Detection of *Cryptosporidium* sp. in Two New Seal Species, *Phoca vitulina* and *Cystophora cristata*, and a Novel *Cryptosporidium* Genotype in a Third Seal Species, *Pagophilus groenlandicus*, from the Gulf of Maine

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DETECTION OF *CRYPTOSPORIDIUM* SP. IN TWO NEW SEAL SPECIES, *PHOCA VITULINA* AND *CYSTOPHORA CRISTATA*, AND A NOVEL *CRYPTOSPORIDIUM* GENOTYPE IN A THIRD SEAL SPECIES, *PAGOPHILUS GROENLANDICUS*, FROM THE GULF OF MAINE

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ABSTRACT: Data on the geographic distribution and host specificity of *Cryptosporidium* spp. are critical for developing an understanding of likely transmission patterns in nature. During a molecular-based survey of fecal samples from 293 terrestrial and aquatic animals in Maine, USA, we detected *Cryptosporidium* sp. in 11 harbor seals (*Phoca vitulina*), 1 hooded seal (*Cystophora cristata*), and 1 harp seal (*Pagophilus groenlandicus*). None of the terrestrial or freshwater mammal fecal samples or bird samples tested positive for *Cryptosporidium* sp. However, the sequencing results of the small subunit (ssu) rRNA gene indicate that the seals were infected with an undescribed species of *Cryptosporidium*, previously isolated only from ringed seals (*Phoca hispida*) in northern Quebec, Canada. In addition, the *Cryptosporidium* sp. detected in the harp seal is significantly different from the previously observed *Cryptosporidium* sp. in other seals. We confirmed the genetic distinctiveness of this *Cryptosporidium* genotype and the identity of the other *Cryptosporidium* sp. seal ssu rRNA sequences by using data from the 70-kDa heat shock protein gene. Based on phylogenetic reconstructions of both genes, it seems that either *Cryptosporidium canis* or *C. felis* are sister species to the seal associated *Cryptosporidium* spp. Our findings extend the range of ''*Cryptosporidium* sp. seal'' well south of the 55th parallel, add other species to the list of seals affected by *Cryptosporidium* sp., and highlight the presence of unrecognized population and potentially species level variation in *Cryptosporidium*.

Cryptosporidium spp. are frequently linked to gastrointestinal disorders in animals, both human and nonhuman. In humans and domesticated animals, the causative agent of cryptosporidiosis is typically *Cryptosporidium parvum* or *C. hominis* (Rider and Zhu, 2008), but other species can cause similar symptoms in their hosts (Traversa et al., 2008). Approximately 21 species are currently recognized in the literature (Fayer, 2010; Robinson et al., 2010). Many of these species are described on the basis of significant morphological differences, genetic differences, or both, whereas others have simply been isolated from a particular host (Fayer, 2010). An important aspect of descriptions of *Cryptosporidium* species is the demonstration of natural and experimental host specificity (Xiao et al., 2004). Further work on the geographic distribution and host affinities of these species is necessary to detect infection patterns that can motivate more mechanistic studies on transmission dynamics. Different host species that share a common *Cryptosporidium* species, or genotype, are more likely to be regularly exchanging pathogens (or to have done so relatively recently in evolutionary history).

Historically, filtration, immunomagnetic separation, and fluorescent antibodies, coupled with microscopic examination, have been used to detect *Cryptosporidium* sp. in water samples (USEPA, 2005), whereas most animal health diagnostic tools use direct immunofluorescence assays. Molecular techniques such as real-time polymerase chain reaction (PCR), traditional PCR, and isothermal amplification techniques have been developed and successfully used to detect and identify species and subtypes of *Cryptosporidium* (Skotarczak, 2010). Molecular methods are generally more sensitive than fluorescence microscopy, and ultimately they provide more detailed information for source tracking studies.

The first reports of *Cryptosporidium* spp. presence in marine mammals were of *C. parvum* in a dugong (*Dugong dugon*) (Hill et al., 1997) and in California sea lions (*Zalophus californianus*) (Deng et al., 2000). Because *C. parvum* is primarily found in domesticated animals and humans, its presence in marine taxa highlighted the potential for transmission between terrestrial and marine environments. Hughes-Hanks et al. (2005) surveyed whales and pinnipeds using an immunofluorescent-based assay and expanded the taxonomic reach of *C. parvum* to bowhead whales (*Balaena mysticetus*), North Atlantic right whales (*Eubalaena glacialis*), and ringed seals (*Phoca hispida*). Molecular techniques provide finer discrimination of *Cryptosporidium* spp., and allowed Santín et al. (2005) to genetically characterize a new seal-associated species of *Cryptosporidium* in ringed seals from northern Quebec, Canada. These results indicated that marine mammals can host more than 1 *Cryptosporidium* species and that more research is needed to fully describe the taxonomic diversity and ecology of this protozoan. To further explore the diversity of this parasite in the northwest Atlantic, we report the results of a survey of *Cryptosporidium* spp. from both terrestrial and aquatic animals in the waters and watersheds of the Gulf of Maine.

