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ORIGINAL PAPER

Purification and isotopic signatures (δ^{13}C, δ^{15}N, Δ^{14}C) of soil extracellular DNA

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Abstract The aim of this work was to obtain pure extracellular DNA molecules so as to estimate their longevity in soil by an isotope-based approach. Extracellular DNA molecules were extracted from all horizons of a forest soil and purified by the procedure of Davis (Purification and precipitation of genomic DNA with phenol–chloroform and ethanol. In: Davis LG, Dibner MD, Battey JF (eds) *Basic methods in molecular biology.* Appleton & Lange, Norwalk, 16–22, 1986) without (DNA1) or with (DNA2) a successive treatment with binding resins followed by elution. The two differently purified DNA samples were compared for their A_{260}/A_{280} ratio, polymerase chain reaction (PCR) amplification and natural abundance of stable (^{13}C and ^{15}N) and radioactive (^{14}C) isotopes. The purity index and the PCR amplification did not differentiate the efficiency of the two purification procedures. The isotopic signature of DNA was more sensitive and was strongly affected by the purification procedures. The isotopic measurements showed that the major contaminant of extracellular DNA1 was the soil organic matter (SOM), even if it is not possible to exclude that the similar δ^{13}C, δ^{15}N and Δ^{14}C values of DNA and SOM could be due to the use of SOM-deriving C and N atoms for the microbial synthesis of DNA. For extracellular DNA2, extremely low values of Δ^{14}C were obtained, and this was ascribed to the presence of fossil fuel-derived substances used during the purification, although in amounts not revealed by gas chromatography-mass spectrometry analysis. The fact that it is not possible to obtain contaminant-free DNA molecules and the potential use of soil native organic compounds during the microbial synthesis of DNA make it not achievable to estimate the age of soil extracellular DNA by radiocarbon dating.

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Introduction

In soil, hydrolysis of DNA can be reduced by several conditions such as low temperature or high salt concentration (Hofreiter et al. 2001), which can inhibit nucleases activity. In addition, DNA released by plants, animals and microbial cells can be protected against degradation by nucleases if it forms stable complexes with clay minerals and/or humic substances (Crecchio and Stotzky 1998; Demanèche et al. 2001). Extracellular DNA, likely adsorbed on soluble organo-mineral complexes, may migrate from the surface to the deeper soil horizons (Potè et al. 2004; Agnelli et al. 2004). However, the stable complexed DNA can be available and can transform competent bacterial cells (Lorenz and Wackernagel 1994). The uptake of extracellular DNA by competent bacteria is considered a process that allows a spreading of genetic information in soil (Gallori et al. 1994; Nielsen and van Elsas 2001).

Total DNA in the topsoil horizons has been estimated to be about 80 μg g^{-1} of soil (Torsvik and Goksøyr 1978; Paget et al. 1998), whereas the amount of extracellular

DNA in soil was about 1 μg g^{-1} (Selenska and Klingmüller 1992; Ogram et al. 1987). Agnelli et al. (2004) extracted from the whole profile of a forest soil both total and extracellular DNA, which were then characterised by denaturing gradient gel electrophoresis (DGGE) after polymerase chain reaction (PCR) amplification with universal primers for bacterial and fungal communities. The amounts of total DNA were in line with those reported above, at least for the near-surface horizons of the profile, and the percentage of extracellular DNA ranged from about 10 to 60% of the total DNA. The eubacterial DGGE profiles obtained from the extracellular DNA showed the presence of bands not present in the total DNA patterns of the same soil horizon, and this was ascribed to bacterial species not anymore present in the relative horizons. This hypothesis should imply the persistence of extracellular DNA within the soil (Pietramellara et al. 2006).

The persistence of extracellular DNA can be considered as an environmental risk in the case of genetically modified organisms. Indeed, if transgenic genes, such as those encoding for the resistance to antibiotics, herbicides or pests, are persistent in soil, it is possible their transmission to the native soil microflora by natural transformation (Lorenz and Wackernagel 1994; England et al. 1997). Potential disturbances to soil functioning are possible if these genes are expressed once incorporated in the indigenous microflora, as microbial communities contribute to key processes in soil (Hiebert and Bennet 1992; Richter and Markewitz 1995). Therefore, there is the need to determine the longevity of the extracellular DNA in the soil environment.

