Molecular and morphological characterisation of *Sphaeronema alni* Turkina & Chizhov, 1986 (Nematoda: Sphaeronematidae) from Spain compared with a topotype population from Russia

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**Summary** – The occurrence of a male-less population of *Sphaeronema alni* parasitising chestnut (*Castanea sativa*) roots and inducing a stelar syncytium is reported for the first time in Pola de Somiedo (Oviedo province), Spain. Morphometric and molecular characters of the Spanish population matched those of a topotype population from Russia. SEM observations showed swollen females having the first lip annulus wider than the second and appearing as a cap-like, circumoral elevation. The second-stage juveniles, having a single band in the lateral fields, were characterised by a non-annulated dome-shaped lip region derived from the fusion of the oral disc with all the lip sectors and lip annuli, and showing slit-like amphidial apertures and an oval prestoma. The sequences of the D2-D3 expansion segments of 28S rRNA, partial 18S rRNA and ITS rRNA gene for the Spanish and topotype populations of *S. alni* were congruent and matched those deposited in GenBank for another population from Germany, thereby confirming their conspecificity. A PCR-RFLP profile of D2-D3 of 28S rRNA for identification of this species was also provided. The phylogenetic relationships between *S. alni* populations and representatives of the suborder Criconematina, as inferred from analysis of partial 18S rRNA and D2-D3 of 28S gene sequences obtained in this and previous studies, indicated that *S. alni* formed a basal clade on the majority consensus Bayesian phylogenetic trees, standing together with *Meloidoderita* sp. or alone. These findings provide additional evidence of the need to clarify the position of *Sphaeronema* within Criconematina and its relationships with representatives of Tylenchulinae.

**Keywords** – 18S rRNA, 28S rRNA, D2-D3, *Castanea sativa*, histopathology, ITS1-5.8S-ITS2, morphology, morphometrics, new record, PCR-RFLP, phylogeny, SEM, taxonomy.

A population of *Sphaeronema* Raski & Sher, 1952 was found in northern Spain during a nematode survey conducted in natural areas of the country. The nematode was parasitising the feeder roots of chestnut (*Castanea sativa* L.) trees growing in a sandy soil near Pola de Somiedo (Oviedo province) and represents a new record for the Iberian Peninsula. A preliminary morphological identification indicated that this Spanish population resembled *S. alni* Turkina & Chizhov, 1986, a species described in Russia and reported also in Germany, Poland, the Slovak Republic and the UK (Turkina & Chizhov, 1986; Brzeski, 1998; Sturhan & Geraert, 2005; Prior *et al.*, 2009). The
current taxonomic status of S. alni in the family Tylenchulidae has been questioned by Sturhan and Geraert (2005) because the juveniles of this species and other tylenchulids have distinct phasmids which are absent in other genera (Paratylenchus Micoletzky, 1922, Cacopaurus Thorne, 1943 and Tylenchocricenoma Raski & Siddiqi, 1975) from the same family. A subsequent phylogenetic study using the D2-D3 expansion region of 28S rRNA of a Russian S. alni population (Subbotin et al., 2005, 2006) supported these arguments and cast doubt on the monophyly of Tylenchulidae that is accepted in the literature (Siddiqi, 2000). In order to determine the correct identification of S. alni from Spain, to augment the morphological data listed in the original description of this species and to confirm the new taxonomic position of Sphaeronomia in Tylenchulidae as inferred by Subbotin et al. (2005, 2006) and Sturhan and Geraert (2005), a study was done to: i) characterise morphologically and morphometrically the Spanish population and topotypes of S. alni and compare morphological and molecular characters with those of previous descriptions; ii) study the histopathology of nematode feeding sites in chestnut roots naturally infected by S. alni; iii) sequence the Spanish population and topotypes of S. alni using the D2-D3 28S rRNA, ITS1-5.8S-ITS2, and partial 18S rRNA gene of the Spanish population and topotypes of S. alni from Russia; and iv) reconstruct the phylogenetic position of S. alni within the suborder Criconematina Siddiqi, 1980 using D2-D3 expansion segments of 28S rRNA and partial 18S rRNA gene sequences obtained in this and previous studies (Subbotin et al., 2005) for S. alni populations and other Criconematina species.

