

Use of Nuclear Microsatellites in Genetic Variability Assessment of *Trichinella* Isolates

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Abstract. Trichinellosis is a food-borne parasitic zoonosis with a yearly incidence of about 10,000 clinical cases worldwide. It is one of the most serious zoonotic diseases in Romania with more than 28,000 human cases reported over the last 25 years. *Trichinella* species and genotypes are present on all continents, from the tropical regions to the cold ones. Up to date, there have been identified four *Trichinella* species in Europe: *T. spiralis*, *T. nativa*, *T. britovi* and *T. pseudospiralis*. In order to identify a genetic variability in ecologically distinct species of *Trichinella*, which may lead to a potential geographical disposition of *Trichinella* isolates, we test the variability of nine nuclear microsatellite loci, in a small population of *Trichinella* isolates of Romania. As a result, we obtained a positive amplification for most of the microsatellites studied for *Trichinella* strains belonging to different species (*T. spiralis*, *T. britovi*). We noticed a size-difference between the amplicons generated by the DNA of various *Trichinella* species (*T. spiralis* vs *T. britovi*). We identify a genetic variability inside the same *Trichinella* population (*T. spiralis*), differentiating 3 strains (7.97, 7.42, 7.29) by 2 microsatellites (TP43, TP32). For a more detailed analysis of the differences obtained in this study, we plan to use the fluorescently (6-FAM) marked primers, for sequencing and genotyping of the amplification products.

Keywords: Trichinellosis, microsatellites, genetic variability

INTRODUCTION

Trichinellosis is a food-borne parasitic zoonosis with a yearly incidence of about 10,000 clinical cases worldwide (Pozio, 2007). It is one of the most serious zoonotic diseases in Romania with more than 28,000 human cases reported over the last 25 years (Blaga et al., 2007). Up to date, eight species and four genotypes of *Trichinella* have been identified worldwide (Murell et al., 2000; Pozio and Zarlenga, 2005). *Trichinella* species and genotypes are present on all continents, from the tropical regions to the cold ones. Following the great number of epidemiological surveys undertaken in the period of time 2000-2006 within the framework of the European projects TRICHIPORSE and TRICHINET (MEDVETNET), there have been identified four *Trichinella* species in Europe: *T. spiralis*, *T. nativa*, *T. britovi* and *T. pseudospiralis*. *Trichinella spiralis*, with predominant hosts of domestic and sylvatic swine, synanthropic animals and a broad range of sylvatic carnivores (Pozio, 2001; Dick and Pozio, 2001); *Trichinella nativa* affecting mostly the sylvatic carnivores living in the frigid zones of Europe (Pozio and Zarlenga, 2005); *Trichinella britovi* identified mostly in the sylvatic carnivores of the temperate areas of Europe; *Trichinella pseudospiralis* with domestic hosts and sylvatic mammals as well as birds, and is present in Finland, France, Georgia, Germany, Italy, Lithuania, Netherlands, Slovakia, Sweden and Russia (Pozio and Zarlenga, 2005; Nockler et al., 2006). In Romania, during various epidemiological surveys, only two *Trichinella* species have been identified in domestic and wild animals: *T. spiralis* and *T.*

britovi. (Blaga et al.; 2009). Simple-sequence repeats (SSRs) or microsatellites are to be found in the polymorphic loci within the nuclear DNA and consist of repeating units of 1-6 base pairs. (Turnpenny and Ellard, 2005). They are usually neutral and co-dominant and are used as molecular markers which have wide-ranging applications in the field of genetics, including gene dosage and population studies. Such an example is the recent study published by Rosenthal et al., (2008), where they used nine nuclear microsatellite loci and sequenced one-fifth of the mitochondrial genome from the broadest population sample yet attempted for species of *Trichinella*, involving twenty-eight countries on four continents, in order to compare the extent and pattern of genetic variability in ecologically distinct species of *Trichinella*.

The geographical assignment of *Trichinella* isolates could simplify the field-work of veterinaries in the case of trichinellosis outbreaks, given the globalization and intensification of world-wide trade. In order to identify a genetic variability in ecologically distinct species of *Trichinella*, which may lead to a potential geographical disposition of *Trichinella* isolates, we test the variability of nine nuclear microsatellite loci, in a small population of *Trichinella* isolates of Romania.

MATERIALS AND METHODS

Specimens

In the period of time March 2008 - January 2009, meat samples were taken from 230 pigs suspected of being infected with *Trichinella* and originating from several different farms, households, as well as meat samples taken from wild animals, such as 34 foxes, 34 rats, 24 wild-boars, 9 wolves, 4 jackals, 2 bears and 1 lynx. Out of them, 11 isolates of *Trichinella* were picked up randomly to be used in this study. They are described in detail (code, original host, country of origin) in Tab.1. They were maintained by serial passages in OF1 female mice under specific quality control. Muscle larvae (ML) were recovered from muscle tissue of infected mice by a standard pepsin-HCl digestion method (Gamble et al., 2000).

