‘Direct PCR’ optimization yields a rapid, cost-effective, nondestructive and efficient method for obtaining DNA barcodes without DNA extraction

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Abstract

Macroinvertebrates that are collected in large numbers pose major problems in basic and applied biodiversity research: identification to species via morphology is often difficult, slow and/or expensive. DNA barcodes are an attractive alternative or complementary source of information. Unfortunately, obtaining DNA barcodes from specimens requires many steps and thus time and money. Here, we promote a short cut to DNA barcoding, that is, a non-destructive PCR method that skips DNA extraction (‘direct PCR’) and that can be used for a broad range of invertebrate taxa. We demonstrate how direct PCR can be optimized for the larvae and adults of nonbiting midges (Diptera: Chironomidae), a typical invertebrate group that is abundant, contains important bioindicator species, but is difficult to identify based on morphological features. After optimization, direct PCR yields high PCR success rates (>90%), preserves delicate morphological features (e.g. details of genitalia, and larval head capsules) while allowing for the recovery of genomic DNA. We also document that direct PCR can be successfully optimized for a wide range of other invertebrate taxa that need routine barcoding (flies: Culicidae, Drosophilidae, Dolichopodidae, Sepsidae; sea stars: Oreasteridae). Key for obtaining high PCR success rates is optimizing (i) tissue quantity, (ii) body part, (iii) primer pair and (iv) type of Taq polymerase. Unfortunately, not all invertebrates appear suitable because direct PCR has low success rates for other taxa that were tested (e.g. Coleoptera: Dytiscidae, Copepoda, Hymenoptera: Formicidae and Odonata). It appears that the technique is less successful for heavily sclerotized insects and/or those with many exocrine glands.

Keywords: Chironomidae, DNA barcodes, DNA extraction, macroinvertebrates

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‘Any study in community ecology requires, ideally, that the individuals in a sample be properly counted and identified to species’ Gotelli 2004

Introduction

DNA barcoding based on COI (5′ cytochrome *c* oxidase subunit 1) has been promoted as a method for species identification for many taxa (Hebert *et al.* 2003; Vences *et al.* 2005; Ward 2009; Dincă *et al.* 2011; Zou *et al.* 2011; Keskin & Atar 2013). Arguably, DNA barcodes are particularly useful when morphological identification is

difficult, time-consuming and/or error-prone (Meier *et al.* 2008; Valentini *et al.* 2009; Schlick-Steiner *et al.* 2010). This is often the case for invertebrate taxa that are small and very abundant. Such hyperabundant yet hard-to-identify taxa are the main obstacle to species-based bioassessment, biodiversity monitoring and community ecology. For these taxa, DNA barcoding can provide an alternative or complementary method to morphological identification (Pfenninger *et al.* 2007). However, this requires that the barcodes accurately reflect species boundaries for at least most species in a sample and that the sequences can be obtained rapidly and at low cost. We here address the latter two issues by promoting the use of ‘direct PCR’, a method that makes it cheaper and faster to obtain DNA barcodes.

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In direct PCR, the time and cost for obtaining DNA barcodes are reduced by placing tissue directly into a PCR master mix without DNA extraction prior to amplification of the target gene. This aids in reducing the time taken (4–16 h depending on extraction protocol) and cost by eliminating the usage of kits and decreasing manpower needs. Direct PCR procedures were proposed as early as 1993 (Panaccio *et al.* 1993), but success rates were either unreported or low. For example, Panaccio *et al.* (1993) and Grevelding *et al.* (1996) did not mention amplification success rates nor did they discuss whether the amplified specimens were suitable for morphological study and vouchers. Rochlin *et al.* (2007) evaluated the method by placing one *Culex* mosquito leg into the well containing PCR reagents and distilled water but reported low success rate (<50%) that were not likely to encourage the use of this method. Yet, our experience with the method indicates that it can yield consistently high success rates (>80%) for a wide range of taxa including flies (Culicidae, Drosophilidae, Dolichopodidae, Sepsidae) and sea stars (Oreasteridae). Key to success is the optimization of a few parameters. We thus consider direct PCR a convenient method for taxonomists and molecular ecologists who routinely have to obtain DNA barcodes for taxa, which are used widely for bioassessment or studies of genetic variability (e.g. Chironomidae, Culicidae, Drosophilidae).

