Genetic variation in mitochondrial DNA among *Enterobius vermicularis* in Denmark

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SUMMARY

Despite being the most prevalent nematode infections of man in Western Europe and North America, our knowledge of the genetic variability in *Enterobius vermicularis* is fragmented. We here report on a genetic study of pinworms in Denmark, performed using the cytochrome oxidase I (*cox1*) gene, with DNA extracted from individual eggs collected from clinical (human) samples. We collected cellophane-tape-test samples positive for pinworm eggs from 14 Departments of Clinical Microbiology in Denmark and surface-sterilized the eggs using a 1% hypochlorite solution before performing conventional PCR. Twenty-two haplotypes were identified from a total of 58 Danish patients. Cluster analysis showed that all Danish worms grouped together with human samples from Germany and Greece and with samples from Japanese chimpanzees designated as 'type B'. Analysis of molecular variance showed no significant difference or trends in geographical distribution of the pinworms in Denmark, and several haplotypes were identical or closely related to samples collected in Germany, Greece and Japan. However, worms from the 4 countries were found to belong to different populations, with Fst values in the range of 0·16 to 0·47. This study shows pinworms in Denmark to be a homogenous population, when analysed using the *cox1* mitochondrial gene.

Key words: pinworm, *Enterobius vermicularis*, PCR, *cox1*, phylogeny.

INTRODUCTION

Enterobius vermicularis is an intestinal parasite of cosmopolitan distribution, and is considered to be one of the most prevalent nematode infections of man in developed countries (Zelck *et al*. 2011). In Denmark, the prevalence of pinworms was found to be 29% among children aged 5–12 admitted to a pediatric ward (Lacroix and Sorensen, 2000).

Infection normally occurs by fecal-oral transmission, with ingested eggs hatching in the intestine and larvae migrating to the ileocecal junction. Gravid females deposit eggs in the perianal zone, a process that causes pruritus, and subsequent scratching traps eggs under fingernails and in clothes, etc. The method of choice for diagnosis is the cellophane tape test, by which a piece of adhesive tape is pressed onto the perianal region and the eggs are identified by light microscopy (Garcia, 2001). Underestimation of incidence may occur due to low diagnostic sensitivity (Roberts and Janovy, 2009).

The mitochondrial genome of *E. vermicularis* was sequenced in 2009 (Kang *et al*. 2009), although only limited sequence data are available to date, especially on nuclear DNA. One phylogenetic study from Japan identified 3 different clusters (designated as type A, B and C) based on mitochondrial cytochrome c oxidase subunit 1 (*cox1*) sequenced in samples collected from captive chimpanzees and humans (Nakano *et al*. 2006). A recent study found that all ($n=36$) analysed pinworm samples obtained from humans in Greece clustered together with the Japanese identified as type B (Piperaki *et al*. 2011). In contrast to these two studies using *cox1* sequencing, no diversity in the ribosomal DNA region of pinworms ($n=37$) from Germany were observed (Zelck *et al*. 2011).

Both mtDNA and ITS regions are commonly used in phylogenetic analyses, with mitochondrial genes being more appropriate for population genetic studies, whereas Internal transcribed spacer (*ITS*) remains better for identification (Blouin, 2002). The *cox1* gene has also previously been used with other nematodes (Blouin *et al*. 1998; McDonnell *et al*. 2000; Traversa *et al*. 2008).

This paper describes the first genetic study on *Enterobius vermicularis* in Denmark, conducted on DNA isolated from individual surface-sterilized eggs.

MATERIALS AND METHODS

Samples

We collected 131 Danish clinical samples (cellophane tape test) from 14 different departments of clinical

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microbiology in several areas of Denmark between 1 February 2011 and 1 May 2011. The presence of *E. vermicularis* in tape samples was microscopically confirmed before and after being sent to the Danish State Serum Institute, where the study was conducted. We also analysed DNA extracted from adult worms collected from stool samples and cellophane tapes of children in different areas of Germany.

