

Bacterial communities in the hepatopancreas of different isopod species

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ABSTRACT:

This study aims to describe animal bacterial associations with culture independent methods. Bacterial communities in the hepatopancreas of the following 7 species of isopods (Pericaridea, Crustacea, Arthropoda) from 3 habitat types were investigated: 2 subtidal species *Idotea baltica* (IB) and *I. wosnesenskii* (IG); 2 intertidal species *Ligia occidentalis* (LO) and *L. pallasii* (LP), and 3 terrestrial species *Armadillidium vulgare* (A), *Oniscus asellus* (O) and *Porcelio scaber* (P). CARD FISH and 16S clone libraries from environmental samples isolated from the hepatopancreas of isopods were used to describe the bacterial communities. Previous work has described two species of symbionts of terrestrial isopods and found very low diversity (predominately only one species per host). Clone libraries from some but not all species in this study included sequences closely related to previously described isopod symbionts with the majority clustering around *Candidatus Hepatoplasma crinochetorum* (Firmicutes, Mollicutes). These sequences clustered by host species confirming published results of host specificity. Closely related sequences to the other described symbiont '*Candidatus Hepatincola porcellionum*' (α -Proteobacteria, Rickettsiales) were only obtained from the hepatopancreas of *L. pallasii*. Counts of bacterial abundance obtained with CARD FISH (Probe EUB I-III) ranged between 1.9×10^3 – 1.7×10^4 bacteria per hepatopancreas, this numbers are 2 to 3 orders of magnitude lower than previously published counts of DAPI stained cells.

INTRODUCTION:

Bacterial associations with animal hosts are important on host functioning. Eukaryotes in a number of phyla have overcome their limitations in nutritional capabilities by associating with microorganisms. Nutritional symbioses are particularly common in animals that feed on diets that are either limited in essential amino acids and vitamins or that are otherwise difficult to digest (Buchner 1965, Douglas 1994). For example, lignin, cellulose and other polymers - resistant to degradation by animal digestive enzymes - are digested by bacterial and fungal enzymes in the gut of herbivorous mammals and arthropods (Douglas 1994, Zimmer et al. 2001, 2002). Novel metabolic capability acquired by one organism from its partner can allow the partners of the symbiosis to expand on their distribution and occupy hitherto unfilled niches (Douglas 1994). While the importance of gut microbes has received much consideration in terrestrial species, little attention has been given to the role of these microbes in aquatic and marine invertebrates (Harris 1993).

Isopods (Eukaria, Animalia, Arthropoda, Crustacea, Malacostraca, Pericaridea) are a good system to study changes in bacterial gut community composition, because they can be found from strictly marine to strictly terrestrial habitats (Abbott 1939, Morris et al. 1980). Within the isopods only isopods in the family Oniscidea important in litter decomposition on land, however have become completely independent of the aquatic environment. This transition from sea to land is supposed to have occurred through the high intertidal or supra-littoral zone (Sutton & Holdich 1984). Recent studies have investigated isopod bacterial associations in a small group of isopods and have described

two bacterial symbionts from the hepatopancreas of the isopod *Porcelio scaber* (Wang et al. 2004a,b). The separation of the isopod digestive system into different microhabitats is relevant in terms of functional significance. Ecdysis is a disruptive force for the development of stable bacteria-host association as both fore and hindgut of crustaceans are molted and need to be re-colonized with a bacterial community following ecdysis. The possibility of establishment of a more stable bacterial community can be expected to form in the midgut that does not undergo ecdysis. In Oniscidean isopods the midgut is greatly reduced but forms the digestive midgut gland or hepatopancreas, with 2 or 3 large paired lobes (Zimmer 2002). The hepatopancreas is involved in the secretion of digestive enzymes and the absorption of nutrients.

Previous research reports high numbers of bacteria in the order of $10^6 - 10^8$ bacteria per hepatopancreas as determined by DAPI counts (Zimmer et al. 2001), diversity has been described as low with only one or two species present (Wang et al. 2007). No bacteria have been described for subtidal isopods (Zimmer et al. 2001). Zimmer and colleagues (2001, 2002) suggested a possible role of these symbionts in cellulose digestion and phenol degradation. These authors considered symbiotic bacteria in the hepatopancreas of isopods as a key evolutionary step and pre-adaptation for the digestion of land-based food sources. More data from isopods in different species and from different habitats in different geographic locations is needed for a better understanding of isopod bacterial associations.

