Bacteria and Fungi

Epifluorescence Staining Protocals

Reagents

Filtered (0.2um) Deionized Water (FDIW)
70% ethanol (ETOH)
Immersion oil (Fisher, type FF)
Phosphate Buffered Saline (PBS)
  Stock solution: 7.8 g Sodium Phosphate Dibasic per liter deionized water
    8.5 g NaCl per liter of deionized water
  Working solution: 100 ml stock solution in 1 liter deionized water
DTAF stain (5-(4, 6 dichlorotriazin-2-yl) aminofluorescein) “Bacteria”
  Working solution 2 mg stain per 10 ml PBS and then filtered
Calcoflour stain (fluorescent brightener) “Fungi”
  Working solution: 2 mg stain per 1 ml FDIW and then filtered

Procedure

1. Wash slides (frosted end, 6-mm, 10-well, glass microscope slides) in Alconox detergent, rinse well with tap water followed by deionized water, rinse with 70% ETOH, rinse with tap water, and again with deionized water. Air-dry in sterile fume hood.

2. Weigh out 5g soil, and record the weight on a data sheet.

3. Homogenize 5g soil and 45 ml of FDIW in Warning blender on high for 1 minute.

4. Immediately remove 1 ml and add to test tube containing 9 ml FDIW. Thoroughly mix test tube by placing on a vortex.

5. Pipette 10 µl of the dilution into each of the 5 wells on a labeled microscope slide.

6. Prepare one slide for bacterial staining and one for fungal staining.

7. Air dry slides in fume hood completely.

Bacteria Staining Process

1. Flood each well on the slide with 10 – 20µl of DTAF stain.

2. Store slides in the dark in a covered container (e.g. a plastic Rubbermaid container, or a cooler) with wet paper towel to prevent drying for 30 minutes.

3. Place slides in staining rack and rinse in PBS for 30 minutes.

4. Repeat rinses twice more for a total of three, 30 minute rinses.

5. Rinse for 30 minutes in FDIW.

6. Remove slides from final rinse and air dry completely in sterile fume hood.

7. Add a small drop of immersion oil (Fisher, type FF) to each well then cover with a large cover slip (22mm by 50 mm). Attach ends of cover slip to slide with clear nail polish.(DO NOT USE QUICK DRY POLISH).

8. Let polish dry on all slides and place in a slide box.

Fungi Staining Process

1. Flood each well on the slide with 15-20µl of Calcoflour stain.

2. Store slides in dark covered container with wet paper towel to prevent drying for 2 hours.

3. Place slides in rack and rise in FDIW for 30 minutes.

4. Repeat rinse twice more for a total of three, 30 minute rinses.

5. Remove slides from final rinse and air dry completely in sterile fume hood.

6. Add a small drop of immersion oil (Fisher type FF) to each well cover with large cover glass. Attach ends of cover slip to slide with clear nail polish.(DO NOT USE QUICK DRY POLISH).

7. Let polish dry on all slides and place in a slide box.

Protozoa

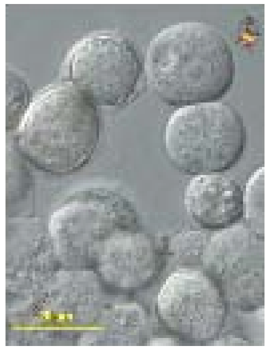
Traditionally protists were treated as a separate kingdom. Molecular characterization of members of this group has made it clear that protists should not be considered as a valid taxonomic group as they are not monophyletic. For the purpose of the workshop, we will separate the protozoa along traditional taxonomic lines of amoebae, flagellate, and ciliates.

Listed below are protists that are found in soils, including Fungi, with locomotion and trophic functional groups represented. From: M.S. Adl and V.V.S.R. Gupta (2006) Protists in soil ecology and forest nutrient cycling. Can. J. For. Res. 36: 1805–1817.

