

A deeply divergent lineage of *Walkerana* (Anura: Ranixalidae) from the Western Ghats of Peninsular India

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Abstract

The frog family Ranixalidae is endemic to the Western Ghats of Peninsular India and contains two genera, *Indirana* and *Walkerana*. The three known species of *Walkerana* are restricted to different hill ranges south of the Palghat Gap, an ancient valley in the Western Ghats. In this study, we report the discovery of a deeply divergent lineage of *Walkerana* from the high elevations of the Elivalmalai hill range. This finding extends the geographic range of the *Walkerana* clade to the north of the Palghat Gap. The new species *Walkerana muduga* **sp. nov.** is genetically and morphologically divergent, and geographically isolated from its sister lineages. We also recovered a potential new lineage in the adjoining hill ranges suggesting the presence of additional new species in this genus north of the Palghat Gap.

Key words: Elivalmalai, Mudugar, Palghat Gap, *Walkerana*, Western Ghats

Introduction

The Western Ghats (WG) escarpment is a global biodiversity hotspot known for its amphibian diversity and endemism (Biju *et al.* 2011; Bocxlaer *et al.* 2012; Vijayakumar *et al.* 2016; Dahanukar *et al.* 2016). It harbors around 250 species of amphibians, with more than 90% of the species endemic to this escarpment (Dinesh *et al.* 2019; Frost, 2019). A number of these have narrow ranges or are confined to massifs within the WG (Vijayakumar *et al.* 2016).

The genus *Walkerana* (Dahanukar *et al.* 2016) is a monophyletic group within the ancient Natatanuran family Ranixalidae (Bocxlaer *et al.* 2006) which is endemic to the Western Ghats of Peninsular India (Nair *et al.* 2012a, 2012b; Dahanukar *et al.* 2016; Garg & Biju, 2016). This genus was recently described based on phylogenetic studies (Nair *et al.* 2012a; Dahanukar *et al.* 2016) with a limited range of distribution and three extant species in the southern Western Ghats (Dahanukar *et al.* 2016).

Until recently, the three species of *Walkerana* were assigned to the genus *Indirana* Laurent, 1986 (Dahanukar *et al.* 2016). These descriptions were based on the collections of Richard Henry Beddome, a British Forest Officer in the Madras Presidency of erstwhile British India (Günther, 1875; Boulenger, 1882). Among the three known *Walkerana* species, Günther first described *Walkerana diplosticta* in 1875 based on Beddome's collection from 'Malabar' (as *Ixalus diplostictus*). Boulenger (1882) considered *Polypedates brevipalmatus* described by Günther in 1875 (based on the collections of Beddome from 'Malabar' and 'Anamallays') as *Rana leptodactyla* (now *Walkerana leptodactyla*). Later, Boulenger (1882) described *Walkerana phrynoderma* (as *Rana phrynoderma*) based on Beddome's collection from 'Anamallays'.

Later studies based on morphological and molecular work recognized these three species as members of the ancient clade Ranixalidae (Dubois, 1992; Roelants *et al.* 2004). Early systematic work on the *Indirana* group recovered a clade composed of *Indirana diplosticta* and *I. leptodactyla* which was sister to the rest of the (*Indirana*)

lineages within Ranixalidae (Nair *et al.* 2012a) and in the study, *I. diplosticta* was inferred to be a polyphyletic taxon in the Western Ghats. However, Dahanukar *et al.* (2016) included individuals of the species *Indirana phrynoderma* and found them to be nested within the clade containing *I. diplosticta* and *I. leptodactyla*. Based on extensive type examination and morphological and phylogenetic analysis, they assigned the clade comprising *I. diplosticta*, *I. leptodactyla* and *I. phrynoderma* to the genus *Walkerana* and retained the name *Indirana* for the members of the sister clade comprising 11 species.

Thus far, all known species of *Walkerana* were found south of the Palghat Gap, a major biogeographic barrier in the WG (Vijayakumar *et al.* 2016). As a part of our large-scale sampling for anurans in the Western Ghats (Vijayakumar *et al.* 2014; Dinesh *et al.* 2015; Vijayakumar *et al.* 2019), we recovered multiple populations of leaping frogs resembling *Walkerana* north of the Palghat Gap. Our phylogenetic and morphological analysis confirmed that they represented a new lineage that is distinct from the three known extant lineages. This deeply divergent lineage is diagnosed and described here as a new species, *Walkerana muduga* **sp. nov.**, extending the distribution range of *Walkerana* north of the Palghat Gap.

