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MORPHOLOGICAL AND BIOLOGICAL VARIABILITY OF *STEINERNEMA FELTIAE* (NEMATODA, STEINERNEMATIDAE) ITALIAN STRAINS (1)

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Clausi M., Troccoli A., Leone D., De Luca F., Rappazzo G., Fanelli E., Ravlic J., Tarasco E. - Morphological and biological variability of *Steinernema feltiae* (Nematoda, Steinernematidae) Italian strains.

Steinernema feltiae belongs to the feltiae-kraussei-oregonensis group, clade III, and is an ubiquitarian species of entomopathogenic nematode. It is found in all types of soil and in all types of habitat. Species identification in the entomopathogenic nematodes genera *Steinernema* is a very complex task, given the broad variability of both morphological and biological traits within populations of a single species. To accomplish this, molecular techniques have been adopted which, however, require additional knowledge. Particularly relevant would be the possibility of testing in a reliable way the variability between different populations of the same species, which might represent different strains with different biological properties. During numerous samplings in Italy, several strains of *S. feltiae* were isolated. In this paper we analyze the intraspecific variability of the main morphometric and biological data of juveniles and males of 50 Italian populations of *S. feltiae.* The aim of our work was to determine if morphometric and biological analysis were useful to identify characters having significant diagnostic value, allowing to reliably discriminate among strains. Seven characters routinely computed for morphology (5 morphometrics for infective juveniles, spicula and gubernaculum shapes for males) and 2 biological performances (time to achieve adult stage, reproduction and progeny) were considered. The results showed extreme variability from both morphological and biological points of view

KEY WORDS: morphometrics, biological characterization, entomopathogenic nematode

INTRODUCTION

The cosmopolitan species *Steinernema feltiae* (Filipjev, 1934) (Nematoda, Steinernematidae) is probably the most famous and widespread entomopathogenic nematode (EPN) in the world (HOMINICK *et al.,* 1997; ADAM & NGUYEN, 2002). In Italy it was isolated from 53 localities of different habitats (CLAUSI & VINCIGUERRA, 2005; TARASCO & TRIGGIANI, 1997; TARASCO *et al.,* 2015) and resulted as the most common EPN in the Country. The aim of this work was to continue the study of the biodiversity of Italian EPNs analyzing intraspecific variability of *S. feltiae*. In particular, the morphological and biological variability were studied by comparing the data of 50 *S. feltiae* Italian populations isolated from different habitats in the southern Italian regions of Apulia, Campania, Basilicata, Calabria and Sicily (table1).

MATERIALS AND METHODS

The study considered a total of 50 populations of *S. feltiae* of which 28 from Apulia, 3 by 3 from Basilicata, Campania and Calabria, and 13 from Sicily.

MORPHOLOGICAL STUDY

Infective juveniles (IJs): Morphometric analysis of *Steinernema feltiae* Italian populations was processed with a non-parametric method using the Kruskal–Wallis test by ranks (Kruskal–Wallis *H* test, or one-way ANOVA on ranks - SPSS Statistics, 2019). A minimum of 20 IJs were observed for each *S. feltiae* population. The analysis focused on the main morphometric characters of the IJs (Body Lenght, Maximum body diameter, Anterior End-Pharynx base distance, Anterior End-Excretory Pore distance, Tail lenght). Males: A minimum of 10 males were observed for each *S. feltiae* population. The analysis focused on the main morphological characters of the spicules and gubernacula shapes (NGUYEN & SMART, 1996; HUNT & NGUYEN, 2016).

BIOLOGICAL OBSERVATIONS

Times to reach the adult stage, reproduction and emergence of the progeny were observed and measured using the "Hanging drop" Technique (POINAR, 1975), a well-established method for examining living, unstained, very small organisms (Fig. I). Our procedure employs a glass slide with a circular concavity in the centre into which a drop of fluid, containing the 'micro-

[1] Original scientific contribution presented and discussed at the XIII National Congress of the Italian Society of Nematology (SIN), Catania - Italy, October 2-4, 2019.

- Received 21 September 2020 Accepted 8 October 2020

Table 1 – Steinernema feltiae Italian populations. *In parenthesis the Province acronym
**Abbreviations of the Italian regions: CAM = Campania, APU = Apulia, BAS = Basilicata, CAL = Calabria, SIC = Sicily, SIC-ai = Aeolian islands, SIC-pi = Pantelleria island