The aim of this project was to characterize bacterial communities in the hepatopancreas of isopods from different habitats by molecular methods and compare

bacterial communities from different species and habitats. I hypothesize to find greater overall bacterial diversity in the hepatopancreas of marine and intertidal isopods that feed on a mixed algal diet than in terrestrial isopods.

MATERIAL AND METHODS:

Sample collection:

Seven different species of Isopods (*Armadillidium vulgare* (A), *Idotea baltica* (IB), *I. wosnesenskii* (IG), *Ligia occidentalis* (LO), *L. pallasii* (LP), *Oniscus asellus* (O) and *Porcelio scaber* (P) were collected between 7/6/2008 and 7/16/2008 either on the Pacific Coast around the Bodega Marine lab in Bodega Bay (38°19'28"N, 123°02'19"W) or in different locations around Woods Hole (41°31'36"N, 70°39'48"W) between 7/6/2008 and 7/16/2008. Isopods were collected from different habitats subtidal (*Idotea 2* species), high intertidal (*Ligia 2* species) and terrestrial (3 species) (Table 1). Isopods were identified to species based on morphology.

Table 1: overview of isopod species from different locations and habitats investigated for bacterial diversity in their hepatopancreas

Species	ID	location	habitat	16S clone library	CARD FISH
<i>A.vulgare</i>	A	Woods Hole, Quisset	terrestrial	✓	
<i>I. baltica</i>	IB	Woods Hole, Vineyard sound	subtidal	✓	
<i>I. wosnesenskii</i>	IG	Woods Hole, Vineyard sound	subtidal	✓	✓
<i>L. occidentalis</i>	LO	Bodega Bay, Campbell Cove	intertidal	✓	✓
<i>L. pallasii</i>	LP	Bodega Bay, Campbell Cove	intertidal	✓	✓
<i>O. asellus</i>	O	Woods Hole bikepath	terrestrial	✓	
<i>P. scaber</i>	P	Woods Hole bikepath	terrestrial	✓	✓

Isopods were kept alive in the laboratory without feeding for 2 to 5 days either in flowing seawater (*Idotea* spp.) or in small plastic containers with moist paper towels

before processing. Prior to dissection isopods were washed 3 times in sterile water followed by a wash in 70% ethanol. Isopods were dissected with sterile techniques using flame sterilized dissecting tools; the intact hepatopancreas was carefully separated from the digestive tract to minimize loss of bacteria and rinsed in 1xPBS before further processing.

Enrichments and Colony PCR:

Subsamples of a portion of the hepatopancreas of *P. scaber* and *L. pallasii* were homogenized in 500ul ddH2O and 50-100ul were spread either on nutrient agar plates or plates with SWLTR. If colonies were formed plates were restreaked. Either white shiny, yellow or orange colonies were isolated. Colony PCR as described in Course manual were performed on isolates, unfortunately none resulted in clean sequences.

DAPI staining and CARD FISH

To optimize sample preparation for DAPI staining and CARD FISH different treatments were used (Table 2) on samples of *P. scaber* and *L. pallasii*.

Table 2: different treatments to test for optimal sample preparation

Fixation	[PBS]	homogenization	filter volume/filter size
1% paraformaldehyde	1x	pre-fixation	3 ml/small
1% paraformaldehyde	1x	post-fixation	3 ml/small
1% paraformaldehyde	1x	pre-fixation	5ml/large
1% paraformaldehyde	1x	post-fixation	5ml/large
2% paraformaldehyde	5x	pre-fixation	5ml/large
2% paraformaldehyde	5x	post-fixation	5ml/large

The best results as determined by DAPI staining were obtained when samples of the whole hepatopancreas were fixed with 1% paraformaldehyde in 1xPBS, stored at 4 °C for 12 to 14 hours, washed 3 times in 1x PBS and homogenized gently with a glass tissue

homogenizer. The homogenized sample was diluted in 1xPBS to a final volume of 5ml and filtered onto a 47mm Milipore filter with a 0.2µm pore size. This method was used for all subsequent samples. CARD- FISH protocols were chosen over monolabelled FISH as this technique provides a much stronger signal than standard mono-labelled FISH (Pernthaler et al. 2004). Greater signal strength is an advantage in samples where high background from host tissue was expected. The CARD FISH protocol from the microbial diversity course 2008 lab binder was followed with the probes described in Table 3.

