Sea-land transitions in isopods: pattern of symbiont distribution in two species of intertidal isopods *Ligia pallasii* and *Ligia occidentalis* in the Eastern Pacific

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Abstract Studies of microbial associations of intertidal isopods in the primitive genus *Ligia* (Oniscidea, Isopoda) can help our understanding of the formation of symbioses during sea-land transitions, as terrestrial Oniscidean isopods have previously been found to house symbionts in their hepatopancreas. *Ligia pallasii* and *Ligia occidentalis* co-occur in the high intertidal zone along the Eastern Pacific with a large zone of range overlap and both species showing patchy distributions. In 16S rRNA clone libraries mycoplasma-like bacteria (Firmicutes), related to symbionts described from terrestrial isopods, were the most common bacteria present in both host species. There was greater overall microbial diversity in *Ligia pallasii* compared with *L. occidentalis*. Populations of both *Ligia* species along an extensive area of the eastern Pacific coastline were screened for the presence of mycoplasma-like symbionts with symbiont-specific primers. Symbionts were present in all host populations from both species but not in all individuals. Phylogenetically, symbionts of intertidal isopods cluster together. Host habitat, in addition to host phylogeny appears to influence the phylogenetic relation of symbionts.

Keywords Isopod · Firmicutes · Sea-land transition · Symbiont · Hepatopancreas

1 Introduction

Eukaryotes in a number of phyla have overcome their limitations in nutritional capabilities by associating with microorganisms. Nutritional symbioses are particularly common in animals feeding on diets that are either limited in essential amino acids and vitamins, or that are otherwise difficult to digest (e.g.Buchner 1965; Breznak and Brune 1994; Douglas 1994; Lau et al. 2002; Dillon and Dillon 2004). 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 (Breznak and Brune 1994; Douglas 1994; Zimmer et al. 2001, 2002). Novel metabolic capabilities acquired by one organism from its microbial partner can allow the hosts to expand their distribution and occupy hitherto unfilled niches (Douglas 1994). Thus animal microbe associations can be important drivers of evolution (Doebeli and Dieckmann 2000; Buckling and Rainey 2002; Leonardo and Muiru 2003).

Because they can be found in habitats ranging from strictly marine to strictly terrestrial (Abbott 1939; Edney 1968), isopods (Arthropoda, Crustacea, Malacostraca, Pericaridea) are a good system to study changes in bacterial gut community composition between related taxa living in different environments. Oniscidean isopods—important in litter decomposition on land—are the only one of ten isopod suborders where the majority of species have become independent of the aquatic environment. This transition from sea to land is believed to have occurred through the high intertidal or supra-littoral zone (Sutton and Holdich 1984), and today primitive oniscidean isopods in the Ligiidae remain in intertidal habitats (Schmalfuss 1989). Thus *Ligia* spp. can serve as model organisms for the transition from a marine to a terrestrial existence (Schmalfuss 1978; Carefoot and Taylor 1995).

The separation of the isopod digestive system into different microhabitats is relevant in terms of its functional

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significance (Hames and Hopkin 1989). In terrestrial isopods, bacteria have been found closely associated with the hepatopancreas, or digestive midgut gland involved in the secretion of digestive enzymes and the absorption of nutrients (Hopkin and Martin 1982; Wood and Griffiths 1988; Fraune and Zimmer 2008). No such symbionts have been found in subtidal isopods (Zimmer et al. 2001, 2002). Two novel symbionts from the hepatopancreas of the terrestrial *Porcelia scaber* (Oniscidea) have been described (Wang et al. 2004a, 2004b); but these bacteria remain uncultured and were thus assigned Candidatus status (Neimark et al. 1998). Ecdysis is a disruptive force for the development of a stable bacteria-host association, as both the fore- and hindgut of isopods are shed and need to be re-colonized with a bacterial community following this process. The establishment of a more stable bacterial community can be expected in the midgut that does not undergo ecdysis (Zimmer 2002). Symbiotic bacteria in the hepatopancreas of Oniscidean isopods have been associated with cellulose digestion and phenol oxidation (Zimmer and Topp 1998; Zimmer et al. 2002), and appear to confer increased survival under conditions of low quality diet (Fraune and Zimmer 2008).

