Microarray-based analysis of changes in diversity of microbial genes involved in organic carbon decomposition following land use/cover changes

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Abstract

To increase our understanding of the impact of land use/cover changes on soil microbial decomposition genes involved in organic carbon decomposition, we analyzed soil samples in four sites with different land cover/use histories in a subalpine region of western Sichuan. One site was in a primitive *Abies faxoniana* forest, the second and the third sites were spruce plantations established in 1960's and 1980's, respectively, and the fourth site was in a cropland dating back to 1960's. The genomic DNA from the microbial community was isolated and hybridized against a functional gene microarray containing 1,961 probes. There were 39, 62, 41, and 28 gene probes with statistically significant positive signals and the gene diversity index (H′) values were 3.59, 4.04, 3.70 and 3.16 in primitive forest, spruce plantations established in 1960s and 1980s and cropland, respectively. The results suggested that the number of functional genes and the gene diversity index were correlated with increasing amounts of soil organic carbon, except in the primitive *Abies faxoniana* forest site. CLUSTER analysis demonstrated that primitive forest soil was clustered more closely to soil from the spruce plantation established in 1960s.

Introduction

Soil is the major reservoir of terrestrial organic carbon (Bouwman, 1990; Post *et al.*, 1990; Schlesinger, 1990), with levels of organic carbon in soil dependent on environmental, biogeochemical, and anthropogenic factors (Ross *et al.*, 1999). Changes in land use/cover can have marked effect on organic carbon present in soil as a result of the interactions between changes in plant residues and the immobilization of organic carbon mediated by microorganisms (Tate, 1987). The impact land use/cover changes have on microbial communities are important in terms of soil fertility, long term sustainability, atmospheric CO_2 concentrations, and may possibly influence the rate of global warming (Bouwman, 1990). Different organic carbon compounds present in soils with different land use/cover require different enzymes for decomposition. Specific enzymes are produced by a limited number of microbial species, and land use/cover changes may increase the competitive ability of microorganisms with adaptive organic carbon decomposition pathways (Jensen & Nybroe, 1999). The majority of microbial studies have monitored the relationship between organic carbon in soil, CO_2 release, and microbial biomass following land use/cover changes (Masaki *et al.*, 1998; Lundquist *et al.*, 1999; Wang *et al.*, 2003; Michelsen *et al.*, 2004). Although these studies have revealed a great deal of information on soil organic carbon dynamics, they approached microbial biomass as a whole and have not examined the structure of the microbial community actively involved in carbon cycling (Kirk *et al.*, 2004).

The spatial and temporal heterogeneity of soil microbial communities have made the study of microbial functional gene diversity difficult. Recent advances in molecular biology offer new opportunities for analysis of species composition and microbial community structure. The microarray is a powerful genomic tool that is widely used in studying biological processes. Microarray technology has been used successfully to analyze global gene expression in cultured microorganisms (Wodicka *et al.*, 1997; Ye *et al.*, 2000). Microarray-based genomic techniques have attracted much interest among microbial ecologists; however, it has only recently been extended to study microbial communities in

the environment (Loy *et al*., 2004; Rhee *et al*., 2004; Tiquia *et al*., 2004; Franke-Whittle *et al*., 2005; Saleema *et al*., 2005; Neufeld *et al*., 2006). Functional gene arrays (FGAs) contain probes corresponding to genes encoding key enzymes involved in various ecological and environmental processes, such as carbon fixation, organic carbon decomposition, and denitrification. These arrays are useful in studying the physiological status and functional activity of microbial communities in natural environments (Zhou & Thompson, 2002; Rhee *et al*., 2004).

In this study, we designed and developed an oligonucleotide FGA array comprising of probes complementary to 1961 metabolic genes. This FGA contained 50 bp nucleotide probes corresponding to bacterial genes involved in organic carbon decomposition, carbon fixation, metal resistance, and sulfur, nitrogen, and phosphorous cycling. We applied this microarray to analyze soils from four different land use/cover histories in subalpine forest of western Sichuan, China. Our goal was to elucidate the effect land use/cover changes on soil microbial communities and gene diversity involved in organic carbon decomposition.