Table 3: Probe name, specificity and Sequence information of oligo-nucleotide probes used in this study

Probe name	Specificity	Sequence (5'-3')
Eubl-III	Bacteria	GCWGCCWCCCGTAGGWGT
Non338	negative control	ACTCCTACGGGAGGCAGC
Arch915	Archaea	GTGCTCCCCCGCCAATTCT
Alf986	Alphaproteobacteria	GGTAAGGTTCTGCGGTT
Gam42a + comp	Gammaproteobacteria	GCCTTCCCACATCGTTT

Sectioning of hepatopancreas for CARD FISH

A subsample of dissected hepatopancreas from *L. pallasii*, *L. occidentalis* and *Porcelio scaber* was fixed in 1% by volume formaldehyde in 1xPBS, stored at 4°C for 12 hours and washed 3x in 1xPBS. Samples were embedded in OCT (Optical Cutting Solution, Tissue –Tek ®, Sakura) and frozen immediately on a metal stub used for cryosectioning. Samples were kept frozen for 2- 4 hours and thin sections (10 to 25 µm thick) were cut with a cryotome at the Electron Microscope facility and immediately mounted on clean microscope slides. Slides were kept overnight at -20°C and stained with DAPI in order to investigate suitability of section procedure for further CARD FISH staining. It was determined that this method did not result in sections of sufficient quality

to warrant staining with CARD FISH. Embedding in paraffin following previously described protocols for the identification of invertebrate bacterial symbions (Dubillier et al.1995) should lead to improved results but was not attempted due to time constraints.

16S rRNA clone libraries:

The dissected hepatopancreas of 5 individual isopods of each species were pooled and DNA was extracted with Mobio Power Soil DNA isolation kit. Genomic DNA was quantified on 1% agarose gel stained with Ethidium bromide. PCR of a portion of the 16S rRNA gene with the primer pair 8F/1492R was performed as described in the course manual using PCR conditions described in the course manual. Based on positive results determined by gel electrophoresis cleaned PCR products were cloned with a TOPO TA cloning kit (Invitrogen) following the manufacturer's protocol. 40 and 80ul of each cloning reaction were spread on kanamycin plates treated with Xgal and incubated overnight at 37°C. Sixteen to 48 transformed colonies per isopod species were picked and transferred to 96 well plates for sequencing at the MBL core sequencing facility, replicate libraries were constructed for *L. pallasii* and *P. scaber*..

Analysis:

A sub sample of sequences were viewed and screened for quality with the freeware 4Peaks Version 1.7.2 (A. Griekspoor and Tom Groothuis). Due to time constraints the decision was made to accept pre-trimmed sequences as obtained from the MBL sequencing facility. Sequences from each clone library were aligned with SILVA (<http://www.arb-silva.de>) and aligned sequences in FASTA format were imported into arb database (<http://www.arb-home.de>). The alignment was modified as necessary by hand based on secondary structure. Neighbor joining trees of symbiont species were built

in arb. Distance matrices were built (for each host species clone library separately, for clone libraries combined for the 3 different host habitats (subtidal, intertidal and terrestrial), as well as one matrix for all libraries combined) following instructions provided in the tutorial handout from Pat Schloss (MBL diversity 2008) for further analysis with the program DOTUR (<http://www.plantpath.wisc.edu/fac/joh/dotur.html>). DOTUR takes a distance matrix of DNA sequences and assigns sequences to operational taxonomic units (OTUs). The number of OTU's in individual and combined libraries was obtained and rarefaction curves built to detect possibility of under-sampling in individual libraries. Shannon index and Chao 1 estimator were used to estimate the species richness of each bacterial community in the hepatopancreas. The Shannon index of species diversity, takes into account the number of species and the evenness in the community. The Chao estimator is a tool to infer the actual species richness of a community from a finite number of samples (Chao, 1984).

