Prevalence and molecular characterisation of Acanthocephala in pinnipedia of the North and Baltic Seas

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ABSTRACT

Harbour seals (Phoca vitulina) and grey seals (Halichoerus grypus) are final hosts of acanthocephalans in the German North and Baltic Seas. Parasitic infections in seals can cause pathological changes, which may result in deteriorated health of the host. Common gastrointestinal parasites of harbour and grey seals are acanthocephalans and a number of 275 of 2460 (11.2%) investigated seals from 1996 to 2013 were infected with Corynosoma spp. (Acanthocephala, Polymorphidae). The prevalence showed a wave-like pattern: it increased from 1.2% and 0.4% in 1996 and 1997, respectively, to 23.9% during the second phocine distemper epizootic in 2002 and decreased to 6.2% in 2004. In 2005, prevalence peaked again with 25.0% followed by a decrease to 9.3% in 2009 and an increase to 38.5% in 2012. Statistical analysis revealed that harbour seals originating from the North Sea showed a higher prevalence than grey seals, whereas no significant difference between grey and harbour seals from the Baltic Sea was observed. Furthermore, juvenile pinnipedia from the North Sea were significantly less infected with Corynosoma spp. than seals older than seven month. Molecular species identification as well as phylogenetic relationship analysis among the detected Corynosoma species were achieved by sequencing and comparisons of the ribosomal ITS1-5.8S-ITS2-complex and cytochrome-c-oxidase I gene. Molecular analysis resulted in a newly arranged distribution of Acanthocephala in the North Sea as in contrast to previous studies, C. strumosum could not be confirmed as predominant species. Instead, C. magdaleni and a C. magdaleni isolate (isolate Pv1NS) with an atypical number of longitudinal rows of hooks at the proboscis were detected. Furthermore, morphological and molecular analyses indicate the possible finding of a cryptic species (Candidatus Corynosoma nortmeri sp. nov.).

1. Introduction

Indigenous seals of the North and the Baltic Seas are harbour seals, Phoca vitulina (Linnaeus, 1758), grey seals, Halichoerus grypus (Fabricius, 1791) and ringed seals, Phoca hispida (Schreber, 1775). After the first PDV epizootic in 1988/89, a stranding network along the coast of the German federal state Schleswig-Holstein and an extensive assessment of health status of seals, including parasitic infections, was established (Siebert et al., 2007). Parasites can cause pathological changes, affecting the health of seals (Bergeron et al., 1997; Lehnert et al., 2007). The class of Acanthocephala (Kohlreuter, 1771), also known as thorny headed worms, represent intestinal parasites associated with focal eosinophilic, granulomatous inflammation and enteritis as well as gastric ulcers and even perforation of the intestinal wall (Bergman and Olsson, 1985; Lehnert et al., 2007; Siebert et al., 2007). The genus Corynosoma (Lühe, 1904) as a member of the family Polymorphidae (Meyer, 1931) is with 38 species the most speciose acanthocephalan genus (Galván, 1994). The trunk of Corynosoma is described as small to medium sized, covered with a characteristicistically distribution of trunk spines. The proboscis is armed with 14–28 longitudinal rows of hooks. The life cycle is heteroxenous, containing usually an amphipod or isopod as first intermediate host, fishes as second intermediate hosts and waterfowl, sea birds or marine mammals as final hosts (Petrochenko, 1971; Crompton and Nickol, 1985; Nickol et al., 2002; García-Varela et al., 2005).

Examinations after the PDV epizootic in 1988/89 revealed that 70.2%–94.5% of the investigated harbour seals were infected with acanthocephalans, namely Corynosoma strumosum (Rudolphi, 1802) (Borgsteede et al., 1991; Strauss et al., 1991). Further prevalence determined for the North Sea at the same time period was 32% in the Kattegat-Skagerrak (Lanneryd, 1992), whereas the infection rate of harbour seals in the North Sea between 1997 and 2000 was 23% with C.
strumosum as predominant species, but also C. semerme (Forsnell, 1904) was detected in one animal (Lehnert et al., 2007). Before the second PDV epizootic occurring between 1996 and 2002, 12% of examined seals were infected with Corynosoma spp., while the prevalence increased to 20% after the second PDV epizootic between 2002 and 2005 (Siebert et al., 2007). In contrast, 100% of investigated ringed seals of the Bothnian Bay in Finland, as part of the Baltic Sea, were infected with C. semerme between 1988 and 1999, while 58.0% of seals were infected with C. strumosum and 81.0% with C. magdaleni (Valtonen et al., 2004). For the same region, Nickol et al. (2002) reported a species-specific tropism in the intestine of grey seals, with C. strumosum and C. magdaleni preferring the small intestine while C. semerme was found primarily in the rectum.

