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An Improved Method for Extracting Bacteria from Soil for High Molecular Weight DNA Recovery and BAC Library Construction

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Separation of bacterial cells from soil is a key step in the construction of metagenomic BAC libraries with large DNA inserts. Our results showed that when combined with sodium pyro-phosphate and homogenization for soil dispersion, sucrose density gradient centrifugation (SDGC) was more effective at separating bacteria from soil than was low speed centrifugation (LSC). More than 70% of the cells, along with some soil colloids, were recovered with one round of centrifugation. A solution of 0.8% NaCl was used to resuspend these cell and soil pellets for purification with nycodenz density gradient centrifugation (NDGC). After purification, more than 30% of the bacterial cells in the primary soil were extracted. This procedure effectively removed soil contamination and yielded sufficient cells for high molecular weight (HMW) DNA isolation. Ribosomal intergenic spacer analysis (RISA) showed that the microbial community structure of the extracted cells was similar to that of the primary soil, suggesting that this extraction procedure did not significantly change the the soil bacteria community structure. HMW DNA was isolated from bacterial cells extracted from red soil for metagenomic BAC library construction. This library contained DNA inserts of more than 200 Mb with an average size of 75 kb.

Keywords: improved extraction method, sucrose, RISA, HMW DNA, metagenomic BAC library

Soil, one of the main reservoirs of miacrobial diversity, supports an immense diversity of microbes that remain largely unexplored (Torsvik and Øvreås, 2002). It is generally thought that less than 1% of microbes are readily culturable with known cultivation techniques (Rosselló-Mora and Amann, 2001). Metagenomics, an alternative strategy to cultivation, is based on the recovery of DNA from entire microbial communities and cloning the DNA into appropriate vectors. Metagenomics has emerged as a powerful tool for gaining access to the physiology and genetics of uncultured micro-organisms, as well as for exploring the functional genes and novel metabolites (Daniel, 2004; Handelsman, 2004). To improve cloning efficiency so that the clones in a gene library provide an acceptable representation of the entire metagenome, and to maximize the probability of cloning entire gene clusters encoding biosynthetic pathways of secondary metabolites (Bertrand *et al.*, 2005), HMW DNA extraction methods and cloning strategies have been developed.

The pivotal step for isolation of HMW DNA from soil is to obtain a sufficient quantity of bacterial cells. However, many soil microorganisms are closely associated with the organic matrix of soil particles, and they produce extracellular polymeric substances (EPS) that promote the irreversible adhesion of cells to soil particles (Bockelmann *et al.*, 2003). All the described methods for bacterial extraction from soil have two steps in common: dispersion of the soil followed by separation of cells from soil particles (Lindahl and Bakken, 1995). First, maximum dispersion of the soil by physical and chemical procedures is crucial for releasing bacteria entrapped within soil aggregates and thus increasing the yield of extracted bacteria (enzymatic treatment is also used sometimes) (Bockelmann *et al.*, 2003; Ehlers *et al.*, 2008). Second, separation of cells from soil particles by centrifugation is also important. Usually, the separation is conducted either on the basis of sedimentation rate with LSC, or on the basis of buoyant density with NDGC (Bakken, 1985).

Bacterial cells extracted from soil for HMW DNA isolation must be pure enough so that the isolated DNA can ensure optimal efficiency of the subsequent enzymatic reactions (Maron *et al.*, 2006). Compared to LSC, NDGC is preferable for two reasons: one is that the yield is similar to or even slightly higher than that obtained with LSC, and the other is that the purity is dramatically improved (Lindahl and Bakken, 1995). For different soil types, the cell yields vary from 3% to 36% of total soil bacteria with the NDGC method (Lindahl and Bakken, 1995; Lindahl, 1996; Mayr *et al.*, 1999; Berry *et al.*, 2003; Bertrand *et al.*, 2005; Bakken and Frostegård, 2006). Usually, the extraction procedure involves either directly extracting bacterial cells from soil particles with NDGC after soil dispersion, or separating the released bacteria from soil with repeated LSC first, and then concentrating them, and finally using NDGC for purification of these cells (Bertrand *et al.*, 2005; Maron *et al.*, 2006). For both methods, much time and labor is required and the cell yields are low, especially from highly weathered, clayey and acidic tropical soils (e.g., red soil in south China), from which the cell yields are no more than 10% (Ehlers *et al.*, 2008). How to get high cell yields from this type of soil with a simple method is a problem

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that needs to be solved.