SONS (<http://www.plantpath.wisc.edu/fac/joh/sons.html>) was used to estimate similarity between communities through use of non-parametric estimators. I determined the fraction of shared OUT's between libraries for distance levels of 0.03 (97% similarity) and 0.05 (95% similarity) (Schloss and Handelsman, 2006).

The program S-Libshuff (<http://www.plantpath.wisc.edu/fac/joh/s-libshuff.html>) was used to obtain p-values of community comparisons (Schloss et al. 2004). The program tests the Null hypothesis of no difference between communities and uses a Bonferoni correction to adjust p-values for multiple comparisons. If either one of the two comparisons was significant, the two communities were considered different (Schloss pers. comm.).

RESULTS AND DISCUSSION:

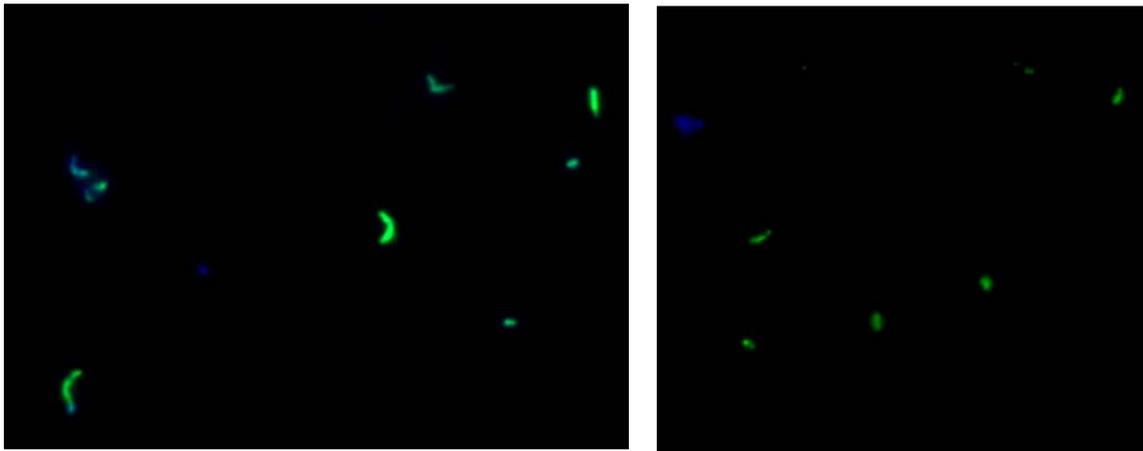
During dissections terrestrial isopods in the Oniscidea were all found to have 2 pairs of lobes, whereas *Ligia* and *Idotea* had 3 pairs of lobes in the hepatopancreas. The color of the hepatopancreas varied between light beige (terrestrial isopods) to yellow and orange brown (*Ligia* sp.). (Figure 1).

Figure 1: The hepatopancreas of *P. scaber* (2 pairs of lobes, white) and *L. pallasii* (3 pairs of lobes, yellow)



CARD-FISH:

