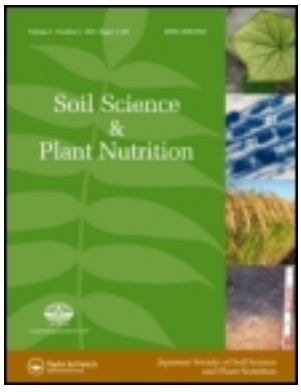

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Comparison of Bacterial Communities Associated with Microcrustaceans from the Floodwater of a Paddy Field Microcosm: Estimation Based on DGGE Pattern and Sequence Analyses

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Comparison of Bacterial Communities Associated with Microcrustaceans from the Floodwater of a Paddy Field Microcosm: Estimation Based on DGGE Pattern and Sequence Analyses

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Bacterial communities associated with five kinds of microcrustaceans (*Tanycypris* sp., *Moina* sp., *Mesocyclops* sp., *Cypretta* sp. and *Heterocypris* sp.) from the floodwater of a paddy field microcosm were examined by the application of denaturing gradient gel electrophoresis (DGGE) to PCR-amplified 16S rDNA products with universal bacterial primers and by sequencing of characteristic DGGE bands. The number of DGGE bands of the associated bacteria was small, indicating the association of specific bacterial members with the microcrustaceans studied, among which *Tanycypris* sp. showed the smallest number of bands. Principal component analysis (PCA) demonstrated that the community structure of the associated bacteria could be divided into three groups: Podocopida (*Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp.), *Moina* sp. and *Mesocyclops* sp., and further analysis separated *Tanycypris* sp. and *Heterocypris* sp. into different clusters. The duration of the incubation period affected the bacteria associated with *Tanycypris* sp., *Moina* sp. and *Cypretta* sp. only. Nearly all of the associated bacteria belonged to Gram-negative bacteria, especially the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group. Closest relatives of the DGGE bands common to three Podocopida and *Mesocyclops* sp. belonged to an invertebrate endosymbiont.

Key Words: **bacteria, CFB, DGGE, floodwater, microcrustacean.**

Epibionts such as microorganisms, protozoa, and algae commonly occur on the exoskeleton of microcrustaceans in the marine environment (Ho and Perkins 1985; Nagasawa 1986a; Chiavelli et al. 1993; Threlkeld et al. 1993; Dumontet et al. 1996; Carman and Dobbs 1997). Although the incidence of copepods (*Acartia* spp.) with bacteria from coastal areas was generally lower than 10%, extremely high incidences over 80% were reported for copepods collected in Tokyo Bay, Japan, and Woods Hole, USA (Nagasawa 1986a, b). Specific bacteria grow at specific sites on the exoskeleton of marine microcrustaceans. Main sites of epibiont attachment included appendages, oral region, egg sac, joints of segments and legs, swimming legs, and depressed parts of the body surface (Johnson et al. 1971; Huq et al. 1983; Nagasawa et al. 1985; Nagasawa 1989; Carman and Dobbs 1997). Rigid host preference was also observed in the interrelations between microcrustaceans and diatom epibionts (Hiromi et al. 1985; Chiavelli et al. 1993; Threlkeld et al. 1993). *Vibrio* spp. were the most common bacterial epibionts of marine copepods. *Pseudomonas*, *Cytophaga*, *Flavobacterium*, *Acinetobacter*, *Leucothrix* and *Aeromonas* were also found as copepod epibionts (Johnson et al. 1971; Kaneko and Colwell 1975; Sochard et al. 1979; Huq et al. 1983; Carli et al. 1993; Dumontet et al. 1996; Hansen and Bech 1996; Carman and Dobbs 1997).

Identification of these epibiont bacteria has been performed by conventional culture techniques. However, most of the bacteria in freshwater require specific nutrients for growth, and they cannot be cultured on media (McCoy and Sarles 1969). Bacterial symbionts of a marine nematode *Laxus* sp., for example, were not successfully cultured (Polz et al. 1994). Recent rapid progress in molecular ecological techniques may enable to elucidate the community structure of copepod epibionts without the bias from culture, and determine the phylogenetic positions of unculturable bacteria, as in the case of Bivalvia *Solemya velum* (Eisen et al. 1992) and for a bryozoan *Bugula neritina* (Haygood et al. 1999).

