Cryptic Diversity in Metropolis: Confirmation of a New Le Species (Anura: Ranidae) from New York City and Surro Coast Regions

Jeremy A. Feinberg , Catherine E. Newman, Gregory J. Watkins-Colwell, Matthew D. Schlesinger, Brian Zarate
Joanna Burger

Published: October 29, 2014 • DOI: 10.1371/journal.pone.0108213

Abstract

We describe a new cryptic species of leopard frog from the New York City metropolitan area and surrounding coastal similar to two largely parapatric eastern congeners, *Rana sphenocephala* and *R. pipiens*. We primarily use bioacoust species, but also examine other lines of evidence. This discovery is unexpected in one of the largest and most densel demonstrates that new vertebrate species can still be found periodically even in well-studied locales rarely associated species typically occurs in expansive open-canopied wetlands interspersed with upland patches, but centuries of loss for conservation concern. Other concerns include regional extirpations, fragmented extant populations, and a restricte type locality within New York City and report a narrow and largely coastal lowland distribution from central Connecticu data) and south to North Carolina (based on call data).

Figures

Citation: Feinberg JA, Newman CE, Watkins-Colwell GJ, Schlesinger MD, Zarate B, et al. (2014) Cryptic Diversit Leopard Frog Species (Anura: Ranidae) from New York City and Surrounding Atlantic Coast Regions. PLoS ONE doi:10.1371/journal.pone.0108213

Editor: Helge Thorsten Lumbsch, Field Museum of Natural History, United States of America

Received: June 21, 2014; **Accepted:** August 18, 2014; **Published:** October 29, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All Supporting Information files.

Funding: Financial support for field work and analysis was primarily provided by a Rutgers Graduate School of N Fellowship and a New Jersey Department of Environmental Protection Conserve Wildlife Matching Grant to JAF, a to JB, and a Hudson River Foundation grant to JB and JAF. Partial support was also provided by the Rutgers Gra Brookhaven National Laboratory, the Foundation for Ecological Research in the Northeast, and the Tiko Fund. Fun

holotype, performed at the Louisiana State University Museum of Natural Science, was provided by NSF DEB-114
funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manusc

Competing interests: The authors have declared that no competing interests exist.

Introduction

In order to develop clear understandings of species and their ecologies, distributions, and conservation needs, they m
delimited [1]. Such efforts can be complicated, however, by the presence of cryptic species – species that, due to mo
included with one or more other species under a single species classification [2]. Identifying cryptic species can be dif
conservation challenges. These challenges can be further exacerbated in heavily altered environments and areas whe
insufficient numbers of individuals or populations for sampling. Nonetheless, a cryptic species discovery can have impo
the new species itself and its cryptic congeners [1]. Further, cryptic species can be found in unexpected locales [3], a
levels of diversity [4]. Left undetected, however, cryptic species can remain concealed among other species, which ca
widespread nominal species actually contain hidden component species that are range-restricted, rare, or even extinc

Considerable effort has been given to identifying and cataloging new species, cryptic and otherwise, over the past fev
efforts carry added urgency in the face of severe global declines and extinctions and also reveal strongholds of undoc
species richness or poorly known composition [4], [5]. In contrast, far less attention or discovery has been associated
or well-documented regions, especially those outside the tropics. Among anurans, for example, only two truly novel sp
recognized as subspecies) have been reported from the continental United States (US) and Canada since 1986 [3], [6
recent of these, a cryptic leopard frog lineage that was first identified from the New York City region in 2012 [3]. Few
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 m
occupies parts of the lower Northeast and mid-Atlantic US within the densely populated and heavily industrialized Inter
largest human population centers on earth [8] and a region where endemic vertebrate species are rare. The long-term
anuran here is both surprising and biogeographically significant, and illustrates how new species can occur almost any
conservation concerns: amphibians can be sensitive to disease, contaminants, and environmental perturbations, and th
in fragmented and urban landscapes [9]. Also worrisome are enigmatic declines that have led to disappearances of le
mid-Atlantic US [10]–[13]; this includes some relatively non-urbanized coastal, suburban, and agricultural regions in so
Connecticut (CT) [11], and presumably parts of northeastern Pennsylvania (PA) where they were reported historically,

Here, we expand upon the initial genetic results presented by Newman *et al.* [3] to name, diagnose, and describe the
supporting evidence, but focus on bioacoustic signals and molecular data. We also provide a brief history of relevant t
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 be
nomenclatural debate in eastern North America [21]–[27]. Our work resolves some of this confusion. In this section w
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 la
species concepts across those years. This has led to numerous synonyms and conflicting species frameworks over ti
R. sphenocephala and *R. pipiens*, received lasting consideration and taxonomic recognition in the east [26], [29]. *Ran*
has a reported range from extreme southeastern NY to Florida (FL) and west from Texas to Iowa [30]. *Rana pipiens*,
eastern Canada, New England, and the northern mid-Atlantic, west to the Pacific Coast states and British Columbia [3
along the US East Coast [29], [30], although Pace [26] reported one possible example of sympatry from Bronx County

Much of the historical discord and confusion surrounding the *R. pipiens* complex can be traced to the Northeast and m greater New York City metropolitan area [11], [33], [34] (referred to hereafter as the NY/NJ-metro area and defined t New Jersey [NJ], and extreme eastern PA). This relatively small region has been associated with longstanding ambigu 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, cen frog in the NY/NJ-metro area, between the recognized East Coast ranges of *R. sphenocephala* and *R. pipiens* at tha examinations with subspecies descriptions by Cope [36] and putative type localities for *R. pipiens* to conclude that thr Northeast and mid-Atlantic US. He classified the northernmost species as *R. brachycephala* and reassigned *R. pipien* northernmost species – to his proposed central species (occupying much of the NY/NJ-metro area and mid-Atlantic re plain and west to Texas); *R. sphenocephala* was maintained as the southernmost species. Despite acknowledging the this could cause, Kauffeld [33] proposed these changes to reflect his conclusion that the type locality for *R. pipiens* fe 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 to garner lasting support [23]–[25]. His proposals also provided the impetus for several studies that led to more conse predominant mid-20th Century single-species interpretation that classified all North American leopard frogs as *R. pipie* based on inconsistent differences among purported species and successful cross-breeding experiments with frogs fro decades later, relying primarily on morphology and bioacoustics, Pace [26] presented a detailed treatment of the *R. p* arrangement in the eastern US, echoing arrangements prior to Kauffeld's work [43]–[45]. This included *R. sphenocepl* the south, and *R. pipiens* to the north, with a species boundary centered in the NY/NJ-metro area. Pace's arrangeme decades, particularly across the eastern US.

Occasional discussion of distinct populations, potential intergradation, and cryptic species in the NY/NJ-metro area co largely speculative [11], [46], [47]. More recently, however, advances in molecular methods utilizing nuclear and mitoc sophisticated species delimitations and analyses of phylogenetic and population genetic relationships. Initial molecular suggesting that an undescribed cryptic leopard frog lineage, termed *R.* sp. nov., does indeed occur between populatic NY/NJ-metro area. They also reported mitochondrial data showing this species to be most closely related to the picke and readily identifiable species [29], rather than to *R. sphenocephala*, the species to which it had been included base 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 occurring in close proximity to several similar congeners. For example, in the Philadelphia region – an area replete wit among leopard frogs [26], [27], [48] – all four regional spotted congeners are now known to occur; *R. pipiens*, *R. pal* occur in succession along a narrow 90-km west-to-east transect between Berks County, PA and Burlington County, N

Materials and Methods

Ethics Statement

The species described here was discovered during research activities conducted under an Institutional Animal Care a Rutgers University (#07-024). Additional field work and collection of the holotype specimen occurred under New York 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 versus a recently proposed replacement name, *Lithobates*, which has been applied to a number of North American ra remains largely unsettled, we have followed the conservative taxonomic practice of continuing to use *Rana* for all Nort complex.

