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Research Article

A protocol specialized for microbial DNA extraction from living poplar wood

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Abstract

Microbial DNA extraction is a critical step in metagenomic research. High contents of chemical substances in wood tissues always cause low microbial DNA yield and quality. Up to date, almost no specialized methods involved in microbial DNA extraction from living wood were reported. In this study, an improved protocol (M1) concerning microbial DNA extraction from living poplar wood was developed. We compared microbial DNA yield and quality by M1 with those by other seven methods, including PowerSoil DNA isolation kit (M2), two soil microbial DNA extraction methods (M3 and M4), poplar genomic DNA extraction method from wood (M5), and microbial DNA extraction method from herb stems (M6), isolating bacteria (M7) and isolating fungus (M8). Results showed that M1 yielded much better quality and concentration of microbial DNA than the other methods (M2-M8) from both poplar wetwood and sapwood tissues. Following M1 protocol, 1 g of wetwood sample could yield 272.27 ng/ul (vol=50 ul) pure microbial DNA with the absorption ratios of 1.87 (A260/A230) and 1.66 (A260/A280). For 1 g of sapwood sample, these values were 361.83 ng/ul, 1.85 and 2.24, respectively. These DNA could be stably visualized by agarose gel electrophoresis and amplified by primer sets of bacteria (16S V3-V4, 16S-V4, 16S V4-V5) and fungus (ITS1, ITS2). While, the other seven methods only obtained less or contaminated microbial DNA, which could not be amplified stably by aforementioned primer sets. Our protocol provided an approach for microbial community study in living poplar wood in a more accurate way by molecular biology techniques.

Keywords: bacterial DNA; DNA quality; fungal DNA; *Populus*; wet heartwood

Introduction

Poplar is one of the most fast-growing timber species in the northern hemisphere, but wetwood occurred seriously and adversely affected its wood processing and utilization (Johansson and Hjelm, 2013; Wang *et al*., 2008). Wetwood, a worldwide disease, is a kind of abnormal phenomenon of heartwood during tree growth (Jeremic *et al*., 2004; Martin *et al*., 2021), with characteristics of high moisture contents, deep color, rot, and acid or alkaline pH value of the extract, as well as alterative physical and chemical wood properties etc. (Grîu

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and Lunguleasa, 2016; Nakada *et al*., 2019; Wang *et al*., 2016). Till now, the dominant factors of onset of wetwood are still unclear. But most scholars considered pathogenic microorganisms played an important role in wetwood formation (Johansson and Hjelm, 2013). A lot of studies on endophytic bacterial communities have been carried out, and many types of bacteria have been isolated from living wetwood based on traditional method of tissue culture, such as *Xanthomonas, Agrobacterium*, *Corynebacterium*, and *Erwinia* etc. (Magnani *et al*., 2013; Pereira *et al*., 2011; Sessitsch *et al*., 2012). However, the global composition of microorganisms in living wood is still ambiguous. In previous studies, almost all researchers adopted traditional method of tissue culture to isolate pathogenic microorganisms from wetwood (Amann *et al*., 1995; Sakamoto and Kato, 2002; Schink and Ward, 1984). But this kind method owns some disadvantages, since only few varieties of microbes (0.01%-1%) in wood environment could be isolated, cultured and purified (Verma and Satyanarayana, 2011). Therefore, it is very difficult to obtain global information of microbial community diversity in living wood tissues by the traditional method of tissue culture. With the development of high-throughput sequencing, metagenomic libraries have been exploited to study microbial diversities in the environment (Daniel, 2004; Torsvik and Ovreas, 2002). It allows researchers to avoid the traditional method of isolation and culture, and directly to investigate the microbial community diversity at the molecular level (Maropola *et al*., 2015).

