Building Capacity in Amphibian Research in Bhutan.

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Zoogeographic location of Bhutan

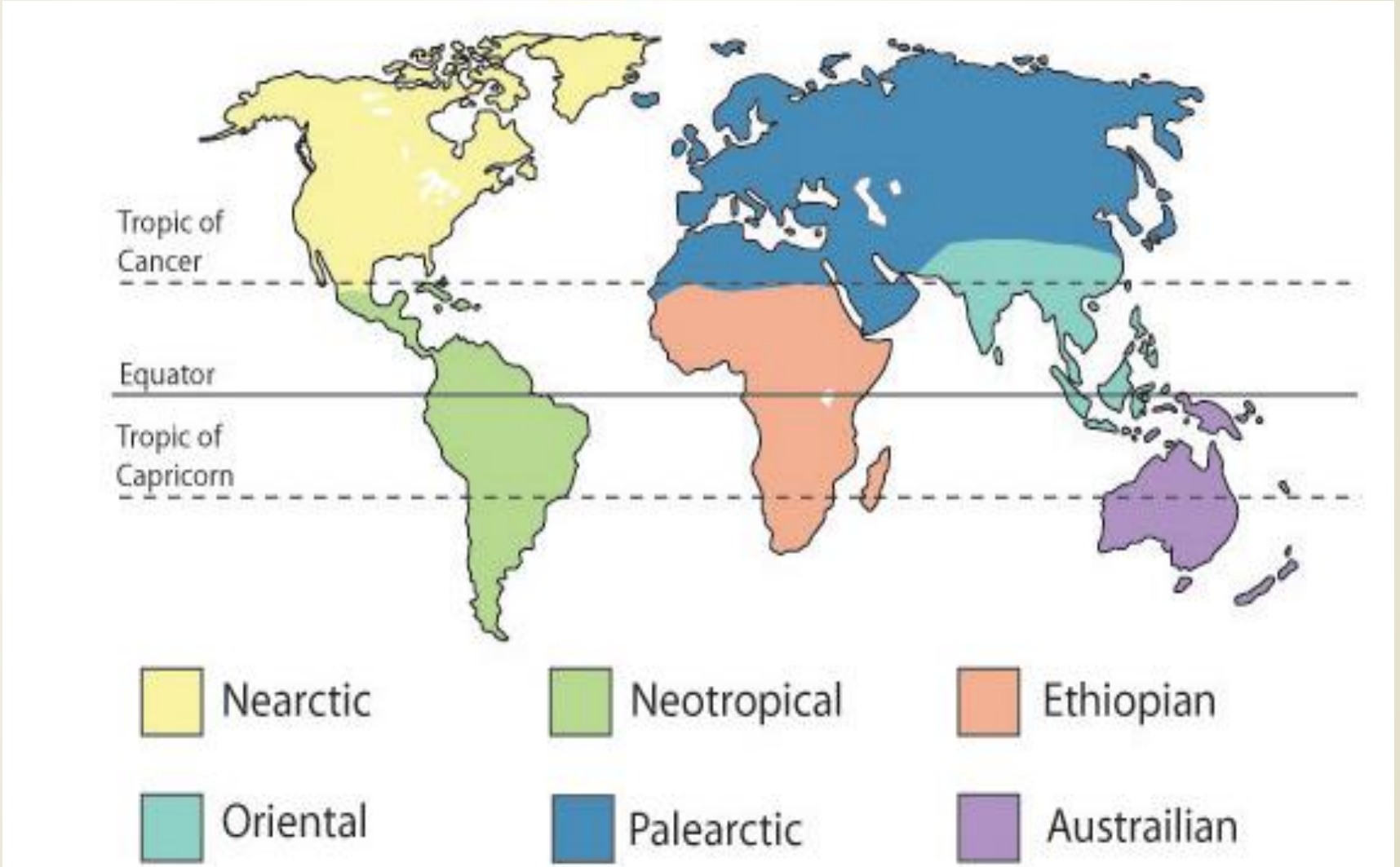
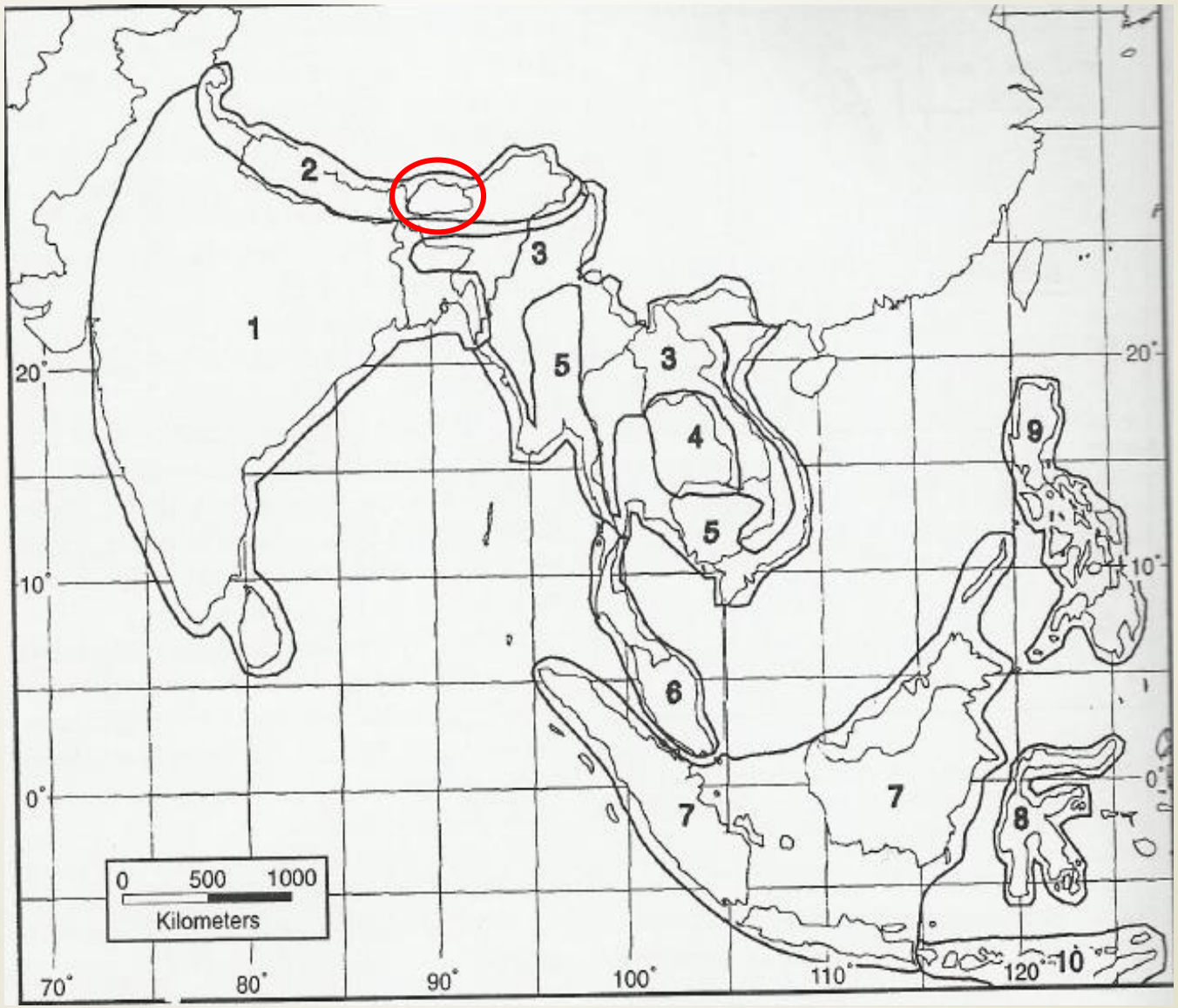


