

Parvicapsula bicornis n. sp. and *P. limandae* n. sp. (Myxozoa, Parvicapsulidae) in Pleuronectidae (Teleostei, Heterosomata) from Denmark

Marianne Køie^{1,*}, Egil Karlsbakk^{2,3}, Are Nylund²

¹Marine Biological Laboratory, University of Copenhagen, 3000 Helsingør, Denmark

²Department of Biology, University of Bergen, PO Box 7800, 5020 Bergen, Norway

³Present address: Institute of Marine Research, PO Box 1870 Nordnes, 5817 Bergen, Norway

ABSTRACT: Two species of *Parvicapsula* were found in the kidney tubules and the urinary bladder of 2 pleuronectid fish from the northern Øresund, Denmark. The coelozoic, spherical, disporic trophozoites of both species are 10 to 12 µm in diameter. The myxospores of both species are elongate, asymmetrical and slightly curved, and have spherical polar capsules. *Parvicapsula bicornis* n. sp. (6–8 × 5–6 µm, polar capsule 2.5 µm in diameter) occurs in *Pleuronectes platessa*. The polar capsules of *P. bicornis* are arranged symmetrically on either side of the longitudinal axis and its spores differ from other species of *Parvicapsula* in having two 2–3 µm long posterior processes of different length. *Parvicapsula limandae* n. sp. (8–11 × 4–5 µm, polar capsule 1.6 µm in diameter) is found in *Limanda limanda*. The polar capsules are arranged along the longitudinal axis. It differs from *Parvicapsula unicornis* Kabata, 1962, recorded from *L. limanda*, in the arrangement of the polar capsules and in the absence of a posterior horn-like projection. The phylogenetic relationship between *P. bicornis* n. sp., *P. limandae* n. sp. and other *Parvicapsula* spp. was examined with their partial small subunit rDNA (SSU rDNA) sequences. *P. limandae* n. sp. and *P. asymmetrica* appear to be closely related, while *P. bicornis* n. sp. and *P. minibicornis* are the most divergent members of the genus.

KEY WORDS: Myxozoan · Parasites · *Parvicapsula bicornis* n. sp. · *Parvicapsula limandae* n. sp. · *Sphaerospora irregularis* · *Parvicapsula unicornis* · *Pleuronectes platessa* · *Limanda limanda* · SSU rRNA gene sequences · Denmark

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INTRODUCTION

Eleven species of *Parvicapsula* Shulman, 1953 (Parvicapsulidae) have been described in marine fish (Køie 2003). The small myxospores develop in disporic trophozoites, likely pseudoplasmodia, mostly in the urinary system, with other sites being the epithelia of the gall bladder, the intestine and the pseudobranch. Since members of *Parvicapsula* are often pathogenic to marine fish, it is important to increase knowledge of the biology, including the life cycles, of members of this genus. Some species are pathogenic in mariculture of salmonid fish (Hoffman 1984, Kent et al. 1997, Karlsbakk et al. 2002, Sterud et al. 2003, Yasutake & Elliott 2003, Nylund et al. 2005).

Plaice *Pleuronectes platessa* and dab *Limanda limanda* are commercially important pleuronectid flat-

fish in northwestern Europe. Several myxozoan species from these hosts have been described (see e.g. Auerbach 1912, Bazikalova 1932, Shulman 1953, Shulman & Shulman-Albova 1953, Polyanskii 1955, Kabata 1962, Gaevskaya & Kovaleva 1984, Køie 2005). The aim of the present paper is to examine the myxozoan fauna of these pleuronectids; in particular potential pathogens in the myxosporean genus *Parvicapsula*.

MATERIALS AND METHODS

Pleuronectes platessa L. and *Limanda limanda* (L.) were bottom-trawled in the northern Øresund, Denmark, at depths of 2 to 22 m. The plaice and dab, 42 and 39 specimens, respectively, were examined fresh. Most specimens were examined immediately upon

*Email: mkoie@bi.ku.dk

capture, but some were kept in aquaria for up to 1 wk. Most organs including the musculature, the eyes, the gall bladder and urinary system were examined. Fresh squash preparations of the urinary bladder and various parts of the kidney were examined for Myxosporea. Smears were air-dried, methanol-fixed, stained with Giemsa and embedded in DPX. Measurements ($n = 10$) are based on fresh smears and are given as the mean with the range in parenthesis.

