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.

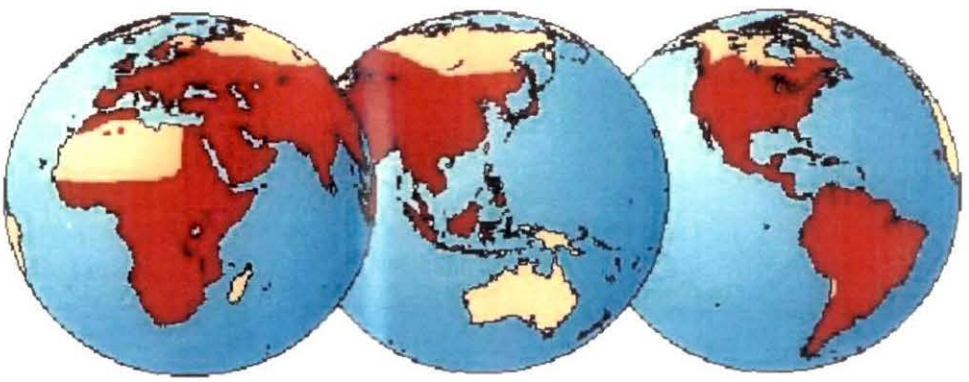
Relation to Humans

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.

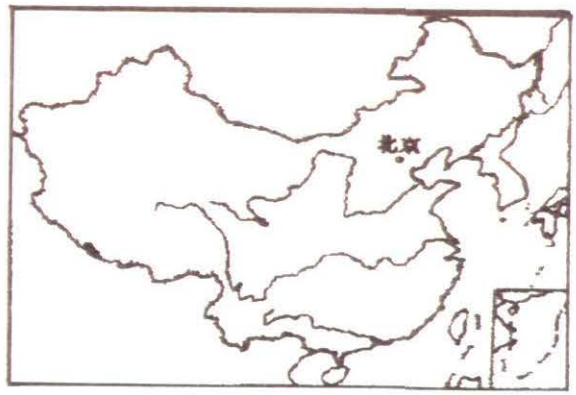
The gregarious habit of this tadpole continues up to the 36th Stage of development (Khan 1965), later the tadpole becomes solitary and when disturbed, takes to the deep water (Khan 1991a; Khan and Mufti 1994b).

Trends and Threats

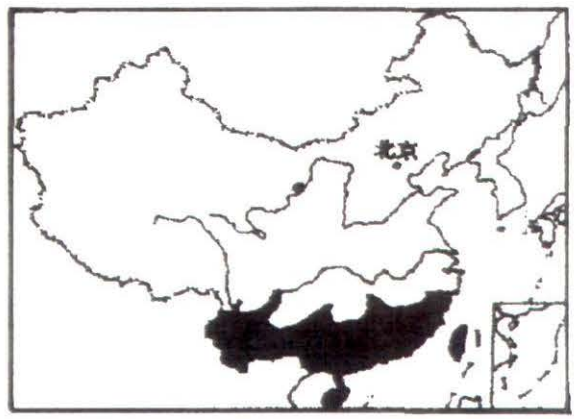
Run off from chemicals used as fertilizers and pesticides against crop pests, is causing death and deformities in tadpoles. Mortality due to increasing traffic is high. The spray of insecticides on crops is causing havoc to the population of this toad, by being directly getting the spray, and by eating 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

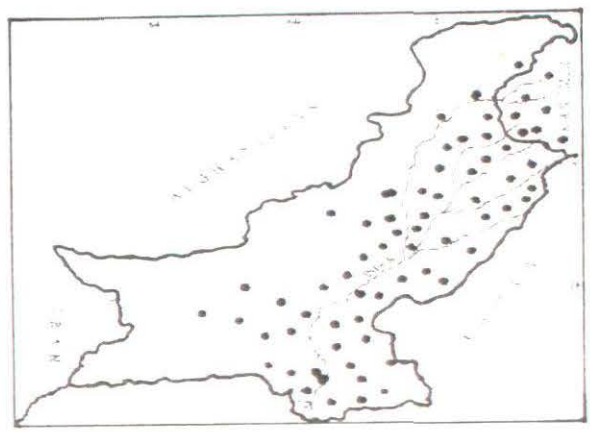
The toad extends in inhabited houses, hiding under household articles, feeding on photophilic insects etc. When handled by children or adults carelessly, cause irritation and boils on skin. Dogs playing with toads are often killed or sickened. The toad is a voracious feeder, devouring different types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

