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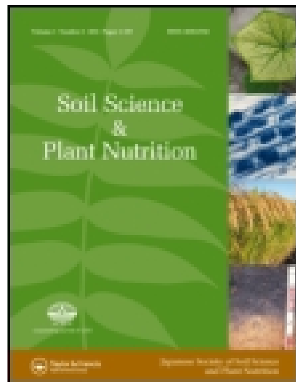

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Bacterial Communities Associated with Aquatic Organisms in the Floodwater of a Japanese Paddy Field Estimated by RFLP Pattern Analysis

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Bacterial communities associated with crustacean (*Tanycypris*, Cladocera, Cyclopoida), Diptera (larvae of *Cricotopus* sp.), and roots of duckweed (*Lemna paucicostata* Hegelm) in the floodwater of a paddy field were analyzed based on the RFLP patterns of 16S rDNA. A larger number of RFLP bands was obtained from the bacterial communities in the floodwater and that associated with Cyclopoida than from the bacterial community associated with the other host organisms. The bacterial community associated with *Tanycypris* gave the smallest number of bands compared with the other habitats. DNA fragments common to *Tanycypris*, Cladocera, Cyclopoida, and Diptera and specific to the respective organisms were recognized. The RFLP pattern of the bacterial communities in the floodwater was markedly different from and more complex than those from aquatic organisms. The RFLP pattern of the bacterial community associated with *Lemna* roots was also specific. Cluster analysis and principal component analysis enabled to clearly separate the RFLP patterns of the bacterial communities associated with aquatic organisms from one another. The effect of sampling time on the bacterial communities was not appreciable compared to the effect of host organisms.

***Key Words*: aquatic organisms, bacterial community, floodwater, paddy field, RFLP pattern.**

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Many kinds of aquatic organisms are known to be present in the floodwater of paddy fields (Miyashita 1929; Kikuchi et al. 1975; Heckman 1979; Ban and Kiritani 1980; Mogi 1993; Yamazaki et al. 2001). The interaction between bacteria and aquatic organisms may play an important role in the ecology of the floodwater of paddy fields, especially in terms of the decomposition of organic materials, nutrient cycling, symbiosis, and food web (Berghe and Bergmans 1981; Fisher 1990; Polz et al. 1992; Kivi et al. 1996). Microcrustaceans are common inhabitants of the floodwater. Kuwabara (1999) and Yamazaki et al. (2001) identified five kinds of crustaceans (Anostraca, Podocopida, Cyclopoida, Harpacticoida, and Cladocera) in the floodwater of a Japanese paddy field.

There are many reports on the bacterial communities associated with aquatic organisms in marine, hot spring, and lake environments (Nagasawa et al. 1985; Ferris et al. 1996; Carman and Dobbs 1997). There is, however, no report on the bacterial association with the zooplankton in the floodwater of paddy fields except for the microscopic studies conducted by Taniguchi et al. (1997a, b, 1999), in which they investigated epibiotic microorganisms associated with microcrustaceans in the floodwater of Japanese and Philippine paddy fields by scanning electron microscopy, and observed the colonization of microbial epibionts at specific sites. Similar specific colonization of many bacterial epibionts on seawater crustaceans was reported using microscopic techniques by Nagasawa and Nemoto (1988), Nagasawa (1989), and Carman and Dobbs (1997). However, these techniques only provided morphological information on the epibiont microbiota without indicating their phylogenetic positions. Although in several reports culture techniques were used to identify the bacterial epibionts associated with marine crustaceans (Sochard et al. 1979; Huq et al. 1984; Carli et al. 1993), the few studies on the phylogenetic lineage of bacteria associated with aquatic organisms were limited to marine environments (Polz et al. 1994, 1999).