So far, Corynosoma species were mainly differentiated by morphological characteristics, but subtle differences between species may result in less reliable data (Van Cleave, 1953; García-Varela et al., 2005). However, reliable species discrimination is crucial for the understanding parasite epidemiology and its impact on the health status of the host. Therefore, the usage of molecular markers is beneficial to discriminate between species with identical or very similar morphological characteristics. According to Hebert et al. (2003), the mitochondrial gene cytochrome c oxidase I (COI) can serve as reliable DNA marker to analyse the systematic relationships between closely related species. Consequently, this marker has been used in several studies to discriminate species in, including Corynosoma spp. and related acanthocephalan genera (García-Varela et al., 2009). In addition, Blouin (2002) described the internal transcribed spacer (ITS)-1–5.8S rRNA-ITS2-complex (ITS-complex) as an excellent tool for DNA diagnostics to distinguish between species. The ITS-complex has already been used successfully to differentiate acanthocephalans including Corynosoma spp. in harbour and grey seals (García-Varela et al., 2005).

The aim of the present study was to compare the occurrence and distribution of acanthocephalans in harbour and grey seals of the German North and Baltic Sea between 1996 and 2012. Furthermore, acanthocephalan specimens of the seal carcasses were analysed using the ITS1-complex and COI as molecular markers to investigate their phylogenetic relationship.

2. Material and methods

2.1. Sample origin

The carcasses of harbour seals (Phoca vitulina), grey seals (Halichoerus grypus) and ringed seals (Phoca hispida) originating from the North and Baltic Sea coasts of the German federal state Schleswig-Holstein were examined as part of the regional monitoring program (Siebert et al., 2007). Additionally, 17 seals from the Polish Baltic Sea were included in the analysis. Included animals were either found dead or mercy killed by officially appointed seal rangers and stored at −20 °C until further investigated. Necropsies were performed as described by Siebert et al. (2007). Specimens of acanthocephalans were collected during necropsy from the digestive tract of examined seals, washed with 0.9% NaCl solution and preserved in 70% ethanol for morphological characterisation and molecular differentiation. Results of the parasitological examination, combined with data comprising sex, age, sampling location, sampling date as well as body length and weight were noted. For this study, a dataset of 2460 seals examined between 1996 and 2013 were used for the epidemiological analyses on acanthocephalan infections. As data were not always completely available, sample sizes vary for below described investigations. Parts of the dataset were already analysed for different pathological aspects and published by Lehnert et al. (2007) and Siebert et al. (2007).

Age group classification of seals was determined by sampling date, body length and weight, as well as the navel and canine development in young seals (Mansfield and Fisher, 1960). Age group 1 included animals between 0 and 6 months, age group 2 those between 7 and 18 months and age group 3 seals older than 18 months.

2.2. Surveillance of Acanthocephala infections in seals

The prevalence of intestinal acanthocephalan infection in the necropsied seals was compared between years 1996 and 2012. Furthermore, prevalence differences between harbour and grey seals from the North and Baltic Sea as well as between age groups were analysed.

2.3. Morphological characterisation of acanthocephalans

Morphological species discrimination of adult acanthocephalans collected from the intestine of necropsied seals was conducted according to criteria described by Delyamure (1955), Petrochenko (1971) and Nickol et al. (2002) using a stereomicroscope (Olympus SZH10 Research Stereo, Olympus, Japan).

Furthermore, 50 morphologically assigned acanthocephalans (35 specimens of C. strumosum, 10 of C. semerme and 5 of C. magdaleni) were characterised in detail by measuring the length and width of the body, fore- and hindtrunk and proboscis as well as determination of the trunk spine coverage and, if possible, the number of the proboscis’ longitudinal hooks. Specimens were photographed with a digital camera (Olympus SC30 camera, Olympus, Japan) attached to a stereomicroscope (Olympus SZH10 Research Stereo, Olympus, Japan) or light microscope (Olympus Cx41, Olympus, Japan). Measurements were conducted with CellSens Entry software (version 1.5, Olympus, Japan).

