NPPO-Nepal, 2025

7.

Detection Survey Protocol for *Pratylenchus brachyurus* (Godfrey) Filipjev & Schuurmans Stekhoven in Nepal

Government of Nepal
Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
Hariharbhawan, Lalitpur

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1. Background Information

With entry into the WTO, Nepal gets the opportunity to export its produce to the international markets. However, the exports from Nepal have not escalated to the same proportion as trade between developed nations. Developed countries have increased exports by using the rules of the SPS Agreement. At the moment, the Government of Nepal is obliged to use the SPS rules to exclude commodities that are posing a threat to the related industries within the country. Nepal should provide an adequate description of the health status of plant-based industries, while negotiating access to foreign trade. Prospective importers of Nepalese agriculture-related commodities assess the risk of introducing new pests based on the authentic pest information provided. Prospective importers also assess the phytosanitary measures being practiced in Nepal to reduce risk to an acceptable level. Extensive specimen-based records are the key for Nepal to negotiating with importing countries on a fair trading system. This document gives detailed guidelines for detection surveys of the nematode *Pratylenchus brachyurus* in the field of agriculture. Besides, it will be applicable for monitoring, surveillance, import inspection and export certification and is the basis for specimen-based records to be developed by the NPPO-Nepal.

Under the Plant Quarantine and Protection Act, 2064, article 6(2), survey and surveillance functions and responsibilities are designated to NPPO-Nepal as per the sub-clause (i) "To perform such other functions as prescribed". This technical guideline to undertaking a pest detection survey of *Pratylenchus brachyurus* has been prepared with a view to guiding the survey activity. This protocol is prepared for researchers, plant protectionists, teachers, and other concerned professionals. This document will be a guide to submitting specimens to a laboratory for diagnosis and preservation.

1.1 About the pest (Root-lesion nematode)

Pratylenchus brachyurus (Godfrey) Filipjev & Schuurmans Stekhoven, also called as root-lesion nematode, is a migratory endoparasitic nematode that moves intercellularly through the root cortex, disrupting and destroying cells, facilitating secondary infection by fungi and bacterial pathogens (Castillo & Vovlas, 2007; Davis & MacGuidwin, 2000). *P. brachyurus* have great economic importance due to its wide geographical distribution, polyphagous nature, and high

population densities, which can result in crop losses of up to 50% in early stages of maize cultivation (Oliveira & Inomoto, 2023). Environmental conditions that are favorable to nematodes or unfavorable to the crop, such as soil compaction, low fertility, high sand content, and use of host crops (e.g., soybean–maize rotation systems), are known to be associated with increased nematode populations (Favoreto et al., 2019).

Pratylenchus spp. is a quarantine pest for China. As such, to comply with the protocol between the Ministry of Agriculture and Livestock Development of Government of Nepal and the General Administration of Customs of the People's Republic of China on The Safety and Health Condition of Haylage Export from Nepal to China the exported haylage must be free from *Pratylenchus* spp.

In Nepal, root-lesion nematode (*Pratylenchus* spp.) have been found associated with various crops, including maize, potato, tomato, cauliflower, chilly, carrot, pea, and papaya crops grown in various districts, including Chitwan (Amatya & Shrestha, 1969; PPD, 2009; Baidya, 2023).

1.2 Identity and taxonomy of target pest (CABI, 2021; PWKB, 2021)

1.2.1 Identity

Preferred scientific name: *Pratylenchus brachyurus* (Godfrey, 1929) Filipjev & Schuurmans Stekhoven, 1941

Preferred common name: Root-lesion nematode

Other scientific names

Anguillulina (*Pratylenchus*) *brachyura* (Godfrey, 1929) Goodey, 1932 (W. Schneider, 1939)

Anguillulina brachyura (Godfrey, 1929), Goodey, 1932

Pratylenchus leiocephalus Steiner, 1949

Pratylenchus pratensis Thorne, 1949

Pratylenchus steineri Lordello, Zamith & Boock, 1954

Tylenchus (*Chitinotylenchus*) *brachyurus* Godfrey, 1929 (Filipjev, 1934)

Tylenchus brachyurus Godfrey, 1929

EPPO code: PRATBR

1.2.2 Taxonomy

Taxonomic tree of the nematode is presented below (CABI, 2021)