Egg isolation and surface sterilization

Since the same patient may be infected by multiple pinworm haplotypes, data based on pools of eggs will give the 'consensus' sequence from that host. So as to work on individual eggs, DNA extraction and PCR were conducted on surface-sterilized eggs using a solution of hypochlorite (APPLICHEM 13% active chlorine, diluted to 1%) previously shown to induce damage to DNA (Hawkins and Davies, 2002). In order to determine the threshold concentration of hypochlorite needed to damage DNA on the outside of the eggshell, we performed a validation substudy testing different concentrations of hypochlorite in succession. We prepared 8 solutions of hypochlorite ($1\% \times 10^{0}$–10^{-7}), added *E. vermicularis* DNA extracted from adult worms (Qiagen tissue kit) and incubated the samples for 10 min. at room temperature. The *E. vermicularis*-hypochlorite solutions were used undiluted and in 3 subsequent dilutions, each time by a factor of 10, after which PCR was performed on all solutions. We determined the threshold solution of hypochlorite to be 0·01%, i.e. the solution at which bands appear despite the hypochlorite, and also showed that subsequent dilution did not allow for positive bands in PCR, i.e. that the DNA-induced damage was only due to the initial (higher) concentration of hypochlorite.

In order to isolate the eggs, the cellophane tape was scraped using a scalpel and the sample was incubated in 1 ml of hypochlorite, sterilizing the egg-surface DNA contaminants. A single egg, visually confirmed to contain a pinworm larva, was transferred with 1 μl of fluid to a 1·5 ml Eppendorf tube, using a micropipette and a light microscope, collecting 3 tubes from each individual cellophane tape sample. The amplified sequences were included in the analysis, selecting the best one from each patient. There were not enough data to study internal variation of amplified sequences from individual patients.

Crushing of eggs and DNA isolation

We added 180 μl of ATL buffer and 20 μl of the proteinase K (as per QIAamp DNA Mini kit protocol) to each of the Eppendorf tubes, now containing a single surface-sterilized pinworm egg. In the remaining steps, we followed the manufacturer's instructions, although 20 μl instead of 100 μL of AE buffer was used to elute the DNA in the final step in order to increase the concentration.

DNA amplification

We designed primers targeting conserved regions of the *E. vermicularis cox1* gene (Accessions no. EU281143) using Primer3 (Rozen and Skaletsky, 2000), which amplified a 636 bp fragment of the gene.

The forward primer (Ent1F) read: 5′-TTG GTT TCT TTA CCT GTG TTG G-3′and the reverse primer (Ent1R) read: 5′- CTT TGA TAA AAC ACA ACC AGT CAT-3′. We used the following conditions for PCR: initial denaturation at 95 °C for 15 min, followed by 36 cycles of 94 °C for 45 sec, 55 °C for 1 min and 72 °C for 45 sec; and a final elongation step at 72 °C for 5 min (PTC-200 Peltier Thermal cycler, MJ Research®). We used 2 μl of template DNA for each PCR with the components as follows: 2·5 μl of 10 × PCR Rxn Buffer (Invitrogen®), 1 μl of dNTPs (1·25 mM/base, Rosche®), 1·75 μl of $MgCl_2$ (50 mM, SIGMA®), 1 μl of each primer (10 pmol/μl, TAG Copenhagen A/S), 0·2 μl of Platinum® Taq DNA Polymerase 500 rxn (5U/μl, Invitrogen®) in a total volume of 25 μl. Positive (DNA) and negative (H_2O) controls were included in all runs. Mean time from sampling until PCR was performed was 36 days.

Electrophoresis and transilluminator

We electrophoresed the PCR products in a 1·5% agarose gel (Invitrogen®) and visualized with EZ-Vision™ reagent (AMRESCO®) in UV transilluminator (Bio Doc-It®). GeneRuler™ 100 bp DNA Ladder (Fermentas®) was also included in the electrophoresis.

