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Diversity and distribution of endophytic bacterial community in the Noni (*Morinda citrifolia* L.) plant

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Noni (*Morinda citrifolia* L.) is a plant used by traditional cultures and also in modern health care products. Various chemical substances are derived from the plant and include, but are not limited to anthraquinone flavonol glycosides, iridoid glycosides, lipids glycosides and triterpenoids. Also commonly found on the plant are endophytic bacteria however, there are no reports on endophytic bacterial community of Noni. We collected samples from five sites of Noni plant (roots, branches, leaves, fruits and seeds) and performed 16S rDNA analysis. Results show that these five parts harbor a highly similar bacterial composition with the top four being *Sphingomonas*, *Pseudomonas*, *Halomonas* and *Geobacillus*. *Sphingomonas* and *Pseudomonas* were found to be widely distributed in plant endophytic biotope; while there are little reports on plant-associated *Halomonas* and *Geobacillus,* indicating distribution in the plant hosts. Unknown genus also is abundant in five sites of Noni, ranging from 26.70 to 33.66%, implicating necessity to reveal them. This study provides information on endophytic bacteria in the Noni for future analysis based on a metagenome strategy.

Key words: Noni, endophytic bacteria, diversity, metagenome.

INTRODUCTION

Plants host an abundant microbial community in rhizosphere, phyllosphere and endosphere areas as previous research has reported and the noni plant microbiome has received significant attention in recent years (Lebeis et al., 2012; Turner and James, 2013; Bulgarelli et al., 2013; Berg et al., 2014). Microbes colonizing plant surfaces and interior areas are vital for plant health and productivity (Bonfante, 2010; Berendsen et al., 2012; Ferrara et al., 2012; Monteiro et al., 2012), but some of them could lead to disease development of plants (James and Olivares, 1998; Monteiro et al., 2012; Van Overbeek et al., 2014). Prior reports indicate that

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plant microbiome could be beneficial for human health through consumption of raw plants (Blaser et al., 2013; Van Overbeek et al., 2014). Therefore, understanding the microbial composition of plants may assist in the development of sustainable agriculture (Berg, 2009; Lugtenberg and Kamilova, 2009).

Several scientific teams documented the feasibility of promoting environment-friendly agriculture through manipulation of plant microbiome (Bloemberg and Lugtenberg, 2001; Philippot et al., 2009; Adesemoye et al., 2009; Singh et al., 2010; Bakker et al., 2012). Bloemberg et al. (2001) revealed that plant microbiome could reduce incidence of plant disease, and research conducted by Bakker et al. (2012) showed contribution of plant microbiome to agricultural production.

Plant microbiome also holds the potential to keep plant productivity with decreased chemical inputs (Adesemoye et al., 2009) and function as a key player in biogeochemical cycles (Philippot et al., 2009; Singh et al., 2010). Although endophytic microbes were ever considered contaminants in some of prior reports (Ryan et al., 2008; Reinhold-Hurek and Hurek, 2011; Mitter et al., 2013), they should be the most stable microbial partners of plants.

Various researchers have identified endophytic bacteria in plants (Hallmann et al., 1997; Compant et al., 2010; Monteiro et al., 2012; Sessitsch et al., 2012) and implicated their significance in promoting plant growth and the ability to control phytopathogens (James, 2000; James et al., 2002; Compant et al., 2010; Reinhold-Hurek and Hurek, 2011; Sessitsch et al., 2012; Suarez-Moreno et al., 2012). However, it is challenging to isolate and inoculate these inner bacteria, making it difficult to get a whole-picture of interaction network among various bacteria and between their hosts. A culture-independent strategy is increasingly used to uncover the endophytic bacterial community such as those in rice and sugarcane (Sessitsch et al., 2012; Fischer et al., 2012).

