PHYLOGENY OF BUFONIDAE
ON THE BASIS OF
SPERM ULTRASTRUCTURE AND DNA ANALYSIS STUDIES

CHANDRANATH CHATTERJEE, M.Sc.
CELL AND MOLECULAR BIOLOGY LABORATORY
P.G. DEPARTMENT OF ZOOLOGY
DARJEELING GOVERNMENT COLLEGE
DARJEELING - 734101
WEST BENGAL
INDIA

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY (SCIENCE)
AT
UNIVERSITY OF NORTH BENGAL
2003

Prof. Dipak Ray
Principal
Taki Govt. College
Taki - 743429

Tel. No. 913-47226 (O)
033-6833960 (R)
033-6628511 (Fax)
033-2662 1153 (Fax)

Ref. No. .....
Date .....

CERTIFICATE FROM RESEARCH GUIDE

This is to certify that Mr. Chandranath Chatterjee, M.Sc., carried the investigations incorporated in the Thesis entitled "Phylogeny of Bufonidae on the basis of Sperm Ultrastructure and DNA Analysis Studies" under my supervision and guidance. This thesis, which embodies the results of original investigations made by Mr. Chatterjee, has been carried out during the period 1999-2003. He has fulfilled all the requirements and regulations relating the nature and prescribed period of research.

This thesis has not been submitted previously any where for any degree what so ever either by him or anyone else.

It may please be noted that he shares equal authorship for all the published works appended to this thesis.

3/12/2003

PROF. DIPAK RAY
Principal
Taki Govt. College
Taki - 743429
West Bengal, India
and
Former Professor of Zoology and Principal
Darjeeling Government College
University of North Bengal

DR. DIPAK RAY, Ph.D., FZA.
West Bengal Senior Education Service
Professor of Zoology & Principal
Taki Govt. College, Taki, 24 Pgs. (N)

ACKNOWLEDGEMENT

I am indebted to my mother whose inspiration along with physical and mental support provided me the energy to cross the long twenty five years of my academic career.

I wish to express my deep sense of gratitude to all my family members for their encouragement and inspiration without which this dissertation would have not taken the present shape.

I express my heartfelt gratitude and indebtedness to my research guide Prof. Dipak Ray, Principal , Taki Government College (Former Head & Professor of Zoology, Post Graduate Department of Zoology, Darjeeling Government College, Darjeeling-734101, West Bengal, India) for suggesting the problem, constant guidance and valuable advice during the entire period of work.

I am ever grateful to Dr. T. C. Nag, Assistant Professor, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, for his valuable suggestions and encouragement towards the fulfillment of this work.

I behold to Dr. S.K. Das for his friendly attitude in teaching me the molecular techniques and his constant guidance in the laboratory.

I am grateful to Dr. D.K. Sarkar, former Principal, Darjeeling Government College, for permitting me to use all the laboratory and library facilities of the college and true encouragement towards this work.

I wish to express my sincere thanks to all the staffs of Department of Electron Microscopy, All India Institute of Medical Sciences, New Delhi, for providing me Transmission Electron Microscopy facilities and technical advice for tissue processing and sample preparation.

I am also grateful to Dr. Srikanta Chakraborty of Central Instrumentation Center, University of Burdwan, West Bengal, for providing me the Scanning Electron Microscope facilities and technical advice.

I am thankful to Dr. S. Bandopadhyay, Indian Institute of Chemical Biology, Kolkata, for providing me the laboratory facilities for FACS studies along with his valuable technical advice.

I like to pay my gratitude to the Libarians of National Medical Library, New Delhi; Bose Institute, Kolkata; Indian Institute of Chemical Biology, Kolkata; Institute for Research in Reproduction, Mumbai and All India Institute of Medical Sciences, New Delhi.

My heartfelt thanks to my dadu (Sri. Tarapada Roy), dida (Smt. Malati Roy), baba (Sri Dharmadev

Chatterjee) and my dada (Dr. Biswanath Chatterjee) for their active cooperation and mental support.

My special regards to Miss Kakali Sarkar, my closest friend, for her encouragement, which has insisted me to join the research project, and her constant moral support and guidance towards the completion of the work.

I also wish to thanks Sri Sudipta Patra, Sri Saurabh Chakraborty, Dr. Diptiman Chandra, Sri Debasish Ghosh, Sri Rupak Chatterjee, Sri Sanjit Chatterjee, Sri Sudarsan Layak, Sri Ananda Mandal, Sri Gautam Chakraborty, Sri Probodh Mondal, Prof. J.K. Chattaraj, Dr. T.B. Mandal, Prof. S. Basak and Dr. Richard R.X. Bajpai, for their active support and cooperation, without which it would have been impossible for me to complete the project.

Chandranath Chatterjee
03/12/03

CONTENTS

PLATES

FIGURES

TABLES

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 necessiated 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 . Skill 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 enlargememts 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 salamenders) 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 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 (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 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 (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 Bufonidae (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 squamata reptiles (Jamieson, 1995; Teixeira et al., 1999b) and fishes (Tanaka et al., 1995), 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 (Frost, 1985; Duellman and Trueb, 1986; Duellman, 1993). 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 (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:

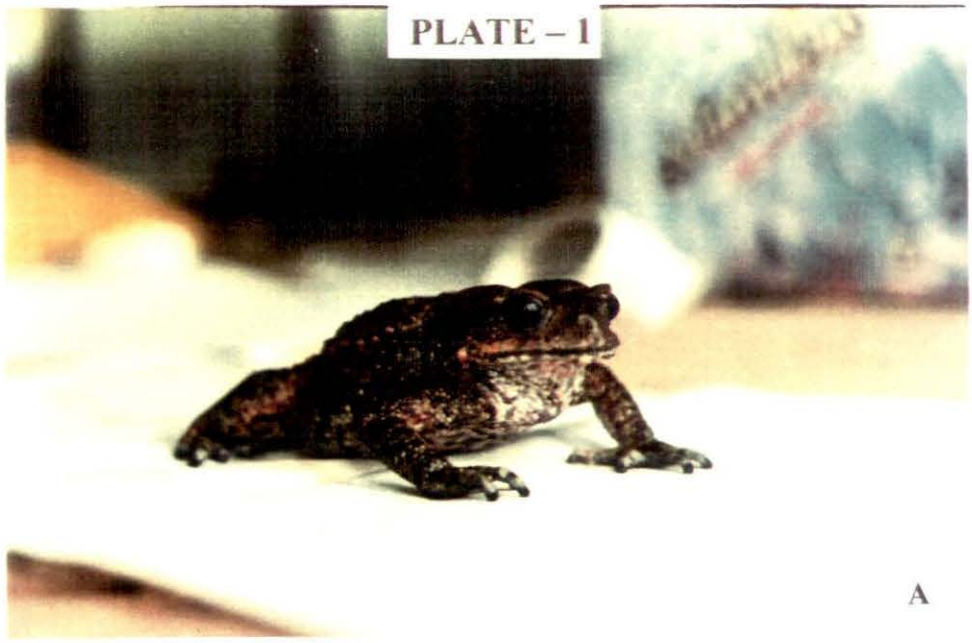
Experimental animals.

(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.

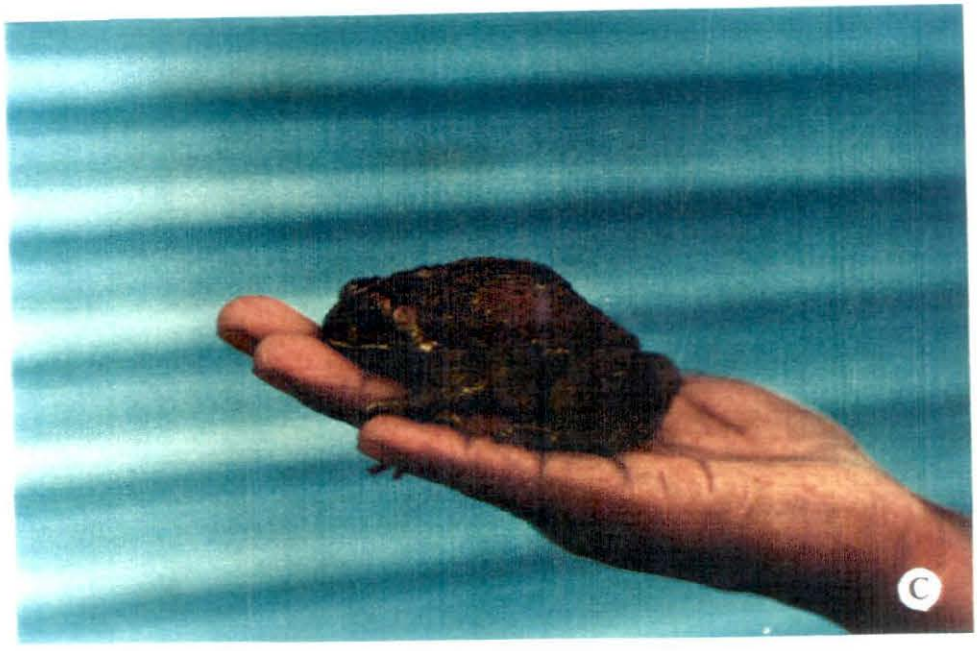
(C)- *Bufo melanostictus* – medium size black body with reddish tinch, distinct black warts and well formed parotid gland.

PLATE – 1



A

B



C

MATERIALS AND METHOD

MATERIALS

Three species of toads under the family Bufonidae were selected as experimental study materials

1. *Bufo himalayanus* Gunther, 1864. (Himalayan Toad)

2. *Bufo stomaticus* Lütken, 1862. (Indus Valley Toad)

3. *Bufo melanostictus* Schneider, 1799. (Southeast Asian Toad)

The details about the individual species are as follows

1. Bufo himalayanus Gunther, 1864.

Description

Head deeply concave with only supraorbital ridges; interorbital space broader than the upper eyelid; tympanum very small or indistinct; first finger does not extend beyond second; toes with single subarticular tubercle, no tarsal fold; parotid is as long as the head; body with irregular porous tubercles.

Snout-vent length 130-132 mm.

Color: Uniform brown. Cranial crest and tips of digits dark brown.

Distribution and Habitat

Country distribution: China, India, Nepal, Pakistan. (Figure:2A)

This toad has been recorded from Himalayas at 2000-3500 m of elevation, from Nepal, Bhutan (Khan, 1968 b). In Pakistan it has been

recorded from Azad Kashmir, Hazara Division, and Northwestern Frontier Province.

Life History, Abundance, Activity, and Special Behaviors

Bufo himalayanus is a mountain species; primarily nocturnal; however, it is often seen moving about in broad day light among rocks and vegetation feeding on grasshoppers, moths, ants, and other invertebrate animals. It rests during the day under stones or in fissures and holes in the ground.

Breeding activity starts after a downpour, during May-July; males croak in low tone with "curr, curr" repeated several times. Eggs are laid in a double string of jelly in shallow pools along torrents.

Tadpole: Head flat, body darker, belly bulging, tail weak, low fins; naris slightly nearer to eye than snout; eyes small and sunken; oral disc anteroventral, labial tooth row formula typically bufonid: 2(2)/3, beak serrated, oral papillae lateral; color uniformly black, ventrum lighter. The tadpoles are found, at a high elevation in the Himalayan range, in small, calm pools along torrents, with algal vegetation.

Total length of tadpole 28-30; tail 19-20 mm.

The toad hibernates during the winter under stones and in fissures in the ground from September to March. The karyotype number recorded for this species is 22 (Chatterjii and Barik, 1970).

Trends and Threats

Inhabits side pools of torrents. Mostly terrestrial.

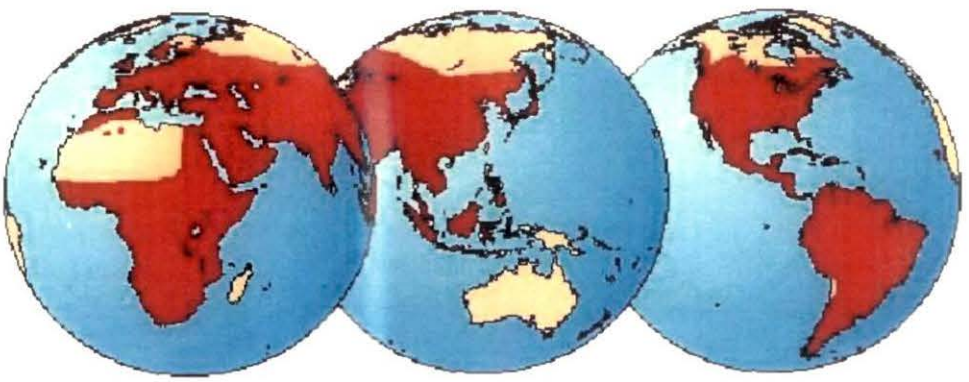
Relation to Humans

No record.

Comments

A very rare highland species.

Possible reasons for amphibian decline



Red indicates the distribution of living members of the family Bufonidae.

Figure:-1 Distribution of the members of the family Bufonidae (the red indicated portion of the globe)

Prolonged drought

Local pesticides, fertilizers, and pollutants

2. *Bufo stomaticus* Lütken, 1862. (Indus Valley toad)

Description

This toad does not have cranial crests; interorbital space a little broader than the upper eyelid; tympanum distinct, round, its diameter two third that of eye; first and second fingers subequal; toes with single subarticular tubercles; a tarsal spinulated ridge; the parotid gland is longer than broad; a distinct tibial gland is present.

Color: Dorsum light gray or olive to almost black, with dark mottling or gray to dark reticulation; upper lip cream. Ventrum dirty white, dark mottling on throat, 3 dark transverse bands on anterior aspect of forearm. Tips of digits are dark brown.

Distribution and Habitat

Country distribution: Oman, Sri Lanka, Nepal, India, Pakistan, Iran, Islamic Republic of, Afghanistan. (Figure: 2C)

In Pakistan: Widely distributed throughout the Indo-Pakistan subcontinent. It is collected from upper and lower Indus Valleys, Balochistan, from plains to an elevation of 1800 m in the northern and western hilly tracts of Pakistan. Outside Pakistan: from Bangladesh through the Ganges Plain, peninsular India, Afghanistan, Iran, and Muscat.

Life History, Abundance, Activity, and Special Behaviors

Tadpole: Schools of dark tadpoles of this species are a common sight in ponds and puddles in the plains of Punjab and Sindh during monsoons. In the hills the tadpoles are confined to the side pools of

torrents. Daniel (1963a) recorded this toad as breeding in pooled water about 90 m away from the sea in Mumbai, India.

The tadpole is typically bufonid, with an oval bulging body, weak tail, high dorsal, and narrower ventral fin. The oral disc is anteroventral with a 2(2)/3 labial tooth row formula; the beak is finely serrated with lateral oral papillae. The body and fins of the tadpole are speckled with light brown (Khan 1968a; Khan and Mufti, 1994a,b). Total length of tadpole is 30-31 mm and the tail is 20 mm.

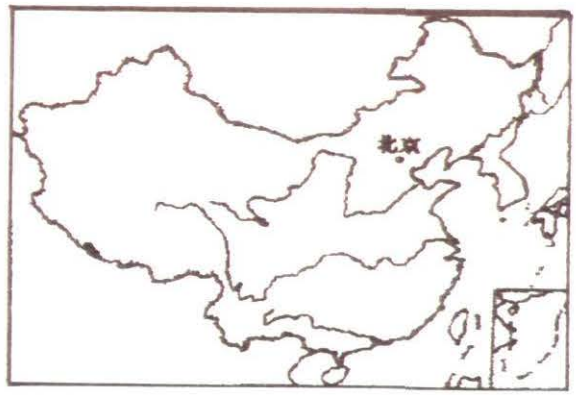
The gregarious habit of this tadpole continues up to the 36th Stage of development (Khan 1965), later the tadpole becomes solitary and when disturbed, takes to the deep water (Khan 1991a; Khan and Mufti 1994b).

Trends and Threats

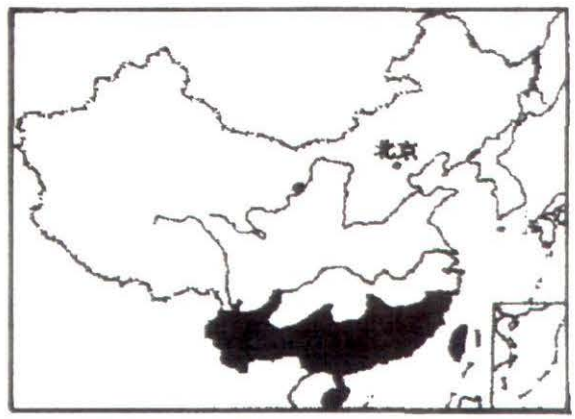
Run off from chemicals used as fertilizers and pesticides against crop pests, is causing death and deformities in tadpoles. Mortality due to increasing traffic is high. The spray of insecticides on crops is causing havoc to the population of this toad, by being directly getting the spray, and by eating spray-killed insects and their larvae. Increasing atmospheric temperatures and decreasing rainfall, has affected population of this toad enormously. The ponds and puddles where it breeds during early breeding period (March to May) are mostly dried out killing eggs and tadpoles. Mostly this toad lives in crevices and holes in fields. The use of heavy tractors with long shears, earth out the toads most of them are killed by trampling over.

Relation to Humans

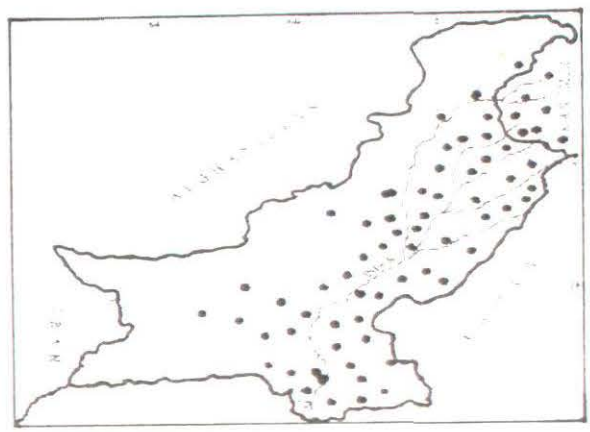
The toad extends in inhabited houses, hiding under household articles, feeding on photophilic insects etc. When handled by children or adults carelessly, cause irritation and boils on skin. Dogs playing with toads are often killed or sickened. The toad is a voracious feeder, devouring different types of insects and larvae, which are mostly crop pests (Khan, 1990).



A. Distribution map of *Bufo himalayanus*



B. Distribution map of *Bufo melanostictus*



C. Distribution map of *Bufo stomaticus*

Figure:- 2

Possible reasons for amphibian decline

General habitat alteration and loss
Habitat modification from deforestation, or logging related activities
Intensified agriculture or grazing
Urbanization
Disturbance or death from vehicular traffic
Prolonged drought
Habitat fragmentation
Local pesticides, fertilizers, and pollutants
Long-distance pesticides, toxins, and pollutants
Climate change, increased ultraviolet beam or increased sensitivity to it, etc.

3. Bufo melanostictus Schneider, 1799. (Southeast Asian Toad)

Description

Head with distinct rostral, preorbital, supraorbital, postorbital and a short orbito-tympanic, cranial crests; no temporal ridge; interorbital space much broader than upper eyelid; tympanum very distinct, at least two third the diameter of the eye; first finger generally but not always extends beyond second; double subarticular tubercles only under third finger. Toes with single subarticular tubercle; parotid elipticle, with dark brown scattered branching concretions; skin heavily tuberculated on flanks, tubercles usually tipped with dark brown spines; a lateral dorsal staggered row of 8-9 enlarged tubercles; cranial crests, lips, digit tips, metacarpal and metatarsal tubercles are cornified with dark brown, which tend to peal off in preserved specimens; head is almost smooth.

Recently Khan (2001) has distinguished Pakistani population of this toad as a new subspecies *Bufo melanostictus* hazarensis, on the basis of kidney shaped parotid glands; double subarticular tubercles under penultimate phalanx of all fingers; rostral ridge absent from head; temporal ridge present; light brown dorsum.

It is the largest toad in Pakistan, female exceeds 150 mm in snout-vent length (Khan,1979).

Color: Dorsum uniform gray of various shades, brown or reddish with dark spots, ventrum uniform dirty white, speckled with light brown on chin and throat.

The throat of breeding male is light orange or yellow. It develops cornified pads on inner side of first and second fingers.

Distribution and Habitat

Country distribution: Bangladesh, China, Hong Kong, India, Indonesia, Laos People's Democratic Republic, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka, Taiwan, Province of China, Thailand, Vietnam. (Figure:2)

Until recently *Bufo melanostictus* was reported to as the common toad of Indo-Pakistan subcontinent (Günther, 1864; Murray, 1884; Boulenger, 1890; Annandale and Rao, 1918). However, in Pakistan, this toad is confined to District Hazara, Northwestern Frontier Province, Alpine Punjab and Azad Kashmir (Mertens, 1969a; Khan, 1972a).

Life History, Abundance, Activity, and Special Behaviors

A rare toad in Pakistan mostly confined to the low northern hilly ranges and Azad Kashmir. Nocturnal, appears soon after sunset; during day hides under stones, logs, piles of vegetation, holes and crevices among

stones and in ground. Once a suitable place is selected, it is permanently shared with several toads.

The toad is lethargic timid animal. It moves about with deliberate hops from place to place in search of insects on which it feeds. In tropical southeast Asia it is most common amphibian, comes out after sunset in large numbers and frequents mostly the human habitations, where it congregate under street lamps to feed on photophilic insects (Church, 1960).

In temperate environs of western Himalayas, the breeding is initiated by monsoon rains, from July to August. Males, gather in shallow side-pools along torrents and ponds. The call in low melodious "curr, curr, curr" repeated several times ending in a whistling note. The calling males become quite aggressive, tugging and jumping over each other, males for exceed female in numbers. It breeds in every available space containing some water from first showers of monsoon rains in the southern India (McCann, 1938). Males are much smaller than females. However, in tropical southeast Asia, the toad is known to breed throughout the year (Church, 1960).

Calling males occasionally jump over each other and try to secure a nuptial hold on each other, however, kicks and zestful wriggling dislodge them from each other and soon they resume calling. The females lurch round, as soon one comes close, a male jumps over it and quickly tightens it nuptial clasp, the other suitors are shaken off as the nuptial pair moves to a quitter place away from the site.

The eggs are laid in a double jelly string, generally in deep quieter water, where the egg-string is entangled in the vegetation or female moves round the submerged vegetation to wound the egg string round it. An egg is enclosed in a double gelatinous capsule (Khan, 1982b).

The tadpoles are uniform dark, inhabits side pools along hilly torrents, schools of them swarm along the marginal waters of ponds and puddles feeding on any type of algal material. The body is typically bufonid, globular with weak tail, dorsal fin is broad while ventral is narrow. The oral disc is typically bufonid, with 2(2)/3 labial tooth row formula, the oral papillae are lateral. The snout is finely serrated and sharp (Khan, 1991a, 1997).

Total length of the tadpole 26-27 mm, tail 19-20 mm.

The swarms of recently metamorphosed toadlets from synchronised pairings leave water, many fall pray to several kind of predators, while several are crushed under feet and passing traffic. Karyotype number recorded for this species is 22 (Nataranjan, 1953).

Trends and Threats

Species of rugged mountains. Breeds in paddy fields, where pollution by chemicals affect its eggs and larvae.

Relation to Humans

Exterminates crop pests and other insects.

Possible reasons for amphibian decline

General habitat alteration and loss
Habitat modification from deforestation, or logging related activities
Prolonged drought
Drainage of habitat
Local pesticides, fertilizers, and pollutants
Long-distance pesticides, toxins, and pollutants

The live specimens were collected from their natural habitats and some specimens were reared in the laboratory maintaining the optimal ambient condition for further study.

The respective specimens were narcotized and their testes were dissected out in amphibian saline. Before going to further processing a metric analysis of the testes were made every time with every type of specimens to get a comparative idea of the shape and size of the testes of different species at different period of the breeding season.

METHODS

A. Study of morphological variation in testes shape and size

Every individual specimens from 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 nonbreeding season.

B. Collection of testicular samples and preparation for Simple Microscopy

The testis were dissected out from individuals of respective species in amphibian saline . Then the testis were bisected with a sharp blade so

the edge of the tissue do not get distorted. 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.

C. Preparation for Scanning Electron Microscopy

After dissecting out the testis, they 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 (Fujita et al., 1970). The materials were dried at critical point (Tanaka et al., 1981) and coated with colloidal gold (Echlin et al., 1975) 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 at Central Instrumentation Center of The University of Burdwan, West Bengal, India, where the subsequent process was carried out.

D. Preparation for Transmission Electron Microscopy

The testes were surgically removed and dissected out in amphibian saline (pH-7.1). They were reduced into fragments (1-3mm) and fixed overnight in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer at 4°C (Karnovsky, 1965). 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 (Reynolds, 1963). The grids were observed under a Philips CM-10 Transmission Electron Microscope at Sophisticated Analytical Instrumentation Facility, All India Institute of Medical Science (AIIMS), New Delhi, India.

E. Isolation and Purification of the DNA from sperm cells

Materials

A. LYSIS BUFFER I – Sucrose : 5.1gm
Tris HCl(pH-7.5) : 0.5 ml from 1M stock
$MgCl_2$: 0.05gm
Triton X100 : 0.5ml
Final volume is made 50 ml by adding distilled water

B. LYSIS BUFFER II – NaCl : 0.219gm
Na EDTA : 0.446gm
Final volume is made 50 ml by adding distilled water. (The chemicals were first dissolved in 40ml of water, pH is adjusted at

8 by adding 4M NaOH, then the volume is made 50 ml)

C. 10% SDS

D. 5M Na-PARAFORMALDEHYDE

E. 6M NaCl

F. ISOPROPANOL

G. 70% ETHANOL, 95% ETHANOL

H. PERCOLL

I. APBS (Amphibian Phosphate Buffer Saline)

J. PROTEINASE K (10 %)

Method

- 200 µl of isolated sperm cells were layered over 1ml 70% Percoll solution in APBS.
- Resultant is centrifuged at 5000 rpm for 10 minutes
- Supernatant is discarded and the precipitate is collected
- Lysis buffer I is added to the precipitate (1ml in each tube)
- Kept in 4°C overnight
- Centrifuged at 3000 rpm for 10 minutes
- Supernatant is discarded, precipitate is containing the nucleus
- Nucleus + Lysis buffer II (225µl) +6.25µl SDS + 2.5µl Proteinase K (10%)
- Heated at 50°C for 1 hour or incubated at 37°C for overnight
- 6M NaCl (100µ) is added to the mixture
- Centrifuged at 8000 rpm for 10 minutes (the protein will precipitate down)
- Soup is collected
- Volume is doubled by adding 95% ethanol
- DNA will precipitate down as visible white network
- Isolated DNA were transferred to TAE buffer and stored at -20°C for further use

- 1 ml of cold (-20°C) 100% ethanol is added and mix well by repeated inversion of the tube (30 sec).
- The DNA is pelleted by centrifugation at 14000 rpm for 10 minutes (4°C).
- The supernatant fluid is carefully discarded by pipeting with out disturbing the DNA pellet.
- 500 µl of 70% ethanol (RT) is added to wash residual salts out the DNA pellet. Mixed by flicking tube gently and inverting a few times.
- Centrifuged at 14000 rpm for 3 min.
- Repeated 70% ethanol wash at RT.
- After centrifugation, ethanol is removed as much as possible using a pipette and taking care not to disturb the DNA pellet.
- Re-spinned the tube in the microcentrifuge for 10 sec and a P200 pipettor is used to eliminate the last remnants of ethanol.
- Allowed the pellet to air-dry in a 37°C bath for 5 min.
- The DNA pellet is resuspend in 48 µl of TE buffer (pH 8.0) and 2 µl of a 2 mg/ml RNase solution (80 µg/ml final concentration). The solution is pipeted in and out 6 times while gently teasing the pellet with a wide bore pipette tip to aid in dissolving the pellet.
- Incubated at 37°C for 30 min. DNA is now ready for further experiments and stored at 4°C.

F. Quantitation of Nucleic Acid Concentration by Optical Density readings

The absorbance of UV light at 260 nm wavelength by nucleic acids gives an estimate of concentration, assuming firstly that there are no protein or phenol contaminants in the solution and secondly, that the concentration of the nucleic acid is greater than 250 µg / ml. The ratio of readings taken at

260 nm and 280 nm wavelengths (both in the UV range) gives an indication of the purity of the nucleic acid.

An OD unit corresponds to the amount of nucleic acid in µg in a 1 ml volume using a 1 cm path length quartz cuvette that results in an OD260 reading of 1.

For DNA OD 260 1 = 50 mm / ml,

For RNA OD 260 1 = 40 mg / ml,

The ratio of readings taken at 260 nm and 280 nm wavelengths indicates of the purity of the nucleic acid :-

For pure DNA OD 260 : OD 280 = 1.8

Ratios less than these values indicate contamination of the solutions with protein, phenol or guanidinium and the estimates of concentration will be inaccurate. Some impurities which interfere with UV OD readings can be removed by extraction of the preparation with n-butanol.

Method

To measure the absorbance of a solution in the short-wave length range (<300 nm) the quartz cuvettes are used. Disposable plastic cuvettes are available for reading in the visible range.

Spectrophotometer is turned on - the switch is on the right in the back.

The instrument is allowed to calibrate. The chamber is not to be opened during this time. The deuterium lamp is OFF by default. To read absorbance in the UV range, the deuterium lamp is turned on after the machine has completed its calibration. For best accuracy, the deuterium lamp should be warmed up for 20 minutes.

The function key is pressed until Fn0 is displayed. Pressed enter. Using the up or down arrow keys, the desired wavelengths are entered (260nm and 280 nm).

A reference cuvette is prepared containing the same dilution as my experimental sample.

The reference cuvette is placed in cell #1 and the experimental samples were placed in cells #2-6.

The reference key is set to blank against the appropriate buffer. The cell key is pressed to advance to read the next sample.

The process is continued.

For pure solutions of DNA, the simplest method of quantitation is reading the absorbance at 260 nm where an OD of 1 in a 1 cm path length = 50 µg/ml for double-stranded DNA, 40 µg/ml for single-stranded DNA and RNA and 20-33 ug/ml for oligonucleotides. An absorbance ratio of 260 nm and 280 nm gives an estimate of the purity of the solution. Pure DNA and RNA solutions have OD260/OD280 values of 1.8 and 2.0, respectively (Davis et.al., 1986).

The DNA sample is diluted in TE buffer (10 µl in 1.0 ml) and the concentration of DNA is determined by using the following formula :-

$$\text{DNA Content} = (50\ \mu g / ml) \times 1.0\ A_{260}$$

G. DNA separation by Gel Electrophoresis :-

Electrophoresis is a technique used in the laboratory that results in the separation of charged molecules. DNA is a negatively charged molecule, and is moved by electric current through a matrix of agarose.

Purified agarose is in powdered form, and is insoluble in water (or buffer) at room temperature. But it dissolves in boiling water. When it starts

to cool, it undergoes what is known as polymerization. Rather than staying dissolved in the water or coming out of solution, the sugar polymers crosslink with each other, causing the solution to "gel" into a semi-solid matrix much like "Jello" only more firm. The more agarose is dissolved in the boiling water, the firmer the gel will be. While the solution is still hot, we pour it into a mold called a "casting tray" so it will assume the shape we want as it.

Now it's time to take the Purified undigested DNA to load the whole genomic DNA and run our gel.

We may digest a piece of DNA with Restriction Enzymes, separate the digested fragments by Agarose Gel Electrophoresis on a gel and analyze and document the results.

The restriction enzymes recognize short sequences of double stranded DNA as target for cleavage. Different enzyme recognize, different but specific sequences, each ranging in length from 4 to 8 base pair. For our experiment we have selected three different restriction enzymes with different digesting site.

*Eco*R I ---------- 5' G↓ AA TTC 3'
3' CTT AA ↑ G 5'

Hind II ---------- 5' G T Py ↓ Pu A C 3'
3' C A Pu ↑ Py T G 5'

*Bam*H I ---------- 5' G↓ G A TCC 3'
3' C C T AG↑ G 5'

If we take a particular DNA sample, digest it with a specific restriction enzyme and subject the sample to gel electrophoresis, we will notice a series of band on the gel slab. The position of different bands will depend on DNA fragment size, such as the smaller fragment will move more rapidly, crossing a greater distance, and the larger fragment will move slowly, crossing a relatively lesser distance.

Materials and Method for Gel Electrophoresis

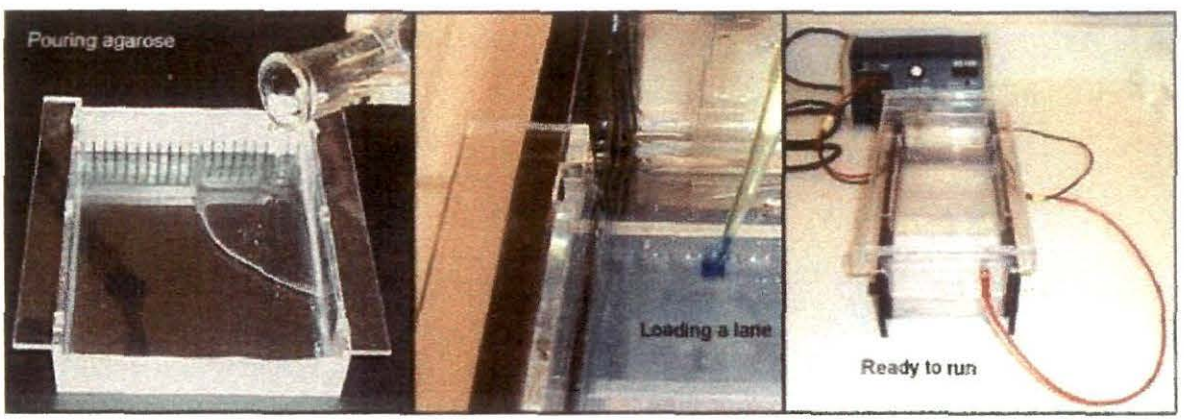
The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:

- An electrophoresis chamber and power supply
- Gel casting trays, which are available in a variety of sizes and composed of UV-transparent plastic.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.
- Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow monitoring or how far the electrophoresis has proceeded.
- Ethidium bromide, a fluorescent dye used for staining nucleic acids.
- Transilluminator (an ultraviolet light box)

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide (final concentration 0.5 ug/ml) is added to the gel at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a

casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipeted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. We can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the anode, which is usually colored red.



1. To prepare 100 ml of a 0.7% agarose solution, measure 0.7 g agarose into a glass beaker or flask and add 100 ml 1X TBE or TAE.
2. Microwave or stir on a hot plate until agarose is dissolved and solution is clear.
3. Allow solution to cool to about 55°C before pouring. (Ethidium bromide can be added at this point to a concentration of 0.5 µg/ml)
4. Prepare gel tray by sealing ends with tape or other custom-made dam.
5. Place comb in gel tray about 1 inch from one end of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray.

6. Pour 50C gel solution into tray to a depth of about 5 mm. Allow gel to solidify about 20 minutes at room temperature.

7. To run, gently remove the comb, place tray in electrophoresis chamber, and cover (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose)

8. Excess agarose can be stored at room temperature and remelted in a microwave.

9. To prepare samples for electrophoresis, add 1 µl of 6x gel loading dye for every 5 µl of DNA solution. Mix well. Load 5-12 µl of DNA per well (for mini gel).

10. Electrophorese at 50-150 volts until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

11. If the gel was not stained with ethidium during the run, stain the gel in 0.5 µg/ml ethidium bromide until the DNA has taken up the dye and is visible under short wave UV light, if the DNA will not be used further, or with a hand-held long-wave light if the DNA is to be cloned.

50x TAE

242 g Tris base

57.1 g glacial acetic acid

100 ml 0.5 M EDTA

10x TBE

108 g Tris base

55 g boric acid

40 ml 0.5 M EDTA, pH=8

distilled water to 1 liter

6x gel loading buffer

0.25% Bromophenol blue

0.25%Xylene cyanol FF

15% Ficoll Type 4000

120 mM EDTA

The distance, DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dye Bromophenol blue. Dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.

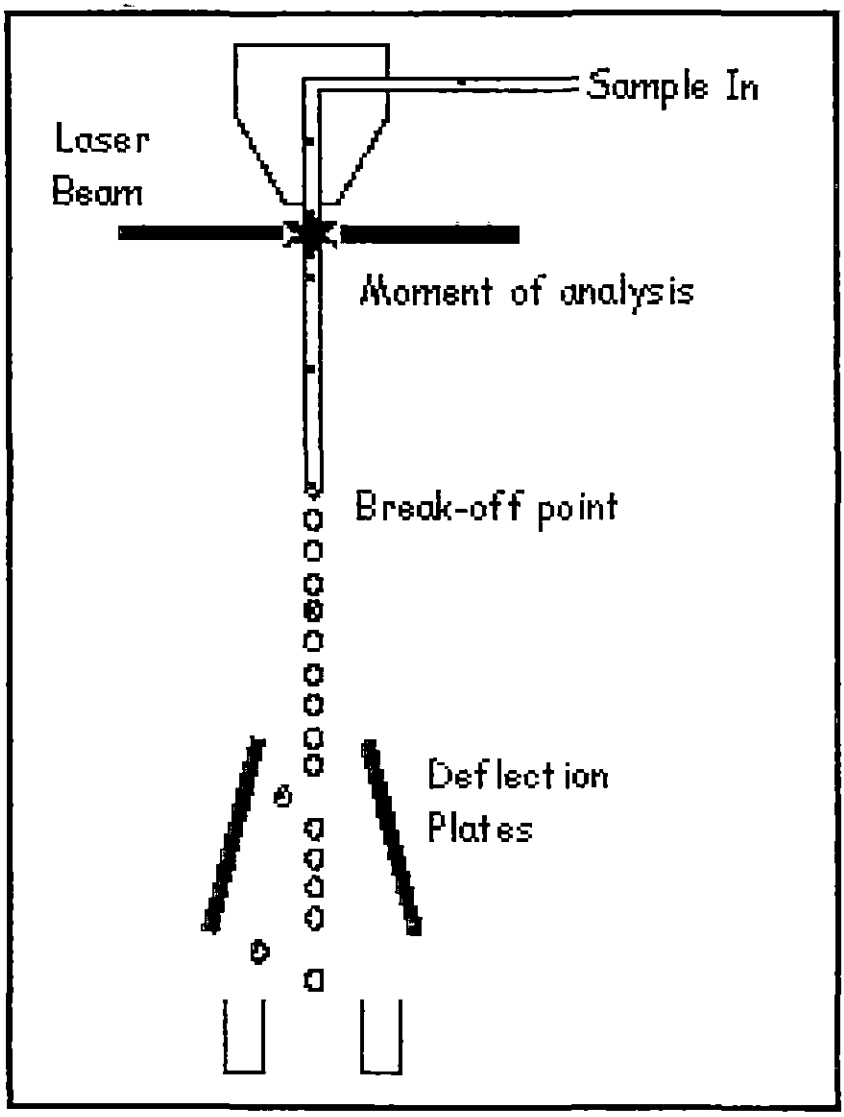
When adequate migration has occured, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA, the gel is placed on a ultraviolet transilluminator. After getting satisfactory result photograph is taken quickly because DNA will diffuse within the gel over time.

H. Fluorescence Activated Cell Sorting :-

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension one by one past a sensing point. In one way flow cytometers can be considered to be specialised fluorescence microscopes. The modern flow cytometer consists of a light source, collection optics, electronics and a computer to translate signals to data. In most modern cytometers the light source of choice is a laser, which emits coherent light at a specified wavelength. Scattered and emitted fluorescent light is collected by two lenses (one set in front of the light source and one set at right angles) and by a series of optics, beam splitters and filters, specific bands of fluorescence can be measured. We can measure physical characteristics such as cell size, shape and internal complexity and, of course, any cell component or function that can be detected by a fluorescent compound can be examined (Robinson, 1993).

Flowcytometry is a technique for making rapid and symultaneous measurement of several parameters of a cell or a subcellular particle as

FIGURE-7



Operating Principle of FACS

they flow in a fluid stream, one by one, through a sensing point (a spot where a beam of laser or arc light is focussed on the stream). With modern cytometers, upto six parameters of every cell can be measured at a time, and as many as 25,000 cells can be analysed per second. Since all parameters of each cellare measured individually, it is possible to estimate the relative variation in every parameter of every cell with the help of modern flowcytometry computer software (Fraker et.al.,1994 ; Telford et.al., 1994).

In general, flow cytometers use a principle involving the electrostatic deflection of charged droplets similar to that used in ink-jet printers. Cells are aspirated from a sample and ejected one by one from a nozzle in a stream of sheath fluid, generally PBS.

All streams are unstable with respect to time and will eventually break up into droplets. It is possible to stabilize this break-off point by applying a stationary wave of vibration of known frequency and amplitude to the stream.

As the cell intercepts with the laser beam, scattered light and fluorescence signals are generated and the sort logic boards make a decision whether the cell is to be sorted or not (Figure- 7).

The distance between the laser intercept and the break-off point is called the drop delay. If a cell of interest i.e. one to be sorted, has been detected, the cytometer waits until that cells has travelled from the intercept to the break-off point and then charges the stream. So as the drop containing the cell of interest leaves the solid fluid stream it will carry a charge, either positive or negative. A further distance downstream the charged drop passes through two high voltage deflection plates and will be attracted to towards the plate of opposite polarity. So it is possible to sort two separate populations from the same sample. By applying two different levels of charge to the left or the right streams it is actually possible to sort

two streams either side and both the Cytomation MoFlo and the Becton Dickinson DiVa are capable of this.

