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NEW LOCALITIES, DISTRIBUTION AND HABITAT MODELING OF THE CRITICALLY ENDANGERED SRI LANKAN FROG *Nannophrys marmorata*

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Nannophrys marmorata is a critically endangered species of frog known only from a few locations in the Knuckles Conservation Forest (KCF) in the Knuckles mountain range of Sri Lanka. Here, we report new localities outside its known range in the KCF and examine the distribution of *N. marmorata* providing the most extensive distribution map for this species. Furthermore, using the distribution information, we model the fundamental ecological niche of this species through Ecological Niche Modeling (ENM) to predict suitable habitats and fine-scale distribution patterns. ENM predicted eastern slopes of the Knuckles mountain range in central Sri Lanka as areas with high habitat suitability (suitability >75%) for *N. marmorata*. Predicted Extent of Occurrence for *N. marmorata* was 831 km² and predicted Area of Occupancy was 275 km². We discuss these findings in light of conservation for this species threatened with extinction.

Keywords: area of occupancy; conservation; DNA barcoding; ecological niche modeling; extent of occurrence; Maxent; VRR sanctuary.

INTRODUCTION

Amphibians are the most threatened group of vertebrates in the world with nearly 41% of the global diversity at risk of extinction (Hoffmann et al., 2010). Sri Lanka is an amphibian hotspot (Meegaskumbura et al., 2002) which is home to 119 species with 105 species being endemic to the island (Wickramasinghe et al., 2015; Senevirathne et al., 2018). Of this, 65% of the diversity is threatened with extinction, while approximately 34% are categorized as critically endangered and 23% categorized as endangered species (Manamendra-Arachchi and Meegaskumbura, 2012). Unfortunately, 18 of the 119 species have already become extinct (Wickramasinghe et al., 2012, 2013a, 2013b). Thus, conservation measures are

urgently necessary to stem the rate of amphibian population declines of many threatened species. Fundamental to the conservation of amphibians is an understanding of fine-scale distribution patterns and identification of isolated populations (Marsh and Trenham, 2001). However, determining the fine scale distribution and identification of isolated populations of species is time consuming and costly; hence computational methods such as ecological niche modeling (ENM) have proved to be useful alternatives in determining the potential areas of occupancy of species (Peterson et al., 2000).

Nannophrys is a genus of frogs endemic to Sri Lanka, which consists of four species (Fernando et al., 2007). All species of the genus are threatened with extinction while a single species (*N. guentheri*) is already extinct (Manamendra-Arachchi and Meegaskumbura, 2012). Members of this genus are closely associated with wet rock surfaces and boulders and are characterized by flattened bodies that enable them to live under boulders and in rock crevices and thus have earned the name “Rock Frogs” (Dutta and Manamendra-Arachchi, 1996; Senevirathne and Meegaskumbura, 2015). The Marbled or Kirthisinghe’s Rock Frog, *Nannophrys marmorata* is a criti-

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cally endangered species of frog known only from a few locations in the Knuckles Conservation Forest (KCF) of the Knuckles mountain range. Here, we report new localities and examine the distribution of *N. marmorata*. Further, we model the fundamental ecological niche of *N. marmorata* using published and new locality data to unravel potential areas of occupancy and fine scale distribution patterns of this critically endangered frog.

MATERIAL AND METHODS

New localities and distribution. We examined the distribution of *N. marmorata* through field surveys conducted in the Knuckles mountain range and the adjacent areas since 2006. The species was identified using the descriptions available in Dutta and Manamendraarachchi (1996) and Fernando et al. (2007). Whenever a positively identified *N. marmorata* was observed, the coordinates of the location were recorded using a Garmin etrex GPS (Garmin International, USA) and the habitat characteristics were noted. We then mapped the distribution of *N. marmorata* using these locations and locations taken from published literature (Clarke, 1983; Dutta and Manamendra-Arachchi, 1996; de Silva et al., 2005; Fernando et al., 2007).