DNA was extracted from kidney tissue or urinary bladders containing *Parvicapsula* spp. trophozoites or spores, using the DNeasy protocol for animal tissues (Qiagen). The DNA were eluted in 100 μ l AE buffer supplied with the Qiagen-kit and stored at -20°C before use in a PCR.

The PCR primers used were Ecf and Myxgen4r (Kent et al. 2000, Nylund et al. 2005) with additional primers constructed based on small subunit rDNA (SSU rDNA) sequences from *Parvicapsula* spp. and other members of the marine group of Myxosporea: forward MarF1 (RRG CGT GCC TTG AAT AAA GC) and MyxF2 (CGC GCA AAT TAC CCA ATC CAG AC), reverse LyR2 (CCT TGC GAT TGT ACT CTC CC) and MarR2 (STA GCG ACG GGC GGT GTG).

The PCR amplifications were performed in a total volume of 50 μ l, using 2 μ l of template DNA and a reaction mixture consisting of 5 μ l $10\times$ PCR buffer, 5 μ l 10 mM dNTP, 2 μ l (10 mM) of the reverse and forward primer, 2 U of thermostable DNA polymerase (Qiagen) and 34 μ l dH_2O . The PCR conditions were as follows: after an initial 5 min denaturation step at 95°C , samples were taken through 35 amplification cycles, each consisting of a 30 s denaturation step at 94°C , a 30 s primer annealing step at a temperature dependent of the primer combination used, and a 90 s extension step at 72°C . A prolonged extension step of 10 min at 72°C completed each reaction.

The PCR products were purified on Qia-quick PCR Purification columns (Qiagen) and then sequenced using the BigDye Terminator Sequencing kit. The sequencing was performed using the amplification primers described above.

The sequence data were assembled with the help of VECTOR NTI software (InforMax) and GeneBank searches were done with BLAST (2.0). The VECTOR NTI Suite software package was used for multiple alignments of the partial sequences. The partial sequences of *Parvicapsula bicornis* (EF429097), *P. limandae* (EF429096) and *Parvicapsula spinachiae* Køie, 2003 (EF431928) (from the type host *Spinachia spinachia* (L.) and the type locality, northern Øresund), were aligned with homologous SSU rDNA sequences from a selected number of myxosporeans already available on the EMBL nucleotide database. In pair-wise comparisons between the different species SSU rDNA, the multiple sequence alignment editor GENEDOC (avail-

able at: www.psc.edu/biomed/genedoc) was used, but the variable regions in the SSU were manually aligned to minimum divergence.

The phylogenetic relationship between *Parvicapsula* spp. was examined with an alignment of selected members of the marine clade (Fiala 2006). Alignments were performed with ALIGNX (VECTOR NTI) and manually edited in GENEDOC. Hypervariable or ambiguous regions were deleted from the alignment to ensure comparison of homologous positions. Phylogenetic analysis of the data sets were performed using PAUP* (4.0b10) (Swofford 2000) and TREEPUZZLE (5.0) (Schmidt et al. 2002), using the maximum likelihood method with 100 and 10 000 replicates respectively. A general time reversible (GTR) model of nucleotide substitution with a 6 category gamma distribution (GTR+ Γ +I) was used following the examination of the data set with the program MODELTEST (Posada & Crandall 1998). The PAUP maximum likelihood analysis used a heuristic search algorithm with 10 random sequence additions and TBR branch swapping. Phylogenetic trees were drawn using TREEVIEW (Page 1996).

RESULTS

Two new species of *Parvicapsula* were found in the urinary systems of *Pleuronectes platessa* and *Limanda limanda*. All measurements below ($n = 10$) give the mean with the range in parenthesis. The prevalence levels of *Parvicapsula bicornis* and *P. limandae* are reported in Table 1.