MATERIALS AND METHODS

Fecal samples were collected from terrestrial and marine mammals and birds (Table I) during 2010–2011 within an area bounded by latitude 42–46°N and longitude 67–71°W. Samples were stored at −20 C before the application of DNA isolation protocols. We used a modified version of protocols developed by Yu and Morrison (2004) and Salonen et al. (2010). This modified protocol uses a repeated bead beating methodology in conjunction with the inhibitor removal methodology of the QIAmp DNA Stool Mini kit (QIAGEN, Valencia, California). The small subunit rRNA gene (18S) was targeted using a nested PCR approach (Xiao et al., 1999) with the following thermal profile: initial denaturation at 98 C for 30 sec + (98 C for 10 sec, 55 C for 20 sec, and 72 C for 40 sec) × 35 cycles, and a final extension at 72 C for 1 min. The primers (XIAO1F, 5′-TTCTAGAGCTAATACATGCG-3′ and XIAO1R, 5′-CCCTAATCCTTCGAAACAGGA-3′) used in the primary reaction amplified a 1,325 nucleotide (nt) fragment (Xiao et al., 1999). The primary reaction was conducted in a 25-µl volume containing 1× ThermoPol II buffer (New England Biolabs, Ipswich, Massachusetts), 200 µM dNTPs, 4.0 mM Mg^{+}, 0.2 µM of each primer, 5% 20 mg/ml bovine serum albumin, 0.625 units of *Taq* DNA polymerase (New England Biolabs), and 1–2 µl of DNA template. The nested reaction targeted an ~825-nt fragment and differed only in the use of 0.5 µl of the primary reaction template and the primer pair (XIAO2F, 5′-GGAAGGGTTGTATTTATTAGATAAA-3′ and XIAO2R, 5′-AAGGAGTAAGGAACAACCTCCA-3′; Xiao et al., 1999). The 70-kDa heat shock protein (HSP-70) gene was targeted using a nested PCR approach (Morgan et al., 2001) with the following thermal profile: initial

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TABLE I. List of birds, terrestrial or freshwater mammals, and marine mammals tested for *Cryptosporidium* sp. presence during this study. All birds and terrestrial and freshwater mammals were collected from various locations within Maine. All of the marine mammal samples came from animals admitted to the rehabilitation facility at the University of New England MARC except for 126 harbor seal samples that were collected from Mount Desert Rock (MDR), Maine.

Species, common name	No. of individuals	No. positive
Birds	10	0
Anas platyrhynchos, mallard duck	1	—
Bubo scandiacus, snowy owl	1	—
Meleagris gallopavo, turkey	4	—
Branto canadensis, Canada goose	1	—
Gallus gallus, chicken	1	—
Passerculus sandwichensis, savannah sparrow	1	—
Tyto alba, barred owl	1	—
Terrestrial and freshwater mammals	75	0
Canis latrans, coyote	8	—
Felis rufus, bobcat	1	—
Odocoileus virginianus, white-tailed deer	12	—
Lontra canadensis, river otter	1	—
Castor canadensis, beaver	1	—
Capra aegagrus hircus, goat	5	—
Ovis aries, sheep	3	—
Mephitis mephitis, skunk	1	—
Lama glama, llama	1	—
Vulpes vulpes, red fox or *Urocyon cinereoargenteus*, gray fox	5	—
Bos taurus, cow	8	—
Blarina brevicauda, short-tailed shrew	1	—
Peromyscus leucopus, white-footed mouse	25	—
Canis lupus familarus, dog	1	—
Tamias striatus, eastern chipmunk	2	—
Marine mammals	205	13
Pagophilus groenlandicus, harp seal	24	1
Phoca vitulina, harbor seal	50(MARC): 126(MDR)	11
Halichoerus grypus, gray seal	2	—
Cystophora cristata, hooded seal	1	1
Lagenorhynchus acutus, white-sided dolphin	1	—
Phocoena phocoena, harbor porpoise	1	—