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Nematoda

Family: Pratylenchidae

Genus: *Pratylenchus*

Species: *P. brachyurus*

1.3 Host range

P. brachyurus is polyphagous, with a very wide host range including fruit trees, forest trees, cereals, vegetables, tuber crops, oil seed crops, fibre crops, woody ornamentals, plantation crops, grasses as well as medicinal plants. Fruit crops such as pineapple (*Ananas comosus*), citrus, strawberry (*Fragaria* sp.), mango (*Mangifera indica*), peach (*Prunus persica*) and avocado (*Persea americana*); graminaceous crops such as oats (*Avena sativa*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa)*, maize (*Zea mays*), sugarcane (*Saccharum officinarum*); oil seed crops such as groundnut (*Arachis hypogaea*), soyabean (*Glycine max*) etc are major hosts of *P. brachyurus* (CABI, 2021; PWKB, 2021).

1.4 Nematode biology and damage symptoms

1.4.1 Life cycle and field identification

As a migratory endoparasite, all juvenile stages and adults of *P. brachyurus* are capable of invading the roots while constantly migrating in and out. However, adults are generally more effective than juveniles in establishing infestations (CABI, 2021). The life cycle can range from four to eight weeks, depending on the environmental factors and the availability of suitable plant hosts (Davis & MacGuidwin, 2000; Pretorius, 2018). However, in maize, it may vary from four weeks at 30-35°C to 14 weeks at 15°C (Olowe & Corbett, 1976). Similar to other nematodes, the

life cycle of *Pratylenchus brachyurus* includes an egg, four juvenile stages (J1 to J4) and the adult stage (males and females).

a) Egg stage:

The ideal temperature for egg production is 35°C. Eggs are laid individually in the root tissues and soil, and the larvae undergo four stages of molting (CABI, 2021).

b) Juvenile stage:

The first-stage juvenile (J1) develops inside the egg, then molts into the second-stage juvenile (J2), which hatches from the egg (Davis & MacGuidwin, 2000; Nemaplex, 2024). Each subsequent stage progresses to the next through a molt of its cuticle (Nemaplex, 2024). Afterwards the J2 juvenile molts and becomes third-stage juvenile (J3), then fourth-stage juvenile (J4) expanding the root lesions.

c) Adult stage:

The fourth-stage juvenile (J4) molt to become adult males and females (Davis & MacGuidwin, 2000). Females are small, measuring less than 1 mm in length (Fig 1). Since males are extremely rare (CABI, 2021), *P. brachyurus* is monosexual which reproduces through mitotic parthenogenesis (Roman & Triantaphyllou, 1969; Davis & MacGuidwin, 2000). When both females and males are present, they have the same form, though the males are smaller (CABI, 2021).

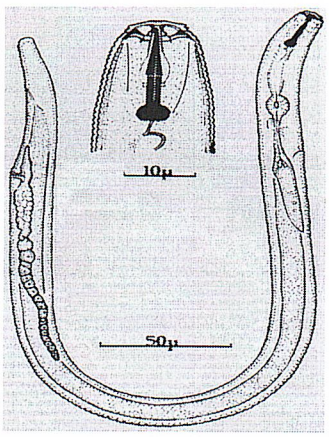

Figure 1. A female *Pratylenchus brachyurus* (Source: Nemaplex, 2024)

1.4.2 Damage symptoms

According to CABI (2021), the typical symptom of *P. brachyurus* attack is the formation of characteristic lesions on the roots, hence commonly referred to as lesion nematodes. The affected plants often appear in patches, which are not uniformly distributed across the field. The above-ground symptoms closely resemble those caused by other root parasitic nematodes (Pretorius, 2018), which include stunted growth, reduced plant vigor, chlorosis on leaves, defoliation and gradual decline in yield (PWKB, 2021). Generally, the lesions formed are brown in colour, though their appearance may vary in different host plants. For maize and rice, the lesions are small and blackish on the root surface (CABI, 2021; PWKB, 2021) (Fig 2). However, in maize roots, the necrosis caused by *P. brachyurus* is greater in the stelar region than in the cortex (Olowe & Corbett, 1976). Infection by root-lesions nematodes provides opening points for entry of bacterial and fungal pathogen, leading to severe plant damage (Back et al., 2002).

