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.

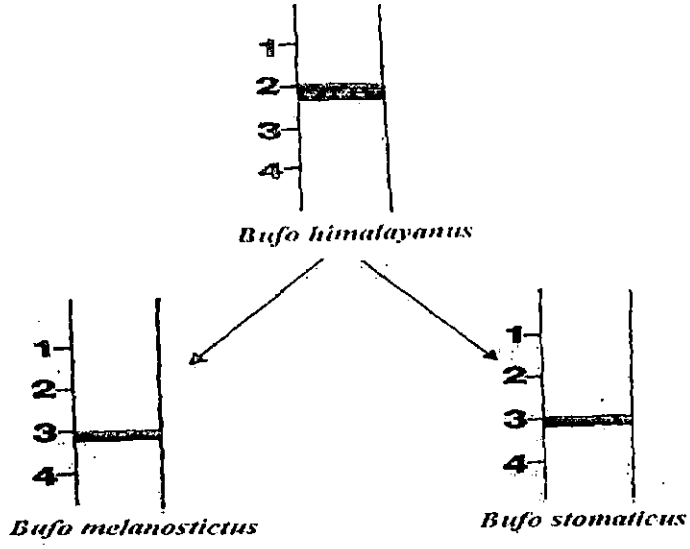
There are three distinct options to explain the situation-

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).

