

Characterization of *Taenia madoquae* and *Taenia regis* from carnivores in Kenya using genetic markers in nuclear and mitochondrial DNA, and their relationships with other selected taeniids

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Abstract

In the present study, we have extended earlier taxonomic, biochemical and experimental investigations to characterize two species of *Taenia* from carnivores in Kenya by use of the sequences of a variable domain (D1) of nuclear ribosomal DNA and the cytochrome *c* oxidase subunit 1 and NADH dehydrogenase 1 genes of mitochondrial DNA. Emphasis was placed on the characterization of *Taenia madoquae* from the silver-backed jackal (*Canis mesomelas*) and *Taenia regis* from the lion (*Panthera leo*), given the previous absence of any DNA sequence data for them, and on assessing their genetic relationships with socioeconomically important taeniids. The study showed that *T. regis* was genetically most closely related to *T. hydatigena*, and *T. madoquae* to *T. serialis*, *T. multiceps* or *T. saginata*. The present findings provide a stimulus for future work on the

systematic relationships and epidemiology of lesser-known taeniid cestodes in Africa and other continents, employing mitochondrial sequence data sets.

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1. Introduction

Species of *Taenia* (Cestoda: Taeniidae) are harboured, as adult tapeworms, in the small intestines of carnivorous or human definitive hosts, and are transmitted to intermediate mammalian hosts where they become established as larval stages in tissues, causing significant disease (cysticercosis or coenuriasis) [1]. A number of species of *Taenia* have public health and economic impact, because they are zoonotic and cause losses to the meat industry due to the condemnation of infected offal or meat [2], [3], [4] and [5]. The specific identification of taeniids is central to their control as well as to investigating their life cycles, ecology and epidemiology, and is usually based on a combination of ecological, biological and morphological criteria, including the morphology of the adult stage (such as the number, size and shape of the rostellar hooks, the distribution of the testes, the shape of the cirrus-sac and its extent relative to the longitudinal osmoregulatory canals, the presence or absence of a vaginal sphincter, the location of the genital pore along the segment margin and the number of principal lateral branches of the gravid uterus), the morphology and type of asexual reproduction of the larval stage, and

the level of host specificity in different geographical regions [6], [7], [8], [9], [10] and [11]. However, unequivocal identification based on these criteria is often difficult. Biochemical and traditional molecular approaches (e.g., multilocus enzyme electrophoresis (MEE) and restriction fragment length polymorphism (RFLP) combined with Southern blot) have assisted in the genetic characterization and identification of *Taenia* spp. from different hosts [12] and [13]. Recently, techniques, such as partial or whole mitochondrial genome sequencing, based on the use of the polymerase chain reaction (PCR) [14] have found broader applicability, mainly because their sensitivity permits the analysis of particular genes from tiny amounts of DNA from fresh, frozen or even ethanol fixed parasite material [15] and [16]. While there is significant DNA sequence information for taeniids of socioeconomic importance, there are limited data for the lesser-known species, particularly those from Africa (reviewed in [6] and [11]). In the present study, we extend earlier studies [17] and [18], in order to characterize different species of *Taenia* from carnivores in Kenya using sequences from one nuclear ribosomal and two mitochondrial DNA regions. Emphasis was placed on the characterization of *Taenia madoquae* from the silver-backed jackal (*Canis mesomelas*) and *Taenia regis* from the lion (*Panthera leo*), and on assessing their genetic relationships with socioeconomically important taeniids.

2. Materials and methods

2.1. Parasites

Adult specimens of *Taenia* ($n=175$) were collected, as described previously [17], from carnivores in Kenya using the methods employed by Macpherson et al. [19]. The subset ($n=160$) included in the present study comprised 65 specimens from *C. familiaris* (dog; $n=21$), 42 from *C. aureus* (golden jackal; $n=2$), 16 from *C. mesomelas* (silver-backed jackal; $n=4$), 14 from *Homo sapiens* (human; $n=4$) and 23 from *P. leo* (lion; $n=1$). The worms were washed in physiological saline, and a central portion of each worm was rewashed four times in distilled water before being stored (in 1 ml cryotubes) in liquid nitrogen or at $-20\text{ }^{\circ}\text{C}$ for subsequent molecular analysis. The remainder of each worm was relaxed in water, preserved in 70% ethanol and then identified morphologically,

according to a combination of characters [6], [7], [8], [20] and [21] and identified/characterized biochemically [17] and [18].

