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Genetic diversity within the genus *Trichinella* as shown by cleavage fragment length polymorphism analysis

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Abstract

The genetic diversity within the genus *Trichinella* was studied using cleavage fragment length polymorphism (CFLP) analysis. The CFLP method generates specific fingerprints based on single nucleotide mutations. By this method the amplified intergenic regions of the 5S rRNA genes of the eight different genotypes of *Trichinella* were analysed. The CFLP pattern of *T. spiralis* was completely different compared with the sylvatic species *T. britovi*, *T. nativa*, *T. nelsoni*, and the genotypes *Trichinella* T5, *Trichinella* T6 and *Trichinella* T8. The *T. pseudospiralis* intergenic region can be differentiated by size from the other species of *Trichinella*.

Introduction

Based on biological, phenotypic and isozyme analysis, the genus *Trichinella* is divided into eight different genotypes (Fukumoto *et al.*, 1987, 1988; Pozio *et al.*, 1989, 1992a,b; La Rosa *et al.*, 1992; Bandi *et al.*, 1995; Wakelin & Goyal, 1996). Several studies have been conducted to analyse the genetic information of the genus *Trichinella* with the aim to identify *Trichinella* isolates to their specific genotypes. Random amplified polymorphic DNA (RAPD) (Bandi *et al.*, 1995), restriction fragment length polymorphism (RFLP) (Klassen *et al.*, 1986; Dame *et al.*, 1987; Nagano *et al.*, 1999), Southern and dot blot analysis (Boyd *et al.*, 1989; Zarlenga *et al.*, 1991) and specific PCRs (Soulé *et al.*, 1993; Wu *et al.*, 1998; Appleyard *et al.*, 1999) have been described to study and to identify the different genotypes. Of these, only RAPD–PCR is able to identify single larvae, although a major drawback is the high variability in the banding patterns (Penner *et al.*, 1993; Bandi *et al.*, 1995; Pozio *et al.*, 1999).

In the present paper, cleavage fragment length polymorphism (CFLP) analysis (Third Wave Technologies, Inc., Madison, Wisconsin) was applied to study the genetic diversity of members of the genus *Trichinella*. This method is based on the specific cleavage of hairpins formed by single-stranded DNA (ssDNA). The enzyme, endonuclease cleavase I, can recognize and cleave the ssDNA folded secondary structures at the 5′ side of the junctions between the ssDNA. Because the formation of these secondary structures is highly dependent on the nucleotide sequence, differences herein will give different DNA fragments typical and reproducible for each selected target DNA. Mutations may result in the appearance or loss of DNA fragments or a change in the signal intensity of one of more bands. The DNA fragments can be detected by polyacrylamide gel electrophoresis (PAGE) and visualized by using a radioactive or non-radioactive end-labelled primer or by silverstaining (Marshall *et al.*, 1997; Rossetti *et al.*, 1997). As a target DNA, amplified ribosomal RNA genes and intergenic regions, which contain both conserved and highly variable nucleotide sequences, can be used to study interspecies variability (Brow *et al.*, 1996). In nematodes, the small 5S rRNA gene is extremely conserved, but the intergenic region between repeating 5S rRNA coding regions vary greatly in size and nucleotide composition (Liu *et al.*, 1996). In the present study, we propose to amplify the intergenic region between the repeating 5S rRNA gene among the members of the genus *Trichinella* and analysed variation of this region using the CFLP method.

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Table 1. *Trichinella* strains used in this study.

