Chapter 7

DNA Barcoding Birds: From Field Collection to Data Analysis

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Abstract

As of February 2011, COI DNA barcode sequences (a 648-bp segment of the 5′ end of the mitochondrial gene cytochrome *c* oxidase I, the standard DNA barcode for animals) have been collected from over 23,000 avian specimens representing 3,800 species, more than one-third of the world's avifauna. Here, we detail the methodology for obtaining DNA barcodes from birds, covering the entire process from field collection to data analysis. We emphasize key aspects of the process and describe in more detail those that are particularly relevant in the case of birds. We provide elemental information about collection of specimens, detailed protocols for DNA extraction and PCR, and basic aspects of sequencing methodology. In particular, we highlight the primer pairs and thermal cycling profiles associated with successful amplification and sequencing from a broad range of avian species. Finally, we succinctly review the methodology for data analysis, including the detection of errors (such as contamination, misidentifications, or amplification of pseudogenes), assessment of species resolution, detection of divergent intraspecific lineages, and identification of unknown specimens.

Key words: Birds, Cytochrome *c* oxidase I, DNA barcodes, Collection, DNA extraction, Neighbor joining, Polymerase chain reaction, Pectoral muscle, Sequencing, Toe pad

1. Introduction

Taxonomy and phylogenetic affinities are better understood in birds than in any other large group of organisms. Additionally, they are probably the best represented group of vertebrates in frozen tissue collections, with more than 300,000 tissue samples covering nearly 75% of known bird species (1). These characteristics make birds an ideal group to analyze the effectiveness of a standardized genetic method for species identification—i.e., DNA barcoding. Consequently, they were the first taxonomic group for which a large-scale barcoding study was performed (2) and were the focus of one

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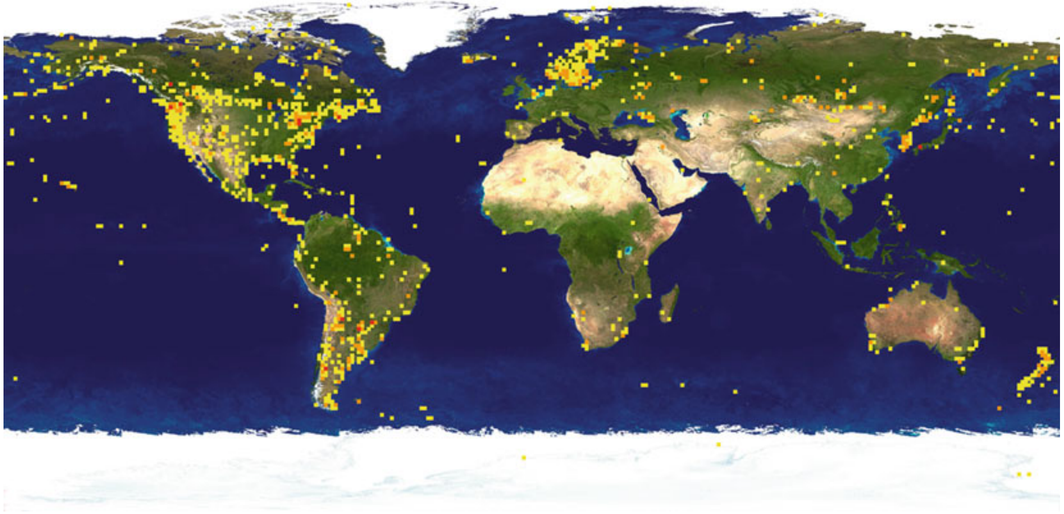

Fig. 1. Localities of collection of bird tissue samples that have been DNA barcoded so far as of February 2011.

of the first global barcoding campaigns, the all birds barcoding initiative (ABBI), which was launched in September 2005 with the goal of obtaining barcodes for the ca. 10,000 extant bird species.

The initial avian barcoding study (2), in which roughly 40% of the North American species were examined, showed that a 648-bp segment of the 5′ end of the mitochondrial gene cytochrome *c* oxidase I (COI) was highly effective for species identification, presaging similar findings in diverse animal groups. Following this preliminary analysis, coverage of North American species expanded to near completion (3) and avian barcode surveys commenced in other regions, including the Neotropics (4–6), the Palearctic (7–9), the Indomalayan region (10, 11), and Australasia (12). As of February 2011, more than 23,000 avian barcode sequences from more than 3,800 species had been obtained, representing more than one-third of the world's avifauna (Fig. 1).

These studies demonstrated that a COI barcode effectively distinguishes among known species of birds (see also ref. 13) and additionally highlights species and species groups in which further analyses of taxonomic boundaries are necessary. Even in a well-studied group such as birds, barcoding projects have revealed previously undetected lineages, many of which could represent new species (e.g., ref. 14). Increasingly comprehensive public libraries of bird barcodes also enable new lines of scientific inquiry and practical applications, including analyses of diversification patterns at continental scales (15), identification of species involved in bird-strikes (16–18), studies of avian reservoirs for human and animal diseases from blood meals of arthropod vectors (19), and identification of bird species occupying cavity nests (20).

Avian barcoding projects also present challenges. Approximately 25% of species are not represented in frozen tissue collections, and obtaining high-quality DNA from historical specimens, such as study skins or skeletons, is arduous and often unsuccessful. Collecting new specimens is relatively expensive and time consuming as compared to collecting other animal groups, such as insects. This is particularly challenging in the context of ABBI's goal of obtaining barcodes from multiple individuals—ideally belonging to geographically distant populations—of all avian species. In addition, many of those species absent from tissue collections are rare or endangered, limiting the possibility of field collection (1). Blood is often collected from threatened species to avoid sacrificing individual birds, but this adds another challenge because avian red cells are nucleated and contain few mitochondria, making blood a relatively poor source of mitochondrial DNA. This results in a higher risk of amplifying nuclear-mitochondrial pseudogenes, or numts, instead of the desired COI mitochondrial copy (21). Finally, necessary permits for collecting specimens and transporting samples within and across national borders are often particularly complex for birds (see Note 1).

In this chapter, we detail the methods for barcoding birds, covering the entire process from field collection to data analysis. We focus on the methods that have been most widely used, and include a brief description of other options where appropriate. Most of the methods that are used to barcode birds are also used for other animal organisms and in many other molecular studies. Therefore, although we outline all the information that a reader needs to barcode avian samples, we emphasize those aspects that are specific to avian barcoding, addressing the aforementioned challenges posed by birds.