2.2. Isolation of genomic DNA

A packed volume ($\sim 200 \mu\text{l}$) of each adult worm was suspended in $\sim 400 \mu\text{l}$ of 20 mM Tris-HCl, pH 8.0, 100 mM EDTA, 1% sodium dodecyl sulphate (SDS) containing 20 mg/ml proteinase K (Boehringer) and incubated at 40 °C for 6 h. Following this incubation, the homogenized suspension was centrifuged (10,000g) for 5 min, genomic DNA isolated from the supernatant using a minicolumn (Wizard DNA Clean-up, Promega), eluting in 30 μl of H₂O.

2.3. Amplification by the PCR

Nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) regions (~ 300 –520 bp) were amplified separately from individual genomic DNA samples (10–20 ng template) by the PCR. From the nuclear genome, the D1 rDNA region was amplified using the oligonucleotide primers JB10 (forward: 5'-GATTACCCGCTGAACTTAAGCATAT-3') and JB9 (reverse: 5'-GCTGCATTCACAAACACCCCGACTC-3') [22]. From the mitochondrial genome, part of the NADH dehydrogenase 1 mitochondrial gene (*nad1*) was amplified with the primers JB11 (5'-AGATTCGTAAGGGGCCTAATA-3') and JB12 (5'-ACCACTAACTAATTCACCTTTC-3') [23], and another from the cytochrome *c* oxidase subunit 1 (*cox1*) was amplified with primers JB3 (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and JB4.5 (5'-TAAAGAAAGAACATAATGAAAATG-3') [23]. PCR reactions (25 μl) were performed in 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 3.5 mM MgCl₂, 250 μM of each dNTP, 25 pmol of each primer and 1 U *Taq* polymerase (*GoTaq*, Promega) under the following conditions: 94 °C, 30 s (denaturation); 55 °C, 30 s (annealing); 72 °C, 30 s (extension) for 35 cycles, followed by a final extension at 72 °C for 5 min. For each set of PCRs, negative (no-DNA) and positive controls were included. Prior to further molecular analyses, the quality and intensity of individual aliquots (5 μl) from amplicons were verified following electrophoresis in ethidium bromide-stained 1.5–2% agarose-TBE

(65 mM Tris–HCl, 22.5 mM boric acid, 1.25 mM EDTA, pH 9) gels, using Φ X174-*Hae* III as the size standard (Promega), under ultraviolet transillumination.

2.4. Mutation scanning, targeted sequencing and analyses

The D1 amplicons produced from all 160 samples were subjected to single-strand conformation (SSCP) analysis [24] to screen for sequence variation within each of the species of *Taenia*. In brief, 10 μ l of individual amplicons were mixed with an equal volume of ‘sequencing-stop’ buffer (10 mM NaOH, 95% formamide, 0.05% of both bromophenol blue and xylene cyanole). After denaturation at 94 °C for 15 min and snap cooling on a freeze block (–20 °C), individual samples (\approx 12 μ l) were loaded into the wells of precast GMA™ S-2 \times 25 gels (96 \times 261 mm; product no. 3548, Elchrom Scientific AG) and subjected to electrophoresis for 14 h at 74 V and 7.4 °C (constant) in a horizontal SEA2000™ apparatus (Elchrom Scientific AG) connected to a MultiTemp III (Pharmacia) cooling system. After electrophoresis, gels were stained for 15 min with ethidium bromide (0.5 μ g/ml), destained in water for the same time and then photographed upon ultraviolet transillumination using a digital camera.

Selected amplicons (\approx 40 μ l remaining for each) representing each unique SSCP profile and each host species were purified over minicolumns (Wizard™ PCR Preps, Promega, WI, USA), eluted in 30 μ l H₂O and then subjected to automated sequencing (BigDye® chemistry, Applied Biosystems), in both directions, using the same primers as for the primary PCR. The *cox1* and *nad1* amplicons produced from the same samples were sequenced in the same way. The electropherogram of each sequence was verified by eye, and the sequences were aligned manually. Reference sequences were used for comparative purposes: D1 sequences were determined from *T. multiceps*, *T. ovis*, *T. pisiformis*, *T. solium*, *T. taeniaeformis* and *Echinococcus granulosus* (samples Tm2, To1, Tp5, Tso15, Tt1 and Eg3, respectively; cf. [25]; *cox1* and *nad1* sequences representing the same species were available from previous publications [22], [23], [26], [27] and [28]. The levels of sequence difference (*D*), based on pairwise comparison, were calculated using the formula $D=1-(M/L)$ [29], where *M* is the number of alignment positions at which the two sequences had a base in common and *L* is the total number of alignment positions over which the two sequences were compared. Since no single tree-building