Methods

As a part of our anuran explorations in the Western Ghats, field sampling was carried out in the hill ranges of the Western Ghats from 2008 to 2014 (Fig. 1). We collected individuals of the genera, *Indirana* and *Walkerana*, from multiple locations. Natural history and habitat type were noted in the field and adult individuals were photographed in controlled conditions. Specimens were euthanized in MS222. Tissue samples were stored in molecular grade 95% ethanol; the specimens were initially fixed in 4% formalin for 24 hours and then preserved in 70% ethanol. Morphological measurements were taken using Mitutoyo vernier calipers (to the nearest 0.1 mm). Molecular protocols followed Vijayakumar *et al.* (2014) and Dinesh *et al.* (2015).

DNA Extraction and sequencing. DNA extraction and sequencing were carried out as part of a larger study on the genera *Indirana* and *Walkerana*, selecting forty-four individuals. Of these, 14 belonged to the genus *Walkerana* and the rest belonged to the sister genus, *Indirana*. Liver and muscle tissues were extracted and stored in ethanol. Extraction and isolation of DNA was performed using the standard phenol-chloroform method. The primers used by Simon *et al.* (1991) were used to amplify the 16S mitochondrial gene.

PCR amplifications for a 16S rRNA gene fragment were performed in 25 µl reactions consisting of 2.5 µl each of 10x PCR buffer, MgCl₂ (25 mM) and deoxyribonucleotide triphosphates (2.5 mM), 0.25 µl of each primer (10 mM) 16Sar (Forward Primer): (5'-CGC CTG TTT ATC AAA AAC AT-3') and 16Sbr (Reverse Primer): (5'-CTC CGG TTT GAA CTC AGA TCA-3'), 0.667 µl of Taq DNA polymerase (Amnion Biosciences), 15.333 µl of dH₂O and 1 µl of template DNA (25ng). The following thermocycling conditions were used: 94°C for 3 min, followed by 35 cycles of 94°C for 50s, 46.8°C for 1 min, 72°C for 40s, followed by a final extension step at 72°C for 5 min. The PCR products were purified using Qiagen purification kits© (Qiagen, New Delhi, India). The purified products were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems).

Phylogenetic Analyses. 16S rRNA sequences were generated for 44 individuals in this study and aligned using the MUSCLE algorithm in the R programming environment (R Core Team, 2017). To this, we added available 16S sequences from recent studies (Padhye *et al.* 2014; Modak *et al.* 2015; Dahanukar *et al.* 2016; Garg & Biju, 2016). The final alignment of 16S gene sequences was 483 bp long and composed of a total 92 individuals which included all individuals of *Indirana* (our own and those published elsewhere) and *Walkerana* (GenBank Accession Numbers for specimens sequenced in this study: KX098602 to KX098645 (partial sequences used in the current study)).

A phylogenetic tree was generated by concatenating a multigene dataset using our own and published sequences. For the multigene tree, we used two mtDNA genes (12S and CO1), and two nuclear genes (Rag1 and Rhodopsin) from earlier studies (Padhye *et al.* 2014; Modak *et al.* 2015; Dahanukar *et al.* 2016; Garg and Biju 2016; Nair *et al.* 2012b), along with our 16S gene data. The concatenated multigene dataset was partitioned by different genes. The analysis was performed using the GTR+G+I model using ML+ through bootstrap method with 1000 replicates. Bayesian phylogenetic analysis was performed using MrBayes v3.2.2 on the concatenated dataset (Ronquist *et al.* 2012). The software jModelTest2 was used to obtain the substitution model that best fits the multi-gene dataset according to AIC criteria (Darriba *et al.* 2012; Akaike, 1974). The dataset was independently run twice for 20 million generations with a random starting tree and four markov chains (three hot and one cold), sampling every 2000th generation. The dataset was divided into five partitions and the likelihood parameters and priors obtained from

jModelTest 2 were used while running MrBayes v3.2.2. The substitution parameters for each data partition were unlinked. Adequate burn-in was determined using a plot of the likelihood scores of the heated chain for convergence in TRACER v1.6 (Rambaut *et al.* 2018). The trees obtained were visualized in FIGTREE v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>) and R to show only individuals of the genus *Walkerana* and the position of the new lineage (Fig. 2) (R Core Team, 2017).

