all variables from Figure S4, except pulse rate. Black symbols twice as large in the morphological DFA represent group centroids.

Figure S3. Box and whisker plots comparing spot features between *Rana kauffeldi* (kauf) and *R. sphenocephala* (sphe). Left: total number of dorsal spots. Right: proportion of dorsal surface covered by spots. For whisker plots, black bars = median, boxes = 25^{th}–75^{th} quartiles, whiskers = minimum and maximum values but exclude outliers (represented by open circles). Side notches in boxes indicate significantly different medians.

Figure S4 Box and whisker plots comparing seven bioacoustic characters among five *Rana* species. Species include *R. kauffeldi* (kauf), *R. palustris* (palu), *R. pipiens* (pipi), *R. sphenocephala* (sphe), and *R. sylvatica* (sylv). For whisker plots, black bars = median, boxes = 25^{th}–75^{th} quartiles, whiskers = minimum and maximum values but exclude outliers (represented by open circles). For each character, species whose measurements differed significantly ($P<0.05$) in a one-way ANOVA are denoted with different letters atop the plot. Call length and call rate were temperature-corrected.

Figure 1

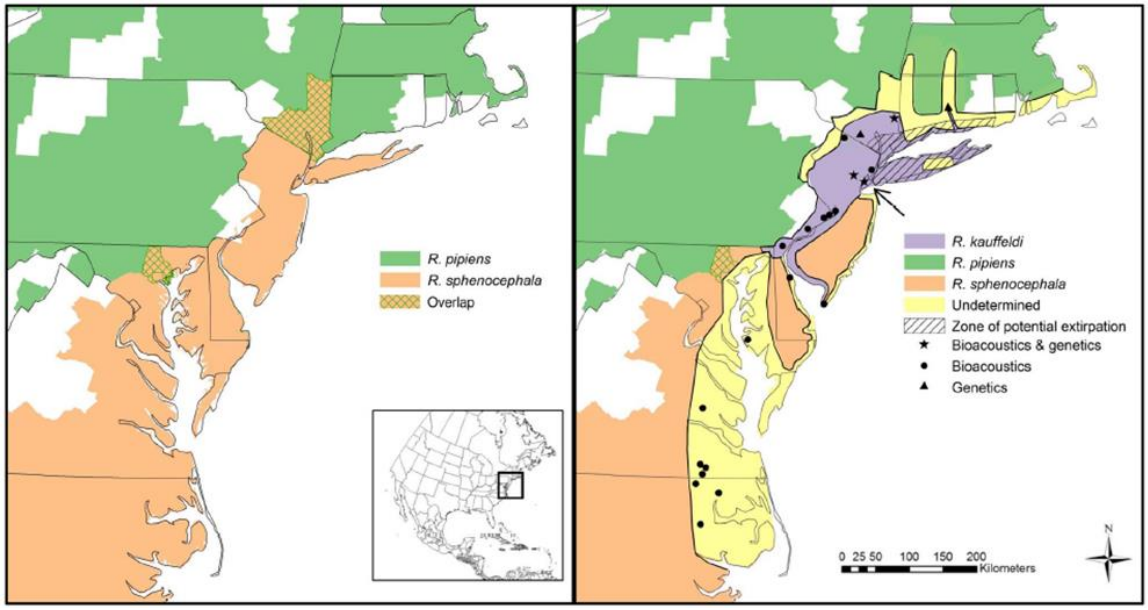

R. pipiens
R. sphenocephala
Overlap
R. kauffeldi
R. pipiens
R. sphenocephala
Undetermined
Zone of potential extirpation
Bioacoustics & genetics
Bioacoustics
Genetics
0 25 50 100 150 200
Kilometers
N

Figure 2

Figure 3

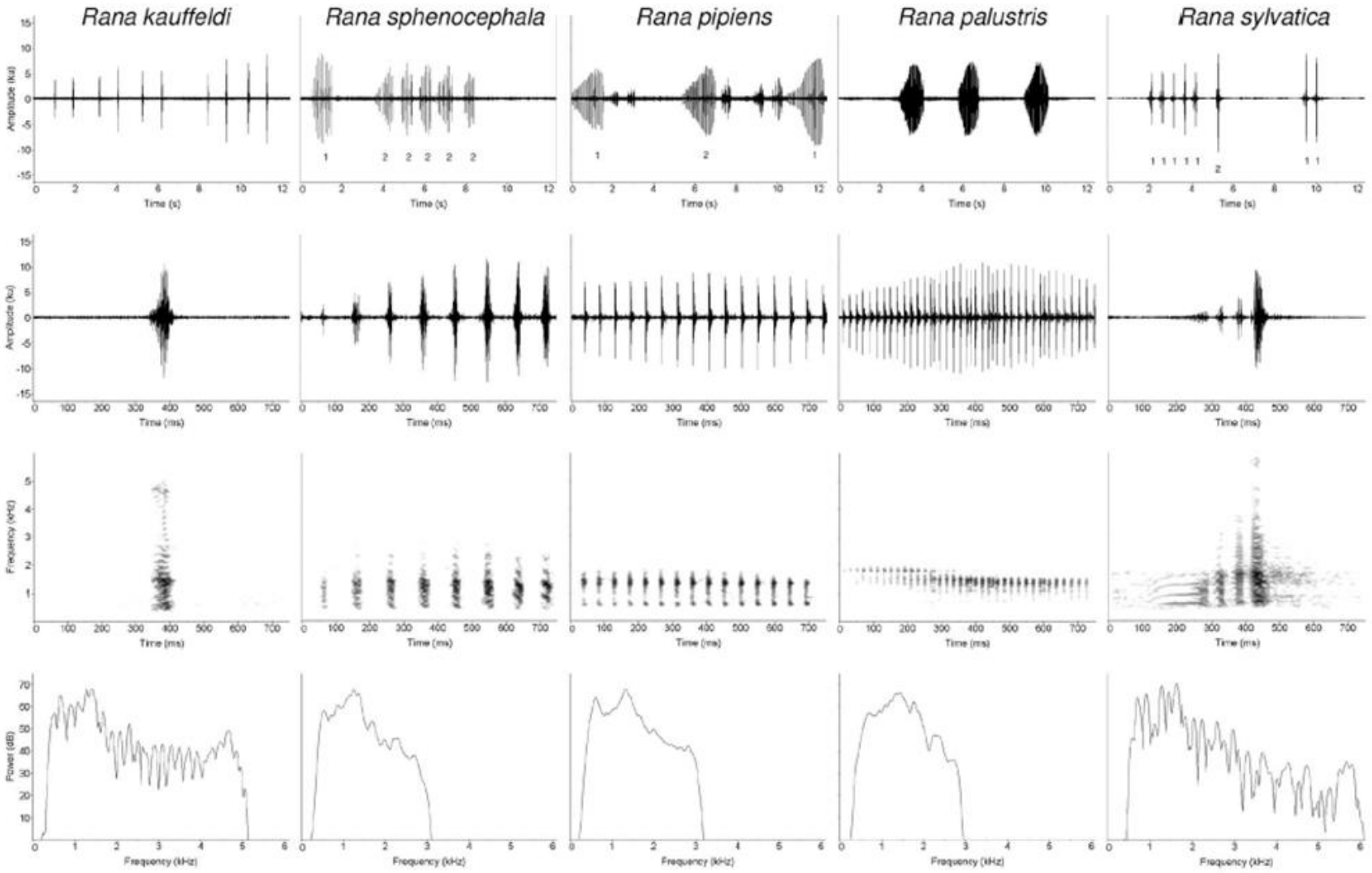

Rana kauffeldi
Rana sphenocephala
Rana pipiens
Rana palustris
Rana sylvatica
Amplitude (kU)
Time (s)
Time (ms)
Frequency (kHz)
Power (dB)
Frequency (kHz)