It is difficult and not sufficient to study the community structure of the microbiota associated with aquatic organisms by culture techniques. For example, the bacterial symbionts of a marine nematode were not success-

fully cultured, which indicated the complexity of their physiological niche (Polz et al. 1994). Haygood et al. (1999) also could not culture bacterial symbionts of a marine invertebrate, *Bugula neritina*, on standard culture media. Recent rapid progress in molecular ecological techniques has enabled to elucidate the community structure of symbionts without the bias from culture, and to determine the phylogenetic positions of unculturable bacteria (Eisen et al. 1992; Amann et al. 1995). Polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) method is a sensitive method for characterizing the changes in the bacterial communities in environmental samples such as water (Martinez-Murcia et al. 1995) and soils (Smit et al. 1997; Toyota et al. 1999). The stepwise strategy of this method is to extract DNA from the microbial communities, and to use it as a template for PCR amplification of 16S rDNA genes with appropriate primers. The amplified DNA is digested with restriction enzymes and the fingerprints of the DNA fragments can be used for the estimation of community structure of microbiota. The abundance and the size distribution of DNA fragments are used to estimate the genetic diversity of the community organisms, although the method cannot be applied to the estimation of phylogenic positions of community members. This method can be applied with a small investment of time and cost, allowing several samples to be analyzed simultaneously.

The present study was the first to use the PCR-RFLP method to elucidate the bacterial communities associated with common microcrustaceans (*Tanycypris*, Cladocera, and Cyclopoida), Diptera, and duckweed roots in the floodwater of a Japanese paddy field during the period of rice cultivation.

MATERIALS AND METHODS

Study field. The study field was located at the Aichi-ken Anjo Research and Extension Center, Central Japan (E2 field; latitude 34°48′ N, longitude 137°30′ E). The soil of the field was an Anthraquic Yellow Soil (Oxiaquic Dystrochrepts). Some of the soil properties 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. Rice plants (*Oryza sativa* L. cv. Asahino-yume) were cultivated in the field by conventional practices (Table 1).

Sampling of aquatic organisms. Aquatic organisms more than 500 μm in size were selected in the present study. They included *Tanycypris*, Cladocera (mainly *Moina* sp.), Cyclopoida (mainly *Mesocyclops*), Diptera (mainly *Cricotopus* sp.), and duckweed (*Lemna paucicostata* Hegelm). The sampling of the aquatic organisms started from the second week after transplanting of rice seedlings. They were collected with a plankton net (pore size: 210 μm), and stored in a 100 mL

Table 1. Field management, sampling date, and organisms trapped in the paddy field studied.

Management	Date	Sampling date	Organisms collected[g]
Plowing	April 25		
Basal fertilization[a]	May 11		
Flooding	May 15		
Puddling	May 18		
Transplanting	May 22		
Herbicides[b]	May 29		
		June 6	*Tanycypris*, Cladocera, Diptera, Heterocypris[h]
		June 19	Cladocera, Cyclopoida,[h] Diptera,[h] *Lemna* roots[h]
Midseason drainage	June 28 to July 10		
		July 12	Cladocera, Cyclopoida, Turbellaria,[h] Ploima,[h] Archiphora[h]
Topdressing[c]	July 21		
		July 25	Cladocera, Cyclopoida, Diptera, *Lemna* roots
Topdressing[d]	July 31		
		August 1	Cladocera, Cyclopoida
Insecticides and Fungicides[e]	August 4		
		August 15	Cyclopoida, *Lemna* roots
		August 29	Cyclopoida, *Lemna* roots
Insecticides and Fungicides[f]	September 7		
		September 14	Cyclopoida, Diptera, *Lemna* roots
Drainage	September 18		
Harvesting	September 28		

Amounts of application (ha^{-1}): [a] 56 kg N, 64 kg P_2O_5, and 56 kg K_2O; [b] 210 g Caphensutol, 450 g Daimuron, and 51 g Bensulfuron-methyl; [c] 22.4 kg N and 22.4 kg K_2O; [d] 24 kg N and 24 kg K_2O; [e] Kasugamycin 57 g, $CuCl_2$ 760 g, Phenthoate 500 g, and Flutolanil 250 g; [f] Phenthoate 500 g and Fenobucarb 500 g. [g] Organisms that were trapped by plankton net. [h] The amount of the organisms collected was not sufficient for DNA extraction.

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plastic bottle. One hundred milliliters of the floodwater was also collected, and carried back to the laboratory in a cooling container to avoid changes in the bacterial communities during transportation.

