

INTRODUCTION

Amphibians occupy a unique place in the evolutionary history for being the first vertebrates to establish life on land, though they prefer to settle on the edge of water. The successful perpetuation of a species and its survival in the terrestrial environment necessitated the development of structures like limbs, lungs and many other anatomical modifications and mainly on its evolution of new reproductive strategies. Amphibians have survived over several million years. During these years, as a consequence of interactions with nature, different mode of reproductions has been evolved such as oviparity, ovo-viviparity and even viviparity. They enjoy diverse habitats and play a very important role in maintaining ecological balance.

The class Amphibia is uniquely characterized by -

- Smooth (i.e., scales), moist skin
- Gills, lungs, and skin as organs of respiration
- Metamorphosis from aquatic larval stage to adult form
- Larval heart two-chambered, adult heart three-chambered
- External fertilization

The class "Amphibia" means "**two lives**", indicating that they are able to survive both in water and on land. Amphibians represent an important step in the phylogeny of vertebrates, the bridge from water onto land. The members of this group do not inhabit the ocean, but some are reported to occur in brackish water.

Amphibian lungs are poorly efficient. For this reason, most amphibians also use their moist skin for respiration. Temperature is another key factor in the life of amphibians. Being **ectothermic**, their body temperature changes with the temperature of the environment. Temperature extremes are deadly to amphibians. Toads burrow below the frost line to escape winter temperatures and many frogs and salamanders **hibernate** in the mud at the bottom of

rivers and lakes. Some species actually produce glycerol, a type of "antifreeze", in their cells to resist ice crystal formation.

Hot, dry conditions are deadly to them. Many amphibian species escape these conditions by **aestivating**, the summer equivalent of winter hibernation. They conserve body moisture by retreating into a moist burrow or crevice, curling tightly and remaining inactive, **torpid**, until better conditions return. Some even secrete a **lipid**, fatty substance, and coat their body to prevent water loss during this period of inactivity.

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.

There are three orders of living amphibians (Anura, Urodela, Apoda) containing about 170 genera. The majority of the amphibians are found in tropical regions with abundant rainfall. Correlated with the moisture requirement of amphibians is the fact that most forms are nocturnal.

In India, about 100 species of anurans, over a dozen species of apodans and one species of Urodela are known to exist. However, studies on the reproductive cycles of Indian amphibians are surprisingly limited to half a dozen species (Saidapur, 1989).

The main characteristics of Amphibians are:- they may be, aquatic, terrestrial or both; they are cold-blooded (poikilothermous) animals. Their body temperature is variable and most of them undergo *hibernation* during winter. Some undergo *aestivation* during summer; the shape of body is variable, it may be depressed or cylindrical and divisible into head, neck, trunk and tail or into head and trunk. The skin is thin, moist, glandular and devoid of exoskeleton. There is a pair of nostrils, which open into the buccal

cavity. They are provided with valves to exclude water. The teeth are alike (*homodont*), *acrodont* and polyphyodont ; Endoskeleton is largely bony or ossified . Skull is *dicondylic* i.e. possesses two occipital condyles for the attachment to the vertebral column. Eyes are provided with movable lids and tear glands to clean and protect them in air. The brain has smooth cerebral hemispheres and the cerebellum is poorly developed. Fertilization is generally external, sometimes internal. Eggs are mesolecithal and are covered with gelatinous covering. They are laid in water.

The anurans are tail less amphibians with elongated hind limbs; the foot is lengthened by the elongated proximal tarsal elements, the tibiale and fibulare, which are fused at least proximally and distally. The vertebral column consists of 5-9 presacral vertebrae. All vertebrae bears transverse processes, except the first (atlas) unless it is fused with Presacral II. Ribs are freely associated with, or fused to, the second, third, and fourth (also fifth and sixth in some) presacral vertebrae in some primitive families. The postsacral vertebrae are fused into a rod like coccyx. The otic-optical region is composed of prootics and exoccipitals (fused or unfused). (Duellman and Trueb,1986).

FAMILY BUFONIDAE- Bufonids are the true toads, although some are not particularly toad like. There are 5-8 holochordal, procoelous presacral vertebrae with imbricate neural arches. Variation in the number of presacral vertebrae is the result of fusion of Presacral I and II. The atlantal cotyles of presacral I are juxtaposed. Ribs are absent. The sacrum has dilated diapophyses and a bicondylar articulation with the coccyx, except in those taxa in which there has been a forward shift of the sacral articulation; in these cases the original sacral vertebra is incorporated into the coccyx, and there is a monocondylar articulation or fusion of the coccyx and the functional sacral vertebra.

