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RESEARCH ARTICLE

Levels of intra-host and temporal sequence variation in a large *CO*1 sub-units from *Anisakis simplex sensu stricto* (Rudolphi 1809) (Nematoda: Anisakisdae): implications for fisheries management

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Abstract This paper is the first to address the suitability and potential of the cytochrome oxidase-1 (*CO*1) region of the parasitic marine nematode *Anisakis simplex sensu stricto* as a genetic marker. *A. simplex s.s.* is an ubiquitous parasite of many marine organisms and has been used as a 'biological tag' for population studies of pelagic fish stocks. The *CO*1 marker informs not only about nematode population structure but also about its hosts. The large *CO*1 sub-unit (~800 bp) was analysed from third stage larvae of *A. simplex s.s.* from Atlantic herring, *Clupea harengus* L. caught off the north-west coast of Scotland. In total 161 *A. simplex s.s. CO*1 sequences were analysed from 37 herring that represented three spawning periods over 2 years. Overall very high haplotype and low nucleotide diversities were observed ($h = 0.997$ and $\pi = 0.008$, respectively). These results are in keeping with studies investigating parasitic nematodes of ungulates and are symptomatic of the high rate of substitutions accumulated by mtDNA and effective dispersal strategies of the parasite. The Tamura-Nei $I + \Gamma$ ($\Gamma = 1.2243$) model of nucleotide substitution best suited the present data which were dominated by a high thymine bias and associated transitions. Large within population differences were highlighted by hierarchal AMOVAs with little variation related to spawning events or years which may indicate localised temporal stability. Temporal homogeneity in the *CO*1 gene coupled with the ubiquitous and widespread nature of the parasite indicates both the potential and limitations for its incorporation in stock-separation studies of its hosts.

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Introduction

For a genetic marker to be used with confidence an investigator needs to know that it is temporally stable in the organism of study. If the stability of the marker is unknown, meaningful information regarding geographic differences cannot be properly understood, as the source of variation is unknown. This paper is the first to address the potential and suitability of the cytochrome oxidase-1 (*CO*1) region of the parasitic marine nematode *Anisakis simplex sensu stricto* as a genetic marker. The *CO*1 marker not only serves as an indicator of nematode population structure but also potentially provides knowledge about that of its host, the

Atlantic herring, *Clupea harengus* L. *A. simplex s.s.* is a ubiquitous parasite of many marine organisms and has been used as a 'biological tag' for population studies of pelagic fish stocks.

Molecular biology techniques have been applied to investigate Anisakidae, a family of omnipresent marine nematodes. There is much interest in these parasites because they infect many fish with economic value (Sabater and Sabater 2000), and also because of the human disease anisakiasis (van Thiel 1962). Specifically, molecular markers, such as the ribosomal DNA (rDNA) internal transcribed spacer (ITS-1, ITS-2) and 5.8S regions, have been employed to assist the taxonomic understanding of the *Anisakis simplex* complex of morphologically similar species (D'Amelio et al. 2000; Abollo et al. 2003; Mattiucci et al. 2004, 2005). In the northern hemisphere the most prevalent anisakid is *A. simplex sensu stricto* (Nascetti et al. 1986; Mattiucci et al. 1989). *A. simplex* are generalists with a life cycle infecting a wide range of marine organisms at various levels through the pelagic food chain (Anderson 2000). Adult anisakids are known to infect about 53 species of cetaceans and pinnipeds worldwide. The ubiquitous nature of *A. simplex* is due to the wide range of intermediate and paratenic hosts that include 200 species of pelagic marine fish, 25 cephalopod species and crustaceans such as euphausiids (Anderson 2000; Klimpel et al. 2004; Mattiucci et al. 2004, 2005). Pelagic fish (e.g., herring *Clupea harengus*) acquire infections through the consumption of infected euphausiids or other pelagic invertebrates. Herring are thought to be paratenic hosts, as nematodes undergo no further development until subsequent ingestion by the definitive host. The anisakid larvae (L3) are most commonly found in the internal viscera of herring but may also be found in the musculature and gonads (Smith and Wootten 1978). Parasites have a long history of being utilised as 'biological tags' by biologists seeking to characterise the population structure of their host(s) (MacKenzie 2002). It is of little surprise, therefore, that several multidisciplinary stock identification projects of commercially exploited pelagic fish species (e.g., WESTHER: http://www.clupea.net/westher; HOMSIR: http://www.homsir.com) have used parasites as one of a range of stock identification techniques.

