Pesquisa Florestal Brasileira
Brazilian Journal of Forestry Research
www.cnpf.embrapa.br/pfb

Scientific Note
Individual genomic DNA isolation of Entomogenous nematodes: the Deladenus study case

Guilherme Schnell e Schühlil[1], Susete do Rocio Penteado[1], Daiane Rigoni Kestring[1], Gisleine Jarenko Steill[2], Micheli Cristina Thomas[2]

[1]Embrapa Florestas, Estrada da Ribeira, km 111, CP 319, CEP 83411-000, Colombo, PR, Brasil
[2]Universidade Federal do Paraná, Setor de Ciências Biológicas, Jardim da Américas, CP 19031, CEP 81531-980, Curitiba, PR, Brasil

[*]**Autor correspondente:**
guilherme.schuhli@embrapa.br

Termos para indexação:
Extração de DNA
Protocolo
Vespa-da-madeira
Sirex
Noctilio

Index terms:
DNA extraction
Protocol
Woodwasp
Sirex
Noctilio

Histórico do artigo:
Recebido em 29/05/2012
Aprovado em 20/11/2012
Publicado em 28/12/2012
doi: 10.4336/2012.pfb.32.72.483

Abstract - Populational studies involve single individual DNA extraction in order to grant further genotyping data. In the case of some parasitic nematodes, the reduced dimensions and high individual number per infestation makes individual genotyping a difficult task. Aiming the development of a protocol we performed adjustments in available methods in order to acquire the best gain in purity and concentration of genomic DNA. Single specimens were digested in Worm Lysis Buffer and submitted to PCR amplification as a concept test. It was possible to obtain good amount and concentration of DNA from individuals. Quality was sufficient to grant subsequent ITS1 sequencing.

Isolamento de DNA genômico de indivíduo de nematoide parasita de insetos: estudo de caso de Deladenus

Resumo - Estudos populacionais envolvem a extração de DNA de um único indivíduo para permitir a genotipagem. No caso de alguns nematoides parasíticos, suas dimensões reduzidas e o grande número de indivíduos por infestação tornam a genotipagem difícil. Com o objetivo de desenvolver um protocolo, foram feitos ajustes em métodos já disponíveis, visando melhor ganho em pureza e concentração de DNA genômico. Espécimes foram digeridos em tampão de digestão de verme e submetidos à amplificação por PCR, como prova de conceito. Foi possível a obtenção de boa quantidade e concentração de DNA de indivíduos. A qualidade foi suficiente para garantir o sequenciamento da região ITS1.

Some genetic approaches, such as populational or phylogeographical studies, involve single individual genomic DNA extraction. The extracted genomic material should enable successful amplification of selected genetic markers in order to grant further high quality genotyping data.

In the case of entomogenous nematodes the development of protocols for individual genomic isolation is problematic. This is due to their reduced dimensions and to the number of individuals obtained from a single sample. Contamination and reduced amount of DNA is often impeditive to conducing molecular screenings.

The parasitic nematode *Deladenus* (*Beddingia*) *siricidicola* is recognized as an effective biological control agent against the woodwasp *Sirex noctilio* (Bedding & Iede, 2005). The parasite has been subject of studies in Brazil since it was first introduced from Australia in 1989 (Iede et al., 1989). Embrapa Forestry is a Governmental agency that maintains the biological

control of the woodwasp as one of its mains activities within its broad forestry scope.

Geographical variation in level of parasitism was detected and explored to develop an effective protocol for biological control of the woodwasp (Zondag, 1971, 1979; Bedding & Akhurst, 1974, 1978; Bedding & Iede, 2005; Hurley et al., 2008). Two strains were largely employed. The Sopron strain (Hungary) obtained the highest levels of parasitism when applied in Australia. A second strain was needed since the Sopron lost its high parasitism levels during Green Triangle outbreak (1987-1990). Researchers returned to evaluate areas previously introduced with Sopron strains. The result led to the proposition of Kamona strain (Tasmania) as a functional second option to the compromised effectiveness of Sopron strain.The loss of effectiveness after subsequent laboratory cycles showed to be a concern.

The molecular screening of wild populations would grant sensible recognition of geographical variants and, therefore, recognition of different levels of parasitism or effectiveness in biological control.