Fig: Biogeographic realms; Source: the nature education.com

Biogeographic region of South Asia based on Amphibian distribution



Physiography of Bhutan

- Mountainous country with elevation between 160 m- 8000 m asl
- Experience distinct four seasons within the co-ordinates of 88°45' and 92°00'E and 26°45' and 28°30'N (Sargent *et al*, 1985).
- Rainfall approximate to 40mm-1000mm (Wangyal and Das, 2012)
- Forest consists of 11 *noda* (Sargent *et al*, 1985)
- Subtropical to icecap mountain

Regional species richness and similarity

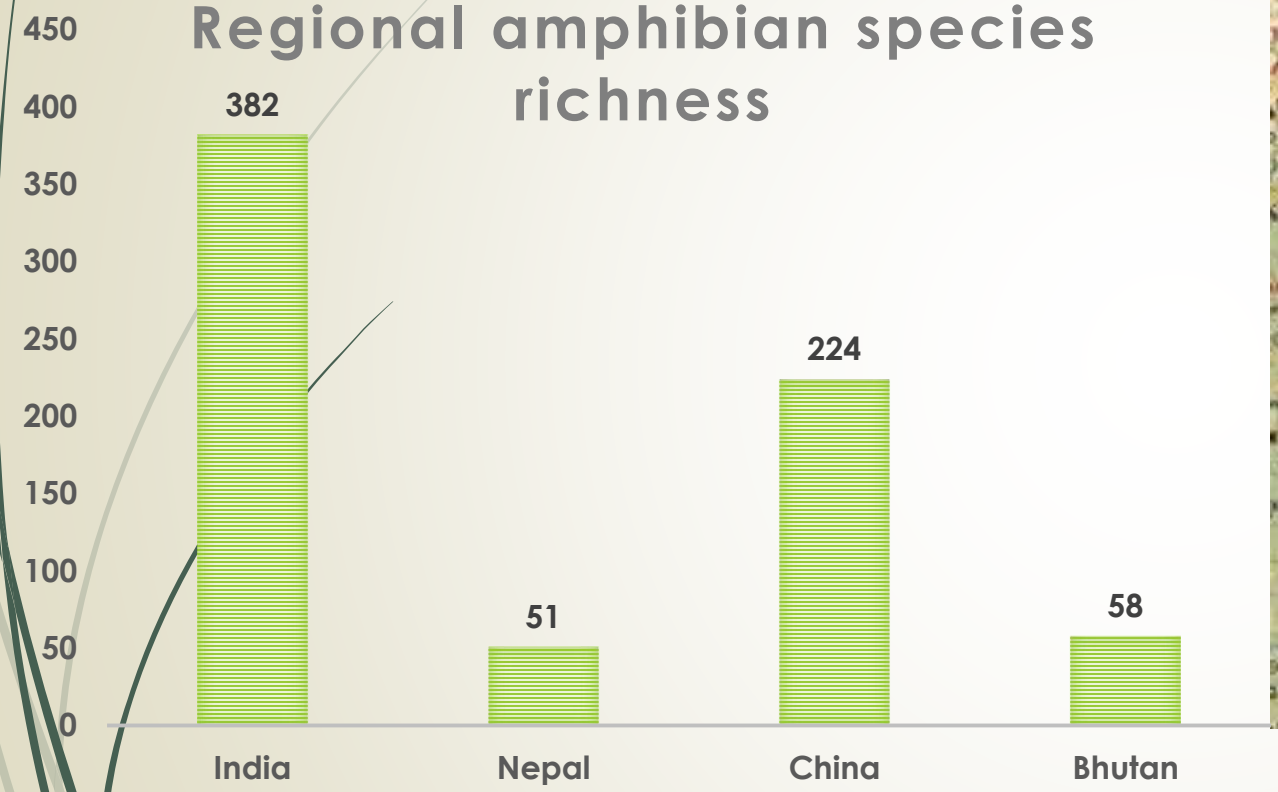


Fig: Species similarity within Himalayan regions; Source: ICIMOD

Concerns and conservations

- 1/3 global amphibian taxa are threatened, in 2004 168 species were extinct (Chen and Bi, 2007; IUCN, 2004).
- Reports on amphibian research in Bhutan is scarce and in-country expert is lacking with acute need of capacity building
- Public awareness of this group of animals is low and public education might reasonably be expected to improve the impact of conservation efforts
- A timely opportunity exists to document the amphibian composition of an endangered but still relatively intact area of the Himalayas and to gather baseline data that will inform both national and international conservation agencies.

