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ANNEX 18

ENG

DP 18: *Anguina* spp.

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ISPM 27
Diagnostic protocols for regulated pests

DP 18: *Anguina* spp.

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1. Pest Information

The Anguinidae family of nematodes contains both mycophagous species and species that parasitize bulbs, tubers and aerial parts of plants. The gall-forming nematodes of the subfamily Anguininae are obligate parasites of plants. More than 40 nominal species of gall-forming Anguinidae have been described. The seed gall nematodes, *Anguina* spp., inhabit the aerial parts of cereals and forage grasses.

Species of *Anguina* invade ovules where they induce galls, lay eggs and reside as second-stage juveniles. This juvenile stage can remain as anhydrobiotes within dried seed galls for many years. A single wheat gall formed by *Anguina tritici* (Steinbuch, 1799) Filipjev, 1936 usually contains 11 000–18 000 nematodes, although galls with as many as 90 000 have been recorded (Decraemer and Hunt, 2013). The nematodes can be retrieved from galls for up to 30 years after forming if kept dry.

Three *Anguina* species, *A. tritici* (Steinbuch, 1799) Filipjev, 1936, *A. agrostis* (Steinbuch, 1799) Filipjev, 1936 and *A. funesta* Price, Fisher & Kerr, 1979 are considered of economic importance as agricultural and quarantine pests in various countries (Chizhov and Subbotin, 1990; Krall, 1991) This diagnostic protocol covers morphological and molecular identification of the genus and these species of major economic importance. Other species with importance in a limited geographical range include:

- *A. agropyri* Kirjanova, 1955
- *A. agropyronifloris* Norton, 1965
- *A. amsinckiae* (Steiner & Scott, 1935) Thorne, 1961
- *A. australis* Steiner, 1940
- *A. balsamophila* (Thorne, 1926) Filipjev, 1936
- *A. caricis* Solovyeva & Krall, 1982
- *A. cecidoplastes* (Goodey, 1934) Filipjev, 1936
- *A. graminis* (Hardy, 1850) Filipjev, 1936
- *A. microlaenae* (Fawcett, 1938) Steiner, 1940
- *A. pacificae* Cid del Prado Vera & Maggenti, 1984 (Ferris, 2013).

A. tritici (wheat gall nematode, bunted wheat) has been recorded in major wheat growing areas on all continents (Southey, 1972) and was historically widely distributed (EPPO, 2015). This species can cause severe crop losses to *Secale cereale* L. (rye) (35–65%) and *Triticum aestivum* L. (wheat) (20–50%) (Leukel, 1929, 1957; Anwar *et al.*, 2001). However, the use of modern seed cleaning methods that separate galls from healthy grains has almost eliminated this species from commercial wheat production in developed countries. For example, recent surveys for *A. tritici* in stored grain harvested from states of the United States of America with records of this nematode did not provide any evidence that it was still present in the country (CABI, 2001, 2014b). Recorded hosts are *T. aestivum*, *Triticum dicoccum* Schrank, *Triticum durum* Desf., *Triticum monococcum* L. (emmer), *Triticum spelta* L. (spelt), *Triticum ventricosum* Ces and *S. cereale*. *Hordeum vulgare* L. (barley) is a very poor host (Southey, 1972). There is little evidence that this nematode reproduces on *Avena sativa* L. (oat) and other grasses; although there are some reports of damage to oat at seedling stage by second-stage juveniles, no galls have been observed on this host.

Clavibacter tritici, the bacterium that is the causal agent of yellow ear rot or “tundu” of *T. aestivum*, is associated with the presence of *A. tritici.* Freshly harvested infected wheat cockles containing the bacterium are toxic to cattle and sheep (Anwar *et al.*, 2001). *A. tritici* has been shown to be vector of *Rathayibacter toxicus* (toxic yellow slime bacterium) under experimental conditions (Riley and McKay, 1990).

A. agrostis (bentgrass nematode) has been reported from Asia, Australia, Europe, New Zealand, North America and Republic of South Africa (CABI, 2014a). It is considered to be a species complex with pathotypes differing in host range (Krall, 1991; Brzeski, 1998). Subbotin *et al.* (2004) supported the concept of narrow specialization of seed gall nematodes, concluding that *A. agrostis* occurs in only

one host, *Agrostis capillaris*, and that other *Agrostis* species are hosts for two further undescribed species of *Anguina*. The type host of *A. agrostis* is *Agrostis tenuis* Sibth. In addition to various bentgrass species, *A. agrostis sensu lato* has been reported from other grass genera, including *Apera*, *Arctagrostis*, *Calamagrostis*, *Dactylis*, *Eragrostis*, *Festuca*, *Hordeum*, *Koeleria*, *Lolium*, *Phalaris*, *Phleum*, *Poa*, *Puccinellia*, *Sporobolus* and *Trisetum* (although certain records may relate to *A. funesta* (CABI & EPPO, 2004)).

A. agrostis has been shown to vector *R. toxicus* under experimental conditions (Riley and McKay, 1990). Several older references (e.g. Goodey, 1960) to this species being the causal agent of disease in livestock relate to galls on *Festuca* spp. *A. agrostis* may actually refer to the species *A. funesta* (Southey, 1973).

A. funesta has been described from Australia, and recently has been reported from Oregon in the United States of America (Meng *et. al.*, 2012). The principal host of *A. funesta* is *Lolium rigidum* (annual ryegrass).

A. funesta is recorded as a vector of *R. toxicus*, which causes the disease annual ryegrass toxicity when consumed by livestock. Annual ryegrass toxicity is responsible for severe losses in the livestock industry in Australia (Price *et al.*, 1979). Rangeland infested by the nematode and bacterium is unusable for grazing (Figures 1 to 3).

2. Taxonomic Information

Name: *Anguina* Scopoli, 1777

Synonyms: *Angvina* (= original spelling, amended to *Anguina* by later workers); *Anguillulina* Gervais & Van Beneden, 1859; *Anguillulina* (*Anguina* Scopoli) (Schneider, 1939); *Paranguina* Kirjanova, 1954; *Paranguina* Kirjanova, 1955

Taxonomic position: Nematoda, Chromadorea, Chromadoria, Rhabditida, Tylenchina, Tylenchomorpha, Sphaerularioidea, Anguinidae, Anguininae (after Decraemer and Hunt, 2013)

Common names: Seed and leaf gall nematode, seed-gall nematode. Other common names in various languages are listed in CABI (2013).

Name: *Anguina tritici* (Steinbuch, 1799) Chitwood, 1935

Synonyms: *Vibrio tritici* Steinbuch, 1799; *Rhabditis tritici* (Steinbuch) Dujardin, 1845; *Anguillula tritici* (Steinbuch) Grube, 1849; *Anguillulina tritici* (Steinbuch) Gervais & Van Beneden, 1859; *Tylenchus tritici* (Steinbuch) Bastian, 1865; *Anguillula scandens* Schneider, 1866; *Tylenchus scandens* (Schneider) Cobb, 1890; *Anguillulina scandens* (Schneider) Goodey, 1932

Common names: Wheat seed gall nematode, wheat cockle nematode

Name: *Anguina agrostis* (Steinbuch, 1799) Filipjev, 1936

Synonyms: *Vibrio agrostis* Steinbuch, 1799; *Anguillula agrostis* (Steinbuch) Ehrenberg, 1838; *Tylenchus agrostis* (Steinbuch) Goodey, 1930; *Anguillulina agrostis* (Steinbuch) Goodey, 1932; *Vibrio phalaridis* Steinbuch, 1799; *Anguilllula phalaridis* (Steinbuch) Ehrenberg, 1838; *Tylenchus phalaridis* (Steinbuch) Örley, 1880; *Anguillulina phalaridis* (Steinbuch) Goodey, 1932; *Anguina phalaridis* (Steinbuch) Chizhov, 1980; *Tylenchus agrostidis* Bastian, 1865; *Anguillula agrostidis* (Bastian) Warming, 1877; *Tylenchus phlei* Horn, 1888; *Anguina poophila* Kirjanova, 1952; *Anguina lolii* Price, 1973

Common names: Bentgrass nematode, grass seed nematode

Name: *Anguina funesta* Price, Fisher & Kerr, 1979

Synonyms: *A. lolii* Bird & Stynes, 1977 (in part)

Common name: Seed gall nematode

3. Detection

3.1 Symptoms specific to *Anguina* species

3.1.1 *Anguina tritici* (after Southey, 1972; Krall, 1991)

A. tritici incites seed galls (ear cockles) in cereals (Figure 4(A)). Invasive juveniles emerge from the seed galls in the soil and attack newly germinated seedlings. They establish infection on the tissues of young leaves near the growing point where they feed as an ectoparasite causing leaf distortion and crinkling (Figure 4(B)). Infected hosts become stunted and exhibit shorter and deformed stems and leaves (Figures 4(B) and 4(C)). Severely infected plants do not form ears or form only stunted ears on stunted stems. A diseased ear is much wider and shorter than a normal ear and has short deformed awns (Figure 4(C)). Slight elevations occur on the upper leaf surface with indentations on the lower side. Other leaf symptoms include wrinkling, twisting, curling of the margins towards the midrib, distortion, buckling, swelling and bulging. A tight spiral coil evolves, and dwarfing, loss of colour or development of a mottled yellowed appearance, and stem bending may also occur. In severe infection, the entire above-ground plant is distorted to some degree and therefore the disease is usually obvious (CABI, 2015). The second-stage juveniles stimulate the formation of galls in floral tissues in place of seed development. Galls vary from light and dark brown to almost black (Figure 5(A)). They are smaller than healthy grains (Figure 5(B)). The nematodes can survive in a quiescent stage in seed galls (Figure 6).

