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New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes)

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Abstract

Digeneans and cestodes are species-rich taxa and can seriously impact human health, fisheries, aqua- and agriculture, and wildlife conservation and management. DNA barcoding using the COI Folmer region could be applied for species detection and identification, but both 'universal' and taxon-specific COI primers fail to amplify in many flatworm taxa. We found that high levels of nucleotide variation at priming sites made it unrealistic to design primers targeting all flatworms. We developed new degenerate primers that enabled acquisition of the COI barcode region from 100% of specimens tested (n = 46), representing 23 families of digeneans and 6 orders of cestodes. This high success rate represents an improvement over existing methods. Primers and methods provided here are critical pieces towards redressing the current paucity of COI barcodes for these taxa in public databases.

Keywords: Cestoda, COI, Digenea, DNA barcoding, Platyhelminthes, Primers

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Introduction

Digenea (flukes) and Cestoda (tapeworms) are among the most species-rich groups of parasitic metazoans. Although involved in major disease in humans and wildlife, the identity of pathogenic species is often poorly characterized (Fürst *et al.* 2012; Thétiot-Laurent *et al.* 2013). Traditional morphology-based detection and identification is often hampered by the small size and inaccessibility within hosts in these organisms. A lack of distinctive morphological features in larval stages and even adults in some groups (e.g. see Kolářová 2007 and references therein) further confound identification to species level.

DNA barcoding is a widely used tool for specimen identification to species level, but despite early success with 'universal' Folmer primers (Folmer *et al.* 1994) in a diverse range of animal taxa, including 14 flatworms (six digeneans and eight cestodes; Hebert *et al.* 2003), it was soon recognized that primer modification would be needed for reliable amplification of the COI barcode in many taxa (Hajibabaei *et al.* 2005). Primer development efforts in COI barcoding thus far have only involved a limited number of flatworm taxa and low representation of nucleotide variation.

Moszczynska *et al.* (2009) developed degenerate primers targeting the Folmer region for digeneans and cestodes that were reasonably successful within a limited number of groups therein. These included the Strigeida (particularly Clinostomidae, Diplostomidae and Strigeidae; Locke *et al.* 2010; Caffara *et al.* 2011; Locke *et al.* 2011) and isolated taxa within the Echinostomida (Psilostomidae; Bergmame *et al.* 2011) and Plagiorchiida (Heterophyidae and Paragonimidae; see Ferguson *et al.* 2012; López-Caballero *et al.* 2013). However, the success rate was only 5% in cestodes (1/20 specimens) and 40% in digeneans (231/572 specimens; see Table S1 (Supporting information) in Moszczynska *et al.* 2009). Moreover, important lineages of medical, veterinary or zoonotic importance, either were not tested (e.g. Hemiuridae, Bucephalidae, Proteocephalidea, Caryophyllidea) or failed to amplify (e.g. *Taenia*, *Diphyllobothrium*, Fasciolidae, Schistosomatidae).

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Vanhove *et al.* (2014) showed considerable variation in the amino acid alignment of the annealing site of the forward Folmer primer, both among flatworms as well as between flatworms and other metazoans. This suggests the poor success of universal COI barcoding primers and those developed by Moszczynska *et al.* (2009) in flatworms may be due to primer-template mismatches. Platyhelminthes comprise the fourth most speciose animal phylum and parasitic flatworms in particular are relatively well studied, yet only 3% of species have COI barcodes (Kvist 2013). Considering that a 95% success rate has been recommended for high-throughput DNA barcoding (Hajibabaei *et al.* 2005), methodological improvements are clearly needed.

Other taxon-specific obstacles also confound COI barcoding across flatworms. First, there is the challenge of obtaining DNA of sufficiently high quality and quantity when starting with single eggs or microscopic larval stages (reviewed by Beltran *et al.* 2008; but see Webster 2009). Second, COI barcoding methods for flatworms must avoid co-amplification of template from the host or associated organisms (e.g. prey in host gut). Third, mononucleotide repeats that are sufficiently long as to hinder successful sequencing occur in some flatworm taxa (Locke *et al.* 2010), but the extent of this is unknown.