Why conserve amphibians?"

- ✓ Indirect development (Amphibious)
- ✓ Plays the role of both the predator and prey in the food web.
- ✓ Indicators of environmental conditions
- ✓ Amphibians can also directly aid humans like food, pest control, etc...
- ✓ Scientific research
- ✓ More risked of extinction due to various reasons like habitat loss, over exploitations, pollutions etc. (Stuart *et al*., 2004)

Objectives:

- Build and Increase in-country capacity in Amphibian research
- Identify threats to amphibian diversity in SNRSA

Ecology and behaviors of amphibian

- Complex life cycle
- Availability of water affects nearly every aspect of the lives of amphibians. The availability of aquatic breeding sites undoubtedly is a major determinant of the geographic and local distribution of species that rely on standing water to breed.
- Amphibians are ectotherms, nearly every aspect of their physiology and behavior is affected by temperature
- Movement and orientations

Wells, 2007

Contd.

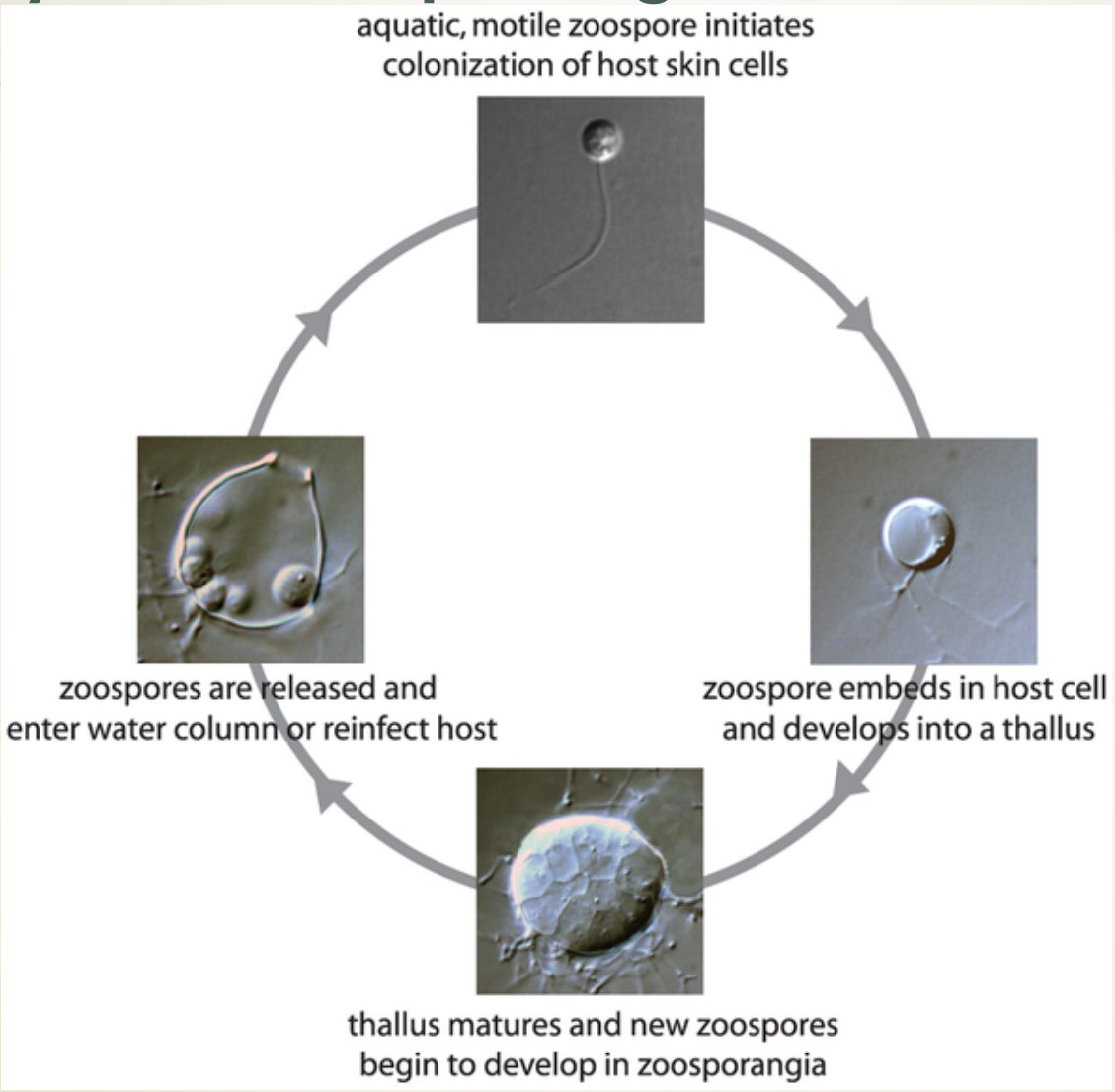
- Vocal communication: (1) advertisement/mating calls, (2) Aggressive/territorial calls, (3) male courtship calls, (4) female courtship calls, (5) Alarm calls, and (6) warning calls, (6) release calls.
- Mating system: (prolonged and explosive breeders) scrambled, resource defense and choruses
- Parental care: Attendance of eggs, attendance of larvae or young, transport of eggs, and transport of larvae (Wells 1981a).

Wells, 2007

Bd detection

***Batrachochytrium dendrobatidis*.**

Life cycle of the pathogenic *Bd*



Rosenblum EB, Voyles J, Poorten TJ, Stajich JE (2010) The Deadly Chytrid Fungus: A Story of an Emerging Pathogen. PLoS Pathog 6(1): e1000550. doi:10.1371/journal.ppat.1000550
http://127.0.0.1:8081/plospathogens/article?id=info:doi/10.1371/journal.ppat.1000550

Swab sample collections

- Requirements
  - 70% ethanol
  - Cotton swap
  - Hand gloves
  - Zip-lock plastics
  - Vials
  - Scrap plastics
  - Stationaries (water proof pen)
  - Bleach solution (Brem et al, 2007).

