The seal louse (*Echinophthirius horridus*) in the Dutch Wadden Sea: investigation of vector-borne pathogens

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Abstract

Background: Belonging to the anopluran family Echinophthiriidae, *Echinophthirius horridus*, the seal louse, has been reported to parasitize a broad range of representatives of phocid seals. So far, only few studies focused on vector function of echinophthiriid lice and knowledge on their role in pathogen transmission is still scarce. The current study aims to investigate the possible vector role of *E. horridus* parasitizing seals in the Dutch Wadden Sea.

Methods: More than 1200 *E. horridus* seal lice were collected from 54 harbour seals (*Phoca vitulina*) and one grey seal (*Halichoerus grypus*) during their rehabilitation period in the Sealcentre Pieterburen, the Netherlands. DNA was extracted from pooled seal lice of individual seals for molecular detection of the seal heartworm *Acanthocheilonema spirocauda*, the rickettsial intracellular bacterium *Anaplasma phagocytophilum*, and *Mycoplasma* spp. using PCR assays.

Results: Seal lice from 35% of the harbour seals (19/54) and from the grey seal proved positive for *A. spirocauda*. The seal heartworm was molecularly characterised and phylogenetically analysed for the first time (rDNA, *cox1*). A nested PCR was developed for the *cox1* gene to detect *A. spirocauda* stages in seal lice. *A. phagocytophilum* and a *Mycoplasma* species previously identified from a patient with disseminated 'seal finger' mycoplasmosis were detected the first time in seal lice.

Conclusions: Our findings support the potential vector role of seal lice in transmission of *A. spirocauda*, and reveal new insights into the spectrum of pathogens occurring in seal lice. As these pathogens might have detrimental effects on the health of seal populations further epidemiological investigations on infections due to these pathogens in seals should be conducted.

Background

Representatives of the family Echinophthiriidae belong to the phthirapteran suborder Anoplura, the sucking lice, and exhibit a host range solely infesting semiaquatic mammals, such as pinnipeds (Otariidae, Phocidae, Odobenidae) and the North American river otter (*Lontra canadensis*) [1]. Within the Echinophthiriidae, five genera (*Antarctophthirus, Lepidophthirus*, *Echinophthirius*, *Proechinophthirus*, and *Latagophthirus*) and 13 species are described [1, 2]. While many echinophthiriid species display a strict major host specificity, the seal louse *Echinophthirius horridus* exhibits the broadest host range among echinophthiriid lice parasitizing eight different species of Phocidae, the earless or true seals, with a geographical distribution confined to the Northern Hemisphere, including harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) [1].

Due to their obligate and permanent hematophagous feeding habits [1], members of the Echinophthiriidae have the potential to play an important role in the epidemiology of vector-borne diseases in free-ranging pinniped populations. Nonetheless, studies on possible vector function of echinophthiriid species are scarce, but some of them already highlighted their role in pathogen transmission (Table 1). The bacterium *Salmonella enteritidis* was isolated from blood and tissue of

Northern fur seals (*Callorhinus ursinus*) and from the echinophthiriid lice *Antarctophthirus callorhini* and *Proechinophthirus fluctus* collected from the same individuals. Additionally, these infections were also associated with a mortality event of *C. ursinus* pups [3]. A *Rickettsia* species closely related to the human pathogen *Rickettsia rickettsi* was isolated from *P. fluctus* collected from *C. ursinus* [4]. *Bartonella henselae* was detected in *E. horridus* and spleen samples of dissected harbour seals [5]. Southern elephant seal virus (SESV), an *Alphavirus*, was isolated from the echinophthiriid species *Lepidophthirus macrorhini* parasitizing the Southern elephant seal (*Mirounga leonina*) [6].

Furthermore, *E. horridus* has been suggested to serve as the obligate intermediate host for the seal heartworm *Acanthocheilonema spirocauda*, i. e. *A. spirocauda*-microfilariae (L1), taken up by a blood meal of the louse, develop into infective L3-stages, which are inoculated to seals during the blood meals [7, 8, 9]. The proposed role of *E. horridus* as intermediate host for *A. spirocauda* was supported by the finding of different developmental stages in dissected lice [7, 9] and the significant positive correlation of infestation of seals with *E. horridus* and seal infection with *A. spirocauda* [9, 10].

Echinophthiriid species exhibit special morphological characteristics, which are unique among anopluran lice and are the basis of their evolutionary success being influenced by heterogeneous environmental conditions. Thus, distinct spiracles are equipped with special occlusions allowing for gas exchange. Abdominal segments are characterized by a general loss of sclerotization and by a distinct trimming with modified setae [11, 12], sensorial organs, that are classified into spines, scales and hairs [13]. Furthermore, the last two pairs of legs for fixation to the host surface is another characteristic of echinophthiriid lice. *E. horridus* differs from other members of the family by the absence of abdominal scales and the restructuring of all three pairs of legs into claws and the absence of abdominal scale manifestation [14, 15, 16].

Due to these morphological adaptions and a strict specifity to semiaquatic host species, a coevolution of echinophthiriid lice with their hosts has been suggested [12, 17, 18]. Thereby, it has been proposed that the ancestors of pinnipeds must have already been infested with ancestral sucking lice before they ventured into the marine habitat [8, 19]. As a consequence of their growing specialization to ancestral pinnipeds, ancestors of *E. horridus* lice might have evolved a vector role in the transmission of ancestors of the heartworm *A. spirocauda*, which is also believed to have undergone a coevolution with its host species [8]. Although filarial infections are usually transmitted by mosquitoes and ticks [20], this filarial heartworm nematode is believed to complete its life cycle in the seal louse *E. horridus* [7, 14]. Nevertheless, it cannot be excluded that other intermediate hosts are involved in transmission of *A. spirocauda*, although suitable mosquito or tick vectors parasitizing marine mammals were not reported.

