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Plant n-alkane production from litterfall altered the diversity and community structure of alkane degrading bacteria in litter layer in lowland subtropical rainforest in Taiwan

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10 **Abstract.** n-Alkane and alkane-degrading bacteria have long been used as important biological indicators in paleoecology, petroleum pollution and oil and gas prospecting. However, their relationships in natural forests are still poorly understood. In this study, long chain n-alkane (C_{14}-C_{35}) levels of plants in litterfall, litter layer and topsoil and the diversity and abundance of n-alkane-degrading bacterial community in litter layer were investigated in 3 habitats across a lowland subtropical rainforest in southern Taiwan, i.e. the ravine habitat, the windward habitat and leeward habitat in Nanjenshan. Our results
15 demonstrated that the litterfall production and flux of long chain n-alkane in ravine area were highest among all habitats. However, long chain n-alkane concentration formed a steep gradient to a similar level from the litterfall to the bulk soil in all habitats, suggesting a higher degrading rate of long chain n-alkane in ravine habitat. The operational taxonomic unit (OTU) analysis from next generation sequencing data revealed that the relative abundance of microbial communities in windward and leeward habitats were similar to each other and different from ravine habitat. Metagenomic data mining by NCBI
20 database revealed that alkB gene associated bacterial (95% similarity to alkB contained bacteria in DNA sequence) were highest in ravine area compared to other habitats. Empirical testing of litter-layer samples by semi-quantitative PCR in alkB gene levels confirmed that ravine habitat had higher alkB gene levels than windward and leeward habitats. Heat map analysis revealed a parallel in the color pattern between plant vegetation and microbial species-composition of habitats, suggesting a causal relationship between the plant n-alkane production and the diversity of microbial communities. This
25 finding indicated that the diversity and relative abundance of microbial communities in litter layer were affected by the n-alkane composition in litterfall derived by plant vegetation.

Introduction

Alkanes are saturated hydrocarbons that accounted for small fraction of total organic carbon in natural habitats. In non-
30 contaminated habitats, alkanes are produced by plants or algae as chemo-attractants or as protecting agents against microbial invasion or water loss (Feakins et al., 2016; Koch et al., 2009). Alkanes are especially stable molecules that can survive in

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soils and sediments, which made them useful as biomarkers in paleoecology (Sachse et al., 2012). Since alkanes are the major component in petroleum, nature gas, and diesel fuel, the existence of alkanes in environmental was also served as an index of oil contamination (Afzal et al., 2013). Although alkanes are very inactive, most of them can be degraded by several microorganisms. As alkane-degrading bacteria are commonly up-regulated by the increases of alkanes, they have been suggested for using as a biological indicator for oil and gas prospecting (Xu et al., 2013; Rasheed et al., 2012).

Extensive knowledge on the degrading of n-alkane by microorganisms has been accumulated during the past decades. The first step of n-alkane oxidization is the catabolizing to the corresponding primary alcohol by alkane terminal hydroxylases. Despite that some of the alkane-degraders of algae and fungi have been discovered, bacteria are one of the most important microorganisms in long chain n-alkane degradations (Rojo, 2010; Rojo, 2009; Singh et al., 2012). Although bacteria can degrade alkanes under aerobic or anaerobic conditions, the decomposition of leaves in litter layer probably dominated by aerobic conditions (Wentzel et al., 2007). The alkane monooxygenase gene (alkB) encoded membrane-bound homologous protein is an inducible integral membrane-bound alkane hydroxylase that play an important role in aerobic alkane degradation (Beilen et al., 2003; Nie et al., 2014). Studies have showed that the level of alkB bacteria are highly dynamic in response to the fluctuation of environmental alkanes. In soil or water environments when polluted with oil, the alkane-degrading bacteria were up-regulated (Afzal et al., 2013). Besides, in oil and gas reservoirs, it has been showed that the abundance of alkB bacteria increased substantially in the surface soils with no significant detection of soil alkane (Xu et al., 2013). The dynamic relationship of alkane and alkB bacteria in soil from agriculture research farm or in lab under controlled conditions has been carefully studied before. Giebler et al. has showed that in controlled environment sizeable alkane-degrading bacteria were detected from soil-litter interface when enriched with artificial alkane supplements, especially in Proteobacteria and Actinobacteria (Giebler et al., 2013). They suggested that some alkane degradation bacteria in soil might represent as a seed bank and could grow opportunistically. Since substrate can selectively grow certain microorganisms and control the biomass based on its initial concentration (Schmidt, 1992), it is reasonable to assume that litterfall might up-regulate the levels of alkB degrading bacteria. The relationship of abundance of alkB bacteria and alkane substrate are more complicated in natural habitats. It has been showed that the dynamic changes of alkB degrading bacteria were also driven by many factors such as different sources of alkane and soil type (Schulz et al., 2012). Although the knowledge of the regulatory mechanism of environmental plant litter on soil bacterial levels and alkane-degrading bacterial dynamic in natural habitats has been very useful and important in many aspects, researches on their dynamic changes are largely lacking currently and should be established.

The Nanjenshan Reserve, a lowland subtropical rainforest in southern Taiwan, consists of several forest dynamics plots where the data of forest structure, vegetation pattern, climatic and topography in permanent study sites have been well established. Up to date, four forest dynamics plots and a transect zone in the habitat have been documented since 1989 (Chao et al., 2010; Chao et al., 2007; Chao et al., 2008; Fan et al., 2005; Hsieh et al., 2000; Tsui et al., 2004). Surveys conducted from past decade in the area have shown that the annual amount of litterfall in ravine habitat where located across plot I (120º 50’ 51” E, 22º 04’ 54” N) and plot II (120º 50’ 36” E, 22º 04’ 52” N) was higher than in windward and leeward

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habitats where both located in Lanjenchi plot (120º 51' 38" E, 22º 03' 23" N). The alkane distributions and concurrent alkane-degrading bacteria have not been uncovered yet. Since the litterfall in those habitats was different, it was applicable to investigate the effects of litterfall on the corresponding level of microbial community in these habitats.

