Applied Soil Ecology 42 (2009) 176–181

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Short communication

Sequential extraction and genetic fingerprinting of a forest soil metagenome

J. Ascher [a,*], M.T. Ceccherini [a], O.L. Pantani [a], A. Agnelli [b], F. Borgogni [a], G. Guerri [a], P. Nannipieri [a], G. Pietramellara [a]

[a] Dipartimento di Scienza del Suolo e Nutrizione della Pianta, Università degli Studi di Firenze, Piazzale delle Cascine 28, 50144 Florence, Italy
[b] Dipartimento di Scienze Agrarie ed Ambientali, Università degli Studi di Perugia, Borgo XX Giugno, 06121 Perugia, Italy

ARTICLE INFO

Article history:
Received 15 October 2008
Received in revised form 23 March 2009
Accepted 23 March 2009

Keywords:
Soil metagenome
DNA extraction efficiency
DNA fractions
Compositional genetic differences
Community level DGGE

ABSTRACT

The soil DNA pool consists of an intracellular (iDNA) and extracellular fraction (eDNA). Challenging to improve the extraction efficiency of soil DNA, and to quantitatively and qualitatively characterize both DNA fractions, we set up a molecular approach consisting of sequential and comparative DNA extraction and microbial community fingerprinting. eDNA was extracted by alkaline soil washings (ASW); iDNA by mechanical chemical cell lysis (MCCL) of the residual soil pellet after the extraction of eDNA (ASW–MCCL). The molecular approach was compared in terms of quantity (fluorometer) and quality (agarose gel electrophoresis; small subunit rRNA-denaturing gradient gel electrophoresis) to directly extracted soil DNA comprising both eDNA and iDNA (tDNA; FastDNA Spin Kit for Soil, BIO101). The proposed method appeared to be a potential tool to separately extract and analyze eDNA (6.07 $\mu g\, g^{-1}$ soil) and iDNA (11.46 $\mu g\, g^{-1}$ soil) and to obtain a greater amount of DNA from soil with broader genetic information about eubacterial and fungal communities with respect to directly extracted tDNA (8.79 $\mu g\, g^{-1}$ soil). Our results revealed the extracellular fraction to be quantitatively and qualitatively important of the soil metagenome. As the sequential DNA extraction method not only increased the total amount of extractable soil DNA (17.53 $\mu g\, g^{-1}$ soil) but also that of iDNA, it is suggested to be suitable for extracting the soil metagenome.

1. Introduction

The soil metagenome is defined as the entirety of the microbial genomes found in the soil environment (Rondon et al., 2000; Daniel, 2004), or as the entire genetic material recovered directly from soil samples (Daniel, 2005). The present study has been made "in the optic of" the latter definition considering the soil DNA pool (tDNA) which is made of an intracellular (iDNA) and an extracellular fraction (eDNA). Many direct and indirect methods are available to extract and purify tDNA and iDNA (for review see Robe et al., 2003; Bakken and Frostegård, 2006; Nielsen et al., 2007) whereas few protocols are available for the extraction of eDNA from soil (Niemeyer and Gessler, 2002; Agnelli et al., 2004, 2007). Only recently has it been suggested that eDNA in soil is quantitatively important (Agnelli et al., 2004; Levy-Booth et al., 2007). Soil eDNA can persist (Nielsen et al., 2006; Pietramellara et al., 2006; Agnelli et al., 2007) and can be transported by water through the soil profile by vertical advection (Ceccherini et al., 2007) or by leaching (Potè et al., 2003; Agnelli et al., 2004). Soil eDNA can be used in gene transfer, in the formation of bacterial biofilm and as a source of nutrients by soil microorganisms (Finkel and Kolter, 2001; Matsui et al., 2003; Wackernagel, 2006; Levy-Booth et al., 2007; Pinchuk et al., 2008).

Genetic fingerprinting studies on the composition of microbial communities have been mainly performed on tDNA or iDNA (Muyzer, 1999; Robe et al., 2003), whereas eDNA has been considered to bias microbial community studies and thus has been eliminated prior to the extraction and analyses of iDNA from soil (Courtois et al., 2001; Bakken and Frostegård, 2006). Agnelli et al. (2004) conducted and compared denaturing gradient gel electrophoresis (DGGE) fingerprints of tDNA and eDNA extracted from soil, to evaluate the composition and distribution of soil bacterial and fungal communities, suggesting genetic microbial diversity of the extracellular soil DNA fraction. No studies are available on sequential extraction and comparative DGGE analyses of soil tDNA, eDNA and iDNA. We hypothesized that these analyses not only could provide quantitative and qualitative information of the eDNA and iDNA fractions of the soil metagenome but also more insights into the composition of soil microbial communities.