Morphology

Fieldwork to collect an adult male holotype was conducted in Richmond County, NY. The specimen was preserved in
70% ethanol and deposited at the Yale Peabody Museum of Natural History (YPM). We collected morphometric meas
the holotype (YPM 13217) and 282 other museum specimens across four species (*R*. sp. nov., *R. sphenocephala, R.*
seven eastern states, and Quebec, Canada (Table S1). When genetic data were not available to confirm species iden
morphology and location to classify preserved specimens based on our knowledge of species habitat preferences anc
measurements were taken to the nearest 0.01 mm with Mitutoyo Digimatic calipers. We measured 13 characters, 11
(SVL; anterior end of snout to posterior end of urostyle), head length (HL; anterior end of snout to occiput), head widt
diameter (ED; at widest point of eye), tympanum diameter (TD; at widest point of tympanum), foot length (FOL; tip of
(END; anterior eye to naris), naris to snout distance (NSD; naris to anterior end of snout), thigh length (THL; anterior k
(IND; closest distance between nares), and interorbital distance (IOD, closest distance between the eyes). We also ir
Heyer *et al.* [52] and dorsal snout angle (DSA; [arcsine ((HW/2)/HL) ×2) following Lemmon *et al.* [6].

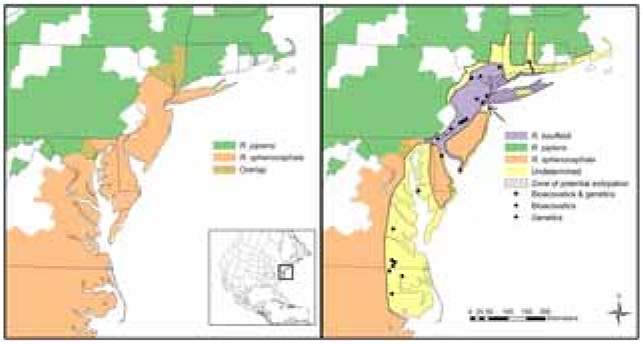


Figure 1. Leopard frog distributions in the Northeast and mid-Atlantic US.
Left: currently recognized IUCN (2012) range maps for *R. pipiens* (green) and *R. sphenocephala* (red) with areas
interpreted distributions for all three leopard frog species including *R. kauffeldi*. Symbols indicate known *R. kauffe*
areas where our field work has confirmed the occurrence of *R. kauffeldi*. Yellow shading indicates areas of less in
kauffeldi may occur in these areas based on habitat and proximity to known populations. Potential sympatry is als
R. sphenocephala (from Long Island southward), or *R. pipiens* (north and west of Long Island). The type locality f
doi:10.1371/journal.pone.0108213.g001

We looked for univariate differences in species morphology using boxplots and one-way ANOVAs followed by Tukey H
discriminant function analysis (DFA) to examine variation in multivariate space and determine which variables best disc
by a MANOVA to look for multivariate differences among species, and then Tukey HSD post-hoc pairwise comparison
among specimens, we removed this effect in our statistical analyses by using the residuals of a regression of snout-ve
length was not available for some specimens (*n* = 19), reducing the number of frogs with complete measurements to
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 (numbe
between the new species and its closest morphological congener, *R. sphenocephala*, following Platz [54]. For spot cc
(*R*. sp. nov., *n* = 22; *R. sphenocephala*, *n* = 18) into ArcMap 10.0 [55] and digitized polygons representing the dorsun
in order to calculate the proportion of the dorsal surface covered by spots. We examined both variables using boxplots
differences. We also conducted several categorical comparisons between *R*. sp. nov. and *R. sphenocephala*, includin
snout spot (present or absent), and 3) skin color (three color categories). We categorized a dorsal spot as ‘elongate’
its widest point, but excluded eyelid spots from this analysis because the curvature of the eye made them difficult to a
the posterior dorsal surface of the femur (thigh) among specimens of *R*. sp. nov., *R. sphenocephala*, and *R. pipiens*.
distinguish leopard frogs in regions where *R*. sp. nov. occurs [24], [32]. We follow Moore [24] in referring to it as the “
light (light ground color with dark spots) or dark (dark ground color with light spots). All specimens used in spot and cc
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 12S–16S regions of the mitochondrial genome, including intervening and flanking tRNAs (1444 bp), and the nuclear ge (Tyr, 557–585 bp), Rag-1 (647–683 bp), seven-in-absentia (SIA, 362–393 bp), and chemokine receptor 4 (CXCR4, 5 Beckman Coulter Genomics (Danvers, MA, USA). All sequences generated in this study were uploaded to GenBank (JX867559-JX867563). Data from the present study were added to the Newman *et al.* [3] data set, and Bayesian phyl [56], [57] for each locus following the analyses described in Newman *et al.* [3] to verify the species identity of the holo

Bioacoustic Analysis

We recorded calls of the new species with an Olympus DS-40 digital voice recorder and Sennheiser MKE 400 directic and 16-bit sampling size. We converted files to.wav format using Roxio Sound Editor (Sonic Solutions, Novato, CA, US [58] using the following settings: spectrogram FFT length 2048, Hanning window size 1024, amount of overlap betwee length 2048. We analyzed calls from three populations (two in Richmond County, NY; one in Bergen County, NJ). For c calls from four congeners using these same methods unless otherwise stated (Table S2); these included *R. sphenoce* acoustically similar species outside the leopard frog complex, *R. sylvatica*. We examined two populations of *R. sphen* Co., NJ), one population of *R. pipiens* (Columbia Co., NY), one population of *R. palustris* (Suffolk Co., NY), and three Suffolk Co., NY, and Larimer Co., Colorado). We did not collect frogs used in our call analysis, but deposited call vouc

We measured seven variables: call length (CL; time from beginning to end of a single call), call rate (CR; based on tim time (CRT; time from call start to maximum amplitude), call duty cycle (CDC; call length/[call length + time to next call a call), pulse rate (PR; based on time between start of first and last pulse), and dominant frequency (DF; frequency o parameters and terminology from Cocroft and Ryan [59] but follow Lemmon *et al.* [6] for CDC and PN. We derived tra individual unless otherwise noted (Table S2). For the purposes of this study, we examined only the primary mating call call by Heyer *et al.* [52]. This approach provided a clear means for comparing species and minimized confusion prese secondary repertoires were considered to fall outside the scope of our objectives and were not analyzed here. We co the same univariate and multivariate statistical procedures described for our morphological analyses. Call rate and cal temperature, so we adjusted these two parameters to a common water temperature of 14°C for our statistical analys regression equations from *R.* sp. nov. in place of *R. pipiens* and *R. palustris* because both species were recorded at 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 No contained herein are available under that Code from the electronic edition of this article. This published work and the r registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for thi urn:lsid:zoobank.org:pub:2E7F07A6-19B1-4352-B5B7-A227A93A37CD.The electronic edition of this work was publish archived and is available from the following digital repositories: PubMed Central and LOCKSS.

Results

Diagnosis and Description

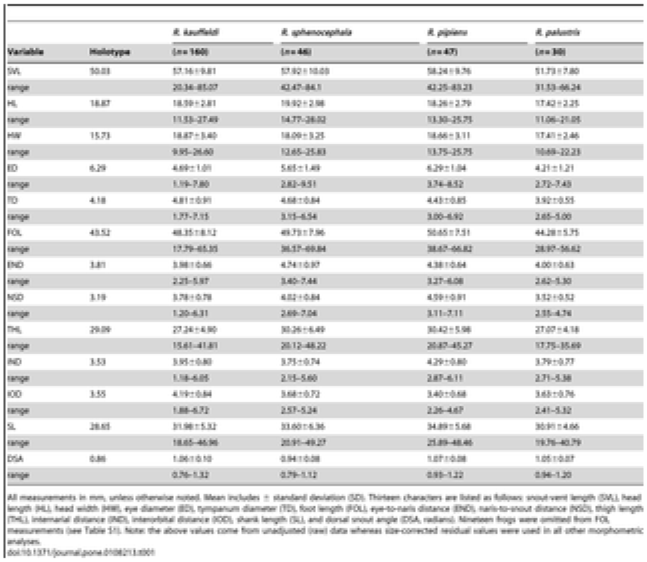
***Rana kauffeldi* sp. nov.**

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, Unit
Curry.

