Zootaxa 4083 (2): 297–300
http://www.mapress.com/j/zt/

Correspondence

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

http://doi.org/10.11646/zootaxa.4083.2.9
http://zoobank.org/urn:lsid:zoobank.org:pub:9D9FFAE0-B5C2-4B30-AC1E-7C8D35C96996

The effect of two fixation methods (TAF and DESS) on morphometric parameters of *Aphelenchoides ritzemabosi*

ANETA CHAŁAŃSKA[1,3], ALEKSANDRA BOGUMIŁ[1], TADEUSZ MALEWSKI[2] & KATARZYNA KOWALEWSKA[2]

[1]*Research Institute of Horticulture, Konstytucji 3 Maja 1/3 Skierniewice, Poland.*
E-mail: aneta.chalanska@inhort.pl; aleksandra.bogumil@inhort.pl
[2]*Museum and Institute of Zoology, Wilcza 64, 00-679 Warsaw, Poland.*
E-mail: tmalewski@miiz.waw.pl; k.kowalewska@miiz.waw.pl
[3]*Corresponding author*

Identification of nematode species by using conventional methods requires fixation of the isolated material and a suitable preparation for further analyses. Tentative identification using microscopic methods should also be performed prior to initiating molecular studies. In the literature, various methods are described for the preparation of nematodes from the genus *Aphelenchoides* for identification and microscopic studies. The most commonly used fixatives are formalin (Timm 1969; Szczygieł & Cid del Prado Vera 1981, Crozzoli *et al*. 2008, Khan *et al*. 2008), FAA (Wasilewska 1969; Vovlas *et al*. 2005, Khan *et al*. 2007) and TAF (Hooper 1958, Chizhov *et al*. 2006, Jagdale & Grewal 2006).

Few studies have focused on the effects of preparation on nematodes. Comparison of techniques suggests that both the method of killing and of fixing nematodes affects the quality of mounted specimens, as well as their morphometric parameters (Lamberti & Sher 1969, Grewal *et al*. 1990). According to Brown & Topham (1984), the same method of preparation should be used for comparing morphometry between closely related species. However, the variety of techniques applied by different authors often makes it impossible to compare new observations with literature data. According to research conducted by Grewal *et al*. (1990), morphology of nematodes after TAF fixation was changed the least, implying that measurements are more reliable than those obtained using other processing techniques, including the commonly applied FAA method. However, for a simultaneous morphological and molecular analysis, where only one specimen is examined, the most appropriate technique is to use DESS (Yoder *et al*. 2006).

For molecular analyses, nematodes are commonly rinsed in water without any fixative (Jiang *et al*. 2007, Khan *et al*. 2012). However, subsequent procedures must be carried out immediately after nematode isolation. DESS (a solution of dimethyl sulphoxide, disodium EDTA and NaCl) allows one to perform DNA isolation even after several months of storage at room temperature (Yoder *et al*. 2006). We thought it important to investigate whether material fixed in this solution could be also used for classical identification methods. The aim of the present study was to compare the morphometric characteristics of female specimens of *Aphelenchoides ritzemabosi* (Schwartz, 1911) Steiner & Buhrer, 1932 taken from the same population, by using two methods of fixation: in TAF and in DESS solution.

Female *A. ritzemabosi* were isolated from the leaves of *Weigela florida* DC. variety 'Bristol Ruby', from Poland. Nematodes were isolated using a modified Baermann tray extraction method (Luc *et al*. 2005), and then killed with hot water at a temperature of 60–70 °C. For fixation, TAF (Courtney *et al*. 1955) and DESS (Yoder *et al*. 2006) were used. After a one year storage of preserved nematodes at room temperature, glycerin mounts were prepared using the Seinhorst method (Seinhorst 1962, 1966). A Nikon Eclipse 80*i* light microscope was employed for nematode species determination, using differential interference contrast (Nomarski DIC). Morphometric measurements were performed at 100X magnification. After identification of specimens preserved in DESS genomic DNA from three specimens was extracted using a Nucleo Spin Tissue XS (Macherey-Nagel, Germany) following the manufacter's instructions. For each sample, DNA was eluted in 30 µl H_2O. 3 µl of extracted DNA was used as template for polymerase chain reactions (PCR). The 28S rDNA sequence fragment was amplified with primers Aph_F (5'-AGAGAGTGCAAGAGAACGTGA-3') and Aph_R (5'-ACCATCTTTCGGGTCTCAAA-3'). PCR primers were designed using the online PRIMER 3 software (http://primer3.ut.ee) after aligning sequences of closely related in GenBank. Primers were purchased from Sigma-Aldrich (Sigma-Aldrich, Milwaukee, WI, USA). Amplification of 28S rDNA and sequencing was performed as described previously (Dobosz *et al*. 2013) with modified polymerase (12.5µl Taq PCR Master Mix Gdansk, Poland) and