The theory and the practicalities of cell sorting are rather more complicated than this simple overview. However, it is also useful to know that we can alter the mode of sorting to give maximum purity, maximum recovery (if a small, precious population is required) or maximum count accuracy (for cloning).

The Fluorescence Activated Cell Sorting Facility contains a laser activated, state-of-the-art cell analysis and sorting machine; the BD FACSVantage SE with DiVa and TurboSort options is one of the most advanced flow cytometers in the world. The BD FACS Vantage SE System is equipped with an air-cooled Spectra-Physics HeNe Laser (633nm) and a water cooled Coherent Enterprise II laser. The Enterprise II is a small frame ion laser that has multiple wavelength outputs, providing simultaneous lines at 488nm and 351-364nm.

The BD FACSVantage SE System is able to collect 2 scatter and 8 fluorescent signals per cell and can achieve sort speeds of up to 7,000 cells per second on high speed and up to 25,000 cells per second using the BD TurboSort option in analogue mode. The FACSDiVa option provides zero dead time, allowing faster sorts (up to 60,000 events per second) and higher yields than the standard Vantage SE, and enables 4-way sorting with the QuadraSort functionality. The DiVa option uses matrix algebra for fully independent compensation of all channels. Pulse processing allows the measurement of area, width and ratio of detector pulses, and can be used to detect doublets in DNA analysis or the ratio of two fluorescence signals for use in calcium flux measurements.

In the present work we have examined living sperm cells from three different species of toad under flowcytometer and analysed the FACS result

The work was conducted by FACS Calibur 2 (Becton Dickinson) in the Indian Institute of Chemical Biology, Kolkota.

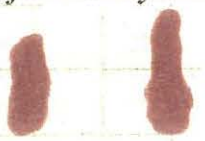
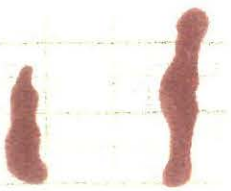
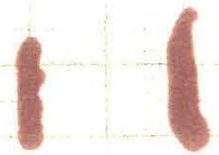
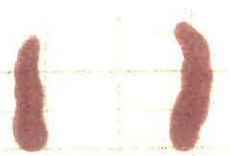
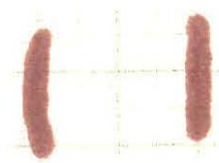
METHOD :

- Sperm cells are are isolated and collected in eppendorf.
- The cells are suspended in 1ml PBS.
- Fixed in 80% ethanol for overnight.
- Fixed cells are centrifused and the supernatant is decanded.
- The cells are washed in 38mM citrate buffer.
- The cells are resuspended in 0.5ml solution of 69µg ethedium bromide in 38mM Na-citrate.

Kept in room temperature for atleast 30 minutes and examined under FACS Calibur 2 (Becton Dickinson).

FIGURE - 3

Study of Morphological and Metric variation in testis according to the changing seasons in three species of Bufo

Bufo himalayanus	*Bufo melanostictus*	*Bufo stomaticus*
 May , L-15.5mm,R-19.0mm	 May, L-13.0mm, R-16.75mm	 May, L-10.0mm,R-10.0mm
 June, L-17.0mm, R-26.0mm	 June, L-15.5mm, R-20.75mm	 June, L-13.5mm, R-12.5mm
 August, L-15.25mm, R-17.0mm	 August, L-18.0mm, R-17.75mm	 August, L-13.25mm, R-13.0mm
 September, L-12.75mm, R-13.5mm	 September, L-10.5mm, R-12.0mm	 September, L-7.0mm, R-7.5mm

OBSERVATION

Study of Morphological and Metric variation in the testis at different Seasons in three species of *Bufo*

Generally in case of family Bufonidae, the breeding season is June to August, April-May is the prebreeding season and September-October is the postbreeding season. From my observation on testicular morphology and metric studies it is clear that in all the three species of *Bufo* there is a generalized increase in size of the testis with the arrival of breeding season. The testes size reaches the peak in the June-August season, indicating their maximum breeding activity at this season. In this period, the testes are engorged with mature and healthy sperms which are to be utilized for reproduction. Of the three species, maximum morphological and metrological variation is observed in the testes of *Bufo melanostictus*, while the minimum variation is observed in *Bufo stomaticus*. (Figure- 3)

From the above observation one particular feature is to be noted that in every species, in every season the size of the right testicular lobe becomes slightly larger than the left. This is a peculiar characteristic feature, which needs further detailed study in anatomical and endocrinological level.

PLATE – 2:

Individual sperm of three different species under light microscope at a magnification of X6000.

A & B→ *Bufo himalayanus*,

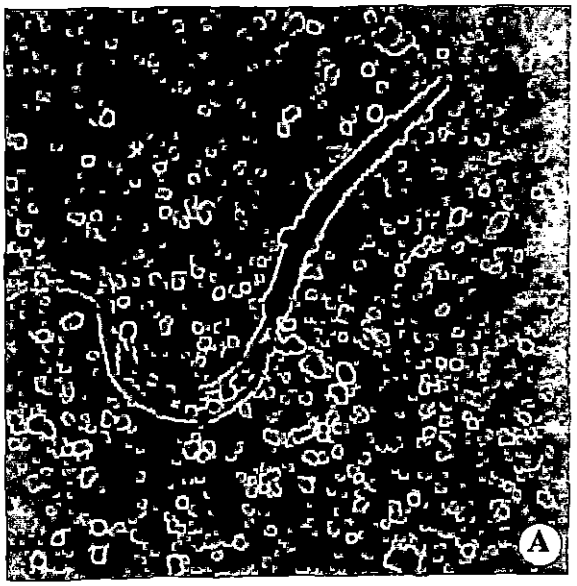
C & D→ *Bufo stomaticus*,

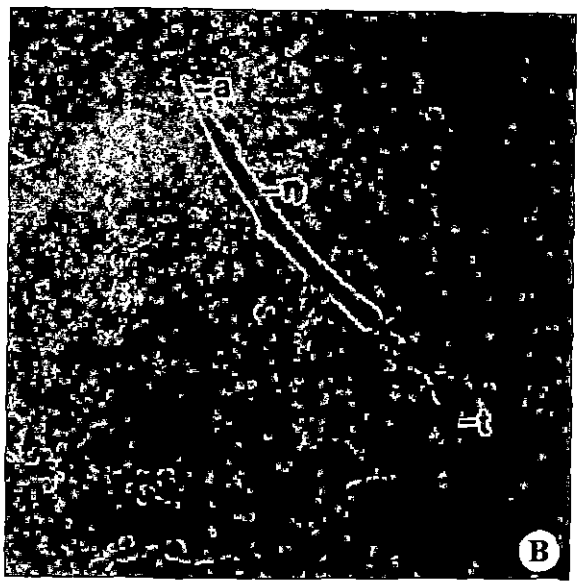
E & F→ *Bufo melanostictus*.

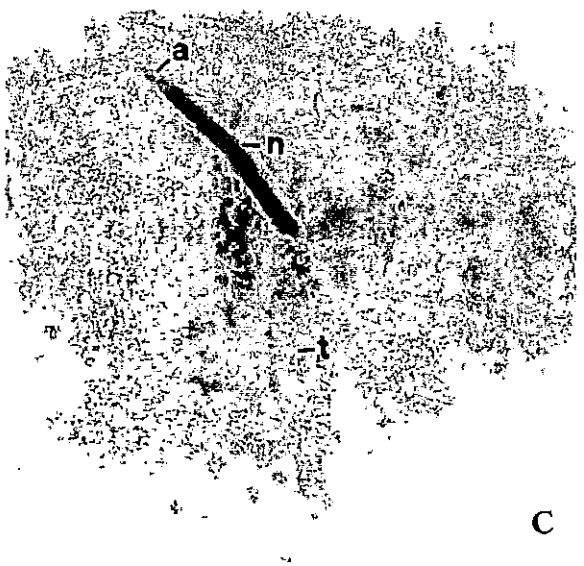
Indication: a- acrosome, n- nucleus, mp- middle piece, t- tail.

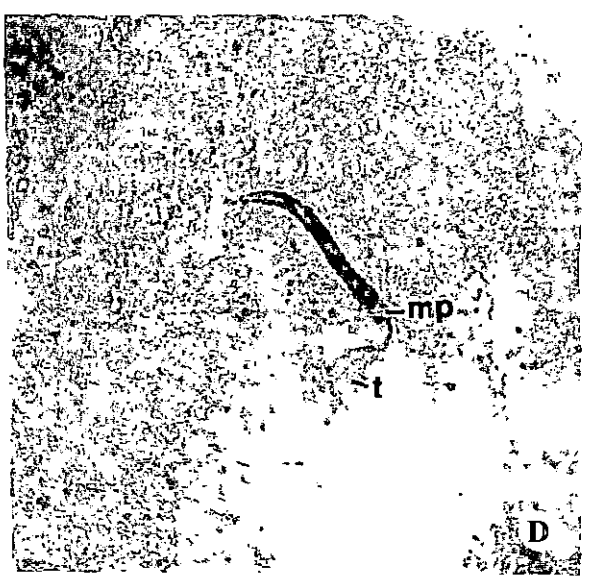
All the species shows biflagellate tail, a distinct acrosome and a flattened post-nuclear middle piece

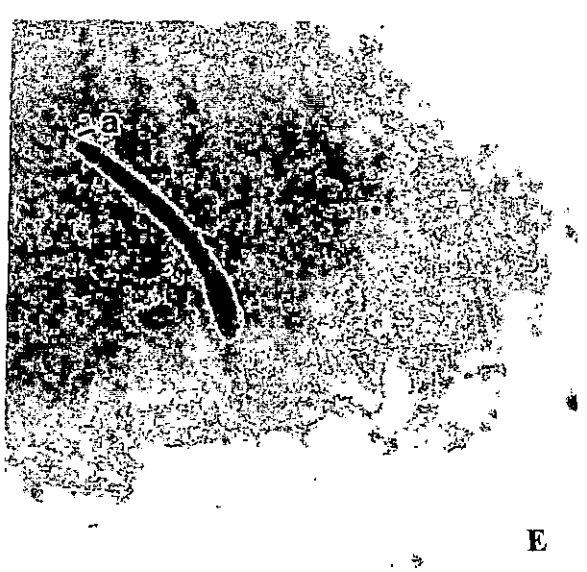
PLATE – 2

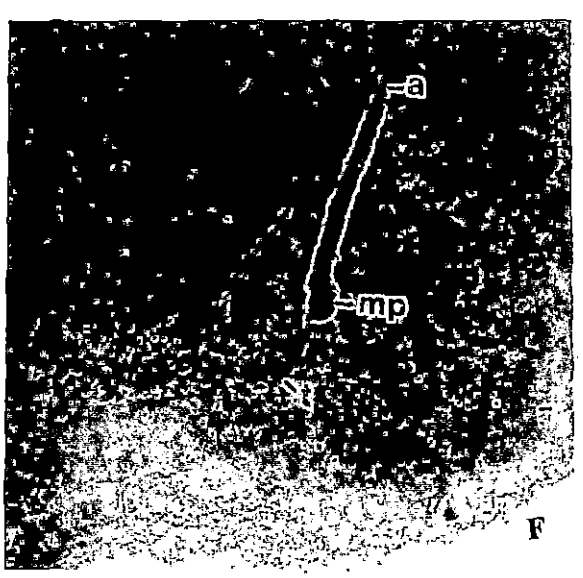












Light Microscopic Observation

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

B*ufo himalayanus*

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 (Plate-3, Fig. D). The head of *Bufo himalayanus* is about 29x2µm in size, larger than that of other two species studied and have a sword like appearance. (Plate-2, Fig. A, B ; Plate-3, Fig. A)

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 (Plate-3, Fig. A).

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 out (Plate-2, Fig. A, B ; Plate-3, Fig. A, G).

Bufo stomaticus

The sperm head of Bufo stomaticus is deeply stained nucleus with a pointed less stained needle like acrosome at its anterior tip (Plate-

PLATE – 3:

Light microscopic pictures of individual sperm at X4000

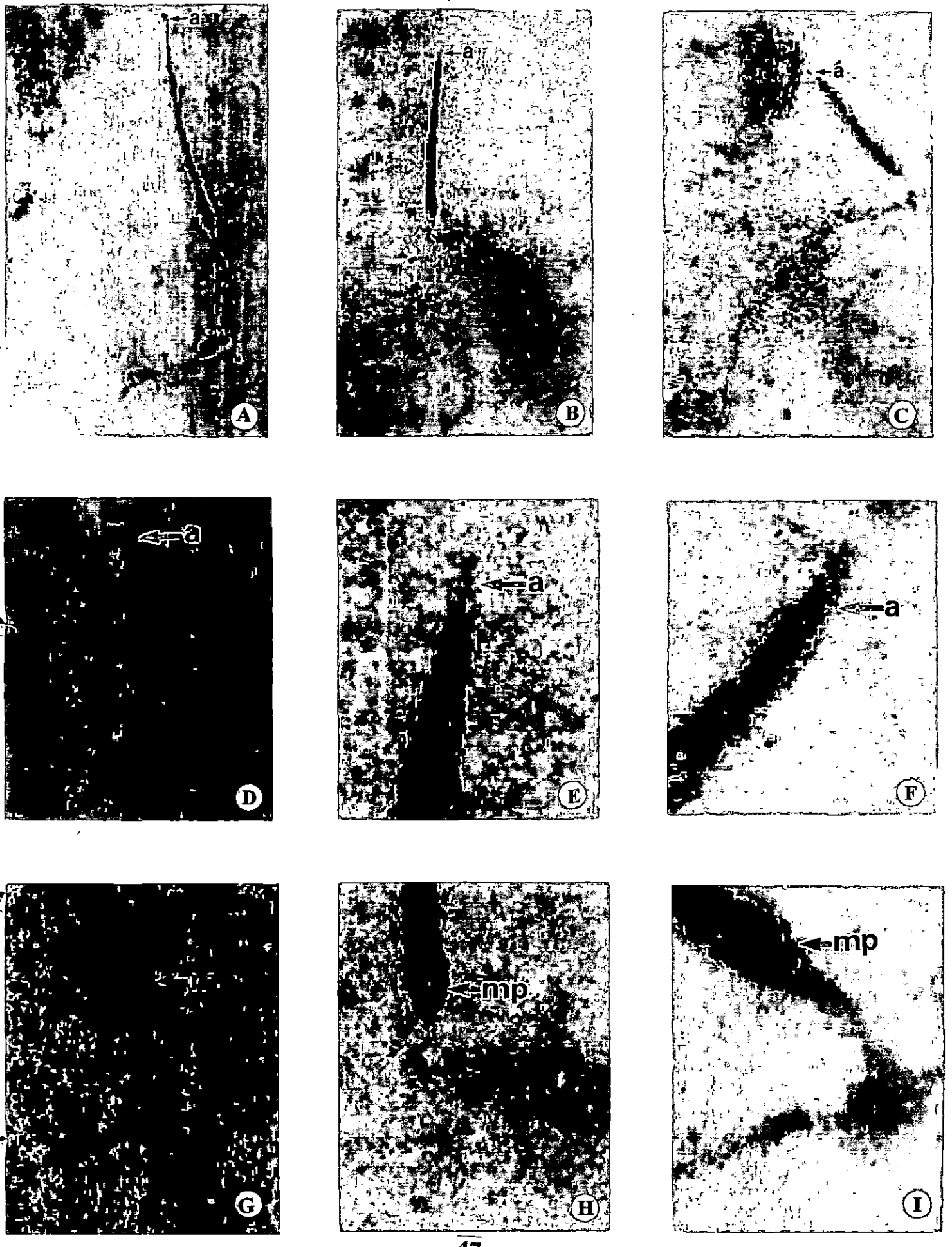
A & B→ *Bufo himalayanus*;

C & D→ *Bufo stomaticus*;

E & F→ *Bufo melanostictus*.

Indication :- a- acrosome; n- nucleus; mp- middle piece; t- tail filament. All the species showing biflagellate tail, a distinct acrosome and a flattened postnuclear middle piece.

PLATE – 3



2, Fig. C, D ; Plate-3, Fig. B, E). 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 (Plate-3, Fig. B).

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 (Plate-3, Fig. B, H).

Bufo melanostictus

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 (Plate-3, Fig. F). Surface morphology of the head is smooth (Plate-2, Fig. E, F ; Plate-3, Fig. C).

The head is followed by an equally stained middle piece from where the tail filament arises (Plate-.3, Fig. C, I).

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 (Plate-2, Fig. E, F ; Plate-3, Fig. I).

A wide range of morphological variations are observed in the three species of bufonids (Table- 1,2,3).

Using the detailed metric study, following classes of sperm morphology can be recognized.

In ***Bufo himalayanus,*** (Table-1) about 66% of the sperm head is about 28x3um in size while 28% of the sperm head class has the

TABLE - 1

Metric details of *Bufo himalayanus* sperm (in μm)

SL No	Head Lth.	Head Dtr.	Tail Lth.	SL No	Head Lth.	Head Dtr.	Tail Lth.
1	32	3	40	26	28	2	40
2	32	2	48	27	28	2	24
3	28	2	56	28	32	2	48
4	28	3	56	29	28	2	60
5	32	2	52	30	32	2	56
6	24	2	56	31	32	2	40
7	32	2	60	32	28	2	52
8	32	2	52	33	24	2	32
9	32	2	56	34	28	2	48
10	28	2	48	35	28	2	48
11	28	2	48	36	28	4	40
12	32	2	56	37	28	2	56
13	32	2	40	38	28	2	48
14	28	3	48	39	28	2	56
15	24	2	48	40	28	2	56
16	28	2	56	41	28	2	56
17	28	2	20	42	28	2	48
18	28	2	56	43	28	2	32
19	32	2	64	44	28	2	56
20	28	2	28	45	28	2	56
21	28	2	36	46	28	2	56
22	32	2	24	47	28	2	32
23	28	2	60	48	28	2	56
24	32	2	48	49	28	2	40
25	32	2	48	50	28	2	48

CALCULATION

Head length	Occurance
32	28%
28	66%
24	6%

Tail length	Occurance
I I-20	2%
21-30	6%
31-40	20%
41-50	26%
51-60	44%
61-70	2%

Total length	Occurance
21-40	0%
41-60	14%
61-80	44%
81-100	42%

MEAN HEAD LENGTH- 28.88μ m ± 2.16

MEAN TAIL LENGTH- 46.00μ m ± 10.63

MEAN TOTAL LENGTH- 75.64μ m ±13.88

TABLE - 2

Metric detalis of *Bufo stomaticus* sperm (in μm)

SL No.	Head Lth.	Head Dtr	Tail Lth.	SL No.	Head Lth.	Head Dtr.	Tail Lth.
1	24	8	20	26	28	2	12
2	24	4	40	27	28	2	20
3	8	4	60	28	28	3	40
4	28	3	48	29	28	2	40
5	28	3	16	30	28	2	48
6	28	3	40	31	28	3	20
7	28	3	40	32	28	6	40
8	28	3	44	33	28	2	20
9	12	4	12	34	28	2	40
10	24	3	48	35	28	2	36
11	28	2	32	36	28	2	16
12	24	3	48	37	28	2	40
13	12	4	20	38	28	2	24
14	28	2	32	39	28	2	40
15	24	3	48	40	28	2	36
16	28	3	40	41	24	2	40
17	8	5	32	42	24	2	32
18	28	3	40	43	28	2	60
19	28	3	32	44	24	2	40
20	28	3	40	45	28	2	44
21	28	2	24	46	28	3	40
22	24	3	40	47	20	4	40
23	6	6	28	48	24	2	20
24	24	3	36	49	28	2	40
25	24	3	32	50	28	2	42

CALCULATION

Head length	Occurance	Tail length	Occurance	Total length	Occurance
28	64%	11-20	22%	21-40	10%
24	24%	21-30	06%	41-60	32%
2O	02%	31-40	54%	61-80	56%
12	04%	41-50	14%	81-100	02%
08	02%	51-60	04%		
		61-70	00%		

MEAN HEAD LENGTH-25 μ m ± 5.6 ±
MEAN TAIL LENGTH – 32.2 μ m ±10.78
MEAN TOTAL LENGTH -60.16 μ m ±14.00

TABLE - 3

Metric details of *Bufo melanostictus* sperm (in μm)

SL No	Head Lth.	Head Dtr.	Tail Lth.	SL No.	Head Lth.	Head Dtr.	Tail Lth.
1	32	3	60	26	32	3	48
2	28	3	16	27	28	2	40
3	28	3	40	28	32	3	56
4	28	3	48	29	32	3	48
5	28	3	60	30	32	3	48
6	16	5	56	31	32	4	40
7	24	2	48	32	32	3	32
8	28	3	32	33	32	3	56
9	32	3	40	34	32	3	40
10	32	2	40	35	32	2	32
11	28	2	64	36	28	3	48
12	20	2	44	37	32	4	56
13	32	3	32	38	28	4	56
14	32	3	48	39	32	3	48
15	28	3	32	40	32	4	52
16	32	3	48	41	32	3	56
17	32	3	40	42	28	3	40
18	24	2	44	43	32	3	64
19	32	3	40	44	28	3	48
20	32	3	56	45	28	4	48
21	24	2	40	46	28	3	40
22	32	3	40	47	28	3	56
23	28	4	52	48	32	4	48
24	32	3	56	49	32	3	40
25	32	3	56	50	32	4	40

CALCULATION

Head length	Occurance
32	58%
28	32%
24	6%
20	2%
16	2%

Tail length	Occurance
I I - 20	2%
21-30	0%
31-40	38%
41-50	28%
51-60	28%
61-70	4%

Total length	Occurance
21-40	0%
41-60	6%
61-80	64%
81-100	30%

MEAN HEAD LENGTH - 29.52 μm ± 3.40
MEAN TAIL LENGTH - 44.26 μm ± 9.97
MEAN TOTAL LENGT - 74.84 μm ± 10.99

FIGURE - 4

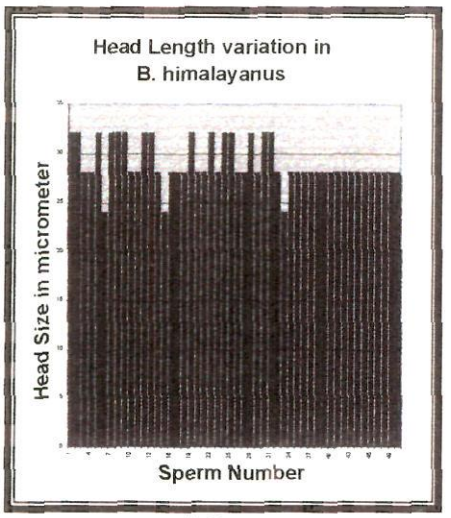
Histogram of the head length variation in the three *Bufo* species.

A-I and A-II showing the occurrence of head length in *Bufo himalayanus* – The maximum head length is 32µm which occupies only 28% of the total sperm count, but the majority of the sperm (66% of the total sperm count) has the size of 28µm.

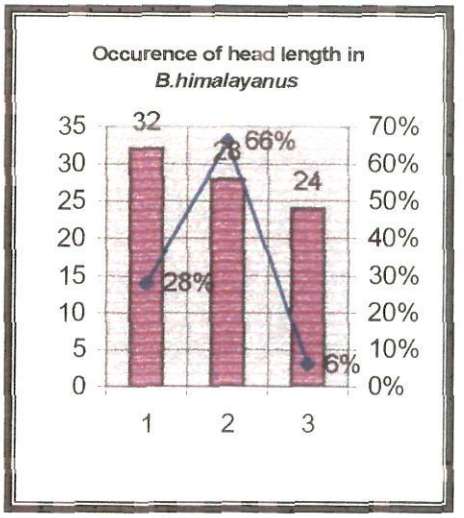
B-I and B-II showing the occurrence of head length in *Bufo stomaticus* - The maximum head length is 28µm which occupies 64% of the total sperm count.

C-I and C-II showing the occurrence of head length in *Bufo melanostictus* – The maximum head length is 32µm which occupies 58% of the total sperm count.

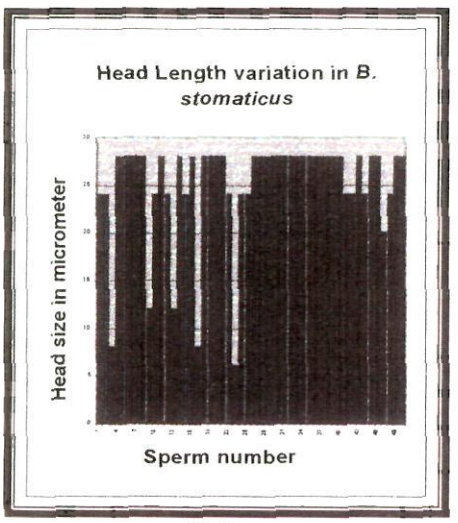
FIGURE-4



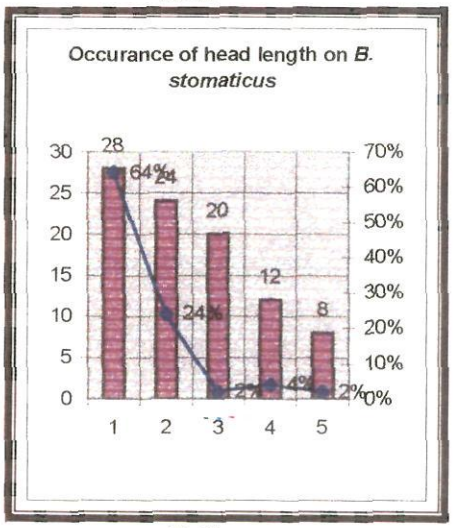
A-I



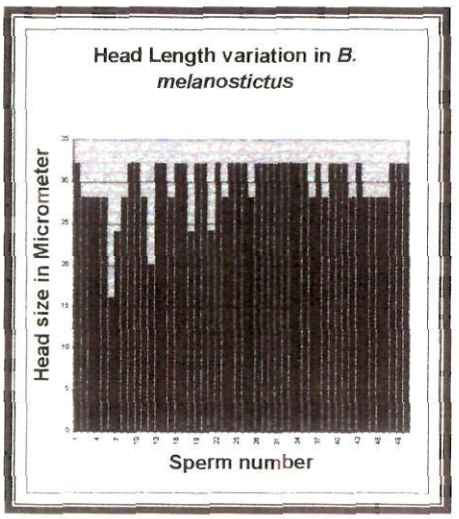
A-II



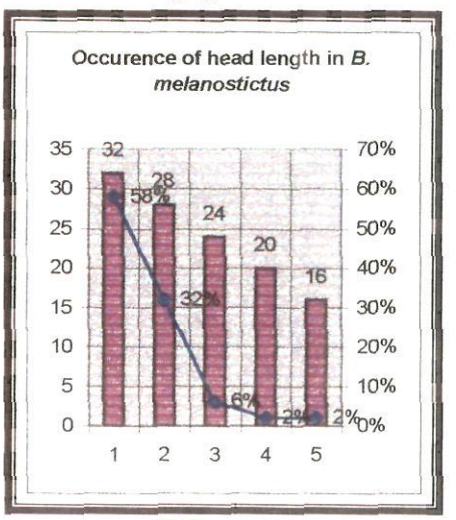
B-I



B-II

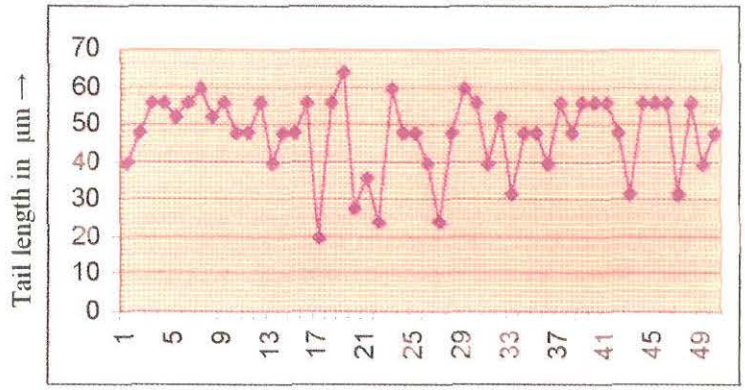


C-I

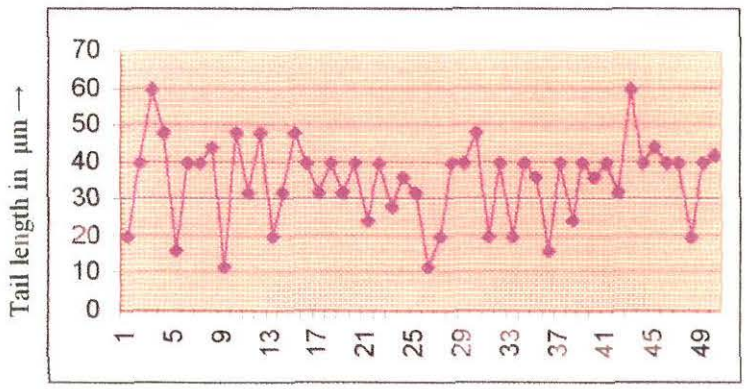


C-II

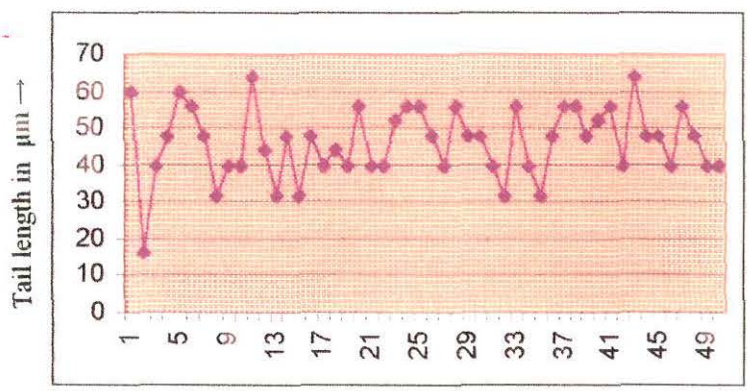
FIGURE-5



B himalayanus tail Sperm number →



B.stomaticus tail Sperm number →



B.melanostictus tail Sperm number →

FIGURE – 5

Frequency curve showing the occurance of sperm tail length in µm

A. Showing the tail length variations in 50 different sperms of *Bufo himalayanus.*

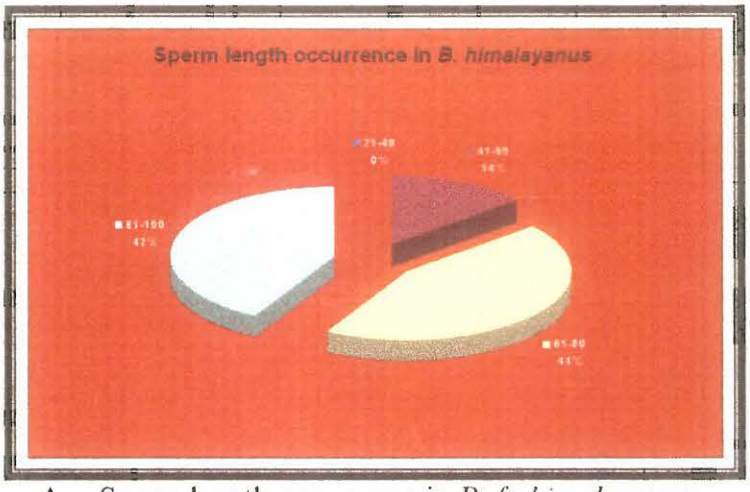
B. Showing the tail length variations in 50 different sperms of *Bufo stomaticus.*

C. Showing the tail length variations in 50 different sperms of *Bufo melanostictus.*

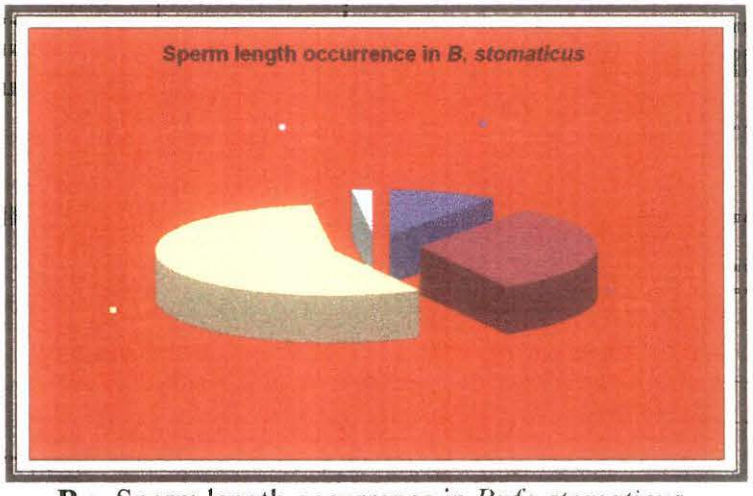
FIGURE – 6

Pie diagram of the occurrence of different sperm length group in the three different *Bufo* species studied.

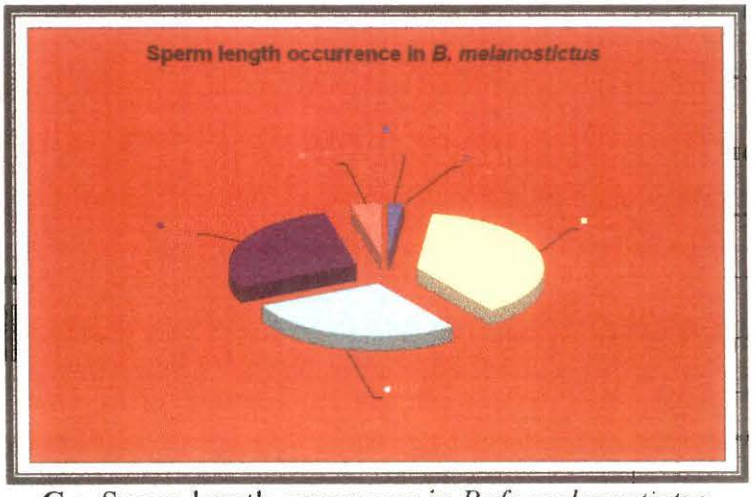
FIGURE- 6



A :- Sperm length occurrence in *Bufo himalayanus*



B :- Sperm length occurrence in *Bufo stomaticus*



C :- Sperm length occurrence in *Bufo melanostictus*

larger dimension (32x2µm). A very few, 6% has the smaller dimension (24x2µm). (Fig.- 4AI, 4AII)

In ***Bufo stomaticus,*** (Table- 2) four distinct head classes are observed. The majority of the sperms (64%) are about 28x2µm in size. About 26% of the sperms of the same species are between 20-24x2µn in size, while about 10% of the sperms are microcephalic and are about 5-8x4µm and almost oval in shape. (Fig.- 4BI, 4BII)

Similarly in ***Bufo melanosticus*** (Table- 3) three sperm classes has been recognized. 80% of the total sperm head are 28-32x2µm in size and rod like in appearance, while about 08% is 22x3µm in size. A very few sperms, 2% are micro-cephalic and heads are about 16x2µm in size. (Fig.- 4CI, 4CII)

The neck region is morphologically not sharply distinct.

Like the head morphology, the tail part exhibits great variations within a species studied.

In ***Bufo himalayanus*** the majority of the sperm (45%) have the tail length of 52-62 µm.(mean -56µm). However the maximum length of the tail is 64µm, while a very few have extremely short tail whose length is about 20µm. An intermediate class having 31-50µm has also been observed.

In ***Bufo stomaticus*** majority (68%) of sperm tails lie between 32µm and 48µm (mean 40µm). However the maximum tail length is 60um, having 4% occurance. A tail class is also observed (28%) which has the smallest tail size with mean value 20µm.

In ***Bufo melanostictus,*** (64%) of the sperm tail lies between 32µm & 48µm (mean 40µm). The largest tail is 64µm in length, which occupies 4% of the total sperm population and the smallest tail size is 16µm, which is 2% of the total cell counted.

The total length variation in all the three species can be clearly observed from the figures 5A,5B and 5C.

Scanning Electron Microscopic Observation

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 (Plate-4, Fig. A,B,C).

Bufo himalayanus

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 (Plate-5, Fig. A).

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 (Plate-6, Fig. A).

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 (Plate-7, Fig. A).

Bufo stomaticus

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 (Plate-5, Fig. B).

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 (Plate-6, Fig. B).

The tail is biflagellate , but both the flagella are free from one another i.e. not connected by proteinacious sheath as found in *B.*

PLATE – 4:

Scanning electron micrographs of the whole sperm of three different species.

A→ *Bufo himalayanus*

B→ *Bufo stomaticus*

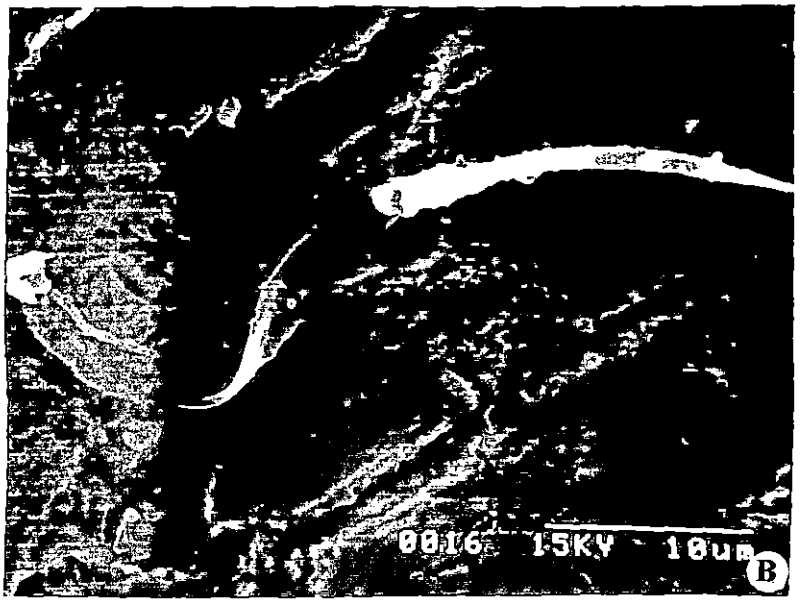
C→ *Bufo melanostictus*

A→ Sperm of *B.himalayanus* showing an anterior bulb like acrosomal tip, swollen middle piece and distinct tail filaments attached at the lower extreme tip by end bulb.

B → Sperm of *B.stomaticus* showing pointed tip and short tail filament with end bulb. Rough head surface morphology is a distinctive feature.

C→ Sperm of *B.melanostictus* showing enlarged, curved head with niddle like pointed acrosomal tip and distinct middle piece.

PLATE – 4



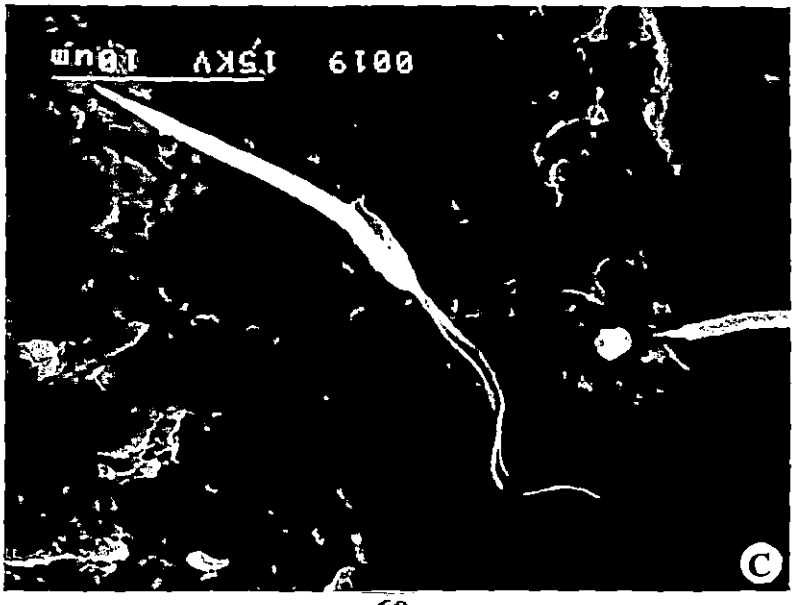


PLATE – 5:

Scanning Electron micrographs of the head region of sperm of the three different species studied.

A→ Sperm head of *Bufo himalayanus*

B→ Sperm head of *Bufo stomaticus*

C→ Sperm head of *Bufo melanostictus*

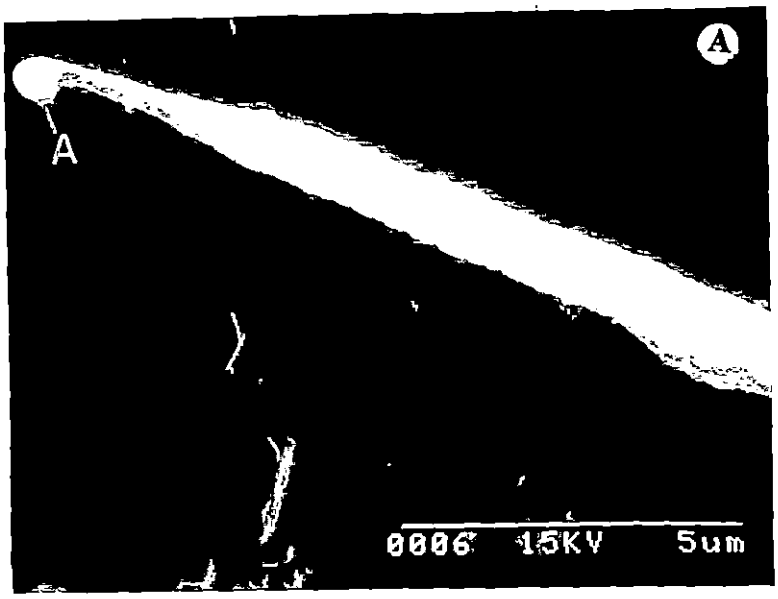
Figure A. shows the swollen cup like acrosomal tip with smooth head surface morphology.