Materials and methods

Study sites

The study sites were located in Miyaluo county (102°35′-103°40′ E, 31°24′-31°55′ N) in subalpine forest area of western Sichuan, China. The mean annual precipitation ranged between 600 and 1100 mm, and the mean annual temperature ranged between 6 and 12 °C. Subalpine coniferous forests (*Abies* sp.) dominate most of the primitive forested areas in western Sichuan, including the upper reaches of the Yangtze River. Since the 1950s, primitive coniferous forests have been felled and replanted with spruce plantations of different age classes and converted to cropland.

Four land use/cover types were selected in this study, including primitive *Abies faxoniana* forest (31°47′46″ N, 102°41′53″ E, elevation 3162 m), spruce plantations (*Picea likiangensis var balfourianan*) established in 1960s (31°47′46″ N, 102°42′03″ E, elevation 3233 m) and 1980s (31°47′32″ N, 102°42′04″ E, elevation 3300 m) following the harvest of the primitive forests, and cropland (31°47′48″ N, 102°41′56″ E, elevation 3168 m) created by the conversion of the primitive forests in 1960s. The spruce plantations and cropland are located on the same mountain and are located less than 10 km from the primitive forest. The primitive forest was undisturbed by human activities with a mean tree density of 587 trees ha^{-1} and a mean tree height of 38.15 m. The mean tree density and mean tree height in the 1960s spruce plantation were 1860 trees ha^{-1} and 14.3 m and 3760 trees ha^{-1} and 5.17 m in 1980s spruce plantation, respectively. The dominant vegetation in the cropland was taro and yam before 1999, and Chinese cabbage from 2000 to present.

Sampling scheme and analysis

Soils were sampled at 1–20 cm in depth in each of the four land use/cover types in August 2005. Soil samples from each site were randomly taken from 5–10 different locations using a sterile hand trowel and were homogenized. Samples were sieved at 20 mm under field soil moisture and stored in plastic bags at room temperature. For the DNA analysis, an aliquot of each sample was obtained at the time of collection and stored at 0–4 °C during transportation to the laboratory where samples were stored at −70 °C.

DNA extraction and purification

The bulk community genomic DNA was extracted directly from 2 g of soil. Samples were mechanically ground in liquid nitrogen and cells were lysed with sodium dodecyl sulfate (SDS) treatment (Richard *et al*., 2001). The extracted DNA was purified by 0.8% electrophoresis and recovered with a Wizard DNA Clean-up Kit (Promega). The DNA was quantified using a fluorometer (Table 2).

Oligonucleotide probe design

The name of the organic compounds known to be present in soil and their metabolities were used as keywords for identifying appropriate genes in GenBank database through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The gene identification (gi) numbers for the genes of interest were retrieved along with the gene sequences. Fifty bp oligonucleotide probes were designed (http://blog.sina.com.cn/u/1463136195) using the software PRIMEGENS (Xu *et al*., 2002) with the modified parameters (Rhee *et al*., 2004). A total of 1961 oligonucleotide probes of 123 genes were designed (Table 1).

Table 1. Summary of designed gene probes in this study

Functional group	No. of genes represented on array*	No. of gene probes†
Organic carbon decomposition	99	1145
Carbon fixation	4	131
Phosphorous cycling	4	81
Nitrogen cycling	13	319
Sulfur cycling	2	204
Metal resistance	1	81
Totals	123	1961

*Orthologs of the same gene are only counted once. For example, the *nifH* gene is only listed as one gene regardless of how many times it's represented by probes to different sequences.

†Each gene probe is complementary to one ortholog of a given gene sequence.

Table 2. Location characteristics of the studied soils

Land use/cover type	No. of sample	SOC ($g\,kg^{-1}$)	TN ($g\,kg^{-1}$)	SOC/TN ratio	DNA yields ($\mu g\,g^{-1}$[dry wt] of soil)
Primitive fir forest	M-Y	141.74	7.37	19.23	3.50
Spruce plantation established in 1960s	M-60	58.05	4.96	11.70	3.08
Spruce plantation established in 1980s	M-80	51.04	3.72	13.72	3.32
cropland	M-C	27.82	4.26	6.53	2.85

The content of different substances is expressed per kilogram (dry weight) of soil. SOC, soil organic carbon; TN, total nitrogen; SOC/TN ratio, soil organic carbon to total nitrogen ratio.

