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ORIGINAL PAPER

Characterization of *Fasciola* samples from different host species and geographical localities in Spain by sequences of internal transcribed spacers of rDNA

S. Alasaad · C. Q. Huang · Q. Y. Li · J. E. Granados · C. García-Romero · J. M. Pérez · X. Q. Zhu

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Abstract In the present study, 25 samples representing *Fasciola* (Platyhelminthes: Trematoda: Digenea) from nine host species and 19 geographical locations in Spain were characterized genetically by sequences of the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA). The ITS rDNA was amplified from individual liver flukes by polymerase chain reaction (PCR), and the amplicons were sequenced directly. The lengths of the ITS-1 and ITS-2 sequences were 422 and 362 bp, respectively, for all Spanish liver fluke samples sequenced. Comparison of the ITS sequences of the Spanish *Fasciola* samples examined in the present study with that of *Fasciola hepatica*, *Fasciola gigantica* and the "intermediate *Fasciola*" revealed that all Spanish *Fasciola* samples examined represent the single species of *F. hepatica*, with only slight sequence variation in the ITS-2 (1/362, 0.3%) among the sequenced samples, but the sequence variation was not related to particular host species and/or geographical origins of the samples. The Spanish *F. hepatica* examined differed from *Fasciola* from elsewhere by two nucleotides in the ITS-2, which provided genetic marker for the differentiation of Spanish *F. hepatica* from *Fasciola* from other geographical localities. These results have implications for studying the population genetic structure of the Spanish *F. hepatica* and for the diagnosis and control of the disease it causes.

S. Alasaad · J. M. Pérez
Departamento de Biología Animal, Biología Vegetal y Ecología,
Universidad de Jaén,
Campus Las Lagunillas, s. n.,
E-23071 Jaén, Spain

S. Alasaad · C. Q. Huang · Q. Y. Li · X. Q. Zhu (✉)
College of Veterinary Medicine,
South China Agricultural University,
483 Wushan Street, Tianhe District,
Guangzhou, Guangdong Province 510642,
People's Republic of China
e-mail: xingquanzh@scau.edu.cn

J. E. Granados
Espacio Natural de Sierra Nevada,
Carretera Antigua de Sierra Nevada,
Km 7.5,
E-18071 Pinos Genil, Granada, Spain

C. García-Romero
Laboratorio de Parasitología Animal, Servicio de Investigación Agraria (SIA), Consejería de Agricultura,
C/ Pintor Matías Moreno,
E-45002 Toledo, Spain

Introduction

Digenean trematodes of the genus *Fasciola* (Platyhelminthes: Trematoda: Digenea) are the common liver flukes of a range of animals with a global geographical distribution (Spithill and Dalton 1998). Fasciolosis caused by *Fasciola* spp. is a significant animal health problem, which causes substantial economic losses estimated at US$2 billion per annum worldwide (Spithill and Dalton 1998). Human infection with *Fasciola* spp. has been reported in a number of countries, millions of people are estimated to be infected, and hundreds of millions of people are at risk throughout the world (Mas-Coma et al. 1999, 2005; Haseeb et al. 2002; Ishii et al. 2002).

Several species have been described within the genus *Fasciola*, but only two species, *Fasciola hepatica* and *Fasciola gigantica*, are commonly recognized as taxonomically valid occurring in animals and humans (Yamaguti 1958; Mas-Coma et al. 2005), with *F. hepatica* mainly occurring in temperate areas, *F. gigantica* in tropical zones,

and both overlapping in subtropical areas (Krämer and Schnieder 1998; Mas-Coma et al. 2005). In addition to *F. hepatica* and *F. gigantica*, several recent studies using the first and/or second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) as genetic markers have identified a so-called "intermediate *Fasciola*" between *F. hepatica* and *F. gigantica* from Japan, Korea, and China (Itagaki and Tsutsumi 1998; Agatsuma et al. 2000; Huang et al. 2004; Itagaki et al. 2005a, b; Lin et al. 2007).