Taxonomic group	Example	Locomotion, morphology	Functional groups
Amoebozoa	Acanthamoebidae	Amoeboid	Bacterivory
	Flabellinea	Amoeboid	Bacterivory, cytotrophy, detritivory, fungivory, invertebrate consumers
	Tubullinea	Amoeboid, flagellate stages	Bacterivory, cytotrophy,
	Mastigamoebidae	Amoeboid flagellates,	Bacterivory, detritivory, fungivory
	Eumycetozoa	Amoeboid, flagellate stages	Bacterivory
Fungi	Ascomycota	Filamentous and yeasts	Primary saprotrophs, lichens, mycorrhizae
	Basidiomycota	Filamentous	Primary saprotrophs, ectomycorrhizae
	Chytridiomycetes	Cilium, thallus	Predators, primary saprotrophs
	Glomeromycota	Filamentous	Root symbionts
	Urediniomycetes	Filamentous and yeasts	Endophytes, pathogens, rhizosphere species
	Ustilaginomycetes	Filamentous and yeasts	Parasites
	Zygomycota	Filamentous	Predators, primary saprotrophs, endomycorrhizae
Parabasalia	Cristamonadida	Flagella	Symbionts
	Spirotrichonymphida	Amoeboid, flagella	Symbionts
	Trichomonadida	Amoeboid, flagella	Symbionts
	Trichonymphida	Amoeboid, flagella	Symbionts
Preaxostyla	Oxymonadida	Flagella	Symbionts
Euglenozoa	Euglenida	Flagella	Bacterivory, cytotrophy, photosynthetic
	Kinetoplastea	Flagella	Bacterivory, parasites
Heterolobosea	Acrasidae	Amoeboid	Bacterivory
	Gruberellidae	Amoeboid	Bacterivory
	Vahlkampfiidae	Amoeboid, flagella	Bacterivory, cytotrophy, Detritivory, fungivory
Cercozoa	Cercomonadidae	Amoeboid with flagella	Bacterivory
	Silicofilosea	Amoeboid	Bacterivory, cytotrophy, Detritivory, fungivory
Peronosporomycetes		Flagella, thallus	Predators, primary saprotrophs
Ciliophora		Flagella	Bacterivory, cytotrophy, detritivory, fungivory
Apicomplexa		Dispersal spores	Parasites

Flagellates - The term “Flagellates” refers to protists that possess one or more flagella. A flagellum (plural: flagella) is a tail-like structure that projects from the cell of certain pro and eukaryotes where it functions as a whip-like propulsive organ. The flgellates encountered in CPER soils feed on bacteria.

Amoebae – among Amoebae usually two groups are distinguished: the naked and the testate amoebae (=shelled amoebae).

Acanthamoeba (trophic stage) has numerous, slender and tapering pseudopodia (acanthopodia) giving the cell a spiny appearance. *Acanthamoeba* cysts are shown on the right. Picture from Marine Biological Laboratory, Woods Hole (MA) USA .

Naked amoebae (= gymnamoebae) have been recognized as a major component in soil litters, comprising 50% to 90% of the protozoa. Members of the genus *Acanthamoeba* are the most frequently isolated and probably the most common genus of gymnamoebae. Gymnamoebae have a trophic, actively feeding life stage, whereas cysts (dormant survival stage) are formed under adverse conditions. Gymnamoebae cysts may survive for years, and when conditions improve cysts germinate and will give rise to trophic amoebae again.

Testate amoebae ((Protozoa: Testacea) are single-celled organisms enclosed in an organic **test (= *shell***). Certain testate amoebae (Amoebozoa: Testacealobosia) are abundant in surface litter and organic horizons (around 100 individuals per gram), where they contribute to tissue decomposition. Mostly, testate amoebae are less numerous than gymnamoebae. The genus *Arcella* is one of the most abundant testacean genera. Most representatives of this genus are cosmopolitan, and they inhabit freshwater pools and streams. In soil they tend to be rare, except for moist forested areas.

