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# DNA Barcoding of Parasitic Nematodes: Is it Kosher?

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ABSTRACT: Nematode parasites were encountered in kosher-certified fish meat and roe, and the question was raised as to whether or not these food products were kosher as concerns food preparation standards—a matter that pertains to the identity and, by extension, the life cycle of the parasites. To ascertain the identities of parasitic nematodes, given the distorted or damaged nature of the specimens, molecular techniques were applied in the form of DNA barcoding. To our knowledge, this is the first application of this technique to an obviously cultural concern as opposed to one of health or economic significance. Results, based both on cytochrome *c* oxidase subunits I and II, suggested that the parasite species found in the fish products are anisakine species that do not inhabit the intestinal lumen of the fish hosts examined. Thus, there was no evidence of failure to adhere to food preparation practices consistent with the proscriptions of Orthodox Judaism. Notwithstanding the success of DNA barcoding in determining at least the higher taxonomic identities of the parasites, some shortcomings of the DNA barcoding pipeline as it pertains to nematode parasites were encountered; specifically, the paucity of data available for the DNA barcoding locus, even for very common nematode taxa.

DNA barcoding promises to allow the rapid and accurate identification of all animal species on the basis of 1 locus, cytochrome *c* oxidase subunit I (COI), to the extent that the locus is unique to all species (Hebert et al., 2003; Hebert and Gregory, 2005; Hajibabaei et al., 2007). While the strategy is not without pitfalls or controversies (e.g., Moritz and Cicero, 2004; DeSalle et al., 2005; Siddall et al., 2009), it has quickly captured the imagination of even the non-academic public. Already, DNA barcoding has uncovered contraband bushmeat (Eaton et al., 2009) and violations of labeling requirements for marketed fish (Wong and Hanner, 2008; Lowenstein et al., 2009) and has allowed accurate identification of potentially toxic tuna (Lowenstein et al., 2010). With a few exceptions (e.g., Besansky et al., 2003; Hansen et al., 2007; Ogedengbe et al., 2011), this technique has not been widely adopted by the parasitological community. In a compelling manifesto, Besansky et al. (2003) argued that "nowhere is the gap in taxonomic knowledge more urgent" than with parasites and that DNA barcoding holds great potential to "boost the rate of discovery" of species diversity and of life cycles. For example, Hansen et al. (2007) were able to distinguish among virulent and non-virulent *Gyrodactylus* specimens, whereas Ferri et al. (2009) called for a comprehensive barcoding study of spiruroid nematodes in light of their economic impact and health significance, noting that it "allows correlating any life stage of an organism, or a small part of it, to a single molecular entity."

In March 2011, 3 rabbinical experts from the Orthodox Union approached us with a problem. Canned sardines and preserved capelin eggs were being found by consumers to be infested with worms. The Orthodox Union is the largest organization that certifies food products as kosher and pareve for the Jewish community. The strict supervision of food preparation that is entailed in such certification extends an assurance of food quality beyond the immediate orthodox Jewish community. Approximately 75% of all pre-packaged food has a kosher certification, and loss of such certification can hamper the financial viability of a food processing company or restaurant. Fish holds a special place in the kosher diet as it can be consumed either with meat products (fleishik) or with milk products (milchik). Thus, prepared fish and fish roe represent sources of protein that are eminently flexible during travel or in the context of functions and events outside of the kosher-adherent home. While the mere presence of microscopic crustaceans in unfiltered New York City tap water renders it non-kosher, such is not the case for nematode worms, against the consumption of which there is no kashrut proscription. Rather, the presence of worms portends of improper handling during which intestinal contents have been allowed to co-mingle with sardine meat or preserved capelin eggs in a manner that would compromise kosher certification. Fish can harbor nematode life-history stages in musculature and elsewhere besides the intestinal lumen; the difference in tissue location is predicated on the nematode species in question and its life cycle. As such, in what we believe is the first application of DNA barcoding to a matter of cultural import, as opposed to one of health or economic significance, the Orthodox Union asked us to bring systematic expertise and technology to provide definitive data and analysis regarding the worms' taxonomic identities.

Sardines, including 6 whole frozen Portuguese *Sardina pilchardus* and 7, 4-ounce tins of prepared fish from Portugal (*Sardina pilchardus*) and Scotland (*Clupea harengus*) as well as 2, 1-lb samples of Icelandic capelin (*Mallotus villosus*) roe labeled "masago," each bearing the mark of Orthodox Union certification, were examined for nematode worms. A 1-lb batch of capelin eggs dyed black yielded 4 worms. A 1-lb batch of capelin eggs dyed orange yielded 53 worms. The numbers of nematodes among clupeiform fish meat from 4-oz tins were 12, 3, and 1 from Scotland and 5, 2, 1, and 0 from Portugal. Whole frozen sardines were parasite-free in musculature, visceral organs, and intestines.