Therefore, the present study aimed to investigate the possible role of *E. horridus* in transmission of pathogens, revealing new insights into the spectrum of potential vector-borne diseases circulating in free-ranging seals in the Dutch Wadden Sea.

Methods

2.1. Sample collection

During routine diagnostics at the Sealcentre Pieterburen (the Netherlands), sucking lice were sampled from 54 hospitalized harbour seals (*Phoca vitulina*) and from one greys seal (*Halichoerus grypus*) admitted along the coast of the Dutch Wadden sea (between 51° 42' 6.8" N, 3° 40' 42.1" E and 53° 27' 57.6" N, 5° 37' 40.8" E) between 6th of May and 16th of August 2012. Thereby, lice of harbour seals were collected opportunistically using lice combs and forceps and seals were not inspected thoroughly in order to minimize these potential stress factors. Care was taken to ensure that handling times were reduced to a minimum. If the lice infestation was severe, seals were treated with selamectin (topical) or ivermectin (subcutaneous). Regarding the age structure of the harbour seals, two animals were estimated at around one year of age, and all other seals were classified as pups (< 6 months), including eight weaners (< 4 weeks). Thirty of 54 harbour seals were males and 22 females. The grey seal examined in this study showed severe cachexia and weakness at its admission to Sealcentre Pieterburen, and died despite intensive medical care during its rehabilitation period. In this case, lice were collected at necropsy.

Collected seal lice specimens as well as eggs (nits) from seal fur, mainly from head and neck, were fixed in 70% ethanol and morphologically determined by light microscopy. All seal lice stages examined here were classified according to morphological features [14, 15, 16]. Additionally, few nasal mites were collected from the nasal cavity of another grey seal during necropsy. The nasal mites were identified as *Halarachne* sp. using a morphological identification key [21], but could not be determined on species level. All fixed specimens were kept at room temperature and delivered to the Institute of Parasitology, Justus Liebig University Giessen, Germany, for further processing.

2.2. Anaplasma phagocytophilum*-,* Acanthocheilonema spirocauda*- and* Mycoplasma *spp.-PCR analyses*

Seal lice were grouped from each seal and collection date separately, resulting in 64 pools from the 54 harbour seals (*P. vitulina*) containing 1-20 lice and one pool of more than 1000 lice from the highly infested grey seal pup divided into pools of 15 lice (Additional file 1). In addition, eight mites obtained from the nasal cavity of another grey seal were pooled. DNA was extracted separately from each seal lice pool/batch. Entire seal lice were washed in distilled water overnight, sliced to minute pieces with sterile scalpel blades in 100 µl of phosphate-buffered saline, and DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instruction. PCR assays were performed to detect *A. phagocytophilum*-, *A. spirocauda*- and *Mycoplasma* spp.-DNA using as template 500 ng DNA extracted from the lice. For *A. phagocytophilum* detection, a partial gene sequence of the *msp2* gene was amplified by real time PCR according to Courtney et al. [22]. *Mycoplasma* was investigated by conventional PCR amplifying a partial 16S ribosomal DNA sequence with primers MGS0 and GP03 [23]. From the filarial species *A. spirocauda* no sequences were available in NCBI GenBank at time of this study (GenBank release 193, 12/2012). In a first attempt to obtain sequence data we used pan-filarial primers COIintF/COIintR [24] of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox1*). The obtained sequences from five seal lice pools differed by only one nucleotide position and had a sequence identity of 90% to an *A. viteae cox1*-sequence (HQ186249) in GenBank database. The *cox1*-sequence from one

seal lice pool was submitted to GenBank (accession number HG005138). To validate the sequences from the lice pools and to obtain further sequences from *A. spirocauda,* we then extracted DNA from morphologically confirmed *A. spirocauda* adult nematodes (identified according to Leidenberger and Boström [25]; please see Additional file 2), which were collected from necropsied harbour seals at Sealcentre Pieterburen between 2009-2011. Further, we amplified, sequenced and assembled a partial sequence of the ribosomal DNA region (ITS1, 5.8S, ITS2, and partial 28S) using primers NC5/NC2 [26] and the above-mentioned *cox1*-primers.

Sequencing was performed by an external service provider (LGC Genomics, Berlin, Germany) and the new sequences were deposited in GenBank database (accession numbers, *A. spirocauda*: HF583266, *Mycoplasma* sp.: MK953546). The cox1 amplicon sequence from the adult *A. spirocauda* (accession number: MW033199) had an additional single nucleotide polymorphism (not shown) compared to the sequences obtained from the DNA extracted from pools of infested lice.

For the molecular detection of *A. spirocauda larvae* in seal lice a more sensitive nested-PCR (COIintF/COIintR; AspF/AspR) was established. Based on the alignment of cox1 sequences from *A. spirocauda*, *A. viteae* and *Dirofilaria immitis*. Primers AspF (5´-TGCTGTTACTTTGGACCAGGT-3´) and AspR (5´-ATGATGGCCCCACACAGAAG-3´) were designed with Primer3 software [27]. The first PCR (COIintF/COIintR) was performed in a reaction volume of 50 µl containing 5 µl of template DNA, 5 µl of 10x buffer, 1 µl of each primer, 1 µl of dNTPs, 1 µl of Taq, and 36 µl of H_20. The following conditions were used: 2 min 95 °C initial denaturation, 35 cycles of 30s 94 °C, 30s annealing at 50 °C, 45s 72 °C, and 5 min 72 °C final extension. For the second PCR (AspF/AspR), 1 µl of the first PCR served as template in a 50 µl reaction containing 5 µl of 10x buffer, 1 µl of each primer, 1 µl of dNTPs, 1 µl of Taq, and 36 µl of H_20, under the following conditions: 2 min 95 °C initial denaturation, 35 cycles of 30s 94 °C, 30s annealing at 58 °C, 30s 72 °C, and 5 min 72 °C final extension. PCR products were separated on a 2% agarose gel. The achieved sensitivity of this method was determined using counted number of microfilariae (see Additional file 3) isolated from gravide *A. spirocauda*.

