SSCP-based identification of members within the *Pseudoterranova decipiens* complex (Nematoda: Ascaridoidea: Anisakidae) using genetic markers in the internal transcribed spacers of ribosomal DNA

X. Q. ZHU[1]†, S. D'AMELIO[2], H. W. PALM[3], L. PAGGI[2], M. GEORGE-NASCIMENTO[4] *and* R. B. GASSER[1]*

[1] *Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia*
[2] *Department of Sciences of Public Health, Parasitology Section, Università di Roma 'la Sapienza', P.le Aldo Moro, 5 - Box 6 Rome 62, 00185 Rome, Italy*
[3] *Bogor Agricultural University, Darmaga Campus IPB, 16680 Bogor, Indonesia, and Institute of Zoomorphology, Cell Biology and Parasitology, University of Düsseldorf, Germany*
[4] *Facultad de Ciencias, Universidad Católica de la Santísima, Concepción, Casilla 297, Concepción, Chile*

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SUMMARY

The anisakid nematodes morphologically corresponding with *Pseudoterranova decipiens sensu lato* (*s.l.*) (Krabbe, 1878) from different seal or sea lion hosts and geographical origins, previously identified as *Pseudoterranova krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa*, *P. azarasi* and *P. cattani* by multilocus enzyme electrophoresis, were characterized using a DNA approach. Also a population of *P. decipiens* (*s.l.*) from *Chaenocephalus aceratus*, the blackfin icefish, from Antarctica and another from *Osmerus eperlanus*, the European smelt, from Germany were included in the study. The first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of ribosomal DNA (rDNA) were amplified by PCR from individual nematodes and analysed by single-strand conformation polymorphism (SSCP), followed by selective sequencing. While no variation in single-stranded ITS-1 and ITS-2 profiles was detected among samples representing each of the species or populations (with the exception of slight microheterogeneity), SSCP analysis of the ITS-2 amplicons allowed the unequivocal differentiation of all of the 5 sibling species of *P. decipiens* (*s.l.*) examined, which was supported by sequence differences in ITS rDNA. Samples representing the *P. decipiens* (*s.l.*) population from *O. eperlanus* had the same SSCP profile as those of *P. decipiens* (*s.s.*), which was supported by a lack of nucleotide difference in the ITS between them, suggesting that the former represented *P. decipiens* (*s.s.*). Based on SSCP results and ITS sequence data, *P. decipiens* (*s.l.*) from *C. aceratus* was genetically most distinct with respect to all other members of *Pseudoterranova* examined, which indicated that it may represent *P. decipiens* E (based on geographical origin) or a distinct species. These findings and the molecular approach taken should have important implications for studying the life-cycles, transmission patterns, epidemiology and population genetics of these anisakid nematodes, and the diagnosis of their infections.

Key words: internal transcribed spacer, polymerase chain reaction, *Pseudoterranova decipiens sensu lato*, ribosomal DNA, single-strand conformation polymorphism, species identification.

INTRODUCTION

The morphospecies *Pseudoterranova decipiens sensu lato* (*s.l.*) (Krabbe, 1878) is a common anisakid nematode of many aquatic hosts and has global geographical distribution (Palm *et al.* 1994; Palm, 1999; Paggi *et al.* 2000). Crustaceans, such as copepods, amphipods, shrimps and isopods, serve as intermediate hosts. Fish of various species serve as intermediate or paratenic hosts, and pinnipeds, including seals and sea lions, serve as definitive hosts (Adams, Murrell & Cross, 1997; Palm, 1999; Anderson, 2000). As seals are regarded as the most frequent definitive hosts, and cod among the most frequent intermediate or paratenic hosts, *P. decipiens* (*s.l.*) is also referred to as the 'sealworm' or 'codworm'. Importantly, humans can also become infected with *P. decipiens* (*s.l.*) larvae by eating raw or inadequately cooked fish, such as cod (Desowitz, 1986; Kliks, 1986; Oshima, 1987; Adams *et al.* 1997). The disease is referred to as codworm anisakiasis or pseudoterranoviasis and is recognized as an important fish-borne zoonosis (Oshima, 1987;

* Corresponding author: Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia. Tel: +61 3 97312283. Fax: +61 3 97312366. E-mail: robinbg@unimelb.edu.au

† Present address: College of Veterinary Medicine, South China Agricultural University, Wushan, Tianhe District, Guangzhou 510642, Guangdong Province, People's Republic of China.

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Table 1. Adult and larval nematodes representing *Pseudoterranova decipiens* (*s.l.*), including *Pseudoterranova krabbei* (Pk), *P. decipiens* (*s.s.*) (Pd), *P. bulbosa* (Pb), *P. azarasi* (Pa), *P. cattani* (Pc) and 2 previously uncharacterized populations (PdCa and PdOe) from different host species

Species/population	Sample code	No. of samples	Host species	Geographical origin
Previously characterized by multilocus enzyme electrophoresis				
P. krabbei	Pk1-Pk15	15	*Halichoerus grypus* (Grey seal)	Froya Island, Norway
P. decipiens (*s.s.*)	Pd1-Pd9	9	*Phoca vitulina* (Harbour seal)	Newfoundland, Canada
P. bulbosa	Pb1-Pb10	10	*Erignathus barbatus* (Bearded seal)	Newfoundland, Canada
P. azarasi	Pa1-Pa8	8	*Eumetopias jubata* (Steller's sea lion)	Iwanai, Japan
P. cattani	Pc1-Pc8	8	*Otaria byronia* (South American sea lion)	Concepción, Chile
Previously uncharacterized by multilocus enzyme electrophoresis				
P. decipiens (*s.l.*)	PdCa1-PdCa29	29	*Chaenocephalus aceratus* (Blackfin icefish)	South Shetland Islands, Antarctica
P. decipiens (*s.l.*)	PdOe1-PdOe27	27	*Osmerus eperlanus* (European smelt)	Elbe estuary, Germany

Ko, 1995; Pinel *et al.* 1996; Adams *et al.* 1997; Takahashi, 1998). The apparent increase in prevalence and intensity of codworm infection and subsequent condemnation of infected fish can cause substantial economic losses to the fishing industry (Brattey & Davidson, 1996; Rohlwing, Palm & Rosenthal, 1998).