3.1.2 *Anguina agrostis* (after Southey, 1973; Krall, 1991)

A. agrostis is considered to be an economically important nematode pest of bentgrass. In grasses, seed galls are difficult to detect as they are covered by lemmas and paleae. A small scarifier can be used to remove lemmas and paleae without damage to seeds or galls. This allows visual identification of galls (Alderman *et al.*, 2003). Galled flowers have glumes of two or three times the normal length, lemmas five to eight times the normal length, projecting beyond the glumes as a sharp point, and paleae developing to about four times the normal length. Galls are at first greenish, and later become dark purple–brown. They reach 4–5 mm long (Figures 7 and 8). Lodicules, stamens and sometimes other flower parts are suppressed in parasitized flowers. Symptoms of the inflorescence also include elongated flower galls, which are modified ovaries that look greenish or purple and may be 4–5 mm long. Seed galls containing the nematodes are dark brown. They may look similar to normal seeds but are less heavy and hence can be separated mechanically from them.

3.1.3 *Anguina funesta*

The life cycle of *A. funesta* is similar to that of *A. agrostis*. During dry summers, *A. funesta* survives within seed galls as anhydrobiotic second-stage juveniles. During winter, the nematodes are released from decaying galls and via water droplets in moist soil they invade new host seedlings, where they feed upon the young leaves. The nematodes congregate near the apical meristems until ovary initiation then stimulate ovary primordia to develop into galls. Occasionally, galls are produced in stamen primordia or, in very heavily infested plants, on glumes or rachis (McCay and Ophel, 1993). Information on the biology of this species can be found in Price *et al.* (1979). Symptoms of infestation are shown in Figures 1 to 3.

3.2 Nematode extraction

3.2.1 Direct examination

Symptomatic foliage and seed suspected to be infested with Anguininae can be processed by dissecting foliage tissue and galls immersed in tap water in a Petri dish. Specimens of motile and immotile stages may be observed under a stereomicroscope, usually within 30 min if the host plant is heavily infested.

Seed cleaning can be achieved most effectively by modern equipment used for this purpose. Galls may be removed by a salt brine method in which the seeds are stirred into a 20% salt solution. Galls and debris float to the surface from where they are skimmed then steamed, boiled or chemically treated to kill the nematodes. The salt solution containing healthy seeds is drained and the seeds are rinsed several times in freshwater to remove the salt, then spread in thin layers on a clean surface to dry.

3.2.2 Extraction from soil and plant material

Detailed descriptions of extraction equipment and procedures can be found in the European and Mediterranean Plant Protection Organization (EPPO) standard on nematode extraction (EPPO, 2013a).

All stages of anguinid nematodes can be extracted from plant tissue, and infective juveniles can also be isolated from soil or growing medium, using the Baermann funnel technique, the modified Baermann tray method (Hooper and Evans, 1993), an adapted sugar flotation method (Coolen and D'Herde, 1972) or the mistifier technique (Hooper *et al.*, 2005). These extraction methods should be conducted for 48 h at room temperature to detect low levels of infestation. Any plant material to be tested should be cut into pieces or sliced before extracting, for increased efficacy of extraction. The number of infective juveniles that may be recovered from soil depends on soil type, sampling depth, host plant and seasonal factors (Hooper, 1986). A large amount of fresh organic matter in the soil sample (e.g. plant residue after harvest) can influence nematode numbers because of its decomposition process, which might be toxic to nematodes or increase the number of saprophytic nematodes, or because the organic matter hampers extraction by clogging sieves or contaminating the supernatant obtained in density-based methods.

The Baermann funnel technique (and modifications of it, such as the tray method, or Seinhorst mistifier, described by Hooper (1986)) is a reference technique for extraction of nematodes from soil and plant material. A piece of rubber tubing is attached to the stem of a glass funnel (with a recommended slope of approximately 30 degrees) and is closed by a spring or screw clip. The funnel is placed in a support and filled almost to the top with tap water. A plastic sieve or wire basket with a large enough aperture size to allow nematodes to actively pass through is placed just inside the rim of the funnel. Plant tissue cut into small pieces or soil is placed either directly onto the mesh or onto a single-ply tissue supported by the mesh, and the water level is adjusted so the substrate is only just submerged. Active nematodes pass through the mesh and sink to the bottom of the funnel stem. Alternatively, funnels made of plastic or stainless steel, or tubing made of silicone, can be used. However, regarding the latter, diffusion of oxygen into water is lower than for polyethylene (Stoller, 1957), which can lead to slow asphyxiation of the nematodes. Depending on the plant tissue, most (50–80%) of the motile nematodes present will be recovered within 24 h; however, samples can be left on the funnel for up to 72 h to increase the recovery rate. For longer extraction periods, regular tapping of the funnel and addition of freshwater increases nematode motility and compensates for evaporation and lack of oxygen, thereby improving the recovery rate. The efficacy of extraction can also be improved by adding 1–3% hydrogen peroxide (H_2O_2) for oxygen supply (Tarjan, 1967, 1972). Following the extraction period, a small quantity of water containing the nematodes is run off and observed under a stereomicroscope (Flegg and Hooper, 1970).

Motile and immotile nematodes can be extracted from plant material by the sugar flotation method (Coolen and D'Herde, 1972). The plant material is washed, cut into pieces of about 0.5 cm, and 5 g portions are macerated in 50 ml tap water in a domestic blender at the lowest mixing speed for 2 min. The suspension of nematodes and tissue fragments is washed through a 750 µm sieve placed on top of

a 45 µm sieve. The residue on the 45 µm sieve is collected and poured into two 50 ml centrifuge tubes. About 1 ml kaolin is added to each tube, the mixture is thoroughly stirred and then it is centrifuged at 1500 *g* for 5 min. The supernatant is decanted and sucrose solution (density 1.13 g/cm^3) is added to the tubes. The mixture is thoroughly stirred and centrifuged at 1800 *g* for 4 min. The supernatant is washed through a 45 µm sieve, the residue is collected and the nematodes are studied under a stereomicroscope. Instead of sucrose, zinc sulphate ($ZnSO_4$), magnesium sulphate ($MgSO_4$) or colloidal silica can be used.

3.2.3 Extraction from seed

Infective juveniles can be extracted from infested seed using a number of methods, including the Baermann funnel technique, which is summarized in section 3.2.2. A comparative study of the efficacy of various methods of extraction from seed, including water-agar blend, sieve blend, misting and blender-funnel-host stimulant, are presented by Griesbach *et al.* (1999).

In the blender-funnel-host stimulant method, which is described in Griesbach *et al.* (1999) as the most effective method for the recovery of *A. agrostis* from *Dactylis glomerata* and *Agrostis* spp. seed, 50 g seed is placed in a blender with 300 ml tap water and blended for 15 s, shaken, and blended again for 15 s. The mixture is placed on a single tissue draped on an 850 µm pore size sieve supported over a large funnel containing tap water. Approximately 0.1 g orchardgrass leaves are added to the funnel as a stimulant, and the water column is aerated. Check valves at the funnel base enable the suspension to be drawn off after 24–48 h. The suspension is finally passed through a 25 µm pore size sieve before examination.

4. Identification

The scope of this diagnostic protocol is to facilitate identification of *Anguina* to the genus level. Both morphological (section 4.1) and molecular (section 4.2) approaches are presented.

4.1 Morphological identification

Information for morphological identification of valid genera within the Anguinidae is provided in section 4.1.2.1. Species of Anguinidae are probably one of the most variable groups among the Tylenchina regarding morphological characters (Brzeski, 1998). Identification to species can be unreliable if morphological characters are used in isolation; information regarding biology, host plant and symptoms of infection should also be taken into consideration. Often, only juveniles are found in seed galls, which can further complicate identification as important morphological features in adult specimens cannot be observed. Morphological information for the three species of economic importance is provided in section 4.1.2.2; however, this information should be used in combination with other sources to confirm diagnosis. Keys to species have been provided by Krall (1991) (ten species) and Brzeski (1998) (four species recorded from temperate Europe).

4.1.1 Preparation of nematode specimens

As with other species of plant-parasitic nematodes, morphological observation should be carried out on as many adult specimens as possible. There are numerous published methods for fixing and processing nematode specimens for study, most recently summarized in Manzanilla-López and Marbán-Mendoza (2012). Nematodes processed with anhydrous glycerol are recommended for examination as important taxonomic features can be obscured if specimens are not cleared sufficiently.

If possible, permanent slides should be prepared for future reference and deposited in nematode reference collections. Methods of preparing permanent slide mounts of nematodes have been described in detail elsewhere (Seinhorst, 1962; Hooper, 1986). The slow evaporation method as described by Hooper (1986), which preserves the structures and characteristics of the nematodes, is outlined in section 4.1.1.2.

Temporary microscope slide preparations can be made quickly for instant examination but such slides may remain usable for only a few weeks.

4.1.1.1 Temporary preparations

A small drop of water is placed on a glass cavity slide, enough to sufficiently fill the well. The nematode specimens are transferred to the water and heated to 65 °C. It is critical that the heat should be applied just long enough to kill the nematodes, as prolonged heating will result in their distortion and deterioration. In practice, 10–15 s on a hotplate will be sufficient time for most species, but it is recommended that the slide be checked at intervals to monitor progress and removed from the heat only when movement of all the nematodes has ceased.

A glass slide, free of dust, is placed on the side of the microscope stage. A small drop of single strength triethanolamine and formalin (TAF) fixative (10 ml formalin[1] (35% formaldehyde in water) mixed with 1 ml triethanolamine and 89 ml distilled water) or another appropriate fixative is put in the centre of the slide and an appropriate amount of paraffin wax shavings is positioned around the drop (the wax will help support the coverslip and seal it to the slide).