Example of a testate amoebae: *Arcella gibbosa* with a light brown chitinous "test" (shell). Picture taken by Wim van Egmond, The Netherlands

Ciliates – ciliates are characterized short, dense hair-like structures on the cell surface (cilia) that serve to propel the ciliate and are used to draw in food particles. Most ciliates are free-living forms, only a few representatives show a parasitic lifestyle. Free-living ciliates may feed on bacteria, algae, or on other ciliates. As compared to their aquatic relatives, ciliates in soils tend to be small. Drought will lead to the formation of the survival stage - cysts. As compared to the Amoebae the typical number of ciliates tends to be relatively low in the range of 10-500 individuals per g soil or litter.

Incubating Protozoa

Materials needed:

Soil Samples 10g
Metal Coffee can (or 2 L beaker)
Beakers for decanting
Balance
Distilled water
Calcium carbonate ($CaCO_3$)
Medium (see recipe below)

For each soil sample will need

24 well plate (4x6 wells)
Sterile biotips
Biotip gun
1 milk dilution bottles with 90 ml medium
5 Test tubes filled with 9 ml soil extract

Preparing Soil Extract

1. Place 1 liter of soil in metal coffee can (or 2 L beaker). Add 1 liter of distilled water. Make sure the mixture does not fill the container more than half full.
2. Autoclave the mixture at 21 psi for 30 minutes and allow to settle over night in a sterile hood.
3. Decant liquid (soil extract) from the top of the autoclaved mixture into a clean beaker with as little soil as possible repeat until soil extract is relatively clear of all soil.

If soil is high in organic and or clay matter:

Do steps 1-2 as above
Decant liquid (soil extract) from the top of the autoclaved mixture into a clean beaker with as little soil as possible.
Add a pinch of calcium carbonate to soil extract allow to sit overnight and decant again into another beaker, again with as little soil a possible.
Autoclave again at 21 psi for 30 minutes and allow to settle for at least 1 hour longer if possible.

Protozoa Medium Preparation

5% soil extract and 0.9% NaCl in aqueous solution
Mix:
50 ml soil extract
9 g NaCl

950 ml distilled water
The medium may be dispensed in milk dilution bottles and screw top test tubes as required, autoclaved and stored for future use

Preparing Dilutions (10^{-1} to 10^{-6})

1. Mark the plate you are going to use with the sample number both on the lid and on the side of the plate.
2. Weigh out 10 g of soil sample.
3. Place 10g of soil into the milk dilution bottle with 90ml of medium, make sure the lid is tight. Shake the bottle vigorously for 1 minute
4. Using a sterile biotip, place 0.5 ml of this dilution into each of the 4 wells on the left row of the plate
5. Take 1 ml of this dilution from the milk bottle (with the same biotip) and put it into s test tube with 9 ml of protozoa medium.
6. Seal the Test tube tightly and shake for 1 minute (or vortex for 10 sec).
7. Using a new, sterile biotip place 0.5 ml of this solution in each of the second row of 4 wells.
8. Take 1 ml of the solution in the test tube, with the same biotip, and put it into a test tube with 9 ml of protozoa medium. Change to a new biotip.
9. Repeat steps 1-8 until all wells are full.

Feeding and Incubation

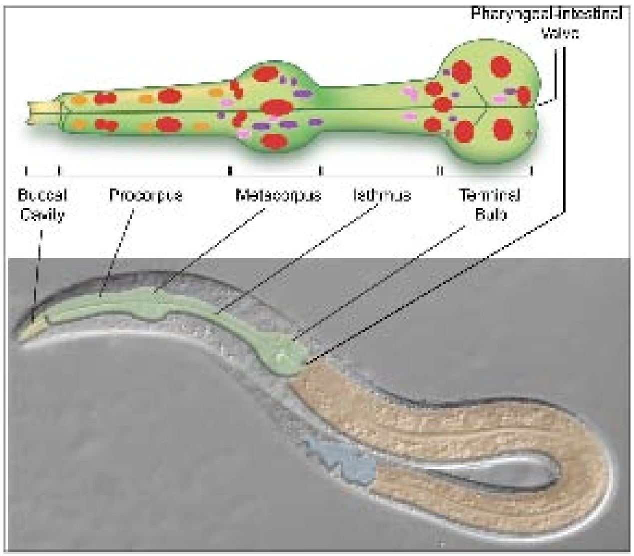
Feed the protozoa by placing 1 drop of any appropriate bacteria in aqueous suspension into each well.