Materials and methods

Nematode populations

Specimens of S. alni from Spain were obtained from moist sandy soil in the rhizosphere and feeder roots of chestnut (C. sativa) in Pola de Somiedo (Oviedo province), northern Spain (43°06′28.84″N latitude, 6°15′40.87″W longitude) at an altitude of 781 m a.s.l. The topotype population from Russia was obtained from feeder roots of Alnus glutinosa (L.) Gaertn., A. incana (L.) Moenchand and Populus tremula L. in Maikovo village, Pushkin district, Moscow region, Russia (56°04′9.50″N latitude, 37°54′155″E longitude) at an altitude of 195 m a.s.l. In addition, a German population from A. glutinosa previously studied by Subbotin et al. (2005) was used for further molecular analyses. Nematodes were extracted from soil samples by magnesium sulphate centrifugal flotation (Coolen, 1979).

Light and scanning electron microscopy

Specimens for light microscopy (LM) were killed by gentle heat, fixed in a solution of 4% formaldehyde + 1% propionic acid and processed to pure glycerin using Seinhorst’s (1966) method. Specimens were examined using a Zeiss III compound microscope with Nomarski differential interference contrast at up to ×1000 magnification. Measurements were done using a camera lucida attached to a light microscope. Morphometric data were processed using Statistix 9.0 (NH Analytical Software, Roseville, MN, USA). For SEM studies, fixed specimens were dehydrated in a graded ethanol series, critical point dried, sputter-coated with gold and observed with a JEOL JSM-5800 microscope (Abolafia et al., 2002).

Histopathology

Naturally infected chestnut root segments were gently washed free of adhering soil and debris and individual infected root portions were selected together with healthy roots. Root tissues were fixed in formaldehyde chromo-acetic solution for 48 h, dehydrated in a tertiary butyl alcohol series (40-70-85-90-100%), and embedded in 58°C (melting point) paraffin wax for histopathology observations. Embedded tissues were sectioned with a rotary microtome. Sections of 10-12 μm thickness were placed on glass slides, stained with safranin and fast-green, mounted permanently in 40% xylene solution of a polymethacrylic ester (Synocril 9122X, Cray Valley Products, NJ, USA), examined microscopically and photographed (Johansen, 1940).

DNA extraction, PCR, cloning, sequencing and RFLP

Nematode DNA from the Spanish and Russian S. alni populations was extracted from single individuals as described by Castillo et al. (2003) and Palomares-Rius et al. (2009). The following primers were used for amplification in the present study: D2-D3 of 28S rRNA: D2A (5′-ACAAATACGGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCGCTACTA-3′) (Subbotin et al., 2006); partial 18S rDNA: (5′-GCTTGTCTCAAAGATA-3′) and SSU_R_81 (5′-TATCCWKCYGGCAAGGTCAC-3′) primers (Palomares-Rius et al., 2008); the
Sphaeronema alni from Spain and Russia

ITS1-5.8S-ITS2: TW81 (5'-GTTTCCGTAGGTGAACC TGC-3') and AB28 (5'-ATATGCTTAAGTTCCGCG GT-3')(Tanha Maafi et al., 2003).

PCR products obtained from Spanish samples were purified after amplification with AccuPrep (Bioneer, CA, USA) gel extraction kits, quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and used for direct sequencing (ITS and D2-D3). For the 18S gene both amplification primers and the internal primer SSU_R_23 (5'-TCTCGCTCGTTATC GGAAT-3') (sequence available at http://www.nematodes.org/barcoding/sourhope/nemoprimers.html) was also used. DNA fragments from two independent PCR amplifications from two different samples were sequenced in both directions with a terminator cycle sequencing reaction kit (BigDye; Perkin-Elmer Applied Biosystems, Warrington, UK) according to the manufacturer’s instructions. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3100 genetic analyser; Applied Biosystems, Foster City, CA, USA) at the University of Córdoba, Spain.

PCR products of ITS and D2-D3 of 28S rRNA obtained from German and Russian samples were purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned as described by Tanha Maafi et al. (2003). Several clones were sequenced in the Genomic Center, University of California, Riverside, CA, USA.

The newly obtained sequences were submitted to the GenBank database under accession numbers: 18S rRNA, GU253916; ITS rRNA, GU253917-GU253921; D2-D3 of 28S rRNA, GU253922, GU253923.

Purified PCR product (10 μl) of D2-D3 of 28S rRNA was digested by one of following restriction enzymes: HinII, MvaI, PstI, or RsaI, in the buffer stipulated by the manufacturer. The digested DNA was run on a 1% TAE buffered agarose gel, stained with ethidium bromide, visualised on UV transilluminator and photographed.

