SUMMARY

Amphibians are intermediate in some ways between the fully aquatic fishes and the terrestrial amniotes. However, they are not simply terrestrial in their morphology, life history, ecology, and behavior. In the successful attainment of independence from water and colonization of land, amphibians have undergone a remarkable adaptive radiation, and the living groups exhibit a greater diversity of modes of life history than any other groups of vertebrates.

Striking differences occur in the tail structure among anurans. Two or more tail filaments are present in all primitive anurans (discoglossoids, pipoids, and pelobatoids). Two tail filaments occur in some members of the Hylidae and Leptodactylidae and in nearly all centrolenids and bufonids. Many leptodactylids, most hylids, all pseudids, and nearly all ranids and microhylids have a single tail. Careful study of the structure of spermatozoa reveals interspecefic differences in the shape and proportional length of the head and neck, and also variation in tail structure.

Results of these studies on spermatozoa of relatively few taxa suggests that certain morphological characters are consistent with classification and that characters of the spermatozoa may contribute to understanding of the phylogenetic relationships among groups of amphibians.

The phylogenetic relationships among families of Anura are still largely unresolved. Groups widely accepted as monophyletic have often been challenged with new phylogenetic reconstructions and the continuous accumulation of new informations. For example, Hillis et al. (1993) using 28S fragments of rRNA found *Neobatrachia* to be polyphyletic. Contents of groups such as Bufonoidea and Ranoidea are in

a constant state of flux because of the addition and exclusion of families, such as Dendrobatidae. At the family level, the resolution of most phylogenetic trees is very poor, and relations between most clades being largely unresolved, while two of the major families (Leptodactylidae and Ranidae) are generally considered polyphyletic.

Analyses using alternative data set, such as molecular markers have slowly added new insights to the problems but also have refuted well-established clades. Filling the gaps on existing data sets and exploring new kinds of characters are important ways to improve phylogenetic hypotheses among anurans.

The ultrastructure of spermatozoon has been used as an alternative data set to investigate the phylogeny of many taxa such as fishes, amphibians, reptiles, and invertebrates. An advantage of sperm ultrastructure data is that they provide more conservative characters for groups with highly derived body plans, such as Amphisbaenia, which cannot be scored for some traditional morphological traits. Spermatozoon ultrastructure data have also been useful in clarifying relationships among Polyplacophora, where traditionally used characters are either too conserved or too variable. Spermatozoon morphology, therefore seems to be useful for groups where external morphology cannot be scored, either because of evolutionary conservativeness (as in some traits of Polyplacophora) or specialization (as for Amphisbaenia).

Some conjectures on anuran phylogeny have been made based upon spermatozoon ultrastructure and the cladistic significance of some characters has been investigated. For example, the conical perforatorium has been proposed as a tentative synapomorphy of Bufonidae, whereas, the presence of an undulating membrane or a rod-shaped perforatorium have been scored as symplesiomorphies of Anura. Yet, due to paucity of data, such as those made for squamata reptiles and fishes has not been conducted for anurans.

The family Bufonidae, with more than 350 species distributed among 33 genera, is one of the most species rich amphibian families. Members of this family occur throughout the world, except for Madagascar, New Guinea and adjacent islands, and the Arctic regions.

The number of species in bufonid genera varies significantly. One genus, *Bufo,* contains over half the species, while most of the remaining genera contain fewer than 10 species each. This distribution of species reflects the preponderance of phenotypic diversity among bufonids and the degree to which the group has been studied. However, the current taxonomy is likely a poor reflection of the phylogenetic relationships within the family. Within bufonids, the genus *Bufo* is the most problematic group. Evidence suggests that *Bufo* is paraphyletic with respect to either some or all of the remaining bufonid genera.

Because bufonids are a major component of the amphibian fauna of East Asia, they have been the subject of many investigations. The majority of previous studies have focused on anatomical characters. Because of the paucity of phylogenetically informative anatomical characters, due to an apparent conservation of the bufonid body plan, many questions remain open.