Take a gallon zip lock type bag, moisten 2 paper towels and wrap 4 plates in each one. Place the protozoa plates into the bag 2 stacks of 4 will fit easily. DO NOT SEAL THE BAG!!

Place the bags with plates into the incubator. You should start seeing protozoa activity within 5 to 7 days.

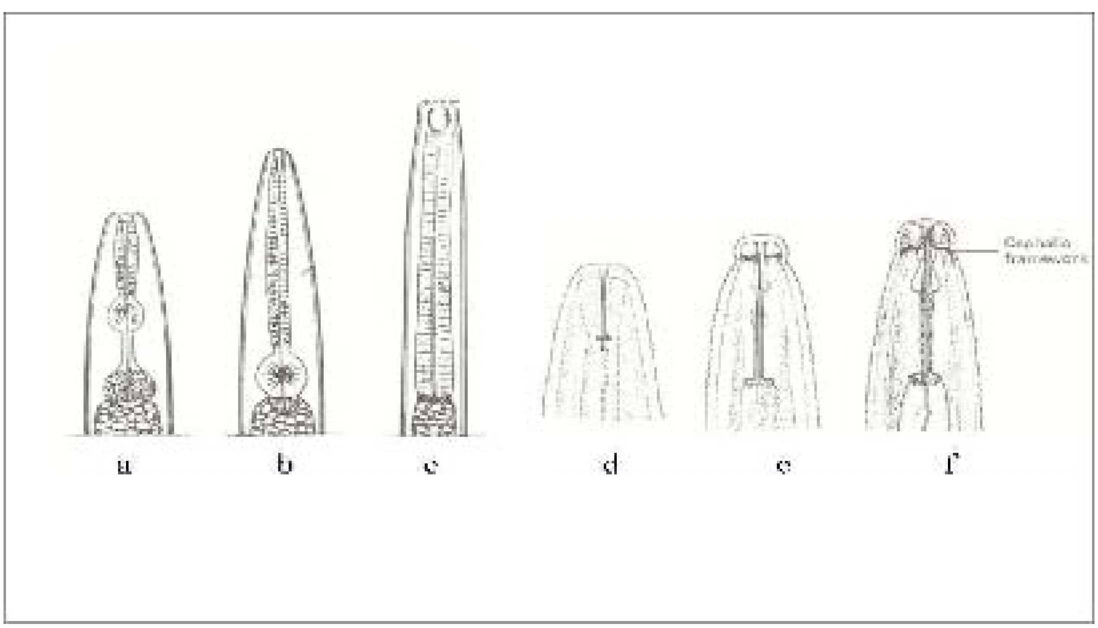
Nematodes
(Phylum Nematoda)

Nematodes constitute one of the largest and most widely distributed groups of animals in marine, freshwater, and terrestrial habitats. Their numerical dominance, exceeding often more than 1 million individuals per square meter and accounting for about 80% of all individual animals on earth (Platt 1994), their diversity in lifestyles, and their presence at various trophic levels point at an important role in many ecosystems. In soil food webs five functional groups are distinguished: plant parasitic nematodes, bacterivorous nematodes, fungivorous nematodes, omnivorous nematodes nematodes, and predaceous nematodes.



(Left). The *C. elegans* digestive tract is an epithelial tube consisting of the buccal cavity (lower panel, yellow), foregut or pharynx (green), midgut or intestine (orange) and hindgut (blue). (source: Suzan E. Mango, The *C. elegans* pharynx: a model for organogenesis. WormBook)

(Lower) Various types of buccal cavities (a, *Diplogaster*; b, *Plectus*; c, *Mononchus*; d, *Ditylenchus myceliophagus*; e, *Tylenchorhynchus* sp.; f, *Rotyenchus*). A,b: bacterivorous nematodes, c: nematophagous predator, d: fungivore, e and f: plant parasites.



