Establishment of the onset of host specificity in four phyllobothriid tapeworm species (Cestoda: Tetraphyllidea) using a molecular approach

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SUMMARY

A parasitological survey in the Bay of Fundy, Canada, resulted in the recovery of mature specimens from 5 species of phyllobothriid tapeworms (Cestoda: Tetraphyllidea) from 4 rajid skates: *Echeneibothrium canadensis* and *E. dubium abyssorum* specimens from *Amblyraja radiata*; *E. vernetae* and *Pseudanthobothrium* n.sp. from *Leucoraja erinacea* and *L. ocellata*; and *P. hanseni* from *A. radiata* and *Malacoraja senta*. Partial sequence data of a variable region (D2) from the large subunit ribosomal DNA (LSU) were used here to determine the host distribution of immature specimens for 4 of these 5 species (*E. d. abyssorum* was not included in the analyses). Immature specimens from both *Pseudanthobothrium* spp. were identified in the same hosts as recorded previously for mature specimens, thus suggesting that there are mechanisms that prevent the attachment of the parasite in an 'unsuitable' host species. Immature *E. canadensis* specimens were recovered exclusively from *A. radiata*, whereas immature *E. vernetae* specimens were recovered from *L. erinacea* and *A. radiata*, despite the latter host species not harbouring mature *E. vernetae* specimens. Their presence in the latter host species may be explained by host restriction or resistance, which allows the attachment of the parasites in the 'wrong' host species, but not establishment or development.

Key words: host specificity, onset of host specificity, Tetraphyllidea, Phyllobothriidae, Rajidae, molecular approach, D2 domain, large subunit of nuclear ribosomal DNA.

INTRODUCTION

Cestodes belonging to the order Tetraphyllidea have been considered to be oioxenous (exhibiting strict host specificity) (e.g. Williams, 1960, 1961, 1964, 1966, 1968, 1969). Williams (1966) noted that no mature *Echeneibothrium* spp. have been reported from more than 1 rajid host species, and only on rare occasions have immature specimens been recovered from 2 rajid host species. For the sister genus *Pseudanthobothrium* Baer, 1956 (Caira *et al*. 1999, 2001), we were unable to identify published accounts regarding host specificity. However, in an earlier study, we assessed the host specificity of mature specimens of 2 *Pseudanthobothrium* spp., using anatomical observations and partial sequence data of a variable region (D2) from the large subunit of nuclear ribosomal DNA (LSU), and established that *Pseudanthobothrium* n.sp. and *P. hanseni* infect different ecological pairs of host species (Randhawa *et al*. manuscript submitted). Randhawa *et al*. (manuscript submitted) also reported, on the basis of morphological features, that *E. vernetae* also occurs in 2 different host species (*Leucoraja erinacea* and *L. ocellata*). These findings question the strictness of the host-parasite relationship for adult *Pseudanthobothrium* and *Echeneibothrium* species.

Assessments have been made from mature specimens possessing the necessary morphological characters for species-level identification and subsequently confirmed by the molecular data (Randhawa *et al*. manuscript submitted). On the other hand, the identity of immature specimens can only be determined using molecular tools, since species-diagnostics are based solely on adult features (e.g. Euzet, 1994). For studies investigating the specificity of parasites to be comprehensive, however, accurate species-level identifications of immature specimens are necessary to determine at what stage of the host-parasite interaction does specificity occur. Molecular markers are invaluable tools for measuring and assessing the specificity patterns of host-parasite relationships (Anderson *et al*. 1998). In cestodes, the D1–D3 region of the LSU is useful for discriminating among species (Mariaux and Olson, 2001; Olson *et al*. 2001; Reyda and Olson, 2003), and the D2 domain, a divergent and rapidly evolving region of the LSU (Harper and Saunders, 2001), has been used previously to differentiate between tetraphyllidean species (Brickle *et al*. 2001; Agusti *et al*. 2005). This molecular marker

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allows us to identify accurately immature specimens to species, therefore determining when host specificity is established in these host-parasite relationships.

There are 2 main views of host specificity: host range and quantitative measure. The first, host range, is the most commonly used concept (Poulin, 1998) and relates to the number of different host species infected by a single parasite species at a given stage of its life-cycle (Euzet and Combes, 1980; Holmes, 1987; Lymbery, 1989; Combes, 1995, 2001; Poulin, 1998). The concept of filters was introduced by Euzet and Combes (1980) to illustrate the 4 parameters responsible for delimiting the host range of parasites, thus defining the degree of specificity of these parasites. The 'encounter filter' is defined as the probability of contact between a given parasite species and potential hosts and includes a biodiversity parameter (geographical component) and a behaviour parameter (spatial component). Host species absent from the ecosystem of a parasite (biodiversity parameter) are excluded from the host range of the parasite. Similarly, host species whose behaviour (behaviour parameter) renders contact with infective stages of the parasite impossible are excluded from the host range of the parasite. The 'compatibility filter' is defined as the probability of a parasite establishing in the host following encounter and includes a resource parameter and a defence parameter. Host species not providing the adequate spatial resources (e.g., attachment surface or interspecific competition) or metabolic resources (e.g., glucose) to meet the needs of the parasite are excluded from the host range of the parasite (resource parameter). This type of exclusion is also referred to as host unsuitability. Host species, whose immune factors or other mechanisms prevent the establishment of the parasite, are excluded from the host range of the parasite (defence parameter). This type of exclusion is also referred to as host resistance or host restriction (e.g. Rohde and Rohde, 2005). Therefore, of all potential host species, only a subset is encountered by the parasite, and of that subset, only the parasite species compatible with the host can establish.