Little is known about the association of primitive intertidal oniscidean isopods with microbes despite the premise that the acquisition of symbionts was a key step in the sea-land transition of isopods (Zimmer et al 2002). Isopods in the Ligiidae are living today in the high intertidal, a transition zone between marine and terrestrial habitats. Findings of microbial associations of isopods in the Ligiidae so far have been inconclusive (Wang et al. 2007; Fraune and Zimmer 2008), and the occurrence of symbionts in this family has been considered rare based on limited data (Fraune and Zimmer 2008). The current study investigates the presence, identity, and distribution of microbial symbionts in the hepatopancreas of two oniscidean isopods *Ligia pallasii* and *Ligia occidentalis*. This genus is considered primitive among Oniscidean isopods (Mattern and Schlegel 2001), and *Ligia* species worldwide are confined to the extreme high intertidal or supralittoral zone (Carefoot and Taylor 1995; Schmalfuss 2003). It is unknown whether all *Ligia* species have symbionts in the hepatopancreas, and whether specificity exists between host and symbionts for this group. Existing studies of symbiont distribution in isopods cover only small geographic areas (Zimmer et al. 2002; Wang et al. 2007; Fraune and Zimmer 2008). This study addresses the question of symbiont prevalence by screening for symbionts in two closely related and ecologically similar isopod hosts over a large geographic range of partially overlapping host distributions.

2 Materials and methods

2.1 Sample collection

Both *Ligia pallasii* and *Ligia occidentalis* occur in the Eastern Pacific and are typically encountered in the upper reaches of the intertidal zone. The northern *L. pallasii* can be found from Alaska to central California, and the southern *L. occidentali*s from southern Oregon to Baja California (Schmalfuss 2003). A wide geographic region of sympatry occurs between southern Oregon and central California (Fig. 1).

Adult isopods were collected between November 2008 and June 2009 in the high rocky intertidal at 20 locations along an approximately 1,900 km stretch of the Eastern Pacific coastline (from Eagle Cove (EC) in the San Juan Islands, Washington, USA 48.46°N, 123.03°W to Erendira (ER) in northern Baja California, Mexico 31.87°N, 116.38°W) (Table 1). Ten sampling stations were located in the presumed zone of range overlap of both *Ligia* species (Schmalfuss 2003). Whenever possible, isopods were collected from multiple locations within a site, usually within 500 meters of each other. All isopods were collected by hand and kept alive in isolated containers in
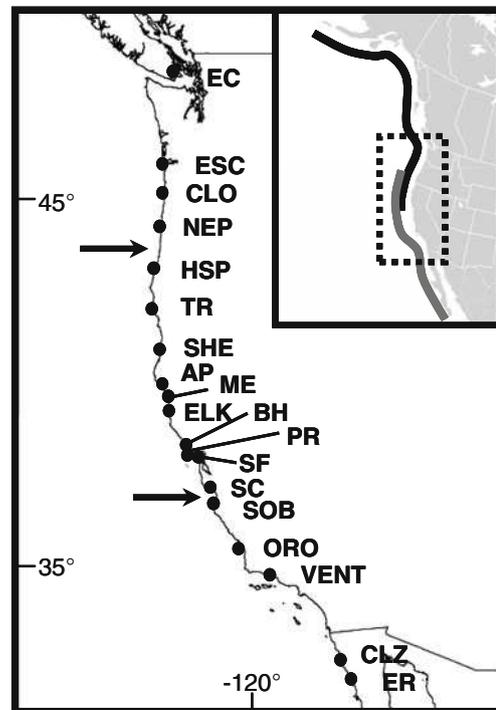

Fig. 1 Sampling sites for symbiont screens of *Ligia pallasii* and *L. occidentalis* hosts. Station IDs identify sampling locations as used in Table 1. Area between arrows marks the sympatric range of the host species. The range distribution of *Ligia pallasii* (black line) and *Ligia occidentalis* (grey line) is shown in inset with area of sampling location marked by dotted line

the laboratory for a maximum of three days prior to dissection.

2.2 DNA extraction, PCR amplification and cloning

Ligia pallasii and *L. occidentalis*, collected from Bodega Bay (38.32°N, 123.06°W) in the central range of the distribution, were used to create 16S rRNA clone libraries of microbial symbionts. The hepatopancreas of five individual isopods of each species was dissected using sterile techniques. Each isopod was washed 5x in distilled water and the outside sterilized with 70% ethanol (2 rinses) prior to dissection. Only healthy adult individuals, both male and female, that were not molting were used for dissection. Genomic DNA was extracted with a MoBio Power Soil® DNA isolation kit (MoBio Laboratories, Carlsbad, CA), checked for quality and quantified against a standard of known concentration on a 1% agarose gel stained with ethidium bromide. DNA was polymerase chain reaction (PCR) amplified with universal bacterial 16S rRNA gene primers 8f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′). PCR reactions contained 12.5 μl Promega master mix (2x) (Promega, Madison WI), 2 μl of each primer (15 pmol), approximately 25 ng of template DNA and nuclease free water to a final reaction volume of 25 μl and were run on a Bio-Rad Thermocyler (Bio-Rad, Hercules, CA) with the following profile: Initial Denaturation 95°C for 5 min, 30 cycles of 95°C for 30 s, 46°C for 30 s, 72°C for 1.5 min, followed by a final extension of 72°C for 5 min. PCR products were visualized on 1.2% agarose gels and products of correct size were purified with ExoSAP-IT® (USB Corporation, Cleveland, OH). Purified PCR products from each species were pooled (5 individual reactions for each species) and cloned into the pCR 2.1 TOPO vector, transformed into TOP10 *Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and plated onto LB agar plates containing 50 μg/ml kanamycin. Positive clones from each species were randomly selected and sequenced on an ABI 3730 capillary sequencer with ABI BigDye Terminator v3.1 Cycle Sequencing chemistry.

