National Diagnostic Protocol

Cyst Nematodes (The genus *Heterodera*)

Photo by Akshita Jain. Specimens from the Nematology section of the Australian National Insect Collection, CSIRO.

NDP 53 V1

This publication (and any material sourced from it) should be attributed as: Subcommittee on Plant Health Diagnostics (October 2024). National Diagnostic Protocol for Cyst Nematodes (The genus *Heterodera*)– NDP 53 V1 (Eds. Subcommittee on Plant Health Diagnostics) Authors Daniel C. Huston, Manda Khudhir, Mike Hodda; Reviewers Akshita Jain, Dylan Corner, Katherine Linsell, Monica Kehoe, Samantha Bond, Sarah Collins, and Stefan Harasymow. ISBN 978-0-6481144-9-9 CC BY 3.0.

Cataloguing data
Subcommittee on Plant Health Diagnostics (October 2024). National Diagnostic Protocol for Cyst Nematodes (The genus *Heterodera*) – NDP 53 V1 (Eds. Subcommittee on Plant Health Diagnostics) Authors Daniel C. Huston, Manda Khudhir, Danièle Giblot-Ducray, Mike Hodda; Reviewers Akshita Jain, Dylan Corner, Katherine Linsell, Monica Kehoe, Samantha Bond, Sarah Collins, and Stefan Harasymow. ISBN 978-0-6481144-9-9.

ISBN; 978-0-6481144-9-9.

Internet
Report title is available at: https://www.plantbiosecuritydiagnostics.net.au/resources/?category=national-diagnostic-protocols

Department of Agriculture, Fisheries and Forestry

Street Address: Agriculture House, 70 Northbourne Avenue, Canberra 2601, Australia
Postal Address: GPO Box 858, Canberra City ACT 2601
Switchboard Phone: 02 6272 3933
Web: http://www.agriculture.gov.au

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs, unless it is shown that the NDP has improved procedures for Australian conditions. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/work/subcommittee-on-plant-health-diagnostics/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for Cyst Nematodes (The genus *Heterodera*) is current as at the date contained in the version control box below.

PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP 53
VERSION NUMBER	V1
PROTOCOL STATUS	Endorsed
ISSUE DATE	2024
REVIEW DATE	2029
ISSUED BY	SPHD

The most current version of this document is available from the SPHD website: https://www.plantbiosecuritydiagnostics.net.au/resources/?category=national-diagnostic-protocols

Further information

Inquiries regarding technical matters relating to this protocol should be sent to: sphd@aff.gov.au

Contents

1 INTRODUCTION

1.1 Protocol scope

This diagnostic protocol follows a system of *primary* identification (molecular methods) followed by *secondary* identification using morphological methods. This is because the difficulties inherent in morphological identification of species of *Heterodera* make this method of identification less reliable than molecular methods. This protocol covers primary molecular identification for most species of the genus *Heterodera* (those species for which molecular data are available), and secondary morphological identification for all presently valid species of the genus. All species of *Heterodera* currently listed as National Priority Plant Pests (NPPPs) in Australia can be diagnosed following this protocol. This diagnostic protocol does not cover cyst nematodes of other genera, such as the potato cyst nematodes (genus *Globodera*).

1.2 The genus *Heterodera*

The genus *Heterodera* Schmidt, 1871 includes 88 species of obligate plant-parasitic 'cyst nematodes', many of which are devastating pests of important crops (Nicol et al. 2007; Subbotin et al. 2010a, b; Toumi et al. 2013; Smiley et al. 2017). Cyst nematodes occur on all continents except Antarctica, and various species parasitise all of the most globally important agricultural crops. When penetrating roots, larval cyst nematodes create wounds on the roots, leaving not only vascular tissue damage but also making the plant's roots vulnerable to infection by other disease-causing organisms in the soil. Furthermore, feeding by the cyst nematode through to the adult female stage induces the formation of specialised feeding cells, known as syncytia, from which the nematode can draw a continuous source of nutrients. Thus, cyst nematodes cause direct and indirect damage to plants. Cyst nematodes have a life cycle involving multiple life stages, the egg, four juvenile stages (J1-J4) and the stage 5 adult (Moens et al. 2018; Smiley et al. 2017). The J2 larval nematodes hatch from eggs in soil. Hatching is stimulated by chemicals produced by susceptible plant roots or specific environmental conditions; however, hatching may also occur spontaneously (Masler and Perry 2018; Smiley et al. 2017). The J2 larval stages infect roots, form specialised feeding sites and progress through larval stages J3 and J4 to stage J5 where males and females mature. Males are mobile in soil and fertilise females which remain attached to roots. After fertilisation, mature females die and the body toughens around the fertilised eggs forming a cyst. These eggs within cysts can remain viable for decades (Back et al. 2018; Smiley et al. 2017).

The following species are presently known, or have previously been reported to occur, in Australia: *Heterodera australis, Heterodera cruciferae*, *Heterodera daverti, Heterodera fici, Heterodera graminis*, *Heterodera humuli*, *Heterodera mani*, *Heterodera schachtii* and *Heterodera trifolii* (Jain et al. 2022; Kempster et al. 2001; Jain et al. 2023; Subbotin et al. 2002; Stirling and Wicks 1975; Davidson 1930; McLeod et al. 1994; Hay and Pethybridge 2003). An additional, undescribed species of the *Humuli*-species group has been reported from Australia, although the collection locality is uncertain (Subbotin et al. 2022). In this protocol, *Heterodera avenae* is treated as presently not occurring in Australia (see taxonomy section for explanation). A full list of species in the genus can be found in Appendix 1.

The following species of *Heterodera* are presently listed as NPPPs in Australia: *Heterodera carotae*, *Heterodera filipjevi*, *Heterodera glycines*, *Heterodera latipons*, *Heterodera sorghi* and *Heterodera zeae*.

1.3 Host range

Most species of cyst nematode are fairly host specific and can only complete their life-cycles on a small number of host plants, however, a few species have a broader host range (Subbotin et al. 2010a, 2010b). The most devastating species of *Heterodera* are those which parasitise cereals (commonly called 'cereal cyst nematodes'), which commonly cause yield losses up to 50% with even higher losses reported occasionally (Toumi et al. 2018). Other species cause significant damage to oilseeds, legumes, fruits, and vegetables, while about half of the species in the genus parasitise native plants or weeds and do not impact agricultural crops (Appendix 1). The identification of the host species is useful for cyst nematode identification but should not be overly relied upon; rather it should be used as supporting data to augment identifications based upon molecular and/or morphological data.

2 TAXONOMIC INFORMATION

Kingdom: Animalia

Phylum: Nematoda

Order: Tylenchida

Superfamily: Tylenchoidea Örley, 1880

Family: Heteroderidae Filipjev & Schuurmans Stekhoven, 1941

Subfamily: Heteroderinae Filipjev & Schuurmans Stekhoven, 1941

Genus: *Heterodera* Schmidt, 1871

Synonymised generic names: *Afenestrata* Baldwin & Bell, 1985; *Afrodera* Wouts, 1985; *Bidera* Krall & Krall, 1978; *Brevicephalodera* Kaushal & Swarup, 1989; *Ephippiodera* Shagalina & Krall, 1981; *Heterobolbus* Railliet, 1896; *Heterodera* (*Heterodera*) Schmidt, 1871; *Sarisodera* Wouts & Sher, 1971; *Tylenchus* (*Heterodera*) Schmidt, 1871

Common name: cyst nematodes

List of species: Appendix 1

The tylenchid family Heteroderidae Filipjev & Schuurmans Stekhoven, 1941, includes eight genera known as 'cyst nematodes', a group of obligate plant parasites for which females, at the end of their lives, form hardened sacs containing eggs (Luc et al. 1986; Baldwin 1992; Subbotin et al. 2001; Bert et al. 2008; Subbotin et al. 2010a, 2010b; Moens et al. 2018). The first genus of cyst nematodes in this family proposed was *Heterodera* with *Heterodera schachtii* as its type species (Schmidt 1871). Later, the genus *Globodera* was proposed for the potato cyst nematodes which were distinguished from species of *Heterodera* in having spherical, rather than lemon-shaped, cysts (Behrens 1975; Mulvey and Stone 1976). Both genera share species which either have or lack vulval fenestration which can be ambi- or bifenestrate. Six additional genera have also been proposed, including *Punctodera* which include species with significant anal fenestration, and *Cactodera* for species that have a circumfenestrate vulva and a distinct posterior protuberance (Mulvey and Stone 1976; Krall and Krall 1978). The four remaining genera (*Betulodera*, *Dolichodera*, *Paradolichodera* and *Vittatidera*) are all monotypic, having been proposed based on unique morphological features (Handoo and Subbotin 2018). Of these genera, those with the most economically important species are *Globodera* and *Heterodera*.

The taxonomy of *Heterodera* is fairly complex due to the large number of species and subtle morphological differences between them. The various species can be divided into nine morphological species groups (see Appendices 1, 2) based on some general morphological characters, however, beyond this, morphological identification is difficult as adults are sexually dimorphic and multiple life-cycle stages are usually required for identification (Handoo and Subbotin 2018; Subbotin et al. 2010b, a). Notably, several species of *Heterodera* cannot be distinguished from one another morphologically at any life-cycle stage (e.g. Subbotin et al. 2002). Thus, molecular methods are important for species identification in this group.

Of particular relevance to Australia is the distinction between *H. avenae* and *H. australis*. Historically, all Australian populations of cereal cyst nematode have been considered to be of the species *H*. *avenae* (Brown 1984; Stirling and Kerry 1983; Riley and McKay 2009), which is common in cereal fields throughout North America, Europe and Asia (Handoo and Subbotin 2018). However, Subbotin et al. (2002) noted a molecular difference between Australian cereal cyst nematodes and those from other regions, and, despite reporting no morphological differences between the Australian population and populations of *H*. a*venae* from elsewhere, described the Australian populations as *H*. *australis*. Australian plant pathologists have generally not accepted the validity of *H*. *australis*, most likely due to the lack of morphological differences noted in the original description. However, since the original description, a number of additional studies have supported the validity of *H*. *australis* through further phylogenetic analyses (Subbotin et al. 2003; Subbotin et al. 2010a, 2010a; Subbotin 2015; Subbotin et al. 2018). Furthermore, based on barcoding data of cereal cyst nematodes from across cereal growing regions in Australia, Huston et al. (2024) failed to detect any populations of *H*. *avenae*; rather, all populations were found to represent *H*. *australis*. This strongly suggests that *H. avenae* is not present in Australia and thus here *H*. *australis* is considered a valid species, and *H*. *avenae* is considered as exotic to Australia.

3 DETECTION

3.1 Symptoms

Symptoms of *Heterodera* infestation are typically subtle and are often mistaken for other issues such as nutrient deficiency, soil compaction or chemical toxicity (Moore 1984; Baldwin and Mundo-Ocampo 1991). The most common manifestation is irregular patches of stunted growth across a paddock (e.g., Fig. 1A, B) which may be more susceptible to water or heat stress (Baldwin and Mundo-Ocampo 1991; Steele 1984). Infected plants may exhibit yellowing of leaves, browning of leaf margins and roots may be stunted (Fig. 1C), deformed or necrotic, with death occurring in some cases (Baldwin and Mundo-Ocampo 1991; Smiley et al. 2017).

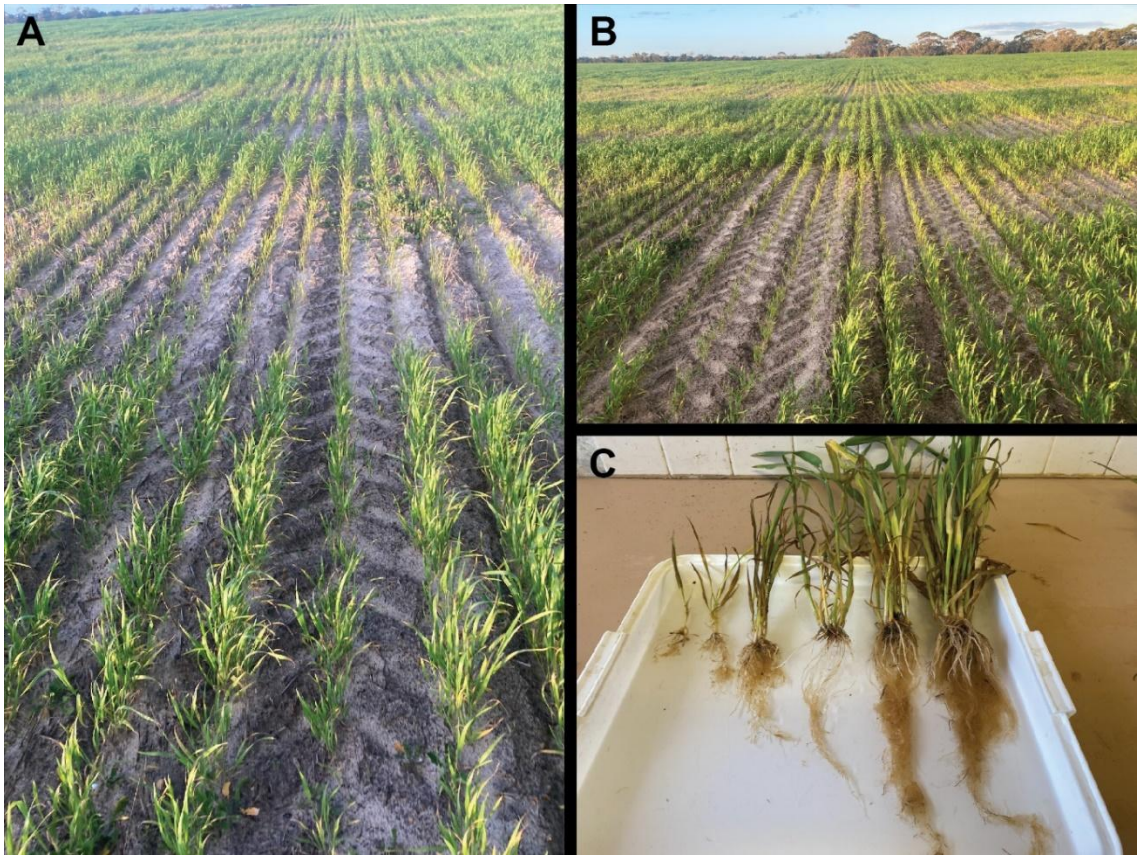

Figure 1. Wheat showing the signs of *Heterodera australis* (Australian cereal cyst nematode) infestation. A, B, wheat fields showing irregular patches of stunted growth. C, wheat plants showing stunted root growth. Images A, B, courtesy of Clancy Thompson, Nutrien Ag Solutions; C, courtesy of Dr Sarah Collins, Department of Primary Industries and Regional Development, Western Australia.

3.2 Soil and root sampling

Cyst nematode infestation can be assessed through examination of soil samples for cysts and free-living vermiform stages (J2s and males), or by examining the roots of infected plants for white females, cysts and vermiform stages.

Soil samples can be collected all year round as cysts persist in soil. However, sampling during or just after a host crop optimises the chance of detection as the numbers of cysts/juveniles increases. The best sampling time will vary with each species/crop combination.

Equipment recommended:

- GPS units to record property/paddock location.
- Soil corer (10 mm diameter x 100 mm or 150 mm long tip), metal bucket to collect cores and screwdriver or scraper to dislodge soil from corer. Small shovel or handheld trowels are also suitable for sample collection, particularly for root samples.
- Sample bags (zip lock or with cable ties).

Before sampling, determine the number of paddocks and sections per paddock to be sampled. If visible, identify sections of the paddock to sample that have sandy soils or well-structured clay soils, where crop performance was poor. In vegetable production, it is recommended to collect one sample per hectare. Higher sampling intensity can be used to confirm a positive result and determine the extent of spread. A lower sampling intensity may be considered for large scale surveillance of broadacre crops. A zig zag pattern in the target area is a good sampling method.

If using a soil corer, an ideal individual sample is a composite of 45 cores of 10 mm × 100 mm or 30 cores of 10 mm × 150 mm; choice of core length is determined by soil depth, but most cysts are found in the top 10–15 cm of soil (Smiley et al. 2017). Combine all 45 (or 30) soil cores in a single sample bag; samples should be approximately 500 g. If using a shovel or trowel, take samples to 10–15 cm depth until an estimate of 500 g is achieved. There is no need to clean the corer or shovel between individual samples in the same paddock, but they should be cleaned and disinfected when moving between paddocks. Store soil bags in an empty cool box (no ice) to protect from direct sunlight; do not leave moist samples in plastic bags exposed to the sun.

Root samples can be taken in the same manner as soil samples, but are best dug up using a trowel or shovel and placed directly into plastic bags (e.g., Fig. 2). Above-ground plant leaves and stems are not needed when sampling for *Heterodera* spp. and can thus be discarded at the time of sampling if desired.

Figure 2. Collecting soil and root samples for cyst nematode screening. Photo courtesy of Dr Sarah Collins, Department of Primary Industries and Regional Development, Western Australia.

3.3 Collection of *Heterodera* specimens from soil and root samples

3.3.1 Visual root inspection

Roots should be gently rinsed in water to remove soil and other attached debris. Roots can then be examined directly under a magnifying lens or a dissecting microscope. White female and brown mature *Heterodera* cysts can be seen attached to the surface of roots (Fig. 3). These female cysts can be picked off from the roots using tweezers.

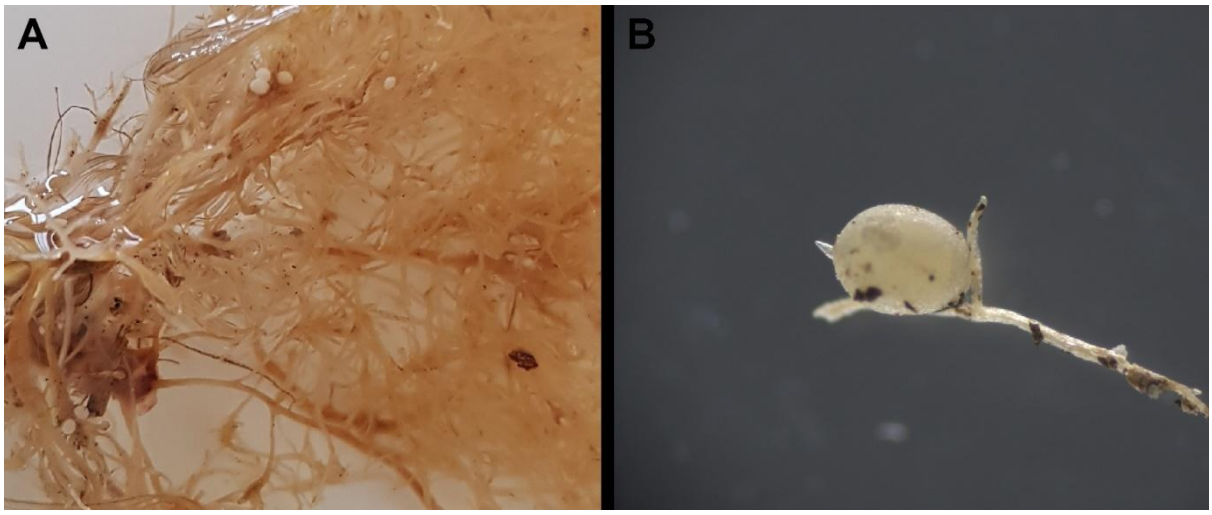

Figure 3. *Heterodera australis* white females attached to the roots of wheat. A, courtesy of Dr Sarah Collins, Department of Primary Industries and Regional Development, Western Australia. B, Photo by Daniel C. Huston (CSIRO).

3.3.2 Isolation of cysts from soil

A number of methods can be used to separate *Heterodera* cysts from soil, such as the Fenwick Can (Fenwick 1940), or Cobbs Sieving and Decanting (Cobb 1918). Both methods involve washing the soil sample and utilising the density and size of the nematodes to isolate them from larger, smaller, and heavier soil particles. The Fenwick can is a specialised piece of equipment and may not be readily available commercially; many Fenwick cans found in diagnostic laboratories were custom made. Cobbs's sieving and decanting is a quick and simple method requiring only a sink, a squirt bottle, some sample containers, a sieve with an 850 µm mesh and a sieve with a 250 µm mesh (Fig. 4.1) and is thus the method recommended here. The procedure is as follows:

1. Place the soil sample into a container (Fig. 4.2).
2. Soak the soil sample in water for 30 min to several hours. This helps with the washing of the soil and hydrates *Heterodera* cysts, making them easier to decant and find (Fig. 4.3).
3. Wash the soil sample through the 850 µm sieve nested on top of the 250 µm sieve, removing large particles. Only small parts of the sample should be washed at a time to prevent the lower sieve from becoming clogged (Fig. 4.4,5).
4. Discard the material in the top screen, and wash the sample remaining in the bottom screen into a large sample container such as a beaker or pitcher. Repeat the process described from step 3-4 until the entire sample has been washed through and all of the remaining sample is in the larger sample container (Fig. 4.6).

5. Spray water at high velocity into the sample container to suspend particles (Fig. 4.7). As the high-density material such as sand quickly drops to the bottom, pour the water off carefully into the 250 µm sieve (Fig. 4.8) to collect the lower density material.
6. Concentrate the sample in the bottom the sieve (Fig. 4.9) using a hose or squirt bottle, and transfer to a smaller sample cup (Fig. 4.10).
7. Pour the sample cup onto a mesh screen, filter paper or other absorbent material (Fig. 4.11, 4.12). This causes the sample to spread in a relatively even layer, facilitating examination.
8. Examine the sample under a dissecting scope for cysts (Fig. 4.13). Collect cysts observed (Fig 4.14) and place them in a drop of water in a petri dish or other container (Fig. 4.15) (because the cysts are wet it is much easier to get them off tweezers undamaged if they are placed in a drop of water).
9. Leave cysts in petri dishes overnight with lids half-off so that the cysts can dry out. Dried cysts can be stored for decades.

Figure 4. Pictorial guide to Cobb's Sieving and Decanting method (Cobb, 1918); Steps described in section 3.3.2. Images by Manda Khudhir & Daniel C. Huston, CSIRO.