Abbreviations. ED—eye diameter; EN—anterior corner of eye to nostril distance; ES—eye to snout tip distance; f1, f2, f3, f4 —finger length; FEL—femur length; FOL—foot length; HL—head length; HW—head width; IN—internarial distance; IO—interorbital distance; PAL—palm length; SVL—snout to vent length; t1, t2, t3, t4, t5—toe length; TAR—tarsus length; TBL—tibia length; TYH—tympanum diameter; UAL—upper arm length; UEW—maximum upper eyelid width; CESF—Centre For Ecological Sciences, IISc, Bangalore; ZSI/WRC/A—Zoological Survey of India, Western Regional Centre, Amphibia.

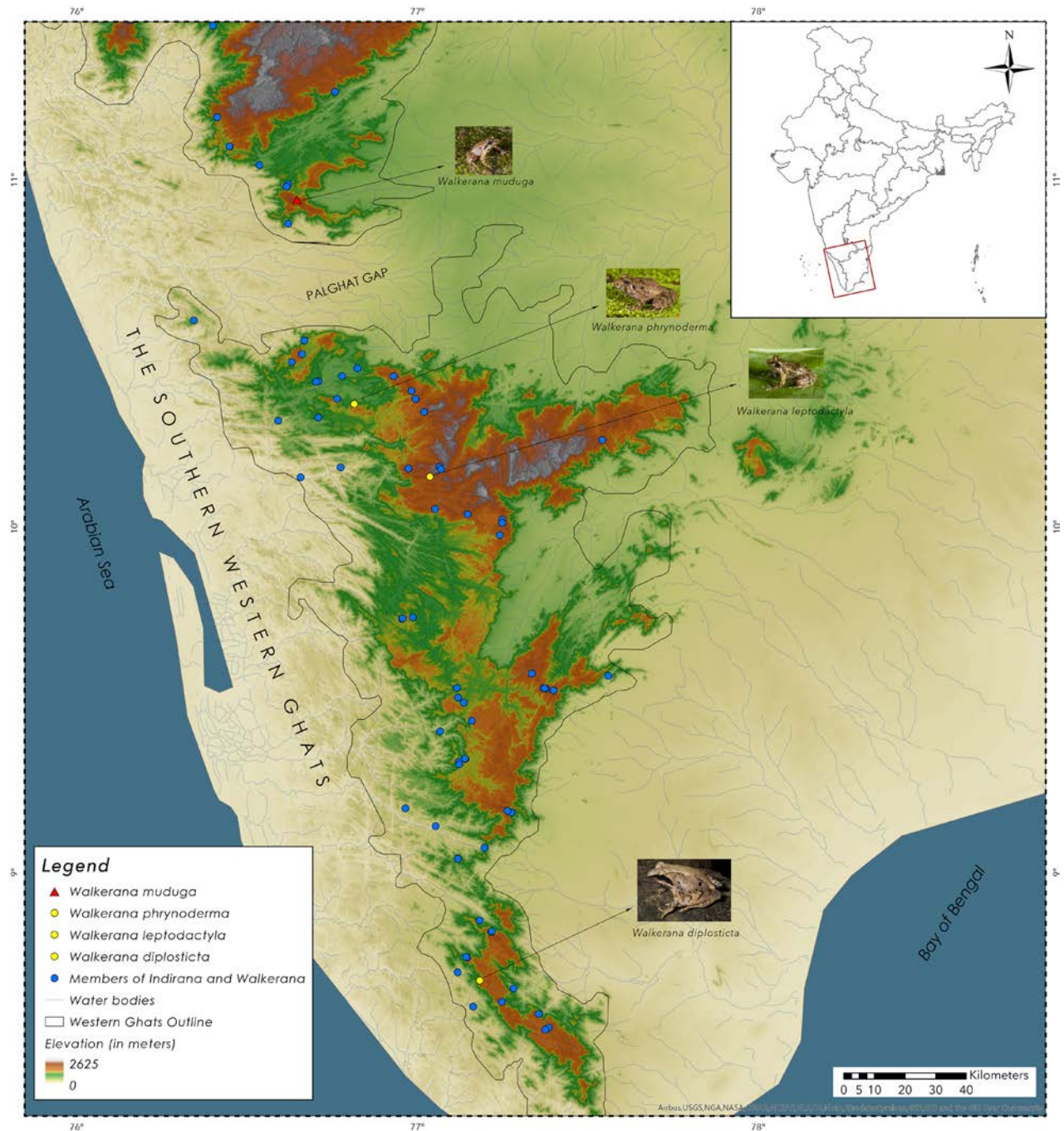


FIGURE 1. Sampling localities for the members of Ranixalidae in the Western Ghats (yellow circle represents the type locality of *W. diplosticta*, *W. leptodactyla* and *W. phrynoderma*; red triangle represents the type locality of *Walkerana muduga* sp. nov.; blue circles represents the collection localities for other members of Ranixalidae).

