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# Molecular Systematics of Pinniped Hookworms (Nematoda: *Uncinaria*): Species Delimitation, Host Associations and Host-Induced Morphometric Variation

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## Molecular systematics of pinniped hookworms (Nematoda: *Uncinaria*): species delimitation, host associations and host-induced morphometric variation

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## ABSTRACT

Hookworms of the genus *Uncinaria* have been widely reported from juvenile pinnipeds, however investigations of their systematics has been limited, with only two species described, *Uncinaria lucasi* from northern fur seals (*Callorhinus ursinus*) and *Uncinaria hamiltoni* from South American sea lions (*Otaria flavescens*). Hookworms were sampled from these hosts and seven additional species including Steller sea lions (*Eumetopias jubatus*), California sea lions (*Zalophus californianus*), South American fur seals (*Arctocephalus australis*), Australian fur seals (*Arctocephalus pusillus*), New Zealand sea lions (*Phocarctos hookeri*), southern elephant seals (*Mirounga leonina*), and the Mediterranean monk seal (*Monachus monachus*). One hundred and thirteen individual hookworms, including an outgroup species, were sequenced for four genes representing two loci (nuclear ribosomal DNA and mitochondrial DNA). Phylogenetic analyses of these sequences recovered seven independent evolutionary lineages or species, including the described species and five undescribed species. The molecular evidence shows that *U. lucasi* parasitises both *C. ursinus* and *E. jubatus*, whereas *U. hamiltoni* parasitises *O. flavescens* and *A. australis*. The five undescribed hookworm species were each associated with single host species (*Z. californianus*, *A. pusillus*, *P. hookeri*, *M. leonina* and *M. monachus*). For parasites of otarids, patterns of *Uncinaria* host-sharing and phylogenetic relationships had a strong biogeographic component with separate clades of parasites from northern versus southern hemisphere hosts. Comparison of phylogenies for these hookworms and their hosts suggests that the association of *U. lucasi* with northern fur seals results from a host-switch from Steller sea lions. Morphometric data for *U. lucasi* shows marked host-associated size differences for both sexes, with *U. lucasi* individuals from *E. jubatus* significantly larger. This result suggests that adult growth of *U. lucasi* is reduced within the host species representing the more recent host-parasite association. Intraspecific host-induced size differences are inconsistent with the exclusive use of morphometrics to delimit and diagnose species of *Uncinaria* from pinnipeds.

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## 1. Introduction

Hookworm disease can have a significant deleterious impact on juvenile pinnipeds and their populations, and although estimates of mortality in pups due to *Uncinaria* hookworm infections vary substantially, one recent study reported that 13% of deaths in New Zealand sea lion pups (*Phocartos hookeri*) were attributable to hookworms (Castinel et al., 2006). The relationship between hookworm infection intensity and clinical hookworm disease in pinnipeds is not entirely clear, and host body condition is inversely correlated with the number of hookworms present (Sepúlveda, 1998; Lyons et al., 2001), perhaps due to the linkage between nursing and transmammmary transmission of these hookworms. Irrespective of questions regarding the exact relationship between the intensity of hookworm infection and pathology, it is clear that *Uncinaria* spp. can cause serious health problems in pinniped pups (Spraker et al., 2004). Recently, hookworms have been reported to interact synergistically with bacterial pathogens, causing enteritis and bacteremia; this emerging disease complex was responsible for 72% of deaths in rookeries of California sea lion pups (*Zalophus californianus*) in one investigation (Spraker et al., 2007). Unexpectedly, California sea lion hosts with enteritis have been found to have hookworm adults deep within muscle layers of the intestine and numerous nematodes free in the peritoneal cavity (Spraker et al., 2004, 2007; Lyons et al., 2005). Hookworm disease in pinnipeds appears to be influenced by host genetics, with homozygosity at a single locus predisposing California sea lion hosts to hookworm anaemia (Acevedo-Whitehouse et al., 2006, 2009). In contrast, genetic studies of different pinniped species offer conflicting results concerning whether increased average homozygosity of hosts is correlated with increased hookworm disease (Acevedo-Whitehouse et al., 2006, 2009). Differences in host responses to hookworms owing to variations in host genetics may help explain the poor predictive value of hookworm infection intensity for pathogenicity and pup condition in certain studies (Lyons et al., 1997, 2001, 2005).

The first species of pinniped hookworm to be formally described was *Uncinaria lucasi* (Stiles, 1901) from the northern fur seal (*Callorhinus ursinus*). This original description was later considered unsatisfactory and because the type specimens were damaged, Baylis (1947) redescribed *U. lucasi* using new specimens obtained from *C. ursinus* collected from the topotype locality (Pribilof Islands, USA). *Uncinaria lucasi* is the only pinniped hookworm for which the life cycle has been experimentally completed (Olsen and Lyons, 1965), and many aspects of its biology have been revealed through subsequent investigations (Olsen, 1958; Lyons and Keyes, 1978, 1984; Lyons and Biggs, 1983; Lyons et al., 1997). Unlike hookworms from many terrestrial mammalian hosts, adult *U. lucasi* establish in hosts only from transmammmary transmission of parasitic L<sub>3</sub>s acquired by nursing pups from their mother's milk. In northern fur seals, adult *U. lucasi* are eliminated spontaneously from juvenile hosts a maximum of 3 months p.i. (Olsen and Lyons, 1965), and adult seals are not parasitised by adult hookworms. Hookworm eggs in rookery soil hatch as free-living L<sub>3</sub>s, and these larvae can penetrate the skin of seals, or enter orally, and persist in tissues as parasitic L<sub>3</sub>s. The life cycle is completed when parasitic L<sub>3</sub>s are reactivated within lactating fur seals and migrate to the mammary glands (pre-parturition) before transmission to pups through nursing.

The only other species of hookworm from pinnipeds that has been formally described is *Uncinaria hamiltoni*, obtained from the South American sea lion, *Otaria flavescens* (syn *Otaria byronia*) in the Falkland Islands (Baylis, 1947). Baylis (1933) originally suggested that specimens of *U. hamiltoni* from *O. flavescens* were conspecific with hookworms recovered from a California sea lion, *Z.*

*californianus* (see Nadler et al., 2000), and this has led to representation of hookworms from *Z. californianus* as *U. hamiltoni*. However, other researchers (Dailey and Hill, 1970) reported that specimens of *Uncinaria* from *Z. californianus* had morphometric characteristics intermediate between *U. lucasi* and *U. hamiltoni*, thereby questioning the conspecificity of hookworms from South American and California sea lions.

Morphological, mainly morphometric, differences have been reported between *Uncinaria* individuals from different pinniped species, but it is unclear whether these are species-level differences, reflect intraspecific variation or are host-induced morphological differences (George-Nascimento et al., 1992; Nadler et al., 2000; Castinel et al., 2006; Ramos et al., 2013). Based on their formal descriptions, morphological differences between the two described species, *U. lucasi* and *U. hamiltoni*, are minor (Baylis, 1933, 1947; Nadler et al., 2000). Nadler et al. (2000) reported statistically significant differences in some morphometric features (e.g., total body length, spicule length) of male *U. lucasi* from northern fur seal pups obtained from two different geographic regions. This observation is consistent with previous suggestions that differences in nematode body size and certain characteristics of infection may reflect host-induced variation. For example, Olsen (1952) noted the larger body size of hookworms from Steller sea lions (*Eumetopias jubatus*) versus hookworms from northern fur seals, although he believed both host species were infested with *U. lucasi*. Similarly, George-Nascimento et al. (1992) reported that differences in nematode body size, prevalence of host skin lesions and infection intensity for adult hookworms parasitising South American sea lions (*O. byronia*) and South American fur seals (*Arctocephalus australis*) represented host-induced variation within one hookworm species.

The species-level systematics of some pinniped *Uncinaria* has recently been investigated using a molecular systematic approach (Nadler et al., 2000; Nadler, 2002; Ramos et al., 2013), yielding evidence independent of morphology for delimiting species and providing a phylogenetic framework for understanding intraspecific and interspecific morphological variation. This approach has been used to evaluate the specific status of *Uncinaria* parasitising California sea lions and northern fur seals (Nadler et al., 2000), with lineage exclusivity and species status determined by molecular phylogenetic analysis. These studies revealed that northern fur seals and California sea lions, species that share the same rookery space in parts of their breeding ranges, host different *Uncinaria* spp. (Nadler et al., 2000; Nadler, 2002). Similarly, molecular characterisation of *Uncinaria* sp. from Australian fur seals, Australian sea lions, and New Zealand fur seals indicates that these three hosts share a distinct species of hookworm that, based on morphology, most closely resembles *U. hamiltoni* (Ramos et al., 2013). However, developing a more complete understanding of the species diversity of *Uncinaria* in pinniped hosts and investigation of their host ranges requires molecular comparisons of hookworms from many additional host species, together with characterisation of hookworms representing both described species.

In the present study, we investigate the specific status of *Uncinaria* parasitising nine pinniped species using evolutionary analysis of nuclear and mitochondrial gene sequences amplified from more than 100 individual hookworms. Phylogenetic trees were reconstructed for the hookworms and compared with published phylogenies for their hosts, yielding new hypotheses for pinniped hookworm evolution and host associations. In addition, an evaluation of the utility of morphometrics is investigated through comparisons of a hookworm species that infects two host species. In addition to providing specific conclusions regarding pinniped hookworms, the approaches used herein are applicable to other investigations of parasites designed to test hypotheses concerning species and to evaluate their relationships.

## 2. Materials and methods

### 2.1. Nematode collection

*Uncinaria* specimens infecting pinniped juveniles were obtained from nine host species that were necropsied at field collection sites (rookeries), with some host species represented by multiple geographic sites and broad geographic ranges (Table 1). The hosts included both otarids (Australian fur seal (AFS) (*Arctocephalus pusillus doriferus*), South American fur seal (SAFS) (*A. australis*), northern fur seal (NFS) (*C. ursinus*), Steller sea lion (SSL) (*E. jubatus*), California sea lion (CSL) (*Z. californianus*), New Zealand sea lion (NZSL) (*P. hookeri*), South American sea lion (SASL) (*O. flavescens*)) and phocids (southern elephant seal (SES) (*Mirounga leonina*), Mediterranean monk seal (MMS) (*Monachus monachus*)). Specimens from *M. leonina* and *A. p. doriferus* represent hookworm samples previously sequenced for the ITS-1 and ITS-2 genes by Ramos et al. (2013). Nematodes were preserved in 90–100% ethanol in the field and stored at  $-20^{\circ}\text{C}$  when returned to the laboratory. As a representative outgroup (see Nadler et al., 2000), *Uncinaria stenocephala* Railliet, was collected from Island foxes (*Urocyon littoralis*) from San Miguel Island, California, USA. To obtain morphometric data relevant to potential host-induced size differences, 54 intact adult hookworms (all females gravid) from Steller sea lions and northern fur seal hosts were measured using a Nikon E600 interference contrast microscope and SPOT image analysis software. Prior to measurement, specimens were rehydrated using the procedure of Naem et al. (2010) to reduce shrinkage artifacts caused by fixation in high-percentage ethanol. Rehydrated specimens were then fixed in alcohol formalin acetic acid fixative (AFA) and mounted temporarily in lactophenol for microscopy. In some cases, features were not measured because they were obscured from view, preventing accurate size determination (e.g., one of the spicules; individual eggs in the uterus). Anterior and posterior ends of specimens used for molecular analysis were placed in 95% ethanol and deposited as vouchers in the University of California Davis, USA, Nematode Collection (referenced using specimen ID numbers in Table 1). These partial specimens were not rehydrated prior to storage as vouchers.