2.4. Molecular and phylogenetic analyses of acanthocephalan specimens

Genomic and mitochondrial DNA of the 50 worms characterised in detail was extracted individually using QiAamp DNA Micro Kits (Qiagen, Germany) according to the manufacturer’s instructions. Isolated DNA was amplified by PCR using the GeneAmp® RNA PCR Core Kit (Applied Biosystems, USA). Amplification of the ribosomal internal transcribed spacer (ITS) complex (ITS1-5.8S rRNA-ITS2) was carried out in a 50 μl reaction mixture containing 5 μl 10x PCR buffer II, 4 μl dNTP mix (1 mM each), 4 μl MgCl2 (5 mM), each 5 μl primer FWcor2 and REVcor2 (0.2 μmol) (García-Varela et al., 2005; Luton et al., 1992), 0.25 μl AmpliTaq DNA Polymerase (0.025 μU/μl), 10 μl template DNA and 16.75 μl DEPC-treated water. PCR cycling parameters comprised an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for min and subsequent final elongation at 72 °C for 5 min.

Parts of the mitochondrially encoded cytochrome c oxidase I (COI) DNA were amplified using 5 μl 10x Taq PCR buffer II (1 mM), 2.25 μl dNTP mix (0.9 mM each), each 5 μl primer Cox1FW and Cox1Rev (0.2 pmol) (Folmer et al., 1994; García-Varela et al., 2009), 0.25 μl PerfectTag DNA Polymerase (5 PRIME GmbH, Germany) (0.025 U/μl), 5 μl template DNA and 27.5 μl DEPC treated water. Cycling conditions comprised an initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and subsequent final elongation at 72 °C for 5 min.

Obtained amplification products were sequenced with the Sanger method (Seqlab, Germany and Eurofins MWG Biotech, Germany). If sequencing of PCR products was not successful, PCR products were ligated into 4-TOPO™ vector and inserted in chemically competent TOP10 E. coli cells (OneShot™ TOP10) using the TOPO® TA Cloning™ Kit (Life Technologies Inc., USA) and purified plasmids (NucleoSpin™ Plasmid DNA Purification Kit, Macherey-Nagel, Germany) were used for Sanger sequencing. Alignment and analysis of obtained sequences were carried out using BioEdit, version 7.2.5 (Hall, 1999). For species identification, obtained sequences were compared to published ITS (Table 1) and COI sequences (Table 2) of Acanthocephala. Primer sequences were removed from the sequences before submission to GenBank (accession nos. MF001277-MF001280 and MF078642-
Phylogenetic analyses were conducted using MEGAS5 software (Tamura et al., 2011) with 1000 bootstrap replicates to test for reliability. For nucleotide alignment of the ITS-complex, a phylogenetic tree was constructed using maximum likelihood (ML) method, based on the Jones-Taylor-Thornton (JTT) model (Jones et al., 1992). Calculations of the pairwise distances were carried out using the Maximum Composite Likelihood model (Tamura et al., 2011). To eliminate silent mutations from the COI, nucleotide sequences were translated into amino acid sequences by using the ExPaSy translate tool provided by SIB Swiss Institute of Bioinformatics for invertebrate mitochondrial genes. Phylogenetic analysis of the COI sequences was conducted using the maximum likelihood (ML) method, based on the Jones-Taylor-Thornton (JTT) model (Jones et al., 1992). Calculations of the pairwise distances were used for species discrimination based on the ITS1-5.8S rRNA-ITS2-region.

Table 1
Acanthocephala species, origin and GenBank accession numbers used for species discrimination based on the ITS1-5.8S rRNA-ITS2-region.

