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Extracting DNA of nematodes communities from Argentine Pampas agricultural soils

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ABSTRACT

We examined four strategies (Tris/EDTA, sodium dodecyl sulfate, Chelex 100 resin and cetyltrimethylammonium bromide -CTAB-) for extracting nucleic acid (DNA) from communities of nematodes. Nematodes were isolated from an agricultural area under different management of long-term crop rotation experiment from Argentina during three seasons. After DNA extraction, Polymerase Chain Reaction-amplifications were performed and considered as indicators of successful DNA extraction. The CTAB combined with proteinase K and phenol-chloroform-isoamyl alcohol was the unique successful method because positive amplifications were obtained by using both eukaryotic and nematode specific primers. This work could contribute to biodiversity studies of nematodes on agroecosystems.

Key words: Agroecosystems, nematodes, nucleic acid extraction, CTAB.

INTRODUCTION

Nematodes are an evolutionarily successful group of organisms and represent an important part of the soil microfauna that affect the soil microflora as they occupy positions at primary, secondary and/or tertiary consumer level in soil food webs (Moore and de Ruiter 1991). Despite the relevance of nematodes, the current adoption of this groups as a bioindicator is limited mainly due to difficulties in obtaining fine-scale taxonomic resolution (genus level or below) based on morphological traits, in addition to often being limited by systematic expertise (Yeates 2003).

Molecular assays utilizing the conserved 18S rDNA [small subunit: SSU] gene have been developed as an alternative to study the diversity of nematodes as a monitoring tool (Chen et al. 2010). Molecular analysis of nematode soil community DNA involves recovery and purification of nucleic acids, followed by PCR amplification. However, the key step is the efficient extraction of high-quality DNA that is representative of the nematode community and its variations within

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the environment. When DNA extraction is done on samples collected from soil it is difficult to completely remove the humus with a routine total DNA extraction procedure which can inhibit the activity of *Taq* DNA polymerase in the PCR reaction (Steffan et al. 1988). Although DNA extraction kits from soil samples are available and successful, the few milligrams of soil that are required for DNA extraction may not be representative of the environmental diversity of nematodes in the sample. Our objective was to asses a reliable protocol for DNA extraction of nematodes communities from agricultural environmental samples.

MATERIALS AND METHODS

Study Area, Soil Collection and Nematode Extraction

Fifteen surface soil samples selected to obtain a wide range in agricultural management and climatic seasons were used in the development of the method (Table I). Soil samples were taken with a soil sampler (2.5 inner diameter x 20 cm long) from the top 20 cm from a long-term crop-pasture rotation experiment at the INTA-FCA, UNMdP Balcarce station, Argentina (37°45' S, 58°18' W; 130 m above sea level; 870-mm mean annual rainfall; 13.7°C mean annual temperature). Soil was a complex of Typic Argiudoll and Petrocalcic Paleudoll soils, pH 5.7 in water, loamy texture, 33.1 cmol kg^{-1} cation exchange capacity, and 5.0 mg kg^{-1} Bray and Kurtz P, organic matter content of 62.0 g kg^{-1}).

Field-crop management conditions where the soil was collected in winter, summer and spring (w, u and s, respectively) were: Pasture (P), Crop-pasture rotation with conventional tillage -CT- (Cpct), Crop-pasture rotation with no tillage -NT- (Cpnt), Crop rotation (30 years) with CT (Cct), Crop rotation (30 years) with NT (Cnt) (Table I). Eight sub-samples were collected from each experimental unit (5 x 25m). These were thoroughly mixed to obtain a single composite sample of approximately 1kg. Nematodes were extracted from 100g of fresh soil from each sample by the centrifugal-flotation method (Caveness and Jensen 1955) and placed in 25ml containers (4°C until use 24h later).

DNA Extraction of the Soil Nematode Community

Each sample was homogenized with a magnetic shaker (2 min) and immediately 4 subsamples of 5 ml each were collected. Nematodes of each subsample were individually collected (at least 20 nematodes) with the help of an "L"-shaped needle and placed in 1.5 ml Eppendorf tubes with 100 µl of milliQ autoclaved water and stored at -20 °C until use. Each subsample was processed according to one of four DNA extraction methods as described.