Many environmental microbial communities are highly complex and diverse and recalcitrant to culture under laboratory conditions. With the recent advent of next generation sequencing (NGS) technology and computational methods, we can conduct genome studies of microbes on these habitats that were known to have many variables such as annual litterfall productivity and vegetation (Degnan and Ochman, 2012). We aimed to investigate the flux of long chain n-alkane in the pristine natural habitats of Nanjenshan Reserve, and explore their relationship with microbial communities, particularly emphasizing the correlation between n-alkane production and bacteria contained alkB gene. In this one-year follow-up study, GC-FID was employed for determination of n-alkane (C14-C35) concentration. The bioinformatics analyses and metagenomic data mining were carried out to reveal the microbial communities and the numbers of read of bacterial lineages that carried alkB genes in the three habits of rainforest. Semi-quantitative PCR was performed to test the results of bioinformatics analysis.

2 Materials and methods

2.1 Sample description

Twenty-two custom-made aluminum stands with frame (0.71 m × 0.71 m) that covered by nylon mesh (1 mm mesh) were used to collect litter-fall in this study. Twelve stands were located at ravine habitat, while 10 stands were built at Lanjenchi plot. Among Lanjenchi plot, six are in windward habitat, whereas 4 are in leeward habitat. Each nylon mesh with litter-fall was collected and replaced with new one monthly form early October 2012 to late September 2013. After the collection of litterfall samples, they were sorted out into litter-leaf, litter-branches, litter-flowers, litter-fruits, and miscellaneous fractions. Sorted samples were weighted after oven-dried at 40°C for 14 days. The litter-leaf were further identified into genus and species. Samples of litter-layer and soils from the three habitats were collected in 2015 and 2016, respectively. All collected samples, including litter-leaf, litter-layer, and soils, were subjected to n-alkane analysis and litter-layer samples in each habitat were further used for the DNA extraction and consequential assays.

The monthly yields of litterfall in each habitat was calculated by dividing the total weighs of litterfall collected monthly in mesh nets by the area of the frame. Calculation of the annual yields of litterfall in each habitat was done by averaging the monthly yields of litterfall calculated.

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2.2 Soxhlet extraction of n-alkanes

All samples from litter-leaf (42 species plants), litter-lay, and soils are dried and well homogenized. Approximately 0.5 g of powder samples were dissolved in dicholromethane (250 ml) and Soxhlet extracted for 16 hours. A surrogate standard of 5a-cholestane (Sigma) was added to each sample before extraction. The extracts were concentrated to the volumes of 5-10 ml by a rotary evaporator with water bath and further concentrated to reduce in volume to approximately 1 ml and were fractionated by silica gel chromatography (Silica gel 60, 3%). The n-alkane fraction was eluted with 4 ml hexane and reduced volume to 1 ml under a stream of nitrogen. An internal standard of squalene (Acros) was added to the concentrated 1-ml hexane fractions prior to instrumental Gas Chromatography (GC) analysis. Quantification of the n-alkanes was performed on a PerkinElmer Clarus 500 gas chromatograph equipped with an autosampler, PerkinElmer Elite-5 CB fused silica capillary column (30 m length, 0.32 mm i.d., film thickness 0.25 μm), and flame ionization detector (FID). The GC oven temperature was programmed from 70 °C to 310 °C at the rate of 4°C/min. The n-alkanes were identified and quantified by calibration standards that contained known concentrations of 5a-cholestane, squalene, and all of the n-alkanes (Dr. Ehrenstorfer) of interest in this study. The concentrations reported in this study were not corrected by the recoveries of the standards.

The measured n-alkane concentrations of the 42 litter-leaf species were further used to estimate the n-alkane concentrations and fluxes of litter-leaf in the three habitats. The average of n-alkane concentrations of litter-leaf in a habitat were summed up the weighting n-alkane concentrations of each litter-leaf species by their ratios of the litter-leaf yield collected to the total litter-leaf yield collected in a habitat. The monthly average fluxes were the products of the estimated average monthly n-alkane concentrations of litter-leaf and the monthly yield of litter-leaf in the habitat. Calculation of the annual n-alkane of litter-leaf flux in each habitat was done by averaging the monthly average n-alkane fluxes calculated of litter-leaf.

2.3 DNA extraction

Leaves of litter-layer were cut into pieces for extracting the DNA of bacteria. The procedure for DNA extracting was described in detail in the user manual Genomic DNA from soil (Macherey-Nagel). Briefly, a bulk of leaf fraction (0.1-0.2 g approximately) was transferred into tube containing the ceramic beads. Lysis buffer (lysis buffer SL1, 700 μl) and enhancer SX (150 μl) were added to each sample and subjected to vortex for 5 min at room temperature. Precipitate contaminants were removed by centrifuge for 2 min at 11,000 g. Supernatant with DNA was transferred to a new tube, added with 150 μl SL3 and subjected to vortex for 5 sec prior to the precipitation by centrifuge for 1 min at 11,000 g. The inhibitors in supernatant were removed by inhibitor removal column. The column that bound with DNA was washed four times and dried out before DNA elution. DNA elution was performed by centrifuging for 30 secs at 11,000 × g after the incubation with Buffer SE (50 μl).