Microarray construction and processing

Oligonucleotides probes (50-mers) were synthesized in the 96-well plate format. The probes were arrayed with 16 pins at a spacing of 210 μm onto a 25- by 75-mm Superamine glass slide using a PixSys 5500 Printer (Cartesian Technologies, Inc.) at a relative humidity of 55–58%. Each probe set was printed in duplicate on a different section of the slide. The slides were cross-linked by exposure to 600 mJ of UV irradiation in a UV Stratalinker 1800 (Stratagene) and stored in a clean slide box at room temperature. Rhee *et al.* (2004) details the process and detection of specificity, sensitivity, and quantification of this method.

Fluorescent labeling of target DNA with Cy3-dUTP

Genomic DNA (3 μg) was mixed with 1.5 μg of random primer (Invitrogen), denatured by boiling for 5 min, and then immediately chilled on ice. The denatured DNA solution was mixed with 15 μL of labeling reaction solution containing 5 mM dATP, 5 mM dTTP, 5 mM dGTP, 2.5 mM dCTP; 1 mM Cy3-dUTP (Amersham Pharmacia Biotech); and 80 U of Klenow fragment (Invitrogen). The reaction mixture was incubated at 37 °C for 3–6 h. The labeled target DNA was purified using a QIAquick PCR purification column (Qiagen) according to the manufacturer's protocol.

Microarray hybridization

All hybridizations were carried out in triplicate, and multiple soil preparations were independently processed for each replicate array. The hybridization solution contained 50% formamide, 3 × SSC (1 × SSC is 150 mM NaCl and 15 mM trisodium citrate), 0.31% SDS, 1 μg unlabeled herring sperm DNA in a total volume of 30 μL. The hybridization solution was denatured at 95 °C for 5 min, and then transferred to > 50 °C heat block to prevent cross-hybridization. The hybridization mixture was deposited directly onto slides, which were prewarmed to 50 °C, and covered with a coverslip. The microarray was immediately placed into a watertight hybridization chamber, sealed and plunged into the 50 °C water bath for overnight hybridization away from light. After hybridization, each microarray slide was taken out, and the coverslip was immediately removed in wash solution 1 (1 × SSC and 0.2% SDS) at 50 °C. The slides were washed using the method of Rhee *et al.* (2004).

Microarray scanning and data analysis

The microarrays were scanned with a GenePix 4100A Microarray scanner (Axon Instruments Inc.) at a resolution of 10 μm. The scanned fluorescent images were analyzed using GENEPIX PRO 5.0 software (Axon Instruments Inc.) and saved as multilayer TIFF images. The signal to noise ratio (SNR) was also calculated based on the following formula (Franke-Whittle *et al.*, 2005): SNR = [(signal intensity-background intensity)/(SD of background intensity)], in which the background measurement refers to the local spot background intensity, and the SD of background was calculated across all pixels measured by the software. Spots that appeared to be lower than the threshold value were removed from the data set. A commonly accepted criterion was used for the threshold for accurate quantification and SNR ≥ 2 (Loy *et al.*, 2002; Franke-Whittle *et al.*, 2005).

SPSS (11.5 version) and Sigmaplot (9.0 version) were used for statistical analyses. The relationships of the genes involved in organic carbon decomposition detected in various soil samples were determined using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW.

Results

The contents of soil organic carbon and total nitrogen

The amount of organic carbon and total nitrogen present in soil samples from each land use/cover types are shown in Table 2. The soil organic carbon and the total nitrogen differed among the four land use/cover types. The sites M-Y and M-C had the highest and lowest content of soil organic carbon, respectively, and sites M-Y and M-80, had the highest and lowest content of total nitrogen, respectively. The soil organic carbon content in cropland was 80%, 52%, and 45% lower than primitive fir forest, spruce plantations established in 1960s and 1980s, respectively.

