Distribution, Identification, Landscape Setting, and Conservation of Rana kauffeldi in the Northeastern U.S.

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in the Coastal Northeast")
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Cover photo: Atlantic Coast leopard frog (*Rana kauffeldi*) by Matthew D. Schlesinger. All photographs within by the authors and field staff.

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Authors' Note: As in some of the authors' previous publications on this newly described species (Newman *et al.* 2012, Feinberg *et al.* 2014), we retain the genus *Rana* for North America's ranid frogs, which include the leopard frogs. While some herpetological societies have adopted the genus *Lithobates*, others still use *Rana* and recent research (Yuan *et al.* 2016) has rejected *Lithobates* as a proper genus.

Executive Summary

Biological inventories aimed at enumerating a region's species, combined with detailed natural history observation, can reveal evidence of cryptic species: overlooked species incorrectly grouped under a single taxonomic name. The identification of cryptic species raises fundamental questions about each species' distribution, identification, and conservation status. Leopard frogs in the northeastern United States have faced this situation since the recent discovery of *Rana* (= *Lithobates*) *kauffeldi*, the Atlantic Coast leopard frog, as distinct from *R. sphenocephala* (southern leopard frog) and *R. pipiens* (northern leopard frog). Following on this discovery, the objectives of our study were to 1) Determine conclusively which leopard frog species occur presently and occurred historically in ten eastern U.S. states; 2) Refine the range of *R. kauffeldi* relative to the two other leopard frog species; 3) Map new, potentially reduced, ranges for the two congeners; 4) Assess the species' conservation status, particularly in areas where *R. kauffeldi* is already known to be of concern; 5) Contrast multi-level habitat associations among the three species; and 6) Improve upon the separation of species using acoustic and morphological field characters to facilitate future inventory, monitoring, and status assessments of the new species.

Our field work in 2014 and 2015 consisted of call and visual surveys to identify populations of each species, followed by sampling frog tissue for genetic analysis. We supplemented this work with examination of museum specimens and compilations of older survey data. Our study area was the northeastern portion of the Atlantic Coastal Plain, from southern New York and Massachusetts to northern North Carolina. We conducted call surveys at 1,004 point locations and collected tissue samples from 254 individual frogs of three species. All told, 244 of the original samples plus 50 individuals determined to be pure *R. kauffeldi*, *R. pipiens*, or *R. sphenocephala* from an earlier study went into the final genetic analysis, for a total of 294 frogs. Bayesian cluster analysis in Structure resolved three clusters. A total of 262 individuals fell into one of the three clusters unambiguously, including 111 *R. kauffeldi*, 79 *R. sphenocephala*, and 72 *R. pipiens*, with the remainder representing varying degrees of admixture.

We confirmed *Rana kauffeldi* in eight eastern US states: CT, NY, NJ, PA, DE, MD, VA, and NC. Eighty-nine percent of *R. kauffeldi* locations were within 20 km, 77% were within 10 km, and just under 50% were within 1 km of coastal waters. The range of *R. kauffeldi* that we drew covers just over 46,500 km^2. Our survey data also support the notion that *R. kauffeldi* has disappeared from a large part of its historical range in southern NY and CT, including much of the Hudson Valley and all of Long Island. We also report an apparent disappearance of *R. pipiens* from a large swath of the southern portion of its range from PA east through northwestern NJ, southeastern NY, southern CT, southern RI, and coastal MA, from where we confirmed several historical museum specimens as *R. pipiens*. In addition, we report a new northern range limit for *R. sphenocephala* in central NJ.

We found a near-perfect match of population-level calling with genetics of individual frogs, demonstrating that the unique call identified previously is reliably associated with genetic identity. To better understand morphological and color differences, we examined 912 photographs of 220 leopard frogs. *Rana kauffeldi* was readily distinguished from *R. pipiens* by its smaller spots, usual absence of a snout spot, and reticulum characterized as predominantly dark. No single character reliably distinguished *R. kauffeldi* and *R. sphenocephala*, but characters used in combination allowed reliable identification in most cases. Nearly all *R. kauffeldi* reticula were predominantly dark with small, unconnected dots of light pigment, while most *R. sphenocephala* reticula were predominantly light with large, connected splotches of dark pigment. Random forest analysis using 144 individuals correctly classified *R. kauffeldi* and *R. sphenocephala* in over 90% of cases.

In the southern portion of its range, *R. kauffeldi* occurs primarily in riparian cypress-gum swamps, and on the Delmarva Peninsula it occupies large coastal cattail (*Typha*) and common reed (*Phragmites australis*) marshes that may be subject to salinity intrusions. In the northern portion of its range, *R. kauffeldi* occupies large freshwater wetlands, typically with open canopies, that otherwise are indistinguishable from similar large, open, freshwater wetlands where it was not detected. A distribution model built from random forest analysis shows that suitable habitat for *R. kauffeldi* is concentrated along the coast and in riparian corridors. The most important variables shaping the distribution of suitable habitat were elevation, impervious surface and wetlands in the surrounding landscape, and distances to calcareous bedrock, saltwater or freshwater emergent wetlands, freshwater forested wetland, and lakes and rivers.

Our multi-year, 10-state project demonstrated conclusively that *R. kauffeldi* is a habitat specialist with a small range centered in the most densely populated region of the United States. In several northern states it is extremely rare, while in the southern portion of its range it can be broadly distributed and abundant. In the core of its range in the mid-Atlantic U.S., south of the glaciated region, *R. kauffeldi* is a species exclusively of the Coastal Plain physiographic province. Since the last glacial maximum was reached over 20,000 ybp, *R. kauffeldi* has colonized some previously glaciated regions along major river valleys to the north and east, and now occurs in the Piedmont, New England, and Valley and Ridge provinces. *Rana kauffeldi* overlaps with, and at the northern edge of its range, supplants the previously known species, *R. sphenocephala*.

While *R. kauffeldi* is locally abundant and likely secure at the core of its range, it is vulnerable in places. *Rana kauffeldi* appears to have the smallest range of any ranid frog on the East Coast, and only two anurans along the East Coast north of Florida have smaller ranges. A small range may make a species more susceptible to stochastic events, and for frogs, may exacerbate the impact of fungal pathogens like *Batrachochytrium dendrobatidis*. Further, most populations of *R. kauffeldi* exist within a highly developed urban and suburban matrix, and the frog's need for larger wetlands (as opposed to *R. sphenocephala*, which may occupy small ponds) may render it vulnerable to habitat fragmentation that results in inhospitable dispersal habitat. Another point of concern for *R. kauffeldi* is the coastal proximity of many populations. Coastal populations of wetland organisms may be threatened by rising sea levels and increasing frequency and intensity of coastal storms, two threats that have been connected to climate change.

As with any newly described species, there is still much to learn about *R. kauffeldi*'s ecology and natural history. In addition to continued research into distinguishing morphological features, descriptions of *R. kauffeldi* egg masses and tadpoles are lacking. Many states have gaps in local distributional information which, along with an understanding of population vulnerability in habitat patches of different sizes and degrees of urbanization, are a critical need for a better understanding of the conservation status of *R. kauffeldi*.

Introduction

Conservation biologists are in broad agreement that a clear understanding of a region's species is necessary for biodiversity conservation (Savage 1995, Wheeler 1995, Balmford and Gaston 1999). Most also recognize that knowledge of the status and distribution of individual species within a region is vital for management, as species have distinct habitat needs, ecologies, and behaviors that often require specific policies and management considerations (Tracy and Brussard 1994, Mace 2004, Lindenmayer *et al.* 2007, White *et al.* 2013). Biological inventories aimed at enumerating a region's species, combined with detailed natural history observation, can reveal evidence of cryptic species, defined as overlooked species incorrectly grouped under a single taxonomic name (Bickford *et al.* 2007). In most cases, cryptic species represent two or more species that are morphologically similar to one another but phylogenetically distinct. For some frogs and birds, otherwise cryptic species often can be distinguished by their vocalizations, and thus bioacoustical analysis is a commonly used tool for recognizing new or overlooked species (Forti *et al.* 2017). Investigations have uncovered cryptic species in a variety of settings, revealing hidden diversity in surprising locations and among presumably well-known taxa (Bickford *et al.* 2007, Angulo and Icochea 2010, Gehara *et al.* 2013, Rodríguez *et al.* 2017).

The identification of cryptic species raises certain fundamental questions for conservation: 1) What is the distribution of each species? 2) How reliably can the species be distinguished from one another in the field? 3) What bearing does the discovery of one have on our knowledge of the other(s)? 4) Can we reconstruct the historical distributions of each species from museum specimens? 5) What is the conservation status of each species? and 6) Does the discovery upend our interpretation of the scientific literature on the complex of species? Thus, the clarification of a cryptic species complex is often followed by further investigation to answer these questions. For instance, the recent split of trilling chorus frogs and identification of a new species (Lemmon *et al.* 2007, 2008) prompted status assessments based on the newly identified species limits (Corser *et al.* 2012, Seburn *et al.* 2014). Leopard frogs in the northeastern United States have faced a similar situation since the recent discovery of *Rana* (= *Lithobates*) *kauffeldi*, the Atlantic Coast leopard frog, as distinct from *R. sphenocephala* (southern leopard frog) and *R. pipiens* (northern leopard frog) (Newman *et al.* 2012, Feinberg *et al.* 2014).

Although herpetologists had suggested that there might be undocumented leopard frog species in the northeastern U.S., (Kauffeld 1936, 1937, Klemens 1993), all the region's leopard frogs were considered *R. pipiens* for much of the previous century until that single-species framework gave way to a two-species taxonomy in the east (adding *R. utricularia*, later *R. sphenocephala*) following Pace (1974). Decades later, Newman et al. (2012) documented a distinct genetic lineage in leopard frogs from northern New Jersey, southern New York, and central Connecticut, and Feinberg et al. (2014) formally described this new species as *R. kauffeldi*, reporting a broader range (Connecticut to North Carolina) along with differences in the primary mating call, morphology, and patterning from those of *R. pipiens*, mainly to the north, and *R. sphenocephala*, whose more southerly range appeared to overlap partially with that of *R. kauffeldi*.

The preliminary range map in Feinberg et al. (Feinberg *et al.* 2014) was based on several lines of evidence: genetics from the New York City metropolitan area, museum specimens whose locality and physical appearance could be reliably associated with each species, and bioacoustic

sampling from as far south as North Carolina. They used data from three sites with co-located bioacoustics and genetic information to support their conclusion that the distinct mating call they documented was from a genetically distinct species. In addition, the Feinberg et al. (2014) paper built on previous observations of leopard frog patterning and morphology (Kauffeld 1936, 1937, Moore 1944, Pace 1974) to propose a set of characteristics for reliable distinction among the three species. Feinberg et al. (2014) concluded with a call for additional research to ensure that the patterns reported with few samples could be reproduced rangewide, to resolve areas of uncertainty across the entire putative range of *R. kauffeldi*, and to confirm characters that could be used to distinguish animals in the field without genetic analysis and outside the calling season.