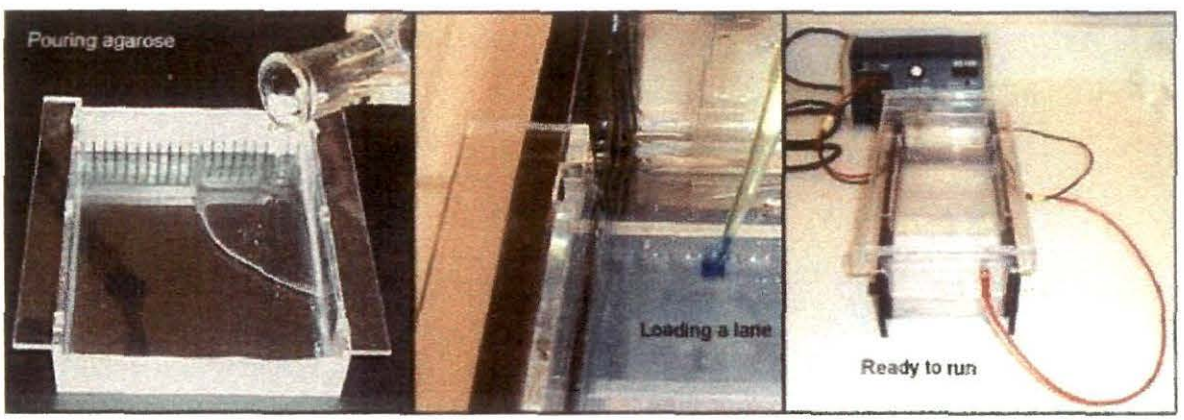
The equipment and supplies necessary for conducting agarose gel electrophoresis are relatively simple and include:

- 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.

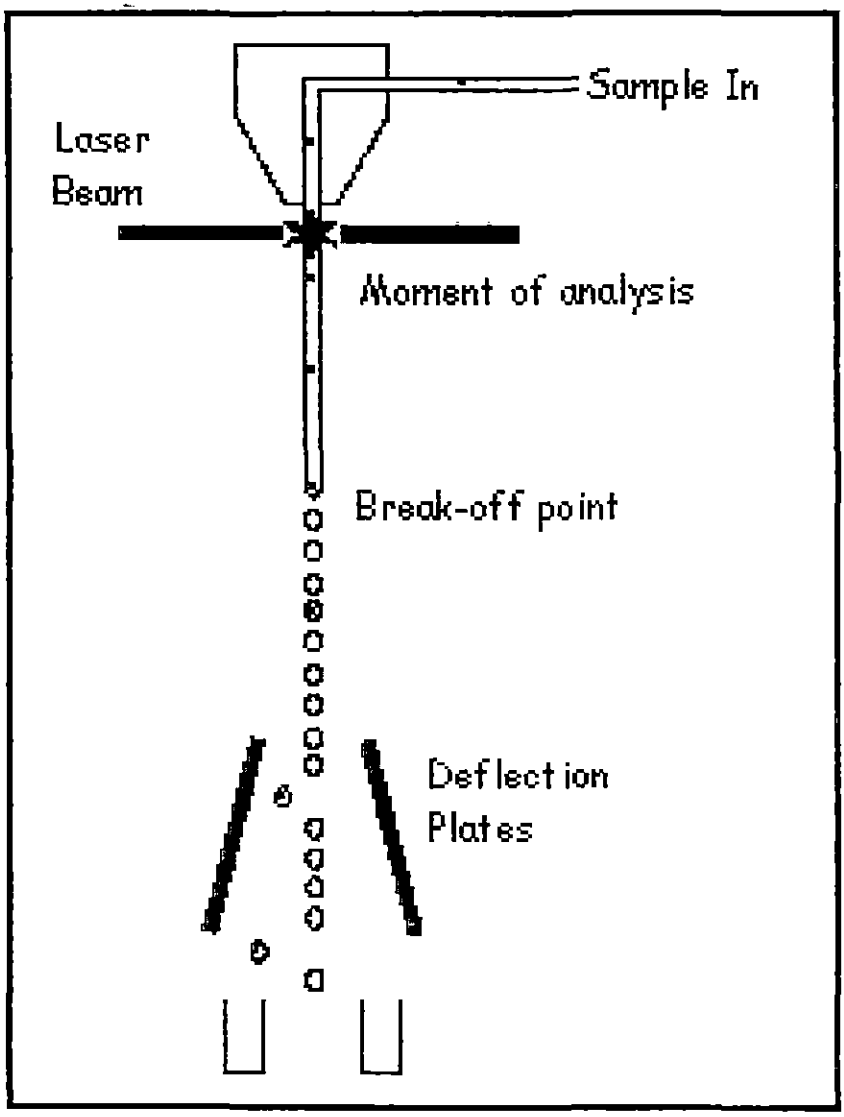
When adequate migration has occured, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking 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.

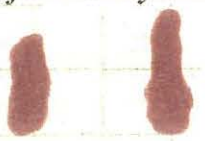
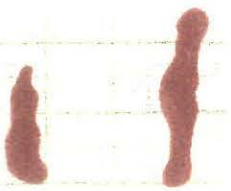
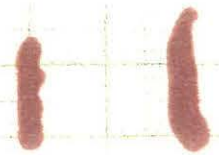
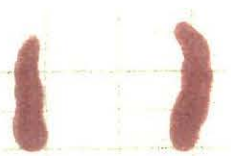
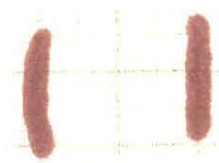
METHOD :

- 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