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An Improved Protocol for Extraction of Metagenomic DNA from High Humus, Alkaline and Saline Soil of Chinampa for T-RFLP Fingerprinting Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SEJ and ETW performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SEJ, FLY, RFH, YTC, FNRO, HLY and ETW managed the experiments of the study. Authors SEJ and ETW managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The principle aim of this study was to obtain high quality metagenomic DNA from the high humus-containing, alkaline soils of the chinampas, an artificial sustainable agro-ecosystem.
Study Design: Different protocols reported previously were tested and were modified to extract the metagenomic DNA. Quality of the DNA samples was evaluated by amplification of 16S rRNA gene with PCR and T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis.
Place and Duration of Study: This study was performed in Department of Microbiology, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional during 2011-2012.
Methodology: Four soil samples were collected from two chinampas at the depth of 0-30

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cm and 30-60cm. A protocol started with repeated prewashing before the direct cell lysis with lysozyme followed by SDS treatments, frozen and melting cycling was developed which combined the DNA isolation and purification procedures. The 16SrRNA genes were amplified from the extracted metagenomic DNAs and were used for T-RFLP fingerprinting analysis.

Results: The 16SrRNA genes were amplified from all the DNA extracts corresponding to the four soil samples and were successfully used in the T-RFLP analysis, which generated 25 to 109T-RFs in the four soil samples digested separately with the restriction endonucleases *Hae*III, *Hha*I and *Msp*I.

Conclusion: The protocol developed in the present study could generate high molecular weight and high quality metagenomic DNA from soils with high content of humic materials, for which the other reported protocols were not functioned. This soil harboured very diverse and unique bacterial communities belonging to at least nine phyla that might contribute to the high soil fertility.

Keywords: Metagenomics; DNA extraction; humic acids; T-RFLP fingerprinting; bacterial communities.

1. INTRODUCTION

The diversity and functions of microorganisms in different environments are two principle aspects in the study of microbial ecology. In the last decades, the application of genomic tools have dramatically improved microbial ecological studies and drastically expanded our knowledge about microbial world [1]. It has been estimated that microorganisms constitute two third of the Earth's biological diversity, and about 86% of existing species on Earth and 91% of species in the ocean still remain unrecognized [2]. Metagenomics is the genomic analysis of microorganisms based upon the DNAs extracted directly from their natural environment, through which the unexplored microbial diversity can be captured [3] and specific functional genes can be screened [4].

In the study of metagenomics, the first and basic step is the extraction of pure metagenomic DNA and/or RNA from the environmental samples, which are subsequently used for PCR analysis. For this purpose, distinct protocols of metagenomic DNA extraction have been developed referring to different environment samples [5-11], or in order to detect some special microorganisms, such as *Mycobacterium avium* [12] or fungi [13]. Some of these methods have been compared experimentally [14] or reviewed [15-17]. Although many methods about the extraction of metagenomic DNA have been described, they cannot fit all the environmental samples, because the characteristics of this material varied dramatically, and different organisms have distinctive cellular structures and chemical compounds. Therefore, some novel or modified methods specific for different environmental samples are still emerging [18,19], and optimized methods are screened for some specific soils [13,20].

The chinampas refer to the artificial floating islands started about 3000 years ago in the Valley of Mexico, including Xochimilco in Mexico City, in which trunks and twigs of trees were used to construct the basis to support the soil composing of vegetable wastes and sediment of the lake. Water channels surround the islands [21]. They are still used as a sustainable agro-system to produce vegetables and ornamental flowers without or with little apply of chemical fertilizers and pesticides. Since 1989 Xochimilco is a site included into World Heritage List of UNESCO. In relation to its chemical composition and floating environment, the mature soil of chinampa is rich in humic material, high pH and high salinity.

Many studies have been done to evaluate the physicochemical characters of the chinampa soil [21], but little microbiological information is available about this soil [22], although the microbiota is an important factor to maintain the productivity of soil.

Aiming at investigating the bacterial diversity in soil of chinampa with metagenomic study, we realized the extraction of DNA from this soil with various methods and an improved protocol was established.

