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Trichinella patagoniensis n. sp. (Nematoda), a new encapsulated species infecting carnivorous mammals in South America ☆

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Until a few years ago, *Trichinella spiralis* was the only taxon of the genus *Trichinella* detected in both domestic and wild animals of South America. Recently, a new genotype, named *Trichinella* T12, was identified in cougars (*Puma concolor*) from Argentina, on the basis of molecular studies using mitochondrial and nuclear ribosomal markers. In the present study, cross-breeding experiments indicated that *Trichinella* T12 is reproductively isolated from all other encapsulated *Trichinella* spp. and suggested that it is biologically more similar to *Trichinella britovi* and *Trichinella murrelli* than to the other encapsulated species/genotypes. Biological assays revealed that the reproductive capacity index of *Trichinella* T12 was ~4 and >2000 times lower than those of *T. spiralis* in mice and rats, respectively. The reproductive capacity index of *Trichinella* T12 in domestic pigs ranged from 0.0 to 0.05. Larvae parasitising the muscles of carnivores were infective to mice after freezing at −5 °C for 3 months, but they lost infectivity after freezing at −18 °C for 1 week. The region within the rDNA, known as the expansion segment V, showed a unique sequence which differs from those of all other known *Trichinella* spp./genotypes. The biological, geographical and molecular data support the classification of the genotype *Trichinella* T12 as a new species widespread in the Neotropical region, for which we propose the name *Trichinella patagoniensis* n. sp.
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1. Introduction

Nematodes of the genus *Trichinella* are zoonotic parasites with a cosmopolitan distribution. Two main clades have been identified in this genus: the encapsulated clade with five species (*Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi*, *Trichinella murrelli* and *Trichinella nelsoni*) and four genotypes (*Trichinella* T6, *Trichinella* T8, *Trichinella* T9 and *Trichinella* T12) infecting only mammals; and the non-encapsulated clade with one species (*Trichinella pseudospiralis*) infecting mammals and birds and two species (*Trichinella papuae* and *Trichinella zimbabwensis*) infecting mammals and reptiles (Pozio et al., 2009). Until recently, only *T. spiralis* had been identified in domestic and wild animals in South America (Pozio, 2000; Krivokapich et al., 2006; Ribicich et al., 2010). This parasite is of European origin, as indicated by microsatellite studies (Rosenthal et al., 2008; La Rosa et al., 2012). Larvae of a *Trichinella* sp. isolated from a cougar (*Puma concolor*) in Argentina in 2004 showed the mitochondrial cytochrome *c*-oxidase (cox-1) gene and the nuclear 5S ribosomal intergenic spacer region (5S-ISR) to be different in sequence from those of the other *Trichinella* taxa, and was thus named *Trichinella* T12 (Krivokapich et al., 2008).

In the present paper, we describe the morphological, biological and molecular characteristics of three isolates of the *Trichinella* T12 genotype from cougars. Reproductive and molecular data provide support for the recognition of these isolates as a separate species within the genus *Trichinella*.

2. Materials and methods

2.1. Parasite origin and collection

Larvae of *Trichinella* spp. were collected from the striated muscles of four cougars hunted in Argentina: (i) near the locality of Trapalcó (Rio Negro province, 66°59′W, 39°34′S) in 2004. Only

☆ New nucleotide sequence data reported in this paper are available in the GenBank database under Accession codes JF260985–JF2601000 and HE819395.

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dead larvae (worm burden in the hind limb muscles, one larva/g) were collected (isolate code ISS1826), preserved in absolute ethanol, and then identified by sequencing of the cox-1 gene and the 5S-ISR as a new genotype, *Trichinella* T12 (Krivokapich et al., 2008); (ii) near the locality of El Calafate (Santa Cruz province, 72°16′W, 50°20′S; five larvae/g in the intercostal muscles) in 2008. This *Trichinella* isolate (code ISS2311) is maintained by serial passage in laboratory mice at the International *Trichinella* Reference Centre (ITRC), Rome, Italy; (iii) from the district of La Paz (Catamarca province, 65° 4′W, 29°17′S) in 2009. Only dead larvae (worm burden in the tongue, 6.6 larvae/g) were collected (isolate code ISS3559) and preserved in absolute ethanol; and (iv) from the district of La Paz (Catamarca province, 65°4′W, 29°17′S; nine larvae/g in the tongue) in 2009. This isolate was identified as *T. spiralis* (code ISS3558) and was used for comparative biological study. *Trichinella* larvae from natural and laboratory hosts were obtained by the standard artificial digestion method (Gamble et al., 2000).

2.2. PCR

Genomic DNA was extracted from single muscle larvae following a previously published protocol (Krivokapich et al., 2006). DNA from single larvae from the isolates ISS2311, ISS3558 and ISS3559 was subjected to multiplex PCR for identification (Zarlenga et al., 1999; Pozio and La Rosa, 2010). For the sequence analysis of the cox-1 and 5S-ISR markers, DNA was isolated from a pool of muscle larvae and then amplified by PCR according to published protocols (Nagano et al., 1999; Rombout et al., 2001). The region within the lsrDNA, known as the expansion segment V (ESV), was amplified using the primer pair TS10177-F (5′-TAAGAAAACGGCGAAAGC-3′) and TS10177-R (5′-AACCCCACAGAGAGATTAAAG-3′). PCR amplifications were performed in 30 μL of a standard reaction mix (Qiagen, Germany), 10 pmol of each primer and 10 μL out of 100 μL of a DNA sample purified from a single larva. The amplification was carried out for 37 cycles as follows: 95 °C for 30 s, 56 °C for 60, and 72 °C for 30 s, plus a pre-step at 95 °C for 5 min and a post-step at 72 °C for 5 min. Twelve larvae from each of the *Trichinella* isolates ISS2311 and ISS3559 were tested individually. The PCRs were performed in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA).

