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J. Parasitol., 91(6), 2005, pp. 1435–1443
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SYSTEMATICS OF *MESOCESTOIDES* (CESTODA: MESOCESTOIDIDAE): EVALUATION OF MOLECULAR AND MORPHOLOGICAL VARIATION AMONG ISOLATES

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ABSTRACT: A hypothesis-based framework was used to test if 3 genetic strains of *Mesocestoides* (clades A, B, and C) are distinct evolutionary lineages, thereby supporting their delimitation as species. For comparative purposes, 3 established cestode species, *Taenia pisiformis*, *Taenia serialis*, and *Taenia crassiceps* were assessed using the same methods. Sequence data from mitochondrial rDNA (12S) and the second internal transcribed spacer of nuclear rDNA (ITS-2) revealed derived (autapomorphic) characters for lineages representing clade A (n = 6 autapomorphies), clade B (n = 4), and clade C (n = 9) as well as *T. pisiformis* (n = 15) and *T. serialis* (n = 12). Furthermore, multivariate analysis of morphological data revealed significant differences among the 3 genetic strains of *Mesocestoides* and between *T. pisiformis* and *T. serialis*. The level of phenotypic variation within evolutionary lineages of *Mesocestoides* and *Taenia* spp. tapeworms was similar. Results from this study support recognizing *Mesocestoides* clades A, B, and C as separate species, and provide evidence that clade B and *Mesocestoides vogae* are conspecific.

Traditionally, parasite species have been described using the Linnaean or similarity species concept, which is based primarily upon differences in morphological characteristics or host identity (Mayr, 1963). Although this species concept and its methods have proven useful for delimiting species of many macroparasites, it has been unsuccessful for certain other common parasites, including *Mesocestoides* spp. tapeworms, because of difficulties presented by morphological plasticity or, conversely, cryptic species. Identification and species delimitation of *Mesocestoides* spp. tapeworms is difficult because of conflicts among key morphological characters (Loos-Frank, 1987) and a high degree of host-induced nonspecific morphological variation (Rausch, 1994). For example, Voge (1955) examined 6 putative *Mesocestoides* species from North America and concluded that the high degree of morphological variability warranted a complete revision of the genus. Furthermore, adult *Mesocestoides* spp. with similar genetic characteristics have been isolated from a wide host and geographic range (Crosbie, Nadler et al., 2000; Crosbie, Padgett et al., 2000; Padgett and Boyce, 2004), thus indicating that there is reduced specificity for final hosts in some species of this genus. Clearly, *Mesocestoides* is a particularly problematic genus, but more broadly, there is limited value in using only similarity measures for delimiting parasite species.

Because efforts to distinguish among *Mesocestoides* species using morphological similarities have often failed, other approaches are needed. In an attempt to reduce the systematic error in nematode species delimitation, Adams (1998) recommended an amalgamation of phylogenetic species concepts (e.g., Cracraft, 1983) and evolutionary species concepts (e.g., Wiley, 1978). Using this methodology, species are recognized based upon evidence of independent evolutionary lineages reflected in the form of autapomorphies, i.e., derived character states unique to individuals of a species. This phylogenetic approach to delimiting species allows for hypothesis testing and potential refutation. For example, Nadler et al. (2000) used this theoretical framework to evaluate *Uncinaria* spp. hookworms from 2 sympatric host species (California sea lions, *Zalophus californianus*, and northern fur seals, *Callorhinus ursinus*, collected from San Miguel Island, Channel Island National Park). They found that hookworms from each host species had autapomorphies characteristic of separate evolutionary lineages, indicating that each pinniped species hosted distinct hookworm species.

Recent molecular phylogenetic studies of *Mesocestoides* spp. revealed at least 3 distinct monophyletic groups (clades A, B, and C) in western North America (Crosbie et al., 1998, Crosbie, Nadler et al., 2000, Crosbie, Padgett et al., 2000). Clade A included acephalic metacestodes and adult tapeworms from dogs and coyotes, respectively. Clade B was composed of tetrathyridia from a dog and a western fence lizard (*Sceloporus occidentalis*), and clade C was composed of adult tapeworms from dogs. Although these studies showed *Mesocestoides* spp. isolates belong to separate clades, evolutionary species delimitation would benefit from population-level sampling of individuals and analysis of multiple genetic loci.

Compared to *Mesocestoides*, the species-level systematics of taeniid tapeworms is better resolved. Based on morphological analysis of adult specimens, there are approximately 40 recognized species in the genus *Taenia* (Hoberg et al., 2000). Many recent studies have shown high levels of interspecific variation for molecular characters that are useful for distinguishing species of *Taenia* (Bowles and McManus, 1994; Gasser and Chilton, 1995; De Queiroz and Alkire, 1997; Nickisch-Rosenegk et al., 1999). Because of the comparatively advanced state of species-level taeniid systematics, *Taenia* species provide a good standard for comparative analysis with other problematic cestode species such as *Mesocestoides*.

The objectives of this study were (1) to determine whether *Mesocestoides* clades A, B, and C are distinct evolutionary lineages that should be delimited as separate species; (2) to determine whether established species of *Taenia* show evidence of lineage independence using the same genetic loci and methods; and (3) to assess the extent of morphological variation among and within each clade of *Mesocestoides* and each species of *Taenia*.

Received 9 July 2004; revised 29 March 2005; accepted 29 March 2005.