method is considered optimal under all circumstances, it is prudent to infer phylogenetic relationships using distance and character state analyses and to search for congruence between or among the trees generated. Phylogenetic analyses of the D1, *cox1* and/or *nad1* data sets were carried out using the neighbour-joining (NJ) and maximum parsimony (MP) methods in PAUP v.4.0b2 [30] according to Chilton et al. [31], using *E. granulosus* as the outgroup. The relative support for clades in the NJ and MP analyses was determined using 1000 bootstrap replicates.

3. Results and discussion

Based on the SSCP analysis of all 160 D1 amplicons (259 bp) for sequence variation (determined from profiles), 17 samples representing *T. hydatigena* from *C. familiaris* or *C. mesomelas* (sample codes Thy29, Thy65, Thy124 and Thy183), *T. madoquae* from *C. mesomelas* (Tma117, Tma118, Tma120 and Tma122), *T. serialis* from *C. aureus* (Tse79, Tse88 and Tse91 and Tse105), *T. regis* from *P. leo* (Tre152, Tre154 and Tre157) and *T. saginata* from *H. sapiens* (Tsa136 and Tsa141) were selected for the sequencing and analyses. The D1 (259 bp), *cox1* (396 bp) and *nad1* (488 bp) sequences were obtained from the 17 samples.

While no sequence variation in D1 was detected within any of the five species of *Taenia* from Africa, within-species variation of 0.25–1.0% was detected in *cox1*, and 0.2–2.1% in *nad1* (Table 1). For *cox1*, sequence variation of 0.25–0.76% (*T. hydatigena*), 0.25–1.0% (*T. madoquae*), 0.50% (*T. regis*), 0.5% (*T. serialis*) and 0% (*T. saginata*) was recorded within individual species (Table 1) compared with 0.3–4.1% in *T. taeniaeformis* (see [32]) and 1.7% in *T. solium* (see [33]). For *nad1*, within-species variation was 0.2–0.6% (*T. hydatigena*) compared with 0.5–5.5% reported by Kedra et al. [34], 0.2–0.8% (*T. madoquae*), 0.6–2.1% (*T. regis*), 0.4–2.1% (*T. serialis*) and 0.41% (*T. saginata*). Among species, sequence differences in D1 varied from 0% to 6.6%, with *T. hydatigena* and *T. regis* having the same sequence. The sequence differences in each of the two mitochondrial loci among all five species from Africa were considerably greater, ranging from 6.3% to 15.2% in *cox1* to 6.6–24.4% in *nad1* (Table 1). Therefore, the level of difference among species was usually the lowest for D1, followed by *cox1* and *nad1*, similar to results for different species or genotypes of *Echinococcus* [23] and [35].

Table 1.

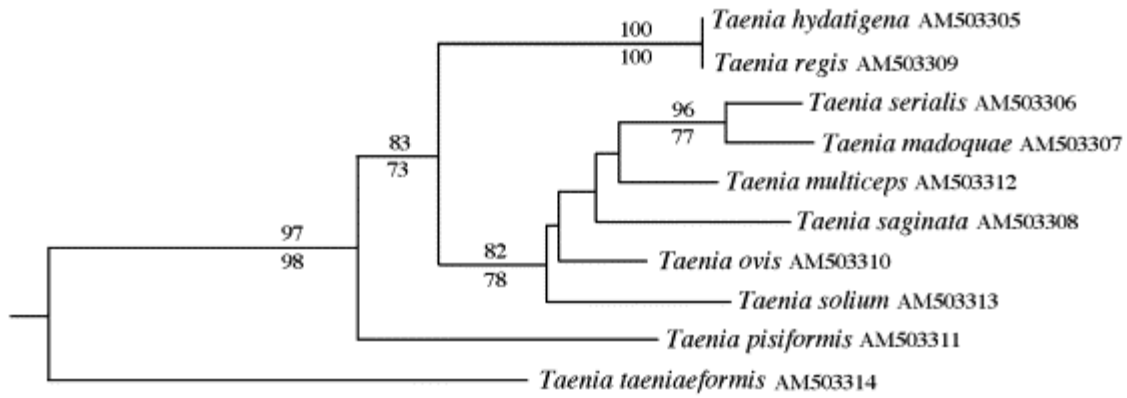
Pairwise comparison of nucleotide sequence differences (%) in the *coxI* (above the diagonal) and *nadI* (below the diagonal) among *Taenia* specimens from Kenya

