Chapter 2

Introduction to Animal DNA Barcoding Protocols

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Abstract

Procedures and protocols common to many DNA barcoding projects are summarized. Planning for any project should emphasize front-end procedures, especially the "genetic lockdown" of collected materials for downstream genetic procedures. Steps further into the DNA barcoding process chain, such as sequencing, data processing, and other back-end functions vary slightly, if at all, among projects and are presented elsewhere in the volume. Point-of-collection sample and tissue handling and data/metadata handling are stressed. Specific predictions of the future workflows and mechanics of DNA barcoding are difficult, so focus is on that which most or all future methods and technologies will surely share.

Key words: DNA barcoding, Animals, ATBI, Genetic preservation

1. Introduction

The Smithsonian's LAB, part of the National Museum of Natural History, in partnership with scientists from our own museum and around the world, have generated DNA barcodes from 22 of the 32 recognized phyla of animals (1–8). We have been actively heading the Leading Labs Network of the Consortium for the Barcode of Life (CBOL) and are participating in multiple DNA barcoding campaigns. These efforts have afforded us the opportunity to be heavily involved in all aspects of DNA barcoding for many different groups of organisms—from sample collection in the field, through lab processes and data analysis, to publication. We work on projects that range from a few to thousands of specimens and on projects taking environmental sampling approaches. When we started this work, we were frustrated by the numerous inefficiencies in all steps of the process, from field collections to database submission. We have invested much effort in an attempt to make things less cumbersome—simplifying or eliminating renaming photographs, minimizing the

W. John Kress and David L. Erickson (eds.), *DNA Barcodes: Methods and Protocols*, Methods in Molecular Biology, vol. 858,
DOI 10.1007/978-1-61779-591-6_2, © Springer Science+Business Media, LLC 2012

number of times a specimen is handled, developing field notebook to lab notebook to final database interconnectivity, and improving lab processes and monitoring. Redundant efforts take time and money and introduce opportunities for human error. We have worked to avoid or eliminate many of these from our barcoding pipeline. After all—how many times do you want to type and retype (accurately) the scientific name of the scrawled cowfish (*Acanthostracion quadricornis*) or repeatedly try to find the correct well in a 384-well plate with a single pipet tip?

Now that many DNA barcoding campaigns and large-scale projects are well underway, where are we, what have we learned, where are we going, and what advice do we need to heed going forward? Who could have predicted the technological fallout resulting from the genomic revolution? Where will we be in terms of biotechnology and the application of new instrumentation to biodiversity documentation in 10 or 20 years?

Even attempting to answer some of these questions promises to be a near-sighted or short-term effort at best. Our goal here is to present an overview of approaches with a view to the long-term. We want to emphasize aspects not only specific to the current methods, but that reflect our current best knowledge on how to enable future research, procedures, and techniques. In this light, the overall guiding principle of all efforts to acquire tissues should stress the critical nature of what we call "genetic lockdown"—stabilizing and securing the specimens, tissues, and DNA extracts for future genetic work as early as possible in the process chain and keeping them stable, secure, and safe from that point on. This will require different procedures in different collecting circumstances: preserving an entire specimen (or environmental sample) in such a way as to enable downstream DNA applications; or rough sorting and tissue subsampling in the field; or taking along an automated DNA extractor for on-site DNA extractions. Although new techniques for recovery of DNA from formalin-fixed and/or ethanol preserved specimens continue to be evaluated (9), most of these are limited in terms of amplicon size, success rates, and parts of the genome that are accessible, and they are not amenable to high-throughput methods. Therefore, they are to be avoided if better quality specimens can be obtained. Better quality specimens will also have greater future utility beyond present DNA barcoding methods. DNA extractions can be performed with many specific protocols highlighted in other chapters in this volume, but we indicate what to strive for in your method of choice, and point out strengths and weaknesses of various approaches.

Subsequent to a high-quality, high molecular weight, archival-quality DNA extract free of secondary compounds and other PCR inhibitors, PCR for DNA barcoding for most animal groups follows very similar procedures. The primary difference is primer selection, and group-specific primers which enhance success for the

barcoding of many animal groups are already available. We will present generalities, what we use as controls if primers are not working for your group, and provide some common primers and PCR optimization strategies.

Once you have a successful PCR product (a clean, single-band of the target size), most animal processes converge onto a similar path—(1) purification of the PCR product; (2) cycle sequencing and subsequent reaction purification; (3) running the cleaned reaction product on an "automated sequencer" or genetic analyzer; (4) processing and quality control of the raw sequence data; (5) submission to databases and repositories. Therefore, we do not spend any time or space on these post-PCR steps.