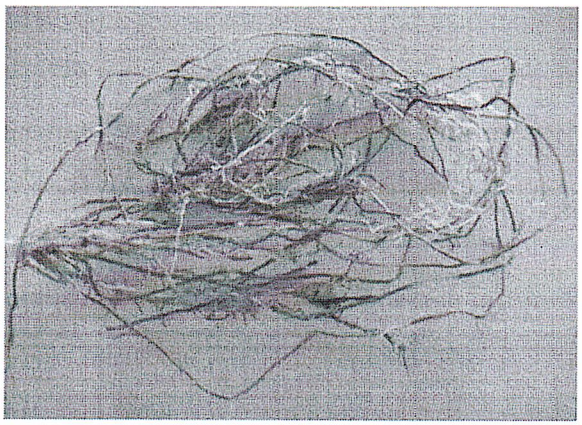

Figure 2. Maize root infected with root-lesion nematodes showing brownish to blackish necrotic tissue (Source: Danny Coyne, IITA)

1.5 Mode of dispersal

Root-lesion nematodes are readily dispersed by movement of infected propagative plant materials, soil when host roots are available and are passively moved with water (irrigation or rainfall) (Duncan & Moens, 2013).

2. Detection survey

A detection survey is conducted in an area to determine if pests are present (FAO, 1990; revised FAO, 1995). These surveys are more frequently carried out to determine pest status in an area, and they follow a definite survey plan, which is approved by NPPO-Nepal. These surveys

are carried out either seasonally or annually and / or following the eradication measures applied to a pest in a given area or production sites. These surveys are organized following a definite survey methodology based on statistical sampling, which are determined after taking into account the biology of the pest and employing appropriate detection techniques such as field diagnostic kits, traps etc. The results of the survey are documented and communicated (PPD/NPPO-Nepal, 2071 BS).

2.1 Purpose and scope of detection survey

The purpose of the detection survey is to determine the presence or absence of *Pratylenchus brachyurus* in a given area or production sites. The scope will be limited to maize and other defined crops to be grown for haylage/silage production for export to China and other concerned countries.

2.2 Timing of survey

Conduct the survey twice during the cropping season of maize (CropWatch, 2015):

- **At V6 growth stage** (within 4-8 weeks after planting), when the nematodes first establish themselves in the root zone.
- **At the time of harvest**

2.3 Selection of survey area

Field plots of maize and concerned crops in the target areas.

2.4 Materials required for survey

- Soil auger or hand corer for soil sampling
- Gloves
- Hand lens
- Sample collection bags
- Zip lock plastic bags
- Plastic sampling containers
- GPS device

- Field notebook for recording observations
- Microscope for nematode identification
- Nematode extraction kit (Baermann funnel, sieves of different mesh sizes, including Whitehead tray, or centrifuge setup)
- Labels/tags
- Data sheets (with different formats for field recording and lab recording separately)
- Permanent markers
- Rubber bands

2.5 Sample size and sampling methods

Select 5-10 random sampling points per maize field, ensuring that sampling spreads across the entire field while avoiding the field edges or areas with obvious damage. Collect samples in a random pattern for small area or systematic pattern for large area from the root zone of the crop to account for field variability (Coyne et al., 2014) (Fig. 3).