Although a large amount of information on the cope-

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pod-bacterial associations has accumulated, scant attention has been paid to the associations in freshwater environments (Carman and Dobbs 1997). Holland and Hergenrader (1981) examined calanoid copepods from three Nebraska lakes by scanning electron microscopy (SEM) and found a specific attachment of the bacteria to the abdominal appendages and genital opening. They isolated *Pseudomonas*, *Bacillus*, *Acinetobacter* and *Flavobacterium* from *Diatomus nevadensis*. This is the only information available about bacterial epibionts of freshwater copepods. By using a SEM, Taniguchi et al. (1997a) observed different colonization patterns of epibiotic microorganisms among microcrustaceans (*Moina macrocopa*, Cyclopoida, *Cypretta* sp. and *Tanycypris* sp.) that were obtained from the floodwater of a paddy field microcosm. The bacterial epibionts, the sites they colonized, and their density depended on the kinds of microcrustaceans. The commonality of the colonization pattern of epibionts on the respective microcrustaceans in the case of Philippine and Japanese paddy fields was also recognized by SEM observation; rod-shaped bacteria on Cyclopoida and Cypridopsida, and sparse presence of microorganisms on *Simocephalus* spp. (Taniguchi et al. 1997b, 1999). Not only individual epibionts but also the community structure seemed to be specific for each microcrustacean in the floodwater. Niswati et al. (2002, 2003) compared the community structure of bacteria (epibiotic and intestinal bacteria) among microcrustaceans in the floodwater of a paddy soil microcosm based on the PCR (polymerase chain reaction)-RFLP (restriction fragment length polymorphism) pattern analysis, and revealed the presence of statistical differences in the bacterial communities associated with a cladoceran (*Moina* sp.), a cyclopoid (*Mesocyclops* sp.), and three podocopids (*Heterocypris* sp., *Tanycypris* sp. and *Cypretta* sp.) and free-living bacteria. In contrast, ammonia oxidizers and methanotrophs that were associated with *Moina* sp., *Mesocyclops* sp. and *Heterocypris* sp. in the floodwater of the same microcosm were limited and common (Niswati et al. 2004). These findings indicated that microcrustaceans were a specific habitat for bacteria in the floodwater of paddy fields.

The objectives of the present study were to compare bacteria associated with five kinds of microcrustaceans (a cladoceran, *Moina* sp.; a cyclopoid, *Mesocyclops* sp.; and three podocopids, *Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp.) in the floodwater of a paddy field microcosm and to determine the phylogenetic position of bacterial associates specific to the respective microcrustaceans for confirming the specific association of eubacteria with microcrustaceans phylogenetically. We first performed a PCR-DGGE pattern analysis of the bacterial communities associated with microcrustaceans in the floodwater of a paddy soil microcosm to determine the specific community structure of the bacteria for the respective microcrustaceans that had been estimated based on the PCR-RFLP pattern analysis (Niswati et al. 2002, 2003). Then, the DGGE bands that were common to every microcrustacean and specific to each microcrustacean were sequenced to determine their phylogenetic positions. Microcrustacean samples in the present study were the same as those used in the PCR-RFLP pattern analysis (Niswati et al. 2003) for direct comparison of the PCR-DGGE and PCR-RFLP methods to estimate the community structure of associated bacteria. The bacterial communities in the present study included both epibiotic and intestinal communities.

MATERIALS AND METHODS

Preparation of microcrustaceans. Three kinds of Podocopida (*Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp.), one kind of Cladocera (*Moina* sp.) and Cyclopoida (*Mesocyclops* sp.) were collected from the floodwater of a paddy field located at Aichi-ken Anjo Research and Extension Center, Central Japan. Each microcrustacean was cultured separately in an aqueous medium containing 5 g L^{-1} chicken manure (N: 2.58%, P_2O_5: 8.11%, K_2O: 3.37%, CaO: 19.21%) and 0.1 g L^{-1} baker yeast with periodical supply of *Chlorella* sp.

Set-up of a paddy field microcosm. Soil samples used in the microcosm experiment were taken from a paddy field located at Aichi-ken Anjo Research and Extension Center, Central Japan (latitude 34°48′ N, longitude 137°30′ E). Properties of the soil sampled (Anthraquic Yellow Soil; Oxiaquic Dystrochrept) were as follows: total C content, 13.3 g kg^{-1}; total N content 0.9 g kg^{-1}; pH(H_2O), 6.0; pH(KCl), 4.9. One kilogram of soil that had passed through a 4-mm mesh sieve was mixed thoroughly with chemical fertilizers consisting of $(NH_4)_2SO_4$, $Ca(H_2PO_4)_2 \cdot H_2O$ and KCl at the rates of 0.5, 0.5 and 0.2 g kg^{-1}, respectively. Rice straw segments about 2 cm long with a C/N ratio of 40 were mixed thoroughly into the fertilized soil (10 g kg^{-1} on a dry weight basis). To avoid the development of indigenous microcrustaceans, the soil was heated at 80°C for 2 h. The soil was put into a container (20 cm long, 13 cm wide and 14 cm high) and was submerged with 3 L of distilled water.

Incubation. About 150 individuals of adult microcrustaceans were inoculated to the paddy field microcosms 1 day after the set-up. The containers were placed in a plant growth chamber cabinet (Koito Industries, Ltd., Yokohama, Japan) at temperatures ranging from 30°C in the daytime (4 a.m. to 8 p.m.) to 20°C at night (8 p.m. to 4 p.m.). During the incubation period, the depth of water was maintained at 10 cm with distilled

water.

Sampling of microcrustaceans. One hundred adult individuals of microcrustaceans were collected weekly with a plankton net. They were washed at least three times in sterile distilled water to minimize the bacterial contamination from bulk water, and stored in a 1.5 mL tube with 1 mL of sterile ultra-pure water at −20°C until DNA extraction.

Nucleic acid extraction and PCR amplification. DNA was extracted from the microcrustacean samples at a high temperature according to the lysis method based on salt and sodium dodecyl sulfate (SDS) (Zhou et al. 1996) with slight modifications (Niswati et al. 2002). The DNA samples were stored at 4°C for immediate use or at −20°C for prolonged storage.