The objectives of our study were as follows: 1) Determine conclusively which leopard frog species occur presently and occurred historically in ten eastern U.S. states; 2) Refine the range of *R. kauffeldi* relative to the two other leopard frog species; 3) Map new, potentially reduced, ranges for the two congeners; 4) Assess the species' conservation status, particularly in areas where *R. kauffeldi* is already known to be of concern; 5) Contrast multi-level habitat associations among the three species; and 6) Improve upon the separation of species using acoustic and morphological field characters to facilitate future inventory, monitoring, and status assessments of the new species. This paper is, in part, the follow-up study proposed by Feinberg et al. (2014).

Methods

Our field work in 2014 and 2015 consisted of call and visual surveys to identify populations of each species, followed by sampling frog tissue for genetic analysis. We supplemented this work with examination of museum specimens and compilations of older survey data. Our study area was the northeastern portion of the Atlantic Coastal Plain, from southern New York and Massachusetts to northern North Carolina. This study area was defined by the range map in Feinberg et al. (2014), focusing on areas of uncertainty.

Call and visual surveys

To locate populations of leopard frogs and identify sites for subsequent sampling, we conducted call surveys in a variety of wetland habitats throughout the study area. Dates of surveys ranged from late February to late March in southern latitudes and from late April to early June in northern latitudes in both 2014 and 2015. Because many survey partners had existing frog monitoring programs, and volunteers comprised a portion of the workforce, we allowed for considerable flexibility in survey methodology. At a minimum, observers recorded the GPS coordinates of sampling locations, time spent listening, and species detected. Sampling locations were selected by observers based on habitat suitability, access, and safety considerations. Observers were asked to spend a minimum of 3 minutes at each sampling location and record the survey duration. If survey duration was not recorded, we assumed the duration was 3 minutes. Surveys began no earlier than one half-hour after sunset and ended by 1:00 a.m. Surveys were not conducted in temperatures below 40 degrees Fahrenheit or in heavy rain or high wind. Observers made audio recordings of suspected leopard frog and the acoustically similar wood frog (*R. sylvatica*) calls for subsequent confirmation from members of the team most familiar with the species.

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From spring through fall in both 2014 and 2015, we visited sites where we had confirmed the presence of leopard frogs bioacoustically to capture frogs for photographic and genetic analysis. We also visited sites of unknown occupancy and suspected historical occupancy for visual surveys to determine presence. If we encountered leopard frogs, we captured them and clipped the last digit of one toe after following standardized photographical protocols (below). Tissue samples were stored in 95% ethanol and shipped to HBS's lab at the University of California, Los Angeles (UCLA) for genetic analysis. We followed the State University of New York College of Environmental Science and Forestry's Institutional Animal Care and Use Committee protocol #140102 for care and handling of captured frogs.

Photography

To aid in the identification of potentially reliable field characters, we photographed captured frogs from several specific angles (Figure 1) to clearly show the dorsal surface, snout profile, femoral reticulum, tympanum (right and left), and hind foot toe webbing, and matched each image with its corresponding tissue sample.

Figure 1. Example of photographs of a captured leopard frog taken from prescribed angles.

Earlier work (Feinberg *et al.* 2014) identified a series of morphological characters that differentiated early initial samples of *R. kauffeldi* from *R. pipiens,* and *R. sphenocephala* and formed part of the basis of the description of *R. kauffeldi.* We used our expanded photographic data set to test the validity of these characters across the (currently unknown) range of the species. A naïve single observer (ELW) with no explicit knowledge of the character states suspected to differentiate each species evaluated photos of each genotyped frog to assess 1)

number of dorsal spots from snout to vent occurring between the dorsolateral folds (conjoined spots were counted as two distinct spots); 2) snout spot (large, small, or absent); 3) snout shape (blunt, pointed, or intermediate); 4) reticulum coloration (predominantly dark, predominantly light, or intermediate); 5) reticulum pattern (mostly large, connected splotches; or mostly small, unconnected dots); 6) left and right tympanum spot pattern (sharp dot, sharp blotch, or present but indistinct); 7) left and right tympanum spot color (white/cream, green, or brown/bronze); and 8) webbing on the first toe of the left and right hind food (curves all the way to the tip or stops about halfway to the tip). For all characters evaluated qualitatively, the observer had exemplar photographs to guide in interpretation (Table 1).

We also used our dorsal photographs of genetically confirmed *R. kauffeldi* and *R. sphenocephala* to quantify snout shape and reticulum coloration, since these characters had been suggested previously to help distinguish the two species (Porter 1941, Feinberg *et al.* 2014). For snout shape, we imported each photo into ArcGIS 10.3 (ESRI 2014) and drew lines in four locations (Figure 2): A) along the posterior edge of the eyes, perpendicular to the spine of the frog, connecting the visible edges of the head ("head width"); B) perpendicular from the tip of the snout to line (A) ("head length"); C) along the anterior edge of the eyes but otherwise as in (A) ("snout width"); D) from the tip of the snout to line (C) 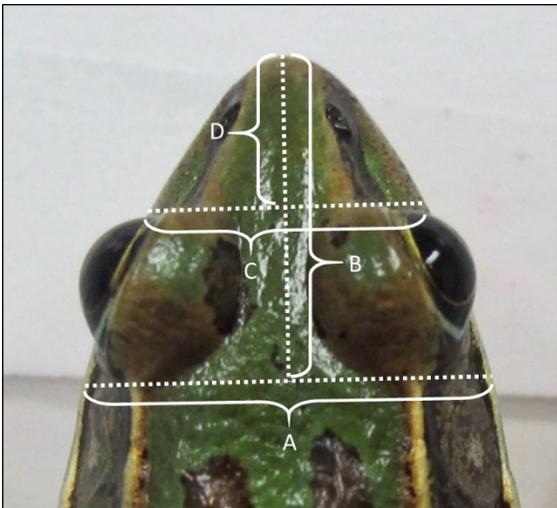("snout length"). We recorded the length of each line in arbitrary map units, and calculated three ratios: head length to head width (B/A), snout length to snout width (D/C), and snout width to head length (C/B). For femoral reticula coloration, we imported photographs with a clear view of the reticulum into ImageJ (Rasband 2016) and selected the area from the vent to the knee from the leg of the frog that had the greatest area exposed. We excluded photos with flash glare on the reticulum, ones that did not show approximately 75% of the reticulum, and ones depicting the reticulum as so dark that the software did not distinguish light and dark. We processed the selected area with the binary function to determine the percent of the reticulum that was dark.

Figure 2. Measurements of head width (A), head length (B), snout width (C), and snout length (D) in leopard frog photographs.

Table 1. Exemplar photographs of characters used in analysis of leopard frog morphology and patterning.

Dorsal spotting				Snout shape
Area of dorsum within which to count spots: between dorsolateral folds and from snout to vent, outlined in orange.	Large snout spot, spots larger than or equal to eye	Snout spot absent, spots larger than or equal eye	Small snout spot, spots smaller than eye	Blunt (left), pointed (right)

Tympanum spots				
Sharp dot, white/cream	Sharp blotch, white/cream	Present; indistinct, white/cream	Present; indistinct, white/cream	Sharp blotch, green
None	None	None	Sharp dot, white/cream	Present; indistinct, green

Femoral reticulum		
Predominantly dark, many small splotches, cream	Predominantly dark, many small splotches, cream	Predominantly light, mostly large connected splotches, cream

Predominantly light, mostly large connected splotches, cream	Predominantly light, mostly large connected splotches, green	Predominantly dark, many small splotches, green
Webbing of first toe on hind foot		
Webbing on first toe (at bottom) stops midway up	Webbing on first toe (at top) curves to tip	Photo inconclusive

Genetic analysis

Genomic DNA extraction and PCR amplification were performed at UCLA following the methods in Newman et al. (2012). We amplified 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 in two directions at Beckman Coulter Genomics (Danvers, MA, USA). Contigs were assembled and trimmed in Geneious version 6.1.6 (Kearse *et al.* 2012). Consensus sequences for each locus were aligned with sequences from Newman et al. (2012) using ClustalW in Geneious and manually adjusted.

To determine how all of the samples grouped into genetic population clusters, we used Structure version 2.3.2 (Pritchard *et al.* 2000, Falush *et al.* 2003) with an allelic data set (12 % missing data) derived from our sequence data. We inferred haplotypes for each locus in the five-locus sequence data set with a Bayesian algorithm using Phase version 2.1.1 (Stephens *et al.* 2001, Stephens and Donnelly 2003). Each allele represented a single haplotype. Phase input files were formatted from nexus files using a Perl script (RC Thomson, unpublished).

Following the same parameters as Newman et al. (2012), we used Structure to determine the number of genetically distinct clusters (K) in our complete data set. We used the admixture model (Pritchard *et al.* 2000) and assumed correlation of allele frequencies among clusters and no other *a priori* population information. We ran 20 iterations of K values from 1 to 10. Iterations had 100,000 generations and a burn-in of 100,000 generations. We chose the appropriate K value by obtaining likelihood scores using the Evanno method (Earl and vonHoldt 2012).

Observation data and museum records

To supplement our field work and inform our depiction of the historical and current ranges of each species, we gathered observational and survey data from a variety of sources. We compiled survey data from sources outside the project team in cases where call recordings were available

to confirm species identification. To determine the historical ranges of the three leopard frogs, we (MDS, JAF, KG, BZ, DQ) examined 1,944 museum specimens from the following institutions: American Museum of Natural History, Cornell University Museum of Vertebrates, North Carolina Museum of Natural Sciences, Smithsonian National Museum of Natural History, Harvard Museum of Comparative Zoology, and Carnegie-Mellon Museum of Natural History. Based on identification characters from Feinberg et al. (2014), we determined the species to the best of our ability. Several features that we characterized in our photographic analysis (for instance, presence and distinctness of tympanum spots) were difficult to discern on preserved specimens. We took photographs of all specimens but did not conduct the full photographic protocol in the interest of time.

Data analysis

Call surveys and genetics

To test whether the unique call described in Feinberg et al. (2014) was conclusively associated with frogs genetically confirmed to be *R. kauffeldi*, we examined the results of call surveys in the vicinity of locations of genetic samples for the three species. It was not feasible to match calls to genetics at the level of the individual frog because of the difficulty in collecting tissue from specific, elusive callers in often impenetrable coastal marshes. Therefore, we matched calls to genetics at the population level by comparing the genetic identity of sampled frogs ("pure" individuals only) to the results of nearby call surveys (within 100 m and 300 m of each sampled frog).