Persistence of specific DNA sequences in soil, usually determined by PCR amplification with specific primers, can be variable. Romanowski et al. (1992, 1993) found that plasmid DNA persisted in soil for at least 60 days; England et al. (1997) documented the persistence of recombinant bacterial DNA for 24 weeks. Persistence of DNA from genetically engineered tobacco plants in soil under field conditions ranged from several months (Widmer et al. 1997) to over 1 year (Paget et al. 1998); Gebhard and Smalla (1999) detected construct-specific sequences for up to 6 months in soil microcosms and for up to 2 years under field conditions. All these reports do not show if the target DNA was partially degraded by enzymes, associated with non-target DNA naturally present in the soil, and stabilised by soil colloids. These three processes might reduce the DNA recovery and PCR detection and, hence, influence the results about the longevity of DNA in soil (England et al. 1997, 2004). As far as we know, no data have been published about persistence of extracellular DNA in the soil profile assessed by ^{14}C natural abundance.

The aim of this research was to obtain pure extracellular DNA molecules so as to allow the estimation of their longevity in soil by an isotope-based approach. To achieve this goal, the extracellular DNA was extracted from all the horizons of a forest soil and purified according to two different procedures; the two differently purified DNA samples were compared for their purity index (A_{260}/A_{280} ratio), PCR amplification and natural abundance of radioactive (^{14}C) and stable isotopes (^{13}C and ^{15}N). As a reference, we also run the isotopic measurements on total DNA and soil organic matter (SOM), both extracted from the same soil.

Materials and methods

The soil, a fine loamy, mixed, mesic, Humic Dystrudept (Soil Survey Staff 2003), is located in the Vallombrosa National Reserve (Florence, Italy). Detailed description of the area and the main soil characteristics are reported elsewhere (Agnelli et al. 2002, 2004). The soil is made by a modern soil (from A1 to Bw2 horizon) that rests on a buried and truncated paleosol (BCb1 and BCb2 horizons; Corti et al. 2001). The soil samples were collected during spring 2002 from the middle portion of each horizon and sieved at 4 mm at field moisture; the less than 4-mm fraction was stored in plastic bags and refrigerated at −20°C.

The organic C content was determined, after washing soil with 0.2 M HCl solution by Carlo Erba NA 1500 combustion analyser.

The SOM was extracted from soil samples by 0.1 M NaOH solution (solid/liquid ratio 1:10 under N_2 atmosphere). After 24 h of shaking, the suspension was centrifuged (1,5000×*g*) to recover the supernatant, which was then filtered at 0.45 μm, brought to pH<4.0 with 6 M HCl solution, dialysed with 100 Da molecular weight cut off membranes (Spectra/Por Biotec CE) and then freeze-dried. After this procedure, we obtained all the extractable bulk organic matter made of humic acids, fulvic acids and non-humic material; this latter, according to Stevenson (1994), comprises compounds such as low molecular weight organic acids, amino acids, nucleic acids, simple sugars and polysaccharides.

Whole-community DNA (total DNA) was extracted from soil using a bead-beating method (FastDNA SPIN Kit for soil, Bio101, USA; Fig. 1). The amount of the extracted DNA was estimated by a fluorometer (Hoefer™ DyNA Quant™ 200) using bisbenzimide dye (Hoechst H 33258).

Extracellular DNA was extracted by a modification of the method of Ogram et al. (1987): several aliquots (10 g each) of soil were sequentially (three times) washed and gently shaken at room temperature for 30 min with 0.12 M Na_2HPO_4 solution at pH 8.0 (solid/liquid ratio 1:1), followed by centrifugation at 3,700×*g* at 20°C for 30 min. The crude DNA extracts were concentrated and purified according to two different methods (outlined in Fig. 1): the commonly adopted one proposed by Davis (1986) and a

Fig. 1 Schematic representation of the procedures used for extraction, concentration and purification of total DNA, extracellular DNA1 and extracellular DNA2 from the horizons of the soil of Vallombrosa forest (Firenze, Italy)

