Collection & Preservation of Bryophytes

Bryophyte species exhibit a high specificity to meso-and microhabitat conditions and, although some can be observed all year-round, many are annual and/or can be identified only during a short period of the year. Bryophytes are collected from their natural habitats using scalpel and forceps. They are thoroughly washed in water and cleaned properly. Finally they are preserved in 10% formalin solution. Sometimes bryophytic thallus, after cleaning, are dried at 50°C and then stored for future study.

Mosses can be preserved as museum specimen in the following way:

(a) Fresh specimens are wiped of all water and then kept in a glass trough filled with a pre-treating solution (Sodium sulphite–16 gms., Conc. H_2SO_4–20 ml. and Distilled water–1,000 ml.) overnight in air tight condition.

(b) Then the specimens are washed in distilled water and treated in saturated solution of $CuSO_4$ for 24 hours.

(c) Finally, the specimens are washed in distilled water and tied on glass plates and preserved in glass bottles using formalin solution (4%).

Where and when to collect bryophytes?

Bryophytes are generally seen as small plants confined to humid habitats, avoiding exposure to direct sunlight. In temperate and tropical rain forests, bryophytes, and especially liverworts, compose luxuriant epiphytic communities. The diversity of bryophytes is correlated with habitat heterogeneity. In a forested landscape, mesohabitats are arranged into a mosaic of dominant mesohabitats (*e.g.* forests), wherein restricted mesohabitats (*e.g.* streams, seeps, cliffs) exist.

In many other habitats, bryophyte species can be observed during a short period of the year only. In fact, plants have to cope with unstable habitats in time (*e.g.*, seasonal climate variations) and space (*e.g.*, habitat degradation or destruction).

Collecting techniques

Scientific collecting is essential for a number of reasons, including specimen identification, herbarium collections for taxonomic studies, and, more recently, constitution of banks of DNA. This is especially true for bryophytes because, although the larger species can often be named in the field with a 10-20x hand lens, many are distinguished based on microscopic characters.

Reference collections of specimens are thus invaluable in the study of bryology, but in order to obtain useful specimens for research, the correct techniques for collecting and processing should be employed. It must also be emphasized that, although bryophyte species rarely legally protected, it is necessary to obtain permits to collect bryophytes and an export license if the material is to be taken out of the country.

Packeting

Bryophytes are among the easiest plants to collect. Since they lack roots, they can often be readily collected by hand, although some species closely attached to their substrate will have to be scratched using a knife. Specimens should be selected to include all the parts of the plant needed for identification. Sporophytes are often useful, if not necessary, for identification, and should be searched for. Several mosses from unstable habitats, *e.g.* riverbanks, arable fields, have rhizoidal tubers buried in the soil. As these are often diagnostic, these bryophytes should be collected with 1-3 cm of the substrate.

Individual species within a collection should be packed-up separately, so far as this is possible. It is in fact generally easier when the material is still fresh than later, when several collections jumbled together in a single packet have to be separated. The specimens are normally put into envelopes. A standard envelope can be folded from an A4 paper to be (10-)12 x 14 cm in size. Particularly small specimens should be wrapped separately in mini-packets before being put into normal size packets. If sporophytes or fertile structures are rare, these should also be placed in mini-packets, but attached to a piece of the gametophyte to avoid any subsequent confusion. If specimens are very wet, as is often the case with *Sphagnum*, they should be gently pressed to remove most of the water, and packed into a double thickness packets. As for ground dwelling species, it is often more appropriate to keep them in stiff boxes for transportation and storage to avoid ending up with a mixture of soil particles and plant fragments.

For collecting of epiphyllous bryophytes in tropical rain forest, whole leaves on which the epiphylls are growing are collected in news papers in a plant press, lightly pressed and dried.

Plant Press

The epiphyllous species are subsequently sorted, and leaves cut up, in the laboratory using a dissecting microscope. For collecting of thalloid liverworts and hornworts it may also be recommendable to dry the specimens in a plant press instead of in collecting bags, in order to keep them flat and avoid them from becoming rolled inwards. Pressing of the specimens should be lightly only, to avoid damage to the plants.

How much to collect?