Strain	*Locality	**Italian regions	Alt.	Date	Vegetation	Habitat	Soil text	Species	Accession number
1.SA1	Sammichele (BA)	APU	280	Jun 1990	Cherry	Orchard	Sandy loam	*S. feltiae*	n.a.
2.CO1	Corato (BA)	APU	230	Feb 1992	Wild vegetation	Uncultivated land	Silt	*S. feltiae*	HQ412814.1
3.GR1	Grassano (MT)	BAS	300	Nov 1995	Tomato	Field	Clay loam	*S. feltiae*	HQ412818.1
4.G4	Gravina (BA)	APU	380	Oct 1995	Pine	Pinewood	Silty loam	*S. feltiae*	n.a.
5.G8	Gravina (BA)	APU	380	Dec 1995	Pine	Pinewood	Sandy loam	*S. feltiae*	n.a.
6.CE2	Cerignola (FG)	APU	120	Oct 1996	Olive	Orchard	Sandy loam	*S. feltiae*	HQ412813.1
7.MO1	Melfi (PZ)	BAS	500	Sep 1996	Apple	Orchard	Sandy loam	*S. feltiae*	HQ416966.1
8.CL2	Celzi (AV)	CAM	650	Oct 1996	Pear	Orchard	Sandy loam	*S. feltiae*	HQ412830.1
9.MU1	Mugnano del Cardinale (AV)	CAM	550	Oct 1996	Kaki	Orchard	Clay loam	*S. feltiae*	HQ412841.1
10.MV1	Montevergine (AV)	CAM	700	Oct 1996	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	n.a.
12.LE1	Tricase (LE)	APU	50	Oct 1997	Meadows	Grassland	Silty loam	*S. feltiae*	HQ412819.1
13.TE1	Terlizzi (BA)	APU	200	Feb 1998	Cherry	Orchard	Clay loam	*S. feltiae*	HQ412831.1
14.MF1	Martina Franca (TA)	APU	350	Mar 1998	Oak	Broadleaf wood	Silty loam	*S. feltiae*	HQ412826.1
15.CS6	Brindisi (BR)	APU	20	Apr 1998	Artichoke	Field	Sand	*S. feltiae*	HQ412815.1
16.CZ19	Giamberga (CS)	CAL	800	May 1998	Pine	Pinewood	Sandy loam	*S. feltiae*	HQ412816.1
17.CZ23	Cecita lake (CS)	CAL	1100	May 1998	Pine	Pinewood	Sandy loam	*S. feltiae*	HQ412817.1
18.MA12	Chiese Rupestri Park (MT)	BAS	400	Sep 1998	Cave	Cave	Sandy loam	*S. feltiae*	HQ412827.1
19.G16	Gravina (BA)	APU	380	Mar 1999	Pine	Pinewood	Silty loam	*S. feltiae*	HQ412828.1
20.Q1	Quasano (BA)	APU	150	May 1999	Meadows	Grassland	Silty loam	*S. feltiae*	HQ412829.1
21.MSA3	Monte S. Angelo (FG)	APU	790	Mar 1999	Meadows	Grassland	Silt	*S. feltiae*	HQ412811.1
22.MSA4	Monte S. Angelo (FG)	APU	790	Nov 1999	Meadows	Grassland	Silt	*S. feltiae*	HQ412825.1
23.BQ1	Monte S. Angelo (FG)	APU	787	Dec 1999	Meadows	Grassland	Silty loam	*S. feltiae*	n.a.
24.B6	Bitonto (BA)	APU	118	Dec 1999	Olive	Orchard	Silt	*S. feltiae*	n.a.
25.TG4	Brindisi (BR)	APU	20	Jan 2000	Swamp	Wetland	Sandy loam	*S. feltiae*	HQ412824.1
26.OT2	Alimini Lake (LE)	APU	25	Apr 2000	Pine	Pinewood	Sand	*S. feltiae*	HQ412820.1
27.ESA	S.Alfio (CT)	SIC	530	Sep 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599911.1
28.RC8	Aspromonte (RC)	CAL	680	Jul 2005	Oak	Broadleaf wood	Sandy loam	*S. feltiae*	n.a.
29.ESC2	Salto del cane (CT)	SIC	1200	Sep 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599905.1; GU599907.1
30.EPP	Piano Porcheria (CT)	SIC	1000	Sep 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599908.1
31.EMM1	Mt. Monaco (CT)	SIC	820	Oct 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599904.1; GU599910.1
32.EPC	Pietracannone (CT)	SIC	1000	Oct 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599912.1
33.ETA	Tarderia (CT)	SIC	750	Oct 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599906.1; GU599909.1
34.EMA	Triciala (CT)	SIC	950	Oct 2005	Chestnut	Broadleaf wood	Sandy loam	*S. feltiae*	GU599913.1
35.OT9	Otranto (LE)	APU	20	May 2006	Oak	Broadleaf wood	Sandy loam	*S. feltiae*	HQ416967.1
36.OT10	Otranto (LE)	APU	20	May 2006	Pine	Pinewood	Sand	*S. feltiae*	n.a.
37.OT11	Otranto (LE)	APU	20	May 2006	Pasture	Grassland	Sand	*S. feltiae*	HQ412821.1
38.OT14	Otranto (LE)	APU	20	Jun 2006	Pine	Pinewood	Sand	*S. feltiae*	HQ412822.1
39.OT15	Otranto (LE)	APU	20	Jun 2006	Artichoke	Field	Sand	*S. feltiae*	HQ412823.1
40.M31	Canale di Pirro (BR)	APU	300	May 2007	Wheat	Field	Silty loam	*S. feltiae*	n.a.

