DNA and the museum tradition

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SUMMARY

DNA analysis is now a cost-effective and routine technique in systematics, taxonomy and population biology. Natural history museums need to maintain their relevance to these advances by expanding and diversifying their collection holdings. We describe different DNA-based techniques, the type of material required, and the most appropriate methods of storage. The needs of DNA-based research point the strategic direction for future collection development in museums. Traditional specimens (e.g. skins and skeletons) as sources of material for DNA-based studies are also discussed. We identify areas of concern associated with the use of DNA material for systematic and taxonomic studies, e.g. voucher specimens, museum accession numbers and other information standard in morphological studies but often lacking in DNA-based analyses. Museums must play a key role in ensuring that the necessary specimen information is included in publications. The relationships and obligations of museums and the researchers who obtain material from them are explored.

Introduction

Museum collections have traditionally been wholly specimen-based. In the case of birds these include skins, skeletons and spirit specimens. Such collections have been essential to the study of the systematics, evolution, biogeography and functional morphology of birds. Skin collections in particular have been important in documenting regional variation in avian species. While skins dominate most museum avian collections, they are not as adequate as we would like when it comes to documenting such things as geographical, age and sex variation (Zusi 1982, Winker *et al.* 1996, Schodde & Mason 1999). Skeletal and spirit specimen holdings are very small by comparison (Jenkinson & Wood 1985, Livezey 2003, Olson 2003). These traditional museum collections are, and will continue to be, important resources for evolutionary, biogeographical and systematic studies of birds. However, the development of new biological tools means that museums need to expand and adapt their scope of holdings in order to continue to be at the forefront of relevance for such studies. The most significant developments have been in molecular genetics, given the ease with which we can now obtain protein allozyme and DNA sequence data for phylogenetic and population studies (Richardson *et al.* 1986, Avise 1994).

Maintenance of material for genetic studies

The earliest widely used molecular technique was protein allozyme electrophoresis, which required freshly collected tissue samples or blood that was then stored in ultra-cold freezers (e.g. Richardson *et al.* 1986). Proteins degrade relatively rapidly when stored in standard freezers (-20°C), and denature when stored in ethanol.

Consequently, the growth of protein electorophoresis as an evolutionary tool started the development of ultra-cold tissue banks in universities and museums.

With the advent of the polymerase chain reaction (PCR) (Saiki *et al.* 1988), techniques such as direct sequencing of DNA became relatively fast and affordable. The tissue banks first established for protein allozyme studies now became just as important for DNA sequencing and other DNA based studies. DNA is much more robust than proteins and can be obtained from samples stored in ethanol (Houde & Braun 1988), buffers (Seutin *et al.* 1991, Amos & Hoelzel 1991, Arctander & Fjeldså 1994), standard freezers, as well as from traditional museum specimens such as feathers (Ellegren 1991, Leeton *et al.* 1993), skin (Thomas *et al.* 1989), scrapings from foot pads (Mundy *et al.* 1997) and bone (Cooper *et al.* 1992).

The ability to use a variety of specimen materials for DNA study has meant that natural history museums now need to address the following issues:

- What sorts of research will in the future require DNA samples?
- What are the most common and widely applicable molecular techniques relevant to museum holdings?
- What sorts of samples are required for these techniques and how do these relate to current museum practices regarding specimen collections?
- What are the future directions that museums should go down to make their collections useful for systematic and evolutionary studies using molecular approaches?
- How can museums use these advances to enhance the information content of their existing collections?

What sorts of research are being conducted using DNA samples?

One of the appeals of using DNA sequence data to address systematic questions is that they can be used at a range of taxonomic levels, from the status of particular taxa (e.g. Norman *et al.* 1998, Alström & Olsson 1999, Irwin *et al.* 2001, Norman *et al.* 2002) through to the systematic relationships between species and genera (e.g. Espinosa de los Monteros 1998, Omland *et al.* 1999, Johnson *et al.* 2001, Whittingham *et al.* 2002), families (e.g. Moore & DeFilippis 1997, Johnson *et al.* 2000, Barker *et al.* 2002, Ericson *et al.* 2002) and orders (e.g. Mindell *et al.* 1997, Paton *et al.* 2002). These DNA-based approaches should not be viewed as a replacement for morphological studies but rather as a complement. Nevertheless, in many cases where the systematics were based on characters associated with feeding and locomotion, molecular approaches have provided strong evidence that numerous examples of convergence had been overlooked (e.g. van Tuinen *et al.* 2001). As the number of nuclear and mitochondrial DNA sequences used to reconstruct phylogenies increases, these will become robust frameworks from which morphological variation can be interpreted. Molecular phylogenies combined with comparative approaches (Brooks & McLennan 1991, Harvey & Pagel 1991) have been used to trace the evolution of features such as breeding behaviour (Poiani & Pagel 1997), sexual dimorphism (Burns

1998), plumage patterns (Espinosa de los Monteros 1998, Omland & Lanyon 2000) and ecology (Richman & Price 1992, Richman 1996, Cicero & Johnson 1998).