The aim of this work was to develop an effective method for extracting bacteria from clayey soil for HMW DNA recovery and BAC library construction. Two clayey soils differing in their chemical properties were used as sources for bacterial cell extraction. Different soil dispersion treatments and bacterial extraction methods were compared by estimating bacterial abundance with 4,6-diamidino-2-phenylindole (DAPI). Additionally, RISA was used to determine whether the extracted cells were qualitatively representative of the total soil bacterial community. Finally, HMW DNA was isolated from bacteria extracted from red soil and cloned into BACs to construct a metagenomic library.

Materials and Methods

Soil samples

Soil samples were collected from depths of 5-20 cm in October 2008 from Yingtan Red soil Ecological Station of the Chinese Academy of Science and Zijin Mountain in Nanjing, China. After root removal, soil samples were sieved through a 2-mm mesh and stored at 4°C.

Evaluation of soil dispersion methods

Samples of all the treatments were centrifuged at a speed of 250×g for 2 min, then the supernatants and sediments, as well as the primary soil, were stained with DAPI after dilution for cell counts as described by Furtadoa (Furtadoa and Casper, 2000). The extraction efficiency was calculated as the number of separated cells in the supernatant as a percentage of the total number of bacterial cells in the primary soil.

Physical dispersion: Different physical dispersion methods were tested in sterilized distilled water with a tissue homogenizer (Joyoung JYL-350, fixed speed of 22,000 rpm), an ultrasonic probe (Kubota, Insonator-201M) and an ultrasonic bath (Autoscience, AS2060B), as described previously (Buesing and Gessner, 2002; Falcioni *et al.*, 2006). Numbers of viable bacterial cells were determined by colony counting of soil bacteria as described by Maron (Maron *et al.*, 2006) to monitor cell damage during dispersion.

Chemical dispersant: For homogenization, 10 g of soil was suspended in 100 ml of various chemical dispersants including sterilized distilled water (Bååth, 1996), 0.2% sodium pyro-phosphate (Bakken and Lindahl, 1995), 0.8% NaCl (Yu *et al.*, 1995), TN disruption buffer (0.2 M NaCl, 50 mM Tris·HCl) (Berry *et al.*, 2003), 10% methanol (Lunau *et al.*, 2005), 25 mg/ml PEG6000 (Lindahl and Bakken, 1995), 0.5% sodium citrate (Katayama *et al.*, 1998), 0.05% TritonX-100 (Katayama *et al.*, 1998), 0.1% Tween-80 (Bakken and Lindahl, 1995), and 50 mM Tris HCl (Niepold *et al.*, 1979), adjusting the solution pH to 7.5.

Enzymatic treatment: An enzymatic treatment to release bacterial cells from soil particles was performed following a protocol described previously (Bockelmann *et al.*, 2003), but using the enzyme cellulase (pH 5.5, Worthington) instead of lipase.

Bacterial extraction with SDGC

Sucrose solution (100 ml of 1.33 g/ml) was poured into a 250-ml polypropylene centrifuge tube, and then the same volume of soil homogenate in 0.2% sodium pyro-phosphate was carefully layered onto the sucrose. This biphasic gradient was centrifuged at 5,500×g for 2 min in an R12A3 rotor (Hitachi CR-21GII centrifuge) with slow acceleration and deceleration. The clear diluted upper sucrose fraction containing the bacterial cells was poured into a new 250-ml polyallomer centrifuge tube, diluted with 1/3 volume of 0.8% NaCl and centrifuged at 20,000×g for 10 min at 4°C. The bacterial cells, along with soil colloids of a similar density, were concentrated and resuspended in a small volume for purification with NDGC.

Different resuspension solutions for purification with NDGC

After SDGC, the cell and soil pellets were resuspended in sterilized distilled water, 0.8% NaCl, or 0.2% sodium pyro-phosphate. Twenty milliliters of each concentrate was transferred to a 40-ml sterilized centrifuge tube and 7 ml of nycodenz (Axis-Shield, Norway, density 1.310±0.002 g/ml) (Rickwood *et al.*, 1982) was carefully pipetted to form a layer below the homogenate. The tubes were placed in a Hitachi CR-21GII centrifuge R20A2 swing-out rotor and centrifuged at 10,900×g for 30 min at 4°C with slow acceleration and deceleration. A faint whitish band containing bacterial cells was resolved at the interface between the nycodenz and the aqueous layer. This band was recovered with a pipette and transferred into a new 40-ml sterilized centrifuge tube. Sufficient phosphate buffered saline (PBS) was added to resuspend the cells, and then the cells were pelleted by centrifugation at 20,000×g for 10 min at 4°C. Finally, the cell pellets were resuspended in 0.5 ml PBS and saved for RISA and HMW DNA isolation.

