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Morphological and molecular characterization of foliar nematodes of the genus *Aphelenchoides*: *A. fragariae* and *A. ritzemabosi* (Nematoda: Aphelenchoididae) from the Main Botanical Garden of the Russian Academy of Sciences, Moscow

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Summary. During surveys in 2003-2004 conducted in glasshouses in the Main Botanical Garden of the Russian Academy of Sciences several fern plants infected by the strawberry crimp nematode, *Aphelenchoides fragariae*, were found. The infected ferns belong to the following species: *Aneimia rotundifolia, Blechnum occidentale, B. gibbum, Pteris longifolia, P. cretica cv. wimsetti, P. cretica* and *Stenochlaena tenuifolia*. Nematode infection produced water-soaked bands that become dark brown to black, usually between the leaf veins. Several plants of *Sambucus racemosa* attacked by the chrysanthemum foliar nematode *A. ritzemabosi* were found in an outdoor area near the glasshouses. Nematodes were extracted from the leaves and growing points of *S. racemosa*. Morphological and morphometrical diagnostic characters and descriptions are given for *A. fragariae* and *A. ritzemabosi*. Differences in the 18S rRNA gene sequences allow a clear separation of these species from each other and from other *Aphelenchoides* species. The phylogenetic relationships of these species with other aphelenchids as inferred from analysis of the 18S-rRNA gene are presented and discussed.

Key words: 18S-rRNA, fern, morphometrics, phylogeny, Russia, *Sambucus racemosa*.

During the summers of 2003 and 2004, nematological surveys to detect foliar nematodes were conducted in the Main Botanical Garden of the Russian Academy of Sciences. Results from the glasshouses revealed several fern plants infected by the strawberry crimp nematode *Aphelenchoides fragariae* (Ritzema Bos, 1891) Christie, 1932. In 1995, in an area about 50 m from the same glasshouses, several plants of red elderberry *Sambucus racemosa* L. infected by the chrysanthemum foliar nematode *Aphelenchoides ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932 were detected by Dieter Sturhan. This paper includes morphometrical characterization of populations of these two species as well as an analysis of phylogenetic relationships of these nematodes with other species of *Aphelenchoides* using 18S rRNA gene sequences.

MATERIAL AND METHODS

Nematode populations. *Aphelenchoides fragariae* and *A. ritzemabosi* were collected from plants from the Main Botanical Garden of the Russian Academy of Sciences. Nematodes were extracted from the leaves and growing points. For comparison, two additional *Aphelenchoides* species were obtained from the nematological collection of the University of

