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Techniques for clearing and mounting Collembola from old ethanol collections

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Abstract

Soil mesofauna was collected between 1983 and 2000 from a successional site in Bremen, Germany. The recent analysis of Collembola from this collection posed great challenges in clearing of the specimens stored in 70% ethanol for 15–32 years for microscopical inspection. For this purpose, five media for permanent slide mounts with different clearing properties were tested: Marc André 2, Huether's E65, Polyvinyl lactophenol (PVA), Rusek's and Gisin's mixture. None of these media cleared the old specimens sufficiently, therefore, pretreatment procedures had to be applied. Two out of eight tested pretreatment methods cleared the aged Collembola successfully: treatment in 10% NaOH solution at 60°C for 10 minutes, which, however, makes the specimens very fragile. Another pretreatment method originally developed for extracting DNA from Collembola cleared the aged specimens very well and kept them sufficiently stable for further handling. For permanent mounts, Marc André 2 was used, as it has good optical properties, is easy to apply and quickly yields ready-to-handle mounts, especially when dealing with high numbers of preparations for ecological research.

Keywords gum-chloral mounting media | high throughput slide mounts | clearing agents | DNA extraction solution

1. Introduction

The determination of soil inhabiting microarthropods to species level by their external morphological features requires examination under a high quality microscope at high magnification. Remains of internal tissue interfere with the detectability of taxonomic characters. Body contents should always be removed or cleared so that the morphological characters used in taxonomy become clearly visible using transmitted light with phase contrast or differential interference contrast (DIC or Nomarski interference contrast). The determination of microarthropods includes mounting for documentation and review. There are lots of considerations in making permanent mounts – ease of use, availability of ingredients, drying time, clearing effect and longevity (e.g. Schauff 1986, Rusek & Greenslade 1992, Gunter & Brown 2004, Glime & Wagner 2013, Rondon & Corp 2015). The most important procedures and materials for fixation, clearing and mounting microarthropods are summarized by Dunger & Fiedler (1997). Here we investigate the applicability of such media on long-time stored Collembola samples.

Collembola communities of two different plots (undisturbed succession and recultivated) were collected between 1980 and 2000 on the plateau of the Siedenburg rubble and debris dump in the vicinity of Bremen (Koehler & Müller 2003). From these collections, only those from 1980 to 1982 had been mounted and studied by Berendt (1985). The material from 1983 to 2000 has been preserved in 70% ethanol for 15–32 years, and meanwhile poses great difficulties and challenges in the clearing process with standard methods. For specimens hard to clear due to long preservation in 70% ethanol,

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various authors proposed to soak them in cold or warm 7–10% potassium hydroxide or sodium hydroxide solution, or to heat the specimens in lactic acid or lactoglycerol (Gisin 1960, Mari-Mutt 1979, Fjellberg 1980, Brown 1997, Gunter & Brown 2004, Brown & De Boise 2006, Greenslade 2010). However, these methods were not successful in clearing our material.

The objective of the experiments reported here is to prepare specimens of Collembola for microscopic identification that have been stored for more than 15 years in 70% ethanol, and to find effective methods to clear large numbers of aged specimens during and/or before permanent mounting.

2. Materials and Methods

2.1. Sampling and fixation

The Collembola specimens had been sampled between 1983 and 2000 from the Siedenburg experimental site (Bremen-Walle, Germany) (Koehler 1998 & 2000, Koehler & Müller 2003). Collembola were extracted with a Macfadyen-canister-type apparatus (10 days, temperature increase 5°C/day, *T*max= 60°C) either into picric acid (from 1980 to 1990) or into ethylene glycol (from 1991 onwards), and afterwards transferred to 70% ethanol through a 25 µm sieve and washed back into the preserving glass with 70% ethanol. Therefore, the Collembola were preserved in 70% cold ethanol including minor impurities from the fixation fluids (Berendt 1985, Koehler 1998 & 2000). Collembola samples were heated in a water bath in 60°C for 5 minutes and shaken for better immersion and fixation (Fjellberg 1980), and afterwards stored at room temperature (22°C).

