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FULL LENGTH RESEARCH PAPER

Virtual Barcoding using LATE-PCR and Lights-On/Lights-Off probes: identification of nematode species in a closed-tube reaction

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Abstract

The present study describes a rapid, universal, easy-to-use, closed-tube, non-sequencing method that should also be able to uniquely identify almost any animal species on earth. The approach, called Virtual Barcoding, is illustrated using five species of nematodes from three genera.

Linear-After-The-Exponential (LATE) PCR was used to amplify a portion of the CO1 gene for each of five commercially available, beneficial species of soil nematodes. A set of ten low temperature Lights-On/Lights-Off consensus probes were included in the reaction mixture and were used at end-point to coat the accumulated single-stranded amplicon by dropping the temperature. Because each of the probes is mis-match tolerant, the temperature at which it hybridizes to its complementary region within the target is sequence dependent. As anticipated, each species had its own unique fluorescent signature in either three different colors, or a single color depending on which fluorophores were used to label the Lights-On probes. Each fluorescent signature was then mathematically converted to a species-specific Virtual Barcode.

Keywords

Barcode of life, CO1 gene of mitochondria, LATE-PCR, Lights-On/Lights-Off probes, nematodes, parasites, species identification, Virtual Barcoding

History

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Introduction

Currently there is no comprehensive understanding of the breadth of species diversity since many species are yet to be described (Pimm et al., 1995). A modern classification system, based on objective DNA sequence information, would allow taxonomists to identify each of the extremely diverse species of living organisms on earth. A 648-bp region known as the ''Folmer Region'' (Folmer et al., 1994) within the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene changes its sequence at a rate commensurate with the rate of speciation in animals (Margoliash, 1965; Marshall, 2005). This sequence has therefore been chosen for amplification and sequencing in an effort to ''barcode'' each animal species on earth (Clark & Whittam, 1992; Hebert et al., 2003).

Information about geographic, morphological, and molecular characteristics of each species is being amassed, organized and stored in the Barcode for Life data system (BOLD; Yeager, 2012). This database aims to improve overall understanding of the scope of biodiversity (Ratnasingham & Herbert, 2007). It also has practical value for identification of invasive and pathogenic species which endanger native species, cause diseases in plants, animals, and humans, and are costly to control or treat. The current BOLD strategy to establish a molecular barcode for each of 500,000 species by 2015 via amplification and sequencing of the CO1 gene target is expensive and labor intensive. Here we describe a novel technology, called Virtual Barcoding that is compatible with the goals of BOLD, but is more rapid and less expensive. Moreover, as more and more specimens are examined using Virtual Barcoding it will only be necessary to actually sequence only those that have not been identified previously. Virtual Barcoding is also ideally suited for future handheld devices that can be used on location.

Nematodes, arthropods, fungi, and microorganisms are considered to be ''hyperdiverse'' groups of terrestrial organisms (Colwell, 1994). Nematodes were chosen as the model organism for these proof-of-principle experiments described here because they are the most abundant multicellular animals on earth, being found in every environment from the tropics to the deep sea to the arctic (Coghlan, 2005). Also many studies have provided important information on nematode species diversity and classification, including attempts to barcode a growing number of species (Abebe et al., 2011; Bhadury et al., 2006; De Ley et al., 2005; De Oliveira et al., 2011; Floyd et al., 2002; Powers, 2004; Powers et al., 2005; The *C. elegans* Sequencing Consortium, 1998). Five different species of Nematodes from three genera were tested here, simply because they are commercially available: *Heterohabditis bacteriophora*, *Caenorhabditis briggsae*, *Steinernema feltiae, Caenorhabditis elegans*, and *Steinernema carpocapse*. The assay uses LATE-PCR (Pierce et al., 2005; Sanchez et al., 2003) and Lights-On/Lights-Off probes (Rice et al., 2012). The resulting fluorescent signatures are species specific reflecting differences in the five different amplified sequences. The fluorescent signatures can be converted to species specific five-bit Virtual Barcodes. This particular assay could readily be used for identification of pathogenic helminths and filarasis species by simply changing the primers. Further extension of the above logic to the use of multiple initiator primers (Pierce & Wangh, 2014) raises the possibility that each species

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within an entire phylum can rapidly be assigned its own Virtual Barcode using this closed-tube approach.