The aim of this study was to reduce obstacles to COI barcoding of parasitic flatworms by designing new degenerate primers. Multiple sequence alignments showed high levels of sequence variation that precluded the development of primers to amplify the Folmer region across all flatworms. Monogeneans and 'turbellarians' were therefore excluded and digeneans and cestodes became the target taxa of this study. New degenerate COI primers were designed to amplify across these two groups: the performance of these primers was tested on 46 specimens (23 digenean families and 6 cestode orders).

Materials and methods

Flatworm sequence alignments

To aid with primer design, publicly available (BOLD/NCBI) flatworm COI sequences were assembled into four alignments (Fig. S1, Supporting information). Taken together, these sequences represented 18 families of digeneans, 6 orders of cestodes, 6 families of monogeneans and two groups of 'turbellarians' (polyclads and triclads); however, sequence and taxon numbers varied among alignments. Aspidogastrean COI sequences were not present in public databases and so could not be included in alignments. Although alignments included only a fraction of extant flatworm diversity – there are over 150 digenean families (Littlewood 2008) and 19 cestode orders (Caira *et al.* 2014) – a very broad phylogenetic range was represented therein, so they were expected to be a reasonable basis for primer design.

Sequences were aligned in GENEIOUS v. 6.1.6 (Biomatters, New Zealand) using default parameters. Visual inspection of the alignments including all platyhelminth taxa showed that levels of polymorphism were so high that designing COI primers to amplify this entire taxonomic breadth was unrealistic. Monogeneans and 'turbellarians' were therefore excluded from alignments used for primer design (see Results and discussion). Sliding window analyses were used to explore nucleotide diversity (with the exclusion of gapped sites, which were outside the regions for primer development; Fig S1, Supporting information). These were performed using DNASP v. 5.10.01 (Librado & Rozas 2009) with window/step sizes of 20/1 in correspondence with the length of typical primers. Results were visualized into graphs with Microsoft Excel (Fig. 1).

COI primer design

Degenerate primers targeting the Folmer region of all digeneans and cestodes were designed by eye. Over 30 primers were tested in many iterative PCR rounds and a successful pair, Dice1F and Dice11R, was found and optimized. This primer pair amplifies the first 570–585 bp of the Folmer region (Table 1). Dice1F is a slightly modified version of Moszczynska *et al.*'s (2009) forward primer Mplatcox1dF, the only change being that the inosine was replaced by 'N'. Dice1F ends 8 bp upstream of the forward Folmer primer LCO1490 (Folmer *et al.* 1994). Dice11R is a newly designed primer that starts 72 bp upstream of HCO2198 (Folmer *et al.* 1994) and 39 bp upstream of Mplatcox1dR (Moszczynska *et al.* 2009). To facilitate sequencing, shortened T3 (16 bp) and T7 (17 bp) primer tails, called 'T3s' and 'T7s' here, were attached to the 5′ ends of Dice1F and Dice11R, respectively. Initial testing suggested that the use of either M13 tails or full-length T3 (20 bp) and T7 (20 bp) tails led to nonspecific amplification products, whereas T3s and T7s did not.

A second degenerate and T7s-tailed reverse primer, Dice14R, was developed for specimens that did not amplify well with Dice11R. This reverse primer starts 162 bp downstream of HCO2198, so when combined with Dice1F generates a ~800- to 820-bp product that requires internal sequencing primers for full bidirectional sequencing (SeqF1/2 and SeqR1/2, Table 1).