	Total DNA	Extracellular DNA1	Extracellular DNA2
Extraction	Soil + Na_2HPO_4 0.12 M + MT buffer (Bio101) soil:liquid ratio 1:2 Cell disgregation (Fast Prep^{TM} Instrument, BIO101) for 30 sec Centrifugation at 3,700*xg* for 10 min	Soil + Na_2HPO_4 0.12 M soil:liquid ratio 1:1 Gentle agitation on horizontal shaker for 30 min Centrifugation at 3,700*xg* for 30 min	Soil + Na_2HPO_4 0.12 M soil:liquid ratio 1:1 Gentle agitation on horizontal shaker for 30 min Centrifugation at 3,700*xg* for 30 min
Concentration		DNA precipitation with ethanol at –20º C and 5M NaCl solution DNA suspension (distilled H_2O)	Freeze - drying DNA suspension (distilled H_2O) Dialysis (100 Da MWCO) Freeze - drying DNA suspension (distilled H_2O)
Purification and concentration	Protein precipitation (PPS, BIO101) DNA adsorption (Binding Matrix, BIO101) DNA washing (SEWS, BIO101) DNA elution (ultra pure water)	Protein precipitation (phenol-chloroform-isoamilic alcohol) DNA precipitation with ethanol at –20º C and 5M NaCl solution DNA suspension (ultra pure water)	Protein precipitation (phenol-chloroform-isoamilic alcohol) Filtration at 0.2 µm (Quiafilter, Qiagen) DNA adsorption (G-500 columns, Qiagen) DNA washing+elution (QC + QF Buffer, Qiagen) DNA adsorption (Binding Matrix, BIO101) DNA washing (SEWS, BIO101) DNA elution (ultra pure water)
	Freeze-drying	Freeze-drying	Freeze-drying

combined procedure. The procedure of Davis (1986) involved the precipitation of the crude DNA by cold 96% (v:v) ethanol and 5 M NaCl solution (DNA extract/ethanol/NaCl ratio 1:2:0.3). The resulting pellets were suspended in appropriate volumes of sterile distilled water and processed with phenol–chloroform and chloroform–isoamilic alcohol to eliminate protein residues. The DNA was then concentrated by further precipitation as described above, incubated at 37°C to evaporate residual ethanol and re-suspended in sterile ultra pure water. The resulting DNA sample was named extracellular DNA1. The yield of extracellular DNA1 was estimated by fluorometer.

The combined procedure involved concentration of the crude DNA extracts by freeze drying, suspension in appropriate volumes of sterile distilled water and successive dialysis with 100 Da molecular weight cut-off membranes (Spectra/Por Biotec CE) to eliminate the exceeding salt. After dialysis, the extracts were concentrated by freeze drying, re-suspended in sterile distilled water and treated with phenol–chloroform and chloroform–isoamilic alcohol to eliminate protein residues. The DNA was then concentrated with cold ethanol and 5 M NaCl solution and re-suspended in sterile distilled water. A further purification was then performed sequentially by DNA-binding resins

using the G-500 columns (Qiagen), which allowed to process and concentrate large volumes of DNA extracts (up to 250 ml) and the DNA-Binding Matrix (Bio101) by "DNA bind–wash–elute" procedures. These latter steps of the purification are described here below in detail. The DNA water suspension was filtered at 0.2 μm (QIAfilter Cartridges, Qiagen) and passed through a G-500 column (Qiagen), allowing the binding of the DNA to the resin. The resin was then washed two times with Buffer QC (Qiagen), containing 1.0 M NaCl, 50 mM MOPS at pH 7 and isopropanol 15%, and then the DNA was eluted from the resin by Buffer QF (Qiagen), containing 1.25 M NaCl, 50 mM Tris, Tris-Cl at pH 8.5 and isopropanol 15%. The eluted DNA was precipitated by mixing the DNA solution with isopropanol by inversion (DNA/isopropanol ratio 1:0.7) at room temperature for 20 min; then, the precipitated DNA was separated by high-speed centrifugation (20,800×*g*) at 4°C for 30 min. The DNA pellets were washed several times with appropriate volumes of room-temperature 70% ethanol (v:v) so as to remove excess salt and residual isopropanol, incubated at 37°C to evaporate the residual ethanol and dissolved in 5 ml of sterile ultra pure water. The DNA solution was transferred to sterile centrifuge tubes, treated with an equal volume of DNA-Binding Matrix solution, containing guanidine isothiocyanate, and incubated at room temperature for 15 min. The DNA-silica matrix complex was separated from the liquid phase in sterile tubes provided with filters (Spin Filters, Bio101) by high-speed centrifugation (20,800×*g*) at room temperature for 5 min. The matrix-bound DNA was washed three times with 500 μl of SEWS (Salt–ethanol wash solution, Geneclean®, Bio101), made of 70% ethanol and 0.1 M of CH_3COONa solution, and then high-speed centrifuged (20,800×*g*). The pellet was dried at 37°C to evaporate the residual ethanol, and finally, the DNA was recovered by elution with sterile water. The resulting DNA was named extracellular DNA2. The yield of extracellular DNA2 was estimated by fluorometer.

