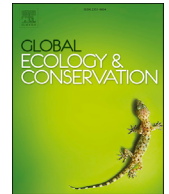




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Original Research Article

Integrating bioacoustics, DNA barcoding and niche modeling for frog conservation – The threatened balloon frogs of Sri Lanka

Nayana Wijayathilaka^{a, b}, Gayani Senevirathne^{c, b}, Champika Bandara^d, Sanath Rajapakse^b, Rohan Pethiyagoda^e, Madhava Meegaskumbura^{f, b, *}^a Department of Zoology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka^b Department of Molecular Biology & Biotechnology, Faculty of Science, University of Peradeniya, KY20400, Sri Lanka^c Department of Organismal Biology and Anatomy, University of Chicago, IL 60637, USA^d Faculty of Science and Technology, Uva Wellassa University, Badulla, Sri Lanka^e Ichthyology Section, Australian Museum, 1 William Street, Sydney, NSW 2010, Australia^f Guangxi Key Laboratory in Forest Ecology and Conservation, College of Forestry, Guangxi University, Nanning 530004, PR China

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ABSTRACT

Discovering and monitoring anuran populations that are in decline, and ascertaining boundaries for cryptic and rare species, is a challenge for their conservation management. Here, we integrate three techniques, bioacoustics (call), niche modeling and DNA barcoding as a test case to investigate how the combination of these methods can enhance search efficiency for previously unknown populations, especially for those species that are threatened. As a focal group, we considered a clade in the genus *Uperodon* earlier referred to as *Ramanella*, represented by four endemic species in Sri Lanka (*U. nagaoui* – Endangered; *U. palmatus* – Critically Endangered; *U. obscurus* – Vulnerable and *U. rohani* – possibly Least Concern); we focus on the two highly threatened species (*U. nagaoui* and *U. palmatus*). We used mitochondrial DNA barcodes (16S rRNA) to link species accurately to their call and subsequently predicted species distributions using MaxEnt-based niche modeling of known species locations and forest cover data to increase the efficiency of searching for new populations. Lastly, we analyzed call data for accurate and rapid identification of new and viable populations. Following enhanced predicted distribution models, we visited 14 potential sites and sampled for calls of the two highly threatened species. Within a period of two weeks of fieldwork, we discovered two new populations of *U. nagaoui* and one population of *U. palmatus* by identifying their calls in areas predicted by niche modeling; we also confirm species identities at several previously unconfirmed locations. Finally, we included the new locations to enhance the distributional predictions for the threatened species. We discuss our results in the context of integrating methods to facilitate conservation of rare and threatened frog species.

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* Corresponding author. Guangxi Key Laboratory in Forest Ecology and Conservation, College of Forestry, Guangxi University, Nanning 530004, PR China.
E-mail address: madhava_m@mac.com (M. Meegaskumbura).

1. Introduction

Threatened species are characterized by reductions in range and population size (IUCN, 2012). The efficient monitoring of populations of such species requires rapid detection and identification of individuals as well as the prediction of habitats in which populations are most likely to persist. The small size of many anurans (frogs and toads), the seasonal variation of their populations, and the structurally complex habitats (e.g., rainforests) in which many threatened species occur, present formidable challenges to such monitoring. Given that the proportion of amphibians threatened with extinction exceeds those of all other vertebrate classes (Collins and Crump, 2009; Hof et al., 2011; Houlahan et al., 2000; Sodhi et al., 2008; Stuart et al., 2004, 2008), rapid monitoring methods that accurately determine their distributions and populations are needed. While the presence of a species in a habitat predicted to be suitable can be determined using a variety of techniques, it is traditionally achieved through collection and identification of specimens by means of external morphology. Biologists involved in the conservation and monitoring of anurans, however, try to reduce stress by minimal handling of specimens, and only carry out destructive sampling for identification in exceptional cases, especially in species that are suspected of being in decline. Monitoring often requires quick evaluation through morphological identification, which could be subjective for species that are cryptic or difficult to locate. Detection is further confounded by many anuran species being only nocturnally active, and often only when it is raining or misty, conditions that further reduce the effectiveness of the identification process. However, if a call can be precisely matched to a specific species, whose identification is confirmed through genetic methods (barcoding), it can effectively be used for discovery, quantification and monitoring of populations, including previously unknown ones.

Most male anurans call, and such calls in most cases have species-specific acoustic features that make them identifiable (Gerhardt and Huber, 2002; Wells, 1977). Such identification, however, requires that calls be precisely documented and accurately analyzed so as to be differentiated from those of closely-related species (Bee et al., 2013; Borzee et al., 2018; Wijayathilaka et al., 2016; Wijayathilaka and Meegaskumbura, 2016). Amphibian biologists have, accordingly, for some time used vocalizations as a proxy for the occurrence of anuran species (Heyer et al., 1994; Rödel and Ernst, 2004).

While bioacoustics is used to determine the location of a species, niche modeling is used to evaluate broad-scale species distribution patterns (Carroll, 2010; de Pous et al., 2010; Guisan et al., 2006; Pawar et al., 2007; Rödder et al., 2008; Urbina-Cardona and Flores-Villela, 2010). Predictive modeling based on species occurrence data can be used to identify regions with bioclimatic conditions conducive to species sustenance (Sandoval-Comte et al., 2012). Such models can then be enhanced through the inclusion of habitat layers to define optimal habitat conditions, such as forest cover or the presence of water-bodies (Carroll, 2010), allowing species distributions to be predicted at the landscape scale.