To duplicate the method performed on rice and sugarcane to evaluate bacterial types and concentrations, we performed 16S rRNA analysis on five different plant parts of the medicinal plant named Noni (*Morinda citrifolia* L.) (Chan-Blanco et al., 2006). These endophytic bacteria could produce various bioactive compounds (Su et al., 2005), that may improve immunity and anti-tumor activity (Furusawa et al., 2003; Brown, 2012). Others have reported that lignin is associated with antioxidant activity (Kamiya et al., 2004). This work will provide an informative reference on this “accessory organ” of Noni, and the first summary of endophytic bacterial community of Noni.

MATERIALS AND METHODS

Sample collection and sterilization

Roots, branches, leaves, seeds and fruits of Noni (*Morinda citrifolia* L.) (Supplementary Figure 1) were randomly collected from mature Noni trees, which were growing in cultivation field of Hainan Noni Biological Engineering Development Co., Ltd. in Sanya, Hainan (18°18′01″N, 109°31′36″E, South China), and stored at the temperature of 4°C.

The samples were washed with sterile water, immersed in 70% alcohol for 3 min, washed with fresh sodium hypochlorite solution (2.5% available Cl^-) for 5 min, rinsed with 70% alcohol for 30s, and finally washed five to seven times with sterile water. Aliquots of the final rinsing water were spread on Luria-Bertani (LB) solid medium plates and cultured for 3 days at 28°C for detection of bacterial colonies (Liu et al., 2013). The samples without bacteria on the surface were used for subsequent analysis.

DNA extraction, amplification, and sequencing

All selected roots were pooled as a single sample to average the deviations in the endophytic bacterial community, which was also done for branches, leaves, fruits and seeds. Then about 5.0g of surface-sterilized samples of each site were frozen with liquid nitrogen and quickly ground into a fine powder with a precooled sterile mortar. Then, the CTAB procedure was used to extract bacterial DNA (Liu et al., 2012), which was used as template to amplify V6 region of the 16S rDNA by primers (967F5’-CAACGCGAAGAACCTTACC-3’) and 1046R (5’-CGACAGCCATGCANCACCT-3’). The purified PCR products were mixed in equal concentration, and sequenced by HiSeq 2000 (Illumina, USA.) following the manipulation instructions at BGI Shenzhen (China).

Acquisition of unique tags and OTUs

The reads with more than 2 bases (quality value lower than 20) were filtered. Then the reads with more than 3 mismatches within amplification primers region were removed, and the low quality bases which is located at the 3’ end were trimmed. Besides these, the reads which contained more than 15 bases of adapter sequences (3 mismatches allowed), 9 N bases or 10 consecutive same bases were removed.

The processed paired-reads were overlapped with each other to form V6 tags under following standard: minimum overlapping length was 30 bp without mismatch or N base. Non-redundant tags were produced by Mothur (version 1.27.0), and the unique tags were the typical tags representing all the similar tags. Unique tags were listed based on abundance and pre-clustered by single-linkage pre-clustering (SLP) following 98% similarity. Then the unique tags were annotated and clustered into operational taxonomy units (OTUs) following 97% identity.

Taxonomy assignment and abundance analysis

Unique tags were classified by alignment to Silva RefSSU database using BLAST (version 2.2.23, and the key parameters were ‘-p blastn -m 8 -F F -a 2 -e 1e-5 -b 50’), and the best alignments were selected. If more than 66% of the unique tags in OTUs were aligned to the same species, the OTUs was assigned to the species and then the analysis went into the next taxonomic rank. The abundance of tags in different classification levels was calculated according to the alignment results.

Analysis of sample complexity and similarity

Alpha diversity was measured by indexes including chao1, ACE, Shannon and Simpson. Values of rarefaction was calculated by

Table 1. Data statistics on different tissue regions of Noni.

Sample name	Data production (M)	Tag number	Unique tag number	OTU Number
Root	497.99	340,000	22,290	3,697
Branch	443.19	350,092	18,482	2,608
Leaf	538.07	339,441	19,753	2,853
Fruits	488.82	346,141	19,611	2,871
Seed	488.47	345,173	19,724	2,951

Table 2. Sample complexity indexes for different samples.