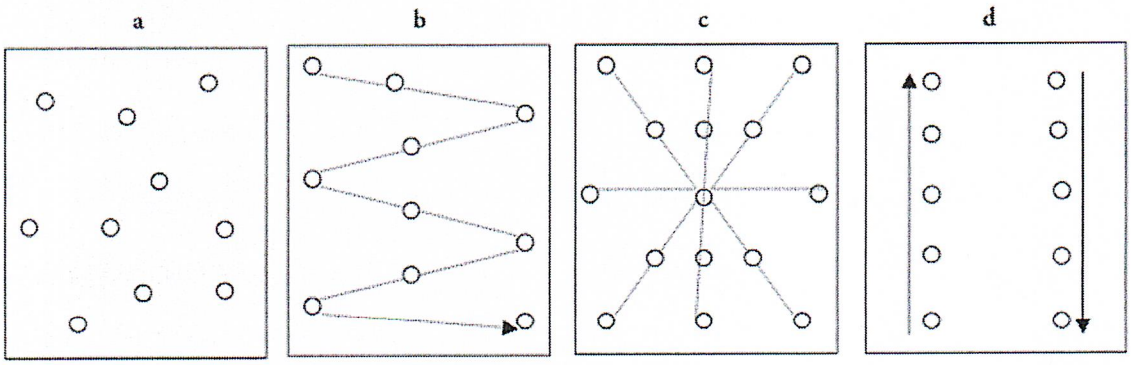

Figure 3. Sampling pattern for nematodes. (a) Random sampling; (b-d) Systemic sampling (Coyne et al., 2014)

2.6 Data recording and mapping

- Data should be recorded in several respects like
  - ✓ Date of collection
  - ✓ Collection number
  - ✓ Locality

- ✓ GPS coordinates
- ✓ Elevation
- ✓ Host plant type and infestation severity
- ✓ Plant growth stage
- ✓ Local name(s)
- ✓ Habit and habitat

- Use mapping tools like GIS to create infestation distribution maps

2.7 Collection of samples and nematode extraction

According to Davis & MacGuidwin (2000), root-lesion nematodes complete their life cycle and subsequent colonization within host roots rather than infesting the soil. Therefore, collect root samples to confirm the presence of nematodes in the field and send to laboratory for further morphological and molecular identification.

2.7.1 Root Samples

- Select 6 random sampling points per field. Carefully uproot 4-6 maize plants (up to V6 growth stage and/or at harvest stage) (CropWatch, 2015) per sampling point with the tops discarded and the soil shaken from the root systems, ensuring minimal damage to the roots (DCS, 1996).
- Check the roots for the characteristic lesions or necrotic spots indicative of *P. brachyurus* damage.
- Collect 5-10 cm segments of roots from each plant to get a representative sample from each sampling point to make a composite sample of 50-100 g (Coyne *et al.*, 2014; Pretorius, 2018).
- Place the root samples in a clean container or plastic bag and label each root sample with field information (e.g., field ID, GPS coordinates and location, date of sampling, name of crop).
- Ensure roots are kept cool (but not frozen) and transported to the lab within 24-48 hours for nematode extraction and analysis.

2.7.2 Nematode extraction from roots

Follow extraction tray method (also known as modified Baermann technique or Whitehead tray method) described by Coyne *et al.* (2014) for nematode extraction from roots as follows:

- Wash roots to remove adhering soil and cut the infected root into 1-2 cm pieces with a knife or scissors.
- Spread the tissue paper inside plastic sieve placed on extraction plates (plastic plate, extraction tray, plastic tray).
- Take 10 g of chopped maize root from composite sample and spread uniformly over the tissue paper.
- Add clean tap water to the extraction plate carefully, enough to moisten the root tissue without covering it. Label the extraction tray and the sieves to identify the samples.
- Leave the extraction sets for 48 hours and move motile juveniles from sample to the extraction tray (Fig. 4).
- After 48 hours, lift up the plastic sieves with roots to drain the water into the extraction tray, and remove the roots.
- Pour the water with nematodes into a labeled beaker, rinse the plate, and let the sample settle.
- To concentrate the nematodes, reduce the water volume or pass the extract through a fine sieve 30 µm mesh. Finally, wash the nematodes into a beaker or tube for counting or preservation.
- Diagnose the nematodes numbers and genera/species under compound stereo-microscope and compound microscope (Baidya, 2023).

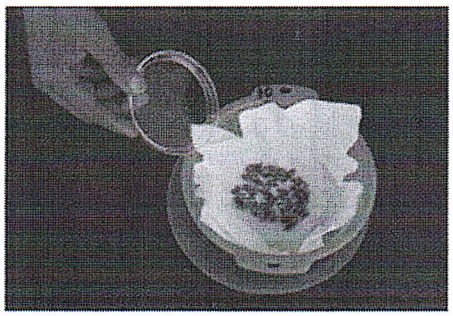

Figure 4. Extraction tray method (Coyne *et al.* (2014)

2.7.3 Fixing and mounting technique

Fix the nematodes with a hot 4% formaldehyde solution and transfer to anhydrous glycerin (De Grisse, 1969). Prepare microscope slides using a paraffin wax ring to mount the nematodes. Position a paraffin ring in the center of the glass slide followed by a small drop of glycerin. Place the nematodes on the glycerin drop, and position a cover slip carefully on top of the ring. Heat the slide on a hot plate until the paraffin melts, allowing the cover slip to settle securely in place (Ryss, 2017).

