Accurate enumeration and identification of *Testacea* (Protozoa, Rhizopoda) in forest soil using scanning electron microscopy

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Abstract

A new procedure is used for separating *Testacea* from soil, based on dispersion, sequential centrifugation-settling and subsequent filtration of the soil suspension. Combined with scanning electron microscopy (SEM), this procedure allows accurate enumeration of *Testacea*, with a recovery rate of 90%) and more precise counting (RSD: 5%) of *Testacea* than in previous works.

Keywords: Dispersant, Enumeration of *Testacea*, SEM observation, Soil particles

Testacea are abundant in soils, and contribute much to the carbon, nitrogen and phosphorus cycles by feeding on organic substances such as bacteria, fungi and

humus. Many methods exist for the enumeration of *Testacea*, including the watered soil suspension method and the Jones and Mollison slide method. In a study of *Testacea* in litter and soil, Coûteaux (1967, 1975) refined the membrane filter technique. However, this technique still suffers from two problems. First, optical microscopy restricts the size of soil samples to approximately1 mg (Coûteaux 1985). Such small samples may lead to inaccuracies in the abundances of rare species, such as *Centropyxis oomorpha* (Foissner, 1987). Second, identification of testacean species is difficult under a light microscope, owing to low resolution (Lousier and Parkinson, 1981).

The present study of the forest-soil ecosystem overcomes the problem of small sample size by repeating the suspend-settle-decant procedure of the soil suspension using a larger amount of sample, resulting in a high recovery rate of *Testacea,* and overcomes the identification problem using a scanning electron microscope (SEM). Some testacean population densities in forest-soils are evaluated below using these more accurate methods.

Soil was taken from a 60-year-old pine-oak forest dominated by *Pinus densiflora* and *Quercus variabillis* on the campus of Nagoya University, Nagoya, using a core sampler (5 cm diameter, 2.5 cm depth). Core samples were taken from the A-horizon of brown forest soil, Cambisols (Ohta, 1976 a,b), on five occasions between November 2000 and April 2001. Three cores were collected on each occasion and each core was put into a polyethylene bag and sealed in the field. Each set of three cores was mixed uniformly in the laboratory and was used in the experiments detailed below.

Separation of *Testacea* from the soil samples was carried out by the membrane filter technique as modified by Coûteaux (1967, 1975). A soil subsample (250 mg wet wt., moisture content about 20%) was suspended in 80 ml dispersant solution in a 100-ml beaker, and was stirred at 600 rpm for 90 min using a magnetic stirrer. The dispersant solution was Na-phosphate buffer, pH6.5, that contained 2 mM $Na_4P_2O_7$ at the final concentration. The pH of the dispersant solution increased to around 6.8. The soil suspension was centrifuged (175×g, 20 min) to remove fine particles of clay

minerals, bacterial cells and other contaminants of the supernatant. Less than 1% of *Testacea* was detected in the supernatant. The precipitate was resuspended in 10 ml distilled water and transferred to a 10-ml test tube. The suspension in the test tube was allowed to settle for either 3 or 5 minutes, and then decanted. Supernatant (sup fraction No.1) and precipitate (ppt fraction) were thereby obtained. The ppt fraction was then suspended and decanted again after either 3 or 5 minutes settling. This suspend-settle-decant procedure was repeated twice, to give the sup fractions Nos.2 and 3 and the final ppt fraction (suspend-settle-decant procedure). The sup fractions No.1-3 and the final ppt fraction were each diluted by the same dilution factor (V_{final}/V_{sample}) to an optical density at 660 nm (OD_{660}) of between 0.1 and 0.2. Ten ml of each diluted fraction were passed through a membrane filter (ADVANTEC, 25 mm diameter, 0.2 μm pore size, effective filtration area: 296 mm^2) using a suction pressure of 21kPa. *Testacea* on the membrane filter were not covered by fine or coarse particles at this OD condition. *Testacea* were observed with a SEM (HITACHI, S-2300) at an accelerating voltage of 10kV. Individual Testacea were counted over 10 randomly selected scan fields (each field 2 mm square) at 600× magnification. For comparison, 20 scan fields of 1 mm square on the membrane filter were surveyed at 800× magnification.

The population density (g^{-1} dry soil) of *Testacea* in the soil was calculated as:

$$\text{Population density}=(N_{total}\times A_t\times d)/(W_{soil}\times f\times A_{field})$$

where N_{total} is the total number of *Testacea* counted for the sup fractions, Nos.1-3, and the final ppt fraction. Also A_t is the effective area of the filter, A_{field} is the area of a single scan field, d is the dilution factor of the soil suspension, W_{soil} is the dry weight of soil sample, and f is the number of scan fields on one membrane filter. To find the distribution of *Testacea* in each fraction, the recovery rate was calculated as

follows:

$$\text{Recovery rate} = N_x / N_{total} \times 100\ (\%)$$

where N_x is the number of *Testacea* counted in sup fraction No.x. The relative standard deviation (RSD) of each data set was calculated to compare the precision with data given by other researchers as mentioned in Table 2.