3.3.3 Isolation of vermiform stages from plant roots and soil

Isolation of *Heterodera* vermiform stages (males and J2s) from plant roots and soil can be achieved using passive extraction methods which utilise the mobility of these nematodes. A number of variations exist (see Hooper 1986) but the basic principle is to place the sample on a filter which is soaked in water, allowing the nematodes to migrate through the filter into the clean water sample over the course of several days. Here we describe one of the most widely used methods, the Whitehead-Hemming tray (Whitehead and Hemming 1965).

1. A Whitehead-Hemming tray consists of a basin and perforated container; anything will do, from custom made containers to colanders or plant trays (Fig. 5.1).
2. The perforated container is placed in the basin and a filter is applied to the perforated container (Fig. 5.2). In this case we are using single-ply facial tissue paper, which works very well.
3. The sample is spread evenly over the tissue paper (Fig. 5.3).
4. Water is added to the basin until it just soaks the sample – the water level should not rise above the sample (Fig. 5.4).
5. The sample is left at room temperature for 2–4 days. Following this period, the soil and paper is discarded and the water from the basin is passed through a 20 µm mesh sieve. The isolated sample is then washed into a petri dish for examination under a dissecting microscope.
6. Extraction efficiency is proportional to basal area, tray mesh size and smoothness of bottom tray.

Note: The Whitehead-Hemming tray method is designed to separate the nematodes from a large amount of material and isolate them in clear water. Clean plant roots can be soaked directly in water in small lots (such as in petri dishes) and then removed.

Males and J2 larvae are best picked from the water using fine needles with a hook at the end, or with a fibre (e.g., a paintbrush with all but a few fibres removed).

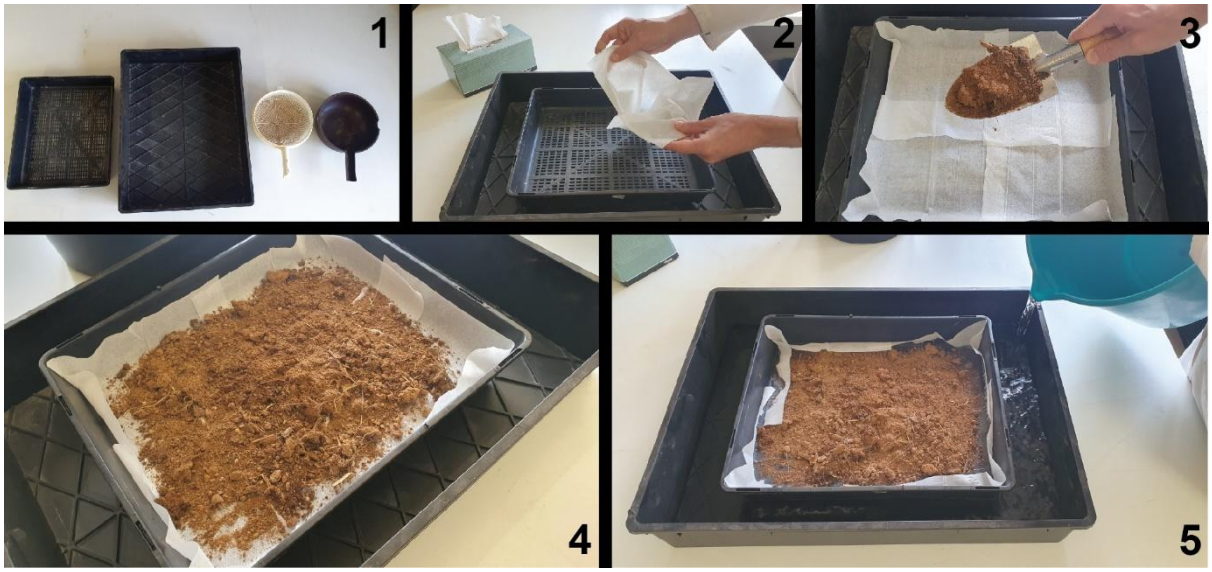

Figure 5. Pictorial guide to setting up Whitehead-Hemming trays (Whitehead & Hemming, 1965) for nematode extraction from soil. Steps described is section 3.3.3. Images by Manda Khudhir & Daniel C. Huston, CSIRO.

3.3.4 Collection of J2 larval stages from cysts

Heterodera cysts are easily cut or broken open. In many cases, gravid cysts will contain live J2s which have already hatched but have not yet left the cyst. J2 hatching and emergence from the cysts may also be stimulated by placing the cysts in dishes of water and refrigerating them for several weeks to several months. The dishes should be checked every few days to see if the J2s have emerged.

3.3.5 Preservation of specimens

For *Heterodera*, vermiform specimens that will be used for morphological study should be fixed and preserved in 4–5% formalin. Specimens that will be used for molecular analyses can be preserved in ethanol for storage, or frozen in a drop of water or DNA extraction buffer if DNA is not going to be extracted immediately.

White females should be placed into a preservative for long term storage, either 80% ethanol or greater, or 4–5% formalin.

Brown cysts can be kept dried for decades, and still remain viable. For long term storage of species of phytosanitary concern, cysts should be stored in 80% or greater ethanol or 10% formalin.

Vermiform stages to be used for morphological study should first be killed in either near boiling water or near boiling 5% formalin, and then rapidly cooled by adding cold water or cold 5% formalin. This causes the nematode body to fix into a consistent position across specimens. After killing, the nematodes should be preserved in 5% formalin. If the specimens are to be used for molecular analyses, they can be placed directly into 80% or greater ethanol, or frozen in a drop of water or DNA extraction buffer. It is still sometimes advantageous to first kill the nematodes in near-boiling water as this is helpful for identifying morphotypes in case of mixed samples. Killing with near-boiling water does not negatively

impact downstream DNA barcoding as described in this document, although it may negatively impact NGS deep-level sequencing efforts.

3.4 Phytosanitary considerations when sampling

Prior to collecting, (i) contact the state Biosecurity department to check permit and other biosecurity requirements in place, (ii) if you intend to send samples to a diagnostic laboratory, provide prior notice of your intention to submit samples of biosecurity concern and (iii) seek permission to access the property to be surveyed.

Appropriate disinfestation procedures must be followed both when entering and leaving a property with suspected cyst nematode infestation. All equipment used and clothing worn on a property must be disinfested or disposed of. Set up a disinfestation station using undiluted Dettol™ (commercial liquid antiseptic) or a freshly prepared 2% sodium hypochlorite solution (chlorine). Use appropriate safety precautions when handling and using hazardous chemicals. This solution can be used multiple times in one day but should be replaced if soil or debris accumulates in the solution and in any case prior to moving between properties. Prior to entering a property – Footwear and sampling tools must be thoroughly cleaned of soil and plant material using water and scrubbing brush. No soil or plant residue should be visible after cleaning. Immerse footwear (ideally rubber boots) in the disinfection solution for at least 60 seconds, making sure to brush the solution up the boot. Do not rinse after immersion. Cover the disinfection tub between use to limit breakdown; replace solution daily or more frequently if it gets dirty. On exit, use water and scrubbing brush (or screwdriver) to remove all soil and plant material from tread of footwear and sampling tools. Disinfest by immersion as described above, including the cleaning tools (e.g., scrubbing brush). Soiled clothing should not be worn between paddocks. It is highly recommended to wear disposable overall suits and change these between each paddock/property when undertaking a survey. Used disposable clothing should be placed inside a plastic bag for appropriate disposal as biosecurity waste. Vehicles should not be driven on the property or remain on designated roads to avoid the pickup of soil on tyres. If the vehicle must be driven to paddocks, ensure it is cleaned thoroughly with a pressure washer to remove any soil and plant material prior to leaving the property. Cleaning is to occur away from the paddock and on a hard stand area with wastewater capture to prevent runoff onto paddock or trafficked areas.

Note: *Heterodera* spp. cysts are very stable and resistant to external stress; they may not be killed by the disinfectant. The scrubbing and removal of debris are critical.

4 IDENTIFICATION

Due to the specialised skills and methodology required for morphological identifications, molecular identification is considered the easiest and most reliable method for identification of species of *Heterodera* and includes extraction of DNA, followed by amplification via the polymerase chain reaction (PCR) and sequencing of the resulting PCR product. However, molecular sequence data are available for only about 66% of species of *Heterodera* (57 out of 88 at the time of the analyses), and eight of these species were represented by just a single sequence (Huston et al. 2022). Fortunately, species of *Heterodera* which are of concern in agriculture are generally well-represented in public sequence databases and can be readily identified, although in some cases interpretation of the diagnostic marker(s) available can be problematic.

Huston et al. (2022) evaluated publicly available barcoding data for species of the genus *Heterodera* to determine the reliability of these data (in terms of the number of sequences on GenBank which are labelled as a species which they are clearly not) and the utility of these data (ability of these data for distinguishing species). This included analyses of all available sequences (up to August 2021) of the small subunit ribosomal RNA (18S rDNA), internal transcribed spacer (ITS; comprising ITS1-5.8S-ITS2), large subunit ribosomal RNA (28S rDNA), and the mitochondrial cytochrome oxidase subunit one (cox1) gene regions.

In terms of sequence reliability, Huston et al. (2022) found that the number of obviously erroneous sequences on GenBank was fairly low and provided a list of these problematic sequences (as supplemental data available on the CSIRO data portal at: https://doi.org/10.25919/2bqn-ya80). This list is also provided here as a table in Appendix 2. We advise diagnosticians to download this list and refer to it when identifying cyst nematodes with molecular methods.

In terms of the utility of barcoding, Huston et al. (2022) recommended a combination of the four markers evaluated for identification confidence. Although Huston et al. (2022) found the cox1 marker showed the most utility for species differentiation (followed by, in order of utility, the ITS, 28S and 18S markers), less than half of the valid species of *Heterodera* have cox1 sequences available. Thus, for some species additional markers may be required, and because some markers may lack the power of discrimination for identification confidence, multiple markers may be necessary, followed by morphological characterisation in rare cases. Given that Huston et al. (2022) found that the 18S rDNA barcode had the lowest utility for species identification of the four evaluated, here we recommend that for molecular identification of *Heterodera* spp. with high confidence, a sequence of the cox1, ITS, and 28S marker be obtained, to avoid identification issues of individual markers (see section 4.6).

4.1 Molecular Identification

Molecular sequence data are not available for all species of *Heterodera*, however molecular identification can be achieved for most species of *Heterodera*, including most of those of agricultural concern (as well as all species of *Heterodera* on the list of Australian NPPPs), using a sequence of the cox1. The cox1 barcode is robust for species identification and differentiation in this genus apart from two specific issues which are mentioned below (see section 4.1.6). For those species which do not have cox1 data available, sequences of the ribosomal ITS region (consisting of ITS1-5.8S-ITS2) or the 28S rDNA may be used for species identification. In some cases, a combination of the two latter markers may

be required, and here we recommend acquiring sequences of the cox1, ITS and 28S rDNA markers to make a definitive molecular identification.

4.1.1 DNA extraction

DNA can be easily extracted from both *Heterodera* spp. cysts and vermiform stages using commercially available extraction kits which utilise proteinase K tissue digestion and spin-column binding (e.g., Qiagen DNeasy® Tissue Kit).

Although vermiform stages can be directly transferred to extraction buffer, *Heterodera* cysts should be carefully broken open in a drop of water under a dissecting microscope to ensure that they contain enough eggs or larvae for DNA extraction. This also presents the opportunity to retain the vulval cone, and potentially some J2 larvae, as morphological specimens (see section 4.2; morphological methods). The cyst and contents can then be transferred directly to extraction buffer in 1.5 mL Eppendorf tube using forceps and a micropipette. If specimens have been stored in ethanol previously, they should be rehydrated in sterile water to remove the ethanol before being transferred to extraction buffer for tissue digestion. Adequate DNA for downstream sequencing can be successfully extracted from a specimen as small as an individual J2 larva, although white females, gravid cysts and males provide larger amounts of DNA.

The basic procedure is to digest the specimen in extraction buffer with proteinase K for several hours, transfer the sample to a spin column, where it is washed and eluted, following the instructions provided by the manufacturer of the DNA extraction kit used. For all samples we recommend performing a double elution as the last step, using 15 µl of elution buffer or DNA-free water such that the final elution volume is no more than 30 µl.

4.1.2 PCR amplification

A broad range of primer sets can be used to amplify the cox1, ITS and 28S rDNA gene regions for species of *Heterodera*. Table 1 details primer pairs which are highly robust for the amplification of *Heterodera* DNA and are among the most commonly used. These primer pairs are also robust for other plant-parasitic nematodes.

Table 1. Primer pairs for the amplification and sequencing of the cox1, ITS and 28S rDNA gene regions for species of the genus *Heterodera*.

Gene Region	Primer	Primer reference
Cox1	JB3 (forward) 5'-TTTTTTGGGCATCCTGAGGTTTAT-3'	Bowles et al. (1992)
	JB5 (reverse) 5'-AGCACCTAAACTTAAAACATAATGAAAATG-3'	Derycke et al. (2005)
ITS	TW81 (forward) 5'-GTTTCCGTAGGTGAACCTGC-3'	Curran et al. (1994)
	AB28 (reverse) 5'-ATATGCTTAAGTTCAGCGGGT-3'	Curran et al. (1994)
28S	D2A (forward) 5'-ACAAGTACCGTGAGGGAAAGTTG-3'	De Ley et al. (1999)
	D3B (reverse) 5'-TCGGAAGGAACCAGCTACTA-3'	De Ley et al. (1999)

4.1.2.1 PCR master mix

No specific PCR master mix is required, and multiple commercially available mixes should be suitable. An example of the master mix used routinely in the CSIRO Insect and Nematode Biosecurity Team, based on Bioline® and Kapa Biosystems® reagents is as follows: For a 25 µL reaction using 2 µL DNA template, combine 17.9 µL of water, 2.5 µL of 10X buffer, 1 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 0.5 µL of each primer (10 pmol), 0.1 µL of 5 U/µL KAPA taq polymerase. All reagents should be thawed completely and thoroughly mixed before use; $MgCl_2$ for example, defrosts in a gradient and if not thoroughly mixed can impair PCR.

4.1.2.2 PCR cycling conditions

A variety of cycling conditions can amplify the cox1, ITS and 28S rDNA gene regions for species of *Heterodera*. Here we recommend using a hot-start, 55–45 °C touch-down cycling profile which is highly successful for amplification of all barcoding regions mentioned in this NDP: Preheat machine to 94 °C, add samples, then 25 cycles of: 94 °C for 30 seconds, 55 °C (- 0.4 °C each cycle) for 30 seconds and 72 °C for 60 seconds (+ 2 seconds each cycle), followed by 13 cycles of 94 °C for 30 seconds, 45 °C for 30 seconds, 72 °C for 120 seconds (+ 3 seconds each cycle), followed by a ten minute extension at 72 °C.

Alternative PCR cycling conditions are provided in Appendix 4 for those with PCR machines that cannot be programmed for touch-down cycling profiles.

4.1.2.3 Visualisation, purification, and quantification of PCR products

Amplicons should be visualised through a method such as a fragment analyser or gel agarose electrophoresis using a florescent dye which binds to DNA (e.g., SYBR™ Safe - Invitrogen, Australia) with the gel subsequently examined on a transilluminator. PCR products which appear as clean bands can be used for sequencing.

Depending on the sequencing service, PCR products may or may not require purification. Some sequencing services will accept unpurified PCR products and will purify them as part of the service. This usually incurs a higher cost than sending purified product. PCR products can be purified in several ways, including spin-column based (e.g., QIAquick PCR Purification Kit; Qiagen®, Australia) and enzymatic methods (e.g., ExoSAP-Clean Up; New England BioLabs®). The standard instructions which come with these commercial kits are suitable for *Heterodera* but double-check with the sequencing service in case any modifications to the protocol are required.

DNA concentrations in purified products should be quantified before sending the samples for sequencing. This can be achieved using a fluorometer such as the Invitrogen™ Qubit™ Fluorometer or the NanoDrop® UV Visible Spectrophotometer following the manufacturer's instructions. Sample concentrations of at least 20 ng/µL are ideal for barcoding *Heterodera*, however, concentrations of less than this still routinely produce successful sequencing reactions. Sequencing of samples with concentrations below 3 ng/µL is typically not successful.

4.1.3 DNA Sequencing

Purified cox1, ITS and 28S rDNA PCR products should be sequenced in both forward and reverse directions using the same primers used in amplification. There are many commercial sequencing services available, such as the Australian Genome Research Facility (AGRF) and Macrogen (Korea) and many universities, research institutions and government laboratories have in-house sequencing

facilities. The data returned by these services should include raw trace files which need to be assembled. There are many options for viewing trace files, including numerous free options (e.g., FinchTV); however, few software packages are capable of assembling forward and reverse sequences automatically to produce a consensus sequence while simultaneously incorporating base-call quality scores. Commercial software packages, such as Sequencher® or Geneious®, are recommended as they are capable of the above and have user-friendly GUI interfaces. If these programs are not available, there are freeware options that can be used to automatically assemble sequences (e.g., Genestudio Pro), and programs such as MEGA or BioEdit can be used to manually assemble sequences.

4.1.4 Identification based on barcodes

Assembled sequences can be quickly compared against the entire GenBank sequence database using the online nucleotide BLAST (Altschul et al. 1990) tool on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Results of the BLAST analysis will include a variety of information, including a % identity match to other sequences in the database. Matches with less than 2% divergence for the cox1, ITS and 28S rDNA barcodes will suggest conspecificity. However, it is important to keep in mind the previously mentioned list of erroneously labelled sequences provided by Huston et al. (2022) (https://doi.org/10.25919/2bqn-ya80; Appendix 2) and shortcomings of molecular identification as detailed in section 4.1.6. Thus, barcoding should be followed up by tree-based phylogenetic methods to confirm species identifications.

4.1.5 Identification based on phylogenetic methods

The basic procedure for tree-based identification is to first align the sequence(s) to be identified with already identified sequences of interest available on public databases such as GenBank, and then use the alignment to construct a phylogenetic tree.

There are many sequence alignment algorithms and many software packages which can perform multiple methods. For very large datasets, MAAFT (Katoh et al. 2019) is a good choice as it is very fast and robust and is easy to use through an online portal. Other methods such as MUSCLE (Edgar 2004) are also suitable and are often integrated into stand-alone software packages such as MEGA (Tamura et al. 2021). As with alignment algorithms, there are a number of suitable desktop and online commercial and freeware software applications that can construct phylogenetic trees from text-based sequence data, e.g., MEGA (freeware). Multiple phylogenetic methods can be used to determine the phylogenetic position of the sequences in question, however, the Neighbour Joining and Maximum Likelihood methods are among the quickest and most robust.

Determining the sequences to include in the alignment and downstream phylogenetic analyses is not always straightforward and often is an iterative process. The results page from the initial BLAST analyses is a good place to start and offers the option to download the first 100 closest matching sequences. However, it is important to carefully examine downloaded sequences from public databases and curate the dataset as some sequences of *Heterodera* in public databases are mislabelled or misidentified (see section 4.1.6) and many sequences are only identified to the genus level (i.e., *Heterodera* sp.). Customs sequence libraries can be manually constructed by downloading species of interest. In the supplemental data files provided by Huston et al. (2022) on the CSIRO data access portal (https://doi.org/10.25919/2bqn-ya80) there are a number of curated FASTA formatted sequence files which can be downloaded and used for alignment. Under the 'Assessment of Utility' folder each of the barcodes (cox1, ITS, 28S) has a folder, within which are unaligned FASTA files including all sequences

of the respective barcode for all species of *Heterodera* available on GenBank (up to August 2021) but excluding those sequences which the analyses of Huston et al. (2022) showed to be erroneous or otherwise unreliable. Users of these files may need to update them to include novel sequence data which has become available since August 2021.

Sequences to be identified should be added to the curated sequence file, followed by sequence alignment and construction of a phylogenetic tree which will show the phylogenetic position of the sequence in question relative to the others in the dataset, and will hopefully show clear conspecificity with named species. Phylogenetic trees should be constructed for each of the three barcodes to acquire a consensus identification across multiple genetic markers.

4.1.6 Shortcomings of molecular identification

Beyond cataloguing sequences on GenBank which were erroneously labelled (see list at: https://doi.org/10.25919/2bqn-ya80; Appendix 2), the study of barcoding data for the genus *Heterodera* by Huston et al. (2022) noted a number of instances where one of the four markers evaluated (cox1, 18S rDNA, ITS, 28S rDNA) failed to differentiate between individual species pairs (Appendix 4). This was especially apparent among species of the *Avenae*, *Cyperi*, *Goettingiana*, and *Schachtii* species groups. Notably, there are several economically important species that cannot be differentiated using the ITS barcode despite this marker being the most widely used for barcoding purposes in the genus *Heterodera* to date (Subbotin et al. 2001; Tanha Maafi et al. 2003; Subbotin et al. 2017; Huston et al. 2022). Thus, for confidence in molecular identification, multiple markers should be sequenced, with the cox1 marker being the most important.

Although Huston et al. (2022) noted two instances where the cox1 marker failed to distinguish between a species pair, we think this marker can still be considered to be able to distinguish all species. The first instance was the small level of difference between *H. avenae* and *H. arenaria*. Sequences of *H. arenaria* differ from some of those of *H. avenae* by only two base pairs. However, these two differences appear to be unique changes, and are consistent. Thus, *H. arenaria* can be differentiated from *H. avenae* from a barcoding perspective, even if the species does not appear monophyletic in phylogenetic analyses (Huston et al. 2022). The second instance involves differentiating *H. cruciferae* from *H. carotae*. Based on the argument presented by Huston et al. (2022), and a number of sequences generated by us, here we consider the cox1 sequences of *H. cruciferae* from the study of Escobar-Avila et al. (2018) to be misidentified and are instead representative of *H. carotae*. Thus, we consider all species of *Heterodera* to be identifiable through cox1 barcoding, and although less than half of all species of *Heterodera* have cox1 sequence data available, most species of agricultural concern (and all Australian *Heterodera* NPPPs) do have such data. Additional cox1 data for further species have also been added to GenBank since the publication of Huston et al. (2022).