Table 3. The detected gene probe number at each functional gene group

		Detected gene probe number				
No. sample sites	Organic carbon decomposition	Carbon fixation	Nitrogen cycling	Sulfur cycling	Phosphorous cycling	Metal resistance
M-Y	32	1	2	1	1	2
M-60	37	3	10	8	0	4
M-80	30	1	6	0	1	3
M-C	22	0	3	1	0	2

Gene diversity and distribution

Overall, the numbers of arrayed probes with statistically significant positive signals (SNR $\geq$ 2) were 39 in site M-Y, 62 in site M-60, 41 in site M-80 and 28 in site M-C (Table 3). To evaluate gene diversity, the Shannon–Weaver index was calculated as follows: $H' = -\Sigma(p_i) \log (p_i)$, where p_i is the signal proportion of the ith gene relative to the sum of all detected genes. The H' values were 3.59, 4.04, 3.70, and 3.16 at the sites M-Y, M-60, M-80, and M-C, respectively. The detected functional gene probes were different among all sites. Site M-60 had the highest number of organic carbon decomposition gene probes with significant positive signals (37). Site M-80 had 30 gene probes involved in organic carbon decomposition and one phosphorous cycling gene probe with significant positive signals.

Analysis of organic carbon decomposition gene orthologs

A total of 121 gene probes involved in organic carbon decomposition produced significant positive signals (SNR $\geq$ 2) in all four samples. These gene probes were related to 23 different gene orthologs, including genes involved in decomposition of alkane, lignin, xylanse, chitinase, endoglugan, hydroxybenzoate, naphthalene, aniline, benzoate, biphenyl, catechol, dibenzofuran, and salicylate (Fig. 1). The mean variation in signal intensity among all six replicates in these samples was 31%, with a SD of 14.4%. The number of detected functional gene probes and gene ortholog were 32 and 18, 37 and 14, 30, and 11, 22 and 13 in sites M-Y, M-60, M-80, and M-C, respectively. Furthermore, the numbers of functional gene probes in each gene ortholog were diverse. There were six gene probes related to catechol decomposition in site M-Y, eight in site M-60, 10 in site M-80, and five in site M-C. The numbers of gene probes related to endoglucan decomposition were two in site M-Y, two in site M-60, seven in site M-80, and one in site M-C. Some gene orthologs involved in organic carbon decomposition were only detected in one or two sites. For example, gene orthologs related to alkane decomposition was only detected in site M-60; however, the gene orthologs related to propionate, hydroxybenzoyl, toluene, and tolenenesulfonate decomposition were not detected in this site.

Cluster analysis of organic carbon decomposition genes

Functional genes involved in organic carbon decomposition were analyzed by cluster analysis using CLUSTER and TREEVIEW software (Fig. 2). Related functional genes clustered together. For example, the genes involved in catechol decomposition (4-hydroxy-2-ketovalerate aldolase, 2-hydroxypenta-2, 4 dienoate hydratase, 4-hydroxy-2-oxovalerate aldolase, muconate cycloisomerase, and muconolactone isomerase) grouped together and were observed only in M-80 soil. Genes related to naphthalene decomposition (2-carboxy benzaldehyde dehydrogenase, large subunit aromatic oxtgenase, and putative 2-hydroxychromene-2-carboxylate isomerase) also clustered together and were detected only in M-60 soil. Genes involved in large subunit of terminal dioxygenase (GenBank no. 1841362) and hydroxylase decomposition (GenBank no. 3059185) were relatively abundant among all the soil samples. Genes detected in site M-Y more closely clustered with site M-60 than with sites M-C and M-80, suggesting that the community structure in terms of the organic carbon decomposition components in site M-Y soil was most similar to that of site M-60 site.

