stored in separate containers, if possible, even when the level of analysis to be examined does not require it (e. g., DNA restriction site analysis among populations). Future studies involving single-copy genes, introgression, hybridisation or recombination require knowledge of specific sources of the molecules. If storage of samples in the same container is unavoidable, polyethylene sacks may be used to separate individual samples [92].

An attempt should be made at the outset to remove all dirt and contaminating organisms (e. g., epiphytes, fungi, ectoparasites) from the specimen or tissue sample. It may not be possible to remove all such organisms (e. g., endoparasites and gut flora), but this problem can be circumvented by judicious tissue choice (see above) and the use of taxon-specific primers in the PCR. Mites are a particularly problematic contaminant of field-collected fungal and algal samples. Not only can these arthropods destroy specimens, they can also cross-contaminate a culture collection as they move about [93]. Additionally, the removal of contaminating organisms is important for preventing the introduction of foreign organisms across international borders (see below).

Labelling and documentation

Investigators should meticulously label all materials they collect. Labels should include species (if identified), collection locality, brief habitat description (including reference to collection method used), date of collection, collector, voucher number, etc. [94]. When working in a team, the principle investigator should allocate documentation duties at the outset and assign a backup. All team members should be conversant with the system used to identify and inventory samples that have been collected, including unambiguous abbreviations, the order in which information should appear on the labels and other critical information about the provenance of the sample, which may disappear when field notes are transcribed.

Permanent ink markers should be used for labelling, but must be tested beforehand in water, ethanol and extremes of temperature [95, 96]. Labels should be written directly on the sample bag or container or, if affixed, tied as well as taped, since tape is liable to come loose during handling, freezing and thawing. Alternatively, labels can be etched into glass or plastic tubes with a diamond-tipped pen [97]. Permanent ink marker or heavy lead-pencil labels, written on roughened paper or card [98, 99] and tied to the specimen itself or placed inside the container, are the safest guarantee that collection and provenance data will remain with appropriate samples. The use of synthetic, polypropylene paper (e. g., Kimdura or Tyvek, available from Kimberly-Clark, Dallas, TX) prevents tearing of labels [92, 100, 101].

It is prudent to test all vials, tubes or other containers of unknown composition before departure, to ascertain if they are unbreakable, solvent-resistant and leakproof (for fluid-preserved samples) or can withstand ultracold conditions (for frozen samples). Fragile glass tubes (e. g., hematocrit tubes) can be inserted into the slots of corrugated cardboard for protection during storage [45].

Water-resistant pocket notebooks and pens with waterproof (and solvent-resistant) ink are as essential to successful collecting as sterile vials and bags [97]. The use of traditional collector's catalogues (e.g., [102, 103]) organised such that each specimen receives a unique number preceded by the collector's initials, is recommended [3, 4]. Catalogue entries should record the type(s) of tissues to be sampled and catalogue numbers should be recorded on the vials containing tissues.

Modern biorepositories often use standardised specimen vials with bar-coded labels printed on thermal- and solvent-resistant material suitable for long-term storage. Advance planning with regard to tissue voucher disposition may allow a researcher to obtain a series of numbered sample vials from the repository prior to the outset of fieldwork. The researcher may then refer to the vial number in the field notebook with regard to collection data, thereby circumventing the need to label vials in the field and facilitating the access to specimens in the repository and its associated database on completion of the study. Advance knowledge of the repository accession numbers associated with specimens also facilitates the preparation of manuscripts.

Primary documentation in the field will always be done by hand (if for no reason besides the fact that the backup systems for field computers are subject to failure). Nevertheless, notebook computers have provided an additional innovation to the documentation of field data. Battery operation for more than three hours, with one-hour recharge times from 110 V outlets or car batteries, together with the ability to link via internal or facsimile modems to remote laboratory computer facilities, has allowed the maintenance of up-to-date database files [2]. For further suggestions on methods of cataloguing in the field, refer to Baker and Hafner [97] and Dessauer et al. [3, 4].

Hazardous organisms

Preventative measures may be needed to protect the researcher working with certain organisms. Investigators should not attempt to work with these taxa without proper equipment and a thorough appreciation of the risks involved. For example, investigators working with venomous animals should wear protective clothing and carry antivenom or venom aspirators, in the event of accidental envenomation. These precautions are especially obvious for those involved in the "milking" of venom for their investigations [3, 4].

Investigators working with some mammals and their parasites should be vaccinated against rabies or tuberculosis. Animal necropsy should also include protective clothing and the containment of possible biohazards, in order to avoid contracting diseases such as psittacosis, erysipelas, rickettsial infections and brucellosis [104]. The National Wildlife Health Center (Madison, WI) has a Resource Health Team available to examine animal specimens for possible diseases (Appendix 2).