One hundred individuals of the respective aquatic organisms were collected, cleaned at least three times in sterile distilled water to minimize the microbial contamination from bulk water, and stored in 1 mL of sterile ultra-pure water at − 20°C until DNA extraction.

The floodwater sample was filtered first with a sieve (38 μm pore size) in order to separate suspended clogs, and collected on a PTFE membrane filter with a pore size of 0.2 μm (ADVANTEC, Tokyo, 47 mm diameter). The membrane filters were kept at − 80°C until DNA extraction. *Lemna* roots were separated from the stem with small scissors and forceps, cleaned several times in sterile distilled water, and stored in sterile ultra-pure water at − 20°C.

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Extraction of DNA from aquatic organisms. The samples of *Tanycypris*, Cladocera, Cyclopoida, and Diptera were separately macerated with a sterile pipette tip in 1.5 mL tube and immediately suspended in 700 μL DNA extraction buffer (100 mM Tris HCl, 100 mM Na_2EDTA, 1.5 M NaCl, and 10 g L^{-1} cetyltrimethylammonium bromide (CTAB) at pH 8.0; Zhou et al. 1996), and 5.2 μL proteinase K (10 g L^{-1}; GIBCOBRL®, Paisley, Scotland) was added. They were mixed, and incubated for 30 min at 37°C with inversion at 10 min intervals. After shaking, 160 μL of 100 g L^{-1} sodium dodecyl sulfate (SDS) was added, mixed, and incubated again for 2 h at 65°C in a water bath with inversion at 30 min intervals. An equal volume of phenol–chloroform–isoamylalcohol (25 : 24 : 1, v / v / v) was added, and the solution was mixed thoroughly, followed by centrifugation for 5 min at 14,000 × *g*. The supernatant phase was transferred to a new microtube, and used for DNA purification twice with an equal volume of chloroform–isoamylalcohol (24 : 1, v / v) by centrifugation for 5 min at 14,000 × *g*. The aqueous phase was transferred to a new tube containing 0.6 volume of isopropanol, and kept for 10 min statically at room temperature. The pellet of crude DNA was obtained by centrifugation for 20 min at 14,000 × *g*, washed with ice-cold 0.7 L L^{-1} ethanol, and suspended again in 100 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was extracted in duplicates. The suspension was stored at 4°C for immediate use or at − 20°C for long storage.

The extraction of DNA from *Lemna* roots was conducted by the same method as that described above using about 100 mg roots per tube. The root sample was suspended in 800 μL DNA extraction buffer, and 6 μL proteinase K was added. It was mixed, and incubated for 30 min at 37°C with inversion at 10 min intervals. After shaking, 180 μL of 100 g L^{-1} SDS was added, mixed, and incubated again for 2 h at 65°C in a water bath with inversion at 30 min intervals. The following procedures for extraction of DNA and storage were the same as those described above.

Microorganisms in the floodwater that were collected on the membrane filter were extracted as follows. Two membrane filters were cut into pieces of ca. 10 mm in size, and placed in a 10 mL tube. The filter papers were suspended in 2 mL DNA extraction buffer, and 20 μL proteinase K was added. The suspension was mixed, and incubated for 30 min at 37°C with inversion at 10 min intervals. After shaking, 600 μL of 100 g L^{-1} SDS was added, mixed, and 3 cycles of freeze-thaw treatment were performed to break the cells mechanically by 30 min incubation at 65°C in a water bath and 15 min freezing at − 80°C. An equal volume of phenol–chloroform–isoamylalcohol (25 : 24 : 1, v / v / v) was added, and the solution was mixed thoroughly, followed by centrifugation for 5 min at 14,000 × *g*. The supernatant phase was transferred to a new tube, and DNA was purified twice with an equal volume of chloroform–isoamylalcohol (24 : 1, v / v) by centrifugation for 5 min at 15,000 × *g*. The supernatant was precipitated by 0.6 volume of isopropanol at room temperature for 10 min. The pellet of crude DNA was obtained by centrifugation for 20 min at 15,000 × *g*, washed with ice-cold 0.7 L L^{-1} ethanol, and resuspended in 100 μL of TE buffer (pH 8.0), and stored at 4°C for immediate use or at − 20°C for long storage.