In the Iberian Peninsula, *F. hepatica* has been found parasitizing domestic animals and wild animals, including European rabbit (*Oryctolagus cuniculus*), hare (*Lepus granatensis*), donkey (*Equus asinus*), horse (*Equus caballus*), pig and wild boar (*Sus scrofa*), fallow deer (*Dama dama*), cattle (*Bos taurus*), domestic sheep (*Ovis aries*), goat (*Capra hircus*), Iberian ibex (*Capra pyrenaica*), and man (Cordero del Campillo et al. 1994; Lavín et al. 1995; Pérez et al. 2006). In addition to *F. hepatica*, *F. gigantica* was reported to infect sheep in central Spain and Portugal (Cordero del Campillo et al. 1994).

However, before the present study, there had been no reports characterizing *Fasciola* from Spain using well-defined DNA sequences. Therefore, the objective of the present study was to characterize *Fasciola* samples from Spain from different host animals and geographical localities by sequences of the ITS-1 and ITS-2 rDNA because these sequences have been shown to provide specific markers for the identification of *F. hepatica*, *F. gigantica* and the "intermediate *Fasciola*".

Materials and methods

Parasites

Adult trematodes were collected from the livers of infected hosts (all of them were local animals) at necropsy from nine host animal species and 19 geographical locations in Spain between 2002 and 2006. Individual worms were washed extensively in physiological saline, identified morphologically as *Fasciola* according to existing keys and descriptions (Yamaguti 1958), and fixed in 70% ethanol until extraction of genomic DNA. Their codes, host species, and geographical origins are listed in Table 1 and Fig. 1.

Extraction and purification of genomic DNA

Genomic DNA was extracted from a portion of individual adult trematodes by treatment with sodium dodecyl sulphate/proteinase K (Zhu et al. 2002), column-purified

Table 1 Geographical locations in Spain and host origins of *Fasciola* samples used in this study

Sample codes	Host species and codes		Geographical locations and codes	
FhCp1	Iberian ibex (*Capra pyrenaica*)	Cp	Granada (Sierra Nevada)	1
FhRp2	Chamois (*Rupicapra pyrenaica*)	Rp	Lérida	2
FhCc2	Roe deer (*Capreolus capreolus*)	Cc	Lérida	2
FhDd2	Fallow deer (*Dama dama*)	Dd	Lérida	2
FhDd3	Fallow deer (*Dama dama*)	Dd	Cuenca	3
FhCe3	Red deer (*Cervus elaphus*)	Ce	Cuenca	3
FhCe4	Red deer (*Cervus elaphus*)	Ce	Andorra	4
FhOa3	European mouflon (*Ovis aries*)	Oa	Cuenca	3
FhEc5	Horse (*Equus caballus*)	Ec	Valencia	5
FhOa6	Ovine (*Ovis aries*)	Oa	Bilbao	6
FhBt6	Bovine (*Bos taurus*)	Bt	Bilbao	6
FhBt5	Bovine (*Bos taurus*)	Bt	Valencia	5
FhBt7	Bovine (*Bos taurus*)	Bt	Barcelona	7
FhBt8	Bovine (*Bos taurus*)	Bt	Mallorca (Balearic Islands)	8
FhBt9	Bovine (*Bos taurus*)	Bt	Tenerife (Canary Islands)	9
FhBt10	Bovine (*Bos taurus*)	Bt	Ávila	10
FhBt11	Bovine (*Bos taurus*)	Bt	Lugo	11
FhBt12	Bovine (*Bos taurus*)	Bt	Cádiz	12
FhBt13	Bovine (*Bos taurus*)	Bt	Oviedo	13
FhBt14	Bovine (*Bos taurus*)	Bt	Castellón	14
FhBt15	Bovine (*Bos taurus*)	Bt	Segovia	15
FhBt16	Bovine (*Bos taurus*)	Bt	Logroño	16
FhBt17	Bovine (*Bos taurus*)	Bt	Pamplona	17
FhBt18	Bovine (*Bos taurus*)	Bt	La Rioja	18
FhBt19	Bovine (*Bos taurus*)	Bt	Valladolid	19

