Single-strand conformation polymorphism-based analysis reveals genetic variation within *Spirometra erinacei* (Cestoda: Pseudophyllidea) from Australia

X. Q. Zhu, I. Beveridge, L. Berger, D. Barton and R. B. Gasser

Department of Veterinary Science, The University of Melbourne, 250 Princes Highway, Werribee, Victoria, Australia, CSIRO Australian Animal Health Laboratory, Geelong, Victoria, Australia, School of Tropical Biology, James Cook University, Townsville, Queensland, Australia

(Received 1 October 2001, Accepted for publication 14 January 2002)

This study examined genetic variability within *Spirometra erinacei* (Cestoda: Pseudophyllidea) from different host species and geographical origins in Australia using a polymerase chain reaction (PCR)-based mutation detection approach, followed by DNA sequencing. Part of the cytochrome c oxidase subunit 1 gene (*pcox*1) was amplified by PCR, scanned for sequence variation by single-strand conformation polymorphism (SSCP), and representative samples from different host species were selected for DNA sequencing. While no variation in SSCP profiles was detected among *S. erinacei* samples from dog, fox, cat, tiger snake and python, they differed in profile from 5 specimens from the green tree frog (*Litoria caerulea*). This was supported by sequence data which demonstrated that *pcox*1 sequences of samples from the latter host species differed at 8 of 393 (2%) nucleotide positions from those from the non-amphibian host. Using a nucleotide difference in the *pcox*1 sequence, a PCR-linked restriction fragment length polymorphism (RFLP) could be employed to unequivocally delineate between samples from non-amphibian and amphibian hosts. These findings demonstrate the existence of at least two genotypes within *S. erinacei*, which may have important implications for studying the epidemiology, ecology and systematics of this cestode.

© 2002 Elsevier Science Ltd

**KEYWORDS:** cytochrome c oxidase subunit 1 gene, PCR, restriction fragment length polymorphism (RFLP), single-strand conformation polymorphism (SSCP), *Spirometra erinacei*.

**INTRODUCTION**

*Spirometra erinacei* (Cestoda: Pseudophyllidea), also known as *Spirometra erinaceieuropaei*, occurs as an adult tapeworm in the small intestine of canid and felid definitive hosts and infects intermediate hosts where it develops as a procercoid (in the invertebrate) or plerocercoid (in the vertebrate) larval stage in tissues. The plerocercoid stage in intermediate or paratenic hosts causes sparganosis. Humans may become infected by ingesting uncooked mammalian, reptilian or amphibian flesh or by the practice of placing frog poultices on open lesions or inflamed areas. When the plerocercoid infects humans, it can migrate to and proliferate in a range of tissues. In some cases, plerocercoids can localize in vital organs, such as brain or spinal cord, and cause serious clinical disease. Proliferative sparganosis has also been reported from a range of carnivorous mammal species.
Table 1. DNA samples used in this study. Each sample represents an individual adult or multiple plerocercoids of *Spirometra erinacei*