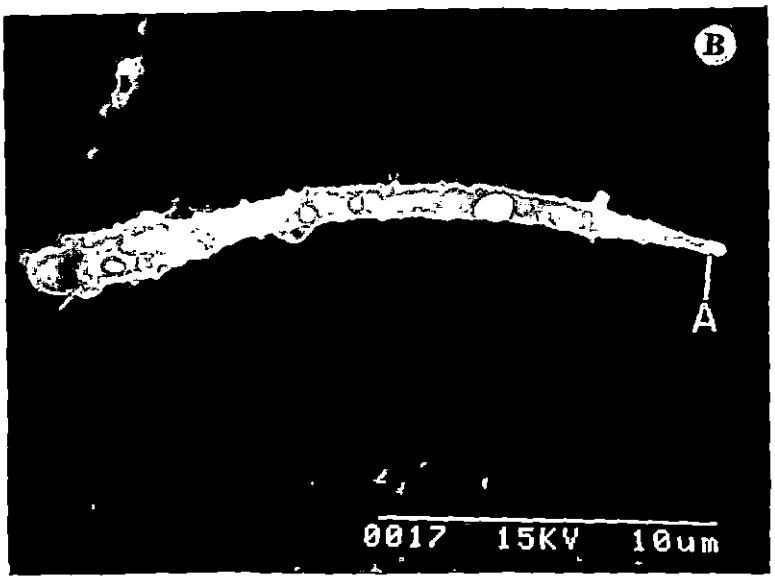
Figure B. shows the rough surface morphology of the *B. stomaticus* head with a pointed acrosomal tip.

Figure C. shows the pin pointed acrosomal tip of *Bufo melanostictus* head with a smooth surface morphology.

A- indicates the acrosome in all the figures.

PLATE – 5





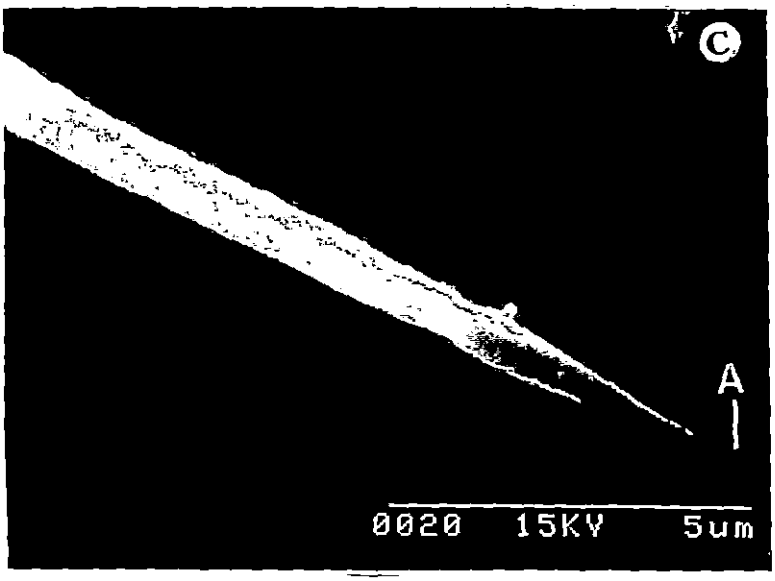


PLATE – 6:

Scanning Electron micrographs of the sperm mid-piece and the arising tail filament of the three different species studied.

A→ Sperm head of *Bufo himalayanus*

B→ Sperm head of *Bufo stomaticus*

C→ Sperm head of *Bufo melanostictus*

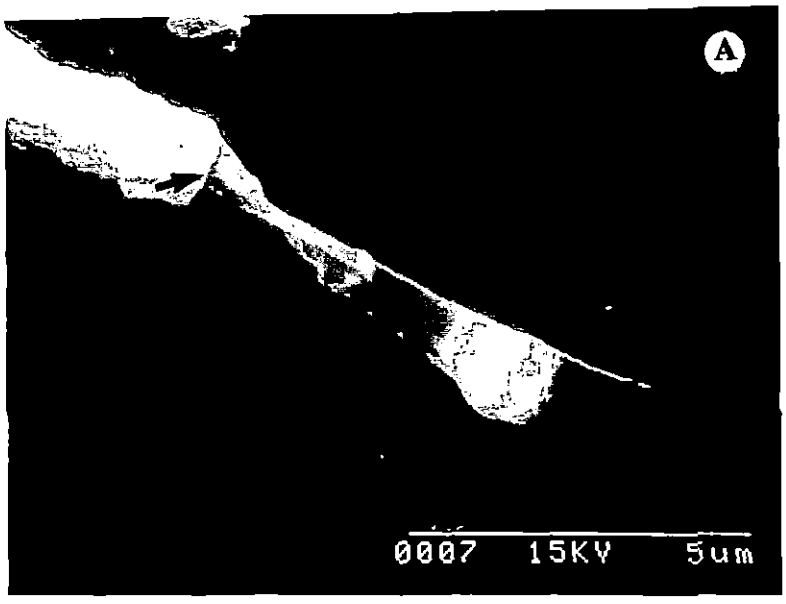
Figure A. shows that in *B. himalayanus* the tail filament arise as a flat process from the middle piece.

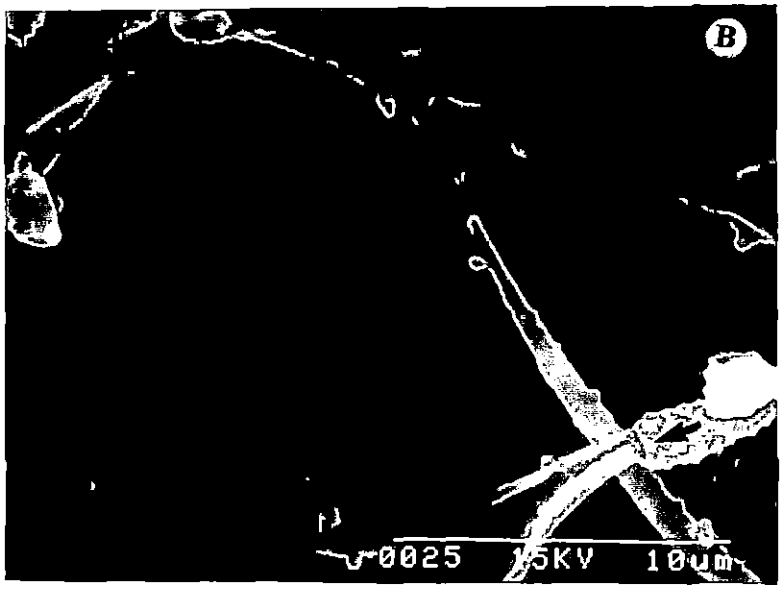
Figure B. shows that in *B. stomaticus* the tail filament and the undulating membrane arise separately from the mid-piece.

Figure C. shows that the tail filament of *B. melanostictus* arises as a slender process from the mid-piece, after a certain distance the undulating membrane projects out from the tail filament.

The black arrow in all the figures indicates the junctional region of the mid-piece and the tail filament.

PLATE – 6





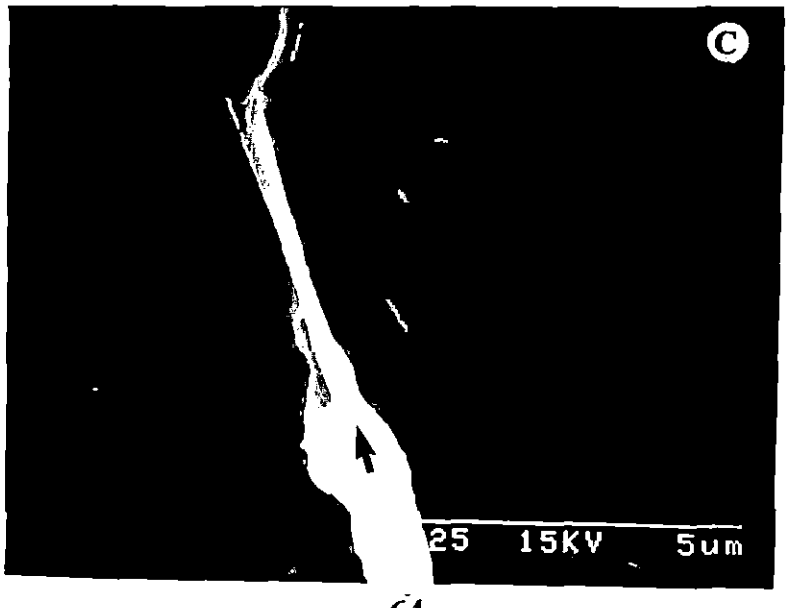


PLATE – 7:

Scanning Electron micrographs of the sperm tail of the three different species studied.

A→ Sperm head of *Bufo himalayanus*

B→ Sperm head of *Bufo stomaticus*

C→ Sperm head of *Bufo melanostictus*

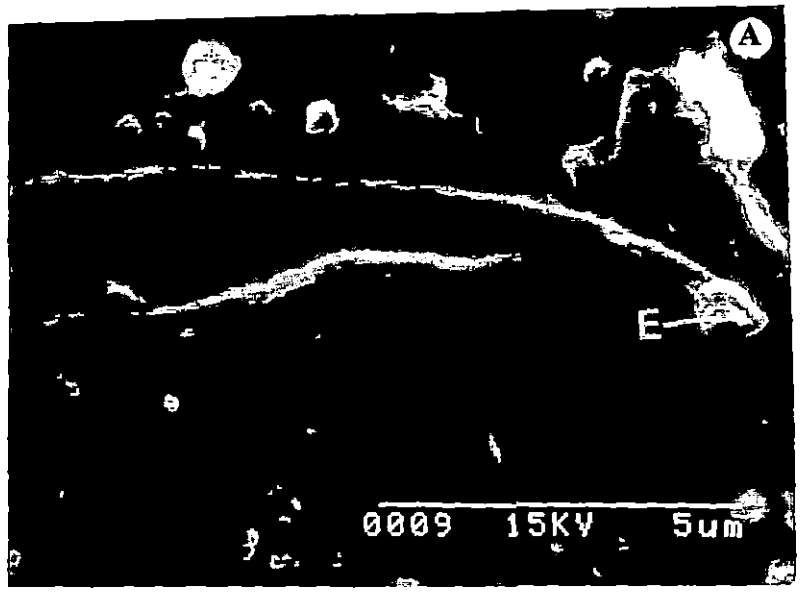
Figure A. shows that in *B. himalayanus* the sperm tail ends in a button like knob.

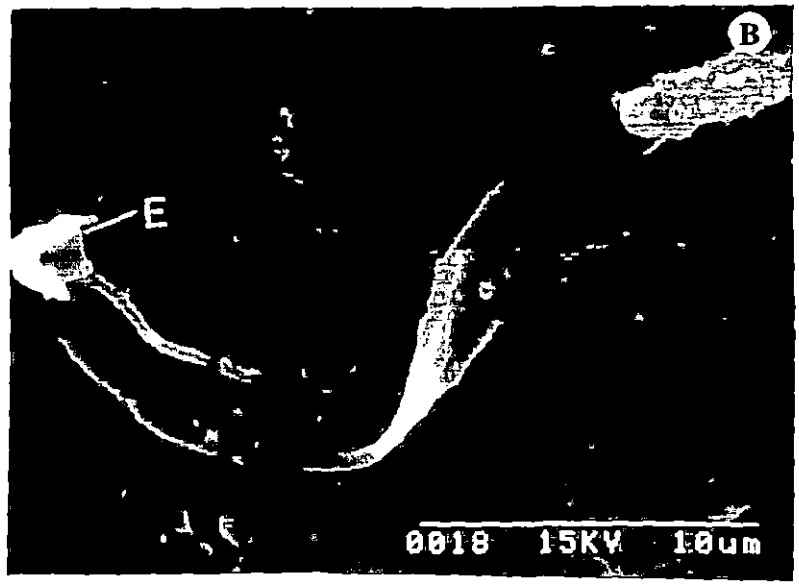
Figure B. shows that in *B. stomaticus* the end bulb of the sperm tail is somewhat flat with a bi-lobed appearance.

Figure C. shows that in *B. melanostictus* the sperm tail also ends in a flattened extreme end but it is not so distinct.

The tail end is marked as E in all the figures.

PLATE – 7





himalayanus. However at the posterior end of the tail filament, both the flagellum unite as a bi-lobed knob (Plate-7, Fig. B).

Bufo melanostictus

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 (Plate-5, Fig. C).

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 (Plate-6, Fig. C).

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 (Plate-7, Fig. C).

As observed under the Scanning Electron Microscope, the sperm morphology exhibits a wide range of variation in all the three species studied.

The spermatogenetic pattern and the release of is almost similar in all the three species.

Unlike other vertebrates, in amphibians, sperm matures in groups occupying numerous pockets in the testis. From the scanning electron micrographs it is observed that the inner morphology of the testes are not smooth. Instead, pouch like pockets appear throughout the testes body. These pockets are mainly of two types; one, the empty pockets and the other, the sperm pockets. The sperm pockets are filled with mature sperms, which are ready to be released. The empty pockets are those which have released their mature sperms and contain only underdeveloped gonial cells and developing spermatocytic cells.

It is also observed that majority of the empty nests are lying in the periphery and the sperm pockets towards the center indicating the gradual maturation of sperms from the center to the periphery.

Transmission Electron Microscopic Observation

Bufo himalayanus

Under TEM the anterior and middle portion of the *Bufo himalayanus* sperm is well differentiated with conical sharp head, tapering towards the anterior end (Plate-11, Fig. a). The nucleus is elongated and with electron dense granules as seen in transverse and longitudinal sections (Plate-11, Fig. B, C; Plate-13, Fig. A; Plate-12, Fig. D). 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 (Plate-12, Fig.-D).

The anterior tip of the head terminates in an electron lucent acrosomal cap. The acrosomal cap sends off acrosomal barb at the anterior end (Plate-12, Fig. C). 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 (Plate-12, Fig. B).

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 (Plate- 12, Fig. D; Plate-13, Fig. B).

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 (Plate- 12, Fig. D). The distal centriole lies perpendicular to the nuclear axis, slightly away from the proximal centriole. Microfilaments of

PLATE – 8:

Scanning Electron micrographs of the longitudinal section of *Bufo himalayanus* testis.

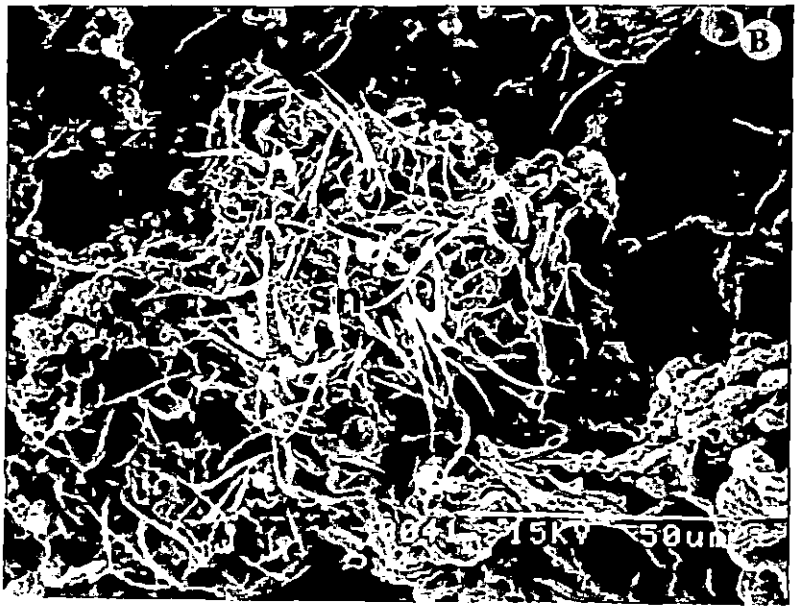
Figure A. Longitudinal section of *B. himalayanus* testis at low magnification showing the inner surface morphology; sperm cluster and the empty nests are well visualized.

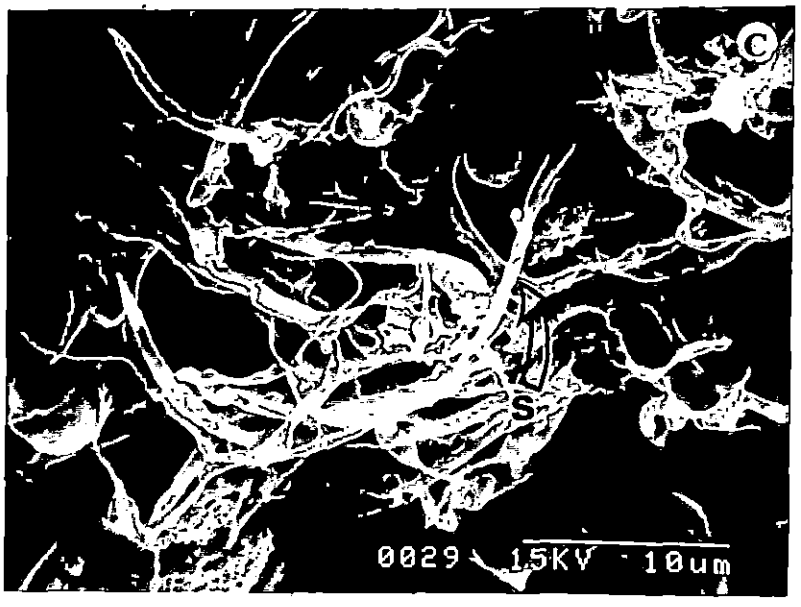
Figure B. Sperm nest, showing cluster of mature sperm.

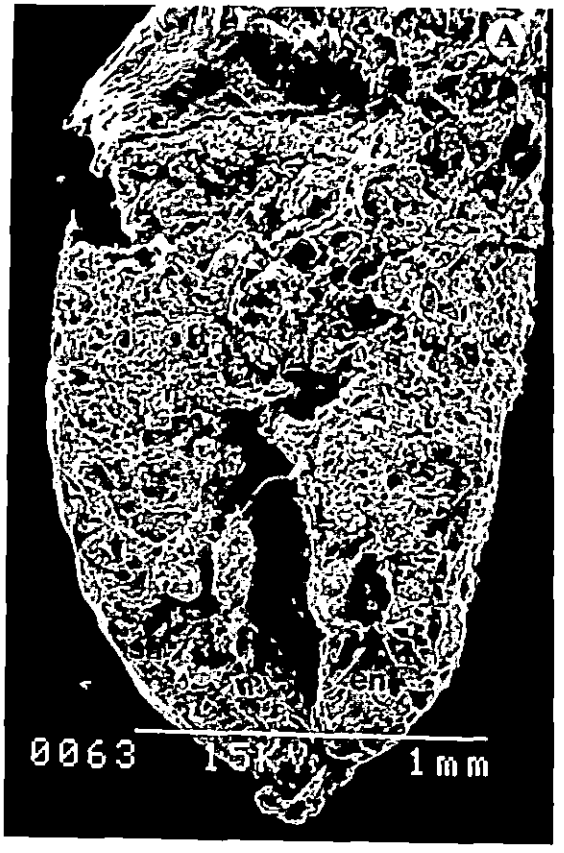
Figure C. Magnified view of sperm nest.

Figure D. Empty nest indicating clearly that the mature sperms has been released.

Indication: SN - sperm nest; S – mature sperm; EN – empty nest.


B
0041 15KV 50um


C
S
0029 15KV 10um


A
0063 15KV 1mm

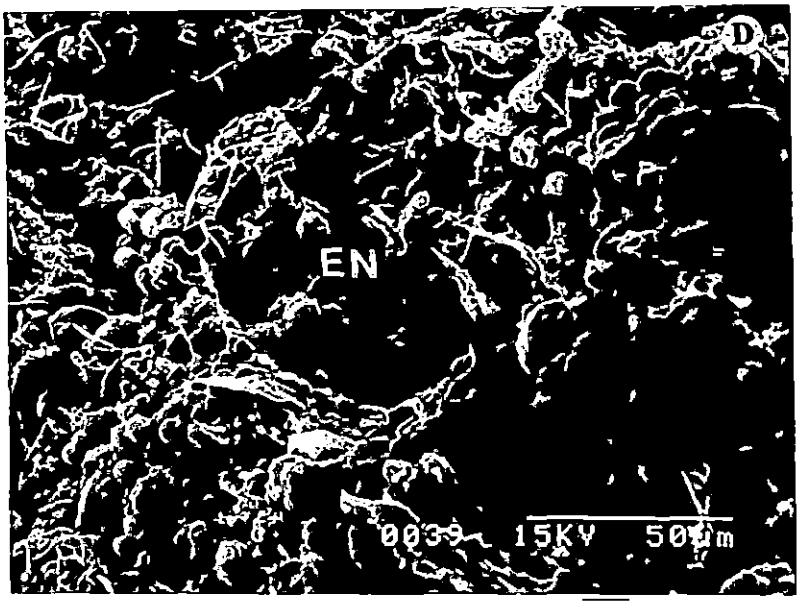

D
EN
0039 15KV 50um

PLATE – 9:

Scanning Electron micrographs of the longitudinal section of the testis of *Bufo stomaticus.*

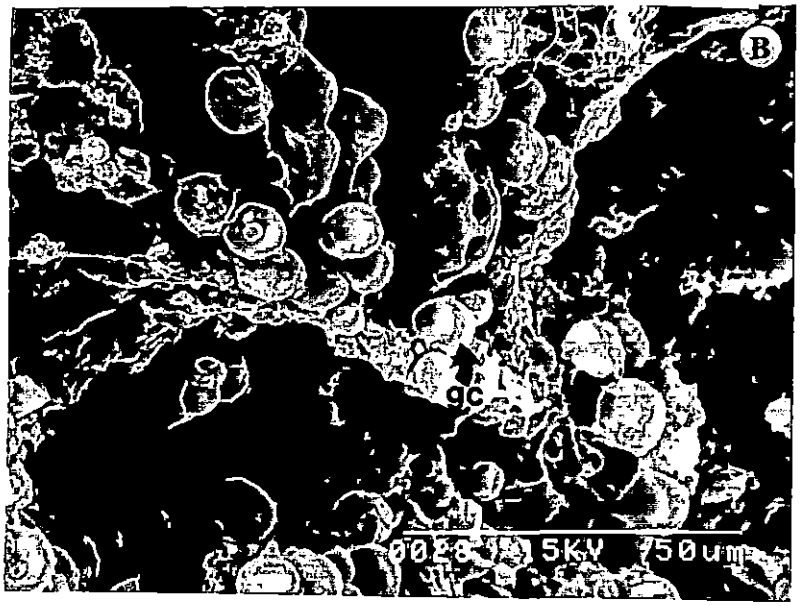
Figure A. Longitudinal section of *B. stomaticus* testis at low magnification showing the inner surface morphology; sperm cluster and the empty nests are well visualized.

Figure B. Cluster of goneal cells in a developing nest.

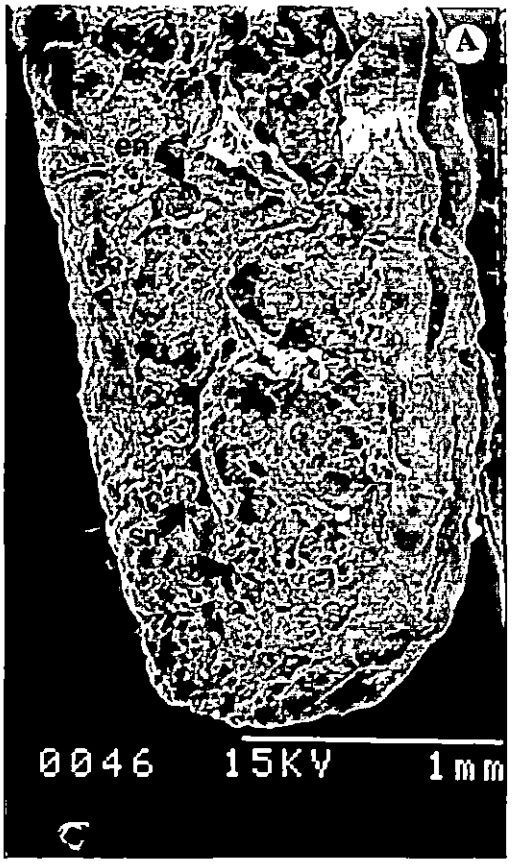
Figure C. Mature sperm cluster in a sperm nest, which is ready for being released.

Figure D. Empty nest showing no mature sperm. All the sperm of that nest has been released.

Indication: SN - sperm nest; EN – empty nest.


B
gc
0028 15KV 750um


C


A
en
0046 15KV 1mm

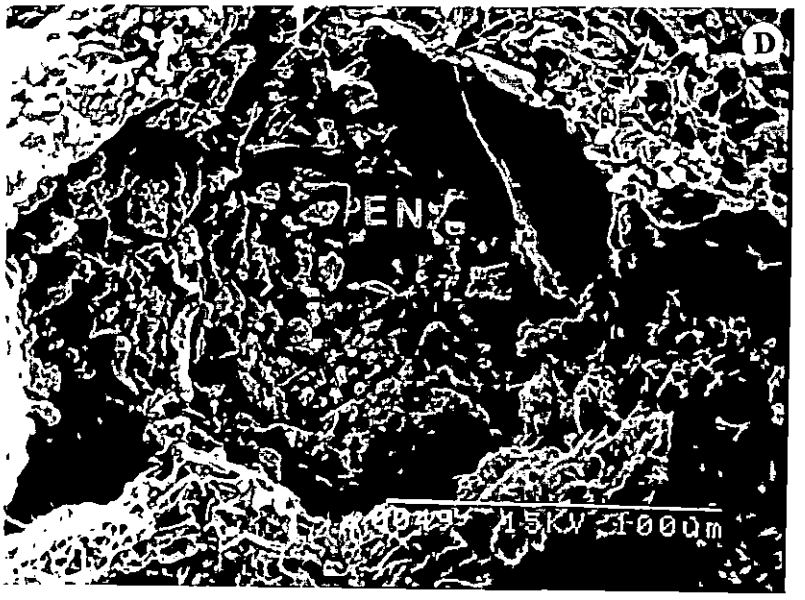

D
OPEN
15KV 100um

PLATE – 10:

Scanning Electron micrographs of the longitudinal section of the testis of *Bufo melanostictus*.

Figure A. Longitudinal section of *B. melanostictus* testis at low magnification showing the inner surface morphology. Distinct patches of sperm nests and empty nests are well visualized.

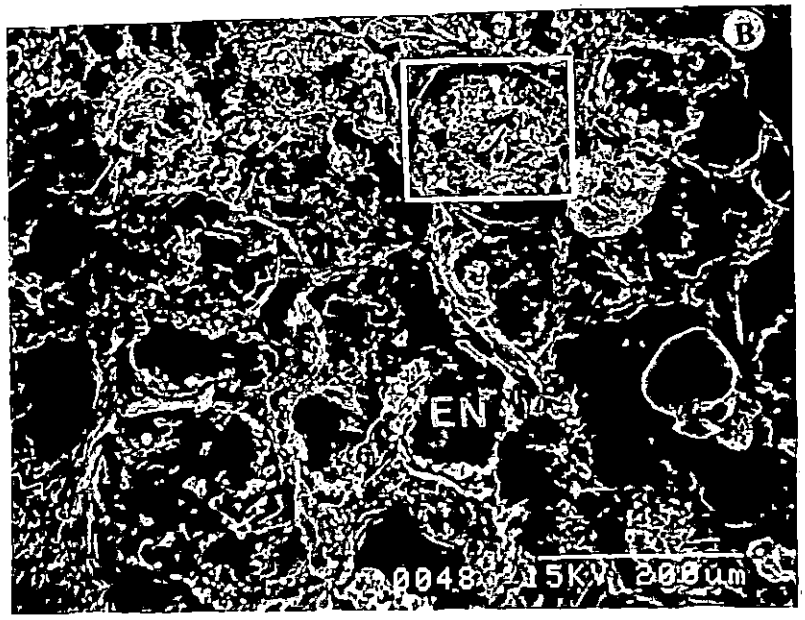
Figure B. Distinct empty and sperm nest; a sperm nest has been highlighted by white rectangular block.

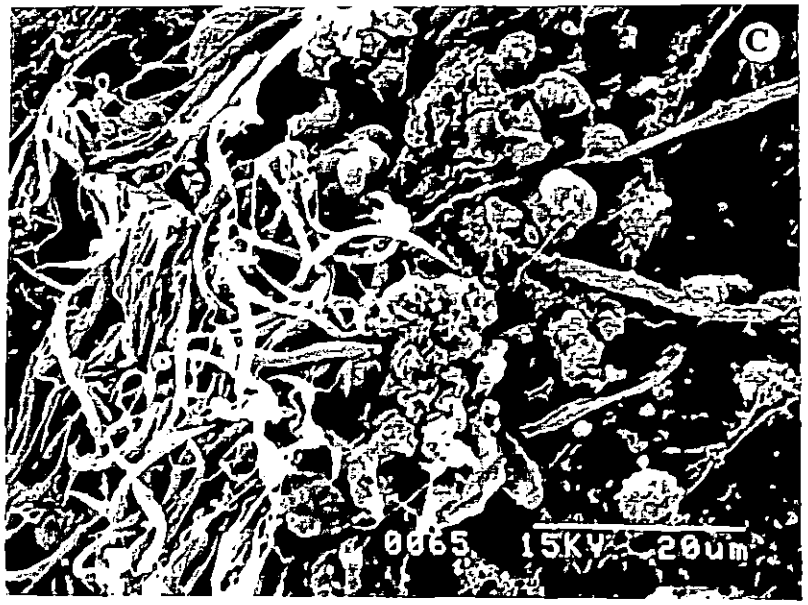
Figure C. Magnified view of the white block in figure A. showing the sperm nest where the mature sperm are well documented.

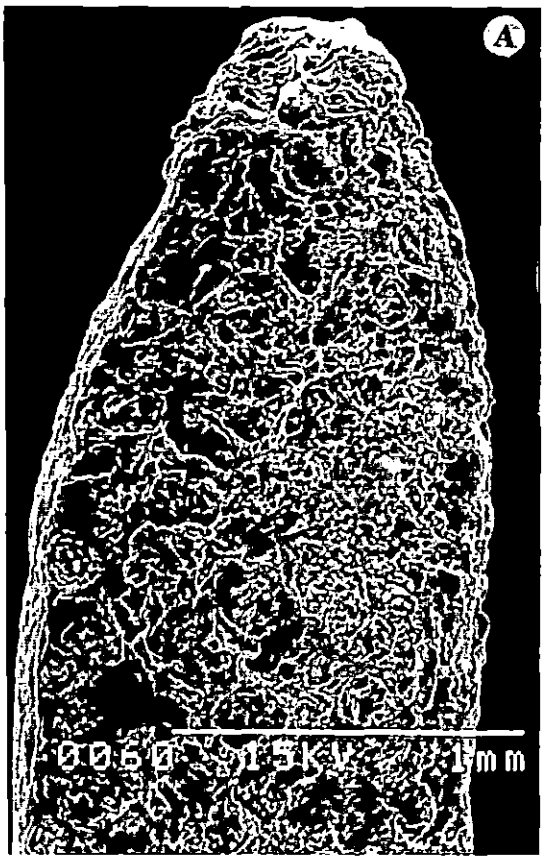
Figure D. Empty nest showing the presence of no mature sperm. All the sperm of that nest has been released.

Indication: SN - sperm nest; EN – empty nest.

PLATE – 10







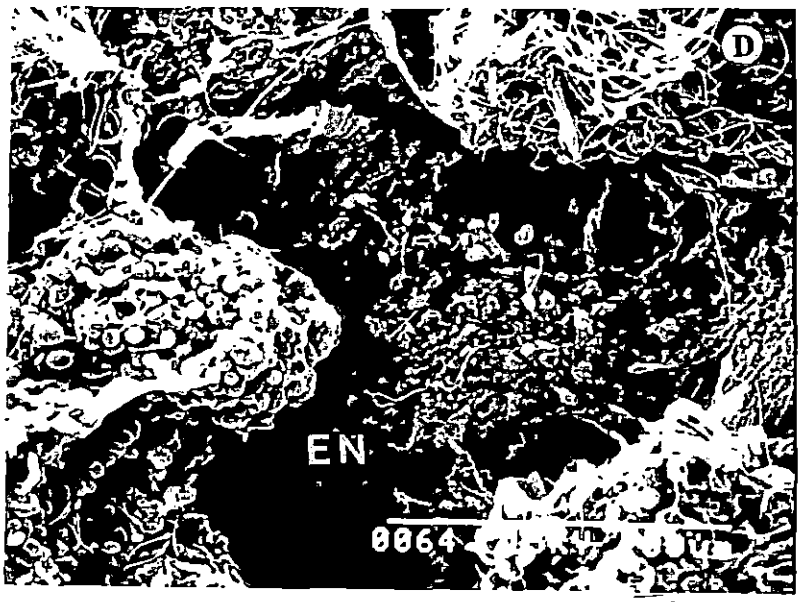


PLATE – 11:

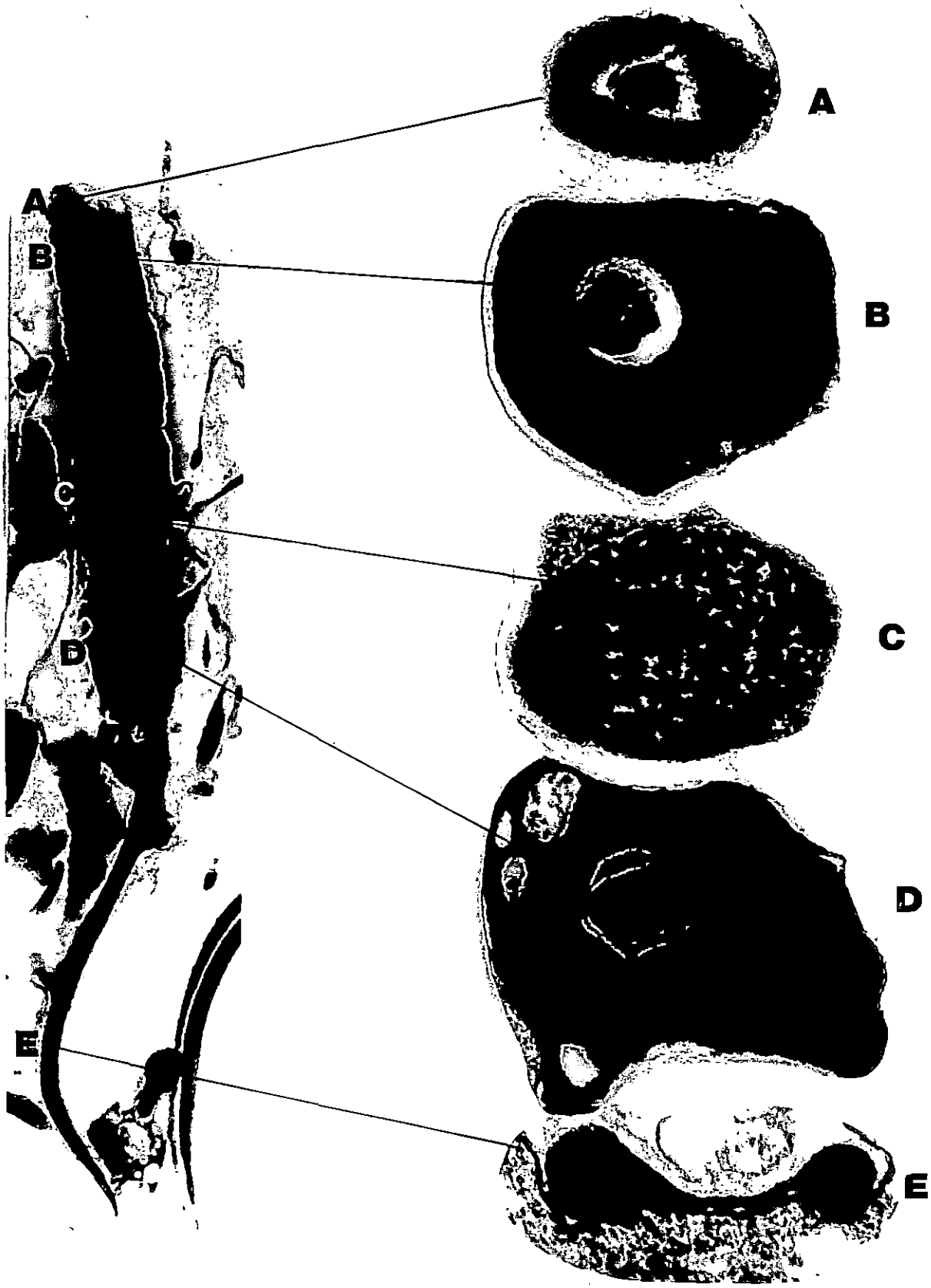
Transmission Electron micrographs of the longitudinal (a) and transverse (b) sections of the *Bufo himalayanus* sperm at different levels, showing the cellular organelles and structural organization.

Figure a. Longitudinal section of the sperm of *B. himalayanus* whose different parts have been marked as A- terminal tip, B- sub-terminal end, C- head nucleus, D- middle piece, and E- tail filament.

Figure b. Transverse section of different parts, which have been marked in the longitudinal section.

A. Transverse section through the acrosomal tip.
B. Grains in the middle circle indicating the presence of nuclear mass encircled by acrosomal part.
C. The middle portion of the nucleus indicated by the presence of pigmented granules throughout the diameter.
D. The middle piece with the peripheral mitochondria region and a central region from where the tail filament arises.
E. Transverse section of tail showing the axial filament with peripheral and central microtubules; an undulating membrane arises from the periphery of the axial filament and extend rightwards to form a button like knob.

PLATE – 11



(a) LONGITUDINAL SECTION (b) TRANSVERSE SECTIONS

PLATE – 12:

Transmission Electron micrographs of different parts of *Bufo himalayanus* sperm.

Figure A. Whole sperm of *Bufo himalayanus*. x2040

Figure B. Lightly pigmented distinct conical perforatorium (p). x8200

Figure C. Sperm head showing lightly pigmented acrosome (a), a distinct subacrosomal space (sa), a darkly pigmented nucleus (n) and an outer cytoplasmic layer (cp). x8200

Figure D. Darkly pigmented granules throughout the nucleus (n). At the subnuclear notch lies both the centrioles (c), proximal centriole (pc) and the distal centriole (dc) are located perpendicular to each other. x8200

PLATE – 12

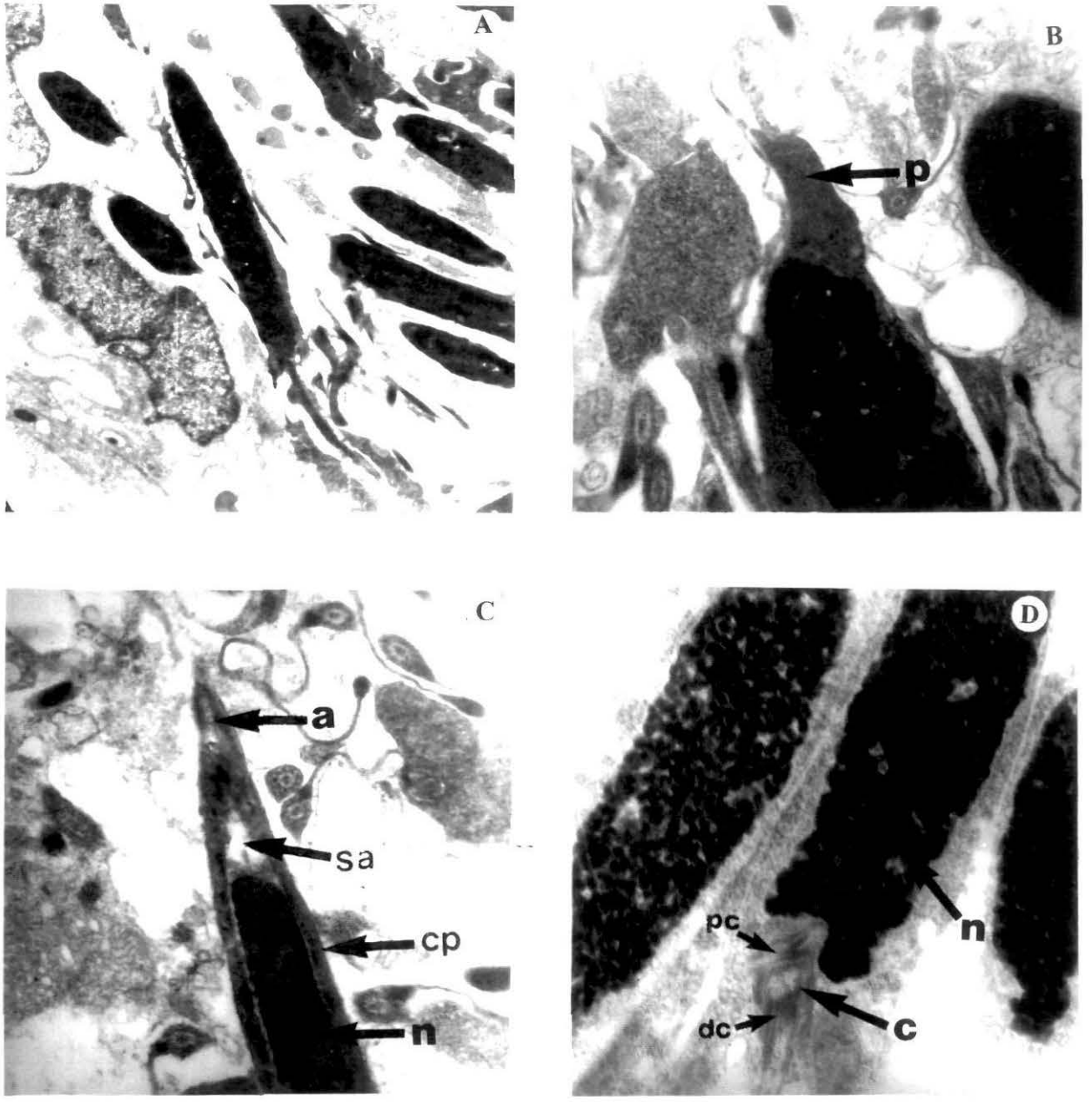


PLATE – 13:

Transmission Electron micrographs of different parts of *Bufo himalayanus* sperm.