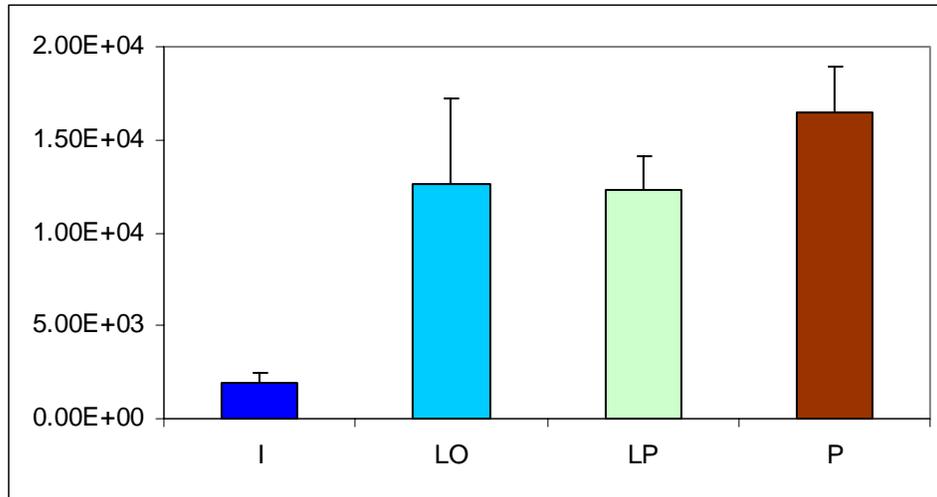
Figure 2: CARD FISH (EUBI-III) of *L. pallasii* showing characteristic morphology of *Candidatus Hepatincola pocellionum* and EUBI-III of *P. scaber*



Counts of bacterial abundance obtained with CARD FISH (Probe EUB I-III)

ranged between 1.9×10^3 – 1.7×10^4 bacteria per hepatopancreas (Figure 3).

Figure 3: CARD FISH counts of different isopod species, numbers are average counts from filters stained with EUB I-III for (I) *Idotea sp.*, (LO) *Ligia occidentalis*, (LP) *Ligia pallasii* and (P) *Porcelio scaber*, 2 filters and 20 fields (2 to 6 rows per field) per filter were counted for each species. Error bars are standard deviations of all the fields combined.



The Archeal probe showed no hybridization signal, and probes for Alpha and Gamma proteobacteria hybridized but no more than 5 cells per filter were counted. I am therefore concluding that these probes did not fail but that these groups were not dominant in the samples investigated. A probe for Firmicutes that were expected to be present in large numbers (confirmation in results of clone libraries) was not available during the course.

Different morphologies were observed in most of the filters suggesting more than one species per host. A curved rod shaped bacterium was repeatedly observed in filters from *L. pallasii* that matched the morphology of *Candidatus Hepatincola pocellionum* – described from the hepatopancreas of *P. scaber* (Wang et al. 2004b) (Figure 2).

Bacterial abundance in this study was 1 to 2 orders of magnitude lower than counts from DAPI stained cells in different studies for the same or a comparable isopod species (Zimmer et al. 2002, Wang et al. 2007). It is possible that DAPI counts lead to inflated numbers of bacterial abundance and/or that CARD FISH counts are overly conservative with EUB I-III probes. Furthermore loss of bacteria during dissection is possible. The lowest bacterial abundances were obtained from the subtidal *Idotea* species. This genus was previously found to contain no bacteria in their hepatopancreas (Zimmer et al. 2002).

16S Clone Libraries:

A total of 321 sequences were obtained in the clone libraries with a total number of 45 different OUT's (defined by a 0.03 distance determined in Dotur). The number of OTU's per library varied between species from 1 to 17, with *L. occidentalis* (# OUT = 1) and *P. scaber* (# OUT = 2) having the lowest and *L. pallasii* (# OUT = 17) the highest number of OTUs (Table 4). The fact that the number of clones sequenced per library varied greatly from 15 to 133, and lack of replicates for all species makes a comparison across habitats and species difficult. The terrestrial species *P. scaber* and *O. assellus* have lower bacterial diversity as measured by the number of different OUT's found in the libraries, than the two subtidal and the other intertidal species. Highest Chao1 measure of diversity is shown for *I. baltica* whereas *L. pallasii* has the highest Shannon Index. The number of OTU's per library varied between species from 1 to 17 with P being the lowest and LP the highest (Table 4). The numbers of clones per library were not even so a comparison across habitats and species is difficult. The lowest diversity was found in *L. occidentalis* with only 1 OUT found in the clone library.

Table 4: Diversity of clone libraries showing number of clones and OTUs; measures of diversity are given by the Chao1 and Shannon Index with 95% Confidence limits.