Plant parasitic nematodes

Phylogenetic analysis of the phylum Nematoda revealed that plant parasitism has independently evolved at least three times. Plant parasites have common features that probably arose by convergent evolution within the constraints by the plant cell morphology. For instance, they all have a hollow protrusible stylet in their mouth, which is used to puncture cell walls, to withdraw nutrients from the plant cells, or to inject secretions. These stylet secretions are synthesized in single-celled pharyngeal glands, and include effectors of which some target the plant's innate immunity.

Bacterivorous nematodes

Bacterivorous nematodes are deposit feeders which mainly appear in spots with high microbial activity. They are thought to enhance decomposition processes through stimulation of the microbial community. Three main ways of stimulation have been proposed: (i) bioturbation resulting in a higher diffusion of oxygen and nutrients, (ii) secretion of nutrient rich compounds such as root diffusates and (iii) grazing, which implies keeping the bacterial community active and re-mineralising nutrients. Bacteriophorous nematodes (*e.g. Plectus* sp. and *Cephalobus* sp.) have a cylindrical, barrel-shaped, or conical shaped buccal cavity that is permanently opened and through which fluid containing bacteria enters.

Fungivorous nematodes

Molecular phylogenetic studies suggest that the three major families harboring fungivorous nematodes - Aphelenchidae, Aphelenchoididae and Diphtherophoridae - are phylogenetically distinct groups. Remarkably, they all evolved phylogenetically close to plant parasitic relatives. This is supporting an longstanding hypothesis suggesting that plant parasitic nematodes evolved from fungal feeding ancestor, or common ancestors feeding both on fungi and – if present – on plant material. As these are distinct lineages, three separate DNA barcodes are to be employed to determine the presence of fungivorous nematodes in a given soil sample. Fungivorous nematodes tend to be polyphagous, feeding on a wide range of species of soil fungi including saprophytic, pathogenic, beneficial and mycorrhizal fungi.

Predaceous Nematodes

Just like any other trophic group, the ability to use other nematode and other small microfauna members as a food source arose several times during nematode evolution. It is easy to recognize predaceous nematodes in a suspension as they are relatively large, possessing a wide U-shaped stoma with sclerotized walls armed with teeth and denticles. Small preys are swallowed whole, and the body contents of large ones are sucked out. Mononchids are free- living predatory nematodes that inhibit soil and freshwaters, where they feed on small invertebrates including protozoans, rotifers, enchytraeids and other nematodes.

Nematode Extraction

Materials

20 g (at least) pre-weighed soil placed in Petri dish and labeled
De-chlorinated tap water
Glass funnels
Screen
Rack
Clamps
Kim-wipes
Scissors
Rubber tubing
Formalin
Hot plate with stir bar
Beaker (500ml)
Scintillation vials
Sharpie
Pipette

Methods

1. At least 24 hours before the nematodes are to be placed in rack, fill a carboy or flasks with tap water and leave open to de-chlorinate.
2. Place funnels on rack and set screen inside. Funnels should have rubber tubing on the ends already, if not, place it over the ends now.
3. Clamp the end of the tube and fill funnel with water
4. Let out a little water to make sure water has filled the entire tube. Make sure water level is just touching the bottom of the screen.
5. Place soil on half a Kim-wipe on top of a screen. Make sure water begins to soak into the Kim-wipe and the cover with Petri dish. Repeat for all samples.
6. Monitor water levels for 3 days keeping water level right under the screen after 3 days they are ready to be removed.
7. Mix a 10% formalin solution and heat on a hot plate stirring while heating (DO UNDER A HOOD).
8. Label scintillation vials with sample number.
9. With the correct vial, carefully remove the clamp from the tubing and fill the vial half full with the liquid in the funnel.
10. Repeat for remaining samples
11. After the formalin has been heated, fill the vial until ¾ full.
12. Fill other ¼ of vial with room temp or chilled formalin.
13. Clean out funnels and screens.