Sample name	Chao1	ACE	Shannon	Simpson
Root	14,451.321	33,017.373	4.348	0.039
Branch	10,357.145	23,206.406	3.965	0.051
Leaf	11,374.413	27,411.963	4.009	0.045
Fruits	10,964.413	22,452.468	3.907	0.052
Seed	11,973.855	24,230.996	3.944	0.051

The bigger chao1, ACE, and Shannon are the more complex the sample was; the smaller Simpson is the more complex the sample was.

Mothur (version 1.27.0) and the rarefraction curve was drawn by R (version 2.11.1) in which the extracted tags were used as X-axis and the OTUs number was used as Y-axis.

Phylogenetic analysis was executed based on species abundancy at the genus level, and the samples were clustered by their phylogenetic distance. The representative sequence for each OTUs was selected at random, and the neighbor-joining phylogenetic tree (1,000 bootstraps) was constructed by combining all OTUs using MEGA4.

RESULTS

Species classification and complexity

V6 region of 16S rDNA was amplified and sequenced to produce 339,441 to 350,092 tags for root, branch, leaf, fruit and seed samples (Table 1). Using the protocol summarized in the methods, the unique tags were clustered into Operational Taxonomic Units (OTUs) (Table 1) and aligned with Silva108 database to identify the bacterial community of each tissue region in Noni. Arranged from 66.34 to 73.30% in different tissues, tags could be classified at the genus level (less than 5.63% at the species level) as Supplementary Figure 2A showed, making it feasible to analyze bacterial composition at this taxonomic level.

Various indexes including chao1, ACE, Shannon and Simpson were employed to evaluate the complexity of samples, indicating that root harbored the most abundant species in comparison to other four sites (Table 2). This was also documented in rarefaction analysis summarized in Supplementary Figure 2B.

Discrepancy of species composition in different sites of Noni

As Figure 1 showed, five sites are colonized by the same most abundant phyla: Proteobacteria (67.90 to 72.67% relative abundance), Firmicutes (19.39 to 22.26%) and Actinobacteria (5.95 to 6.87%) accounting for more than 95% of the species components for each site.

To understand the specific compositions, the bacterial community was profiled according to their relative abundance at the genus level (Figure 2). Among those taxa examined, *Sphingomonas* was the most abundant genus in all samples, ranging from 19.88 to 25.97%. The second abundant genus was *Halomonas* in the endophytic communities of fruits, leaves and seeds, while it *Pseudomonas* and *Geobacillus* were predominant in the branches and roots respectively. Genus *Pseudomonas and Halomonas* were widely distributed over all five parts with high relative abundance. Tags which could not be classified hold 26.70 to 33.66% abundance suggesting necessity to disclose them in the follow-up study.

Similarity analysis on bacterial community of five tissues

The dendrogram (Supplementary Figure 3) indicated that Noni fruits hold more similar bacterial types than the leaves or root. In fact, the leaves and root held the endophytic bacterial community under the similarity higher than 97%. Five sites shared 46 genus with high relative abundance (Supplementary Figure 4), including