2.2. Optimization of preparation and mounting of Collembola from old collections

2.2.1. Pretreatment before permanent mounting

Eight pretreatment methods were applied prior to permanent mounting (Tab. 1). All pretreatment methods were applied systematically to three orders of Collembola available from samples, Poduromorpha, Entomobryomorpha, and Symphypleona. In order to check the clearing properties of the various methods, for method 1 to 7 (Tab. 1) six Collembola from four different families (Entomobryidae Isotomidae, Onychiuridae, Sminthurididae) were transferred by a pipette to a small glass vial. Vials were filled with the respective liquids and kept at temperatures and with time periods shown in Table 1.

The technique developed for DNA extraction (method 8, Tab. 1) was applied by placing eight old Collembola specimens from four different families (Brachystomellidae, Entomobryidae, Isotomidae, Sminthurididae) in small glass vials. Most remaining ethanol was removed with a pipette, and 200 µl of SNET buffer solution (Laird et al. 1991) plus 10 µl of proteinase

Table 1. Pretreatment methods in the optimization experiment.

method	pretreatment	mounting medium	temperature (˚C)	time of pretreatment (min)	heating device
1A	ethanol 70% + NaOH 10% (1: 1)	Marc André 2	40	10	thermomixer 1)
1B	ethanol 70% + NaOH 10% (1: 1)	Marc André 2	45	10	thermomixer 1)
1C	ethanol 70% + NaOH 10% (1: 1)	Marc André 2	50	10	thermomixer 1)
1D	ethanol 70% + NaOH 10% (1: 1)	Marc André 2	55	10	thermomixer 1)
1E	ethanol 70% + NaOH 10% (1: 1)	Marc André 2	60	10	thermomixer 1)
2	ethanol 70% + Toerne (1: 1)	Marc André 2, Huether's E65, PVA	60	25	water bath 2)
3	ethanol 99%	PVA	100	4	thermomixer 1)
4	Huether 1	Huether's E65	22*	24 h (1 day)	–
5	Huether 1	Huether's E65	22*	120 h (5 days)	–
6	Lactic acid + glycerol (3: 1) (Lactoglycerol)	Marc André 2	45	4	thermomixer 1)
7	Nesbitt's fluid	Marc André 2	22*	72 h	–
8	DNA extraction solution (SNET)	Marc André 2	55	3-5 h	lab drying oven 3)

* Room temperature, 1) Eppendorf thermomixer compact, 2) JUERGENS, 3) JUERGENS Heraeus electronic-B5042E. See Table 2 for further details.

K solution (600 mAU, QiaGen Hilden, Germany) were added. This mixture was kept at 55°C for 3–5 hours in a lab drying oven (JUERGENS Heraeus-electronic-B5042E). Then 500 µl ethanol (35 %) and 1.5 µl formol (3 %) were added directly to the mixture, and the samples were then stored at room temperature (22°C) for at least 3 days to remove remaining buffer salts from the body. After that step the specimens were mounted permanently on microscope slides with Marc André 2 mixture (Tabs 1–3).

2.2.2. Permanent mounting without pretreatment

In order to test whether extensive pretreatment methods could be avoided, the clearing potential of five embedding media was tested without pretreatment: Marc André 2, Huether's E65, Polyvinyl lactophenol (PVA), Rusek's and Gisin's mixtures (Tab. 2). It was done for 6 specimens per treatment from three different families: Entomobryidae, Isotomidae and Onychiuridae. Three drying temperatures of 55 ± 5°C, 65 ± 5°C and 75 ± 5°C were applied in a lab drying oven (JUERGENS Heraeus-electronic-B5042E) for 72 hours before microphotographs were taken. Two to three specimens were mounted between a large coverslip (24 × 40 mm) and a small coverslip (18 × 18 mm) to enable dorsal and ventral inspection with equal optical quality by simply turning the slide. Two microscope slides were prepared per treatment.

2.2.3. Assessment of clearing

To assess the efficiency of the clearing methods, the Collembola were retrieved under a LEICA MS5 stereomicroscope. The respective results of clearing were documented photographically (Olympus microscope BX60, SONY camera U-SMAD). All pictures of the whole body, mouthparts, thorax, furca and tibiotarsi were taken with 100, 200 and 400 fold magnification. According to the visibility of organs documented in the microphotographs, clearing quality scores were allocated as follows: 1 = excellent, 2 = very good, 3 = good, 4 = bad, 5 = very bad (Figs 1–3).