denaturation at 98 C for 30 sec + (98 C for 10 sec, 56 C for 20 sec, and 72 C for 40 sec) × 35 cycles, and a final extension at 72 C for 1 min. The primers (HSPF4, 5′-GGTGGTGGTACTTTTGATGTATC-3′ and HSPR4, 5′-GCCTGAACCTTTGGAATACG-3′) used in the primary reaction amplified a 448-nt fragment (Morgan et al., 2001). The primary reaction was conducted in a 25-µl volume containing 1× ThermoPol II buffer (New England Biolabs), 200 µM dNTPs, 2.0 mM Mg^{+}, 0.5 µM of each primer, 4% 20 mg/ml bovine serum albumin, 0.625 units of *Taq* DNA polymerase (New England Biolabs), and 3 µl of DNA template. The nested reaction targeted a 325-nt fragment and differed only in the use of 0.5 µl of the primary reaction template and the primer pair (HSPF3, 5′-GCTGSTGATACTCACTTGGGTGG-3′ and HSPR3, 5′-CTCTTGT-CCATACCAGCATCC-3′; Morgan et al., 2001). To ensure reaction integrity, we included both positive and negative controls in both the 18S and HSP-70 sets of reactions. Putative *Cryptosporidium* sp. 18S and HSP-70 fragments were cleaned using an ExoSAP protocol before sequencing at Macrogen Corporation (Rockville, Maryland).

Both forward and reverse sequences were generated and aligned to generate a consensus sequence within Sequencher version 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan). Each sequence was then checked for homology with other sequences using NCBI/MegaBlast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the nucleotide query. Similar sequences and others of interest were downloaded for further analysis. Putative 18S sequences were aligned using T-Coffee (http://www.tcoffee.org; Notredame et al., 1998; Tommaso et al., 2011), and changes within hypervariable regions were minimized by eye using MacClade version 4.08 (Maddison and Maddison, 2003). HSP-70 sequences were aligned using Sequencher version 4.10.1 (Gene Codes Corporation).

Evolutionary relationships among, and within, species were initially described using MEGA5 version 5.02 (Tamura et al., 2011), and an appropriate model of DNA substitution was selected using the Akaike Information Criterion within MEGA5 for the individual 18S and HSP-70 data sets. Both the maximum likelihood (ML) and neighbor-joining (NJ) options within MEGA5 were used to reconstruct evolutionary relationships. Nodal support was estimated using 500 and 1,000 bootstraps, respectively (Felsenstein, 1985). A third phylogenetic reconstruction was conducted using a Bayesian approach as implemented in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). For the Bayesian searches, 2 runs of 2 million generations were conducted simultaneously and sampled every 1,000 generations. To assess convergence of the Markov Chain Monte Carlo sampling runs, the average standard deviation of the split frequencies of the 2 runs was inspected and if it was greater than 0.01, an additional 1 million generations were run. This process was repeated until the average standard deviation of the split frequencies of the 2 runs was less than 0.01. To determine the ''burnin'' number, the parameters of all individual runs were summarized, and the initial runs below the asymptote were discarded. In general, only the last 500 samples of generations from both runs were used to determine parameter and posterior probability values.

RESULTS

In total, 293 fecal samples were tested for *Cryptosporidium* presence (Table I). None of the 78 terrestrial or freshwater animals or 10 birds tested positive. Of the 205 samples from marine mammals, 13 tested positive for *Cryptosporidium*. The positive samples originated from 11 harbor seals (*Phoca vitulina*), 1 hooded seal (*Cystophora cristata*), and 1 harp seal (*Pagophilus groenlandicus*). Positive samples included 6 of 50 harbor seals that had stranded and were being rehabilitated at the University of New England's Marine Animal Rehabilitation Center (MARC, Biddeford, Maine) and 5 of 126 harbor seal samples collected from a haul out at Mount Desert Rock in the Gulf of Maine. In total, 24 harp seals were tested; 1 was positive, but the single hooded seal sample in our collection also tested positive.

Our putative 18S sequences were edited and a consensus sequence was generated for each positive sample. The alignment with other *Cryptosporidium* sequences resulted in a region of 855 nt (including gaps) for further analysis. Before conducting any model selection analysis, we conducted a pairwise analysis to determine the number of differences among the sequences (Fig. 1). Eight of the sequences generated from the harbor seal samples and the 1 sequence generated from the hooded seal sample were an exact match to 2 *Cryptosporidium* sequences obtained from ringed seal hosts and designated as *Cryptosporidium* sp. seal 1 and *Cryptosporidium* sp. seal 2 (Santín et al., 2005). In addition, 3 other harbor seal samples exhibited 1–2 nt differences from the aforementioned *Cryptosporidium* sp. seal sequences, whereas the harp seal sample sequence exhibited 7–8 nt differences (Fig. 1). Because of the relatively large number of differences between the harp seal *Cryptosporidium* 18S sequence and all other *Cryptosporidium* 18S seal sequences, we reamplified and resequenced to confirm that we were not amplifying a paralog of 18S. We were able to independently generate 2 other complete matches to the initial harp seal sequence using new DNA isolations from both a fecal and an intestinal sample from the same animal (the affected harp seal had died during rehabilitation, and