2.3. Nucleotide sequencing and sequence analysis

The PCR products from the ESV region of the 12 larvae from the *Trichinella* isolates ISS2311 and ISS3559 were sequenced in both directions using the same primers as those employed for the amplification. The amplification products of the cox-1 and 5S-ISR of the two isolates (ISS2311, ISS3559) were cloned using a Topo TA Cloning Kit into a pCR 2.1-TOPO vector (Invitrogen, Brazil). Three positive clones from each of the two markers (cox-1, 5S-ISR) were sequenced in both directions, using universal M13 forward and reverse primers, by an ABI PRISM 3100-Avant sequencer (Applied Biosystems). Sequences were analysed with the Chromas Lite 2.0 (Technelysium Pty Ltd, Australia) software. Multiple sequence alignments, representing each of the three markers, were performed using ClustalW (Thompson et al., 1994) using the default settings. DNA sequences for cox 1, ESV and 5S-ISR were deposited in GenBank under the Accession Nos. JF260987–JF260992, HE819395 and JF260995–JF261000, respectively (Table 1).

2.4. Phylogenetic analysis

A total of 21 and 20 sequences were used for the phylogenetic analysis of cox-1 and 5S-ISR genes, respectively, corresponding to all of the *Trichinella* taxa known to date and the new *Trichinella* isolates reported herein (Table 1). Sequences of the nematode

Table 1
GenBank accession numbers of cytochrome *c*-oxidase subunit I (cox-1) gene and 5S rDNA intergenic spacer region (5S-ISR) gene sequences of all *Trichinella* taxa used for the phylogenetic analysis in this study.

Trichinella taxa	Isolate[a]	GenBank accession number for cox-1	Isolate[a]	GenBank accession number for 5S-ISR
Trichinella spiralis	ISS248	DQ007890[b]	NA	AY009946[c]
Trichinella nativa	ISS70	DQ007891[b]	NA	AY009944[c]
Trichinella britovi	ISS271	DQ007892[b]	NA	AY009943[c]
Trichinella pseudospiralis	ISS13	DQ007893[b]	NA	AY009950[c]
Trichinella murrelli	ISS470	DQ007894[b]	NA	AY009947[c]
Trichinella T6	ISS40	DQ007895[b]	NA	AY009948[c]
Trichinella nelsoni	ISS37	DQ007896[b]	NA	AY009945[c]
Trichinella T8	ISS149	DQ007897[b]	NA	AY009949[c]
Trichinella T9	ISS408	DQ007898[b]	NA	NA
Trichinella papuae	ISS572	DQ007899[b]	ISS 572	AY845861
Trichinella zimbabwensis	ISS1029	DQ007900[b]	ISS1029	AY845862
Trichinella T12	ISS1826	EU161657[d]	ISS1826	EF694983[d]
Trichinella T12	ISS1826	JF260985[e]	ISS1826	JF260993[e]
Trichinella T12	ISS1826	JF260986[e]	ISS1826	JF260994[e]
Trichinella T12	ISS2311	JF260987[e]	ISS2311	JF260995[e]
Trichinella T12	ISS2311	JF260988[e]	ISS2311	JF260996[e]
Trichinella T12	ISS2311	JF260989[e]	ISS2311	JF260997[e]
Trichinella T12	ISS3559	JF260990[e]	ISS3559	JF26098[e]
Trichinella T12	ISS3559	JF260991[e]	ISS3559	JF26099[e]
Trichinella T12	ISS3559	JF260992[e]	ISS3559	JF261000[e]

NA: isolate and/or sequence data not available in GenBank.
[a] Isolate code of the International Trichinella Reference Centre, Rome, Italy (www.iss.it/site/Trichinella/index.asp).
[b] Zarlenga et al. (2006).
[c] Rombout et al. (2001).
[d] Krivokapich et al. (2008).
[e] Present work.

Enterobius vermicularis, of the family Oxyuridae, were used as an outgroup for comparison (GenBank Accession Nos. EU281143 for cox-1 and U65496 for 5S-ISR).

The nucleotide divergence was based on the proportion of nucleotide sites at which the compared sequences were different (*p*-distance); the analysis was conducted using the program MEGA v. 4.0.1 (Tamura et al., 2007). Neighbour-Joining (NJ) trees were constructed from matrices of genetic distances in MEGA v. 4.0.1 (Tamura et al., 2007).

2.5. Morphological study

Muscle larvae and adults of isolate ISS2311 were fixed in 70% ethanol. Measurements were taken and averaged from: (i) 30 male and 30 female muscle larvae recovered from the fifth passage in mice, obtained by artificial digestion of the carcass of a CF-1 mouse that had been infected for 35 days; and (ii) 30 male and 30 female adult worms recovered from the gut of a CF-1 mouse 6 days p.i., which had been infected with 1000 larvae from the fifth passage in mice. The following morphometric variables were examined in muscle larvae: total body length, width at mid-body, length of oesophagus, length of stichosome, length of genital primordium, length of rectum, and distance from posterior margin of genital primordium to posterior extremity of body. Morphometric variables taken from adults were: total body length, width at mid-body, length of oesophagus, length of stichosome, length of uterus, length of ovary, length of testis, and length and width of copulatory appendages. These measurements were compared with those reported for other species of *Trichinella* in the literature (Dick, 1983; Pozio et al., 1992a; Murrell et al., 2000; Pozio and La Rosa, 2000).