Figure 4

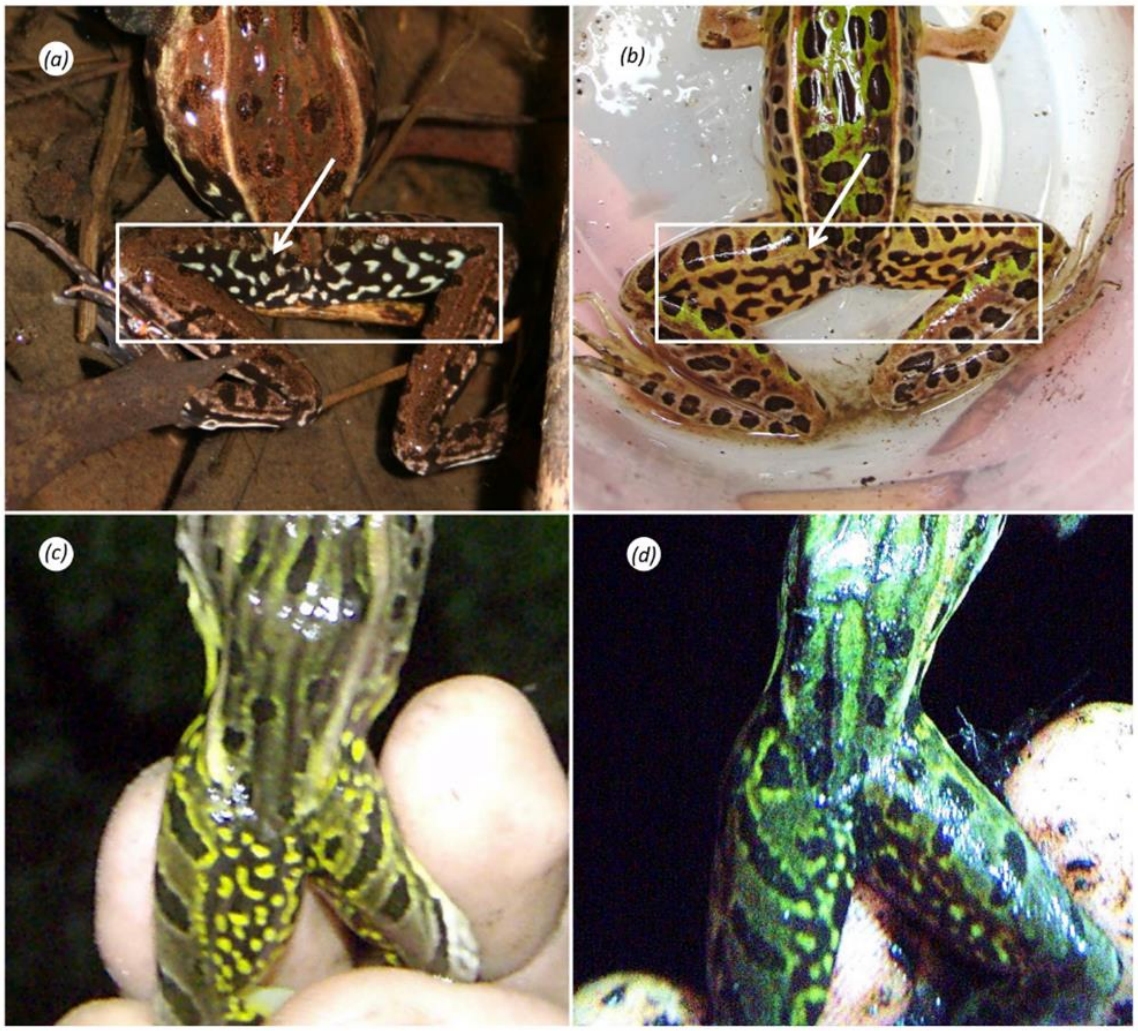


Figure S1

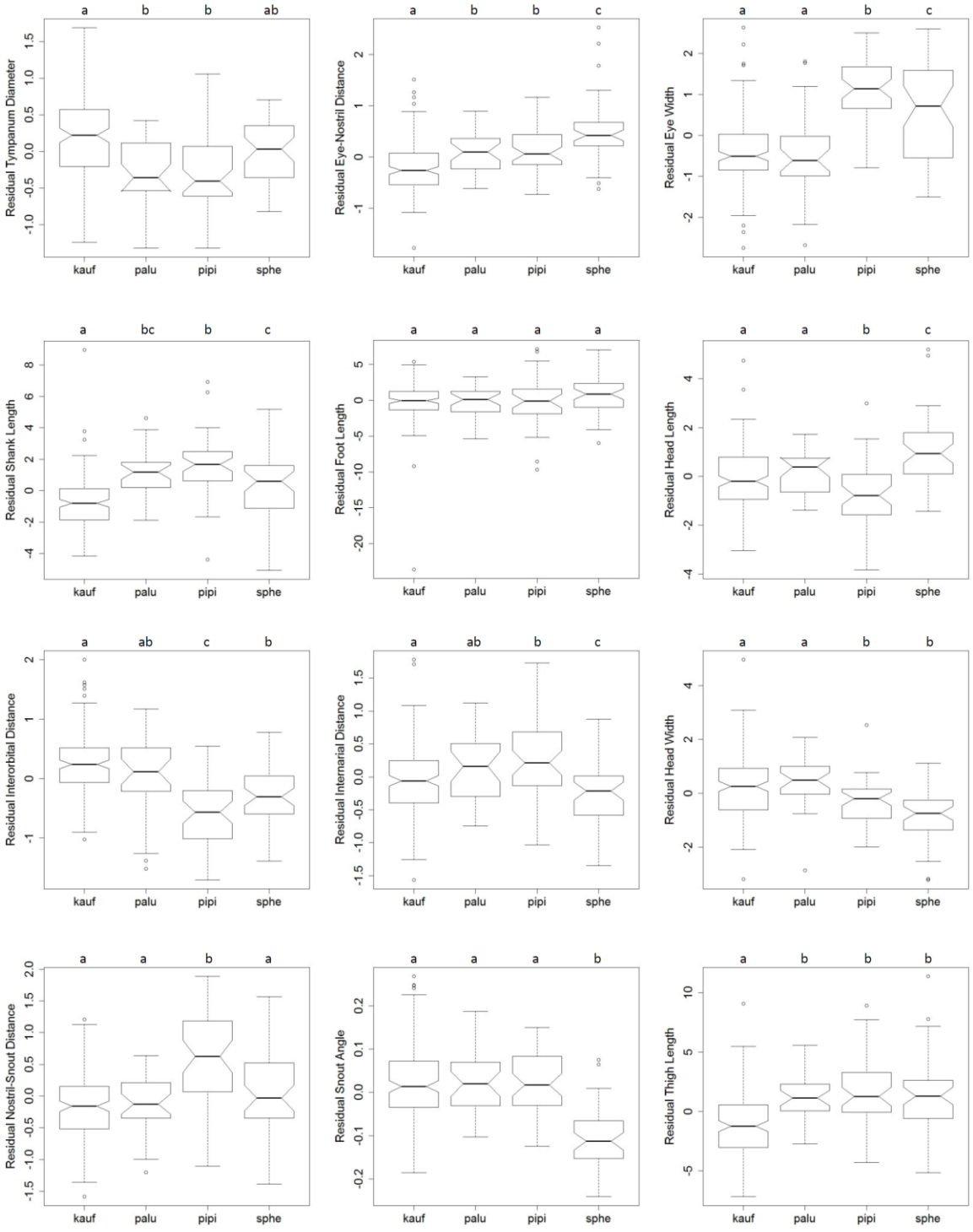

a b b ab
Residual Tympanum Diameter
kauf palu pipi sphe
a b b c
Residual Eye-Nostril Distance
kauf palu pipi sphe
a a b c
Residual Eye Width
kauf palu pipi sphe
a bc b c
Residual Shank Length
kauf palu pipi sphe
a a a a
Residual Foot Length
kauf palu pipi sphe
a a b c
Residual Head Length
kauf palu pipi sphe
a ab c b
Residual Interorbital Distance
kauf palu pipi sphe
a ab b c
Residual Internarial Distance
kauf palu pipi sphe
a a b b
Residual Head Width
kauf palu pipi sphe
a a b a
Residual Nostril-Snout Distance
kauf palu pipi sphe
a a a b
Residual Snout Angle
kauf palu pipi sphe
a b b b
Residual Thigh Length
kauf palu pipi sphe

Figure S2

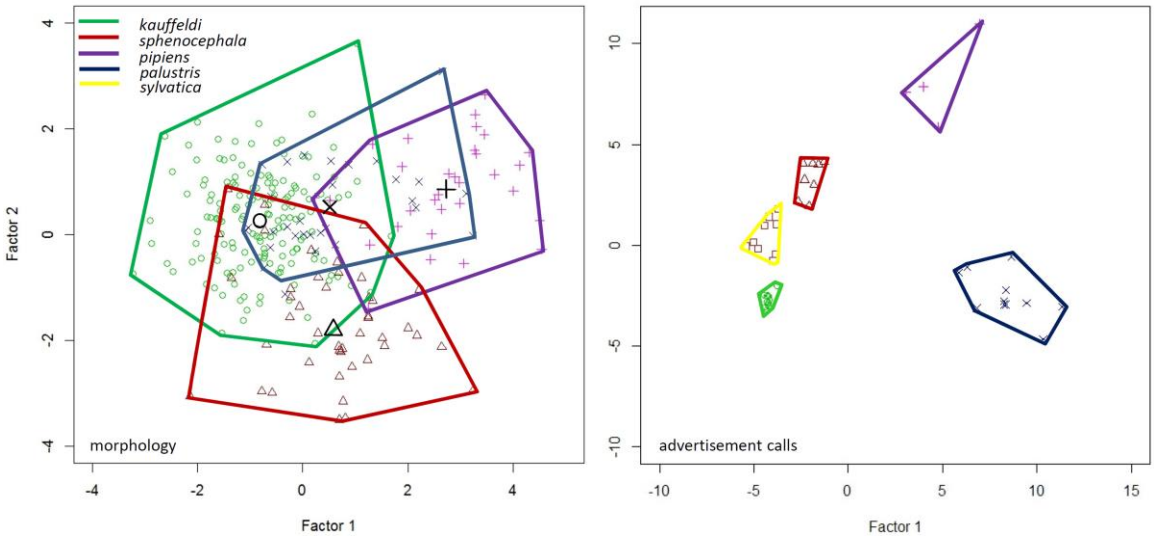

kauffeldi
sphenocephala
pipiens
palustris
sylvatica
morphology
Factor 1
Factor 2
advertisement calls
Factor 1

Figure S3

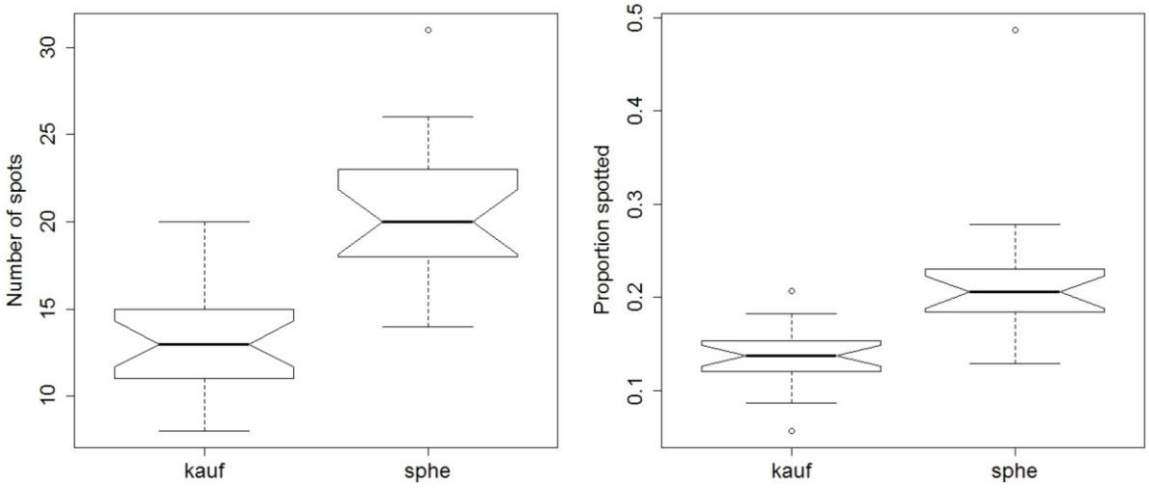
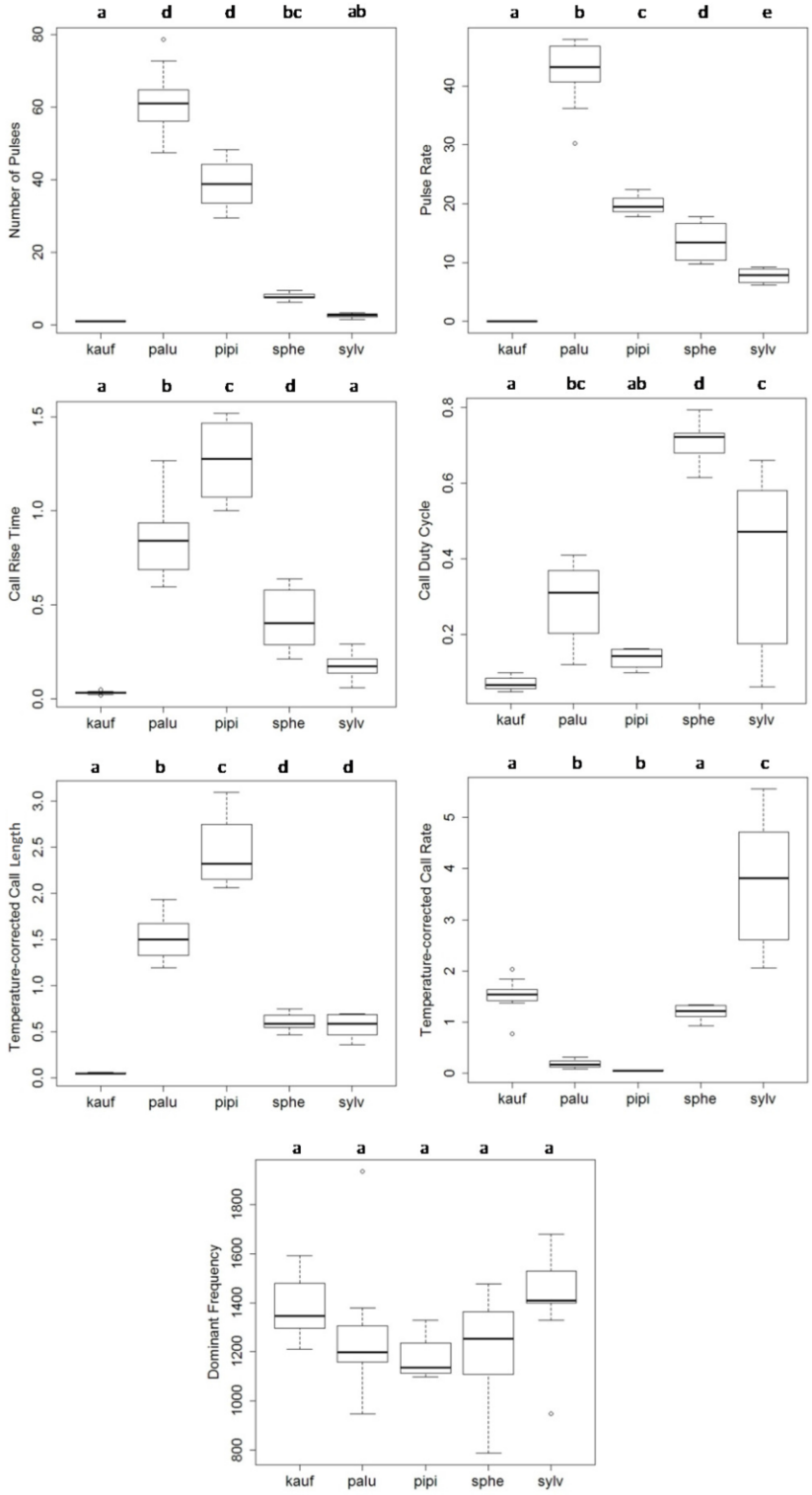

Number of spots
10
15
20
25
30
kauf
sphe
Proportion spotted
0.1
0.2
0.3
0.4
0.5
kauf
sphe