Anisakis simplex have been used as biological tags to help uncover the population structure and behaviour of their hosts, for example herring (McGladdery and Burt 1985; Moser and Hsieh 1992; Grygiel 1999; Tolonen and Karlsbakk 2003). However, these studies have only investigated the diversity, abundance and intensity of anisakid occurrence.

More recent molecular studies on other parasite species have substantially improved our understanding of the population structure and co-evolution of many organisms (Mattiucci et al. 2000; Donald et al. 2004; McCoy et al. 2005). Thus the levels of genetic variability and spatial structure of parasite populations may impart important information about the population biology of their hosts. The population structure of a parasite should reflect not only its own life history and genetic structure but also that of its host (Mulvey et al. 1991). Various investigations have shown the ability of parasites to indicate the more subtle population structure of their host where analysis of said host revealed none. Mitochondrial DNA (mtDNA) regions such as *CO*1 have been widely used to investigate intraspecies population structure of nematodes due to higher mutation rates compared to rDNA (Blouin 2002). The majority of work on mtDNA has been carried out on parasitic nematodes, which infect domestic and livestock animals and pose a potential human health risk (Blouin et al. 1995; Peng et al. 1998; Zhu et al. 2000; Zhan et al. 2001; Hu et al. 2002, 2003). To date molecular studies on marine parasites have focused on the biogeographic distribution of closely related, cryptic species, to infer relationships between host populations (Derycke et al. 2005; Hansen et al. 2003; Martin-Sanchez et al. 2005; Mattiucci et al. 2005).

To our knowledge mtDNA has rarely been used to investigate the genetic diversity in a marine nematode, so that its potential to discriminate between populations of these parasites, and therefore their host populations, is unknown despite its ability to be a good indicator of intra-population difference (Mattiucci et al. 2000; Blouin 2002).

Population studies require variables under investigation to be stable over time (Brown et al. 1996). There has been little work investigating temporal stability within populations of parasite nematodes. Thus the objectives of this study were to investigate variation and local temporal stability in the *CO*1 sub-unit of *A. simplex s.s.* mtDNA and assess its suitability as a genetic marker that could assist investigation of movements of its host, the herring. Despite the increasing use of parasite genetics studies for assessment of host population dynamics, no one has addressed whether sampling should address the level of variation within hosts or should focus on increasing the number of hosts at the expense of understanding patterns of diversity within a host. Moreover, few studies have confirmed temporal stability of the mtDNA region. Temporal stability of target DNA sequence is clearly of interest and importance and our data have important implications for future biogeographic studies.

Materials and methods

Sampling

Between October 2003 and 2004 three samples of spawning herring (*C. harengus*) were collected, on commercial vessels, from the north west coast of Scotland (Table 1) in autumn 2003 and 2004, and spring 2004. Herring were caught as part of the EU project WESTHER, a multi-disciplinary stock identification project which applied a range of stock identification techniques to samples of juvenile, non-spawning and spawning herring from sites to the west of the United Kingdom and Ireland on a number of occasions between 2002 and 2005. Spawning herring (herring exhibiting gonads in maturity stages V and VI) were collected due to their putative philopatric behaviour (McQuinn 1997) that reduced the likelihood of more than one population being sampled. Individual fish were given a unique code and the internal viscera and associated parasites were removed and preserved in absolute ethanol.

In the laboratory L3 *A. simplex* larvae were identified, counted and dissected from the herring viscera using a light microscope. *A. simplex* specimens from individual herring were stored in 2.5 ml sample tubes, containing liberal amounts of absolute ethanol and labelled with the corresponding unique fish identifier. A sub-sample of *Anisakis* larvae was identified as *A. simplex sensu stricto* (Rudolphi 1809) by PCR–RFLP of an rDNA fragment (~1 kb) spanning the ITS-1, ITS-2 and 5.8S region following the methods published by (D'Amelio et al. 2000) (data not shown but available from M.A. Cross on request).