High-quality microbial DNA extraction is the first and important step of the metagenomic research (Maropola *et al*., 2015). Whereas, tree tissues, especially for wood tissues, often contain large amounts of chemical substances, such as polysaccharides, organic acids and phenolic compounds, which are difficult to be separated from DNA, and always cause low microbial DNA yield and quality (Thomas, 1975; Verbylaite *et al*., 2010). Up to date, almost no specialized methods involved in microbial DNA extraction from living wood were reported. We tested many methods to extract microbial DNA from living poplar wood, such as microbial DNA extraction methods from soil (Tsai and Olson, 1991; Verma and Satyanarayana, 2011), herbs stem (Maropola *et al*., 2015), isolating bacteria (Pindi *et al*., 2013) and isolating fungus (Motková and Vytrasová, 2011), as well as PowerSoil DNA isolation kit (Mobio, USA), but no high-quality microbial DNA was obtained. In the present study, we aim to setup a specialized protocol for microbial DNA extraction from poplar wetwood and healthy sapwood to facilitate the research of microbial community diversity in polar wood.

Materials and Methods

Plant material and wood sample collection

Wood samples were collected in July from a poplar plantation located in Huazhong Agricultural University (30°28′ N, 114°21′ E), Wuhan, China. The region has a warm, temperate climate, with an annual average of 240 frost-free days, 1269 mm of rainfall, and a mean yearly temperature of 16.3 °C. Most rainfall occurs in June-August. 15-year-old trees of *Populus deltoides* cv. 'Lux ex. I-69/55' with average diameter of 35.7 cm at 1.3 m of trunk were adopted as the plant materials in this study. Columnar wood samples with 4.3 mm diameter were taken out by increment borer 500 mm (Mora, Sweden) from the trunk of trees (1-1.3 m height from stump). Healthy sapwood and wetwood were collected and stored in sterilized 50 ml tube, separately (Figure 1). Wetwood is collected from central zone of trunk with brown color. Healthy sapwood is around 1 cm far from trunk outer edge with normal white color. The wood samples were frozen immediately in liquid nitrogen, and subsequently broken into small pieces with a sterilized hammer, and further ground into powders using mortar and pestle in liquid nitrogen and mixed well. Finally, these wood powders were stored in sterilized tubes under -80 °C until utilization. Between each sampling, the borer was washed once by 70% ethanol and three times by sterilized water to avoid interaction between different wood samples. Five biological replicates were adopted in this study, and each biological replicate included five well-mixed individual plants.

Figure 1. Sample collection by dividing wood tissues into two parts: (1) SW: sapwood, located in the outer edge of trunk with normal white color; (2) WW: wetwood, located in the central zone of trunk with brown color.

Measurements of pH values and absolute water contents of wood samples

2 g of air-dried sapwood and wetwood powders were added into 30 ml cold boiled ddH_2O, respectively, mixed intermittent for 50 min, and then to stand for 10 min. Thereafter, pH values were measured by pH meter PB-21 (Sartorius, Germany). Absolute water contents of wetwood and sapwood were, respectively, measured by oven-drying method. Fresh wood samples were dried at 105 °C for 30 min followed by 65 °C for 1 week, and weighed thereafter (Jeremic *et al.*, 2004). Water content (%) = (fresh weight - dry weight)/ dry weight×100%. Measurements of pH values and absolute water contents were carried out with five replicates in sapwood and wetwood, respectively.

DNA extraction procedures

We developed a protocol for microbial DNA extraction from living poplar wetwood and sapwood (M1), and compared it with other seven methods (M2-M8) in efficiency of extracting microbial DNA, respectively, i.e. PowerSoil DNA isolation kit (M2) (Mobio, USA), two microbial DNA extraction methods from soil (M3-M4) (Tsai and Olson, 1991; Verma and Satyanarayana, 2011), a poplar genomic DNA extraction method from wood (M5) (Verbylaite *et al.*, 2010), and microbial DNA extraction methods from herbs stems (M6) (Maropola *et al.*, 2015), isolating bacteria (M7) (Pindi *et al.*, 2013), and isolating fungus (M8) (Motková and Vytrasová, 2011). For each method, same amount of wood powders (1 g) was used, except protocol of PowerSoil DNA isolation kit (0.25 g), which was performed according to the manufacturer's instructions. All of the final purified DNA was eluted in 50 ul TE buffer for normalization purposes. All DNA extraction methods were performed in triplicate.

