ZOOLOGIA 40: e23021
ISSN 1984-4689 (online)
scielo.br/zool



SHORT COMMUNICATION

# A reliable and efficient BioPulverizer method in preparing and grinding nematodes for nucleic acid extraction and molecular identification

Ye Jiang[1,2], Ruifeng Qin[1,2], Chunjie Li[1], Minghui Huang[1], Dan Jiang[1,2], Doudou Chang[1,2], Yifan Xie[1,2], Yanan Zhao[1], Congli Wang[1]

[1]*State Key Laboratory of Black Soils Conservation and Utilization, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences. Harbin 150081, Heilongjiang, China.*
[2]*University of Chinese Academy of Sciences. Beijing 100049, China.*
*Corresponding author: Congli Wang (wangcongli@iga.ac.cn)*

*https://zoobank.org/502616B5-0278-48B7-97B0-4DFF933004A8*

ABSTRACT. Due to their tiny size, soil nematodes and plant-parasitic nematodes are challenging to homogenize and collect using traditional mortar and pestle methods. To overcome this, we developed a reliable and efficient nematode preparation and grinding method using the BioPulverizer. The method involves creating a modified sample 'sandwich' with liquid nitrogen-frozen nematode samples placed between two layers of aluminum foil. This 'sandwich' facilitates complete homogenization through cryogenic grinding without any sample loss during transfer. The powerful nematode grinding yields high-quality and high-quantity samples, suitable for nucleic acid release and subsequent molecular identification and other downstream applications.

KEY WORDS. Cryogenic grinding, homogenization, plant parasitic, soil nematodes.

Obtaining large amounts of nematodes for nucleic acid (DNA or RNA) extraction, particularly in the case of plant parasitic nematodes, poses challenges due to their tiny size. General methods commonly used for animal and tissue nucleic acid extraction are not as efficient for nematodes as they are for larger animals like rats, fish, and shrimp. The tiny size of nematodes makes them difficult to homogenize, impacting the extraction process. Orlando et al. (2020) compared the efficiency of six DNA extraction methods using root-lesion nematodes (*Pratylenchus* spp.) and found that the glass bead with tissue disruptor method yielded high-quality DNA.

In our own study, we encountered difficulties with traditional methods like the mortar and pestle, even the mini-pestle and mortar for grinding plant or animal tissues in liquid nitrogen. These tools were too large to effectively collect all materials after grinding small nematodes. Similarly, using the pellet pestle in microcentrifuge tubes did not lead to complete tissue disruption, even with the tissue homogenizer. Moreover, the molecular identification of nematode species or genera from single or multiple nematodes required picking and cutting the nematodes into pieces with a sharp surgical blade (scalpel) under a microscope, demanding specialized techniques. Additionally, the use of plastics led to static buildup, resulting in nematodes being lost by adhering to the tube walls when they were removed for freezing. These limitations collectively contributed to unsuccessful nematode preparation for nucleic acid extraction.

To prevent nematodes from adhering to the plastic pipette tip wall during transfer, we utilized a metal syringe needle, which was made by cutting a 5 mm tip from a 20 ml syringe needle (1.6 mm in diameter, 50 mm in length, SUS 316 L, Shandong Weigao Group Medical Polymer Co., Ltd., China) and smoothed the cut end using a grinding wheel. This modified needle, along with its holder, could be directly installed into a 50 µl pipette (Thermo Labsystems Inc., USA), with a maximum volume capacity of 46.5 µl (Fig. 1).

For complete DNA or RNA release, we employed the BioPulverizer equipment (Biospec Products INC., Bartlesville, OK USA) (Fig. 1) to grind the nematode materials thoroughly. The BioPulverizer comprises a two-component mortar with handles, a pestle, and a hammer, specifically designed for pul-

verizing 10–1000 mg of tissue. This equipment is highly useful for homogenizing hard-frozen tissues into a fine powder, enabling subsequent rapid and complete cell disruption with proteinase K or nucleic acid extraction media. The mechanical disruption strength from the hammer under super low temperatures is theoretically much stronger than that generated by glass beads and tissue disruptors at room temperature.

To avoid contamination between samples and minimize sample loss during grinding, we created a 'sandwich' arrangement by placing the sample between two layers of aluminum foil or tinfoil. Moreover, this setup reduces the need to wash the mortar between samples, as the equipment cannot be washed under extreme temperatures. The combination of these components establishes a reliable and efficient nematode preparation and grinding method (Fig. 1), which is described in detail below.