We set up a molecular approach of sequential and comparative DNA extraction and genetic fingerprinting in order to separately extract and analyze the extracellular and intracellular fraction of a forest soil metagenome. The principal aim of the present study was

* Corresponding author. Tel.: +39 055 3288219; fax: +39 055 333273.
E-mail address: judith.ascher@unifi.it (J. Ascher).

0929-1393/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2009.03.005

to quantitatively and qualitatively characterize the different fractions of the soil DNA pool and to investigate the inherent genetic information for microbial communities. This sequential approach was evaluated by comparing it to a direct extraction and fingerprinting approach of soil DNA in terms of quantity and quality of extracted soil DNA.

2. Materials and methods

2.1. Study site, soil sampling and sample preparation

The study site is located in the Vallombrosa Forest Reserve (Florence, Italy) with soil covered by either *Pinus nigra* Arn. ssp. laricio (soil pH_{H_2O} 5.3, pH_{KCl} 4.3) or *Abies alba* Mill. (soil pH_{H_2O} 4.5, pH_{KCl} 3.5) about 60 and 80 years old, respectively (Agnelli et al., 2004). The mean annual air temperature and precipitation of the area are about 10 °C and 1400 mm, respectively. The soil, developed on a set of thick turbiditic strata of quartz-feldspathic sandstone intercalated by thin layers of siltstone, has been classified as a Cambic Umbrisol (Arenic) (IUSS, 2006). The site is on a moderate west-facing slope (about 15%), at 950 m a.s.l.

In May 2006, three subjects (S1, S2 and S3) of *P. nigra* (PN) and *A. alba* (AB) trees were selected; bulk soil (500 g) was taken from 0 to 15 cm soil layer at the base of the trees in three different holes (H1, H2 and H3) for each tree; the distance between trees of the same species was about 20 m (Fig. 1). The soil samples were sieved at 2 mm and aliquots (0.5 g soil) were weighed and stored in Microfuge® tubes (2 ml) at −20 °C for DNA extraction.

2.2. Direct extraction of soil DNA

Total DNA (tDNA), comprising eDNA and iDNA, was extracted directly from 0.5 g of soil with 978 μl 0.12 M Na_2HPO_4 at pH 8 and 122 μl MT buffer (1% SDS, sodium dodecyl sulfate, 0.5% Teepol, and PVP40 with EDTA and Tris) under mechanical-chemical cell disruption (FastPrep System and FastDNA Spin Kit for Soil, BIO101, Qbiogene, Inc., USA) according to the manufacturer's instructions. We define this tDNA extraction method as mechanical chemical cell lysis (MCCL) and, due to its widespread application in molecular ecology, it is considered as reference method for evaluating the extraction efficiency of our proposed sequential method.

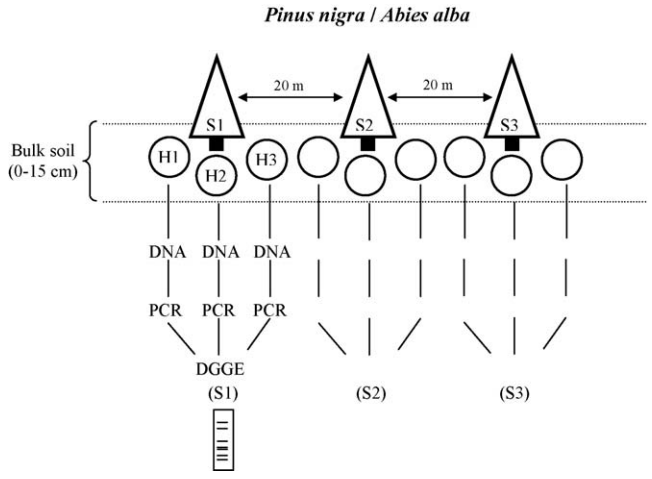

Fig. 1. Experimental set up designed to fulfill statistical requests (mixed-effects model) to determine the extraction efficiency of the proposed method. Bulk soil was sampled under three subjects (S1, S2 and S3) of *P. nigra* and *A. alba* in three holes (H1, H2 and H3) on the base of each tree. PCR replicates of each soil DNA fraction (directly extracted tDNA; sequentially extracted eDNA and iDNA) were homogenized to investigate the DNA fraction as main parameter influencing DGGE community analysis.

2.3. Sequential extraction of soil DNA

Extracellular DNA (eDNA) was extracted by gentle soil washings; 0.5 g soil was amended with 500 μl of 0.12 M Na_2HPO_4 at pH 8 in Microfuge® tubes and horizontally shaken for 30 min (100 movements/min). The soil slurry was then centrifuged (4 °C, 30 min, at 7500 × *g*) and the supernatant was collected. The soil pellet was subjected to the same procedure for two more cycles; the supernatants were pooled together, resulting in a final volume of 1.5 ml of *crude* DNA. We define this eDNA extraction method as alkaline soil washing (ASW).

