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Mitochondrial genomesof *Anisakis simplex*and*Contracaecum osculatum*(*sensu stricto*) - comparisonswith selected nematodes

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ABSTRACT

Anisakid nematodesparasitizemainly fish, marine mammals and/orfish-eating birds, and can be transmitted to a range of fish-eating mammals, including humans, where theycan cause gastrointestinal disease linked to larval infection or allergic responses.In spite of the animal and human health significance of these parasites, there are still gaps in our understanding of thesystematics, biology, epidemiologyand ecology of anisakids.Mitochondrial (mt) DNA provides useful genetic markers for investigations in these areas, but complete mt genomic data have been lacking for most anisakids. In the present study, the mt genomesof *Anisakis simplex sensu stricto* and *Contracaecum osculatumsensu stricto*were amplified from genomic DNA by long-range polymerase chain reactionand sequenced using 454 technology. The circular mt genomesof these species were 13926 and 13823 bp, respectively, and each of them contained 12 protein-coding, 22 transfer RNA, and 2 ribosomal RNA genes consistent for members of the Ascaridida,Oxyurida, Spirurida, Rhabditida and Strongylida.Thesemt genomes provide a stepping-stone for future comparative analyses among a range of anisakids and a basis for reinvestigating their genetic relationships. In addition, these markers might be used inprospecting for cryptic species and exploring host affiliations.

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1. Introduction

The Nematoda (roundworms) is a large phylum in the animal kingdom, and contains a wide range of species with exceptionally varied life histories(Hugot et al., 2001). Many nematodes are parasites of animals and cause diseases of major socio-economic importance globally (Anderson, 2000). Central to studying nematodes is an ability to accurately identify them to species andknowledge of their systematics (Anderson et al., 1998; Blouin, 1998; Viney, 1998; Gasser, 2006). However, given the limited number of informativemorphological characters for nematodes, nuclear and/or mt DNA sequence data have been employed for taxonomic, phylogeneticand population genetic studies(e.g., Hu et al., 2004;Jex et al., 2010a; Mattiucci and Nascetti, 2008). In particular, barcodes inferred from whole mt genome sequences of parasitic nematodes have been used to infer robust systematic relationships(Jex et al., 2010b; Park et al., 2011).

In spite of the availability of advanced sequencing technologies, there is still relatively limited knowledge of mt genomes for many parasitic nematodes of socio-economic importance, such as members of the family Anisakidae (superfamily Ascaridoidea), which parasitizemainly fish and marine animals(cetaceans and pinnipeds)and, in some cases, cause disease (anisakidosis or anisakiasis) in humans(Mattiucci and Nascetti, 2008; Hochberg and Hamer, 2010).Thisfamilyincludesthe genera *Anisakis*, *Contracaecum* and *Pseudoterranova*, which are considered of most zoonotic importance. Currently, the genus *Anisakis*is represented by two main clades; cladeIincludes species of the *A. simplex*complex (i.e. *A. simplex* (*sensu stricto, s.s.*), *A. pegreffii* and *A. simplex* C) as well as*A. typica*, *A. ziphidarum*and *Anisakis*sp.; clade IIincludes *A. physeteris*, *A. brevispiculata* and *A. paggiae* (reviewed by Mattiucci and Nascetti, 2008). In addition, within the genus *Contracaecum*, the *Contracaecum osculatum* complex alone includes three Arctic sibling species called *C. osculatum*A, *C. osculatum*B and *C. osculatum* (*sensu stricto, s.s.*) and two Antarctic members, *C. osculatum* D and *C. osculatum* E (reviewed by Mattiucci and Nascetti, 2008).

To date, complete mt genomes sequences are available for some members of the Asacrididae, including *Ascaris suum, As. lumbricoides*(see Liu et al., 2012), *Toxocara canis, T. cati, T. malaysiensis*(see Jex et al., 2008; Li et al., 2008); *Baylisascaris ailuri*, *B. transfuga*, *B. procyonis* and *B. schroederi*(see Xie et al., 2011a,b). For the Anisakidae, mt genomes have been characterizedonly for an indeterminatememberwithin the *Anisakis simplex* complex(see Kim et al., 2006) and *Contracaecumrudolphi* B (see Lin et al., 2012), but those of individual members of the *Anisakis*, *Contracaecum* and *Pseudoterranova* species complexes have not yet been sequenced.*A. simplex*(*s.s.*)isof particularzoonotic importance in countries in which humans consume uncooked(infected) fish, such as sushi or sashimi. Following ingestion, viable third-stage larvae (L3s) released from such fish tissues can penetrate the stomach, intestines, liver and/or other organs, causing tissue damage, inflammation and/or hypersensitivity(Sakanari and McKerrow 1989). An allergic response can also ensue the consumption of fish containing dead L3s(Audicana and Kennedy, 2008; Valero et al., 2003).Although adequate freezing or cooking kills anisakid larvae (Wharton and Aalders, 2002), parasiteallergens are thermostable (Audicana et al., 2002; Moneo et al., 2005).

Although there has been an increase inunderstanding of the biology of anisakids,through the combined use of morphological and molecular approaches, there are still some gaps in our knowledge of the genetics, ecology and epidemiology of this parasitegroup(Mattiucci and Nascetti, 2008). In addition, the prospecting for cryptic species within the Anisakidae andtheir genetic characterization (Mattiucci and Nascetti, 2008; Kijewska et al., 2009; Dzido et al., 2012)emphasize a need andutility ofwhole mt genomic data sets.Studying the mt genomes of members of this family alsoprovides a basis for assessing genetic relationships and, more broadly, forcomparative analyses of mt genomes amongmembersof the superfamily Ascaridoidea.Using long-range PCRcoupledwith massively parallel sequencing (Jex et al., 2010b), the present study determined the sequences and structures of the mt genomes for representative individuals of*A.simplex s.s.* and*Contracaecum osculatums.s.*,and compared them with currently available mt genome sequences forascaridoids and selected nematodes representing the orders Ascaridida, Oxyurida, Spirurida, Rhabditida and Strongylida.

2. Materials and Methods

2.1. Parasites, DNA isolation and identification

Larval specimens of*A. simplex* (sample AchvL1) and *C. osculatum*(sample Cosc35) were collected at autopsy from the body cavity of *Clupea harengus*from the Vistula Lagoonand the liver of *Gadus morhua*from Gdansk Basin(Baltic Sea, Poland), respectively(Zhu et al., 2007). Initially, the morphological identification of theseanisakids was based largely on the (1) morphological characteristics of lips; (2) position of the excretory pore; (3) length and shape of the ventriculus; (4) presence, length and position of the anterior intestinal caecum and posterior ventricular appendix; (5) shape of the tail, and (6) cloacal papillae(Cannon, 1977; Deardorff and Overstreet, 1980; Fagerholm, 1988; Bruce and Cannon, 1989; Fagerholm, 1990, 1991).Total genomic DNA was extracted from a small (2 mm) mid-body portion of each specimen by sodium dodecyl-sulphate/proteinase K treatment and purification over a mini-spin column (Wizard, Clean-Up; Promega) (Zhu et al., 1998; Gasser et al., 2006). In order to independently verify the identity of each specimen as *A. simplex s.s.* and *C. osculatums.s.* (cf. Mattiucci and Nascetti, 2008), the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA was amplified by the polymerase chain reaction (PCR) and sequenced according to an established method (Zhu et al., 1998, 2002).