PCR conditions. The primer set used for PCR amplification of the eubacteria was 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1401r (5′-GCGTGTGTACAAGACCC-3′) (Heuer and Smalla 1997). Fifty microliters of a PCR mixture contained appropriate amounts of DNA template, 0.5 μL of 0.2 μM of each primer, 5 μL of a 2.5 mM dNTP mixture, 5 μL of 20 mM TaKaRa *Ex Taq*™ (Takara Shuzo, Co., Ltd., Otsu, Japan) buffer, 1.25 U *Ex Taq*™ polymerase, and ultrapure water. A hot start PCR was performed. Amplification conditions were 94°C for 1 min (initial denaturation), followed by 25 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. PCR procedure was performed on TaKaRa PCR thermal cycler Model TP240 (Takara Shuzo Co., Ltd.). An experiment with negative control was carried out during the procedure, in which water instead of DNA template was used to exclude the possibility of cross-contamination. The presence of the PCR product was confirmed by the analysis of 3 μL of the product on 7 g L^{-1} agarose gels containing 2% of 50× TAE buffer (40 mM Tris-acetate, 2 mM EDTA) after staining with ethidium bromide (10 g L^{-1}). Electrophoresis of agarose gels was run with 1× TAE buffer in a Mini Gel electrophoretic System (Advance Co., Ltd., Tokyo) at 100 V

for 30 min. The gel was scanned under UV illumination and photographed.

A 100 μL aliquot of PCR product was precipitated with 200 μL of pure ice-cold ethanol and 10 μL of 3 M NaOAc at pH 5.2, centrifuged, washed with 1 mL 0.7 L L^{-1} ice-cold ethanol, dried up, and resuspended in 40 μL ultra-pure water.

RFLP analysis. Four kinds of restriction enzymes (*Eco*R I, *Hae* III, *Hinf* I, and *Sau*3A I: Toyobo, Tokyo) were selected based on the studies conducted by Toyota et al. (1999) and Wang et al. (2001), in which they used these enzymes to estimate the impact of fumigation with Metam Sodium on the microbial community structure and of fungicide Thiram on the microbial community of the surface of rice seed sown in soil, respectively. The purified PCR products were digested by each restriction enzyme. Before analysis, the products were checked for the band intensity to determine the DNA concentration. Each sample contained 8 μL of a PCR product, 10 U of *Hae* III, *Hinf* I, *Sau*3A I or 15 U of *Eco*R I, and 1 μL of 10× M buffer (100 mM Tris-HCl at pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol, and 500 mM NaCl) for buffering *Hae* III, or 10× H buffer (500 mM Tris-HCl at pH 7.5, 100 mM $MgCl_2$, 10 mM dithiothreitol, and 1,000 mM NaCl) for buffering *Hinf* I, *Sau*3A I, and *Eco*R I. Incubation was performed at 37°C for 1 h. The digested sample was injected to 20 g L^{-1} MetaPhor® agarose (FMC Co., Rockland, Maine, USA) with 50× TAE buffer, staining with ethidium bromide and electrophoresed at 50 V for 1 h. The 100 bp ladder was used as a marker. The gel was scanned by UV illumination and photographed. The RFLP analysis was conducted in triplicates. The length and amount of the fragments that were visualized by UV illumination were estimated according to the mobility of the marker and the band intensity based on 4 grades (0, no band; 1, weak; 2, intermediate; and 3, strong), respectively. Reliable comparison of size equivalence or difference of DNA fragments among the samples was deemed more important than the precise determination of the fragment size in the present study. As it was difficult to detect small differences in the fragment size among the samples that were run on different gel plates, a representative sample was selected from the samples that had been run together, and representative samples were run again on the same gel plate to determine the size equivalence or difference in the DNA fragments in all the samples studied. Fragments shorter than 100 bp were not considered in the following analysis because their diffusion was too wide to determine their fragment sizes.

Statistical analysis. Cluster analysis was performed by the Blackbox program (Aoki 1996). Ward method was used in this analysis. All the fragments obtained by four kinds of restriction enzymes were used in the calculation. The values were normalized for principal component analysis and cluster analysis. Principal component analysis was performed using Sristat program in EXCEL STATISTICS 97 for Windows (SRI, Tokyo). Correlation matrix was used for this analysis.

