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Taenia spp.: 18S rDNA microsatellites for molecular systematic diagnosis

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Abstract

The 18S rDNA gene of adult worms of *Taenia parva* found in *Genetta genetta* in the Iberian Peninsula and larval stages of *T. pisiformis* from the wild rabbit (*Oryctolagus cuniculus*) in Tenerife (Canary Islands) were amplified and sequenced. The sequences of the 18S rDNA gene of *T. parva* (1768 bp) and *T. pisiformis* (1760 bp) are reported for the first time (GenBank accession nos. AJ555167–AJ555168 and AJ555169–AJ555170, respectively). In 168 alignment positions microsatellites in the 18S rDNA of both taxa were detected for the first time (TGC in *T. parva* and TGCT in *T. pisiformis*) and differences in their sequences with different repetition numbers were observed. The use of nucleotide sequences of this gene in the resolution of systematic problems in cestodes is discussed with reference to the systematic status of *Taenia* spp. and mainly in human taeniids such as *T. solium*, *T. saginata*, and Asian human isolates of *Taenia*.

Introduction

Cestodes of the genus *Taenia* are parasites of mammals. There are more than 35 described species in the genus (Verster, 1969) but the morphological limits between the species are problematic (Hoberg *et al.*, 2000). In spite of the considerable taxonomical work on the genus and systematic revisions (Abuladze, 1964; Verster, 1969; Q1 Rausch, 1994), few data are available on the phylogenetical relationships within *Taenia*. Moore & Brooks (1987) and Hoberg *et al.* (2000) used a variety of morphological characters to infer a comprehensive phylogenetical hypothesis of *Taenia* spp. Okamoto *et al.* (1995) estimated relationships among seven species and variants of taeniids using nucleotide sequences of the cytocrome *c* oxidase subunit I (COI) gene. Queiroz & Alkire (1998) inferred phylogenetical relationships within *Taenia* spp. using COI and 28S large subunit rDNA sequences. COI and 5′28S rDNA were used by Bowles & McManus (1994) and Fan & Chung (1998) to distinguish between *T. saginata* and *T. asiatica*. Variable regions of ribosomal DNA, such as the internal transcribed spacer (ITS) and others, have been used in phylogenetic studies and species identification of *T. crassiceps* and Asian taeniids (Zarlenga *et al.*, 1991; Zarlenga & George, 1995; Eom *et al.*, 2002). The 12S rDNA gene has been used by Nickisch-Rosewegk *et al.* Q2 (Nickisch-Rosenegk, 1999) in 11 *Taenia* species, together with *Echinococcus granulosus* and *E. multilocularis* to test the phylogenetical classification based on the morphology of *Taenia* spp. described by Verster (1969). Gonzalez *et al.* (2002) confirmed inter-species differences based on the sequence of the HDP2DNA fragment, specific for *T. saginata* and *T. solium*, and the sequence of the rDNA internal transcribed spacers 1 and 2.

The 18S rDNA gene has not previously been used in the genetic characterization of taeniids. The nucleotide sequence of this gene can significantly help in the resolution of systematic problems in cestodes (Mariaux, 1998) and no completed sequences of 18S rDNA of any species of *Taenia* is currently available. In the present work, the sequences of 18S rDNA from two species of *Taenia* are presented and compared and the presence of

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microsatellites in these sequences is discussed from a systematic point of view.

Materials and methods

Taenia parva Baer, 1926 is a parasite in adult stage of the common genet, *Genetta genetta* (Carnivora, Viverridae) in Africa and the Iberian Peninsula. The larval stage of *T. pisiformis* (Bloch, 1780) is parasitic in the liver of Lagomorpha, and different species of Canidae act as definitive hosts. These two species of taeniids, *T. parva* and *T. pisisformis*, adult and larvae tapeworms respectively, were collected from the small intestine of *G. genetta* and liver of *Oryctolagus cuniculus* (Lagomorpha: Leporidae). Twelve specimens of *T. parva* were collected from Spain (Montseny, Catalonia, in the north-east of the Iberian Peninsula) and 15 specimens of *T. pisiformis* from Canarian rabbits in Tenerife (Canary Islands).