### 2.2. Statistical analysis

A *t*-test was used to compare all mean measurements of adult hookworms collected from northern fur seals versus Steller sea lions. Female and male worms were analysed separately. To determine whether there was an interaction between worm sex and host species, a two-way analysis of variance was conducted with sex and host as the main effects. Only measurements from both sexes were used in this analysis; specifically, both spicule lengths and the vulva to posterior end measurements were excluded. All analyses were conducted with JPM<sup>®</sup> 9 software (SAS Institute Inc., 2010).

### 2.3. PCR amplification

DNA was extracted from the excised mid-body (2–4 mm cross-section) of individual hookworms using the sodium hydroxide method (Floyd et al., 2002). Three regions of nuclear ribosomal DNA were amplified individually by PCR using primers and cycling conditions described previously (Nadler et al., 2000). These regions included the internal transcribed spacers and 5.8S subunit (ITS-1, 5.8S, ITS-2), the lsrDNA D2/D3 domains, and the lsrDNA D18/19 domains. In addition, approximately 78% of the mitochondrial 12S rDNA corresponding to positions 1,027–1,568 in *Caenorhabditis elegans* was amplified using forward primer #505

(5'-GTTCAGAATAATCGGCTAGAC) and reverse primer #506 (5'-TCTACTTTACTACAACCTACTCCCC). For the mitochondrial amplification, PCR parameters included denaturation at  $94^{\circ}\text{C}$  for 3 min, followed by 37 cycles of  $94^{\circ}\text{C}$  for 30 s,  $50^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 45 s, followed by a post-amplification extension at  $72^{\circ}\text{C}$  for 7 min. Mitochondrial 12S PCRs (25  $\mu\text{l}$ ) included 0.5  $\mu\text{M}$  of each primer, 200  $\mu\text{M}$  deoxynucleoside triphosphates, 0.5 units of Finnzymes DNAzyme EXT proofreading polymerase (New England Biolabs, USA) and 3 mM  $\text{MgCl}_2$ .

### 2.4. DNA sequencing

DNA templates for direct sequencing of amplified DNA from individual hookworms were prepared by enzymatic treatment of PCR products using exonuclease I and shrimp alkaline phosphatase (US Biochemical, Affymetrix Pre-Sequencing kit, USA). Sequences were obtained using an ABI 3730 DNA Sequencer (Perkin-Elmer Applied Biosystems, USA). All sequences were completely double-stranded for verification using reactions primed using the two PCR primers (D2/D3 lsrDNA, 12S rDNA) or the PCR primers plus internal primers (ITS-1, 5.8S, ITS-2 and D18/D19 lsrDNA); internal sequencing primers were described previously (Nadler et al., 2000). CodonCode Aligner (version 2.06, CodonCode Corporation) and Phred base-calling were used for contig assembly. Site polymorphisms in directly sequenced PCR products were recorded only when both alternative nucleotide peaks were present in sequences representing both DNA strands. If the heights of the alternative nucleotide peaks at polymorphic sites were not equal, the height of the minor peak was required to exceed background terminations significantly and comprise at least 25% of the major peak to be scored as a polymorphism. Sequences corresponding to the PCR primers were removed prior to analysis.

### 2.5. Phylogenetic analyses

Each sequenced region was aligned separately using CLUSTAL-X (Thompson et al., 1997) and concatenated subsequently for combined analysis. Sequence data were analysed separately by locus (i.e., nuclear ribosomal DNA (rDNA) versus mitochondrial rDNA), and as a combined or total evidence dataset. Phylogenetic trees were rooted using the outgroup *U. stenocephala*. Justification for use of a single outgroup is based on previous analysis of *Uncinaria* spp. (Nadler et al., 2000), which showed that including sequences of other hookworm genera introduced substantial alignment ambiguity, resulting in loss of the phylogenetic resolution necessary for species delimitation when the ambiguous alignment regions were excluded from tree reconstruction. Maximum parsimony (MP) analysis was conducted using PAUP\* (Swofford, 1998). Heuristic parsimony searches were conducted using tree-bisection-reconnection branch-swapping and 500 replicates of random-taxon addition, saving a maximum of 20 trees per replicate. Bootstrap MP searches were conducted using 1,000 pseudoreplicate datasets, each with 10 replicates of random-taxon addition, saving a maximum of 10 trees per pseudoreplicate, and a search time limit of 1 min per pseudoreplicate. When more than one most parsimonious tree was found, a strict consensus of the most parsimonious trees was produced. Bayesian tree inference (BI) was performed using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). The best-fit model of nucleotide substitution for each of the four sequenced regions (ITS-1, 5.8S, ITS-2; D2/D3 lsrDNA; D18/19 lsrDNA; 12S rDNA) was selected using the Akaike Information Criterion (AIC) as implemented in Modeltest version 3.7 (Posada and Crandall, 1998). For combined Bayesian analysis, these substitution models were then applied to each respective partition, with the model parameters unlinked across partitions and estimated as part

**Table 1**  
Hookworm specimen identifier, nematode sex (F, female; M, male), host identifier, collection locality and GenBank accession numbers for sequences used for phylogenetic analyses. The hookworm identifier is used as the terminal taxon label in Fig. 1. Host abbreviations: AFS, Australian fur seal (*Arctocephalus pusillus doriferus*), SAFS, South American fur seal (*Arctocephalus australis*), NFS, northern fur seal (*Callorhinus ursinus*), SSL, Stellar sea lion (*Eumetopias jubatus*), CSL, California sea lion (*Zalophus californianus*), NZSL, New Zealand sea lion (*Phocarcos hookeri*), SASL, South American sea lion (*Otaria flavescens*), SES, southern elephant seal (*Mirounga leonina*), and MMS, Mediterranean monk seal (*Monachus monachus*).

Hookworm identifier	Sex	Host identifier	Host collection locality	ITS rDNA Acc. No.	D2/D3 rDNA Acc. No.	D18/D19 rDNA Acc. No.	12S mtDNA Acc. No.
X2361	F	CSL 99ZC-1	San Nicolas Island, CA, USA	HQ262056	HQ261907	HQ261942	HQ262169
X2741	F	CSL 00ZC-32	South Cove, San Miguel Island, CA, USA	HQ262063	HQ261915	HQ261946	HQ262170
X2742	F	CSL 00ZC-32	South Cove, San Miguel Island, CA, USA	HQ262062	HQ261914	HQ261947	HQ262171
X2743	F	CSL 00ZC-32	South Cove, San Miguel Island, CA, USA	HQ262061	HQ261913	HQ261948	HQ262172
X2744	F	CSL 00ZC-32	South Cove, San Miguel Island, CA, USA	HQ262060	HQ261912	HQ261949	HQ262173
X2745	F	NFS 00CU-16	Adams Cove, San Miguel Island, CA, USA	HQ262087	HQ261904	HQ261975	HQ262183
X2791	F	NFS 00CU-16	Adams Cove, San Miguel Island, CA, USA	HQ262042	HQ261903	HQ261974	HQ262184
X2792	F	NFS 00CU-16	Adams Cove, San Miguel Island, CA, USA	HQ262086	HQ261902	HQ261973	HQ262185
X2793	M	NFS 00CU-16	Adams Cove, San Miguel Island, CA, USA	HQ262085	HQ261901	HQ261972	HQ262186
X2795	M	NFS 00CU-16	Adams Cove, San Miguel Island, CA, USA	HQ262088	HQ261900	HQ261971	HQ262155
X2987	M	NFS RC06	Commander Islands, Russia	HQ262070	HQ261882	HQ261955	HQ262180
X2988	F	NFS RC06	Commander Islands, Russia	HQ262071	HQ261883	HQ261956	HQ262181
X2989	F	NFS RC06	Commander Islands, Russia	HQ262072	HQ261884	HQ261957	HQ262182
X2990	F	NFS RC06	Commander Islands, Russia	HQ262073	HQ261885	HQ261958	HQ262201
X2993	F	NFS RC06	Commander Islands, Russia	HQ262067	HQ261888	HQ261953	HQ262187
X2994	M	NFS RC06	Commander Islands, Russia	HQ262068	HQ261889	HQ261954	HQ262188
X2995	M	NFS RC06	Commander Islands, Russia	HQ262069	HQ261899	HQ261970	HQ262189
X3029	F	NFS 01CU-53	Reef Rookery North, St. Paul Island, AK, USA	HQ262075	HQ261890	HQ261960	HQ262191
X3030	F	NFS 01CU-60	Reef Rookery South, St. Paul Island, AK, USA	HQ262074	HQ261886	HQ261959	HQ262190
X3031	F	NFS 01CU-60	Reef Rookery South, St. Paul Island, AK, USA	HQ262076	HQ261887	HQ261961	HQ262192
X3066	F	FOX Zor-249	San Miguel Island, CA, USA	HQ262052	HQ261919	HQ261940	HQ262165
X3067	F	FOX Zor-249	San Miguel Island, CA, USA	HQ262053	HQ261918	HQ261939	HQ262166
X3068	M	FOX Zor-249	San Miguel Island, CA, USA	HQ262054	HQ261917	HQ261938	HQ262167
X3069	M	FOX Zor-249	San Miguel Island, CA, USA	HQ262055	HQ261916	HQ261941	HQ262168
X3537	F	SASL OF26	Punta León, northern Patagonia, Argentina	HQ262111	HQ261850	HQ261998	HQ262224
X3539	M	SASL OF30	Punta León, northern Patagonia, Argentina	HQ262112	HQ261851	HQ261999	HQ262225
X3556	M	SASL OF26	Punta León, northern Patagonia, Argentina	HQ262115	HQ261852	HQ262000	HQ262226
X3558	M	SASL OF26	Punta León, northern Patagonia, Argentina	HQ262113	HQ261853	HQ262005	HQ262231
X3585	F	SASL OF26	Punta León, northern Patagonia, Argentina	HQ262118	HQ261856	HQ262001	HQ262230
X3586	F	SASL OF26	Punta León, northern Patagonia, Argentina	HQ262117	HQ261857	HQ262002	HQ262229
X3587	F	SASL OF30	Punta León, northern Patagonia, Argentina	HQ262116	HQ261858	HQ262003	HQ262227
X3589	F	SASL OF30	Punta León, northern Patagonia, Argentina	HQ262114	HQ261859	HQ262004	HQ262228
X3620	M	SAFS, #1	Cabo Polonio, Rocha, Uruguay	HQ262110	HQ261870	HQ261987	HQ262213
X3621	F	SAFS, #1	Cabo Polonio, Rocha, Uruguay	HQ262100	HQ261863	HQ261997	HQ262214
X3729	F	SAFS, #1	Cabo Polonio, Rocha, Uruguay	HQ262101	HQ261864	HQ261988	HQ262215
X3802	M	SSL Hazy5-2003	Hazy Island, Southeast AK, USA	HQ262131	HQ261818	HQ262018	HQ262244
X3803	M	SSL Hazy5-2003	Hazy Island, Southeast AK, USA	HQ262132	HQ261819	HQ262019	HQ262245
X3804	M	SSL Hazy5-2003	Hazy Island, Southeast AK, USA	HQ262133	HQ261820	HQ262020	HQ262246
X3805	F	SSL Hazy5-2003	Hazy Island, Southeast AK, USA	HQ262134	HQ261821	HQ262021	HQ262247
X3806	F	SSL Hazy5-2003	Hazy Island, Southeast AK, USA	HQ262135	HQ261839	HQ262022	HQ262248
X3807	F	SSL Hazy5-2003	Hazy Island, Southeast AK, USA	HQ262136	HQ261838	HQ262023	HQ262249
X3877	M	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262089	HQ261872	HQ261976	HQ262207
X3878	M	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262091	HQ261871	HQ261982	HQ262202
X3879	M	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262090	HQ261879	HQ261977	HQ262203
X3891	F	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262092	HQ261873	HQ261978	HQ262204
X3892	F	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262093	HQ261880	HQ261979	HQ262205
X3893	F	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262099	HQ261881	HQ261980	HQ262206