Previous studies employing multilocus enzyme electrophoresis (MEE) have shown that *P. decipiens* (*s.l.*) comprises at least 6 sibling species (which are morphologically very similar but genetically differentiated), with distinct host preferences and geographical distributions (Paggi *et al.* 1991, 1998; Bullini *et al.* 1994, 1997; George-Nascimento & Llanos, 1995; Mattiucci *et al.* 1998; George-Nascimento & Urrutia, 2000). Three of these sibling species, *P. krabbei*, *P. decipiens* (*s.s.*) and *P. bulbosa*, have been reported in the North Atlantic, Norwegian and Barents Seas (Paggi *et al.* 1991), while *P. azarasi* has been identified in the Arctic-Boreal and Japanese waters, *P. decipiens* E in Antarctic waters (Bullini *et al.* 1992, 1997; Mattiucci *et al.* 1998), and *P. cattani* in the Southeastern Pacific Ocean (George-Nascimento & Llanos, 1995; George-Nascimento & Urrutia, 2000).

While useful for taxonomic and population genetic studies, the technique of MEE (reviewed by Andrews & Chilton, 1999) has a limitation in that it is reliant on the use of relatively large amounts of fresh or fresh/frozen parasite material for analysis. Polymerase chain reaction (PCR) (Saiki *et al.* 1988) techniques have the advantage over MEE in that their sensitivity allows the analysis of minute amounts of fresh, lyophilized or ethanol-fixed parasite material. Recent PCR-based studies have shown that the first (ITS-1) and/or second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) can provide genetic markers for the accurate identification of a number of morphospecies or cryptic species of ascaridoid (Jacobs *et al.* 1997; Zhu & Gasser, 1998; Zhu *et al.* 1998*a–c*, 1999, 2000*a*, *b* 2001*a*, *b*). Importantly, a study of the *Contracaecum osculatum* complex (see Zhu *et al.* 2000*b*) indicated that most of its members could be identified and differentiated by single-strand conformation polymorphism (SSCP) analysis of their ITS, suggesting that this rDNA region may also provide specific markers for individual members of the *P. decipiens* complex. In the present study, we employed SSCP to measure the magnitude of sequence variation in the ITS-1 and ITS-2 rDNA within and among 5 recognized members and 2 previously uncharacterized populations of *P. decipiens* (*s.l.*) from different host species and geographical origins.

MATERIALS AND METHODS

Parasites

Adult and larval nematodes corresponding morphologically with *P. decipiens* (*s.l.*) (see Paggi *et al.* 1991, 2000; Palm *et al.* 1994; Mattiucci *et al.* 1998; Palm, 1999; George-Nascimento & Urrutia, 2000) were used in this study. Their codes, numbers, host species and geographical origins are listed in Table 1. Adult nematodes were collected at necropsy from the stomachs of seal or sea lion definitive hosts, and larval nematodes from the liver, body cavity or musculature of the teleost intermediate or paratenic hosts. Parasites were washed extensively in physiological saline and then frozen at −80 °C or fixed in 50% ethanol. Adult specimens representing the sibling species *P. krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa*, *P. azarasi* and *P. cattani* were identified previously by MEE analysis (Paggi *et al.* 1991, 1998; George-Nascimento & Llanos, 1995; Bullini *et al.* 1997; Mattiucci *et al.* 1998). Larval specimens representing *P. decipiens* (*s.l.*) from the teleost hosts *Chaenocephalus aceratus* and *Osmerus eperlanus*, not previously characterized by MEE and thus designated as distinct operational taxonomic units or populations (PdCa and PdOe, respectively), were also included in the study (Table 1).

DNA isolation and enzymatic amplification

Genomic DNA was isolated from individual nematodes by sodium dodecyl-sulphate/proteinase K treatment (Gasser *et al.* 1993), column-purified (Wizard™ DNA Clean-Up, Promega) and eluted into 40 μl of H_2O. Seal DNA was also isolated from liver tissue using the same method. PCR was used to amplify the ITS-1 (plus ~ 80 bp of flanking sequence) with primers NC5 (forward; 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC13R (reverse; 5′-GCTGCGTTCTTCATCGAT-3′), and the ITS-2 (plus ~ 120 bp of flanking sequence) with primers XZ1 (forward; 5′-ATTGCGCCATCGGGTTCATTCC-3′) and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′) (Zhu *et al.* 2000*b*). PCR reactions (50 μl) were performed in 10 mM Tris–HCl, pH 8·4, 50 mM KCl, 3 mM $MgCl_2$, 250 μM each of dNTP, 50 pmol of each primer end-labelled with [γ^{33}P]ATP (NEN, DuPont) and 2 U *Taq* polymerase (Promega) in a thermocycler (Perkin Elmer Cetus) under the following conditions: 94 °C for 5 min (initial denaturation), followed by 30 cycles of 94 °C, 30 sec (denaturation), 55 °C, 30 sec (annealing), 72 °C, 30 sec (extension) and a final extension of 72 °C for 5 min. One microlitre (5–10 ng) of genomic DNA was added to each PCR reaction. Samples with seal DNA or without genomic DNA were included in each amplification run as ‘negative’ controls. An aliquot (4 μl) of each PCR product was examined by agarose gel electrophoresis (Sambrook, Fritsch & Maniatis, 1989).

Single-strand conformation polymorphism (*SSCP*) *analysis*

SSCP analysis was carried out as described previously (Zhu & Gasser, 1998). In brief, 10 μl of each PCR product were mixed with an equal volume of loading buffer (10 mM NaOH), 95 % formamide, 0·05 % bromophenol blue and 0·05 % xylene cyanole). After denaturation at 94 °C for 5 min and snap-cooling on a freeze-block (−20 °C), 3 μl of individual samples were loaded into the wells of a 0·4 mm thick, 0·6 × MDE (= mutation detection enhancement, BMA) gel and subjected to electrophoresis in a conventional sequencing rig (Base Runner™, IBI). The conditions for electrophoresis (7 W for 12 h at 20 °C) were standardized for optimal resolution of bands (Zhu & Gasser, 1998). Gels were subsequently dried on to blotting paper and exposed to X-ray film (RP1, Agfa).