Variable region No. 3 of the 16S rDNA was amplified by PCR using the primer set for eubacteria 357f-GC (5′GCCCCGCCGCGCGCGGCGGGGCGGGGCACGGGGGGGCCTACGGGAGGCAGCAG-3′) and 517r (5′-ATTACCGCGGCTGCTGG-3′), with the *Escherichia coli* positions of 341–357 and 533–517 bp (Muyzer et al. 1993), respectively. The PCR mixture (50 μL) contained DNA template (25–35 ng), 0.5 pmols of each primer, 0.25 mM dNTP mixture, 0.2 mM TaKaRa *Ex Taq*™ (TaKaRa Shuzo, Otsu, Japan) buffer and 1.25 U *Ex Taq*™ polymerase. Amplification conditions were as follows: 94°C for 1 min (initial denaturation), followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR was performed on TaKaRa PCR thermal cycler Model TP240 (TaKaRa Shuzo). The presence of the PCR product (2 μL) was confirmed on 2% (w/v) agarose gel containing ethidium bromide (10 g L^{-1}).

DGGE analysis. DGGE was performed using the Dcode™ System (BioRad, Hercules, CA, USA), as described in the manufacturer's instructions and by Muyzer et al. (1993). PCR products (about 300 ng) were loaded onto 8% (w/v) acrylamide gels (containing a linear denaturant gradient of 25–65% (100% denaturant contained 7 M urea and 40% (v/v) formamide)). DGGE was conducted at a constant voltage of 100 V at 60°C for 14 h in 7 L of 1× TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. After completion of electrophoresis, the gels was stained for 30 min with 1:10,000 (v/v) SYBR Green I nucleic acid stain solution (BMA, Rockland, ME, USA) and visualized with UV transilluminator.

Statistical analysis of DGGE patterns. The photograph of the DGGE band patterns was statistically analyzed for mobility and intensity. Respective band positions were identified and the banding patterns of the different samples were calculated from the band intensity in each lane. The intensity of the DGGE bands was classified into 4 grades (0, no band; 1, weak (<40 ng); 2, medium (40–95 ng); and 3, strong (>95 ng). The values were normalized for cluster analysis and principal component analysis. DGGE bands which did not show the closest relation with bacterial 16S rDNA sequences were excluded from the analysis. Cluster analysis was performed using the Blackbox program according to the Ward method (Aoki 1996). Principal component analysis was performed using the Sristat program in EXCEL STATISTICS 97 for windows (SRI, Tokyo). Correlation matrix was used for this analysis.

Cloning and sequencing of DGGE fragments. The DGGE bands were carefully excised under UV illumination and then placed in 100 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was extracted from the gel piece by over-night incubation at 4°C, and the supernatant was used as template DNA in the re-amplification by PCR with the same primers as those described above. The amplicons were electrophoresed again on a DGGE gel to confirm that the mobility was the same as that of the original bands. This operation was repeated until the band appeared to be single. The distinctly separated bands were directly sequenced. Sequence reactions were performed using Thermo Sequenase™ II Dye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA) with primers 357f and 517r, according to the manufacturer's instruction. The PCR products were analyzed with an automatic sequencer (model 373s, Applied Biosystems). When the resultant sequencing failed due to the presence of several ambiguous peaks, the amplified DNA from the DGGE gel was cloned into the pT7Blue T-vector (Novagen, Darmstadt, Germany). The plasmids were transformed into competent cells of *E. coli* XL I-blue (Toyobo, Osaka, Japan) and the transformants were selected based on the presence of blue-white colonies. Plasmid DNA was extracted from the clones using a QIAprep spin miniprep kit (Qiagen, Crawley, UK). The clones with correct inserts that showed the same mobility as that of the original DGGE bands were sequenced as described above. M13 (5′-GTTTTCCCAGTCACGAC-3′) and RV (5′-CAGGAAACAGCTACGAC-3′) primers were used for sequencing.

Phylogenetic analysis of characteristic DGGE bands. Sequences were compared with the sequences available in the GenBank database. The BLAST search option of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) was used to determine the closest relatives of the 16S rDNA sequences (Altschul et al. 1997). Sequences were then aligned using CLUSTAL W and distances were determined by a neighbor-joining algorithm with the same software. The sequences obtained in this study are available in DDBJ under accession numbers from AB104657 to AB104685.

RESULTS

Number of DGGE bands

The DGGE pattern of the bacterial communities associated with five kinds of microcrustaceans is shown in Fig. 1. A total of 35 different bands differing in their mobility appeared, among which 11, 15, 17, 13 and 12 different DGGE bands were observed during the incubation period for the bacterial communities of *Tanycypris* sp., *Moina* sp., *Mesocyclops* sp., *Cypretta* sp. and *Heterocypris* sp., respectively. Among them, 3, 3, 3, 3 and 5 bands were constantly present in the bacterial communities of *Tanycypris* sp., *Moina* sp., *Mesocyclops* sp., *Cypretta* sp. and *Heterocypris* sp., respectively (Fig. 1). As shown in Fig. 2, the average number of bands from *Tanycypris* sp. (5.67 ± 1.49) during the incubation period was significantly ($p<0.05$) smaller than that from *Moina* sp. (7.78 ± 2.44) and *Heterocypris* sp. (7.50 ± 1.22) but not significantly different from that of *Mesocyclops* sp. (6.56 ± 1.50) and *Cypretta* sp. (6.44 ± 0.83).