2.2. PCR-based sequencing and assembly of mt genomes

Using each of the primer pairs MH39F-MH42R and MH5F-MH40R (Hu et al., 2007), two regions of the entire mt genome (of ~5 and 10 kb, respectively) were amplified by the long PCR (BD Advantage 2; BD BioSciences) from 20 ng of genomic DNA from each DNA sample. The cycling conditions in a ABI 2720 thermal cycler (Applied Biosystems) were: one cycle at 95°C for 1 min (initial denaturation); followed by 35 cycles of 95 °C for 15 s (denaturation); 52°C for20 s (~5 kb region) or 53 °C for 15 s (~10 kb region) (annealing); and 62°C (~5 kb region) or 68°C (~10 kb region) for6 min (extension), followed by a final extension at 62°C or 68°C for 6min (Hu et al., 2007). Each PCR yielded a single amplicon detected by agarose gel electrophoresis (Jex et al.,

2010b). Amplicons were consistently produced from the positive control sample (total genomic DNA of *Ascarissuum*); in no case was a product detected for any of the negative (no-template) controls. Amplicons were then treated with shrimp alkaline phosphatase and exonuclease I and quantified in a spectrophotometer(ND-1000 UV-VIS v.3.2.1, NanoDrop Technologies). Following an electrophoretic analysis of quality and integrity, the two amplicons (2.5 µg of each) from each of the two worms from each host species were pooled and then sequenced using the 454 Genome Sequencer FLX (Roche) (Jex et al., 2010b). The mt genome sequences (GenBank accession nos. KC965056 and KC965057) were each assembled from (~300 bp) reads using the program CAP3 (Huang and Madan, 1999).

2.3. Annotation

Each mt genomewas annotated using an established computational pipeline (Jex et al., 2010b). In brief, each protein-encoding mt gene was identified by local alignment comparison (six reading frames) using amino acid sequences conceptually translated from corresponding genes from the mt genome of *A.simplex (s.l.)*(GenBank accession no.NC_007934). The large and small subunits of the mt ribosomal RNA genes (*rrn*S and *rrn*L, respectively) were identified by local alignment of nucleotide sequence data. The tRNA genes were predicted (from both strands) based on their structure, using scalable models, based on the standard nematode mt tRNAs (Hu and Gasser, 2006). All predicted tRNA genes were then grouped, based on their anti-codon sequence, and identified based on the amino acid encoded by this anti-codon. Two separate tRNA gene groups were predicted each for serine (one each for the anticodons AGN and UCN, respectively) and leucine (one each for the anticodons CUN and UUR, respectively), because these tRNA genes are duplicated in many invertebrate mt genomes (Hu and Gasser, 2006). All tRNAs within each amino acid group were ranked based on "structural strength" (as inferred by the number of nt mismatches in each stem), and the 100 best-scoring structures for each group were compared by BLASTn against a custom database representing all published nematode mt genome sequences available in the GenBank database (accessible *via*www.ncbi.nlm.nih.gov).All tRNA genes of each mt genome were then identified and annotated based on maximum sequence identity to known nematode tRNAs. Annotated sequence data were imported using the program SEQUIN (available *via* http://www.ncbi.nlm.nih.gov/Sequin/) for the final verification of the mt genome organization/annotation prior to submission to the GenBank database.

2.4. Sliding window analysis

This analysis was performed on the aligned, complete mt genome sequences of the three species of anisakid sequenced here and other ascaridoids studied to date (Table 1) using DnaSP v.5 (Rozas et al., 2003). The alignment of these sequences was achieved using MUSCLE v.3.8 (Edgar, 2004), as implemented in SeaView v.4 (Gouy et al., 2010). Keeping the nucleotides in frame, there were no ambiguously aligned regions. A sliding window of 300 bp (steps of 10 bp) was used to estimate nucleotide diversity (π) within and between members of each genus and across the Anisakidae; indels were excluded using DnaSP. Nucleotide diversity for the entire alignments was plotted against midpoint positions of each window, and gene boundaries were defined.Separating the analyses this way allowed a comparison of general patterns across the ascaridoids, across the anisakids

and between members of each species to highlight regions with potential for the design of additional mitochondrial markers for which high variability is desirable.

2.5. Phylogenetic analysis

The amino acid sequences conceptually translated from individual genes of each of the two mt genomes were concatenated. Selected for comparison were concatenated amino acid sequences predicted from published mt genomes from key nematodes, including *A. simplex (s.l.)*,*C. rudolphi* B,*Cucullanus robustus*;*As. suum*, *As. lumbricoides*, *T. canis, T. cati, T. malaysiensis,B. ailuri*, *B. transfuga*, *B. procyonis* and *B. schroederi* (order Ascaridida) and selectednematodes from other orders, including*Enterobiusvermicularis, Wellcomia siamensis* (Oxyurida); *Heliconema longissimum*, *Brugia malayi*, *Dirofilaria immitis* and *Onchocerca volvulus* (Spirurida); *Ancylostoma duodenale* and*Necator americanus* (Strongylida); and *Caenorhabditis elegans*(Rhabditida) (see Table 1). All amino acid sequences (considering all homologous characters) were aligned using MUSCLE and then subjected to phylogenetic analysis. For this analysis, best-fit models of evolution were selected using ProtTest 3.0 (Darriba et al., 2011) employing the Akaike information criterion (AIC) (cf. Akaike, 1974). Bayesian inference analysis was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001), with a fixed JTT amino acid substitution model (Jones et al., 1992) using four rate categories approximating a Γ distribution, four chains and 200,000 generations, sampling every 100th generation. The first 200 generations were removed from the analysis as burn-in.

3. Results

3.1. Features and organization of the mt genomes

The complete mt genomesof*A. simplex s.s.* and*C. osculatums.s.*were13926 and 13823bp in length (see Fig. 1; Table 1), respectively, being comparable (13,916 to 14,322 bp) to those published for other ascaridoids, including *A. simplex s.l.*(Kim et al., 2006), *Ascaris suum, As. lumbricoides*(see Liu et al., 2012), *Toxocara canis, T. cati, T. malaysiensis* (see Jex et al., 2008; Li et al., 2008),*Baylisascaris* spp. (see Xie et al., 2011a,b)and *C. rudolphi* B (see Lin et al., 2012).The mt genomes of all three species contained36 genes: 12 protein-coding genes (adenosine triphosphatase subunit 6[*atp6*], the cytochrome *c* oxidase subunits 1, 2 and 3[*cox*1-*cox*3], cytochrome b(*cytb*)and the nicotinamide dehydrogenase subunits 1-6[*nad*1-*nad*6and *nad*4L]), 22 tRNA genes (two coding for leucine and two coding for serine) and the small [*rrn*S] and large [*rrn*L] subunits of rRNA. Each protein-coding gene had an open reading frame (ORF), and all genes were located on the same strand and transcribed in the same direction (5' to 3'), consistent with the mt genomes of ascaridoid nematodes characterized to date (cf. Hu and Gasser, 2006; Jex et al., 2010a). Specifically, the gene arrangement for the mt genome of the two anisakids was consistent with that of GA2 (Hu et al., 2003a), has been reported previously for other members of the Ascaridoidea, including *A. simplex s.l.*,*As. suum*,*T. canis* and *B. procyonis*(see Table 1).Consistent with the mtgenomes characterized to date for other Ascaridida, the AT-rich region for the *A. simplex s.s.*and *C. osculatums.s.*was located between *rrn*Sand *nad*1.As expected based on knowledge of other nematode mt

genomes (Wolstenholme et al., 1992; Hu and Gasser, 2006), the overall A+T content was high (Table 2) with mean A+T contents of 70.8% for *A. simplex s.s.* (48.2% T, 22.6% A, 9.9% C and 19.4% G) and70.2% for *C. osculatums.s.*(47.5% T, 22.7% A, 10.0% C and 19.8% G).