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MATERIALS AND METHODS

Primary characterization of samples

With the exception of specimens acquired from museum or personal collections (certain *Mesocestoides* spp., *T. pisiformis*, and *T. serialis*), all tapeworm specimens used in this study were identified in the following manner. Metacestode and adult tapeworm samples were identified to genus using morphological characters. Because many *Mesocestoides* spp. metacestodes from domestic dogs lack apical suckers (''acephalic metacestodes''), morphological identification of these specimens was not possible. Instead, all tapeworms were tested independently by a PCR–RFLP assay targeting the second internal transcribed spacer region (ITS-2) of nuclear rDNA (Crosbie et al., 1998) and grouped according to distinct restriction fragment patterns, i.e., clade A, clade B, clade C, *T. crassiceps*, *T. pisiformis*, and *T. serialis*.

Source of tapeworm material

Mesocestoides spp. metacestodes (acephalic and tetrathyridial morphotypes) were obtained from domestic carnivores diagnosed with peritoneal cestodiasis throughout California and other locations in western North America (Table I). Tetrathyridia also were obtained from wild-caught deer mice (*Peromyscus maniculatus*) at 2 California sites (Table I). All larvae were preserved in 70% ethanol or frozen in sterile saline solution before genetic analysis.

Intact clade A *Mesocestoides* spp. adult tapeworms were obtained by necropsy from wild-caught coyotes (*Canis latrans*) killed by county trappers in 4 northern California counties (Mendocino, Napa, Sutter, and Yuba). In addition, proglottids were acquired from dogs experimentally or naturally infected with adult *Mesocestoides* spp. tapeworms (clades B and C). Intact adult tapeworms of *Mesocestoides* spp. (clades A and C) were also obtained from island fox (*Urocyon littoralis*) during necropsy of animals that died of natural causes at 4 California Channel Islands (San Nicolas, San Miguel, Santa Catalina, and San Clemente). These adult tapeworms were preserved in 70% ethanol for genetic analysis. The anterior and posterior ends were removed from adult *Mesocestoides* that were used for morphological analysis (Table II). A subset of these tapeworms was deposited in the U.S. National Parasite Collection (USNPC), Beltsville, Maryland (accession numbers: clade A, 093311; clade B, 093312; clade C, 093313). For comparison, voucher specimens from the USNPC were obtained of *Mesocestoides kirbyi* Chandler, 1944 (36898, type specimen), *Mesocestoides variabilis* Mueller, 1927 (51171, cotype), *Mesocestoides manteri* Chandler, 1942 (44941, type specimen), and *Mesocestoides bassarisci* MacCallum, 1921 (45604 and 45933).

Metacestodes of *T. pisiformis* and *T. serialis* were obtained from rabbits. Adult *T. pisiformis* were obtained from coyotes in Mendocino, Yuba, and Sutter counties; adult *T. serialis* were obtained from coyotes in Mendocino and Napa counties. Adult *T. serialis* also were obtained from an experimentally infected dog and a wild-caught mountain lion. For morphometric analyses, voucher specimens of adult *T. pisiformis* and *T. serialis* were obtained from the USNPC (*T. pisiformis* accession numbers: 77533, 77801, 78502, 78503, 78511, 78734, 82562, and 78511; *T. serialis* accession numbers: 15794, 18104). Measurements were made for a total of 32 *T. pisiformis* and 31 *T. serialis* specimens.

Adult tapeworms were stained with acetocarmine, dehydrated in ethanol, cleared in methyl salicylate, and mounted in Canada balsam. Measurements were made using a compound microscope and ocular micrometer. For adult *Mesocestoides* spp., the following characters were measured: width of the scolex at the widest point, diameter of the suckers, and diameter of the paruterine organ in mature proglottids. For adult *Taenia* spp., measurements included scolex width, sucker diameter, and total length of the small and large rostellar hooks. Multivariate statistics (MANOVA) were used for comparison of these measurements among *Mesocestoides* spp. clades A, B, and C (Wilk's Lambda) and between *Taenia* species (F test). In addition, a 1-way ANOVA was performed on each character. Analyses were performed with the JMP IN statistical program (SAS Institute, 2000).

PCR amplification and sequence analysis

DNA was extracted from whole metacestodes or proglottids with the Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany) using the manufacturer's protocol. Polymerase chain reaction (PCR) amplifications were performed on 2 gene fragments. A 314-bp fragment of mitochondrial 12S rDNA was amplified using cestode-specific primers 60.for. (5′-TTAAGATATATGTGGTACAGGATTAGATACCC-3′) and 375. REV. (5′-AACCGAGGGTGACGGGCGGTGTGTACC-3′) (Nickisch-Rosenegk et al., 1990). As previously reported (Gasser and Chilton, 1995; Crosbie et al., 1998), amplification of a 600–700-bp region of ITS-2 nuclear rDNA was accomplished using primers NC-6 (5′-ATC GACATCTTGAACGCACATTGC-3′) and NC-2 (5′-TTAGTTTCTTT TCCTCCGCT-3′). Total volume for PCR reactions was 100 μl, comprising 20 μl of DNA extract, 54.6 μl of sterile water, 5 μl of each primer, 2 μl of 10 mM dNTP mix, 10 μl 10x PCR buffer, 3.0 μl $MgCl_2$ (1.5 mM final concentration), and 0.4 μl (5 units/μl) *Taq* Polymerase (Invitrogen, Carlsbad, California). Automated nucleotide sequencing was performed by Davis Sequencing (Davis, California) and University of California Davis, Division of Biological Sciences sequencing facilities. Both strands were sequenced for all PCR products. Complementary sequences were assembled and edited using Sequencher version 3.0 (Gene Codes, Ann Arbor, Michigan). Regions that corresponded to the PCR primers were removed from the sequences before sequence alignment and phylogenetic analysis.