The nematodes are transferred from the cavity slide to the fixative so that they are positioned beneath the meniscus in the centre of the drop and not overlapping one another. The number of specimens able to fit on a slide will vary according to the size of the nematodes.

An appropriately sized coverslip is carefully cleaned with lens tissue. It is gently lowered onto the wax shavings so that contact is made with the drop of fixative. The slide is placed on a hotplate and monitored until the wax has just melted; the air that may be lodged under the coverslip is removed by gently tapping the slide. The slide is then removed from the heat and examined.

There should be a clear area of fixative containing the nematodes in the centre and a complete ring of wax to seal the slide.

Should the seal be broken or the nematodes become embedded in the wax, the slide is heated again, the coverslip carefully removed, and the nematodes recovered and remounted on a new slide. If the wax has spread beyond the coverslip, it is cleared away with a fine blade.

The coverslip is sealed with a ring of clear nail varnish. When the varnish has dried, the specimens are ready for study.

4.1.1.2 Permanent preparations

A small drop of water is placed on a glass cavity slide, enough to sufficiently fill the well. The nematode specimens are transferred to the water and heated to 65 °C. It is critical that the heat should be applied just long enough to kill the nematodes, as prolonged heating will result in their distortion and deterioration. In practice, 10–15 s on a hotplate will be sufficient time for most species, but it is recommended that the slide be checked at intervals to monitor progress and removed from the heat only when movement of all the nematodes has ceased.

The nematodes are transferred to an embryo dish or suitable watch glass half full of single strength TAF fixative (7 ml formalin[1] (40% formaldehyde in water) mixed with 2 ml triethanolamine and 91 ml distilled water). The dish is covered and left to fix for a minimum of one week.

The specimens are transferred to a watch glass containing a 3% glycerol solution with a trace amount of TAF fixative. The nematodes should be submerged. A coverslip is placed over the watch glass and left on it overnight.

The coverslip is slightly moved so that a small gap is produced to allow evaporation, and the watch glass is left in an incubator (approximately 40 °C) until all the water has evaporated (this may take one week or longer). At the same time, a small beaker of glycerol is placed in the incubator to ensure it becomes anhydrous.

[1] Formalin comprises 35–40% formaldehyde in water.

A small drop of the anhydrous glycerol is dispensed using a syringe or dropper onto the centre of a glass slide and the nematodes are transferred to this, arranged centrally.

Three coverslip supports, such as glass beads, of similar diameter to that of the nematodes are placed at intervals in the margin of the glycerol drop so that they form an even support.

Small amounts of paraffin wax shavings are placed at regular intervals around the circumference of the glycerol drop.

A coverslip is heated on a hotplate for a few seconds. The coverslip is cleaned with lens tissue and gently lowered on to the wax, so that contact is just made between coverslip and glycerol.

The slide is placed on the hotplate and as soon as the wax has melted and any air bubbles have been expelled by the settling coverslip, the slide is removed from the heat and the wax is allowed to reset.

When the wax is completely hard, any excess wax is removed from around the coverslip with a scalpel.

The coverslip is sealed with a ring of sealant such as Glyceel or clear nail varnish. The slide is labelled with an indelible marker, or affixed with a slide label. Information includes classification, date of slide preparation, host, locality, sample number (if applicable) and method of preservation.

4.1.2 Morphological identification

4.1.2.1 Morphological identification at the genus level

Comparative morphology of genera assigned to the Anguininae is presented in Table 1. Definitions of terminology used in the following sections can be found in EPPO's *Diagnostic protocols for regulated pests: Pictorial glossary of morphological terms in nematology* (EPPO, 2013b).

Diagnosis of the Anguininae and the genus *Anguina* has been described by Siddiqi (2000), as follows and as described in Table 1, with key characters for identification shown in **bold**. Medium to large in size (1.0–2.7 mm), obese; **mature female curved generally in one to one-and-a-half spirals. Metacorpus muscular.** Basal bulb in adults enlarged, continuous or offset from isthmus by a constriction, base usually extending over anterior end of intestine. Ovary with one or two flexures anteriorly due to excessive growth; **oocytes in multiple rows, arranged about a rachis. Crustaformeria a long tube formed by a large number of cells in multiple irregular rows.** Spermatogonia in multiple rows. **Bursa subterminal.** Second-stage juveniles generally resistant, and the infective stage. Obligate plant parasites incite galls in seeds of cereals and grasses as well as stems, leaves and inflorescence of various monocotyledonous plants; type species causes wheat seed galls (ear cockles); only *A. amsinckiae* and *A. balsamophila* are known to parasitize dicotyledonous plants.

There has been little recent morphological work on the genus and no reliable and up-to-date morphological keys to species are available. Therefore, identification at the genus level is described with summary information only for the three economically important pest species *A. tritici*, *A. Agrostis* and *A. funesta*.

Genus	Galls	Female body shape	Crustaformeria Quadri-columella	Rachis	Vulval flaps	Post-uterine sac	Median pharyngeal bulb	Terminal bulb	Incisures in lateral fields	Excretory pore	Guber-naculum	Bursa	Tail shape
Litylenchus Zhao *et al*., 2011	–	Slender and semi-obese	+	–	–	+	+, non-muscular	Abuts	4	Posterior to nerve ring	+	Extends to near tail tip	Conoid
Anguina Scopoli, 1977	+	Obese, spiral	–	+	–	+	+, non-muscular or muscular	Base extends over intestine	4, unclear	Posterior to nerve ring	+	Small, subterminal	Conoid
Diptenchus Khan, Chawla & Seshadri, 1969	? (roots)	Slender	+	–	–	–	+, muscular	Abuts	5	At base of pharynx	+	To two-thirds tail length	Conical
Ditylenchus Filipjev, 1936	– (+ from India)	Slender	+	–	–	+	+, non-muscular or muscular	Abuts or overlaps a little	4 or 6	Posterior to nerve ring	+	Adanal to subterminal	Elongate conoid to filiform
Indotylenchus Sinha, Choudhury & Baqri, 1985	? (mangroves)	Slender	Not available	–	–	+	+, muscular	Offset	4	Anterior to median bulb	+	To two-fifths tail length	Elongate conoid
Nothanguina Whitehead, 1959	+	Obese, spiral	–	+	–	+	–	Offset or small dorsal overlap	Not available	Posterior to nerve ring	-	To half tail length	Conoid
Nothotylenchus Thorne, 1941	–	Slender	+	–	–	+	+, non-muscular	Offset from intestine	4 or 6	Posterior to nerve ring	+	To half tail length	Elongate conoid
Orrina Brzeski, 1981	+	Slender	+	–	–	+	+, non-muscular	Overlaps a little	4	Posterior to nerve ring	+	To two-thirds tail length	Conoid
PseudhalenchusTarjan, 1958	Not available	Slender	+	–	–	+	+, muscular	Overlaps	4	Posterior to nerve ring	+	To one-third tail length	Elongate conoid
Pterotylenchus Siddiqi & Lenné, 1984	+	Slender	–	–	+	+	–	Overlaps dorsally	4	Posterior to nerve ring	Not available	Not available	Elongate conoid
Safianema Siddiqi, 1980	– (fungal feeder)	Slender	+	–	–	+	+, muscular	Overlaps laterally	6	Posterior to nerve ring	+	Adanal to subterminal	Elongate conoid to filiform
Subanguina Paramonov, 1967	+	Slender or semi-obese	–	–	–	+	+, muscular	Abuts or overlaps	Not available	Posterior to nerve ring	+	Subterminal	Conoid
Zeatylenchus Zhao *et al*., 2013	–	Slender to semi-obese	+	–	–	+	+, non-muscular, fusiform	Subventral glands overlap intestine	Unable to discern in ♀, 3 at mid-body in ♂	In region of retracted stylet	+	Leptoderan, to*ca* 30% distance to tail tip	Conoid, terminus with a ventral spike

Table 1. Comparative morphology and feeding habits of Anguininae

Source: After Zhao *et al.* (2011, 2013).

4.1.2.2 Morphological identification of selected **Anguina** *species*

Anguina tritici

Description after Southey (1972) and Krall (1991). Refer to Table 2, and Figure 9 and Figure 10.

Mature females. Body obese, spirally coiled ventrally. Lip region low and flattened, slightly offset, cephalic framework weak. Cuticle very finely annulated. Procorpus often swollen by gland secretions but constricted at junction of metacorpus. Isthmus sometimes posteriorly swollen, offset from pharyngeal glands by a deep constriction. Pharyngeal glands not overlapping, or with very slight overlap of intestine. Excretory pore near junction of pharynx and intestine or slightly more posterior. Vulval lips protruding, orifice of small glands visible on vulval lips anterior and posterior to vulva. Ovary with two or more flexures, often reaching to pharyngeal region with oogonia in multiple rows arranged around a rachis. Spermatheca pyriform, separated from oviduct by a sphincter. Postvulval uterine sac present. Tail conoid, tapering to an obtuse or rounded tip, not mucronate.

Males. More slender than females. Habitus upon heat relaxation curved either ventrally or dorsally. Testis with one or two flexures. Spicules stout, arcuate, with two ventral ridges running from tip to widest part. Capitulum with distinct ventral folding at anterior. Gubernaculum simple, trough-like. Bursa leptoderan. Tail conoid, tip rounded or obtuse.

Second-stage juveniles. Body slender, not spirally coiled. Tail conoid, pointed (Figure 10). For measurements, see Table 2.

Anguina agrostis

Description after Southey (1973) and Krall (1991). Refer to Tables 1 and 2, and Figures 11 and 12.