We integrate DNA barcoding, bioacoustics and niche models for a closely related group of frogs endemic to Sri Lanka to accurately and rapidly determine their distributions to facilitate conservation. A nested clade within the balloon frogs of the genus *Uperodon* (previously assigned to *Ramanella*: Peloso et al., 2015), is restricted to South Asia. Five of these species are endemic to Indian peninsula and four to Sri Lanka (Garg et al., 2018). The threat-status of the Sri Lankan species is variable, three of them are threatened with extinction, while the fourth remains to be assessed; their natural history may explain much of their distributions.

Uperodon nagaoui is an Endangered species inhabiting the lowland's rainforests of the island's south-western 'wet zone' (Manamendra-Arachchi and Pethiyagoda, 2006; MOE, 2012). It deposits eggs overhanging phytotelms 0.5–9 m above ground (Manamendra-Arachchi and Pethiyagoda, 2001). *Uperodon obscurus* is a habitat-generalist facultative tree-hole breeder, which lays floating eggs (Meegaskumbura, 1999; Bowatte and Meegaskumbura, 2011), assessed as Vulnerable (MOE, 2012). *Uperodon palmatus*, a Critically Endangered species, is similar in morphology and breeding behaviour to *U. obscurus*, except for its high-elevation distribution (MOE, 2012). The fourth species, *U. rohani* (earlier *U. variegatus*) occurs widely in the lowland dry zone of Sri Lanka, depositing floating masses of eggs in ground pools (Garg et al., 2018). In future assessments, due to its high area of occupancy and extent of occurrence, *U. rohani* will be likely evaluated as Least Concern. All four species have filter-feeding tadpoles, some with behavioral and morphological specializations.

We describe the calls of these species and generate their DNA barcodes while using bioacoustic surveys to locate new populations of the two most threatened species. We discuss predicted and actual species distribution boundaries, and the usefulness of integrating rapid, remote and accurate methods for threatened-species conservation.

2. Materials and methods

2.1. Niche modeling for predicting suitable habitats

We collated distribution records of the four Sri Lankan species of *Uperodon* from the published literature (Manamendra-Arachchi and Pethiyagoda, 2006; Karunaratne and Amarasinghe, 2009) and our own field records (Table A1). The program MaxEnt, version 3.3.3 k (Philips et al., 2004) was used to estimate the potential geographic distribution of each species. MaxEnt is an ecological niche-modeling program (Philips et al., 2006), which uses a machine-learning algorithm and maximum entropy technique to determine the best possible probability distribution. The program needs two input resources: 1) Presence-only records of the species, and 2) digital layers of environmental variables in a selected geographic area. In this study, for the predictive distribution models for *U. nagaoui*, *U. palmatus*, *U. obscurus*, and *U. rohani*, 17, 8, 14 and 13 location-records were used, respectively (Table A1). We used 19 environment layers, in addition to the altitude layer, at 0.0083° (~1 km²) resolution, from WorldClim (<http://www.worldclim.org>). All layers were clipped to our study region bounded by

Table 1

Values extracted from the distribution models produced using MaxEnt software for the four *Uperodon* species. LPT = Lowest Presence Threshold, PDF = Predicted distribution within tree covered habitats, PDP = Predicted distribution within the protected area network, AOO = Area of occupancy (each cell is 100 km²), EOO = Extent of occurrence.

Species	LPT	Tree cover (%)	Predicted area (km ²)	PDF (km ²)	PDP (km ²)	AOO (km ²)	EOO (km ²)	Habitat (breeding)
<i>U. nagaoui</i>	0.332 (43.2%)	>78	6222	3640	1086	1300	2690	Exclusively in tree-holes in canopy covered forests
<i>U. palmatus</i>	0.242 (32%)	NA	982	NA	455	600	330	Small pools, not observed in tree-holes. Open areas and forests in high hills
<i>U. obscurus</i>	0.222 (28%)	NA	12544	NA	2082	1200	5700	Tree-holes, rock pools and temporary pools in wet zone.
<i>U. rohani</i>	0.306 (49%)	NA	50882	NA	16488	1300	36310	Pools in dry zone

5.908°–9.842° N and 79.516°–81.891° E (which envelopes Sri Lanka). Highly correlated variables ($r \geq 0.8$ Pearson correlation coefficient) were eliminated from the analysis, following Graham (2003); altogether 9, 8, 8 and 10 variables were selected to generate the predictive models of *U. nagaoui*, *U. palmatus*, *U. obscurus*, and *U. rohani*, respectively (Table A2). To generate the predictive models for all species, the automatic mode with jackknife validation approach was used as proposed by Pearson et al. (2007), which performs well even when the sample size is small. The random seed option was used for all sample points to train the model and 25% of the records to test it. We ran 10 replicates using the bootstrap function and selected the average model. To obtain the values of habitat suitability we use the logistic method, in which the probability values ranged from 0 to 1. The results were then recorded as binary 'presence' and 'absence' values. Minimum training presence (LPT) was used as the threshold value for each model (see Table 1). The tree cover layer was downloaded from the Google Earth engine database (Hansen et al., 2013). The layer contains tree cover in the year 2000, defined as canopy closure for all vegetation taller than 5 m (more recent tree cover data for Sri Lanka is not available). This data is encoded as a percentage value and has a spatial resolution of approximately 30 m at the equator. Predicted distribution was filtered using the tree-cover layer for improved prediction of forest niches for the forest-dependent species. Further, we use this layer to obtain the percentage forest cover at the presence locations of the four species. The protected areas network layer was downloaded from the World Database on Protected Areas (<http://www.protectedplanet.net>); this was used to calculate the potential distribution within protected areas. The Extent of Occurrence (EOO) and Area of Occupancy (AOO) were calculated for each species following the definitions of IUCN (2012) and using the Geospatial Conservation Assessment Tool (Bachman et al., 2011). To estimate the AOO, a uniform grid of 10 × 10 km representing a cell area of 100 km² was used (see Table 1). Following the discovery of new populations of *U. nagaoui* and *U. palmatus*, the predicted distributions and areas of overlap of distributions were recalculated using ArcGIS 10.2.