Phylogenetic Analyses

Sequences of *Mesocestoides* spp. clades A, B, and C and *T. crassiceps*, *T. pisiformis*, and *T. serialis* were aligned for both 12S and ITS-2 gene regions using Se-Al version 2.0 (Rambaut, 1996) and checked for variable nucleotides. Trees were constructed by maximum parsimony using PAUP*4.0 (heuristic search with 1,000 replicates of random stepwise addition). Support for clades was assessed by bootstrap resampling (1,000 replicates) and maximum-parsimony inference. All trees were rooted using *Taenia crassiceps* as the out-group. This out-group choice was based on morphological evidence that Taeniidae and Mesocestoididae are closely related families (Hoberg et al., 1999) and that *T. crassiceps* is sister to *T. pisiformis* and *T. serialis* (Hoberg et al., 2000). Certain published 12S rDNA sequences from GenBank were also included in phylogenetic analyses: *M. vogae* (L49448), *Mesocestoides lineatus* (L49450), and *Mesocestoides leptothylacus* (L49451) (Nickisch-Rosenegk et al., 1999).

RESULTS

Morphometrics

Analyses of morphological data revealed significant differences among all *Mesocestoides* clades and between *Taenia* species for all characters ($P < 0.0001$) (Tables III, IV). The results were identical for each character analyzed separately by ANOVA.

Morphometric analyses suggest that variation in the morphological characters analyzed is similar in *Mesocestoides* and *Taenia* spp. Coefficients of variation (CV) for morphological character measurements showed moderate variation within *Mesocestoides* clades (CV for clades A, B, and C for scolex: 14.8%, 19.5%, 17.3%; sucker: 10.2%, 13.6%, 15.7%; paruterine organ: 14.4%, 13.0%, 18.1%). Not surprisingly, the CV for pooled *Mesocestoides* spp. clades was greater than for individual clades (scolex: 22.1%, sucker: 24.5%, paruterine organ: 21.3%). For *T. pisiformis* and *T. serialis*, the scolex was more variable than were other characters, such as the rostellar hooks (CV for *T. pisiformis* and *T. serialis* scolex: 24.0%, 26.5%; sucker: 15.6%, 14.5%; external rostellar hook: 5.3%, 15.5%; internal rostellar hook: 12.4%, 11.4%). As in *Mesocestoides*, the CV for pooled *Taenia* spp. measurements was greater (scolex: 35.2%, sucker: 22.5%, external rostellar hook: 23.6%, internal rostellar hook: 27.0%).

Morphometric variation within clades of *Mesocestoides* spp. was no more variable than within *Taenia* spp. However, it was difficult to make systematic assessments based on morphomet-

TABLE I. DNA sequence data from *Mesocestoides* and *Taenia* spp.

	Host	Location	Stage	ITS-2	12S
Clade A					
	Coyote 65	Hopland, CA	Adult	DQ078756	DQ102739
	Coyote 222	Hopland, CA	Adult	DQ096544	DQ102740
	Dog	Red Bluff, CA	Adult	DQ096545	DQ102741
	Dog	Garden Grove, CA	Adult	DQ096546	DQ102742
	Island fox	Santa Catalina Island, CA	Adult	DQ096547	DQ102743
	Island fox	San Clemente Island, CA	Adult	DQ096548	DQ102744
	Island fox	San Miguel Island, CA	Adult	DQ096549	DQ102745
	Deer mouse	San Miguel Island, CA	Tetrathyridia	DQ096550	DQ102746
	Dog	Willits, CA	Tetrathyridia	DQ096551	DQ102747
	Dog	Inglewood, CA	Acephalic metacestode	AF119698	DQ102748
	Dog	Davis, CA	Acephalic metacestode	AF119699	DQ102749
	Dog	Weaverville, CA	Acephalic metacestode	AF119700	DQ102750
Clade B					
	Coyote 347	Hopland, CA	Adult	DQ096552	DQ102751
	Coyote 347	Hopland, CA	Adult	DQ096553	DQ102752
	Deer mouse	Hopland, CA	Tetrathyridia	DQ096554	DQ102753
	Dog†	Auburn, CA	Adult	DQ096555	DQ102754
	Dog	Auburn, CA	Tetrathyridia	AF119697	DQ102755
	Mouse*	U. of Berne, Switzerland	Tetrathyridia	AF119696	DQ102756
	Dog	Phoenix, AZ	Tetrathyridia	DQ096556	DQ102757
Clade C					
	Dog	Sumpter, OR	Adult	DQ096558	DQ102758
	Dog	Telluride, CO	Adult	DQ096558	DQ102759
	Dog	Midpines, CA	Adult	AF119708	DQ102760
	Dog	Mt. Shasta, CA	Adult	AF119707	DQ102761
	Island fox 108	San Nicolas Island, CA	Adult	DQ099557	DQ102762
	Island fox 180	San Nicolas Island, CA	Adult	DQ099558	DQ102763
	Island fox 180	San Nicolas Island, CA	Adult	DQ099559	DQ102764
	Island fox 180	San Nicolas Island, CA	Adult	DQ099560	DQ102765
	Island fox 180	San Nicolas Island, CA	Adult	DQ099562	DQ104224
	Island fox 251	San Nicolas Island, CA	Adult	DQ099561	DQ104225
	Island fox 066	San Nicolas Island, CA	Adult	DQ099563	N/A‡
Taenia crassiceps					
	Mouse	UC Riverside, CA	Metacestode	DQ099564	DQ104226
Taenia pisiformis					
	Rabbit	Central Valley, CA	Metacestode	DQ099565	DQ104227
	Coyote 88	Yuba County, CA	Adult	N/A‡	DQ104228
	Coyote 90	Yuba County, CA	Adult	DQ099566	DQ104229
	Coyote 90	Yuba County, CA	Adult	DQ099567	DQ104230
	Coyote 96	Yuba County, CA	Adult	DQ099568	DQ104231
	Coyote 89	Sutter County, CA	Adult	DQ099569	DQ104232
	Coyote 350	Mendocino County, CA	Adult	DQ099570	DQ104233
Taenia serialis					
	Rabbit	Vancouver, BC	Metacestode	DQ099571	DQ104234
	Coyote 222	Mendocino County, CA	Adult	N/A‡	DQ104235
	Coyote 63	Mendocino County, CA	Adult	DQ099572	DQ104236
	Coyote 179	Mendocino County, CA	Adult	DQ099573	DQ104237
	Coyote 94	Mendocino County, CA	Adult	DQ099574	DQ104238
	Coyote 117	Mendocino County, CA	Adult	DQ099575	DQ104239
	Coyote 162	Napa County, CA	Adult	DQ099576	DQ104240

