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Phylogenetic study of *Setaria cervi* based on mitochondrial *cox*1 gene sequences

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Abstract The objective of the present study was to examine the phylogenetic position of *Setaria cervi* based on sequences of mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene. A fragment of the *cox*1 gene from two morphologically identified *S. cervi* collected from red deer (*Cervus elaphus*) from Italy were amplified, sequenced, and compared with corresponding sequences of other filarioid nematode species. Phylogenetic studies using Bayesian analysis revealed *S. cervi* as monophyletic with other *Setaria* species, confirming *S. cervi* as a member of the *Setaria* genus. *S. cervi* appeared to be sister species to *Setaria labiatopapillosa* and *Setaria digitata*. *Setaria tundra* and *Setaria equina*, the other two *Setaria* species presented in the Italian fauna, formed a sister group to the clade consisting of *S. cervi*, *S. labiatopapillosa*, and *S. digitata*. In addition to phylogenetic clarification, our study is the first molecular identification of *S. cervi*, which may be useful for further molecular identification and differentiation of this filarial worm from other filarioid nematode species, especially in the earlier developmental stages of its life cycle.

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Introduction

Filarioid nematode parasites are considered as major health hazards with important medical, veterinary, and economic ramifications, affecting millions of people and animals globally (World Health Organization 2007). Filarioid parasites are transmitted by various haematophagous arthropods (Anderson 2000), and some have recently been considered to be indicators of climate change (Genchi et al. 2009; Laaksonen et al. 2010). Due to the mobility of the vectors and/or the risk of resistance to drugs used for etiological treatments (Yatawara et al. 2007), these parasites are difficult to control.

Traditionally, the morphological characters were used to establish the phylogenetic relationships and position of filarial nematodes (Chabaud and Bain 1994; Bain 2002). Molecular studies are needed to evaluate and confirm the morphological description or taxonomy, since the similar morphological characters among filarial lineages weakened the proposed evolutionary pattern (Yatawara et al. 2007). Also, the correct identification of particular species of *Setaria* worms is of great importance and has further health implications, e.g., for confirming the antigen source from mixed infections in cattle (Almeida et al. 1991).

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Three species within the genus *Setaria* are known to occur in Italy, namely *Setaria equina, Setaria labiatopapillosa*, and *Setaria tundra* (Pietrobelli et al. 1995; Giannetto et al. 1996; Favia et al. 2003). An additional single citation refers to *Setaria cervi* in the red deer, *Cervus elaphus* (Manfredi et al. 2003). *S. cervi* is a filarial nematode parasite transmitted by mosquitoes during a blood meal when infective larvae leave the vector to enter the vertebrate host. The adult worm inhabits the peritoneal cavity of bovine and cervid hosts, causing little or no harm (Urquhart et al. 1996). The distribution of *S. cervi* is reportedly cosmopolitan, but it remains a matter of debate if worms infecting bovines in Asia and those infecting cervids (mainly) in Europe are conspecific (Böhm and Supperer 1955; Shoho 1967; Becklund and Walker 1969). *S. cervi* (from bovine hosts) is also considered as a suitable model for screening anti-filarial agents (Singhal et al. 1972; Nayak et al. 2011). To our knowledge, no molecular studies have been carried out to characterize this parasite and to determine its phylogenetic position in relation to other filarial worms. Therefore, the objective of the present study was to examine the phylogenetic position of *S. cervi* based on sequences of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene.

Materials and methods

Sample collection and morphological examination

One adult male *Setaria* was collected from the peritoneal cavity of an adult female red deer (*C. elaphus*) from Magliano dei Marsi L'Aquila province (Abruzzo Region, Central Italy), and one adult male *Setaria* was collected from the peritoneal cavity of a 10-month-old male red deer from Pescasseroli L'Aquila province (Abruzzo Region, Central Italy) which were presented to the Istituto "G. Caporale" for necropsy. Nematodes were conserved in ethanol (70%) at room temperature before DNA extraction. *Setaria* specimens were identified as *S. cervi* based on morphological characteristics (Shoho and Uni 1977; Almeida et al. 1991).

DNA extraction

Genomic DNA was extracted from each of the two *S. cervi* specimens following standard phenol/chloroform procedures (Sambrook et al. 1989). DNA extractions were carried out in a sterilized laboratory exclusively for low DNA concentration samples. Two blanks (reagents only) were included in each extraction to monitor for contamination.