2. Materials

2.1. Collecting, Documenting, and Storing Tissue Samples and Their Associated Vouchers

Materials needed for the first steps of the pipeline are not specific to barcoding and include general equipment for collecting specimens (see Note 2), dissecting tools for obtaining tissue samples, materials for storing tissue samples, taxidermy materials for preparing vouchers, and imaging equipment for documenting specimens. See refs. 22–28 for a general description of materials needed to collect, prepare and store avian tissue samples and their associated vouchers.

2.2. DNA Extraction

The following are the working solutions for the manual glass fiber (GF) protocol for DNA extraction using 96-well plates (29, 30) or individual tubes. The volumes described are those needed to extract ten 96-well plates or approximately 1,000 samples (see the protocol available from the Canadian Centre for DNA Barcoding (CCDB)

website (www.dnabarcoding.ca) for recipes for preparing stock solutions and for descriptions and suppliers of reagents, disposables, and equipment 30).

1. Vertebrate lysis buffer (VLB): 0.5% SDS, 100 mM NaCl, 50 mM Tris–HCl pH 8.0, and 10 mM EDTA pH 8.0. To prepare 50 ml of VLB, mix 0.25 g of SDS, 5 ml of 1 M NaCl, 2.5 ml of 1 M Tris–HCl pH 8.0, and 1 ml of 0.5 M EDTA pH 8.0, and then add double-distilled water (ddH_2O) to reach the final volume.
2. Binding buffer (BB): 6 M guanidine thiocyanate, 20 mM EDTA pH 8.0, 10 mM Tris–HCl pH 6.4, and 4% Triton X-100. To prepare 100 ml of BB, mix 70.9 g of guanidine thiocyanate, 4 ml of 0.5 M EDTA pH 8.0, 10 ml of 0.1 M Tris–HCl pH 6.4, and 4 ml of Triton X-100, and then add ddH_2O to reach the final volume (see Note 3).
3. Wash buffer (WB): 60% ethanol, 50 mM NaCl, 10 mM Tris–HCl pH 7.4, and 0.5 mM EDTA pH 8.0. To prepare 750 ml of WB, mix 475 ml of 96% ethanol, 37.5 ml of 1 M NaCl, 7.5 ml of 1 M Tris–HCl pH 7.4, and 0.75 ml of 0.5 M EDTA pH 8.0, and then add ddH_2O to reach the final volume. Mix well and store at −20°C.
4. Binding mix (BM): To prepare 100 ml of BM, mix 50 ml of BB and 50 ml of 96% ethanol. BM is stable for about 1 week at room temperature.
5. Protein wash buffer (PWB): To prepare 180 ml of PWB, mix 47 ml of BB and 126 ml of 96% ethanol, and then add ddH_2O to reach the final volume. PWB is stable for about 1 week at room temperature and it should be discarded if any crystallization occurs.

2.3. PCR Amplification of Cytochrome *c* Oxidase I

The following are the reagents needed to amplify the COI barcode region via Polymerase Chain Reaction (PCR) according to the protocol generated at the CCDB (31).

1. Taq polymerase: We recommend Platinum Taq polymerase (Invitrogen), which is the one currently used at the CCDB (see Note 4).
2. 10× PCR buffer (for Platinum Taq). Store at −20°C.
3. 50 mM $MgCl_2$. Store at −20°C.
4. 10 mM dNTP mix. Store at −20°C.
5. 10 μM primer solution (obtained by diluting ten times the 100 μM stock solution, which should be prepared from the desiccated primer by dissolving each nmol of the primer in 10 μl of ultrapure water). Store at −20°C.
6. 10% Trehalose (obtained by dissolving each gram of D-(+)-trehalose dihydrate in 10 ml of ultrapure water). Approximately

6.25 ml of 10% trehalose are used for 1,000 PCRs. Store at –20°C (see Note 5).

7. Ultrapure water.

2.4. Sanger Sequencing

The following are the reagents needed to obtain the COI barcode sequence following the protocol developed at the CCDB (32).

1. BigDye Terminator v3.1 reaction mix (ABI).
2. 5× sequencing buffer (400 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$).
3. 10% Trehalose (see above).
4. 10 μM primer solution (see above).
5. Ultrapure water.
6. Sephadex G-50 (Sigma-Aldrich) for purification of sequence products.

3. Methods

3.1. Collecting, Documenting, and Storing Tissue Samples and Their Associated Vouchers

Avian barcoding projects often rely on tissue samples already deposited in museums (3, 7–9). However, current collections do not always provide adequate coverage of all bird species or regions. In these cases, barcoding projects are a catalyst for the growth of bird tissue collections (e.g., ref. 6). Collecting avian specimens, obtaining and storing tissue samples, and preparing vouchers are complex tasks. Here, we focus on the key points and emphasize those that are particularly relevant in the context of barcoding.

Regardless of whether tissue samples are collected for the project or obtained from museums, it must be borne in mind that they should be sampled broadly across the geographic range of species so that intraspecific genetic variation is accurately represented. To achieve this objective, as a rule of thumb, it is recommended to analyze five to ten specimens per species selected from sites distributed throughout its range. However, this number varies depending on various factors, such as the complexity of the species population structure, availability of specimens, permits, and financial limitations (see Note 6).

3.1.1. Obtaining and Storing Tissue Samples

1. Collection of specimens should follow standard procedures (e.g., ref. 26) in accordance with the collection permits obtained for the project (see Note 1). Ideally, tissue samples should be obtained immediately after collecting the specimen. If this is not possible, the intact specimen should be kept frozen until tissue sampling can be performed (see Note 7). The most widely used tissue source is pectoral muscle (which is large and easy to access), but other sources of muscle, such as the heart, are also commonly used. The liver has also been sampled

traditionally, but, likely due to its high enzymatic activity, liver samples are not as good as muscle samples for amplifying mitochondrial DNA (A. Borisenko, personal communication).

2. For long-term preservation of tissue samples, immediate freezing is recommended. The use of nitrogen tanks is a popular method to achieve this while in the field. Once the sample is deposited in a permanent collection, it will likely be kept in either nitrogen or an ultracold freezer at −80°C (see Note 8). Regular microtubes can be used for storing the samples in ultracold freezers, but cryogenic tubes, which are thicker walled, are required if samples are stored in nitrogen, even if only for a few days. If samples cannot be frozen either in the field or permanently, then they should be fixed (see Note 9). The most common fixative is ethanol (96% or higher, see Note 10), but there are other options, such as DMSO buffer. In this case, regular microtubes can be used (see Note 11).
3. When only a blood sample is obtained (e.g., with rare or endangered species), it is also recommended to freeze the sample, although blood is usually immersed in ethanol or lysis buffer (see Note 12) regardless of the temperature of storage.