Species	Name[a]	Source	Country
T. spiralis (T1)	ISS 3	*Sus scrofa*	Poland
T. spiralis	RIVM (ISS 14)	*Rattus norvegicus*	The Netherlands
T. spiralis	CO77 (ISS 88)	*S. scrofa fera*	Spain
T. spiralis	MAD83 (ISS 92)	*S. scrofa fera*	Spain
T. britovi(T3)	ISS 2	*Vulpus vulpes*	Italy
T. britovi	ISS 324	*V. vulpes*	France
T. britovi	ISS 327	*V. vulpes*	France
T. britovi	ISS 384	*V. vulpes*	Switzerland
T. britovi	Monegrillo (ISS 89)	*V. vulpes*	Spain
T. britovi	ZI91	*V. vulpes*	Spain
T. nativa (T2)	ISS 10	*Thalarctos maritimus*	Norway
T. nativa	ISS 296	*V. vulpes*	Finland
T. nativa	ISS 335	*Martes americana*	USA
T. nelsoni (T7)	ISS 29	*Crocuta crocuta*	Kenya
T. nelsoni	ISS 232	*Panthera leo*	South Africa
T. pseudospiralis (T4)	ISS 13	*Nyctereutes procyonoides*	Caucasus
T. pseudospiralis	ISS 141	*Dasyurus maculatus*	Australia
T. pseudospiralis	ISS 470	*Caragypus atratus*	USA
Trichinella T5	ISS 103	*Equus caballus*	France
Trichinella T5	ISS 224	*Procyon lotor*	USA
Trichinella T5	ISS 346	*Ursus americanus*	USA
Trichinella T6	ISS 34	*U. arctos*	USA
Trichinella T6	ISS 334	*Felis concolor*	USA
Trichinella T6	ISS 456	*F. concolor*	USA
Trichinella T8	ISS 124	*Crocuta crocuta*	South Africa
Trichinella T8	ISS 148	*C. crocuta*	South Africa
Trichinella T8	ISS 149	*Panthera leo*	South Africa

[a] ISS: Istituto Superiore di Sanità, Rome, Italy; RIVM: National Institute of Public Health and the Environment, the Netherlands.

Materials and methods

Trichinella *strains*

From each of the five *Trichinella* species and the three additional genotypes, different isolates were used in this study (table 1). *Trichinella* strains were identified according to their alloenzyme grouping (La Rosa *et al.*, 1992) and the strains were maintained in mice. Larvae were isolated by artificial digestion in 5 g pepsin and 30 ml 2.4 N HCl in 400 ml water by the Trichomatic35® (Foss electronics, Denmark).

DNA isolation and PCR

For isolation of genomic DNA from a single *Trichinella* larva, one individual larva was heated in Tris-HCl (pH 7.6) at 90°C for 10 min and treated with proteinase K (100 μg ml^{-1} in a total volume of 10 μl) at 48°C for at least 3 h. After inactivation of the proteinase K, 5 μl was amplified by PCR (Bandi *et al.*, 1995).

A sense primer 5′-GCGAATTCTTGGATCGGAGACG-GCCTG-3′ and antisense primer 5′-GCTCTAGACGA-GATGTCGTGCTTTCAACG-3′ as described by Liu *et al.* (1996) were used to amplify the 5S rDNA intergenic region of *Trichinella*. The forward PCR primer was biotin-labelled at the 5′-site, which made it possible to detect this strand of the 5S rDNA intergenic region PCR product by the CFLP method. PCR amplification was carried out with 5 μl crude DNA of one individual larva in a total volume of 50 μl. The reaction mixtures contained 1.5 mM $MgCl_2$, 200 μM dNTPs each, 1.5 U of *Taq* DNA polymerase (Perkin Elmer) and 25 pmol of each primer. Thermal cycling conditions were 94°C for 90 s, followed by 94°C for 30 s, 48°C for 1 min and 72°C for 1 min for 40 cycles followed by a 10 min extension at 72°C (Perkin Elmer 480). After gel-electrophoresis on a 1.5% agarose gel (Sambrook *et al.* 1989), the amplified product was isolated and purified using the GeneClean® II kit (Bio101, Inc.).

CLFP

Approximately 100 fmol of each purified PCR-product was used for the CFLP method in a total volume of 15 μl. The target DNA was heated at 95°C for 10–20 s and then cooled down to the reaction temperature in a Perkin-Elmer 480 thermocycler. A mix of 5 μl prewarmed (at reaction temperature) reaction mix was added containing 2 μl 10× CFLP-buffer (100 mM MOPS (pH 7.5), 0.5% Tween®20, 0.5% Nonidet™P-40), 25 U cleavase I and 2 μl 2 mM $MnCl_2$. After 6 min, the reaction was stopped by adding 16 μl of stop solution (95% formamide, 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0)). The DNA fragments were denatured at 80°C for 2 min and 20 μl of the reaction mixture was resolved by electrophoresis in a 0.3 cm 8% denaturing polyacrylamide gel containing 7 M urea in 0.5× TBE at 20 watt. Biotinylated Φ174/HinfI fragments, 0.1 μg, were used as a marker (Gibco BRL). After electrophoresis, the gel was blotted on a positively charged nylon membrane (Boehringer Mannheim,