The presence of diethylaminoethanol (DEAE; a component of the G-500 resin), guanidine isothiocyanate (used as chaotropic salt for the DNA-Binding Matrix resin), and CH_3COONa (a component of SEWS) as contaminants in the extracellular DNA2 was checked by gas chromatography-mass spectrometry (GC-MS). The DNA2 extracts were analysed by an Agilent 5971 GC-MS after the following treatments: DEAE was extracted after alkalinisation to pH 11 with dichloromethane (v:v 1:1), guanidine isothiocyanate was derivatised with acetylacetone (Palaitis and Curran 1984), acetate ions were extracted by SPME (solid phase micro-extraction) after acidification at pH 2 with H_3PO_4 and derivatised according to Aikawa and Burk (1997) and Godoi et al. (2005).

The purity of the total and extracellular DNA was monitored by measuring the absorbances at 260 and 280 nm and calculating the purity index A_{260}/A_{280} ratio (Tien et al. 1999) and by PCR amplification of eubacterial 16S rDNA (Agnelli et al. 2004) using a dilution ratio for the reaction mixture of 1:6.25 for all the DNA samples, with the exception of the extracellular DNA1 extracted from A1 and A2 horizons (ratio of 1:62.5).

Natural abundance of ^{13}C, ^{15}N and ^{14}C were determined at the Keck Carbon Cycle Accelerator Mass Spectrometry Laboratory, Earth System Science Department, University of California, Irvine (USA).

The abundance of ^{13}C and ^{15}N were measured by stable isotope mass spectrometry, and the values were expressed, respectively, in

$$\delta^{13}C = \left({}^{13}C/{}^{12}C_{sample} - {}^{13}C/{}^{12}C_{standard}\right)/{}^{13}C/{}^{12}C_{standard} \times 10^3,$$

where $\delta^{13}C$ represents the ‰ deviation from the $^{13}C/^{12}C$ ratio of the Pee Dee Belemnite standard (PDB), and

$$\delta^{15}N = \left({}^{15}N/{}^{14}N_{sample} - {}^{15}N/{}^{14}N_{standard}\right)/{}^{15}N/{}^{14}N_{standard} \times 10^3,$$

where $\delta^{15}N$ represents the ‰ deviation from the $^{15}N/^{14}N$ ratio of atmospheric air.

Radiocarbon data were obtained by accelerator mass spectrometry (AMS) and expressed in $\Delta^{14}C$, the ‰ deviation from the $^{14}C/^{12}C$ ratio of oxalic acid standard in 1950 (Modern) corrected for a $\delta^{13}C$ of −25‰, which takes into consideration the mass dependent isotopic fractionation effects (Stuiver and Polach 1977). Positive $\Delta^{14}C$ indicate the presence of ^{14}C produced by nuclear weapons testing, meaning that a considerable amount of the C in the analysed material was fixed by plants since 1950 (Trumbore 1996); negative values indicate that the C in the material has resided in the soil long enough for significant radioactive decay of ^{14}C (half life 5,730 years).

On the basis of the radiocarbon data and according to the model of Trumbore et al. (1996) and Gaudinski et al. (2000), reported in detail in Agnelli et al. (2002), we calculated the mean residence time (MRT) of the C in the analysed samples.

Each sample was analysed at least in duplicate, and the standard errors (Webster 2001) were calculated accordingly.

Results

Organic C content and total DNA, extracellular DNA1 and extracellular DNA2 yield

The largest content of organic C was in the A horizons, 91.0 and 31.4 mg g^{-1} in the A1 and A2 horizons, respectively

Table 1 Amounts of organic C, total DNA, extracellular DNA1 and extracellular DNA2 from the horizons of the soil of Vallombrosa forest (Firenze, Italy)

Horizons	Depth (cm)	Organic C (mg g^{-1})		Total DNA (μg g^{-1})		Extracellular DNA1 (μg g^{-1})		Extracellular DNA2 (μg g^{-1})	
A1	0–11	n=3	91.0 (2.2)	n=8	92.6 (23.6)	n=4	18.9 (2.9)	n=6	17.9 (5.7)
A2	11–23	n=3	31.4 (0.8)	n=6	68.5 (21.4)	n=2	41.1 (0.9)	n=6	39.1 (1.2)
Bw1	23–42	n=3	12.7 (0.1)	n=6	44.9 (18.3)	n=5	10.8 (1.5)	n=6	10.5 (3.2)
Bw2	42–71	n=3	5.9 (0.2)	n=6	39.2 (12.9)	n=5	5.6 (2.2)	n=6	5.4 (4.8)
BCb1	71–109	n=3	4.3 (0.2)	n=6	36.8 (13.8)	n=7	3.8 (0.7)	n=6	3.6 (1.8)
BCb2	109–149+	n=3	3.1 (0.5)	n=6	20.4 (9.0)	n=14	2.2 (0.3)	n=6	2.0 (1.0)

Numbers in parentheses are the standard errors.
n Number of replicates for organic C and extractions for DNA

(Table 1). The organic C concentration decreased steeply with depth reaching 3.1 mg g^{-1} in the BCb2 horizon.