Soil microbial community structure determination by RISA

To determine for either soil whether the extracted cells were qualitatively representative of the soil bacterial community, the community structure of the extracted cells was compared to that of the primary soil.

The total DNA of the extracted bacterial cells and primary soil was extracted by the direct lysis method described by Zhou *et al.* (1996), and the crude DNA was purified using the dialysis method.

RISA was performed following the protocol described by Ranjard *et al.* (2000). Band detection and analysis was performed by Quantity One 4.4.0 (Bio-Rad).

HMW DNA isolation and BAC library construction

HMW DNA was isolated as described by Berry *et al.* (2003). BAC library construction and analysis were conducted following a previously described protocol (Stein *et al.*, 1996; Luo and Wing, 2003). Pulsed field gel electrophoresis (PFGE) of DNA fragments was achieved with a PFGE system (Bio-Rad) following the instructions.

Results and Discussion

Evaluation of soil dispersion methods

Homogenization and sonication are commonly used methods for soil dispersion (Furtadoa and Casper, 2000; Buesing and Gessner, 2002; Falcioni *et al.*, 2006). Because of the strong bonds between soil particles and bacteria, disrupting them might result in severe cell damage (Bakken and Lindahl, 1995). Therefore, the dispersion procedure must consider cell survival as well as dispersion efficiency. Due to its higher dispersion efficiency and lower cell damage compared to sonication (Table 1), homogenization was chosen for soil dispersion.

Dispersion efficiency was better with the use of sodium pyro-phosphate compared to the other chemical dispersants (Table 2). The reason might be that sodium pyro-phosphate alters the structure of extracellular polymers and extracts some of the polysaccharides with dispersive effects on soil, and the separated bacterial cells thus fail reassemble into large aggregates (Oades, 1984; Lindahl, 1996).

Table 1. Effects of different physical dispersion procedures on extraction efficiency and cell damage

Dispersion procedure	Yield×10^9 cells/g soil		Yield (%)		Vital bacteria×10^7 cells/g soil		Cell damage (%)	
	a	b	a	b	a	b	a	b
Primary soil	6.76±0.14	4.39±0.22			6.07±0.22	3.34±0.21		
Tissue homogenizer	3.61±0.15	2.26±0.19	53.40	51.48	6.02±0.31	3.22±0.27	0.82	3.59
Ultrasonic probe	2.97±0.22	1.78±0.23	43.93	40.55	5.41±0.27	2.83±0.19	10.87	15.30
Ultrasonic bath	1.72±0.17	1.05±0.11	28.25	23.92	4.65±0.22	2.17±0.14	23.39	35.03

a, Red soil; b, Sandy clayey silt soil.

The enzymatic treatment had little effect on extraction efficiency compared to the conventional methods (data not shown). There is no doubt that this method has made a great contribution to the quantification of soil bacteria, but due to the high cost of the enzymes and the complexity of the protocol (Lunau *et al.*, 2005), it could not be widely used for mass bacterial cell extraction.

Bacterial extraction with SDGC

We found that centrifugation at 600×g (which is often used to separate bacteria from soil particles with LSC (Riis *et al.*, 1998) caused most of the bacteria to enter the sediment. However, if the centrifugal force was below 200×g, the soil particles could not be removed effectively. Therefore, we considered using SDGC instead of LSC to separate bacterial cells from soil.

Sucrose solution with a density of 1.33 g/ml should be a suitable matrix for the separation of bacteria from soil, because this density is between that of bacterial cells and soil particles. Experiments with pure cultures showed that more than 90% of the bacterial cells were retained in the solution (data not shown). But sucrose solution has a high osmotic pressure that might cause cell dehydration, so the centrifugation time was shortened to 2 min to maintain cell integrity. After centrifugation, the bacterial extracts and the primary soil were stained with DAPI for cell counting. The results showed that compared to LSC (600×g, 2 min), the use of SDGC improved the extraction efficiency markedly from 25.74% to 72.78% for red soil; whereas for sandy clayey silt soil, the extraction efficiency increased from 23.23% to 70.16% (Table 3).