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 an
BRC (a), BZ (b), and GWC (c–d).
doi:10.1371/journal.pone.0108213.g002



		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	14.77–26.02	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.
doi:10.1371/journal.pone.0108213.t001

Table 1. Mean morphological parameters for four species of *Rana*.
doi:10.1371/journal.pone.0108213.t001

Paratypes.

YPM 13559, subadult male (paragenetypes: GenBank accession numbers JN227403, JN227458, JN227127, JN2271
YPM 13560, adult male (paragenetypes: GenBank accession numbers JN227404, JN227459, JN227128, JN227181,
collected from Wangunk Meadows in Portland, CT by T. Mahard and M. Blumstein on 15 September 2010; genetically
holotype [3].

Referred material.

YPM 13920, juvenile (GenBank accession numbers JN227377, JN227432, JN227102, JN227155, JN227209, JN2273
Feinberg from the type locality on 27 March 2009 (hatched in captivity and raised *in situ* within a field enclosure on Lo
project); genetically confirmed within the same clade as the holotype [3]. AMNH 121857–121858, juveniles; collected
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-r
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 synonyr
assigning a binomial and identified five candidates: *R. pipiens* Schreber [60], *R. halecina* Daudin [61], *R. utricularius*
and *R. brachycephala* Cope [36] as elevated to species rank by Kauffeld [33]. Based on our review and commentary
determined that none of these candidates has clear unequivocal support or the precise locality information or type spe
the new species. Most recently, Frost *et al.* [50] proposed *Lithobates pipiens* as a systematic replacement for *Rana*
and, as noted earlier, disagreements in the herpetological community as to the utility and appropriateness of *Lithobate*

We include *R. pipiens* as a synonym because its type locality has been restricted to various parts of the NY/NJ-metro
[63], [64]. However, given the lack of precision, geographic consensus, or a physical type specimen, Pace [26] desigr
central NY (UMMZ 71365). We follow Pace, and thus consider *R. pipiens* to be removed from further geographic con
Pace [26] that the frog illustrated by Schreber [60] most resembles the northernmost species, not the species describ
geographic range of *R. pipiens* is unwarranted and, despite the confusion and numerous synonymies from the NY/NJ-
warrants resurrection. We also refer briefly to Lavilla *et al.* [62] and point out that *R. halecina* was introduced to trans
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
genetics [3], 3) habitat (see *Distribution*), 4) geographic distribution (Fig. 1), and 5) a combination of morphological ch

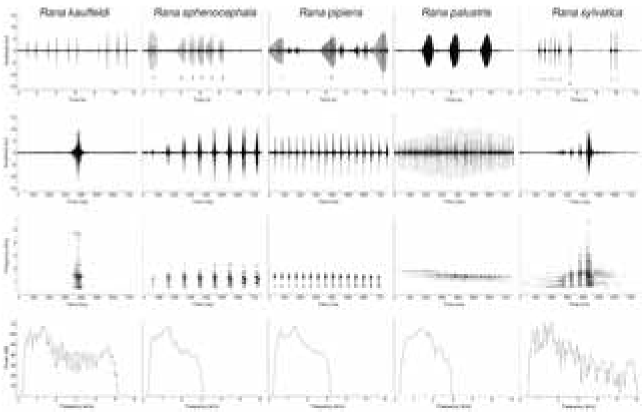
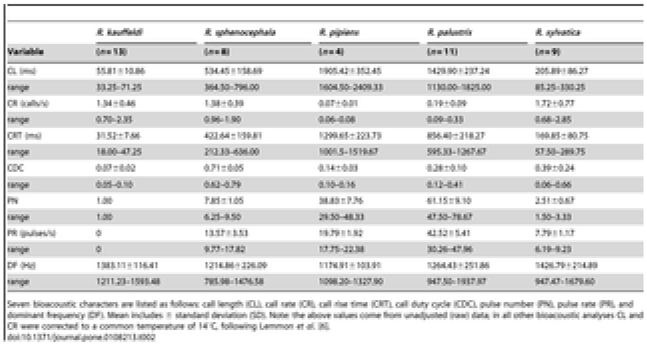


Figure 3. Primary (advertisement) calls of five *Rana* species from the study region.
Species include *R. kauffeldi* (column 1), *R. sphenocephala* (column 2), *R. pipiens* (column 3), *R. palustris* (columr
individuals were recorded within 8°C of each other at 10.0, 11.0, 18.0, 15.0, and 10.1°C, respectively. Row 1 shov
(12 s scale) (note: *R. pipiens* contains secondary grunts). Rows 2 and 3 show single-call waveforms and spectrog

shows power spectra for each single call. Numbers assigned to waveforms in row 1 indicate and identify different
et al. [6].
doi:10.1371/journal.pone.0108213.g003



	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	1216.86 ± 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].
doi:10.1371/journal.pone.0108213.t002

Table 2. Mean primary (advertisement) call parameters for five species of *Rana*.
doi:10.1371/journal.pone.0108213.t002

The advertisement call is a single-noted unpulsed 'chuck' (Video S1) that is distinct from the pulsed 'ak-ak-ak' of *R. sp*
pipiens and *R. palustris*. The quivering 'quack' of *R. sylvatica* is superficially similar but consists of discrete bouts of 2
accompanied by secondary 'groans' as occasionally emitted by *R. kauffeldi*. Although sympatric with *R. kauffeldi*, *R.*
distinct and typically calls from smaller canopied wetlands and forested pools whereas *R. kauffeldi* usually calls from

Adult male *R. kauffeldi* possess very large, laterally paired external vocal sacs that distinguish them from all similar co
Additionally, *R. kauffeldi* has a dark femoral reticulum (Fig. 4*a*) whereas northeastern populations of *R. sphenocepha*
(Fig. 4*b*). This diagnostic was 100% consistent in *R. kauffeldi* from NY and NJ (*n* = 27) and *R. pipiens* from the north
88.6% consistent in *R. sphenocephala* from NJ (*n* = 35). The diagnostic value of this character may be limited to nort
that leopard frogs predominantly exhibit a dark reticulum across portions of the Southeast where *R. sphenocephala* is

Figure 4. Reticulum shading patterns.
Examples include (a) dark state, *Rana kauffeldi* (YPM 14143); (b) light state, *R. sphenocephala* (YPM 14097); (c
(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

Rana kauffeldi may be further distinguished from *R. sphenocephala* by a tympanic spot that is typically duller, less we
sphenocephala); from *R. pipiens* by a light spot in the center of the tympanum that is often small and faint (but occasi
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. Nare
around two-thirds closer to tip of snout than anterior corner of eye. Canthus rostralis distinct and angular; loreal regior
protuberant; diameter slightly less than combined eye-to-naris and naris-to-snout distances. Internarial distance nearly
distinct and relatively large (>65% diameter of the eye); bordered dorsally and posteriorly by faint supratympanic fold.
from posterior eye to pelvic insertion of femur. Forearms relatively short and robust; unwebbed fingers; relative length
without expansion; subarticular tubercles small, round, and moderately prominent. No palmer tubercles appear preser
nuptial pad; all other fingers slender. Hindlimbs relatively long, moderately robust; thigh and shank length nearly equal.
rounded tips without expansion; subarticular tubercles small, round, and prominent. Inner tarsal fold connects tarsus tc
metatarsal tubercle. Indistinct, small outer metatarsal tubercle faintly evident. Toe IV very long and slender; toe V sligl
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 f
dorsolateral folds. Flanks, thighs, and shanks smooth. Ventral surface mostly smooth with papillae-like granulation on
external vocal sacs.

Color in life.

