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Jurassic frogs and the evolution of amphibian endemism in the Western Ghats

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The diversity of frogs and toads (Anurans) in tropical evergreen forests has recently gained importance with reports of several new species[1]. We describe here a fossorial frog taxon related to the African Heleophrynidae and Seychellian Sooglossidae from the Western Ghats of India. This frog possesses a suite of unique ancient characters indicating that it is a transitional form between Archaeobatrachians and Neobatrachians. Molecular clock analysis based on the nucleotide diversity in mitochondrial 12S and 16S genes dates this frog as a Gondwana relic, which evolved 150–195 Mya during the mid-Jurassic period. With this taxon, the evolution of endemism in the Western Ghats and other Gondwana break up landmasses is now dated much before the Cretaceous–Tertiary boundary. We propose that sea level surges in the late Jurassic[2] isolated tablelands creating insular amphibian fauna. Reduction in area may have promoted stochastic extinctions and resulted in amphibian endemism. Our study reinforces the conservation significance of the Western Ghats as major global hotspot of biodiversity. The habitat of this endemic amphibian lineage is currently endangered due to various upcoming dam projects, which is a cause of serious conservation concern.

THE Western Ghats mountain range in Southern India, one of the Gondwanaland breakup landmasses, is amongst the most important hotspots of biodiversity[3]. Recently the Western Ghats and Sri Lanka have gained attention as hotspots of amphibian diversity due to recent reports of several undescribed taxa[1,4,5]. We present here an ancient amphibian taxon, which provides new insights in our present day understanding of the endemism and distribution of fauna and flora in the Western Ghats and other Gondwanaland breakup landmasses and puts the evolution of several other anuran lineages in the middle Jurassic. A similar frog taxon has recently been described under a newly erected anuran family from Western Ghats[6]. In this emerging scenario, resolving the biogeography and the evolution of endemism in these landmasses becomes crucial in developing preemptive conservation strategies. Our findings imply that a large amount of the evolutionary history of amphibians is encompassed within the Western Ghats, making it an important region for conservation.

We provide here a detailed morphological and ontogenic description of the taxon based on many specimens and resolve its phylogeny using molecular data. We also put forth a hypothesis on the evolution of endemism of amphibians in the Gondwana breakup landmasses.

The study is based on three adult specimens: one male, two females and four tadpoles. Adult specimens (BNHM 4214 to 4216) are deposited in the Bombay Natural History Museum, Mumbai and tadpoles (WII 659 to 662) in the Wildlife Institute of India Museum, Dehradun. Tissue samples from the male and one female were used for genotyping. BNHM 4214, a mature male, SVL 52.8 mm was found in Sankaran Kudi ca 600 ha rainforest fragment in the Anamalais (10°14′46″N, 76°55′55″E) Tamil Nadu, by K.V. on 20 May 2001 at 1730 h. BNHM 4215 (Figure 1), a gravid female, SVL 89.9 mm was collected along with BNHM 4214; BNHM 4216, a gravid female was SVL 78.3 mm, collected from Murikkassery, Kothamangalam, Ernakulam District, Kerala by Sr. Jose Mary, Alphonsa College, Palai, Kerala. Additional specimens examined were as follows: (i) A mature male, SVL 67.0 mm, collected during July 1994 from Manimala River at Erumely, Kottayam District, Kerala. This specimen is available at

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the Zoology Museum, St. Thomas College, Kozhencherry, Kerala. (ii) An immature male, SVL 45.0 mm, collected from Najayappilly, a small village near Thattakad Bird Sanctuary, Ernakulam District, Kerala (10°07′10″N, 76°40′E), collected on 13 January 1998. This specimen has been utilized for alcian blue and alizarin red preparation of bones stored in the auxillary collections at the Wildlife Institute of India (WII), Dehradun. (iii) A mature male collected from the Indira Gandhi Wildlife Sanctuary, Pollachi, Tamil Nadu. This specimen is available at the AVC College, Mayiladuthurai, Mannapandal, Tamil Nadu. The exact locality of the specimen is not known. Four tadpoles (WII 659–662) were collected from the floor of a fast-flowing stream in a coffee estate in Manamboli, Anamalais (10°22′59″N, 76°55′23″E) on 22 May 1998 at 1600 h. Tissues (pf1 and pf2) stored at the Centre for Cellular and Molecular Biology, Hyderabad, are from BNHM 4214 and BNHM 4215. Based on the current knowledge of amphibian biogeography in the region the rough distribution limits of this taxon are thought to be forested habitats in the southern Western Ghats along the western coast of India, between 8 and 11°N latitude.