RESULTS

Bacterial communities associated with aquatic organisms

The RFLP patterns of sample DNA were reproducible enough to detect differences in the patterns between the bacterial communities associated with aquatic organisms and floodwater at each sampling time. The analysis of the RFLP patterns using four restriction enzymes resulted in a specific pattern for each bacterial community associated with aquatic organisms and the floodwater (Fig. 1). Although we surveyed the field 8 times in total, a sufficient quantity of *Tanycypris*, Cladocera, Cyclopoida, Diptera, and *Lemna* roots was collected for RFLP analysis only 1, 5, 6, 3, and 4 times, respectively. The successions of dominant aquatic organisms during the rice growth periods were in agreement with the observations of Yamazaki et al. (2001). The types of aquatic organisms were more numerous after midseason drainage but only the number of Cladocera and Cyclopoida was sufficient for the analysis. More numerous bands were obtained from the bacterial communities in the floodwater and from that associated with Cyclopoida than from those associated with the other host organisms. The bacterial community associated with *Tanycypris* gave the smallest number of bands compared with the other habitats.

Fragments common to the bacterial communities associated with aquatic organisms and specific to the respective organisms were recognized. *Sau*3A I 550 and *Sau*3A I 240 (restriction enzyme used and fragment size (bp)) were common to every organism, and *Hae* III 630, *Hae* III 500, *Hinf* I 400, and *Sau*3A I 350 were specific to Cladocera, *Hinf* I 420, *Hinf* I 140, *Hinf* I 120, and *Hinf* I 100 were specific to Cyclopoida, and *Hae* III 470 was specific to Diptera, respectively (Fig. 1). *Hae* III 520 and *Hae* III 320 that were found in the bacterial community associated with *Lemna* roots were not present in those of *Tanycypris*, Cladocera, Cyclopoida, and Diptera.

The RFLP patterns of the bacterial communities in the floodwater were markedly different from and more complex than those from the aquatic organisms studied. And the bacterial communities in the floodwater had specific fragments (*Hae* III 720, *Hae* III 120, *Hinf* I 650, and *Sau*3A I 370) that were not found in the bacterial communities associated with aquatic organisms as well

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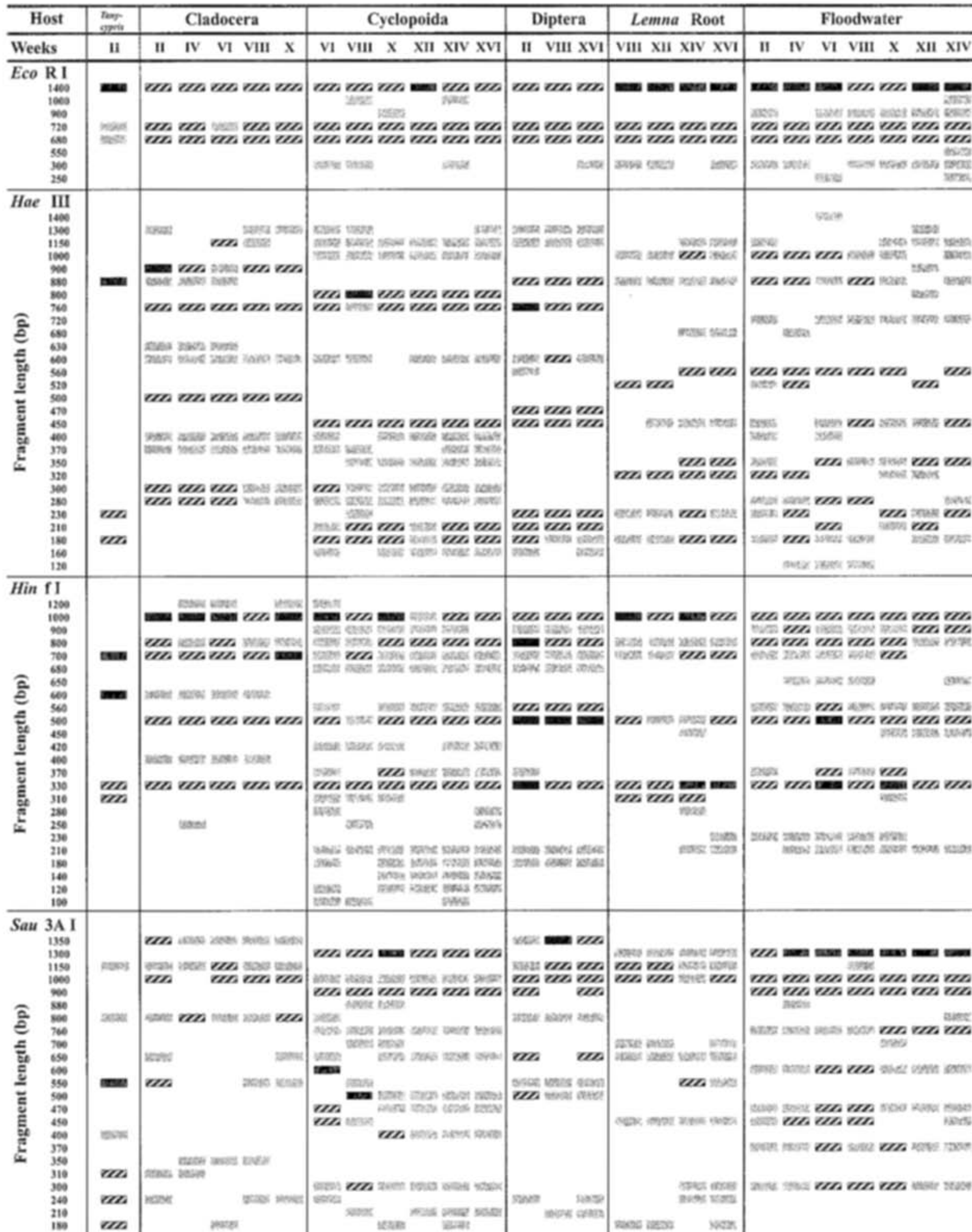