Strain	*Locality	**Italian regions	Alt.	Date	Vegetation	Habitat	Soil text	Species	Accession number
41.M23	Pianelle Park (TA)	APU	200	May 2007	Oak	Broadleaf wood	Sandy loam	*S. feltiae*	n.a.
42.M50	Canale di Pirro (TA)	APU	300	May 2007	Artichoke	Filed	Sandy loam	*S. feltiae*	n.a.
43.M62	Galeone Forest (TA)	APU	300	Jun 2007	Oak	Broadleaf wood	Sandy loam	*S. feltiae*	n.a.
44.FF1	Francavilla Fontana (BR)	APU	150	Sep 2008	Wheat	Field	Clay loam	*S. feltiae*	n.a.
45.MUE3	Pantelleria (TP)	SIC-pi	50	Jun 2010	Mulberry	Broadleaf wood	Sandy loam	*S. feltiae*	n.a.
46.PR4	Gornalunga Lake (CT)	SIC	800	Nov 2010	Pine	Pinewood	Sandy loam	*S. feltiae*	n.a.
47.CTSA18	San Leonardo River (SR)	SIC	5	Dec 2008	Eucalyptus	Pinewood	Sand	*S. feltiae*	HQ412832.1
48.CT27	Mt. Serra (CT)	SIC	450	Apr 2009	Oak	Broadleaf wood	Sandy loam	*S. feltiae*	HQ412834.1
49.VE1	Oasi di Vendicari (SR)	SIC	23	May 2009	Olive	Orchard	Clay loam	*S. feltiae*	HQ412835.1
50.SAL3	Salina (ME)	SIC-ai	130	Sep 2009	Pine	Pinewood	Sandy loam	*S. feltiae*	HQ412836.1

organisms', hangs from a coverslip. The hemolymph was taken from the *Galleria mellonella* L. (Lepidoptera, Pyralidae) larva: the middle legs of the insect were cut and 2 drops of hemolymph coming out were placed on the square coverslip (20×20 mm). Meanwhile, the entomopathogenic nematodes from different populations of *S. feltiae* were kept in beakers (a beaker for each different population). Later, a few nematodes (4-6 IJs) were taken with a thin needle to another beaker containing hyamine solution (Hyamine 1622 solution

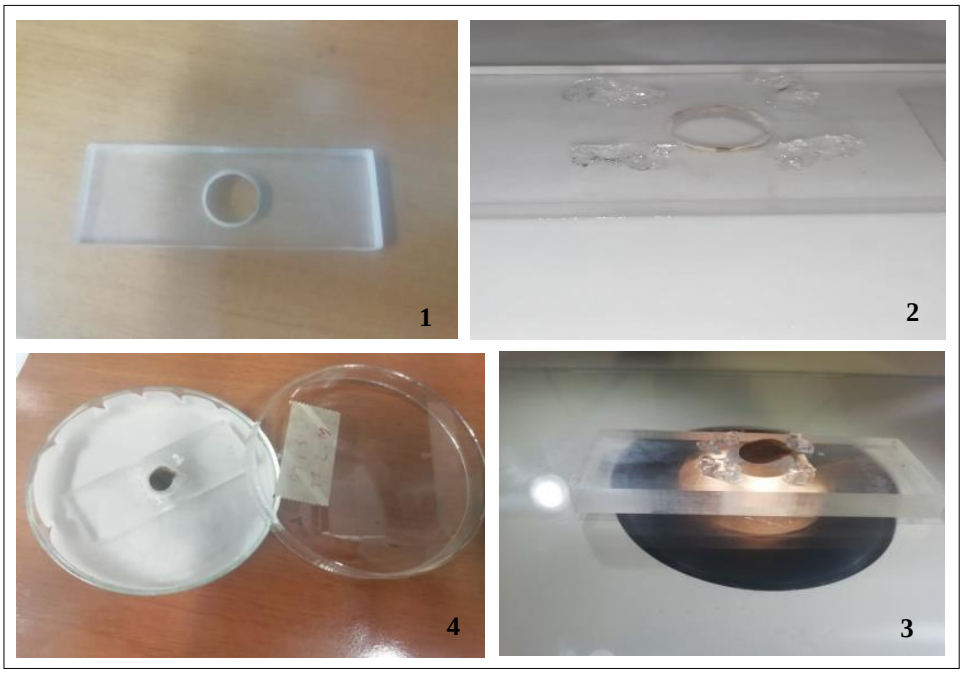

Fig. I – Biological observations - ''Hanging drop" technique: 1. Perforated slide; 2. Perforated slide with gel to keep the cover slide with the haemolymph drop; 3. Petri dish with humid filter paper and slide with haemolymph drop; 4. slide with haemolymph drop under microscope for observation.