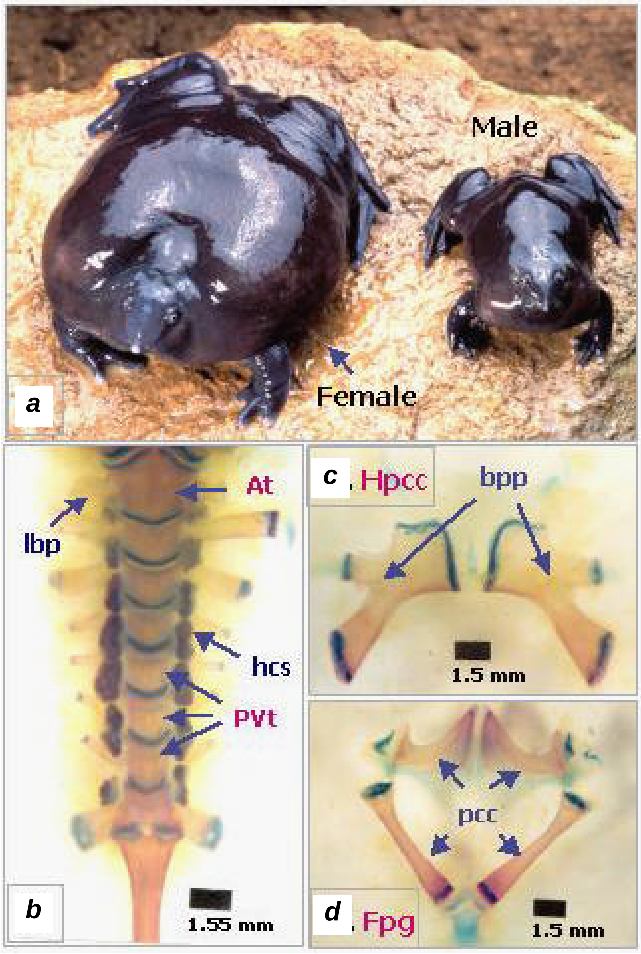

Figure 1. ***a***, Important primitive and unique features of the pignose frog. A. BNHM4214 male and BNHM4215 female; ***b***, Vertebrae column showing undivided atlas (At), lateral bony processes (1 bp), hard calcareous substance (hcs), procelous vertebrae (PVt); ***c***, Hyoid apparatus with poorly developed cartilaginous cornua (Hpcc) and prominent branched posteromedial processes (bpp). ***d***, Firmisternal pectoral girdle without cartilaginous plates (Fpg) and prominent clavicle and corocoid bones (pcc). Colour code for characters: red labels-primitive; blue for unique.

The specimens (BNHM 4215 and BNHM 4216) were collected from the breeding habitat, photographed, euthenized and preserved in 10% formaldehyde after extracting the tissue samples. Similarly, four tadpoles were collected and preserved. The following measurements were obtained from the preserved specimens of adults and tadpoles: SVL (snout-vent length), IOD (inter-orbital distance), IND (inter-narial distance), ED (eye diameter), END (eye-to-nostril distance), TIL (tibia length), TL (tail length), IMTTL (inner metatarsal tubercle length), IMTTW (inner metatarsal tubercle width) and WM (width of tadpole mouth). The measurements were made using dial vernier calipers (Mitutoyo™) to the nearest 0.1 mm. One of the adult specimens and a metamorphosing tadpole from the cohort were examined for their morphological and anatomical similarities. The specimens were stained using alcian blue and alizarin red and their features were compared using photographs of the specimens with a Leica trinocular stereoscope microscope. The tadpoles were staged[7] and compared with the adult specimens.

Total genomic DNA was isolated from alcohol-preserved liver and muscle tissues taken from BNHM 4214 and BNHM 4215, following standard proteinase-K, phenol–chloroform–isoamyl extraction method. The isolated samples were analysed for nucleotide diversity across two taxonomically informative domains of the mitochondrial genome, namely 12S and 16S rDNA, to ascertain the phylogenetic position of the taxon described here. In each case, the target mitochondrial sequences were PCR amplified using standard protocols on a PE9600 thermocycler. The PCR products were sequenced for both strands on an automated ABI-3700 DNA sequencer using the Big dye terminator ready reaction kit as per the manufacturer's instructions (Applied Biosystems, USA). Each sample was sequenced three times to confirm the sequence data.