Some fungi or their secondary products can also cause virulent or chronic disease in animals and may require special procedures [105]. Culture collection staff (see below) may be able to advise on the necessary procedures for pathogenic fungi.

4 Other Sources of Specimens and Tissue Samples

There are many ways in which tissues can be obtained without even going into the field. Stock centers and culture collections, commercial supply companies, cell line centers, seed/spore banks, botanical gardens and zoological parks are the most frequently used sources for fresh tissues. The primary consideration in obtaining tissues from any of these sources is the reliability of the identification of the organisms that are used for the tissues. If the species designation is suspect, the specimen may not be worth the trouble. In addition, the exact origin of organisms from these collections is often unknown or ambiguous, such that caution should be exercised with specimens obtained from these sources if precise location data are needed for a particular study.

In addition to these sources of fresh samples, vast numbers of frozen, desiccated and fluid-preserved samples exist in natural history museums, herbaria and frozen tissue collections. Such samples are generally accurately identified, documented and, if collected in series, vouchered. However, some (e. g., desiccated and fluid-preserved samples) may provide considerable challenges to protein or nucleotide extraction, depending on the method and extent of preservation.

4.1 Sources of Fresh Specimens and Tissue Samples

Stock centers and culture collections

Stock centers exist for several animal taxa (e. g., *Caenorhabditis*, *Drosophila*, *Peromyscus* and *Tribolium*), from which particular strains or species may be ordered at cost (Appendix 3). The services of such centers, including preparation, testing, preservation, maintenance and shipping of the cultures, are expensive, hence user fees must defray costs [106].

Animal stock centers are often limited in the diversity of taxa available, although some hold a large variety. For example, the Jackson Laboratory (Bay Harbor, ME) maintains more than 2500 strains of genetically defined mice for biomedical research, while the *Drosophila* Species Stock Center (Tucson, AZ) can supply approximately 300 species of *Drosophila*. Samples of bacteria, fungi, algae and other unicellular protists may similarly be obtained at cost from culture collections, where they are maintained in pure culture. Lyophilised cultures of fungi may be shipped immediately on order, but algae must usually be grown out on agar or in liquid medium so that shipping may be delayed several weeks.

Detailed information on worldwide culture collections is available from the World Federation for Culture Collections (WFCC) which maintains the World Data Center on Microorganisms (WDCM) and is a component of the UNESCO Microbial Resources Centers (MIRCEN) network. As part of their mission to disseminate information on culture collections, the WDCM has produced two useful publications: the *World Directory of Collections of Cultures of Microorganisms* [107], which includes 345 fungal collections, and the *World Catalogue of Algae* [108]. In addition, users may search the listings of all the collections for individual species on the WDCM electronic database (Appendix 4). According to this database, 472 culture collections in 62 countries are currently registered in WDCM. A guide to the database (*Guide to World Data Center on Microorganisms – A List of Culture Collections of the World*) is also available. The websites of other culture collections and germplasm repositories are listed in Appendices 3 and 4.

In addition to the major collections with broad general holdings, many smaller collections occur throughout the world, which might be of interest for providing samples for particular studies or geographic regions. Medical research institutes, which include departments specialising in parasitology, toxicology and medical entomology (e.g., Instituto Butantan, São Paolo, Brazil; South African Institute for Medical Research, Johannesburg, South Africa), often stock permanent cultures of common medically important local taxa (e.g., mosquitoes, spiders, scorpions, snakes and unicellular protists), from which samples may be supplied for molecular studies, on request. Insect fungal pathogens can be obtained from the specialist collection of entomopathogenic fungi maintained by the U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) at the U.S. Plant, Soil and Nutrition Laboratory (Ithaca, NY). Cultures of many isolates of wood-rotting basidiomycetes are available from the USDA Forest Service at the Forest Products Laboratory and Center for Forest Mycology (Madison, WI), which maintains voucher specimens for every culture. The *Chlamydomonas* Genetics Center at Duke University (Durham, NC) houses a large collection of *Chlamydomonas reinhardtii* mutants and numerous strains of other *Chlamydomonas* species and provides a printout of file information on each strain. The Soil Microbiology Division of the International Rice Research Institute (Manila, Philippines) maintains a collection of prokaryotic nitrogen-fixing blue-green algae, and a collection of algae with high potential for use in biomass energy production is available at the National Renewable Energy Laboratory (Golden, CO).