Soil Arthropods

Mites

Subphylum: **Chelicerata** (phylum Arthropoda)
Subclass: Acari (superorder Actinotrichida)

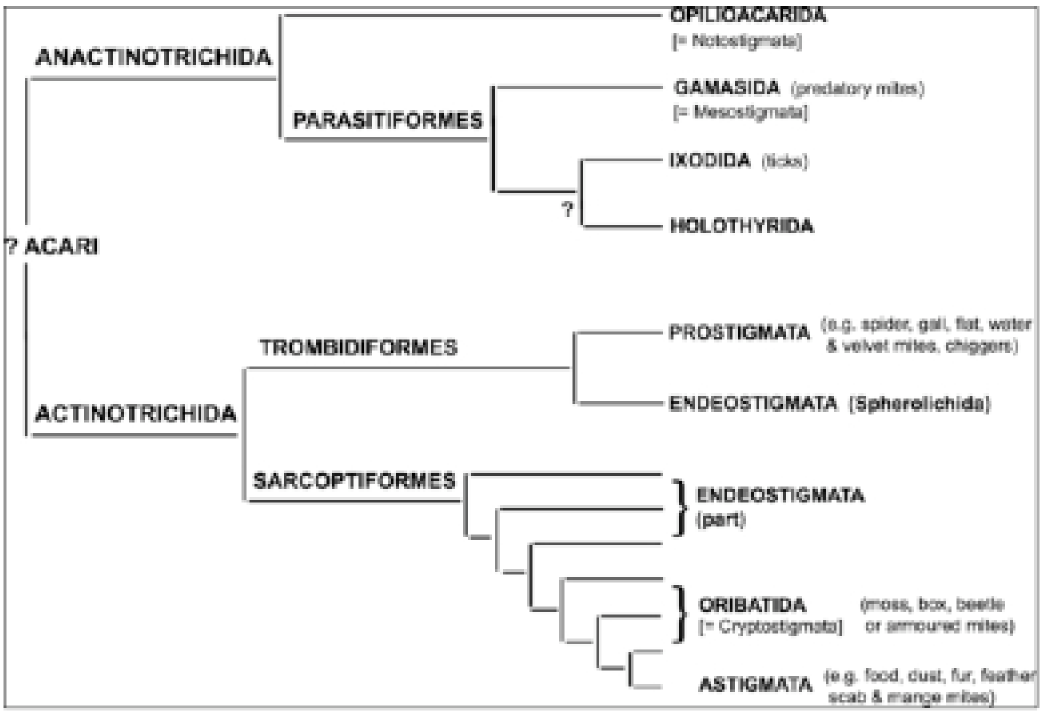


Fig. 3.3. A possible outline phylogeny for the mites illustrating major taxa (see text) and some of their alternative names and/or common members of the group. Oribatids themselves also appear to resolve as a paraphyletic assemblage with respect to the astigmatics. From: *J. A. DUNLOP And G. ALBERTI (2007) Journal of Zoological Systematics and Evolutionary Research Vol. 46, 1 Pages: 1-18*

Cryptostigmatid Mites: Oribatida (formerly **Cr**yptostigmata, phylum Arthropoda), also called oribatid mites or *armored* mites (they have sclerotized, often calcareous exoskeleton). Consequently adult oribatid mites experience relatively little predation. Oribatida are the most numerous of the micro-arthropods (up to several hundred thousand individuals per m2 occurring mainly in theorganic horizons of most soils. Oribatid mites have five active postembryonic instars: larva, 3 nymphal instars and the adult form. They show (extreme) juvenile polymorphism: immature stadia do not resemble the adults (this is why "juvenile mites" and "adult mites" are in different boxes). Oribatids reproduce relatively slowly; usually there are only one or two generations per year. Please note that among oribatid mites ("cr. mites" in this figure) various feeding habitats are distinguished: fungivorous (TL2), predaceous (TL3) and nematophagous (TL3) mites. These groups do not cover all feeding types; other taxa consume living and dead plant and fungal material, lichens and carrion.

Non-Cryptostigmatid Mites: this term meanly refers to the Prostigmata. They belong to the clade "Trombidiformes". About 50 families live in soil environments. Numbers vary dramatically per major habitat: up to 100,000 per m2 in pine forest in Northern Europe to 7,500 in moss turf banks in Antarctica. In grasslands Prostimata mites may form 40-50% of the total mites population, whereas this percentage may drop to 10% in temperate forests.