Cryptosporidum spp.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *C.* sp. seal 1		14-16	-	1	-	2	1	1	1	1	-	-	-	1	32	51	45	66
2. *C.* sp. seal 2	0		-	13-15	-	14-16	13-15	13-15	13-15	13-15	-	-	-	13-15	34-36	50-52	49-51	69-70
3. Cc444	0	0		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. Pv327	0	0	0		-	0	0	0	0	0	-	-	-	0	30	50	44	64
5. Pv312	0	0	0	0		-	-	-	-	-	-	-	-	-	-	-	-	-
6. Pv278	0	0	0	0	0		0	1	0	0	-	-	-	0	31	51	45	65
7. Pv275	0	0	0	0	0	0		0	0	0	-	-	-	0	30	50	44	64
8. Pv270	1	1	1	1	1	1	1		0	0	-	-	-	0	31	50	44	65
9. Pv268	0	0	0	0	0	0	0	1		0	-	-	-	0	31	50	44	64
10. Pv245	1	1	1	1	1	1	1	2	1		-	-	-	0	30	49	44	63
11. Pv200	0	0	0	0	0	0	0	1	0	1		-	-	-	-	-	-	-
12. Pv151	0	0	0	0	0	0	0	1	0	1	0		-	-	-	-	-	-
13. Pv141	0	0	0	0	0	0	0	1	0	1	0	0		-	-	-	-	-
14. Pv140	1	1	1	1	1	1	1	2	1	2	1	1	1		31	50	44	64
15. Pg453	7	7	7	7	7	7	7	7	7	8	7	7	7	8		50	45	58
16. *C. felis*	33	35	33	33	33	33	33	32	33	34	33	33	33	34	34		51	64
17. *C. parvum*	31	32	34	33	33	33	33	32	33	32	33	33	33	34	34	25		55
18. *C. bovis*	36	36	36	36	36	36	36	35	36	37	36	36	36	37	35	28	32	

FIGURE 1. Number of pairwise differences between seal associated *Cryptosporidium* DNA sequences and select described species (Pv = *Phoca vitulina*, Cc = *Cystophora cristata*, and Pg = *Pagophilus groenlandicus*). Below the diagonal, number of nucleotide differences in 855 nt of 18S gene sequences. Above the diagonal, number of nucleotide differences in ~324 nt of HSP-70 gene sequences. Cells with 2 numbers represent the 2 alleles identified by Santin et al. (2005) for *Cryptosporidium* sp. seal 2. Dashes indicate no gene sequence for that individual.

we were able to collect an intestinal scraping during the necropsy). Further analyses were based only on those sequences that either exhibited some degree of variation or originated from different seal species. Therefore, we included the hooded seal sample sequence in the phylogenetic reconstruction, even though it matched other harbor seal samples, and the 3 harbor seal samples that differed by 1–2 nt. Finally, the harp seal sequence that differed by 7–8 nt also was retained for further analysis.

The evolutionary distances were computed using the Tamura 3-parameter (T92; Tamura, 1992) method with the rate variation among sites modeled as a gamma distribution ($\alpha = 0.81$). Distance comparisons including the seal samples and all other described species of *Cryptosporidium* ranged from 0 to 13.3% for the 18S gene region analyzed. 18S distances within the seal associated sequences ranged from 0 to 1.1%. The same T92 + Γ model was used to run both a NJ and ML analyses of the limited 18S sequences data set, plus previously described genotypes or species. For the Bayesian analysis, 4 million generations were required before the split standard deviation between the 2 runs was <0.01. The resulting consensus phylogram from the Bayesian analysis grouped all seal associated sequences into 1 clade but clearly separated the 18S sequence of the harp seal from the other seal samples as did the NJ analysis (Fig. 2). Of the 3 phylogenetic analyses conducted, only the ML analysis resulted in a slightly different topology in the positioning of the harp seal sequence. In the ML analysis, the harp seal was not distinguishable from the other seal sequences. Only the Bayesian analysis provided strong nodal support for *C. canis* as a sister taxa to the seal associated *Cryptosporidium*. In the ML analysis, the bootstrap support for this node was <60 and the branching order in the NJ analysis placed the seal associated *Cryptosporidium* in a position basal to the large, mostly unresolved *Cryptosporidium* clade containing *C. felis*, *C. parvum*, *C. wrairi*, and others (Fig. 2).