Materials and methods

Design features of LATE-PCR assay

Linear-After-the-Exponential (LATE-PCR) is an advanced form of non-symmetric PCR for efficient production of single-stranded DNA amplicons. Primers specific sequences within the Folmer Region located at the 5′ end of the CO1 mitochondrial gene were designed based on LATE-PCR melting temperature, Tm, criteria for a Limiting Primer (L) and an Excess Primer (X) which states that Tm^L-$Tm^X \geq 0$ (Pierce et al., 2005; Sanchez et al., 2003). Multiple sequence alignments (CLUSTAL 2.0.10) were taken into account to choose specific conserved primer regions based on 264 nematode species sequences of many genera from BOLD. Each primer (Table 1) covers 36 base pairs and was built through inspection of each nucleotide of the 264 species and then checked for melting temperature, minimization of primer interactions, and secondary structure using Visual OMP (VOMP), a nucleic acid software design program (DNA Software, Ann Arbor, MI). Three limiting primers were designed to insure that any one of the 264 available CO1 nematode sequences in the data base at that time would amplify if added to a reaction.

Lights-On/Lights-Off probes are pairs of hybridization probes (Rice et al., 2012). Each Lights-On probe has a fluorophore at the 5′ end and a BHQ at the 3′ end, while each Lights-Off probe only has a BHQ at the 3′ end (Table 1). Sets of Lights-On/Lights-Off probe pairs can be used to cover an extended DNA sequence (Carver-Brown et al., 2012; Rice et al., 2012). In the present study a total of ten (five pairs) of Lights-On/Lights-Off probes were designed to cover 411 base pairs of the amplified Folmer Region target (Table 1). In the first experimental design, three different fluorophores were used to label sub-sets of Lights-On probes covering different sections of the target sequence: Quasar 670 two pairs of probes, Cal-Red two pairs of probes, Cal-Orange one pair of probes. In a second experimental design, all five pairs of probes were labeled with Quasar 670.

Nematode DNA extraction and purification

Nematode species *H. bacteriophora, S. carpocapse, S. feltiae*, were purchased (Buglogical Control Systems, Tucson, Arizona) and individual worms of each, as well as *C. briggsae*, and *C. elegans,* (Provided by the laboratory of Piali Sengupta, Brandeis University) were placed in 25 µl volume of lysis buffer containing 100 µg/ml proteinase K, 10 mM Tris-Cl pH 8.3 m and 5 µM sodium-dodecyl-sulfate (SDS). The DNA was then extracted by heating to 50 °C for 30 min followed by 95 °C for 10 min then stored at −20 °C. Three different samples of each nematode species were used in this assay.

Assay composition

Every reaction had a final volume of 25 µL containing: 1X PCR buffer (cat. no. 10,966–034; Invitrogen, Carlsbad, CA), 3 mM $MgCl_2$ (cat. no. 10,966–034; Invitrogen, Carlsbad, CA), 250 nM of each dNTP (Invitrogen, Carlsbad, CA), PCR Grade Water (cat.no. BP2819-1; Fisher Scientific), 150 nM of each Lights-Off probe and 50 nM of each Lights-On probe (Biosearch Technologies, Novato, CA). Each mixture also contained 100 nM of each limiting primer (Sigma-Aldrich, St Louis, MO), 1000 nM of each excess primer (Sigma-Aldrich, St Louis, MO), 1.25 units of Platinum Taq DNA polymerase (cat. no. 10,966–-034; Invitrogen, Carlsbad, CA) and 1 µL extracted nematode DNA containing approximately 10,000 mitochondrial genomes as determined by real time LATE-PCR amplification with SYBR Green (results not shown).