Direct PCR of Chironomidae

We here use nonbiting midges (Diptera: Chironomidae) to describe how to optimize direct PCR. These midges are extremely abundant in many freshwater samples and routinely used as key bioindicator taxa for water quality assessment (Ferrington 2008; Luoto 2011; Milošević *et al.* 2013) as different species have distinct ecological preferences (Punti *et al.* 2009; Krosch & Cranston 2012; Molozzi *et al.* 2012). However, accurate identification of chironomid larvae is difficult because the morphological features are often minuscule and easily obscured (Epler 2001; Sharley *et al.* 2004; Hajibabaei *et al.* 2011; Kim *et al.* 2012). These factors can lead to high error rates when the larvae are identified by parataxonomists (Cranston & Hillman 1992; Krell 2004), thus impacting subsequent water quality analyses. In addition to identification problems, the cost of morphological identification is high because microscopic slides have to be prepared (Cranston 1994; Epler 2001; Carew *et al.* 2007; Cranston *et al.* 2013), which requires approximately 15–20 min per specimen. Yet, it is not unusual to find thousands of chironomid larvae in one environmental sample. It is therefore not surprising that the high cost of morphological identification has led to a debate whether chironomid larvae should be included in bioassessment of aquatic environments

(Kerans & Karr 1994; Cranston 2000; Rabeni & Wang 2001; Nijboer *et al.* 2005; Roque *et al.* 2010) although recent studies have shown that inclusion improves accuracy and signal-to-noise ratio (Ferrington 2008; Raunio *et al.* 2011; Brodin *et al.* 2013; Milošević *et al.* 2013). In addition, the high abundance of chironomids in urban aquatic systems can degrade the recreational value of waterfront areas. Thus, their mass occurrence can have serious economic implications and correct species-level identification for their control is crucial (Cranston *et al.* 2013).

Given the problems with morphology in this case, DNA barcodes are an obvious alternative or complementary tool as long as one can demonstrate that at least most midge species have discrete barcodes. Currently, the chironomid literature indicates that this is the case. Most barcoded species are separated by genetic distances of >4% at the DNA barcode region while intraspecific variation rarely exceed 2% (Ekrem *et al.* 2007, 2010; Sinclair & Gresens 2008; Cranston *et al.* 2013). Beyond their use in routine identification, DNA barcodes have other important uses in Chironomidae. First, species are traditionally described based on adults, rendering larvae unidentifiable until they are associated with adults via rearing or DNA barcoding. Making such associations is important because larvae are the more important stage for freshwater assessment (Ferrington 2008; Marziali *et al.* 2009; Luoto 2011; Nandi *et al.* 2012; Milošević *et al.* 2013) and nowadays DNA barcodes are the choice method for this purpose (Carew *et al.* 2005; Ekrem *et al.* 2007). Second, DNA barcodes have been important for the discovery of putatively cryptic species (Ekrem *et al.* 2007; Sinclair & Gresens 2008).

Here, we describe how an optimized direct PCR procedure without prior DNA extraction can yield success rates of 80–100% for a range of taxa. Furthermore, we document that direct PCR preserves the cuticle such that diagnostic morphological features can be studied and allows for the recovery of genomic DNA for the amplification of additional mitochondrial and nuclear genes. We also discuss other invertebrate taxa for which we were able to optimize direct PCR successfully.