Mature females. Based on specimens from the type host. Body obese, C-shaped to spirally coiled ventrally. Lip region low and flattened, offset by a fine constriction. Cuticle marked by fine annulations. Lateral fields not discernible on fully developed adult females, six incisures are visible on immature specimens. Neither procorpus nor isthmus exhibiting marked swellings, the former slightly contracted at its junction with the metacorpus. Isthmus occasionally folded in mature specimens by forward pressure of the gonad. Pharyngeal glands not overlapping, or with very slight overlap of intestine. Vulval lips prominent. Ovary usually with two flexures, often reaching to pharyngeal region with oogonia in multiple rows arranged around a rachis. Spermatheca pyriform, separated from oviduct by a constriction. Postvulval uterine sac present, 36–63% of vulva–anus distance. Tail conoid with an acute terminus.

Males. Smaller and more slender than females. Habitus upon heat relaxation curved ventral to almost straight. Lateral field difficult to discern on mature specimens, reported to have six incisures. Testis usually reflexed once, spermatocytes arranged about a rachis. Spicules more slender in build than those of *A. tritici*, the capitulum showing little or no ventral folding at anterior. Two ventral ridges running from tip of each spicule to widest part, before converging and joining the capitulum. Gubernaculum simple, bursa leptoderan, ending just short of acute or finely mucronate tail tip.

Second-stage juveniles. Body slender, not spirally coiled; tail conoid, pointed (Figure 12(A)). For measurements, see Tables 2 and 3.

Anguina funesta

Description after Price *et al.* (1979). Refer to Table 1 and Table 2, and Figure 13.

Mature females. Length of postvulval uterine sac 62–112 µm; stylet length 7–10 µm. Young females are fully motile, but older females, in which gross expansion of the ovary has occurred, are strongly ventrally curved and capable of only weak movements of head and tail. Habitus following heat relaxation ventrally curved forming a complete circle, with head and tail overlapping. Lips slightly offset and rounded in front, cephalic framework lightly cuticularized. Stylet with conus and shaft of

roughly equal length and with well-developed knobs. Pharynx 64–178 µm long with wide procorpus opening to a muscular metacorpus, ovate to spheroid, 17–25 µm long. Pharyngeal–intestinal junction obscured by the large dorsal gland, 45–72 µm in length, ovate to spatulate with a prominent nucleus. Pharyngeal glands slightly overlapping the intestine. Hemizonid between base of metacorpus and anterior end of dorsal pharyngeal gland, at 80–100 µm from anterior. Excretory pore located more posteriorly, 105–155 µm from anterior. Lateral field difficult to discern. Vulva with prominent lips. Anterior ovary with one or two flexures. Rarely, the gonad not reflexed but rather extended anteriorly to the base of the procorpus. Oocytes arranged in three or four rows about a rachis, except near the base of the ovary where this increases to five or six rows. Spermatheca ovate or elongated, long and 25–40 µm wide. Crustaformeria long and slender, made up of more than four columns of cells and up to 350 µm long, separated from spermatheca and uterus by short constrictions, 10–15 µm long. Crustaformeria often containing sperm cells and up to eight eggs. Uterus thick-walled, 70–100 µm long. Postvulval uterine sac approximately the same length as the uterus, 62–112 µm. Tail 48–112 µm long, vulva–anus distance 86–73 µm. Body width at anus approximately half that at vulva. Tail occasionally bluntly rounded, more usually conically pointed, sometimes with a mucronate tip.

Males. Males shorter and thinner than females. Habitus upon heat relaxation straight or slightly curved. Lip and pharyngeal regions of the male similar to that of the female, although dorsal pharyngeal gland larger, almost rectangular, 50–78 µm long. Hemizonid and excretory pore 71–90 µm and 102–147 µm from anterior, respectively. Lateral field with five or six incisures, broken at intervals and occupying one-quarter to one-third of body width. Testis nearly always reflexed once. Testis with spermatocytes in multiple rows about a rachis. Spicules paired, non-fused and arcuate, each 16–28 µm long with characteristic bulges on the manubrium and where the manubrium and shaft join. Gubernaculum slim and trough-like. Bursa leptoderan, extending almost to tail tip, 44–114 µm in length. Tail 43–72 µm long with terminus conically pointed. Body width at cloaca 17–43 µm. For measurements, see Tables 2 and 3.

Table 2. Morphometric data for *Anguina tritici*, *Anguina agrostis* and *Anguina funesta*

Morphometric characters	**Range, numerous populations**		
	A. tritici	***A. agrostis* (restricted to *Agrostis* spp.)**	***A. funesta***
Mature females			
L (mm)	3.0–5.2	1.3–2.7	1.65–2.44
Stylet (µm)	8–11	8–12	7–10
a (nematode body length/greatest width (usually at mid-body))	13–30	13.8–25.4	16.8–20.1
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	9.8–25.0	8.0–28.7	9.3–34.0
c (body length/tail length)	24–63	25.2–44.0	18.1–41.2
V (%)	70–95	87–92	86.9–94.0
Males			
L (mm)	1.9–2.5	1.05–1.68	0.78–1.52
Stylet (µm)	8–11	10–12	7–10
a (nematode body length/greatest width (usually at mid-body))	21–30	23–38	20.3–30.9
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	6.3–13.0	6–9	6.3–9.5
c (body length/tail length)	17–28	20.0–28.4	16.1–24.9
Spicules (µm)	35–40	25–40	16–28
Gubernaculum (µm)	Approximately 10	10–14	9–14
Second-stage juveniles			
L (mm)	0.75–0.95	0.55–1.25	0.81–0.87
Stylet (µm)	Approximately 10	Approximately 10	7–10
a (nematode body length/greatest width (usually at mid-body))	47–59	44–71	48–53
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	4.0–6.3	3.2–6.1	4.2–4.6
c (body length/tail length)	23–28	11.7–20	12.3–15.1

Source: After Southey (1972, 1973), Price *et al.* (1979), Chizhov (1980), Krall (1991), Brzeski (1998) and Meng *et al.* (2012).
L, length; V, distance from the anterior end to the vulva divided by nematode body length (%).

Additional data for populations of *A. agrostis* and *A. funesta* (infective juveniles) are presented in Table 3.

Table 3. Morphometric data of juveniles of an *Anguina funesta* population from annual ryegrass, an *Anguina agrostis* population from bentgrass and an *Anguina agrostis* population from orchardgrass from commercial seed production fields in Willamette Valley, Oregon, the United States of America

Characters (*n* = 20)	**Mean ± standard deviation (range) (µm)**		
	***A. funesta* (annual ryegrass)**	***A. agrostis* (bentgrass)**	***A. agrostis* (orchardgrass)**
Body length	836.2 ± 14.6 (815.9–865.7)	795.0 ± 33.8 (726.4–875.6)	739.8 ± 20.0 (726.4–796.0)
Genital primordium to posterior	396.7 ± 31.1 (351.5–480.2)	354.9 ± 25.0 (311.9–415.8)	350.0 ± 21.6 (297.0–381.2)
Tail length	63.3 ± 3.3 (55.9–68.0)	57.7 ± 2.1 (53.5–60.8)	61.5 ± 4.2 (55.9–70.5)
Anterior to excretory pore	122.5 ± 2.5 (119.1–128.8)	122.2 ± 2.1 (116.6–126.4)	122.5 ± 2.5 (119.1–128.8)
Pharyngeal length	183.0 ± 7.1 (172.5–194.4)	186.9 ± 10.5 (158.0–199.3)	183.7 ± 8.8 (167.7–194.4)
Genital primordium length	20.4 ± 1.5 (18.0–23.0)	16.5 ± 1.5 (13.5–19.0)	18.1 ± 1.8 (15.0–23.0)
Genital primordium width	8.8 ± 1.2 (6.0–11.0)	7.5 ± 0.9 (6.0–10.0)	8.1 ± 0.7 (7.0–10.0)
Body width	16.6 ± 0.7 (15.0–18.0)	14.0 ± 0.5 (13.0–15.0)	14.6 ± 0.6 (14.0–15.0)
Metacorpus length	17.0 ± 1.1 (15.0–19.0)	16.2 ± 1.6 (12.0–19.0)	15.9 ± 1.0 (15.0–17.0)
Metacorpus width	8.5 ± 0.5 (8.0–9.0)	8.7 ± 0.8 (7.5–10.0)	7.9 ± 0.7 (7.0–9.0)
Stylet length	8.0 ± 1.0 (7.0–10.0)	8.0 ± 1.0 (7.0–10.0)	8.0 ± 0.7 (7.0–10.0)
Anterior to base of stylet	10.1 ± 0.3 (10.0–11.0)	10.1 ± 0.5 (8.0–11.0)	10.5 ± 0.4 (10.0–11.0)
Pharyngeal length	0.9 ± 0.4 (0.5–1.5)	1.2 ± 0.4 (0.5–1.5)	1.0 ± 0.3 (0.5–1.5)
a (nematode body length/greatest width (usually at mid-body))	50.4 ± 1.8 (48.0–52.9)	56.9 ± 3.7 (49.1–62.8)	50.6 ± 2.1 (45.4–54.7)
b (nematode body length/pharynx length from lips to pharyngo-intestinal valve)	4.6 ± 0.2 (4.2–4.6)	4.3 ± 0.2 (4.0–4.7)	4.0 ± 0.2 (3.8–4.4)
c (body length/tail length)	13.2 ± 0.8 (12.3–15.1)	13.8 ± 0.6 (12.8–15.0)	12.1 ± 1.0 (11.0–15.4)
Anterior to excretory pore as % of length	14.6 ± 0.3 (14.4–16.0)	15.4 ± 0.6 (14.2–16.7)	16.6 ± 0.5 (15.3–17.1)
Genital primordium to tail as % of length	47.4 ± 3.6 (43.2–56.8)	44.7 ± 2.7 (36.2–49.7)	47.3 ± 3.0 (39.8–51.8)

Source: Reproduced from Meng *et al.* (2012), courtesy Plant Management Network, *Plant Health Progress*.