The amount of total DNA followed the same trend of the organic C and ranged from the surface to the bottom of the profile from 92.6 to 20.4 μg g^{-1} (Table 1). The extracellular DNA was present in greater amounts in the A2 horizon (41.1 μg g^{-1} for DNA1 and 39.1 μg g^{-1} for DNA2) than in the A1 horizon (18.9 μg g^{-1} for DNA1 and 17.9 μg g^{-1} for DNA2). Below, the A2 horizon the quantity of both extracellular DNA1 and DNA2 decreased with increasing depth and was as low as about 2.2 μg g^{-1} in the BCb2 horizon (Table 1).

Efficiency of the purification procedures of extracellular DNA tested by purity index and PCR amplification

The extracellular DNA2 presented purity indices (A_{260}/A_{280} ratio) between 1.8 and 2.0, which are similar or higher than those generally obtained for the total DNA (Table 2). On this basis, extracellular DNA2 appeared better purified than extracellular DNA1, which gave values between 1.6 and 1.9. As reference, we determined the purity indices of some commercial pure DNAs from different sources, and we obtained values that were similar to those of extracellular DNA2 (Table 3).

Table 2 Purity index (A_{260}/A_{280} ratio) of total DNA, extracellular DNA1 and extracellular DNA2 extracted from the horizons of the soil of Vallombrosa Forest (Florence, Italy)

Horizons	Total DNA	Extracellular DNA1	Extracellular DNA2
A1	1.7 (0.07)	1.6 (0.03)	1.8 (0.03)
A2	1.8 (0.06)	1.6 (0.02)	1.9 (0.03)
Bw1	1.8 (0.04)	1.7 (0.03)	1.9 (0.08)
Bw2	1.9 (0.06)	1.8 (0.02)	1.9 (0.04)
BCb1	1.9 (0.03)	1.7 (0.06)	2.0 (0.08)
BCb2	1.9 (0.04)	1.9 (0.05)	1.9 (0.03)

Numbers in parentheses are the standard errors (n=5).

All the samples responded positively to the 16S rDNA-PCR assay (Fig. 2), as total DNA and both extracellular DNA1 and DNA2 were amplified, although extracellular DNA1 from A1 and A2 horizon required a tenfold higher dilution than the other samples.

Isotopic analyses

Figure 3 shows the isotopic signatures for ^{13}C (δ^{13}C) and ^{15}N (δ^{15}N) of total and extracellular DNA and SOM. The δ^{13}C and δ^{15}N of extracellular DNA1 were very close to those of the SOM (Fig. 3a, b). On the contrary, extracellular DNA2 showed a strong depletion in ^{13}C content with respect to those of SOM and extracellular DNA1 (Fig. 3a). The total soil DNA showed δ^{13}C values lower than those of the other fractions extracted from A1 to Bw2 horizons (from −37.3 to −43.4‰); in the BCb horizons, the lowest δ^{13}C values were shown by extracellular DNA2: −46.0 and −46.6‰ in the BCb1 and BCb2, respectively. The extracellular DNA2 showed δ^{15}N values higher than those of total DNA only in the A1 and A2 horizons: −4.9 and −0.6‰, respectively (Fig. 3b). In the B horizons, the trend reversed, and the δ^{15}N values of extracellular DNA2 were lower than those of total DNA of the same horizons. In any case, the δ^{15}N of extracellular DNA2 and total soil DNA were always lower than those of extracellular DNA1 and SOM.

The radiocarbon analysis of SOM extracted from the A horizons showed positive Δ^{14}C values, 171.6‰ in the A1 and 30.7‰ in the A2 (Table 4). Further, this C pool was probably dominated by fast cycling compounds, as shown

Table 3 Purity indices of commercial pure DNAs

Commercial DNA	
Calf Thymus (Hoefer, Amersham)	1.8 (0.04)
Felix Marker (Quantum, Appligene)	1.9 (0.02)
1-kb Plus DNA Ladder (Gibco BRL, Life Technologies)	1.8 (0.04)
Gene Ruler 1 kb (Fermentas)	1.9 (0.08)

Numbers in parentheses are the standard errors (n=5).

