Chapter 7

Organizing specimen and tissue preservation in the field for subsequent molecular analyses

by

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Abstract

During the last decades DNA-based methods have revolutionized almost all areas of biological research. While DNA isolation techniques are continuously being improved, the impact and importance of adequate pre-DNA-isolation treatment are still largely underestimated. In the present review, we present some guidelines on how to organize specimen and tissue preservation in the field for optimized subsequent molecular analyses. Recommendations are given on how to set up a collection plan and sampling strategy, how to gather information on the environment, habitat and taxa to be collected, and how to deal with legal issues. Furthermore, we review currently used field tissue storage methods and their efficiency for different types of samples and organisms, taking into account the available resources and the intended use of the sampled material. We also make suggestions about logistics, precautions, and safety as well as on how to carry out field-work and how to prevent contamination. When collecting specimens (vouchers) and parts of specimens (DNA, tissue) both the short-term and long-term preservation of the samples and their subsequent storage in natural history collections must be guaranteed. Checklists of documentation essentials and equipment for collection trips are appended.

Key words: DNA, collection, silica gel, documentation, storage

1. Introduction

During the last decade, DNA-based analyses have radically influenced nearly all areas of biological research and most strongly influenced our understanding of evolutionary mechanisms, population dynamics, phylogenetic relationships, and systematics. While DNA isolation techniques are continuously being improved and standardized during the past few years, related protocols of voucher compilation, issues of documentation and tissue collection prior to DNA isolation have widely been neglected. The aim of the present chapter is to give some guidelines for streamlining, optimizing and standardizing pre-DNA treatments of sampled specimens.

Streamlining will become increasingly important, as DNA-based analyses have not only become an essential part of fundamental research but also hold the potential for fast, standardized and cheap species identification and comparison for rapid biodiversity assessments.

Even conservative guesstimates stress that the vast majority of the Earth's biodiversity is still unknown and undescribed. Knowledge of species diversity and sufficient capacity for its rapid assessment are crucial for tackling numerous research questions, including the impact of global change and conservation considerations. It has often been postulated that global warming will lead to massive waves of species declines and extinctions. Yet, for the most diverse groups of organisms the extent of such changes will remain speculative as no baseline data on current diversity are available. The importance of species diversity for ecosystem services and function may be paramount but at present can only be addressed for a restricted set of model organisms, or by subsuming several species as "functional groups". In evolutionary biology, patterns and mechanisms of species-rich adaptive radiations will only be understood once complete inventories of the radiations have been made. However, our knowledge about the true extent of biodiversity will stay fragmentary unless traditional methods for organism identification and description are complemented by more sophisticated techniques to allow increased speed and capacity.

To help accelerate and standardize species inventories by means of mechanical or electronic systems, new methods like DNA barcoding, DNA taxonomy, and e-taxonomy have been proposed. DNA barcoding, first suggested by Hebert *et al.* (2003) involves the comparison of a short pre-defined stretch of the DNA of unknown organisms to a database of sequences from the same DNA region from verified reference specimens for identification (for recent reviews see Hajibabaei *et al.*, 2007; Valentini *et al.*, 2009; Fazekas *et al.,* 2009; Chase & Fay, 2009; for plants, see also CBOL Plant Working Group, 2009). The method allows for fast, cheap, standardized, automated species identification and has the potential to flag new and undescribed species. Attempts to establish DNA barcoding for all organisms on a highly coordinated world-wide scale are in progress (*e.g.*, CBOL (http://www.barcoding.si.edu/), BOLD (http://www.barcodinglife.org)). Once the techniques are firmly established and become a routine application, they will assist and greatly accelerate biodiversity assessments and species inventories. However, standardized procedures are required.

Historical background

First protocols for plant DNA isolation from small tissue samples became established in the 1980s (*e.g.*, Dellaporta *et al.,* 1983; Rogers & Bendich, 1985; Doyle & Doyle 1990) and have been improved since for different groups of organisms and numerous applications. By now, hundreds of DNA isolation protocols can be found in the literature, many of which merely represent slight modifications of existing standard procedures (see Weising *et al.*, 2005 for an extensive survey of plant DNA extraction methods). Furthermore, various commercial DNA isolation kits are now on the market and manufacturers proclaim rapid and efficient isolation of genomic DNA with high yield. However, the impact of adequate pre-DNA-isolation treatment is most often underestimated; even though the state of knowledge has progressed in this area. For example, we know now that treating the sampled tissues with certain fixatives (*e.g.* alcohol, formalin) or poisons (*e.g.* mercurichloride, arsenic) can greatly decrease the success rates of subsequent molecular studies, while novel DNA-protecting/preserving measures are available that make use of, *e.g.*, inert beads and trehalose. Furthermore, next-generation sequencing technologies enable us to perform “environmental” or mixed-sample sequencing, with a strong impact on current collection strategies, but not always for the global good (some “second-generation” procedures are tolerant of sheared or small DNA fragments).

For earlier work on specimen collection and tissue preservation strategies for molecular projects and biorepository issues, the reader should also refer to the excellent reviews of Dessauer & Hafner (1984), Simione (1992), Guarino *et al.* (1995), Dessauer *et al.* (1996), Prendini *et al*. (2002), Hanner & Gregory (2007) and ISBER (2008).

2. Before you go - pre-expedition preparations

Before embarking on a collection trip one has to

- set up an adequate collection plan/sampling strategy and organize the logistics
- gather ample information on the environment, habitat and taxa to be collected from the literature and other sources
- find out if collection permits are required (also consider permits for transport, export and import) and obtain permit(s) (see *e.g.* Convention on Biological Diversity, www.cbd.int; Anonymous, 2002 and CITES, www.cites.org). Each country may have its own legislation!
- determine and test the most suitable field tissue storage method for the samples. "Optimal" and "best" is not always the same (*e.g.* in remote areas with limited labour liquid nitrogen tanks are not feasible)
- prepare your collecting protocol

2.1. Collection plan/Sampling strategy

The optimal strategy to collect biological specimens for molecular analyses is mainly determined by the aims of the particular project. However, the decision of how many individuals and populations should ideally be sampled also depends on logistical issues such as financial support, team size, and the locally available resources. The optimal sampling strategy is often a compromise between scientific needs and financial constraints. Nature conservation issues may become a limiting factor as well. To reduce possible negative impacts on any wild plant, animal or fungal populations, sampling designs should be clearly defined and analysed prior to field collecting.