4.2 Morphological Methods

Molecular data are not available for all species of *Heterodera* (Appendix 1) and thus these species can only be identified with morphological methods. Furthermore, in some cases molecular data may not provide a definitive identification due to a lack of sequences of multiple markers for some species. Thus, some molecular identifications may also require supplemental morphological data.

Morphological identification usually requires multiple life-cycle stages, typically the female cyst, J2 larvae and sometimes, the free-living male.

4.2.1 Morphological features of Heterodera spp.

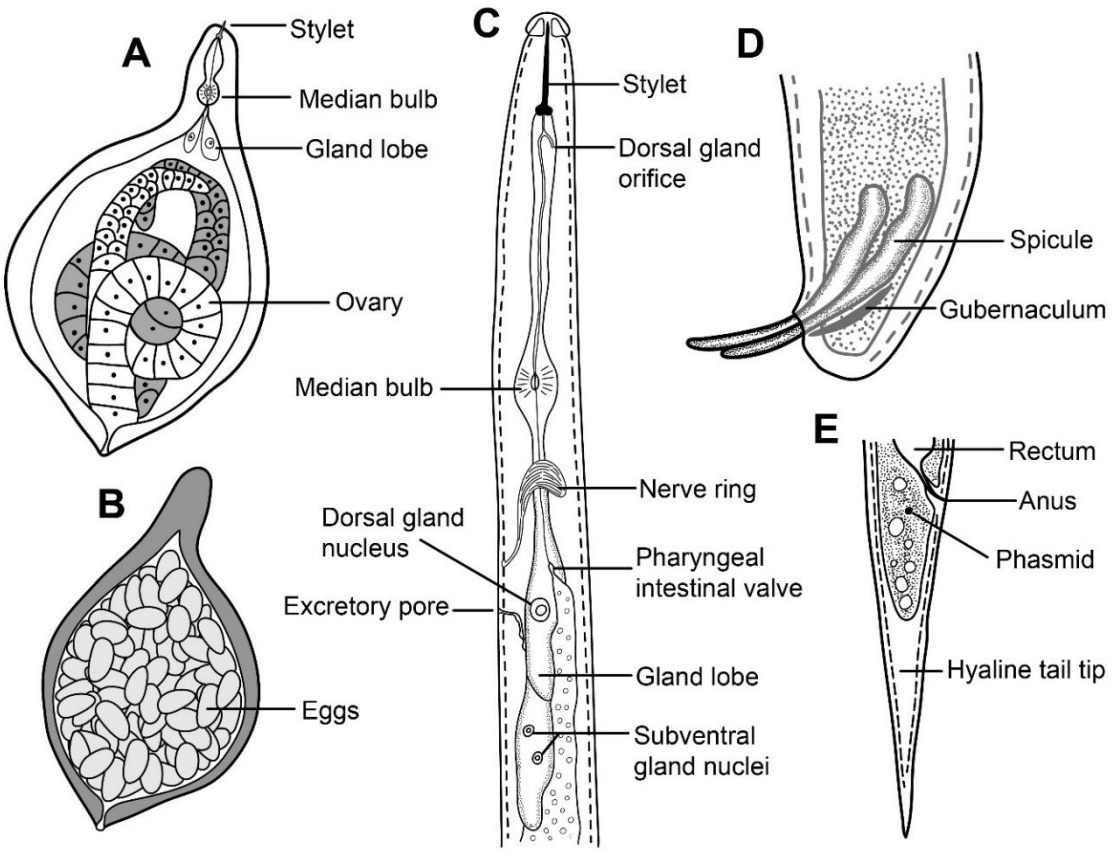

Figure 6. Generalised diagrams of the various life-cycle stages of cyst nematodes of the genus *Heterodera*. A, female. B, cyst. C, Generalised anterior of vermiform stages (Males and J2 larvae); D, male posterior end. E, J2 posterior end. C, D, and E after Williams and Siddiqi (1972).

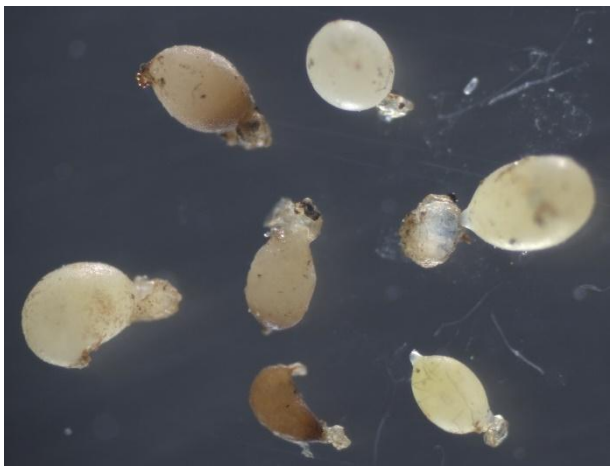

Figure 7. White females of *Heterodera trifolii* with attached egg sacs. Photo by Daniel C. Huston, CSIRO. Specimens from Australian National Nematode Collection.

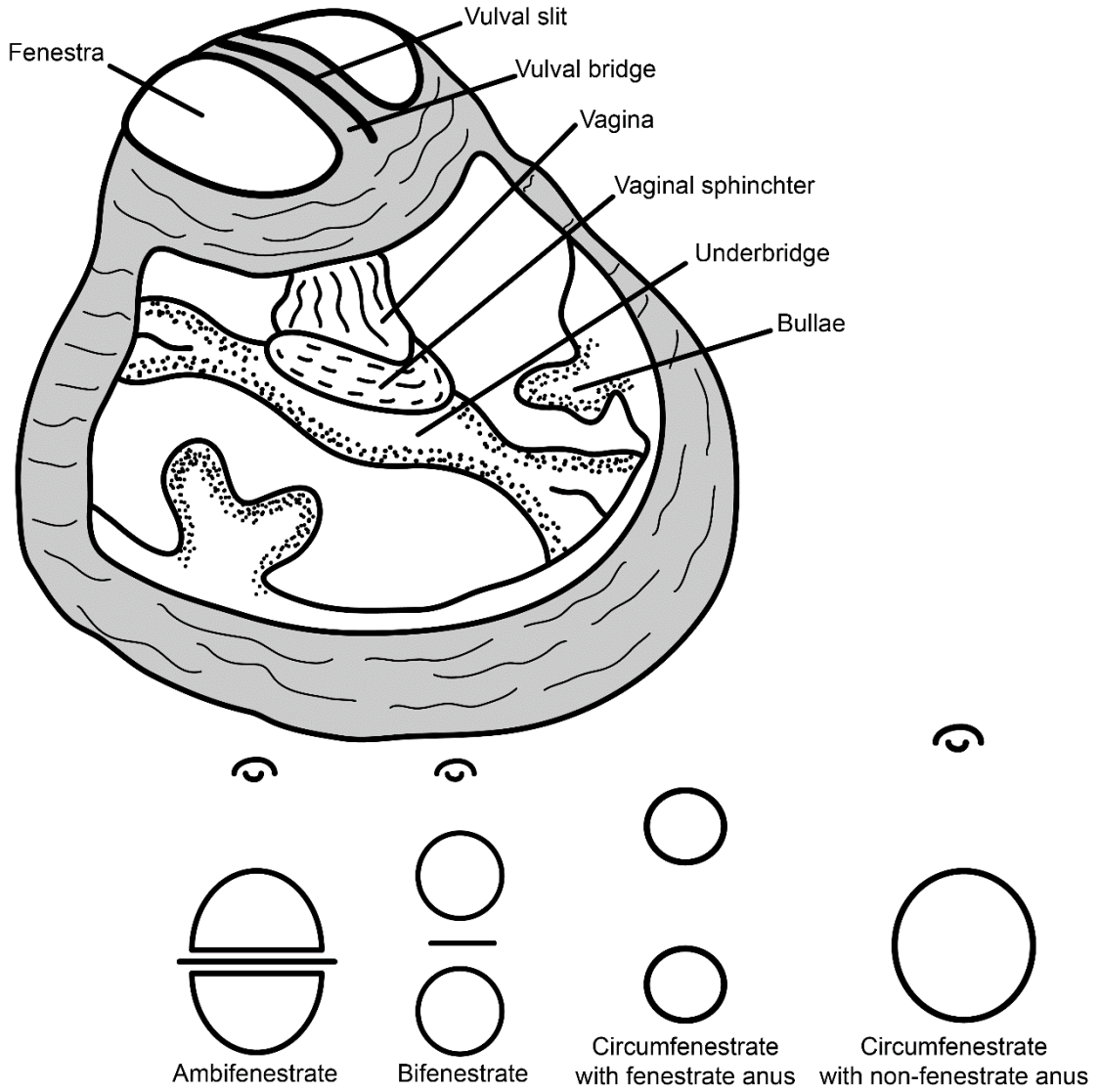

Figure 8. Generalised diagram of the terminal region of the cyst stage of females of the genus *Heterodera*, including various fenestration types of cyst nematodes. Note: Circumfenestrate vulval cones are found in the related genera *Cactodera*, *Globodera* and *Punctodera.*

4.2.1.1 Female adults and cysts

Adult females (Fig. 6A) are sedentary and generally appear as swollen, ovoid nodules with a narrow neck (Baldwin and Handoo 2018). They may or may not have egg sacs encircling their terminal end (Fig. 7). White females have less features of diagnostic value than the cyst stage (Fig. 6B). In *Heterodera*, the cyst stage has a 'vulval cone' (Fig. 8) manifesting as a terminal protrusion at the posterior end, giving the distinctive 'lemon' shape characteristic of cysts of most species of this genus (Baldwin and Handoo 2018; Subbotin et al. 2010a). This contrasts with cysts from some of the other cyst-forming genera *Dolichodera*, *Globodera*, *Punctodera* and *Paradolichodera* where the female stage is globular and lacks a posterior terminal protrusion (Subbotin et al. 2010a). The terminal region of the vulval cone have apertures called fenestra, and the vulval slit is bordered on either side by narrow strips of tegument forming the vulval bridge. These characters are taxonomically informative. Except for species in the Afenestrata group (Appendices 1, 2) which do not develop fenestral openings, cysts of the genus *Heterodera* have fenestration of two types: Ambifenestrate and Bifenestrate (Fig. 8). In older cysts, the

vulval bridge may become damaged and the fenestration may be mistakenly interpreted as circumfenestrate; although close study should permit detection of the remains of the vulval bridge (Subbotin et al. 2010a). This is important as cysts of the species of the genera *Cactodera* and *Betulodera* are circumfenestrate. In some species of *Heterodera* cysts exhibit an 'underbridge' which is located beneath the vulval bridge and 'bullae' which appear as finger or knob-like protrusions. Presence or absence of the above two structures, as well as morphometric data related to the underbridge are taxonomically important (Subbotin et al. 2010a; Baldwin and Handoo 2018).

4.2.1.2 Male and J2 vermiform stages

Males are vermiform and occur free in the soil near roots and females. They are often abundant although some species are parthenogenic and lack males (Subbotin et al. 2010a). Like males, J2 larvae are vermiform and are found in soil; they may also be recovered directly from cysts. Males are significantly larger than J2s (~900–1600 µm in length vs ~300–700 µm), but both share the same general arrangement of features in the anterior region of the body (Fig. 6C). The morphometrics of these features are important for species delineation. Males have a developed reproductive system consisting of a single, elongate testis (see Fig. A5.7) vs an indistinct oval reproductive primordium in J2s (Subbotin et al. 2010a). Males and J2s differ obviously in the structure of the tail (Figs. 6D, E). Males possess copulatory spicules and a gubernaculum (Fig. 6D) whereas J2s include a distinct hyaline region in the tail tip (Fig. 6E). The shape and morphometrics of these features are taxonomically informative.

4.2.1.3 Measurements and morphometric ratios

As the morphology of the various stages of *Heterodera* is relatively conserved, especially among vermiform stages, comparison of morphometric data is important for species identification. For vulval cones measurements of fenestral length, fenestral width, vulval slit length, vulval bridge width, and underbridge length are all important including various ratios comparing these features. Note that measurements of the fenestrae length can be inconsistent across different taxonomic papers; some papers report measurement of individual fenestral openings, other report a length that includes both fenestral openings. For vermiform stages, the following measurements are typically taken: total body length, the maximum body diameter or that at mid body, tail length, diameter of the body at the anus or cloaca, labial region height, labial region diameter, stylet length, distance between the dorsal gland opening and the stylet base, the distance between the anterior end to the dorsal gland opening, the distance between the anterior extremity and the median bulb valve, the distance between the anterior end and the excretory pore, the oesophagus length (tip of stylet to end of gland lobes) and the oesophagus length from tip of stylet to the oesophageal-intestinal valve. The spicules and gubernaculum are measured in males and the hyaline region is measured in J2s.

A number of standard ratios, based on the de Man formulae (de Man 1884) are also important in the morphometric analyses of *Heterodera* specimens, and include:

- a = overall body length divided by greatest body width
- b = overall body length divided by the oesophagus length cut off at the oesophageal intestinal valve
- b' = overall body length divided by the oesophagus length from tip of stylet to end of the oesophageal glands
- c = overall body length divided by the length of the tail
- c' = tail length divided by the tail diameter at anus or cloaca

4.2.1.4 Locating and interpreting morphological features

A number of morphological features important in the taxonomy of *Heterodera* spp. are subtle and can be difficult to observe when presented with actual specimens as compared to what is shown in morphological diagrams (e.g., the dorsal-oesophageal gland opening and oesophageal-intestinal junction). It is also fairly rare for an individual specimen to present quality views of all morphological features; many specimens will have obscured views of some features, and lower quality specimens may provide only basic morphometric data. This is one of the reasons that morphological characterisations should be based on as many specimens as possible. A number of images of *Heterodera* specimens indicating and/or highlighting specific morphological features have been included in Appendix 6.

4.2.2 Preparation of specimens for study

4.2.2.1 Temporary slides

Nematodes can be mounted on a temporary slide in a drop of water under a coverslip for all but the highest magnifications – this is especially useful for studying live nematodes. Objectives that touch a temporary mount are likely to push the coverslip and at very high magnifications the refraction of light through water produces poor views of informative features. For some specimens, most taxonomically informative features will be visible under a 40X objective, although some features, such as the dorsal oesophageal gland orifice, and the oesophageal intestinal valve, are better viewed under oil immersion.

4.2.2.2 Permanent slides

Nematodes previously fixed in formalin should be placed in a dish and a small amount of 10% glycerol (10% glycerol / 90% H_2O) is added to the solution. The dish is then placed under a fume hood with the lid slightly ajar, and the formalin is allowed to evaporate off over the course of two days (the formalin should have all evaporated within 24 hr, but 48 hr is used for caution). Several times during the evaporative process additional glycerol/water solution is added to the dish. After the two days, additional glycerol is added to the dish, and then the water is allowed to evaporate off the dish over the course of 2–4 weeks. Additional drops of pure glycerol should be added as the water evaporates to prevent the volume of solution from becoming too low. Nematodes can be stored in pure glycerol near-indefinitely. However, samples stored long-term in aqueous glycerol should be periodically examined for fungal growth and in some cases a fungicide may need to be added.

4.2.2.3 Mounting vermiform nematodes in glycerol

Equipment needed: Dissecting microscope (for working under), compound microscope (for viewing completed slides), glass microscope slides, glass coverslips, hot plate, paraffin wax such as orthodontal wax, glass dish for heating wax in, short piece of copper pipe with insulation added to one end, 100% glycerol, fine needles, or fibre for moving nematodes.

Nematodes are best mounted on microscope slides, using the 'wax ring' method. Briefly, a good quality paraffin wax is melted in a glass dish on a hot plate. A piece of copper pipe with a diameter slightly larger than the glass cover slips to be used is placed on the hotplate to heat, along with a number of microscope slides (Fig. 9A). After the wax has melted, the copper pipe is dipped into the wax and 'stamped' onto the microscope slide; the slide is then removed from the heat and allowed to harden (Fig. 9B). It is most convenient to produce a large number of wax slides prior to mounting nematodes.

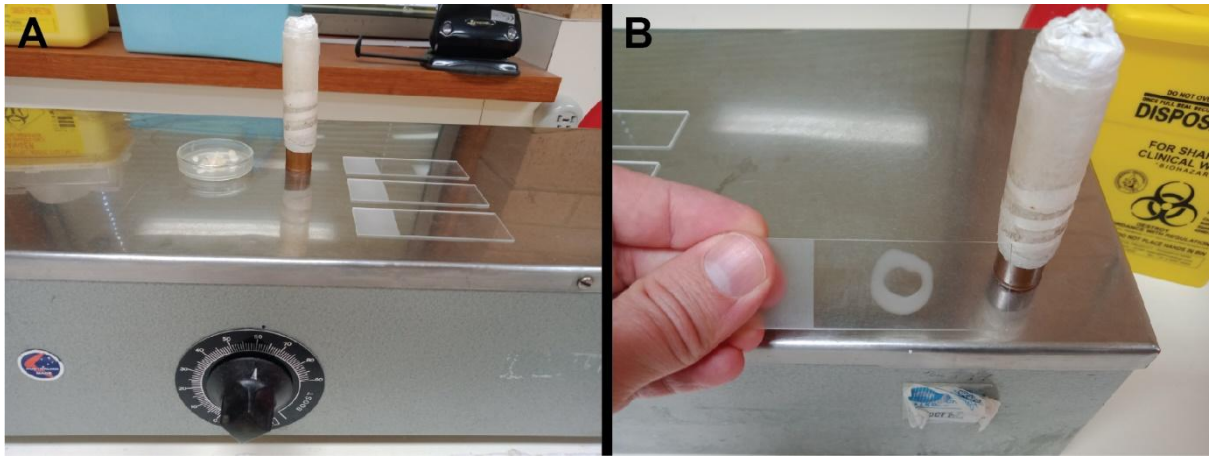

Figure 9. Preparation of wax ring slides for mounting nematodes in glycerol. A, preparing the slides and wax. B, completed slide with cooled wax ring. Photos by Daniel C. Huston, CSIRO.

To mount nematodes, a small drop of glycerol is added into the centre of the wax ring. Using a dissecting microscope and a fine needle or fibre one or more specimens are placed in the drop of glycerol. A coverslip is then added, and the slide is moved to a hotplate heated to about 60 °C; the wax will then melt, and the coverslip will settle into position. Sometimes bubbles will be present, waiting a few minutes will help disperse them. The slide can then be moved away from the hot plate to cool. After the slide is completely cooled the coverslip should be sealed with three successive rings of clear fingernail hardener or polish.

4.2.2.4 Preparing cyst vulval cone mounts

Lateral mounts of the terminal region of *Heterodera* cysts can be useful for viewing bullae, the underbridge and the position of the anus (Baldwin and Handoo 2018). More important, however, are mounts of the terminal cone of the female cyst with the vulva facing upwards toward the observer, which facilitates views of the fenestrae, vulval bridge and vulval slit, as well as the bullae, underbridge and anus, all important features for heteroderid taxonomy and identification (Baldwin and Handoo 2018; Subbotin et al. 2010b, a).

Mounting upward facing vulval cones is a specialized and relatively difficult technique. Here we describe the process using a non-toxic glycerol jelly mounting media modified from the original formulation of Kaiser's Glycerin Jelly (Formulation described in Appendix 6).

Equipment needed: dissecting microscope (two dissecting microscopes is ideal), fine dissecting forceps, scalpel blade, fine dissecting needle or fibre mounted on a long handle, hot plate, pipette.

Cysts are best soaked in water for an hour or more before they are processed for terminal mounts; swollen cysts are easier to clean out and work with than shrunken or shrivelled cysts. Using fine dissecting forceps, the cyst is taken and placed in a drop of water under a dissecting microscope. It is best if the cyst sinks in the water, however, they often float regardless of how long they have been soaking in water. If the cyst floats, then it should be placed on the petri dish in just a trace of water film to facilitate the cyst adhering to the surface of the dish. The cyst is then oriented with the tweezers, and, using a scalpel blade, the terminal region of the cyst is cut off in one slice. This is often difficult, and fine forceps or a needle can be used in the off-hand to help keep the cyst in the correct orientation when

making the cut. Smaller cysts may simply be cut in half, whereas larger cysts will need to be cut closer to the vulval cone.

After the vulval cone is cut, the interior may need to be cleaned of eggs and other debris. If too much material remains inside the cone when it is mounted, this will obscure most features of interest in light microscopy. Cleaning out the cone is best accomplished with a fine needle or fibre. The vulval cones are easily damaged so patience is essential while cleaning the specimen.

After the vulval cone is prepared, a small piece of Kaiser's glycerin jelly is placed in the centre of a microscope slide. The microscope slide is then placed on a hot plate, heated to about 60 °C. Allow the piece of jelly to melt completely and for any large bubbles to dissipate; this should take no more than 1–2 minutes. The size of the drop should be just larger than the coverslips that will be used (10 mm coverslips are sufficient). After the drop of jelly is ready, quickly move the slide to the stage of a dissecting microscope. Gently pick up the vulval cone with forceps and place it into the drop of jelly. Using forceps or a dissecting needle, gently move the cone down into the media and around in a tight circle to mix any water on or in the cone into the jelly media. Next use the needle to place the cone in the correct orientation – with the vulva facing directly up. **Place the slide aside and allow to cool for several minutes or until the jelly has hardened**. Next, place a coverslip over the top of the jelly and tap it down gently to centre it. Finally, place the slide back on the hot plate just long enough for the jelly to re-liquify and bind to the entire coverslip. Remove the slide from the heat and allow slide to completely cool before sealing the slide with three rings of fingernail hardener.

4.2.3 Morphological identification

After specimens are prepared for study, the first step of identification is typically determination of the species group to which the specimens belong, using cyst vulval cones, and in some cases J2 larvae (Appendix 3). Following this, complete morphometric characterisation of the nematodes should be performed for cysts, J2s and males (where available) and the data is compared to that of the other members of the determined species group. General morphological characteristics useful for narrowing down tentative identifications are presented in Appendix 8. Preliminary identifications should then be compared against the original, and any subsequent, descriptions of that species.

Identifications may be performed using the key to species provided in the pages that follow (Section 4.2.3.2).