PCR and sequencing of mitochondrial *cox*1 gene

PCR for amplification of a fragment of the *cox*1 gene followed the methods of Casiraghi et al. (2001): The 30-μL PCR mixture contained 2 μL of template DNA, 0.25 μM of the primers cox1intF (5′-TGATTGGTGGTTTTGGTAA-3′) and cox1intR (5′-ATAAGTACGAGTATCAATATC-3′), 0.12 mM of each dNTP, 3 μL of PCR buffer (Bioline), 1.5 mM $MgCl_2$, 0.4% BSA, 1.5 μL DMSO, and 0.2 μL (0.2 U/reaction) *Taq* polymerase (Bioline). Samples were subjected to the following thermal profile for amplification in a PTC0200 thermal cycler (Bio-Rad): 4 min at 94°C (initial denaturation), followed by 30 cycles of three steps of 1 min at 94°C (denaturation), 1 min at 52°C (annealing), and 50 s at 72°C (extension), before a final elongation of 5 min at 72°C. PCR blanks (reagents only) were included.

Following the PCRs, excess primers and dNTPs were removed using enzymatic reaction of *Escherichia coli* exonuclease I, Antartic phosphatase, and Antartic phosphatase buffer (all New England Biolabs). Sequencing was carried out in both directions using the BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Labeled fragments were resolved on an automated A3130*xl* genetic analyzer (Applied Biosystems). Incomplete terminal sequences and PCR primers were removed.

Molecular analysis

Templates were sequenced on both strands, and the complementary reads were used to resolve rare, ambiguous base-calls

Table 1 Nucleotide substitutions (above diagonal) and *p*-uncorrected distances (%) (below diagonal) for each pairwise comparison between *Setaria cervi* and the other available *Setaria* species

	S. cervi	*S. digitata*	*S. labiatopapillosa*	*S. equina*	*S. tundra*
S. cervi	–	8.09	9.22	10.03	10.19
S. digitata	50	–	9.54	11	10.51
S. labiatopapillosa	57	59	–	9.7	11.32
S. equina	62	68	60	–	10.03
S. tundra	63	65	70	62	–

Table 2 Best six BIC models selected; −lnL (negative log likelihood), *K* (number of estimated parameters), BIC (Bayesian information criterion), delta (BIC difference), weight (BIC weight), and cumWeight (cumulative BIC weight)

Model	−lnL	*K*	BIC	Delta	Weight	cumWeight
TIM1+I+G	4,063.508	50	8,448.3405	0	0.9819	0.9819
TPM3uf+I+G	4,071.5073	49	8,457.9125	9.572	0.0082	0.9901
TrN+I+G	4,071.5108	49	8,457.9196	9.5791	0.0082	0.9983
TIM2+I+G	4,070.641	50	8,462.6064	14.2659	0.0008	0.9991
TIM3+I+G	4,070.6949	50	8,462.7142	14.3737	0.0007	0.9998
GTR+I+G	4,065.9505	52	8,466.0785	17.738	0.0001	1

in Sequencher v.4.9. Sequences were aligned in Seaview v.4.2.12 (Gouy et al. 2010) under ClustalW2 (Larkin et al. 2007) default settings. Nucleotide substitutions and *p*-uncorrected distances (percent) were performed with PAUP*4.b.10 (Swofford 2002), and phylogenetic analyses were performed with MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001). The GenBank entries used by Yatawara et al. (2007) and the GenBank accession numbers with more than 92% similarity to our sequences were used in phylogenetic analysis.

The most appropriate substitution model for the Bayesian maximum likelihood analyses was determined by the Bayesian Information Criterion (BIC) in jModeltest v.0.1.1 (Posada 2008). MrBayes was used with default priors and Markov chain settings and with random starting trees. The gamma shape parameter and proportion of invariant sites were estimated from the data. Each run consisted of four chains of 10,000,000 generations, sampled each 10,000 generations for a total of 1,000 trees. A plateau was reached