Common DGGE bands that appeared in every microcrustacean sample and in the respective microcrustaceans

In general, the DGGE pattern was specific to each microcrustacean host and it was almost similar during the incubation period among the samples from each microcrustacean host. However, some of the DGGE bands were common to all the microcrustacean hosts. Closest relatives of the DGGE bands are listed in Table 1 with their appearance frequency. Out of 35 bands differing in mobility, 32 bands were successfully sequenced. The Com1 and Com2 bands appeared in the *Tanycypris* sp., *Mesocyclops* sp., *Cypretta* sp. and *Heterocypris* sp. samples throughout the incubation period. The closest relatives belonged to the endosymbiont bacteria that were found in an invertebrate host, suggesting that these bacteria developed as symbionts of the microcrustacean host. Most of the closest relatives of the bands which frequently appeared for the respective microcrustaceans belonged to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group. The density of these bands was higher than that of other bands suggesting the predominance of the CFB group in the bacterial communities associated with the microcrustaceans. The closest relatives of the T30 and T31 bands which appeared for every microcrustacean belonged to α-*Proteobacteria*, although the frequency and density of the bands were lower than those of the bands commonly observed in the CFB group. The T17 and T18 bands were observed in every sample of *Tanycypris* sp. and *Heterocypris* sp.

Bacteria specific to the respective microcrustaceans

Principal component analysis was performed for the DGGE band patterns to characterize the bacterial communities associated with the respective microcrustaceans (Fig. 3). Percentage contributions were 16.9 and 16.0% for the first and second components, respectively. Bacterial communities associated with *Moina* sp. formed a distinct group and were characterized by the T15, T12, T8, T5 and T10 bands (CFB members and β-

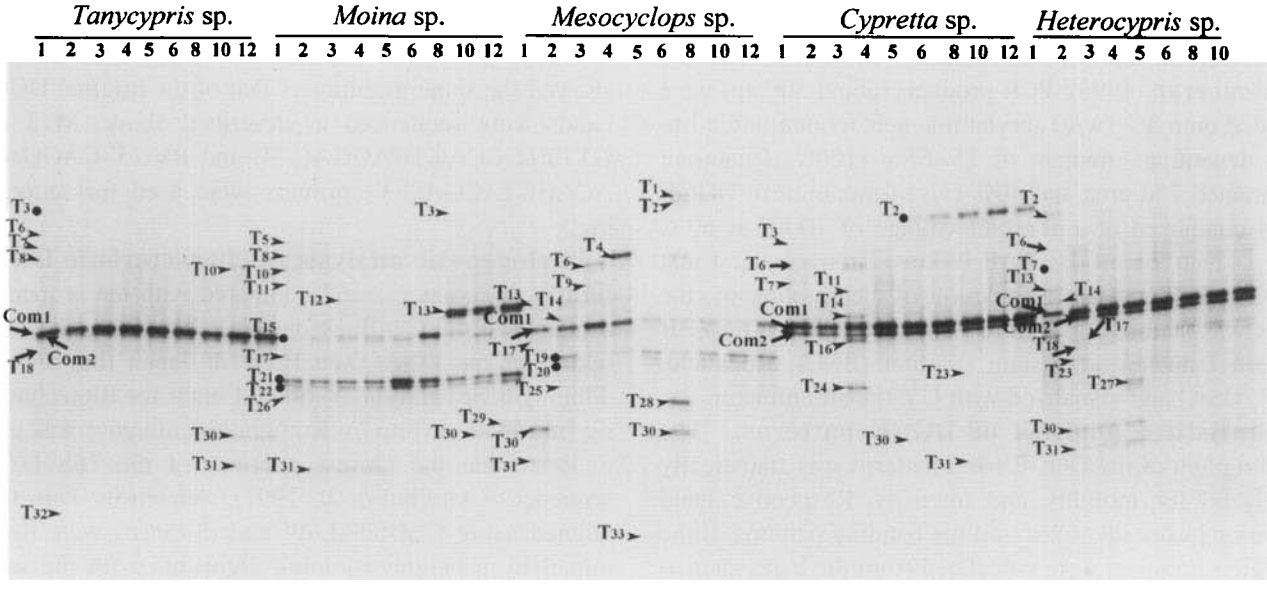

Fig. 1. PCR-DGGE bands patterns from five kinds of microcrustaceans during different incubation periods. →, common band to more than two kinds of microcrustaceans; ●, common band to the respective microcrustaceans; ➤, band number differing mobility. Lane numbers correspond to the incubation period (weeks).

