A Comparison of Preparation Techniques in Taxonomic Studies of *Longidorus africanus* Merny

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Abstract: A comparison was made of ten different techniques for killing, fixing, and mounting *Longidorus africanus* Merny for microscopic study. The most satisfactory specimens were those killed by Seinhorst's method, fixed in FAA and mounted in glycerin by the slow method. Specimens killed by "gentle heat," fixed in FAA and mounted in glycerin were also acceptable as were those killed by hot formalin and mounted in glycerin or processed by Baker's method. Less satisfactory were: nematodes killed by "gentle heat," fixed in formalin and mounted in glycerin and specimens killed by vapor phase perfusion or by Hopper's lethal stain, both latter groups were mounted in glycerin after fixation in formalin. Killing with cold formalin, gradual heat (60 C for 15 min), or by storage in distilled water produced poorly defined specimens. Nematodes killed by hot formalin, and processed to glycerin by the slow method, maintained their live dimensions. Reduction in length occurred in specimens killed by cold formalin, by storage, or treated with solutions containing acetic or propionic acids. Nematodes processed by Baker's method increased in size. Other minor modifications occurred in specimens processed by the different methods. Esophageal definition was best in nematodes killed with formalin, hot or cold. There is no correlation between position of the posterior part of the esophagus and position of the onchiostyle.

Various methods have been used for the preparation of whole mounts of nematodes for identification and microscopic studies. No single technique, however, preserves all the morphological characters in lifelike form. The purpose of this investigation was two-fold: to find the most appropriate technique for the study of morphological characters useful in identifying *Longidorus africanus* and to determine whether different processing techniques cause morphological changes that might prevent correct specific identification.

MATERIALS AND METHODS

For the purpose of comparing ten different techniques for killing, fixing and mounting nematodes, aliquants (about 20 females each) of *Longidorus africanus* Merny, collected at Brawley in southern California, were used. The nematodes were extracted from the soil by Cobb's screening and gravity method (3). Techniques employed for killing and fixing were as follows: Seinhorst's method (8) with fixation in FAA (6); vapor-phase perfusion method (formalin-water 3 : 1 for 24 hr), fixation in 4% formalin (6); gentle heat (3) and fixation in 4% formalin; storage in distilled water; Hopper's lethal stain (4) followed by fixation in 4% formalin; hot 10% formalin (2); gentle heat (3) with fixation in FAA; cold formalin; and gradual heat at 60 C. Nematodes mounted by slow glycerin method were placed in 2.5% glycerin and 0.5% formalin in distilled water, until the water evaporated. To slow evaporation and avoid collapsing the cuticle, the watch glasses containing the nematodes were kept in a Petri dish with a moist paper disc for about 4 weeks. After this the nematodes were desiccated for a few days over calcium carbonate before being mounted in anhydrous glycerin on Cobb aluminum slides. To determine the effect of the glycerin on the specimens, and for comparative purposes, aliquants of nematodes were mounted directly in 2.5% formalin or in distilled water. Another group of specimens was processed by Baker's method (3) after being killed by

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TABLE 1. Evaluation of characters in *Longidorus africanus* processed by different techniques.

Technique (Killing, fixing, mounting)	Characters and their evaluation[a]						
	Amphidial pouches	Nerve ring	Esophagus[b]	Nerve cells surrounding the esophagus behind the nerve ring	Hemizonid	Female gonads	Large cells in the anal and caudal region
Seinhorst's method, fixed in FAA, mounted in glycerin	2	2	5	2	3	2	2
Vapor-phase perfusion method, fixed in formalin, mounted in glycerin	4	1	3	4	3	4	4
"Gentle heat," fixed in formalin mounted in glycerin	3	4	3	4	3	4	4
Baker's method	1	3	4	4	3	3	3
Storage in distilled water, mounted in glycerin	4	4	5	4	3	4	4
Hopper's stain, fixed in formalin, mounted in glycerin	3	3	5	1	1	3	1
Hot formalin, mounted in glycerin	3	2	2	4	3	2	4
"Gentle heat," fixed in FAA, mounted in glycerin	3	3	5	5	3	3	3
Cold formalin, mounted in formalin	3	3	3	4	3	4	4
Gradual heat at 60C, mounted in distilled water	3	3	5	4	3	4	4