Most curators of culture collections ensure that cultures are correctly identified, even returning transferred cultures to the depositing scientist for authentification before cataloguing [106]. Accordingly, the majority of samples obtained from such cultures will be correctly identified (although collection data may be unavailable). Where multiple strains of species are offered by culture collections, informed choices should be made, depending on the research question. Strains may differ in metabolism and other genetic characters, which may be important in selecting a culture. When strains are intended to be

representative of the species, so-called type cultures (i. e., those isolated from the type collection of a species at the time of its description) should be ordered preferentially. Two useful publications of the American Type Culture Collection (ATCC) list processes and products associated with many isolates in their collection [109, 110] and additional information may be found on their website (Appendix 4). Similarly, some culture collection catalogues contain information, including literature references to strains [111, 112].

Ploidy number should be a further consideration when selecting fungal cultures. Highly variable DNA regions in single-copy or repetitive genes (e. g., ribosomal RNA genes) may vary at single base positions in the homologous chromosomes of diploid or dikaryotic isolates [113]. Haploid cultures from many fungi with gametic meiosis can be readily acquired from single spores, whereas isolates from basidiomata, ascomata or mass spore cultures of heterothallic basidiomycetes and ascomycetes provide strains with nuclear variation [93]. Diploid material is difficult to avoid in some organisms (e. g., oomycetes), but it is important to recognise the possibility of variation. DNA extraction from single spores [114] may eliminate some ploidy problems and provide a means for a variety of intraspecific studies. The ploidy level of most algae in culture collections is known or can be inferred from the life cycle (information available in general texts such as Bold and Wynne [115]).

Cell line centers

Cell lines offer an alternative source of fresh tissues for comparative research. A large selection of cell lines are held in storage by a variety of different centers (Appendix 4), e. g., viral infected cell lines, cell lines for large mammals and cell lines for human diseases [106, 116, 117]. Further examples of the breadth of taxonomic representation are provided on the websites of the ATCC (American Type Culture Collection) and the ECACC (European Collection of Cell Cultures). The CRES (Center for Reproduction of Endangered Species, Zoological Society of San Diego, CA) supplies a variety of endangered vertebrate cell lines and tissues (Appendix 5).

Many centers store multiple different lines derived from different tissues for a variety of taxa, thus allowing specific tissues to be selected for studies in which tissue specificity is a concern [106]. The major cell line centers such as the ATCC and ECACC are easily accessible via email or their websites, which provide online order forms to facilitate the rapid purchase of cell lines. These centers also offer advice on the materials and methods of cell culturing, once the cell lines have been shipped to researchers. Each cell line center has specific terms of usage that the researcher should consult before placing an order (see below).

Commercial Sources

Live representatives of most animal phyla and pure cultures of some protists can be obtained relatively cheaply from biological supply companies (Appendix 6). Ordering specimens is convenient insofar as they are shipped direct to the laboratory, thereby saving considerable time and effort. However organisms

should be ordered as far in advance as possible, since their appearance in the field may be seasonal or unpredictable. Identifications are usually correct (common, well-known taxa are usually stocked), but voucher specimens should still be preserved (see below).

The commercial pet trade provides yet another convenient source of specimens or tissue samples for some animal taxa, notably birds, fish, herpetofauna and selected terrestrial and marine invertebrates. Prices of commonly available species acquired from these sources are usually reasonable, but less commonly available taxa may be expensive. Moreover, although certain common or captive-reared species are available year-round, the availability of most field-collected species is highly unpredictable and requires constant vigilance on the part of the investigator. Apart from the commonly available species, identifications of most specimens acquired through the pet trade should be viewed with suspicion, especially where reptiles, amphibians and invertebrates are concerned. Besides general identification errors made by untrained individuals (especially common with sexually dimorphic taxa), deliberate misidentification is used by certain dealers to enhance the apparent "diversity" of taxa on offer and hybrids are often sold as "new species." Collection data, if available (seldom more than country of origin, which is acquired third-hand by the dealer from the importer, who received it from the collector!), should be mistrusted for the same reasons. Finally and most importantly, it behooves the investigator intending to use the pet trade as a source of specimens to ensure that the specimens to be purchased were acquired legally by the dealer (addressed further below).

Botanical and zoological gardens

Botanical gardens (including arboreta and university greenhouses) are an established source of land plant tissue, whereas zoological gardens (including aquaria, aviaries, butterfly gardens and snake parks) are just emerging as a source of tissue from vertebrates (and certain invertebrates). Currently, CRES is the only zoological institution to make their resources formally available. The large diversity of plant and animal families and genera maintained by botanical and zoological gardens is ideal for higher level molecular studies using exemplar taxa. More specialised collections (e.g., certain arboreta, butterfly gardens and snake parks) may also be useful for molecular studies at the genus or species level. Most directors of botanical or zoological gardens are very willing to provide access to their collections for molecular study as it enhances the role, and thus continued support, of the garden. However, since animal tissues may have to be sampled when animals are sedated for medical examination, investigators should request samples well in advance.