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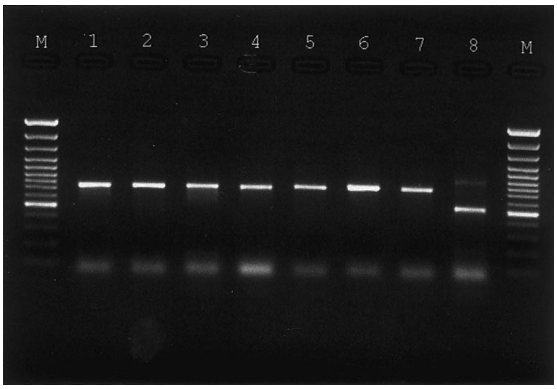

Fig. 1. PCR products of eight identified *Trichinella* species. Lane 1: *T. spiralis* ISS 3; lane 2: *T. britovi* ISS 2; lane 3: *T. nativa* ISS 10; lane 4: *T. nelsoni* ISS 29; lane 5: T5; lane 6: T6; lane 7: T8; lane 8: *T. pseudospiralis* ISS 13; lane 9: molecular weight marker VI Boeringer.

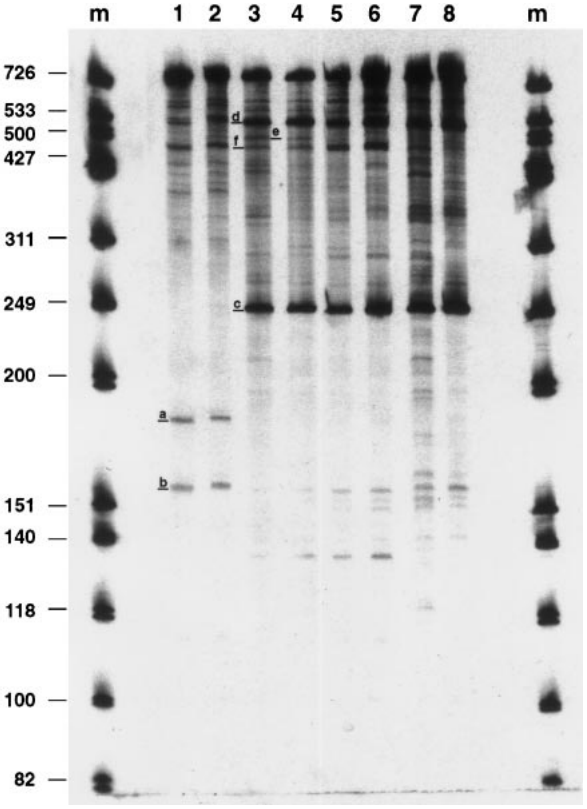

Fig. 2. CFLP fingerprints of the sense strand of the 5S rDNA intergenic region PCR-products of different *Trichinella* species. Lane M, molecular weight marker ΦX174/HinfI; lane 1, *T. spiralis* strain ISS 3; lane 2, *T. spiralis* strain RIVM; lane 3, *T. britovi* strain ISS 2; lane 4, *T. britovi* strain ISS 384; lane 5, *T. nativa* strain ISS 10; lane 6, *T. nativa* strain ISS 296; lane 7, *T. nelsoni* strain ISS 29; lane 8, *T. nelsoni* strain ISS 232.