Materials and methods

Chironomidae

Larvae and adults of various sizes and species were collected from three freshwater habitats (Bedok Reservoir—1°20'32"N 103°55'30"E, Upper Seletar Reservoir—1°24'04"N 103°48'14"E, Pandan Reservoir—1°18'50"N 103°44'30"E) in Singapore via sediment grab sampling (larvae) or light trapping (adults) and preserved in 70–100% ethanol or isopropanol (Cranston *et al.* 2013).

Larvae and adults were presorted into three size classes (Fig. 1). For adults, we consider 'small' midges to range from (anterior to posterior) 1.5–2.4 mm, 'medium' from 2.5 to 3.4 mm and 'large' >3.5 mm. The corresponding size classes for larvae are small (3.0–4.4 mm), medium (4.5–6.4 mm) and large (6.5–8.0).

Direct PCR optimization

We initially used whole specimens for direct PCR. The success rates with primer pair LCO 1490/HCO 2198 (Folmer *et al.* 1994) were as low as reported in the literature (<50%; Rochlin *et al.* 2007; small adults, $n = 8$, 87.5% successful; medium adults, $n = 4$, 0%; large adults, $n = 4$, 0%; small larvae, $n = 8$, 50%; large larvae, $n = 8$, 37.5%). Exceptions were the smallest adults (1.5–2.4 mm) that yielded high success rates. We thus adjusted the tissue amount for medium- and large-sized adults (2.5–3.4 mm and >3.5 mm, respectively), and all three size classes of larvae (3.0–4.4 mm, 4.5–6.4 mm and 6.5–8.0 mm, respectively). High success rates were obtained when using (i) three legs from medium-sized adult and two legs from large-sized adults ($n = 4$: 100% successful), (ii) the dissected anterior body segments from larvae of all three size classes (small larvae, $n = 5$: 80% successful; medium larvae, $n = 10$: 90% successful; large larvae, $n = 5$: 60% successful). We also experimented with another tissue source (dissected abdomen of mid- and large-sized adults; $n = 4$: 0% successful). After these trials with small numbers of specimens, we applied the methods to larger numbers of speci-

mens (see below). In addition to tissue amount and source, the type of polymerase influenced PCR success rates. Four different polymerases were tested (TaKaRa ExTaq, Qiagen HotStar Taq, KAPA Taq and a homemade Taq). The total reaction volume was 20 μ L, with 2 μ L of Taq 10 $\times$ buffer, 1.5 μ L of 2 mM dNTPs, 1 μ L of 10 μ M primers and varying volume of Taq- and DNase-free sterile water depending on the Taq brand (0.15 μ L TaKaRa ExTaq; 0.5 μ L Qiagen HotStar Taq; 0.5 μ L KAPA Taq; 1 μ L homemade Taq). The highest success rates were achieved using TaKaRa ExTaq while KAPA Taq and the homemade Taq gave successful amplifications, but the products were often 'smeary' under UV light. Qiagen HotStar Taq did not yield successful amplifications. Given that the removal of legs requires time, we tested also if whole specimens for midges and larvae in the mid- and large-sized classes could be used if a larger reaction volume (40 μ L) was used, but success rates were not as consistent and predictable as with 20 μ L and the tissues described above. Lastly, the addition of 1 μ L (1 mg/mL) of bovine serum albumin (BSA) did not influence success rates.

Optimized protocol for direct PCR and sequencing

After optimizing direct PCR for chironomids, we tested the procedure with a new and larger batch of specimens (Table 2: 30–60). Whole specimens (small adults), three legs (medium adult), two legs (large adult), anterior half of larval body (small larvae), or head capsule and 2–3 anterior 'segments' (total length = ~1 mm) for the

Fig. 1 Sizes of specimens and the corresponding parts (indicated by arrows) used for direct PCR. Larvae are shown in the top row: (A) small-sized larvae, (B) medium-sized larvae and (C) large-sized larvae. Adults are shown in the bottom row: (D) small-sized adults, (E) medium-sized adults and (F) large-sized adults.