The ideal scenario to capture the maximum genetic diversity of a species under investigation would include sampling of as many individuals as possible over an area as wide as possible, without endangering the species or population (Groves 2003; Neel & Cummings 2003). For reasons outlined above, this is most often not possible or even desirable, especially as the molecular technique to be used will often limit the amount of genetic screening that is possible. Nevertheless, more than one individual per taxon should definitely be sampled even for phylogenetic studies, because a single individual does not represent the genetic diversity of a species or population (though it is preferable to none for some studies). Genetic diversity depends on inherent aspects, such as breeding system and population size, but it is also conditioned by biotic and abiotic factors of the environment. The differences in environmental conditions at different geographic locations are likely to impose different selection pressures on populations and thereby promote genetic differentiation. The availability of only a single individual per taxon will also limit the opportunities to discover problems associated with misidentifications, cryptic species, or related issues.

Recommendations given in the literature on how to collect plant genetic resources mainly deal with crop species and their wild relatives (*e.g.*, Marshall & Brown 1975; Guarino *et al.,* 1995; Singh *et al.,* 2006). For population level analyses, Marshall & Brown (1975) proposed the capture of at least one copy of 95% of all alleles that occur at frequencies greater than 5% in the target population. To achieve this, the authors estimated that the minimum number of randomly chosen individuals per population to be sampled should be 30 (outbreeders) or 59 (inbreeders) while the Center for Plant Conservation in the USA recommends the sampling of between 10-50 plants per population (Falk & Holsinger, 1991). Singh *et al.* (2006) stressed that between 5 and 12 samples for some wild wheat species would be needed to obtain a standard error equal to 10% of the diversity in the population of the species. However, the exact value depends on the species. So far, published recommendations are based on the investigations of only a few species and no generalities can be proposed. Knowledge is especially scarce in tropical regions, where data about animal or plant population structure are rarely available!

To capture the genetic diversity within a species, the more information that collectors have at hand, the better is their decision-making with regard to sampling. However, collectors are increasingly working against a background of rapid population loss and relatively meagre resources. Therefore, in the absence

of better advice, a good start would be to sample individuals from five populations from across the geographical range of the taxon (see Falk & Holsinger, 1991). Obviously, the fewer individuals or populations sampled, the less genetic diversity is likely to be captured. Selection of individuals or populations to be collected should follow economic (distance from base, and time for collection) as well as eco-geographical criteria. In essence, there is no problem in collecting within a population until an obvious barrier to genetic exchange (likely to lead to genetic isolation) is encountered. It would then be advisable to keep samples from either side of this barrier separate. However, care should be taken in regions where past barriers, *e.g.* glaciation, can easily be overlooked. This could lead to an underestimation of the present-day genetic diversity, when only one population from a restricted area is collected. One has also to consider that some species occur in fragmented habitats, like forests (at least in Europe), rivers, moors etc. In plants, the nature of barriers will depend on the pollen and fruit/seed dispersal strategy of the species - in animals it is dependent on means of migration and availability of past and extant migration routes. Most of the dispersal will usually be local. As a practical approach, and when there is insufficient information on dispersal of the targeted species, the boundary between two adjacent populations could be arbitrarily established as the absence of individuals between them over a certain distance. However, one has to keep in mind that there will be considerable differences between species in this respect. With sufficient sampling and geo-referencing of all samples, the data can help to determine, post-facto, where interesting groupings occur, and thereby help to direct future sampling efforts.

Collectors also need to gather information on other biological characteristics of the targeted species. In some cases knowledge may already exist about intraspecific morphological variation, breeding system, ecological specialisation and distribution patterns, and assumptions can then be made about patterns of gene flow and the numbers of individuals and populations that should be sampled. For instance, outcrossing, wind-pollinated woody perennial plant species usually have a high proportion of their gene diversity within populations. Consequently, fewer populations may have to be sampled from these species as compared with, *e.g.*, selfing annuals where a high proportion of the total gene diversity is usually found between populations (Hamrick *et al.*, 1995). Similarly, highly fragmented distribution patterns are often indicators for high levels of genetic differentiation between the isolated populations. In general, one should always attempt to collect the broad diversity of a species or population. For large populations in a uniform landscape, it may be advisable to sample at regular intervals along transects.

Before embarking on a field trip, a **collecting protocol** should be set up to ensure that all collectors or collector teams will sample with comparable efforts, independent of time and location. This becomes critically important if collection efforts and/or occurrence data need to be quantified. The data should be recorded in a way that is as objective as possible and will be easy to comprehend several decades from now. Full documentation allowing for verification and re-sampling of the material is a crucial requirement for any collection (for examples see Table 1).

Working protocols must include the locality – this is the singular most important information, without it any other information is of lesser value. Protocols should also include columns for taxon-specific information (see also chapters on taxa oriented methods further in this manual), for DNA data that will be entered at a later stage (see Table 1), and for other kinds of annotations. In some cases (especially in tropical countries) it may be necessary to collect duplicate vouchers because local authorities may request one set of specimens to be kept in the country of origin.

In summary:

- Before planning a collection trip one should bear in mind the questions being asked, the budget that is available for the project, the rarity of the species to be collected, and the ease/likelihood of future collecting opportunities.
- A well-defined sampling strategy has to be set up prior to the collection trip. Most importantly, it must be estimated how many populations/individuals need to be sampled to capture the inherent genetic diversity. In addition, the ultimate uses of the samples beyond the immediate project aim need to be considered. Not all specimens collected need to be analyzed immediately, but an important factor is the cost associated with long-term storage.
- A collection protocol needs to be established prior to going into the field and changes need to be annotated as necessary.