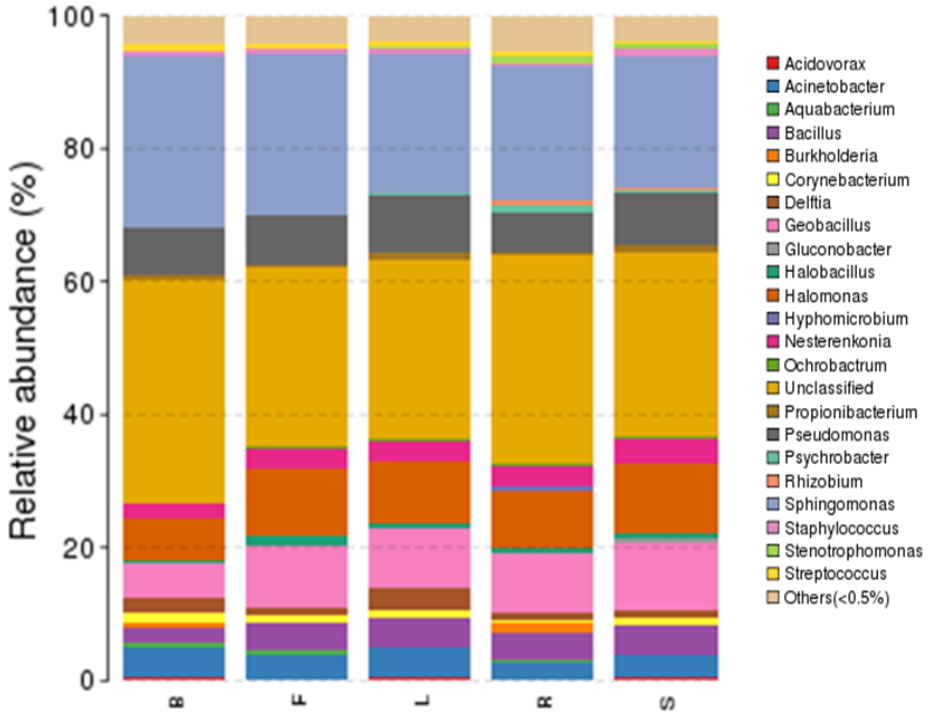

Figure 2. Distribution of bacteria in different parts of Noni at the genus level. F: fruit; L: leaf; B: branch; R: root; S: seed.

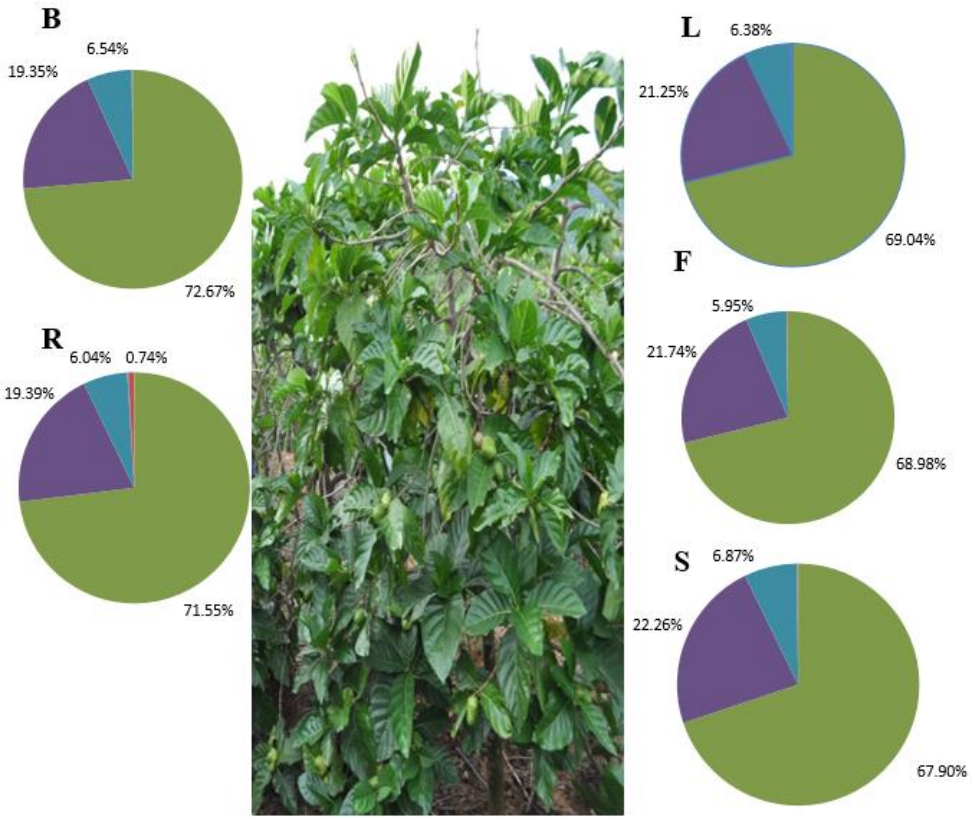

Figure 1. Distribution of bacteria in different parts of Noni at the phylum level. Green color represents Proteobacteria, purple stands for Firmicutes, Actinobacteria and Acidobacteria are highlighted in blue and red respectively. F: fruit; L: leaf; B: branch; R: root; S: seed.