Another broad area of DNA-based research focuses on documenting genetic variation within species. This includes looking at the taxonomic status of isolated populations (e.g. Norman *et al.* 1998, Irwin *et al.* 2001), the partitioning of genetic variation across the distributional range of species (e.g. Edwards 1993, Rising & Avise 1993, Baker & Marshall 1997, Milá *et al.* 2000, Liebers *et al.* 2001) and its relationship to conservation (Avise & Nelson 1989, Norman & Christidis 1997, Robinson & Matthee 1999, Pestano *et al.* 2000, Zink *et al.* 2000). Repetitive DNA markers such as multilocus fingerprints and microsatellites can also be used in behavioural studies (Burke 1989, Queller *et al.* 1993, Painter *et al.* 2000, Semple *et al.* 2001, Conrad *et al.* 2001).

Comparative genomics is a new and rapidly developing field of research which may increasingly rely on museum collections as a source of material. Comparative genomics involves the isolation and characterisation of specific genes from different organisms in an effort to understand their structure, function, mechanisms of regulation and evolution. Although domesticated species have been the major focus of research to date, there is increasing interest in studying the genomes of native species (Couzin 2002).

What are the most common and widely applicable molecular techniques?

The molecular techniques used commonly today in evolutionary and systematic studies can be divided into two groups: those that are PCR-based and those that are not. These two broad groups relate directly to the types of tissue or specimen material that can be used, amount required and preferred method of preservation.

The most widely used non-PCR techniques have been protein allozyme electrophoresis (e.g. Richardson *et al.* 1986), multi-locus DNA fingerprinting (e.g. Jeffreys *et al.* 1985), and DNA-DNA hybridisation (e.g. Sibley & Ahlquist 1990, Sheldon *et al.* 1995). Here a limited number of tissue sources can be used. For example, protein allozyme analysis requires frozen tissues while good-quality DNA is required for DNA fingerprinting.

The most widely used PCR-based techniques are direct sequencing of mitochondrial (e.g. Mindell *et al.* 1997, Paton *et al.* 2002) and nuclear (e.g. Prychitko & Moore 1997, Groth & Barrowclough 1999, Barker *et al.* 2002, Ericson *et al.* 2002) DNA, and microsatellite analysis (e.g. Queller *et al.* 1993, Painter *et al.* 2000, Semple *et al.* 2001, Conrad *et al.* 2001). PCR-based techniques only require small amounts of intact or even degraded DNA (Pääbo 1989). Consequently, there is great flexibility on the types of material that can be used with such techniques.

In the new field of comparative genomics the primary demand will be for high-quality frozen tissues from which intact DNA and RNA molecules can be isolated. RNA is more susceptible to damage than DNA and must be stored under ultra-cold conditions. RNA is used to isolate specific gene sequences using reverse-transcriptase PCR and to establish gene libraries containing all expressed DNA sequences (i.e.

those portions of the DNA that encode genes). These are termed Expression Sequence Tagged or EST libraries.

DNA from museum specimens: advantages

As discussed above, DNA that is suitable for analysis can be obtained from bone, feather bases, scrapings of foot pads and pieces of skin from museum specimens. The success of this is related to the age of the specimen. The older the specimen the more degraded is the DNA (Pääbo 1989). However, there is also variation between similarly aged specimens. Moreover, from some individuals it almost impossible to extract DNA. This might be a reflection of the types of preservatives used (Cooper 1993). DNA can sometimes be obtained from formalin preserved material (Shibata 1994), but formalin fixation causes significant sequence alterations (Williams *et al.* 1999) which can be misinterpreted as genetic variation. Nevertheless, protocols for obtaining good-quality DNA from formalin-fixed specimens are continually being developed (e.g. Coombs *et al.* 1999, Shi *et al.* 2002).