2.6. Cross-breeding experiments

Muscle larvae were sorted by sex (Pozio et al., 1999) and five mice (CD1 females of 25 g each) were inoculated by gavage

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connected to a 1-ml syringe, with one male and one female muscle larva belonging to different species in each direction (i.e., male of isolate ISS2311 per female of other species and male of the other species per female of isolate ISS2311). Two mice were inoculated with a single male and female muscle larva belonging to the same species as a control. To ensure that no larvae remained in the syringe or tube, both were rinsed twice with water and then examined under an inverted microscope. All mice were immunosuppressed with 4 mg each of cyclophosphamide at 0, 4 and 8 days p.i. to improve the probability of producing offspring, since laboratory mice do not serve as natural hosts for some *Trichinella* spp. Mice were killed 40 days p.i., and the entire skinned and eviscerated carcasses were digested individually. The fertility of the F1 generation produced in these experiments was assessed by cross-breeding experiments involving single female and male larvae, and 100 pooled larvae of both sexes in mice.

2.7. Reproductive capacity index in mice and rats

Five female Wistar rats and six male CF-1 mice were inoculated per os with 2000 and 300 muscle larvae of the *Trichinella* isolate ISS2311, respectively. As controls, five rats and three mice were infected with the same number of *T. spiralis* larvae (isolate ISS3558). Larvae of both isolates, from the third passage, were isolated by artificial digestion of the carcasses of CF-1 mice. The rats were euthanised at 30 days p.i. and mice at 5 weeks p.i. Each animal was skinned, eviscerated and the entire carcass was digested individually and muscle larvae were counted. The reproductive capacity index (RCI) was calculated as the number of muscle larvae recovered divided by the number of larvae inoculated. Arithmetic averages and S.D.s of larvae per gram of muscle (LPG) and RCI were calculated for mice and rats.

2.8. Infectivity to domestic pigs

Four male pigs (Yorkshire × Landrace), 5 weeks of age (average weight 9.25 kg), were inoculated per os with 20000 muscle larvae of the *Trichinella* isolate ISS2311. Two pigs were used as uninfected controls. Necropsies were performed 5 weeks p.i., when the pigs weighed between 20 and 23 kg. Muscle samples were minced using a grinder and the LPG was assessed after the digestion of 100 g of each of six muscle groups: shoulder, foreleg, intercostals, abdomen, hind leg and filet, and 33–88 g of each of three other muscles: diaphragm, tongue and masseter. To evaluate the RCI of the *Trichinella* isolate in each pig, all of the striated muscles were mixed together except for those already collected to determine LPG (see above). The muscle mixture from each pig weighed 4183, 5634, 5708 and 5818 g, and their homogenisation was performed by mincing. Then, a sample of 500 g was randomly collected from each muscle mixture and tested by artificial digestion. The LPG of each muscle pool was calculated by multiplying the number of larvae counted in the 500 g sample by the weight of the muscle pool. The total number of larvae per pig was then calculated by adding the amount of larvae recorded in every pool of muscles to that of the individual muscle samples. Finally, the RCI was calculated for each animal.

2.9. Resistance of muscle larvae to freezing

Two female domestic cats, weighing 3.3 and 3.7 kg, were infected with 500 and 1000 larvae per kg of the *Trichinella* isolate ISS2311, respectively. Both animals were necropsied, skinned and eviscerated at 90 days p.i., and the muscle tissues were minced and pooled. Twenty-four cat muscle samples of 30 g each, containing approximately 40 LPG, were stored in plastic bags and flattened to 1 cm thickness. Half of the samples were stored at −5 °C and the other half at −18 °C. Temperatures at the cores of the meat samples were recorded at 15 min intervals using a digital thermometer with an accuracy of 0.5 °C and a precision of 0.1 °C. Before and after 1 week, 2 weeks, 1 month, 2 months, 3 months and 6 months of freezing, two subsamples representing each storage temperature were thawed overnight at 5 °C, allowed to stand at room temperature for 1 h and then digested as described above. Larvae were considered alive if they displayed some motility or dead if they were still coiled after 30 min at 37 °C. Larval viability, defined as the percentage of live larvae, was assessed for each subsample. To test for infectivity, male CF-1 mice were inoculated orally with 200 live larvae from each subsample. If the number of live larvae was insufficient, the number of larvae was divided into two aliquots and inoculated into two mice. At 35 days p.i., mice were checked for *Trichinella* infection after artificial digestion as described above, and the RCI was calculated.

2.10. Production of newborn larvae (NBL)

A total of 67 adult female worms were recovered from the duodenum of a CF-1 mouse which had been infected 6 days earlier with 1000 muscle larvae of *Trichinella* isolate ISS2311. As controls, 36 adult females of *T. spiralis* isolate ISS3558 were recovered from the duodenum of a CF-1 mouse at 6 days p.i. with 1,000 muscle larvae. One female was cultured per well in 400 µl of DMEM, high glucose (4.5 g/l) (Gibco, USA) supplemented with 10% FCS (PAA Laboratories, Austria), 1% penicillin–streptomycin solution (10,000 units/ml of penicillin, 10 mg/ml of streptomycin) (Sigma, USA) and 1% amphotericin B (250 µg/ml) (Sigma USA) in 24-microwell plates (Falcon 3047, Becton Dickinson, USA), and incubated at 37 °C, in a 5% CO_2 atmosphere for 72 h. The culture medium was renewed after 24 and 48 h and NBL were counted daily (Pozio et al., 1992b).