Figure A. Transverse section of head region showing lightly pigmented cytoplasm and deeply pigmented nucleus. Pigmented granules (PG) in the nucleus are well documented. x8200

Figure B. Transverse section of middle piece showing peripheral layers of mitochondria (mc) and the central axial filament (af). x13500

Figure C. Transverse section of flagella showing distinct 18 peripheral and 2 central microtubule (mt). x21500

Figure D. Transverse section of flagella showing the projecting undulating membrane (u) from axial filament. x31000

PLATE – 13

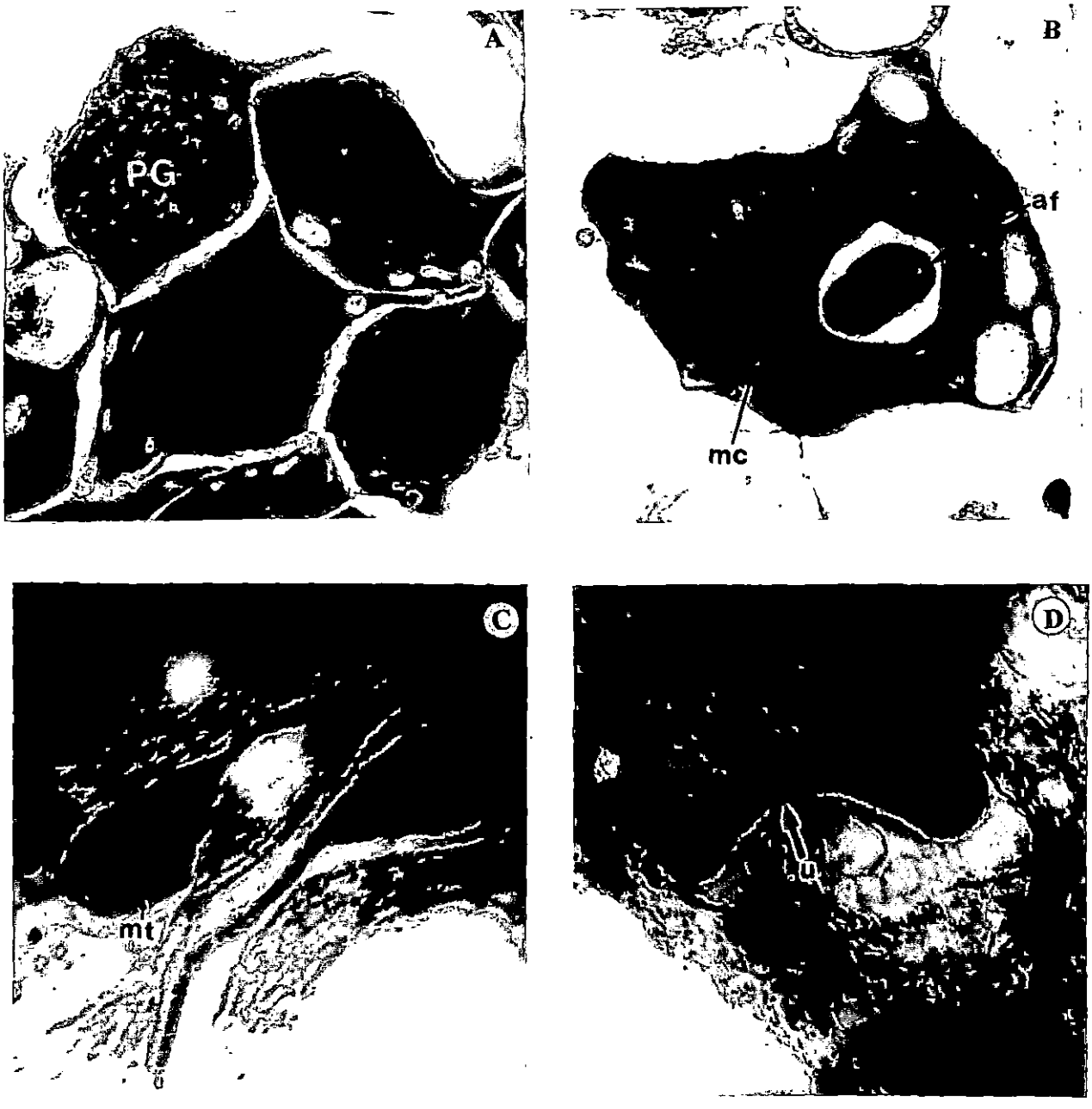


PLATE – 14:

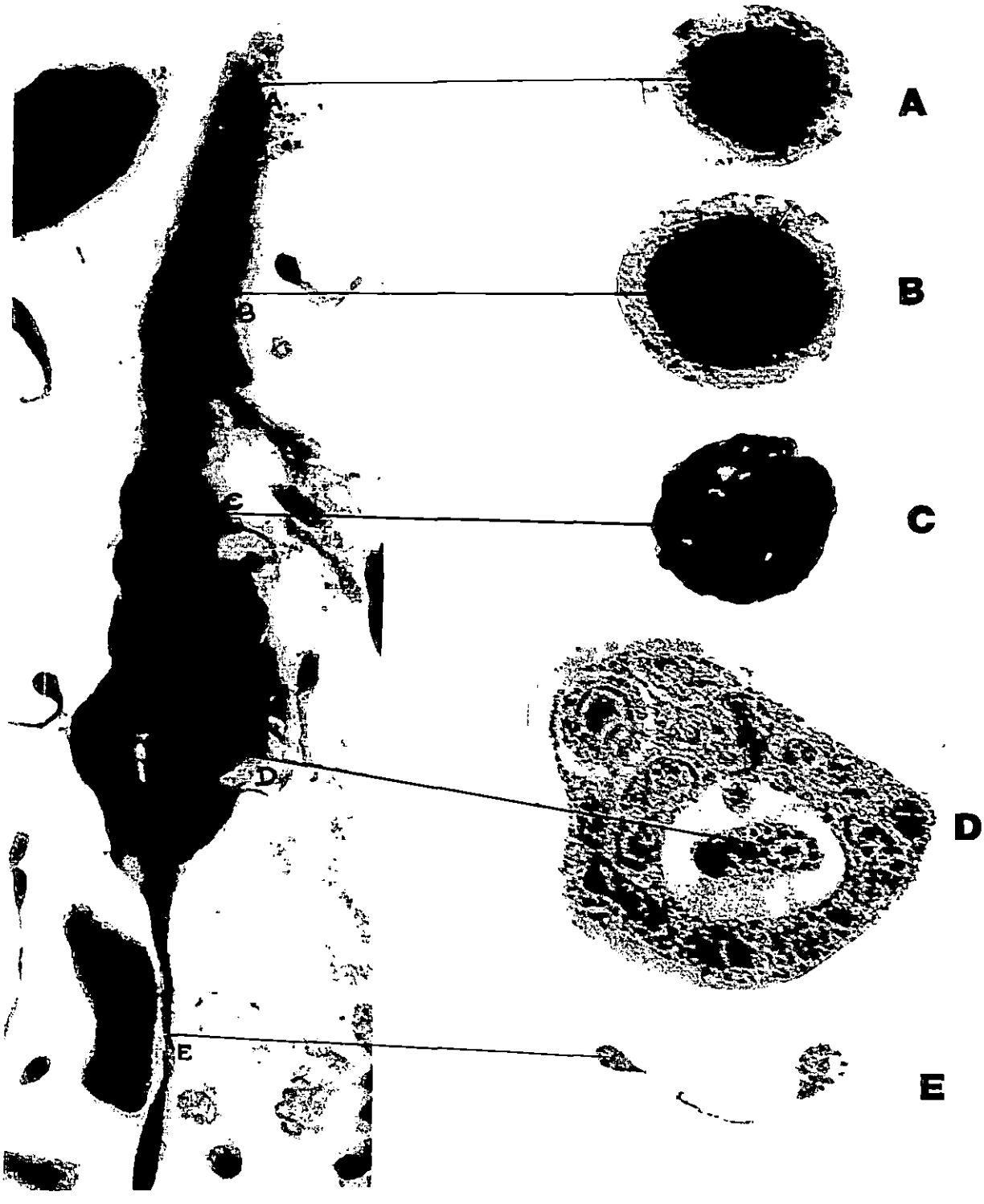
Transmission Electron micrographs of the longitudinal (a) and transverse (b) sections of the *Bufo stomaticus* sperm at different levels, showing the cellular organelles and structural organization.

Figure a. Longitudinal section of the sperm of *B. stomaticus* whose different parts have been marked as A- terminal tip, B- sub-terminal end, C- head nucleus, D- middle piece, and E- tail filament.

Figure b. Transverse section of different parts, which have been marked in the longitudinal section.

A. Transverse section through the acrosomal tip.
B. Showing deeply stained central nucleus encircled by lightly pigmented peripheral cytoplasmic material.
C. The middle portion of the nucleus indicated by the presence of pigmented granules throughout the diameter.
D. The middle piece with the peripheral mitochondria region and a central region from where the tail filament arises.
E. Tail consisting of a distinct axial filament containing 18+2 microtubules and an undulating membrane, which form a bulb like swollen part at the extreme tip.

PLATE – 14



(a) LONGITUDINAL SECTION (b) TRANSVERSE SECTIONS

PLATE – 15:

Transmission Electron micrographs of different parts of *Bufo stomaticus* sperm.

Figure A. Longitudinal section of the whole sperm. x2900

Figure B. Head nucleus showing distinct nuclear granules (ng). x5400

Figure C. Middle piece showing the axial filament (af) arising from the centriole; elongated mitochondria (m) are arranged haphazardly on both the sides of the middle piece. x8200

Figure D. Distinct proximal centriole (pc) located at the subumbrellar nuclear notch, containing 18 peripheral microtubules but no central microtubule. x10500

PLATE – 15

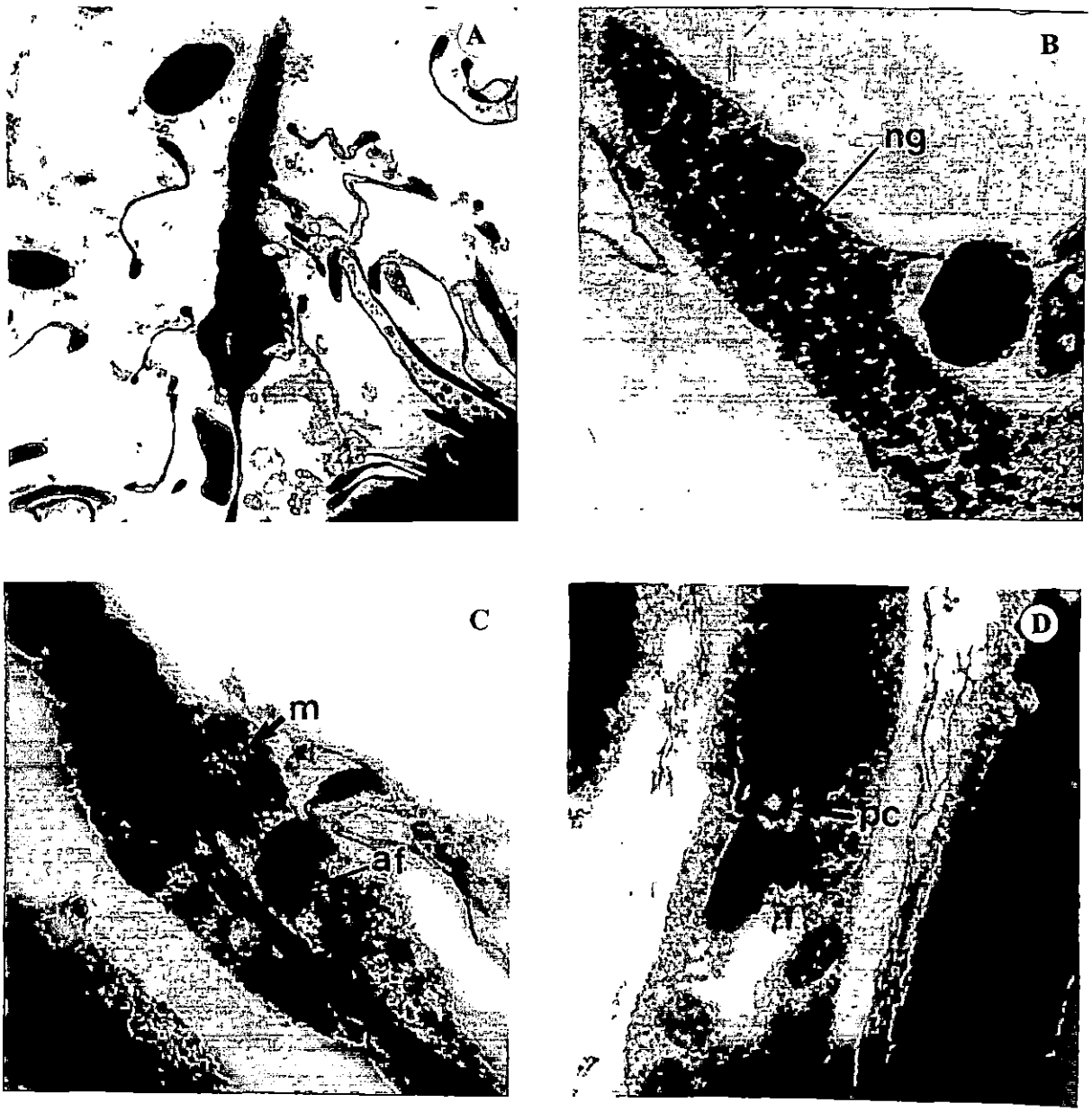


PLATE – 16:

Transmission Electron micrographs of different parts of Bufo stomaticus sperm.

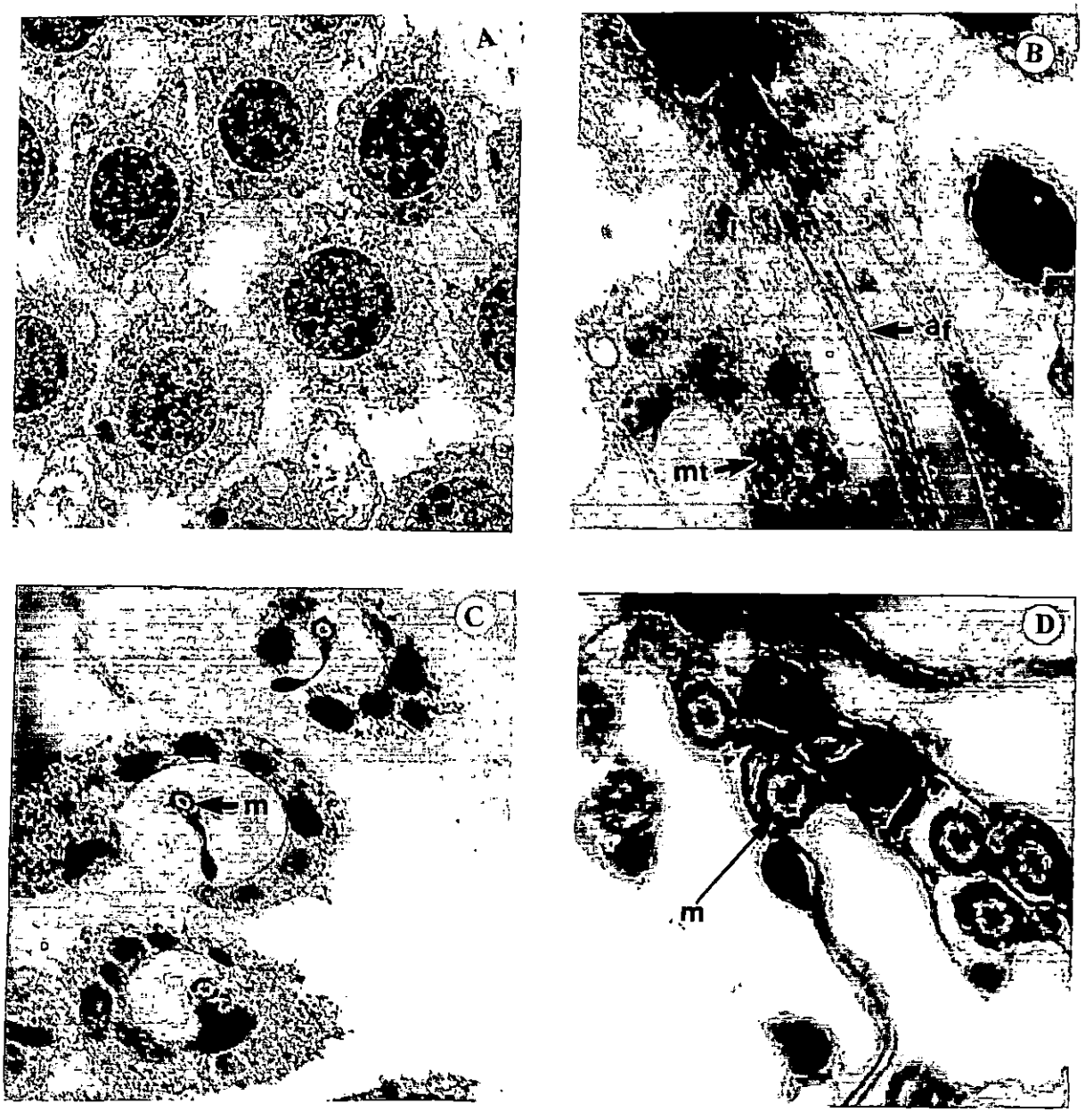
Figure A. Transverse section of the head region of the sperm showing numerous nuclear granules (ng). x2900

Figure B. Axial filament arising from the centriole of the middle piece; numerous oval mitochondria (mc) are located throughout the middle piece. x8200

Figure C. Transverse section of midpiece showing central microtubule encircled by peripheral mitochondrial rim. x5400

Figure D. Transverse section of the axial filament showing distinct 18 peripheral and 2 central microtubules (m). x21500

PLATE – 16



the centrioles are not very distinct as found in other two species described.

The tail is filamentous in appearance with a central flagellum (Plate-13, Fig. B). 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 (Plate-13, Fig. C). Well visible undulating membrane present, which terminates in a thick broad end (Plate-13, Fig. D).

Bufo stomaticus

In *Bufo stomaticus,* the head and middle piece are clearly demarcated (Plate-14, Fig. a). The head is elongated, slightly curved and circular in cross section (plate-14, Fig. b.B, bC) with rounded base and tapering tip. However the nucleus exhibits irregular thickening resulting in a rough surface morphology (Plate-15, Fig. A). Electron dense particles are well visualized throughout the head length in transverse and longitudinal section (Plate-16, Fig. A; Plate-15, Fig. B), 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 (Plate-15, Fig. B).

Well formed acrosome is visible with external acrosomal vesicle and internal subacrosomal cone (Plate-15, Fig. A, B). 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 (Plate-14, Fig. b.D; Plate15, Fig. C). 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 (Plate-15, Fig. C, D). Proximal centriole is situated within the posterior notch of the nucleus. It is round in appearance with nine peripheral submicroscopic tubules (Plate-15, Fig. D). In longitudinal section the posterior centriole is elongated in appearance (Plate-15, Fig. C; Plate-16, Fig. B).

The tail is filamentous in appearance with a central core made of flagella (Plate-16, Fig. B, C). 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 (Plate-16, Fig-D). 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 (Plate-16, Fig-C, D). This type of organization gives a biflagellar appearance of the tail (Plate-14, Fig.-b.D). (However, it may be mentioned that under light and electron microscopic observation the sperm tail has been described as biflagellate).

Bufo melanostictus

In *Bufo melanostictus* the head nucleus is elongated, slightly curved and circular in cross section (Plate-17, Fig. a, b). 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 (Plate-18, Fig. A).

The acrosome cap is smooth in morphology with distinct acrosome and subacrosomal cone over the pointed head (Plate-18. Fig. C). Distinct niddle shaped acrosomal barb is visible in the extreme tip of the head (Plate-18, Fig. B).

PLATE – 17:

Transmission Electron micrographs of the longitudinal (a) and transverse (b) sections of the *Bufo melanostictus* sperm at different levels, showing the cellular organelles and structural organization.

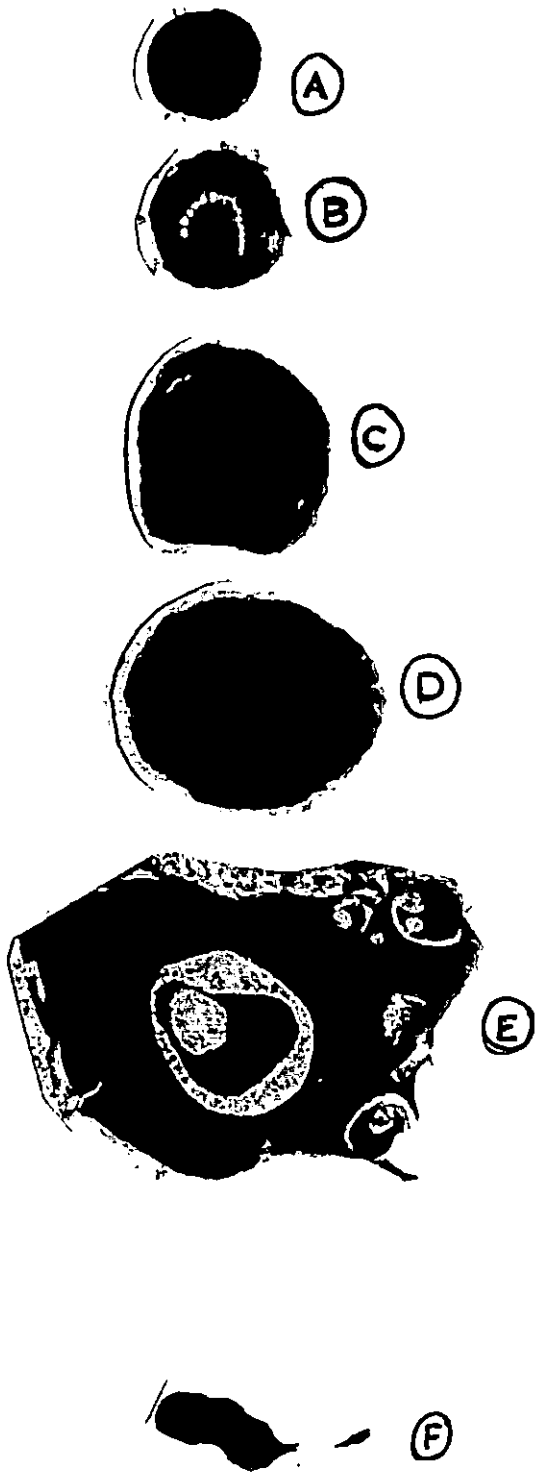
Figure a. Longitudinal section of the sperm of *B. melanostictus* whose different parts have been marked as A- terminal tip, B- sub-terminal end, C- anterior head nucleus, D- Posterior head nucleus, E. middle piece, and F- tail filament.

Figure b. Transverse section of different parts, which have been marked in the longitudinal section.

A. Transverse section through the acrosomal tip.
B. Grains in the middle circle indicating the presence of nuclear mass encircled by acrosomal part.
C. The deeply pigmented central nuclear region gradually increase in diameter indicating the anterior region of the nucleus.
D. The posterior portion of the nucleus indicated by the presence of pigmented granules throughout the diameter.
E. The middle piece with the peripheral mitochondria region and a central region from where the tail filament arises.
F. Transverse section of tail showing the axial filament with peripheral and central microtubules; an undulating membrane arises from the periphery of the axial filament and extend rightwards to form a button like knob.

PLATE – 17

(a) LONGITUDINAL SECTION



(b) TRANSVERSE SECTIONS

PLATE – 18:

Transmission Electron micrographs of different parts of *Bufo melanostictus* sperm.

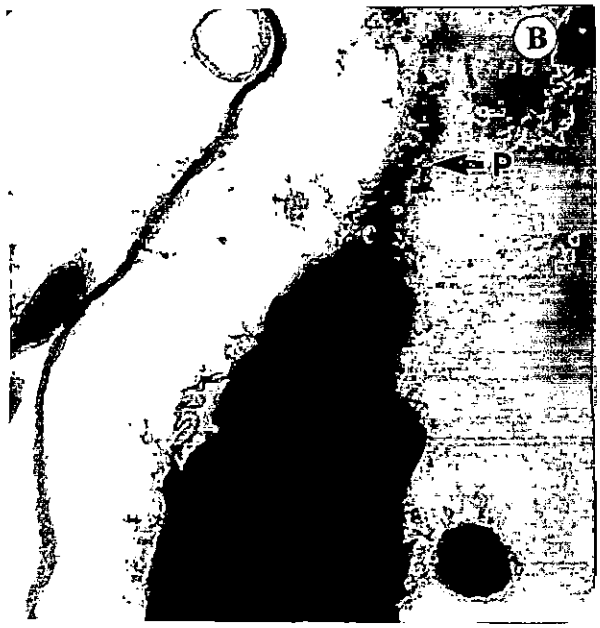
Figure A. Longitudinal section of the whole sperm showing the proximal (PC) and distal centriole (DC). x3400

Figure B. Head portion showing distinct pinpointed perforatorium (P). x13500

Figure C. Distinct acrosome (A) and subacrosomal space is well visualized. The acrosomal membrane is continuous with the nuclear membrane. x8200

Figure D. Numerous sperms remail embedded in the Sertoli cell. x2050

PLATE – 18



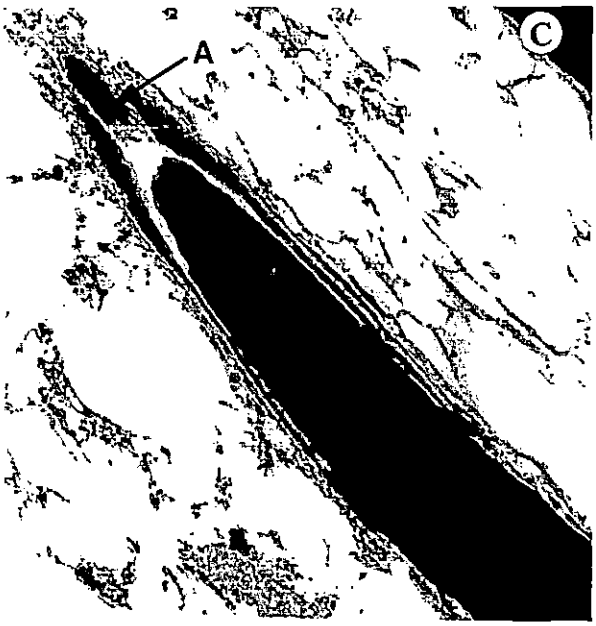


PLATE – 19:

Transmission Electron micrographs of different parts of *Bufo melanostictus* sperm.

Figure A. Transverse section of nucleus showing numerous electron dense granules (NG). x10500

Figure B. Tail filament arising from the distal centriole; proximal centriole (pc) distinct and located in the subumbrellar nuclear notch. x13500

Figure C. Middle piece showing central axial filament (af) surrounded by numerous mitochondria. x5400

Figure D. Distinct axial filament with 18 peripheral and 2 central microtubule; undulating membrane arising from this axial filament. x21500

PLATE – 19

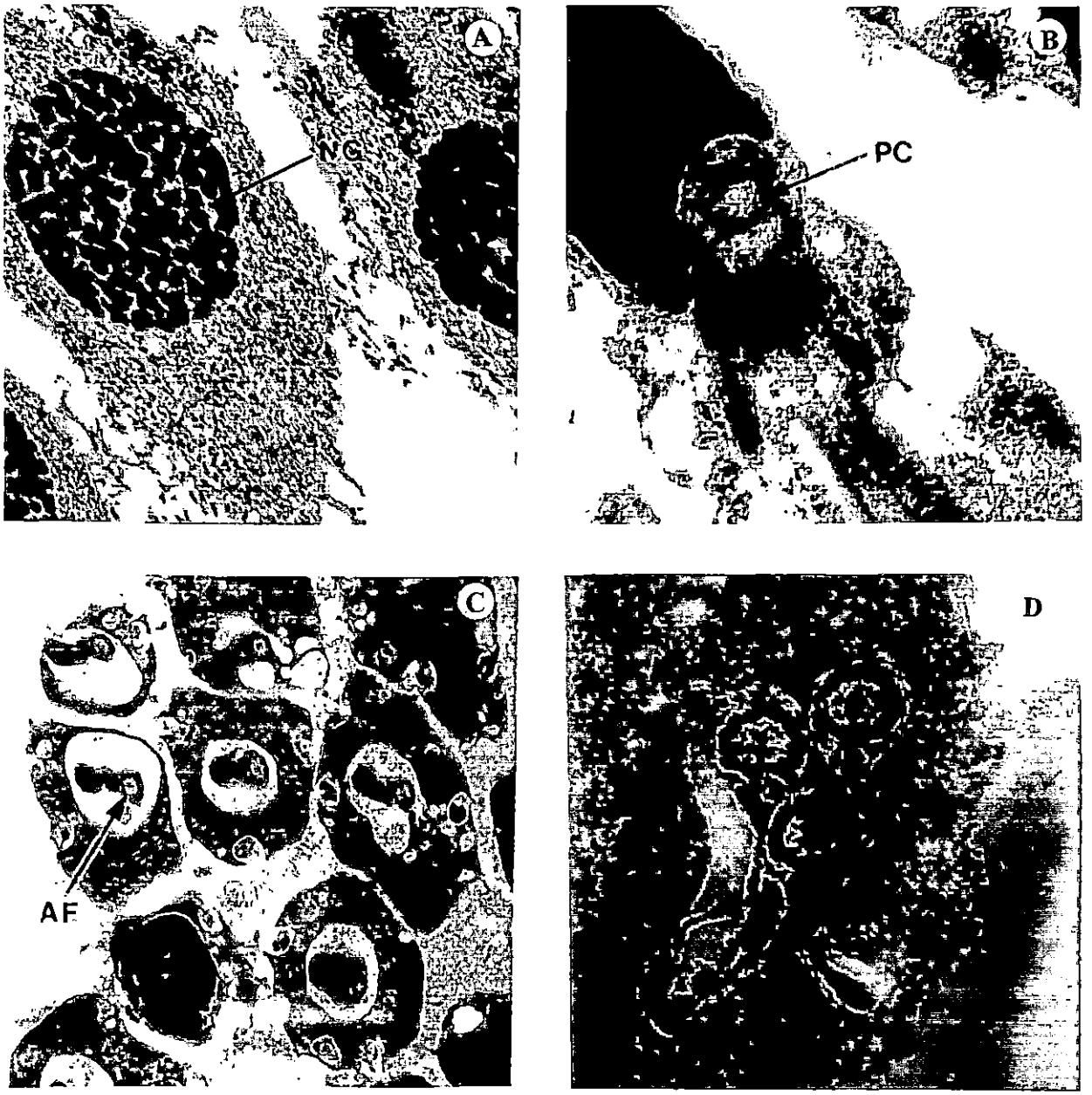


PLATE – 20:

Light microscopic picture of sperm of *Rana cyanophlyctis* at x3000 magnification.

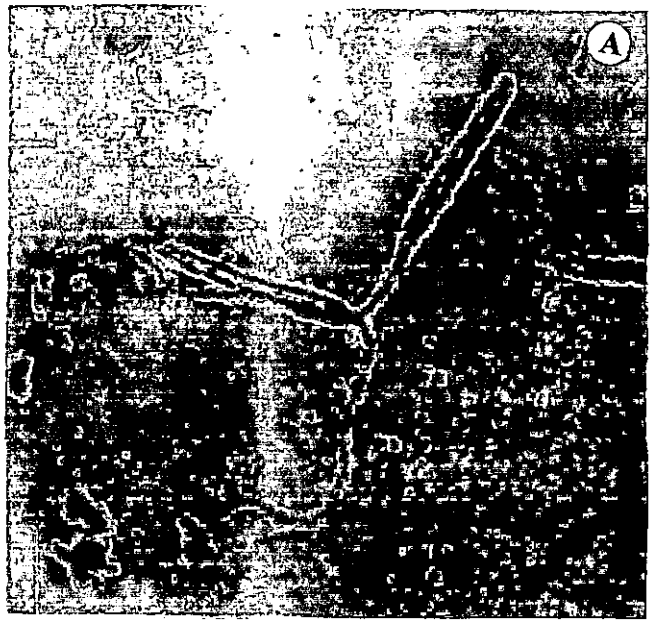
A, B, C shows normal sperm with elongated nucleus and single tail filament.

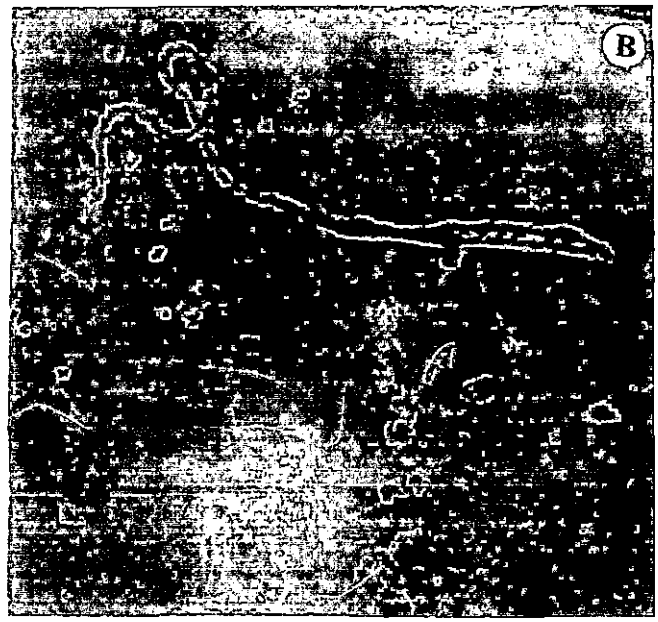
D- Megacephalic sperm

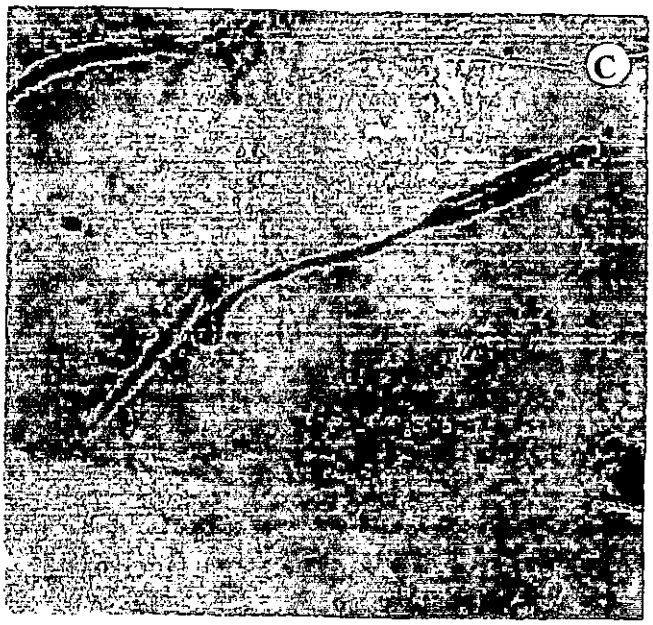
E- Interstitial cells of Leydig

F- Sperms hanging from the Sertoli cell

PLATE – 20







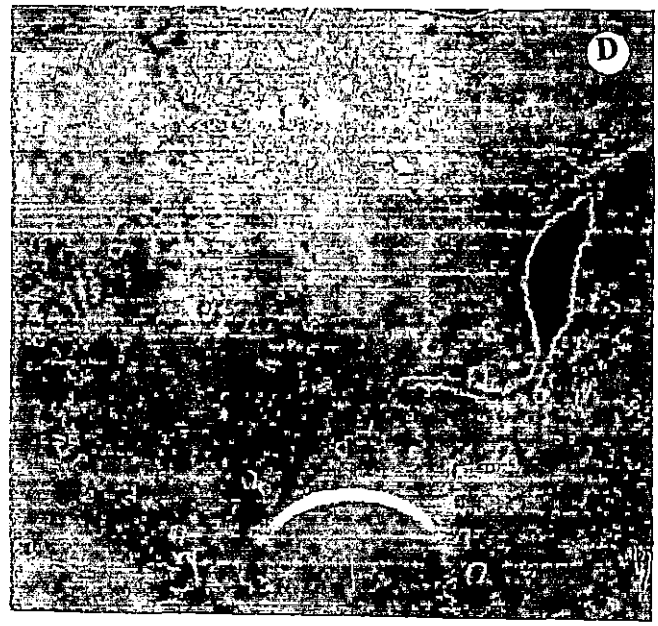


PLATE – 21:

Scanning and Transmission Electron micrographs of sperm of *Rana cyanophlyctis*.

A. Longitudinal section of *R. cyanophlyctis* testis. (SEM)

B. Sperm nest. (SEM)

C. Single normal sperm. (SEM)

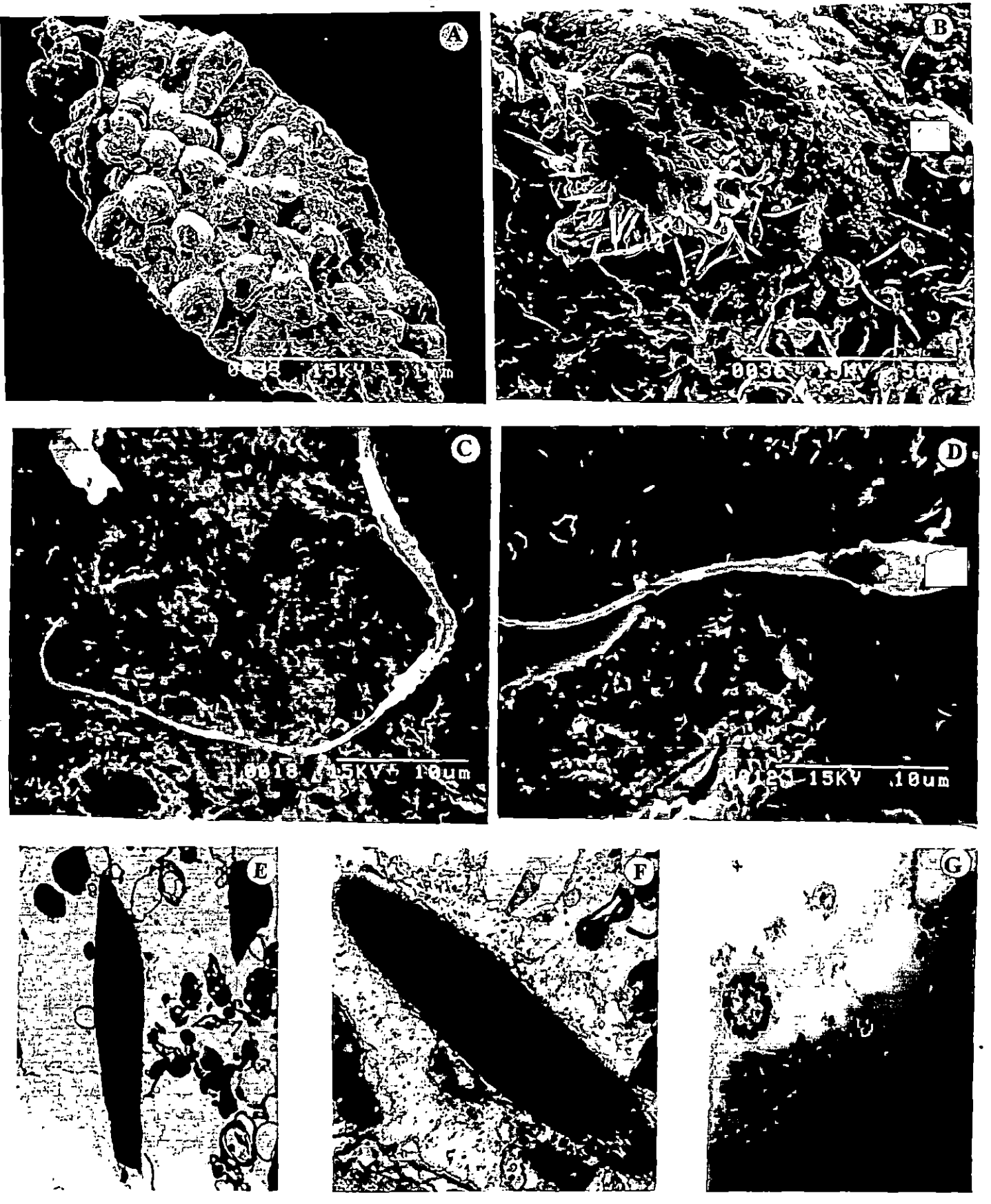
D. Megacephalic sperm (SEM)

E. Pointed acrosomal tip. x3400

F. Electron dense head nucleus. x4600

G. Axial filament of the tail with 18 peripheral and 2 central microtubule but without undulating membrane. x21500

PLATE – 21



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 (Plate-17, Fig. a). 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 (Plate18, Fig. A; Plate-19, Fig. B).

