CHAPTER 19

Sampling Vertebrate Collections for Molecular Research: Practice and Policies

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Abstract.—With the widespread use of protein electrophoresis in vertebrate systematics in the 1960s and 70s, several museums and universities established extensive collections of frozen tissues. Use of these special collections expanded exponentially with the development of the polymerase chain reaction (PCR) and improvements in obtaining nucleotide sequences. Moreover, using PCR, DNA sequences can now be routinely obtained directly from traditional voucher collections (skins, bone, alcohol-preserved specimens, etc.), accentuating the issue of destructive sampling of this material. Herein we briefly review: the development of special tissue collections for consumptive use; the rationale for and methods of collecting both voucher specimens and tissue samples amenable to genetic analyses; tissue collection storage and management; the suitability of voucher collections as direct sources of DNA; and policies on the consumptive sampling of special collections and destructive sampling of voucher collections.

Museums have the fundamental role of building and maintaining collections of biological specimens for documentation of biodiversity, studies of evolutionary pattern and process, and other evolutionary research. With the advent of the polymerase chain reaction (PCR; Mullis and Faloona, 1987) and other analytical tools for directly assaying genetic variation, use of these collections
has rapidly shifted to include molecular-oriented research in addition to traditional morphological studies. In vertebrate collections, this change in orientation has been accompanied by the development of ancillary special collections used mainly for direct genetic assays. These special collections, such as frozen tissues, samples of isolated DNA, and ethanol-preserved tissues, have posed novel challenges for collection development and management, especially as they are ultimately designed for consumptive use. The rationale and intent behind these special collections contrasts sharply with those for traditional voucher collections. Accordingly, institutional policies for consumptive sampling of special collections will differ from the necessarily more restrictive policies associated with destructive sampling of voucher collections.

Extensive use of comparative molecular methods in population genetics and systematics of vertebrates has occurred only in the past 30 years (see reviews in Dessauer and Hafner, 1984; Honeycutt and Yates, 1994). In particular, the wide adaptation of protein electrophoresis in the 1960s and 70s (see Richardson et al., 1986; Murphy et al., 1996) provided the impetus for assembling frozen tissue collections. This was the first comparative technique that allowed routine, direct assessment of genetic variation within and between populations. Focus on the microevolutionary level, including the examination of large numbers of individuals to estimate parameters such as heterozygosity, polymorphism, and population subdivision led to the development of large, comprehensive tissue collections from wild populations (Baverstock and Moritz, 1996). More recently, these collections have proven invaluable for direct extraction of nucleic acids (DNA or RNA) used in a variety of studies, including molecular systematics and phylogenetics. For example, the number of comparative molecular papers using these collections and published in the Journal of Mammalogy increased from zero in 1954 to two in 1964, six in 1974, 14 in both 1984 and 1994, and 15 in 1996. Further, there has been a decided shift in techniques from karyology, immunology, and protein electrophoresis in the early part of this period to the current emphasis on comparative studies of DNA. These collections, together with the voucher specimens from which they were derived, also serve as baseline samples in forensic studies by law enforcement arms of agencies such as the US Fish and Wild-
life Service, responsible for monitoring traffic in animals and their products (Dessauer and Goddard, 1984).

Concomitant with the acceptance, development, and routine application of molecular techniques, special collections have grown rapidly. In 1984, there were five North American collections with over 10,000 frozen tissue samples of vertebrates, all in the United States (Dessauer and Hafner, 1984). By 1994, there were 13 North American collections of this size and large collections were established both in Canada (Dessauer et al., 1996) and in México (F. Cervantes, Universidad Nacional Autónoma de México, pers. comm.). Several of these collections, such as the amphibian and reptile collections in the Royal Ontario Museum (ROM), now have more than 30,000 individual tissue samples (Dessauer et al., 1996). Growth of these collections has been facilitated by requirements of granting agencies. For example, the National Science Foundation and the National Geographic Society now routinely require that tissue samples be taken and deposited in major museums during the course of biological survey work.