During the course of the survey, several frogs belonging to the genus *Nannophrys* were observed on wet rock surfaces on the northern portion of the Victoria-Randenigala-Rantambe (VRR) Sanctuary on the 19 April 2008 and the 14 August 2008. Though they generally resembled *N. marmorata*, their coloration was somewhat different to the typical coloration of *N. marmorata* seen in the Knuckles mountain range. We revisited the site on the 13 February 2016 and observed a total of 32 frogs in four different nearby locations. Four specimens were photographed in several different angles and a total of 12 morphometric measurements were taken using a Mitutoyo digital caliper (Mitutoyo Corp, Kanogawa, Japan) and released back to the same site. The 12 morphometric measurements were taken following Fernando et al. (2007): snout to vent length (SVL), head length (HL), head width (HW), head depth (HD), eye diameter (ED), inter orbital length (IO), tympanum width (TW), upper arm (UA); lower arm (LA), femur length (FL), tibia length (TL), tarsus length (TAL).

To confirm the identity of the frogs through DNA barcoding, 0.5 mm parts of the fingertip was clipped from two individual frogs and were preserved in 70% ethanol solution. No specimens were collected and preserved during the study. For DNA barcoding, we PCR amplified and sequenced an approximately 550 bp length region of the small subunit of the 16S rRNA gene. Whole

genomic DNA was extracted from the two fingertips using Promega Wizard[®] genomic DNA purification kit (Promega Corporation, Madison, Wisconsin, USA) following manufacturer's protocols. 16S ribosomal RNA gene region was amplified using the primers 16SA-L (CGCCTGTTTATCAAAAACAT and 16SB-H (CCGGTCTGAACTCAGA TCACGT) (Vences et al., 2005). The PCR amplification employed 35 cycles with an annealing temperature of 55°C (Vences et al., 2005) following standard PCR protocols with Promega PCR master mix (Promega Corporation, Madison, Wisconsin, USA). The PCR products were purified and sequenced in both directions at the Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka (IBMBB). Consensus sequences from forward and reverse reads were assembled in GENEIOUS PRO 5.6 software (Drummond et al., 2009). The newly generated DNA sequences have been submitted to the genbank under the accession numbers MN022541-MN022542. We downloaded several DNA sequences belonging to *N. ceylonensis* and *N. marmorata* from the Genbank (www.ncbi.nlm.nih.gov/genbank). *Nannophrys marmorata* reference sequence available in the Genbank has been collected from Laggala, Matale in the northern KCF (Vences et al., 2000). All the sequences were aligned using the GENEIOUS PRO 5.6 software (Drummond et al., 2009) and then manually edited and refined by eye. A neighbor-joining tree was constructed to examine the identity of the two DNA sequences in MEGA version 7.0 (Kumar et al., 2016). *Hoplobatrachus rugulosus* was used as an outgroup since it has been shown that *Nannophrys* is sister to both *Euphlyctis-Hoplobatrachus* clade (Pyrone and Wiens, 2011). The branch support for the Neighbor-Joining tree was assessed by implementing 200 bootstrap pseudoreplicates. Average pairwise genetic distances were calculated in MEGA version 7.0 using K2P substitution model.

Ecological Niche modeling (ENM). ENM was done using coordinates of the occurrence localities of *N. marmorata* obtained from literature ($n = 7$) (Dutta and Manamendra-Arachchi, 1996) and from our own observations ($n = 4$) with environmental variable layers. We obtained environmental variable layers ($n = 55$) for South Asian region from BIOCLIM (<http://www.worldclim.org/bioclim>), a portal that provides free climate data. The data layers were cropped to a smaller size to only include Sri Lanka (9.9433 – 5.86810° N 79.31250 – 82.2285° E) from its larger tile size that included the Indian subcontinent. All the environmental variable layers were re-sampled into 1 km² resolution using ArcMap 10.3 software (ESRI, 2014). ENM was done using maximum-entropy techniques implemented in the software Maxent (Phillips et al., 2006) with 15 replicates and 5000 itera-