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Life cycle stage</th>
<th>Host</th>
<th>Geographical origin in Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1p</td>
<td>Plerocercoid</td>
<td>Dog</td>
<td>Melbourne, Victoria</td>
</tr>
<tr>
<td>S5p</td>
<td>Plerocercoid</td>
<td>Dog</td>
<td>Wodonga, Victoria</td>
</tr>
<tr>
<td>Se3</td>
<td>Adult</td>
<td>Fox</td>
<td>Victoria</td>
</tr>
<tr>
<td>Se4</td>
<td>Adult</td>
<td>Fox</td>
<td>Victoria</td>
</tr>
<tr>
<td>Se6</td>
<td>Adult</td>
<td>Fox</td>
<td>Victoria</td>
</tr>
<tr>
<td>S8a</td>
<td>Adult</td>
<td>Fox</td>
<td>Victoria</td>
</tr>
<tr>
<td>S8b</td>
<td>Adult</td>
<td>Fox</td>
<td>Victoria</td>
</tr>
<tr>
<td>S9</td>
<td>Adult</td>
<td>Cat</td>
<td>Werribee, Victoria</td>
</tr>
<tr>
<td>S20</td>
<td>Plerocercoid</td>
<td>Tiger snake (<em>Notechis scutatus</em>)</td>
<td>Melbourne, Victoria</td>
</tr>
<tr>
<td>S21</td>
<td>Plerocercoid</td>
<td>Python (<em>Antaresia maculosus</em>)</td>
<td>Townsville, Queensland</td>
</tr>
<tr>
<td>S4</td>
<td>Plerocercoid</td>
<td>Green tree frog (<em>Litoria caerulea</em>)</td>
<td>Emerald Beach, New South Wales</td>
</tr>
<tr>
<td>S10</td>
<td>Plerocercoid</td>
<td>Green tree frog (<em>Litoria caerulea</em>)</td>
<td>Emerald Beach, New South Wales</td>
</tr>
<tr>
<td>S12</td>
<td>Plerocercoid</td>
<td>Green tree frog (<em>Litoria caerulea</em>)</td>
<td>Coffs Harbour, New South Wales</td>
</tr>
<tr>
<td>S13</td>
<td>Plerocercoid</td>
<td>Green tree frog (<em>Litoria caerulea</em>)</td>
<td>Coffs Harbour, New South Wales</td>
</tr>
<tr>
<td>S15</td>
<td>Plerocercoid</td>
<td>Green tree frog (<em>Litoria caerulea</em>)</td>
<td>Queensland</td>
</tr>
</tbody>
</table>

*Cases of proliferative sparganosis.*

The precise identification and characterization of cestodes is important in studying their epidemiology as well as in controlling the diseases they cause. Traditionally, cestodes have been identified using a combination of ecological, biological and/or morphological criteria, but these can have limitations. This is also the case for members of the genus *Spirometra*. A number of species of *Spirometra* have been described based on the morphology of the adult stage, but the identity of some of them is controversial because the morphological characters used to separate them have been found to be variable, and some species have been recognized as synonyms of previously described species.

DNA techniques provide useful complementary tools for the identification and genetic characterization of cestodes. In particular, polymerase chain reaction (PCR)-linked mitochondrial DNA (mtDNA) sequencing has been successful for analyzing genetic variation within and among cestode populations and for studying the genetic relationships of taeniid cestodes of human and animal health importance. For instance, using cytochrome c oxidase subunit 1 gene (*cox1*) and subsequently NADH dehydrogenase subunit 1 gene (*nad1*) sequence data, Bowles *et al.* defined distinct genotypes within the hydatid tapeworm, *Echinococcus*, and analyzed genetic relationships among species and genotypes of this genus. More recently, mutation scanning approaches such as single-strand conformation polymorphism (SSCP) have been employed to genotype *Echinococcus* and other members of the cestode family Taeniidae.

Currently, *S. erinacei* is the only species of *Spirometra* recorded in Australia. As is the case with *Echinococcus*, genetic variation may exist within *S. erinacei*, given its broad host range, wide geographical distribution and biological variability (Stephanson, J. M. 1985. Biology and immunobiology of *Spirometra* in Western Australia, PhD thesis, Murdoch University), but to date, this has not been investigated using molecular approaches. The aims of this study were to characterize part of the *cox1* sequence for *S. erinacei* from different host species and geographical locations in Australia, including material from clinical cases of canine proliferative sparganosis, and to assess the extent of sequence variability within and among individual isolates using a mutation scanning approach combined with DNA sequencing.