Parvicapsula bicornis n. sp. (Figs. 1 & 2)

Syn. part. *Sphaerospora irregularis* Kabata, 1962 from *Pleuronectes platessa*.

Description: Trophozoites with mature myxospores, coelozoic, disporic and subspherical, 12 μ m (11 to 13 μ m) in diameter. Myxospores most often orientated in the same direction (Fig. 1A,B). Immature trophozoites, transparent and spherical, 2 to 10 μ m in diameter.

Myxospores (Figs. 1C–E and 2 A,B) 6.8 (5.5 to 7.7) μ m in length (exclusive of posterior processes), and 5.3 (4.6 to 5.9) μ m in maximum width. Nearly square (exclusive of posterior processes) in sutural view, i.e. from the concave (or convex) side (Figs. 1C,D & 2A). Curved, slightly flattened in frontal view (Fig. 2B). Asymmetrical due to 2 posterior processes of different length, 2.3 (2.0 to 2.5) μ m and 3.3 (3.0 to 3.5) μ m in length. Processes concealed when surrounded by crystalline structures (Fig. 1E). Suture line passes on both sides from between the polar capsules along concave and convex side to half way down the longest process.

Table 1. Prevalences (%) of *Parvicapsula bicornis* n. sp. and *P. limandae* n. sp. in *Pleuronectes platessa* and *Limanda limanda* from the northern Øresund, Denmark, September to December 2005. For *P. platessa* 0-group: 5–12 cm; 1-group and older: >23 cm. For *L. limanda* 0- and 1-groups: 7–14 cm; older: >16 cm. Numbers of fish examined in each size group are in parenthesis

Parasite	Host			
	<i>Pleuronectes platessa</i> 0-group (20)	<i>Pleuronectes platessa</i> 1-group and older (22)	<i>Limanda limanda</i> 0- and 1- groups (15)	<i>Limanda limanda</i> Older (24)
<i>Parvicapsula bicornis</i>	15	55	0.0	0.0
<i>Parvicapsula limandae</i>	0.0	0.0	47	79

Valve nuclei at extreme posterior end of each process. Unequal valves thin-walled and smooth. Sporoplasm binucleate.

Equal spherical polar capsules close to each other 2.5 (2.3 to 2.6) μm in diameter. Plane of polar capsules perpendicular to longitudinal axis. No 'head organ'. Polar capsules open at concave side. Number of coils of polar filaments: 6 to 8.

Type host: *Pleuronectes platessa* L. (Pleuronectidae).

Type locality: The northern Øresund between Helsingør and the island of Ven, 2 to 20 m depth, Denmark, 55° 58' N, 12° 38' E.