DNA studies based on frozen tissue samples can suffer from a lack of coverage of species and geographical areas in current collections. The availability of appropriate samples has been identified as a severe bottleneck for molecular evolutionary studies (Arctander & Fjeldså 1994, Winker *et al.* 1996). Omland *et al.* (1999) pointed out the importance of comprehensive species coverage, including subspecies, in constructing well-resolved molecular phylogenies. The ability to use existing museum skins (e.g. Dumbacher & Fleischer 2001) and skeletons for DNA-based studies (e.g. Paxinos *et al.* 2002, Shapiro *et al.* 2002) therefore provides an enormous resource in terms of species coverage, localities, the number of specimens and temporal sampling. Using museum skins is often the only way of working on rare, endangered (Norman & Christidis 1997) or extinct (Christidis *et al.* 1996) species. Instead of leaving out such critical species, museum skins allow their inclusion in molecular phylogenetic studies.

Museum collections also provide an historical perspective, as the specimen series from some regions can span decades. Such temporal series can be used to investigate the onset and impact of recent hybridisation events, range expansions and contractions, and allow us to investigate temporal variation in levels of genetic diversity (e.g. Thomas *et al.* 1990, Lambert *et al.* 2002, Paxinos *et al.* 2002).

DNA from museum specimens: disadvantages

Despite the advantages of coverage provided by existing skin and skeletal collections, there are several limitations and problems associated with only using such material for DNA studies.

The most obvious concern is that such sampling requires the removal of feathers, skin or other material from fragile and valuable specimens (Graves & Braun 1992). It is a form of destructive specimen sampling. While this may not be a problem for common species where numerous specimens will exist in collections, it will be of

concern when rare, extinct or unique material is sampled. Furthermore, it is with these latter taxa that tissue material will most probably be unavailable and taxonomic or evolutionary questions will exist. Consequently, it is on such valuable specimens that most pressure will be placed (Graves & Braun 1992).

Another problem with using museum specimens relates to the quality of the DNA obtained. DNA from museum skins and skeletons is degraded (Pääbo *et al.* 1989, Handt *et al.* 1994). Therefore, the DNA fragments that can be amplified using PCR will often be small, around 200 base pairs or fewer (Handt *et al.* 1994). From fresh material it is possible routinely to amplify fragments of 1,000 to 2,000 base pairs. Another limitation of using museum skins and skeletons is that only relatively small amounts of DNA can be obtained. Both these factors will increase the time and costs of a DNA study. With the relatively small amounts of DNA that will be obtained there will be a limit as to the number of PCR reactions that can be performed from any one extraction.

There are also age-related artefacts where post-mortem changes in the DNA can be misinterpreted as genetic variation. This is most likely to be a problem when analysing microsatellites (Gagneux *et al.* 1997).

A bigger concern with using museum skins and skeletons for DNA study is ensuring that the correct genome is being sampled. There are two problems here. The first is contamination. Because only small amounts of degraded DNA will be obtained from museum specimens there is a greater risk that extraneous DNA from other sources will be preferentially amplified (Handt *et al.* 1994). Controls and stringent laboratory techniques are critical to avoiding contamination in such studies.

The second problem, harder to control, is validation of the sequences. Most phylogenetic studies concentrate on the rapidly evolving mitochondrial genome (Avise 1994). However, it is now well established that multiple copies of mitochondrial genes can exist in the nuclear genome (Sorenson & Quinn 1998, Nielsen & Arctander 2001). Using PCR there is always the possibility that a nuclear copy of a mitochondrial gene will be amplified instead. Comparing a mixture of mitochondrial and nuclear sequences in a phylogenetic analysis will lead to highly misleading results (e.g. Arctander 1995).

With frozen tissue samples it is possible to purify mitochondrial DNA (Tamura & Aotsuka 1988), thereby decreasing the chance of amplifying nuclear copies of mitochondrial genes. Unfortunately, this is not possible with museum skins given the degraded nature of the DNA. One approach is to obtain sequences from purified mitochondrial DNA and from total DNA extracted from museum skins for the same species, and then compare the two to confirm that similar sequences are being obtained from both DNA sources (e.g. Norman *et al.* 1998).

Museums and tissue collections

It is clear from the previous discussion that traditional skin and skeletal collections alone are not sufficient for DNA-based evolutionary studies. These collections should be seen as a supplement to continued tissue bank development. Even so, museums

are generally the most appropriate institutions for establishing specialist tissue collections. They have the expertise and facilities for long-term taxonomic research, collection and data management, and are often the official regional faunal repositories.

In establishing tissue collections, issues that need to be considered include:

- types of tissues to be stored
- whether to link all tissues to vouchers
- methods of preservation
- storage facilities

Obtaining tissue samples

When collecting specimens, skeletal muscle, heart and liver provide excellent sources of DNA. However, liver should only be collected from freshly dead birds as the DNA in liver degrades more rapidly. When sampling DNA from specimens that have been dead for some weeks, feathers or foot pads provide the best source of relatively undegraded DNA.