Fig. 1. RFLP patterns of the bacterial communities associated with *Tanycypris*, Cladocera, Cyclopoida, Diptera, and *Lemna* roots in the floodwater of a paddy field. ▬, strong; ▨, intermediate; ▭, weak. Roman letters indicate the duration (week) after transplanting.

as common fragments (*Eco*R I 720, *Eco*R I 680, *Hin*f I 700, and *Hin*f I 330) that were found in the RFLP patterns of the bacterial communities associated with aquatic organisms (Fig. 1).

Community structure of the microbiota associated with aquatic organisms

As shown in Fig. 2, cluster analysis enabled to clearly separate the RFLP patterns of the bacterial communities

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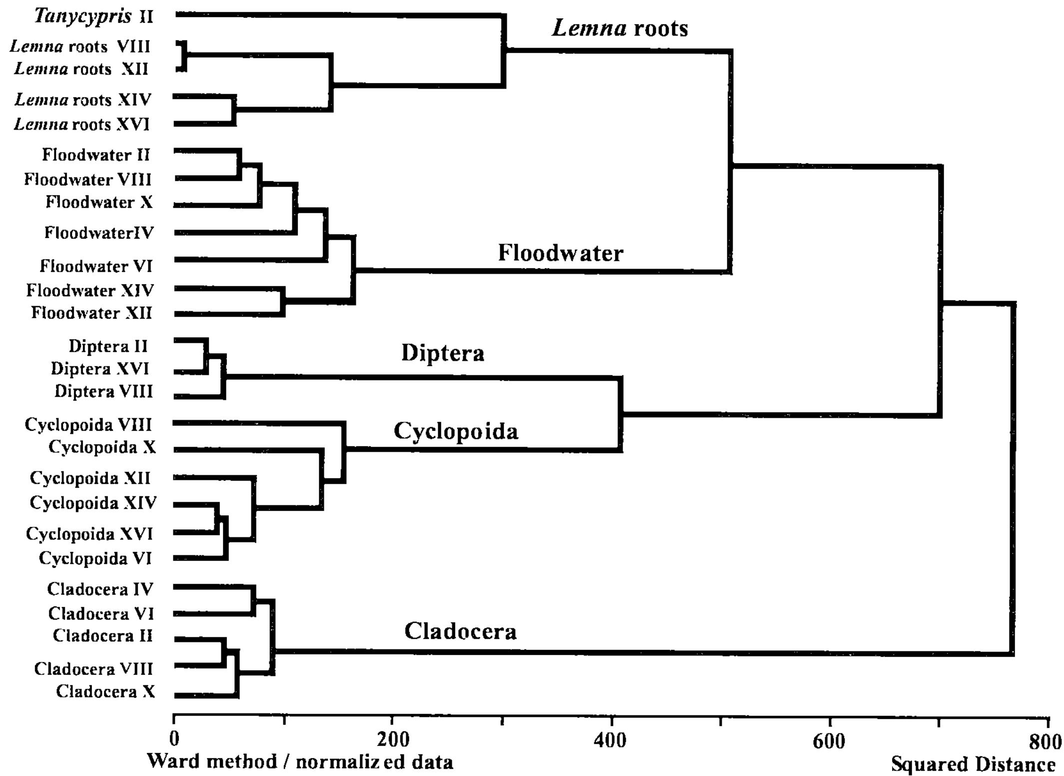