medium- and large-sized larvae were used. Relevant body parts intended for direct PCR were removed, dried briefly, then placed into the PCR wells and dried further for 1 min using a Speed-Vac. PCR reaction mixtures were prepared (20 μ L volume: 2 μ L of 10 $\times$ ExTaq Buffer, 1.5 μ L of 2 mM dNTP mixture, 0.15 μ L of TaKaRa ExTaq polymerase, 1 μ L of 10 μ M primers for the forward and reverse direction, DNase-free sterile water) and subsequently pipetted into the wells containing the dried specimens. The DNA barcoding region consisting of a 658-bp fragment of *COI* was amplified using the general invertebrate primers LCO 1490—5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and HCO 2198 5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3' (Folmer *et al.* 1994). Cycling conditions were: denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s and extension at 72 °C for 1 min. The cycle was repeated for 41 times. After PCR, the body parts inside the PCR wells were removed, preserved as vouchers in 70% or 100% ethanol and stored in –20 °C. The amplification results were checked with electrophoresis on a 1% agarose gel stained with GelRed (Biotium Inc.). Successfully amplified products were purified with SureClean™ (Bioline) according to the manufacturer's instructions. Purified products of small- and large-sized adults and larvae were sequenced in both directions with BigDye (PerkinElmer) terminator reactions and analysed on the ABI Avant 3130xl Genetic Analyzer. Medium-sized adults and larvae were sequenced in single direction (forward) after we found that single direction sequencing can yield >500 bases which is sufficient for routine species delimitation.

The chromatograms were assembled and edited using Sequencher ver. 4.6. Alignment of sequences was performed in MAFFT version 7 with default options. The alignments were translatable to amino acid sequences free of stop codons and the sequences checked via MEGABLAST in GenBank and compared to sequences in our own local database for chironomids of Pandan, Bedok, and Upper Seletar Reservoirs in Singapore (Cranston *et al.* 2013). The sequences were assigned to species clusters as described in Meier *et al.* (2006) using uncorrected pairwise distances (4%) as recommended in Srivathsan & Meier (2012).

Retrieval of body parts for morphological study

Adult chironomids are identified based on male genitalia, structures of the thorax and wing patterns, while larvae are distinguished based largely on characters of the head capsule (Cranston 1994; Epler 2001; Cranston *et al.* 2013). It was thus important to ascertain that specimens that had undergone direct PCR could still be used as vouchers. Slide mounts of specimens that had undergone direct PCR were prepared using Hoyer's mounting

agent. For medium- and large-sized adult, this test was not needed because the diagnostically important body parts were not used during direct PCR and even the legs could be retrieved after direct PCR.

Recovery of genomic DNA

We tested two methods for recovering genomic DNA from the small adults that were exposed to direct PCR. First, the specimens from the PCR wells were taken out and subjected to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. For the second method, we performed genome amplification for 14 *COI* PCR products obtained with direct PCR earlier. The DNA was successfully amplified using the Repli-G Mini Kit (Qiagen) according to manufacturer's protocol but with halved reaction volumes (from 50 to 25 μ L). Subsequently, PCRs were performed for three genes using the genomic DNA obtained by both methods (*COI*: nonbarcode segment; *18S*: nuclear rDNA; *AATS*: segment 1, nuclear protein-encoding). These genes were used previously in phylogenetic studies of Chironomidae (Cranston *et al.* 2010, 2012; Dahle 2012; Krosch & Cranston 2012). Primer sequences and PCR cycling conditions are provided in Table 1.

Direct PCR of other taxa

We subsequently used the same optimization procedures described here in an attempt to optimize direct PCR for other taxa (Copepoda; Coleoptera: Dytiscidae; Hymenoptera: Formicidae and Odonata; Diptera: Culicidae, Dolichopodidae, Drosophilidae and Sepsidae; Valvatida: Oreasteridae).

