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Endosymbionts of Two Species of Mediterranean Lucinid Clams:
A Molecular, Microbial and Histological Analysis

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Verfasserin:	Mauß Michaela
Matrikel-Nummer:	0126288
Studienrichtung /Studienzweig (lt. Studienblatt):	Diplomstudium Ökologie
Betreuer:	Steiner Gerhard Ao. Univ.-Prof. Dr.

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“Nothing in biology makes sense

except in the light of evolution.”

Theodosius Dobzansky (1919 – 1975)

“Nature does nothing uselessly.”

Aristotle (384 – 322 bf. Chr.)

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Introduction

Chemosymbiosis in Marine Invertebrates

The phenomenon of chemosymbiosis was first discovered in the hydrothermal vent tube worm *Riftia pachyptila* Jones, 1981 (Vestimentifera, Polychaeta) (Cavanaugh et al., 1981; Felbeck, 1981). During more than 25 years that passed since the discovery, this association of Metazoa with chemoautotrophic bacteria was described for numerous invertebrates from diverse habitats (reviewed in Cavanaugh, 1983; 1994; Dubilier et al., 2008).

Despite of the high variety of phyla and habitats, the partners in all of these symbioses profit from similar benefits. But dependent on characteristics of the chemosymbiosis one might benefit more. The prokaryote provides an additional food source for its invertebrate host or even takes over the whole nutrition (Dando et al., 1986; Schweimanns & Felbeck, 1985). This is made possible by the process of chemosynthesis during which reduced sulphur or methane compounds from the environment become oxidised while producing ATP (Distel, 1998). Following the chemical reaction the reducing substance is used as energy source in the Carbon-Benson cycle for the fixation of inorganic carbon (Felbeck et al., 1981; Schweimanns & Felbeck, 1985). Further the role of the bacterial symbionts in sulphide detoxification is discussed but less evident (Anderson, 1995; Gros et al., 2000).

On the other hand the bacterial partner seems to benefit from increased availability of substrates necessary for chemosynthesis, especially oxygen and sulphide or methane (reviewed in Cavanaugh, 1994). The host either pumps these substances to its symbiont via the inhalant tube like in bivalves (e.g. Dando & Southward, 1986) or the animal itself functions as a transporter for the bacterium (Katz et al., 2006). Thus, for sulphur-oxidising chemoautotrophs the range of habitats is expanded (Cavanaugh, 1994). The invertebrate host certainly profits from nutrition, whereas if symbionts are digested, as often proposed, they benefit less.

The spectrum of invertebrate hosts participating in this type of symbiosis ranges from vestimentiferan, pogonophoran, annelid and nematode worms to bivalves, gastropods (reviewed in Distel, 1998) and solenogastres (Katz et al., 2006). According to Bright and Giere (2005), chemosymbioses in annelids cover all expressions of the association from loose and occasional in tubificids over regular ectosymbiosis in alvinellids to obligatory extra- and intracellular incorporation like in

gutless oligochaetes. An example of the latter provides *Inanidrillus leukodermatus*, which harbours two morphotypes of a dominant symbiont (Dubilier et al., 1995). For a long time it was believed that, although structural polymorphism is possible, only one prokaryotic symbiont species is present in each host species. Further, the association was thought to be species specific (Distel et al., 1988), but advances in methods brought a change in this paradigm. Using DNA-DNA hybridisation, Edwards and Nelson (1991) found that the symbionts of *Riftia pachyptila* and *Tevnia jerichonana* belonged to the same bacterial species. The same was found in lucinid bivalves, where several species from the same habitat harboured the same symbiont species (Durand & Gros, 1996; Durand et al., 1996; Gros et al., 2000; 2003a).

Due to 16S rRNA sequence analysis and fluorescence *in situ* hybridisation it became obvious that in some host species involved in chemosymbiosis not a single, but two or even more prokaryotic species functioned as symbionts. In the gutless marine oligochaete *Olavius loisae*, for example Dubilier et al. (1999) discovered three potential symbiont phylotypes between cuticle and epidermal cells, belonging to the α- and γ-Proteobacteria, and spirochetes, respectively. A similar situation was observed in Mollusca as well. For example Katz et al. (2006) reported a large variety of α- and γ-Proteobacteria symbionts living epibiotic, endocuticular or even intracellular on *Helicoradomenia* spp. (Solenogastres) or γ- and ε-Proteobacteria were detected in gills of the gastropod *Alviniconcha* spp. (Urakawa et al., 2005).

In *Maorithyas hadalis*, a thyasirid clam inhabiting cold-seeps, two different symbiont phylotypes were detected in separate compartments of the gill filaments (Fujiwara et al., 2001). Outer regions of bacteriocytes contained a potential thioautotroph, further inward parts a symbiont of unknown physiological function, which is distantly related to free-living chemoautotrophic bacteria (Fujiwara et al., 2001). Other deep-sea bivalves like *Bathymodiolus* spp. and *Idas* sp. (Mytilidae) were reported to harbour up to 6 different intercellular bacterial species of γ-Proteobacteria parallel with thioautotrophic, methanotrophic or another unknown physiology (Duperron et al., 2005; 2007b; 2008).

In contrast to these multi-species symbioses the bivalve families Solemyidae, Vesicomyidae and Lucinidae harbour just a single symbiont strain. Apart from *Maorithyas hadalis* for other investigated Thyasiridae like *Thyasira flexuosa* only one extracellular prokaryotic symbiont is reported (Distel & Wood, 1992). Independent of the number of symbionts involved, the strategy of chemosymbiosis is most

widespread in bivalve molluscs (reviewed in Distel, 1998). Phylogenetic relations between symbiotic strains imply that chemosymbiosis independently developed several times within different lineages of invertebrates and bivalves (Distel et al., 1988; Eisen et al., 1992).

Chemosymbiosis in Lucinidae (Bivalvia: Mollusca)

Within the Bivalvia the family Lucinidae Flemming, 1828 belongs to the Heterodonta and therein to the superfamily Lucinoidea. The family Lucinidae branches off early within the Heterodonta. Phylogenetic analyses rooted the lineage either between Anomalodesmata and (*Hiatella arctica* + Pharidae) or it formed the sistergroup of the Anomalodesmata (Dreyer et al. 2003). The monophyly of the superfamily Lucinoidea was refuted in several studies. Steiner & Hammer (2000) showed that members of the Lucinidae and Ungulinidae group at different positions within a tree containing heterodonts. Further, analysis of genomic data from four families of the Lucinoidea revealed that Ungulinidae and Thyasiridae were unrelated to the Lucinidae (Williams et al., 2004) which is supported by morphological data (Taylor & Glover, 2006).

The diversity of Lucinidae is probably underestimated. For example 34 lucinid species with 18 of them being previously unknown were described in water depth less than 200 m for New Caledonia, the Loyalty Islands and Chesterfield Bank (Glover & Taylor, 2007). This can be seen as an indication of their potential diversity. The authors of the study even spoke of the most diverse assemblage of chemosymbiotic bivalves yet recorded and confirmed empirical observations estimating highest diversity of Lucinidae to occur in habitats associated with coral reefs of the Indo-West Pacific (Glover & Taylor, 2007). A total of 500 extant lucinid species worldwide are estimated (Taylor & Glover, 2006). At higher latitudes the family is less diverse. This for example becomes obvious by only eight records of shallow-water species from the Mediterranean Sea (CLEMAM, Checklist of European Marine Mollusca, http://www.somali.asso.fr/clemam).

Additionally to their species diversity lucinid clams were found burrowing in the sediment of a variety of habitats (Figure 1). Environments occupied by members of this family range from near shore shallow-water habitats (e.g. Durand et al., 1996) to bathyal depths (e.g. Duperron et al, 2007a). The occurrence of lucinids is often associated with sites of high organic input as it can be found in sea-grass beds (Durand & Gros, 1996), mangroves (Frenkiel et al., 1996; Schweimanns & Felbeck,

1985) and oxygen minimum zones (Cary et al., 1989). According to recent reports they are also present at cold seeps (Duperron et al., 2007a) or hydrothermal vents (Glover et al., 2004).

Many unusual anatomic features of the family Lucinidae were largely unexplained or misunderstood for a long time. They possess simplified, but thick and large gills, a highly reduced labial palp consisting of a small fold at the edge of the lips, a simple stomach and a short gut. These morphological features can be attributed to the nutritional dependence of the clam on thioautotrophic symbionts and are therefore adaptations to chemosymbiosis (Taylor & Glover, 2000; 2006). Along with anatomical simplification, physiological reliance on their endosymbiotic bacteria increases, which is supported by $\delta\ ^{13}C$ values (Le Pennec et al., 1995).

As suggested by their anatomical adaptations, all Lucinidae studied so far harbour intracellular sulphide-oxidising, chemoautotrophic prokaryotes within specialised cells, so called bacteriocytes, located in their gill filaments (Anderson, 1995; Taylor & Glover, 2000). Among the five bivalve families possessing chemosymbiosis, lucinid clams are by far the most diverse and geographically widespread ones (Distel, 1998). Further, Schweimanns & Felbeck (1985) considered endosymbiotic bacteria to be a species characteristic of the lucinid family. Analysing bacterial 16S rRNA Distel et al. (1988) were the first to show that prokaryotic endosymbionts fell within a limited group of the γ-subdivision of Proteobacteria.

The physiological importance of the endosymbionts being sulphur-oxidising chemoautotrophs was assigned in several studies (e.g. Anderson, 1995; Le Pennec et al., 1995; Schweimanns & Felbeck, 1985). Several studies located sulphur globules in *Codakia orbicularis* in the periplasmic space (Vetter, 1985) or in periplasmic vesicles (Lechaire et al., 2008) of bacteria. These sulphur deposits were interpreted as inorganic energy reserves allowing endosymbiotic bacteria to function even during temporary sulphur depletion (Vetter, 1985; Lechaire et al., 2008). Further activities of enzymes associated with the Calvin-Benson cycle like ribulose-1,5-bisphospate carboxylase (Schweimanns & Felbeck, 1985) or sulphide oxidation as APS reductase α subunit (Duperron et al., 2007a) were analysed. Positive records were understood as indication for the presence of endosymbiotic thioautotroph prokaryotes.

The phylogeny of endosymbionts based on 16S rRNA was the subject of a variety of studies. Two distinct clades, one consisting of bacterial symbionts of

Lucinidae (three species) and the vestimentiferan *Riftia pachyptila*, the other of a symbiont from a Vesicomyidae and Mytilidae each, were assigned (Distel et al., 1988). Analysis including more sequences from endosymbiont species of bivalves and other invertebrates support these first findings, since lucinid endosymbionts formed a clade with symbionts of Vestimentifera, Solemyidae and a thyasirid bivalve (Duperron et al., 2007a; Durand & Gros, 1996; Durand et al., 1996; Gros et al., 2003a). Distel et al. (1994) speak of a congruence between host and symbiont phylogeny which indicates shared evolutionary history of hosts and symbiont lineages and suggests an ancient origin for chemosymbioses in Bivalvia.

In contrast to the protobranch bivalve *Solemya velum,* where symbionts seem to be transmitted vertically (Krueger et al., 1996), transmission of lucinid endosymbionts is suggested to be environmental. Hints could be found in *Codakia orbicularis* using PCR amplifications of 16S rDNA, which were successful in metamorphosed larvae cultured on unsterilised sand, but failed with the ovar, egg, veligers and metamorphosed larvae cultivated on sterilised sand (Gros et al., 1996a). A similar experiment with other Lucinidae species harbouring the same bacterial symbiont was unsuccessfully in amplifying symbiont DNA targets from ovaries or testis (Gros et al., 1998a), which further supports the hypothesis of environmental transmission. Finally for *C. orbicularis* incorporation of bacteria could be shown (Gros et al., 1998b). Newly settled juveniles take up bacteria from the sediment by endocytosis at the apical poles of undifferentiated cells which afterwards differentiate into bacteriocytes (Gros et al., 1998b). Although none of the symbionts were cultivated so far, free-living forms of the endosymbionts of *C. orbicularis* could be detected in sediment samples from the sea-grass habitat of the bivalve host using fluorescence *in situ* hybridisation (Gros et al., 2003b).

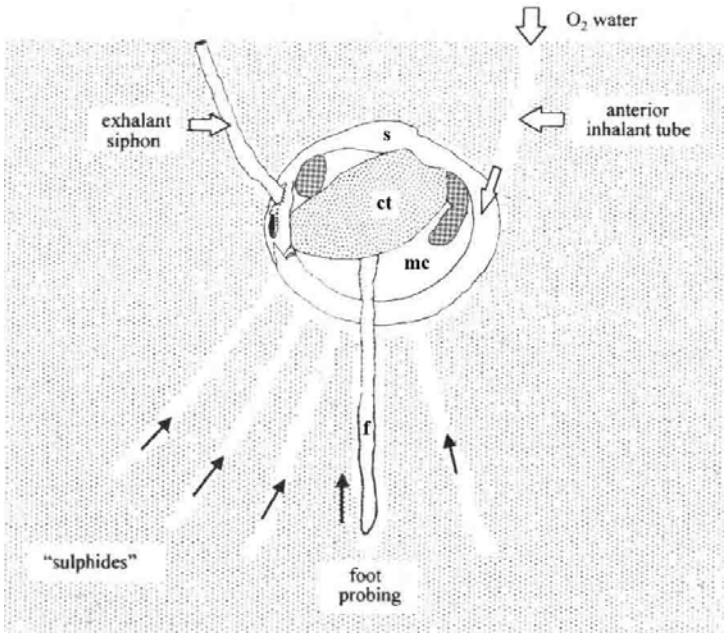


Figure 1: Diagram summarising the life position and major water currents of a typical lucinid based on *Codakia* spp.; ctenidium (ct), foot (f), mantle cavity (mc), shell (s) (adapted from Taylor & Glover, 2000).

Gill Anatomy and Endosymbiont Morphology

Ctenidia of lucinid clams, consisting of the inner demibranchs only, are large and thick compared to other heterodont bivalves (Taylor & Glover, 2000). Gill filament structure looks alike in all examined species with a ciliated zone similar to other Heterodonta (Taylor & Glover, 2006). Inwards an intermediary zone is located followed by a broad lateral zone (Taylor & Glover, 2000). The enlarged subfilamentar region consists of interfilamentar bridges between neighbouring filaments and connects inner and outer lamellae via interlamellar bridges (Dando et al., 1985) for a higher stability. In symbiont-bearing bivalves this subfilamentar region constitutes the lateral zone (Dando et al., 1985; Southward, 1986) (compare Figure 2).

The lateral zone comprises of different cell types varying in examined species (e.g. Frenkiel & Mouëza, 1995; Gros et al., 2003a; Southward, 1986). But the used nomenclature slightly differs in various studies, for example in Distel & Felbeck (1987) the intermediary zone is referred to as transition zone.

Chemoautotrophic sulphur-oxidising symbionts were only reported from the lateral zone (e.g. Frenkiel et al., 1996; Gros et al., 2000). Cell types other than bacteriocytes and intercalary cells, which are consistent within Lucinidae (Taylor & Glover, 2006), vary dependent on metabolic requirements of host and symbiont (Gros et al., 1996b).

Examples of different morphological features within various Lucinidae species are mucocytes (e.g. Distel & Felbeck, 1987; Frenkiel et al., 1996), granule cells (e.g. Frenkiel & Mouëza, 1995; Southward, 1986) or large granular inclusions (Herry et al., 1989; Dando et al., 1985), hemocytes (Frenkiel & Mouëza, 1995) and peroxisomes (Gros et al, 1996b). Intercalary cells are always found interspersed between bacteriocytes (e.g. Frenkiel & Mouëza, 1995), covering neighbouring cells with a thin epithelium densely packed with microvilli (Distel & Felbeck, 1987). Gros et al. (2000) distinguished two types based on their apical expansions.

In most Lucinidae species bacteriocytes form the dominant cell type within the lateral zone (e.g. Distel & Felbeck, 1987; Frenkiel & Mouëza, 1995). They are described as large rectangular to spherical cells with a basal, often irregular shaped nucleus and few cellular organelles (e.g. Distel & Felbeck, 1987; Johnson & Fernandez, 2001). They contain a single bacterium enclosed within a vacuole (Gros et al., 2000) in most cases. The actual structural details of the bacteriocytes slightly vary between species.

The most conspicuous inclusions of bacteriocytes, the bacteria, are enclosed within vacuoles (Gros et al., 2003a). The bacteria vary in outline, some being rod-shaped (e.g. Distel & Felbeck, 1987; Gros et al., 2000), others ovoid (e.g. Frenkiel & Mouëza, 1995; Herry et al., 1989) or coccoid (e.g. Cavanaugh, 1983; Southward, 1986) and possess the typical double membrane of Gram-negative bacteria (e.g. Gros et al., 2000; Herry et al., 1989). Bacterial cytoplasm may contain different structures like rosette-shaped particles in *Loripes lucinalis* (Herry et al., 1989) or clear vesicles, thought to function as storage of sulphur (e.g. Frenkiel & Mouëza, 1995; Herry et al., 1989) or glycogen-like particles (Gros et al., 2000).

Different bacterial endosymbionts seem to possess slightly different morphology dependent on species as well as on the bivalve host. This reflects the situation regarding cell types, where each host species holds unique features determining for their metabolic relationship with the endosymbiont (Gros et al., 1996b).

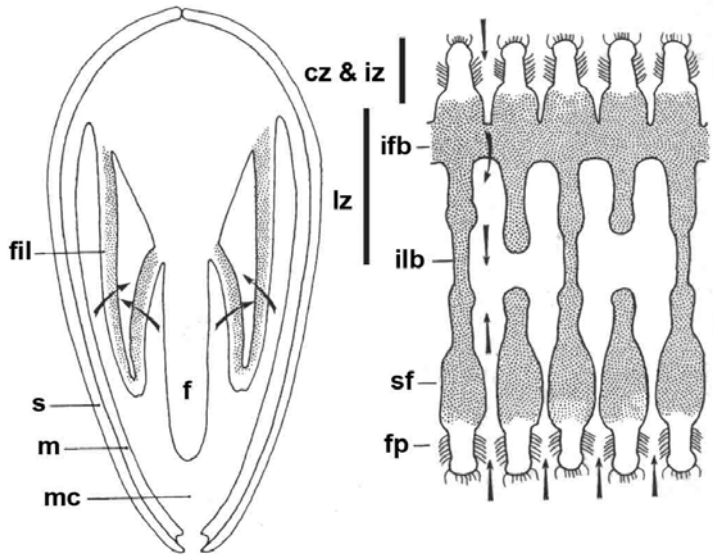


Figure 2: Diagram of the ctenidium based on *Myrtea spinifera*. (A) Vertical section through the animal. (B) Section through both gill lamellae. Arrows indicate direction of water flow, stipples the position of bacteriocytes; Foot (f), filament (fil), filament proper (fp), interfilamentar bridge (ifb), interlamellar bridge (ilb), mantle (m), mantle cavity (mc), shell (s), subfilamentar region (sf) (adapted from Dando et al., 1985).

Fluorescence in situ Hybridisation (FISH)

As a technique allowing simultaneous evaluation of phylogenetic identity, morphology, number and localisation of individual microbial cells, fluorescence *in situ* hybridisation (FISH) of whole cells using 16S rRNA targeted oligonucleotides is useful for many applications in all fields of microbiology (Amann et al., 1995; Moter & Göbel, 2000). Besides culturable even so far unculturable microorganisms can be detected which is helpful in understanding complex microbial communities (Moter & Göbel, 2000). FISH has been applied in natural as well as artificial ecosystems (Amann et al., 1995).

The method bases on oligonucleotide probes complementary to a specific target sequence in the ribosome which permeabilise microbial cells and hybridise to the target region. Since unbound probes are removed by a wash step, only specifically targeted cells retain probes (Hugenholz et al., 2001). 5'-end labelling with fluorochrome reporters enables a direct observation of the hybridisation signal (Hugenholz et al., 2001) using epifluorescence microscope, confocal laser scanning microscope or similar devices (Moter & Göbel, 2000). Limitations of this method are due to poor cell permeability, ribosome accessibility and content, and autofluorescence of the sample (Amann et al., 1995).

The identification of unknown bacterial cells within environmental samples remains the most important task for FISH. For example a new type of bacteria could be detected in a sample from hydrothermal vents representing about 40 % of the bacterial population (Harmsen et al., 1997). Regarding host organisms as a small ecosystem inhabited by few or only one well-adapted bacterial species the approach can also be applied for symbiosis (Amann et al., 1995). In bivalves the technique was first used in Solemyidae to confirm that the detected sequences belonged to the bacteria residing in the hosts gills (Krueger & Cavanaugh, 1997). In symbiosis with two or more chemoautotroph symbionts, FISH was used for local assignment (e.g. Fujiwara et al, 2001; Duperron et al., 2007b; 2008) and 3-dimentional FISH enabled quantification of volumes occupied by each symbiont type in bacteriocytes of *Bathymodiolus azoricus* (Halary et al., 2008).

Within this study the technique was applied to confirm that obtained sequences belonged to endosymbionts and to ascertain their number. Further endosymbionts could be located within the gill tissue (compare below).

Introduction of the Organisms and Previous Studies

The two investigated bivalve species *Loripes lacteus* (Linnaeus, 1758) and *Anodontia* (*Loripinus*) *fragilis* (Philippi, 1836) belong to the family Lucinidae. Both species occur in the Mediterranean (Riedl, 1983, p. 361; Taylor & Glover, 2005). *Loripes lacteus* has circular, milky-white and spiral sculptured shells that reach a maximum size of 20 mm. It inhabits muddy bottoms (Riedl, 1983) and *Cymodocea nodosa* sea-grass beds (Johnson et al., 2002). In comparison, the shells of the subcircular (maximum 11.8 x 13.5 mm), globose and semi-transparent *A. fragilis* can mainly be found in muddy sand and sea-grass beds from the intertidal to around 100 m depth (Taylor & Glover, 2005).

Several biodiversity investigations in the Mediterranean report the two species. Fischer (2005) counted 89 mollusc species at the Amvrakian Gulf, Greece, and among the 43 bivalves, *L. lacteus* was present at three, *A. fragilis* at one sampling site. Koulouri et al. (2006) found both species present when measuring molluscan diversity for a soft bottom sublittoral ecotone at Heraklion Bay, Greece, and described them as deposit feeders. Further, *A. fragilis* was found to be among the dominant species in the transition zone (20 - 35 m) between the shallow benthic biocoenosis and the deeper biocoenosis of coastal terrigenous mud.

L. lacteus was used for investigating the ecological importance of invertebrate chemoautotrophic symbiosis to phanerogam sea-grass beds. Johnson et al. (2002) estimated net primary production and autotrophic potential. It was concluded that this species plays an important role in the carbon cycle at *Cymodocea nodosa* beds at Corsica, France, arranging the return of symbiotically fixed carbon originating from mineralised organic material to the atmosphere via predation. Upon this path the carbon sink of the sea-grass community would be reduced.

Taylor and Glover (2005) gave a detailed description of anatomy, morphology, phylogeny and systematic taxonomy of the genus *Anodontia* including a discussion of *A. fragilis*. The bivalve was found to be the only Mediterranean species of the genus, so confusion with the similar *A. subfragilis* or any other *Anodontia* species can be excluded.

So far no description of the endosymbiosis by the means of molecular or morphological analysis of either species is available.

Aim of the Study

The aim of the present study was the description of endosymbionts of the two Mediterranean bivalve species *Loripes lacteus* (Linnaeus, 1758) and *Anodontia (Loripinus) fragilis* (Philippi, 1836) belonging to the family Lucinidae. The questions of concern were:

- How many different symbiotic bacterial strains occur in each bivalve species and to which bacterial taxon do they belong?
- Do the co-occurring bivalve species *L. lacteus* and *A. fragilis* harbour the same endosymbiont species?
- Where are the endosymbionts located?
- Are symbiosis-related gill structures of *L. lacteus* and *A. fragilis* similar or different to those of other examined lucinids?
- Are the new endosymbionts phylogenetically related with already described sulphur-oxidising ones?
- Do different bacterial cohorts settling in consecutive host generations cause the once observed gill colour variation in *L. lacteus*?

To answer these questions, different methods were used. All potential endosymbiotic bacterial species should be detected by sequence analysis of specimens collected at two different sampling times. Further, generated sequences allow the design of specific oligonucleotides for fluorescence *in situ* hybridisation (FISH), which is used for direct detection and localisation of the endosymbionts. The appliance of phylogenetic methods should show the relationship of detected to previously described endosymbionts. Endosymbiosis-related gill filament structures are examined by light and electron microscopy and compared to the literature.