| Species | Sample origin | Origin | GenBank acc. no. | Reference |
|---------|---------------|--------|-----------------|-----------|
| Applancha sieboldi | n.d. | n.d. | AF416411 | Not published |
| Bolbosoma cf. capitatum | Homo sapiens | Kyoto, Japan | AY706182 | García-Varela et al., 2013 |
| B. nipponicum | Balaenoptera acutorostrata | North Pacific Ocean | AY706183 | García-Varela et al., 2013 |
| Brachionus patulus | n.d. | n.d. | AF416412 | Not published |
| Corynosoma australe | Otaria byronia | n.d. | AF286307 | García-Varela et al., 2005 |
| C. bulbosum | Mirounga leonina | n.d. | AF286308 | García-Varela et al., 2005 |
| C. cetaceum | Pontoporia blainvillei | n.d. | AF286310 | García-Varela et al., 2005 |
| C. capricornis | Phoca capricornis | n.d. | AF286309 | García-Varela et al., 2005 |
| C. enhydra | Enhydra lutris | n.d. | AF286311 | García-Varela et al., 2005 |
| C. hainani | Leptonychotes weidelli | n.d. | AF286312 | García-Varela et al., 2005 |
| C. neglectus | Phoca hispida | n.d. | AY532065 | García-Varela et al., 2005 |
| C. neglectus isolate Pv1NS | Phoca vitulina | North Sea | MF078643 | this study |
| C. negletus sp. nov. | Phoca vitulina | North Sea | MF001280 | this study |
| C. semmerme | Halichoerus grypus | Baltic Sea | MF001279 | this study |
| C. strumosum | Phoca hispida | n.d. | AF286313 | García-Varela et al., 2005 |
| C. validum | Odobenus rosmarus | n.d. | AF286314 | García-Varela et al., 2005 |
| C. vollegrum | Eumetopias jubatus | n.d. | AF286315 | García-Varela et al., 2005 |
| Macracanthorhynchus ingens | n.d. | n.d. | AF416414 | Not published |
| Moniliformis moniliformis | n.d. | n.d. | AF416415 | Not published |
| Neochoepistomum goslini | Dorsimactar maculatus | Mexico | FJ968109 | Martínez-Aquino et al., 2009 |
| Octopusoides sp. | Pteria splendidia | n.d. | FJ388978 | Not published |
| Okracanthorhynchus tortuosa | n.d. | n.d. | AF416417 | Not published |
| Oncicola sp. | n.d. | n.d. | AF416416 | Not published |
| Polymorphus sp. | Anas platyrhynchos | n.d. | AF461421 | García-Varela et al., 2005 |
| P. altmani | Enhydra lutris | n.d. | AY532066 | García-Varela et al., 2005 |
| P. brevis | n.d. | n.d. | AF286306 | Not published |
| P. minutus | Gammarus pulex | n.d. | AY532067 | García-Varela et al., 2005 |
| Pompobroduus tereticalis | Platichthys flesus | Straitland, Baltic Sea | JF706705 | Špákovalová et al., 2011 |
| Tempiolobius sp. | n.d. | n.d. | JF694277 | Not published |

n.d.: no data available.

Table 2
Acanthocephala species, origin and GenBank accession numbers used for species discrimination based on partial COI sequences.

| Species | Sample origin | Origin | GenBank acc. no. | Reference |
|---------|---------------|--------|-----------------|-----------|
| Bolbosoma sp. | Callirhina urinum | St. Paul Island, Alaska, USA | JX442190 | García-Varela et al., 2013 |
| Corynosoma australe | Phocarctos hookeri | New Zealand | JX442191 | García-Varela et al., 2013 |
| C. enhydra | Enhydra lutris | Monterey Bay, California, USA | DQ689719 | García-Varela et al., 2013 |
| C. neglectus isolate Pv1NS | Phoca hispida saimensis | Lake Saimaa, Finland | EF467872 | García-Varela et al., 2011 |
| C. neglectus sp. nov. | Phoca vitulina | North Sea | MF078642 | this study |
| C. neglectus sp. | Phoca vitulina | North Sea | MF001278 | this study |
| C. neglectus sp. | Phoca vitulina | North Sea | MF001277 | this study |
| C. neglectus sp. | Phoca vitulina | North Sea | MF001276 | this study |
| C. neglectus Sp. | Ewegianus abrus | Veracruz, Mexico | GQ681438 | García-Varela et al., 2013 |
| Macracanthorhynchus ingens | n.d. | n.d. | AF416997 | Not published |
| Mediorychon gammarus | n.d. | Indonesia | KC261352 | Not published |
| Moniliformis moniliformis | n.d. | n.d. | AF416998 | Not published |
| Olcathorhynchus tortuosa | n.d. | n.d. | AF416999 | Not published |
| Oncicola sp. | n.d. | n.d. | AF417000 | Not published |
| Polymorphus brevis | Zoogeonicus purpureus | Central Mexico | KC549497 | Alcántar-Escalera et al., 2013 |
| P. obtusus | Aythyia affinis | Baja California Sur, Mexico | JX442195 | García-Varela et al., 2013 |
| P. trochaus | Fulica americana | Sinaloa, Mexico | JX442196 | García-Varela et al., 2013 |
| Pompobroduus tereticalis | Gammarus pulex | Chevigny, France | AY422363 | Perrot-Minot 2004 |
| Pseudocorynosoma sp. | Oxylariaаксericanensis | Durango, Mexico | JX442198 | García-Varela et al., 2013 |
| Tempiolobius sp. | Limnas argenteiculatus | n.d. | JF694276 | Not published |

n.d.: no data available.
achieved using the JTT matrix-based model.

2.5. Statistical analyses

The χ²-test was performed to test for significant differences between the infection rates over years and between the three age groups. Holm–Bonferroni method was used to control the familywise error rate.