In photographs taken before preservation, dorsal ground color of holotype varies from mint-gray in bright lighting (Fig.
(Fig. 2*b*). Medium to dark brown spots irregularly distributed across dorsum and lateral body; more elongate or barre
encompasses dorsal and posterior tympanum along the supratympanic ridge. Labial margins slate gray with light mott
margin; terminates under the tympanum (continues to anterior forearm in females). Dark canthal band runs from snout
of dorsolateral fold; terminates above the arm. On snout, inner edge of canthal band is paralleled by light brown band
a dorsolateral fold that varies from gold (Fig. 2*a*) to bronze (Fig. 2*b*) in different lighting. Iris gold with dark intrusions a
surrounding skin. Lower flank of holotype pale with light yellowish-green hues and smaller, lighter spots and mottles; tl
body in some individuals. Tympanum finely granulated brown color with black flecks; central spot creamy and subtly de
entirely absent in some individuals. Reticulum and anterior ventral margin of thigh dark with distinct light flecks or mottl
bone-white (Fig. 4*a*), light yellow (Fig. 4*c*) or green (Fig. 4*d*) in some individuals. Ventral limbs of holotype pinkish-gray
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
specimens (as in paratypes YPM 13559 and 13560). Colored flecks and mottles in life appear white in preservative. V
mottling behind knees (Fig. 2*d*). Dorsolateral fold of holotype rust brown (Fig. 2*c*); off-white to brown in other individua
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 phyloge
nuclear haplotypes are identical to other *R. kauffeldi* samples. As reported by Newman *et al.* [3], *R. kauffeldi* is genet
spotted ranid frogs (*R. sphenocephala, R. pipiens, and R. palustris*). The mitochondrial phylogeny suggests that *R. k*
Average pairwise mitochondrial sequence divergence (uncorrected p) is similar to genetic divergences between other
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, I
and North Carolina [NC]) based on bioacoustic sampling reported here. The estimated range from these samples is a
central CT to northeastern NC (Fig. 1). The range is narrow, however, east-to-west, occurs almost entirely within the
than most if not all other ranid frogs along the eastern North American seaboard. Within the presented range, we depi
shading) where gaps in genetic and bioacoustic information were filled by other lines of evidence (e.g., specimens, ph
Rana kauffeldi appears to occur parapatrically in this core area. Beyond the core area, we depict an extended area c
based on habitat features and proximity to known bioacoustic confirmations in DE, MD, VA, and NC. Within the yellow
sympatry with *R. sphenocephala* (in the south) and *R. pipiens* (in the north) based on genetic, bioacoustic, and speci

Rana kauffeldi has a mesic distribution that is wider in the north and narrows from Trenton, NJ, to the Delmarva Penir
the Delaware River floodplain and the Atlantic Fall Line – the geologic interface between the relatively xeric Atlantic co
and more interior and upland regions to the west – where *R. pipiens* occurs. This species is usually abundant where i
area tend to be disjunct and isolated from one another and often occur in highly fragmented landscapes with limited co
kauffeldi was generally included within the range of *R. sphenocephala* prior to its discovery, but northern mainland po
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 m
We used multiple lines of evidence to inform this conclusion, including historical locality information [11], [33], photogra
personal communications (A. Sabin and F. C. Schlauch), and museum specimens (Table S1). Our assessment of mus
from Long Island (*n* = 27) and Bronx County, NY (*n* = 7). Based on our examination, 29 of these 34 frogs were *R. ka*
of Long Island, NY (Suffolk County), appeared to be *R. sphenocephala* (AMNH 125956, 176153). The remaining thre
106549, 106550) came from the Bronx County site previously noted by Pace [26] and Klemens *et al.* [31], where spe
106551–10654) were also collected historically. The third was a lone individual from western Long Island, in Queens
specimens (*n* = 9) from two presumably extirpated sites in southeastern CT (New Haven County) (Table S1). All were
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* a
palustris (Fig. S1). *Rana kauffeldi* had 1) the shortest eye-to-naris distance ($F_{3,279}$ = 28.41, $p<0.0001$), 2) shortest th
shortest shank length ($F_{3,279}$ = 27.95, $p<0.0001$) of the four species examined. *Rana kauffeldi* had 4) narrower eyes
($F_{3,279}$ = 14.59, $p<0.0001$), 6) and longer interorbital distance ($F_{3,279}$ = 35.02, $p<0.0001$) than *R. sphenocephala* and
shorter head than *R. sphenocephala* and a longer head than *R. pipiens*, ($F_{3,279}$ = 16.00, $p<0.0001$), 8) a longer intern
shorter internarial distance than *R. pipiens* ($\mathrm{F}_{3,279}$ = 8.48, $p<0.0001$), 9) a larger tympanum diameter than *R. pipiens*
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.*
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.
detected ($F_{3,260}$ = 120.0, $p<0.0001$). The DFA correctly classified 78.0% of specimens (Table S3). Post-hoc Tukey's
be significantly different from one another ($p<0.0001$) except for *R. sphenocephala* and *R. palustris* (p = 0.9966). The
58.4% of the variation in the data with tympanum diameter loading most heavily, while the second function accounted
distance having the greatest load (Table S4).

Previous studies report fewer and smaller dorsal spots among leopard frogs from areas where *R. kauffeldi* occurs [2
has fewer dorsal spots than *R. sphenocephala* (mean = 13.18±3.22 SD vs. 20.44±4.10 SD, respectively) (t = −4.32,
covered by spots (mean = 13.56%±3.29 vs. mean = 22.13%±7.76, respectively) (t = −6.12, two-tailed $p<0.0001$) (Fi
35.71% (*n* = 42) of *R. kauffeldi* had one or more elongated spot compared to 61.16% (*n* = 67) of *R. sphenocephala*
32.86% (*n* = 70) of *R. kauffeldi* versus 16.88% (*n* = 77) of *R. sphenocephala*. Lastly, we found considerable categori
75) (74.7% = dark olive to mint-gray, 24.0% = green to light brown, and 1.3% = bright green) and *R. sphenocephala*
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 recorded at multiple locations within the type locality. Five males (YPM 14137–14140; Table S2) were recorded at the heard calling and collected (but not recorded). These frogs were recorded between 2028 and 2042 h on 15 March 20 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 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 from one of these frogs (YPM 14137 and 14172) were used to represent temporal and spectral features for *R. kauffe* *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 opportunisti to 25.6°C (Table S2), reflecting the different geographies and phenologies among species. The temperature range wa 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* (9.68°C±0.94).

Our univariate analysis revealed significant differences among species in 6 of 7 call parameters (Fig. S4). *Rana kauffe* $p<0.0001$) and 2) shorter call duration than all other species ($F_{4,40} = 171.0$, $p<0.0001$), and 3) a lower pulse number (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 cy 37.8, $p<0.0001$), and 6) a call rate that was higher than *R. pipiens* and *R. palustris* and lower than *R. sylvatica* ($F_{4,40}$ 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 c 323.7, $p<0.0001$). The only classification errors were two *R. sylvatica* classified as *R. kauffeldi* (Table S5). Post-hoc comparisons to be significantly different from one another ($p<0.001$) except for *R. kauffeldi* and *R. sylvatica* ($p = 0.9$ because *R. kauffeldi* has only one pulse per call. The first discriminant function accounted for 61.0% of the variation i heavily, while the second function accounted for 24.3% of the variation with call length contributing the greatest load (T