4.2.3.1 Reference material

The Australian National Insect Collection (ANIC) at CSIRO Black Mountain in Canberra, Australian Capital Territory, has specimens of many of the species of the genus *Heterodera*, including most which appear on Australia's NPPP List. CSIRO will maintain these specimens and the expertise to identify nematodes within this group. Vouchers of any specimens of *Heterodera*, or other cyst forming nematodes, should be deposited in ANIC for future reference, and any submitted for identification will be included in the collection.

4.2.3.2 Key to species of the genus Heterodera

Modified from Subbotin et al. (2010b). Refer to figures 6 and 8 for illustrations of morphological features; further explanation of some characters are provided where needed in the key below. Note, *Heterodera sojae* (Kang et al. 2016), described as a parasite of soybean in Korea, could not be included in the key because no information on the bullae and underbridge in cysts or incisures in the lateral fields were provided in the original description. This species occurs on soybean and should be compared directly to *H. glycines* if using morphological identification; it can also be identified using molecular data.

1-	Cyst without fenestration (No openings around vulva)	2
-	Cyst bifenestrate	7
-	Cyst ambifenestrate	24
2-	Female vulval cone absent	*H. bamboosi*
-	Female vulval cone present	3
3-	Vulval lips (vulval bridge narrow) of female close together, vulval slit slightly sunken, J2 with three incisures	4
-	Vulval lips of female widely separated (vulval bridge broad), vulval slit deeply sunken, J2 with four incisures	5
4-	Stylet length of J2 > 20 µm, knobs with flat anterior surface	*H. axonopi*
-	Stylet length of J2 ≤ 20 µm, knobs rounded	80
5-	Underbridge present, J2 with three or four labial annuli *(labial region refers to the offset 'head' portion of the anterior of the nematode)* *(annuli are circumferential ridges occurring along the entire nematode body)*	*H. saccharophila*
-	Underbridge absent, J2 with two labial annuli	6
6-	Male absent. Cuticular patterns of vulval cone with tuberculate cuticular ridges *(tuberculate cuticular ridges refers to distinct protrusions seen at either end of the vulval slit of Heterodera orientalis)*	*H. orientalis*
-	Male present. Cuticular patterns of vulval cone without ornamentation	*H. africana*
7-	Vulval slit < 25 µm	8
-	Vulval slit ≥ 25 µm	21
8-	Semifenestrae widely separated (average vulval bridge width > 12 µm) *(fenestrae refer to both openings, semifenestrae refer to individual openings)*	9

-	Semifenestrae not widely separated (average vulval bridge width ≤ 12 µm)	11
9-	Bullae surrounding vagina as an almost regular ring, no underbridge, means of tail in J2 < 40 µm, stylet ≤ 20 µm	*H. turcomanica*
-	Bullae few or absent, strong underbridge, means of tail in J2 ≥ 40 µm, stylet > 20 µm	10
10-	Vulval slit < 15 µm	*H. latipons*
-	Vulval slit > 15 µm	*H. hordecalis*
11-	Cyst spherical, with very low vulval cone and distinct underbridge in the configuration of a 'bow-tie', juvenile tail terminus sharply pointed	*H. spinicauda*
-	Cyst lemon-shaped, with distinct vulval cone and no bowtie-shaped underbridge; juvenile tail not sharply pointed	12
12-	J2 with three incisures	*H. bifenestra*
-	J2 with four incisures	13
13-	Underbridge present	14
-	Underbridge absent or if present it is slender	15
14-	J2 with average tail length ≥ 80 µm and hyaline region ≥ 50 µm	*H. ustinovi*
-	J2 with average tail length < 80 µm and hyaline region < 50 µm	*H. filipjevi*
15-	J2 with average body length > 585 µm and hyaline region > 48 µm	*H. arenaria*
-	J2 with average body length ≤ 585 µm and hyaline region ≤ 48 µm	16
16-	Average fenestral length ≤ 42 µm, J2 with average body length < 460 µm	*H. riparia*
-	Average fenestral length > 45 µm, J2 with average body length ≥ 460 µm	17
17-	Stylet knobs flat or moderately concave anteriorly in J2	18
-	Stylet knobs deeply concave anteriorly in J2	*H. mani*
18-	Average tail length usually < 69 µm in J2, parasitising only grasses	*H. pratensis*
-	Average tail length usually < 69 µm in J2, parasitising only cereals	*H. sturhani*
-	Average tail length usually ≥ 69 µm in J2, parasitising cereals or grasses	19
19-	Average cyst length ≤ 690 µm and J2 body length ≤ 508 µm	*H. aucklandica*
-	Average cyst length > 690 µm and J2 body length > 508 µm	20
20-	Average stylet of J2 = 26–28 µm	*H. avenae*
-	Average stylet of J2 = 25–26 µm	*H. australis*

21-	Average body length ≤ 460 µm and average tail ≤ 53 µm in J2	22
-	Average body length > 460 µm and average tail > 53 µm in J2	*H. litoralis*
22-	Average tail > 47 µm and average hyaline region > 23 µm in J2	23
-	Average tail ≤ 47 µm and average hyaline region ≤ 23 µm in J2	*H. ripae*
23-	Average fenestral length ≥ 48 µm and L/W cyst ratio *ca* 1.4	*H. humuli*
-	Average fenestral length 46 µm and L/W cyst ratio *ca* 1.1	*H. vallicola*
-	Average fenestral length < 46 µm and average L/W cyst ratio 1.3	*H. amaranthusiae*
24-	J2 with four incisures (aka lateral lines) in lateral field *(Incisures, or lateral lines, are lines on the outside of the cuticle, which run longitudinally throughout the length of the nematode body)*	42
-	J2 with three incisures in lateral field	25
25-	Underbridge with dorsoventral finger-like projections	26
-	Underbridge without dorsoventral finger-like projections	28
26-	Average hyaline region < 34 µm in J2	*H. sacchari*
-	Average hyaline region ≥ 34 µm in J2	27
27-	Average fenestral length ≤ 55 µm; cyst length > 700 µm	*H. leuceilyma*
-	Average fenestral length > 55 µm; cyst length > 700 µm	*H. goldeni*
-	Average fenestral length 40–65 µm; cyst length < 700 µm	*H. fengi*
28-	Bullae present	29
-	Bullae absent	34
29-	Average fenestral length > 45 µm	30
-	Average fenestral length ≤ 45 µm	31
30-	Body length = 461 (420–525) µm and average hyaline region = 31 (25–35) µm in J2	*H. sorghi*
-	Body length = 552 (508–591) µm and average hyaline region = 37.5 (30–43) µm in J2	*H. sinensis*
31-	Underbridge strongly developed	32
-	Underbridge weakly developed	33
32-	Average stylet > 21 µm, average hyaline region < 32 µm	*H. gambiensis*
-	Average stylet ≤ 21 µm, average hyaline region ≥ 32 µm	*H. oryzae*

33-	Fenestra round, average fenestral length = 29–40 µm, few bullae, average J2 body length = 367–402 µm	*H. elachista*
-	Fenestra oval, average fenestral length = 27–32 µm, many bullae, average J2 body length = 392–440 µm	*H. oryzicola*
34-	Cysts spherical but with distinct vulval cone, average L/W ratio = 1.05; strongly developed underbridge; tail = 115 (110–120) µm, hyaline region = 60 (55–65) µm in J2	*H. canadensis*
-	Cysts lemon-shaped to elongate, average L/W ratio = 1.3–1.9; underbridge present but weak or rare in some species; tail < 75 µm, hyaline region < 40 µm in J2	35
35-	Average J2 length usually ≤ 395 µm, knob-like structures usually present at ends of vulval bridge	36
-	Average J2 length usually > 395 µm, knob-like structures absent at ends of vulval bridge	39
36-	Average stylet of J2 *ca* 18 µm	37
-	Average stylet of J2 > 18 µm	38
37-	Average body length for male = 784 µm, spicules = 25–28 µm	*H. longicolla*
-	Average body length for male = 844 µm, spicules = 29–32 µm	*H. phragmitidis*
38-	Underbridge strongly developed	79
-	Underbridge weakly developed	*H. guangdongensis*
39-	Underbridge with a subcircular mass attached to its centre	*H. delvii*
-	Underbridge without a subcircular mass attached to its centre	40
40-	Labial region (offset portion of anterior body) of J2 with three annuli and average tail > 67 µm	*H. pakistanensis*
-	Labial region of J2 with four to five annuli and average tail ≤ 67 µm	41
41-	Average fenestral length *ca* 37 µm and vulval slit *ca* 31 µm	*H. cyperi*
-	Average fenestral length *ca* 50 µm and vulval slit *ca* 45 µm	*H. graminophila*
42-	Bullae and underbridge prominent and well developed	43
-	Bullae and underbridge weak or absent	60
43-	Average J2 tail > 59 µm	44
-	Average J2 tail ≤ 59 µm	49
44-	Average fenestral length ≤ 36 µm	*H. salixophila*
-	Average fenestral length > 36 µm	45
45-	Average underbridge length > 105 µm	46

-	Average underbridge length ≤ 105 µm	48
46-	Average J2 tail ≥ 69 µm	*H. betae*
-	Average J2 tail < 69 µm	47
47-	Average hyaline region < 38 µm in J2	*H. ciceri*
-	Average hyaline region ≥ 38 µm in J2	*H. rosii*
48-	Average hyaline region = 35–40 µm in J2, parasitising *Galeopsis*	*H. galeopsidis*
-	Average hyaline region = 32–37 µm in J2, parasitising *Trifolium*	*H. trifolii*
49-	Four finger-like bullae present immediately beneath underbridge, which is < 58 µm and not forked at ends	*H. zeae*
-	Four finger-like bullae absent immediately beneath underbridge, which is ≥ 58 µm and forked at ends	50
50-	Average tail ≥ 54 µm in J2	51
-	Average tail < 54 µm in J2	53
51-	J2 with average body length < 440 µm	*H. agrostis*
-	J2 with average body length ≥ 440 µm	52
52-	Average hyaline region = 30–33 µm in J2	*H. daverti*
-	Average hyaline region = 26–30 µm in J2	*H. lespedezae*
53-	Average stylet ≤ 23 µm in J2	54
-	Average stylet > 23 µm in J2	56
54-	Average vulval slit < 42 µm and two or three annuli in labial region of J2	55
-	Average vulval slit ≥ 42 µm and two or three annuli in labial region of J2	*H. glycines*
55-	Average distance from anterior end to excretory pore for J2 = 98 µm	*H. swarupi*
-	Average distance from anterior end to excretory pore for J2 = 81 µm	*H. spiraeae*
56-	J2 with average body length < 410µm and for hyaline region ≤ 22 µm	*H. mediterranea*
-	J2 with average body length > 411µm and for hyaline region > 22 µm	57
57-	Average cyst length < 700 µm	58
-	Average cyst length ≥ 700 µm	59
58-	Average length of hyaline region ≥ 29 µm in J2	*H. medicaginis*
-	Average length of hyaline region < 29 µm > 23 µm in J2	*H. cajani*

	Average length of hyaline region < 23 µm in J2	*H. dunensis*
59-	Average length of hyaline region > 27 µm and average tail > 50 µm in J2	*H. sonchophila*
-	Average length of hyaline region ≤ 27 µm and average tail ≤ 50 µm in J2	*H. schachtii*
60-	Average stylet ≤ 20 µm in J2	61
-	Average stylet > 20 µm in J2	64
61-	Average tail ≥ 60 µm in J2	62
-	Average tail < 60 µm in J2	63
62-	Bullae present, L/W ratio for cysts < 2.7	*H. mothi*
-	Bullae absent, L/W ratio for cysts ≥ 2.7	*H. raskii*
63-	J2 with average body length < 350µm and for hyaline region = 17 µm	*H. turangae*
-	J2 with average body length ≥ 350µm and for hyaline region = 25 µm	*H. skohensis*
64-	Average tail ≥ 57 µm in J2	65
-	Average tail < 57 µm in J2	67
65-	Average body length ≥ 500 µm in J2	*H. urticae*
-	Average body length < 500 µm in J2	66
66-	Average length of hyaline region ≥ 30 µm, underbridge length ≥ 70 µm	*H. goettingiana*
-	Average length of hyaline region < 30 µm, underbridge length < 70 µm	*H. plantaginis*
67-	Average length of hyaline region ≤ 17 µm	*H. bergeniae*
-	Average length of hyaline region > 17 µm	68
68-	Average body length ≥ 480 µm in J2	*H. amygdali*
-	Average body length < 480 µm in J2	69
69-	Bullae present	70
-	Bullae absent	72
70-	Average stylet ≥ 25 µm in J2	*H. glycyrrhizae*
-	Average stylet < 25 µm in J2	71
71-	Average body length *ca* 440 µm in J2 and average fenestral width *ca* 44 µm	*H. persica*
-	Average body length = 377-419 µm in J2 and average fenestral width = 25–37 µm	*H. fici*
72-	Average tail < 40 µm in J2	*H. menthae*
-	Average tail ≥ 40 µm in J2	73

73-	Average hyaline region usually ≥ 28 µm	*H. carotae*
-	Average hyaline region < 28 µm	74
74-	Average cyst length usually ≤ 530 µm	75
-	Average cyst length > 530 µm	76
75-	Average tail for J2 = 41–50 µm	*H. cruciferae*
-	Average tail for J2 = 54 µm	*H. uzbekistanica*
76-	L/W ratio for cyst usually *ca* 1.1	*H. johanseni*
-	L/W ratio for cyst usually *ca* 1.3–1.7	77
77-	Average fenestral length ≥ 50 µm	*H. kirjanovae*
-	Average fenestral length < 50 µm	78
78-	Stylet knobs concave anteriorly in J2	*H. scutellariae*
-	Stylet knobs rounded and sloping posteriorly in J2	*H. circeae*
79-	Average J2 length ≤ 388 µm, average hyaline region < 26 µm	*H. cardiolata*
-	Average J2 length > 388 µm, average hyaline region ≥ 26 µm	*H. graminis*
80-	Underbridge absent; vulval slit > 40 µm	*H. koreana*
-	Underbridge present; vulval slit ≤ 40 µm	*H. hainanensis*

5 CONTACTS FOR FURTHER INFORMATION

5.1 Primary contacts

Dr Daniel Huston
Insect and Nematode Biosecurity, Australian National Insect Collection, CSIRO, Canberra, ACT
Postal Address: GPO Box 1700, Canberra ACT 2601
Street Address: Clunies Ross Drive, Acton ACT 2601
Phone: +61 2 6218 3476, email: daniel.huston@csiro.au

Dr Mike Hodda
Insect and Nematode Biosecurity, Australian National Insect Collection, CSIRO, Canberra, ACT
Postal Address: GPO Box 1700, Canberra ACT 2601
Street Address: Clunies Ross Drive, Acton ACT 2601
Phone: 6246 4371, Fax: (02) 6246 4000, email: mike.hodda@csiro.au

Dr Danièle Giblot-Ducray
South Australian Research and Development Institute (SARDI), Department of Primary Industries and Regions
Address: SARDI Plant Research Centre, 2a Hartley Grove, Urrbrae, SA 5064
Phone: +61 8 8429 2229, email: Daniele.giblot-ducray@sa.gov.au

5.2 Jurisdictional contacts

The following individuals are diagnostic experts and participated in a training workshop led by the NDP authors focused on cyst nematodes and the use of this NDP:

Akshita Jain, Agriculture Victoria, Victoria (akshita.jain@agriculture.vic.gov.au)
Sarah Collins, DPIRD, Western Australia (Sarah.Collins@dpird.wa.gov.au)
Stefan Harasymow, DPIRD, Western Australia (Stefan.Harasymow@dpird.wa.gov.au)
Dylan Corner, QDAF, Queensland (Dylan.Corner@daf.qld.gov.au)
Samantha Bond, DITT, Northern Territory (Samantha.Bond@nt.gov.au)

6 ACKNOWLEDGEMENTS

We thank Dr Ian Riley and his team (Osameh, Mustafa, Eniola and Fakurdeen), Ellie Darling, Timothy Paulitz, Nuan Wen, Lester Núñez-Rodríguez, Eric Grenier, Hiromichi Sakai and Shun Yosano for providing exotic cyst nematode specimens. We thank John Lewis, Katherine Linsell, Sarah Collins, Andrea Hills, Stefan Harasymow, Akshita Jain, John Wainer, Simon Whittock and Oliver Hayes for assisting with domestic sampling. We thank Sarah Dunstan for her extensive efforts investigating *Heterodera graminis*.

The development of this NDP was supported by Grains Research and Development Corporation, through funding from the Australian Government Department of Agriculture, Fisheries & Forestry, as part of its Rural R&D for Profit program and along with Cotton Research and Development Corporation, Hort Innovation Australia, Wine Australia, Sugar Research Australia and Forest and Wood Products Australia.

Special additional thanks are due to Akshita Jain, Sarah Collins, Stefan Harasymow, Dylan Corner and Samantha Bond for participating in a workshop centred on the use and verification of this NDP document. Exceptional advice and constructive criticism were received during the workshop, which significantly improved the present NDP.

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8 APPENDICES (OPTIONAL)

Appendix 1. List of currently accepted species of *Heterodera*, including species grouping, agricultural impact, molecular barcoding sequence availability, and geographic regions where the species is known.

Species name	Species group	Agricultural impact	Barcodes available	Geographic Region
Heterodera africana	Afenestrata	Non-crop host		Kenya, South Africa, Tanzania
Heterodera agrostis	Schachtii	Non-crop host		Russia
Heterodera amaranthusiae	Humuli	Non-crop host	ITS, 28S, COI	China
Heterodera amygdali	Goettingiana	Nuts		Tajikistan
Heterodera arenaria	Avenae	Non-crop host	ITS, CO1	UK, Germany, Denmark, Netherlands, France, Italy
Heterodera aucklandica	Avenae	Non-crop host	ITS, CO1, 28S	New Zealand, Belgium, UK
Heterodera australis	Avenae	Cereals	ITS, CO1	Australia, China
Heterodera avenae	Avenae	Cereals	ITS, 18S, 28S	Belgium, Bulgaria, Czech R. , Denmark, Estonia, France, Germany, Greece, Ireland, Italy, Latvia, Malta, Netherlands, Norway, Poland, Portugal, Slovakia, Spain, Sweden, Switzerland, UK, Yugoslavia, China, India, Iran, Iraq, Israel, Japan, Pakistan, S. Arabia, Türkiye, Algeria, Libya, Morocco, South Africa, Tunisia, Canada, USA, Peru, New Zealand
Heterodera axonopi	Afenestrata	Non-crop host		Brazil
Heterodera bamboosi	Afenestrata	Non-crop host		India
Heterodera bergeniae	Goettingiana	Non-crop host		Pakistan
Heterodera betae	Schachtii	Sugar crops, Fruits and Vegetables	ITS, 18S, 28S, CO1	Belgium, France, Germany, Italy, Cotroneo, Lithuania, Netherlands, Sweden, Switzerland
Heterodera bifenestra	Bifenestra	Cereals	ITS, 18S	Belgium Bulgaria, Germany, Netherlands, Poland, Slovakia, Sweden, UK, Ukraine
Heterodera cajani	Schachtii	Pulses, Oilseeds	ITS, 28S	India, Pakistan, Egypt
Heterodera canadensis	Cyperi	Non-crop host		Canada

Heterodera cardiolata	Cardiolata	Non-crop host	CO1 (28S and ITS as *H. cynodontis*, jun. syn.)	India, Kazakhstan, Pakistan, Nepal, Tajikistan, Madzhidov, Uzbekistan, South Africa
Heterodera carotae	Goettingiana	Fruits and Vegetables	ITS, 18S, 28S, CO1	Denmark, France, Germany, Hungary, Italy, Ireland, Netherlands, Portugal, Poland, Russia, Serbia, Slovenia, Slovakia, Sweden, Switzerland, UK, USA, Cyprus, South Africa
Heterodera ciceri	Schachtii	Pulses	ITS, CO1	Jordan, Lebanon, Syria, Türkiye
Heterodera circeae	Goettingiana	Non-crop host	ITS	Germany
Heterodera cruciferae	Goettingiana	Fruits and Vegetables	ITS, 18S, 28S, CO1	Belgium, Bulgaria, France, Germany, Hungary, Ireland, Italy, Latvia, Lithuania, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, UK, Ukraine, F. Yugoslavia, Armenia, Azerbaijan, Iran, Pakistan, Türkiye, Libya, USA, Australia
Heterodera cyperi	Cyperi	Non-crop host	ITS, CO1	USA, Spain
Heterodera daverti	Schachtii	Pulses	ITS, 28S, CO1	France, Germany, Italy, Netherlands, UK, Egypt, Tunisia, Australia
Heterodera delvii	Cyperi	Cereals		India
Heterodera dunensis	Schachtii	Non-crop host	ITS, CO1, 18S, 28S	Gran Canaria, Canary Islands, Spain
Heterodera elachista	Cyperi	Cereals	ITS, 18S, 28S, CO1	Japan, Iran, China
Heterodera fengi	Cyperi	Non-crop host	ITS, 28S	China
Heterodera fici	Humuli	Fruits and Vegetables	ITS, 18S, 28S, CO1	Belarus, Belgium, Estonia, France, Germany, Greece, Hungary, Italy, Netherlands, Norway, Poland, Portugal, Russia, Spain, F. Yugoslavia, China, Georgia, Iran, Türkiye, Uzbekistan, Australia, New Zealand, USA, Brazil, Algeria, South Africa
Heterodera filipjevi	Avenae	Cereals	ITS, CO1, 28S, 18S	Belarus, Bulgaria, Estonia, Germany, Italy, Latvia, Norway, Poland, Russia, Spain, Sweden, UK, Ukraine, China, India, Kazakhstan, Syria, Tajikistan, Türkiye, Uzbekistan, Iran, USA
Heterodera galeopsidis	Schachtii	Non-crop host	ITS, CO1	Bulgaria, Denmark, Estonia, Germany, Hungary, Latvia, Lithuania, Netherlands, Norway, Poland, Russia, Slovenia, Sweden, UK, Ukraine, Kazakhstan
Heterodera gambiensis	Sacchari	Cereals		Gambia, Niger, India