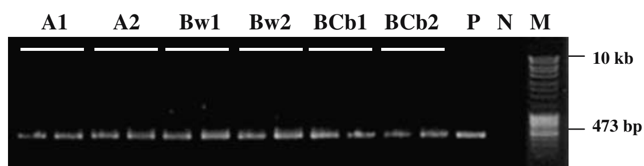

Fig. 2 Determination of purity of extracellular DNA1 by 16S rDNA-PCR assay. The presence of positive signals (473 bp) on the agarose gel indicates the PCR amplification of the DNA1 extracted from the horizons of the soil of Vallombrosa forest (Florence, Italy). *P*, positive control (PCR product of DNA of *B. subtilis*); *N*, negative control (no DNA); *M*, high mass DNA ladder (10 kb–80 bp; Fermentas)

by the MRT values ranging from 13 to 207 years (Table 4). SOM showed a Δ^{14}C of −135.8‰ in the Bw1 horizon and −282.5‰ in the Bw2, with radiocarbon-based ages of 1,120 and 2,615 years before present (YBP), respectively; the estimated MRT for Bw1 and Bw2 horizons rose to 1,360 and 3,230 years, indicating a turnover of the organic C slower than that in the A horizons. At deeper horizons, the Δ^{14}C signatures were −270.9 for the BCb1 horizon and −256.2‰ for the BCb2, with a radiocarbon-based age of 2,485 and 2,325 YBP and a MRT of 3,050 and 2,830 years, respectively.

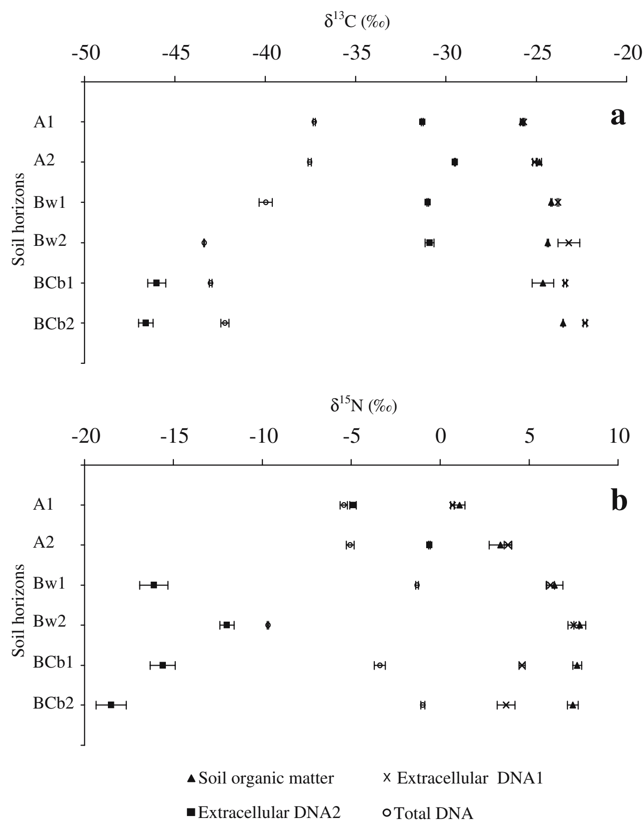

Fig. 3 δ^{13}C (**a**) and δ^{15}N (**b**) of soil organic matter, total DNA, extracellular DNA1, and extracellular DNA2 extracted from the horizons of the soil of Vallombrosa forest (Firenze, Italy). The *horizontal bars* represent the standard errors (n=3)

Table 4 Δ^{14}C, ^{14}C-based age and mean residence time (MRT) of soil organic matter, extracellular DNA1 and extracellular DNA2 extracted from the horizons of the soil of Vallombrosa forest (Florence, Italy)

	Δ^{14}C (‰)	^{14}C age (years before present)	MRT (year)
Soil organic matter			
A1	171.6 (2.8)	nd	13
A2	30.7 (2.3)	nd	207
Bw1	−135.8 (1.7)	1,120 (20)	1,360
Bw2	−282.5 (1.7)	2,615 (20)	3,230
BCb1	−270.9 (1.7)	2,485 (20)	3,050
BCb2	−256.2 (1.8)	2,325 (20)	2,830
Extracellular DNA1			
A1	150.7 (3.5)	nd	11
A2	4.2 (3.3)	nd	300
Bw1	−161.0 (2.2)	1,360 (25)	1,633
Bw2	−257.6 (2.1)	2,340 (25)	2,854
BCb1	−333.3 (2.1)	3,205 (30)	4,073
BCb2	−320.0 (2.2)	3,108 (38)	3,840
Extracellular DNA2			
A1	−983.2 (0.6)	32,790 (270)	470,000
A2	−985.4 (0.8)	32,862 (180)	545,000
Bw1	−987.4 (2.6)	35,090 (1650)	630,000
Bw2	−991.0 (0.5)	37,760 (490)	900,000
BCb1	−927.6 (0.7)	21,040 (80)	103,000
BCb2	−933.7 (0.8)	21,750 (100)	112,000