3.2. Protein genes and codon usages

The initiation and termination codons predicted for the protein encoding genes were compared among the three anisakids investigated (Table 3). The commonest start codon for *A.simplex s.s.*was TTG (for eight of 12 proteins each), followed by ATT (three genes), TTA and ATG (one gene each in each genome); for *C. osculatums.s.*,it was TTG (seven genes), followed by ATT (five genes).Seven to nine mt protein genes had a TAA or TAG translation termination codon; the other protein genes ended in an abbreviated stop codon, such as TA or T (Table 3). For all two anisakid species studied, the 3'-end of these genes was immediately adjacent to a downstream *trn* gene (Table 3), consistent with the arrangement for *Toxocara* species(Jex et al., 2008; Li et al., 2008), *As. suum*, *As. lumbricoides* (see Liu et al., 2012), *B. ailuri*, *B. transfuga*, *B. procyonis* and *B. schroederi* (see Xie et al., 2011a,b)

The codon usages for the 12 protein genes were also compared between the anisakids (Table 4); 63of 64 possible codons were used in the mt genome of *A. simplex s.s.*, whereas all 64 codons were used in *C.osculatums.s*.Codon CGC (Arg) was not utilized in the mt genome of *A. simplex s.s*. The preferred nucleotide usage at the third codon position of mt protein genes of both anisakids studied reflects the overall nucleotide composition of these mt genomes. At this position, T is the most frequently, and C the least frequently used. For both species, the codons ending in G have higher frequencies than the codons ending in A, which is similar to, for example,*Toxocara* species and*As. suum* (Ascaridida) and *O. volvulus* (Spirurida) but distinct from representatives of the orders Strongylida (*An.duodenale* and *N. americanus*) and Rhabditida (*C. elegans*). As the usage of synonymous codonsis proposed to be preferred in gene regions of functional significance (Sharp and Matassi, 1994; Durent and Mouchiroud, 1999), codon bias might be linked to selection at silent sites.

The AT bias in the two mt genomes characterizedwas also reflected in a bias in the amino acid composition of predicted proteins. The AT-rich codons represent the amino acids Phe, Ile, Met, Tyr, Asn or Lys, and the GC-rich codons represent Pro, Ala, Arg or Gly. The most frequently used codons were TTT (Phe), TTA (Leu), ATT (Ile), TTG (Leu), TAT (Tyr) and GTT (Val). The least frequently used codons were CTA, CTG (Leu), ATC (Ile), GTC (Val), AGC (Ser), CCC (Pro), GCC (Ala), TAC (Tyr), CAC (His), AAC (Asn), CGA (Arg), TCC (Ser) and GGC (Gly). All four GC-rich only codons are represented here, and every codon had at least one C. When the frequencies of synonymous codons within the AT-rich group, such as Phe (TTT:13.0% to 13.3%; TTC: 0.7% to1.1%), Ile (ATT:6.3% to6.4%; ATC: 0.5% to 0.9%), were compared between the mt genomes, the frequency was always less if the third position was a C.

3.3. Ribosomal and transfer RNA genes

The small and large subunitribosomal RNA genes (*rrn*S and *rrn*L, respectively) of

the mt genomes of *A. simplex s.s.* and*C. osculatums.s.*were identified by comparisons with those of *A. simplex s.l.* and *Contracaecumrudolphi* B. The *rrn*S and *rrn*L genes were 699 bp and957bp in length, respectively. The predicted secondary structures of 22 tRNAs for each of the three species were similar to those reported from a range of nematode species, with the exception of species of *Trichinella*(Lavrov and Brown,2001; Webb and Rosenthal, 2011). For instance, all tRNAs, except for *trn*S1 and *trn*S2, lacked a TΨC and, instead, had a TV replacement loop; also, *trn*S (UCN) and *trn*S (AGN) lacked a dihydrouridine (DHU) arm, but had a TΨC stem-loop structure (Hu and Gasser, 2006).

3.4. Non-coding regions

The AT-rich non-coding regions, between *trn*S (UCN) and *trn*N,were 513bp and 365 bp in length for *A. simplex s.s.* and*C. osculatum s.s.*, respectively (Table 2). With A+T contents of 79.2-82.4%, these non-coding regions were richer in A+T than any other regions within these mt genomes (Table 2).

3.5. Sliding window analysis

Results of the sliding window analyses are shown in Fig. 3 each for (i) *Ascaris*, (ii) *Baylisascaris*, (iii) *Toxocara*, (iv) *Contracecum* and (v) *Anisakis* species, the latter including a plot of nucleotide variability across the Anasakidae (*Contracaecum* and *Anisakis* species). For each ascaridoid, the AT-rich (AT) regions provided the greatest source of diversity, largely because unambiguous alignments could not be established between taxa within these regions. Although the AT-rich region is common to all nematode mtDNAs, its non-coding nature and relatively fast evolutionary rate, makes base positional homology difficult, if not impossible, to establish here. Elsewhere in the mtDNAs, base positional homology could be established readily and unambiguously, reflecting conservation of gene length and relative gene order; peaks and troughs of nucleotide diversity reflect predominantly transitions and transversions, with the occasional indel (mostly amongst RNA and short non-coding regions).

Nucleotide diversity patterns are broadly similar across the sliding window analyses, with greater amplitude of diversity between more distantly related species within genera. Diversity within and between genes is relatively consistent across the genera, with no single gene region appearing as markedly more variable than any other. In anisakids (Fig. 3), the following genes consistently showed regions of high variability: *rrn*L, 3'-end of *nad*5, *nad*6, *nad*2, *cyt*b and the 3'-ends of *cox*3 and *nad*4.

3.6. Comparative analysis with other nematodes

Pairwise comparisons in the amino acid sequences inferred from individual protein-coding genes and the nucleotide sequences of the rRNA genes betweenthe mt genomesof *A. simplex s.s.* or *C. osculatum s.s.*and 18other nematodes (of the orders Ascaridida, Oxyurida, Spirurida and Strongylida) (Table 5). The amino acid sequence similarities in individual inferred proteins ranged from 48.0% (NAD2) to 100.0% (ATP6, NAD3 and NAD4L) among species of Ascaridida. Upon pairwise comparison, the amino acid

sequence similarities between *A.simplex s.s.* or*C. osculatums.s.*and selected Spirurida (*B. malayi*, *D. immitis* and *O. volvulus*) and Strongylida (*A. duodenale* and *N. americanus*) ranged from 19.4-21.6% (ATP6) to 51.4-90.9% (COX1) and from 49-53% (NAD2) to 89-94.3% (COX1), respectively. The nucleotide sequence similarities (Table 5) in *rrn*S were 80.5-81.1%between *A. simplex s.s.* and *C. osculatums.s.*, 53.0-55.5% compared with the three members of the order Spirurida, and 53.2-66.0% compared with the two species of Strongylida. In addition, the nucleotide sequence similarities in *rrn*Lranged from 57.4-97.3%, 51.7-55.9% or 58.0-64.2% between*A. simplex s.s.* or*C. osculatums.s.* and selected species representing the order Ascaridida, Spirurida or Strongylida, respectively (Table 5).

The phylogeny of amino acid sequence data (Fig. 2) shows a robust estimate of interrelationships of the selected nematodes, with each node fully supported, as estimated by posterior probabilities. The ascaridoid nematodes form a monophyletic clade, with each genus also strongly supported as monophyletic. *Baylisascaris* and *Ascaris* are resolved as sister taxa, in turn forming a clade with *Toxocara*, and all three genera comprise a clade sister to a monophyletic grouping of *Contracaecum* and *Anisakis*. The relatively short branch lengths separating each *Ascaris* species and each of the two members of *Anisakissimplex* reflect the high sequence similarity recorded.

4. Discussion

The superfamily Ascaridoidea comprises more than 50 valid genera that parasitize all major groups of vertebrates (see Hartwich, 1974; Sprent, 1983; Fagerholm, 1991; Sprent, 1992). Within this superfamily are many species of major animal and/or human health importance (e.g., Cheng, 1982; Bernardo and Dohoo, 1988; Stewart and Hale, 1988; Kazacos and Boyce, 1989; Dick et al., 1991; Crompton, 2001). The identification of these nematodes at any life cycle stage in any host to the species level is central to the diagnosis of infection and is also the basis for studying their life cycles, epidemiology, population biology and systematics. However, there are considerable obstacles in the specific identification of some developmental stages based on their morphology because of a lack of distinctive characters. This statement applies particularly to nematodes of the family Anisakidae, which parasitize fish, mammals, fish-eating birds and reptiles (Hartwich, 1974).