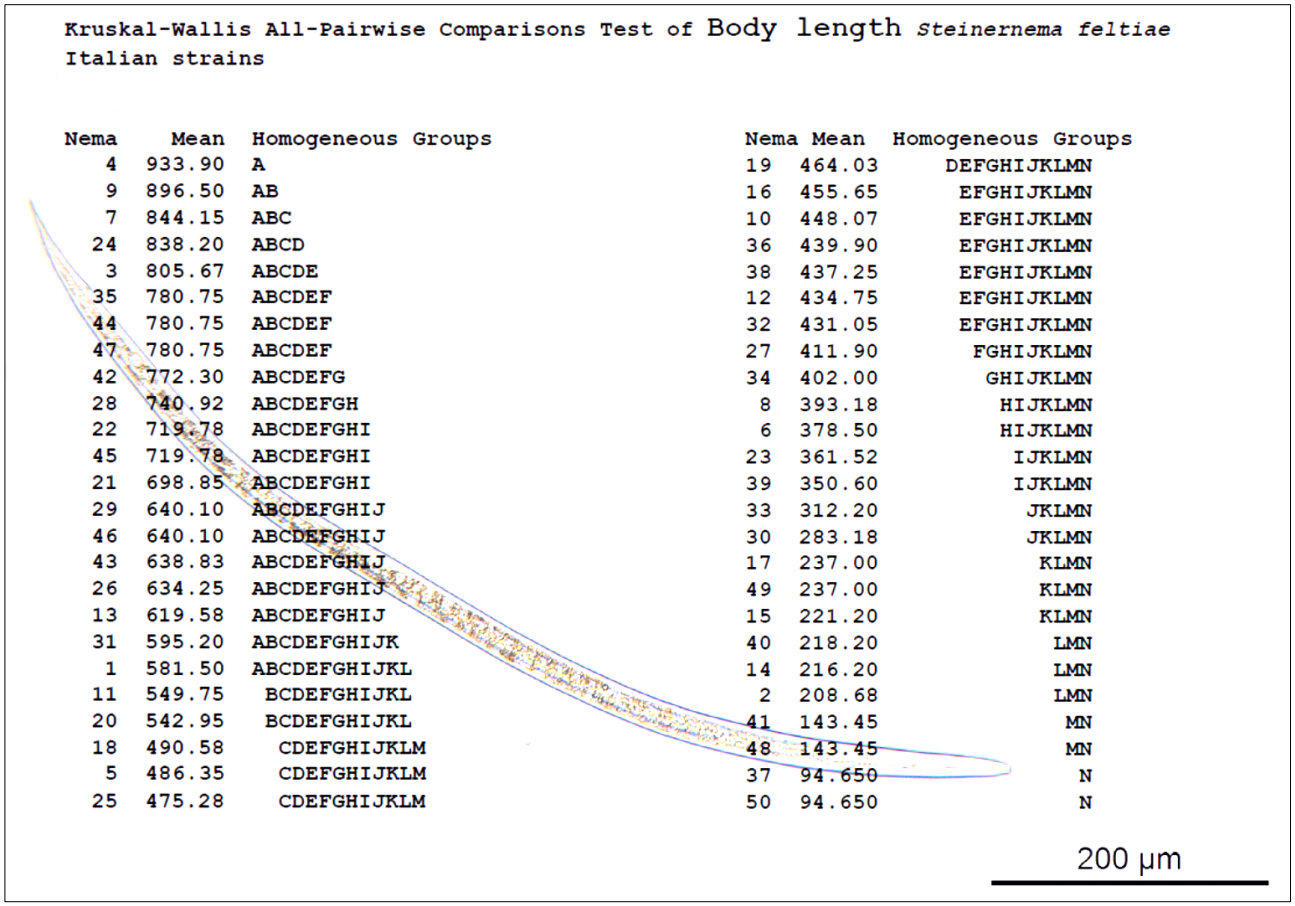

Fig. II - Variability of Body Length (BL): homogeneous morphometrics in comparison for 50 strains of Italian *S. feltiae*. For strain number see Table 1.