Protocol for microbial DNA extraction from living poplar wood

1 g of wood samples were evenly suspended with 0.6 g of powdered activated charcoal (PAC) and 75 ul of proteinase K (20 mg/ml) in a 10 ml centrifuge tube with 5 ml of extraction buffer [1% CTAB+2% polyvinylpolypyrrolidone (PVPP)+1.5 M NaCl+100 mM EDTA+100 mM TE (pH 8.0) +0.1 M sodium phosphate buffer (pH 8.0)] (Desai and Madamwar, 2007; Verma and Satyanarayana, 2011). 1 ml of SDS (10%) was added to the homogenate and homogenized again. Then the samples were incubated in water bath at 60 °C for 2 h with intermittent shaking. The supernatant was collected by centrifugation at 12000 rpm under 4 °C for 20 min. DNA was precipitated by adding its 1/2 volume of PEG 6000 (30% in 1.6 M NaCl), and standing at room temperature for 1 h (Yeates and Gillings, 1998). The precipitated DNA was collected by centrifugation at 12000 rpm under 4 °C for 5 min. The supernatant was discarded, and the pellet was dissolved in 1 ml of TE buffer (pH 8.0). Thereafter, 100 ul of 5M potassium acetate were added and incubated at 4 °C for 15min. The supernatant was collected after centrifugation at 10000 rpm under 4 °C for 15 min, and treated with equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and stood at room temperature for 10 min, followed by centrifugation at 10000rpm for 10min under 4 °C. The aqueous layer was transferred into a new tube and treated with its 0.7 volume of isopropanol for 1 h at room temperature. DNA was precipitated

by centrifugation at 10000rpm for 10 min under 4 °C. The pellet was washed with 1 ml of 75% (v/v) ethanol, followed by 1 ml of anhydrous alcohol, and then centrifuged at 10000 rpm for 10 min under 4 °C, and dried at room temperature. The dried pellet was dissolved in 50 µl of TE buffer, and kept at 37 °C for 15 min after adding 2 ul of RNase A (10 mg/ml) to remove RNA (Zhou *et al.*, 1996). Finally, the DNA solution was stored under -20 °C.

M2: PowerSoil DNA isolation kit

The steps were performed following the manufacturer's instructions (Mobio, USA).

M3: Soil microbial DNA extraction method_1

Microbial DNA was extracted following the protocol of Verma and Satyanarayana (2011) based on the use of powdered activated charcoal.

M4: Soil microbial DNA extraction method_2

Microbial DNA extraction was conducted according to the protocol of Tsai and Olson (1991).

M5: Poplar genomic DNA extraction method from wood samples

Microbial DNA was extracted following the steps of Verbylaite (2010) based on the CTAB protocol.

M6: Microbial DNA extraction method from herbs stem

Microbial DNA extraction was executed according to the steps of Maropola (2015) based on the SDS protocol.

M7: DNA extraction method from isolating bacteria

Microbial DNA extraction was performed following the steps of Pindi (2013) based on the ethanol protocol.

M8: DNA extraction method from isolating fungus

The steps were conducted following the protocol of Motková and Vytrasová (2011) based on a classic method in combination with cell wall disruption by liquid nitrogen according to Cenis (1992).

DNA quantification

Concentrations of the extracted DNA were measured by NanoDrop 2000 UV-Vis spectrophotometer (Thermo, USA) at 260 nm wave length. Purity investigation was conducted as well to estimate possible contaminants. The purity was determined at A260/A280 and A260/A230, respectively.

Gel electrophoresis of the extracted DNA

To determine the quality, the extracted DNA was analysed on 40 ml of 0.8% agarose gel containing 1ul nucleic acid dye. λ/ Hind III digest (Takara clontech, Japan) was adopted as marker, and electrophoresis condition was set at 120 v for 75 min.

PCR amplification and gel electrophoresis analysis

To validate the availability of the extracted DNA, PCR amplification was performed in total 10ul volume, including 0.5 µl extracted DNA (diluted to 75 ng/ul), 3.5 µl ddH_2O, 5 µl 2×TSINGKE Master mix (Beijing TsingKe Biotech Co., Lid., China), as well as 0.5 µl forward primer and 0.5 µl reverse primer. The primer sets consisted of five pairs (Table 1), i.e., three pairs of bacterial 16S (16S V4, 16S V3-V4, 16S V4-V5) and two pairs of fungal ITS (ITS1, ITS2) (Větrovský and Baldrian, 2013; Verma and Satyanarayana, 2011; White *et al.*, 1990). PCR products were analysed on 40 ml of 1.5% agarose gel containing 1 µl nucleic acid dye.