Karyological studies using conventional and banded chromosomes reveal that the karyotypes of Asian bufonids are highly conservative. All species, except for *Bufo danatensis,* have a karyotype consisting of 22 chromosomes, including six large and five small pairs. *Bufo danatensis* is a tetraploid species that evolved from a 22-chromosome ancestor. Although the karyological studies have contributed to resolving some questions of species identity in Asian bufonids, the data have been phylogenetically uninformative at higher taxonomic levels.

Recently, mitochondrial DNA sequences have been used to reconstruct the history of bufonids. Graybeal (1997) examined species that represent most of the geographic and taxonomic groups, as an effort

to provide the overall pattern of the family. Another study focused on the *Bufo bufo* species group from the eastern Tibetan Plateau.

In the present work I have worked out the detailed sperm ultrastructure and DNA content of three species of toad belonging to the family Bufonidae of Indian continent. Based on the observed data on sperm ultrastructure and DNA studies an attempt has been made to comment on their evolutionary correlation and phylogenetic status of three anuran species (*Bufo himalayanus, Bufo stomaticus* and *Bufo melanostictus*).

Therefore, the aims and objectives of the present dissertation can be boiled down as –

1. To examine the morphological features and structural peculiarities of sperm in three species of Bufonidae.
2. How does the sperm morphology aid in recognizing a particular genus or species?
3. Is there any genetic background for the sperm morphology or is it just a structural peculiarity?
4. How can sperm ultrastructure be a basis for understanding phylogenetic status of the Bufonids?

To study the morphological variation of testes shape and size, every individual specimen of each species group is marked first. Throughout the prebreeding, breeding and nonbreeding season the testicular activity of every experimental specimen is noted by the morphological and metrological changes in the testes. Before proceeding to microscopic tissue preparation, the individual species samples were narcotized and their testes (Right and Left) were taken out and placed in a clear blotting paper to get an impression of the individual testis. The sizes were measured along with an outline sketch of their shape. After that the testes samples were transferred to amphibian saline for further processing. This experiment was conducted for every specimen sacrificed

at every season so that a clear idea can be gained about the testicular change in breeding and non-breeding season.

For light microscopic study the testes were dissected out. One half of the testis is processed further for electron microscopic studies and the other half is teased in 0.1 (M) Cacodylate buffer. After teasing, the buffer turns milky white. This solution is centrifuged in 3000 RPM for five minutes, as a result mature sperms precipitate down. These sperms are smeared over slide, fixed in methanol, stained with Giemsa and viewed under compound microscope.

For Scanning electron microscopy the testes were bisected in APBS. The two halves were processed separately, one for direct testis study and the other for individual sperm study. The testis and extracted sperms were washed in Cacodylate buffer and fixed in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium caccodylate buffer (pH 7.1) for 4 hours at 4°C. After fixation the materials were washed and transferred to 2% osmium tetroxide solution in cacodylate buffer for 2 hours. The tissues and sperm samples were washed in cacodylate buffer and dehydrated through ascending grades of ethanol. After complete dehydration the samples were treated with a mixture of absolute alcohol and amyl acetate (1:1) for 30 minutes. Finally the samples were transferred to amyl acetate and kept at 4°C. The materials were dried at critical point and coated with colloidal gold for getting better resolution. The sperm samples were dropped on copper stub and coated with gold after air drying. Surface morphology of the testis and also the sperm cells were studied under Hitachi S-530 Scanning Electron Microscope.

For Transmission electron microscopy the testes lobes were reduced into fragments (1-3mm) and fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer at 4°C. After fixation the materials were washed in cacodylate buffer (pH 7.1) and postfixed in 1% osmium tetroxide in the same buffer for 2 hours in the dark at 4°C, contrasted en block with aqueous solution of 5% uranyl

acetate for 2 hours, dehydrated in acetone and embedded in araldite. Thick sections (1μm) were cut, stained with aqueous toluidine blue and observed under a light microscope. Thin sections (60-70 nm) were cut in an L.K.B. ultra-microtome, mounted on copper grid and stained with a saturated solution of uranyl acetate in 50% alcohol and lead citrate. The grids were observed under a Philips CM-10 Transmission Electron Microscope.