Intracellular DNA (iDNA) was extracted from the residual soil pellet after the ASW procedure. The pellet was resuspended in 978 μl 0.12 M Na_2HPO_4 at pH 8 and 122 μl MT buffer, transferred to tubes containing a lysing matrix (BIO101), and processed by the MCCL method as described above. We define this extraction method as ASW–MCCL.

If the eDNA extraction is considered as a pre-treatment of the soil for the iDNA extraction, the set up sequential method can be defined as a modification of a standard method of tDNA extraction.

2.4. DNA purification of tDNA, eDNA and iDNA

Crude DNA extracts were amended with a protein precipitating solution (PPS, 250 μl; Geneclean® BIO101) and the mixture was centrifuged (20 °C, 5 min, 7500 × *g*) to eliminate proteins. DNA was then adsorbed on an anion exchange resin (AER, 1 ml; Geneclean® BIO101) and collected in spin columns (centrifugation at 20 °C, 2 min, 7500 × *g*; SPIN™ Filters); washed three times with 500 μl guanidine isothiocyanate (5.5 M), followed by three washings with 500 μl salt ethanol wash solution (SEWS; Geneclean® BIO101). The purified DNA was eluted in sterile ultrapure water (100 μl), collected by centrifugation (20 °C, 2 min, 7500 × *g*) and stored at −20 °C.

2.5. Quantitative and qualitative DNA analyses

The purified DNA was quantified by fluorimetric measurements (Hoefer DyNA Quant® 200, Hoefer Pharmacia Biotech, San Francisco, CA, USA) using Hoechst 33258 bisbenzimide as fluorochrome and DNA of calf thymus as standard.

The molecular weight of DNA and its fragment length distribution were assessed by agarose gel electrophoresis (1× Tris acetate EDTA buffer; 1× ethidium bromide, EtBr; 0.8% (w/v); 100 V; 60 min) after staining with EtBr (1:10 000, 10 mg ml^{-1}) and DNA mass ladder mix (Fermentas, 80–20 000 bp) for comparison.

2.6. SSU rRNA-PCR-DGGE of eubacterial (16S rRNA genes) and fungal (18S rRNA genes) communities

16S rRNA genes of directly extracted tDNA and sequentially extracted eDNA and iDNA were amplified by polymerase chain reaction (PCR) as reported by Agnelli et al. (2004), using the GC-clamped eubacterial specific primers GC968*f*/UNI1401*r* (Nübel et al., 1996).

The amplicons of the three holes (H1, H2 and H3) of each DNA type (tDNA, eDNA and iDNA) extracted from soils under three subjects (S1, S2 and S3) of *P. nigra* (PN) and *A. alba* (AB) were pooled together in order to obtain a representative sample of target sequences for each of the tree replicates (S1, S2 and S3) (Fig. 1); the homogenization of the samples at the amplicon-level was also made to reduce the number of samples in order to analyze them together in one DGGE-gel so as to have as main variable the DNA

fraction (tDNA *vs.* eDNA *vs.* iDNA) which could be covered by differences in the composition of microbial communities due to microtopography. DGGE was performed with 100 ng of amplicons (473 bp) on a 6% polyacrylamide gel (acrylamide/bisacrylamide 37.5:1; 20 cm × 20 cm; 1 mm) with a urea–formamide-denaturing gradient of 46–56% (100% denaturant contains 7 M urea and 40% formamide) at 60 °C, 70 V for 16 h using the DCode system (Bio-Rad, Hercules, CA, USA).

18S rRNA genes were amplified by a nested PCR approach. The first round of PCR was performed on 40 ng target DNA by amplifying a 1700 bp portion, almost the whole 18S gene, using the primer set NS1*f*/NS8*r* (Kowalchuk, 1999). The resulting PCR products (2 μl) were subsequently amplified with the GC-clamped primer set EF4*f*/NS3*r*GC (Brodie et al., 2003). First and second round PCR and DGGE conditions are described by Kowalchuk (1999) and Brodie et al. (2003). The 500 bp amplicons (150 ng) were analyzed by DGGE on a 10% polyacrylamide gel with a denaturing gradient of 30–45% at 60 °C, 85 V for 17 h. The fingerprints were visualized with UV light gel transillumination (254/497 nm) after a 2 h staining with SybrGreen I (1:10 000; FMC Bio Products, Rockland, ME, USA), and photographed with Polaroid Gel Cam (Elect; Polaroid Type 667 Film ISO 3000). Similarities between the generated profiles were determined by UPGMA (unweighted pair group method with arithmetic mean) cluster analyses based on Dice similarity coefficients (Dice, 1945) using the software GelCompare II (Applied Maths BVBA).

The composition of eubacterial and fungal communities was analyzed for differences in DGGE profiles of directly (tDNA) and sequentially extracted DNA (eDNA *vs.* iDNA), and for the selective effect of arboreal species (*P. nigra vs. A. alba*).