Procedure

- **Person 1:**
  - ✓ Handle specimens.
  - ✓ Carry gloves.
  - ✓ Waste bag/container (Brem *et al.*, 2007).
- **Person 2:**
  - ✓ Responsible for data entry
  - ✓ labeling vials
  - ✓ Carrying equipment (Brem *et al.*, 2007).


Photo: Tshering Nidup

Preparation:

- ✓ Thorough equipment checking is advised.
- ✓ Organized equipment in field for easy access.
- ✓ Prepare data sheets ready.
- ✓ One person (person 1) should put on clean gloves to capture amphibian
- ✓ Second person (person 2) should wear clean gloves in case of accidental contamination of hands.
- ✓ Proceed to visually encounter the amphibians (Brem et al, 2007)

Capture:

- ✓ Only one person should attempt to capture the specimen
- ✓ Efficient capture is recommended
- ✓ Minimize the handling duration of the specimen
- ✓ Rinsing free zoospores off skin may reduce chances of detecting light infections.
- ✓ Washing off an animal risk the introduction of pathogen into an aquatic environment facilitating transmission.
- ✓ Washing specimen in potentially contaminated water might introduce zoospores onto animal body (Brem, Mendelson & Lips, 2007).

Swabbing and sample preservation

- ✓ Person 1 swabs each: **rear foot, ventral surface of thigh and abdominal surface**

- Preservation of the sample
  - ✓ Person 2 holds an open, alcohol filled, pre-labeled vial as Person 1 carefully inserts the swab in the alcohol containing vial.
  - ✓ Person 1 then carefully break the end of the swab off into the vial
  - ✓ Person 2 secure the cap to the vial
  - ✓ Assures all information is recorded, and store the sample in a closeable bag labeled with date, time, and location (Brem et al, 2007).

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- ✓ Samples should be stored in +4 degree temperature (Voros *et al*., 2008)
- ✓ These samples are then used to test for the presence of *Bd* using a diagnostic PCR assay/real-time PCR.
- ✓ PCR(polymerase chain reaction), is a critical part of the molecular technique to test for the presence of DNA from *Bd*.

Histological examination

- ✓ Skin samples (approx. 1 cm2) from the venter just anterior to the groin, approximately along the midline of the body.
- ✓ Toe webs can also be clipped and used.
- ✓ Skin samples were fixed in 10% buffered formaldehyde for 24 hours, washed with 70% ethanol, dehydrated and cleared with a series of ethanol and xylene solutions.
- ✓ Samples can be paraffin embedded, sectioned at 5 µm thickness with a microtome, and stained with hematoxylin and eosin (McLeod *et al*., 2008)

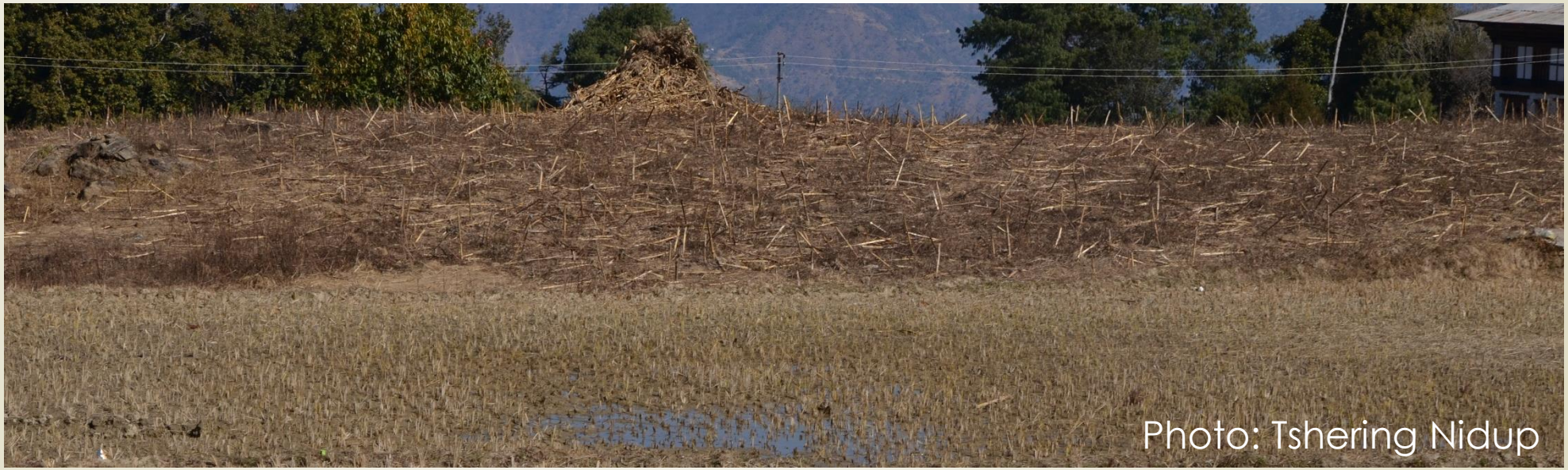
Diversity inventorying

Xenophrys sp. Photo: Tshering Nidup

Habitats selection and considerations

a) Terrestrial habitat

- ✓ Kind of vegetation (evergreen low- tropical forest, temperate deciduous forest, thorn scrub).
- ✓ Climate and topography
- ✓ Degree of disturbance.
- ✓ Other habitat factor like soil type, water holding capacity, frequency of flooding (Heyer *et al.*, 1994).



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b) Aquatic Habitats

- ✓ surrounding vegetation.
- ✓ Water temperature and water turbidity.
- ✓ Lentic- ponds and lakes.
- ✓ Lotic- streams and rivers.
- ✓ Streams, ponds, fields (paddy fields), swamps etc.

Sample size:

- Consider at least two aspects of sample size
  - ✓ The numbers of quadrats, transects, or trips to the site.
  - ✓ The numbers of specimens collected and species sampled (Heyer, *et al.*, 1994).