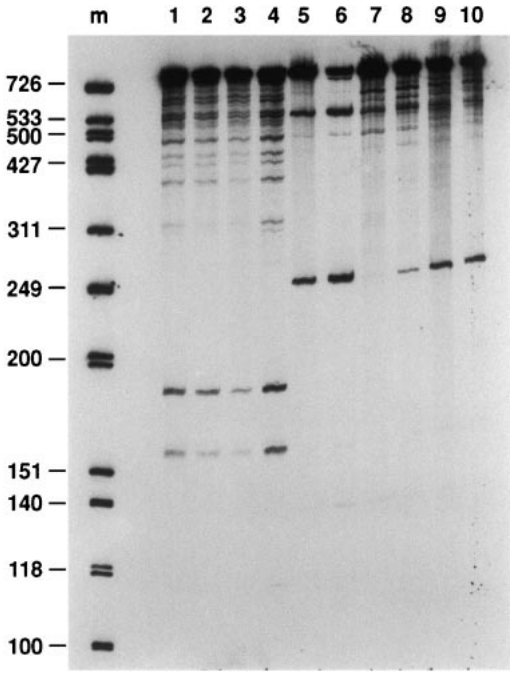

Fig. 3. CFLP fingerprints of the sense strand of the 5S rDNA intergenic region PCR products of *T. spiralis* and three unidentified *Trichinella* genotypes. Lane 1, *T. spiralis* ISS3; lane 2, *T. spiralis* ISS 14; lane 3, *T. spiralis* ISS 88; lane 4, *T. spiralis* ISS 92; lane 5, T5 ISS 103; lane 6, T5 ISS 346; lane 7, T6 ISS 34; lane 8, T6 ISS 334; lane 9, T8 ISS 148; lane 10, T8 ISS 149.

Germany). The DNA fragments were detected by chemiluminescent detection with ECL (Amersham).

Results

Except for *T. pseudospiralis*, the PCR resulted in an amplified product of approximately 750 bp for all *Trichinella* genotypes. The amplified fragment of *T. pseudospiralis* had a length of 522 bp (fig. 1). The optimal reaction conditions of the CFLP method, 6 min at 48°C, resulting in a partial digested ssDNA, were determined according to the directions of the manufacturer (data not shown). CFLP of *T. spiralis* resulted in two prominent bands of approximately 181 nucleotides (a) and 157 nt (b), which were absent in the sylvatic *Trichinella* genotypes (fig. 2). The CFLP fingerprints of the sylvatic species, *T. nelsoni*, *T. nativa* and *T. britovi* and the *Trichinella* genotypes T5, T6 and T8 showed a prominent fragment at approximately 250 nt (c), which make the CFLP patterns of these genotypes very distinct from the domestic species *T. spiralis* that lacks this fragment (figs 2 and 3). Although the CFLP banding pattern among the different sylvatic *Trichinella* genotypes resulted in slight differences in the high molecular banding region, these differences were less prominent compared with the differences with *T. spiralis* (fig. 4). Therefore, it was difficult to use the high molecular banding pattern with the aim to identify sylvatic *Trichinella* genotypes to individual genotypic level.

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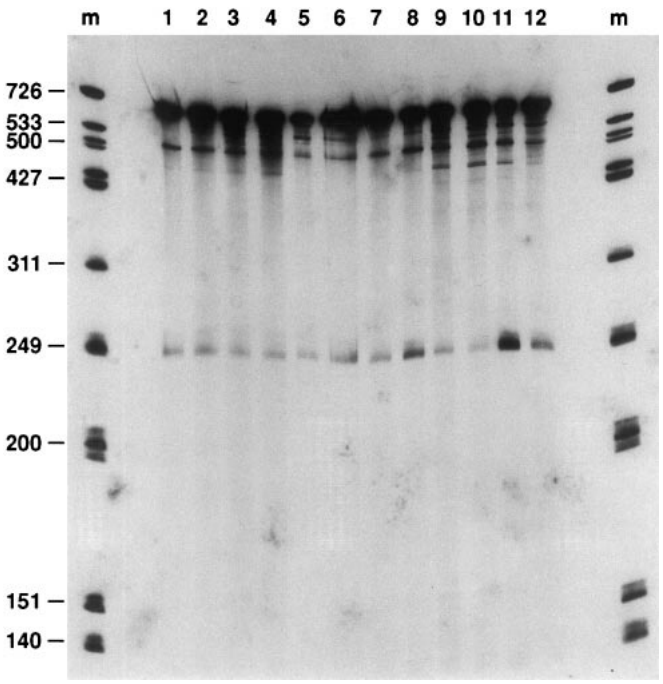

Fig. 4. CFLP fingerprints of the sense strand of the 5S rDNA intergenic region PCR products of all sylvatic *Trichinella* genotypes. Lane 1, *T. britovi* ISS 2; lane 2, *T. britovi* Monogrillo; lane 3, *T. nativa* ISS 10; lane 4, *T. nativa* ISS 335; lane 5, *T. nelsoni* ISS 29; lane 6, *T. nelsoni* ISS 232; lane 7, T5 ISS 103; lane 8, T5 ISS 346; lane 9, T6 ISS 34; lane 10, T6 ISS 334; lane 11, T8 ISS 148; lane 12, T8 ISS 149.