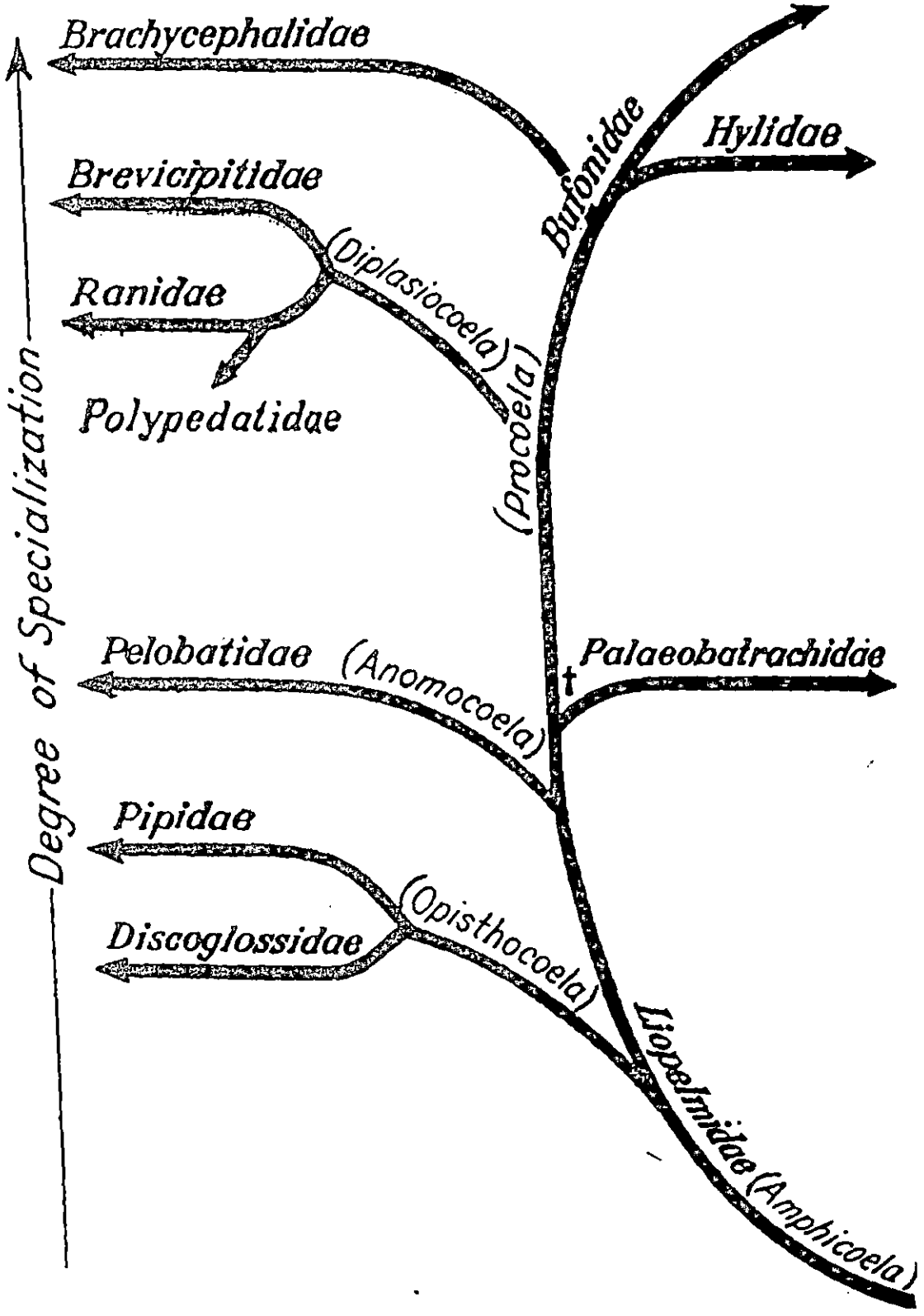
TABLE – 6

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.