4.2 Molecular identification

This section provides information on molecular tests that allow the identification of isolated nematodes of the major *Anguina* species. The tests are generally performed following a morphological examination in order to confirm the results obtained.

Molecular diagnosis of *Anguina* spp. is based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) (Powers *et al.*, 2001), real-time PCR (Ma *et al.*, 2011; Li *et al.*, 2015) or sequencing of the internal transcribed spacer (ITS) region of ribosomal (r)RNA (Subbotin *et al.*, 2004). The choice of test depends on whether identification requires confirmation of both the presence and the absence of particular species, and on the availability of species standards for controls. The method described by Ma *et al.* (2011) is limited to positive identification of *A. agrostis*, while the other methods are able to simultaneously distinguish multiple species within the same test. PCR combined with analysis of RFLP is the most common way in which to simultaneously distinguish a range of *Anguina* species from each other (Powers *et al.*, 2001).

Powers *et al.* (2001) first sequenced the ITS1 region for *Anguina* spp. Subbotin *et al.* (2004) subsequently sequenced 58 populations of *Anguina*, *Ditylenchus*, *Heteroanguina* and *Mesoanguina* for phylogenetic analysis. There are 71 sequence accessions of rRNA fragments obtained from *Anguina* spp. collected from different localities and host plants presently available in the United States National Center for Biotechnology Information (NCBI) public database.

ITS DNA PCR fragments may also be used for DNA sequence analysis, as described in section 4.2.5.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.1 DNA extraction

A single juvenile (or adult, if available) is processed for PCR by placing it in a 15 µl drop of double distilled sterile water on a glass slide and manually disrupting it by cutting it into several pieces with a knife under a stereomicroscope (Powers and Harris, 1993).

The nematode pieces in 8 µl double distilled sterile water are transferred to a microcentrifuge tube containing, for example and depending on size and number of nematodes, 10 µl nematode extraction buffer (10 mM Tris, pH 8.2; 2.5 mM $MgCl_2$; 50 mM KCl; 0.45% Tween 20; 0.05% gelatin; 60 µg/ml proteinase-K) (Thomas *et al.*, 1997) and frozen at −70 °C for 15 min or until needed. The extract is thawed and incubated at 60 °C for 60 min then the proteinase-K is denatured by heating at 95 °C for 15 min. In the protocol by Ma *et al.* (2011) the nematode is cut in 8 µl double distilled water and this suspension is transferred to a tube containing 1 µl PCR buffer with 1 µl proteinase-K (1 µg/ml), with freezing as described above and incubation at 65 °C for 60 min followed by 95 °C for 10 min.

There are no published protocols designed specifically for bulk DNA extraction from *Anguina* spp.; however, methods described for other nematodes can be adapted as required. For example, the commercially available QIAamp DNA Micro Kit (Qiagen[2]) was used for DNA extraction from reniform nematodes following Baermann extraction and sugar centrifugal flotation to isolate nematodes from soil (Sayler *et al.*, 2012). Quantification of extracted DNA is measured with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific[2]).

[2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.2 ITS1 rRNA PCR-RFLP for identification of *Anguina* spp. (Powers *et al.*, 2001)

The ITS rRNA universal primers described in this test are:

rDNA2 (forward): 5′-TTGATTACGTCCCTGCCCTTT-3′ (Vrain *et al.*, 1992)

rDNA1.58S (reverse): 5′-ACGAGCCGAGTGATCCACCG-3′ (Cherry *et al.*, 1997)

The PCR and the cycling parameters as described by Szalanski *et al.* (1997) are presented in Table 4. Alternatively, the amplification can be conducted according to Meng *et al.* (2012) (Table 5). After PCR, 5 µl of the product is analysed electrophoretically on a 1.5% agarose gel in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer. The gel can be stained with ethidium bromide and photographed using a gel imaging system with an ultraviolet light filter.

The PCR products are purified with the Geneclean II Kit (MP Biomedicals[2]) or a similar PCR purification kit. The restriction enzymes *Alu*I, *Bsr*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I and *Taq*I are required for identifying *Anguina* spp. The reactions are conducted separately (one tube for each enzyme) and according to the individual enzyme manufacturer's recommendations (Table 6). A positive restriction control should be included in the RFLP step to confirm the success of the enzymatic digestion.

The lengths of the restriction fragments generated by these diagnostic enzymes and the restriction pattern of each species are given in Table 7.

Table 4. ITS1 rRNA conventional PCR master mix composition, cycling parameters and amplicons (after Szalanski *et al.*, 1997)

Reagent	Final concentration
PCR-grade water	–†
PCR buffer (including $MgCl_2$)	1×
dNTPs	0.8 mM
Primer rDNA2 (forward)	0.4 mM
Primer rDNA1.58S (reverse)	0.4 mM
DNA polymerase	2.5 U
DNA (volume)	1 µl
Cycling parameters‡	
Initial denaturation	94 °C for 3 min
Number of cycles	40
Denaturation	94 °C for 45 s
Annealing	55 °C for 1 min
Elongation	72 °C for 2 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	From 547 to 553 bp for *Anguina* spp. (except for *Astrebla* genus: 575 bp)

† For a final reaction volume of 50 µl.

‡ According to the DNA polymerase manufacturer's instruction.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

Table 5. ITS1 rRNA conventional PCR master mix composition, cycling parameters and amplicons (after Meng *et al*., 2012)

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
$MgCl_2$	1 mM
dNTPs	0.4 mM
Primer rDNA2 (forward)	0.2 mM
Primer rDNA1.58S (reverse)	0.2 mM
DNA polymerase (GoTaq Flexi (Promega[2]))	2.0 U
DNA (volume)	15 µl
Cycling parameters‡	
Initial denaturation	94 °C for 3 min
Number of cycles	40
Denaturation	94 °C for 1 min
Annealing	55 °C for 1 min
Elongation	72 °C for 1 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	From 547 to 553 bp for *Anguina* spp. (except for *Astrebla* genus: 575 bp)

† For a final reaction volume of 50 µl.

‡ According to the DNA polymerase manufacturer's instruction.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rRNA/DNA, ribosomal RNA/DNA.

Table 6. Master mix composition, template, reaction conditions and amplicons for RFLP

Reagent	Final concentration
PCR-grade water	–†
Enzyme mix	1×
Restriction enzyme	10 U
PCR product (volume)	6 µl
Reaction conditions‡	37 °C or 65 °C for 8 h
Expected amplicons	
Size	See Table 7

† For a final reaction volume of 14 µl.

‡ 37 °C for all enzymes except *Taq*I, for which incubation should be performed at 65 °C.

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table 7. Restriction fragment sizes for *Anguina* species and associated restriction fragment length polymorphism (RFLP) patterns (after Powers *et al.*, 2001)

Species	*Alu*I	*	*Bsr*I	*	*Eco*RI	*	*Hae*III	*	*Hha*I	*	*Hinf*I	*	*Taq*I	*
A. agrostis	548	A	295, 238, 15	B	299, 249	A	548	A	548	A	448, 100	B	355, 135, 58	A
A. agropyronifloris	547	A	547	C	547	B	317, 230	B	547	A	447, 100	B	489, 58	B
A. funesta	548	A	295, 238, 15	B	299, 249	A	548	A	548	A	448, 100	B	490, 58	B
A. graminis	548	A	310, 238	B	548	B	548	A	548	A	249, 199, 100	C	490, 58	B
A. microlaenae	550	A	550	C	301, 249	A	550	A	550	A	449, 52, 49	D	357, 135, 58	A
A. pacificae	549	A	239, 225, 85	D	549	B	319, 230	B	549	A	448, 101	B	491, 58	B
A. tritici	277, 274	B	550	C	550	B	550	A	462, 88	B	550	E	492, 58	B
A. sp. / *Dactylis*	550	A	297, 253	B	550	B	320, 230	B	550	A	252, 198, 100	C	357, 135, 58	A
A. sp. / *Agrostis*	553	A	301, 237, 15	B	553	B	333, 220	B	303, 250	C	454, 99	B	360, 135, 58	A
A. sp. / *Polypogon*	553	A	301, 237, 15	B	553	B	333, 220	B	553	A	454, 99	B	360, 135, 58	A
A. sp. / *Stipa*	548	A	467, 81	A	299, 249	A	548	A	548	A	375, 98, 46, 29	F	355, 135, 58	A
A. sp. / *Astrebla*	359, 216	C	575	C	575	B	575	A	575	A	401, 128, 46	A	517, 58	B
A. sp. / *Holcus*	550	A	310, 240	B	301, 249	A	550	A	550	A	450, 100	B	303, 135, 58, 54	C

* Code for the RFLP profile for each restriction enzyme.

4.2.3 TaqMan real-time PCR for identification of *Anguina agrostis* (Ma *et al.*, 2011)

This test developed by Ma *et al.* (2011) was designed as a species-specific real-time PCR to identify juveniles of *A. agrostis.* It was evaluated against the target species *A. agrostis* as well as non-target species *A. tritici*, *A. wevelli* and *Ditylenchus destructor.* While described for the "detection" of nematodes isolated from seed galls or plant material, the test was not specifically evaluated for its ability to quantify nematodes from quarantine samples.

The ITS rRNA species-specific primers described in this test are:

PF: 5′-GTTTGCCTACCGGTTGTTTACG-3′

PR: 5′-CCACATGCAGTCGGTGTGAA-3′

TaqMan probe Pb: 5′-FAM-TCATGTCTTGGCTATTGTAGACGTATCTGA-TAMRA-3′

The amplification is performed in a real-time PCR using the LightCycler (Roche[2]) according to the cycling parameters described in Table 8.