DNA sequencing and analyses

Representative amplicons were purified over spin columns (Wizard™ PCR-Preps, Promega) and subjected to manual sequencing using the *fmol*™ cycle-sequencing kit (Promega), employing the same primers (individually) as used for primary amplification. The 5′ and 3′ ends of the ITS sequences were determined by comparison with those of a range of ascaridoid nematodes, including those within the *C. osculatum* complex (Jacobs *et al.* 1997; Zhu *et al.* 1998*a*, *c*, 1999, 2000*a*, *b*, 2001*a*, *b*), and sequences were aligned manually. Pairwise comparisons were made of the level of sequence difference (D) using the formula $D = 1-(M/L)$ (Chilton, Gasser & Beveridge, 1995), where M is the number of alignment positions at which the 2 sequences have a base in common, and L is the total number of alignment positions over which the 2 sequences are compared. A phenogram was constructed using the unweighted pair group method using arithmetic averages (UPGMA; Sneath & Sokal, 1973).

RESULTS

The ITS-1 and ITS-2 rDNA (including flanking sequences) were amplified individually from genomic DNA prepared from 106 individual worms (Table 1) representing 5 sibling species (*P. krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa*, *P. azarasi* and *P. cattani*) and 2 previously uncharacterized populations of *P. decipiens* (*s.l.*). Both the ITS-1 and ITS-2 amplicons were ~ 430 bp in length, and no size variation was detected on agarose gels among any of the samples for both rDNA regions. In no case were amplification products detected in the host or no-DNA control samples (not shown).

The analysis of sequence variation by SSCP has significant technical advantages over other methods, particularly when relatively large numbers of amplicons require analysis in a sequence-dependent manner (Gasser & Zhu, 1999). Hence, this technique was used to screen both the ITS-1 and ITS-2 amplicons for sequence variation within and among the 106 individual nematodes. SSCP analysis of the ITS-1 amplicons resolved 2–3 single-stranded bands per individual, whereas 4–5 bands were displayed for the ITS-2. Except for slight microheterogeneity (Fig. 1, arrowed), no variation in single-stranded ITS-1 and ITS-2 profiles was detected within each of the sibling species or populations, whereas significant differences in SSCP profiles were detected among most of them. For example, *P. cattani*, *P. krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa* and *P. azarasi* each had unique ITS-2 SSCP profiles, which allowed their identification and differentiation from one another (Fig. 1). All individuals of the *P. decipiens* (*s.l.*) population PdOe had the same ITS-2 SSCP profile as those representing *P. decipiens* (*s.s.*), whereas samples representing the population PdCa all had an ITS-2 SSCP profile which was distinct from all species examined and from population PdOe (Fig. 1).

Of the 212 amplicons scanned by SSCP, the 20 (10 for each rDNA spacer) which represented profiles

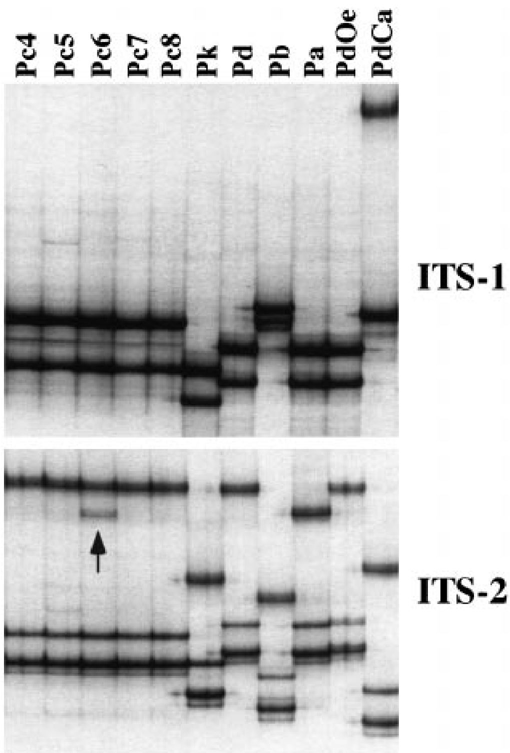

Fig. 1. Representative SSCP gel displaying sequence variation in the ITS-1 (top) or ITS-2 (bottom) within *Pseudoterranova cattani* (samples Pc4–Pc8), and among *P. cattani*, *P. krabbei* (Pk), *P. decipiens* (*s.s.*) (Pd), *P. bulbosa* (Pb), *P. azarasi* (Pa), and *P. decipiens* (*s.l.*) from *Osmerus eperlanus* (PdOe) or *Chaenocephalus aceratus* (PdCa). The arrow indicates an example of microheterogeneity in the SSCP profiles among samples within a species.

displaying the full extent of variation among all individuals examined were subjected to sequencing (Table 2). The lengths of the ITS-1 and ITS-2 sequences ranged from 343 to 357 bp and 296 to 306 bp, respectively (Table 2). While no variation in sequence length was detected within any species or population for which multiple samples were sequenced, length differences varied from 0 to 14 bp in the ITS-1 and 0 to 10 bp in the ITS-2 between samples representing each of the 7 species or populations (Table 2).

In accordance with the SSCP results, no substitutional changes were detected in either the ITS-1 or the ITS-2 sequences between multiple samples sequenced for each *P. cattani*, *P. bulbosa* and population PdOe, although 2 polymorphic positions were detected in the ITS-1 of *P. bulbosa* (alignment positions 211 and 233, respectively; Fig. 2) and one in the ITS-2 of both *P. cattani* and PdOe (alignment positions 62 and 157, respectively; Fig. 2), which was consistent with the microheterogeneity detected in SSCP profiles (Fig. 1). While samples representing *P. decipiens* (*s.s.*) and population PdOe had the same ITS sequences, those representing *P. cattani*, *P. krabbei*, *P. bulbosa*, *P. azarasi* and PdCa each had their unique ITS sequence, allowing their unequivocal molecular identification (Table 3). The ITS-2 representing *P. azarasi* differed from that of both *P. decipiens* (*s.s.*) and PdOe by a single nucleotide, reinforcing the high mutation detection rate of the SSCP method used (Zhu & Gasser, 1998). The G+C content of the sequences varied from 46·4 to 47·9% for ITS-1 and from 43·5 to 45·6% for ITS-2.