LATE-PCR protocol and thermal amplification parameters

Each reaction was run in triplicate in the Bio-Rad IQ5 Multicolor Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA). Three no template controls (NTCs) were also run in parallel in every experiment. An initial denaturation step at 95 °C for 3 min was followed by 5 amplification cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 45 s. This was further followed by 55 cycles with an increase in anneal temperature from 55 °C for 10 s to 64 °C for 10 s. At the end of the amplification, the temperature was dropped from 72 °C to 25 °C. Beginning at 25 °C, the temperature was increased in 56 cycles of 30-s steps and 1 °C increments to 80 °C. Empirical observation suggests that this down-up process improves the reproducibility of the replicate samples (data not shown). Data collection was then carried out by decreasing the temperature, annealing in 1 °C decrements at 30 s intervals for 71 steps between 95 °C and 25 °C. Fluorescence was measured in the Cal Orange 560, Cal Red 610 and Quasar 670 channels at each anneal step.

Data analysis

The fluorescent signatures were generated as follows: the first derivatives of the raw fluorescent data at endpoint, generated using the algorithms for each temperature step and each dye

Table 1. Primer and probe sequences used in the three color Lights-On/Lights-Off nematode identification assay.

Primer name			Primer sequences
Limiting 1			5′-GGTTATACCTAGTATAATTGGTGGTTTTGGTAATTG-3′
Limiting 2			5′-GGTTATACCTAGTATAATTGGTGGTTTTGGTAACTG-3′
Limiting 3			5′-GGTTATACCTAGTATAATTGGTGGTTTTGGCAATTG-3′
Excess			5′-ACTAGGATCAAAAAAAGAAGTATTAAAATTACGATC-3′
#	Length	Color & Type	Probe sequences
1	40	Off	AATATTACCTTTGATGTTAGGGGCTCCTGATATAAGTTTT-BHQ1
2	41	Cal-Org On	CalOrg-ATCCTCGTTTAAATAATTTAAGTTTTTGATTATTACCTACTTCAT-BHQ1
3	45	Off	TTTGTTTTGTTGTTGGGATTCTTGTTTTGTTGATATAGGTGGTGGAA-BHQ2
4	34	Cal-Red On	CalRed- AACTGGTTGAACTGTTTACCCTCCTTTAAGAACTT-BHQ2
5	43	Off	AAGTAGGTCATCCTGGTAGTACTGTAGATTTTGTTATTTTTACTT-BHQ2
6	35	Cal-Red On	CalRed- ATGCATGGTGCTGGTTTTAGTTCTATTTTGGGTGCTAT-BHQ2
7	40	Off	ATTAATTTTATGGGTACTACTGTTAAGAATCTGCGTAGTTAT-BHQ2
8	39	Quasar On	Quasar-TT CTATTTCTTTGGAACATATGAGTCTGTTTGTTTGGACTGAA-BHQ2
9	35	Off	TTTTTGTGACTGTTTTTTTGTTGGTTCTGTCTCTAA-BHQ2
10	36	Quasar On	Quasar-TTCCTGTTTTAGGTGGGGCTATTACTATATTGTTAACTAA-BHQ2

channel collected on the Bio-Rad IQ5, were exported to Microsoft Excel 2007. Each set of data was normalized by subtracting all values by the value of the signal at 65 °C in that data set. This was because no probe was bound to any target at this temperature and the fluorescence intensity in each color was at the background level for all probes of that color. At this point all positive fluorescent signals different from background for each nematode in their respective color were re-scaled by dividing all fluorescent values in a data set by the highest absolute fluorescent value in that data set and then multiplied by −15. The resulting fluorescent values describe a temperature-dependent fluorescent signature in relative fluorescent unit values on a scale of −15 to 15 and to generate numerical values further used in a five-bit barcoding format.

Results

Lights-On/Lights-Off probes have recently been described for mutation scanning of relatively long target sequences (Rice et al., 2012). For instance, three Lights-On probes labeled in the same fluorophore, together with their adjacent Lights-Off probes were used to scan a single-stranded target 101 nucleotides long in the rpoB gene of tuberculosis, for mutations responsible for rifampin resistance. The resulting unique fluorescent signatures can then be compared to the wild type (drug sensitive) strain to detect the presence and location of virtually any nucleotide change in the target.

