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Molecular Phylogenetics – What can Museums Contribute?

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Abstract. With the rapid increase of molecular applications in systematics and evolutionary biology, the role of museum collections is changing and broadening. In addition to specimen collections, museums should strive to build up tissue (including blood) collections specifically aimed at providing material for DNA typing or sequencing. Technical requirements for preservation and storage of tissues are trivial compared to traditional specimens. Obtaining suitable material is also much easier than obtaining material for specimens or skeletal preparations, so large numbers of samples can be stored (without freezing). Some recommendations are given on how to collect, preserve, label and store such material. Blood or soft tissue are best stored in 95-98 % ethanol, EDTA (10 %)-thymol buffer or DMSO-NaCl, none of which requires freezing for long-term storage. Fixation in formalin or blood sampling with heparin are to be avoided. Free availability of such samples to the scientific community and efficient exchange of information (e.g. via internet homepages of museums) on which species are available where are important prerequisites to make fuller use of existing collections.

Key words. Techniques for tissue collection, DNA analysis, avian collections

1. INTRODUCTION

With the technical innovations in molecular biology in the 1980s and 1990s, the use of DNA sequences and other types of molecular markers has become routine in fields such as systematics, phylogenetics, population genetics and behavioural ecology. In fact, most major advances in these fields over the past 20 years were entirely dependent on novel molecular methods such as polymerase chain reaction (PCR), DNA sequencing, single- or multi-locus genotyping and, coming into the fore just now, microarrays for the study of gene expression (e.g. ENARD et al. 2002). Enormous progress has been made in understanding the phylogeny of all kinds of organisms and many questions once thought to be unsolvable are now being tackled or can already be answered with a high degree of confidence. Prominent examples include the phylogeny of vascular plants (PRYER et al. 2001), in particular angiosperms (KUZLOFF & GASSER 2000), and of mammalian orders (MURPHY et al. 2001).

With these recent developments, the role of collections in natural history museums and the kinds of material they preserve has to be viewed in a new light. In many quarters of the biological sciences, especially in Germany, there is a general feeling that specimen collections are no longer needed for active research or, to put it another way, that no major advances in biology based on specimen collections are to be expected. This highly biased and pessimistic view must certainly change, but with it museum collections must also change to meet the demands of modern systematics and evolutionary research. Since it is no longer just the skin or the skeleton of a bird or mammal that researchers need to make full use of a specimen, museums should make every effort to also preserve samples of soft tissue suitable for extraction of high molecular weight DNA.

Below I will give some recommendations for sample preservation from my own experience with DNA sequencing from avian material. Some opinions regarding the role of museum collections and ways of exchanging material follow.

2. PRESERVATION OF SAMPLES SUITABLE FOR DNA TYPING AND SEQUENCING

Under favourable conditions it is possible to extract amplifiable DNA from museum specimens up to the age of several decades (COOPER 1993, ELLEGREN 1993). However, such DNA is always degraded to various extents (depending on storage conditions) and obtaining the desired information from DNA of specimens not preserved for this purpose is technically much more demanding and more error-prone than from DNA of freshly preserved tissue. Collections of stuffed specimens, therefore, can be no substitute for a tissue collection specifically aimed at providing material for molecular studies.

Technical requirements for adequate preservation of tissue samples for DNA studies are rather trivial: 0.5 – 1 gram of fresh – preferably muscle – tissue should be cut up into small pieces and stored in 95-98% ethanol. No particularly high grade of ethanol is needed, most commercially available kinds will do. Samples should be stored in screw-top vials (glass or durable plastic) with a rubber-sealed screw-top. To minimize demand for space, 2 ml vials are ideal. If tissue is stored in small pieces in a ratio of 1 vol. tis-

sue to 1 vol. 98 % ethanol, sufficient material for dozens of DNA extractions (aliquots to different laboratories) can be stored in a 2 ml vial.

For optimal preservation of DNA, short post-mortem times are crucial, i.e. samples should be placed in ethanol as soon after death of the animal as possible (normally in the field just after collecting). Contamination can best be avoided by cutting tissue from inside the animal excluding parts that were exposed to the outside such as skin or feathers.