Nematodes from two different sources were selected to assess the effectiveness of this method: one group was obtained from soil samples, and the other from plant tissues. Soil samples were collected from the field and nematodes were isolated using the shallow water-filled tray or container method (Van Bezooijen 2006). A 200 g soil sample was placed on a two-layer tissue paper and soaked in autoclaved distilled water. After three days, nematodes that had moved into the water were collected through a 500 mesh (25 μm) sieve. Subsequently, the nematodes were thoroughly rinsed with autoclaved Millipore water and left to settle in a 100 ml glass beaker overnight. A vacuum filtration system was then used to remove the water, retaining approximately 5 ml at the bottom. The nematodes were transferred to a 15 ml centrifuge tube and allowed to settle for two hours. After removing the supernatant, the concentrated nematode sample was transferred into a 1 ml glass tube (Fig. 1) to prevent nematode adhering to the tube wall (Haimen Minlei Glass Instrument Factory, Jiangsu, China) using the syringe needle. The glass tube was subsequently placed into a 15 ml centrifuge tube and centrifuged at 3500 rpm (1974 g) for two minutes, and the supernatant was removed via pipetting. If the nematode solution appeared contaminated, an additional washing step with water was conducted. Finally, approximately 100 μl of the sample was preserved for high-throughput sequencing to study nematode diversity. Meanwhile, five nematode samples from soil without initial BioPulverizer processing were prepared as controls.

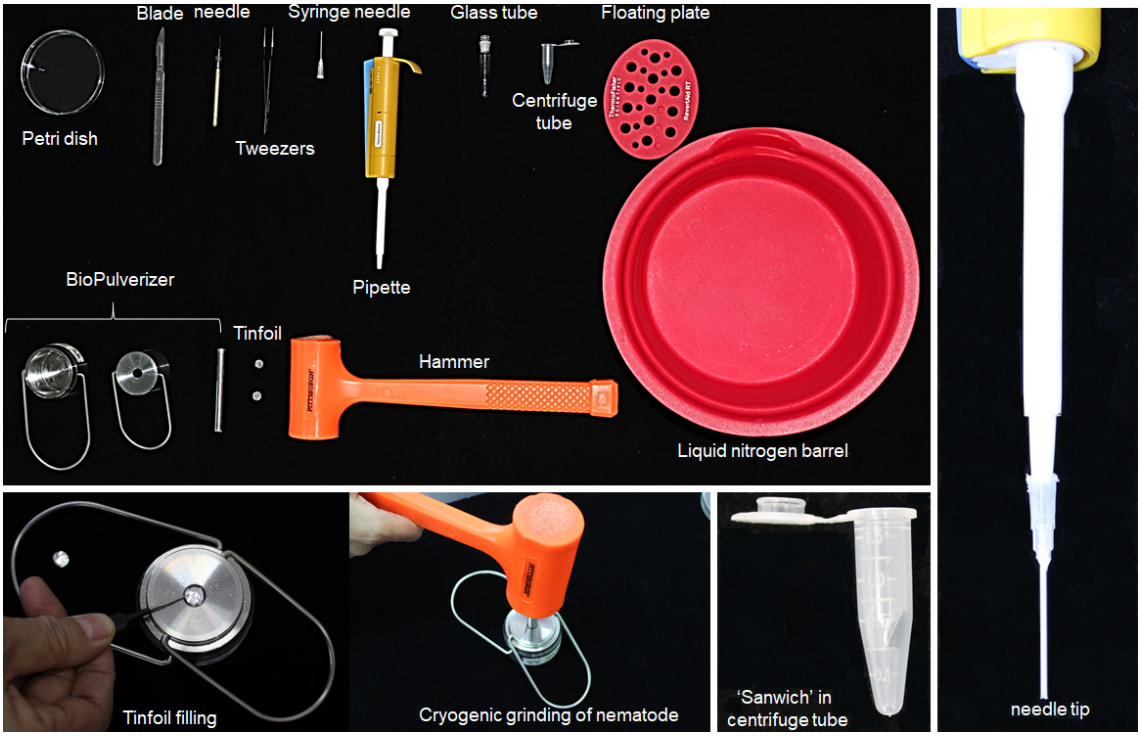



Figure 1 Equipment used for nematode preparation and grinding. The names of all the equipment are listed above or under each respective one.

Additionally, the extracted DNA from soil samples can be employed for the identification of specific nematodes of interest. In this study, as the samples were collected from soybean or corn fields in Northeast China, we utilized species-specific primers (GlyF1/rDNA2 and SCNF1/SCNR1) designed for soybean cyst nematodes, *Heterodera glycines* Ichinohe, 1952 (Baidoo et al. 2017). By amplifying the samples with these primers, we aimed to confirm the presence or absence of this particular nematode species.