Table 8. Real-time PCR master mix composition, cycling parameters and amplicons (after Ma *et al.*, 2011)

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
$MgCl_2$	1.25 mM
dNTPs	0.2 mM
Primer PF (forward)	0.4 µM
Primer PR (reverse)	0.4 µM
Probe Pb	0.02 µM
DNA polymerase	0.5 U
DNA (volume)	1 µl
Cycling parameters	
Initial denaturation	94 °C for 3 min
Number of cycles	45
Denaturation	94 °C for 10 s
Annealing	60 °C for 30 s
Expected amplicons	
Size	88 bp

† For a final reaction volume of 10 µl.

bp, base pairs; PCR, polymerase chain reaction.

4.2.4 Real-time PCR for identification of *Anguina agrostis*, *A. funesta*, *A. pacificae* and *A. tritici* (Li et al., 2015)

Li *et al.* (2015) designed a TaqMan real-time PCR to identify *A. agrostis*, *A. funesta*, *A. pacificae* and *A. tritici.* This test includes forward and reverse genus-specific primers combined with a fluorescent probe (modified with TET dye and BHQ-2 Black Hole Quencher[2]). This primers and probe set was designed to serve as an internal control for confirming the presence of *Anguina* spp. as well as the integrity of the PCR components and user performance. The test also includes primers and probe sets specifically designed for the detection of each of the target species mentioned above and is intended for identification of single juveniles. Species-specific probes were modified with 6-FAM and BHQ-1 and were simultaneously detectable on a different fluorescent channel in duplex PCRs (i.e. with the species-specific and genus-specific primers and probe sets). The sensitivity of the test was demonstrated through construction of standard curves from reactions using serially diluted nematode DNA: the test was able to detect as little as 1.25 copies of the ITS rDNA. The specificity of each primers and probe set was demonstrated in singleplex and duplex reactions (i.e. with the species-specific and genus-specific primers and probe sets) tested against all of the target species as well as several non-target nematodes including *Anguina* spp., *Meloidogyne* spp., *Pratylenchus* spp. and *Ditylenchus* spp.

The ITS rRNA genus- and species-specific TaqMan primers and probes described in this test are:

***A. agrostis* (AAfpr primers-probe set)**

AAf (forward): 5′-CGGTTGTTTACGGCCGT-3′

AAr (reverse): 5′-ATGTAGTCGGTGTGAAAACAGCCAT-3′

AAp (probe): 5′-6-FAM/ATCATGTCTTGGCTATTGTAGACGTATCTG/BHQ-1-3′

***A. funesta* (AFfpr primers-probe set)**

AFf (forward): 5′-GGTTGCTTACGGCCC-3′
AFr (reverse): 5′-GTGTAATCGATGTGATACAGCCCC-3′
AFp (probe): 5′-6-FAM/ATCATGTCTTGGCTATTATAGACGTATCTG/BHQ-1-3′

***A. pacificae* (APfpr primers-probe set)**

APf (forward): 5′-ACCGGTTGAATATTGGCTGT-3′
APr (reverse): 5′-ATGTAATCGATGTGAAACAGCCGT-3′
APp (probe): 5′-6-FAM/ATCATGTCTTGGAAAGTTTAGACGTATCTG/BHQ-1-3′

***A. tritici* (ATfpr primers-probe set)**

ATf (forward): 5′-GTTGCCTACGGCCGT-3′
ATr (reverse): 5′-ATGTAATCGATGTGGTACAGCCAT-3′
ATp (probe): 5′-6-FAM/ATCATGTCTTGGCTAGTGTAGACGTATCTG/BHQ-1-3′

***Anguina* spp. (ASfpr primers-probe set)**

ASf (forward): 5′-GTCTTATCGGTGGATCACTCGG-3′
ASr (reverse): 5′-TGCAGTTCACACCATATATCGCAG-3′
ASp (probe): 5′-TET/TCATAGATCGATGAAGAACGCAGCCA/BHQ-2-3′

The amplification reaction is performed in a real-time PCR using the SmartCycler II real-time PCR system (Cepheid[2]) according to the cycling parameters described in Table 9.

Table 9. Real-time PCR master mix composition, cycling parameters and amplicons (after Li *et al.*, 2015)

Reagent	**Final concentration**
PCR-grade water	–†
PCR buffer (including $MgCl_2$)	1×
$MgCl_2$	6.0 mM
dNTPs	0.24 mM
Species-specific primer (forward)	240 nM
Species-specific primer (reverse)	240 nM
Species-specific probe	120 nM
ASf internal control primer (forward)	160 nM
ASr internal control primer (reverse)	160 nM
ASp internal control probe	120 nM
DNA polymerase (Platinum Taq (Invitrogen[2]))	1.0 U
DNA (volume)	1 µl
Cycling parameters	
Initial denaturation	95 °C for 20 s
Number of cycles	40
Denaturation	95 °C for 1 s Optics OFF
Annealing	60 °C for 40 s Optics ON
Ramp	5 °C per s

Expected amplicons	
Size	74–85 bp

† For a final reaction volume of 25 µl.

bp, base pairs; PCR, polymerase chain reaction.

4.2.5 DNA sequence analysis of ITS1 and ITS2 rRNA

Molecular phylogenies of *Anguina* spp. provide a foundation for species identification based on alignment of rDNA sequences, including ITS1-partial 5.8S as described by Powers *et al.* (2001) or ITS1-5.8S–ITS2 as described by Subbotin *et al.* (2004). Sequences obtained from new isolates or unknown species are thus placed within the context of known species boundaries and phylogenetic relationships.

For amplification of complete ITS1, 5.8S rDNA and ITS2, the primers used are:

TW81 (forward): 5′-GTTTCCGTAGGTGAACCTGC-3′ (Joyce *et al.*, 1994)

AB28 (reverse): 5′-ATATGCTTAAGTTCAGCGGGT-3′ (Howlett *et al.*, 1992)

Alternatively, the following primer pair can be used:

rDNA2 (also known as 18S) (forward): 5′-TTGATTACGTCCCTGCCCTTT-3′ (Vrain *et al.*, 1992)

rDNA1 (also known as 26S) (reverse): 5′-TTTCACTCGCCGTTACTAAGG-3′ (Vrain *et al.*, 1992)

The PCR is run with the composition and cycling parameters shown in Table 10. The PCR products are analysed by agarose gel electrophoresis. DNA fragments are extracted from the gel using commercially available reagents (e.g. Qiaquick Gel Extraction Kit (Qiagen[2])) and sequenced with the same primers as for PCR. Alternatively, PCR products are cloned into a plasmid vector (e.g. TOPO TA cloning vector (Thermo Fisher Scientific[2]) or StrataClone vector (Agilent Technologies[2])) and transformed into competent *Escherichia coli*. Plasmid clones are isolated from transformed bacteria using blue-white colony selection and sequenced using universal vector primers (Zheng *et al.*, 2000).

The size of the complete ITS1-5.8S–ITS2 region is approximately 675 base pairs for *Anguina* spp.

DNA sequence alignment methods are numerous and rapidly evolving. DNA alignments with sequences obtained from GenBank are constructed with ClustalW, Clustal Omega or MAFFT from the European Bioinformatics Institute (available from http://www.ebi.ac.uk/Tools/msa) or by alignment plug-in modules within the commercial software packages Geneious (Biomatters[2]) and Chromas (Technelysium[2]). Pairwise genetic distances are calculated for all sequence combinations and expressed as percentage similarity or absolute number of nucleotide differences per aligned pair. Interspecific variation that exceeds the intraspecific variation generally indicates separation of species. A high degree of sequence similarity to named species should confirm results from PCR-RFLP and yield a definitive species diagnosis. A match to a previously identified unnamed population may yield the best possible conclusion for the circumstances.

Table 10. DNA sequence analysis of ITS1 and ITS2 rRNA: PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–†
Taq incubation buffer (Taq PCR Core Kit (Qiagen[2]))	1×
5× Q-solution (Taq PCR Core Kit (Qiagen[2]))	1×
dNTPs	0.2 mM
TW81 (forward)	1.5 µM
AB28 (reverse)	1.5 µM
DNA polymerase (Taq PCR Core Kit (Qiagen[2]))	0.8 U
DNA (volume)	10 µl
Cycling parameters	
Initial denaturation	94 °C for 4 min
Number of cycles	35
Denaturation	94 °C for 1 min
Annealing	55 °C for 1 min 30 s
Elongation	72 °C for 2 min
Final elongation	72 °C for 10 min
Expected amplicons	
Size	675 bp

† For a final reaction volume of 100 µl.

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

4.2.6 Controls for molecular tests

For the test result to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For PCR a positive nucleic acid control, a negative amplification control (no template control) and a negative extraction control are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction), and specifically the amplification. Pre-prepared (stored) nucleic acid of the target nematode may be used.

Negative amplification control (no template control). This control is necessary for conventional PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction. The control comprises nucleic acid extraction and subsequent amplification of extraction buffer only. It is recommended that multiple controls be included when large numbers of positive samples are expected.

Positive digestion control (for RFLP only). This control is used to monitor the efficiency of the enzymatic digestion. Amplicon obtained from pre-prepared (stored) nucleic acid of target nematode may be used, as long as the nematode has been accurately identified as belonging to one of the species described in the RFLP patterns.

4.2.7 Interpretation of results from PCR

4.2.7.1 Conventional PCR and PCR-RFLP

The pathogen-specific PCR will be considered valid only if these criteria are met:

- the positive control produces the correct size amplicon for the target nematode species
- the negative extraction control and the negative amplification control produce no amplicons of the correct size for the target nematode species
- the restriction enzyme patterns reveal only the bands expected for the species, with no additional bands and no missing bands.