Morphological and color characters

We report simple summary statistics and frequencies of different character states for each species. To test for differences among species for particularly important or challenging characters, we used one-way ANOVAs in R version 3.0.2 (R Development Core Team 2013). In case no single character was diagnostic in distinguishing among species, we used multivariate methods to explore the value of combinations of characters in identifying the species. We used the randomForest package (Liaw and Wiener 2002) in R version 3.0.2 (R Development Core Team 2013) to run a random forests classification analysis (Breiman 2001) on the 16 characters obtained from photos with the genotyped species identification as the dependent variable. Only frogs with 90% or greater genetic composition of a single species (i.e., no admixed individuals) were included. Missing values resulted from photos of certain angles not being submitted and from photos where characters could not be discerned. We treated missing values in two ways, each in a separate analysis: by imputing them using the function rf.impute and by omitting cases with missing values for some characters. For each analysis, we built 1,000 trees and determined variable importance as the mean decrease in model accuracy without each variable following Strobl et al. (2008).

Species distribution mapping and modeling

Our final maps of species occurrence are based upon genotyped frogs from this study and Newman et al. (2012), call surveys from this study, documentation of calling frogs by the authors, and other confirmed calls or visual identification mainly from 2005-2013 but as far back as the 1990s. We drew updated range maps for each of the three leopard frog species in our region using these presence points in combination with earlier range maps (IUCN, Conservation International, and NatureServe 2013, Feinberg *et al.* 2014) and constrained by watershed

boundaries. We based historical ranges of *R. kauffeldi* and *R. pipiens* on these maps and museum specimens that we confirmed to species by photograph or by examination of the physical specimen. In some cases, this represented our "best guess" based on characters typically associated with the three species as described here and in Feinberg et al. (2014).

We built a "presence-only" species distribution model for *R. kauffeldi* following methods in Howard and Schlesinger (2013). In brief, we attributed 169 presence points and 10,000 background points with 81 environmental layers representing climate, geology, topography, and land cover using 30-m grid cells (T. Howard, NY Natural Heritage Program, unpublished). Background points were restricted to a 171,704-km^2 modeling area that encompassed the area of known presence, matching 8-digit Hydrological Unit Code boundaries (USGS and USDA 2013). We used the randomForest package (Liaw and Wiener 2002) in R version 3.0.2 (R Development Core Team 2013) to run a classification analysis (Breiman 2001) to distinguish areas of predicted presence of suitable habitat from areas of predicted lack of suitable habitat. We used the "out-of-bag" estimate of the error rate (Breiman 2001) and the confusion matrix as measures of the model's accuracy. We determined variable importance following Strobl et al. (2008). We used the results of the analysis to predict the probability of suitable habitat for the modeling area.

Determining conservation status

To aid managers in prioritizing their conservation and management attention, we suggested conservation status ranks for *R. kauffeldi* for its entire range and for each state in which it was documented. We used the NatureServe and Natural Heritage Program methodology (Master *et al.* 2012, Faber-Langendoen *et al.* 2012), which determines conservation status through an evaluation of a suite of factors representing rarity, threats, and trends to arrive at a G-rank ("global" rank, for the entire range; Table 2) or S-rank (for a state or other subnational jurisdiction). We focused on three rarity factors we believed our data could inform—range extent (area encompassed by the outer boundary of presence points), area of occupancy (number of 4-km^2 grid cells occupied), and number of occurrences (estimated by counting a detection as a separate occurrence if separated by 5 km of suitable habitat or 1 km of unsuitable habitat; NatureServe 2016). For each of these categories, the methodology uses wide ranges and provides the opportunity to select multiple scores to encapsulate uncertainty.

Table 2. Conservation status ranks used in the NatureServe methodology (Master *et al.* 2012, Faber-Langendoen *et al.* 2012). From http://www.natureserve.org/conservation-tools/conservation-status-assessment. At the subnational level, S-ranks are used.

Global Rank	Definition
G1	Critically Imperiled—At very high risk of extinction due to extreme rarity (often 5 or fewer populations), very steep declines, or other factors.
G2	Imperiled—At high risk of extinction or elimination due to very restricted range, very few populations, steep declines, or other factors.
G3	Vulnerable—At moderate risk of extinction or elimination due to a restricted range, relatively few populations, recent and widespread declines, or other factors.
G4	Apparently Secure—Uncommon but not rare; some cause for long-term concern due to declines or other factors.

Global Rank	Definition
G5	Secure—Common; widespread and abundant.
GX	Presumed Extinct— Species not located despite intensive searches and virtually no likelihood of rediscovery. Ecological community or system eliminated throughout its range, with no restoration potential.
GH	Possibly Extinct (species) — Known from only historical occurrences but still some hope of rediscovery. There is evidence that the species may be extinct or the ecosystem may be eliminated throughout its range, but not enough to state this with certainty.

Our data were less well suited to addressing threats factors and trends factors. Given the lack of species-specific information on threats, we characterized intrinsic vulnerability, a surrogate for threats, as the combination rating "High or Moderate" for all jurisdictions. Amphibians are highly sensitive to aquatic pollutants (Taylor *et al.* 2005, Egea-Serrano *et al.* 2012 p.) and *R. kauffeldi*'s proximity to the coast makes it vulnerable to habitat degradation and loss from coastal storms and rising sea levels (Feinberg *et al.* in prep., 2014). On the other hand, *R. kauffeldi* is known from wetlands near heavy industry (Feinberg *et al.* 2014), suggesting it may be less sensitive to environmental toxins if suitable freshwater habitat exists. Information on trends was mostly lacking given the recent discovery of this species and the difficulty of identifying older museum specimens with certainty. We calculated a G-rank for the overall range and S-ranks for each state in two ways: using NatureServe's element rank calculator (NatureServe 2015) and also based on expert interpretation of the data.

Results

Call surveys

We conducted call surveys at 1,004 point locations throughout the northeastern U.S. (Figure 3). The majority of points were surveyed once, with some surveyed multiple times, for a total of 2,159 surveys. Survey durations ranged from 1 to 110 minutes (mean = 5.76 minutes) and totaled approximately 207 hours.

Calls from *R. kauffeldi* were documented as early as March 10, 2014 in Maryland and March 14, 2015 in Delaware. Outside the formal window of this study, calls were documented on February 3, 2016 in North Carolina. The last call dates in the two project years were April 23, 2014 in Pennsylvania and April 15, 2015 in New Jersey. Calls have been heard sporadically in late April and May in New York (JAF, unpublished data), and June 12, 2006 in New Jersey's Meadowlands (Kiviat 2011).
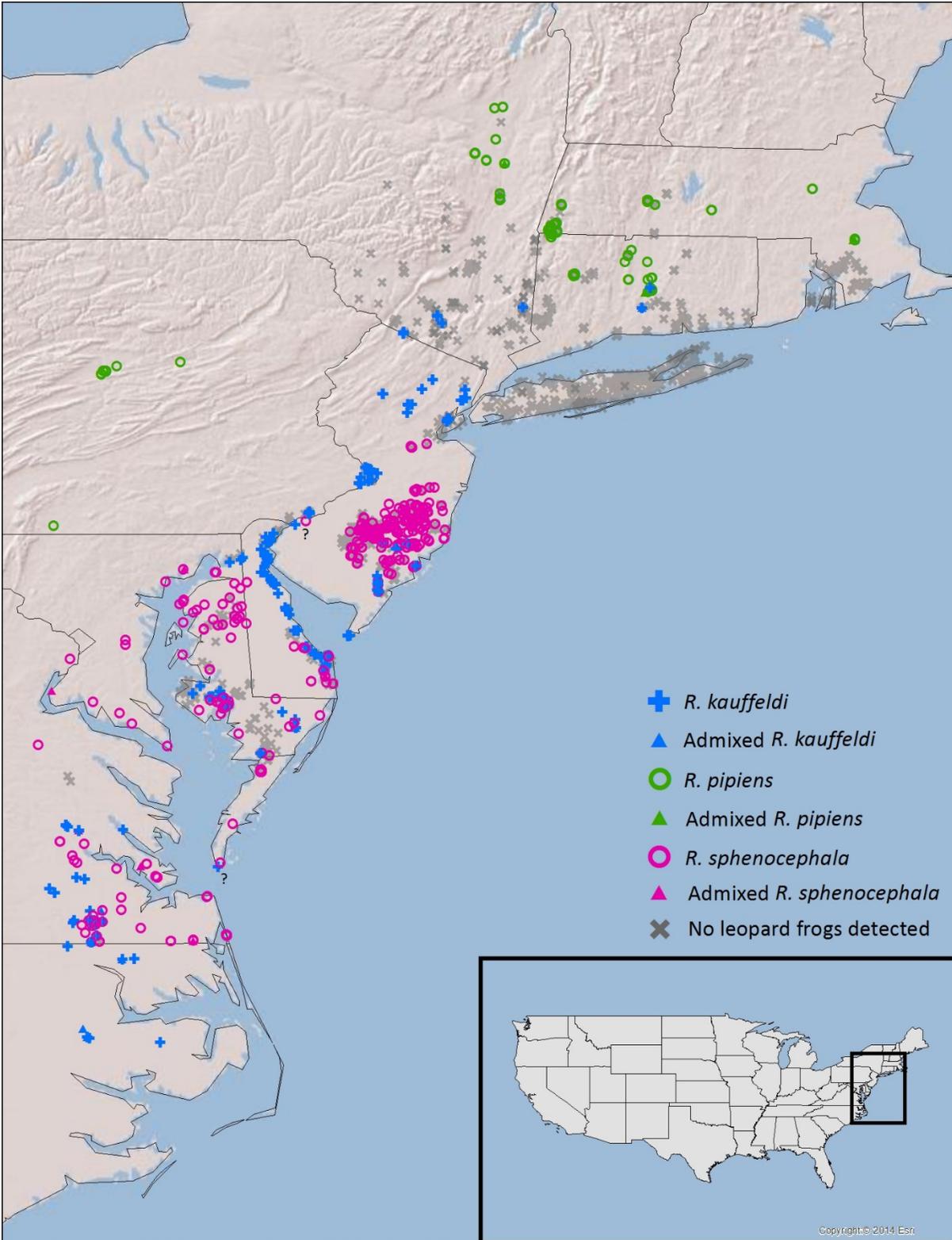
Figure 3. Leopard frog presence and nondetections from call surveys and genetic analysis in the northeastern U.S. Question marks are placed in two locations directly below presence points where identifications from genetic analysis were questionable.