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2.4 Sequencing via an Illumina MiSeq platform

We carry out PCR amplification of 16S rRNA gene sequences at V3-V4 regions using Illumina's MiSeq system to create paired-end sequencing data. The experimental protocol was modified from the Illumina manual. The target sequence was amplified by PCR using mixed forward and reverse primers. The sequences of forward primers are: 5'-TCGTC GGCAG CGTCA GATGT GTATA AGAGA CAGCC TACGG GNGGC WGCAG, 5'-TCGTC GGCAG CGTCA GATGT GTATA AGAGA CAGAC CTACG GGNGG CWGCA G, 5'-TCGTC GGCAG CGTCA GATGT GTATA AGAGA CAGTD CCTAC GGGNG GCWGC AG, and 5'-TCGTC GGCAG CGTCA GATGT GTATA AGAGA CAGGD RCCTA CGGGN GGCWG CAG. The sequences of reverse primers are: 5'-GTCTC GTGGG CTCGG AGATG TGTAT AAGAG ACAGG ACTAC HVGGG TATCT AATCC, 5'-GTCTC GTGGG CTCGG AGATG TGTAT AAGAG ACAGT GACTA CHVGG GTATC TAATC C, 5'-GTCTC GTGGG CTCGG AGATG TGTAT AAGAG ACAGA CGACT ACHVG GGTAT CTAAT CC, and 5'-GTCTC GTGGG CTCGG AGATG TGTAT AAGAG ACAGG TTGAC TACHV GGGTA TCTAA TCC. After separation by electrophoresis in agarose gel, PCR products with expected sizes were purified from the matrix. Illumina Nextera XT index kit was used in the second-stage PCR for addition of the index. Capillary electrophoresis followed by a fluorescence-based method were employed to qualify and quantify the libraries respectively. Nucleotides will be sequenced by Miseq sequencer for 18 dark cycles and 350 read cycles in the forward read and 18 dark cycles and 250 read cycles in the reverse read. The data of forward and reverse reads were aligned using CLC bio's analysis platform (Genomic Workbench v.8.5) with Q20 as a threshold to generate output fasta files.

2.5 Metagenomics library construction and analysis

Fasta files were further processed by sequence analysis tool USEARCH. We merged all sequence fills together, removed duplicates, and clustered the sequences into operational taxonomic units (OTUs) at 97% pairwise identity with a minimum cluster size of 2 to construct a OTU-reference library. Then, a comparison between samples and the reference library at a level of 97% sequence identity was made to create a OTUs table, which contained the numbers of DNA sequence read of each OTU. A 16s UTAX reference database was employed as blast (Basic Local Alignment Search Tool) library. Finally, these data were combined together to determine the relative abundances and subsequent visual patterns of heat map. Principal coordinate analysis (PCoA) was used in this study for knowing the relative distance between OTUs. For plotting the PCoA figures, a calc_distmx command together with a phylip_lower_triangular parameter was used to calculate the distance of OTUs. The axes file contains the plotting coordinates for graphing the results was derived from mothur with PCoA command. The PCoA figures were plotted by R.

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2.6 Estimation of the portion of alkB genes contained bacteria in the habitats by NCBI database

Metagenomic data mining was carried out to look for the microbial communities which might contain alkB gene. Since the bacteria in the same genus may contain similar gene sequences and functions, nucleotide blasting was carried out to search for the homogeneous sequences in OTUs. We defined the lineage-like bacteria of a known bacterium were the microbes which the DNA sequences were 95% or more similar to a known gene. NCBI database was used to make an alkB gene reference library. To investigate the populations of alkB gene family in different habitats, we first collected the representative 16s gene sequences of all known alkB contained bacteria in phyla Actinobacteria and Proteobacteria from currently NCBI database. The alkB gene sequences in 16s rRNA of bacteria were downloaded to a reference-nucleotide file and combined with the OTUs library of Actinobacteria and Proteobacteria.

To search the alkB family gene-like lineage from current OTUs database, sequence alignment and phylogenetic analysis of alkB nucleotide sequence were conducted using the Molecular Evolutionary Genetics Analysis 7 (MAGA 7) program. The MAGA 7 using parsimony, neighbor joining, and maximum likelihood analyses was used to create 16S rRNA gene phylogenetic tree. The nucleotide blast program of NCBI was carried out to test the similarity of DNA sequences between OTUs and adjacent references. DNA sequences with similarity more than 95% were manually selected to determine the numbers of DNA sequence read of alkB-lineage OTUs in the library. The Shannon-Wiener diversity index was used in order to calculate the diversity of alkB-lineage bacteria and it was generated in PRIMER-5 software (Plymouth Routines in Multivariate Ecological Research).

2.7 Semi-quantitative PCR for alkB gene level from litter-layer samples

Since alkB was highly abundant and diverse, a standard method for gene quantification was not available. We employed semi-quantitative PCR for measuring environmental alkB modified from previous studies since the alkB-targeting primer had coverage among the bacterial strains of Proteobacteria and Actinobacteria (Jurelevicius et al., 2013; Kloos et al., 2006). Litter-layer samples from 3 habitats in Nanjenshan were subjected to semi-quantitative PCR study for quantifying the DNA levels of alkB genes in natural habitats. Following DNA extraction of litter-layer samples, DNA eluate (2μl) were mixed with alkB gene primer set (forward primer: 5'- AAY ACN GCN CAY GAR CTN GGN CAY AA -3', reverse primer: 5'-GCR TGR TGR TCN GAR TGN CGY TG -3' ,1μl, 0.4μM), 5μl Fast-Run Taq Master Mix with Dye, and 16μl ddH2O with the final reaction volume of 25μl. Thermal cycling conditions for PCR included an initial temperature of 95℃ for 5 min and followed by 30, 27, or 24 cycles of 95℃ for 30 sec, 55℃ for 30 sec, 72℃ for 1 min and finial of 72℃ for 5 min. The expected amplicons of the PCR are 548 bps. Following PCR, aliquots (5μl) of each amplicon were confirmed by electrophoresis on a 1.5% agarose gel followed by 0.5μg/mL SYBR Safe DNA staining, and imaged by ultraviolet trans illumination system.