X3935	M	SSL Hazy1-2003	Hazy Island, Southeast AK, USA	HQ262141	HQ261816	HQ262024	HQ262250
X3936	F	SSL Hazy1-2003	Hazy Island, Southeast AK, USA	HQ262140	HQ261823	HQ262028	HQ262260
X3938	F	SSL Hazy2-2003	Hazy Island, Southeast AK, USA	HQ262137	HQ261817	HQ262025	HQ262251
X3940	F	SSL Hazy3-2003	Hazy Island, Southeast AK, USA	HQ262138	HQ261824	HQ262027	HQ262252
X3942	F	SSL Hazy4-2003	Hazy Island, Southeast AK, USA	HQ262139	HQ261822	HQ262026	HQ262253
X3944	M	NZSL	Sandy Bay beach, Enderby Island, New Zealand	HQ262094	HQ261874	HQ261981	HQ262208
X3986	F	SASL #3	Cabo Polonio, Rocha, Uruguay	HQ262119	HQ261854	HQ262006	HQ262232
X3988	F	SASL #3	Cabo Polonio, Rocha, Uruguay	HQ262120	HQ261855	HQ262007	HQ262233
X3989	F	SAFS #2	Lobos Island, Maldonado, Uruguay	HQ262102	HQ261860	HQ261989	HQ262216
X3990	F	SAFS #2	Lobos Island, Maldonado, Uruguay	HQ262103	HQ261861	HQ261990	HQ262217
X3991	F	SAFS #2	Lobos Island, Maldonado, Uruguay	HQ262104	HQ261862	HQ261991	HQ262218
X4066	F	SAFS #3	Lobos Island, Maldonado, Uruguay	HQ262105	HQ261865	HQ261992	HQ262219
X4067	F	SAFS #4	Lobos Island, Maldonado, Uruguay	HQ262106	HQ261866	HQ261993	HQ262220
X4068	F	SAFS #5	Lobos Island, Maldonado, Uruguay	HQ262107	HQ261867	HQ261994	HQ262221
X4069	F	SAFS #6	Lobos Island, Maldonado, Uruguay	HQ262108	HQ261868	HQ261995	HQ262222
X4070	F	SAFS #7	Lobos Island, Maldonado, Uruguay	HQ262109	HQ261869	HQ261996	HQ262223
X4072	F	NZSL E03/04-58	Sandy Bay beach, Enderby Island, New Zealand	HQ262095	HQ261875	HQ261983	HQ262209
X4073	F	NZSL E03/04-71	Sandy Bay beach, Enderby Island, New Zealand	HQ262096	HQ261876	HQ261984	HQ262212
X4077	F	NZSL E03/04-67	Sandy Bay beach, Enderby Island, New Zealand	HQ262097	HQ261877	HQ261985	HQ262210
X4078	F	NZSL E03/04-67	Sandy Bay beach, Enderby Island, New Zealand	HQ262098	HQ261878	HQ261986	HQ262211
X4080	F	SSL Low-1-03	Lowry Island, Forrester Islands, AK, USA	HQ262142	HQ261825	HQ262029	HQ262254
X4081	F	SSL Low-1-03	Lowry Island, Forrester Islands, AK, USA	HQ262143	HQ261826	HQ262041	HQ262255
X4083	M	SSL Low-2-03	Lowry Island, Forrester Islands, AK, USA	HQ262144	HQ261827	HQ262030	HQ262256
X4084	F	SSL Low-2-03	Lowry Island, Forrester Islands, AK, USA	HQ262145	HQ261828	HQ262031	HQ262257
X4085	F	SSL Low-2-03	Lowry Island, Forrester Islands, AK, USA	HQ262146	HQ261829	HQ262032	HQ262258
X4086	F	SSL Low-2-03	Lowry Island, Forrester Islands, AK, USA	HQ262147	HQ261830	HQ262033	HQ262259
X4115	F	CSL 00ZC-27-30	Northwest Cove, San Miguel Island, CA, USA	HQ262059	HQ261911	HQ261945	HQ262174
X4116	F	CSL 00ZC-27-30	Northwest Cove, San Miguel Island, CA, USA	HQ262064	HQ261910	HQ261944	HQ262177
X4117	F	CSL 00ZC-27-30	Northwest Cove, San Miguel Island, CA, USA	HQ262058	HQ261909	HQ261943	HQ262175
X4118	F	CSL 00ZC-27-30	Northwest Cove, San Miguel Island, CA, USA	HQ262057	HQ261908	HQ261950	HQ262176
X4267	F	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262148	HQ261831	HQ262034	HQ262261
X4268	F	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262149	HQ261832	HQ262035	HQ262262
X4269	F	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262150	HQ261833	HQ262036	HQ262263
X4270	F	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262151	HQ261834	HQ262037	HQ262264
X4271	F	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262152	HQ261835	HQ262038	HQ262265
X4272	F	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262153	HQ261836	HQ262039	HQ262266
X4277	M	SSL 1	Iony Island, Sea of Okhotsk, Russia	HQ262154	HQ261837	HQ262040	HQ262267
X4609	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262077	HQ261891	HQ261962	HQ262193
X4610	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262078	HQ261892	HQ261963	HQ262194
X4612	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262079	HQ261893	HQ261964	HQ262195
X4613	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262080	HQ261894	HQ261965	HQ262196
X4614	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262081	HQ261895	HQ261966	HQ262197
X4615	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262084	HQ261896	HQ261967	HQ262198
X4616	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262082	HQ261897	HQ261968	HQ262199
X4617	F	NFS 05Cu-73	Reef Rookery, St. Paul Island, AK, USA	HQ262083	HQ261898	HQ261969	HQ262200
X5304	M	AFS	Lady Julia Percy Island, Victoria, Australia	HQ262043	HQ261927	HQ261936	HQ262156
X5306	M	AFS	Lady Julia Percy Island, Victoria, Australia	HQ262044	HQ261925	HQ261934	HQ262157
X5307	F	SES	Macquarie Island, Tasmania, Australia	HQ262121	HQ261848	HQ262016	HQ262234
X5308	F	SES	Macquarie Island, Tasmania, Australia	HQ262122	HQ261843	HQ262011	HQ262235
X5309	F	SES	Macquarie Island, Tasmania, Australia	HQ262123	HQ261849	HQ262017	HQ262236
X5310	F	SES	Macquarie Island, Tasmania, Australia	HQ262124	HQ261846	HQ262014	HQ262237
X5311	F	SES	Macquarie Island, Tasmania, Australia	HQ262125	HQ261845	HQ262013	HQ262238
X5312	F	SES	Macquarie Island, Tasmania, Australia	HQ262126	HQ261847	HQ262015	HQ262239
X5313	F	AFS	Lady Julia Percy Island, Victoria, Australia	HQ262045	HQ261928	HQ261937	HQ262158
X5315	F	AFS	Lady Julia Percy Island, Victoria, Australia	HQ262046	HQ261926	HQ261935	HQ262159
X5316	F	AFS	Lady Julia Percy Island, Victoria, Australia	HQ262051	HQ261920	HQ261929	HQ262160
X5317	F	AFS	Lady Julia Percy Island, Victoria, Australia	HQ262047	HQ261922	HQ261931	HQ262161
X5319	F	AFS	Seal Rocks, Phillip Island, Victoria, Australia	HQ262048	HQ261924	HQ261933	HQ262162

(continued on next page)

Table 1 (continued)

Hookworm identifier	Sex	Host identifier	Host collection locality	ITS rDNA Acc. No.	D2/D3 rDNA Acc. No.	D18/D19 rDNA Acc. No.	12S mtDNA Acc. No.
X5320	F	AFS	Seal Rocks, Phillip Island, Victoria, Australia	HQ262049	HQ261923	HQ261932	HQ262163
X5322	M	AFS	Seal Rocks, Phillip Island, Victoria, Australia	HQ262050	HQ261921	HQ261930	HQ262164
X5324	F	SES	Macquarie Island, Tasmania, Australia	HQ262127	HQ261844	HQ262012	HQ262240
X5325	F	SES	Macquarie Island, Tasmania, Australia	HQ262128	HQ261842	HQ262010	HQ262241
X5326	F	SES	Macquarie Island, Tasmania, Australia	HQ262129	HQ261840	HQ262008	HQ262242
X5327	F	SES	Macquarie Island, Tasmania, Australia	HQ262130	HQ261841	HQ262009	HQ262243
X5551	F	MMS MM061105	Orei, North Eria, Greece	HQ262065	HQ261905	HQ261951	HQ262178
X5552	F	MMS MM061105	Orei, North Eria, Greece	HQ262066	HQ261906	HQ261952	HQ262179

of the analysis. The standard deviation of split frequencies was used to assess whether the number of generations completed was sufficient; MrBayes was run using four Markov chain Monte Carlo (MCMC) chains for  $10^6$  generations. The chains were sampled every 1,000 generations and burn-in was determined empirically by examination of the log likelihood values of the chains. Pairwise sequence divergence was calculated for the each sequenced region using the *p*-distance in PAUP\* and hookworm sequences representing each evolutionary lineage.