**MATERIALS AND METHODS**

**Parasites and DNA isolation**

Specimens of *S. erinacei* (larval or adult stages) were collected from various definitive, intermediate or paratenic hosts from different geographical origins in Australia (Table 1). They were identified based on their host and tissue origins, and morphological features, washed extensively in physiological saline and frozen (−70°C) or fixed in 50% ethanol. Genomic DNA was isolated from individual adults or multiple plerocercoids by sodium dodecyl-sulphate/proteinase K treatment, column-purified (Wizard™ DNA Clean-Up, Promega) and eluted into 40 µl H₂O. DNA from dog, frog, snake or cat muscle, or human blood was also prepared using the same method.
Enzymatic amplification

The PCR\textsuperscript{28} was used to amplify a ~450 bp portion (pcox1) of the cox1 from 10–20 ng of genomic DNA with primers JB3 (5’-TTTTTTGGGATCTGTAGGTTTAT-3’) and JB4-Le (5’-TAAAGAAAGACATAATTGAAAAATG-3’).\textsuperscript{17} Reactions (50 µl) in 10 mM Tris-HCl (pH 8-4), 50 mM KCl, 3·5 mM MgCl\textsubscript{2}, 250 µM of each dNTP, 50 pmol of each primer and 2 U Taq poly-
merase (Promega) were performed in a thermocycler (Perkin Elmer Cetus) using the following cycling conditions: an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing) and 72°C for 30 s (extension), followed by a final extension at 72°C for 5 min. Host DNA samples and samples without DNA were included as controls. An aliquot (4 µl) of each PCR product was subjected to agarose gel electrophoresis, carried out as described previously.\textsuperscript{28}

Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis of the pcox1 amplicons was carried out using an optimized protocol.\textsuperscript{28} In brief, 10 µl of each PCR product were mixed with an equal amount 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), 4 µl of individual samples were loaded into the wells of a 0·4 mm thick, 0·6 × mutation detection enhancement gel (MDE, FMC Bio-Products) and subjected to electrophoresis in a sequencing rig (Base Runner\textsuperscript{TM}, IBI) at 7 W for 15 h at 20°C. Gels were subsequently dried on to blotting paper and exposed to X-ray film (Curix-Blue, Agfa).

DNA sequencing and analyses

Selected amplicons were purified over spin columns (Wizard\textsuperscript{TM} PCR-Preps, Promega) and subjected to manual sequencing with the fmol\textsuperscript{TM} cycle sequencing kit (Promega) using the same primers (individually) and cycling conditions as for primary amplification. The sequences were aligned manually. Pairwise comparisons were made of the level of sequence differences (D) using the formula \( D = 1 - (M/L) \),\textsuperscript{30} where \( M \) is the number of alignment positions at which two sequences have a base in common, and \( L \) is the total number of alignment positions over which the two sequences are compared. Restriction mapping of the pcox1 sequences for ~40 common restriction endonucleases was conducted using a computer program (MacVector\textsuperscript{TM} 4.1.4, Kodak).

Restriction fragment length polymorphism (RFLP) analysis

Spin column-purified pcox1 amplicons (10 µl) were digested with 10 units (1 µl) of the restriction endonuclease Nla III (Promega) at 37°C for 1 h in a volume of 20 µl, according to the manufacturer’s recommendations. Digests were resolved in ethidium bromide-stained agarose (2·5% w/v) gels and photographed (667 film, Polaroid). Fragments sizes were estimated using the FX 174-Hae III size marker (Promega).

RESULTS

The pcox1 amplicons were of the expected size (~450 bp), and no size variation was detected on agarose gels among any of the 15 S. erinacei samples from various host species and geographical locations in Australia (Table 1). Given the technical advantages and high mutation detection capacity of SSCP for the analysis of genetic variation, this method was used to scan for sequence variation in the pcox1 within and between samples of S. erinacei. Except for a faint band of unique position for the dog DNA sample, no band was detected for any other host- and no-DNA control samples by SSCP (Fig. 1). No variation in SSCP profiles was detected among 10 samples (representing different developmental stages) from canids, cat and snake hosts. While no variation in profiles was detected among the five S. erinacei samples from the green tree frog, Litoria caerulea, there was a distinct difference in SSCP profiles between all S. erinacei samples from this amphibian host and those from non-amphibian hosts (downward shift for the former in Fig. 1).