Data	**Example**	**Comments**	**Obligatory**
Name of Expedition	"Greece, Kykades 14.-20.6.2009"		Yes
Country	"Canada"		Yes
Date	"15.10.2000"		Yes
Coordinates	"40° 22' 5"N 44° 2' 49"E"	as precise as possible	Yes
Location	"Vayots Dzor province, mainroad to south Armenia, W of Yeghegnadzor, SE of crossroad to Erechgnadzor"		Yes
Location description	"slope S of river", "pine forest", "fresh water lake"	precise information is helpful	Yes
Altitude	"1050 m s.m."	sometimes GPS is not very accurate - indicate this	Yes
Collector/ collection team	"Ch. Brown"	indicate if you collected in a team	Yes
Collection strategy	"Plot sampling of 12 individuals per population", "transect along an east-west	be as precise as possible	Yes

	gradient of xxx km/miles", "all catches of trap 10 between 11-12 pm on 6th of June 2009",		
Tissue ID	B GT 0003256	One unique identifier or code for each individual tissue. For population samples combine a unique identifier of the locality with one of the taxon	Yes
Tissue type	"Leaf", "Root", "Seed", "Leg", "Toe", "Blood"	Indicate if mixed tissue types have been collected, if possible contamination/ symbiosis/infection has been detected and special post collection treatments needs to be carried out	Yes
Relation tissue to voucher	"tissue and voucher from the same in situ individual", "tissue and voucher from the same in situ population"		Yes
Pre-preservation	"Anaesthesia", "Fixatives",	Chemicals used prior to tissue preservation	Optional
Tissue preservation	"Silica gel", "Alcohol", "Air dried", "Lyophylised"	The preservation/fixation of the tissue material	Yes
Transportation	"cooled throughout", "continuously dry", "evaporated during transport"	Rapid climatic changes support DNA degradation and might necessitate different laboratory treatments (*e.g.* usage of DNA repair kits)	Optional
Place of tissue deposit	Botanic Garden and Botanical Museum Berlin-Dahlem/D		Yes
Place of voucher deposit	Royal Botanic Gardens, Kew/GB		Yes
Notes	"female/male", "heavily grazed meadow"	Additional information of potential interest	Yes

Table 1. Example of a data collection sheet for DNA specific documentation.

2.2. Gathering information on the taxon to be collected

Local and regional floral and faunal listings, checklists, monographs, and databases are useful references in order to find detailed descriptions and information on where potentially to find and how to differentiate between related taxa. Further platforms that should be screened are the EDIT specimen and observation explorer for taxonomists (http://search.biocase.org/edit/) as well as the websites of the Global Biodiversity Information Facility (http://www.gbif.org). Flora Europaea (now available on CD) is the primary reference for the European flora. Euro+Med PlantBase (http://www.euromed.org.uk/) as well as the website at the Royal Botanic Gardens, Kew (http://www.kew.org), and Index Herbariorum (http://sciweb.nybg.org/science2/IndexHerbariorum.asp) are also useful sources of information. A detailed compilation of genera and families of flowering plants is provided by Kubitzki *et al.* (from 1990). The Guide to Standard Floras of the World (Frodin, 2001) gives an overview of available floras all over the World. For animals there might be similar literature available and proper homework should be done in advance.

Specific instructions on where, when, and how to get the specific organisms of interest can be obtained from experts or from people being familiar with the localities and/or the taxa. Precise information about localities where a particular taxon can be found may also be obtained from genetic resource centres, natural history associations, governmental agencies, species monitoring projects (for rarities), eco-geographic surveys (occasionally available), inventories (national and local), natural history collections (which give a historical perspective of the distribution), chorological accounts in botanical and zoological journals and distribution maps in revisions. However, often data might need to be verified from a number of sources especially when they are old. Sometimes there is a large variation between species as to what is known about their geographical distribution and their known populations. Local botanists and zoologists as well as ecologists might have a more detailed knowledge and may also be able to assist. Frequently, collections are made in an opportunistic way at a particular (perhaps remote) site, and more than one taxon is sampled. However, one should always know what to expect and what not to expect (*e.g.* species assemblages, phenology) when collecting with a specific method in a specific habitat at a specific time.

Information on putative diseases or pests that might infect the targeted species may also be useful. Collection time has to be kept in mind as well; flowering time or breeding season may differ within or between species and this can affect the sampling strategy. The same is true for animals with strong seasonal activity (*e.g.* many insects) – which only emerge as imago at a certain season each year.

In summary:

- Get ample information about your taxon prior to going out in the field to optimize collection success.

2.3. Collecting with permission

Collecting organisms - be they plants, animals or microorganisms in soil or water samples - must be in accordance with national and international legal aspects. Unauthorised collection can damage populations of native species, leading to potentially adverse effects and may have serious legal consequences.

Be aware that in several countries you need to apply for permissions (including collecting, export, CITES, and import permits, phytosanitary certificates containing the identification and description of the purpose of the tissue, etc.) several months before you go out in the field. Plan a minimum of 6 months ahead. Often cooperation with local scientists is mandatory to receive permissions - so try to establish contacts well in advance. Here the Index Herbariorum may be a good guide to localize botanists (http://sciweb.nybg.org/science2/IndexHerbariorum.asp). In some countries you must visit official authorities personally prior to going out in the field. Consider this in your schedule.

The following aspects should be considered in the context of doing legal collections:

- Information about how to collect legally can be obtained from institutes and/or official administrations. To find the relevant addresses in your country of interest contact the national CBD focal points (http://www.cbd.int/countries/).
- Permission from the land-owner/manager of the site/national park authorities and, in the case of protected species, the relevant government authority must be obtained.
- Permissions (permits) should preferably be in your hands before starting your travel.
- Permission should cover voucher specimens, tissue material and DNA of as broad a range of species (including those targeted) as possible.
- Obtaining permission can take a long time. General collection permits are often much more difficult to obtain than specific ones.
- Check whether target species are listed in international agreements or directives that give them special status. Of particular note are:
  - ✓ CITES (http://www.cites.org)
  - ✓ European Council Regulation (EC) No. 338/97 incl. Annexes http://ec.europa.eu/environment/cites/legis_wildlife_en.htm).
  - ✓ The Bern Convention (http://www.ecnc.nl/doc/europe/legislat/bernconv.html).
  - ✓ Habitat Directive (http://europa.eu.int/comm/environment/nature_conservation/eu_nature_legislation/habitats_directive/index_en.htm).