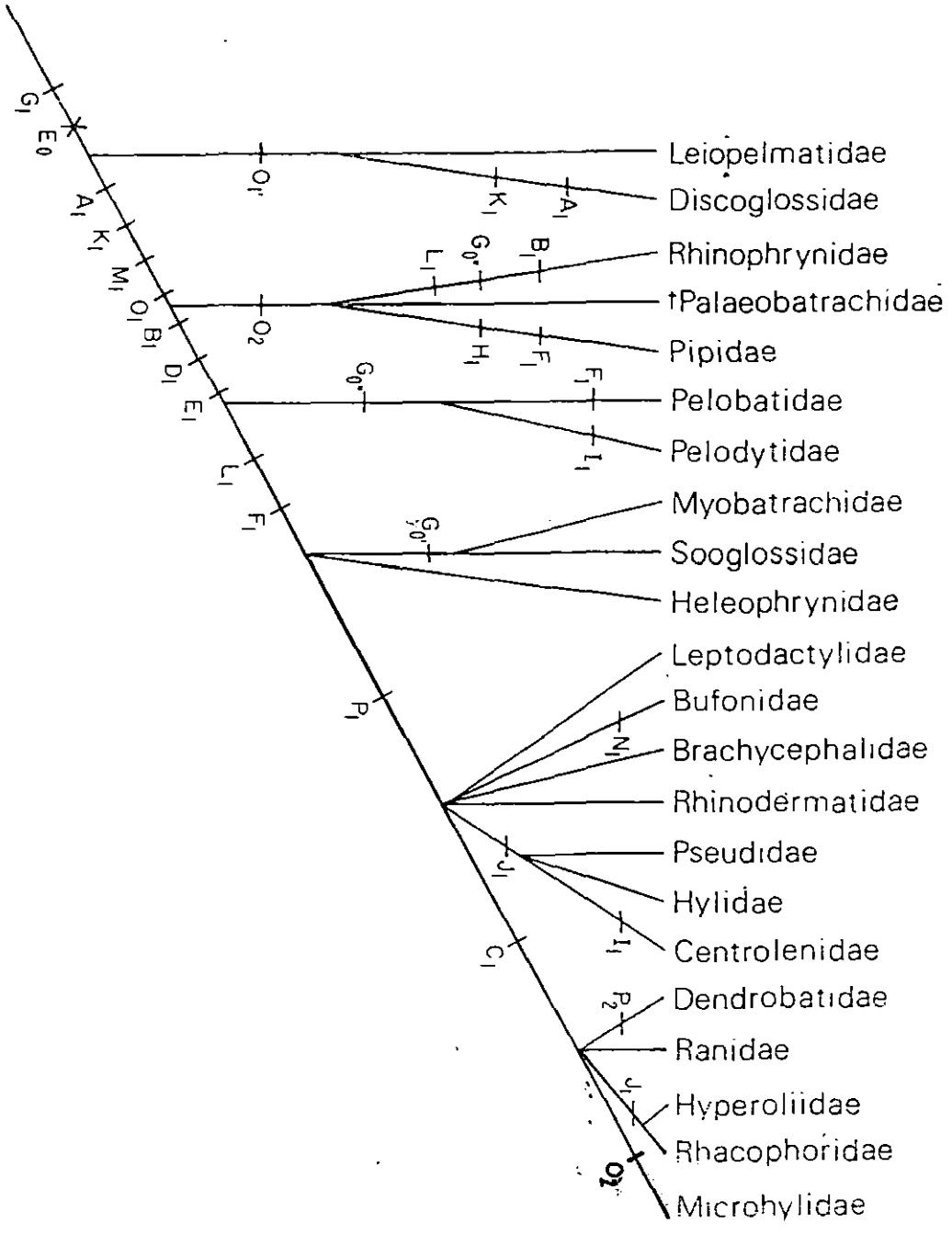
Comparison of Three Recent Classifications of Anurans