Genetics

We collected tissue samples from 254 individual frogs for genetic analysis from throughout the putative range of *R. kauffeldi* and beyond to include samples of all three species across the region. Of these samples, 251 were successfully extracted, amplified, and sequenced. Three samples had DNA concentrations that were too low for successful PCR amplification and were removed from the analysis. Aligned sequence lengths for nuclear loci were similar to those for a broader set of leopard frog samples for the same loci (Newman *et al.* 2012): 527 bp (CXCR4), 530 bp (NTF3), 648 bp (Rag-1), 388 bp (SIA), and 549 bp (TYR). There were between 31 and 49 variable sites per locus. Haplotypes receiving probability scores of less than 80% after phasing were treated as null. Phase output consisted of 189 individuals with no missing haplotypes, 65 individuals with one null haplotype, 20 with two null haplotypes, and 20 with three null haplotypes. Seven individuals received greater than three null haplotypes and were removed from the analysis. In total, 244 of the original samples plus 50 individuals determined to be pure *R. kauffeldi*, *R. pipiens*, or *R. sphenocephala* from Newman et al. (2012) went into the final analysis, for a total of 294 frogs.

Bayesian cluster analysis in Structure resolved three clusters (lnL = 591.04, DK = 33.36). Individuals with a cluster probability greater than 90% were assigned to that cluster, and individuals with a cluster assignment between 10% and 90% were designated as admixed. We assigned species identification to each cluster using the individuals from Newman et al. (2012) as controls. A total of 262 individuals fell into one of the three clusters unambiguously, including 111 *R. kauffeldi*, 79 *R. sphenocephala*, and 72 *R. pipiens* (Figure 3). The remaining 32 individuals were considered potential hybrids and were identified by the dominant species (based on admixture proportions (e.g., "admixed *Rana kauffeldi*").

We identified and omitted from further analysis four samples that appeared, upon genotyping, to have identification or locality errors. Two samples from one site in central CT appeared to have their labels switched: one was visually *R. pipiens* but was genotyped as *R. kauffeldi*, while the other was visually *R. kauffeldi* but was genotyped as *R. pipiens*. Both species occur at this site based on other individuals. Two additional samples are identified with question marks in Figure 3. One frog from the southern tip of the Delmarva Peninsula (Northampton Co., VA) appeared visually to be *R. sphenocephala* but was genotyped as *R. kauffeldi*. Only calls of *R. sphenocephala* have been documented in the area and appropriate habitat for *R. kauffeldi* does not appear to exist at that locality. An additional frog in western NJ near the Delaware River (Gloucester Co.) was genotyped as *R. sphenocephala*, but no other *R. sphenocephala* are known from that mesic area of the state. No photographs were associated with this sample, but another frog from the site appears visually to be *R. kauffeldi*. Leopard frogs from nearby Little Tinicum Island in the Delaware River (Delaware Co., PA) show field characters of both species, so we cannot rule out either species on the NJ side. These may prove to be admixed frogs.
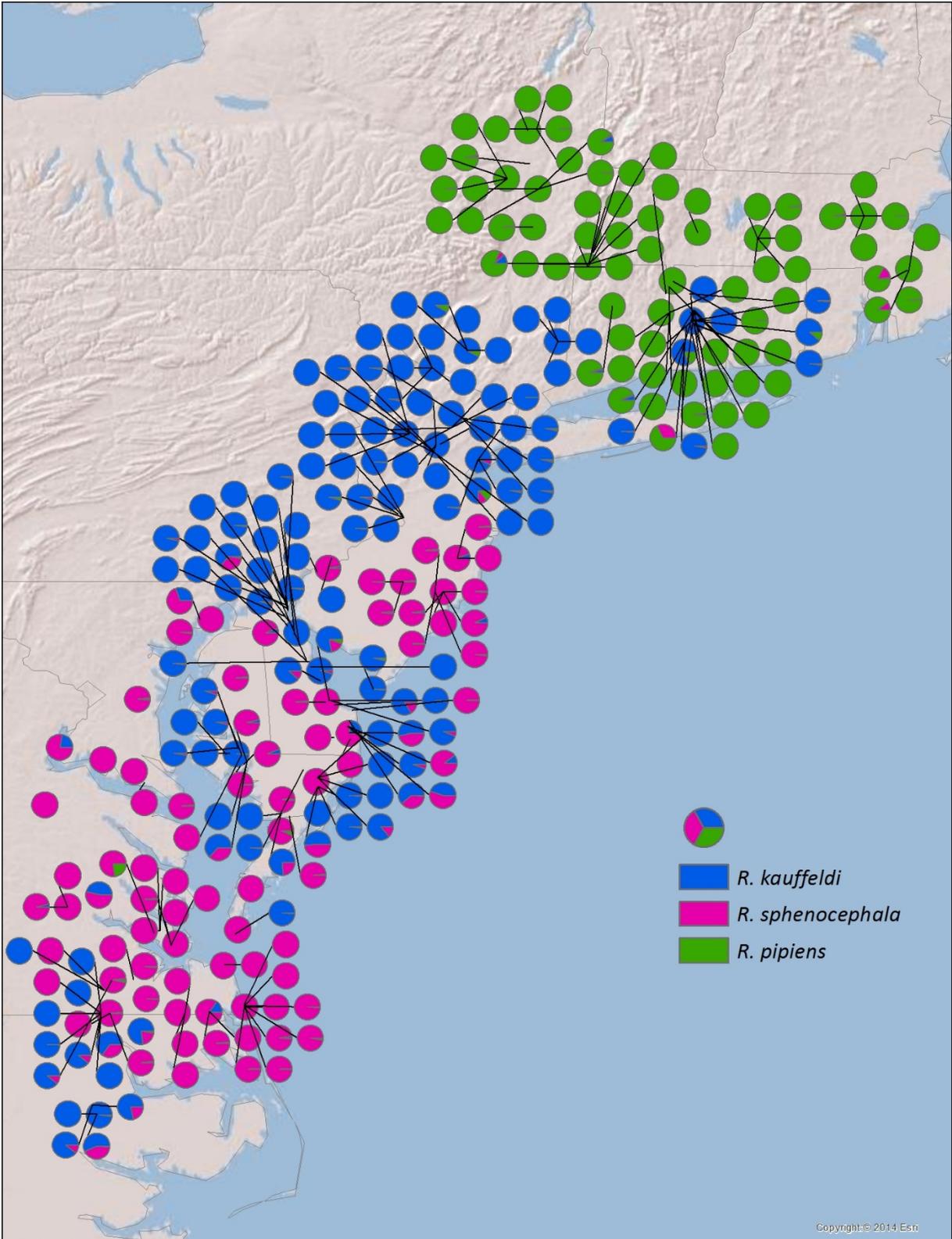
Figure 4. Distribution of genetic variation in leopard frogs in the northeastern U.S. The pie charts, with leader lines pointing to actual sampling locations, represent the probability of a frog belonging to one of three species: *Rana pipiens*, *R. kauffeldi*, and *R. sphenocephala*.

Distributions of northeastern leopard frogs

Rana kauffeldi

We confirmed *Rana kauffeldi* in eight eastern US states: CT, NY, NJ, PA, DE, MD, VA, and NC. We did not detect *R. kauffeldi* in MA or RI, where only *R. pipiens* was detected. The two locations farthest from one another in CT and NC are 746 km apart, close to the 780 km estimated by Feinberg et al. (2014). The range of *R. kauffeldi* that we drew (Figure 5), covers just over 46,500 km^2. The highest elevation where the species was confirmed was 208 m in northern New Jersey, and the lowest was at sea level in multiple locations. No *R. kauffeldi* were detected south of the 17°C isocline (i.e., annual average temperature greater than 17°C). Confirmed extremes of the range of *R. kauffeldi* were as follows (in WGS84): northernmost—Middlesex County, CT (lat 41.63, long -72.62); southernmost—Washington County, NC (latitude 35.79, longitude -76.41); easternmost—Middlesex County, CT (lat 41.60, long -72.61); and westernmost—Sussex County, VA (lat 36.98, long -77.27). Our survey data also support the position of Feinberg et al. (2014) that *R. kauffeldi* has disappeared from a large part of its historical range in southern NY and CT (Figure 5), including much of the Hudson Valley and all of Long Island. We could not verify recent reports of leopard frogs from these areas despite considerable survey effort.

The species does not occur far from coastally influenced habitats. The maximum distance the species was documented from the ocean, bays, and estuaries was 40 km near the border of New Jersey and New York. Eighty-nine percent of *R. kauffeldi* locations were within 20 km, 77% were within 10 km, and just under 50% were within 1 km of coastal waters.

In portions of its range, *R. kauffeldi* overlaps with its close congeners. *Rana pipiens* and *R. kauffeldi* were documented to be syntopic at a single site in CT, and the ranges of *R. sphenocephala* and *R. kauffeldi* overlap broadly from central New Jersey south to North Carolina, including several instances of syntopy. In central NJ, DE, and MD they are less frequently sympatric than in VA and NC.