PHYLOGENETIC ANALYSES

The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson et al., 1997) with default parameters with their corresponding published gene sequences, respectively (Subbotin et al., 2005, 2006; Holterman et al., 2006; Vovlas et al., 2006; Bert et al., 2008; van Megen et al., 2009). Outgroup taxa for each dataset were chosen according to the results of previous published data (Subbotin et al., 2005, 2006; Holterman et al., 2006; Bert et al., 2008). Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The best fit model of DNA evolution was obtained using the program MrModeltest 2.2 (Nylander, 2002) with the Akaike Information Criterion in conjunction with PAUP* 4b4a (Swofford, 2003). BI analysis using the GTR + I + G model for each gene was initiated with a random starting tree and was run with four chains for 1.0 × 10⁶ generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 10³ generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades.

Description

Sphaeronema alni Turkina & Chizhov, 1986

(Figs 1-3)

MEASUREMENTS

See Table 1.

DESCRIPTION

Female

Spanish population characterised by translucent white to brown, sub-spherical to lemon-shaped, body with an elongate neck curved ventrally and a protruding vulval cone. First lip annulus 3.3-3.5 μm diam., wider than second annulus, appearing as a cap-like circumoral elevation, set off from neck, prestoma rectangular. Cuticle finely annulated, 6.5-10.0 μm thick at mid-body. Excretory pore located near or at level of pharyngeal median bulb. Stylet robust, nearly twice as long as shaft, knobs small, rounded, 2-3 μm wide. Dorsal pharyngeal gland orifice (DGO) at 3.4-3.7 μm from stylet base. Median bulb prominent rounded to elongated in shape (32-34 × 38-40 μm). Basal bulb enlarged posteriorly, extending into anterior portion of swollen body cavity. Pharyngeal-intestinal junction indistinct. Intestine obscure. Anus pore-like, dorsally situated, 40-58 μm from vulva. Vulva slit-like, large, 25-30 μm long, with prominent protruding, unsculptured vulval lips.
Fig. 1. Light micrographs of *Sphaeronema alni* from Spain. A: Coiled second-stage juveniles (J2) partially released from egg shell; B: Entire J2 body; C, D: Anterior and posterior body portions of J2 with anus (arrow); E-G: Anterior body portion of J2 showing DGO, pharynx and stylet; H, J: Swollen female; I: Excretory pore of adult female (arrow); K: Detail of excretory pore; L, M: Female anterior region and terminal cone. (Scale bars: A, B, H, I, J = 50 μm; C-G, K-M = 20 μm.)
Fig. 2. SEM micrographs of Sphaeronema alni from Spain. A-C: Anterior, entire body and vulval region of adult female. Note the circumoral elevation (A: arrow); D, G, I: Lateral, en face and profile views of second-stage juvenile (J2); E: J2 anus (arrow); F: Detail of annulation and anus position; H: Detail of lateral fields of J2. (Scale bars: A, D, G-I = 5 μm; B = 100 μm; C, E = 20 μm; F = 10 μm.)
Fig. 3. Sphaeronema alni. A-D: Second-stage juvenile. A: Pharyngeal region; B: Entire body; C, D: Tail region. E-I: Mature female. E, F: Entire body; G: Pharyngeal region; H: Ventral view of vulval region; I: Adult parasitising chestnut root.
Table 1. Morphometrics of females and second-stage juveniles (J2) of *Sphaeronema alni* from Spain and Russia. All measurements are in μm and in the form: mean ± s.d. (range).