Specimen collection and vouchering

A total of 32 digenean specimens belonging to 23 families and 14 cestode specimens belonging to 6 orders were

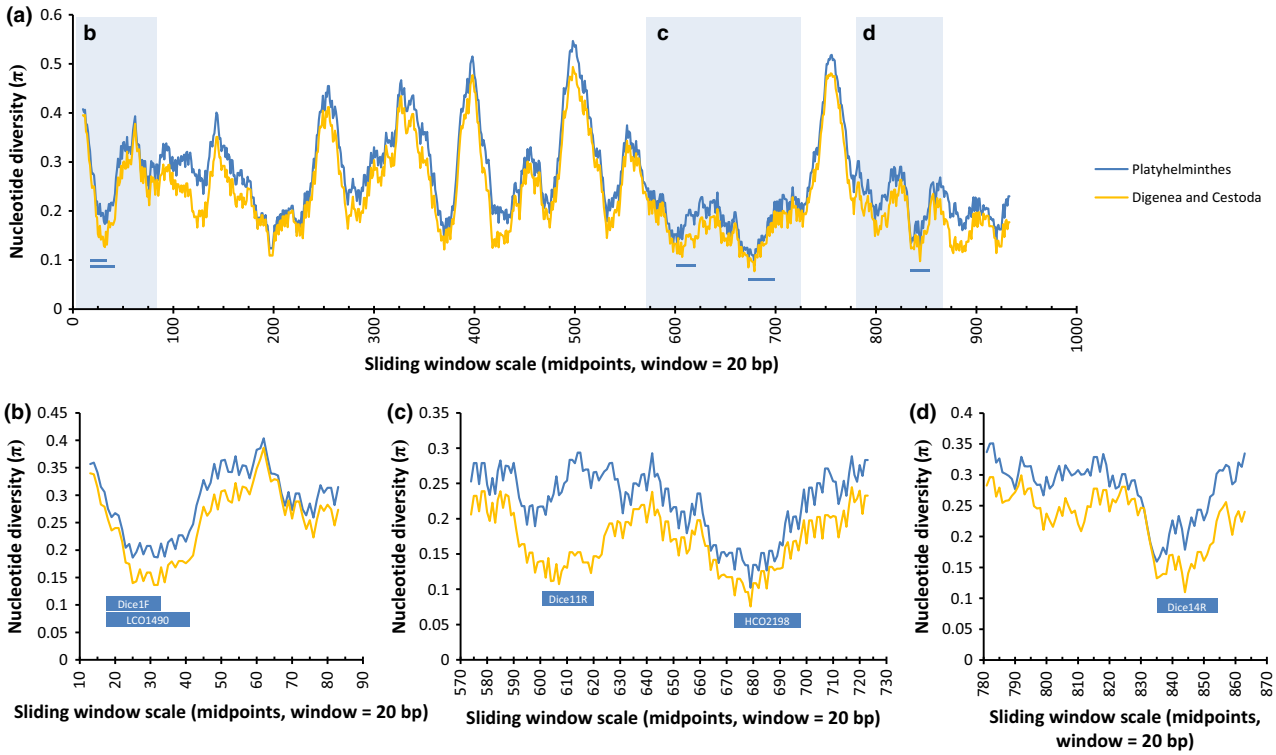

Fig. 1 Sliding window analyses showing levels of nucleotide diversity in: (a) the first 1000 bp of the COI gene (alignment Fig. S1a, Supporting information); (b) the priming region and flanking regions of LCO1490 and Dice1F (alignment Fig. S1b, Supporting information); (c) the priming region and flanking regions of HCO2198 and Dice11R (alignment Fig. S1c, Supporting information); (d) the priming region and flanking regions of Dice14R (alignment Fig. S1d, Supporting information). Blue lines represent sliding window analyses performed on alignments including all flatworms (i.e. digeneans, cestodes, monogeneans and 'turbellarians'); yellow lines represent sliding window analyses performed on alignments including only digeneans and cestodes. Priming sites of the forward (LCO1490) and reverse (HCO2198) Folmer primers and the new primers developed here (Dice1F, Dice11R, Dice14R) are shown.