With LSC, repeated dispersion-centrifugation procedures can increase the extraction efficiency to 70-80% (Lindahl, 1996), but the process consumes more time and labor. The SDGC method has proven to be a simple, quick, and reliable alternative to the current methods for bacterial extraction from soil. It can be used to separate bacteria from a variety of soils, because setting times and centrifugation speeds can be adjusted to achieve optimum layering of the bacterial cells, and the procedure avoids the labor-intensive and time-consuming published methods (Pillai *et al.*, 1991).

Table 2. Effects of different chemical dispersants on extraction efficiency

Dispersant	Yield×10^9 cells/g soil		Yield (%)	
	Red soil	Sandy clayey silt soil	Red soil	Sandy clayey silt soil
Primary soil	6.76±0.14	4.39±0.22		
Water	3.61±0.15	2.26±0.19	53.40	51.48
Sodium pyrophosphate	4.26±0.23	2.68±0.17	63.02	61.05
Sodium chloride	2.45±0.18	0.54±0.07	36.24	12.38
Sodium citrate	3.79±0.22	2.48±0.25	56.07	56.44
TN buffer	1.57±0.23	0.48±0.16	23.22	10.89
Tris.HCl	3.66±0.24	0.83±0.09	54.14	18.81
PEG6000	2.84±0.17	1.49±0.21	42.01	33.91
TritonX-100	2.37±0.18	1.35±0.17	35.06	30.69
Tween-80	2.81±0.20	1.81±0.19	41.57	41.34
Methanol	3.14±0.24	1.22±0.12	46.45	27.72

NaCl (0.8%) as a resuspension solution for purification with NDGC

The influence on extraction efficiency for nycodenz purification of different resuspension solutions are presented in Table 4, including sterilized distilled water, 0.8% NaCl and 0.2% sodium pyro-phosphate.

The cell yields were more than 60% with sodium pyrophosphate or water as the resuspension solutions. However, this resulted in too much contaminant capable of affecting the subsequent enzymatic reactions. Therefore, we finally chose NaCl as the resuspension solution for NDGC purification and modified its pH to 7.5. It has been reported that NaCl can remove more than 99% of the Al+Fe contamination from bacterial extracts, and the combination of modified pH and NaCl ensured reasonable cell yields and low contamination. The effects were probably due to changes in variable charge (by pH) and ion distribution (NaCl) around interacting particles (soil and bacteria), thus affecting their flocculation (Ehlers *et al.*, 2008). The cells obtained by NDGC with NaCl

Table 3. Comparison of the extraction efficiency of SDGC and LSC

Treatments	Yield×10^9 cells/g soil		Yield (%)	
	Red soil	Sandy clayey silt soil	Red soil	Sandy clayey silt soil
Primary soil	6.76±0.14	4.39±0.22		
With sucrose (5550×g 2 min)	4.92±0.28	3.08±0.19	72.78	70.16
Without sucrose (5550×g 2 min)	0.17±0.07	0.11±0.05	2.53	2.51
Without sucrose (600×g 2 min)	1.74±0.19	1.02±0.12	25.74	23.23

Table 4. Effects of different resuspension solutions on extraction efficiency during nycodenz purification

Resuspended dispersant	Yield×10^9 cells/g soil		Yield (%)	
	Red soil	Sandy clayey silt soil	Red soil	Sandy clayey silt soil
Soil pellet	4.92±0.28	3.08±0.19		
Water	3.11±0.15	1.97±0.22	63.28	63.96
Sodium pyrophosphate	3.24±0.22	2.19±0.21	66.04	71.10
Sodium chloride	2.09±0.16	1.52±0.17	42.53	49.35

as the resuspension solution could be directly used for lysis and restriction enzyme digestion without any additional purification.

It is worth noting that 0.8% NaCl should be used to resuspend the cell and soil pellets after SDGC for nycodenz purification. When it was used as the dispersant in homogenization, only about 10-30% of the bacterial cells in soil were separated with SDGC, and after nycodenz purification, the extraction efficiency was only about 5-10% (data not shown). This result is consistent with the report of Ehlers (Ehlers *et al.*, 2008). This is most likely due to the higher ionic strength leading to thinner diffuse double layers, which enhances soil particle flocculation as well as reattachment of bacteria due to van der Waals forces (Bolan *et al.*, 1999). During centrifugation, these aggregates sedimented to the bottom of the tube, and this led to decreases in cell yield and impurities. In contrast, sodium pyro-phosphate was a good chemical dispersant for the reasons mentioned above. For the same reason, however, the bacterial suspension extracted with sodium pyro-phosphate as the resuspension solution was highly contaminated and could not be used for HWM DNA isolation. It is remarkable that NaCl addition reduced the soil particle contamination in the extracted bacterial suspension to a greater extent than it reduced the cell yield during NDGC (Ehlers *et al.*, 2008). Thus, we decided to use sodium pyro-phosphate as the dispersant to separate bacteria from soil particles with SDGC first, and then to use NaCl as the resuspension solution for purification of the separated bacteria with NDGC.