The alignment of the ITS-1 and ITS-2 sequences representing the 5 *Pseudoterranova* species and 2 populations of *P. decipiens* (*s.l.*) is shown in Fig. 2. Comparison among all of the sequences (Fig. 2) revealed a total of 65 nucleotide differences (i.e. 27 in the ITS-I and 38 in the ITS-2, respectively). Of these, 35 (53·8%) were single-base substitutions, representing 25 (38·5%) transitional changes between either purines (A↔G; $n = 13$) or pyrimidines (C↔T; $n = 12$), and 9 (13·8%) transversions (i.e. substitutions between a purine and a pyrimidine). One position (1·5%) represented a multiple-substitution event. The remaining 30 differences (46·2%) represented deletions or insertions.

Pairwise comparisons of the 7 ITS sequences revealed nucleotide differences ranging from 0 to 6·8% (Table 3), which were within the range (0–2·3%) among members of the *C. osculatum* complex (Zhu *et al.* 2000*b*). With the exception of *P. decipiens* (*s.s.*) and population PdOe which had identical ITS sequences, each of the 5 currently recognized sibling species of *P. decipiens* (*s.l.*) (based on MEE analysis) could be identified by the presence of at least 1 unequivocal nucleotide difference (Fig. 2). For instance, the G at alignment position 50 in the ITS-2 was unique to *P. azarasi*, whereas the T's at both alignment positions 84 and 86 in the ITS-2 allowed the differentiation of *P. krabbei* from the other taxa (Fig. 2).

The phenogram (Fig. 3) constructed based on the pairwise sequence difference data (Table 3) showed that *P. azarasi* was genetically more similar to both *P. decipiens* (*s.s.*) and population PdOe than it was to any other member of *Pseudoterranova*, whereas population PdCa was genetically most distinct with respect to all other members of *Pseudoterranova* examined herein.

DISCUSSION

Using MEE analysis, 6 genetically distinct and reproductively isolated species have been detected previously within the morphospecies *P. decipiens* from the Arctic-Boreal, Boreal and Antarctic waters, and the Southeastern Pacific Ocean (Paggi *et al.* 1991, 1998; George-Nascimento & Llanos, 1995; Bullini *et al.* 1997; Mattiucci *et al.* 1998). A recent study of *P. decipiens* (*s.l.*) samples from Atlantic Canada, by restriction fragment length polymorphism analysis of genomic DNA followed by

Table 2. Length (in bp) and GenBank™ accession numbers of the ITS-1 and ITS-2 sequences representing *Pseudoterranova krabbei* (Pk), *P. decipiens* (*s.s.*) (Pd), *P. bulbosa* (Pb), *P. azarasi* (Pa), *P. cattani* (Pc) and *P. decipiens* (*s.l.*) from *Chaenocephalus aceratus* (PdCa) or *Osmerus eperlanus* (PdOe)

(Samples sequenced were selected based on SSCP analysis.)

Species/population	Samples sequenced	ITS-1	Accession no.	ITS-2	Accession no.
P. krabbei	Pk1	353	AJ413965	306	AJ413966
P. decipiens (*s.s.*)	Pd1	353	AJ413968	299	AJ413967
P. bulbosa	Pb1	353	AJ413969	299	AJ413971
	Pb5	353	AJ413970	299	AJ413972
P. azarasi	Pa1	353	AJ413973	299	AJ413974
P. cattani	Pc1	343	AJ413981	299	AJ413983
	Pc6	343	AJ413982	299	AJ413984
PdCa	PdCa1	357	AJ413979	296	AJ413980
PdOe	PdOe1	353	AJ413975	299	AJ413978
	PdOe5	353	AJ413976	299	AJ413977

Southern blotting using an 'heterologous', cloned rDNA repeat unit as a probe, revealed the presence of *P. decipiens* (*s.s.*) and *P. bulbosa* in Canadian Atlantic marine fishes and seals (Brattey & Davidson, 1996). This finding was in agreement with results of a previous MEE study (Paggi *et al*. 1991) which reported the existence of *P. decipiens* (*s.s.*) and *P. bulbosa* in Atlantic Canada. In the present study, 106 individual nematodes representing 5 currently recognized sibling species within the *P. decipiens* complex (*P. cattani*, *P. krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa* and *P. azarasi*) and 2 *P. decipiens* (*s.l.*) populations (PdCa and PdOe) of unknown taxonomic status were characterized by SSCP analysis of their ITS-1 and ITS-2. Each of the 5 sibling species had a unique SSCP profile and ITS sequence, allowing their unequivocal identification and differentiation from one another, which was consistent with previous MEE studies (Paggi *et al*. 1991, 1998; George-Nascimento & Llanos, 1995; Bullini *et al*. 1997; Mattiucci *et al*. 1998). Individuals representing population PdOe from the host *O. eperlanus* had the same SSCP profile and ITS sequence as *P. decipiens* (*s.s.*), suggesting that this population represented *P. decipiens* (*s.s.*). This proposal is supported by the geographical origin of PdOe, but independent genetic analysis (e.g. using MEE analysis) is required to verify this. Based on SSCP profile and ITS sequence, population PdCa from the host *C. aceratus* is likely to represent a distinct species. Geographical and host origins as well as its genetic distinctiveness with respect to all other members of the *P. decipiens* complex would support the proposal that it represents *P. decipiens* E. A direct genetic comparison of individuals representing PdCa with those of *P. decipiens* E using the present molecular approaches is warranted when samples representing the latter become available.

P. decipiens E is a parasitic nematode occurring in Antarctic waters (Palm, 1999). Its main definitive host is the widely-distributed Weddell seal, *Leptonychotes weddelli* (see Bullini *et al*. 1992, 1997), and its 3rd-stage larvae occur in a number of fish species in the Weddell Sea and South Shetland waters (Palm *et al*. 1994, 1998; Palm, 1999). A previous study (Palm *et al*. 1998) has indicated that most parasite species of the Antarctic fish, *Notothenia coriiceps*, appear to be broadly distributed within Antarctic waters, which would correspond to a low host specificity in higher latitudes. However, to date, it remains unclear whether all *P. decipiens* (*s.l.*) in the Antarctic belong to a single sibling species (i.e. *P. decipiens* E). Employing the SSCP-sequencing approach, this question could be readily addressed by genetic comparison of *P. decipiens* (*s.l.*) specimens from a range of intermediate, paratenic and definitive host species and localities within the Antarctic waters.