Table 2. Materials used in the optimization experiment (recipes given in Dunger & Fiedler 1997, Sambrook & Russell 2001).

	ingredients																					
Name of material	Chloral hydrate (g)	Diethyl ether (ml)	Ethanol 98%-100% (ml)	EDTA (g)	Formol 40 % (ml)	Glacial acetic acid (ml)	Glycerol (ml)	Glycerol, saturated picric acid (ml)	Gum arabic (g)	H2O dest. (ml)	HCl (ml)	Isopropanol (ml)	Karion (Sorbitol) P 300 (g)	Karion (Sorbitol) F liquid (ml)	Lactic acid (ml)	NaCl (g)	Phenol (g)	Polyvinyl alcohol (g)	SDS (sodium dodecyl sulfate) (%)	Sodium hydroxide (g)	Tris-Cl (g) (pH: 7.5-8.5)
NaOH 10% (pretreatment)										90										10		
Toerne (pretreatment)					3	30						1000										
Huether (1) (pretreatment)		25	75		0.3																	
Lactoglycerol (pretreatment)							10			10					30							
Nesbitt´s fluid (pretreatment)	40									12.5	2.5											
Marc André 2	200						30		20	50												
PVA (Polyvinyl lactophenol)	20						10			71					35		3.8	10				
Huether's E65	60								24	16			12	12			95					
Rusek	50					5	5		15	20												
Gisin					2.6		13	10							65							
SNET buffer solution				0.19						97.2						1.2			0.2		1.2	