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3 Results and Discussion

3.1 Quantitative estimates of Annual litterfall from the gathering in Nanjenshan Reserve

The estimation of annual average litterfall was calculated from the litterfall gathering at sampling sites. The annual yields of
5 litterfall from 3 habitats in Nanjenshan was showed in figure 1. As showed on the records of the past decade, the litterfall was higher in ravine habitat than in the other habitats. Our results on the litterfall of Nanjenshan have indicated the annual productivity of weight in ravine habitat was higher than in leeward or windward habitat, which is consistent with previous studies. According to records from 1999 to 2007, seasonal litterfall in Nanjenshan was ranged from 2 to 7 ton/ha, which was affected by typhoon, precipitation, and monsoon seasons. In this study, the litterfall was about 7 to 10 ton/ha, which was due
10 to the effects of typhoon. Still, the change of weather did not affect the order of litterfall output in the habitats. One of the reasons may lie on the topography of ravine habitat which was more suitable for the growth of plants. In fact, the average height and diameter of cross section of plants in ravine habitat were highest. Since leaves were the major parts of the litterfall in weight and in the average n-alkane concentration levels, we focused on the n-alkane levels of leaves in these habitats.

15
3-2 Typical example of n-alkanes measurement in Nanjenshan Reserve

The n-alkane concentration in leaves from various kind of plants that harvested in this study were assayed. Figure 2 showed a representative GC traces in leaves of *Bischofia javaniva*, *Cyclobalanopsis championii*, and *Lithocarpus amygdalifolius*, which were the most dominant tree species regarding the contribution of litterfall amount in ravine, windward and leeward habitats respectively. The recovery rate of the experiment was determined to be 99.3 ± 6.9%. All samples exhibited an odd-
20 even odd/even carbon-number predominance, with maximum at C_{31}, C_{29} and C_{33}. The distribution of aliphatic fraction showed the major n-alkanes components were from n-C_{27} to n-C_{25} with a distinctive n-alkane distribution pattern of leaves in each species. For example, the ratio of C_{29}/C_{33} was different in all three plants. N-alkane of C_{14} to C_{26} was only present in relatively lower amount. This study was the first to reveal the n-alkane levels of plants in Nanjenshan. More analysis was need to impose on the relationship between plant species and n-alkane distribution pattern.

25

3-3 Annual Plant leaf productivity and their n-alkane productivity

A total of forty-two plant species were identified in this study. Data of the list of plant species, the total amount of annual leaf in litterfall, and the ranking order of leaf n-alkane concentration of these plants were given in table 1. The data of leaf n-alkane concentration and dry weight were employed to make a gross estimate of the n-alkane flux of litterfall in Nanjenshan
30 Reserve. It has been showed that the n-alkane concentration of leaves was dynamic during leaf development and was

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affected by environmental parameters (Kahmen et al., 2011; Jetter and ̈ffer, 2001; Hoffmann et al., 2013). Since old leaves were affected less by environment variable, the internal variations within species might be slighter in leaves of litterfall. Besides, the leaves were gathered from habitats in the same area in a geographic scale, thus the differences of climate and geography were hardly to remarkably affect the n-alkane concentration of leaves within samples of the same species. In this
5 study, the ranges of leaf n-alkane concentration between species were from 3000 (μg /g) to 200 (μg /g). We assume that the variable of n-alkane concentration within species can be ignored when compared with the variable between species. Interestingly, the leaves of plants which were of high leaf n-alkane concentration tended to grow at ravine habitat, such as *Ficus benjamina L* and *Iles rotunda*, to name a few.

10

3-4 Estimates of annual n-alkane productivity in three habitats

Figure 3 showed the results of n-alkane concentrations from litterfall to bulk soil (fig 3A) and average n-alkane flux of litterfall in the three habitats (fig 3B). The average n-alkane concentration of litterfall was highest in ravine area when compared with other habitats. Besides, the n-alkane concentrations of different layer formed a steep gradient from the
15 litterfall layer to the bulk soil. The n-alkane flux of litterfall in ravine habitat was about twice as much as other habitats (fig 3B). We concluded that the ravine habitat has higher n-alkane input than the other habitats. This character offered us an example of higher n-alkane input in the natural habitat.

Several effects such as decomposition, erosion and sediment transportation might play roles to govern the organic resource such as n-alkane (Kirkels et al., 2014; Quinton et al., 2006). A study in these habitats has shown that the total organic carbon
20 of litter layer and bulk soil in the ravine habitat was equal or lower than in the windward and leeward habitats (Kuo, 2010). Those evidences did not favor the possibility of high mineralization of hydrocarbon in ravine habitats. Microorganisms were known to play many key roles in degrading the plant substratum. We carried out NGS and bioinformatics studies to unearth the roles of microbial communities on the degradation of n-alkane.

25 **3-5 Bacterial composition in Nanjenshan Reserve**

NGS was carried out to reveal the microbial community of litter layer from 3 habitats. Figure 4 showed the relative abundance of Metagenomic data of OTUs that grouped in phylum. Interestingly, the microbial communities in windward and leeward habitats were similar to each other. Since the windward and leeward habitats were both located in the same plot of different orography, several parameters such as plant vegetation and soil properties were also similar between both habitats.
30 The most significant differences between windward and leeward habitats were the average height of plants and number of trees per hectare. Study has showed that in the leeward habitat, the height of plants was higher and the plant density was

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lower, when compared with the windward habitat (8.41±1.73 meter vs. 4.63±0.88 meter; 7,505 tree/ha vs. 20,065 tree/ha). The plant height and density in the ravine habitat were 9.45±1.35 meter and 4257 tree/ ha (Chin, 2008). Apparently, physical parameters that affected the growth of plants in mountain area didn't affect the relative abundance of microbial communities. On the other hand, the microbial relative abundance in ravine habitat was different from windward and leeward habitats, with more members in phyla of *Actinobacteria* and less members in *Proteobacteria* and *Acidobacteria*. The up-regulation of might be due to the effects of increasing n-alkane input in the ravine habitat. Although many phyla have been identified to carry alkB gene, our data revealed that the microbial communities which related to alkB gene were *Proteobacteria*, and *Actinobacteria*.

