6.

Detection Survey Protocol for

Longidorus spp. in Nepal

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

March, 2025

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March 12, 2025

Detection Survey Protocol for *Longidorus* spp.

NPPO-Nepal, 2025

Endorsed by NPPO-Nepal on March 12, 2025

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 *Longidorus* sp. 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 *Longidorus* sp. 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 (Needle nematode)

Needle nematodes (also known as longidorid nematodes), members of the genus *Longidorus* Micoletzky, 1922, are migratory ectoparasitic nematodes (Archidona-Yuste et al., 2016), which feed the plant exclusively at, or just behind, the root tips resulting in the production of root-tip galls (PWKB, 2021). These nematodes can cause substantial yield reductions in crops by their direct feeding on plant roots (CABI, 2021). Longidorus nematodes are distributed world widely, from within the Arctic circle to tropical regions (CABI, 2021; PWKB, 2021) and

associated with a large variety of plants (Archidona-Yuste et al., 2016; Cai et al., 2020). Feeding by *Longidorus* spp. leads to significant reduction in the root system of the host plant, severely stunting both lateral and tap roots (CABI, 2021; PWKB, 2021); and induces gall formation with hypertrophy of the procambial cells in the host root tip (CABI, 2021). Additionally, several *Longidorus* species are natural vectors of nepoviruses which cause severe economic damage to a wide range of crops, especially high-value fruits, ornamentals and vegetables (CABI, 2021; Taylor & Brown, 1997). Mueller *et al.* (2016, 2020) reported significant yield losses in maize caused by *Longidorus*, indicating that the extent of damage by these nematodes may vary depending on geographical locations and other influencing factors.

Longidorus 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 *Longidorus* spp.

In Nepal, *Longidorus elongatus* was first reported in 1967 (Bhatta, 1967). *Longidorus* spp. are most commonly prevalent in vegetable crops such as chilly, cabbage, cauliflower and potato in various districts, including Chitwan (Amatya & Shrestha, 1969; PPD, 2009) followed by rice (Amatya & Shrestha, 1969; Baidya, 2013) and papaya (Baidya, 2023).

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

1.2.1 Identity

Preferred scientific name: *Longidorus* Micoletzky, 1922 (Filipjev, 1934)

Preferred common name: Needle nematodes

Other scientific names

Brevinema Stegarescu, 1980

Neolongidorus Khan, 1987

EPPO code: LONGSP

1.2.2 Taxonomy

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

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Nematoda

Family: Longidoriae

Genus: *Longidorus*

Species: *Longidorus africanus, L. attenuatus, L. cohni, L. elongatus, L. fasciatus, L. intermedius, L. pauli, L. pisi, L. proximus, L. vinecola* etc.

1.3 Host range

The genus *Longidorus* consists of more than 180 species worldwide (Kantor et al., 2024); many are polyphagous and some have restricted reported host range (CABI, 2021). *Longidorus* spp. has a very wide host range including onion (*Allium cepa*), pineapple (*Ananas comosus*), asparagus (*Asparagus officinalis*), sugarbeet (*Beta vulgaris* var. *saccharifera*), cabbage (*Brassica oleracea* var. *capitata*), bell pepper (*Capsicum annuum*), lime (*Citrus aurantiifolia*), lemon (*Citrus limon*), sweet orange (*Citrus sinensis*), grapefruit (Citrus x paradisi), coffee, melon (*Cucumis melo*), carrot (*Daucus carota*), yam (*Dioscorea*), strawberry (*Fragaria ananassa*), soyabean (*Glycine max*), lettuce (*Lactuca sativa*), barley (*Hordeum vulgare*), sunflower (*Helianthus*), pea (*Pisum sativum*), potato (*Solanum tuberosum*), wheat (*Triticum*), maize (*Zea mays*) etc. as main host (PWKB, 2021).

1.4 Nematode biology and damage symptoms

1.4.1 Life cycle and field identification

The life cycles of *Longidorus* spp. may vary from a few months to several years depending on the species capable of surviving for several years (Flegg, 1968). *Longidorus africanus* completed its life cycle in less than four months on a suitable host at constant temperature range of 20–23°C (Cohn & Mordechai, 1969).