Table 1 Primers developed and used for the amplification and sequencing of mitochondrial COI and nuclear 18S rDNA gene fragments from digenean and cestode samples. Shortened T3 (T3s) and T7 (T7s) tails at the 5′ end of Dice1F and Dice11R/Dice14R, respectively, are underlined and were used for sequencing. Additional sequencing primers (SeqFx and SeqRx) were used in combination with Dice14R

Primer name	Direction	Primer sequence (5′-3′)	Usage	Gene	Reference
Dice1F	Forward	ATTAACCCTCACTAAATTWCNTTRGATCATAAG	PCR	COI	Moszczynska *et al.* (2009)
Dice11R	Reverse	TAATACGACTCACTATAGCWGWACHAAATTTHCGATC	PCR	COI	This study
Dice14R	Reverse	TAATACGACTCACTATACCHACMRTAAACATATGATG	PCR	COI	This study
SeqF1	Forward	AATGCTTTAAGTGCTTG	Sequencing	COI	This study
SeqF2	Forward	AATGCNTTRAGKGCDTG	Sequencing	COI	This study
SeqR1	Reverse	CAAGCACTTAAAGCATT	Sequencing	COI	This study
SeqR2	Reverse	CAHGCMCTYAANGCATT	Sequencing	COI	This study
18S9modF	Forward	GATCCTGCCAGTAGTCATATGCTTG	PCR/Sequencing	18S	Moszczynska *et al.* (2009)
18S637modR	Reverse	TACGCTWYTGGAGCTGGAGTTACCG	PCR/Sequencing	18S	Moszczynska *et al.* (2009)

used to evaluate the performance of the COI barcoding primers and methods recommended here (Table S1, Supporting information). This represents a broad phylogenetic coverage of both groups (Olson *et al.* 2003; Olson & Tkach 2005; Caira *et al.* 2014). Specimens were preserved in 95–100% ethanol and in some cases were subsampled such that a portion of the specimen was used for molecular work, and the remaining material

was retained as a voucher (hologenophore, sensu Pleijel *et al.* 2008). Complete specimens were used for DNA extraction when their small size necessitated this; in these cases, one or more individual worms that were morphologically indistinguishable from the sequenced specimen and inhabiting the same site within the same host individual were retained as vouchers (paragenophores, sensu Pleijel *et al.* 2008); in 13 cases, it was not possible to obtain a morphological voucher. Vouchers were deposited at the Canadian Museum of Nature's Parasite collection (see Table S1, Supporting information for accessions) after being stained in acetocarmine, mounted on slides in Canada balsam and identified using keys in Khalil *et al.* (1994), Gibson *et al.* (2002), Jones *et al.* (2005), Bray *et al.* (2008) and the primary literature.

DNA extraction, PCR and sequencing

DNA extraction methods varied depending on the size of the specimen and minor alterations were made to the manufacturer's protocols, as detailed in Appendix S1 (Supporting information). The first ~560–580 bp of 18S ribosomal DNA were amplified to verify that all DNA extracts used for COI primer testing were of suitable quality and quantity for PCR. Several combinations of existing primers from the literature (Littlewood & Olson 2001) and slightly modified versions of the 18S primers of Moszczynska *et al.* (2009) were tested. The latter were most successful and so are presented here. The primer 18S9modF is one nucleotide shorter than 18S9F, while 18S637modR is a degenerate version of 18S637R (Table 1).

PCRs were 25 μL in volume and typically contained: 3.5 mM $MgCl_2$, 0.5 μM each primer, 0.2 mM dNTPs, 0.6 U Platinum® *Taq* polymerase (Invitrogen) in 1× PCR buffer. DNA template was 5 and 1 μL for COI and 18S, respectively. In some taxa, COI sequencing failed due to long poly-T runs, which can cause *Taq* slippage leading to PCR products of varying length and unusable sequence traces. Using Phusion® Hot Start Flex DNA polymerase (New England Biolabs, Inc.) during PCR prevents this (see Fazekas *et al.* 2010). These PCRs used the following: 0.5 μM each primer, 0.2 mM dNTPs, 0.5 U of Phusion® Hot Start Flex DNA polymerase in 1× Phusion HF buffer. Thermocycling conditions were as follows: 94 °C for 2 min; 3 cycles of 94 °C for 40 s, 51 °C for 40 s, 72 °C for 1 min; 5 'touchdown' cycles of 94 °C for 40 s, 50 °C to 46 °C for 40 s (dropping 1 °C per cycle), 72 °C for 1 min; 35 cycles of 94 °C for 40 s, 45 °C for 40 s, 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were visualized on 1.5% TBE agarose gels stained with SYBR® Safe (Invitrogen).