DNA purification and sequencing

Cleanup was performed on all PCR positive samples using QIAquick® PCR Purification Kit (250) and PCR products were sequenced (Sanger's method) in both directions by Eurofins MWG Operon (Ebersberg, Germany).

Sequence and data analysis

Bioedit (Hall, 1999) and EditSeq programs were used to analyse and manually edit sequences, and SeqMan to align them (latter two from Lasergene 5.0 from DNAstar). We used Arlequin 3.5.1.2 (Excoffier *et al.* 2005) to conduct analyses of molecular variance (AMOVA) to estimate the partitioning of genetic variation within and between populations. The fixation index, F_{ST} (Excoffier *et al.* 1992) was estimated taking the relationships between haplotypes

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Table 1. Molecular data of the samples from the largest geographical regions in Denmark, Jutland and Zealand, together with the entire population from Denmark and Germany

		Jutland	Zealand	Denmark	Germany
Sample size		18	32	58	15
Haplotypes		10	15	22	5
Polymorphic sites		16	22	28	8
Gene diversity		0·92 ± 0·04	0·91 ± 0·03	0·90 ± 0·02	0·48 ± 0·12
Nucleotide diversity (π)		0·014 ± 0·008	0·014 ± 0·008	0·014 ± 0·008	0·004 ± 0·003
Mean number of pairwise differences (K)		4·58 ± 2·36	4·69 ± 2·36	4·60 ± 2·29	1·41 ± 0·91
Transitions		15	22	27	8
Transversions		1	0	1	0
Indels		0	0	0	0
Nucleotide composition	%C	26·54	26·53	26·56	26·41
	%T	20·32	20·32	20·30	20·44
	%A	44·19	44·17	44·16	44·66
	%G	8·94	8·98	8·99	8·49

into account. F_{ST} was computed using pairwise differences for the distance matrix and 1023 permutations were performed to test for significant differentiation between populations. Arlequin was likewise used to conduct a Mantel test to assess the correlation between the logarithm of linear geographical (ln distance) and genetic distances. We employed MEGA 5.0 (Tamura *et al*. 2011) to calculate p-distances between populations and to construct a Neighbour-joining (NJ) tree based on Kimura 2-parameter model (Kimura, 1980). The topology of the tree was evaluated using the bootstrap test (Felsenstein, 1985) with 1000 re-samplings. *Cox1* sequence from *Enterobius anthropopitheci* (AB254450) obtained from a chimpanzee was included in the tree as well.

RESULTS

Danish samples

Of the 131 Danish clinical samples, 58 proved of sufficient quality and with sufficient number of eggs to harvest, and for these we obtained 58 sequences from individual eggs, each one from a different patient. Sequences were aligned and trimmed to a length of 333 bp according to previous published *cox1* sequences (Nakano *et al*. 2006; Piperaki *et al*. 2011). Twenty-two haplotypes were identified in the 58 Danish samples (deposited in in Genbank with Accession nos. JQ411483-JQ411504) whereas 5 haplotypes were identified in the 15 German samples (Accession nos. JQ411505-JQ411509). Polymorphic sites were found in 28/333 (8·4%) sites in the Danish clinical samples with 22 of them being located in the third position of the open reading frame of the region PCR amplified. In the German clinical samples, polymorphic sites were found in 8/333 (2·4%), and all but 1 varied in the 3rd position. Only 1 transversion was found in a Danish haplotype, the rest of the substitutions in both Danish and German samples were transitions. No indels were identified (Table 1).

Most of the amino acid sequences (88%) of the Danish samples are in accordance with type B (Nakano *et al*. 2006) (Table 2). Variations were found in 5 positions: 3 haplotypes had 3 single amino acids not found previously in either Japanese or Greek data. DK19 and DK 20 had a valine for isoleucine substitution as found in type C and 2 haplotypes (DK10 and DK18) had a methionine instead of threonine as found in type A.