Genomic DNA was extracted from ten worms of each species. Specimens were homogenized and incubated in 700 μl of extraction buffer (10 mM Tris, 100 mM Na_2EDTA, 100 mM NaCl, pH 8, 0.05%, w/v SDS, 200 μg proteinase K) overnight at 55°C. Samples were then incubated with RNAse-A (100 $\mu g\,ml^{-1}$) for 30 min followed by a phenol/chloroform extraction. DNA was precipitated with ethanol, dried and resuspended in 50 μl TE.

The 18S rDNA gene was totally amplified from genomic DNA by Mariaux (1998) in two overlapping fragments, in 50 μl volumes containing 20 and 50 ng template DNA, 50 mM KCl, 1.5 mM $MgCl_2$ pH 8.3, 10 mM Tris-HCl, dNTP 200 mM, 20 pmol of each primer, and 1 unit of Taq DNA polymerase (Applied Biosystems, New Jersey, USA). The cycling conditions were: an initial denaturing phase of 94°C for 2 min, 35 cycles at 94°C for 30 sec, 55°C for 40 sec, and 72°C for 1.5 min, and a final extension phase at 72°C for 5 min. Polymerase chain reactions (PCR) were performed in a Perkin Elmer GeneAmp PCR System 9600 thermal cycler, using the oligonucleotides 81 + 83 and 82 + 84 designed by Mariaux (1998). Sizes of fragments were fractionated by 1% agarose electrophoresis stained with ethidium bromide and the band was further purified using QIAEX II Gel Extraction kit (150) (Qiagen, Hilden, Germany).

Nucleotide sequences were determined directly from PCR products by automated sequencing using Thermo Sequenase Fluorescent mix (Pharmacia Biotech, Cambridge, UK), according to the instructions of the manufacturer and using an automated sequencer (ALF-Express, Pharmacia). Five internal primers (87–91) [15] were used to sequence the 18S gene in addition to the four PCR primers.

Sequences from the 18S gene were aligned using Clustal X (Thompson *et al.*, 1997) and minor corrections were made by hand. The obtained sequences were used for designing the flanking primer pair microsatellite, using the Gene-Runner (Hastings Software INC) computer programme. The PCR mixture and cycling conditions with microsatellite primer were identical to these used for the first PCR with an annealing temperature of 66°C.

For restriction fragment analysis, 5 μl of PCR products with microsatellite primers were digested in a 10 μl reaction mixture containing 2 U of *SphI* restriction endonuclease and 5 μl of the appropiate restriction buffer at 37°C for 4 h. The digested products were fractionated on a 2% agarose gel, stained with a solution of ethidium bromide and visualized and photographed under an UV transilluminator.

Results and discussion

Sequences from the 18S rDNA of *T. parva* (1768 bp) and *T. pisiformis* larvae (1760 bp) were identified and submitted to GenBank under the accession nos. AJ555167–AJ555168 and AJ555169–AJ555170 respectively. These sequences have not been previously reported. We detected microsatellites in the 18S rDNA gene sequence, in 168 alignment positions in both taxa (fig. 1). Differences in the repetition number and in the sequences of microsatellites between the two species were observed. *Taenia parva* presents eight repetitions of three base pairs (TGC), while the microsatellite in *T. pisiformis* is constituted by seven repetitions of four base pairs (TGCT).