Long-term storage of ethanol-preserved samples is feasible without freezing. If cooling space is limited, storage at 4 °C is fully acceptable. Whether freezing at -18 ° to -22 °C provides any advantage justifying the additional cost is debatable, but it will not do any harm and minimizes potential problems of ethanol evaporation (which may occur even from rubber-sealed vials). Failure of freezers (e.g. due to a power failure) should not affect ethanol-preserved tissue samples. Apart from ethanol, various buffers, e.g. EDTA (10 %) – NaF (1 %) – Thymol (trace) solution (ARCTANDER 1988), DMSO (Dimethylsulfoxid) saturated with NaCl (ARCTANDER & FJELDSA 1994) or even laundry detergent (BAHL & PFENNINGER 1996) are probably equally suited for blood and soft tissue preservation. Deep-freezing is not necessary for any of these, but long-term storage at 4 °C is recommended. Unfortunately, high molecular-weight DNA can usually not be extracted from specimens or tissues fixed in formalin (or other histological fixatives) prior to storage in alcohol (CANN et al. 1993; personal experience). Fixation should, therefore, be avoided with material intended for later DNA analysis.

A seemingly trivial but often neglected issue is the labelling of samples. Lables should not be immersed with the tissue sample. Also, writing onto the vial with any kind of („permanent") feltpen or other type of marker is not the ideal way of labelling, because such writing does tend to come off in the long term, especially if it comes into contact with alcohol or other solvents. In our tissue collection we use laser-printed labels taped with clear tape all around the (clean and dry) vial.

3. TYPES OF TISSUE TO BE PRESERVED

All types of tissues including blood or feathers can be preserved in the way described above. Preservation in ethanol is preferable over air-drying (of blood drops) or just keeping feathers sealed in a plastic bag. DNA can be isolated from most tissues and body components of an animal including skin, hair (HIGUCHI et al. 1988), feathers (TABERLET & BOUVET 1991; ELLEGREN 1993; LEETON et al. 1993), bones, teeth, egg shell membranes (STRAUSBERGER & ASHLEY 2001), blood, semen, saliva and even cells in faeces (SEGELBACHER & STEINBRÜCK 2001). However, there are huge differences in the amount and quality of DNA that can be recovered and in the amount and technical sophistication of work required to extract DNA from the various sources. For these reasons, if there is a choice, one should preserve those tissues that are ideal for DNA isolation (muscle, blood), not necessarily those that are easiest to obtain or to store.

A major consideration, of course, is whether the animal needs to be killed. Apart from the fact that it is always preferable to have a complete voucher specimen along with a tissue sample, sampling for DNA analysis is possible in most cases without harming the animal and without compromising the efficiency of DNA extraction. In birds, there are two main options: blood or feathers. Blood has the advantage of yielding much greater quantities of DNA, which is important if several or many molecular analyses (e.g. sequencing of a number of different genes, each of which may require several PCR reactions) are to be conducted and if samples are to be collected for distribution to multiple laboratories. Some researchers advocate collection of feathers rather than blood on the grounds that (co-) amplification of nuclear copies of mitochondrial DNA („numts") is less likely from feather DNA than from blood-derived DNA (PAYNE & SORENSEN, this issue). Although this is true, the argument is a weak one since avoidance of 'numts' should never rely primarily on the source of DNA. Mitochondrial sequence-specific primers and verification of sequence by amplification with several different primer combinations (preferably „long-fragment PCR") are mandatory anyway to exclude amplification of non-target sequences. Given such precautions, mitochondrial sequences can be obtained from fresh, total DNA extracted from blood just as easily as from DNA of feathers (or internal body tissues).

Feathers, although easier to collect, have the drawback of containing very few (usually dead and dried) cells and correspondingly little DNA. In theory, this should not be a problem since a PCR reaction needs only a few target molecules to work. However, the smaller the amount of target DNA available, the fewer PCR reactions can be run, thus compromising the option to verify a sequence with alternative amplification primers. Also, the danger of (co-)amplifying contaminant sequences is inversely related to the amount of target DNA available to the PCR reaction. The fewer target molecules a PCR has to start from, the greater can be the relative proportion of non-target molecules that may be coamplified. This is a problem especially if PCR products are to be cloned, less so, if direct sequencing is intended. Thus, sampling for purposes of DNA typing or sequencing should be done in

a way that does not unnecessarily limit the amount of available target DNA.