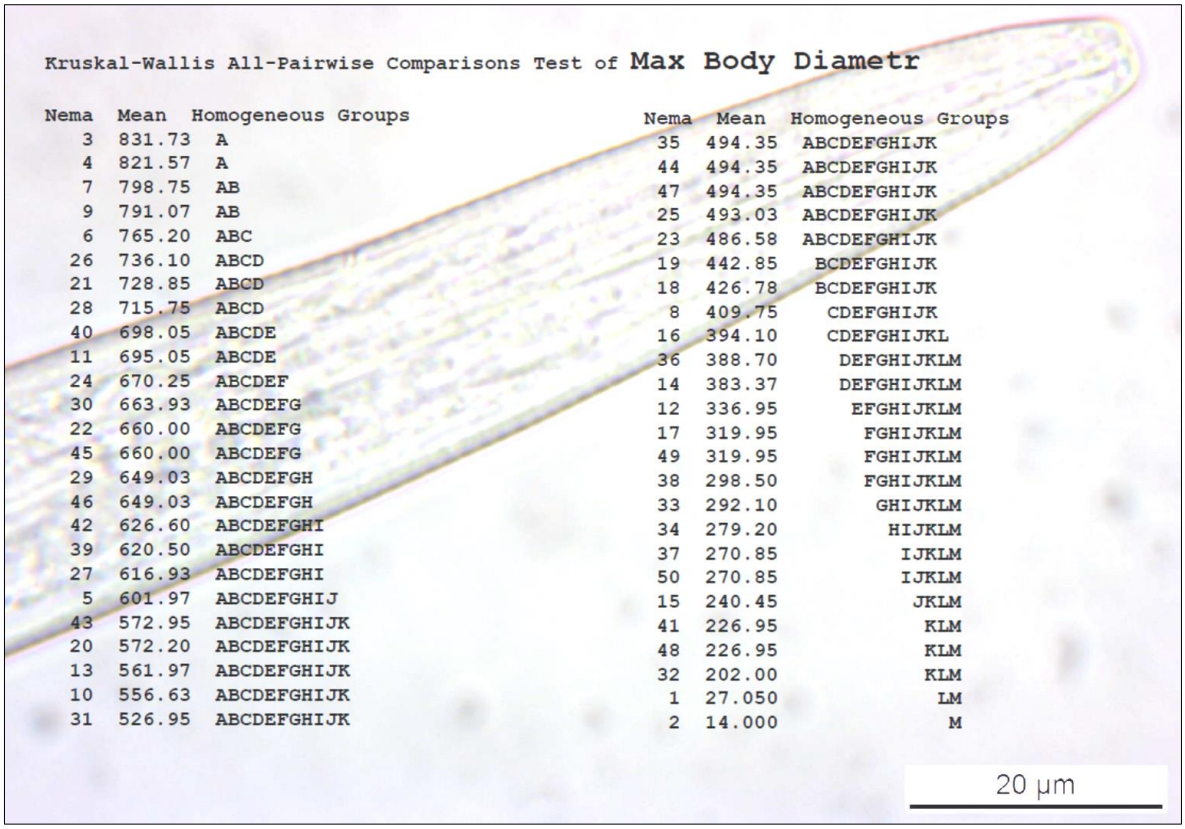

Fig. III –Variability of Maximum Body Diameter: homogeneous morphometrics in comparison for 50 strains of Italian *S. feltiae*. For strain number see Table 1.

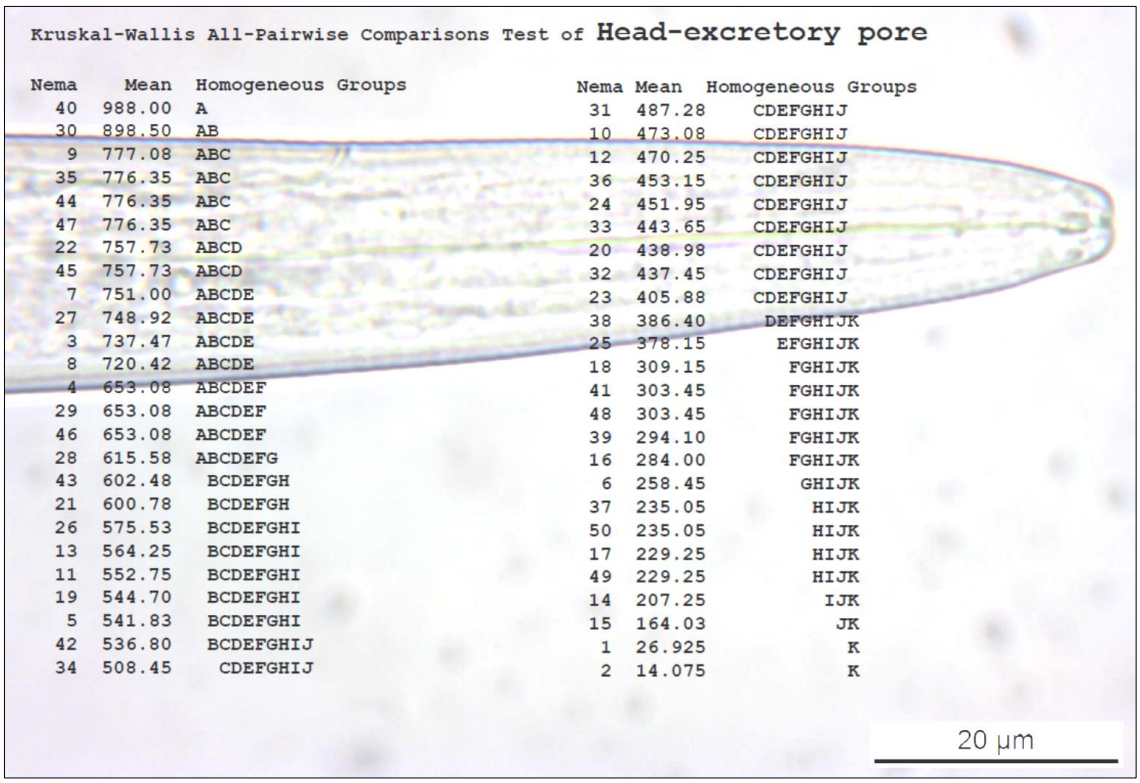

Fig. IV –Variability of Head-excretory pore distance: homogeneous morphometric's groups in comparison for 50 strains of Italian *S. feltiae*. For strain number see Table 1.