One-way ANOVA and Kruskal-Wallis-test were used to test for statistically significant differences of morphological measurements between the identified groups. Statistical calculations were conducted with SigmaStat™ (version 3.11, Systat Software, Germany). In all analyses, P ≤ 0.05 was considered statistically significant.

3. Results

3.1. Surveillance of Acanthocephala infections in seals

Throughout the study period from 1996 to mid-2013, a dataset including 2293 harbour seals (2120 from the North Sea, 54 from the Baltic Sea and 119 with unknown origin), 161 grey seals (67 from the North Sea, 76 from the Baltic Sea and 18 with unknown origin) and 6 ringed seals (5 from the Baltic Sea and 1 with unknown origin) was analysed for intestinal Acanthocephala infections. In total, 11.2% (275/2460) of investigated seals were infected. Since numbers of examined ringed seals were low (n = 6; one infected animal), data were excluded from subsequent statistical analyses.

3.1.1. Annual prevalence

At the beginning of the study period in 1996/1997, the annual prevalence of thorny headed worms in seals was 1.2% (2/170) and 0.4% (1/224). As of 1998, the infection rate increased in three waves up to a maximum of 38.5% (47/121) in 2012. An overview of the annual prevalence of thorny headed worms in seals was 1.2% (2/170) and 0.4% (1/224). As of 1998, the infection rate increased in three waves between the North- and Baltic Seas was significantly higher compared to harbour seals from the North Sea (P = 0.013 and P ≤ 0.001, respectively), whereas no significant differences between grey and harbour seals from the Baltic Sea were observed.

3.1.2. Harbour and grey seals in North- and Baltic Seas

Overall, 10.5% (223/2120) harbour seals originating from the North Sea carried an infection with Corynosoma spp, whereas harbour seals from the Baltic Sea were significantly less frequently infected (7.4%; 4/54; P ≤ 0.001). In contrast, no statistically significant differences between grey seals from the North- (20.9%; 14/67) and Baltic Sea (34.2%; 26/76) were found. The infection rate of grey seals from the North- and Baltic Seas was significantly higher compared to harbour seals from the North Sea (P = 0.013 and P ≤ 0.001, respectively), whereas no significant differences between grey and harbour seals from the Baltic Sea were observed.

3.1.3. Age class distribution

Detailed data on prevalences in different age groups of seals from the North and the Baltic Seas are shown in Table 3. Harbour seals of age group 1 originating from the North Sea were significantly less infected with Corynosoma spp. than harbour seals of age group 2 (P ≤ 0.0001) and age group 3 (P = 0.0001) from the same habitat. In addition, grey seals belonging to age group 1 from the North Sea were also significantly less frequently infected than grey seals of age group 2 (P ≤ 0.0001) and age group 3 (P = 0.0004) from the same habitat. Grey seals of age group 1 from the North Sea were significantly less frequently infected than grey seals of age group 1 of the Baltic Sea (P = 0.0002); however, no statistically significant differences between grey seals of age group 2 and 3 between North Sea and Baltic Sea were determined. Furthermore, comparisons of harbour seals originating from the North Sea vs. Baltic Sea revealed no statistically significant differences between any of the age groups.

3.2. Morphological characterisation of acanthocephalans

Morphological discrimination of Corynosoma species was possible for 217 of the 275 infected seals, from the remaining 58 seals no specimens were preserved. The specimens of 191 seals (177 harbour seals from the North Sea, 11 grey seals from the North Sea and 3 harbour seals from the Baltic Sea) were morphological identified as C. strumosum. Interestingly, some specimens identified as C. strumosum showed an atypical number of 16 longitudinal hook rows as described by Amin et al. (2011). Specimens of 5 seals (4 grey seals and 1 ringed seal, all originating from the Baltic Sea) were identified as C. magdalenii. C. semerm was identified in 26 seals (1 harbour seal originated from the North Sea and 25 grey seals from the Baltic Sea). Coinfections of C. semerm and C. strumosum occurred in 1 harbour seal from the North Sea, whereas 4 grey seals from the Baltic Sea were coinfected with C. semerm and C. magdalenii.