In an effort to develop methods of isolation from a single specimen (for subsequent PCR and sequencing) we investigated available methods in phylogenetically related taxa. We based our approach in the method proposed by Williams et al. (1994). In their protocol, the single nematode was digested in a lysis buffer (worm lysis buffer – WLB) to receive the PCR master mix directly over the extract (nematode + WLB). The procedure did not allow further examination of the same individual. The method proved to be very effective but was limited in providing enough material to process more than one amplification. Williams method was also designed to address *Caenorhabditis elegans* (Rhabditina) a different evolutionary lineage than our study object *Deladenus siricidicola* (Tylenchomorpha) (*sensu* Meldal et al., 2007).

Adjustments were performed in order to acquire the best gain in purity and concentration of genomic DNA from a single specimen. The settings and options for these protocols were presented here as a way to turn our findings available for further molecular approaches within this nematode group.

Strains of *Deladenus siricidicola* were obtained from pure laboratory cultures isolated from *Sirex noctilio* in the Laboratory of Forest Entomology. We tested strains from five different geographical origins within the Brazilian woodwasp geographical distribution. Specimens are usually cultured in a Petri dish with culture media. The culture dish was washed with ca. 50 ml of ethanol 98 °GL and the resultant solution was kept in plastic vials. A drop from this solution was deposited in an excavated slide where nematodes were sorted individually with an entomological dissection pin under a stereomicroscope.

Instead of direct processing of the worm and WLB into the PCR tube (as proposed by Williams et al., 1994) we tried to provide a tube with the extracted DNA in order to grant subsamples for further multiple PCRs with different markers.

We tested the digestion of single nematodes in 2.5 ul of the suggested WLB (Williams et al., 1994), 5, 10, 15, and 20 ul. Digestion products were subsequently cooled in -70 °C overnight aiming cell membranes thermal disruption. The product of the digestion and thermal disruption were incubated at 60 °C for one hour and heated at 95 °C for 15 minutes and cooled to 4 °C in a thermal block.

The digestion product was submitted to PCR amplification as template with a previously known nematode marker. The internal transcribed spacer region of the ribosomal DNA (ITS) was chosen for a concept test with the following primer set: Forward $^{5'}$ GTT TCC GTA GGT GAA CCT GC $^{3'}$ and Backward $^{5'}$ GTG ATC CAC CGC TAA GAG T $^{3'}$(Wu et al., 2009). Amplification followed: 10X PCR Buffer; 4mM $MgCl_2$; 0,2 mM dNTPs; 0,5 uM each primer; 0,6U ul^{-1} Taq and dd Water to the final volume of 25 ul.

Programmable termocycler had the following conditions: initial denaturation at 94 °C for 2 min, followed by 40 cycles (composed by three different annealing temperatures – Touchdown: Don et al., 1991)of 45s of denaturation at 94 °C, 30s of annealing temperature at 51 °C (3 cycles), 49 °C (3 cycles), and 47 °C (34 cycles), 1 min and 30s of elongation at 72 °C and a final elongation time of 10 min at 72 °C.

To ensure maximum gaining, concerning volume and concentration, weak products (30<ng ul^{-1}) went into a re-amplification (rePCR) based on the same concentrations presented for the first PCR. Template DNA was the product of the first PCR diluted to compose final 0,1 to 0,3ng ul^{-1}.

DNA concentrations were evaluated roughly by direct comparisons with mass ladder with the software GelAnalyzer (Copyright 2010 by Dr. Istvan Lazar).

PCR (or rePCR) product was then purified with ethanol precipitation and ressuspended in TE.

To proceed with the automated sequencing of the target region (sequencing in both directions), products were processed with the Big Dye terminator Cycle Sequencing Kit (Applied Biossystems).

The resultant material was purified with a simple PEG based protocol. The purified products were then inspected in an automated genetic analyzer (3130 Applied Biosystems).

Electrophoretograms were transformed, analyzed and verified in the Staden Package (Staden, 1996). Contigs were only considered when resulted at least one sequence from each direction (Forward and Backward).

A final indicative of a good genomic DNA extract was evaluated considering the following criteria:

a) PCR and rePCR final amount and concentration.

b) Intensity of peaks (electrophoretograms) and quality of readings.

c) Coincidence with deposited sequences expected for this primer set in databanks (BLAST searches) based on e-values and hits.

A large collection of nematodes was obtained regarding the five different geographic origins. In all cases, nematodes isolation from the collected mass was not demanding with the help of an entomological dissection pin and a micropipette. Nematodes in an excavated microscope slide were pushed away into the proper amount of WLB and captured into a micropipette tip.