P. cattani is a recently described anisakid of the South American sea lion, *Otaria byronia*, which is distinct with respect to other known *Pseudoterranova* species based on allozymic and morphological characters (George-Nascimento & Llanos, 1995; George-Nascimento & Urrutia, 2000). The adult and larva of this species from the south-eastern Pacific Ocean were recognized previously as *Phocanema decipiens* (see George-Nascimento & Urrutia, 2000). The results of the present genetic study support the specific taxonomic status of *P. cattani*, given its distinct SSCP profile and ITS sequence with respect to all other members of *Pseudoterranova* examined. Moreover, in addition to *P. decipiens* (*s.l.*) and *P. cattani*, *P. kogiae* and *P. ceticola* have also been described in the literature (Anderson, 2000). Therefore, the SSCP-sequencing approach employed in this study should be useful for a direct genetic comparison of these *Pseudoterranova* species for which no molecular data are currently available.

Although morphometric keys are available for the identification of adult males of *P. krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa* and *P. azarasi* (see Di Deco *et al*. 1994; Mattiucci *et al*. 1998; Paggi *et al*. 2000), no

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      ITS-1--->
Pk    ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCGCATAGAAACTCATACACGTGT 90
Pd    ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCACATAGAAACTCATACACGTGT
Pb    ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCACATGGAAACTCATACACGTGT
Pa    ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCACATAGAAACTCATACACGTGT
PdOe  ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCACATAGAAACTCATACACGTGT
PdCa  ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCGCATAGAAACTCATACACGCGT
Pc    ATCGAGCGAATCCAAAACGAAAAAGTCTCCCAACGTGCATACCATCCATTTGCATGTTGTTGTGAGCCACATAGAAACTCATACACATGT
                                                                          *   *             **

Pk    GGTGGCAGCCGTCTGATGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT 180
Pd    GGTGGCAGCCGTCTGATGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT
Pb    GGTGGCAGCCGTCTGCTGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT
Pa    GGTGGCAGCCGTCTGATGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT
PdOe  GGTGGCAGCCGTCTGATGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT
PdCa  GGTGGCAGCCGTCTGCTGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT
Pc    GGTGGCAGCCGTCTGATGTGCTTTATCGTGCAGACAATGGCTTATGAGTGGCTGTGTGATTGTTGAACAACGGTGACCAATTTGGCGTCT
                    *

Pk    ACGCCGTATCTAGCTTCTGCCTGGACCGTCGGTAGCGATGAAAGATGCGGAGAAAGTTCCTCTGTTTTGGT----ACGCTAACGCAGAGT 270
Pd    ACGCCGTATCTAGCTTCTGCCTGGACCGTCGGTAGCGATGAAAGATGCGGAGGAAGTTCCTCTGTTTTGGT----ACGCTAAAGCAGAGT
Pb    ACGCCGTATCTAGCTTCTGCCTGGACCGTCRGTAGCGATGAAAGATGCGGAGRAAGTTCCTCTGTTTTGGT----ACGCCAGCGCAGAGT
Pa    ACGCCGTATCTAGCTTCTGCCTGGACCGTCGGTAGCGATGAAAGATGCGGAGGAAGTTCCTCTGTTTTGGT----ACGCTAAAGCAGAGT
PdOe  ACGCCGTATCTAGCTTCTGCCTGGACCGTCGGTAGCGATGAAAGATGCGGAGGAAGTTCCTCTGTTTTGGT----ACGCTAAAGCAGAGT
PdCa  ACGCCGTATCTAGCTTCCGCCTGGACCGTCGGTAGCGATGAAAGATGCGGAGGAAGTTCCTCTGTTTTGGTTTCAACGCTAACGCAGAGT
Pc    ACGCCGTATCTAGCTTCTGCCTGGACCGTCAGTAGCGATGAAAGATGCGGAGGAAGTTCCTCTGTT--------------AACGCAGAGT
                       *            *                     *             ************** **

Pk    TGAGCAGACTTAATGAGCTACGCTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA 357
Pd    TGAGCAGACTTAATGAGCCACGCTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA
Pb    TGAGCAGACTTAATGAGCCACGCTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA
Pa    TGAGCAGACTTAATGAGCCACGCTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA
PdOe  TGAGCAGACTTAATGAGCCACGCTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA
PdCa  TGAGCAGACTTAATGAGCCACTGTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA
Pc    TGAGCAGACTTAATGAGCCACGCTTGGTGGCCGCCAAAACCCAAAACACAACCAGTCTATTTTAACGTTTGTTGATATGTTAATGTA
                        *  **

      ITS-2--->
Pk    TTATAGTAAACTATCTTCGAGTACTTTTTATGGTCGTGAAGTATTCGGCAAGTAGTGCAGTCGGAT---TGTTTTTGTTGGTCTTTCGGT 90
Pd    TTATAGTAAACTATCTTCGAGTACTTTTTATGGTCGTGAAGTATTCGGCAAGTAGTGCAGTCGGAT---TGTTTTTGTTGGTCGTCCG-T
Pb    TTATAGTAAACTATCTTCGAGTACTTTTTATGGTCGTGAAGTATCCGGCAAGTAGTGCAGTTGGAT---TGTTTTTGTTGCTTGTCCG-T
Pa    TTATAGTAAACTATCTTCGAGTACTTTTTATGGTCGTGAAGTATTCGGCGAGTAGTGCAGTCGGAT---TGTTTTTGTTGGTCGTCCG-T
PdOe  TTATAGTAAACTATCTTCGAGTACTTTTTATGGTCGTGAAGTATTCGGCAAGTAGTGCAGTCGGAT---TGTTTTTGTTGGTCGTCCG-T
PdCa  TTGTAGTAAACTGTCTTCAAGTACTTTTTATGGTCGTGAAGTATTCGGCAAGCAGT--TGTCGGATAGTTGTTTTTGTTGATCGTCCG-T
Pc    TTATGGTAAACTATCTTCGAGTACTTTTTATGGTCGTGAAGTATTCGGCAAGTAGTGCAGTYGGAT---TGTTTTTGTTGGTCGTCCG-T
        *         *     *                         *    *  *   ***  *    ***           * ** *  *