Numbers in parentheses are the standard errors (n=2). Positive values of Δ^{14}C indicate the presence of ^{14}C produced by nuclear weapons testing, meaning that a considerable amount of the C in the analysed material was fixed by plants in recent times (since 1950).
nd Not determinable

The extracellular DNA1 displayed Δ^{14}C values close to those of SOM for A and Bw horizons (Table 4), with signatures that decreased by increasing depth. As for SOM, extracellular DNA1 had positive Δ^{14}C values in the A1 and A2 horizons and negative values in the deeper horizons. In the BCb horizons, DNA1 had Δ^{14}C values lower than those of the SOM.

The extracellular DNA2 samples showed low abundances of ^{14}C (Table 4); the Δ^{14}C values decreased slightly from the surface to the Bw2 horizon and increased in the BCb horizons. The calculated age of extracellular DNA2 ranged from about 33,000 YBP in the A horizons to about 38,000 YBP in the Bw2 horizon and from around 21,000 to 21,750 YBP in the BCb horizons. As expected from the low abundance of radiocarbon, the calculated MRT of extracellular DNA2 gave very high values, ranging from about 100,000 to 900,000 years.

The GC-MS analysis of the DNA2 samples, ran to assess the presence of some reagent residues (DEAE, guanidine isothyocianate, CH_3COONa) used in the last steps of the purification procedure, showed the absence of these compounds, at least considering the detection limit of the instrument (less than 0.1 ppm).

Discussion

Total and extracellular DNA were recovered from all the soil horizons, up to about 1.5 m depth. Such a depth is very common in this kind of soils (Inceptisols), among the most diffused forest soils in Mediterranean regions. The extraction of intracellular and extracellular DNA up to the depth of 1.5 m confirms that microbial communities inhabit all the soil profile and not only the superficial horizons.

The two procedures used to purify the crude extracellular DNA extracts gave similar yields for DNA1 and DNA2 (Table 1), probably because the unavoidable DNA losses because of purification (Rose-Amsaleg et al. 2001; Braid et al. 2003; Robe et al. 2003) occurred during the steps shared by the two methods (Fig. 1). The “DNA bind–wash–elute approach,” the last purification steps of extracellular DNA2, did not produce detectable losses of DNA.

The purity index and the PCR assay did not give clear-cut results on the efficiency of the used purification procedures. By comparing the purity indices of commercial, total and extracellular DNA samples, the combined procedure (extracellular DNA2) was more effective than the procedure of Davis (1986; extracellular DNA1). However, differences between the purity indices of the two purified extracellular DNA samples were small. Both extracellular DNA samples were successfully amplified by PCR, although the dilution needed to amplify the extracellular DNA1 extracted from the A horizons was tenfold higher than that used for the DNA2 of the same horizons. This fact would confirm the better efficacy of the combined procedure. Nevertheless, PCR amplification cannot be taken as conclusive evidence of the absence of organic contaminants such as humic acids in the samples. The lowest amount of humic acid inhibiting the Taq-polymerase, the key enzyme of PCR, was 0.08 μg per ml of reaction mixture (Tebbe and Vahjen 1993), and the inhibition does not depend on the amount of DNA present in the solution (Tsai and Olson 1992). Consequently, the presence of smaller amounts of humic substances could not inhibit the PCR amplification, but they could affect C and N isotopic signature of DNA samples. The δ^{13}C, δ^{15}N and Δ^{14}C values of extracellular DNA1 were very close to those of SOM, indicating that DNA1 was contaminated by SOM, although in low amounts to allow PCR amplification. These isotopic data may also suggest that the use of ethanol and phenol–chloroform in the purification procedure did not contaminate, or only slightly (and, in this case, they were masked by the SOM), the DNA samples. Similar results were reported by Créach et al. (1999), who did not observe any contamination by using ethanol and phenol–chloroform during the purification procedure of DNA extracted from sediments. If those fossil fuel-derived reagents had been present as main contaminants of the DNA samples, the radiocarbon signatures should have been more negative than those measured for extracellular DNA1. In fact, small amounts of very old C atoms can greatly influence the signature and/or the estimated age of the samples (Paul et al. 1997). In the BCb1 and BCb2 horizons of the Vallombrosa soil, the low yield of extracellular DNA (Table 1) and the possible presence of small amounts of fossil fuel-derived reagents as contaminants in the extracts could be responsible for the fact that the Δ^{14}C value of extracellular DNA1 was considerable lower than that of SOM (Table 4).

