SOIL MICROBIAL COMMUNITY CHANGE DURING NATURAL FOREST CONVERSION TO RUBBER PLANTATIONS

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Abstract. Extensive conversion of natural forest to monoculture rubber plantation in Xishuangbannan. It is the 'non-traditional' rubber plantation areas in southwest (SW) of China has resulted in the soil microbial community change. The aim of our study was to identify the impacts of conversion of natural forests into rubber plantations on soil microorganisms. Soil microbial community from 10 old years rubber plantation (10aRP), 20 old years rubber plantation (20aRP), 30 old years rubber plantation (30aRP), Lower hill seasonal rainforest (LRF) and tropical ravine rainforest (RRF) were analysed by Illumina MiSeq technology. The results showed that changes in soil physical and chemical properties were strongly correlated with available phosphorus (AP), total nitrogen (TN), available potassium (AK) and total potassium (TP) which were lower in natural forests than in rubber plantations. And the dominant phylum were *Acidobacteria* (44.67%), *Proteobacteria* (13.92%), *Chloroflexi* (13.13%), *Verrucomicrobia* (9.99%) and *Planctomycetes* (5.23%) in 45 soil microbial communities. Microbial community in rubber plantations were less abundant than in natural forests. In addition, TP, AK, TN and AN were the main environmental factors which affected soil microbial composition. The soil health of rubber plantations were impacted by soil bacterial community.
Keywords: *Hevea brasiliensis plantation, land-use change, soil microorganism, biodiversity, 16S rRNA gene*

Introduction

Due to demand rapid growing in the rubber market, more than 500,000 ha of rubber plantation (*Hevea brasiliensis)* has been planted into tropical forest in Southeast Asia including southwest of China for decades (Kumagai et al., 2015). About 40% of the rubber plantations have grown in the Xishuangbanna, Southwest China. Land use Changes cause negative influence of ecosystem services which associated with the conversion to natrual rainforests on monoculture rubber plantations, inculding loss of biodiversity, reduction of carbon pools, increased soil degradation, soil erosion and reduced soil fertility (Ahrends et al., 2015; Warren-Thomas et al., 2015; Drescher et al., 2016).

Plant rhizosphere microorganisms play crucial role in soil regulation which controlled the decomposition of organic matter, nutritional cycling and energy metabolism of soil (Godin et al., 2019). Indeed, soil microbial communities were closely related to the vegetation types. It has been suggested that vegetation types change in land would have further impacts on diversity, composition and activity of soil microbial communities (Crowther et al., 2014; Lagerlöf et al., 2014). Whereas, the

microbial community of soil is the most sensitive indicator of soil quality and fertility which have greatly affected by the soil microbial community during the conversion of vegetation in land. Also, soil microbial community is a vital reflection of human disturbance and land use change. It can reflected the benefits of land use because of the close relationship with soil quality and fertility (Cardoso et al., 2013).

Furthermore, soil ecosystem service would be significant influenced by rubber plantations after conversion from natural forests because of the change of vegetation types, soil microbial community and human activities like exploring, cleaning and fertilizing. Some researchers have shown that the soil microbial biomass decreased with rubber tree-age and afforestation activities increase (Guo et al., 2015). Moreover, prokaryotic community was significantly influenced during the conversion from natural forests to rubber plantations (Krashevska et al., 2015; Schneider et al., 2015). Although the result of soil microbial diversity and environmental factors have be known during those natural forests conversion (Docherty et al., 2015), the gradient change of soil microbial community structure of rubber plantation with different forest ages in not traditional rubber growing environments need further research (Allen et al., 2015).

Previous researches have carried on the scientific planting techniques in the "traditional" rubber areas (Kerfahi et al., 2016; Wang et al., 2017). However, the soil microbial communities and environmental factors were impacted after the conversion from natural forest in 'non-traditional' rubber plantation areas are lacking. Xishuangbanna, Southwest of China, is rubber plantations highland. It is a biodiversity hotspot because of the extreme dry and rainy seasons where a 'non-traditional' rubber plantation areas beyond 477 m asl. About 54.17% of natural forests were converted into rubber plantation from 1990 to 2014. Rubber plantations were planted over about 500 m to 1,300 m a wide range of altitudes (Wu et al., 2001).