### 3. Results

#### 3.1. Sequence characteristics and genetic variation

Intra-individual sequence polymorphisms were found in a very small fraction (0.008%) of the total nucleotides sequenced for these four gene regions. Among all individuals sequenced, 27 sites were polymorphic, with 26 of these sites within nuclear rDNA. Fourteen of these polymorphisms were within the ITS1–5.8S–ITS2 sequences, 10 within the LsrDNA D2–D3 sequences and two within the LsrDNA D18–D19 sequences. Most (81%) of these polymorphisms were transition substitutions, with 48% scored as R (A or G) and 33% as Y (C or T). The number of distinct sequences per gene region (haplotypes for mitochondrial DNA (mtDNA)), considering nematodes from each host species as a separate group a priori, varied between the two loci (nuclear rDNA and mtDNA). Per pinniped host species, there averaged 1.5 distinct *Uncinaria* sequences for nuclear rDNA and 4.4 haplotypes for mitochondrial DNA. Pairwise percent sequence divergence between hookworms from different host species varied substantially according to the region sequenced (Tables 2 and 3), with 12S mtDNA having approximately twice the pairwise sequence divergence of the next most divergent sequence, ITS1–5.8S–ITS2. Differences in pairwise divergence values show that the relative rate of substitution for these four sequence regions varies as follows: 12S rDNA > ITS1–5.8S–ITS2 > LsrDNA D2–D3 > LsrDNA D18–D19. The two described hookworm species from pinnipeds, *U. lucasi* and *U. hamiltoni*, showed pairwise sequence differences for all four sequenced regions, as did these two species versus the outgroup, *U. stenocephala* (Tables 2 and 3). For the fastest evolving regions (12S and ITS regions), pairwise comparisons of other host-associated hookworms showed an absence of sequence differences for Steller sea lion hookworms versus northern fur seal (*U. lucasi*) hookworms (ITS regions) and South American fur seal hookworms versus South American sea lion (*U. hamiltoni*) hookworms (ITS regions); both of these comparisons also showed very low divergence for 12S rDNA (0.2% in each case). For the more slowly evolving nuclear LsrDNA regions, these same two pairwise comparisons also showed either no differences or very low

divergence (no more than 0.2%). Some other pairwise comparisons of host-associated hookworms showed low levels of divergence (e.g., <1%) for particular sequenced regions (Tables 2 and 3), and four other comparisons showed no sequence differences for the more conserved nuclear LsrDNA gene regions including *U. lucasi* versus California sea lion hookworms (D2–D3 LsrDNA), Australian fur seal versus New Zealand sea lion hookworms (D2–D3, D18–D19 LsrDNA), Steller sea lion versus California sea lion hookworms (D2–D3 LsrDNA), and Mediterranean monk seal versus southern elephant seal hookworms (D18–D19 LsrDNA).

#### 3.2. Phylogenetic analysis of *Uncinaria* lineages

Multiple alignments of the separate sequence regions resulted in datasets of 871 characters (ITS1–5.8S–ITS2), 596 characters (D2–D3 LsrDNA), 938 characters (D18–D19 LsrDNA) and 547 characters (12S mtDNA). The number of parsimony informative sites varied among these regions in accordance with pairwise divergence values and relative rates of evolution: 12S mtDNA region (91 informative sites), ITS1–5.8S–ITS2 region (76 informative sites), D2–D3 LsrDNA region (34 informative sites), and D18–D19 LsrDNA region (14 informative sites). There were very few regions of alignment ambiguity due to the low fraction of variable sites and minimal length variation for these sequences. Best-fit nucleotide substitution models, as selected by ModelTest, included GTR+I+G (12S dataset), HKY+G (ITS1–5.8S–ITS2 dataset), HKY+I+G (D2–D3 LsrDNA dataset), and F81+G (D18–D19 LsrDNA dataset). For the 12S mtDNA dataset, the Bayesian search burn-in period was estimated to include the first  $7 \times 10^4$  generations; for the nuclear rDNA datasets it was estimated as the first  $2 \times 10^5$  generations; and for the combined dataset the first  $1 \times 10^5$  generations. The corresponding number of trees associated with each burn-in period was discarded before producing Bayesian consensus trees.

MP analysis of the combined nuclear rDNA regions (113 taxa) reached the set maxtree limit (maximum number of trees saved) of 10,000; each tree had a length of 286 steps and a consistency index, (excluding uninformative characters) of 0.87. The strict consensus of these trees (not shown) yielded seven monophyletic groups of *Uncinaria* individuals (Table 4). Five of these clades represented all host-associated nematodes from each of five different host species. Two clades recovered by MP each included *Uncinaria* individuals from two different host species. Northern fur seal hookworms (*U. lucasi*) and Steller sea lion hookworms were resolved in one of these clades, and South American sea lion (*U. hamiltoni*) and South American fur seal hookworms were grouped in another clade (Table 4). MP bootstrap support for clades in the nuclear rDNA analysis was strong ( $\geq 85\%$ ) in two instances (California



sea lion hookworms, Mediterranean monk seal hookworms), but moderate in most and very low for *U. hamiltoni* (South American sea lion) plus South American fur seal hookworms (Table 4). The Bayesian posterior consensus tree for nuclear rDNA recovered monophyletic groups for six of the same host-associated groups of *Uncinaria*, the exception being Northern fur seal plus Steller sea lion hookworms. Bayesian posterior probabilities (BPP) of clades were high in five of the six clades (Table 4). There were no strongly supported sub-clades (BPP  $\geq$  90%) nested within the six main clades resolved by these nuclear rDNA data, or otherwise grouping individual hookworms in the trees.

MP analysis of the 12S mitochondrial rDNA region (113 taxa) yielded 8,760 trees, each with a length of 177 steps and a consistency index (excluding uninformative characters) of 0.70. The strict consensus of these trees (not shown) yielded four monophyletic groups of *Uncinaria* representing five host species (Table 4), including one with both South American sea lion and South American fur seal hookworms. MP bootstrap support for clades was strong for three groups and moderate in the fourth (Table 4). The Bayesian posterior consensus tree for 12S mitochondrial rDNA recovered the same four monophyletic groups, plus a fifth clade for *Uncinaria* from Australian fur seals (Table 4). BPP exceeded 95% for four of the five clades. There were five supported sub-clades (BPP  $\geq$  90%) grouping individual hookworms within the host-associated clades. With one exception, these sub-clades occurred within larger clades that represented hookworms of single host species. These sub-clades of individual hookworms (X numbers refer to individual specimens, Table 1) included (BPP in parentheses): SES X5307 + SES X5327 (100%), AFS X5316 + AFS X5317 (95%), AFS X5304 + AFS X5306 (98%), SSL X4268 + NFS X4613 (92%), and CSL X2741 + CSL X2743 (95%).

MP analysis of the combined (total evidence) sequence dataset (113 taxa) yielded the maximum number of saved trees (10,000), each with a length of 465 steps and a consistency index (excluding uninformative characters) of 0.77. The strict consensus of these trees (Figs. 1 and 2; Table 4) included seven monophyletic groups of *Uncinaria*. Two of these clades included *Uncinaria* from two different host species: northern fur seal hookworms and Steller sea lion hookworms were members of one clade, and South American sea lion and South American fur seal hookworms were resolved as another clade (Figs. 1 and 2; Table 4). Hookworms from each of the other five host species examined were resolved as separate clades (Figs. 1 and 2; Table 4). MP bootstrap support for these seven clades was strong in five cases and moderate for the other two clades (Table 4). The Bayesian consensus tree for the combined data recovered the same clades of host-associated hookworms as in MP analysis, with one exception: northern fur seal and Steller sea lion hookworms were not resolved as monophyletic, but were instead unresolved within a larger clade that included the

monophyletic group of California sea lion hookworms. For the combined sequence dataset, BPP of clades were high (100%) for the six resolved clades (Table 4). There were seven supported sub-clades grouping hookworms within the larger clades. Four of these were identical to those defined by mtDNA sequences (but not including CSL X2741 + CSL X2743). The other three included (BPP in parentheses): AFS X5316 + AFS X5317 + AFS X5304 + AFS X5306 (90%), NZSL X4072 + NZSL X4077 (93%), and CSL X4118 and CSL X2743 (94%). For these combined data, there were few reliably supported clades nested within the main clades, as assessed by bootstrap resampling or BPP (Fig. 1). Associations and phylogenetic relationships between the pinniped *Uncinaria* lineages and their hosts (reduced host phylogeny from Higdon et al., 2007) are shown in Fig. 2.

### 3.3. *Uncinaria* morphometric comparisons

Fifty-four rehydrated *U. lucasi* specimens were measured (Supplementary Tables S1–S4) for seven features common to both sexes (body length, oesophagus to anterior length, oesophagus length, oesophagus bulb width, width at oesophagus/intestine junction, buccal capsule length and width). Five measurements were specific to one sex (males: spicule lengths; females: egg length, egg width and tail length). Hookworms collected from Steller sea lions were consistently larger in every feature than those collected from northern fur seals (Table 5). Interestingly, two measurements had a significant interaction between the main effects of host and sex. Body length ( $P > t = 0.001$ ) and buccal width ( $P > t = 0.024$ ) measurements were more affected by host for female hookworms than males. Males from Stellar sea lions were just less than twice the body length of those collected from northern fur seals whereas females from Stellar sea lions were nearly three times the length of those collected from northern fur seals (Table 5). A similar relationship was found for buccal width but the difference was not as marked.