Rana pipiens

We report an apparent disappearance of *R. pipiens* from a large swath of the southern portion of its range from PA east through northwestern NJ, southeastern NY, southern CT, southern RI, and coastal MA, from where we confirmed several historical museum specimens as *R. pipiens*. Our surveys and those of the Pennsylvania Amphibian and Reptile Survey (The Mid-Atlantic Center for Herpetology and Conservation 2016), including many historical locations for *R. pipiens*, have not yielded leopard frogs of any species at those locations (Figure 3), with the exception of a single population near Providence, RI, discovered in 2017. The southernmost location at which we documented *R. pipiens* was in Middlesex County, CT (lat 41.63, long -72.62). Locations in lower latitudes have been reported by others in central PA (The Mid-Atlantic Center for Herpetology and Conservation 2016) and farther west.
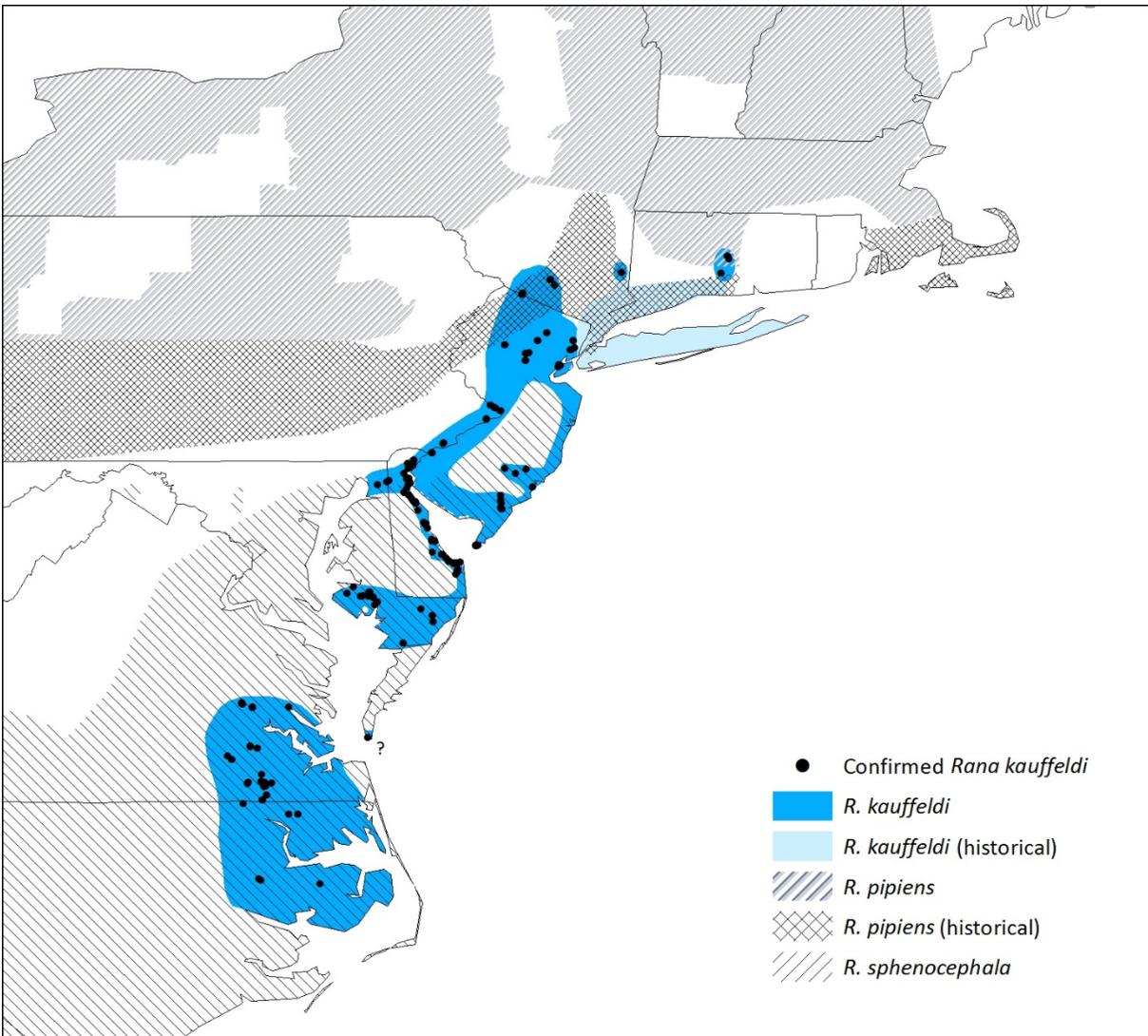
Figure 5. Range of *Rana kauffeldi*, with presence points confirmed by bioacoustics or genetics, compared to ranges of *R. pipiens* and *R. sphenocephala*. Historical ranges for *R. kauffeldi* and *R. pipiens* are based on examination of museum specimens and recent detections.

Rana sphenocephala

Prior range maps of *R. sphenocephala* (e.g., Conant and Collins 1998, IUCN, Conservation International, and NatureServe 2013) included southern NY and northern NJ. As suggested by Feinberg et al. (2014), we consider these areas to be occupied, or have been occupied, by *R. kauffeldi*, not *R. sphenocephala*. The northernmost extant locality for *R. sphenocephala* in our surveys (and thus, apparently in its entire range) is in Middlesex County, NJ (lat 40.42, long -74.35).

Match of calls to genetics

We found a near-perfect match of population-level calling with genetics of individual frogs. At 16 sites for *R. kauffeldi*, 18 sites for *R. sphenocephala,* and 3 sites for *R. pipiens*, genetically pure frogs of each species were confirmed where that species of leopard frog call was documented within 100 m; the only mismatch was one point at which the identification was *R. kauffeldi* based

on calls but *R. spehnocephala* genetically. The same concordance between calls and genetics held for situations where calling frogs were documented within 300 m of genetic samples (23 sites for *R. kauffeldi*, 21 for *R. sphenocephala*, and 11 for *R. pipiens*); the only potential exceptions were three genetically *R. kauffeldi* sites that had both *R. kauffeldi* and *R. sphenocephala* calling, and the above-noted genetically *R. sphenocephala* site with *R. kauffeldi* calls.

Morphological and color characters

We examined 912 photographs of 220 leopard frogs with genetic identities as follows: 80 pure *R. kauffeldi*, 16 admixed *R. kauffeldi*, 45 pure *R. pipiens*, 5 admixed *R. pipiens*, 64 pure *R. sphenocephala*, and 10 admixed *R. sphenocephala*. Not all 220 frogs had suitable photographs of specific characters, so sample sizes for each character were different.

Rana kauffeldi had fewer dorsal spots on average than both *R. sphenocephala* and *R. pipiens* in a one-way ANOVA ($F_{174,2} = 15.08$, $P < 0.0001$, Tukey HSD post-hoc tests), although there was considerable overlap among species (Table 3; Figure 7). *Rana sphenocephala* and *R. pipiens* had similar numbers of spots.

Rana kauffeldi was readily distinguished from *R. pipiens* by its smaller spots (85% of frogs with spots smaller than the eye vs. 36%), usual absence of a snout spot (15% of frogs vs. 71%), and reticulum characterized as predominantly dark (97% of frogs vs. 0%) (Table 3; Figure 6).

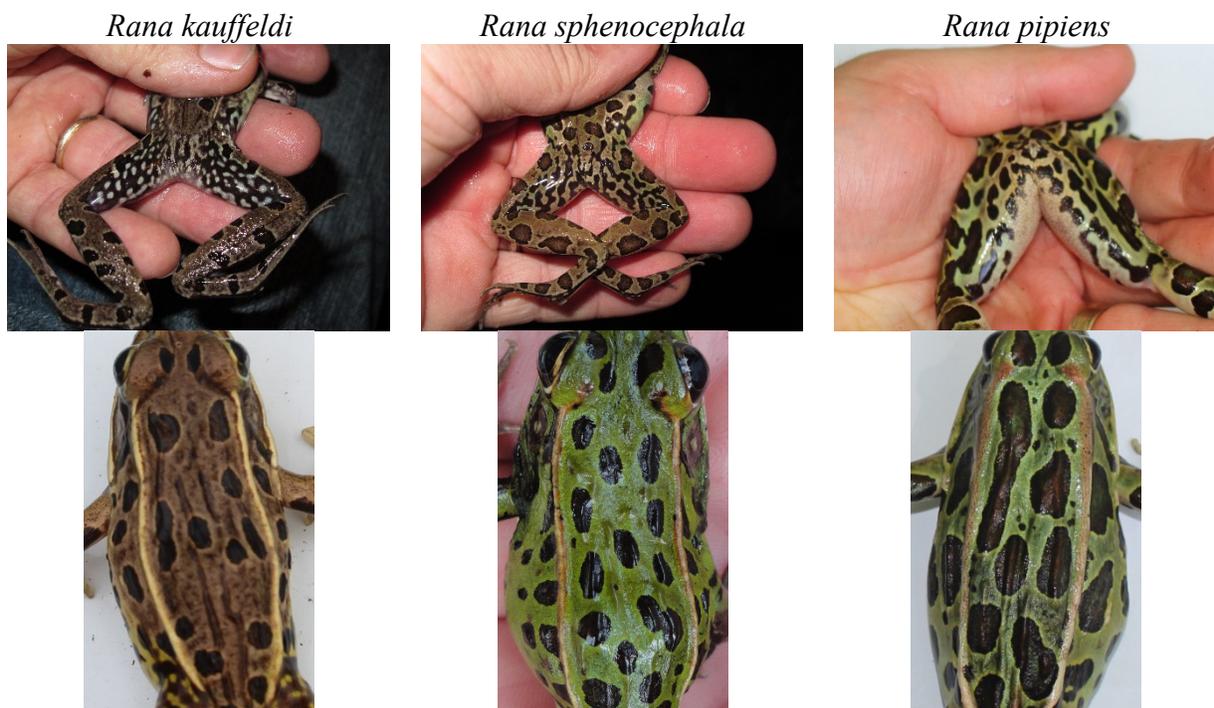| *Rana kauffeldi* | *Rana sphenocephala* | *Rana pipiens* |

Figure 6. Typical patterns in femoral reticula (top row) and dorsal spotting (bottom row) in three species of leopard frogs in the northeastern United States.

Rana kauffeldi and *R. sphenocephala* were more challenging to differentiate. No single character reliably distinguished the two species, but characters used in combination allowed reliable

identification in most cases. Nearly all *R. kauffeldi* reticula were predominantly dark with small, unconnected dots of light pigment, while most *R. sphenocephala* reticula were predominantly light with large, connected splotches of dark pigment (Table 3; Figure 6). *Rana kauffeldi* had sharp tympanum spots less frequently (58% of frogs) than *R. sphenocephala* (91%). Overall, the pattern of *R. kauffeldi* could be described as typically "duller" than that of *R. sphenocephala*. The snout of *R. kauffeldi* was more frequently characterized as "blunt" (62% of frogs) than that of *R. sphenocephala* (13%), which was usually characterized as "pointed." The three head measurement ratios were not significantly different between the two species in a one-way ANOVA, although the ratio of snout width to head length was nearly so ($F_{121,1} = 3.899$, $0.05 < P < 0.10$).