The life cycle of *Longidorus* nematodes consists of six stages: the egg (or embryo), four juvenile stages (J1 to J4) and the adult.

a) Egg stage: In temperate climates, egg laying occurs in spring and early summer under annual host plants when the plants produce new roots. With perennial host plants, egg laying mainly occurs early in the growing season, with a second period of egg laying coinciding with a secondary root production phase during the latter part of the season (CABI, 2021).

b) Juvenile stage: Eggs hatch shortly after being laid, with the first-stage juveniles contributing to the population of adults and juveniles that overwintered. If provided with adequate environmental stimulation such as adequate moisture, hatching typically occurs once the first-stage juvenile is fully developed. Each juvenile stage is separated by a molt, involving the separation of the cuticle from the underlying hypodermis (apolysis), formation of a new cuticle, and shedding of the old one (ecdysis), including the oesophagus lining and odontostyle. In the first-stage juvenile, the position of the replacement odontostyle is unique as it lies within the odontophore, just posterior to the base of the functional odontostyle (CABI, 2021).

c) Adult stage: After undergoing several molts, juvenile nematodes mature into adults. Males are rare in most species and presumably unnecessary for reproduction (Raski, 1988), though amphimictic population can be found (Ye & Robbins, 2004).

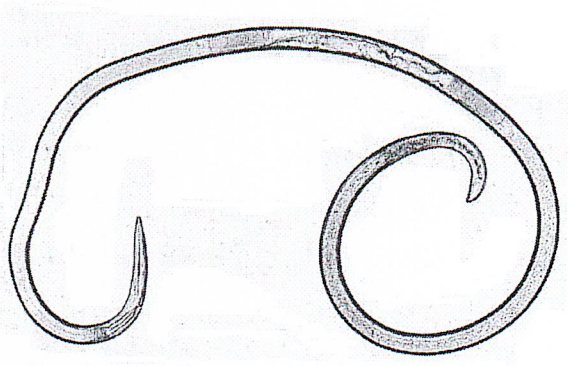

Figure 1. Adult *Longidorus* nematode (Source: Scottish crop research institute, PWKB, 2021)

1.4.2 Damage symptoms

Longidorus nematodes cluster at the tip of the roots (Askary et al., 2018), and feed exclusively at or just behind the root tips usually causing galling (Askary et al., 2018; CABI, 2021). According to Askary et al. (2018) and CABI (2021), feeding by *Longidorus* spp. significantly reduce root systems, with lateral and sometimes tap roots being severely stunted. Damage caused by *Longidorus* spp. is most noticeable in seedlings and young cuttings (Askary et al., 2018), leading to cessation of main root growth and formation of short, swollen lateral roots with enlarged, swollen (galled) root-tips (CABI, 2021; PWKB, 2021) (Fig. 2). Crop damage caused by these nematodes is often visible as patches of stunted plants, which expand slowly by a few centimeters each year and may eventually cover an entire field. Affected plants are usually susceptible to drought and often exhibit leaf malformations or discoloration, typically chlorosis (PWKB, 2021).

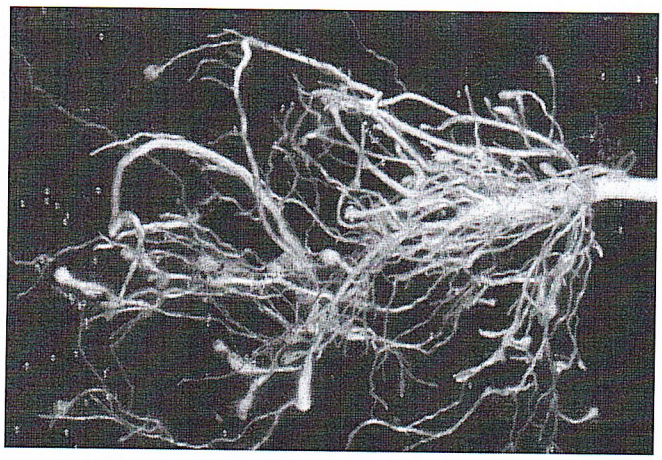

Figure 2. Root-tip galls on *Petunia hybrida* caused by the feeding of *Longidorus macrosoma* (Source: Derek J.F. Brown, PWKB, 2021)

1.5 Mode of dispersal

Longidorid nematodes are primarily dispersed over long distances when plants with soil adhering to the roots are transported by man, and may occasionally be dispersed by water, wind, birds and animals (McNamara & Flegg, 1981; Boag, 1985; Sturhan, 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 *Longidorus* spp. 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).