It is wellrecognized that some genera of anisakids are transmissible to humans, where they can cause significant clinical diseases (Cheng, 1982; Dick et al., 1991; Yagi et al., 1996). Since the first reports describing the pathogenic effects of *Anisakis* species in humans (van Thiel et al., 1960, van Thiel, 1962), there has been increasing awareness of fish-borne parasitic diseases (Smith and Wootten, 1978;Hochberg and Hamer, 2010; Pravettoni et al., 2012).Although*A. simplex* has been reported as the main cause of human anisakiasis (Mattiucci and Nascetti, 2008), other species can also represent a potential hazard.For instance, some recent studies (Mattiucci et al., 2011, 2013) have reported that *A. pegreffii* was associated with a number of anisakiasis cases and gastrointestinal/allergic reactions in humans in Italy. Moreover,human anisakidosis caused by *P. decipiens*has been recordedinFrance (Pinel et al., 1996), Iceland (Skirnisson, 2006), Korea (Koh et al., 1999, Yu et al., 2001), Chile (Mercado et al., 1997, 2001; Jofré et al., 2008) andPeru (Cabrera et al. 2003), and a case caused by *Contracaecum* sp. was reported recently from Australia (Shamsi and Butcher, 2011).

Human anisakidosis can be classified into several different clinical forms,based on the locations and histopathological lesions caused by anisakid larvae (Cheng, 1982; Olson et al., 1983). Although some gastric anisakidosis cases can be diagnosed by endoscopic examination, clinical diagnosis is often challenging (Pinkus et al., 1975;Hochberg and Hamer, 2010). Serodiagnosis has some potential for diagnosis when larvae cannot be detected in the stomach or small intestine (Akao et al., 1990), but antigenic cross-reactivity among different species/genotypes of anisakids can lead to misdiagnoses.There are also considerable obstacles in the accurate identification of anisakids due to limitations in identifying larval stages based on morphological features (e.g., Cheng, 1982; Olson et al., 1983; Fagerholm, 1988; Dick et al., 1991; Mattiucci and Nascetti, 2008) or when only fragments of worms from infected hosts are available for identification (Dick et al., 1991). PCR-based methods, utilizing genetic markers in first (ITS-1) and second (ITS-2) internal transcribed spacers, have usually overcome some of these obstacles in that these techniques allow the specific detection of parasite DNA from minute (nanogram to picogram) amounts of parasite material (Gasser, 2006).However, for some species of anisakids, there is insufficient sequence variation in ITS to allow accurate delineation among some sibling species. For instance, while the unequivocal identification of *A. pegreffii* is not possible based on the ITS-2 sequence alone, because this species has the same sequence as *A. simplexs.s.* (accession nos. AY826720, AB196670 and AB196672), differentiation appears to be possible based on diagnostic nucleotides C and C at alignment positions 280 and 296 in *A. pegreffii* in ITS-1 (D'Amelio et al., 2000; Abollo et al., 2003; Abe et al., 2005). Given this low level of sequence variation in ITS-1, mt genomic markers are likely to provide improveddiagnostic signals.In addition, recently, Abollo et al. (2003) identified two distinct sequence types (with C/T and C/T polymorphisms at nucleotide positions 280 and 296, respectively) in the ITS-1, in order to characterize the genotype of *Anisakis* hypothesized to be a hybrid between *A. pegreffii* and *A. simplexs.s.* Although the "hybrid proposal" has been adopted, it is possible that "mixed" ITS-1 sequences might relate to lineage sorting and retention of ancestral polymorphism (Elder and Turner, 1995; Anderson, 2001).For the Pacific region, current evidence (Umehara et al., 2006) suggests that *A. simplexs.s.* is mainly distributed in the North PacificOcean and *A. pegreffii* in the southern Sea of Japan. Therefore, more evidence is required to support to the proposal that *A. pegreffii* x *A. simplexs.s.* "hybrids" occur in natural populations in Asian waters. Mitochondrial genomic and proteomic datasets might be employed in a complementary way, together with morphological study and with ITS-1 and ITS-2 sequence data sets, to retest the hybrid proposal, and might assist future studies of members within the *A. simplex* complex in natural populations.

Based on sliding window analysis across available mt genomes of anisakids, primers can now be rationally and selectively designed to relatively conserved regions flanking "variable tracts" considered as most informative for population genetic investigations (cf.Fig. 3). Using such primers, single-strand conformation polymorphism (SSCP) analysis (Gasser et al., 2006) can be applied to screen large numbers of individuals representing different populations for haplotypic variability in a range of genes. This approach has been utilized, for instance, in a study of the genetic structures of *Ascaris* populations in pigs and humans in six provinces in China (Peng et al., 2005);this study provided support for the proposals that there is restricted gene flow in *Ascaris* between these two host species and that pigs are not a significant source of human ascariasis in endemic regions (Anderson, 2001; Peng et al., 2007). Moreover, Hu et al.

(2001) employed SSCP-based analyses of three different regions, including cytochrome *c* oxidase subunit I [*cox*1], and the small and large subunits of rRNA [*rrn*S and *rrn*L] to define 46 haplotypes among 62-66 individuals representing six different members of the *C. osculatum* complex. For all three mtDNA regions, 4 (10.5%), 7 (18.4%), 15 (39.5%) and 11 (28.9%) of 38 nucleotide positions were considered diagnostic (fixed) and could thus unequivocally delineate *C. osculatum*A, B, C (now*C. osculatum s.s.*) and *C. osculatumbaicalensis*. The lack of an unequivocal nucleotide difference in any of the three mtDNA regions used between *C. osculatum*D and *C. osculatum*E was in accordance with previous nuclear ribosomal DNA sequence data (Zhu et al., 2000), but inconsistent with multilocus enzyme electrophoretic data (Orecchia et al.,1994;D'Amelio et al., 1995). Using more variable mt gene regions, such as *rrn*L, *nad*5, *nad*4, *nad*2 and *cyt*b (Fig. 3), we hope that it might be possible to reliably delineate all sibling species. Although highly variable in length and sequence composition, it is unlikely the AT-rich regions will be of use except perhaps for fine-scale population genetics within species.

Utilizing a range of mt gene markers with differing degrees of within-specific variability, such a mutation scanning-targeted approach is also readily and directly applicable to anisakid populations. This is particularly relevant, given the evidence that *A.simplex* and*C. osculatum*represent species complexes(Mattiucci and Nascetti, 2008), and there are still some considerable gaps in our knowledge ofthe biology, life cycles and ecology of parasites within these complexes as well as precisely which species and genotypes are transmissible to humans. For instance, although it is wellestablished that some *Anisakis*members, such as *A. simplex s.s.*, are transmissible to humans, in which they can cause significant clinical diseasecharacterized by colic (abdominal pain) and/or allergic responses(Hochberg and Hamer, 2010; Pravettoni et al., 2012), therehave been limitations in the specific identification of larval stages of members of the *A. simplex* complex using morphological features (Mattiucci and Nascetti, 2008).

From epidemiological and ecological perspectives, it would be interesting, utilizing mt genomic data, to confirm or refute the involvement of particular species or genotypes of anisakids in human anisakidosis cases, currently considered to be caused mainly by *A. simplex s.s.* based on serological evidence (e.g., Toro et al., 2004), as there has been controversy as to the specific identity of the causative agent of this disease in many instances and in a number of countries (Mattiucci and Nascetti, 2008). It would also be relevant to explore whether larvae of particular sibling species within the *A. simplex* and othercomplexes have a particular affinity to the human host and/or cause disease, and whether there are specific genotypes that cause allergic disease *visa vis* infection. Indeed, some studies (Suzuki et al. 2010;Quiazon et al. 2011;Abattouyet al. 2012; Arizonoet al. 2012) suggest that *A pegreffii* might be less pathogenic than*A simplex s.s.*This proposal remains to be testedusing a combination of molecular tools with traditional parasitological and immunological techniques, it should also be possible to investigate the ecology of anisakids in marine environments, and also to characterize in detail experimental infections in "model hosts" (e.g., mice) (Cho and Lee, 2006). Furthermore, mt barcodes might be useful for exploring the zoonotic risk of anisakid larvae in fish hosts, complementing the use of other genetic markers, such as ITS-1 and ITS-2 rDNA regions (Gasser, 2006; Gasser et al., 2006).