2.2. Bioacoustics: recording and characterizing frog calls

To describe the vocalizations of each species, we recorded calling males of *Uperodon nagaoui* from a population in Hiyare (135 m asl., 6.0618° N, 80.3198° E, WGS84 datum) between 19 and 21 October 2013. *Uperodon palmatus* was recorded from a population in a shallow pool in Nuwara-Eliya (1910m asl., 6.9717° N, 80.7866° E, WGS84 datum) between 20 and 22 December 2013 and *U. rohani* from a population in Mihinthale (120 m asl., 8.3550° N, 80.5055° E, WGS84 datum) between 26 and 28 September 2013. We recorded *U. obscurus* in Peradeniya (480 m asl., 7.2564° N, 80.5986° E, WGS84 datum) on 6 May 2013 (refer supplementary audio files).

Vocalizations were recorded between 18:00 and 01:00 h using a Marantz PMD 620 MKII solid-state digital recorder (sampling rate 44.1 kHz, 16-bit resolution) equipped with a directional Sennheiser ME66 microphone with a foam wind-screen. The microphone was held by hand or mounted on a tripod, and the recording tip of the microphone maintained approximately 1 m from the focal frog. The gain setting of the recorder was adjusted to optimize a high signal-to-noise ratio prior to each recording, and the same setting maintained throughout each recording without changing the distance between the frog and the microphone.

Raven Pro 1.4 was used to measure the following call characters: call duration; call rise-time; call fall-time; 50% call rise-time; 50% call fall-time; pulses per call; pulse rate; dominant frequency; and peak power. Call characterization and measuring procedure is outlined using a call of *Uperodon palmatus* as an example (Figure A2), which follows Wijayathilaka and Meegaskumbura (2016).

Vocalizations for three individuals from each species were recorded and 50 calls were measured from each species (a minimum of 16 calls from each individual). The snout-vent length (SVL), body weight and wet-bulb air temperature at the frog's calling site were measured after each recording. Tissues from two recorded specimens were collected, and representative voucher samples preserved for morphological studies. A digital caliper and a portable digital balance were used to determine SVL and body weight of the call recorded animals, to the nearest 0.01 mm and 0.01 g, respectively. A Zeal[®] thermometer (76 mm immersion, 305 mm length) was used to measure air temperature to the nearest 0.1 °C.

Call properties (Table 2) were measured using Raven Pro 1.4 software (Cornell Laboratory of Ornithology, Ithaca, NY, USA) to the nearest 0.01 ms using Raven's waveform display, while spectral properties were measured by averaging the spectrum

Table 2Description of acoustic properties measured for the four *Uperodon* species.

Properties of calls	
Call duration (ms)	Time between onset of first pulse and offset of last pulse in a call
Call rise time (ms)	Time between onset of first pulse and pulse of maximum amplitude
Call fall time (ms)	Time between pulse of maximum amplitude and offset of last pulse
50% Call rise time (ms)	Time between call onset and the half-amplitude point of earliest maximum peak in the call waveform
50% Call fall time (ms)	Time between the half-amplitude point of the last maximum peak in the call waveform and pulse offset
Pulses per call	Count of pulses (k)
Pulse rate (pulses/s)	$(k - 1)/t$, where t is the time between onset of first pulse and onset of last pulse
Dominant frequency (Hz)	Maximum frequency using Raven's selection spectrum function over the duration of the entire call
Peak power	Maximum power of the call amplitude measured in dB.

over the entire duration of a call (256 pt fast Fourier transform; Fig. 1 C1-D4). We selected calls with high signal-to-noise ratios that were free from overlap with other calling males.

We calculated the mean (\bar{X}) standard deviation (SD) and coefficients of variation ($CV = (SD/\bar{X})100$), using Systat version 11.0 (Systat Software, Inc. 2009. SYSTAT 11. Systat) to conduct principal component analysis (PCA) on the correlations matrix for all acoustic characters measured, which were common to all four species. Temperature adjustments outlined by [Platz and Forester \(1988\)](#) were not applied because recordings were made only for three specimens of each species at any one location within absence of or narrow temperature variations at calling sites.

2.3. DNA barcoding analysis for confirming calls to the species

To validate their identity, tissues were collected only from call recorded frogs. Samples were collected as outlined in [Garg et al., \(2018\)](#). Collected samples were stored at Department of Zoology, University of Peradeniya (DZ). Tissue samples from two individuals were used from each species for the molecular analysis. Additional sequences were downloaded from GenBank ([Table A3](#)). DNA was extracted from ethanol-preserved tissues using Qiagen DNeasy blood and tissue kit following manufacturer's protocols. A total of 543 base pairs (bp) were sequenced. Portions of the mitochondrial 16S ribosomal RNA (16S) gene were amplified by PCR and sequenced directly using dye-termination cycle sequencing. The primers 16Sar and 16Sbr were used for both PCR and sequencing of mitochondrial genes ([Palumbi, 1996](#)). PCR conditions for amplification were as follows: denaturation at 95 °C for 40s, annealing at 45 °C for 40s, and extension at 72 °C for 50s, 35 cycles, with a final extension of 72 °C for 5 min. All products were purified using Qiagen Qiaquick PCR purification kit and sequenced on an ABI 3100 automated sequencer following manufacturers' protocols.

Chromatograms were edited using ChromasPro (v. 1.34). The 16S dataset was aligned using the ClustalW method in MEGA v. 5.2.2 ([Tamura et al., 2011](#)), and was improved by eye. Genetic distances between the 8 individuals of *U. nagaoui*, *U. palmatus*, *U. obscurus* and *U. rohani* were calculated using PAUP*v.4.0b10 ([Swofford, 2002](#)). *Lankanectes corrugatus* (Nictibatrachidae) was designated as the outgroup in the analysis ([Frost et al., 2006](#)).