3.6 Prediction of bacteria carrying alkB gene in Nanjenshan Reserve

The relative abundance of bacteria contained AlkB gene in phyla *Actinobacteria* and *Proteobacteria* from different habitats were shown in figure 5. The relative abundance of DNA sequence read in OTUs of alkB lineage-like bacteria in phylum *Actinobacteria* were limited in leeward and windward habitats. On the other hand, a significant amount of sequence read in OTUs of that kind was found in the ravine habitat. Although the relative abundance of *Proteobacteria* in the ravine habitat was 20% less than the other habitats, the relative abundance of DNA sequence read of alkB lineage-like bacteria in the ravine habitat were more than 1.5 times higher than the windward and leeward areas. To sum up, the DNA sequence read of alkB lineage-like bacteria in the ravine habitat were twice higher than the other habitats.

3.7 The principal coordinate analysis of bacteria that carried alkB genes

PCoA was used to visualize the similarities of DNA sequences in *Proteobacteria* and *Actinobacteria* from 3 habitats. Figure 6 showed the distribution of a total of 240 OTUs in phyla *Proteobacteria* and *Actinobacteria*. The phylum *Actinobacteria* was circled in pink except the one in blue while the OTUs not included in the red circle were belong to phylum *Proteobacteria*. At most OTUs (>90%), sequences can be found from 2 or 3 habitats. An initial capital letter was given to denote OTUs according to the largest source in read numbers of the habitats. The nomenclature of OTUs of alkB lineage-like bacteria were showed on the figure. There were 34 OTUs that contained alkB lineage-like bacteria. Around 70 percent of the OTUs of them were clustered together at location A, B and C. The corresponding numbers of OTUs that contained alkB lineage-like bacteria in ravine, windward, and leeward habitat were 31, 31, 26. The Shannon-Weiner index in OTUs that contained alkB-like bacteria in ravine, windward, and leeward habitats were 2.55, 2.39 and 2.46 respectively. Although some nomenclature of OTUs were given by a specific genus or species; such as, *alpha-Proteobacteria* in location A, *Stenotrophomonas* in location B, and *Mycobacterium* in location C to name a few, most of nomenclature of OTUs were

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pointed to uncultured bacterium clones. We concluded that the diversity of OTUs associated with alkB lineage-like were greater in ravine habitat when compared with windward and leeward habitat.

3.8 Empirical testing of bacteria carrying alkB gene

5 We carried out semi-quantity and heat map analysis of annual litterfall yield of individual species in each habitat to explore the relationship between microbial communities and plant vegetation patterns. The study of semi-quantitative PCR in alkB gene was carried out to verify the alkB gene numbers in those habitats. Figure 7A showed a triplicate study of DNA staining in amplicons from the three habitats after various semi-quantitative PCR cycles. No bands were detected if the cycles of amplification were less than 24. Marginal PCR products can be detected with increasing cycle number to 27. Figure 7B 10 showed the statistic results of alkB gene levels in the three habitats of Nanjenshan, which confirmed that the bacterial alkB numbers were higher in ravine habitat than the other two habitats. Alkane degradation bacteria were known to exist ubiquitously in natural habitats. We provided evidence that the communities of alkane-degrading bacteria could be upregulated by litterfall and plant vegetation in natural habitats.

15

3.9 Exploring the relationship of plant vegetation and microbial communities by heat map analysis

Heat map analysis of plant species and read-number of OTUs versus habitats were carried out to investigate the relationship between the plant vegetation patterns and microbial communities in different habitats. The map of figure 8 was based on the microbial community abundance (A) and plant productivity of litterfall (B) in different habitats, with the color intensity 20 indicating the numbers of read or weight of litterfall (in log). In figure 8B, the color patterns of litterfall levels of showed the microbial abundance are more similar to each other in windward and leeward habitats, indicating the similarity of composition of microbial communities in these habitats. Interestingly, the litterfall data were also presented in a parallel manner, suggesting an existence of a connection between plant species and microbial compositions. The similarity in the pattern of plant species and bacterial species can be easily explained by substrate-induced growth-response in nature habitats. 25 The data provided a supportive evidence that the diversity of bacteria in all OTUs were affected by the plant vegetation. A litter-bag study can be tested in future for leading us to a better understanding on the relationship between a specific leaf and the genus of n-alkane degradation bacteria or microbial community in general in different habitats.

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4 Conclusions

The bacteria contained alkB gene was known to play crucial roles in degrading n-alkane, which have been applied in many sectors such as soil remediation and oil gas prospecting. In this study, the effects of n-alkane in litterfall input on the abundance of n-alkane degrading bacterial community in litter layer in natural habitats were investigated using GC/FID and
5 NGS. We revealed that the plant vegetation not only affect the n-alkane input but also have profound impact to the microbial community of litter layer. In ravine habitat, high productivity of litterfall and n-alkane production in plants have rendered the changes of microbial community and up-regulation of n-alkane degrading bacterial in litter layer. Although alkB gene associated bacteria were identified in many phyla, they were primarily found in *Proteobacteria* and *Actinobacteria* in the lowland subtropical rainforest in Taiwan. Increasing of n-alkane input caused the rising of diversity and the shift of relative
10 abundance in *Actinobacteria* and *Proteobacteria*, which have been confirmed by NGS and semi-quantitative PCR for alkB gene level. To our knowledge, this is the first report on the regulation of n-alkane degrading bacteria by litterfall in rainforest.