The primer pair flanking microsatellite sequences for PCR reactions were 5′AAGCCATGCATGTCTCAGTTCAG and 5′GCCCTCCAATTGATCCTCGTG, and were designed from regions of 18S rDNA that are highly conserved in a wide range of cestodes between the cyclophyllids deposited in the GenBank database, i.e. *Andrya cuniculi* AJ555162, *Anoplocephaloides dentata* Z98356–Z98358, *Mosgovoyia ctenoides* AJ555164 (Anoplocephalidae), *Choanotaenia infundibulum* AJ555171 (Dilepididae), *Lyruterina nigropunctata* AJ555173 (Paruterinidae), *Raillietina australis* AF286980, *Raillietina micracantha* AJ555178, *Pseudidiogenes nana* AJ555175 (Davaineidae) [Cyclophyllidea]; and between the tetrabothrids, i.e. *Tetrabothrius* sp. AJ555181, *Tetrabothrius* sp. AJ287582, *Tetrabothrius erostris* AJ287581, *Tetrabothrius forsteri* AF124473 (Tetrabothriidae) [Tetrabothriidea].

The PCR products that were amplified with this primer pair contain a highly conserved region and another hypervariable region, both in sequence and length. The microsatellites are located inside this second variable region in the two *Taenia* species and are not present in cestode sequences deposited in the Gen Bank data base.

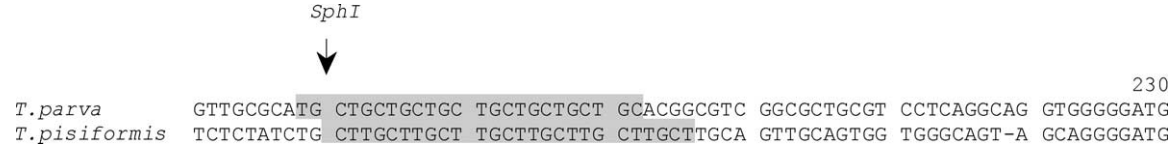

Fig. 1. Fragment of the alignment of 18S rDNA sequences of *Taenia parva* and *T. pisiformis*. The number (230) refers to positions obtained in the alignment made with CLUSTAL X. Shaded regions correspond to the microsatellites.

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For these primers, the PCR products were 640 and 636 bp long for *T. parva* and *T. pisiformis*, respectively. The PCR products were digested with *SphI*. *Taenia parva* generated two visible bands of 170 and 470 bp, and *T. pisiformis* generated one visible band (fig. 2). Differences in the restriction site in this region permit a distinction between the two species with a *SphI* restriction enzyme.

Species of the genus *Taenia* are cestodes with much medical and veterinary significance. In the present study, adult worms in *G. genetta* were compared with larval stages of *T. pisiformis* from the wild rabbit. *Taenia parva* is a typical parasite of *Genetta* spp. in Africa and *G. genetta* in the Iberian Peninsula. *Taenia parva* was introduced with the common genet from Africa to Iberia (Casanova *et al.*, 2000). Species of Canidae harbour adult stages of *T. pisiformis* (Verster, 1969) in Tenerife (Canary Islands) and dogs are definitive hosts of this species (Valladares *et al.*, 1985). The rabbit, *Oryctolagus cuniculus*, was introduced from the Iberian Peninsula into the Canary Islands (Foronda *et al.*, 2003) and has been reported as an intermediate host of *T. pisiformis* in Tenerife Island. Both *T. parva* and *T. pisiformis* are clearly differentiated at the morphological, biological and phylogeographical levels (Verster, 1969; Opuni, 1970; Casanova *et al.*, 2000).

Sequences analysed in this study of 18S rDNA gene from the two species of taeniids are useful for species differentiation, and the clade *Taenia* spp. derives from other cyclophyllidean cestodes in the phylogenetic study, inferred by the 18S rDNA (Foronda, 2002). Microsatellites found in both *T. parva* and *T. pisiformis* sequences could be used as specific markers in *Taenia* systematics as they are found in the same position in the two phylogenetically diverse species.

Microsatellites, tested by PCR, are currently used for analyses in human genetics (Weissenbach *et al.*, 1992). In vertebrates and invertebrates, microsatellites in the 18S rDNA are distributed in constant regions of this gene (Bretagne *et al.*, 1996). Differences in the number and sequences of the repeated unit copy in mammalian microsatellites have been also observed (Bretagne *et al.*, 1996). A number of mechanisms such as replication slippage, unequal sister chromatid exchange, and unequal recombination between alleles, may currently be proposed to account for length-change mutations (Arnour *et al.*, 1993). Little is known about the microsatellites in parasitic helminths. There are a few reports on their presence and use in systematics and ecology (Bretagne *et al.*, 1996). Moreover, this technique is more reliable for typing parasites (Bretagne *et al.*, 1996).