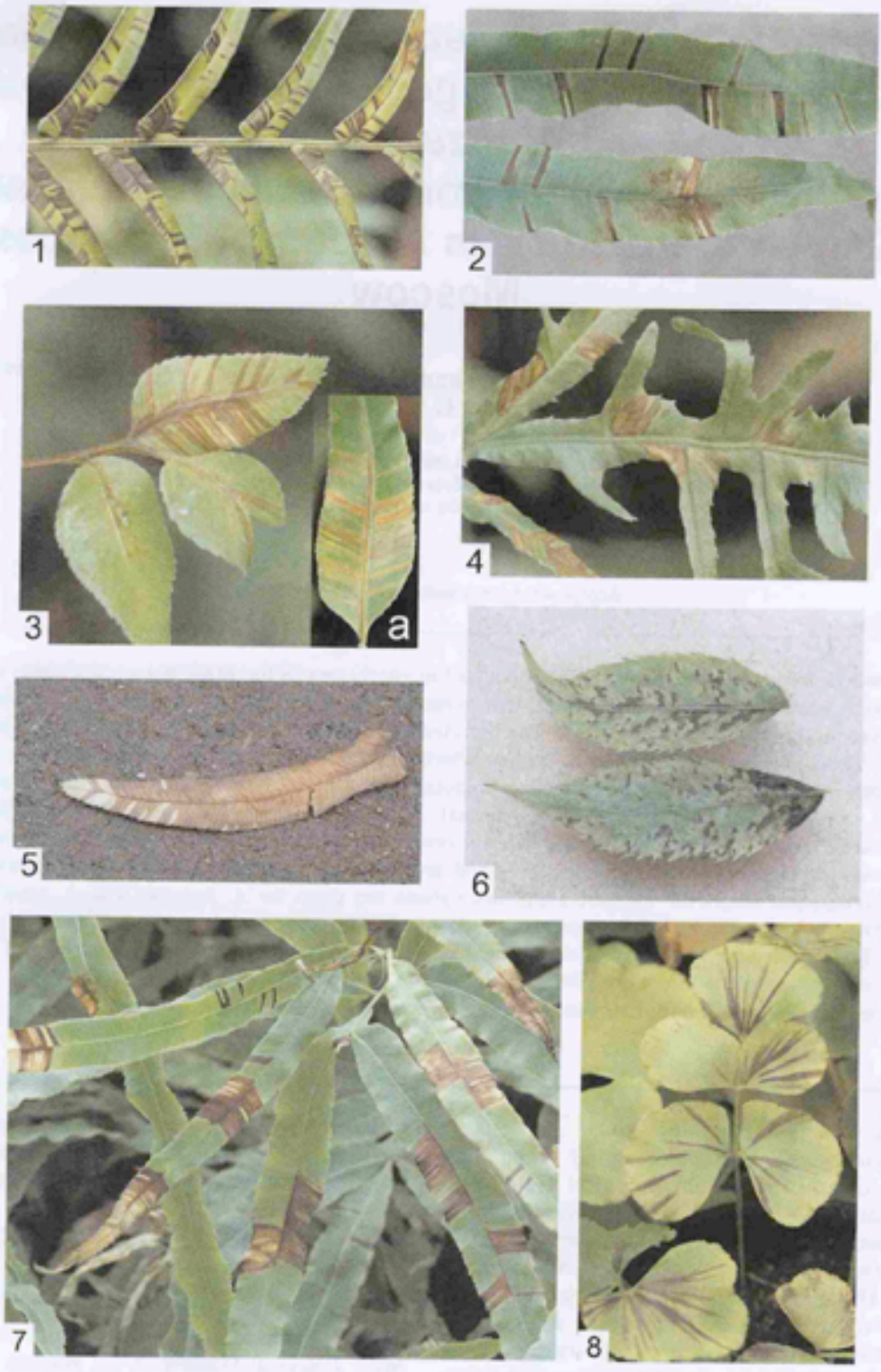

Fig. 1. Symptoms of infection induced by foliar nematodes of the genus *Aphelenchoides*. *Aphelenchoides fragariae*: 1 – *P. longifolia*, 2 - *P. cretica*, 3 – young leaves of *S. tennifolia* (a – old leaf), 4 – *P. cretica wimsetti*, 5 – old leaves of *P. cretica*. 7- infected fronds with *A. fragariae*, 8 – *Aneimia rotundifolia*; *A. ritzemabosi*: 6 - infected leaves of *Sambucus racemosa*.

