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SUBJECT : **ADOPTING THE PROTOCOL ON THE COLLECTION OF TISSUE SAMPLES FOR DNA BARCODING**

Pursuant to Republic Act No. 9147 (Wildlife Resources Conservation and Protection Act of 2001) and DAO 2016-12 (Adopting the Philippine Biodiversity Strategy and Action Plan), the attached "Protocol on the Collection of Tissue Samples for DNA Barcoding/Analysis" is hereby adopted. This protocol will facilitate the proper and efficient collection of tissue samples of wild flora and fauna towards building the DNA reference collection of the country's wildlife resources for research and development, wildlife law enforcement, among other purposes relevant to conservation and harnessing the economic benefits of Philippine biodiversity.

This Technical Bulletin is issued for the information and guidance of all concerned.

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United Nations **Decade on Biodiversity**

PROTOCOL ON THE COLLECTION OF TISSUE SAMPLES FOR DNA BARCODING

I. Rationale

DNA barcoding is one way of identifying species by using genetic sequences from a standard or known taxon/genome or its closest relative. Species are routinely identified using morphological features, like color, size and shape of body parts. Guides and various references are readily available to facilitate this procedure. However, identification becomes difficult if an organism is in its immature state or if the specimen is already damaged/broken to pieces. DNA barcoding becomes an effective tool in this aspect.

DNA barcoding is similarly an important tool in the identification of vector species that can cause serious infectious diseases to animals and humans thereby understand the disease and administer appropriate medication to cure them.

The technology is also being used to establish authenticity of health products to determine safety and as well as in verifying mislabeled cheap products being sold as the expensive ones.
DNA analysis also play an important role in forensics/wildlife crime investigations especially since accurate and correct species identification are crucial factors in the filing of cases against perpetrators.

The application of DNA barcoding is dependent upon the availability of reference library of DNA barcodes to which case samples can be compared to for their identification. Thus, the continuous collection of tissue samples is necessary to build the country's reference collection of Wildlife DNA barcodes. This Protocol thus provides the standard procedures in the collection of wildlife fauna and flora samples for DNA barcoding purposes.

II. General Guidelines

1. Prioritize the collection of samples from endemic and indigenous species of wild fauna. Samples may be collected on live wild fauna turned over to Regional Wildlife Rescue Centers or from the rescued animals prior to releasing them in their natural habitat.
2. Samples may also be collected from dead wild fauna or their by-products.
3. Record in a datasheet morphological data (e.g. weight, body length, sex, etc.) of the animal from which the tissue samples were taken;
4. Identification mark/tag must be attached to the individual samples bearing pertinent data (i.e. Species/taxon group, type of sample, number of samples, name of collector, collection site, GPS coordinates.);
5. Always take pictures of the sample with its unique individual identification mark/tag. Also take pictures from different angles and positions, of the wildlife from which the samples were collected especially if its identification is uncertain.
6. Prevent cross contamination of samples. Samples should as much as possible be placed in individual containers. NEVER handle samples with bare hands; always wear powder-free gloves during collection of samples. ALWAYS flame-sterilize collection instruments in-between sampling.
7. Whenever possible, use disposable single-use items/materials, especially for evidence collection.

8. Ensure that the sample collected is kept in the appropriate preservative and properly stored (i.e. blood sample in EDTA kept frozen).
9. To facilitate collection of samples, prepare a tool kit containing all materials needed in the collection of specimen samples. Always bring this tool kit during field work.
10. Ensure that the turn-over of samples is properly documented using an Acknowledgement Form ("Annex A").
11. Collected samples shall initially be deposited to the Wildlife Resources Division of the Biodiversity Management Bureau for recording and data basing. Samples will be turned-over to the appropriate laboratory thereafter.

III. SAMPLE COLLECTION FOR LIVE WILD FAUNA

This portion of the Protocol shall discuss covers the collection of specimens from live animals being turned over to wildlife rescue centers, or those animals being kept captive in wildlife facilities, zoological parks, or those animals intended to be released.

Materials needed:

1. Surgical scissors / Scalpel with disposable blade
2. Thumb forceps
3. 1.5 - 2 ml microcentrifuge tubes half-filled with 95-100% ethanol
4. Unwaxed brown envelops
5. Self-sealing bags
6. Alcohol burner / candle and matches
7. Permanent markers

A. AMPHIBIANS: Toe clipping

Toe clipping is a standard method for sampling amphibians and it can be used as a tag for distinguishing sampled individuals. However, some amphibians have been known to fully regenerate appendages after a few years.

Sampling

To sample the toe, secure the animal in one hand. Hold one of the feet and, using surgical scissors, cut about three fourths of the middle toe. Place the cut portion in a microcentrifuge tube with ethanol.

