Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) for the identification of *Trichinella* isolates

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SUMMARY

In the present study, polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis was developed to identify 5 species (*Trichinella spiralis*, *Trichinella britovi*, *Trichinella nativa*, *Trichinella nelsoni* and *Trichinella pseudospiralis*) and 3 phenotypes of uncertain taxonomic status (*Trichinella* T5, T6, and T8). Eleven restriction endonucleases were used to restrict 3 DNA fragments (1) a 2800 bp fragment of the 43 kDa excretory–secretory (E–S) protein gene, (2) a 1250 bp fragment amplified with the primer pair SB147A and (3) a 372 bp fragment amplified with the primer pair SB372A. This RFLP method allows the identification of the 8 *Trichinella* phenotypes as follows: *T. spiralis* by the *Hinf* I or *Dde*I endonuclease restriction of the 2800 bp fragment; *T. nativa* by the *Rsa*I restriction of the 2800 bp fragment, or by the *Alu*I restriction of the 1250 bp fragment; *T. britovi* and *Trichinella* T8 by the *Alu*I restriction of the 1250 bp fragments, and can be discriminated between them by the *Ssp*I restriction of the 2800 bp fragment; *T. pseudospiralis* by the *Msp*I restriction of the 372 bp fragment; *T. nelsoni* by the *Hha*I or *Alu*I restriction of the 2800 bp fragment; *Trichinella* T5 by the *Hha*I restriction of the 2800 bp fragment; *Trichinella* T6 by the *Alu*I restriction of the 1250 bp fragment; and *Trichinella* T8 by the *Ssp*I or *Rsa*I restriction of the 2800 bp fragment. This study reveals also an intraspecifies polymorphism in the 2800 bp and 1250 bp fragments for *T. britovi*, *Trichinella* T5 and T6.

Key words: PCR, RFLP, *Trichinella* spp.

INTRODUCTION

The genus *Trichinella* has been considered to be monospecific (Dick, 1983). Evidence in the last 2 decades, however, has suggested that this genus exists as a species complex. Experiments of cross-hybridization, phenotypic and isozyme data support this notion (Pozio, 1987; Fukumoto *et al*. 1988; La Rosa *et al*. 1992; Pozio *et al*. 1992*a*). In a recent taxonomic revision, Pozio *et al*. (1992*b*) identified in this genus 8 phenotypes, 5 at the species level (*Trichinella spiralis*, *Trichinella britovi*, *Trichinella nativa*, *Trichinella nelsoni* and *Trichinella pseudospiralis*), and 3 of uncertain taxonomic status (*Trichinella* T5, T6 and T8).

Progress in molecular biology has allowed the identification of *Trichinella* phenotypes by PCR using single larva (Bandi *et al*. 1995; Dick *et al*. 1992) or isolated genomic DNA (Dupouy-Camet *et al*. 1991; Soule *et al*. 1993; Wu *et al*. 1997; Wu, Nagano & Takahashi 1998). At the present time, specific primers can identify some *Trichinella* phenotypes, but cannot differentiate closely-related species and phenotypes, such as *T. nativa*, *T. britovi* and *Trichinella* T6 (Wu *et al*. 1998).

Restriction fragment length polymorphism (RFLP) is another method with an excellent discriminating power in genetic analysis, allowing the establishment of taxonomic relationships (De Jonckheere, 1994; Clark, Martin & Diamond 1995) and the characterization of species and/or strains (Vodkin *et al*. 1992). This method relies on the highly specific recognition of base sequences by endonuclease within DNA fragments. Restriction with an endonuclease often results in the generation of a unique set of DNA fragments of different sizes, which are recognized by a unique DNA pattern to the corresponding genotype. Thus studies on RFLP afford a useful approach to discriminate *Trichinella* phenotypes.

In the present study, we developed a scheme of RFLP for the identification of *Trichinella* phenotypes that may be used as a gene fingerprint of these parasites.