4.2.7.2 Real-time PCR

The real-time PCR will be considered valid only if these criteria are met:

- the positive control produces an amplification curve with the species-specific primers and probe
- the negative extraction control and the negative amplification control produce no amplification curve or no exponential curve
- in the case of the Li *et al.* (2015) test, the genus-specific primers and probe produce an amplification curve in the presence of test sample DNA, indicating the presence of intact nematode DNA and integrity of the PCR components.

A sample will be considered positive if it produces an exponential amplification curve. If a cycle cut-off value is needed, its value has to be verified in each laboratory when implementing the test for the first time.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability: preserved or slide-mounted specimens, photographs of distinctive taxonomic structure, DNA extracts and photographs of gels.

For morphological evidence, critical features as outlined in the morphological section should be drawn or photographed while fresh material is available, and relevant measurements should be included.

Good photomicrographs (or scanning videos) of key morphological features are likely to be important for record keeping.

6. Contacts Points for Further Information

Further information on this protocol can be obtained from:

Nematology Laboratory, United States Department of Agriculture (USDA), Agricultural Research Service (ARS), 10300 Baltimore Ave., Bldg 010A BARC West, Rm 113, Beltsville, MD 20705, United States of America (Andrea Skantar; e-mail: Andrea.Skantar@ars.usda.gov; tel.: +1 301 504 5917).

Agri-Food and Biosciences Institute, Newforge Lane, Belfast BT9 5PX, United Kingdom (Colin Fleming; e-mail: Colin.Fleming@afbini.gov.uk).

Nematology Unit, Fera Science Limited, National Agri-Food Innovation Campus, Sand Hutton, York, YO41 1LZ, United Kingdom (Thomas Prior; e-mail: Colin.Fleming@afbini.gov.uk; tel.: +44 1904 462206).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on

Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by Andrea Skantar (Nematology Laboratory, USDA-ARS, United States of America (see preceding section)), Colin Fleming (Agri-Food and Biosciences Institute, United Kingdom, Northern Ireland (see preceding section)) and Thomas Prior (Nematology Unit, Fera Science Limited, United Kingdom (see preceding section)).

8. References

The present annex may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. Healthy *Lolium rigidum* seed (left), *Anguina funesta* gall (centre) and nematode gall colonized by *Rathayibacter toxicus* (right).
Photo courtesy I. Riley, South Australian Research and Development Institute (SARDI), Adelaide, Australia.

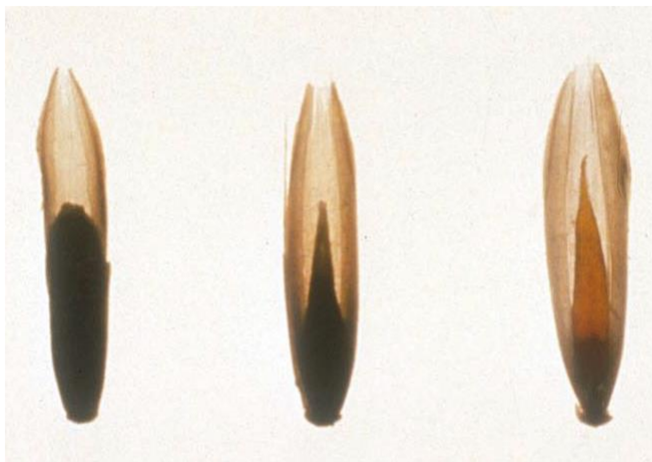


Figure 2. Healthy *Lolium rigidum* seed (left), *Anguina funesta*-infested nematode gall (centre) and *Anguina funesta*-infested bacterial gall (right).
Photo courtesy J. Allen, Western Australia Department of Agriculture and Food, Perth, Australia.

Figure 3. Gumming disease of *Lolium* due to *Rathayibacter toxicus*.
Photo courtesy J. Allen, Western Australia Department of Agriculture and Food, Perth, Australia.

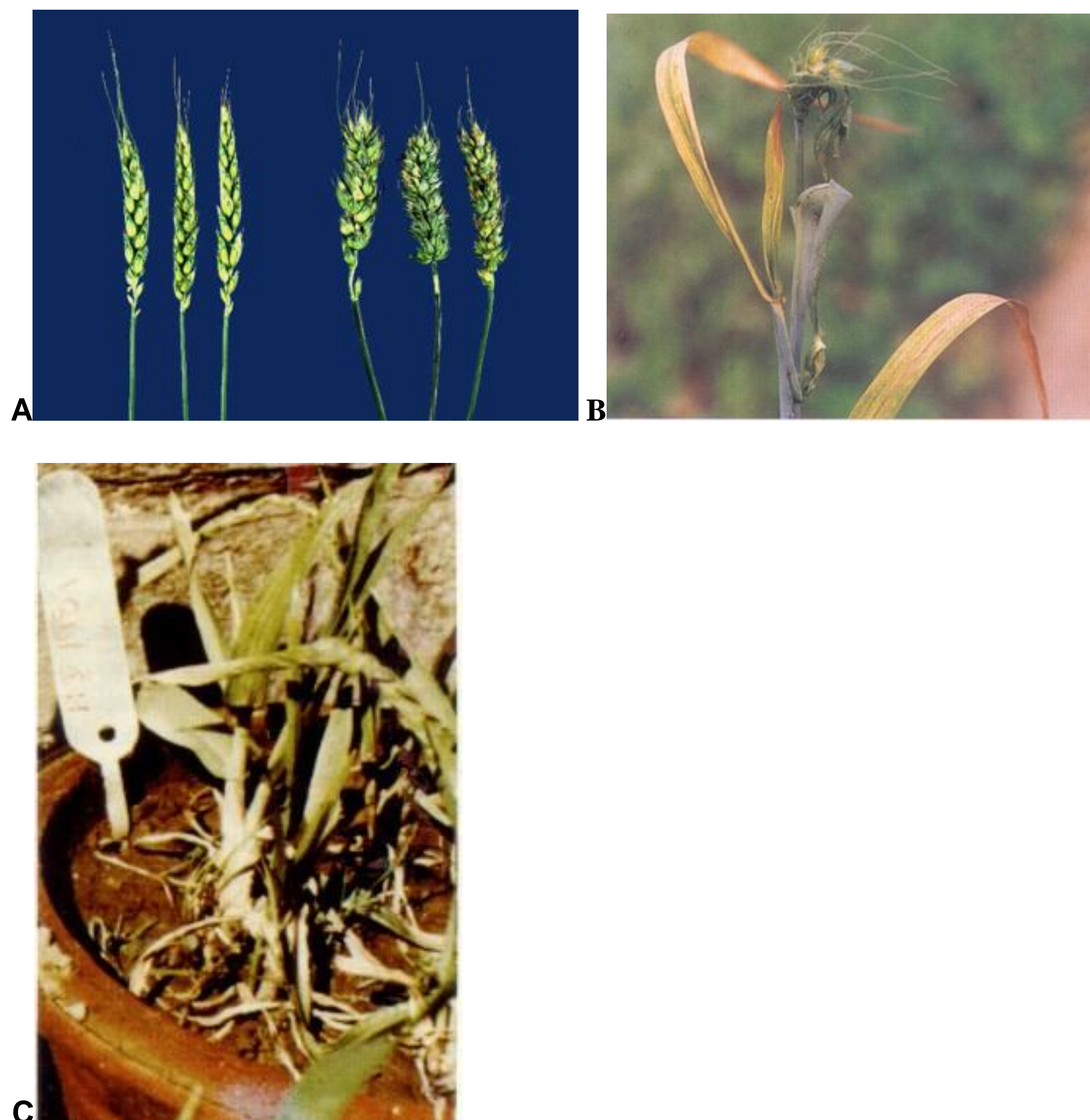


Figure 4. (A) Healthy *Triticum aestivum* ears (left) and ears infested with *Anguina tritici* (right). (B) and (C) Symptoms of infestation of *T. aestivum* seeds with *A. tritici.*
Photos (A) © Howard Ferris, University of California, Davis, CA, United States of America, 1999; (B) courtesy Fera, United Kingdom; and (C) courtesy J. Swarup, India.

Figure 5. (A) Healthy *Triticum aestivum* seeds (left) and seeds infested with *Anguina tritici* (right).
Photo © Ulrich Zunke, University of Hamburg, Germany.

Figure 5. (B) Comparison of colour and shape of healthy *Triticum aestivum* seeds (left) and seeds infested with *Anguina tritici* (right).
Photo courtesy T. Kościuch, Poland.

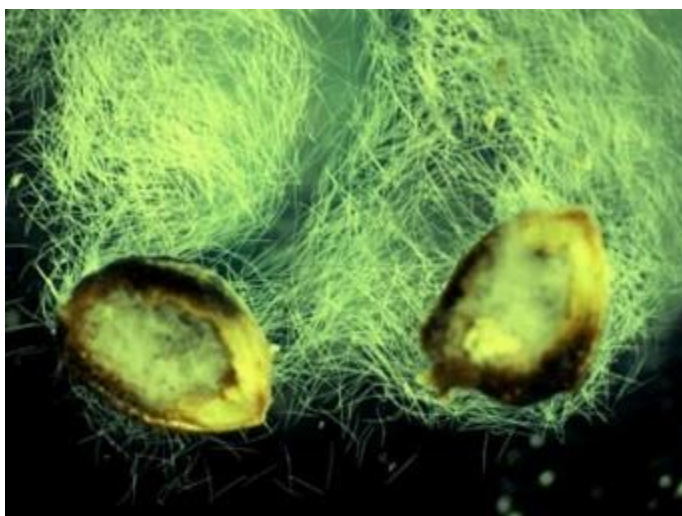


Figure 6. Invasive juveniles of *Anguina tritici* survive in a quiescent state within a seed gall, emerging to infest germinated seedlings.
Photo © Howard Ferris, University of California, Davis, CA, United States of America, 1999.