Toads and their allies generally have thick, glandular skin with or without postural warts; *Bufo* and some other genera have parotoid glands, and some species has large glands on the limbs. Most species are terrestrial or fossorial and have short limbs; the digits are reduced and shortened with a thick interdigital pad in *Osornophryne*. Bufonids vary in size from the minute *Oreophrynella* (20mm) to the gigantic toad *Bufo blombergi* (250 mm). Most Bufonids hav extensively ossified skull and in most of them the skin is co-ossified with the skull.

The large genus *Bufo* has about 200 species and is naturally cosmopolitan except for Australia. The other genera are distributed in three tropical areas: South America, Africa, and Southeast Asia. Some of these other genera are clearly derived from *Bufo*, but others belong to a major evolutionary lineage that is distinct from *Bufo* and its allies.

Bufonids range in size from 20 to more than 200 mm. No bufonids have teeth, although the absence of teeth occurs sporadically in other frog groups. Also, many bufonids (but not all) have a Bidder's organ, which is a mass of gonadal tissue in males that has the appearance of an immature testis. If the testis of a male is surgically removed, the Bidder's organ will enlarge and differentiate into a functional ovary.

Most toads of the genus *Bufo* are dull. However, *Bufo periglenes* is brightly colored and exhibits extreme color dimorphism between males and females. This rare toad lives in the cool wind-swept cloud forests of Costa Rica near Monteverde. It has not been observed in several years and may be extinct.

Species of the genus *Atelopus*, also called Harlequin Frogs, are brightly colored. *Atelopus zeteki* from Panama have skin toxins (Brown et al. 1977). *Bufo spinulosus* is among the highest ranging amphibians; it is known from about 5000 m elevation in the Andes of South America.

Ford and Cannatella (1993) defined Bufonidae as the node-based name for the most recent common ancestor of living bufonids (*Bufo frostius*, etc., as listed in Frost [1985]), and all its descendants. Putative synapomorphies of Bufonidae are the presence of Bidder's organ (Duellman and Trueb, 1986); a unique pattern of insertion of the hyoglossus muscle; absence of the posterior constrictor muscle (Trewavas, 1933); the absence of teeth; origin of depressor mandibulae muscle solely from the squamosal, and associated angle of orientation of the squamosal (Griffiths, 1954; Starrett, 1968); and the presence of the "otic element," an independent ossification in the temporal region that fuses to the otic ramus of the squamosal (Griffiths, 1954).

The distribution of Bidder's organ was summarized by Roessler et al. (1990). No bufonids are known to have teeth, but teeth are absent in unrelated taxa, including some basal telmatobiine leptodactylids with no clear relationships to other taxa. Barring the close relationship of any of these taxa to Bufonidae, the absence of teeth is tentatively considered a synapomorphy of Bufonidae. The conformation of the hyoglossus muscle and absence of the constrictor posterior muscle were listed by Trewavas (1933) as possible diagnostic features of Bufonidae. These characters are virtually unique in bufonids among frogs, and Cannatella has confirmed the presence of these in several other bufonid genera, but greater taxonomic coverage is needed. Griffiths (1963) stated that the "otic element" is diagnostic of bufonids, but his observations on its development were limited to seven species of *Bufo* and two species of *Atelopus*.

AMPHIBIAN TESTIS- While the utilitarian value of teleosts as food and game has promoted much interest in their reproduction, amphibians have received less consistent attention. On the other hand, their easy maintenance in the laboratory and the relatively large or small size of their germ cells have made amphibians attractive objects from the very beginning of the cytological and genetic investigations, and recently they have proved to be excellent

experimental animals for research in the endocrinology and reproduction. A number of important contributions to basic biology have been achieved in the course of investigations of spermatogenesis in amphibians (Roosen-Runge, 1977).

The morphology of the amphibian testis is similar to that of the teleosts. Spherical or tubular compartments are radially arranged and open separately into a more or less peripheral duct system which runs longitudinally towards the efferent at the caudal end of the gonad. The compartments are usually called "ampullae" in urodeles and "seminiferous tubules" or rarely "lobules" in anurans. Within them the germ cells develop in cysts (Humphrey, 1921; Lofts & Boswell, 1960; Lofts, 1964).

SECONDARY SEXUAL CHARACTERS- The amphibians possess a great diversity of secondary sexual characters that are closely associated with reproductive function. The tegumentary callosities are most commonly encountered secondary sexual structures in the anurans. These are found on the fingers and/or the ventral regions of the forearms. The epidermis of this pad is papillate during the breeding period and enables the males to firmly hold on to the slippery surface of the female during amplexus.