Marker 1 (Dongsheng Biotech Co., Lid., China) was adopted as marker, and electrophoresis condition was set at 120 v for 47 min.

Table 1. Information of the primer sets

Type of primer	Primer sequences (5'-3')
Bacterial 16S V4	F: GTGCCAGCMGCCGCGGTAA R: GGACTACHVGGGTWTCTAAT
Bacterial 16S V3-V4	F: CCTAYGGGRBGCASCAG R: GGACTACNNGGGTATCTAAT
Bacterial 16S V4-V5	F: GTGCCAGCMGCCGCGGTAA R: CCGTCAATTCCTTTGAGTTT
Fungal ITS1	F: GGAAGTAAAAGTCGTAACAAGG R: GCTGCGTTCTTCATCGATGC
Fungal ITS2	F: GCATCGATGAAGAACGCAGC R: TCCTCCGCTTATTGATATGC

PCR conditions of primer 16S V4 and 16S V3-V4 were set as: (1) initial denaturation step at 94 °C for 5 min; (2) denaturation step at 94 °C for 45 s; (3) primer annealing step at 56 °C for 35 s; (4) elongation step at 72 °C for 90 s; (5) final elongation step at 72 °C for 5 min. The step 2-step 4 were repeated 35 times in 16S V4, and 38 times in 16S V3-V4, respectively.

PCR condition of primer 16S V4-V5 was as follows: (1) initial denaturation step at 94 °C for 5 min; (2) denaturation step at 94 °C for 45 s; (3) primer annealing step at 62 °C for 35 s; (4) elongation step at 72 °C for 90 s; (5) final elongation step at 72 °C for 5 min. The step 2-step 4 were repeated 32 times.

PCR condition of primer fungal ITS1 and ITS2 were set as: (1) initial denaturation step at 94 °C for 5 min; (2) denaturation step at 94 °C for 45 s; (3) primer annealing step at 60 °C for 35 s; (4) elongation step at 72 °C for 90 s; (5) final elongation step at 72 °C for 5 min. The step 2-step 4 were repeated 32 times.

Results

pH values and absolute water contents of sapwood and wetwood

In wetwood and sapwood samples, pH values were 8.41 and 7.31, respectively, and absolute water contents were, respectively, 193.74% and 69.88% in this study. It indicated that poplar wetwood was more alkaline than normal sapwood, accompanied with higher water content, identical to those reported from many other species (Moya *et al*., 2009). While, pH value is not a consistently reliable indicator of wetwood, because wetwood is generally more acidic than adjacent normal sapwood in conifers, but is usually more alkaline than normal sapwood in hardwoods. Sometimes both acidic and alkaline wetwood may be found in the same tree species (Jeremic *et al*., 2004; Schink and Ward, 1984).

Microbial DNA extraction from wetwood and sapwood

To compare efficiency of the eight methods (M1-M8) in microbial DNA extraction from poplar wetwood and sapwood, amount and purity of the extracted DNA were determined by spectrophotometry and agarose gel electrophoresis (Terrat *et al*., 2012). An efficient method should provide a sufficient amount of pure DNA which could be further amplified by PCR without containing inhibitors (Demeke and Jenkins, 2010). In the present study, only M1 provided pure DNA with high concentrations, the others (M2-M8) all displayed unsatisfactory results (Table 2).

Table 2. DNA concentrations and purities of the eight methods (vol: 50ul)

DNA extraction method	Wetwood			Sapwood		
	DNA concentration (ng/ul)	Purity A260/A280	Purity A260/A230	DNA concentration (ng/ul)	Purity A260/A280	Purity A260/A230
M1	272.27±99.39	1.87±0.02	1.66±0.05	361.83±54.72	1.85±0.04	2.24±0.06
M2	8.20±1.34	1.77±0.15	——	4.83±0.38	2.19±0.16	——
M3	33.73±14.12	1.60±0.08	0.68±0.10	126.00±40.36	1.73±0.04	1.52 ±0.23
M4	381.00±142.90	1.49±0.05	1.77±0.26	805.70±175.66	1.46±0.02	1.40±0.03
M5	6.43±1.34	1.22±0.03	——	1.83±0.57	0.97±0.20	——
M6	250.30±58.20	1.12±0.09	0.77±0.15	459.90±38.84	0.94±0.03	1.00±0.11
M7	455.90±76.22	1.29±0.05	0.85±0.10	477.37±145.95	1.26±0.05	0.84±0.08
M8	219.50±56.87	1.24±0.03	0.46±0.04	130.27±19.46	1.43±0.02	0.63±0.04