2.8 Diagnostic laboratories

- National Plant Pathology Research Centre, Nepal Agricultural Research Council, Khumaltar, Lalitpur
- Central Agricultural Laboratory, Department of Agriculture, Hariharbhawan, Lalitpur
- National Herbarium and Plant Laboratories, Department of Plant Resources, Godawari, Lalitpur
- Natural History Museum, Swayambhu, Kathmandu
- Private laboratories – Center for Molecular Dynamics Nepal (CMDN), Thapathali, Kathmandu; Nepal Plant Disease and Agro Associates (NPDA), Balaju, Kathmandu, and others if any.

2.9 Identification of *Pratylenchus brachyurus*

2.9.1 Morphological identification

According to Loof (1978), the main diagnostic characters that distinguish *P. brachyurus* from other *Pratylenchus* species include the shape of the head, the shape of the lip region (with *P. brachyurus* having conspicuous angular outer edges of the first annule), the presence of two annules in the lip region, length of stylet (>19 µm for both *P. brachyurus* and *P. macrostylus*), shape of stylet knobs, absence of spermatheca (indicating the absence of males), shape of female tail and tail tip, and position of the vulva (which is approximately 82-89% for both *P. brachyurus* and *P. macrostylus*).

a) Body length

Body of *P. brachyurus* is small, slender, spindle-shaped, finely annulated with a thin cuticle, typically ranges from 0.5 mm (Nemaplex, 2024) to 1 mm in length (CABI, 2021). Lateral field usually consists of four lines in mid-body, occasionally dividing into five or six in vulval region and terminating into two lines near the tail (CABI, 2021; Kolombia et al., 2021).

b) Head and Lip morphology

Labial or Lip region is set off from body, with angular anterior margin having two distinct annules (CABI, 2021; Kolombia et al., 2021; Machado et al., 2015) (Fig. 5). Front is undivided, with six papillae surrounding the oval, oral aperture and two large amphid openings (CABI, 2021). Two large sunken areas extend laterally from the amphidial apertures (Lopez & Salazar, 1990).

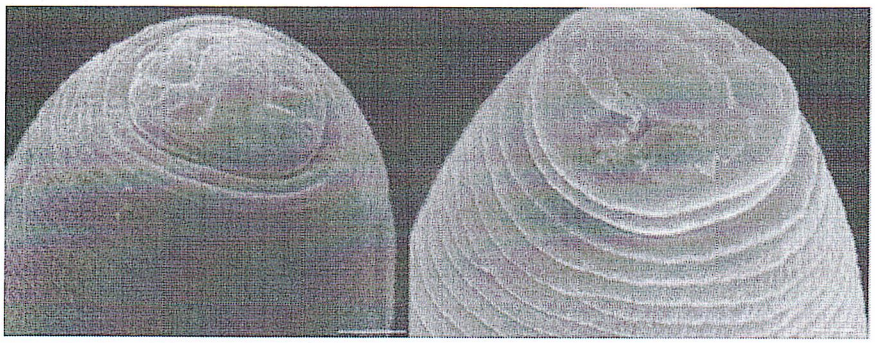

Figure 5. Labial region of *Pratylenchus brachyurus* (Source: Machado et al., 2015)

c) Stylet morphology

Stylet of *P. brachyurus* is characteristic of root-lesion nematodes and is about 20 µm long, usually ranges from 17 to 22 µm (Loof, 1960), moderately sclerotized with stout basal knobs which are rounded above and below (CABI, 2021; Kolombia et al., 2021) (Fig. 6).