2.7. Experimental design and statistical analyses

To account for the variability structure of the experiment, a mixed-effects model was fitted to the data. The direct MCCL method was considered the reference method and *P. nigra* (PN) the reference species.

The experimental design was a randomized block design with DNA (μg g^{-1} soil) as response, "hole" within "subject" as blocking factor, and "method" and "species" as experimental factors. The software R was used for the relative statistical analyses and for the preparation of the graphics (R Development Core Team, 2007; Murrell, 2006). The statistical analyses were performed according to Pinheiro and Bates (2000), using the "nlme" library of the software R (Pinheiro et al., 2007).

3. Results

Quantitative analyses of directly extracted soil tDNA (mechanical chemical cell lysis, MCCL; *b*) and sequentially extracted eDNA (alkaline soil washings, ASW; *a*) and iDNA (combined ASW–MCCL method; *c*) from a forest soil under two tree species revealed different extraction efficiencies equivalent to $a < b < c$ (Fig. 2). This general trend is formally summarized in the t-table of the mixed-effects model (Table 1). The random part of the model was in the form "hole" within "subject"; the fixed part concerned two experimental factors and their interaction ("method" + "species" + "method" × "species"). As the statistical analysis revealed no significant effect of the plant species (*A. alba* and *P. nigra*) on the yields of extracted tDNA ($p = 0.09$), eDNA ($p = 0.38$) and iDNA ($p = 0.45$), the factor "species" was removed from the model. The amount of directly extracted tDNA (comprising eDNA and iDNA) from soil was 8.79 μg DNA g^{-1} soil (Table 1), whereas those of sequentially extracted eDNA and iDNA were 6.07 and 11.46 μg DNA g^{-1} soil, respectively, resulting in a total amount of extractable soil DNA of 17.53 μg DNA g^{-1} soil.

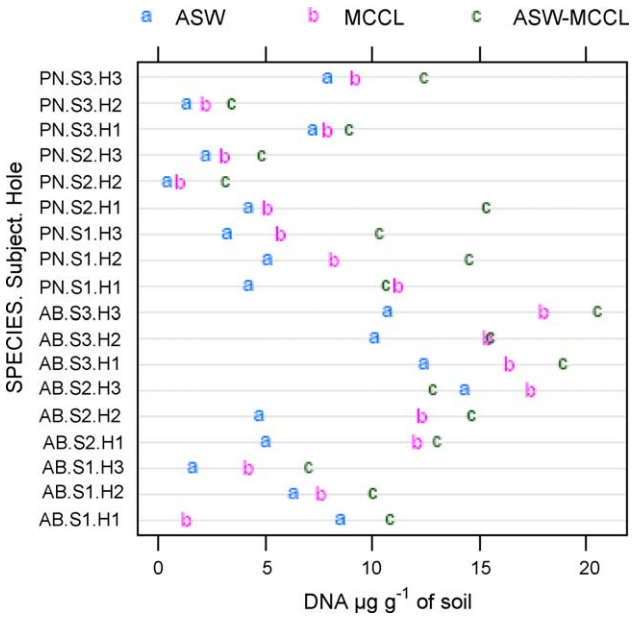

Fig. 2. Amounts of the three DNA fractions (μg g^{-1} soil) extracted from surface soil (0–15 cm) by the ASW method (eDNA), ASW–MCCL method (iDNA) and by the MCCL method (tDNA). Bulk soil was sampled under three subjects (S1, S2 and S3) of *P. nigra* (PN) and *A. alba* (AB) in three holes (H1, H2 and H3) on the base of each tree.

Electrophoresis of tDNA, eDNA and iDNA on agarose gel showed high molecular weight DNA with similar fragment length distribution and molecular masses ranging from about 80–20 000 bp (data not shown).

3.1. Comparative DGGE analyses of directly (tDNA) vs. sequentially (eDNA and iDNA) extracted soil DNA

The DGGE patterns provide information regarding predominant population richness (number of bands) and the composition of microbial communities, whereas the UPGMA-analysis of DGGE profiles provides information about the compositional differences among the DNA fractions (Fig. 3).

Microbial DGGE fingerprinting (Fig. 3) was performed on pooled amplicons (H1 + H2 + H3), representative of the microbial communities under each of the spatially different tree replicates (Fig. 1; S1, S2 and S3), in order to reduce the number of samples for analyses on the same DGGE-gel and to have as main parameter the DNA fraction (tDNA *vs.* eDNA *vs.* iDNA). Both, bacterial and fungal community DGGE of directly (tDNA) and sequentially extracted soil DNA (eDNA and iDNA) generated unique community profiles, suggesting compositional differences among the different DNA fractions (Fig. 3).