Heterodera glycines	Schachtii	Oilseeds, Fruits and Vegetables	ITS, 18S, 28S, CO1	Italy, USA, Egypt, Argentina, Brazil, Chile, Colombia, Ecuador, Paraguay, China, India, Indonesia, Iran, Japan, Mongolia, North Korea, Russia, South Korea
Heterodera glycyrrhizae	Goettingiana	Pulses		Uzbekistan
Heterodera goettingiana	Goettingiana	Pulses	ITS, 18S, 28S, CO1	Belgium, Bulgaria, Denmark, France, Germany, Hungary, Ireland, Italy, Latvia, Malta, Netherlands, Poland, Portugal, Russia, Serbia, Slovenia, Spain, UK, Ukraine, Algeria, Libya, Morocco, Tunisia, China, Iran, Israel, Japan, Jordan, Türkiye, USA
Heterodera goldeni	Sacchari	Cereals	ITS	Egypt, Iran, Israel
Heterodera graminis	Cardiolata	Non-crop host	ITS	Australia
Heterodera graminophila	Cyperi	Cereals	18S	USA
Heterodera guangdongensis	Cyperi	Non-crop host	ITS, COI, 28S	China
Heterodera hainanensis	Afenestrata	Non-crop host	ITS, 28S	China
Heterodera hordecalis	Avenae	Cereals	ITS, 18S, 28S, CO1	Belgium, Denmark, Estonia, France, Germany, Greece, Italy, Netherlands, Poland, Slovakia, Sweden, Ukraine, Tunisia, Iran, Israel, Kazakhstan, Turkmenistan, Uzbekistan
Heterodera humuli	Humuli	Non-crop host	ITS, 28S, CO1	Belgium, Bulgaria, Czech R., Germany, Greece, Italy, Netherlands, Poland, Russia, Slovakia, Slovenia, Spain, Switzerland, UK, Ukraine, F. Yugoslavia, Armenia, China, Japan, Israel, Iran, Canada, Mexica, USA, South Africa, Australia, New Zealand
Heterodera johanseni	Goettingiana	Non-crop host Fruits and Vegetables, Cereals		Nepal
Heterodera kirjanovae	Goettingiana	Non-crop host		Uzbekistan
Heterodera koreana	Afenestrata	Non-crop host	ITS, 18S, 28S, CO1	South Korea, Thailand, USA
Heterodera latipons	Avenae	Cereals	ITS, 28S, CO1	Bulgaria, Cyprus, Czech Republic, Estonia, France, Greece, Italy, Russia, Spain, UK, Ukraine, Armenia, Iran, Israel, Japan, Jordan, Kazakhstan, Lebanon, Syria, Tajikstan, Türkiye, Turkmenistan, Uzbekistan, Algeria, Libya, Tunisia, Canada

Heterodera lespedezae	Schachtii	Sugar crops, Oilseeds		USA
Heterodera leuceilyma	Sacchari	Non-crop host		USA
Heterodera litoralis	Humuli	Non-crop host	ITS, 28S	New Zealand
Heterodera longicolla	Cyperi	Non-crop host		USA- Kansas
Heterodera mani	Avenae	Non-crop host	ITS, CO1, 18S	Belgium, Estonia, France, Germany, Netherlands, Slovakia, UK, South Africa, USA, Australia
Heterodera medicaginis	Schachtii	Non-crop host	ITS, COI, 28S	Russia, Ukraine, Kazakhstan, Uzbekistan
Heterodera mediterranea	Schachtii	Oilseeds, Nuts	ITS, 18S, 28S	Italy, Spain
Heterodera menthae	Goettingiana	Non-crop host		Uzbekistan
Heterodera microulae	Goettingiana	Non-crop host	ITS, COI, 28S	
Heterodera mothi	Cyperi	Sugar crops, Fruits and Vegetables	ITS, CO1	India, Iran, Iraq, Kazakhstan, Nepal, Pakistan, USA
Heterodera orientalis	Afenestrata	Non-crop host	ITS, 28S	Russia
Heterodera oryzae	Cyperi	Cereals, Fruits and Vegetables	ITS, COI, 28S	Bangladesh, India, Iran, Pakistan, Cameroon, Gambia, Liberia, Senegal
Heterodera oryzicola	Cyperi	Cereals, Fruits and Vegetables	ITS, 28S	India
Heterodera pakistanensis	Cyperi	Cereals		Pakistan
Heterodera persica	Goettingiana	Non-crop host	ITS	Iran
Heterodera phragmitidis	Cardiolata	Cereals		Russia
Heterodera plantaginis	Goettingiana	Non-crop host		Uzbekistan
Heterodera pratensis	Avenae	Non-crop host	ITS, COI	Germany, Russia, Estonia, Netherlands
Heterodera raskii	Cyperi	Non-crop host		India
Heterodera ripae	Humuli	Non-crop host	ITS	Russia, Estonia, Latvia, Armenia, Moldova, Ukraine, Uzbekistan, Bulgaria, Germany, Belgium, Greece, Sweden
Heterodera riparia	Avenae	Non-crop host		Russia
Heterodera rosii	Schachtii	Non-crop host		Ireland
Heterodera sacchari	Sacchari	Sugar crops	ITS	Benin, Burkina Faso, Cameroon, Chad, Congo, Gambia, Ghana, Guinea, Liberia, Nigeria, Senegal, Togo, India, Pakistan, Trinidad

Heterodera saccharophila	Afenestrata	Non-crop host		India
Heterodera salixophila	Humuli	Non-crop host	ITS, 28S	Belgium, Estonia, Germany, Poland, Russia, Slovakia, Ukraine
Heterodera schachtii	Schachtii	Sugar crops, Fruits and Vegetables	ITS, 18S, 28S, CO1	Albania, Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Moldova, Netherlands, Poland, Portugal, Romania, Russia, Slovakia, Slovenia, Lithuania, Spain, Sweden, Switzerland, UK, Ukraine, Yugoslavia (former), Armenia, Azerbaijan, China, India, Iran, Iraq, Israel, Jordan, Kazakhstan, Kyrgyzstan, Pakistan, South Korea, Syria, Tajikistan, Türkiye, Uzbekistan, Algeria, Cape Verde, Gambia, Libya, Morocco, Senegal, South Africa, Tunisia, Canada, Mexico, USA, Chile, Peru, Uruguay, Australia, New Zealand
Heterodera scutellariae	Goettingiana	Non-crop host	ITS	Germany
Heterodera sinensis	Sacchari	Non-crop host	ITS	China
Heterodera skohensis	Cyperi	Cereals	ITS	India
Heterodera sojae	Cyperi	Oilseeds	ITS, COI, 28S	South Korea
Heterodera sonchophila	Schachtii	Non-crop host	ITS, CO1	Estonia, Latvia, Poland, Russia
Heterodera sorghi	Sacchari	Cereals	ITS, 28S	India
Heterodera spinicauda		Non-crop host		Netherlands
Heterodera spiraeae	Schachtii	Non-crop host		Russia
Heterodera sturhani	Avenae	Cereals	CO1	China
Heterodera swarupi	Schachtii	Pulses		India
Heterodera trifolii	Schachtii	Pulses, Sugar crops, Oilseeds, Fruits and Vegetables	ITS, 18S, 28S, CO1	Belgium, Bulgaria, Belarus, Denmark, France, Finland, Germany, Hungary, Ireland, Italy, Latvia, Lithuania, Netherlands, Norway, Poland, Portugal, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, UK, Ukraine, India, Iran, Israel, Japan, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan, Egypt, South Africa, Tunisia, Canada, UK, Argentina, Chile, Colombia, Venezuela, Australia, New Zealand
Heterodera turangae		Non-crop host		Uzbekistan
Heterodera turcomanica	Humuli	Non-crop host	ITS	Turkmenistan and Iran
Heterodera urticae	Goettingiana	Non-crop host	ITS, 28S	Belgium, Germany, Slovakia, Spain, UK
Heterodera ustinovi	Avenae	Non-crop host	ITS, CO1	Belarus, Belgium, Estonia, Germany, Lithuania, Poland, Russia, Slovakia, UK, Ukraine, USA

Heterodera uzbekistanica	Goettingiana	Non-crop host		Uzbekistan
Heterodera vallicola	Humuli	Non-crop host	ITS	Russia
Heterodera zeae	Zeae	Cereals, Sugar crops	ITS, 18S, 28S, CO1	India, Nepal, Pakistan, Thailand, Egypt, USA, Greece, Portugal

Appendix 2. List of actually or potentially erroneous sequences of species of the genus *Heterodera* available on GenBank as flagged by Huston et al. (2020). Additional information regarding each sequence is available in the database uploaded to the CSIRO data portal (https://doi.org/10.25919/2bqn-ya80)

GB accession	GenBank sequence species label	Gene region	Notes
MK093059	*Heterodera avenae*	COI	Unreliable sequence; Likely contamination
MZ501493	*Heterodera avenae*	COI	Misidentification
MZ501494	*Heterodera avenae*	COI	Misidentification
MZ501495	*Heterodera avenae*	COI	Misidentification
MZ501496	*Heterodera avenae*	COI	Misidentification
KC172915	*Heterodera daverti*	COI	Unreliable, contamination or poor sequencing results
MH144207	*Heterodera elachista*	COI	Unreliable, lacking close congeners
HM462016	*Heterodera cardiolata*	COI	Unreliable; potentially another region of mitochondria
EU040127	*Heterodera avenae*	18S	Unreliable sequence; base call errors likely
MG550969	*Heterodera avenae*	18S	Unreliable. Inaccurate base calls
AY566814	*Heterodera avenae*	18S	Problematic; short sequence leading to multiple 100% matches
KF241988	*Heterodera glycines*	18S	Unreliable sequence; unexplained insertion
FJ040405	*Heterodera hordecalis*	18S	Unreliable sequence; probable misidentification
AY912045	*Heterodera cf. graminophila*	18S	Unreliable; possible misidentification or contamination
EU040134	*Heterodera schachtii*	18S	Species mislabel
HG738844	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
LT159843	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
LT159844	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068512	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068514	*Heterodera avenae*	ITS	Poor quality; multiple base call errors

HM068515	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068516	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068517	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068518	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068519	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068520	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068521	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068522	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068523	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068524	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068525	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068526	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068527	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068528	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068529	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068531	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068533	*Heterodera avenae*	ITS	Uploaded backwards; OK but requires reverse complementing
HM068534	*Heterodera avenae*	ITS	Poor-quality sequence with multiple base call errors
KF679395	*Heterodera avenae*	ITS	Species Mislabel
KY977413	*Heterodera avenae*	ITS	Poor-quality sequence with multiple base call errors
MK840646	*Heterodera avenae*	ITS	Unreliable. Base call errors? ~20
KX463294	*Heterodera carotae*	ITS	Uploaded backwards; OK but requires reverse complementing
AY045754	*Heterodera carotae*	ITS	Unreliable. Base call errors? ~20
LT996913	*Heterodera fici*	ITS	Uploaded backwards; OK but requires reverse complementing
HG738845	*Heterodera filipjevi*	ITS	Species Mislabel
MG836298	*Heterodera glycines*	ITS	Unreliable. Base call errors ~30
LT159842	*Heterodera hordecalis*	ITS	Uploaded backwards; OK but requires reverse complementing
AY045756	*Heterodera hordecalis*	ITS	Species mislabel
LT159822	*Heterodera avenae*	28S	Unreliable sequence; Probably base call errors
LT159823	*Heterodera avenae*	28S	Unreliable sequence; Probably base call errors
JX402414	*Heterodera cruciferae*	28S	Unreliable; possible mis-id or contamination w/ H. schachtii

HM560851	*Heterodera glycines*	28S	Unreliable sequence; Mis-ID
MH032764	*Heterodera goettingiana*	28S	Unreliable sequence; Mis-ID
FJ151164	*Heterodera latipons*	28S	Unreliable sequence; contamination
FJ151170	*Heterodera latipons*	28S	Unreliable sequence; contamination
AF133304	*Heterodera glycines*	28S	Uploaded backwards; OK but requires reverse complementing

Appendix 3. Morphological characteristics of different *Heterodera* species groups. From Subbotin et al. (2018).

Characters	**Cyst Shape**	**Cyst Fenestration**	**Cyst Bullae**	**Cyst Underbridge**	**Cyst Vulval slit**	**J2 Lateral field**
Group						
Afenestrata	Lemon or rounded	Absent	Absent	Absent or weak	Long	3 or 4
Avenae	Lemon	Bifenenstrate	Well developed	Absent or present	Short	4
Bifenestra	Lemon	Bifenestrate	Absent	Absent	Short	3
Cardiolata	Lemon	Ambifenestrate	Absent	Present	Long	3
Cyperi	Lemon or rounded	Ambifenestrate	Absent or present	Absent or present	Long	3 or 4
Goettingiana	Lemon	Ambifenestrate	Absent or present	Weak	Long	4
Humuli	Lemon	Bifenenstrate (except for *H. fici* - ambifenestrate)	Absent or small	Weak	Long	4
Sacchari	Lemon or rounded	Ambifenestrate	Finger-like	Strong	Long	3
Schachtii	Lemon	Ambifenestrate	Well developed	Strong	Long	4

Appendix 4. Alternative PCR profiles for amplifying the cox1, ITS and 28S barcode regions for *Heterodera* spp.

Profile 1 (Amplifies the ITS region): Initial denaturation of 4 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min 30 s at 55 °C, and 2 min at 72 °C, followed by an extension step of 10 min at 72 °C. From Subbotin et al. (2018).

Profile 2 (Amplifies the ITS and 28S regions): Initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s and extension at 72 °C for 1 min and a final step at 72 °C for 7 min. From Tirchi et al. (2016).

Profile 3 (Amplifies the ITS and 28S regions): Initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C (ITS) or 60 °C (D2-D3) for 30 s, and extension at 72 °C for 1 min. From Sekimoto et al. (2017).

Profile 4 (Amplifies the cox1 region): Initial denaturation of 4 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 45 °C, and 1 min 30 s at 72 °C, with a final extension at 72 °C for 10 min. From Subbotin et al. (2018).

Profile 5. (Amplifies the cox1 region): Initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 46 °C for 15 s, and extension at 72 °C for 30 s. From Sekimoto et al. (2017).

Appendix 5. Species pairs of *Heterodera* which are inadequately or poorly distinguished from one another using one or more of the standard molecular markers evaluated (18S, ITS, 28S, *cox*1), including minimum base pair differences observed between sequences of problematic species pairs. From Huston et al. (2022).

Species group	Species pair	Gene region	Minimum base pair difference	Delineation power	Notes
Avenae	*H. avenae - H. filipjevi*	18S	1	inadequate	
Avenae	*H. avenae - H. hordecalis*	18S	4	weak	*H. hordecalis* not monophyletic in NJ/ML analyses; potentially distinguishable from closely related species in isolated analyses.
Avenae	*H. avenae - H. mani*	18S	1	inadequate	
Avenae	*H. filipjevi - H. hordecalis*	18S	1	inadequate	
Avenae	*H. filipjevi - H. mani*	18S	2	inadequate	
Avenae	*H. hordecalis - H. mani*	18S	3	weak	See above note.
Avenae	*H. arenaria - H. avenae*	ITS	0	inadequate	
Avenae	*H. avenae - H. pratensis*	ITS	0	inadequate	
Avenae	*H. avenae - H. australis*	ITS	2	weak	Although only two bp different, sequences of *H. australis* form clade with some sequences of '*H. avenae*' which have been shown to be *H. australis* (see Subbotin et al., 2018).
Avenae	*H. avenae - H. mani*	ITS	2	weak	Despite only two bp difference, sequences of *H. mani* form monophyletic clade to exclusion of other closely related sequences.
Avenae	*H. aucklandica - H. avenae*	28S	1	inadequate	Only one 28S sequence of *H. aucklandica* available for comparison.
Avenae	*H. aucklandica - H. hordecalis*	28S	1	inadequate	Two 28S sequences of *H. hordecalis* 9 bp different
Avenae	*H. aucklandica - H. pratensis*	28S	1	inadequate	Only one 28S sequence of *H. pratensis* available for comparison.
Avenae	*H. avenae - H. pratensis*	28S	0	inadequate	See above note.
Avenae	*H. avenae - H. hordecalis*	28S	0	inadequate	Two 28S sequences of *H. hordecalis* 9 bp different
Avenae	*H. arenaria - H. avenae*	*cox*1	2	weak	Despite only two bp difference, *H. arenaria* forms monophyletic clade within *H. avenae* group.
Cyperi	*H. elachista - H. oryzae*	ITS	1	inadequate	
Cyperi	*H. elachista - H. oryzae*	28S	0	inadequate	

Goettingiana	*H. carotae - H. cruciferae*	ITS	1	inadequate	
Goettingiana	*H. goettingiana - H. microulae*	28S	0	inadequate	Two 28S sequences of *H. goettingiana* are 19 bp different from one another; one potentially misidentified
Goettingiana	*H. carotae - H. urticae*	28S	2	weak	Only one 28S sequence of *H. urticae* available for comparison.
Goettingiana	*H. carotae - H. cruciferae*	28S	2	weak	Only one 28S sequence of *H. cruciferae* available for comparison (two on GenBank but one appears to actually be *H. schachtii*).
Goettingiana	*H. carotae - H. cruciferae*	*cox*1	1	inadequate	Three *cox*1 sequences available for *H. cruciferae*, one 15 bp different from others.
Schachtii	*H. betae - H. glycines*	18S	2	weak	*H. glycines* forms monophyletic clade to exclusion of *H. betae*, *H. schachtii* & *H. trifolii* in ML, but not NJ, analyses.
Schachtii	*H. betae - H. schachtii*	18S	0	inadequate	
Schachtii	*H. betae - H. trifolii*	18S	0	inadequate	
Schachtii	*H. schachtii - H. glycines*	18S	2	weak	See above note.
Schachtii	*H. schachtii - H. trifolii*	18S	0	inadequate	
Schachtii	*H. trifolii - H. glycines*	18S	2	weak	See above note.
Schachtii	*H. betae - H. daverti*	ITS	0	inadequate	
Schachtii	*H. betae - H. schachtii*	ITS	0	inadequate	
Schachtii	*H. betae - H. trifolii*	ITS	0	inadequate	
Schachtii	*H. ciceri - H. schachtii*	ITS	2	inadequate	
Schachtii	*H. ciceri - H. trifolii*	ITS	1	inadequate	
Schachtii	*H. daverti - H. schacthii*	ITS	0	inadequate	
Schachtii	*H. daverti - H. trifolii*	ITS	0	inadequate	
Schachtii	*H. glycines - H. medicaginis*	ITS	1	inadequate	
Schachtii	*H. schachtii - H. trifolii*	ITS	0	inadequate	
Schachtii	*H. betae - H. daverti*	28S	1	inadequate	
Schachtii	*H. betae - H. schachtii*	28S	2	inadequate	
Schachtii	*H. betae - H. trifolii*	28S	1	inadequate	
Schachtii	*H. daverti - H. schachtii*	28S	3	weak	
Schachtii	*H. daverti - H. trifolii*	28S	1	inadequate	
Schachtii	*H. schachtii - H. trifolii*	28S	1	inadequate	

Appendix 6. Morphological imagery to aid in morphological study and identification

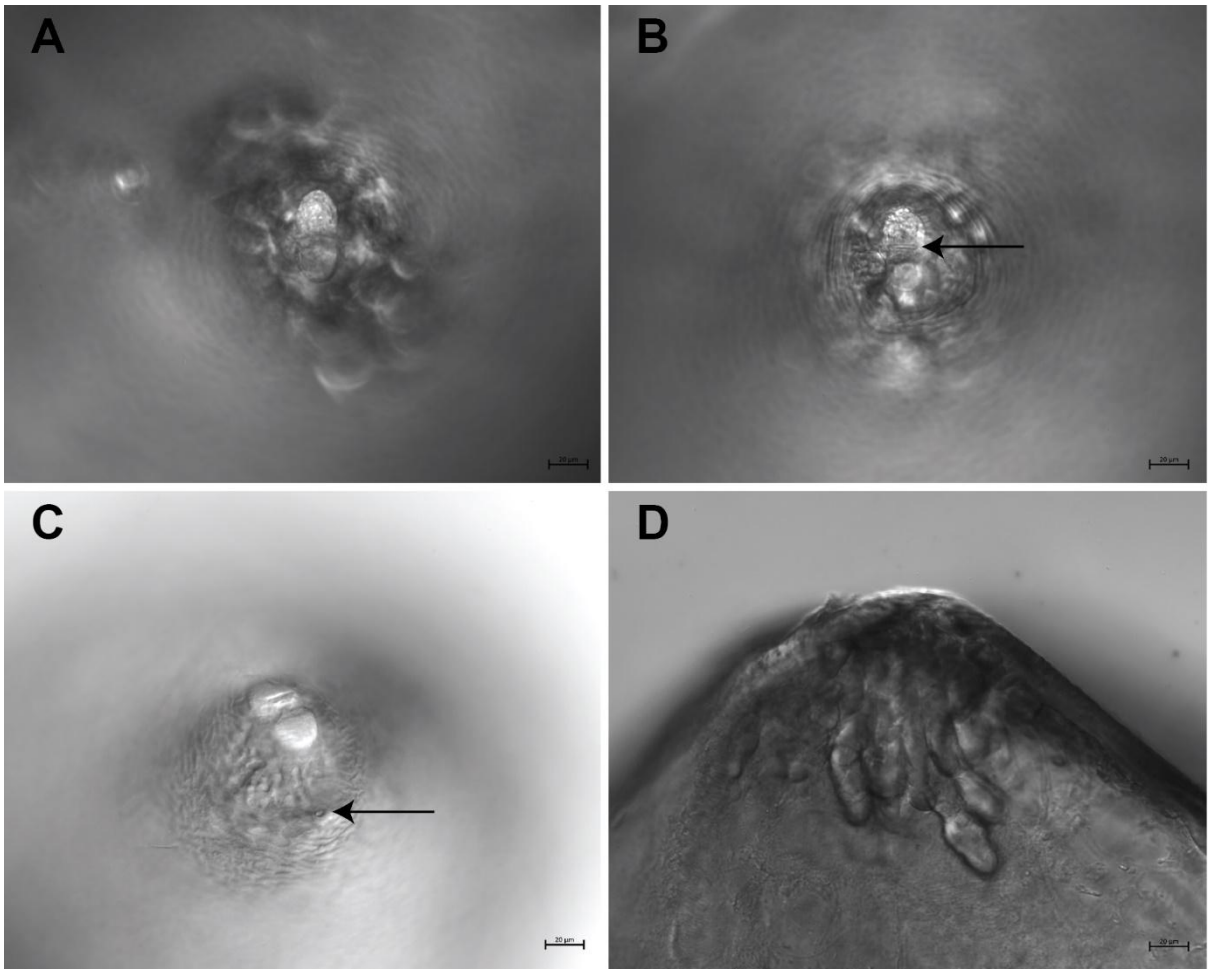

Figure A6.1. Vulval cones of *Heterodera australis*. A, Surface view of vulval plate showing fenestrae. B, surface view of vulval plate showing fenestrae, vulval slit indicated by arrow. C, surface view of vulval plate showing fenestrae, anus indicated by arrow (note: the anus is often difficult to observe, or obscured, in many vulval cone mounts). D, Lateral view of vulval cone showing strongly-developed bullae. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