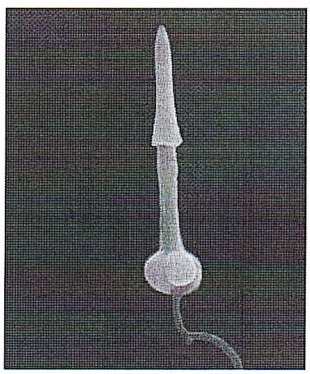

Figure 6. Stylet of *Pratylenchus brachyurus* (Source: J. D. Eisenback, Virginia Polytechnic Institute and State University, bugwood.org)

Oesophagus is well developed with a prominent median bulb (CABI, 2021; Kolombia et al., 2021). The oesophageal gland lobes is approximately 51 µm long, ranging from 31-68 µm (Loof, 1960), and overlap the intestines ventrally and laterally (CABI, 2021; Kolombia et al., 2021). Orifice of dorsal oesophageal gland is located about 2 µm behind spear base (CABI, 2021). Excretory pore is positioned just behind the hemizonid, 57-108 µm from the head, typically located at the oesophageo-intestinal junction which is often indistinct (CABI, 2021; Kolombia et al., 2021). Gonad outstretched, occasionally reflexed and rarely extends past the lobe of the oesophagus (CABI, 2021).

d) Reproductive structures and tail shape

Females: According to CABI (2021), vulva of female *P. brachyurus* is posteriorly positioned (82-89%) and ovary does not extend to oesophageal gland. Ovary consists of a single row of oocytes, though occasionally it may contain one or two oocytes doubled (CABI, 2021; Kolombia et al., 2021). Posterior uterine branch is short, measuring less than one body width (10-30 µm) in length. Spermatheca is inconspicuous and non-functional (CABI, 2021). Tail is broadly conoid, smooth and broadly rounded, truncate (De Araujo Filho et al., 2014; Machado et al., 2015) or spatulate at the tip with 13-24 annules and no striations (Fig. 7). While phasmids are located approximately at the midpoint of the tail (CABI, 2021).

Males: Very rare. If present, with single outstretched testis and phasmids located behind mid tail that do not extend into the bursa. Additionally, the gubernaculum is simple trough-shaped and equipped with arcuate spicules (CABI, 2021).

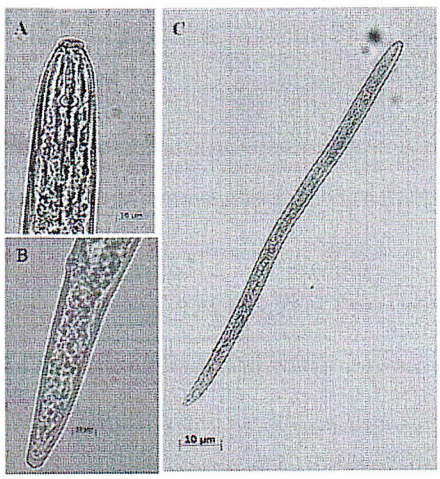

Figure 7. Light micrograph of the anterior region (A), tail (B), and whole body (C) of female *Pratylenchus brachyurus* (Source: De Araujo Filho et al., 2014)

2.9.2 Molecular identification

Due to its morphological similarities with other *Pratylenchus* species, molecular identification techniques are highly desirable for detecting *Pratylenchus brachyurus*.

ITS, rDNA, 18S rDNA, D2-D3 of the 28S rDNA and mitochondrial genes are extensively used techniques in molecular detection of *Pratylenchus* (Castillo & Vovlas, 2007; De Luca et al., 2012).

However, the D3 subunit fragment of 28S rDNA of *Pratylenchus* is more accurate molecular marker for species identification within the genus (Al-Banna et al., 1997; Subbotin et al., 2008; Hodda et al., 2014). Therefore, molecular analysis method using D3 region of the 28S rDNA described by Sandoval-Ruiz et al. (2023) is given below. However, the method is not necessarily mandatory to follow. Any other established/adopted methods may be used alternatively.

DNA extraction

- Select adult *Pratylenchus* nematodes using a metal surgical needle and place it in a Petri dish with distilled water.
- Cut each nematode with a scalpel into three pieces and transfer five nematodes per sample into PCR tubes containing 5 µL of Worm Lysis Buffer + proteinase K for DNA extraction.