the top four abundant genus *Sphingomonas*, *Pseudomonas*, *Halomonas* and *Geobacillus.* Roots harbored 13 special genus and this number were 6, 2, 4, 4 for branches, leaves, fruits and seeds respectively, but the highest relative abundance of these special genus was just 0.12% suggestive of an insignificant role they play.

DISCUSSION

Noni is an important plant providing various -derived health food raw material but the research about Noni mainly concentrated on its efficacy component detection and functional test (Liu et al., 2014a; Xu et al., 2014). Little research exists on the endophytic bacteria which may affect Noni's healthy growth, production of bioactive ingredients and the plant products' quality (Cheng et al., 2013; Cao et al., 2014; Liu et al., 2014b).

In this study, we found that bacterial community in different sites of Noni tend to be stable and most abundant genus for each of the five plant parts were *Sphingomonas*, *Pseudomonas*, *Halomonas*, and *Geobacillus*. Endophyte *Sphingomonas* is widely distributed in various plants including tomato, *Sedum alfredii*, *Dendrobium officinale* (Chen et al., 2014a; Khan et al., 2014; Chen et al., 2014b; Yang et al., 2014). Several reports have proved significant role of *Sphingomonas* and *Pseudomonas* for plant hosts by increasing plant biomass, improving cadmium uptake, fixing nitrogen and producing phytohormone including gibberellins and indole-3-acetic acid (IAA) (Chen et al., 2014a; Khan et al., 2014; Chen et al., 2014b; Yang et al., 2014). Genomic analysis of some *Pseudomonas* strains isolated from plants indicated that they uphold the potential to be involved in plant growth promotion, environmental adaptation and antagonism to fungal pathogens (Duan et al., 2013; Illakkiam et al., 2014). Based on our knowledge, there were no report about function analysis on plant-derived endophytic *Halomonas* and *Geobacillus*, requiring more efforts to elucidate how they contribute to the host. This is also needed for unclassified genus with high abundance.

Culture-independent methods applied in this work will provide a significant reference to reveal micro-ecosystem in Noni and isolate endophytic bacteria with potential value for plant growth, bioactive compounds or pathogen resistance. Findings in this research will be also referable to identify and assemble bacterial genomes from metagenomic samples (Nielsen et al., 2014) of Noni. This will be a significant alternative to understand functional networks for endophytic bacteria in Noni due to difficulties in removing host contamination in metagenome analysis and culturing some endophytes and laid the foundation for better development and use of noni plant resources.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGMENTS