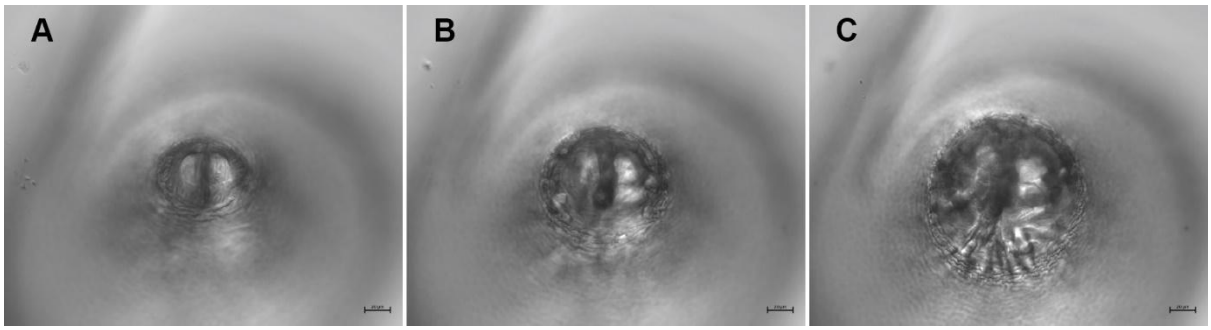

Figure A6.2. Vulval cone of *Heterodera daverti* viewed under light microscopy. Depth of view increasing from A-C. A, view of surface of vulval plate showing fenestrae and vulval bridge. B, increased depth showing underbridge. C, further increase in depth showing bullae. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

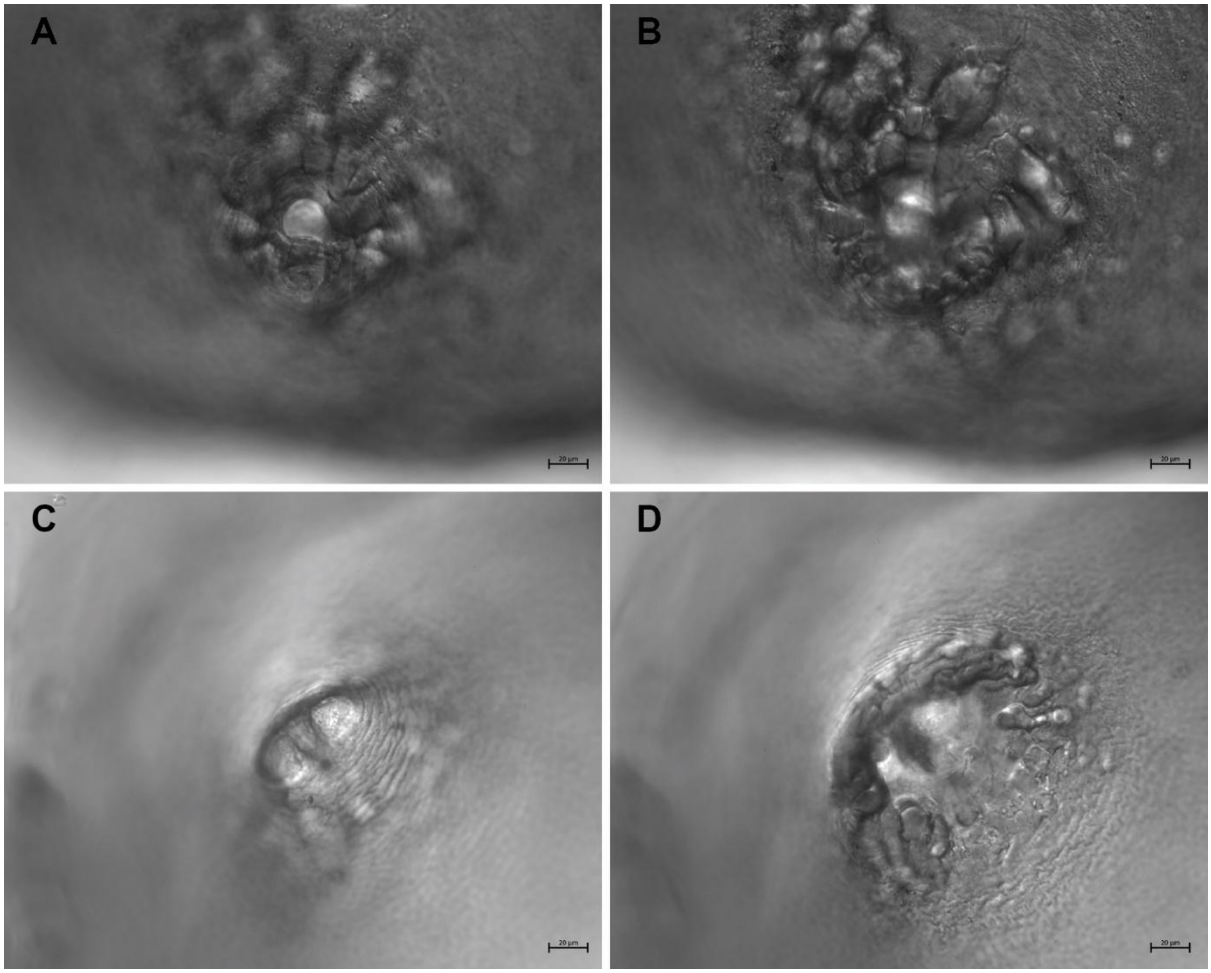

Figure A6.3. Vulval plates of *Heterodera avenae* (A, B) and *Heterodera filipjevi* (C, D). B and D are deeper views of A and C, respectively. Note the lack of underbridge and presence of strong bullae visible in B and D, a characteristic of members of the *Avenae* species group. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

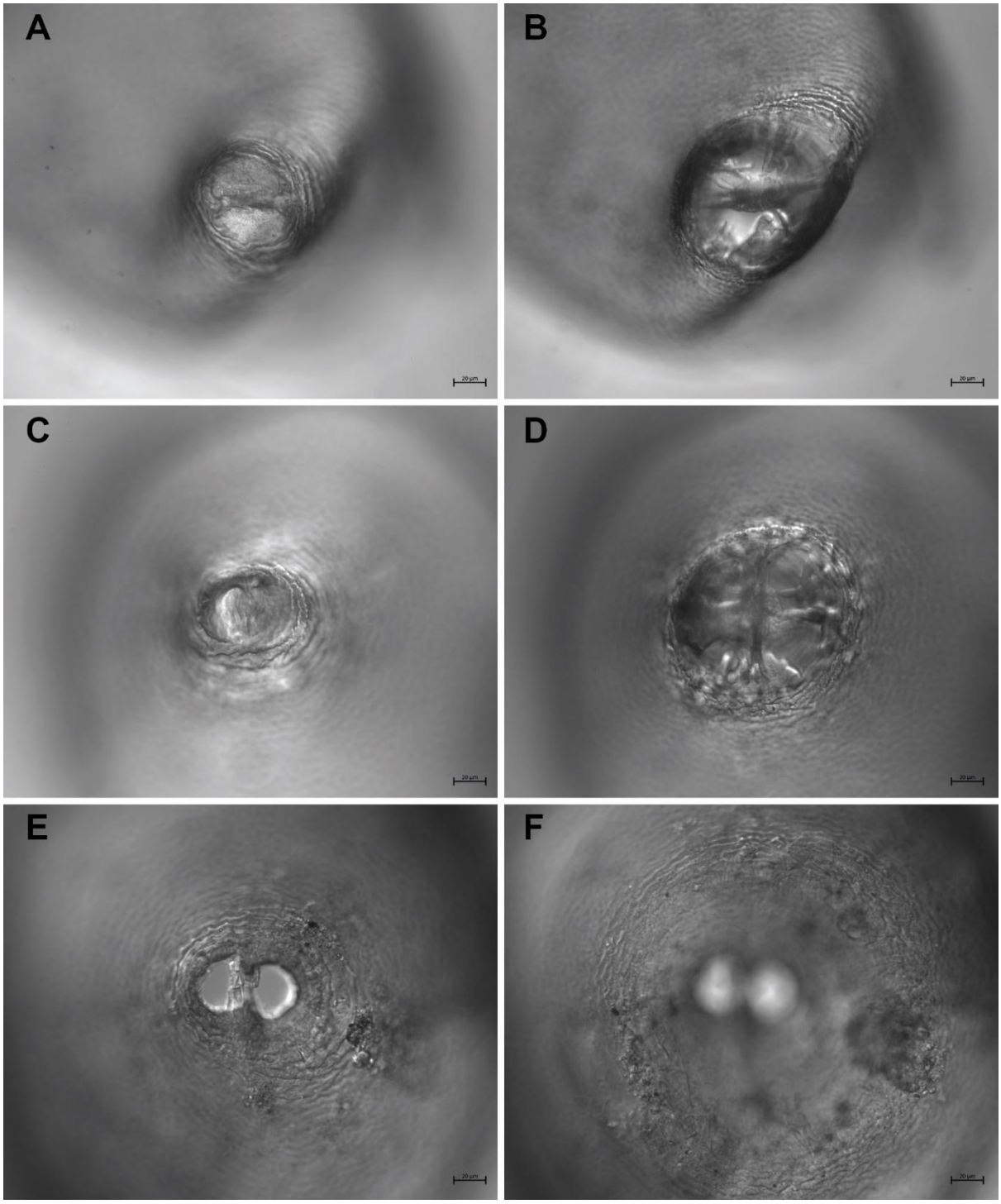

Figure A6.4. Vulval plates of *Heterodera glycines* (A, B), *Heterodera schachtii* (C, D) and *Heterodera humuli* (E, F). B, D, and F are deeper view of A, C and E, respectively. *Heterodera glycines* and *Heterodera schachtii* are both in the *Schachtii* species group, *Heterodera humuli* is in the *Humuli* species group. Note the strong underbridge and bullae present in *H. glycines* and *H. schachtii* vs the barely discernible underbridge and lack of bullae in *H. humuli*. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

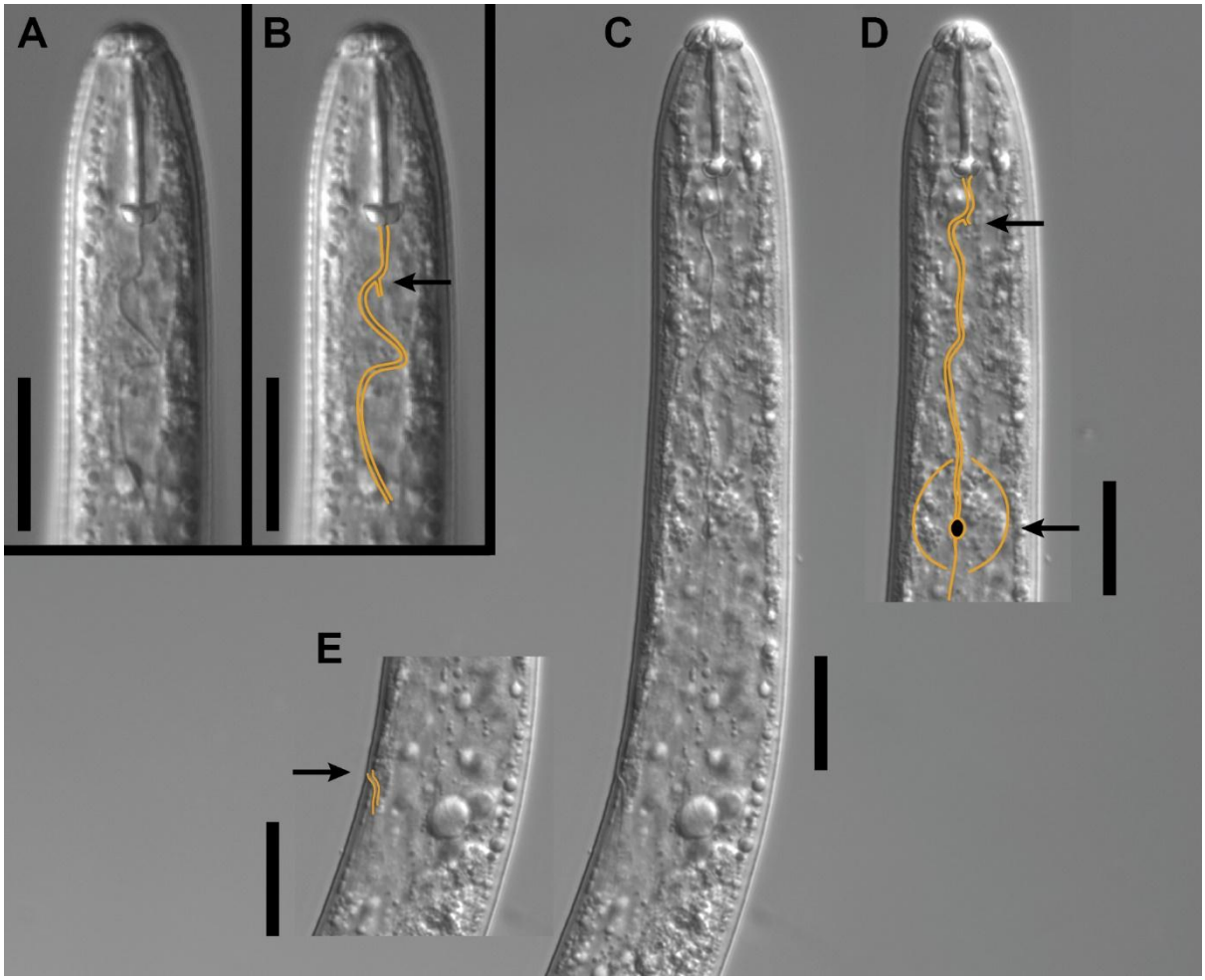

Figure A6.5. J2s of *Heterodera australis* with some difficult to view features highlighted. A, B, oesophagus highlighted with dorsal gland orifice indicated with arrow. D, oesophagus highlighted showing dorsal gland orifice (upper arrow) and median bulb (lower arrow). E, excretory pore indicated with arrow. Scale bars = 20 µm. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

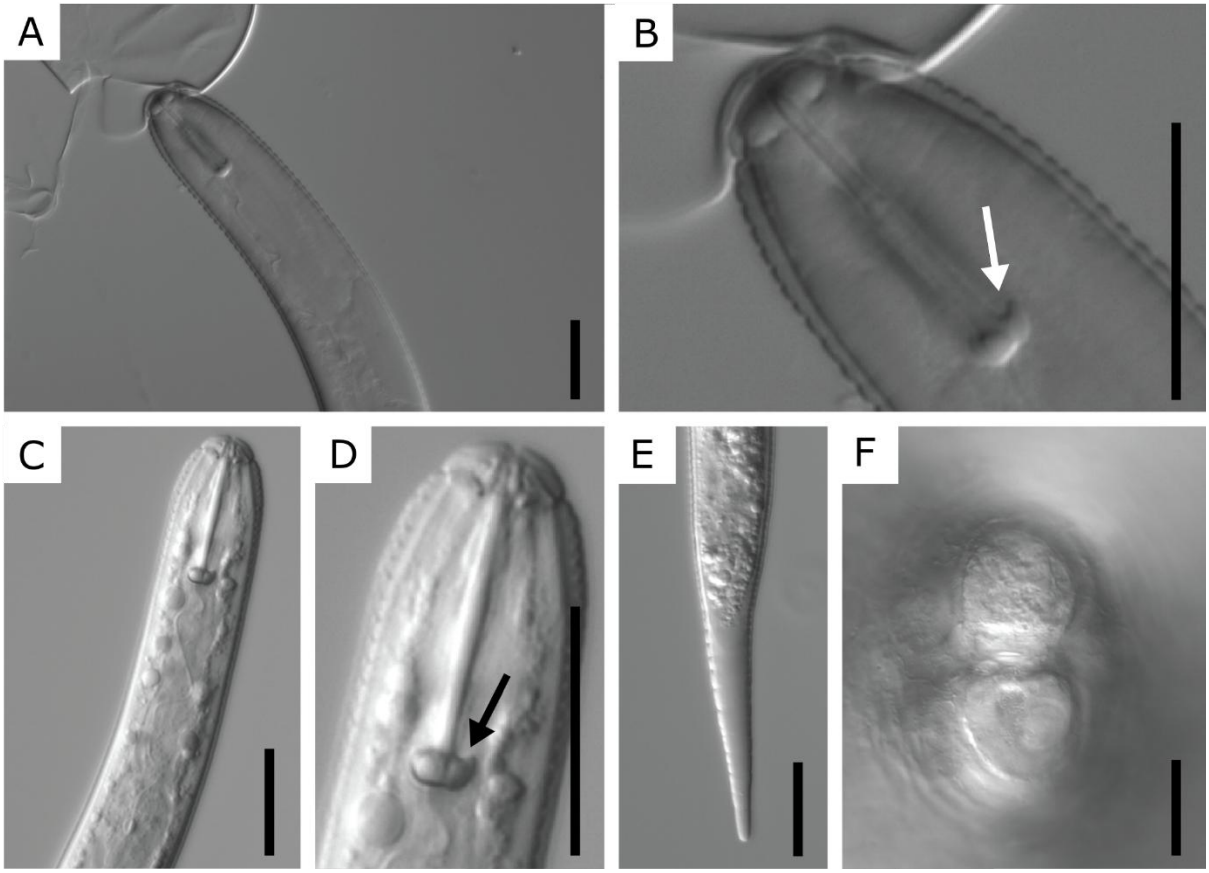

Figure A6.6. *Heterodera mani* second stage juveniles (a-e) and vulval plate (f). (b) and (d) are enlargements of (a) and (c), respectively;(e) hyaline region of tail. Arrows indicating the deeply concave stylet knobs characteristic of *H. mani*. Scale bars = 20 µm. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

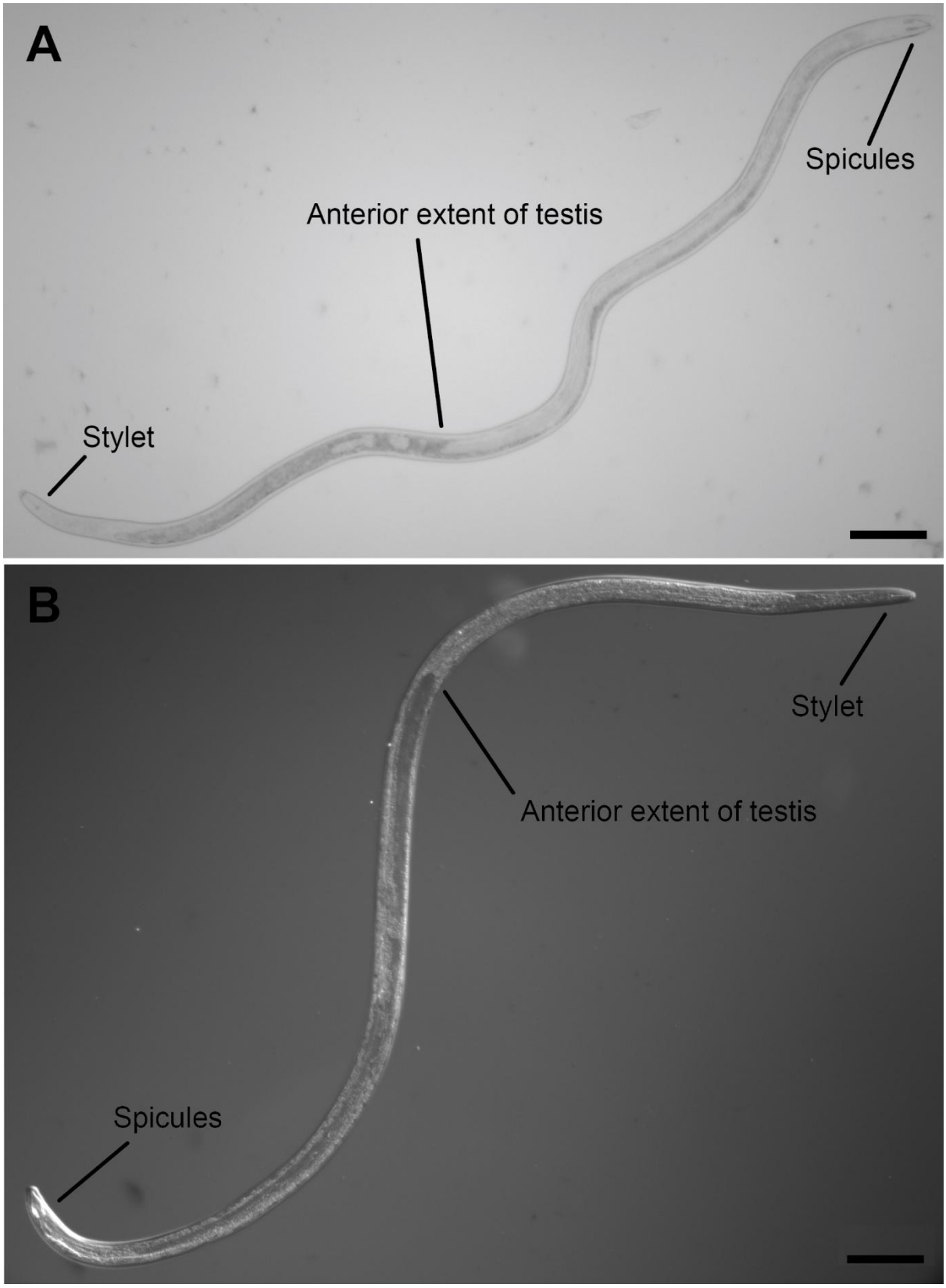

Figure A6.7. Males of *Heterodera australis*. A, B, two separate individuals under different contrast. Note location of spicules at posterior extremity, this is a notable feature of the sedentary plant-parasitic Tylenchida (and members of the Heteroderidae in particular). Scale bars = 100 µm. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