3.3. Molecular and phylogenetic analyses of acanthocephalan specimens

A total of 50 thorny headed worms were used for molecular species determination based on published ITS and COI sequences.
3.3.1. ITS sequence analysis

The 10 sequenced acanthocephalans morphologically identified as *C. semerme* showed identical ITS sequences. However, as no genetic data of *C. semerme* were available, no species-specific percentage identity with sequences deposited in public databases could be determined. The 5 specimens morphologically identified as *C. magdalenii* showed 100% identity with the published ITS sequence of *C. magdalenii* (GenBank acc. no. AY532065). However, none of the 35 specimens morphologically determined as *C. strumosum* was confirmed by molecular species discrimination. Instead, 13 sequenced specimens showed 100% identity with the published ITS sequence of *C. magdalenii*. Further 19 specimens showed 99% identity to *C. magdalenii* due to one transversion (g.288G > C) and one transition (g.304G > A). These samples are referred to as *Corynosoma magdalenii* isolate P1NS in the following. The remaining 3 specimens contained 86 single nucleotide polymorphisms (SNPs) compared to the published *C. magdalenii* ITS sequence as well as 16 nucleotide insertions at six positions (g.611_612ins AAT; g.616_617insACTGT; g.635_636insA; g.649_650insT; g.728_729insGGGCT and g.766_767insG) and 5 nucleotide deletions at three positions (g.522del, g.527_529del and g.565del) compared to the published *C. magdalenii* ITS sequence. Compared to *C. strumosum*, as which the three specimens were determined morphologically, 102 SNPs as well as 17 nucleotide insertions at seven positions (g.611_612ins AAT; g.616_617insACTGT; g.635_636insA; g.649_650insT; g.728_729insGGGCT; g.766_767insG and g.769A) and 5 nucleotide deletions at three positions (g.522del, g.527_529del and g.565del) were detected. As these sequences were substantially different from any other known species, they are referred to as “*Candidatus Corynosoma nortmeri* sp. nov.” in the following.

An alignment of previously published ITS sequences of *C. strumosum* (GenBank acc. no. AF286313) and *C. magdalenii* (GenBank acc. no. AY532065) with selected nucleotide sequences obtained in the present study [C. *semalenni* (GenBank acc. no. MF01279)], *C. magdalenii* isolate P1NS (acc. no. MF078643) and Cand. *nortmeri* sp. nov. (acc. no. MF01280)] is provided in Fig. 2.

Phylogenetic analysis of the ITS-complex was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. This model yielded a single best tree with the highest log likelihood of −16,028.00 (Fig. 3). Analysis involved 25 nucleotide sequences with a total of 636 positions in the final dataset. The genus *Corynosoma* forms a monophyletic group with *C. caspicum* (Golvan and Mokhayer, 1973), *C. magdalenii* and the obtained sequence of *C. magdalenii* isolate P1NS as sister species as well as *C. enhydri* (Morozov, 1940) and *C. strumosum*. By contrast, Cand. *nortmeri* sp. nov. and *C. semerme* showed less close relationships to these species.

The nucleotide distance based on the Maximum Composite Likelihood method between *C. magdalenii* and *C. magdalenii* isolate P1NS was low (0.005) compared to the distance between *C. magdalenii* and *C. strumosum* with a value of 0.033. The class of Cand. *nortmeri* sp. nov. showed the closest relationship to *C. magdalenii* (0.204).

3.3.2. COI sequence analysis

The 10 sequenced acanthocephalans morphologically identified as *C. semerme* showed identical COI sequences. Again, no comparison with previously published *C. semerme* sequences was possible. 5 specimens morphologically identified as *C. magdalenii* showed 100% amino acid identity with the published sequence of *C. magdalenii* (GenBank acc. no. EF467872), confirming molecular ITS species determination. Again, none of the 35 specimens morphologically identified as *C. strumosum* matched perfectly to published COI amino acid sequences, but the 13 specimens identified as *C. magdalenii* on ITS level showed 100% identity with the published COI sequence of *C. magdalenii*. Similarly, COI sequences of the 19 specimens referred to as *C. magdalenii* isolate P1NS (see section above) were identical to those of *C. magdalenii*. The sequences of the three Cand. *nortmeri* sp. nov. specimens contained one amino acid substitution compared to sequences of *C. magdalenii* (p.Ile12Val) as well as *C. strumosum* (p.Leu57Trp). An alignment showing *Corynosoma* COI sequence comparison is provided in Fig. 4.

Construction of the phylogenetic tree was conducted by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) model. The tree with the highest log likelihood (−2479.70) is shown in Fig. 5. In total, 19 amino acid sequences with a total of 201 positions in the final dataset were involved in the analysis. *Corynosoma* species grouped together as a monophyletic assemblage, even though the bootstrap values were moderate. The estimated evolutionary divergence between Cand. *nortmeri* sp. nov. and *C. strumosum* as well as *C. magdalenii* was each 0.005.