Finally, two nice resources: the first is the two-part manual put out in 2010 by ABC Taxa on protocols for All Taxa Biodiversity Inventories (10). It contains many chapters relevant to animal barcoding and includes chapters on all vertebrate groups, insects and canopy arthropods, soil and litter sampling, marine and continental freshwater habitats; the second is the Consortium for the Barcode of Life's social network portal (http://connect.barcodeoflife.net/) that is a fantastic option to get assistance and information prior to setting out on a new project, or if you run into problems. This is your barcoding community resource, and should be a first avenue to seek guidance or answers. An abbreviated general Materials and Methods is presented; specifics should be found in the taxon-specific chapters.

2. Materials

2.1. Sample collection

(a) Proper disposable or easily sterilized tools.

(b) Proper individual storage containers for the organisms and tissues.

(c) Data collection tools to handle specimens, tissues.

(d) Photodocumentation materials (digital camera with appropriate lens(es), memory cards, backup hard drives).

2.2. Storage buffers

(a) VPLN dewar or dry ice and cooler (see Note 1).

(b) Salt solution (11).

(c) EtOH—95% (nondenatured) (see Note 2).

(d) Formalin or other voucher specimen preservation solution(s) (see Chapter 4 for specimen handling solutions and ref. 12).

2.3. Extraction components (see Note 3)
   (a) Lysis buffer for extraction method (see Note 4).
   (b) Proper plates, tubes or storage vessels (see Note 5).
   (c) When possible, on-site portable DNA extractor (see Note 6).

2.4. PCR components
   (a) PCR reaction ingredients and primers (see Note 7).
   (b) Positive control 16S or 18S primers (13).

2.5. Sequencing, data QC, and analysis—see other chapters in this volume.

3. Methods

3.1. Sample collection—methods will vary by taxonomic group and habitat. The EDIT ATBI volumes (10) are a great source of taxon and/or habitat specific methods as well as the chapters that follow. An excellent summary for marine invertebrates is provided in ref. 12 for relaxation, fixation, preservation as well as specific procedures by taxon; similar resources exist for many groups and can be found via networking with active research groups and labs.

The primary goal should always be preserving the integrity of the DNA, and trying to maintain a high-quality voucher specimen. One without the other loses significant value.

Photo documentation—for groups where it is necessary, living color patterns or morphology should be captured prior to tissue subsampling, if that will decrease the value of the image. However, some methods (i.e., fin painting with formalin) can degrade the DNA, so care should be taken to preserve the integrity of the genetic material.

3.2. Tissue subsampling—as soon as possible after collection (and potentially the death of the organism) the tissue subsampling needs to occur in order to stop the degradation process. There are many ways of accomplishing this, and many options for storage, transport, and DNA extraction, but the emphasis should always be to quickly stop degradation, then to create a high-quality, high molecular weight, archival DNA extract that will have maximal utility going forward.

3.3. DNA extraction methods—there are several alternatives for acceptable DNA extraction methods that yield a quality product from multiple sources and taxa that can be useful for decades to come. It is advisable to ensure, via preliminary experimentation on a few samples, that the methods work

prior to destroying the tissues of new taxa for all the specimens on hand.

3.4. PCR methods—see Chapter 4 for a table of primers by taxon.

3.5. Sequencing, data QC, and analysis—see other chapters in this volume.

4. Notes

1. Freezing tissues is frequently optimal, sometimes difficult; vapor phase liquid nitrogen requires proper tanks and materials (−20°C storage for short term can be adequate).
2. 70% ethanol, denatured ethanol, and isopropanol should be avoided if possible because preservation and/or utility might be compromised.
3. It is advisable to pretest different extraction protocols for new taxonomic groups—different methods have different strengths and weaknesses when it comes to yield and to secondary compound and PCR inhibitor carryover.
4. We have found that putting tissues or minced tissues from many phyla directly into the M2 buffer of the Autogen prep protocol for transport back to the lab works well.
5. It is important to use proper seals to avoid well-to-well contamination during transport. One should be cognizant of contamination issues when transporting different vessel types with less than perfect sealing mechanisms—for example, sticky foil and sealing tapes are disastrous, and most of the plate sealers are insufficient—each well of the plate should be sealed independently.
6. When logistics or permits require, it is possible to extract DNA in 96-well plates at many field sites (as long as they have electricity) with instruments, such as the Qiagen BioSprint or Thermo KingFisher and a magnetic bead protocol.
7. Several taxonomic groups have benefited from PCR primer optimization or redesign. Some homework in NCBI's BarStool, on BOLD and literature searches might yield primers that will increase PCR amplification success.

Acknowledgments

All of us work with teams of taxonomists, field assistants, lab technicians, and colleagues in our own institution and departments as well as at numerous other institutions around the globe—this

chapter represents what those people have taught us and learned with us over the years and they share in the effort and credit but are too many to name.

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