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Ranking order of Leaf n-alkane concentration	Plant species	**Relative annual Leaf production levels (by dry weight)**		
		Ravine	Windward	Leeward
1	*Iles rotunda*	++++	++	+
2	*Ficus benjamina* L.	+++++		
3	*Ficus benjamina* L. f.	+++++	+	
4	*Diospyros eriantha*	++		++
5	*Reevesia formosana*	+++		
6	*Celastrus kusanoi*	+++++	+	
7	*Erycibe henryi*	+++		+
8	*Cyclobalanopsis longinux*	+	+	+++++
9	*Archidendron lucidum*		+++++	+++++
10	*Celtis sinensis* Pers.	+++		
11	*Vitis kelungensis* Moriyama	+	++++	++
12	*Ilex cochinchinensis*		+++	+++
13	*Rhaphiolepos indica*		+++++	+
14	*Castanopsis fabri Hance*		+	+++++
15	*Symplocos caudata* Wall.		++++	+
16	*Cryptocarya hainanensis*	++++	+	+
17	*Podocarpus macrophyllus*		+++++	+++
18	*Sapium discolor*	++	+	++++
19	*Illicium arborescens*	+	+++	+
20	*Syzygium formosanum*		+++++	
21	*Syzygium kusususense*	+	+++	+
22	*Machilus thunbergii*	+++++		
23	*Cyclobalanopsis pachyloma*	+	+++	+
24	*Lithocarpus formosanus*	+++++	+	+
25	*Bischofia javaniva*	++++	+	+
26	*Machilus zuihoensis*	++		+++++
27	*Ficus aurantiaca*	++++	+	
28	*Helicia formosana*	+++	++++	++++
29	*Cyclobalanopsis championii*		+++++	++
30	*Aglaia formosana*		+++	+++++
31	*Turpinia ternata*	+	++++	++++
32	*Aglaia elliptifolia*	++	++	+
33	*Castanopsis indica*	+++	+	+++
34	*Psychotria rubra*	++++	++++	+++
35	*Castanopsis carlesii*			+++
36	*Schima superba*	+	+	++
37	*Antidesma hiiranense*	+	++	+
38	*Lithocarpus amygdalifolius*	+	++	+
39	Schizostachyum diffusum	+	+	++++

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40	*Beilschmiedia fordii*	+	+	++++
41	*Beilschmiedia erythrophloia*	+	+	++
42	*Alniphyllum pterospermum*	++	++	+

Table 1: The n-alkane levels of leaves, plant nomenclatures and their ranking in order of annual production in its habitat. The leaf n-alkane concentrations in each species were presented in descending order. The number of plus sign characters (+) indicated the semi-quantitative score, which added information of the ranking in the order of annual litterfall yield of particular species in a habitat. Five plus sign (+++++) denoted the ranking number of 1to 5. That ranking of 6 to 10 denoted 4 plus sign (++++); that ranking of 11 to 15 denoted 3 plus sign (+++); that ranking of 16 to 20 denoted 2 plus sign (++); and that ranking after 21 denoted one plus sign (+). A blank space denoted none of leaf was harvest for entire year.

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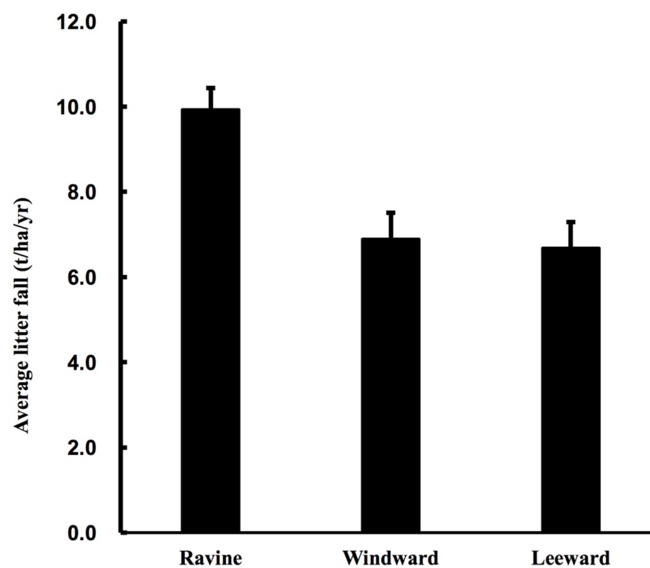

Figure 1. Annual litterfall in 3 habitats of Nanjenshan Reserve. Annual productions of litterfall in ravine habitat were higher than windward and leeward habitats ($p < 0.05$).

Figure 1

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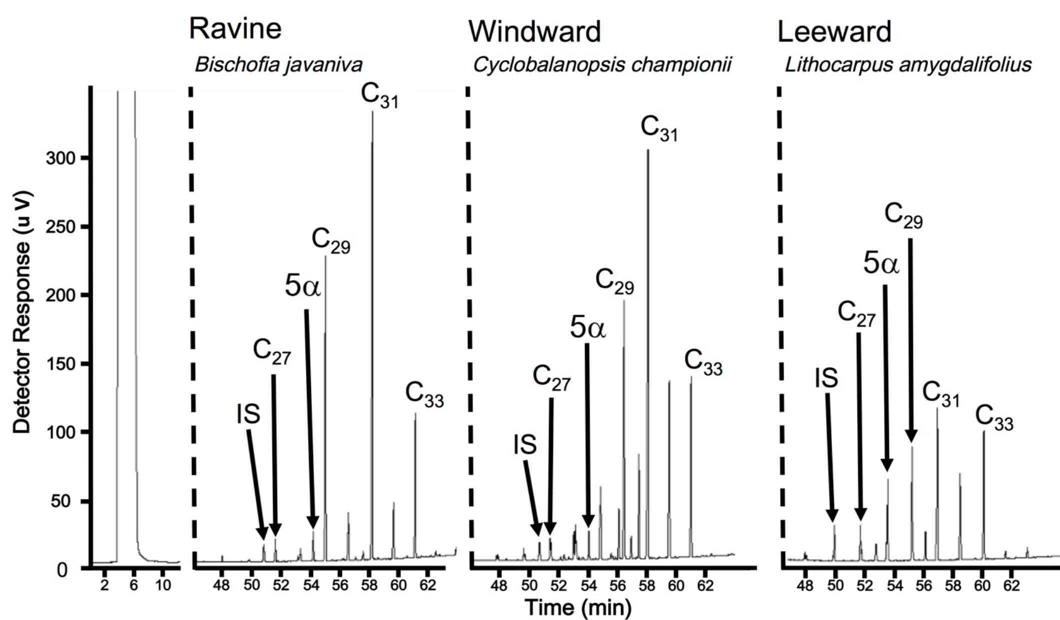

Figure 2. Representative GC-FID traces of aliphatic hydrocarbons of the dominated species in each habitat.

