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Morphological and molecular differentiation between *Dicrocoelium dendriticum* (Rudolphi, 1819) and *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 (Platyhelminthes: Digenea)

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Abstract

Dicrocoelium dendriticum (Rudolphi, 1819) and *Dicrocoelium hospes* (Looss, 1907) are recognised to affect the liver of domestic and wild ruminants. A third species, *Dicrocoelium orientalis* which was described from musk deer in the Baikal region of the former Soviet Union and re-named to *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 was isolated from other species of deer in Asian countries and from mouflon and roe deer in Europe. Scant information is available for *D. chinensis*, including the range of species that act as definitive and intermediate hosts. To provide morphological and molecular evidences differentiating *D. chinensis* versus *D. dendriticum*, 239 *Dicrocoelium* spp. specimens were collected from sheep, cattle and sika deer from different localities in Austria, Germany and Italy. Specimens were morphologically identified based on the testes orientation, overall size, and level of maximum body width and other morphometric measurements. From this sample, 10 specimens of *D. chinensis* and 25 of *D. dendriticum* from different hosts and geographical localities were characterized molecularly through sequencing of partial 18S rDNA (∼1400 bp) and ITS-2 (including the 5.8S and 28S flanking regions; ∼600 bp). Interspecific differences between *D. dendriticum* and *D. chinensis* of 0.14% and 3.8% were recorded in 18S rRNA and ITS-2 sequences, respectively. Phylogenetic analyses via Bayesian inference were conducted using sequences of ITS-2 (276 bp) and partial 28S (221 bp) of the above species of *Dicrocoelium* together with 20 species belonging to the Xiphidiata within the Plagiorchiida available in GenBank. Both gene regions were strongly concordant in differentiating the Dicrocoeliidae, Gorgoderidae and Plagiorchiidae and were in agreement with their current classification. Morphological and molecular characterization clearly differentiate *D. dendriticum* and *D. chinensis* as two distinct digeneans infecting ruminants. The implications on the separate status of *D. chinensis* on the etiology, biology and diagnosis of dicrocoeliosis are discussed.
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Keywords: *Dicrocoelium dendriticum*; *Dicrocoelium orientalis*; *Dicrocoelium chinensis*; *Dicrocoelium hospes*; 18S rRNA; 28S rRNA; ITS rRNA; rDNA; Xiphidiata; Plagiorchiida; Digenea

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1. Introduction

Dicrocoelium spp. (Trematoda, Dicrocoeliidae) live in the bile ducts and gall bladder of domestic and wild ruminants (e.g. sheep, goats, cattle, buffaloes, roe-deer, camels) and exhibit low host specificity (occasionally parasitizing rabbits, pigs, dogs, horses and humans). Dicrocoeliosis is transmitted in lowland or mountain pastures that provide adequate conditions for the survival and development of snails and ants which act as intermediate hosts (reviewed in Otranto and Traversa, 2003). The causative agents of dicrocoeliosis are *Dicrocoelium dendriticum* (Rudolphi, 1819), which is reported in Europe, Asia, northern Africa and North America, and *Dicrocoelium hospes* Looss, 1907 found exclusively in Africa (Soulsby, 1968). A third species, originally isolated from musk deer (*Moschus moschiferus*) from the Baikal region of the former Soviet Union, named *Dicrocoelium orientalis* by Sudarikov and Ryjikov (1951) and re-named to *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 (syn. *Dicrocoelium suppereri* Hinaidy, 1983) because of pre-occupation of the first name and morphological identity to *D. chinensis* described from sheep in China (Hinaidy, 1983; Schuster, 1998) has been recorded from several species of cervids in former Soviet Union (Sudarikov and Ryjikov, 1951; Oshmarin, 1963; Gubanov, 1964) and sika deer (*Cervus nippon centralis*) in Japan (Taira et al., 2006). In Europe, *D. chinensis* has been found in mouflon (*Ovis ammon musimon*) and roe deer (*Capreolus capreolus*) in Austria (Hinaidy, 1983) and from mouflon in northern Italy (Poglayen et al., 1996). In contrast to our knowledge of the ecology, biology and pathogenicity of *D. dendriticum*, information for *D. chinensis* is only available for China (Tang et al., 1983, 1985). Nevertheless, *D. chinensis* versus *D. dendriticum* are readily distinguished by size and orientation of the testes (Sudarikov and Ryjikov, 1951; Tang and Tang, 1978; Hinaidy, 1983). Molecular and biochemical studies on the population structure of *Dicrocoelium* spp. both within and among host individuals are restricted to *D. dendriticum* (e.g. Campo et al., 1998; Sandoval et al., 1999). For example, an isoenzyme study on *D. dendriticum* from cattle, sheep and goats revealed little phenotypic variation among flukes from a single host individual or from a single host species collected from the same geographic area (Campo et al., 1998). However, genetic analysis based on randomly amplified polymorphic DNA revealed considerable intra-population variability among specimens collected from sheep in a relatively small geographic area (Sandoval et al., 1999).