DNA was extracted from sperm cells and purified as per standard techniques and the DNA concentration was measured by optical densitometry.

Isolated DNA were electrophorosed in native condition and also after restriction endonuclease treatment using three separate restriction enzymes (Bam HI, Hind II and Eco RI).

The living sperm cells from three different species were examined and analyzed by FACS following standard technique.

The light microscopic observation shows a typical anuran morphology of the sperm, i.e. with a slender head, small middle piece and long tail.

The ripe spermatozoon of *Bufo himalayanus* possesses a deeply stained head consisting of a cylindrical nucleus and a pointed needle-like acrosome situated at its anterior tip. The acrosomal tip is slightly curved like a notch. The head of *Bufo himalayanus* is about 29x2μm in size, larger than that of other two species studied and have a sword like appearance. Immediately behind the head and in intimate contact with it is a small and equally deeply stained middle-piece where the mitochondria are clumped together. The tail is biflagellate and about 46μm in length. There are two axial filaments joined together by a rudimentary undulating membrane. Each filament arises from its own centriole, but as both the centrioles side by side at the base of the small deeply staining middle-piece, they cannot always be clearly made.

The sperm head of *Bufo stomaticus* is deeply stained nucleus with a pointed less stained needle like acrosome at its anterior tip It measures 25x3µm in size. The cylindrical head region shows irregular surface morphology. The deeply stained head is followed by an equally stained slightly flat mid piece from which the tail filaments arises. Mid piece contains irregularly arranged groups of mitochondria, which helps in flagellar movement. The tail of *B. stomaticus* is biflagellar in appearance and 40µm in length. Both the filaments arise as a single fiber and after a distance both the fibers separate distinctly and runs parallel to each other. A fine membrane connects both the fibers throughout the length

In *Bufo melanostictus* the head is cylindrical elongated having a size of 28x3µm. The head hears a darkly stained nucleus and a pointed acrosome at its tip. Surface morphology of the head is smooth. The head is followed by an equally stained middle piece from where the tail filament arises. The tail in *B. melanostictus* if biflagellar in organization and both the filaments arise from a single point in the middle piece. The tail length is 46µm, but the undulating membrane in between the tail filaments is not well visualized.

The Scanning Electron Microscopic Observation supports the light microscopic features mentioned earlier. The head, neck, and tail parts of individual species are well documented and easily recognized.

In ***Bufo himalayanus,*** the head is elongated, slender with a cup like depression at the acrosomal end. Surface morphology of the head piece is smooth. The neck region is not morphologically differentiable under SEM, as it is over-flanked by the posterior portion of the head and is only recognizable by a swollen portion at the posterior most part of the head. The tail is biflagellate and are connected by a membranous sheath medially, however such membranous sheath is not visible at the posterior end of the tail, which terminates in a knob like structure.

In case of ***Bufo stomaticus,*** the head is slightly curved with a pointed anterior portion as a barb. The head surface is not smooth and

bears small wart like projections of variable shape and size along its length. Like *B. himalayanus,* the neck region is not morphologically visible and is over-flanked by the posterior end of the head. Mid piece is slightly flattened than the head. The tail is biflagellate, but both the flagella are free from one another i.e. not connected by proteinacious sheath as found in *B. himalayanus*. However at the posterior end of the tail filament, both the flagellum unite as a bi-lobed knob.

The head morphology of *Bufo melanostictus* strongly resembles with that of *B. stomaticus*. The head is elongated, with anterior barb like projection but without warts on the head surface. It is cylindrical in shape and pointed at its anterior end. The neck, unlike other two species studied is morphologically visible as elongated rod like region. The tail filament appears from this thread like middle piece. Tail is bi-flagellate, the flagella are free from one another throughout the entire length and each terminates as small knob at the posterior end.