2.3 Phylogenetic analysis

Sequences were first checked for chimeras using the online program CHECK-CHIMERA at the Ribosomal Database Project (Cole et al. 2003). Putative chimeric sequences were removed from phylogenetic analyses. Hepatopancreas bacterial sequences were submitted to BLAST searches (Altschul et al. 1997) to find closely related sequences in public databases and grouped by phyla according to the search results. All sequences in the phylum Firmicutes from

the clone libraries, and closely related sequences from public databases (including previously published symbiont sequences from other isopod host species, Fraune and Zimmer 2008), were aligned with the NAST algorithm on the greengenes website (DeSantis et al. 2006); the resulting alignment was checked manually. A neighbor-joining tree combining representative sequences of both hosts obtained in this study and sequences from public databases was created with the software *MEGA* v. 4 (Tamura et al. 2007) with Kimura 2-parameter distances (Kimura 1980) and bootstrapping of 1,000 replicates (Felsenstein 1985). For the maximum likelihood (ML) analysis models of molecular evolution were selected on the basis of MODELTEST (Posada and Crandall 1998) as implemented by FindModel (http://hcv.lanl.gov/content/hcvdb/findmodel/findmodel.html). The ML parameter values were optimized with a BIONJ tree as a starting point in PhyML (Guindon and Gascuel 2003). Support values of nodes on the ML tree were estimated with 500 bootstrap replicates.

2.4 Population screening with Symbiont specific primers

Isopods of *Ligia pallasii* and *Ligia occidentalis* were collected at 20 different locations (Table 1) and the hepatopancreas of isopods was dissected as described above. DNA was extracted either with Mo Bio Power Soil® DNA isolation kit (Mo Bio Laboratories, Carlsbad CA) or with a CTAB extraction protocol (Schulenburg et al. 1999). DNA was checked for quality and quantified against a standard of known concentration on a 1% agarose gel stained with ethidium bromide. Diagnostic PCRs for the presence of Mycoplasma-like symbionts were performed using the primer pair PsSym372f (5′-CAG CAG TAG GGA ATT TTT CAC-3′) (Wang et al. 2007) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′) (Weisburg et al. 1991) targeting a portion of approximately 1130 bps of the bacterial 16S rRNA gene.

Each PCR reaction contained: 2.5 μL of 10X reaction buffer, 200 μM of each dNTP, 2 mM MgCl₂, 20 pmol of each primer, 0.2 μg/μl BSA, 0.5U Taq polymerase (Qiagen, Valencia, CA), 5–10 ng of DNA template, and water to a total reaction volume of 25 μl. The PCR was performed at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 40 s at 52°C, and 50 s at 72°C, with a final extension at 72°C for 3 min. All PCR reactions were run with a positive control (DNA extracted from the hepatopancreas of *Porcelio scaber*—previously shown to contain Mycoplasma-like symbionts) and negative controls (DNA extracted from periopods of *Ligia pallasii* and *Ligia occidentalis*, no DNA). PCR reactions were screened for positive bands on a 1.5% agarose gel. Samples were considered positive for symbionts when they showed a band of the correct size and negative in the absence of such a band. All samples

Table 1 Sampling stations along the Eastern Pacific Coastline indicating the presence of host species *Ligia pallasii* (LP) and *L. occidentalis* (LO) as well as the number of individuals of each species collected at a site that were used for symbiont screens. Stations are ordered from North to South, stations in areas of range overlap of both species are highlighted in bold, but individuals of both species were not found in all of these stations. * indicates that hepatopancreas of multiple individuals was pooled prior to PCR screens