Part of 16S rDNA (~ 575 bp) was amplified and sequenced using the primers 16Sar-L [5′-cgcctgtttatcaaaaacat-3′] and 16Sbr-H [5′-ccggtctgaactcagatcacgt-3′]. Similarly, approximately 435 bp of 12S rDNA was amplified and sequenced using the primers 12SUI-L1091 [5′-aaagcttcaaactgggattagataccccactat-3′] and 12SUII-H1478 [5′-tgactgcagagggtgacgggcggtgtgt-3′].

The GenBank database (GenBank release 113.0 http://www.ncbi.nlm.nih.gov) was searched for reference anuran mitochondrial DNA sequences corresponding to the two analysed domains. Based on the searches and initial sequence analysis, 62 reference sequences (Table 1) representing both recent and ancient anuran species were retrieved from the database for ascertaining the phylogenetic status of the frog taxon described in the study.

The sequences were aligned using the CLUSTAL program[8] and also checked manually for large gaps. The aligned sequences were flushed at the ends to avoid missing information for any compared reference entries. This resulted in a final 674-bp long combined aligned sequence of 12S and 16S rDNA domains for further comparisons. Nucleotide diversity was used to derive corrected Kimura two-parameter distance estimates[9] and infer phylogenetic relationships using the neighbour-joining method[10], with analytical routines available in the software packages PHYLIP 3.6 (ref. 11) (http://evolution.genetics.washington.edu/phylip.html) and MEGA2 (ref. 12). In order to test for earliest branching patterns, all substitutions were considered and the transition/transversion rate ratio (k) was assumed to be equal to four. Support for nodes on the shortest tree and estimates of divergence time were derived using 1000 bootstrap replications. Relative rate test was performed to test molecular clock hypothesis with MEGA2 (ref. 12) using Tajima's algorithm for clock hypothesis[13]. The phylogenetic tree was rooted using *Ichthyophis* spp. (order Gymnophiona), which represents one of the outgroups for anurans.

Table 1. Details of 12S and 16S rDNA sequences of reference anuran species, obtained from GenBank database, for use in the final phylogenetic analysis

Reference Species	GenBank accession numbers 12s rDNA	GenBank accession numbers 16s rDNA	Family
Limnodynastes peronii	AJ440770	AJ269756	Myobatrachidae
Neobatrachus pelobatoides	X86245	X86279	Myobatrachidae
Atelopus varius	U52736	U52779	Bufonidae
Ansonia muelleri	U52740	U52784	Bufonidae
Bufo himalayanus	AF160762	AF160780	Bufonidae
Bufo melanostictus	AY180227	AF249061	Bufonidae
Dendrobates ventrimaculatus	AF482784	AF482797	Dendrobatidae
Pseudis paradoxa	X86250	X86283	Pseudidae
Leptodactylus pentadactylus	U52741	U52790	Leptodactylidae
Rhinoderma darwinii	X86233	X86267	Rhinodermatidae
Allophryne ruthveni	AF364511	AF364512	Allophrynidae
Centrolene geckoideum	X86230	X86264	Centrolenidae
Hyla labialis	AF308084	AF308118	Hylidae
Ichthiophis spp.	AY101221	AY101239	Icthyophidae
Scaphiopus holbrookii	X86226	X86260	Pelobatidae
Ascaphus truei	X86225	X86259	Acsaphidae
Leiopelma hamiltoni	AF231464	X86275	Leiopelmatidae
Bombina orientalis	X86227	AF224728	Leiopelmatidae
Rhinophrynus dorsalis	X86246	X86280	Rhinophrynidae
Xenopus laevis	Y10943	M10217	Disscoglossidae
Heleophryne natalensis	X86237	AY176690	Heleophrynidae
Hemisus marmoratus	AF095349	AF215342	Hemisotidae
Lalistoma labrosa	AF026354	Y11980	Ranidae
Mantella expectata	AF215173	AF215295	Ranidae
Sphaerotheca dobsoni	AF215212	AF215418	Ranidae
Fejervarya limnocharis	AF215210	AF215416	Ranidae
Dyscophus guineti	AF124099	AF215368	Microhylidae
Kaloula taprobanica	AF249004	AF249057	Microhylidae
Micrixalus fuscus	AF249024	AF249056	Ranidae
BNHM-4215	AY425726	AY425725	Nasikabatrachidae
Crinia parinsignifera	AJ269698	Personal Communication[a]	Myobatrachidae
Nesomantis thomasseti	X86254	X86288	Sooglossidae

[a] 16S sequence obtained from Dr Scott Keogh, Australian National University, Canberra, Australia.