TRIS-EDTA BUFFER. Nematodes were crushed in 500 µl of TE buffer (10 mM Tris-HCl; EDTA 1 mM [pH 8.0]) and β-mercaptoethanol Sigma 1% solution with a sterile micropestle in an ice bath. The pellet was dried and resuspended in 50 µl PCR-quality (nuclease-free) water after centrifugation (13,000 rpm, 4 min, 4 °C) and stored at -20 °C until use.

SODIUM DODECYL SULFATE. Nematodes were crushed with a sterile micropestle in an ice bath in 500 µL of SDS (1% sodium dodecyl sulfate), 50 mM EDTA, 100 mM NaCl, 100 µg K proteinase ml^{-1} (Invitrogen), 1% β-mercaptoethanol (Sigma) and 100 mM Tris-HCl pH 8.5 following Donn et al. (2008). The material was frozen in liquid nitrogen, followed by thawing and heating to 60 °C for 30 min. The supernatant was transferred to a fresh tube, and subjected to phenol:chloroform:isoamyl alcohol (25:24:1, pH=8) extraction, with a subsequent chloroform extraction. The DNA was then precipitated (cold ethanol and 1/10 volume sodium acetate) and the resulting pellets were washed twice with 70% cold ethanol, dried, resuspended in 50 µl PCR-quality water and stored at -20 °C until use.

CHELEX RESIN. Nematodes were crushed in 240 µl TE buffer with 160 µl of 20% Chelex 100 (Bio-Rad Laboratories, Hercules, Calif.), with a sterile micropestle in an ice bath as a modification of the method described by Stock (2009). The samples were then subjected to two cycles of

TABLE I
Treatments description, yield and purity of DNA extracted by the Cetyltrimethylammonium Bromide Buffer (CTAB) method. Genomic DNA were analyzed on the Epoch (Biotech) Spectrophometer. The DNA concentration was determined by measuring absorbance at 260nm. The purity was determined by calculating the ratio of the absorbance at 260nm and 280nm.

Treatment (Nomenclature)	Season (Nomenclature)	Crop Characteristics	ADN ng.µL^{-1}	A260/A280
	Winter (w)			
Pasture (P)	(Pw)	*Dactylis glomerata*, *Phalaris tuberose, Festuca arundinacea*], *Lolium perenne*, *Trifolium repens*, and *Triflolium pratense*	66.345	1.592
Crop-pasture rotation with conventional tillage (Cpct)	(Cpctw)	Plowed soil (disk harrow and chisel one month before sampling)	15.345	0.941
Crop-pasture rotation with no-tillage (Cpnt)	(Cpntw)	Wheat (*Triticum aestivum* L.) fallow	31.295	1.468
Continuous cropping with conventional tillage (Cct)	(Cctw)	Plowed soil (disk harrow and chisel one month before sampling)	24.345	1.580
Continuous cropping with no-tillage (Cnt)	(Cntw)	Wheat (*Triticum aestivum* L.) fallow	14.345	1.258
	Summer (u)			
(P)	(Pu)	Same as (Pw)	29.845	0.935
(Cpct)	(Cpctu)	Pasture (5 month) *Dactylis glomerata*, *Phalaris tuberose, Lolium perenne*, *Trifolium repens*, and *Triflolium pratense*	20.845	0.918
(Cpnt)	(Cpntu)	Same as (Cpctu)	23.977	1.181
(Cct)	(Cctu)	Maize (*Zea mays*, 4 month)	31.845	1.684
(Cnt)	(Cntu)	Maize (*Zea mays*, 4 month)	15.345	1.153
	Spring (s)			
(P)	(Ps)	Same as winter	83.345	1.606
(Cpct)	(Cpcts)	Pasture (11 month), same as (Cpctu)	37.845	1.776
(Cpnt)	(Cpnts)	Pasture (11 month), same as (Cpctu)	54.345	1.455
(Cct)	(Ccts)	Maize (*Zea mays*) stover	50.643	1.412
(Cnt)	(Cnts)	Maize (*Zea mays*) stover	31.845	1.427

heating (95 °C, 10 min) / freezing in liquid nitrogen and then centrifuged at 13,000 rpm for 2 min at 4 °C. The supernatant was carefully transferred to a new tube and stored at -20 °C until use.