2. MATERIALS AND METHODS

2.1. Soil Sampling

Bulk soils were sampled in 2012 from two chinampas far away from the touristic zone in Xochimilco district, Mexico City. In each chinampa, the surface (0-30cm depth) and subsurface (30-60cm) were sampled. The soils samples were recollected from five points (four corners and the centre), stored in black plastic bags, and transported directly to the laboratory, where the five soil samples from each chinampa at each depth were pooled to form a compiled sample. The sampled soils showed a black colour, indicating the high content of organic matter, mainly humus with high melanisation and high molecular weight [21]. All soil samples were kept at 4°C until their utilization for soil physicochemical characterization by the routine methods [22] and for DNA extraction.

2.2 Quantification of Culturable Mesophilic Aerobic Bacteria

For each soil sample, decimal dilutions were prepared in sterilized NaCl solution (0.85%). An aliquot of 0.1mL of the dilutions 10^{-4}, 10^{-5} and 10^{-6} was spread in duplicate in Petri dishes containing PY medium (peptone, 5g; yeast extract, 3g; $CaCl_2$, 0.6g; agar, 18g; distilled water, 1L; pH8.0). The plates were incubated at 28°C during 48 h. Colonies were counted manually and abundance of mesophilic aerobic bacteria was calculated as colony forming units (CFU) in each gram of dry soil.

2.3 Extraction of Metagenomic DNA from the Soil Samples

In this study, several protocols [7,9,11,23,24] were employed previously, and the protocol of Ceja-Navarro et al. [23] modified by adding prewashing and substituting the flocculant agent was finally used. In starting, 5.0g of soil were suspended with 25ml of pyrophosphate sodium ($Na_4P_2O_7$,0.15M) in a 50ml Oak Ridge tube (Nalgene™, Sigma-Aldrich®) to dissolve the humic material. The tube was vortexed for 1 min, following by 10min of precipitation and then centrifuged 10min at 7,700×g under room temperature. The supernatant was discarded and the washing procedure was repeated 5 times. The sediment was re suspended in 5ml of phosphate buffer (0.15M NaH_2PO_4; pH8.0). After 10min of precipitation, the suspension was vortexed for 1min and centrifuged 10min at 7,700×g under 25°C, then the supernatant was discarded and this washing procedure was repeated 4 times.

The washed sediment was suspended in 5ml of lysis solution I (NaCl 0.15M; EDTA 0.1M; pH=8.0; 10mgml^{-1} of lysozyme), vortexed and incubated at 37°C for 1h, with briefly vortexing each 20min. Then, 5ml of the lysis solution II (NaCl 0.1 M; Tris-HCl 0.5M; 12% SDS; pH 8.0) were added and the mixture was maintained at -20°C for 20min, and at 65°C for another 20min. This frozen-melting cycle was repeated two times and in the first cycle, 5ml of $Al_2(SO_4)_3$ (0.3M) as flocculant were added to eliminate the humic material. The mixture was

vortexed and the frozen-melting cycle was repeated as mentioned above. The mixture was centrifuged at 7,700×g during 10min. The supernatant was transferred into a new sterilized Oak Ridge tube and was mixed with 2.7ml of NaCl (5M) and 2.1ml of Triton X-100 (10% in 0.7M NaCl solution). After a incubation at 65°C for 10min, 12ml of a chloroform: isoamylic alcohol (24:1, v/v) solution were added and the tube was mixed gently by inversion and centrifuged at 3000×g for 30min. The aqueous phase was transferred into a clean tube and mixed with 12ml of Polyethylenglycol (PEG 13%; NaCl 1.6M) and maintained on ice overnight. The precipitated metagenomic DNA was collected by centrifugation at 12,000×g for 30 min at 4°C. The DNA pellet was resuspended in 500µl of deionized sterile water, transferred into a sterilized Eppendorf tube, and mixed with 1 volume of absolute ethanol. After incubated at 4°C for 30min, the tube was centrifuged at 12,000×g for 30min at 4°C. The supernatant was discarded and the precipitated DNA was washed with 500µl of cold ethanol (70%, v/v). The DNA pellet was dried by incubating the tube at 65°C for 10min. Then it was dissolved in 500µl of deionized sterile water and was stored at -20°C.