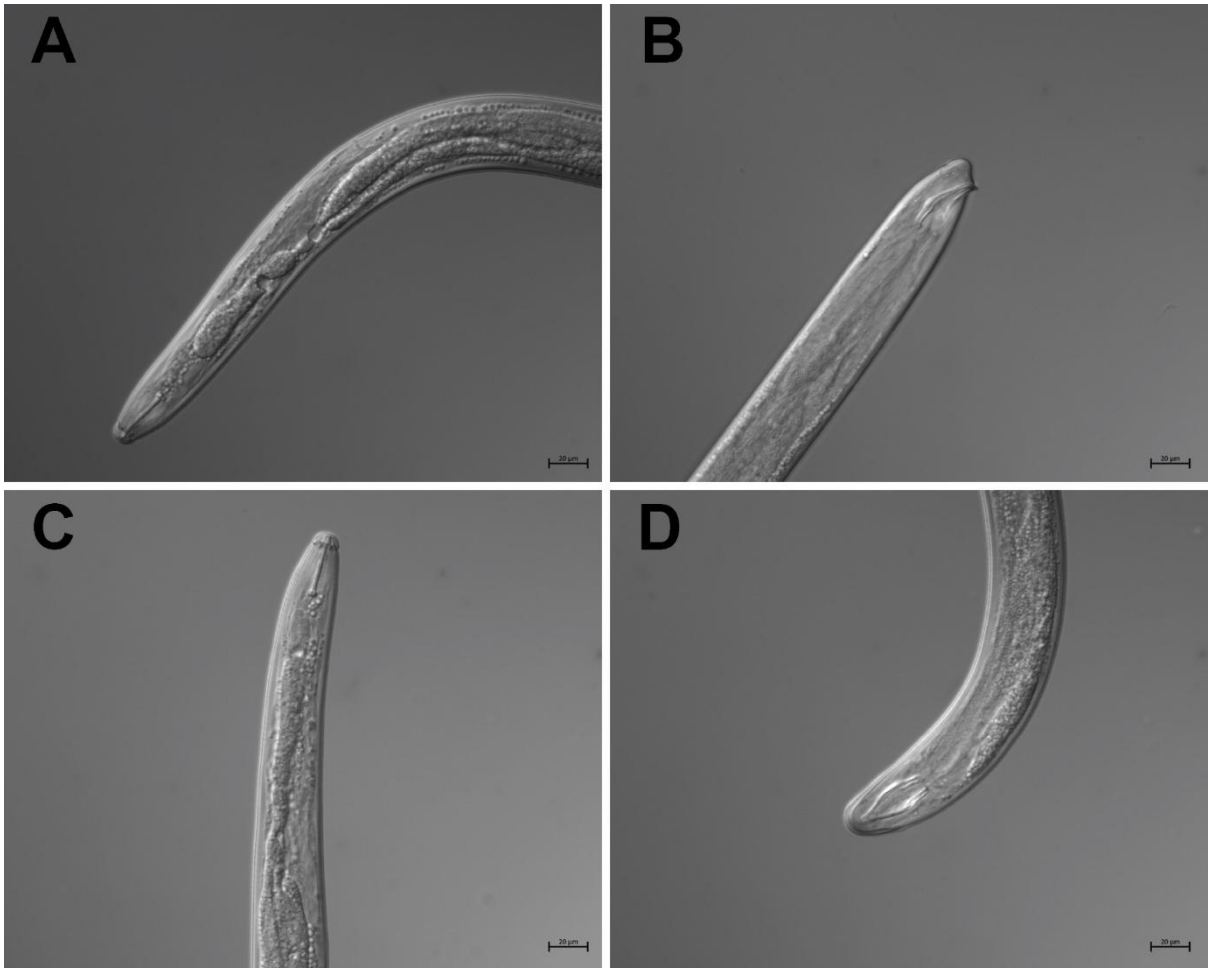

Figure A6.8. Males of *Heterodera australis*. A, C; anterior end. B, D, posterior ends of A and C, respectively. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

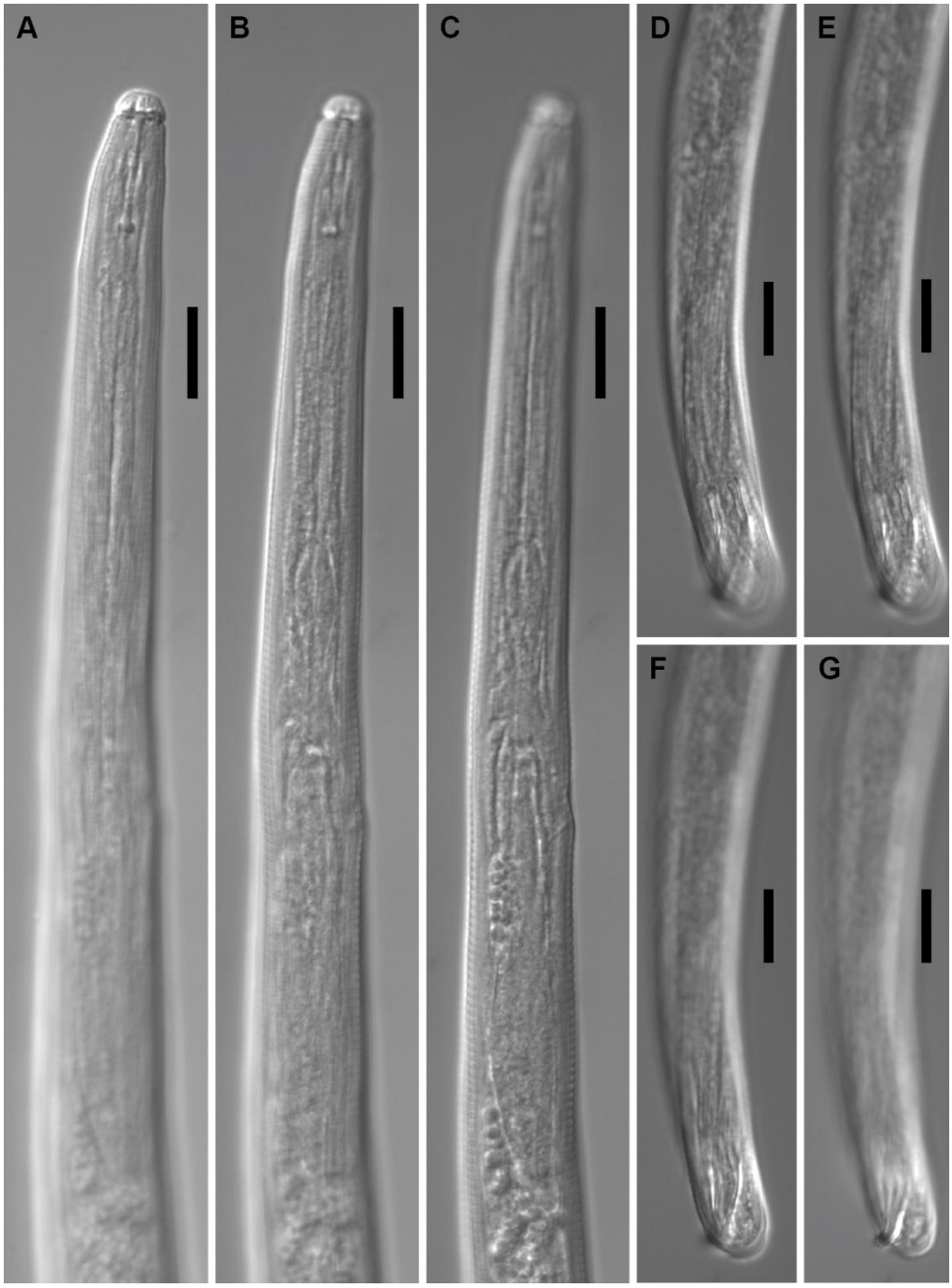

Figure A6.9. Male of *Heterodera carotae*. A, B, C, Anterior end at increasing depth of focus. D, E, F, G, Posterior end at increasing depth of focus. Photos by Daniel C. Huston, CSIRO; specimens from Australian National Nematology Collection.

Appendix 7. Background and recipe for non-toxic Kaiser's Glycerin Jelly

Heterodera vulval cones can be mounted in a variety of media, such as Canada balsam, however glycerin-based media have the advantage over balsam in that they are ready for study immediately. Traditional formulations have used phenol as an antiseptic to prevent the growth of bacteria and fungi. However, phenol is carcinogenic, and as the jelly needs to be heated, media including phenol should only be used under fume hood. This can be restrictive if a fume hood is not available and is often logistically challenging considering the delicate procedures and various pieces of equipment required to successfully mount vulval cones. Dioni (2003) argued that the primary reason phenol was included in traditional formulations of various microscopy media was to serve as an antiseptic and suggested that dental mouthwash products (such as Listerine®) include several antiseptic ingredients, and thus mouthwash can serve as a non-toxic substitute for Phenol. Following the recommendations of Dioni (2003) we have used the following non-toxic formula, modified from the original formula for Kaiser's Glycerin Jelly (Kaiser, 1880), to mount hundreds of *Heterodera* vulval cones. The medium is easy to work with, has a good refractive index and keeps well at room temperature.

Modified Kaiser's Glycerin Jelly
Ingredients:

- 7 grams gelatine. Standard culinary packets from any supermarket are suitable.
- 42 mL water. Tap water is acceptable, distilled is preferable.
- 50 mL Glycerol. Glycerol is also known as glycerin.
- 4 mL Listerine mouthwash. The green variety is needed, as it includes alcohol.

Procedure:
In your final container (wide-mouth glass jar is best):

1) Add gelatine to H_2O, leave for 5–10 min
2) Stir
3) Stir in Glycerol, stir well until solution appears homogenised
4) Begin heating gently by placing glass jar into a hot water bath
5) Stir and heat until all gelatine is dissolved (this may take some time)
6) Stir in Listerine, allow to remain heating until all bubbles have risen to the top and out of the main solution
7) Allow to cool to room temperature (Leaving the jar in the hot water bath as it cools down helps to get any remaining bubbles out).
8) The media is now ready to use. Should keep for a long time. Examine every now and then to make sure that fungi have not started to grow on it. If problems with microbial growth occur, try increasing concentration of Listerine.

References

Dioni W. (2003) Safe microscopical reagents for amateurs. Mounting microscopic subjects. Part 4 -Glycerine jellies. Part 5 - Mountant summary. Micscape Magazine. Microscopy, UK.

Appendix 8. Morphological and morphometric characteristics useful for the identification of species of *Heterodera*

Table A8.1. Characteristics of the female cyst useful for identification.

Species	Length	Width	L/W ratio	Fenestral length	Fenestral width	Underbridge	Vulval slit length	Bullae	Fenestration type	Group
H. africana	600–616	366–459	1.4–1.7	-	-	Absent	64	Absent	Absent	*Afenestrata*
H. agrostis	625	383	1.6	45	18	Strong	39	Prominent	Ambifenestrate	*Schachtii*
H. amaranthusiae	431–611	360–563	1.1–1.5	35–52	17–24	Absent or weak	28–32	Absent	Bifenestrate	*Humuli*
H. amygdali	546	302	1.8	37–54	32–48	Absent	51	Few	Ambifenestrate	*Goettingiana*
H. arenaria	693–925	503–706	1.3–1.4	45–51	21–26	Absent	9 to 10	Prominent	Bifenestrate	*Avenae*
H. aucklandica	480–690	346–460	1.4	47–49	24–28	Absent	6 to 9	Prominent	Bifenestrate	*Avenae*
H. australis	734–780	528–564	1.4	47–52	23–25	Absent	7 to 8	Prominent	Bifenestrate	*Avenae*
H. avenae	600–845	470–601	1.3–1.5	43–55	20–25	Absent	8 to 10	Prominent	Bifenestrate	*Avenae*
H. axonopi	558	459	1.2	-	-	Absent	47	Absent	Absent	*Afenestrata*
H. bamboosi	609	370	1.6	-	-	Absent	55	Absent	Absent	*Afenestrata*
H. bergeniae	440	325	1.3	50	42	Present	48	Absent	Ambifenestrate	*Goettingiana*
H. betae	830–878	455–518	1.6–1.9	44–55	38.43	Strong	48–57	Prominent	Ambifenestrate	*Schachtii*
H. bifenestra	431–530	324–432	1.3	45–55	25–30	Absent	12 to 15	Absent	Bifenestrate	-
H. cajani	448–670	209–422	1.4–2.1	27–65	25–30	Strong	43–55	Prominent	Ambifenestrate	*Schachtii*
H. canadensis	580	543	1.0	51	45	Strong	47	Absent	Ambifenestrate	*Cyperi*
H. cardiolata	485–818	385–509	1.2–1.4	52–61	25–39	Present	34–43	Absent	Ambifenestrate	*Cyperi*
H. carotae	408	309	1.3	31	39	Slender	47	Absent	Ambifenestrate	*Goettingiana*

H. ciceri	796	452	1.8	40	27	Strong	50	Prominent	Ambifenestrate	*Schachtii*
H. circeae	555	397	1.4	43	41	Slender	48	Absent	Ambifenestrate	*Goettingiana*
H. cruciferae	429–557	333–399	1.3–1.4	35–41	32–38	Slender	37–46	Absent	Ambifenestrate	*Goettingiana*
H. cyperi	581–640	325–405	1.4	37	28	Present	31	Absent	Ambifenestrate	*Cyperi*
H. daverti	650–749	380–491	1.4–1.5	42–54	31–40	Strong	47–52	Prominent	Ambifenestrate	*Schachtii*
H. delvii	617	355	1.7	40–56	36–45	Present	36–45	Absent	Ambifenestrate	*Cyperi*
H. dunensis	316–610	241–478	1.2–1.9	37–51	34–53	Strong	35–63	Prominent	Ambifenestrate	*Schachtii*
H. elachista	431–446	311–323	1.4	29–40	30–32	Slender	36–40	Few	Ambifenestrate	*Cyperi*
H. fengi	280–465	226–335	1.2–1.4	40–65	45–63	Present	40–60	Absent	Ambifenestrate	*Cyperi*
H. fici	503–561	370–424	1.3–1.5	46–62	25–37	Slender	43–48	Small	Ambifenestrate	*Humuli*
H. filipjevi	597–928	437–685	1.3–1.4	51–59	28–30	Present	7–10	Prominent	Bifenestrate	*Avenae*
H. galeopsidis	576–797	408–556	1.4–1.5	41–50	31–38	Strong	39–50	Prominent	Ambifenestrate	*Schachtii*
H. gambiensis	834	524	1.6	38	44	Strong	54	Present	Ambifenestrate	*Sacchari*
H. glycines	474–709	327–535	1.3–1.7	34–58	16–41	Strong	38–50	Prominent	Ambifenestrate	*Schachtii*
H. glycyrrhizae	764	438	1.7	57	44–45	Absent	?	Prominent	Ambifenestrate	*Goettingiana*
H. goettingiana	521–650	372–420	1.6–1.7	35–61	37–43	Weak	40–53	Few	Ambifenestrate	*Goettingiana*
H. goldeni	707–766	437–481	1.6	60–61	42–52	Prominent with finger–like projections	42–49	Prominent	Ambifenestrate	*Sacchari*
H. graminis	561	441	1.3	50	32	Strong	38	Absent	Ambifenestrate	*Cyperi*

H. graminophila	498–710	331–489	1.5	50	43	Prominent	45	Absent	Ambifenestrate	*Cyperi*
H. guangdongensis	266–415	207–361	1.0–1.5	22–39	27–36	Weak	31–41	Absent	Ambifenestrate	*Cyperi*
H. hainanensis	337–560	232–450	1.1–1.6	-	-	Weak	25–40	Absent	Absent	*Afenestrata*
H. hordecalis	533–563	355–454	1.2–1.3	54–71	22–25	Strong	16–21	Absent	Bifenestrate	*Avenae*
H. humuli	418–540	310–345	1.4	48–61	24–27	Slender	34–39	Absent	Bifenestrate	*Humuli*
H. johanseni	534	509	1.1	32	?	Absent	?	Absent	Ambifenestrate	*Goettingiana*
H. kirjanovae	618	326	1.7	52	37	Absent	39	Absent	Ambifenestrate	*Goettingiana*
H. koreana	840	520	1.5	-	-	Absent	49	Absent	Absent	*Afenestrata*
H. latipons	544–584	413–447	1.2–1.3	53–67	14–22	Absent	6 to 8	Absent or present	Bifenestrate	*Avenae*
H. lespedezae	678–719	371–522	1.4	50	35–41	Strong	45–47	Prominent	Ambifenestrate	*Schachtii*
H. leuceilyma	830–845	480–579	1.7	48–50	38–40	Prominent with finger-like projections	50–65	Absent	Ambifenestrate	*Sacchari*
H. litoralis	700	370	1.9	34	27	Present	40	Absent	Bifenestrate	*Humuli*
H. longicolla	496	359	1.4	37	25	Present	32	Absent	Ambifenestrate	*Cyperi*
H. mani	580–624	468–507	1.2–1.3	52–55	25–27	Absent	7–9	Prominent	Bifenestrate	*Avenae*
H. medicaginis	635	466	1.4–1.5	47	34	Strong	40–45	Prominent	Ambifenestrate	*Schachtii*
H. mediterranea	573	380	1.4	42	40	Strong	43	Prominent	Ambifenestrate	*Schachtii*
H. menthae	637	411	1.5	52	33	Present	41	Absent	Ambifenestrate	*Goettingiana*
H. microulae	414–543	305–456	1.12–1.5	28–33	35–40	Weak	35–44	Absent	Ambifenestrate	*Goettingiana*
H. mothi	588–694	292–360	1.9–2.1	40–44	29–30	Short	34–40	Rounded dark-brown	Ambifenestrate	*Cyperi*

H. orientalis	576–727	458–583	1.2–1.3	-	-	Absent	50	Absent	Absent	*Afenestrata*
H. oryzae	405–715	394–460	1.2–1.7	24–39	27–52	Present	39–50	Few	Ambifenestrate	*Cyperi*
H. oryzicola	500	366	1.4	27–32	25–30	Slender	39–43	Present	Ambifenestrate	*Cyperi*
H. pakistanensis	560	320	1.7	28	19	Slender	48	Absent	Ambifenestrate	*Cyperi*
H. persica	533	380	1.4	47	44	Slender	49	Present	Ambifenestrate	*Goettingiana*
H. phragmitidis	488–679	394–585	1.2	33–37	31–32	Present	37–41	Absent	Ambifenestrate	*Cyperi*
H. plantaginis	575	362	1.6	55	28–31	Slender	38	Absent	Ambifenestrate	*Goettingiana*
H. pratensis	650–760	490–570	1.2–1.4	47–49	22–25	Absent	9– 11	Prominent	Bifenestrate	*Avenae*
H. raskii	760	280	3	50	43	Slender	49	Absent	Ambifenestrate	*Cyperi*
H. ripae	415–505	307–375	1.3–1.4	46–52	25–27	Slender	33–37	Absent	Bifenestrate	*Humuli*
H. riparia	519–582	347–428	1.4	40–42	18–22	Slender	8–11	Prominent	Bifenestrate	*Avenae*
H. rosii	840	512	1.0–1.7	48–65	40–55	Strong	45–59	Prominent	Ambifenestrate	*Schachtii*
H. sacchari	550–735	430–470	1.5–1.7	50–53	31–48	Strong with finger-like projections	50–53	Present	Ambifenestrate	*Sacchari*
H. saccharophila	577	498	1.2	-	-	Present	?	Absent	Absent	*Afenestrata*
H. salixophila	873	591	1.5	22–36	28–42	Present	40–62	Present	Ambifenestrate	-
H. schachtii	768–815	512–529	1.5–1.6	35–38	25–31	Strong	41–44	Prominent	Ambifenestrate	*Schachtii*
H. scutellariae	560	424	1.3	35	39	Narrow	43	Absent	Ambifenestrate	*Goettingiana*
H. sinensis	723	513	1.4	62	53	Weak	46	Present	Ambifenestrate	*Sacchari*
H. skohensis	553	372	1.4	55	30	Present	32	Absent	Ambifenestrate	-
H. sojae	435–664	343–567	1.1–1.8	23–54	22–51	Absent	9 to 25	Absent	Ambifenestrate	-

H. sonchophila	853	520	1.6	44	40	Strong	48	Prominent	Ambifenestrate	*Schachtii*
H. sorghi	739	492	1.4	47	38	Strong	32	Prominent	Ambifenestrate	*Sacchari*
H. spinicauda	475	422	1.1	48	23	Distinct	4 to 9	Absent	Bifenestrate	-
H. spiraeae	631	444	1.4	47	29	Strong	41	Present	Ambifenestrate	*Schachtii*
H. sturhani	504–696	384–504	1.2–1.5	43–50	19–31	Absent	6 to 12	Prominent	Bifenestrate	*Avenae*
H. swarupi	589	366	1.7	45	35	Rod-like	41	Prominent	Ambifenestrate	*Schachtii*
H. trifolii	608–841	341–536	1.3–1.8	43–53	33–44	Strong	40–53	Prominent	Ambifenestrate	*Schachtii*
H. turangae	487	267	1.8	53	32	Slender	38	Absent	Ambifenestrate	*Goettingiana*
H. turcomanica	416–661	355–551	1.2	58–69	15–16	Absent	4 to 5	Prominent	Bifenestrate	*Humuli*
H. urticae	492	435	1.1	38	37	Slender	42	Absent	Ambifenestrate	*Goettingiana*
H. ustinovi	610–675	459–520	1.3	42–51	24–26	Present	9 to 11	Prominent	Bifenestrate	*Avenae*
H. uzbekistanica	475	308	1.5	66	31–35	Prominent	40	Absent	Ambifenestrate	*Goettingiana*
H. vallicola	468	419	1.1	46	21	Slender	31	Absent	Bifenestrate	*Humuli*
H. zeae	501–636	347–455	1.3–1.7	40–50	19–30	Slender	37–44	Prominent	Ambifenestrate	-