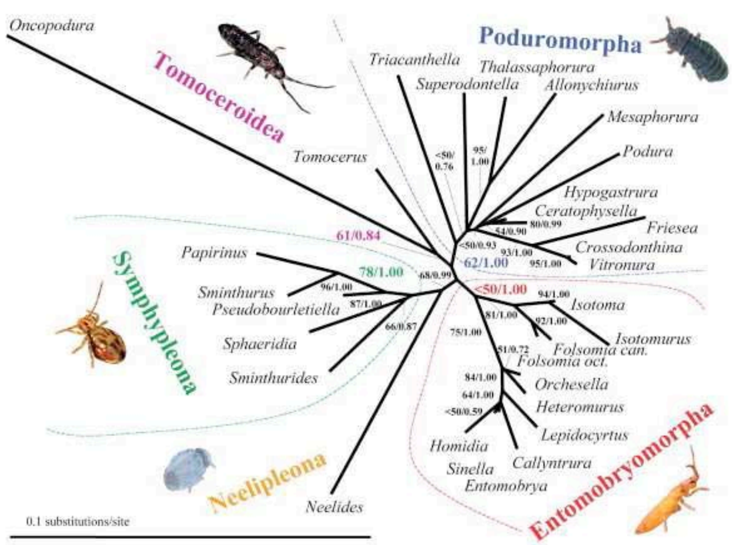
Predatory Mites

Predatory mites include most of the Gamasida and many Prostigmata.
Many gamasid mites are parasites of vertebrates or invertebrates. However, the ones found in soil invariably are predators feeding on small arthropods or nematodes. Most soil inhabiting gamasid mites are heavily sclerotized and highly mobile, the latter characteristic allows them to avoid unfavorable microclimatic conditions. Many of the prostigmata are not as heavily sclerotized and are less mobile



Collembola – springtails
Subphylum: Hexapoda (phylum **Arthropoda**)
The subphylum **Hexapoda** (from the Greek for *six legs*) constitutes the largest (in terms of number of species) grouping of arthropods and includes the insects as well as three much smaller groups of wingless arthropods: Collembola, Diplura and Protura (all of these were once considered insects)
Class: Entognatha (apterous = lacking wings) (Entognatha is a polyphyletic group)
Subclass: Collembola (springtails), Diplura (two-pronged bristletails) and Protura (coneheads)
Orders:
- Poduromorpha (left): generally stout, with short antennae, short legs, and robust elongated body
- Entomobryomorpha (middle): long antennae and legs, and elongated bodies
- Symphypleona (right): almost globular in shape, brightly colored, active and easily noticed.





Arthropod Extraction

Materials

70% ethanol

Jars with lids (Urine sample jars work best)

Aluminum foil

Plastic funnels with wire screen inside

Light (heat) source

Rack

Cheese cloth (non synthetic)

Scissors

Data sheets

Methods

1. Make sure the rack and funnels are clean and free from dirt. Place each funnel on the rack and put a wire screen inside the funnel.
2. Cut cheese cloth into squares three layers thick.
3. Write sample numbers once on each side of jar and lid and place on rack. (NOT UNDER SOIL SAMPLES OR THEY CAN FILL WITH SOIL DURING SET UP).
4. Place 1 soil core or 30 g of soil for cave samples into cheese cloth weigh cheese cloth and soil and record combined mass.
5. Wrap the cheese cloth around the soil then place into funnel with wire screen. Place funnel on rack by corresponding jar number (NOT OVER)
6. Repeat steps 1-5 for all soil samples
7. Once all samples are up, wrap the aluminum foil around the top of the funnel and the light source.
8. Fill each jar half way with alcohol and place under corresponding funnel (MAKE SURE FUNNEL IS POINTED INTO JAR).
9. Turn on rack.
10. The jars will need to be checked every day to every other day for alcohol evaporation (DO NOT LET JARS DRY OUT).
11. After 5 days, place the lids on the jars and remove.
12. Reweigh soil and cheese cloth and record.
13. Throw away the soils and cheese cloth and clean rack and funnels so they are ready for next time.