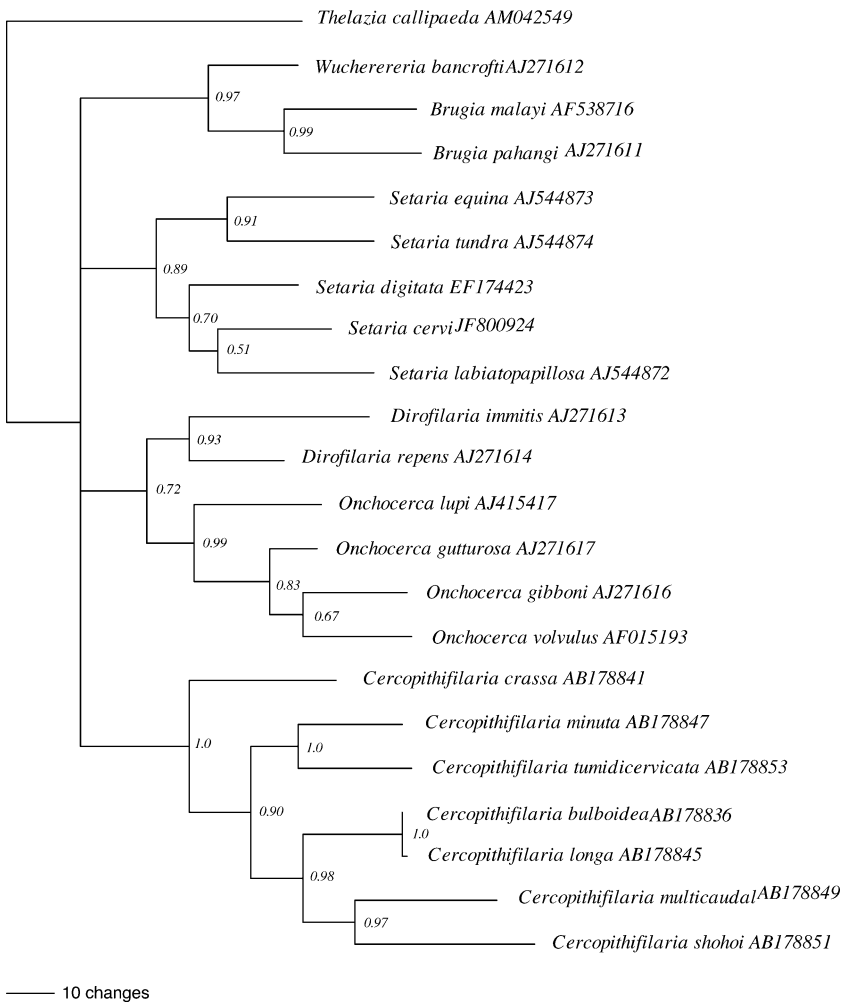

Fig. 1 Bayesian maximum likelihood 50% consensus cladogram of filarial worms including *S. cervi*. *Values by nodes* are the posterior probabilities recovered from the Bayesian analysis

after 5,000 generations with 10% (200 trees) of the trees resulting from the analyses discarded as "burn in."

Results and discussion

The *S. cervi cox*1 fragment was 680 bp in length. The two examined specimens had identical sequences, which was deposited in the GenBank under the accession number JF800924. The uncorrected *p*-distances among species from the *Setaria* genus ranged between 8% (between *S. cervi* and *S. digitata*) and 11% (between *S. equina* and *Setaria labiatopapillosa*). The uncorrected *p*-distance between *S. cervi* and other *Setaria* species ranged between 8% (with *S. digitata*) and 10% (with *S. equina*) (Table 1). The best-fitting model (Table 2) for the BML tree was the TIM1+I+G (−lnL=−4,063.5080). Based on *cox*1 sequences of *S. cervi* and the other 21 parasite nematode specimens downloaded from GenBank, the Bayesian 50% consensus tree supports the monophyly of the *Setaria* genus, thus confirming *S. cervi* as a member of the *Setaria* genus. *S. cervi* is grouped with *S. labiatopapillosa* and *S. digitata*. *S. tundra* and *S. equina*, the other two *Setaria* species presented in the Italian fauna, formed a sister clade well separated from the clade consisting of *S. cervi*, *S. labiatopapillosa*, and *S. digitata* (Fig. 1)

The present study is the first molecular characterization of *S. cervi*, which is of interest, since an important requirement to plan effective control strategies for the emerging parasite infections is the correct identification of the parasite species involved (Madathiparambil et al. 2009; Srinivasan et al. 2009). Larval stages of filarial species usually cannot be identified by classical morphology (Cancrini and Kramer 2001). Hence, molecular characterization allows the identification of the parasites throughout all their developmental stages. Future sequence comparison of other morphologically similar specimens to the bar code sequence of this study (GenBank accesion number JF800924) may prove important to determine the identity of such parasite specimens and to assess the molecular diversity within the species and the genus. The molecular characterization of *S. cervi* is, therefore, advantageous and particularly suitable for epidemiological studies which require the analysis of large numbers of samples to assess the level of genetic divergence between specimens and hosts and the haplotypic variation between and within regions (Favia et al. 1997).

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