The optimal procedure for bacterial extraction and purification

After evaluating the various bacterial extraction and purify-cation methods, we determined the optimal procedure, as follows (Fig. 1).

The bacteria extracted for HMW DNA recovery must be pure enough to allow subsequent enzymatic reactions. NDGC was preferable in this situation for bacterial extraction with low impurities (Furtadoa and Casper, 2000). Traditional bacterial extraction with NDGC is either ineffective or troublesome. SDGC was selected (instead of LSC) to extract the separated bacteria from the soil first, and then the upper sucrose fractions containing the cell and soil pellets were concentrated, and finally the cell and soil pellets were resuspended in 0.8% NaCl for purification with NDGC. With this procedure, more than 30% of the bacterial cells were recovered with high purity. The extraction efficiency of the separation and purification steps are listed in detail (Table 5).

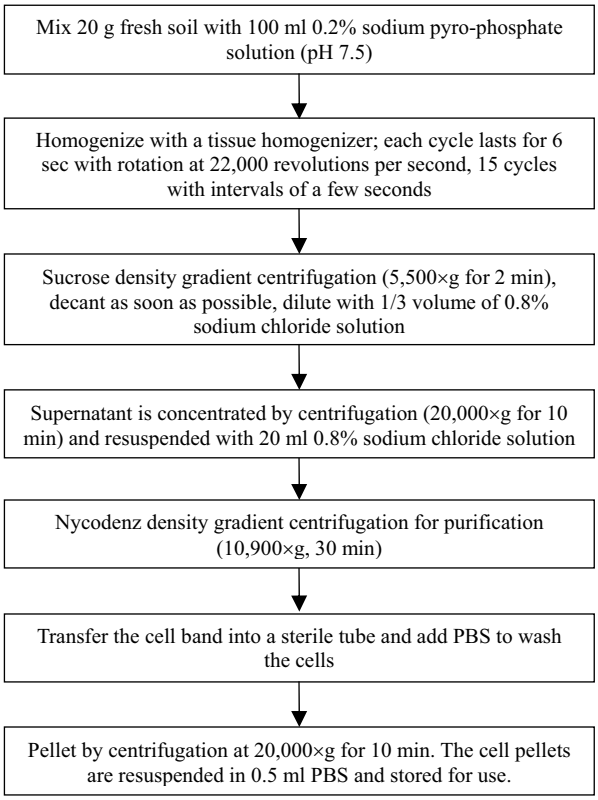

Fig. 1. Optimal procedure for bacterial extraction and purification.

Structure of the soil microbial community determined by RISA

The level of potential bias caused by the bacterial extraction steps on the bacterial community structure was studied by the RISA approach with template DNA originating from both extracted bacterial cells and the primary soil samples. Analysis of the results in Fig. 2 shows that for red soil the two conditions tested had 80.85% similar RISA fingerprints, while for sandy clayey silt soil, the similarity was 66.67%. This meant

Table 5. DAPI and colony forming unit (CFU) counts of primary soil, cell and soil pellets after SDGC and of extracted cells after NDGC

	Primary soil	Cell and soil pellets after SDGC	Extracted cells after NDGC	Cell recovery (%)
DAPI×10^9 cells/g soil				
Red soil	6.76±0.14	4.92±0.28	2.09±0.12	30.92
Sandy clayey silt soil	4.39±0.22	3.08±0.19	1.52±0.17	34.62
CFU×10^7/g soil				
Red soil	6.02±0.37	4.32±0.24	1.94±0.18	32.23
Sandy clayey silt soil	3.34±0.21	2.40±0.27	1.23±0.19	36.75