Ribosomal DNA (rDNA) has been widely used for phylogenetic and diagnostic studies of parasitic nematodes (see Gasser and Newton, 2000; Blouin, 2002) and flatworms (see Olson and Tkach, 2005), albeit molecular investigations of the order Plagiorchiida are few (e.g. Tkach et al., 2000a, 2001). It was the aim of the present work to provide morphological and molecular differentiation of *D. chinensis* versus *D. dendriticum* and to investigate the level of inter- and intraspecific variation using ribosomal markers among specimens of the two species above collected from different localities and hosts (i.e. sika deer, cattle and sheep). These data are also used to assess the phylogenetic affinities of the species and to examine their position within the Plagiorchioidea.

2. Materials and methods

2.1. Source material and morphological identification

Two hundred thirty-nine specimens of *Dicrocoelium* were collected via necropsy of livers and gall bladders from sheep (*Ovis aries*), cattle (*Bos taurus*), sika deer (*Cervus nippon*) and mouflon (*Ovis ammon musimon*) from different localities in Austria, Germany and Italy (Table 1). Specimens were identified morphologically based on the descriptions of Yamaguti (1971), Euzéby (1971), Hinaidy (1983) and Taira et al. (2006) after staining with 10% carmine and stored in 70%

Table 1
Host and locality of *Dicrocoelium chinensis* and *Dicrocoelium dendriticum* specimens

Species	Host	Collection locality	No. of specimens examined morphologically	No. of specimens characterized molecularly
D. chinensis	*Cervus nippon*	Lower Austria	89	10
D. dendriticum	*Ovis aries*	Basilicata, Italy	90	10
		Swabia, Germany	8	2
	Ovis ammon musimon	Thuringia, Germany	5	3
	Bos taurus	Apulia, Italy	47	10

ethanol. Diagnostic morphological characters (i.e. overall size, level of maximum body width with respect to the middle of the fluke body, testes orientation, oral and ventral sucker diameters and vitelline glands) differentiating *D. chinensis* and *D. dendriticum* were recorded and described.

2.2. Extraction of genomic DNA, PCR and sequencing

Ten specimens of *D. chinensis* and 25 of *D. dendriticum* from different hosts and geographic localities were characterized molecularly (Table 1). Genomic DNA was isolated using a QIAamp tissue kit (Qiagen GmbH, Germany) after washing three times in cold distilled water to remove traces of the preservative (i.e. EtOH). Partial 18S rDNA (∼1400 bp) was amplified in fragments using primers Dd18SF1 (5′-GATAACGGGTAACGGGGAAT-3′) – Dd18SR1 (5′-AACCTCTGACTTTCGCTCCA-3′) and Dd18SF2 (5′-TGGAGCGAAAGTCAGAGGTT-3′) – Dd18SR2 (5′-GTACAAAGGGCAGGGACGTA-3′). The ITS-2, plus 5.8S and 28S flanking regions (∼600 bp), were amplified using Dd58SF1 (5′-ATATTGCGGCCATGGGTTAG-3′) – Dd28SR1 (5′-ACAAACAACCCGACTCCAAG-3′). All primers were designed using the criteria of Sharroks (1994) and based on previously characterized 18S (GenBank accession no. Y11236) and 5.8S-28S (GenBank accession nos. DQ379986 and AF151939) sequences of *D. dendriticum*. Genomic DNA (4 μL) was added to PCR mix (46 μL) containing: a 10 mM Tris–HCl, pH 8.3 and 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM of each dNTPs, 50 pM of each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems). PCR was validated and performed using the following cycling protocol: 94 °C for 10 min, followed by 40 cycles of 98 °C for 30 s; 53 or 55 °C for 60 s (for ITS-2 and 18S, respectively); 72° C for 90 s, followed by 7 min at 72 °C. Amplicons were purified using the Jetquick PCR spin kit (GenoMed) and sequenced directly using the BigDye cycle sequencing kit (v.2, ABI), cleaned and run on an ABI-Prism 377 automated sequencer. Sequences were determined in both directions using the same primers as used for PCR, and the electro-phenograms verified by eye. Sequences have been deposited in GenBank under accession numbers EF547131 and EF547132.