3.4. Morphological characteristics of Corynosoma spp. determined by molecular analysis

Analysed specimens were assigned to the *Corynosoma* species resulting of the molecular species discrimination. Determined morphological parameters are shown in Table 4. Due to the small sample size of Cand. *nortmeri* sp. nov. (n = 3), One-way ANOVA was conducted only between *C. magdalenii* and *C. magdalenii* isolate P1NS. The latter had a significant larger body size than *C. magdalenii* (P ≤ 0.001), associated with a significantly longer and wider fore- (P ≤ 0.001 and P ≤ 0.001, respectively) and hindtrunk (P ≤ 0.001 and P ≤ 0.001, respectively).

4. Discussion

An extensive assessment of health status of seals has been undertaken since the first phocine distemper virus (PDV) epizootic in 1988/89 (Siebert et al., 2007). Besides histological alterations and microbiological infections, special attention of the assessment has been given to reveal the parasitic burden of seals in German waters (Lehnert et al., 2007; Siebert et al., 2007). One frequently occurring class of parasites are thorny-headed worms, scientifically known as Acanthocephala. Within the health monitoring, Siebert et al. (2007) reported an acanthocephalan prevalence of 12% between 1996 and 2002 and 20% between 2002 and 2005 for seals originating from the coast of the German federal state Schleswig-Holstein. Lehnert et al. (2007) found 23% of harbour seals of the North Sea to be infected with *Corynosoma* spp between 1997 and 2000. To extend obtained data, this study focused on the investigation of annual prevalences over an extended period of time (1996–2012), taking host preferences and age dependent infection patterns of acanthocephalans in seals of the North and Baltic Seas into account. While the seal populations in the German Wadden Sea grew constantly (Galatius et al., 2016), the prevalence of acanthocephalan infections fluctuated during this period. In the beginning of the study, the annual prevalence of infected seals was low, but increased continuously until the second PDV epizootic in 2002, when 23.9% of the seals were infected. Afterwards, the Acanthocephala prevalence decreased to 6.2% in 2004. The PDV infection could have led to a decreased immune response, which may contribute to a predisposition for parasitic infections (Borgsteede et al., 1991), but as this was not confirmed during the second PDV epidemic in 2002, it is assumed that the PDV infection progresses too rapidly and severely to influence the parasitic burden (Siebert, 2003). In the following years, this wavy pattern continued by increasing again to high levels (25.0%) in 2005 decreasing to 9.3% in 2009 and reaching the overall highest prevalence level of 38.5% in 2012. Influential factors on marine parasite populations, which may contribute to the observed wave-like pattern of the annual Acanthocephalan prevalences, are the abundance of intermediate host like amphipods and fishes as well as abiotic factors such as the pH, temperature and phosphate level in the environment (Marengliese and Cone, 1997; Poulin, 2006; Sinisalo, 2007). However, limited information is available on the intermediate hosts of *Corynosoma* spp. in the North Sea.

It might be assumed that acanthocephalan prevalences in harbour
and grey seals, as trophically transmitted pathogens, are almost identical due to their overlapping prey spectrum (King, 1983). However, in the presented study, grey seals of the North Sea were more frequently infected (20.9%; 14/67) than harbour seals in the same habitat (10.5%; 223/2120). However, in the Baltic Sea no statistically significant differences between both seal species were determined, but rather low sample sizes from the Baltic Seas (54 harbour seals; 7.4% positives vs. 76 grey seals, 34.2% positives) may have hampered statistical significance.

Parasitic infections with pulmonary nematodes, heartworms and seal lice in harbour seals seem to be age-dependent (Claussen et al., 1991; Lehnert et al., 2007, 2016; Ulrich et al., 2016), probably due to development of protective immunity and/or a shift in the prey spectrum during adolescence (Lehnert et al., 2007, 2016; Lundström et al., 2010). The latter might also be assumed for acanthocephalan infections, as studies during the first PDV epidemic revealed that young seals were less frequently infected with Corynosoma spp. than subadult and adult seals, which did not show differences between their age classes (Borgsteede et al., 1991; Strauss et al., 1991). Similar results were observed in the presented study. However, the juvenile age group includes data of a number of seals which died during their weaning period and were consequently not exposed to intermediate hosts. Nevertheless, subadult seals from the North Sea were more often infected with thorny-headed worms than adults.