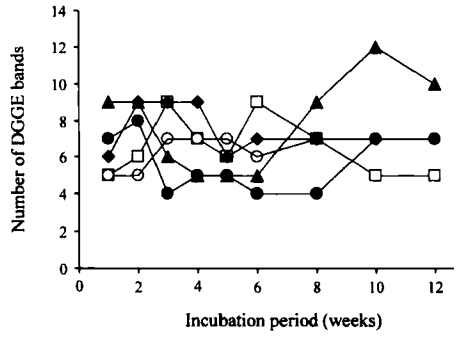

Fig. 2. Changes in the number of PCR-DGGE bands obtained from five kinds of microcrustaceans with the duration of the incubation period. ●, *Tanycypris* sp.; ▲, *Moina* sp.; □, *Mesocyclops* sp.; ○, *Cypretta* sp.; ◆, *Heterocypris* sp.

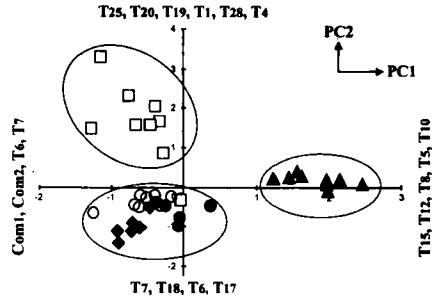

Fig. 3. Principal component analysis of the DGGE band patterns of the bacterial communities associated with microcrustaceans in the floodwater of a paddy field microcosm. Band names in the respective corners show those with high values of eigenvector (refer to Fig. 1). ●, *Tanycypris* sp; ▲, *Moina* sp.; □, *Mesocyclops* sp.; ○, *Cypretta* sp.; ◆, *Heterocypris* sp.

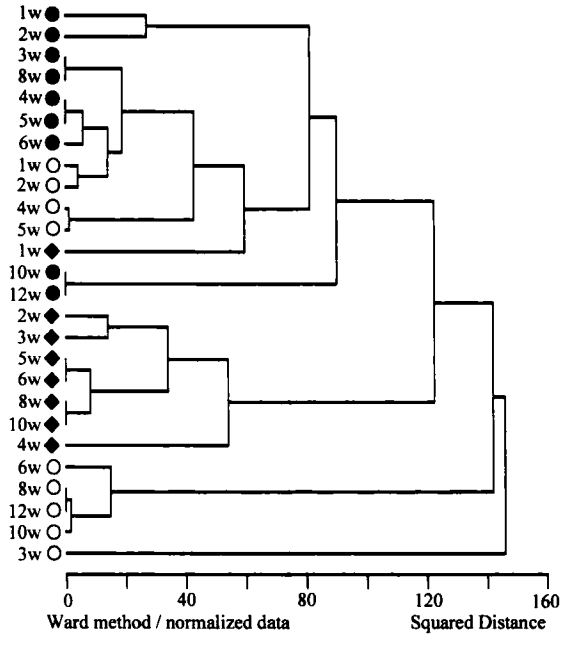

Fig. 4. Cluster analysis of DGGE band patterns of the bacterial communities associated with *Tanycypris* sp., *Heterocypris* sp. and *Cypretta* sp. Figures before the letter w indicate the duration of the incubation period after flooding (week). ●, *Tanycypris* sp.; ○, *Cypretta* sp.; ◆, *Heterocypris* sp.

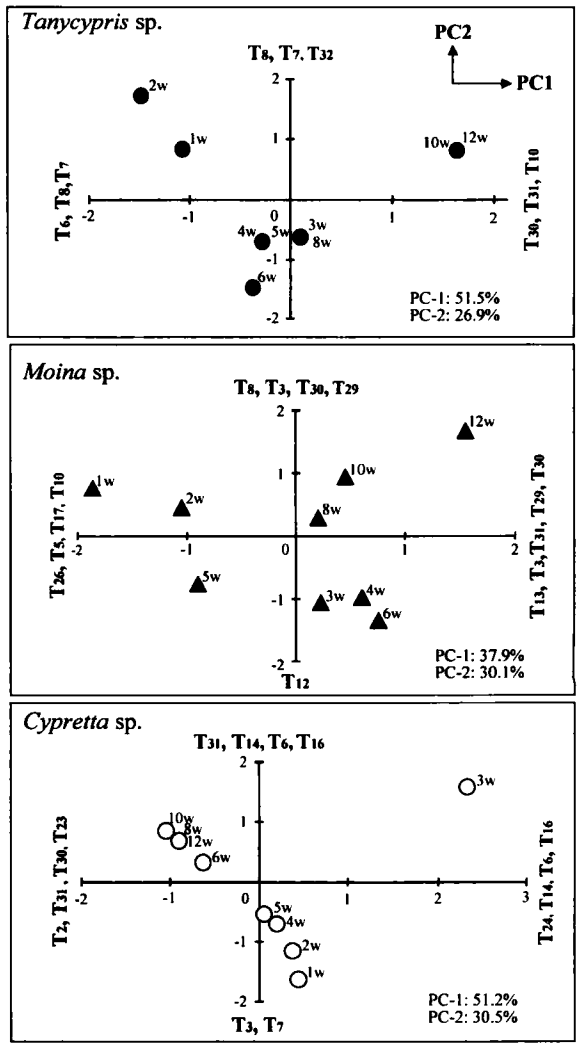

Fig. 5. Principal component analysis of the effect of the duration of the incubation period on the bacterial communities associated with *Tanycypris* sp., *Moina* sp. and *Cypretta* sp. Band names in the respective corners show those with high values of eigenvector (refer to Fig. 1). Figures before the letter w indicate the duration of the incubation period after flooding (weeks).

and γ-*Proteobacteria*).*Mesocyclops* sp. samples were also different from those of other microcrustaceans and were characterized by the T25, T20, T19, T1, T28, T4 and Com1 bands (mainly CFB members). The bacterial communities associated with three Podocopida (*Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp.) were relatively similar to each other and were characterized by the Com1, Com2, T6, T7, T18 and T17 bands (CFB members and β-*Proteobacteria*). Cluster analysis of the DGGE band patterns supported these findings based on principal component analysis where the bacterial communities associated with five kinds of microcrustaceans were separated into three clusters (*Moina* sp., *Mesocyclops* sp. and Podocopida, data not shown).