Despite the obvious benefits of obtaining tissue samples from botanical or zoological gardens (large diversity, ease of collection and transport, saving in cost and time), potential problems remain. Foremost is the lack of complete voucher information (collector, date and exact locality), attached directly or indirectly in the records, to the specimen. A more serious problem is the

possibility of misidentifications and label switches, although these can be obviated by making vouchers for subsequent identification by specialists in each group. A further problem concerns collection and transport of the material. Many gardens have neither time nor personnel to oversee the multitude of requests for shipment of samples and should not be expected to bear the associated expenses. Personal contact with scientists and managers at the gardens is recommended.

Henderson and Prentice [118] provide a worldwide listing of botanical gardens. Various other sources, e.g., the teaching guide to the text *Biology* [119], also contain extensive lists of zoos and botanical gardens. Recent listings can be found at the websites provided in Appendix 7.

Seed and spore banks

Seed banks are becoming an increasingly important source of plant tissue in molecular studies, especially where agronomically important groups, including cultivated species and their wild relatives (e.g., *Brassica*, *Solanum*, *Triticum*, *Zea*, *Glycine*), are involved (Appendix 3). The U.S. National Plant Germplasm System (NPGS), coordinated by the National Germplasm Resources Laboratory (Beltsville, MD), comprises 22 repositories that are annually updated and planted out to maintain their collections. One of these, the U.S. Potato Gene Bank (Sturgeon Bay, WI), was essential for a large molecular research program on the cultivated potato and its wild relatives [2]. Besides maintaining an extensive collection of seeds, the National Seed Storage Laboratory (Fort Collins, CO), another repository of the NPGS, is investigating pollen storage as a means of storing germplasm of clonally held species [120].

In addition to such formal repositories, many botanical gardens maintain seed exchange lists (e.g., *Index Seminum* of the Modena Botanical Garden, Italy). The Millenium Seed Bank Project of the Royal Botanical Gardens, Kew has the most extensive listing of fully documented plant species, including many families of noncultivated plants.

Although fronds and rhizomes are most often used as tissue sources from ferns or fern allies, spores and resultant gametophytes are equally suitable. The American Fern Society maintains a spore exchange program and information can be obtained from the *Bulletin of the American Fern Society* or their website (Appendix 3).

Potential problems with seed or spore sources, whether from seed banks or university greenhouse collections, include contaminated seed, errors in handling and labelling, and misidentification. Such problems can be avoided by vouchering all plant tissue grown from seed.

Gifts or exchanges with scientific colleagues

A commonly used alternative source of field-collected material is through gifts or exchanges with colleagues in foreign countries, who are either specialists in the group under study or knowledgeable about specific collection sites. Although the use of local scientists can add greatly to the breadth of the study,

several practical issues remain, including the provision of detailed instructions concerning shipment of material and the necessity for providing permits if crossing international borders (see below). A further issue to consider is whether the support in obtaining samples from colleagues has been so extensive and critical to the success of the project that nothing short of co-authorship would be adequate compensation.

4.2 Frozen Tissue Collections

Many natural history museums have created, and continue to support, frozen tissue collections or collections that harbor tissues in ethanol or lysis buffers [121, 122]. Two directories of frozen tissue collections ([4, 31], reproduced and updated in Appendix 5) list public and private institutions in the U.S.A. and abroad that have holdings of frozen tissues. Several frozen tissue collections are now online while others are in the process of producing websites for easy electronic access via the internet.

Some requests are easily fulfilled by these facilities, as with commonly available tissues of which large quantities exist in storage. Other requests are more difficult to fulfill, as with tissues of endangered species of which precious little remains. Consequently many frozen tissue facilities require a formal application from the researcher wishing to obtain loans, especially for the more difficult requests (for examples of frozen tissue collection policy, refer to the websites in Appendix 2). First, the loan request must be accompanied by some assurance that the applicant is proficient in isolating the molecules of interest from frozen specimens [97, 123]. Such assurance is usually demonstrated in a short proposal that the applicant submits to the repository for curatorial approval. Second, many frozen tissue facilities request that a proportion of the DNA or proteins isolated from the frozen tissue be deposited back in the frozen tissue facility in return for the loan.

Shipments of frozen tissues are costly to package and transport hence payment of tissue shipment costs is normally requested of the recipient [97]. Investigators planning to obtain samples from frozen tissue collections should provide for these costs in their grant budgets.

