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Primitive breeding in an ancient Indian frog genus *Indirana*

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The Western Ghats biodiversity hotspot is rich in herpetofauna and harbours numerous endemic species. Unfortunately, many of these understudied species are threatened due to habitat loss, pollution, infectious diseases and climate change. *Indirana* (family Ranixalidae) is an ancient frog genus, endemic to the Western Ghats of India. Unlike most amphibians, it lays terrestrial eggs and has semi-terrestrial tadpoles. We barely have any knowledge about their development, life history, mating systems and reproductive ecology. Such information is crucial to design and implement successful conservation programmes. Hence, we stud-

ied the courtship, spawning behaviour and reproductive mode of an *Indirana* sp. from Amboli Reserve Forest located in the northern Western Ghats, Maharashtra, India. This species showcases a primitive type of inguinal amplexus and exhibits pronounced sexual size dimorphism, where females are significantly larger than the males. Average clutch size was 226 ± 41.5 eggs, with an egg diameter of 3.25 ± 0.32 mm. Fertilization rate was 87% with 100% hatching success. Additionally, this frog has evolved terrestrial eggs without the dependent traits like parental care and large egg size/small clutch size witnessed in other terrestrially egg-laying anurans (frogs and toads). This frog has reproductive mode 19, with its characteristic semi-terrestrial tadpoles. This genus represents the extreme of the trend (from obligatory aquatic to completely terrestrial) that amphibians show towards terrestriality.

Keywords: Amplexus, anuran, *Indirana*, reproductive mode, terrestrial eggs.

AMPHIBIANS exhibit a great diversity of reproductive modes, more than any other vertebrate group¹. Diversification in reproductive modes reflects the environmental challenges that various species have overcome to successfully propagate a succeeding generation^{1,2}. Behaviours associated with reproduction are usually species-specific and the defined set of courtship rituals usually concludes in the union of the gametes. In anurans (frogs and toads), fertilization being external, numerous ways such as peculiar sexual embrace and size assortative mating have evolved to achieve high fertilization success^{3–5}. Anurans have various ways in which the male clasps the female during mating and spawning. This sexual embrace also known as ‘amplexus’ has evolved to juxtapose the male and female cloacae facilitating fertilization³. In primitive frogs like the Archeobatrachians, Myobatrachians, some Leptodactylids and Sooglosids, the amplexus is inguinal, where the male holds the female at the waist, anterior to her hind limbs. This type of amplexus is not as efficient as the axillary amplexus seen in Neobatrachians, where the male clasps the female near the arm pits and their cloacae are juxtaposed synchronizing semen ejaculation with oviposition, thus ensuring a high rate of fertilization^{1,3}. Depending on the species, relative body size of the sexes, parental care and mode of oviposition, modifications of inguinal and axillary amplexus exist among species and are known as the cephalic, the straddle, the glued or the independent type¹.

Reproductive mode on the other hand is a combination of oviposition and developmental factors such as oviposition site, ovum and clutch characters and type of parental care^{1–3}. Any particular type of reproductive mode seems to have evolved according to abiotic factors like temperature, precipitation, altitude and biotic factors such as predators or parasites^{2,6}. Reproductive mode is also an integral part of the reproductive strategy employed by the species or the individual³. A large part

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of information on different types of modes is based on studies focused on amphibians of temperate regions. Very few studies have been carried out in the species-rich tropical regions and many new reproductive patterns hitherto remain undiscovered^{3,4}.

The Western Ghats is a chain of mountains that runs parallel to the west coast of peninsular India. This region is a globally recognized biodiversity hotspot, especially rich in herpetofauna with numerous endemic species^{7,8}. The genus *Indirana* (family Ranixalidae) is endemic to the Western Ghats and has evolved during the late Cretaceous⁹. Members of *Indirana* habituate the evergreen and semi-evergreen forests. Currently, there are 11 recognized species from this genus¹⁰. Recently, there have been many attempts to resolve the taxonomy and biogeography of the amphibians of the Western Ghats; yet the systematics of *Indirana* remains unresolved and the species complex comprises many cryptic species¹¹. Even some of the known species show a high degree of genetic divergence/cryptic diversity and are polyphyletic¹¹. This genus is expected to undergo substantial taxonomic revision¹¹; therefore, we refrain from confirming species and identifications were done till the genus level. Based on species distribution data, this putative species may belong to the *Indirana beddomii* species complex.