2.11. Experimental animals

The study protocols for animals (pigs and cats) was approved under permit numbers 2008/30 and 2009/16 by the Institutional Committee for Use and Care of Laboratory Animals of the Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina and the animals were sacrificed according to the American Veterinary Medical Association (http://www.avma.org/KB/Policies/Documents/euthanasia.pdf). In Italy, the study protocol for mice was approved by the Italian Ministry of Health according to the European directive 8/609 EEC.

3. Results

3.1. Molecular identification and phylogenetic analysis

A multiplex PCR from single muscle larvae of isolates ISS2311 and ISS3559 generated single bands similar to those of *T. nativa*, *T. britovi*, *T. murrelli* and *Trichinella* T6, and was thus of no diagnostic value for *Trichinella* T12. However, the PCR amplification and sequencing of the ESV of *Trichinella* T12 using the TS10177 primer pair yielded a fragment of 161 bp (GenBank Accession No. HE819395), which was distinct from the ESV of the other taxa in the genus.

PCR amplification and sequencing of the 5S-ISR and cox-1 genes from the isolates ISS2311 and ISS3559 yielded fragments of 749 and 419 bp, respectively. Pairwise nucleotide distances (*p*-distances) were estimated among all *Trichinella* sequences available for these two markers (Table 2). The average *p*-distance among the nine cox-1 sequences of the three isolates ISS2311, ISS3559 and ISS1826 of *Trichinella* T12 was estimated as

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0.004 ± 0.004, which is much lower than the value among all encapsulated taxa (0.088 ± 0.019). The same pattern of variation was observed for the 5S-ISR sequences, with an average *p*-distance among the nine sequences of the three isolates of *Trichinella* T12 of 0.015 ± 0.009. This value was almost three times lower than the average *p*-distances calculated among all encapsulated taxa (0.040 ± 0.013). The NJ trees constructed from both gene fragments (Fig. 1) showed that all *Trichinella* T12 sequences clustered together, with bootstrap support of 94% for 5S-ISR and 100% for cox-1. These results indicate that although there is some genetic variation among T12 isolates, they grouped together in the phylogenetic tree to the exclusion of other *Trichinella* spp.

3.2. Morphological and biological studies

The morphological features of male and female muscle larvae and adult worms of the *Trichinella* T12 isolate are shown in Table 3. Female larvae and adults were longer than male larvae and adults, but their morphological features were within the range of those reported for other recognised *Trichinella* spp. (Pozio, 2007).

Single females and males of *Trichinella* T12 crossed successfully only with *T. britovi* and *T. murrelli* in both directions (Table 4). However, no offspring were produced in mice infected with the F1 generation obtained from either a single mating pair or multiple mating pairs of *T. britovi* × *Trichinella* T12 and *T. murrelli* × *Trichinella* T12, in both directions (data not shown). Only four dead hybrid larvae were recovered from a mouse infected with a male larva of *T. nativa* and a female larva of *Trichinella* T12.

Both mice and rats were less susceptible to infection with *Trichinella* T12 than with *T. spiralis*. The average RCI in CF-1 mice was 65.07 ± 28.91 and 251.44 ± 63.49 for *Trichinella* T12 and *T. spiralis*, respectively. In Wistar rats, the RCI was 0.02 ± 0.02 and 44.47 ± 21.27 for *Trichinella* T12 and *T. spiralis*, respectively. These data suggest that the infectivity of *Trichinella* T12 was 3.9 and 2223.5 times lower than that of *T. spiralis* in mice and rats, respectively.

Larvae of *Trichinella* T12 were detected in three of the four inoculated pigs, albeit at a very low level (Table 5). No larvae were recovered from the two control pigs (data not shown). The three infected pigs displayed an average LPG and RCI of 0.0027, 0.0067, 0.1569 and 0.0008, 0.0014, 0.0448, respectively. In all of these pigs, the tongue had the highest number of larvae. In only one pig were all muscles or groups of muscles tested separately and the pool of muscles positive (Table 5).

Larvae of *Trichinella* T12 present in cat muscles frozen at −5 °C for 3 months were infective to laboratory mice. The RCI in mice was reduced from 63.03 to 7.18 with 1 week to 3 months of freezing, respectively (Table 6). Larvae of *Trichinella* T12 present in cat muscles frozen at −18 °C did not infect mice, independently of the length of time of freezing.

Adult females of *Trichinella* T12, cultured in vitro for 72 h, released 21.85 ± 7.47, 3.36 ± 2.62 and 0.70 ± 1.00 NBL on the first, second and third days, respectively, (average NBL production per female: 25.91 ± 8.86). Under the same experimental conditions, adult females of *T. spiralis* cultured in vitro for 72 h produced 81.56 ± 22.63, 30.58 ± 20.03 and 4.44 ± 5.88 NBL, on the first, second and third days, respectively (average NBL production per female: 116.58 ± 40.21).