Figure 2

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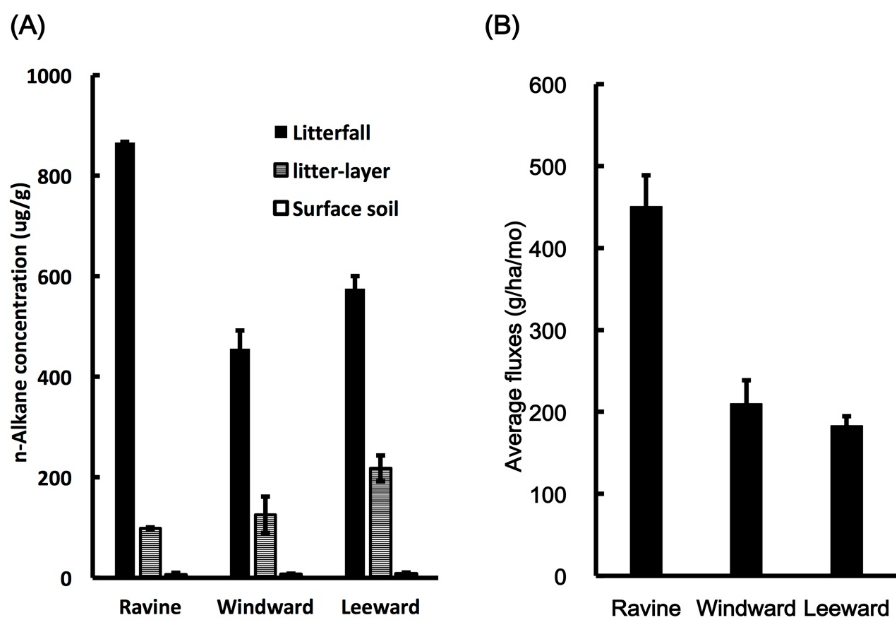

Figure 3. (A) Dynamic n-alkane level changes from litterfall, litter-layer to surface soil. (B) Estimated annual n-alkane flux generated by leaf of litterfall in 3 habitats.

Figure 3

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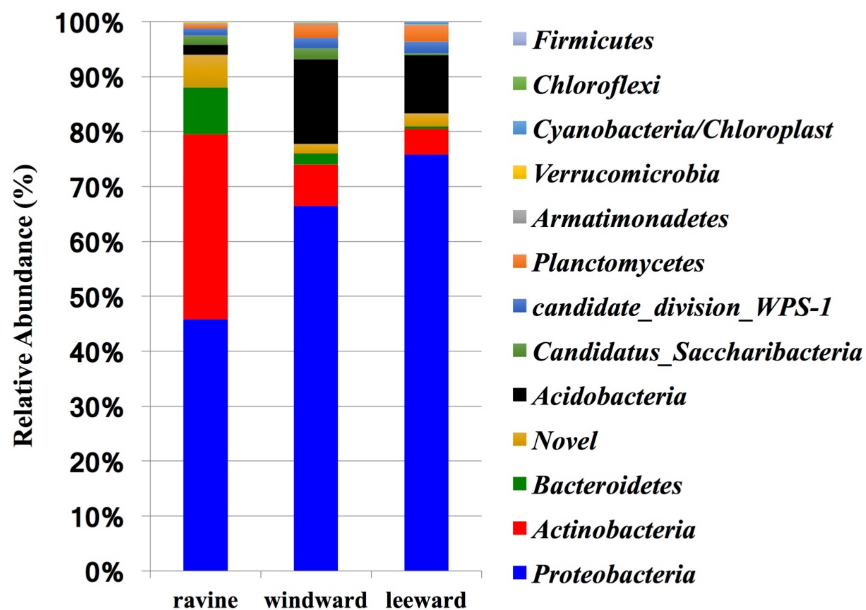

Figure 4. Microbial community structure in the three habitats of Nanjenshan Reserve. Bacterial lineages were indicated by phylum.

Figure 4

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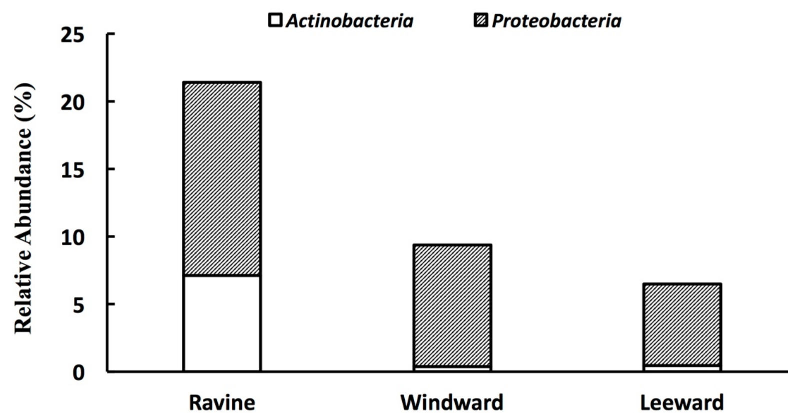

Figure 5 The relative abundance in microbial community of alkB gene-lineages. White block indicated the relative abundance of alkB gene in phylum *Actinobacteria* while the stripe block was in *Proteobacteria.*