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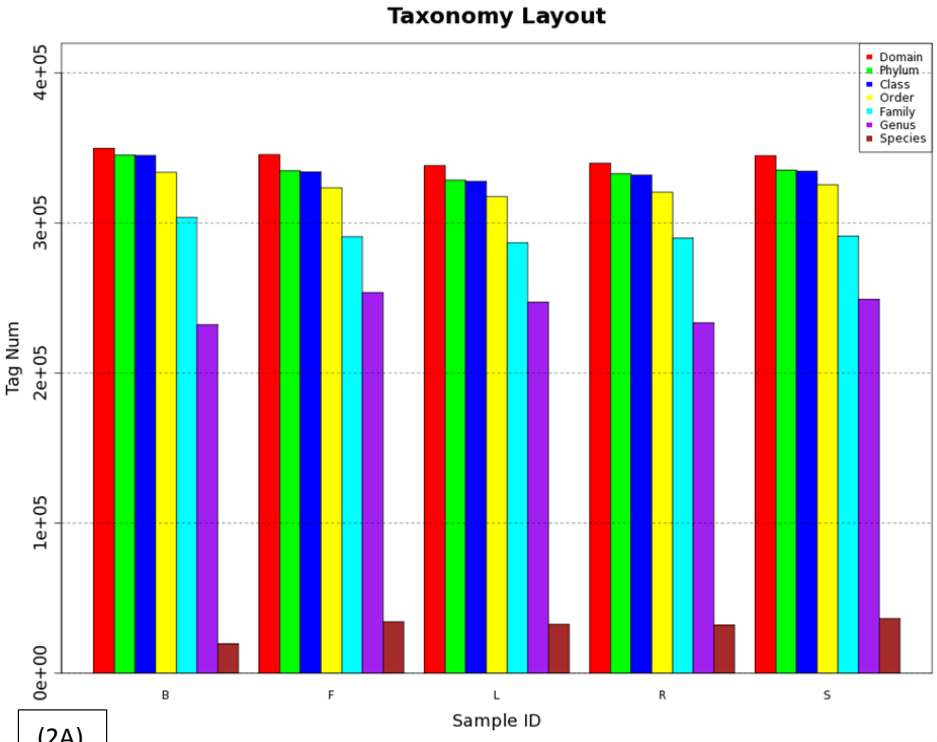
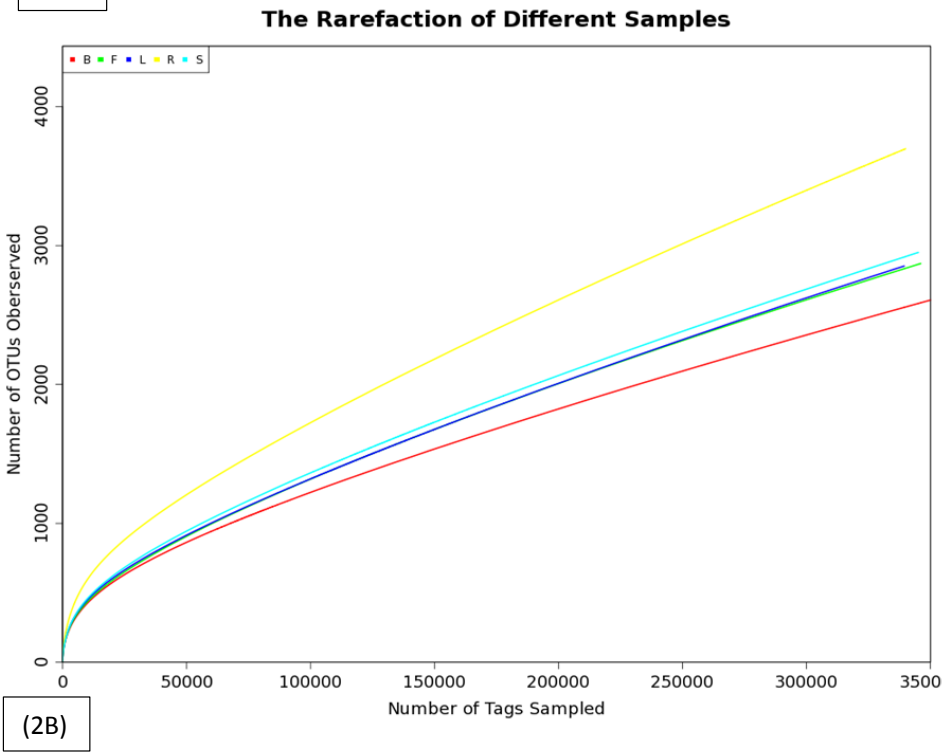
Supplementary Figure 1. Base of Noni cultivation and samples collected from five sites. F: fruit; L: leaf; B: branch; R: root; S: seed.