We sampled in 10 years old rubber plantation (10aRP), 20 years old rubber plantation (20aRP), 30 years old rubber plantation (30aRP), lower hill seasonal rainforest (LRF) and tropical ravine rainforest (RRF) in Xishuangbanna. Our objectives were: (1) to investigate the infulence of soil microbial community to the conversion from natural forests to rubber plantations; (2) to research on the difference of microbial community in different ages rubber plantation; (3) to find out the major environmental factors cause soil microbial community change. It is critical for the sustainable management and restoration of soil to study on how the soil microbial community change during the conversion.

Materials and methods

Study site and soil sampling

Our research was carried out in Mengla, Xishuangbanna, Yunnan province, southwest of China. All the chosen sites were at elevations between 600 and 800 m asl. Soil samples mainly collect the upper layer 0~20 cm (Schulz et al., 2019). Due to the highest microbial density and activity can be observed in the surface soil where accurately representation of the microbial community inhabit (Kerfahi, 2016).

Three sampling sites were selected for each same type of forest. The samples collected from each site consisted of 3 parallel samples. So there were 15 soil profiles and 45 groups of soil samples in total. Soil were sampled on April 10, 2015. The 30aRP were marked A-C, the 20aRP were marked D-F, the 10aRP were marked G-I, the RRF were marked J-L, the LRF were marked M-O (21°15'7.20"N-22°5'56.54"N,

101°14'25.52"E-101°42'36.07"E). The sample sites of soil samples in our study were listed in *Figure 1*.

All soil samples were filtered through a 2 mm mesh to remove root and plant materials. Then, each soil sample was classified into two groups. One group was air-dried at room temperature before physical and chemical analysis. The other part was placed in a freezer at -4 °C which was then transported to the laboratory for DNA extraction within several hours.
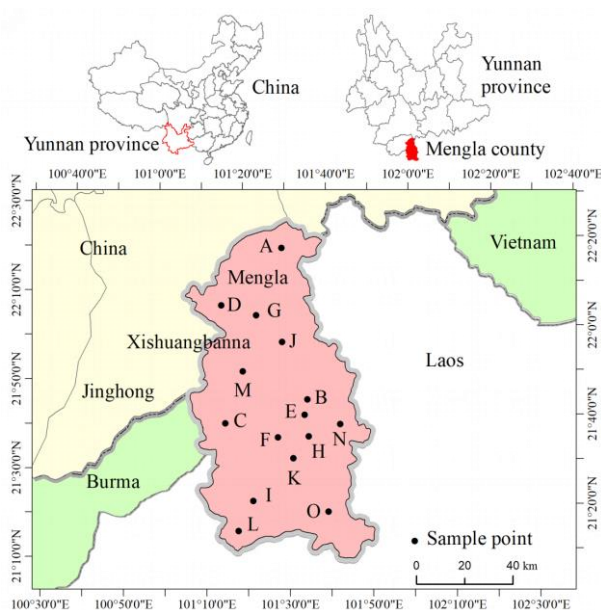

Figure 1. *Map of study area located in Mengla, Xishuangbanna, China. There were 15 soil sites that A-C (30aRP), D-F (20aRP), G-I (10aRP), J-L (RRF), M-O (LRF)*

Soil physical and chemical properties

Soil samples were sent to plateau environmental changes key laboratory of Yunnan for physical and chemical analysis. There are 8 indicators including pH, organic matter (OM), total potassium (TK), total nitrogen (TN), available potassium (AK), total phosphorus (TP), available phosphorus (AP), available nitrogen (AN), were determined. PH of soil was measured potentiometrically at a soil: water ratio of 1:2.5 in H_2O. OM was quantified by oxidation with a potassium dichromate solution in sulfuric acid (H_2SO_4-$K_2Cr_2O_7$). TK was determined by NaOH melting atomic absorption spectrophotometers; TN was determined by semi-micro-kjeldahl method. AK was determined by ammonium acetate extraction atomic absorption spectrophotometer; TP was determined by NaOH fused molybdenum-antimony anticolorimetric method. AP was determined by sodium bicarbonate extraction molybdenum blue colorimetric method; AN was determined by NaOH diffusion method (Bao, 2000).

DNA extraction and PCR amplification

The total DNA genome was extracted from 0.5 g of samples using the E.Z.N.A.®-Soil DNA Isolation Kit (Omega Bio-tek, Norcross, GA, U.S.). Monitoring the DNA concentration and purity on a 1% agarose gel and diluting the DNA was to 20 ng/mL with TE buffer.