Table 1
The t-table for the mixed-effects models used to analyze the quantitative experimental data. For the direct MCCL method (mechanical chemical cell lysis; total tDNA; reference method) the amounts of extracted tDNA are reported as true values. For the ASW method (alkaline soil washing; extracellular eDNA) and the combined ASW–MCCL method (intracellular iDNA), the values are expressed as differences with respect to the MCCL method. The *p*-values indicate the probability that the value reported in the third column (DNA, μg g^{-1} of soil) is equal to zero.

Extraction method	DNA	DNA, μg g^{-1} of soil	Std. Error	df	*t*-value	*p*-value
MCCL	tDNA	8.79	1.624	34	5.42	$<10^{-3}$
ASW	eDNA	−2.72	0.817	34	−3.33	0.0021
ASW–MCCL	iDNA	2.67	0.817	34	3.27	0.0025

Number of observations: 54; number of groups: 6 subject; 18 holes within subject; AIC 293, BIC 305, logLik-141 (AIC, Akaike information criterion; BIC, Bayesian information criterion; df, degrees of freedom).

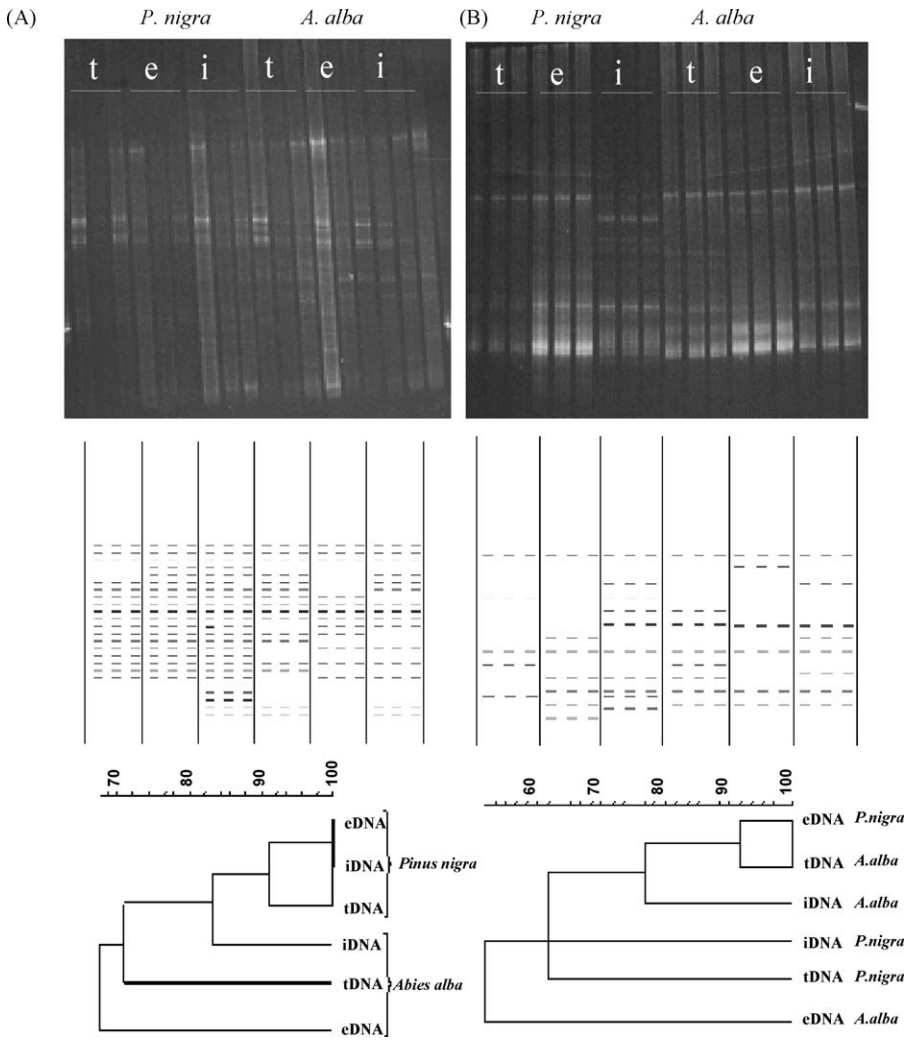

Fig. 3. Eubacterial (16S rRNA; A) and fungal community (18S rRNA; B) structures as revealed by DGGE fingerprinting of directly extracted tDNA (t), and sequentially extracted eDNA (e) and iDNA (i) from soil under *P. nigra* and under *A. alba*. DGGE was performed on pooled PCR samples of DNA from three different holes (H1, H2 and H3) for each tree subject (S1, S2 and S3)/species, in order to investigate on the DNA fraction as main parameter determining DGGE community analysis. Dice-UPGMA-cluster analysis was performed to assess differences in the genetic information of the various DNA fractions about the composition of bacterial and fungal communities. Percentage similarity is shown on the scale above the dendrogram.