Specimens of Drosophilidae and Sepsidae were obtained from reared cultures at Temasek Life Sciences Laboratory and Evolutionary Biology Laboratory (National University of Singapore), respectively. Culicidae specimens were either obtained from Malaise traps or from cultures at Duke-NUS Graduate Medical School insectary, while all specimens of Dolichopodidae and Formicidae were collected from Malaise traps. The reared culture specimens were preserved in 100% ethanol, while the Malaise trap specimens were preserved in 70% ethanol. The Oreasteridae were sampled at Chek Jawa, Pulau Sekudu, Cyrene Reef, Pulau Semakau and Beting Bronok, while the Odonata samples came from Golf Link Marsh, Kent Ridge Park, Bishan Park, Nee Soon Swamp Forest and Pulau Ubin and the Copepoda samples from Pandan and Upper Seletar Reservoirs. The specimens were preserved in 100% ethanol. All locations mentioned are in Singapore. The Dytiscidae specimens were collected in Brandenburg, Germany, and preserved in 96% ethanol.

Table 1 Primers and cycling conditions used for PCRs of genome-amplified PCR products

Locus/Primer	Primer sequence	Reference	Cycling condition
<i>COI</i>			Denaturation: 94 °C, 30 s
s2183	5' CAA CAT TTA TTT TGA TTT TTT GG 3'	Simon <i>et al.</i> (1994)	Annealing: 51–54 °C, 30 s
a3014	5' TCC AAT GCA CTA ATC TGC CAT ATT A 3'	Simon <i>et al.</i> (1994)	Extension: 72 °C, 1 min 30 s
			Final extension: 72 °C, 3 min
			Number of cycles: 41
18S			Denaturation: 94 °C, 1 min
18S_ai	5' CCT GAG AAA CGG CTA CCA CAT C 3'	Whiting <i>et al.</i> (1997)	Annealing: 55–58 °C, 1 min
18S_bi	5' GAG TCT CGT TCG TTA TCG GA 3'	Whiting <i>et al.</i> (1997)	Extension: 72 °C, 1 min 30 s
			Final extension: 72 °C, 3 min
			Number of cycles: 41
AATS			Denaturation: 94 °C, 30 s
A1-92F	5' TAY CAY CAY CAN TTY TTY GAR ATG 3'	Regier (2008)	Annealing: 51–54 °C, 30 s
A1-244R	5' ATN CCR CAR TCN ATR TGY T 3'	Feng-Yi Su <i>et al.</i> (2008)	Extension: 72 °C, 1 min
			Final extension: 72 °C, 3 min
			Number of cycles: 41

Results

Amplification success rate

DNA barcodes were sequenced for three different size classes of chironomid adults and larvae. We tested ≥ 30 specimens for each size class and stage. The direct PCR success rates were high, ranging from 90% to 100% for all size classes and stages (Table 2; see Fig. 2A). DNA sequence quality was comparable to what would have been obtained using traditional techniques involving DNA extraction (Fig. 2B). Sequence clustering at 4% using SpeciesIdentifier ver. 1.7.9 (Meier *et al.* 2006) revealed that the adult DNA barcodes belonged to eight molecular operational taxonomic units (MOTUs), while larval barcodes belonged to 10 MOTUs. According to our morphological and molecular databases for Singapore's reservoirs, the MOTUs belong to the following species/genera: *Tanytarsus oscillans* Johannsen, 1932; *Tanytarsus ovatus* Johannsen, 1932; *Cladotanytarsus* sp.1, *Cladotanytarsus* sp. 2, *Paratanytarsus* sp.1, *Polypedilum nubifer*

Skuse, 1889; *Polypedilum leei* Freeman, 1961; *Polypedilum* (*Pentapedilum*) *nodosum* Johannsen, 1932; *Polypedilum griseoguttatum* Kieffer, 1921; *Polypedilum* sp. 1 and *Chironomus circumdatus* Kieffer, 1916.

