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Critical factors for assembling a high volume of DNA barcodes

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Large-scale DNA barcoding projects are now moving toward activation while the creation of a comprehensive barcode library for eukaryotes will ultimately require the acquisition of some 100 million barcodes. To satisfy this need, analytical facilities must adopt protocols that can support the rapid, cost-effective assembly of barcodes. In this paper we discuss the prospects for establishing high volume DNA barcoding facilities by evaluating key steps in the analytical chain from specimens to barcodes. Alliances with members of the taxonomic community represent the most effective strategy for provisioning the analytical chain with specimens. The optimal protocols for DNA extraction and subsequent PCR amplification of the barcode region depend strongly on their condition, but production targets of 100K barcode records per year are now feasible for facilities working with compliant specimens. The analysis of museum collections is currently challenging, but PCR cocktails that combine polymerases with repair enzyme(s) promise future success. Barcode analysis is already a cost-effective option for species identification in some situations and this will increasingly be the case as reference libraries are assembled and analytical protocols are simplified.

Keywords: museum specimens; DNA isolation; PCR; species identification; taxonomy; cox1

1. ASSEMBLING DNA BARCODES: THE CHALLENGE

DNA barcoding promises fast, accurate species identifications by focusing analysis on a short standardized segment of the genome (Hebert et al. 2003). Several studies have now established that sequence diversity in a ~650 bp region near the 5' end of the mitochondrial cytochrome oxidase subunit I (cox1; also referred to as COI) gene provides strong species-level resolution for varied animal groups including birds (Hebert et al. 2004b), fishes (Ward et al. 2005), springtails (Hogg & Hebert 2005), spiders (Barrett & Hebert 2005) and moths (Hebert et al. 2003; Janzen et al. 2005). These early results have provoked larger-scale barcoding efforts and global projects for fishes and birds have now been initiated (Marshall 2005). These projects represent the first wave in a series of initiatives which will demand the capability to assemble barcodes rapidly and cost-effectively. As one looks further to the future, the need for substantial analytical capacity looms. For example, an effort to barcode the 1.7 million described species (Hawsworth 1995) would require the assembly of some 20 million barcodes, given a target of about 10 barcodes per species. This total will rise fivefold if barcode coverage is desired for all 10 million eukaryote species (e.g. Hammond 1992), producing a sequence library of 65 billion base pairs, approximately twice the current size of GenBank (April 2005). This task could be completed within a decade by establishing 50 core laboratories, each producing 200 000 barcode records per year. When viewed from the perspective of major genomic facilities, some of which generate more than 50 million sequences a year, the production goals for barcode facilities may seem modest. However the business of generating barcodes is complex; each record represents a sequence derived from a specimen that had to be collected, archived and databased.

In the balance of this paper, we direct most of our attention to an evaluation of how the primary steps in the analytical chain extending from specimens to barcode records can be optimized, scaled up and economized. Because the single most critical step to achieve high production involves a move from protocols based on single specimens to those compatible with 96-well format, we only consider methods compliant with this approach.

2. SPECIMENS

(a) Sourcing specimens

Specimens are the raw material for any barcode facility. This need can be met most easily by sequencing all specimens encountered, but because of the lognormal distribution of species abundance (May 1975), most of the resultant sequences will derive from a few common species. Collaborations with taxonomists represent a far more effective strategy for provisioning the analytical chain with specimens (Janzen et al. 2005; Smith et al. 2005; Ward et al. 2005). With this approach, it is...
feasible to assemble a library of sequences that provides both broad species coverage and similar sampling intensity across species (e.g. 10 barcodes each). Moreover, sample sizes can be increased in cases where complexities, such as cryptic species, are encountered in the first pass (Hebert et al. 2004a; Janzen et al. 2005).