Fig. 2. Cluster analysis of the RFLP patterns of the bacterial communities associated with *Tanycypris*, Cladocera, Cyclopoida, Diptera, and *Lemna* roots in the floodwater of a paddy field. Roman letters indicate the duration (week) after transplanting.

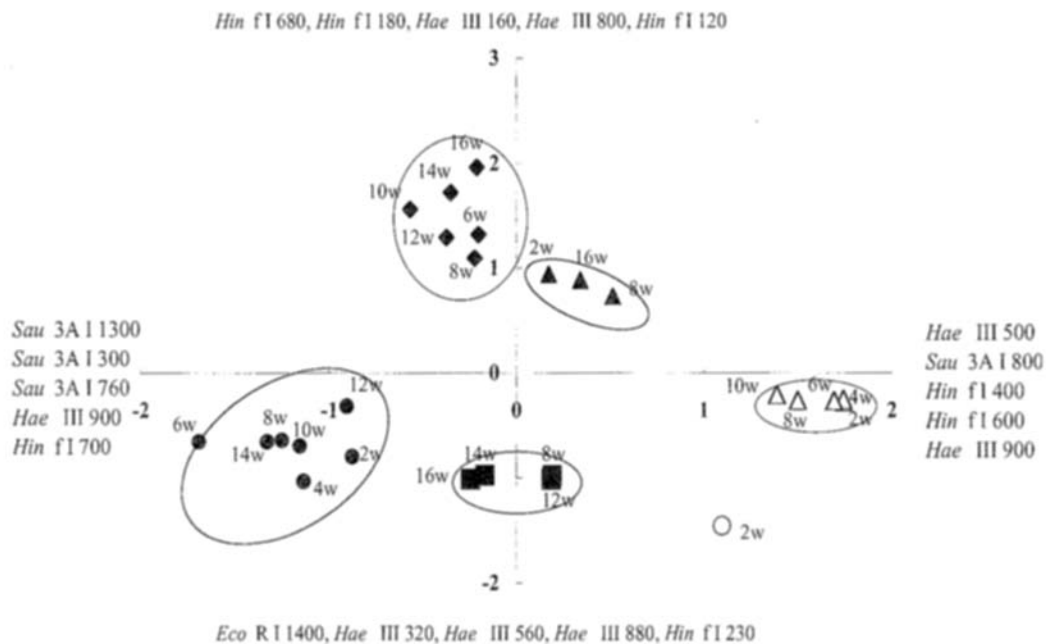

Fig. 3. Principal component analysis of the RFLP patterns of the bacterial communities associated with *Tanycypris*, Cladocera, Cyclopoida, Diptera, and *Lemna* roots in the floodwater of a paddy field. ○, *Tanycypris*; △, Cladocera; ◆, Cyclopoida; ▲, Diptera; ■, *Lemna* roots; ●, floodwater. DNA fragments on each side show those with high values of eigenvector.

associated with aquatic organisms from one another. The bacterial community associated with *Tanycypris* was closest to that of *Lemna* roots. The effect of the sampling time on the community structure was not appreciable compared to the effect of host organisms.