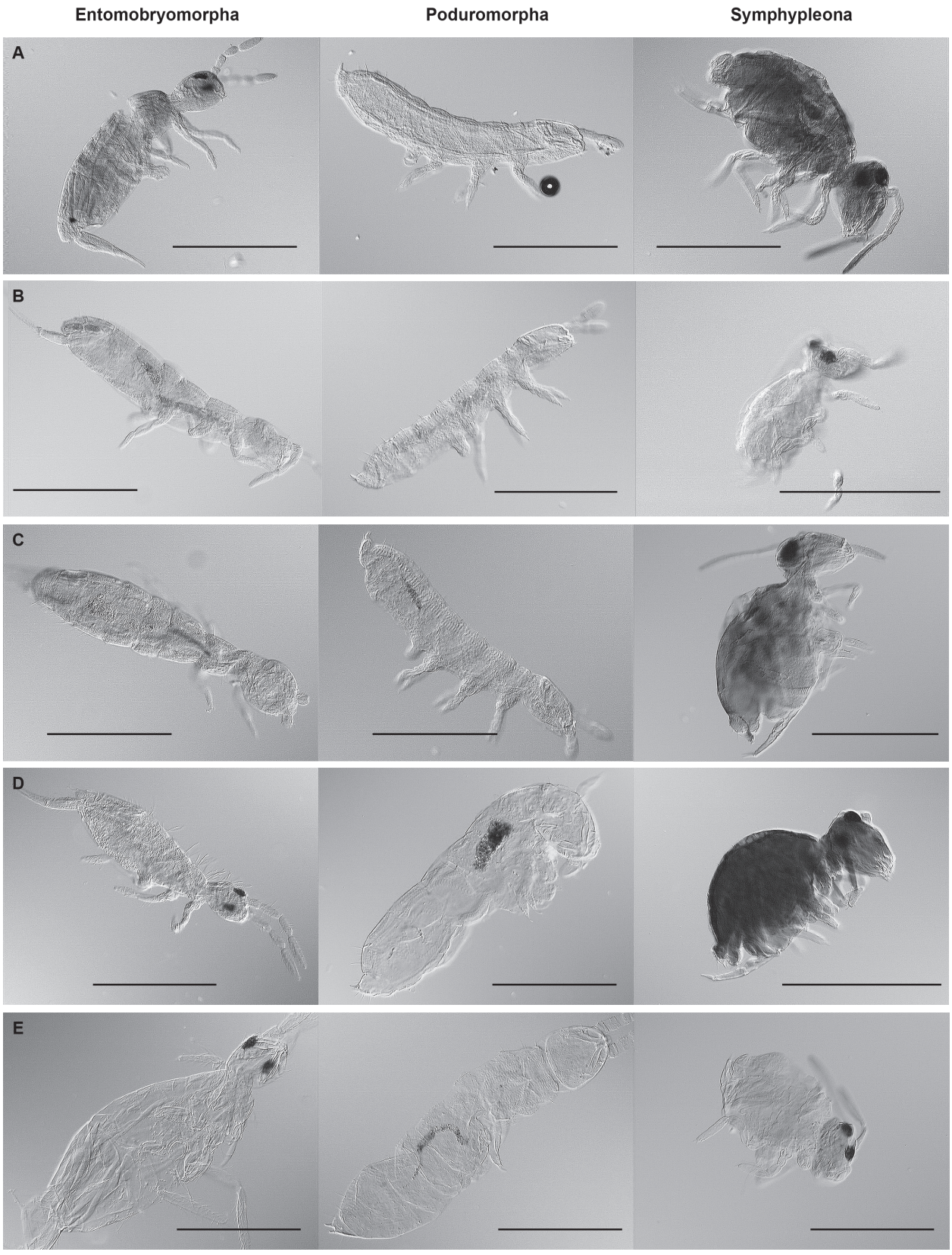

Figure 1. Representative pictures of three orders of Collembola after clearing in 10% NaOH for 10 minutes at five different temperatures and subsequent mounting in Marc André 2 medium. (**A**) 40°C, (**B**) 45°C, (**C**) 50°C, (**D**) 55°C, (**E**) 60°C. Pictures correspond to methods 1A to 1E in Table 1, respectively. All scale bars 0.5 mm.

3. Results

3.1. Pretreatment before permanent mounting

As shown in Table 3 and the microphotographs (Figs 1–3) the old Collembola specimens were not cleared sufficiently, except for method 1 (pretreating with 10% NaOH solution, see also Fig. 1, with five different temperature regimes) and method 8 (a technique developed for DNA extraction). Among the five temperature regimes in 10% NaOH solution, heating at 60°C for 10 minutes was the only method tested that could clear the Collembola sufficiently.

Treatment at 45°C yielded more feeble results, followed by 40°C, 50°C, 55°C and 60°C, without any significant differences among the three investigated orders.

Heating in a 10% NaOH solution at a temperature of 60°C resulted in sufficient clearing of specimens, however, those specimens became very soft and fragile, and therefore were difficult to mount. All the more, they lost some important identification characteristics, especially chaetae (Fig. 3). The technique developed for DNA extraction cleared the specimens very well, while the chaetal pattern remained intact and the cuticle retained good shape, so that specimens could easily be transferred to coverslips and mounted.