All nematode worms found were larval and in various states of distortion or damage and, thus, were refractory to definitive morphological identification. All 4 worms from black capelin roe, 10 worms from orange capelin roe, and all worms from tinned fish were cut in half with the posterior portion subjected to DNA extraction with a DNeasy® Blood and Tissue (Qiagen Ltd., Valencia, California) protocol. Amplification of the barcoding locus was attempted with both universal COI primers (Folmer et al., 1994) and with anisakid-specific COI primers (Cross et al., 2006). Following the attempts at molecular identification using this barcoding locus, additional amplifications were performed using primers no. 210 and 211 for COII (Nadler and Hudspeth, 2000). The methods used for extraction, amplification, and sequencing of these loci followed those of Phillips et al. (2010) for COI; identical procedures were followed for both COI and COII. None of the larval worms from tins of sardines yielded amplifiable DNA. To enable taxonomic identification of successfully sequenced amplicons, comparative COI and COII sequences of representative nematode taxa were obtained from the National Center for Biotechnology Information on the basis of highest-scoring BLASTn (Altschul et al., 1990) matches. Alignment of sequences was performed using MUSCLE v3.7 (Edgar, 2004) on the European Bioinformatics Institute server, applying default settings. Phylogenetic analyses were performed in TNT (Goloboff et al., 2008) using a heuristic search of 5 random taxon addition sequences, each followed by ratcheting and tree fusing, stipulating that the shortest tree must be found at least 3 times.

The resulting DNA barcoding tree for COI (Fig. 1A) suggested the presence of at least 4, and perhaps 5, species of parasitic nematodes co-mingling with capelin roe. Only 1 cluster of these unknown worms grouped definitively with a previously barcoded species: ORW4 and ORW6 were molecularly equivalent on COI to *Anisakis simplex*. The remainder, while clearly grouping among anisakine species as expected, did not convincingly associate monophyletically with any of those species for which barcodes were available (Fig. 1A).

The overall inconclusive results of DNA barcoding, i.e., employing COI, forced our consideration of COII for species identification because

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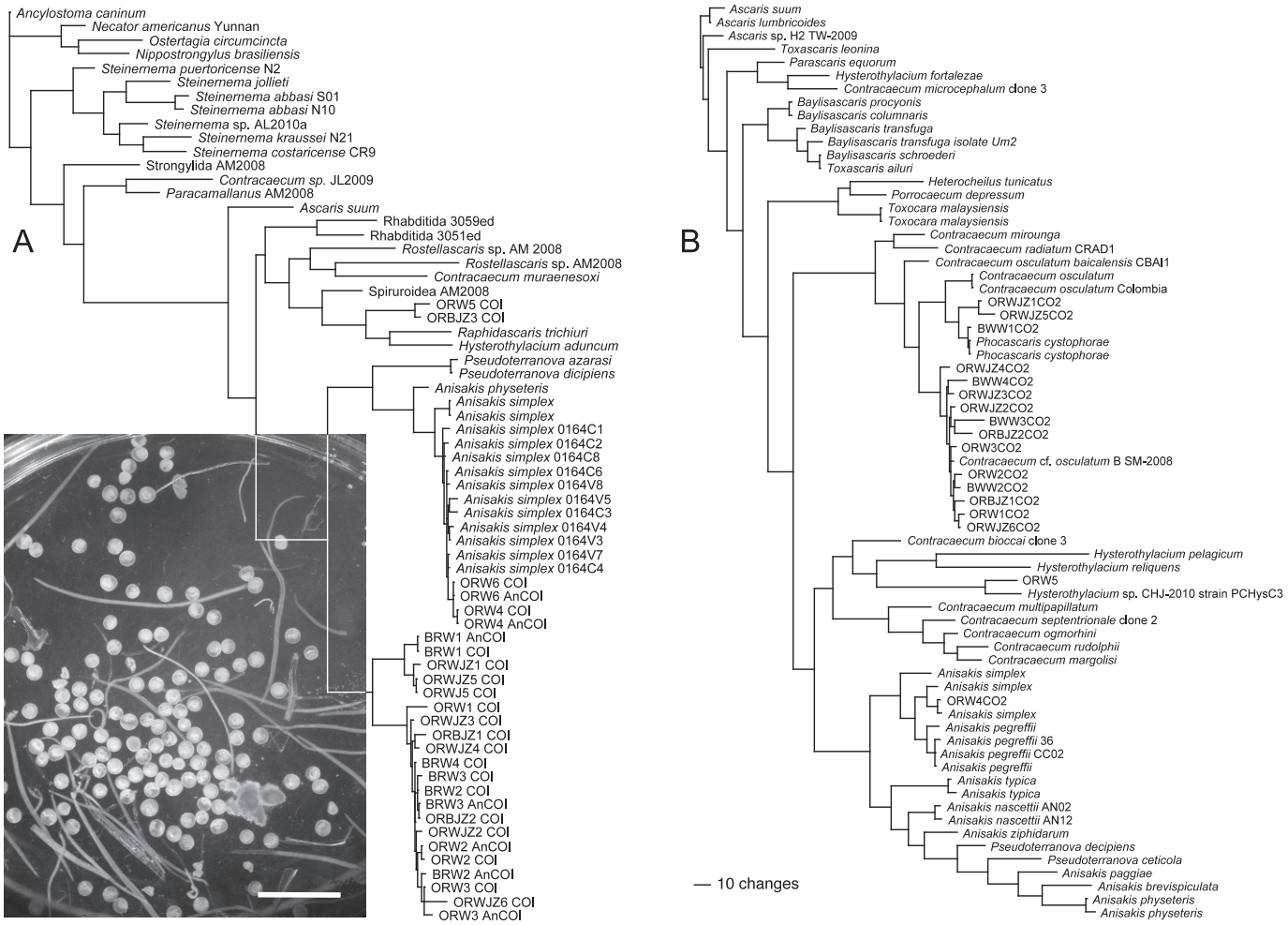