FIGURE – 8A



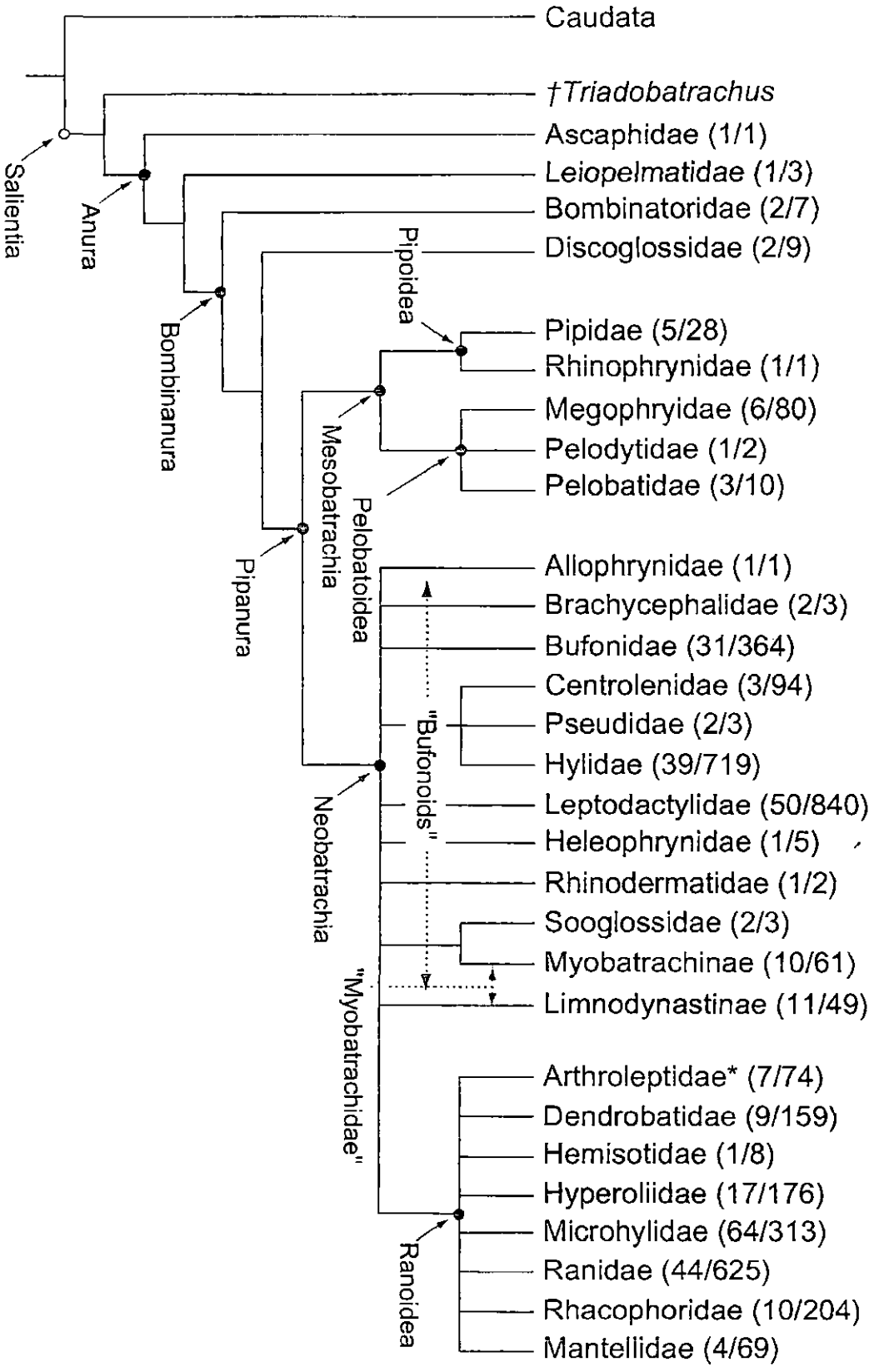
Phylogenetic relationship of Anura (Adapted from Noble, 1932)