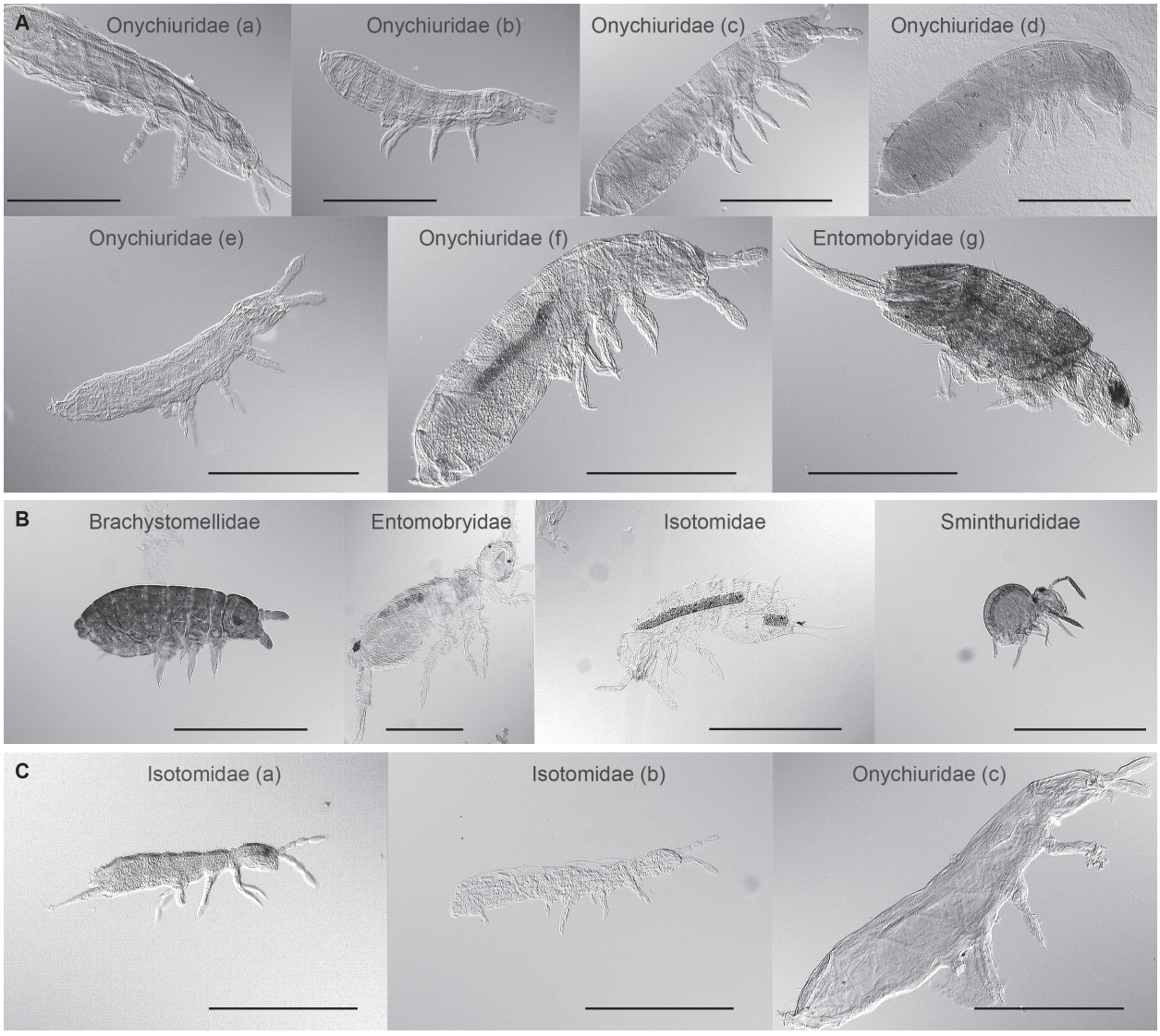

Figure 2. (**A**) **a, b**: Collembola mounted in Marc André 2 at 75 ± 5°C for 72 hours; pretreatment: **a** – Toerne, **b** – Lactoglycerol. **c–e**: Collembola mounted in Huether's E65 at 75 ± 5°C for 72 hours; pretreatment: c – Toerne, d – Huether 1 five days, e – Huether 1 one day. **f, g:** Collembola mounted in PVA at 75 ± 5°C for 72 hours; pretreatment: **f** – Toerne, **g** – ethanol 99%. (**B**) Collembola mounted in Marc André 2 at 55°C for 72 hours; pretreatment: DNA extraction method. (**C**) **a–c**: Collembola mounted in **a** – Marc André 2, **b** – PVA, **c** – Rusek's mixture without pretreatment at 75 ± 5°C for 72 hours. All scale bars 0.5 mm.