Note: Mean value of triplicate data ± standard error (Mean ± *S.E.*, $n = 3$); "——" means undetectable. M1: Protocol for microbial DNA extraction from living poplar wood; M2: PowerSoil DNA isolation kit (Mobio, USA); M3: Soil microbial DNA extraction method_1 (Verma and Satyanarayana, 2011); M4: Soil microbial DNA extraction method_2 (Tsai and Olson, 1991); M5: Poplar genomic DNA extraction method from wood sample (Verbylaite *et al.*, 2010); M6: Microbial DNA extraction method from herbs stems (Maropola *et al.*, 2015); M7: DNA extraction method from isolating bacteria (Pindi *et al.*, 2013); M8: DNA extraction method from isolating fungus (Motková and Vytrasová, 2011). The same below for the methods of M1-M8.

The concentrations of DNA extracted by M1 varied from 100.5 to 444.8 ng/ul in wetwood tissues, with an average of 272.3 ng/ul, and in sapwood these values were 287.6, 468.6 and 361.8 ng/ul, respectively. All DNA exhibited good purity with acceptable absorbance ratios of A260/A230 (DNA/humic acid) and A260/A280 (DNA/protein). In wetwood, the ratios were 1.66 and 1.87, and in sapwood were 2.24 and 1.85, respectively. That means the DNA extracted by M1 was suitable for downstream processing, and thus confirmed the producibility of M1 protocol (Table 2). Whereas, the other methods (M2-M8) did not meet the concentration and purity requisites. In terms of M2 and M5, microbial DNA was hardly detected in both wetwood and sapwood (Table 2). For M3, M4, M6, M7 and M8, high DNA concentrations were observed in both wetwood and sapwood, but their purities were unacceptable for further application.

Agarose gel electrophoresis visualized qualities of the extracted DNA, and further manifested efficiencies of the eight methods (M1-M8) in isolating microbial DNA from poplar wetwood and sapwood (Figure 2). In wetwood samples, most sizes of the DNA extracted by M1 and M3 were around 23 kb. Smearing of the smaller fragments should result from the DNA-shearing effect (Tsai and Olson, 1991). In sapwood samples, only M1 showed visualized microbial DNA, and the other methods showed nothing.

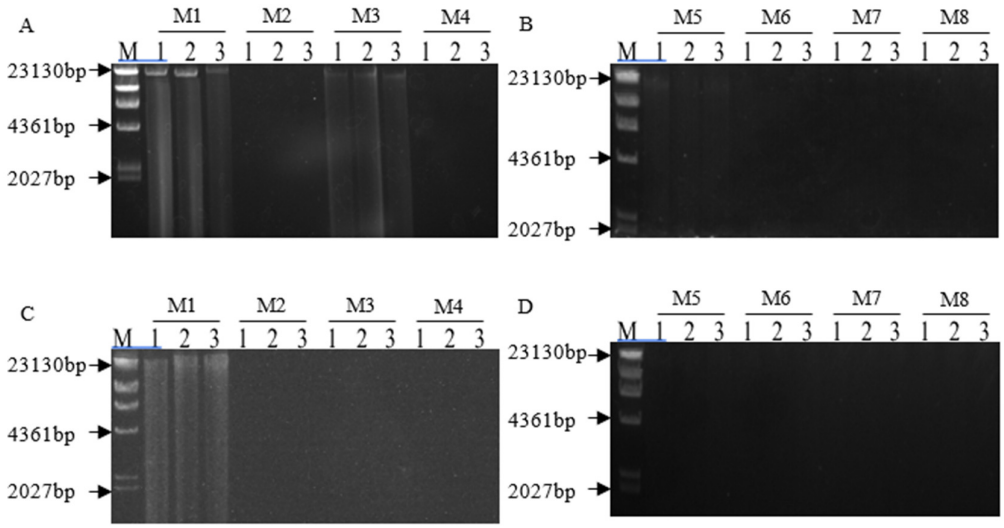

Figure 2. Agarose (0.8%, w/v) gel electrophoresis (120 V, 75 min) of microbial DNA extracted by the eight protocols