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 (Plate-19, Fig. D). 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 (Plate-19, Fig. C, D).

DNA Analysis Observation

Through Gel Electrophoresis

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} .

The obtained results are detailed in Table-4.

TABLE - 4

Spectrophotometric results of isolated DNA

Serial Number	Sample Number	OD at 260 nm	OD at 280 nm	OD260/OD280		DNA Content
01.	Bh1a	0.26	0.14	1.86	✔	**13.0 µg / ml**
02.	Bh1b	0.17	0.09	1.88	✔	**08.5 µg / ml**
03.	Bh1c	0.28	0.18	1.55	✘	14.0 µg / ml
04.	Bh2a	0.22	0.12	1.83	✔	**11.0 µg / ml**
05.	Bh2b	0.24	0.13	1.85	✔	**12.0 µg / ml**
06.	Bh2c	0.22	0.12	1.83	✔	**11.0 µg / ml**
07.	Bh3a	0.16	0.13	1.23	✘	08.0 µg / ml
08.	Bh3b	0.25	0.14	1.78	✘	12.5 µg / ml
09.	Bh3c	0.17	0.10	1.70	✘	08.5 µg / ml
10.	Bs1a	0.15	0.08	1.87	✔	**17.5 µg / ml**
11.	Bs1b	0.22	0.16	1.37	✘	14.5 µg / ml
12.	Bs1c	0.17	0.10	1.70	✘	11.0 µg / ml
13.	Bs2a	0.16	0.09	1.77	✘	08.0 µg / ml
14.	Bs2b	0.16	0.08	2.00	✔	**08.0 µg / ml**
15.	Bs2c	0.21	0.14	1.50	✘	10.5 µg / ml
16.	Bs3a	0.16	0.08	2.00	✔	**08.0 µg / ml**
17.	Bs3b	0.15	0.08	1.87	✔	**07.5 µg / ml**
18.	Bs3c	0.22	0.12	1.83	✔	**11.0 µg / ml**
19.	Bm1a	0.37	0.18	2.05	✔	**18.5 µg / ml**
20.	Bm1b	0.32	0.18	1.77	✘	16.0 µg / ml
21.	Bm1c	0.35	0.15	2.33	✔	**17.5 µg / ml**
22.	Bm2a	0.28	0.18	1.55	✘	14.0 µg / ml
23.	Bm2b	0.28	0.16	1.75	✘	14.0 µg / ml
24.	Bm2c	0.35	0.22	1.59	✘	17.5 µg / ml
25.	Bm3a	0.32	0.18	1.80	✔	**16.0 µg / ml**
26.	Bm3b	0.32	0.17	1.88	✔	**16.0 µg / ml**
27.	Bm3c	0.35	0.21	1.67	✘	17.5 µg / ml

PLATE – 23:

Photographs of DNA after gel electrophoresis-

A. Genomic DNA of three different species of family Bufonidae ;

Lane 1- *Bufo himalayanus*

Lane 2- *Bufo stomaticus*

Lane 3- *Bufo melanostictus*

B. Bam H_1 digested DNA of three different species of family Bufonidae ;

Lane 4- *Bufo himalayanus*

Lane 5- *Bufo stomaticus*

Lane 6- *Bufo melanostictus*

C. Eco R I digested DNA of three different species of family Bufonidae ;

Lane 4- *Bufo himalayanus*

Lane 5- *Bufo stomaticus*

Lane 6- *Bufo melanostictus*

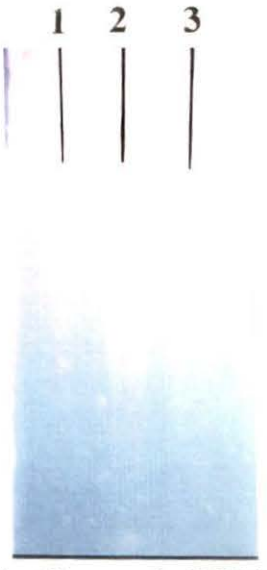
D. Hind II digested DNA of three different species of family Bufonidae ;

Lane 4- *Bufo himalayanus*

Lane 5- *Bufo stomaticus*

Lane 6- *Bufo melanostictus*

PLATE – 23



A. Genomic DNA

B. Bam H_1 digest

C. EcoR I digest

D. Hind II digest

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.

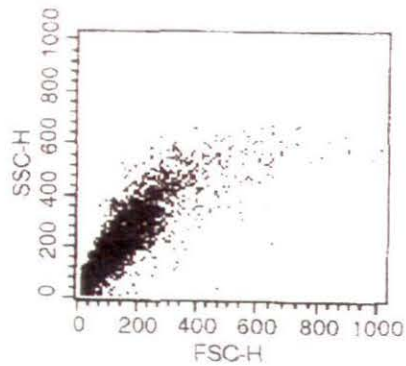
From the gel pictures (Plate-23) it can be analysed as follows :-

In Gel A (genonic DNA) it can be seen that all the genomic DNA bands were almost in the same level with the *B.himalayaus* is at slightly upper level than the other two (*B. stomaticus and B. melanostictus).* This indicates that the genomic DNA of *B.himalayanus* is slightly greater in size than *B. stomaticus and B. melanostictus.*

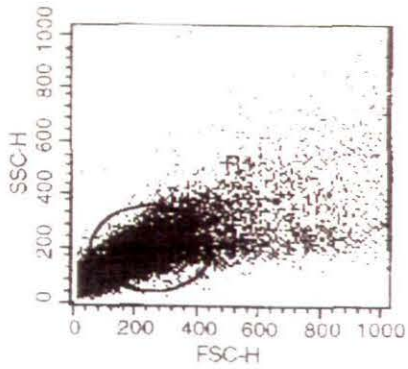
Analysing Gel A we can see that after Bam H_1 digest all the DNA samples in the gel has moved down indicating the restriction cut of the DNA and fragmentation has taken place. Here also we can see that the DNA of *B. himalayanus* is running behind the other two, while that of *B. stomaticus* and *B. melanostictus* are running neck to neck indicating quantative similarity in the genomic DNA if the two (*B. stomaticus* and *B. melanostictus*).

Lane 7, 8, & 9 of Gel C is showing the restriction digest of genomic DNA of all the three species by EcoR I. and it is seen that this particular enzyme is more sensitive to *B. stomaticus* than the other two. But a different situation arises in case of Hind Iiof Gel D . 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.

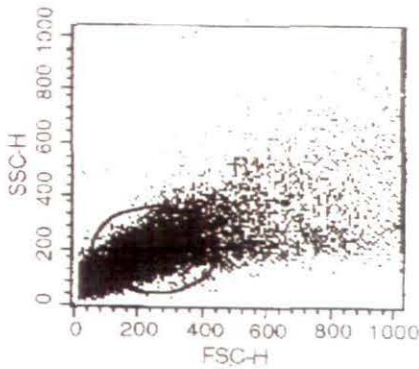
PLATE – 24



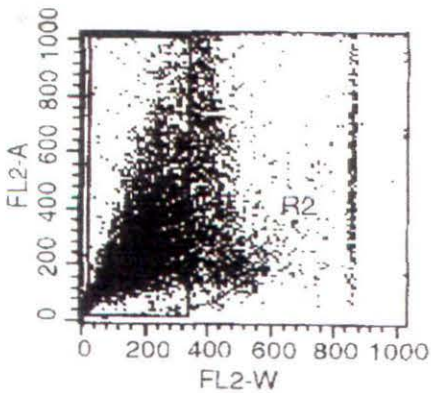
Bufo himalayanus



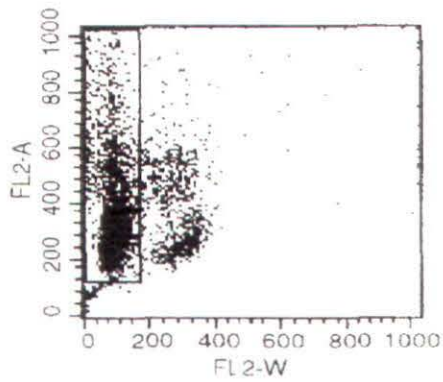
Bufo stomaticus



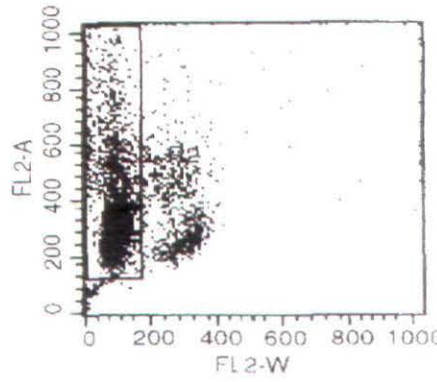
Bufo melanostictus



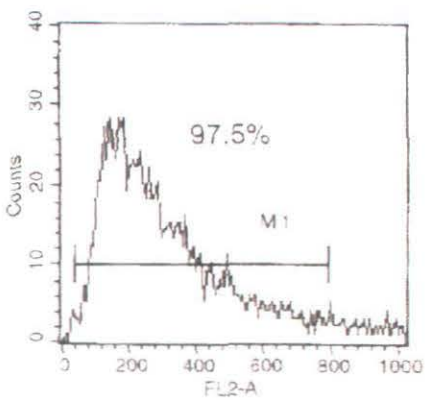
Bufo himalayanus



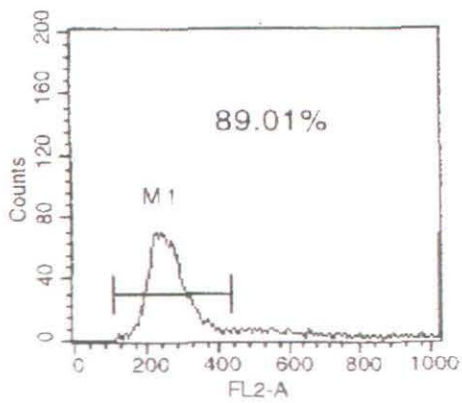
Bufo stomaticus



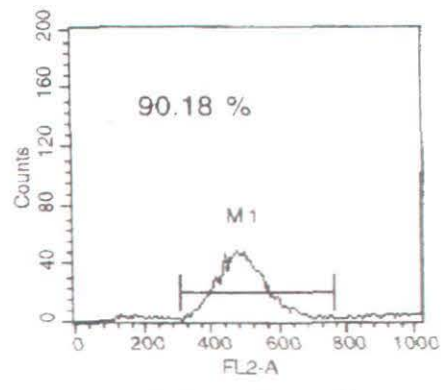
Bufo melanostictus



Bufo himalayanus



Bufo stomaticus



Bufo melanostictus

Result of FACS study for measuring DNA content of sperm of three different species of the family Bufonidae.

Through Fluorescence Activated Cell Shorting

From the graph result (Plate-24), 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.

DISCUSSION

A. Testicular size and composition

In Amphibia the gonadal activity is highly dependent on environmental cues like light, rainfall, temperature etc. (Jorgensen et.al.,1979 ; Lofts,1984 ; Rastogi and Iela,1980 ; Saidapur, 1983). In tropical areas, rainfall and temperature together interplay a vital role in the overall testicular as well as ovarian activity. Endocrine regulation is also influenced by these factors (Jorgensen et.al.,1979 ; Lofts,1984 ; Rastogi and Iela,1980 ; Saidapur, 1983).

The relative increase in gonadal size in males in the species studied is confirmatory to the earlier observations (Ray et.al., 1989 ; Ray et.al.,1999 ; Mukhopadhyay,2002). 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 (Basu ,1962,1969 ; Basu and Mondal, 1960 ; Loft,1976).

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. This observation is usually collelated with difference in activity of the two testis as observed in mammals (Pierce and Breed, 2001). However, here it can be viewed in a different angle. Harms (1926), Ponse (1926) first observed that in the tadpole of Bufo, the anterior portion of developing gonad become enlarge and in the adults form the Bidder's organ. However, the gonadal differentiation and appearance of Bidder's organ is not synchronized in the developing gonads (Mukhopadhyay,

2002). Paladhi et.al. (2003) in their observation on Bidders organ and testicular activity in *Bufo himalayanus* and *Bufo melanostictus* have shown that the left Bidder's organ exhibit less ovarian specialization than the right one, suggesting that the left testis (Anlagen) slowly differentiate in to the male gonad than the right one. Pronounced ultrastructural changes have been noted in the species studied. The endocrine control of the phenomenon is under study (Gomes, 2003).

B. Sperm morphology

The sperm morphology is conventionally studied by using double staining technique (Haematoxylin-Eosin, PAS-Haematoxylin) or by using metachromatic dye (Giemsa). Sharma and Dhindsha first described the light microscopic structure of *Bufo stomaticus* sperm together with the whole spermatogenesis of *B.stomaticus*. Accordingly the ripe spermatozoon of the toad posses a deeply stained head and a pointed niddle like acrosome situated at its anterior tip. Immediately behind the head and in intimate contact with it a deeply stained small middle piece was noted. The middle piece was found to be stud with mitochondria and two centrioles lying side by side. There are two axial filaments jointed together by a rudimentary undulating membrane. Each filament arises from its own centriole. Similar observation was made by Bowen in *Bufo arenarum*. Similalarly, the light microscopic structure has been described in *Bufo melanostictus* (Bandhyopadhyay, et.al.,1977) and *Bufo himalayanus* (Mukhopadhyay,2002). The present observation is also confirmatory to the earlier observations. But it is to be noted that the double axial filaments as observed under light microscopy is somewhat erroneous and under electron microscope it is revealed as a single axial rod as because one of the filaments is not containing submicroscopic tubules and that is the extended folded part of the undulating membrane.

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. However, there is more or less constancy in the middle piece architecture in all the three species studied. (Fig. 4,5)

The variation in sperm length should be viewed under high magnification using scanning electron micrographic technique.

Scanning electron microscopy provides a scope to study the surface topography of the parts described under light microscopy. The scanning electron microscopic observation strongly supports the light microscopic features mentioned earlier. In *B.himalayanus* and *B.melanostictus* the surface morphology of headpiece is smooth while in *B.stomaticus* the head surface bears small wart like projections of variable shape and size along its length. Such topological variation may be equated with the mode of fertilization in this species. B.stomaticus being a representative of arid and valley region needs more support to bind tightly with egg membrane surface. Semik and Kilarsky (1998) using scanning electron microscopy revealed that there is a significant difference of surface topography of unfertilized and fertilized eggs of common toad *Bufo bufo*. In unfertilized eggs two types of surface protrusions were found: microvilli and microfolds. As fertilization involves a "lock and key" interaction between sperm and ova the surface protrusion of *Bufo stomaticus* sperm may be considered as complementary binding sites of egg surface. Soon after fertilization it has been found that the whole egg surface of *Bufo bufo* becomes surrounded by a group of small depressions which gradually spread over the whole surface, presumably to prevent polyspermy. However,

no such literature is available on the fertilization event of *B.himalayanus, B.melanostictus* and *B.stomaticus* under high resolution.

The variation in sperm morphology as revealed under light microscope has been confirmed by scanning electron microscopy.

The polymorphism of sperm morphology is not a monopoly of anuran species. Similar polymorphism has been noted in urodeles (Wortham.1977 ; Wortham et.al.,1982 ; Roy,1989 ; Patra,2003; etc) and caecilians (Seshachar, 1939).

The polymorphism in sperm structure is a subject of debate since 1836 when Siebold recorded the existence of two types of spermatozoa in *Paludina* (a pond snail). Since then many cases of conspicuous polymorphism have been found in invertebrates and in some vertebrates (Ankel, 1924,1930,1933, 1958; Heberer,1932; Gupta,1964; Koehler and Birky,1966; Macleod,1970; Ray,1978; Roy and Ray,1989; Chatterjee et.al.,2002; Patra et.al.,2003).

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 (Koehler and Birky,1966; Macleod,1970; Ray,1978; Roy and Ray,1989; Chatterjee et.al.,2002; Patra et.al.,2003).

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 (Zhao et.al.,1996). 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.(Burgos and Fawcett,1956). Therefore, sperm abnormality represents either "switching off" of some sperm specific genes or duplications as found in some amphibians (Roy and Ray,1989).

C. Sperm Ultrastructure

Burgos and Fawcett (1956) first provide a remarkable account of sperm ultrastructure while narrating the spermiogenesis of *Bufo arenarum* (this description has been considered as out group in my dissertation to consider phylogenetic relationship using sperm ultrasructure as a tool). The description includes the formation of acrosome, changes in the nucleus, a perforatorium, centrioles, tail and undulating membrane.

Burgos and Fawcett (1956) noted that at the very early stage, the spermatids of toad lack a conspicuous Golgi complex such as occurs in mammalian spermatids. The Golgi material is represented by one or more small aggregations of spherical vesicles of varying size. Associated with these there are parallel double membranes represent actually the section of extremely thin, flattened vesicles. In the differentiation of the spermatids certain of the vesicles of Golgi complex appear to coalesce, giving rise to a acrosomal vacuole. This vacuole applies itself to the anterior pole of the nucleus. The vacuole gradually increases its area of contact with the nuclear membrane and extends further down over the elongated nucleus at its anterior pole. Burgos and Fawcett (1956) also provided an excellent account of nucleus condensarion due to which round or spherical nucleus of spermatid transforms into rod like nucleus in ripe spermatozoon. During these changes of the nucleus striking change takes place in the fine structure of the nuclear material. The homogeneous nuclear material of the spermatid representing fine granules (100-150 A°) coalesce and give rise to dense granules as well as appearance of nuclear lacunae. In recent years only the molecular background of this compactation process has been worked out. Risley et.al.(1986) have studied changes in DNA topology during spermatogenesis in few species of Anura viz. *Xenopus, Bufo* and *Rana*. DNA topology during spermiogenesis had been studied using histone and protamin depleted nuclei (nucleoids) from somatic cells, sperm and spermatogenic cells to visualize configuration of DNA loop domains during spermatogenesis. The study indicates that the nucleus remains in two different states called

condensed-relaxed-condensed called as biphasic change. DNA in sperm nucleoids from *Xenopus laevis* and *Bufo fowleri* remain in relaxed and expanded state at low EB (ethidium bromide) concentration and gradually condensed as the EB concentration was increased. On the other hand *Rana catesbeiana* sperm DNA exhibited biphasic change only at higher EB concentration. When sperm DNA was exposed to UV light, DNAse I, proteinase K or urea no such biphasic changes were observed. These results demonstrate that DNA in sperm nucleoid is constrained in domains of supercoiling by nonbasic nuclear proteins. Negatively supercoiled DNA is present in nucleoids from cells with a full complement of histones, including *Rana* sperm, but not in nucleoids from *Xenopus* and *Bufo* sperm in which histones are replaced by intermediate protamins. Result suggests an important role of the basic nuclear proteins of sperm in the morphogenesis of nucleus and the arrangement of DNA. Therefore, the different degree of compactation of head nucleus in the species studied can be viewed as difference in DNA supercoiling in the three species mentioned during the spermiogenesis.

Burgos and Fawcett (1956) for the first time 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.

Burgos and Fawcett (1956) also described the existence of two cylindrical centrioles in the middle piece with typical resemblance with the basal body of cilia as described by Fawcett and Porter(1956).

Burgos and Fawcett (1956) also described the ultrastructure of undulating membrane and tail fiber. According to their description flagellum consists of a bundle of nine perepheral and two central pairs of fibrils and that have a core of low density giving them a tubular appearance. The undulating membrane exhibits the existence of thickened material along the free edge of the membrane and along its base. The base of the undulating membrane is in

TABLE – 5

Summarized ultrastructural features of sperm of Bufo species –

Plesiomorphies	Apomorphies	Autapomorphies
• Conical perforetorium i.e. pointed end of acrosomal cap. • Head nucleaus cylindrical or rod shaped. • Nuclear material compact with electron dense granules and electron lucent lacunae • Middle piece with rows of mitochondria arranged as mitochondrial collar. Mitochondria uniform and appear spherical or oval in cross section. • Presence of two centrioles in the middle piece, arranged perpendicular to each other. • Proximal centriole beset at the notch of the head nucleus. • Each centriole exhibits nine perepheral doublets. • Axial filament with typical 18+2 arrangements of microfilaments.	• Perforatorium conical with dense strands of fibers. (Bh, Bm). • Head nucleus slender with uniform compactation of chromatin material, lacunae regular.(Bh, Bm) • Head surface smooth i.e. without protrusions and tubercles. (Bh, Bm) • Nuclear space distinct. (Bh, Bm) • Extreme end of tail filament assume bulb like structure as undulating membrane duses with the axial filament. (Bh, Bm) • Uniflagellar tail. (Bh, Bm, Bs)	• Conical perforatorium hook like. (Bh) • Conical perforatorium needle like. (Bm) • Conical perforatorium indistinct and without fibrous strand i.e. electron lucent. (Bs) • Head surface rough with regular distribution of protrusions and tubercles (Bs) • Mitochondtral collar extended back as flap on either side. (Bm) • Mid piece broad and distinct. (Bs)

Bh- ***Bufo himalayanus*****,** **Bs-** ***Bufo stomaticus*****,** **Bm-** ***Bufo melanostictus***

close relation with one of the peripheral pairs of tail fibrils and is always in line with the central pair. The axis of symmetry therefore passes through the central pair, through one of the peripheral pairs and through the base of the undulating membrane.

My observations on sperm ultrastructure strongly upholds the Bufonid lineage as described by Burgos and Fawcett. The variations noted at individual level clearly suggest the differences among the species. Such ultrastructural differences along with consistent ultrastructural features(Table-5) would be used to describe phylogenic relationship among the taxa.

D. DNA analysis studies

The DNA content of the isolated nuclei or ethanol fixed cells can be measured by flowcytometry. Fluorescence dyes like propidium iodide, mithramycin or ethedium bromide are commonly used for this purpose. Each of the dye binds to DNA stoichiometrically and therefore emits a fluorescent signal that is proportional to the amount of DNA in the nucleus.

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. Thetefore 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.

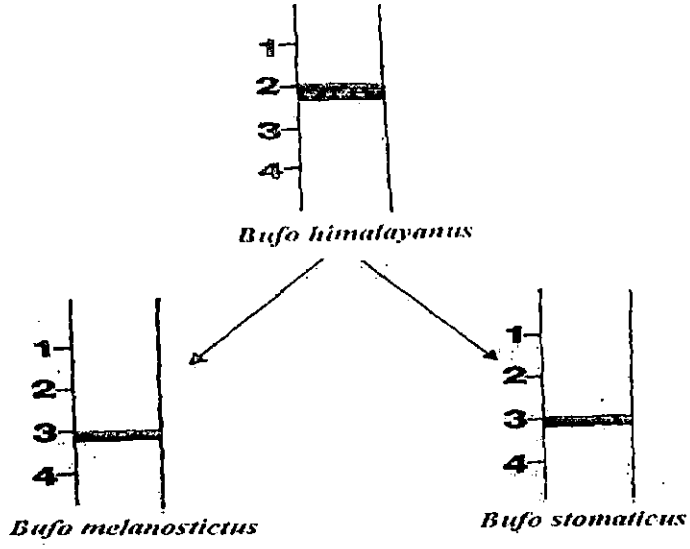
There are three distinct options to explain the situation-

1. If *B.somaticus* is considered as the ancestral species for *B.melanostictus* and *B.himalayanus* then the polarity of evolution suggests a reduction of genomic size towards the *B.himalayanus* lineage, while a dramatic increase of genome size in *B.melanostictus*.
2. If *B.himalayanus* is considered as ancestral species (more plausible from ultrastructural study) then the polarity of evolution has a bi-directional mode. In one lineage (*B.melanostictus*) the genome size increases significantly, while in another lineage (*B.stomaticus*) increase in genome size follows a gradual increase.
3. If *B.melanostictus* is considered as ancestral species then there is a significant reduction in both the lineage (i.e. *B.stomaticus* and *B.himalayanus*) suggesting an unidirectional polarity of evolution.

This contention has been supported by Bachmann et.al.,(1972) and Bachmann and Blommars-Schlosser (1975). They were of openion that generalized species of anurans have genome size near the mode value, while highly specialized species tends to have extremely low or high amounts of DNA.

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 restriction cut analysis, it can be suggested that *B.himalayanus* has a greater value than *B.stomaticus* and *B.melanostictus* and a tentative suggestion as follow be made.



Rough diagram showing greater electrophoretic mobility of DNA, of *B.melanostictus* and *B.stomaticus* than in *B.himalayanus*.

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.

E. Phylogeny of Bufonidae

The evolutionary or phylogenetic relationship among the families of living amphibians, are basic to an interpretation of their biogeography and to contrasting a meaningful classification. Helling (1938), first proposed a testable hypotheses that based shared character states phylogenetic relationship of the families can be derived. Wiley (1981), however, has proposed an alternative hypothesis.

The Henning's hypothesis is based on the identification of homologous structures and evolutionary direction (Polarity) of transformation series from a

primitive character (Plesiomorphy) to a derived character. It states that a nested sets of derived characters states (Apomorphies) show phylogenetic relationship, whereas primitive characters (Plesiomorphies) shared by two or more taxa do not show relationships. Characters that are unique to a given linage (Autapomorphies) are useful in recognizing a particular lineage but not in determining the relationship of that lineage with any other one.

Conventionally the phylogenetic relationships among the living groups of amphibians are constructed by using the WAGNER 78 computer program written by J.S. Farris (1969,1982). Depending on the number of convergences or reveals (Homoplasies) the cladogram or phylogenetic trees have been generated. The most parsimonious cladogram is called preferred arrangement, i.e. the one having the highest consistency index. The consistency index is determined by the following formula:

$$\frac{\text{Minimal number of possible changes in character states}}{\text{Actual number of changes}}$$

For each of the living orders of amphibians, the transformation series used in a phylogenetic reconstruction at the family level are described and their characters noted as primitive (0) or derived (1). In all the cases, the polarity, i.e., direction of evolutionary change is 0→1. In those cases, where there is more than one derived characters are found, they are represented sequentially or serially (i.e., 0→1→2 etc). if certain character is specified as derived independently, is expressed as 1' ←0 → 1→ 2, etc. The direction (polarity) of evolutionary change is determined by the characters in an out group. For most characters the out group is other Lissamphibia or tetrapods (Duellman & Trueb,1986).

The taxonomy of anurans is not well established. Duellman (1975), Laurent (1979), Dubois (1983), represented a meaningful classification based on the polarity or direction of evolutionary change, and have maintained paraphyletic groups. A comparative statement of such classification is summarized in the table (Table- 6).

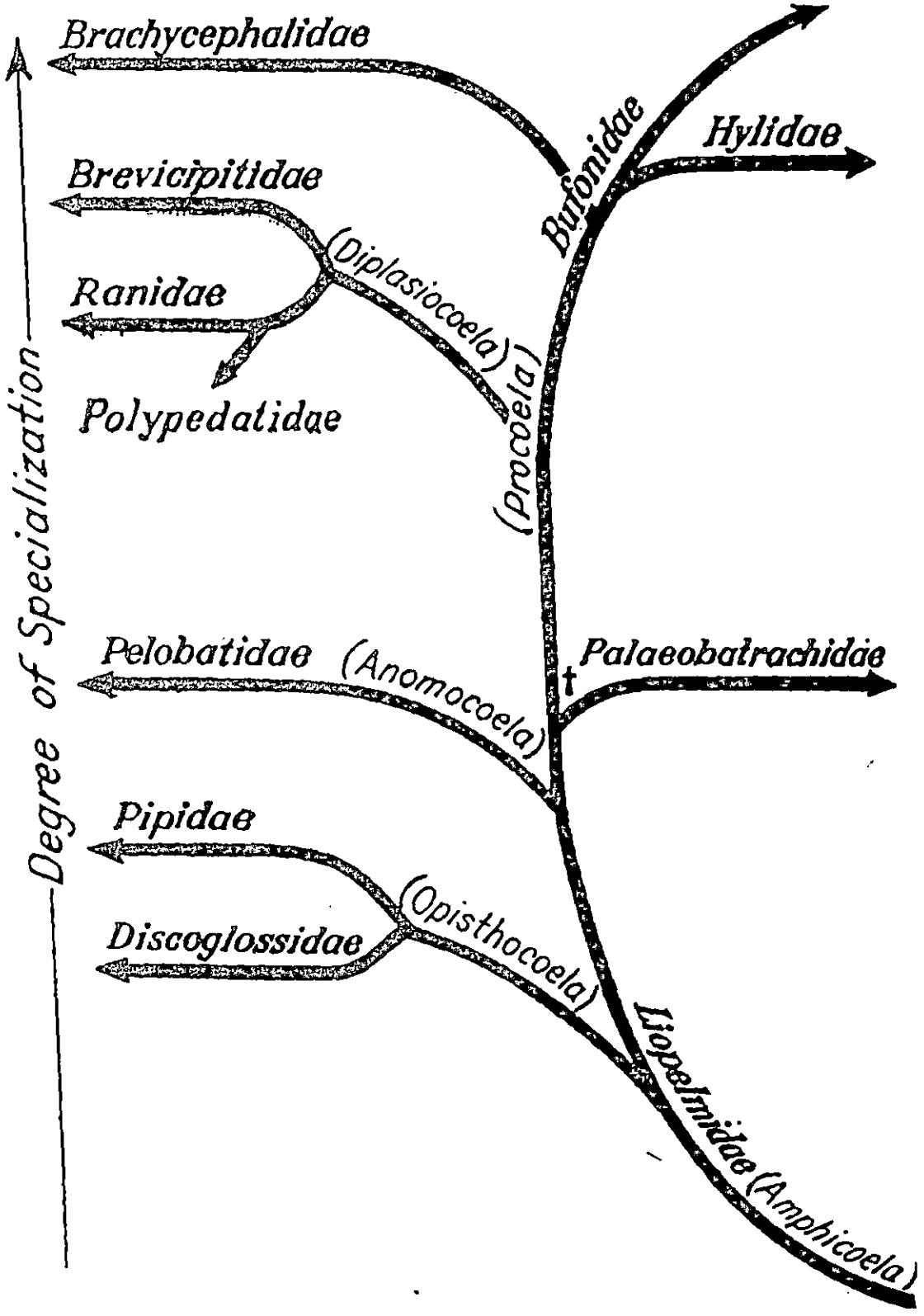
TABLE – 6

Duellman (1975)	Laurent (1979)	Dubois (1983)
Suborder Archaeobatrachia	Suborder Archaeobatrachia	Suborder Discoglossoidei
Superfamily Discoglossoidea	Superfamily Discoglossoidea	Superfamily Discoglossoidea
Family Leiopelmatidae	Family Leiopelmatidae	Family Discoglossidae
Discoglossidae	Discoglossidae	Leiopelmatidae
Superfamily Pipoidea	Suborder Mesobatrachia	Suborder Pipoidei
Family †Palaeobatrachidae	Superfamily Pipoidea	Superfamily Pipoidea
Pipidae	Family Pipidae	Family Pipidae
Rhinophrynidae	†Palaeobatrachidae	Rhinophrynidae
Superfamily Pelobatoidea	Rhinophrynidae	Superfamily Pelobatoidea
Family Pelobatidae	Superfamily Pelobatoidea	Family Pelobatidae
Pelodytidae	Family Pelobatidae	Pelodytidae
Suborder Neobatrachia	Pelodytidae	Superorder Ranoidei
Superfamily Bufonoidea	Suborder Neobatrachia	Superfamily Hyloidea
Family Myobatrachidae[a]	Superfamily Bufonoidea	Family Rheobatrachidae
Leptodactylidae	Family Rheobatrachidae	Myobatrachidae
Bufonidae	Myobatrachidae	Sooglossidae
Brachycephalidae	Sooglossidae	Leptodactylidae
Rhinodermatidae	Leptodactylidae	Dendrobatidae
Dendrobatidae	Phyllobatidae[b]	Bufonidae
Pseudidae	Bufonidae	Brachycephalidae
Hylidae[c]	Brachycephalidae	Rhinodermatidae
Centrolenidae	Rhinodermatidae	Pseudidae
Superfamily Microhyloidea	Pseudidae	Hylidae
Family Microhylidae	Hylidae	Centrolenidae
Superfamily Ranoidea	Centrolenidae	Pelodryadidae
Family Sooglossidae	Pelodryadidae	Superfamily Microhyloidea
Ranidae[d]	Superfamily Microhyloidea	Family Microhylidae
Hyperoliidae	Family Microhylidae	Superfamily Ranoidea
Rhacophoridae	Superfamily Ranidae[e]	Family Ranidae
	Family Hyperoliidae[f]	Rhacophoridae
	Ranidae	Arthroleptidae
	Hemisidae	Hyperoliidae
		Hemisidae

[a]Includes Rheobatrachidae.
[b]Equals Dendrobatidae.
[c]Includes Pelodryadidae.
[d]Includes Arthroleptidae and Hemisidae.
[e]Includes Rhacophoridae.
[f]Includes Arthroleptidae.

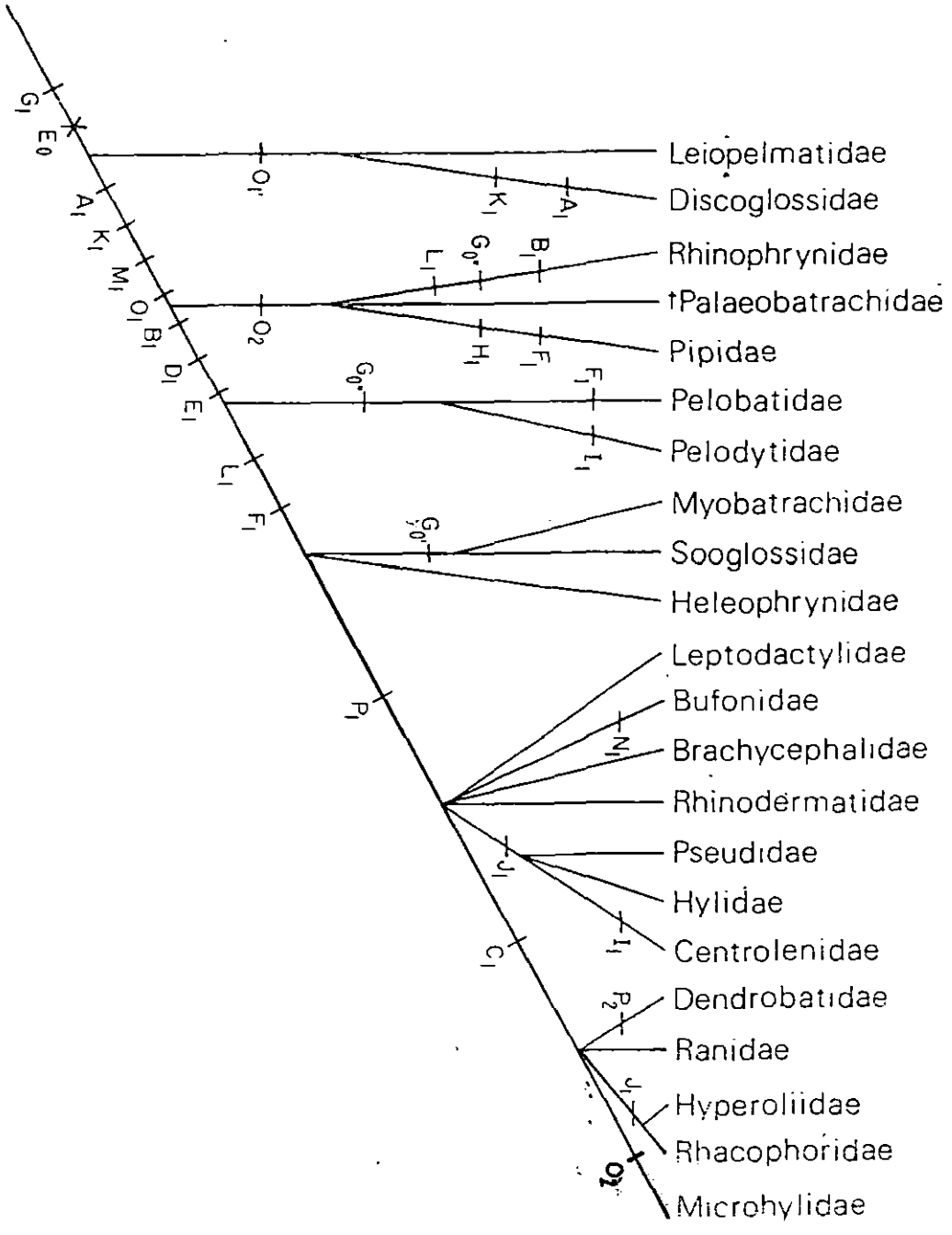
Comparison of Three Recent Classifications of Anurans

FIGURE – 8A



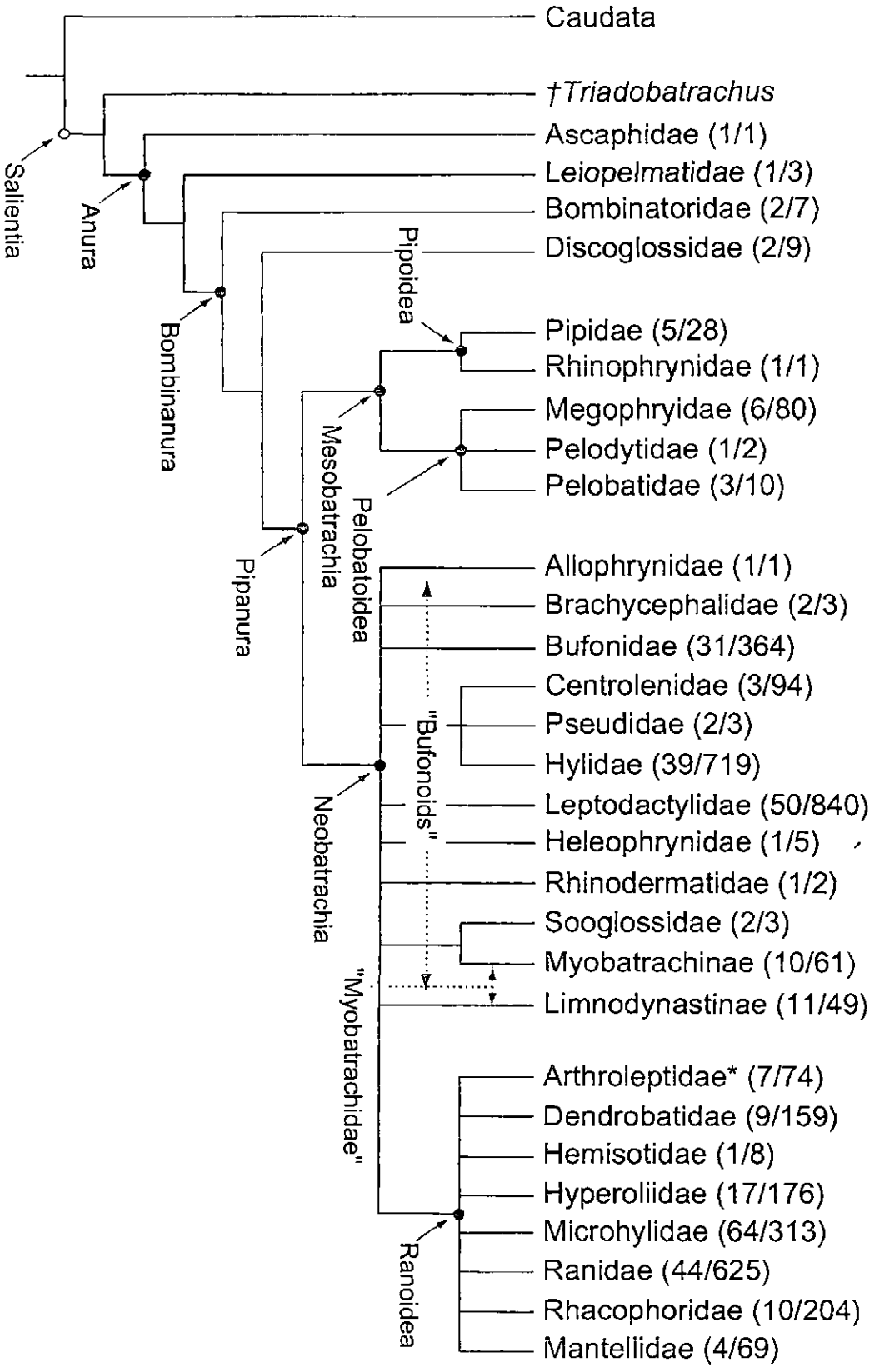
Phylogenetic relationship of Anura (Adapted from Noble, 1932)

FIGURE – 8B



Hypothesized phylogenetic relationships of the families of anurans based on 16 characters and reconstructed by the WAGNER78 program. For purposes of analysis, the states of characters, G, L, M, and O of the existing family Palaeobatrachidae were coded the same as those of Pipidae; also character N in the Brachicephalidae and Sooglossidae was coded the same as other bufonids. Tick marks indicate places of shifts of characters to states indicated by subscripts. Two convergences exist in each of seven characters (A, B, G, I, J, K, and L), and three convergences occur in character F. consistency index =65.6%. (After Duellman and Trueb, 1986)

FIGURE – 8C



Phylogeny of Bufonidae by David Cannatella, 1997

On the basis of the obove classification, it is acceped that the order Anura contains twentyone living and one extinct families. These contains 301 living genera with 3438 living species, plus 98 extinct species (Duellmann & Trueb, 1986). However, there is constant influx of living genera and species on the basis of intensive research on the field (Inger,1967 ; Lee and Jamieson, 1992, 1993 ; Ford,1993 ; Know and Lee,1995 ; Meyer et.al.,1997 ; Selmi et.al.,1997 ; Lourenco et.al.,2000 ; Masta et.al.,2002)

Using WAGNER 98 computer program, only 16 characters were coded for phylogenetic analysis (Table - 7). A cladogram generated from these character states that (Fig- 8A, 8B, 8C), this cladogram has eight homoplasious characters and a constancy index of 65.6%.