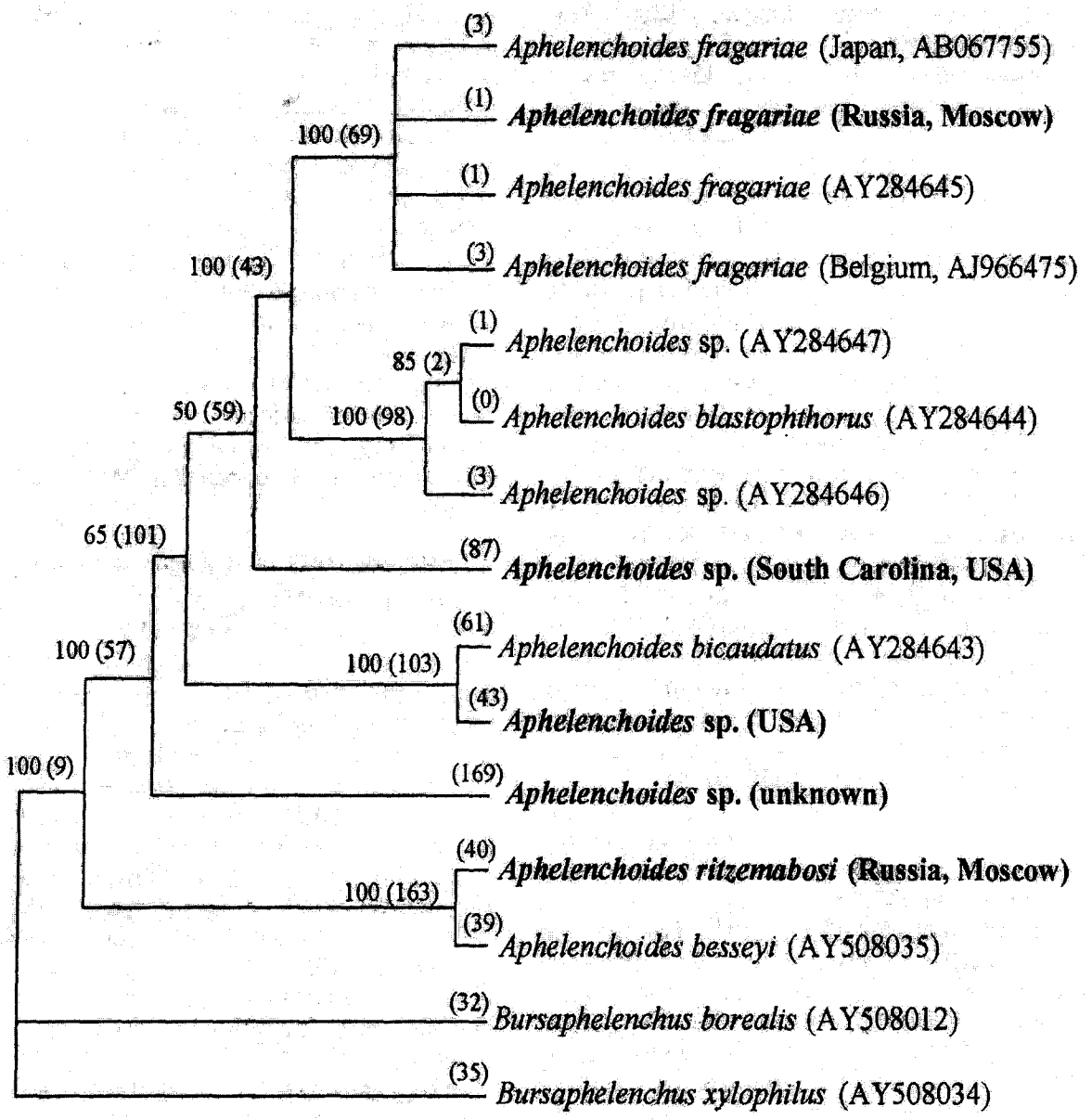

Fig. 2. Phylogenetic relationships between *Aphelenchoides* species as inferred from maximum parsimony analyses of the 18S rRNA gene sequences. One of the fifteen maximum parsimonious trees (tree length = 910; CI = 0.7692; HI = 0.2308; total characters = 1709; informative characters = 364). Bootstrap value and number of nucleotide changes are given in brackets for appropriate clades. Bold letters indicate new sequences.

California, Riverside and one additional species was from Clemson University, South Carolina.

Morphological study. Nematodes were killed and fixed in hot 4% TAF and processed to glycerin by a modified slow evaporation method (Seinhorst, 1959). Specimens were mounted in dehydrated glycerin on permanent slides and examined and measured with a light microscope, Axio Imager, Carl Zeiss MicroImaging GmbH.

Molecular study. To obtain DNA, several nematode specimens of each sample were transferred to an Eppendorf tube containing 16 µl ddH2O, 2 µl 10X PCR buffer and 2 µl Proteinase K (600 µg ml-1) (Promega, Benelux, The Netherlands) and crushed with an ultrasonic homogenizer. The tubes were incubated at 65°C (1 h) and then at 95°C (15 min). Detailed protocols for PCR, cloning and automated sequencing are as described by Tanha Maafi *et al.* (2003). The 18S rRNA gene was amplified using two sets of primers (two overlapping fragments): (i) forward G18SU (5'- GCT TGT CTC AAA GAT TAA GCC-3') (Blaxter et al., 1998) and reverse R18Tyl1 (5'-GGT CCA AGA ATT TCA CCT CTC-3') (ii) forward F18Tyl2 (5'-CAG CCG CGG TAA TTC CAG C-3') and reverse R18Tyl2 (5'-CGG TGT GTA CAA AGG GCA GG-3') or forward F18Tyl1 (5'-GTG CCA GCA GCC GCG GTA ATT CC-3') and reverse DF18A-16 (5'-CAC CTA CTC GYA CCT TGT TAC GAC T-3') (Kiontke *et al.*, 2004). Five new sequences of the 18S rRNA gene reported here have been deposited in the GenBank under the accession numbers: DQ901550-DQ901554.

New and known sequences of *Aphelenchoides* were aligned using the ClustalX program with two *Bursaphelenchus* species as outgroup taxa. Maximum parsimony (MP) analysis was performed with PAUP* 4b4a (Swofford, 2003). For MP, the gaps were coded as missing data and molecular characters were assessed as independent, unordered and equally weighted. Heuristic search settings were 10 replicates of random taxa, addition, branch swapping, multiple tree retained and without steepest descent. Robustness of the clades was assessed by bootstrap analysis yielding bootstrap percentage (BS) for each node estimated from 1000 replicates.