We have adopted the TrakMates micro-plate system (Matrix Technologies, Hudson, NH, USA) to force the organization of specimen shipments into the blocks of 96 needed for the later stages of analysis. One micro-plate holds 96 vials (94 specimens, two controls), each uniquely barcoded on the bottom of the vial. These barcoded vials can be rapidly scanned, aiding the tracking of specimens as they enter the analytical chain. Aside from an organized flow of specimens to the barcode facility, there is a critical need for the firm connection of specimens to their collaterals. To facilitate this, we have developed a spreadsheet that organizes key specimen information. We have, as well, developed web-based software to both organize the specimen information and to connect each barcode sequence with its source specimen (see below).

3. DNA ISOLATION

(a) Different strategies

Methods for DNA isolation fall into two broad categories: DNA release and DNA extraction. DNA release protocols aim to rapidly release DNA into solution, making it accessible for downstream applications such as PCR. Release-based methods also enable DNA isolation from samples without their physical disruption. In this case, the entire specimen can be removed after DNA isolation, allowing the retention of a voucher in cases where this would not otherwise be possible. Release methods are, however, not very sensitive and do not produce high purity DNA suitable for long-term storage (e.g. more than 1 year). By contrast, DNA extraction methods aim to purify DNA, often by binding it to a membrane (e.g. silica) or by chemical fractionation. Some classical methods, such as phenol/chloroform extractions (Sambrook et al. 1989), are not attractive because they are time consuming and involve toxic materials. The type and condition of specimens is a key factor in selecting a DNA isolation method. For fresh or recently collected tissue, a release-based DNA extraction usually provides sufficient DNA for barcoding. However, for archival material, more sensitive approaches should be used. Because little DNA is needed for barcode analysis, the amount of tissue used in DNA isolation is usually minute. Figure 1 shows four typical tissue samples for barcode analysis.

(b) Comparing DNA isolation techniques

In order to determine an optimal procedure for high volume barcoding, we compared five DNA isolation methods on four sets of specimens (birds, fish, recent and archival moths—see Electronic Appendix part 1A for details). The major criterion for the inclusion of methods in this performance test was their capacity for high-throughput analysis, but we also considered cost and sensitivity. These methods included an artisanal (=homemade) DNA release method, called DryRelease, which employs Chelex resin as a DNA release agent (Walsh et al. 1991). We also examined three DNA extraction methods that use silica to bind DNA: Silitom, an artisanal method based on the protocols of Elphinstone et al. (2003) and Boom et al. (1990), NucleoSpin96 tissue kit (Machery-Nagel, Düren, Germany) and DNeasy96 tissue kit (QIAGEN, Hilden, Germany). Finally, we tested a DNA extraction method that uses magnetic beads to bind DNA: ChargeSwitch Forensic kit (Invitrogen, Carlsbad,
(c) Which DNA isolation method is best for archival specimens?

In our studies, the NucleoSpin96 kit produced the highest amplification success for the full-length barcode region of cox1 in archival moths (31%) followed by Silitom (20%), DNeasy96 kit (18%), DryRelease (8%) and ChargeSwitch kit (1%; figure 2). These results make it clear that silica-based methods should be used for DNA isolation from archival specimens.

4. PCR AMPLIFICATION

(a) Primer design is critical for high success

Before starting a barcode project on any new taxonomic group, it is essential to test the performance of existing primers on fresh specimens from a range of species in the target group. If one or two current primer sets do not deliver more than 95% amplification success for the test assemblage, primer redesign should be undertaken. Our past studies on varied taxonomic assemblages have shown that minor adjustments in
primer sequences can have a large impact on barcode recovery. Primer reconfiguration begins by aligning all available sequences for the target taxonomic group. Subsequent adjustments in sequence to maximize matches have enabled the development of effective primer sets (more than 95% amplification across species) for large taxonomic assemblages, such as Lepidoptera (Janzen et al. 2005), birds (Hebert et al. 2004) and fish (Ward et al. 2005). In most cases, effectively complete barcode recovery for all species in a group can be achieved with two sets of non-degenerate primers. Primers with degenerate positions or modified bases such as inosine (which can form base pairs with all four nucleotides) can help with recalcitrant groups where variable nucleotide positions across taxa compromise amplification (Candrian et al. 1991). Using primers with degenerate positions may also reduce the chance of preferential amplification of nuclear pseudogenes (Sorenson et al. 1999). Many software packages are available to aid primer design, but we recommend PRIMER3 (Rozen & Skaletsky 2000) for designing non-degenerate primers and CODEHOP (Rose et al. 2003) for degenerate primers.