4.3 Sources of Desiccated or Fluid-preserved Specimens and Tissue Samples

Natural history museums

Museum collections represent a tremendous resource for molecular studies. The world's research collections contain vast amounts of material amenable to molecular analysis that may be otherwise unavailable due to extinction or

collection difficulties. This can be especially acute in higher level studies of geographically diverse taxa. Such collections can make possible, in time and resources, studies for which it would take years to gather material.

Zoological collections are composed predominantly of skeletal remains, dried skins, dried insects and fluid-preserved specimens. Skeletal material, which makes up a large proportion of vertebrate collections, is usually devoid of amplifiable DNA [19, 21, 22, 124]. Nevertheless, Hagelberg et al. [125] amplified DNA from human bone samples 300–5500 years old. More recently, methods developed for protein extraction from ancient bone were found to yield large amounts of DNA with molecular weights much higher than those seen from ancient soft tissue [126–128]. Dried, untanned skins routinely yield DNA sequences, as do dried, pinned insects, although the DNA may be extremely degraded [38, 64, 124].

The vast majority of helminth and arthropod specimens are preserved in ethanol, in which DNA preserves fairly well (depending on collection practices), although it is usually degraded to < 2 kb, especially if 70% ethanol was used, as is prevalent in older collections [65]. However, other fixatives, e. g., glycerin and 2-propanol (in arachnid collections), may have been added to the ethanol in an effort to harden, soften or maintain the colour of the specimens. For example, terrestrial planarians, stored long-term in 80% ethanol, must first be fixed in a formaldehyde calcium cobalt solution [62].

Fluid-stored insect collections may contain similar fixatives, including formamide, picric acid, formalin and glycerin. Any of these fixatives may likewise affect DNA, hence it is crucial to know how samples were collected and maintained. For example, DNA isolated from insects preserved in acetone, ethyl acetate, formal saline, Carnoy's solution (ethanol:acetic acid, 3:1), methanol, or propanol was highly degraded, compared with that extracted from insects preserved in liquid nitrogen, preserved in ethanol (stored at 4°C or at ambient temperature), sun-dried, or dried over silica gel [15, 38, 65].

Vertebrates and marine invertebrates (e. g., cnidarians, ctenophores and ctenostome bryozoans) have traditionally been preserved in solutions of 10% buffered-neutral formalin (BNF) (v/v) with implications for DNA isolation from such samples. PCR is greatly affected by the amount of time spent in formalin, the temperature at fixation and the method of buffering. For example, Greer et al. [64] found that fragments of DNA, approximately 1 kb in length, were impossible to amplify from formalin-fixed tissues after only 24 hours at ambient temperature. However, high molecular weight DNA was obtained from tissues fixed at 4°C in formalin buffered with 4 M urea [23, 129]. Similarly, Noguchi et al. [63] established that fixation in formalin at 4°C and in formalin containing 5 mmol/L EDTA at ambient temperature preserved significantly more high molecular weight DNA than fixation in formalin at ambient temperature. Savioz et al. [130] recently provided protocols for the isolation of high molecular weight DNA from tissues stored in formalin for nearly 50 years. A simple test for the identification of formalin *versus* ethanol-preserved specimens, based on a colour change in the acid-base indicators sodium sulphite and sodium metabi-

sulphite, was reported by Waller and McAllister [131]. Tests for other fixatives were outlined by Moore [132].

Greer et al. [64] and Criscuolo [133, 134] evaluated the ability of various other fixatives, traditionally used in vertebrate collections, to yield DNA fragments of moderate size on PCR amplification. Most fixatives gave poor results, e. g., acetone, Zamboni's solution, Clarke's solution, methylated spirits, phenol, chloroform, glutaraldehyde, paraformaldehyde, formalin-ethanol-acetic acid (FAA) and metacarn. The worst results were obtained with highly acidic fixatives such as Carnoy's solution, Zenker's solution and Bouin's solution. Zenker's solution is a common tissue fixative (5 parts glacial acetic acid mixed with 95 parts saturated mercuric chloride and 5% potassium dichromate) which affects the stability of DNA in tissues preserved in it, owing to high heavy metal concentrations which promote phosphodiester breakage. Bouin's solution was formerly the fixative of choice for many studies of infectious or parasitic diseases in animals, where a large piece of tissue was preserved in excess solution. However, parasitic samples preserved even under these conditions may be useful if investigators design PCR primers to yield small (< 200 bp) fragments.

Overall, most of the wet and all of the dry samples in museum collections are potentially useful to molecular studies. Given the resources of some of the large institutional collections, some intractable problems may be resolved and some groups whose molecular biology is poorly known may be sampled more completely (e. g., [135]). Readers should consult the *Ancient DNA Newsletter* for updates on protocols suitable for the successful isolation and amplification of DNA from museum specimens.