Table 2
Classification and accession numbers of species included in the phylogenetic analysis

Family	Genus	Species	ITS-2	28SrDNA
Dicrocoeliidae	*Dicrocoelium*	*D. dendriticum*	DQ379986	AF151939
		D. chinensis	EF547131	EF547132
		D. hospes	EF102026	EF102025
Gorgoderidae	*Phyllodistomum*	*P. folium*	AF533015	AY288828
Plagiorchiidae	*Haematoloechus*	*H. abbreviatus*	AF316157	AF184251
		H. breviplexus	AF387796	AF387800
		H. coloradensis	AF133190	AF133108
		H. complexus	AF316155	AF387797
		H. medioplexus	AF316161	AF387799
		H. pulcher	AF133189	AF531866
		H. variegates	AF316160	AF151916
		H. varioplexus	AF316163	AF387798
	Glypthelmins	*G. brownorumae*	AY278062	AY875674
		G. californiensis	AY278065	AY278052
		G. facioi	AY278060	AY875675
		G. hyloreus	AY278066	AY278050
		G. quieta	AY278063	DQ452537
		G. tuxtlasensis	AY278061	AY278047
	Plagiorchis	*P. elegans*	AF151952	AF151911
		P. koreanus	AF151946	AF151930
		P. muelleri	AF151948	AF184250
		P. vespertilionis	AF151951	AF151931
	Skrjabinoeces	*S. similes*	AF316168	AY222279
Echinostomatidae	*Isthmiophora*	*I. hortensis*	AB189982	AB189982

2.3. Alignment and analyses

Individual sequences of *D. dendriticum* and *D. chinensis* were aligned using ClustalX (Thompson et al., 1997) and compared within and between species. The 5′ and 3′ boundaries of the ITS-2 were determined by comparison with the ITS-2 sequence of *D. dendriticum* (GenBank accession no. DQ379986). *D. dendriticum* and *D. chinensis* sequences for ITS-2 plus the flanking regions were also compared with those of *D. hospes* (GenBank accession nos. EF102026 and EF102025 for ITS-2 and 28S, respectively).

Pairwise comparisons of nucleotide sequence differences (*D*) were made (excluding primer sequences) using the formula $D = 1 - (M/L)$ (Chilton et al., 1995), where *M* is the number of alignment positions at which the two sequences have a base in common, and *L* is the total number of alignment positions over which the two sequences are compared.

Separate alignments of the ITS-2 and partial 28S genes were constructed using the *Dicrocoelium* spp. and 20 representatives of the Xiphidiata (Plagiorchiida) *sensu* Olson and Tkach (2005) as shown in Table 2. These alignments were used for phylogenetic analysis, whereas 18S data corresponding to the same Xiphidiata taxa were not available and thus these data were used solely for pairwise estimates of divergence. Nucleotide substitution models were selected for each alignment independently using MrModelTest 1.1b (Nylander, 2004) resulting in a general time reversible model incorporating the gamma estimate of among-site rate variation (i.e. GTR + G) providing the best fit in both partitions. Phylogenetic analysis via Bayesian inference was conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with analyses run for 1 million generations with default prior probabilities and topologies sampled every 100th generation. Consensus trees were constructed using the 'sumt' command with the variables burnin = 250(28S)/300(ITS-2) and contype = allcompat. Nodal support was assessed both through consensus posterior probabilities and through parsimony-based bootstrapping (1000 replicates) using PAUP* 4.0b10 (Swofford, 2001).