Materials & Methods

Sampling

Specimens used for this study were collected in about three meters depth from a sea-grass bed in Val Vaborsa, Rovinj, (Croatia, Figure 3) in the Northern Adriatic from 27th – 29th of April and 2nd – 10th of July 2007. Sediment was put into a bucket, transported to the beach and sieved through a sieve of 1 mm mesh size from which lucinid clams could be sorted out. Two species could be distinguished. The first was identified from literature (Riedl, 1983, p. 361) as *Loripes lacteus* (Linnaeus, 1758). The second was recognised as *Anodontia (Loripinus) fragilis* (Philippi, 1836) by John Taylor (Natural History Museum, London) from photographs of the shells (inside, outside and side view) using anterior and posterior adductor muscle scars. Specimens were either processed further shortly after collection in the field or transported to the laboratory at the University of Vienna. Then specimens were opened by cutting the adductor muscles to be fixed or processed according to the methods applied. Samples collected in July were fixed in 70 °C ethanol only and used for molecular processing as described below.

Figure 3: Map of Rovinj (Croatia), the surrounding villages and bays. The Northern Adriatic Sea is coloured in black, land in shades of grey. A white cross indicates the sample site, the bay at Val Vaborsa. (Image adapted from Google Earth 2008.)

Overall Morphology of the Two Lucinids

Shells of individuals of both bivalve species whose gills were fixed for fluorescent *in situ* hybridisation (FISH) and transmission electron microscopy (TEM) (see below) were exposed to caustic potash solution to corrode rests of muscle tissue attached to them. After this procedure the anterior and posterior adductor muscle scars could be used for identification. Under a binocular (Macroscop M420, Wild), cleaned shells were photographed inside, outside and from the side using a Nikon Coolpix 4500 digital camera.

Morphological Analysis of the Endosymbionts

Endosymbionts were analysed morphologically using light and transmission electron microscopy (TEM). The structure of the gills is described, with focus on the parts containing endosymbionts.

a) Light Microscopy

Sections of gill tissue mounted on optic slides not used for FISH were stained with haemalaun-eosin solution after Meyer (Romeis, 1989) for light microscopic analysis. For removal of paraffin, slides were treated two times for 5 min with 100 % xylene followed by two treatments with isopropanol (5 min). Afterwards, rehydration was performed in a descending ethanol series of 90 °C, 80 °C and 70 °C and distilled water (dH_2O) (5 min each). Tissue sections were stained in haematoxylin for 15 min and differentiated in running tap water for 17 min followed by a short rinse in dH_2O and a second staining in eosin for 5 min. Next, sections were shortly rinsed in dH_2O and differentiated in 70 °C of ethanol before they were dehydrated in an ascending ethanol series (96 °C for 5 min, 2 x 100 °C for 3 min). Finally tissue sections were treated two times with 100 % xylene before covering with Biomount 2 mounting medium (British Biocell International) and a cover slip.

Light microscopic analyses of haemalaun-eosin as well as toluidine blue (see preparations for electron microscopy) stained sections were performed using an Eclipse E800 microscope (Nikon) and a Digital Sight DS-U1 digital camera with a FDX-35 Data Mask (Nikon). Adobe Photoshop CS2 v9.0 and CorelDraw v11.0 were used for processing the digital images.

b) Transmission Electron Microscopy (TEM)

Part of the specimens of *L. lacteus* (five individuals) and *A. fragilis* (four individuals) collected in April 2007 (see above) were used for TEM analysis. Gills were cut into small pieces (approximately 1 mm^2) and prefixed in cooled 2.5 % glutaraldehyde with 0.1 M sodium cocadylate buffer (pH 7.3) and 5 % sucrose over night (about 18 hours) at approximately 4 °C. After fixation the gill pieces were rinsed three times for 10 min in 0.1 M Na-cocadylate buffer with 5 % sucrose (pH 7.3) and samples processed in the field were transported in the third buffer to the laboratory at the University of Vienna. Samples were postfixed for 2 hours in 1 % osmium tetroxide in the same buffer. Afterwards, gill pieces were rinsed three times in dH_2O (10 min) followed by an ascending ethanol series with repetition of each step (30 °C, 50 °C, 90 °C, 96 °C and 100 °C for 15 min each) for dehydration. Then samples were treated two times for 15 min with a mixture of 100 °C ethanol and 100 % acetone followed by treatment with 100 % of acetone for another 15 min.

Samples were embedded in Agar Low Viscosity Resin (LV, Agar Scientific), after infiltration of gill tissues in a mixture of 100 % acetone and resin at a ratio of 1 : 1 for 2.5 hours followed by infiltration in pure resin for 4 hours. Afterwards samples were maintained in pure resin over night (approximately 17 hours) and thereafter embedded in fresh resin. The resin containing gill samples were polymerised for 24 hours at 60 °C in the oven.

Gills were sectioned on an Ultracut E ultramicrotome (Reichert & Jung) using glass knives. These knives were broken from glass blocks (Leica) using a Knifemaker II with Symparter (Reichert & Jung) or an EM KMR2 (Leica). Semi-thin sections (1 µm) were stained with 0.1 % toluidine blue in 2.5 % borax solution, differentiated under running tab water, covered with Biomount 2 mounting medium (British Biocell International) and a cover slip and analysed on an Eclipse E800 microscope (Nikon) as described above. Ultra-thin sections (70 nm) were mounted on grids (G202 Old 200 copper 3.05 mm grids, Athene®) or formvar coated slot grids (G2500C 2 mm x 1 mm slot copper 3.05 mm grids, Agar Scientific; formvar solution: 0.2 g formvar in 100 ml dioxin). Prior to analysis ultra-thin sections were contrasted for 30 min in 2 % aqueous uranyl acetate, rinsed three times in dH_2O, contrasted another 6 – 7 min in lead citrate and rinsed again three times in dH_2O. After grids dried at least for 30 min, sections were examined with a TEM 902 microscope

(Zeiss). Pictures were processed using Adobe Photoshop CS2 v9.0 and CorelDraw v11.0 as for light microscopic images.

DNA-Isolation, PCR and Sequence Analysis

Specimens were partly frozen in liquid nitrogen and partly conserved in 70 °C ethanol, which was changed two times. Total DNA was isolated from gill tissue using peqGOLD Tissue DNA Mini Kit (PEQLAB Biotechnologie GmbH) following the manufacturer's instructions with slight modifications: for drying, samples were spinned at 14.000 rpm instead of 10.000 rpm and finally eluted in 100 µl of elution buffer pre-heated to 70 °C. Concentrations of total DNA were measured on a BioPhotometer 6131 (Eppendorf) to estimate the right concentrations of total DNA used in PCR amplification.

The 16S rDNA gene encoding rRNA (further referred to as 16S rRNA) of the bacterial endosymbionts was amplified from *Loripes lacteus* (three individuals from April and July each) and from *Anodontia fragilis* (three individuals from April and two from July) using the specific primer 616V, 630R (Juretschko et al., 1998) and 1492 R (Lane, 1991; compare Table 1). The 25 µl reaction for PCR contained 2.5 µl of 10 x buffer, 0.8 µl of 50 mM MgCl, 2.5 µl of 2.5 mM dNTP mix (each dNTP at 370 µl; dNTP-Set, Bioline), 0.2 µl of 50 pmol forward and reverse primers and 0.75 µl of Taq polymerase (1 unit/µl Mango Taq DNA Polymerase, Bioline) and was filled up with double distilled water (dH_2O) to the final volume. Between 10 and 15 ng/µl of total DNA were applied to the reaction buffer. For PCR a thermo cycler Primus 96 advanced (PEQLAB, Biotechnologie GmbH) was used. The cycler protocols are presented in Table 2.

The obtained PRC products were separated by electrophoresis using a 1.5 % agarose gel (peqGOLD universal Agarose, PEQLAB Biotechnologie GmbH) at 100 V. Resulting gels were stained with ethidium bromide and visualized under UV transillumination. Afterwards amplified products of the correct fragment length of approximately 1500 bp (Durand et al., 1996) were purified using peqGOLD Cycle-Pure Kit (PEQLAB Biotechnologie GmbH) following manufacturer's instructions with slight modifications. These were: two times washing of the filters with 700 µl of SPW-wash buffer followed by drying through spinning at 14.000 rpm in the centrifuge instead of 10.000 rpm. The sample was eluted in 30 µl of elution buffer. Purified amplificates were controlled again on a 1.5 % agarose gel at 100V.

Purified PCR products were cloned into a plasmid with a TOPO-TA Cloning® Kit (Invitrogen) using pCR® II TOPO Vector and TOPO 10 potent cells (Invitrogen) as described in the manufacturer's instructions. After growth over night at 37 °C, several randomly chosen single colonies were transferred from the plate into 25 µl reactions for PCR. These consisted of 2.5 µl of 10 x buffer, 0.8 µl of 50 mM MgCl, 2.5 µl of 2.5 mM dNTP mix (each dNTP at 370 µl; dNTP-Set, Bioline), 0.5 µl of 0.1 µg/µl vector specific forward and reverse primers (see Table 1) and 0.75 µl of Taq polymerase (1 unit/µl Mango Taq DNA Polymerase, Bioline) and was filled up with dH_2O to the final volume. The PCR protocol is presented in Table 2. Clone products were checked on

Table 1: Primers used for amplification and sequencing.

	Name	Sequence	Comments	Reference
PCR				
	616V	5'-AGAGTTTGATYMTGGCTC-3'	most eubacteria, archaebacteria	Juretschko et al., 1998
	630R	5'-CAKAAAGGAGGTGATCC-3'	most eubacteria, archaebacteria	Juretschko et al., 1998
	1492R	5'-GGYTACCTTGTTACGACTT-3'	most eubacteria, archaebacteria	Lane, 1991
Cloning				
	M13F	5'-GTAAAACGACGGCCAG-3'	vector specific	manufacturer's protocol
	M13R	5'-CAGGAAACAGCTATGAC-3'	vector specific	manufacturer's protocol
Sequencing				
	TopoSeq-F	5'-TCTAGATGCATGCTCGA-3'	vector specific	not published
	TopoSeq-R	5'-AGCTTGGTACCGAGCT-3'	vector specific	not published

Table 2: PCR-protocols for amplification of 16S rRNA, direct PCR after cloning, and the sequencing reaction.

	Process	Temperature	Duration
16S rRNA			
initial denaturation		94 °C	3 min
35 cycles	denaturation	94 °C	30 sec
	annealing	50 °C	30 sec
	extension	72 °C	1:30 min
final extension		72 °C	15 min
Screening[a]			
initial denaturation		94 °C	3 min
30 cycles	denaturation	94 °C	15 sec
	annealing	55 °C	20 sec
	extension	72 °C	1 min
final extension		72 °C	10 min
Sequencing reaction			
25 cycles	denaturation	96 °C	20 sec
	annealing	48 °C	10 sec
	extension	60 °C	4 min

[a] direct PCR after cloning

a 1.5 % agarose gel at 100 V and amplificates of the accurate length purified using peqGOLD Cycle-Pure Kit as described above.

PCR products from cloning reactions were directly sequenced on the capillary sequencer ABI 3130xl Genetic Analyser applying Data Collection Software v3.0. The 10 µl sequencing reaction consisting of 1 µl Big Dye v3.1 (Bioline), 1 µl of further inward vector specific forward and reverse primers (see Table 1), 2 µl of cloning product, filled up with double distilled water to the final volume was provided.

Sequenced fragments were edited with Finch TV v1.4.0 (Geospiza Inc.) and afterwards checked for similarities using a BLAST search (Basic Local Alignment Search Tool; Altschul et. al., 1997) (http://blast.ncbi.nlm.nih.gov). The overlap of forward and reverse fragments was controlled with an internet tool for pairwise alignment (http://pir.georgetown.edu\pirwww\search\pairwise.shtml). For further processing and contig construction, sequences were imported into ARB (from Latin *arbor*, tree; Ludwig et al., 2004), a software environment created for processing sequence data.

Fluorescence in situ Hybridisation (FISH)

Fluorescence *in situ* hybridisation was used to detect the bacterial endosymbionts in gill tissues directly based on the generated sequence data. Therefore the design of symbiont specific probes based on the 16S rRNA gene sequence (encoding for rRNA) gained from *Loripes lacteus* and *Anodontia fragilis* was necessary. Parallel gill tissue needed to be prepared for hybridisation.

a) Sequence Processing and Probe Design

Sequences of the 16S rRNA gene (encoding for rRNA) obtained from gill tissue of *L. lacteus* and *A. fragilis* were imported into ARB (from Latin *arbor*, tree; Ludwig et al., 2004), where they could be processed further. Additionally the eleven published sequences from the NCBI database (National Center for Biotechnology Information, U.S.; http://www.ncbi.nlm.nih.gov/) showing highest similarities in a BLAST search with obtained sequences were added to the ARB database.

First forward and reverse fragments of the obtained sequences were combined creating contigs from the partial sequences. Next newly generated complete sequences (approximately 1500 bp long) and the eleven published ones were aligned by the Editor of the ARB software and corrected manually. Therefore

the alignment was controlled in ARB and for questionable regions the corresponding chromatogram files of obtained sequences were checked using Finch TV v1.4.0 (Geospiza, Inc.).

After correction sequences were added to an existing tree consisting of Gamma-Proteobacteria in the ARB database. A similarity matrix was calculated from pairwise distances between all generated sequences from endosymbionts of the two bivalve species using ARB phylogenetic tools. The similarity matrix was exported into Microsoft Exel 2003 for further analysis. For each of the host species P-distances indicated monophyly for all obtained endosymbiont sequences. P-distances within endosymbiont sequences from *L. lacteus* were smaller than P-distances to published sequences with highest similarities. The same was true for P-distances calculated for endosymbiont sequences of *A. fragilis*. According to this finding two symbiont sequences of *L. lacteus* and *A. fragilis* (one from April and July each) were chosen randomly and used to calculate a similarity matrix from P-distances with 42 published sequences from GenBank (Benson et al., 2008; http://www.ncbi.nlm.nih.gov; compare phylogenetic analysis).

Clone specific probes for fluorescent *in situ* hybridisation were designed using the Design Probes tool in ARB as described by Kumar et al. (2005). Probe design was restricted to 16S rRNA sequences from gill tissue of *L. lacteus* and *A. fragilis* that clustered with published sequences of other bivalve or vestimentiferian endosymbionts in the database and could therefore be identified as endosymbiotic of the two investigated clam species. Sequences not clustering with published ones (from *L. lacteus* only) and therefore probably belonging to environmental bacteria were ignored for the probe design. Possible probes returned by the program were manipulated to increase weighted mismatches with non-target species and afterwards tested to determine the best fitting ones engaging the Check Probe feature. This program element controlled the probes for possible non mismatches or low weighted mismatches with sequences from the database. The results had to be evaluated depending on their relationship to the sequences from *L. lacteus* and *A. fragilis*.

Although ARB provides tools showing secondary structure and estimating accessibility of the probe region (Kumar et al., 2005) those were not employed, because the secondary structure is not primarily restricting probe access. Ribosomal proteins constitute the main reason for inaccessibility. The model for probe

accessibility prediction in ARB bases on a flow cytometry analysis of *Escherichia coli* 16S rRNA performed by Fuchs et al. (1998). Since the endosymbionts of *L. lacteus* and *A. fragilis* are no close relatives of *E. coli*, this model does not give a good estimation of probe accessibility for the investigated symbionts and was not used in this study. Instead, designed probes were tested empirically performing hybridisations to select the best ones and excluding those giving no or weak signals.

Further an existing probe for *Codakia orbicularis* (Gros et al., 2003b) was compared with and adapted to the generated sequences from endosymbionts of the bivalve species. Moreover, the best fitting probes were controlled by the internet tool probeCheck (http://131.130.66.200/cgi-bin/probecheck/probecheck.pl) for additional non mismatches or low weighted mismatches with other sequences from different databases. Last, by applying probeBase (Loy et al., 2003; 2007), generated sequences were searched for additional, less specific probes from the internet database, which could be combined with the specific ones in the hybridisation. Newly designed as well as other used oligonucleotide probes are listed in Table 3.

The generated oligonucleotide probe SymLl1251 was found to be the best fitting for *L. lacteus* out of three probes tested. This probe was based on the *C. orbicularis* endosymbiont-probe from literature (Gros et al., 2003b), but had to be modified to fit for the analysed species. It consisted of 18 bp (nucleotide position 1251 – 1268 based on *Escherichia coli* numbering) and had the sequence 5'- CGC GGG TTC GCG GCT CTC -3'. This newly designed probe was also specific for endosymbionts of the lucinids *C. costata* and *Lucina floridana*.

For *A. fragilis* two probes were selected from a set of six tested ones. The probe SymAf576 consisted of 20 bp (nucleotide position 576 - 595) with the sequence 5'- GAC TTG GCC GCC TAC GCA CG -3' and was also specific for endosymbionts of the near relative *A. phillipiana*. Parallel, a second probe, which was not specific for the relative's endosymbionts but for *Achromatium* sp. and an unknown γ-Proteobacteria as well as bacterium UMB8C was used. This other probe was 23 bp long (nucleotide position 1272 - 1294) and was called SymAf1272. The position was chosen according to the same probe sequence as for *L. lacteus*, but moved further back to exclude several non-specific matches. The probe sequence was 5'- CCG GTT TTG TGA GAT TAG CTC CC -3'.

All three probes were specific for all sequences of potential endosymbionts of either species regardless of sequences quality. Even clone sequences originally

excluded from probe design due to low sequences quality were indicated to be accessible by designed probes according to ARB.

Newly designed probes for endosymbionts of both lucinid species were hybridised together with probes for all bacteria (Eub338-Mix) and γ-Proteobacteria (Gam42a, which needed the competitor Bet42a) as well as a probe for negative control (Nonsense, compare Table 3). It was found that the bacteria-probe gave the best impression, if all bacterial cells in the tissue belonged to one endosymbiont or if there was another one missing, which might have been overlooked with the γ-Proteobacteria probe.

Since fluos-dye did not give a well detectable signal in the gill tissue, only the stains Cy3 and Cy5 could be used. The specific probe designed for *L. lacteus* was labelled with the fluorescent dyes Cy3 (red) and Cy5 (blue) whereas the *A. fragilis* specific probes were labelled with Cy5 only. Oligonucleotide probes were manufactured by Thermo Electron GmbH (Thermo Fischer Scientific, Inc.). For the final analysis the endosymbiont specific probes stained with Cy5 and the bacteria probe stained with Cy3 were found to give the best observable signals. This setting was used for the pictures presented in the results.

b) Gill Tissue Preparation and Hybridisation

Both gills of some specimens of each clam species collected in April 2007 (as described above) were fixed in 4 % paraformaldehyde/0.1 M phosphate buffer after Sorensen (PBS) (pH 7.4) containing 10 % (w/v) sucrose at approximately 4 °C for 12 hours as described by Nussbaumer et al. (2006). They were rinsed three times in 0.1 M PBS for 10 min and samples processed in the field were kept in the last phase of PBS for transportation. All samples were then partially dehydrated in an ascending ethanol series of 30 °C, 50 °C, 70 °C, 80 °C and 90 °C for 15 min each, followed by three times 15 min in 100 °C ethanol. For better infiltration the gills were transferred into 100 % xylene for 15 min + 20 min + 20 min.

Two changes of paraffin at 58 °C (45 min and 120 min) preceded the over-night infiltration. After about 17 hours they were embedded in paraffin using paper boxes of 1.5 cm x 2 cm x 1.5 cm in size. For embedding gill tissues were orientated upright along the longer axis of a cross. Paraffin was then cooled at room temperature and after a few minutes at approximately 4 °C in the refrigerator until the paraffin was completely solid.

Horizontal sections (thickness 4 µm) of the gills were cut using a Biocut 2030 microtome (Reichert & Jung) and mounted on Teflon and poly-L-lysine coated optic slides with differently sized wells (Ø 14 mm: Diagnostic Microscope Slides, Apogent, Erie Scientific Company, Portsmouth; Ø 6 mm: Microscope slides with black epoxy resin colour mask, Marienfeld GmbH & Co. KG). Slides with sectioned gill tissue were further hybridised with fluorescent labelled oligonucleotide probes or stained for light microscopic analysis (see above).

Preparations for hybridisation including deparaffinisation and permeabilisation were performed as described by Duperron et al. (2005) with slight modifications. Paraffin was removed from sections using three 10 min treatments of xylene followed by a rehydration in a decreasing ethanol series of 96 °C, 80 °C and 70 °C of ethanol for 10 min. Tissues were then permeabilised for 12 min (instead of 10 min) in 0.2 M HCl, rinsed in 20 mM Tris-HCl buffer (pH 8), permeabilised again for 5 min in Tris-HCl buffer containing 0.5 µg of proteinase K ml^{-1} at 46 °C (instead of 37 °C) and at last rinsed for 10 min in 20 mM Tris-HCl buffer. After drying at 46 °C in the oven sections on slides with large wells (Ø 14 mm) were circled with a Liquid Blocker Super Pap Pen (Daido Sangyo Co., Ltd. Tokyo, Japan) to prevent the hybridisation buffer from running off.

Hybridisation of tissue sections of *L. lacteus* (nine individuals) and *A. fragilis* (four individuals) was performed in hybridisation chambers containing one optic slide and a paper tissue wetted with hybridisation buffer consisting of 180 µl of 5 M NaCl,

Table 3: Oligonucleotide probes applied in this study with necessary additional information. All probes were used with 10 % of formamide.

Probe	Specificity	Probe-Sequence	Target Site	Reference
Target 16S rRNA				
Nonsense	negative control	5'- AGA GAG AGA GAG AGA GAG -3'	-	not published
Eub338	most Bacteria	5'- GCT GCC TCC CGT AGG AGT -3'	338-355	Amann et al., 1990
Eub338 II	Planctomycetales	5'- GCA GCC ACC CGT AGG TGT -3'	338-355	Daims et al., 1999
Eub338 III	Verrucomicrobiales	5'- GCT GCC ACC CGT AGG TGT -3'	338-355	Daims et al., 1999
SymLl1251	*L. lacteus* symbiont	5'- CGC GGG TTC GCG GCT CTC -3'	1251-1268	this study
SymAf576	*A. fragilis* symbiont	5'- GAC TTG GCC GCC TAC GCA CG -3'	576-595	this study
SymAf1272	*A. fragilis* symbiont	5'- CCG GTT TTG TGA GAT TAG CTC CC -3'	1272-1294	this study
Target 23S rRNA				
Gam42a	γ-Proteobacteria	5'- GCC TTC CCA CAT CGT TT -3'	1027-1043	Manz et al., 1992
Bet42a	β-Proteobacteria, CompetitorGam42a	5'- GCC TTC CCA CTT CGT TT -3'	1027-1043	Manz et al., 1992

20 µl of 1 M Tris-HCl at pH 8, 699 µl dH_2O, 10 % formamide and 1 µl of 10 % sodium dodecyl sulphate (SDS). Hybridisation buffer was applied on the tissue sections within the wells (10 µl on small, 20 µl on large wells) and the probes added (2 µl/probe on small, 4 µl/probe on large wells). The used oligonucleotide probes are listed in Table 3. Incubation of the slides within the hybridisation chamber was performed for 4 hours at 46 °C in the oven.

After incubation slides were washed in washing buffer (4.5 ml of 5 M NaCl, 1 ml of Tris-HCl at pH 8.0 filled up to a final volume of 50 ml with dH_2O) preheated to 48 °C in the water bath for 10 min, briefly rinsed with Milli-Q water and dried under compressed air. Hybridised slides were either mounted in anti-fading glycerol/PBS medium (Citifluor AF1, Citifluor) and covered by a cover slip for analysis or stored at -25 °C until examination. Visual section analysis was carried out on a LSM 510 Meta confocal laser scanning microscope (Zeiss). For picture processing and measuring of endosymbiont size LSM Image Browser v4.2 (Zeiss) was applied. Scale bars were included in Adobe Photoshop CS2 v9.0.

Phylogenetic Analysis

For phylogenetic analysis 42 sequences of the 16S rRNA gene with at least 1250 bp length were chosen from the NCBI database (http://www.ncbi.nlm.nih.gov/). 21 sequences belonged to endosymbionts of bivalves (five different families), nine originated from vestimentiferian tubeworms, two from oligochaetes, one from a nematode and four were free-living γ-Proteobacteria (compare Table 4). Five free-living bacteria (*Agrobacterium thumefaciens, Rickettsia rickettsii, Cytophaga* sp., *Chlamydia trachomatis* and *Clostridium butyricum*) served as outgroup.

All sequences were imported into ARB (Ludwig et al., 2004), aligned and integrated into an existing tree containing sequences of free-living and symbiotic γ-Proteobacteria, as is the common method when working with ARB. Chosen published sequences together with two randomly picked endosymbiont sequences of both investigated bivalve species (one collected in April and July 2007 each) were used for the calculation of a similarity matrix based on P-distances (see above). Additionally, a phylogenetic analysis using maximum parsimony with bootstrap analysis (100 replicates) and maximum likelihood using PHY ML (DNA) with a HKM-model and AxML were calculated with the ARB program package.