Pk    TCGTTGGGTCGGTCGATCAACAACAATATTCTGAGGCTCCTTGCTTAGTTGTGTTCTAGTAGACGTTAACACCTGAACAATATGTGGTGG 180
Pd    TCGTTCGGTCGGTC----AACAACAATATTCTGAGGCTCCTTGCTTAGTTGTGTTCTAGTAGACGTTAACACCTGAACAATATGTGGTGG
Pb    TCGTTCGGTCGGTT----AACAACAATATTCTGAGGCTCCTTGCTTAGTTGTGTTTTAGTAGACGTTAACACCTGAACAATATGTGGTGG
Pa    TCGTTCGGTCGGTC----AACAACAATATTCTGAGGCTCCTTGCTTAGTTGTGTTCTAGTAGACGTTAACACCTGAACAATATGTGGTGG
PdOe  TCGTTCGGTCGGTC----AACAACAATATTCTGAGGCTCCTTGCTTAGTTGTGTTCTAGTAGACGTWAACACCTGAACAATATGTGGTGG
PdCa  TCGTTCGGTCGGTC----CAGAGCAATATTCTGAGGCTCCTTGCTTAGTTGCGTTTTGGTAGACGTTAACACCCGAACAATATGTGGTGG
Pc    TCGTTCGGTCGGTC----AACAACAATATTCTGAGGCTCCTTGCTTAGTTGTGTTCTAGTAGACGTTAACACCTGAACAATATGTGGTGG
           *       ****** * *                            *   * *               *

Pk    TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATTGTGAAGCCAGGGCA 270
Pd    TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATT--GAAGCCAGGGCA
Pb    TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATT--GAAGCCAGGGCA
Pa    TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATT--GAAGCCAGGGCA
PdOe  TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATT--GAAGCCAGGGCA
PdCa  TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATT--GAAGCCAGGGCA
Pc    TGATATTGGTGATGGCGAGAATCATGCCGCTTCAATGGGGCAGCAACCAGCATACGCTAATGACAGTTGGTTGATT--GAAGCCAGGGCA
                                                                                  **