[a] 1—Very well defined; 2—Well defined; 3—Defined; 4—Poorly defined; 5—Undefined.
[b] In the evaluation of the esophagus is meant the general shape of the posterior portion as well as the definition of the nuclei.

gentle heat and fixation in 4% formalin. A subjective evaluation of all specimens processed by the various methods was made by observing the preservation of certain morphological characters (Table 1) under oil immersion.

The diagnostic characters for species separation in *Longidorus* are not well defined because no comparative studies have been made on the important characters for species identification. Differentiation between *Longidorus* and *Xiphinema* is based on the position and structure of the guiding ring, and on the structure of the spear extension (1). We believe that certain characters useful for the specific identification in the genus *Xiphinema* (1, 5) may also be important for *Longidorus*.

All specimens were evaluated using the following measurements taken by camera lucida technique (200×): length of body, length of esophagus, and anterior extremity-vulva distance. The distance of the guiding ring from the anterior extremity, the onchiostyle and its extension lengths, the length of tail and the width of body at vulva and anus were measured under oil immersion (2,000×). The ratios of the deMan formula

(12) as well as the V percentage (12) and the "c′" (9), were calculated.

To establish a standard method (SM) as control for comparison purposes, 12 live females were first measured in a drop of egg white hanging from the lower surface of a cover-slip inverted over the cavity of a deep-well slide. The viscosity of the egg white reduced nematode movement. These nematodes were then individually killed by immersion in fuming 4% formalin (56–58 C) in a deep-well slide, fixed 24 hr in 4% formalin, and then remeasured in 4% formalin. This operation was repeated in glycerin and anhydrous glycerin after slow glycerin infiltration followed by 2 weeks in a desiccator. Final measurements were taken from permanent slide mounts. The differences between the measurements were compared for statistical significance by Student's "t" test.

To determine whether different methods of killing affect the position of the esophagus, *L. africanus* (ten specimens for each treatment) were individually placed in a drop of egg white hanging into the cavity of a deep well slide and the posterior part of the esophagus sketched with the aid of a camera lucida (800×). The nematodes were then killed using one of the following methods: (i) vapor phase perfusion (formalin-water 3 : 1, 24 hr) (6); (ii) cold aqueous 5% formalin; (iii) hot aqueous 5% formalin; (iv) "gentle heat" according to Goodey (3); (v) Seinhorst's method (8); (vi) Hopper's lethal stain (4). The posterior part of the esophagus was again sketched and the drawings compared with those of the live nematodes.

The relationship between esophagus and onchiostyle position of over 100 specimens was studied by comparing the distance between the anterior tip of the onchiostyle and the anterior body extremity with changes in the position of the posterior esophagus.

RESULTS

The morphological characters were preserved best in nematodes killed by Seinhorst's method and fixed for 24 hr in FAA. In these specimens the amphidial pouches, nerve ring, the nerve cells surrounding the esophagus posterior of the nerve ring and some large cells in the caudal and anal region were clearly visible. The female gonads were also well defined. Some adverse effects were observed: a slight collapsing of the somatic muscles in the anterior region, and a lack of visible esophageal nuclei (Table 1).

In specimens prepared by the vapor phase perfusion method the gonads were not well defined and the esophagus appeared somewhat contracted and slightly distorted. Nerve cells surrounding the esophagus, cells in the caudal and rectal region and amphidial pouches were not detected. The nerve ring was clearly visible (Table 1).

Nematodes killed by Hopper's lethal stain revealed the most satisfactory after a 5 min treatment period followed by 24 hr fixation in 4% formalin. This method favored definition of certain structures (e.g., nerve cells surrounding the esophagus, hemizonid and cells in the caudal region), but the somatic musculature was slightly collapsed in the anterior region and the nuclei were not discernible in the slightly contracted esophagus. The female gonad and nerve ring were well defined in some specimens but obscure in others (Table 1).