(b) PCR optimization

An optimized PCR for the barcode region of cox1 should yield a single sharp amplicon, with no more than minor sub-banding when examined on an agarose gel. This can often be achieved by optimizing cycling conditions, especially the annealing temperature, and by altering the concentration of PCR reagents such as magnesium, dNTPs and primers through pilot studies on a few taxonomically divergent members of the target assemblage. Optimization often also dramatically increases amplification success and can eliminate the need for PCR cleanup prior to the sequencing reaction. PCR amplification can also be enhanced with additives such as bovine serum albumin, betaine, DMSO (Abu Al-Soud & Radstrom 2000) and trehalose. Trehalose is especially useful because it acts as a potent PCR enhancer by both lowering the DNA melting temperature and stabilizing Taq polymerase (Spiess et al. 2004). Trehalose can also overcome the effect of PCR inhibitors that are often present in crude DNA extracts (e.g. DNA release methods). Minimalization of the volume of each PCR reaction is also important to reduce reagent use and cost; 10 µl reactions should be employed.

(c) Evaluation of different polymerases

Taq DNA polymerase from Thermus aquaticus (Saiki et al. 1988) is standard for PCR, but a wide variety of other polymerases have higher fidelity or processivity (e.g. Cline et al. 1996). As well, more complex PCR cocktails that include one or more repair enzymes offer new hope for the amplification of degraded DNA (Di Bernardo et al. 2002; Mitchell et al. 2005). Restorase (Sigma-Aldrich, St. Louis, MO, USA) represents one recently introduced commercial enzyme cocktail that couples AccuTaq, a high accuracy polymerase, with a repair enzyme.
We evaluated the effectiveness of four polymerases on DNA isolated using the NucleoSpin96 kit from two sets of specimens: recent moths (90 samples, six negative controls) and archival moths (84 samples, 12 negative controls). The recent moths were all less than 1 year old, whereas the archival moths included 14 specimens from each of six age groups (2, 4, 8, 16, ~32, ~64 years; See Electronic Appendix part 1A for details). We tested amplification of the DNA extract from each specimen using: Taq polymerase, Diamond DNA polymerase (Bioline, Randolph, MA, USA), AccuTaq (Sigma-Aldrich, St. Louis, MO, USA) and Restorase. Each enzyme was used according to the manufacturer’s instructions, but the amount of template DNA was constant across all four enzymes (See Electronic Appendix part 1D for details). We tested these enzymes for their ability to amplify the full-length cox1 barcode (658 bp), as well as partial barcode sequences of 407 and 155 bp (see Electronic Appendix part 1E for sequences of primers).

As expected, positive PCR results were much higher for recent than archival specimens (figure 3). For recent moths, the highest overall success was obtained with Taq polymerase (86%, 93% and 87% success for 658, 407 and 155 bp amplicons, respectively). For archival moths, Restorase performed best overall (44%, 50% and 26% success for 658, 407 and 155 bp amplicons, respectively), but the Diamond and Taq polymerases outperformed it for the smallest amplicon. This latter result was not wholly surprising as Restorase is not recommended for the amplification of small targets. Our results indicate that standard Taq polymerase provides both high performance and low cost for specimens whose DNA has not been degraded, while the use of Restorase merits consideration in archival specimens.