B. ARTHROPODS: Appendage/Leg

Sampling

Obtain a leg from the arthropod and place it in a microcentrifuge tube with ethanol.

C. AVIAN:

C.1. Feather and blood-feather samples

Blood feathers are growing feathers characterized by having blood within its shaft. Search the bird for blood feathers. If blood feathers are not present, flight or tail feathers may be sampled.

Sampling

Using forceps, pluck 2-3 blood feathers or 5-10 flight or tail feathers and place them inside a microcentrifuge tube with ethanol. Make sure that the root is intact. If the feather is bigger than the microcentrifuge tube, cut the vane leaving only the calamus inside the tube.

Collected flight or tail feathers may also be placed in a clean, dry unwaxed envelop or a clear, self-sealing bag. Place desiccant (silica gel beads) before sealing the envelop or plastic bag.

C.2. Blood

Collection of blood shall only be done by a veterinarian or a certified personnel with experience in blood collection in birds. The collection site is prepared aseptically by cleaning and wiping the site with alcohol.

Sampling

Depending on size of bird, obtain about 0.5-1 ml of blood. Mix blood thoroughly inside a microcentrifuge tube with an anti-coagulant (violet-capped bottle).

D. MAMMALS:

D.1 Fur/hair

Sampling

Look for coarse, clean, and hard to remove hair. Pluck about 10-15 strands, making sure that the root or bulb is intact. Always handle the hair by the tip not by the roots. Place the hairs in unwaxed brown envelop before sealing in a self-sealing plastic bag with desiccant (silica gel beads).

D.2 Blood

Collection of blood shall only be done by a veterinarian or a certified personnel with experience in blood collection in mammals. The collection site is prepared aseptically by cleaning and wiping the site with alcohol. Sometimes, especially for thick-haired species, the hair is trimmed to provide a clearer view of the blood vein to facilitate collection.

Sampling

Obtain about 0.5-1 ml of blood. Mix blood thoroughly inside a microcentrifuge tube with an anti-coagulant (violet-capped bottle).

D.3 Ear punches and Wing punches

Biopsy punches are handy tools used to punch out two or three-millimeter circular tissues from the ear pinna. They can also be used to obtain samples from the wing membranes of bats.

The holes produced on the ears and wings can be used as a tag for sampled individuals. Varying the position and number of holes may also help identify an individual.

Sampling

Restrain the animal and ensure the ear or wing membrane lies securely on a clean, flat surface. Using a disposable biopsy punch, bore a hole on the least vascularized part of the ear pinna or wing membrane. As the punched-out tissue usually attaches to the biopsy punch, carefully dip the device in a microcentrifuge tube with ethanol. Ensure that the cut tissue is found inside the tube. In case the cut tissue remains on the flat surface, use sterilized forceps to collect the tissue and place inside the tube. Never hold the cut tissue with your hands. Do not re-use the biopsy punch; remember to use only one biopsy punch per individual.

If bleeding occurs at the cut portion, dab and apply pressure on the wound with clean, dry cotton and disinfect the wound.

E. REPTILES (SNAKES, LIZARDS, TURTLES):

E.1 Tail snip

Sampling

Secure the animal's body and stretch out the tail. Cut a two millimeter length of tissue from the end of the tail. Place the cut tail piece in a microcentrifuge tube with ethanol. Use cotton and disinfectant if bleeding occurs.

E.2 Scale snip

This sampling method can be used on crocodiles. The cut scales can similarly be used to identify individual crocodiles.

Sampling

Secure the animal's head and body and stretch out the tail. Cut a two millimeter length of tissue from the protruding vertical scales along the tail. Place the cut scale in a microcentrifuge tube with ethanol. Use cotton and disinfectant if bleeding occurs.

E.3 Skin

This method is used to collect samples from marine turtles. It may also be used for terrapins but retraction of the head and limbs inside the shell must be prevented.

Sampling

Secure the turtle's head while carefully stretching out a fore flipper. The collection area should be disinfected properly, algae and barnacles removed. A tissue sample is collected on the skin between the head and the fore flipper using a disposable biopsy punch. As the punched-out tissue usually attaches to the biopsy punch, carefully dip the device in a microcentrifuge tube with ethanol. Ensure that the cut tissue is found inside the tube. In case the cut tissue remains attached to the turtle's skin, always use a sterilized forceps and scissors to free its attachment before placing in a microcentrifuge tube with ethanol. Never hold the cut tissue with your hands. Do not re-use the biopsy punch; remember to use only one biopsy punch per individual.

In case a biopsy punch is not available, sterilized forceps and scissors may be used to cut a skin sample, approximately 1 x 1 cm, from the same site. The skin at the posterior edge of a hind flipper (avoiding the scale) may similarly be utilized as a collection site.