For non-destructive sampling, blood and feathers are both suitable (Arctander & Fjeldså 1994). The removal of body feathers is preferred as it is much simpler, and adequate DNA samples can be obtained from one or two body feathers of larger species. While pin feathers provide the best source of DNA these are not always easy to obtain. To minimise harm to the bird it may also be better to obtain several body feathers than one or two primaries.

Taking blood samples is more complicated as it causes stress to the bird and requires certain skills on the part of the field researcher. With blood there is also a greater chance of amplifying nuclear copies of mitochondrial genes because avian red cells are nucleated and have low concentrations of mitochondria (Quinn 1992, Sorenson & Quinn 1998).

The need for linkage to vouchers

Concerns have been raised on the use of tissues or feathers that are not linked to voucher specimens in DNA studies (Winker *et al.* 1996). These are valid for phylogenetic studies where each species may only be represented by one individual. Misidentification of a specimen can have serious effects here. Consequently, vouchers are necessary for species that are hard to identify from other similar-looking species, where hybridisation is an issue, and where cryptic species may be suspected to exist.

For population studies of easily identified birds the need for vouchers may not be as great. Even for difficult species misidentification may be a minor problem if material is obtained from experienced researchers dealing with a local population study. Researchers studying the ecology and behaviour of a particular species can provide non-destructive samples of feathers and blood without the risk of misidentification. In fact, this is one fruitful way of obtaining material from highly threatened species. Wildlife managers involved in the translocation of individuals can collect non-destructive samples, which can then be used for DNA study. It is

relatively easy to carry appropriate sample tubes routinely, so that material suitable for DNA study can be obtained whenever a bird is being handled.

Museums need to develop strategies on how to deal with such non-voucher-based samples. Rejecting all tissue/feather material without vouchers may appear to be a sound scientific policy but it is also highly restrictive, particularly for population studies. Museum curators and collection managers are in the best position to ascertain the quality of material, in terms of identification and use, before incorporating it into tissue banks. Such material should be flagged as lacking a voucher as this may impact on the type of study for which it is later used.

Methods of tissue preservation

The most effective long-term method of storing tissue for molecular analyses is ultra-cold freezing. This requires a significant commitment in facilities for a museum. Ultra-cold freezers are expensive and require some form of back-up system. Field collecting for ultra-cold storage is also difficult. Obtaining and transporting liquid nitrogen and dry ice is not practical in many field situations. Servicing loans is also a complicated process in terms of the practicalities of transporting frozen material and quarantine issues. Standard freezers are not a good option for long-term tissue storage, as some enzyme activity will continue at that temperature which will lead to degradation of the DNA.

There are alternative storage methods for tissues. Tissue samples can be stored in ethanol and used for DNA analyses (e.g. Houde & Braun 1988). While ethanol may not be an ideal system for long-term storage compared to ultra-cold freezers, it does have many practical advantages. It is more cost-effective for smaller museums and those in less developed countries. No additional storage facilities are required, as most museums already have ethanol-preserved specimens. Ethanol storage is also highly convenient for field collecting, although this may not be the case in countries where alcohol is prohibited on religious grounds.

The DNA from ethanol-preserved specimens will degrade to some extent due to endonuclease activity (Houde & Braun 1988). Although ethanol stops endonuclease activity, it is important that the ethanol permeate the tissue sample completely and rapidly. Tissue samples should be sectioned into small portions so that this can occur. It is also important that the samples be stored in such a way that evaporation of the ethanol is minimised, and that only highly pure ethanol is used.

Tissues can also be stored in a variety of buffer solutions at room temperature (Amos & Hoelzel 1991, Seutin *et al.* 1991, Arctander & Fjeldså 1994). For long-term storage refrigeration is probably better. The drawback with buffers is that access to distilled water, fine balances and autoclaves is required to prepare the buffer solutions. Although Arctander & Fjeldså (1994) were able to extract DNA with no detectable degradation from material stored in buffers for five years, our experience has been that buffer-stored material provides variable results.

One limitation of both ethanol- and buffer-stored material is that it may not always be possible to obtain purified mitochondrial DNA. This can be a problem where nuclear copies of mitochondrial genes are an issue.

Storage facilities

As not all museums will have the capabilities to establish ultra-cold tissue banks, alternative systems need to be considered. The pooling of resources across museums and establishing centralised ultra-cold tissue collections is a possible solution. An advantage to users is that it is easier to source material from a single centralised collection. For the participating museums issues relating to individual roles, responsibilities, databasing, acknowledgment and benefits would need to be addressed.