We approached the analysis of the HSP-70 sequences in the same manner as described for the 18S sequences. HSP-70 sequences were generated for 8 of the 13 positive *Cryptosporidium* seal samples. We were not able to amplify a clean HSP-70 fragment from the hooded seal sample, but we were able to amplify HSP-70 fragments from all samples that exhibited nucleotide polymorphisms in the 18S sequences (Fig. 1). Forward and reverse sequences from HSP-70 amplifications were edited, and a consensus sequence was generated for each individual. The HSP-70 seal sequences were easily aligned with the GenBank sequences from other described or undescribed species of *Cryptosporidium*. Pairwise analysis of nucleotide differences indicated that all harbor seal sequences were either exact matches or differed by 1–2 nt from the *Cryptosporidium* sp. seal 1 sequence (Fig. 1). The nucleotide differences of all harbor seal sequences to *Cryptosporidium* sp. seal 2 sequences (isolates 1 and 2) of Santín et al. (2005) were greater, ranging from 13 to 16 nt differences. However, the harp seal HSP-70 sequence differed from all other seal sequences by twice the number of

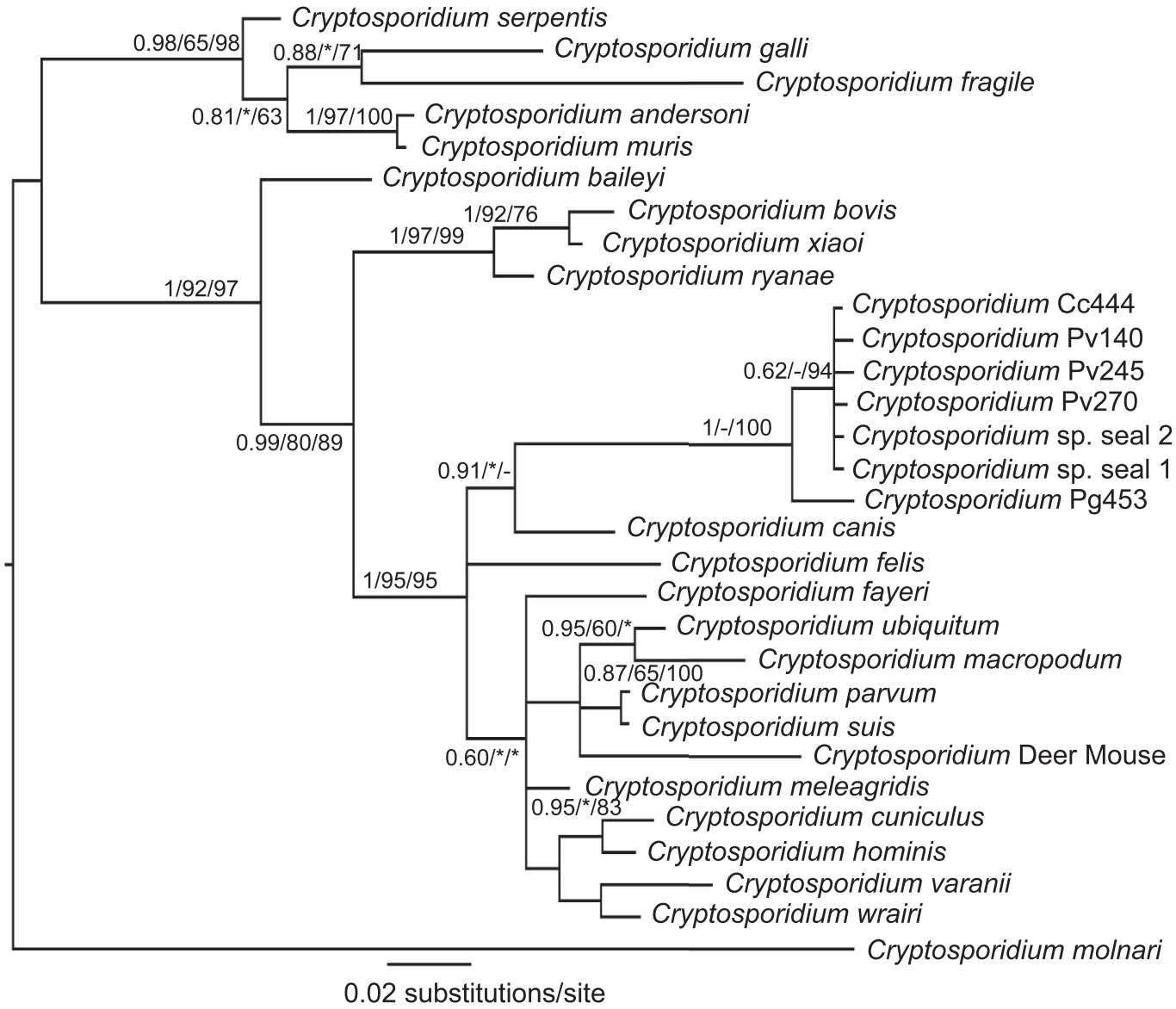

FIGURE 2. Reconstruction of the evolutionary relationships of *Cryptosporidium* spp. plus the isolates from seals sampled in New England inferred using the Bayesian method and 855 nt of the 18S ribosomal gene. New sequences reported here are indicated as *Cryptosporidium* sp. + host species initials (Pv = *Phoca vitulina*, Cc = *Cystophora cristata*, and Pg = *Pagophilus groenlandicus*) + sample number. The tree shown is a Bayesian consensus phylogram. Numbers indicate the nodal support values from 3 separate phylogenetic analyses in the following order: posterior probability value/ML bootstrap value/NJ bootstrap value. Dash (-) indicates different branching order and asterisk (*) indicates bootstrap values < 60. Accession