During the breeding season the epidermis is highly papillate and keratinized. The thumb pads are regressed in other months. The development of thumb pads in Anura is controlled by the testicular androgens.

Vocalization plays an important role in the reproductive biology of anurans. The males of most anurans possess well developed and variety of species-specific vocal sacs that enable them to produce mating calls. The calls are species-specific and often act as a primary isolating mechanism in speciation since females are only attracted to calls of males of their own species.

There is little or no color dimorphism in anurans, but special nuptial coloration is common in many urodeles. In all species forelimbs and

shoulders of the males are more muscular. In Indian apodans no visible secondary sexual characters are reported.

AMPHIBIAN SPERMATOGENESIS WITH SPECIAL REFERENCE TO ANURANS- In amphibians, as in all vertebrates, spermatogenesis occurs in cycles. In the majority of tropical and in a few non-tropical species the cycles are continuous and non-seasonal, but species with seasonal cycles of the temperate zones, particularly anurans, have been more thoroughly investigated. In most of these species the spermatogenic cycle is of the "postnuptial" type, i.e. spermatogenesis begins immediately after spawning and is completed in a few months so that the tubules contains spermatozoa in an advanced state of maturity for long periods before spawning. The pattern of cyclical activities, and their variations, and distributions have been comprehensively reviewed by Van Oordt (1960).

The amphibian testes are usually simple ovoid or elongate structures, but in some urodele species, e.g. in *Salamandra maculosa* (Meves, 1896), they are distinctly lobed, i.e. there is a tandem arrangements of three to five successive enlargements which appears as though multiple testes were lined up along the gonadal axis. The mode of testis formation is due to a special modification of the process of spermatogenesis (Humphrey, 1922). In all urodeles there is a so-called "spermatogenic wave" along the length of the testis which results in the most progressive developmental stages being located in the caudal region.

The anuran testes are paired, compact and ovoid structures situated in close proximity to the kidneys. They are attached to the dorsal body wall by a short mesorchium through which run the vasa efferentia containing the seminiferous tubules and the modified nephric elements running to the Wolffian ducts. In anurans the germ cells are derived from the endoderm. The two principal elements of the testes are the seminiferous tubules and the intestinal tissue consisting of blood capillaries and closely packed small ovoid steroid secreting Leydig cells (Saidapur, 1989).

In most animals it is difficult to observe the release of spermatozoa from the somatic cells, but in Amphibia the cyst cells and the germ cells are so large that they can be resolved easily by light microscope. In frogs, the cyst wall ruptures as soon as the flagella of the spermatids begin to grow, and opens into the lumen of the tubule. At the peripheral pole of the cyst the cyst cells aggregate and then transform into large, pillar like cells called sertoli cell. There are on the average 12 of these in every cyst and to each attaches a bundle of 60-150 spermatids. Each bundle, with the heads of the spermatids pointing towards the periphery of the cyst, is deeply embedded in a cup like depression of the supporting cell.

AMPHIBIAN SPERM WITH SPECIAL REFERENCE TO ANURANS-

The basic morphology of an amphibian spermatozoon contains the following structures in a linear, antero-posterior sequence.

Acrosome: The tip of the head piece is formed by transformation of linear parts of Golgi bodies during spermatogenesis. This is properly referred to as an acrosomal cap.

Head: The head and acrosome may not be entirely distinct externally in amphibians. The head contains closely packed chromosomes covered by a thin layer of cytoplasm.

Neck or Middle Piece: Formed by cytoplasmic material, the neck contains one (in salamanders) or two (in anurans) centrioles anteriorly next to the nuclear material of the head and at the proximal end of the axial filament. Mitochondria spiral around the axial filament.

Tail: The tail is long, usually variable and contains of an axial filament covered by a very thin layer of cytoplasm, which does not reach the tip of the tail. The axial filament or rod consists of several longitudinal fibers with the usual vertebrate microtubule arrangement (9 pairs surrounding a single median pair). The cytoplasm is expanded into a fanlike structure, the flagellum, but the distal part of the axial rod, the end piece, is always naked. (Broman, 1900; Furieri, 1975).

Striking differences occur in the tail structure among anurans (Fouquette and Delahoussaye, 1977). 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 (Duellman and Trueb, 1986). Careful study of the structure of spermatozoa reveals interspecific 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 (Duellman and Trueb, 1986).