Pairwise comparisons of the amino acid sequences conceptually translated from protein-coding genes as well as the nucleotide sequences of the ribosomal RNA genes indicated that the mt genomesof *A. simplex s.s.* and*C. osculatums.s.* most closely resemble those of other members of the order Ascaridoidea. However, analyses of sequence data

indicatedthat the next most similar nematode orders were the Strongylida and Rhabditida, but not the Spirurida. This result is consistent with previous phylogenetic analyses of mt datasets, such as concatenated amino acid sequences for all 12 protein-coding genes (Hu et al., 2004; Park et al., 2011) and gene arrangements (Hu et al., 2003a). These studies placed the Spirurida in a clade separate from the other nematodes for which data were available, being consistent with the relationships based on the analysis of classical taxonomic data sets(Skrjabin, 1991, 1992; Skrjabin et al., 1991, 1992). The placement of the Ascaridida and Strongylida relative to Spirurida contrasts that based on phylogenetic analysis of sequence data for the small subunit (SSU) of the nuclear rRNA gene (Blaxter et al., 1998), indicating that members of the orders Ascaridida and Spirurida are within "clade III", and those of the Strongylida represent "clade V".The distinct taxonomic placement the Ascaridida and Strongylida relative to the Spirurida is further supported by variation in anti-codon usage in some mt tRNA genes of some Strongylida and Ascaridida studied compared with members of the Spirurida (i.e. *Brugia malayi*, *Dirofilaria immitis* and *Onchocerca volvulus*) (Hu et al., 2003b, 2004). The incongruence in the relationships inferred for these three nematode orders (i.e. Ascaridida, Strongylida and Spirurida) in relation tothe phylogeny proposed for the Nematoda using SSU sequence data (cf. Blaxter et al., 1998)begs further investigation of a wide range of nematodes.

In conclusion, the present study emphasizes the importance and utility of the mt genomes of anisakids as a basis for systematic, ecological and biological studies, and the specific diagnosis of infections. In particular, studying members of the Anisakidae provides a stimulus to re-explore the relationships of cryptic species using whole mt genomic datasets. PCR-coupled sequencing and bioinformatic platform employed for the direct sequencing of mt genomes from tiny body sections from singlelarvae or adults provides the prospect for large-scale population genetic and mt genomic studies of anisakids.

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Authors' contributions

RBG, ARJ and DTJL conceived and planned the research, and participated in data collection. AJ conducted long PCR-coupled sequencing, NM and DTJL conducted the analyses, RBG, ARJ and DTJL interpreted the data, and RBG, NM, DTJL, AJ and ARJwrote the manuscript. All authors read commented on and approved the final version of the paper.

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FIGURES

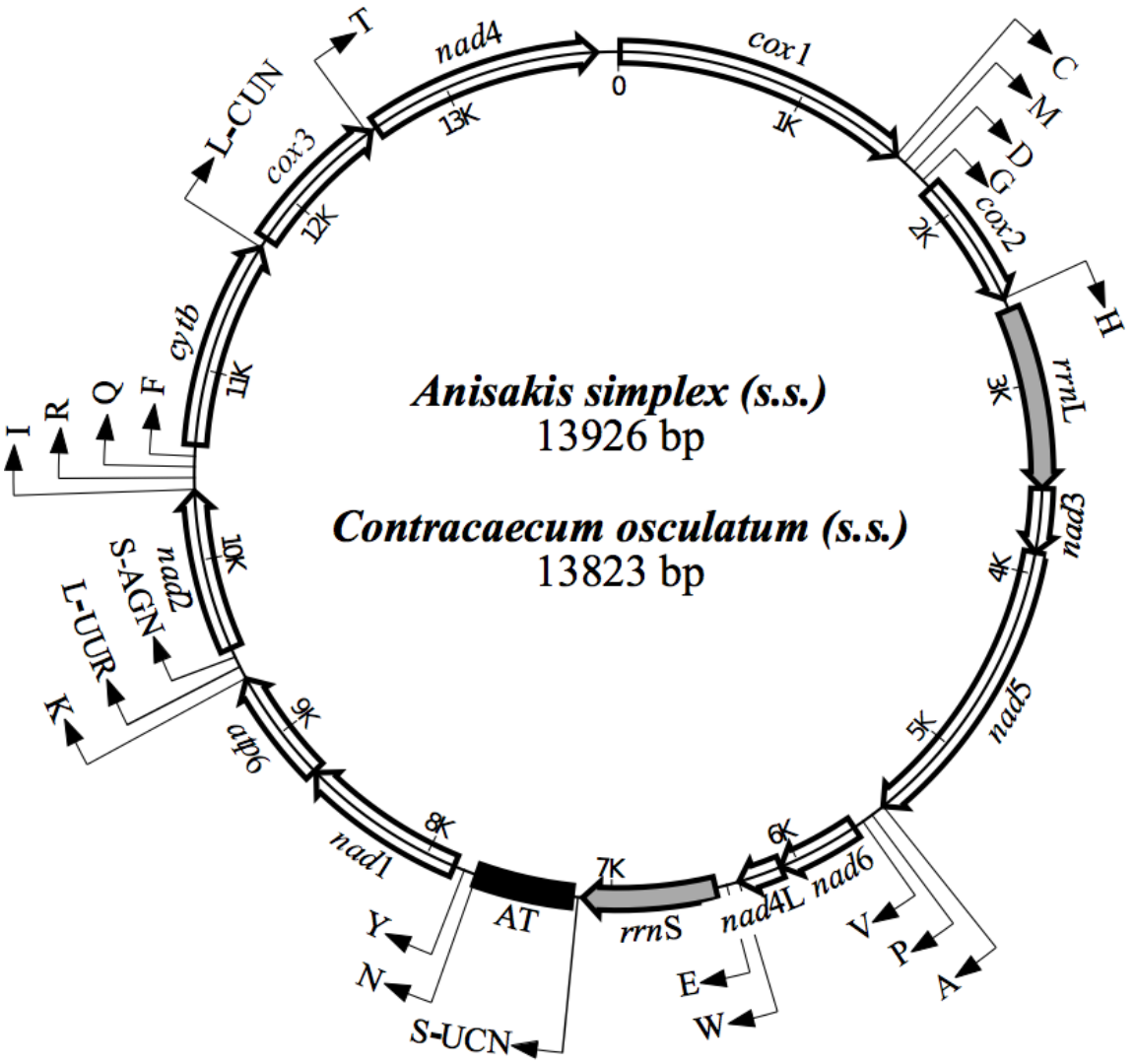

Fig. 1.A circular mt genome map representing *Anisakis simplex s.s.* and *Contracaecum osculatums.s.*All 12 protein-coding genes and the large and small ribosomal subunits of the rRNA genes are indicated in italics. Each tRNA gene is identified by its one letter symbol. Transcription is clockwise.