Contact the national custom authorities if specimens are to be moved between the EU and other countries. You will probably need both, an export permit from the country of origin and an import permit from your own country or place of residence.

In summary:

- Do not collect without permissions (collecting, exporting, importing, transporting).
- Plan well ahead as it is often time consuming to get the necessary permits.
- Contact local scientists with knowledge of and experience with the national or local administrative organisations. In some countries such contacts are mandatory.

2.4. Methodological considerations

Specific methodologies exist for sampling environmental, soil, bacterial, fungal, and algal specimens, and we will not focus on those here as most world-wide collection efforts tend to focus on major plant and animal groups.

2.4.1. Tissue collecting for plants

The best tissue for DNA analyses of plants is a piece of leaf, either from leaf buds or very young leaves as they feature many cells with high DNA content. Be aware that this does not account for the surrounding bud scales which are often lignified and may contain high amounts of secondary compounds for protection against predators. If no buds are available then tissue material of young leaves should be collected. For plants with pruinose or hairy leaves the surface (epidermis) sometimes has to be removed (*e.g.* Boraginaceae) as the silica on the leaf surfaces interacts with many DNA isolation kits (silica binds the DNA). Hard leathery leaves with few stomata as well as succulent leaves will not dry properly in silica gel as the stomata close after removal from the plant and the DNA in the mesophyll will be degrading fast during the slow tissue drying process. This often is the case for tropical or Mediterranean plants. In such cases, the leaves have to be cut into small stripes or pieces before their preservation in silica gel to ensure fast drying processes throughout the DNA containing tissue.

If leaves are soft and juicy or even succulent the DNA content per square centimetre is low in comparison to the vacuole content. This often causes low yields in DNA extraction. If this is the case, either larger amounts of leaf material have to be collected - then the tissue has to be dried fast since tissue with high water content is subject to fast degradation - or other plant parts should be considered for collection. Sepals and petals of the flowers as well as fruits feature larger cells with anthocyans, carotinoids, flavons, flavonols or other secondary compounds in the vacuole or the chromoplasts, and/or starch and sugar in the amyloplasts. Using these tissues for DNA extraction will usually provide less DNA per cubic centimetre due to the enlarged cells, and purifying

the DNA might be more problematic as compared with leaf material. Nevertheless, Lin & Ritland (1995) reported high yields and good PCR amplification of DNA preparations from petals of several species. Thus, it may be worth trying petals as an alternative source of DNA. Alternatively, soft and juicy or succulent leaf parts may be directly put into saturated NaCl-CTAB buffer in the field (Rogstad, 1992). Pollen has rarely been used for DNA isolation (*e.g.* Simel *et al.,* 1997) though featuring only a haploid chromosome set which is sometimes advantageous for subsequent analyses.

Seeds are the life preservation stage for plants. In seeds, DNA is usually well-preserved in the long term, but accessibility may be difficult if seeds are surrounded by a large endosperm. Several groups have reported successful DNA isolation from seeds of various plant species (*e.g.* Wang *et al.,* 1993; Krishna & Jawali 1997; Kang *et al.,* 1998; von Post *et al.*, 2003). For large seeds, the endosperm should be removed prior to DNA extraction and only the embryo (which contains high amounts of DNA) should be used for isolation. For larger DNA yields it may be desirable to germinate seeds prior to DNA extraction; however, attention has to be paid to potential fungal contamination. This can, to some extent, be prevented by washing the seeds in a hypochlorite-solution prior to germination on sterilized media. Each seed represents a single individual; therefore, seeds should not be pooled prior to DNA extraction as this than presents a multi-individual community.

In general it is not recommended to collect lignified plant material for DNA extraction as lignin also hampers extraction efficiency. DNA isolation from wood is principally possible (*e.g.*, Dumolin-Lapègue *et al.*, 1999; Deguilloux *et al.*, 2002), and some DNA isolation kits are especially designed for wood and lignified plant material. However, these DNA extractions can often be tricky, requiring large amounts of primary material and usually resulting in low yields. If only lignified stem material is available, or routine collection of leaf material is difficult, it is recommended to scratch off the bark and collect the cambium. For example, we obtained good yields of well-amplifiable DNA from cambium and cortical tissues of *Macaranga* trees from SE Asia (Weising, unpublished results). Thorns and spines should not be used for DNA extraction as the DNA content is usually too low. If you collect freshwater or marine plants, be very careful to remove epiphytes which often cover the leaf surfaces. As most plant species are associated to mycorrhizal fungi and some to rhizobia it is also not recommended to collect roots as DNA samples for plants. Try to avoid tissue that might be host to parasites (*e.g.* mildew) or other potential contaminants. If specific PCR primers are used for subsequent analyses such material can still be appropriate. However, potentially contaminated material should neither be used for restriction fragment analyses nor for any PCR assays with unspecific, arbitrary primers.

2.4.2. Tissue collecting for animals

Vertebrate DNA can be obtained from blood, and from a large variety of other tissues, including muscle, heart, liver, kidney, testes, bone, nail, embryonic tissue from placentas or eggs, pulp of feathers, skin and hair follicles. The mitochondrial DNA (mtDNA) can even be obtained from single hair shafts (Wilson *et al.*, 1995;

Gilbert *et al.*, 2007). Non-invasive samples (Smith & Wayne, 1996) such as hair, feather, foot pads, buccal or skin cell (swabs), faeces, urine, moulted skins, fish scales or fin clippings can all be useful for molecular genetic analysis, but a wide variety of problems can be encountered and appropriate solutions have to be found with such kind of material (for a recent review see Beja-Pereira *et al.*, 2009). However, invertebrate (terrestrial as well as aquatic) diversity is so great that generalities about tissue and extraction methods are very difficult to make. For minute organisms, more than one specimen can often constitute the tissue sample. For larger organisms various body parts can be selected, including legs, abdomen, feet, muscle biopsy etc. Care should be taken to avoid known problematic tissues, *e.g.,* tissues rich in muco-polysaccharides, "slime" and hardened exoskeletons, guts and associated gut contents.