* Laboratory strain of *Mesocestoides vogae*; original host a western fence lizard from California (Specht and Voge, 1965)
† Dog experimentally infected using *Mesocestoides* tetrathyridia from dog (Padgett and Boyce, 2004).
‡ Sequence not included due to repeated failure of PCR amplification.

TABLE II. Host and collection locality of adult tapeworms used in morphometric analyses.

Clade/Species	Number	Host	Locality	Collector	Accession no.
Mesocestoides					
A	10	Island fox	Santa Catalina Island, CINP	Dr. Linda Munson	N/A
A	9	Island fox	San Clemente Island, CINP	Dr. Linda Munson	N/A
A	12	Coyote	Mendocino Co., CA	K.A. Padgett	N/A
B	25	Domestic dog*	Placer Co., CA	K.A. Padgett	N/A
C	18	Island fox	San Nicolas Island, CINP	Dr. Linda Munson	N/A
Taenia					
pisiformis	14	Coyote	Sutter Co., CA	K.A. Padgett	N/A
pisiformis	9	Coyote	Yuba Co., CA	K.A. Padgett	N/A
pisiformis	1	Coyote	Webb Co., TX	L. Windberg	77533
pisiformis	1	Gray fox	Escamia Co., FL	J.A. Conti	77801
pisiformis	2	Bobcat	Brown Co., NE	K.L. Tiekotter	78502
pisiformis	1	Bobcat	Brown Co., NE	K.L. Tiekotter	78503
pisiformis	2	Bobcat	Pawnee Co., NE	K.L. Tiekotter	78511
pisiformis	1	Coyote	Muhlenberg Co., KY	R.P. Myers	82562
pisiformis	2	Domestic dog	NWT, Fort Smith, Canada	W.E. McIntyre	78734
serialis	7	Coyote	Napa Co., CA	K.A. Padgett	N/A
serialis	3	Coyote	Mendocino Co., CA	K.A. Padgett	N/A
serialis	6	Domestic dog	Athenia Co., NJ	A.C. Carlton	18104
serialis	10	Domestic dog*	Alaska	Dr. Robert Rausch	N/A
serialis	5	Mountain lion	Union Co., OR	Dr. Robert Rausch	N/A

* Experimental infection.

rics because of the extent of variation among *Mesocestoides* spp. clades and the overlap in range of values for characters measured. In comparative analysis with museum type and voucher specimens, clade B adult tapeworms were found to be most similar to *M. variabilis* and *M. bassarisci* in measurements of 3 morphological characters (scolex width, sucker diameter, and paruterine organ). Measurements for *M. variabilis* (n = 4): scolex width (mean = 353.3, range: 328.4–408.0, SD = 37.7), sucker diameter (mean = 145.0; range: 140–150, SD = 5.8), paruterine organ (mean = 326.0; range: 298.5–348.3, SD = 20.6). Voucher specimens of *M. bassarisci* (n = 22) measured as follows: scolex width (mean = 331.3, range: 275.0–380, SD = 31.7), sucker diameter (mean = 118.2, range: 100–140, SD = 11.0), paruterine organ (mean = 143, range: 90–175, SD = 33.7). *Mesocestoides bassarisci* were small and appeared immature; only 6 of 22 had paruterine organs, and these did not appear fully developed. There was no statistical difference between *M. bassarisci* and clade B in the size of the scolex ($P = 0.68$) and the sucker ($P = 0.0063$). Scolex, sucker, and paruterine organ measurements of *M. kirbyi* and *M. manteri* were both outside the range of clade B and fit most closely within the range of measurements for clade A (*M. kirbyi* type specimen [497.5; 189.1; 447.8] and *M. manteri* type specimen [497.5; 179.1; 358.2]). Measurements of morphological characters of clade C did not match any type specimen included in these analyses.

TABLE III. Measurements of adult *Mesocestoides* spp. from North American dogs and wildlife.