Several unresolved polytomies exist in this cladogram and on that basis several groups to be rearranged and thus phylogeny based on morphological characters have raised questions (Fig- 8A, 8B, 8C).

The Bufonidae (as family) deserves an independent and intensive research. It contains twentyfive genera with 335 living and 20 extinct species (Duellman & Trueb, 1986). Some of the genera that are now contained in the Bufonidae, formerly were placed in the Atelopopidae. On the basis of the presence of Bidder's organ all of them were placed in the Bufonidae, except *Brachycephalus* (Bidder's organ absent), is now placed in the Brachycephalidae (McDiarmid, 1971).

More than half of the species within the family Bufonidae are contained within the genus *Bufo*, the 205 species of this genus are distributed throughout major land- masses of the Australo-Papuan Realm and Madagaskar (Duellman and Sweet, 1999), the phylogenetric analysis of the species within the genus *Bufo* has been reviewed by Blair (1972).

The cladogram documented on the basis of morpho-anatomical traits is not widely accepted and thus the phylogenetic relationship among families are still unresolved and phylogenetic trees is very poor and doubtful. Contents of the group such as Bufoniae and Ranidae are in a constant state of flux because of the addition and exclusion of families, such as Dendrobatidae

TABLE – 7

Character	Brachycephalidae	Bufonidae	Centrolenidae	Dendrobatidae	Discoglossidae	Heleophrynidae	Hylidae	Hyperoliidae	Leiopelmatidae	Leptodactylidae	Microhylidae	Myobatrachidae	†Palaeobatrachidae	Pelobatidae	Pelodytidae	Pipidae	Pseudidae	Ranidae	Rhacophoridae	Rhinodermatidae	Rhinophrynidae	Sooglossidae
A. Vertebral column	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1
B. Ribs	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1
C. Basic pectoral girdle architecture	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0
D. Other features of the pectoral girdle	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1
E. Cranium	0	0	0	0	1	0	0	0	1	0	0	1	1	0	0	1	0	0	0	0	1	0
F. Parahyoid	1	1	1	1	0	1	1	1	0	1	1	1	0	1	0	1	1	1	1	1	0	1
G. Cricoid cartilage	1	0	1	0	1	0	0	0	1	0	0	0	?	0	0	1	0	0	0	0	0	0
H. Tongue	0	0	0	0	0	0	0	0	0	0	0	0	?	0	0	1	0	0	0	1	0	0
I. Astragalus and calcaneum	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
J. Hands and feet	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
K. Caudalipuboischio-tibialis muscle	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1
L. Semitendinosus-sartorius muscle complex	1	1	1	1	0	1	1	1	0	1	1	1	?	0	0	0	1	1	1	1	1	1
M. Trigeminal and facial ganglia	1	1	1	1	0	1	1	1	0	1	1	1	?	1	1	1	1	1	1	1	1	1
N. Bidder's organ	0	1	0	0	0	0	0	0	0	0	0	0	?	0	0	0	0	0	0	0	0	0
O. Larval types	—	1'	1'	1'	1	1'	1'	1'	1	1'	2'	1'	2	1'	1'	2	1'	0	0	1'	2	—
P. Amplectic position	1	1	1	2	0	0	1	1	0	1*	1	0'	?	0	0	0	1	1	1	1	0	0

[a] 0 = primitive; 1, 2, etc.; ? = unknown; — = not applicable.
[b] See text for definitions of states.
[c] Derived state in phrynomerines.
[d] Derived state in mantellines.
[e] Primitive state in some telmatobiines.
[f] Derived state (1) in limnodynastines.

Distribution of Character States in Families of Anurans
(After Duellman and Trueb, 1986)

(Ford, 1993 ; Ford and Cannatella, 1993). It is generally considered that the Bufonoidae, is a monophyletic lineage while the Ranidae and Leptodactylidae are generally considered polyphylectic.

The debate, doubts and counter arguments on the phylogenetic status of the Anura prompted the search for alternative tools and methods for a well convincing view on the issue. The tools that are now used are cyto-genetic study, genome size and characteristics, biochemical studies, mitochondrial DNA analysis etc.

Amphibians are exceptionally good specimens for karyological studies because most have few, comparatively large chromosomes. Most of the work on amphibian cytogenetics has been with conventionally stained chromosomes (Bogart,1972 ; Morescalchi, 1973 etc). Newly developed techniques have been employed to provide an insight into the chromosomal location of constitutive heterochromatin, nuclear organizer regions and rRNA genes etc (Schmid,1978 ; Bristein,1982 ; Yang,1983 ; Kuramota, 1990 ; Liu and Yang, 1997 ; Lourenco et.al., 2000 etc). On the basis of these studies following generalizations can be made on amphibian karyological characteristics –

1. A tendency towards genome hypertrophy.
2. High degree DNA spiralization.
3. A gross morphological difference in chromosomes in terms of size (Microchromosomes which are extremely small and macrochromosomes which are relatively larger and variable in size) and centromeric position.
4. Great interspecific differences in the amount of nuclear DNA.

The Karyotypes of the living orders of Amphibia have been worked out of which the Anura includes hundreds of species representing all families (Table - 8). The data available poised to suggest that

a) Supposed microchromosomes have been found only in members of primitive families viz. *Ascaphus truei*, *Leiopelma hochsterri, Discoglossus pictus,* etc.

TABLE – 8

Taxonomic group	Diploid number
Leiopelmatidae	
Ascaphus truei	46[a]
Leiopelma hochstetteri	22–30[a]
Leiopelma archeyi and *hamiltoni*	18
Discoglossidae	
Alytes obstetricans	38[a]
Discoglossus pictus	28
Bombina (3 species)	24
Rhinophrynidae	
Rhinophrynus dorsalis	22
Pipidae	
Xenopus (6 species)[b]	36
Xenopus tropicalis	20
Hymenochirus boettgeri	24
Pipa carvalhoi	20
Pipa pipa	22
Pipa parva	30
Pelobatidae	
All genera and species,	26
except *Leptolalax pelodytoides*	24
Pelodytidae	
Pelodytes punctatus	24
Myobatrachidae	
All genera and species,	24
except 4 species of *Limnodynastes*	22
Sooglossidae	
Nesomantis and *Sooglossus*	26
Heleophrynidae	
Heleophryne	26
Leptodactylidae	
Ceratophryinae[b]	26
Telmatobiinae	
Eupsophus	28, 30
Other genera	26
Odontophrynini[b]	22
Grypiscini	26
Eleutherodactylini	
Eleutherodactylus	18–36
Sminthillus limbatus	32
Syrrhophus (2 species)	26, 30
Holoaden bradei	18
Other genera	22
Hylodinae	
Crossodactylus and *Hylodes*	26
Leptodactylinae	
Limnomedusa (1 species)	26
Adenomera (2 species)	26
Adenomera marmorata	24
Paratelmatobius lutzi	24
Pseudopaludicola (2 species)	18–22
Lithodytes lineatus	18
Other genera[b]	22
Bufonidae	
All genera and species,[b]	22
except *Bufo regularis* group[b]	20
Brachycephalidae	
Brachycephalus ephippium	22
Rhinodermatidae	
Rhinoderma (2 species)	26

Taxonomic group	Diploid number
Pseudidae	
Pseudis paradoxa	24
Hylidae	
Pelodryadines,	26
except *Litoria infrafrenata*	24
Phyllomedusines[b]	26
Hemiphractines	
Fritziana (3 species)	26–30
Gastrotheca (12 species)	26, 28
Other genera and species	26
Hylinae	
Osteopilus brunneus	34
Hyla "leucophyllata complex"	30
Acris crepitans	22
Other genera and species[b]	24
Centrolenidae	
Centrolenella	20
Dendrobatidae	
Colostethus	24
Dendrobates (6 species)	18–22
Ranidae	
Raninae[b]	26
except *Ptychadena* (5 species),	24
Rana (3 species),	24
and *Rana kuhlii* and *namiyei*	22
Petropedetinae	
Anhydrophryne and *Petropedetes*	26
Dimorphognathus africanus	24
Phrynobatrachus (6 species)	16–20
Mantellinae	
All genera and species,	26
except some *Mantidactylus*	24
Arthroleptinae	
Arthroleptis (3 species)	14
Cardioglossa (2 species)	16
Astylosterninae	
Astylosternus diadematus	54
Nyctibates corrugatus	28
Hemisinae	
Hemisus (1 species)	24
Hyperoliidae	
All genera and species,	24
except *Leptopelis* (10 species)	22, 24, 30
Rhacophoridae	
All genera and species	26
Microhylidae	
Asterophryinae	26
Genyophryninae	26
Phrynomerinae	26
Dyscophinae	26
Cophylinae	26
Microhylinae	
Kaloula[d]	28
Other Asian genera	26
Glossostoma and *Otophryne*	26
Chiasmocleis	24
Other New World genera	22

b) With exception of Xenopus, the basic karyotype of other anurans seems to be 26 bi-arm chromosomes.

c) Diverse lineages of Anura show a reduction from the basic number of 26. This is especially evident in the Leptodactylidae, Hylidae, Ranidae, and is characteristics of all Bufonids.

The reduction presumably has occurred not through the loss of genomic material, but by the rearrangement of materials by centrifusion, specially of the smaller telometic chromosomes (Morescalchi,1973).

d) In addition to normal diploid chromosomes, some species exhibits polyploidy (Bogart,1967; Fischberg and Kobel, 1978; Mahony and Robinson, 1980) and such polyploidy may be the result of hybridization of two or more species (Allopolyploidy) or may have occurred spontaneously in a single species (Autopolyploidy) .

Karyological studies using conventional and banded chromosomes reveal that the Karyotypes of bufonoids are conservatives. All species, except for *Bufo danatensis*, have a karyotype consisting 22 chromosomes, including 6 large and 5 small pairs. A very few (viz. *Bufo danatensis, Bufo viridis)* is tri or tetraploid species (Mazik et.al., 1976; Pisanetz, 1978; Liu et.al., 2000).

Although the karyological studies have contributed to resolving some question of species, the data have been phylogenetically uninformative at higher taxonomic levels (Liu et.al., 2000).

Genome Size

Phylogenetic relationship among the families or species group has been tried to establish through the study of the genome size or DNA content / nuclei / species. The accepted principle is that within the related species or families the "genome size" exhibit constancy at an expected level.

The amount and replication sequences of nuclear DNA are highly variable among amphibians. In caecilians, it varies from 7.4 - 27.9 picograms of nuclear DNA/ diploid nucleus (Morescalchi, 1973). On the other hand anurans and salamanders have much greater amount 2-36 pg/N (Morescalchi, 1977) and 33-192 pg/N (Morescalchi, 1977, Morescalchi et.al.,1979), respectively.

The modal genome size in anurans is 9 or 10 pg /N (Bachmann and Blommers-Schlosser, 1975). Bachmann et.al.(1972) suggested that each group of organism has a minimum amount of DNA representing the genetic information necessary for expressing the group specific characteristic; the amount of DNA beyond this minimum codes for species specific characters. Further more high values of DNA provide a reservoir of raw materials for production of new gene(s).

Generalized species of anurans usually have genome sizes near the mode, whereas high specialized species tend to have extremely low or high amount of DNA (Bachmann and Blommers-Schlosser, 1975).

Baldari and Amaldi (1976) from the comparative study on DNA reassociation and genome size revealed that each of the species had about the same absolute amount of unique DNA. The differences in the total nuclear DNA in the species studied by Baldani and Amaldi (1976) tend to lie in the repetative sequence classes or due to variation of number of copies of a sequence class.

On the basis of these observations the authors suggested that the greater difference between salamanders and anurans involve all sequence classes in parallel.

The pattern that has been emerged from the studies on genome size is highly impressive. It seems that genome size has particular co-relation with development pattern, mutation rate and selective pressure under which the species was subjected (Bush et.al., 1977).

Biochemical Studies

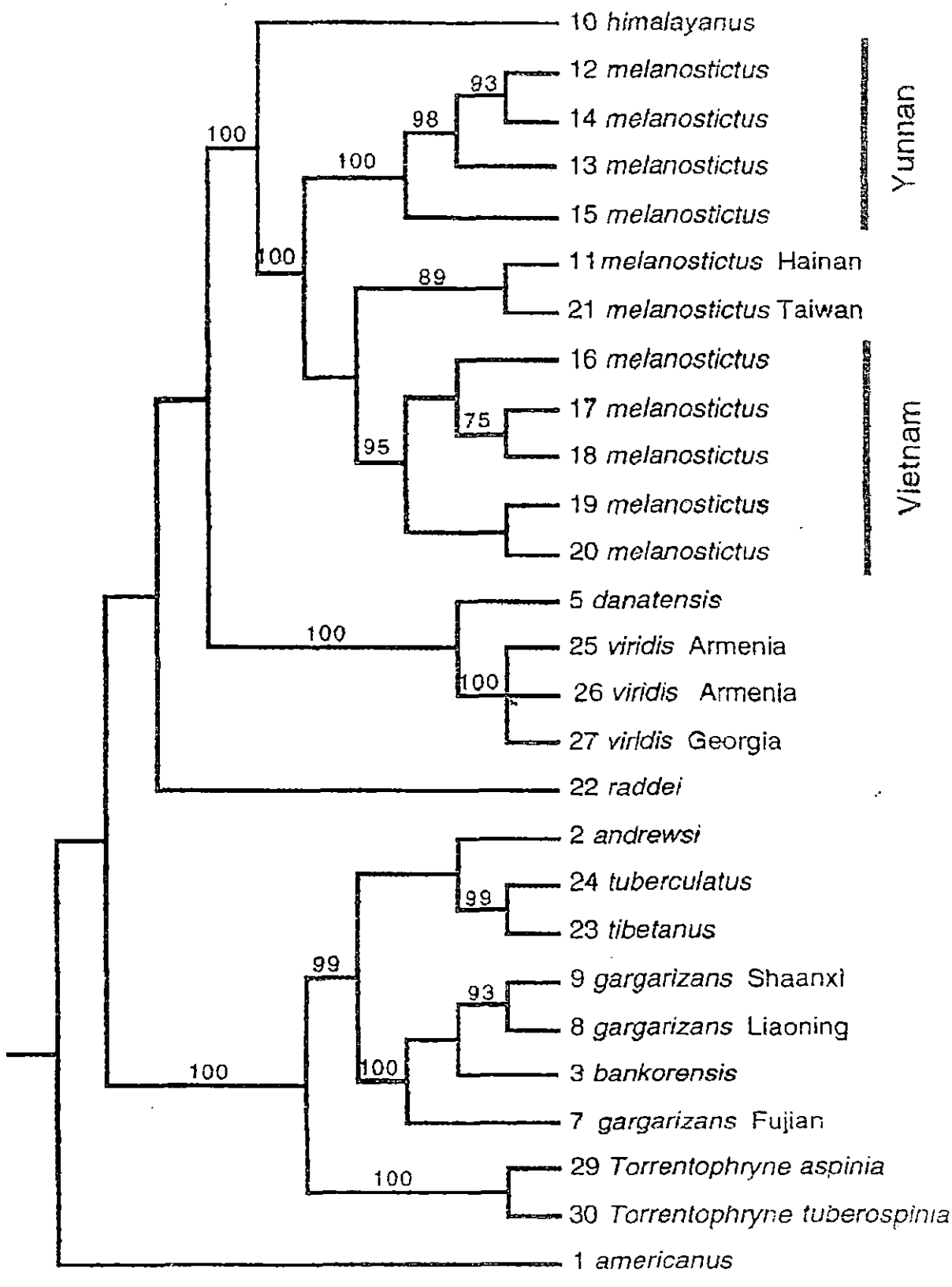
a) Ribosomal RNA :- the nucleolar organizer region (NOR) is the region of the chromosome that produces the nucleolus. In interphase through late prophase these region lack DNA but are rich in RNA. NOR is regarded as differentiated region of chromatin (DNA) which transcribe the larger species of RNA i.e., 18s and 28s ribosomal RNA (rRNA). However, the other regions of the genome produce the 5s RNA and code for the ribosomal proteins.

Quantitative relationship exists between chromosomal DNA and rDNA. DNA-RNA hybridization in three species of anurans and nine of salamanders revealed that the proportion of rDNA decreases with increasing DNA content. Furthermore, the total amount of rDNA complementary to the 18s and 28s rRNA is much less in anurans. Although the urodels have much larger genome sizes than anurans (Vlad,1977). Subsequent studies have confirmed a positive correlation between rRNA and rDNA loci in haploid set of chromosome (Batistoni et.al.,1980; Vitelli et.al.,1982; Macgregor and delPino,1982 etc).

b) Proteins:- Determination of homogeneity and heterogeneity at loci for a variety of genes coding for certain proteins provide evidence for the genetic composition of the organism. Proteins subjected to electrophoresis can be identified chemically and different alleles can be determined. Estimates of genetic identity (I) and genetic distance (D) between samples can be calculated by the methods of Nei (1972), Rogers (1972), Hillis (1984).

Molecular divergence is measured by immunological distance (ID) by means of microcomplement fixation of serum albumin. It is usually denoted that one unit of immunological distance is approximately equivalent to a single amino acid difference between the samples compared (Maxon and Wilson, 1974).

FIGURE – 9

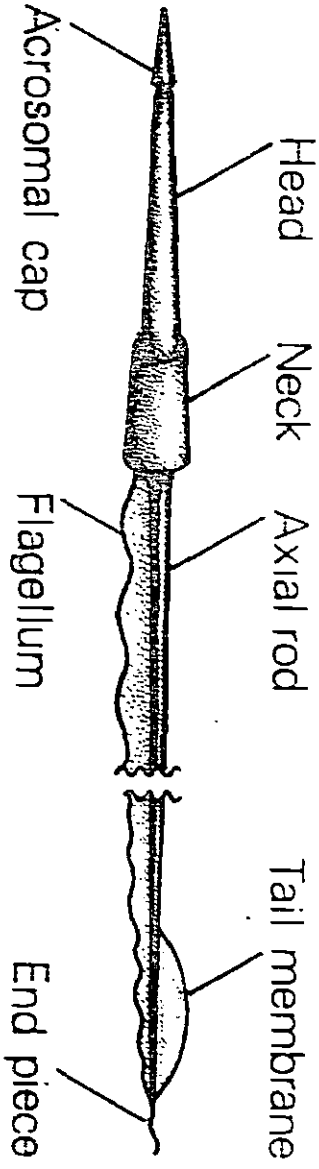


Phylogenetic tree of Bufonidae as proposed by Liu et.al., 2000.

The protein structure of amphibian spermatozoa is highly variable like that in fishes and unlike that in reptiles, birds and mammals. Interspecific differences in histones have been noted for a variety of anurans and their hybrids (Kasinsky et.al., 1978; Kasinsky et.al., 1981). Kasinsky et.al (1981) suggested that histone diversity among the species reflects their interrelation and they hypothesized that histone diversity in spermatozoa declines in vertebrate evolution as sex determination becomes increasingly chromosomally based. In recent years sperm specific histones (SP_1-SP_6) are used to interpreter the phylogenetic relationship or akinness (Abe and Hiyoshi, 1991; Mita et.al., 1991; Mita et.al., 1995; Hars et.al., 2001).

c) Mitochondrial DNA :- In recent years mitochondrial DNA (mtDNA) is employed to determine the phylogenetic relationship among the species of a particular genera (Graybeal, 1997; Liu et.al., 2000; Pramuk et.al., 2001) have used mtDNA sequences to reconstruct the history of Bufonids particularly that of Asian bufonids. Liu et.al.(2000) in there extensive work used 26 samples representing 14 species of *Bufo* from China, Vietnam Armenia, Georgia and Canada. They have worked out the relationship of Asian bufonids using partial sequences of mtDNA genes. Sequences from 12s rRNA, 16s rRNA, cytochrome B and the control region were analyzed using parsimony. The study indicates that East Asian bufonids can be grouped into two major clades. The first clade included *B.andrewsi*, *B.bonkorenis*, *B.gargarizans*, *B.tibetanus*, *B.tuberculatus*, its sister clade *B.cryptotympanicus* and the 2 species of *Torrentophryne*. The second clade consisted of *B.geleatus*, *B.himalayanus*, *Bufo melanostictus*, and a new species from Vietnam. The placement of three taxa (*B.raddei*, *B.viridis* and its sister species *B.danatensis*) was problematic. The genus *Torrentophryne* should be synonymized with *Bufo* to remove Paraphyly (Figure - 9).

FIGURE – 10



Generalized amphibian spermatozoon showing morphological structures

Study 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.

Sperm Ultrastructure

The basic morphology of an amphibian spermatozoon contains of the following structures in a linear, antero-posterior sequence(Figure – 10)- a) Acrosome, b) Head, c)Neck or middle piece, d)Tail. However, the morphology of spermatozoa is highly variable in anurans and salamanders and too little is known about the morphology of caecilians.

The biological significance of different size and structure of spermatozoa is still unknown. Species specific differences in spermatozoa may be correlated with difference in structures of egg membrane (Kawamura, 1953 ; Nelson and Humphrey, 1972 ; Semik and Kilarski, 1998; etc). It may also be related with mode of fertilization or evolutionary plasticity of the taxon (Wortham et.al. 1982).

Martan and Wortham (1972); Wortham *et.al.* (1982) first advocated that variation in sperm morphology are consistent with classification and that such characters may contribute to understanding of the phylogenetic relationship among groups of amphibians. For example, the presence of tail membrane in Ambystomatids that is unknown in other salamanders (Martan and Wortham, 1972). Similarly the presence of barb on the acrosome is an unique feature of all salamander spermatozoa, but its length and position are variable (Wortham *et.al.*, 1982). There are interspecific differences in length of parts of spermatozoa in *Ambystoma* and the hybrids are intermediate between those of paternal species (Brandon *et.al.*, 1974). The same is true for species of *Hynobius* and their hybrids (Kawamura, 1953). The structure of

the junction of the head and neck in spermatozoa is different among genera of salamander (Fawcett, 1970 ; Werner, 1970) and among the species of *Ambystoma* (Brandon *et.al.,* 1974). In *Tylototriton verrucosus* similar differences have been noted (Roy, 1989 ; Patra, 2003).

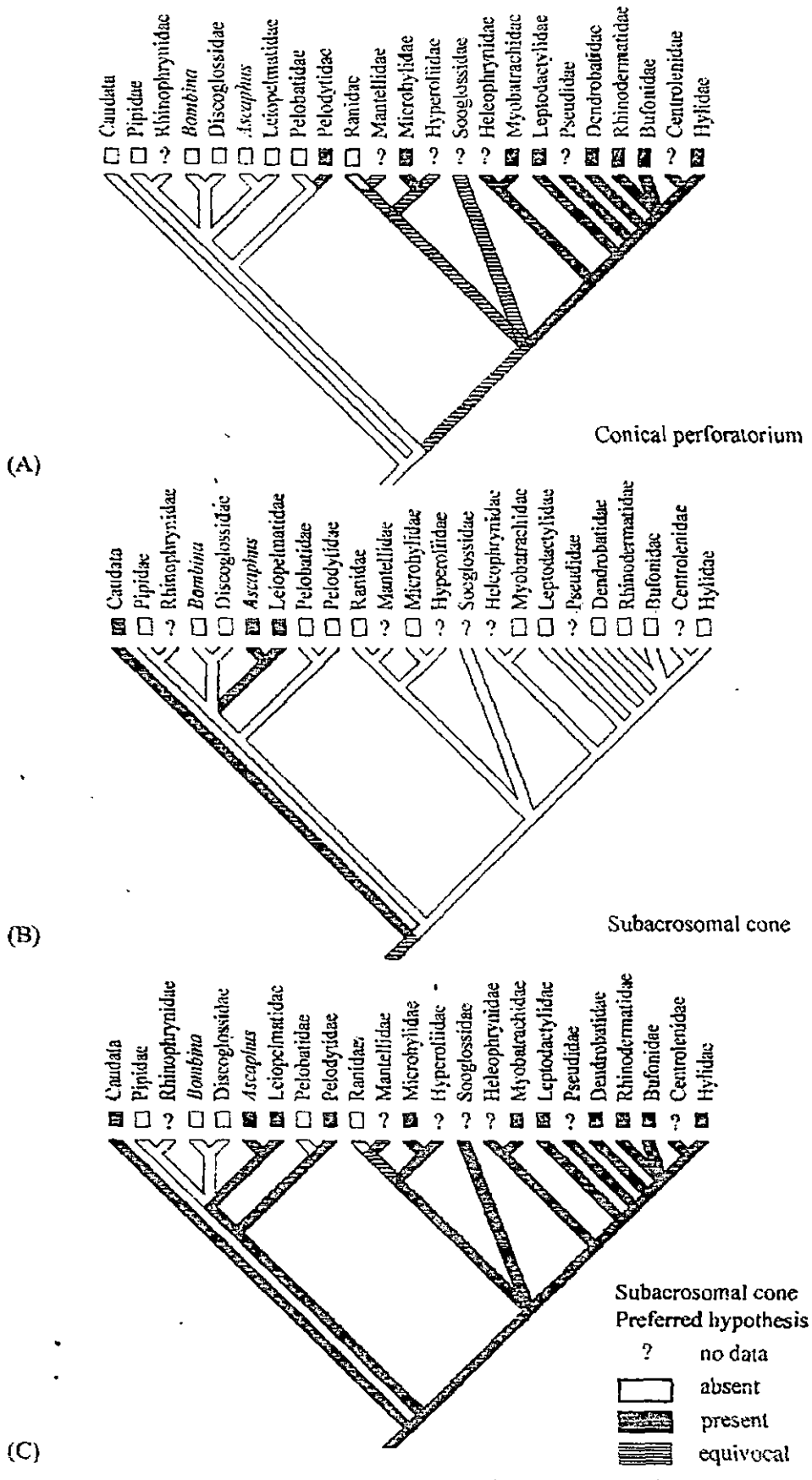
Wortham *et.al.* (1977) have opined that proportional in length of different parts of spermatozoa of plethdontids are consistent with the taxonomy of the group except for *Aneides*. Moreover, Wortham *et.al.* (1977) suggested that the long heads with more nuclear material of plethodontine spermatozoa may be related to the evolutionaty plasticity of that subfamily.

Striking differences are found among Anurans (Fouquette and Delhoussaye, 1977; Yoshizaki, 1987; Rastogi *et.al,* 1988; Lee and Know, 1992; Lee and Jamieson, 1993, Amaral *et.al.*, 1999; Bao *et.al.*, 2001). It has been generalized that in primitive group of Anura (viz. Discoglossoids, Pipoids, Pelobatoits, etc.) two or more tail filaments are present. Two tail filaments occur in some member of the Hylidae and Leptodactylidae, in nearly all Centrolemids and Bufonids. On the other hand, all Ranids, Microhylids and Pseudids; many Leptodactylids have a single tail. Similarly striking differences are found in the shape and proportional length of the head and neck among these species.

Result of these studies 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 (Poinier and Spink, 1971; Jamieson,1987; Jamieson,1991; Jamieson *et.al.*,1993; Jamieson,1995; Jamieson,1999; Lee and Jamieson,1992; Lee and Jamiesin,1993; Lee and Know,1996 ; Know and Lee, 1995 ; Meyer *et.al.*,1997 ; Scheltinga *et.al.*,2001; Tanaka *et.al.*,1995; Teixeira *et.al.*,1999a,1999b; Garda *et.al.*,2002).

FIGURE- 11



Phylogeny of Bufonidae from Hay et al. (1995).

(A) Evolution of conical perforatorium, according to Jamieson et al. (1993) and Lee and Jamieson (1993), number of steps = 4.

(B) Evolution of subacrosomal cone, according to Jamieson et al. (1993) and Lee and Jamieson (1993), number of steps = 2.

(C) Preferred hypothesis for the evolution of the subacrosomal cone, when considering the conical perforatorium homologous to the subacrosomal cone in anurans, number of steps = 4.

Based on the unique study of Garda et.al. (2002) (Figure - 11), the evolution of ultrastructural characters in Anura *vis.a.vis* of Bufonidae can be resolved as follows—

a) Subacrosomal Cone - The subacrosomal cone was originally absent in Anura and evolved independently twice in the group. James (1972) considered the subacrosomal cone as plesiomorphic for Bufonids. However, Burgos and Fawcett (1956) stated that subacrosomal cone like structure is present in *Bufo arenarum* and ultimately regarded it as perforatorium. James (1970) and Jamieson (1999) stated that subacrosomal cone and conical perforatorium are homologous and is considered as feature of Bufonid lineage. According to Hay et.al. (1995) the conical perforatorium was absent in the common ancestor of anurans and salamanders and evolved as a feature of Bufonoid lineage.

b) Acrosome - Acrosome in bufonids appear as a cap like structure filled with electron lucent materials. Burgos and Fawcett (1956) first observed the coarse strands of dense materials around the tapering end of the nucleus in the spermatozoon of *Bufo arenarum.* Similar structures have been observed in other bufonoid species studied (Lauder, 1994 ; Lee and Jamieson, 1992, 1993 ; Jamieson et.al., 1993). Therefore, the acrosome vesicle with its higher electron density due to the presence of fibrous material may be considered as a typical feature of bufonids. However, in some species (*Ascaphus trui, Myxophyes faciculatus* etc.) an intermediate condition is found.

c) Nuclear Space - In most Bufonids the nucleus is highly compact and a little space is left in between the acrosomal vesicle and head nucleus called nuclear space. This unique feature is considered to be a characteristics of Bufonid lineage (Rastogi *et.al.*, 1988 ; Lee and Jamieson, 1993 ; Meyer *et.al.*, 1997).

d) Nucleus - The nucleus is highly compact in mature spermatozoa with nuclear lacunae and inclusions. The nuclear lacunae are probably

formed during the condensation of chromatin. They are typically electron lucent with no material inside and are of small diameter, adistinct feature different from Ranoid lineage (Poirier and Spink, 1971).

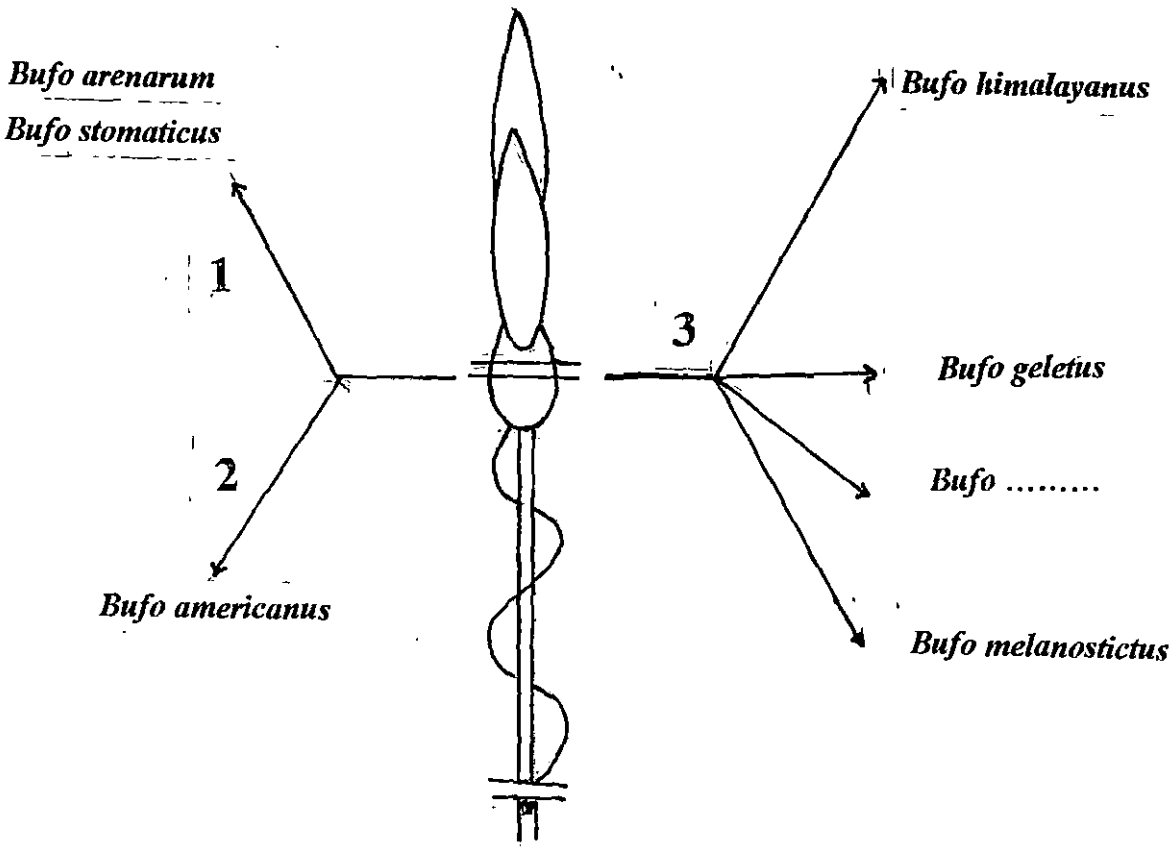
e) Middle Piece - Numerous and randomly arranged mitochondria in the mid piece usually form a mitochondrial collar in Bufonidae and Leptodactylidae (Burgos and Fawcett, 1956 ; Swan *et.al.,* 1980 ; Pugin-Rios, 1980 ; Bao *et.al.*, 1991 ; Lee and Jamieson, 1993). The presence of mitochondria creates a large separation between the axial sheath and the plasma membrane. However, in some Bufonids the mitochondrial collar is distinctly separated from the middle piece. Burgos and Fawcett (1956) described two typical centrioles in maturing spermatid of toad (*Bufo americanus*). They are not spherical but cylindrical like typical centrioles under electron microscope. One, which lies in the axis of the sperm is called axial centriole while the other lies oblique to the long axia of the cell is called oblique centriole or distal centriole. The centriole exhibits nine peripheral microtubular structures.

f) Axial Filament - In all bufonids the axial fibre is atypical flagellar structure containing two central microtubules encircled by nine peripheral doublets. This condition is also found in other families, viz. Ranidae, Leptdactylidae, Myobatrachidae.

g) Undulating Membrane:- Undulating membrane is a membranous structure which is very much pronouncing in Bufonidae. It is made up of thin ribbon like dense fibrous substance and arises in close relation in the oblique centriole and projects perpendicular from the side of the axial filament. This extended axial sheath takes a folded flap like structure at the terminal end. All ranoids so far studied (Ranidae, Rhacophoridae and Microhylidae) posseses a tail with only the axoneme, i.e. undulating membrane is absent.

h) End Bulb:- At the extreme end portion of the tail filament the undulating membrane becomes discontinuous and gives rise to a swollen base of

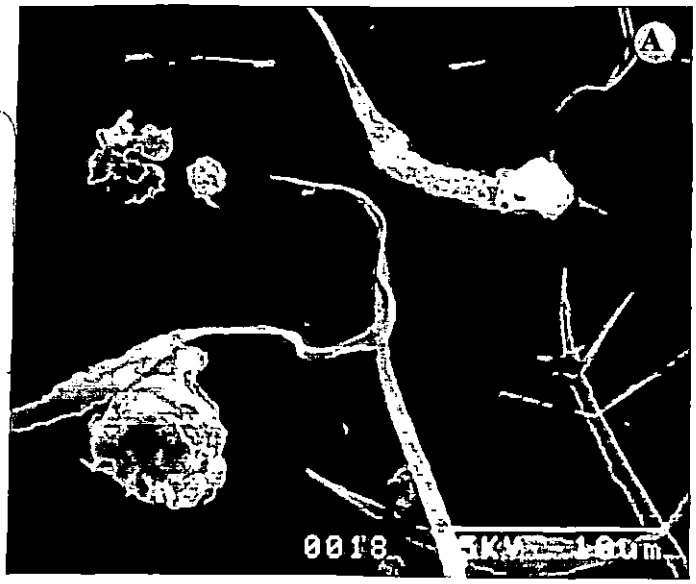
FIGURE – 12

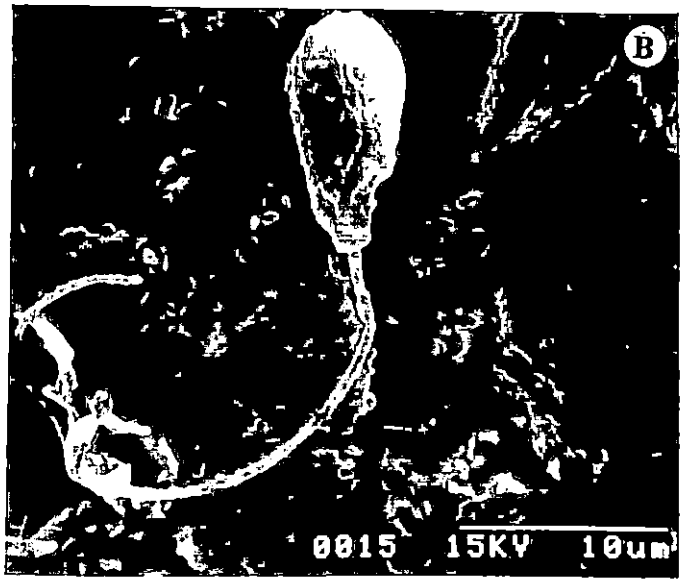


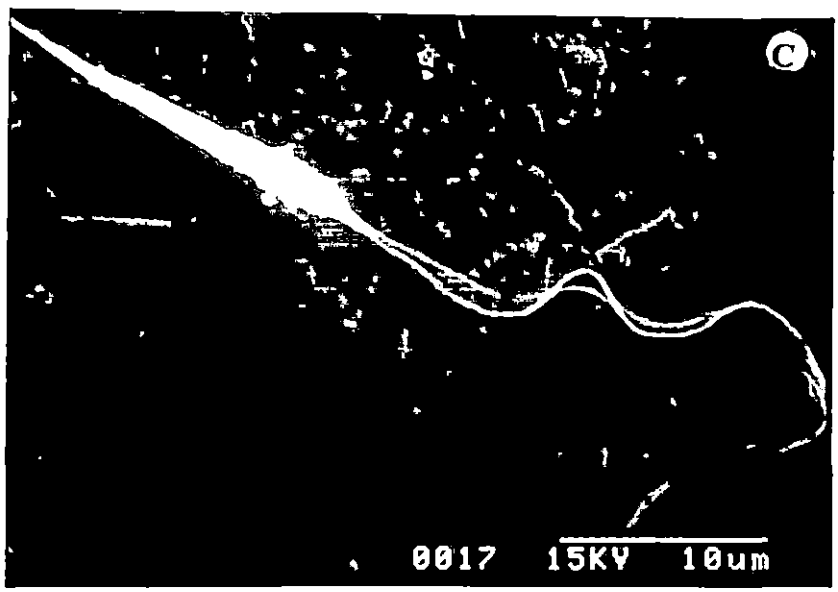
Typical *Bufo* Sperm

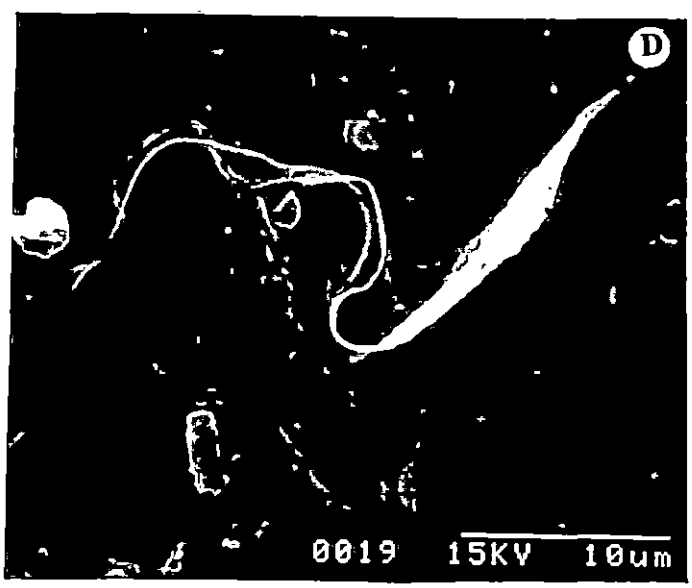
Self proposed Cladogram of Bufonidae

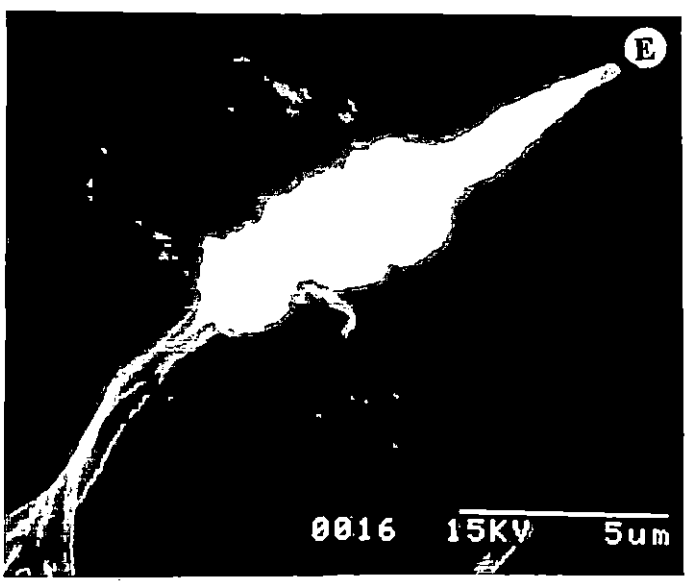
PLATE – 22











Sperm polymorphism in *Bufo stomaticus*

tail fiber called end bulb. However, such structures are not equivocal in all bufonid species so far studied.