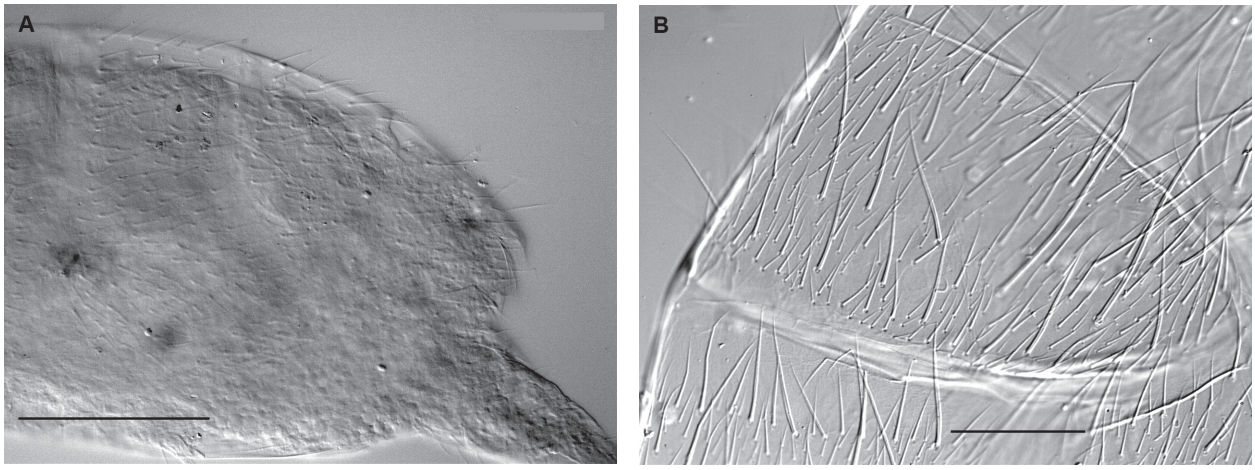

Figure 3. Pictures of chaetal pattern (here using the example of Isotomidae). Collembola mounted in Marc André 2 at 55°C for 72 hours; (**A**) pretreatment: 10% NaOH (poor result), (**B**) pretreatment: DNA extraction method (good result). All scale bars 0.1 mm.

Table 3. Results of pretreatment (see methods in Table 1) before permanent mounting (see details in Table 2; microphotographs were taken after 72 hours; see examples in Figs 1–3).

method	permanent mounting fluid	temperature (lab drying oven) °C	result
1A	Marc André 2	55 ± 5	not cleared, shrinking, remnants of fat and filament
1B	Marc André 2	55 ± 5	not cleared, shrinking, remnants of fat and filament
1C	Marc André 2	55 ± 5	not cleared, shrinking, remnants of fat and filament
1D	Marc André 2	55 ± 5	not sufficiently cleared, remnants of filaments
1E	Marc André 2	55 ± 5	cleared and features are visible, but very soft and inappropriate to handle for mounting
2	Marc André 2	55 ± 5	not cleared, shrinking, remnants of fat and filament
2	Marc André 2	65 ± 5	not cleared, shrinking, remnants of fat and filament
2	Marc André 2	75 ± 5	not cleared, shrinking, remnants of fat and filament
6	Marc André 2	75 ± 5	not cleared, shrinking, remnants of fat and filament
7	Marc André 2	55 ± 5	not cleared, shrinking, remnants of fat and filament
7	Marc André 2	65 ± 5	not cleared, shrinking, remnants of fat and filament
7	Marc André 2	75 ± 5	not cleared, shrinking, remnants of fat and filament
8	Marc André 2	55	sufficiently cleared, features are visible and appropriate to handle for mounting
2	Huether's E65	55 ± 5	not cleared, shrinking, remnants of fat and filament
2	Huether's E65	65 ± 5	not cleared, shrinking, remnants of fat and filament
2	Huether's E65	75 ± 5	not cleared, shrinking, remnants of fat and filament
4	Huether's E65	55 ± 5	not cleared, shrinking, remnants of fat and filament
4	Huether's E65	65 ± 5	not cleared, shrinking, remnants of fat and filament
4	Huether's E65	75 ± 5	not cleared, shrinking, remnants of fat and filament
5	Huether's E65	75 ± 5	not cleared, shrinking, remnants of fat and filament
2	PVA	55 ± 5	not cleared, shrinking, remnants of fat and filament
2	PVA	65 ± 5	not cleared, shrinking, remnants of fat and filament
2	PVA	75 ± 5	not cleared, shrinking, remnants of fat and filament
3	PVA	55 ± 5	not cleared, shrinking, remnants of fat and filament
3	PVA	65 ± 5	not cleared, shrinking, remnants of fat and filament
3	PVA	75 ± 5	not cleared, shrinking, remnants of fat and filament

3.2. Permanent mounting without pretreatment

The selected permanent mounting media without applying pretreatment methods did not clear the old specimens sufficiently (Tab. 5). As visible in the microphotographs (Fig. 2C), best, but suboptimal results were achieved when applying Marc André 2 at high temperature (75°C ± 5°C).

4. Discussion

Standard methods in the lab, such as mounting in Marc André 2 without pretreatment, poorly clear old Collembola specimens and thus hamper a proper microscopic identification.

Therefore, an appropriate pretreatment method is needed that can be applied prior to mounting. In the current study, we tested eight different pretreatment methods. Since heating in lactic acid was recommended for deeply pigmented or old specimens (e.g. Bellinger 1954, Greenslade 2010), all pretreatment methods except for Huether 1, were tested by heating the specimens in different clearing mixtures (Tab. 1). However, heating the specimens in ethanol 99% (Berch et al. 2001), Toerne mixture (Dunger & Fiedler 1997), Lactoglycerol (Fjellberg 1980) and warm lactic acid on a cavity slide proved unsuccessful for clearing our old Collembola specimens.