Blood sampling, e.g. from the ulnar vein as described by ARCTANDER (1988), can be done even in small birds without harming the animal. Two points are important here:

(1) In small birds one should not try to insert a syringe into the blood vessel. Rather, one should puncture the vessel, wait for a drop of blood to form, and take up the blood into a glass capillary by capillary action. Alternatively, a few drops of blood can be soaked up on a piece of clean tissue which is then immersed in ethanol (or an appropriate buffer).
(2) Syringes and capillaries should not be heparinized and preservation buffer should not contain heparin. Clotting of blood, which occurs immediately in ethanol, is not a problem for later DNA extraction, so any anticoagulent is superfluous. Heparin will intercalate with the DNA double helix and thus block the PCR reaction. Although heparin can enzymatically be removed from blood samples if needed, this is expensive and should be avoided.

Taking blood form a bird can be done very quickly by a single person, although two people, one holding the bird, the other taking the blood, are preferable with large birds. Careful studies have shown that, if done properly, blood sampling does not have any negative effects even in small birds (ARDERN et al. 1994). In nestlings or moulting birds it is possible, of course, to obtain tissue by plucking a growing feather and preserving the basal part of the feather that contains highly vascularized living tissue. In that case the tissue-containing tip of the feather shaft should be cut off, split open and then be submerged in ethanol (or buffer).

4. ROLE OF MUSEUMS

Museum collections will be increasingly important in providing material not only for morphological, but also for molecular studies. As with specimens, they should function as repositories of material that is freely available to the scientific community. Obtaining adequate material for tissue collections is much easier than obtaining animals appropriate for specimen preparation. Animals collected or sampled in the wild, those that died in zoos or bird parks, victims of collisions with human-made structures (windows, wires) or even fresh road kills are a perfectly good sources of material for molecular analysis. Many species can be sampled at bird-ringing stations or on ringing expeditions. It is worth keeping in mind, though, that samples from birds of known breeding status with exact geographic origin are scientifically more valuable than samples from migrants caught during routine operations at fixed ringing stations.

Given the minimal technical requirements and cost involved, I strongly encourage all museums to build up tissue collections in addition to their specimen holdings. Whenever possible, the entire animal should be kept as a voucher specimen. However, there are many reasons why this may not be feasible: conservation or ethical considerations (especially in large and rare species), lack of permits to kill an animal, taxidermists' work hours involved in preparing a specimen, space requirements for storage, labour of collection maintenance etc. Also, in population-genetic and phylogeographic studies requiring large sample sizes it may not be feasible to preserve all specimens from which samples are taken. For instance, in a phylogeographic study of the *Larus argentatus - fuscus* group we have sequence DNA of over 1200 individuals (LIEBERS et al. 2001; LIEBERS & HELBIG 2002). No matter how desirable it would be to have voucher specimens of all these birds, few museums would be able and willing to prepare and store such a number of large-bodied specimens. Depending on the species involved, alternative kinds of voucher material can be archived, e.g. identifiable body parts (a set of feathers or dried wings in case of birds, skulls), alcohol-preserved whole specimens or photographs (labelled with sample identification number).

5. AVAILABILITY OF MATERIAL

The building up of tissue collections should not be limited by the research interests of their curators, but should ideally be targeted at the requirements of the scientific community as a whole. This means that taxonomical collections should be as broad as possible and material should be freely available to any researcher requesting it (perhaps against an appropriate fee, if necessary). In most cases, many aliquots can be obtained from a single sample so that repeated study of material from the same source is possible.

Museums should build up an information system enabling researchers to find out where they can obtain material of a particular species of interest. Several large museums already have publicly accessible inventories of their holdings that can be searched via the internet. It would be desirable to establish a universal information system among museums of a particular country or within Europe to enable a quick overview over collection holdings. But even before such a system is in place, simple lists on a museum's home page are an essential step in the right direction and do not require much work to be established and kept up to date.