MATERIALS AND METHODS

DNA specimens

Genomic DNA was prepared according to the conventional method (Dupouy-Camet *et al*. 1994)

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Table 1. Template DNA source of *Trichinella*

Isolate code	Species	Original host	Geographical origin
ISS408	*T. britovi*	Black bear (*Ursus thibetanus japonicus*)	Japan
ISS409	*T. britovi*	Raccoon dog (*Nyctereutes procyonoides viverrinus*)	Japan
ISS63	*T. britovi*	Stray dog (*Canis familiaris*)	Italy
ISS271	*T. britovi*	Red fox (*Vulpes vulpes*)	Italy
ISS99	*T. britovi*	Wolf (*Canis lupus*)	Italy
ISS173	*T. britovi*	Wild boar (*Sus scrofa*)	Slovak Republic
ISS119	*T. britovi*	Red fox (*Vulpes vulpes*)	Italy
ISS201	*T. britovi*	Wild boar (*Sus scrofa*)	Spain
ISS234	*T. britovi*	Brown bear (*Ursus arctos*)	Slovak Republic
ISS2	*T. britovi*	Red fox (*Vulpes vulpes*)	Italy
ISS410	*T. nativa*	Polar bear (*Ursus maritimus*)	Arctic
ISS70	*T. nativa*	Wolf (*Canis lupus*)	Russia
ISS10	*T. nativa*	Polar bear (*Ursus maritimus*)	Svalbard Island
ISS181	*T. nativa*	Fox (*Vulpes corsac*)	Kazakhstan
ISS72	*T. nativa*	Racoon dog (*Nyctereutes procyonoides*)	Russia
ISS413	*T. spiralis*	Wild boar (*Sus scrofa*)	Poland
ISS412	*T. spiralis*	Domestic pig (*Sus scrofa*)	USA
ISS411	*T. spiralis*	Human (*Homo sapiens*)	Thailand
ISS407	*T. spiralis*	Domestic dog (*Canis familiaris*)	China
ISS248	*T. spiralis*	Wild boar (*Sus scrofa*)	Spain
ISS160	*T. spiralis*	Wild boar (*Sus scrofa*)	Spain
ISS154	*T. spiralis*	Domestic pig (*Sus scrofa*)	Egypt
ISS37	*T. nelsoni*	Wart hog (*Phacochoerus aethiopicus*)	Tanzania
ISS13	*T. pseudospiralis*	Raccoon dog (*Nyctereutes procyonoides*)	Caucasus
ISS35	*Trichinella* T5	Black bear (*Ursus americanus*)	Pennsylvania
ISS178	*Trichinella* T5	Horse (*Equus caballus*) (imported from Connecticut, USA)	France
ISS415	*Trichinella* T5	Raccoon (*Procyon lotor*)	Indiana, USA
ISS40	*Trichinella* T6	Mountain lion (*Felis concolor*)	Montana, USA
ISS339	*Trichinella* T6	Wolverine (*Gulo gulo*)	Montana, USA
ISS124	*Trichinella* T8	Spotted hyena (*Crocuta crocuta*)	South Africa
ISS272	*Trichinella* T8	Lion (*Panthera leo*)	Etosha Park, Namibia

from 31 strains. Detailed data (code, original hosts, locality and country of origin) of these strains are shown in Table 1. The reference code is that of the *Trichinella* Reference Center in Rome, Italy (Pozio, La Rosa & Rossi 1989).

PCR primer and DNA fragments

Three DNA fragments (Ts43CA, SB147 and SB372) were used for RFLP experiments. DNA fragment Ts43CA was produced by the amplification of genomic DNA with the primer pair Ts43CA constructed by Wu *et al*. (1998) from the cDNA sequence encoding a 43 kDa excretory–secretory (E–S) glycoprotein of *T. spiralis*. The size of the amplicon was 2800 bp which was longer than the expected size (1024 bp) from the cDNA sequence.

DNA fragment SB147 was produced by the amplification of genomic DNA with the primer pair SB147A developed by Wu *et al*. (1998), and it produced an expected band of 1250 bp.

DNA fragment SB372 was produced by the amplification of genomic DNA with the primer pair SB372A developed by Wu *et al*. (1998), and it produced an expected band of 372 bp.

PCR

PCR was performed according to the method described by Wu *et al*. (1997). The condition of PCR was as follows: step 1 – one cycle at 92 °C for 3 min; step 2 – 30 cycles at 92 °C for 30 sec, 51 °C for 30 sec and 72 °C for 60 sec; step 3 – one cycle at 72 °C for 10 min for the final extension.