The main phylogenetic analysis was performed using the program package PAUP* 4.0b10 (Swofford, 1998) and an exported alignment from ARB. For all analysis characters from the beginning and the end of the alignment were excluded (position 1 - 40 and 1617 - 1677). For maximum parsimony (MP) analysis uninformative characters were excluded. A heuristic search strategy with an initial search using 500 random addition sequences and branch swapping keeping the 100 most parsimonious trees of each replicate was performed. This search was repeated by branch swapping without restriction of the numbers of trees. Additionally a bootstrap analysis (10.000 replicates) was calculated using a heuristic search with three random replicates.

For maximum likelihood (ML) analysis the best fitting model from Modeltest 3.6 (Posada and Crandall, 1998) being the GTR+I+G model selected by ACI was applied for the data set. Branch swapping employing the strict and majority rule consensus trees gained by parsimony analysis as starting trees was performed. (For commands used in PAUP* see Figure 4).

Table 4: Sequences from NCBI database used for phylogenetic analysis with NCBI accession number. Sequences mainly belong to endosymbionts and a few free living sulphide oxidising bacteria. Bacteria other than Gamma-Proteobacteria were used as out-group taxa.

NCBI Accession No.	Host Name	Taxonomy	Sequence Length
L25711	*Anodontia phillipiana*	Lucinidae, Bivalvia	1463 bp
L25712	*Codakia costata*	Lucinidae, Bivalvia	1493 bp
X84979	*Codakia orbicularis*	Lucinidae, Bivalvia	1502 bp
L25707	*Lucina floridana*	Lucinidae, Bivalvia	1359 bp
X95229	*Lucina nassula*	Lucinidae, Bivalvia	1482 bp
X84980	*Phacoides (Lucina) pectinata*	Lucinidae, Bivalvia	1502 bp
M99448	*Lucinoma aequizonata*	Lucinidae, Bivalvia	1340 bp
AM236336	*Lucinoma aff. kazanii*	Lucinidae, Bivalvia	1505 bp
AJ441189	*Archarax johnsoni*	Solemyidae, Bivalvia	1371 bp
U41049	*Solemya occidentalis*	Solemyidae, Bivalvia	1374 bp
U62130	*Solemya pusilla*	Solemyidae, Bivalvia	1282 bp
L25709	*Solemya reidi*	Solemyidae, Bivalvia	1413 bp
U62131	*Solemya terraeregina*	Solemyidae, Bivalvia	1505 bp
M90415	*Solemya velum*	Solemyidae, Bivalvia	1460 bp
AB042413	*Maorithyas hadalis*	Thyasiridae, Bivalvia	1467 bp
L01575	*Thyasira flexuosa*	Thyasiridae, Bivalvia	1497 bp
AB036709	*Bathymodiolus septemdierum*	Mytilidae, Bivalvia	1455 bp
AM402956	*Idas sp.*	Mytilidae, Bivalvia	1492 bp
AB044744	*Calyptogena fossajaponica*	Vesicomyidae, Bivalvia	1467 bp
AF035725	*Ectenagena ectenta*	Vesicomyidae, Bivalvia	1429 bp
AF035726	*Vesicomya gigas*	Vesicomyidae, Bivalvia	1429 bp
AY129107	*Escarpia laminata*	Vestimentifera, Siboglinidae	1374 bp
AF165909	*Escarpia spicata*	Vestimentifera, Siboglinidae	1461 bp
U77481	*Lamellibrachia columna*	Vestimentifera, Siboglinidae	1459 bp
AY129114	*Oasisia alvinae*	Vestimentifera, Siboglinidae	1374 bp
U77478	*Riftia pachyptila*	Vestimentifera, Siboglinidae	1461 bp
DQ66082	*Rigdeia piscesae*	Vestimentifera, Siboglinidae	1463 bp
AY129105	*Seepiophila jonesi*	Vestimentifera, Siboglinidae	1374 bp
AY129118	*Tevnia jerichonana*	Vestimentifera, Siboglinidae	1374 bp
AF165907	Vestimentiferian endosymbiont	Vestimentifera, Siboglinidae	1449 bp
U24110	*Inanidrilus leukodermatus*	Oligochaeta, Annelida	1482 bp
AF104472	*Olavius loisae*	Oligochaeta, Annelida	1487 bp
U14727	*Laxus* sp.	Nematoda	1401 bp
	free living Bacteria		
AF069959	*Thiomicrospira crunogena*	Gamma-Proteobacteria	1491 bp
AF016046	*Thiomicrospira thyasirae*	Gamma-Proteobacteria	1424 bp
L40993	*Thiotrix nivea*	Gamma-Proteobacteria	1421 bp
U32940	*Thiotrix ramosa*	Gamma-Proteobacteria	1429 bp
EU592041	*Agrobacterium tumefaciens*	Alpha-Proteobacteria	1519 bp
U11021	*Rickettsia rickettsii*	Alpha-Proteobacteria	1479 bp
AJ431238	*Cytophaga sp.*	Sphingobacteria	1504 bp
D89067	*Chlamydia trachomalis*	Chlamydiae	1548 bp
M59085	*Clostridium butyricum*	Clostridia	1516 bp

```
[! ---*** parsimony analysis ***---]
begin paup;
log start file=alle_pars.log;
exclude 1-40 1617-1677;
exclude uninf;
set increase=auto tcompress=yes;
hsearch addseq=random nreps=500 nchuck=100 chuckscore=10;
hsearch start=current nchuck=0 chuckscore=0;
describe 1 /plot=no;
savetrees all file=alle_pars.tre;
contree all / majrule=yes file=alle_pars_con.tre;
log stop;
end;
[! ---*** parsimony bootstrap ***---]
begin paup;
log start file=alle_pars_boot.log;
bootstrap nreps=10000 search=heuristic / addseq=random nreps=3;
savetrees from=1 to=1 file=alle_pars_boot.tre savebootp=nodelabels;
log stop;
end;

[! ---*** likelihood analyse ***---]
begin paup;
log start file=alle_ml.log;
include all;
exclude 1-40 1617-1677;
set increase=auto tcompress=yes;
set crit=like;
[!
Likelihood settings from best-fit model (GTR+I+G) selected by AIC in Modeltest 3.6
]
Lset Base=(0.2536 0.2103 0.3034) Nst=6 Rmat=(0.9752 2.9852 1.3602 1.0277 4.4970)
Rates=gamma Shape=0.4446 Pinvar=0.2235;
gettrees file=alle_pars_con.tre;
hsearch start=current;
describe 1 /plot=phylo;
savetrees all file=alle_ml.tre brlens=yes;
log stop;
end;
```

Figure 4: Commands included in the NEXUS-file executed in PAUP* for phylogenetic analyses.

Results

Aspects of the General Morphology

Loripes lacteus is characterised by its typical cradle-shaped umbo, and the white nearly circular shell with concentric growth increments. The shell diameter varies from a maximum of 14 mm to a minimum of 6.5 mm. In the side view shells appear flat and less bulging than those of *Anodontia fragilis*. The anterior adductor scar on the inside of the shells is quite long and slim; whereas the oval posterior adductor scar is shorter and broader (Figures 5A - C).

The subcircular, slightly longer than high shells of *Anodontia fragilis* (Figures 5D - F) show a fine sculpture resulting from concentric growth and are thinner and more fragile than those of *L. lacteus*. Their colour ranges from beige to some kind of dirty yellow or white with a yellow umbonal area. Especially on the inside, but also slightly on the outside of the shells, little filled or circled darker dots can be noticed (Figure 5F). Shells vary from 9 mm by 7.5 mm to 6 mm by 5 mm. In the side view shells are more tumid than those of *L. lacteus* (Figure 5D). In comparison to *L. lacteus* the anterior adductor scar also appears oval but shorter and broader whereas

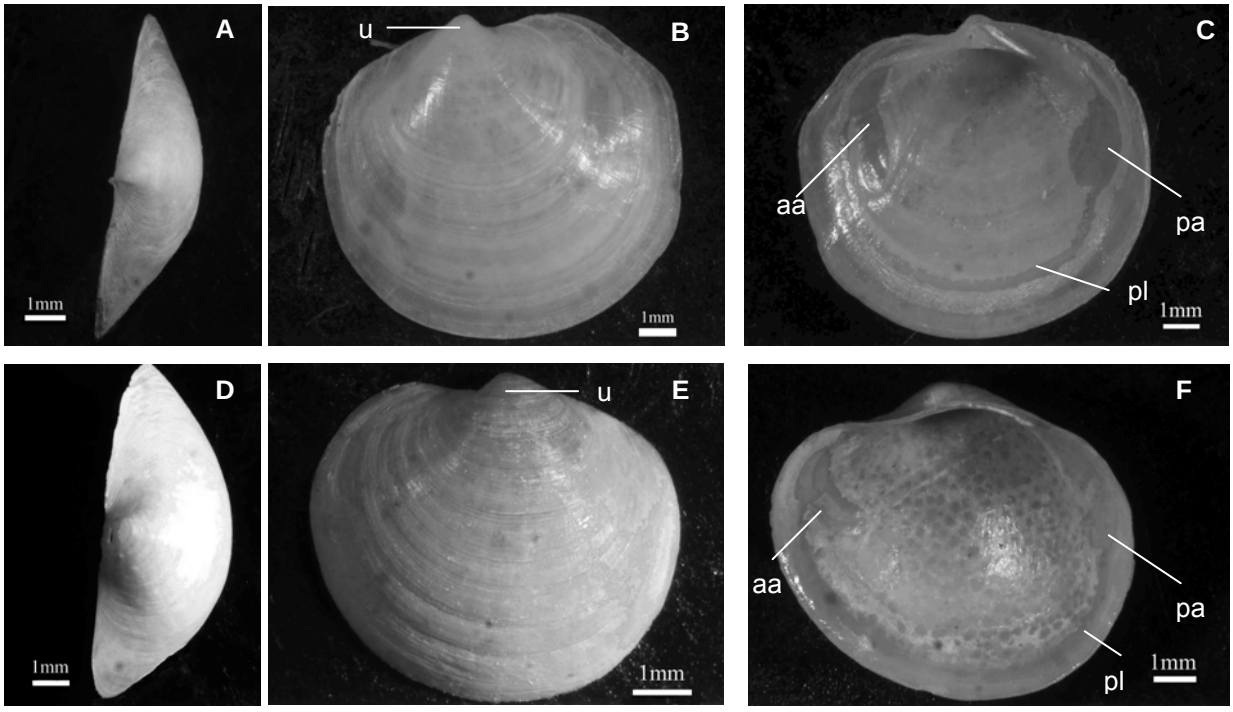


Figure 5: Shells of *Loripes lacteus* (A – C) and *Anodontia fragilis* (D – F). (A) Side view of the right valve, (B) outside view of the right valve, (C) inside view of the right valve of *L. lacteus*, (D) side view of the right valve, (E) outside view of the left valve and (F) inside view of the right valve of *A. fragilis*; umbo (u), anterior (aa) and posterior (pa) adductor scars and pallial line (pl).

the posterior adductor scar is smaller and drop shaped.

The most important and interesting aspect of the bivalve morphology is the proportional large size of the gill, which varies in colour. In the case of *L. lacteus* gills show either whitish or grey colour, whereas *A. fragilis* displays a dark red to brownish colour. In both clams the large and fleshy gills cover the whole body. Ctenidia consist of a single, homorhabdic, thickened inner demibranch on either side of the body. The foot of both species looks bean-shaped, being cylindrical and narrow with a slightly broader, glandular-looking tip.

Further aspects of the inner morphology are not addressed here. Figure 6 shows pictures of the bodies of both species.

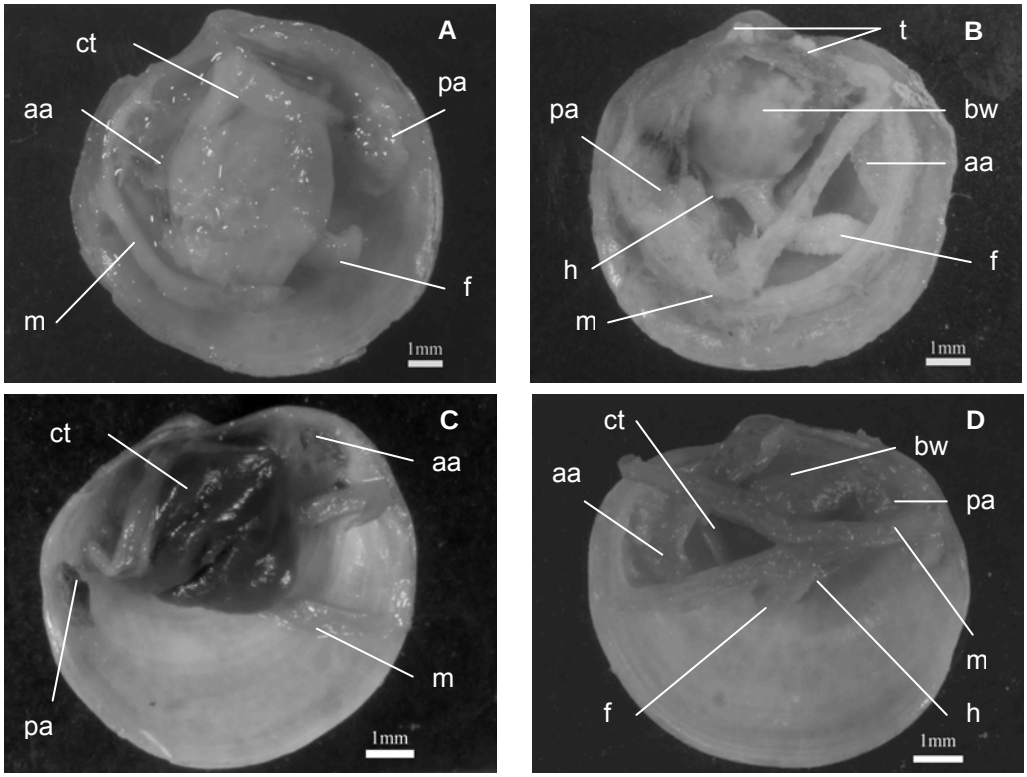


Figure 6: General anatomy of *L. lacteus* (A, B) and *A. fragilis* (C, D). (A) *L. lacteus* with left valve removed, and (B) with right valve and ctenidium removed, (C) *A. fragilis* with right valve removed, mantle detached and, (D) with left valve and ctenidium removed, mantle detached; anterior adductor muscle (aa), body wall (bw), ctenidium (ct), foot (f), heel of foot (h), mantle (m), posterior adductor muscle (pa), tooth (t).

Morphological Analysis of the Endosymbionts

a) Gill and Gill Filament Morphology

General gill and filament structure were assessed by light microscopy. The ctenidia consist of a single inner demibranch formed by two lamellae of filaments. They are rather thick compared to ctenidia of bivalve species without endosymbionts. In horizontal sections an interlamellar space between the lamellae is visible at the centre of the demibranch, interrupted by some connecting filaments of the inner and outer lamellae (Figure 7).

Each filament is composed of three distinct regions: (1) a ciliated zone on the outside, (2) an intermediary zone of variable size below, and (3) a lateral zone containing endosymbiont bacteria towards the centre (Figures 8A, B). Further, each filament consists of a single epithelium. At the area of the ciliated zone this epithelium has a tightened basal lamina, which is often referred to as collagen axis in the literature, and along the lateral zone encloses a blood lacuna (Figure 9).

The ciliated zone consists of different cells (frontal, latero-frontal and lateral cells) organised along a collagen axis as was previously described for various Lucinidae. The intermediary zone is formed by large, rather empty appearing cells with only a nucleus and few organelles (Figures 9A, C), determined to be mitochondria under the electron microscope. These two zones together with the neighbouring filaments constitute a narrow canal allowing a permanent flow of seawater along the lateral zone containing the bacteriocytes. This channel is either referred to as bacteriocyte channel or as interfilamental space by different authors.

While the ciliated zone looks similar in both investigated bivalve species the intermediary zone slightly differs. In *L. lacteus* the two zones more or less resemble each other in length and the large clear cells end with a rather slender profile (Figures 8A, 9B). In contrast the intermediary zone of *A. fragilis* appears considerably longer than the ciliated zone. Along the transition to the lateral zone the large, clear cells constituting the intermediary zone are partly covered by the first bacteriocytes giving them a longer, drop shaped profile. Thus, the transition between the two zones is gradual (Figures 8B, 9D).

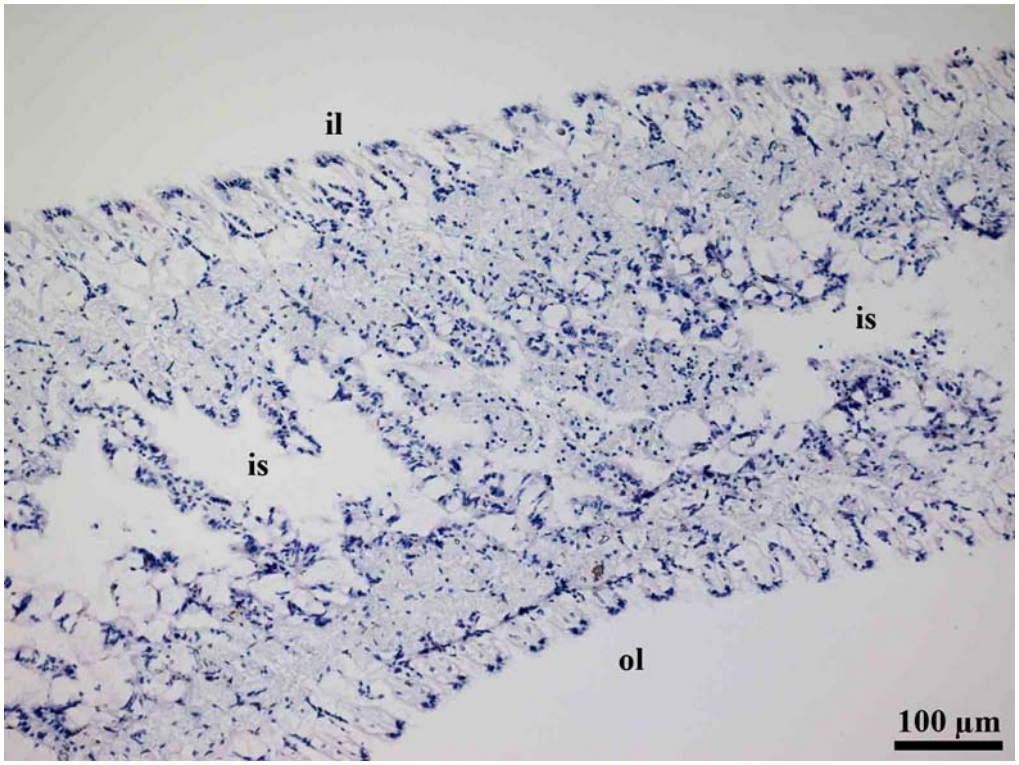


Figure 7: Horizontal section of the central part of one ctenidium of *L. lacteus* stained with haemalaun-eosin showing inner (il) and outer (ol) lamellae with interlamellar space (is) in between.

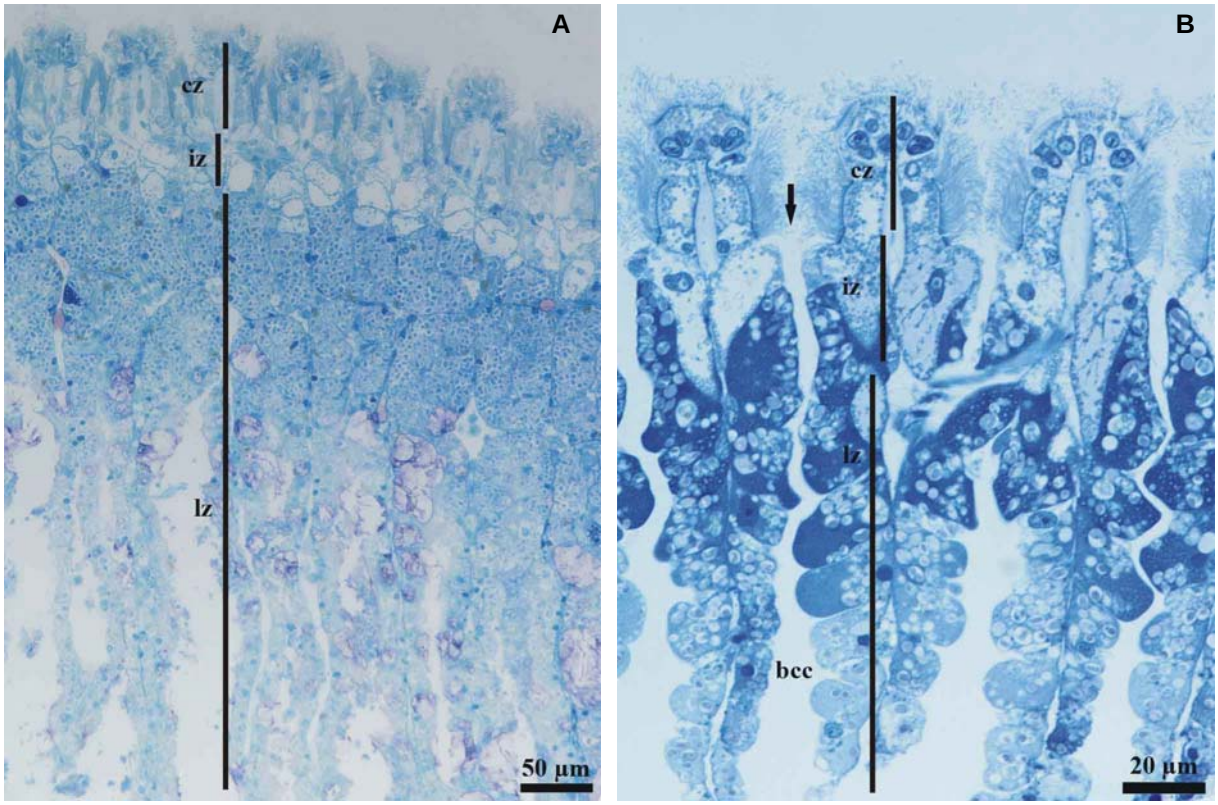


Figure 8: Images of horizontal ctenidia sections stained with toluidine blue of *L. lacteus* and *A. fragilis* showing gill and filament structure. (A) Overview of the outer lamellae with ciliated zone (cz), intermediary zone (iz) and lateral zone (lz) of *L. lacteus* and (B) three gill filaments of *A. fragilis* showing the same zoning; bacteriocyte channel (bcc), an arrow indicates the direction of water flow along the filament from the ciliated and intermediary zone to the lateral zone.