For sampling in micromammals, the Institutional Animal Care and Use Committee (IACUC) recommends ear punch, toe clipping and tail clipping. The ear punch method involves punching a hole or making a notch in the ear. Ear punch samples collected on animals do not require the use of anaesthesia or analgesics, but the ear punch must be disinfected between animals. Toe clipping involves removal of the distal phalange bone of one or more limbs. Tail clipping involves amputating a minute portion of the distal tail. A pair of sterile sharp scissors or scalpel can be used for this procedure and must be disinfected in between uses. After taking the sample, it should be either frozen or transferred to a sterile vial containing a minimum of 70% alcohol, or DMSO/EDTA/salt buffer (Seutin *et al.*, 1991).

For sampling in amphibians and reptiles we refer to chapter 20.

Bird blood can be collected from the jugular vein (right side of the bird's neck), brachial/ulnar vein (wing vein) or medial metatarsal vein (leg vein) using a hypodermic needle or butterfly needle, and a syringe, depending on the size of the bird and the amount of blood to be collected. In general, it is safe to collect 0.3-0.6 ml of blood per 100 g of body mass from living birds. However, it is always advisable to collect the minimum amount of blood necessary for the investigation. For some investigations blood spots (FTA, see chapter 1.5) are sufficient. The blood should immediately be transferred from the syringe to a sterile vial containing EDTA solution (*e.g.* purple top) and this should promptly be refrigerated then frozen when possible. More information on sampling in birds is available in chapter 21 of this manual.

Fish in the field are best euthanized in tricaine methane sulphonate. Care should be taken to avoid changes to acidic pH at high concentrations of the solute (see protocols on tricaine use, Alpharma Animal Health (2001) and Brown (2003)). Fish are frequently sampled using muscle biopsy from the right side of the body (left side preserved intact for photodocumentation and morphological examination whenever possible), right eye removal, right side pectoral fin clips and occasionally gill material. Very small larvae and juveniles are sometimes dissected in half, with the caudal end being sacrificed for molecular analyses. Tissue explants can either be held dry in sealed vials along with, but separated from a moist tissue pad at 0-4^{o}C underneath melting ice, or in cryoprotectant solution (L-15 medium, 10% Fetal Bovine Serum (FBS),125 mM sucrose, 10%

DMSO, for further information see Moritz & Labbe 2008). More information on sampling in fish is available in chapter 22 of this manual.

Tissues that might contain parasites or other potential contaminants, such as gut contents, should be avoided whenever possible. One can get around contamination problems with specific PCR primers, but when generic fish primers are used on fish samples that included gut contents from fish that eat other fish, it is entirely possible to get both the predator and the prey amplified.

2.5. Tissue/DNA collection techniques

➔ Remember that when collecting tissue/DNA from voucher specimens.

Water (at any temperature) and temperature (depending on moisture) cause the highest DNA degradation. *Warm and moist is bad. Cold and dry is good.*

Certain analysis techniques demand certain collecting techniques.

Certain collecting techniques are advantageous under certain climatic conditions.

For optimal results, it is recommended to use one of the following four strategies of tissue and DNA preservation in the field:

- **Freezing**
- **Fast drying**
- **Storage in liquid media**
- **DNA isolation in the field**

The "gold standard" is to immerse all specimens/tissues immediately into vapor-phase liquid nitrogen (VPLN) upon collecting using dry-shippers or cryotanks in the field. Everything short of this represents some sort of compromise. It should be noted that freezing can decrease yields of mitochondrial DNA if this is the focal point – but if genomic DNA with just some mtDNA is desired, freezing will produce adequate quantities. Many of the compromises described below are either necessary or acceptable, or both. Depending on how many compromises are introduced, the samples might not subsequently be amenable to protein, RNA, genomic or other studies. Ancient DNA studies frequently take advantage of samples that have undergone significant degradation, yet are still amenable to *e.g.* mtDNA analysis. Taking large liquid nitrogen tanks or dry ice to the field could result in logistical obstacles that could compromise the collection effort. If any of the alternative collection techniques described below would enable orders of magnitude more samples to be collected in perfect conditions for most mitochondrial, chloroplast and nuclear DNA work, then the choice about how to collect is obvious.

The rapid drying of plant tissues with desiccating agents was first suggested by Liston *et al.* (1990) and Chase & Hills (1991). At present, **silica gel** is the most common fast drying procedure for plant collecting (*e.g.* Cliquet & Jackson, 1997). It is especially recommended in temperate regions, for plants which are non-succulent, non-woody, with a non-waxy epidermis. The leaf material is collected in paper bags (preferably tea bags as these allow evaporation) along with at least 10 times the weight of dry silica gel. The silica gel must remain dry during the

whole storage process. It should be exchanged when the colour of the moisture indicator dye changes (2-3 times; approximately every 6-24 hours, depending on the tissue). Under humid conditions the use of screw-capped vials for storing the tea bags may be preferable as these effectively exclude external moisture.

Dried samples are easy to handle, require no cooling devices in the field, and can be stored for years at room temperature under desiccated conditions. Problems may arise if the drying process is not fast enough, *e.g.*, in xeromorphic plants with a fleshy mesophyll and a thick, leathery and highly cutinized and waxy epidermis. Such leaves tend to close their stomata after first contact with silica gel, considerably slowing down the drying process with a negative influence on the quality and quantity of DNA retrieval. Problems of this kind can be circumvented by cutting the leaf tissue into smaller pieces before placing it into the paper bag (see also point 2.4.1 above).