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from the mid body region of individual nematodes, with the anterior and posterior regions of the nematodes retained in 100% ethanol to allow subsequent morphological examination if necessary. Tissue samples were digested using 10 μg of proteinase-K (30 mg μl^{-1}) (Promega) and incubation at 50°C overnight in 500 μl of 5% Chelex-100 (BioRad) solution that was subsequently heated to 95°C for 5 min to denature the Proteinase-K (protocol modified from Walsh et al. 1991). DNA was extracted from 161 *A. simplex s.s.* specimens from 37 fish representing three discreet samples that comprised several year classes. Multiple nematodes taken from a single herring host were uniquely labelled to reflect their particular origin.

PCR amplification of the *CO*1 region from *Anisakis* was initially performed using the primers LCO1490 and HCO2198 (Folmer et al. 1994), however, these primers were also found to amplify herring DNA. *Anisakis* sequence generated by LCO1490 and HCO2198 was aligned with other nematode *CO*1sequences, and with Clupeidae sequences, from public databases and degenerate primers designed. Anisakid sequence generated using these primers were used to design the primers AnCO1 F (5′-ATTTGGTC TTTGATCTGGTATGG-3′) and AnCO1 R (5′-TGG CAGAAATAACATCCAAACTAG-3′) used in the current study.

An approximately 1,030 bp partial fragment of the large sub-unit of the *CO*1 gene of the mtDNA was amplified by PCR in a Dyad thermocycler (MJ-Research) using the primers: AnCO1 F and AnCO1 R. About 5 μl of the DNA extract was used to seed a 15 μl PCR containing 75 mM Tris–HCl pH 8.9, 20 mM $(NH_4)_2SO_4$, 0.01% v/v Tween-20, 0.2 mM each dNTP, 50 mM $MgCl_2$, 10 pmol each primer and 0.25 U *Taq* polymerase (ABgene). Thermal cycling conditions were: 94°C for 5 min, 40 × [94°C for 60 s, 54°C for 60 s, 72°C for 2 min 30 s], and a final extension of 72°C for 5 min. The PCR fragments were treated with EXOSAP-IT (USB) and then cycle sequenced using Big Dye™ chemistry (PE Applied Biosystems) and electrophoresis on an ABI3100 automated sequence following the manufacturers' recommended protocol, but with a reduced sequencing reaction volume of 10 μl. Cycle sequencing conditions were: 94°C for 5 min, 39 × [95°C for 30 s, 50°C for 15 s, 72°C for 2 min 30 s], and a final extension of 72°C for 5 min. In addition to using the two terminal primers for sequencing, an internal primer (Reverse 2 5′-GCATA CACCATCCCCAAAGAACC-3′), designed using PRIMER3 (Rozen and Skaletsky 2000), was used to obtain full-length, overlapping sequences. The nucleotide positions of the forward (AnCO1 F) and reverse primers (AnCO1 R) in the full *A. simplex* mitochondrial genome (Genbank accession no. AY994157) are 6,171–6,193 and 7,187–7,210, respectively, and nucleotides 87–109 and 1,103–1,126 of the *CO*1 gene (Kim and Eom 2006).

Table 1 Locations where *Clupea harengus* were caught from which *Anisakis simplex sensu stricto* were sampled

	Collection date	Latitude	Longitude
Autumn 2003	01 Sep 2003	58°62′N	4°01′W
Spring 2004	01 Mar 2004	57°50′N	6°15′W
Autumn 2004	22 Aug 2004	58°60′N	5°40′W

Sequence analysis

Consensus sequences were aligned using SEQMAN in the DNASTAR software (DNASTAR, Madison, WI, USA) and cropped to an unambiguous common length of 799 bp. No insertions or deletions were observed in the 161 sequences aligned. Sequences were converted to a FASTA format, and MODELTEST ver. 3.06 (Posada and Crandall 1998) was used to find the best model fit for analysis of *A. simplex s.s. CO*1. All subsequent analysis was carried out using the assumptions of the best model and the parameter values specified using the Akaike Information Criterion (AIC).