Discussion

Soils with different land use/covers have been shown to diverge markedly in their soil organic carbon and total nitrogen (Ross *et al.*, 1999). We observed a similar pattern in our study and found that soil from cropland had lower organic carbon content than the other three sites with different histories. The impact of land use/cover changes on soil properties and soil organic matter content was consistent with other studies focusing on the upper soil layer (Ross *et al.*, 1999; Scott *et al.*, 1999; Murty *et al.*, 2002). The changes that occur in soil composition after land use/cover alterations often lead to changes in microbial biomass and activity which in turn can significantly affect atmospheric

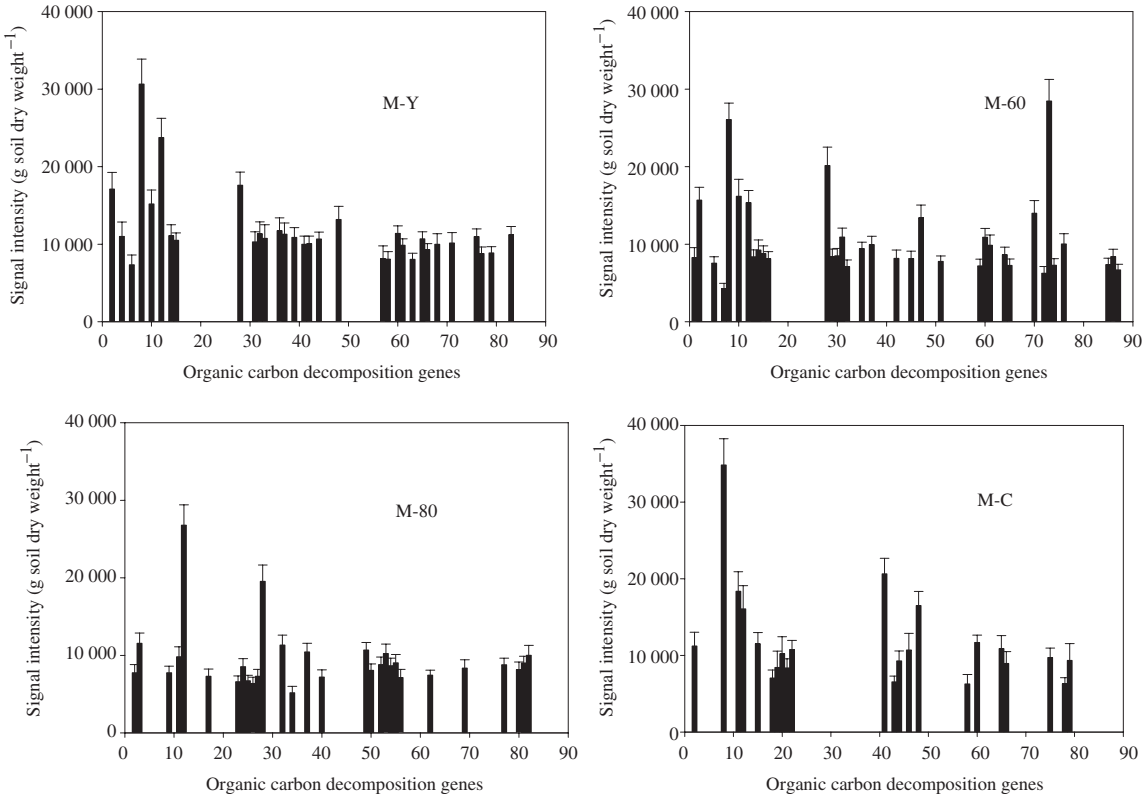

Fig. 1. The normalized distribution of signal intensity levels for organic carbon decomposition genes as determined by microarray-based analysis of community DNA from M-C, M-60, M-80, and M-C soil environments. The data represent mean values obtained from six replicates after subtracting background hybridization signals. Bars represent the functional gene involved in organic carbon decomposition. Bar 1 represent alkane, bar 2 represent aniline, bars 3–8 represent benzoate, bars 9–16 represent biphenyl, bars 17–38 represent catechol, bars 39–42 represent chitinase, bars 43 represent chlorobenzene, bars 44–47 represent dibenzofuran, bar 48 represent dioxin, bars 49–59 represent endoglucan, bar 60 represent fluorene, bar 61 represent hydroxybenzoate, bar 62 represent propionat, bars 63–67 represent lignin, bar 68 represent methane, bar 69 represent methyl, bars 70–73 represent naphthalene, bar 74 represent phenol, bar 75 represent phenol-TCE, bar 76 represent phosphite, bar 77 represent salicylate, bars 78 and 79 represent toluene, bars 80 and 81 represent toluenesulfonate, bars 82–87 represent xylanse.