2.3. Phylogenetic analyses

For molecular phylogenetic analyses datasets of highly matching sequences to *A. spirocauda* and *Mycoplasma* sp. sequences were obtained from BLAST searches of the GenBank database (GenBank release 238, 06/2020), sequences were trimmed to homologous ends and realigned using the multiple sequence alignment program MAFFT 7 [28] with the L-INS-i method for the cox1 and 16S sequence data sets and the structure-aided Q-INS-i method for the ITS2 sequence data set. Phylogenetic trees were constructed using Bayesian analysis (MrBayes 3.2) (10.000 tree generations, sampling each 10, discarding first 250 trees) and TreeDyn for tree drawing at the phylogeny.fr platform [29]. The datasets included sequences homologous to nucleotides (nt) 122–624 of the *A. spirocauda cox1* sequence (HF583266K), nt 466–942 of-the *A. spirocauda* ITS2 sequence (HG005138), and nt 1–217 of the *Mycoplasma* sp. 16S ribosomal RNA gene sequence (MK953546). The *A. spirocauda cox1* sequence of an isolate from *Erignathus barbatus* (bearded seal) in Barrow (Alaska, USA) (KF038155) [30] was not

included because the overlapping sequence to the other *A. spirocauda cox1* sequences (excluding KT899872) was too short (169 nt, intraspecies variation 2.4% i.e. 4/169 nt).

2.4. Scanning electron microscopy (SEM) of Echinophthirius horridus*-adult- and -egg stages*

E. horridus adults and eggs glued to harbour seal fur hair were fixed with 1.5% paraformaldehyde glutaraldehyde in 0.15 M Hepes buffer (Merck) for 15 min at RT and afterwards washed carefully with 0.1 M cacodylate (Merck) buffer. All specimens of *E. horridus* were then post-fixed in 1% osmium tetroxide (Merck) diluted in 0.1 M cacodylate buffer at RT, washed three times in distilled water, dehydrated in ascending ethanol concentrations, critical point dried with CO_2 and sputtered with gold particles. Specimens were examined at the Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Germany, by using a Philips XL30® scanning electron microscope (Fig. 1).

Results

3.1. Seal lice and vector-borne pathogens

In total, 200 adult *E. horridus* seal lice were collected separately from 54 harbour seals (infestation 1-20 lice/seal, median 2; see Additional file 1) and more than 1000 lice from a single grey seal (*H. grypus*) pup. Seal lice from 19 of the 54 infested harbour seals (prevalence 35.2%; 95% CI: 22.5–47.9%) and from the highly infested grey seal pup proved positive for *A. spirocauda* (Fig. 2). In addition, seal lice (eight pools of 15 specimens) from the grey seal were found positive for *A. phagocytophilum* and seal lice from three harbour seals were found positive for a single species of *Mycoplasma*. Comparison of partial 16S sequence indicated that the *Mycoplasma* species found in the seal lice pools of the habour seals was identical to a *Mycoplasma* sp. from an Alaska Native hunter, who suffered from disseminated seal finger mycoplasmosis [31].

In an intial cox1-PCR using pan-filarial primers and sequencing the obtained sequences from lice pools and morphological confirmed *A. spirocauda* adults differed by only 3/649 nt (intraspecies variation 0.5%). We compared the cox1 single and nested PCR on ten pools. The nested PCR was more sensitive for these pools and therefore we decided to apply the nested PCR as diagnostic PCR for all pools. It had a consistent minimal detection of 10 microfilariae (see Additional file 3) and sufficient to detect *A. spirocauda* larvae in a single louse (seven positive lice pools from the harbour seals contained only one seal louse; see table in Additional file 1).

3.2 Phylogenetic analysis of A. spirocauda and the Mycoplasma isolate

Phylogenetic analysis of the partial *cox1* sequence showed that *A. spirocauda* was most closely related to *A. odendhali* (Fig. 3), a filarial parasite of the Northern fur seal (*Callorhinus ursinus*) and the California sea lion (*Zalophus californianus*) occuring in subcutaneous and intermuscular sites and considered to be non-pathogenic [30, 32]. Interestingly, one *A. spirocauda* isolate (PPr11-007; accession nos. *cox1* KT899872, ITS2 MG581463) collected from a stranded, deceased harbour seal from the USA west coast

(Stinson beach, California) [33] differs from all other *A. spirocauda* isolates by some indels in the ITS2 region and point mutations in the partial *cox1* gene resulting in only 95% and 93% sequence identity respectively (not shown). The partial 16S sequence of the *Mycoplasma* species detected in seal lice of three harbour seals in this stuy, was identical with the sequence from an infected patient. This zoonotic *Mycoplasma* species forms a new phylogenetic group together with isolates from dolphins and from a California sea lion (Fig. 4). It is positioned next to the elephantis-equigenitalium group and clearly separated from other Mycoplasma species previously described infecting pinnipeds, such as *M. phocidae*, *M. phocirhinis*, *M. phocicerebrale* and *M. zalophi*.

3.3. Severe Echinophthirius infestation in a grey seal

Investigation of a grey seal (*H. grypus*) which was found stranded on the beach revealed a severe *E. horridus* infestation (>1000 specimens). The animal weighted 15 kg at admission to the Sealcentre Pieterburen and was estimated on its teeth development as a few week old male pup. On arrival to the Sealcentre Pieterburen the pup was cachectic, showed fever and severe dehydration. After 14 days of intensive medical care treatments, the pup did not improve and died.