By meeting the new demands of molecular systematic research, museums may counter the unfortunate trend of decreasing public awareness of the importance of their collections to modern science. In the long run, this may also foster an improved cooperation between „traditional" museum systematists working with morphological characters and molecular phylogeneticists.

LITERATURE

ARCTANDER, P. (1988): Comparative studies of avian DNA by restriction fragment length polymorphism analysis: convenient procedures based on blood samples from live birds. J. Ornithol. **129**: 205-216.

ARCTANDER, P. & FJELDSÅ, J. (1994): Avian tissue collections for DNA analysis. Ibis **136**: 359-360.

ARDERN, S.L., MCLEAN, I.G., ANDERSON, S., MALONEY, R. & LAMBERT, D. M. (1994): The effects of blood sampling on the behavior and survival of the endangered Chatham Island Black Robin *(Petroica traversi)*. Conserv. Biol. **8:** 857-862.

BAHL, A. & PFENNIGER, M. (1996): A rapid method of DNA isolation using laundry detergent. Nucl. Acid Res. **24**: 1587-88.

CANN, R. L., FELDMAN, R. A., FREED, L. A., LUM, J. K. & REEB, C. A. (1993): Collection and storage of vertebrate samples. In: Methods in Enzymology **224**: 38-51.

COOPER, A. (1993): DNA from museum specimens. - In: Herrmann, B. & S. Herrmann (eds.): Ancient DNA. Pp. 149-165. Springer Verlag, New York.

ELLEGREN, H. (1993): Genomic DNA from museum bird feathers. - In: B. Herrmann & S. Hummel (eds.): Ancient DNA. Pp. 211-217. Springer-Verlag, New York.

ENARD, W., KHAITOVICH, P., KLOSE, J. et al. (2002): Intra- and interspecific variation in primate gene expression patterns. Science **296**: 340-343.

HIGUCHI, R., VON BEROLDINGEN, C. H., SENSABAUGH, G. F. & ERLICH, H. A. (1988): DNA typing from single hairs. Nature **332**: 543-546.

KUZOFF, R. K. & GASSER, C. S. (2000): Recent progress in reconstructing angiosperm phylogeny. Trends Plant Sci. **5**: 330-336.

LEETON, P., CHRISTIDIS, L. & WESTERMAN, M. (1993): Feathers from museum bird skins – a good source of DNA for phylogenetic studies. Condor **95**: 465-466.

LIEBERS, D. & HELBIG, A. J. (2002): Phylogeography and colonization history of Lesser Black-backed Gulls *(Larus fuscus)* as revealed by mtDNA sequences. J. Evol. Biol. **15**.

LIEBERS, D., HELBIG, A. J. & DE KNIJFF, P. (2001): Genetic differentiation and phylogeography of gulls in the *Larus fuscus – cachinnans* group (Aves: Charadriiformes): inferences from mitochondrial control region sequences. Molecular Ecology **10**: 2447-2462.

MURPHY, W. J. et al. (2001): Resolution of the early placental mammal radiation using Baysian phylogenetics. Science **294**: 2348-2351.

PAYNE, R. B. & SORENSEN, M. D. (2003): Museum collections as sources of genetic data. Bonn. zool. Beitr.**51**: 9-16.

PRYER, K. M., SCHNEIDER, H., SMITH, A. R., CRANFILL, R., WOLF, P. G., HUNT, J. S. & SIPES, S.D. (2001): Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. Nature **409**: 618-621.

SEGELBACHER, G. & STEINBRÜCK, G. (2001): Bird faeces for sex identification and microsatellite analysis. Vogelwarte **41**: 138-142.

STRAUSBERGER, B. M. & ASHLEY, M. V. (2001): Eggs yield nuclear DNA from egg-laying female cowbirds, their embryos and offspring. Conserv. Gen. **2**: 385-390.

TABERLET, P. & BOUVET, J. (1991): A single plucked feather as a source of DNA for bird genetic studies. Auk **108**: 959-960.

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