Bacterial 16S rRNA genes in the V3-V4 region were exaggerated by using the bacterial primers 341F (5′-CCTACGGGRSGCAGCAG-3′) and 806R (5′-GGACTACCAGGGTATCTAAT-3′) with labelled barcodes sequence. All PCR reactions were performed in 30 μL of solution using Kapa HotStart HiFi 2 × ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, UK), forward and reverse primers (0.2 mM), and approximately 10 ng of template DNA. First, the cycling conditions were denatured at 95 °C for 3 min and then 30 cycles were performed which were denatured at 95 °C for 30 s, annealed at 55 °C for 30 s and extended at 72 °C for 45 s. In the end, the sample was held at 72 °C for 5 min (Xu et al., 2016). For the convenience of qualitative and quantitative analysis on PCR products, fair volume of loading buffer (with SYBR green) and PCR solution were mixed before performing the electrophoresis detection on a 1% agarose gel. Samples that demonstrated a clear major of about 400-450 bp were applied to further experiments.

Illumina MiSeq sequencing

Sequence reads was carried out using a paired-end Illumine MiSeq sequencing method on an Illumine MiSeq device (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. Subsequently, reads from all samples were combined into a single dataset for processing with QIIME. All original datasets obtained in our research has been deposited to NCBI SRA repository and can be obtained by sequence number of PRJNA574017.

Diversity estimations

An open reference method of combination of de novo and reference based OTU identification was carried out in Qiime platform. RDP Classifier was using to determine taxonomic classification for each OTU of bacterial community at similarity level of 97%. On the basis of OTU results, a sample of alpha diversity was computed, which is the analysis of species diversity in a single sample, where ACE values, chao1, Shannon and the Simpson indices are included (Edgar, 2017).

Statistical analysis

Data for soil physical-chemical properties were conducted using the program SPSS 20.0 (SPSS Inc., Chicago, USA). Correlation analysis among all soil variables was performed. Duncan's test was used to compare the significant differences between land use types (30aRP, 20aRP, 10aRP, RRF and LRF) for each parameter. Data were tested for normal distribution and if needed log transformation was done in order not to violence the assumptions of normality and equal variances. A principal component analysis (PCA) was done to investigate differences in microbial community composition among land cover types.

Results

Soil physical and chemical properties

Land use change usually affected most soil physical and chemical parameters (*Table 1*). The pH of soil of different land cover types (30aRP, 20aRP, 10aRP, RRF and LRF) between 4.2 and 4.8. The content of OM was 0.35 ± 0.43 and AK was

113.54 ± 5.46 mg/kg in 20aRP. The OM and AK in 20aRP were significantly higher than others, While AK was 37.98 ± 4.61 mg/kg in RRF which was the lowest value of 5 sample sites. In LRF, TK and OM showed lowest value were 5.78 ± 0.67 g/kg and 0.03 ± 0.0012, respectively. It suggested the soil physical and chemical properties content increased with land use change and increased human disturbance.

Table 1. *Physical and chemical properties at each sample sites. Values are means ± SD (n = 9)*

Soil properties	30aRP	20aRP	10aRP	RRF	LRF
pH	4.48 ± 0.10a	4.72 ± 0.09a	4.20 ± 0.04a	4.80 ± 0.17a	4.70 ± 0.25a
OM (%)	0.15 ± 0.16a	0.35 ± 0.43a	0.04 ± 0.004b	0.05 ± 0.001b	0.03 ± 0.0012b
AN (mg/kg)	10.77 ± 0.49b	10.53 ± 1.80b	10.89 ± 1.32b	11.11 ± 0.64ab	13.10 ± 2.81a
TN (g/kg)	0.94 ± 0.035c	1.36 ± 0.089ab	1.26 ± 0.028b	1.39 ± 0.55ab	1.43 ± 0.39a
TP (g/kg)	0.11 ± 0.006a	0.12 ± 0.022a	0.13 ± 0.008a	0.12 ± 0.001a	0.11 ± 0.025a
AP (mg/k g)	2.38 ± 0.24c	3.91 ± 0.31b	4.85 ± 0.85a	4.36 ± 0.56ab	4.89 ± 2.47a
TK (g/kg)	7.16 ± 1.48b	13.94 ± 2.59a	11.54 ± 1.87ab	11.02 ± 0.61ab	5.78 ± 0.67c
AK (mg/kg)	50.78 ± 1.63bc	113.54 ± 5.46a	75.57 ± 3.54b	37.98 ± 4.61c	68.73 ± 6.61b