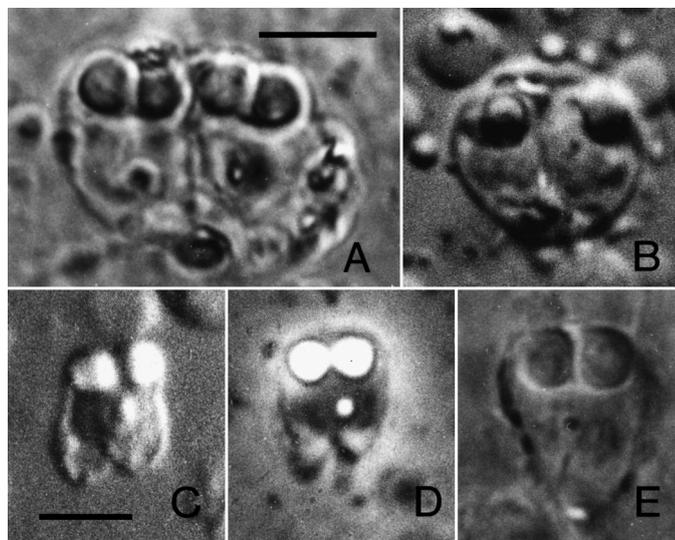


Fig. 1. *Parvicapsula bicornis*. Fresh squash preparation of trophozoites and myxospores in kidney tubules and urinary bladder of *Pleuronectes platessa* using (A–C & E) interference contrast and (D) phase contrast. (A) Disporic trophozoite in kidney tubule. (B) Trophozoite free in urinary bladder. (C–E) Myxospore, sutural view. (C,D) Myxospores free in urinary bladder. (E) Myxospore in kidney tubule. Refractive granules adhere to the surface of the spore. Note that A & E and B–D are to the same scale. Scale bars = 5 μm

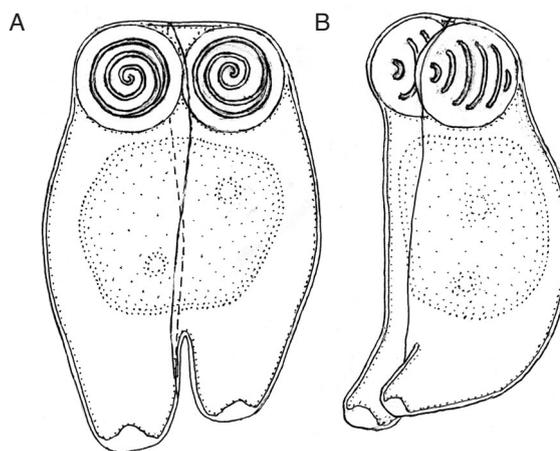


Fig. 2. *Parvicapsula bicornis*. Myxospore. (A) Sutural view, seen from the concave side. (B) Frontal view. Scale bar = 5 μm

Prevalence: 36% (15 of 42) of plaice examined from September to December 2005 (Table 1). Only 20% of the infected fish had mature spores in the urinary bladder.

Type material: Giemsa-stained smears are deposited at the Zoological Museum, Copenhagen, Denmark. The museum does not use catalogue numbers.

Etymology: The species name refers to the 2 posterior processes.

Site and host reaction: All developmental stages, coelozoic in the kidney tubules and Bowman's capsules, were surrounded by elongated, highly refractive crystalline granules of host origin (Fig. 1A,E). The granules sometimes completely covered trophozoites or myxospores. The chemical nature of these granules and their effect on the parasites are unknown.

Comments: Based on spore morphology, host, and geographical location, the myxosporean reported here is a new species of *Parvicapsula*, *Parvicapsula bicornis* n. sp. It has the characteristics of the family Parvicapsulidae Shulman, 1953 (Lom & Dyková 1992, Køie 2003). *P. bicornis* differs from all known species of *Parvicapsula* (see Køie 2003) in having both spherical polar capsules and 2 posterior processes of different length.

The partial SSU rDNA sequence of *Parvicapsula bicornis* shows highest identity to *P. unicornis* and *P. pseudobranchicola*, with highest percent overall identity (including indels) with the former, and highest percent identity by aligned bp with the latter (Table 2). However, the *P. bicornis* sequence differs in a number of substitutions at positions conserved across the other *Parvicapsula* spp., reflected in the phylogenetic analysis by a basal position in the *Parvicapsula* clade (see Fig. 5).

Table 2. Comparison of the partial SSU rRNA gene sequences from *Parvicapsula bicornis* (EF429097) and *P. limandae* (EF429096) with other *Parvicapsula* spp. Percent sequence identity (PID) was calculated as identity by aligned bp (PIDa) and overall identity (including indels) (PIDo). nt: nucleotides