At necropsy, gross pathology findings included consolidation of large portions of lungs and presence of foam and fluids in trachea and main bronchial trees. Histopathological examination of lungs revealed acute interstitial pneumonia, which was diagnosed as cause of animal death. No *A. spirocauda* infection was detected during necropsy. PCRs for Herpes as well as Morbili viruses were performed on tissue samples from lungs, liver, spleen, brain, kidney, and bladder. Virology results were all negative. Unfortunately, no blood samples were left from this grey seal in order to perform blood smears and additional diagnostic PCRs to determine whether the grey seal was positive for *A. phagocytophilum* too.

Discussion

The present study reports on the presence of pathogens in the seal louse *E. horridus* and discusses the potential vector role of this ectoparasite in the marine habitat. The presence of *A. spirocauda* larvae in *E. horridus* was already described in the past [7, 8, 9] and, for first time, molecularly confirmed in the present study. Thereby, our results strongly support the hypothesis that *E. horridus* functions as intermediate host of *A. spirocauda*. Furthermore, *A. spirocauda,* the only filarial nematode parasite of phocid seals [8, 34], was molecularly characterized for the first time. Before this survey started no molecular data were available from the filariae *A. spirocauda*.

Moreover, our results represent the first detection of *A. phagocytophilum* and *Mycoplasma* sp. in seal lice. However, based on the limited knowledge of vector-borne pathogens occurring in marine habitats, our findings of *A. phagocytophilum* and *Mycoplasma* sp. should not directly be equated with the presence of vector-borne pathogens in seal lice. Analog to mosquitoe vectors [35], evidence of vector competence of *E. horridus* and the ability of transmitting these pathogens could only be proved by experimental infections or the morphological verification of pathogens in salivary glands of seal lice under controlled settings. Using molecular methods, it cannot be excluded that pathogens detected were located in the

gastro-intestinal tract of *E. horridus* individuals after blood-consumption of infected seals, but are not able to be transmitted to other host individuals. Nevertheless, molecular surveys of the present study constitute important baseline studies in the field of marine mammal parasitology to initially reveal a spectrum of pathogens, which could possibly be transmitted by seal lice. Thereby, our results can also help to encourage other researchers to extend the knowledge of vector-borne pathogens in the field of marine mammal parasitology.

To our current knowledge, there are neither reports on *A. phagocytophilum* or *Mycoplasma* spp. in any other ectoparasite affecting marine mammals nor evidence of anaplasmosis occurring in stranded phocid seals [36, 37]. *A. phagocytophilum* (Rickettsiales, Anaplasmataceae) constitutes an emerging globally distributed pathogen transmitted mainly by *Ixodes* ticks and leads to granulocytic anaplasmosis, which is one of the most relevant tick-borne diseases of veterinary and public health significance worldwide [38]. Practically, nothing is known on anaplasmosis in seals as nobody has investigated clinical relevance of *A. phagocytophilum* in determining marine seal population health status, like the ones occurring in the Dutch Wadden Sea. No tick infestations are known to occur in pinnipeds, nonetheless it might be speculated that haemotophagous ectoparasites other than ticks might become involved in transmission of *A. phagocytophilum* among marine mammals.

Variable clinical signs of granulocytic anaplasmosis can include high fever, lethargy, inappetence, anorexia, dullness, reduced weight gain, coughing and abortion in different animal species in Europe, including domestic ruminants, horses, dogs and cats [39, 40]. In humans, symptoms were reported as non-specific and include influenza-similar symptoms with fever and myalgia. In addition, leucopenia, thrombocytopenia and/or anaemia have been frequently reported to occur in certain *A. phagocytophilum* strain-infections [39]. Many of these clinical signs coincided well with the ones observed in the highly *E. horridus* infested grey seal pup at Sealcentre Pieterburen and might have been linked to an acute granulocytic anaplasmosis infection. However, the zoonotic potential of the *A. phagocytophilum* genetic variant detected here cannot be assessed by the *msp2* sequence and needs further characterisation. In this context, it would also be interesting to investigate if red foxes — hosts of *A. phagocytophilum* — living in the Dutch coastal dune area occasionally feed on dead seal pups and get infested by *E. horridus* as had been reported for Arctic foxes from Alaska and the fur seal louse (*Antarctophthirus callorhini*) [1]. Thereby, red fox activity was observed in UK within a mainland grey seal breeding colony [41] and satellite tracking showed that tagged grey seals in the Netherlands leave to breed in the UK [42].

Mycoplasma sp. was also molecularly identified in collected seal lice specimens in the current study, and in contrast to *A. phagocytophilum* there are previous reports on occurrence of mycoplasmal infections in pinnipeds [43, 44, 45, 46, 47, 48]. Thus, *Mycoplasma* spp. are common inhabitants of respiratory, gastrointestinal and genital tract of marine mammals, and a study on Australian fur seals (*Arctocephalus pusillus doriferus*) demonstrated the presence of different *Mycoplasma* species such as *M. zalophi, M. phocidae*, *M. phocicerebrale*, and *Mycoplasma* sp. in tested animals [45]. Furthermore, PCR testing of nasal swabs detected presence of *Mycoplasma* spp.-DNA in South American fur seal (*Arctocephalus australis*) populations in Peru with an estimated prevalence of 37.9% [49], evidencing rather

cosmopolitan distribution of mycoplasmas in pinnipeds. For universal detection of mycoplasmas the highly specific and sensitive PCR assay of van Kuppenveld [23] was used in the current study based on the conserved region of the 16S gene. Direct sequencing and sequencing of several clones indicated a single species only. However, better species differentiation would be possible selecting further housekeeping genes (e.g. *rpoB*, *rpoC*) and culturing for phenotypical characterisation and serological testing. Therefore, our findings on *Mycoplasma*-positive *E. horridus* lice might suggest presence of these bacterial infections in pinnipeds of the Dutch Wadden Sea. Whether seal lice might be potentially involved in the transmission of *Mycoplasma* needs further investigations.