To test the reproducibility of the CFLP method, all *Trichinella* strains listed in table 1 were tested. The 5S rDNA intergenic region amplified products of all *T. spiralis* isolates showed the same characteristic banding pattern, compared with the sylvatic species, *T. nelsoni*, *T. nativa* and *T. britovi* (fig. 2) and the *Trichinella* T5, T6 and T8 genotypes tested (figs 2–4). This confirmed the reproducibility and usability of the CFLP to clearly distinguish *T. spiralis* from the other seven genotypes. In contrast to the other *Trichinella* genotypes, which all showed the identical banding pattern within the same genotype, the CFLP banding pattern of the two *T. nelsoni* strains were, except for the characteristic 250 nt band, not identical (fig. 2).

Discussion

In this study, the genetic diversity among different *Trichinella* genotypes was analysed by the CFLP method. This method has been described as a sensitive tool to distinguish a number of bacterial genera (Brow *et al.*, 1996), hepatitis C virus genotypes (Marshall *et al.*, 1997) and mutations in human genes (Rossetti *et al.*, 1997). For this CFLP application, we used the 5S rDNA intergenic region as a target, which is described to vary in nucleotide sequence and size among nematodes (Liu *et al.*, 1996). This region was amplified using primers, which were selected in a conserved region, the tandem repeated 5S rRNA gene of nematodes (Liu *et al.*, 1996). This resulted in a single prominent PCR product of approximately 750 bp for the *Trichinella* species tested in this study, except for *T. pseudospiralis*.The 522 bp PCR product of *T. pseudospiralis* directly showed the difference between this species and the other *Trichinella* genotypes at molecular level.

The CFLP analysis showed a major difference between *T. spiralis* and the sylvatic *Trichinella* genotypes. Although differences in the high molecular banding pattern among the sylvatic *Trichinella* genotypes were detected, identification of these genotypes was difficult using CFLP. Therefore, it was concluded that the genetic variation within the intergenic region of the 5S rRNA gene of all sylvatic genotypes was less diverse. Although both *T. nelsoni* strains tested had the characteristic 250 nt band of the sylvatic *Trichinella* genotypes, the CFLP pattern showed diversity within the *T. nelsoni* strains. La Rosa (1993) also described that the *T. nelsoni* ISS 232 isolate was different from other *T. nelsoni* strains, indicating the presence of genomic polymorphism within the species *T. nelsoni*.

Application of the CFLP method, with limited digestion of a specific region of DNA under defined annealing conditions, yields a distinct series of DNA fragments revealed by gel-electrophoresis without prior knowledge of the precise nucleotide sequence. We showed that this method can be used to distinguish *T. spiralis* from the

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sylvatic strains of *Trichinella*. Other methods, e.g. random amplified polymorphic DNA analysis (Penner *et al*., 1993; Bandi *et al*., 1995), PCR using species-specific primers (Appleyard *et al*., 1999) and allozyme analysis (Fukumoto *et al*., 1987, 1988; La Rosa *et al*., 1992; Bandi *et al*., 1995) have been described for this purpose. Until now, the amount of DNA needed, the cumbersome procedures and the results, which are sometimes not reproducible, make it difficult to rapidly and reproducibly identify *Trichinella* isolates. Identification of *T. spiralis* might be important to provide information about the possible source of infection and to estimate the importance for public health. The CFLP pattern of *T. spiralis*, which is the most important species with respect to human infections, could be easily identified compared with CFLP patterns of the sylvatic species *T. britovi*, *T. nativa* and *T. nelsoni*. Therefore, the use of CFLPA seems to be a rapid and very reproducible marker to identify *T. spiralis*.

In conclusion, this study indicates that the intergenic region of the 5S rRNA genes is an appropriate genetic marker to study genetic diversity among the genus *Trichinella* and to differentiate the domestic *T. spiralis* from the sylvatic *Trichinella* genotypes. The CFLP fingerprint of *T. spiralis* is clearly recognizable and characteristic for this species.

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