Potato roots or tubers infected with root-knot nematodes (RKN) *Meloidogyne* spp. were utilized for species identification as well. Various nematode stages, including eggs, second-stage juveniles (J2), J3-J4, and female stages, were obtained from thin pieces of potato tuber (0.5–1.0 mm) cut with a sharp blade. Egg masses were carefully collected using a needle and tweezers under a microscope and transferred into autoclaved Millipore water for hatching, resulting in the emergence of J2s. The volume of the final sample depended on the number of juveniles present. For instance, if only a few juveniles were obtained, 10 µl of the solution was retained; if the number of nematodes ranged from 10 to several hundred, 20 µl of the solution was kept. Larger J3, J4, or female stages were visually identifiable at the bottom of the tube, and individual females could be frozen in liquid nitrogen for further analysis.

Using the syringe needle pipette, the collected nematode sample was then transferred into a prelabeled 1.5 ml-microcentrifuge tube that was placed in a cryogenic floating rack inside the benchtop liquid nitrogen container. As soon as the drop of nematode sample touched the liquid nitrogen, it formed a nematode 'ball' or 'pellet', making it easy to remove the nematodes from the tube for the subsequent grinding step.

To ensure efficient chilling, the BioPulverizer cryogenic tissue crusher was prechilled in a -86 °C freezer one day before the experiment. During the actual experiment, the prechilled BioPulverizer was placed in liquid nitrogen for an additional three minutes for even deeper chilling. If the BioPulverizer is not prechilled in the freezer, a longer time is required to chill it effectively.

For the grinding process, a 1 cm-diameter aluminum foil or tinfoil, matching the size of the center hole of the mortar, was prepared and prechilled in liquid nitrogen for three minutes or kept in liquid nitrogen throughout the experiment. Similarly, the tweezers were also prechilled in liquid nitrogen to maintain low temperature during the grinding process.

The BioPulverizer, taken from the liquid nitrogen, was placed on a table covered with tinfoil. Using tweezers, a 1 cm-diameter tinfoil piece from the liquid nitrogen was carefully positioned horizontally into the center hole of the BioPulverizer. The frozen nematode balls were then poured into the center hole, and another tinfoil piece was placed horizontally on top, creating a 'sandwich' arrangement (Fig. 1).

Once the 'sandwich' was formed, the pestle was immediately inserted into the hole, and the hammer was used to hit the pestle several times, effectively grinding the nematode 'ball' into a powder. The pestle and the inner cup of the mortar were removed, and the 'sandwich' was picked up with tweezers and transferred into either the original tube or a newly labeled tube filled with liquid nitrogen. By gently shaking the two pieces of tinfoil with tweezers, the powdered material fell into the liquid nitrogen, and then the two foil pieces were removed and discarded. Once the liquid nitrogen had evaporated, the sample could be stored in a -80 °C freezer for long-term storage or used for the next step of DNA extraction or for direct enzyme reaction with proteinase K.

The nematode DNA from soil samples was extracted using the TGuide S96 Magnetic Soil/Stool DNA kit following the manufacturer's instructions (Tiangen Biotech Co., Ltd., Shanghai, China). Initially, the samples were lysed by adding buffers with grinding beads to the tubes, and homogenization was achieved using a TGrinder H24 Tissue Homogenizer. For high-throughput sequencing, the seminested procedure (Sapkota and Nicolaisen 2015) was employed. The primer pair NemF (GGGGAAGTATGGTTGCAAA) and 18Sr2b (TACAAAGGGCAGGGACGTAAT) was used for the preamplification step, followed by amplification with the primer pair NF1 (GGTGGTGCATGGCCGTTCTTAGTT) and 18Sr2b, which was tailed with sample-specific Illumina index sequences to enable deep sequencing by Biomarker Technology Co., Ltd. (Beijing, China).

To extract genomic DNA for RKN species identification, a mixture of 1 µl 10 × Taq Buffer ($Mg^{2+}$ plus) (Nanjing Vazyme Biotechnology Co., Ltd., China) and 1 µl of 20 $mg{\cdot}ml^{-1}$ proteinase K (Thermo Fisher Scientific Inc., USA) was added to the nematode sample using a 10 µl collection volume by pipetting. Since the sample volume slightly decreased after grinding, an 8 µl sample was considered. For a 20 µl nematode sample, 2 µl 10 × Taq Buffer and 2 µl of 20 $mg{\cdot}ml^{-1}$ Proteinase K were added. The volumes of the buffer and enzyme were adjusted based on the sample volume. The samples were thoroughly mixed and then frozen at -80 °C for 30 minutes for enzyme lysis with proteinase K. Subsequently, PCR for species identification followed previously established methods (Jesus et al. 2016, Mao et al. 2019).