MOLECULAR APPROACH TO PHYLOGENY:-

The phylogenetic relationships among families of Anura are still largely unresolved (Duellman & Trueb, 1986; Ford & Cannatella, 1993). Groups widely accepted as monophyletic have often been challenged with new phylogenetic reconstructions and the continuous accumulation of new information. 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 (Ford, 1993; Ford & Cannatella, 1993). 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 (Ford & Cannatella, 1993).

Analyses using alternative data set, such as molecular markers (Hillis et al., 1993; Hay et al. 1995), 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 (Ford, 1993).

The ultrastructure of spermatozoon has been used as an alternative data set to investigate the phylogeny of many taxa such as fishes (Jamieson, 1991; Tanaka et al., 1995), amphibians (Lee & Jamieson, 1992, 1993; Jamieson et al., 1993; Scheltinga et al., 2001), reptiles (Jamieson, 1995; Teixeira et al., 1999a,b), and invertebrates (Jamieson, 1987). 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 (Teixeira et al., 1999b). Spermatozoon ultrastructure data have also been useful in clarifying relationships among *Polyplacophora*, where traditionally used characters are either too conserved or too variable (Buckland-Nicks, 1995). 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 *Bufo* (Lee & Jamieson, 1993), 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 squamate reptiles (Jamieson, 1995; Teixeira et al., 1999b) and fishes (Tanaka et al., 1995), has not been conducted for anurans.

The family *Bufo*, 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 (Frost, 1985; Duellman and Trueb, 1986; Duellman, 1993). The number of species in *Bufo* 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 (Graybeal, 1993). 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 (Maxson, 1984; Lynch and Renjifo, 1991; Graybeal, 1993).

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. Inger (1972) recognized five species groups in East Asia. Hu *et al.* (1984) reviewed Chinese *Bufo* and formed two additional species groups: the *cryptotympanicus* and the *galeatus* groups. Recently, Yang *et al.* (1996) described a new genus, *Torrentophryne*, based on the morphology of both adults and in particular the tadpoles. Despite these contributions, the phylogenetic relationships among East Asian bufonids are far from resolved. 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 (Bogart, 1972; Moreschalchi, 1973; Schmid, 1978; Yang, 1983; Kuramoto, 1990; Liu and Yang, 1997). 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 (Pisannitz, 1978). 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 (Macey *et al.*, 1998).

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?

PLATE – 1:

Experimental animals.

(A)- *Bufo himalayanus* – largest in size of the three species studied, with distinct black and red warts all over the body.

(B)- *Bufo stomaticus* – smallest in size of the three species studied, with yellowish coloration and relatively blunt snout.

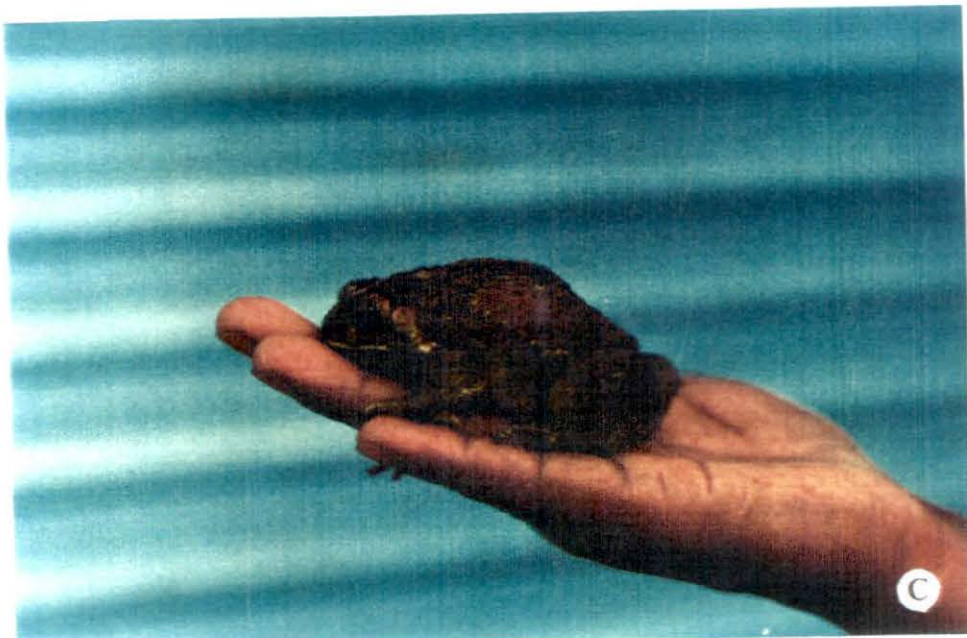
(C)- *Bufo melanostictus* – medium size black body with reddish tinch, distinct black warts and well formed parotid gland.



A



B



C