The second view measures specificity by quantifying prevalence, abundance and mean intensity of infection by parasites in different host species (Rohde, 1980, 1994, 2005; Lymbery, 1989; Rohde and Rohde, 2005) and relating these parameters to the phylogenetic relatedness between infected host species (e.g. Poulin and Mouillot, 2003, 2004; Krasnov *et al*. 2004; Rohde and Rohde, 2005). These measurements would provide information on host preference of parasites and accidental infections. Although the traditional view of host specificity (i.e. host range) is adhered to here, prevalence and intensity of infection data are presented in recognition of their utility in discussing the ecological implications of our findings on the host distribution of mature and immature specimens of the four parasite species studied herein.

From June 2002 to September 2004, 84 *L. erinacea* (Mitchill, 1825), 25 *Malacoraja senta* (Garman, 1885), 11 *Amblyraja radiata* (Donovan, 1808), and 7 *L. ocellata* (Mitchill, 1815) were collected from Passamaquoddy Bay and waters surrounding the West Isles of the Bay of Fundy, NB, Canada. As a result of our parasitological survey: mature specimens of *Pseudanthobothrium* n.sp. and *Echeneibothrium vernetae* Euzet, 1956 were recovered from *L. erinacea* and *L. ocellata*; *P. hanseni* Baer, 1956 was recovered from *A. radiata* and *M. senta*; *E. dubium abyssorum* Campbell, 1977 and *E. canadensis* Keeling and Burt, 1996 were recovered from *A. radiata*; *Zyxibothrium kamienae* Hayden and Campbell, 1981 was recovered from *M. senta*, and *Grillotia* sp. was recovered from all 4 rajid skate species. Approximately 250 immature specimens of *Pseudanthobothrium* spp. and *Echeneibothrium* spp. were also recovered from the 4 rajid skate hosts.

In this study, the host distribution of both mature and immature specimens of 2 *Pseudanthobothrium* spp. and two *Echeneibothrium* spp. was assessed, using the partial sequence of the D2 domain of the LSU as a molecular marker, to gain insights into the stage of the host-parasite relationship where specificity becomes apparent (onset). The results indicate that, for the species studied here, specificity in *Pseudanthobothrium* specimens occurs prior to attachment, whereas specificity in *Echeneibothrium* specimens occurs post-attachment.

MATERIALS AND METHODS

Collections and examination of material

Skates were collected during Otter trawls on board the W. B. Scott R/V and CCGS Pandalus III, and identified using keys and descriptions from Scott and Messieh (1976) and Scott and Scott (1988). Skates were maintained in a holding tank or 'live well' on the vessels and subsequently kept live at the research facilities of the Huntsman Marine Science Centre (HMSC) in St Andrews, NB, until examination. This generally occurred within 24 h of their capture. Skates were pithed and access to internal organs was achieved by cutting out the ventral body wall. Spiral valves from 24 *L. erinacea*, 11 *A. radiata*, ten *M. senta*, and seven *L. ocellata* were retrieved and examined immediately by making a mid-ventral incision through the whorls of the mucosal sheet, from the rectum straight up to the pyloric stomach along the ventral blood vessel, thus exposing 2 surfaces with distinct chambers separated by a mucosal flap. These spiral valves were then placed in saline in a large Petri, or culture, dish and examined using a binocular dissecting microscope. Both mature and

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Table 1. Inventory of specimens used for assessment of host distribution with voucher and GenBank Accession numbers