RESULTS AND DISCUSSION

Aphelenchoides fragariae (Ritzema Bos, 1891) Christie, 1932

The ferns plants infected by *A. fragariae* and detected during our surveys belong to the following species: *Aneimia rotundifolia, Blechnum occidentale, B. gibbum, Pteris longifolia, P. cretica cv. wimsetti, P. cretica* and *Stenochlaena tenuifolia*. The plant-parasitic nematodes of the genus *Aphelenchoides* have been recorded in glasshouses of the Main Botanical Gardens since the 1950s (Sveshnikova, 1956; Ilinskaja, 1963; Kirjanova & Krall, 1971; Matveeva, 1974; Matveeva & Shakhova, 1980). According to Matveeva & Shakhova, (1980), infection by strawberry crimp nematode was recorded from the nine fern species: *Aneimia phyllitidis, Aneimia* sp., *Blechnum brasiliense, B. occidentale, Callipteris prolifera, Dryopteris sieboldii, Phanerophlebia falcata, Pteris longifolia* and *Stenochlaena tenuifolia*.

Symptoms of nematode diseases of fern leaves are given in Fig 1. In the infected plants necrotic fields with nematode infection were mostly limited to veins. The colour of infected parts of leaves varies from light brown to dark depending on infection age. Over an eight month period, our observation of infected leaves of *P. longifolia* revealed that necrotic parts extended from a small spot to large fields occupying 40-60% of the total foliage.

Morphometrics of *A. fragariae* from fern plants:

Females (n=25): L = 0.525-0.685 (0.579 ± 0.043) mm; a = 37.1-59.8 (48.7 ± 4.8); b = 7.6-9.1 (8.1 ± 0.3); c = 15.2-20.6 (17.0 ± 1.2); c' = 3.6-5.7 (4.7 ± 0.3); V% = 65-74 (69 ± 2); stylet length = 8-11 (9) µm; head region width = 4-5 µm, head region high = 3 µm; distance from anterior end to medial bulb base = 52-64 (58) µm, nerve ring = 63-78 (72) µm, excretory pore = 68-85 (76) µm, oesophageal gland base = 100-150 (128) µm; post uterine sac length = 58-98 (77) µm; tail length = 28-40 (34) µm; body width at vulva level = 10-16 (12) µm and anus level = 6-8 (7) µm. Lateral field with 2 incisures. Postvulval uterine sac extending for more than half vulva-anus distance. Tail conoid, acute terminus without mucro.

Males (n = 24): L = 0.435-0.562 (0.493 ± 0.037) mm; a = 41.2-54.8 (46.8 ± 3.1); b = 6.5-8.1 (7.2 ± 0.4); c = 15.9-24.1 (18.5 ± 1.8); stylet length = 8-10 (9) µm; head region width = 4-5 µm, head region height = 3 µm; spicule length = 10-13 (12) µm; distance from anterior end to: medial bulb base = 52-62 (57) µm, oesophageal gland base = 100-135 (118) µm, nerve ring = 68-77 (71) µm, excretory pore = 70-82 (76) µm; testis length = 204-289 (250) µm; maximal body width = 10-13 (11) µm; tail length = 21-33 (27) µm.

The description provided here generally corresponds to that published for this species by Siddiqi (1975).

Aphelenchoides ritzemabosi (Schwartz, 1911) Steiner & Buhrer, 1932

Only plants of *Sambucus racemosa* were found to be infected by this nematode. Extensive surveys did not reveal any infection of other neighbouring plant species by *A. ritzemabosi*.

Morphometrics of *A. ritzemabosi* from leaves of *S. racemosa*:

Females (n=15): L = 0.768-1.027 (0.916 ± 0.067) mm; a = 43.4-60.5 (51.2 ± 3.7); b = 8.1-9.5 (9.1 ± 0.3); c = 16.8-21.2 (19.3 ± 1.1); c' = 4.0-5.1 (4.6 ± 0.2); V% = 68-71 (69 ± 0.2); stylet length = 9-11 (10) µm, head region width = 6-7 µm, head region height =3 µm; distance from anterior end to medial bulb base = 71-77 (74) µm, nerve ring = 95-108 (100) µm, excretory pore = 108-130 (121) µm, oesophageal gland base = 145-185 (170) µm; postuterine sac length = 105-160 (134) µm; tail length = 41-54 (48) µm; body width at vulva level = 16-23 (18) µm and anus level = 8-12 (10) µm.