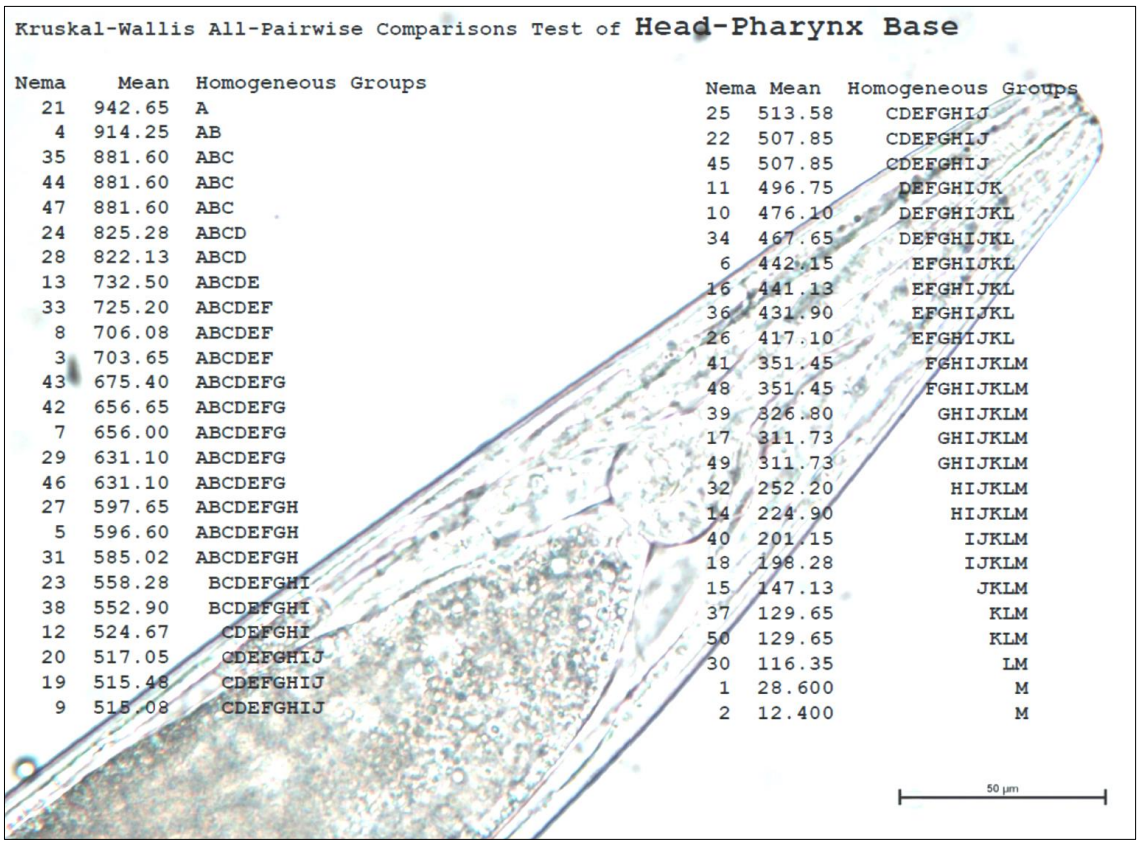

Fig. V – Variability of Head-pharynx base distance: homogeneous morphometric's groups in comparison for 50 strains of Italian *S. feltiae*. For strain number see Table 1.

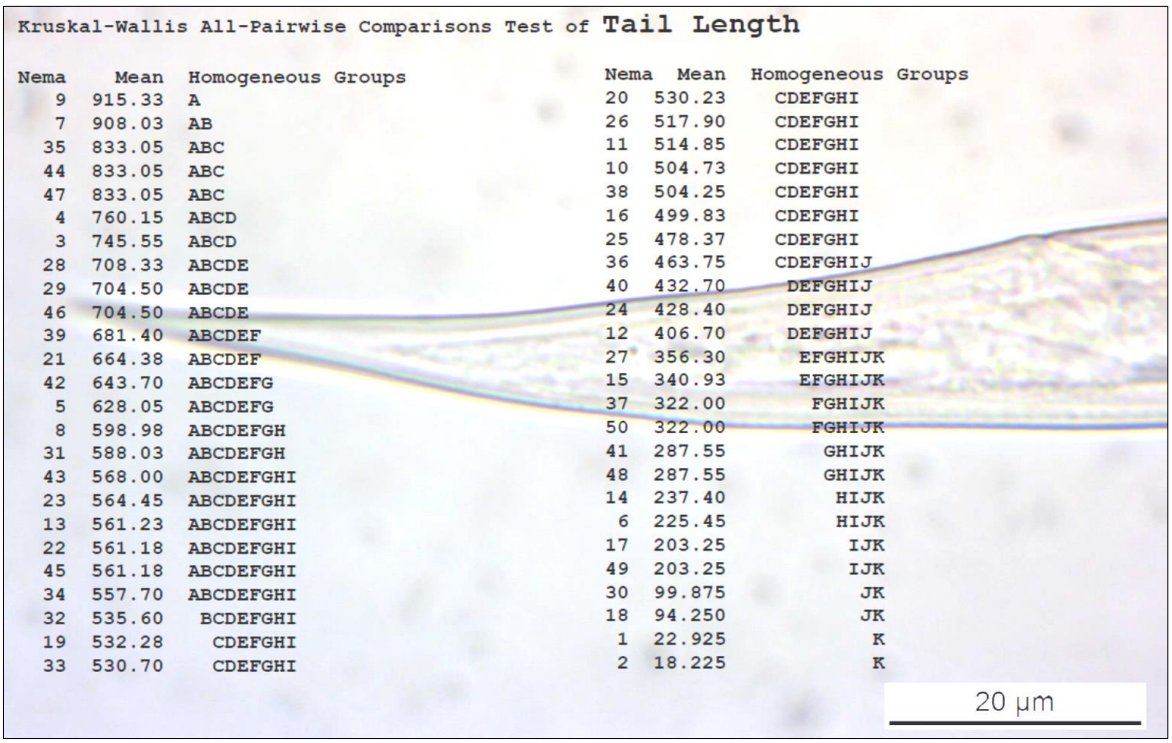

Fig. VI - Variability of Tail lenght: homogeneous morphometric's groups in comparison for 50 strains of Italian *S. feltiae*. For strain number see Table 1.