Table 1. Closest relatives of the sequenced DGGE bands of 16S rDNA products from five kinds of microcrustaceans.

Band name[a]	Microcrustacean host[b]					Seq. bp	Align-ment	Closest relative	Taxonomic description	Simi-larity (%)	Accession number	Sources
	Ta	Cy	He	Me	Mo							
Com1, Com2	+++[c]	+++	+++	+++	−	155	149/155	*Ixodes scapularis* endosymbiont	CFB group	96	AB001518	Tick *Ixodes scapularis*
T6	++	+++	+++	+	−	121	118/121	Rhizosphere soil bacterium RS1-24	CFB group	97	AJ252591	Soil
T17, T18	+++	−	+++	+	+	126	97/99	Uncultured bacterium clone FW119	β-*Proteobacteria*	97	AF523954	Surface sediment
T3	+++	+	−	−	+	135	93/98	Uncultured Crater Lake bacterium CLO-79	CFB group	94	AF316797	Lake water
T7	+	+	+++	−	−	125	84/89	*Flavobacteriurn johnsonae*	CFB group	94	D12664	
T19	−	−	−	+++	−	155	150/153	*Runella slithyformis*	CFB group	98	M62786	
T20	−	−	−	+++	−	155	143/155	Bacteroidales str. WB4	CFB group	92	AB078842	Rice straw in paddy soil
T25	−	−	−	+++	−	155	149/155	Uncultured CFB group bacterium	CFB group	96	AY038777	River Taff epilithon
T15	−	−	−	−	+++	154	153/154	Uncultured Cytophagales bacterium	CFB group	99	AF361200	Lake water
T2	−	++	+	+	−	125	123/123	*Chryseobacterium* sp. AU939	CFB group	100	AY043370	Respiratory secretion
T30	+	++	+	+	+	136	132/134	*Sphingomonas paucimobilis*	α-*Proteobacteria*	98	X94101	Contaminated soil
T31	+	+	++	+	++	135	135/135	Uncultured bacterium IAFESC6	α-*Proteobacteria*	100	AF273323	Groundwater
T4	−	−	−	++	−	155	140/156	Uncultured bacterium	CFB group	89	AF268289	River water
T8	+	−	−	−	++	155	153/155	Uncultured Bacteroidetes bacterium	CFB group	98	AY038764	River Taff epilithon
T12	−	−	−	−	++	129	129/129	Uncultured *Acinetobacter* sp.	γ-*Proteobacteria*	100	AY129795	
T5	−	−	−	−	++	160	160/160	*Pseudomonas* sp.	γ-*Proteobacteria*	100	AX175614	
T10	+	−	−	−	+	160	156/160	*Variovorax paradoxus*	β-*Proteobacteria*	97	AF288730	Polluted soil
T23	−	+	+	−	−	144	142/144	Uncultured bacterium fukuS12	β-*Proteobacteria*	98	AJ290075	Lake water
								Uncultured beta proteobacterium		98	U85124	Lake water
T24	−	+	−	−	−	138	137/138	Uncultured bacterium	β-*Proteobacteria*	99	AY168738	Bioreactor processing
T32	+	−	−	−	−	139	129/131	*Actinobacterium* PI GH.1.C2	High G+C	98	AY162041	Soil
								Uncultured earthworm intestine bacterium		98	AY154545	Earthworm
T27	−	−	+	−	−	138	133/138	*Aeromonas* sp.	γ-*Proteobacteria*	96	AF539686	Wastewater
								Aeromonas hydrophila		96	X74677	
T1	−	−	−	+	−	160	148/160	Bacterium str. 82348	β-*Proteobacteria*	92	AF227863	Environment
							147/160	*Chromobacterium violaceum*		91	U65966	
T28	−	−	−	+	−	135	135/135	Uncultured bacterium	α-*Proteobacteria*	100	AF422681	Contaminated soil
								Blastobacter aggregatus		100	X73041	
								Rhizobium vitis		100	X67225	
T26	−	−	−	−	+	135	129/135	Uncultured α-*Proteobacterium*	α-*Proteobacteria*	95	AF287028	Seawater with high DOM
T29	−	−	−	−	+	160	156/160	*Brachymonas denitrificans*	β-*Proteobacteria*	97	D14320	
T11	−	+	−	−	++	137	125/130	*Pyramimonas parkeae*	Plastid, Chloroplast	96	AF393608	Green algae
T9	−	−	−	+	−	136	123/127	*Euglena anabaena*	Plastid, Chloroplast	96	AF289240	Green algae
T13	−	−	−	++	+	138	130/138	*Chlamydomonas applanata*	Plastid, Chloroplast	94	AF394204	Green algae
T21, T22	−	−	−	−	+++	136	135/136	*Bosmina caregoni*	—	99	AY075093	Cladoceran
								Polyphemus pediculus		99	AY075080	

[a] see Fig. 1. [b] Ta, *Tanycypris* sp.; Cy, *Cypretta* sp.; He, *Heterocypris* sp.; Me, *Mesocyclops* sp.; Mo, *Moina* sp. [c] Appearance frequency: +++, high (>67%; underlined, 100%); ++, medium (<67%, >33%); +, low (<33%); −, no (0%).