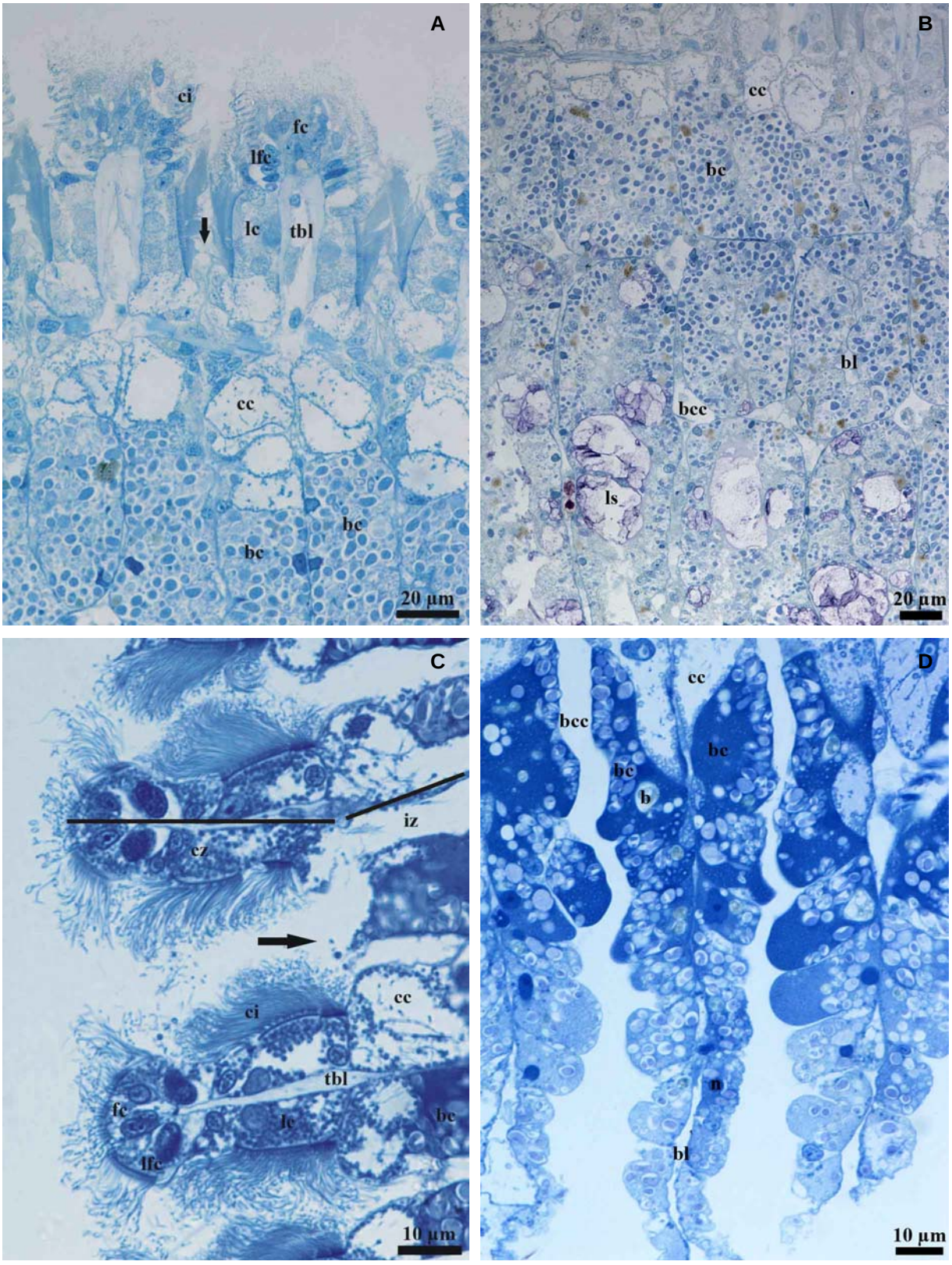


Figure 9: Toluidine blue stained horizontal sections through the gill of *L. lacteus* (A, B) and *A. fragilis* (C, D) showing (A) cell types of the ciliated and intermediary and parts of the lateral zone, and (B) the transition between intermediary and lateral zone of *L. lacteus* with nearly transparent cytoplasm; (C) cell types of the ciliated (cz) and intermediary (iz) zone and (D) the transition to the lateral zone of *A. fragilis* with stained cytoplasm. Bacteria (b), bacteriocyte channel (bcc), bacteriocytes (bc), blood lacuna (bl), tightened basal lamina (tbl), clear cells (cc), cilia (ci), frontal cells (fc), lateral cells (lc), latero-frontal cells (lfc), lysosomal substances (ls), an arrow indicates the direction of water flow through the lateral zone.

b) The Endosymbionts Containing Lateral Zone

The lateral zone, which constitutes the main part of the gill filament, consists of different cell types, the most abundant and obvious being the large bacteriocytes. In the filaments of both clam species these bacteriocytes are rather large and throughout the whole cell they contain lots of endosymbionts enclosed in vacuoles. A visible difference between the two host species is the staining of the cytoplasm. While in *L. lacteus* it is light to nearly stainless (Figure 9B), cytoplasm of *A. fragilis* bacteriocytes stains heavily in dark blue using toluidine blue (Figure 9D).

Cells of the lateral zone are coated by microvilli and connected by a fibrous glycocalyx along the surface area of both investigated clams. Since especially in *L. lacteus* the cytoplasm of the cells in the lateral zone appears light and transparent, endosymbionts, nuclei and cell organelles were easily detectable. An overview of the lateral zones of *L. lacteus* and *A. fragilis* showing the cell types detected in each species is presented in Figure 10.

Apart from the obvious, large and bacteria containing bacteriocytes, that are most abundant along the lateral zone, three other cell types can be found in *L. lacteus*. Between each of the large bacteriocytes a smaller, trumpet-shaped cell is visible. These so called intercalary cells (Figure 11A) are, besides their shape, characterised by an oval or flattened nucleus in apical position. Along the surface, intercalary cells are covered by longer microvilli when compared to bacteriocytes but without a linking glycocalyx. They cover part of neighbouring bacteriocytes, which leads to the typical trumpet-shape. Intercalary cells contain mitochondria, but lack envacuolated bacteria.

Along or within the blood lacuna cells filled with lysosomes can be found. These cells are smaller than bacteriocytes, do not contain bacteria, but in addition to lysosomes and a nucleus mitochondria, lots of vacuoles and small Golgi bodies are visible. According to the organelles found and according to literature they are addressed as hemocytes (Figure 11C).

Some of the bacteriocytes of *L. lacteus* contain electron-dense inclusions at a basal position. They vary in size, but mainly are larger than envacuolated bacteria. Several look like electron-dense, amorphous material; others resemble membrane-like structures of whorls and strands. Some of these cells contain substances of lytic appearance. Based on their structure these cells can be either called “granule-cells” or bacteriocytes containing electron-dense granules (Figure 11D).

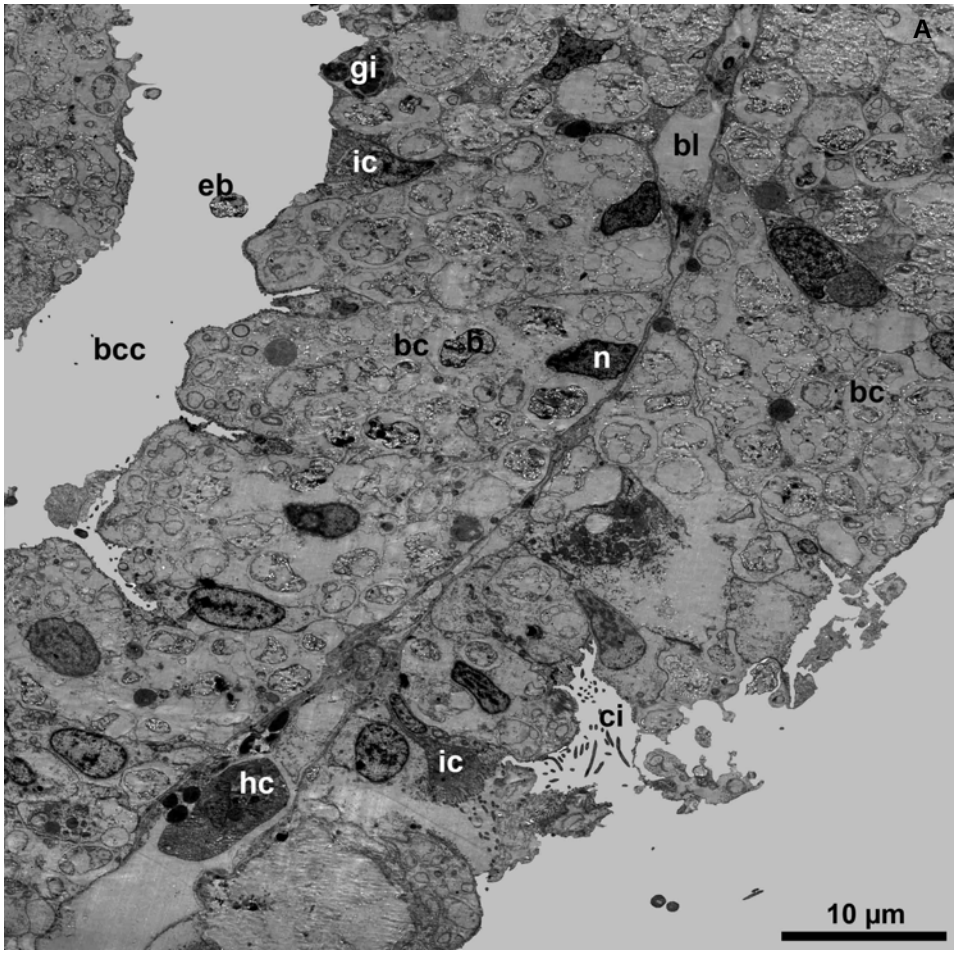
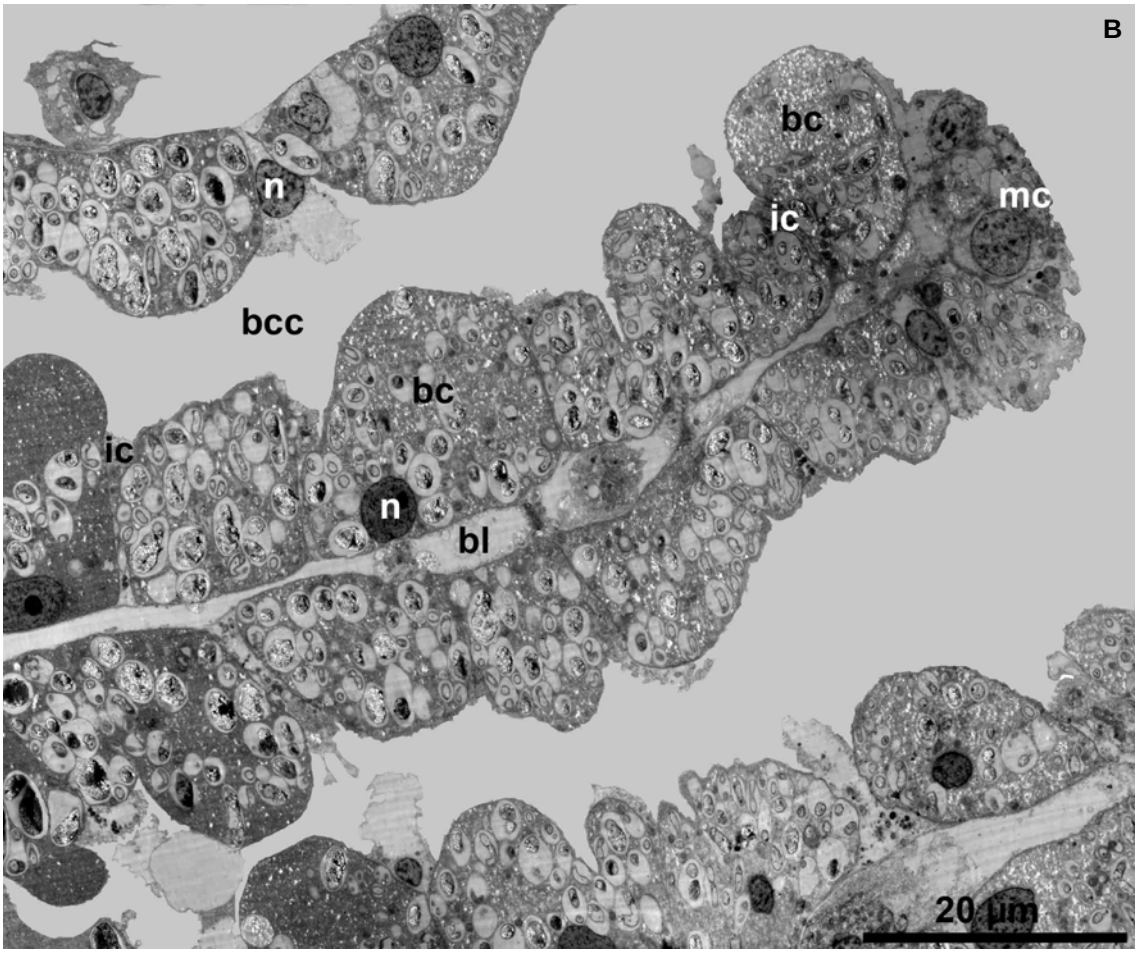


Figure 10: TEM images showing parts of the lateral zones of gill filaments from *L. lacteus* (A) and *A. fragilis* (B) containing different cell types. Bacteria (b), bacteriocyte (bc), bacteriocyte channel (bcc), blood lacuna (bl), cilia (ci), extra-cellular bacterium (eb), granular inclusion (gi), hemocyte (hc), intercalary cell (ic), mucocytes (mc), nucleus (n).



Bacteriocytes in the transition area between intermediary and lateral zone of *A. fragilis* harbour less envacuolated bacterial endosymbionts and show a darker cytoplasm staining than bacteriocytes further along the filament (compare Figures 8B, 9D and 10B). Like in the case of *L. lacteus* the surface of the lateral zone is covered by microvilli and glycocalyx. Including bacteriocytes, a total of three different cell types are present in *A. fragilis*.

Trumpet-shaped intercalary cells (Figure 11B) intermingled with large bacteriocytes can be found in *A. fragilis* as well, but they seem smaller and do not reach as far over the neighbouring bacteriocytes as it was the case for *L. lacteus*. Their microvilli are long and lack a linking glycocalyx. Nucleoli, in addition to mitochondria the only detectable organelles in rather electron-lucent looking cells, are hardly visible, but then they have an apical or lateral position.

Occasionally, bacteriocytes of *A. fragilis* near the transition to the intermediary zone contain lysosomes and a small vacuole the same size as bacteria containing ones enclosing electron-dense inclusions. They are composed of thicker membrane-like structures in basal position, but these are rather few (Figure 12B). Real “granule-cells” comparable to *L. lacteus* or other lucinids were not detected during this study.

But *A. fragilis* shows another cell type lacking in *L. lacteus*: Mucocytes (Figure 11E) can only be observed in the abfrontal parts of the lateral zone of *A. fragilis*. These cells are a bit smaller than bacteriocytes with a lighter cytoplasm. In addition to the nucleus, mucocytes contain quite a lot of mitochondria, Golgi bodies and vacuoles for mucus production and storage.

The bacteriocytes, constituting the main part of the lateral zone in the gill filaments of both bivalves, is described in detail below.

Bacteriocytes represent the dominant and largest cell type of the lateral zone in both examined clams. They vary from nearly round or oval to drop-shaped and harbour quite a lot of bacterial endosymbionts in vacuoles. The rounded, apical surface of bacteriocytes is covered by microvilli linked via a fibrous glycocalyx. Besides envacuolated symbionts only a few regular organelles can be detected, which consist of mitochondria and sometimes a small number of lysosomes in part of the bacteriocytes (compare above). Other organelles are scarce. In addition to these common characteristics *L. lacteus* and *A. fragilis* also show some differences in bacteriocytes.

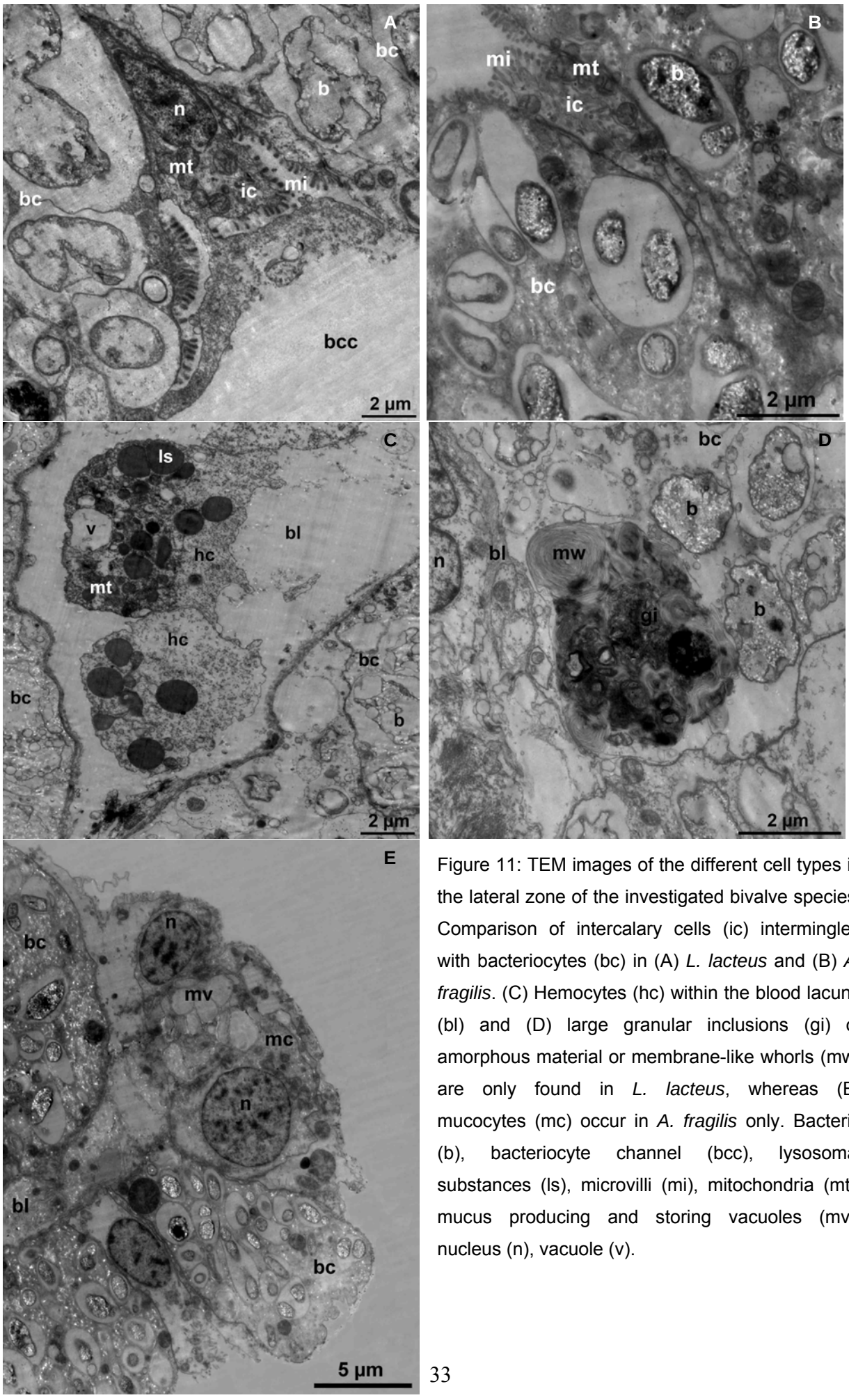


Figure 11: TEM images of the different cell types in the lateral zone of the investigated bivalve species. Comparison of intercalary cells (ic) intermingled with bacteriocytes (bc) in (A) *L. lacteus* and (B) *A. fragilis*. (C) Hemocytes (hc) within the blood lacuna (bl) and (D) large granular inclusions (gi) of amorphous material or membrane-like whorls (mw) are only found in *L. lacteus*, whereas (E) mucocytes (mc) occur in *A. fragilis* only. Bacteria (b), bacteriocyte channel (bcc), lysosomal substances (ls), microvilli (mi), mitochondria (mt), mucus producing and storing vacuoles (mv), nucleus (n), vacuole (v).

In *L. lacteus* the cytoplasm of the bacteriocytes is electron-lucent as already mentioned before. The mostly oval or irregularly shaped nucleus holds a basal position within the cell. In contrast, the cytoplasm of *A. fragilis* shows the above described electron-density, which becomes more lucent towards the centre of the filament. Within electron-dense bacteriocyte cytoplasm very small electron-lucent, crystal like structures are visible (Figure 12B). The basal nucleus of this species often has a round, nearly circular or sometimes oval shape. Bacteriocytes of *A. fragilis* seem to be more crowded with envacuolated bacteria compared to those of *L. lacteus* (Figures 12A & B).

In analysed samples of both species all prokaryotic endosymbionts are enclosed by a vacuole with mostly one symbiont in each. Sometimes dividing stages or two symbionts per vacuole can be observed, but especially in *L. lacteus* this is rather rare. The endosymbionts themselves possess the typical double membrane of Gram-negative bacteria and vary in size and shape depending on sectioning orientation and positioning within the bacteriocytes.

Endosymbionts of *L. lacteus* (Figure 12C) are often small near the apical part of the bacteriocytes with a firm outline, but become larger with invaginations along the surface towards more basal regions. Bacteria can resemble ovoid to coccoid or long rod-shaped forms, dependent on sectioning orientation. All endosymbionts contain one or more vacuoles in the granular looking bacterial cytoplasm with the inner bacterial membrane enclosing these vesicles whereas the outer bacterial membrane always outlines the whole bacterium.

In *A. fragilis* endosymbionts also vary in firmness, shape and size, but larger and smaller ones here seem intermingled with each other. Shapes rang from nearly circular over coccoid or rod-shaped to long oval sections. In contrast to *L. lacteus* hardly any or only very small vacuoles or vesicles are visible in bacterial endosymbionts of *A. fragilis*. The bacterial cytoplasm showed a differentiation into more and less electron-lucent regions (Figure 12D).

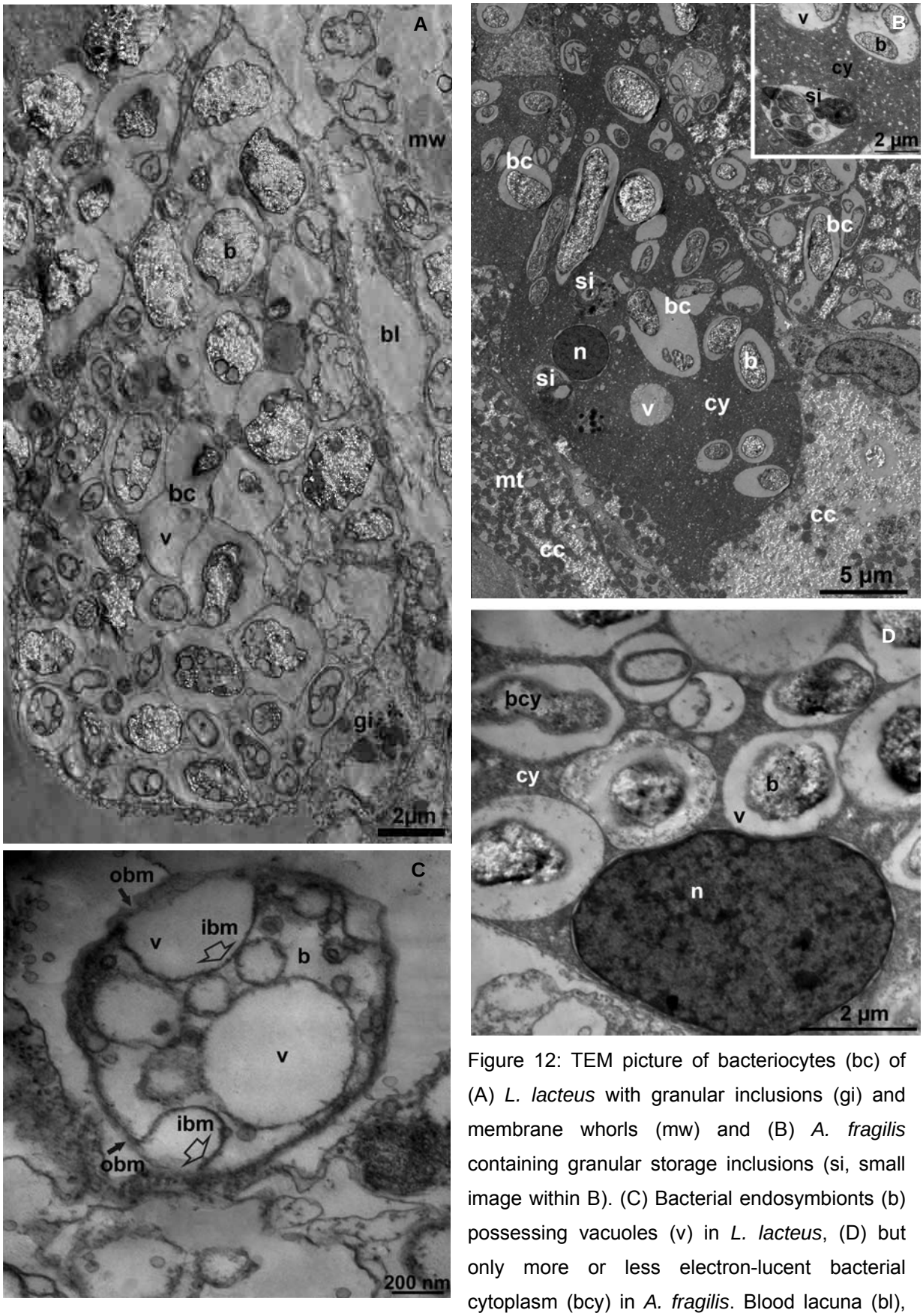


Figure 12: TEM picture of bacteriocytes (bc) of (A) *L. lacteus* with granular inclusions (gi) and membrane whorls (mw) and (B) *A. fragilis* containing granular storage inclusions (si, small image within B). (C) Bacterial endosymbionts (b) possessing vacuoles (v) in *L. lacteus*, (D) but only more or less electron-lucent bacterial cytoplasm (bcy) in *A. fragilis*. Blood lacuna (bl), large, clear cells (cc) of the intermediary zone, cytoplasm (cy), nucleus (n), inner (ibm, empty arrow) and outer (obm, filled arrow) bacterial membrane.