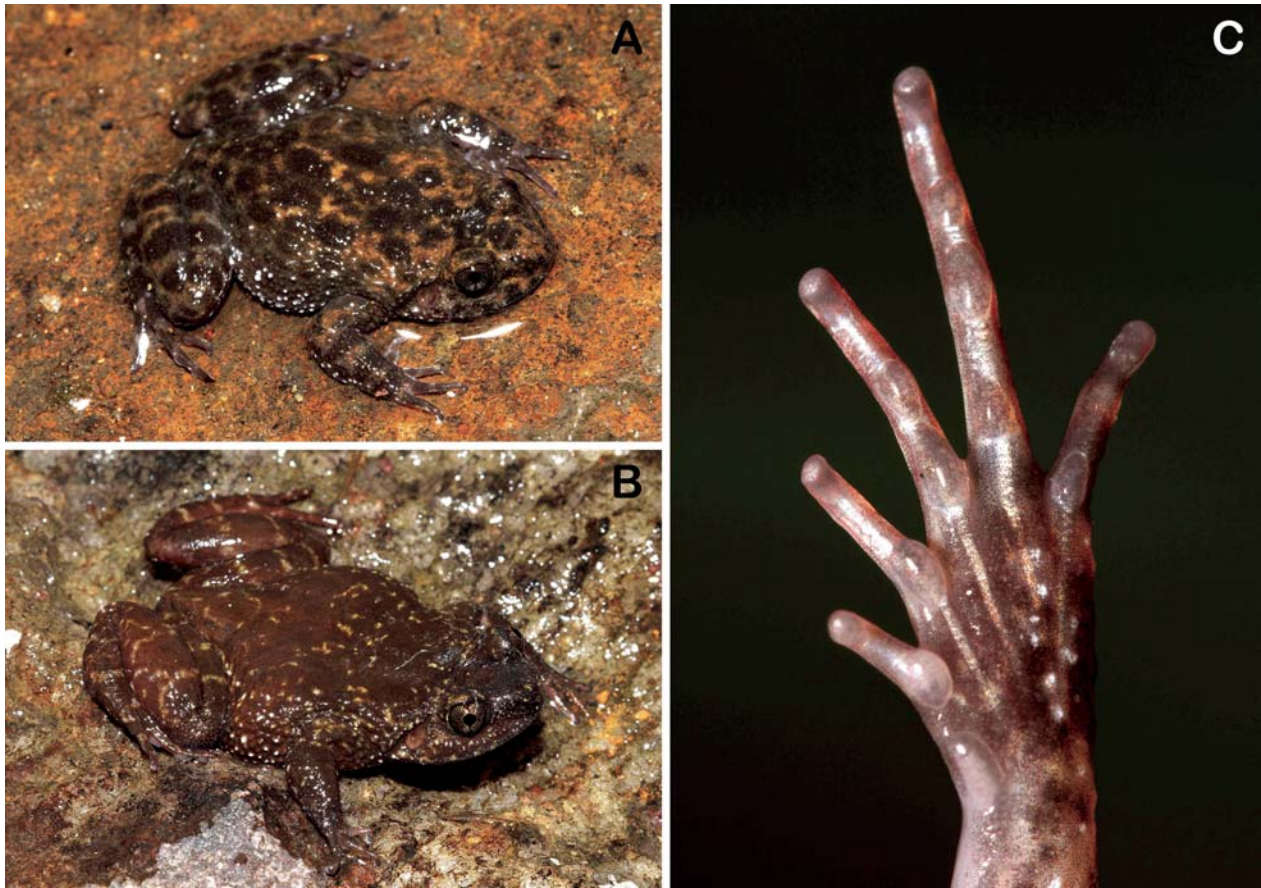


Fig. 1. *Nannophrys marmorata* observed in the VRR Sanctuary, Sri Lanka: A, male; B, female; C, ventral aspect of the left foot showing the closely placed distal subarticular and penultimate subarticular tubercles on the 4th toe.

tions while keeping all other parameters default. The output map was processed and interpreted using ArcMap 10.3 software (ESRI, 2014). The habitat suitability layer properties of the predicted map were classified into five classes (100 – 75%, 75 – 50%, 50 – 30%, 30 – 15%, 15 – 0%) using ArcMap 10.3.

We calculated the predicted Extent of Occurrence (EOO) for *N. marmorata* using the predicted distribution map by drawing a polygon connecting all the outer grids which has over 75% habitat suitability and calculating the area within the polygon using ArcMap 10.3 software. We calculated the predicted Area of Occupancy (AOO) by counting the number of 1 km² grids over 75% habitat suitability using ArcMap 10.3.