More recently, the issue of destructive sampling of existing traditional collections of vertebrates as direct sources of DNA, where special collections are not available, has reopened an old problem for those responsible for maintaining the archival quality of those collections. Destructive sampling of voucher specimens for anatomical, physiological, medical, and other avenues of research has been a long-standing issue but has been brought to the fore by the sheer volume of requests (occasionally demands) from the molecular research community. All research collections are designed to be used, and curators and collection managers are faced with the conundrum of balancing present research needs with the charge of preserving the collections for future research (Cato, 1993). Although the prospect of using traditional specimens as sources of DNA has added an unforeseen research dimension, further justifying their archival value to sometimes sceptical administrations, this use must be carefully regulated. The widespread availability of special collections designed for such consumptive use in molecular systematics has helped to ameliorate, but not eliminate, these pressures for vertebrate collections.

The aim of this paper is to: 1) briefly review techniques for field collection, storage, and collection management of special
collections for vertebrates (mainly frozen tissue collections); 2) discuss methodology and policies for destructive sampling of traditional, existing collections of voucher specimens; and 3) discuss institutional policies for consumptive use of special collections and destructive sampling of traditional voucher collections. It is hoped that this review will provide some background into the debate on consumptive sampling as it has evolved in the vertebrate museum community.

SPECIAL COLLECTIONS

Field Methods

Field preservation, transport, and storage of tissue collections were recently reviewed by Dessauer et al. (1996), and will be only briefly summarized here. For genetic analyses, tissues and their included polymers need to be collected and maintained in a biochemically active form. This necessity poses a series of challenges for field collecting, depending on location of the work and the types of molecular studies to be pursued. For vertebrates, samples are most often quick-frozen in either liquid nitrogen (LN$_2$: -196°C) or dry ice (-60°C) in the field and later stored at ultra-low temperatures (near -80°C or colder) upon return to the laboratory. For certain molecular approaches, such as studies of cellular DNA content (Sharbel et al., 1997) or isolation of whole, intact, mitochondrial DNA, initial freezing in LN$_2$ is required or highly preferable. Other approaches, such as DNA sequencing, pose fewer obstacles. Macromolecules can be preserved partially intact in a variety of solutions (see below), or even in dried pieces of skin or muscle (the latter enabling extraction of small segments of DNA from traditional specimens). To minimize financial and logistic constraints on field work and collection storage and maintenance, it is critical to determine at the outset which molecular approaches are to be supported by the collections. We routinely save tissue samples from most specimens collected.

A variety of tissue types can be collected in the field, and that variety may be affected by the intended molecular approach. For vertebrates, the array of tissues most commonly preserved includes blood, heart muscle, skeletal muscle, kidney, and liver, but may also include brain, spleen, stomach, eye (in fishes), and testes (in
birds and mammals). Tissues should be dissected from appropriately euthanized specimens as soon after death as possible. Skin biopsies, blood and other tissues can sometimes be removed without killing the animal (e.g., Amos and Hoelzel, 1991; Seutin et al., 1991; Whitmer and Barratt, 1996) but we recommend preparation of at least some specimens of each taxon as vouchers. For protein electrophoretic studies, the selection of tissues is critical because the enzymatic expression of individual protein loci is often restricted to specific tissues. Tissue-specific expression of proteins varies widely among species (Matson, 1984; Murphy and Crabtree, 1985; Murphy and Matson, 1986) with the result that this information is sometimes useful in reconstructing phylogeny (Fisher et al., 1980; Buth, 1984; Murphy and Crabtree, 1985). In this regard, taking a greater diversity of tissues makes the collection more useful. For extraction of DNA, tissue type is less important, although mitochondrial-rich tissue such as liver or spleen is best for extraction of whole mtDNA molecules. Longmire et al. (1997) noted that for partially decomposed specimens of mammals, DNA in brain may be less subject to degradation than that in some other tissue types.