Sequence Analysis

Sequences obtained from clones of the 16S rDNA gene (encoding for rRNA) vary slightly in length depending on the reverse primer used for amplification. The shorter sequences have about 1503 bp (1492R), longest clones included 1543 bp (630R).

Clones of the 16S rRNA gene of several specimens of *Loripes lacteus* and *Anodontia fragilis* were sequenced to generate a set of endosymbiont sequences. The numbers of clone sequences obtained from each individual vary depending on the success of the 16S rRNA PCR amplifications and sequencing reactions as well as on the number of potential endosymbionts found within each individual. The lower the number of these candidates is, the more sequences are generated.

Altogether 102 complete sequences of 16S rRNA were obtained from *L. lacteus* (6 individuals) and 63 from *A. fragilis* (5 individuals). From these a total of 38 from *L. lacteus* and 56 from *A. fragilis* are identified as potential endosymbionts (see below). Clones of low sequence quality of both species are excluded from the data set.

BLAST searches return for 92.1 % γ-Proteobacteria as closest matches to the sequences obtained from both species. Only a few sequences resemble bacteria belonging to other groups (α- and β-Proteobacteria, Bacillales). Within *L. lacteus* even 93.1% of the generated sequences group with γ-Proteobacteria which is slightly higher than 90.5 % in *A. fragilis*. Sequences not belonging to γ-Proteobacteria were not taken into further account for analyses.

Table 5: List of sequences obtained from *L. lacteus* and *A. fragilis*, with number of clones sequenced for each investigated individual and groups of highest similarities in the BLAST search. Potential symbionts are highlighted. (γ -Proteobac. – γ-Proteobacteria, pot. Symb. – potential endosymbionts)

			Sequences					
Species	**Month**	**Individual**	**total No.**	**total γ-Proteobac.**	**pot. Symb.**	**within *Marinobacter***	**other γ-Proteobac.**	**other Groups**
L. lacteus	April	Ind. 1	8	7	**3**	3	1	1
		Ind. 2	17	14	**7**	5	2	3
		Ind. 3	22	22	**8**	10	4	0
	July	Ind. 1	22	22	**7**	10	5	0
		Ind. 2	18	16	**5**	9	2	2
		Ind. 3	16	15	**9**	5	1	1
A. fragilis	April	Ind. 1	7	4	**3**	0	1	3
		Ind. 2	6	6	**6**	0	0	0
		Ind. 3	17	17	**17**	0	0	0
	July	Ind. 1	21	20	**20**	0	0	1
		Ind. 2	12	10	**10**	0	0	2

Potential endosymbiotic sequences were easier obtained from *A. fragilis*. In *L. lacteus* less than half of the sequences (40.0 % of all sequences grouping within the γ-Proteobacteria) show highest similarities with other lucinid or sulphide oxidising endosymbionts. Nearly all γ-Proteobacteria sequences obtained from *A. fragilis* cluster with other lucinid symbionts in the BLAST search. Only Individual 1 collected in April 2007 forms a potential outlier with 25 % of the sequences belonging to non-endosymbiotic groups. The high number of clone sequences not clustering with potential endosymbionts in *L. lacteus* can probably be assigned to extracellular bacteria. These free-living bacteria resemble species belonging to the *Marinobacter*-group, the genus *Shewanella* (mainly terrestrial bacterium with species from deep sea locations) or a few others. For detailed numbers of clones sequenced in each group compare Table 5.

Pairwise-distances of potential endosymbiont sequences in *L. lacteus* range from 96.7 – 100 % identity, with only one specimen remaining in the data set being responsible for the value of 96.7 %. All other 16S rRNA sequences resemble each other for at least 98.6 % (compare Table 6). Potential endosymbiont sequences obtained from *A. fragilis* show a similarity of 98.1 % and higher.

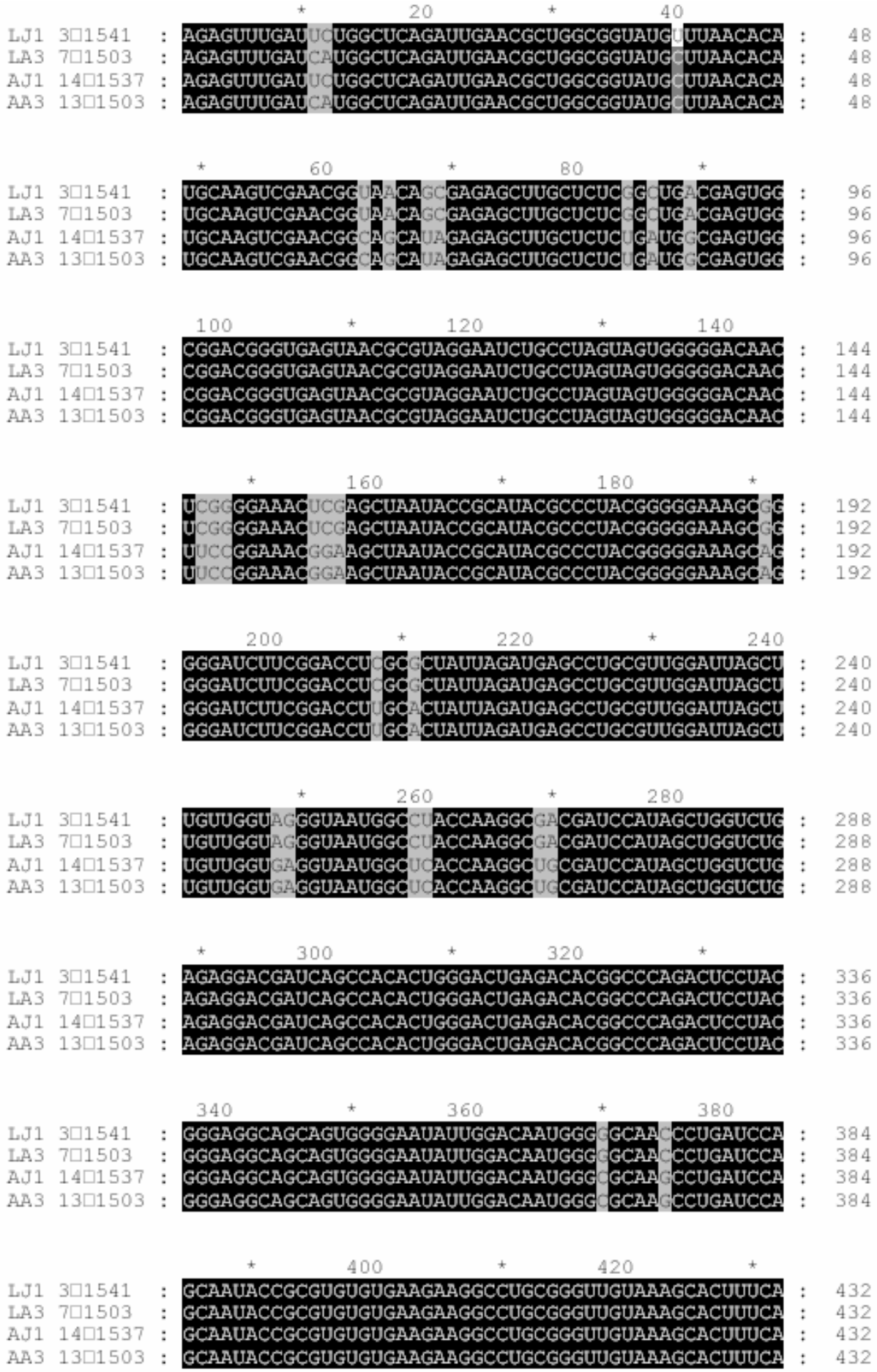
No differences in the bacterial population between the two sampling dates (April and July 2007) can be detected in either species. The similarity matrix shows comparable P-distances for individuals from the different sampling times. Sequence variability within the two dates does not diverge from variability between them.

Since clones of potential endosymbionts exhibit such high similarities (see above), two sequences (one from April and July each) were chosen randomly for each species. The selected sequences show high identities with other ones and are therefore thought to be good representatives for all others contained within the data set. Chosen sequences are used for phylogenetic analysis (see below). For analysis, the 16S rRNA sequences were transcribed into rRNA. Figure 13 presents an alignment of the selected endosymbiont sequences from *L. lacteus* and *A. fragilis* highlighting differences.

P-distances of the 16S rRNA of the potential endosymbionts of *L. lacteus* and *A. fragilis* are rather low. The two potential clam symbionts shared about 94 % of the nucleotides of this gene, which is below the species border of 97.5 % nucleotide similarity for the 16S rRNA.

Table 6: Similarity matrix based on P-distances calculated for endosymbiont clone sequences of the 16S rRNA gene from *L. lacteus* using ARB programme package, representative for P-distances of both investigated species. One specimen showing lower similarity is highlighted. *L. lacteus* collected in April, Ind. 1 (LA1), Ind. 2 (LA2), Ind. 3 (LA3); *L. lacteus* collected in July, Ind. 1 (LJ1), Ind. 2 (LJ2), Ind. 3 (LJ3). Index numbers represent number of the clone sequence.

	LA1_1	LA1_2	LA1_3	LJ1_1	LJ1_2	LJ1_3	LJ1_4	LJ1_5	LJ1_6	LJ1_7	LA2_1	LA3_1	LA3_2	LA3_3	LJ3_1	LJ3_2	LJ3_3	LJ3_4	LJ3_5	LA2_2	LA2_3	LA2_4	LJ2_1	LJ2_2	LJ3_6	LA3_4	LA3_5	LJ2_3	LJ2_4	LJ2_5	LJ3_7	LJ3_8	LA2_5	LA2_6	LA2_7	LA3_6	LA3_7	LA3_8	LJ3_9
LA1_1	100																																					
LA1_2	99.2	100																																				
LA1_3	99.2	99.6	100																																			
LJ1_1	99.1	99.5	99.3	100																																		
LJ1_2	98.7	98.6	98.6	98.6	100																																	
LJ1_3	98.8	98.8	98.7	98.7	99.7	100																																
LJ1_4	98.9	98.7	98.8	98.7	99.5	99.6	100																															
LJ1_5	96.8	96.8	96.7	96.8	97.7	97.9	97.7	100																														
LJ1_6	98.7	98.7	98.6	98.7	99.7	99.6	99.6	97.7	100																													
LJ1_7	98.7	98.7	98.6	98.7	99.5	99.7	99.5	97.7	99.7	100																												
LA2_1	99.1	99.1	99.1	99.3	99.3	99.3	99.3	97.3	99.5	99.3	100																											
LA3_1	99.5	99.3	99.4	99.3	99.5	99.7	99.6	97.6	99.7	99.7	99.7	100																										
LA3_2	99.3	99.4	99.2	99.4	99.5	99.6	99.4	97.5	99.6	99.6	99.7	99.8	100																									
LA3_3	98.6	98.6	98.6	98.6	98.8	98.8	98.7	96.7	98.9	98.8	99.0	99.1	99.1	100																								
LJ3_1	99.3	99.3	99.2	99.3	99.4	99.4	99.5	97.4	99.5	99.4	99.6	99.7	99.7	99.0	100																							
LJ3_2	99.3	99.4	99.2	99.4	99.5	99.6	99.4	97.5	99.6	99.6	99.7	99.8	99.9	99.1	99.7	100																						
LJ3_3	99.0	99.1	98.9	99.1	99.2	99.3	99.1	97.3	99.5	99.3	99.4	99.5	99.6	98.9	99.4	99.6	100																					
LJ3_4	99.3	99.4	99.2	99.4	99.4	99.5	99.5	97.5	99.7	99.5	99.6	99.7	99.8	99.0	99.7	99.8	99.7	100																				
LJ3_5	99.3	99.4	99.2	99.4	99.5	99.6	99.4	97.5	99.7	99.6	99.7	99.8	99.9	99.1	99.7	99.9	99.7	100	100																			
LA2_2	99.3	99.4	99.2	99.5	99.4	99.5	99.5	97.5	99.5	99.5	99.7	99.7	99.8	99.0	99.7	99.8	99.5	99.9	99.8	100																		
LA2_3	99.2	99.2	99.1	99.3	99.4	99.4	99.3	97.3	99.5	99.4	99.7	99.7	99.7	99.0	99.6	99.7	99.4	99.6	99.7	99.7	100																	
LA2_4	99.2	99.3	99.1	99.5	99.4	99.5	99.3	97.5	99.5	99.5	99.8	99.7	99.8	99.0	99.6	99.8	99.5	99.7	99.8	99.9	99.9	100																
LJ2_1	99.1	99.3	99.1	99.4	99.3	99.5	99.3	97.4	99.6	99.5	99.7	99.7	99.7	98.9	99.5	99.7	99.6	99.8	99.9	99.8	99.8	100	100															
LJ2_2	99.2	99.2	99.1	99.3	99.3	99.3	99.4	97.3	99.5	99.3	99.7	99.7	99.6	98.9	99.7	99.6	99.3	99.7	99.6	99.8	99.7	99.7	99.6	100														
LJ3_6	99.3	99.3	99.2	99.3	99.4	99.4	99.5	97.4	99.7	99.4	99.6	99.7	99.7	99.0	99.7	99.7	99.5	99.9	99.8	99.7	99.6	99.6	99.7	99.7	100													
LA3_4	98.9	99.1	98.9	99.1	99.1	99.2	99.1	97.2	99.2	99.2	99.3	99.4	99.5	98.7	99.4	99.5	99.2	99.5	99.5	99.5	99.3	99.4	99.3	99.3	99.5	100												
LA3_5	99.3	99.3	99.3	99.3	99.3	99.6	99.5	97.6	99.5	99.6	99.5	99.8	99.7	98.9	99.7	99.7	99.5	99.8	99.7	99.8	99.5	99.7	99.6	99.6	99.8	99.6	100											
LJ2_3	99.5	99.5	99.4	99.5	99.3	99.5	99.4	97.5	99.5	99.5	99.4	99.7	99.6	98.8	99.5	99.6	99.5	99.7	99.7	99.6	99.4	99.5	99.6	99.4	99.6	99.3	99.7	100										
LJ2_4	99.2	99.2	99.1	99.2	99.5	99.5	99.4	97.4	99.7	99.5	99.7	99.8	99.7	99.1	99.7	99.7	99.6	99.8	99.9	99.7	99.7	99.7	99.7	99.6	99.8	99.3	99.6	99.7	100									
LJ2_5	99.2	99.2	99.1	99.2	99.3	99.5	99.3	97.5	99.5	99.5	99.5	99.7	99.7	98.9	99.5	99.7	99.5	99.7	99.8	99.6	99.5	99.6	99.7	99.4	99.6	99.3	99.7	99.8	99.8	100								
LJ3_7	99.1	99.3	99.1	99.3	99.3	99.5	99.3	97.4	99.6	99.5	99.5	99.7	99.7	98.9	99.5	99.7	99.6	99.8	99.9	99.7	99.5	99.7	99.7	99.5	99.7	99.3	99.6	99.6	99.7	99.7	100							
LJ3_8	99.3	99.3	99.3	99.3	99.4	99.7	99.5	97.6	99.7	99.7	99.6	99.9	99.8	99.0	99.6	99.8	99.7	99.9	100	99.7	99.6	99.7	99.8	99.5	99.7	99.4	99.8	99.8	99.8	99.9	99.8	100						
LA2_5	99.5	99.5	99.5	99.6	99.4	99.4	99.4	97.4	99.5	99.3	99.7	99.7	99.6	98.9	99.6	99.6	99.3	99.6	99.6	99.7	99.7	99.7	99.6	99.7	99.6	99.3	99.5	99.3	99.6	99.4	99.5	99.5	100					
LA2_6	99.5	99.3	99.4	99.3	99.4	99.5	99.6	97.5	99.5	99.5	99.6	100	99.7	99.1	99.8	99.7	99.5	99.8	99.7	99.8	99.7	99.7	99.6	99.7	99.8	99.5	99.9	99.7	99.7	99.7	99.6	99.8	99.7	100				
LA2_7	99.4	99.4	99.3	99.4	99.5	99.7	99.5	97.7	99.6	99.7	99.7	100	99.9	99.1	99.7	99.9	99.6	99.8	99.9	99.8	99.7	99.8	99.7	99.6	99.7	99.5	99.9	99.7	99.7	99.8	99.7	100	99.6	99.9	100			
LA3_6	99.4	99.4	99.3	99.4	99.5	99.7	99.5	97.7	99.6	99.7	99.7	100	99.9	99.1	99.7	99.9	99.6	99.8	99.9	99.8	99.7	99.8	99.7	99.6	99.7	99.5	99.9	99.7	99.7	99.8	99.7	100	99.6	99.9	100	100		
LA3_7	99.4	99.4	99.3	99.4	99.5	99.7	99.5	97.7	99.6	99.7	99.7	100	99.9	99.1	99.7	99.9	99.6	99.8	99.9	99.8	99.7	99.8	99.7	99.6	99.7	99.5	99.9	99.7	99.7	99.8	99.7	100	99.6	99.9	100	100	100	
LA3_8	99.4	99.4	99.3	99.4	99.5	99.7	99.5	97.7	99.6	99.7	99.7	100	99.9	99.1	99.7	99.9	99.6	99.8	99.9	99.8	99.7	99.8	99.7	99.6	99.7	99.5	99.9	99.7	99.7	99.8	99.7	100	99.6	99.9	100	100	100	100
LJ3_9	99.3	99.3	99.2	99.3	99.4	99.4	99.5	97.4	99.7	99.4	99.6	99.7	99.7	99.0	99.7	99.7	99.5	99.9	99.8	99.7	99.6	99.6	99.7	99.7	99.9	99.4	99.7	99.6	99.8	99.6	99.7	99.7	99.6	99.8	99.7	99.7	99.7	99.7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AJ1 14□1537 : AGAGUUUGAUCUGGCUCAGAUUGAACGCUGGCGGUAUGCUUAACACA : 48
AA3 13□1503 : AGAGUUUGAUCAUGGCUCAGAUUGAACGCUGGCGGUAUGCUUAACACA : 48
LJ1 3□1541 : UGCAAGUCGAACGGUAACAGCGAGAGCUUGCUCUCGGCUGACGAGUGG : 96
LA3 7□1503 : UGCAAGUCGAACGGUAACAGCGAGAGCUUGCUCUCGGCUGACGAGUGG : 96
AJ1 14□1537 : UGCAAGUCGAACGGCAGCAUAGAGAGCUUGCUCUCUGAUGGCGAGUGG : 96
AA3 13□1503 : UGCAAGUCGAACGGCAGCAUAGAGAGCUUGCUCUCUGAUGGCGAGUGG : 96
LJ1 3□1541 : CGGACGGGUGAGUAACGCGUAGGAAUCUGCCUAGUAGUGGGGGACAAC : 144
LA3 7□1503 : CGGACGGGUGAGUAACGCGUAGGAAUCUGCCUAGUAGUGGGGGACAAC : 144
AJ1 14□1537 : CGGACGGGUGAGUAACGCGUAGGAAUCUGCCUAGUAGUGGGGGACAAC : 144
AA3 13□1503 : CGGACGGGUGAGUAACGCGUAGGAAUCUGCCUAGUAGUGGGGGACAAC : 144
LJ1 3□1541 : UCGGGGAAACUCGAGCUAAUACCGCAUACGCCCUACGGGGGAAAGCGG : 192
LA3 7□1503 : UCGGGGAAACUCGAGCUAAUACCGCAUACGCCCUACGGGGGAAAGCGG : 192
AJ1 14□1537 : UUCCGGAAACGGAAGCUAAUACCGCAUACGCCCUACGGGGGAAAGCAG : 192
AA3 13□1503 : UUCCGGAAACGGAAGCUAAUACCGCAUACGCCCUACGGGGGAAAGCAG : 192
LJ1 3□1541 : GGGAUCUUCGGACCUCGCGCUAUUAGAUGAGCCUGCGUUGGAUUAGCU : 240
LA3 7□1503 : GGGAUCUUCGGACCUCGCGCUAUUAGAUGAGCCUGCGUUGGAUUAGCU : 240
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AA3 13□1503 : AGAGGACGAUCAGCCACACUGGGACUGAGACACGGCCCAGACUCCUAC : 336
LJ1 3□1541 : GGGAGGCAGCAGUGGGGAAUAUUGGACAAUGGGGGCAACCCUGAUCCA : 384
LA3 7□1503 : GGGAGGCAGCAGUGGGGAAUAUUGGACAAUGGGGGCAACCCUGAUCCA : 384
AJ1 14□1537 : GGGAGGCAGCAGUGGGGAAUAUUGGACAAUGGGCGCAAGCCUGAUCCA : 384
AA3 13□1503 : GGGAGGCAGCAGUGGGGAAUAUUGGACAAUGGGCGCAAGCCUGAUCCA : 384
LJ1 3□1541 : GCAAUACCGCGUGUGUGAAGAAGGCCUGCGGGUUGUAAAGCACUUUCA : 432
LA3 7□1503 : GCAAUACCGCGUGUGUGAAGAAGGCCUGCGGGUUGUAAAGCACUUUCA : 432
AJ1 14□1537 : GCAAUACCGCGUGUGUGAAGAAGGCCUGCGGGUUGUAAAGCACUUUCA : 432
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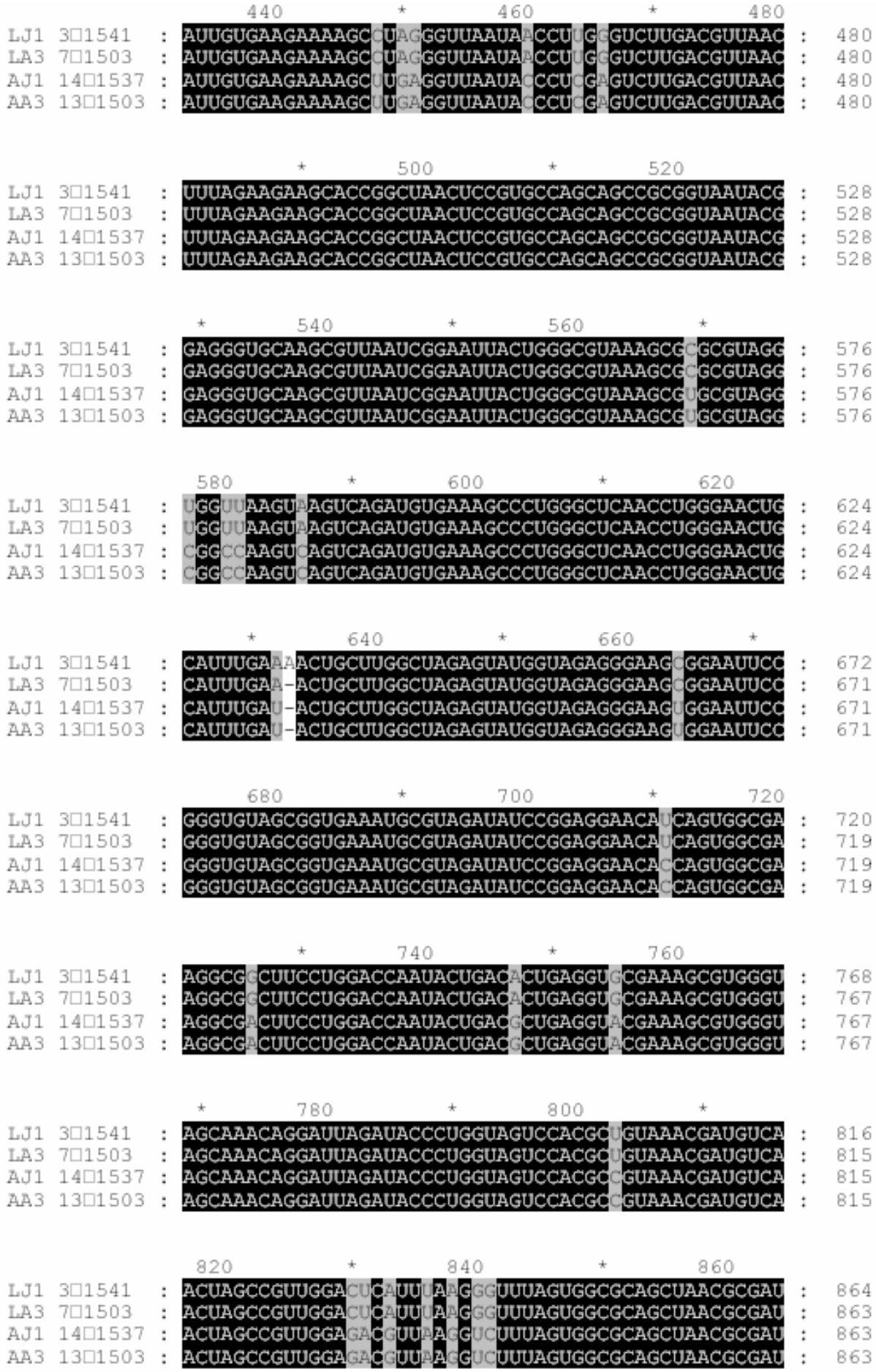
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AJ1 14□1537 : AUUGUGAAGAAAAGCUUGAGGUUAAUACCCUCGAGUCUUGACGUUAAC : 480
AA3 13□1503 : AUUGUGAAGAAAAGCUUGAGGUUAAUACCCUCGAGUCUUGACGUUAAC : 480
* 500 * 520
LJ1 3□1541 : UUUAGAAGAAGCACCGGCUAACUCCGUGCCAGCAGCCGCGGUAAUACG : 528
LA3 7□1503 : UUUAGAAGAAGCACCGGCUAACUCCGUGCCAGCAGCCGCGGUAAUACG : 528
AJ1 14□1537 : UUUAGAAGAAGCACCGGCUAACUCCGUGCCAGCAGCCGCGGUAAUACG : 528
AA3 13□1503 : UUUAGAAGAAGCACCGGCUAACUCCGUGCCAGCAGCCGCGGUAAUACG : 528
* 540 * 560 *
LJ1 3□1541 : GAGGGUGCAAGCGUUAAUCGGAAUUACUGGGCGUAAAGCGCGCGUAGG : 576
LA3 7□1503 : GAGGGUGCAAGCGUUAAUCGGAAUUACUGGGCGUAAAGCGCGCGUAGG : 576
AJ1 14□1537 : GAGGGUGCAAGCGUUAAUCGGAAUUACUGGGCGUAAAGCGUGCGUAGG : 576
AA3 13□1503 : GAGGGUGCAAGCGUUAAUCGGAAUUACUGGGCGUAAAGCGUGCGUAGG : 576
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LA3 7□1503 : UGGUUAAGUAAGUCAGAUGUGAAAGCCCUGGGCUCAACCUGGGAACUG : 624
AJ1 14□1537 : CGGCCAAGUCAGUCAGAUGUGAAAGCCCUGGGCUCAACCUGGGAACUG : 624
AA3 13□1503 : CGGCCAAGUCAGUCAGAUGUGAAAGCCCUGGGCUCAACCUGGGAACUG : 624
* 640 * 660 *
LJ1 3□1541 : CAUUUGAAAACUGCUUGGCUAGAGUAUGGUAGAGGGAAGCGGAAUUCC : 672
LA3 7□1503 : CAUUUGAA-ACUGCUUGGCUAGAGUAUGGUAGAGGGAAGCGGAAUUCC : 671
AJ1 14□1537 : CAUUUGAU-ACUGCUUGGCUAGAGUAUGGUAGAGGGAAGUGGAAUUCC : 671
AA3 13□1503 : CAUUUGAU-ACUGCUUGGCUAGAGUAUGGUAGAGGGAAGUGGAAUUCC : 671
680 * 700 * 720
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LA3 7□1503 : GGGUGUAGCGGUGAAAUGCGUAGAUAUCCGGAGGAACAUCAGUGGCGA : 719
AJ1 14□1537 : GGGUGUAGCGGUGAAAUGCGUAGAUAUCCGGAGGAACACCAGUGGCGA : 719
AA3 13□1503 : GGGUGUAGCGGUGAAAUGCGUAGAUAUCCGGAGGAACACCAGUGGCGA : 719
* 740 * 760
LJ1 3□1541 : AGGCGGCUUCCUGGACCAAUACUGACACUGAGGUGCGAAAGCGUGGGU : 768
LA3 7□1503 : AGGCGGCUUCCUGGACCAAUACUGACACUGAGGUGCGAAAGCGUGGGU : 767
AJ1 14□1537 : AGGCGACUUCCUGGACCAAUACUGACGCUGAGGUACGAAAGCGUGGGU : 767
AA3 13□1503 : AGGCGACUUCCUGGACCAAUACUGACGCUGAGGUACGAAAGCGUGGGU : 767
* 780 * 800 *
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LA3 7□1503 : AGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCUGUAAACGAUGUCA : 815
AJ1 14□1537 : AGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCCGUAAACGAUGUCA : 815
AA3 13□1503 : AGCAAACAGGAUUAGAUACCCUGGUAGUCCACGCCGUAAACGAUGUCA : 815
820 * 840 * 860
LJ1 3□1541 : ACUAGCCGUUGGACUCAUUUAAGGGUUUAGUGGCGCAGCUAACGCGAU : 864
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AJ1 14□1537 : ACUAGCCGUUGGAGACGUUAAGGUCUUUAGUGGCGCAGCUAACGCGAU : 863
AA3 13□1503 : ACUAGCCGUUGGAGACGUUAAGGUCUUUAGUGGCGCAGCUAACGCGAU : 863