ARLEQUIN ver. 2.001 (Schneider et al. 2001) software was used to calculate haplotype and nucleotide intra-species diversities within each temporal sample. The level of spatial structure between haplotypes from several individual nematodes from a single herring within a group was pooled for analysis of molecular variance (AMOVA) using ARLEQUIN ver. 2.001. This allowed a three-tier investigation: intra-host diversity within a single fish (FST), between hosts from a single period (FSC) and overall (FCT). In total 132 sequences were analysed from eight herring from three samples collected over a 2 year period (Table 2). Haplotype and nucleotide diversities were computed using ARLEQUIN ver. 2.001 (Schneider et al. 2001). Samples were pooled within sample group to investigate temporal stability and population structure. Differences were measured using pairwise F_{ST}s between pooled samples. To increase the number of host fish sampled single *A. simplex s.s.* were analysed from ($n = 29$) individual fish to provide a total of 161 individually sequenced specimens.

Results

Sequence analysis

Inspection of 161 sequences from *A. simplex s.s.* revealed 135 unique haplotypes. The best model of evolution of the *A. simplex s.s. CO*1 was that of Tamura-Nei + I + Γ (Tamura and Nei 1993) (Γ = 1.2243, AIC = 5,487.6934, −ln L = 2,736.8467), and the assumptions of this model and the specified parameters were used throughout the analysis. The Tamura-Nei + I + Γ model specifies unequal base frequencies and a lower rate of transversion mutations than transitions, and a different rate of transition between purines and pyrimidines. Thus, on average our sequence data exhibited a substantial skew towards thymine ($\pi_T = 0.444$) compared to cytosine, adenine and guanine ($\pi_C = 0.141$, $\pi_A = 0.197$, $\pi_G = 0.218$). This was highlighted by the exceptionally high number of transitions ($n = 115$) compared to transversions ($n = 14$). The nucleotide sequences have been submitted to Genbank under accession nos. DQ994243 and DQ994403.

Intraspecific analysis

In total 132 nematode partial *CO*1 sequences were generated from a total of eight herring. *A. simplex s.s. CO*1 gene is characterised by very high levels of overall haplotype diversity with 115 of these sequences unique ($h = 0.997$; Table 2). Conversely, the nucleotide diversity in the sequenced region was very low ($\pi = 0.008$; Table 2.). A plot of unique haplotypes against number of parasite specimens analysed, from a single herring exhibits an almost 1:1 ratio linear regression gradient

Table 2 Samples analysed with the year and season of capture and number of individual *Anisakis simplex sensu stricto* (*n*) per single host (*Clupea harengus*)

Sample	*N*	n_h	n_p	*T*s	*T*v	*h*	SE	π	SE
Autumn 2003 fish 1	21	20	41	38	3	0.995	0.017	0.009	0.005
Autumn 2003 fish 2	10	10	26	26	0	1.000	0.045	0.009	0.005
Autumn 2003 fish 3	8	8	22	21	1	1.000	0.063	0.009	0.005
Autumn 2003All fish combined	39	37	56	52	4	0.997	0.007	0.008	0.004
Spring 2004 fish 4	18	18	46	44	3	1.000	0.019	0.009	0.005
Spring 2004 fish 5	15	15	31	30	1	1.000	0.024	0.008	0.004
Spring 2004 fish 6	29	25	38	37	3	0.988	0.013	0.008	0.004
Spring 2004All fish combined	62	55	75	72	7	0.995	0.004	0.008	0.004
Autumn 2004 fish 7	8	8	27	27	1	1.000	0.063	0.010	0.006
Autumn 2004 fish 8	23	23	45	44	1	1.000	0.013	0.009	0.005
Autumn 2004All fish combined	31	31	59	57	2	1.000	0.008	0.009	0.005
Total	132	115	118	110	12	0.997	0.002	0.008	0.004

The table includes the genetic characteristics: number of unique haplotypes (*n*h), polymorphisms (*n*p), observed transcriptions (*T*s), tranversions (*T*v) events, haplotype (h) and nucleotide-diversity (π) with their respective standard errors (SE) were appropriate

(see Fig. 1; gradient = 0.8671). Both logarithmic and linear regressions show high R^2 values (0.9789 and 0.988, respectively; Fig. 1). Nucleotide transitions were an order of magnitude larger than the respective transversions in comparable samples (Table 2). Sequence analysis of single nematodes from individual herring collected in the Autumn 2003, Spring and Autumn 2004 revealed no common haplotypes within or between samples periods ($h = 1.00$, $\pi = 0.008$). Structuring of *A. simplex s.s.* within a single host was analysed using hierarchal AMOVA. The large majority of variation was observed within a population (single herring host) with only a small percentage among population (hosts) (Table 3).