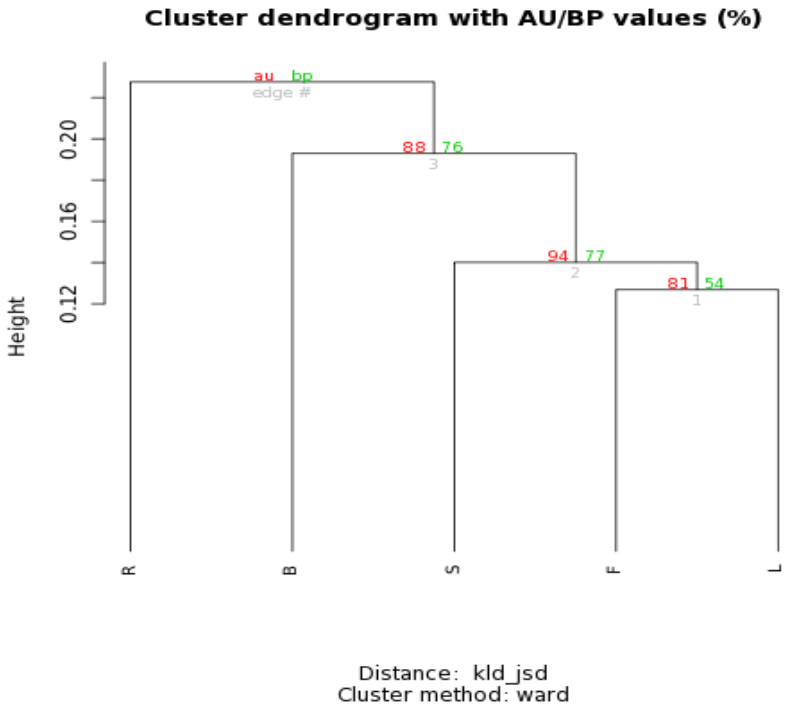


(2A)

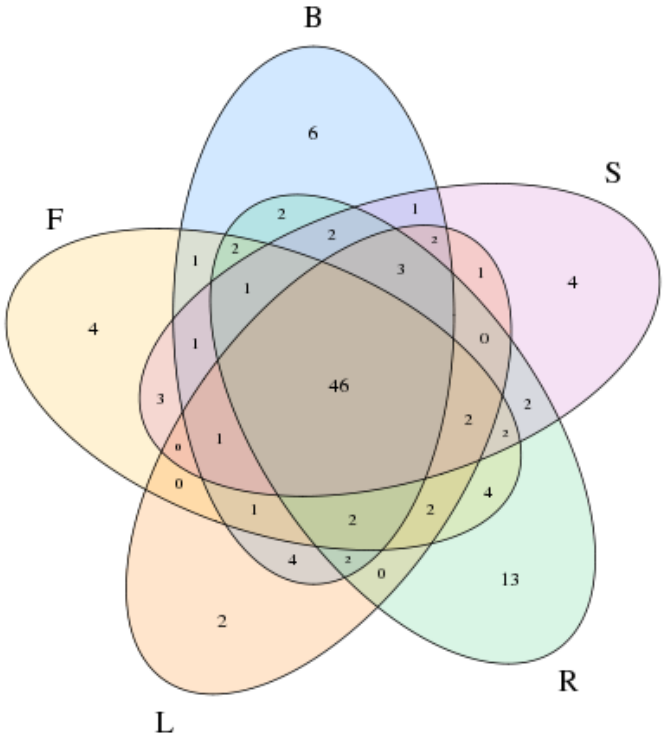


(2B)

Supplementary Figure. (2A) Number of tags that could be assigned to specific taxonomic level. F: fruit; L: leaf; B: branch; R: root; S: seed. (2B) Rarefaction of different sites of Noni. F: fruit; L: leaf; B: branch; R: root; S: seed.



Supplementary Figure 3. Community clustering results of different parts of Noni. In the picture, the communities of fruit and leaf exhibited a close relationship, and the community components of root was far from other samples. Five samples were compared together and the general relationship was exhibited.



Supplementary Figure 4. Venn diagram describing discrepancy of species similarity of five parts at the genus level five samples were compared together and the general relationship was exhibited.