An alternative method of desiccation involves the crushing of the leaf tissue onto FTA paper, which is a commercial medium initially developed for long-term storage of blood spots. **FTA cards** are patented by Whatman to simplify the handling and processing of nucleic acids under ambient temperatures (Smith & Burgoyne, 2004) and are suitable for both plant and animal tissues. FTA cards facilitate sample collection in remote locations and simplify sample transportation. According to the manufacturer, virtually any type of organismic material can be used and a variety of configurations are available to meet specific requirements. We recommend using the FTA method for juicy tissue. The FTA card contains chemicals that lyse cells, denature proteins and protect nucleic acids from nucleases as well as from oxidative and UV damage. The released nucleic acids are entrapped in the fiber matrix and remain immobilized and stabilized for transport, immediate processing or long-term storage at ambient temperature. The amount of DNA that can be stored on an FTA card is limited. According to the manufacturers, FTA cards rapidly inactivate microorganisms, including blood-borne pathogens, and prevent the growth of bacteria. Up to now, 20 years of experience for DNA storage on FTA cards exist but the DNA elution efficiency has been much improved only recently. DNA released from FTA cards proved to be a suitable substrate for PCR-based methods (Mas *et al.*, 2007), whereas restriction enzyme applications were less satisfactory because of low yields (Gemeinholzer, unpublished information). Continuous efforts by the company to overcome this problem are in progress. Contamination is one of the biggest problems when using FTA cards, which have to be handled with special care. Special storage and transportation equipment is available from the manufacturer.

Another substance, not commonly used for fast drying specimens for scientific purposes yet but patented as desiccant, *e.g.* for drying of biomass, is **Zeolite**. It is a silicate made from equal parts of silicon tetroxide and aluminum tetroxide which might hold some potential for very rapid drying (Silva *et al.*, 2007). However, to date none of the authors of the present survey has any experience with Zeolite.

Tissue collection **in liquid** media can be advantageous if fast drying or freezing in the field is not possible. In plants, however, early work has shown that treatments with different types of organic solvents like ethanol, methanol, or

formaldehyde are unsuitable and result in DNA degradation after a few days (Doyle & Dickson, 1987; Pyle & Adams, 1989). Nevertheless, in later studies the successful use of 95% or absolute ethanol to preserve leaves from various plant species was reported (Murray Pitas, 1996; Flournoy *et al.,* 1996). For example, Flournoy *et al.* (1996) showed that leaf tissue of spinach, juniper and broccoli gave good yields of high molecular weight DNA after almost one year of storage in ethanol, provided that a proteinase was included in the DNA extraction buffer. Rogstad (1992) described the preservation of small pieces of leaf tissue in NaCl-saturated solutions of cetyltrimethylammonium bromide (CTAB) at room temperature. This technique has been used quite often since then, and has been effective in our hands for *Suaeda*, *Salicornia* and other genera with succulent species of the Chenopodiaceae, as well as Bromeliaceae. However, samples should be processed as soon as possible after returning to the laboratory, and a CTAB-based DNA isolation protocol (*e.g.*, Doyle & Doyle, 1987) should be applied. For animal tissue, storage in ethanol is most advisable. The higher the alcohol concentration the better, absolute alcohol is best. The ratio of ethanol to the sample volume should be about 3:1 (Seutin *et al.,* 1991; Presnell *et al.,* 1997). Alternatively, animal tissues can be stored in salt-EDTA-DMSO solutions that also hamper degradation processes, than however care has to be taken concerning subsequent DNA extraction as many kits are based upon silica (salt) binding membranes not determined for the salt buffer solution.

In some instances, logistical problems in the field, or problems with permits make it nearly impossible to transport tissues. To circumvent these problems, Nickrent (1994) suggested to prepare a raw extract using a standard CTAB DNA extraction buffer in the field, and to store the homogenized and filtered extract at ambient temperature until returning to the laboratory for completing the isolation procedure. A more recent alternative is the use of automated **field DNA extractions**. Several companies sell an instrument capable of taking a 96-well plate of digested tissues (for which you need a small incubator/shaker) and turning it into a plate of extracted, archivable DNA using magnetic beads in about 30 minutes – on a robotic platform that will fit into a case for many commercial airlines, weighs less than 45 kg, and runs on standard power.

If you extract DNA in the field or store tissue in vials, be aware that qualitative differences between different vial manufacturers exist. It is important to prevent evaporation, *e.g.* during exposition of the vials to low air pressure in planes. One should also be aware that cryo-tubes are designed for contraction during cooling processes, but might not be the best choice for short-term storage above room temperature.

In general, we strongly recommend testing the planned tissue preservation strategy and DNA extraction methods on your group of organisms well before going to the field. Especially in plants, DNA extraction can be tricky because of the frequent presence of diverse polysaccharides, polyphenols and other secondary compounds that may severely hamper molecular analyses (for a review, see Weising *et al.*, 2005). Optimize your technique before large scale collecting in remote areas. While considering the different options for tissue collection, also think beyond your own study to other potential uses of the material.

Silica gel is made of hygroscopic sodium silicate which is non-toxic, non-flammable, non-reactive and stable with ordinary usage; however, it might be irritating to the respiratory tract, may cause irritation of the digestive tract, and dust from the beads may irritate the skin and the eyes, so precautions for handling should be taken. Most often silica gel is pre-mixed with a visible indicator of the moisture content. Previously cobalt chloride ($CoCl_2$) was added, which causes the indicator to change from blue to pink when hydrated. Cobalt chloride is toxic and may be carcinogenic - only handle with gloves! Recently, the indicator has been substituted by the less dangerous ammonium iron sulphate ($NH_4Fe(SO_4)_2$) which causes the gel to change from orange (anhydrous) to colourless (hydrated). Crystalline silica powder or silica dust are colourless, have a higher hygroscopic capacity than silica gel and need to be mixed with some moisture indicator, too. Crystalline silica dust can cause silicosis and should only be used with face masks or handled under a laboratory hood or laminar flow. Once silica gel is saturated with water, the gel can be re-used after desiccation. This can be achieved by heating to 120°C (250°F) for two hours or even using a frying pan). It is not recommended to use a microwave oven as too high temperatures can lead to melting processes. If silica gel is to be reused, care must be taken to ensure that no fragments of previously dried tissues are carried over.

2.6. Logistics, precautions and safety

If possible, make a prior visit to the site to confirm the identity of the taxa to be sampled and to choose an adequate season for collecting. Such a pre-visit also provides an opportunity to collect additional voucher specimens in a different life stage, to study maps of the area and to set up a rough timetable for the collecting trip. Detailed climatic data and recent weather reports (particularly important when collecting in mountainous areas) are available from the internet for most places in the world. Check the area for accommodation and service stations, particularly in remote regions. Think through contingency plans in the event of an emergency and carry appropriate telephone numbers with you. Where telephone signal coverage is limited, radio communication may be necessary. Do not collect all by yourself in isolated areas. Local guides can provide invaluable help in finding your way and avoiding problems of any kind. Before you leave, give your itinerary to someone who will take care and appropriate action if regular pre-arranged contact is lost.