The eubacterial 16S rRNA-PCR-DGGE fingerprints (Fig. 3A) of directly extracted tDNA from soil under *P. nigra* showed 17 bands representing predominant populations. The fingerprints of sequentially extracted eDNA (19 bands) and iDNA (23 bands) revealed the presence of two and six additive bands. The compositional similarity between the different DNA fractions was about 90% as shown by UPGMA-cluster analysis (Fig. 3A). In soil under *A. alba* the highest number of bands was generated by tDNA (16) and iDNA (15); the eDNA fraction showed a less rich (12) fingerprint. In contrast to *P. nigra*, the DGGE fingerprinting of sequentially extracted eDNA and iDNA did not increase the species richness (number of detected bands) revealed for tDNA. Because some bands were present in the separate fingerprints of eDNA and iDNA that were not present in the tDNA fingerprint, the overall genetic information of the composition of the bacterial community was approved by the sequential extraction and fingerprinting approach. The compositional similarity between the different DNA fractions was lower, about 72%, with respect to *P. nigra*.

The fungal DGGE fingerprints (Fig. 3B) of directly *vs.* sequentially extracted DNA from soil under both tree species showed less rich and more variable communities with respect to the eubacterial communities (Fig. 3), but a similar trend with higher band richness generated by eDNA and iDNA with respect to tDNA under *P. nigra*. In soil under *P. nigra* DGGE fingerprints of iDNA generated the highest number of dominant bands (10), and tDNA the lowest number of bands (5). In soil under *A. alba* both tDNA and iDNA generated richer DGGE patterns than eDNA and each DNA fraction yielded unique fungal community profiles (Fig. 3B). The selective effect of the arboreal species on the fungal communities is less pronounced than that of the genetic differences of the different DNA fractions with similarities ranging from 92% to 50%.

4. Discussion

The proposed sequential extraction and genetic fingerprinting approach of the soil DNA pool proved to be a potential tool to quantitatively and qualitatively characterize the extra- and intracellular DNA fraction. The sequential extraction demonstrates that, by a preliminary alkaline washing of soil, one can separately collect eDNA, and the following standard DNA extraction provides a greater amount of iDNA, compared with the direct extraction of tDNA from a non-washed soil, which provided a mixture of eDNA and iDNA. If the eDNA extraction is considered as a pre-treatment of the soil for the iDNA extraction, the significant increase of extractable iDNA by the sequential method suggests the modification of a standard method of direct tDNA extraction (MCCL;

BIO101) to be an optimization; furthermore, the more than double amount of sequentially extractable soil DNA (the sum of eDNA + iDNA) suggests an underestimation of the soil metagenome when the direct standard method is used. In addition, the eDNA extracted from soil represents 34.6% of the sum of eDNA and iDNA, suggesting the extracellular fraction to be an important portion of the soil metagenome. The soil washing with alkaline buffer (ASW), used for the extraction of eDNA, probably made microbial cell aggregates more accessible to the successive mechanical lysis, performed to extract iDNA; this could explain the significantly higher yield of iDNA obtained by the sequential method (ASW–MCCL) than that obtained by the direct method (MCCL).

The presence of Na^+ and the alkaline pH (8.0) of the ASW buffer probably disrupted soil aggregates by increasing the number of microbial cells exposed to the MCCL procedure. In addition, the ASW buffer probably saturated surface-reactive particles, avoiding the adsorption of DNA molecules released after cell lysis. Our findings are in accordance to He et al. (2005), who reported a 24% increase of extractable DNA from forest soils when pre-lysis buffer washings with sodium phosphate buffer (0.1 M, pH 7.5) or EDTA (20 mM, pH 7.5) were performed prior to cell lysis. The pre-lysis washing procedure was hypothesized to promote soil dispersion and homogeneity, to decrease DNA adsorption by soil components and to remove covalent cations such as iron oxides, and those easily dissolving organic compounds, prevalently humic acids, from soil. However, because they removed the supernatants of the soil washings which contained eDNA and performed cell lysis on the soil remnants, in the light of what we found in the here described study, their resulting DNA has to be defined as intracellular iDNA rather than total soil tDNA.