CO_2 concentrations (Dhillion, 1997; Kandeler *et al.*, 1999; Wang *et al.*, 2003).

Community genomic DNA was isolated from the upper soil layer in the Miyaluo subalpine forest to assess the impact land use/cover changes have on microbial metabolic potential of organic carbon decomposition bacterial communities. Our experimental results showed that land use/cover changes influenced hybridization signal intensity and functional gene diversity. A total of 170 functional gene probes distributed broadly in six functional groups were detected in the 4 sites. However, the observed numbers and groups of the functional gene probes detected differed among the sites. Although there was not a significant relationship between soil organic carbon and functional gene distribution, the general trend showed an increase in gene number and gene diversity index (H′) with an increase in soil organic carbon content, except in the primitive fir forest. Land use/cover affected community structure and functional microbial diversity. Differences in the composition of organic matter and available substrates are likely to explain the differences in microbial community structure observed in the present study (Lettau & Kuzyakov, 1999). Differences in microbial community composition and functional gene diversity influence the soil cycle and respiration at these sites. It must be noted that, in the current study, the use of H′ was not intended to describe the total diversity of the sites, but merely to provide an indicator for comparing changes in the structure of the microbial community (William & Ronald, 2002).

In our study, 121 gene probes related to organic carbon decomposition were detected in all sites. These genes are involved in the decomposition of soil lignin, endoglucan, chinitase, xylanse, etc.. The gene for conversion of aniline to catechol originally found in *Pseudomonas putida* and the gene associated with biphenyl decomposition in an indigenous plasmid of *Rhodococcus erythropolis* were detected among all the soil samples. Thus, the *Pseudomonas putida*-type and *Rhodococcus erythropolis*-type microorganisms