We further compared PCR and sequencing results for different age groups of the archival moths (figure 4). For all four enzymes, success in both PCR and sequencing declined with specimen age for all three amplicons. Restorase delivered the highest PCR success for the full-length product, but none of the enzymes produced 658 bp amplicons from samples older than 8 years (figure 4). For the 407 bp amplicon, all four enzymes performed well, amplifying almost 100% of the samples 8 years and younger. In samples older than 8 years, Taq polymerase showed lower success compared to the other three enzymes (figure 4). Restorase, AccuTaq and Diamond polymerase performed similarly and produced 407 bp amplicons from about 70% of the samples as old as 32 years (figure 4). However, results with Diamond polymerase were inflated by two sequence contaminations. Surprisingly, for the smallest amplicon (155 bp), all four enzymes performed poorly for samples older than 8 years (figure 4).

Direct sequencing of all PCR reactions revealed an interesting result: sequences were sometimes recovered from samples where no PCR product was evident on the agarose gel. This was particularly the case for samples 8 years and older. For example, agarose gels revealed only two of 14 positive PCR products in Restorase amplification of 32 year old moths, but we obtained 11 sequences (with no sign of contamination) from the same reactions (figure 4). Visualization of PCR products on agarose requires a product concentration of at least 1–2 ng μl⁻¹ (Sambrook et al. 1989; White & Wu 2001), while capillary sequencers are known to be more sensitive. This fact suggests that all PCR products from archival specimens should be sequenced.

(d) Archival specimens and DNA repair
AccuTaq is the polymerase present in the Restorase enzyme blend so a comparison of results using Restorase versus those using AccuTaq can indicate if the repair mechanism in Restorase aids barcode recovery from archival specimens. We found that Restorase produced more PCR positives on agarose gels for both the 658 and 407 bp amplicons than AccuTaq (figure 4). However, we found no difference once these samples were sequenced (figure 4). This result suggests that Restorase aids PCR yield, perhaps by repairing template damage, but that the effect is small. In earlier experiments with Restorase, we were able to amplify full-length 658 bp cox1 barcodes from moths up to 70 years old. However, this success required extensive optimizations that are not time- or cost-effective when the goal is high production rates. However, in the case of extremely rare or extinct species, this capacity could be valuable.

5. SCREENING PCR PRODUCTS
When working on a new taxonomic group or on specimens where PCR success is uncertain, it is helpful to screen PCR reactions for product. This has traditionally been a laborious task involving gel casting and the loading of individual reactions onto the gel. We have explored two options to accelerate this process: microfluidic devices and pre-cast agarose gels. Microfluidic devices ‘sip’ small volumes of the PCR reaction from each of the 96 wells on a plate and then run electrophoresis on a very small scale to determine both the size and concentration of the PCR

Figure 3. Evaluation of different PCR enzymes for the amplification of cox1 for recent and archival specimens. Four enzymes including Taq polymerase (Taq), Restorase (Res), AccuTaq (Acc) and Diamond polymerase (Dia) were compared for the amplification of full-length and partial cox1 barcodes (658, 407, 155 bp). Results are shown as % PCR success.

Figure 4. Screening PCR products.
product (e.g. Greiner et al. 2004). Unfortunately, current devices have several limitations for DNA barcoding; they are expensive (more than $100K, €80K), have high operating costs and are relatively slow. These disadvantages are not offset by any gain in sensitivity: the detection limits for these systems mirror those that can be achieved through agarose gels. Pre-cast agarose gels represent a second option: they are fast, require little capital investment (less than $1K, €0.8K) and have modest operating cost. We regularly employ the E-Gel 96 system (Invitrogen, Carlsbad, CA, USA) to screen PCR products, but similar gels are manufactured by several other suppliers.