For the nonchironomid taxa tested, the initial amplification success rates were low, but rose to $>80\%$ for most taxa after optimizing tissue quantity, tissue type, primer pair and Taq polymerase (Table 3). Once optimized for a particular taxon, the same recipe worked consistently and could be used for subsequent samples. However, certain taxa appeared unsuitable for direct PCR. Regardless of body parts used (head, legs and thorax), Formicidae yielded low success rates of $<20\%$, possibly due to glands that may produce PCR inhibitors (Billen 2009; Schrader *et al.* 2012). Optimization was also unsuccessful for the heavily sclerotized Dytiscidae and Copepoda. Of the taxa with less sclerotized legs/bodies, only Odonata failed to yield high success rates. The taxa that failed to amplify via direct PCR could be amplified with extracted DNA when using normal PCR recipes with the same primer pairs thus suggesting that direct PCR was the source of the problem.

Table 2 Chironomidae: number of direct PCR reactions and associated success rates. Refer to Dryad doi in data accessibility for full specimen information

Morphospecies	Sample size	Successful attempts	Success rate for first amplification attempt (%)	GenBank Accession no.
Small adult	62	59	95.2	KJ530765–KJ530823
Medium adult	33	31	93.9	KJ530824–KJ530854
Large adult	31	28	90.3	KJ530855–KJ530882
Small larvae	30	30	100	KJ530883–KJ530912
Medium larvae	30	29	96.7	KJ530913–KJ530941
Large larvae	30	28	93.3	KJ530942–KJ530969

Fig. 2 (A) Agarose gel electrophoresis of direct PCR product from small-sized larvae 1 (L1–L8) using LCO-1490 and HCO-2198. WL, DNA weight ladder; N, negative control, (B) DNA sequence with BigDye nucleotide peaks visualized in DNA Sequencer (position 78 to position 224).

Recovery of morphological features and genomic DNA in Chironomidae

Direct PCR does not impair the morphologically important diagnostic characters. Male genitalia characters such as anal tergite bands, medium volsella, superior volsella, and gonostylus shapes, among others were preserved during direct PCR. Similarly, the characters of larvae such as the mentum, mandible, antenna etc were intact after the procedure (Fig. 3).

It is shown that genomic DNA can be recovered after direct PCR via one of the two methods tested. Of the two genomic DNA recovery methods described above, genome amplification of the direct PCR products proved more effective because the success rates for PCR of three nonbarcoding markers were significantly higher: *COI*: 100% (14/14), *AAT51*: 100% (14/14), *18s rDNA*: 78.6% (11/14) than those

with extracted DNA: *COI*: 64.3% (9/14), *AAT51*: 0% (0/14), *18s rDNA*: 21.4% (3/14).

Discussion

Direct PCR has high success rates for a wide range of taxa once a few key parameters are optimized. The most critical factor is the amount of tissue because too little or too much template released during the initial heating step of PCR often leads to PCR failure, presumably due to a suboptimal reagent ratio (Kramer & Coen 2001). Fortunately, nowadays PCR is successful across a fairly wide range of reagent ratios so that, for example, for chironomids we only have to differentiate between three different size classes in order to obtain high success rates. Note that high success rates for direct PCR are clade-independent because the tested specimens belong to five genera

Table 3 List of taxa and success rates for direct PCR

Taxa	Life stages	Body parts used*	Primer Pairs (<i>COI</i>)	Sources	Type of polymerase	Number of successful trials	Success rate (%)
Culicidae	Adult	2–3 legs	LEP F1/LEP R1	Hebert <i>et al.</i> (2004)	TaKaRa ExTaq	27/30	90
Drosophilidae	Adult	2–3 legs/ whole body	LCO-1490/HCO-2198 or LEP F1/LEP R1	Folmer <i>et al.</i> (1994)/ Hebert <i>et al.</i> (2004)	TaKaRa ExTaq	25/30	83.3
Dolichopodidae	Adult	1–3 legs	LCO-1490/HCO-2198	Folmer <i>et al.</i> (1994)	homemade Taq	17/20	85
Sepsidae	Adult	1–3 legs	mtD4/mtD9	Simon <i>et al.</i> (1994)	TaKaRa ExTaq	77/95	81.1
Oreasteridae	Adult	1 mm ³ tube foot	tRNAasn42F/ ValvaCOI-770R	Crandall <i>et al.</i> (2008)	homemade Taq	78/83	93.9

*Depends on the size of specimens, *c.f.* Chironomidae's size.