Molecular strain	Scolex width (*u*m)	Sucker diameter (*u*m)	Paruterine organ diameter (*u*m)
Clade A			
Mean	405.5	178.1	327.4
Range	(245.0–497.5)	(149.0–200.0)	(230.0–398.0)
Standard deviation	59.8	18.2	47.1
Number	25	26	18
Clade B			
Mean	325.0	130.7	299.9
Range	(219.0–407.0)	(100.0–180.0)	(200.0–365.0)
Standard deviation	63.4	17.8	39.1
Number	24	25	25
Clade C			
Mean	466.3	222.1	446.4
Range	(325.0–587.0)	(150.0–278.0)	(298.0–547.3)
Standard deviation	80.7	34.8	80.9
Number	16	14	8

12S mitochondrial sequence data and analysis

The amplified region of *Mesocestoides* spp. and *Taenia* spp. 12S sequences were variable in length (primer sequences removed): clade A (347 nt), clade B (352 nt), clade C (349 nt), *T. pisiformis* (320 nt), *T. serialis* (324 nt), and *T. crassiceps* (321 nt). The average base composition of *Mesocestoides* spp. was 0.29 (A), 0.13 (C), 0.19 (G), and 0.39 (T). The average base composition of *Taenia* spp. was 0.26 (A), 0.16 (C), 0.18 (G), and 0.40 (T). There were no significant differences in base composition among *Mesocestoides* spp. clades or *Taenia* spp. species. There was a slightly higher frequency of the nucleotide T in *Taenia* than in *Mesocestoides* ($P = 0.0004$).

Analysis of *Mesocestoides* spp. 12S data yielded a bootstrap consensus tree that represented *M. lineatus* and *M. leptothyla-*

TABLE IV. Measurements of adult *Taenia pisiformis* and *T. serialis* from North American dogs and wildlife.

Species	Scolex width (*u*m)	Sucker diameter (*u*m)	Total length of rostellar hooks	
			External (*u*m)	Internal (*u*m)
Taenia pisiformis				
Mean	940.2	300.8	140	235.1
Range	(537.3–1552.0)	(200.0–378.1)	(129.0–149.3)	(139.0–268.7)
Standard deviation	225.6	46.9	7.44	29.1
Number	30	30	29	20
Taenia serialis				
Mean	547.7	268	86.4	145.3
Range	(250.0–815.9)	(115.0–268.0)	(60.0–110.0)	(90.0–175.0)
Standard deviation	145.1	30.9	13.34	16.5
Number	20	20	18	21

cus as sister to North American *Mesocestoides* spp. (Fig. 1A). Within North American *Mesocestoides* spp. there was strong bootstrap support for clades A and B as sister taxa (100%), with clade C sister to A plus B (100%). Analysis of aligned 12S sequences revealed sequence variation, partitioned as apomorphies for each *Mesocestoides* spp. clade (clade A, n = 4; clade B, n = 2; clade C, n = 4) (Table V; Fig. 2). These clade-specific sequence states involved 5 transition and 5 transversion substitutions.

For *Taenia* species, the tree based on 12S data placed *T. pisiformis* and *T. serialis* as sister species with *T. crassiceps* sister to these species (Fig. 3); these relationships received strong bootstrap support. Analysis of *Taenia* spp. sequences revealed derived nucleotide character states for each species (*T. pisiformis*, n = 10; *T. serialis*, n = 7) (Table VI; Fig. 4). Differences between *T. pisiformis* and *T. serialis* for the 12S sequence data involved 6 transition and 8 transversion substitutions, and 3 indels.

ITS-2 sequence data and analysis

The sequenced region of *Mesocestoides* spp. and *Taenia* spp. ITS-2 was length-variable: clade A (472 nt), clade B (494 nt), clade C (482 nt), *T. pisiformis* (538 nt), *T. serialis* (516 nt), and *T. crassiceps* (611 nt). Although there were no significant differences in the average base composition among *Mesocestoides* spp. clades (A = 0.19, C = 0.23, G = 0.34, and T = 0.27) or *Taenia* species (A = 0.27, C = 0.34, G = 0.24, and T = 0.15),