The tested pretreatment method with 10% NaOH yielded sufficient clearing but has some disadvantages, as NaOH is a harsh chemical, and heating the specimens in 10% NaOH solution makes them very soft and fragile. Heating the solution to more than 55°C improves the clearing process but tends to damage important identification characteristics such as chaetae, and does not keep the cuticle in a good shape. Therefore the specimens become inappropriate for handling and difficult to mount on slides. Our modified DNA extraction technique overcomes the disadvantages of other pretreatment methods. As common DNA extraction buffers for eucaryotic genomic DNA include tissue lysis properties (e.g. Laird et al. 1991), they cannot only be used to bring DNA into solution but as well to dissolve

Table 4. Condition of Collembola after pretreatment in 10% NaOH at different temperatures and subsequent mounting in Marc André 2 at 65 ± 5°C for 48 hours. Scores: 1= excellent, 2= very good, 3= good, 4= bad, 5= very bad.

temperature (°C)	Poduromorpha	Entomobryomorpha	Symphypleona
40	5	5	4
45	5	5	5
50	4	4	5
55	3	5	4
60	2	3	2

Table 5. Details and results of permanent mounting without pretreatment. Clearing time = time between mounting and taking photos.

permanent mounting fluid	drying temperature (°C)	clearing time	result
Marc André 2	46	4 weeks	not cleared
Marc André 2	60	1 day	not cleared
Marc André 2	75 ± 5	2 days	little improvement (proper to interior study)
Polyvinyl-lactophenol	50	2 days	not sufficiently cleared
Polyvinyl-lactophenol	60	one day	not cleared
Polyvinyl-lactophenol	75 ± 5	2 days	not sufficiently cleared
Huether's E65	50	2 days	not sufficiently cleared
Huether's E65	60	2 days	not sufficiently cleared
Huether's E65	75 ± 5	2 days	not sufficiently cleared
Rusek	55 ± 5	2 or 3 days	not cleared, shrinking, remnants of body fluids and tissue
Rusek	65 ± 5	2 or 3 days	not cleared, shrinking, remnants of body fluids and tissue
Rusek	75 ± 5	2 or 3 days	not cleared, shrinking, remnants of body fluids and tissue
Gisin	55 ± 5	2 or 3 days	not cleared, shrinking, remnants of body fluids and tissue
Gisin	65 ± 5	2 or 3 days	not cleared, shrinking, remnants of body fluids and tissue
Gisin	75 ± 5	2 or 3 days	not cleared, shrinking, remnants of body fluids and tissue

the inner body contents while keeping the animal cuticle in good condition for subsequent identification. As the method can be applied to all individuals of one sample in a single vial, it enables preparation also of large numbers of specimens as common in ecological studies, and with the same quality in the clearing process for different orders of Collembola. According to microphotographs, we consider this method the most appropriate pretreatment when preparing old Collembola specimens for mounting and identification (see 2.2.1; Figs 2B & 3B).

Among five different permanent mounting media used in this investigation, Marc André 2 gave best results compared to other mixtures (Tab. 2). It possesses good clearing and optical properties, enables delicate and thin mounts and quickly yields ready-to-handle mounts. It should, however, be noted that chloral hydrate, as in all gum-chloral media tends to crystallize, due to evaporation of the solvent (water). The longevity of these media is hard to predict, while sealing the coverslip may prevent evaporation for a longer time (Gunter & Brown 2004). The other permanent mounting media tested here have some disadvantages: PVA very quickly gives well-cleared specimens but the appendages, e.g., legs and palpi, tend to shrink after a few weeks, and the specimens become distorted and fade over time. Besides, it is highly toxic because it contains phenol and the correct formulation of PVA is critical. Rusek's medium gives thicker mounts, limiting its suitability for preparation of small specimens, and specimens mounted in Gisin's mixture are often in very poor condition, as this is a liquid medium not appropriate for permanent mounting (Martin 1977, Rusek & Greenslade 1992, Huether 1993, Upton 1993, Halliday 1994, Bernard 2006).

As such, to achieve good clearing of old ethanol collection material, we recommend to incubate it in DNA extraction buffer such as SNET plus proteinase K as described in 2.2.1, method 8, and to mount it in Marc André 2 for best optical results.

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