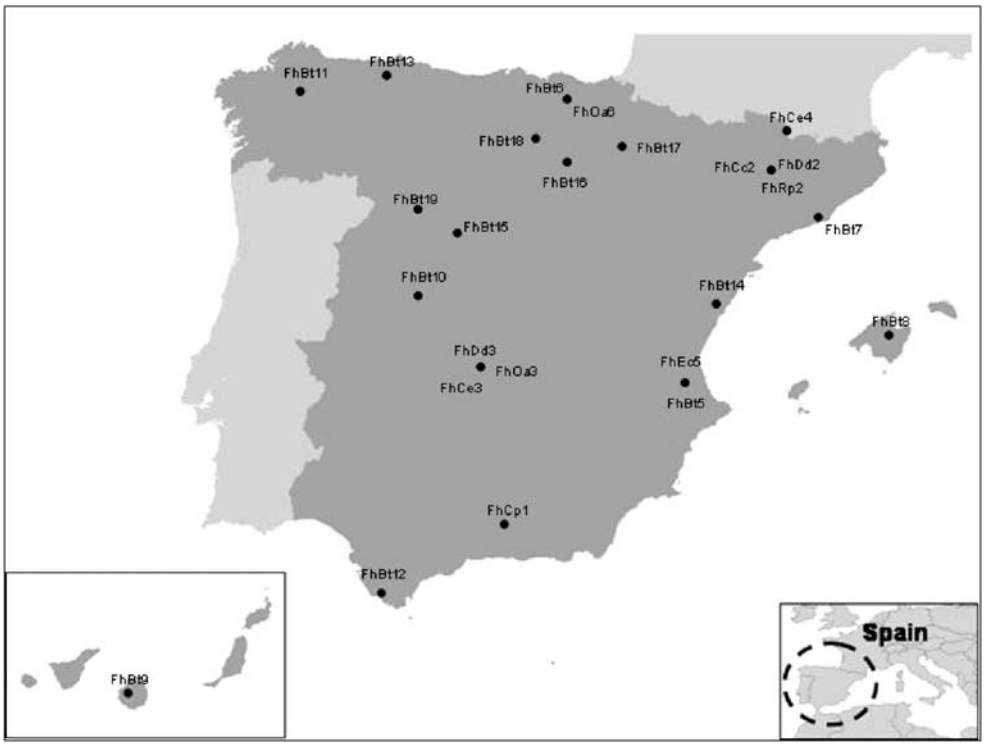

Fig. 1 Spain map showing approximate sites for sample collection. Codes next to the sites represent the sample codes in Table 1

using Wizard™ DNA clean-up system (Promega) and then eluted into 65 μl H_2O according to the manufacturer's recommendations. DNA was also isolated from livers from cattle, buffalo or goat using the same method as for trematode samples. All the DNA samples were stored at −20°C until further use.

Enzymatic amplification of the ITS-1, 5.8S rDNA, and ITS-2

The DNA region comprising ITS-1, 5.8S rDNA, and ITS-2 plus primer flanking sequences (ITS+) was amplified by polymerase chain reaction (PCR) from trematode DNA using primers BD1 (forward; 5′-GTCGTAACAAGG TTTCCGTA-3′) and BD2 (reverse; 5′-TATGCTTAAA TTCAGCGGGT-3′; Luton et al. 1992). PCR reactions (25 μl) were performed in 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 200 μM each of dNTP, 50 pmol of each primer and 0.25 U *Taq* polymerase (TaKaRa) in a thermocycler (Biometra) under the following conditions: 95°C for 5 min (initial denaturation) followed by 30 cycles of 95°C, 30 s (denaturation), 55°C, 30 s (annealing), 72°C, 30 s (extension), and a final extension of 72°C for 7 min. One microliter of genomic DNA was added to each PCR reaction. Samples with host DNA or without genomic DNA were included in each amplification run as "negative" controls. An aliquot (5 μl) of each amplicon was examined on 1% agarose-TBE (65 mM Tris–HCl, 22.5 mM boric acid, and 1.25 mM ethylenediamine tetraacetic acid, pH 9.0) gels, stained with ethidium bromide, and photographed using a gel documentation system (UVITEC). The DNA size marker DL2000 was used to estimate the length of the ITS+ amplicons.