Bacterial communities associated with Podocopida

Although the bacterial communities of three microcrustaceans belonging to Podocopida seemed to be similar in Fig. 3, cluster analysis of the bacterial communities among *Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp. indicated differences in their communities (Fig. 4). The bacterial communities associated with *Tanycypris* sp. and *Heterocypris* sp. formed different clusters, and some samples from *Cypretta* sp. were distributed into the cluster of *Tanycypris* sp.

Effect of the duration of the incubation period on DGGE patterns

Principal component analysis within each microcrustacean revealed the existence of a temporal shift of the bacterial communities of *Tanycypris* sp., *Moina* sp. and *Cypretta* sp. (Fig. 5). For the bacterial community of *Tanycypris* sp., the early period was characterized by the T6, T8 and T7 bands (CFB members) and the late period by the T30, T31 and T10 bands (α- and β-*Proteobacteria*), respectively. For the bacterial community of *Moina* sp., the T26, T5, T17 and T10 bands (*Proteobacteria*) characterized the early period, T12 band the middle period, and the T13, T30, T29, T31 and T3 bands (mainly *Proteobacteria*) the late period, respectively. For the bacterial community associated with *Cypretta* sp., the T3, T7, T24, T14 and T16 bands characterized the early period, and the T2, T31, T30 and T23 bands the late period.

The bands that characterized the early and late periods belonged to the CFB group and *Proteobacteria* (α- and β-groups) for *Tanycypris* sp. and *Proteobacteria* (α-, β- and γ-groups) and *Proteobacteria* (α- and β-groups) for *Moina* sp., respectively. In contrast, some members belonging to the CFB group and *Proteobacteria* (α- and β-groups) characterized the early and late periods for *Cypretta* sp.

Closest relatives of other DGGE bands

Besides bacterial 16S rDNA, the DGGE bands that were related to the 18S rDNA (T21 and T22) and plastid chloroplasts of green algae (T9, T11 and T13) were also identified from *Moina* sp., *Mesocyclops* sp. and *Cypretta* sp. (Table 1).

DISCUSSION

Bacterial communities associated with microcrustaceans. As shown in Fig. 2, the number of DGGE bands associated with microcrustaceans in the floodwater of a paddy field microcosm (ranging from 4 to 12) was remarkably smaller than that in the floodwater of paddy fields (Kimura et al. 2002), sea water (Riemann et al. 1999) and rice roots (Ikenaga et al. 2003), indicating that the bacterial communities associated with microcrustaceans are specific and less diverse. By SEM observation, Taniguchi et al (1997a) found that morphology of epibiotic microorganisms was very limited compared with the communities of free-living bacteria in the floodwater. The presence of few bands with a strong intensity in every microcrustacean indicated the predominance of few specific bacteria in association with each microcrustacean.

The number of DGGE bands was smaller for *Tanycypris* sp. than for *Moina* sp., *Mesocyclops* sp. and *Heterocypris* sp. (Fig. 2), which coincided with the larger number of RFLP bands (Niswati et al. 2002, 2003). Taniguchi et al. (1997a) also reported a sparser colonization of microorganisms on *Tanycypris* sp. than on *Moina macrocopa*, a Cyclopoida, and *Cypretta* sp.

Clear differences among the community structure of the bacteria associated with *Moina* sp., *Mesocyclops* sp. and three kinds of Podocopida (*Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp.) were observed (Fig. 3), which is also in agreement with the grouping of the bacterial communities estimated by RFLP pattern analysis (Niswati et al. 2003). Similar characteristics to those of epibiont colonization among three Podocopida members were presumably due to a similar behavior, as well as morphological and histochemical properties among them, although their communities were different from each other in more detailed analysis (Fig. 4), which was also found in the cluster analysis of their RFLP patterns (Niswati et al. 2003). Thus, host preference was in some cases very rigid in the interactions between microcrustaceans and epibionts. For example, euglenoid *Colacium calvum* and cyanobacterium *Synedra cyclopum* were reported to prefer *Daphnia* (Cladocera) to Cyclopoids, and *Colacium vesiculosum* preferred cyclopoids to *Daphnia* as their epibiotic hosts (Chiavelli et al. 1993). Hiromi et al. (1985) observed host-specific relations between diatoms and copepods: *Pseudohimantidium pacificum* on corycaeid copepods and *Protoraphis atlantica* on pontellid and candacid copepods. In contrast, no differences in specific colonization of seven bacteria were observed among four planktonic copepods (*Temora stylifera*, *Acartia clausi*, *Centropages typicus* and *Paracalanus parvus*) (Dumontet et al. 1996). The reason for the host-specific relations between bacteria and microcrustaceans has not yet been elucidated.