CETYLTRIMETHYLAMMONIUM BROMIDE BUFFER. Extraction of nucleic acids was performed following a method described by Vierstraete (2009) and Sambrook et al. (1989), somewhat modified: Nematodes were crushed in 500 µl of TE buffer with 2% CTAB (cetyltrimethylammonium bromide), 100 µg/ml K proteinase, and 1% β-mercaptoethanol with a sterile micropestle in an ice bath. Each microcentrifuge tube was subjected to three cycles of heating (95 °C, 10 min) / freezing in liquid nitrogen with 15 sec vortexing. After centrifugation (13,000 rpm, 7 min, 4 °C), the upper phase (without any solid material) was transferred to a fresh tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH=8) extraction solution was added and finger vortexed. This step was repeated, an equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and the sample was vortexed and centrifuged to remove any residual phenol. After centrifugation (13,000 rpm, 15 minutes), the upper phase (without chloroform) was transferred into a new 1.5 ml tube containing 750 µl cold isopropanol and 1/10 volume sodium acetate.

The tubes were maintained at -20 °C overnight. After centrifugation (13,000 rpm, 30 min, 4 °C), the supernatant was decanted into a beaker and the DNA pellet was washed in 200 µl cold 70% ethanol. The DNA pellet was air-dried and re-suspended in 50 µl PCR-quality water and stored at -20 °C until use.

Assessing the Quantity/Quality of Extracted DNA and the Success of the Extraction

The DNA concentration was quantified in all extracts by measuring absorbance at 260 nm with the Epoch (Biotech) spectrometer. The quality of the extract was also assessed by the 260/280 nm absorbance ratio (Sambrook et al. 1989). All experimental samples were subjected to PCR before any purification, to establish whether a purification step was essential for the successful amplification of gDNA.

To test the suitability of the DNA extracts for downstream molecular applications, the 18S small subunit ribosomal gene was amplified twofold using the primer pairs NEMF1/S3 (5'-CGCAAATTACCCACTCTC-3'/5'-AGTCAAATTAAGCCGCAG-3') and NS1/NS4 (5'-GTAGTCATATGCTTGTCTC-3'/5'-GGCTGCTGGCACCAGACTTGC-3'). For PCR amplification 2 µl DNA of template were used directly in a 25 µl reaction mixture in the presence of Taq Polymerase (Invitrogen, Carlsbad, CA). PCR was performed in an automated thermal cycler (PTC-200, MJ Research Inc.) with an initial 94 °C denaturation for 2 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1.5 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. Each reaction included a negative control without DNA.

When the PCR amplification failed, the extracts were purified with the Wizard® SV Gel and PCR Clean-Up System and each purified DNA sample was then subjected to PCR as described above. Agarose gels were stained with GelRed® and visualized with UV transillumination.

RESULTS AND DISCUSSION

The disruption of nematodes in TE buffer, in SDS, and in Chelex Resin methods, failed in the subsequent PCR amplification, even though the DNA samples were purified with the Wizard® kit. The validity of using molecular techniques for environmental studies depends on obtaining representative extracts of nucleic acids from an environmental sample. The DNA extraction protocols used for soils usually include from one to all three of the following basic elements: physical disruption, chemical lysis, and enzymatic lysis (Sambrook et al. 1989). In our experiment, we performed the same physical disruption of nematodes but we changed the buffer for chemical extraction and/or enzymatic lysis. Nucleic acid extraction methods are often inefficient because of coextraction of enzymatic inhibitors from the soil together with the DNA.