FIGURE 1. Phylogenetic trees derived from parsimony analyses of COI (**A**) and COII (**B**) mitochondrial gene sequences. The prefix "OR" indicates that isolates were collected from orange capelin roe; the prefix "BR" indicates that isolates were from black capelin roe. Isolates from tinned fish muscle were refractory to amplification of either locus. Differential representation of isolates in A or B is indicative of success of amplification and sequencing of the 2 loci. Branches are drawn proportional to amount of change. Inset: nematode larval stages among capelin eggs. Scale bar = 1 cm.

more comparative data are available for the latter. The COII tree (Fig. 1B) was considerably more informative as to identities. As with COI, ORW4 (and by implication ORW6 in Fig. 1A) was reliably identified as *Anisakis simplex*. BRW1 was identified as *Phocascaris cystophorae*. ORW5 appeared to be a species of *Hysterothylacium*, while the largest assemblage of isolates formed a monophyletic group with European isolates of *Contracaecum* c.f. *osculatum* (Fig. 1B).

While COII suggests a second species of *Phocascaris*, presently there are insufficient comparative data in public databases to convincingly identify either ORWJZ1 or ORWJZ5 to species.

With the exception of some species of *Hysterothylacium* (Køie, 1993; Adroher et al., 1996), none of which infect clupeiforms as adult worms, none of the species or genera of nematodes identified herein are expected to be present in the lumena of fish serving as definitive hosts. As such, there was no positive evidence of intestinal worms (and by implication, intestinal contents) co-mingling with capelin roe. Furthermore, *Hysterothylacium aduncum* is the only nematode known to use Iberian sardines (Rello et al., 2008) as an intermediate host, while this species and *Anisakis* spp. are all that is to be expected from extraintestinal sites of North Atlantic herring (McGladdery and Burt, 1985). Based on existing knowledge of the life cycles (e.g., Mattiucci and Nascetti, 2008; Moravec, 2009), we inferred that none of the worms found occur in the intestinal lumena of the hosts examined. Provided with the foregoing, the Orthodox Union issued a decision in June 2011 that, whereas the increased prevalence of nematodes in these samples might be alarming and perhaps unsightly, the products nonetheless were kosher and there was no evidence of any failure to adhere to food preparation practices consistent with Orthodox Judaism (Daf Hakashrus, 2011).

To our knowledge, this is the first case in which molecular taxonomy and an attempt at DNA barcoding have been brought to bear on an issue of cultural or religious significance. As satisfying as that is, it remains that COI, the central tenet of DNA barcoding, is obviously under-utilized by nematode parasitologists. The fact that species as ubiquitous as *H. aduncum* and *C. osculatum* each await even 1 definitive DNA barcode is as remarkable a finding, as is the presence of so many nematode species (and specimens) among capelin eggs that one may find on a grocer's shelf. The task before the parasitological community is as clear as is the potential for funding. Echoing each of Ferri et al. (2009) and Besansky et al. (2003), now is the time to create a large molecular taxonomic database for parasitic worms, particularly those that are more likely to be encountered in identification-refractory larval stages such as those in fish tissues. These efforts should simultaneously adhere to several desiderata, i.e., reference barcodes from adult worms from definitive type hosts from type localities (Kvist et al., 2010), representative barcodes of larval stages from the widest range of intermediate hosts, and representative barcodes from known or suspected geographic ranges. The promise that DNA barcoding holds for distinguishing virulent and non-virulent species and strains has already been demonstrated (Hansen et al., 2007), with obvious implications for treatment costs in aquaculture. The potential of this tool for discovering intermediate hosts and elucidating parasite life cycles is as great as is its potential to suggest levels of species diversity as-yet uncovered by traditional methods. Parasite systematics, ecology, ethology,

and epidemiology each stand to benefit enormously from a concerted effort to characterize organisms at a molecular level.

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