Sampling location	Sample ID	Latitude (°N)	Longitude (°W)	*Ligia* at site	LP (n)	LO (n)
San Juan Islands, WA	EC	48.46	123.03	LP	8	
Ecola State Park, OR	ESC	45.93	123.97	LP	16	
Cape Lookout, OR	CLO	45.35	123.98	LP	8	
Newport, OR	NEP	44.26	124.11	LP	18	
Cape Arago, OR	ARA	43.31	124.4	LP	12	
Harris State Park, OR	**HSP**	**42.1**	**124.35**	**LP**	**7**	
Trinidad, CA	**TR**	**41.06**	**124.15**	**LO, LP**	**17**	**10**
Shelter Cove, CA	**SHE**	**40.03**	**124.08**	**LO**		**5**
Westport, CA	**AP**	**39.67**	**123.79**	**LO, LP**	**2**	**9**
Mendocino, CA	**ME**	**39.3**	**123.8**	**LO, LP**	**14**	**3**
Elk, CA	**ELK**	**39.12**	**123.71**	**LP**	**5**	
Bodega, CA	**BH**	**38.32**	**123.06**	**LO, LP**	**10**	**14**
Point Reyes National Seashore, CA	**PR**	**38.18**	**122.97**	**LO, LP**	**18**	**10**
San Fransisco Bay, CA	**SF**	**37.82**	**122.49**	**LO, LP**	**15**	**12**
Santa Cruz, CA	**SC**	**36.97**	**121.95**	**LP**	**14**	
Soberones, CA	SOB	36.45	121.93	LO		8
Morro Bay, CA	ORO	35.27	120.89	LO		11
Ventura, CA	VENT	34.25	119.27	LO		7
Campo Lopez, Baja California	CLZ	32.16	116.9	LO		10*
Erendira, Baja California	ER	31.87	116.38	LO		10*

that showed no band in the initial PCR reaction were run at least twice to assure that absence of a band was not due to technical problems. The percentage of individual *Ligia* positive for the symbiont in each sample location was determined. Initially, all samples independent of outcome were run multiple times to determine reliability of results. A random sub-sample of positive bands was cleaned with ExoSAP-IT® and sequenced in both directions on an ABI 3730 capillary sequencer to confirm the specificity of the primers to mycoplasma-like symbionts. Sequences were checked against known sequences in GenBank.

3 Results

3.1 16SrRNA clone libraries from the hepatopancreas of Ligia pallasii and Ligia occidentalis

Analysis of partial 16S rRNA sequences indicated that bacteria in the hepatopancreas of *L. occidentalis* and *L. pallasii* belonged to 3 phyla: Cytophaga-Flavobacteria-Bacteroidetes, Proteobacteria and Firmicutes, and the clone library of *L. pallasii* also contained an additional representative sequence from Verrucomicrobia and Actinobacteria (Table 2). In both libraries, mycoplasma-like sequences

(Firmicutes) were the most abundant sequences with 45 clones out of 47 clones (96%) from the hepatopancreas of *L. occidentalis* and 45 of 87 clones (52%) from the hepatopancreas of *L. pallasii*. In the *L. pallasii* library Bacteroidetes (16 of 87 clones, 18%), Gammaproteobacteria (17 of 87 clones, 20%) and Betaproteobacteria (seven of 87 clones, 8%) had additional representative clones. Clones in the Firmicutes were 98% similar within each host species and were also 98% similar between *L. pallasii* and *L. occidentalis* host species. The closest representatives in public databases were symbionts obtained from the hepatopancreas of other isopod species (90 to 91% similarity to clones from the intertidal isopods *Tylus europeaus* and *Ligia oceanica*). Sequences were submitted to GenBANK under the Accession numbers GU111260-GU111306 and GU211017-GU211104.

3.2 Phylogenetic tree of mycoplasma like symbionts

Both neighbor-joining and maximum likelihood methods revealed essentially the same tree topology as shown in the neighbor-joining tree in Fig. 2. Bacteria from different individuals in both *L. pallasii* and *L. occidentalis* clustered together and were distinct from sequences obtained from other host species. Host specificity could not be confirmed