Sampling Design:

- ✓ How are we going to survey?
- ✓ Different types of methods to survey: *following slides*

I. Visual encounter survey (VES)

- Field personnel walk through an area or habitat for a prescribed time period/distance systematically.
- Can be done along a transect, in a plot, along a stream, around a pond etc.
- Use for rapid inventorying of large forest areas.
- Suitable method to record rare species/unlikely to be caught with trapping methods (Heyer *et al*, 1994).

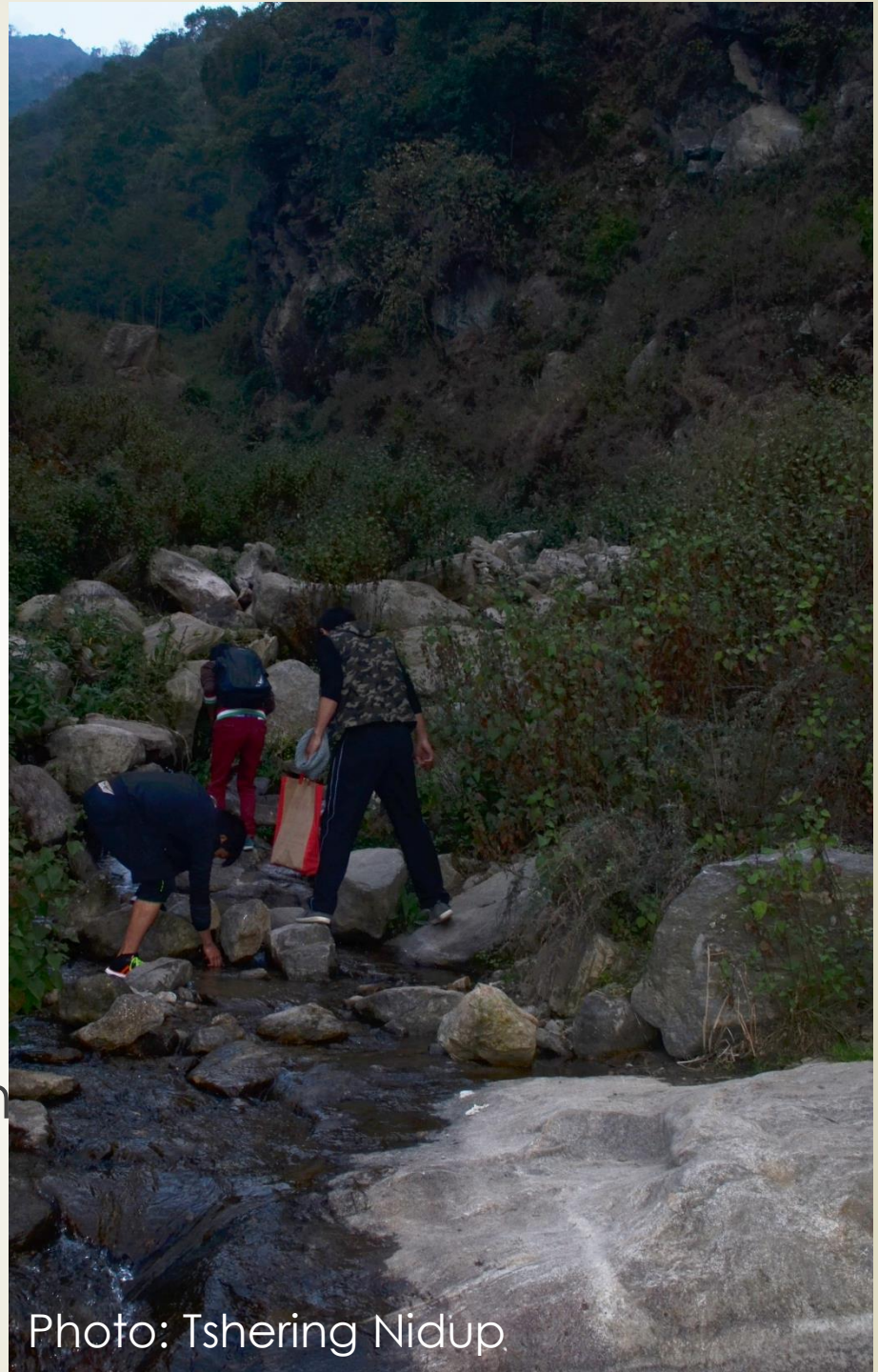


Photo: Tshering Nidup

II. Pitfall trap with drift fence

- ✓ Drilled a hole, approximately 21cm in diameter and 19.5cm deep (Enge, 1997).
- ✓ A wetted sponge was placed in each trap to prevent desiccation (Enge, 1997).