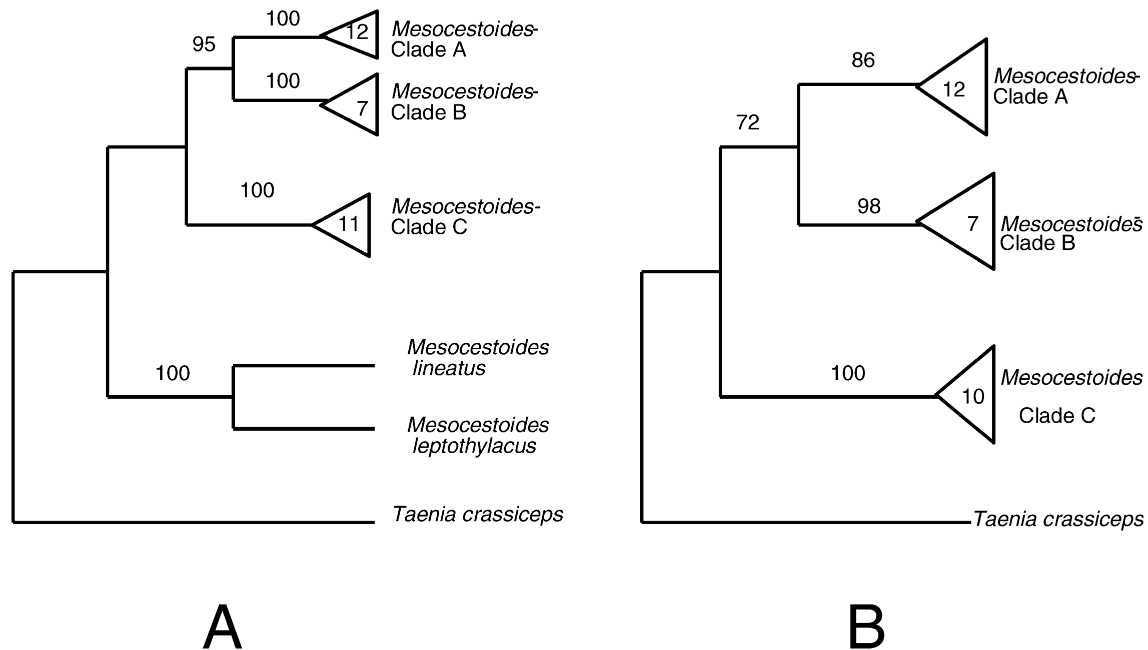

FIGURE 1. **A.** Phylogenetic relationships of *Mesocestoides* clades A, B, C, *M. lineatus*, *M. leptothylacus*, and *Taenia crassiceps* based on maximum parsimony using partial sequences of 12S gene (strict consensus of 40 trees, tree length = 172, CI = 0.82, RI = 0.90, HI = 0.18; 109 parsimony-informative/34 variable parsimony-uninformative sites). **B.** Maximum parsimony (strict consensus of 24 trees, tree length = 638, CI = 0.90, RI = 0.89, HI = 0.10; 269 parsimony-informative/254 variable parsimony uninformative sites) based on partial sequences of the ITS-2 gene for *Mesocestoides* spp. clades A, B, C, and *T. crassiceps*. All trees were rooted with *T. crassiceps* as out-group; numbers above branches represent bootstrap support (1,000 replicates). Number within clade triangle indicates the number of individuals sampled.

TABLE V. Diagnostic sequence data from *Mesocestoides* tapeworms, Clades A, B, and C in relation to *Taenia crassiceps* (outgroup); apomorphies of *T. crassiceps* are not shown.

	Position of variable characters for 12S									
	55	79	88	99	106	166	202	251	259	260
Taenia crassiceps	G	T	T	T	A	T	T	T	T	T
Clade A	**A**	T	T	**C**	**G**	**A**	T	T	T	T
Clade B	G	T	T	T	A	T	T	T	**A**	**A**
Clade C	G	**A**	**C**	T	A	T	**A**	**G**	T	T
	Position of variable characters for ITS-2									
	37	41	42	52	60	74	108	143	668	
Taenia crassiceps	G	T	A	T	T	G	G	G	C	
Clade A	G	**A**	A	T	T	**A**	G	G	C	
Clade B	G	T	A	T	T	G	**A**	**A**	C	
Clade C	**C**	T	**C**	**C**	**G**	G	G	G	**A**	

the difference in base frequency between *Mesocestoides* and *Taenia* was significant for all nucleotides ($P < 0.0001$). Strict consensus trees constructed using ITS-2 sequence data had trees with the same topology as trees constructed from 12S sequence data (Fig. 1B). All individuals of clade A, B, and C *Mesocestoides* clustered into monophyletic groups as did *T. pisiformis* and *T. serialis* with bootstrap support from 86–100%.

Autapomorphies also were recovered within ITS-2 sequences for *Mesocestoides* spp. clades and *Taenia* species. *Mesocestoides* spp. ITS-2 sequences had derived nucleotide differences (2 transitions and 7 transversions): clade A (n = 2), clade B (n = 2), and clade C (n = 5) (Table V; Fig. 2). Similarly, *Taenia* species had fixed nucleotide character autapomorphies (2 transitions and 8 transversions) for *T. pisiformis* (n = 5) and *T. serialis* (n = 5) (Table VI; Figure 4). Hence, the distribution of these autapomorphies, representing separate nuclear and mitochondrial loci, are inconsistent with the null hypothesis that these *Mesocestoides* isolates represent a single species.

DISCUSSION

This study is the first to address the taxonomic status of *Mesocestoides* spp. cestodes using data from multiple genetic loci and a hypothesis-testing framework. Systematics is a vibrant component of parasitological research, with new species described frequently, e.g., an average of 5 new species per issue in the *Journal of Parasitology* during 2003. Although most parasite species are successfully delimited by the similarity species concept, some species are clearly less amenable to this approach. Here, an objective hypothesis-testing approach was applied and the results strongly support species status for each of 3 clades that were previously recognized (Crosbie, Nadler et al., 2000.). This methodology is applicable to many organisms and has been applied to systematic questions involving entomopathogenic and parasitic nematodes (Adams, 1998; Nadler et al., 2000; Nadler, 2002).

Although several *Mesocestoides* species have been described based on qualitative morphologic features (e.g., Voge, 1955; Loos-Frank, 1990), some qualitative characters are potentially

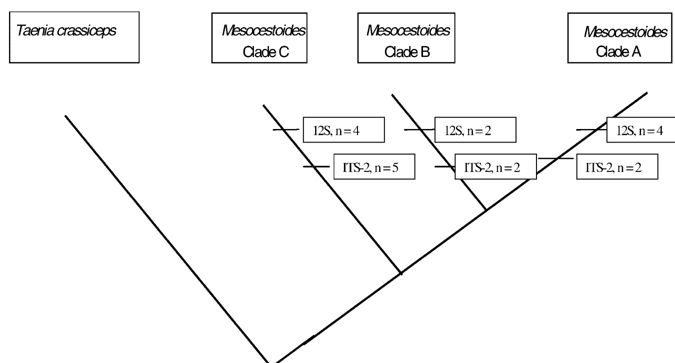

FIGURE 2. Autapomorphies of *Mesocestoides* clades A, B, and C based on out-group comparison (*Taenia crassiceps*) of 12S mitochondrial and ITS-2 nuclear DNA data sets. For each DNA region (12S or ITS-2), the number within each box represents the corresponding number of character state changes; see Table V for specific changes.