Taxonomic summary

Trichinella patagoniensis n. sp. Biological features: RCI at the third passage in CF-1 mice 5 weeks p.i., 65.07 ± 28.91; RCI in Wistar rats 1 month p.i., 0.02 ± 0.02; RCI in domestic pigs 5 weeks p.i., from 0.0008 to 0.05. Resistance to freezing in cat muscles at −5 °C, 3 months; no resistance to freezing in cat muscles at −18 °C. Average NBL production per female in vitro 25.91 ± 8.86 in 3-day culture.

Table 2

Pairwise comparisons of cytochrome *c*-oxidase subunit I (cox-1) gene and 5S rDNA intergenic spacer region (5S-ISR) gene markers from members of the genus *Trichinella*. The values represent the percentage of nucleotide sites at which two DNA sequences differ in pairwise comparisons (*p*-distance) of cox-1 (below the diagonal) and 5S-ISR (above the diagonal) gene fragments from all of the recognised members of the genus *Trichinella*, including the *Trichinella* T12 isolates ISS1826, ISS2311 and ISS3559 from Argentina. The numbers following the isolate codes indicate the three different DNA sequences obtained from each *Trichinella* T12 isolate. ISS1826.1, ISS1826.2, ISS1826.3, ISS2311.1, ISS2311.2, ISS2311.3, ISS3559.1, ISS3559.2 ISS3559.3 represents the Genbank cox-1 sequences JF260985, JF260986, EU161657, JF260987, JF260988, JF260989, JF260990, JF260991 and JF260992 and the Genbank 5S-ISR sequences JF260993, JF260994, EF694983, JF260995, JF260996, JF260997, JF260998, JF260999 and JF261000, respectively. *Enterobius vermicularis* was used as an outgroup for both markers.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1		1.18	1.19	1.20	1.32	1.17	1.18	1.19	1.15	n.a.	1.18	1.22	1.10	1.13	1.11	1.10	1.10	1.10	1.12	1.12	1.14
2	27.75		0.05	0.05	0.46	0.05	0.06	0.05	0.05	n.a.	0.38	0.41	0.05	0.05	0.05	0.06	0.05	0.06	0.05	0.05	0.06
3	27.76	0.11		0.02	0.47	0.01	0.01	0.04	0.01	n.a.	0.39	0.41	0.04	0.04	0.04	0.05	0.04	0.05	0.04	0.04	0.05
4	27.33	0.11	0.06		0.46	0.01	0.03	0.04	0.01	n.a.	0.38	0.40	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.05
5	27.33	0.11	0.13	0.15		0.46	0.48	0.45	0.45	n.a.	0.23	0.25	0.44	0.46	0.45	0.46	0.45	0.46	0.46	0.46	0.47
6	27.33	0.11	0.04	0.07	0.14		0.02	0.03	0.00	n.a.	0.38	0.40	0.04	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.05
7	27.75	0.08	0.01	0.06	0.13	0.04		0.05	0.02	n.a.	0.40	0.42	0.05	0.04	0.05	0.06	0.05	0.05	0.05	0.05	0.06
8	27.34	0.08	0.07	0.07	0.15	0.08	0.07		0.03	n.a.	0.38	0.40	0.04	0.04	0.04	0.05	0.04	0.05	0.04	0.04	0.05
9	27.33	0.10	0.06	0.05	0.15	0.07	0.06	0.07		n.a.	0.37	0.40	0.04	0.03	0.03	0.04	0.04	0.04	0.04	0.03	0.04
10	27.33	0.08	0.05	0.07	0.13	0.05	0.04	0.08	0.08		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
11	27.75	0.13	0.14	0.14	0.14	0.12	0.14	0.13	0.14	0.13		0.03	0.37	0.38	0.37	0.38	0.38	0.38	0.39	0.38	0.40
12	27.31	0.16	0.14	0.15	0.12	0.15	0.15	0.14	0.16	0.16	0.07		0.39	0.40	0.39	0.41	0.40	0.41	0.41	0.41	0.42
13	26.92	0.10	0.11	0.10	0.15	0.11	0.11	0.08	0.10	0.10	0.13	0.16		0.01	0.00	0.01	0.01	0.01	0.02	0.02	0.03
14	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.07	0.09	0.09	0.12	0.16	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.02
15	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.08	0.09	0.09	0.12	0.15	0.01	0.00		0.01	0.00	0.01	0.02	0.01	0.03
16	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.08	0.10	0.09	0.12	0.15	0.01	0.01	0.01		0.01	0.02	0.03	0.02	0.04
17	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.08	0.10	0.09	0.12	0.15	0.01	0.01	0.01	0.00		0.01	0.02	0.02	0.03
18	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.08	0.10	0.09	0.12	0.15	0.01	0.01	0.01	0.00	0.00		0.02	0.02	0.03
19	26.92	0.10	0.10	0.10	0.16	0.10	0.10	0.08	0.10	0.09	0.13	0.16	0.01	0.01	0.01	0.00	0.00	0.00		0.01	0.02
20	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.08	0.10	0.09	0.12	0.15	0.01	0.01	0.01	0.00	0.00	0.00	0.00		0.02
21	26.92	0.10	0.10	0.10	0.15	0.10	0.10	0.08	0.10	0.09	0.12	0.15	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	

Enterobius vermicularis, 1; *Trichinella spiralis*, 2; *Trichinella nativa*, 3; *Trichinella britovi*, 4; *Trichinella pseudospiralis*, 5; *Trichinella murrelli*, 6; *Trichinella* T6, 7; *Trichinella nelsoni*, 8; *Trichinella* T8, 9; *Trichinella* T9, 10; *Trichinella papuae*, 11; *Trichinella zimbabwensis*, 12; *Trichinella* T12, ISS1826.1, ISS1826.2, ISS1826.3, ISS2311.1, ISS2311.2, ISS2311.3, ISS3559.1, ISS3559.2, ISS3559.3, from codes 13 to 21, respectively. n.a., data not available.