	<i>Parvicapsula minicornis</i> (AF201375)	<i>Parvicapsula spinachiae</i> (EF431928)	<i>Parvicapsula unicornis</i> (AY584190)	<i>Parvicapsula pseudobranchicola</i> (AY308481)	<i>Parvicapsula asymmetrica</i> (AY584191)	<i>Parvicapsula limandae</i>
<i>Parvicapsula bicornis</i>						
Alignment, length (nt)	1410	1368	1274	1303	1297	1230
Aligned bp	1237	1235	1238	1236	1237	1169
Identities (nt)	1063	1059	1049	1070	1063	1001
Insertions/deletions (nt)	4/169	6/127	3/33	2/65	1/59	1/60
PIDo (%)	75.4	77.4	82.3	82.1	82.0	81.4
PIDa (%)	85.9	85.7	84.7	86.6	85.9	85.6
<i>Parvicapsula limandae</i>						
Alignment, length (nt)	1361	1322	1239	1232	1226	
Aligned bp	1229	1242	1210	1224	1225	
Identities (nt)	1060	1098	1115	1166	1216	
Insertions/deletions (nt)	13/119	9/71	29/0	2/6	1/0	
PIDo (%)	77.9	83.1	90.0	94.6	99.2	
PIDa (%)	86.2	88.4	92.1	95.3	99.3	

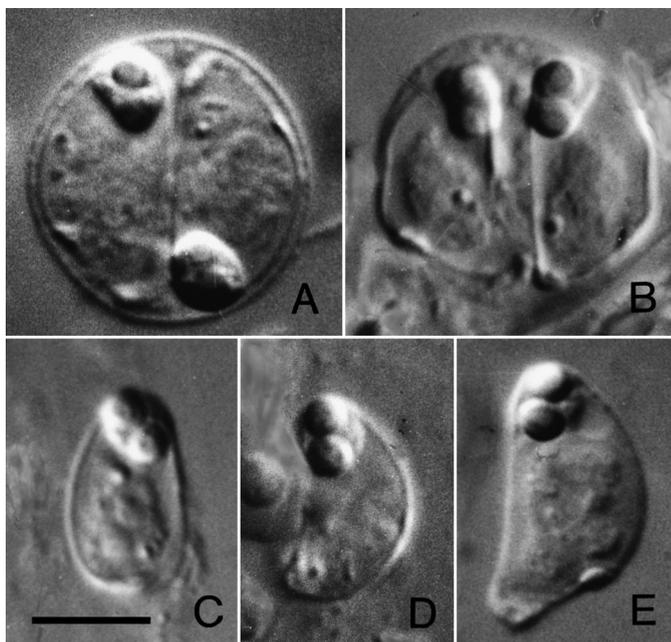


Fig. 3. *Parvicapsula limandae*, fresh squash preparation of trophozoites and myxospores in the urinary bladder of *Limanda limanda* using interference contrast. (A,B) Trophozoites. (C) Myxospore, frontal view. (D) Myxospore, oblique view, close to blood cell. (E) Myxospore, lateral view. Scale bar = 5 µm

Parvicapsula limandae n. sp. (Figs. 3 & 4)

Syn. part. *Parvicapsula unicornis* Kabata, 1962 from *Limanda limanda*.

Description: Trophozoites with fully developed myxospores, coelozoic, dispodic and spherical, 10.5 (10 to 11) µm in diameter. Spores most often oriented in same direction and with concave sides facing each other

(Fig. 3A,B). Immature trophozoites, transparent and spherical, 2 to 8 µm in diameter. Myxospores 9.5 (8.3 to 10.8) µm in length and 4.7 (4.3 to 5.4) µm in maximal width. Ovoid to pyriform in frontal view or medial plane, i.e. from the concave (or convex) side (Figs. 3C & 4A). Polar capsules on longitudinal axis. Frontal view of spores occasionally slightly asymmetrical as polar capsules may be displaced from longitudinal axis.

Spores asymmetrical (curved) in lateral or sutural view (Figs. 3D,E & 4B). Suture line from between polar capsules along lateral sides to convex side. Suture lines unite 3 to 4 µm from posterior end and slightly posterior to nucleus of anterior valve cell. Nucleus of posterior valve cell at extreme posterior end. Unequal valves, thin-walled and smooth. Sporoplasm binucleate.

Spherical polar capsule, 1.6 (1.5 to 1.6) µm in diameter close to each other. Polar capsules in a prominent spherical 'head organ', twice the diameter of polar capsules. Polar capsules open at concave side. Polar filaments with 6 to 8 coils.