Of the 15 amplicons examined by SSCP (Fig. 1), 1–2 samples representing each of the host species (samples S1 and S5, Se3 and Se6, S9, S20, S21, S4 and S12, Table 1) were subjected to sequencing. A 393 bp sequence was obtained for each of these samples. The G + C content of this sequence for S. erinacei samples from dog, fox, cat, tiger snake and python was 38·2%, while that of the samples from the green tree frogs was 37·7%. In accordance with SSCP analysis, no nucleotide difference in the pcox1 sequence was detected among S. erinacei samples from canid, felid and snake hosts. Also, samples S4 and S12 from green tree frogs both had the same pcox1 sequence but differed from that of the non-amphibian hosts by 8 of 393 (2%) nucleotides, all of which represented transitions (C<->T, A<->G). Hence, two distinct pcox1 sequence haplotypes, designated SH1 and SH, were detected among the 15 S. erinacei samples. Haplotype SH1 represented S. erinacei samples from dog, fox, cat, tiger snake...
Fig. 1. Single-strand conformation polymorphism analysis of sequence variation in a ~450 bp portion of the cytochrome c oxidase subunit 1 gene (pcox1) among different samples (S1, S5, Se3, Se4, Se6, S8a, S8b, S9, S20, S21, S4, S10, S12, S13 and S15) of *Spirometra erinacei* from different hosts and geographical locations in Australia (cf. Table 1). Lanes D, F, H, S, C and N represent DNA from dog, frog, human, snake, cat and no-DNA control, respectively.

Fig. 2. Alignment of the pcox1 sequences (393 bp) representing the two distinct genetic variants within *Spirometra erinacei*, designated haplotypes SH1 and SH2. Haplotype SH1 represents *Spirometra erinacei* samples from dog, fox, cat, tiger snake and python, while SH2 represents those from the green tree frog (cf. Table 1 and Fig. 1). Sequences have been deposited in the EMBL, GenBank™ and DDJB databases under the accession numbers AJ308257–AJ308265. Nucleotide differences between the two haplotypes are indicated by asterisks. Underlined are the locations of the NlaIII restriction sites.
Genetic variation within *Spirometra erinacei*

**Fig. 3.** Differentiation of *Spirometra erinacei* samples representing haplotype SH1 (samples S1, S5, Se3, Se4, Se6, S8a, S8b, S9, S20 and S21) from those representing haplotype SH2 (samples S4, S10, S12, S13 and S15) by restriction fragment length polymorphism analysis of a ~450 bp portion of the cytochrome c oxidase subunit 1 gene (pcox1) using endonuclease NlaIII. M represents the FX174-HaeIII size marker (bp).

and python, while SH2 represented those from green tree frogs.

The T at alignment position 192 (Fig. 2), underlined in the pcox1 sequence of *S. erinacei* samples from green tree frogs resulted in the loss of a restriction site for endonuclease Nla III. Hence, PCR-linked restriction fragment length polymorphism (RFLP) could be used for the delineation of the samples representing the two pcox1 haplotypes (Fig. 3). Three bands of ~180 bp, 110 bp and 50 bp were detected for samples from non-amphibian hosts, whereas three bands of ~240 bp, 110 bp and 50 bp were displayed for samples from the amphibian hosts. Based on sequence data, co-migrating fragments of ~50 bp were predicted for samples from both non-amphibian and amphibian hosts. The sizes of the digestion fragments were in accordance with calculations based on the restriction maps (not shown).

**DISCUSSION**

In this study, two pcox1 sequence haplotypes were detected among a range of *S. erinacei* samples examined. Haplotype SH1 represented *S. erinacei* from non-amphibian hosts (dog, fox, cat, tiger snake and python), whereas SH2 represented those from the green tree frog. The sequence difference of 2% in the pcox1 between the two haplotypes was within the range (0·3–9·3%) detected among the genotypes of *Echinococcus granulosus*, a cyclophyllidean cestode of the family Taeniidae. Together with the absence of nucleotide variation among samples representing each of the two pcox1 haplotypes, these results support the existence of at least two populations within *S. erinacei* in Australia. The ability to detect such genetic variation using PCR-based SSCP analysis is likely to have important implications for studying the taxonomy, population genetics, biology and epidemiology of *Spirometra* (considered to represent *S. erinacei*), which would underpin specific diagnosis of sparganosis and its control.