Long and accurate PCR (LA–PCR) was adapted to amplify genomic DNA with the primer pair Ts43CA. The PCR reaction mixture was composed of 2 μl of dNTPs, 2 μl of 10 X Ex PCR buffer, 0·08 μl of TaKaRa Ex *Taq* polymerase (5U/μl) (TaKaRa Shuzo Co. Ltd, Kyoto, Japan), 2 μl of template DNA 4 μg/ml), 4 μl of primers (5 μM) and 9·92 μl of distilled water. LA–PCR was carried out as follows: step 1 – one cycle at 94 °C for 1 min; step 2 – 30 cycles at 98 °C for 20 sec and at 60 °C for 15 min; step 3 – one cycle at 72 °C for 10 min for the final extension.

RFLP analysis

After amplification, PCR products were restricted by different restriction endonucleases (*Rsa*I, *Hha*I, *Ssp*I, *Dde*I, *Hinf*I, *Alu*I, *Hae*III, *Hind*III, *Mva*I, *Pst*I and *Msp*I; Promega, Madison, WI, USA) in a

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reaction mixture of 2 μl of 10 X buffer, 0·2 μl of BSA solution (10 mg/ml), 0·5 μl (4–5 U) of restriction endonuclease, 8 μl of PCR product, and 9·7 μl of distilled water to a final volume of 20 μl, at 37 °C for 2 h. The restricted products were analysed by electrophoresis in 1·0 % or 1·5 % agarose gels (SEAKEM GTG, FMC Bio-products, Rockland, ME, USA) and were detected by staining with ethidium bromide. The 100 or 500 Base-Pair Ladder of molecular weight marker (Pharmacia Biotech, Tokyo, Japan) was electrophoresed in parallel to estimate the size of DNA fragments.

RESULTS

RFLP of the gene encoding 43 kDa E–S protein

The primer pair Ts43CA can amplify the gene encoding 43 kDa E–S protein. When the amplicons (2800 bp) from 30 different DNA specimens belonging to *T. spiralis*, *T. britovi*, *T. nativa*, *T. nelsoni*, *Trichinella* T5, T6 and T8 were restricted with 10 endonucleases, it was possible to differentiate them by the band profiles (Table 2).

Rsa*I restriction*

Five types of band patterns were produced (Fig. 1A): *Trichinella nativa* had a pattern characterized by a 800 bp band (lanes 11–15), which was not shared by the other phenotypes; *Trichinella* T8, had a pattern of 1400, 500 and 450 bp bands (lanes 29–30); *Trichinella spiralis* and *T. nelsoni*, showed a similar pattern of 2000 and 900 bp band (lanes 16–22 and lane 23); *T. britovi*, except the 2 Japanese strains of *T. britovi* (ISS408, ISS409, lanes 1 and 2), had a pattern of 4 bands of 1400, 900, 500 and 450 bp (lanes 3–10). The 2 Japanese strains of *T. britovi*, and T5 and T6 showed 3 bands of 1400, 900, and 500 bp. A strain of *Trichinella* T6 (lane 27) had an extra band of 1000 bp, which was not shared by the other T6 strain (lane 28).

Hha*I restriction*

Trichinella nelsoni showed a prominent band of 1250 bp (lane 23) (Fig. 1B). T5 was characterized by a 1500 bp band (lanes 24–26). The other phenotypes shared a similar band pattern. This restriction enzyme showed variability in the T5 phenotype: the ISS35 strain (lane 24) was characterized by 3 bands of *ca*. 1600, 550 and 450 bp, whereas the 2 other T5 strains (ISS178, ISS415, lanes 25 and 26) were characterized by 4 bands of *ca*. 1800, 1600, 550 and 500 bp.

Ssp*I restriction*

Trichinella T8 could be discriminated from the other phenotypes by the presence of 2300 and 2100 bp bands (Fig. 1C). The 2 Japanese strains of *T. britovi* exhibited unique RFLP patterns which were different from that of other strains of *T. britovi* and other *Trichinella* phenotypes. The 2 strains of T6 could be also discriminated from each other by a 1800 bp band (lane 27).

Dde*I restriction*

This RFLP method produced 3 band patterns (Fig. 1D) that can be summarized as follows: *T. spiralis*, a unique pattern composed of 3 bands of 1400, 750 and 650 bp; *Trichinella* T8, a pattern of 3 bands of *ca*. 750, 650 bp and *ca*. 400 bp, the last band was not shared by the other phenotypes. *Trichinella britovi*, *T. nativa*, *T. nelsoni*, T5, and T6 shared a similar pattern.