Table A8.2. Morphological characteristics of the larval J2 useful for species identification

Species	Body Length	Stylet Length	Stylet Knob Shape	Tail Length	Hyaline Region	Incisure Number	Group
H. africana	375–420	22–26.5	Strongly anteriorly projected	55	31–32	3	*Afenestrata*
H. agrostis	427	26	Moderately anteriorly projected	54	30	4	*Schachtii*
H. amaranthusiae	384–430	22–25	Rounded to slightly projecting anteriorly	48–58	20–27	4	*Humuli*
H. amygdali	500	25	?	35–42	30	4	*Goettingian a*

H. arenaria	587–663	28–29	Distinctly anteriorly projected	74–82	48–55	4	*Avenae*
H. aucklandica	494–508	24–25	Slightly anteriorly projected	69–76	46–48	4	*Avenae*
H. australis	562–583	25–26	Slightly to moderately anteriorly projected	68–71	43–45	4	*Avenae*
H. avenae	535–572	26–27	Anteriorly flattened to projected	61–70	38–47	4	*Avenae*
H. axonopi	384	22	Anteriorly flattened	54	34	3	*Afenestrata*
H. bamboosi	472	20	Almost roundish knobs	63	41	3	*Afenestrata*
H. bergeniae	396	24	Rounded	36	16	4	*Goettingiana*
H. betae	547–607	29–31	Anteriorly projected	70–74	38–42	4	*Schachtii*
H. bifenestra	426–489	23–24	Distinctly hollow posteriorly	70–81	39–49	3	-
H. cajani	420–519	22–27	Anteriorly projected or rounded	42–52	23–31	4	*Schachtii*
H. canadensis	558	24	Rounded	115	60	3	*Cyperi*
H. cardiolata	340–387	19–21	Anteriorly projected	46–49	18–24	3	*Cyperi*
H. carotae	411–429	23–24	Proconcave anterior faces	49–53	28–31	4	*Goettingiana*
H. ciceri	525	29	Strongly anteriorly projected	60	36	4	*Schachtii*
H. circeae	434	25	Rounded and sloping posteriorly	52	26	4	*Goettingiana*
H. cruciferae	351–438	21–25	Anteriorly flattened to projected	41–50	21–29	4	*Goettingiana*
H. cyperi	441–480	20	Rounded	60–66	25–29	3	*Cyperi*
H. daverti	457–476	25–26	Slightly anteriorly projected	54–57	30–33	4	*Schachtii*
H. delvii	480	18–20	Anteriorly projected	49–60	29–36	3	*Cyperi*
H. dunensis	426–520	27–31	Rounded and strongly anteriorly projected	35–45	16–23	4	*Schachtii*
H. elachista	367–402	19–20	Rounded or anteriorly projected	52–59	31–32	3	*Cyperi*

H. fengi	440–520	22–24	Slightly projecting or flat anteriorly	62–77	35–45	3	*Cyperi*
H. fici	377–419	21–23	Rounded and directed slightly anteriorly	47–54	22–29	4	*Humuli*
H. filipjevi	505–552	24–27	Moderately anteriorly projected	55–62	33–39	4	*Avenae*
H. galeopsidis	485–553	26–28	Anteriorly projected	68	35–40	4	*Schachtii*
H. gambiensis	403–489	22–23	Rounded or anteriorly projected	49–57	25–33	3	*Sacchari*
H. glycines	386–471	21–23	Anteriorly projected	39–51	22–30	4	*Schachtii*
H. glycyrrhizae	424	26	Moderately anteriorly projected	50	23	4	*Goettingiana*
H. goettingiana	454–486	24–25	Rounded to slightly projecting anteriorly	58–66	33–38	4	*Goettingiana*
H. goldeni	503–546	23–24	Moderately anteriorly projected	59–65	34–38	3	*Sacchari*
H. graminis	391	22	Anteriorly projected	56	29	3	*Cyperi*
H. graminophila	391–508	18–23	Rounded	56–62	27–33	3	*Cyperi*
H. guangdongensis	318–410	19–21	Slightly projecting or flat anteriorly	42–61	18–34	3	*Cyperi*
H. hainanensis	337–411	16–18	Rounded, sloping slightly posteriorly	47–63	27–41	3	*Afenestrata*
H. hordecalis	436–474	23–26	Strongly anteriorly projected	52–63	34–40	4	*Avenae*
H. humuli	364–425	23–24	Rounded	49–50	26–29	4	*Humuli*
H. johanseni	452	22	Slightly to moderately projected	53	27	4	*Goettingiana*
H. kirjanovae	398	23	Anteriorly projected	40–64	22	4	*Goettingiana*
H. koreana	446	18	Rounded	66	40	3	*Afenestrata*
H. latipons	454–518	22–26	Anteriorly projected	47–57	27–34	4	*Avenae*
H. lespedezae	457–481	24–25	Slightly anteriorly projected	54–56	26–30	4	*Schachtii*
H. leuceilyma	550–594	24–26	Rounded	61–67	37–43	3	*Sacchari*

H. litoralis	520	30	Almost flat anteriorly	56	28	4	*Humuli*
H. longicolla	353	18	Anteriorly projected	50	27	3	*Cyperi*
H. mani	526–578	24–26	Strongly anteriorly projected	59–68	37–42	4	*Avenae*
H. medicaginis	462–483	25	Anteriorly projected	52	29	4	*Schachtii*
H. mediterranea	405	25	Anteriorly projected	40	22	4	*Schachtii*
H. menthae	307	23	Anteriorly projected	39	24–26	4	*Goettingiana*
H. microulae	505–628	24–29	Rounded and flat, or slightly concave anteriorly	49–61	24–31	4	*Goettingiana*
H. mothi	401–465	17–18	Nearly rounded; somewhat anteriorly projected	62–73	29–37	3 or 4	*Cyperi*
H. orientalis	428–504	23–24	Rounded or anteriorly projected	47–53	25–30	3	*Afenestrata*
H. oryzae	390–554	20–21	Anteriorly projected	55–59	31–38	3	*Cyperi*
H. oryzicola	392–440	18–20	Rounded	55–60	28	3	*Cyperi*
H. pakistanensis	446	17	Anteriorly projected	70	32	3	*Cyperi*
H. persica	440	23	Rounded or anteriorly projected	47	24	4	*Goettingiana*
H. phragmitidis	363–398	18–19	Anteriorly projected	52	28–30	3	*Cyperi*
H. plantaginis	442–474	23–26	Slightly anteriorly projected	68–71	23–26	4	*Goettingiana*
H. pratensis	504–560	24–25	Slightly projected or almost flat anteriorly	60–69	38–45	4	*Avenae*
H. raskii	500	19	Rounded	80	34	4	*Cyperi*
H. ripae	350–373	22–24	Slightly anteriorly projected	40–47	19–23	4	*Humuli*
H. riparia	446–452	22–24	Slightly anteriorly projected	57–61	36–37	4	*Avenae*
H. rosii	550	31	Slightly anteriorly projected	66	41	4	*Schachtii*
H. sacchari	460–592	21–24	Anteriorly projected	50–62	26–32	3	*Sacchari*
H. saccharophila	449	25	Slightly anteriorly projected	51	24	4	*Afenestrata*
H. salixophila	453–508	25–30	Rounded or slightly anteriorly projected	58–67	32–36	4	-

H. schachtii	436–489	25–26	Moderately or strongly anteriorly projected	45–49	24–27	4	*Schachtii*
H. scutellariae	408	24	Slightly anteriorly projected	47	25	4	*Goettingiana*
H. sinensis	552	25	Anteriorly projected	65	38	3	*Sacchari*
H. skohensis	380	20	Slightly anteriorly projected to rounded	41	25	4	-
H. sojae	412–478	24–25	Oval	54–75	33–46	?	-
H. sonchophila	469	26	Anteriorly projected	52	28	4	*Schachtii*
H. sorghi	461	21	Anteriorly projected	52	31	3	*Sacchari*
H. spinicauda	445	23	Flattened or slightly anteriorly projected	64	34	4	-
H. spiraeae	420	23	Rounded and anteriorly projected	46	24	4	*Schachtii*
H. sturhani	480–537	24–25	Slightly concave, sloping slightly posteriorly	55–72	34–45	4	*Avenae*
H. swarupi	420	22	Flattened to anteriorly projected	48	24	4	*Schachtii*
H. trifolii	492–613	25–28	Anteriorly projected	60–72	32–37	4	*Schachtii*
H. turangae	337	19	Anteriorly projected	31	17	4	*Goettingiana*
H. turcomanica	386–396	18–19	Rounded to slightly projecting anteriorly	30–37	19–21	4	*Humuli*
H. urticae	541	27	Slightly anteriorly projected	58	29	4	*Goettingiana*
H. ustinovi	560–638	25–27	Anteriorly projected	80–94	52–62	4	*Avenae*
H. uzbekistanica	377	25	Anteriorly projected	54	15–23	4	*Goettingiana*
H. vallicola	383	25	Flattened anteriorly or slightly projected	49	29	4	*Humuli*
H. zeae	381–453	20–23	Rounded or slightly anteriorly projected	41–46	19–24	4	-

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE

This section was originally prepared by Dr Danièle Giblot-Ducray. Additional information was subsequently incorporated from previous sections of this NDP, written in 2023.

9.1 Introduction

Cyst nematodes (*Heterodera* spp.) infect a wide range of pulses (chickpea, lentil, broad bean), cereals (oats, wheat, barley, soybeans, rye, maize, sorghum), and vegetables (carrots, beans, peas, beetroot). Most species are exotic to Australia including cereal cyst nematodes (*Heterodera avenae*, Heterodera *filipjevi*, *H. latipons*), chickpea cyst nematode (*H. ciceri*), maize cyst nematode (*H. zeae*) and soybean cyst nematode (*H. glycines*). If introduced, they can spread easily via movement of infested plant roots and soil, contaminated machinery, and footwear. The cysts of most species can persist in soil for many years.

This section outlines a protocol to undertake surveillance for cyst nematodes. It is based on the protocol developed by SARDI Molecular Diagnostic to monitor the Australian cereal cyst nematode *H. australis* and includes sampling and diagnostic methods recommendations. *Heterodera australis* was considered to be *H. avenae* prior to 2002 (Subbotin et al. 2002), and it was subsequently determined that *H. avenae* does not likely occur in Australia (Huston et al. 2024). The SARDI protocol can detect one *H. australis* cyst in a 500 g soil sample. Other protocols may be available but have not been evaluated. Sampling recommendations may need to be adjusted to optimise the chance of detection for each species.

This protocol is recommended to support surveillance in case of a suspected incursion of *Heterodera* sp. It can also be used for background surveillance to support area freedom. Practices to avoid contamination of soil between properties, including cleaning of tools, footwear, and vehicle to remove excess dirt are recommended. It is advised that surveillance teams consult with the diagnostic laboratory prior to planning the surveillance program to discuss capacity and containment.

9.2 Sampling

Prior to collecting, (i) contact the state Biosecurity department to check permit and other biosecurity requirements in place, (ii) inform the diagnostic laboratory of your intention to submit samples of biosecurity concern and (iii) seek permission to access the property to be surveyed.

9.2.1 Disinfestation procedures

Appropriate disinfestation procedures must be followed both when entering and leaving a property with suspected cyst nematode infestation. All equipment used and clothing worn on a property must be disinfested or disposed of using appropriate methods such as bagging and burial or autoclaving.

Set up a disinfestation station using a freshly prepared 2% sodium hypochlorite solution ('chlorine'), methylated spirit and water (80/20 mix) or other approved disinfestation chemical solution. Use appropriate safety precautions when handling and using hazardous chemicals. This solution can be used multiple times in one day but should be replaced if soil or debris accumulates in the solution. Foot baths should be covered when not in use to prevent evaporation or dilution by rain. Discard the solutions on site prior to moving to a new property and rinse the containers. Make new solutions on arrival at a new site.

9.2.2 Footwear and small tools

Prior to entering a property – Footwear and sampling tools must be thoroughly cleaned of soil and plant material using water and scrubbing brush. No soil or plant residue should be visible after cleaning.

On arrival at a new property immerse footwear (ideally rubber boots) in disinfestation solution by stepping gently into the footbath for at least 60 seconds, making sure to brush the solution up the boot. Do not rinse after immersion. Cover the tub containing the disinfestation solution between use to limit breakdown or dilution; replace solution daily or more frequently if it gets dirty.

On exit – Use water and scrubbing brush (or hoof pick) to remove all soil and plant material from tread of footwear and sampling tools. Disinfest by immersion as described above, including the cleaning tools (e.g., scrubbing brush, hoof pick).

Note: *Heterodera* spp. cysts are very stable and resistant to external stress; they may not be killed by the disinfectant. Physical removal of debris by scrubbing is critical and leave discarded sterilising solutions and debris within the property boundaries. Do not pour sterilising solutions and debris into drainage channels.

9.2.3 Clothing

Soiled clothing should not be worn between paddocks. It is highly recommended to wear disposable overall suits and change these between each paddock/property when undertaking a survey. Used disposable clothing should be placed inside a plastic bag for appropriate disposal as biosecurity waste. Disinfest boots between paddocks.

9.2.4 Vehicle

Vehicles should not be driven on the property or remain on designated roads to avoid the pickup of soil on tyres. If the vehicle must be driven to paddocks, ensure it is cleaned thoroughly with a pressure washer to remove any soil and plant material prior to leaving the property. Cleaning is to occur away from the paddock and on a hard stand area with wastewater capture to prevent runoff onto paddock or trafficked areas. When cleaning the vehicle ensure that there is no soil or vegetable matter up under the wheel arches or stuck in the tyres.

9.3 Soil sampling

Soil samples can be collected all year round, as cysts persist in soil. However, sampling during or just after a host crop optimises the chance of detection as the numbers of cysts/juveniles increase. The best sampling time will vary with each species/crop combination.

Different diagnostic laboratories may have different capacity and requirement to analyse soil samples for exotic cyst nematodes. The below is recommended when submitting samples to SARDI Molecular Diagnostic (Adelaide, SA). Check with the laboratory for appropriate sampling before submitting samples.

Figure 1. A soil corer.

9.3.1 Equipment required

- GPS units for recording property/paddock location.
- Soil corer with a 10 mm diameter by 100 mm or 150 mm long tip, metal bucket to collect cores and screwdriver or scraper to dislodge soil from corer.
- Sample bags (zip lock or with cable tie), double bags (some diagnostic laboratories may provide sampling kits) and sturdy box or other container for shipping.
- Bar-coded labels (recommended; maybe supplied by diagnostic laboratory) or other way to label the samples for tracking.
- Documentation required by plant quarantine standards of the state in which the diagnostic laboratory is located.

9.3.2 Where to take a sample

Before sampling, determine the number of paddocks and sections per paddock to be sampled. If visible, identify sections of the paddock with sandy soils or well-structured clay soils, where crop performance was poor to collect samples from. In vegetable production, it is recommended to collect one sample per hectare. Higher sampling intensity can be used to confirm a positive result and determine the extent of spread. A lower sampling intensity may be considered for large scale surveillance of broadacre crops.

The soil sample should be a composite of 45 cores of 10 mm × 100 mm cores or 30 cores of 10 mm × 150 mm cores; choice of core length is determined by soil depth.

- Adopt a zig zag sampling pattern in the target area

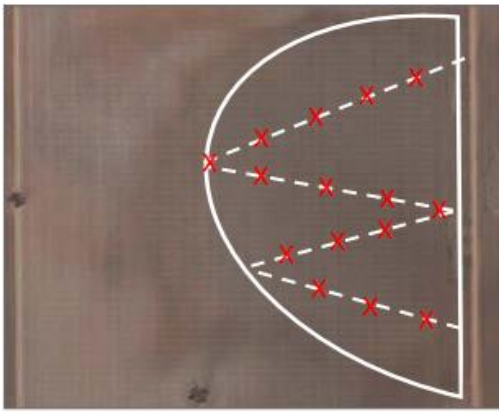

Figure 2. Zig-Zag sampling method.

- Collect three cores at each of 15 locations if using a 100 mm long core, or two cores per location if using a 150 mm long core. Core directly from the root zone of the most recent host crop, if visible; do not remove any roots or plant debris. Transfer soil from each core into the bucket, lined with a sample bag. The soil can be removed by tapping on the edge of the bucket or scraped from the corer tip.
- There is no need to clean the corer between individual samples in the same paddock (i.e. between composite samples).

Figure 3. Soil corer being used to take soil samples in vicinity of crop.

- Combine all 45 (or 30) soil cores in a single sample bag; sample should be approx. 500 g.
- Clean and disinfest the corer, scraper and bucket between paddocks.
- Store soil bags in an empty cool box (no ice) to protect from direct sunlight; Do not leave moist samples in plastic bags exposed to the sun.
- Send to diagnostic laboratory as soon as possible after collection.

Note: Root systems from plants showing typical symptoms can be examined visually by a nematologist and can also be tested using DNA assays.

9.3.3 Sending sample to laboratory

Ensure labelling is adequate for complete traceability of the sample.

Securely seal each sample bag, then place in another plastic bag and seal tightly.

Place all double bagged samples in a sturdy box. Complete all quarantine documentation and affix to the box. Ensure all biosecurity packaging requirements are met.

Dispatch samples to the diagnostic laboratory via express courier (ensure tracking of parcel) and notify laboratory. Ensure that samples are not in transit over the weekend.

9.4 Diagnostic methods

9.4.1 *Visual root inspection*

Please refer to Section 3.3.1 for further information on visual root inspection.

9.4.2 Isolation of cysts and juveniles from soil

Please refer to Section 3.3.2 for further information on isolation of cysts and juveniles from soil.

9.5 Laboratory Tests

When available, quantitative PCR (qPCR) is the recommended method for surveillance. It allows for a high throughput and fast turnaround specific diagnostic. qPCR can detect the DNA of any life forms of the nematode. In addition, the DNA extracted from soil can be used to monitor other soil borne pests and pathogens, be they endemic or exotic.

9.5.1 DNA extraction

For the development of this protocol, DNA was extracted using the commercial DNA extraction method developed by SARDI Molecular Diagnostic, Adelaide (Ophel-Keller et al. 2008). This method is adapted to extract DNA from large soil samples (200–500 g). The efficiency and consistency of SARDI's method to extract DNA from soil in comparison with commercial kits has been demonstrated previously (Haling et al. 2011).

Commercial kits to extract DNA from soil or infected root tissues are available. These kits will need to be validated for cyst nematode surveillance prior to using.

After extraction, store the DNA at -20 °C until ready to analyse using qPCR.

9.5.2 qPCR

Cereal cyst nematode *Heterodera filipjevi* and carrot cyst nematode *H. carotae* can be quantified using qPCR tests developed by SARDI Molecular Diagnostic. This method has been used by the SARDI Molecular diagnostic service, but has not been verified in an independent laboratory.

The primer and probe (TaqMan MGB) sequences and target regions are listed in table 1.

Table 1. *Heterodera filipjevi* and *H. carotae* qPCR tests

	H. filipjevi	*H. carotae*
Forward primer	GCTTTGGGGTGTTCTCCGT	CATATGTTTGTAGTTGGGATAGATTTAGATAGA

Reverse primer	CAACGAGCGTGCTCCCTT	TAATCAAGAAAAAACCTTTACCGCC
Probe	AGTTGGTGGCGGACCG	TGCTTATTTTAGTGCTGCCAC
Target region	Internal Transcribed Spacer (ITS)	Mitochondrial Cytochrome C Oxidase I (COXI)

qPCR conditions are the same for both tests. The 10 µL qPCR reaction mixture consists of 4 µL DNA, 200 nmol/L TaqMan probe and 400 nmol/L each primer in 1X Quantitect Probe PCR master mix (Qiagen, Hilden, Germany). DNA extracted from soil samples needs to be diluted 1/5 prior to qPCR when using the method above. The dilution factor for commercial kits may vary. Cycling conditions are 15 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Each PCR plate needs to include positive and negative controls (for DNA extraction and PCR). It is recommended to use synthetic DNA as positive control. Calibration standards can be used to calculate the amount of cyst nematode DNA/g sample. The use of internal control to monitor DNA extraction yield and presence of PCR inhibitors is highly recommended.

In combination with the DNA extraction protocol developed by SARDI Molecular Diagnostic, the two qPCR tests described in this protocol can detect the equivalent of 1 cyst per Kg of soil with 95–100% confidence.

A SYBR Green qPCR test has been published for the specific detection of soybean cyst nematode *H. glycines* (Baidoo and Yan, 2021). It has not been validated as part of this work.

Any positive result should be followed-up to confirm detection.

9.6 Acknowledgements

We acknowledge the assistance of Dr Eric Grenier (National Research Institute for Agriculture, Food and Environment (INRAE), France), for providing soil samples infected with various exotic cyst nematode species to support evaluation of DNA extraction method from soil. We thank Dr Alan McKay and Michael Rettke (South Australia Research and Development Institute, SARDI) for their expert contribution and critical review of the surveillance protocol. Development of this surveillance protocol was supported by Grains Research and Development Corporation, through funding from the Australian Government Department of Agriculture, Fisheries & Forestry, as part of its Rural R&D for Profit program and along with Cotton Research and Development Corporation, Hort Innovation Australia, Wine Australia, Sugar Research Australia and Forest and Wood Products Australia.

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