Host species	Mature/ Immature	Parasite species (n)	Voucher numbers	GenBank Accession numbers
Amblyraja radiata	Mature	*Echeneibothrium canadensis* ($n=3$)	P34, P36, P147[a,b]	EF207935 – EF207937
	Mature	*Pseudanthobothrium hanseni* ($n=12$)	P3, P6, P9, P12, P148, P149, P151, P152, P153, P154, P157, P158[c]	EF207818 – EF207829
	Immature	($n=11$)	P41, P80, P104, P109, P110, P111, P145, P146, P150, P155, P156[a,d]	EF207842 – EF207852
Leucoraja erinacea	Mature	*Echeneibothrium vernetae* ($n=7$)	P26, P30, P134, P199, P200, P204, P207[a]	EF207938 – EF207944
	Mature	*Pseudanthobothrium* n.sp. ($n=21$)	P1, P4, P10, P11, P13, P14, P15, P16, P17, P18, P19, P21, P22, P176, P178, P194, P196, P198, P201, P205, P208[c]	EF207788 – EF207808
	Immature	($n=54$)	P46, P47, P48, P49, P51, P52, P60, P61, P62, P63, P64, P65, P66, P67, P68, P69, P70, P71, P72, P73, P74, P76, P77, P79, P81, P82, P83, P84, P90, P92, P93, P94, P95, P97, P98, P99, P102, P103, P113, P114, P115, P116, P117, P136, P140, P141, P142, P143, P144, P159, P160, P202, P203, P206[a]	EF207853 – EF207906
L. ocellata	Mature	*Echeneibothrium vernetae* ($n=2$)	P32, P33[a]	EF207945, EF207946
	Mature	*Pseudanthobothrium* n.sp. ($n=9$)	P5, P138, P139, P162, P163, P169, P174, P175, P182[c]	EF207809 – EF207817
	Immature	($n=3$)	P43, P44, P45[a]	EF207907 – EF207909
Malacoraja senta	Mature	*Pseudanthobothrium hanseni* ($n=10$)	P23, P24, P187, P188, P189, P190, P191, P192, P193[c]	EF207833 – EF207841
	Mature	*Pseudanthobothrium hanseni* ($n=1$)	P27[a]	EF207934
	Immature	($n=24$)	P50, P53, P54, P55, P56, P57, P58, P59, P85, P86, P87, P88, P89, P105, P106, P107, P108, P112, P118, P119, P120, P121, P122, P123[a]	EF207910 – EF207933

[a] Sequences obtained during this study.
[b] Includes 1 specimen from 1997 collections.
[c] Sequences obtained from Randhawa *et al*. (manuscript submitted).
[d] Includes 6 specimens from 1997 collection.

immature parasites were removed from the spiral valves, and cleaned in fresh saline prior to being processed. For the purpose of this study, immature worms are defined as those encompassing a morphological gradient between that resembling a plerocercoid to that of specimens with evident strobilation, but lack of sexual features. Only scoleces, both attached and detached, were counted to determine the number of parasites present in each individual spiral valve and the attachment-site of those attached was noted. Both mature and immature worms were fixed in hot, almost boiling 70% ethanol and stored in fresh 95% or absolute ethanol. Scoleces of both mature and immature specimens were retained (stored in 70% ethanol) as vouchers for each of the specimens. Other spiral valves, preserved for later examination, were injected with 10% formalin and treated as described by Randhawa *et al*. (manuscript submitted).

This study included sequence data from 64 mature specimens, including the 51 mature *Pseudanthobothrium* specimens from the Northwest Atlantic sequenced by Randhawa *et al*. (manuscript submitted), and 92 immature specimens (Table 1). Immature *Echeneibothrium* specimens were not recovered from *L. ocellata*. Additionally, since the 3 mature *E. d. abyssorum* specimens recovered from *A. radiata* were used for physiological experiments (results to be published elsewhere), this parasite species was not included in the analyses. Voucher

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material for immature worms is deposited with the NB Museum (Table 1).

Molecular characterization and analysis

Genomic DNA was extracted using standard techniques (Devlin *et al*. 2004). The 5′ end of the large subunit ribosomal DNA (LSU) was amplified as reported by Harper and Saunders (2001), using the Ex-*Taq* polymerase PCR kit (Takara Bio Inc., Otsu, Shiga, Japan). The amplicons were purified from 0·8% electrophoresis grade agarose (MP Biomedicals, Aurora, OH, USA) gels as described by Saunders (1993) and sequenced using the T16 forward primer (Harper and Saunders, 2001), following the method of Randhawa *et al*. (manuscript submitted), the 'ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit v.3.1' in a 16 capillary 3100 Genetic Analyzer (Applied Biosystems). In order to test the accuracy of the data obtained from a single primer, the other strand (T30 reverse primer; Harper and Saunders (2001)) was sequenced for 2 mature *E. vernetae* from *L. erinacea* and 1 from *L. ocellata*; 1 mature *E. canadensis* from *A. radiata*; and 1 immature specimen from each of the 4 host species. This region included the D2 domain of the LSU.

Sequence data were edited using Sequencher 4.5 (Gene Codes Corporation, ©1991–2005) and subsequently aligned using MacClade 4.07 (Maddison and Maddison, 2005). The transversional model (TVM) was determined to provide the best fit to the data based on Modeltest 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). The neighbour-joining algorithm, implemented in PAUP v.4.0b10 (Swofford, 2002), was used for the visual display of the within-species variation *versus* the between-species differences. The purpose of this analysis was not to construct phylogenies; rather it served to assign specimens to a particular cluster, each of which represents a different species.