In contrast, the Folmer Region of the CO1 gene used to barcode animal species is about 648 nucleotides long and no particular sequence from any species can be regarded as the ''wild type'' for all other species. In the first configuration of the assay described here, we interrogated a 411 nucleotide long sequence within the Folmer Region using five pairs of Lights-On/Lights-Off probes labeled with three differently colored fluorophores. In the second configuration of the assay, we analyzed the same target using the same five pairs of probes, but all Lights-On probes were labeled with the same fluorophore. Three samples of each nematode were analyzed independently showing the same Lights-On/Lights-Off fluorescent signature and five bit barcodes. Figure 1 illustrates the steps involved in constructing the multicolored fluorescent signature for the *Steinernema feltiae* CO1 target and also demonstrates that pairs of Lights-On/Lights-Off probes generate much more detailed information about a target sequence than Lights-On probes alone. Figure 1(A) shows the melt curves generated by these probes over a temperature range of 25–65 °C in each color, and Figure 1(B) shows the first derivatives for each of those curves, normalized to their highest positive values. We call the melt curves ''fluorescent contours'' and the normalized first derivatives ''fluorescent signatures'' (Rice et al., 2012). Fluorescent signatures analyzed in several colors are said to be comprised of sub-signatures, each one of which corresponds to an adjacent region of the CO1 target sequence. As shown below, each species has its own unique fluorescent signature, even when two sub-signatures are nearly identical because the regional sequences are virtually the same. Moreover, the fact that each species has its own characteristic signature does not require that every probe in the set binds to the target sequence of that species, since some probes may be too mis-matched to hybridize even at 25 °C.

Virtual barcoding in three colors

Figure 2 shows how each normalized fluorescent sub-signature can be mathematically converted into a five-bit barcode. The first bit encodes whether the fluorescent signal at each degree of temperature is either positive, negative, or zero. If the value is zero or above the first bit is filled in. Conversely, if the value of the normalized fluorescent signature is below zero the first bit is not filled in. The next four bits encode integers one, two, four, and eight to convert the analogue value of normalized fluorescent signature into a binary value. For instance, at 47 °C in Figure 2 (arrow) the integer from the CalRed610 fluorescent is −15, and bits two, three, four and five are therefore filled, while bit 1 is not filled. We call this representation of the underlying target sequence the Virtual Barcode of that species.

Figure 3 shows both the three colored fluorescent signatures and the Virtual Barcode for each of the five species of nematodes analyzed in this study. All five species was amplified and analyzed using a single reaction mixture that contained three Limiting Primers and five pairs of Lights-On/Lights-Off probes labeled in three fluorescent colors. The use of three Limiting Primers guaranteed that amplification occurred even though the species came from three genera with significantly different CO1 sequences. Moreover, amplification for 60 cycles guaranteed that all reactions accumulated single-stranded amplicons even if they differed in the efficiency with which amplification got started (Sanchez et al., 2006). Two of the species *C. elegans* and *C. briggae* are members of same genus, as are *S. feltiae,* and *S. carpocapse,* while *H. bacteriophora* is a representative of a third genus. Despite these taxonomic similarities and distances