Postvulval uterine sac extending for more than half vulva-anus distance. Lateral field with 4 incisures. Tail elongate-conoid having terminus with 2-4 minute processes.

Males (n = 15): L = 0.625-0.852 (0.721 ± 0.053) µm; a = 36.9-53.3 (46.3 ± 3.3); b = 6.5-9.4 (7.9 ± 0.6); c = 17.3-22.4 (19.9 ± 1.1); stylet length = 9-11 (10) µm; head region width = 6-7 µm; head region height = 3 µm; spicule length = 15-18 (16) µm; distance from anterior end to medial bulb base = 67-72 (69) µm, nerve ring = 85-108 (93) µm ; excretory pore = 92-118 (105) µm, oesophageal gland base = 156-180 (169) µm; testis length = 353-512 (442) µm; tail length = 34-39 (36) µm.

Morphometrics of *A. ritzemabosi* from wintering buds of *S. racemosa*:

Females (n=15): L = 0.972-1.165 (1.076 ± 0.048) mm; a = 36.7-46.4 (41.3 ± 2.5); b = 13.2-15.1 (13.8 ± 0.3); c = 18.9-21.9 (20.5 ± 0.4); c' = 3.9-4.9 (4.1 ± 0.2); V% = 69-70; stylet length = 10-11 µm; head region width = 6-7 µm; head region height =3 µm; postuterine sac length = 125-192 (171) µm; maximum body width = 21-30 (27) µm; body width at anus level = 11-15 (13) µm; tail length = 45-57 (53) µm; eggs in uterus = 78-85 x 20- 24 µm.

The description of the *A. ritzemabosi* population from *S. racemosa* is similar to that provided by Siddiqi (1974). Infected leaves are easily distinguished by the presence of necrotic spots of irregular forms and different sizes (Fig. 1). Nematodes overwinter in leaf and flower buds. From each bud more than 15 specimens have been recovered with the following ratio: 60% females, 30% juveniles and 10% males. Young and old stems are free from nematode infection.

Phylogenetic relationships between *Aphelenchoides* species as inferred from the 18S rRNA gene sequences.

Ibrahim *et al.* (1994) used PCR-ITS-RFLP for identification of *Aphelenchoides* species and clearly showed the utility of this approach for diagnostics of species of this genus. The present study also confirms utility of the 18S rRNA gene sequence for separation of agricultural important species of *Aphelenchoides* from each other and from closely related species. Analysis of phylogenetic relationships between species of the genus indicated the presence several clades, and two of these unite plant-parasitic aphelenchids: (i) *A. ritzemabosi* and *A besseyi* and (ii) *A. fragariae* and *A. blastophthorus*, together with two *Aphelenchoides* sp., which seems to be a co-specific with the last species (Fig. 2). Additional sequences and taxa should be included in new analyses to create a more complete picture of phylogenetic relationships within the genus *Aphelenchoides*.

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Chizhov V.N., Chumakova O.A., Subbotin S.A., Baldwin J.G. Морфологическая и молекулярная характеристика листовых нематод рода *Aphelenchoides*: *A. fragariae* и *A. ritzemabosi* (Nematoda: Aphelenchoididae) из Главного Ботанического сада Российской Академии наук, Москва.

Резюме. Проведенные в 2003-2004 г. обследования папортников из теплиц Главного ботанического сада РАН выявили присутствие нематод *Aphelenchoides fragariae*. Пораженные папортники принадлежали к видам: *Aneimia rotundifolia, Blechnum occidentale, B. gibbum, Pteris longifolia, P. cretica cv. wimsetti, P. cretica* и *Stenochlaena tenuifolia*. Нематодное поражение приводило к образованию наполненных водянистой жидкостью полостей между жилками листьев, принимавших затем темно-коричневый или черный цвет. Некоторые растения бузины *Sambucus racemosa*, произраставшие под открытым небом рядом с теплицами, подверглись поражению нематодой *A. ritzemabosi*. Этих нематод извлекали из листьев и точек роста бузины. Приводятся описания и диагностические признаки для *A. fragariae* и *A. ritzemabosi*. Различия в последовательностях 18S ДНК позволяют четко разграничить эти виды друг от друга и от других видов *Aphelenchoides*. На основе анализа 18S-ДНК выявлены и обсуждены филогенетические отношения этих видов с другими видами рода.