Figure 5

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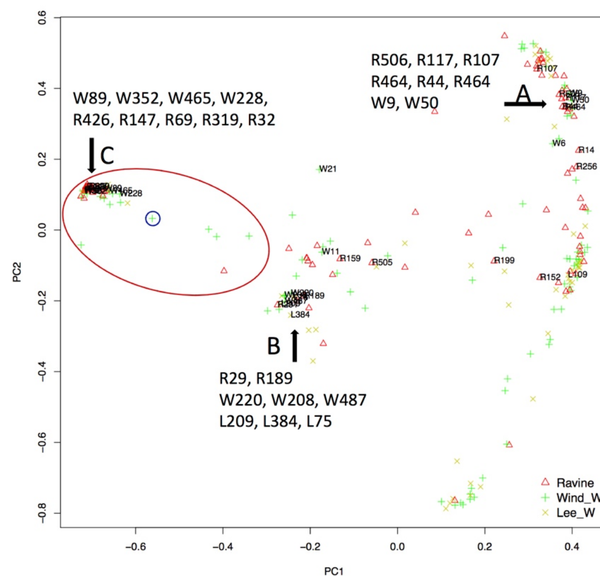

Figure 6. The PCoA plot of OTUs data in phylum *Actinobacteria* and *Proteobacteria*. The circle area in red was the phyla of *Actinobacteria* except the OTU with blue circle. The arrow bars indicated the OTUs of alkB gene lineage which clustered together.

Figure 6

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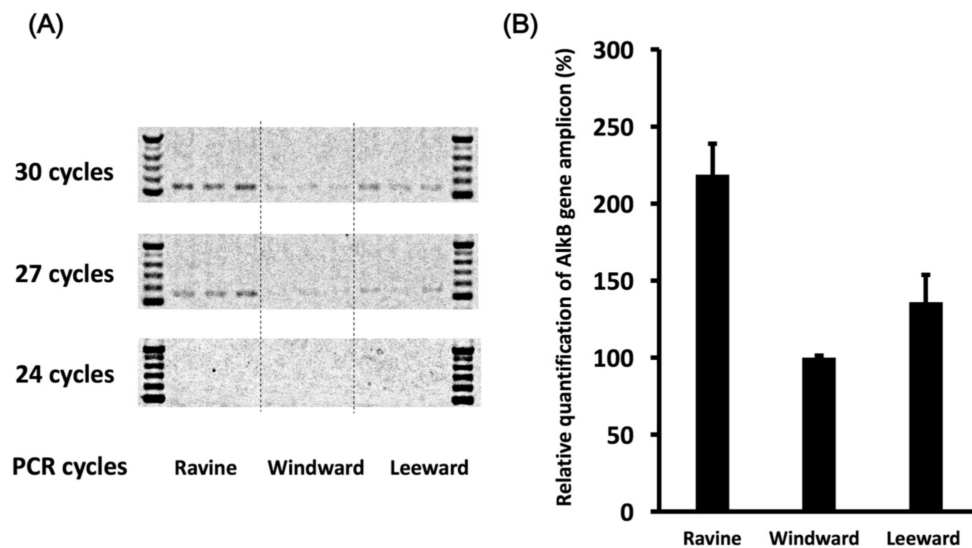

Figure 7. Semi-quantitative PCR of alkB gene results from samples of 3 habitats in Nanjenshan Reserve. (A) Agarose electrophoresis of alkB genes in different amplified cycles. Standard markers were loaded at both sides of samples. The samples from different habitats were separated by dash line. (B) The statistic results of semi-quantitative PCR after amplifying 30 cycle. The read numbers of alkB gene were significant higher in ravine habitat than windward and leeward habitats accordingly.

Figure 7

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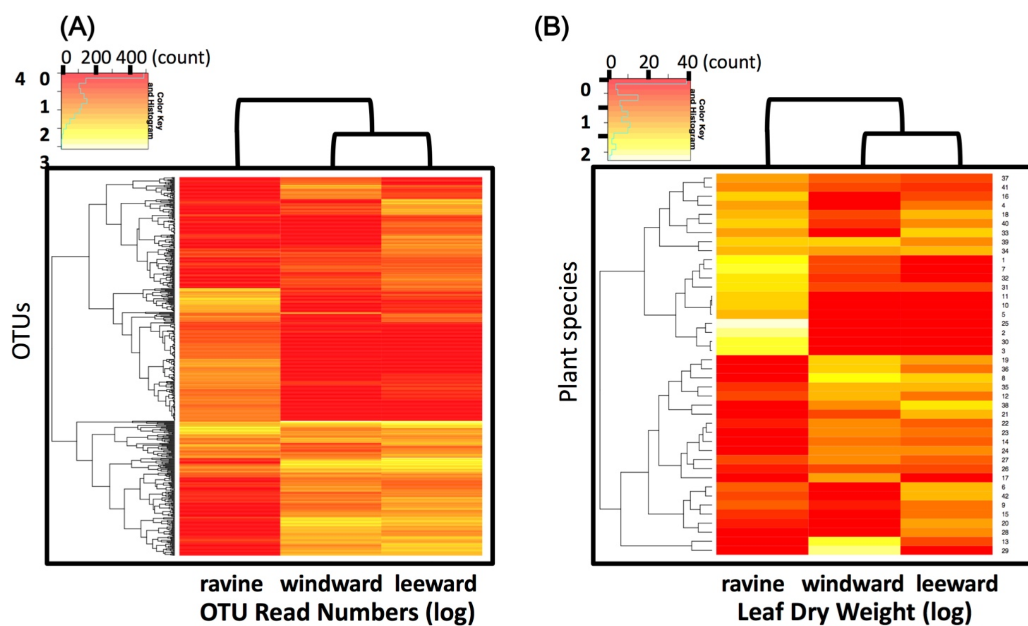

Figure 8. Heat map analysis of read-number of OTUs and annual litterfall productions of each plant species in 3 habitats. (A) Heat map of read-number of OTUs in 3 habitats. (B) Heat map of litterfall productions of plants in 3 habitats.

Figure 8