As a consequence of increased extractable soil DNA, also the encoding genetic information on soil microbial communities increased. The separate and comparative DGGE fingerprinting of the sequentially extracted soil DNA fractions revealed dominant populations that were not assessable by fingerprinting of the directly extracted tDNA. The general higher number and/or different electrophoretic mobility of bands revealed by DGGE of eDNA respect to tDNA and/or iDNA could be explained in different ways. The eDNA fraction, actively extruded or released after cell death from soil microorganisms, can persist over time by interaction with soil colloids (Agnelli et al., 2004, 2007; Pietramellara et al., 2007) or can move with the soil water by advection movements (Potè et al., 2003; Agnelli et al., 2004; Ceccherini et al., 2007); thus eDNA bands not present in the respective tDNA and/or iDNA fingerprints could also be interpreted as eDNA residuals, reminders of absent microorganisms in the studied environment (Agnelli et al., 2004). Because most of the eDNA bands are also present in the fingerprints of iDNA and tDNA, eDNA could be considered to represent soil microbial communities and for the fate of DNA in soil. The presence of additive and/or different bands in the iDNA DGGE patterns could be attributed to the increased efficiency of the sequential extraction method; in fact, as stated above, a higher amount of iDNA could contain more genetic information. For a more complete interpretation of DGGE fingerprints we suggest, together with Kozdrój and van Elsas (2000) and Heuer and Smalla (1997), considering the possible effect of different DNA extraction methods. In our opinion, differences in DGGE patterns of the different DNA fractions are probably due to genetic differences of the DNA fractions. These facts, raised in a previous study by Agnelli et al. (2004), have been the driving factor for the present work. To our knowledge, only Steinberger and Holden (2005) investigated genetic differences of tDNA, eDNA and iDNA, and then in a multiple-species biofilm in laboratory conditions, rather than soil. Up to now no studies are available on comparative DGGE analyses of soil tDNA, eDNA and iDNA. The UPGMA-cluster analysis of DGGE fingerprints confirmed a lower similarity among the fungal DNA fractions than among eubacterial DNA fractions in soil under both tree species. In addition, lower genetic similarities of eubacterial DNA fractions (tDNA *vs.* eDNA *vs.* iDNA) were detected in soil under *A. alba* (70%) than under *P. nigra* (92%) (Fig. 3). The comparison of the bacterial communities under both tree species revealed a similarity of 74%, and this may depend on the different composition and amount of rhizodeposition of the two plant species (Lejon et al., 2005).

5. Conclusion

The proposed molecular approach of sequential extraction and genetic fingerprinting of soil DNA showed that extracellular eDNA is a quantitative and qualitative important fraction of the soil metagenome. We suggest considering these findings in defining the soil metagenomic DNA. If the definition of the soil metagenome is restricted to the entirety of genomes of the living microbiota, the sequential DNA extraction should also be considered as it significantly increased the amount of intracellular iDNA with respect to the net amount of directly extracted total DNA, which contains both eDNA and iDNA, and also contained greater genetic information about the composition of microbial communities.

Acknowledgements

The authors are very grateful to Prof. Federico M. Stefanini and to Dr. A. Mengoni for their help in statistical and DGGE-cluster analyses. This work was partially supported by the Ministry of Environment through the Special Integrative Fund for Research, FISR—"Soil Sink" and by the "Cassa di Risparmio di Firenze".

References

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M.T., Nannipieri, P., Pietramellara, G., 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. Soil Biol. Biochem. 36, 859–868.

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M.T., Pietramellara, G., Nannipieri, P., 2007. Purification and isotopic signatures (δ13C, δ15N Δ14C) of soil extracellular DNA. Biol. Fertil. Soils 44, 353–361.

Bakken, L.R., Frostegård, A., 2006. Nucleic acid extraction from soil. In: Nannipieri, P., Smalla, K. (Eds.), Soil Biology, Nucleic Acids and Proteins in Soil, 8. Springer-Verlag, Heidelberg, Germany, pp. 49–73.

Brodie, E., Suzanne, E., Clipson, N., 2003. Soil fungal community structure in a temperate upland grassland soil. FEMS Microbiol. Ecol. 1523, 1–10.

Ceccherini, M.T., Ascher, J., Pietramellara, G., Vogel, T.M., Nannipieri, P., 2007. Vertical advection of extracellular DNA by water capillarity in soil columns. Soil Biol. Biochem. 39, 158–163.

Courtois, S., Frostegård, A., Göransson, P., Depret, G., Pascale, J., Simonet, P., 2001. Quantification of bacterial subgroups in soil: comparison of DNA extracted directly from soil or from cells previously released by density gradient centrifugation. Environ. Microbiol. 3, 431–439.

Daniel, R., 2004. The soil metagenome—a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. 15, 199–204.

Daniel, R., 2005. The metagenomics of soil. Nat. Rev. Microbiol. 3, 470–478.

Dice, L.R., 1945. Measures of the amount of ecologic association between species. Ecology 26, 297–302.

Finkel, S.E., Kolter, R., 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J. Bacteriol. 83, 6288–6293.

He, J., Xu, Z., Hughes, J., 2005. Pre-lysis washing improves DNA extraction from a forest soil. Soil Biol. Biochem. 37, 2337–2341.

Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: van Elsas, J.D., Wellington, E.M.H., Trevors, J.T. (Eds.), Modern Soil Microbiology. Marcel-Dekker, Inc., NY, pp. 353–373.

IUSS, Working Group, WRB, 2006. World reference base for soil resources. World Soil Resources Reports, vol. 103. FAO, Rome.