numbers of sequences used in the phylogenetic reconstruction: *Cryptosporidium andersoni* (AY954885), *C. baileyi* (L19068), *C. bovis* (AY120911), *C. canis* (AF112576), *C. cuniculus* (W17211), *C. fayeri* (AF112570), *C. felis* (AF112575), *C. fragile* (EU162751), *C. galli* (HM116388), *C. hominis* (AB369994), *C. macropodum* (AF513227), *C. meleagridis* (AF112574), *C. molnari* (HM243548), *C. muris* (AF093498), *C. parvum* (L16996), *C. ryanae* (AY587166), *C. serpentis* (AF093501), *C. suis* (AF108861), *C. ubiquitum* (AF442484), *C. varanii* (AF112573), *C. wrairi* (AF115378), *C. xiaoi* (FJ896050), *Cryptosporidium* sp. seal 1 (AY731234), *Cryptosporidium* sp. seal 2 (AY731235), *Cryptosporidium* sp. deer mouse (AY120905), *Cryptosporidium* Cc444 (JN858905), *Cryptosporidium* Pv140 (JN858906), *Cryptosporidium* Pv245 (JN858907), *Cryptosporidium* Pv270 (JN858908), and *Cryptosporidium* Pg453 (JN858909).

nucleotide differences (30–36 nt; Fig. 1). Further analysis was based on 2 of the harbor seal sequences and the harp seal sequence.

The ML and NJ reconstructions were computed using a T92 + Γ ($\alpha = 0.25$) model and, as with the 18S analysis, no priors were assumed for the Bayesian analysis. Only 2 million generations was necessary for the average split deviations between the 2 runs to fall below 0.01. The relationship among the HSP-70 sequences is illustrated using a Bayesian consensus phylogram (Fig. 3). Individual branching patterns within the HSP-70 topology do not agree with the 18S topology; however, all seal associated sequences clustered together, with the harp seal sequence clearly separated from other seal sequences in both the ML and Bayesian analyses. The NJ analysis resulted in a bootstrap value <60 for this node (Fig. 3). In addition, *C. felis* or *C. canis* were implicated as sister species to the seal sequences in all 3 analyses, although the branching order was not resolved.