Microsatellites can therefore be used for addressing some epidemiological questions in parasitology (Bretagne *et al.*, 1996). The present results suggest that the use of 18S rDNA microsatellites as diagnostic tools for larval and adult taeniasis should be taken into consideration.

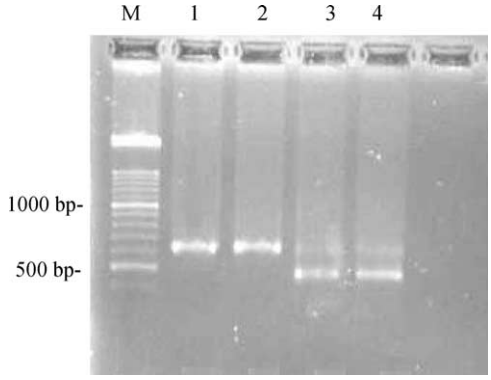

Fig. 2. Differentiation of *Taenia parva* and *T. pisisformis* by *SphI* digestion of the polymerase chain reaction products. Lane M molecular weight marker; lanes 1 and 2 *T. pisiformis* from *Oryctolagus cuniculus*, Tenerife, Canary Islands; lanes 3 and 4 *T. parva* from *Genetta genetta*, Barcelona, Spain.

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References

Abuladze, K.I. (1964) *Essentials of cestodology, vol. IV.* (English translation, Israel Programme for Scientific Translations, Jerusalem, 1970) Moskva.

Arnour, J.A.L., Monckton, D.G., Neil, D.L., Tamaki, K., McLeod, A., Allen, M., Croiser, M. & Jeffreys, A.J. (1993) Mechanisms of mutations at human minisatellite loci. pp. 43–57 *in* Davies, K.E. & Warren, S.T. (*Eds*) *Genome rearrangement and stability.* New York, Cold Spring Harbor Laboratory Press.

Bowles, J. & McManus, D.P. (1994) Genetic characterization of the Asian *Taenia*, a newly described taeniid cestode of humans. *American Journal of Tropical Medicine and Hygiene* **50**, 33–34.

Bretagne, S., Assouline, B., Vidaud, D., Houin, R. & Vidaud, M. (1996) *Echinococcus multilocularis*: microsatellite polymorphism in U1 snRNA genes. *Experimental Parasitology* **82**, 324–328.

Casanova, J.C., Feliu, C., Miquel, J., Torres, J. & Spakulová, M. (2000) Faunistic and ecological trenes on the helminthic community of *Genetta genetta* Linnaeus 1758 (Carnivora: Viverridae) in the Iberian Peninsula. *Helminthologia* **37**, 223–228.

Eom, K.S., Jeon, H.K., Kong, Y., Hwang, U.W., Yang, Y., Li, X., Xu, L., Feng, Z., Pawlowski, Z.S. & Rim, H.J. (2002) Identification of *Taenia asiatica* in China: molecular, morphological, and epidemiological analysis of a Luzhai isolate. *Journal of Parasitology* **88**, 758–764.

Fan, P.C. & Chung, W.C. (1998) *Taenia saginata asiatica*: epidemiology, infection, immunological and molecular studies. *Journal of Microbiology, Immunology and Infection* **31**, 84–89.

Foronda, P. (2002) *Estudio faunístico y sistemático de helmintos de aves de Canarias*. PhD thesis, Universidad de La Laguna, Spain.

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Foronda, P., Valladares, B., Lorenzo-Morales, J., Ribas, A., Feliu, C. & Casanova, J.C. (2003) Helminths of the wild rabbit (*Oryctolagus cuniculus*) in Macaronesia. *Journal of Parasitology* **89**, 952–957.