Even though direct lysis techniques have been used frequently because they yield more DNA, in our study this did not provide the expected successful result. The major drawback of direct lysis methods is that more substances PCR-inhibitory of the Taq polimerase are extracted along with the DNA (Leff et al. 1995). Although we obtained DNA product after direct physical disruption in TE buffer we found no visible PCR products. The efficacy of diverse chemical lysis components remains largely unknown. Donn et al. (2008) reported that DNA extraction of nematodes from environmental samples with lysis mixtures containing the detergent SDS yielded both quantifiable DNA and PCR product. However, in our experiment, the SDS procedure resulted in DNA product but failed to obtain a nematode DNA extract that could be amplified by PCR. We made modifications to the basic chemical lysis techniques, which included high-temperature (90 °C to boiling) incubation (Stock 2009) and incorporation of the chelating agent Chelex resin, to inhibit nucleases and

disperse soil particles (Jacobsen and Rasmussen 1992). However, this method also completely failed and no PCR products were obtained after direct amplification or amplification after purification.

The CTAB/phenol chloroform extraction was the only DNA extraction method that provided positive PCR amplicons of 18S rDNA. The PCR amplifications using the primer pairs NEMF1/S3 and NS1/NS4 resulted in expected fragments of about 700 bp and 1,000 bp, respectively (Figs. 1 and 2). The NEMF1 primer, targets the variable V3 and V5 regions of the 18S rDNA and is widely used in molecular-biodiversity studies of nematodes; S3 is a "universal" primer for eukaryotes that targets with highly a conserved region of the 18S rDNA (Waite et al. 2003). The NS1 and NS4 hybridized with highly-conserved regions of the eukaryote 18S rDNA molecule (White et al. 1990).

CTAB is known to help remove humus materials from most soils that contain high concentrations of humus acids which inhibit PCR enzymes. It has been reported that phenol/chloroform helps deproteinize the homogenate and remove a good deal of the humus and pigmented components

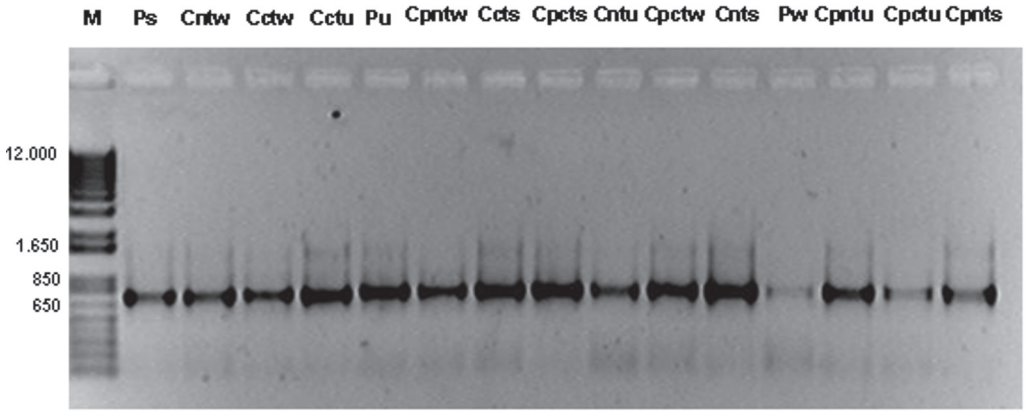

Figure 1 - A representative sample of 18S rDNA PCR product by using the NEMF1/S3 primer pair for each habitat and season. P = pasture; Cpnt = Crop-pasture rotation with no-tillage; Cpct = Crop-pasture rotation with conventional tillage; Cnt = Continuous cropping with no-tillage, Cct = Continuous cropping with conventional tillage; w = winter; s = spring; u = summer; M= 1 kb ladder (Invitrogen).