3. Results

3.1. Morphological analysis

Specimens of *D. dendriticum* ($n = 150$) were identified from sheep and cattle from different localities (Table 1) whereas *D. chinensis* ($n = 89$) was found only in sika deer in Austria. All specimens were identified

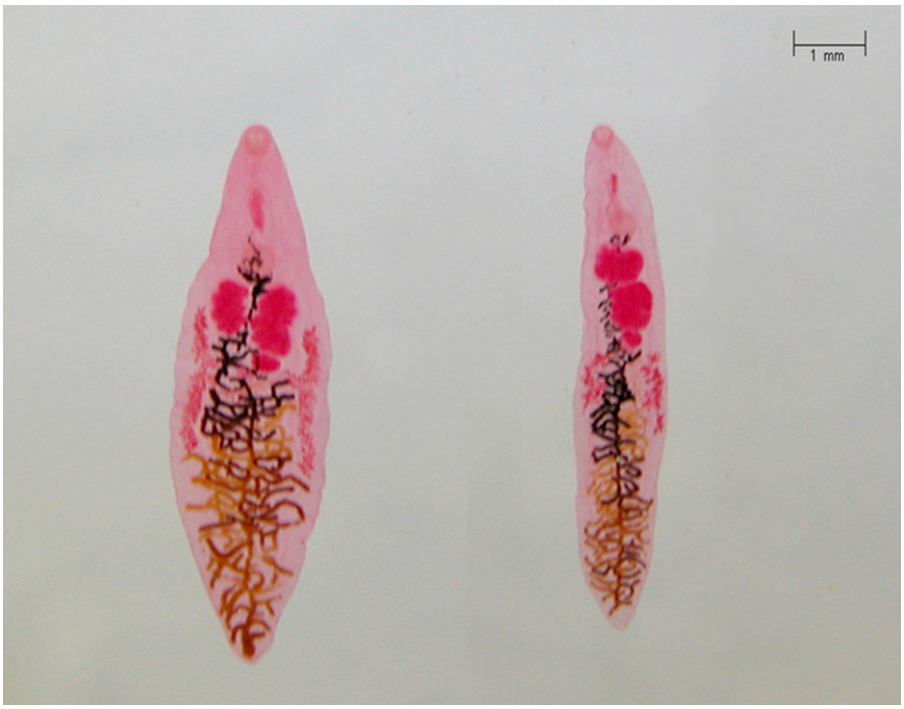

Fig. 1. Light micrographs of carmine-stained *Dicrocoelium chinensis* (left) and *D. dendriticum* (right). Note differences in overall size and the orientation of the testes.

Table 3
Morphometric data of *Dicrocoelium chinensis* (from sika deer) and *Dicrocoelium dendriticum* (from different hosts)

	Dicrocoelium chinensis	*Dicrocoelium dendriticum*	
		Sheep	Cattle
	Min/max (mean ± S.D.)	Min/max (mean ± S.D.)	Min/max (mean ± S.D.)
Body			
Length	7.33–9.20 mm (8.08 ± 0.55)	4.70–5.60 mm (5.05 ± 0.33)	6.10–8.07 mm (6.78 ± 0.63)
Width	1.90–3.10 mm (2.24 ± 0.46)	1.30–1.80 mm (1.47 ± 0.29)	1.52–1.94 mm (1.69 ± 0.13)
Oral sucker diameter			
Internal	210–270 μm (246.20 ± 25.24)	180–195 μm (187.50 ± 10.61)	137–256 μm (184.33 ± 36.90)
External	400–494 μm (438.80 ± 40.95)	310–348 μm (329 ± 26.87)	279–386 μm (330.33 ± 41.54)
Ventral sucker diameter			
Internal	175–220 μm (203.75 ± 19.74)	210–254 μm (236.25 ± 19.36)	119–199 μm (162.17 ± 29.13)
External	370–396 μm (381.50 ± 13.50)	398–454 μm (426 ± 23.57)	301–436 μm (361.11 ± 51.16)
Testes			
Length	641–1250 μm (1037 ± 200.39)	502–638 μm (561 ± 54.68)	504–917 μm (740.25 ± 87.51)
Width	550–724 mm (595.33 ± 53.70)	436–544 μm (480.40 ± 47.43)	475–747 mm (630.70 ± 69.81)
Vitelline glands			
Length	1.20–2.60 mm (1.90 ± 0.51)	0.60–1.30 mm (0.97 ± 0.27)	1.40–2.22 mm (1.83 ± 0.24)

using light microscopy as *D. dendriticum* or *D. chinensis* (Fig. 1) based on the testes orientation (bilateral *D. chinensis* and tandem in *D. dendriticum*), overall size (see Table 3) and level of maximum body width with respect to the middle of the fluke body (wider in *D. chinensis* than in *D. dendriticum*, see Table 3). Although there was minor morphometric variation among specimens of *D. dendriticum* from different hosts and localities of collections, it was consistent with being intraspecific. Morphometric measurements are given in Table 3.