The current characterization based on clutch and egg characters of anurans, defines terrestrial eggs as those that are deposited at arboreal, fustorial and sub-terrestrial sites and are not exposed to free water at the time of oviposition¹². This species lays terrestrial eggs in crevices or depressions on rocks and on tree barks that are not adjacent to any water body and hatch into semi-terrestrial tadpoles which feed on the algae growing on the substrate.

We have little knowledge about their development, life history, mating systems and reproductive ecology. We are barely scratching the surface of reproductive ecology of tropical amphibians, many of which are endemic and critically endangered, and hence vulnerable to extinction. Understanding their reproductive biology and autecology will not only provide crucial insights into the ecology and evolution of these amphibians, it will also enable us to conserve them better. With this view, we studied the breeding ecology and reproductive behaviour of *Indirana*.

Field observations were carried out from June to August 2013 at Amboli (15°57'45"N 73°59'52"E/15.96250°N 73.99778°E) Maharashtra, India. Amboli is a hill station, situated in the Western Ghats at an altitude of 720 m amsl. It receives heavy rainfall from the southwest monsoon with average annual precipitation of 7446 mm. Forest type is wet evergreen to semi-evergreen with thick undergrowth¹³. Intense observations were taken from a forest patch from July to August 2013.

Field observations were carried out every day from 9.30 pm to 12.30 am during the study period consisting of 28 days. Egg size, male and female snout vent length (SVL) were measured using a digital calliper to the near-

est millimetre. Freshly laid *Indirana* eggs are partially pigmented and have a black and white hemisphere (Figure 1b). They develop fast and hatch within four days. Fertilized eggs reach gastrulation stage 5–6 h after spawning (pers. obs.) and adorn a greyish colour, and the two poles are no longer distinct. Fertilization success was recorded the next day of spawning by counting the number of eggs that reached gastrulation (fertilized) and the number of eggs that still had distinct hemispheres (unfertilized) with no change in colour even after 12 h. Hatching success and duration were calculated from the day the eggs were laid till all of them hatched out, observing the eggs at the field site every 12 h. Courtship and spawning behaviour was photographed using a digital SLR camera, Nikon D4. Male and female SVL was compared by the Mann–Whitney test performed using the freeware PAST¹⁴.

Courtship and spawning behaviour of two pairs was observed on 16 July and 2 August 2013. Egg clutch and oviposition site parameters were taken on five other occasions. Several tadpoles at various developmental stages were observed.

Males started vocalizing at the onset of the monsoon and reached their peak in mid July. They perched on rocks covered with moss, usually near a crevice at the height of 0.3–1 m. The advertisement call of this frog is a short and very soft 'kruuk'. Inflated vocal sacs of calling males were not visible even after observations were made from close proximity. This species apparently lacks vocal sacs. Males were observed calling from a particular site for a day or two, but later disappeared even if mating and spawning had occurred. They did not display any site fidelity with respect to the calling site, suggesting that territoriality may be absent in this species.

Calling males positioned on rocks or on tree barks were approached slowly by females. In one instance, it took the female 15 min to cover a distance of 15 cm to get close to the male. Once the female was in sight, the male gave continuous vigorous calls. This call drastically differed from the intermittent advertisement call both in its frequency and intensity. The female approached the male from the front and he quickly grasped her with his forelimbs around her waist and their cloacae were not juxtaposed. The amplexus in this frog is of inguinal type. This may be due to sexual size dimorphism (SSD) where females are significantly larger than the males. The frogs remained in amplexus for 12 ± 0.07 min, $N = 2$.