Accepted by P. Mullin: 31 Dec. 2015; published: 19 Feb. 2016

primer concentrations (1µl of 10 µM each primer). Cycling was performed by a Veriti 96-Well Thermal Cycler as follows: an initial denaturation step of 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min and a final extension step of 72°C for 5 min. 28S rDNA sequences were aligned in MEGA ver. 6.06 program software using default parameters (Tamura *et al*. 2011). The sequences reported in this study have been deposited in Gen Bank with accession numbers KR261601, KR261602, KR261603. The length of sequences for *A. ritzemabosi* ranged from 453 bp to 528 bp. Sequences were compared to each other and deletion of one nucleotide in one of the samples was observed.

TABLE 1. Morphometric measurements of females of *A. ritzemabosi*. Measurements are in µm and in the form: range, mean±SD.

	♀♀-DESS n=30, mean±SD	♀♀-TAF n=30, mean±SD
L (µm)	683.7–830.3 767.7±33.2	693.7–824.0 774.1±30.0
head-vulva length	471.0–562.0 526.9±23.5	483.7–566.0 535.3±21.4
vulva-anus length (v-a)	174.2–224.9 199.5±11.7	166.5–217.8 198.9±12.7
maximum body diameter	15.6–19.4* 17.9±0.9	14.2–18.1 16.3±1.0
tail length	36.5–47.0 41.3±2.9	35.2–45.9 40.0±2.9
anal body diameter	9.2–12.* 10.8±0.8	8.2–11.1 9.9±0.8
postvulval uterine sac (PUS)	73.6–136.6 109.2±14.3	96.8–134.1 111.5±11.2
PUS - % v-a	42.3–66.5 54.7±6.6	46.4–62.7 56.1±4.3
PUS - xV	4.0–7.5* 6.1±0.8	6.0–8.2 6.9±0.7
V%	66.9–70.8 68.6±1.0	67.1–70.8 69.2±0.9
a	36.9–47.2* 43.0±2.3	42.2–54.8 47.6±3.3
c	17.1–20.4 18.7±1.1	16.0–22.5 19.5±1.6
c'	3.2–4.5 3.9±0.3	3.2–5.5 4.1±0.5
b	7.4–8.6 8.1±0.4	7.3–9.2 8.1±0.6
b’	4.4–5.2* 4.7±0.2	4.6–5.5 5.0±0.3
pharynx length	89.7–99.8 95.0±3.9	85.7–103.5 95.6±6.4
pharynx length (with glands)	148.1–179.7* 164.4±8.0	140.1–168.3 154.1±7.9
stylet length	10.8–13.4 12.5±0.8	11.6–13.2 12.5±0.5
excretory pore from head end	82.7–96.5* 88.8±4.7	76.3–103.9 97.2±6.1
lateral field width	2.6–4.4 3.5±0.5	2.7–3.8 3.2±0.3

* values marked with a star in columns are significantly different according to Tukey test ($p<0.05$)

Analysis of variance (ANOVA) was applied to the morphometric results. For comparison averages, a Tukey test was used, assuming a $p = 0.05$ level of significance. The analysis was performed in STATISTICA v. 10 (Stat Soft Inc., 2011). Statistical analysis revealed significant differences between methods of fixation in 7 out of 20 tested parameters. Females preserved in DESS were characterized by a greater body width, both in the widest part of the body and at the level of the anus, as well as a longer pharynx with glands compared to females fixed in TAF. An inverse dependency was observed in the position of excretory pore from the head end (Table 1).