For mass collections, hot (90 C) 10% formalin was poured into aqueous nematode suspension in equal volume (the final temperature was about 60 C). Nematodes so prepared were fixed and preserved in 5% formalin. Morphological features of these specimens were well defined and most organs were easily visible. Amphidial pouches were visible but not distinct. The esophagus was relaxed with observable nuclei (Table 1). Internal organs of nema-

todes killed by "gentle heat" and fixed for 24 hr in 4% formalin, and then processed with the glycerin slow method were not always well defined. Esophageal nuclei were sometimes visible. Amphidial pouches could be seen (Table 1).

The condition of nematodes killed by "gentle heat" fixed 24 hr in 4% formalin and processed by Baker's method, were good although not as satisfactory as those obtained with the Seinhorst method. Gonads, large caudal cells and nerve ring were visible; the esophagus was poorly defined and its nuclei were not evident. The nerve cells surrounding the esophagus were not easily detectable. Baker's method was the best method for detecting the amphidial pouches (Table 1).

Nematodes killed by "gentle heat," and fixed for 24 hr in FAA were better defined than those killed by the same method and fixed in formalin. Gonads, nerve ring and caudal cells were poorly defined, the esophagus was contracted, and the esophageal nuclei were not detectable. The amphidial pouches were well defined (Table 1).

According to Thorne (12), cold fixatives induce distortions and contractions in specimens that may be advantageous for certain characters. Because of this possibility, the effects of both cold formalin and gradual heating on a hot plate were tested. To accomplish this, an equal volume of 10% cold formalin was poured into a 25 ml nematode-water suspension. The nematodes were left for fixation a few days in the same medium and then were mounted in 2.5% formalin. The specimens were badly distorted and twisted, and morphological characters were difficult to resolve. The esophagus did not appear altered and in some cases a few nuclei were seen. Most nematodes were reduced in length and increased in width (Table 1).

Live nematodes were placed in 10 ml of tap water on a hot plate at 60 C for 15 min, then mounted and examined in distilled water without prior fixation; morphological characters were difficult to resolve. The esophagus, vagina and uterus were distorted and badly twisted (Table 1).

In all of these methods, death of the nematodes occurred relatively abruptly. To investigate the effect of a more "natural death," several specimens, either larvae or females, were stored in a small beaker in distilled water at ambient temperature. The water was changed daily to reduce contamination. All of the nematodes appeared to be dead after 2 weeks, and were processed by the slow glycerin method. Fixation was omitted to eliminate possible effects on the structures. The internal structures were difficult to resolve and the somatic musculature had collapsed in many areas under the cuticle. Moreover, the esophagus was contracted and the nuclei were undefined (Table 1).

No statistically significant differences were found between the means for the measurements made on the same specimens alive and subsequently processed to permanent mounts in glycerin by the slow method after killing in hot 4% formalin (Table 2). Small variations reported in the intermediate steps are probably attributable to experimental errors. Therefore, in the study on the effect of the various techniques on the dimensions of the nematodes, the standard technique was altered by pouring hot 10% formalin into an equal volume of aqueous nematode suspension instead of individually immersing the nematodes in fuming 4% formalin. The killing temperature was the same in both methods.

Significant differences were detected in the dimensions (length, esophagus and width) of nematodes processed by different techniques (Table 3). Body length increased significantly in Baker's method, remained essentially the same in vapor phase perfusion and "gentle heat" followed by formalin fixa-

TABLE 2. Variations in measurements at different steps during preparation of specimens of *Longidorus africanus* by the standard method (SM) and the slow glycerin infiltration method.