The female immediately started depositing eggs after initiating amplexus in discrete packets. The male caught a few eggs in his hind limbs and may have fertilized them (Figure 1b). The amplexed pair moved a little forward after they laid an egg clump. A large number of eggs were laid by the female and the mean clutch size was 226 ± 41.5 eggs, $N = 5$. The egg diameter was 3.25 ± 0.32 mm with the egg jelly and 1.95 ± 0.08 mm without the jelly, $N = 25$. The eggs were exposed to the environment and did not have a nest or any vegetation

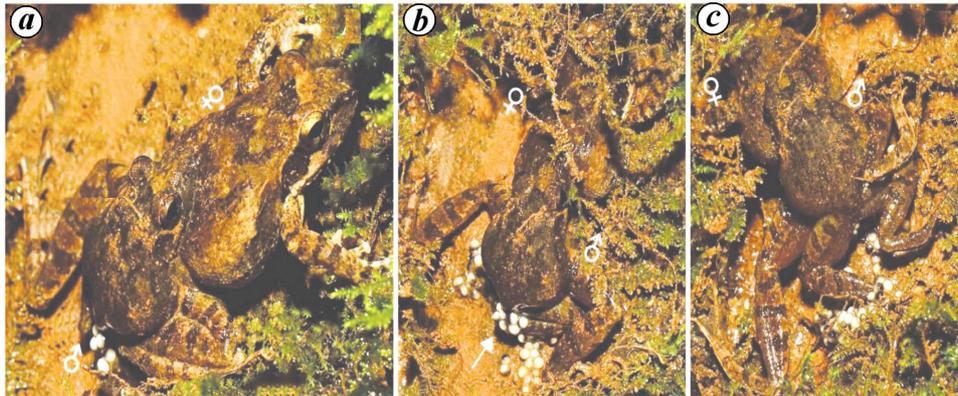


Figure 1. Various postures of *Indirana* sp. during courtship and spawning. **a**, An *Indirana* sp. pair initiated amplexus and started laying eggs immediately. The noticeably small male clasps the larger female around the waist, exhibiting inguinal amplexus. **b**, The male caught a packet of eggs and may have fertilized them. **c**, After successful spawning, the pair is ready to disengage.

cover to protect them. They were loosely attached to the substratum (rocky depression or tree bark covered with moss) that was always moist due to frequent rain.

Five different clutches were observed during the study period. Two of which were deposited under moss growing on tree barks, while the other three were found in small depressions on rocks. Frequent rain maintained a thin film of water, keeping the eggs hydrated. Out of the total 1356 eggs from 5 clutches examined, 177 eggs were found to be unfertilized; 11–13% of eggs from each clutch was left unfertilized. The remaining 1179 fertilized eggs hatched within 4 days. Though the fertilization success was 87%, the hatching success was 100%. With the hatching duration of 4 days, they are one of the fastest developing terrestrial eggs¹. The tadpoles did not have much yolk and started feeding within a day.

This species exhibits pronounced SSD. Females were found to be significantly larger than the males ($U = 0$, $P = 0.0004$). Males had an average SVL of 28.8 ± 0.3 mm, $N = 9$; whereas females had a mean SVL of 45.0 ± 0.6 mm, $N = 9$.

Most terrestrial breeding anurans have a resource defence type of mating system with some lek-like characters. Here males monopolize certain resources such as oviposition sites, that are required by the females for successful reproduction³. Males establish multipurpose territories that harbour a suitable calling post, oviposition sites and serve as a place for the tadpoles to develop^{3,15}. They also display some sort of parental care; in many species it is obligatory^{3,16}. Parents often care for the eggs by avoiding desiccation or protect them from predators by attending to them^{3,17}. *Indirana* sp. does not offer any sort of parental care by either sex as neither parent was observed near the eggs during the day or at night.