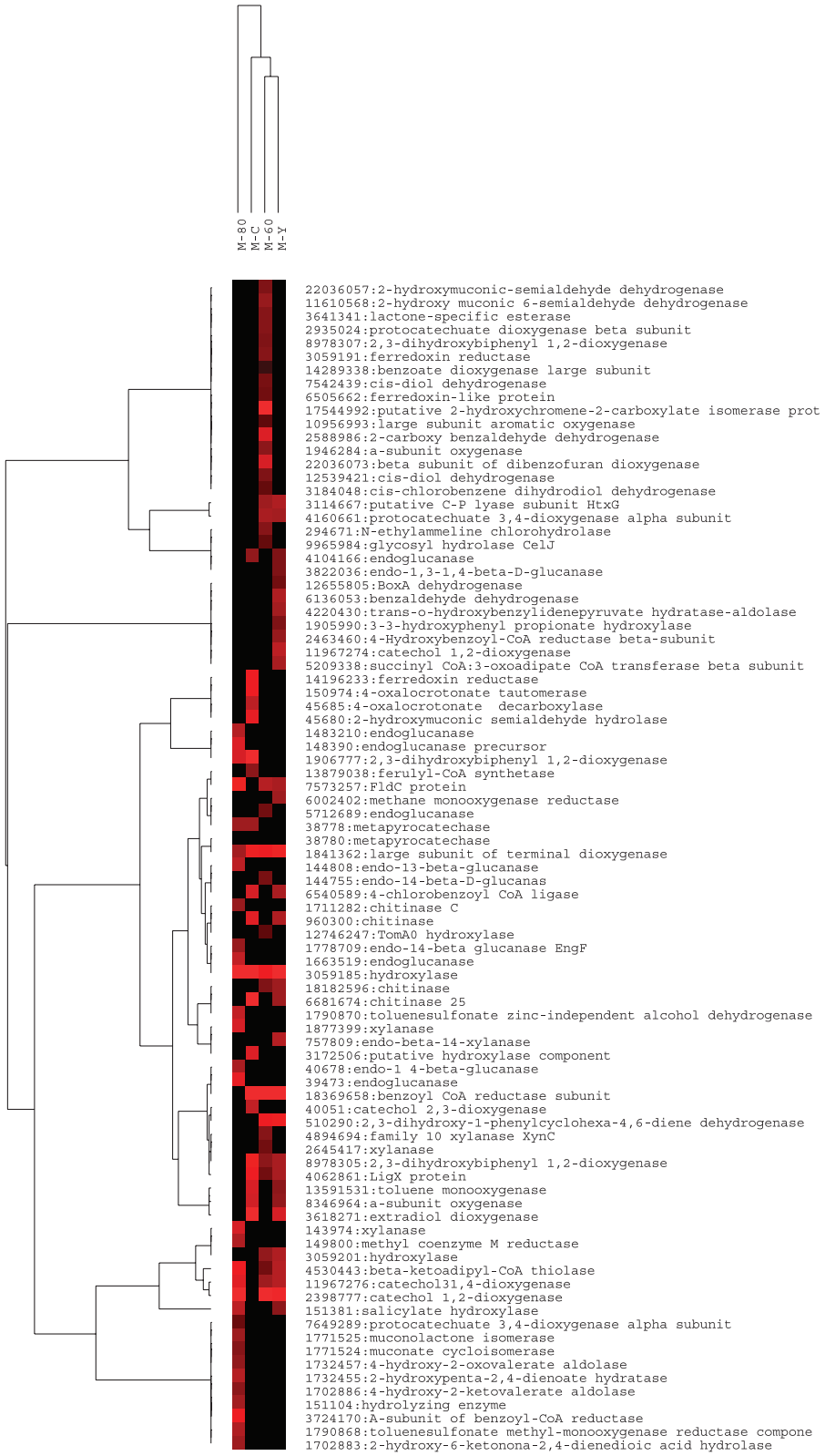

Fig. 2. Hierarchical cluster analysis of community relationships of organic carbon decomposition genes based on hybridization signal intensity ratios for genes showing SNRs ≥ 2. The figure was generated using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW. The hybridization signals of genomic DNA from each of the four land use soils were divided by the sum hybridization signals from corresponding genomic DNA. Microarray hybridization patterns with the labeled genomic DNA from the soils are shown in each column. Each row represents hybridization signal observed for each gene when the genomic DNA from the soil indicated above the column was used for hybridization. Black represents no detected the hybridization signal, while red represents a significant hybridization signal.

might be actively involved in organic carbon decomposition sites with different land use/cover. However, the individual gene orthologs associated with organic carbon decomposition were abundant and diverse. For example, the gene related to the benzoate decomposition originally found in *Azoarcus evansii* was the most abundant in sites M-Y and M-C, and second most abundant in site M-60. The gene of putative 2-hydroxychromene-2-carboxylate isomerase of *Ralstonic solanacearum* was only observed in site M-60 and was most abundant among all the detected genes. Therefore, the *Azoarcus evansii*-type, *Ralstonic solanacearum*-type and *Rhodococcus erythropolis*-type microorganisms may be the most active microbial groups involved in organic carbon decomposition in sites M-Y, M-C, M-60, and M-80, respectively. Thus, the microbial communities involved in organic carbon decomposition were different in these four land use/cover soil samples. The influence of land use/cover changes on soil microbial community structure and microorganisms involved in organic carbon decomposition are likely to strongly affect the organic carbon dynamics and increasing atmospheric CO_2 concentrations.

Altogether, the oligonucleotide array developed in this study contained 1961 unique probes to target a variety of genes involved in the decomposition of organic carbon and nitrogen cycling and others. To our knowledge, this is one of the most comprehensive arrays containing organic carbon decomposition genes available for environmental studies. In addition, the probes should be a good representation of known microbial-gene diversity involved in matter decomposition because the probes were designed based on the most recent data available in GenBank. The developed microarray is a useful tool for monitoring the composition, structure, activity, and dynamics of microbial populations involved in organic carbon decomposition across different environments. In addition, the arrays contain probes from different steps of the catabolic pathways involved in organic carbon decomposition. The developed arrays should be useful for assessing carbon decomposition populations in a variety of environments. However, the probes on arrays may not represent the indigenous microbial communities present in sites since the majority of microorganisms present in natural environments have not been cultivated. Additional studies that increase our understanding of the genetic diversity of decomposition pathways in different environments are needed.

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