Species	# clones	# OTUs	Chao 1	95%lCI	95%hCI	Shannon	95%lCI	95%hCI
<i>A. vulgare</i>	16	8	15.5	9.32	50.53	1.65	1.11	2.18
<i>I. baltica</i>	24	15	81	37.12	211.90	2.46	2.08	2.83
<i>I. wosnesenskii</i>	24	6	16	7.94	57.58	0.85	0.35	1.35
<i>L. occidentalis</i>	47	1	1	1.00	1.00	0.00	-0.03	0.03
<i>L. pallasii</i>	133	39	22.6	18.16	44.14	3.22	3.06	3.39
<i>O. asellus</i>	15	4	2	2.00	2.00	0.38	0.04	0.72
<i>P. scaber</i>	62	2	7	4.37	28.09	0.69	0.17	1.21

The terrestrial species *P.scaber* and *O. assellus* had lower diversity in the gut than the two subtidal and the other intertidal species. Measures of diversity Shannon Index and Chao1 show similar rankings of diversity with highest diversity found in *I. baltica*.

Table 5: number of sequences with >97% identity shared among different clone libraries

IB	IG	LO	LP	A	O	P
1	-	1	1	2	-	-
-	-	-	2	1	-	-
-	-	-	6	8	-	-
3	-	-	-	1	-	-
-	-	-	2	1	-	-
6	19	-	-	-	-	-
-	-	-	-	-	13	53

In a comparison of symbiont OTU's shared between different hosts (table 5), it is interesting to note that *A. vulgare* and *L. pallasii* share sequences in 4 OUT's especially since they were collected at different geographic regions and in different habitats. Based on S-Lipshuff results no statistically significant differences could be observed between

the bacterial communities in the hepatopancreas of *A. vulgare* and *L. pallasii*, *O. asellus* and *L. pallasii*, *O. asellus* and *P. scaber*, and *I. baltica* and *I. wosniezki*.

Clone libraries from some but not all species in this study included sequences closely related to previously described isopod symbionts (Wang et al. 2004a,b) with the majority (66 sequences) clustering around *Candidatus Hepatoplasma crinochetorum* (Firmicutes, Mollicutes) (Figure 4,5). Closely related sequences to the other described symbiont '*Candidatus Hepatincola porcellionum*' (α -Proteobacteria, Rickettsiales) were only obtained from the hepatopancreas of *L. pallasii* and *A. vulgare*, but not from *P. scaber* libraries (Figure 6).

Sequences obtained in the Mollicutes clustered by host (Figure 5); these findings confirm host specificity of this group of symbionts as discussed in a recent extensive study on the evolution of the association between isopod and symbionts (Fraune and Zimmer 2008). These authors suggested an ancient association between host and this type of symbiont and make a case that this association increases host survival in nutrient poor conditions. Investigation of how nutrient condition affects the species of isopods and their symbionts investigated in this study would be interesting to follow up especially in light of seasonally varying resource availability (e.g. algal wrack for *Ligia*).

The greater bacterial diversity in the hepatopancreas of the isopod species than previously reported is interesting especially in light of community dynamics within the host and is an interesting topic for future research.

Figure 4: Neighborjoining tree of sequences from all clone libraries color-coded by host habitat (blue subtidal, light blue intertidal, brown terrestrial) with the two previously described symbionts marked in red boxes.

