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Short communication

Morphology and genetic variability within *Taenia multiceps* in ruminants from Italy

Antonio Varcasia[a,*], Anna Paola Pipia[a], Giorgia Dessì[a], Antonella Zidda[a], Claudia Tamponi[a], Marco Pau[a], Antonio Scala[a], Belgees Boufana[b]

[a] *Laboratorio di Parassitologia, Ospedale Didattico Veterinario, Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Via Vienna 2, Sassari, 07100, Italy*
[b] *Department of Zoology, University of Benghazi, P.O. Box 1308, Benghazi, Libya*

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ABSTRACT

The aim of this study was to investigate the genetic variability and population structure of *Taenia multiceps*, and to correlate morphological features of individual coenuri with haplotypes. A total of 92 animals (86 sheep; 4 goats; 1 cattle; 1 mouflon, *Ovis musimon*) aged between 6–36 months showing clinical symptoms of cerebral coenurosis were included in this study. *T. multiceps* coenuri (n = 118) sampled from live animals during routine surgery procedures or at post-mortem inspections were examined morphologically and molecularly identified. Morphological features of the 52 coenuri selected for this study (number and size of large and small hooks) were within the range reported in the literature. Fifty-two of the molecularly confirmed *T. multiceps* coenuri harboured by 47 animals (sheep = 41; cattle = 1; goats = 4; mouflon = 1) were used to determine gene genealogies and population genetic indices and were compared to the 3 *T. multiceps* genetic variants, Tm1–Tm3 previously described from Sardinia, Italy. For the 379 bp *cox1* dataset we identified 11 polymorphic sites of which 8 were parsimony informative. A high haplotype diversity (0.664 ± 0.067) was recorded for the *cox1* sequences defining 10 haplotypes (TM01–TM10). The comparison of haplotypes generated in this study with published *T. multiceps* Tm1 variant pointed to the possible existence of a common lineage for *T. multiceps*. No correlation was detected between the size of the small and large hooks and the *cox 1* haplotypes. Polycystic infestation (2–9 coenuri) was recorded in 27.7% of animals (13/47). No statistical correlation between polycystic *T. multiceps* infection and haplotypes was detected.

1. Introduction

Coenurosis is a parasitic disease caused by the metacestode stage of *Taenia multiceps* (Cestoda, Taeniidae) commonly known as *Coenurus cerebralis* (Rostami et al., 2013). The adult tapeworm inhabits the small intestine of a number of domestic and wild carnivores, including dogs, jackals, foxes and coyotes (Varcasia et al., 2015) and eggs are excreted into the environment with the faeces of the definitive hosts and ingested by intermediate herbivorous hosts including sheep, goats, horses, cattle, camels, deer and pigs (Scala et al., 2007; Varcasia et al., 2013). Following the ingestion of the eggs, the oncosphere hatches, burrows its way through the intestinal wall and reaches the central nervous system (CNS) and other organs via the bloodstream (Paltrinieri et al., 2010). Infection is often lethal in intermediate hosts and the parasite is a cause of significant economic losses in many parts of the world (Varcasia et al., 2011). Coenurosis is also a zoonosis and there are several human coenurosis reports from Europe, Africa, Brazil, Israel and the United States (Hermos et al., 1970; Ing et al., 1998; El-On et al., 2008).

One of the most important aspects of taeniid biology and epidemiology is the existence of intraspecific variants. Understanding the extent and nature of genetic variation within individual cestode parasites is essential for the implementation of control and prevention programs. Mitochondrial DNA has been widely used for the genetic investigation of helminth parasites mainly because of its conserved nature and its relatively high evolutionary rate (Gasser, 2006; Liu et al., 2011). The existence of genetic diversity within taeniid cestodes is probably best exemplified by the genus *Echinococcus* within which several genotypes (currently separate species) have been identified worldwide (reviewed in Thompson, 2008). In addition, current knowledge relating to several *Taenia*

* Corresponding author at: Università degli Studi di Sassari, Laboratorio di Parassitologia, Ospedale Didattico Veterinario, Dipartimento di Medicina Veterinaria, Via Vienna 2, 07100 Sassari, Italy.
E-mail addresses: varcasia@uniss.it, antonio@varcasia.com (A. Varcasia).

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species indicate the existence of a remarkable degree of genetic variation within most of its members (Allsopp et al., 1987; Kedra et al., 2001; Boufana et al., 2015; Scala et al., 2015). However the significance and extent of intraspecific variation within different *Taenia* species remains to be determined.

The genetic characterization of *T. multiceps* from sheep, goat and cattle intermediate hosts has been the subject of several studies. Using 37 sheep-derived coenuri, Varcasia et al., 2006 provided the first evidence of genetic variation within *T. multiceps* from sheep in Sardinia and identified three variants which were designated as Tm1, Tm2 and Tm3. Coenurosis due to *T. multiceps* was also investigated in goats from Iran and the United Arab Emirates using the mitochondrial CO1 and ND1 genes (Oryan et al., 2010; Varcasia et al., 2012; Akbari et al., 2015). In addition a recent study investigated for the first time the correlation between haplotypes of *T. multiceps* and morphology of these metacestodes in sheep from Iran (Rostami et al., 2013).

Information on the genetic variability of this economically important parasite species is necessary for both epidemiological studies and for the implementation of control programs. This study was therefore undertaken to investigate the genetic variation and population structure of *T. multiceps* and also to explore the correlation of morphological features of individual coenuri with haplotypes.