A number of species of *Spirometra*, described based on morphological features of the adult stage, are of uncertain taxonomic status because of considerable variability in these features. The only species which can be defined reliably is *S. mansonoides*, in which the C-shaped outer loop of the uterus with its anterior limb constricted in the midline to form a lateral expulsion chamber, is a constant feature. In earlier descriptions, emphasis was placed on geographical and host origins as well as definitive and intermediate host uniqueness, but these are probably not valid since different species of *Spirometra* can be found in the same geographical regions. While the adults of *Spirometra* are relatively host specific, plerocercoids are known to occur in a broad range of vertebrate paratenic hosts. Therefore, the use of a molecular approach for the genetic characterization of *S. erinacei* isolates from a wide range of host species and geographical locations should enable specific identification, which would provide a foundation for population genetic, epidemiological and biological investigations.

Biological variation within *S. erinacei* has been documented by a number of authors. For instance, Odening and Bockhardt identified two different biological forms of *Spirometra* (both corresponding morphologically with *S. erinaceieuropaei*), based on the presence or absence of a specific effect of the sparganum growth factor (SGF) on the growth of hypophysectomized male rats. While implantation with *S. erinaceieuropaei* plerocercoids from Bulgaria induced a positive effect on the growth in male rats, typical of the *S. mansonoides* SGF, this was not the case for *S. erinaceieuropaei* plerocercoids from Poland, Thailand or Burma. Also, significant morphological variation has been detected both
within and among isolates of *S. erinacei* from different hosts in Australia (Stephanson, J. M. 1985. Biology and immunobiology of *Spirometra* in Western Australia, PhD thesis, Murdoch University). It is therefore possible that the difference in the ability of plerocercoids to produce SGF, and possibly other biological characteristics (e.g., life cycles and host preference) relate to haplotypic variability in the cox1 gene.

The analysis of genotypic variation within *S. erinacei* should also provide insights into the transmission patterns of sparganosis and may establish whether particular genotypes relate to human infection. For instance, the occurrence of human sparganosis in parts of southeast Asia has been attributed to the practice of eating frog meat and/or applying raw frog flesh as a poultice to skin lesions. In a recent study in Taiwan, Ooi et al. (1998) reported a high (32%) prevalence of *S. erinacei* (referred to by the authors as *S. erinaceieurupaei*) in the frog species, *Rana limnocharis*, suggesting the potential for this parasite to infect humans. Genetic comparison of plerocercoid isolates from humans in Taiwan (and other countries) with adult and larval stages from vertebrate and invertebrate (intermediate or paratenic) hosts, respectively, may identify which and how many genotypes relate to human infection(s) and which reservoir hosts pose a public health threat. The molecular tools established herein could be used effectively to address such epidemiological questions and elucidate whether multiple different transmission cycles exist.

The definition of genetic markers in the cox1 of *S. erinacei* also has diagnostic implications. Traditionally, diagnosis of human sparganosis has relied solely on the microscopic detection of plerocercoid stages isolated from body cavities or tissues, but this is seriously compromised by the considerable variability in the parasite’s morphology at this developmental stage. Although immunoassays, ultrasonography, computed tomography and magnetic resonance imaging have been evaluated as alternative tools for pre-operative diagnosis, there are significant problems with their diagnostic specificity or sensitivity. Such services are also costly to provide or inaccessible to patients in some countries. Hence, the present PCR-based RFLP and SSCP assays utilizing cox1 markers should have considerable advantages over previous methods (i.e., increased specificity, sensitivity and relatively labour and time effective) for the specific identification of plerocercoids (or their DNA) obtained by pre-operative paracentesis or from biopsy material aspirated during exploratory surgery.

ACKNOWLEDGEMENTS

Project support to RBG was provided by the Australian Companion Animal Health Foundation and the Australian Research Council (ARC).

REFERENCES

Genetic variation within *Spirometra erinacei* 165