Different letters indicate significant differences between land cover types ($P < 0.05$). pH: pH, OM: organic matter, AN: available nitrogen, TN: total nitrogen, TP: total phosphorus, AP: available phosphor, TK: total potassium, AK: available potassium, 30aRP: 30 old years rubber plantation, 20aRP: 20 old years rubber plantation, 10aRP: 10 old years rubber plantation, RRF: tropical ravine rainforest), LRF: lower hill seasonal rainforest

The soil physical and chemical properties at each sampling site have significant differences except pH and TP. There were distinct differences in AP, TK and AK ($P < 0.05$). AP in LRF (a) and RRF (a) were significant differences between 30aRP (c). TK in LRF (c) differs from 30aRP (b), but the largest difference was between LRF (c) and 20aRP (a). AK was the largest difference in RRF (c) and 20aRP (a). Meanwhile, AK and TK were significant differences between soil samples of 30aRP, 20aRP and 10aRP.

Microbial community composition

Sequencing 45 soil samples using Miseqa produced 1,738,755 high-quality tags. The endophytic bacteria with abundant diversity in 5 sample sites by the Alpha diversity estimation. As results shown in *Table 2*.

Table 2. *Number OUTs and Alpha diversity of prokaryotic in soils*

Sample ID	OTU number	Chao1	Ace	Shannon	Simpson
30aRP	1933	2139	2139	5.869	0.0083
20aRP	2040	2197	2197	5.946	0.0073
10aRP	1443	1625	1639	5.798	0.0087
RRF	1542	1726	1726	6.099	0.0067
LRF	1230	1511	1511	5.802	0.0083

The community richness index of Chao1 and Ace were from 1511 (LRF) to 2197 (20aRP). These can estimate the number of OTUs in the soil samples. The average number of OTUs ranging from 1230 (LRF) to 2040 (20aRP). The total number of OTUs

detected in different ages rubber plantations (97% sequence similarity) were much higher than in natural forests. The average of rubber plantations was 1850 and the natural forests was 1380. The average of Shannon index of rubber plantation was 5.871 and the natural forest was 5.924, respectively. The rubber plantation's was 0.053 lower than the natural forest. The Shannon index of RRF was 6.099 which was the highest value of 5 sample sites, and the lowest was 5.798 of 10aRP. On the contrary, the highest Simpson index was 0.0087 of RRF and the lowest was 0.0067 of 10aRP.

The whole sequences were categorized from phylum to genus. There were 42 different phyla, 42 classes or 48 orders were found by using the program RDP classifier Bayesian algorithm. 45 samples showed very different 16Sr RNA profiles at the phylum level (*Fig. 2*). About 17 different phylas occupy for 97.73% to 99.50% were common. The dominant phyla in each sample composed over 86% of the microbial community. There were *Acidobacteria* (44.67%), *Proteobacteria* (13.92%), *Chloroflexi* (13.13%), *Verrucomicrobia* (9.99%) and *Planctomycetes* (5.23%). *Acidobacteria* mainly contains *acidobacteraceae*.

Alphaproteobacteria was the main group of proteobacteria, while the rest were Myxococcales, Syntrophobacterales, Betaproteobacteria, Deltaproteobacteria.

Chloroflexi were mainly *Anaerolineae* (*Fig. 2*). As the hierarchical heat map shown in *Figure 2* that the microbial community structure of RRF was different from other samples.
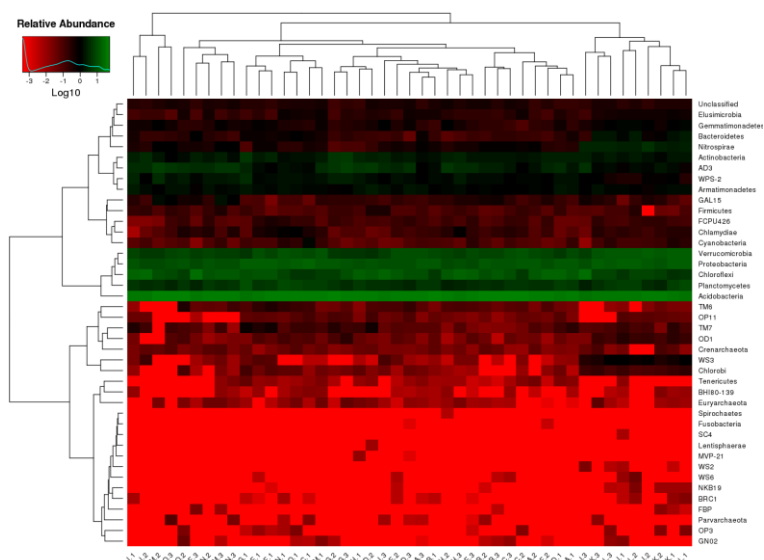