Type host: The only known host is the common dab *Limanda limanda* (L.) (Pleuronectidae).

Type locality: The northern Øresund between Helsingør and the island of Ven, 16–20 m, Denmark, 55° 58' N, 12° 38' E.

Type material: Giemsa-stained smears are deposited at the Zoological Museum, Copenhagen, Denmark. The museum does not use catalogue numbers.

SSU rDNA sequence: EF429096 (partial).

Etymology: The species name refers to the name of its fish host.

Prevalence of infection: 67% (26 of 39) specimens of *Limanda limanda* examined from September to

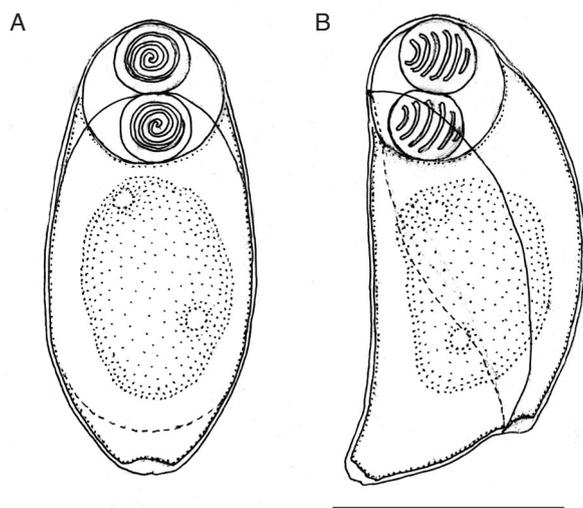


Fig. 4. *Parvicapsula limandae*. Myxospore in (A) frontal view, seen from the concave side and (B) lateral view. Scale bar = 5 μ m

December 2005 (Table 1). Less than 10% of the infected fish had mature spores in the urinary bladder.

Site and host reactions: Immature, spherical, transparent trophozoites 2 to 8 μ m in diameter occur coelozoic in kidney tubules and Bowman's capsules. Trophozoites with mature spores and free myxospores in kidney tubules and urinary bladder. Even though the kidney tubules and Bowman's capsules were dilated due to numerous small transparent trophozoites, no host reaction was observed.

Comments: *Parvicapsula limandae* n. sp. has the characteristics of the family Parvicapsulidae Shulman, 1953 (Lom & Dyková 1992, Køie 2003). Only 2 of the previously described species of *Parvicapsula* have (sub)spherical polar capsules which are arranged at the longitudinal axis of the spore (see Køie 2003). *P. limandae* is most similar to *Parvicapsula* sp. in the kidney of *Oncorhynchus kisutch* from the northern US Pacific (Hoffman 1984), as described from the appearance of the spores in sections. Since *Parvicapsula* sp. has recently also been detected in pseudobranch tissue (Yasutake & Elliott 2003), that species may be closely related to the similar *P. pseudobranchicola* Karlsbakk et al. 2002, which typically occurs in the pseudobranchs of *Salmo salar* in Norway (Karlsbakk et al. 2002, Sterud et al. 2003). *P. pseudobranchicola* also has subspherical polar capsules and shows a prominent 'head organ', but is larger than *P. limandae* sp.n. and clearly distinct from it by the low SSU rDNA sequence similarity (95%).

Parvicapsula limandae n. sp. has a superficial resemblance to *P. unicornis* Kabata, 1962 found in—among others—*L. limanda* from the northern North Sea (Kabata 1962). *P. unicornis* differs from the present

species primarily in that it has a long posterior projection and in that the polar capsules are arranged transverse to the longitudinal axis.