Ecology, Behavior, and Natural History

Rana kauffeldi inhabits a restricted range of mesic lowland habitats that primarily includes coastal freshwater wetland riparian valley floodplains. This species is typically associated with large wetland complexes composed of open-canop systems with ample open upland and early-successional habitats. Aquatic conditions are usually clear, shallow, and sc 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 o observed migratory activity on rainy nights with above-average temperatures in early February, and have documented above-average temperatures in early-to-mid March. Choruses are most consistent nocturnally, with air temperatures r and nocturnal chorusing is common early in the season and through the initial 2–3 week peak breeding period (late Ma warmer days. Thereafter, chorusing tapers to a more episodic nocturnal and precipitation-based regime from mid-Apr observed opportunistic mid-summer chorusing as we and others [26], [71] have for *R. sphenocephala*, but we have ol 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 fro noted that leopard frogs (*sensu lato*) tend towards darker nocturnal shading and brighter, more vivid diurnal colors (as degree of seasonal color change also appears to exist in *R. kauffeldi*; we often observed frogs with darker, drabber c 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 fro Males call while floating in shallows with emergent vegetation and as little as 20 cm of water. As stated by Mathewso carry far. This is especially apparent in the presence of louder, higher pitched sympatric species like spring peepers (may have compensatory value, especially when faced with noisy conditions [74] or acoustic competition from other an clustered in groups or deposited near one another. Porter [32] and Moore [77] discussed eggs and embryonic develo *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 bein work, we observed individuals on land later in the season, but also noted periods, typically in summer and early fall, w is not specifically known, but is 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*) ar
from the US mainland and Canada to 30 [7]. Despite the vast size of this area, new frog discoveries north of Mexico a
significant. For example, *R. kauffeldi* and the Cajun chorus frog, *P. fouquettei,* [6] are the only newly described anurar
nearly three decades (since 1986) [7], and *R. kauffeldi* is the first anuran from the US Atlantic coast since the New Je
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 le
also significant. It provides an example of new species discovery, not from a tropical biodiversity hotspot or poorly stu
urban Northeast; one of the most developed, heavily settled, and well-inventoried places on earth. Novel and undescril
(particularly amphibians) and thus carry considerable interest and value. The last amphibian described from NY or Nev
in 1882 [78], and *R. kauffeldi* follows the northern cricket frog, *Acris crepitans*, in 1854 [79], as the seventh amphibiar
warrant consideration. For one, this discovery clearly demonstrates that human knowledge of the natural world remair
Second, although new frog discoveries are generally uncommon north of Mexico, they do still occur periodically. Third,
and *P. fouquettei* [6]) are both cryptic species. Taken together, these points suggest that occasional future discoveries
probably in the form of additional cryptic species rather than morphologically distinct taxa (which are likely already cat

Although *R. kauffeldi* is a cryptic species, it is a relatively large, conspicuous, non-fossorial species nonetheless, and
and poorly documented within one of the largest population centers on earth [8] spanning eight eastern US states and
remarkable. As a point of comparison, we consider another cryptic species group from the eastern US, the gray treef
Despite being arboreal, smaller, and less conspicuous than leopard frogs, these two congeners were recognized as s
earlier (in 1966) by differences in their calls [7], [80].

In part, the sustained concealment of *R. kauffeldi* may have been due to its narrow and fragmented range, short and
(less audible) call. Repeated acoustic misidentification may have also played a concealing role; many colleagues with
from frog populations now known to be *R. kauffeldi*. Some attributed these calls to *R. sylvatica* in unusual habitats; ot
sphenocephala. Given these examples and the generally stereotyped and species-specific nature of frog calls [4], [9]
in identifying species, we encourage greater scrutiny and examination of aberrant calls elsewhere, especially when en
populations or regions. Such efforts may reveal additional diversity, especially in areas of systematic uncertainty or co
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 grou
case of *R. kauffeldi*, its discovery from the Northeast and mid-Atlantic US has direct consequences for three species
entirely from two cryptic congeners, *R. sphenocephala* and *R. pipiens*. Thus, the recognized distributions of both con
kauffeldi occurs alone. These changes will refine certain ecological understandings and distributional patterns too. For
statewide distribution in NJ, *R. sphenocephala* is now exclusively restricted to xeric habitats such as the Pine Barrens
from a previous range over a wide variety of habitats and geologies to a newly defined range that conforms to the coa
herpetofaunal species.

Distributional relationships vary between *R. kauffeldi* and its close congeners. The general distributions of *R. kauffeld*
reported in Newman *et al*. [3]) overlap broadly [29], [30], though we did not find them together in the field and noted d
keep the two species ecologically isolated. Conversely, the distribution of *R. kauffeldi* is generally parapatric with *R. s*
sympatry do exist with both species. Newman *et al.* [3] provided genetic evidence of sympatry without hybridization w
specimens noted by both Pace [26] and Klemens *et al.* [31] that suggest additional potential sympatry in northwesterr

AMNH 13114, 35139). We also identified areas of sympatry between *R. kauffeldi* and *R. sphenocephala* in southeast
American Amphibian Monitoring Program), and suspect additional overlap in southern locales. Lastly, based on museu
are now extirpated, we note several isolated examples of possible *R. sphenocephala* from xeric eastern Long Island,
Counties, NY (Table S1). Historical species composition in these areas remains unclear, however. These sparse samp
(and potential areas of overlap with *R. kauffeldi*) or possible human introductions; isolated geographic records can su
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 bio
species, identifying regional compositions and reassigning museum specimens can be challenging but important, espe
extirpations or species overlap. In our work, leopard frogs were simply unavailable across vast landscapes due to hab
were available, differentiating similar-looking congeners was difficult. To overcome such challenges, several strategies
using genetic and bioacoustic methods at sites where new species and their cryptic congeners still occur to delineate
hybridization; 2) using genetics and morphology to identify subtle physical differences, if any, between species; and 3)
and extirpated locales to help assess historical compositions and distribution where populations no longer exist. These
archival specimens when possible) can link genetic and bioacoustic tools with museum specimens and morphology an
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
management levels. This will include possible threatened or endangered species considerations in certain areas, and
R. kauffeldi and its cryptic congeners in some of these impacted areas. It may also provide further opportunity to inve
boundaries throughout different parts of the range. This may be challenging, however, especially in states where leopa
protections and in areas where multiple species are found to co-occur. Thus, reliable, field-ready characters that disti
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
proper conservation measures [2], [3]. It also reinforces the critical role that basic natural history and alternative meth
in distinguishing potentially rare cryptic species. Lastly, it demonstrates that undocumented species can still reside in
inhabited parts of the world; these areas can harbor significant biodiversity and, with proper management, simultaneo
educational opportunities to urban communities. The United Nations Environment Programme and US Fish and Wildlife
both focused recent efforts on protecting urban biodiversity and enhancing the value and scope of urban wildlife refuge
important observation to the growing consensus that we must protect sensitive species where they occur, not just in p
provide invaluable opportunities to highlight and enhance access for increasingly urban societies to experience new sp
concern firsthand.

The overall conservation status of *R. kauffeldi* awaits further definition of distribution and habitat use and should be co
classification system. On-the-ground assessments, coupled with genetic and bioacoustic data, will be critical to this a
boundaries and overlap with related taxa. If the distribution is indeed narrow and fragmented (as reported here), it ma
geographically restricted species are often at risk of extinction due to demographic stochasticity [81]. Several other c
First, survival prospects of *R. kauffeldi* populations in the NY/NJ-metro area vary from tenuous to stable, with the mos
small and isolated and threatened by succeeding canopy closure and development. Second, dense breeding groups a
essential features of *R. kauffeldi* demography, but may also represent key vulnerabilities in the face of habitat impacts
in noting metapopulation susceptibility, habitat impacts, and canopy closure as potential threats for *R. pipiens*. Lastly,
sea levels, increased storm frequencies and intensities) have the ability to alter coastlines and threaten proximate low
populations therein with potentially harmful saline inundation.