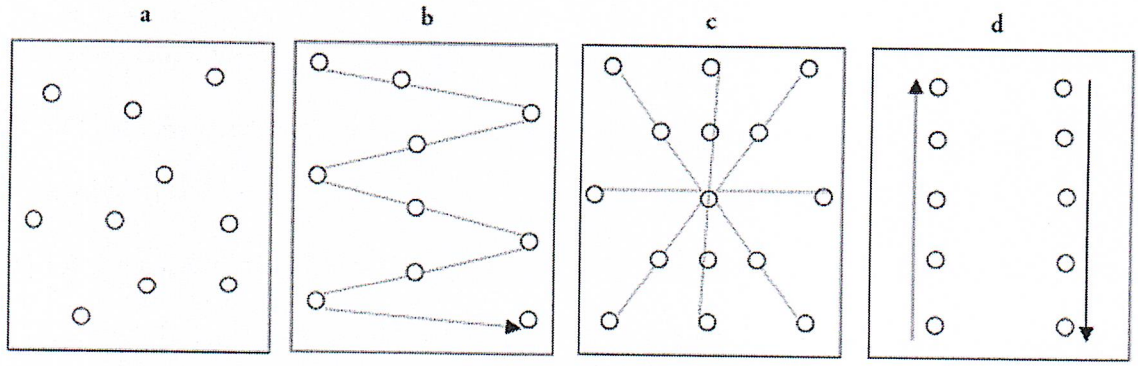

Figure 3. Sampling pattern for nematodes. (a) Random sampling; (b-d) Systemic sampling (Coyne et al., 2014)

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

Being migratory ectoparasitic nematodes, reliable detection of *Longidorus* spp. requires their recovery/extraction from moist soil by various extraction methods such as decanting and sieving, centrifugal floatation and elutriation (Southey, 1986).

2.7.1 Soil samples

- Select 6 random sampling points per field. At each sampling point, collect soil cores around 200 g from the surface to a depth of 15-20 cm, close to the plant roots (root zone), with the help of a soil auger. Mix the soil samples from all sampling points to prepare a final composite sample of 200 g (Baidya, 2023).
- Take about 20 soil cores from an area of 10 acres or less and cores within a single area. Mix them thoroughly to make a composite sample. Soil should not be overly wet or dry when sampling (Grabau & Vann, 2024).
- Place soil samples in sterile plastic bags, close them tightly with rubber bands to prevent moisture loss and label each sample with field information (e.g., field ID, GPS coordinates and location, date of sampling, name of crop).
- Store the soil samples in a cool, dry place to avoid direct sunlight and transport them to the laboratory as soon as possible for nematode extraction.

2.7.2 Nematode extraction from soil

Extract the Longidorus nematodes from moist soil using modified sieving and decanting technique described by Brown & Boag (1988) as follows:

- Take 200 g of soil in a 1-liter beaker, fill it with water, crumble soil lumps and stir into suspension.
- After 15 seconds sedimentation, slowly decant the suspension into another bucket through a sieve of 2.0 mm aperture and stir the suspension carefully again.
- Wash the residue on the sieve carefully over the bucket then discard.
- After 15 seconds sedimentation, slowly decant the suspension through a 150 µm sieve.
- Wash the residue on the sieve carefully over the bucket and after 15 seconds decant the suspension through a 95 µm sieve.
- Allow the water to settle for 24 hours to let nematodes and fine particles sink. Transfer the concentrated residue then to a glass beaker for counting or preservation.
- Diagnose the nematodes numbers and genera/species under compound stereo-microscope and compound microscope (Baidya, 2023).

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 *Longidorus* spp.

2.9.1 Morphological identification

Accurate identification of *Longidorus* spp. requires careful examination under a dissecting microscope or compound microscope focusing on key morphological features that distinguish Longidorus from other nematodes. Chen *et al.* (1997) provided a polytomic key for *Longidorus* species identification which include a large body (length between 3.0-9.0 mm) and odontostyle (length between 80-160 µm), a lip region continuous or offset by depression or expanded anteriorly with a border rounded or flattened, amphidial fovea usually more or less pouch-like between shallowly or distinctly bilobed symmetrical or asymmetrical, and a tail usually short, which varies between hemispheroid and elongate-conical.