Figure 2. Soil microbial community distribution of the 42 Phylum among the 15 sample sites which marked A to O. The 30aRP were A-C, the 20aRP were D-F, the 10aRP were G-I, the RRF were J-L and the LRF were M-O, respectively. And 3 parallel samples collected from each sites like A1, A2, A3, so there were 45 soil samples in all. The relative values for bacterial family were inferred by color intensity with the legend indicated at the top left corner distribution

Differences in soil microbial community between natural forests and rubber plantations

In *Figure 3* shown the relative abundances of different phylum in soil microbial community in 30aRP, 20aRP, 10aRP, RRF and LRF. Only a small amount of

Fusobacteria 0.0018% and *Lentisphaerae* 0.0084% was found in 20aRP. And a few of *Spirochaetes* 0.0043% was found in 10aRP. *Tenericutes* in RRF was 0.0127% which was less than that of other samples. Meanwhile, compared with other groups, the content of *Flavobacteriales* and *Cytophagales* were very low in 10aRP which only 0.0056% and 0.0074%, respectively.

Classify all sequences using the RDP Classifier at similarity level of 97%. It indicated that relative abundance of soil microbial communities in the RRF were significantly higher than in the rubber plantations. For example, *Verrucomicrobia*, *Nitrospirae*, *Bacteroidetes*, *Gemmatimonadetes* and *Elusimicrobia* were 3.74%, 1.42%, 0.62%, 0.31% and 0.18% higher than those in rubber plantations.

By difference analysis between each phylum microorganisms in the 5 sample sites. It was found that *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Actinobacteria*, *WPS2*, *ADS3*, *Bacteroidetes*, *Gemmatimonadetes*, *Elusimicrobia*, *Nitrospirae*, *WS3* and *OD1* were extremely significant differences (P < 0.01). *WS25* was significant differences (P < 0.05).

Analyzing the differences between natural forests and rubber plantations, it was found that *Chloroflexi*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Gemmatimonadetes*, *Nitrospirae*, *WS3* and *OD1* were extremely significant differences (P < 0.01). *GAL15* and *OP112* were significant differences (P < 0.05).
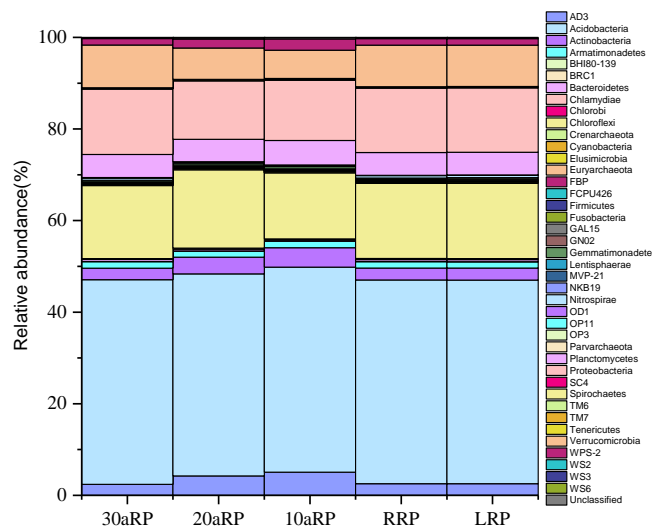

Figure 3. *Abundances of different phylums in soil microbial community in the 5 different land cover types*

As shown in *Figure 4a* and *b,* The LRF samples (purple points) on the PC1 axis were significant different from other samples by PCA. Compared with LRF (purple points), no significant difference between RRF (yellow points) and RP were found. Natural forests (red points) which were distributed to the left of the RP (blue points) were significant difference between them.