Leopard frogs (*sensu lato*) have already vanished from some parts of North America [30] including several areas spe
[10], [11], [13]. Some of these disappearances were likely caused by direct habitat loss or alteration, especially in urb
occurred enigmatically within less-developed coastal, suburban, and semi-rural areas (Fig. 1); this includes Long Islan
US and a former leopard frog stronghold [10] where potential causes of extirpations (e.g., disease, invasive species,

A. Feinberg and J. Burger, unpublished data). Counterintuitively, *R. kauffeldi* persists in several locales within New Yor Meadowlands. These sites are heavily industrialized and have endured severe long-term anthropogenic impacts and ir *australis*. Most offer large habitat areas, however, which may provide an important clue to survival. The surprising per landscapes, while not completely understood, is encouraging and may have implications for management and restorat

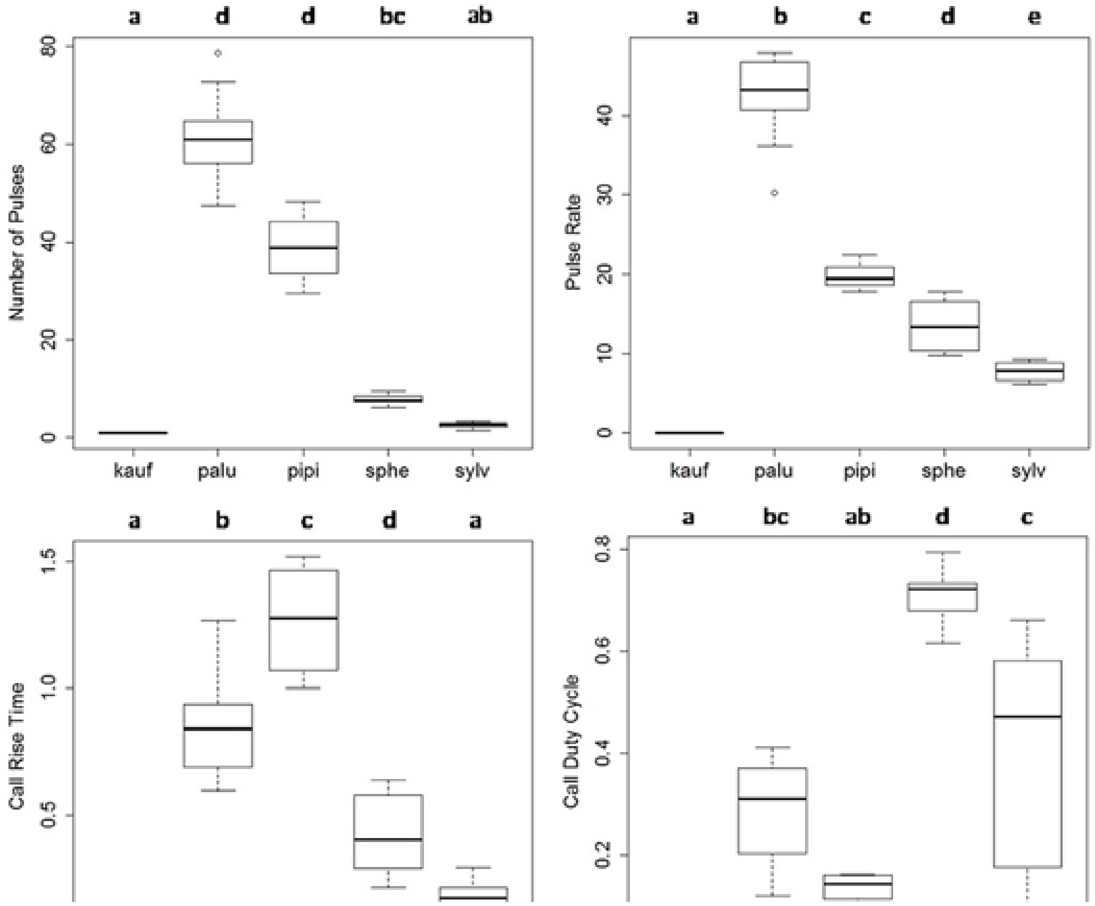
We finish with a cautionary note regarding reintroductions, repatriations, and translocations. Moving species to restore conservation and management practice, but one that can have unintended risks and consequences. For example, had on Long Island before the 2007 discovery of extant populations on nearby Staten Island (that were later found to be R *sphenocephala*) would have been moved from known populations farther to the south that harbor *R. sphenocephala*, systematics and population genetics at both donor and recipient site ends is critical to responsibly conducting any sucl

Conclusions

In diagnosing, describing, and defining the Atlantic Coast leopard frog, *R. kauffeldi*, we add a new and potentially at-r northeastern and mid-Atlantic US fauna. *Rana kauffeldi* can be characterized as 1) potentially vulnerable with highly sp locally abundant where present, but often only occurring in isolated and scattered locales; 3) having a restricted distrib regions; and 4) having suffered extirpations from certain areas. Concerns over habitat loss and degradation continue t disease, contaminants) that may pose additional future challenges.

Supporting Information

Figure_S1.tif



figshare 1 / 14

download

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 = 25th–75th quartiles, whiskers = minimum and maximum values but exclude outliers (represented by open circles). For each character, species whose measurements differed significantly (*P*

Figure S1.

Box and whisker plots comparing the size-corrected residuals of 12 morphological characters among four *Ra*
(kauf), *R. palustris* (palu), *R. pipiens* (pipi), and *R. sphenocephala* (sphe). For whisker plots, black bars = median, bo
and maximum values but exclude outliers (represented by open circles). For each character, species whose measurer
one-way ANOVA are denoted with different letters atop the plot. Side notches in boxes indicate significantly different r

doi:10.1371/journal.pone.0108213.s001

(TIF)

Figure S2.

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

doi:10.1371/journal.pone.0108213.s002

(TIF)

Figure S3.

Box and whisker plots comparing spot features between *Rana kauffeldi* (kauf) and *R. sphenocephala* (sphe).
proportion of dorsal surface covered by spots. For whisker plots, black bars = median, boxes = 25th–75th quartiles, v
exclude outliers (represented by open circles). Side notches in boxes indicate significantly different medians.

doi:10.1371/journal.pone.0108213.s003

(TIF)

Figure S4.

Box and whisker plots comparing seven bioacoustic characters among five *Rana* species. Species include *R. k*
(pipi), *R. sphenocephala* (sphe), and *R. sylvatica* (sylv). For whisker plots, black bars = median, boxes = 25th–75th c
values but exclude outliers (represented by open circles). For each character, species whose measurements differed
denoted with different letters atop the plot. Call length and call rate were temperature-corrected.

doi:10.1371/journal.pone.0108213.s004

(TIF)

Table S1.

List of *Rana* specimens examined.

doi:10.1371/journal.pone.0108213.s005

(DOC)

Table S2.

List of *Rana* primary (advertisement) calls measured for bioacoustic data.

doi:10.1371/journal.pone.0108213.s006

(DOC)

Table S3.

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

doi:10.1371/journal.pone.0108213.s007

(DOC)

Table S4.

Coefficients for three discriminant functions (from four species of *Rana*) for each of 12 morphological charac
diameter (ED), tympanum diameter (TD), foot length (FOL), eye-to-naris distance (END), naris-to-snout distance (NS
(IND), interorbital distance (IOD), shank length (SL), and dorsal snout angle (DSA).
doi:10.1371/journal.pone.0108213.s008
(DOC)

Table S5.

Classification matrix for five *Rana* species using discriminant function analysis on bioacoustic variables.
doi:10.1371/journal.pone.0108213.s009
(DOC)

Table S6.

Coefficients for four discriminant functions (from five species of *Rana*) for each of six bioacoustic characters
(CRT), call duty cycle (CDC), pulse number (PN), and dominant frequency (DF).
doi:10.1371/journal.pone.0108213.s010
(DOC)

Table S7.

Underlying (raw) morphometric data.
doi:10.1371/journal.pone.0108213.s011
(XLSX)

Table S8.

Underlying (raw) bioacoustic data.
doi:10.1371/journal.pone.0108213.s012
(XLSX)

Table S9.

Underlying (raw) data for color and pattern analyses.
doi:10.1371/journal.pone.0108213.s013
(XLSX)

Video S1.

A male *Rana kauffeldi* emitting its primary (advertisement) call in foreground with several other males calling
***Pseudacris crucifer*).**
doi:10.1371/journal.pone.0108214.s014
(MOV)

Acknowledgments

We extend thanks to Chris Camacho, Erik Kiviat, Ellen Pehek, Susan Stanley, Ed Johnson, Jay Westerveld, Karena D
Gruner for critical field assistance; John Burnley, Frederick C. Schlauch, Erik Kiviat, Eric Klaastad, Robert Zappalorti,
feedback on distributions and natural history for Long Island and the NY/NY-metro area; and Tim Green, Michael Goc
logistical and technical support. We acknowledge John Bunnell, Jason Tesauro, Nate Nazdrowicz, John F. White Jr., a
unusual attributes or the potential for cryptic species among regional leopard frogs in the years prior to or during our
Nazdrowicz, Scott Bush, Michael Toroco, Jennifer Tennessen, Holly Niederriter, Kyle Loucks, Dave Golden, Chris Hob
for sending audio recordings. We also extend thanks to Julie Lockwood for editorial assistance and David Wake, Davi
thoughtful reviews and helpful comments. We are grateful for assistance provided by the following museums and their
of Drexel University (Ned Gilmore, Ted Daeschler), American Museum of Natural History (David Kizirian, Darrel R. Fro
Natural History (Stephen P. Rogers), and Sam Noble Oklahoma Museum of Natural History (Jessa Watters, Janlee P.
Philip Kuchuk at Yale Peabody Museum of Natural History for photographic assistance.