Dividing our samples geographically in 2 main regions in Denmark, we used AMOVA to compare 18 sequences from Jutland (Danish peninsula) with 32 sequences in samples from Zealand (largest island of the country, harbouring the capital Copenhagen). The remaining 8 samples were from other regions or unknown origin and were not included in this analysis. We did not find any population differentiation between the two regions (F_{ST}: −0·02, $P = 0·92$) and the worms from Denmark were therefore found to belong to the same population.

Danish, German, Greek and Japanese samples

There was significant ($P < 0·001$) differentiation between the *E. vermicularis* populations from the different countries (Table 3). There was high ($F_{ST} > 0·25$) genetic structure between all the populations when compared, except between pinworms from the 2 neighbouring countries, Denmark and Germany, where a moderate F_{ST} of 0·16 was obtained. German, Greek and Japanese populations showed an increasing F_{ST} value when compared with the Danish one, matching the geographical distance (Table 3). However, overall the Mantel test did not suggest an 'isolation by distance' as there were only weak correlations between geographical and genetic distances (coefficient correlation = 0·57; determination of F_{ST} by geographical distance = 32%;

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Table 2. Different amino acid sequences among the 58 Danish samples (22 haplotypes as indicated by numericals), compared with type A, B and C (Nakano *et al.* 2006)

(51 of the 58 Danish clinical samples had the same amino acid sequence as type B. Denmark (DK) and Japan (JP). A dot denotes identical amino acid to top sequence, and a slash denotes compression of the amino acid sequence.)

	I	L	P	A	F	G	I	V	S	#16	G	M	I	Y	A	#16	F	T	I	G	F	#46	F	I	F	M	F	T	T	G	G	L	T	G	I
DK 1,2,3,4,5,6,7, 8,9,11,12,15,16,17,18 and 22 (n = 51)										/						/						/												
DK 10,18 (n = 2)	.	.	.	.	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	M	.	.	.	.	.	.
DK 13 (n = 1)	.	.	.	.	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	/	.	.	L	.	.	.	.	.	.	.	.	.
DK 14 (n = 1)	.	.	.	.	.	.	.	.	.	/	.	.	V	.	.	/	.	.	.	.	.	/	.	.	.	.	.	.	.	.	.	.	.	.
DK 19,20 (n = 2)	.	.	.	.	.	.	.	.	.	/	.	.	.	.	.	/	.	.	V	.	.	/	.	.	.	.	.	.	.	.	.	.	.	.
DK 21 (n = 1)	.	.	.	.	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	S	.	.	.	.	.	.
JP cluster C	.	.	.	.	.	.	V	.	.	/	.	.	.	.	.	/	.	.	V	.	.	/	.	.	.	.	.	.	L	.	.	.	.	.
JP cluster B	.	.	.	.	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	.	.	.	.	.	.	.
JP cluster A	.	.	.	.	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	/	.	.	.	.	.	.	M	.	.	.	.	.

Table 3. Pairwise estimate of population differentiation (Fst) between the different countries

(High significance is denoted by *** ($P<0{\cdot}001$).)

	Denmark	Germany	Greece
Germany	0·160***		
Greece	0·356***	0·468***	
Japan	0·402***	0·344***	0·418***

$P=0{\cdot}16$). In contrast, if the German samples were excluded the coefficient correlation would be 0·98 and the determination of F_{ST} by geographical distance is 96% but still not significant ($P=0{\cdot}18$).

The percentage of sequence difference (p-distance) increased gradually with the geographical distance when the Danish samples were compared with the German, Greek and Japanese data (1·2%, 1·5% and 4·2% respectively) in agreement with the AMOVA analysis. However, when the Japanese pinworms were divided in the 3 types proposed by Nakano *et al.* (2006), the distance changed considerably, becoming 1%, 4·1% and 5·4% for the B, A and C types, respectively (Table 4).