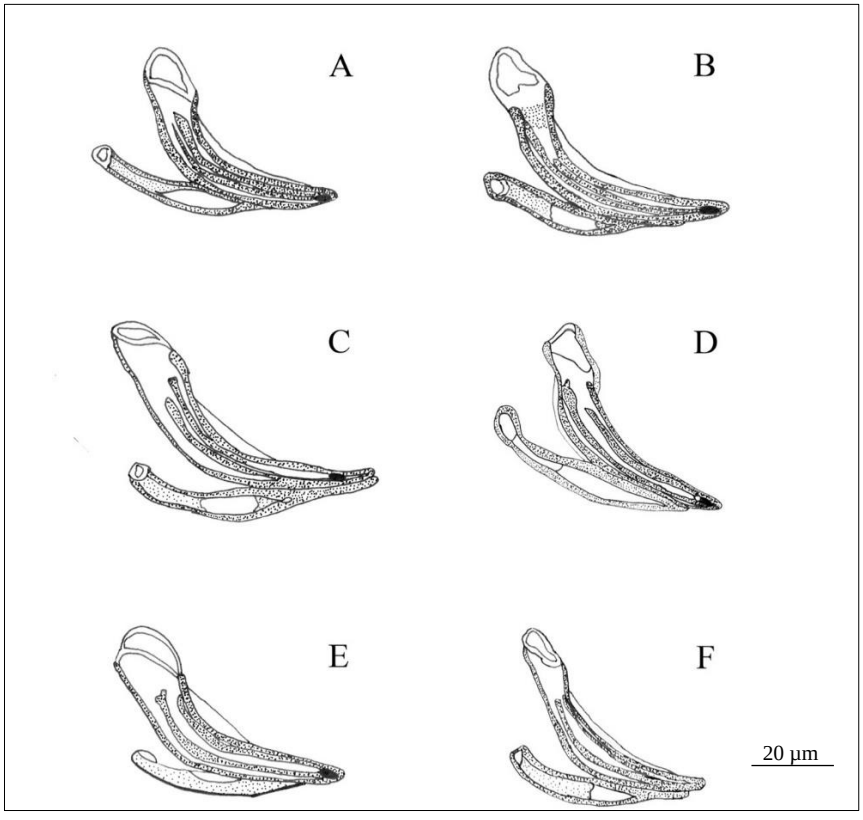

Fig. VII - Morphology of spicules and gubernacula of Italian *Steinernema feltiae* strains: 6 different typologies were recognized.

0.004M), for 5" for disinfection of the nematodes. Then the disinfected nematodes were taken carefully with a needle and placed on the coverslip inside the drop of hemolymph. The slide with the hole in the middle was used for easier and clearer observation of the nematodes. A gel suspension was settled on the four corners of the coverslip around the hole to keep the coverslip apart from the slide. The next step was to carefully invert the coverslip, trying not to disturb the hemolymph drop with nematodes inside, and mount it exactly above the hole situated in the middle of the slide. The hole in the middle of the slide was used to keep the droplet intact and to facilitate viewing under the microscope (Fig. I). Then the slide was placed inside a Petri dish containing a wet filter paper to maintain the required moisture. Water was added in the Petri dishes every two days to keep a saturated level of moisture to prevent the drops of hemolymph from dehydration. Petri dishes with slides were kept in the dark at 24 °C. The aim of this method was to monitor the nematode development and mating inside the hemolymph drops. The emergence of the new offsprings was also observed.

RESULTS

With the morphometric analysis we highlighted different major groupings of the EPN. Within the variability of the populations, some showed interesting morphometric similarities and there were groups showing homogeneous morphometrics in comparison to the others. For Body Length (BL) there were up to 14 similarity degrees with a maximum of 28 populations in the same homogenous group (Fig. II); for Maximum Body Diameter (MBD) there were 13 similarity degrees with up to 31 populations in the same homogenous group (Fig. III); for Head-Excretory pore (He) 11 similarity degrees were observed with up to 32 populations in the same homogenous group (Fig. IV); for Head-Pharynx base distance (H-P) the similarity degrees were 13 with up to 29 populations in the same homogenous group (Fig. V). Finally, for Tail Length (TL) 11 similarity degrees were observed with up to 31 populations in the same homogenous group (Fig. VI).
Spicule morphology of *S. feltiae* isolates showed some differences, basically attributable to at least 6 typologies