In order to estimate the divergence time, three different time estimates were used as calibration points: (i) the split between Gymnophiona and Salientia dating back to 240 Mya[14]; (ii) the split between Arheobatrachia and Neobatrachia, 200 Mya[15,16] and (iii) the origin/evolution of the Gondwanan genera *Fejervarya* and *Mantella* during the Cretaceous–Tertiary boundary before 88 Mya[17]. The corrected Kimura distance estimates for pairwise comparisons of sequence data were used to derive the estimate of evolutionary time separating the two, using the conservative estimates of sequence divergence rates reported for mtDNA divergence[18,19]. Sequences used for comparison and their accession numbers are as follows: *Ichthyophis* (AY101221, AY101239) – BNHM4215 (AY425726, AY425725), *Ascaphus* (X86226, X86260) – BNHM4215, *Scaphiopus* (X86225, X86259) – BNHM4215, *Fejervarya* (AF215210, AF215416) – BNHM4215, *Mantella* (AF215173, AF215295) – BNHM4215.

The taxon possesses a combination of unique, derived and primitive characters (Figure 1). Derived primitive characters shared with early neobatrachids are: basally attached fluted shape of the tongue; hyoid cornua discontinuous and sparsely developed; no maxillary teeth; vertebrae procoelous; inguinal amplexus; tadpole dorsoventrally flat with reduced tail fin; tadpole beak with keratinized labial teeth rows and sinistral spiracles; adult with diminutive head and small eyes. Unique neobatrachian characters are: ventrally positioned narial opening and mouth; snout with a knob at the anterior end; metatarasal tubercle with three bony elements comprising modified centrale, prehallux and the distal tarsus; atlas with transverse processes; hyoid apparatus poorly developed; hyoid plate has two prominent branched posteromedial processes; firmisternal pectoral girdle with a prominent clavicle and coracoid bones; sternum absent; tadpole with funnel-shaped cloacal tube with flap-like extensions ventral to the tail fin.

The taxon is characterized by its large size (Table 2) globular body; small head, acutely pointed with a hard knob-like projection; mouth ventral, not extending beyond eye; ventrally placed nostril, nearer to the tip of the snout than to the eye; diminutive eye, diameter of the eye smaller than the distance between anterior edge of the eye and the nostril; inter-orbital distance more than thrice the width of upper eyelid; tympanum absent; without vomerine teeth; tongue small with rounded tip. The stomach contents of BNHM 4214 and 4215 contained only termites. Forelimbs with short unwebbed fingers; tips of fingers rounded; without subarticular tubercles; palm hard without palmar tubercle; relative length of the fingers, 3 > 2 > 1 = 4. Hindlimbs small; tibia short; tips of toes rounded, fully webbed, webbing in the form of a thick skin fold; no subarticular tubercles; large, elongated, shovel-

Table 2. Morphometry of adults, tadpoles and egg diameter of pignose frog

Measurement (mm)	Adult BNHM4214 male	 BNHM4215 female	 BNHM4216 female	Tadpole Stage 38 WII659	 Stage 42 WII660	 Stage 44 WII661	 Stage 44 WII662
SVL	52.77	89.9	78.3	28.2	28.0	29.3	28.8
IOD	8.3	11.13	11.2	4.6	4.8	4.3	4.0
IND	3.32	5.4	3.4	2.0	2.1	2.1	1.9
ED	2.78	3.89	3.3	–	–	–	–
END	6.69	9.41	8.5	–	–	–	–
TIL	22.71	31.15	26.4	–	–	–	–
IMTTL	8.84	14.47	11.4	3.1	3.1	3.6	3.5
IMTTW	3.86	5.9	4.3	1.1	1.1	1.3	1.2
WM	–	–	–	10.1	10.3	10.4	10.1
TL	–	–	–	34.2	34.7	30.5	26.5

Average egg diameter ($N = 20$), 3.32 mm.