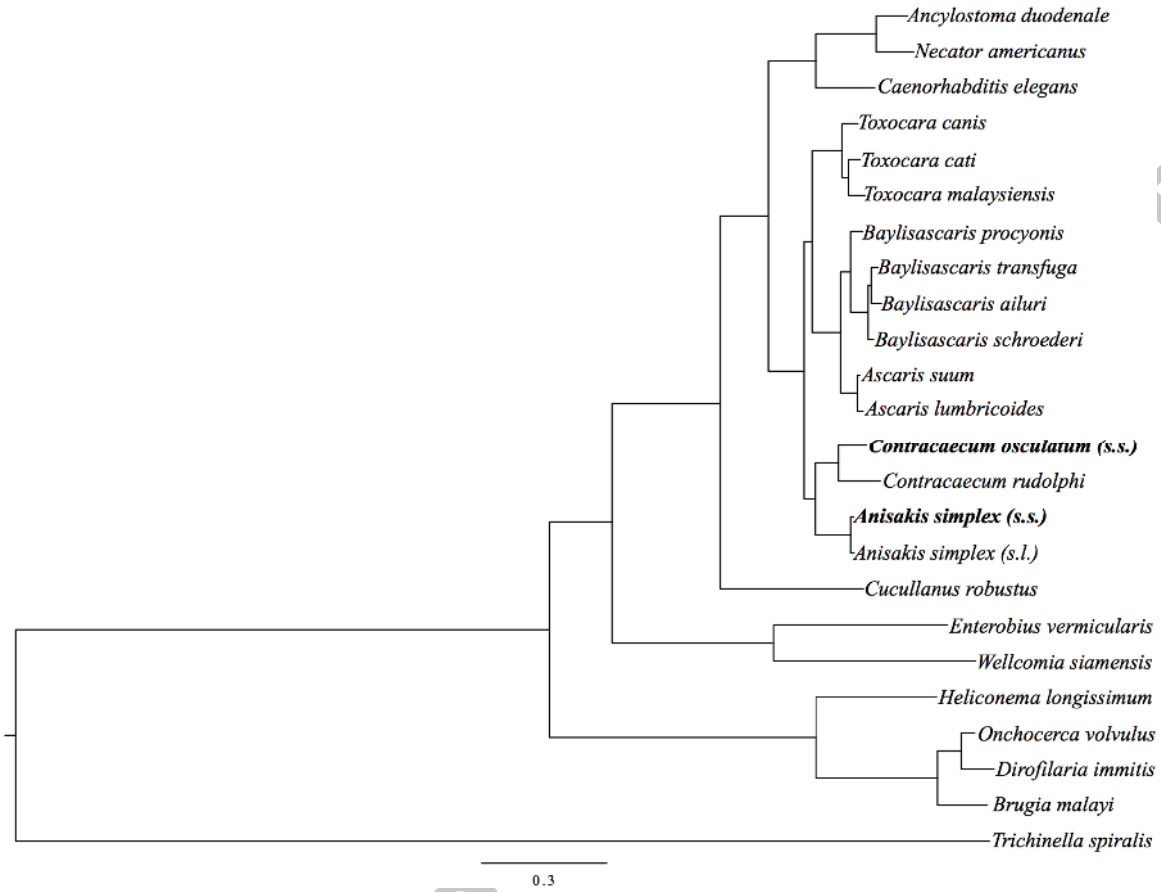

Fig. 2. Relationship of *Anisakis simplex s.s.* and *Contracaecum osculatum s.s.*with *A. simplex s.l.*, *Contracaecum rudolphi* B, *Cucullanus robustus*; *Ascaris suum*, *A. lumbricoides*, *T. canis, T. cati, T. malaysiensis*; *Baylisascaris ailuri*, *B. transfuga*, *B. procyonis* and *B. schroederi* (order Ascaridida) and selected nematodes from other orders, including *Enterobiusvermicularis* and *Wellcomia siamensis* (Oxyurida); *Brugia malayi*, *Dirofilaria immitis, Heliconema longissimum* and *Onchocerca volvulus* (Spirurida); *Ancylostoma duodenale* and *Necator americanus* (order Strongylida); and *C. elegans* (Rhabditida), based on a phylogenetic analysis of concatenated amino acid sequence data for the 12 inferred mt proteins. A multiple sequence alignment was performed using MUSCLE; the best-fit models of amino acid substitution were selected based on the Akaike Information Criterion (AIC) using ProtTest 3.0. There was absolute support (pp = 1.00) at each individual node.

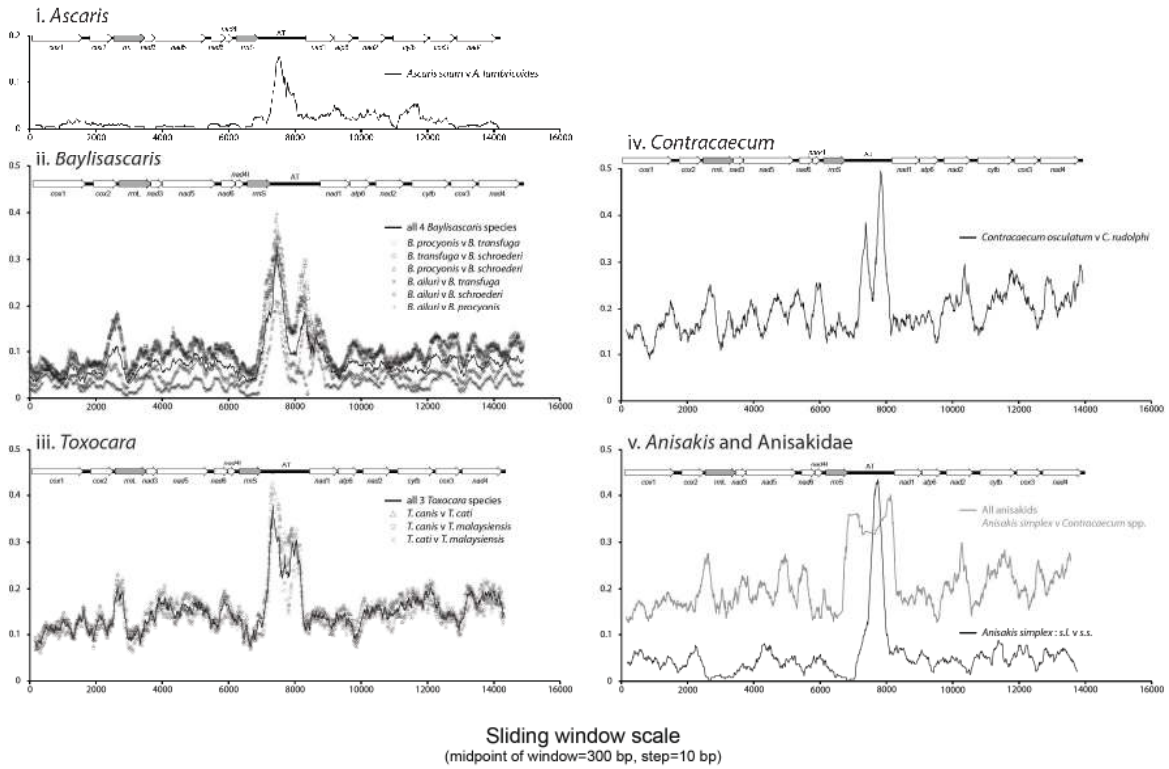

Fig. 3. Sliding window analyses of mt genomes between or among species of (i) *Ascaris*, (ii) *Baylisascaris*, (iii) *Toxocara*, (iv) *Contracaecum* or (v) *Anisakis*, and a plot across anisakid species. Nucleotide diversity, measured iteratively every 10 bp over 300 bp windows of aligned sequence data indicate peaks and troughs of sequence variability. Linearized maps of the genomes are provided for each aligned data set, with the highest peaks of variability falling within the non-coding AT-rich regions.

Table 1
Accession numbers, mt genome size, host(s) and geographical origins as well as gene order for the whole mt genome sequences characterized in this study as well as reference sequences.

Species	Accession no.	Mt genome size	Host and geographical origins	Gene order[a]	Reference
Anisakissimplex s.s.	KC965056	13926	Atlantic herring (*Clupea harengus*), Baltic Sea, Poland	GA2	This study
A. simplex (s.l.)	NC_007934	13916	Whitespotted conger (*Conger myriaster*), Korea	GA2	Kim et al. (2006)
Ascaris suum	HQ704901	14311	Pig (*Sus scrofa*), China	GA2	Liu et al. (2012)
As. lumbricoides	HQ704900	14303	Human (*Homo sapiens*), China	GA2	Liu et al. (2012)
Contracaecum osculatums.s.	KC965057	13823	Atlantic cod (*Gadus morhua*), Baltic Sea, Poland	GA2	This study
C. rudolphi B	FJ905109	14022	Cormorant, China	GA2	Lin et al. (2012)
Cucullanus robustus	GQ332426	13972	Whitespotted conger, South Korea	GA2	Park et al. (2011)
Baylisascaris ailuri	NC_015925	14657	Red panda (*Ailurus fulgens*), China	GA2	Xie et al. (2011a)
B. procyonis	JF951366	14781	Raccoon (*Procyon lotor*) in a Zoo, China	GA2	Xie et al. (2011b)
B. schroederi	NC_015927	14778	Giant panda (*Ailuropoda melanoleuca*), China	GA2	Xie et al. (2011a)
B. transfuga	NC_015924	14898	Polar bear (*Ursus maritimus*), China	GA2	Xie et al. (2011a)
Toxocara canis	NC_010690	14322	Dog*(Canis familiaris),* China	GA2	Li et al. (2008)
T. cati	NC_010773	14029	Cat*(Felis cati),* China	GA2	Li et al. (2008)
T. malaysiensis	NC_010527	14266	Cat, China	GA2	Li et al. (2008)
Enterobiusvermicularis	NC_011300	14010	Human, China	Unique	Kang et al. (2009)
Wellcomia siamensis	NC_016129	14128	Malayan porcupine (*Hystrix brachyura*)	Unique	Park et al. (2011)
Brugia malayi	NC_004298	13657	Human, Malaysia (1950's)	GA3	Ghedin et al. (2007)
Dirofilaria immitis	NC_005305	13814	Dog, Australia	GA3	Hu et al. (2003b)
Onchocerca volvulus	NC_001861	13747	Human, Liberia	GA3	Keddie et al. (1998)
Heliconema longissimum	NC_1016127	13610	Japanese eel (*Anguilla japonica*), South Korea	GA3	Park et al. (2011)
Ancylostoma duodenale	NC_003415	13721	Human, China	GA2	Hu et al. (2002)
Necator americanus	NC_003416	13605	Human, China	GA2	Hu et al. (2002)