Samples	Thy 29	Thy 65	Thy 124	Thy 183	Tma 117	Tma 118	Tma 120	Tma 122	Tre 152	Tre 154	Tre 157	Tsa 136	Tsa 141	Tse79	Tse88	Tse91	Tse105
Thy29		0.76	0.76	0.50	13.64	13.89	13.64	13.64	8.33	8.08	8.08	12.88	12.88	14.65	15.15	15.15	15.15
Thy65	0.41		0.50	0.25	13.38	13.64	13.38	13.38	7.58	7.32	7.32	13.13	13.13	13.89	14.39	14.39	14.39
Thy124	0.20	0.61		0.25	13.38	13.64	13.38	13.38	8.08	7.83	7.83	13.13	13.13	14.39	14.90	14.90	14.90
Thy183	0.20	0.61	0.41		13.13	13.38	13.13	13.13	7.83	7.58	7.58	12.88	12.88	14.14	14.65	14.65	14.65
Tma117	19.47	19.88	19.88	19.67		1.01	1.01	0.76	12.63	12.88	12.88	7.58	7.58	6.31	6.57	6.57	6.57
Tma118	19.47	19.67	19.67	19.47	0.61		0.50	0.25	12.88	13.13	13.13	8.08	8.08	6.82	7.07	7.07	7.07
Tma120	19.67	19.88	19.88	19.67	0.82	0.20		0.25	12.63	12.88	12.88	7.58	7.58	6.31	6.57	6.57	6.57
Tma122	19.88	19.88	19.67	19.67	0.82	0.20	0.41		12.63	12.88	12.88	7.83	7.83	6.57	6.82	6.82	6.82
Tre152	7.17	6.56	7.38	6.97	22.34	22.13	22.34	23.34		0.50	0.50	13.38	13.38	13.64	14.14	14.14	14.14
Tre154	7.38	7.17	7.58	7.17	22.75	22.54	22.75	22.75	0.84		0.00	13.64	13.64	13.89	14.39	14.39	14.39
Tre157	7.38	6.97	7.38	7.17	22.54	22.34	22.54	22.54	2.05	0.61		13.64	13.64	13.89	14.39	14.39	14.39
Tsa136	19.26	19.47	19.47	19.26	9.43	9.22	9.43	9.43	21.52	21.93	22.13		0.00	6.31	6.82	6.82	6.82
Tsa141	19.67	19.88	19.67	19.67	9.43	9.22	9.43	9.43	21.52	21.93	22.13	0.41		6.31	6.82	6.82	6.82
Tse79	22.34	22.34	22.13	22.34	11.68	11.48	11.68	11.27	23.16	24.18	24.39	12.09	12.09		0.50	0.50	0.50
Tse88	21.72	21.52	21.52	21.72	11.27	11.07	11.27	10.86	22.95	23.77	23.98	11.07	11.48	1.64		0.00	0.00
Tse91	21.72	21.52	21.52	21.72	11.27	11.07	11.27	10.86	22.95	23.77	23.98	11.07	11.48	1.64	0.00		0.00

Samples	Thy 29	Thy 65	Thy 124	Thy 183	Tma 117	Tma 118	Tma 120	Tma 122	Tre 152	Tre 154	Tre 157	Tsa 136	Tsa 141	Tse79	Tse88	Tse91	Tse105
Tse105	22.13	21.93	21.93	22.13	11.68	11.48	11.68	11.27	23.36	24.18	24.39	11.48	11.89	2.05	0.41	0.41	