## 4. Discussion

The question of how many different hookworm species infect pinnipeds is a longstanding one, dating back to the original scientific descriptions of *U. lucasi* (Stiles, 1901) from the northern fur seal (*C. ursinus*) and *U. hamiltoni* (Baylis, 1947) from the South American sea lion *O. flavescens* (syn. *O. byronia*). No new species of pinniped hookworm has been described since 1947, although Botto and Mañé-Garzón (1975) proposed subspecies status for hookworms from *O. flavescens* from Uruguay (*U. hamiltoni platensis*) versus those from the type locality in the Falkland Islands (*U. hamiltoni hamiltoni*). Baylis' publications (Baylis, 1933, 1947) also

**Table 2**

Pairwise percent uncorrected sequence divergence ( $p$ -distance  $\times$  100) for the ITS1–5.8S–ITS2 region (listed first), followed by the value for mitochondrial 12S rDNA. Abbreviations indicate the host source and species identification of the hookworms: California sea lion (CSL), northern fur seal (NFS), South American sea lion (SASL), New Zealand sea lion (NZSL), Australian fur seal (AFS), southern elephant seal (SES), Steller sea lion (SSL), South American fur seal (SAFS), and Mediterranean monk seal (MMS). *Uncinaria stenocephala* is the outgroup (canid) hookworm.

	CSL	NFS	<i>U. stenocephala</i>	SASL	NZSL	AFS	SES	SSL	SAFS	MMS
CSL	–									
NFS ( <i>Uncinaria lucasi</i> )	0.75/1.3	–								
<i>U. stenocephala</i>	6.7/11.6	6.1/12.0	–							
SASL ( <i>Uncinaria hamiltoni</i> )	1.6/4.4	1.1/4.7	5.9/11.1	–						
NZSL	1.5/5.0	1.1/5.3	5.6/10.8	0.50/3.9	–					
AFS	1.5/5.4	1.0/5.3	5.7/10.5	0.50/4.2	0.40/0.7	–				
SES	4.4/9.4	3.7/8.9	4.6/9.8	3.6/7.8	3.5/6.4	3.3/5.8	–			
SSL ( <i>U. lucasi</i> )	0.74/1.1	0/0.2	6.1/11.7	1.1/4.5	1.1/5.1	1.0/5.0	3.7/8.9	–		
SAFS ( <i>U. hamiltoni</i> )	1.6/4.8	1.1/5.0	6.1/11.3	0/0.2	0.50/4.3	0.5/4.1	3.7/7.6	1.1/4.9	–	
MMS	4.5/7.6	3.7/7.2	4.6/11.4	3.6/6.8	3.6/7.6	3.4/6.9	0.50/5.4	3.7/7.3	3.7/7.1	–

**Table 3**  
Pairwise percent uncorrected sequence divergence ( $p$ -distance  $\times$  100) for nuclear large-subunit regions. Value for D2–D3 IsrDNA region is listed first, followed by the D18–D19 IsrDNA region. Abbreviations indicate the host source and species identification of the hookworms: California sea lion (CSL), northern fur seal (NFS), South American sea lion (SASL), New Zealand sea lion (NZSL), Australian fur seal (AFS), southern elephant seal (SES), Steller sea lion (SSL), South American fur seal (SAFS), and Mediterranean monk seal (MMS). *Uncinaria stenocephala* is the outgroup (canid) hookworm.

	CSL	NFS	<i>U. stenocephala</i>	SASL	NZSL	AFS	SES	SSL	SAFS	MMS
CSL	–									
NFS ( <i>Uncinaria lucasi</i> )	0/0.16	–								
<i>U. stenocephala</i>	2.4/0.35	2.7/0.54	–							
SASL ( <i>Uncinaria hamiltoni</i> )	1.2/0.16	1.5/0.32	2.7/0.18	–						
NZSL	0.97/0.34	0.96/0.51	3.6/0.36	0.57/0.17	–					
AFS	0.77/0.32	1.1/0.49	2.8/0.36	0.37/0.16	0/0	–				
SES	1.5/0.47	1.9/0.65	1.3/0.19	2.2/0.33	2.5/0.51	1.8/0.47	–			
SSL ( <i>U. lucasi</i> )	0/0.2	0.4/0	2.5/0.5	1.1/0.3	1.0/0.5	0.7/0.5	1.5/0.7	–		
SAFS ( <i>U. hamiltoni</i> )	1.2/0.2	1.5/0.3	2.8/0.2	0/0	0.6/0.2	0.4/0.2	2.2/0.3	1.1/0.3	–	
MMS	2.1/0.47	2.4/0.65	1.5/0.19	2.8/0.33	3.3/0.51	2.3/0.47	0.53/0	2.0/0.7	2.7/0.3	–

**Table 4**  
Monophyletic host-associated groups of *Uncinaria* as inferred by maximum parsimony and Bayesian inference. Numbers refer to bootstrap percentages of clades (maximum parsimony) and Bayesian posterior probabilities, respectively. Rows preceded by 1 are nuclear ribosomal DNA, rows preceded by 2, 12S mtDNA, and rows preceded by 3 the combined data.

Monophyly of <i>Uncinaria</i> from	MP clade, bootstrap	Bayesian clade, BPP
1 California sea lion	Yes, 100	Yes, 100
1 Northern fur seal + Steller sea lion	Yes, 65	No
1 South American sea lion + S. American fur seal	Yes, 51	Yes, 100
1 New Zealand sea lion	Yes, 66	Yes, 100
1 Australian fur seal	Yes, 61	Yes, 75
1 Mediterranean monk seal	Yes, 99	Yes, 100
1 Southern elephant seal	Yes, 66	Yes, 95
2 California sea lion	Yes, 81	Yes, 100
2 Northern fur seal + Steller sea lion	No	No
2 South American sea lion + S. American fur seal	Yes, 100	Yes, 100
2 New Zealand sea lion	No	No
2 Australian fur seal	No	Yes, 61
2 Mediterranean monk seal	Yes, 100	Yes, 100
2 Southern elephant seal	Yes, 93	Yes, 99
3 California sea lion	Yes, 100	Yes, 100
3 Northern fur seal + Steller sea lion	Yes, 80	No
3 South American sea lion + S. American fur seal	Yes, 99	Yes, 100
3 New Zealand sea lion	Yes, 86	Yes, 100
3 Australian fur seal	Yes, 73	Yes, 100
3 Mediterranean monk seal	Yes, 100	Yes, 100
3 Southern elephant seal	Yes, 100	Yes, 100

contributed some ambiguity to subsequent interpretations of pinniped hookworm systematics. For example, prior to the formal description of *U. hamiltoni*, Baylis (1933) suggested that hookworms from *O. flavescens* were conspecific with nematodes recovered from what he later asserted (Baylis, 1947) was a California sea lion (*Z. californianus*). However, in this 1933 paper, Baylis indicated uncertainty as to whether the source host of these hookworms was a California sea lion or a Steller sea lion. Furthermore, Baylis (1947) suggested that specimens from *Z. californianus* had characteristics intermediate between *U. lucasi* and *U. hamiltoni*. In support of this observation, Dailey and Hill (1970) also concluded that hookworms from California sea lions did not fit the original descriptions of *U. lucasi* or *U. hamiltoni* and instead had an intermediate morphology. This ambiguity and the high degree of overall similarity between *U. lucasi* and *U. hamiltoni* have hindered precise morphological identification of pinniped hookworm species.

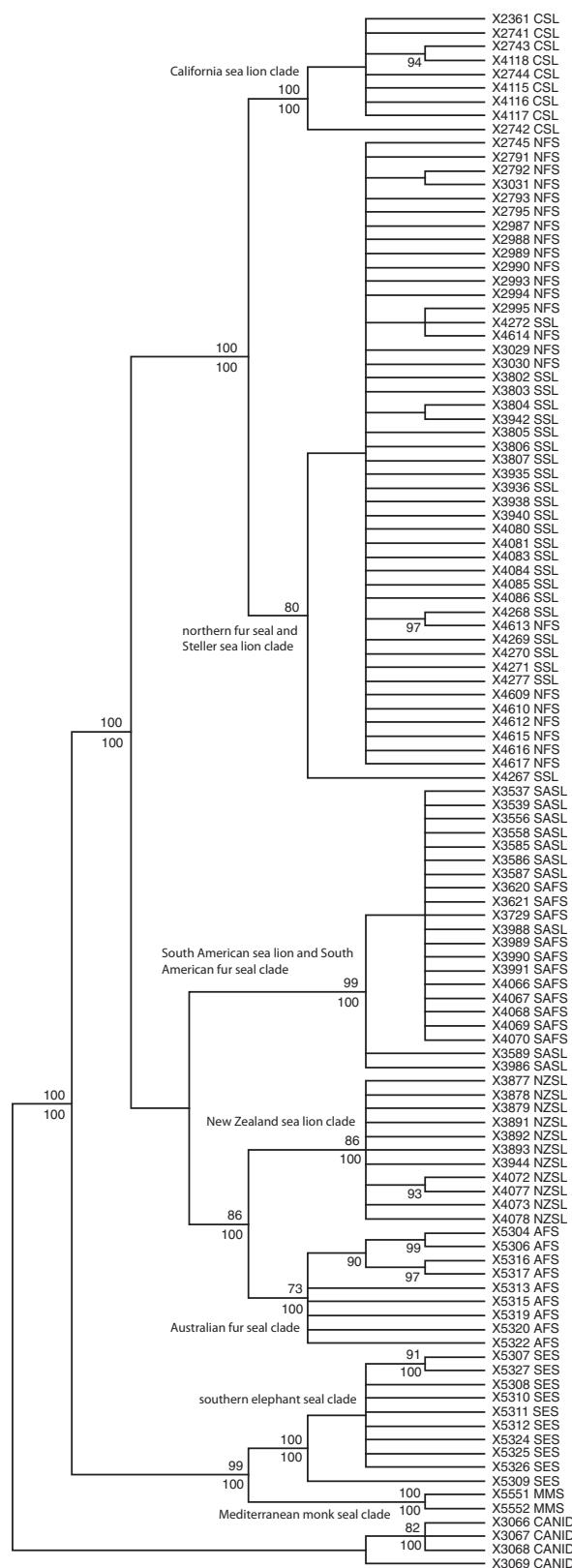
According to Baylis (1947), morphological differences between *U. lucasi* and *U. hamiltoni* include six morphometric and two qualitative features (see Nadler et al., 2000). The qualitative differences reported were the absence of a thickening in the base of the buccal capsule wall for *U. lucasi*, and the absence of tooth-like structures in the border of the capsule in this species (Baylis, 1947). However, interpretation of these structures is difficult, as they appear to vary based on the angle of view (Nadler et al., 2000); for example, the

buccal capsule wall thickening was reported to be present in *U. lucasi* by Olsen (1952). Morphometric differences between these species as originally reported by Baylis (1933, 1947) cannot be evaluated statistically due to small or unknown sample sizes. In addition, Castinel et al. (2006) indicated that measurements once considered important for comparing *Uncinaria* spp. often show very broad ranges, at least in hookworms from New Zealand sea lions. Intraspecific morphometric variation has also been documented, including significant differences in certain measurements within sexes of single species. For example, four of five measurements of male *U. lucasi* from *C. ursinus* hosts collected in two different geographic localities (California and Alaska) showed statistically significant differences (Nadler et al., 2000). Several investigators have concluded that differences in hookworm size are host-induced. Olsen (1952) reported differences in measurements for male and female hookworms from northern fur seals and Steller sea lions, but concluded that both hosts were infected with the same species, *U. lucasi*. The statistical comparison of *U. lucasi* adults from northern fur seals and Steller sea lions herein supports this conclusion, with every measurement significantly different between host-associated worms of each sex. With respect to this host-induced variation, the two-way analysis of variance reveals that increases in female worm size (total body length, buccal capsule width) were disproportionately greater than those for male