3.1.2. Preparing, Documenting, and Storing Vouchers

1. The presence of vouchers associated with tissue samples is critical when establishing a barcode library (see Notes 13–17) and their preparation and storage should follow standard procedures (22–28).
2. Documentation of vouchers is an important step in the barcode pipeline; photographs of specimens should be included with the information uploaded to the Barcode of Life Data Systems (BOLD; www.boldsystems.org; ref. 33). BOLD is an online repository of barcode records and a workbench for DNA barcoding projects. Photographs typically document prepared specimen skins (which should also feature a scale and the voucher number, Fig. 2) but alternatively could feature the living bird (see Note 18) or the specimen prior to preparation if only a skeleton will be prepared (both photographs of the nonprepared specimen and the skeleton can be uploaded). When a project uses samples already deposited in museum collections, photographs of vouchers should still be included as data to support the DNA barcode sequence.

3.1.3. Data Associated to the DNA Barcode Sequence

1. The collection information associated with a barcode record is vital for its use either for identification purposes, aiding species discovery, or any evolutionary or ecological study. Several information fields can be uploaded to BOLD, and increasing the amount of information enhances the value of the barcode record. The information about the data that should be included in each barcode record and the spreadsheet used to upload the data can be obtained from the BOLD website (see Note 19).

Fig. 2. Photographs of vouchers (particularly museum skins) add value to BOLD records. Picture taken by K. Kerr.

2. As previously mentioned, images of each specimen should also be uploaded to BOLD. The image submission protocol can also be obtained from the BOLD website.

3.2. DNA Extraction

A homemade GF DNA extraction method has been developed by Ivanova et al. (29) with the objective of providing high-quality DNA extracts at low cost and thus increasing the efficiency of the barcoding process. This protocol can be applied to either vertebrates or invertebrates with only slight modifications, and has already been used on thousands of bird specimens (6, 8). Several different types of tissue samples can be used with this method, including muscle, blood, feathers, and toe pads (see Note 20). This method can also be adapted to various scales, from single reactions to 384-well plates. We describe below a version of the protocol for 96-well plates, which is a commonly used scale when developing large bird barcoding surveys, and a small-scale version for individual tubes. Both utilize the working solutions listed in Subheading 2.2. In high-throughput laboratories, an automated version of the protocol may be employed (this version is not described here; see ref. 29 for a detailed protocol).

3.2.1. 96-Well Plate Format

1. For each plate, mix 5 ml of VLB and 0.5 ml of Proteinase K (20 mg/ml) and dispense 50 µl of this Lysis Mixture in each well.

2. Add a small piece of tissue (approx. 1–2 mm^3, see Note 21) to each well of the plate (see Notes 22–24). To avoid cross contamination, it is highly recommended to gently cover the plate with caps before transferring the tissue and only uncover the row that is being used (see Notes 25 and 26). After transferring all the samples into the plate, the caps need to be fully inserted to avoid evaporation during incubation.
3. To allow digestion, incubate at 56°C overnight (or for a minimum of 6 h, see Note 27).
4. Centrifuge the plate at 1,500 × *g* force for 15 s to remove any condensate from the caps.
5. Remove the caps and add 100 μl of BM to each sample using a multichannel pipette. Mix by pipetting.
6. Transfer the lysate (about 150 μl) from the wells of the microplate into the wells of a GF plate (e.g., AcroPrep 96 1 ml filter plate with 1.0 μm GF media) placed on top of a square-well block (e.g., PP MASTERBLOCK, 96-well, 2 ml) to be used as a catch plate, using a multichannel pipette. Seal the plate with self-adhering cover (e.g., Axyseal sealing film).
7. Centrifuge the GF plate with the square-well block at 5,000 × *g* force for 5 min to bind DNA to the GF membrane.
8. Remove the cover and add 180 μl of PWB to each well of the GF plate. Seal the plate with a new cover and centrifuge at 5,000 × *g* force for 2 min.
9. Add 750 μl of WB to each well of the GF plate. Seal with a new cover and centrifuge at 5,000 × *g* force for 5 min.
10. Discard the square-well block and the flow through (see Note 28). Remove the cover film of the GF plate, place it on the lid of a tip box, and incubate at 56°C for 30 min to evaporate residual ethanol.
11. Place the GF plate on top of the microplate that will be used to collect the DNA. Dispense 30–60 μl of ddH_2O (prewarmed to 56°C) directly onto the membrane in each well of the GF plate and incubate at room temperature for 1 min before sealing the plate.
12. Place the assembled plates on a clean square-well block (to prevent cracking of the collection plate) and centrifuge at 5,000 × *g* force for 5 min to elute the DNA into the microplate (see Note 28). Remove the GF plate and discard it.
13. Cover the microplate containing the DNA extracts with caps or aluminum PCR foil. Extracts can be temporarily stored in a refrigerator (4°C), but a freezer (–20°C) is recommended for long-term storage. Between 1 and 5 μl of the DNA extract should be used for PCR depending mainly on the quality of the DNA source (e.g., fresh tissue sample vs. toe pad sample).