When single, clearly visible COI PCR products were obtained, they were enzymatically purified prior to sequencing using Illustra™ ExoStar (GE Healthcare). When there were nonspecific products, the targeted product was isolated by running it on an E-Gel® CloneWell™ 0.8% SYBR® Safe precast agarose gel (Invitrogen). When PCR products were weak or absent, re-amplification was tried using PCR product as template in a second round of PCR.

Sequencing reactions were 10 μL and contained 1 μL BigDye Terminator (BDT) v3.1 (Applied Biosystems), 2 μL BDT buffer, 0.16 μM primer and 1–2 μL PCR product. Sequencing products were purified with the DyeEx® 2.0 Spin Kit (Qiagen) and run on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequences were viewed and edited in GENEIOUS v. 6.1.6 and subjected to an identification request for COI sequences in the Public Record Barcode Database on the BOLD website (http://www.boldsystems.org) and/or a BLAST search for 18S sequences on the NCBI website (http://www.ncbi.nlm.nih.gov) to check for possible contamination (i.e. sequence from nontarget organism) and any inconsistencies with morphological identifications.

Results and discussion

Nucleotide variation among flatworms and COI primer development

The alignment of the first 1000 bp of the COI gene for digeneans, cestodes, monogeneans and 'turbellarians' revealed regions with high nucleotide diversity interspersed with more conserved regions (Fig. 1a). The first ~100 bp of the COI region in flatworms has a single conserved region that contains the annealing sites for forward primers LCO1490 and Dice1F, as depicted visually by the sliding window analyses (Figs. 1a, b). The latter portion of the barcode region and downstream flanking region (between ~600 and 1000 bp) contain multiple regions with higher sequence conservation and these correspond to the annealing sites of reverse primers Dice11R, HCO2198 and Dice14R (Figs. 1a, c and d). By comparing sliding window analyses as well as levels of primer degeneracy between alignments containing all flatworm diversity versus alignments excluding monogeneans and 'turbellarians', it was clear that the inclusion of the latter two taxa caused a substantial increase in nucleotide diversity at four of the five primer annealing sites (the exception being HCO2198; Fig. 1, Table 2). While the LCO1490 priming site is highly variable among flatworms, as shown by the high level of primer degeneracy needed to accommodate that variation (Table 2), the HCO2198 priming site is relatively conserved among flatworms and other metazoans (see

Table 2; Geller *et al.* 2013; Vanhove *et al.* 2014). However, using HCO2198 paired with the relatively conserved Dice1F would risk of co-amplifying host DNA; hence, more specific reverse primers were designed.

Primer performance on a broad taxonomic diversity of digeneans and cestodes

The overall sequencing success using primers Dice1F/Dice11R on our taxonomically diverse set of specimens was 91% (42 of 46 specimens). The four specimens (3 digeneans and 1 cestode) that failed to amplify with this primer pair were successfully amplified using primer pair Dice1F/Dice14R (Table S1, Supporting information). This high success rate is an improvement compared to the 39% overall success rate in the study of Moszczynska *et al.* (2009). In addition, this relatively low success rate was biased by overrepresentation of 3 diplostomid (*Ornithodiplostomum*, *Posthodiplostomum* and *Diplostomum*) and 2 strigeid genera (*Apatemon* and *Ichthyocotylurus*); excluding these genera, the success rate dropped to 26%.

Our approach differed from that of Moszczynska *et al.* (2009) in that we designed primers using much larger alignments and tested them on a larger phylogenetic diversity of samples. Admittedly, our results are based on a much lower overall number of samples because only a single specimen was tested for each species. Nonetheless, methods presented here yielded a completely sequenced set of samples and results suggest that the use of alternative primers is only necessary in a minority of taxa given the high (91%) success rate using Dice1F/Dice11R.