Based on the information available on ultrastructural details of different species of *Bufo,* I have considered following characters of *Bufo* as plesiomorphic, apomorphic, autapomorphic (Table - 5). Depending on the homoplasies a cladogram has been proposed. (Figure - 12). It shows that the species available in the experimental study area (Eastern India) has above characters unique to them (Table - 5).

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.*

During this study a polymorphism in sperm structure has been noted in light, scanning and transmission electron microscopic observations (Plate –22). Such deviations could easily be ignored as aberrant features. But Patra (2003) based on his study on *Tylototriton verrucosus* (Urodela) has suggested that intraspecies polymorphism of sperm has a direct bearing with the group (lineage) from which the species has actually been evolved or likely to be evolved subsequently during the course of evolution. In my observations the megacephalic sperm may easily be compared with the sperm of *Bufo melanostictus* (derived group) and *Bufo arenarum* (ancestral group). This ultrastructural features is also confirmatory to my observations based on DNA analysis study, restriction endonuclease digestion and flow cytometry using FACS technique (See observation for detailed description).

F. Concluding Remark

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 (Figure – 1). *Bufo merinus* has been introduced into Australia and New Guinea and many other islands (Duellman and Trueb, 1986).

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 (Savage, 1973).
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 (Duellman and Trueb, 1986).
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 (Duellman and Trueb,1986).

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

An immunological study carried out in our laboratory (Das and Banerjee, 2003) tend to suggest that ID between *B.himalayanus* and *B.melanostictus* is comparable than that of *B.stomaticus*, suggesting a close phylogenetic relationship between the two species (*B.melanostictus* and *B.himalayanus*) than with the *B.stomaticus*.

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

REFERENCES

Annandale, N. (1907). "The distribution of *Bufo andersoni*." ***Rec. Indian Mus.*, 1, 1:** 171-172.

Ankel, W.E. (1930). Die atypische Spermatogenese von *Janthina* (Prosobranchia, Ptenoglossa). *Z. Zellforsch mikrosk. **Anat.*** **11:** 491-608.

Asana, J.J. and Khardi, R. G. (1937). "The chromosomes of *Rana tigerina*." ***Curr. Sci.*, 5:** 649.

Abdulali, H (1962). "An account of a trip to the Barapede Cave, Talewandi, Belgaum District, Mysore State, with some notes on reptiles and amphibians." ***J. Bombay. Nat. Hist. Soc.*, 59:** 228-237.

Anderson, J. (1872). "On some Persian, Himalayan and other reptiles." ***Proc. Zool. Soc. London*, 1872:** 417-498.

Auffenberg, W. and Rehman, H. (1977). "Geographic variations in *Bufo stomaticus*, with remarks on Bufo olivaceus: biogeographical and systematic implications,." ***Biodiversity of Pakistan.*** S.A. Mufti, C.A. Woods, and S.A. Hasan Eds., eds., Mus. Nat. Hist. Islamabad, Pakistan., pp. 351- 372.

Adamson, H. and Shaw, I. (1981). ***A Traveller's Guide to Pakistan.*** The Asian Study Group, Islamabad.

Abe. S.I. and Hiyoshi, H. (1991). Synthesis of sperm-specific basic nuclear proteins (SPs) in cultured spermatids from *Xenopus laevis*. ***Exp. Cell Res.*, 194:** 90-94.

Amaral, M.J.L.V., Fernandes, A.P., Báo, S.N. and Recco-Pimentel, S.M. (1999). An ultrastructural study of spermiogenesis in three species of *Physalaemus* (Anura, Leptodactylidae). ***Biocell*, 23:** 211–221.

Broman, I. (1900). Ueber Bau und Entwicklung der Spermien von Bombinator igneus, ***Anat. Anz.*, 17:**129-145.

Boulenger, G.A. (1920). "A monograph of the south Asian, Papuan, Melanesian and Australian frogs of the genus Rana." ***Rec. Indian Mus.*, 20:** 1-226.

BoleGowda, B.N. (1948). "The spermatogenesis of Uperodon systoma." *M.Sc. Dissertation*,. Mysore University, Mysore.

Bhaduri, J.L. (1953). A study of the urinogenital system of Salientia. ***Proc. Zool. Soc. Bengal*, 6:** 1-111.

Burgos, M.H. and Fawcett, D.W.(1956). An electron microscope study of spermatid differentiation in the toad *Bufo arenarum* Hensel. ***J. Biophys. Biochem. Cytol.*, 2:** 223–239.

Basu, S.L. and Mondal, A. (1960). Spermatogenetic cycle in *Bufo melanostictus* Schneid. ***Indian Journal of Physiology and Allied Sciences*, 14(2):** 43-46.

Basu, S.L. (1962) Impact of some endo and exogenous factors on the spermatogenesis of common Indian toad *Bufo melanostictus*. ***Folia-Biol. (Warsaw)*, 12:** 203-210.

Barker, K.R. and Biesele, J.J.(1967). Spermateleosis of salamander *Amphiuma tridactylum* Cuvier. A correlated light and electron microscopic study. ***La Cellule*, 57:** 91-118.

Bogart, J.P. (1967). Chromosomes of the South American amphibian family Ceratophridae with a reconsideration of the taxonomic status of *Odontophrynus americanus*. ***Canadian J. Genet. Cytol.*, 9:** 531-542.

Basu, S.L. (1969) Effect of hormones on the salient spermatogenesis in vivo and in vitro. *Gen. Comp.* ***Endocrinol. Suppl.*, 2:** 203-213.

Bachmann, K., Goin, O.B. and Goin, C.J. (1972). Nuclear DNA amounts in vertebrates. ***Brookhaven Symp. Biol.*, 23:** 419-447.

Blair, W.F. (1972). Evolution in the Genus *Bufo,* Austin: Univ. Texas Press.

Brown, P.S.; Hastings, S.A. and Frye, B.E. (1977). A comparison of water-balance response in five species of plethodontid salamander, ***Physiol. Zool.*, 50:** 203-214.

Bogart, J. P. (1972). Karyotypes. *In* "Evolution of the Genus *Bufo*"(W. F. Blair, Ed.), Univ. of Texas Press, Austin. pp. 171–195.

Blanford, W.T. (1974). "Descriptions of new reptilia and amphibia from Persia and Balochistan." ***Ann. Mag. Nat. Hist. London*, 4,14:** 31-35.

Brandon, R.A., Martan, J., Wortham, J.W.E. and Englert, D.C. (1974). The influence of interspecific hybridization on the morphology of the spermatozoa of Ambystoma (Caudata, Ambystomatidae), ***J. Reprod. Fertil.*, 41:** 275-284.

Bachmann, K. and Blommers-Schlosser (1972). Nuclear DNA amounts of frogs of Madagascar. ***Zool. Anz.*, 194:** 13-21.

Baldari, C.T. and Amaldi, F. (1976). DNA reassociation kinetics in relation to genome size in four amphibian species. ***Chromosoma,* 59:** 13-22.

Bandopadhyay, T.K. (1977). The chronology of meiosis and spermatogenesis in *Heteropneustes fossilis, Bufo melanostictus, Calotes versicolar* and *Bos indicus*. Ph.D. Thesis. Burdwan University, Burdwan.

Bush, G.L., Case, S.M., Wilson, A.C. and Patton, J.L. (1977). Rapid speciation and chromosomal evolution in mammals. ***Proc. Natl. Acad. Sci. U.S.A.,* 74:** 3942-3946.

Brown, G.B., Kim, Y.H., Kuntzrl, H., Mosher, H.S., Fuhrman, G.J. and Fuhrman, F.A. (1977) Chemistry and pharmacologyof skin toxins from the frog *Atelopus zeteki* (atelopidtoxin; zetekitoxin), ***Toxicon,* 15:** 115-128.

Beveridge, A.S. (1979). *Babur-Nama.* Sangemeel Publication, Lahore.

Batistoni, R.., de Lucchini, S., Vitelli, L., Andronico, F., Nardi, J. and Barsacchi-Pilone, G. (1980). Karyological analysis and distribution of ribosomal genes in *Hynobius keyserlingii* (Amphibia Urodela) and *Alytes obstetricans* obstetricans (Amphibia, Anura). ***Atti. Assoc. Genet. Italiano*, 25:** 31-35.

Bremer, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. ***Evolution,* 42:** 795–803.

Buckland-Nicks, J. (1995). Ultrastructure of sperm and sperm–egg interaction in Aculifera. In: Jamieson, B.G.M., Ausio, J. and Justine, J. (eds) Advances in Spermatozoal Phylogeny and Taxonomy. ***Muséum National d'Histoire Naturelle,*** Paris, France, pp. 129–153.

Baig, K.J. and Gvozdik, L (1998). *"Uperodon systoma* (Schneider):record of a new microhylid frog from Pakistan." ***Pakistan J. Zool.*, 30:** 155-156.

Báo, S.N., Vieira, G.H.C. and Fernandes, A.P. (2001). Spermiogenesis in *Melanophryniscus cambaraensis* (Amphibia, Anura, Bufonidae): ultrastructural and cytochemical studies of carbohydrates using lectins. ***Cytobios*, 106:** 203–216.

Church, G. (1959). "Size variation in Bufo melanostictus from Java and Bali (Amphibia)." ***Treubia* , 25:** 113-126.

Church, G (1960). "Annual and lunar periodicity in the sexual cycle of the Javanese toad, Bufo melanostictus Schneider." ***Zoologica*, 44:** 181-188.

Chatterjee, K., and Barik, S.K. (1970). "A study of chromosomes of both sexes of the Himalayan toad, *Bufo himalayanus*." ***Genen phaenen.*, 14:** 1-3.

Callard,I.P., Callard, G.V., Lance, V., Bolaffi, J.L. and Rossett, J.S. (1978). Testicular regulation in nonmammalian vertebrates. ***Biol. Reprod.*, 18:**16-43.

Chatterjee, C., Ray, D. and Patra, S. (2002). Sperm Polymorphism in Amphibia, a Fluorescence Activated Cell Sorting Study. ***Bulletin of Pure and applied Sciences,* 21A(2):** 63-66.

Daniel, J.C. (1963). "Field guide to the amphibians of western India." *Part 2. J. Bombay Nat. Hist. Soc.*, **60:** 690-702.

Dubois, A. (1975). "Un nouveau sous-genre (Paa) et trois nouvelles especes du genere Rana. Remarques sur la phylogenie des Ranides (Amphibiens, Anoures)." ***Bull. Mus. Nat. D'Histor. Natur. 3e ser*, 231:** 1093-1115.

Duellman, W.E. (1975) On the classification of frogs. ***Occ. Pap. Mus. Nat. Hist.*** Univ. Kansas, **42:** 1-14.

Daniel, J.C. (1975). "Field guide to the amphibians of western India." *Part 3. **J. Bombay Nat. Hist. Soc.***, 72: 506-522.

Dubois, A., and J. Martens, (1977). "Sur les crapauds du groupe de Bufo viridis (Amphibiens,Anoures) de l'Himalaya Occidental (Cachemire et Ladakh)." ***Bulletin de la Societe Zoologique de France*** , **102:** 459-465.

Dubois, A., M.S. Khan (1979). "A new species of frog (genus Rana, subgenus Paa) from northern Pakistan (Amphibia, Anura)." ***J. Herpetol*****, 13:** 403-410.

Dubois, A. (1983) Classification et nomenclature supragenerique des amphibiens anoures. ***Bull. Men. Soc***. Linneenne Lyon, **52:** 270-276.

Davis, L.G., Dibner, M.D., Battey JF. (1986). Basic Methods in Molecular Biology. ***Elsevier.*** New York.

Duellman, W.E. and Trueb, L. (1986). ***Biology of Amphibians***. McGraw-Hill, New York.

Duellman, W. E. (1993). Amphibian species of the world: Additions and corrections. ***Univ. Kansas Publ. Mus. Nat. Hist.*****, 21:**1–372.

Das, I., and S.K. Dutta (1998). "Checklist of the amphibians of India, with English common names." ***Hamadryad*****, 23:** 63-68.

Duellman, W. E., and Sweet, S. (1999). Distribution patterns of amphibians in the Nearctic region of North America. *In* "Patterns of

Distribution of Amphibians: A Global Perspective" (W. E. Duellman, Ed.), Johns Hopkins Press, Baltimore, pp. 31–110.

Das, S.K.; Banerjee, A. and Pradhan, A. (2003). Comparative study of Serum of *Bufo himalayanus* and *Bufo stomaticus* by Gel Electrophoresis. Recent Advances in Animal Science Research Vol. 3, Orion Press International.

Eiselt, V.J. and J.F. Schmidtler (1973). "Froschlurche aus dem Iran unter Berucksichtigung ausseriranischer Populationsgruppen." ***Ann. Naturhistor. Mus. Wien.,*** **77:** 181-243.

Echlin, P. (1975). Sputter coating techniques for scanning electron microscopy. In ***Scanning Electron Microscopy*** edited by Johari, O. and Corvin, I. IIT Research Institute, Chicago, p.p. 217-224.

Edward C. Roosen-Runge (1977). Amphibia *The process of Spermatogenesis in Animals* Cambridge University Press, p.p.115-121.

Farris. J.S. (1969) A successive approximations approach to character weighting. ***Syst. Zoo.l,*** **18:** 374-385.

Fawcett, D.W. (1970). A comparative view of sperm ultrastructure. ***Biol. Reprod. Supple.*** **2:**90-127.

Fujita, T. Miyoshi, M. and Tokunaga, J. (1970). Scanning and transmission microscopy of human ejaculated spermatozoa with special reference to their abnormal forms. ***Z. Zellforsch.*** **105:** 483-497.

Furieri, P. (1975). The peculiar morphology of the spermatozoon of *Bombina variegata* (L.), ***Monit. Zool. Italiano*** (N.S.), **9:**185-201.

Fouquette, M. J. Jr. and Delahoussare, A. J. (1977). Sperm morphology in the *Hyla rubra* group (Amphibia, Anura, Hylidae), and its bearing on genetic status, ***J. Herpetol.***, **11:** 387-396.

Fischberg, M. and Kobel, H.R. (1978) Two new polyploid *Xenopus* species from western Uganda. ***Experientia*, 34**: 1012-1014.

Farris, J. (1982). The logical basis of phylogenetic analysis. In: Platnick, N. and Funk, V. (eds) ***Advances in Cladistics***: Proc. Second Meet. Willi Hennig Soc. Columbia University Press, New York, USA. pp 7-36.

Frost, D. R. (Ed.) (1985). "Amphibian Species of the World: A Taxonomic and Geographical Reference", Allen Press and Assoc. ***Syst. Coll.***, Lawrence.

Ford, L.S. and Cannatella, D.C. (1993). The major clades of frogs. ***Herpetol. Monogr.***, 7: 94-117.

Ford, L.S. (1993). The phylogenetic position of dart poison frogs (Dendrobatidae) among anurans: an examination of competing hypothesis and their characters. ***Ethol. Ecol. Evol.***, **5:** 219-231.

Ford, L.S. and Cannatella, D.C. (1993). The major clades of frogs. ***Herpetol. Monogr.***, **7:** 94–117.

Fraker, P.J., King, L.E., Lill-Elghanian, D. and Telford, W.G. (1994). Quantitation of apoptotic events in pure and heterogeneous populations of cells using flow cytometry. In ***Methods in Cell Biology*** *Volume 46: Cell Death,* B. Osborne and L. Schwartz, eds., Academic Press, New York, NY, pp. 57-76.

Griffiths, I. (1954). The phylogenetic status of the Sooglossinae. ***Ann. Mag. Nat. Hist.,*** **13(2):** 626-640.

Griffiths, I. (1963). The phylogeny of the Salientia, ***Biol. Rev.,*** **38:** 241-292.

Gupta, B.L. (1964). Cytological studies of the male germ cells in some fresh water ostracods and copepods. Ph.D. Thesis, Cambridge University.

Graybeal, A. (1993). The phylogenetic utility of cytochrome *b:* Lessons from bufonid frogs. ***Mol. Phylogenet. Evo,l.*** **2:** 256–269.

Graybeal, A. (1997). Phylogenetic relationships of bufonid frogs and tests of alternate macroevolutionary hypotheses characterizing their radiation. ***Zool. J. Linn. Soc.,*** **119:** 297–338.

Gvozdik, L. and H. Radek (1997). "A small collection of amphibians from Baluchistan and Punjab, Pakistan in the Silesian Museum,." ***Opava. Cas. Slez. Muz., Opava*** , **(A)46:** 203-208.

Garda, A.A.; Colli, G.R.; Aguiar Jr, O; Recco-Pimental; Bao, S.N. (2002) The ultrastructure of the spermatozoa of *Epipedobates flavopictus* (Amphibia, Anura, Dendrobatidae), with comments on its evolutionary significance. ***Tissue & Cell,*** **34(5):** 356-364.

Gomes, A. (2003) Personal communication.

Humphrey, R. R. (1921) The intestinal cells of the urodele testis. ***Am. J. Anat.*** **29:** 213-279.

Humphrey. R. R. (1922). The multiple testes in urodeles. ***Biol. Bull.,*** **43:** 45-67.

Helling, H. (1938). Das Geruchsorgan der Anuren, vergleichendmorphologisch betrachtet. ***Zeit. Ges. Anat.,*** **108:** 587-643.

Hemmer, H., J.F. Schmidtler, W. Böhme. (1978). "Zur Systematik zentralassiatischer Grunkroten (Bufo viridis-Komplex) (Amphibia, Salientia, Bufonidae)." ***Abh. Mus. Tierkd. Dresden,*** **34:** 349-384.

Hu, Q.-x., Jiang, Y.-m., and Tian, W.-s. (1984). Taxonomic studies on genus *Bufo* of China. ***Acta Herpetol. Sinica Chengdu*** [New Ser.], **3:** 79–85. [in Chinese].

Hillis, D.M., (1984). Misuse and modification of Nei's genetic distance, ***Syst, Zool.,*** **33:** 238-240.

Hillis, D.M., Ammerman, L.K., Dixon, M.T. and DeSá, R.O.(1993). Ribosomal DNA and the phylogeny of frogs. ***Herpetol. Monogr.,*** **7:**118-131.

Hay, J.M., Ruvinsky, I., Hedges, S.B. and Maxson, L.R. (1995). Phylogenetic relationships of amphibian families inferred from DNAsequences of mitochondrial 12S and 16S ribosomal RNA genes. ***Mol. Biol. Evol.,*** **12:** 928–937.

Hass, C. A., Maxson, L. R., and Hedges, S. B. (2001). Relationships and divergence times of West Indian amphibians and reptiles: Insights from albumin immunology. *In* "Biogeography of the West Indies: Patterns and Perspectives" (C. A. Woods and F. E. Sergile, Eds.). CRC Press, Boca Raton, FL.

Inger, R.F. (1967) The development of a phylogeny of frogs. *Evolution*, **21**: 369-384.

Inger, R. F. (1972). *Bufo* of Eurasia. *In* "Evolution of the Genus *Bufo*"(W. F. Blair, Ed.),. Univ. of Texas, Austin. pp. 102–118.

James, W.S. (1970). The Ultrastructure of Anuran Spermatids and Spermatozoa. Unpublished Doctor in Philosophy, University of Tennessee, Knoxville.

Jorgensen, C.B., Larsen, L.O. and Lofts, B. (1979). Annual cycle of fat bodies and gonads in the toad *Bufo bufo bufo* (L) compared with cycles in other temperate zone anurans. ***Biol. Skr.*, 22(5):** 1-37.

Jamieson, B.G.M. (1987). The Ultrastructure and Phylogeny of Insect Spermatozoa. Cambridge University Press, Cambridge, UK, pp 1–320.

Jamieson, B.G.M. (1991). *Fish Evolution and Systematics: Evidence from Spermatozoa.* Cambridge University Press, Cambridge, UK, pp 1-319.

Jamieson, B.J.M., Lee, M.S.Y. and Long, K. (1993). Ultrastructure of the spermatozoa of the internally fertilizing frog *Ascaphus truei* (Ascaphidae: Anura: Amphibia) with phylogenetic consideration. ***Herpetologica*,** 49**:** 52-65.

Jamieson, B.G.M. (1995). The ultrastructure of spermatozoa of the Squamata (Reptilia) with phylogenetic consideration. In: Jamieson, B.G.M., Ausio, J. and Justine, J (eds) Advances in Spermatozoal Phylogeny and Taxonomy. ***Museum National d'Histoire Naturelle,*** Paris, France, pp 359-383.

Jamieson, B.J.M. (1999). Spermatozoal phylogeny of vertebrates. In Gagnon, C. (ed) ***The male gamate: From Basic Science to Clinical Applications.*** Cache River Press, Vienna, IL, pp 303-331.

Kawamura, T. (1953). Studies on hybridization in amphibians. V. Physiological isolation among four Hynobius species. ***J. Sci. Hiroshima Univ. (Zool.),*** **14:** 93-116.

Kushida, H. (1963). An improved epoxy resin 'Epok 533' and polyethylene glycol 200 as a dehydrating agent. **J. *Electronmicroscopy* 12:** 167-174.

Karnovsky, J.M. (1965). A formaldehyde – glutaraldehyde fixative of high osmolality for use of electron microscopy. ***J.Cell Biol.,*** **27:** 127-137.

Khan, M.S. (1965). "A normal table of *Bufo stomaticus*." ***Biologia*** , **11:** 1-39.

Khan, M.S. (1968a). "Morphogenesis of digestive tract of Bufo stomaticus." ***Pakistan J. Scient. Res.*,** **20:** 93-106..

Khan, M.S. (1968b). " Amphibian fauna of District Jhang with notes on habits." ***Pakistan J. Sci.*,** **20:** 227-233.

Khan, M.S (1969). "A normal table of *Rana tigrina* Daudin. 1.Early development (Stages 1-27)." ***Pakistan J. Sci.*, 21:** 36-50.

Khan, M.S. (1972 a). "The "commonest toad" of West Pakistan and a note on *Bufo melanostictus* Schneider." ***Biologia*,** **18:** 131-133.

Khan, M.S. (1973). "Food of tiger frog *Rana tigerina* Daudin." ***Biologia*** , **19:** 93-107.

Khan, M.S. (1974). "Discovery of *Microhyla ornata* (Dúmeril & Bibron) from the Punjab, Pakistan." ***Biologia*** **, 20:** 179-180.

Kullmann, E. (1974). "Die Tierwelt Ostafghanistans in ihren geographischen Beziehungen." ***Kol. Zoo,*** **13:**13-25.

Khan, M.S. (1976). " An annotated checklist and key to the amphibians of Pakistan." ***Biologia*** **, 22:** 201-210.

Kasinsky, H.E.; Huang, S.Y.; Kwauk, S.; Mann, M.; Sweeney, J. and Yee, B. (1978). On the diversity of sperm histones in the vertebrates. III. Electrophoretic variability of the testis-specific histone patterns in Anura contrast with relative constancy in Squamata. ***J. Exp. Zool.,*** **203:** 109-126.

Khan, M.S. (1979). "On a collection of amphibians from northern Punjab and Azad Kashmir, with ecological notes." ***Biologia*** **, 25:** 37-50.

Khan, M.S. (1980). "Affinities and Zoogeography of herpetiles of Pakistan." ***Biologia*** **, 26:** 113-171.

Kasinsky, H.E.; Mann, M.; Pickerill, L.; Gutovich, L. and Byrd Jr, E. W. (1981). Sperm histone diversity in the vertebrates: an evolutionary trend. ***J. Cell Biol.,*** **91:** 187.

Khan, M.S. (1982 a). "Key for the identification of amphibian tadpoles from the plains of Pakistan." ***Pakistan J. Zool.,*** **14:** 133-145.

Khan, M.S. (1982 b). "Collection, preservation and identification of amphibian eggs from the plains of Pakistan." ***Pakistan. J. Zool.,*** **14:** 241-243.

Khan, M.S. (1985). "An interesting collection of amphibians and reptiles from Cholistan Desert, Punjab, Pakistan." ***J. Bombay. Nat. Hist. Soc.*, 82:** 144-148.

Khan, M.S. (1986). "A noteworthy collection of amphibians and reptiles from northwestern Punjab, Pakistan." ***The Snake*, 18:**118-125.

Khan, M. S. and S. A. Malik, (1987). "Buccopharyngeal morphology of tadpole larva of *Rana hazarensis* Dubois and Khan 1979, and its torrenticole adaptations." ***Biologia*, 33:** 45-60.

Khan, M. S. and S. A. Malik, (1987). "Reproductive strategies in a subtropical anuran population in arid Punjab, Pakistan." ***Biologia* , 33:** 279-303.

Khan, M.S. (1987). "Checklist, distribution and zoogeographical affinities of amphibians and reptiles of Balochistan." ***Proc. Pakistan Zool. Congr.***, 105-112.

Khan, M.S. and Ahmed, Naeem (1987). "On a collection of amphibians and reptiles from Baluchistan, Pakistan." ***Pakistan J. Zool.*, 19:** 361-370.

Khan, M. S. and K.J. Baig, (1988). "Checklist of the amphibians and reptiles of District Jhelum, Punjab, Pakistan." ***The Snake*, 20:**156-161.

Khan, M. S. and Tasnim, Rashida, (1989). "A new frog of the genus Rana, subgenus Paa, from southwestern Azad Kashmir." ***J. Herpetol.*, 23:** 419-423.

Kuramoto, M. (1990). A list of chromosome numbers of anuran amphibians. ***Bull. Fukuoka Univ. Edu,*. 39:** 83–127.

Khan, M.S (1990). "The impact of human activities on the status and distribution of amphibians in Pakistan." ***Hamadryad*** **, 15:** 21-24.

Khan, M.S. (1991a). "Morphoanatomical specialization of the buccopharyngeal region of the anuran larvae and its bearing on the mode of larval feeding." *Ph.D. diss.*

Khan, M.S., and S.A. Mufti, (1994 a). "Oral disc morphology of amphibian tadpole and its functional correlates." ***Pakistan J. Zool.*****, 26:** 25-30.

Khan, M. S. and S.A. Mufti, (1994 b). "Buccopharyngeal specializations of tadpole of *Bufo stomaticus* and its ecological correlates." ***Pakistan J. Zool.*****, 26:** 285-292.

Khan, M.S., and S.A. Mufti, (1995). "Oropharyngeal morphology of detritivorous tadpole of Rana cyanophlyctis Schneider, and its ecological correlates." ***Pakistan J. Zool.*****, 27:** 43-49.

Kwon, A.S. and Lee, Y.H. (1995). Comparative spermatology of anurans with special references to phylogeny. In: Jamieson, B.G.M., Ausio, J. and Justine, J. (eds) Advances in Spermatozoal Phylogeny and Taxonomy. ***Mémoires du Muséum National d'Histoire Naturelle,*** Paris, France, pp 321–332.

Khan, M.S. (1996). "Oropharyngeal morphology of tadpole of southern cricket frog Rana syhadrensis Annandale, 1919; and its ecological correlates." ***Pakistan J. Zool.*****, 28:** 133-138.

Khan, M.S. (1996). "The oropharngeal morphology and feeding habits of tadpole of tiger frog Rana tigerina Daudin." ***Russian J. Herpetol*****, 2:** 163-171.

Khan, M.S. (1997). "A new subspecies of common skittering frog Euphlyctis cyanophlyctis (Schneider 1799) from Balochistan, Pakistan." ***Pakistan J. Zool.*, 29(2):** 107-112.

Khan, M.S. (1997). "A new toad from the foot of Siachin Glacier, Baltistan, northeastern Pakistan." ***Pakistan J. Zool.*, 29(1):** 43-48.

Khan, M. S. (2001). "Recent advances in the taxonomic status of ranid frogs of Pakistan." ***Pakistan J. Zool.*, 33(3):** 169-171.

Lofts. B. & Boswell. C. (1960). Cyclical changes in the distribution of testis lipids in the common frog. *Rana temporaria*. ***Nature,*** Lond. **187:** 708-709.

Lofts. B. (1964). Seasonal changes in the functional activity in the intestinal and spermatogenic tissueof the green frog *Rana esculenta*. ***Gen.Comp. Endocrin***. **4:** 550-562.

Lofts, B. (1976) Physiology of the Amphibia. Vol.3, Academy press, New York. USA.

Laurent, R.F. (1979). Esquisse d'une phylogenese des anoures. ***Bull. Soc. Zool.*** France, **104:** 397-422.

Lofts, B. (1984) Reproductive Cycles in Vertebrates- Amphibians. In, Marshall's Physiology of Reproduction (G. E. lamming, ed.), Vol. I, Churchill Livingston, London. pp. 127-205.

Lynch, J. D., and Renjifo, J. M. (1991). Two new toads (Bufonidae: *Rhamphophryne*) from the northern Andes of Columbia. ***J. Herpetol.*** **24:** 64–371.

Lee, Y.H. and Kwon, A.S.(1992). Ultrastructure of spermiogenesis in *Hyla japonica* (Anura, Amphibia). ***Acta Zool.*** (Stockholm), **73:** 49–55.

Lee, M.S.Y. and Jamieson, B.G.M. (1992). The ultrastructure of the spermatozoa of three species of myobatrachid frogs (Anura, Amphibia) with phylogenetic considerations. ***Acta Zool.*** (Stockholm), **73:** 213–222.

Lee, M.S.Y. and Jamieson, B.G.M. (1993). The ultrastructure of the spermatozoa of bufonid and hylid frogs (Anura, Amphibia): implication of phylogeny and fertilization biology. ***Zool. Src.,*** **22:** 309-323.

Lauder, G.V.(1994). Homology, form, and function. In: Hall, B.K. (ed) The Hierarchical Basis of Comparative Biology. Academic Press, San Diego, pp 151–196.

Lee, Y.H. and Kwon, A.S. (1996). Ultrastructure of spermatozoa in urodela and primitive anura (Amphibia) with phylogenetic considerations. ***Korean J. Syst. Zool.,*** **12:** 253–264.

Liu, W., and Yang, D. (1997). A study on karyotype, C-banding and Ag-NORs of two species of *Bufo* in Gaoligongshan, Yunnan. ***Acta Herpetol. Sinica (Zunyi),*** **6–7:** 1–4. [in Chinese]

Lourenco, L.B., Garcia P.C. and recco-Pimentel, S.M. (2000). Cytogenetics of two species of Paratelmatobius (Anura: Leptodactylidae), with phylogenetic comments. ***Hereditas,*** **133(3):** 201-209.

Liu, W., Lathrop, A., Fu, J., Yang, D., Murphy, R.W. (2000). Phylogeny of East Asian Bufonids Inferred from Mitochondrial DNA sequences (Anura: Amphibia). ***Molecular Phylogenetics and Evolution,*** **14(3):** 423-436.

Mukerji, D.D. (1931). "Some observations on the burrowing toad Cacopus globulosum Günther." ***J. Proc. Asiatic Soc. Bengal,*, 27:** 97-100.

McCann, C. (1938). "The reptiles and amphibians of Cutch State." ***J. Bombay Nat. Hist. Soc.*, 40:**425-427.

Minton, S.A. (1962). "An annotated key to the amphibians and reptiles of Sind and Las Bela, West Pakistan." ***Am. Mus. Novit No.*, 2081:** 1-21.

Minton, S.A. (1966). "A contribution to the herpetology of west Pakistan." ***Bull. Amer. Mus. Nat. Hist.*** , **134(2):**31-184.

Mertens, R. (1969). "Die Amphibien und Reptilien West-Pakistans." ***Stuttg. Beitr. Naturk.*, 197:** 1-96.

Mertens, R (1970). "Die Amphibien und Reptilien West-Pakistans.1." ***Nachtrag. Stuttg. Beitr. Naturk.*, 216:**1-5.

McDiarmid, R.W. (1971). Comparative morphology and the evolution of frogs of the Neotropical genera *Atelopus, Dendrophryniscus, Melanophryniscus,* and *Oreophrynella, Nat. Hist. Mus. Los Angeles Co. Sci. Bull.*, **12:** 1-66.

Mertens, R. (1971). "Die Amphibien und Reptilien West-Pakistans. 2." ***Nachtrag. Senckenb. Biol.*, 52(1-2):** 7-15.

Mertens, R. (1972). " Nachtrage zum Krokodil-Katalog der senckenbergischen Sammlungen. 3." ***Nachtrag. Senckenb. Biol.*, 53:** 21-35.

Martan, J. and Wortham, E. (1972). A tail membrane on the spermatozoa of some ambystomatid salamanders. ***Anat. Rec.,* 172:** 460-472.

Morescalchi, A. (1973). Amphibian. *In* "Cytotaxonomy and Vertebrate Evolution" (A. B. Chiarelli and E. Capanna, Eds.), Academic Press, New York, pp. 233–348.

Maxon, L.R., and Wilson, A.C. (1974). Convergent morphological evolution detected by studying proteins of tree frogs in the *Hyla eximia* species group. ***Science,* 185:** 66-68.

Mertens, R (1974). " Die Amphibien und Reptilien West-Pakistans." ***Senckenb. Biol.*, 55(1-3):** 35-38.

Morescalchi, A. and Olmo, E. (1974). Sirenids: a family of polyploid urodeles?. ***Experientia*, 30:** 491-492.

Mazik, E.Y., Kadyrova, B.K. and Taktosunov, A.K. (1976). Peculiarities of the karyotype of the green toad (*Bufo viridis*) of Kirghizia. ***Zool. Zhur.*, 55:** 1740-1742.

Morescalchi, A. (1979). "Amphibia" *In* Cytotaxonomy and vertebrate evolution. Ed. A. Chiarelli E. Capanna. Academic Press, New York, pp. 233-248.

Mahony, M.J. and Robinson, E.S. (1980). Polyploidy in the Australian leptodactylid frog genus *Neobatrachus*. ***Chromosoma*, 81:** 199-212.

Macgregor, H.C., and Del Pino, E.M. (1982). Ribosomal gene amplification in multinucleate oocytes of the egg brooding hylid frog *Flectonotus pygmaeus. Chromosoma,* **85:** 475-488.

Maxson, L. R. (1984). Molecular probes of phylogeny and biogeography in toads of the widespread genus *Bufo*. ***Mol. Biol. Evol.*, 1:** 47–350.

Mita, K. Takamune and Katagiri, C. (1991) Genes for Sperm- specific Basic Nuclear Proteins in *Bufo* and *Xenopus* Are Expressed at different Stages in Spermatogenesis. ***Develop. Growth & Differ.*, 33(5):** 491-498.

Mita, K., Ariyoshi, N., Abe, S.I., Takamune, K., Katagiri, C. (1995) Structure of genes for sperm-specific nuclear basic protein (SP4) in *Xenopus laevis*. ***Biochemica et Biophysica Acta*, 1245:** 430-438.

Meves. F. (1896). Ueber die Entwicklung der mannlichen Geschlechtszellen von Salamandra maculosa. ***Arch. Mikrosk. Anat.* 48:** 1-83.

Meyer, E., Jamieson, B.G.M. and Scheltinga, D.M. (1997). Sperm ultrastructure of six Australian hylid frogs from two genera (*Litoria* and *Cyclorana*): phylogenetic implications. ***J.Submicrosc. Cytol. Pathol.*, 29:** 443-451.

Macey, J. R., Schulte II, J. A., Larson, A., Fang, Z.,Wang, Y., Tuniyev, B. S., and Papenfuss, T. J. (1998). Phylogenetic relationships of toads in the *Bufo bufo* species group from the eastern escarpment of the Tibetan Plateau: a case of vicariance and dispersal. ***Mol. Phylogenet. Evol.*, 9:** 80–87.

Masta, .E., Sullivan, B.K., lamb, T. and Routman E.J. (2002). Molecular systematics, hybridization, and phylogeography of *Bufo americanus* complex in Eastern North America. ***Mol. Phylogenet. Evol.*, 24(2):** 302-314.

Mukhopadhyay, S. (2002). Gonadal Differentiation and Spermatogenetic activity in Himalayan toad, *Bufo himalayanus* (Gunther): Anura, Amphibia. An Ultrastructural study. Ph.D. Thesis, University of North Bengal.

Natarajan, R. (1953). " A note on the chromosomes of Cacopus systoma." *Proc. 40th Indian Sci. Cong.* , **3:**180-181.

Natarajan, R. (1958). "Contribution to the cytology of Indian Anura: II. On the number and morphology of the chromosomes of three species of Rana (Ranidae)." *J. Zool. Soc. India,* **9:** 114-119.

Nelson, C.E., and Humphrey (1972). Artificial interspecific hybridization among *Ambystoma*. ***Herpetologica,*** **28:** 27-32.

Nei, M., (1972). Genetic distance between populations, ***Amer. Nat.,*** **106:** 283-292.

Oordt, P.G., van, W.J (1960). The influence of internal and external and external factors in the regulation of spermatogenetic cycle in Amphibia. *Symp. Zool. Soc.* London, **2:** 29-52.

Poirier, G.R. and Spink, G.C. (1971). The ultrastructure of testicular spermatozoa in two species of *Rana*. ***J. Ultrastruct. Res.***, **36:** 455–465.

Pisannitz, E. M. (1978). On a new polyploid species of *Bufo danatensis* Pisanetz, sp. N., from Turkmenistan. ***Dokl. Adad. Nauk Ukran. SSR. (Ser. B)*** **3:** 280–284. [in Russian]

Pisanetz, Y.M. (1978). New polyploid species of *Bufo danatensis* Pisanetz. Sp. Nov., from the Turkmen SSR (in Russian), ***Sopov. Akad. Nauk. Ukr. Rsp., Heol. Biol. Nauky,*** **3(B):** 277-282.

Pugin-Rios, E. (1980). Étude comparative sur la structure du spermatozoïde des amphibiens anoures. Comportament des gamètes lors de la fécondation. Unpublished Université de Rennes, France, Rennes.

Prakash, S. (1988). "Genetic studies on *Rana limnocharis*". Ph.D.Thesis, North Eastern Hill University, Shillong.

Pierce, E.J. and Breed, W.G. (2001). Comparative study of sperm production in two species of Australian arid zone rodents (*Pseudomys australis, Notomys alexis*) with marked differences in testis size. ***Reproduction*, 121:** 239-247.

Patra, S. (2003). An ultrastructural study on seasonal testicular changes of Himalayan Newt (Urodela, Amphibia). Ph.D. Thesis. North Bengal University.

Patra, S. and Ray, D. (1993). Scanning electron micrographic observation of spermatogenesis of Himalayan Newt, *Tylototriton verrucosus* Anderson (Urodela: Amphibia). ***Recent advances in Animal Science Research,*** Vol. 3, Orion Press Internatiolal.

Pramuk, J.B.; Hass, C.A. and Hedges, S.B. (2001). Molecular Phylogeny and Biogeography of West Indian Toads (Anura: Bufonidae). ***Moloecular Phylogeny and Evolution,* 20(2):** 294-301.

Paladhi, M.; Kaur Chetri, S.; Singh, R. (2003) Ultrastructure of Bidder's Organ of *Bufo himalayanus* during Breeding Season. ***Journal of Experimental Zoology*** (India) (In Press).

Rao, C.R.N. (1917). "On the occurance of iridocytes in the larva of *Microhyla ornata* Boulenger." ***Rec. Indian Mus.*, 13:** 281-292.

Rao, C.R.N. (1918). "Notes on the tadpoles of Indian Engystomatidae." ***Rec. Indian Mus.*** , **15:** 41-45.

Reynolds, E.S. (1963). The use of lead citrate at high pH as electron opaque stain in electron microscopy. ***J. Cell Biol.,*** **17 :** 208-218.

Rogers, J.S. (1972) Measures of genetic similarity and genetic distance. ***Univ. Texas Publ.*** **7213:** 145-153.

Roosen-Runge, E.C. (1977). The process of spermatogenesis in animals, Cambridge University Press. pp-115-121

Ray, D. (1978). Autoradiographic studies on spermatogenesis of some vertebrates with biochemical effects of induced spermatogenesis in mammals. Ph.D. Thesis, University of Burdwan.

Rastogi, R.K. and Iela, L. (1980). Steroidogenesis and spermatogenesis in anuran amphibians: a brief survey. In, Steroids and their Mechanism of Action in Nonmammalian Vertebrates (G. Delroi and J. Brachet edts.), Ravan Press. pp. 131-146.

Ray, D., Roy, R.., Paul, K., Henry, W. and De, A. (1986). Mitotic and meiotic chromosomes of *Philautus annandalli* (Boulenger) [Anura: Amphibia]. ***J. Sci. Res.,*** **1:** 33-39.

Rastogi, R.K., Bagnara, J.T., Iela, L. and Krasovich, M.A.(1988). Reproduction in the Mexican leaf frog, *Pachymedusa dacnicolor*. IV. Spermatogenesis: a light and ultrasonic study. ***J. Morphol.,*** **197:** 277–302.

Risley, M.S.; Einheber, S. and Bumerot, D.A. (1989). Changes in DNA topology during spermatogenesis. ***Chromosoma,*** **94(3):** 217-227.

Roy, R. and Ray, D. (1989). Chromosomal polymorphism in spermatogonial cells of Himalayan toad, *Bufo himalayanus* (Gunther) [Anura: Amphibia]. ***Perspectives in Cytology and Genetics*, 6:** 443-447.