RESULTS

The size of the amplicons was ~1850 bp. The region sequenced was ~800 bp, of which 541 bp (central) were used for sequence alignment and analysis. Sequences resolved as 4 distinct clusters assignable to: *Pseudanthobothrium* n.sp., *P. hanseni*, *E. vernetae* and *E. canadensis* (Fig. 1). The dissimilarity between species, expressed as percentage of nucleotide difference, was between 2·59 and 8·69%, whereas within-species variation was 0–0·74%. All 9 sequences assignable to those from mature *E. vernetae* specimens were identical, regardless of whether specimens were recovered from *L. erinacea* or *L. ocellata* (Figs 1 and 2). Additionally, all 3 sequences assignable to those from mature *E. canadensis* were identical (Figs 1 and 2). The genetic distance between mature specimens of both *Echeneibothrium* spp. was 2·96%, whereas that between mature specimens of both *Pseudanthobothrium* spp. was 2·59–3·33%. The within-species variation among the 30 mature specimens of *Pseudanthobothrium* n.sp. was <0·55%, whereas no variation was observed among the 22 mature specimens of *P. hanseni*.

Of the 38 sequences from immature *Pseudanthobothrium* recovered from *L. erinacea*, all were assignable to *Pseudanthobothrium* n.sp., as were the 3 sequences for immature *Pseudanthobothrium* isolates recovered from *L. ocellata* (Figs 1 and 2). Of the 24 sequences from immature *Pseudanthobothrium* recovered from *M. senta*, all were assignable to *P. hanseni*, as were the 3 sequences of immature *Pseudanthobothrium* recovered from *A. radiata* (Figs 1 and 2). The sequences of all 16 immature *Echeneibothrium* specimens recovered from *L. erinacea* were assignable to those from *E. vernetae*; of the sequences from 8 *Echeneibothrium* specimens recovered from *A. radiata*, 5 were assignable to those from *E. vernetae* and 3 were assignable to those from *E. canadensis* (Figs 1 and 2).

The genetic distance between immature and mature specimens for all 4 species was <0·74% for *Pseudanthobothrium* n.sp., <0·18% for *P. hanseni*, 0% for *E. canadensis*, and <0·18% for *E. vernetae*. These values are all within the range expected for the variable D2 domain of the LSU within tetraphyllidean species as recorded here and reported previously (Brickle *et al*. 2001; Reyda and Olson, 2003; Agusti *et al*. 2005; Randhawa *et al*. manuscript submitted). A summary of genetic differences is presented in Fig. 2.

Prevalence, defined as the proportion of hosts examined infected with one or more individuals of a given parasite species (Margolis *et al*. 1982; Bush *et al*. 1997), of *Pseudanthobothrium* spp. is high among all 4 rajid host species (60·0–85·7%), whereas that of *Echeneibothrium* spp. is lower and more variable (18·2–52·4%). The prevalences of *E. canadensis* in *A. radiata* and *E. vernetae* in *L. ocellata* are <30% (Table 2). The intensity of infection, defined as the mean number of parasites of a given species per infected host (Margolis *et al*. 1982; Bush *et al*. 1997), of *Pseudanthobothrium* spp. is almost double that of *Echeneibothrium vernetae* (9·9–14·2 *versus* 6·6–8·5 per infected host, respectively). The intensity of infection of *E. canadensis* is 1·5, with a range of 1 or 2 specimens per infected *A. radiata*. All prevalence, intensity of infection and range data are presented and summarized in Table 2.

DISCUSSION

Using a variable region of the LSU, we were able to assign unequivocally immature tetraphyllideans to known species. The present results indicate that mature *E. vernetae* specimens are found in both