The first amplifications of ITS from single nematodes digested in 2.5, 5 and 10 ul WLB for *D. siricidicola* were not successful. The remaining tested volumes (single nematodes digested in 15 and 20 ul of WLB) yielded very low DNA amounts (>10ng ul^{-1}).

Re-amplification was effective for templates from individuals digested in 15 and 20 ul of WLB. No measurable differences were noticed in the amount of amplified DNA obtained in both samples. The product of the ITS re-amplification for five reactions is shown in Figure 1. The amount of ITS amplified from digestions with 15-20 ul ranged from 50 to 60 ng ul^{-1} for most of the amplification.

Quality score for each nucleotide is presented below for each sequence in both senses (forward and backward primer) (Table 1). Evaluation of both senses allowed unambiguous reading of a region with 472 bp. Sequences were submitted to GenBank (ID 1541150).

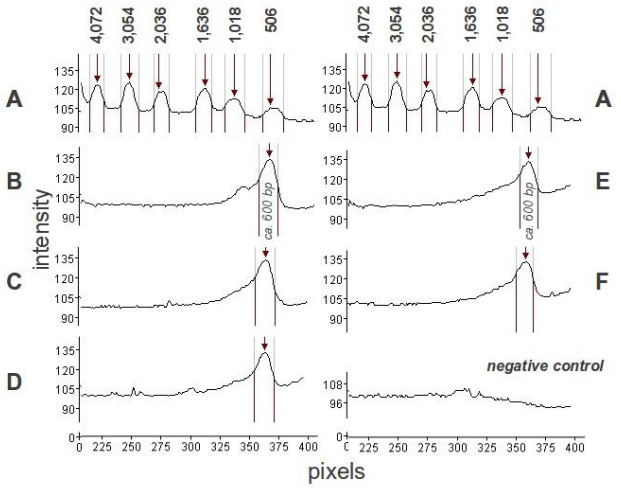

Figura 1. Graphical representation of a gel electrophoresis presenting PCR amplification for internal transcribe spacer (ITS) in *D. siricidicola* specimens from different geographic origins. Axis X presents the position in gel (pixels) while axis Y presents intensity of light reading (intensity). **A**: 1Kb Ladder – fragment size is presented over each peak; **B-F**: different specimens collected from different insects. The setae points to the ITS amplified fragment (ca. 600bp).

Table 1. Nucleotide quality scores for each sequence. Sequences were generated in both senses for each sample (F stands for Forward primer sequence and R stands for Backward primer sequence in geographic origin 1, 2, 3 and 4).

Sequence	Nucleotide quality score		
	A	C	G
P1F	88	82	94
P1R	86	116	120
P2F	75	67	72
P2R	64	107	101
P3F	144	144	120
P3R	139	181	182
P4F	141	159	137
P4R	118	190	146

Table 2. Nucleotide basic local alignment search tool performed (nBLAST- query 34299) identified our samples with deposited *D. siricidicola* ITS sequences with high bit scores and absolute e-value.

Subject ids	% Identity	Alignment lenght	Mismatches	Gap opens	e-value	Bit score
gi|198387451|gb|FJ004889.1|	99.36	468	3	0	0.0	848
gi|158702785|gb|EU200775.1|	99.36	468	3	0	0.0	848
gi|158702784|gb|EU200774.1|	99.36	468	3	0	0.0	848
gi|158702783|gb|EU200773.1|	99.36	468	3	0	0.0	848
gi|158702782|gb|EU200772.1|	99.36	468	3	0	0.0	848
gi|158702781|gb|EU200771.1|	99.36	468	3	0	0.0	848
gi|158702780|gb|EU200770.1|	99.36	468	3	0	0.0	848
gi|158702779|gb|EU200769.1|	99.36	468	3	0	0.0	848
gi|198387452|gb|FJ004890.1|	99.15	468	4	0	0.0	843
gi|158702787|gb|EU200777.1|	99.15	468	4	0	0.0	843
gi|119865941|gb|EF122861.2|	99.15	469	3	1	0.0	843
gi|171674351|gb|EU545475.1|	98.72	468	6	0	0.0	832
gi|324331644|gb|JF304744.1|*	95.77	426	12	5	0.0	682

All the compared subjects were identified in GenBank as *D. siricidicola* except for *D. proximus* (marked with*).