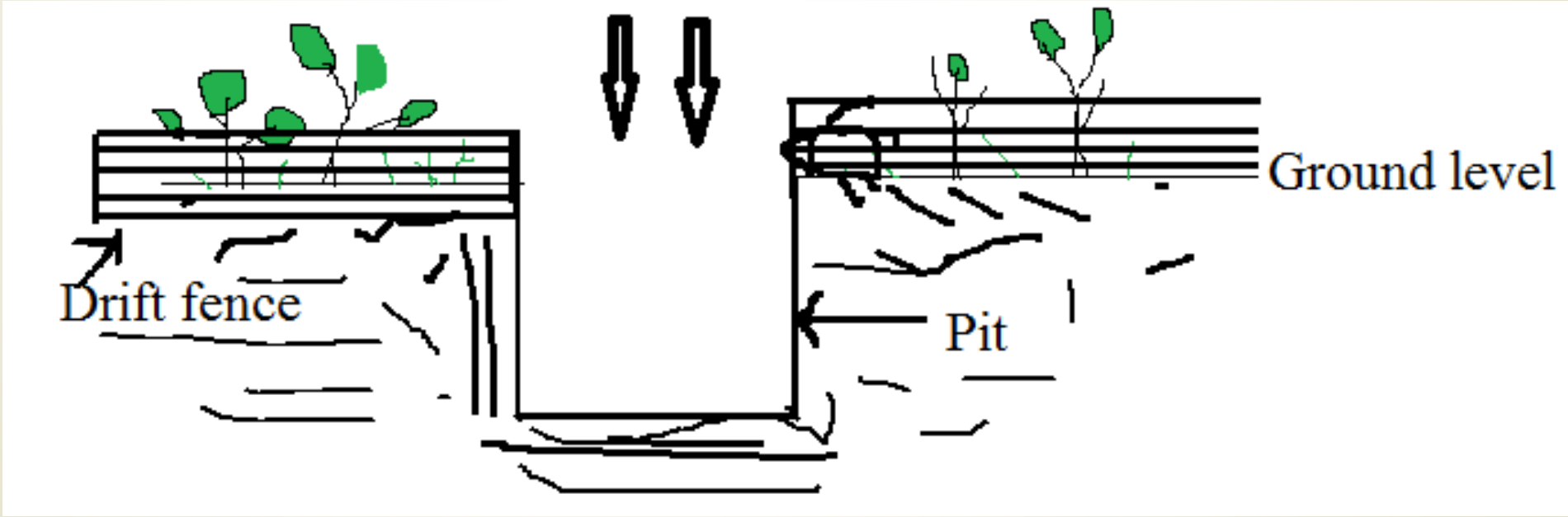


Figure: Drift fence array (modified from Strain & Rasely, 2006).

IV. Leaf litter bag survey

- Constructed of 61x40cm plastic netting with 2.5cm mesh (Jung & Pauley, 2003).
- Each bags filled with leaf litter debris and small pebbles.
- Bags were then placed in the stream (Strain & Rasely, 2006).

III. Cover board survey

- Consisted of pine boards of approximately 30x30x5cm
- Leaf litter and debris was cleared away and the boards were placed upon bare dirt (Marsh and Goicochea, 2003).

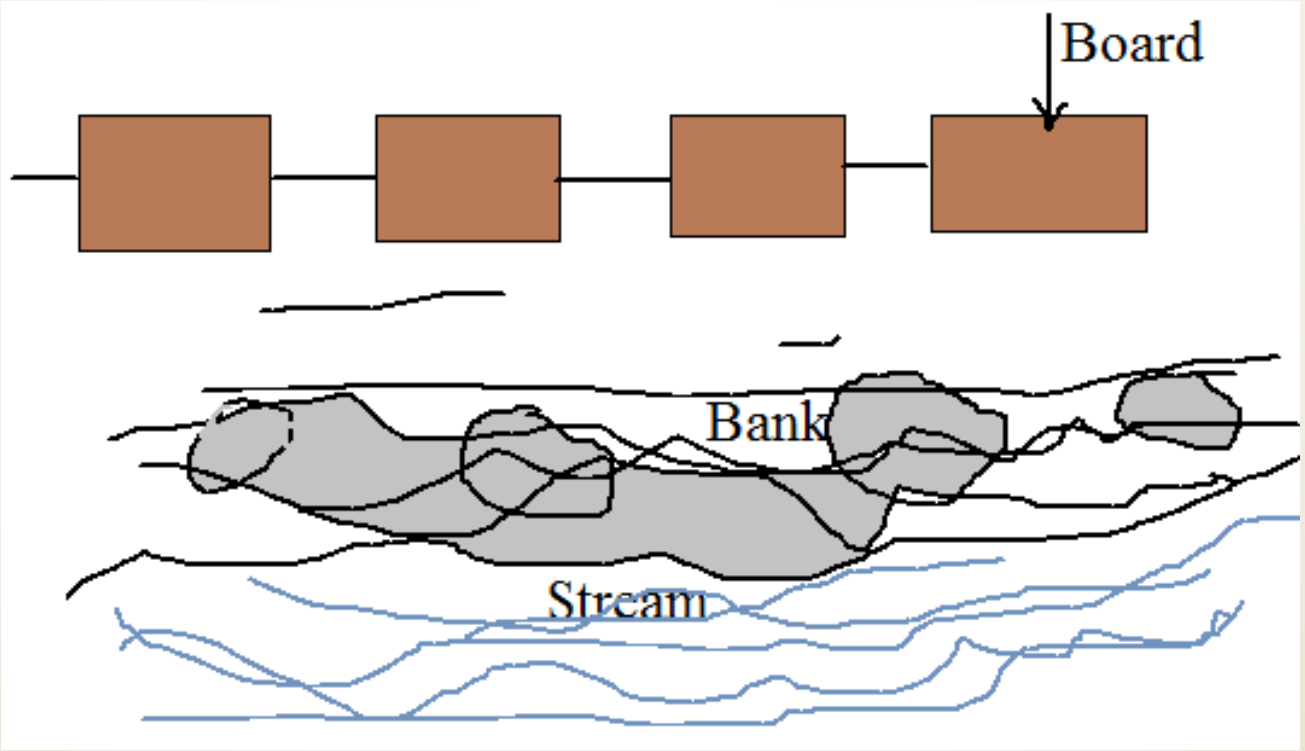


Figure. Cover board array (modified from Strain & Rasely, 2006).

V. Funnel traps

- Constructed of aluminum window screening with double-ended funnel traps (Enge, 1997).
- Sponges are placed in each trap to prevent from desiccation (Enge, 1997).
- Four funnel traps were used in each array.

VI. Quadrate leaf litter searches

- Construct a quadrat
- Randomly select the sites within a habited and thoroughly searching those squares for amphibians (Heyer, *et al.*, 1994).