Table 2 Bacterial diversity in 16S rRNA clone libraries of *L. occidentalis* and *L. pallasii* hosts indicating the number of bacterial clones represented in the library as well as the identity and % similarity of the closest sequence found in public databases
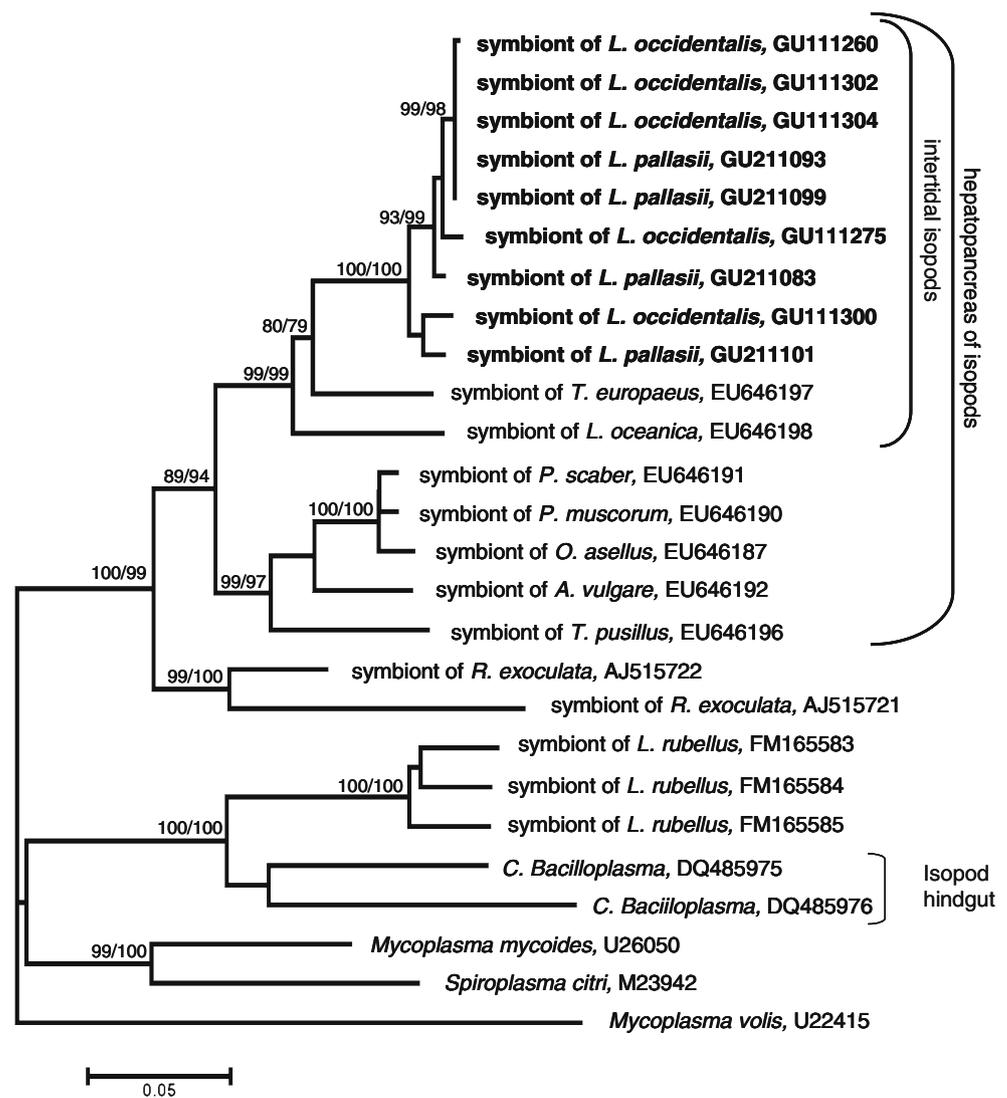
Host	representative clone	# of clones	% similarity between clones	closest relative in database	% similarity with database sequence
L. occidentalis					
	clone LO_G03	45	98	clone Ty_Hep1.19, EU646197.1 Firmicutes	90–91
	clone LO_H04	1	–	*Ralstonia pickettii*, CP001644.1 β-Proteobaceria	99
	clone LO_ C01	1	–	*Vibrio penaeicida*, EU0-73023.1 γ-Proteobacteria	96
L. pallasii					
	clone LP_A1	45	98	clone Ty_Hep1.19, EU646197.1 Firmicutes	90–91
	clone LP_C9	9	97	clone 01D2Z34, DQ330310.1 Bacteroidetes	92–94
	clone LP_A10	4	97	bacterium P100, EU195944.1 Bacteroidetes	95
	clone LP_A11	2	98	uncultured bacterium, AM930453.1 Bacteroidetes	92
	clone LP_D9	1	–	*Lacinutrix* sp., EU581705.1 Bacteroidetes	96
	clone LP_A1	7	96	*Vibrio rumoiensis*, DQ530289 γ -Proteobacteria	95
	clone LP_B8	4	97	uncultured bacterium, G0349987.1 γ -Proteobacteria	89
	clone LP_A8	3	99	uncultured bacterium, AB294932.1 γ -Proteobacteria	91
	clone LP_C1	1	–	*Vibrio penaeicida*, EU0-73023.1 γ -Proteobacteria	96
	clone LP_E11	1	–	unculltured isolate, DQ831101.1 γ -Proteobacteria	90
	clone LP_H11	1	–	*Cobetia* sp. FJ210806.1 γ -Proteobacteria	99
	clone LP_A12	7	99	*Ralstonia pickettii*, CP001644.1 β-Proteobacteria	99
	clone LP_C12	1	–	uncultured bacterium, FN424398.1 Actinobacteria	94
	clone LP_H10	1	–	clone HAL-SW-5, EU350894.1 Verrucomicrobia	94