RESULTS

New localities. The specimens observed in the VRR sanctuary were assigned to the genus *Nannophrys* by the following combination of characters: dorso-ventrally

flattened body; horizontal pupil; blunt digit tips; partially webbed toes; well developed supra-tympanic fold; bound outer metatarsals; fourth toe webbing between penultimate subarticular tubercle and antepenultimate subarticular tubercle; presence of scattered white-tipped tubercles on the upper surface of head, shoulders and limbs; presence of an elongate and a compressed inner metatarsal tubercle (Clarke, 1983; Dutta and Manamendra-Arachchi, 1996) (Fig. 1). The specimens from the VRR sanctuary were distinguished from *N. ceylonensis* and *N. guentheri* by the smoothly rounded snout when viewed laterally and the presence of scattered white tipped tubercles on head and by the presence of marbled markings on legs (Fig. 1A, B). They were differentiated from *N. naeyakai* by the presence of closely placed distal subarticular and penultimate subarticular tubercles on the 4th toe (vs. well separated in *N. naeyakai*) tubercle (Clarke, 1983; Dutta and Manamendra-Arachchi, 1996) (Fig. 1C).

Description of the specimens. Dorsum heavily tuberculated in males and subadults but smooth in the two

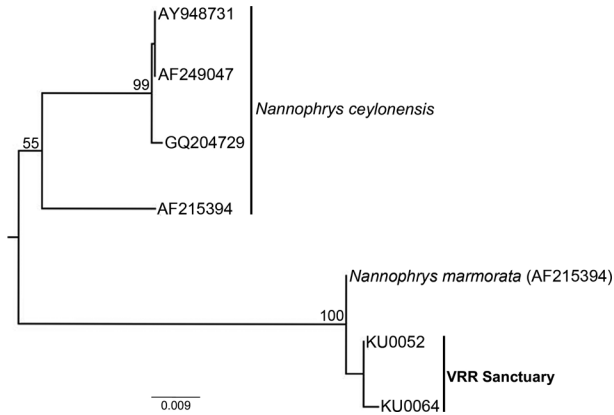


Fig. 2. Neighbor-Joining tree of 16S rRNA gene sequences showing the affinities of the two *Nannophrys* specimens collected from VRR Sanctuary. The outgroup, *Hoplobatrachus rugulosus* is not shown. Values at the nodes indicate bootstrap support. Scale is in number of substitutions per site.

females observed, venter smooth in all the observed individuals. Warty pustules present on the sides of the belly in all. Dorsum yellow ochre to light brown with oval dark brown spots in subadults and males and it was brown with yellow marbled pattern on the female. Limbs marbled, belly whitish yellow in all specimens. Snout to vent length ranged from 25.32 to 34.89 mm (Table 1).

Molecular diagnosis. Neighbor-Joining tree placed the two sequences generated from the samples collected from the VRR Sanctuary with *N. marmorata* reference sequence from the Genbank (AF215394) in a strongly supported (bootstrap support >70) reciprocally monophyletic group (Fig. 2). There was 0.4% average genetic distance between the two sequences generated from the

TABLE 1. Morphometric Measurements (mm) and the Sex of *Nannophrys marmorata* Specimens Observed from the VRR Sanctuary, Sri Lanka

Morphometric measurement	Specimen 1	Specimen 2	Specimen 3	Specimen 4
SVL	34.9	26.5	28.2	25.3
HL	13.3	11.3	11.8	10.1
HW	14.2	11.6	13.0	10.8
HD	7.6	5.2	4.4	4.2
ED	4.3	3.7	3.8	3.7
IO	4.0	2.9	3.0	2.4
TW	3.4	2.0	2.4	1.9
UA	6.3	4.8	6.1	4.2
LA	6.9	4.3	5.1	3.5
FL	13.0	11.3	11.5	10.2
TL	13.8	11.3	12.1	11.9
TAL	11.0	7.8	8.1	7.2
Sex	Female	Male	Male	Male

samples collected from VRR Sanctuary and *N. marmorata* sequence from the Genbank in the 16S rRNA gene while it was 9.3% between the former and *N. ceylonensis*.

Description of the new locations and habitat. Individuals of *N. marmorata* were found on exposed wet rock surfaces, roadside drains, rock crevices, under boulders and along streams between Udadumbara and Gurulupotha in three locations along the Kandy – Mahiyangana – Padiyathalawa highway (coordinates 7°19'56" N 80°54'10" E – 7°19'40" N 80°53'41" E) (Fig. 3). The elevation of these locations ranged between 801 – 834 m a.s.l. The surrounding habitat consisted of an ecotone between tropical dry-mixed evergreen forests and submontane forests.