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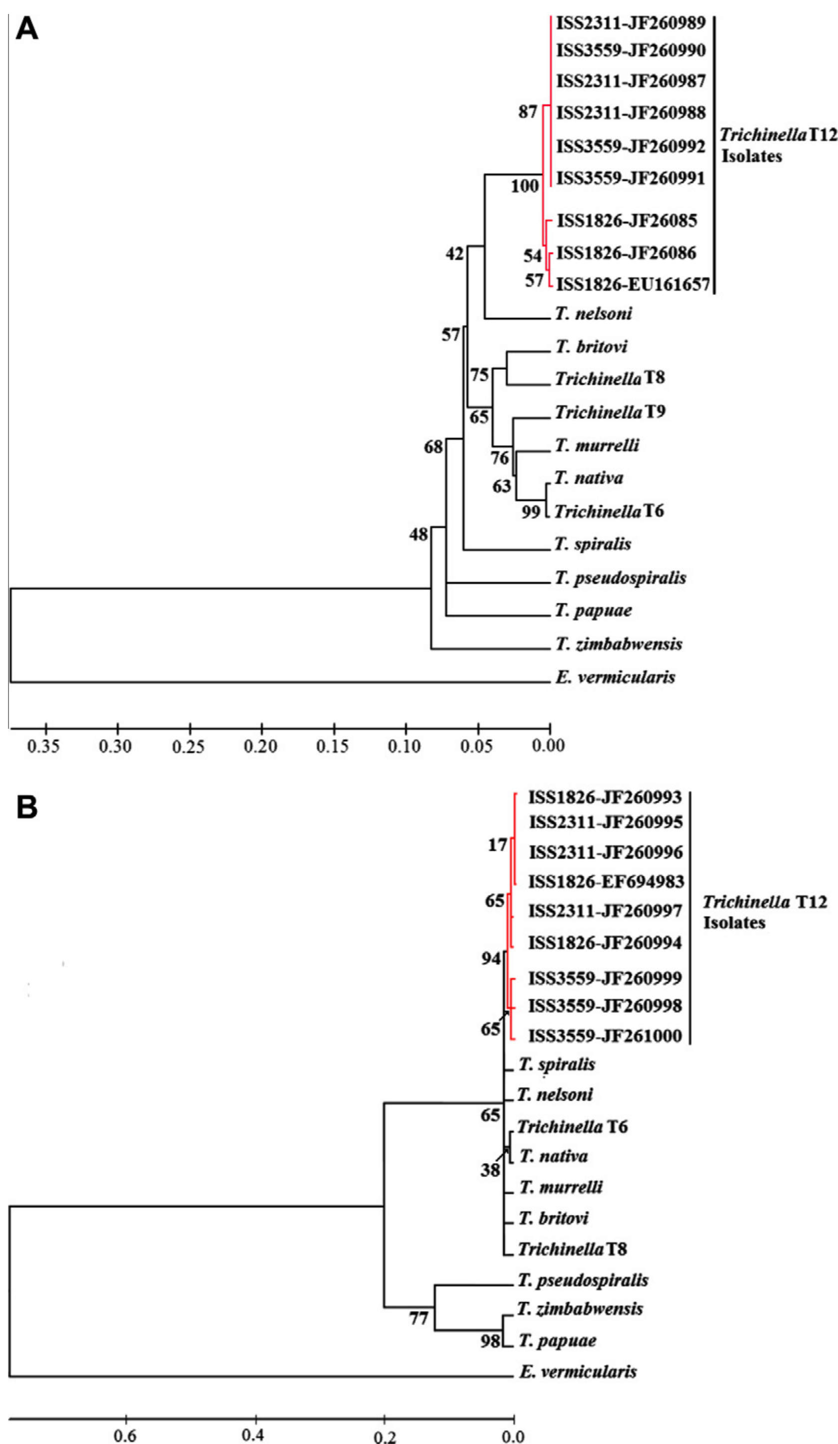

Fig. 1. Neighbour-joining distance tree based on the cytochrome *c*-oxidase subunit I gene sequences (A) or the nuclear 5S rDNA intergenic spacer region sequences (B) from all of the recognised *Trichinella* taxa, including nine clone sequences of three *Trichinella* T12 isolates (indicated in the tree by the isolate codes followed by their GenBank accession number). The number at the node indicates the level of bootstrap support (out of 1000 replicates). The scale bar indicates the evolutionary distance (*p*-distance) measured as nucleotide substitutions per position.

Reproductive isolation: single male and female adults of *T. patagoniensis* cross with single female and male adults of *T. britovi* and *T. murrelli* in both directions, but they produce infertile F1 offspring.

Type host: Puma concolor.

Experimental hosts: CF-1 and Swiss mice (*Mus musculus*), Wistar rats (*Rattus norvegicus*), cats (*Felis silvestris*), pigs (*Sus scrofa*)

Site: small intestine (adults) and skeletal muscle (larvae).

Morphological features: average total length of muscle larvae, 824 μm (range 641–915 μm) for males and 871 μm (range 748–1020 μm) for females; average total length of adults, 767 μm (range 663–997 μm) for males and 1386 μm (range 952–1601 μm) for females.