Hinf*I restriction*

Trichinella spiralis exhibited a unique RFLP pattern (Fig. 1E) characterized by a broad band (probably composed of 2 bands of 1420 and 1380 bp). A strain of T5 (lane 24) showed an RFLP pattern characterized by 2 bands of *ca*. 1550 and 1250 bp, different from those belonging to the same and from the other phenotypes.

Alu*I restriction*

Trichinella nelsoni had a prominent band pattern characterized by a 1200 bp band (lane 23) (Fig. 1F). The other phenotypes showed a similar band pattern, but *T. spiralis* was devoid of the 350 bp band.

Hae*III restriction*

Trichinella nativa and T6 shared the same RFLP pattern, whereas the other phenotypes shared a different RFLP pattern (Fig. 1G). The 2 Japanese strains of *T. britovi* showed 2 bands of 1100 and 950 bp, whereas the other strains of *T. britovi* showed an additional band of 800 bp. The enzyme restriction also produced a different RFLP pattern among T5 strains: a strain (lane 24) showed 2 bands of 1100 and 800 bp, whereas the other 2 strains (lanes 25 and 26) showed an additional band of 950 bp.

Hind*III restriction*

Trichinella spiralis and *Trichinella* T6 shared a similar band pattern characterized by a 2700 bp band (Fig. 1H). The other phenotypes produced 2 main bands of 1700 and 1000 bp.

Mva*I restriction*

There were differences within species, which may be used as a gene fingerprint (Fig. 1I). *Trichinella britovi* strains from Italy (ISS63, ISS99 and ISS2 in

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Table 2. RFLP analysis of the 43 kDa E–S protein gene, showing used restriction enzymes and bp size of resulting bands

Species (ISS)*	*Rsa*I	*Hha*I	*Ssp*I	*Dde*I	*Hinf*I	*Alu*I	*Hae*III	*Hind*III	*Mva*I	*Pst*I
T. br (408)	1400, 900, 500	1800, 550, 500	1400, 1100, 650	750, 650	2800	750, 550, 450	1100, 959, 550	1700, 1000	1100, 700	1500, 1300
T. br (409)	1400, 900, 500	1800, 550, 500	1400, 650	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. br (63)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1700, 1100, 700	1500, 1300
T. br (271)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750,550, 450	1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. br (99)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1700, 1100, 700	1500, 1300
T. br (173)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. br (119)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. br (201)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. br (234)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. br (2)	1400, 900, 500, 450	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1100, 950, 550	1700, 1000	1700, 1100, 700	1500, 1300
T. na (410)	1400, 900, 800, 500	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1500, 1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. na (70)	1400, 800, 500	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1500, 1100	1700, 1000	1100, 700	1500, 1300
T. na (10)	1400, 800, 500	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1500, 1100	1700, 1000	1100, 700	1500, 1300
T. na (181)	1400, 800, 500	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1500, 1100	1700, 1000	1100, 700	1500, 1300
T. na (72)	1400, 900, 800, 500	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1500, 1100, 950, 550	1700, 1000	1100, 700	1500, 1300
T. sp (413)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. sp (412)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. sp (411)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. sp (407)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. sp (248)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. sp (160)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. sp (154)	2000, 900	1800, 550, 500	2100	1400, 750, 650	1420, 1380	750, 550, 450	1100, 950, 800, 550	2700	1100, 700	2800
T. ne (37)	2000, 900	1250, 550, 500	2100	750, 650	2800	1200, 750, 550, 450	1100, 950, 550	1700, 1000	1100, 700	2800
T5 (35)	1400, 900, 500	1600, 550, 450	2100	750, 650	2800, 1550, 1250	750, 550, 450	1100, 800, 550	1700, 1000	1700, 1100	1500, 1300
T5 (178)	1400, 900, 500	1800, 1600, 550	2100	750, 650	2800	750, 550, 450	1100, 950, 800, 550	1700, 1000	1700, 1100, 700	1500, 1300
T5 (415)	1400, 900, 500	1800, 1600, 550	2100	750, 650	2800	750, 550, 450	1100, 950, 800, 550	1700, 1000	1700, 1100, 700	1500, 1300
T6 (40)	1400, 1000, 900, 500	1800, 550, 500	2100, 1800	750, 650	2800	750, 550, 450	1500, 1100	2700	1100, 700	1500, 1300
T6 (339)	1400, 900, 500	1800, 550, 500	2100	750, 650	2800	750, 550, 450	1500, 1100	2700	1100, 700	1500, 1300
T8 (124)	1400, 500, 450	1800, 550, 500	2300, 2100	750, 650, 400	2800	750, 550, 450	1100, 950, 550	1700, 1000	1700, 1100, 700	1500, 1300
T8 (272)	1400, 500, 450	1800, 550, 500	2300, 2100	750, 650, 400	2800	750, 550, 450	1100, 950, 550	1700, 1000	1700, 1100, 700	1500, 1300