[a] Gene order according to Hu et al. (2003a)

Table 2
Nucleotide composition of *Anisakis simplex s.s.* and *Contracaecum osculatums.s*.

Parasite	Nucleotide	Length (bp)	A (%)	C (%)	G (%)	T (%)	A+T (%)
A. simplex s.s.	Entire sequence	13926	22.6	9.9	19.4	48.2	70.8
	Protein genes	10326	20.0	10.4	20.0	49.6	69.6
	RNA genes	1658	30.2	8.3	17.4	44.1	74.3
	AT-rich region	513	36.2	6.2	14.4	43.2	79.4
C. osculatums.s.	Entire sequence	13823	22.7	10.0	19.8	47.5	70.2
	Protein genes	10359	20.4	10.5	20.2	49.0	69.4
	RNA genes	1658	29.0	8.3	17.9	45.0	74.0
	AT-rich region	365	39.7	5.5	12.0	42.7	82.4

Table 3
Organization of mt genomes of *Anisakis simplex s.s.* and *Contracaecum osculatums.s*.

Gene	*Anisakis simplex (s.s.)*		*Contracaecum osculatum* C	
	Positions and lengths of nucleotide sequences (bp)	Initiation and Termination codons	Positions and lengths of nucleotide sequences (bp)	Initiation and Termination codons
*cox*1	10 - 1585 (1575)	TTG - T (525)	1 - 1576 (1575)	TTG/T
*trn*C	1586 - 1641 (55)		1577 - 1632 (55)	
*trn*M	1643 - 1701 (58)		1632 - 1694 (62)	
*trn*D	1710 - 1768 (58)		1698 - 1753 (55)	
*trn*G	1775 - 1830 (55)		1757 - 1812 (55)	
*cox*2	1831 - 2530 (699)	TTG - TAG (232)	1813 - 2509 (696)	TTG/TAA
*trn*H	2528 - 2585 (57)		2508 - 2562 (54)	
*rrn*L	2593 - 3550 (957)		2567 - 3524 (957)	
*nad*3	3551 - 3887 (336)	TTG - TAG (111)	3521 - 3857 (336)	TTG/TAA
*nad*5	3887 - 5468 (1581)	ATT - T (527)	3856 - 5437 (1581)	ATT/T
*trn*A	5469 - 5525 (56)		5438 - 5492 (54)	
*trn*P	5542 - 5598 (56)		5495 - 5548 (53)	
*trn*V	5601 - 5656 (55)		5549 - 5603 (54)	
*nad*6	5657 - 6092 (435)	TTG - TAA (144)	5604 - 6039 (435)	TTG/TAA
*nad*4L	6093 - 6324 (231)	ATT - T (77)	6038 - 6272 (234)	ATT/TAG
*trn*W	6325 - 6382 (57)		6273 - 6329 (56)	
*trn*E	6391 - 6450 (59)		6334 - 6390 (56)	
*rrn*S	6451 - 7150 (699)		6394 - 7093 (699)	
*trn*S (UCN)	7153 - 7206 (53)		7098 - 7151 (53)	
AT - rich	7207 - 7718 (513)		7152 - 7515 (365)	
*trn*N	7719 - 7784 (65)		7516 - 7562 (46)	
*trn*Y	7788 - 7848 (60)		7562 - 7613 (51)	
*nad*1	7845 - 8721 (876)	TTA - TAA (290)	7770 - 8643 (873)	TTG/TAA
*atp*6	8734 - 9334 (600)	TTG - TAA (199)	8646 - 9246 (600)	ATT/TAA
*trn*K	9340 - 9401 (61)		9249 - 9313 (64)	
*trn*L (UUR)	9408 - 9462 (54)		9316 - 9371 (55)	
*trn*S (AGN)	9463 - 9515 (52)		9371 - 9423 (52)	
*nad*2	9515 - 10361 (846)	TTG - TAA (281)	9423 - 10269 (846)	ATT/TAG
*trn*I	10372 - 10434 (62)		10269 - 10329 (60)	
*trn*R	10434 - 10493 (59)		10329 - 10386 (57)	
*trn*Q	10491 - 10545 (54)		10383 - 10438 (55)	
*trn*F	10546 - 10605 (59)		10439 - 10494 (55)	
cob	10606 - 11703 (1097)	ATT - TAA (366)	10494 - 11601 (1107)	TTG/TAA
*trn*L (CUN)	11705 - 11759 (54)		11602-11656 (54)	
*cox*3	11760 - 12546 (786)	TTG - T (255)	11657 - 12422 (765)	ATT/T
*trn*T	12526 - 12581 (55)		12423 - 12478 (55)	
*nad*4	12582 - 13812 (1230)	TTG - TAA (409)	12478 - 13708 (1230)	TTG/TAG

Table 4
Number of codons and codon usages (%) in mt protein genes of *Anisakis simplex s.s.* and*Contracaecum osculatums.s.*

Amino acid	Codon	*A. simplexs.s.*	*C. osculatums.s.*
Non-polar			
Alanine	GCN	125 (3.64)	118 (3.42)
Isoleucine	ATY	232 (6.75)	243 (7.05)
Leucine	CTN	156 (4.54)	137 (3.97)
Leucine	TTR	368 (10.7)	384 (11.13)
Methionine	ATR	177 (5.15)	174 (5.04)
Phenylalanine	TTY	482 (14.02)	488 (14.15)
Proline	CCN	86 (2.5)	85 (2.46)
Tryptophan	TGR	76 (2.21)	73 (2.12)
Valine	GTN	300 (8.73)	324 (9.39)
Polar			
Aspargine	AAY	122 (3.55)	124 (3.6)
Cysteine	TGY	60 (1.75)	55 (1.59)
Glutamine	CAR	42 (1.22)	43 (1.25)
Glycine	GGN	201 (5.85)	201 (5.83)
Serine	AGN	196 (5.7)	202 (5.86)
Serine	TCN	167 (4.86)	161 (4.67)
Threonine	ACN	124 (3.61)	115 (3.33)
Tyrosine	TAY	171 (4.97)	167 (4.84)
Acidic			
Aspartate	GAY	71 (2.07)	74 (2.15)
Glutamate	GAR	81 (2.36)	76 (2.2)
Basic			
Arginine	CGN	31 (0.9)	34 (0.99)
Histidine	CAY	60 (1.75)	59 (1.71)
Lysine	AAR	100 (2.91)	100 (2.9)

Table 5

Percentage of similarity in the amino acid sequences inferred from the 12 mt protein-coding genes and in the nucleotide sequence of each of the two mt ribosomal genes (*rrn*L and *rrn*S) upon pairwise comparison between *Anisakis simplexs.s.*and*Contracaecum osculatums.s.*,and 18other parasitic nematodes (representing the orders Ascaridida, Spirurida, Oxyurida and Strongylida).