The final 16S rRNA dataset consisted of 540 bp. This was used to construct a Neighbor Joining (NJ) tree using PAUP* v. 4.0b10 under the Kimura-2-Parameter (K2P) model, a common procedure in barcoding studies. To determine node support and clade stability, a Maximum Parsimony (MP) bootstrap analysis was conducted using PAUP with 1000 replicates with a random stepwise addition set to 100 replicates.

Additionally, a Maximum Likelihood analysis was run on the Cipres Science Gateway Server ([Miller et al., 2010](#)) using the 16S rRNA dataset; model parameters, were given as estimated from the jModelTest (v.2.1.4 ([Darriba et al., 2012](#)); model parameters are given in Supplementary Material) and was run with 1000 bootstrap pseudoreplicates. Clade stability was assessed using ML bootstrapping and Bayesian Posterior Probability (PP) values. Bootstrapping was estimated in a ML framework using RAxML 7.2.8 ([Stamatakis, 2006](#)). The GTRGAMMA model was used with one thousand RAxML searches and 1000 iterations. MrBayes (v. 3.1.2; [Huelsenbeck and Ronquist, 2001](#)) was used to infer trees using a Bayesian criterion and assess posterior probability at each node. The model of sequence evolution was the same as that used in the ML analysis. Four Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) chains were run for ten million generations and uniform priors were used throughout, with branch lengths, topology, and nucleotide substitution parameters unconstrained. Burnin was defined as 0.1% generations by observing the log-output file in Tracer v.1.6 ([Rambaut et al., 2014](#)) (refer to the [Supplementary Figure A3](#) for the ML tree with clade support indicated).

2.4. Predicting new populations based on niche models and bioacoustics

Between 14 and 28 November 2014, we conducted a rapid survey using the predicted distribution model. We visited 14 arbitrarily-selected sites (Morningside, Bambarabotuwa, Ballahela, Kitulgala, Rangala, Madamahanuwara, Hantana, Agarapatana, Moray estate, Talawakele, Lindula, Nanuoya, Keerthibandrapura, Athweltota) (*U. nagaoui* and *U. palmatus*; [Table A4](#)). We searched for calling males on rainy nights and, when they were located, we proceeded to record their calls following the same procedure. We analyzed a minimum of 10 calls from 1 to 3 individuals from each new population and conducted a PCA to ascertain their identity ([Fig. 2](#)).