It is possible to adapt the Williams et al. (1994) protocol to obtain good genomic DNA from single individuals of *D. siricidicola*.

The extracted genomic DNA from a single individual is enough (amount and concentration) to allow several amplifications of average size PCR markers (ca. 500 bp). In our demonstration, quality was sufficient to grant subsequent sequencing producing electrophoretograms with regular intensity of peaks and quality reading.

The method made it possible to obtain a precise taxonomic identity at species level based on ITS sequences suggesting that the employed marker when associated to this protocol is useful as a molecular identification aid.

References

BEDDING, R. A.; AKHURST, R. J. Use of the nematode Deladenus siricidicola in the biological control of Sirex noctilio in Australia. **Journal of the Australian Entomological Society**, Birsbane, v. 13, p. 129–135, 1974.

BEDDING, R. A.; AKHURST, R. J. Geographical distribution and host preferences of Deladenus species (Nematoda: Neotylenchidae) parasitic in siricid woodwasps and associated hymenopterous parasitoids. **Nematologica**, Leiden, v. 24, p. 286–294, 1978.

BEDDING, R. A.; IEDE, E. T. Application of beddingia siricidicola for sirex woodwasp control. In: GREWAL, P. S.; EHLERS, U.; SHAPIRO-ILAN, D. I. (Ed.). **Nematodes as biocontrol agents**. Wallingford: CAB International, 2005. p. 385-399.

DON, R. H.; COX, P. T.; WAINWRIGHT, B. J.; BAKER, K.; MATTICK, J. S. "Touchdown" PCR to circumvent spurious priming during gene amplification. **Nucleic Acids Research**, Oxford, v. 19, n. 14, p. 4008, 1991.

HURLEY, B. P.; SLIPPERS, B.; CROFT, P. K.; HATTING, H. J.; LINDE, M. van der; MORRIS, A. R.; DYER, C.; WINGFIELD, M. J. Factors influencing parasitism of Sirex noctilio (Hymenoptera: Siricidae) by the nematode Deladenus siricidcola (Nematoda: Neotylenchidae) in summer rainfall areas of South Africa. **Biological Control**, Orlando, v. 45, p. 450–459, 2008.

IEDE, E.T.; BEDDING, R.A.; PENTEADO, S. R. C.; MACHADO, D. C. **Programa nacional de controle da vespa-da-madeira: PNCVM**. 1989. Curitiba: EMBRAPA-CNPF, 1989. 10 p.

MELDAL, B. H. M.; DEBENHAM, N. J.; LEY, P. de; MELDAL, B. H. M.; DEBENHAM, N. J.; LEY, P. de; VANFLETEREN J. R.; VIERSTRAETE, A. R.; BERT, W. BORGONIE, G.; MOENS, T.; TYLER, P. A.; AUSTEN, M. C.; BLAXTER, M. L.; ROGERS, A. D.; LAMBSHEAD, P. J. An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. **Molecular Phylogenetics and Evolution**, Orlando, v. 42, n. 3, p. 622-36, 2007.

STADEN, R. The staden sequence analysis package. **Molecular Biotechnology**, Totowa, v. 5, p. 233-241, 1996.

WILLIAMS, B. D.; SCHRANK, B.; HUYNH, C.; SHOWNKEEN, R.; WATERSTON, R. H. A Genetic mapping system in Caenorhabditis elegans based on polymorphic sequence-tagged sites. **Genetics**, v. 131, p. 609-624, 1994.

WU, S. G.; WANG, G. T.; XI, B. W.; XIONG, F.; LIU, T.; NIE, P. Population genetic structure of the parasitic nematode Camallanus cotti inferred from DNA sequences of ITS1 rDNA and the mitochondrial COI gene. **Veterinary Parasitology**, Amsterdam, v. 164, n. 2-4, p. 248-56, 2009.

ZONDAG, R. Control of Sirex noctilio (F.) with Deladenus siricidicola Bedding. Part I-1967 field trial. **New Zealand Journal of Forestry Science**, Rotorua, v. 1, p. 5–14, 1971.

ZONDAG, R. Control of Sirex noctilio (F.) with Deladenus siricidicola Bedding. Part II. Introductions and establishments in the South Island 1968–75. **New Zealand Journal of Forestry Science**, Rotorua, v. 9, p. 68–76, 1979.