In a subsequent analysis using ImageJ, we quantified the degree of darkness of femoral reticula of 55 frogs genetically identified as *R. kauffeldi* and 50 frogs genetically identified as *R. sphenocephala*. Reticula of *R. kauffeldi* were determined to be 70% dark on average, significantly darker than those of *R. sphenocephala*, which were around 55% dark on average ($F_{103,1} = 52.38$, $P < 0.0001$), although there was considerable overlap between species (Figure 7). We also compared these values against our categorical ratings. While we underestimated the percent darkness overall in our ratings—both species on average were over 50% dark—our ratings generally matched up with the actual percent dark. Reticula we called "predominantly dark" ($n = 65$) averaged 69.5% dark, while those we called "predominantly light" ($n = 36$) averaged 50.0% dark. There were a few frogs whose measured reticular darkness contradicted their categorical placement, but this comparison was imperfect because the categorical method may have relied on different or additional photographs than the ones used in the ImageJ analysis.
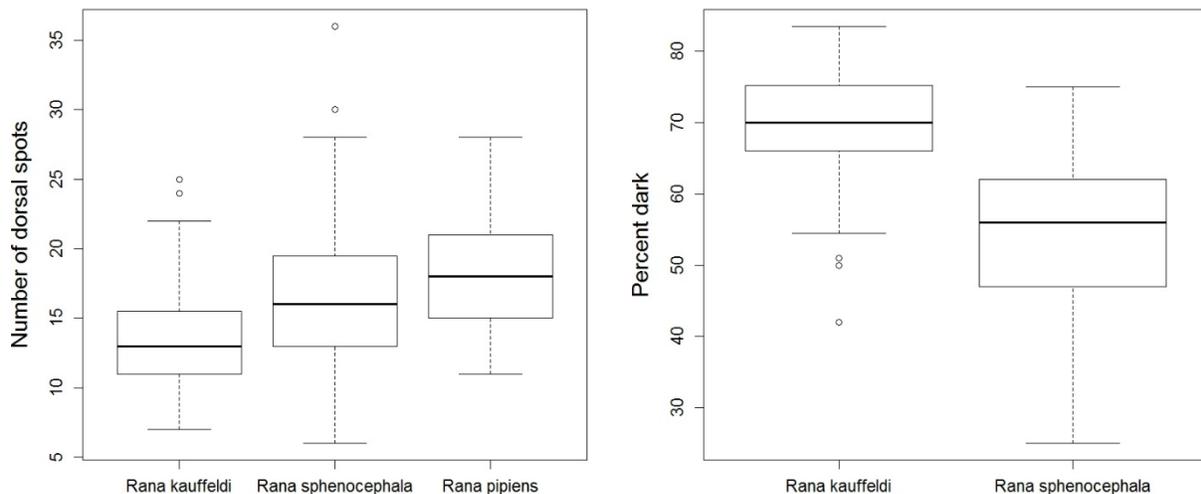

Figure 7. Boxplots of number of dorsal spots (left) and percent of femoral reticulum classified as "dark" by ImageJ software (right) for *Rana* kauffeldi (n = 48) and *R. sphenocephala* (n = 50) in the coastal northeastern U.S. Open circles are statistical outliers.

The extent of toe webbing and the color of reticulum spots and blotches did not yield any consistent pattern for distinguishing among the three species.

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Using combinations of characters proved more reliable. Random forest analysis using 144 individuals with missing values imputed correctly classified *R. kauffeldi* and *R. sphenocephala* in over 90% of cases (out-of-bag estimate of error rate = 9.72%). The most important field characters in distinguishing between species were reticulum color, reticulum pattern, and snout shape. When the analysis was run with cases with missing values omitted (leaving n = 33 individuals), the error rate was similar (9.09%), although the order of important variables shifted somewhat, with the ratio of snout width to head length, reticulum color, reticulum pattern, and the ratio of head length to head width being most important.

Habitat association and distribution model

Our surveys found that the basic habitat description in Feinberg et al. (2014) holds. South of the glacial maximum, *R. kauffeldi* is a habitat specialist restricted to large coastal and riparian wetlands. In the southern portion of its range, it occurs primarily in riparian cypress-gum swamps, and on the Delmarva Peninsula it occupies large coastal cattail (*Typha*) and common reed (*Phragmites australis*) marshes that may be subject to salinity intrusions. In the northern portion of its range, *R. kauffeldi* occupies large freshwater wetlands, typically with open canopies, that otherwise are indistinguishable from similar large, open, freshwater wetlands where it was not detected. Where *R. sphenocephala* is sympatric with *R. kauffeldi*, it is a generalist, being found in similar habitats to those of *R. kauffeldi*, but also nearly any semi-permanent (isolated) or permanent wetlands, created or natural, including tire ruts, fish hatchery ponds, waterfowl impoundments, and cypress-gum swamps. It also appears to be less restricted to xeric habitats in the southern portion of its range.

Our distribution model shows that suitable habitat for *R. kauffeldi* is concentrated along the coast and in riparian corridors (Figure 8). The model overall was very accurate, with an out-of-bag error estimate of 1.11%, although absences were predicted with greater accuracy (99%) than presences (34%). The most important variables shaping the distribution of suitable habitat were elevation, impervious surface and wetlands in the surrounding landscape, and distances to calcareous bedrock, saltwater or freshwater emergent wetlands, freshwater forested wetland, and lakes and rivers.

Conservation status

Because we recognized that a two-year survey, even when supplemented with some pre-survey data, could not reveal all locations of *R. kauffeldi*, we embraced the uncertainty allowed by the NatureServe approach. For example, when our counts of number of grid cells or number of occurrences fell near the boundaries of ranking categories, we selected both categories. We identified a considerable decline in New York based on our surveys and Feinberg et al. (2014), but the evidence for decline in other northeastern states was weaker, so we did not use the Trends factor in those states. We estimated a slight rangewide decline based on our surveys (Table 4).

Table 3. Morphological and color characters from photographic analysis of 220 leopard frogs (*Rana* spp.) in the eastern U.S. Frogs were identified using genetics and designated as "pure" if they had a 90% or greater match with a single species; individuals with a species match between 10% and 90% were designated as admixed. Sample sizes for each character are given because not every frog had photographs suitable for examination of that character.

Character	Character state	Pure *R. kauffeldi* (n = 80)	Admixed *R. kauffeldi* (n = 16)	Pure *R. sphenocephala* (n = 64)	Admixed *R. sphenocephala* (n = 10)	Pure *R. pipiens* (n = 45)	Admixed *R. pipiens* (n = 5)
Dorsal spots	Min	7	6	6	8	11	12
	Max	25	29	36	24	28	23
	Mean ± sd	13.6 ± 3.7	15.5 ± 6.3	17.0 ± 5.7	14.0 ± 4.7	18.1 ± 4.2	16.7 ± 5.7
	Mainly larger than eye	12	5	19	1	27	3
	Mainly smaller than eye	66	11	43	9	15	0
Snout spot	Absent	67	12	54	9	12	2
	Small	6	2	8	0	0	0
	Large	6	1	1	1	30	2
Snout	Blunt	28	4	6	1	16	2
	In between	3	2	7	0	7	1
	Pointed	14	5	32	2	16	1
Reticulum	Predominantly dark	75	12	14	5	0	0
	About 50/50	1	0	6	3	42	5
	Predominantly light	1	1	44	1	0	0
	Mostly large, connected splotches	12	3	55	5	34	4
	Mostly small, unconnected dots	64	8	9	4	8	1
Tympanum spots	None	2	1	0	0	2	0
	1 sharp, 1 indistinct	4	1	3	2	4	1
	2 indistinct	9	1	2	0	5	0
	2 sharp	21	7	53	6	17	0

| Character	Character state	Pure *R. kauffeldi* (n = 80)	Admixed *R. kauffeldi* (n = 16)	Pure *R. sphenocephala* (n = 64)	Admixed *R. sphenocephala* (n = 10)	Pure *R. pipiens* (n = 45)	Admixed *R. pipiens* (n = 5)
				Species			
	Left spot absent	8	2	4	0	1	0
	Left spot indistinct	18	3	3	2	6	1
	Left sharp blotch	12	5	30	3	21	1
	Left sharp dot	18	4	26	5	4	0
	Right spot absent	8	2	0	0	4	2
	Right spot indistinct	15	1	4	1	16	0
	Right sharp blotch	12	5	27	2	17	2
	Right sharp dot	25	5	32	5	4	0
	Left spot brown/bronze	12	3	3	3	15	1
	Left spot green	11	4	11	2	6	0
	Left spot white/cream	22	5	44	5	9	1
	Right spot brown/bronze	14	3	2	3	19	1
	Right spot green	13	6	11	2	10	0
	Right spot white/cream	23	2	48	4	5	1
Combinations	Dark reticulum, large splotches	10	2	8	1	0	0
	Dark reticulum, small dots	64	8	6	4	0	0
	Dark retic, 1 tymp spot sharp, 1 blurry	3	0	0	2	0	0
	Dark retic, 2 tymp spots sharp	21	5	12	2	0	0
	Dark retic, 2 tymp spots blurry	9	1	0	0	0	0
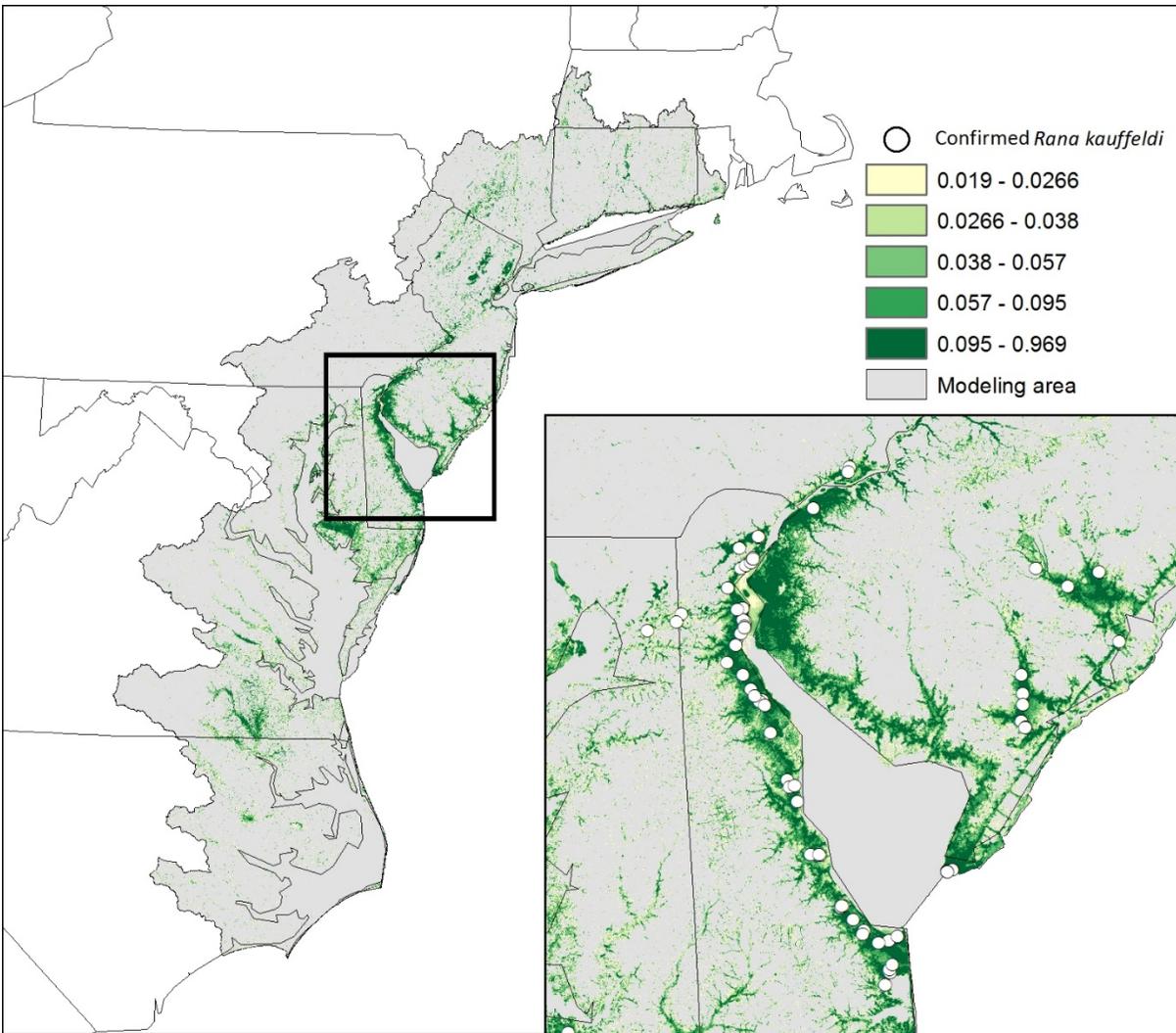
Figure 8. Distribution model for the full range of *R. kauffeldi* along the coastal northeastern U.S. Inset: Delaware Bay and surrounding states. Values reflecting habitat suitability were broken into 10 quantiles, with the top five displayed.