Figure 7. *Agrostis* plants infested with *Anguina agrostis*.
Source: Pscheidt and Ocamb (2015, part of Ohio State University Extension Plant Pathology Slide).

Figure 8. Agrostis plants infested with *Anguina agrostis*.
Photo © Malcolm Storey, 2011–2015.

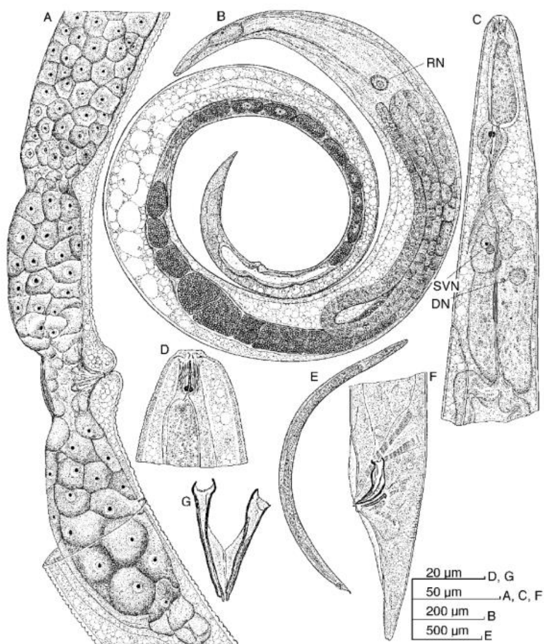


Figure 9. *Anguina tritici* from wheat grain: (A) vulval region showing a surface view of the uterus and postvulval uterine sac; (B) female; (C) oesophageal region of male; (D) head end of female; (E) male; (F) tail end of male; and (G) spicules. DN, nucleus of dorsal oesophageal gland; SVN, nuclei of subventral glands; RN, nucleus of renette cell.
Reproduced from Siddiqi (2000), courtesy CABI.

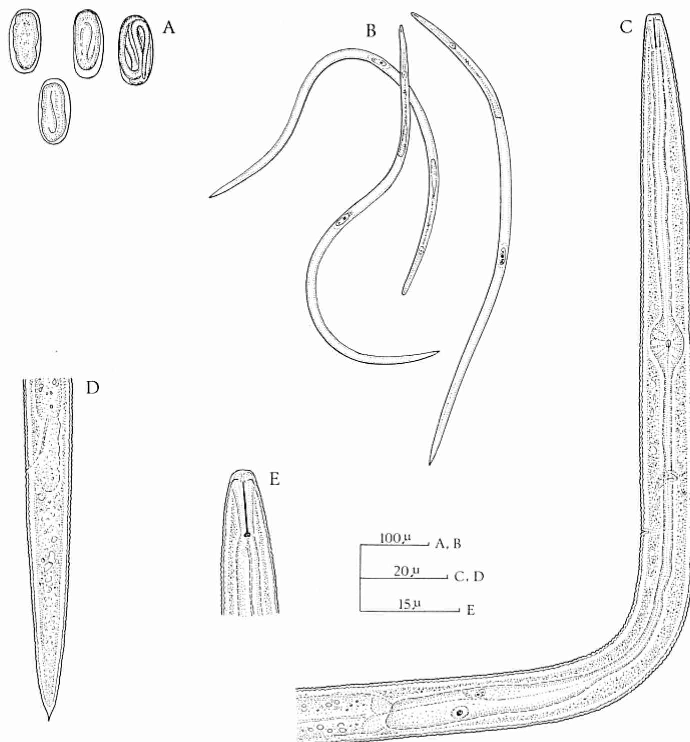


Figure 10. *Anguina tritici* from wheat: (A) eggs and (B–E) second-stage juveniles showing (C) pharyngeal region, (D) tail and (E) lip region.
Reproduced from Southey (1972), courtesy CABI.

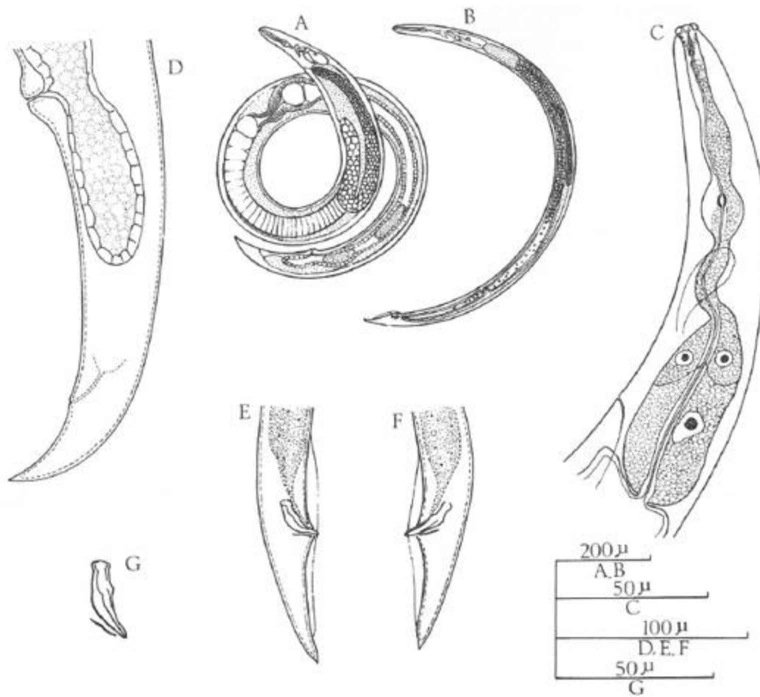
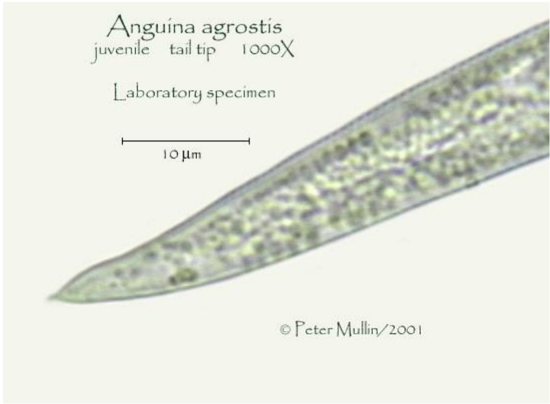


Figure 11. *Anguina agrostis*: (A) female; (B) male; (C) female pharyngeal region; (D) female tail; (E–F) male tails; and (G) spicule and gubernaculum.
Reproduced from Southey (1973), courtesy CABI.

A

B 

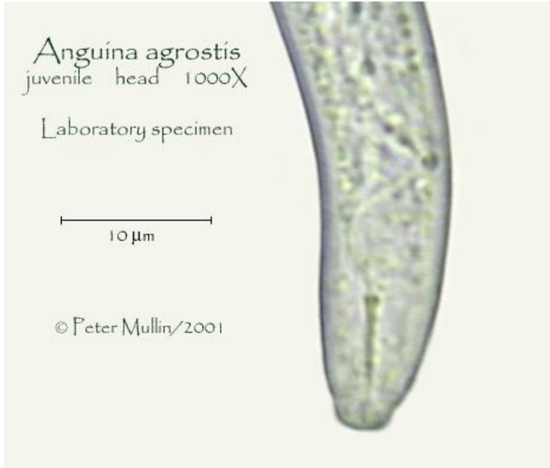
C 

Figure 12. *Anguina agrostis* juvenile: (A) whole nematode; (B) tail; and (C) head.
Source: University of Nebraska-Lincoln Nematology Lab (n.d.). Photo © Peter Mullin, 2001.

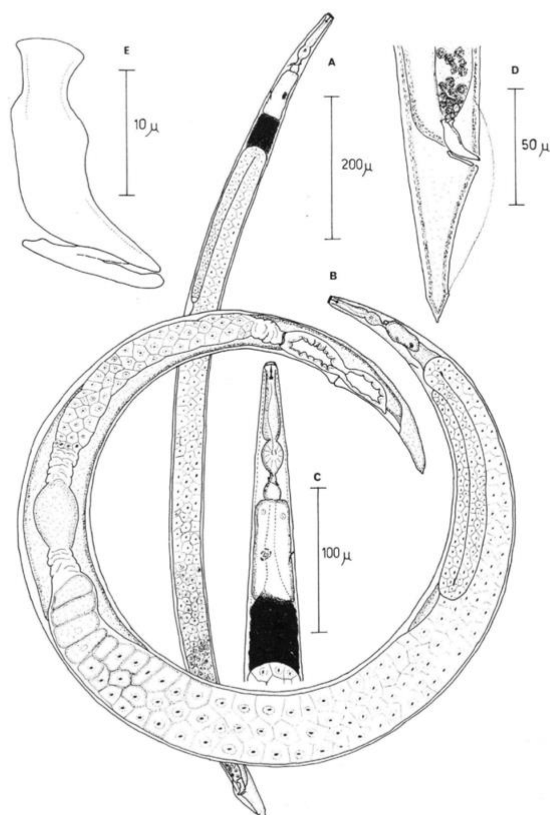


Figure 13. *Anguina funesta*: (A) adult male; (B) adult female; (C) male anterior; (D) male tail; and (E) spicule and gubernaculum.
Reproduced from Price *et al.* (1979), courtesy *Nematologica*.

Publication history

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2013-05 SC added subject *Anguina* spp. (2013-003) to the work programme under topic *Nematodes* (2006-008).

2015-03 Expert consultation on draft DPs.

2015-06 TPDP revised draft.

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2016-02 First consultation.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

International Plant Protection Convention (IPPC)

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