LJ1 3□1541 : AAGUUGACCGCCUGGGGAGUACGGCCGCAAGGUUAAAACUCAAAGGA : 912
LA3 7□1503 : AAGUUGACC-GCCUGGGGAGUACGGCCGCAAGGUUAAAACUCAAAGGA : 910
AJ1 14□1537 : AAGUUGACC-GCCUGGGGAGUACGGCCGCAAGGUUAAAACUCAAAGGA : 910
AA3 13□1503 : AAGUUGACC-GCCUGGGGAGUACGGCCGCAAGGUUAAAACUCAAAGGA : 910
LJ1 3□1541 : AUUGACGGGGGCCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUG : 960
LA3 7□1503 : AUUGACGGGGGCCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUG : 958
AJ1 14□1537 : AUUGACGGGGGCCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUG : 958
AA3 13□1503 : AUUGACGGGGGCCCGCACAAGCGGUGGAGCAUGUGGUUUAAUUCGAUG : 958
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LA3 7□1503 : CAACGCGAAGA-ACCUUACCAGCCCUUGACAUCCUGGAAACUUACUAG : 1005
AJ1 14□1537 : CAACGCGAAGA-ACCUUACCAGCCCUUGACAUCCAGAGAAUUUUCUAG : 1005
AA3 13□1503 : CAACGCGAAGA-ACCUUACCAGCCCUUGACAUCCAGAGAAUUUUCUAG : 1005
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LA3 7□1503 : AGAUAGUUUGGUGCCUUCGGGAAUCCAGUGACAGGUGCUGCAUGGCUG : 1053
AJ1 14□1537 : AGAUAGAUUAGUGCCUUCGGGAACUCUGUGACAGGUGCUGCAUGGCUG : 1053
AA3 13□1503 : AGAUAGAUUAGUGCCUUCGGGAACUCUGUGACAGGUGCUGCAUGGCUG : 1053
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LA3 7□1503 : UCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGUAACGAGCGC : 1101
AJ1 14□1537 : UCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGUAACGAGCGC : 1101
AA3 13□1503 : UCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGUAACGAGCGC : 1101
LJ1 3□1541 : AACCCUUGUCCCUAGUUGCCAGCACAUAAUGGUGGGAACUCUAGGGAG : 1152
LA3 7□1503 : AACCCUUGUCCCUAGUUGCCAGCACAUAAUGGUGGGAACUCUAGGGAG : 1149
AJ1 14□1537 : AACCCUUGCCCUUAGUUGCCAGCACGUAAUGGUGGGAACUCUAGGGGG : 1149
AA3 13□1503 : AACCCUUGCCCUUAGUUGCCAGCACGUAAUGGUGGGAACUCUAGGGGG : 1149
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LA3 7□1503 : ACUGCCGGUGACAAACCGGAGGAAGGUGGGGAUGACGUCAAGUCAUCA : 1197
AJ1 14□1537 : ACUGCCGGUGAUAAACCGGAGGAAGGUGGGGAUGACGUCAAGUCAUCA : 1197
AA3 13□1503 : ACUGCCGGUGAUAAACCGGAGGAAGGUGGGGAUGACGUCAAGUCAUCA : 1197
LJ1 3□1541 : UGGCCCUUAUGGGCUGGGCUACACACGUGCUACAAUGGCCGGUACAGA : 1248
LA3 7□1503 : UGGCCCUUAUGGGCUGGGCUACACACGUGCUACAAUGGCCGGUACAGA : 1245
AJ1 14□1537 : UGGCCCUUAUGGGCUGGGCUACACACGUGCUACAAUGGCCGGUACAGA : 1245
AA3 13□1503 : UGGCCCUUAUGGGCUGGGCUACACACGUGCUACAAUGGCCGGUACAGA : 1245
LJ1 3□1541 : GAGCCGCGAACCCGCGAGGGGAAGCAAUCUCAGAAAACCGGUCGUAG : 1296
LA3 7□1503 : GAGCCGCGAACCCGCGAGGGGAAGCAAUCUCAGAAAACCGGUCGUAG : 1293
AJ1 14□1537 : GGGCCGCAAACCCGCGAGGGGCAGCUAAUCUCACAAAACCGGUCGUAG : 1293
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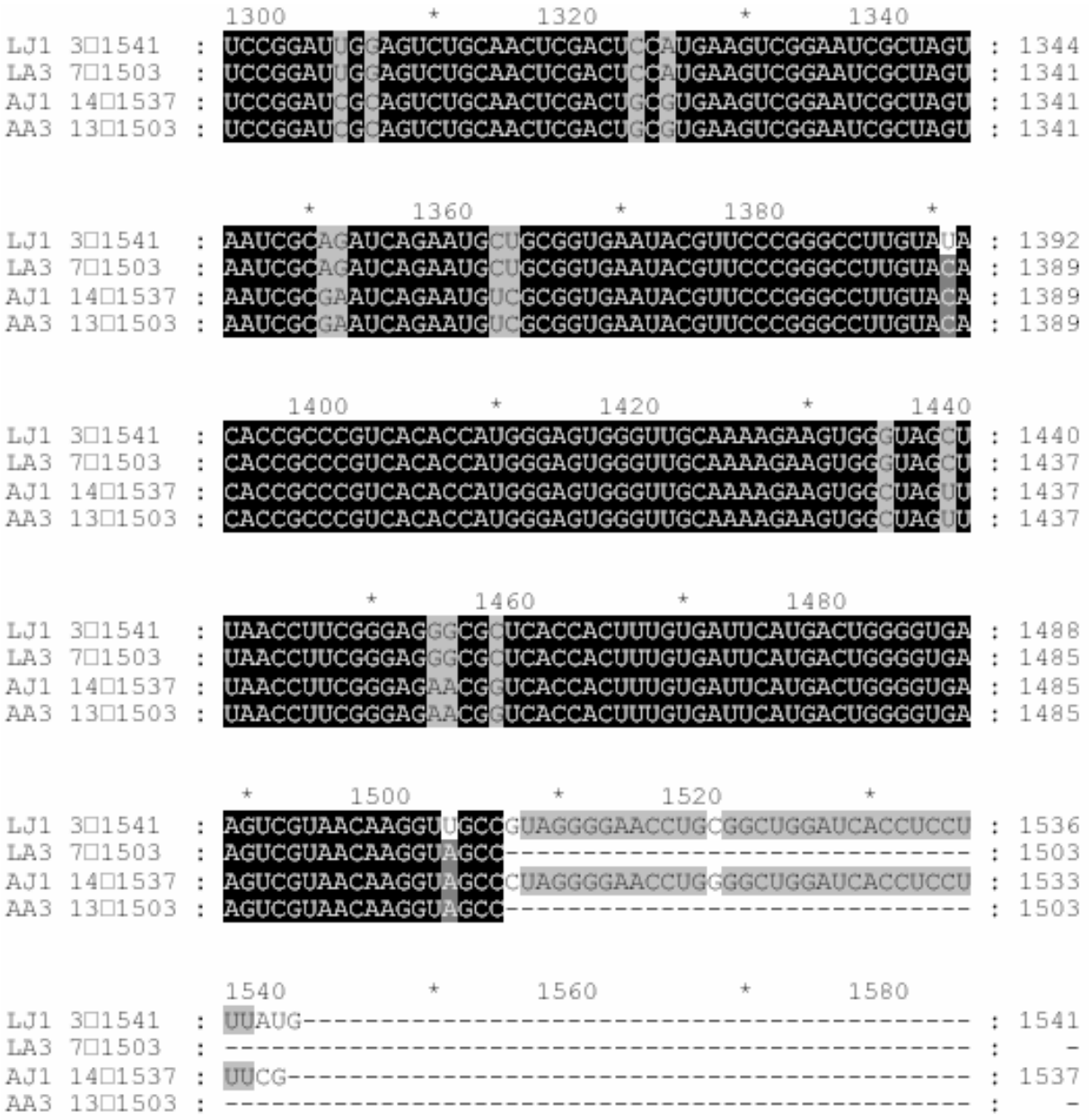

Figure 13: Alignment of selected endosymbiont 16S rRNA sequences obtained from *L. lacteus* and *A. fragilis* (one collected in April and July 2007 each) highlighting differences in nucleotides in grey (50 % difference) or white (> 50 % difference). Similarities between all four sequences are shaded with black. Numbers at the end of the lines (right) count the base pairs of each sequence and numbers on the left indicate the total number of base pairs obtained for each sequence (e.g. 1541 bp for sequence LJ1 3). Gaps are indicated by "-" in the alignment. LJ1 3: *L. lacteus* collected in July, individual number 1, clone number 3; LA3 7: *L. lacteus* collected in July, individual number 3, clone number 7; AJ3 14: *A. fragilis* collected in July, individual number 3, clone number 14; AA3 13: *A. fragilis* collected in April, individual number 3, clone number 13. The figure was constructed using GeneDoc (Nicholas & Nicholas, 1997).

Fluorescence in situ Hybridisation (FISH)

Signals from fluorescence *in situ* hybridisation (FISH) show low signal intensity in *L. lacteus*, but have been achieved from all bacteria containing cells of nine different individuals. The endosymbiont specific probe (SymLl1251) hybridises to small rod-shaped or coccoid bacteria with a length of 1.84 ± 0.31 µm.

Bacteria-containing cells are only found in the lateral zone, whereas cells of the ciliated and intermediary zone are free of fluorescent signal. In the lateral zone endosymbionts fill most of the bacteriocytes and show their highest abundance in the apical regions.

The general bacterial probe (Eub338-Mix) also reveals a high bacterial presence within the gill tissue. A combination of the two channels with the specific (blue) and the general bacterial (red) signal results in a pink colour. Within the tissue no blue or red sections remain (Figure 14).

Besides these intercellular bacterial signals some extracellular signals are also observed in *L. lacteus*. But in contrast to the endosymbionts, extracellular bacteria only hybridise with the general bacterial probe and not with the specific one. Therefore in the combination of the channels some red fluorescence signals between the filaments can be noticed (Figure 15). Hybridised extracellular bacteria are rod-shaped and larger than endosymbionts (3.00 ± 0.55 µm in length). Usually they occur in single lines between the gill filaments, but can also form clustered groups in the interfilamental channels.

Compared to *L. lacteus* signal intensity after FISH is much stronger in gill filaments of *A. fragilis*. For this species only gills of five individuals have been hybridised and the signal is observable in each of them. Small rod-shaped or coccoid bacteria measuring 1.79 ± 0.29 µm in length are detected by both endosymbiont specific probes (SymAf576 and SymAf1271) used.

Hybridisation is observable in gill filaments of *A. fragilis*, but the endosymbiont signals are not evenly distributed. Like in *L. lacteus* bacteriocytes are visible in the lateral zone only, whereas the ciliated and intermediary zones are free of fluorescent signals. The bacterial signals are located in most parts of the cells, but were especially abundant along the apical regions (Figure 16).

Concerning the general bacterial probe (Eub338-Mix) the hybridisation shows fluorescent signals within the bacteriocytes as well. In *A. fragilis* a combination of the channels for the specific and the general bacterial signals results in pink colour (Figure 14D). No regions remained without this mixing colour in all investigated individual gill tissues of this species.

In contrast to *L. lacteus* only intracellular bacteria with endosymbiont signals can be detected. There are no obvious concentrations of extracellular bacteria in lines or groups along the bacteriocyte channels. Only a few single extracellular bacteria were observed, but these were rather rare.

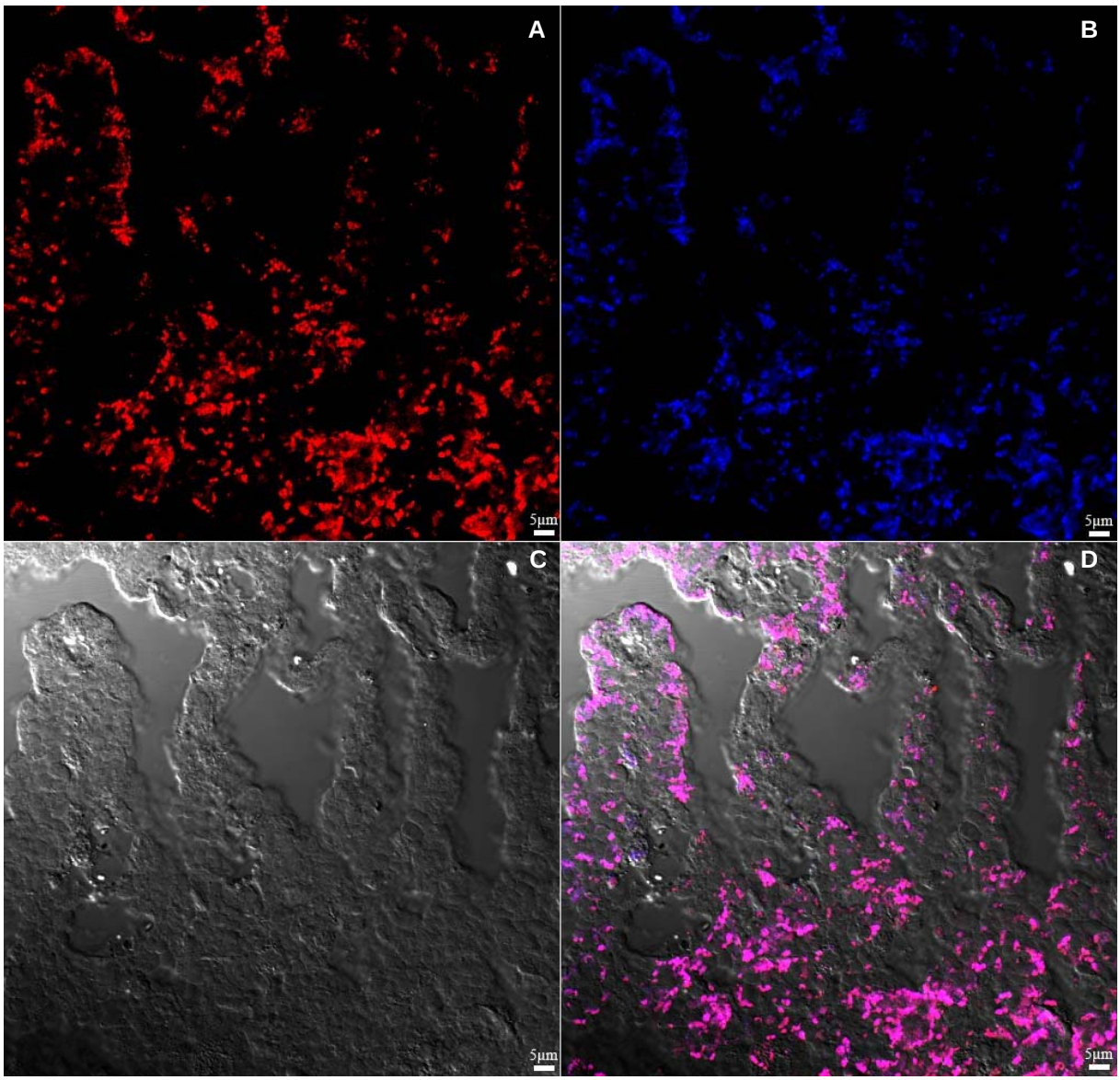

Figure 14: FISH signal from the gill tissue of *L. lacteus*. (A) Signal of the bacterial probe Eub338-Mix in red, (B) signal of the endosymbiont specific probe SymLl1251 in blue, (C) picture of the transmitted light channel and (D) combined picture of all three channels with a pink signal of endosymbionts with highest abundance in the apical cell regions of the lateral zone.

The probe used for negative control (Nonsense) does not show any fluorescent signal after hybridisation with gill tissue of *L. lacteus* or *A. fragilis*, when the same laser intensities are applied as for all other probes and stains. Only the bacterial probe, which was applied parallel, can be detected (data not shown). Therefore hybridisations with the used clone specific probes and the general bacterial probes are unambiguous.

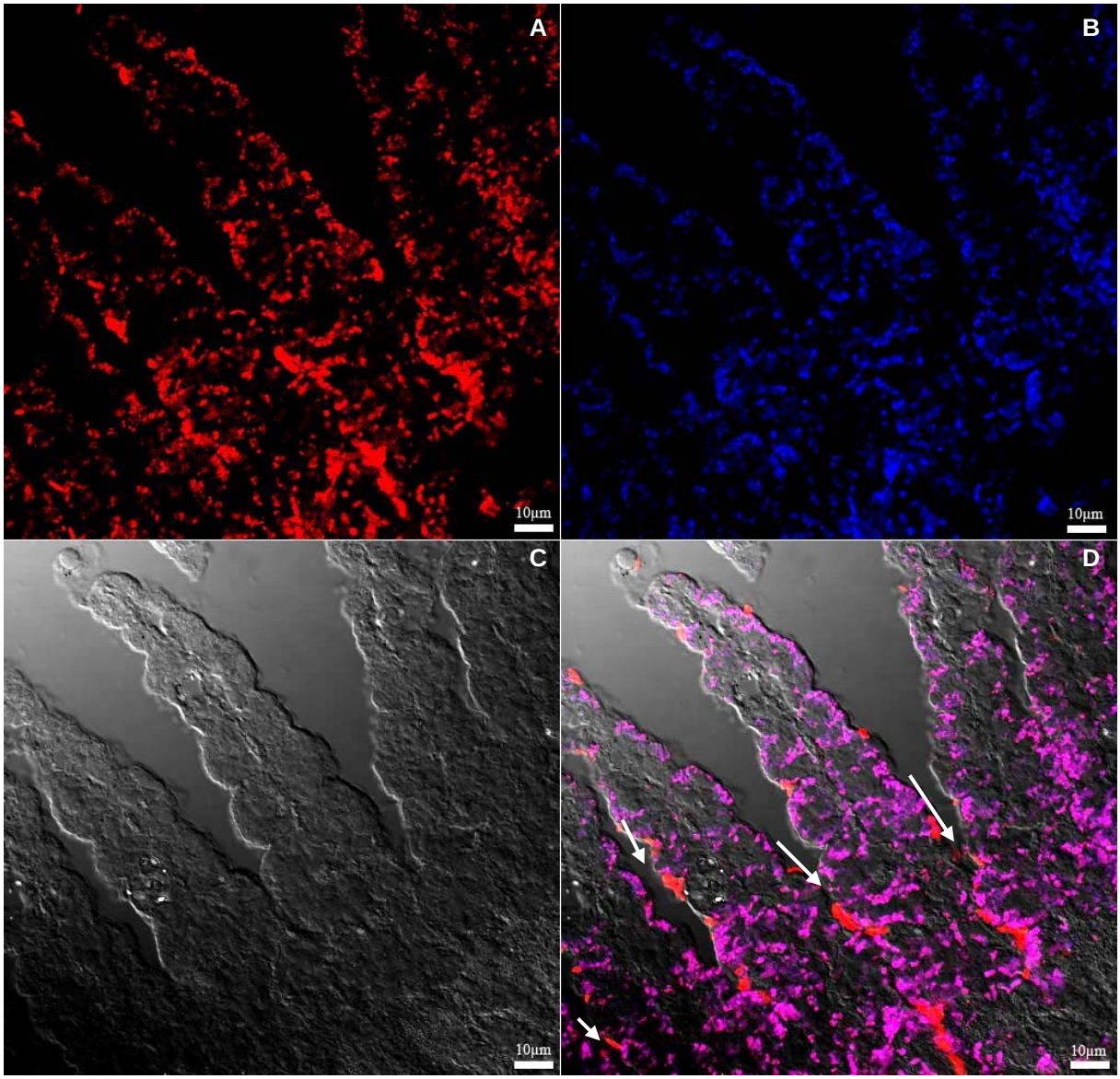

Figure 15: Images of FISH of *L. lacteus* endosymbionts in gill filaments. (A) Red signal of the bacteria specific probe Eub338-Mix, (B) endosymbionts of the bivalve giving a blue signal after hybridisation with the specific probe SymLI1251, (C) transmitted light channel outlining gill filaments and (D) combination of the three channels representing endosymbionts in pink and extracellular bacteria in red. Arrows indicating the extracellular bacteria lined along the interfilamental space.