For the determination of soil nematode diversity through high-throughput sequencing, we assessed the

quality and quantity of DNA and PCR products for each sample. All samples (more than 200) met the sequencing requirements, with an average OD260/280 ratio of 1.81 (data not shown). Some samples, characterized by low nematode density, displayed low DNA amounts (< 5 ng $\mu l^{-1}$, even as low as 0.25 ng $\mu l^{-1}$), but still exhibited high-quality DNA (OD260/280 ~1.80).

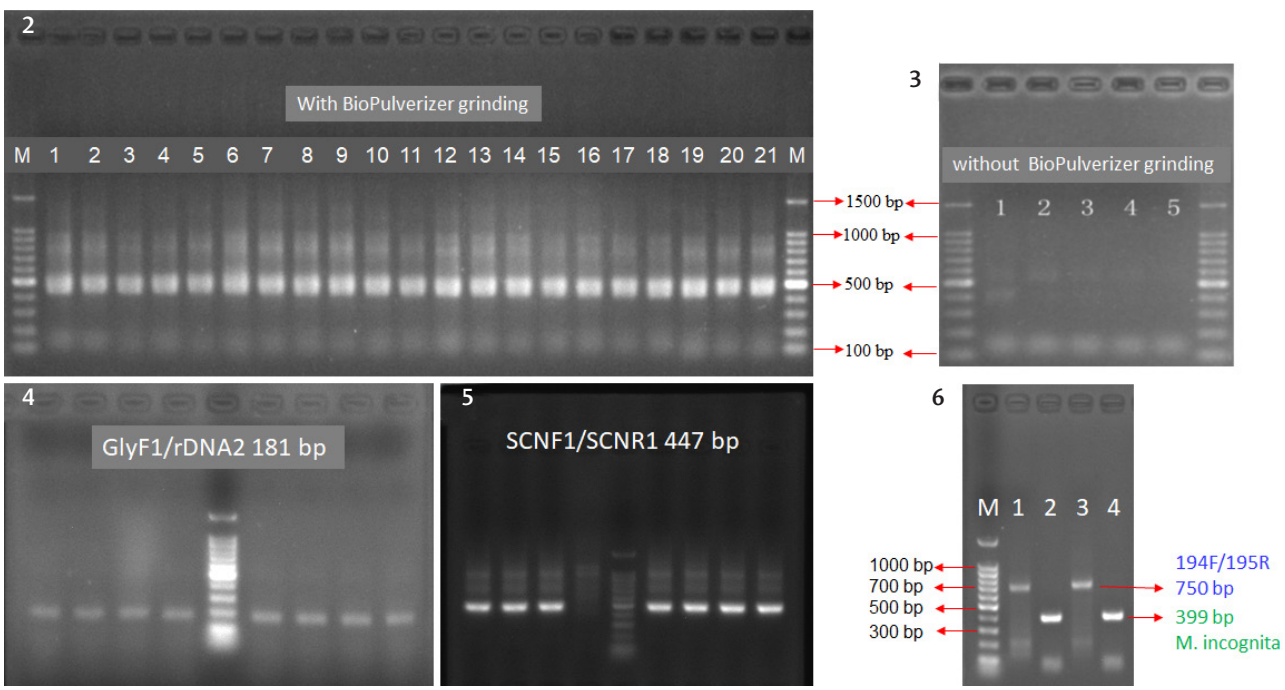
Examples of amplification bands obtained through the seminested procedure in the agarose gel are presented in Fig. 2, with amplicon sizes ranging from 420 to 550 bp, consistent with the sequence assembly results. However, we observed that PCR amplification of the five samples without liquid nitrogen grinding failed with the first pair of amplifications (NemF and 18Sr2b) due to low DNA quality or quantity (OD260/280 <1.8; < 5 ng $\mu l^{-1}$). Upon reamplification of PCR products, a very weak band was observed, which did not meet the requirements for high-throughput sequencing (Fig. 3). This issue might be attributed to the low number of nematodes and incomplete cell lysis with grinding beads.