The ranks generated by the rank calculator tended toward a greater degree of concern than those based on our expert opinion (compare the last two columns in Table 4), but we still report both ranks here to be transparent about our process. In the core of *R. kauffeldi*'s range (NJ, DE, VA, and perhaps MD), we believe it to be secure, with many apparently large populations in protected wetlands. At the northern edge of its range (CT, NY), *R. kauffeldi* is exceedingly rare and appears to have declined substantially. In NY, for example, the species once occurred across 11 counties and likely more than 100 populations; today it is known from only three counties and fewer than 10 populations. While the species is common along the Delaware River, only a sliver of its range falls within PA, hence its suggested S1 status in that state. At the southern edge of its range (NC), it may be rare, but this area needs additional field surveys to confirm the species' status. Throughout its range a rank of G3G4 (Vulnerable to Apparently Secure) seems appropriate; determining the most appropriate G-rank with greater precision would require a better understanding of short- and long-term trends.

Table 4. Conservation status ranking of *Rana kauffeldi* using the NatureServe methodology (Master *et al.* 2012, Faber-Langendoen *et al.* 2012). Range extent is defined by the smallest polygon that encapsulates known occurrences. Area of occupancy is the area within the range in which the species actually occurs. Number of occurrences is intended to reflect number of populations, based on taxon-specific distances within which animals are assumed to be interacting. Long-term trend is estimated based on historical literature or museum specimens. Calculated rank is generated by NatureServe's element rank calculator (Faber-Langendoen *et al.* 2012), while Expert rank is assigned by people familiar with the species in the jurisdiction of interest.

Assessment area	Range Extent (km^2)	Area of Occupancy (no. 4-km^2 cells)	# Occurrences	Long-term Trend[a]	Calculated Rank[b]	Expert Rank[b]
Rangewide	20,000-200,000	26-125	21-80	10-30% decline	G2G3	G3G4
CT	<100	3-5	1-5	NA	S1	S1
PA	100-250	2	1-5	NA	S1	S1
NJ	5,000-20,000	26-125	6-80	NA	S2S3	S4
NY	250-5,000	6-25	1-20	50-90% decline	S1S2	S1S2
DE	1,000-5,000	26-125	6-80	NA	S2?	S4
MD	5,000-20,000	6-25	6-20	NA	S2	S3
NC	1,000-20,000	6-25	1-20	NA	S1S2	S1S3
VA	1,000-20,000	6-125	6-80	NA	S1S3	S4

[a] NA = not assessed; [b] Status rank definitions are in Table 2.

Discussion

Our multi-year, 10-state project demonstrated conclusively that *R. kauffeldi* is a habitat specialist with a small range centered in the most densely populated region of the United States. In several northern states it is extremely rare, while in the southern portion of its range it can be broadly distributed and abundant. We have a much better idea of its distribution than we did just a few years ago, but some unexplained gaps remain. Fortunately, for those interested in surveying for this frog, methods for its identification in the field are also better understood now. The unique breeding call identified by Feinberg et al. (2014) was reliably associated in our study with frogs genetically determined to be *R. kauffeldi*. And separation from *R. pipiens* based on morphology and color patterns is nearly foolproof while separation from *R. sphenocephala* can be correct as much as 90% of the time. We hope that our study spurs further research into *R. kauffeldi*'s ecology and conservation needs.

Distribution and biogeography

In the core of its range in the mid-Atlantic U.S., south of the glaciated region, *R. kauffeldi* is a species exclusively of the Coastal Plain (Figure 9), and the degree to which its apparent western range margin matches that of the Coastal Plain is striking. The Coastal Plain physiographic region of the United States covers the Gulf and Atlantic coasts from southern Texas east to Florida and north to Long Island, New York (Fenneman and Johnson 1946). The region is characterized by low elevation, minimal topography, and unconsolidated sediments (Fenneman 1938) and has recently been recognized as a global biodiversity hotspot (Noss 2016). Within this

physiographic region, several ecoregions—areas with similar geology, soils, climate, and vegetation (Bailey 1998)—have been identified, of which the Mid-Atlantic Coastal Plain (MACP), Chesapeake Bay Lowlands (CBY), and North Atlantic Coast (NAC) are occupied in part by *R. kauffeldi*. The MACP has been described as "a factory for the generation of new and novel species" because of its dynamism and juxtaposition of natural communities (The Nature Conservancy 2001).

Since the last glacial maximum was reached over 20,000 ybp, *R. kauffeldi* has colonized some previously glaciated regions along major river valleys to the north and east, and now occurs in the Piedmont, New England, and Valley and Ridge physiographic provinces. These ecoregions are characterized by greater topographic variation and a greater diversity of habitat types and soils.

Gaps in the range in areas with at least some suitable habitat—southern coastal NJ, the northwestern shore of the Delmarva Peninsula, and from Baltimore, MD to mid-coastal VA—may be clarified with further sampling and continued examination of museum specimens and historical recordings. Note, however, that the distribution model found little suitable habitat in the MD-to-VA gap. Our range map was based on confirmed observations, with the expectation that new observations would add to the map of the known range. Our distribution model represents suitable habitat at a finer scale, with coastal impoundments and river valleys standing out and a clear signature of having sampled in part along roads in both the map and the variable importance rankings. Future distribution modeling could use likely absence points instead of random background points, which may allow for more accurate predictions of presence points. The combined use of modeling, interpretation of aerial imagery, and field survey may help fill these gaps over time.

Field identification and genetics

While our study did not identify a definable single morphological or color character for distinguishing *R. kauffeldi* from *R. sphenocephala*, we did find that using a combination of characters greatly improved reliable identification. Characterization of the femoral reticulum, tympanum spots, size and number of dorsal spots, snout shape, and overall coloration provided the correct identification more than nine out of ten times. Additional research into potentially diagnostic features or combinations of features may reveal an even more reliable method for distinguishing between the two species. For now, the primary mating call described by Feinberg et al. (2014) remains the only truly diagnostic feature in the field, and our results strongly suggest that the three species as defined genetically are reliably distinguishable based on calls. In continuing to be visually cryptic but bioacoustically distinguishable, *R. kauffeldi* and *R. sphenocephala* join the treefrogs *Hyla versicolor* and *H. chrysoscelis* (Johnson 1966, Conant and Collins 1998) as species pairs that cannot always be reliably discerned without vocalizations. But if anything, the leopard frogs may be "less cryptic" than the tree frogs given the high success rate made possible by using the characters reported here.
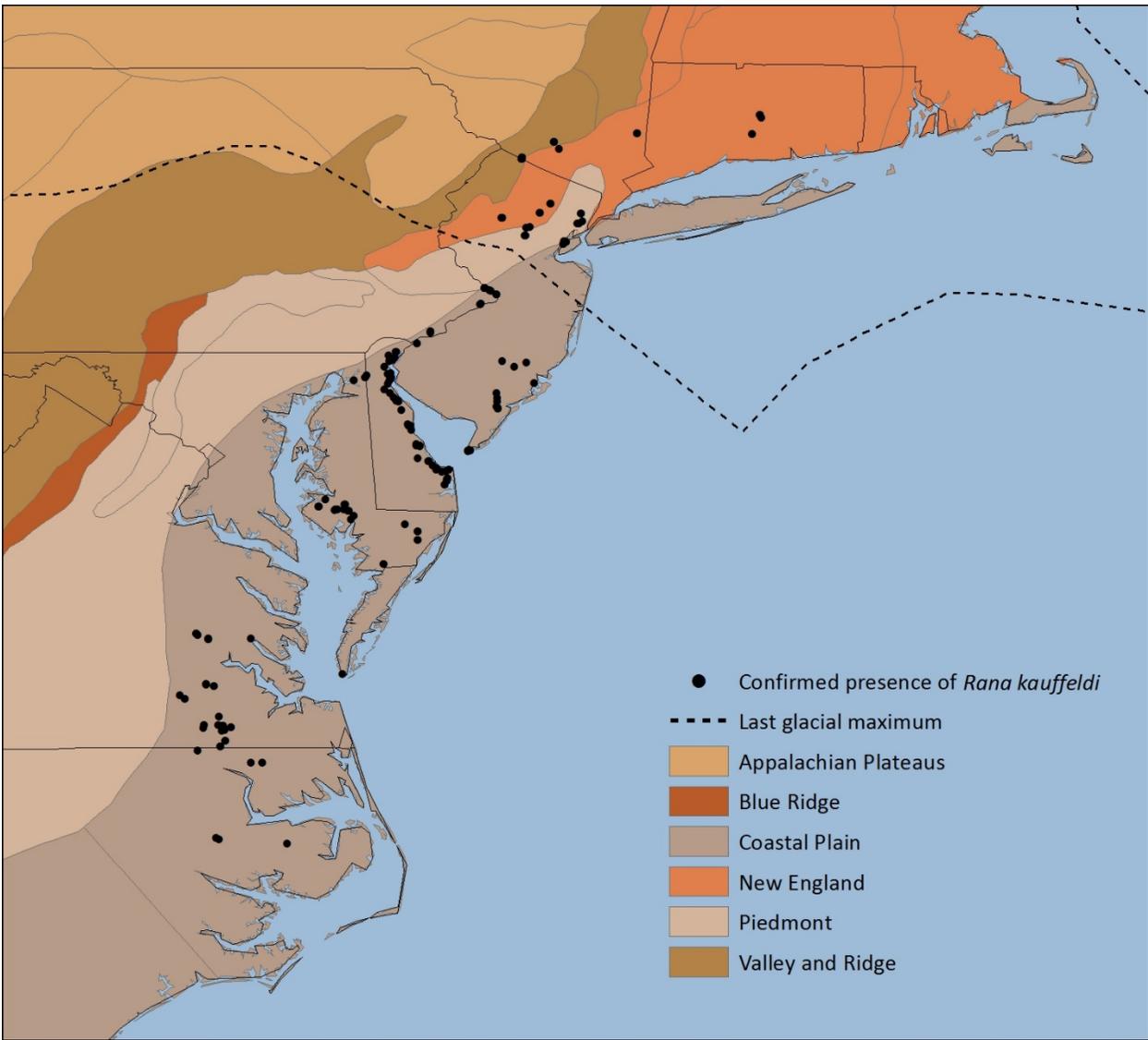
Figure 9. Physiographic regions of coastal northeastern U.S. (Fenneman and Johnson 1946) with presence points for *Rana kauffeldi* confirmed by bioacoustics and/or genetics. Last glacial maximum is from Ray and Adams (2001).