Herbaria

As with museum collections, there is increasing interest in herbaria because they can potentially provide DNA from identified, fully vouchered species, including types and species that are rare or nonculturable [136–138]. Like museums, the role of herbaria will undoubtedly become more important as PCR and sequencing technology allows more rapid and extensive surveys at all taxonomic levels.

Herbarium specimens of fungi as old as 50 years [139] and nearly 100 years [140] have provided adequate templates for PCR of ribosomal genes. Several studies have also successfully extracted DNA from plant [48, 50, 51] and algal [141] specimens, which similarly proved to be good templates for PCR.

Douglas and Rogers [142] assessed the effects of seven cytological fixatives (3.7% formaldehyde at pH 3.0 and 7.0, FAA at pH 3.0 and 7.0, 1% glutaraldehyde at pH 3.0 and 7.0, and Lavdowsky's fluid, containing mercuric chloride, at pH 3.0) and one storage buffer (SED = NaCl-EDTA-DMSO, pH 7.0) on the DNA of nine plant and fungal species. DNA from untreated tissue and SED-treated tissue was of high molecular weight (> 50 kb). DNA from glutaraldehyde-treated tissues averaged 20 kb in length, while DNA from all other treatments averaged < 8 kb in length. Nearly all attempts to amplify from specimens treated with 3.7% formaldehyde (at pH 3.0 and 7.0) failed.

Although DNA from desiccated herbarium specimens may be partially degraded, primer-directed amplification, especially of multiple copy genes, can overcome the problem. However, the relatively poor quality precludes efficient restriction site analysis [55]. Generally, specimens fast-dried at moderate temperatures, or the edges of large specimens, provide the least-degraded DNA [93]. It is unlikely that isozymes will ever be routinely extracted in an active state from herbarium specimens, although certain desiccated tissues can provide good activity for some systems [58], discussed above. In addition to providing DNA from tissue, herbarium specimens of fungi and algae may contain viable propagules for establishing cultures [143] or extracting DNA [141]. Selected websites, containing recent listings of natural history museums and herbaria, are provided in Appendix 8.

Medical facilities

Archival collections of paraffin-embedded tissues prepared for histology [144–146] and collections of dried blood spots on Guthrie cards stored by state neonatal-screening laboratories [41, 147, 148] represent valuable resources for retrospective studies in clinical pathology and molecular epidemiology (Appendix 4).

Protocols for DNA extraction from paraffin-embedded tissues up to 40 years' old were provided by Frank et al. [149], Morgan et al. [150] and Pavelic et al. [151]. Pavelic et al. [151] assessed the effects of different fixatives on paraffin-embedded tissues, noting that 10% formalin caused irreversible DNA damage that was greater with prolonged fixation time, while tissues stained with Carnoy's, AmeX and Papanicolaou fixatives resulted in consistent yields of high molecular weight DNA. Makowski et al. [41], Ostrander et al. [42] and McCabe et al. [43] provided methods for extracting DNA from blood spots on paper.

5 Transportation, Long-term Storage and Archiving of Specimens and Tissue Samples

Whether specimens and tissue samples are collected in the field or acquired from other sources, they will have to be transported back to the laboratory for processing. Transportation methods must aim to retain the samples as intact as possible during transit, which may require rapid delivery and be subject to a variety of regulations, depending on the nature of the samples (e. g., live *versus* preserved) and the mode of shipping (e. g., airline, courier, mail).

On arrival in the laboratory, samples must be processed (including identification, if this has not already been conducted), excess tissues stored for future use, voucher specimens retained (or deposited in appropriate institutions) and collection data archived in a computerized database. A variety of approaches to long-term storage and archiving are available to the investigator, the choice among which depends both on the taxa and molecules of interest.

5.1 Transportation

Protocol 4 lists considerations relevant to the transportation of a variety of preserved specimens and tissues. Specific recommendations for packing and labelling are provided in Protocol 5.

Protocol 4 Common transportation methods

1. *Live specimens and cultures.* Invertebrate material is best shipped live from supply houses. However, transit times and export/import regulations may prevent this option when international shipping is involved. Small insects such as *Drosophila* or *Tenebrio* can be sent in vials containing limited amounts of food. Larger arthropods (e.g., scorpions) and many reptiles will tolerate several days without food or water. Most freshwater and marine organisms (adults or larvae) can be shipped live in sealed plastic bags.

Packages containing live specimens should be well padded, slightly humid and insulated with Styrofoam against external temperature fluctuations. Live field-collected invertebrates may also be transported as airline hold or carry-on baggage, again subject to the necessary export and import permits for live animals and the airline regulations.