Figure S4


a d d bc ab
Number of Pulses
0 20 40 60 80
kauf palu pipi sphe sylv
a b c d e
Pulse Rate
0 10 20 30 40
kauf palu pipi sphe sylv
a b c d a
Call Rise Time
0.0 0.5 1.0 1.5
kauf palu pipi sphe sylv
a bc ab d c
Call Duty Cycle
0.2 0.4 0.6 0.8
kauf palu pipi sphe sylv
a b c d d
Temperature-corrected Call Length
0.0 0.5 1.0 1.5 2.0 2.5 3.0
kauf palu pipi sphe sylv
a b b a c
Temperature-corrected Call Rate
0 1 2 3 4 5
kauf palu pipi sphe sylv
a a a a a
Dominant Frequency
800 1000 1200 1400 1600 1800
kauf palu pipi sphe sylv

CHAPTER 3

A rapid assessment of post-hurricane impacts on the new leopard frog, *Rana* (*Lithobates*) *kauffeldi*, in the New York City Metropolitan Region

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Abstract

In 2013, we investigated impacts of Hurricane Sandy on coastal populations of a newly described frog species in the New York-New Jersey-Connecticut Tri-State area. This extreme weather event occurred only months after the species was first recognized, in 2012. At the time, approximately three-quarters of all known regional populations were located within vulnerable low-lying parts of the tidal storm-surge floodplain. This created cause for concern that was exacerbated by a still-tenuous understanding of the range and ecology of the new species itself and the potential loss of all remaining populations within the unique urban landscapes in and around New York City. Thus, we conducted rapid survival assessments at several of the most at-risk populations, all within the lower Hudson River Estuary watershed. Additionally, we sought to estimate the size and intensity of breeding choruses from any surviving populations and measure salinity and other water quality metrics for comparisons to pre-storm conditions. Our results confirmed survival at all five of our focal study areas. We also identified several new areas of habitation and noted potential increases at some populations, but failed to detect frogs at three previously documented sub-locations and noted several other populations that may be in decline or alarmingly small already. Mean salinity increased significantly, by 207% across sampled wetlands, but we did not find significant changes in any other water quality metrics we examined. Our study suggests that the new frog can survive occasional large-scale coastal storms and associated salinity increases. None of our study areas was destroyed, however, and the impacts they sustained may have been less severe than wetlands closer to the immediate Atlantic coastline.

Keywords: Atlantic Coast Leopard Frog; *Rana kauffeldi*; *Lithobates kauffeldi*; Urban Biodiversity; Climate Change; Hurricane Sandy; Sea-level Rise; Resilience

1. Introduction

In 2012, a new species of leopard frog was first documented from Staten Island and surrounding parts of New York (NY), New Jersey (NJ), and Connecticut (Newman et al. 2012), referred to hereafter as the Tri-State area. This frog, initially designated as *Rana* sp. nov. when first identified, was named and formally described as the Atlantic Coast leopard frog, *Rana* (*Lithobates*) *kauffeldi*, in 2014 (Feinberg et al. 2014). The species is characterized by its unique call, secretive lifestyle, and restricted habitat affinities; it occurs almost exclusively in expansive open-canopy coastal freshwater marshes and low-lying riparian floodplain corridors.

Months after the initial discovery, the Tri-State area was struck by Hurricane Sandy, on 29 October 2012. This major climatic event brought severe flooding and damage to coastal lowland areas throughout the region (Brandon et al. 2014). This put many *R. kauffeldi* populations at risk of potentially harmful saline intrusion (Christy and Dickman 2002; Gunzburger et al. 2010; Karraker et al. 2008; Kiviat, 2012) or outright extirpation due to critical habitat destruction. At the time, there were 23 known extant *R. kauffeldi* populations in the Tri-State area; 74% (n=17) were situated within the hurricane floodplain (Fig. 1). This situation, compounded by an incomplete overall understanding of the new species or its status beyond the Tri-State area, presented immediate cause for concern.

In response, we conducted rapid impact assessments at five of the highest-risk population areas in the lower Hudson River watershed. We surveyed each of these areas for vocalizing frogs (breeding choruses) in early spring 2013 to see if populations had persisted and to estimate the size and intensity of choruses and compare the results to pre-

storm data from the same localities, where possible. We also measured salinity and several other water chemistry attributes and compared those to pre-storm data. Finally, we searched for new *R. kauffeldi* populations farther from the coast in the lower Hudson River Valley. This inland region represents a potentially important and presumably more secure outpost from future sea-level rise or major coastal storm and flood events.

Rana kauffeldi is one of only two completely new frog species (not previously recognized as subspecies) north of Mexico in nearly 30 years (Feinberg et al. 2014). It is the first amphibian described from any northeastern US state since 1882 and the first described specifically from New York State since 1854. Ironically, the type-locality—the specific geographic area where *R. kauffeldi* was formally described—is on Staten Island, one of areas hardest hit by Hurricane Sandy.

2. Materials and methods

The methodologies described below are specific to our post-storm work at five focal study areas within the New York City vicinity and lower Hudson River watershed (Fig. 1, inset). We regarded these as the highest priority sites in the Tri-State area, including two areas in New York City (Staten Island) and three in New Jersey (the Meadowlands). We examined these sites to test the null hypotheses that (1) there would be no post-storm changes to the status of *R. kauffeldi* populations, and (2) there would be no post-storm changes to mean salinity and other basic water quality attributes. To examine the status of populations, we looked at both basic occupancy (present or absent) and estimates of population size and intensity. In this paper, we also consider and include some relevant findings from other regional populations and studies, but such information, where noted,

may have been collected outside the direct scope of this study and specific methods described below.

2.1. Population Survival Surveys

We conducted basic presence-absence surveys to assess *R. kauffeldi* survival at our study areas. We relied upon early spring breeding choruses as the principal indicator of survival. Acoustic surveys are an effective means of rapidly detecting and assessing *R. kauffeldi*, but the species only has a 2-3 week peak calling period that becomes increasingly sporadic as the season wanes (Feinberg et al. 2014). Thus, we visited each study area at least once under peak conditions during the proper time of year (late March to mid-April) to maximize the likelihood of acoustic detection. If we detected one or more calling frogs, a population was considered to have persisted. If no calls were detected, we employed visual surveys for frogs, tadpoles, and egg masses as secondary lines of detection. We also examined each site for physical impacts that could have impaired long-term breeding suitability or the overall health and survival of populations.

As allowed by weather, we conducted survival surveys across all five study areas on the evenings of 8-9 April 2013. At two of the Meadowlands study areas (Teterboro Airport and Little Snake Hill) we examined several distinct sub-locations as per earlier work by Kiviat (2011; 2012). Kiviat (2012) had also examined multiple sub-locations at the third NJ study area (Upper Penhorn Marsh), but we were only able to access one of those two previous locations. However, one of us (EK) resurveyed the site, including both previous sub-locations, on 3 April 2014. In NY, we surveyed and examined single locations at both Staten Island study areas (North and South) but these locations provided

full acoustic coverage of the publicly accessible extents of each site. *Rana kauffeldi* occurs within additional locations around the North study area, but they are privately owned, and as such, were not included in this study.

2.2. Frog Call Analyses

We estimated the size and intensity of *R. kauffeldi* choruses at our study areas and compared those data to pre-storm data from the same areas, where available. In contrast to the coarse-grain (binary) presence-absence data, these assessments provided a finer-grain approach for pre- and post-storm comparisons within and between sites. We recorded choruses as encountered during our survival surveys at all sites except for Staten Island North, where we instead recorded calls several days earlier, on 30 March 2013 (from 1930-1940h). Choruses were recorded with an Olympus DS-40 digital recorder and a Sennheiser MKE 400 directional microphone to document populations and allow for direct comparisons between them. To estimate chorus size—the general size of calling groups—we approximated the total number of frogs heard within a particular breeding aggregation. We estimated chorus intensity using rank methods by Lepage et al. (1997) and Kiviat (2011): 0=no calls heard, 1=calls heard individually and distinctly, 2=individual calls heard with some overlap between callers, and 3=calls heard but too many callers to distinguish individuals. Comparative pre-storm data came either from recordings by Feinberg (unpub. data) or Kiviat (2012), or in-field estimates by Kiviat (2011; unpub. data). All acoustic data were collected under typical calling conditions in their respective years of collection. Thus, we consider them readily comparable between

years. In cases where site data were collected on multiple dates within a year, we selected the date with the most robust calling for that year.

2.3. Water Chemistry

As with call analyses, we also sampled water chemistry during our survival surveys. At one study area (Staten Island North), sampling extended into a second day on 11 April 2013. For salinity—of particular interest given coastal flooding across our study areas—we collected three 100-ml water samples per wetland for lab analyses (S. Findlay, Cary Institute of Ecosystem Studies); this included direct measurements of conductivity (using a YSI EC300 conductivity meter) and chloride (using an Accumet chloride combination ion-specific electrode). Conductivity provides a common way to estimate salinity, and chloride analysis provides a means of examining the reliability of such estimates (Gunzburger et al. 2010; Karraker et al. 2008). We did this by plotting the values from our salinity estimates against our direct chloride measurements to see how well the values correlated. A weak correlation suggests the potential for non-target ions that can influence conductivity and, in turn, confound salinity estimates in some cases. We also used the individual samples to calculate average chloride and salinity values from each wetland for use in our general site summaries and between-site comparisons.

Additionally, we measured several other water quality parameters in the field with a YSI 650 MDS water quality meter and 6920 multi-parameter sonde; parameters included conductivity (mS/cm), water temperature (C), dissolved oxygen (ppm and % saturation), pH, and turbidity (NTU). We took 1-3 measurements per wetland and calculated average values when more than one measurement was taken.

Pre-storm data were available from at least part of all three NJ study areas as sampled by Kiviat (2011; 2012). This allowed for direct pre- and post-storm comparisons between four wetlands where *R. kauffeldi* had been documented. Additional pre-storm data were also available from another nine wetlands where Kiviat had not found *R. kauffeldi* (2011; 2012; unpub. data). Combining all of the pre-storm samples provided a more robust data set and allowed for broader unpaired comparisons with our post-storm data. The nine additional locations—referred to as unoccupied wetlands hereafter—include proximate sites within or near our study areas that we did not sample, but consider because of their geographic relevance. They all occur within viable habitat areas of the Meadowlands; two (at Teterboro Airport) were examined in 2006 (Kiviat, 2011; unpub. data), the other seven (two at Little Snake Hill plus Kingsland 1-3 and Kearny 1-2) were examined in 2012 (Kiviat 2012; unpub. data).

We also calculated the nearest straight-line distance (in meters) to subtidal estuarine water bodies for all wetlands included in our water chemistry analyses. We were unable to sample water quality at several locations across our study areas; these include the Marsh 2 and West Pond sub-locations at Little Snake Hill and both North Channel sub-locations at Upper Penhorn Marsh.

2.4. New Population Surveys

We surveyed three areas within the lower Hudson River Valley, between Rockland and Orange counties (NY), that had been associated with recent sightings of leopard frogs. Each site was monitored with a froglogger – a digital device that records ambient sounds over several days or weeks. Each unit comprised an Olympus DM-620

digital voice recorder coupled with an Olympus ME-52W noise cancelling microphone; both components were encased in waterproof housing and mounted on trees next to the target wetland. Each unit was set to record three-minute sound segments three times per day (before, during, and after sunset, when *R. kauffeldi* is most likely to call). Frogloggers were deployed one per site on 17 April 2013 and allowed to run until out of power, providing 12-17 days of consecutive recordings between the three sites. We initiated our sampling slightly after the peak calling period in 2013. To compensate for this delay, we deployed a fourth froglogger at a known *R. kauffeldi* reference area on Staten Island to provide a control for calling activity that we could later use to interpret the results from the three exploratory sites.

2.5. Statistical Analyses

We conducted several analyses to compare water quality before and after the storm. At a broad level, we compared the means from all pre-storm wetlands, regardless of occupancy, to those from all post-storm wetlands; because the samples from these two datasets were largely unpaired, we used Welch's t-tests for unequal variance to compare means. We did, however, separately examine four frog-occupied wetlands from the broader group for which both pre- and post-storm data were available; we used paired t-tests for these direct comparisons of means.

Also, looking only at frog-occupied wetlands, we searched for relationships between water quality and frogs to see what factors best explained post-storm changes in our frog populations. Because of small sample sizes, our exploration of post-storm water quality compared to presence-absence data was limited to examination of summary

statistics. However, we were able to use our chorus size estimates from nine wetlands with water quality data to run a multiple linear regression for identifying important relationships between abundance and post-storm water quality. To do this, we assigned chorus size values to continuous categories (0 callers=0, 1-5=1, 6-14=2, 15+=3) and then subtracted the pre-storm values (using the largest estimate where multiple estimates existed) from their associated post-storm values to generate an index of change along an ordinal scale from -2 to 2.