Distribution. The type locality of *N. marmorata* is Maussakanda, Gammaduwa (7°34' N 80°42' E) at an elevation of 915 m in the northwestern area of the Knuckles Mountain range, which lies outside the KCF (Kirtisinghe, 1946). Clark (1983) reported it from Laggala (7°33' N 80°44' E) at an elevation of 1145 m. In addition to these locations, Dutta and Manamendra-Arachchi (1996) reported it from Gonewela (near Pallegama) in the eastern slopes of the Knuckles mountain range. At an elevation of 200 m a.s.l., Gonewela seems to be the lowest elevation this species has been recorded so far. Fernando et al. (2007) reported it near Rathna Ella falls (7°22'35" N 80°55'06" E, elevation: 572 m a.s.l.) in the Kandy district, which also lies outside the KCF. Further, the species has been reported from Pitawala pathana (7°32'51" N 80°45'18" E, elevation: 858 m a.s.l., near Lakegala (7°26'52" N 80°49'23" E, elevation: 688 m a.s.l., Emmadawa (7°30'50" N 80°49'38" E, elevation: 478 m a.s.l. and Kahatagolla (7°18'19" N 80°52'08" E, elevation: 918 m a.s.l. (de Silva et al. 2005). More recently the species was reported from an unspecified location (thus not shown in map) in the Moragahakanda reservoir construction site (Pollonnaruwa district, North Central Province) (IUCN, 2007). These locations suggest that the species is distributed between an elevation range of 200 – 1200 m a.s.l. Observations made by us and previous authorities indicate that the frogs are mostly seen on wet rock surfaces along streams. In Maussakanda, Gammaduwa, we found the specimens along a stream that flows through a tea estate and in Pitawala pathana the frogs occur on wet rocks, under boulders and crevices along a seasonal stream surrounded by grassland. Habitats the specimens reported by us and other studies include tropical submontane forests, pathana grasslands and tropical moist evergreen forests. Extent of occur-