3.2.2. Individual Tubes Format

1. Mix 50 µl of VLB and 5 µl of Proteinase K (20 mg/ml) multiplied by the number of samples to process. Put 50 µl of this Lysis Mixture in each tube.
2. Add a small piece of tissue (approx. 1–2 mm^3, see Note 21) to each tube (see Notes 22–24 and 26).
3. To allow digestion, incubate the tubes at 56°C overnight (or for a minimum of 6 h) (see Note 27).
4. Centrifuge the tubes at 5,000–8,000 × *g* force for 15 s to remove any condensate from the walls and lids.
5. Add 100 µl of BM to each tube.
6. Mix the tubes by pipetting and transfer the lysate into spin columns (e.g., Epoch Biolabs spin columns with attached Lid) placed on top of collection tubes.
7. Centrifuge the columns and tubes at 5,000–6,000 × *g* force for 2 min to bind DNA to the GF membrane.
8. Add 180 µl of PWB to each column and centrifuge the columns and tubes at 5,000–6,000 × *g* force for 2 min. Pour out the contents of each collection tube or replace it with a new tube.
9. Add 700 µl of WB to each column and centrifuge the columns and tubes at 5,000–6,000 × *g* force for 4 min. Pour out the flow through from each collection tube or replace it with a clean one and centrifuge the columns and tubes at 10,000 × *g* force for an extra 4 min.
10. Replace the collection tubes with 1.5 ml tubes with removed lids and incubate the columns (with the lids opened) and tubes at 56°C for 15–30 min (incubation can alternatively be done at room temperature).
11. Add 50–80 µl of ddH_2O (prewarmed to 56°C) directly onto the membrane of each column and incubate the columns and tubes at room temperature for 1 min before closing the lids of the columns.
12. Centrifuge the columns and tubes at 10,000 × *g* force for 5 min to collect the DNA eluate.
13. Transfer the DNA extract into a clean tube. Extracts can be temporarily stored in a refrigerator (4°C), but a freezer (–20°C) is recommended for long-term storage. Between 1 and 5 µl of the DNA extract should be used for PCR depending mainly on the quality of the DNA source (e.g., fresh tissue sample vs. toe pad sample).

3.3. PCR Amplification of COI

3.3.1. Primer Selection

1. The target region in birds is the standard animal COI barcode, a 648-bp fragment of the 5′ end of COI. Primer pairs that have been used to amplify this gene region are shown in Table 1, and corresponding amplicon sizes in Table 2 (see also Fig. 3). For efficient analysis of large numbers of samples, it is desirable to use "universal" primers, i.e., those which work with a broad taxonomic range of birds. To date, the largest number of successful amplifications have utilized the forward BirdF1 and the reverse COIBirdR2 primers (3, 6, 8, 9). Other primer pairs generally effective for avian samples include CO1F + CO1R (16), PasserF1 + PasserR1 (10), and AWCF1 + AWCR6 (12). These four sets of primers should be considered as first-choice primer pairs (combinations of them could also give good results, such as the pair AWCF1 + COIBirdR2 for passerines; ref. 12). Other primers should be considered when these recommended first options fail. The forward primer FalcoFA, for example, has been shown to work well in cases in which the combination BirdF1 + COIBirdR2 tended to fail (3). In situations where DNA yields are low (e.g., degraded samples), one of us (KCRK) found that a nested PCR approach often succeeds when standard amplification attempts fail. The primer pair LTyr + COI907aH2 (13) can be used for an initial PCR and the amplified fragment can then be used as a template for a second PCR using the primer pair COIaRt + COI748Ht (13). The latter primer pair is also used for sequencing.
2. The aforementioned primers are designed to amplify as a single fragment the entire barcode region (or an even longer portion of the mitochondrial genome) and are generally effective when using high-quality DNA (i.e., obtained from frozen or ethanol-preserved tissue samples). For many species or geographic regions, however, these kinds of tissue samples are not available. In such cases, traditional museum collections of study skins or skeletons can be sampled. The drawback of using historical samples is that their DNA is usually highly degraded making full-length barcode amplification usually unsuccessful. In these cases, short, overlapping fragments (each of them not longer than 300–400 bp) must be amplified using internal primers. Two groups of primers used to amplify relatively short fragments are shown in Table 3 and Fig. 4, one consisting of four primers that generate the barcode in two fragments (6) and the other consisting of six primers that generate the barcode in three fragments (12).

3.3.2. PCR mixture

1. The ingredients of the PCR mixture are detailed in Table 4 (using the reagents listed in Subheading 2.3). This recipe should be followed when performing PCR reactions in 12.5 μl volumes, which has the advantage of reducing the total amount

Table 1
Avian DNA barcode primers [primer name, orientation, sequence, length, position of 3′ nucleotide relative to COI start codon [designated position 0], and references are shown]

Name	Orientation	Sequence (5′–3′)	Length	Location	Primary reference	Additional references
BirdF1	F	TTCTCCAACCACAAAGACATTGGCAC	26	51	(2)	(3, 6–9, 37)
BirdR1	R	ACGTGGGAGATAATTCCAAATCCTG	25	747	(2)	(3, 7, 9)
BirdR2	R	ACTACATGTGAGATGATTCCGAATCCAG	28	747	(2)	
BirdR3	R	AGGAGTTTGCTAGTACGATGCC	22	1,064	(2)	
FalcoFA	F	TCAACAAACCACAAAGACATCGGCAC	27	51	(3)	
VertebrateR1	R	TAGACTTCTGGGTGGCCAAAGAATCA	26	707	(3)	
COIbirdR2	R	ACGTGGGAGATAATTCCAAATCCTGG	26	746	(6)	(8, 9)
CO1-ExtF	F	ACGCTTTAACACTCAGCCATCTTACC	26	–2	(9)	
CO1-ExtR	R	AACCAGCATATGAGGGTTCGATTCCT	26	1,551	(9)	
PasserF1	F	CCAACCACAAAGACATCGGAACC	24	52	(10)	(11)
PasserR1	R	GTAAACTTCTGGGTGACCAAAGAATC	26	708	(10)	
AWCF1	F	CGCYTWAACAYTCYGCCATCTTACC	25	–2	(12)	
AWCR6	R	ATTCCTATGTAGCCGAATGGTTCTTT	26	794	(12)	
LTyr	F	TGTAAAAAGGWCTACAGCCTAACGC	25	–24	(13)	
COIaRt	F	AACAAACCACAAAGATATCGC	21	49	(13)	
COI748Ht	R	TGGGARATAATTCCRAAGCCTGG	23	746	(13)	
COI745h2	R	ACRTGNGAGATRATTCCRAANCCNG	25	747	(13)	
COI907aH2	R	GTRGCNGAYGTRAARTATGCTCG	23	905	(13)	
CO1F	F	TTCTCGAACCAGAAAGACATTGGCAC	26	51	(16)	
CO1R	R	ACTTCTGGGTGGCCAAAGAATCAGAA	26	704	(16)	
L6615	F	CCYCTGTAAAAAGGWCTACAGCC	23	–30	(46)	(4, 5)
H10884	R	GGRTCRAANCCRCAYTCRTANGG	23	4,260	(46)	
H8121	R	GGGCAGCCRTGRATTCAYTC	20	1,478	(47)	(4, 5)