We conducted our analyses in R, v. 2.15.2 and v. 3.2.2 (R Core Team 2012; 2015). Where we provide summary statistics, we include ± standard deviations (SD) with the mean. In cases where a specific wetland was sampled more than once in different pre-storm years, we only used the most recent data for statistical analyses.

3. Results

3.1. Survival of Populations

We detected *R. kauffeldi* within all five focal study areas (Fig. 1, Table 1). At Teterboro Airport (surveyed 2000-2300h), we only detected frogs at one of three previously detected locations. At Little Snake Hill (surveyed 2100-2320h), where *R. kauffeldi* was only first documented several months prior to Hurricane Sandy (Kiviat 2012), we detected frogs at all previous locations plus one other location that had been examined previously without detection. At Upper Penhorn Marsh (surveyed 2350-0110h), we did not hear any calls but observed one adult male frog; we examined only one of two previously documented sub-locations, however, as shallow water prevented

canoe access to the second location. During a 2014 resurvey of Upper Penhorn Marsh by EK, no frogs were detected at either sub-location, but *R. kauffeldi* calls were detected from a new and separate (third) part of the study area (Table 1). We detected frogs at both the Staten Island North (surveyed 1650-1720h) and South (surveyed 1800-1830h) study areas.

Post-storm work by colleagues in the region revealed survival of *R. kauffeldi* from at least five other previously identified at-risk areas (Fig. 1, Table 1), including Philadelphia and central Connecticut. This also includes three populations of initially undetermined species composition along the Southeast NJ coast (Fig. 1); *R. kauffeldi* was later confirmed from all three areas (Fig. 2). At two of these areas (Cape May and Tuckahoe), we first identified *R. kauffeldi* through online site-videos of choruses posted to YouTube. This was corroborated through bioacoustic field sampling at both sites, by B. Zarate and J. Bunnell, respectively (unpub. data). The third site (Oceanville) was confirmed through bioacoustic sampling alone, by J. Bunnell (unpub. data) (Table 1).

Lastly, we report nine completely new Tri-State area *R. kauffeldi* populations found during or after this study (Fig. 2, yellow points). Among these, three occur within high-risk parts of the hurricane floodplain (Fig. 3, red points); two on Staten Island—found during surveys for this project by JAF—and a third in the Delaware River floodplain of southwestern NJ, found outside the scope of this project (B. Pitts, unpub. data). The remaining six populations all occur farther inland, and as such, were deemed low-risk sites (Fig. 3, green points). Among these sites, four were found in northern NJ (B. Zarate and R. Zappalorti, unpub. data) and two in southeastern NY (M. Klemens and J. Westerveld, unpub. data).

None of the sites examined directly or reported on here were extirpated by Hurricane Sandy. Further, the final number of regional populations (*n*=31) (Fig. 3) is greater than our original estimate before this study (*n*=23) (Fig. 1). Nonetheless, the majority of *R. kauffeldi* populations in the Tri-State area still occur within vulnerable coastal and low-lying floodplains and remain at-risk of future climate-change impacts and events. This majority, however, is less than originally estimated: the final percentage of at-risk sites is 65% (20 of 31 sites) compared to 74% (17 of 23 sites) at the start of this study. We briefly point to one site from our original map in Fig. 1—a low-risk site in western Connecticut—that was included in error. This site never harbored leopard frogs, insofar as we know, and was thus removed from all considerations and figures thereafter, but kept in Figure 1 for consistency.

3.2. Population Size and Stability

Our examination of *R. kauffeldi* chorus size and intensity revealed a varied picture across locations and populations (Table 2). At Teterboro Airport, we heard very few calls (*n*=4) at the one sub-location where we did find frogs (Southeast Pond). This is fairly consistent with pre-storm data from that sub-location (Table 2), though more individuals (*n*=10) were heard in 2012 (Kiviat 2012) and greater chorus intensity was reported in both 2006 and 2012 (Kiviat 2011; unpub. data). However, when we consider the Teterboro Airport study area as a whole, our cumulative results from the three sub-locations therein, suggest that this area has undergone a marked decline since 2006 (Table 2), including possible extirpations at two sub-locations (West Gate and West Pools).

At Little Snake Hill, we found increases in both the number of callers and chorus intensity at Marshes 1 and 2 (Table 2). At Marsh 1, we also noted expansion beyond the original 2012 calling area (Kiviat 2012) into a second area in an adjacent wetland 35-m to the southeast, separated only by a narrow berm. At Marsh 3, we found very few calling frogs (*n*=3) and low chorus intensity, consistent with pre-storm data from that sub-location. At the final sub-location, West Pond, where frogs were first documented during this study, we found very few callers (*n*=3) and low chorus intensity.

At Upper Penhorn Marsh, where we only accessed one sub-location (Central Channel) and observed only one frog in 2013, we did not hear or record any calls for analysis that year. In 2014 no frogs were detected or recorded at either original sub-location (Central Channel and North Channel-West End). However, calls were detected and assessed (directly in the field) at the new (third) sub-location found by EK (North Channel-East End). This sub-location had many callers and a high intensity chorus that exceeded peak levels from either original sub-location (Table 2).

On Staten Island, we documented moderate chorus size and intensity at the North study area. These levels actually reflect slight decreases from pre-storm estimates in 2012 (Feinberg, pers. obs.) and other years between 2008 and 2011 (Feinberg, unpub. data), and may in part be associated with recent development and habitat impacts in and around the sampling area, but are still relatively strong compared to many of the other chorus groups examined here (Table 2). At the South study area, we documented very few calling frogs (*n*=4) and low chorus intensity, reflecting a considerable decrease in both estimates compared to pre-storm data from 2010 (Feinberg, unpub. data). Lastly, we consider the two new sites where *R. kauffeldi* was detected during our 2013 surveys

(Table 2). At the Teleport site, we recorded frogs on 11 April 2013 (from 2130-2140h), approximately two weeks after their initial discovery, and found moderate calling numbers and chorus intensity. The other site (Office Wetland) was identified solely by a single calling frog on 1 April 2013 that was not recorded. A second frog (an adult female) was later observed and photographed from this location by D. Eib, (unpub. data) in July 2013. Both of these new areas had been surveyed without detection in previous years (Feinberg, pers. obs.), but not necessarily during the most appropriate month or time of day.

3.3. Water Quality Impacts and Comparisons

We ran a preliminary linear regression of our post-storm salinity and chloride values prior to our general water quality analyses, and the two attributes were highly correlated (n=24, r=0.904, p<0.0001). This suggests that conductivity, as measured and applied from the wetlands sampled here, provides a reliable estimate of salinity. Thus, we included salinity with our other water quality measurements and present them in Table 3 along with comparative pre-storm data. We also added one of our new sites to the sampling regimen (Staten Island Teleport) several weeks after finding *R. kauffeldi* there; we collected those samples during our visit to record frog calls at that site.

We present detailed within-and-between-site comparisons of salinity, dissolved oxygen (DO, part-per-million), pH and turbidity from all sites in Figure 4; however, we excluded DO (% saturation), conductivity, and water temperature because of redundancies or interrelatedness with the other metrics. Figure 4 shows that post-storm salinity increased fairly consistently across most wetlands, with the highest overall

salinity coming from Marsh 2 at Little Snake Hill (5.50 parts-per-thousand). This wetland is also closer to subtidal estuarine waters than any other wetland (Table 3). Note that Figure 4 includes two pre-storm sampling periods for Teterboro Southeast Pond (2006, 2012), but we did not include the earlier values in our quantitative dataset (see Methods).

When we combined the means of all pre-storm wetlands and compared them to those of all post-storm wetlands (n=14; mean=0.89 ppt±0.64 SD vs. n=10; mean=2.74 ppt±1.56 SD, respectively) we found a threefold increase in salinity (207%) that was significant (t=3.55, two-tailed p <0.01). We did not find similar significant differences for any of the other water quality metrics. Among the pre-storm data, all of the 2006 samples we analyzed (n=4) were collected during a warmer time of year (mid-June) than the other samples (late March to mid-April), which caused some concern about potential correlations with water temperature. Thus, we conducted individual linear regressions for each water quality attribute; we plotted each of the sample values against their associated water temperatures. Only DO (ppm) was significantly correlated with water temperature (n=24, r=0.436, p<0.05), so we removed the 2006 DO values from that dataset before running tests on it. We did not do this for the three other attributes (salinity, pH, and turbidity) as we did not find significant correlations with water temperature.

We also examined the four paired frog-occupied sites. Among them, mean salinity was again the only variable for which we found a significant difference between pre- and post-storm conditions (mean=1.12±0.86 vs. mean=3.15±1.43, respectively) (t=-5.60, two-tailed p <0.01). The largest percent-wise increase was at Southeast Pond (Teterboro Airport); salinity increased 1011.1% between 2006 and 2013 samples (Table 3), however, when we consider only the most recent pre-storm values (2012) as per our

statistical methodology (see Methods), the salinity increase was less, 483.3%, but still greater than at any other wetland.

We were unable to statistically test for differences between frog-occupied and unoccupied sites due to an insufficient number of post-storm unoccupied sites, but we briefly note and summarize key differences in an exploratory context (with 2006 DO samples excluded). The only attribute that showed a clear and consistent difference was salinity, which was 130% higher in occupied wetlands than unoccupied wetlands (n=9; mean=2.28±1.62 vs. n=11; mean=0.99±0.67, respectively). Turbidity also showed a moderate difference between groups (46.5% higher in unoccupied wetlands), but this was largely attributable to one outlying value (Kingsland-1) that, once removed, dropped the difference to only 1.2%. Although we could not compare occupied and unoccupied sites statistically, we were able to explore statistical relationships between water quality attributes and frog abundance (using changes in pre- and post-storm chorus size estimates) at nine frog-occupied wetlands. Results from the multiple linear regression are presented in Table 4; they include two statistically significant relationships: one between increasing abundance and salinity (p<0.01) and another between increasing abundance and decreasing turbidity (p<0.01).

3.4. Inland Surveys

We did not detect new *R. kauffeldi* populations at any of our three exploratory sites in the lower Hudson River Valley. We did, however, document calls from our froglogger at our Staten Island control reference site, but only during the first week of deployment.

3.5. Incidental Species Observations

We observed a total of 4 other amphibians and 2 reptile species during the general course of work for this study and other incidental post-storm visits to our focal study areas. A full list of these species is provided in Table 5. Additionally, we also documented several incidental species on our inland frog-logger recordings: these include spring peepers (*Pseudacris crucifer*), pickerel frogs (*Rana* [*Lithobates*] *palustris*), and American toads (*Anaxyrus americanus*).

4. Discussion

Rana kauffeldi populations survived Hurricane Sandy at all five focal study areas and several other areas where previously documented populations were examined outside the direct scope of this project. At the wetlands we studied, it is clear that (1) salinity increased considerably—more than threefold across sites—after the storm; (2) despite the increase in salinity, *R. kauffeldi* populations persisted; (3) other species of amphibians and reptiles also survived; and (4) these organisms must have compensatory mechanisms, whether physiological, behavioral, or both, that allow for some degree of tolerance and resilience in the face of a large-scale natural disturbances such as this.

Beyond confirming survival at our study areas, which included some of the most at-risk populations in the Tri-State region, we also found a slightly encouraging picture for *R. kauffeldi* compared to what was known at the time of the storm, in terms of both the known number of regional populations (up from 23 to 31) and the percentage of populations in coastal high-risk areas (down from 74% to 65%). Further, three years after

the storm and initial discovery of *R. kauffeldi*, we know more about the overall distribution of the species: it occurs well beyond the immediate Tri-State area, extending as far north as central Connecticut and as far south as North Carolina (Feinberg et al. 2014). Nonetheless, while the north-to-south range is longer than originally known, the vast majority of populations remain confined to low-lying coastal areas (Feinberg et al 2014; unpub. data). And while a single hurricane could not likely threaten the full extent of these populations, sea-level rise could have range-wide impacts to the species (Maschinski et al. 2011).