Sequencing of the ITS-1, 5.8S and ITS-2 rDNA and analysis

ITS+ products of 25 samples were sent to BioSune Biotechnology Company for sequencing using ABI 377 automated DNA sequencer (BioDye Terminator Chemistry)

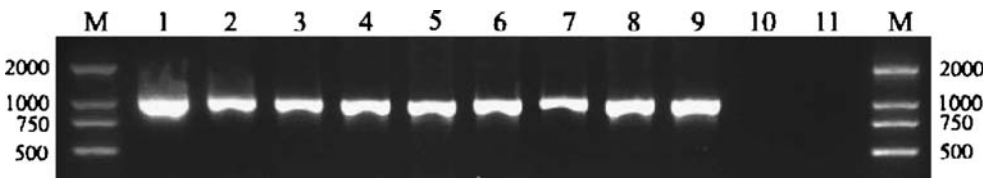

Fig. 2 Agarose gel electrophoresis of ITS+ PCR products of representative *Fasciola* samples from Spain. Lanes *1–9* represent *Fasciola* samples from Iberian ibex, Chamois, Roe deer, Fallow deer, Red deer, European mouflon, Horse, Ovine, and Bovine, respectively (cf. Table 1). Lanes *10* and *11* represent host (bovine) and no DNA control, respectively. *M* represents a DNA size marker (ordinate values in bp)

from both directions using the same primers as used in primary amplification. The 5′ and 3′ ends of the *Fasciola* ITS-1, 5.8S and ITS-2 sequences were determined by comparison with previously published *Fasciola* ITS-1, 5.8S and ITS-2 sequences (Itagaki and Tsutsumi 1998; Agatsuma et al. 2000; Huang et al. 2004; Itagaki et al. 2005a, b; Lin et al. 2007; also see GenBank™ accession numbers AJ557567, AJ557569, AJ557570, AJ557571, AJ628430, AJ628431, and AJ628043). The sequences were aligned and compared among themselves and with those of *F. hepatica*, *F. gigantica* and the "intermediate *Fasciola*". Pairwise comparisons were made of the level of sequence differences (*D*) using the formula $D=1-(M/L$; Chilton et al. 1995), where *M* is the number of alignment positions at which the two sequences have a base in common and *L* is the total number of alignment positions over which the two sequences are compared.

Results and discussion

Genomic DNA was isolated from 25 individuals of *Fasciola* representing nine different host species and 19 geographical locations in Spain (Table 1, Fig. 1). The ITS+ fragment amplified from each sample using primers BD1 and BD2 was approximately 1,000 bp in length, and in no case was product amplified from no-DNA sample or host DNA control. Figure 2 shows the ITS+ fragment amplified from representative *Fasciola* samples.