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

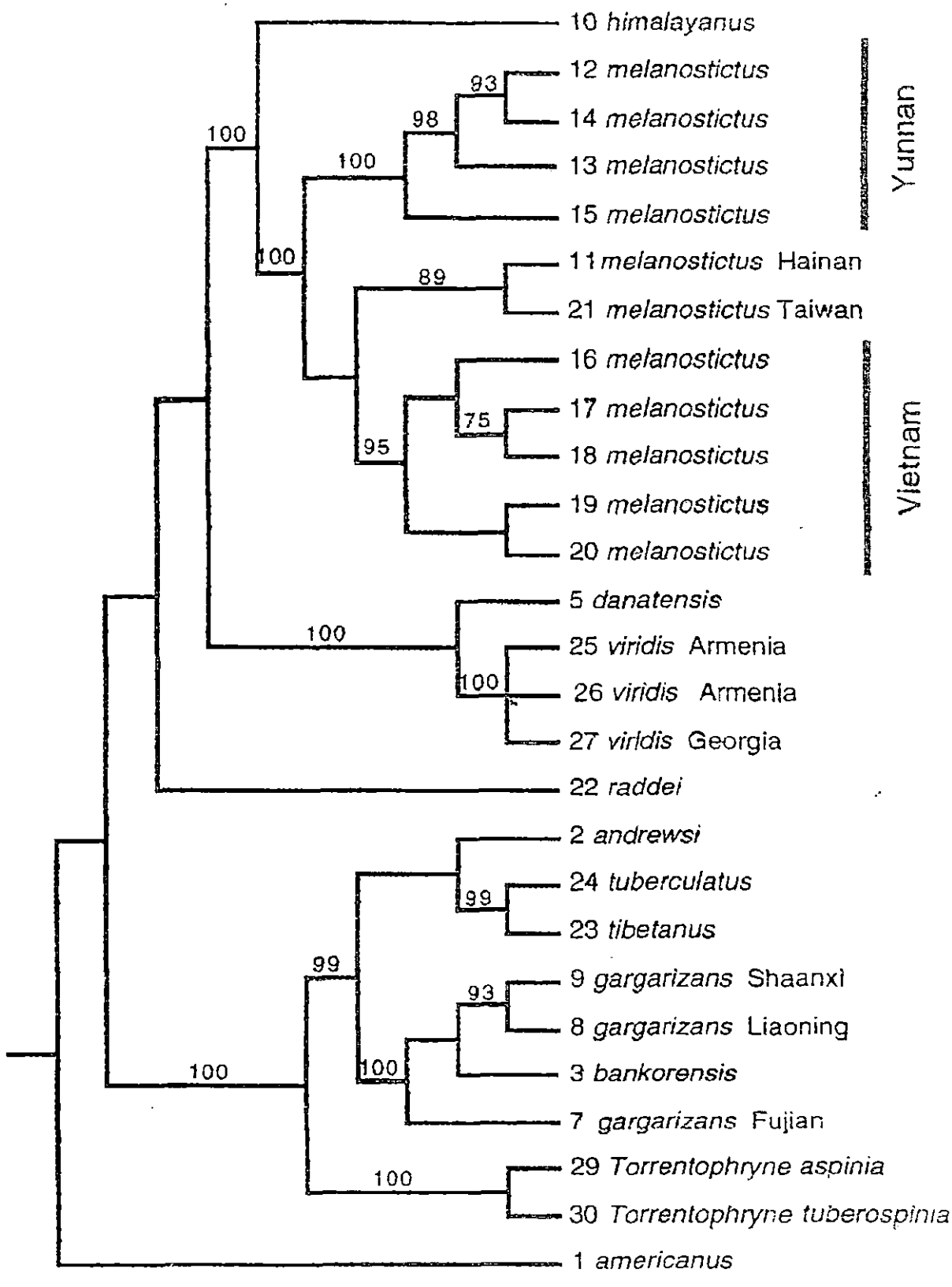
a) Ribosomal RNA :- the nucleolar organizer region (NOR) is the region of the chromosome that produces the nucleolus. In interphase through late prophase these region lack DNA but are rich in RNA. NOR is regarded as differentiated region of chromatin (DNA) which transcribe the larger species of RNA i.e., 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