Although the soils have so far been suspended in water, the sodium pyrophosphate solutions effectively broke soil aggregates and allowed *Testacea* to be detached (Table 1). Griffiths and Ritz (1988) found a similar effect of the dispersant Tris buffer, 50mM, pH 7.5, on mineral soil particles. After exclusion of fine particles by centrifugation, the suspend-settle-decant procedure for the soil suspension was carried out to remove particles larger than *Testacea*. Recovery rates of *Testacea* in sup fraction No.1 were respectively 70% and 57% for settling times of three and five mins (Table 1). These values show that a single cycle of the suspend-settle-decant procedure is inadequate to recover all *Testacea* from the soil suspension. In contrast, the cumulative recovery rates for sup fraction No.3 for settling times of three and five minutes were 90% and 85%, showing that *Testacea* were adequately separated from the soil suspension by repeating the procedure three times. A settling time of 3 min was sufficient. This procedure has the great advantage of using a large amount of soil sample to allow more accurate enumeration of *Testacea*.

Testacea have a shell consisting of a homogeneous layer, or agglutination of self-synthesized regular scales (often siliceous scales = idiosomes) or foreign particles (e.g., mineral particle = xenosomes). Most xenosomes have larger and heavier shells than idiosomes. The *Testacea* observed in this study were idiosomes such as *Trinema* and *Euglypha*, and xenosomes such as *Phryganella*; the rest were

Schoenbornia humicola and *Hyalosphenia subflava*.

Most idiosomes and others in sup fractions Nos.1-3 were located separately on the membrane filters and could be enumerated in both observation scan areas. The cumulative recovery rate of xenosomes in sup fractions Nos.1-3, however, were 46% for the 20-mm^2 scan area and 58% for the 40-mm^2 scan area (Fig. 1). These low recovery rates imply that enumeration of xenosomes should take place not only in the sup fractions but also in the ppt fraction. Also, the 20-mm^2 scan area proved inadequate to count xenosomes because of its large standard deviation. Xenosomes were therefore measured more exactly using a 40-mm^2 scan area.

Table 2 shows examples of the enumeration of *Testacea* and the RSD obtained by the present method. The population density of *Testacea* was estimated seasonally as the mean of five replicates (about 25 mg of air-dried soil was finally mounted on the membrane filter). All RSD values are smaller than those calculated from the data given in previous studies, except for Coûteaux (1985). That work gave accurate measurements using the membrane filter technique for litter- and soil-*Testacea*, but the amounts of soil were very small (1 mg dry soil). However, the data of Lousier and Parkinson (1981) give larger RSD values based on larger soil samples (5 or 10 mg wet soil) using the same method. This discrepancy may be a result of a greater variation among their soil samples, and counting loss. Lousier and Parkinson (1981) stated that the observation of *Testacea* by optical microscopy is difficult because of the low resolution with light transmitted through the membrane filter. The identification of *Testacea* mixed with diverse particles on the membrane filter demands a high-resolution microscope and skill, but SEM observation allows identification of testacean species without skill. If the shell of a testacean faces the membrane filter by its pseudostome, it would be difficult to identify species that

have been classified by the morphology of their pseudostome. Nevertheless, SEM observation should suffice to determine genus. Wilkinson and Davis (2000) showed that determination of genus diversity can act as a rapid assessment of microbial diversity.

Although it is difficult to know whether a testacean shell is empty or not, SEM observation has the following significant advantages: (1) small *Testacea* can be detected and identified from their shell morphology using high-resolution images; (2) there is no need for fixation and staining of *Testacea*; and (3) the gold-coated membrane filters can be stored for long periods without special treatment.

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Figure Captions

Fig. 1. Total recovery rates of *Testacea* (mean ± SD) in different scan areas, under triple repetition of the suspend-stand-decant procedure (settling time 3 min). □: smaller scan area (20 mm^2), ■: larger scan area (40 mm^2)