<table>
<thead>
<tr>
<th>Character</th>
<th>Paratypes (Turkina &amp; Chizhov, 1986)</th>
<th>Spanish population (Pola de Somiedo, Oviedo province)</th>
<th>Topotypes (Maikovo village, Moscow region, Russia)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>J2</td>
<td>Female</td>
</tr>
<tr>
<td>n</td>
<td>19</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>L</td>
<td>252 (185-319)</td>
<td>530 (440-630)</td>
<td>260 ± 19.7 (224-286)</td>
</tr>
<tr>
<td>a</td>
<td>1.5 (1.3-1.8)</td>
<td>31 (23.3-34.6)</td>
<td>1.8 ± 0.28 (1.3-2.8)</td>
</tr>
<tr>
<td>b</td>
<td>– (3.2-4.0)</td>
<td>3.7 (5.8-8.1)</td>
<td>– (5.7-7.6)</td>
</tr>
<tr>
<td>c</td>
<td>– (5.8-8.1)</td>
<td>6.4 (5.7-7.6)</td>
<td>– (5.7-7.6)</td>
</tr>
<tr>
<td>c′</td>
<td>– – (5.7-7.6)</td>
<td>8.5 ± 1.03 (8.2-9.7)</td>
<td>– – (8.2-9.7)</td>
</tr>
<tr>
<td>Max. body diam.</td>
<td>168 (116-232)</td>
<td>150 ± 25.5 (96-206)</td>
<td>16 ± 0.5 (15-17)</td>
</tr>
<tr>
<td>Stylet length</td>
<td>21-23 (19-22)</td>
<td>20.5 ± 0.8 (19.0-22.0)</td>
<td>20 ± 0.5 (19-21)</td>
</tr>
<tr>
<td>O</td>
<td>– – (16.8-19.1)</td>
<td>17.6 ± 2.1 (17.2-17.9)</td>
<td>17.5 ± 1.5 (17.2-17.9)</td>
</tr>
<tr>
<td>Anterior end to excretory pore</td>
<td>– 90-125 (50-80)</td>
<td>69 ± 16.0 (50-80)</td>
<td>96 ± 14.0 (89-115)</td>
</tr>
<tr>
<td>Cuticle thickness</td>
<td>– – (5.7-9.0)</td>
<td>6.5 ± 2.4 (5.7-9.0)</td>
<td>– 5.3 ± 0.9 (5.3-7)</td>
</tr>
<tr>
<td>Pharynx length</td>
<td>– – (131-145)</td>
<td>137 ± 6.0 (131-145)</td>
<td>– 126 ± 4.0 (119-135)</td>
</tr>
<tr>
<td>Vulva-anus distance</td>
<td>– 47 ± 7.3 (40-58)</td>
<td>92 ± 7.3 (40-58)</td>
<td>– 44 ± 4.0 (37-49)</td>
</tr>
<tr>
<td>Tail length</td>
<td>– 65-98 (83-99)</td>
<td>92 ± 7.3 (83-99)</td>
<td>– 87 ± 6.8 (77-96)</td>
</tr>
</tbody>
</table>

**Male**

Not found in Spanish or topotype populations, a fact coincident with data for the British population from downy birch (*Betula pubescens* Ehrh.) in Whitby, UK (Prior et al., 2009).

**Second-stage juvenile (J2)**

Spanish population characterised by a vermiform, translucent white body, tapering at both extremities, but particularly so in tail region. Cuticle marked by fine annuli, 0.70-0.86 μm across. Head cap completely fused with dome-shaped lip region. Lip region lacking annulation. In SEM view, stoma slit-like, prestoma ovoidal. Amphidial apertures slit-like, slightly posterior to lateral edges of prestoma. Lips absent, lip region smooth. Lateral field consisting of two longitudinal incisures delimiting a single lateral band, 2.2-2.5 μm wide at midbody. Labial framework well developed. Stylet strongly developed, cone longer than shaft and knobs. Knobs massive, rounded, posteriorly elongated. Procorpus elongate, muscular, median bulb oval, joined to a pyriform basal bulb by a rather long, slender isthmus. Pharyngeal-intestinal junction conspicuous. Phasmids not observed. Tail very long, often curved ventrally, tapering gradually posterior to anus (c′ = 8.2-9.7), with distinct annulation as seen by SEM. Phasmid-like structure not detected under SEM observations. Tail terminus finely rounded.
Egg

Translucent, oblong, 97 ± 2.6 (93-103) μm long × 47 ± 1.4 (45-50) μm diam., enclosed within a gelatinous matrix of maximum width 349 ± 46 (280-420) μm containing 0-75 eggs/female.

Notes

The morphological and morphometric characters of the females and J2 of the topotype population from Russia did not differ from those of the Spanish and type populations (Table 1).

Remarks

When comparing all the morphometric characters of the S. alni population from Spain, they agree very well with those of the original description, as well as the topotypes collected from Maikovo village, Pushkin district, Moscow region, Russia, a population from Poland (Brzowski, 1998), and a population from downy birch (Prior et al., 2009). Nevertheless, lower values of some characters and ratios such as L, a, b, c, c', total pharynx length, and tail length of J2 were observed in the topotype population compared to those of the type (Turkina & Chizhov, 1986) and the Spanish populations (Table 1). These differences are attributable to intraspecific variability, as confirmed by molecular analyses. The failure to detect phasmids in the studied S. alni populations may be due to inappropriate fixation which obscured these organs. Sturhan and Geraert (2005) observed phasmids in juveniles of all S. alni populations examined and other Sphaeronema sp. from north-eastern Europe and for the first time emphasised their taxonomic relevance. However, these caudal sense organs have been reported in the past in many Sphaeronema species, e.g., S. rumicis Kirjanova, 1970 from USA, S. sasseri and S. whittoni (Sledge & Christie, 1962; Eisenback & Hartman, 1985; Vovlas & Insera, 1986), although their taxonomic value was not properly remarked upon.