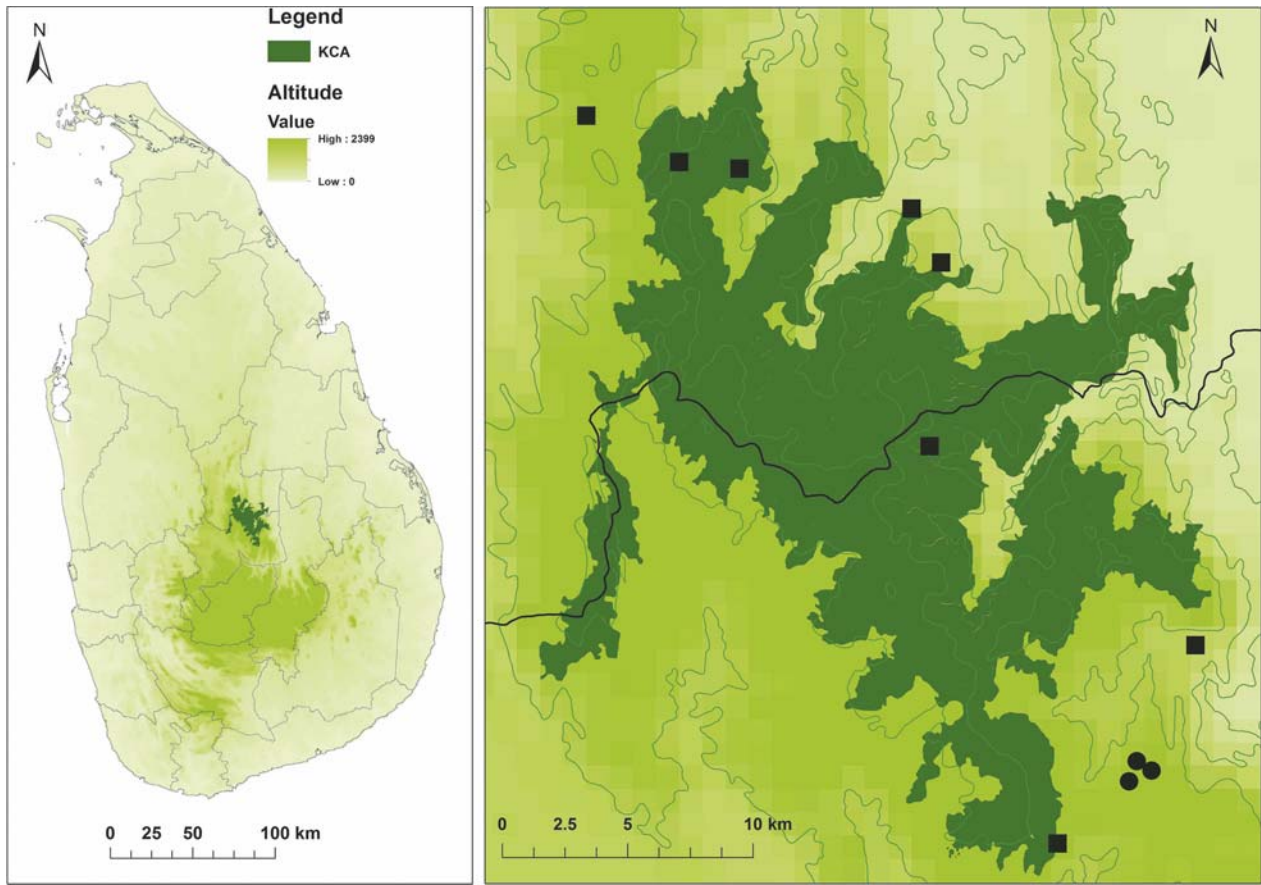


Fig. 3. Distribution of *Nannophrys marmorata*, filled squares represent known locations of *N. marmorata* from the northern Knuckles mountain range and the filled circles depict new localities. Dark green area represents the Knuckles Conservation Forest (KCF). Dark line illustrates the district boundary that separates the Matale and Kandy districts.

rence with new locality data is 255.65 km² while the AOO is 11 km².

Ecological Niche modeling. ENM mostly predicted eastern slopes of the Knuckles mountain range as areas with high habitat suitability (suitability >75%) for *N. marmorata* (Fig. 4). Predicted EOO for *N. marmorata* was 831 km² and predicted AOO was 275 km². The high Area Under the Curve value (0.995, SD = ±0.004) suggested good model performance and high accuracy of the prediction. The environmental variable layer “Mean Diurnal Range (Mean of monthly (maximum temperature – minimum temperature))” had the most useful information for the model by itself as it had the highest gain when used in isolation. Similarly, “Mean Diurnal Range (Mean of monthly (maximum temperature — minimum temperature))” had the most information for the model that is not present in the other environmental variables as the gain decreased the most when omitted.

DISCUSSION

Our morphological comparisons and molecular diagnosis strongly confirmed that the individuals of *Nannophrys* observed in VRR Sanctuary are conspecific with *N. marmorata*. The morphological characters and coloration of the specimens from the new locality reported here were generally in agreement with that for the previously reported specimens from the northern Knuckles mountain range (Clarke, 1983; Dutta and Manamendra-Arachchi, 1996). Though, the dorsal color was lighter in a few specimens observed, the two large females observed had the marbled coloration, which is the typical dorsal coloration for the species (Dutta and Manamendra-Arachchi, 1996).

The presence of *N. marmorata* in the VRR Sanctuary reported here makes it the fourth (other three locations Gammaduwa, Rathna Ella and Moragahakanda) population of this species known outside the Knuckles Conservation Forest. Our findings and published data indicate

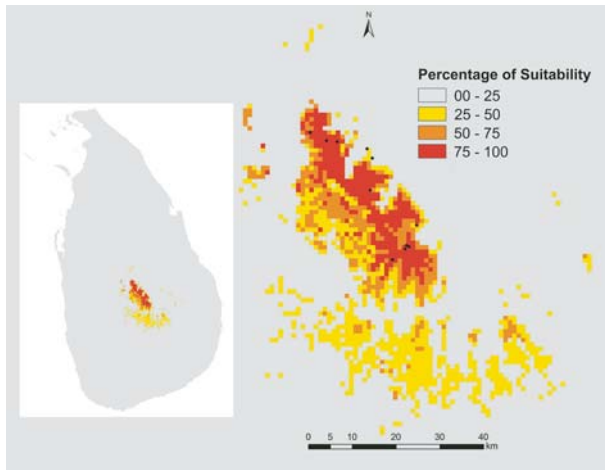


Fig. 4. Suitable habitats for *N. marmorata* predicted through ecological niche modeling in MAXENT. Model predicted most of the habitats on the eastern slopes of the Knuckles mountain range as highly suitable habitats (suitability >75%) for *N. marmorata*. Small black dots indicate occurrence localities of *N. marmorata*, which were used in ENM.