Characterizing the darkness of the femoral reticulum is critical to identifying *R. kauffeldi* correctly, especially in areas of overlap with *R. sphenocephala*. *Rana kauffeldi* always had a reticulum with light spotting on a dark background, although reticula of *R. sphenocephala* could have either pattern. Our analysis using ImageJ showed that reticular darkness averaged around 70% for *R. kauffeldi* and 55% for *R. sphenocephala*, showing that despite the categories used in our analysis, both frogs' reticula can be more appropriately characterized as "mostly dark." In most cases, characterizing the reticulum as "mostly dark" or "as much light as dark" will be sufficient. Because this feature is typically hidden when frogs are at rest, identification from photographs of sitting frogs will remain challenging.

Further challenging the correct field identification of non-calling frogs is apparent hybridization among these sister taxa, in particular between *R. kauffeldi* and *R. sphenocephala*. Over 10% of

frogs we subjected to DNA testing showed admixture. Hybridization has been studied extensively in leopard frogs and documented in the wild for certain species pairs (Platz 1972, Platz and Frost 1984, Parris 2001). The small number of genes we had to work with and the sequencing methodology we used did not allow for detailed genetic study beyond simple typing. And the small sample size might also explain some of the odd admixtures of *R. kauffeldi* with *R. pipiens* in the south and *R. sphenocephala* in the north (Figure 4). Further research using next-generation sequencing (Shendure and Ji 2008) would be a logical and important next step in understanding the genetic structure of these occasionally syntopic species.

Conservation, management, and information needs

Often when cryptic species are first discovered, little is known about their distribution or conservation status (e.g., Esselstyn *et al.* 2014, Brown 2015, Howlader *et al.* 2016). As more thorough studies accumulate, and particularly as their ranges and habitat preferences are determined, these cryptic taxa are often determined to have very limited distributions and be of conservation concern (e.g., Jones *et al.* 2005, Venchi *et al.* 2015, Clulow *et al.* 2016). Often this is because they have small populations, which likely hindered their discovery in the first place. The identification of cryptic species as a special case of new species discovery likewise can yield species of concern, for the simple reason that a species' range, or a species' population, turns out to be two smaller ranges, or two smaller populations (Lemmon *et al.* 2007, Bickford *et al.* 2007). For example, several species of genetically distinct leopard frogs in the American Southwest of the *R. pipiens* "complex" (Frost and Bagnara 1976, Platz and Mecham 1979, Platz 1993) are now known to be of conservation concern (Clarkson and Rorabaugh 1989, Lannoo 2005). And in the southeastern U.S., Pauly et al. (2007) determined that a single Threatened salamander species was in fact two species, one of which was quickly upgraded to Endangered by the U.S. Fish and Wildlife Service (2009).

In the situation described here, the newly described species, *R. kauffeldi*, overlaps with, and at the northern edge of its range, supplants the previously known species, *R. sphenocephala*. It has a far smaller range than *R. sphenocephala*, and while it is locally abundant and likely secure at the core of its range, it is vulnerable in places. Along with its small range, *R. kauffeldi*'s largely coastal distribution is a major reason for conservation concern (Feinberg *et al.* 2014). Most populations of *R. kauffeldi* exist within a highly developed urban and suburban matrix, and the frog's need for larger wetlands (as opposed to *R. sphenocephala*, which may occupy small ponds) may render it vulnerable to habitat fragmentation that results in inhospitable dispersal habitat. For species such as leopard frogs that spend considerable time in the uplands, the landscape surrounding the aquatic breeding habitat may be crucial to long-term persistence (Pope *et al.* 2000, Semlitsch and Bodie 2003). Based on typical migration distances reported in the literature on other species (Semlitsch and Bodie 2003), a terrestrial buffer around breeding habitat of several hundred meters is likely necessary to ensure suitable upland habitat, although in some highly urban settings the species appears to survive with little surrounding upland greenspace (Kiviat 2011). Studies specific to *R. kauffeldi* are needed, including studies to determine genetic connectivity among populations to ensure that the isolated nature of some populations has not led to inbreeding depression (Franklin 1980). Fragmentation has more immediate, direct effects on mobile individuals in the form of road mortality. Highways and other major roads bisect leopard frog habitat throughout the northeast, and frogs are often killed when crossing them. In fact, many of our samples were obtained from road-killed frogs. High-

volume or multi-lane roads may serve as permanent barriers to dispersal, which along with roadkill can have considerable impacts to anuran richness and abundance (Cosentino *et al.* 2014, Marsh *et al.* 2017).

A small geographic range is associated with greater risk of extinction across taxa and in amphibians specifically (Purvis *et al.* 2000, Sodhi *et al.* 2008, Cooper *et al.* 2008, Bielby *et al.* 2008). *Rana kauffeldi* appears to have the smallest range of any ranid frog on the East Coast, as posited by Feinberg et al. (2014). Several other species range from the mid-Atlantic south to Florida, and in some cases west along the Gulf Coast (e.g., *R. virgatipes*, *R. grylio*, *R. capito*) but none is as restricted as *R. kauffeldi*, with the exception of *R. sevosa*, known from a single site in Mississippi (Richter and Jensen 2005). Apart from the pine barrens treefrog (*Hyla andersoni*) and New Jersey chorus frog (*Pseudacris kalmi*), no anuran along the East Coast north of Florida has as small a range as *R. kauffeldi*. A small range may make a species more susceptible to stochastic events, and for frogs, may exacerbate the impact of fungal pathogens like *Batrachochytrium dendrobatidis* (Bd; Bielby *et al.* 2008). Bd has recently been documented in *R. kauffeldi* (JAF, unpublished data) as it has in *R. pipiens* (Voordouw *et al.* 2010, Chatfield *et al.* 2013) and many other ranids.

Another point of concern for *R. kauffeldi* is the coastal proximity of many populations. Coastal populations of wetland organisms may be threatened by rising sea levels and increasing frequency and intensity of coastal storms, two threats that have been connected to climate change (Scavia *et al.* 2002, Hopkinson *et al.* 2008, Pacifici *et al.* 2015). Feinberg et al. (2014) expressed concern for *R. kauffeldi* in this regard, but they had not confirmed the degree to which *R. kauffeldi* is a species of coastal ecosystems. While the presence of *R. kauffeldi* was not strongly tied to climate parameters in our distribution model, the model was not designed to forecast distributional shifts with changing climate or its corollary effects like sea-level rise and storms. Storms may cause saltwater overwash into freshwater habitats, and ongoing research (Feinberg et al., unpubl. data) is addressing the tolerance of *R. kauffeldi* to brackish conditions and persistence in coastal sites after a major storm event. In Delaware, large calling chorus of *R. kauffeldi* disappeared from freshwater impoundments along the Delaware Bay following storms that altered the coastline creating inlets that allowed for inflow of saltwater (J. White, pers. comm.). More inland (typically large-river riparian) populations of *R. kauffeldi* may be less vulnerable to changing coastal conditions, but also possibly less adapted to storm-related flooding.

We recommend some additional field inventory, especially during the late winter and early spring calling season when frogs are most easily identified, to clear up a few remaining uncertainties in the broad-scale distribution of *Rana kauffeldi*. Our understanding of the frog's distribution at the edges of its range is poorest, perhaps unsurprisingly, given the typical pattern at range edges, compounded by the urban landscape of the northern edge. In New York and Connecticut, populations are highly disjunct, a function of the likely riparian dispersal corridors and heavily developed landscape now dividing surviving populations. In between these populations, there are scattered reports of leopard frogs that are undocumented by recordings or photographs, and our field work did not confirm many of these reports. At the apparent southern edge of the range in North Carolina, the distribution of *R. kauffeldi* is just beginning to be understood, and it is not yet clear whether the frog is rare or common in the state and whether the

southern range margin is set by physiography, climate, interspecific interactions, or other factors, or extends farther south than our efforts demonstrated. Many states have gaps in local distributional information which, along with an understanding of population vulnerability in habitat patches of different sizes and degrees of urbanization, are a critical need for a better understanding of the conservation status of *R. kauffeldi*.

We also wish to call attention to the apparent extirpation of *R. pipiens* from a huge swath near the southern edge of its former range. This species can no longer be found in many locations in PA, NJ, NY, CT, RI, and MA where it once occurred based on museum specimens and historical literature. The recent (April 2017) discovery of a population near Providence, RI (CR, unpublished data) is one bright spot. Many extirpations of this species have been documented from western North America (Rorabaugh 2005) and local declines have been noted in the Northeast (Klemens 1993) but widespread extirpation in this region has not previously been reported. Whether extirpations are a result of habitat loss, range shifting with a warming climate, introduced populations failing to sustain themselves, or some other factor is a topic for further research.

As with any newly described species, there is still much to learn about *R. kauffeldi*'s ecology and natural history. In addition to continued research into distinguishing morphological features, descriptions of *R. kauffeldi* egg masses and tadpoles are lacking (Altig and McDiarmid 2015). The susceptibility to environmental contaminants of the various life stages is of great interest given the highly urban setting of many populations. Given the overlap with *R. sphenocephala* and the suggestion of hybridization in both the genetic and morphological data, research into possible competitive interactions may shed light on any niche separation. Finally, additional taxonomic research using the powerful combination of genetics, morphology, and bioacoustics may yet reveal more cryptic diversity within the North American leopard frog complex.

Acknowledgments

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