4.1. Methodological Issues

Studies in natural systems often face a variety of external factors that can limit, confound, and impede ideal approaches to data collection (Gotelli and Ellison 2004). This can be particularly problematic with before-and-after studies of natural disturbances, especially when pre-disturbance monitoring was not designed with the future disturbance in mind. Such was the case in this study, but the situation nonetheless provided a unique opportunity to look at the impacts of a powerful storm on a new species in a major urban system, all under overarching concerns of climate change and related future risks to human and non-human species alike. Thus, we discuss the significance of our results while addressing some of the uncertainties associated with our methodologies.

Further, this study involved multiple observers, variation among whom can confound observational data (Marsh 2009). This was a concern for our chorus size and intensity estimates, but we were confident that such variations did not exceed slight differences in the estimated number of frogs or chorus intensity rankings (not exceeding

more than one rank-level difference at most). To address this in our statistical analysis, we used only our chorus size data but converted them to categorical values that we felt provided a safe buffer from inter-observer discrepancies.

We also faced several issues specifically related to the ecology and behavior of *R. kauffeldi*. For one, the species exhibits a relatively short chorusing period that limits its acoustic detectability (in part, this also likely explains why the species remained undocumented for so long, despite occurring across one of the most densely populated and well-studied places on earth [Feinberg et al. 2014]). Further, its calling window is highly sporadic, both within and between the days of the calling season, and varies by different parts of the calling season, diurnal or nocturnal settings, and weather conditions. This in turn, highlights another issue – weather, which also varies within and between years, making consistent and broad-scale surveys quite challenging across time and space. To compensate for this and minimize potential variability, we conducted most of our work over two consecutive days early in the season when calling tends to be most reliable (Feinberg et al. 2014). Lastly, we point to one additional basic challenge we faced: working with a newly described species for which little natural history information was available to guide our work.

To illustrate several of the above issues, we provide an example from the Staten Island South site. Our survey results there suggested a fairly substantial population decline (Table 2). However, our methods reflected a somewhat incomplete understanding of the diel calling patterns and overall breeding ecology of the species at that time. Although we knew *R. kauffeldi* tended toward more consistent calling at night versus day (Feinberg et al. 2014), and incorporated that understanding into our methodological

framework, we did not consider the time immediately before sunset to be problematic. As a result, and given our ambitious survey schedule, we surveyed this site slightly before sunset—the only such case among our 2013 surveys. We now know (unpub. data) that calling typically peaks only after skies have completely darkened. Thus, our results may have under-estimated chorus size and intensity and should be regarded as tentative pending a future re-survey of the site under ideal post-sunset conditions.

Finally, we address issues related to our rapid assessments and one-time surveys at most of the study areas during we examined. While this approach was done to minimize sampling variation (as stated above) and as a cost-and-time efficient way of covering a lot of ground during a short calling season, there is still inherent variation in call levels within and between sites, and even within peak parts of the breeding season. One concern is that we may have missed choruses altogether, if frogs were not calling during the time or day of our visit. This could potentially apply to the three sub-locations we report as extirpated, and is a recognized concern in amphibian breeding studies (Marsh and Trenham 2001). It is also possible that at locales were we did hear frogs, they were calling at below-maximum levels that under-represented the true size of the calling group (as discussed above for Staten Island South above). These concerns can also be applied in the reverse; that is, some of the new sites we reported herein may have in fact been surveyed and missed during pre-storm surveys.

4.2. Survey Outcomes and Considerations

Although *R. kauffeldi* persisted at all of the study areas we examined, our chorus size and intensity data show a variety of post-storm changes across the locations we

examined (Table 2), including apparent extirpations at three sub-locations. Two of these extirpations occurred at one study area alone, Teterboro Airport. Given the number of years between full surveys (2006 to 2013), however, it is difficult to determine what role, if any, Hurricane Sandy played in the apparent declines at Teterboro Airport. Regardless, the post-storm results suggest the potential loss of two sub-locations (West Gate and West Pools) and very few callers at the remaining sub-location, Southeast Pond. These results paint a seemingly bleak outlook for an overall population that may be imperiled. In some cases, however, subpopulation extirpations may occur as normal or cyclical events within the greater metapopulation dynamic (Sjögren-Gulve 1994; Marsh and Trenham 2001) while other subpopulations persist and help maintain the overall population and allow for potential future recolonizations (Griffiths et al. 2010; Cosentino et al. 2011). At Teterboro Airport, recolonization may be unlikely as Southeast Pond is small, fairly isolated, and appears to harbor only a sparse number of frogs.

Elsewhere in the Meadowlands, at Upper Penhorn Marsh, both original 2006 sub-locations (Kiviat 2011) appear to be in severe decline. One of these (North Channel-West End) was already small (Table 2) and is likely extirpated based on a 2014 re-survey by EK. We heard no calls at the other location (Central Channel) in 2013, on a night when leopard frogs were calling extensively elsewhere, although we did observe one frog (visually); no frogs were detected here at all in 2014. These results suggest a steep decline or possible extirpation within this sub-location as well. As with Teterboro Airport, the amount of time between surveys at Upper Penhorn Marsh makes it difficult to determine what role, if any, Hurricane Sandy may have played in these changes. This area was visited once between 2006 and 2013 (by EK on 4 April 2012), however, without

detection, but the site was only assessed casually and peripherally during the day when frogs are less likely to call. Thus, these findings are tenuous at best and were not included or considered in our results. The discovery of a robust chorus in a third and entirely new sub-location (North Channel-East End) suggests that (1) the possible loss of both original sub-locations and considerations of imperiled site status may be offset by the robust chorus found at the new sub-location, and (2) subpopulations may not necessarily vanish in all cases, but rather migrate to different locations over time; this may also be something to consider at Teterboro Airport, where there is considerable additional wetland habitats that we did not examine.

At the last NJ study site, Little Snake Hill, our results suggest a stable outpost with populations that may be more extensive and perhaps larger than originally thought (Kiviat 2012), or possibly even increasing and expanding over time. For example, at Marsh 1, we report frogs calling from two discrete areas, suggesting a potential expansion from only one area in 2012 (Kiviat 2012). This further highlights the possibility that subpopulations may not remain static over time. Rather, as documented among multiple amphibians elsewhere (e.g., Skelly et al. 1999), they may expand and decline in certain areas, perhaps with no substantial net change to the overall population. That is, at least in some cases, choruses and subpopulations may shift normally between years (e.g., Heard et al. 2012) and possibly even at different times within years (e.g., Randall et al. 2015), in response to environmental conditions or other preferences or cues. Further, large-scale flooding from natural events such as hurricanes may actually facilitate or allow for expansion, migration, and colonization of new sub-locations across the greater overall landscape (Wassens et al. 2008; Schalk and Luhring 2010). Such

dynamics might also explain detection of several new calling areas during the course of this study, including one new site on Staten Island (Office Wetland) where frogs had not previously been documented despite several years of pre-storm surveys (Table 1).

4.3. Insights from Water Quality

Our exploratory results from water-quality comparisons between frog-occupied and unoccupied sites are interesting and show higher salinities at occupied sites versus unoccupied sites (130% higher, on average). This suggests that *R. kauffeldi* might tend towards wetlands with relatively higher salinities than other available wetland habitats, but such a conclusion must be considered tentative. For one, some sites were only measured before the storm (n=10) others only after (n=6), and others still were paired and measured in both conditions (n=4); this confounds direct comparisons between occupied and unoccupied sites. Further, the sample size among unoccupied sites was unbalanced in the direction of pre-storm measurements (n=9) over post-storm measurements (n=2). Thus, given the effects of the storm and the small number of measurements from post-storm unoccupied site, we were unable to conduct any meaningful statistical analyses to compare occupied and unoccupied sites and draw further conclusions beyond the exploratory results presented here.

We were, however, able to statistically examine those sites where frogs were present to look for relationships between water quality and frog-abundance changes between pre-storm to post-storm conditions. Our results indicate a significant positive relationship between salinity and abundance. We know of few other examples of this type of relationship (but see Rios-López 2008), and *R. kauffeldi* may be somewhat unique in

this regard, at least where it occurs along the coast. This might also have some explanatory value in the context of two larger regional congeners, green frogs, *R. clamitans*, and bullfrogs, *R. catesbeiana*. These species are widely distributed throughout the Tri-State area (Feinberg pers. obs.) but absent from four of our five *R. kauffeldi* study areas. We cannot say whether their absence is a direct result of salinity or if that absence provides *R. kauffeldi* with a competitive or predatory release, but recommend that future research look into this by examining the relationships, distributions, and salt-tolerances of all three species. We also found a significant negative relationship between decreasing turbidity and increasing abundance, but this was not surprising as *R. kauffeldi* tends to occur in clear waters with low currents and disturbance (Feinberg et al. 2014).

We also briefly explore the possibility that a tendency toward more-saline habitats may confer some level of protection against pathogenic diseases. Recent work by others has shown that wetland salinity can be an important factor in reducing both the presence and intensity of chytridiomycosis, a disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (BD) (Bramwell 2011; Heard et al. 2014; Heard et al. 2015). Again however, without direct study into this question here, any discussion relative to *R. kauffeldi* is strictly speculative, but provides another important area of consideration for future research pathways. Interestingly, the aforementioned congeners, *R. clamitans* and *R. catesbeiana* have been documented as carriers of BD (Richards-Hrdlicka et al. 2013; but see Gervasi et al. 2013) and research on other frog species has shown that pathogens can have strong influences on the interactions between different species (Kiesecker and Blaustein 1999). Thus, future work should look not only at what role salinity may or may not play in limiting disease in *R. kauffeldi*, but also at the

relationship of congeners, both in terms of vector potential and interactive influences on *R. kauffeldi*.

Our water quality results indicate that *R. kauffeldi* can tolerate certain levels of elevated salinity within breeding wetlands. The highest salinity measured among wetlands with breeding choruses (at our five focal study areas) was 5.50 ppt, and the mean among all seven active breeding wetlands we measured was 3.30 ppt (see Table 3). These salinities are within the lethal range of values reported among several other regional amphibians (Collins and Russell 2008). However, despite the general conception that amphibians are broadly intolerant of saline habitats, a number of species—especially those with coastal populations—have been documented from environments with considerable salinity (Hopkins and Brodie 2015). Several of the most tolerant and well-documented examples include the natterjack toad (*Epidalea calamita*), which has been found to breed in salinities as high as 22 ppt in Europe (Gomez-Mestre 2003), the marine toad (*Rhinella marina*), adults (only) of which have been documented in salinities of 20.5 ppt in the Caribbean, and the Crab-eating Frog (*Fejervarya cancrivora*)—generally considered the most salt tolerant frog (Karraker 2007)—the larvae of which have been found in salinities reaching 35 ppt in Asia (Gordon et al. 1961).

It remains unclear as to whether *R. kauffeldi* can tolerate only temporary salinity increases or long-term and permanent salinity increases as well. The particular mechanism that allows *R. kauffeldi* to tolerate saline habitats also remains unclear, as our work here did not look into this question. Possibilities could include some sort of physiological adaptation or behavioral response in which the frogs move to lower salinity parts of wetlands or uplands. Whatever the mechanism may be, it allows *R. kauffeldi* to

survive in coastal habitats and may also allow the species to survive in unlikely habitats across the New York City urban landscape (Feinberg et al. 2014). That is, the same salinity tolerance that helps *R. kauffeldi* in the face of storms may also help in the face of anthropogenic inputs from road de-icing and runoff.

4.4. Future Implications

Our work here focused largely on adult frogs and did not consider the effects of water-quality changes on eggs or larvae. Presumably, most if not all the frogs we encountered during our 2013 surveys metamorphosed prior to the arrival of Hurricane Sandy (and may have sought upland refuge during the storm itself). As such, monitoring survival outcomes among post-storm eggs and larvae would have increased our confidence in the results. To an extent, the results from the 2014 re-survey of Upper Penhorn Marsh are encouraging, as a continued population more than a full year after Hurricane Sandy almost certainly contained some post-storm young adults. Additional positive survey results in 2014 at Little Snake Hill (by EK) and 2015 at Staten Island North (by JAF) provide other examples and demonstrate even longer persistence.

Following the recommendations above, we generally encourage future resurveys of all locations to check for long-term persistence. Further, we encourage additional sampling at places such as Teterboro Airport and the Staten Island South study area to gain greater certainty on the putative declines observed at those locations. This study highlights several important lessons learned, including (1) the value of strong baseline data and well-balanced measurements as a critical foundation for conducting species assessments after major weather events; (2) the idea that frogs may be more tolerant of

salinity and associated “natural disasters” than typically presumed; and (3) the role that comprehensive arrays of frog-call recording devices can play in providing detailed bioacoustic data beyond what a single-night survey can provide.