Gonzalez, L.M., Montero, E., Puente, S., Lopez-Velez, R., Hernandez, M., Sciutto, E., Harrison, L.J.S., Parkhouse, R.M.E. & Garate, T. (2002) PCR tools for the differential diagnosis of *Taenia saginata* and *Taenia solium* taeniasis/cysticercosis from different geographical locations. *Diagnostic Microbiology and Infectious Disease* **42**, 243–249.

Hoberg, E.P., Jones, A., Rausch, R.L., Eom, K.S. & Gardner, S.L. (2000) A phylogenetic hypothesis for species of the genus *Taenia* (Eucestoda: Taeniidae). *Journal of Parasitology* **86**, 89–98.

Mariaux, J. (1998) A molecular phylogeny of the Eucestoda. *Journal of Parasitology* **84**, 114–124.

Moore, J. & Brooks, D.R. (1987) Asexual reproduction in cestodes (Cyclophyllidea: Taeniidae): ecological and phylogenetic influences. *Evolution* **41**, 882–891.

Nickisch-Rosenegk, M., Lucius, R. & Loos-Frank, B. (1999) Contributions to the phylogeny of the Cyclophyllidea (Cestoda) inferred from mithocondrial 12S rDNA. *Journal of Molecular Evolution* **48**, 586–596.

Okamoto, M., Bessho, Y., Kamiya, M., Kurosawa, T. & Horii, T. (1995) Phylogenetic relationship within *Taenia taeniformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome *c* oxidase subunit I gene. *Parasitology Research* **81**, 451–458.

Opuni, E.K. (1970) Asexual multiplication in *Cysticercus pisiformis* (Cestoda). *Journal of Helminthology* **44**, 321–322.

Queiroz, A. de & Alkire, N.L. (1998) The phylogenetic placement of *Taenia* cestodes that parasitize humans. *Journal of Parasitology* **84**, 379–383.

Rausch, R.L. (1994) Family Taeniidae Ludwig, 1886. pp. 665–672 *in* Khalil, L.F., Jones, A. & Bray, R.A. (*Eds*) *Keys to the cestodes parasites of vertebrates*. Wallingford, Oxon, CAB International.

Thompson, J.D., Gibson, F.P., Jeanmongin, F. & Higgins, D.G. (1997) The CLUSTAL X windows interfase: flexible strategies for multiple sequence alignment aided by quality analysis tool. *Nucleic Acids Research* **24**, 4876–4882.

Valladares, B., Gijón, H. & López-Román, R. (1985) Estudio epidemiológico del parasitismo intestinal del perro (*Canis familiaris*) en la isla de Tenerife. *Revista Ibérica de Parasitología* **45**, 41–48.

Verster, A. (1969) A taxonomic revision of the genus *Taenia* Linnaeus, 1758, s. str. *Onderstepoort Journal of Veterinary Research* **36**, 3–58.

Weissenbach, J., Gyapay, G., Dib, C., Virginal, A., Morisette, J., Millasseau, P., Vaysseix, G. & Lathrop, M. (1992) A second-generation linkage map of the human genome. *Nature* **359**, 794–801.

Zarlenga, D.S. & George, M. (1995) *Taenia crassiceps*: cloning and mapping of mitochondrial DNA and its application to the phenetic analysis of a new species of *Taenia* from Southeast Asia. *Experimental Parasitology* **81**, 604–607.

Zarlenga, D.S., McManus, D.P., Fan, P.C. & Cross, J.H. (1991) Characterization and detection of a newly described Asian taeniid using cloned ribosomal DNA fragment and sequence amplification by polymerase chain reaction. *Experimental Parasitology* **72**, 174–183.

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Author Queries

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Q1 In reference Raush, 1994 has been changed to Rausch, 1994 kindly check.

Q2 Please note that et al. has been added to Nickisch-Rosenegk, 1999.

Q3 Bloch, 1780 has not cited in the reference list.