However, some mycoplasmas (e.g. *M. phocicerebrale*) are associated with seal mortality and zoonotic 'seal-finger' infection, a disease known among people who handle seals for more than hundred years [50]. Seal-finger lesions could progress to septic arthritis of joints if tetracycline-based treatment is not received. Accordingly, in more recent published studies on a series of bites and contact abrasion in open-water swimmers caused by California sea lions and harbour seals revealed presence of *Mycoplasma* spp. in human wounds [48, 51], demonstrating its zoonotic potential. GenBank database search using the partial *Mycoplasma* sp. 16S sequence, detected in seal lice collected from three harbour seals in the current study, resulted in 100% identity to the sequence of an unnamed *Mycoplasma* species (GenBank accession no. KP292569) obtained from a patient with 'seal finger' and infected hip joint. The patient previously hunted and harvested ringed seals (*Phoca hispida*) without protective gloves in an area where *Mycoplasma* infected seals were noticed before [31].

Molecular analyses on collected seal lice from harbour and grey seals revealed presence of DNA of the seal heartworm *A. spirocauda*. In this context, *E. horridus* has previously been proposed to be the natural obligate intermediate host of *A. spirocauda* [7, 8, 9], and different stages of *A. spirocauda* larvae were found in dissected *E. horridus* seal lice [7, 9]. So far, the heartworm *A. spirocauda* has been reported from different phocid species such as harbour seals, hooded seals (*Cystophora cristata)*, bearded seals, ribbon seals (*Phoca fasciata*), harp seals (*Phoca groenlandica*), ringed seals, spotted seals (*Phoca largha*), monk seals (*Monachus monachus*), and recently from grey seals [8, 33, 52]. Furthermore, there is a significant positive correlation between heartworm infection and infestation of harbour seals with seal lice [9, 10]. Tested on 10 pools the designed nested PCR on basis of the mitochondrial cox1 gene was more sensitive in detection of *A. spirocauda*-DNA in lice pools compared to a single PCR and had a confident sensitivity of 10 microfilariae. This number will correspond to approximately 1.0 ng DNA [53]. Recently Keroack et al. [33] developed a more sensitive *A. spirocauda* real-time quantitative PCR based on a highly repetitive genomic DNA repeat identified using whole genome sequencing which will improve future monitoring of seal heartworm infections. These authors also identified the first time an *A. spirocauda* adult worm in a presumed grey seal carcass from the coast of Cape Cod (Massachusetts, USA). However, the authors mentioned that the carcass was in very poor condition due to extensive decomposition and could not be fully identified and reported just the evidence for possible *A. spirocauda* infection in the grey seal. Nevertheless, the results of Keroack et al. [33] support our detection of *A. spirocauda*-DNA in the seal lice collected from the grey seal which implied that grey seals could also get infected.

Previous SEM studies of *E. horridus* described the antennal structures [15, 16]. Our SEM analysis confirmed unique morphological adaptation features of *E. horridus* as for presence of strong legs with potent and well-developed claws acting almost as padlocks, allowing this marine seal louse to hold tight to the host fur coat while diving activities as previously postulated [10, 13, 14].

Regarding epidemiology and pathogenicity of echinophthiriosis, it is more frequently reported in young and weak animals [7, 8, 54], showing no seasonal variations for adult seals, but pups and immature seals have higher prevalences in spring. In contrast, Dailey and Fallace [10] reported highest prevalence in autumn and winter months, but no significant differences between examined age classes of seals and their seal lice burden were detected. Interestingly, in closely related species *A. microchir* from the South American sea lion over 60% of 1-day-old pups were infested with lice, and recruitment increased in pups up to three days old and leveled off onwards. In 1-day-old pups, significantly more adults than nymphs were found, but this pattern was reversed in older pups, documenting importance of vertical transmission most probably through their mother [55].

Conclusions

In conclusion, this study shows occurrence of potential pathogens within seal lice of infested pinnipeds in the Dutch Wadden Sea and reveals new insights into the potential role of *E. horridus* as vector. Thereby, the current study molecularly confirms former suggestions that E. horridus functions as intermediate host for the seal heartworm *A. spirocauda*. Whether *E. horridus* plays a role in the transmission of granulocytic anaplasmosis and *Mycoplasma* spp. infections in seals remains to be clarified by future studies, and it is thus clear that further epidemiological research on the occurrence of vector-borne pathogens in marine ecosystems is needed.

Declarations

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Authors' contributions

CH designed the study. CH, JH and DE drafted the manuscript. JH conducted molecular analyses of lice samples and *A. spirocauda*. JH and DE contributed equally to this work. GSC and ARG provided support during sample taking at Sealcentre Pieterburen. GM and UG provided scanning electron microscopic images. AT essentially revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

All parasite specimens analyzed in the current study (*E. horridus* and adult *A. spirocauda* individuals) were exclusively provided by the Sealcentre Pieterburen, Netherlands, sampled from free-ranging harbour and grey seals during a rehabilitation period or necropsies. Seal sampling procedures were conducted in strict accordance to the permission of the government of the Netherlands (valid permission ID at time of study: FF/75/2012/015), which approves admission and rehabilitation of free-ranging seals at the Sealcentre Pieterburen, as well as collection of dead seals. In the present study, non-invasive lice sample-taking process (using lice combs) was conducted during routine veterinary diagnostic and therapy to minimize additional stress factors and sampling duration. No seals were euthanized for research purposes. All samples of the present study were exclusively taken on territory of the Netherlands. The Sealcentre Pieterburen granted permission to the Institute of Parasitology (Justus Liebig University Giessen) for further analyses of parasite samples to perform the present study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Table 1 Pathogens transmitted by Echinophthiriidae