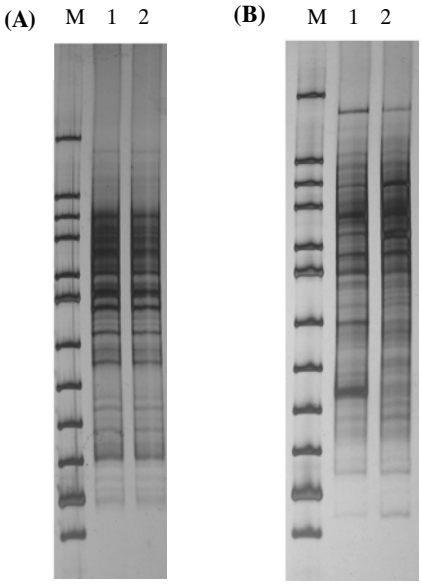

Fig. 2. Bacterial community structure of extracted bacteria and primary soil evaluated by RISA. (A) Red soil, (B) Sandy clayey silt soil. Lanes: M, 50-bp DNA ladder (TaKaRa); 1, RISA of total community DNA extracted from primary soil; 2, RISA of DNA extracted from bacterial cells obtained by the sucrose method and nycodenz purification.

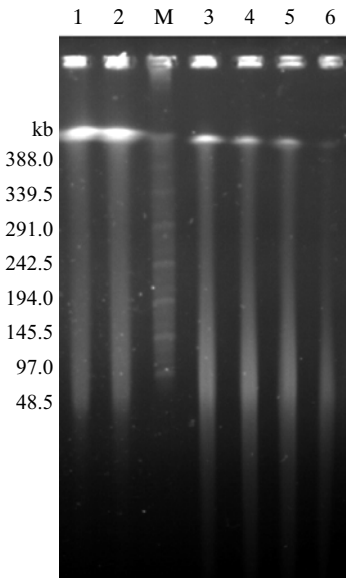

Fig. 3. PFGE of HMW DNA isolated from bacteria extracted from red soil immobilized in LMT agarose plugs. Lanes: M, Lambda ladder PFGE marker; 1-2, HMW DNA isolated from extracted bacterial cells; 3-6, *Sau*3AI restriction digestion of HMW DNA (0.625 U, 1.25 U, 2.5 U and 5 U *Sau*3AI in 100 μl buffer, respectively).

that the extraction procedure did not change the community structure of the soil bacteria significantly, and that the extracted bacteria qualitatively represents the soil bacterial community to a certain extent.

HMW DNA extraction and BAC library construction

PFGE analysis of the DNA fragments obtained from the bacteria extracted from red soil showed that the DNA fragments were in excess of 400 kb (Fig. 3: lanes 1 and 2), and that the HMW DNA could be effectively digested with *Sau*3AI (Fig. 3: lanes 3-6).

A metagenomic library containing 3,024 BAC clones was constructed with the HMW DNA isolated from the bacteria extracted from red soil in south China. *Dra*I restriction analysis of 30 randomly selected plasmids showed that the insert sizes were in the range of 25-165 kb, and 70% of them were in the range of 50-100 kb (Fig. 4). The average size was about 75 kb, and the total capacity of the library was estimated to be about 200 Mb. *Hin*dIII restriction analysis showed that these clones had different restriction patterns (data not shown), indicating that the library presented a good random representation of the cloned DNA. BAC terminal sequence analysis of ten randomly selected clones from the BAC library was conducted using BlastN and BlastP. The results showed that no nucleotide homology was found in the GenBank database for nine of the clones. BlastP analysis showed that the putative proteins from these clones had 25-69% identity to known proteins.

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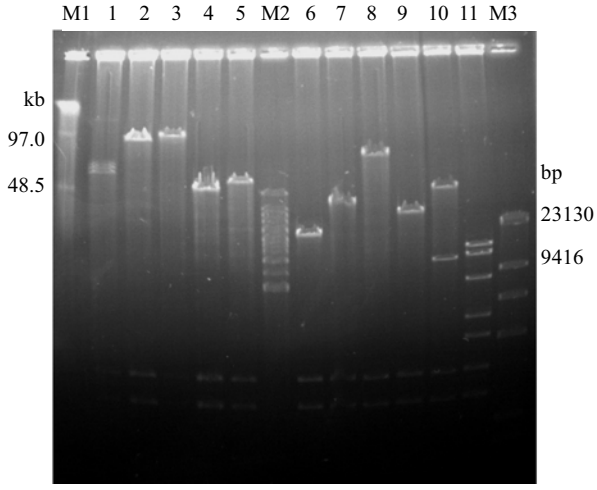

Fig. 4. *Dra*I restriction digestion of eleven randomly picked BAC clones from red soil metagenomic library. Lanes: M1, Lambda ladder PFGE marker; M2, Lambda mixed ladder; M3, Lambda/*Hin*dIII ladder; 1-11: *Dra*I restriction digestion of eleven randomly picked BAC clones.

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