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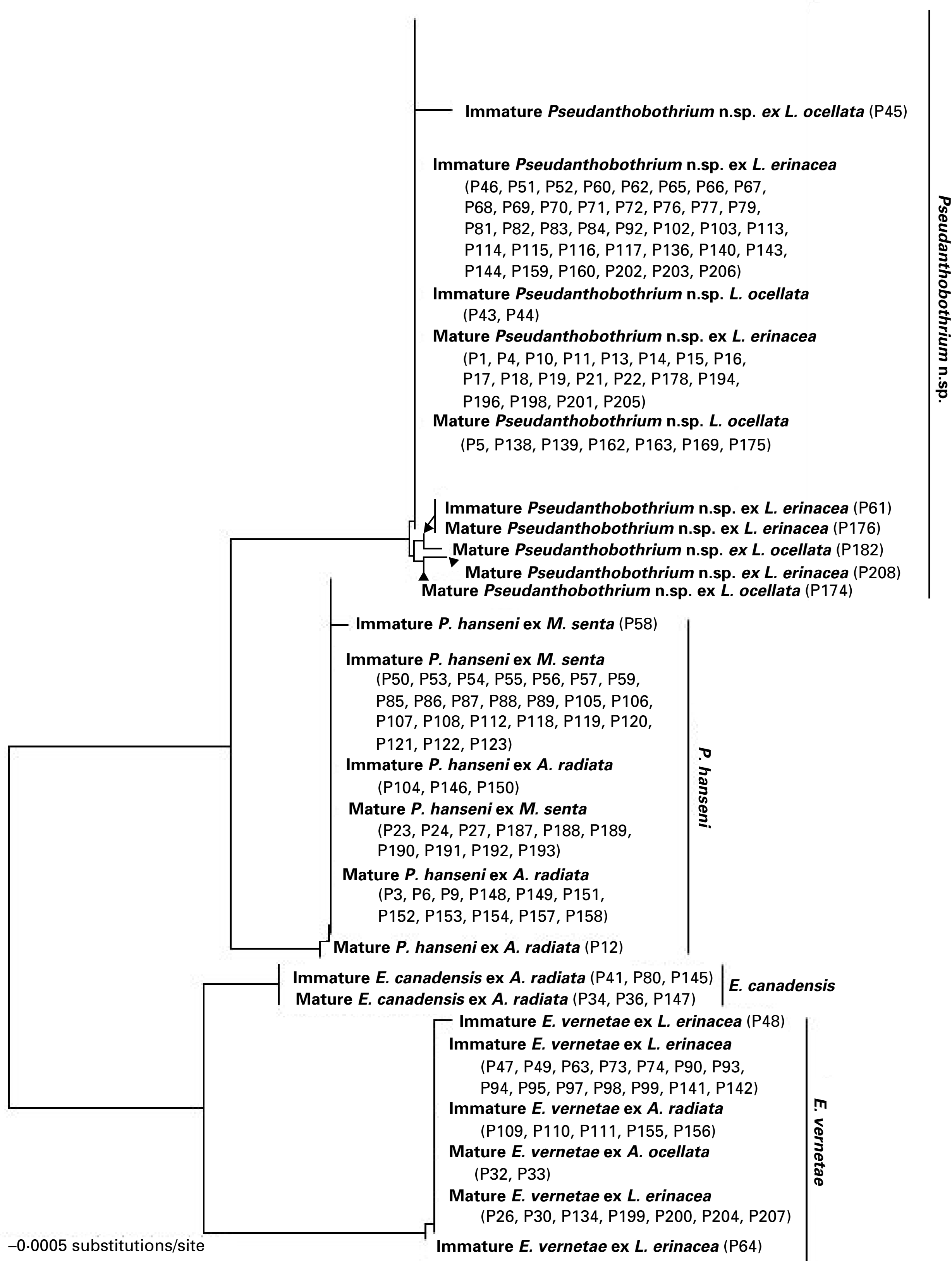

Fig. 1. Phylogram (neighbour-joining) displaying four clusters: one for the included specimens for *Pseudanthobothrium* n.sp., one for *P. hanseni*, one for *Echeneibothrium canadensis*, and one for *E. vernetae*. Each cluster is accompanied by maturity level (mature or immature), host species, voucher numbers and GenBank Accession numbers for individual isolates.

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	IPpLe	PpLe	IPpLo	PpLo	IPhAr	PhAr	IPhMs	PhMs	IEcAr	EcAr	IEvLe	EvLe	EvLo	IEvAr
IPpLe	**0-1**				Immature and mature *P. purtoni* from *L. erinacea* and *L. ocellata*									
PpLe	**0-3**	**0-3**										Immature and mature *E. vernetae* from *A. radiata*, *L. erinacea* and *L. ocellata*		
IPpLo	**0-3**	**0-4**	**0-2**											
PpLo	**0-2**	**0-2**	**0-4**	**0-2**										
IPhAr	15-16	15-17	15-17	15-17	**0**				Immature and mature *P. hanseni* from *A. radiata* and *M. senta*					
PhAr	14-16	14-17	14-17	14-17	**0**	**0**								
IPhMs	15-17	15-18	15-18	15-18	**0-1**	**0**	**0-1**							
PhMs	15-16	15-17	15-17	15-17	**0**	**0**	**0-1**	**0**						
IEcAr	35-36	35-38	36-38	36	31	30-31	31-32	31	**0**		Immature and mature *E. canadensis* from *A. radiata*			
EcAr	35-36	35-38	36-38	36	31	30-31	31-32	31	**0**	**0**				
IEvLe	43-46	43-47	43-47	43-47	39-41	39-41	39-42	39-41	16-17	16-17	**0-1**			
EvLe	44-45	44-46	44-46	44-46	40	40	40-41	40	16	16	**0-1**	**0**		
EvLo	44-45	44-46	44-46	44-46	40	40	40-41	40	16	16	**0-1**	**0**	**0**	
IEvAr	44-45	44-46	44-46	44-46	40	40	40-41	40	16	16	**0-1**	**0**	**0**	**0**