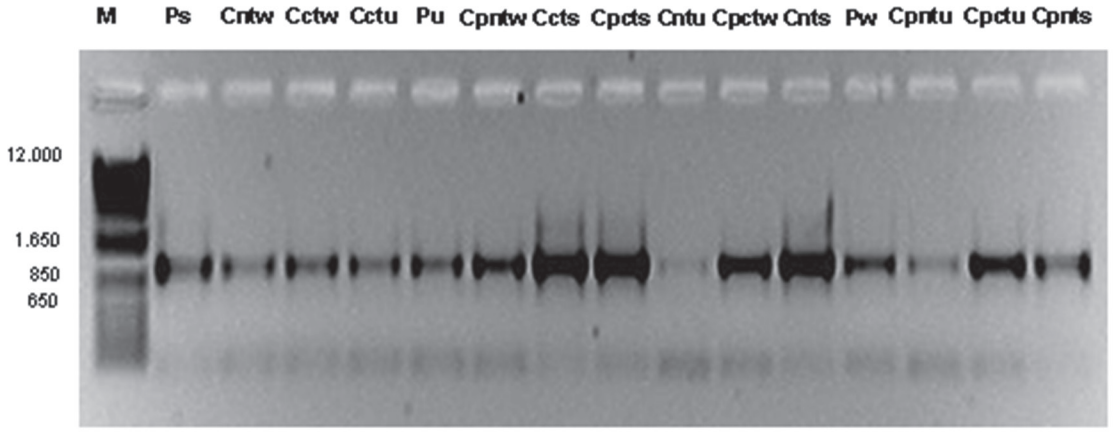

Figure 2 - A representative sample of 18S rDNA PCR product by using the NS1/NS4 primer pair for each habitat and season. P = pasture; Cpnt = Crop-pasture rotation with no-tillage; Cpct = Crop-pasture rotation with conventional tillage; Cnt = Continuous cropping with no-tillage, Cct = Continuous cropping with conventional tillage; w = winter; s = spring; u = summer; M= 1 kb ladder (Invitrogen).

of soil (Schneegurt et al. 2003). In our experiment, the DNA extracts obtained by the other (TE, SDS and Chelex) methods were highly pigmented (data not shown), but the extract obtained by the CTAB method combined with phenol/chloroform/isoamyl alcohol was clear and free of pigments.

The concentration of DNA of extracts from the CTAB/phenol chloroform method, ranged from about 20 ng μl^{-1} to 80 ng μl^{-1} (Table I). This could probably be associated with both, the variable abundance of nematodes communities in a sample and to the different contribution of DNA associated to the size of nematodes in each condition/station of sample collection. This wide range of DNA yield confirms the efficiency of the CTAB/phenol chloroform extraction method for a variable abundance of DNA in environmental samples. The highest purity of the extracted DNA was recorded in samples in which extraction was performed by the CTAB/phenol chloroform method (with values of absorbance A260/A280 ratio, close and higher to 1,5, Table I). While other tested methods of extracting DNA resulted in high yield, the quality of the extractions was lower than those obtained by the CTAB method (data not shown). Although the CTAB in the chemical lysis buffer and the proteinase K in the enzymatic lysis, combined by the phenol, chloroform and isoamyl alcohol extraction, was the most time-consuming method, it yielded both quantifiable DNA and amplifiable by PCR reaction. Used primers resulted in the expected amplicon`s sizes and served to test the success of the DNA extraction methods used.

In conclusion, we developed a simple and relatively affordable extraction method (CTAB combined with proteinase K and phenol-chloroform-isoamyl alcohol) for the extraction of DNA of nematodes communities from agricultural soils. The proposed protocol was successful for all analyzed samples of nematodes which were representative of different agricultural management and environmental stations. This provides the opportunity for studying nematodes as indicators of disturbances associated with agricultural management. After the successful extraction of DNA from nematodes communities, fingerprinting strategies (such as DGGE, SSCP, t-RFLP, etc.) or cloning-sequencing, may help define the role of the communities of nematodes as bioindicators of edaphic disturbances.

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RESUMO

Foram examinadas quatro estratégias (Tris / EDTA, dodecilsulfato de sódio, resina Chelex 100 e brometo de cetiltrimetilamónio -CTAB-) para a extração de ácido nucleico (DNA) das comunidades de nematóides. Os nematóides foram isolados a partir de uma área agrícola sob manejo diferente num experimento de rotação de culturas de longo prazo, durante três temporadas na Argentina. Após a extração de DNA, a reação em cadeia da polimerase e amplificações foram realizadas e consideradas como indicadoras da extração de DNA com sucesso. O CTAB combinada com proteinase K e álcool fenol-clorofórmio-isoamílico foi o único método bem sucedido porque as amplificações positivas foram obtidas utilizando ambos os primers específicos para os eucariótas e nematóides. Este trabalho poderá contribuir para o estudo da biodiversidade de nematóides em agroecossistemas.

Palavras-chave: Agroecossistemas, nematóides, extração de ácido nucleico, CTAB.

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