Parvicapsula limandae n. sp. shows a high sequence similarity with a *P. asymmetrica* Shulman, 1953 sequence from the type host *Cyclopterus lumpus* (cf. Nylund et al. 2005) (see Fig. 5 which also shows the phylogenetic position of *P. bicornis*). *P. asymmetrica* is a larger species, with spherical trophozoites 17–21 μ m in diameter, spores 10.5–17 \times 6–9 μ m, pyriform polar capsules, rounded posterior end and a suture line that posteriorly unites terminally or subterminal-ventrally (Shulman 1953, Shulman & Shulman-Albova 1953). Based on spore morphology, and our knowledge on intraspecific variation in myxospores, conspecificity of *P. limandae* and *P. abyssorum* is highly unlikely. We recently reproduced the *P. asymmetrica* sequence from a *Parvicapsula*-infected *C. lumpus* caught near Bergen (W. Norway). Identical *P. limandae* sequences were obtained from 3 individual *Limanda limanda* only infected with *P. limandae* in their urinary system and caught at the same locality at different times of the year. Hence, we appear to have a case with 2 morphologically dissimilar *Parvicapsula* spp. that show related SSU rDNA sequences. The unambiguous differences found represent 4 pyrimidine transitions, one purine transition and one transversion in the V4 region, 2 transversions in the V7 region and a purine transition in the V8 region.

DISCUSSION

Parvicapsula bicornis n. sp. and *P. limandae* n. sp. are members of the family Parvicapsulidae and the genus *Parvicapsula* (Lom & Dyková 1992). Both new species of *Parvicapsula* have apparently been recorded from their respective hosts previously.

Sphaerospora irregularis Kabata, 1962 was recorded in 0- and 1-group *Pleuronectes platessa* from farm environments off western Scotland (MacKenzie et al. 1976). It is likely that this myxozoan should be identified as *Parvicapsula bicornis*, which has large polar capsules and a superficial resemblance to *S. irregularis* Kabata, 1962 described from the urinary bladder of *Hippoglossoides platessoides* (Fabricius) (syn. *Drepanopsetta platessoides*) from the northern North Sea (Kabata 1962). Kabata (1962) placed the new species tentatively in the genus *Sphaerospora*. Gaevskaya & Kovaleva (1984) transferred *S. irregularis* to the genus *Myxoproteus* based on the original description by Kabata (1962). However, Arthur & Lom (1985), without having reexamined the species from the type host, transferred *S. irregularis* to the genus *Ortholinea*. *S. irregularis* has apparently only been found in *H.*