Extended field trips in remote regions sometimes preclude the use of LN$_2$ and other cryogenic options for tissue collection. Although freezing tissues remains the most reliable and versatile method of preserving tissue for long-term storage, cryopreservation is not required for some molecular studies. DNA can be preserved (although not wholly intact) in either 95% ethanol or in 35% isopropyl alcohol. It is best to mince tissues to allow quick penetration by alcohol, although whole specimens can also be preserved in ethanol. Long-term stability of DNA molecules may also be enhanced by adding ethylenediaminetetraacetic acid (EDTA) which inhibits nucleases. Given that DNA is preserved in alcohol, several institutions such as the Museum of Vertebrate Zoology, University of California, Berkeley, and the ROM ornithology collection, now maintain alcohol-fixed tissue collections. Voucher collections never preserved in formaldehyde (e.g., Zoological Institute of St. Petersburg, Russia) also have been used as sources of material for molecular research, although their value in anatomical or histological studies may be compromised. Macerated tissues initially preserved in lysis buffer in the field (Seutin et
al., 1991; Longmire et al., 1997), a preliminary step in DNA isolation, can be stored at room temperature, refrigerated, or frozen for several years prior to use. Finely minced tissues can also be saved in a saturated salt solution containing dimethyl sulfoxide (DMSO) and EDTA (Amos and Hoelzel, 1991; Seutin et al., 1991; Whitmer and Barratt, 1996) and stored at room temperature for six months or more. Preservation in either lysis buffer or DMSO salt solution produces larger yields of high molecular weight fragments of DNA than fixation in alcohol (Seutin et al., 1991).

Transportation and regulations for importation of animals and tissues to the United States are discussed in Dessauer and Hafner (1984) and Dessauer et al. (1996). In addition to obtaining any required research, collecting and/or export permits from the country of origin, Canadian regulations require a “Permit to Import Material of Animal or Microbial Origin Into Canada,” obtained in advance from Agriculture and Agri-Food Canada (present fee, Can$21.00), to import tissues and specimens. Within Canada, written authorization is also required before exchanging imported tissues among institutions. If return airline schedules include a stop and customs clearance in the United States, the material must be declared there and all United States regulations satisfied (see Dessauer and Hafner, 1984) before proceeding.

Curation

Unlike traditional voucher specimens, it is usually impractical to re-number tissue vials once they have been returned from the field. Thus the field number initially written on the tissue container is the number used to identify the tissue and retrieve it from storage. Regardless of the numbering system, the sample should always be cross-referenced to the museum catalogue number of the voucher specimen from which it was taken.

The problem of field cataloguing has been handled in a variety of ways. Some institutions use a separate hard-bound field catalogue for tissues, wherein every tissue sample is assigned a unique sequential number, in addition to the field collector’s number. These tissue catalogue pages are subsequently annotated with the permanent museum catalogue number of the voucher, thus cross-referencing the voucher with parts derived from it. A second system
uses a single field number for both the tissue sample(s) and the voucher, and this number is recorded directly on the vial. On return to the lab, these samples are assigned another frozen tissue number and cross-referenced to the voucher collection. In both of these systems, three numbers are associated with each tissue sample: the collector's field number, the tissue catalogue number, and the catalogue number of the voucher specimen (Baker and Hafner, 1984).

Alternatively, the tissue catalogue can be eliminated altogether. In the ROM mammal and herpetology collections, collectors use pre-printed field catalogue pages and rolls of unique, sequential field numbers. These field numbers are used for both the voucher specimens and tissue samples. On return to the museum, the collection data are immediately entered into a temporary file and voucher specimens are assigned a permanent catalogue number. Thus, like the voucher specimen, only two numbers are associated with the tissue samples: the field number (used for retrieval purposes in the frozen tissue collection) and the permanent museum catalogue number of the voucher. Once field identifications are verified, the temporary database is updated and specimen information is sent to two permanent databases: the database for the main specimen collection and the frozen tissue database, each of which contains some unique fields (Woodward and Hylwka, 1993). The advantage of this system is that final determinations of the voucher and its collection catalogue number are automatically updated and cross-referenced to the tissue sample.