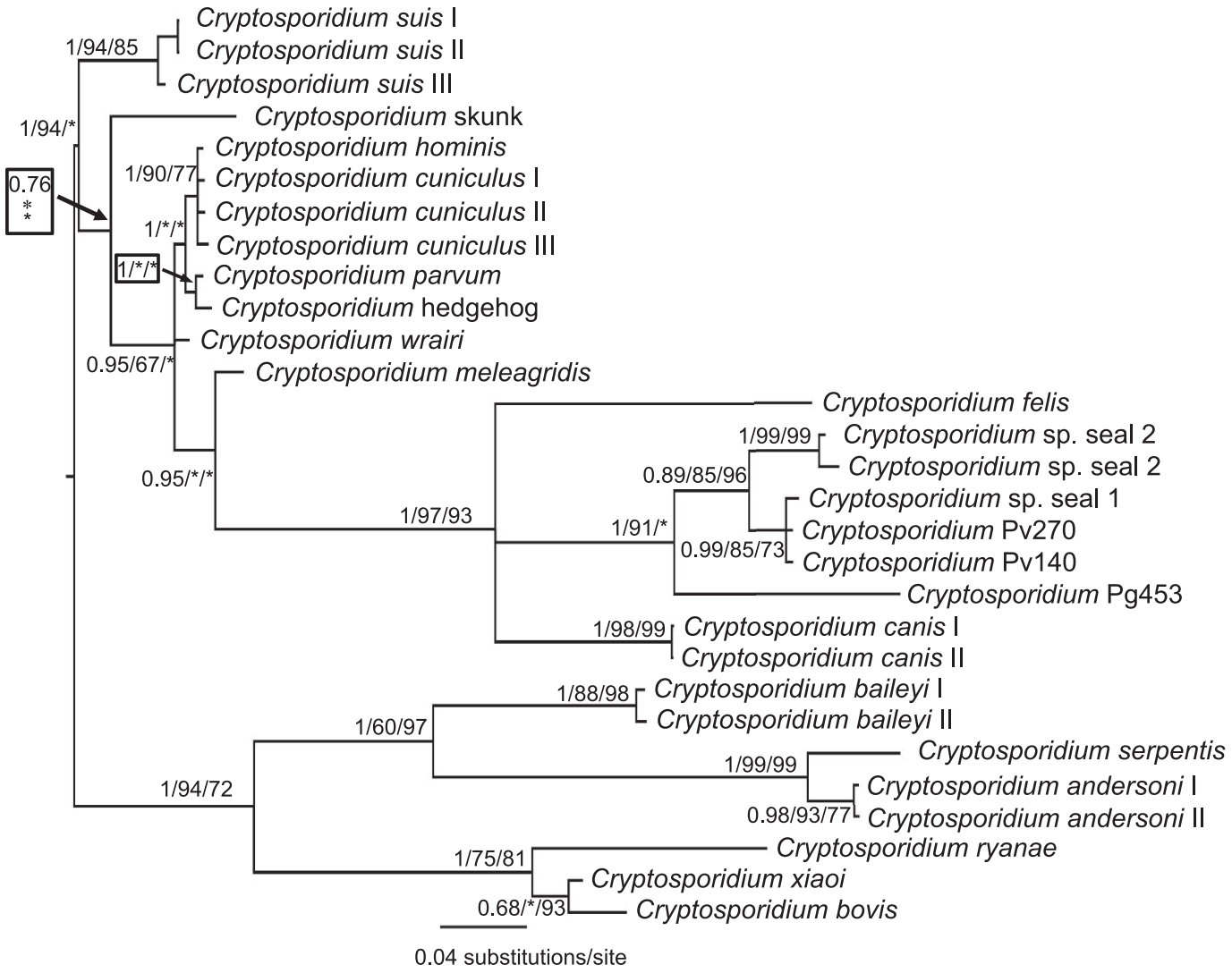

FIGURE 3. Reconstruction of the evolutionary relationships of *Cryptosporidium* spp. plus the isolates from seals sampled in New England inferred using the Bayesian method and 1,857 nt of the HSP-70 gene. New sequences (only ~300–324 nt) reported here are indicated as *Cryptosporidium* sp. + host species initials (Pv = *Phoca vitulina*, Cc = *Cystophora cristata*, and Pg = *Pagophilus groenlandicus*) + sample number. The tree shown is a Bayesian consensus phylogram. Numbers indicate the nodal support values from 3 separate phylogenetic analyses in the following order: posterior probability value/ML bootstrap value/NJ bootstrap value. Dash (-) indicates different branching order and asterisk (*) indicates bootstrap values < 60. Accession numbers of sequences used in the phylogenetic reconstruction: *Cryptosporidium andersoni* I (AJ567390), *C. andersoni* II (AJ567391), *C. baileyi* I (AF221539), *C. baileyi* II (AF316634), *C. bovis* (AY741306), *C. canis* I (AF221529), *C. canis* II (EU754843), *C. cuniculus* I (FJ262728), *C. cuniculus* II (FJ262729), *C. cuniculus* III (GU967462), *C. felis* (AF221538), *C. hominis* (AF221535), *C. meleagridis* (AF221537), *C. parvum* (U69698), *C. ryanae* (EU410346), *C. serpentis* (AF221541), *C. suis* I (AF221533), *C. suis* II (DQ898164), *C. suis* III (DQ833281), *C. wrairi* (AF221536), *C. xiaoi* (FJ896041), *Cryptosporidium* sp. seal 1 (AY731236), *Cryptosporidium* sp. seal 2 I (AY731237), *Cryptosporidium* sp. seal 2 II (AY731238), *Cryptosporidium* sp. skunk EU437414), *Cryptosporidium* sp. hedgehog (GQ259143), *Cryptosporidium* Pv140 (JN860883), *Cryptosporidium* Pv270 (JN860882), and *Cryptosporidium* Pg453 (JN860884).

DISCUSSION

The lack of any *Cryptosporidium* spp. positives in the nonmarine mammal samples was surprising, because 93 cryptosporidiosis cases were reported in Maine in 2010 alone (Maine CDC, 2010 Epidemiology Report). The Maine CDC reported an increasing trend in the incidence of cryptosporidiosis over the past 2 yr. Approximately 40% of the reported cases were from 3 counties, but no pattern of association between location and incidence was indicated. The majority of our samples were collected in the southern, more coastal, York County; this county was fourth in terms of number of cases reported, but still low at 3.5/100,000 persons. The lack of detection in the terrestrial and avian fauna may be an artifact of sample sizes. Because we applied the same molecular techniques to all fecal samples analyzed, we do not believe that the lack of detection was related to our technique.

The phylogenetic analyses of the 18S sequences confirmed that the sequences obtained from harp, harbor, and hooded seals in the New England collections were most closely related, or exact matches, to the genotype(s) of *Cryptosporidium* from ringed seals (Fig. 1; Santín et al., 2005). Our phylogenetic analyses of both 18S and HSP-70 gene fragments indicate that the *Cryptosporidium*

DNA we isolated from the harp was significantly different not only from the other seal associated *Cryptosporidium*, but also from all other described *Cryptosporidium* species. These findings document undescribed variation in *Cryptosporidium* affecting seals and support the findings of Santín et al. (2005) on the presence of undescribed species.