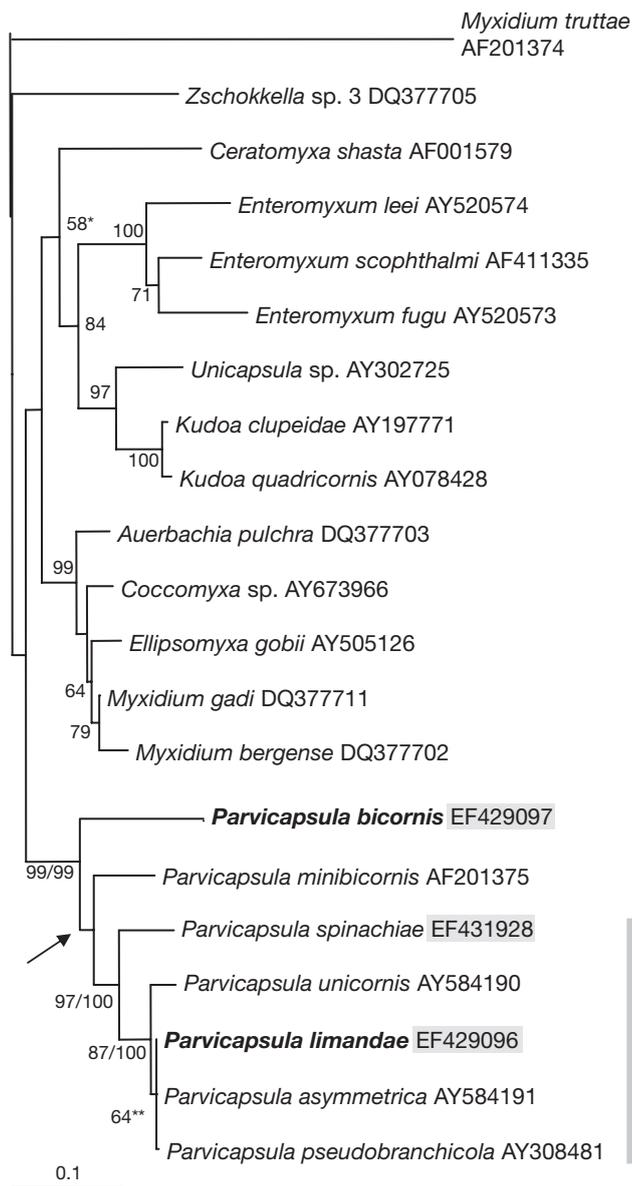


Fig. 5. Maximum likelihood distance tree, based on alignment of SSU rDNA sequences from 21 taxa with 965 characters, constructed using PAUP 4.0b10. Rooted with *Myxidium truttae*. Support values at nodes; ML bootstrap (PAUP). In the *Parvicapsula* clade, quartet puzzling (QP) support values (SV) are also shown except in ambiguous nodes (*). Only support values above 50% shown. The topology of the QP tree did not agree in the position of *Ceratomyxa shasta*, placed as a sister group to the others (DQ377705) with poor support (SV 58), or in placing *P. minibicornis* as a sister group to the other *Parvicapsula* spp. with strong support (SV 99). Arrow: the interrelationship between *P. bicornis*, *P. minibicornis* and the branch with the remaining *Parvicapsula* spp. ('core' group, gray vertical line) is not resolved in PAUP; *P. bicornis* sister group to the core group with SV 91 in QP. (**) the interrelationship between the 3 species in the terminal node (QP) is as follows: *P. pseudobranchicola* sister to *P. limandae* and *P. asymmetrica* (SV 74), the latter 2 species grouped with SV 90. Bar: 0.1 substitutions per site

platessoides by Kabata (1962). It is possible that re-examination of *S. irregularis* will show this species to belong to *Parvicapsula*.

Myxoproteus caudatus Shulman, 1953 in *Limanda limanda* has, apart from the filamentous caudal projection and some distance between the polar capsules, also some resemblance to *Parvicapsula bicornis*. This species has only been found in the White Sea and the Barents Sea (Shulman 1953, Polyanskii 1955).

Kabata (1962) described *Parvicapsula unicornis* from *Callionymus lyra* L. (Callionymidae), *Limanda limanda* L. (Pleuronectidae) and *Lepidorhombus whiffiagonis* (Walbaum) (Scophthalmidae). Kabata (1962) found the new species in 4 of 60 *C. lyra*, but in only 1 of 55 and 1 of 46 *L. limanda* and *L. whiffiagonis*, respectively. The genus *Parvicapsula* is apparently highly host specific, probably to genus and sometimes to species level. It is unlikely that *P. unicornis* occur in 3 different fish families. It is expected that the description by Kabata (1962) is based on specimens found in the first mentioned and most heavily infected host. Since *Callionymus lyra* is the first listed host, this species should be regarded as the type host of *P. unicornis*. The specimens found in *L. limanda* by Kabata (1962) might be identical with *P. limandae*, which superficially has some resemblance to *P. unicornis*. It would be interesting to know whether *L. whiffiagonis* is infected with a third species of *Parvicapsula*.

Plaice *Pleuronectes platessa* and dab *Limanda limanda* are common in the northern Øresund. The 0-groups of plaice were infected with *Parvicapsular bicornis*, indicating that these fish acquire the infection in shallow water above the halocline, which occurs at depths of 10 to 15 m, and that the invertebrate hosts probably live here. The salinities above and below the halocline are 12 to 20‰ and 25 to 30‰, respectively. On the other hand, the small specimens of dab live on the muddy bottom below the halocline and since they are infected with *Parvicapsula limandae*, the invertebrate hosts probably live in this area.

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Editorial responsibility: Dieter Steinhagen,
Hannover, Germany

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