Table 2
Primer pairs that have been successfully used to obtain bird barcodes and sizes of the amplified fragment in base pairs (Sizes of fragments obtained with first-choice primer sets are in bold)

Forward	Reverse CO1R	VertebrateR1	PasserR1	COIbirdR2	BirdR1	BirdR2	COI748Ht	AWCR6	COI907aH2	BirdR3	H8121	CO1-ExtR	H10884
L6615											1,551		4,336
LTyr									976				
CO1-ExF												1,605	
AWCF1				799				**847**					
COIaRT							740						
BirdF1		707		**746**	746	749				1,060			
CO1F	**704**												
FalcoFA					747	750							
PasserF1			**705**										

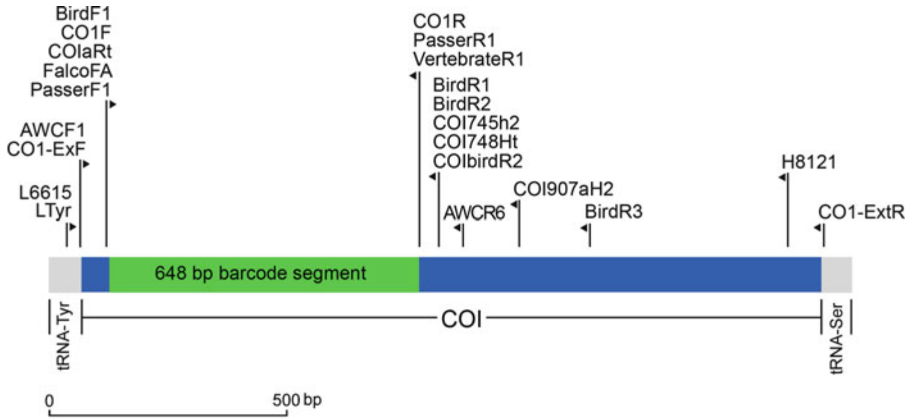

Fig. 3. Schematic of avian DNA barcode primers and COI landmarks.

Table 3
Primers used to obtain the avian DNA barcode when working with degraded DNA

Primer	Primer sequence (5′–3′)	Primer length	Primer 3′ end location relative to COI start codon	Original reference for the primer
BirdF1	TTCTCCAACCACAAAGACATTGGCAC	26	52	(2)
AvMiF1	CCCCCGACATAGCATTCC	18	285	(6)
AvMiR1	ACTGAAGCTCCGGCATGGGC	20	411	(6)
COIbirdR2	ACGTGGGAGATAATTCCAAATCCTGG	26	747	(6)
AWCF1	CGCYTWAACAYTCYGCCATCTTACC	25	−2	(12)
AWCintF2	ATAATCGGAGGCTTCGGAAACTGA	24	245	(12)
AWCintF4	TCCTCAATCCTGGGAGCAATCAACTT	26	493	(12)
AWCintR2	ATGTTGTTTATGAGTGGGAATGCTATG	27	275	(12)
AWCintR4	TGGGAKAGGGCTGGTGGTTTTATGTT	26	510	(12)
AWCintR6	GGATTAGGATGTAGACTTCTGGGTG	25	720	(12)

The top four primers generate two overlapping fragments and the bottom six primers generate three shorter, overlapping fragments (see Fig. 4 for a scheme showing how to combine each set of primers)

of reagents needed (and thus the cost of each reaction) and allows sequencing without cleanup because primers and dNTPs are consumed during the PCR reaction. Always prepare more volume than needed to account for pipetting error and dead volumes (e.g., prepare enough PCR mix for 100 reactions to

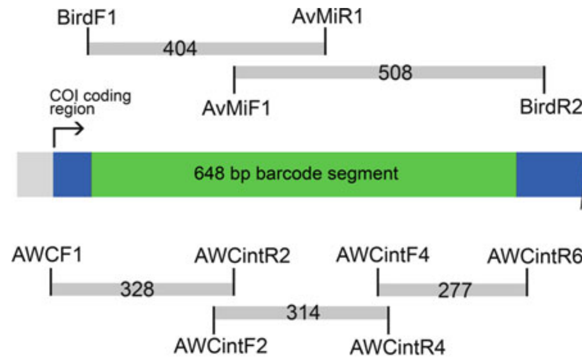

Fig. 4. Schematic of avian DNA barcode primers that can be used to amplify short, overlapping fragments when dealing with degraded DNA.

Table 4
Recipe for the PCR mix (this recipe is for using 2 µl of DNA extract, but the volume of ddH_2O can be adjusted to use between 1 and 4 µl of DNA template)

Reagent	Amount for 1 reaction (µl)	Amount for 100 reactions (one plate) (µl)
10% Trehalose	6.25	625
ddH_2O	2	200
10× Buffer	1.25	125
50 mM $MgCl_2$	0.625	62.5
10 µM forward primer	0.125	12.5
10 µM reverse primer	0.125	12.5
10 mM dNTPs	0.0625	6.25
Taq polymerase (5 U/µl)	0.06	6
Total	10.5	1,050
DNA template	2 µl per well or tube	

amplify an entire 96-well plate, see Table 4). Due to the high efficiency of the Platinum taq, it is important to minimize the risks of contamination (see Note 29 for recommendations).

3.3.3. Thermal Cycling Programs

1. The amplification parameters for first-choice primer sets applied to high-quality DNA are outlined in Table 5. The thermal cycle parameters for primer sets used for degraded DNA are outlined in Table 6. For programs used for other primer pairs, please check the references listed in Table 1.
2. PCR success can be visualized on 2% agarose gels. If working with 96-well plates, we recommend the use of the Invitrogen E-gel 96 system.