Pk    ACGGAGTGATGTACGTGTGGCGATCATTTAACGTTTATA 309
Pd    ACGGAGTGATGTACGTGTGGCGATCATTTAACGTTTATA
Pb    ACGGAGTGATGTACGTGTGGCGATCATTTAACGTTTGTA
Pa    ACGGAGTGATGTACGTGTGGCGATCATTTAACGTTTATA
PdOe  ACGGAGTGATGTACGTGTGGCGATCATTTAACGTTTATA
PdCa  ACGGAGTGATGT----GTGGCGATCATTTAACGTTTGTA
Pc    ACGGAGTGATGTACGTGTGGCGATCATTTAACGTTTATA
                  ****                    *
```

Fig. 2. Alignment of the ITS-1 and ITS-2 sequences representing *Pseudoterranova krabbei* (Pk), *P. decipiens* (*s.s.*) (Pd), *P. bulbosa* (Pb), *P. azarasi* (Pa) and *P. decipiens* (*s.l.*) from *Osmerus eperlanus* (PdOe) or *Chaenocephalus aceratus* (PdCa) as well as those of *P. cattani* (Pc). Refer to Tables 1 and 2 for samples sequenced. Sequences have been deposited in the EMBL, GenBank™ and DDBJ databases. Nucleotide differences among the aligned sequences are indicated by asterisks. Polymorphic sites in the sequences are designated with IUPAC codes.

Table 3. Percentage of nucleotide difference in the entire ITS ribosomal DNA region (consensus sequence) among *Pseudoterranova krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa*, *P. azarasi*, *P. cattani* and *P. decipiens* (*s.l.*) from *Chaenocephalus aceratus* (PdCa) or *Osmerus eperlanus* (PdOe)

Species/population	Pk	Pd	Pb	Pa	PdCa	PdOe	Pc
P. krabbei (Pk)	–						
P. decipiens (*s.s.*) (Pd)	1·8	–					
P. bulbosa (Pb)	3·5	1·8	–				
P. azarasi (Pa)	2·3	0·2	2·0	–			
PdCa	6·5	5·2	5·6	5·3	–		
PdOe	2·1	0	1·8	0·2	5·2	–	
P. cattani (Pc)	3·9	2·1	3·2	2·3	6·8	2·1	–

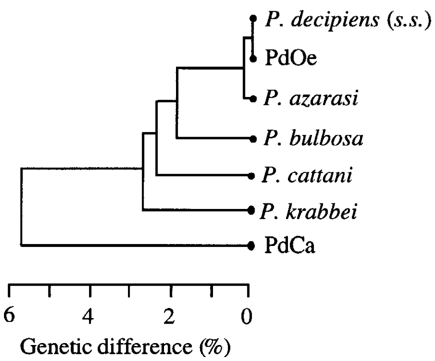

Fig. 3. Phenogram depicting the genetic differences (upon pairwise comparison) among *Pseudoterranova krabbei*, *P. decipiens* (*s.s.*), *P. bulbosa*, *P. azarasi*, *P. cattani* and *P. decipiens* (*s.l.*) from *Chaenocephalus aceratus* (PdCa) or *Osmerus eperlanus* (PdOe) for the entire ITS rDNA sequence.

such keys are available for the specific identification of larval stages. A number of crustacean and fish species have been identified as the intermediate and intermediate/paratenic hosts of *P. decipiens* (*s.l.*), respectively (George-Nascimento & Llanos, 1995; Palm, 1999), but relatively little is currently known about the distributions and role(s) of each host species in the transmission of each sibling species (Paggi *et al.* 1991). Therefore, the establishment of molecular-genetic tools for the precise identification of sibling species within *P. decipiens* (*s.l.*) is of significance for further studies of their life-cycles, host preference, transmission patterns and population genetic structures.

The molecular tools should also have significant implications for the diagnosis of pseudoterranoviasis in humans. To date, clinical diagnosis of human gastric and intestinal pseudoterranoviasis has relied mainly on the detection of nematodes upon endoscopy, followed by morphological identification of the larvae recovered (Oshima, 1987; Ko, 1995; Pinel *et al.* 1996; Adams *et al.* 1997). However, given the limitations associated with the morphological identification of larval stages or portions thereof, SSCP analysis of ITS-2 can achieve their specific identification from minute amounts of parasite material extracted during endoscopic examination. Indeed, a recent study (D'Amelio *et al.* 1999) has demonstrated the usefulness of PCR-linked restriction fragment length polymorphism analysis for the specific identification of *Anisakis* larva endoscopically recovered from the stomach of a woman in Southern Italy.

There is a distinct difference between the clinical syndrome of human pseudoterranoviasis in Japan and that in the USA (Oshima, 1987). In Japan, the disease is quite severe, whereas in the USA, it is milder and without clear symptoms attributable to gastro-intestinal invasion (Oshima, 1987). While the reason(s) for this variation in pathogenic effect is currently unclear, Desowitz (1986) suggested that it may be attributable to different 'strains' of *P. decipiens* (*s.l.*) with differing virulence. It would be interesting to genetically compare *P. decipiens* larvae from humans from Japan with those from the USA, employing the SSCP approach. Also, genetic comparison of *P. decipiens* (*s.l.*) larvae from humans with adult or larval stages from a range of intermediate, paratenic and definitive hosts may establish which species is/are the causative agent(s) for human pseudoterranoviasis and thus pose a public health threat.

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REFERENCES

ADAMS, A. M., MURRELL, K. D. & CROSS, J. H. (1997). Parasites of fish and risks to public health. *Revue Scientifique et Technique* **16**, 652–660.

ANDERSON, R. C. (2000). *Nematode Parasites of Vertebrates. Their Development and Transmission*, 2nd Edn. CAB International, Wallingford, UK.

ANDREWS, R. H. & CHILTON, N. B. (1999). Multilocus enzyme electrophoresis: a valuable technique for providing answers to problems in parasite systematics. *International Journal for Parasitology* **29**, 213–253.

BRATTEY, J. & DAVIDSON, W. S. (1996). Genetic variation within *Pseudoterranova decipiens* (Nematoda: Ascaridoidea) from Canadian Atlantic marine fishes and seals: characterization by RFLP analysis of genomic DNA. *Canadian Journal of Fisheries and Aquatic Sciences* **53**, 333–341.

BULLINI, L., ARDUINO, P., CIANCHI, R., NASCETTI, G., D'AMELIO, S., MATTIUCCI, S., PAGGI, L. & ORECCHIA, P. (1994). Genetic and ecological studies on nematode endoparasites of the genera *Contracaecum* and *Pseudoterranova* in the Antarctic and Arctic-Boreal regions. In *Proceedings of the Second Meeting on Antarctic Biology* (ed. Battaglia, B. Bisol, P. M. & Varotto, V.), pp. 131–146. Edizioni Universitarie Patavine, Padova, Italy.

BULLINI, L., ARDUINO, P., CIANCHI, R., NASCETTI, G., D'AMELIO, S., MATTIUCCI, S., PAGGI, L., ORECCHIA, P., PLOTZ, J., BERLAND, B., SMITH, J. W. & BRATTEY, J. (1997). Genetic and ecological research on anisakid endoparasites of fishes and seals in the Antarctic and Arctic-Boreal regions (Nematoda, Ascaridoidea). In *Antarctic Communities: Species, Structure and Survival* (ed. Battaglia, B., Valencia, J. & Walton, D. W. H.), pp. 39–44. Cambridge University Press, Cambridge.

CHILTON, N. B., GASSER, R. B. & BEVERIDGE, I. (1995). Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). *International Journal for Parasitology* **25**, 647–651.

D'AMELIO, S., MATHIOPOULOS, K. D., BRANDONISIO, O., LUCARELLI, G., DORONZO, F. & PAGGI, L. (1999). Diagnosis of a case of gastric anisakidosis by PCR-based restriction fragment length polymorphism analysis. *Parasitologia* **41**, 591–593.

DESOWITZ, R. S. (1986). Human and experimental anisakiasis in the United States. *Hokkaido Journal of Medical Science* **61**, 358–371.