PCR amplification using species-specific primers (GlyF1/rDNA2, 181 bp, and SCNF1/SCNR1, 447 bp) confirmed the presence of soybean cyst nematodes *H. glycines* (Figs 4–5). Additionally, a 750 bp amplification band from the universal primer pair 194F/195R confirmed the sample as RKN, and a 399 bp band from the *Meloidogyne* species-specific primer pair indicated the sample as *Meloidogyne incognita* (Kofoid & White, 1919) (Fig. 6). These results were consistent across all stages, including eggs, J3-J4, and females.

All the results obtained from our lab experiments consistently demonstrate that the modified BioPulverizer grinding method using liquid nitrogen is highly reliable and efficient for the subsequent extraction of high-quality and high-quantity DNA or RNA from nematodes.

## LITERATURE CITED

Baidoo R, Yan G, Nelson B, Skantar AM, Chen S (2017) Use of chemical flocculation and nested PCR for *Heterodera*




Figures 2–6 PCR amplification product with primers: (2) PCR products from the seminested primer pairs (NemF and 18Sr2b; NF1 and 18Sr2b) obtained from soil nematode samples with BioPulverizer grinding; (3) PCR amplification with the first cycle of the primer pair (NemF and 18Sr2b) from soil samples without BioPulverizer grinding; (4–5) Two amplification bands, 181 bp with the species-specific primer pair GlyF1/rDNA2 (4) and 477 bp with SCNF1/SCNR1 (5), were amplified for *Heterodera glycines*; (6) PCR products with the universal primer pair 194F/195R and the species-specific primer pair (*Meloidogyne incognita*) from potato tuber samples. All molecular markers (M) are 100 bp DNA ladders. The concentrations of agarose gel are 1.8% in Figs 2–3 and 1% in Figs 4–6.

*glycines* detection in DNA extracts from field soils with low population densities. Plant Disease 101(7): 1153–1161. https://doi.org/10.1094/PDIS-08-16-1163-RE

Jesus DS de, Oliveira CMG, Roberts D, Blok V, Neilson R, Prior T, Balbino HM, MacKenzie KM, Oliveira RDD (2016) Morphological and molecular characterisation of *Aphelenchoides besseyi* and *A. fujianensis* (Nematoda: Aphelenchoididae) from rice and forage grass seeds in Brazil. Nematology 18(3): 337–356. https://doi.org/10.1163/15685411-00002962

Mao Y, Hu Y, Li C, Zhang H, Yu D, Liu X, Yang G, Wang C (2019) Molecular identification of *Meloidogyne* species isolated from potato in China and evaluation of the response of potato genotypes to these isolates. Nematology 21: 847–856. https://doi.org/10.1163/15685411-00003259

Orlando V, Edwards SG, Neilson R, Prior T, Roberts D, Back M (2020) Comparing the efficiency of six common methods for DNA extraction from root-lesion nematodes (*Pratylenchus* spp.). Nematology 23(4): 415–423. https://doi.org/10.1163/15685411-BJA10049

Sapkota R, Nicolaisen M (2015) High-throughput sequencing of nematode communities from total soil DNA extractions. BMC Ecology 15: 3. https://doi.org/10.1186/s12898-014-0034-4

Van Bezooijen J *(*2006*)* Methods and techniques for nematology. J. van Bezooijen, Wageningen, unpublished data, 112 pp. Available online at: https://www.wur.nl/upload_mm/f/9/3/10aac0cb-8289-400a-a6e5-c4687461d138_MethodsandTechniquesforNematology.pdf

Submitted: July 30, 2023
Accepted: August 14, 2023
Editorial responsibility: Walter A.P. Boeger

Author Contributions

YJ: Data curation, Formal analysis, Methodology, Investigation, Writing – original draft. RQ: Investigation. CL: Resources, Data curation. MH: Investigation. DJ: Investigation. DC: Investigation. YX: Investigation. YZ: Investigation. CW: Conceptualization, Resources, Data curation, Supervision, Validation, Methodology, Project administration, Writing – review & editing.

**Competing Interests**

The authors have declared that no competing interests exist.

**Funding**

This work was supported by the National Natural Science Foundation of China (32272501) and the National Science & Technology Fundamental Resources Investigation Program of China (2018FY100304) to CW.

**How to cite this article**

Jiang Y, Qin R, Li C, Huang M, Jiang D, Chang D, Xie Y, Zhao Y, Wang C (2023) A reliable and efficient BioPulverizer method in preparing and grinding nematodes for nucleic acid extraction and molecular identification. Zoologia 40: e23021. https://doi.org/10.1590/S1984-4689.v40.e23021

**Published by**

Sociedade Brasileira de Zoologia at Scientific Electronic Library Online (https://www.scielo.br/zool)

**Copyright**