Regarding Corynosoma species identification, the intraspecific variance and the interspecific conformance of morphological characteristics between C. strumosum and C. magdalenis is a barrier to reliably distinguishing them morphologically. Therefore, molecular species differentiation using the ITS1-5,8S rRNA-ITS2-complex as well as COI was additionally conducted for selected individuals. To our knowledge,
this study provides the first genetic sequences of *C. semerme* and their phylogenetic classification. All sequenced worms morphologically identified as *C. semerme* resulted in identical sequences. In terms of the ITS-complex, *C. semerme* did not show any closer relationship to another known species of the genus *Corynosoma*. In contrast, the translated amino acid sequence of the COI was identical to a published sequence of *C. obtuscens*. This species is widely distributed along North and South America with a broad range of definitive hosts (Lincicome, 1943; Tantaleán et al., 2005). Moreover, terrestrial animals like the Andean fox, *Pseudalopex culpaeus* (Molina, 1782), are described as hosts (Tantaleán et al., 2007). *C. obtuscens* and *C. semerme* are morphologically similar and can be differentiated only by subtle differences in the spination of males and the number of longitudinal hook rows at the proboscis (Neiland, 1962). Unfortunately, no ITS-sequence of *C.*
obtusca has been published so far, so that it remains unknown whether this gene region is a suitable marker for reliable differentiation of these two species.

Samples of *C. magdaleni* isolate Pv1NS showed the same COI amino acid sequence as *C. magdaleni*, but two polymorphisms in the ITS-complex were detected. Furthermore, there were remarkable differences in morphological characteristics. According to the emended descriptions of Nickol et al. (2002), the body of *C. magdaleni* is 3.6–5.3 (mean 4.7) mm long. Individuals of this study determined as *C. magdaleni* by molecular analysis exhibited a slightly longer body length of 4.0–6.3 (mean 5.3) mm, whereas *C. magdaleni* isolate Pv1NS was with 6.4–8.3 (mean 7.6) mm significantly longer than those of *C. magdaleni*. In addition, proboscises of *C. magdaleni* isolate Pv1NS were armed with 16–18 longitudinal hook rows while *C. magdaleni* is commonly described with 17–23 rows (18–19 in the presented study, cf. Table 4).

From obtained results it remains questionable whether *C. magdaleni* isolate Pv1NS represents a variant occurring simultaneously with *C. magdaleni* in the same habitat or a different species. Future investigations using further genetic markers such as the small (SSU) and large (LSU) subunits of nuclear ribosomal DNA (García-Varela et al., 2011) are needed to answer this question. However, if proven to be a variant only, the morphological description of *C. magdaleni* needs extension by parameters of the presented study, in particular body length and the proboscis armature.

According to DeSalle et al. (2005), boundaries of a taxon are determined by geographical, morphological, ecological, reproductive and behavioural information. Consequently, morphologically cryptic species can only be detected at the DNA sequence level. The ITS-region of
Cand. *C. nortmeri* sp. nov. revealed considerable nucleotide substitutions, insertions and deletions compared to other published *Corynosoma* ITS-sequences. The phylogenetic analysis resulted in an apparent evolutionary distance between Cand. *C. nortmeri* sp. nov. and *C. magdalenii*, the closest related species. Additionally, on COI amino acid level, Cand. *C. nortmeri* sp. nov. differed from other *Corynosoma* species and showed a substitution of Leucine to Tryptophan (p.Leu5Trp) compared to *C. nortmeri* sp. nov. 4.7–7.8 mm) and *C. hadweni* was only described once, when discovered in Alaska in 1953 (Van Cleave, 1953).

Even though only three Cand. *C. nortmeri* sp. nov. individuals were available, results indicate the potential finding of a cryptic species. Nevertheless, further studies including on an appropriate sample size are crucial to confirm this potential new species. When these data are provided, we propose the name *Corynosoma nortmeri* sp. nov., named after Nortmer, a late Middle High German notation for the North Sea.

The high interspecific conformation of *Corynosoma* spp. complicates discrimination between some species and Van Cleave (1953) criticised the high rate of incorrectly determined samples as *C. strumosum*. Specimens of *Corynosoma* spp. with 16–18 longitudinal hook rows and remarkable alterations in body size and shape originated from the Caspian Sea were determined as *C. strumosum* (Amin et al., 2011), even though *C. strumosum* is described with 18 longitudinal hook rows only (Nickol et al., 2002). Previous studies on the acanthocephalan distribution in seals of the North Sea during the study period of 1996–2012, with annual prevalences ranging between 0.4% and 38.5%. For the first time, molecular species differentiation of acanthocephalan species in the North and Baltic Sea was performed, resulting in an altered distribution of acanthocephalans in German parts of the North and the Baltic Sea compared to previous morphology-based studies: None of the examined parasites was identified as *C. strumosum*, but rather *C. magdalenii* was found in seals from both, the North and Baltic Seas. The combination of morphology and molecular analysis indicate the potential finding of a cryptic species, for which as the name Candidatus *Corynosoma nortmeri* sp. nov. is proposed.

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