Molecular features: locus ESV, sequence of 161 bp (GenBank ID HE819395) amplified by the primer pair TS10177-F (5́-TAA-GAAAACGGCGAAAGC-3́) and TS10177-R (5́-AACCCCACAGAGA-GATTAAAG-3́).

Type locality: El Calafate, Santa Cruz, Argentina, 72°16′W, 50°20′S.

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Table 3
Morphological features (μm) of muscle larvae and adult worms of the *Trichinella* T12 genotype.

Features[a]	Male larva ± S.D. (range)	Female larva ± S.D. (range)	Adult male ± S.D. (range)	Adult female ± S.D. (range)
TL	824 ± 59 (641–915)	871 ± 72 (748–1020)	767 ± 75 (663–997)	1386 ± 148 (952–1601)
MW	32 ± 2 (29–36)	31 ± 2 (27–34)	30 ± 2 (27–37)	33 ± 3 (26–43)
OE	140 ± 23 (103–181)	145 ± 28 (101–200)	84 ± 14 (51–111)	89 ± 16 (62–121)
ST	413 ± 51 (294–515)	456 ± 56 (361–558)	273 ± 25 (219–313)	285 ± 35 (222–346)
UT	–	–	–	904 ± 121 (572–1093)
OV	–	–	–	107 ± 19 (79–144)
TS	–	–	339 ± 40 (276–445)	–
CA	–	–	14 ± 2 × 8 ± 1(10–17 × 6–11)	–
GP	216 ± 26 (137–254)	245 ± 31 (174–314)	–	–
RE	47 ± 3 (41–53)	23 ± 4 (17–35)	–	–
GPE	59 ± 9 (45–82)	34 ± 7 (25–56)	–	–

[a] TL, total length; MW, width at the mid-body; OE, length of the oesophagus; ST, length of stichosome; UT, length of the uterus; OV, length of the ovary; TS, length of the testis; GP, length of the genital primordium; RE, length of the rectum; and GPE, distance of the posterior margin of the genital primordium from the posterior extremity of the nematode; CA, length and width of copulatory appendages.

Table 4
Cross-breeding experiments with single female and male larvae of the *Trichinella* T12 genotype with single female and male larvae of the five encapsulated species of the genus *Trichinella*.

Parents			No. of *Trichinella* positive/infected mice	Average number of larvae per mouse
1 ♂		1 ♀		
Trichinella T12	×	*T. spiralis*	0/5	0
Trichinella T12	×	*T. nativa*	0/5	0
Trichinella T12	×	*T. britovi*	5/5	500
Trichinella T12	×	*T. murrelli*	5/5	260
Trichinella T12	×	*T. nelsoni*	0/5	0
Trichinella spiralis	×	*Trichinella* T12	0/5	0
Trichinella nativa	×	*Trichinella* T12	1/5	4
Trichinella britovi	×	*Trichinella* T12	5/5	220
Trichinella murrelli	×	*Trichinella* T12	5/5	120
Trichinella nelsoni	×	*Trichinella* T12	0/5	0

Table 5
Number of larvae per gram of muscle from three out of four *Trichinella* T12 gentotype-infected pigs inoculated with 20000 muscle larvae of the isolate ISS2311. No larvae were detected in the pig 3. Muscles are reported in a rank order according to the level of infection.

Muscles	Pig 1	Pig 2	Pig 4
Tongue	0.0870	0.0375	0.2443
Intercostals	0.0000	0.0000	0.2000
Diaphragm	0.0000	0.0000	0.1975
Hind leg	0.0000	0.0000	0.1400
Shoulder	0.0000	0.0100	0.1200
Masseter	0.0000	0.0000	0.1205
Filet	0.0000	0.0000	0.1000
Abdomen	0.0000	0.0000	0.0500
Foreleg	0.0000	0.0000	0.0300
Pooled muscle samples	0.0067	0.0027	0.1280

Table 6
Percentage of viable larvae and reproductive capacity index (RCI) of *Trichinella* T12 genotype larvae in cat muscles frozen at −5 °C for different lengths of time.

Length of freezing	% of viability ± S.D.	RCI ± S.D.
Day 0	98.24 ± 0.83	66.13 ± 36.92
1 week	86.53 ± 6.51	63.03 ± 33.84
2 weeks	93.85 ± 3.81	68.92 ± 16.86
1 month	98.26 ± 0.11	89.93 ± 51.87
2 months	68.14 ± 1.00	6.55 ± 7.10
3 months	82.16 ± 22.20	7.18 ± 5.01
6 months	5.04 ± 2.66	0.00

Distribution: three localities in the provinces of Rio Negro, Santa Cruz and Catamarca of Argentina.

Specimens deposited: syntypes, male and female adults and larvae, International *Trichinella* Reference Centre (ITRC) at the Istituto Superiore di Sanità, Rome, Italy (Ref. No. ISS2311) and Parasitic Worms Division, Department of Zoology at The Natural History Museum, London (male and female adults, BMNH 2011.4.15.1-2). Muscle larvae of this species are also maintained in CD1 female mice at the ITRC, Rome, Italy.

Etymology: the species is named for the Argentinean region where it was found.

4. Discussion

The *Trichinella* larvae isolated from a cougar from the locality of Trapalcó (Rio Negro, Argentina) and identified as a *Trichinella* T12 genotype were genetically distinct from those identified as *T. spiralis* in South America (Krivokapich et al., 2008). The muscle larvae of this isolate were non-viable and their classification into a new genotype was only based on cox-1 and 5S-ISR markers (Krivokapich et al., 2008).