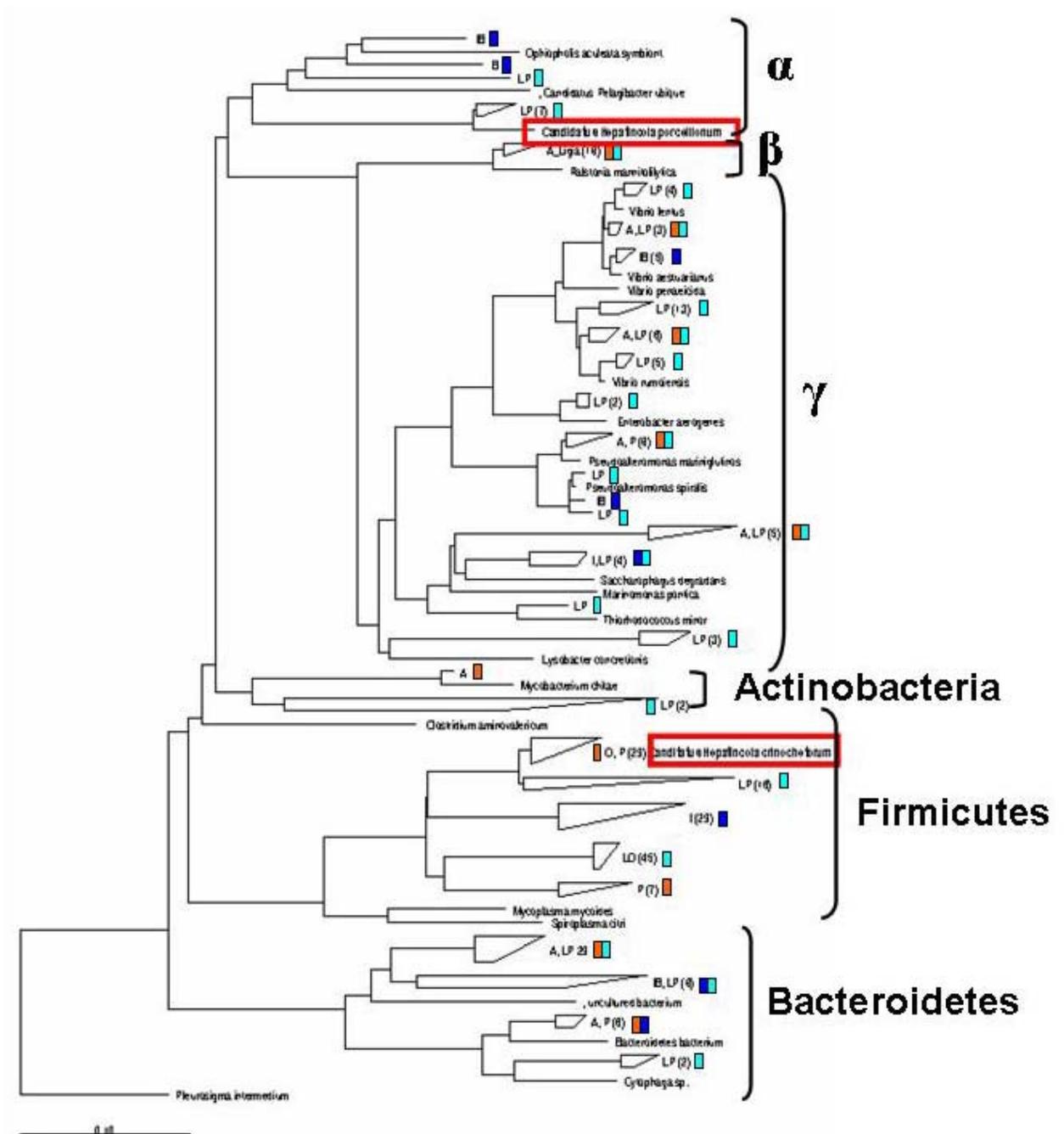


Figure 5: Neighbor joining tree of sequences clustering around the previously described *Candidatus Hepatoplasma crinochetorum*. Species ID of clones as previously described. Numbers in parentheses indicate numbers of sequences in this group