FAA is one of the more commonly used fixatives. Grewal *et al*. (1990), comparing different fixation methods, showed that FAA causes many changes in morphometric parameters. According to these authors, TAF causes shrinking of the specimens, although to a lesser extent than other fixatives. In the present study, there were 7 disagreements in parameters between TAF and DESS, which indicate that DESS had less affect on in the morphometric changes than TAF. Furthermore, as shown by Yoder *et al*. (2006), morphological characteristics of nematodes preserved in DESS are comparable to those fixed in formalin, which is employed frequently in the methodology of nematode fixation. The utility of DESS in both morphological examination and molecular analyses was also shown for animal-parastic nematodes (Naem et al. 2010) and other groups of invertebrates (Strona et al. 2009). It should be noted, however, that a disadvantage of this fixation method is reduced sharpness of specimen morphological characteristics as relates to nematodes with gently outlined diagnostic features. This does not change the fact that fixation in DESS after killing nematodes with hot water allows one to perform both regular morphometric measurements of nematodes and molecular analyses.

Acknowledgements. This work was done as part of the Innovative Economy Operational Programme conducted by the Museum and Institute of Zoology, Polish Academy of Sciences. Project No. WND-POIG.01.03.01-00-133/09 co-financed by the European Union from the European Regional Development Fund.

References

Brown, D.J.F. & Topham, P.B. (1984) A comparison of reported variation in the morphometrics of *Xiphinema diversicaudatum* (Nematoda: Dorylaimida) and the effects of some methods of reparing specimens for examination by optical microscopy. *Nematologia Mediterranea*, 12, 169–186.

Chizhov, V.N., Chumakova, O.A., Subbotin, S.A. & Baldwin, J.G. (2006) Morphological and molecular characterization of foliar nematodes of the genus *Aphelenchoides*: *A. fragariae* and *A. ritzemabosi* (Nematoda: Aphelenchoididae) from the Main Botanical Garden of Russian Academy of Science, Moscow. *Russian Journal of Nematology,* 14 (2), 179–184.

Courtney, W.D., Polley, D. & Miller, V.L. (1955) TAF, an improved fixative in nematode technique. *Plant Disease Reporter,* 39, 570–571.

Crozzoli, R., Hurtado, T., Perichi, G. & Arcia, A. (2008) Characterization of a Venezuelan population of *Aphelenchoides ritzemabosi* on chrysanthemum. *Nematologia Mediterranea,* 36, 79–83.

Dobosz, R., Winiszewska, G., Malewski, T., Rybarczyk-Mydłowska, K., Tereba, A., Kowalewska, K., Gawlak, M. & Bogdanowicz, W. (2013) Morphological and molecular features of *Punctodera stonei* Brzeski, 1998 (Nematoda: Heteroderidae) – species associated with roots of grasses. *Annales Zoologici,* 63, 157–162.
http://dx.doi.org/10.3161/000345413X669487

Grewal, P.S., Richardson, P.N. & Wright, D.J. (1990) Effects of killing, fixing and mounting methods on taxonomic characters of parthenogenetic adult female *Caenorhabditis elegans* (Nematoda: Rhabditidae). *Revue de Nématologie,* 13 (4), 437–444.

Hooper, D.J. (1958) *Aphelenchoides dactylocercus* n. sp. and *A. sacchari* n. sp. (Nematoda: Aphelenchoidea). *Nematologica,* 3, 229–235.
http://dx.doi.org/10.1163/187529258X00454

Jagdale, G.B. & Grewal, P.S. (2006) Infection behaviour and overwintering survival of foliar nematodes, *Aphelenchoides fragariae*, on Hosta. *Journal of Nematology,* 38 (1), 130–136.

Jiang, L., Li, X. & Zheng, J. (2007) First record of *Bursaphelenchus rainulfi* on pine trees from eastern China and its phylogenetic relationship with intro-genus species. *Journal of Zheijang University SCIENCE B,* 8 (5), 345–351.
http://dx.doi.org/10.1631/jzus.2007.B0345

Khan, M.R., Handoo, Z.A., Rao, U., Rao, S.B. & Prasad, J.S. (2012) Observations on the foliar nematode, *Aphelenchoides besseyi*, infecting Tuberose and Rice in India. *Journal of Nematology,* 44 (4), 391–398.