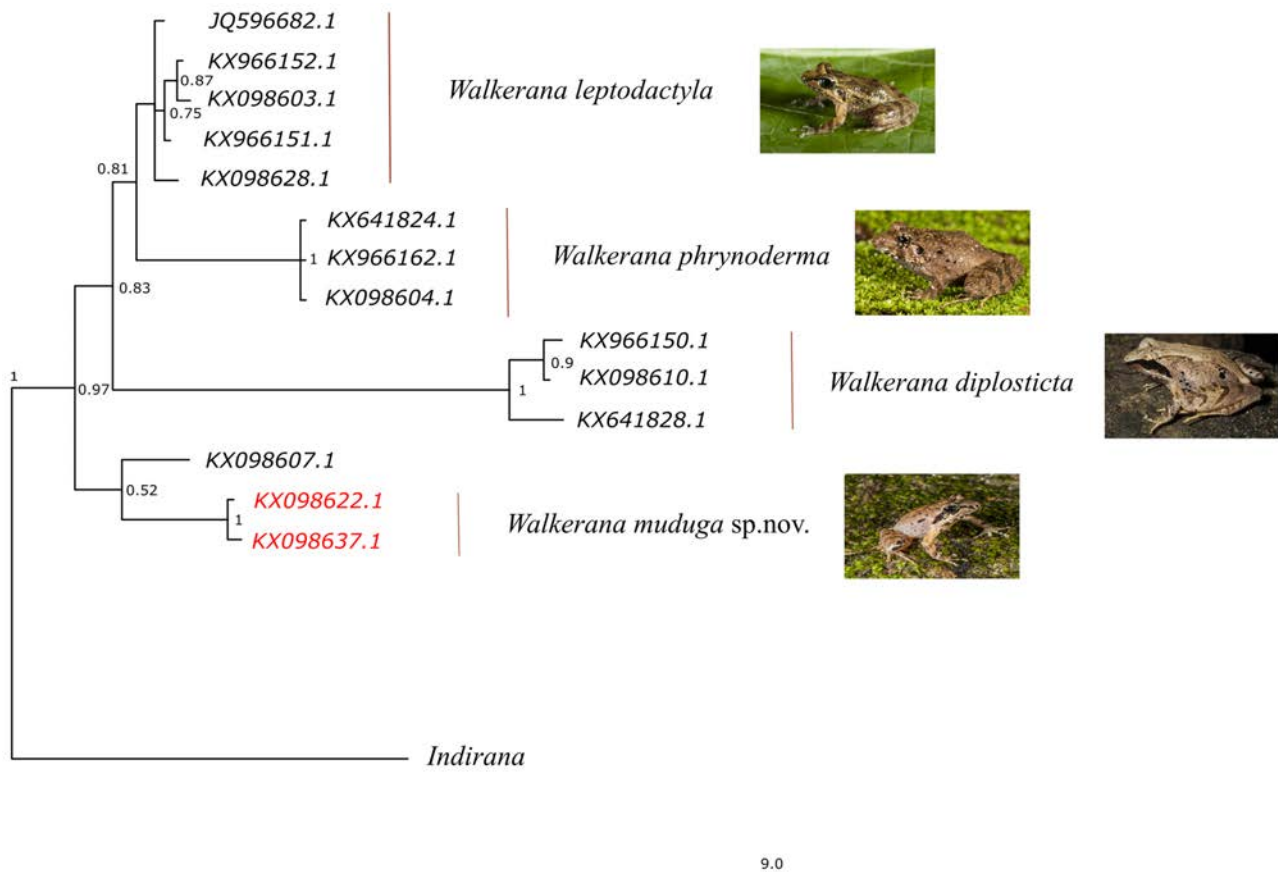


FIGURE 2. Bayesian tree of the genus *Walkerana* with posterior probability values indicated on the respective nodes.

Results

Phylogeny of *Walkerana* and generic allocation of the new lineage

Among the members of Ranixalidae, we recovered two well supported clades assignable to the genera, *Walkerana* and *Indirana*, as in the studies of Dahanukar *et al.* (2016) and Garg & Biju (2016). The new deeply divergent lineage is assigned to the genus *Walkerana*, based on its phylogenetic position within Ranixalidae (Fig. 2).