Frozen samples are permanently stored in cardboard boxes with dividers, which in turn are arranged in a system of metal racks in an ultra-cold freezer. Samples preserved in ethanol, lysis buffer or DMSO-salt solutions can be kept in the dark at room temperature, but DNA is more stable if tissues are kept cool. In the ROM ornithology collection, ethanol-preserved samples are stored at -20°C for short intervals and between -70°C and -80°C for long term storage.

Two systems for sorting samples for retrieval are often used, either numeric, where samples are placed in order of field or tissue catalogue numbers (the system used in the ROM mammal collection), or taxonomic, where samples are arranged in the same order as the voucher collection (the system used by the ROM
herpetology collection). The numeric system is more space efficient, as samples are simply added to the end of the numeric sequence. However, retrieval of specific taxa is more time consuming. Regardless of the system used, random gaps can occur in the boxes as samples are consumed.

Another challenge to the management of tissue collections is that samples are often completely consumed. To maintain a running inventory in the ROM mammal collection, we record the number of tissue vials originally present and update the database records any time a sample is granted to an outside investigator or used in-house. When the vial number equals zero, the sample has been totally consumed and it is effectively deaccessioned. However, records of how the sample was used and by whom are still maintained. One person (preferably a tissue collection manager) is assigned to maintain the tissue database, store and retrieve samples, and process inquiries and loan requests. Tissue grants are made only on the approval of the curator responsible for that collection.

DESTRUCTIVE SAMPLING OF VOUCHER COLLECTIONS

Tissues do not necessarily need to be frozen or preserved in solution if collections are solely for studies of DNA. One of the great advantages of PCR is its ability to produce large quantities of DNA from very small numbers of target nucleic acids. Short fragments can be recovered from skin, bone, feathers, teeth, and other dried body parts that are hundreds and, in some cases, thousands of (or even more) years old (Paabo et al., 1988; Ellegren, 1991; Hagelberg and Clegg, 1991).

Traditional voucher collections are now recognized by molecular systematists not only as the primary repository of information on morphological characters and relationships but also as storehouses of short strands of DNA. Although these specimens can be used, they present many problems for the recovery of DNA not experienced with frozen tissues. For example, the autolytic processes that follow cell death degrade nucleic acids. The resulting low concentration of target DNA is very susceptible to contamination from extraneous DNAs in the laboratory or from other sources. For example, Haddrath recently amplified a mitochondrial
cytochrome b sequence from bird lice, only to find that the sequence recovered was actually that of the host (a kiwi) on which the lice had most recently fed. Inhibitors of the polymerase chain reaction that are found in traditionally preserved skins and tissues also tend to co-purify with DNA, often resulting in no or low yields (Hummel and Herrmann, 1994). Given these problems, frozen or otherwise preserved tissues from the special collections are much preferred as sources of DNA for PCR. However, for rare, endangered or, especially, extinct species, the original voucher specimens may be the best or only practical alternative. These factors should be considered in both the investigator’s initial selection of source materials, and in the curatorial decision whether or not to grant a request to destructively sample specimens.

Although there are a variety of protocols for removing samples from museum specimens, several aspects are universal. When handling a specimen, latex gloves must be worn and any cutting should be done with sterile utensils. The smallest and least conspicuous tissue sample possible should be removed and then placed in a sterile plastic tube to lower the chance of contamination. It is best to remove two samples from separate places on the specimen. Both samples can then be processed independently to confirm the authenticity of any resulting DNA sequences and to minimize the possibility that any aliquots of DNA returned to the granting institution are contaminated. Double sampling and amplification is now required by some journal editors to confirm the repeatability of experiments involving ancient DNA. In the case of soft tissues, samples should be removed from subsurface areas when possible to limit the effects of contamination and action of preservatives (e.g., formaldehyde). In dried or mummified tissues, sampling near the extremities of the specimen enhances the chances of recovering DNA because these regions dehydrate quickly, limiting degrading autolytic processes.