Under TEM the anterior and middle portion of the *Bufo himalayanus* sperm is well differentiated with conical sharp head, tapering towards the anterior end. The nucleus is elongated and with electron dense granules as seen in transverse and longitudinal sections. However, the concentration of electron dense granules is not uniform throughout the length of the nucleus. At the proximal end of the nucleus the concentration of electron dense material is less than the distal end. The middle portion of the head exhibits a maximum concentration of electron dense granules. The anterior tip of the head terminates in an electron lucent acrosomal cap. The acrosomal cap sends off acrosomal barb at the anterior end. The acrosomal barb is wooly in appearance. In light magnification it takes a cup shaped rigid flattened structure. However in higher magnification such rigidity is not observed. The middle piece is elongated with moderately electron lucent vacuoles surrounded by heavily packed multi-layers of mitochondria. In longitudinal section mitochondria takes circular shapes. However mitochondria are not arranged in any ordered fashion, and as a

result, appears to be randomly arranged in longitudinal and transverse section. Two distinctly visible centrioles are present - proximal and distal. The proximal centriole is located in close proximity to the nuclear notch at the basal end of the head. It lies in a parallel fashion to the head nucleus. The distal centriole lies perpendicular to the nuclear axis, slightly away from the proximal centriole. Microfilaments of the centrioles are not very distinct as found in other two species described. The tail is filamentous in appearance with a central flagellum. Each flagellum consists of an axial filament made up of 20(18+2) submicroscopic longitudinal fibrils arranged in typical outer circlet of nine doublets and inner core of two singlets, which are situated distantly from each other. Thin proteinous filaments connect the central filaments with the peripheral filaments and giving a cart-wheel appearance. Well visible undulating membrane present, which terminates in a thick broad end.

In *Bufo stomaticus,* the head and middle piece are clearly demarcated. The head is elongated, slightly curved and circular in cross section with rounded base and tapering tip. However the nucleus exhibits irregular thickening resulting in a rough surface morphology. Electron dense particles are well visualized throughout the head length in transverse and longitudinal section but the granules are of smaller size. The concentration of such electron dense granules are lower in the posterior end than in the middle and anterior end. Well formed acrosome is visible with external acrosomal vesicle and internal subacrosomal cone. The acrosomal surface is almost smooth with no surface projections. Acrosomal barb is present but with no distinct conformation. In *Bufo stomaticus* the middle piece is broad with extended flap like projections on both the sides. Mitochondria are layered on the flaps. Two types of mitochondria are visible *i.e.* they appear elongated and circular in cross section. The mitochondria are arranged in dispersed manner. Two centrioles are present- proximal is round and distal is elongated. Both of them lie perpendicular to each other Proximal centriole is situated within

the posterior notch of the nucleus. It is round in appearance with nine peripheral submicroscopic tubules. In longitudinal section the posterior centriole is elongated in appearance. The tail is filamentous in appearance with a central core made of flagella. Each flagellum is made up of microtubules, which are arranged in usual 9+2 fashion. There are nine peripheral doublets and two distinctly separated central singlet filaments. Both types of filaments are interconnected by protein fibers giving the appearance of a wheel. Undulating membrane originates from the axial filaments but the extend of the membrane is variable. The undulating membrane terminates in an end bulb of medium size, superficially resembling the axial filament. This type of organization gives a biflagellar appearance of the tail. However, it may be mentioned that under light and electron microscopic observation the sperm tail has been described as biflagellate.