A and B showed microbial DNA from wetwood; C and D showed microbial DNA from sapwood. M stood for molecular size marker (λ/ Hind III, ladders from top to bottom were 23130 bp, 9416 bp, 6557 bp, 4361 bp, 2322 bp and 2027 bp, respectively). Lane 1, 2 and 3 showed three repeats of each method.

PCR amplification

To further test the quality of the extracted microbial DNA, PCR amplifications with five primer sets (Table 1) were performed in the present study (Karakousis *et al*., 2006). For M1, all primer sets showed stable fine PCR products with right sizes in both wetwood and sapwood. While, in microbial DNA from M2, M3 and M4, target DNA fragments could not be stably amplified by all of the five primer sets (Figure 3). For instance, the DNA from M2 was totally or partly failed to be amplified by primer 16S V4, ITS1 and ITS2. In terms of M5, M6, M7 and M8, no PCR amplicons were obtained successfully, except of fungal ITS2 primer in sapwood extracted by M5 (Figure 3). Therefore, the PCR amplification results further confirmed that M1 was the most effective extraction protocol, in agreement with the results of DNA quantification.

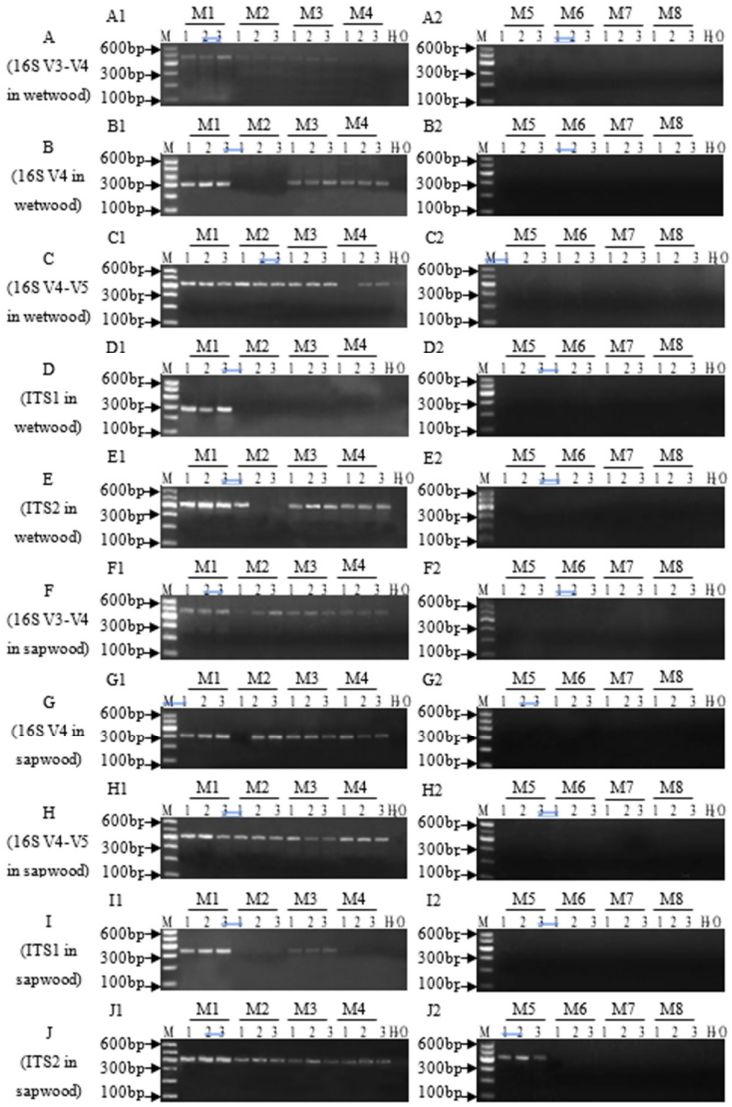

Figure 3. Agarose (1.5%, w/v) gel electrophoresis (120 v, 47 min) of the PCR amplicons (6 primer sets) from microbial DNA extracted by the eight protocols