2.4 Evaluation of DNA Concentration and Quality

The integrity and concentration of DNA sample was estimated by electrophoresis in 1% (w/v) agarose gel with 0.5×TBE as electrode buffer as described elsewhere [25]. The DNA sample (1µl) was loaded in the gel, and the 1Kb ladder (Invitrogen™) was included to estimate the molecular size and concentration of extracted DNA. After the electrophoresis and stained by ethidium bromide (0.5µg ml^{-1}), the gel was observed under UV light and was photographed with Multimage™ Light Cabinet (Alpha Innotech Corporation). DNA concentration was measured by UV- spectrophotometry: 1µL of DNA solution was diluted to 50µL with sterilized deionized and the concentration of DNA was read at 260nm directly from the spectrophotometer (Epoch-Biotek™, with software Gen5 take 3).

2.5 T-RFLP Analysis

For T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis, the fragments of 16S rRNA gene were amplified from the metagenomic DNA extracts by PCR as described by Sun et al. [26], with the primers 27f (5´-AGA GTT TGA TCM TGG CTC AG-´3) marked with fluorescently carboxyfluorescein (6-FAM) and 1495r (5´-CTA CGG CTA CCT TGT TAC GA-3´). Then, 10µL of each PCR product were digested with 10U of the restriction endonucleases *Msp*I, *Hha*I and *Hae*III, respectively, at 37°C for 3h. The digests were purified and electrophorized as reported previously [26]. The electrophoretic patterns were analyzed with the GeneMarker software (SoftGenetics®) and only the terminal restriction fragments (T-RF) between 50 and 500pb were chosen for further analysis. The Shannon-Weaver (*H´*) and Simpson (1-*D*) indices were calculated by using PAST 2.17c software [27] for estimation of the bacterial diversity in each soil. The T-RF patterns were used to identify the bacteria by using the data in National Center for Biotechnology Information Database (http://trflp.limnology.wisc.edu/index.jsp) [28]. Only the unambiguous fragments were designed into the bacterial taxa (phylum and/or class) [29].

3. RESULTS AND DISCUSSION

3.1 Soil Characterization and Quantification of Mesophilic Aerobic Bacteria

The physicochemical characteristics of the chinampa soils are presented in Table 1. In general, the soils are sandy clay loam, with alkaline pH ranged between 8.0 and 8.8, with

high salinity (2054-4468mgKkg^{-1}), and rich in organic materials (4.6-7.5%). The abundance of mesophilic aerobic bacteria was about 10^7 CFU g^{-1} of dry soil, implying that the major form of organic matter in the chinampa soils was humus, which is difficult for degradation and does not support the growth of high numbers of heterotrophic bacteria.

3.2 Extraction of Metagenomic DNA from Soil

In our study, the DNA extracted from the chinampa soils with commonly used protocols either showed brown colour, or did not produce PCR fragments, even after the post purification procedures. The post purification procedures were 1) using of the silica columns (QUIAGEN ® and MO BIO ®) as recommended by the manufacturer; 2) purifying the DNA band recovered from the agarose gel (0.7%) after electrophoresis with QIAamp DNA Mini Kit (QUIAGEN) following the manufacturer´s guide; and 3) flocculation of 500 µl DNA solution with 250µl of $Al_2(SO_4)_3$ (10mM) and 50µl of phosphate buffer (0.15M NaH_2PO_4; pH8.0). These procedures could eliminate the brown colour, but the purified DNA samples were still not good for amplification by PCR. With the modified protocol of Ceja-Navarro et al. [23], the humic acids were eliminated gradually from the soil samples, because the brown colour in the supernatants was decreased time by time, until disappeared completely. The DNA extracts had high molecular size (>12 kb) without degradation Fig. 1. and the yield was 18 to 34ngg^{-1} soil. The obtained DNA samples were good for gene amplification by PCR using BSA (Bovine Serum Albumine) as PCR additive.

Table 1. Characteristics of soil samples collected from two chinampa soils

Soil feature	Soil samples *			
	Ch Is	Ch Id	Ch IIs	Ch IId
Organic matter (%)	7.5	4.6	7.5	6.4
Total N (%)	0.80	0.56	0.75	0.58
Total P (mgkg^{-1})	22.4	17.4	3.7	19.6
Total K (mgkg^{-1})	4468	3539	3728	2054
pH	8.6	8.2	8.7	8.0
Water Retention Capacity (%)	120.2	116.7	148.0	112.1
Humidity (%)	24	44	38	48
Humidity/ WRC (%)	19.97	37.70	25.68	42.82
CFU g^{-1} dry soil	5.07×10^7	3.45×10^7	3.45×10^7	4.04×10^7