3.2. Molecular analysis

Comparison of the 18S sequences (~1440 bp) showed only two transversions (A ↔ T), or a raw divergence of 0.14%. No intraspecific difference was observed in either species. The ITS-2 region was 234 bp in *D. dendriticum* and 233 bp in *D. chinensis*. Among the isolates of *D. dendriticum*, intraspecific variation was observed in three positions (1.3% divergence) which did not correlate with either host or locality. In contrast, interspecific divergence between the species was 3.8% (four transitions, four transversions and one indel). Comparison of the ITS-2 with the more distantly related *D. hospes* showed divergences of 12.8% and 13.3% in *D. dendriticum* and *D. chinensis*, respectively. The 5.8S and 28S flanking regions did not present any intraspecific variation and only one interspecific difference (i.e. 0.4% mutational rate). Comparison with *D. hospes* of the 28S flanking region showed divergences of 1.4% and 1.7% for *D. dendriticum* and *D. chinensis*, respectively. Phylogenetic analysis of the ITS-2 and partial 28S data (Fig. 2) resolved the genera *Glypthelmins*, *Haematoloechus* and *Plagiorchis*, but was inconsistent in their interrelationships and in the relative position of *Skrjabinoeces*. The Dicrocoeliidae was supported as a separate lineage from members of the Plagiorchiidae, with *D. dentriticum* and *D. chinensis* forming a sister clade to *D. hospes*.

4. Discussion

Morphological and molecular characterisation clearly differentiate *D. dendriticum* and *D. chinensis* as two distinct species infecting ruminants. Whereas *D. dendriticum* is found throughout Europe (reviewed in Otranto and Traversa, 2003), *D. chinensis* is present primarily in East Asia (Sudarikov and Ryjikov, 1951; Oshmarin, 1963; Gubanov, 1964; Tang and Tang, 1978; Tang et al., 1983, 1985) including Japan (Taira et al., 2006) and has been reported only recently from Europe (i.e. Austria; Hinaidy, 1983, and northern Italy; Poglayen et al., 1996). In particular, *D. chinensis* has been here firstly described from sika deer in Europe. It is likely that *D. chinensis* has been introduced through sika deer imported to Europe from Asian countries. For example, the sika deer population in the county of Tulln (Austria), where *D. chinensis* was detected, was settled in the first half of the 20th century with a few animals of Japanese origin imported through an enclosure in the former Czechoslovakia (Bauer, 2001). Unfortunately, the scant data regarding the epidemiology, ecology and biol-