Table 5
Thermocycler parameters for first-choice primer sets for high-quality DNA

Primer pair	Thermocycle program
BirdF1 + COIbirdR2	94°C for 1 min, 5 cycles (94°C for 1 min, 45°C for 40 s, 72°C for 1 min), 35 cycles (94°C for 1 min, 51°C for 40 s, 72°C for 1 min), 72°C for 5 min
CO1F + CO1R	94°C for 2 min, 25 cycles (94°C for 20 s, 48°C for 45 s, 72°C for 30 s), 72°C for 3 min
PasserF1 + PasserR1	95°C for 3 min, 40 cycles (94°C for 1 min, 58°C for 40 s, 72°C for 1.5 min), 72°C for 5 min
AWCF1 + AWCR6	94°C for 2 min, 35 cycles (94°C for 30 s, 57.5°C for 30 s, 72°C for 30 s), 72°C for 4 min

Table 6
Thermocycle parameters for primer sets used to amplify degraded DNA

Primer set	Thermocycle program
BirdF1 + AvMiR1 AvMiF1 + COIbirdR2	94°C for 1 min, 25 cycles (94°C for 1 min, 45°C for 1.5 min, 72°C for 1.5 min), 35 cycles (94°C for 1 min, 55°C for 1.5 min, 72°C for 1.5 min), 72°C for 5 min
AWCF1 + AWCintR2 AWCintF2 + AWCintR4 AWCintF4 + AWCR6	94°C for 2 min, 10 cycles (94°C for 20 s, 55°C for 20 s, 72°C for 20 s), 30 cycles (94°C for 20 s, 50°C for 20 s, 72°C for 20 s), 72°C for 4 min

3.4. Cycle Sequencing

3.4.1. Sequencing Mixture

The recipe for the sequencing mix is detailed in Table 7. This recipe includes a stabilizer (i.e., 10% trehalose) so that premade mixes can be stored in a −20°C freezer for up to 3 months. The primer used depends on those used during PCR. For some primer pairs, unique primers are introduced for sequencing (see ref. 13). For high-throughput sequencing, M13 tails can be added to PCR primers to streamline cycle sequencing reactions (34).

3.4.2. Thermal Cycling Program

Cycle sequencing should generally involve the following thermal cycle: 96°C for 2 min; 30 cycles of 96°C for 30 s, 55°C for 15 s, and 60°C for 4 min; then hold at 4°C until samples are removed from the thermocycler.

Table 7
Recipe for the sequencing mix (this recipe is for using 1 µl of DNA extract)

Reagent	Amount for 1 reaction (µl)	Amount for 100 reactions (one plate) (µl)
BigDye Terminator v3.1	0.25	25
5× ABI sequencing buffer	1.875	187.5
10% Trehalose	5	500
10 µM sequencing primer	1	100
ddH_2O	0.875	87.5
Total	9	900
DNA template	1 µl per well or tube	

3.4.3. Sequencing Clean-up

The following protocol, adapted from that developed at the CCDB (32), details how to clean cycle sequencing reactions using the Sephadex method in 96-well plate format (plates are best prepared two at a time to provide a balance for centrifugation steps).

1. Measure out Sephadex G-50 proportions for a 96-well plate using a multiscreen column loader (Millipore), and then invert over a 96-well filter plate with 0.45 µm pore size.
2. Add 300 µl of ultrapure water to each well, and then allow the plate to sit for at least 1 h (alternatively, plates can be left overnight in a refrigerator at 4°C).
3. Assemble the filter plate with hydrated Sephadex into a sandwich with a 96-well microtiter catch plate. Centrifuge at 750 × *g* force for 3 min to remove the water from the well. Discard the flow through (the collection plate can be reused for this wash step in the future).
4. Pipette the entire contents of the sequence reaction onto the centre of the Sephadex columns.
5. Add 25 µl of 0.1 mM EDTA pH 8.0, to each well of a final collecting 96-well plate.
6. Place the filter plate over the final collecting plate (pay careful attention to plate orientation), and then centrifuge at 750 × *g* force for 3 min.
7. Discard Sephadex from the filter plate. Dry the contents of the final collection plate and seal once completely dry.

3.5. Data Analysis

3.5.1. Sequence Assembly and Verification

Bidirectional sequencing—a requisite for high-confidence base calls and GenBank keyword barcode compliance—necessitates contig assembly from forward and reverse sequencing reads. Several programs exist that can facilitate this process through automation (e.g., Sequencher or CodonCode). Assembled contigs should then be aligned. If no ambiguous base calls occur at the end of the sequences (i.e., sequences are of full length between primer-binding sites) or are relatively few, then sequences may be easily aligned by eye. Otherwise, sequence alignment software may be required (e.g., MEGA). Amino acid translations should be carefully scrutinized for rare mutations, which may be indicative of errors introduced during contig assembly.

3.5.2. Tree Based Verification

Newly obtained sequences should be reviewed for possible sources of error, including contamination, misidentifications, and pseudogenes. Most of these are most readily identified via phylogenetic methods, such as tree construction. Sequences uploaded to BOLD may be easily reviewed using the "Taxon ID Tree" tool under the "Sequence Analysis" menu, which generates a neighbor-joining tree of the selected sequences. The more species—and specimens per species—one includes in this analysis, the better chance one has to detect possible sources of error. When including large numbers of sequences, trees can be colorized based on the sequence age to highlight the specimens that were recently added to BOLD and one wants to check.

3.5.3. Contamination

Contamination may originate from lab products (which involve common laboratory species, such as *Mus musculus* or *Sus domestica*), from cross-well contaminants when using 96-well plates, or from diverse sources when using samples obtained from historical specimens (such as toe pad samples from museum skins). The former is readily identifiable, while cross-well contamination can usually be detected when the sequences obtained from two samples of the plate that belong to different species are identical, particularly if these species are quite different from each other and not likely to be confused (Fig. 5). However, cross-well contamination might be more challenging to confirm when closely related species are processed in the same plate (see Note 25). Sequences obtained from degraded DNA should be carefully scrutinized for accuracy because contaminant DNA can outperform that which is targeted—contamination should be suspected if there is an unexpected result.

3.5.4. Misidentifications

If lab errors and contamination have been ruled out, disagreements between specimen identification and barcode results should prompt the inspection of voucher materials. This is most pivotal when the species indicated by the barcode is similar to the one originally identified based on morphology (Fig. 6). In these cases, the identification of the specimen should be carefully reviewed.