Species diagnosis and description

Walkerana muduga sp. nov.

(Table 1, 2; Fig. 1,2,3,4,5)

Holotype: ZSI/WRC/A/2211 (CESF 1607), an adult male (SVL 27.9 mm) collected by S.P. Vijayakumar and team in October 2010 from Muthikulam, (N 10.945; E 76.644) a high elevation site (1544 amsl) in the hill range Elival-mala / Elivalmalai located north of Palghat Gap.

Paratype: ZSI/WRC/A/2212 (CESF 1606), an adult female (SVL 44.9 mm), collection data same as the holotype.

Etymology: The new species is named after the “Mudugar” indigenous community of Palghat district, Kerala who speak ‘Muduga’ language (Menon, 1996). The species epithet is used as an invariable noun in apposition to the generic name. The suggested common name is ‘Muduga mountain leaping frog’.

Lineage diagnosis: *Walkerana muduga* sp. nov. can be diagnosed phylogenetically as a member of the ‘*Walkerana* clade’ (Fig. 2), and exhibits 5.7% genetic divergence from *W. leptodactyla*, 7.5% divergence from *W. phrynoderma* and 11.7% divergence from *W. diplosticta* at the 16S rRNA fragment. The species is highly divergent in

morphology from all known species (see comparison section). Phylogenetic analysis also shows another undescribed lineage from the adjacent massif (north of Elival Mala), exhibiting high genetic divergence of 3.8 % for 16S rRNA (CESF 1554). However, due to the availability of only a single poorly preserved specimen (CESF 1554), we defer the description of this lineage.

Description of Holotype ZSI/WRC/A/2211 (CESF 1607) (Fig. 2, 3). A medium sized species of *Walkerana* (SVL = 27.9 mm) with squat, raised body; head width (HW = 11.3 mm) sub equal to head length (HL = 10.3 mm); snout bluntly pointed (ES = 4.7 mm) in both dorsal and ventral view and more than 1.2 times of eye diameter (ED = 3.9 mm); canthus rostralis angular, loreal region concave, inter orbital space flat (IO = 3.0 mm) and sub equal to upper eye lid (UEW = 2.4 mm); nostrils oval, nearer to tip of snout than to eye; eyes relatively smaller (EL/HL = 0.37), protruding out of the head; tympanum (TYH = 2.2 mm) distinct below the supratympanic fold and 1.7 times of eye diameter; minute vomerine ridges present with 2 to 3 spinular teeth; tongue bifid with a raised papilla; symphyseal knob weak.



FIGURE 3. Preserved holotype of *Walkerana muduga* sp. nov. (A) dorsal view. (B) ventral view.

Fore arm slender (UAL = 4.7 mm) and sub equal to hand (PAL = 7.6 mm); fingers short and thin (dermal fringes distinct in the ventral view on the finger 2, 3 and 4); first finger shorter than second (f1 = 3.7 mm and f2 = 4.2 mm); finger tips with truncated enlarged discs with minute dorsoterminal grooves; fingers free without webbing between fingers; rounded subarticular tubercles moderate (one each on finger 1 and 2, and two each on finger 3 and 4); prepollex indistinct and palmar tubercle distinct, supernumerary tubercles absent.

Hind limbs moderately long, strongly overlap when folded at right angles to the body and tibio-tarsal articulation reaches beyond the snout tip; femur length less than tibia length (FEL = 14.9 mm; TBL = 18.2 mm); foot length is 1.8 times more than the tarsus length (FOL = 14.9 mm, TAR = 8.1 mm), relative toe length I<II<V<III<IV; tips of toes with truncated enlarged discs having minute dorsoterminal grooves; webbing between toes reduced, webbing just touches the last subarticular tubercle of toe IV and V; inner metatarsal tubercle moderate, bulbous; outer metatarsal tubercle and supernumerary tubercles absent.

Overall skin on the dorsum smooth glandular with scattered small ridged longitudinal glandular folds, folds are discontinuous without any pattern; supratympanic fold distinct; on the ventral side of the body, region of throat smooth and belly glandular.

In life, colour on the dorsum light fleshy brown, base of the raised ridges dark brown and the inter orbital space with light brown blotches; lower lip barred, dark brown streak from the tip of snout till the end of the supratympanic fold in front of shoulders; side of the body uniform light brown without any markings; fore and hind limbs barred; ventrally, region of throat yellowish brown, belly whitish grey and region of thighs flesh-coloured.