Echinophthiriid species	Detected pathogen	References
Echinophthirius horridus	*Acanthocheilonema spirocauda*	[7, 9, **Present study**]
	Bartonella henselae	[5]
	Anaplasma phagocytophilum	[**Present study**]
	Mycoplasma sp.	[**Present study**]
Antarctophthirus callorhini	*Salmonella enteritidis*	[3]
Proechinophthirus fluctus	*Salmonella enteritidis*	[3]
	Rickettsia sp.	[4]
Lepidophthirus macrorhini	Southern Elephant seal virus (SESV)	[6]

Figures

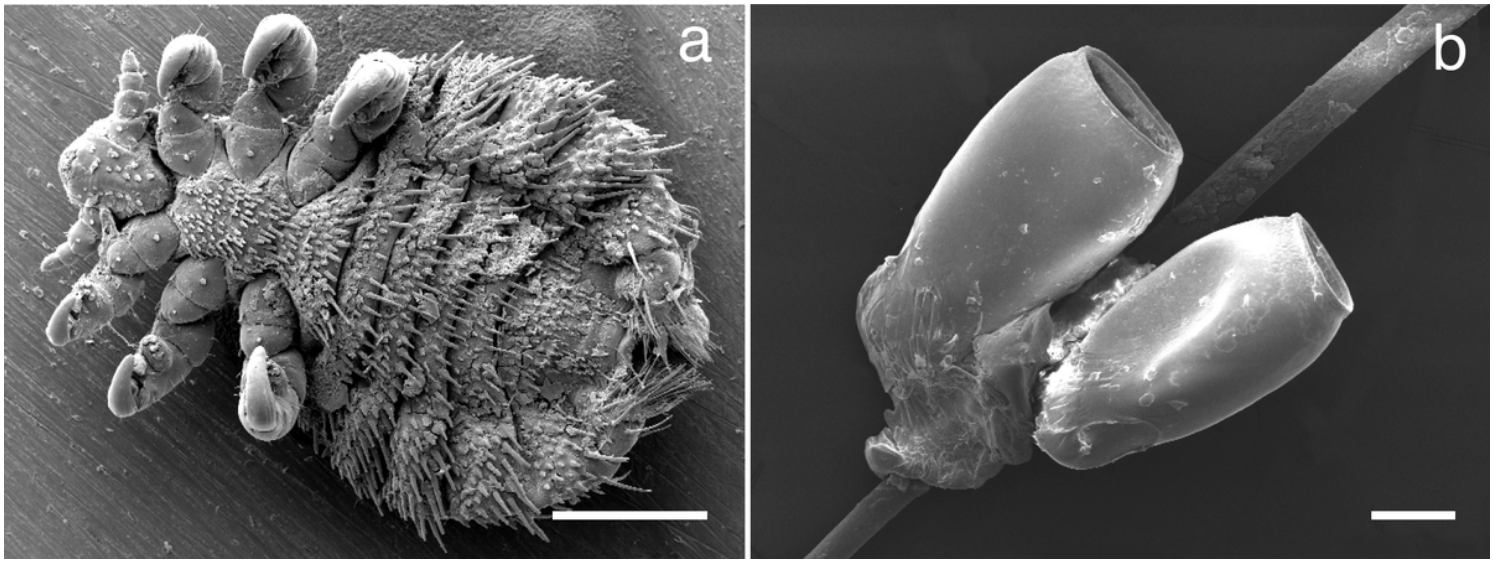

Figure 1

Ultrastructural illustration of Echinophthirius horridus adult- and egg (nit)-stages. (a) ventral view of adult E. horridus, (b) two opened nits lacking opercula firmly glued to seal fur hair. Scale bars: (a) 500 µm, (h) 200 µm.