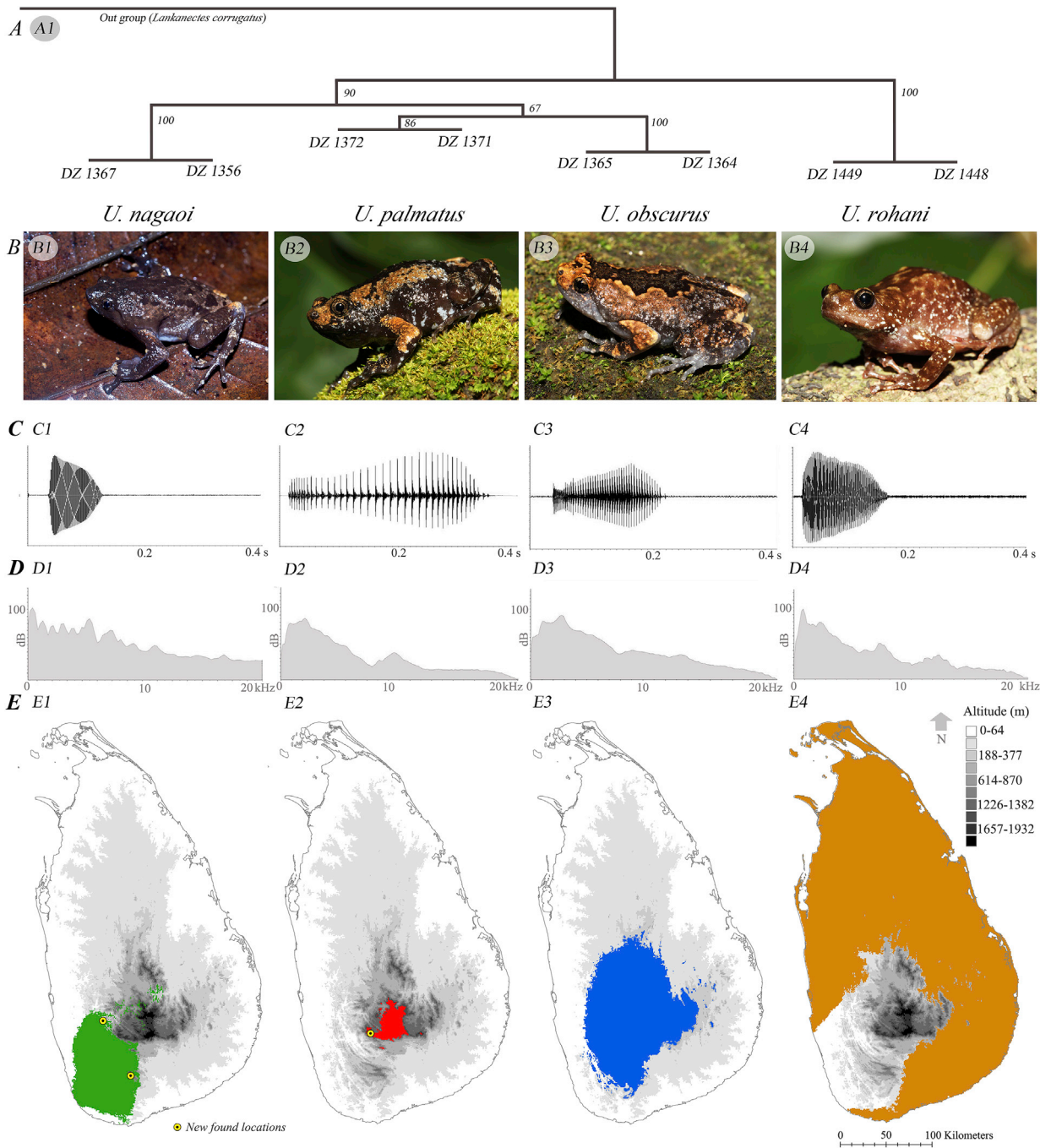


Fig. 1. Neighbor joining tree constructed using 16S gene for four species of *Uperodon* (*Ramanella*) for validating the call. Values at nodes represent the bootstrap support (A); four *Uperodon* species in life (B); Waveform (C) and power spectrum (D) of the advertisement call of four *Uperodon* species. Time shown 0.4 s. Note: To show the waveform clearly, Bandstop filter was used for the *U. nagaoui* call; Predicted distribution of the four species of *Uperodon* (*Ramanella*) in Sri Lanka based on maximum entropy (Maxent) models constructed using climate data, using Lowest Presence Threshold (LPT) criteria (E).

Maxent modeling was repeated including the newly-discovered populations to redefine the distribution models for the two Endangered species, *U. nagaoui* and *U. palmatus*. Predictions for new AOOs and EOOs were generated based on the newly discovered populations.

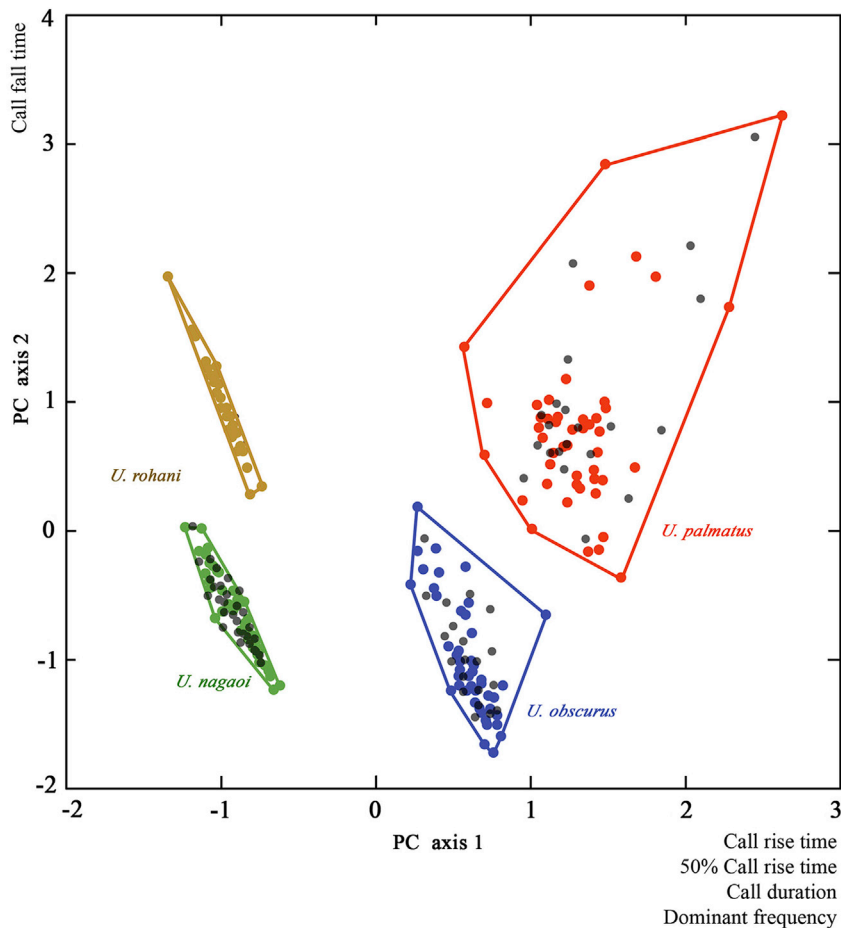


Fig. 2. Factor scores of PC1 vs. PC2 of the principal components analysis of measurements of 50 calls from each species of *Uperodon* (*Ramanella*) based on the measurements of six call characters (Call duration, Dominant frequency, Call rise time, 50% call rise time, Call fall time, 50% call fall time). Gray dots indicate the calls recorded from newly discovered populations.

3. Results

3.1. Potential distributions based on niche models

Predicted distributions of four species of *Uperodon* in Sri Lanka based on the maximum entropy models constructed using 19 bioclimatic variables, in addition to altitude, are shown in Fig. 1 and A1 (see supplementary Google Earth maps). We used mean predicted probability of presence (averaged from 10 replicates) while implementing 25% random test points for each dataset. We received better AUC values for the species (*U. nagaoui*: 0.958, *U. palmatus*: 0.996, *U. obscurus*: 0.936, *U. rohani*: 0.759) and used the Lowest Presence Threshold (LPT) criterion to select the best suitable habitat for each species (*U. nagaoui*: 0.332 (43%), *U. palmatus*: 0.242 (32%), *U. obscurus*: 0.222 (28%), *U. rohani*: 0.306 (49%)).