6. SEQUENCING

(a) Sequencing reaction optimization

Sequencing reactions employ standard chemistry, but reactions can be run in low volume format with diluted sequencing mix (i.e. BigDye; Applied Biosystems, Foster City, CA, USA) without compromising sequencing success or quality. By employing a 10 μl reaction volume containing 0.25 μl BigDye (1/16 of standard reaction), the cost of each sequencing reaction can be substantially reduced. Before the reaction product is submitted for sequencing, it must be cleaned up. There are a variety of solutions for this step and several are scalable to very large production rates. Ethanol precipitation and magnetic bead protocols are widely used by major genomic facilities, but column-based approaches are also effective. Any high volume DNA barcoding facility requires access to one or more capillary sequencers, such as the ABI 3730 DNA Analyser (Applied Biosystems, Foster City, CA, USA). Based on a bidirectional sequencing each barcode record represents two ‘reads’ (see below). Operating seven days a week, a single ABI 3730 can generate just 200K reads or 100K barcode records per year, setting a production threshold for the facility.

(b) Sequence assembly and edit

A bidirectional sequencing strategy has the advantage of enabling the use of automated sequence assembly software to both assign quality values like PHRED scores (Ewing et al. 1998) for each base position and produce a consensus barcode sequence from the reads. It also enhances the quality of the final barcode and ensures its compliance with the minimum read length (i.e. 550 bp) needed to gain barcode designation (by avoiding signal deterioration that often occurs at the end of the reads). Manual inspection and editing of the barcode sequence at the electropherogram level are still required to validate sequence quality and to check for

Figure 4. Effectiveness of four PCR enzymes for the amplification of cox1 in archived specimens of varied ages. Taq polymerase (Taq), Restorase (Res), AccuTaq (Acc) and Diamond polymerase (Dia) were compared for the amplification of full and partial cox1 barcodes (658, 407, 155 bp) in archival moth specimens from six age groups (2, 4, 8, 16, ~32, ~64 years). Results are shown as (a), % PCR success and (b), % sequencing success.
possible polymorphic sites. Their presence, which is often overlooked by sequence assembly software, can indicate the co-amplification of nuclear pseudogenes (Bensasson et al. 2001) along with the authentic mitochondrial sequence. Several software packages are available for visualizing, editing and assembling mitochondrial sequence. Several software packages such as Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and SEQSCAPE (Applied Biosystems, Foster City, CA, USA) are the most popular commercial software options and include features such as internal basecallers, automatic alignment, contig assembly and trimming of sequences.

### 7. BARCODE OF LIFE DATA SYSTEMS

Large-scale DNA barcoding projects will create a substantial number of sequence records that must each link to a voucher specimen, as well as to its collateral data. These records need to be organized and analyzed. In addition, for the barcode database to be useful for species identification, it must be searchable by sequence, as well as by species name and higher taxonomic categories. The Barcode of Life Data Systems (BOLD), see http://www.barcodinglife.org, provides varied support for a large-scale barcode project. It is an online repository for Cox1 sequences as well as a workbench for barcode analysis that includes three components: a laboratory information management system (LIMS), a data management and analysis system (DMAS), and a species identification engine.

#### (a) Laboratory information management system

The assembly and storage of hundreds of thousands of barcode records requires a LIMS to ensure the accurate tracking of all specimens passing through the multistep analytical chain. Commercial LIMS are available, but they typically cost $50–100K ($40–80K) per site license, and they lack some key functionality required to support the DNA barcoding initiative.

#### (b) Data management and analysis system

The DMAS of BOLD provides support for both the warehousing and analysis of barcode records. It includes a simple interface enabling the upload of new sequences to password-protected projects. Its web-based delivery allows work to proceed simultaneously in different labs while being managed from a centralized location, improving communication and preventing data loss or duplication. Moreover, because the DMAS includes information on where each specimen was collected, where it is currently deposited, copies of sequence traces, and high-resolution photographs of each specimen, it allows straightforward traceability of the data stream back to the source. The DMAS was designed to operate at a global scale, ideally supported by mirror sites based at facilities active in barcode analysis.

The DMAS also includes a suite of analysis tools that allow processing or visualization of data. Sequence records, which can be submitted via a simple interface, are automatically aligned. Specimen pages are created automatically from the user-defined data, including an automated plot of GIS coordinates on high-resolution, multi-scale geographic maps. BOLD also includes programs for assembling and exporting neighbouring trees (Saitou & Nei 1987), which include color coding to indicate taxonomic affiliation or other user-defined parameters, as well as tools for specimen display. Finally the DMAS includes an interface that allows the bulk submission of barcode records to GenBank.