for these two closely related species. Sequences obtained in this study were most closely related to those recovered from the hepatopancreas of other isopods from intertidal habitats (*Tylos europeaus* and *Ligia occidentalis*) with strong bootstrap support for this clustering. A cluster of all symbionts from the hepatopancreas of different isopod species was supported by our analysis, whereas *Candidatus Bacilloplasma*, a Firmicute obtained from the hindgut lining of *P. scaber* (Kostanjsek et al. 2007) differed from this grouping (Fig. 2).

3.3 Symbiont screens

A total of 273 *Ligia* hosts from 20 stations (165 *L. pallasii* from 14 stations and 109 *L. occidentalis* from 10 stations) were screened for the presence of mycoplasma-like symbionts (Table 1); the decision was made to focus on mycoplasma-like symbionts since these were the most abundant symbionts in the clone libraries of both *L. pallasii* and *L. occidentalis*. Positive bands were obtained for all populations checked for both host species. The percentage

Fig. 2 Neighbor-joining tree shows Mycoplasma-like symbionts of *Ligia pallasii* and *L. occidentalis* cluster together with symbionts from other intertidal isopods. All symbiont sequences from the hepatopancreas from isopods (sequences in bold this study, other isopod sequences previously published in Fraune and Zimmer 2008) form a group that is different from bacterial sequences in public databases recovered from other host species. Bootstrap support values are given above branches first values for ML analysis and 2nd value neighbor joining. GenBank accession numbers are given for all sequences

of positive individuals varied between stations from 38% to 100% for *L. pallasii* and 33% to 100% for *L. occidentalis* with overall similar prevalence of infection (Fig. 2). No clear evidence for a latitudinal trend in prevalence of infection could be detected for either species. Infection prevalence differed between *L. pallasii* and *L. occidentalis* at stations where both species could be collected. BLAST searches confirmed that all randomly sequenced PCR products (submitted to GenBank under accession numbers GU197554-GU197586) were related to symbiont sequences from isopods.

4 Discussion

4.1 Occurrence of symbionts in intertidal Ligia

The present study showed that sequences obtained from the clone libraries of both *Ligia pallasii* and *L. occidentalis*

contained mycoplasma-like symbionts that were 90–91% similar to those previously described from *Tylos europeaus* (Tylidae) collected in Italy and *Ligia oceanica* collected in Germany (Fraune and Zimmer 2008). This cluster was strongly supported by bootstrap values on the phylogentic tree obtained in this study. Three of the host species in this cluster belong to the primitive Ligiidae, but *T. europeaus* is a member of a different oniscidean family (Schmalfuss 2003). This finding confirms the lack of strict co-evolutionary history between host and symbionts as has been previously discussed (Fraune and Zimmer 2008). Host habitat may play an important role, as all *Ligia* species as well as *T. europeaus* occur in intertidal habitats with access to similar food sources (Kensley 1974; Pennings et al. 2000; Laurand and Riera 2006). It appears that the type of preferred substrate within the intertidal habitat (rock for *L. pallasii*, *L. occidentalis*, and *L. oceanica* versus sand for *T. europeaus*) does not matter. This makes sense, since similar food sources (algal wrack, diatoms and bacteria) are available at both habitats, and

isopods are highly mobile and can easily reach preferred food. Mycoplasma-like symbionts appear to be restricted to terrestrial and intertidal isopods and to be lacking in marine isopods, as to date no bacteria were found in the hepatopancreas of two suborders of marine isopods, the Valvifera and Sphaeromatidea (Zimmer et al 2001; Wang et al. 2007). These subtidal isopods tend to feed on fresh macroalgae (Zimmer et al. 2001; Orav-Kotta and Kotta 2004), rather than algal wrack and diatoms.