The similar isotopic signatures between extracellular DNA1 and SOM can also depend on the fact that the source of C and N atoms of DNA is SOM. Indeed, most of the soil DNA is of bacterial origin (Torsvik et al. 1995), and SOM is the main C and N source of soil microorganisms. Several authors showed a direct link between SOM and soil microbial community by using isotopic probes. Coffin et al. (1990) measured the ^{13}C natural abundance in the nucleic acids extracted from estuarine bacterial community to trace sources of organic matter for bacteria in aquatic environments; Pelz et al. (1998) found that D-alanine, a specific bacterial amino acid, has similar isotopic signature of the bacterial substrate, and it could be used as biomarker to trace bacterial carbon sources in soils. Créach et al. (1999) found that the ^{13}C/^{12}C ratios of DNA extracted from sediments of two different vegetation zones of a salt marsh were very close to those of the plant-derived organic matter. Boschker et al. (1999) used ^{13}C ratios of bacterial phospholipid fatty acids to reveal the contribution of plant-derived material to microbial biomass in salt-marsh sediments.

However, the occurrence of an isotopic fractionation during the synthesis of the DNA cannot be excluded, as heavy isotope discrimination occurs in several microbial biosynthetic pathways, such as the synthesis of amino acids (Macko et al. 1987), lipids (Summons et al. 1994; Abraham et al. 1998), fatty acids and in the Krebs cycle (Blair et al. 1985). Therefore, the signatures of extracellular DNA1 could be the result of the use of both C and N atoms of SOM during bacterial growth, with a preferential incorporation of the lighter isotopes, and the co-presence in DNA extracts of native organic matter compounds as contaminants. Because of this, the dating and the estimation of the persistence of extracellular DNA1 in soil by radiocarbon data have to be considered with caution.

The differences in δ^{15}N values between DNA1 and SOM in the BCb horizons could be ascribed to the microbial utilisation of inorganic N, which is less enriched in heavy isotope than organic N and, as NH_4^+, is mostly stabilised by 2:1 phyllosilicates (Delwiche and Steyn 1970; Krull and Skjemstad 2003). In the Vallombrosa soil, $NH_4^+ - N$ trapped in the interlayer of micas is present in considerable amounts in the B horizons (Corti et al. 1999). The fixed $NH_4^+ - N$, although not promptly available, can satisfy the

N needs of the microorganisms inhabiting the deeper horizons of this soil (Marzadori et al. 1994; Paris et al. 1995; Scherer and Werner 1996).

Extracellular DNA2 was likely better purified from the humic material than extracellular DNA1. However, the low isotopic signatures, and in particular those of radiocarbon, suggested that DNA2 was probably contaminated by fossil fuel-derived substances. The source of this contamination is not clear, as the ethanol and the phenol–chloroform–isoamilic alcohol, used respectively for DNA precipitation and protein removal, might have not affected the isotopic measurements (as discussed for extracellular DNA1); in addition, the other organic compounds (DEAE, guanidine isothyocianate, CH_3COONa) used in the purification procedure were not detected by GC-MS analysis. However, the low signatures showed by extracellular DNA2 and total DNA suggested that the contamination could be occurred in the last purification steps (the treatments with resins and the following washings of the resin-bound DNA), which were used for both types of DNA (Fig. 1).

Our results showed that the isotopic signature of extracellular DNA extracted from soil was strongly affected by the purification procedures. In the case of extracellular DNA1, the major contaminant, at least in the upper horizons, was SOM, even if we cannot exclude that the similar isotopic signatures of DNA1 and SOM can also derive from the use of C and N atoms of SOM during the microbial synthesis of DNA. In the case of the total and extracellular DNA2, the isotopic signatures were possibly affected by contaminants (not detected) such as fossil fuel-derived substances used during the purification or present in the purification kits.

In conclusion, the unfeasibility to obtain contaminant-free DNA molecules and/or the possible use of native soil organic compounds in the microbial synthesis of DNA make not possible the estimation of age and persistence of extracellular DNA in soil by a radiocarbon-based approach. However, future research addressed toward extraction methods of soil DNA that minimize the co-release of humic substances and purification procedures based on fossil fuel-not-derived reagents could improve the use of isotopes natural abundance in the study of soil DNA.

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