Predominant bacteria associated with respective microcrustaceans. Nearly all the bacteria that were associated with the tested microcrustaceans belonged to Gram-negative bacteria, especially to the CFB group (Table 1). Simidu et al. (1971) studied the generic composition of aerobic bacteria that were isolat-

ed from the phytoplankton and zooplankton from the Pacific coast near Tokyo, Japan, by the culture method, and also observed that more than 80% of them were Gram-negative bacteria, mainly *Vibrio*, *Aeromonas* and *Pseudomonas*. The CFB group members were abundant in both freshwater and marine environments (Kirchman 2002), and were known to form symbiosis and associations with invertebrate hosts (Dugas et al. 2001; Horn et al. 2001; Zchori-Fein et al. 2001; Kostanjšek et al. 2002; Lau et al. 2002). Some of the CFB members were chitinoclastic (Reichard et al. 1983) and could metabolize organic matter with a high molecular weight (Cottrell and Kirchman 2000). Decomposition of organic materials by *Cytophaga* and *Flavobacteria* was observed in aquatic environments with a high organic matter input such as during the bloom of diatoms (Riemann et al. 2000; Jaspers et al. 2001). These traits of the CFB members were attributed to preferential colonization to microcrustaceans. Among the CFB members listed in Table 1, there was no specific phylogenetic distribution in relation to the microcrustacean hosts.

There are many studies on the epibionts of marine crustaceans, and *Vibrio* spp. were detected as the most common epibionts in a wide spectrum of crustaceans in marine environments, including estuaries and lagoons (Carman and Dobbs 1997). However, the members of the genus *Vibrio* were not identified as the closest relatives in the present study, probably because they are halophilic (Singleton et al. 1982; Huq et al. 1984) and cannot grow well in the floodwater of paddy fields.

In addition to the CFB members, *Chromobacterium violaceum*, *Pseudomonas* sp. and uncultured *Acinetobacter* were also identified as closest relatives (Table 1). They were commonly isolated from copepods (Sochard et al. 1979; Holland and Hergenrader 1981). *Moina* sp. was also colonized by *Rhizobium* sp. (Table 1). Proctor (1997), Zehr et al. (1998) and Braun et al. (1999) reported the association of nitrogen-fixing bacteria with calanoid and cyclopoid copepods from seawater.

In the present study, the association of *Euglena anabaena* with *Mesocyclops* sp., *Chlamydomonas applanata* with *Moina* sp. and *Pyramimonas parkeae* with *Moina* sp. and *Cypretta* sp. was observed (Table 1). Chang and Jenkins (2000) also isolated the plastid of eukaryotic algae from *Daphnia obtusa*, which indicated the close association of eukaryotic algae with microcrustaceans. As the soil for the microcosm was pre-heated for 2 h at 80°C to exclude indigenous microcrustaceans, it may be necessary to confirm in paddy fields the predominant bacterial associates that were identified in the present study.

Changes in associated bacteria with the duration of the incubation period. Eubacterial communities associated with the respective microcrustaceans were not determined for the microcrustacean samples cultured in aqueous medium. However, the effects of these eubacterial communities on those of week 1 were considered to be negligible, because eubacterial communities at week 1 and afterwards were not appreciably different from one another for every microcrustacean, as shown in Fig. 3. Different bacterial communities appeared depending on the duration of the incubation period for the samples taken from *Tanycypris* sp., *Moina* sp. and *Cypretta* sp. (Fig. 5). Although the changes in the chemical properties of the floodwater appeared to have led to changes in the bacteria-host association, the effect of the nutrient status of the euglenoid and diatom epibionts on the microcrustaceans was not found to be significant for lake water (Chiavelli et al. 1993). As Dawson et al. (1981) pointed out that *Vibrio* showed a strong affinity to the solid surface, when the nutrient status was not favorable, the surface of microcrustaceans might have acted as a niche rich in substrates. The changes in the age distribution with the duration of the incubation period appeared to be the factor responsible for the changes in the bacterial composition, because suctorian *Tokophrya quadripartita* on a calanoid *Limnocalanus* sp. was only observed in adult calanoids, and not in immature forms in Lake Michigan (Evans et al. 1979).

In conclusion, DGGE analysis enabled to identify the bacterial communities specific to the respective microcrustaceans (*Tanycypris* sp., *Moina* sp., *Mesocyclops* sp., *Cypretta* sp. and *Heterocypris* sp.) and the relative similarity of the communities among Podocopida (*Tanycypris* sp., *Cypretta* sp. and *Heterocypris* sp.) in the floodwater of a paddy field microcosm. Sequence analysis of the DGGE bands that were common and specific to the bacterial communities associated with the respective microcrustaceans indicated the close association of Gram-negative bacteria classified into the CFB group and α-, β- and γ-*Proteobacteria* with the microcrustaceans studied. The incubation period affected the associated bacterial communities only for some microcrustaceans examined (*Tanycypris* sp., *Moina* sp. and *Cypretta* sp.).

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