Correlation between microbial community structure and environmental factors

Environmental factors such as soil nutrient elements and organic matter have significant effects on soil microbial community (Docherty et al., 2015). The

environmental elements were influenced by the conversion from natural rainforests to rubber plantations. Thus it is critical to figure out the effects of environmental factors on soil microbial community during the conversion.
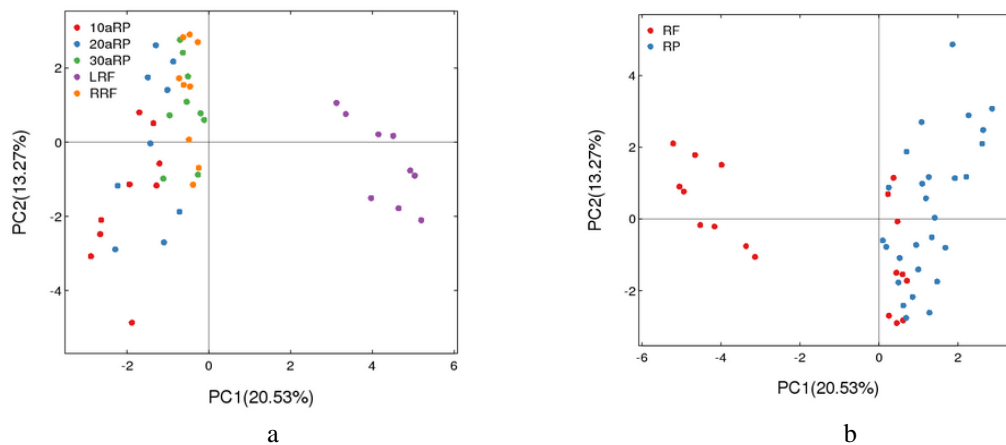

Figure 4. *(a, b) Principal component analysis with similarity as instrumental variables was carried out with 45 soil samples from natural rainforests and rubber plantations*

As results shown in *Table 3*. We can found *Chlamydiae* and *GAL15* were significantly correlated with AN. *Bacteroidetes*, *Gemmatimonadetes*, *Nitrospirae* and *Verrucomicrobia* were significantly correlated with TN. *Chloroflexi* was negatively significantly correlated with AK. While, *Proteobacteria* were negatively correlated with TK. *Planctomycetes* was negatively correlated with AK.

Discussion

The response of nature forest transformation to rubber tree plantations on soil microbial community

Illumina Mi Seq technology was used to figure out the reactions of soil microbial community in "Non-traditional" rubber plantation areas where with altitudes above 300 m asl and sharply sloping land. Alpha diversity estimation indicated microbial diversity richness in 5 sample sites shown in *Table 2*. The Shannon and Simposn index suggested that diversity of microbial community in rubber plantations was less abundant than natural forests. The diversity of microbial community of RRF was more abundant than LRF. And it showed 20aRP > 30aRP > 10aRP in rubber plantations. The 20aRP was the most abundant microbial community diversity of rubber plantations.

Prokaryotes in the soil were significant differences between natural forests and rubber plantations (Kerfahi et al., 2016; Schneider et al., 2015). Because the surface soil of rubber plantations were usually disturbed by human activities such as fertilizing and weeding. The living situation of soil microorganisms would be change by human activities to lead great influence of the quantity and structure of microbial community. Moreover, the leaf litter and soil nutrient cycle reduction lead to the diversity of native plants and the availability of nutrients decrease during the conversion of natural forests to monoculture rubber plantations (Kerfahi et al., 2016). Previous researchers also have proved that soil microorganisms change largely due to land use change. It caused many

negative ecological effects that the soil quality in the natural forests area were degraded (Guillaume et al., 2016), the decomposition rate of litter is slowed down, the degradability of organic matter was reduced and the total organic carbon, microbial biomass carbon and bioactive organic carbon were reduced (Kerfahi et al., 2016; Zhang et al., 2013).

Table 3. *Pearson correlation coefficient analysis of soil microbial community structure and environmental factors in natural rainforests and rubber plantations*

	Bacteroidetes	Chlamydiae	Chloroflexi	GAL15	Gemmatimonadetes	Nitrospirae	Planctomycetes	Proteobacteria	Verrucomicrobia
pH	0.59	0.488	-0.461	0.343	0.423	0.579	0.312	0.361	0.682
OM	-0.295	-0.206	0.522	-0.369	-0.352	-0.31	-0.811	-0.194	-0.193
AN	-0.318	.952*	-0.213	.965**	-0.123	-0.333	0.407	-0.497	-0.289
TN	.992**	-0.101	-0.461	-0.127	.892*	979**	0.51	0.648	.885*
TP	0.026	-0.423	0.793	-0.451	0.454	-0.049	-0.522	-0.481	-0.278
AP	0.08	0.379	0.243	0.424	0.588	0.008	0.245	-0.579	0.217
TK	-0.749	0.108	0.834	0.049	-0.379	-0.799	-0.725	-.911*	-0.828
AK	-0.222	-0.582	.906*	-0.665	0.028	-0.275	-.887*	-0.427	-0.38