Roy, R. (1989). Some aspects of spermatogenesis in Indian Salamander, *Tylototriton verrucosus* Anderson (Urodela, Amphibia). Ph.D. Thesis, University of Burdwan.

Robinson, J Paul. (1993). Handbook of Flow Cytometry Methods. Wiley-Liss, New York.

Ray, D and Mukhopadhyay, S. (1999). Kinetics of meiosis and spermatogenesis in Himalayan toad, *Bufo himalayanus* (Gunther). ***Indian J. Applied and Pure Biol.*, 14:** 174-177.

Steindachner, F. (1867). *Amphibien.* Novara-Expedition, ***Zoologisches Theil.*** Vienna **I:** 1-70.

Stoliczka, F. (1872). "Notes on some new species of Reptilia and Amphibia, collected by Dr. W. Waagen in North-Western Panjab." ***Proc. Asiatic Soc. Bengal*, 39**: 124-131.

Stoliczka, F. (1872). "Observation on some Indian and Malayan Amphibia and Reptilia." ***Proc. Asiatic Soc. Bengal*, 39:** 134-228.

Seshachar, B.R. (1939). The spermatogenesis of *Uraetyphlus narayani* Seshachar. ***La Cellule*, 48:** 63-76.

Starrett, P.H. (1968). The Phylogenetic Significance of the Jaw Musculature in Anuran Amphibians, Ann Arbor: Univ. Michigan, Ph.D. Dissertation.

Schmidtler, J.J. and J.F. Schmidtler, (1969). "Uber Bufo surdus; mit einem Schlussel und Anmerkungen zu den ubrigen Kroten Irans und West-Pakistans." ***Salamandra*** **, 5:** 113-123.

Savage, J. M., (1973). "The geographic distributiion of frogs patterns and predictions." *Evolutionary Biology of the anurans: contemporary research and major* Selmi, M.G., Brizzi, R. and Bigliardi, E. (1997) Sperm morphology of salamandrids (Amphibia, Urodela): implications for phylogeny and fertilization biology. ***Tissue & Cell,*** **29:** 651-664.

Schneider, H. (1977). "Acoustic behavior and physiology of vocalization in the European tree frog, *Hyla arborea* (L)" in D. H. Taylor and S. I. Guttman (eds.), The Reproductive Biology of Amphibians, New York, Plenum Press, pp.-295-335.

Schmid, M. (1978). Chromosome banding in Amphibia I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla.* ***Chromosoma,*** **66:** 361–388.

Swan, M.A., Linck, R.W., Ito, S. and Fawcett, D.W.(1980). Structure and function of the undulating membrane in spermatozoan propulsion in the toad *Bufo marinus.* ***J. Cell Biol.,*** **85:** 866–880.

Saidapur, S.K. (1983). Patterns of testicular activity in the Indian amphibians. ***Indian Rev. Life Sci.,*** **3:** 157-184.

Saidapur, S. K. (1989). Reproductive Cycles of Amphibians in *Reproductive Cycles of Indian Vertevrates* by Allied Publishers Limited, pp.166-223.

Semik, D and Kilarski, W. (1988). Scanning electron microscopy of the egg surface of the common toad, *Bufo bufo,* following fertilization

and during first cleavage. ***Folia Histichem. Cytobiol.*, 36(1):** 29-34.

Seshachar, B.R. (1994). The intestinal cells in the testis of *Icthyophis glutinosus* Linn. ***Proc. Indian Acad. Sc.*, 13:** 244-254.

Selmi, M.G., Brizzi, R. and Bigliardi, E. (1997). Sperm morphology of salamandrids (Amphibia, Urodela): implications for phylogeny and fertilization biology. ***Tissue Cell*, 29:** 651–664.

Semik, D. and Kilarski, W. (1998). scanning electron microscopy of the egg surface of common toad, Bufo bufo, following fertilization and during first cleavage. ***Folia Histochem Cytobiol.*, 36(1):** 29-34.

Stock, M., M. Schmid, C. Steinlein, and W-R. Grosse, (1999). "Mosaicism in somatic triploid specimens of the Bufo viridis complex in the Karakoram with examination of calls, morphology and taxonomic conclusions." ***Ital. J. Zool.*, 66:** 215-232.

Scheltinga, D.M., Jamieson, B.G.M., Eggers, K.E. and Green, D.M. (2001). Ultrastructure of the spermatozoon of *Leiopelma hoschostetteri* (Amphibia, Anura, Leiopelmatidae). ***Zoosystema*, 23:** 157–171.

Trewavas, E. (1933). The hyoid and larynx of the Anura, ***Phil. Trans, Roy. Soc.*** London, **222(B):** 401-527.

Tanaka, K. and Naguro .(1981). High resolution scanning electron microscopy of cell organelles by a new specimen preparation method. ***Biomed.Res 2(Suppl)*,** p.p. 63-70.

Telford, W.G., King, L.E. and Fraker, P.J. (1994). Rapid quantitation of apoptosis in pure and heterogeneous cell populations using flow

cytometry (review article). ***Journal of Immunological Methods,*** **172:**1-16.

Tanaka, S., Kurokawa, H. and Masako, H. (1995). Comparative morphology of the sperm in chondrichthyan fishes. In: Jamieson, B.G.M., Ausio, J. and Justine, J (eds) *Advances in Spermatozoal Phylogeny and Taxonomy.* ***Museum National d'Histoire Naturelle,*** Paris, France, pp 313-320.

Teixeira, R.D., Colli, G.R. and Báo, S.N. (1999a). The ultrastructure of the spermatozoa of the lizard *Micrablepharus maximiliani* (Squamata Gymnophthalmidae) with considerations on the use of sperm ultrastructure characters in phylogenetic reconstruction. ***Acta Zool.*** (Stockholm), **80:** 47–59.

Teixeira, R.D., Colli, G.R. and Báo, S.N.(1999b). The ultrastructure of the spermatozoa of the worm-lizard *Amphisbaena alba* (Squamata, Amphisbaenidae), and the phylogenetic relationships of amphisbaenians. ***Can.J.Zool.,*** 77**:**1254–1264.

Vlad, M. (1977). Quantitative studies of rDNA in amphibians. ***J. Cell Sci.,*** **24:** 109-118.

Vitelli, L., Batistoni, R., Andronico, F., Nardi, I. and Barsacchi- Pilone (1982). Chromosomal localization of 18S+28S and 5S ribosomal RNA genes in evolutionary diverse anuran amphibians. ***Chromosoma,*** **84:** 475-491.

Werner, C. (1970). "On the development and structure of the head and neck in urodele sperm." in B. Baccetti (ed.), Comparative Spermatology, New York: academic Press, pp. 85-92.

Wiley, E.O. (1981). Phylogenetics. *The Theory and Practice of Phylogenetic Systematics,* John Wiley and Sons, New York, USA.

Wortham, J.W.E. Jr.; Brandon, R.a. and Martan, J. (1977). Comparative morphology of some plethodontid salamander spermatozoa. ***Copeia,* 1977**: 666-680.

Wortham, J.W.E.Jr., Murphy, J.A., Martan, J. and Brandon, R.A. (1982). Scanning electron microscopy of some salamander spermatozoa. ***Copeia,* 1982:** 52-55.

Yadav, J.S. and R.K. Pillai, (1975). "Somatic karyotypes of two Indian species of frogs (Anura, Amphibia)." ***Cytobios*, 13:** 109-115.

Yang, Y. (1983). A comparative study on three subspecies of *Bufo bufo* from China using techniques of C-banding, Ag-NORs and electrophoresis of sera and lactate dehydrogenase (LDH) isozyme. ***Acta Herpetol. Sininca* 2:** 1–9. [in Chinese]

Yoshizaki, N. (1987). Isolation of spermatozoa, their ultrastructure and their fertilizing capacity in two frogs *Rana japonica* and *Xenopus laevis*. ***Zool. Sci.*, 4:** 193–196.

Yang, D., Liu, W., and Dingqi, R. (1996). A new toad genus of Bufonidae—*Torrentophryne* from trans-Himalaya mountain of Yunnan of China with its biology. ***Zool. Res.* 17:** 353–359. [in Chinese]

Zhao, E., and Adler, K. (Eds.) (1993). "Herpetology of China," SSAR, St. Louis, MO.

Bulletin of Pure and Applied Sciences. Vol. 21A (No. 2) 2002; P. 63-66

SPERM POLYMORPHISM IN AMPHIBIA- A FLUORESCENCE ACTIVATED CELL SORTING STUDY

D. Ray, C. Chatterjee and S. Patra
Cell and Molecular Biology Laboratory, Postgraduate Department of Zoology, Darjeeling Government College, Darjeeling - 734101, (West Bengal), India

ABSTRACT

Spermatogonia and spermatozoan cells from three anuran and one urodele amphibian species were collected by differential centrifugation method and the cells were fixed in 80% ethanol, stained with ethidium bromide in sodium citrate buffer and scanned under FACS. The histograms obtained from *Bufo melanostictus, Bufo himalayanas, Bufo stomaticus*, and *Tylototriton verrucosus* exhibited normal haploid spermatozoa to hypohaploid and hyperhaploid spermatozoan cells. Similarly gonia cells exhibited normal diploid (2n) to hypodiploid (< 2n) and hyperdiploid (>2n) DNA values. The observations strongly support earlier observations on sperm polymorphism in amphibians made by karyological and ultrastructural technique/method.

Key words : Sperm, Polymorphism, FACS, Analysis, Amphibia.

INTRODUCTION

Existence of Sperm Polymorphism has been recorded in a number of invertebrates and vertebrates including Amphibia (Siebold, 1836; Ankel, 1924; Gupta, 1964; Ray *et al*, 1984; Roy and Ray, 1989) reported that in Amphibia there are more than one clone of stem cells and each such clone has different chromosomal set ranging from normal diploid number to hypo and hyper diploid sets . In the present investigation, an additional evidence is provided to substantiate the existence of sperm polymorphism in Amphibia using fluorescence activated cell sorting (FACS) technique.

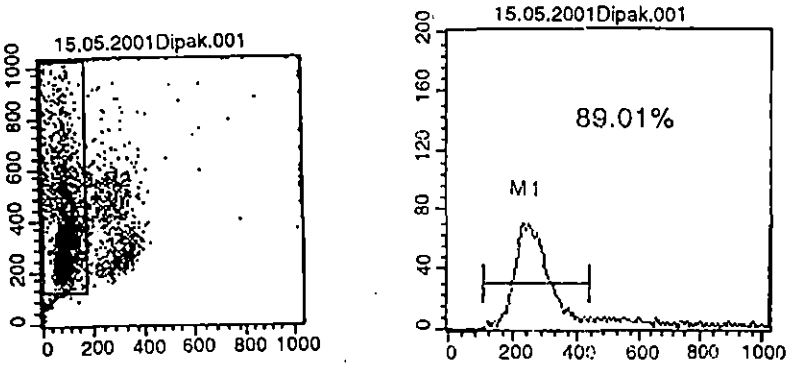
MATERIALS AND METHODS

Male adult toads of Bufo melanostictus, Bufo himalayanus, Bufo stomaticus and the urodele Tylototriton verrucosus were used as experimental animals. Spermatogonic cells were collected by using differential centrifugation technique (Ray *et al.*1984) and the fractions containing pure spermatogonia (2n) and sperm cells (n) were used for FACS study at IICB, Kolkata.

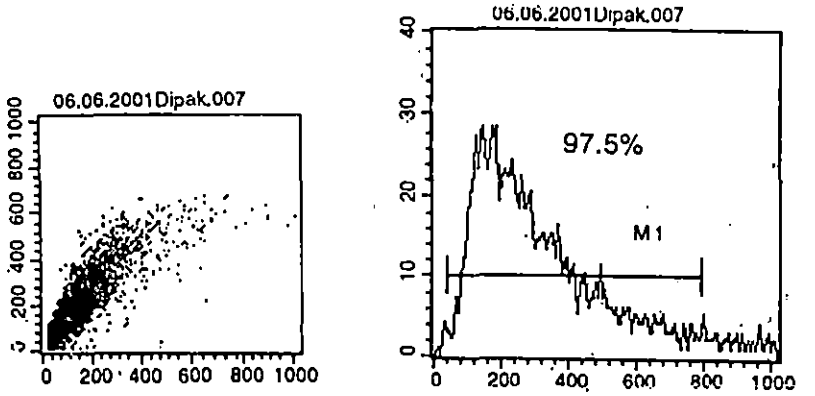
Spermatogonic cells were collected in phosphate buffer saline (PBS) and fixed in 80% ethanol overnight. Cells were then washed with citrate buffer(pH7) and mixed with 1% ethidium bromide in sodium citrate buffer prior to FACS study.

OBSERVATIONS

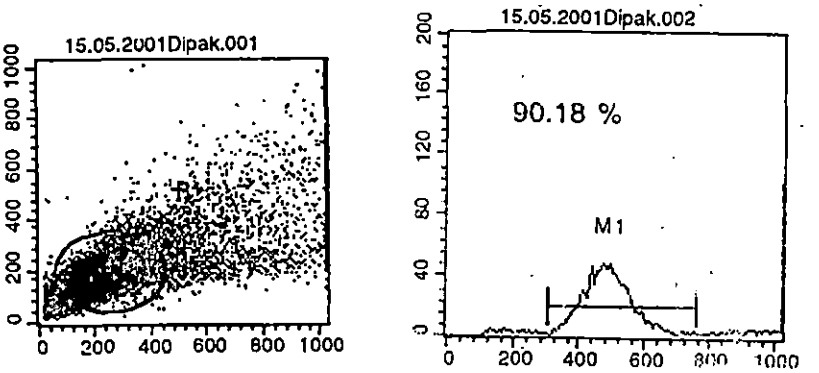
The histograms A, B, C, and D, suggest that in *B. melanostictus* 89.01% cells studied have normal haploid DNA value, while 10.9% cells have a range of below haploid DNA value to hyperhaploid DNA value. In *B. himalayanas* 97.15% cells have normal haploid value, while *B. stomaticus* 90.18% cells have normal haploid value. In *Tylototriton verrucosus*



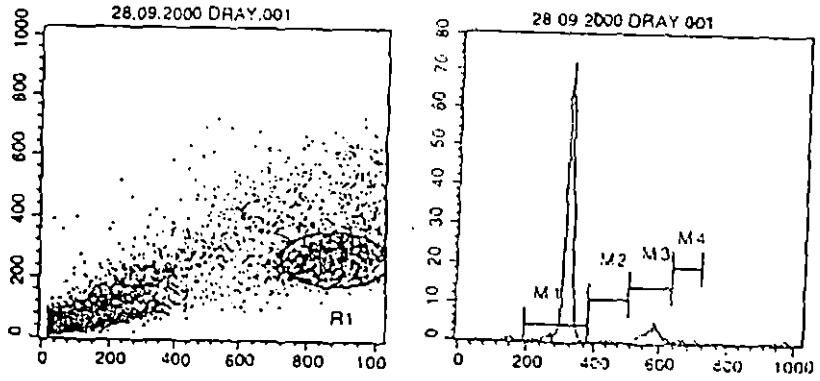
Histogram A - *Bufo melanostictus*



Histogram B - *Bufo himalayanus*



Histogram C - *Bufo stomaticus*



Histogram D - *Tylototriton verrucosus*

91.56% of cells have normal haploid value while 7.04% cells have normal diploid value. A small fraction of cells have hypo diploid (0.30%) and hyperhaploid (0.15%) DNA value. Similarly about 0.30% cells have hyper diploid DNA value.

DISCUSSION

Sperm Polymorphism was first recorded in the pond snail *Paludina* by Siebold (1836) and he described two types of spermatozoa in that species. However, systematic studies on this issue have been made only in recent years. Hendelberg(1969) found two kind of spermatozoa in an acoelan flatworm one with two flagella called 'typical' and a smaller 'atypical' without nucleus. In many other cases of polymorphism the aberrant types are morphologically very characteristic and sometimes show a behavior which suggested that they have become functional components of the reproductive process although they have never been successful to accomplish normal fertilization (Rossen - Runge, 1977).

Origin of sperm polymorphism has been described variously by different authors. Ray & Roy (1984), Roy (1989), Mukhopadhyay *et al*; (1999) reported that in amphibia gonia cells have different cytological and karyological features and gave the concept of 3 different clones of gonial cells; the normal diploid cell, hypo diploid cell and hyper diploid cells. The ultra structural features of such have been described in *B. himalayanus* by Mukhopadhyay (2002) and in *Tylototriton verrucosus* by Patra *et al* (2002).

In the present investigation, gonia cells (2n) were mixed with fractions of spermatozoa collected by differential centrifugation method theoretically to have haploid DNA content. However, the histograms exhibited an array of hypohaploid to hyperhaploid DNA values. Similarly hypodiploid and hyperdiploid DNA values were also recorded, unequivocally suggesting the existence of different gonia as well as spermatozoan cells in the four amphibian species investigated.

A further study is under process to establish the existence of different expression of sperm specific proteins (SP1, to SP6) in gonial as well as spermatozoan cells.

ACKNOWLEDGEMENT

The authors are indebted to Prof. S. Bondypadhyay, Senior scientist, Indian Institute of Chemical Biology, Kolkata, to allowing them to use the FACS facility in his laboratory and Dr. T.C. Nag, Lecturer, P.G. Department of Zoology, Darjeeling Govt College , Darjeeling - 734101, W.B. for his valuable suggestions .

REFERENCES

1. Ankel,W.E.(1924).Der Spermatozoendimorphism bei *Bythinia tentaculata* L.und *Viviparus viviparus* L.Z. *Zellforsch.mikrosk. Anat.* 11; p. 491-166.

2. Gupta,B.L.(1964).Cytological studies of the male germ cells in some freshwater ostracods and copepods. Ph.D. thesis, Cambridge University.

3. Hendelberg, J.(1969). On the development of different types of spermatozoa from spermatids with two flagella in the *Turbellaria* with remarks on the ultrastructure of the flagella. *Zool. Bibr., Uppsala* 38; p. 1-50.

4. Mukhopadhyay, S.; Das, S and Ray, D. (1999). Polymorphism in Sperm Morphology -A scanning electron microscopic study on *Bufo himalayanus* (Anura : Amphibia). *Trans. Zool. Soc. East India*, 3(1); p. 58-60.

5. Mukhopadhyay,S.(2002). Gonadal differentiation and Spermatogenesis of Himalayan toad, *Bufo himalayanus* (Gunther) [Anura: Amphibia] – An ultrastructural study. *Ph.D. Thesis, University of North Bengal.*

6. Patra, S.; Roy, R.R. and Ray, D. (2002). Scanning electron microscopic observation on spermiogenesis of Himalayan newt (Amphibia:Urodela). *Recent Advances in Animal Science Research* (Orion Press International) Vol.2; p. 272-280.

7. Ray, D.; Roy, R.R.; Paul, K.; Henry, W. and De, A. (1984). Mitotic and meiotic chromosomes of *Philautus annandalli* (Boulenger) [Anura: Amphibia]. *J.Sci.Res.*,1; p. 33-36.

8. Roy, R. and Ray, D. (1989). Chromosomal polymorphism in spermatogonial cells of Himalayan toad, Bufo himalayanus (Gunther)[Anura:Amphibia].*Perspectives in Cytology and Genetics*. (Eds. G.K.Manna and U. Sinha). 6; p. 443-447.

9. Roy, R. (1989). Some aspects of Spermatogenesis in Indian Salamander, *Tylototriton verrucosus* (Anderson) [Urodela:Amphibia]. Ph.D. Thesis, University of Burdwan.

10. Rossen-Runge,E.C.(1977).The Prosses of Spermatogenesis in Animals. Cambridge University Press, Cambridge, London.

11. Siebold,C.T.von(1836). Ueber die Spermatogoen der Crustaceen, Insecten, Gastropoden und einiger anderer wirbelloser Thiere. *Muller's Arch.* 6; p. 13-53.

SEXUAL DIMORPHISM IN ENZYME ACTIVITY PATTERN OF *Canis lupus chanco* (TIBETAN WOLF)

C. Chatterjee, K. Sarkar, S. Patra, K. Pradhan
P. G. Department of Zoology
Darjeeling Government College
Darjeeling

A biochemical study was undertaken to reveal enzyme activity pattern of male and female captive wolves reared at Padmaja Naidu Himalayan Zoological Park, Darjeeling. The quantities of total protein, serum albumin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase were determined following standard biochemical techniques. The study indicated a strong sexual dimoephism in enzyme activity at normal physiological condition.

Introduction

Wolves are of generally two types – the Red wolves and the Grey wolves. The Tibetan wolf – *Canis lupus chanco* is one of the grey wolves. This Himalayan creature is found at an altitude between 7000 and 13000 feet in the Western Himalayas. These canines prey upon larger mammals, such as wild goats, sheeps and other high altitude herbivores to smaller ones like rodents besides birds (Annual Report PNHZP, 1995–1996).

Lots of information are available on the blood cells and various blood parameters of diverse vertebrates. Several workers have stressed upon the possibility of employing haematological indices as aids to the diagnosis and assessment of diseases. In the present work blood was collected from some of the captive wolves and was tried to estimate the total protein, serum albumin, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase were determined following standard biochemical techniques. A biochemical study was undertaken to reveal the enzyme activity pattern in male and female wolves.

Materials and methods

Experimental animals

The specimens under experiment were from Padmaja Naidu Himalayan Zoological Park, Darjeeling, situated at an elevation of 2133.5 mts. Three Tibetan wolves were taken for the experiment.

Serum collection

10 ml of blood was drawn from individual wolf and centrifused (1 hr, 37°C) when the blood cells precipitated down. The supernatant was the only source for the serum which thereafter was centrifused (3000 RPM, 5 mins.).Discarding the unwanted materials the collected serum was immediately used for the assay.

Experimental procedure

The commercial kits were used for getting the results without delaying time as because the enzyme activity changes with the change of time. The experiments were done according to the protocols as provided by the kits (Qualigens diagnostics from Glaxo and Dr. Reddy's laboratory).

Results

After using the kits in required proportions and incubating in the prescribed order we recorded the results obtained from the spectrophotometric observations.

	Male	Female
Total Protein	8.29 gm%	8.79 gm%
Serum Albumin	4.13 gm%	3.82 gm%
Alkaline Phosphate	16.22 U/ml	14.24 U/ml
SGOT	16.25 U/ml	14.50 U/ml
SGPT	12.16 U/ml	10.92 U/ml

Discussion

From the observations recorded we noticed that all the blood parameters, except that of the total protein, show a greater value in male than that of the female wolf.

SGPT catalyses transfer of amino group from L–alanine to α–ketoglutarate with formation of pyruvate and glutamate. SGPT catalyses transfer of amino group from L–aspartate to α–ketoglutarate with formation of oxaloacetate and glutamate. Formed products are then used for citric acid cycle and protein biosynthesis (Das, 1995). The experimental result suggests that male wolves are metabolically more active than the females under captive condition.

SGOT, SGPT and alkaline phosphatase are enzymes which are globular in nature and supply a mass to the total protein.

A higher total potein level in female than the male may be due to the fact that the female bears the baby and so she feeds both for herself and the baby (Chatterjee, 1992). Again, a high total protein content in such carnivorous wild animals in respect to a normal human being (6.0–6.9 gm/100 ml, Chatterjee, 1992) suggests that it may help them during starvation period (there may be a chance of not getting adequate food everyday) as protein reservoir.

The serum albumin content in Tibetan wolves is in normal level (54–60% of the total plasma protein, Das, 1995) but the female shows a declined value than the male. We know that when the body fluid discharge or decreases the albumin level also decreases (McMurray, 1982). As the female in our experiment was pregnant, so the decreased albumin may be due to the placental supply to the baby.

References

Annual Report – PNHZP. Darjeeling, 1995–1996.

Annual Report – PNHZP. Darjeeling, 1996–1997.

Chatterjee, C. C. 1992. Human Physiology Vol. 1, Medical Allied Agency, IInd edition.

Das, D. 1995. Biochemistry, Academic Publishers, 8th edition.

Devlin, T. M. 1997. Text Book of Biochemistry, John Wiley and Sons, 4th edition

Doumas, B. T., Arends, R. L. and Pinto, P. C. 1972. Standard Method of Clinical Chemistry, 7: 175–189

Flack, C. P. and Woolen, J. W. 1984. Clin. Chem. 30: 559.

McMurray, W. C. 1989. A Synopsis of Human Biochemistry (19–20). Harper and Row.

Reitman, S. and Frankel, S. 1957. Am. J. Clin. Path. 28:56.

Schlienger, J. L., Pradignae, A. and Grunenlerger, F. 1995. Nutrition of the elderly; a challange between facts and needs. Horm. Res. 43: 46.

Tietz, N. W. 1970. Fundamentals of Clinical Chemistry. p.447.

Tietz, N. W. 1996. Fundamentals of Clinical Chemistry: Philadelphia. W. B. Salinders Company, 240.

RECENT ADVANCES IN ANIMAL SCIENCE RESEARCH : VOL I : PP 101-105
ORION PRESS INTERNATIONAL

Gonadal Differentiation Pattern of Himalayan Toad, *Bufo himalayanas* (Gunther) : ANURA, AMPHIBIA-An Ultrastructural Study

S. Mukhopadhyay*, C. Chatterjee, S. K. Das** and D. Ray****

*Department of Zoology & Biology
Hooghly Mohsin College, Chinsurah-712 101 (India)

**Post-Graduate Department of Zoology
Darjeeling Govt. College, Darjeeling-734 101 (India)

Abstract

Gonadal differentiation pattern during larval development period has been studied from SEM and TEM observations. The larval period has been categorized into four stages, viz., '0' limb stage, '2' limb stage, '4' limb stage and 'sub-adult' stage. The '0' limb stage gonad exhibits no sign of gonadal differentiation and cells exhibit uniformity at ultra-structural levels. Gonadal differentiation becomes evident around '2' limb stage and '4' limb stage. Gonial cells also appear at late '4' limb stage. 'Sub-adult' stage exhibits appearance of primary and secondary spermatids. Appearance of scanty sperms is also evident at this stage which suggest a probable case of progenesis in this species.

Abstrait

Le motif de différentiation gonadale pendant la période du développement larvaire a été étudié des observations SEM et TEM. La période larvaire a été catégorisée en quatre phases, c'est-à-dire, phase '0' membre, phase '2' membre, phase '4' membre et phase 'sous-adulte'. La gonade de la phase '0' membre ne nontre aucun signe de différentiation gonadale et les cellules montrent l'uniformité aux niveaux ultra-structuraux. La différentiation gonadale est évidente vers les phases '2'-membres et '4'-membres. Descellules gonadiques apparaissent aussi vers la fin de la phase '4'-membres. La phase 'sous-adulte' expose l'apparence des spermatides primaives et secondaires. L'apparence des spermes insuffisants est aussi évidente à cette phase, ce qui suggère un cas probable de progénésis dans cette espèce.

* Author for correspondence.

Spermatogenesis in amphibians is usually identified with sexual maturity. However, in several anuran species, spermatogenic waves have been reported well before the sexual characters developed and even in the larval stage. This phenomenon has been named as 'pre-spermatogenesis' or 'precocious spermatogenesis' or 'juvenile spermatogenesis' (5, 6).

In the present text, a phemonon of juvenile spermatogenesis has been described in an anuran species, *Bufo himalayanas*, found at high altitudes of Darjeeling hills. The study has been made under Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

Materials and Methods

Tadpoles of *Bufo himalayanas* were collected from different places of Darjeeling hills at an altitude of 1800-2300 mts. Tadpoles were reared in the laboratory conditions (temperature 20°C). Following the metamorphic events, the tadpoles were classified as '0' limb stage, '2' limb stage, '4' limb stage and 'sub-adult' stage (Plate 1, Fig. a-d). Gonadal masses from each stage were collected by surgical operations for routine histological and ultra-microscopic studies.

A part of gonadal mass were fixed in 2.5% Glutaraldehyde in Cacodylate buffer (pH 7.1) for SEM studies, while the rest mass was fixed in Glutaraldehyde-Paraformaldehyde mixture for TEM observations. In both the cases, the post-fixation was made with 0.01 M Osmium Tetr oxide in cacodylate buffer (pH 7.1). Before critical point drying, the gonadal masses were superficially teased to expose the internal mass for SEM observations. SEM and TEM observations were made under Hitachi S-530 and Philips CM-10 Microscopes, respectively.

Observations and Discussions

The larvae of the *Bufo himalayanas* from hatching to emergence of sub-adults can be categorized into 39 stages. However, the gonadal mass can only be recognized visually on and from stage 21, called '0' limb stage, as a median oblong mass around the developing kidneys. For the convenience of the study, the larval developmental events has been summarized as '0' limb stage (i.e., with limb bud), '2' limb stage (i.e., with hind limbs only), '4' limb stage (i.e., with both hind and fore limbs) and 'sub-adult' stage (i.e., just after culmination of metamorphosis).

At '0' limb stage, the gonadal anlagen is visualized as an oblong mass at the antero-median part of the developing kidneys. Under the SEM, the anlagen mass exhibits primordial germ cells of spherical shape. The gonads originate from an outpocket of cells on the ventral surface of the kidney. There is no observable differences between males and females. The undifferentiated anlagen mass is kept within solid oblong gonads with clear cortex and medulla portion.

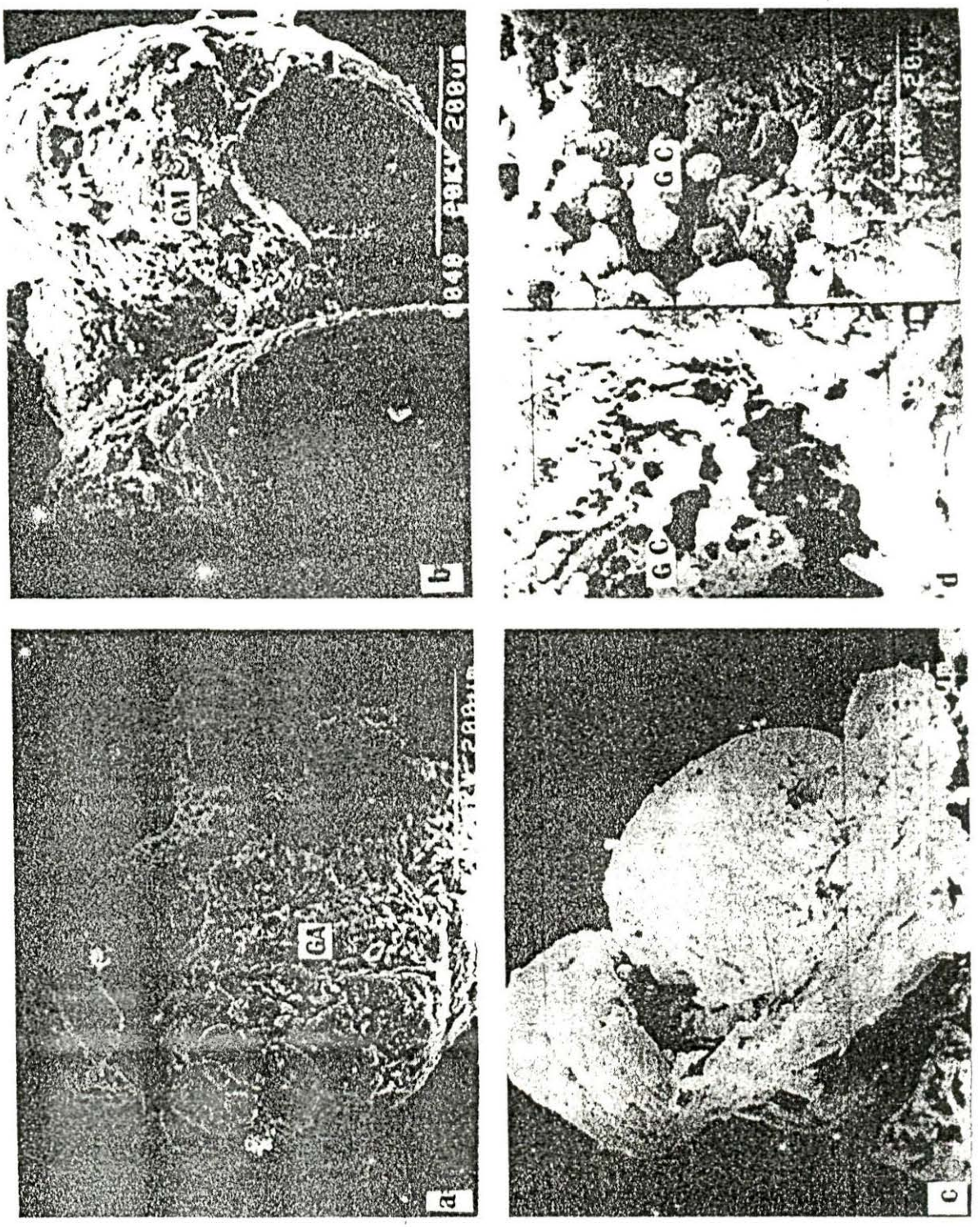
At '2' limb stage, the gonadal mass assumes a globular shape and is lodged at the anterior end of the kidney. The developing gonad is morphologically indistinguishable as Testis or Ovary. However, under SEM, the developing anlagen was exhibiting the appearance of the gonial cells as spherical irregular cells of about 7-10 mμ in diameter. The surface morphology of the gonial cells is characterised by rough and irregular surface with numerous depressions and ridges at regular intervals (Plate 1, Figs. a-d).

The existence of gonial cells in the developing testis has been sustained from TEM observations. The gonial cells exhibit the characteristic features of gonial cells as in other anurans (Plate 2, Figs. a-c). These gonial cells exhibit irregular surface morphology, with myriad convolutions. TEM ultra-structure shows oval or elliptical shape with oval nucleus and uniformly distributed chromatin granules. The nucleolus is electron dense and spherical. Cytoplasm is homogeneous and contains oval mitochondria, free ribosomes, granular and agranular endoplasmic reticulum (Plate 2, Figs a, b & c).

At '4' limb stage, gonial cells show some characteristics as in '2' limb stage. Under SEM observations, gonials cells show irregular surface morphology and oval and spherical shape of about 5-8 mμ in diameter. Here, at this stage, gonials cells along with primary and secondary spermatocytes are seen. TEM observations show gonial cells of similar nature as found in '2' limb stage. Primary and secondary spermatocytes show spherical central nucleus without nucleous. The electron dense heterochromatin masses of nucleoplasm irregularly distributed and condensed at the periphery of the nucleus. The cytoplasm shows characteristic electron dense droplets and vacuoles (Plate 2, Figs. d, e, & f).

Spermatogenesis is usually identified with sexual maturity. However, in several anuran species, spermatogenic activity has been recorded during early development before secondary sexual characters developed. Spermatogenesis has been recorded even in the larval conditions (1, 2, 4).

Plate -1



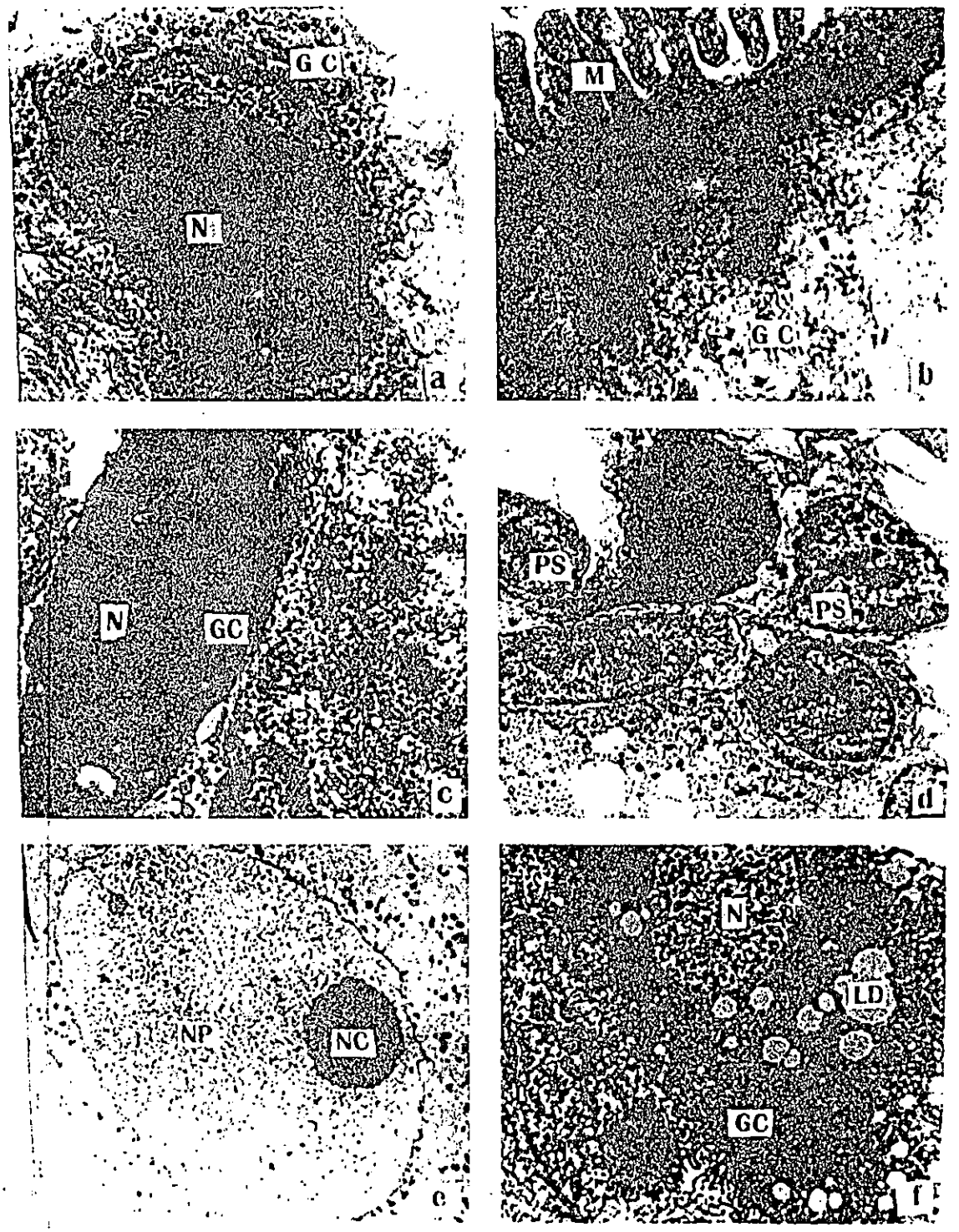
0 limb stage

2 limb stage

4 limb stage

4 limb stage (enlarged)

Plate -2



GC = Gonial cell PS = Primary spermatocyte N = Nucleus M = Micovilli
NC = Nucleolus NP = Nucleoplasm LD = Lipid droplet

Iwasawa and Kobayashi (2) have described that the juvenile spermatogenesis usually ends with degeneration of spermatogenic nests before completion of spermatogenic cycle. However, others (3, 4, 5, 6, 7) have recorded that in some anuran species, the juvenile spermatogenic cycle may proceed to the formation of spermatozoon with no clear difference from the adult spermatogenic cycle.

The present study indicates that in Himalayan toad (*Bufo himalayanas*), the spermatogenic activity starts in the larval stage, when the differentiation of the gonadal anlagen of '0' limb stage takes place into a distinct gonadal fate (i.e., either testis or ovary) around '2' limb stage, which is supported by the experiments of Hayes (8). Therefore, critical point of gonadal differentiation in this species is likely to take place at around '2' limb stage.

Acknowledgements

The authors are indebted to Dr. T. C. Nag, Department of Zoology, Darjeeling Govt. College, Darjeeling, for technical advice for SEM and TEM preparations, Dr. S. Chakraborty, UCIS, University of Burdwan and Dr. T. K. Das, AIIMS, New Delhi, for providing SEM and TEM facilities at their centres.

References

1. Kobayashi, S. (1975). Sexual and bilateral differences in germ cell numbers in course of gonadal development of *Rana nigromaculata*. *Zoology Magazine*, **84** : 1099-114.
2. Kobayashi, I and H. Iwasawa (1984). Effects of fat body excision on testicular ultrastructure in young frogs of *Rana nigromaculata*. *Zool. Sci. (Japan)*, **1** : 972.
3. Iwasawa, H and M. Kobayashi (1976). Development of the testis of the frog *Rana nigromaculata* with special reference to germ cell maturation. *Copeia*, **1976** : 461-467.
4. Swingle, W. W. (1921). The germ cells of Anura : I. The male sexual cycle of *Rana catesbeiana* larvae. *J. Expt. Zool.* **32** : 235-331.
5. Rastogi, R. K. Iela, L., Di Meglio, M., Di Mallee, L., Minucci, S., and Izzo-Vitiello, I. (1983). Initiation and kinetic profiles of Spermatogenesis in the frog, *Rana esculanta* (Amphibia). *J. Zool. (London)*, **200** : 233-247.
6. Jorgensen, C. B. and Billeter, E. (1982). Growth, differentiation and function of the testis in the toad *Bufo bufo bufo* (L.) with special reference to regulatory capacities ; effects of unilateral castration, hypophysectomy, and excision of Bidder's organ. *J. Expt. Zool.*, **221** : 225-236.
7. Jorgensen, C. B. (1986). Growth and Reproduction ; In : Physiology of Amphibian vertebrates, Reinnert Publication, London.
8. Hayes, T. B. (1998). Sex determination and primary sex differentiation in Amphibians : Genetic and developmental mechanism. *J. Expt. Zool.* **281** : 373-399.