* *Trichinella britovi* (T. br), *Trichinella nativa* (T. na), *Trichinella spiralis* (T. sp), *Trichinella nelsoni* (T. ne), *Trichinella* T5 (T5), *Trichinella* T6 (T6), and *Trichinella* T8 (T8).

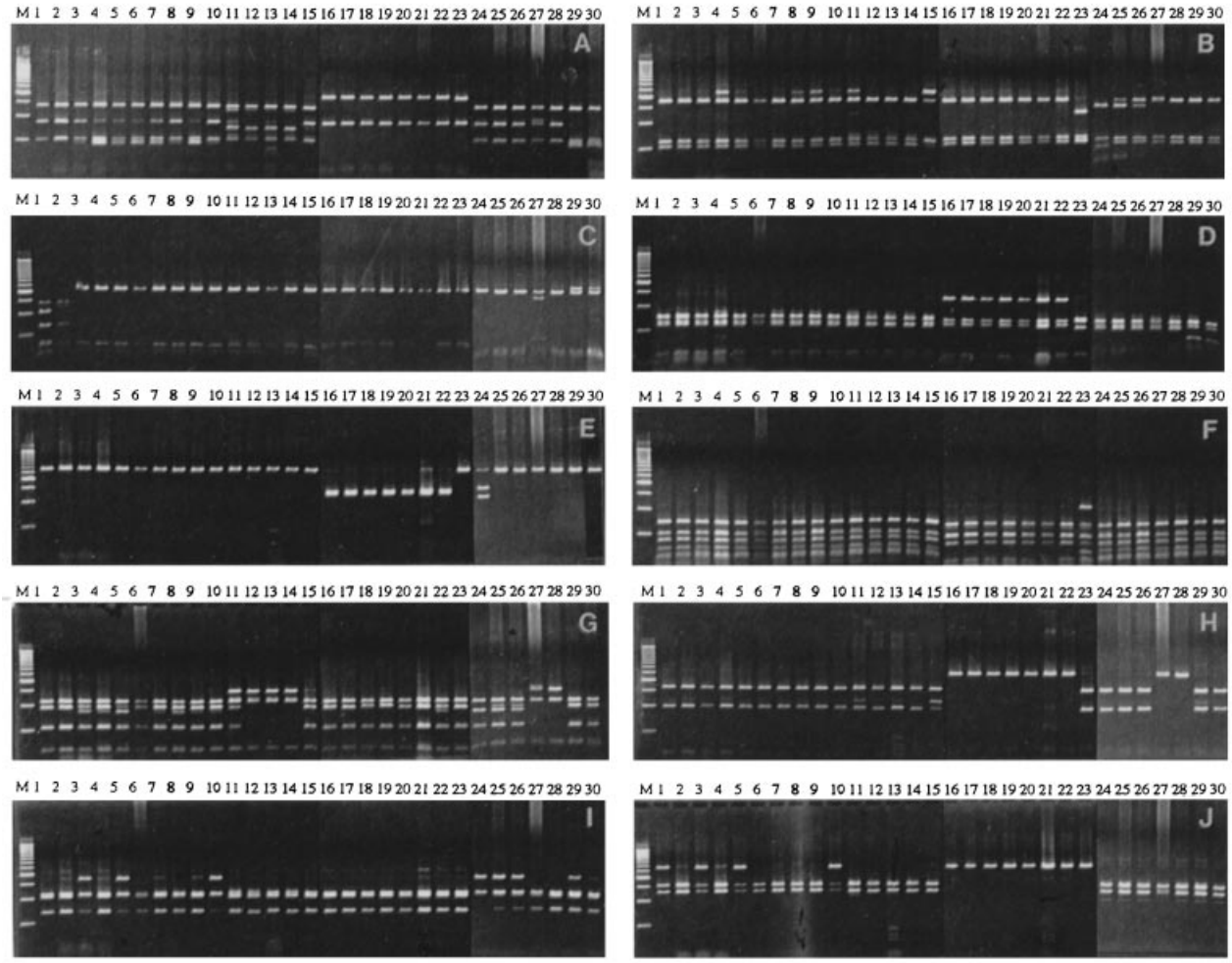

Fig. 1. RFLP analysis of 43 kDa E–S protein gene. M: M_r, 500 Base-Pair Ladder of molecular weight marker; lanes 1–10: *Trichinella britovi* (strain codes: ISS408, ISS409, ISS63, ISS271, ISS99, ISS173, ISS119, ISS201, ISS234 and ISS2); lanes 11–15: *Trichinella nativa* (strain codes: ISS410, ISS70, ISS10, ISS181 and ISS72); lanes 16–22: *Trichinella spiralis* (strain codes: ISS413, ISS412, ISS411, ISS407, ISS248, ISS160 and ISS154); lane 23: *Trichinella nelsoni* (strain code: ISS37); lanes 24–26: *Trichinella* T5 (strain codes: ISS35, ISS178 and ISS415); lanes 27–28: *Trichinella* T6 (strain codes: ISS40 and ISS339); lanes 29–30: *Trichinella* T8 (strain codes: ISS124 and ISS272). (A) *Rsa*I; (B) *Hha*I; (C) *Ssp*I; (D) *Dde*I; (E) *HinfI*; (*F*) *Alu*I; (G) *Hae*I; (H) *Hind*III; (I) *Mva*I; (J) *Pst*I.

lanes 3, 5 and 10) could be discriminated from the other *T. britovi* strains by a 1700 bp band, and a 1100 bp band which was apparently single in Italian strains, but not in the other strains of *T. britovi* with a different geographical origin. A 700 bp band was shared by 2 strains of T5 (lanes 25 and 26), but it was absent in another strain of T5 (lane 24).

Pst*I restriction*

Trichinella spiralis and *T. nelsoni* showed an uncleaved band of 2800 bp, whereas the other 5 phenotypes shared a band pattern (Fig. 1 J) of 2 bands of 1500 and 1300 bp, except some strains of *T. britovi* (lanes 1, 4, 6 and 10).

RFLP of DNA fragment amplified by primer pair SB147A

The SB147 DNA fragment (1250 bp) was produced by the amplification of the genomic DNA from 7 phenotypes (*T. spiralis*, *T. nativa*, *T. britovi*, *T. nelsoni*, T5, T6 and T8) by the primer pair SB147A. The amplicons were restricted by 2 endonucleases (Table 3).