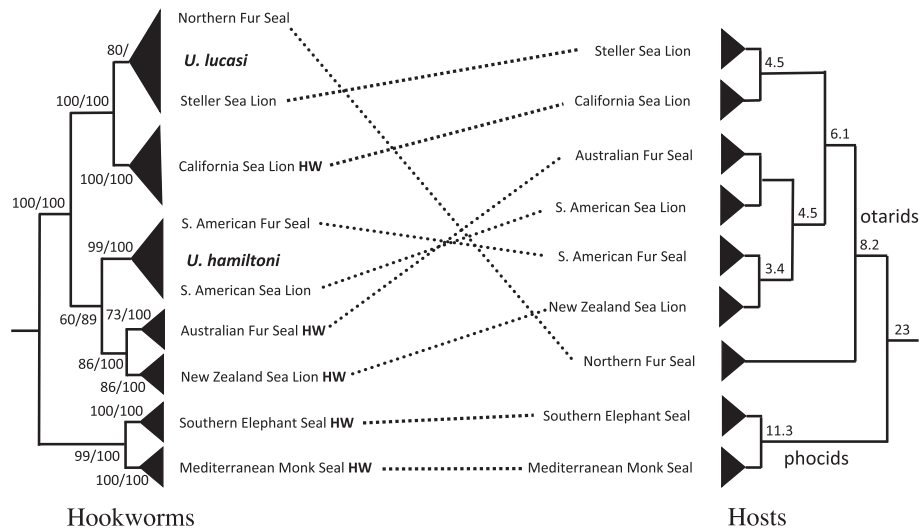
worms. George-Nascimento et al. (1992) also reported differences in measurements between what they inferred to be the same hookworm species (*U. lucasi*) parasitising South American sea lions and South American fur seals; their multivariate (PCA) analyses clearly distinguished hookworms from these hosts, with body size differences accounting for 77% of the total variance. Their results also showed that body size differences between hookworms from these two host species were greater among females than males. The molecular results herein show that although hookworms from South American sea lions and fur seals are the same species, they are not *U. lucasi*. Instead these hookworms, which include specimens from the type host (*O. flavescens*), represent *U. hamiltoni*. Obtaining and sequencing specimens from *O. flavescens* from the type locality would provide additional verification of this result. The presence of host-induced morphometric variation within pinniped hookworm species is inconsistent with the continued use of measurements as a characteristic to discriminate between species. In addition, another potential caveat in comparing morphometrics of adult hookworms from different host species and individuals is size variability among features of mature worms, which appears to be greater in hookworms from southern hemisphere hosts (Castinel et al., 2006).

Hookworms have been reported from several species of otarid pups (fur seals and sea lions), but more rarely from phocids (earless seals). The latter include records from the southern elephant seal (*M. leonina*) and the ringed seal (*Pusa hispida*); hookworms have not been reported from odobenids (walruses) (Dailey, 1975; George-Nascimento et al., 1992; Lyons et al., 2001). Despite these reports, there have been no comprehensive systematic studies of hookworms from pinnipeds. Literature reports have either diagnosed hookworms as *Uncinaria* sp., assigned specimens to one of the two described species (Berón-Vera et al., 2004), or noted that specimens do not fit the description of known species (Dailey and Hill, 1970; Castinel et al., 2006). This potential underestimation of species diversity has led to the suggestion of broad host ranges for *U. lucasi* and *U. hamiltoni*. For example, *U. lucasi* has been ascribed to hookworms from northern fur seals (type host), South American fur seals, Steller sea lions, ringed seals and South American sea lions (Baylis, 1947; Olsen, 1958; Dailey, 1975; George-Nascimento et al., 1992). Likewise, *U. hamiltoni* has been reported from multiple host species including South American sea lions (type host), Steller sea lions (Dailey, 1975), southern elephant seals (Johnston and Mawson, 1945) and Australian sea lions, *Neophoca cinerea* (Beveridge, 1980). Dailey (1975) suggested that the overlapping geographic distribution of South American sea lions and southern elephant seals was responsible for the purported presence of a single hookworm species in both hosts.

Molecular systematic approaches have great potential for discovering and delimiting parasite species, especially those that are difficult or impossible (cryptic species) to distinguish based on morphology (Nadler, 1990; Vilas et al., 2005; Pérez-Ponce de León and Nadler, 2010; Nadler and Pérez-Ponce de León, 2011). Nucleotide sequences represent the most promising source of molecular information for detailed assessments of nematode biodiversity (Baldwin et al., 1999; De Ley, 2000). Species can be delimited using sequence data to test for evidence of independent evolutionary lineages, that is, non-reticulate or phylogenetic relationships among individuals (Adams, 1998, 2002; Nadler et al., 2000; Nadler, 2002), and this is best achieved through use of rapidly evolving gene regions and multiple loci (Nadler and Pérez-Ponce de León, 2011). In this research we applied evolutionary (tree-based) approaches to test the null hypothesis of a single *Uncinaria* species (see Pérez-Ponce de León and Nadler, 2010; Nadler and Pérez-Ponce de León, 2011); the null hypothesis was rejected based on combined analysis of two loci, and the



**Fig. 1.** Maximum parsimony strict consensus tree inferred from the combined dataset (mitochondrial 12S, ITS1–5.8S–ITS2, D2–D3 lsrDNA, D18–D19 lsrDNA). Parsimony bootstrap values  $\geq 70\%$  are shown above internal nodes. Bayesian posterior probabilities  $\geq 90\%$  are shown below internal nodes. Abbreviations indicate the host source of the hookworms: California sea lion, northern fur seal, South American sea lion, New Zealand sea lion, Australian fur seal, southern elephant seal, Steller sea lion, South American fur seal, Mediterranean monk seal, *Uncinaria stenocephala* (canid hookworm) outgroup. The type host of *Uncinaria lucasi* is the NFS, and the type host of *Uncinaria hamiltoni* is the South American sea lion.



**Fig. 2.** Associations between host and parasite species in a phylogenetic context. *Uncinaria* tree depicts relationships among species lineages (triangles) from maximum parsimony and Bayesian inference analyses. Numbers at nodes list maximum parsimony bootstrap values followed by posterior probabilities from BI. The known species *Uncinaria lucasi* and *Uncinaria hamiltoni* are labelled; host associations are shown by dotted lines. The host tree (reduced to show only hosts for hookworms) and divergence times at nodes (million years ago) are redrawn from Higdon et al. (2007).

**Table 5**

Comparison of mean values ( $\pm$ S.E.M.) of morphological measurements from male (M prefix) and female (F prefix) *Uncinaria lucasi* collected from northern fur seal or Steller sea lion pups. Measurements are in micrometers. Means were compared using the pooled *t*-test; statistical information (*T*-ratio, degrees of freedom and Prob > *t*) is provided in the table.

Character	NFS measure	SSL measure	<i>T</i> -ratio	DF	Prob > <i>t</i>
M body length	4741.9 $\pm$ 89.4	8511.2 $\pm$ 602.0	5.43	22	0.0001
M oesophagus to anterior	782.0 $\pm$ 14.3	1288.1 $\pm$ 21.1	18.93	22	0.0001
M oesophagus length	573.6 $\pm$ 16.5	1064.5 $\pm$ 17.8	20.19	22	0.0001
M oesophagus bulb width	107.1 $\pm$ 4.8	172.1 $\pm$ 3.1	12.36	22	0.0001
M width at E/I junction	186.2 $\pm$ 7.7	302.9 $\pm$ 13.6	6.96	22	0.0001
M buccal capsule length	184.6 $\pm$ 7.8	220.1 $\pm$ 3.0	5.02	22	0.0001
M buccal capsule width	149.0 $\pm$ 3.2	161.6 $\pm$ 3.5	2.63	22	0.0154
M spicule length 1	469.3 $\pm$ 6.1	611.0 $\pm$ 10.4	11.21	22	0.0001
M spicule length 2	449.3 $\pm$ 6.8	575.4 $\pm$ 16.7	6.67	16	0.0001
F body Length	5990.3 $\pm$ 260.1	16313.2 $\pm$ 511.3	16.02	28	0.0001
F oesophagus to anterior	930.5 $\pm$ 47.1	1524.9 $\pm$ 38.7	10.08	28	0.0001
F oesophagus length	756.7 $\pm$ 40.4	1257.8 $\pm$ 34.8	9.62	28	0.0001
F oesophagus bulb width	136.8 $\pm$ 8.5	181.2 $\pm$ 4.9	5.07	28	0.0001
F width at E/I junction	221.5 $\pm$ 11.1	381.5 $\pm$ 12.3	9.09	28	0.0001
F buccal capsule length	210.5 $\pm$ 6.3	270.2 $\pm$ 7.2	6.06	28	0.0001
F buccal capsule width	187.9 $\pm$ 5.6	219.6 $\pm$ 4.8	4.38	28	0.0002
F vulva to posterior end	2243.1 $\pm$ 196.3	6759.0 $\pm$ 266.8	12.41	27	0.0001

E/I, oesophagus/intestine.

individuals compared were discovered to represent seven different reciprocally monophyletic groups or species. Formal description and naming of these species must await acquisition of additional specimens permitting detailed morphological comparisons of both sexes. Such integrative molecular and morphological studies are necessary to understand whether these species should be formally described as cryptic (Pérez-Ponce de León and Nadler, 2010) or, conversely, if non-morphometric features serve to differentiate some species for differential diagnosis.