Algal (and occasionally fungal) samples are usually provided from culture collections as liquid- or agar-grown cultures and may require specific light or temperature requirements during shipping. Although most phototrophic algae can survive several days without light, a prolonged delay in shipping time may be fatal, as may exposure to extreme temperatures.

Prompt treatment of organisms is required on receipt of the shipment. Specimens obtained for rearing should be placed on rearing media, whereas those obtained for DNA isolation should be immediately frozen at or below –20°C.

2. *Fresh plant samples.* Fresh plant tissue may be transported in two manners. For short transport duration (2–3 days, e.g., when acquiring samples from botanical gardens), leaf tissue or shoot cuttings in Ziploc bags can be carried at ambient temperature in insulated Styrofoam containers or mailed directly via overnight or express mail couriers, with or without Styrofoam containment.

A more reliable and expensive transport method involves placing the bags of tissue into an ice source inside a Styrofoam container. Wet ice may be used, but will melt and soak the package after a few days. Alternatively, a small amount of dry ice, wrapped in canvas, may be placed with the wet ice, to prevent the latter from melting. To avoid cold temperature burn, the Ziploc bags should be separated from the ice by means of newspaper.

Fresh tissue transport from remote locations requires phytosanitary, export and import permits. Tissue may be carried on wet ice in Styrofoam containers as airline hold or carry-on baggage [2]. Small packages require specific documentation to pass customs and agricultural inspection points in both shipping and receiving countries. Using airlines to ship packages is ill-advised, since many airlines refuse to handle a package originating abroad unless it has cleared

customs, thus requiring a contact person. A copy of the USDA permit, phytosanitary certificate and letter identifying and describing the purpose of the plant tissue should be attached inside the package and outside with the shipping label (see below).

3. *Frozen samples.* Vertebrate tissue samples (e.g., blood and muscle) are routinely transported frozen. Transport of frozen tissue on dry ice or in liquid nitrogen is simple when air shipment is not required. However, authorisation for shipment of these chemicals on airlines is mandatory, since both are classified as "Dangerous Goods" [31]. Policies for packing, labelling and carrying such materials have been promulgated by the International Air Transport Association (IATA) in their *Dangerous Goods Regulations* manual, and general guidelines are provided on their website (Appendix 2). Several courier services have instituted similar policies for the transportation of dangerous goods, e.g. the FX 12 Operator Variation of Federal Express.

Airlines permit no more than 200 kg of dry ice in a single container [3, 4]. The "Shipper's Declaration of Dangerous Goods" is required and the package must be marked as Hazard Class 9 (solid carbon dioxide). Handwritten declarations are not accepted; forms must be typewritten or computer-generated (refer to Appendix 1 for the website of Saf-T-Pak, which provides free online software for computer-generated Shipper's Declaration forms). Since cargo facilities may require pound equivalents, both metric and English equivalents should be used for packages. The special black-and-white sticker is a prerequisite for acceptance as air cargo.

Airlines permit up to 50 kg of liquid nitrogen to be transported in a nonpressurised Dewar container, which must also be marked as hazardous (compressed gas). The Shipper's Declaration is again required and the package must be clearly labelled "NONPRESSURISED LIQUID NITROGEN", "THIS SIDE UP" and "DO NOT DROP – HANDLE WITH CARE" to prevent careless handling and spillage. It may also require a green label stating "NONFLAMMABLE GAS". Alternatively, the liquid nitrogen can be discarded before short flights and "Dry Shipper" (e.g., the Arctic Express Thermolyne CY 50915/50905 available from VWR Scientific Products, San Francisco, CA) used, thereby avoiding liquid nitrogen spillage. This is preferable for cryogenic collections because of the ease with which samples can be transported by air.

Sperm can be shipped on wet ice if it is collected undiluted in water and sodium azide has been added to a final concentration of 0.01–0.02%, in order to prevent bacterial growth [14]. For further guidelines on the transportation of frozen tissues, consult sections E1565 and E1566 in Volume 11.05 of the *Annual Book of ASTM Standards* of the American Society for Testing and Materials (West Conshohocken, PA) (Appendix 1).

4. *Desiccated samples.* Cultures of some fungi are stored lyophilised and can be shipped immediately on order [93]. Dried samples of skin, hair, feathers, muscle and blood from vertebrates may be placed in tubes or envelopes (in the case of blood spotted on paper) and mailed in regular mail [45]. Dried insects and dried plant tissues can be similarly transported: individual samples should be care-

fully wrapped in paper towel to prevent damage and placed inside vials or Ziploc bags. A small amount of desiccant (e. g., silica gel) should be included in the vials or bags. In the case of plant samples, this should be in contact with the leaf tissue. Sabrosky [152] and Piegler [153] provide guidelines for the packing and shipping of pinned insects. Desiccated samples must be shipped in airtight containers to prevent rehydration and indicator silica gel is recommended to verify that the samples have not rehydrated [2].