Kowalchuk, G., 1999. Fungal community analysis using denaturing gradient gel electrophoresis (DGGE). In: Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J. (Eds.), Molecular Microbial Ecology Manual, 3.4.6. Kluwer Academic Publisher, Dordrecht, pp. 1–16.

Kozdrój, J., van Elsas, D.J., 2000. Application of polymerase chain reaction-denaturing gradient gel electrophoresis for comparison of direct and indirect extraction methods of soil DNA used for microbial community fingerprinting. Biol. Fertil. Soils 31, 372–378.

Lejon, D.P.H., Chaussod, R., Ranger, J., Ranjard, L., 2005. Microbial community structure and density under different tree species in an acid forest soil (Morvan, France). Microb. Ecol. 50, 614–625.

Levy-Booth, D.J., Campbell, R.G., Gulden, R.H., Hart, M.M., Powell, J.R., Klironomos, J.N., Pauls, K.P., Swanton, C.J., Trevors, J.T., Dunfield, K.E., 2007. Cycling of extracellular DNA in the soil environment. Soil Biol. Biochem. 39, 2977–2991.

Matsui, K., Ishii, N., Kawabata, Z., 2003. Release of extracellular transformable plasmid DNA from *Escherichia coli* cocultivated with algae. Appl. Environ. Microbiol. 69, 2399–2404.

Murrell, P., 2006. R graphics. In: Computer Science & Data Analysis Series, Chapman & Hall/CRC, Boca Raton, FL, USA.

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. 2, 317–322.

Nielsen, K.M., Calamai, L., Pietramellara, G., 2006. Stabilization of extracellular DNA and proteins by transient binding to various soil components. In: Nannipieri, P., Smalla, K. (Eds.), Soil Biology, Nucleic Acids and Proteins in Soil, 8. Springer-Verlag, Heidelberg, Germany, pp. 141–153.

Nielsen, K.M., Johnsen, P.J., Bensasson, D., Daffonchio, D., 2007. Release and persistence of extracellular DNA in the environment. Environ. Biosaf. Res. 6, 37–53.

Niemeyer, J., Gessler, F., 2002. Determination of free DNA in soils. J. Plant Nutr. Soil Sci. 165, 121–124.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.

Pietramellara, G., Ceccherini, M.T., Ascher, J., Nannipieri, P., 2006. Persistence of transgenic and not transgenic extracellular DNA in soil and bacterial transformation. Biol. Forum 99, 37–68.

Pietramellara, G., Ascher, J., Ceccherini, M.T., Nannipieri, P., Wenderoth, D., 2007. Adsorption of pure and dirty bacterial DNA on clay minerals and their transformation frequency. Biol. Fertil. Soils 43, 731–739.

Pinchuk, G.E., Ammons, C., Culley, D.E., Li, S.M.L., McLean, J.S., Romine, M.F., Nealson, K.H., Fredrickson, J.K., Beliaev, A.S., 2008. Utilization of DNA as a sole source of phosphorus, carbon, and energy by *Shewanella* spp.: ecological and physiological implications for dissimilatory metal reduction. Appl. Environ. Microbiol. 74, 1198–1208.

Pinheiro, J.C., Bates, D.M., DebRoy, S., Sarkar, D., 2007. The R Core team, nlme: linear and nonlinear mixed effects models. R Package Version 3.1-86.

Pinheiro, J.C., Bates, D.M., 2000. Mixed-effects Models in S and S-PLUS. Springer-Verlag, pp. 1–528.

Potè, J., Ceccherini, M.T., van Tran, V., Rosselli, W., Wildi, W., Simonet, P., Vogel, T., 2003. Fate and transfer of antibiotic resistance genes in saturated soil columns. Eur. J. Soil Biol. 39, 65–71.

R Development Core Team, 2007. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, ISBN 3-900051-07-0, http://www.R-project.org.

Robe, P., Nalin, R., Capellano, C., Vogel, T.M., Simonet, P., 2003. Extraction of DNA from soil. Eur. J. Soil Biol. 39, 183–190.

Rondon, M.R., August, P.R., Bettermann, A.D., Brady, S.F., Grossman, T.H., Liles, M.R., Loiacono, K.A., Lynch, B.A., Macneil, I.A., Minor, C., Tiong, C.L., Gilman, M., Osburne, M.S., Clardy, J., Handelsman, J., Goodman, R.M., 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. 66, 2541–2547.

Steinberger, R.E., Holden, P.A., 2005. Extracellular DNA in single- and multiple-species unsaturated biofilms. Appl. Environ. Microbiol. 71, 5404–5410.

Wackernagel, W., 2006. The various sources and the fate of nucleic acids in soil. In: Nannipieri, P., Smalla, K. (Eds.), Soil Biology, Nucleic Acids and Proteins in Soil, 8. Springer-Verlag, Heidelberg, Germany, pp. 117–134.