The establishment of specific microbial communities

in the respective habitats was also suggested from principal component analysis (Fig. 3). The score plots of the RFLP pattern of the bacterial communities in the floodwater were distributed on the left-hand side, and those of the bacterial communities associated with Cladocera and Cyclopoida were distributed on the right-hand and at the upper-central sites, respectively. The effect of the sampling time on the community structure was also not appreciable compared to the effect of host organisms by this analysis.

DISCUSSION

The surface skeleton and the gut are considered to be the sites of colonization of microorganisms in the case of *Tanycypris*, Cladocera, Cyclopoida, and Diptera. In a preliminary investigation, the surface skeleton was separated from the body of the Cladocera (mainly *Moina* sp.) and Cyclopoida (mainly *Mesocyclops*) samples under the microscope, and the RFLP patterns of the bacterial community that colonized separated skeleton were compared with those of the bacterial community collected from the whole body. The RFLP patterns were similar to one another for the Cladocera samples, while they were different for the Cyclopoida samples (data not shown). As we macerated sample organisms before the extraction of DNA, no considerable differences in the efficiency of DNA extraction from gut microorganisms were expected among the studied organisms. Determination of the main sites of bacterial colonization of the respective microcrustaceans, Diptera, and *Lemna*, and relations between the bacterial communities and the sites of colonization should be investigated in future.

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Figure 1 shows that the bacterial community associated with Cyclopoida was more complex than those associated with the other aquatic organisms studied, and it corresponded to the microscopic observation by Taniguchi et al. (1997a), in which a much larger number of epibionts colonized Cyclopoida than *Tanycypris* and *Cypretta*. The surface structure and the chemical composition of the host tissues seemed to have played an important role in bacterial colonization. Montgomery and Kirchman (1993, 1994) who studied the attachment of the genus *Vibrio* to the surface of Crustaceans, detected not only chitinous substances at the surface of the skeleton but also a specific protein of bacterial origin for attachment to the chitinous surface. Dumontet et al. (1996) reported that the colonization process was strongly determined by the kind of aquatic organisms and the bacteria living in the surrounding water.

Although the bacterial communities generally change very rapidly in response to their environmental conditions (Huq et al. 1984; Güde 1988), the variations in the RFLP patterns with the duration of rice cultivation were negligible in the present study for both the floodwater and aquatic organisms studied (Figs. 1–3), which was in agreement with the significant but limited seasonal changes in the phospholipid fatty acid (PLFA) composition of the microbiota in the floodwater of a paddy field under long-term fertilizer trial (Okabe et al. 2000).

The presence of DNA fragments that were common to every habitat indicated the presence of some bacteria that were present commonly not only in the floodwater but also in association with aquatic organisms (Fig. 1). The presence or absence of DNA fragments that were specific to aquatic organisms might imply the selective growth of some bacteria on aquatic organisms. In this context, Hansen and Bech (1996) reported that some genera of bacteria colonizing the fecal pellets of zooplankton arose from seawater. In addition to the presence of DNA fragments such as *Hae* III 470, specific to Diptera, some fragments were absent only in Diptera, which indicated the specific inhibition of some bacteria in the association with Diptera.

Figures 2 and 3 suggested the establishment of a specific bacterial community in the respective habitats. The bacterial communities in the floodwater and those associated with Cladocera and Cyclopoida were characterized by microorganisms with the *Sau*3A I 1300, *Sau*3A I 300, *Sau*3A I 760, *Hae* III 900, and *Hinf* I 700 fragments, microorganisms with the *Hae* III 500, *Sau*3A I 800, *Hinf* I 400, *Hinf* I 600, and *Hae* III 900 fragments, and microorganisms with the *Hinf* I 680, *Hinf* I 180, *Hae* III 160, *Hae* III 800, and *Hinf* I 120 fragments, respectively (Fig. 3). The identification of the bacteria with the DNA fragments mentioned above should be investigated in future.

In conclusion, the presence of the bacterial communities both common and specific to aquatic organisms that were recognized from the RFLP patterns strongly suggested the development of specific bacterial communities in association with aquatic organisms in the floodwater of paddy fields.

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