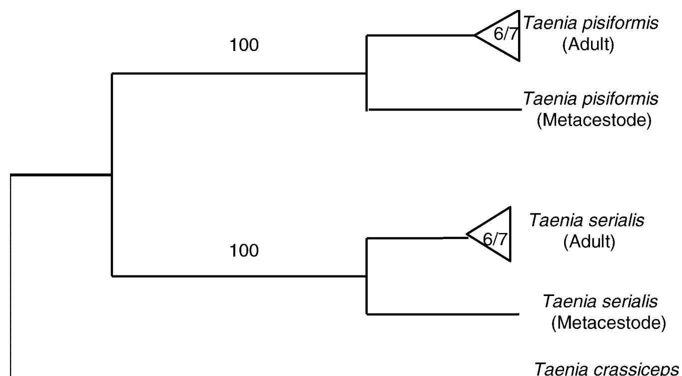

FIGURE 3. Phylogenetic relationships of *Taenia crassiceps*, *T. pisiformis*, and *T. serialis* (metacestodes of *T. pisiformis* and *T. serialis* from rabbit hosts included). Tree based on maximum parsimony using partial sequences of 12S gene (strict consensus of 264 trees, tree length = 101, CI = 0.88, RI = 0.94, HI = 0.12; (42 parsimony-informative/32 parsimony-uninformative sites) and on partial sequences of ITS-2 gene (1 tree, tree length = 765, CI = 0.85, RI = 0.85, HI = 0.15; 216 parsimony-informative/242 variable parsimony uninformative sites). Number within species triangle indicates the number of individuals sampled (6 = ITS-2; 7 = 12S). Trees were rooted with *Taenia crassiceps* as out-group; numbers above branches represent bootstrap support (1,000 replicates). Both data sets yielded identical consensus trees (consensus tree shown here).

TABLE VI. Diagnostic DNA sequence data from *Taenia.* Apomorphies from *Taenia crassiceps* (outgroup) are unlisted except those shared with *T. pisiformis* and *T. serialis.*

	Position of variable characters for 12S																
	3	24	76	144	155	158	218	227	228	230	233	243	247	263	273	279	285
Taenia crassiceps	T	A	G	T	G	T	T	A	T	T	G	GAP	T	T	T	G	A
Taenia pisiformis	**GAP**	A	**T**	T	**T**	**GAP**	A	A	**G**	**A**	**A**	GAP	**A**	T	T	**A**	A
Taenia serialis	T	**G**	G	**G**	G	T	T	**G**	T	T	G	**C**	T	**A**	**A**	G	**T**

	Position of variable characters for ITS-2									
	18	39	55	56	641	689	690	702	711	728
Taenia crassiceps	G	T	C	A	C	T	T	T	A	C
Taenia pisiformis	**A**	T	C	A	**G**	T	**C**	T	**G**	**T**
Taenia serialis	G	**A**	**A**	**G**	C	**C**	T	**C**	A	C

subjective. Thus, there is a clear need to clarify the taxonomy of *Mesocestoides* species by using characters independent of morphology, e.g., molecular sequences, and to assess the extent of morphological variation within this genus. Using morphometric analysis, significant differences were found among *Taenia* species and the *Mesocestoides* spp. clades. The morphometric values for *Taenia* spp. fall within the range given by Verster (in Loos-Frank, 2000), as well as other studies (Esch and Self, 1965; Gubanyi, 1995). *Mesocestoides* spp. clades A and B were more similar morphologically to each other than either was to clade C, consistent with molecular phylogenies that depict clades A and B as sister groups (Crosbie, Nadler et al., 2000; this study). Although it has been suggested that *Mesocestoides* spp. has a comparatively high degree of morphological variability (Voge, 1955; Specht and Voge, 1965; Rausch 1994; Gubanyi and Eszterbauer, 1998), the amount of phenotypic diversity measured within clades of adult *Mesocestoides* spp. was similar to that found within species of *Taenia*. Thus, a high level of intraspecific morphological plasticity per se would not appear to be responsible for the difficulties in *Mesocestoides* species-level systematics. One potential caveat to this interpretation is that a limited range of definitive hosts was used as the source of cestode adults in morphometric analyses, and the influence of a wide range of final host species on *Mesocestoides* spp. morphology remains unexplored.

In addition to being morphologically distinct, cladistic analyses support that these 3 genetically defined clades of *Mesocestoides* spp. are separate evolutionary lineages, consistent with their recognition as separate species. The same evolutionary approach and genetic loci delimited *T. pisiformis* and *T. serialis* as distinct species. Phylogenetic analyses based on both the nuclear ITS-2 and mitochondrial 12S gene fragments agreed with previously published trees for *Taenia* spp. based on morphological characters (Hoberg et al., 2000) and for *Mesocestoides spp.* based on molecular characters (Crosbie, Nadler et al., 2000.). In testing whether there is evidence that *Mesocestoides* spp. clades A, B, and C are independent lineages, the following null hypothesis was tested: ''there are no derived molecular characters unique to each lineage, i.e., autapomorphies.'' Although each lineage must have at least 1 apomorphy to demonstrate evolutionary independence, multiple apomorphies were recovered for each gene and for each clade (*Mesocestoides* spp.) or species (*Taenia* spp.). Thus, the null hypothesis was rejected, resulting in the conclusion that the 3 clades showed evidence of lineage exclusivity and thus represent distinct species.