5. *Fluid-preserved samples.* The most convenient method for transporting tissue samples of most invertebrates, vertebrates and marine algae, is preserved in 70–100% ethanol. However, since ethanol is flammable, highly volatile and a strong solvent (especially at higher concentrations), the use of unbreakable, leak-proof and ethanol-resistant plastic vials is a prerequisite to avoid evaporation or spillage. Sealing the vials inside thick, ethanol-resistant plastic sleeves is advised as a further precaution. Similar precautions against leakage should be ensured when transporting samples stored in other fluids. For further details, refer to McCoy [154].

Protocol 5 Packing and labelling for transportation

1. *Packing.* Sturdy, internally padded packaging should be used when shipping specimens or tissue samples. All external labels should be clearly marked with permanent ink and covered with a plastic sheet. A list of materials included, with relevant names, addresses and telephone numbers should be enclosed for the benefit of customs officials and to help recipients determine if the package was tampered with.
2. *Labelling.* When pertinent, the outside of the package may be marked "BIOMEDICAL SAMPLES FOR SCIENTIFIC RESEARCH – PLEASE RUSH", though for most packages, the words "SCIENTIFIC SPECIMENS FOR RESEARCH" will suffice. Customs forms should clearly indicate that the contents have "NO COMMERCIAL VALUE".
3. *Shipping.* Before shipping, ensure that holidays (civic and religious) will not impede or delay the delivery of packages. It is also prudent to avoid shipping over the weekend. These considerations are especially critical for shipments of live or frozen samples. Parcels of live samples should not be sent during periods of extremely hot or cold weather. Recipients should be called, faxed or contacted via electronic mail on the date of dispatch and notified of the expected arrival of the package and the airbill or tracking number. Recipients should be informed as to whether they can expect delivery or should collect their samples, and are advised to bring multiple copies of any permits or documentation necessary for collection. If transporting across international boundaries, colleagues on both sides should be intimately involved in the process of shipment.

5.2 Long-term Storage

Frozen tissues

Long-term storage conditions should minimise variation in temperature, light and liquid volume. Therefore, most formal frozen tissue repositories maintain samples at –130–150°C in ultracold freezers or in the vapour phase of liquid nitrogen [155, 156]. Nonetheless, most plant, fungal and animal tissues will remain indefinitely stable for extraction of nucleic acids and proteins if maintained at –70–80°C. Furthermore, although long term storage at or below –80°C is the preferred method for animal samples (e. g., [65]), there are alternatives (Protocol 6).

For short-term storage, frozen algal samples can be kept in a standard freezer at –20°C, in a mixture of glycol and dry ice (~ –78°C) or in liquid nitrogen (–196°C). For long-term storage, algal viability is more likely to be maintained at or below –40°C. Routine, periodic monitoring of viability is essential for long-term storage of such samples.

Plant tissue samples have been stored at –20°C, after quick freezing at –70°C, with apparently no DNA degradation after 6–8 months [2]. However, marked degradation has also been observed when leaf tissue was stored at –20°C, compared with –70°C (no difference in DNA quality has been detected between tissue quick frozen in liquid nitrogen before being placed in the ultracold freezer relative to tissue simply placed in the freezer).

Storage at lower temperature is thus recommended and is essential if the tissue is to be used for isozyme analysis. Storage of tissues for RNA preparations, which require fresh or –70°C frozen tissues, similarly necessitates cryogenic conditions for successful nuclease inhibition. When tissues are stored for such purposes, –70°C freezers should be equipped with alarms and backup generators and temperatures should be checked on a regular basis, especially after storms or building maintenance [97]. Protocol 6 discusses additional considerations for the storage of frozen tissues.

If facilities for long-term storage of frozen samples are unavailable, investigators should consider depositing their samples in established frozen tissue collections upon completion of projects, for which guidelines for submission of samples are provided below. Further advice on cryopreservation, sources of the chemicals and the organisation of frozen tissue collections is provided by Dessauer et al. [3, 4], Simione [30], Baker and Hafner [97] and sections E1342, E1564, E1565 and E1566 in Volume 11.05 of the *Annual Book of ASTM Standards* (Appendix 1).

DNA

An alternative approach to tissue storage is to minimise or even eliminate storage in ultracold freezers and store only the DNA permanently (Protocol 6). Tissues are placed in the refrigerator immediately and DNA isolated as soon as possible. This is the preferred method in studies where fast DNA extraction