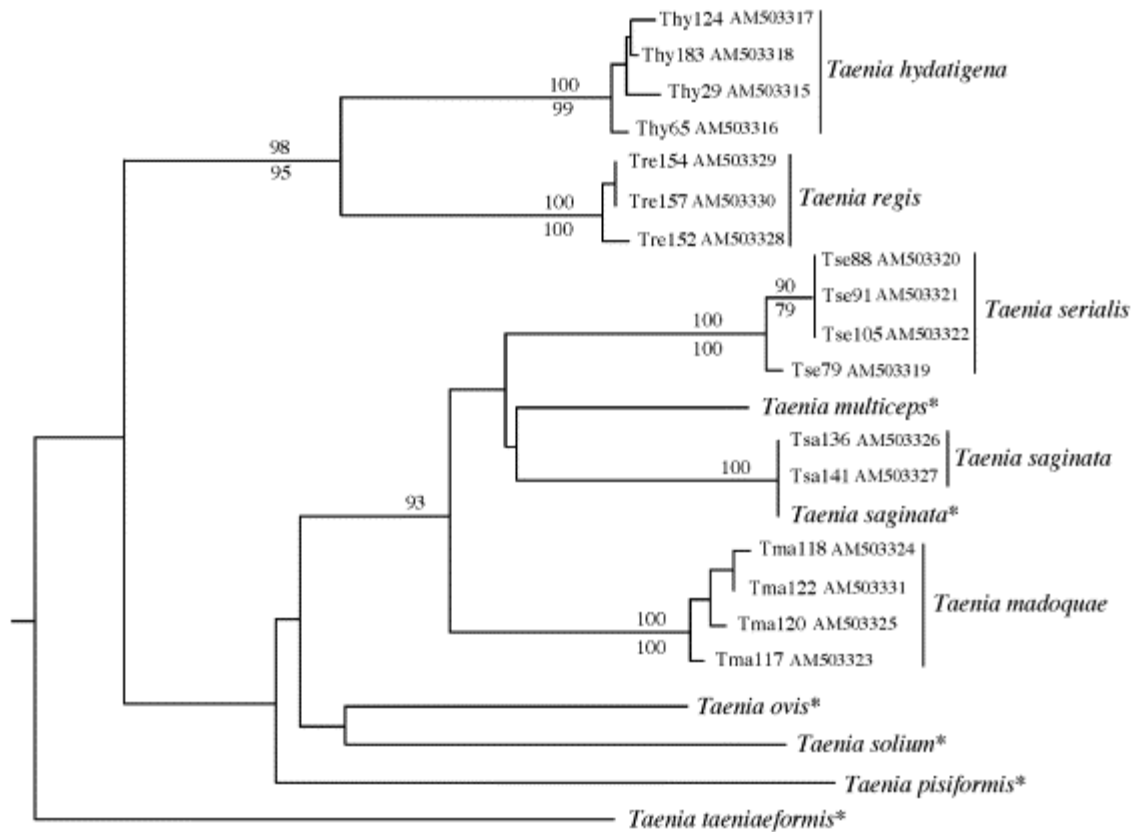
Taenia hydatigena (sample codes Thy29, Thy65, Thy124 and Thy183), *Taenia madoquae* (Tma117, Tma118, Tma120 and Tma122), *Taenia regis* (Tre152, Tre154 and Tre157), *Taenia saginata* (Tsa136 and Tsa141) and *Taenia serialis* (Tse79, Tse88, Tse91 and Tse105). Intraspecific variation (bold). Highest or lowest values (italics).

The three trees constructed using individual sequence data sets were similar in topology (Fig. 1), with the exception of the position of *T. saginata*. This difference among trees was not surprising, as different regions of rDNA and mtDNA can evolve at different rates [36]. There was clear concordance among all trees in that *T. regis* was most similar genetically to *T. hydatigena* and that *T. madoquae* was most similar to *T. serialis*, *T. multiceps* and/or *T. saginata*, which grouped to the exclusion of *T. ovis* and *T. solium*, which are known, also based on previous analyses (e.g., [27]), to be genetically closely related. *T. taeniaeformis* from cats was genetically the most distant from all other *Taenia* species from canids and humans (Fig. 1), consistent with previous evidence (e.g., [27] and [32]). The NJ and MP trees constructed using the combined *cox1+nad1* data set were similar in topology to those constructed using individual sequence data sets (not shown), and both provided moderate to strong bootstrap support (95% and 82%, respectively) for the relationship of *T. madoquae* with *T. serialis*, *T. multiceps* and/or *T. saginata*.