Ethanol tissue storage should be possible for all museums, and is suitable for most samples. However, some material from highly rare species should still be kept in an ultra-cold facility. At least some representation of each species should also be stored in ultra-cold conditions. A combination of centralised ultra-cold and individual ethanol tissue banks is an option worth exploring.

Responsibilities of museums and users

Samples for DNA work are a form of destructive sampling. Therefore it is important that the objectives and scope of the work are defined before loans are approved (Arctander & Fjeldså 1994). With requests for feathers, skin or bone from museum specimens, material should not be provided until a strong case is presented that the requesting researcher has the technical skills, experimental design and facilities to extract and sequence DNA from such material.

For all DNA studies, the material should only be used for the agreed specified project (Arctander & Fjeldså 1994). All excess material and DNA must be sent back and material or DNA should not be given to a third party without prior consent.

It is important that researchers include accession numbers of the tissues and vouchers when they publish sequences. This is needed so that the same bird is not sequenced by different laboratories and then treated as an additional data point. Information on locality and subspecies should be included as well (Hackett *et al.* 1995). The collection and collectors (if appropriate) should be acknowledged in publications. Such information is taken for granted when morphological analyses are published but it has not been common practice in molecular studies. Museums can play a role in introducing such rigour by making it a condition of using either museum specimens or tissues for DNA work.

When using material that is not linked to a voucher, it is still important that there is a tissue accession number and that it be cited in the publication. Again this is to ensure that specimens can be tracked across studies. It is also useful to have researchers provide the sequence information to the museum so that this information can be then linked directly to the specimen's database record.

Researchers not linked to museums but who have collected material for their own studies should be encouraged to lodge any material left after completion of their study with an appropriate museum. Often this excess material lies forgotten in university freezers or drawers and is eventually discarded. This is a loss of potentially

very useful material that should be accessible to the wider scientific community by being incorporated into a museum collection.

Museums which have made an effort to build up DNA collections are often reluctant to provide samples to research groups that consistently make no effort to supplement these collections or to contribute their own resources towards securing material. Some museums have responded to this issue by imposing charges for tissue loans, but this is contrary to the spirit of cooperation that has been the tradition of museum specimen-based research. The imposition of charges ultimately discourages researchers from lodging material in museums. More positive approaches would be to (1) build alliances between institutions and researchers that undertake molecular systematic and evolutionary research *and* are willing to provide material to each other when needed; and (2) encourage non-museum researchers who make loan requests for DNA material to collect (where possible) and lodge material in appropriate museum collections. Museums have a responsibility to ensure appropriate use of their collections. As with any loan request we have the right of refusal if it is deemed to make inappropriate use or places excessive demands on the collection.

Future directions for museum collections and DNA research

In developing tissue collections museums need to identify those areas that are poorly covered in current holdings in terms of species and geographical distributions. It is important that the development of tissue banks be integrated with that of skin and skeletal collections. All specimens collected should have material lodged as either frozen or ethanol-preserved tissue.

Another factor that needs to be considered when developing tissue banks is the type of study the collections are being used for. The most common use for DNA material from museums is for studies addressing systematic and phylogenetic questions. Often the requests for material involve single representatives from species and subspecies. There is less call for material to be used in population studies, even though avian molecular ecology has been a rapidly growing area in recent years. These latter studies require larger numbers of individuals per species, but few species are currently represented by large numbers of frozen tissue samples (Arctander & Fjeldså 1994).

Museums can take active measures to become more relevant for population and ecological studies. Museums are often the regional repositories for fauna found dead. Tissue or feather samples should be routinely obtained from all specimens lodged with a museum. In this way large collections of the commoner species will accumulate.

With most museums that were contacted, the greatest users of the tissue banks were internal staff from the museums themselves. The development of tissue banks and in-house expertise and facilities for molecular-based research were correlated. This is understandable given that collection development is often driven by the research interests of curators. This is a strong reason why the collecting of material suitable for DNA study should be integrated with general collection growth.

Enhancing existing museum collections

Museums should also be aware that DNA technology has the potential to add information to existing collections. Several PCR-based DNA markers have been developed that are sex-linked across a range of taxa (Griffiths *et al.* 1998). Consequently, it might be possible to sex museum specimens using DNA. This would be of benefit with unique or rare unsexed specimens, immatures or those with doubtful sex assignments. Clearly the costs involved would make this avenue only appropriate where sex information is critical. DNA sequencing has also been used to determine the taxonomic status of species based on a single museum specimen (Joseph *et al.* 1999). DNA information can determine whether the unique specimens represent colour variants, preservational artefacts or species hybrids. In all the above examples DNA studies can be used to enhance the information content of collections.

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