Overcoming initial amplification and sequencing failures

A minority of specimens failed in the first PCR or sequencing attempt. Failures were caused by *Taq* slippage during amplification of a long poly-T run, nonspecific primer binding or low-quantity PCR products. Table S1 (Supporting information) lists alternative methods (e.g. alternative enzyme, gel purification and re-amplification) to overcome these failures. A schematic depicting the barcoding workflow recommended here is in Fig. S2 (Supporting information).

While amplification of nontarget DNA and low-quantity PCR products are routinely encountered in DNA barcoding, the presence and frequency of mononucleotide repeats that comprise sequencing success is rarely

Table 2 Consensus sequences of forward and reverse primers based on the COI alignments in Fig. S1 (Supporting information)

		Degeneracy
Forward		
LCO1490	GGTCAACAAATCATAAAGATATTGG	0
Consensus flatworms	DNWSNHYNDVHCAYAAGVRNRTNRG	95 551 488
Consensus Digenea + Cestoda	KNWSNHTDGAYCAYAAGCGNRTNRG	294 912
Consensus Monogenea	TYACNHTDRRHCAYAAGMRBATHGG	62 208
Consensus 'Turbellaria'	WTTCTACHWMWCATAAGGATATWGG	96
Dice1F (without T3s tail)	TTWCNTTRGATCATAAG	16
Consensus flatworms	DNWSNHYNDVHCAYAAG	248 832
Consensus Digenea + Cestoda	KNWSNHTDGAYCAYAAG	4608
Consensus Monogenea	TYACNHTDRRHCAYAAG	1728
Consensus 'Turbellaria'	WTTCTACHWMWCATAAG	48
Reverse		
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	0
Consensus flatworms	TANACYTCNGGRTGNCCRAWRAAYCA	4096
Consensus Digenea + Cestoda	TANACYTCNGGRTSNCCAAWRAAYCA	2048
Consensus Monogenea	TANACYTCNGGRTGNCCRAARAAYCA	2048
Consensus 'Turbellaria'	TAWACYTCNGGRTGNCCRAARAAYCA	1024
Dice11R (without T7s tail)	GCWGWACHAAATTTHCGATC	36
Consensus flatworms	VHNGNNYHRAVDTKNCGRTC	995 328
Consensus Digenea + Cestoda	RHNGHNCHRARDTTHCGRTG	62 208
Consensus Monogenea	GMDGDDYYRAADTTNCGRTC	10 368
Consensus 'Turbellaria'	SWHGTDTTRAMDTKHCGATC	3888
Dice14R (without T7s tail)	CCHACMRTAAACATATGATG	12
Consensus flatworms	CCNVHNRHRWACATRTSRTG	27 648
Consensus Digenea + Cestoda	CCNRYNRYRAACATRTSRTG	4096
Consensus Monogenea	CCHAYDGWRWACATRTGRTG	576
Consensus 'Turbellaria'	CCNVHDRYRTACATRTGRTG	3456

discussed. A 13-bp poly-T run in a highly conserved region 200 bp into the COI amplicon occurs in a broad range of digenean and cestode phylogenetic lineages. Representatives of Diplostomidae, Schistosomatidae, Strigeidae, Azygiidae, Heterophyidae, Plagiorchiidae, Diphyllobothriidea and Trypanorhyncha are known to have this repeat based on results of this study, data mined from GenBank/BOLD, and unpublished data (N. Van Steenkiste and S. Locke). The poly-T run is interrupted by other nucleotides in most digenean and cestode taxa which prevents the problem of polymerase slippage during PCR. Some representatives of Diplostomidae (e.g. *Bolbophorus* sp.) also have a second long poly-T run between 467 and 480 bp of the COI amplicon that only seems to cause a problem for sequencing in some taxa, particularly within Crassiphialinae (N. Van Steenkiste and S. Locke, unpublished data). Amplification with Phusion DNA polymerase significantly improved sequencing success in affected taxa.