Although we cannot claim that the novel genotype from the harp seal is a new species without information regarding morphology and other traditional metrics of taxonomy (Xiao et al., 2004; Fayer, 2010), we suggest that it is at least a unique genotype of seal associated *Cryptosporidium* on an independent evolutionary trajectory. Other interpretations can be rejected on a variety of grounds. First, it is highly unlikely that we amplified a paralog of the 18S gene because we were able to amplify and generate identical sequences from both fecal and intestinal material and the same amplification reaction yielded examples of the C*ryptosporidium* sp. seal types. Therefore, all comparisons of genetic variation are based on analogous regions. Second, the HSP-70 gene data support the distinctiveness of this genotype with 2–3 times the number of nucleotide differences observed among the previously identified *Cryptosporidium* sp. seal isolates 1 and 2 of Santín et al. (2005). Third, one would expect the degree of variation among 18S sequences to be lower if the harp sequence is the same species as that found among other seals. This conclusion is hindered by an absence of information about population genetic variation in 18S but consistent with our understanding from other taxa. Other species level comparisons result in fewer nucleotide differences than those we have observed. Fourth, we have included variants of HSP-70 identified from other described species, e.g., *C. cuniculus* and *C. bovis*, and the degree of variation found within the seal associated *Cryptosporidium* is significantly higher. Finally, although we cannot discount the possibility of recombination (as observed both in natural populations of *C. parvum* [Mallon et al., 2003] and in the laboratory [Tanriverdi et al., 2007]), we found no evidence in the sequence itself or the resulting phylogenetic analyses. In summary, the genetic distinctiveness of this harp seal associated genotype is significant and warrants further investigation.

Of the multiple phylogenetic reconstructions based on the 18S data set, only the Bayesian analysis of the 18S gene sequences indicated a strongly supported sister relationship with *C. canis* and the seal associated *Cryptosporidium* clade (Fig. 2). Our results concur with those of Santín et al. (2005) in that the HSP-70 gene data suggest a sister relationship with *C. canis* or *C. felis* (Fig. 3) but differ in that branching relationships were ambiguous in our analyses. Uncertainty remains as to the true identity of the sister species to seal associated genotypes, but the 2 options provide a plausible source of *Cryptosporidium* in the marine realm. Relationships within this larger clade containing *C. parvum*, *C. felis*, and others are still unresolved, but an analysis of 3 concatenated genes was able to generate strong bootstrap support for most branching patterns within this troublesome clade (Robinson et al., 2010). Although the seal associated *Cryptosporidium* was not included in the analysis, we predict that it would again group with the canid and felid *Cryptosporidium* species. Other genes, such as actin and COWP, have been used independently to resolve some relationships, but they also do not unambiguously resolve relationships within this clade (Sulaiman et al., 2002; Santín et al., 2005; Traversa et al., 2008). It is likely that the use of more quickly evolving genes, a concatenated approach, or both, such as that of Robinson et al. (2010), are necessary to elucidate evolutionary relationships within this speciose clade. Alternatively, there may have been a rapid diversification that we will simply not be able to resolve.

Based on the analyses reported here, the genotype of *Cryptosporidium* that we isolated from harbor and hooded seals in the Gulf of Maine matches *Cryptosporidium* sp. seal 1 and 2 isolated from ringed seals in northern Quebec, Canada. This is the first documentation of this genotype south of the 55th parallel, i.e., between 42 and 43°N latitude, and the first report of its presence in seals other than ringed seals (Santín et al., 2005). Consequently, we have both extended the distribution and described more hosts of this likely species of *Cryptosporidium*. We also have tested gray seals (*Halichoerus grypus*; n = 2) from the Gulf of Maine, but we did not detect this parasite in our samples. The apparent absence of *Cryptosporidium* sp. in gray seals could be an artifact of a small sample size rather than a lack of infection in this species. Gray seals share some haul out sites with harbor seals and also tend to be more generalist predators; accordingly, gray seals are likely to be exposed to the same *Cryptosporidium* genotypes.

The detection of this *Cryptosporidium* type in additional seal species reveals little about the actual mode of transmission. Because ringed seals are considered more solitary and maintain a greater distance among individuals (Riedman, 1990), it is not likely that they are passing this parasite to other species or to non-related con-specifics by direct contact. Therefore, waterborne transmission or consumption of infected prey seems likely in the marine transmissions. Furthermore, the absence of this (or any other) *Cryptosporidium* species in any of our terrestrial samples argues against frequent transmission between marine and terrestrial hosts in this region. Pending *Cryptosporidium* genotype information from terrestrial animals in other northern areas, we tentatively conclude that *Cryptosporidium* sp. seal is widespread in seals in the northwest Atlantic, but that it is not shared with terrestrial hosts.

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