2. Materials and methods

A total of 92 animals (86 sheep; 4 goats; 1 cattle; 1 mouflon, *Ovis musimon*) aged between 6–36 months which were reported by farmers and/or veterinary practitioners between May 2012 and July 2015 as showing clinical symptoms attributable to brain coenurosis were included in this study. Putative *T. multiceps* coenuri (n = 118) were sampled from live animals during routine surgery procedures at the Veterinary Teaching Hospital (University of Sassari, Italy) or at post-mortem inspections in several abattoirs in Sardinia, Italy. Once in the laboratory, metacestodes (n = 52) were examined morphologically using a stereomicroscope (Leica EZ4HD; software: Leica Application Suite, V. 4.2.0) to record their dimensions (length, width) and numbers of clusters (associations/groups of protoscoleces). In addition, the size of protoscoleces (length and width), rostellum (major and minor axis) and suckers (major and minor axis) as well as the number and length of large and small hooks were evaluated using an optical microscope (Olympus BX41TF; software: micro LC 5.2).

Genomic DNA extracted using the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) was used to amplify a fragment within the cytochrome *c* oxidase subunit 1 (*cox1*) gene using published protocols (Bowles et al., 1992; Bowles and McManus, 1993a,b). PCR products were commercially sequenced by MWG-Biotech (Ebersberg, Germany) and the generated data was compared with that on the NCBI database through the use of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Cytochrome *c* oxidase subunit 1 (*cox 1*) nucleotide sequences of 52 molecularly identified *T. multiceps* coenuri harboured by 47 animals (sheep = 41; cattle = 1; goats = 4; mouflon = 1) were used to determine gene genealogies and population genetic indices. The selection of *C. cerebralis* metacestodes to be included in this analysis was based on the animal farm, geographical distribution and host species. In addition, nucleotide sequences of *T. multiceps* variants, Tm1–Tm3 (Accession numbers DQ309767– DQ309769) previously described from Sardinia (Varcasia et al., 2006) were included in this study for comparison.

This was carried out using previously described methodology (Boufana et al., 2015). Briefly, nucleotide DNA sequences were aligned in ClustalX2 (Larkin et al., 2007) and transported into DnaSP 5 (Librado and Rozas, 2009). The calculation of population diversity indices (haplotype and nucleotide diversities) was carried out using Arlequin 3.1 (Excoffier et al., 2005). In order to test for various demographic scenario's we calculated four test statistics, each sensitive to a particular demographic event. The hypothesis of population expansion was tested using Fu's Fs (which is based on the distribution of alleles or haplotypes) (Fu, 1997) and Tajima's *D* (Tajima, 1989) (which incorporates the frequency of segregating nucleotide sites) in Arlequin using 1000 permutations to test for their significance. DnaSP was used to calculate Ramos-Onsins Rozas's R_2 (Ramos-Onsins and Rozas, 2002) and Fu and Li's D* (Fu and Li, 1993; Fu, 1996). The former is sensitive to the detection of recent severe population expansion and the latter detects reduction in population size through the presence of an excess of old mutations. *P*-values for each statistic were determined in DnaSP 5 using coalescence simulations with 1000 replicates. Haplotype networks were drawn in HapView (Salzburger et al., 2011) using maximum likelihood trees constructed using DNAML program (PHYLIP) which was run from HapView (Felsenstein, 1989).

For the correlation between morphology and genetic variability, the number and size of the hooks (small and large) and haplotypes for each *coenurus* (n = 52) sampled from 47 animals (sheep = 41; cattle = 1; goats = 4; mouflon = 1) were considered as described by Rostami et al., 2013. Analysis was conducted using the Student's *t*-test and Pearson's correlation in Microsoft Office Excel 2016 software (Microsoft Corporation, Redmond, WA, USA) and Pearson's correlation in Minitab® 16.2 (2012 Minitab Inc.). Pearson's correlation was also utilised to verify any correlation between haplotypes and polycystic infection.

3. Results

Molecular DNA analysis (using BLAST algorithm) of the 118 putative cysts included in this study confirmed our clinical diagnosis and showed that all the 92 intermediate hosts (sheep, goats, cattle) had suffered from cerebral coenurosis caused by *T. multiceps*. These cysts were localised within the CNS and distributed within the brain (n = 102), spinal cord (n = 2) and the cerebellum (n = 14).

Morphological examination of 52 *coenuri* showed that the minimum and maximum number of clusters per cyst was 1 and 23 respectively. Each cluster had a variable number of protoscoleces with 4 suckers and a mean diameter of 282.9 μm (± 49) and a large rostellum measuring 314.6 μm (± 60) which contained an average of 28 (± 2) large and small hooks arranged in two rows. The average total length of the large and small hooks was 162 μm (± 12.2) and 114.3 μm (± 9.8) respectively.

A total of 55 *cox 1* nucleotide sequences of *T. multiceps* were analysed (52 from this study and the 3 *T. multiceps* variants, Tm1–Tm3). For the amplified 379 bp *cox 1* dataset we identified 11 polymorphic sites of which 8 were parsimony informative. The number of transitions and transversions detected for the *cox 1* sequences of *T. multiceps* were 7 and 4 respectively. No insertions or deletions were detected within the amplified fragments.

Diversity and neutrality indices for *T. multiceps* Sardinian isolates are shown in Table 1. A high haplotype diversity (0.664 ± 0.067) was recorded for the *cox 1* sequences defining 10 haplotypes. Within the *cox 1* parsimony network 56.4% (31/55) of *T. multiceps* isolates including those derived from 2 goats and 1 cattle shared one common haplotype (TM08) (Fig. 1). The remaining 9 haplotypes encompassed between 1–5 *T. multiceps* isolates and were separated from the common haplotype (TM08) by 1–4 mutational steps. In addition, *T. multiceps* Tm1 variant had an identical sequence to isolates that constituted the most common haplotype (TM08) whereas Tm2 shared haplotype TM02 with a single sheep *T. multiceps* isolate and variant Tm3 occupied haplotype TM05 along