Temporal stability analysis

All specimens within a group (e.g., Autumn 2003) were pooled for subsequent analysis. Haplotype and nucleotide diversity for the pooled specimens exhibited a similar trend to those in the intraspecfic analysis (Tables 2, 4), although at least one rare haplotype was present in each temporal group (cf. n and n_h; Table 4). Only six common haplotypes were observed between the 161 pooled sequences (max = five identical sequences) with 135 unique singletons. Pairwise F_{ST}s between pooled groups of *A. simplex s.s.* indicated no significant difference at the 95% confidence level between temporal groups (F_{ST}s: Autumn 2003 v Spring 2004 = 0.00272, Autumn 2003 v Autumn 2004 = 0.00811, Spring 2004 v Autumn 2004 = < 0.00).

Discussion and conclusions

Molecular investigations using parasites increasingly are being used to study the population structure of their host because of the higher mutation rates in their genomic DNA that may infer subtle differences between their host groups (Anderson et al. 1998; Nieberding et al. 2004). The mitochondrial *CO*1 gene was investigated because of the level of divergence and high mutation rates required to compare inter- and intra-specific genetic variation in *A. simplex s.s* (Zhan et al. 2001; Blouin 2002; Hu et al. 2002).

Overall, sequences from individual *A. simplex s.s.* showed a high haplotype diversity and low nucleotide diversity in keeping with previous population studies of nematode mtDNA (Blouin et al. 1995). It has been reported that nematodes exhibit a strong AT bias in their mtDNA (Hyman and Azevedo 1996; Hugall et al. 1997; Blouin et al. 1998). Whilst *A. simplex s.s.* shows a strong T bias the same could not be said for any of the other nucleotides. However, these results are similar to the study by Hu et al. (2002) which investigated the *CO*1 region of three hookworm populations.

The complex life history of *A. simplex s.s.* which involves free-living pelagic, paratenic and parasitised mobile hosts results in large panmictic populations (Klimpel et al. 2004; Mattiucci et al. 2004, 2005). High within-population diversity has been attributed to the rapid evolution of mtDNA, high gene flow and large effective population sizes (Blouin et al. 1992, 1995) and this certainly could be the case with *A. simplex s.s.* Temporal stability of the target gene under investigation is vitally important (Brown et al. 1996) although an often-overlooked aspect of genetic population structure judging by its lack of investigation in many published articles. The lack of genetic differentiation between the temporal samples indicates that the *CO*1 sub-unit may be temporally stable therefore suitable for future investigation of its potential to be a genetic marker. The northern krill (*Meganyctiphanes norvenica*) which has a planktonic larval stage with passive

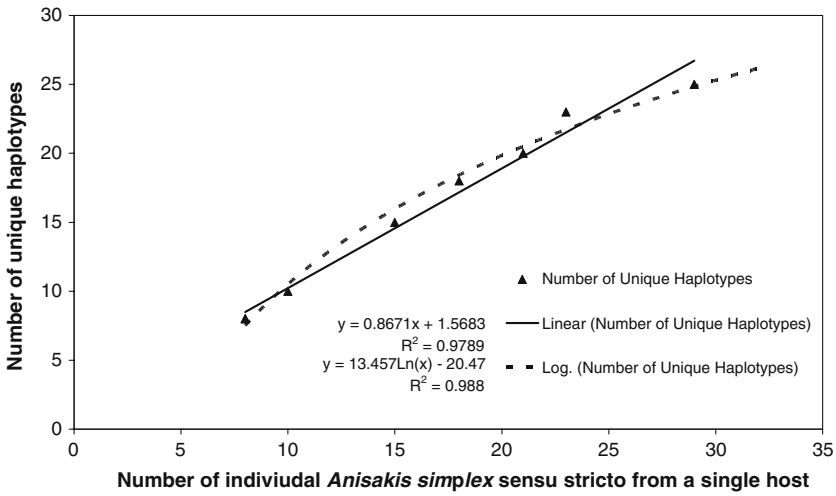

Fig. 1 Number of unique *Anisakis simplex sensu stricto* haplotypes from individual nematodes from a single host (*Clupea harengus*)