The predicted distribution of *U. nagaoui* extends over an area of 6220 km² across the lowland wet zone (mountainous and southwestern quadrant of Sri Lanka receiving more than 2000 mm of rainfall) including parts of the Galle, Matara, Kaluthara, Rathnapura and Kegalle districts (Fig. 1 E1). According to the forest cover percentage analysis at presence locations, this species requires over 78% forest cover. It therefore appears to be an exclusive forest inhabitant; of its predicted range, only 3640 km² has the degree of forest cover required by the species, and only 17% (1086 km²) of its predicted range falls within the protected areas system. The EOO and AOO of *U. nagaoui* were 2690 km² and 1300 km², respectively. The environmental variables that contributed mainly to the predictive model were Precipitation during the Driest Quarter (39%), Temperature Seasonality (21%) and Precipitation during the Warmest Quarter (20%).

The predicted distribution for *U. palmatus* is 982 km², entirely within the central hills at elevations above 1300 m in Nuwara-Eliya, Horton Plains National Park and Peak Wilderness sanctuary (Fig. 1 E2). Our observations suggest that this species is a facultative tree-hole breeder: calling males and tadpoles were found in shallow pools adjacent to forest edges, and in small streams. Of the predicted range, 46% (455 km²) falls within protected areas. The EOO and AOO of *U. palmatus* were

330 km² and 600 km², respectively. The environmental variables that contributed mostly to the predictive model were Mean Altitude (85%) and Precipitation Seasonality (10%).

Our model shows that *U. obscurus* occurs in mid and lower altitudes in the wet and intermediate zones (Fig. 1 E3), extending over an area of 12,544 km² of which 17% (2082 km²) falls under the protected areas network. The EOO and AOO of *U. obscurus* were 5700 km² and 1200 km², respectively. Mean Temperature of Driest Quarter (36%), Mean Diurnal Range (30%) and Precipitation Seasonality (16%) contributed mostly to the distribution model of *U. obscurus*.

Uperodon rohani has the widest distribution of the four species, including the entirety of the dry zone (Fig. 1 E4): an area 51,340 km²; almost 78% of the total terrestrial area of the island. Approximately 16,370 km² of its range lies within the protected areas network. The EOO and AOO of *U. rohani* were 36,310 km² and 1300 km², respectively. Precipitation during Warmest Quarter (16%), Precipitation during Driest Quarter (13%), Mean Annual Temperature (13%), Precipitation of Wettest Quarter (10%) and Altitude (12%) contributed mostly to the distribution model of *U. rohani*.

The predicted range of *U. obscurus* overlapped completely with that of *U. palmatus*, while that of *U. nagaoui* overlapped with *U. palmatus*, *U. obscurus* and *U. rohani* by 250 km², 4045 km² and 300 km², respectively. Only an overlap of 110 km² exists between the predicted distributions of *U. rohani* and *U. obscurus* (Table A5).

3.2. Bioacoustics: characterizing and identifying vocalizations

All four Sri Lankan species of *Uperodon* are chorus callers, calling in small groups (2–5 males). Calls can be categorized into two distinct groups: the advertisement calls of *U. obscurus* and *U. palmatus* consist of multiple pulses, whereas those of *U. nagaoui* and *U. rohani* consist of single pulses (Fig. 1C).

The advertisement calls of *U. obscurus* contain 40 to 53 pulses, with an average pulse rate of 270 ± 21 per second and a call duration of 156–203 ms (179.6 ± 8.5 ms). Dominant frequency ranged between 2.5 and 3.2 kHz (2.8 ± 0.2 kHz), with a call fall time (59 ms) twice as great as the call rise time. However, the advertisement call of *U. palmatus* (phylogenetically closest to *U. obscurus*: Garg et al., 2018) is composed of 28–49 distinct pulses, with a duration between 282 and 548 ms (340 ± 48 ms) and an average pulse rate of 119 ± 15 per second. The dominant frequency ranged between 1.8 and 2.2 kHz (Table 3).

The advertisement call of *U. nagaoui* consists of a single pulse, of duration between 60 and 92 ms (82.9 ± 6.2 ms). The call typically reaches its maximum amplitude within 12 ms (8.2 ± 2.2 ms), decaying over the subsequent 86 ms (74.7 ± 5 ms). The dominant frequency ranged between 0.5 and 0.9 Hz, with four prominent harmonics. The advertisement call of *U. rohani* is about twice as long as that of *U. nagaoui* (164.3 ± 6.2 ms). It has a rapid onset, reaching its peak amplitude in 18.6 ± 6.6 ms and decaying over the subsequent 145.7 ± 9.7 ms. The dominant frequency of all three males of *U. rohani* that were recorded was 0.9 Hz, lacking prominent harmonics. All four species showed little or no frequency modulation.

The PCA of the call characters (Fig. 2), involving six variables (call duration, call rise time, 50% call rise time, call fall time, 50% call fall time and dominant frequency) common to the four species show that they separate clearly in principle component (PC) space. The first two principal components explain more than 86% of the total variance. *Uperodon rohani* and *U. nagaoui* separate well from *U. palmatus* and *U. obscurus* on the PC 1 axis, which is explained mostly by call rise-time, 50% rise-time, call duration and dominant frequency. The PC2 axis, on which *U. nagaoui* and *U. rohani* separate clearly, is explained mostly by call fall-time. *Uperodon palmatus* and *U. obscurus* overlap slightly on both axes.

In PC space, calls from the newly-discovered populations (Fig. 2; grey dots) at Morningside and Ballahela clustered within *U. nagaoui*, while calls recorded from Morningside clustered within *U. palmatus* and those from Hantana (Kandy district) fell within *U. obscurus*.

3.3. DNA barcoding analysis

The percentage uncorrected pairwise genetic distance for 16S rRNA (Table A6) ranged between 2.6 and 8.0 among the four *Uperodon* species. The lowest interspecific genetic divergence, 2.6%, occurs between *U. obscurus* and *U. palmatus*. The genetic distance between *U. rohani* and the three other congeners is more than 7%. The 16S-based phylogeny shows *U. palmatus*, *U. obscurus* and *U. nagaoui* to form a clade separate from *U. rohani* (Fig. 1A).