Check beforehand if electricity, gas, or any other equipment you need is available on the site.

Concerning safety in the field, literature on possible health hazards associated with the collection and handling of post-mortem zoological material does exist (Irvin *et al.*, 1972), also including a checklist of diseases mainly related to collecting samples from vertebrates. Be aware you might need vaccination (*e.g.* hepatitis), especially for collecting animal blood.

3. In the field

On arriving at a collecting site, it is important to first estimate the number, size and distribution of populations of the species under consideration. Any collection is started by filling out the general comments in the collection sheet (who, when, where). A GPS should be used for proper geo-referencing (see also chapter 4). Extra batteries should be brought along, and backups be done if electronic storage of data is the only record. Otherwise, GPS measurements should be recorded in a pocketbook.

Samples are usually identified by morphological characters, and photographed prior to tissue sampling and preparation of a voucher. If this is going to be time-consuming, then care must be taken to minimize degradation of the samples in the interim. DNA degradation starts immediately and the tissue sample designed for DNA analysis should be secured as fast as possible, *e.g.*, by adding silica gel. Be aware that voucher specimens can be very tolerant to conditions that the DNA is not. Depending on the storage process, several changes or iterations might be necessary. For example, the silica gel (for drying plant tissue) or ethanol (for conserving animal tissue) might need changing once or several times after the initial preservation (see also above).

At least one voucher specimen per population should be kept for reference, but frequently vouchers of all specimens turn out to be valuable or necessary. When morphological diversity within the population is large, several individuals should be sampled. Keep a record about which DNA sample is directly associated with the voucher. This cross link is very important for documentation purposes. In case DNA is taken from the same population but not from the actually vouchered individual, then this has to be noted down accordingly. Mixed collections are to be avoided. For some (especially large-sized) animals it is not permitted or otherwise impossible to make a voucher. In these cases, an e-voucher, *e.g.* a photo, should be prepared instead. As herbarium vouchers accompanying the DNA sample serve as evidence for the identification of a sample, they should ideally be taken from a fertile individual, displaying flowers or fruits. Characters that are likely to be lost after processing should be captured, *e.g.* by providing a description on the specimen label or by creating an e-voucher. These characters may include habit, flower or fruit colour, smell, and the presence and colour of sap.

Individuals from populations should be sampled as randomly as possible. Whatever method is used (*e.g.* transect, random), biased sampling (the selection of individuals on the basis of appearance, ease of collection, etc.) should be avoided. Material should be checked for pathogens, fungi or other organisms on the surfaces to avoid contamination.

For collecting plant tissue in silica gel, the specimen is put into paper bags, preferably tea bags, and put in a zip-lock bag or a screw-capped vial with dry silica gel. Bags are then folded and stapled, and seams checked for potential leakage. Each bag is labelled individually with unique identifiers being traceable to the voucher specimen as well as site information, collection date, collector, collection techniques, etc. (see Table 1). On tea bags, labelling with pens or

pencils is most advisable. It is recommended also to label the zip-lock bag. In general, several tea bags can be placed into one zip-lock bag; however, attention has to be paid to the silica gel which must be dry during the whole storage process. During the first days of storage, the silica gel will therefore have to be changed at certain intervals.

A permanent marker should be used for labelling collection tubes or vials. Information on the tube should be precise and reduced to a minimum. Markers should be checked for staying permanent in contact with ethanol and salt solution. The same labelling should be applied to the voucher and tube if the tissue is derived from the same specimen. The unique ID number on the tube must refer to a database where additional information can be found. Filling and labelling vials or at least printing labels prior to field work may substantially save time.

A critical source of potential error is placing tissues into the wrong tube, or multiple tissues into the same tube. This can be avoided by having two working boxes – one that contains the empty (or pre-filled) tubes and another to where the labelled tubes are being transferred after the sample has been added. The tube's identification number and sample ID should be verified in the database.

Much care needs to be taken to avoid sample-to-sample contamination between handling subsequent specimens. Tweezers, scissors, scalpels, etc. used to collect or fragment tissue, should be rinsed shortly in alcohol after handling each individual specimen. Sterilization of instruments in certain intervals, *e.g.* by flame, bleach treatment or use of 96% alcohol is an important precaution to combat contamination.

Samples and chemicals are best stored in a cool, dark, dry place to minimize chemical reactions, and (UV) light exposure. Solutions used (anaesthesia, fixative, CTAB solutions etc.) and the duration of the treatment should be entered in the "tissue preservation" field of the collection spreadsheet in the database.

Before moving to another field site, it is recommended to clean and sterilise all field equipment (clothes, plastic holding jars/bottles/plastic ware) to prevent disease transmission and to minimize cross-contamination of localities. Also check clothing and shoes for attached seeds or fruits before leaving a collecting site. Collectors can unwittingly transfer organic material from one population of a species to another. With regard to plant seeds, this could lead to undesirable out-crossing events in certain narrow endemic species. Human-dispersed biota might also become a serious pest at another locality, or lead to hybridisation with closely related species resulting in loss of genetic integrity of the populations affected.

In the case of higher vertebrates, extra care should be taken because many health hazards for humans are associated with the handling of post-mortem material, like blood. One should also be aware that the plant and animal parts touched during collecting may be poisonous. Care should be taken about irritant hairs; gloves should be worn wherever appropriate.

Particularly when collecting material from rare species at sites close to public areas, attracting attention should be avoided by inconspicuous behaviour. Heavy

trampling around the collecting site, potentially drawing attention to rare plants, is to be avoided as well.

It is advisable to check and complete the field notes after each collection day. Even minor details may be of later interest. The collection database should be updated as soon as possible.