Alu*I restriction*

This enzyme restriction produced 4 RFLP patterns (Fig. 2A) that can be summarized as follows: *T. nativa*, a pattern characterized by 2 bands of 1250 and 1150 bp; *Trichinella* T6, by a band of 1150 bp; *T. spiralis*, *T. nelsoni*, and T5, a similar band pattern characterized by 2 bands of 800 and 300 bp. *Trichinella britovi*, except the 2 Japanese strains and 1 Italian strain (ISS119), could be identified by a 1050 bp. The 2 Japanese strains of *T. britovi* showed a band pattern similar to that of *Trichinella* T8.

Msp*I restriction*

The 2 Japanese strains of *T. britovi* and 2 strains of

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Table 3. RFLP analysis of the SB 147 fragment, showing used restriction enzymes and bp size of resulting bands

Species (ISS)*	*Alu*I	*Msp*I
T. br (408)	1250, 1150, 800, 300	1250
T. br (409)	1250, 1150, 800, 300	1250
T. br (63)	1250, 1150, 1050, 800, 300	750, 500
T. br (271)	1250, 1150, 1050, 800, 300	750, 500
T. br (99)	1250, 1150, 1050, 800, 300	750, 500
T. br (173)	1250, 1150, 1050, 800, 300	750, 500
T. br (119)	1250, 1150, 800, 300	750, 500
T. br (201)	1250, 1150, 1050, 800, 300	750, 500
T. br (234)	1250, 1150, 1050, 800, 300	750, 500
T. br (2)	1250, 1150, 1050, 800, 300	750, 500
T. na (410)	1250, 1150	750, 500
T. na (70)	1250, 1150	750, 500
T. na (10)	1250, 1150	750, 500
T. na (181)	1250, 1150	750, 500
T. na (72)	1250, 1150	750, 500
T. sp (413)	800, 300	750, 500
T. sp (412)	800, 300	750, 500
T. sp (411)	800, 300	750, 500
T. sp (407)	800, 300	750, 500
T. sp (248)	800, 300	750, 500
T. sp (160)	800, 300	750, 500
T. sp (154)	800, 300	750, 500
T. ne (37)	800, 300	750, 500
T5 (35)	800, 300	750, 500
T5 (178)	800, 300	1250, 750, 500
T5 (415)	800, 300	1250, 750, 500
T6 (40)	1150	750, 500
T6 (339)	1150	750, 500
T8 (124)	1150, 800, 300	750, 500
T8 (272)	1150, 800, 300	750, 500