Previous molecular systematic studies of pinniped hookworms (Nadler et al., 2000; Nadler, 2002; Ramos et al., 2013) have used nuclear rDNA sequences, or nuclear rDNA and mtDNA sequences (Nadler, 2002) to test the hypothesis of separate species. Phylogenetic analysis of sequence data (Nadler et al., 2000; Nadler, 2002) provided strong support for two distinct host-associated species of pinniped hookworms, one from northern fur seals and another from California sea lions. In another study based on nuclear rDNA (Ramos et al., 2013), *Uncinaria* specimens from three southern

hemisphere host species (Australian fur seals, Australian sea lions, New Zealand fur seals) were found to be almost identical in sequence for the ITS-1 and ITS-2 genes, but different from hookworms parasitising southern elephant seals, northern fur seals and California sea lions. These results suggest the presence of two previously undescribed hookworm species in these four southern hemisphere pinnipeds. An improvement in the current study is that specimens representing *U. lucasi* and *U. hamiltoni* were available for study, in addition to specimens from many of the pinniped host species from which they have been reported. These specimens included hookworms from the topotype locality for *U. lucasi*. Hosts for which hookworms have been reported, but are not sampled here include the Juan Fernandez fur seal, *Arctocephalus philippii*, (Sepúlveda, 1998), ringed seal, *P. hispida* (Dailey, 1975), Australian sea lion, *N. cinerea* (Beveridge, 1980), New Zealand fur seal, *Arctocephalus forsteri* (Beveridge, 1980), northern elephant seal, *Mirounga angustirostris* (Dailey, 2001), and possibly the Hawaiian monk seal, *Monachus schauinslandi*. However, it is

likely that other pinniped host species may have unreported hookworm infections because diagnosis requires necropsy of pups or examination of their faeces for eggs (Olsen and Lyons, 1965). For example, the *Uncinaria* sp. from the Mediterranean monk seal (*M. monachus*) used for molecular systematics is a new host record.

Phylogenetic analyses of nuclear and mitochondrial sequences for these *Uncinaria* individuals resolved seven hookworm species among the nine pinniped host species from which specimens were collected. The results showed only minor sensitivity to the method of phylogenetic inference. The finding that MP and BI for the same data yielded somewhat different results is not unexpected, given the different assumptions of these methods, differences in sequence characters used by each method (e.g., aligned sites that include gaps are not used in BI), and the complexity of the substitution models, partitioned by gene for BI (maximum likelihood framework) versus equal costs of nucleotide changes in unweighted parsimony. These differences in interpretation also apply to comparing results of bootstrap resampling versus BPP (Alfaro et al., 2003). For the nuclear rDNA dataset, MP analysis recovered one clade (*U. lucasi* from both northern fur seals and Steller sea lions) that was not resolved in the Bayesian analysis. This result for BI was also recovered for the combined nuclear and mitochondrial datasets, however the combined data are dominated by nuclear characters (81% of total). In addition, BI of mitochondrial sequences recovered one clade of host-associated nematodes (Australian fur seal, *Uncinaria*) that was not resolved by MP analysis of these data. Combined analysis of nuclear and mitochondrial sequences (total evidence analysis) provided increased MP bootstrap support for clades already resolved by one or both loci, sometimes markedly so. Similarly, most BPP were increased in the combined analysis when compared with analyses for individual loci. Phylogenetic analysis of the nuclear rDNA data provided greater resolution than the 12S mtDNA data, even though the latter showed greater pairwise sequence divergence and a larger number of parsimony informative sites per aligned sequence character. The lower consistency index of the mtDNA dataset suggests that this difference in resolution is due to greater homoplasy in the 12S dataset compared with nuclear rDNA.

Testing hypotheses of species based on molecular sequences and phylogenetic analysis benefits from the use and comparison of results representing separate loci. However, when time since speciation is relatively short it is unlikely that any one locus will yield shared-derived characters delimiting every species lineage, which argues in favour of the combined analysis of data, provided that different loci do not show major conflicts (Nadler and Pérez-Ponce de León, 2011). In theory, mtDNA genes should be of great value for testing hypotheses of species, due to both a rapid rate of evolution in nematodes (Thomas and Wilson, 1991) and maternal inheritance; the latter results in smaller effective population size than for nuclear genes, so that mtDNA polymorphisms should achieve reciprocal monophyly in descendent species more quickly than autosomal loci (Avise, 1994). For these *Uncinaria* spp., 12S mtDNA does show evidence of a higher evolutionary rate (compared with nuclear rDNA sequences for the same taxa, e.g., Table 2), but this greater level of sequence divergence does not yield greater phylogenetic resolution. One possible explanation is that much more nuclear data (2,405 characters) was obtained than mtDNA sequence (547 characters); accordingly, the number of parsimony informative sites for nuclear rDNA (124) is greater than for mtDNA (91).

Separate analyses of nuclear and mitochondrial sequence datasets provide independent support for most, but not all, of the clades recovered in combined analyses of sequences. For example, nuclear rDNA sequences recovered six or all seven clades (depending on the method of inference). In contrast, mtDNA sequences

recovered a maximum of five of these same clades, and 12S sequences do not provide independent evidence of monophyly for *U. lucasi* or for hookworms from New Zealand sea lions. There were seven minor yet strongly supported sub-clades (consisting of two to four individual hookworms) nested within species lineages resolved by the combined dataset. Four of these intra-specific sub-clades were also resolved by the mtDNA sequences and are consistent with mtDNA haplotype structure within species. One of these sub-clades was composed of two *U. lucasi*, one from a northern fur seal (St. Paul Island, USA and another from a Steller sea lion (Iony Island, Russia), indicating that mtDNA polymorphisms are shared between *lucasi* from these hosts. Investigation of haplotypes, particularly as revealed by more variable mtDNA genes, should be useful for assessing phylogeographic structure within *Uncinaria* spp.

In the absence of topological differences between trees representing different loci, there is little controversy regarding the value of combining datasets for phylogenetic analysis (Kluge, 1998). The combined analysis dataset result differs only slightly between parsimony and Bayesian trees, with the former resolving seven host-associated clades of *Uncinaria* with moderate to high bootstrap support, and the latter resolving six of these same groups, all with high posterior probability. In parsimony analysis of combined data, hookworms from northern fur seals and Steller sea lions were resolved as members of a single evolutionary lineage or species. The northern fur seal hosts included individuals from a broad range of their breeding populations, including a Russian population (Commander Islands), Channel Islands, California, and the type locality of *U. lucasi*, St. Paul Island, Alaska. Similarly, *Uncinaria* sp. from Steller sea lions in this analysis included individuals collected from different geographic regions, including hosts from Russia (Sea of Okhotsk) and two rookeries from south-eastern Alaska. Comparison of these sequences shows that northern fur seals and Steller sea lions are parasitised by the same species, *U. lucasi*, supporting what Olsen (1952) had concluded based on morphology, despite differences in the size of hookworms infecting these hosts.

Hookworms from South American sea lions and South American fur seals were resolved as members of a single evolutionary lineage with very high bootstrap support and BPP. South American sea lions are the type host of *U. hamiltoni* and the hosts of these specimens were obtained from two localities, Uruguay and Argentina (northern Patagonia); however, specimens were not available from the type locality (Falkland Islands). Nevertheless, these molecular results show that South American sea lions and fur seals host the same hookworm species, *U. hamiltoni*, at least for these geographic samples. The finding of a single species of *Uncinaria* in South American sea lions and fur seals is in agreement with the morphologically based conclusions of George-Nascimento et al. (1992).

In addition to documenting host associations for *U. lucasi* and *U. hamiltoni*, phylogenetic analyses of combined sequence data revealed evolutionary lineages representing five additional, previously unrecognised species of *Uncinaria*, each from a different host species. Levels of clade support for these five lineages were high by bootstrap parsimony analysis and BPP, with the exception of the bootstrap value for Australian fur seal hookworms (73%). One of these lineages, *Uncinaria* sp. from California sea lions had previously been recognised through phylogenetic analysis of sequence data (Nadler et al., 2000; Nadler, 2002). In addition, Dailey and Hill (1970) suggested that hookworms from this host species did not fit the morphological descriptions of *U. lucasi* or *U. hamiltoni*, and that this nematode might be a different species. Specimens from New Zealand sea lions were previously studied morphologically and determined to be different in comparison with *U. lucasi* and *U. hamiltoni* (Castinel et al., 2006). In total, these

molecular findings have revealed or corroborated the existence of five new species of *Uncinaria* that require formal description, including taxa from the California sea lion, the New Zealand sea lion, the Australian fur seal, the Mediterranean monk seal and the southern elephant seal.

In addition to delimiting *Uncinaria* individuals from these pinniped hosts as species, the combined data provided robust resolution for most phylogenetic relationships among these species. There was very reliable support for two separate clades of *Uncinaria* spp., one including all hookworms from otarid hosts and the other including the two described species from phocid hosts (Fig. 2). Within the otarid clade, there were two sub-clades separated according to geographic distribution of the host species. Hookworm species from northern hemisphere hosts (*U. lucasi* and the unnamed *Uncinaria* sp. from California sea lions) formed a strongly supported clade. Hookworm species from southern hemisphere otarids (*U. hamiltoni* plus two unnamed *Uncinaria* spp., one each from the Australian fur seal and the New Zealand sea lion) formed a clade, but one that was not as strongly supported by bootstrap resampling or BPP. Within the southern hemisphere otarid clade, the two *Uncinaria* spp. from hosts in Oceania (the Australian fur seal and the New Zealand sea lion) were monophyletic with strong support. These results suggest that biogeography has been an important factor in the evolution of pinniped hookworms.

The strongly supported phylogenetic separation of the otarid *Uncinaria* spp. into northern versus southern hemisphere clades is not unique among parasites of pinnipeds. This history has been found for ascaridoid nematodes and cestodes infecting these hosts; repetition of this pattern among independent parasite groups is consistent with a common causal history, including speciation in potential geographic refugia for hosts and parasites (Hoberg, 1992; Hoberg and Adams, 2000; Hoberg and Klassen, 2002). Phylogenetic investigation of additional phocid hookworm lineages is required to determine whether this clade is also divided into northern and southern hemisphere subclades. Multi-gene molecular phylogenies and associated divergence times for pinnipeds (Higdon et al., 2007) show that the separation between Phocidae and Otarioidea occurred 23 million years ago, (mya) whereas many of the divergence events within the Otariidae are much more recent, mainly within the last 5 million years. There is no independent time calibration to calculate divergence dates for *Uncinaria* parasites of pinnipeds, but it is noteworthy that even the most rapidly evolving gene sequenced (12S mtDNA) shows relatively low levels of pairwise divergence (1–4%) between sister species of hookworms from otarid hosts. Interpretation of this observation depends on the rate of sequence evolution for these genes. However, one possibility is that speciation in these hookworms is quite recent, even postdating the relatively recent speciation of their hosts. Although it is likely that both vicariance and dispersal have been involved in the evolution of the hookworm–pinniped system, the potential disparate ages of these host–parasite biotas in combination with the general absence of cospeciation suggest a larger role for taxon pulses and ecological fitting (Hoberg and Brooks, 2010) in diversification of this host–parasite assemblage.