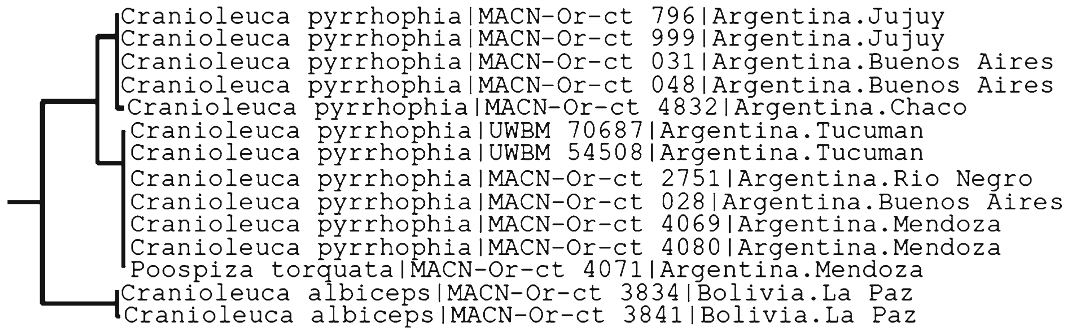

Fig. 5. An example of a BOLD species ID tree that depicts a cross-well contamination. Note the presence of a sequence theoretically belonging to a specimen of *Poospiza torquata* placed within the haplogroup of *Cranioleuca pyrrophia*. The evidence that this is likely a case of contamination includes two aspects: (**a**) the sequence is identical to others in the same plate (note that the process ID of the sample of *P. torquata* is LBARG050-10, and there are two other identical sequences from close wells in the plate: LBARG048-10 and LBARG058-10) and (**b**) *P. torquata*, which belongs to the family Emberizidae, is quite different from *C. pyrrophia*, which is a furnarid, and therefore an identification error can be ruled out.

Fig. 6. An example of a BOLD species ID tree that flags a possible misidentification. Note that *Saltator coerulescens* and *S. similis* are clearly separated in the neighbor-joining tree, but one specimen of *S. similis* (MACN-Or-ct 5072) is placed within the *S. coerulescens* clade. Because juveniles of *S. coerulescens* resemble the adults of *S. similis*, it is likely that a juvenile of *S. coerulescens* was collected and misidentified in the field as *S. similis*. The voucher has to be reanalyzed in cases like this, which is the main reason why it is so important to use vouchered samples for barcoding projects.

3.5.5. Nuclear-Mitochondrial Pseudogenes (Numts)

Numts are copies of mitochondrial genes that have been translocated to the nuclear genome. These copies are typically nonfunctional and, thus, accumulate nonsensical mutations, such as frameshifts and stop codons. These features usually allow pseudogenes to be rapidly identified. Full-length COI pseudogenes are uncommon but might be more frequently encountered when working with avian blood samples. In exceptional cases, characteristic features might be absent from a pseudogene, in which case a more thorough phylogenetic analysis may be required to properly identify it (21).

3.5.6. Assessing Species Resolution of DNA Barcodes

The "Nearest Neighbor Summary" tool, available under the "Sequence Analysis" menu in BOLD, can be used to identify low values of interspecific variation. Alternatively, neighbor-joining trees can also be used to highlight species that fail to produce independent haplogroups. Species pairs with highly similar sequences can be subjected to character-based analysis (35, 36) to identify diagnostic character states (i.e., single-nucleotide polymorphisms) that distinguish otherwise very similar sequences.

3.5.7. Identifying Divergent Intraspecific Lineages

The "Distance Summary" tool, available under the "Sequence Analysis" menu in BOLD, can be used to identify species with high levels of intraspecific variation, which can be indicative of cryptic species. Alternatively, neighbor-joining trees may be generated with bootstrap support to identify lineages that garner stronger statistical support when clustered independently rather than as one cohesive group.

3.5.8. Identifying Unknown Specimens

If barcode sequences have been acquired for the purpose of species identification (as opposed to generating a reference library), then these may be identified singly using either the BOLD Identification Engine (BOLD-IDS) or the Basic Local Alignment Search Tool (BLAST) available from NCBI (37). The main advantage of using the former system is that BOLD includes not only the public sequences that are available in GenBank, but also those that belong to particular projects, which are not visible to all users but are part of the database searched by the identification engine, increasing in this way the chances of a correct identification. In addition, identifications in BOLD can not only be done simply by sequence similarity (as in a BLAST search), but also using a tree-based approach.

4. Notes

1. It is usually necessary to obtain a permit to collect whole bird specimens, blood samples, or even feathers. A transit permit is also generally necessary to move specimens or samples between provinces/states within each country. A permit (or a series of

permits) is usually also needed to export/import tissue samples or DNA extracts. If samples originate from a species protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), then an additional permit is needed. It is essential to check national regulations before starting any barcoding project that potentially includes collection or movement of tissue samples or DNA extracts across borders.

2. Usually small- to medium-sized birds (ranging from hummingbirds to doves) can be captured with mist nets and larger species are hunted using firearms. There are also special types of nets used for particular groups of birds, such as cannon- or rocket-projected nets used for capturing shorebirds.

3. Weigh the guanidine thiocyanate first, add the required volumes of stock solutions and part of the ddH_2O needed to reach the final volume, vigorously mix on magnetic stirrer with heater until the guanidine thiocyanate is fully dissolved, and finally add more ddH_2O if necessary (when working at a smaller scale and preparing smaller volumes in a tube that cannot be placed in a magnetic stirrer, the solution can be warmed at 56°C and mixed with a vortex). If any recrystallization occurs, prewarm the solution at 56°C to dissolve the guanidine thiocyanate before using it.

4. The use of Platinum Taq polymerase (Invitrogen) has several advantages compared to the standard Taq, including higher success rate, higher amount of amplicons produced per sample, the need of a "hot start" for activation (which reduces amplification of nonspecific fragments), and stability at room temperature.

5. The use of trehalose stabilizes the PCR and allows freezing of aliquoted PCR mixes, which can simplify and accelerate workflow in high-throughput facilities.

6. For example, in studies supported by the funding sources of the International Barcode of Life project (iBOL), ten specimens per species is the upper limit. This is because the objective is to maximize the number of species represented in the database. However, in the case of species for which two or more lineages with deep genetic divergence are found, one might want to include more individuals to better study the genetic structure of the species. In fact, if the divergence between lineages suggests that they have been isolated for a long period of time and are evolving independently, up to ten specimens per lineage might be sampled for barcoding.

7. Depending on the characteristics of the collecting trip, specimens can be frozen in a freezer or in a cooler containing dry ice. Wet ice should be avoided because of its humidity and higher temperature, which does not guarantee that all specimens in the cooler will be kept frozen.