A, B, C, D and E showed PCR products amplified by primer of bacterial 16S V3-V4, 16S V4, 16S V4-V5, fungal ITS1 and ITS2 from wetwood, respectively. F, G, H, I and J showed PCR products amplified by primer of bacterial 16S V3-V4, 16S V4, 16S V4-V5, fungal ITS1 and ITS2 from sapwood, respectively. M stood for molecular size marker (Marker 1, ladders from top to bottom were 100 bp, 200 bp, 300 bp, 400 bp, 500 bp and 600 bp, respectively). Lane 1, 2 and 3 showed three repeats of each method. H_2O showed the blank control.

Discussion

Metagenomic DNA yield relies on the efficiency of cell lysis step, which includes mechanical (e.g. grinding and dead-mill) and chemical (e.g. enzymatic lysis) processes for cell disruption (Moré *et al*., 1994). In order to obtain high-quality DNA, it also has to undergo several cleaning steps. In the present study, M1 applied combination of tissue grinding under liquid nitrogen and lysis buffers, followed by a number of cleaning steps. To overcome purity constraints of metagenomic DNA, M1 incorporated PAC and PVPP to remove impurity

substances significantly, such as polyphenolics, tannins and humic substances, which was adopted from Verma and Satyanarayana (2011). But we increased the concentration of PAC compared with them, which absorbed and removed more impurity substances, and did not raise brown colour of the slurry. PAC has a vast surface area and pore volume, which has been extensively used for removal of humic acid, lignin sulfonate, tannic acid, arabic gum polyphenolic compounds, many biodegradable/ non-biodegradable-coloured compounds, and heavy metals (Corapcioglu and Huang, 1987; Logan *et al*., 1997; Seo and Ohgaki, 2001). The precipitation of microbial DNA by using 30% PEG 6000 was the third approach. PEG has been used for the precipitation of soil metagenome (Martin-Laurent *et al*., 2001; Rondon *et al*., 2000), since isopropanol or ethanol favours precipitation of DNA along with humic substances, while PEG does not coprecipitate humic substances (Yeates *et al*., 1997). Verma and Satyanarayana (2011) precipitated pure DNA from alkaline soil and sediment samples by use of 30% PEG 8000. In the present study, 30% PEG 6000 precipitated higher-quality metagenomic DNA than 30% PEG 8000 (data not shown). At this stage, the microbial DNA became almost free from humic substances. As PEG is supposed to be an interfering agent in PCR reactions, DNA was finally precipitated with isopropanol after routine phenol-chloroform-isoamyl alcohol treatment to remove PEG. All DNA extracted by M1 exhibited good purity with acceptable absorbance ratios (Table 2) and visualized agarose gel electrophoresis (Figure 2), and thus confirmed the reproducibility of the protocol.

Conclusions

Poplar wetwood contained more alkaline and higher absolute water contents than normal sapwood. Our protocol (M1) could fully lysed microbial cell, released microbial DNA, removed impurities along with the DNA and finally obtained amount of pure microbial DNA. The extracted DNA was suitable for further PCR amplification and downstream processing. Following the protocol, we have already successfully investigated microbial community diversities in living poplar wetwood and sapwood by metagenomic libraries construction and 16S rDNA/ ITS sequencing. More than 3000 OTUs (Operational Taxonomic Units) were identified from wetwood and sapwood, respectively, by16S rDNA sequencing (such as *Methanobacterium*, *Methanosaeta* and *Bacteroides* etc.), and more than 300 OTUs were identified as well by ITS sequencing (such as *Agaricomycetes*, *Xylaria* and *Devriesia*, and etc.) (data not shown). Therefore, our protocol (M1) provided a possible approach for microbial ecologists to study microbial DNA from wood samples in a more accurate way by molecular biology techniques.

Authors' Contributions

Implementation of the experiment: XLY and XYH; Writing- original draft: XLY, XYH and XXW; Writing- review and editing: XYZ and KBD; Data curation: XLY; Formal analysis: XXW; Funding acquisition: XYZ and KBD.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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