Terrestrial conditions are a stark contrast to aquatic ones and species with non-aquatic eggs are under different selective pressures than those that oviposit under water³. Amphibians accordingly display clutch energy partitioning, i.e. species with terrestrial eggs invest more per egg and have larger eggs compared to those that lay

aquatic eggs. Due to this differential investment, species with aquatic eggs are able to produce twice as many eggs per clutch than their terrestrial counterparts¹⁸. In reproductive modes with terrestrial eggs, tadpoles that hatch out are bigger and at an advanced stage of development than those that hatch from aquatic eggs¹⁸. Two characteristic traits of species with terrestrial eggs are territoriality and small egg clutches with large eggs^{1,3,18}. Astonishingly, these predominant features are deficient in this species. The mean clutch size of 226 eggs is high among other terrestrial egg-layers¹. Thus, *Indirana* shows a unique kind of a mating system where terrestriality has evolved without the dependent characters seen in other terrestrial egg-layers.

Mode of reproduction is a combination of oviposition and developmental factors such as oviposition site, ovum and clutch characters, duration of development, stage and size at hatching and parental care, if any¹. Currently, more than 40 reproductive modes are recognized in anurans^{2,3,19,20}. The sites of oviposition as well as the location of tadpole development are some of the key factors considered during the description of the reproductive mode of a species. In modes 28–33, eggs are terrestrial or arboreal. In all the modes where the eggs are arboreal or terrestrial, tadpoles move into ponds or streams, develop in water-filled cavities on trees, are carried to the water body by the adults or directly develop into froglets^{2,3}. Reproductive mode 19 has the characteristic semi-terrestrial tadpoles. This species therefore showcases reproductive mode 19 as eggs are deposited on rocks, tree barks or in crevices away from water bodies and develop into free-living semi-terrestrial tadpoles. The morphology and development of tadpoles from this genus has been already described^{21,22}. However, there are no reports of a species showcasing reproductive mode 19 from India. In Asia, a couple of genera like *Indirana* and *Nannophrys* exhibit semi-terrestrial larval stages^{16,21}. Other species from these genera may have similar reproductive behaviour.

All orders of amphibians show a clear trend towards terrestriality¹; reproduction and development occurring

further away from water. A few anuran genera in the tropics seem to have attained this summit; they showcase both direct development as well as semi-terrestrial tadpoles³. *Indirana* is one such genus, whose larval stages have adapted to life on land. High atmospheric moisture content and plenty of algal growth on varied terrain of the Western Ghats during the rainy season seem to favour the terrestrial habit of *Indirana* tadpoles. A short hatching period, muscular tails and lack of tail fins are manifestation of adaptation to this exceptional niche. Fast development and a unique style of jumping with the help of their tails, when startled have probably evolved to evade predation²³.

The genus *Indirana* originated during the late Cretaceous⁹ and ancient frog lineages are known to exhibit inguinal amplexus³. The Jurassic frog, *Nasikabatrachus sahyadrensis* also exhibits this type of amplexus²⁴. Inguinal amplexus in *Indirana* validates that this was the predominant means of reproduction in ancient frogs. It is believed that this type of amplexus would be inefficient in fertilization³, this was corroborated in this species as only 87% of the eggs were successfully fertilized, whereas like most anurans, even members of the genus *Nyctibatrachus* such as *N. humayuni* and *N. petraeus* that have a pseudo amplexus achieved 100% fertilization^{15,17}.

The genus *Indirana* shows tremendous genetic diversity and many cryptic species. Such behavioural characters and reproductive descriptions will aid the systematics of this genus. In the Western Ghats, several amphibian species along with *Indirana* are vulnerable to extinction due to habitat destruction, climate change and infectious disease like chytridiomycosis^{10,25}. The present report on the reproductive ecology of this exclusive genus *Indirana* will be crucial for its conservation and to set up captive breeding programmes to save critically endangered species such as *I. gundia* and *I. phynoderma*.

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