#### (c) Species identification engine

The first step in creating a DNA-based species identification system lies in the assembly of a comprehensive barcode sequence library. The second step involves the development of an effective engine for the comparison and matching of sequences from new specimens to the barcode library. The species identification engine, BOLD-ID, includes a simple user interface to allow Cox1 sequences to be entered into a search field and automatically compared against the existing dataset. BOLD-ID makes use of a combination of Local Alignment Search Tool (BLAST; Altschul et al. 1990) and hidden Markov models based on a global protein alignment for the Cox1 gene, which increase both the speed and accuracy of the matching procedure. Using this algorithm, BOLD returns a probability-based match profile indicating the likely identity of the source species. Links to the species page provide additional information about it (e.g. photographs) that can be useful in confirming the identification. Aside from identifying single specimens, BOLD-ID also performs batch identifications on 96-well plates of samples. The current version of BOLD-ID is optimized for Cox1 gene. However, future versions will include the capability to analyse barcode data from other genes or non-coding regions because barcoding systems in some groups (i.e. plants) will use such data (Chase et al. 2005; Kress et al. 2005).

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8. PROSPECTS FOR HIGH VOLUME DNA BARCODING

Few molecular taxonomy and evolution laboratories process more than a few thousand specimens a year, but the assembly of a comprehensive barcode library will require, as noted earlier, 100-fold higher production rates. In one sense, the protocols described in this paper are unproven because no barcoding facility has yet achieved this production target. However, we are confident, based on our own experience (table 2), that these protocols will allow the 100K goal to be achieved (see Electronic Appendix part 2 for routine protocols). We emphasize that there is no single optimized protocol if varied types of specimens are being analysed. For example, our work on recently collected North American Lepidoptera employed the DryRelease protocol for DNA isolation, followed by PCR recovery of the full-length barcode. By contrast, results on Costa Rican Lepidoptera, aged from 1 to 28 years, were greatly improved by using the NucleoSpin96 kit for DNA isolation. Moreover, when a full-length cox1 barcode could not be recovered (mainly in samples more than 10 years old), additional PCRs were used to obtain 400 and 350 bp barcode sequences that were concatenated to produce the full-length sequence. These two examples provide a sense of the methodological flexibility that is critical to achieve high success while minimizing costs. While our work has been mainly focused on animals, we expect that barcode analysis of other organisms, such as plants, will require substantial protocol changes, particularly in the isolation of DNA and in the choice of a target genomic barcode region (Kress et al. 2005).

Although barcoding can be executed in a decentralized fashion, economies of scale are gained by establishing core facilities. The capital costs involved in creating a facility capable of generating 100K barcodes a year will range from US$0.5–0.8M (€0.4–0.6M) with the higher figure allowing the emplacement of two capillary sequencers. However, much smaller capital investments ($50K, €40K) will allow the creation of facilities capable of generating 100K PCR products that might then be submitted to any sequencing facility for analysis. The generation of 100K barcode records based on bidirectional sequencing will require an operating budget of approximately $0.3M (€0.2M) before salaries. Although such investments will allow an impressive advance on past production levels, it may be insufficient as work moves from the construction of barcode libraries to the routine application of DNA barcodes for rapid, large-scale assessments of biodiversity in conservation biology and other ecological contexts (DeSalle & Amato 2004). Fortunately there are prospects for both further reductions in cost and increases in production. Costs will drop as reaction volumes shrink and microfluidic devices, which employ nanolitre reaction volumes for PCR and sequencing, are under development. There is also the potential for robotic intervention, which when coupled with unidirectional short reads for identification (as opposed to reference barcodes) could drive production levels to more than 500,000 specimens per year from a single sequencer. In short, the prospects for both the assembly and use of barcode libraries appear bright enough to expect illumination of many key problems in biodiversity science.

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