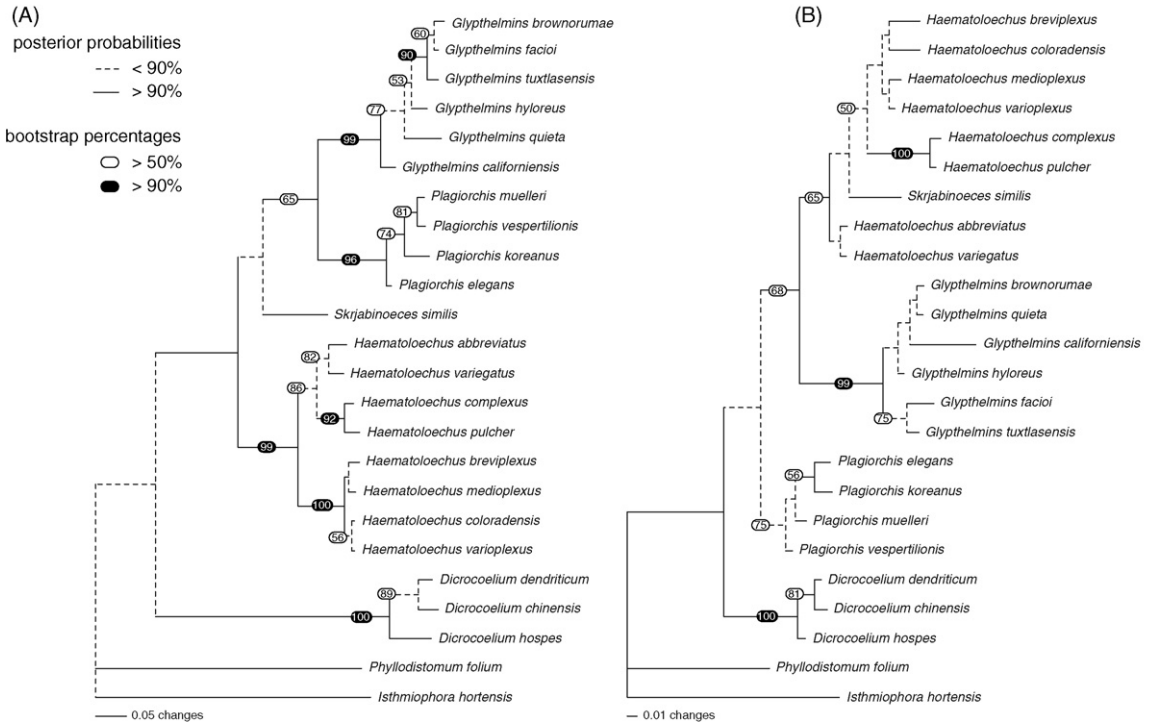

Fig. 2. Phylogenetic analyses of ITS-2 (A) and partial 28S (B) based on Bayesian inference.

ogy of *D. chinensis* do not permit a robust assessment of its potential to spread more widely within Europe or of its pathogenic impact on infected animals. No significant intraspecific morphometric differences were recorded for *D. dendriticum* from different hosts or localities, whereas a low level of intraspecific variation (i.e. 1.3%) was found in the ITS-2 sequences of *D. dendriticum* irrespective of the host species or locality. It is possible that the morphological variability observed in specimens of *D. dendriticum* from cattle and sheep (Table 3) may be influenced by the state of maturation, the intensity of the invasion or by factors linked to the host species as previously described for other plagiorchiid parasites (Tkach et al., 2000b). Interspecific divergence of the ITS-2 between *D. dendriticum* and *D. chinensis* (3.8%) was about three times greater than the maximum value of the intraspecific rate (0–1.3%), demonstrating the utility of ITS-2 as a marker for species circumscription, which is also supported by analyses carried out on ITS-1 and ITS-2 within several groups of digeneans (Morgan and Blair, 1995; Jousson et al., 1998; Tkach et al., 2000a; Casey et al., 2003). In a comprehensive review of the use of ITS data for species circumscription among digeneans, Nolan and Cribb (2005) discuss both examples of geographically widespread taxa showing no variation in their ITS as well as taxa that show small amounts of intraspecific variation (typically <2%) consistent with that reported herein for *D. dendriticum*. In no case did they (Nolan and Cribb, 2005) find intraspecific variation to exceed that of interspecific, albeit investigator error and a failure to recognize cryptic species can confound the issue. Furthermore, by comparing the degree of interspecific difference between *D. dendriticum* and *D. chinensis* with those of both species with *D. hospes* (i.e. 12.8% and 13.3%, respectively), it clearly shows a higher degree of affinity of the European species versus the third which is reflected in the phylogenetic analyses (Fig. 2). Broader phylogenetic implications of the analyses reflect those of previous studies and interested readers are thus referred to the works of Tkach et al. (2000a, 2001) concerning the Plagiorchiida and of Olson et al. (2003) concerning the entire Digenea for such discussions.

The low level of intraspecific differences for *D. dendriticum* irrespective of the host and locality suggests a limited, if any, genetic affinity between *D. dendriticum* and the host species. This finding accounts for a likely genetic stability within *D. dendriticum* population as already shown for other generalist parasites which infect multiple species of animals all over the world (Humbert and Cabaret, 1995; Nolan and Cribb, 2005). However, it cannot be ruled out that a larger number of isolates sam-

pled from different localities and host individuals may show a pattern of population subdivision not found in a more limited sample.

Knowledge regarding several biological aspects of *D. chinensis* is lacking, including the severity of pathology they may induce in infected animals. The use of molecular markers to detect *D. chinensis* is of particular importance in enabling the identification of the species of snail and ant that may act as competent intermediate hosts (as demonstrated for *D. dendriticum*, Heussler et al., 1998), to investigate the pathogenic role of this fluke and to overcome constraints that may occur during the morphological identification which are linked to the operator skills, to the difficulties in retrieval larval stages (including first and second generation sporocysts, cercaria or metacercaria) and to the time consuming dissection procedures.

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