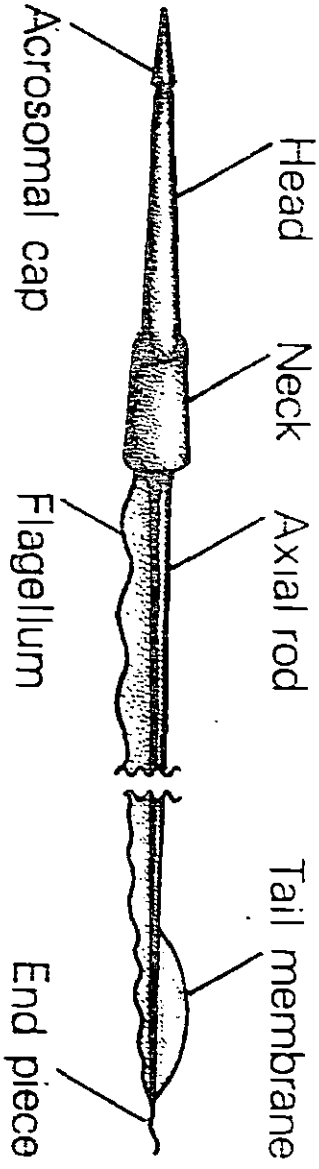


Phylogenetic tree of Bufonidae as proposed by Liu et.al., 2000.

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).

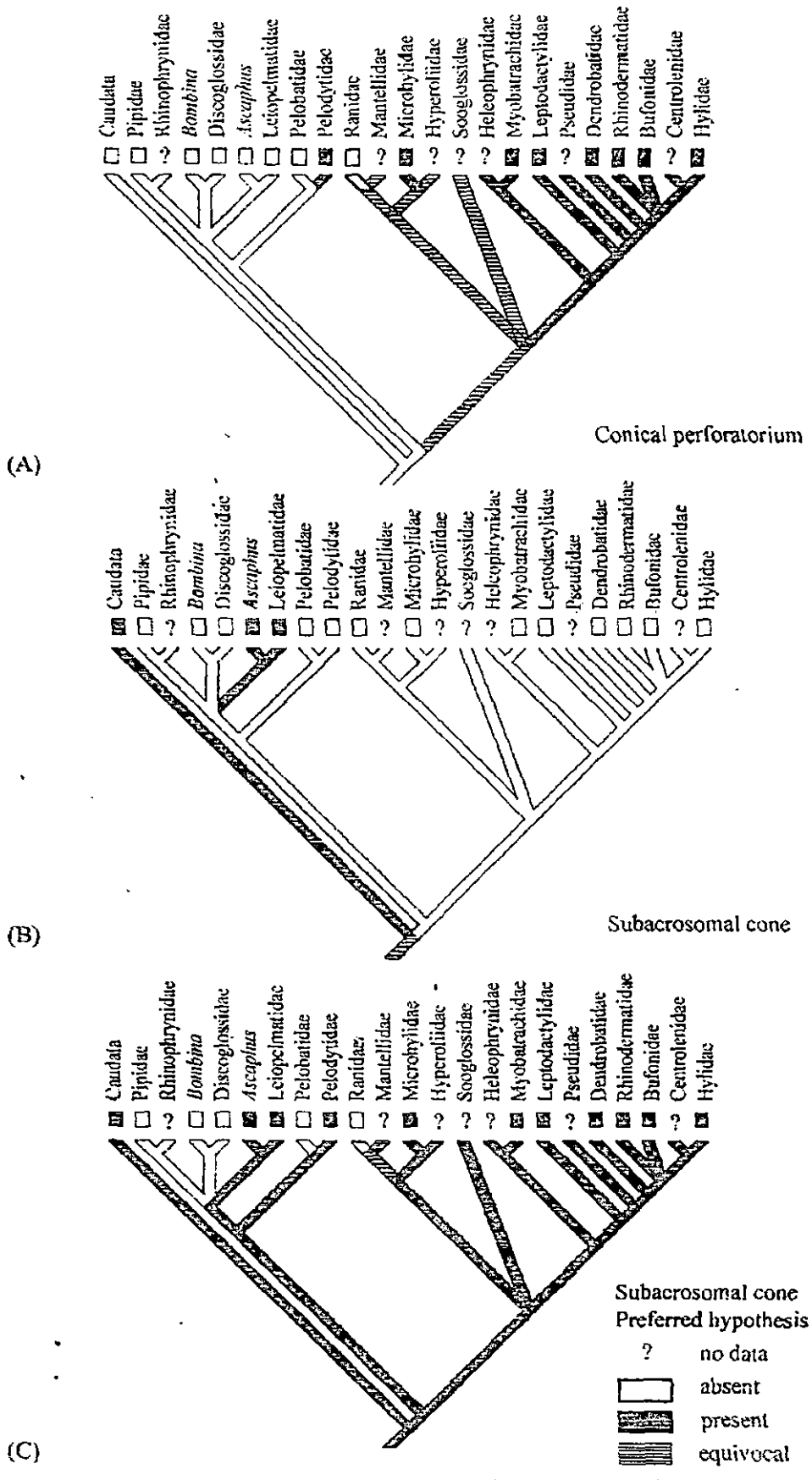
Wortham *et.al.* (1977) have opined that proportional in length of different parts of spermatozoa of plethdontids are consistent with the taxonomy of the group except for *Aneides*. Moreover, Wortham *et.al.* (1977) suggested that the long heads with more nuclear material of plethodontine spermatozoa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