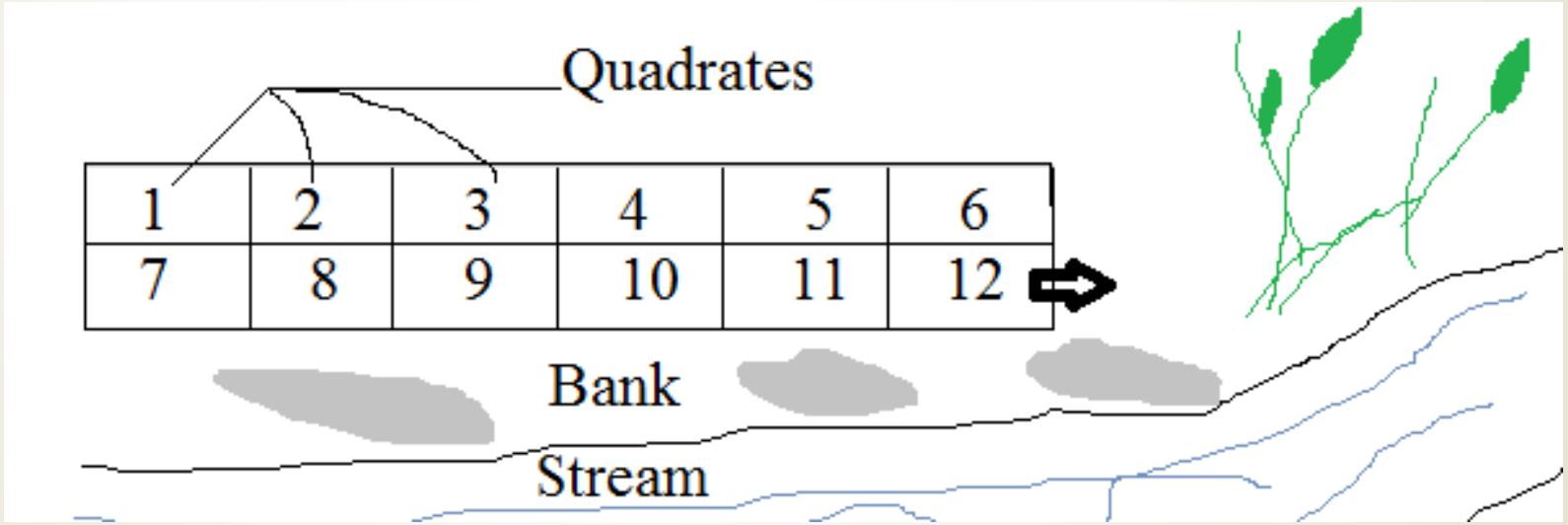


Figure: Arrangement and numbering of quadrats (Strain & Rasely, 2006).

VII. Electrofishing (Salamanders)

- Originally a standard 1/8th inch dipnet was used, but some salamander larvae were able to escape.
- Therefore a dipnet with small mesh was used (approx. 1/6th inch) (Strain & Rasely, 2006).

Specimen preservations of reference collection

- Specimens collected were euthanized with chloroform (90% or above)
- Inject the (70-90%) alcohol in the body (body, limbs, etc.)
- Preserve in 70% alcohol
- Stored in the closed lid container (container depends on availability)
- Label the specimen with the tags (Jiang, *et al*., 2013; Matsui and Panha, 2006)

Taxonomic identification


Photo: Tshering Nidup

- Essential measurements: body measurements (SVL, HL, HW, ED, TL, etc..)
- Characters: color, body granules, foot webbing, etc…
- Calls
- DNA
- Habits
- Literatures

Collection of tissue samples for DNA analysis (Taxonomic identification)

1. **The toe clipping method.**

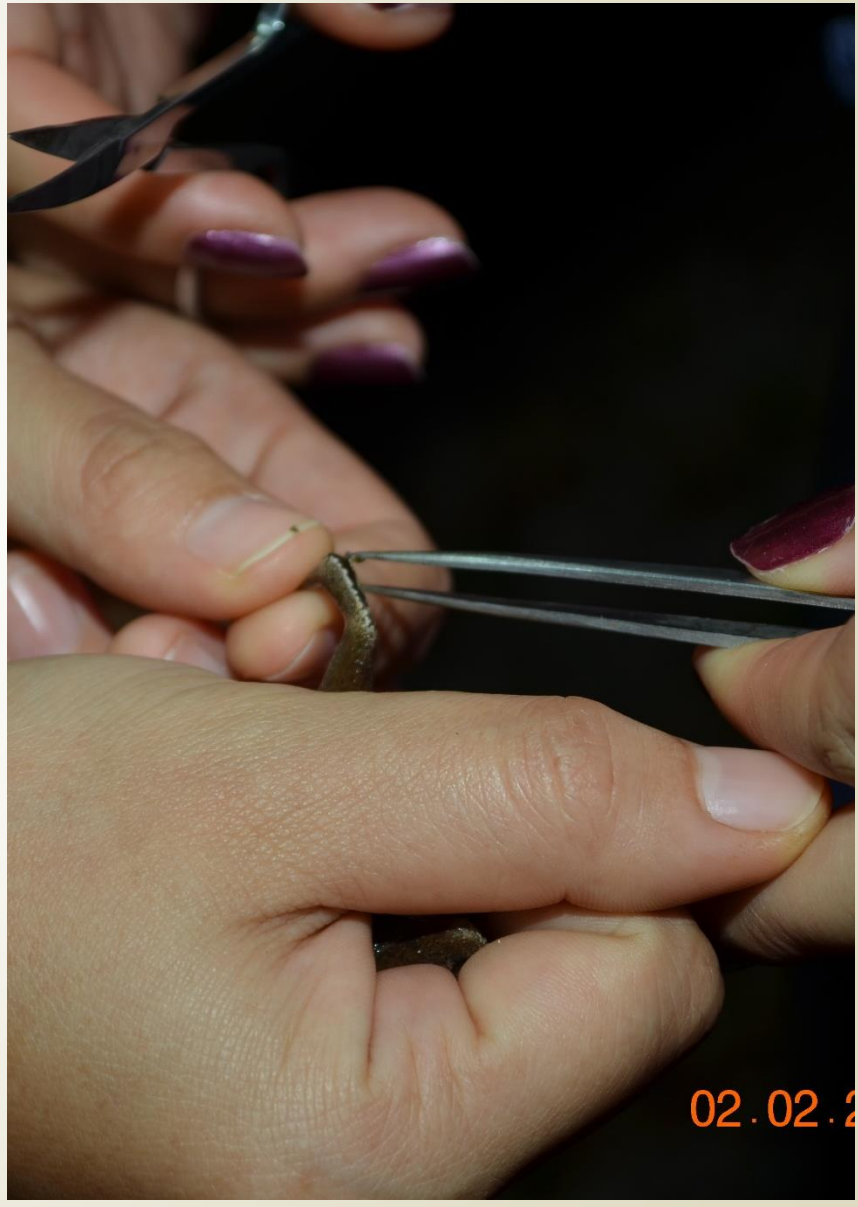
✓ Provide valuable amounts of DNA.