In *Bufo melanostictus* the head nucleus is elongated, slightly curved and circular in cross section. The posterior end of the head nucleus is almost round and anterior tip is pointed. Numerous electron dense granules are present throughout the entire length of the nucleus. The anterior portion of the head bears electron lucent acrosome cap. The acrosome cap is smooth in morphology with distinct acrosome and subacrosomal cone over the pointed head. Distinct niddle shaped acrosomal barb is visible in the extreme tip of the head. Distinct middle piece is visible which starts from the 'n' shaped subumbrellar notch of the nucleus. Numerous mitochondria are packed in this region, which are almost circular in longitudinal section and arranged in a regular fashion on both sides of the anterior end of the tail. Proximal and distal centrioles are well documented and lie perpendicular to one another with a shorter in-between distance. The proximal centriole is located at the base of the head. It is circular in appearance with nine peripheral filaments distinctly visible. The distal centriole is elongated in longitudinal section and continues with the tail filament. The tail has a flagellar structure with 9+2

microtubular arrangement in the axial filament. In cross section the axial filament of the tail contains nine doublets of microtubules encircling closely situated but separate two singlets of microtubules. The central microtubules are connected to the peripheral microtubules by transverse protein fibres. The diameter formed by the peripheral microtubules, are quite large and no distinct membrane is visible on the outer surface of the axial filament. Undulating membrane is present but it is short and stumpy. The extreme end of the undulating membrane is rolled on itself giving a hollow tubular appearance.

The isolated DNA samples from individual specimens were analyzed under UV- Spectrophotometer and their purity along with concentration was observed by using the formula -DNA Content = (50 μg / ml) x 1.0 A_{260} . Of these estimated DNA samples, the pure forms were isolated for further electrophoretic analysis and the rest discarded. Equal amount of DNA from three different species were separated by gel electrophoresis and the banding patterns of the genomic DNA was noted.

After that, equal amount of DNA from three different species were again taken and digested separately by three different restriction endonuclease enzyme. Finally they were passed through gel for electrophoresis and the results noted.

It is seen that Eco RI enzyme is more sensitive to *B. stomaticus* than the other two. But a different situation arises in case of Hind II digest. After Hind II digestion it is observed that the DNA of *B.himalayanus* remains relatively intact but that of other two smeared off after Hind II digestion indicating a massive digestion rate.

Through Fluorescence Activated Cell Shorting it is observed that the peak value for Bufo stomaticus is 309.8, for Bufo melanostictus it is 523.34 and for Bufo himalayanus it is 280.92. As a single curve is obtained in every case it can be said that the examined cells were containing a same quantity of DNA in every case. From these two type of observation it can be concluded that all the three specimens analyzed

under FACS were containing different amount of DNA as the genetic material.

The relative increase in gonadal size in males in the species studied is confirmatory to the earlier observations. It is principally agreed to the fact that the increase in gonadal weight as well as size is linear with breeding activity in the months in which the experiments were carried out. In *Bufo himalayanus,* the variation is relatively lower than in the *B.stamaticus* and *B.melanostictus*. But in all the cases testicular composition and their variations are very much pronounced in the breeding season when compared with non-breeding and post breeding seasons.

The light, scanning electron and transmission electron micrographic observations uphold the fact that during breeding months the endocrine organs which controls the gonadal activity also exhibit hypertrophy and hyperactivity.

In the present dissertation a peculiar observation on testicular size variation is noted. In each species the size of the right testicular lobe is slightly larger than that of the left.

Variation in different parts of spermatozoa is common among the different species of Anura. In my observation such variation can be clustered as head classes and tail classes. In *Bufo stomaticus* four distinct head classes are observed among which the majority of the sperm (64%) are with larger head. Microcephalic sperm are rare in the sample. Similarly, in *B.melanostictus* three sperm head classes can be recognized and such variation in *B.himalayanus* (66%) of sperm head represent the frequent class while the rare class is represented by a sperm population of 6% only. Similarly sperm can be categorized into different classes on the basis of relative length of tail in all the three species studied. The polymorphism of spermatozoa manifests not only gross morphological derivations of nuclear and cytoplasmic characteristics, simple size differences, but also in there genetic and the biological functions.