In preservative, overall colour on the dorsum grayish brown on the dorsum between the inter-orbital space, horizontal light black band connecting the raised eye balls; upper and lower lips barred; a thin blackish band starting from the tip of snout, through the upper edge of canthus rostralis on either side till the posterior end of the supratympanic fold; sides of the body light gray, raised longitudinal glandular folds dark grey; fore and hind limbs barred; ventrally uniform cream white.



FIGURE 4. Holotype of *Walkerana muduga* **sp. nov.** in life (not to scale). (A) dorsal view. (B) ventral view.

Secondary sexual characters: No visible external sexual dimorphism was noticeable except for the amplexed pairs where males are smaller than the females.

Additional information from paratypes and variations: Morphological data are given in Table 1. Paratype ZSI/WRC/A/2212 (CESF 1606), an adult female (SVL 44.9 mm) and other reference collections (CESF 1608, male, SVL 22.7 mm) were similar to holotype in all the external morphological characters.

Distribution: *Walkerana muduga* **sp. nov.** is known from the Elivalmalai range, north of the Palghat Gap and south of Nilgiri Massif.

Comparisons: All the described species of *Walkerana* are known to occur in the high elevation ranges south of the Palghat Gap. There are no congeners for *Walkerana muduga* **sp. nov.** north of the Palghat Gap. However, there is a deeply divergent lineage (CESF 1554) (see diagnosis) recovered from an adjacent massif. *Walkerana muduga* **sp. nov.** differs from the undescribed *Walkerana* sp. (CESF 1554) in having higher HL/SVL ratio of 0.370 to 0.405 (n=2) (vs. lower HL/SVL ratio of 0.342 (n=1)); higher HW/SVL ratio of 0.397 to 0.406 (n=2) (vs. lower HW/SVL ratio of 0.390 (n=1)); lower TYH/SVL ratio of 0.079 to 0.080 (n=2) (vs. higher TYH/SVL ratio of 0.083 (n=1)); higher IO/SVL ratio of 0.107 to 0.109 (n=2) (vs. lower IO/SVL ratio of 0.097 (n=1)); higher PAL/SVL ratio of 0.257 to 0.271 (n=2) (vs. lower PAL/SVL ratio of 0.234 (n=1)); higher f1/SVL ratio of 0.119 to 0.133 (n=2) (vs. lower f1/SVL ratio of 0.112 (n=1)); higher f2/SVL ratio of 0.131 to 0.151 (n=2) (vs. lower f2/SVL ratio of 0.123 (n=1)); lower FEL/SVL ratio of 0.535 to 0.536 (n=2) (vs. higher FEL/SVL ratio of 0.545 (n=1)); lower TAR/SVL ratio of 0.287 to 0.291 (n=2) (vs. higher TAR/SVL ratio of 0.295 (n=1)); higher FOL/SVL ratio of 0.536 to 0.565 (n=2) (vs. lower FOL/SVL ratio of 0.530 (n=1)).

Walkerana muduga **sp. nov.** differs from species found south of the Palghat Gap (see key to the species of *Walkerana* by Dahanukar *et al.* 2016) in having distinct canthus rostralis and smooth skin (vs. indistinct canthus rostralis and warty skin in *W. phrynoderma*); lacking a distinct pair of black patches on the loin (vs. distinct black patch on the loin in *W. diplosticta*) and tympanum relatively smaller than the diameter of eye (vs. tympanum three-fourth of the diameter of eye in *W. leptodactyla*).

TABLE 1. Morphometric data (in mm) for the type series and reference collections of *Walkerana muduga* **sp. nov.**

Reg. No.	ZSI/WRC/A/2211 (CESF1607) [#]	ZSI/WRC/A/2212 (CESF1606) [§]	CESF1608 ^{&}
Sex	Male	Female	Male
SVL	27.9	44.9	22.7
HL	10.3	15.4	9.2
HW	11.3	17.3	9.0
ES	4.7	7.6	4.3
ED	3.9	5.5	4.1
TYH	2.2	3.2	1.8
UEW	2.4	3.4	2.2
EN	2.5	3.6	1.9
IN	3.7	5.4	3.3
IO	3.0	3.9	2.5
UAL	4.7	7.7	4.3
PAL	7.6	11.5	5.8
f1	3.7	5.3	2.7
f2	4.2	5.9	3.0
f3	4.6	7.0	3.5
f4	3.0	4.4	2.4
FEL	14.9	24.8	12.1
TBL	18.2	28.4	14.0
TAR	8.1	13.0	6.5
FOL	14.9	25.6	12.8
t1	2.5	3.8	2.0
t2	3.9	5.6	2.6
t3	5.7	9.6	4.1
t4	8.8	15.2	7.0
t5	4.8	7.4	3.5