Fig. 3 Morphology of adults and larvae after direct PCR (A) Hypopygium morphology of *Tanytarsus* sp. ap, anal point; iv, inferior volsella; sv, superior volsella; gs, gonostylus. (B) Larval head capsule. mt, mentum; vmp, ventromental plate; mdb, mandible.

(*Tanytarsus*, *Cladotanytarsus*, *Paratanytarsus*, *Polypedilum*, *Chironomus*). Adult chironomids like most other arthropods are particularly convenient tissue sources for direct PCR because (i) legs are easily removed thus providing a convenient way for scaling tissue quantity, (ii) they rarely contain large glands that may harbour PCR inhibitors, and (iii) they are bilaterally symmetrical so that diagnostic features can be retained on the specimen by removing only one leg on either side of the body. Should initial direct PCR fail, additional pairs of leg are available for a second trial. Note that the legs can be retrieved after the procedure without significant damages to the structures.

A second factor that influences direct PCR success rates is the choice of Taq polymerase. Over the years, we have observed that some taxa require expensive and high-fidelity Taq polymerases (e.g. Chironomidae) while high success rates can be attained for other taxa even with low-cost and homemade enzymes (e.g. Sepsidae). Given that simultaneous optimization of tissue quantity and enzyme is time-consuming, we recommend first optimizing the former with a high-fidelity Taq polymerase. Once PCR success rates are high, a cheaper and/or more versatile Taq polymerase can be tested. A third factor that influences PCR success rates is the source of tissues. For insects, legs generally work well while abdominal tissues should be avoided. Low success rates for the latter suggest that they may contain PCR inhibitors that could come from the digestive tract (Juen & Traugott 2006). If abdominal tissues cannot be avoided (e.g. chironomid larvae), success rates increase when the anterior-most abdominal segments are used. In addition, we have recently increased success rates further using more specific primer pairs (LEP F1/LEP R1 instead of LCO 1490/HCO 2198 for insect taxa). Lastly, not surprisingly, storage conditions of specimens influence success rates. Fresh specimens that are used immediately after preservation and/or are stored at low temperatures amplify easily, whereas older material with inferior preservation and storage conditions can fail. There are excep-

tions, however, and we routinely use direct PCR successfully for Malaise trap material preserved initially in 70% ethanol (Dolichopodidae).

Direct PCR: why now?

Given that direct PCR procedures have been known for more than 20 years, one has to wonder why they have not been widely adopted. We believe that there are three reasons. First, early publications on direct PCR reported low or no success rates which are unlikely to encourage the adoption of the method. We find that such discouragingly low rates are normal during initial rounds of direct PCR optimization. We believe that some laboratories have tried direct PCR, but abandoned it following low, initial success rates. These laboratories never went to the optimization stage.

Second, scientists may feel uneasy about direct PCR procedures that use whole specimens because the initial barcode may reveal that a specimen belongs to a particularly interesting species. Sequencing of additional genes is then desirable, but it remained untested whether genomic DNA can be recovered from a specimen that had already been used in direct PCR. We here show that this is possible as long as a genome amplification of the original amplification product is performed. Using the amplified genome as template, we were able to sequence additional mitochondrial and nuclear markers. Initially, we worried that such procedure would overamplify the COI sequence because it is already enriched in the direct PCR product, but the primers used by the Repli-G Mini Kit appear to be sufficiently diverse that genomic DNA is amplified effectively. Note that the recovery of template DNA via genome amplification is needed only for small specimens where the whole body is used as template in direct PCR. For most specimens, direct PCR utilizes only legs so that most of the specimen remains in pristine condition and can be used for DNA extraction.