Author Contributions

Conceived and designed the experiments: JAF CEN GJWC MDS BZ HBS JB. Performed the experiments: JAF CEN
MDS. Contributed reagents/materials/analysis tools: JAF GJWC MDS BZ BRC JB. Wrote the paper: JAF CEN GJW

References

1. Angulo A, Icochea J (2010) Cryptic species complexes, widespread species and conservation: lessons from
marmoratus group (Anura: Leptodactylae). Syst Biodivers 8: 357–370 doi:10.1080/14772000.2010.507264.
View Article • PubMed/NCBI • Google Scholar

2. Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, et al. (2007) Cryptic species as a window on diversity a
doi:10.1016/j.tree.2006.11.004.
View Article • PubMed/NCBI • Google Scholar

3. Newman CE, Feinberg JA, Rissler LJ, Burger J, Shaffer HB (2012) A new species of leopard frog (Anura: Ra
Phylogenet Evol 63: 445–455 doi:10.1016/j.ympev.2012.01.021.
View Article • PubMed/NCBI • Google Scholar

4. Funk WC, Caminer M, Ron SR (2011) High levels of cryptic species diversity uncovered in Amazonian frogs.
doi:10.1098/rspb.2011.1653.
View Article • PubMed/NCBI • Google Scholar

5. Köhler J, Vietes DR, Bonett RM, García FH, Glaw F, et al. (2005) New amphibians and global conservation:
endangered vertebrate group. BioScience 55: 693–696 doi:[];10.1641/0006-3568(2005)055[0693:NAAGCA]2
View Article • PubMed/NCBI • Google Scholar

6. Lemmon EM, Lemmon AR, Collins JT, Cannatella DC (2008) A new North American chorus frog species (Amp
central United States. Zootaxa 1675: 1–30.
View Article • PubMed/NCBI • Google Scholar

7. Frost DR (2013) Amphibian species of the world: an online reference, v5.6 (9 January 2013). New York, USA:
Available: http://research.amnh.org/herpetology/amphibia/index.html. Accessed 2013 November 19.

8. Florida R, Gulden T, Mellander C (2008) The rise of the mega-region. Cambridge J Regions Econ Soc 1: 459-
View Article • PubMed/NCBI • Google Scholar

9. Wells KD (2007) The ecology and behavior of amphibians. Chicago, IL: University of Chicago Press. doi:10.72

10. Schlauch FC (1978) Literature review: endangered amphibians and reptiles. Pitch Pine Nat 4: 5–6.
View Article • PubMed/NCBI • Google Scholar

11. Klemens MW (1993) The amphibians and reptiles of Connecticut and adjacent regions. State Geol Nat Hist S
View Article • PubMed/NCBI • Google Scholar

12. Shiels AL (1999) Seeing spots: the northern leopard frog. Pennsylvania Angler and Boater 1999: 58–61.
View Article • PubMed/NCBI • Google Scholar

13. Kiviat E (2011) Frog call surveys in an urban wetland complex, the Hackensack Meadowlands New Jersey, in
http://www.urbanhabitats.org/v06n01/frogcallsurveys_full.html Accessed 2013 March 4..
View Article • PubMed/NCBI • Google Scholar

14. Latham R (1971) The leopard frog on eastern Long Island. Engelhardtia 4: 58.
View Article • PubMed/NCBI • Google Scholar

15. Surface HA (1913) The amphibians of Pennsylvania. Zool Bull Div Zool Pa Dep Agri 3: 66–152.
View Article • PubMed/NCBI • Google Scholar

16. Stanaka W (1933) A preliminary list of the amphibians of Lackawanna County. Proc Penn Acad Sci 7: 96–100
View Article • PubMed/NCBI • Google Scholar

17. Netting MG (1933) The amphibians of Pennsylvania. Proc Penn Acad Sci 7: 100–110.
View Article • PubMed/NCBI • Google Scholar

18. McCoy CJ (1982) Amphibians and reptiles in Pennsylvania: checklist, bibliography, and atlas of distribution. Sp
View Article • PubMed/NCBI • Google Scholar

19. Shaffer LL (1991) Pennsylvania amphibians and reptiles. Harrisburg, PA: Penn Fish Comm.

20. Hulse AC, McCoy CJ, Censky E (2001) Amphibians and reptiles of Pennsylvania and the Northeast. Ithaca, N

21. Holbrook JE (1836) North American herpetology. Philadelphia, PA: J. Dobson and Son.

22. Boulenger GA (1920) A monograph of the American frogs of the genus *Rana*. Proc Am Acad Arts Sci 55: 413
View Article • PubMed/NCBI • Google Scholar

23. Burt CE (1938) The frogs and toads of the southeastern United States. Trans Kansas Acad Sci 41: 331–367.
View Article • PubMed/NCBI • Google Scholar

24. Moore JA (1944) Geographic variation in *Rana pipiens* Schreber of eastern North America. Bull Am Mus Nat
View Article • PubMed/NCBI • Google Scholar

25. Wright AH, Wright AA (1949) Handbook of frogs and toads of the United States and Canada. 3rd edition. Ithac

26. Pace AE (1974) Systematic and biological studies of the leopard frogs (*Rana pipiens* complex) of the United
1–140.
View Article • PubMed/NCBI • Google Scholar

27. Brown LE, Smith HM, Funk RS (1977) Request for the conservation of *Rana sphenocephala* Cope, 1886, an
1826 and *Rana virescens* Cope, 1889 (Amphibia: Salientia). Bull Zool Nomenclature 33: 195–203.
View Article • PubMed/NCBI • Google Scholar

28. Hillis DM (1988) Systematics of the *Rana pipiens* complex: puzzle and paradigm. Annu Rev Ecol Syst 19: 39–
View Article • PubMed/NCBI • Google Scholar

29. Conant R, Collins JT (1998) A field guide to reptiles and amphibians: eastern and central North America. 3rd e

30. Lannoo MJ (2005) Amphibian declines: the conservation status of United States species. Berkeley, CA: Univer
/9780520235922.001.0001.

31. Klemens MW, Kiviat E, Schmidt RE (1987) Distribution of the northern leopard frog, *Rana pipiens*, in the lowe
Northeast Environ Sci 6: 99–101.
View Article • PubMed/NCBI • Google Scholar

32. Porter KR (1941) Diploid and androgenetic haploid hybridization between two forms of *Rana pipiens*, Schrebe

View Article • PubMed/NCBI • Google Scholar

33. Kauffeld CF (1937) The status of the leopard frogs, *Rana b*
Herpetologica 1: 84–87.
View Article • PubMed/NCBI • Google Scholar

34. Schmidt KP (1953) A check list of North American amphibians and reptiles. 6th edition. Chicago, IL: University

35. Kauffeld CF (1936) New York the type locality of *Rana pipiens*. Herpetologica 1: 11.
View Article • PubMed/NCBI • Google Scholar

36. Cope ED (1889) The batrachia of North America. Bull US Nat Mus 34: 1–525. doi: 10.5962/bhl.title.38254
View Article • PubMed/NCBI • Google Scholar

37. Stejneger LH, Barbour T (1939) A check list of North American amphibians and reptiles. 4th edition. Cambridg
doi:10.4159/harvard.9780674592728.