3.1. Standard of work

In zoology, sampling methods and strategies can be very diverse, depending on the taxa under investigation, ranging from large terrestrial mammals, either nocturnal or diurnal, to flying birds, aquatic vertebrates and many different invertebrates. In botany, sampling aquatic plants is different from sampling succulent land plants, and different secondary compound compositions may demand different collecting techniques. It is therefore highly important to develop standard protocols for each of the processes that must apply to each particular type of fauna and flora when working in the field. To make the collecting of samples as efficient, representative, reliable and homogeneous as possible one may not allow too much space for improvisation. This is especially true if the collection procedure needs to be repeated for one reason or the other. As exaggerated sampling will have a negative impact on fauna and flora, it is one´s obligation to make sure that not more samples than absolutely necessary are processed. Before going out to the field, it is therefore highly recommendable to establish written protocols that describe all processes in detail, always based on the existing bibliography and on previous successful experiences.

4. Transportation of samples and arrival at the laboratory

Air and ground carriers have been changing their regulations and requirements for transportation of ethanol, liquid nitrogen, nitrogen dry shippers and dry ice frequently in recent years. Knowledge of current procedures, labelling requirements, etc., will help to avoid catastrophic sample loss due to delays and unexpected storage periods *en route*. Samples should be brought to the laboratory as fast as possible, and under as stable conditions as possible. Try to maintain control/ownership of your specimens. It is recommended to accompany one's specimens personally rather than sending specimens via mail as they might get lost. It is advisable to obtain information about the reliability of mail shipments in the respective countries beforehand. Sometimes it is possible to contract parcel post insurance; however, if the parcel is lost, most often the value is irrecoverable. Sending specimens via ocean freighter from one continent to another is not recommended as long transportation times have a potentially negative effect on the specimens´ DNA quality. If vials are used for transportation, care should be taken that lids are tightly closed, vials don't get squeezed, and changes in air-pressure and temperature won't affect the samples. Sometimes it is advisable to wrap cling-film or Stretch-Tite around the lids of vials before the transport to avoid evaporation. We recommend taking the samples in the cabin when travelling by plane, since temperature and air pressure are more constant there. If silica gel is used, the corresponding material safety data sheet should be carried along if questioned at borders.

Back to the laboratory, samples should be checked and transferred to stable conditions for short, medium or long term storage as soon as possible. Care should be taken about documentation, and missing information about transport and final destination be added.

5. Deposition of material in natural history collections

It is mandatory to guarantee both short-term and long-term preservation of the collected specimens to deposit material in natural history collections. The scientific institutions that will receive the material must therefore ensure that there is sufficient space and budget available to correctly house and maintain the specimens for the long-term. As the samples collected in the field have only been prepared in a provisional way, additional handling and data entry will be necessary once they are deposited in a research collection. In the case of tissue samples, permanent and safe physical space in, *e.g.*, freezers and cryo-vats must be available and accessible. One should be aware that, as a general rule, most natural history collections are only willing to store (tissues and/or DNAs from) vouchered and well documented material.

Once incorporated into a national collection, voucher specimens may be examined by many researchers over time. If the country of origin placed restrictions on the use of voucher material in the collection (or export) permit, such as stipulating that vouchers may not be used for third-party DNA extraction or not to be sent on loan to another institution, then these restrictions need to be noted on the specimen itself (and ideally also in the management system of the collection housing the specimen; compare Savolainen *et al.*, 2006).

Fig. 1. A. Out in the field (Siberia, Altai region) for DNA collection; B. Taking samples in the field; C. Sampling marine organisms; D. Example on how specimens (here *Leuciscus leusciscus* (Linnaeus, 1758)) are photographed before taking tissues; E. Vials of different types; F. Taking tissue samples from a wire; G. Laboratory equipment if tissue is taken in the lab; H. Barcode labelled specimen and the corresponding database;. (Picture A by N. Enke; B by A. Camacho, C. by Panglao Marine Biodiversity Project 2004; D by M. Rawson; E. by G. Droege; F & H. by I. Rey; G. by G. Droege and H. Zetzsche.

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7. Appendices

Appendix 1: Top Ten List – DOs and DON'Ts

1. Try to do what is BEST – NOT what is EASIEST – This starts with tissue sampling for later DNA analysis. Try to get the tissue/DNA preserved in the best state as early in the pipeline as possible so that degraded DNA is not what enters the biorepository.
2. Strive for the GOAL: Specimen vouchered in an accessible collection, tissue and/or DNA extract in an accessible biorepository, sequences in GenBank, all metadata available/included. **ANY MISSING PIECE REDUCES THE VALUE**
3. DO your homework – do you know: what to expect? What to do with it when you get it? How to transport? How to record (what) data?
4. DON'T collect, export, import or transport specimens, tissues, or DNAs without the necessary official permits.
5. DON'T put off metadata documentation until later – it is harder, takes significantly longer to do so, and usually ends up less complete and accurate when postponing it.
6. Do realize the difference (in time, resources, necessary partnerships, etc) between building a reference library of vouchered, high-quality specimens, tissues, DNAs and sequences and just collecting and barcoding to get a quick identification of something you are not going to study any further.
7. Do recognize the limitations of compromising or taking shortcuts on easier/quicker/cheaper methods – use best practices.
8. Do AVOID sample-to-sample contamination.
9. Do ASK FOR HELP or advice if not sure about the best way of how to proceed.
10. OPTIMIZE short- and long-term preservation of the collected specimens.

Appendix 2: Checklist of possible equipment for collection trips

Chemicals and their storage

- 95% (> 70%) ethanol (DO NOT SUBSTITUTE WITH ANY OTHER TYPE OF ALCOHOL)
- DMSO salt buffer
- Saturated NaCl-CTAB buffer
- Distilled water (or deionized water)
- Various plastic jars/bottles (with watertight cap) for transporting chemicals
- Paper envelope which is bleach free
- Silica gel
- FTA paper
- Cling-film or Stretch-Tite for vial wrapping is sometimes advantageous during transport

Capture/storage equipment

- Waterproof plastic jars/bottles or Tupperware
- Plastic Zip-lock bags (various sizes)
- Paper bags
- Scissors
- Scalpel
- Tweezers
- Vials or Microtubes (screw cap with O-ring) – one for each individual sampled (2 ml or various sizes)
- Pipette or medicine dropper – for filling microtubes with solutions

Miscellaneous

- Fine point sharpie markers
- Micron archival ink pens
- Pencils
- Labels