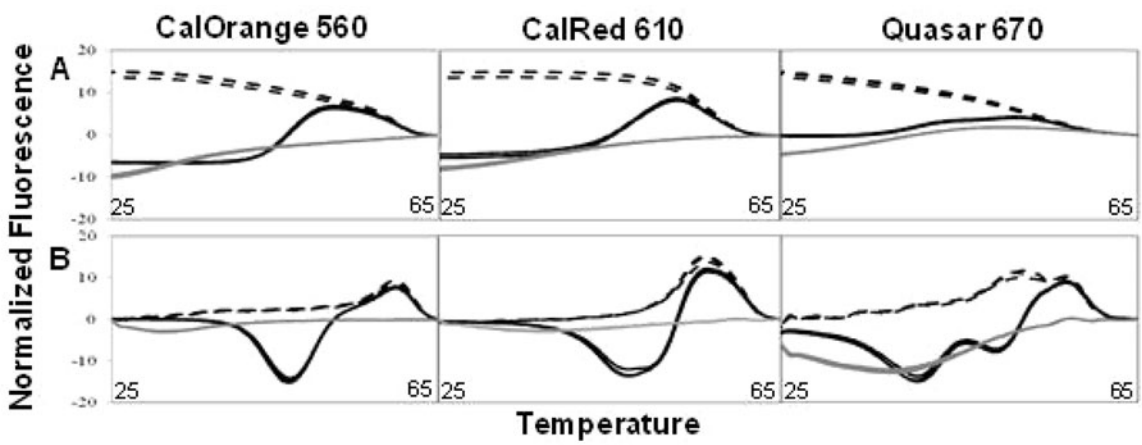

Figure 1. The detection of *S. feltiae* in the fluorescent channels CalOrange560, CalRed610, and Quasar670 as a function of temperature. The signals in the Cal Orange, Cal Red, and Quasar channels were spread over the same broad temperature range although they arose from one, two, and two pairs of Lights-On/Lights-Off probes, respectively. Lights On probes – black dashed, Lights On/Lights Off probes – black solid, No template control – grey solid (A) Normalized anneal curves are fluorescent contours (B) Normalized 1st derivative anneal curves, are fluorescent signatures.

each species has its own unique Virtual Barcode comprised of its own unique sub-signatures because the ten probes in the set match and mis-match to their target sequences differently. As a result each probe in the set of ten probes has a different Tm to its corresponding target variant, Table 2. THE LATE-PCR amplicon of the *C. elegans* nematode species was sequenced and showed the same sequence as that found in the GenBank Database NC_001328 except for a single nucleotide polymorphism.

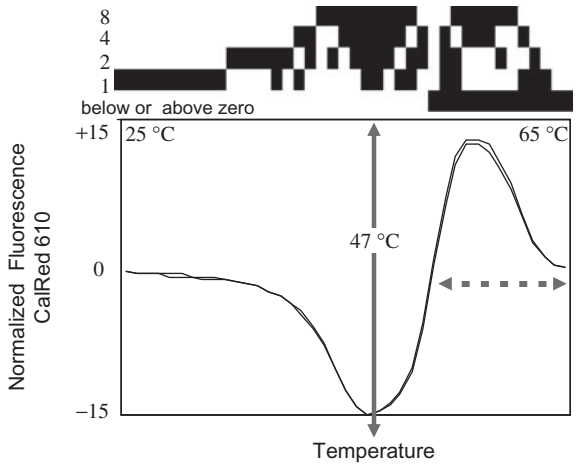

Figure 2. The detection of *S. feltiae* in the fluorescent channel CalRed610 as a function of temperature with its corresponding barcode. Lights On/Lights Off fluorescent signature – black solid. The absolute value of maximum fluorescence and corresponding barcode value of 15 $(1+2+4+8=15)$ is indicated by solid grey arrow. Grey dashed arrow is illustrating the area of the curve above zero, which corresponds to the filled in area for the above zero line of the barcode.

Identification using one fluorescent channel

Virtual Barcoding also offers two strategies for identifying sub-species, or strains within a species. The first approach is to compare the three colored Virtual Barcode of different individuals from the same species. Since the CO1 sequences of varieties within a species generally have a smaller percent differences than those between species, it is likely that these small differences will not be spread across the entire CO1 target. Therefore only one of the three sub-signatures will differ between varieties while the other two sub-signatures will be the same.

An alternative higher resolution approach is to amplify and analyze additional mitochondrial gene targets whose rate of evolution is faster than that of CO1. In order to carry out such an analysis as a multiplex reaction in a single-tube, it will be necessary to economize on the number of fluorescent colors needed to analyze the CO1 target. For this reason, we constructed a second assay using the same primer and probe sequences, but labeled all five Lights-On probes with Quasar 670. Figure 4 shows that each of the five nematode species has its own fluorescent signature. This result immediately suggests that it will be possible to construct duplex, triplex, and even more complex LATE-PCR assays in which each single-stranded target is coated with a differently colored set of Lights-On/Lights-Off probe. Assays designed in this way could be used to confirm the species identify of each specimen under examination and simultaneously scan for