Template quality and taxonomic verification

A total of 45 of 46 samples amplified with 18S primers 18S9modF and 18S637modR, of which 43 yielded 550-to 674-bp-long sequences (see Table S1, Supporting information). Concordance at a high taxonomic level was generally observed between morphological identifications and those inferred by querying COI and 18S sequences against public databases (Table S1, Supporting information). The exception was an 18S sequence of *Megalodiscus* sp., which revealed contamination by its host (sequence identical to that of *Lithobates pipiens*). The degree of similarity returned between COI sequences obtained here and their closest match in public databases was often low (less than 85% in 30 cases), which is in keeping with the poor representation of digenean and cestodes in reference databases.

Present and future challenges in COI barcoding of parasitic flatworms

In this study, three primers (one forward and two reverse) and troubleshooting methods were used to achieve a 100% success rate for COI barcoding of digeneans and cestodes. While this deviates from the ideal methodology of DNA barcoding using a single set of standardized conditions, for some taxa, such an approach may not be practical (Ondrejicka *et al.* 2014). Methods presented here can be readily adapted to high-throughput COI barcoding of digeneans or cestodes. The data also broaden COI sequence libraries, as illustrated by the lack of genus-level matches in public databases in 31 of 46 cases (Table S1, Supporting information). This added coverage is expected to facilitate the generation of taxon-specific primers for particular taxa that prove problematic. For example, the primer combination Dice1F/Dice14R amplifies a ~800- to 820-bp-long COI fragment extending ~160 bp downstream of the Folmer region and thus provides a good option for generating a sufficiently long fragment to enable the development of a more taxon-specific reverse primer.

Only a tiny fraction of parasitic flatworm diversity has been barcoded to date (Kvist 2013). This is, at least to some extent, reflective of the degree to which existing methodological challenges have limited the use of COI barcoding for the identification of flatworms. The COI primers and methods presented here for digeneans and cestodes are expected to facilitate and therefore increase the rate of COI barcoding in parasitic flatworms.

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Data accessibility

All new 18S and COI sequences and their trace files are accessible in GenBank and BOLD (project PRNVS). GenBank accessions (KM538076-KM538164 and KP119664), BOLD sample ID and BIN numbers and voucher accessions are also provided in Table S1 (Supporting information). Sequence alignments used for the sliding window analyses and primer development are provided as Appendices S2–S5 (Supporting information).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Multiple sequence alignments used for exploration of levels of nucleotide variation in flatworms and COI primer development in digeneans and cestodes: (a) sequence alignment of the first ~1000 bp of the COI gene including both forward and reverse Folmer primer annealing sites and flanking regions; (b) sequence alignment of the first ~95 bp of the COI gene including the forward Folmer primer annealing site and flanking regions; (c) sequence alignment of the reverse Folmer primer annealing site and flanking region between ~620 and 790 bp; (d) sequence alignment of a relatively conserved region between ~830 and 930 bp.

Fig. S2 DNA barcoding workflow recommended here to maximize success obtaining COI sequences from any digenean and cestode specimen. (a) Partial 18S is amplified for template quality control. (b) Standard COI amplification starts with Platinum *Taq* and the primers Dice1F and Dice11R. (c–d) For the majority of the tested taxa, this resulted in sequencable amplicons and high quality sequence traces. (e–f) Cases of low or absent amplification are re-amplified using the PCR product as template. (g–h) In the case of multiple bands, isolate the target band with

Clonewell before sequencing. *(i–j)* In the case of poor-quality sequence traces caused by a poly-T repeat, the Platinum *Taq* polymerase was replaced by Phusion Hot Start Flex DNA polymerase. (k–l) If all else failed, the identical workflow was applied with the reverse primer Dice14R.

Table S1. Digenean and cestode specimens used in this study, collection data, vouchering, and comments on 18S amplification and COI barcoding.

Appendix S1. DNA extraction.doc

Appendix S2. Fig.S1a.fasta

Appendix S3. Fig.S1b.fasta

Appendix S4. Fig.S1c.fasta

Appendix S5. Fig.S1d.fasta