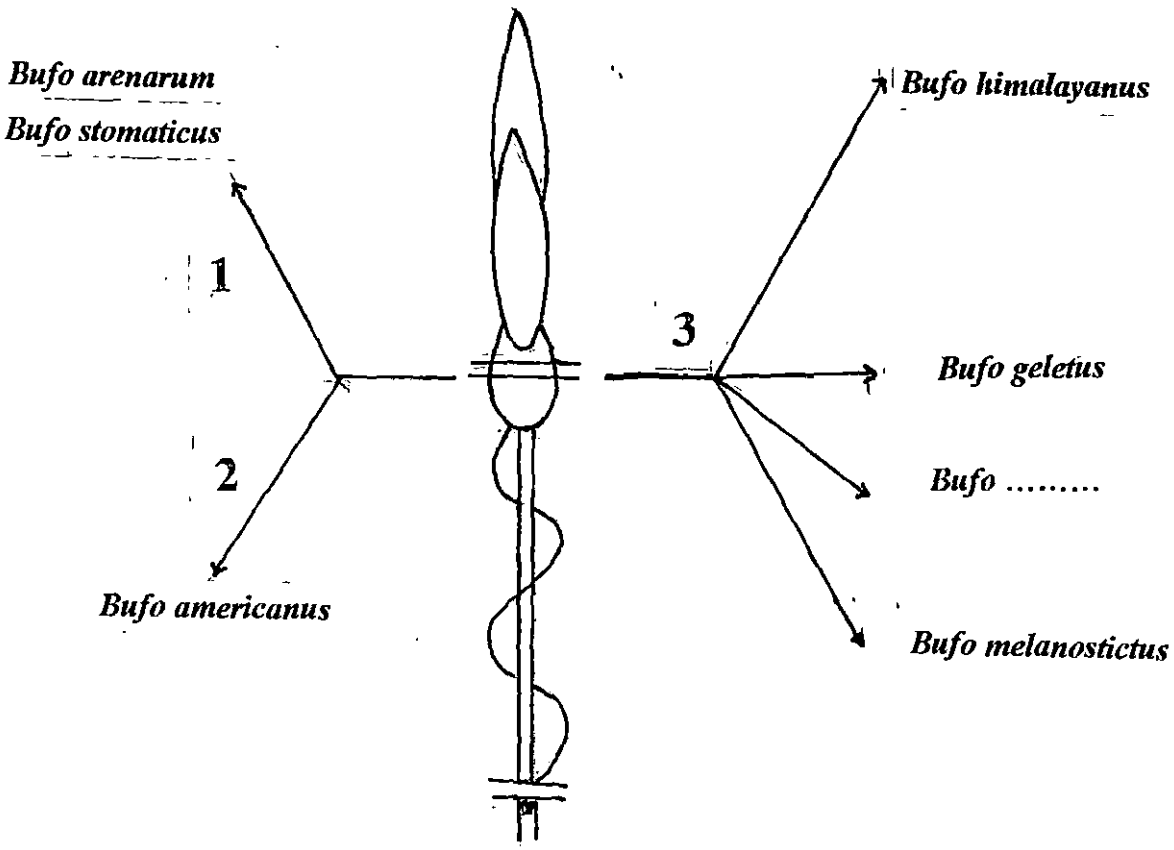
e) Middle Piece - Numerous and randomly arranged mitochondria in the mid piece usually form a mitochondrial collar in Bufonidae and Leptodactylidae (Burgos and Fawcett, 1956 ; Swan *et.al.,* 1980 ; Pugin-Rios, 1980 ; Bao *et.al.*, 1991 ; Lee and Jamieson, 1993). The presence of mitochondria creates a 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