D1

0.01 changes



cox1

0.01 changes

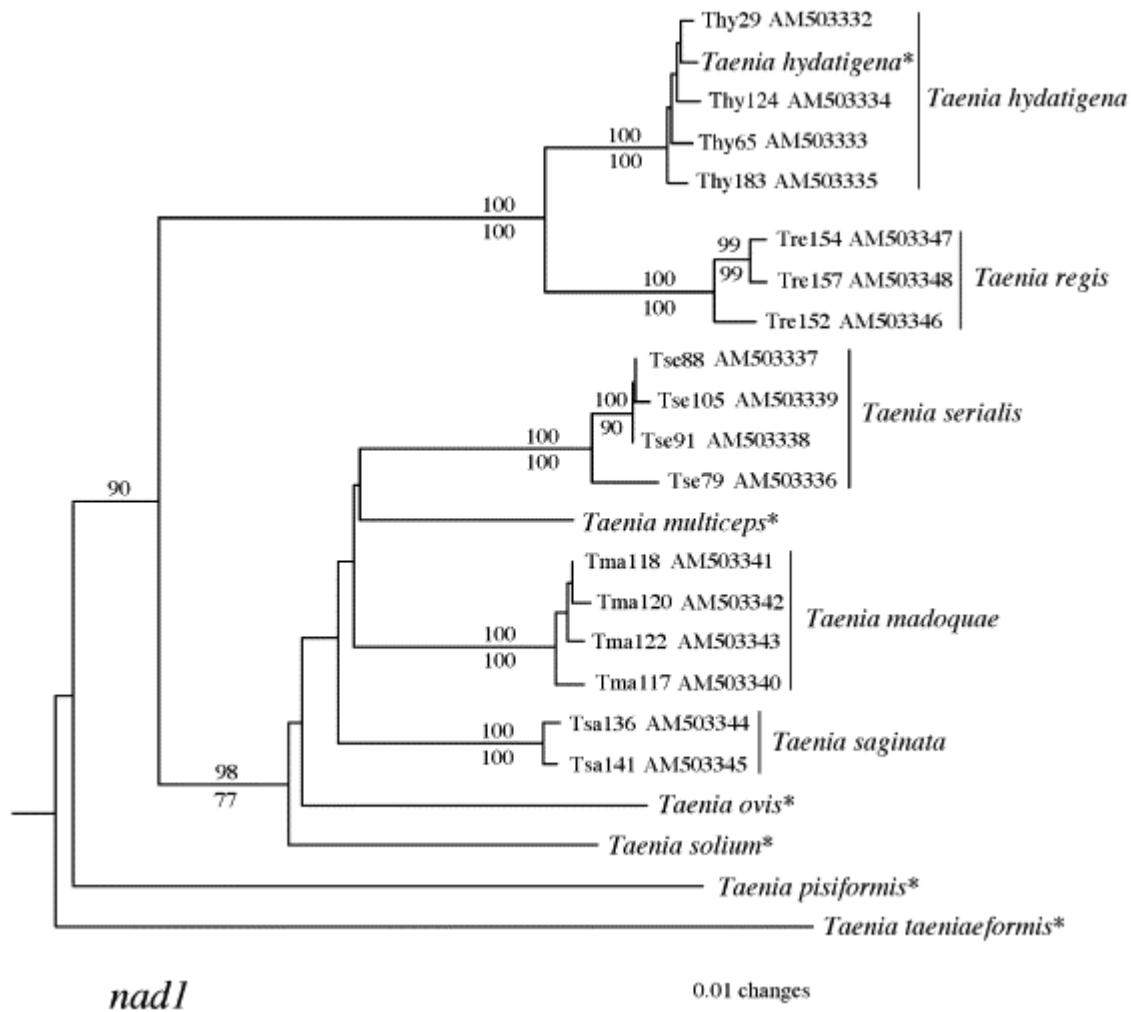


Fig. 1. Relationships of *Taenia madoquae* (from silver-backed jackal) and *T. regis* (from lion) with some other species of *Taenia* based on D1, *cox1* or *nad1* sequence data using the neighbour-joining (NJ) method. The accession numbers of individual sequences determined in the present study are shown in each tree. Marked with an asterisk are selected *cox1* and *nad1* reference sequences from previous studies [22], [23], [26], [27] and [28] used for comparative purposes in the analyses. A scale bar indicates estimated distance. There was concordance in the topology between trees constructed using the NJ and maximum parsimony (MP) methods (1000 replicates). Bootstrap values of >50% are indicated above (NJ) and below (MP) the branches.