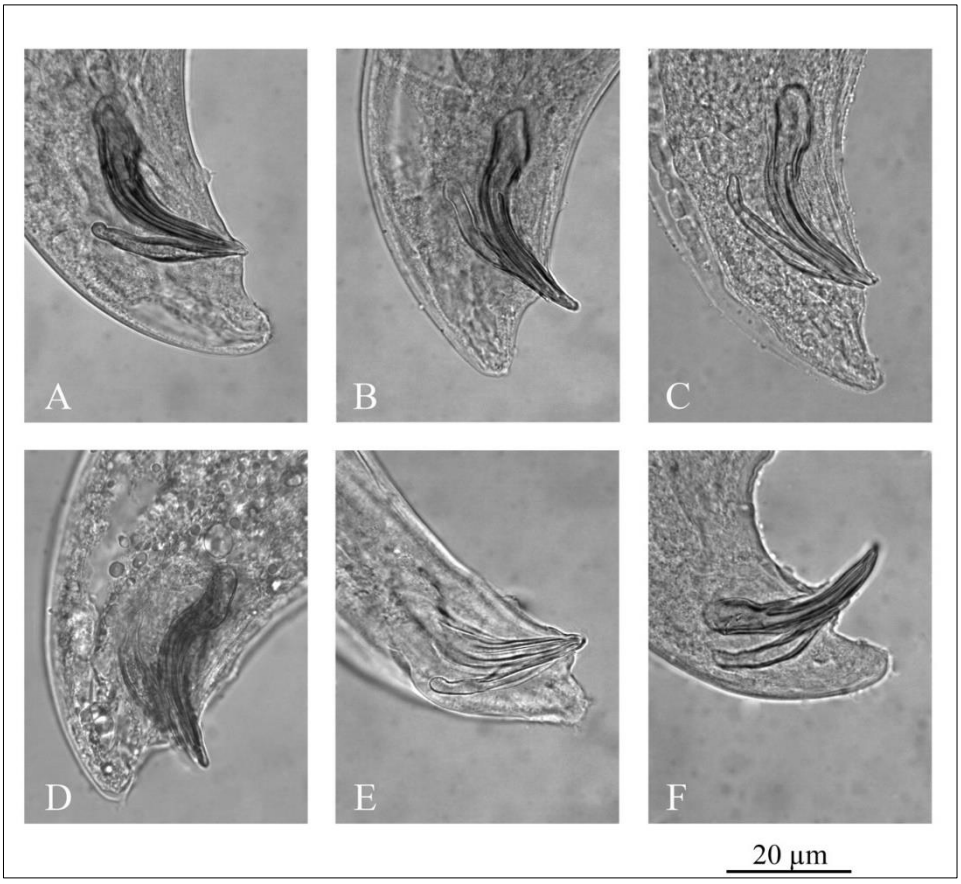

Fig. VIII – Morphology of spicules and gubernacula of Italian *Steinernema feltiae* strains: 6 different typologies were recognized.

(Figs. VII-VIII). Most of the isolates belonged to the A, B (i.e. population n. 32 EPC and 27 ESA, as A-type and B-type respectively) and C (i.e. population n. 29 ESC2) type showing larger spicules with respect to all other isolates, with head longer than wide. Isolates of F-type (i.e. population n. 30 EPP) also showed a head longer than wide, with a thin velum extending almost to the entire length of the blade. In isolates of D-type (i.e.

population n. 31 EMM1) and A-type (i.e. population n. 32 EPC) the head of spicule was as long as wide. The velum was thin and as long as spicule blade, in A, B and F (isolates n. 32 EPC, n. 27 ESA and n. 30 EPP), whereas it was short (about half of blade length) in C and E isolates (n. 29 ESC2 and n. 2 CO1, C-type and E-type respectively). In isolates of D-type (i.e. n. 31 EMM1) the velum was hardly visible. A slightly pronounced rostrum could be observed in isolates of types C and D, being nearly absent in all other spicule typologies. Finally, differences were observed in gubernaculum shape, with the anterior end generally in axis with corpus, except in isolates of C-type which showed an obtuse angle. The first group, common to most of the isolates, presented a slender spicule, with head and foil long and thin, while the second group showed spicules stubbier in all the parts (Figs. VII-VIII).

BIOLOGICAL OBSERVATIONS - Both sexes were obtained in every samples. The adult stages were found from one day (3 populations) to 1.5 day (5 populations), 2 days (12 populations), 2.5 days (20 populations) and 3 days (10 populations). Mating took place shortly after reaching the adult stage (for the majority of the strains two-three days after they were put into the hemolymph drop). The progeny emerged after 4 to 7 days from mating. The nematode specimens were observed on the edge of the hemolymph drop.

DISCUSSION AND CONCLUSIONS

Our study focused on the intraspecific characteristics of 50 populations isolated in Italy and the results showed extreme variability from both morphological and biological points of view. Paraphrasing the original sentence of Aaron Levenstein, related to the statiscts, with reference to the morphological and biological aspects of the nematodes, we could say that “Molecular analysis are like bikinis: what they reveal is suggestive, but what they conceal is vital”. Molecular analyses have now become an indispensable tool for species identification, but the results of our survey show that morphological and biological studies on the variability of individual populations of *S. feltiae* are equally important and provide fundamental data for the characterisation of the species. Morpho-biological and molecular studies are functional to each other and must proceed in parallel for a correct and complete species characterization.

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