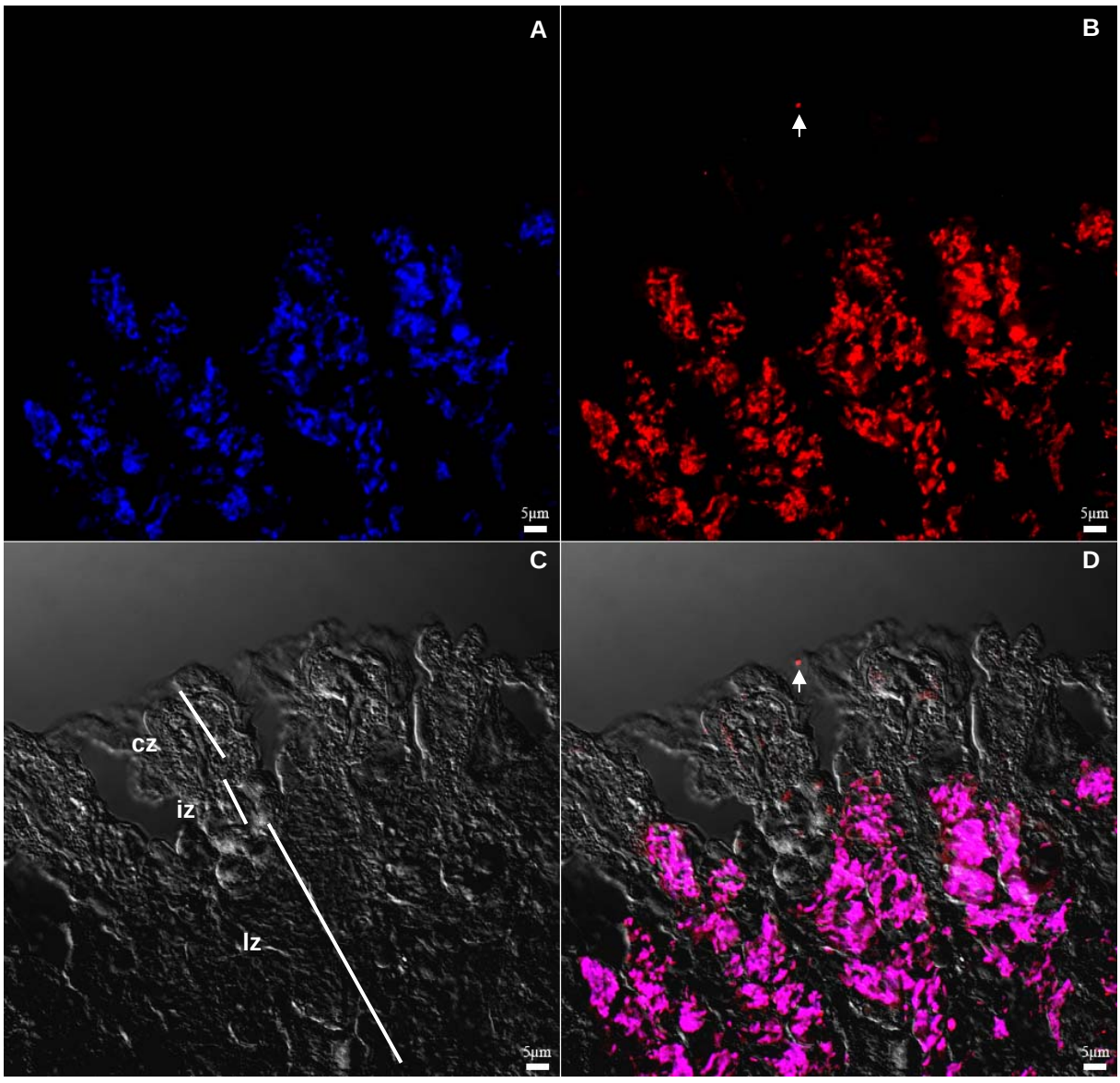

Figure 16: Image of gill filaments of *A. fragilis* after FISH. (A) Specific endosymbiont signal in blue maintained from the probe SymAf1272, (B) red fluorescent signal from the general bacterial probe Eub338-Mix, (C) outlines of the gill filaments in the transmitted light channel and (D) combination of all three channels in one image resulting in a pink endosymbiont signal from the whole lateral zone (lz). Neither the ciliated (cz) nor the intermediary (iz) zone contain any detectable endosymbionts. Arrow indicates an auto fluorescent granule.

Phylogeny of the Endosymbionts

Phylogenetic relationships of the symbionts were inferred from the 16S rRNA gene of two of each investigated bivalve species (one from specimens collected in April and July each), 32 of symbionts from diverse invertebrate hosts, four free-living γ-Proteobacteria and five other bacteria forming the outgroup.

The alignment consists of 1677 characters. Positions 1 - 40 and 1617 – 1677 were excluded from analyses because of a high proportion of missing data. A total of 544 characters are parsimony informative. All obtained trees are rooted with the free-living bacteria *Agrobacterium tumefaciens, Chlamydia trachomalis, Clostridium butyricum, Cytophaga* sp. and *Rickettsia rickettsii*. The maximum parsimony (MP) analysis results in 13 most parsimonious trees of a total length of 2535 [consistency index (CI) = 0.3949, homoplasy index (HI) = 0.6051, retention index (RI) = 0.6090 and rescaled consistency index (RC) = 0.2405] (strict consensus tree Figure 17).

A bootstrap analysis (10.000 replicates) shows low support values for basal clades, but better support for upper branches (Figure 17). In each of the obtained parsimonious trees the endosymbiont of *Loripes lacteus* clusters together with those of other Lucinidae. It forms a sistergroup to endosymbionts of *Codakia costata* and *Lucina floridana* (bootstrap value: 89). However, lucinid endosymbionts are not monophyletic. This becomes obvious by the clade formed of the endosymbionts of the two *Anodontia* species *A. fragilis* and *A. phillipiana* and *Solemya terraeregina* with the latter two forming a sistergroup (bootstrap value: 92). They cluster within a larger, weakly resolved clade of symbionts of Solemyidae (*Solemya terraeregina, S. pusilla*), Thyasiridae (*Thyasira flexuosa*), vestimentiferan tubeworms (*Escarpia spicata*) and the Lucinidae *Phacoides (Lucina) pectinata* (Figure 17).

Modeltest v3.6 returned the (GTR+I+G) model as the best fitting one for maximum likelihood (ML) analysis, which produced a tree with - logL = 14 791.20287. Substitution rates are A – C = 0.9752, A – G = 2.9852, A – T = 1.3602, C – G = 1.0277, C – T = 4.4970 and G – T = 1.00. Assumed nucleotide frequencies are set at A = 0.2536, C = 0.2130, G = 0.3034 and T = 0.2327. The assumed proportion of invariable sites was 0.2235 and the shape parameter (alpha) 0.4446. 101 characters (position 1 – 40 and 1617 - 1677) were excluded from the calculation resulting in a total of 1576 characters. The resulting ML tree is shown in Figure 18.

All the analyses performed using PAUP* resulted in the same clades, which differ only in basal positions. Lucinidae endosymbionts showed a polyphyletic

relationship with one clade of sequences from lucinids only, within which the *L. lacteus* endosymbiont groups. Parallel, a second general symbiont clade exists, since the endosymbiont sequences of the two *Anodontia* species and of *Phacoides* (*Lucina) pectinata* cluster among different other bivalve and vestimentiferan endosymbionts. Some other endosymbionts of diverse Solemyidae group outside forming two sistergroups with weak resolution. In all the analysis endosymbionts of deep-sea bivalves (Mytilidae, Vesicomyidae and the thyasirid *Maorithyas hadalis*) cluster together, but are less closely related to endosymbionts of Lucinidae than symbionts of Solemyidae, Thyasiridae and Vestimentifera (Siboglinidae) (compare Figures 17, 18).

Free-living sulphide oxidising bacteria (*Thiotrix* spp. and *Thiomicrospira* spp.) group rather basal and are only distantly related to from bivalve symbionts. In the case of *Thiomicrospira crunogena* and *T. thyasirae* the positioning varies slightly in the different analyses. Using ML these two free-living bacteria even form the sistergroup of the deep-sea bivalve symbionts.

Phylogenetic analyses performed in ARB show the same tendencies as described for the PAUP*-analyses (data not shown). They resulted in equal clades of Lucinidae, Vestimentifera, deep-sea bivalves and a mixed clade of Lucinidae, Solemyidae, Thyasiridae and Vestimentifera. The tree obtained under maximum parsimony resembled the one generated by PAUP*, but the resolving of the groups slightly differed. The two maximum likelihood methods with other models than the best fitting one resulted in trees being similar to the one from PAUP* with (GTR+I+G) model differing only in the positioning of the deep-sea-bivalve symbionts.

Therefore trees from ARB-analyses are not shown, since the trends are nearly the same and analysis performed in PAUP* present the more proper and higher resolved analyses.

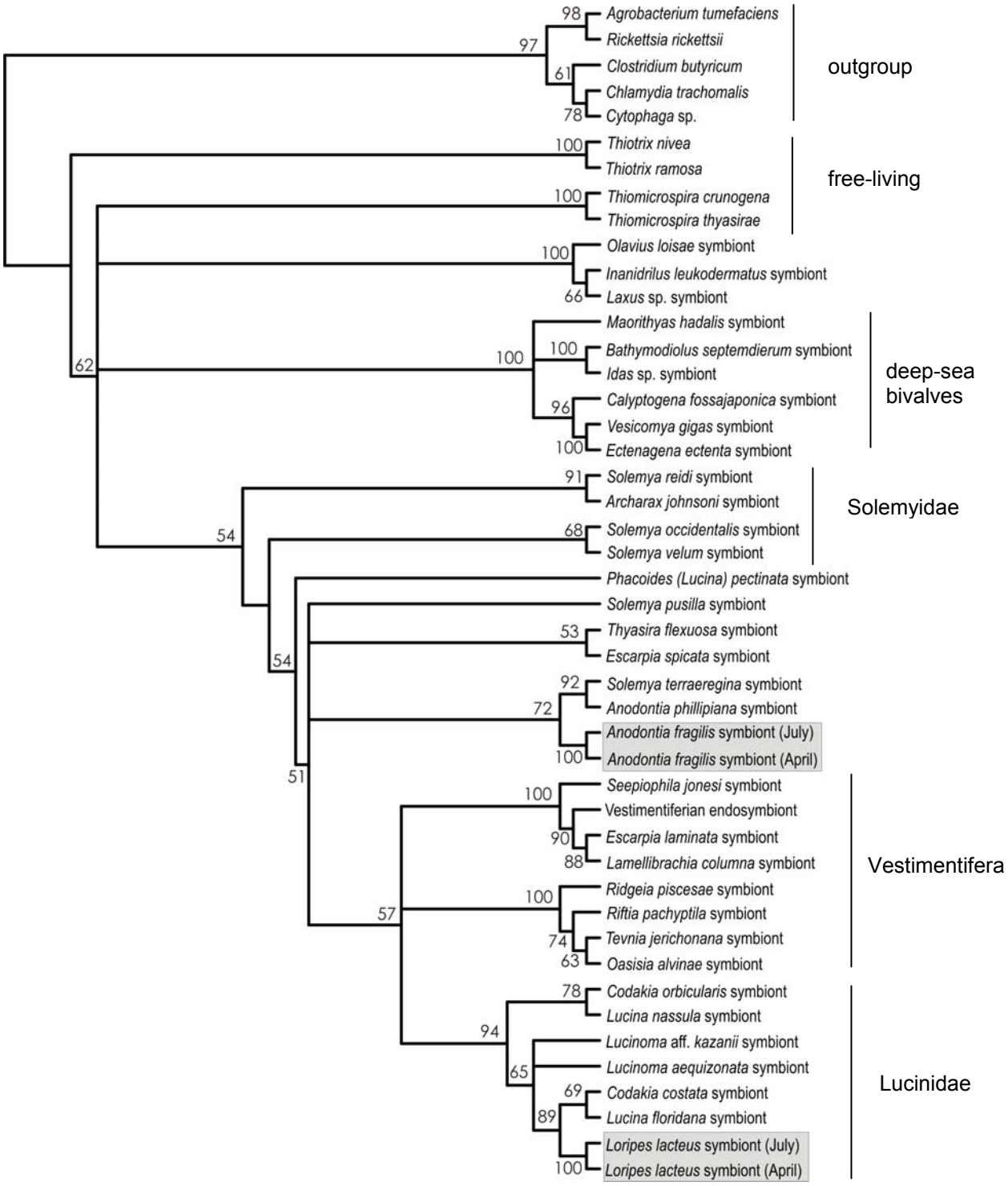

Figure 17: Strict consensus trees of 13 most parsimonious trees inferred from the 16S rRNA gene of diverse sulphide oxidising symbionts and free-living bacteria. Clades of different (symbiont) groups are labelled with family or group name of the hosts. Values indicate bootstrap supports (10.000 replicates) with those below 50 % not shown. The positions of the investigated species *L. lacteus* and *A. fragilis* are highlighted in grey.

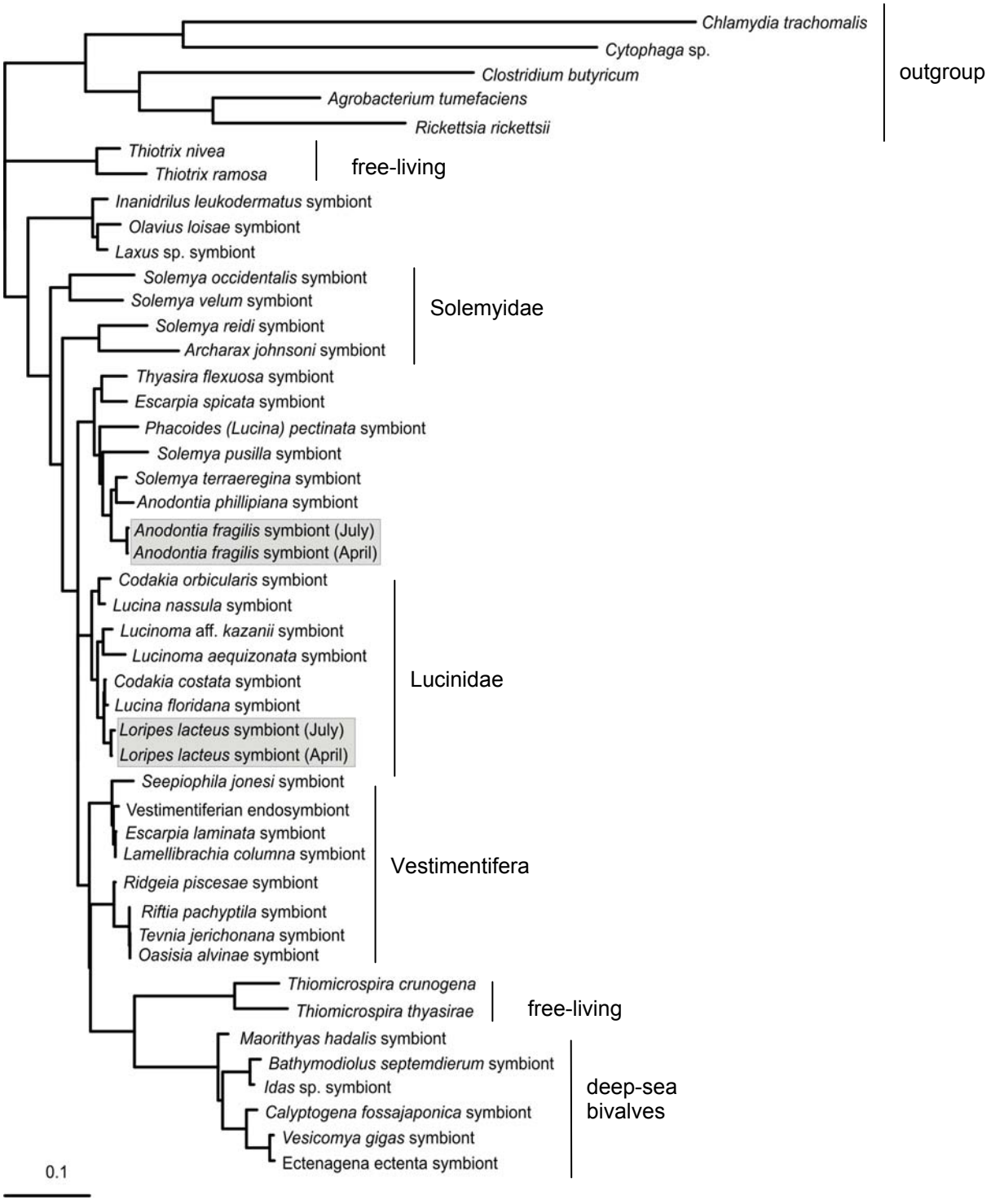

Figure 18: Maximum likelihood tree inferred from maximum likelihood analysis of the 16S rRNA gene of diverse sulphide oxidising symbionts and free-living bacteria [GTR+I+G model, - logL = 14791.20287, substitution rates A – C = 0.9752, A – G = 2.9852, A – T = 1.3602, C – G = 1.0277, C – T = 4.4970 and G – T = 1.00, assumed nucleotide frequencies A = 0.2536, C = 0.2130, G = 0.3034 and T = 0.2327, assumed proportion of invariable sites = 0.2235 and shape parameter (alpha) = 0.4446]. Clades of different (symbiont) groups are labelled with family or group name of the hosts. The positions of the investigated species *L. lacteus* and *A. fragilis* are highlighted in grey.

Discussion

Within this first part of the discussion the aims of the study shall be addressed and answered as far as possible giving a brief summary of the results.

Concerning the question of the number of occurring different symbiotic bacterial strains, sequence analysis and fluorescence *in situ* hybridisation (FISH) prove the existence of one specific endosymbiont species in gill tissue of *Loripes lacteus* and *Anodontia fragilis*. The generated symbiont sequences belong to the γ-Proteobacteria.

Since other co-occurring lucinids harbour the same symbiont species (Gros et al., 2003a), it was of interest, if the sympatric bivalves *L. lacteus* and *A. fragilis* do so as well. The endosymbionts belong to the γ-Proteobacteria, but represent members of two different, closely related lineages of sulphur-oxidising invertebrate symbionts.

To solve the question of endosymbiont location, FISH, light and transmission electron microscopy (TEM) were applied parallel. All these methods detect bacterial endosymbionts located within bacteriocytes in the lateral zone of gill filaments, whereas ciliated and intermediary zone remain symbiont-free.

In total, symbiosis-related gill structures of *L. lacteus* and *A. fragilis* resemble those of other investigated Lucinidae (e.g. Frenkiel et al., 1996; Gros et al., 2003a; Herry et al., 1989) and differ only in details. Cell types vary in each species and electron-lucent vesicles occur in the bacterioplasm of *L. lacteus* endosymbionts.

Addressing the question of phylogenetic relationships, endosymbiont sequences from *L. lacteus* are found to cluster among other lucinid ones. In contrast, endosymbionts of *A. fragilis* group within a general symbiotic clade of symbiont sequences from other lucinids, the bivalve families Solemyidae and Thyasiridae and vestimentiferan tube worms. Thus, phylogenetic analysis supports the existence of two different endosymbiotic species in the examined bivalve species. Further, monophyly of lucinid endosymbionts can be rejected.

Concerning the question, if different bacterial cohorts settling in consecutive host generations may lead to gill colour variation, no population differences are detectable between specimens from April and July 2007. Therefore, the observed colour changes can not be explained by such a phenomenon and are probably due to other reasons that lay beyond the potential of this study.

Symbiont Sequences

Potential endosymbiotic clone sequences belong to the γ-Proteobacteria. Similarity calculation of pairwise distances adds up to 98.6 % - 100 % in potential endosymbionts of *Loripes lacteus* and to 98.1 % - 100 % in *Anodontia fragilis*. Between the two bivalve endosymbionts sequence similarities lie at 94 %.

The γ-Proteobacteria represent the only chemoautotrophic endosymbionts of the bivalve family Lucinidae. Apart from *L. lacteus* and *A. fragilis* this bacterial strain also was endosymbiotic in other lucinid clams (e.g. reviewed in Distel, 1998; Gros et al., 2003a). γ-Proteobacteria are also endosymbiotic in other bivalves. Solemyidae (e.g. Krueger & Cavanaugh, 1997), Thyasiridae (e.g. Fujiwara et al., 2001), Vesicomyidae (e.g. Imhoff et al., 2003) and Mytilidae (e.g. Duperron et al., 2007b) harbour bacterial symbionts belonging to the γ-subdivision of the Proteobacteria.

The endosymbionts of each examined bivalve species belong to a single bacterial strain. Species definition in bacteriology proposes 97.5 % sequence similarity of the 16S rRNA as indication that bacteria represent the same species (Stackebrandt & Goebel, 1994). The endosymbionts of *L. lacteus* (> 98.6 % sequence similarity excluding one low quality sequence) and *A. fragilis* (> 98.1 % sequence similarity) possess sequence homology above this border value suggesting their classification as a single species. Nevertheless, resolution power of sequence analysis is significantly lower than that of DNA-DNA hybridisation (Amann et al., 1992), so latter would have to be applied for a more accurate classification.

So far in all examined Lucinidae only one symbiotic bacterial species was observed (e.g. Distel et al., 1988; Duran and Gros, 1996) and clone sequences obtained from *L. lacteus* and *A. fragilis* cluster among already known sulphur-oxidising symbionts (e.g. Duperron et al., 2007a; Schweimanns & Felbeck, 1985) within the γ-Proteobacteria. Therefore, even without analysing the endosymbionts' physiological function, a thioautotrophic metabolism can be assumed, as this way of indirect evidence was used in most studies (reviewed in Dubilier et al., 2008).

Even though *L. lacteus* and *A. fragilis* occupy the same *Cymodocea nodosa* sea-grass habitat, harboured bacterial endosymbionts represent two different species of distant relation. Sequence similarity between the examined endosymbionts lies below the proposed species border of 97.5 % (Stackebrandt & Goebel, 1994). Since it is unlikely that at sequence similarity below this value two organisms share more

than 60 – 70 % of DNA similarity and hence are unrelated at species level (Stackebrandt & Goebel, 1994), the endosymbionts belong to two different strains. This separation is also supported by the assignment into two phylogenetically different endosymbiotic clades (see phylogenetic analysis). This stands in contrast to studies describing up to six co-occurring bivalves harbouring the same endosymbiont species (Durand & Gros, 1996; Durand et al., 1996; Gros et al, 2000; 2003a).

Variability in gill colour does not result from different bacterial cohorts settling in consecutive host generations. No seasonal differences between bacterial populations of *L. lacteus* or *A. fragilis* were found and it can be assumed that the symbiont population remains stable over several months. Therefore, varying endosymbiont populations did not cause the observed gill colour variations in *L. lacteus*. In aquarium-maintained specimens of *Lucinoma aequizonata* with gill colour varying from yellow to grey, brown and black, darker gills contained fewer bacterial symbionts as well as lower sulphur and total protein concentrations (Hentschel et al., 2000). This is consistent with the finding that the storage of elemental sulphur directly influences colouration within trophosomes of *Riftia pachyptila* (Pflugfelder et al, 2005) and may also explain gill colouration changes in *L. lacteus*.

The rather low differences between all endosymbiotic clone sequences obtained from each bivalve species (*L. lacteus* or *A. fragilis*, respectively) may result from sequencing errors. For example, Distel and Wood (1992) found approximately 99.5 % of all detected differences in sequences of the same symbiont species attributable to sequence ambiguity. Alternatively, few nucleotide substitutions in symbionts of different host specimens might occur. But this stands in contrast to the description of endosymbiont sequences of up to six lucinid species to be identical (Durand & Gros, 1996; Durand et al., 1996; Gros et al., 2003a).

The high number of potential non-symbiotic clone sequences may result from environmental bacteria. A common method to avoid contamination with surface-attached, free-living prokaryotes is keeping living bivalves in 5 µm-filtered sea water prior to fixation (e.g. Gros et al., 2000). Otherwise, removed gills can also be rinsed in filtered sea water (e.g. Distel & Wood, 1992). These procedures were not performed with any of the examined specimens. Additional, non-symbiotic clone sequences match more closely with environmental bacteria of the γ-Proteobacteria and other lineages. It was thus necessary to exclude these sequences from further analyses to avoid misleading results like for *Thyasira flexuosa* (Distel & Wood, 1992).

In situ Detection of Endosymbionts

Using fluorescent *in situ* hybridisation (FISH) specific endosymbionts are detected within the lateral zone of gill filaments of *Loripes lacteus* and *Anodontia fragilis*. They show an even distribution throughout this zone. Negative control with a non-binding probe proved the specificity of hybridisation signals.