Tab. 1

The results of artificial digestion

Nr.	Species numbers	Grame digerate	Larvae numbers	LPG	Origin	Date
1	7.03	39.41	323300	8203.50	Fierbinți, Ialomița County	19.11.08
2	7.18	39.57	97500	2463.99	Fierbinți, Ialomița County	24.11.08
3	7.21	38.7	165000	4263.57	Fierbinți, Ialomița County	17.11.08
4	7.29	49.2	76675	1558.43	Fierbinți, Ialomița County	24.11.08
5	7.30	46.8	95000	2029.91	Fierbinți, Ialomița County	17.11.08
6	7.42	35.05	150000	4279.60	Fierbinți, Ialomița County	20.11.08
7	7.68	43.05	121675	2826.36	Fierbinți, Ialomița County	20.11.08
8	7.97	29.51	40000	1355.47	Fierbinți, Ialomița County	19.11.08
9	Wolf 1	154.55	8000	51.76	Mures County	14.01.09
10	Wolf 4	95.6	2450	25.63	Cluj County	17.02.09
11	jackal	49	6817	139.12	Danube Delta	20.02.09

DNA extraction

Genomic DNA was obtained from small pools (approximately 100 ML) of *Trichinella* isolates, using the “QIAamp DNA” kit (Qiagen, Germany), according to the manufacturer’s instructions for mouse- or rat-tail protocols.

PCR amplification

The primers used in this study, for each locus, and the repeat motif they flank, are specified in Tab. 2. (Rosenthal et al., 2008). PCR reactions were performed using either EX Taq kit (Takara, France) or the Mi Taq Mix (Metabion, Germany). PCR was performed in a 25µl reaction volume containing 2,5 µl buffer; 2,00 µl dNTP; 0,125 µl Taq polymerase; 1,5 µl of each forward and reverse primer; 5 µl AND (50 ng); 12,375 µl ultrapure water for EX Taq kit (Takara, France) and 12,5 µl Mi Taq Mix; 1,5 µl of each forward and reverse primer; 5 µl AND (50 ng); 4,5 µl ultrapure water for Mi-Taq Mix (Metabion, Germany). All reactions were performed in a MyGenie96 Thermal Block (Bioneer, Korea) under the following PCR conditions: one cycle at 94°C for 5 min, 35 cycles at 94°C for 30 seconds, 53°C for 45 seconds, 72°C for 1.5 min, one extension cycle at 72°C for 10 min and storage at +4°C. The amplicons were electrophoresed on a 1% agarose gel (Sigma-Aldrich, St. Louis, Missouri, USA) containing 3.5 µl of Syber-Safe (Invitrogen, USA) and visualized under an ultraviolet transilluminator.

Tab. 2

Primers’ sequences

Locus	Primer	MW	Sequence	Repetitive structure	Bp	GC %	Tm
1	TP1 F	6095	GCGCGATTACGACACTACAA	TTAA	20	50	58
	TP1 R	5979	ATTCGCCACTGTCACTTTCC	TTAA	20	50	58
2	TP5 F	5766	TACATGGCCCACAGCAAAT	TTA	19	47.4	55
	TP5 R	6184	GATGGCCACCAGGTAAGAAA	TTA	20	50	58
3	TP19 F	6208	AGGAAGATCAAGCGGCAATA	CAA	20	45	56
	TP19 R	6141	CACGAGTTTGCCTGATGAAA	CAA	20	45	56
4	TP 26 F	6463	GACGTTCAAGAAACGAATGCT	AAC	21	42.9	57
	TP 26 R	6108	GGATAACCCTCGGCGTATTT	AAC	20	50	58
5	TP28 F	6111	TCGTTTTTCGTGCTTGATTG	TTAAAA	20	40	54
	TP28 R	6179	CGGACTTGGTTGCTGTTGA	TTAAAA	20	50	58
6	TP 32 F	6451	GCGGGTGAAAAATTTCTCTTT	TG	21	38.1	55
	TP32 R	6112	TCAGTCGAAGCAAACCAAAA	TG	20	40	54
7	TP 43 F	6086	TACAGGCGTTCGACACAATC	TA	20	50	58
	TP 43 R	6139	AGCGCTGAGGTGTCTTTCAT	TA	20	50	58
8	TP 47 F	6182	GAACAGCTTCGGTAGGATGC	TA	20	55	60
	TP 47R	6187	TGAATGGCGTGTGTTGACAAT	TA	20	40	54
9	TP 53 F	6110	TTGCACAAGTGCGAAAATC	TG	20	45	56
	TP 53 R	6197	TGGGTGTGATAGCAACCAGT	TG	20	50	58

RESULTS AND DISCUSSION

The results of PCR amplification with EX-Taq kit (Takara, France), using the genomic DNA of isolates 7.03, 7.21 and 7.68, for all nine microsatellites (TP1, TP5, TP19, TP26, TP28, TP32, TP43, TP47, TP53) are summarized in Tab. 3.