All Danish and German haplotypes clustered in the B clade of the NJ tree (Fig. 1) proposed by Nakano *et al.* (2006), along with 3 of the Japanese haplotypes (from chimpanzees: AB221467, AB221468 and AB221469) and all 11 Greek haplotypes (from humans). Danish haplotypes DK1 and DK5 were identical to the Japanese AB221469 and AB221468, while 2 other Danish haplotypes (DK3 and DK17) were identical to the Greek ones HQ317440 and HQ317432. DK8 was identical to a Greek as well as a Japanese haplotype (HQ317429 and AB221467), while DE4 matched with the DK16 haplotype.

DISCUSSION

It was only possible to obtain sequences from 58 of the 131 samples. Some samples contained only very few eggs, and proved impossible to harvest. Other samples failed to produce positive bands in PCR, which we attribute to possible degradation of egg and/or DNA. While we cannot rule out PCR inhibition we consider it unlikely, as we assume the presence of PCR inhibitors in a singular pinworm egg to be small, and because we observed more positive bands when testing on pools of eggs, despite a potentially equal increased level of PCR inhibitors. Using more template and increasing the number of PCR cycles did not allow for better results, although using a (more sensitive) diagnostic real-time PCR did confirm presence of pinworm DNA in a higher fraction of the harvested eggs (data not shown).

Previous studies have successfully amplified multi-copy genes from individual unembyonated *Ascaris* eggs (Carlsgart *et al.* 2009), but to our knowledge no

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Table 4. Nucleotide p-distances between Danish (DK), Greek (GR), German (DE) and Japanese (JP) *Enterobius vermicularis* populations

(Japanese samples were also divided in the types as proposed by Nakano *et al*. (2006), i.e. types A, B and C (JPA, JPB and JPC). *Enterobius anthropopitheci* (E.ant) was also included in this analysis.)

	DK	DE	GR	JP	JPA	JPB	JPC
DK	—						
DE	0·012	—					
GR	0·015	0·008	—				
JP	0·042	0·036	0·039	—			
JPA	0·041	0·034	0·034	0·040	—		
JPB	0·010	0·007	0·012	0·039	0·039	—	
JPC	0·054	0·048	0·051	0·030	0·058	0·053	—
E.ant	0·141	0·136	0·133	0·125	0·132	0·141	0·116