E.4 Blood

Collection of blood shall only be done by a veterinarian or a certified personnel with experience in blood collection in reptiles. The collection site is prepared aseptically by cleaning and wiping the site with alcohol.

Sampling

Obtain about 0.5-1 ml of blood. Mix blood thoroughly inside the microcentrifuge tube with an anti-coagulant (green-capped bottle).

F. SAMPLE COLLECTION ON DEAD WILD FAUNA

Sample collection from dead animals or carcasses follows the procedure for collecting specimens from live animals as described above.

Although the whole carcass can be taken and stored in a facility, it would be practical to take small tissue samples on site and place them in bottles with appropriate preservative to ensure that DNA quality will be high. Small organisms that can fit inside the tubes can be collected as whole specimen.

Sampling

Cut out 1 x 1 cm of tissue from the carcass and cut the piece into 2-3 smaller pieces. Place the tissue inside a tube with ethanol. DO NOT place too much sample inside the tube as this may prevent the tissue from mixing well with the ethanol. Tissues that can be collected from the dead animal may include skin, muscle tissue, kidney and liver.

In case the specimen is at an advanced state of decomposition, take samples from the deeper part of the tissue/organ and follow the same procedure as mentioned above.

G. STORAGE OF THE COLLECTED SAMPLES FROM WILD FAUNA

1. In general, all collected dry samples is kept in envelops or self-sealing bags with desiccant stored at room temperature for months. These can also be stored in a refrigerator or freezer to further prolong the preservation process.
2. All collected samples preserved in ethanol can be stored at room temperature for a few days to one week. Except blood samples, they can also be kept in the refrigerator for three (3) months and in the freezer (at -8°C) for months.
3. Blood samples can be stored at room temperature not more than a day and not more than a week if kept in the refrigerator. Blood samples are best turned over to the laboratory immediately after collection.

IV. SAMPLE COLLECTION FOR WILD FLORA

This covers the collection of specimens from plant materials turned-over to wildlife rescue centers or those intended as evidence for case-building.

Materials needed:

1. Pruning shears or cutter
2. Self-sealing plastic bags
3. Unwaxed brown envelops
4. Desiccant (silica gel beads)
5. Newspaper
6. Denatured alcohol

Sampling for plant materials requires two (2) types of vouchers: DNA and herbarium vouchers. A voucher is all or a portion of a plant that is collected, preserved and maintained as a reference material.

A. DNA Voucher

Sampling

Collect 2 – 3 young leaves of the plant and place in a small self-sealing bag or unwaxed envelop with silica gel beads. Young leaves are the best samples for this procedure as they are softer and usually have less compounds and therefore easier to extract. Other plant parts, like roots, stems, seeds, fruits and flowers may also be collected and should be placed individually in self-sealing bags or unwaxed envelops.

B. Herbarium Voucher

Herbarium vouchers are useful tools for post-identification.

Sampling

Cut a small stem that best represents the leaves and their arrangement. Samples that include flowers and fruits is most ideal. Insert the plant sample into folded newspaper making sure that all parts are neatly arranged in the folded paper. Lightly press the sample and temporarily store in a self-sealing bag with an ample amount of denatured alcohol.

C. STORAGE OR WILD FLORA SAMPLES

1. In general, all collected dry samples kept in envelops or self-sealing bags with desiccant can be stored at room temperature for months. These can also be stored in a refrigerator or freezer to further prolong the preservation process.
2. Fruits and flowers should always be kept in the freezer.
3. Herbarium vouchers should be submitted to a herbarium facility, such as Jose Vera Santos Memorial Herbarium of the University of the Philippines – Diliman and the National Museum of the Natural History, Manila for proper mounting and storage.

SAMPLING SUMMARY

Tissue that can be sampled from wild fauna:

Invertebrates	Fish	Amphibian	Reptile	Birds	Mammals
Appendage	fin clippings	toe clipping	blood	blood	blood
	Intact scales	tail snip	tail snip	blood feathers with intact lower umbilicus or "root"	Fur/hair with intact root
			intact scales	tail feathers with intact lower umbilicus or "root"	ear punches
			skin	Flight feathers with intact lower umbilicus or "root"	(bats) wing membrane punches

"Annex A"

ACKNOWLEDGEMENT FORM

This is to acknowledge receipt of the following wildlife samples turned–over by _____ of (Office) _____ :

Species (if known) **or taxon group**	**sample** (e.g toe clipping, skin, tail snip, scale snip, feather, fur, hair, blood, leaves, etc)	**Preparation** (e.g in microcentrifuge tube, tube, or self-sealing bags)	**Number samples** (i.e number of toe clippins, tail snip, etc.)
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			

Received By:

Name:_____

Office:_____

Date:_____