4.2 Host symbiont specificity

Specificity of host/symbiont interactions appears not to hold true for the congeners *L. pallasii* and *L. occidentalis*, since mycoplasma-like symbiont sequences in this study showed 98% similarity both within as well as between the two host species, and sequences from both host species grouped together on the phylogenetic tree. A recent extensive study on the evolution of isopod symbiont associations (Fraune and Zimmer 2008) suggests high levels of specificity between host and symbionts, but only investigated distantly related isopods. The present study shows that *L. pallasii* and *L. occidentalis* (belonging to the same genus) show more recent diversification that is not yet reflected in their symbionts.

All mycoplasma-like sequences so far recovered from the hepatopancreas of different isopod species, including sequences obtained in this study, are more closely related to each other than mycoplasma-like bacteria from other non isopod hosts (e.g deep sea shrimp, termites and earthworms). Different areas of the digestive system of one isopod host can have different symbionts: *P. scaber* has distinctly different mycoplasma-like bacteria in the hepatopancreas (*Candidatus* Hepatoplasma crinochetorum, Wang et al. 2004a, 2004b) and in the hindgut *Candidatus* Bacilloplasma (Kostanjsek et al. 2007) as shown in Fig. 2. Different areas within the digestive system, with different functions and environmental conditions, are prone to form strong associations with different bacterial types (Eckburg et al. 2005; Kostanjsek et al. 2007; Gong et al. 2007). The phylogenetic association of bacterial sequences from the hepatopancreas of *L. pallasii* and *L. occidentalis* with those from the hepatopancreas of other isopods but not with *Candidatus* Bacilloplasma (Kostanjsek et al. 2007) from the hindgut supports the notion of strict tissue specificities of mycoplasmas in arthropod hosts (Razin 2006) (Fig. 3).

4.3 Prevalence and transmission of mycoplasma-like symbionts in Ligia

Mycoplasma-like symbionts were found in all populations of both *L. pallasii* and *L. occidentalis* screened in this study. This result contradicts the notion that this group of
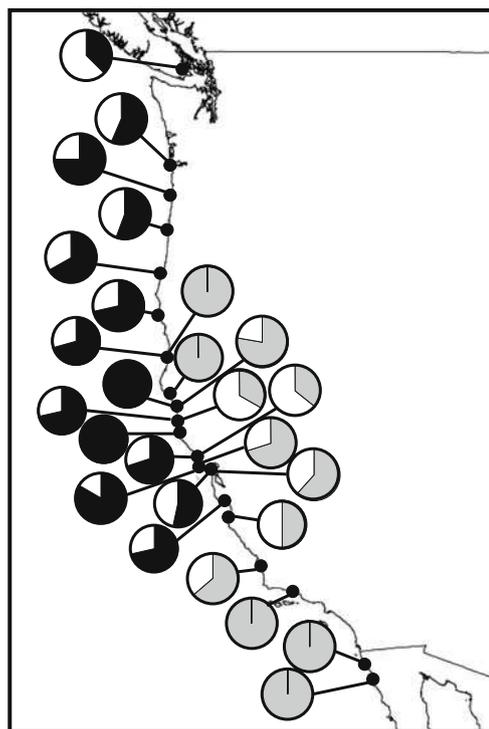

Fig. 3 Presence of mycoplasma-like symbionts in the hepatopancreas of *L. pallasii* and *Ligia occidentalis* along 20 sampling stations. The grey portion of each circle represents the % of positive screens of all the individuals sampled per station (dark grey *L. pallasii,* light grey *L. occidentalis*)

symbionts is absent (Wang et al. 2007) or rare (Fraune and Zimmer 2008) in Ligiidae. Symbiont prevalence, however, varied greatly between locations as was described in previous studies of terrestrial isopods (Wang et al. 2007; Fraune and Zimmer 2008). No clear pattern of variation in infection prevalence along abiotic gradients such as latitude or temperature was found, and further study is necessary to eliminate or identify those ecological factors that influence symbiont occurrence. Associations of facultative symbionts with different hosts have been shown to vary geographically (e.g. Tsuchida et al. 2002; Leonardo and Muiru 2003; Simon et al. 2003), and might confer different benefits under varying environmental stressors such as heat stress (Montllor et al 2002; Berkelmanns and van Oppen 2006) and parasites (Oliver et al. 2003, 2006).

While the specific role of mycoplasma-like symbionts in the hepatopancreas during the digestive process remains unclear, experimental work in *P. scaber* demonstrated increased survival of individuals with *Candidatus* Hepatoplasma crinochetorum under prolonged conditions of severe nutrient limitation (Fraune and Zimmer 2008). The correlation between increased survival and presence of facultative symbionts does not rule out that dietary components may influence the types of bacteria present in the digestive systems, as has been shown for humans (Duncan et al. 2007).