It is now well conceived that sperm morphology is a derivative of a set of genes which are known as sperm specific genes and expressed only in germ cell line. Though sperm are genotypically haploid but phenotypically enjoy the status of a diploid cell due to the presence of cytoplasmic continuity with the clone of cells as found in mammals or Sertoli-spermatozoa cluster in sperm nest as found in Amphibia, Reptilia etc. Therefore, sperm abnormality represents either "switching off" of some sperm specific genes or duplications as found in some amphibians. Burgos and Fawcett (1956) first provide a remarkable account of sperm ultrastructure while narrating the spermiogenesis of *Bufo arenarum* .The description includes the formation of acrosome, changes in the nucleus, a perforatorium, centrioles, tail and undulating membrane. For the first time, they reported the existence of a perforatorium in the developing spermatids and in ripe spermatozoa in *Bufo arenarum* . they also pointed out the existence of slender fibrils within the perforatorium and stated that such fibrils of the perforatorium are not continuous with the nuclear membrane.

My observations on sperm ultrastructure strongly upholds the Bufonid lineage. The variations noted at individual level clearly suggest the differences among the species. Such ultrastructural differences along with consistent ultrastructural features would be used to describe phylogenic relationship among the taxa.

The genome size, ploidy of cells can directly be determined by measuring the DNA content of the nuclei. Nucleated chicken red blood cells (CRBC) which have a known genome size of 2.33 pg are often included as a standard in the sample. The ratio of the mean fluorescent of the unknown nuclei / CRBC nuclei is multiplied by known CRBC value (i.e., 2.33pg) equals to the genome size of the unknown. However in our experiment no CBC were used and instead the gonial cells (theoretically have 2n value) were used as control. The histograms distinctly exhibit that in each case the majority of cells have a single peak suggesting the purity

of the sample. In *Bufo himalayanus* the peak value is 280.92 while in *Bufo stomaticus* and *Bufo melanostictus* peak value recorded 309.8 and 523.35 respectively. Therefore, it can be suggested that the majority of (97.5%) sperms scanned at a time in *Bufo himalayanus* have the haploid value. The deviation that are noted on either side of the peak value suggest sperm DNA polymorphism. Similarly, in *Bufo stomaticus*, the peak value of 309.8 contains only 89.01% of spermatozoal cells suggesting a more deviation of the normal haploid value in the sample. Similarly in Bufo melanostictus, the peak value 523.34 reflects more DNA content in the sperm nuclei as well as large deviation of DNA content in the species. . Therefore from this observation tend to suggest that *B.himalayanus*, *B.stomaticus* and *B.melanostictus* have different genome size and there is a degree of either increase or decrease of genome size.

However, gel electrophoretic studies using total sperm DNA and restriction endonuclease digestion using EcoRI, HindII and Bam H I provide an another picture which is more persimonic to ultrastructural studies. Sperm DNA concentration was recorded highest in *B.himalayanus* as a result exhibits very little electrophoretic migration along the length of the gel slab. On the other hand, *B.melanostictus* and *B.stomaticus* DNA exhibited a more or less same migration rate.

On the basis of above analysis it can be concluded that *B.stomaticus* and *B.melanostictus* are derived species of *B.himalayanus*. However, such a conclusion needs further detailed study at the level of genes and molecules.

Studies on Ribosomal RNA, proteins and mitochondrial DNA recognized *B.himalayanus* and B.melanostictus as sister group along with *B.galeatus* and Bufo sp(?) while other species grouped in an another clade. However, the study did not mention the status *B.stomaticus*, a prevalent species of Indian subcontinent. Similarly Pramuk et.al. (2001) has inferred the phylogeny of West Indian Bufonidae using 2Kb mtDNA

sequence data. The analysis supports the monophyly of native West Indian toad and a New world origin for the group.

Result of ultrastructural study on spermatozoa of relatively few taxa suggest that certain morphological characters are consistent with classification and that characters of the spermatozoa may contribute to understanding of the phylogenetic relationship among groups of amphibians.