Khan, Z., Son, S.H., Moon, H.S., Kim, S.G., Shin, H.D., Jeon, Y.H. & Kim, Y.H. (2007) Description of foliar nematode, *Aphelenchoides fragariae* (Nematoda: Aphelenchida) with additional characteristics from Korea. *Journal of Asia-Pacific Entomology,* 10 (4), 313–315.
http://dx.doi.org/10.1016/S1226-8615(08)60368-4

Khan, Z., Son, S.H., Shin, H.D. & Kim, Y.H. (2008) First report of a foliar nematode *Aphelenchoides fragariae* (Aphelenchidae) on *Stachys riederi* var. *japonica*, a medicinal plant, in Korea. *Plant Pathology Journal,* 24 (1), 97–100.
http://dx.doi.org/10.5423/PPJ.2008.24.1.097

Lamberti, F. & Sher, S.A. (1969) A Comparison of preparation techniques in taxonomic studies of *Longidorus africanus* Merny. *Journal of Nematology,* 1 (3), 193–200.

Luc, M., Sikora, R.A. & Bridge, J. (2005) *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture*, CABI, 871 pp.

http://dx.doi.org/10.1079/9780851997278.0000
Naem, S., Pagan, C. & Nadler, S.A. (2010) Structural restoration of nematodes and acanthocephalans fixed in high percentage alcohol using DESS solution and rehydration. *Journal of Parasitology,* 96 (4), 809–811.
http://dx.doi.org/10.1645/GE-2402.1
Schwartz, M. (1911) Die Aphelenchen der Veilchengallen und der Blattflecken an Farnen und Chrysanthemum. *Arbeiten aus der Kaiserlichen Biologischen Anstalt für Land- und Forstwirtschaft*, 8, 303–344.
Seinhorst, J.W. (1962) On the killing, fixation and transferring to glycerin of nematodes. *Nematologica*, 8 (1), 29–32.
http://dx.doi.org/10.1163/187529262X00981
Seinhorst, J.W. (1966) Killing nematodes for taxonomic study with hot F.A. 4:1. *Nematologica,* 12 (1), 178.
http://dx.doi.org/10.1163/187529266x00239
Steiner, G. & Buhrer, E.M. (1932) Miscellaneous notes on nemic diseases. *Plant Disease Reporter,* 16, 137.
Strona, G., Stefani, F. & Galli, P. (2009) Field preservation of monogenean parasites for molecular and morphological analyses. *Parasitology International*, 58, 51–54.
http://dx.doi.org/10.1016/j.parint.2008.10.001
Szczygieł A. & Cid del Prado Vera, I. (1981) Association of *Aphelenchoides fragariae* and *A. ritzemabosi* with strawberry plants in Mexico. *Zeszyty Problemowe Postępów Nauk Rolniczych*, 249, 81–85.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology and Evolution,* 28, 2731–2739.
http://dx.doi.org/10.1093/molbev/msr121
Timm, R.W. (1969) Two marine species of *Aphelenchoides*. *Nematologica,* 15, 370–375.
http://dx.doi.org/10.1163/187529269X00434
Vovlas, N., Minuto, A., Garibaldi, A., Troccoli, A. & Lamberti, F. (2005) Identification and histopathology of the foliar nematode *Aphelenchoides ritzemabosi* (Nematoda: Aphelenchoididae) on basil in Italy. *Nematology,* 7 (2), 301–308.
http://dx.doi.org/10.1163/1568541054879548
Wasilewska, L. (1969) *Aphelenchoides dubius* sp. n. from Poland (Nematoda, Aphelenchoidea). *Bulletin de L'Academie Polonaise des Sciences*, *Série des Sciences Biologiques,* 17 (7), 455–458.
Yoder, M., Tandingan De Ley, I., King, I.W., Mundo-Ocampo, M., Mann, J., Blaxter, M., Potras, L. & De Ley, P. (2006) DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. *Nematology,* 8 (3), 367–376.
http://dx.doi.org/10.1163/156854106778493448