Measurements[a]	Specimens alive	Killed with hot formalin	Slowly infiltrated with glycerin	Kept 2 weeks in desiccator in dehydrated glycerin	In permanent mounts [b]
Length mm	3.592	3.608	3.592	3.570	3.593
Esophagus length μ	385	391	396	392	391
Distance guiding ring-anterior extremity μ	29	30	29	29	29
Body width at vulva μ	39	38	39	39	40
Body width at anus μ	27	27	28	28	27
Tail length μ	36	36	36	36	36

[a] Average of 12 specimens
[b] $t = 2.074$ ($P = 0.05$) and 2.819 ($P = 0.01$).

tion but decreased significantly from that of the standard techniques (SM) in all other methods. The ratio "a" decreased significantly in cold formalin, Hopper's stain and Seinhorst's method, whereas the ratio "b" increased significantly in the storage, "gentle heat," Baker's method and vapor phase perfusion method. The posterior part of the esophagus moved conspicuously forward in the specimens treated by these latter methods. The tail ratio "c" was significantly reduced from SM, in the Seinhorst's, cold 10% formalin, storage and in those killed by "gentle heat" and fixed in FAA methods. No differences were observed in the location of the vulva and only in the case of specimens killed with Seinhorst's method was "c" statistically different from "c" observed for SM.

It is interesting to note that the mean length of the onchiostyle was statistically shorter in specimens killed by gradual heat, and that the spear extension in specimens killed by the Hopper's stain measured 12.9% less than in SM.

The guiding ring in specimens killed by storage, Seinhorst's method, "gentle heat," and vapor phase perfusion was in a more anterior position, the means for its distance from the anterior extremity ranging 3.4% to 15% less than in the SM nematodes (Table 3).

The SM nematodes were 8% wider than the specimens killed by storage, and 9.7% narrower than those killed either by vapor phase perfusion or "gentle heat" and processed, respectively, through the slow glycerin and Baker's methods (Table 3).

Only small differences were detected in the tail length and anal body width. Since the range is in the order of a few microns, the differences are considered unimportant (Table 3).

The "dorylaim" two-part esophagus is composed, anteriorly, of a slender tube occupying about two-thirds of the length of the esophagus, and posteriorly by an enlarged muscular bulb of rectangular shape containing glandular bodies. Often, the posterior part of the esophagus is projected forward so a loop of esophagus is seen overlapping the bulb at the attachment of the two parts. Since the few accurate studies on the morphology of the esophagus of Longidorinae (7, 13) report no longitudinal muscles connected with the esophagus, the mechanism of this process remains unexplained.

Studies on the position of the esophagus showed only small differences between live specimens and those killed by the vapor phase perfusion method or by either hot or cold formalin, while distortions and contractions occurred in specimens killed either by "gentle heat," or Hopper's lethal stain, and in some of those killed by Seinhorst's method.

Formation of the esophageal loop, due to the forward position of the posterior part of

TABLE 3. Measurements and ratios of *Longidorus africanus* females prepared with different techniques.[a]