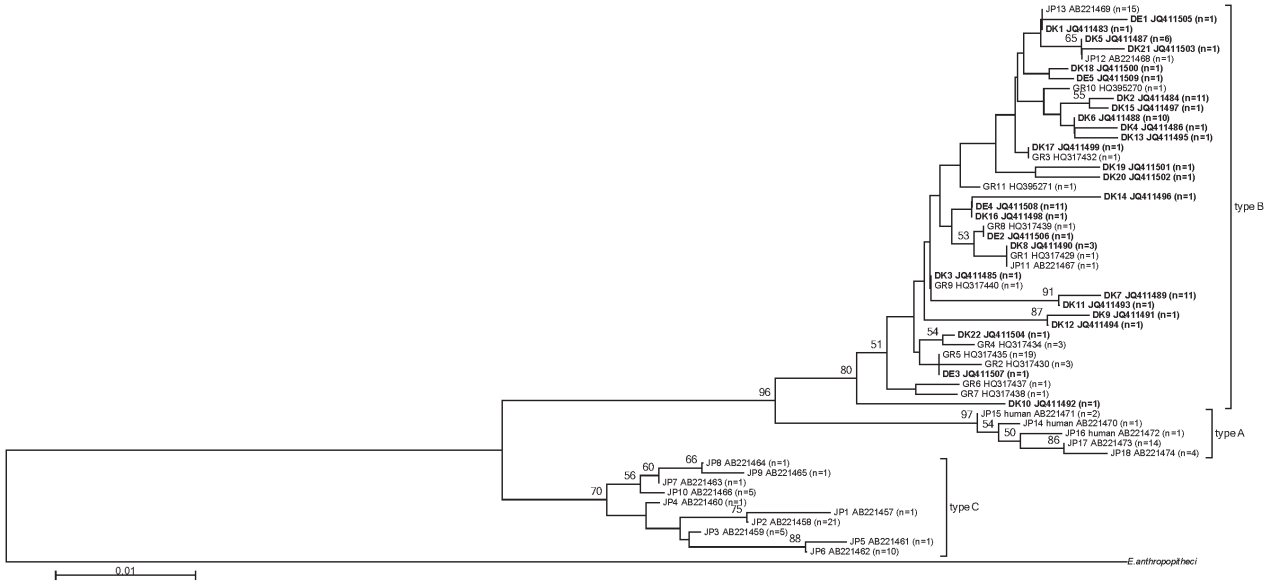

Fig.1. Neighbour-Joining tree based on Kimura 2-parameter distances and with 1000 bootstraps (bootstrap probability of 50% indicated). Twenty-two Danish (DK), 5 German (DE), 11 Greek (G.R) (all collected from human samples) and 18 Japanese (JP) haplotypes (3 from humans, 15 from chimps) based on 333 bp of the cox 1 gene were included in the tree. *E. antropopitheci*, was also included. Samples from this study are indicated in bold. The number of samples of each haplotype is denoted in parentheses.

study has done so on single pinworm eggs. The technique used to surface-sterilize eggs employed hypochlorite, and the concentrations used in the study were validated both with respect to DNA-damaging concentrations and concentrations which did not subsequently inhibit the PCR reaction. Thus amplified sequences represent DNA from within the egg and therefore do not include the genetic variation present in a host harbouring multiple haplotypes of *E. vermicularis*. While we used Qiagen extraction to increase concentration and purity of DNA, and to decrease the number of putative PCR inhibitors, subsequent testing showed that simply treating the egg with proteinase K and performing PCR directly on the egg contents was also possible (data not shown), although only in eggs previously treated with hypochlorite.

Pinworms from the two main regions in Denmark, Jutland and Zealand, were found to belong to the same population, implying high gene flow within the country. This is to be expected due to high mobility of humans in relation to work and vacation among the Danes. In contrast, despite the geographical proximity between Germany and Denmark, there is a moderate differentiation ($F_{ST}=0{\cdot}16$) between pinworm populations in the two countries. We hypothesize that the frequent travelling across the border might allow some gene flow, but only to a limited extent due to little direct interaction between people. Similarly, population differentiation becomes even higher when comparing the Danish population to the Greek and Japanese, reflecting the 'likelihood' of interaction with respect to the geographical distance. In contrast, however, the highest F_{ST} values were

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obtained when comparing German and Greek populations although this might be due to the limited number of German samples included in our analysis. These observations are in agreement with the results obtained by the Mantel test.

All 58 sequences from Denmark clustered with type B identified in Japanese chimpanzees (Nakano *et al*. 2006) together with the human sequences from Greece (Piperaki *et al*. 2011). Since wild chimpanzees are not infected with human pinworms (Nakano *et al*. 2006) our findings support the hypothesis that captive chimpanzees have acquired *Enterobius vermicularis* by exchange with humans. Likewise, *Ascaris suum*, a helminth of pigs has also been transmitted to captive chimpanzees (Nejsum *et al*. 2010).

The p-distances confirmed the relations between the four populations, supporting the F_{ST} values. However, when the Japanese data were split into the types A, B and C, the p-distances varied considerably. The distances between the Danish, German, Greek and Japanese haplotype B were all below 2% which is the normal range for members of the same species (Blouin *et al*. 1998) suggesting that humans in these countries are infected with the same *Enterobius* species despite the AMOVA analysis showing moderate to high population differentiation between the countries. In contrast, the p-distances between type A and C and the other populations are in the range of 3·4% – 5·8% close to 6% which is the maximum range that has been seen within helminth species (Blouin *et al*. 1998). *Enterobius* might therefore harbour cryptic species but more genetics analyses are needed to clarify this (Nakano *et al*. 2006). In addition, it would be interesting to sample pinworms from additional geographical locations in order to explore whether haplotype A and C are circulating in other populations and to see whether type B is the predominant type in humans as seems to be the case presently.

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