Fig. 2. Matrix summarizing the number of actual nucleotide differences (out of 541 bp) among sequences of *Pseudanthobothrium* n.sp., *P. hanseni*, *Echeneibothrium canadensis* and *E. vernetae* for both mature and immature specimens analysed from the four different host species. **IPpLe**, Immature *Pseudanthobothrium* n.sp. ex *Leucoraja erinacea*; **PpLe**, Mature *Pseudanthobothrium* n.sp. ex *L. erinacea*; **IPpLo**, Immature *Pseudanthobothrium* n.sp. ex *L. ocellata*; **PpLo**, Mature *Pseudanthobothrium* n.sp. ex *L. ocellata*; **IPhAr**, Immature *P. hanseni* ex *Amblyraja radiata*; **PhAr**, Mature *P. hanseni* ex *A. radiata*; **IPhMs**, Immature *P. hanseni* ex *Malacoraja senta*; **PhMs**, Mature *P. hanseni* ex *M. senta*; **IEcAr**, Immature *Echeneibothrium canadensis* ex *A. radiata*; **EcAr**, Mature *E. canadensis* ex *A. radiata*; **IEvLe**, Immature *E. vernetae* ex *L. erinacea*; **EvLe**, Mature *E. vernetae* ex *L. erinacea*; **EvLo**, Mature *E. vernetae* ex *L. ocellata*; **IEvAr**, Immature *E. vernetae* ex *A. radiata*.

Leucoraja spp., whereas mature *E. canadensis* specimens are restricted to *A. radiata*. The lack of sequence variation among mature specimens of *Echeneibothrium* spp. is consistent with the within-species variation reported by Randhawa *et al.* (manuscript submitted) among mature specimens of both *Pseudanthobothrium* n.sp. (<0·31%) and *P. hanseni* (<0·47%) over 643 bp. These findings indicate that, similarly to mature isolates of *Pseudanthobothrium* n.sp. and *P. hanseni* (see Randhawa *et al.* manuscript submitted), mature isolates of *E. vernetae* are shared by 2 species of rajid skates. The recovery of mature *Pseudanthobothrium* n.sp., *P. hanseni* and *E. vernetae*, each from 2 host species, challenges the dogma surrounding the strict host specificity of these parasites (generally accepted as 1 tetraphyllidean species being restricted to 1 host species). Recording *E. vernetae* from both *L. erinacea* and *L. ocellata* contradicts Williams (1966), who stated that: "… no mature specimens of any one species of *Echeneibothrium* have been found in more than one host species …" (p. 268). Conversely, *E. canadensis* seems to exhibit strict (or oioxenous) host specificity, however, increased sampling effort is required in order to confirm this observation as only 3 mature specimens were recovered during this study. The present results highlight the need to extend studies investigating the host specificity of tetraphyllidean cestodes to other genera (also see Randhawa *et al.* manuscript submitted).

For both *Pseudanthobothrium* n.sp. and *P. hanseni*, levels of within-species variation (0·18–0·55%) and between-species differences (2·59–3·33%) in sequence for immature specimens were consistent with those reported for adult specimens of the same species (0·31–0·47% and 2·64–3·42%, respectively, for 643 bp.) (Randhawa *et al.* manuscript submitted). Also, levels of within-species variation and between-species differences (0·18% and 2·96–3·14%, respectively) for immature specimens of *E. vernetae* and *E. canadensis* were consistent with those reported from mature specimens in this study (0% and 2·96%, respectively). Thus, all immature specimens were assigned unequivocally to 1 of the 2 *Pseudanthobothrium* spp. or 1 of the 2 *Echeneibothrium* spp. Molecular results indicated that all immature specimens of *Pseudanthobothrium* n.sp. were restricted to *L. erinacea* and *L. ocellata*, whereas those of *P. hanseni* were restricted to *A. radiata* and *M. senta*. These results are consistent with observations of mature parasite-host relationships for these species (Randhawa *et al.* manuscript submitted), which suggests that host specificity for species of the genus *Pseudanthobothrium* is expressed early in this host-parasite relationship. Either plerocercoids of *Pseudanthobothrium* n.sp. are not encountered by *A. radiata* and *M. senta* (and similarly plerocercoids of *P. hanseni* are not encountered by *L. erinacea* and *L. ocellata*) or some mechanism(s) prevents their attachment, and therefore establishment of either plerocercoid in the 'wrong' host. Differing host behaviours, such as substrate preferences and different feeding habits of hosts, close the encounter filter of the host spectrum, whereas the absence of parasite adaptations necessary to overcome host defences or the incompatibility of parasite adaptations to host resources (spatial or metabolic) close the compatibility filter of the host spectrum. All 4 skate species are known to be sympatric over their geographical range (McEachran and Musick, 1975; McEachran *et al.* 1976, and references therein). However, *L. erinacea* and *L. ocellata* prefer sandy and gravely bottoms (Packer *et al.* 2003*a, b*), whereas *M. senta* prefers soft, muddy substrate (Packer *et al.* 2003*c*).