Then, the 25 ITS+ PCR products were subjected to direct sequencing; 25 sequences of 946 bp were obtained and deposited in the GenBank™ (accession numbers AM709498-AM709500, AM707030, AM709609-AM709622, AM709643-AM709649; also see Table 2). The sequence was composed of the complete ITS-1 sequence of 422 bp, complete 5.8S sequence of 162 bp,

Table 2 Comparison of the ITS-1 and ITS-2 sequences of *Fasciola* from different hosts and geographical locations in Spain with that of *F. hepatica*, *F. gigantica* and the "intermediate *Fasciola*" at variable sequence positions

Samples code	Variable positions in the ITS-1 and ITS-2 sequence													
	17	107	201	279	299	791	815	854	860	868	911	918	925	926
F. gigantica[a]	T	T	T	A	T	C	C	T	T	C	–	A	A	T
Fasciola sp.[b]	C/T	A/T	C/T	T/A	C/T	T	T/C	C/T	C/T	C	T/–	G/A	A	T
F. hepatica[c]	C	A	C	T	C	T	T	C	C	C	T	G	A	T
FhCp1	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhRp2	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhCc2	C	A	C	T	C	T	T	C	C	T	T	G	T	A
FhDd2	C	A	C	T	C	T	T	C	C	T	T	G	T	A
FhDd3	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhCe3	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhCe4	C	A	C	T	C	T	T	C	C	T	T	G	T	A
FhOa3	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhEc5	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhOa6	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt6	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt5	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt7	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt8	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt9	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt10	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt11	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt12	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt13	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt14	C	A	C	T	C	T	T	C	C	T	T	G	T	A
FhBt15	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt16	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt17	C	A	C	T	C	T	T	C	C	T	T	G	T	A
FhBt18	C	A	C	T	C	T	T	C	C	C	T	G	T	A
FhBt19	C	A	C	T	C	T	T	C	C	C	T	G	T	A

[a] GenBank™ accession numbers for ITS-1 and ITS-2 were AJ628043 and AJ557569, respectively
[b] GenBank™ accession numbers for ITS-1 and ITS-2 were AJ628430 and AJ557567, respectively
[c] GenBank™ accession numbers for ITS-1 and ITS-2 were AJ628431 and AJ557571, respectively

and complete ITS-2 sequence of 362 bp. While there was no nucleotide variation in the ITS-1 and 5.8S rDNA among the 25 *Fasciola* samples, two different ITS-2 sequences were defined for the examined Spanish *Fasciola* samples, differing at one nucleotide (0.3%, 1/362) in the ITS-2 (sequence position 868, Table 2), indicating the existence of two genotypes among the examined Spanish *Fasciola* samples. However, it appears that this sequence variation was not related to particular host species and/or geographical origins of the samples because it occurred in the examined *Fasciola* samples from different host species and geographical locations in Spain (Tables 1 and 2).

Comparison of the ITS sequences of the Spanish *Fasciola* samples examined in the present study with those of *F. hepatica*, *F. gigantica* and the "intermediate *Fasciola*" revealed that all Spanish *Fasciola* samples examined represent the single species of *F. hepatica* (see Table 2). It is interesting to find that the examined Spanish *F. hepatica* differed from *Fasciola* from elsewhere by two nucleotides in the ITS-2 (sequence positions 925 and 926, Table 2), which provided genetic marker for the differentiation of Spanish *F. hepatica* from *Fasciola* from other geographical localities.

In the present study, 25 *Fasciola* samples from nine host species and 19 geographical locations in Spain were sequenced, and no sequence variation was detected either in their ITS-1 or 5.8S rDNA, while their ITS-2 sequences differed only by one nucleotide (0.3%). The explanation for such a low level of intraspecific variation seems to be that repeated DNA sequences, such as rDNA, have been subject to concerted evolution, which tends to homogenize sequences among individuals and among populations (Dowling et al. 1990). This tendency increases the discriminating power of repeated sequences at the species level by reducing the incidence of intraspecific sequence divergence.

In conclusion, the present study demonstrated that the 25 liver fluke samples from nine host species and 19 geographical locations in Spain represented the single species of *F. hepatica*, and two different ITS-2 sequence types were defined and that *F. hepatica* from Spain differed from *Fasciola* from elsewhere by the two nucleotides in the ITS-2. Further studies using more variable genetic markers, such as the sequence-related amplification polymorphism (Li and Quiros 2001), are warranted to examine the genetic variability and population genetic structure within *F. hepatica* from different hosts and geographical locations in Spain.