3.4. New populations discovered based on niche modeling predictions and bioacoustics

Of the 14 newly-surveyed locations, two new populations of *U. nagaoui* were discovered, from Morningside forest reserve (Rakwana Hills) and Ballahela, Deraniyagala, Kegalle district (Table A1). Though the distribution model sparsely highlighted the central hills above the known northern boundary of *U. nagaoui* (Fig. 1, Fig. A1), we failed to record its call at the five sites we investigated within this region: Agarapatana, Keerthibandrapura (Nuwara-Eliya district), Rangala, Madamahanuwara and Hantana (Kandy district). We confirm their presence in three previously suggested (but not confirmed) locations, Kitulgala (Kegalle district), Athweltota (Kaluthara district) and Bambarabotuwa (Rathnapura district).

We discovered *U. palmatus* at only one new location, well away from its known range: Moray Estate (adjacent to Peak Wilderness Sanctuary, a part of a UNESCO world heritage site). Though we surveyed three locations within its predicted range (Talawakele, Lindula and Nanu Oya), we found no evidence of the species.

Table 3Descriptive statistics for the calls of four *Uperodon* species based on values determined from a sample of 50 calls from 3 males of each species.

	Mean	SD	Range (min-max)	CV (%)
<i>Uperodon nagaoui</i>				
Call duration (ms)	82.9	6.21	60.86–92.52	7.5
Call rise time (ms)	8.18	2.18	3.64–11.8	26.73
Call fall time (ms)	74.7	5.02	56.3–85.6	6.72
50% Call rise time (ms)	3.77	1.05	1.88–5.74	27.82
50% Call fall time (ms)	32.1	10.04	18.16–55.52	31.29
Dominant frequency (Hz)	720	83	516.8–861.3	11.52
Peak power	99.2	2.74	91.8–105.5	2.76
<i>Uperodon rohani</i>				
Call duration (ms)	164.3	6.23	147.8–179.6	3.79
Call rise time (ms)	18.6	6.64	10.87–50.68	35.7
Call fall time (ms)	145.7	9.67	117.33–166.56	6.63
50% Call rise time (ms)	3.91	1.53	1.33–9.97	39.3
50% Call fall time (ms)	38.15	6.58	24.47–63.85	17.26
Dominant frequency (Hz)	861.3	0	861.3	0
Peak power	97.05	0.64	96.3–98.3	0.66
<i>Uperodon palmatus</i>				
Call duration (ms)	340.2	48.6	282.39–548.78	14.3
Call rise time (ms)	258.9	42.7	220.0–464.4	16.5
Call fall time (ms)	81.31	21.16	39.7–161.6	26.0
50% Call rise time (ms)	97.6	33.8	13.79–209.5	34.7
50% Call fall time (ms)	22.23	5.83	7.4–35.76	26.26
Pulses per call	40 ^a	38–43 ^b	28–49	9.9
Pulse rate (pulses/s)	119.4	15.9	55.45–139	13.32
Dominant frequency (Hz)	2166	117.8	1894.9–2239.5	5.44
Peak power	86	2.1	79.4–91	2.46
<i>Uperodon obscurus</i>				
Call duration (ms)	179.6	8.5	156.3–203.3	4.74
Call rise time (ms)	120	14.9	82.5–155	12.4
Call fall time (ms)	59.5	13.8	30.6–95.3	23.1
50% Call rise time (ms)	41	10.8	23.3–94	26.5
50% Call fall time (ms)	18.5	8.5	6.9–44.7	46
Pulses per call	49 ^a	47–51 ^b	40–53	5.9
Pulse rate (pulses/s)	270.7	21.2	197.2–311.3	7.8
Dominant frequency (Hz)	2832	231.3	2584–3273	8.2
Peak power	91.4	1.9	86.1–97.7	2.1

4. Discussion

The integration of niche modeling, bioacoustics and DNA barcoding offers a means of rapidly and accurately assessing anuran species distributions as well as knowledge of finer-scale interactions among species, such as how they partition resources at habitat level, the condition of populations, a proxy for breeding readiness, and their periods of activity and dormancy. Together, these enrich knowledge of factors relevant to species conservation.

The remaining rainforests of Sri Lanka's south-western quarter, which are heavily fragmented, harbor the preponderant majority of Sri Lanka's endemic plant and animal species (Meegaskumbura et al., 2007). In these forest patches, habitat loss has been flagged as a major stressor for almost every one of the island's threatened anuran species (MOE, 2012). In one group of terrestrially-reproducing frogs, *Pseudophilautus*, as many as 19 species are thought to have become extinct during the past 150 years (Meegaskumbura et al., 2007; MOE, 2012). Given that Sri Lanka has lost 95% of its natural forest cover over the last two centuries, together with the high incidence of microendemism, this is not surprising. The loss of forest cover may, however, provide opportunities for some species tolerant of open habitats to increase their range and population relative to forest-specialized species. In the present instance, *Uperodon obscurus* appears to be such a case, with its ability to adapt to anthropogenic habitats enabling it to occupy a much larger range than the forest-dependent *U. nagaoui* and *U. palmatus* (Meegaskumbura et al., 2011). On a global scale, habitat alteration is the biggest threat to frog and toad populations (Gallant et al., 2007).

Uperodon palmatus (high elevations, above 1300 m) and *U. obscurus* (lower elevations) show climatic separation; they occupy similar habitat types within these elevations with both species laying eggs in phytotelms, where available, or else in small pools on the forest floor. Increasing average temperatures almost everywhere in Sri Lanka (de Costa, 2008) may have helped spread of the generalist *U. obscurus* even as the range of the cold-adapted *U. palmatus* populations contracted. Despite this, a previously unknown population of *U. palmatus* was discovered and confirmed (Table A1) through our modeling exercise. These techniques in combination may help in the discovery of remnant populations despite the continuing diminution of their ranges. Indeed, it would be these remnant populations that most urgently call for conservation attention.