As a final consideration, we note that the coastal affinity of *R. kauffeldi*—quantified here as including 65% of all known Tri-State area populations—will put this species on the front lines of future sea-level rise along the US East Coast. And while we found this species and several others to persevere in the face of increased salinity, the continued availability of coast freshwater marshes presumably remains essential for their long-term survival. As such *R. kauffeldi* could be an important model species to consider in the development of managed retreat policies that allow for processes of erosion and landward migration of coastal wetlands. However, such policies would need to account for the continued maintenance of coastal freshwater habitats along with expanding and migrating salt marsh habitats.

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Table 1. Pre-storm and post-storm survival results from at-risk Tri-State populations. Single 'quotes' correspond to locational terminologies from Kiviat (2011, 2012). (a)=acoustic detection; (v)=visual detection. Numbers denote areas where bioacoustic data were collected from unrelated projects by other researchers: [1] Bill Pitts; [2] Jennifer Tennessen; [3] John Bunnell; [4] Brian Zarate; [5] Dennis Quinn (also includes 2014 observations and photographs).

Area Name	**Sub-location Name**	**2006-2010 (Pre-Storm)**			**2012 (Pre-Storm)**		**New**	**2013 (Post-Storm)**	
		Detection	Year	Source	Detection	Source		Detection (this study)	Date
Focal Study Areas									
Teterboro Airport (TET)	'West Gate'	**Detected** (a)	2006	Kiviat (2011)	Not Examined			**Non-detect**	8-Apr
	'West Pools'	**Detected** (a)	2006	Kiviat (2011)	Not Examined			**Non-detect**	8-Apr
	'Southeast Pond'	**Detected** (a)	2006	Kiviat (2011)	**Detected** (a)	Kiviat (2012)		**Detected** (a,v)	8-Apr
Upper Penhorn Marsh (UPM)	'Central Channel'	**Detected** (a)	2006	Kiviat (2011)	Not Examined			**Detected** (v)	8-Apr
	'North Channel' (West End)	**Detected** (a)	2006	Kiviat (2011)	Not Examined			**Non-detect** (2014)	3-Apr
	'North Channel' (East End)	Not Examined			Not Examined		Yes	**Detected** (a) (2014)	3-Apr
Little Snake Hill ('LSH')	'Marsh 1' ('LSH-E'/East Wetlands)	Not Examined			**Detected** (a)	Kiviat (2012)		**Detected** (a)	9-Apr
	'Marsh 2' (Toll Plaza/North Wetland)	Not Examined			**Detected** (a)	Kiviat (2012)		**Detected** (a)	9-Apr
	'Marsh 3' ('LSH-SW'/Central Ash Area)	Not Examined			**Detected** (a)	Kiviat (2012)		**Detected** (a)	9-Apr
	West Pond (South of Laurel Hill')	Not Examined			**Non-detect**	Kiviat (2012)	Yes	**Detected** (a)	9-Apr
Staten Island North		**Detected** (a,v)	Multiple	Feinberg (pers. obs.)	**Detected** (a)	Feinberg (pers. obs.)		**Detected** (a)	9-Apr
Staten Island South		**Detected** (a,v)	Multiple	Feinberg (pers. obs.)	Not Examined			**Detected** (a)	9-Apr
Additional Areas									
Staten Island Teleport		**Non-detect**	2008	Feinberg (pers. obs.)	Not Examined		Yes	**Detected** (a)	30-Mar
Staten Island Office Wetland		**Non-detect**	Multiple	Feinberg (pers. obs.)	Not Examined		Yes	**Detected** (a)	1-Apr
Bridgeport NJ (Delaware River)[1]		n/a			n/a		Yes	**Detected** (a) (2014)	
Philadelphia PA (Delaware River)[2]		n/a			n/a			**Detected** (a)	
Oceanville NJ (Forsythe NWR)[3]		n/a			n/a			**Detected** (a) (2015)	
Tuckahoe NJ (Tuckahoe River)[3]		n/a			n/a			**Detected** (a) (2015)	
Cape May NJ [4]		n/a			n/a			**Detected** (a) (2015)	
Middletown CT (Connecticut River)[5]		n/a			n/a			**Detected** (a) (2015)	

Table 2. Pre-and-post storm breeding chorus estimates. Single 'quotes' correspond to locational terminologies from Kiviat (2011, 2012). Change from previous compares post-storm data (this study) to the most recent pre-storm data. For additional sub-location name details, see Table 1; (v)=visual detection only; *=post-storm results are from 2014, not 2013.

Area Name	Sub-location Name	2006-2010 (Pre-Storm)				2012 (Pre-Storm)			2013 (Post-Storm)		
		Rank	Estimated # callers	Year	Source	Rank	Estimated # callers	Source	Rank	Estimated # callers	Change from previous
Focal Study Areas											
Teterboro Airport (TET)	'West Gate'	2	4	2006	Kiviat (2011, unpub. data)	Not Examined	---	---	0	0	Possible Extirpation
	'West Pools'	1	1	2006	Kiviat (2011, unpub. data)	Not Examined	---	---	0	0	Possible Extirpation
	'Southeast Pond'	2	4	2006	Kiviat (2011, unpub. data)	2	10	Kiviat (unpub. data)	1	4	Slight Decrease
Upper Penhorn Marsh (UPM)	'Central Channel'	2	15+	2006	Kiviat (2011, unpub. data)	Not Examined	---	---	0	1 (v)	Decrease
	'North Channel' (West End)*	1	2	2006	Kiviat (2011, unpub. data)	Not Examined	---	---	0	0	Possible Extirpation
	'North Channel' (East End)*	Not Examined	---	---	---	Not Examined	---	---	3	20+	Increase (new)
Little Snake Hill ('LSH')	'Marsh 1' (East Wetlands)	Not Examined	---	---	---	1	5	Kiviat (unpub. data)	2	10-20	Increase (shift)
	'Marsh 2' (Toll Wetland)	Not Examined	---	---	---	1	5	Kiviat (unpub. data)	2	15-20	Increase
	'Marsh 3' (Central Ash Area)	Not Examined	---	---	---	1	5	Kiviat (unpub. data)	1	3	Similar
	West Pond	Not Examined	---	---	---	0	0	Kiviat (2012)	1	3	Increase (new)
Staten Island North		3	20+	Multiple	Feinberg (unpub. data)	3	20+	Feinberg (pers. obs.)	2	10-15	Decrease
Staten Island South		3	20+	2010	Feinberg (unpub. data)	Not Examined	---	---	1	4	Decrease
Additional Areas											
Staten Island Teleport		0	0	2008	Feinberg (pers. obs.)	Not Examined	---	---	2	5-10	Increase (new)
Staten Island Office Wetland		0	0	Multiple	Feinberg (pers. obs.)	Not Examined	---	---	1	1	Increase (new)

Table 3. Pre-and-post storm water chemistry results from at-risk populations. Single 'quotes' correspond to locational terminologies by Kiviat (2011, 2012); these include several relevant sub-locations adjacent to the areas sampled in this study. 2013 data from this study are shaded in gray; comparative pre-storm data are unshaded. All 2006 data (Kiviat, 2011; unpub. data) were collected with a HydroLab Surveyor 4 portable water-quality probe; all 2012 data (Kiviat, 2012; unpub.data) were collected with a YSI MDS 650 meter and 6600 EDS multi-parameter sonde (except salinity, calculated as described in methods); For additional sub-location name details, see Table 1; *=not detected post-storm (Kiviat, pers. obs.).

Area Name	**Sub-location Name**	**Attributes**											
		Sample Information				Lab Measurements & Estimates		Direct Field Measurements					
		Sample Year	Date	Frog Detection Status	Subtidal Estuary Distance (m)	CL (ppm)	Salinity (ppt)	Conductivity (mS/cm)	H20 Temp (°C)	DO (ppm)	DO (% sat)	pH	Turbidity (NTU)
Focal Study Areas													
Teterboro Airport (TET)	'West Gate'	2013	8-Apr	No (previously detected)	3202.6	1214.2	1.60	2.80	19.04	9.19	100.30	4.61	-1.30
	'West Pools'	2013	8-Apr	No (previously detected)	3186.5	1664.2	2.00	3.53	16.60	13.78	142.90	8.49	43.40
	'Southeast Pond'	2013	8-Apr	Detected	1866.8	1129.7	1.17	2.01	17.21	9.12	95.30	7.22	0.60
		2012	4-Apr	Detected	1866.8		0.20	0.50	16.80	10.80	113.30	7.13	4.30
		2006	12-Jun	Detected	1866.8		0.11	0.23	23.10	1.28	18.00	6.26	
	'Southeast Pond Ditch'	2006	12-Jun	No (undetected adjacent area)	1963.4		0.13	0.26	18.20	1.00	12.70	6.20	
	'Redneck Ave. Ditch'	2006	12-Jun	No (undetected adjacent area)	1850.7		0.66	1.28	18.80	1.02	13.20	6.36	
Upper Penhorn Marsh (UPM)	'Central Channel'	2013	8-Apr	Detected	2365.7	2487.8	3.10	2.74	12.74	3.71	35.30	6.68	38.60
		2006	13-Jun	Detected	2365.7		0.57	1.07	23.20	0.50	7.00	6.86	
	'North Channel' (West End)	2006	13-Jun	Detected (future non-detect)*	2784.2		0.75	1.42	25.25	1.33	17.35	6.80	
Little Snake Hill ('LSH')	'Marsh 1' (East Wetlands)	2013	9-Apr	Detected	25.3	2285.8	4.35	7.29	19.08	10.20	112.65	8.12	1.20
		2012	28-Mar	Detected	25.3		1.90	3.52	15.40	6.50	66.30	7.93	68.00
	'Marsh 2' (North Wetland)	2013	9-Apr	Detected	24.9	2698.5	5.50	9.04	19.66	6.62	74.50	7.67	10.90
	'Marsh 3' (Central Ash Area)	2013	9-Apr	Detected	294.9	2353.0	4.00	6.71	18.07	14.53	158.70	9.36	28.85
		2012	15-Apr	Detected	294.9		1.80	3.20	19.30	5.17	56.80	8.25	5.60
	'Rail Station' Pond	2012	28-Mar	No (undetected adjacent area)	549.8		0.80	1.53	12.40	11.71	109.80	9.14	24.60
	'Turnpike' Pond	2012	28-Mar	No (undetected adjacent area)	607.9		1.20	2.29	13.50	8.33	81.00	7.88	25.00
Staten Island North		2013	9 & 11-Apr	Detected	479.0	2026.5	3.30	5.17	14.83	6.77	69.83	7.21	34.40
Staten Island South		2013	9-Apr	Detected	168.9	1229.5	1.70	3.09	22.25	6.10	71.73	6.66	12.97
Additional Areas													
Staten Island Teleport		2013	11-Apr	Detected	1000.1		0.71	1.37	13.00	6.78	64.60	7.41	-0.40
Kingsland	Kingsland-1	2012	3-Apr	No (undetected regional area)	2430.1		0.40	0.77	14.30	22.26	216.10	10.20	165.10
	Kingsland-2	2012	3-Apr	No (undetected regional area)	2269.2		0.40	0.77	13.70	10.97	107.10	7.90	11.30
	Kingsland-3	2012	3-Apr	No (undetected regional area)	1786.4		0.30	0.61	13.40	11.14	106.80	8.10	15.10
Kearny	Kearny	2012	14-Apr	No (undetected regional area)	1790.8		1.80	1.92	18.20	13.33	142.60	8.42	20.90
	Kearny-2	2012	14-Apr	No (undetected regional area)	1770.8		1.60	3.35	18.40	9.67	104.20	8.18	12.60

Table 4. Multiple linear regression model explaining change in frog abundance (based on number of frog calls estimated before and after Hurricane Sandy) in relation to post-storm water quality attributes measured at those sites.

Variable	**Coefficient**		***t*-value**	***p*-value**	
	ß	Standard Error			
Post-Storm Salinity (ppt)	0.350	0.086	4.062	0.010	**
Post-Storm Dissolved Oxygen (ppm)	-0.129	0.065	-1.979	0.105	
Post-Storm pH	0.129	0.091	1.422	0.214	
Post-Storm Turbidity (NTU)	-0.397	0.075	-5.310	0.003	**

F=12.61 (p=0.008); df=4, adj. R^2=0.838.