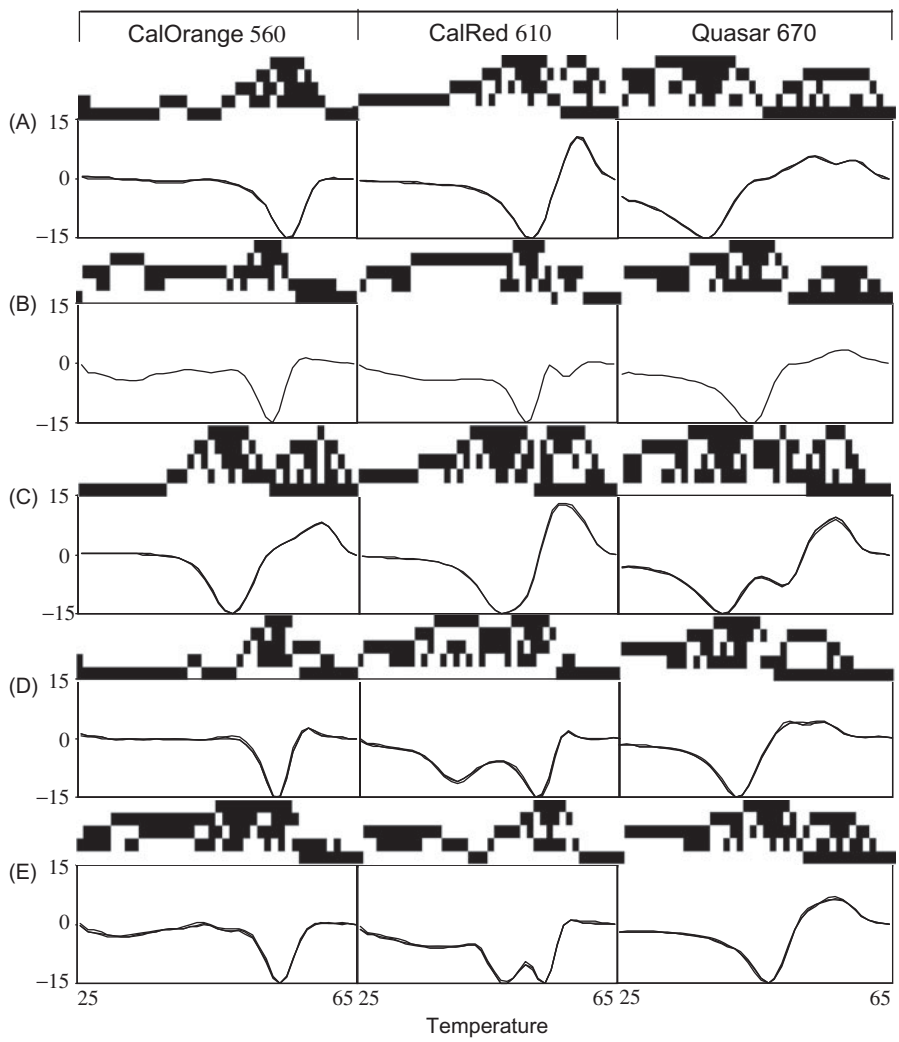

Figure 3. The fluorescent signatures in the channels, CalOrange560, CalRed610 and Quasar670 with their corresponding five-bit barcodes directly translated from each of the normalized fluorescent values (A) – *H. bacteriophora* (B) – *C. briggsae* (C) – *S. feltiae* (D) – *C. elegans* (E) – *S. carpocapse*.

Table 2. Predicted Tm's of probe/targets in the three color Lights-On/Lights-Off nematode species identification assay.

			Number of Probe/Target Mismatches and Tm's of Probe/Target									
Probe	Length	Color & Type	*C. elegans*		*C. briggsae*		*H. bacteriophora*		*S. feltiae*		*S. carpocapse*	
1	40	Off	7	52.3	6	53.8	8	63.0	11	41.6	3	61.7
2	41	Cal-Org On	5	55.3	5	58.3	5	55.2	3	61.3	6	52.3
3	45	Off	12	53.3	12	58.6	12	51.1	14	45.7	12	54.7
4	34	Cal-Red On	6	51.7	3	54.9	3	58.9	3	59.5	5	56.7
5	43	Off	9	43.4	7	53.5	7	52.4	10	42.4	8	50.5
6	35	Cal-Red On	9	28.0	6	55.9	7	51.9	6	53.9	8	25.4
7	40	Off	12	47.2	14	35.0	14	34.7	6	49.8	12	44.9
8	39	Quasar On	7	47.2	6	58.9	6	51.3	4	58.8	4	58.1
9	35	Off	8	33.2	8	48.1	8	37.4	9	22.8	6	46.1
10	36	Quasar On	5	56.1	3	42.9	3	58.8	7	53.9	6	57.7