During our survey, we also discovered two remote populations of *U. nagaoui*, which were well outside the minimum convex polygon that previously circumscribed their distribution (see Table A1), suggesting that other such populations await

discovery. *Uperodon nagaoui* had never previously been reported at elevations above 400 m asl. except Bambarabotuwa-Ratnapura, 950 m asl. (Karunaratne and Amarasinghe, 2009). However, in Morningside, an isolated mountain region with emergent cloud-forest ridges, we discovered a population at ~1000 m asl. They were preliminarily identified based on a prediction of the niche model and their call, later confirmed through the examination of adult and tadpole morphology and observation of gel-encapsulated eggs adhered to the walls of arboreal phytotelms, a life history characteristic of these frogs (Manamendra-Arachchi and Pethiyagoda, 2001). At least six calling males were heard from the short canopy of the montane forest, which suggests the existence of a breeding population at this site. The population found at Ballahela, Deraniyagala was in a mid-elevation canopy-covered habitat, which extends the northern boundary of the previously known range of this species.

Vocalizations inform not only the identity and approximate size of a frog population but also provide a proxy for the breeding condition of such a population. Frog calls serve to advertise the caller's location, signal its reproductive condition and readiness to defend its territory (Gerhardt and Huber, 2002; Narins et al., 2007; Wells and Schwartz, 2007). A calling population signals both the presence of reproductively-mature adults in good health, and optimal environmental and climatic conditions for breeding. This is especially pertinent for *U. nagaoui*, because calling males are associated with arboreal phytotelms (Manamendra-Arachchi and Pethiyagoda, 2001), a microhabitat mostly observed in old growth forests.

Since some phytotelms are located high up on trees, they cannot easily be reached without specialized climbing gear, and at night when the frogs are active, this is an impossibility. But by enumerating calls, the number of such breeding sites in an area can be assessed rapidly. Since 2–5 males usually vocalize from each occupied phytotelm (pers. obs.), an approximate estimation of the male population can be made.

When the newly-discovered populations were added to the niche model, it altered the available predicted habitats for the two threatened species in two different ways: *U. nagaoui* decreased by 673 km² and *U. palmatus* increased by 358,284 km², respectively. The increase in area is expected, but a decrease in predicted area is unexpected. This could be due to the better resolution of the bioclimatic data that formulated the model.

Our analysis of Sri Lankan *Uperodon* suggests that the species of primary conservation concern is *U. nagaoui*. With genetic distances ranging from 3.9 to 8.1% for the 16S gene fragment, this is the most genetically-divergent of the four Sri Lankan species. It is also distinctive in terms of its reproductive biology, bioacoustics, ecology and behaviour. We have up to now not observed *U. nagaoui* breeding in or calling from near in ground pools, which suggests that unlike the other three Sri Lankan species of *Uperodon*, it obligatorily breeds in arboreal phytotelms, adhering its eggs to a woody surface overhanging the water. Moreover, we observed males of *U. nagaoui* hydrating clutches of eggs using their abdomen, a form of parental investment not observed in any other species of *Uperodon*. Populations of this species appears to have declined with the loss of closed-canopy forest cover due to anthropogenic activities (FAO, 2010).

The two sister taxa, *U. palmatus* (CR) and *U. obscurus* (VU), have an uncorrected pairwise distance ranging between 2.4 and 3.1% for the 16S barcoding gene; hence they are closely related to each other genetically. Their calls differ mostly in temporal properties rather than spectral properties; this could be due to the differences in the habitat temperatures experienced by these two frogs. Moreover, *U. palmatus* and *U. obscurus* exhibit facultative tree-hole egg laying, on the surface of the water. Furthermore, the predicted niche of *U. obscurus* completely overlaps that (and the presence locations) of the rarer species. Focused population level genetic studies are hence needed to ascertain the species boundaries and reciprocal monophyly for the two species.

The recently recognized new species, *Uperodon rohani*, showing an extensive distribution across the island's dry and intermediate zones, requires the least amount of conservation attention. It overlaps the predicted distributions of *U. nagaoui* and *U. obscurus*, by 120 and 1140 km², respectively (Table A4). In the likely event of climatic warming, *U. rohani* is ominously predisposed to replace *U. obscurus* populations at least in the lowland-wet zone regions of the island (de Costa, 2008).

Integrating methods while generating new information helps in formulating new questions of value to conservation planning. If a species is not present in a habitat predicted by the models, one can infer reasons for this. Is it because the species was never there? Is the species present but not calling due to unfavorable conditions (i.e. is the population not in breeding condition)? Or did a local population extinction occur?

Through integrating niche modeling, bioacoustics and DNA barcoding, we have generated knowledge that will facilitate conservation of *Uperodon* (*Ramanella*) species in Sri Lanka. We have indicated the following: highlighting the specialized, forest-dependent species that will be affected by primary habitat loss; species that are tolerant to opening up of habitats; remnant populations of threatened species from outside of their known distributions; conformity of genetic species boundaries with that of vocalization patterns and changes in distribution for species in relation to warming events of their respective habitats.

5. Conclusions

Given that many frog and toad populations are in decline across the world, accurate identification of populations that are in breeding condition will greatly facilitate their conservation management. Our study of *Uperodon* suggests that integration of niche modeling and bioacoustics, supported by accurate reference to their calls through DNA barcoding, can be used to search not only widely but also locally for cryptic and threatened frog species. Integration of methods for conservation is especially pertinent in a backdrop where climatic change is threatening to dislodge well established populations to move or be doomed.

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Appendix A. Supplementary data

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