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References

Agatsuma T, Arakawa Y, Iwagami M, Honzako Y, Cahyaningsih U, Kang SY, Hong SJ (2000) Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. Parasitol Int 49:231–238

Chilton NB, Gasser RB, Beveridge I (1995) Differences in a ribosomal DNA sequence of morphologically indistinguishable species within the *Hypodontus macropi* complex (Nematoda: Strongyloidea). Int J Parasitol 25:647–651

Cordero del Campillo M, Castañón-Ordóñez L, Reguera-Feo A (1994) Índice-catálogo de zooparásitos ibéricos, 2nd edn. Universidad de León, León

Dowling TE, Moritz C, Palmer JD (1990) Nucleic acids II: restriction site analysis. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer, Massachusetts, pp 250–317

Haseeb AN, el-Shazly AM, Arafa MA, Morsy AT (2002) A review on fascioliasis in Egypt. J Egypt Soc Parasitol 32:317–354

Huang WY, He B, Wang CR, Zhu XQ (2004) Characterisation of *Fasciola* species from Mainland China by ITS-2 ribosomal DNA sequence. Vet Parasitol 120:75–83

Ishii Y, Nakamura-Uchiyama F, Nawa Y (2002) A praziquantel-ineffective fascioliasis case successfully treated with triclabendazole. Parasitol Int 51:205–209

Itagaki T, Tsutsumi K (1998) Triploid form of *Fasciola* in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA. Int J Parasitol 28:777–781

Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, Fukuda K (2005a) Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. Parasitology 131:679–685

Itagaki T, Kikawa M, Terasaki K, Shibahara T, Fukuda K (2005b) Molecular characterization of parthenogenic *Fasciola* sp. in Korea on the basis of DNA sequences of ribosomal ITS1 and mitochondrial NDI gene. J Vet Med Sci 67:1115–1118

Krämer F, Schnieder T (1998) Sequence heterogeneity in a repetitive DNA element of *Fasciola*. Int J Parasitol 28:1923–1929

Lavín S, Ruiz-Bascarán M, Marco I, Viñas L (1995) Ecopatología del gamo (*Dama dama*) en el Principado de Asturias. Vet Castilla León 9:14–18

Li G, Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theor Appl Genet 103:455–461

Lin RQ, Dong SJ, Nie K, Wang CR, Li AX, Song HQ, Huang WY, Zhu XQ (2007) Sequence analysis of the first internal transcribed spacer of rDNA supports the existence of an intermediate *Fasciola* between *F. hepatica* and *F. gigantica* in mainland China. Parasitol Res (in press, Mar 15)

Luton K, Walker D, Blair D (1992) Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes

(Platyhelminthes: Trematoda: Digenea). Mol Biochem Parasitol 56:323–327

Mas-Coma MS, Esteban JG, Bargues MD (1999) Epidemiology of human fascioliasis: a review and proposed new classification. Bull World Health Organ 77:340–346

Mas-Coma S, Bargues MD, Valero MA (2005) Fascioliasis and other plant-borne trematode zoonoses. Int J Parasitol 35:1255–1278

Pérez JM, Meneguz PG, Dematteis A, Rossi L, Serrano E (2006) Parasites and conservation biology: the 'ibex-ecosystem'. Biodivers Conserv 15:2033–2047

Spithill TW, Dalton JP (1998) Progress in development of liver fluke vaccines. Parasitol Today 14:224–228

Yamaguti S (1958) Systema Helminthum, Vol. I. The digenetic trematodes of vertebrates. Interscience Publishers, Inc., New York

Zhu XQ, D'Amelio S, Palm HW, Paggi L, George-Nascimento M, Gasser RB (2002) SSCP-based identification of members within the *Pseudoterranova decipiens* complex (Nematoda: Ascaridoidea: Anisakidae) using genetic markers in the internal transcribed spacers of ribosomal DNA. Parasitology 124:615–623