The amount of material required from voucher specimens for PCR varies with the type of tissue being sampled. For soft tissues, such as dried skins, small pieces less than 0.1 g (1 or 2 mm²) will often suffice (Thomas et al., 1990; Scott Woodward, Brigham Young University, pers. comm.), whereas for hard tissues, such as bone, between 0.5 g and 1.0 g usually is needed (Hagelberg and Clegg, 1991). One advantage in using bone is that longer fragments
of DNA can be amplified from it, in some cases up to 1,000 base pairs (bp; Hagelberg et al., 1991). DNA in soft tissues is often more degraded and maximum fragment size is usually between 150 bp to 350 bp. In contrast, length of fragments in frozen tissues is limited only by the maximum size that can be amplified using PCR. State of preservation, rather than absolute age of the specimen, is the best indicator of the likelihood of successfully recovering a DNA sample.

We have been able to recover and amplify DNA fragments between 150–350 bp from skin samples in the voucher collection that are up to 100 years old, greater than 500 bp from bone up to 3,000 years old, and fragments greater than 350 bp from bone several thousand years old. For example, Haddrath used a 1 g sample of bone to isolate 400 bp fragments of DNA from a 10,000 year old New Zealand moa, and, using overlapping primers, reconstructed a 1,000 bp sequence from this extinct bird.

Methods of preservation which alter the chemical structure of vouchers may limit their utility in the recovery of DNA. For example, for large mammals it is very difficult to recover DNA from chemically-altered, tanned skins. In cases where large hides are to be tanned, we clip a piece of skin beforehand and store it along with the voucher skeleton. Likewise, dried museum skins are prepared without any preserving agents or insecticides, such as arsenic or mercuric chloride, and bones are rinsed only in water during their final cleaning. Although it is sometimes possible to recover DNA from specimens initially preserved in formalin, this is usually problematic. We have had little success amplifying DNA from well-fixed material, even when specimens have been subsequently transferred to ethanol for long-term storage.

**Policies for Consumptive and Destructive Sampling**

An important collections management issue is to determine under which circumstances it is appropriate to permit consumptive sampling from special collections or destructive sampling of traditional vouchers. Some balance must be struck between the need for access to collections by individual researchers and the long-term responsibility of the holding institution to maintain the future value and integrity of its collections. At the ROM, we are much more strict about destructive sampling of specimens in the
main voucher collection than in loaning samples from special collections for consumptive use. For example, over a recent five year period, the ROM mammalogy collection has granted only three requests for destructive sampling of vouchers, whereas loans from the special frozen tissue collection comprised over 20% of all filled requests.

The issue of both consumptive and destructive sampling should be addressed in the form of a collections policy statement, such as that of the Museum of Vertebrate Zoology (MVZ), University of California, Berkeley (currently posted on the Internet at: http://www.mip.berkeley.edu/mvz/fcpolicy.html). A number of authors have discussed points that should be formalized in a written policy statement (e.g., Pääbo et al., 1992; Hafner, 1994; Whitfield and Cameron, 1994; American Society of Mammalogists, Systematic Collections Committee, F. Villablanca, in litt.), and Cato (1993) provided an example.

The following is a brief summary and amplification of points that we feel merit discussion in any formulation of a policy for both consumptive and destructive sampling.

1. Any request must be made in writing and should include a research proposal. Student proposals must be signed by the supervisor who must accept accountability for the material loaned.

2. The researcher should have demonstrated competency in the proposed methods, and positive results using a common, related taxon might be required before a request is granted.

3. Portions of samples that are not used should be returned to the collection at the conclusion of the project. Transfer of materials from the borrower to a third party should never be allowed, unless expressly authorized in writing by the lending institution. Such third party loans can lead to the loss of the connection between the voucher specimen and the tissue or subsequent DNA extraction. It is very important to avoid the loss of this link because it can result in the original lending institution losing both control of, and the rights to, information derived from its specimens.