** $p < 0.01$

Table 5. Post-storm incidental observations of additional amphibians and reptile species.

Species Name	Class	Observed Locale(s)
Spring peeper (*Pseudacris crucifer*)	Amphibian	Staten Island North Staten Island South Staten Island Teleport Staten Island Office Wetland
Green frog (*Rana* [*Lithobates*] *clamitans*)	Amphibian	Staten Island North Staten Island Teleport
Bullfrog (*Rana* [*Lithobates*] *catesbeiana*)	Amphibian	Staten Island North
Fowler's toad (*Anaxyrus fowleri*)	Amphibian	Staten Island North
Dekay's brownsnake (*Storeria dekayi*)	Reptile	Staten Island North
Painted turtle (*Chrysemys picta)*	Reptile	Staten Island North

Figure Legend

Fig. 1 Map of original (2013) known extant Tri-State area *Rana kauffeldi* populations, showing localities and flood extents from Hurricane Sandy. Red indicates at-risk populations; green indicates low-risk populations; stars indicate focal study areas; black center dots indicate areas of unconfirmed populations or unclear species composition. Inset: focal study areas in New York City vicinity (1=Teterboro Airport [NJ], 2=Upper Penhorn Marsh [NJ], 3=Little Snake Hill [NJ], 4=Staten Island North [NY], 5=Staten Island South [NY]). Base map and flood data layers downloaded from USGS Hurricane Sandy Storm Tide Mapper (http://water.usgs.gov/floods/events/2012/sandy/sandymapper.html).

Fig. 2 Map highlighting new and previously unconfirmed *Rana kauffeldi* populations. Yellow indicates new populations added to the original map (Fig. 1); black center dots were removed from previously unconfirmed sites (Fig. 1) where *R. kauffeldi* was later confirmed.

Fig. 3 Updated map (2015) of Tri-State area *Rana kauffeldi* populations showing post-study localities for both at-risk coastal populations (red) and low-risk inland populations (green).

Fig. 4 Select water quality comparisons between sites and sub-locations where *R. kauffeldi* was documented. Attributes include salinity in ppt (a), dissolved oxygen in ppm (b), pH (c), and turbidity in NTU (d). Comparisons include all 2013 post-storm measurements (blue) and pre-storm data from 2012 (green) (Kiviat, 2012; unpub. data) and 2006 (orange) (Kiviat, 2011; unpub. data). * indicates sites where R. kauffeldi was documented in 2006 (Kiviat, 2011) but not in 2013.

Figure 1

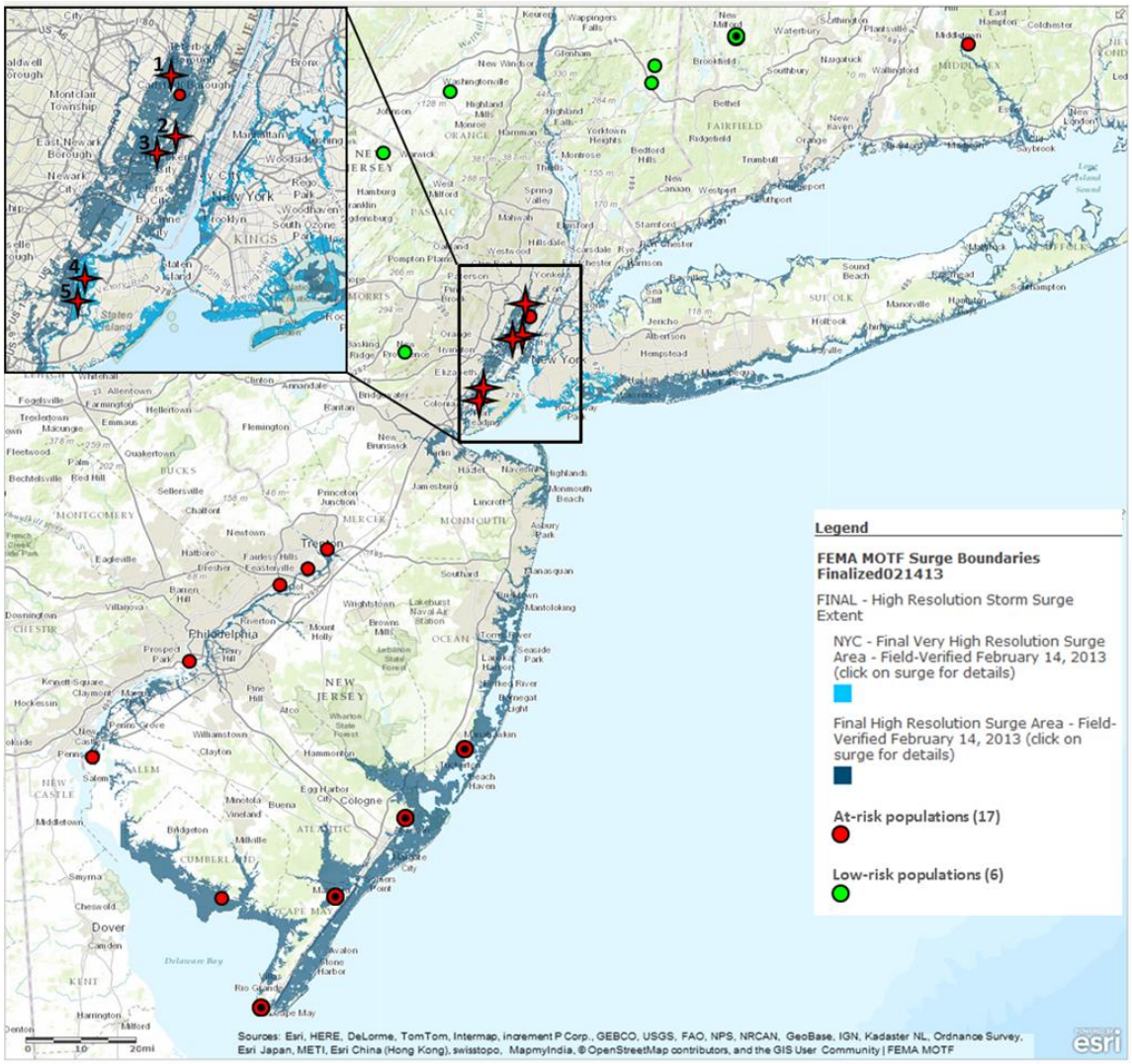
Legend
FEMA MOTF Surge Boundaries Finalized021413
FINAL - High Resolution Storm Surge Extent
NYC - Final Very High Resolution Surge Area - Field-Verified February 14, 2013 (click on surge for details)
Final High Resolution Surge Area - Field-Verified February 14, 2013 (click on surge for details)
At-risk populations (17)
Low-risk populations (6)
Sources: Esri, HERE, DeLorme, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, MapmyIndia, © OpenStreetMap contributors, and the GIS User Community | FEMA MOTF
esri

Figure 2

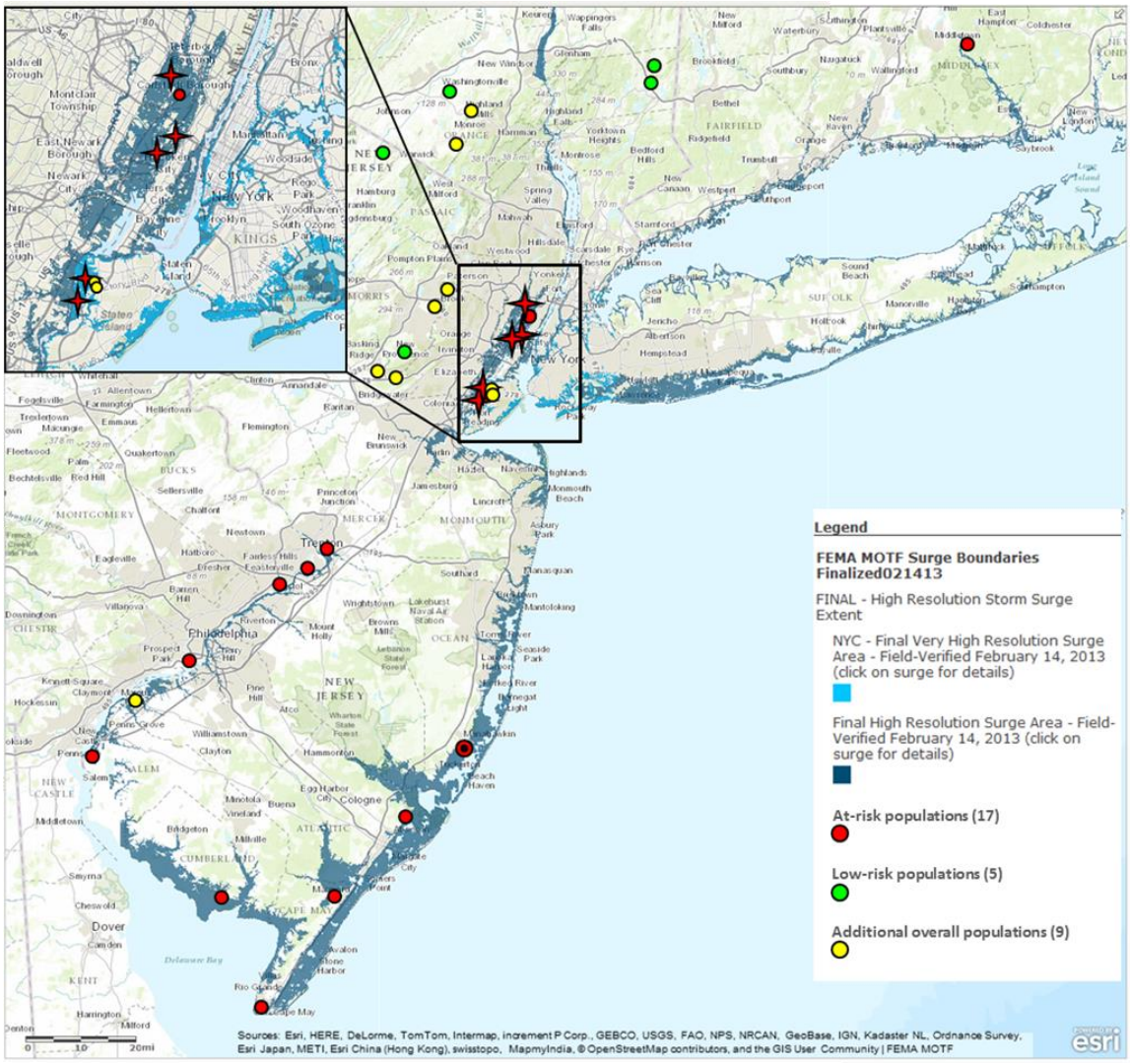
Legend
FEMA MOTF Surge Boundaries Finalized021413
FINAL - High Resolution Storm Surge Extent
NYC - Final Very High Resolution Surge Area - Field-Verified February 14, 2013 (click on surge for details)
Final High Resolution Surge Area - Field-Verified February 14, 2013 (click on surge for details)
At-risk populations (17)
Low-risk populations (5)
Additional overall populations (9)
Sources: Esri, HERE, DeLorme, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, MapmyIndia, © OpenStreetMap contributors, and the GIS User Community | FEMA MOTF
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Figure 3