** Ch Is, Ch Id, Ch IIs, and Ch IId represent soil sampled from chinampa I (0-30cm), chinampa I (30-60cm), chinampa II (0-30 cm), and chinampa II (30-60cm), respectively*

3.3 Analysis of T-RFLP

T-RFLP is a technique widely used for investigation of microbial diversity in environmental samples [30-32]. With the metagenomic DNAs obtained in this study, 25 to 109T-RFs were obtained from the 16S rRNA amplicons digested by the three restriction endonucleases Table 2. The Shannon and Simpson indices were very similar in the four samples Table 2. both the Shannon indices greater than 3 [29] and the Simpson indices near to one [32] indicated that the bacterial communities in the chinampa soils were very diverse and composed of many species.

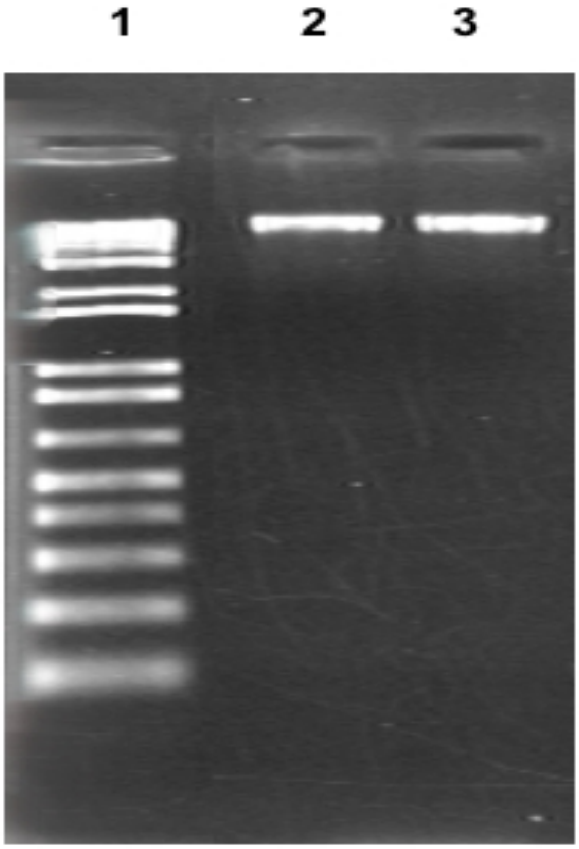

Fig. 1. Metagenomic DNA extracted with the improved method developed in this study: 1, 1 Kb DNA Ladder; 2, DNA from Ch IIs; 3, DNA from Ch IId

Comparing with the database, the T-RFs obtained in this study were defined into nine Phyla Fig. 2. Firmicutes was the most abundant one in all the four samples, with 18.9% to 24.19%. The second abundant group was Phylum *Proteobacteria*, followed by Phylum *Actinobacteria*. The minor groups were Phyla *Chloroflexi*, *Cyanobacteria* and *Planctomycetes* etc. About 25% of the T-RFs could not defined into any phylum, which might imply the existence of novel taxa or the limit of information in the database and of the T-RFLP technique.

Compared with the previous results obtained from other soils with some similar characteristics, the community composition of bacteria in the chinampa soils was unique. Proteobacteria, Firmicutes, Actinobacteria and Bacteoiredetes were the major bacterial groups in alkaline (pH 8.28-8.45) crop soils in China [26]. *Proteobacteria, Verrucomicrobia* and *Chloroflexi* were most abundant phyla in the soils of constructed wetland (pH 6.23-6.59; organic material 13.54-29.75%) in Spain [33]. While *Proteobacteria*, *Acidobacteria*, *Actinobacteria, Bacteroidetes* and Firmicutes made up more than 80% of the bacteria sequences in created wetland (pH4.2-5.8, organic material 3-6%) in Virginia, USA [34]. In addition, *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were the most abundant groups in an alkaline saline soil (pH 9.0, organic material 1.29%) in Mexico [35]. Based upon all the previous studies mention here and the results obtained in the present study, it was clear that the *Proteobacteria* group always exist as one of the predominant group, and the other

dominant groups, as well as the relative abundance of *Proteobacteria*, varied according to the soil characters, like pH, humidity, saline and maybe also the content and type of organic matter.