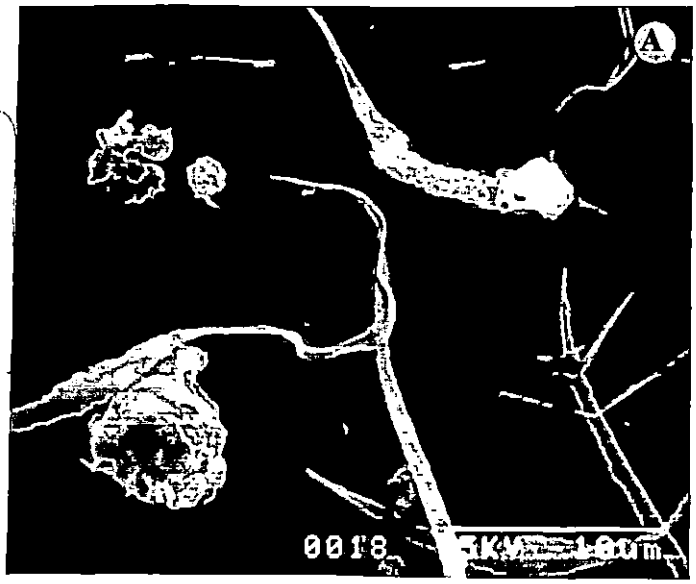
FIGURE – 12

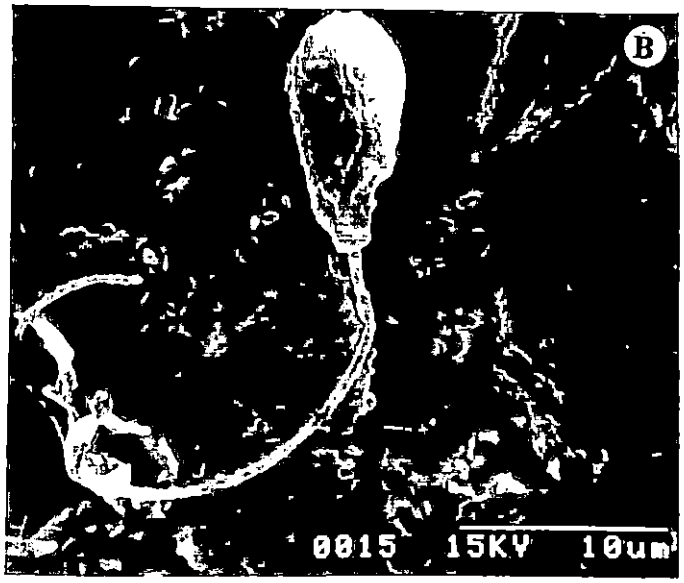


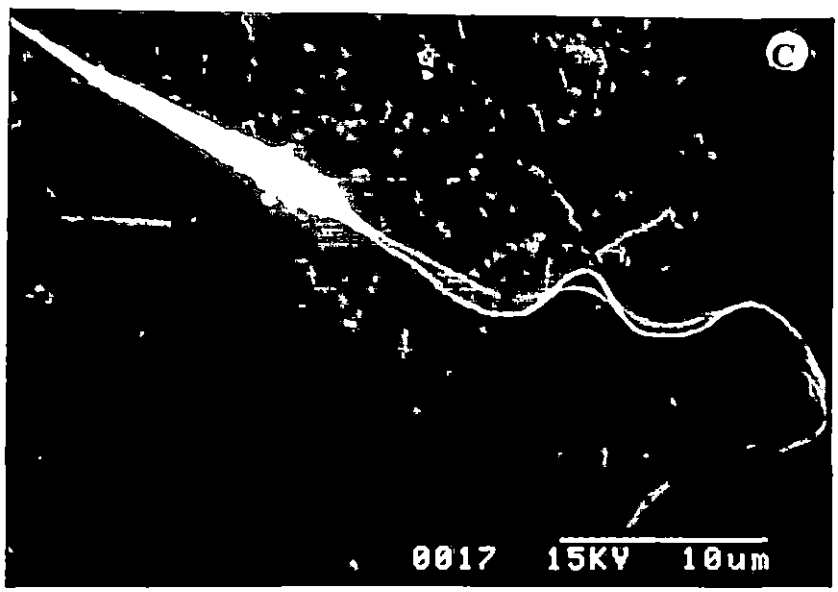
Typical *Bufo* Sperm

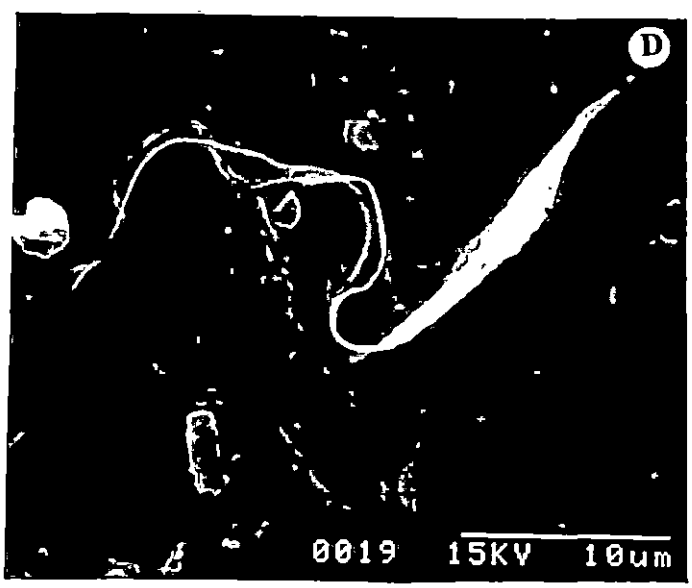
Self proposed Cladogram of Bufonidae

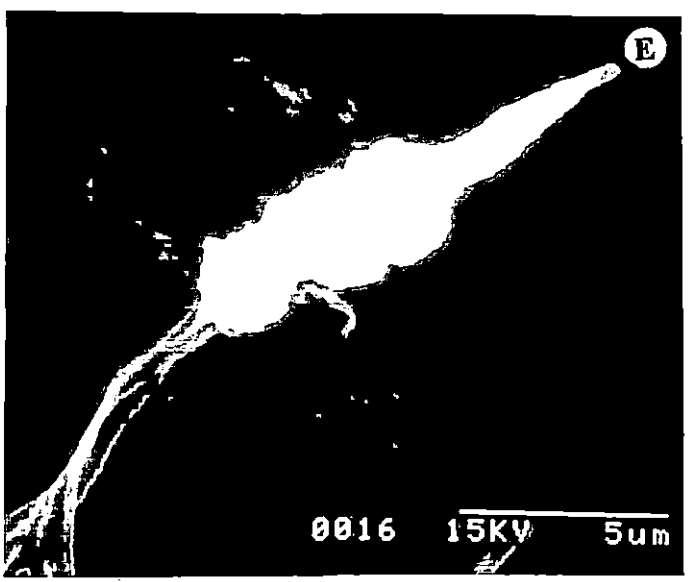
PLATE – 22











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*.