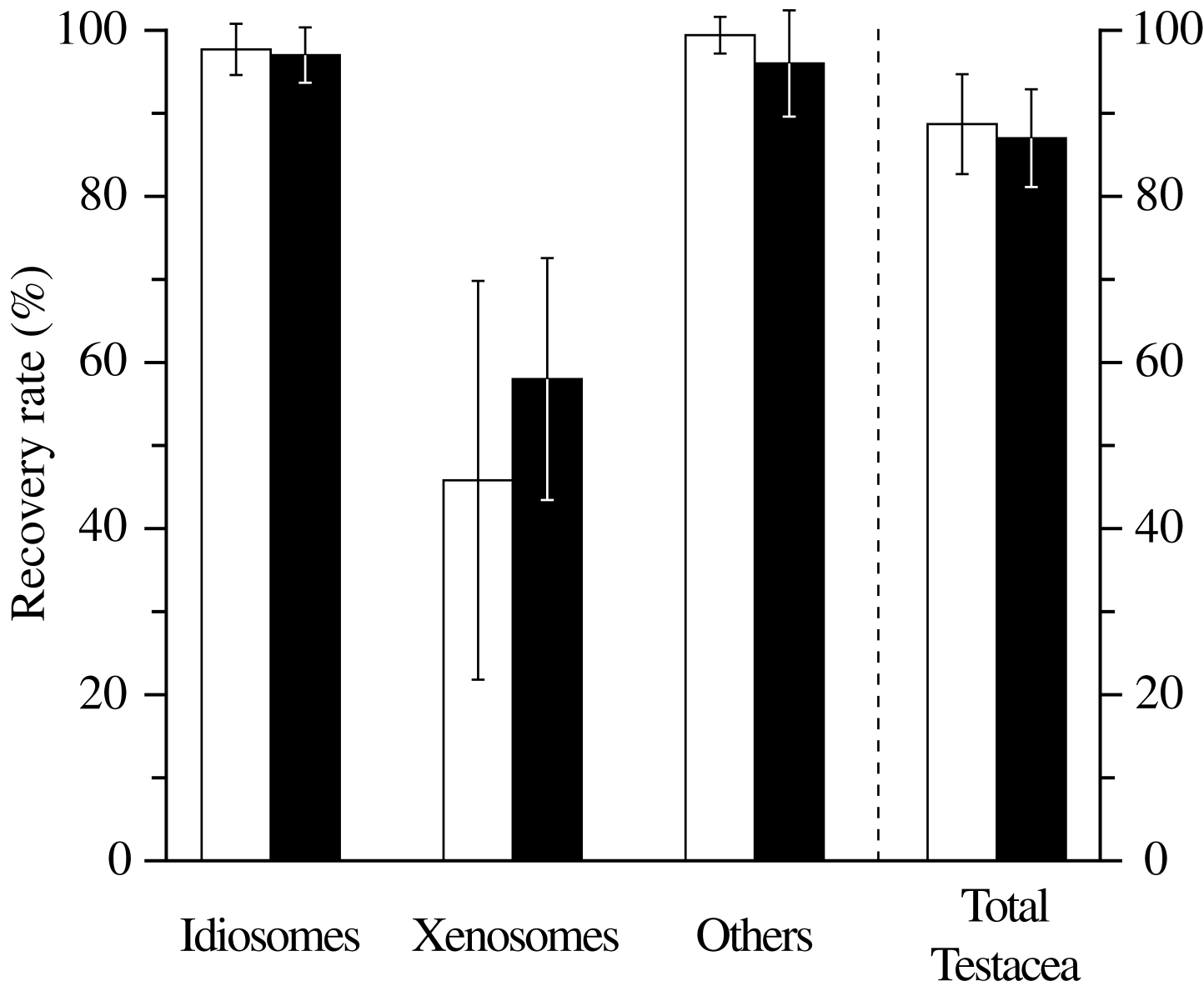
Recovery rate (%)
100
80
60
40
20
0
Idiosomes
Xenosomes
Others
Total
Testacea

Table 1. Recovery rate of each fraction separated by suspend-settle-decant procedure of settling time 3 or 5 min

Settling time (min)	Dispersant solution	Recovery rate (%)			
		sup			final ppt
		1	2	3	
5	without	40	13	7	40
5	with	57	22	6	15
3	with	70	19	1	10

Table 2. Population densities of Testacea and the precisions measured by this and previous

Data set	Population density (g^{-1} dry soil)	Population density ($\times 10^6$ m^{-2})	RSD(%)
Present Data (by SEM)			
Pine-oak forest			
13-Nov-00	13220	277	5
04-Dec-00	11360	176	7
25-Dec-00	6190	107	14
11-Apr-01	11480	243	11
17-Apr-01	13060	274	9
Published Data (by light microscope)			
Watered soil suspensions method			
Balík, 1996*			
Oak-hornbeam forest soil		640 - 970	26 - 33
Oak forest soil		610 - 2260	36 - 56
Beech forest soil		450 - 750	26 - 37
Membrane filter technique			
Lousier and Parkinson, 1981			
Aspen woodland (moder) soil	6159 - 59144		21 - 28
Coûteaux, 1967			
Oak forest (A_0) soil	77050		16
Coûteaux, 1985			
Mixed forest (dysmoder) soil	87370 - 198990		3 - 23
Jones and Mollison slide method			
Heal, 1964			
Mixed forest (mull) soil	6000 - 50000		14 - 80
Mixed forest (moder) soil	10000 - 182000		18 - 40
Oak forest (moder) soil	5000 - 264000		24 - 69
Heal, 1965*			
Grassland soil	40000	890	94
Limestone grassland soil	9000 - 69000	448	39 - 120
Juncuss squarrosus moor soil	25000 - 32000	195	101 - 237
Woodland (moder) soil	4000 - 31000	188 - 465	69 - 135
Lousier and Parkinson, 1981			
Aspen woodland (moder) soil	5900 - 13072		168 - 275

* Counted only living Testacea