To decrease the probability of mistaking the evolutionary history of a single genetic region for that of the taxa involved, both nuclear and mitochondrial sequence data were used in these comparisons. The highly variable ITS-2 region of the nuclear rDNA has been useful for many systematic studies of helminths (Gasser and Chilton, 1995; Crosbie, Nadler et al., 2000; Nadler et al., 2000; Luo et al., 2002). In *Mesocestoides* spp., Crosbie, Nadler et al. (2000) found the ITS-2 region yielded more informative variation than a similar-sized region of 18S rDNA, which placed clades A and B together in a monophyletic group. Likewise, because of its fast evolutionary rate and lack of recombination, mitochondrial DNA is often used in molecular population genetics and phylogenetic studies that involve closely related taxa. The 12S mitochondrial gene fragment used in this study is composed of highly variable noncoding regions, flanked by conserved stretches (Nickisch-Rosenegk et al., 1999). This mitochondrial region provided more apomorphies than did the ITS-2 region, most notably for *Taenia* species.

The hypothesis-based approach to species delimitation requires care in planning and in interpretation of results. Instead of simply measuring the degree of differences, e.g., sequence differences among putative species, hypotheses of species should be tested using an evolutionary, e.g., phylogenetic,

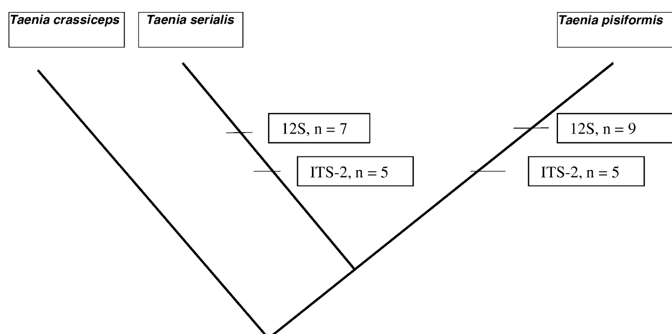

FIGURE 4. Autapomorphies of *Taenia pisiformis* and *T. serialis* based on out-group comparison (*T. crassiceps*) of 12S mitochondrial and ITS-2 nuclear DNA data sets. For each DNA region (12S or ITS-2), the number within each box represents the corresponding number of character state changes; see Table VI for specific changes.

framework, evaluating evidence of lineage independence rather than merely genetic distances. To avoid confounding individual or population level differences with species, multiple samples of each taxon should be included, ideally, samples from a broad geographic range and in the case of generalist parasites, from a broad range of definitive hosts. Finally, evidence of evolutionary history should be acquired from independent genetic loci to avoid mistaking gene trees for species trees. In this study, we addressed these requirements by including 7–12 samples of each taxon in question. Samples were collected from a broad geographic region of western North America, including the California Channel Islands and from different host species. Finally, sequence data from 2 independent loci, i.e., mitochondrial and nuclear genetic regions, were included in these analyses.

Molecular phylogenetic results from this study indicate that tapeworms in clade B represent the species *M. vogae* (previously *Mesocestoides corti*, Etges, 1991). This designation is supported also by morphological data that show clade B individuals fall within the range of morphological values for *M. vogae* (Voge, 1955). Furthermore, morphological evidence presented here, i.e., measurements of scolex width, sucker diameter, and paruterine organ, supports synonym status for *M. vogae* and *M. variabilis* first proposed by Voge (1955). Based on the available morphological data, it is not possible to determine whether clades A and C are previously described *Mesocestoides* species. Clade A specimens closely resemble both *M. kirbyi* (originally isolated from a coyote in California) and *M. manteri* (isolated from a lynx in Nebraska). Voge (1955) noted that *M. manteri* matched *Mesocestoides* from island fox from Santa Rosa Island, Channel Islands N.P. in several key morphological characters (size range of organs and testis number); we were unable to obtain *Mesocestoides* spp. from Santa Rosa island fox to include in these analyses. Clade C individuals are much larger tapeworms than any of the *Mesocestoides* spp. voucher specimens measured in this study and likely represent an undescribed species.

We present evidence that *Mesocestoides* clades A, B, and C are distinct species and that clade B is conspecific with *M. vogae*. This study illustrates the practical application of sequence data to test the hypothesis of lineage independence and species status for cestodes. Assuming that species can be represented as separate evolutionary lineages, phylogenetic methods can be applied to delimit species in nature (Adams, 2001). Other systematically intractable cestode taxa may benefit from this method of species delimitation (i.e. strains/subspecies of *Echinococcus granulosus* (Le et al., 2002)), by using comparative analyses of large datasets of sister taxa. This approach should be applied in concert with comparative morphological analyses to assign species names and to develop standard morphological descriptions, when feasible. The life cycles of *Mesocestoides* spp. remain enigmatic, but future life history studies should recognize clades A, B, and C as separate *Mesocestoides* species, with potentially distinct life histories.

ACKNOWLEDGMENTS

We thank Grace Lee (UC Davis) for enlightening discussions and excellent technical assistance. We are sincerely grateful to all those who provided material: Robert Rausch (University of Washington), Karen Terio (UC Davis), Edward G. Platzer (UC Riverside), Tim Coonan (Channel Islands National Park), Grace Smith (U.S. Navy), Dave Garcelon (Institute for Wildlife Studies), and Janne Potter (West Boulevard Veterinary Clinic, Vancouver, British Columbia, Canada). We would also like to thank Robin Houston for help with mounting tapeworms and Tanya Scharaschkin for helpful comments on phylogenetic analysis. This project was partially supported by a grant from the Center for Companion Animal Health, School of Veterinary Medicine, UC Davis (W. M. Boyce & K. A. Padgett), a grant from the UC Davis Center for Biosystematics (K. A. Padgett), and a Jastro Shields Research Fellowship (K. A. Padgett).

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