Polymerase Chain Reaction (PCR)

- Incubate samples at 80 °C for 30 min, 65 °C for 1 hour, followed by a proteinase K inactivation step at 95 °C for 15 min.
- Amplify the D3 region of the 28S rDNA from the extracted material using species-specific primers such as D3A (5′-GACCCGTCTTGAAACACGGA-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (Al-Banna et al., 1997). The amplification conditions consist of an initial denaturation at 92 °C for 5 min, 40 cycles at 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min.
- Carry out PCR in 25 µL as follows: 2.5 µL of Dream Taq Buffer 10X, 1µL of dNTP′s (2 mM), 2 µL of each primer (10 µM), 1 µL of BSA (20 mg mL-1), 1.5 mM of MgCl2, 0.5 µL of Dream Taq (5 U µL-1, Thermo Fisher Scientific), and 5 µL of the DNA extract.
- Analyze the PCR product by electrophoresis on a 1.6% agarose gel (1.6 g of agarose in 100 mL of TRIS-Borate-EDTA 0.5X buffer).
- Compare resulted sequences with other sequences of *Pratylenchus brachyurus* from the GenBank by BLAST Search.

2.10 Reporting

The responsible or concerned organizations (diagnostic laboratory) or an independent surveyor, after analysis and identification, should submit a report to the NPPO-Nepal for the reporting/declaration of nematode. The reports should include infestation maps, photographs and specimen vouchers. If specimens cannot be identified morphologically, they should be identified by molecular methods.

2.11 Record keeping

NPPO-Nepal, in collaboration with responsible laboratories, will preserve the specimens and keep all the records safely. The documentation system should be well maintained by the NPPO-Nepal and the collaborating institutions will have access to it.

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ANNEXES

Annex- 1: Field datasheet

1. Name of field/Site visited:
2. Date/Time of visit:
3. GPS reference point Latitude:
   Longitude: Altitude:
4. Province: District:
   Municipality: Ward no./Place:
5. Climate data of locality: Average min. temp (in °C):
Average max. temp (in °C): Rainfall (in mm)
6. Survey/Field plot no.
7. Host plant species inspected: Variety:
8. Phenological stage of the plant:
7.1 Description of habitat (such as aspect, slope, vegetation type, soil type)

7.2 Alternate host plant species found infected, if any:
9. Sampling method:
10 Contact details of the local informant involved in the survey:
11. Details of pest recorded

SN	Scientific name	Common name	Plant parts affected	Symptom & Sign	Disease incidence	Severity % / Score

10. Any additional information (including collection of specimens for investigation):

11. Name/Signature of surveyor with date:

Annex 2: Format for forwarding specimens

1. Collection number: 2. Date of Collection:
3. Submitting organization:
4. Name/Address/Contact no. of the sender:
5. Locality of collection (Province / District / Municipality / Ward No. / Place):

6. Reasons for identification:
7. Name of the host plant species (Scientific name / Common name / Variety:

8. Origin of host/commodity (Source of seed/planting materials, if applicable):

9. Plant parts affected:	[ ] roots; [ ] stems; [ ] leaves; [ ] inflorescence; [ ] fruits; [ ] seeds/nuts [ ] others (_____)
10. Category of pest specimen/organism submitted:	[ ] insects; [ ] mites; [ ] nematodes; [ ] fungi; [ ] bacteria; [ ] virus; [ ] others (_____)
11. Life stage of the pest (Applicable to insects):	[ ] egg; [ ] larvae; [ ] pupae; [ ] adult; [ ] nymphs; [ ] juveniles; [ ] anamorphic [ ]; cysts; [ ]others (_____)
12. Type of pest specimen/organism submitted:	[ ] preserved specimen; [ ] pinned/card board mounted specimen; [ ] dry specimen with host; [ ] culture; [ ] disease specimen (fresh); [ ] disease specimen (partially dry); [ ] slide mount; [ ] others (_____)

14. Number of specimens submitted per each collection:
15. Signature/stamp/office seal of the sender with date:

For identifier use

16. Name &address of Diagnostic/Referral Laboratory:
17. Remarks of identifier (condition of receipt of specimens):

18. Pest identification (Common/Scientific name/Taxon):

19. Description notes, if any:

Place: _____

Date: _____

_____

(Signature/Name/Designation of Identifier)

Note: This form should be prepared in duplicate by the sender and forwarded to the identifier/referral laboratory along with each collection of specimens. The identifier should return the original copy after entering the particulars of the pest identified along with description notes and remarks if the identifier will retain any to the sender of the specimen and duplicate the copy.