4. Some institutions require that aliquots of DNA extractions be returned, to be stored by the lender (see discussion between Whitfield and Cameron, 1994; and Hafner, 1994). Return of
DNA extractions may prevent unnecessary resampling of the same specimens or tissue samples in the future since the extraction could be used instead. Our own experience with returned extractions is mixed, however, and we request this only for rare specimens, and then on a case-by-case basis. We have found that the long-term stability of DNA extracts can be affected by either the choice of reagents used to isolate the DNA or by the storage method. For example, while Chelex is very effective at extracting DNA from a wide range of cell types, the resultant high pH environment has been observed to affect the long-term stability of the DNA. Repeated freezing and thawing similarly compromises long-term stability. It should be noted that this can occur when specimens are stored in frost-free freezers due to the oscillation in temperatures of these units. There is also the possibility of the extractions being contaminated with extraneous DNA, a problem mostly found with extractions of ancient DNA. Unfortunately, these are often precisely the rare specimens which one would prefer not to resample.

5. For vertebrates, central depositories are being identified that are willing to accept and store returned extractions when the original lender does not have the facilities or personnel to do so (Hafner, 1994). It is unclear, however, how these central repositories will maintain the link between the original specimen and the extraction of PCR product, and under what circumstances these products might be loaned, given that the original lender should retain the rights to material derived from their specimens. Any repository system should be regulated by a disciplinary body (such as the Society for the Preservation of Natural History Collections or the Association of Systematics Collections) to insure that rights of the original collections are not inadvertently violated.

6. Collections differ in their policies regarding the scope of materials to be loaned (i.e., whether the lending institution is willing to serve as the major source of tissues and/or taxa for an outside study, or whether it is willing only to provide supplementary material to augment existing data). Some institutions, like the MVZ, will only provide supplementary material
whereas others, like some collections in the ROM, may provide the lion's share of the material required.

7. For destructive sampling of study specimens, some institutions allow known and qualified researchers to remove samples of skin, bone, feathers, etc., from specimens either under supervision in the collection at the lending institution or at the borrower's laboratory. Other institutions are more restrictive and insist on removing the samples themselves or in some cases, even performing the DNA extractions (e.g., zoological collections of the Natural History Museum, London). By choosing the specimen(s) and removing the tissue clips in house, the lending institution retains the most control over its material and insures that damage to specimens is minimized. This is the course most often taken at the ROM.

8. As noted by most authors of sequencing studies, DNA sequences should be submitted by the researcher to either GenBank or an equivalent database, and the accession numbers of these sequences should be returned to the lending institution to become a permanent part of the specimen record. Whether or not other results are required to be returned varies among institutions and types of studies. For example, in protein studies the individual genotypic frequency data might be deposited with the lending institution so that these data can be correlated with individual vouchers. This is particularly important if the original genotypic data are not published. However, accumulating, filing, and cross-referencing raw data has its limits. As pointed out by Hafner (1994), museums curate specimens, not reams of unpublished methods and results based on them. In the ROM we require, at minimum, the return of GenBank numbers and reprints of published papers, and ask for other data only in special circumstances. In all cases, any publications resulting from a study of borrowed material should both acknowledge the lending institution and list the individual specimens that were used.

9. Unlike loans of study specimens, reimbursement for shipping costs is usually required, particularly for frozen tissues which are sent by overnight courier. For large collections with relatively liberal grant policies, the cost of shipping soon becomes
insupportable unless the trade of material is evenly reciprocated. We occasionally waive this rule for starving graduate students. Some institutions also charge a retrieval fee, ranging from $10 to $50 per tissue sample, to recover costs associated with assembling and processing loans. Other institutions require that some tissue samples be received in exchange for any tissues granted.

CONCLUSION

The distinction between consumptive sampling of special collections assembled for laboratory analyses and destructive sampling of voucher collections is important and should be considered by both molecular systematists and curators. Voucher collections are preserved, processed, and stored to ensure their long-term value in the documentation and study of biodiversity and related specimen-oriented systematic research, and their utility in molecular systematics is a secondary consideration. Frozen and other special tissue collections designed for consumptive use alleviate the need to extensively sample traditional collections and remain the primary source of materials for molecular systematics. Institutional policies for destructive sampling of vouchers are necessarily more stringent than those regarding sampling of special collections designed for consumption. Although the advent of PCR has resulted in important new uses for both traditional and special collections, there is a continued requirement to balance the need for immediate use with the preservation of their archival value.

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LITERATURE CITED