Isopods appear to obtain their symbionts from the environment (Wang et al. 2007). It has been suggested that mutualistic symbioses evolve when symbionts are transmitted vertically from parents to offspring (Herre et al. 1999), yet many examples of environmental transmission of mutualistic symbionts exist: for example in corals (van Oppen 2004), chemoautrophic tubeworms (Nussbaumer et al. 2006), the squid *Euprymna scolopes* (Nyholm et al. 2000), and in hemiptera (Fukatsu and Hosokawa 2002; Kikuchi et al. 2007). In plataspid stink bugs, symbionts are acquired each generation in the nymphal stage (Kikuchi et al. 2007). Ligiidae brood their young in a ventral brood pouch that is open to surrounding environment. Transmission of symbionts from the mother or other species via the environment is possible. Offspring is potentially exposed to symbionts from isopod excretions while the mother is fanning water through the brood pouch with her legs thereby preventing desiccation of her brood. Future work should investigate symbiont prevalence in different life history stages of *Ligia*. The gregarious lifestyle of *Ligia*, wherein great numbers of individuals congregate to preserve moisture (Carefoot 1973), could also be a means of exchange of symbionts and lead to stable microbial associations. Additionally, transmission could occur through coprophagy, ingestion of bacteria in feces voided by self or conspecifics (Kautz et al. 2002; Carefoot 1973).

4.4 Microbial diversity in 16S clone libraries

Aside from mycoplasma-like symbionts, other microbes were recovered in the clone libraries of both *L. pallasii* and *L. occidentalis*. The role of these microbes is unclear and more work is required to determine their contribution to the host, if any. It is possible that these microbes are transients rather than residents in the hepatopancreas. Host surface sterilization and the use of sterile techniques during dissection makes it unlikely that these bacteria are contaminants.

Methods used in this study do not allow for a distinction between the occurrence of multiple symbionts in one individual, and different bacteria in different individuals for *L. occidentalis*. The microbial diversity recovered exceeded the number of individuals pooled in the creation of the *L. pallasii* library, which indicates that more than one symbiont type was present in individual hosts. While two distinct microbial symbionts *Candidatus* Hepatincola porcellionum (*Rickettsiales*, a-*Proteobacteria*; Wang et al. 2004a) and *Candidatus* Hepatoplasma crinochetorum (*Mycoplasmatales*, *Mollicutes*; Wang et al. 2004a, Wang et al. 2004b) have been described in the terrestrial isopod *Porcelio scaber*, they appear not to co-occur in individuals. An exception to the one host one symbiont rule is the lake isopod *Asellus aquaticus* (Asellidae) that was found to harbor additional microbes from the Rhodobacter,

Burkholderia, Aeromonas or Ricketsiella groups (Wang et al. 2007). While sequences similar to Burkholderia were also found in *Ligia pallasii* there was no overlap with other microbial species between the two hosts. Sequences in the genus *Vibrio* were recovered from *L. pallasii*. *Vibrio* belong to a diverse mostly marine group of bacteria with a few species known as causing disease in stressed organisms, but some others are known to form mutualistic symbioses (Farmer and Hickman-Brenner 2006). Ecological specialization with spatial and temporal resource partitioning has been shown for this group (Hunt et al. 2008).

The significance of multiple bacterial types in the hepatopancreas is unknown but may be beneficial to the host. Both host individuals and populations could benefit from a diversity of symbionts in the hepatopancreas, as diversity would help to protect organisms against unstable environmental conditions, ensuring the ability of the bacteria and their hosts to resist environmental stress (Naeem and Li 1997) as has been shown for biofilm communities (Boles et al. 2004). Further studies are needed to investigate both the stability of multiple species associations and the effects of additional microbes on the widespread mycoplasma-like symbionts in semi-terrestrial isopods.

5 Conclusion

This study clearly shows that facultative symbionts are frequently encountered in *Ligia* and are not as rare as previously believed. Mycoplasma-like bacteria that were previously considered to be common only in fully terrestrial isopods have now been shown to be prevalent also in semi-terrestrial high intertidal isopods. *Ligia* spp. live in the transition zone between marine and terrestrial habitats. In fact all populations of *L. pallasii* and *L. occidentalis* tested over a wide geographic range possessed symbionts, making *Ligia* and their symbionts a good model system for the study of symbiont association during sea-land transitions. Between these two closely related isopod species, which share a broad area of range overlap, no host specificity was discovered. Symbionts from hosts found in intertidal habitats clustered together compared to those from terrestrial hosts. Host habitat in addition to strict host phylogeny appears to influence the phylogenetic relation to symbionts.

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