that *N. marmorata* is mostly scattered in the Knuckles mountain range and in a few adjacent areas. The novel population reported here lies within the already known elevation range of the species, which is between 200 – 1200 m a.s.l. (Dutta and Manamendra-Arachchi, 1996). The Rathna Ella location reported by Fernando et al. (2007) was not clearly depicted in their distribution map and hence the significance of this finding was not highlighted then. The latter location, lies just outside the northeastern border of the VRR Sanctuary in the Kandy district (L. J. Mendis Wickramasinghe, personal communication) and is closer to new locations reported here (a direct distance of 5 km). Moragahakanda is the northern most location reported for this species so far. Though this location is in the Northcentral Province, it actually lies approximately 16 km north of Pitawala Pathana.

The new population reported here was observed in the northern portion of the VRR Sanctuary, which is the largest sanctuary in Sri Lanka. The northern portion of the VRR sanctuary comprises regions of higher elevations (800 – 1000 m a.s.l.) with lower temperatures and consists of ecotone vegetation of dry-mixed evergreen forests and submontane forests. The rest of the sanctuary consists of mid-elevational (300 – 600 m a.s.l.) grasslands, dry-mixed evergreen forests and anthropogenic habitats. Thus it is not known whether the rest of the sanctuary supports suitable habitats for *N. marmorata* other than that of the area reported here. However, the possible existence of *N. marmorata* in these areas cannot be ruled out without a thorough survey of the sanctuary.

Nannophrys marmorata has been occasionally found on exposed habitats such as man made boulders (L. J. Mendis Wickramasinghe, personal communication). However, the species has generally been observed on wet rock surfaces with seepage, in crevices and under boulders in rocky hill streams of submontane forests, grasslands and tropical moist evergreen forests in the Knuckles mountain range (Clarke, 1983; Dutta and Manamendra-Arachchi, 1996). The presence of these frogs adjacent to highly disturbed places such as highways and tea estates may indicate the ability of this frog to tolerate and survive in disturbed habitats up to a certain extent.

Ecological niche modeling suggested highly suitable areas for *N. marmorata* in the eastern slopes of the Knuckles mountain range. However, apart from the few isolated populations, populations in the intervening regions are yet to be found. Ecological niche modeling further indicated that the environmental variable layer “Mean Diurnal Range (Mean of monthly (maximum temperature – minimum temperature))” had the most useful information for the model by itself and had the most information for the model that is absent in the other environmental variables. Both adult frogs and semi-terrestrial tadpoles of *N. marmorata* live on wet rock surfaces (Dutta and Manamendra-Arachchi, 1996; Senevirathne and Meegaskumbura, 2015). High temperatures in the surrounding environment may increase the drying of wet rock surfaces that the adults and tadpoles dwell on. It is well documented that increasing temperatures negatively affect the survival of all stages of amphibians (Blaustein et al., 2010; Li et al., 2013). Thus, prolonged droughts in combination with high temperatures will most likely reduce the most suitable habitats for *N. marmorata* in the future. This could be the reason that the “Mean Diurnal Range (Mean of monthly (maximum temperature – minimum temperature))” was the environmental variable layer that contained the most useful information for the model by itself and had the most information for the model that is absent in the other environmental variables.

According to the IUCN, the EOO of *N. marmorata* is 81.73 km² and the AOO is <10 km² and hence has been placed in the “Critically Endangered” category (criteria B1ab) (Manamendra-Arachchi et al., 2008). The EOO with new locality data has increased to 255 km² and it has further increased to 831 km² according to the predictions through ENM. Since the species was placed in the “Critically Endangered” category based solely on EOO (EOO <100 km²) and AOO (AOO <10 km²) (IUCN, 2012), the species can be now removed from the category as EOO and AOO are higher. The AOO from the prediction of ENM has also increased to an area of 275 km². However, it is necessary to confirm the occurrence of

N. marmorata in the predicted area before this can be revised. Most importantly, the discovery of this critically endangered restricted range species of frog outside its previously known range highlights the importance of taxon specific biodiversity surveys of vertebrate groups in Sri Lanka. Hence, future surveys may even shed light on species that are considered to be extinct such as *N. guentheri* similar to the other studies that have rediscovered other species of amphibians that were thought to be extinct (Meegaskumbura et al., 2012; Wickramasinghe et al., 2012; Wickramasinghe et al., 2013a, 2013b).

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