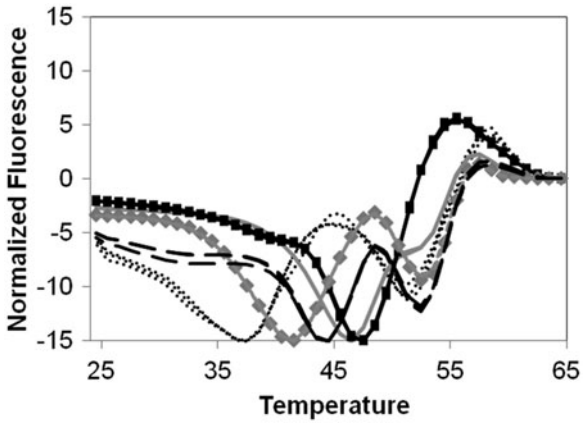

Figure 4. The fluorescent signatures of *C. briggsae* – black dashed, *H. bacteriophora* – black dotted, *C. elegans* – grey cubed, *S. carpocapse* – grey solid and *S. feltiae* – black cubed, in the single fluorescent channel Quasar670.

sequence variations between populations and within populations of these individuals.

Discussion

The Virtual Barcoding assays described here should be regarded as proof-of-principle demonstrations of a highly versatile new method for species identification. In order to standardize the method and make it broadly applicable many more decisions have to be made regarding the optimal length of the target sequence, the number of fluorescent colors used to analyze that sequence and the exact design and placement of Lights-On/Lights-Off probes to coat and distinguish all sequences within a genus. The thousands of COI sequences already available can be used for designing and *in silico* testing a universal set of Lights-On/Lights-Off probes that is likely to generate a unique Virtual Barcode for every amplified CO1 target.

The technical challenge ahead for Virtual Barcoding is how best to define the primers used for amplification. Our experience thus far is that LATE-PCR primers can be designed to be inclusive of all species within a genus, but exclusive of species in an adjacent genus. Alternatively, as explored in the present study, multiple Limiting primers can be combined such that any species within any genus is amplified. Initiator primers described by Pierce & Wangh (2014) suggest an even more inclusive approach to primer design. In this case, low concentrations of initiator primers serve to get amplification of even very divergent targets started. Once started, amplification is driven to completion by a common thermodynamic LATE-PCR that guarantees that a copious supply of single-stranded target is generated. All of these strategies can be used to advantage with Virtual Barcoding and more than one strategy can be used in the same multiplex reaction.

One reason for development of this new method lies in the fact that the current approach to barcoding the tens of millions of eukaryotes on earth is slow, labor intensive and expensive. The fully optimized Virtual Barcoding assay of the future will make it possible to inexpensively, rapidly, and automatically screen new specimens in order to identify and further characterize only those that have not been observed before. Once the resulting amplicons are sequenced using the previously described Dilute'N'Go protocol (Jia et al., 2010; Rice et al., 2007), its new Virtual Barcode along with its nucleotide sequence can be added to in ever expanding Barcode of Life library of information. Multiplexed Virtual Barcoding will also make it possible in the future to screen for strains within a species.

Conclusion

An important reason for developing Virtual Barcoding is to detect and rapidly identify species of animals and plants important to health, commerce, environmental protection, law enforcement, and many other practical pursuits of humankind. In this regard the full potential of Virtual Barcoding will be achieved by implementing it in a rapid, low cost, handheld device. The proof-of-principle assays described here can readily be converted into diagnostic assays for parasitic helminthes by using LATE-PCR primers specific for amplification of the CO1 gene in those pathogenic species. Because each single stranded CO1 gene product is coated with ten different independently-hybridizing probes in this closed tube assay, it is likely that the same set of probes can be used to generate a different Virtual Barcode for every sequence variant of the CO1 gene target amplified from any animal species on earth. We anticipate that Virtual Barcoding will be particularly useful for rapid sensitive identification of small multicellular and single-celled species that are difficult to classify on the basis of morphology.

Declaration of interest

None of the authors have any conflict of interest or financial stake in this research. The research describe here was funded by a grant from Brandeis University to LJW.

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