Taenia madoquae, the adult stage of which is very similar morphologically to that of *T. hydatigena*, was established as a distinct species by Jones et al. [18]. It is intriguing and unexpected that *T. madoquae*, a species with a cysticercus in the skeletal muscles of a small antelope appears to be genetically closely related (of the species studied here) to *T.*

serialis and *T. multiceps*, both of which have a coenurus as the larval stage. Current findings contrast with cladistic analyses by Hoberg et al. [37], [38] and [39], which indicated that *T. madoquae* appeared in a subclade separate from both *T. multiceps* and *T. serialis*. All three species occur as adults in canids, but there are some differences in the intermediate host group and the site occupied by the larval stages. The coenurus of *T. multiceps* occurs in the central nervous system, most frequently in ruminants; that of *T. serialis* occurs in the intermuscular tissues of, predominantly lagomorphs. In the present study, the two clades defined constitute species with cysticerci found predominantly in the peritoneal cavity on the one hand (*T. regis* and *T. hydatigena*) and cysticerci or coenuri found in the musculature, intermuscular/subcutaneous connective tissue or central nervous system (brain) on the other hand (*T. madoquae*, *T. saginata*, *T. serialis* and *T. multiceps*). In a cladistic study based on morphological data [37], this character was homoplasious, with a consistency index of only 0.273 and was thus not examined further. This apparent incongruity may be due to the comparison of morphological with molecular data sets or may be an artefact of sampling in the molecular data as the current molecular tree, as well as all other molecular trees for the genus, includes a limited range of species. The pattern observed here is not replicated, for example, in the molecular studies by Okamoto et al. [32] and de Queiroz and Alkire [40]. However, these trees are also based on a limited number of species. A more careful examination of metacestode localization may be warranted when more complete molecular phylogenetic trees for the genus become available.

The morphology of the larval stage has been used to distinguish taeniid genera, such as *Multiceps*, *Hydatigera* and *Tetratirotaenia*, by Abuladze [20] and others. A close genetic relationship between *T. madoquae* and two species previously allocated to *Multiceps* supports the synonymy of the latter genus and, by implication, the others with *Taenia*; these synonymies (discussed in [9] and [37]) have gradually gained general acceptance. A future line of study might be to examine species, such as *T. taeniaeformis* and *T. parva*, with a strobilocercus/polycephalic strobilocercus metacestode to better clarify their genetic relationships to species with a cysticercus.

Taenia regis, a parasite of felids (lions and leopards), was most closely related to *T. hydatigena*, a parasite of wild and domesticated canids. This finding is similar to the

cladistic study by Hoberg et al. [37], [38] and [39], in which they both appeared in the same subclade. Both species have a relatively large cysticercus (usually up to 2.0–2.5 cm in the case of *T. regis*, and ~2.5–4 cm or more for *T. hydatigena*) in the serosa in the coelom of the intermediate host, but they are clearly distinct based on adult morphology. Allsopp et al. [17] found that these two species differed only in their lactate dehydrogenase (LDH) zymograms. The use of markers to characterize and identify the larval stages of these species would be useful, because the cysticercus of *T. hydatigena* was virtually unknown in large game antelopes (the intermediate hosts of *T. regis*) surveyed in the 1960s in East Africa [41], [42] and [43] but became increasingly common during the 1970s, as grazing domestic stock extended and competed for space with wild predators and their prey (A. Jones, unpublished data, Kenya). It might also provide the means of resolving the validity of *T. simbae*, a rare species from the lion, which can be difficult to separate from *T. regis* based on morphological criteria [17].

In conclusion, the present analyses showed that *T. regis* was genetically closely related to *T. hydatigena*, and *T. madoquae* to *T. serialis*, *T. multiceps* or *T. saginata*. This study provides additional support for the application of the present mitochondrial markers to the characterization, and population genetic, epidemiological and phylogenetic studies of a broad range of species or operational taxonomic units of *Taenia* from Africa and other parts of the world [11]. Importantly, the present findings also provide a ‘stepping stone’ for future work on establishing the systematic relationships of some of the lesser-known taeniid cestodes [11] based on partial or, preferably, whole mitochondrial genome sequence data sets (cf. [15] and [44]), as has been undertaken comprehensively for members of the genus *Echinococcus* (e.g., [15], [35], [45] and [46]).

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The sequences determined in the present study have been deposited under GenBank accession numbers: AM503304–AM503314 (D1), AM503315–AM503331 (*cox1*) and AM503332–AM503348 (*nad1*).

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