[#] holotype; [§] paratype; [&] other referred specimens**TABLE 2.** Extant valid species of *Walkerana* in the Western Ghats and GenBank accession numbers for the Ranixalidae taxa used in the construction of Bayesian tree

Sl. No.	Species	GenBank accession numbers	Voucher Numbers	Broad hill range distribution in the WG
1	<i>Walkerana muduga</i> sp. nov.	KX098622 and KX098637	CESF 1606 and CESF 1607	Elivalmalai
2	<i>Walkerana diplosticta</i> (Günther, 1876)	KX098610	CESF 2045	Agasthyamalai
3	<i>Walkerana leptodactyla</i> (Boulenger, 1882)	KX098603 and KX098628	CESF 698 and CESF 282	Anaimalai
4	<i>Walkerana phrynoderma</i> (Boulenger, 1882)	KX098604	CESF 720	Anaimalai

Key to the species of *Walkerana* in the Western Ghats(modified after Dahanukar *et al.* 2016)

1. canthus rostralis indistinct; skin glandular, warty *W. phrynoderma* (Fig. 5B)
- canthus rostralis distinct, angular; skin smooth 2

2. distinct black band from the tip of snout to end of supratympanic fold through the entire canthus rostralis and black spot on the region of loin (with lumbar spots) *W. diplosticta* (Fig. 5D)
- absence of black spot on the loin and the black band on the face not covering the entire region of canthus rostralis 3
3. head longer than wide; snout longer than eye diameter; tympanum three-fourth of the diameter of eye; a brown streak from tip of snout to anterior corner of eye *W. leptodactyla* (Fig. 5C)
- head length equal to head width; snout equal to eye diameter; tympanum relatively smaller than the diameter of eye; a brown streak from tip of snout to end of supratympanic fold *W. muduga* **sp. nov.** (Fig. 5A)