Techniques	Length mm	a	b	c	V%	Tail length/ body w. at the anus (C[1])	Onchio-stlye μ	Exten-sion μ	Distance anterior extremity-guiding ring μ	Body width at the vulva μ	Tail length μ	Body width at the anus μ
Hot formalin, mounted in glycerin (Standard technique) = (SM)	4.020	97.3	10.6	105.3	46.75	1.30	87	35	30	41	38	29
Cold formalin, mounted in 2% formalin	3.461	79.6	10	93.9	45.8	1.28	85	36	29	43	37	29
% difference with (SM)[b]	−14**	−18**	−6.5	−12**	−2	−1.6	−2.3	+2.8	−3.4	+5	−2.7	0
Storage, mounted in glycerin	3.532	92.7	13.4	94.3	46.43	1.40	86	33	28	38	38	27
% difference with (SM)	−12**	−4.7	+26**	−10.5*	−0.7	+7.7	−1	−6	−7**	−8*	0	−7.4*
Seinhorst's method, fixed in FAA, mounted in glycerin	3.728	87.9	10.5	93.6	46.89	1.44	87	34	29	42	40	28
% difference with (SM)	−7.8**	−10.6**	−1	−12.5**	+0.3	+10.8**	0	−3	−3.4*	+2.4	+5.3	−3.6
Hopper's stain, fixed in formalin, mounted in glycerin	3.730	87.4	10.8	98.9	47.15	1.38	86	31	26	43	38	27
% difference with (SM)	−7.8**	−11.2**	−2	−6.4	+0.8	+6	−1	−12.9**	−15**	+4.9	0	−7.4*
"Gentle heat," fixed in FAA, mounted in glycerin	3.780	92	11.6	95.6	46.85	1.42	84	34	29	41	40	28
% difference with (SM)	−6.3**	−5.7	+8.8	−10*	+0.2	+9.2	−3.6	−3	−3.4*	0	+5.3	−3.6
Gradual heat at 60 C, mounted in distilled water	3.888	98.3	11.5	97.7	46.67	1.35	83	35	30	40	40	29
% difference with (SM)	−3.3**	+1	+7.9	−7	−0.2	+3.7	−4.8*	0	0	−2.5	+5.3	0
"Gentle heat," fixed in formalin, mounted in glycerin	4.079	95	12.3	101	46.88	1.40	87	35	28	43	41	29
% difference with (SM)	+1.5	−2.6	+16**	−4.6	+0.3	+7.7	0	0	−7**	+4.9	+7.8*	0
Vapor-phase perfusion method, fixed in formalin, mounted in glycerin	4.162	92.8	11.8	107.3	46.89	1.33	88	36	29	45	39	29
% difference with (SM)	+3.5	−4.8	+11.5*	+2	+0.3	+2.3	+1	+2.8	−3.4*	+9.7**	+2.6	0
Baker's method	4.302	95.5	12.6	110.6	46.77	1.32	88	35	29	45	39	29
% difference with (SM)	+7**	−1.8	+18**	+5	+0.04	+1.5	+1	0	−3.4*	+9.7**	+2.6	0

[a] Each figure is the mean of 14 observations.
[b] Standard Technique (SM) = 100%.

* Higher than mean for standard technique (P = 0.05).
** Higher than mean for standard technique (P = 0.01).

the esophagus, was not correlated with the onchiostyle position. The onchiostyle, extruded or retracted, was independent of the formation of the esophageal loop.

DISCUSSION

Of the techniques tested, none can be considered the single outstanding method of processing this *Longidorus* species. The characters observed were best preserved in specimens killed by Seinhorst's method. But appreciable reduction in body length occurred in nematodes either killed or fixed with solutions containing acetic or propionic acids. Goodey attributed this effect to ethanol. Specimens killed with hot 10% formalin, then processed to glycerin by the slow method, were satisfactory for the characters considered and were not greatly modified compared with live specimens. Only in those nematodes killed by hot formalin was it possible to determine the distribution of the esophageal nuclei. The amphidial pouches were easily discernible in specimens killed by "gentle heat" and then prepared by Baker's method. Nematodes killed by "gentle heat" and fixed in 4% formalin were darker than those fixed with FAA, or those processed by Baker's method. We believe that the lactophenol and the FAA fixative has a clearing effect on the specimens, possibly due to lipid extraction. Those processed by Baker's method increased in size; this may have been caused by the combined action of lactophenol and heat, which made the tissues more turgid. Other methods such as vapor phase perfusion, Hopper's lethal stain, cold 10% formalin, gradual heat (at 60 C) or storage in distilled water, gave poorly preserved specimens and are not recommended. Most of the morphological characters studied in *Longidorus* species, and the dimensions of the nematodes, underwent significant changes. Consistent modifications of more than one character by more than 10% may result in erroneous identification. Particular attention should be paid to the anterior position of the onchiostyle guiding ring in specimens killed by Hopper's stain. Keys to the species of *Longidorus* use the ratio distance of the guiding ring from the anterior extremity, divided by the width of the lip region as a diagnostic character (10, 11). Changes in this measurement are unimportant in species having the guiding ring in a forward position, but might considerably affect the ratio in those species in which the guiding ring is more posteriorly located.

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