38. Bragg AN (1941) Some observations on Amphibia at and near Las Vegas, New Mexico. Great Basin Nat 2: 1
View Article • PubMed/NCBI • Google Scholar

39. Grant R (1941) Salientia of northern Pontiac County, Quebec. Copeia 1941: 151–153 doi:10.2307/1437739.
View Article • PubMed/NCBI • Google Scholar

40. Trapido H, Clausen RT (1938) Amphibians and reptiles of eastern Quebec. Copeia 1938: 117–125 doi:10.230
View Article • PubMed/NCBI • Google Scholar

41. Ruibal R (1957) An altitudinal and latitudinal cline in *Rana pipiens*. Copeia 1957: 212–221 doi:10.2307/14393
View Article • PubMed/NCBI • Google Scholar

42. Brown LE (1973) Speciation in the *Rana pipiens* complex. Am Zool 13: 73–79 doi:10.1093/icb/13.1.73.
View Article • PubMed/NCBI • Google Scholar

43. Dickerson MC (1906) The frog book: North American toads and frogs, with a study of the habits and life histo
York, NY: Doubleday Page and Company. doi:10.5962/bhl.title.1542.

44. Stejneger LH, Barbour T (1933) A check list of North American amphibians and reptiles. 3rd edition. Cambridg

45. Wright AH, Wright AA (1933) Handbook of frogs and toads of the United States and Canada. 1st edition. Ithac
doi:10.5962/bhl.title.6753.

46. Schlauch FC (1971) The subspecific status of leopard frogs of a region in the Pine Barrens of Long Island. En
View Article • PubMed/NCBI • Google Scholar

47. Moore JA (1975) Rana-pipiens: the changing paradigm. Am Zool 15: 837–849 doi:10.1093/icb/15.4.837.
View Article • PubMed/NCBI • Google Scholar

48. Harlan R (1826) Descriptions of several new species of batrachian reptiles, with observations on the larvae of
View Article • PubMed/NCBI • Google Scholar

49. Conant R (1979) A zoogeographical review of the amphibians and reptiles of southern New Jersey, with emph
editor. Pine Barrens ecosystems and landscapes. New Brunswick, NJ: Rutgers University Press. pp. 467–488

50. Frost DR, Grant T, Faivovich J, Bain RH, Haas A, et al. (2006) The amphibian tree of life. Bull Am Mus Nat Hi
doi:[];10.1206/0003-0090(2006)297[0001:TATOL]2.0.CO;2.

View Article • PubMed/NCBI • Google Scholar

51. Napoli MF (2005) A new species allied to *Hyla circumdata*
southeastern Brazil. Herpetologica 61: 63–69 doi:10.1655/03-41.
View Article • PubMed/NCBI • Google Scholar

52. Heyer WR, Rand AS, Rand C, Cruz AG, Peixoto OL, et al. (1990) Frogs of Boracéia. Arquivos de Zoologia 3
View Article • PubMed/NCBI • Google Scholar

53. R Development Core Team (2011) R: a language and environment for statistical computing. R Foundation for
Available: http://www.R-project.org/.

54. Platz JE (1972) Sympatric interaction between two forms of leopard frog (*Rana pipiens* complex) in Texas. C
View Article • PubMed/NCBI • Google Scholar

55. ESRI (2010) ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute, Inc.

56. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:
View Article • PubMed/NCBI • Google Scholar

57. Ronquist F, Huelsenbeck JP (2003) MRBAYES v.3: Bayesian phylogenetic inference under mixed models. Bioi
doi:10.1093/bioinformatics/btg180.
View Article • PubMed/NCBI • Google Scholar

58. Charif RA, Waack AM, Strickman LM (2010) RAVEN Pro v.1.4, user's manual. Ithaca, NY: Cornell Lab of Orni

59. Cocroft RB, Ryan MJ (1995) Patterns of advertisement call evolution in toads and chorus frogs. Anim Behav
View Article • PubMed/NCBI • Google Scholar

60. Schreber H (1782) Beitrag zur naturgeschichte der frösche. Der Naturforscher 18: 182–193.
View Article • PubMed/NCBI • Google Scholar

61. Daudin FM (1802) Histoire naturelle des rainettes, des grenouilles et des crapauds. Paris, France: Levrault.

62. Lavilla EO, Langone JA, Caramaschi U, Heyer WR, De Sa RO (2010) The identification of *Rana ocellata* Linn
species currently known as *Leptodactylus ocellatus* (Leptodactylidae) and *Osteopilus brunneus* (Gosse, 185
View Article • PubMed/NCBI • Google Scholar

63. Stejneger LH, Barbour T (1923) A check list of North American amphibians and reptiles. 2nd edition. Cambridg

64. Kellogg R (1932) Mexican tailless amphibians in the United States National Museum. US Nat Mus Bull 160: 1–
View Article • PubMed/NCBI • Google Scholar

65. Smith PW (1961) The amphibians and reptiles of Illinois. Ill Nat Hist Surv Bull 28: 1–298.
View Article • PubMed/NCBI • Google Scholar

66. Savage JM (2002) The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, betw
Chicago Press.

67. Overton F (1914a) Long Island fauna and flora III: the frogs and toads. Mus Brooklyn Inst Arts Sci Bull 2: 21–
View Article • PubMed/NCBI • Google Scholar

68. Overton F (1914b) The frogs and toads of Long Island. Brooklyn Mus Q 1: 30–38.
View Article • PubMed/NCBI • Google Scholar

69. Villani R (1997) Long Island: a natural history. New York, NY: Harry N. Abrams.

70. Sherwood WJ (1898) The frogs and toads found in the vicinity of New York City. Proc Linn Soc NY 10: 9–24.
View Article • PubMed/NCBI • Google Scholar

71. Bridges AS, Dorcas MF (2000) Temporal variation in anuran calling behavior: implications for surveys and mor
doi:%%;10.1643/0045-8511(2000)000%5B0587:TVIACB%5D2.0.CO;2.
View Article • PubMed/NCBI • Google Scholar

72. Holmes SJ (1916) The biology of the frog. New York, NY: Macmillan. doi:10.5962/bhl.title.30369.

73. Mathewson R (1955) Reptiles and amphibians of Staten Island. Proc Staten Island Inst Arts Sci 17: 28–50.
View Article • PubMed/NCBI • Google Scholar

74. Wollerman L (1999) Acoustic interference limits call detection in neotropical frog *Hyla ebraccata*. Anim Behav
View Article • PubMed/NCBI • Google Scholar

75. Gerhardt HC, Schwartz JJ (1995) Interspecific interactions in anuran courtship. In: Heatwole H, Sullivan BK, e
behavior. Chipping Norton, NSW, Australia: Surrey Beatty and Sons. pp. 603–632.

76. Penna M, Velasquez N (2011) Heterospecific vocal interactions in a frog from the southern temperate forest, *E*
View Article • PubMed/NCBI • Google Scholar

77. Moore JA (1949) Geographic variation of adaptive characters in *Rana pipiens* Schreber. Evolution 3: 1–24 do
View Article • PubMed/NCBI • Google Scholar

78. Hinckeley MH (1882) On some differences in the mouth-structure of tadpoles of the anourous batrachians four
21: 307–314.
View Article • PubMed/NCBI • Google Scholar

79. Smith HH, Zappalorti RT, Breisch AR, McKinley DL (1995) The type locality of the frog *Acris crepitans*. Herpet
View Article • PubMed/NCBI • Google Scholar

80. Johnson C (1966) Species regulation in the *Hyla versicolor* complex. Texas J Sci 18: 361–364.
View Article • PubMed/NCBI • Google Scholar

81. Lande R (1988) Genetics and demography in biological conservation. Science 241: 1455–1460 doi:10.1126/sc
View Article • PubMed/NCBI • Google Scholar

82. Rorabaugh JC (2005) *Rana pipiens*, Schreber 1782 northern leopard frog. In: Lannoo MJ, editor. Amphibian c
States species. Berkeley, CA: University of California Press. pp. 570–577.

83. Burger J, Feinberg JA, Jeitner C, Gochfeld M, Donio M, et al. (2014) Selenium: mercury molar ratios in bullfro
northeastern United States. Ecohealth 10: 1–10 doi:10.1007/s10393-014-0913-3.
View Article • PubMed/NCBI • Google Scholar