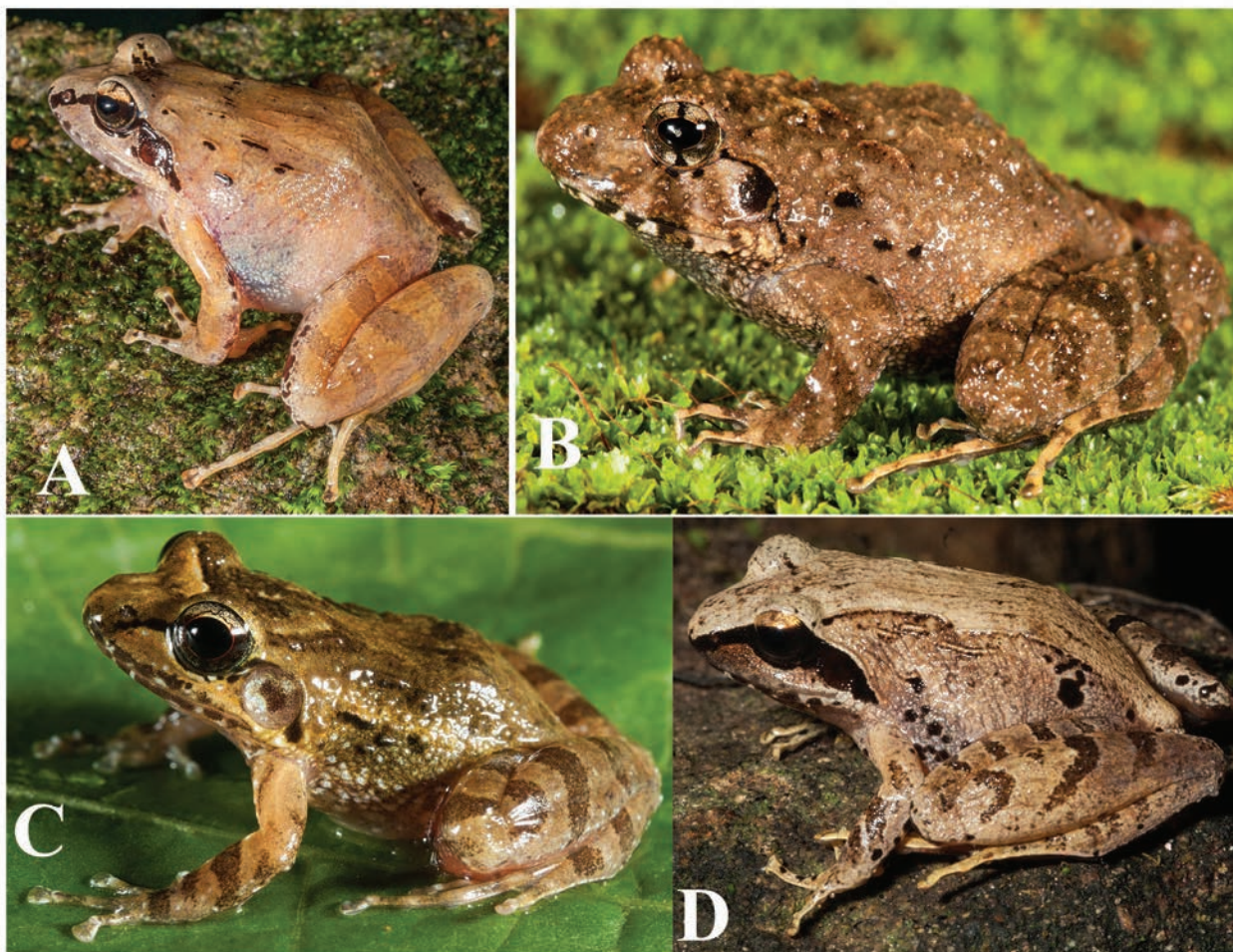


FIGURE 5. Extant congeners of *Walkerana* in life (A) *Walkerana muduga* **sp. nov.**; B) *W. phrynoderma*; C) *W. leptodactyla*; D) *W. diplosticta*.

Discussion

During our field studies, we recovered multiple populations of *Walkerana* on either side of the Palghat Gap. Here, we describe a deeply divergent lineage *Walkerana muduga* **sp. nov.** from the higher elevations of Elivalmalai and report an unidentified lineage of *Walkerana* from the adjacent hill ranges north of the Palghat Gap.

The type localities mentioned in historical descriptions were vague, for e.g. *Walkerana diplosticta* was described by Günther as being from ‘Malabar’. Later, this species was reported by Inger *et al.* (1986) from Agasthyamalai. Recent phylogenetic studies confirmed the occurrence of this species in many sites within Agasthyamalai (from Ponmudi by Nair *et al.* 2012b; from Bonacaud by Dahanukar *et al.* 2016 and from Athirimala, Pondimotta, Pandipath by Garg & Biju, 2016).

The type locality for *Walkerana leptodactyla* was mentioned as ‘Malabar’ and ‘Anamallays’ and subsequent studies have recovered this lineage from different sites in the Anaimalai (from Munnar by Nair *et al.* 2012b and, Kanagavel *et al.* 2018; from Eravikulam by Dahanukar *et al.* 2016; from Anaimudi, Vaguvarai, Eravikulam, Matupetti, Munnar, Ponkalapara, Andiparai shola, Grass Hills by Garg & Biju 2016 and Kanagavel *et al.* 2018). Our

taxon sampling suggests that *W. leptodactyla* is a species complex and that there is a need for further studies. The third known species in this genus, *Walkerana phrynoderma* was described as being from ‘Anamallays’ by Boulenger (1882) and recent phylogenetic analysis based on fresh collections confirmed its range in Anaimalai (Dahanukar *et al.* 2016, Garg & Biju 2016 and Kanagavel *et al.* 2018).

These studies suggested that the range of the clade *Walkerana* is restricted to the mountains south of the Palghat Gap in the Western Ghats. The present discovery extends the distribution range to the north of Palghat Gap and reveals a deeply divergent new species in the *Walkerana* clade after a span of 137 years. Although new species discoveries are being reported across the Western Ghats on a regular basis, many deeply divergent species are now being reported north of the Palghat Gap. Recent discoveries of *Fejervarya marathi* from Bhamburde, northern Western Ghats by Phuge *et al.* 2019 (a deeply divergent lineage of the ‘rufescens clade’ within the larger *Fejervarya* clade); *Mysticellus franki* from Suganthagiri, Wayanad Plateau by Garg & Biju, 2019 (a deeply divergent lineage of ‘*Micryletta* clade’ within Microhylidae) and *Astrobatrachus kurichiyana* from Kurchiyarmala, Wayanad Plateau by Vijayakumar *et al.* 2019 (a deeply divergent lineage within Natatanura) suggest that these regions could harbor hidden diversity which needs to be further explored.

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