In the present study, two additional isolates of *Trichinella* T12 were identified in cougars hunted in geographically distant localities of Argentina. Sequence analyses of these three isolates demonstrated some level of genetic diversity (for cox-1 and 5S-ISR) that is within the range expected for intra-specific variability within the genus (Rosenthal et al., 2008; Pozio et al., 2009). Furthermore, the sequences from the three isolates clustered together with high bootstrap support in both cox-1 and 5S-ISR phylogenetic trees (Fig. 1), indicating that they belong to the same encapsulated *Trichinella* T12 taxon.

These results support the proposal that this taxon is widespread in Argentina, with the cougar serving as a natural reservoir. The presence of a new encapsulated taxon of the genus *Trichinella* in South America adds a new piece to the puzzle of genetic variability and distribution of the genus *Trichinella* (Pozio et al., 2009). Since cougars are broadly distributed throughout the American continent (Culver et al., 2000), it is reasonable to assume that *Trichinella* T12 does not occur only in Argentina but also in other South American countries, thus posing a risk to consumers of game meat (Dworkin et al., 1996).

The ESV product displayed on the agarose gels did not allow the differentiation of *Trichinella* T12 from *T. nativa*. Nonetheless, the difference in the size (in bp) of the ESV sequence between *Trichinella* T12 and the other *Trichinella* taxa, obtained by the primer pair TS10177, suggests that it is a useful molecular diagnostic tool for *Trichinella* T12.

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Larvae and adult worms of *Trichinella* T12 cannot be distinguished from the other recognised taxa by their morphology. The sizes of larval and adult stages are consistent with those of other species of the encapsulated clade. The maximum lengths of *Trichinella* T12 adults were more similar to *T. murrelli*, which is noticeably smaller than those of *T. spiralis*, *T. nativa*, *T. britovi* and *T. nelsoni* (Dick, 1983; Pozio et al., 1992a; Murrell et al., 2000; Pozio and La Rosa, 2000).

The results of the cross-breeding experiments reported in this paper suggest that *Trichinella* T12 is reproductively more closely related to *T. britovi* and *T. murrelli* than to any other encapsulated species. This may be the case even if this taxon is reproductively isolated, as suggested by the infertility of the F1 (Table 4). The production of as few as four dead larvae in only one of five mice infected with a single female larva of *Trichinella* T12 and a single male larva of *T. nativa* may point to the existence of a reproductive barrier, which is stronger between these taxa than between *Trichinella* T12 and *T. britovi* and *T. murrelli*. Similar patterns have been observed for the crosses *T. murrelli* × *T. britovi* and *T. murrelli* × *T. nativa* (Britov, 1995; Pozio and La Rosa, 2000). Although these species are isolated by effective barriers, the production of hybrids, although sterile, would indicate closer relationships among them than to *T. spiralis* and *T. nelsoni*. The relationships of *Trichinella* T12 with *Trichinella* T6, T8 and T9 remain to be established because no cross-infection experiments were performed with those genotypes.

In this study, experimental infections of Wistar rats showed that the RCI of *Trichinella* T12 was much lower than that of *T. spiralis*. This is in agreement with previously published data for experimentally infected rats, which are known to be appropriate hosts only for *T. spiralis* (RCI = 185–237) (Pozio et al., 1992b). In fact, the enteral and parenteral niches of rats are not suitable for the other encapsulated species (i.e., *T. nativa, T. britovi, T. murrelli, T. nelsoni*) which generate a slight infection (RCI = 0.02–1.2) (Pozio et al., 1992b; Pozio and La Rosa, 2000). These results suggest that synanthropic rats cannot act as reservoir hosts of *Trichinella* T12.

Trichinella T12 had low infectivity in pigs, in accordance with all other 'sylvatic' encapsulated species of *Trichinella* which are considered to have radiated more recently (Zarlenga et al., 2006); within this group, this genotype seems to be more closely related to *T. nativa* and *T. murrelli* which show the lowest infectivity in pigs (Dame et al., 1987; Kapel and Gamble, 2000; Nöckler et al., 2005).

In nature, the transmission of *Trichinella* spp. depends on the ability of muscle larvae to withstand environmental temperatures during the interval between host death and ingestion by the next host (Pozio, 2000). Taking into account the fact that *Trichinella* T12 is circulating in Patagonia, where the minimum average temperature in July is −2.8 °C (www.smn.gov.ar/), the survival of larvae was evaluated in carnivore muscles frozen at different temperatures for different periods of time. Larvae in cat muscles remained infective at −5 °C for up to 3 months but died when kept at −18 °C for 1 week, suggesting that the genotype under study survives in frozen host carcasses for a period of time which is shorter than that of *T. britovi* and *T. nativa*, but higher than that of *T. murrelli*, which cannot withstand freezing (Pozio and Murrell, 2006).

The number of NBL produced per *Trichinella* T12 female in vitro over a 72 h period is lower than for *T. spiralis* and more similar to numbers reported previously for other 'sylvatic' taxa (Pozio et al., 1992b; Pozio and La Rosa, 2000). This biological feature further supports the inclusion of *Trichinella* T12 within the group of 'sylvatic' encapsulated species (Pozio et al., 2009).

In brief, on the basis of the geographical, cross-breeding and molecular evidence reported herein, we propose that the genotype *Trichinella* T12 should be assigned to a new species, *Trichinella patagoniensis* n. sp.

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