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Table 2. Summary of the prevalence and intensity of infection (range) for *Pseudanthobothrium* n.sp., *P. hanseni*, *Echeneibothrium canadensis* and *E. vernetae*

Parasite species	Host species	Prevalence	Intensity of infection (Range)
Pseudanthobothrium n.sp.	*Leucoraja erinacea*	83·3% (70 of 84)	14·2 (1–53)
	L. ocellata	85·7% (6 of 7)	11·3 (1–26)
P. hanseni	*Amblyraja radiata*	81·8% (9 of 11)	13·7 (1–55)
	Malacoraja senta	60·0% (15 of 25)	9·9 (1–73)
E. canadensis[a]	*A. radiata*	18·2% (2 of 11)	1·5 (1 or 2)
E. vernetae[a]	*L. erinacea*	52·4% (44 of 84)	6·6 (1–51)
	L. ocellata	28·6% (2 of 7)	8·5 (8 or 9)

[a] Immature *E. vernetae* and *E. canadensis* specimens from *A. radiata* were not included as only a fraction were identified using the molecular marker. Others could not unequivocally be identified due to absence of species-diagnostic features.

Amblyraja radiata shows little substrate preference (Packer *et al*. 2003*d*) but is positively associated to *M. senta* (McEachran and Musick, 1975; McEachran *et al*. 1976), whereas this ecological species-pair is negatively associated with the *L. erinacea* and *L. ocellata* ecological species-pair (McEachran and Musick, 1975). These substrate preferences (behaviour parameter of the encounter filter) and corresponding prey biota explain, at least in part, the presence of *Pseudanthobothrium* n.sp. in 1 ecological species-pair and its absence from the other, and *vice versa* for *P. hanseni*.

Furthermore, molecular results indicated that all immature specimens of *E. canadensis* were specific to *A. radiata*, whereas those of *E. vernetae* were recovered from *A. radiata* and *L. erinacea*. The specificity of immature *E. canadensis* is not surprising, since it reflects that of mature specimens, but should be considered as preliminary for reasons stated earlier. The low prevalence and intensity of infection of immature *Echeneibothrium* specimens in *A. radiata*, and non-recovery from *L. ocellata*, lead to an overestimation of the specificity of *E. canadensis*. High specificity is possibly an artefact of inadequate sampling (Poulin, 1998). Williams (1966) also stated that: "… only on very rare occasions were immature specimens of a [*Echeneibothrium*] species found in two species of *Raja* …" (p. 268). Although the recording of immature *E. vernetae* specimens from *A. radiata* and *L. erinacea* supports the potential for recovering immature *Echeneibothrium* spp. from 2 rajid hosts (Williams, 1966), it questions the rarity of this event. Of the 8 immature *Echeneibothrium* specimens recovered from *A. radiata*, 5 were assignable to *E. vernetae*, a parasite specific to *L. erinacea* and *L. ocellata* once mature. Additionally, the recovery of mature *E. vernetae* from *L. ocellata* implies that immature *E. vernetae* occur in this host and should be recovered with intensified sampling effort.

These results suggest that host specificity of the genus *Echeneibothrium* is not apparent from our sampling of immature specimens and that worms can establish (or attach), but are not able to mature (or develop) in the 'wrong' host species.

Host resistance (restriction) occurs when the host's defence mechanisms (e.g. immune system) prohibit the development of the parasite, i.e., death or detachment of the parasite occurring pre-establishment (or post-attachment) or prohibit the maturation of the parasite. Host resistance, or restriction, has been shown experimentally by exposing *Acanthobothrium quadripartitum*, a tetraphyllidean cestode, to serum from the 'wrong' host, which led to the death of 80% of the worms within 2 h (McVicar and Fletcher, 1970) (an example of death occurring post-attachment); and by transferring the host-specific monogenean *Entobdella soleae* onto the 'wrong' host, which led to it detaching within 30 h (see Rohde and Rohde, 2005) (an example of detachment occurring post-attachment). The presence of immature, and the absence of mature, *E. vernetae* specimens from *A. radiata* caused by host resistance is a plausible hypothesis, however, an experiment exposing immature and mature *E. vernetae* specimens to various rajid skate sera is necessary for its confirmation.

Neither host incompatibility nor host resistance hypotheses have been tested here, therefore, neither can be confirmed nor ruled out as an explanation for the presence of immature *E. vernetae* in *A. radiata* and the specificity of *E. canadensis*. Parasitological surveys of wild rajid skates are inadequate in addressing hypotheses of host resistance or unsuitability. Therefore, experimental infections are the only valid alternatives for testing either of these two hypotheses. Support for the host resistance hypothesis would assume that immature *E. vernetae* in *A. radiata* were recently acquired infections (hours or days) and would not be 'able' or 'allowed' to

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establish over the long term. Host unsuitability would assume that *E. canadensis* larvae are encountered by *M. senta*, but are unable to attach. It is also possible that host resistance or host unsuitability is/are involved in the specificity of *Pseudanthobothrium* spp., but was not observed. Dissections only provide a glimpse into an otherwise dynamic relationship between hosts and parasite populations/communities and do not offer the means necessary to make strong inferences on past assemblages (hours or days), therefore reiterating the importance of experimental infections in order to test these hypotheses.