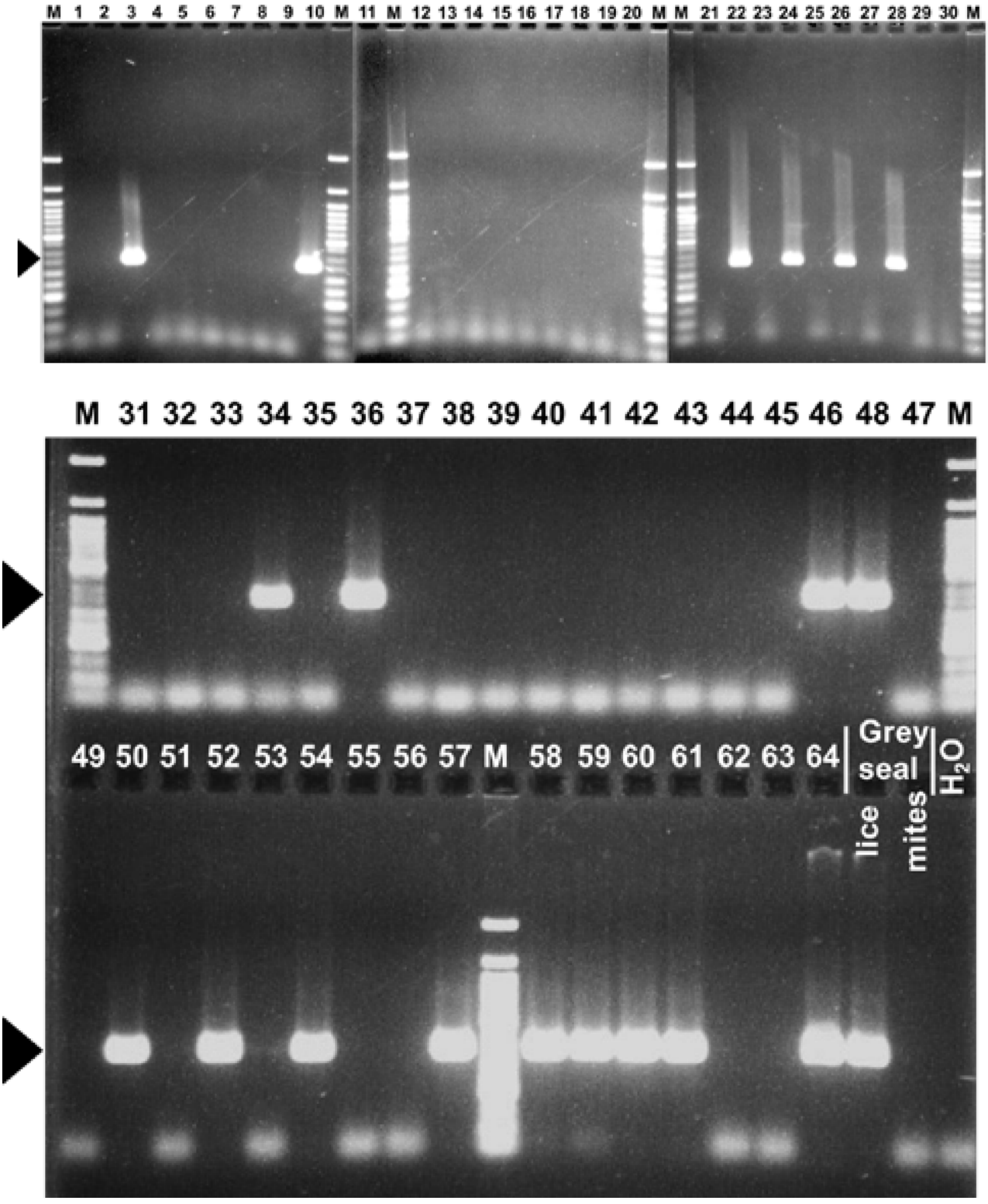

Figure 2

A. spirocauda cox1 nested-PCR using DNA extracted of seal lice collected from 54 harbour seals and one grey seal. Lice-pools 1-64 from harbour seals, one lice pool from a highly infested grey seal pup, and one pool of mites from another grey seal. Specific 351 bp-amplicon (arrow heads), M = molecular weight standard (50 bp-ladder), H2O (no template control). Pools 47 and 48 were swapped during loading on the gel.