8. Other options are available for long-term storage of tissue samples at room temperature or in a refrigerator, such as drying the sample on ceramic beads or using FTA paper, but nitrogen or ultracold freezers are the most widely used and have been demonstrated to be effective for long-term storage.
9. Even though the sample is fixed, it is highly recommended to store the tube in an ultracold freezer or in nitrogen once it is deposited in the permanent collection if this is possible.
10. Ethanol should be replaced a few days after placing the sample inside the tube. This is because the water in the tissue sample is replaced by ethanol and it is released to the tube, thus diluting the ethanol. This is particularly relevant when the sample is large relative to the amount of ethanol.
11. We recommend to write the identification of the tube contents on adhesive labels with a pencil (covering it with tape), given that it is common for inked labeling to wear off after extended periods in an ultracold freezer or liquid nitrogen. In the case of samples fixed with ethanol, it is particularly important to use a pencil or to make sure that the marker is alcohol-proof because most permanent markers are only waterproof and are easily removed by ethanol (even by the ethanol vapors that can accumulate inside the boxes or bags in which the tubes are stored). Scratching the information on the tube is a highly discouraged alternative because it is unpractical and the data is later difficult to retrieve.
12. Freezing the sample is not recommended for some lysis buffers due to the tendency of some of their components to precipitate. In this case, we recommend keeping the samples in a refrigerator (particularly for long-term storage).
13. Vouchers are vital because they allow confirming the identification of species. In addition, they provide important information (e.g., about morphology) that can complement the conclusions reached through the DNA analysis. The importance of collecting bird specimens and preserving vouchers in addition to tissue samples has been emphasized in the literature (38–43).
14. There are different standards for vouchers, depending on the accuracy with which they permit species identification. The "gold standard" for most birds is a study skin from an adult male in breeding plumage, but other options are also valid when these ideal conditions cannot be met, including the use of juveniles or females (which might be as useful as males for species that lack sexual dimorphism) and the preparation of skeletons or ethanol-preserved specimens (ideally, in at least 96% ethanol).
15. We advise to preserve as many sources of information from the specimen as possible. The ideal situation is to prepare a study

skin and keep a partial skeleton, including the bones that are retired from the skin during preparation. Other materials, such as internal organs, the syrinx, stomach contents, or parasites, can also be preserved.

16. When blood samples are obtained and the individuals are not collected (as in the case of rare or endangered species), we recommend taking diagnostic photographs before the bird is released to serve as e-vouchers.
17. For projects using preexisting museum collections, the presence—and type—of vouchers should be considered when selecting samples.
18. This is particularly relevant when obtaining an e-voucher of a specimen that is bled and then released. However, it is also useful to take pictures of the living bird even if it will be collected because some characteristics might be better observed in the individual when it is alive (such as eye color, for example). Pictures can also aid in confirming the identification of the specimen after returning from the field (particularly in cases when preparation of vouchers is not done immediately).
19. It is very useful to store the data associated with the specimens in a database as compatible as possible with the data submission spreadsheet to minimize the work needed to upload this information to BOLD.
20. The toe pad is the best source of DNA from study skins. This is because the contact with preservatives that may damage DNA is reduced in this area and because the risk of cross-contamination is also minimized (particularly if the sample is obtained at some depth and not from the toe pad surface).
21. Use very small amounts of tissue. Larger samples complicate the extraction process rather than facilitating it. A rule of thumb is that the tissue sample is big enough as long as it can be seen with the naked eye.
22. Dissection instruments used for transferring tissue samples to the plate (or tube) should be sterilized between samples. This can be done using a DNA-removing detergent (such as ELIMINase). The instrument should be placed for a few seconds in the detergent and then washed three times using three different water containers (this way, the concentration of detergent decreases in each subsequent container and after the third wash, there should be no detergent left on the instrument). Alternatively, instruments can be sterilized with bleach or a flame (although the latter can damage the instruments much more than the detergent).
23. When working with toe pad samples, it is recommended to rehydrate and soften the tissue before digestion. Samples can be preincubated in phosphate-buffered saline (PBS) for 24 h

before digesting them with the Lysis Mix (44). A recipe for PBS includes the following final concentration of components: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, adjusted to pH 7.4. Note that the same plates or tubes that are used for the extraction can be used for the incubation with PBS, in which case the PBS should be pipetted out of each well or tube before adding the Lysis Mix (it is recommended to pulse centrifuge the plate or tube for a few seconds after pipetting the PBS and then pipette again any leftovers before adding the Lysis Mix).

24. When using feathers as a DNA source, undissolved keratin from feather barbs and barbules may clog the filter plates, obstructing DNA extraction. When working with larger feathers (i.e., rectrices), isolate the section of the feather shaft containing the superior umbilicus and use only this for your sample. When working with smaller feathers (i.e., contour feathers from passerine birds), feather keratin may be digested by adding 20 μl of 1 M dithiothreitol (DTT) solution to the Lysis Mix for each sample. Alternatively, DNA may be extracted from feathers using a Chelex-based method (45), but this results in a poor-quality DNA extract.

25. Because cross-well contamination between conspecific samples is virtually impossible to detect, we recommend not placing tissue samples from the same species in contiguous wells.

26. It is always a good practice to clean the bench top with ethanol before starting the extraction protocol and to change gloves if they touch possible sources of contamination. Furthermore, pipettes used for the extraction protocol should never be used to handle PCR products. These caveats are particularly important when extracting DNA from samples obtained from historical specimens (such as toe pad samples from study skins). In this case, change gloves often, use filter tips, and work as isolated as possible from other areas of the laboratory in which other samples, DNA extracts, or PCR products are handled.

27. In the case of samples obtained from toe pads or feathers in which digestion tends to be more complicated, longer incubation periods might be necessary to completely digest the tissue. Incubate samples for 24 h or until there is no visible undegraded tissue. Adding 2–3 μl of proteinase K (20 mg/ml) after the first 6 h of incubation is recommended.

28. Square-well blocks can be washed with a DNA-removing detergent, autoclaved, and reused.

29. To minimize the risks of contamination, always clean the bench top before preparing the PCR mix, change gloves if they touch any contaminants, add DNA template lastly after reagents have been returned to the freezer, and try not to work with PCR

products in the area where PCR mix is prepared. Ideally, a different set of pipettes should be used for handling PCR reagents and PCR products (and a third one for performing DNA extraction). Contamination is particularly problematic when amplifying the barcode from an extract containing degraded DNA (such as with toe pad samples) because a fragment of the nondegraded, contaminant DNA might have higher chances of being amplified than the target DNA. In these cases, it is vital to be extra careful; change gloves often, always use filter tips to prepare the PCR mix, and work as isolated as possible from other areas of the laboratory in which other samples, DNA extracts, or PCR products are handled. To detect contamination of reagents, always include a negative control.

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