Table 2. T-RFs and diversity estimated from the T-RFLP analysis in the chinampa soil samples

Soil simple	*Hha* I			*Msp* I			*Hae* III			Average		
	T-RFs	H´*	1-D*	T-RFs	H´	1-D	T-RFs	H´	1-D	T-RFs	H´	1-D
Ch Is	89	4.48	0.98	102	4.62	0.99	79	4.36	0.98	90.0	4.49	0.98
Ch Id	80	4.38	0.98	109	4.69	0.99	63	4.14	0.98	84.0	4.40	0.98
Ch IIs	97	4.57	0.98	53	3.97	0.98	70	4.24	0.98	73.3	4.26	0.98
Ch IId	61	4.11	0.98	81	4.39	0.98	25	3.21	0.96	55.7	3.90	0.97
Average	81.8	4.38	0.98	86.2	4.67	0.98	59.2	3.99	0.98	75.7	4.26	0.98

**. H´, Shannon Index; 1-D, Simpson Diversity Index*

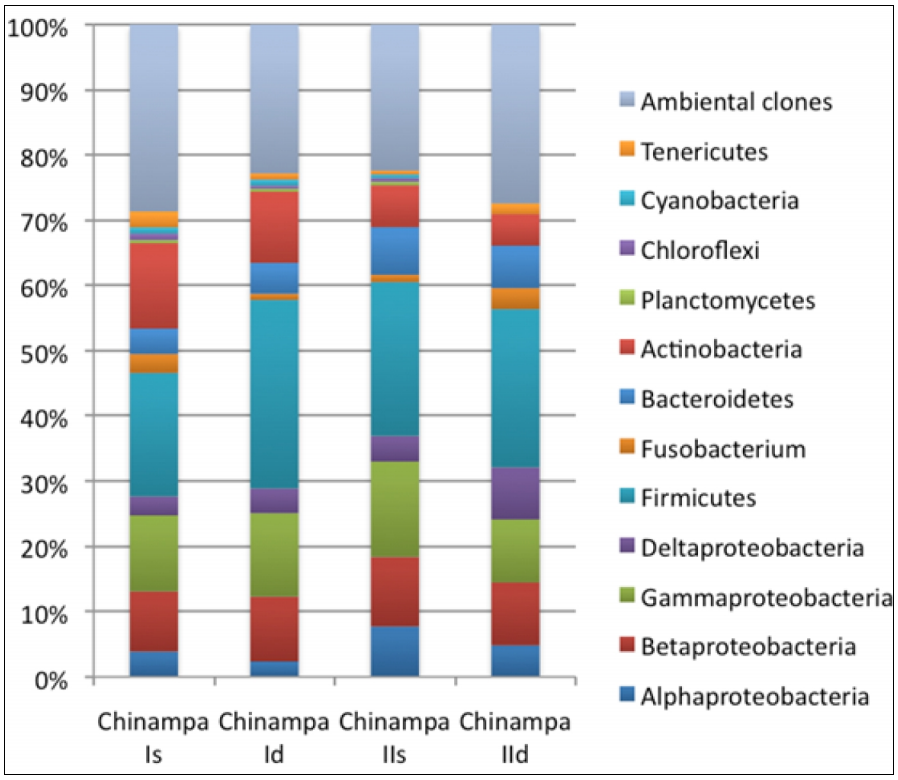

Fig. 2. Community composition of bacteria and relative abundance of different bacteria groups estimated from the T-RFLP analysis in the chinampa soils. Ch Is, Ch Id, Ch IIs, and Ch IId represent soil sampled from chinampa I (0-30cm), chinampa I (30-60cm), chinampa II (0-30cm), and chinampa II (30-60cm), respectively

4. CONCLUSION

The protocol modified in the present study could generate high molecular weight and high quality metagenomic DNA from soils with high content of humic materials, for which the other reported protocols were not functioned. The key points were the prewashing of the soil to eliminate contamination of humic acids and $Al_2(SO4)_3$ as flocculant agent. This protocol is recommendable for soils with similar characters. The DNA samples extracted with this protocol were adequate for PCR amplification and T-RFLP analysis, which revealed that the chinampa soils harboured very diverse and unique bacterial communities belonging to at least nine phyla. Further studies on the diversity are undergoing by other molecular methods.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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