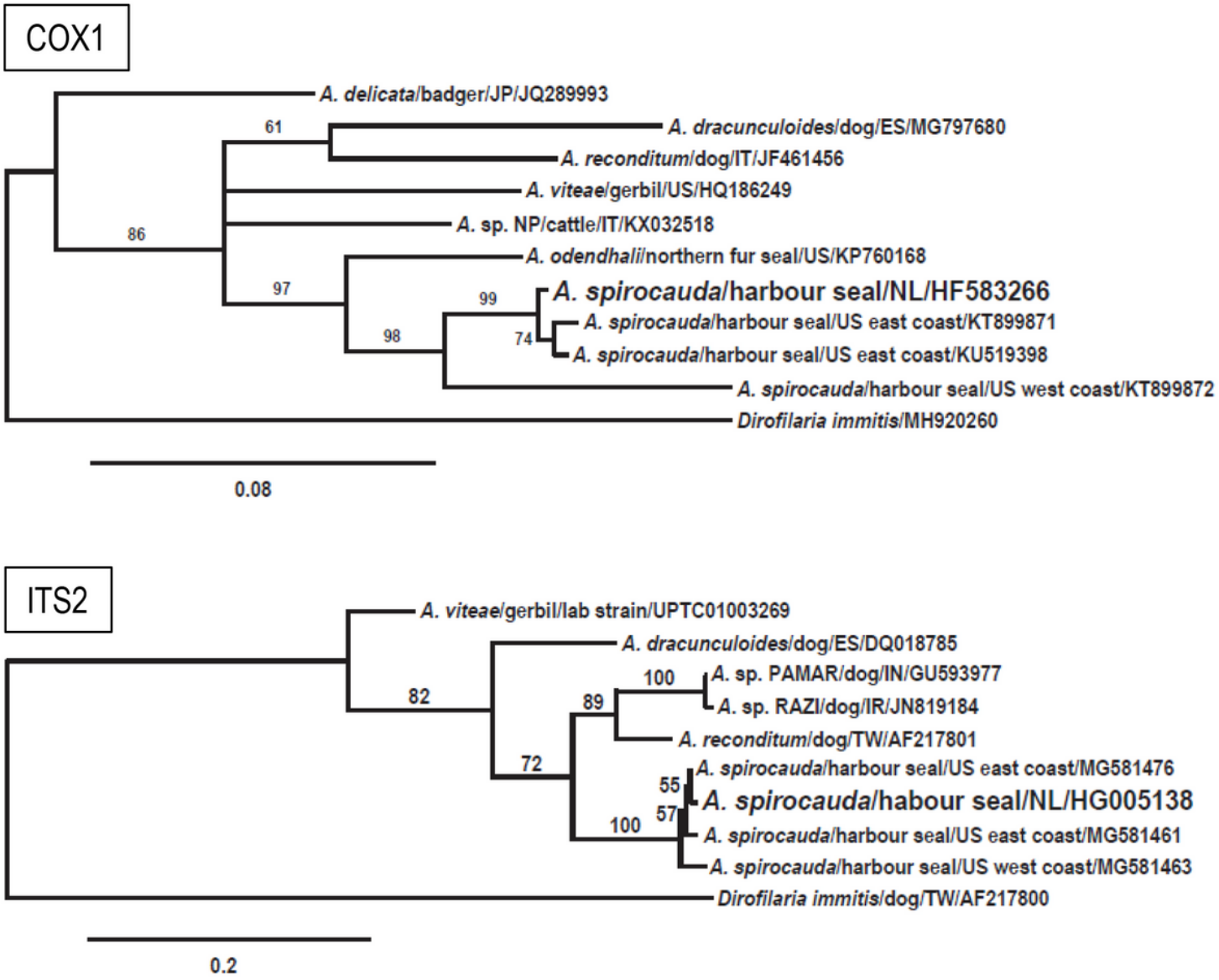

Figure 3

Phylogenetic analysis of A. spirocauda. The phylogeny is based on the MAFFT alignments for a partial cox1 sequence and complete ITS2 rDNA region of A. spirocauda and other Acanthocheilonema species using Bayesian inference (Dirofilaria immitis sequence as outgroup). The A. dracunculoides ITS2 sequence is shorter and does not cover the whole sequence of A. spirocauda. Branch lengths are drawn proportionally to evolutionary distance (scale bar is shown). Numbers adjacent to nodes indicate posterior probabilities in per cent. Branch labels provide species names/host/two-letter country code/GenBank accession number.

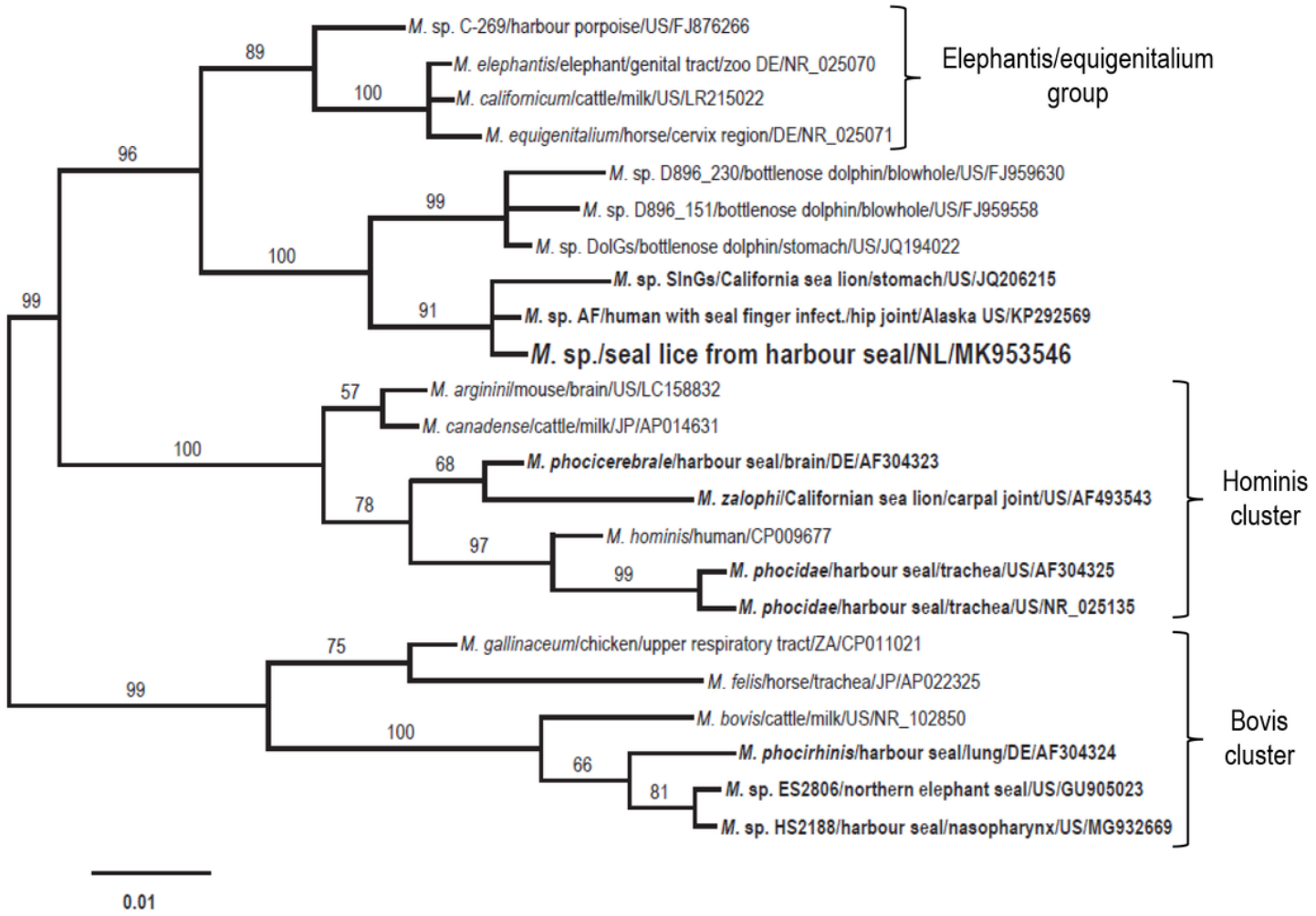

Figure 4

Phylogenetic analysis of Mycoplasma sp. from seal lice collected from harbour seals. Bayesian phylogenetic tree of Mycoplasma sp. of seal lice from harbour seals of the present study and phylogenetically closely related Mycoplasma species based on partial 16S gene sequences. Branch lengths are drawn proportionally to evolutionary distance (scale bar is shown). Numbers adjacent to nodes indicate posterior probabilities in per cent. Branch labels provide species names/host/source/two-letter country code/GenBank accession number. Mycoplasma species from seals highlighted in bold.

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