DI DECO, M. A., ORECCHIA, P., PAGGI, L. & PETRARCA, V. (1994). Morphometric stepwise discriminant analysis of three genetically identified species within *Pseudoterranova decipiens* (Krabbe, 1878) (Nematoda: Ascaridida). *Systematic Parasitology* **29**, 81–88.

GASSER, R. B. & ZHU, X. Q. (1999). Sequence-based analysis of enzymatically amplified DNA fragments by mutation detection techniques. *Parasitology Today* **15**, 462–465.

GASSER, R. B., CHILTON, N. B., HOSTE, H. & BEVERIDGE, I. (1993). Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Research* **21**, 2525–2526.

GEORGE-NASCIMENTO, M. & LLANOS, A. (1995). Micro-evolutionary implications of allozymic and morphometric variations in sealworms *Pseudoterranova* sp. (Ascaridoidea: Anisakidae) among sympatric hosts from the Southeastern Pacific Ocean. *International Journal for Parasitology* **25**, 1163–1171.

GEORGE-NASCIMENTO, M. & URRUTIA, X. (2000). *Pseudoterranova cattani* sp. nov. (Ascaridoidea: Anisakidae), a parasite of the South American sea lion *Otaria byronia* De Blainville from Chile. *Revista Chilena de Historia Natural* **73**, 93–98.

JACOBS, D. E., ZHU, X. Q., GASSER, R. B. & CHILTON, N. B. (1997). PCR-based methods for identification of potentially zoonotic ascaridoid parasites of the dog, fox and cat. *Acta Tropica* **68**, 191–200.

KLIKS, M. M. (1986). Human anisakiasis: an update. *Journal of American Medical Association* **255**, 2605.

KO, R. C. (1995). Fish-borne parasitic zoonoses. In *Fish Diseases and Disorders, Vol. 1: Protozoan and Metazoan Infections* (ed. Woo, P. T. K.), pp. 631–671. CAB International, Wallingford, UK.

MATTIUCCI, S., PAGGI, L., NASCETTI, G., ISHIKURA, H., KIKUCHI, K., SATO, N., CIANCHI, R. & BULLINI, L. (1998). Allozyme and morphological identification of *Anisakis*, *Contracaecum* and *Pseudoterranova* from Japanese waters (Nematoda, Ascaridoidea). *Systematic Parasitology* **40**, 81–92.

OSHIMA, T. (1987). Anisakiasis – is the Sushi bar guilty? *Parasitology Today* **3**, 44–48.

PAGGI, L., NASCETTI, G., CIANCHI, R., ORECCHIA, P., MATTIUCCI, S., D'AMELIO, S., BERLAND, B., BRATTEY, J., SMITH, J. W. & BULLINI, L. (1991). Genetic evidence for three species within *Pseudoterranova decipiens* (Nematoda, Ascaridida, Ascaridoidea) in the North Atlantic and Norwegian and Barents Seas. *International Journal for Parasitology* **21**, 195–212.

PAGGI, L., MATTIUCCI, S., ISHIKURA, H., KIKUCHI, K., SATO, N., NASCETTI, G., CIANCHI, R. & BULLINI, L. (1998). Molecular genetics in anisakid nematodes from the Pacific Boreal Region. In *Host Response to International Parasitic Zoonoses* (ed. Ishikura, H., Aikawa, M., Itakura, H. & Kikuchi, K.), pp. 83–107. Springer-Verlag, Tokyo.

PAGGI, L., MATTIUCCI, S., GIBSON, D. I., BERLAND, B., NASCETTI, G., CIANCHI, R. & BULLINI, L. (2000). *Pseudoterranova decipiens* species A and B (Nematoda, Ascaridoidea): nomenclatural designation, morphological diagnositic characters and genetic markers. *Systematic Parasitology* **45**, 185–197.

PALM, H. W. (1999). Ecology of *Pseudoterranova decipiens* (Krabbe, 1878) (Nematoda: Anisakidae) from Antarctic waters. *Parasitology Research* **85**, 638–646.

PALM, H. W., ANDERSEN, K., KLÖSER, H. & PLÖTZ, J. (1994). Occurrence of *Pseudoterranova decipiens* (Nematoda) in fish from the southeastern Weddell Sea (Antarctic). *Polar Biology* **14**, 539–544.

PALM, H. W., REIMANN, N., SPINDLER, M. & PLÖTZ, J. (1998). The role of the rock cod *Notothenia coriiceps* Richardson, 1844 in the life cycle of Antarctic parasites. *Polar Biology* **19**, 399–406.

PINEL, C., BEAUDEVIN, M., CHERMETTE, R., GRILLOT, R. & AMBROISE-THOMAS, P. (1996). Gastric anisakidosis due to *Pseudoterranova decipiens* larva. *Lancet* **347**, 1829.

ROHLWING, T., PALM, H. W. & ROSENTHAL, H. (1998). Parasitation with *Pseudoterranova decipiens* (Nematoda) influences the survival rate of the

European smelt *Osmerus eperlanus* retained by a screen wall of a nuclear power plant. *Diseases of Aquatic Organisms* **32**, 233–236.

SAIKI, R. K., GELFAND, D. H., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B. & ERLICH, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

SNEATH, P. H. A. & SOKAL, R. R. (1973). *Numerical Taxonomy: The Principles and Practice of Numerical Classification*. W. H. Freeman and Co., San Francisco.

TAKAHASHI, S. (1998). Anisakidosis: global point of view. In *Host Response to International Parasitic Zoonoses* (ed. Ishikura, H., Aikawa, M., Itakura, H. & Kikuchi, K.), pp. 109–120. Springer-Verlag, Tokyo.

ZHU, X. Q. & GASSER, R. B. (1998). Single-strand conformation polymorphism (SSCP)-based mutation scanning approaches to fingerprint sequence variation in ribosomal DNA of ascaridoid nematodes. *Electrophoresis* **19**, 1366–1373.

ZHU, X. Q., JACOBS, D. E., CHILTON, N. B., SANI, R. A., CHENG, N. A. B. Y. & GASSER, R. B. (1998*a*). Molecular characterization of a *Toxocara* variant from cats in Kuala Lumpur, Malaysia. *Parasitology* **117**, 155–164.

ZHU, X. Q., CHILTON, N. B. & GASSER, R. B. (1998*b*). Detection of sequence variation in parasite ribosomal DNA by electrophoresis in agarose gels supplemented with a DNA-intercalating agent. *Electrophoresis* **19**, 671–674.

ZHU, X. Q., GASSER, R. B., PODOLSKA, M. & CHILTON, N. B. (1998*c*). Characterisation of anisakid nematodes with zoonotic potential by nuclear ribosomal DNA sequences. *International Journal for Parasitology* **28**, 1911–1921.

ZHU, X. Q., CHILTON, N. B., JACOBS, D. E., BOES, J. & GASSER, R. B. (1999). Characterisation of *Ascaris* from human and pig hosts by nuclear ribosomal DNA sequences. *International Journal for Parasitology* **29**, 469–478.

ZHU, X. Q., GASSER, R. B., JACOBS, D. E., HUNG, G.-C. & CHILTON, N. B. (2000*a*). Relationships among some ascaridoid nematodes based on ribosomal DNA sequence data. *Parasitology Research* **86**, 738–744.

ZHU, X. Q., D'AMELIO, S., PAGGI, L. & GASSER, R. B. (2000*b*). Assessing sequence variation in the internal transcribed spacers of ribosomal DNA within and among members of the *Contracaecum osculatum* complex (Nematoda: Ascaridoidea: Anisakidae). *Parasitology Research* **86**, 677–683.

ZHU, X. Q., D'AMELIO, S., HU, M., PAGGI, L. & GASSER, R. B. (2001*a*). Electrophoretic detection of population variation within *Contracaecum ogmorhini* (Nematoda: Ascaridoidea: Anisakidae). *Electrophoresis* **22**, 1930–1934.

ZHU, X. Q., GASSER, R. B., CHILTON, N. B. & JACOBS, D. E. (2001*b*). Molecular approaches for studying ascaridoid nematodes with zoonotic potential, with an emphasis on *Toxocara* species. *Journal of Helminthology* **75**, 101–108.