Table 3 AMOVA: partitioning of *CO*I gene sequence from *Anisakis simplex sensu stricto* from infected *Clupea harengus*

Source of variation	Degrees of freedom	Sum of squares	Percentage of variation	*P*
Among groups (FCT)	2	9.26	1.21	0.059
Among populations within groups (FSC)	5	14.95	0	0.829
Within populations (FST)	124	422.3	99.6	0.385

Samples are grouped into multiple *A. simplex s.s* from single host (FST), grouped with in sample (FSC) and between sampling groups (FCT)

Table 4 *Anisakis simplex sensu stricto* (*n*) grouped by sampling period from numerous hosts *Clupea harengus* (n_f)

SAMPLE	n_f	*n*	n_h	n_p	*Ts*	*Tv*	*h*	SE	π	SE
Autumn 2003	15	51	49	64	59	5	0.998	0.004	0.008	0.004
Spring 2004	12	71	61	78	74	8	0.995	0.003	0.008	0.004
Autumn 2004	10	39	38	64	62	3	0.999	0.006	0.009	0.005
ALL	37	161	135	124	115	14	0.997	0.001	0.008	0.004

The table includes a list of suitable genetic characteristics: Number of unique haplotypes (n_h). polymorphisms (n_p), observed transcriptions (*Ts*), tranversions (*Tv*) events, haplotype (h) and nucleotide-diversity (π) with their respective standard errors (SE) where appropriate

distribution, comparable to *A. simplex s.s.*, also exhibited local temporal stability (Papetti et al. 2005). Obviously the temporal stability observed in this present study could be the result of an exceedingly large effective population size, which requires further investigation. However, initial investigations of population sub-divisions should be tested temporally as well as spatially.

Haplotype diversity was very high whether multiple *A. simplex s.s.* were sequenced from a single fish or single nematodes from numerous fish. It is unclear whether the high haplotype diversity between multiple nematode sequences from an individual host was due to multiple *A. simplex s.s.* infections over time or mtDNA's high mutation rate. Future population structure investigations should focus on improving fish sample sizes by analysing fewer worms from a single fish, due to diminishing returns in information gained for the extra time and cost expenditure (Brown et al. 1996). Increasing the number of fish sampled would provide greater information about the stock structure of herring.

To date there have been very few studies utilising the genetic structure of parasites to infer information about the life histories and population structures of their hosts. For example, a large *CO*1 region from two *Gyrodactylus* spp. (class: Monogenea) which infect salmonids suggested multiple introductions of the two morphologically indistinguishable species into Norway (Hansen et al. 2003). Another investigation of parasitic nematodes that infect ungulates in North America found no significant population structure in cattle and sheep but showed evidence of isolation by distance and population subdivision in deer (Blouin et al. 1995). This was due to the widespread movement of cattle and sheep across America through agriculture that resulted in increased gene flow. It is reasonable to suggest that the success of these genetic-population-studies relies on an appropriate scale of sampling to account for the different life histories and geographic distribution of the organisms being studied. Widespread migrations over large geographic distances performed by the numerous hosts of *A. simplex s.s.* provide ample opportunity for gene flow between potentially putative populations. It is of interest that a study of the northern krill, which is an intermediate host of *A. simplex s.s.*, was shown to have genetically and geographically distinct gene pools in samples covering the Atlantic Ocean and Mediterranean Sea (Papetti et al. 2005).

This study has indicated that *CO*1 gene sequences from *A. simplex s.s.* analysed between and within spawning seasons of its host (herring) exhibited a degree of temporal stability, strengthening the advocation of this nematode as a biological-tag and the potential suitability of this region as a genetic marker. *A. simplex s.s.* is a suitable candidate for future studies of its host stock structures, not only herring but other commercially valuable fish populations.

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