Inspired by this doctrine in comparative recent years, the ultrastructural features have been used as a tool to ascertain phylogenetic relationship between the families, genus and even at species level .

Based on the information available on ultrastructural details of different species of *Bufo,* I have considered following characters of *Bufo* as plesiomorphic, apomorphic, autapomorphic. Depending on the homoplasies a cladogram has been proposed. It shows that the species available in the experimental study area (Eastern India) has above characters unique to them.

The species *Bufo himalayanus* and *Bufo melanostictus* have more common features suggesting a close relationship. On the other hand, *B.stamaticus* exhibits some characters, which are not shared by the two species (*B.himalayanus* and *B.melanostictus*) mentioned earlier but with *Bufo arenarum.*

The phylogenetic status of Bufonidae, particularly of the genus Bufo as evidenced from fossil records, morph anatomical features, biochemical and ultra structural studies and mitochondrial DNA sequence analysis tend to suggest the pylogeography of the genus and its descendent species.

The genus *Bufo* is known from the Paleocene of South America and from upper Tertiary and Quaternary deposits of North America, South America, Europe and Africa. The genus in its present form is more or less cosmopolitan in temperate and tropical regions, except for Australo-

Papuan, Madagascar and oceanic region. *Bufo merinus* has been introduced into Australia and New Guinea and many other islands.

The present day distribution of the families of Anura when compared with the fossil history suggests the following -

1. The historical biogeography of Anura is associated mainly with Gondwanaland. However limited fossil evidences and present distribution of some families of Anura indicate that some anuran stocks was associated with initial brake up of Pangaea in the Early Jurassic (160-180 million years). By Late Jurassic, numerous fossils have been recorded from Europe, North America and South America. So it may be assumed that anurans became widespread in the world during the Jurassic.
2. Leiopelmatidae is considered as the most primitive genera of Anura and were distributed widely prior to the breakup of Pangaea and that the living genera are relicts of this ancestral group.
3. The Lauracian fragment of Pangaea include following families- Discoglossidae, Paleobatrachidae. Rhinophyridae, Pelodatidae and Pelodytidae. Whereas,
4. The vicariance of the other family groups of Anura is associated primarily with the breakup of Gondwanaland. Prior to the initial breakup of Gondwanaland in the Late Jurassic (140 million years), the ancestral stock of the Anura differentiated into three major groups- Bufonoids, Ranoids and Microhyloids. The further differentiation of the three lineages took place when the Gondwanaland fragmented into three continental masses.
5. Each such fragment harbored a set of families which can be summarized as follows –

Africa- S. America	Madagascar- Seychelles- India	Australia-Antarctica
Leiopelmatidae	Myobatrachidae?	Leiopelmatidae
Pipidae	Bufonidae?	Myobatrachidae
Leptodactylidae	Microhylidae	Hylidae

Hylidae	Ranidae
Bufonidae	Hyperliidae
Microhylidae	Rhacophoridae
Ranidae	
Hyperliidae	
Rhacophoridae	

6. The anuran fauna of Madagascar, Seychelles-Indian continent that drifted from the rest Gondwanaland about 140 million years ago contained only tropical groups.
7. Savage (1973) suggested that radiation of Bufonoids in Southeastern Asia was a late Cenozoic event following the dispersal of Bufo into that region from North America via Beringia. The diversity of Bufonoid genera in Southeastern Asia and adjacent islands strongly suggests an earlier arrival of a Bufonoid stock. Unfortunately, there is no fossil evidence whatsoever.
8. A further dispersal of Bufonoids to wards Indian subcontinent took place in the Oligocene, when India collided with Asia. Most of the transgression probably took place into the Assam-Burma region in the Late Cenozioc.

This dissertation takes the liberty to comment that introduction of the genus Bufo in eastern India took place from two direction-

a) Burma → Assam → Eastern → Himalayan region → Sub Himalayan region → Gangetic plain → Penninsular India

and / or

b) Africa → Southern India → Penninsular India → gangetic plains → Eastern Himalayan region