a) Body length and shape

Body of *Longidorus* nematodes are long to very long (ranging from 3 to >10 mm) and slender. In heat relaxed state, their form can vary from relatively straight to C-shaped. Its lateral chords are broad with one or two rows of lateral body pores. The cephalic region is rounded, with fused lips and a typical 6 + 10 papillae arrangement (CABI, 2021; Kantor et al., 2024). The amphidial apertures are small, leading to well-developed pouch-like fovea (Askary et al., 2018; CABI, 2021).

b) Stylet characteristics

Odontostyle, the most distinguishing feature of *Longidorus* spp., is needle-like, elongated, and not heavily sclerotized, with a simple guiding apparatus located near the head. Odontophore is moderately sclerotized, about two-thirds the length of the odontostyle (CABI, 2021), and lacks basal flanges (Askary et al., 2018; CABI, 2021). Oesophagus is narrow and cylindrical at the front, looping when the odontostyle is retracted, with a muscular and glandular posterior bulboid expansion. There are three glands: dorsal, and two ventrosublateral (CABI, 2021). The nerve ring is located around the anterior oesophagus, and sometimes a second nerve ring is present more posteriorly (Askary et al., 2018; CABI, 2021).

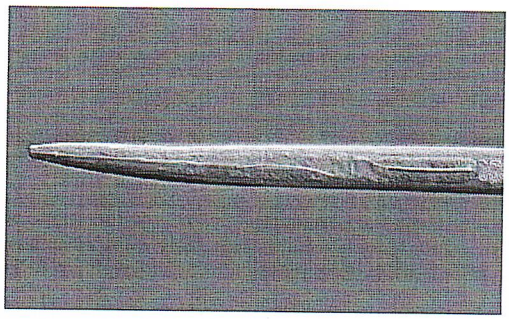

Figure 4. Anterior end of *Longidorus* spp. odontostyle, odontophore, and esophageal glands as seen with differential contrast interference microscopy (Source: U. Zunke/Nemapix vol. 1, PWKB, 2021).

c) Reproductive structures:

Females: Vulva is a median transverse slit, with a well-developed, muscular vagina positioned at right angles to the body axis, leading to a substantial ovejector (CABI, 2021; Kantor et al., 2024). Genital tract is amphididelphic and reflexed. Tail is short, dorsally convex-conoid (Askary et al., 2018), and ends in a finely rounded or broadly rounded terminus (CABI, 2021; Kantor et al., 2024). Several pairs of caudal pores are present (CABI, 2021).

Males: Male genital tract is diorchic, with both testes joined by a common vas deferens anterior to the cloaca. Spicules are paired, ventrally arcuate, and massive, with short accessory guiding pieces. Copulatory muscles are oblique and extend several body widths anterior to the cloaca, while copulatory supplements include an adanal pair and up to 20 ventromedian pairs (CABI, 2021).

2.9.2 Molecular identification

Due to its morphological similarities with other species, molecular identification techniques such as PCR are highly effective for detecting *Longidorus* spp. A number of methods are available for molecular diagnosis of plant nematodes. The method given below is not necessarily mandatory to follow. Any other established/adopted methods may be used alternatively.

DNA extraction

- Select adult nematodes using a metal surgical needle and place it in a Petri dish with distilled water.

- Extract DNA from the nematodes using a DNA extraction kit or standard protocols (such as Worm Lysis Buffer + proteinase K) that involve tissue lysis and purification (Subbotin, 2021; Kantor et al., 2024).

Polymerase Chain Reaction (PCR)

Molecular analysis method described by Kantor et al. (2024) is given below.

- Amplify DNA from the extracted material using following species-specific primers: (i) D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTA CTA-3′) (Subbotin, 2021) for amplification of the D2-D3 expansion segments of the 28S rRNA gene; (ii) TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and rDNA 1.58S (5′-ACGAGCCGAGTGATCCA CCG -3′) (Subbotin, 2021) for amplification of the ITS1 rRNA gene; (iii) Long-COIFmod (5′- GATTYTTTGGDCACCCNGARGT-3′) and Het-CoxiR (5′-CCTAAAACATAATGAAAAT GWGC -3′) (Inserra et al., 2021) for amplification of COI gene.
- Purify the PCR products using the QIAquick Gel Extraction Kit (Qiagen) and submit to direct sequencing.
- Compare the obtained sequences with known *Longidorus* species sequences in genetic databases (e.g., GenBank) using tools like BLAST.

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.