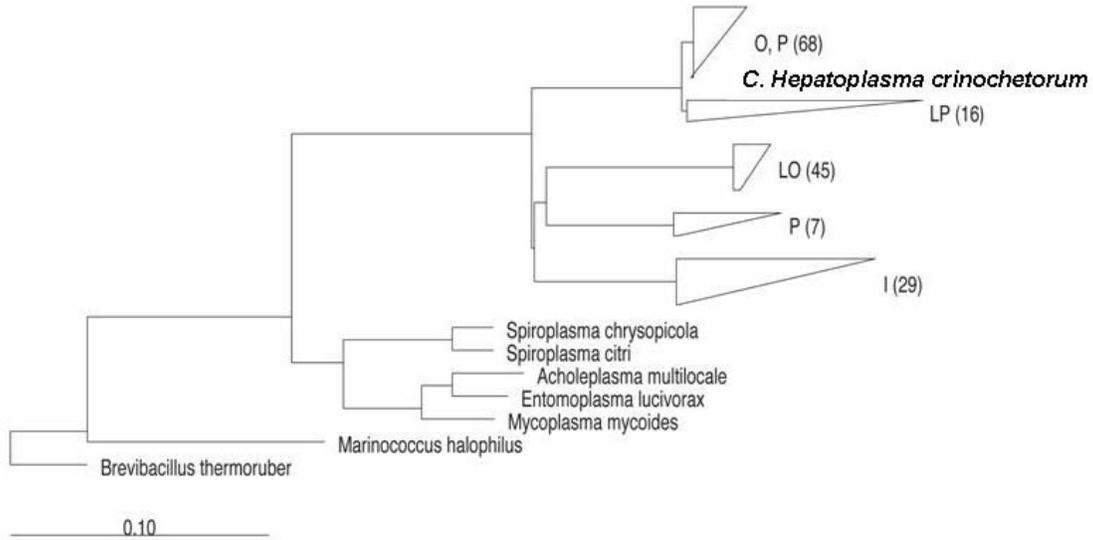
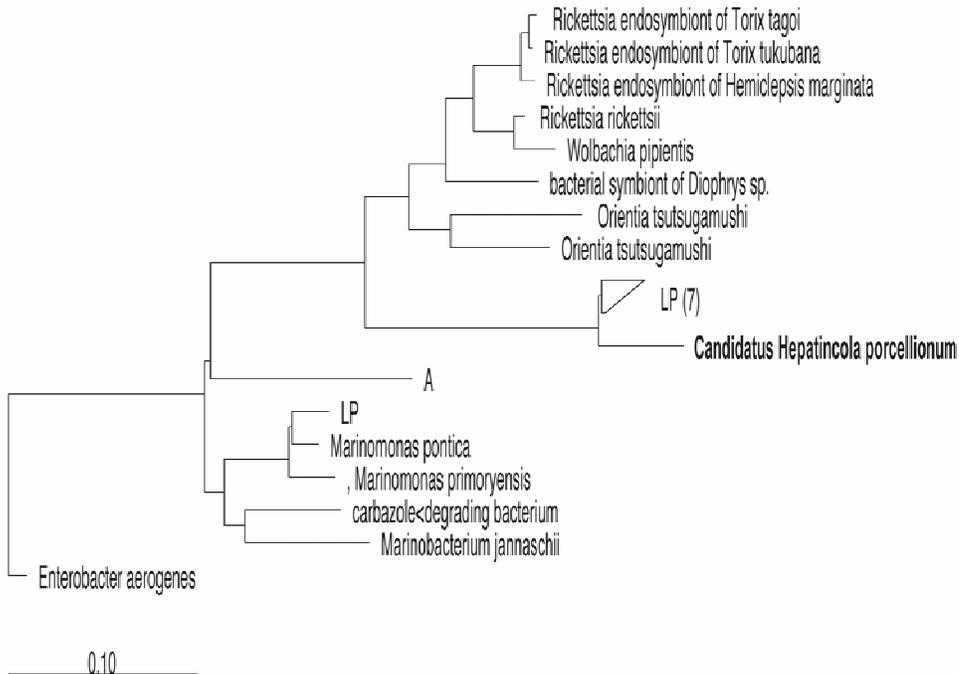


Figure 6: Neighbor joining tree of sequences from clone libraries that cluster around the previously described *Candidatus Hepatincila porcellionum*. Host species identifiers are used as in table 1. Numbers in parentheses behind species identifier represent the number of sequences in that group.



Acknowledgements:

Many thanks to the course instructors Tom Schmidt and Bill Metcalf and all the TAs for their help, especially Dave Ward for help with Arb and Dagmar Wobken for assistance with CARD FISH and to all my class mates for many suggestions and interesting discussions. I am grateful to Amanda Newson (Bodega Marine Lab) and Ed Enos (MBL Aquatic Resources Division) for help with sample collection. Funding for course participation was obtained through a F. R. Lillie Fellowship.

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