* *Trichinella britovi* (T. br), *Trichinella nativa* (T. na), *Trichinella spiralis* (T. sp), *Trichinella nelsoni* (T. ne), *Trichinella* T5 (T5), *Trichinella* T6 (T6), and *Trichinella* T8 (T8).

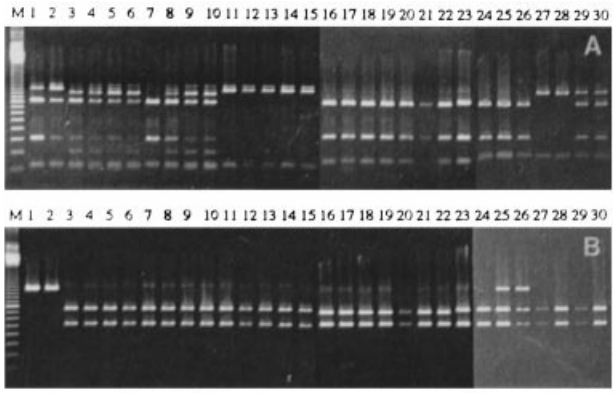

Fig. 2. RFLP analysis of the SB147 RAPD fragment. M: M_r, 100 Base-Pair Ladder of molecular weight marker; lanes 1–30; the arrangement is the same as reported in Fig. 1. (A) *Alu*I; (B) *Msp*I.

T5 (ISS178 and 415) showed a 1250 bp band (Fig. 2B). All phenotypes shared 2 bands of 750 and 500 bp, except the 2 Japanese strains.

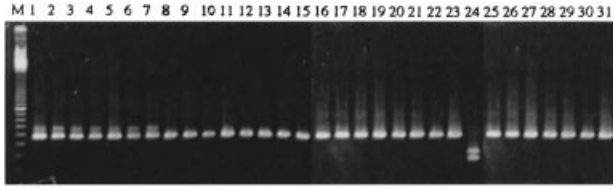

Fig. 3. RFLP analysis of the SB372 RAPD fragment with the *Msp*I restriction enzyme. M: M_r, 100 Base-Pair Ladder of molecular weight marker; lanes 1–24; the arrangement is the same as reported in Fig. 1; lane 24: *Trichinella pseudospiralis* (strain code: ISS13); lanes 25–27: *Trichinella* T5 (strain codes: ISS35, ISS178 and ISS415); lanes 28–29: *Trichinella* T6 (strain codes: ISS40 and ISS339); lanes 30–31: *Trichinella* T8 (strain codes: ISS124 and ISS272).

RFLP of DNA fragment amplified by the primer SB372A

The SB372 DNA fragment (372 bp) was obtained by amplifying the genomic DNA from all *Trichinella* phenotypes by the primer pair SB372A. The amplicons were restricted with *Msp*I, and only the strain of *T. pseudospiralis* produced 2 cleaved bands of 200 and 170 bp (Fig. 3).

Checking the gene flow

There is gene flow within the species (Pozio *et al.* 1997) that disturbs the usefulness of the RFLP analysis of pooled larvae. Therefore, we checked the possibility of gene flow within the species by means of RFLP of the gene encoding 43 kDa ES protein and RFLP of DNA fragment amplified by primer SB147A. Enzymes used included *Rsa*I, *Hha*I, *Ssp*I, *Dde*I, *Hin*fI, *Alu*I, *Hae*III, *Hin*dIII, *Mva*I, *Pst*I and *Msp*I. Single larvae of *T. britovi* (ISS408, Japan; ISS409, Japan; ISS2, Italy; ISS324, France), *T. nativa* (ISS410, Arctic), *T. spiralis* (ISS413, Poland), T5 (ISS178, France), T6 (ISS339, USA) and T8 (ISS124, South Africa) gave the same results as pooled larvae (data not shown), which suggested that these isolates have no gene flow.