Lastly, based on previous studies on direct PCR, it was unclear whether the morphology of specimens

undergoing direct PCR remained intact for vouchering. In this study, we found that even sensitive and small body parts are well preserved. Re-identification and description of specimens that have undergone direct PCR can be performed.

Direct PCR: still relevant?

Molecular ecology and bioassessment are quickly adopting next-generation-sequencing (NGS) (Hajibabaei *et al.* 2011; Carew *et al.* 2013) and one may wonder whether there is still a need for a technique such as direct PCR. However, the analysis of NGS data generally involves the identification of species (e.g. food species) via barcode-like markers (Porazinska *et al.* 2009; Zhou *et al.* 2013). Hence, DNA barcode databases are still very much needed. Unfortunately, these databases remain extremely species-poor for many groups (Harris 2003; Ekrem *et al.* 2007; Begerow *et al.* 2010; Kwong *et al.* 2012; Kvist 2013). Improving species coverage will require a large amount of species-specific Sanger sequencing in order to obtain new DNA barcodes for additional species. Given that many very common species have already been barcoded, most new barcodes will have to be generated for rare species and these are likely to be produced by laboratories that specialize in particular taxa and that thus have an incentive for optimizing direct PCR for these taxa. Indeed, much of the species coverage for COI sequences in GenBank already comes from projects that are unlikely to be associated directly with DNA barcoding studies (Kwong *et al.* 2012).

Direct PCR will be particularly attractive for two types of laboratories. The first type is invertebrate systematics laboratories. Such laboratories routinely acquire large numbers of specimens in target groups. Many such laboratories have already adopted sample processing procedures that consist of rough sorting based on morphology with subsequent testing of the morphological units via COI sequencing for exemplars from the putative morpho-species (Tan *et al.* 2010). Species identification is ultimately achieved through the evaluation of multiple data sources (Tan *et al.* 2010; Yeates *et al.* 2011). This procedure requires that many specimens are barcoded and any shortcut to the process (e.g. direct PCR) is very welcome. The second type of laboratories that should be interested in direct PCR is those that employ parataxonomists in bioassessment. These laboratories are increasingly aware that the accuracy of the sorting by parataxonomist can be improved through a feedback loop that provides information on the validity of the morphospecies that were delimited by parataxonomists (Stribling *et al.* 2008). Ideally, the verification source is taxonomic experts, but they are often not available and

the next best choice would be DNA barcodes. Again, for these laboratories, a cheaper and faster technique for generating barcodes will help with implementing iterative procedures for validating parataxonomists' morpho-species boundaries. Moreover, the DNA barcodes generated can serve as libraries that are subsequently used for NGS-based bioassessment.

Conclusions

Direct PCR is an old technique neglected by most molecular ecologists. However, our study demonstrates that for many taxa, high success rates can be achieved without substantial damage to specimens once PCR conditions have been optimized. This reduces overall sequencing cost by saving time via skipping the DNA extraction step. Based on several years of experience, we only occasionally encounter taxa for which direct PCR optimization fails. Note that further reductions in cost can be achieved through one-directional sequencing, which is sufficient for routine identifications.

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W.H.W. and R.M. conceived the study and designed the experimental setups with input from M.B. and P.S.C. Y.C.T. and J.P. performed direct PCR optimization on Oreasteridae and Dolichopodidae respectively, while optimization of other nonchironomid taxa was performed by W.H.W. Analysis of data and writing of manuscript were done by W.H.W., with input from R.M., M.B. and P.S.C.

Data Accessibility

DNA sequences are uploaded to NCBI and have the following GenBank accession nos KJ530765–KJ530969. Sequence alignment and an Excel file containing individual specimen information were deposited in the Dryad database (doi: 10.5061/dryad.4q4kf).