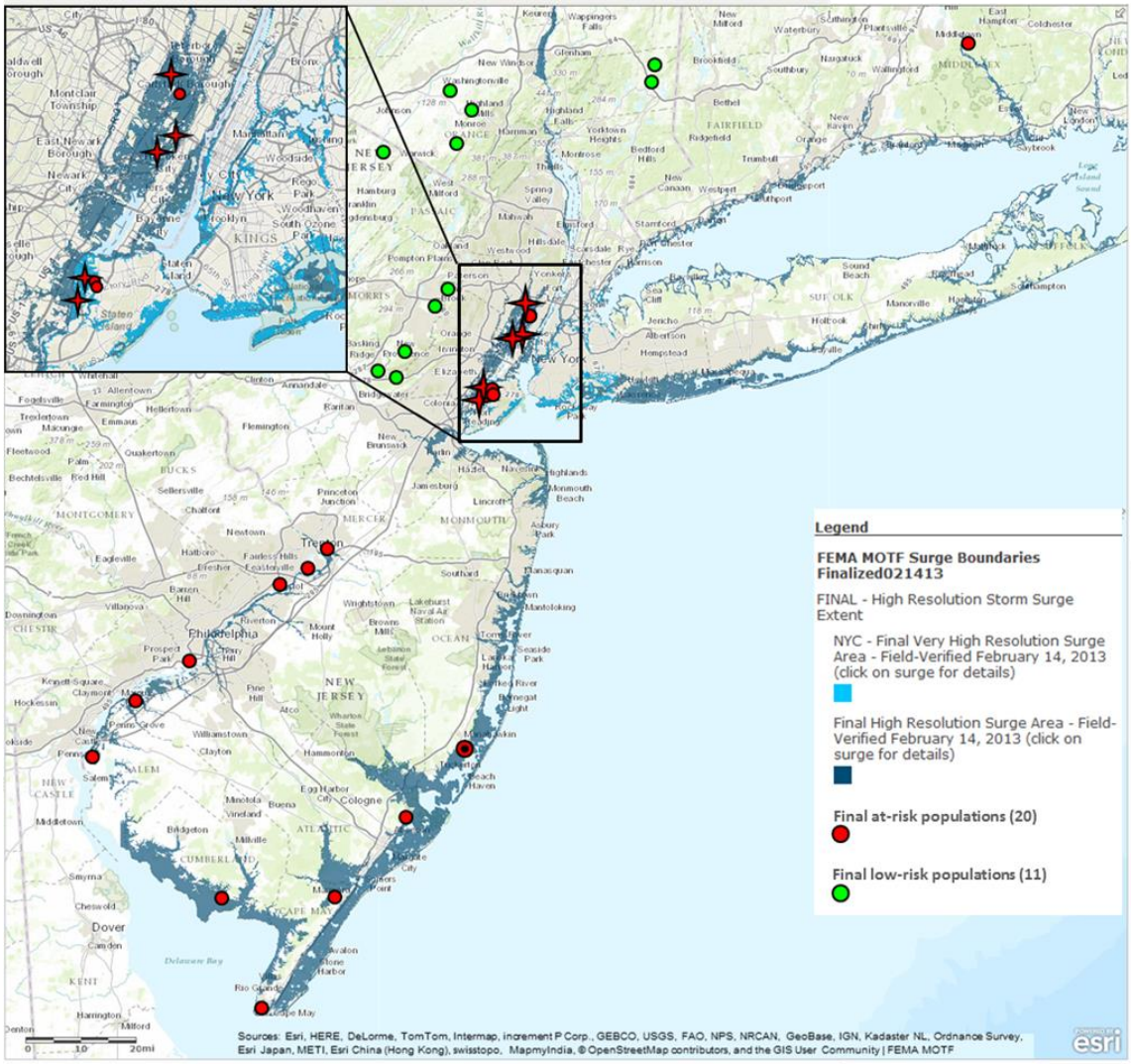
Legend
FEMA MOTF Surge Boundaries Finalized021413
FINAL - High Resolution Storm Surge Extent
NYC - Final Very High Resolution Surge Area - Field-Verified February 14, 2013 (click on surge for details)
Final High Resolution Surge Area - Field-Verified February 14, 2013 (click on surge for details)
Final at-risk populations (20)
Final low-risk populations (11)
Sources: Esri, HERE, DeLorme, TomTom, Intermap, increment P Corp., GEBCO, USGS, FAO, NPS, NRCAN, GeoBase, IGN, Kadaster NL, Ordnance Survey, Esri Japan, METI, Esri China (Hong Kong), swisstopo, MapmyIndia, © OpenStreetMap contributors, and the GIS User Community | FEMA MOTF
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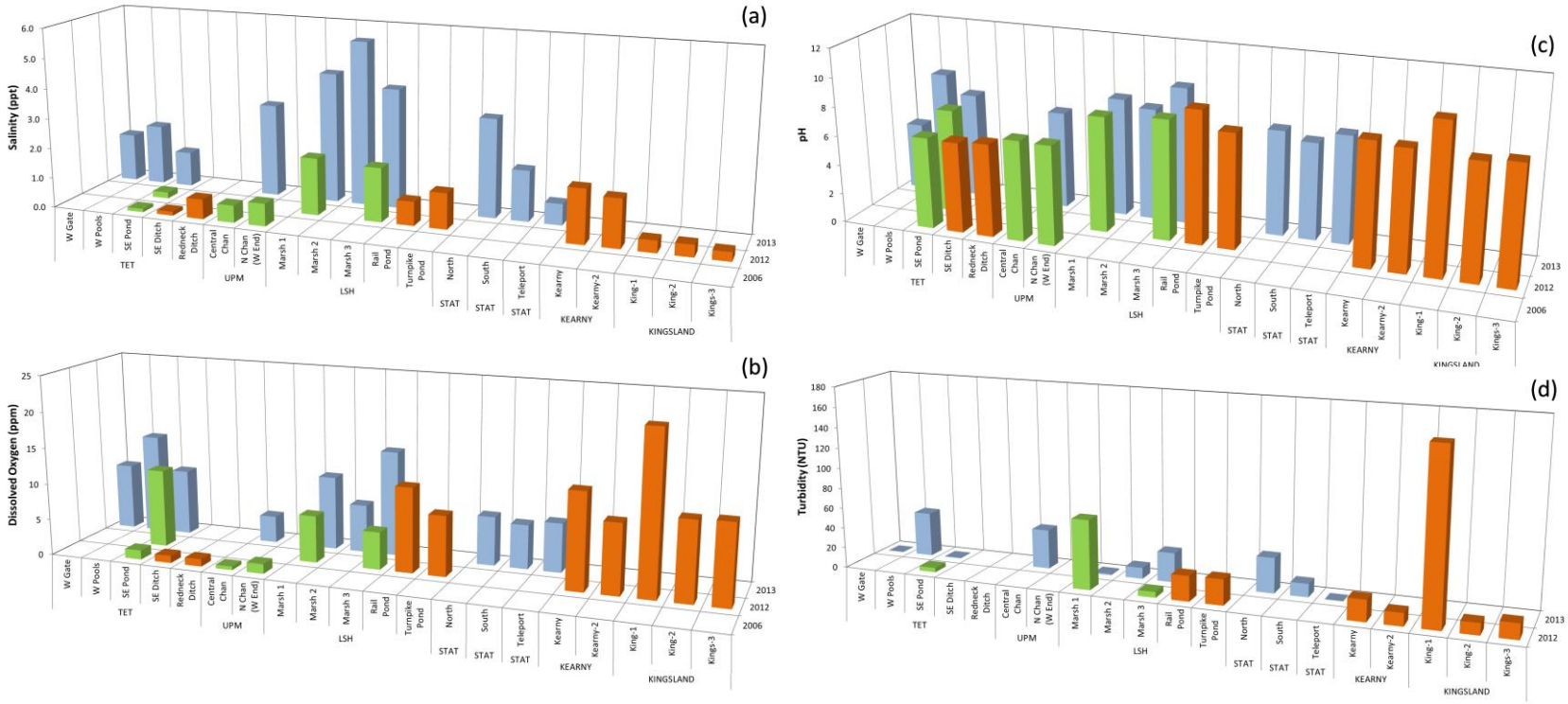
Figure 4


(a)
Salinity (ppt)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
(b)
Dissolved Oxygen (ppm)
25
20
15
10
5
0
(c)
pH
12
10
8
6
4
2
0
(d)
Turbidity (NTU)
180
160
140
120
100
80
60
40
20
0
W Gate
W Pools
SE Pond
SE Ditch
Redneck Ditch
Central Chan
N Chan (W End)
Marsh 1
Marsh 2
Marsh 3
Rail Pond
Turnpike Pond
North
South
Teleport
Kearny
Kearny-2
King-1
King-2
Kings-3
TET
UPM
LSH
STAT
KEARNY
KINGSLAND
2013
2012
2006

CONCLUSIONS

At a time of great concern regarding biodiversity loss and extirpations and extinctions among anurans, the discovery of a new leopard frog from a well-studied and urban region is both surprising and encouraging as it highlights the potential for hidden diversity in unexpected locales. This find, which ironically stemmed from research into a frog disappearance, demonstrates that we should not discount the possibility than new and unique species can still exist in some of the most unassuming and well-studied places on earth.

In chapter 1, leopard frogs from several atypical populations in NY and NJ were found to be genetically different from the widespread southern species, *Rana sphenocephala*, to which they had typically been included. The nuclear and mitochondrial data provided strong genetic evidence indicating that leopard frogs from these four focal study populations represented a new and previously undocumented cryptic genetic lineage, *R.* sp. nov. Further, no evidence of hybridization was found, although one example of sympatry was identified between *R.* sp. nov. and its northern congener, *R. pipiens*, in central CT. The discovery of *R.* sp. nov. in CT is interesting on several levels. For one, the CT locale was sampled as a control-only site for *R. pipiens* and was not thought to include members of the new species too. Second, it provided evidence of a fifth *R.* sp. nov population additional to the four focal populations in this study. Finally, it provided an unexpected and considerable extension of the known range for *R.* sp. nov. at that time, more than 80 km east of the nearest focal population.

In chapter 2, additional lines of evidence, including bioacoustics and morphology, were examined and used to provide the extra support needed to determine if *R.* sp. nov.

was indeed a distinct species. The results from the bioacoustic analysis provided strong evidence of separation between *R.* sp. nov. and its most cryptic congener, *R. sphenocephala*, as well as all three other examined congeners, *R. pipiens*, *R. palustris*, and *R. sylvatica*. The results from the morphometric analysis were less distinct overall, which is not surprising for morphologically similar cryptic species, but still revealed some significant character differences between *R.* sp. nov. and its congeners. Additional external morphological features were also considered, including color patterning on the dorsal surface of the hind legs, as a means of visually distinguishing *R.* sp. nov. from its congeners in the Tri-State area, though not necessarily beyond that region. Ultimately, the results from these analyses combined with genetic confirmation of a proposed Staten Island holotype, provided the necessary evidence to formally diagnose, describe, and name the new species as the Atlantic Coast leopard frog, *R. kauffeldi*. The species name is a patronym recognizing Carl F. Kauffeld, who likely pointed to this frog as a distinct species in the 1930s, but did not have the necessary lines of evidence to support his assertion. This study revealed additional areas of sympatry too, between *R. kauffeldi* and its most cryptic congener, *R. sphenocephala*, in several areas to the south.

The work presented in chapter 3 was conducted at a time when additional *R. kauffeldi* localities had been identified throughout the Tri-State area (after the original five areas from chapter 1). This study examined several population areas in and around New York City—including the type locality on Staten Island—after the impacts of Hurricane Sandy. At the time of the storm, understanding of *R. kauffeldi* and its basic ecology and range-wide distribution was still unclear. Thus, the extent to which the hurricane may or may not have impacted the entire species was not known. In response, I

studied five of the most vulnerable population areas to gauge impacts. Each area was located well within the floodplain extent of the tidal storm surge and showed elevated levels of salinity after the event. I found that *R. kauffeldi* survived at all five areas. In some cases, populations may have declined from pre-storm conditions, although such declines may have been the result of other non-storm factors. In other cases, populations were found to be larger and more extensive than pre-storm levels. On average, salinity increased by more than 200% across examined wetlands. The results from this study show that populations survived both the direct physical impacts from the storm as well as the long-term salinity increases that followed the flooding. These results suggest that *R. kauffeldi* has likely evolved to withstand hurricane-related perturbations in the coastal habitats where it occurs, at least to the extent seen here with Hurricane Sandy.

In summary, my dissertation is based around the revelation of an unexpected yet critical component to the story of leopard frogs from the eastern US. While this work shifted the focus away from my initial research on declines and extirpations, it helped unravel another important mystery and provided valuable insights that may have long-term impacts on how biodiversity is viewed (especially in unexpected areas) and the role of bioacoustics (in helping to identify cryptic species). My dissertation also highlights the role of large-scale collaborations as essential to the completion of ambitious undertakings, especially those with many different research components that require multiple areas of expertise. Hopefully, I will return to my original concerns regarding leopard frog extirpations, to complete the initial research I started. Any such work in the future will now be guided by a new and resolved understanding of the species involved and their distributions and compositions throughout the extirpation zone.