Tab. 3

Amplification results using EX-Taq kit

ADN /Primer	TP1F +TP1R	TP5F +TP5R	TP19F +TP19R	TP26F +TP26R	TP28F +TP28R	TP32F +TP32R	TP43F +TP43F	TP47F +TP47R	TP53F +TP53R
7.03	+	+	+	+	+	+	+	+	+
7.21	+	+	+	+	+	+	+	+	+
7.68	+	+	+	+	+	+	+	+	+

The results of PCR amplification with mi-Taq mix Kit (Metabion, Germany) using the genomic DNA of *Trichinella* isolates 7.97, 7.42, 7.29, 7.30, 7.18, wolf 1, wolf 4 and jackal, for all nine microsatellites, are summarized in Tab. 4.

Tab. 4

Amplification results using mi-Taq Mix kit

AND /Primer	TP1F +TP1R	TP5F +TP5R	TP19F +TP19R	TP26F +TP26R	TP28F +TP28R	TP32F +TP32R	TP43F +TP43F	TP47F +TP47R	TP53F +TP53R
7.97	-	-	-	-	-	-	+	-	-
7.42	-	+	+	+	+	-	+	-	+
7.29	-	+	+	+	+	+	+	-	+
7.30	+	+	+	+	+	+	+	+	+
7.18	+	+	+	+	+	+	+	+	+
Wolf 1	+	+	+	+	+	+	+	+	+
Wolf 4	-	+	+	+	+	+	+	+	+
jackal	+	+	+	+	+	+	+	+	+

The above tables (tab. 3 and tab. 4) indicate that all nine microsatellites can be amplified for both *Trichinella spiralis* and *Trichinella britovi* isolates. At a closer look, the results of the study indicate that strains 7.03, 7.21, 7.68, 7.30, 7.18, wolf 1 and jackal can be amplified by all 9 microsatellites (TP1, TP5, TP19, TP26, TP28, TP32, TP43, TP47, TP53); strain 7.42 can be amplified by 6 of 9 microsatellites (TP5, TP19, TP26, TP28, TP43, TP53); strain 7.29 can be amplified by 7 of 9 microsatellites (TP5, TP19, TP26, TP28, TP32, TP43, TP53), whereas the jackal strain can be amplified by 8 of 9 microsatellites (TP5, TP19, TP26, TP28, TP32, TP43, TP47, TP53). Interestingly, strain 7.97 can only be amplified by microsatellite TP43, even though it originates from the same trichinellosis outbreak as strains 7.03, 7.21, 7.68, 7.30, 7.18, 7.42, 7.29, respectively from the same pig farm (Fierbinți, Ialomița) and from the same pig batch. In order to explain this results two hypothesis may be explored: first of all, this result could be explained by an inappropriate DNA extraction; still, according to the quantification results of genomic DNA extraction of various *Trichinella* isolates, it is obvious that the DNA has a good concentration (25,34 ng/μl). The purity of the sample (ratio 260/230=2.1) shows a certain level of protein contamination which, however, does not fully interfere with the amplification the way it does in case of the other samples (7.21, 7.68, 7.42, 7.30, 7.18) which indicate a similar purity, but show a positive amplification for most of the microsatellites. Secondly, there could be another explanation: the amplification was not performed optimal; still, according to tab. 4, "Amplification results using mi-Taq Mix kit", the results are positive for more than half of the tested samples. Leaving out the two assumptions, we conclude that microsatellite TP43 is specific for strain 7.97.

Furthermore, strains 7.42 and 7.29 are different from each other by one single

microsatellite: both strains can be amplified by microsatellites TP5, TP19, TP26, TP28, TP43, TP53; in comparison to strain 7.42, strain 7.29 can also be amplified by microsatellite TP32 which is peculiar to this strain. In contrast with these two strains, the jackal strain can also be amplified by microsatellite TP47, leading thus to a distinction among these strains.

As to the length of the amplicons, following a comparison using the molecular weight marker, it appeared that the lengths are similar for all *Trichinella spiralis* strains (7.03; 7.21; 7.68; 7.97; 7.42; 7.29; 7.30; 7.18) and for all *Trichinella britovi* strains (wolf 1; wolf 4; jackal). A size-difference has been noticed, however, between the amplicons generated by the DNA of *T. spiralis* and *T. britovi*, difference that might be explained by the existence of a genetic variability between the two *Trichinella* species, mentioned previously (Zarlenga et al., 1996; Pozio and Zarlenga, 2005).

CONCLUSIONS AND PERSPECTIVES

The research undertaken for the identification of a genetic variability inside the genus *Trichinella*, using simple-sequence repeats (microsatellites) as a molecular method to study isolates, shows the following: we obtained a positive amplification for most of the microsatellites studied for *Trichinella* strains belonging to different species (*T. spiralis*, *T. britovi*); we noticed a difference in size between the amplicons generated by the DNA of various *Trichinella* species (*T. spiralis* vs *T. britovi*); we identify a genetic variability inside the same *Trichinella* population, differentiating strains 7.97, 7.42, 7.29, by microsatellites TP43, TP32.

As future goals, we plan a more detailed analysis of the differences obtained in this study by using the fluorescently (6-FAM) marked primers ideal for sequencing and genotyping of the amplification products.

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