**Significantly correlated at the 0.01 level. *Significantly correlated at the 0.05 level

Difference analysis of microbial community structure in rubber plantations of different ages

The Ace and Chao index of 5 different land use in our study were shown that 20aRP have the most community richness than others. Consistent with Kerfahi's recent research that the soil microbial ecosystem would be affected by land use change. During the natural forests converted into rubber plantations in Xishuangbanna, the differences of microbial community structure on the surface soil were affected by human activities. It suggested that the more the human disturb, the more the microbial community richness of rubber plantations have.

Compared with young rubber, mature rubber plantations have more litter decomposition (Puttaso et al., 2015). Since mature rubber plantations suffered more disturbance like harvesting, fertilizing and weeding. Those human activities possibly to lead negative influence on the nutrients in the soil. Thus the composition and structure of soil microbial community would be affected (Kang et al., 2019).

As we known, the 20RP was the most human disturbance sample site because of that large amount of fertilizer intake for rubber growth and latex harvest in the period (Lan et al., 2012). And LRF has less human disturbance than RRF. The rubber plantations basically have the similar community structure even they were different tree-ages. Furthermore, the 10RF and 20RF were more similar which consistent with the results of the intermicrobiome analysis.

Acidobacteria, *Proteobacteria*, *Chloroflexi*, *Verrucomicrobia* and *Planctomycetes* were the 5 microorganisms with high proportion of all sample sites. Others were significant differences except *Planctomycetes*.

Human activities affect soil microbial community structure. There were small amount of *Fusobacterium* and *Lentisphaerae* found in 20RF and a few of *Spirochaetes* were found in 10RF. Those microorganisms were proved from human or animal waste (Nie et al., 2017; Repass et al., 2018; Granja-Salcedo et al., 2017).

Correlation analysis of soil microorganisms and environmental factors

Consistent with correlation analysis results, The contents of TN and AN of soil in natural forests were higher than rubber plantations. The abundance of *Verrucomicrobia*, *Nitrospirae*, *Bacteroidetes*, *Gemmatimonadetes* and *Elusimicrobia* in natural forests were more richer than rubber plantation.

The physical and chemical properties of the soil have significantly changed during the conversion from natural forests to rubber plantations. The contents of AP, TN, AK and TK in soil have significant or very significant differences. Furthermore, there were also significant differences in soil physical and chemical properties between LRF, RRF and Rubber Plantations (10aRP, 20aRP, 30aRP). The differences in soil physicochemical properties would be increased with rubber tree growing.

The 30RF and RRF have the largest difference of soil physical and chemical properties by difference analysis. Meanwhile, the contents of AK, TK, TN, AP and AN in the soil were the main environmental factors which affect the difference of microbial community composition in 5 sample groups. The difference of microbial community composition was mainly affected by the physical and chemical properties of soil related to fertilize. Because it significantly changed the biogenic elements for the microorganisms growth and metabolism such as N, P, K and others which have significant effects on soil microorganisms (Krashevska et al., 2013; Kerekes et al., 2013).

Conclusion

In summary, the diversity of microbial community in rubber plantations were less abundant than in natural forests. Human activities actually affect soil microbial community structure. In addition, the difference of microbial community distribution was strongly affected by the physical and chemical properties of soil related to fertilize. Consequently, the reduced diversity in monoculture rubber plantations and the disturbance affected by the establishment of monoculture rubber plantations were all likely to negatively impact soil microbial community. Nowadays, the mixed rubber cultivations is establishing to reduce negative effects from the monoculture rubber plantations in Xishuangbanna. Further researches are obviously needed to evaluate the response of soil microbial community to land use change in different rubber agroforestry systems like Hevea brasiliensis-Camellia sinensis agroforestry systems, Hevea brasiliensis-Coffea arabica agroforestry systems (CAAs) and Hevea brasiliensis-Theobroma cacao agroforestry systems (TCAs).

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