Furthermore, knowledge of the ecology of skate hosts allows certain inferences to be made. Keeling and Burt (1996) reported that the prevalence and intensity of infection for *E. canadensis* were 13·7% and 1·4 *E. canadensis* per infected *A. radiata* (range of 1 to 2), respectively. Prevalence, intensity of infection and range for *E. canadensis* reported herein are consistent with those published (Keeling and Burt, 1996). Although *A. radiata* and *M. senta* are sympatric and have a high coefficient of association (McEachran and Musick, 1975), *M. senta* has specialized its feeding habits in response to possible competition with *A. radiata* for resources (McEachran *et al*. 1976). This has led to lower diversity of prey species in the diet of *M. senta* (McEachran *et al*. 1976) even though they share some of the more abundant prey species (Packer *et al*. 2003*c*, *d*). This suggests that *P. hanseni* may be transmitted via one of these common prey items (possibly an amphipod), as it is a cestode common to both rajid skates (Randhawa *et al*. manuscript submitted), whereas *E. canadensis* may be transmitted via a larger prey item specific to *A. radiata* (possibly infauna, e.g. polychaete worm). It is assumed here that the larger prey items are less abundant (number of individuals) in the host diet and that both *Pseudanthobothrium* spp. and *Echeneibothrium* spp. are transmitted in similar numbers during each infection event, thus explaining the greater intensity of infection of *Pseudanthobothrium* spp. compared to that of *Echeneibothrium* spp.

Euzet (1956) did not publish prevalence and intensity data for *E. vernetae* when he described this species based on material collected by Linton (1889) from *L. erinacea*, nor did Linton (1924) from material collected between 1905 and 1913 from *L. erinacea*, which he described as *E. variabile* (later recognized as *E. vernetae* by Euzet in 1956), therefore contributing little to the understanding of the ecology of the parasite. Prevalence of infection for *E. vernetae* reported herein (52·4% in *L. erinacea vs* 28·6% in *L. ocellata*) suggests that *L. erinacea* is the preferred host for this cestode in the area sampled and that the relatively high abundance (3·5 in *L. erinacea* and 2·4 in *L. ocellata*) of the parasite indicates that infections are not acquired accidentally in either host species. Experimental infections tracking the development of *E. vernetae* in both host species and investigating host suitability (e.g. stunted growth in 1 host species, lower biotic potential in 1 host species, etc.) would provide useful information for host preference. If differential fitness is observed, then it could be assumed that one host species is the preferred host, whereas the other is less suitable and may provide evidence for the trade-off hypothesis (trade-off between adapting to a new host species *versus* the ability to reach high abundance in that host species) (see Poulin and Mouillot, 2004).

It is generally accepted that ecological factors often drive host specificity (e.g. Holmes, 1990; Rohde and Rohde, 2005) and that the availability of 'suitable' hosts is necessary for the successful colonization of a new host species (Poulin, 1992). *Leucoraja erinacea* is one of the commonest demersal fishes in the Northwest Atlantic (Packer *et al*. 2003*a*, and references therein) whereas *L. ocellata* is sympatric to *L. erinacea* over most of its range (McEachran and Musick, 1975; Packer *et al*. 2003*b*) its abundance is much lower than that of *L. erinacea* in the Bay of Fundy and Passamaquoddy Bay, as shown by number of fish examined for this study (84 *vs*. 7, respectively) and reported in Packer *et al*. (2003*a*, *b*). This relative abundance is consistent with abundance data over the shared range of both sympatric *Leucoraja* spp. as reported in Packer *et al*. (2003*a*, *b*). Higher prevalence of *E. vernetae* in *L. erinacea* may be an artefact of relative abundance of both skate species. Host relative abundance (or host availability) can skew host-use by parasites and render host specificity indices unreliable (Poulin, 1998). It is therefore suggested herein that investigations of host specificity should provide the measures of prevalence and intensity (when available) to gain a better understanding of parasite ecology and so that host specificity can be measured and compared across studies.

In summary, the onset of host specificity differs between *Pseudanthobothrium* spp. and *Echeneibothirum* spp. Specificity in *Pseudanthobothirum* spp. occurs prior to attachment, whereas specificity in *Echeneibothrium* occurs post-attachment. Estimating the onset of host specificity was predicted by determining the specificity of mature specimens of each parasite species and comparing the host distribution of immature specimens of those same species. The host specificity of immature *P. hanseni*, *Pseudanthobothrium* n.sp. and *E. canadensis* mirrors that of mature specimens of their respective host species. The recovery of immature *E. vernetae* from *L. erinacea* and *A. radiata* was unexpected and somewhat surprising. Since *A. radiata* is not host to mature specimens of *E. vernetae*, the presence of immature cestodes of that species in *A. radiata* may indicate that host restriction or resistance is involved, allowing the attachment of the parasite, but not its establishment.

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