The present new record from northern Spain, as well as that from downy birch in UK (Prior et al., 2009), confirm that S. alni is a parasite of woody plants in cool habitats. The soil population densities of this nematode on chestnut were low at the time of our survey. Nematode females protected by a gelatinous matrix and protruding from the root surface (Fig. 4A, B) were easily detected by microscopic examination of the infected roots and provided the specimens used in this study.

Histopathology

Colonies of S. alni were detected on chestnut secondary roots. Swollen females of S. alni protruded from the root surface (Fig. 4A) occurring individually or in clusters without causing distortion of the entire root diameter (Fig. 4A). These females had the anterior body portion inserted into the cortical and periderm tissues and established a permanent feeding site in a multicellular syncytium derived from proliferated phloem tissue (Fig. 4B-C). The mono-nucleate syncytial cells showed granulated cytoplasm and hypertrophied nuclei (7-8 μm diam.) with large nucleoli (3-4 μm diam.). The external cell walls delimiting the mature syncytium were irregularly thickened. These anatomical alterations did not differ from those described for alder roots (Subbotin, 1989), downy birch roots (Prior et al., 2009), or those induced by S. rumicis on cottonwood roots (Vovlas & Insera, 1986).

On the basis of our observations, the host range of S. alni mainly includes the forest trees mentioned above. Unfortunately, biological information on this nematode remains scant.

Molecular characterisation of S. alni and phylogenetic position within Criconematina

Alignments of 18S rRNA gene sequences contained 19 accessions including three outgroup taxa and were 1595 bp in length. Phylogenetic relationships within Criconematina, as inferred from analysis of this gene, are presented in Fig. 5A. Sphaeronema alni formed a highly supported clade with Meloidoderita Poghossian, 1966, which occupied a basal position in the tree.

The sequences of the D2-D3 expansion segments of 28S rRNA of S. alni from Spain and Russia were identical to that of the population from Germany deposited in GenBank. RFLP-D2-D3 of 28S rRNA diagnostic profiles obtained for S. alni is given in Fig. 6. The D2-D3 alignment included 33 sequences and was 585 bp in length. Sphaeronema alni occupied a basal position within Criconematina (Fig. 5B).

The ITS rRNA sequences of S. alni showed high variation in length due to several insertions/deletions and in nucleotide positions. The lengths of analysed fragments for five sequences obtained from three populations varied from 744 to 766 bp with sequence divergence up to 2.7% (20 nucleotides). Differences between two ITS clones obtained from the German population were 2.5% (19 nucleotides) and from the Russian population 0.07% (six nucleotides).
Sphaeronema alni from Spain and Russia

Fig. 4. Histopathology of Sphaeronema alni from Spain infecting chestnut roots. A: Gelatinous matrix, filled with eggs and juveniles, covering mature females; B, C: Histological alterations induced by Sphaeronema alni in chestnut roots, showing syncytium (S). Abbreviations: em = egg mass; j = juvenile. (Scale bars: A = 200 μm; B, C = 100 μm.)

Fig. 5. Phylogenetic relationships within Criconematina: Bayesian 50% majority rule consensus tree from two runs as inferred from (A) partial 18S rRNA gene and (B) D2-D3 of 28S gene sequence alignments using the GTR + I + G model. Posterior probabilities >70% are given for appropriate clades. Newly obtained sequences are indicated by bold letters.
Fig. 6. Restriction fragments of amplified D2-D3 of 28S rRNA gene for Sphaeronema alni. Lane codes: M = 100 bp DNA ladder (Promega); 1 = HinfI, 2 = MvaI, 3 = PstI, 4 = RsaI.

The position of Sphaeronema within Tylenchida and Criconematina was analysed several times and the validity of Tylenchulinae sensu Raski and Luc (1987) was tested using D2-D3 28S rRNA datasets (Subbotin et al., 2005, 2006; Vovlas et al., 2006). Although these analyses either placed Sphaeronema at a basal position to the Criconematina or showed unresolved polytomy with Meloidoderita, maximum likelihood testings could not reject the validity of the Tylenchulinae, where Sphaeronema, Meloidoderita and Tylenchulus Cobb, 1913 formed a clade. Further phylogenetic analysis using longer 28S rRNA fragments and other genes is needed to clarify the position of Sphaeronema within the Criconematida and its relationships with representatives of the Tylenchulinae.

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References


Kirjanova, E.S. (1970). [Sphaeronema rumicis sp. nov. (Nematoda, Sphaeronematidae), the first form of this genus in Europe.] Parasitologiya 4, 489-493.


Spain with approaches to molecular phylogeny of related genera. *Nematology* 11, 343-354.