✓ Its not appropriate method for climbing species like tree frogs (Prunier, *et al*., 2012).

2. **The tail tipping method.**

✓ Applied to DNA sampling in anuran tadpoles.

✓ Leads to death of sampled individuals (Prunier, *et al*., 2012).



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3. **Buccal swabbing.**

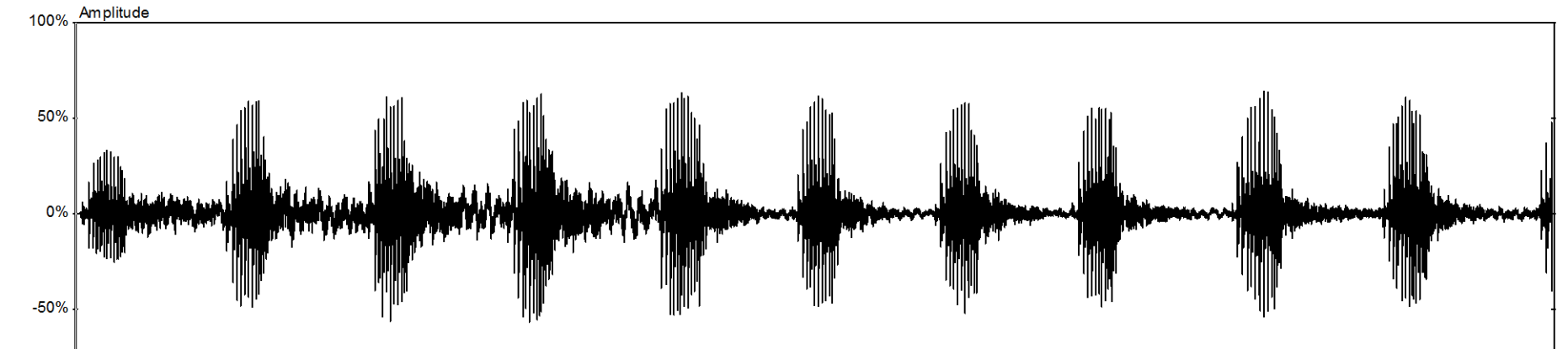
- ✓ Method of genetic sampling that provide enough DNA.
- ✓ Upper and lower jaw are opened with the help of spatula (Prunier *et al*., 2012).

4. **Skin swabs.**

- ✓ Useful for small and vulnerable animals.
- ✓ Dorsal swabs performed better than ventral swabs (Prunier *et al*., 2012).

Recording and analysis of calls

- Frogs does not emit sounds of high frequency.
- Most species of frogs have dominant frequencies of about 4000Hz or less
- Thus, a tape recorder with a frequency range of 5000 to 20000Hz is reasonable(Heyer, *et al.*, 1994).
- Requirements:
  - Portable tape recorder
  - Hygro-thermometer



How to record frog matting calls?

- Record either frog choruses or calling individuals
- Individual calls are recorded at the distances from 0.5 to 1.5 meter.
- Temperature data are critical for comparing different recordings as calling rate, pulse rate, and call length vary with ambient temperature.
- Collect voucher specimen and identify frogs that are recorded.
- The amphibians collected as vouchers should be maintained in plastic bags until processed (Heyer, et al., 1994).

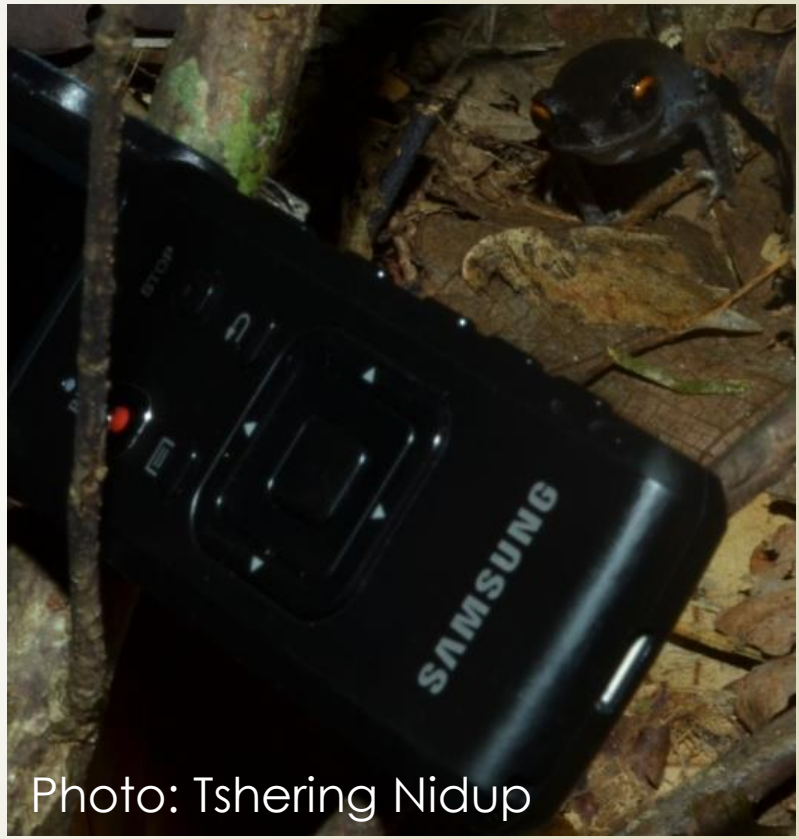


Photo: Tshering Nidup

Review of species diversity in Bhutan

- 58 species recorded till now
- One caecilian species
- one salamander species
- 21 expected species not yet reported (Wangyal, 2014)

Photo: Wangyal & Gurung, 2012

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