This study presents the first application of FISH on gill filaments of the bivalve family Lucinidae. Prior, the method was used for the detection of free-living symbionts of *Codakia orbicularis* in sediment samples (Gros et al., 2003b). Nevertheless, similar approaches were used for symbiosis description in Mytilidae (Duperron et al., 2005; 2007b; 2008), a solenogastre species (Katz et al., 2006) and several other symbiont-bearing invertebrates (e.g. Bright & Giere, 2005; Dubilier et al., 1995; 1999; Rinke et al., 2006).

Like the results from sequence analysis FISH signals support the presence of only a single symbiont species within gill filaments of both investigated bivalves. Detected signals from the general bacterial probe and the specific one, developed from endosymbiotic clone sequences, show an overlap of 100 %, which is expected in endosymbiosis with just one symbiont strain involved (compare Rinke et al., 2006). If another symbiont species were present, some areas showing only the general bacterial signal would remain.

Fluorescent signals are present in the entire lateral zone of both host bivalves indicating an even distribution of active endosymbionts. Since oligonucleotides target ribosomal rRNA, the signal is strong due to the high number of ribosomes in each target cell (Hugenholz et al., 2001). In contrast, no endosymbiont signals are observable from the ciliated and intermediary zone as is confirmed by ultrastructural morphology (e.g. Gros et al., 2003a; Herry et al., 1989).

Lower signal intensity observed in gill sections of *L. lacteus* may have several reasons. If the probe is error-free, which is supported by various tests, the difficulty lies in the target region, the 16S rRNA. Neighbouring proteins may block binding positions in the secondary structure, which leads to low signal intensities (Fuchs et al., 1998). Probes specific for *L. lacteus* and *A. fragilis* endosymbionts vary in their position and thus are differently influenced by surrounding proteins. Additionally, the fixation time (12 hours) being significantly longer than the 2 – 4 hours applied for other bivalves (e.g. Duperron et al., 2005), could have resulted in probe penetration

problems. The quality of preservation is a critical factor. If protein linkages established by the fixation fluid are too strong, probes can neither penetrate the cells nor bind to the target region. Since different tissues react differently to the fixation protocol, preservation quality may vary. This becomes even more evident, when taking into account that signal intensities even vary between different individuals of the same species (*L. lacteus* and *A. fragilis*, respectively). So it is likely that fixation time eventually was too long for *L. lacteus*, although it enabled good observable endosymbiont signals for *A. fragilis*.

Physiological differences of the endosymbiotic bacteria may cause stronger fluorescent signals from the apical regions of bacteriocytes. Newly endocytosed bacteria, as observed during symbiosis establishment in *Codakia orbicularis* (Gros et al., 1996a; 1998b) showing higher activity and older ones being partly digested (Herry et al., 1989), might be a reason for this variation. Comparable heterogeneous patterns were described by Caro et al. (2007) using flow cytometry. Within bacteriocytes of *C. orbicularis*, 7 endosymbiotic subpopulations of different relative nucleic acid content and therefore different activity were found (Caro et al., 2007).

The few detected extracellular bacteria singly lined along interfilamentar channels of gills of *L. lacteus* do not belong to a symbiotic strain. Specific oligonucleotide probes only target specific endosymbionts within the gill filaments (Amann et al., 1995). Since extracellular bacteria show no specific but only the general bacterial fluorescent signal, they can be assigned to surface attached environmental bacteria. Their presence is not unlikely, because the gills were not rinsed with filtered sea-water.

A combination of FISH and ultrastructural analyses like performed in the present study would be advantageous for future investigations of lucinid chemosymbiosis. These methods enable the definite identification and localisation of endosymbionts in gill tissues, but were not previously applied to Lucinidae. Nevertheless, both techniques were used to gain information about other symbiosis like in mussels of the genus *Bathymodiolus* (Duperron et al., 2005; 2007b; 2008). A 3-dimentional analysis of gill filaments and symbionts contained within might further improve our knowledge. Additionally a combination of FISH with infection experiments as performed by Gros et al. (2003a) might give an interesting insight in symbiosis establishment.

Phylogenetic Relationships of Endosymbionts

Phylogenetic analysis grouped endosymbionts of *Loripes lacteus* among other lucinid endosymbionts, whereas those of *Anodontia fragilis* clustered in a general clade of Lucinidae, Solemyidae, Thyasiridae and Vestimentifera symbionts. Monophyly of lucinid endosymbionts was not supported. Symbionts of other Vestimentifera and deep-sea bivalves formed distinct clades.

Lucinidae endosymbionts are polyphyletic according to the performed phylogenetic analyses. Since two separate clades, one consisting of lucinid endosymbionts only and a general one containing other bivalve and vestimentiferan symbionts, were found, monophyly must be rejected. This is in accordance with the results of other phylogenetic analyses (Durand et al., 1996; Duperron et al., 2007a).

Endosymbionts of the two investigated species are only distantly related. *L. lacteus* endosymbionts group within a clade solely containing Lucinidae endosymbionts, lacking those of *A. fragilis*. In contrast, endosymbionts of this clam are more closely relation to other symbionts like those of the protobranch *Solemya terraeregina*, the thyasirid *Thyasira flexuosa* or vestimentiferan tube worms. Anyway, the close relation to endosymbionts of *A. phillipiana* resembles host phylogeny (Distel et al., 1994), where the genus *Anodontia* was found to form a distinct clade within the Lucinidae (Williams et al., 2004). This might indicate co-speciation like proposed for several symbiont-bearing invertebrates (reviewed in Dubilier et al., 2008).

Relationships of symbionts of different bivalve families imply the existence of several symbiotic lineages. Support for this hypothesis is found in each bivalve family. Lucinidae endosymbionts form the two already mentioned clades. Symbionts of Thyasiridae are separated in an extracellular lineage grouping with lucinid endosymbionts, whereas an intracellular, deep-sea line clusters among thioautotroph symbionts of Vesicomyidae and Mytilidae (Fujiwara et al., 2001; Duperron et al., 2007b; 2008) from the same habitat. In the remaining family, the Solemyidae, bacterial endosymbionts of *Solemya velum* and *S. occidentalis* are more closely related to each other than to other symbionts of the family, for which reason a common ancestor was proposed for them (Krueger & Cavanaugh, 1997). Therefore, it is most likely that chemosymbiosis evolved several times and did so independently within bivalve families (and other invertebrates, respectively) as was also suggested by other authors (reviewed in Dubilier et al., 2008).

Free-living chemoautotrophic bacteria included in the analysis are only distantly related to symbiotic lineages. According to their positioning nearest to the clade of deep-sea bivalve symbionts (Vesicomyidae, Mytilidae and the thyasirid *Maorithyas hadalis*), they probably are their closest relatives within symbiotic lineages, which is in congruence with other studies (Distel et al., 1988; Distel et al., 1994; Gros et al., 2000). Therefore, deep-sea bivalve symbionts may represent the youngest of several lineages. An inclusion of more free-living sulphur-oxidising bacteria in the analysis might be of advantage (compare Dubilier et al., 2008), but this lay beyond the prospects of the present study focusing on Lucinidae.

The 16S rRNA gene does not represent a very good phylogenetic marker regarding basal positions. Basal resolution is not optimal and lowly supported in phylogenetic analysis, since in the conservative rRNA gene nucleotide differences are restricted to taxonspecific hypervariable regions (Stackebrandt & Goebel, 1994). Due to the high conservation its ability as a phylogenetic marker is limited in basal positions. Although basal clades in this study might be weakly resolved, at family level groups possess a higher resolution and good support values. The 23S rRNA provides a potential alternative, but presently available sequences are limited.

The inclusion of additional lucinid endosymbiont sequences might be advantageous to further analyse the observe differentiation in two clades. Symbiont sequences of close relatives of *L. lacteus* and *A. fragilis* may increase the knowledge about the symbiotic bacteria of the investigated bivalves. Lucinid endosymbiont sequences obtained from underrepresented habitats like the Mediterranean or other habitats at higher latitudes might answer questions concerning symbiosis evolution.

Morphology of Gills and Bacteria Containing Cells

Gills are enlarged and their filaments can be distinguished into three distinct regions: a ciliated, an intermediary and a lateral zone. The latter mainly consists of bacteriocytes containing endosymbionts interspersed with intercalary cells. In *Loripes lacteus* hemocytes and bacteriocytes with large, granular inclusion are present, whereas *Anodontia fragilis* additionally possesses mucocytes. Prokaryotic endosymbionts are enclosed in vacuoles within bacteriocytes and those of *L. lacteus* contain vesicles in the bacterioplasm.

The subfilamentar region of symbiont-bearing bivalves is enlarged due to the formation of more bacteriocytes, which can be regarded as adaptation to the chemosymbiosis. As a result, the whole gill becomes enlarged and fleshy. This characteristic is shared by bivalves harbouring chemoautotrophic symbionts (Taylor & Glover, 2000; 2006). In bivalve species without symbiotic bacteria, ctenidia mainly consist of the filamentar region, since the subfilamentar region is not engorged. Southward (1986) described this effect comparing gill filaments of symbiont containing Thyasiridae and Lucinidae with non-symbiotic bivalve families.

The conspicuous difference in gill colour between *L. lacteus* and *A. fragilis* is probably due to different contained or stored substances. Elemental sulphur (S^0) stored in gills of *L. lacteus* may lead to white or greyish colouration comparable to juveniles of *Riftia pachyptila* with the content of S^0 directly resembling the colour of the developing trophosome (Pflugfelder et al., 2005). On the other hand, the dark red gills of *A. fragilis* probably result from haemoglobin. In *Myrtea spinifera* and *Phacoides (Lucina) pectinata*, both species with dark coloured gills, haemoglobin was hypothesised to be involved in oxygen tension (Dando et al., 1985; Frenkiel et al., 1996). Differences in colouration are also reflected in ultrastructure by electron-lucent cytoplasm of *L. lacteus* and electron-denser one of *A. fragilis*. Nevertheless, colouring pigments were not analysed in this study and their real nature can not be assessed.

In both investigated species, ciliated and intermediary zone as well as their cell types are similar to those previously described in other members of the Lucinidae (e.g. Dando et al., 1985; Frenkiel & Mouëza, 1995; Frenkiel et al., 1996; Gros et al., 1996b). The length of the intermediary zone varies between species being short in *L. lacteus* as in its relative *L. lucinalis* (Herry et al., 1989), whereas that of *A. fragilis* consists of several large, clear cells similar to *A. alba* (Gros et al., 2003a).

Besides bacteriocytes and intercalary cells the lateral zones of *L. lacteus* and *A. fragilis* possess different cell types, which may indicate dissimilar functional aspects of the symbiosis. Unique features of the lateral zone were interpreted as determinant for the metabolic relationship between host and symbiont (Gros et al., 1996b). Examples for such features are the granule-cells of *Codakia orbicularis* (Frenkiel & Mouëza, 1995), high levels of cytoplasmic haemoglobin in *Phacoides (Lucina) pectinata* (Frenkiel et al., 1996) and *Myrtea spinifera* (Dando et al., 1985) or peroxisomes detected in *Lucina (Linga) pensylvanica* (Gros et al., 1996b).

Cell types detected in *L. lacteus* are widely similar to those found in its relative *L. lucinalis* with only slight differences. Bacteriocytes, intercalary cells, hemocytes and bacteriocytes with large, granular inclusions were found in *L. lacteus*. Herry et al. (1989) additionally reported few mucocytes dispersed among bacteriocytes in *L. lucinalis*, but this cell type was not detected in the examined specimens of *L. lacteus*. Either *L. lacteus* does not possess mucocytes, because of different morphological interactions with its symbionts, or these secretory cells were overlooked due to the lack of special staining techniques for glucose or mucous detection.

Symbiotic relations of *A. fragilis* seem to be similar to those of its relative *A. alba*, since both species share most of their ultrastructural features. Both species possess bacteriocytes alternating with intercalary cells along the lateral zone, whereas mucocytes are restricted to the abfrontal parts (compare Gros et al., 2003a). The concentration of mucocytes may indicate that this cell type plays a minor role within *Anodontia* spp. compared to other lucinids like *Lucina (Linga) pensylvanica* (Gros et al., 1996) or *Phacoides (Lucina) pectinata* (Frenkiel et al. 1996; Liberge et al., 2001). Here, this cell type was found at both ends of the lateral zone.

Although intercalary cells in the lateral zone of *L. lacteus* and *A. fragilis* vary in appearance, their function probably is the same. Intercalary cells were found to be elongated and more or less trumped shaped, overlapping the surface of neighbouring bacteriocytes. This tendency is stronger in *L. lacteus*. Since in neither species pigment granules are present within intercalary cells I disagree with Distel & Felbeck (1987). Based on the appearance of these inclusions, the authors postulated an involvement of intercalary cells in the elimination of waste products from bacterial lysis. But since these cells are always free of endosymbiotic bacteria, they may be involved in the formation of new bacteriocytes as presumed by several other authors (Reid & Brand, 1986; Herry et al., 1989).

The existence of hemocytes within the blood lacuna of *L. lacteus* gill filaments could be a hint for exchanges between the lacunal space and bacteriocytes. Herry et al. (1989) described pseudopodial-like expansions of the hemocyte cytoplasm in contact with the blood lacuna's epithelium in *L. lucinalis*. In juveniles of *Codakia orbicularis* hemocytes and basal membrane inside the lacuna also were in close contact (Frenkiel & Mouëza, 1995). This phenomenon was presumed to result from detoxification processes of lysed products (Herry et al., 1989), but the hypothesis could not be proven. Anyway, in *L. lacteus* the contact between hemocytes and the basal membrane of the blood lacuna is not as extensive. Thus, any occurring exchanges are probably of a lower importance than in *L. lucinalis*.

Since large, electron-dense, granular inclusions of amorphous material or membrane-like whorls appear in basal regions of bacteriocytes of *L. lacteus*, they could originate from lysed bacteria. The observed surface invaginations of basal bacteria may give a further hint for this hypothesis. Similar inclusions containing osmiophilic material (Herry et al., 1989) or myelin-like structures (Johnson & Fernandez, 2001) were detected in *L. lucinalis*. In other lucinids these granules were referred to as "pigment granules" (Distel & Felbeck, 1987) or "storage cells" (Southward, 1986). According to the common opinion they result from lytic activity.

If these granular inclusions present in *L. lacteus* are enough to call the cells "granule-cells" remains with the reader, but their existence indicates a higher lytic activity than in bacteriocytes of *A. fragilis* or even a different way of nutrition. Like specimens examined in this study, other members of the genus *Anodontia* lack granule-cells (Gros et al., 2003a; Taylor & Glover, 2006). In *A. fragilis* the few lysosomes and small electron-dense inclusions mainly found in the transition of intermediary and lateral zone were not as dominant as granular inclusions in gill filaments of *L. lacteus*. Therefore, the assumption of less lysosomal activity in *A. fragilis* bacteriocytes seems justified. If bacteria are lysed for nutrition of *L. lacteus* this food source probably is of lower importance in *A. fragilis*.

Bacteriocytes are the dominant cells in the lateral zone of both investigated species and thus most essential for the maintenance of chemosymbiosis. All lucinids examined so far were found to contain endosymbiotic bacteria within bacteriocytes (e.g. Dando et al., 1985; 1986; Frenkiel & Mouëza, 1995; Taylor & Glover, 2006). This cell type provides the necessary features for a successful function of the symbiotic association.

Round or drop-shaped bacteriocytes increase the volume available for bacterial settlement and parallel the microvilli-covered surface enables exchange with the environment via the bacteriocyte channels. This is important for the functioning of the symbiosis. More endosymbionts provide more nutrition for the host and microvilli were shown to enhance interactions with the water flow along their surface (Herry et al., 1989) by which substances like oxygen and sulphide can be received.

Very small electron-lucent inclusions in the electron-dense bacteriocytes' cytoplasm in *A. fragilis* may provide a variant of sulphur storage. They are present in all examined specimens and looked similar to elemental sulphur stored in *Phacoides (Lucina) pectinata*. This host species produces small vesicles containing elemental sulphur (Liberge et al., 2001; Lechaire et al., 2006) located at the basal pole of bacteriocytes (Frenkiel et al., 1996). But the electron-lucent inclusions in *A. fragilis* may also be normal features of the cytoplasm independent from sulphides or even represent problems within fixation or infiltration during TEM processing. Since no element analysis was performed their real nature cannot be revealed by this study.

Periplasmic, membrane bound vesicles in endosymbiotic bacteria of *L. lacteus* presumably function as sulphur storage. One large or several smaller vesicles were detected in various Lucinidae and regarded as sulphur globules (e.g. Frenkiel & Mouëza, 1995; Gros et al., 2000; Southward, 1986). Lechaire et al. (2008) proved that elemental sulphur was contained in the cytoplasm of bacterial symbionts in *Codakia orbicularis*, suggesting their location in the periplasmic granules inside the endosymbionts. In case of sulphur depletion in the environment these storage compounds probably constitute an important energy source (Lechaire et al., 2008).

Similar vesicles were not present in endosymbionts of *A. fragilis* leading to the conclusion that sulphur storage in the symbionts of the two examined bivalve species is different. Although ultrastructural images from *A. alba* show small vesicles in bacterial cytoplasm (Gros et al., 2003a), sulphur storage is not proven for them. No such vesicles were found in endosymbionts of *A. fragilis*. Together with the above discussed potential storage of sulphur within bacteriocyte cytoplasm it can be assumed that sulphur is stored differently in symbionts of *L. lacteus* and *A. fragilis*.

Elemental analysis of gill filaments or specific staining techniques may answer remaining questions about the nature of inclusions. Nevertheless, this expanded the possibilities of the present study. Thus, the chemosymbiosis needs further attention for a better understanding of the complex interactions between hosts and symbionts.

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Appendix

Abstract

Symbioses are a potent survival solution for organisms in hostile environments like sulphide rich sediments, as was proven in several invertebrates. Among these, the bivalve family Lucinidae was reported to harbour sulphide oxidising endosymbionts to cope with the harsh conditions of their habitats and exploit the energy rich resources. Since mainly large tropical representatives of this family were examined in the past, my diploma thesis focused on the small Mediterranean species *Loripes lacteus* (Linnaeus, 1758) and *Anodontia (Loripinus) fragilis* (Philippi, 1836). The study aimed to provide a description of endosymbionts of both clam species. Therefore, in each of these bivalves collected from sulphide rich sediment layers below a *Cymodocea nodosa* sea-grass bed, sequences of the bacterial 16S rRNA gene were identified. Low sequence similarity based on the calculation of pairwise distances (P-distances) gave a first indication that endosymbionts of *L. lacteus* and *A. fragilis* belong to different bacterial strains. Using clone specific probes, fluorescence *in situ* hybridisation (FISH) proved the specificity of potential endosymbiotic sequences. Further, an even distribution of the symbionts throughout the lateral zone of gill filaments was observed using the confocal laser scanning microscope. *L. lacteus* and *A. fragilis* were shown to harbour a single, specific endosymbiont population of γ-Proteobacteria, which was stable over three months. On transmission electron micrographs, single endosymbionts enclosed by vacuoles located in large bacteriocytes along the lateral zone were detected. Additional cell-types varied slightly between the examined bivalves, probably due to different physiological adaptations of symbiont and host. Endosymbionts of *L. lacteus* further possess several vacuoles within the bacterial cytoplasm, most likely for sulphide storage. Phylogenetic analyses do not supported monophyly of lucinid endosymbionts. Symbiotic sequences originating from *L. lacteus* are related to endosymbionts of *Lucina floridana* and *Codakia costata*. 16S rRNA sequences obtained from *A. fragilis* form a sistergroup with *A. phillipiana* and *Solemya terraeregina* clustering within a general symbiotic clade of symbiont sequences from the lucinid *Phacoides (Lucina) pectinata*, Solemyidae, Thyasiridae and Vestimentifera. Thus, the phylogenetic analysis supported the existence of two different endosymbiotic species in *L. lacteus* and *A. fragilis*.

Zusammenfassung

Symbiosen stellen, wie anhand einiger Invertebraten belegt, eine potentiell wichtige Überlebensstrategie vieler Tiere in unwirtlichen Lebensräumen dar. Solche Lebensräume können etwa schwefelhaltige Sedimentschichten sein. Um mit den lebensfeindlichen Bedingungen hier zu Recht zu kommen, besitzen Muscheln der Familie Lucinidae (dt. Mondmuscheln) Sulfid-oxidierende Endosymbionten. Außerdem schließen die Symbionten durch den chemischen Prozess die energiereichen reduzierten Schwefelverbindungen als zusätzliche Nahrungsquelle auf. Da bislang vorwiegend tropische Vertreter der Familie untersucht wurden, beschäftigt sich meine Diplomarbeit mit den kleineren Mittelmeerarten *Loripes lacteus* (Linneaus, 1758) und *Anodontia (Loripinus) fragilis* (Philippi, 1836). Ziel der Arbeit war eine Beschreibung der Endosymbionten beider Muschelarten. Die analysierten Tiere stammten aus Sulfid-reichen Sedimentschichten unterhalb einer *Cymodocea nodosa*-Seegraswiese. Sequenzanalysen hatten das 16S rRNA Gen des bakteriellen Symibonten zum Ziel. Niedrige Sequenzähnlichkeiten, basierend auf der Berechnung von paarweisen Distanzen (P-distances), gaben einen ersten Hinweis, dass die Endosymbionten von *L. lacteus* und *A. fragilis* zwei unterschiedlichen Bakterienarten angehören. Basierend auf der Sequenz wurden klonspezifische Sonden für die Muschelsymbionten entwickelt und mittels Fluoreszenz *in situ* Hybridisierung (FISH) die Spezifität geprüft. Außerdem konnte mittels confocaler Laser-Scannung Mikroskopie eine gleichmäßige Verteilung des fluoreszierenden Symbiontensignals in der gesamten Lateralzone der Kiemenfilamente beobachtet werden. Die Untersuchung ergab, dass *L. lacteus* und *A. fragilis* eine einzige, spezifische Endosymbiontenpopulation aus der Gruppe der γ-Proteobacteria beherbergen, die über drei Monate unveränderlich blieb. Transmissionselektronenmikroskopische Aufnahmen zeigen einzelne Endosymbionten eingeschlossen in Vakuolen, die innerhalb großer Bacteriocyten über die gesamte Lateralzone verteilt sind. Außerdem variieren die zusätzlichen Zelltypen in den untersuchten Bivalven, was sich vermutlich auf unterschiedliche physiologische Anpassungen an die Symbiose zurückführen lässt. Im Bakterioplasma der Endosymbionten von *L. lacteus* befinden sich zahlreiche Vakuolen, die möglicherweise der Sulfidspeicherung dienen. Phylogenetische Analysen unterstützen eine Monophylie von Endosymbionten der Familie Lucinidae nicht. Stattdessen zeigen symbiontische Sequenzen basierend auf Kiemen-Extrakten

von *L. lacteus* eine nahe Verwandtschaft mit Symbionten von *Lucina floridana* und *Codakia costata*. 16S rRNA Sequenzen aus *A. fragilis* bilden dagegen eine Schwesterngruppe mit *A. phillipiana* und *Solemya terraeregina*. Diese Gruppe clustert innerhalb eines allgemeinen Symbiontenclades, das neben der Endosymbiontensequenz der Lucinidae *Phacoides (Lucina) pectinata* auch die Symbionten der Bivalvenfamilien Solemyidae und Thyasiridae, sowie solche von vestimentiferen Röhrenwürmern enthält. Damit ist durch die phylogenetische Analyse die Existenz zweier unterschiedlicher endosymbiontischer Arten in *L. lacteus* und *A. fragilis* untermauert.

Curriculum Vitae

PERSONAL INFORMATION	
Name	Mauß Michaela
Nationality	Austria
Date of birth	23.10.1982
Place of birth	Melk
SECONDARY EDUCATION	
Dates (from – to)	1997 – 2001
School	BORG Krems
Address	Hainemannstrasse 12, 3500 Krems, Austria
ACADEMIC BACKGROUND	
Dates (from – to)	2002 – 2008
University	University of Vienna
Address	Althanstrasse 14, 1090 Vienna, Austria
Study	Biology/Ecology
TEMPORARY STAY ABROAD	
Dates (from – to)	01.09.2005 – 17.01.2006
University	Lund University
Address	Lund University Box 117, 221 00 Lund, Sweden
Study	Ecology
WORK EXPERIENCE	
Dates (from – to)	01.09.2006 – 31.01.2007 & 01.09.2007 – 31.01.2008
Name and address of employer	Gerhard Steiner Ao. Univ.-Prof. Dr., University of Vienna, Austria
Occupation or position held	Tutor
Main activities and responsibilities	Coaching students in comparative morphology (Porifera – Arthropoda)
	01.09.2008 – 31.01.2009
	Gerhard Steiner Ao. Univ.-Prof. Dr., University of Vienna, Austria
	Tutor
	Coaching students in comparative anatomy and morphology of Mollusca