The comprehensive multi-gene phylogenetic hypothesis for pinniped species (Higdon et al., 2007) is useful as a predictive framework for examining host–hookworm relationships. However, analytical comparative analyses are complicated by the occurrence of the two widespread (i.e., present on multiple host species) hookworms, *U. lucasi* and *U. hamiltoni*, and the relatively small number of *Uncinaria* spp. that have been reported (Dailey, 1975) among the 34 species of extant pinnipeds. For example, the two hookworm species from different phocid host

species are sister taxa in the phylogeny. However, without additional sampling of phocid hosts and hookworms, it is not possible to assess whether this pattern reflects cospeciation or simply phocid hookworm monophyly relative to parasites from otariids. In certain instances, *Uncinaria* spp. relationships have been inferred in cases where there is strong phylogenetic evidence for host species relationships (Fig. 2). The hypothesis of host–parasite cophylogeny makes specific predictions about hookworm relationships. For example, California sea lion and Steller Sea lion hosts are strongly supported as sister taxa in phylogenetic analyses (of 50 genes) for pinnipeds (Fig. 2). Cophylogeny predicts these pinnipeds should host sister-species of hookworms. In the molecular phylogenetic tree for *Uncinaria*, the California sea lion *Uncinaria* sp. is sister to *U. lucasi*, and the latter parasite is currently found associated with two host species (northern fur seals and Steller sea lions). Given the predictive hypothesis of co-speciation, the molecular phylogenies suggest that Steller sea lions are the original host for *U. lucasi*, with a subsequent host-switch to northern fur seals. This implies that any *Uncinaria* sp. originally present in northern fur seals (prior to colonisation by *U. lucasi*) has either not been sampled or became extinct, perhaps as a result of subsequent interspecific competition. According to molecular data, Steller and California sea lions diverged approximately 4.5 mya, whereas the divergence of the northern fur seal dates to the most recent common ancestor of the otarids, approximately 8.2 mya (Higdon et al., 2007). Genetic divergence between *U. lucasi* and its sister species from the California sea lion is low, a result consistent with a more recent speciation event and the cophylogenetic hypothesis. Interestingly, the statistical analysis of *U. lucasi* shows marked host species-associated size differences for both sexes, with *U. lucasi* individuals from Steller Sea lions significantly larger. This result suggests that growth in adult *U. lucasi* is reduced in northern fur seals, the host hypothesised to represent the more recent (colonisation) association. This size differential (total body length) is greater in females than males, which predicts greater fecundity per female worm in *U. lucasi* from Steller Sea lions. If true, host-related intraspecific differences in hookworm fecundity would be expected to have fitness consequences for the parasite.

Developing hypotheses for hookworm–host relationships for southern hemisphere otarids is more difficult. The relationship between South American fur seals and the New Zealand sea lions hosts is strongly supported by molecular data, not as sister-species per se, but as members of a larger clade (but only these two host species are represented by hookworm parasites in the present study). However, the *Uncinaria* phylogeny shows that hookworms from these two hosts do not share a most recent common ancestor, and instead the hookworm species parasitising Australian fur seals and New Zealand sea lions are sister taxa. This incongruence, together with the presence of *U. hamiltoni* in both South American fur seals and South American sea lions, is consistent with host-switching events rather than cophylogeny. It is of note that hookworm relationships for parasites of southern hemisphere otarids reflect the current geographic distribution of their pinniped hosts, a result also consistent with host-switching. Although additional species of *Uncinaria* from pinnipeds need to be discovered to permit a more detailed comparative analysis of host–parasite cophylogeny, at face value the known host–parasite associations, including other evidence of shared species (Ramos et al., 2013), suggest that host-switching events are important. Advancing beyond simple visual comparisons of host and parasite phylogenies, to application of formal analytical methods for developing detailed hypotheses of host–parasite associations (cospeciation,

colonisation), will require continued use of genetic tools to both discover and delimit species of pinniped hookworms and to estimate their evolutionary relationships.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2013.08.006>.

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Supplementary Table S1. Measurements of 18 female *Uncinaria lucasi* specimens from Steller sea lion (SSL) hosts.

Individual hosts of nematodes are indicated in column headers. Measurements are in micrometers.

Measurement	Host 1 F1	Host 1 F2	Host 1 F4	Host 1 F5	Host 1 F6	Host 1 F7	Host 1 F8
Body length	18,417	16,411	11,316	15,871	15,345	15,751	12,100
Vulva to posterior end	7408	6890	3764	6675	6301	6712	4539
Esophagus base to anterior	1492	1614	1168	1794	1458	1383	1285
Esophagus length	1225	1299	994	1462	1161	1075	1044
Esophagus bulb width	179	168	175	173	155	167	145
Width at esophagus/intestine junction	350	386	286	462	442	438	271
Buccal capsule length	267	314	268	332	297	303	237
Buccal capsule width	214	225	205	262	247	236	247
Egg length	118	130	131	120	125	129	122
Egg width	81	77	82	78	80	63	82
Tail length	na	322	264	281	247	264	251

na, measurement not available.

Supplementary Table S1. Measur  
Individual hosts of nematodes are

Measurement	Host 1 F9	Host 4 F1	Host 4 F2	Host 4 F3	Host 4 F4	Host 4 F5	Host 4 F6
Body length	17,880	17,864	16,998	17,255	15,914	15,571	18,387
Vulva to posterior end	7471	7550	7166	6974	6651	6298	8179
Esophagus base to anterior	1459	1606	1477	1528	1665	1686	1667
Esophagus length	1184	1323	1208	1249	1396	1428	1433
Esophagus bulb width	224	183	214	184	177	183	184
Width at esophagus/intestine junction	351	417	376	352	397	422	423
Buccal capsule length	275	283	269	265	262	251	234
Buccal capsule width	211	234	175	216	218	210	208
Egg length	126	115	132	125	129	118	121
Egg width	81	76	60	78	81	68	81
Tail length	239	247	278	339	202	260	339

na, measurement not available.

Supplementary Table S1. Measur  
Individual hosts of nematodes are

Measurement	Host 4 F7	Host 4 F8	Host 4 F9	Host 4 F10
Body length	18,574	17,189	14,181	18,614
Vulva to posterior end	7745	7203	6339	7797
Esophagus base to anterior	1656	1384	1456	1670
Esophagus length	1406	1124	1245	1384
Esophagus bulb width	178	218	174	181
Width at esophagus/intestine junction	395	354	374	371
Buccal capsule length	250	260	211	286
Buccal capsule width	220	204	208	212
Egg length	130	108	118	120
Egg width	79	72	79	68
Tail length	342	249	240	357

na, measurement not available.

Supplementary Table S2. Measurements of 14 male *Uncinaria lucasi* specimens from Steller sea lion (SSL) hosts. Individual hosts of nematodes are indicated in column headers. Measurements are in micrometers.

Measurement	Host 1 M1	Host 1 M2	Host 1 M3	Host 1 M4	Host 1 M5	Host 1 M6	Host 1 M7	Host 1 M8	Host 4 M1	Host 4 M2	Host 4 M3	Host 4 M4	Host 4 M5	Host 4 M6
Body length	9587	9237	8294	9717	9111	8681	8637	8874	10026	9385	9158	8228	9033	1189
Esophagus base to anterior	1227	1212	1304	1202	1290	1435	1443	1284	1291	1205	1276	1286	1238	1341
Esophagus length	1001	937	1069	1061	1061	1105	1219	1067	1059	1042	1048	1070	1025	1139
Esophagus bulb width	159	155	168	171	164	167	166	181	176	183	166	187	171	196
Width at esophagus/intestine junction	298	267	294	283	336	269	356	386	296	251	322	343	343	197
Buccal capsule length	212	220	207	241	229	230	224	217	232	211	228	216	213	202
Buccal capsule width	164	159	171	140	178	141	148	156	161	163	184	169	159	169
Spicule 1 length	634	652	628	629	601	520	658	603	663	591	577	602	599	597
Spicule 2 length	618	619	na	624	581	490	628	527	na	na	na	553	595	519

na, measurement not available.

Supplementary Table S3. Measurements of 12 female *Uncinaria lucasi* specimens from northern fur seal (NFS) hosts. Individual hosts of nematodes are indicated in column headers. Measurements are in micrometers.

Measurement	Host 2 F1	Host 2 F2	Host 2 F3	Host 2 F4	Host 2 F5	Host 2 F6	Host 2 F7	Host 2 F8	Host 2 F9	Host 2 F10	Host 2 F11	Host 2 F12
Body length	7593	5004	6320	4970	4876	5892	6214	7346	5862	5889	6361	5556
Vulva to posterior end	na	1854	2372	1883	1469	3797	2314	2681	2407	1776	2187	1934
Esophagus base to anterior	1228	882	935	1020	615	1033	790	1082	954	892	926	809
Esophagus length	1001	674	637	914	733	806	544	831	805	755	805	575
Esophagus bulb width	211	118	120	151	144	156	104	134	140	125	115	123
Width at esophagus/intestine junction	313	185	192	na	194	231	204	243	245	215	223	192
Buccal capsule length	228	188	225	218	171	212	208	209	185	245	211	226
Buccal capsule width	168	193	210	212	173	203	174	171	161	203	181	206
Egg length	na	na	na	na	na	na	84	na	na	na	93	na
Egg width	na	na	na	na	na	na	46	na	na	na	55	na
Tail length	201	154	174	164	163	156	163	187	190	186	194	181

na, measurement not available.

Supplementary Table S4. Measurements of 10 male *Uncinaria lucasi* specimens from northern fur seal (NFS) hosts. Individual hosts of nematodes are indicated in column headers. Measurements are in micrometers.

Measurement	Host 2 M1	Host 2 M2	Host 2 M3	Host 2 M4	Host 2 M5	Host 2 M6	Host 2 M7	Host 2 M8	Host 2 M9	Host 2 M10
Body length	5155	4937	4958	4815	4246	4816	4809	4479	4497	4707
Esophagus base to anterior	812	820	781	793	775	808	838	692	757	744
Esophagus length	635	612	475	610	551	630	537	549	561	576
Esophagus bulb width	128	116	104	133	93	99	104	104	87	103
Width at esophagus/intestine junction	187	196	177	219	197	218	147	171	161	189
Buccal capsule length	177	191	187	183	171	178	237	182	196	144
Buccal capsule width	140	158	147	170	143	154	139	142	145	152
Spicule 1 length	481	505	468	447	462	478	457	466	458	471
Spicule 2 length	454	483	446	443	461	460	430	na	416	na

na, measurement not available.