DISCUSSION

Results from this and previous papers (Bandi *et al.* 1993, 1995) suggested no evidence of the gene flow in isolates including *T. spiralis* (ISS413, ISS160 and ISS154), *T. britovi* (ISS408, ISS409 and ISS2), *T. nativa* (ISS37), *T. nelsoni* (ISS37), *T. pseudospiralis* (ISS13), T5 (ISS35 and ISS178), T6 (ISS40) and T8 (ISS124 and ISS272). Therefore, RFLP patterns obtained from these samples are supposed to be reference ones.

Previously, we constructed 19 pairs of PCR primers that can be used to identify *Trichinella* phenotypes (Wu *et al.* 1997, 1998). The present study shows that PCR–RFLP is a promising method

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to identify and fingerprint *Trichinella* isolates at the species and/or strain level. *Trichinella nativa* can be identified by the endonuclease *Rsa*I restriction of the 43 kDa ES protein gene (Ts43CA fragment), or by the *Alu*I restriction of SB147 DNA fragment. No single enzyme restriction allows identification of *T. britovi* isolates, although some strains, except the 2 Japanese ones, can be identified by the *Alu*I restriction of the SB147 fragment. The 2 Japanese strains can be identified by *Ssp*I restriction of the Ts43CA fragment or *Msp*I restriction of the SB147 fragment. *Trichinella britovi* and T8 show a specific band pattern by the *Alu*I restriction of the SB147A fragment, and they can be differentiated by the *Ssp*I restriction of the Ts43CA fragment. *Trichinella* T6 can be identified by *Alu*I restriction of the SB147 DNA fragment.

The RFLP method further supports the presence of an intraspecies polymorphism in *Trichinella* phenotypes, previously observed in allozyme patterns by La Rosa *et al*. (1992) for *T. spiralis*, *T. nativa* and *T. britovi*, and by Snyder *et al*. (1993) for *Trichinella* T5, and in DNA patterns by Zarlenga *et al*. (1991) for *T. spiralis*, *Trichinella* T5 and T6, and by Zarlenga *et al*. (1996) for *T. pseudospiralis*, and by Bandi *et al*. (1995) for several phenotypes. Strains of *T. britovi*, T5 and T6 are examples. Seven enzyme restrictions (*Dde*I, *Hha*I, *Hin*fI, *Hin*dIII, *Alu*I, *Pst*I, and *Mva*I) of the Ts43CA fragment confirm that the genotype of the 2 Japanese strains belongs to *T. britovi*, as reported by Pozio *et al*. (1996), whereas 5 enzyme restrictions (*Rsa*I, *Hae*III and *Ssp*I) of the Ts43CA fragment and (*Msp*I, *Alu*I) of the SB147 DNA fragment show differences between the genotype of *T. britovi* strains from Japan and from Europe, suggesting a geographical isolation of these strains. Furthermore, the 3 Italian strains of *T. britovi* also show some genotype differences from the other strains of *T. britovi* when the Ts43CA fragment is restricted by *Mav*I.

Four enzyme restrictions (*Hae*III, *Hha*I, *Hinf*I and *Mva*I) of the Ts43Ca fragment exhibit differences between genotypes of T5 from Pennsylvania and from Connecticut and Indiana (USA).

A genetic variability between 2 strains of T6 is shown by analysing the Ts43Ca fragment with *Ssp*I or *Rsa*I restriction enzymes.

Although these genetic differences are not enough to justify a revision of the taxonomy of the genus *Trichinella*, it may be useful in identifying subspecies or geographical strains. The most useful application of this RFLP method seems to be the identification of routes of infection. For this purpose, fingerprint libraries of *Trichinella* isolates have to be available. Furthermore, testing more restriction endonucleases and a high number of strains, it could be possible to establish such fingerprint libraries.

This study shows that the 8 phenotypes recognized in the genus *Trichinella* previously on allozyme analysis and biological characteristics (Pozio *et al*. 1992), correspond to 8 genotypes that can be identified by the RFLP analysis. The DNA polymorphism revealed by the RFLP method seems to be important for genetic and epidemiological investigations and for the construction of the taxonomy of the genus *Trichinella*.

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