shaped inner metatarsal tubercle; thigh and shank enclosed in body cavity. Dorsum of body and limbs smooth; skin thick. Cloacal opening directed postero-ventrally. The gravid female had large white eggs in the ovarian lobules. Two colour morphs of the species have been found. BNHM 4214 and 4215 are deep brown with pale ventral side. BNHM 4216 is reddish-brown with pale ventral side. The palmar region, the digit tips and the metatarsal tubercle lack pigmentation and are white. Observations made on BNHM 4214 and 4215 in their locality suggest that this species is exclusively burrowing in its habit. The frogs were heard calling from 3 to 4 cm beneath the soil and emerge out during the night to breed. They have a loud call with single notes of 'rraaak' that are repeated. The amplexus is inguinal. The breeding activity was restricted to few weeks when the pre-monsoon showers prevailed in the region during May and June. Tadpoles of the species were found in fast flowing streams, resisting the water current. They had ventrally placed suctorial mouth; flat body; large white head; dorsally placed eyes and nostrils; reduced tail fin; sinistral spiracle, keratinized beaks with teeth rows. The funnel-shaped cloacal tube and flap-like extensions ventrally on the tail fin from the cloaca are unique to this species. Analysis of 12S and 16S rDNA nucleotide diversity revealed that (i) the taxon forms a distinct ancient clade with Seychellian sooglossidae, basal to all Bufonoidea[15] (Figure 2), and (ii) this clade constitutes distinct anuran taxa that diverged 150–195 Mya (Table 3) in the Jurassic period, with possible origin in the Gondwanaland prior to the continental split. We have assigned this taxon, which we call 'pignose frog', to the newly erected anuran family Nasikabatrachidae[6]. The taxon appears similar to *Nasikabatrachus sahyadrensis*[6], indicating the need for further studies with additional samples to address the distribution and diversity within the group.

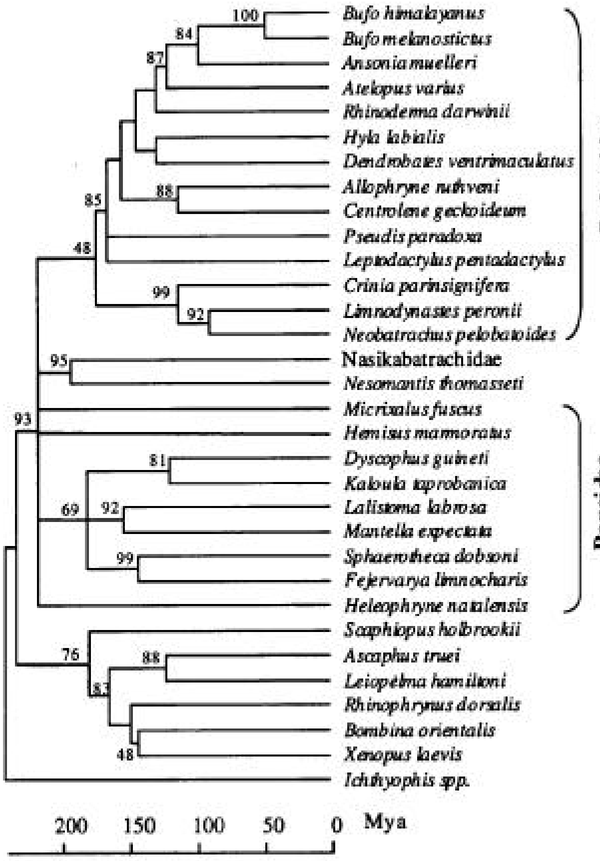

Figure 2. NJ tree based on 12S and 16S rDNA polymorphism with molecular clock estimates. Distance represents gamma corrected K 2-P with 1000 bootstrap replicates obtained using MEGA 2.01.

The taxon possesses a set of morphological, reproductive and ontogenic characters that are combinations of both shared primitive and uniquely derived characters. The pointed snout, diminutive eyes, ventrally placed mouth, absence of sternum and large shovel-shaped inner metatarsal tubercle of the monotypic genus *Hemisus*[20], are also found in this taxon. However, these cannot be ascribed to their relatedness. During the Cretaceous–Tertiary transition, harsh environments prevailed and it is speculated that nocturnal and subterranean modes were among the favoured adaptations[21]. These characters presumably have poor phylogenetic value, and such adapta-

Table 3. Estimated time of divergence of relic frog was computed using Method 1: 0.5% substitution per Mya[32] and Method 2: 0.69 substitutions per Mya[33]