Proteinor rDNA	Species studied	Compared with other parasitic nematodes																	
		Asi (s.l.)	*Asu*	*Alu*	*Cru*	*Cro*	*Ba*	*Bp*	*Bs*	*Bt*	*Tcan*	*Tcat*	*Tm*	*Ev*	*Bm*	*Di*	*Ov*	*Ad*	*Na*
ATP6																			
	A. simplexs.s.	100.0	84.4	83.4	88.4	56.3	85.4	84.9	83.4	84.4	83.4	82.9	84.9	21.6	20.2	19.4	21.1	76.4	73.4
	C. osculatum s.s.	87.4	82.4	80.4	88.9	56.3	84.4	84.4	83.9	84.4	83.9	81.4	83.9	23.1	20.2	19.9	21.6	73.9	71.9
COX1																			
	A. simplex s.s.	95.0	96.4	84.4	87.2	93.7	93.7	93.5	96.2	96.2	95.6	61.1	51.4	52.4	53.0	90.9	90.8	94.3	93.7
	C. osculatum s.s.	94.7	94.3	93.7	96.2	84.2	87.2	93.7	93.7	93.9	95.8	95.8	95.2	61.1	51.4	52.4	52.8	90.5	90.6
COX2																			
	A. simplex s.s.	99.6	90.9	90.9	92.6	78.8	91.8	90.5	91.4	91.8	91.4	90.9	90.5	37.5	41.4	41.8	41.4	83.5	84.0
	C. osculatum s.s.	92.2	93.1	93.1	95.7	79.2	93.1	93.1	93.5	93.5	92.2	91.8	91.3	38.5	42.9	43.3	42.4	84.4	86.6
COX3																			
	A. simplex s.s.	98.0	87.0	87.0	89.0	69.0	88.0	87.0	88.0	80.0	90.0	90.0	89.0	45.0	35.0	35.0	37.0	83.0	81.0
	C. osculatum s.s.	89.0	89.0	89.0	89.0	71.0	87.0	89.0	87.0	78.0	90.0	89.0	88.0	47.0	34.0	34.0	34.0	83.0	82.0
CYTB																			
	A. simplex s.s.	98.6	75.9	74.2	79.0	67.2	74.3	77.3	74.0	75.4	74.9	76.0	76.2	48.6	48.9	48.9	49.7	71.6	69.9
	C. osculatum	78.1	74.8	73.2	84.0	69.9	73.6	75.0	74.7	74.5	72.3	72.6	70.9	49.7	50.0	49.4	50.0	70.7	70.1
NAD1																			
	A. simplex s.s.	94.1	82.8	82.4	81.0	65.5	82.8	82.8	82.4	82.8	84.1	85.5	83.8	56.8	49.8	49.5	49.5	67.9	67.2
	C. osculatum s.s.	88.3	84.5	84.1	91.4	68.6	83.4	84.5	83.4	83.4	86.2	87.6	85.5	59.2	52.8	51.4	50.7	70.7	71.0
NAD2																			
	A. simplex s.s.	99.0	80.0	80.0	71.0	48.0	80.0	80.0	80.0	80.0	76.0	78.0	79.0	35.0	40.0	34.0	38.0	49.0	52.0
	C. osculatum s.s.	76.0	73.0	73.0	76.0	50.0	72.0	72.0	73.0	72.0	74.0	74.0	73.0	34.0	38.0	34.0	36.0	51.0	53.0
NAD3																			
	A. simplex s,s,	100.0	84.7	84.7	83.8	69.1	82.9	82.9	82.9	82.9	88.3	88.3	84.7	57.7	36.9	41.4	44.1	66.7	71.2
	C. osculatum s.s.	83.8	84.7	84.7	85.6	67.3	83.8	84.7	83.8	82.9	85.6	85.6	84.7	55.9	34.2	36.9	40.5	67.6	69.4
NAD4																			
	A. simplex s.s.	99.3	81.2	80.9	81.9	64.1	82.4	81.2	82.4	82.2	82.4	83.1	81.9	52.7	47.2	46.2	45.7	62.8	64.3
	C. osculatum s.s.	80.4	77.0	76.8	83.1	64.5	78.0	78.0	77.5	77.0	78.7	79.5	78.5	48.3	46.5	46.0	45.7	63.8	64.5
NAD4L																			
	A. simplex s.s.	100.0	85.7	85.7	92.2	71.4	87.0	88.3	85.7	87.0	88.3	87.0	84.4	50.6	35.1	41.6	42.9	75.3	76.6
	C. osculatum s.s.	93.5	90.9	90.9	94.8	72.7	92.2	89.6	90.9	92.2	89.6	87.0	84.4	46.8	37.7	44.2	45.5	75.3	76.6
NAD5																			
	A. simplex s.s.	99.0	81.0	81.0	78.0	63.0	81.0	81.0	80.0	81.0	81.0	83.0	83.0	52.0	38.0	37.0	38.0	65.0	66.0
	C. osculatum s.s.	83.0	81.0	81.0	85.0	63.0	80.0	82.0	78.0	80.0	80.0	81.0	80.0	53.0	40.0	38.0	40.0	67.0	67.0
NAD6																			
	A. simplex s.s.	98.0	80.0	80.0	77.0	57.0	78.0	79.0	82.0	79.0	76.0	78.0	77.0	41.0	36.0	31.0	31.0	58.0	59.0
	C. osculatum s.s.	83.0	76.0	76.0	80.0	58.0	75.0	76.0	77.0	76.0	77.0	79.0	80.0	45.0	36.0	30.0	30.0	56.0	56.0
*rrn*L																			
	A. simplex s.s.	97.3	70.4	70.1	67.6	61.4	69.0	67.3	67.8	68.9	68.9	71.4	70.9	53.6	55.9	55.0	55.3	63.5	59.8
	C. osculatum s.s.	70.6	71.2	70.9	72.3	57.4	70.9	70.8	68.9	70.6	69.1	68.0	68.6	46.8	54.4	51.8	54.6	62.4	58.0
*rrn*S																			
	A. simplex s.s.	99.3	78.8	71.0	77.4	63.4	76.5	76.6	77.4	76.5	76.8	79.2	78.3	56.2	54.3	55.2	53.4	63.8	65.7
	C. osculatum s.s.	76.7	75.5	68.9	80.7	63.8	73.7	75.0	73.5	73.6	76.6	76.0	74.9	56.5	53.0	53.7	53.2	64.0	65.7

Abbreviations: *Asi (s.l.) = Anisakis simplex (s.l.)* (Ascaridida: Anisakidae)*; Asu = Ascaris suum* (Ascaridida: Ascarididae)*; Alu = Ascaris lumbricoides*(Ascaridida: Ascarididae)*; Cru = Contracaecum rudolphi* B (Ascaridida: Anisakidae)*; Cro = Cucullanus robustus* (Ascaridida: Cucullanidae)*; Ba = Baylisascaris ailuri*(Ascaridida: Ascarididae)*; Bp = Baylisascaris procyonis*(Ascaridida: Ascarididae)*; Bs = Baylisascaris schroederi*(Ascaridida: Ascarididae)*; Bt = Baylisascaris transfuga*(Ascaridida: Ascarididae)*; Tcan = Toxocara canis*(Ascaridida: Toxocaridae)*; Tcat = Toxocara cati*(Ascaridida: Toxocaridae)*; Tm = Toxocara malaysiensis*(Ascaridida: Toxocaridae)*; Ev = Enterobius vermicularis*(Oxyuroidea; Oxyuridae)*; Bm = Brugia malayi* (Spirurida: Onchocercidae)*; Di = Dirofilaria immitis* (Spirurida: Onchocercidae)*; Ov = Onchocerca volvulus* (Spirurida: Onchocercidae)*; Ad = Ancylostoma duodenale* (Strongylida: Ancylostomatidae)*; Na = Necator americanus* (Strongylida: Ancylostomatidae)

Highlights

The completemt genomes of *Anisakis simplex (sensustricto)* and *Contracaecumosculatum*C were sequenced using 454 technology.

These mt genomes provide a stepping-stone for future comparative analyses of a range of anisakids and for reinvestigating their genetic relationships.

These markers should be useful forprospecting for cryptic species and their biology.

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