Species	Calibration age (Mya)	No. of substitutions	History (w.r.t. target)	Percentage sequence divergence	Estimated time of divergence	
					Method 1[a]	Method 2[b]
Ichthyophis sp.	240	216	Ancient	31.68	176.6	194.1
Scaphiopus holbrooki	200	178	Ancient	26.405	147	161.73
Ascaphus truei	200	169	Ancient	24.995	150	163.77
Mantella expectata	110	211	Recent	32.715	175.42	157.4
Fejervarya limnocharis	110	211	Recent	29.73	169.46	153.1

tions are now considered a case of simple parallel evolution, where burrowing adaptations evolved independently several times during amphibian radiation[22]. The inguinal amplexus is considered as an ancestral character found in both primitive and transitional frogs[23]. Sooglossidae possess inguinal amplexus[24], and the condition in Heleophrynidae is not known. Our taxon also showed inguinal amplexus. The use of this behaviour as an indication of the primitiveness of the taxon is probably erroneous, because *Micrixalus*, which also diverged at about the same time, shows axillary amplexus[25]. The tongue in our taxon has a rounded tip, which is also considered a primitive character[26]. The large suctorial mouth of the tadpole with dorsally placed nostrils and eyes are unique characters that are shared with Heleophrynidae[27]. There is evidence that the taxon belongs to a relic group that diverged in the Jurassic. This indicates that the evolution of faunal endemism in the Gondwana breakaway land masses, especially in the case of amphibians, might have taken place earlier[28,29]. This finding points at the inadequacy of the K/T boundary to explain the present-day endemism and distribution of fauna and flora in the Gondwanaland break-up land masses and dates the evolution of several lineages (e.g. Hemisotidae, Micrixalinae, Heleoprhynidae, Nasikabatrachidae and Sooglossidae) in the middle Jurassic (ca. 180 Mya). We propose that this present day endemism of anuaran lineages can be accounted by vicariant evolution, a hypothesis that has not been considered earlier.

There have been more than 100 global sea-level changes since the Triassic[2]. The Vail and Haq first-order cycles coincide with times of major continental plate break-up and lasted 200–300 Mya. According to Haq *et al.*[2], global sea level last reached its present level during the lower Triassic (ca. 250 Mya) and there has been a steady increase since then. In the early Cretaceous (130 Mya), sea level rose to about 200 m higher than the present level. This probably resulted in submergence of land mass by the Mosambique and Somali basins and the Proto-Wendell Sea, prior to the spreading of the continental shelves between the separating land masses[30]. We hypothesize that the sea-level rise starting 250 Mya could have caused insularization of amphibian fauna on the tablelands of the Gondwana landmasses for long periods (ca. 150 Mya). The endemism of these lineages can be also accounted for by vicariance[31]. *Indobatrachus*, a Gondwana Myobatrachid from the Eocene in western India[32] became extinct and Myobatrachids are now confined to Australia and New Guinea[31]. Similarly, extinction of several representatives of Sooglosidae, Hemisotidae and Nasikabatrachidae in neighbouring land masses due to shrinkage consequent to sea-level rise could have resulted in the present-day endemism. This interpretation has not been validated due to the paucity in fossil records from the Western Ghats.

The cosmopolitanism hypothesized among Gondwanan mammals at the end of the Cretaceous[33], may not be applicable to amphibians and other lower taxa, since they cannot disperse through sea. Palaeo sea-level surges and subsidence have played a role in the geomorphology of the Western Ghats[34]. However, the evidences are few and scattered, such as the Jurassic fossils of marine ostracodes from Gujarat[35,36] and the fossil *Productus*, a marine Brachiopod from Umaria (460 m asl) in Madhya Pradesh[37]. The opening of land bridges by receding sea levels in the late Cretaceous[33] could have enabled more vagile species and salt water tolerant species like cichlids[38], reptiles, mammals and birds to disperse. This conforms to the evidence from fossils[33] and molecular data[38].

Conservation importance can be ascribed on the basis of past evolutionary history or future evolutionary potential[39]. This primitive frog predates any of the frog species from the Indian subcontinent by 80–100 Mya. There is clear indication that a large amount of evolutionary history of amphibians may be encompassed within the Western Ghats. It reinforces the conservation importance of the region, which is among the established global hot spots of biodiversity[3] but under severe threat due to rapid habitat destruction[40]. Within the rough distribution limits of this taxon, about 20 dams are under construction, and it is estimated that a single dam project would submerge ca. 244 ha of forests[41], including the habitat of this relic frog. This could possibly drive this ancient survivor to extinction within a short span of time. The situation warrants prioritization of threats and conservation measures both at the national and international level to protect and manage this rich reservoir of anuran biodiversity.

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