Collecting of specimens for scientific purposes is usually highly selective and seldom constitutes a real threat to the survival of species. The extinction of species by a targeted over-collecting has been, however, already documented. It is difficult to provide exact guidelines since everything depends on species size, local and overall abundance, etc. As a general rule, collecting enough to fill a 12 x 8 cm packet should be plenty for a robust species. On the other hand, too small specimens are of no value if there is insufficient material to allow identification and, perhaps, DNA extraction. In addition, the really important plant in a collection may not be what the collector actually saw in the field, but some minute plant sparsely mixed with it, and only discovered later in the laboratory.

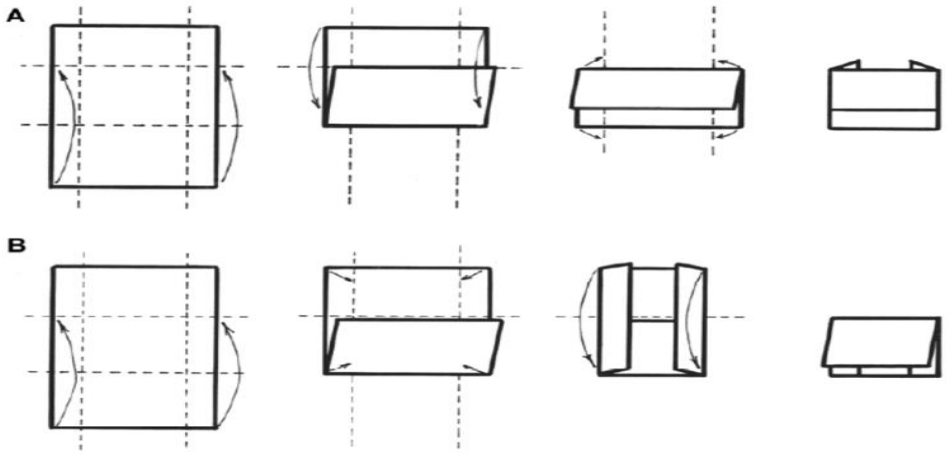

Fig. 4. Folding procedure for packing-up bryophytes.

Data and labelling

The information record is similar to that of other plants, and includes habitat information (for instance, if a species occurs on tree or rock, the tree species or rock type should be recorded), nature of the surrounding vegetation, elevation, and locality details, including GPS coordinates. For rare species, information on population size is often useful but might be difficult to assess in the case of bryophytes. Indeed, many bryophyte species are highly clonal, and several gametophytes can develop from a single protonema following the germination of a single spore.

Drying and processing

The collected specimens should be dried as soon as possible to avoid fungal damage. In most cases, the packets can be left to air-dry. In wet areas during extended expeditions, however, drying might become a major issue and preoccupation, and the use of a plant dryer can sometimes become necessary. As liverwort capsules tend open when drying, releasing their spores, it is recommended that some specimens with capsules be placed in a small paper envelope before drying together with the rest of the sample, to ensure that at least some unopened capsules are preserved.

These is no need to give a descriptive account of the plant, as one does systematically for fungi and sometimes for higher plants, since most bryophyte species recover their primary appearance upon remoistening. A special care must, however, be taken with liverworts. Indeed, the identification of many species relies on the size, shape, number, colour, and distribution of oil bodies, which are unique organelles among land plants. Because of the volatility of the oils they contain, oil bodies progressively disappear upon drying in the laboratory. In some taxa, the process takes only a few hours, so that fresh material must be studied, whereas in other, oil-bodies last for some years and can still be studied on herbarium specimens. In any case, it is advisable to take a micro-photograph of the cells to keep a record of the oil body morphology. For preservation

of DNA, fresh material should be cleaned and quickly air-dried, and subsequently kept dry. Any moistening of the material must be avoided as this might lead to degradation of the DNA, making the material unsuitable for molecular analysis.

Slide preparation and stains

Fresh specimens are the best to work with. They are bright green and require little or no hydration before placing them in a drop of water on a slide. But most often we don't have the pleasure to observe fresh material under the microscope. Instead, we have dry, often brittle, specimens collected in great numbers in a day-long or even months-long collecting trip.

Cleaning Bryophytes

Suggested a cleaning procedure using a net, but suggested placing the netting (mosquito or bridal veil netting) tightly in an embroidery hoop. This is particularly useful for thallose liverworts. They should be collected with ~3 mm substrate to protect rhizoids and scales. The liverworts are placed on the hoop netting with a second net placed over them. They are then washed with a stream of water. This may take some practice because too much water will damage the plants whereas a weak stream will not succeed in removing the soil and debris.

HCl

Zander (1993) suggests using dilute HCl to clean away limy incrustations. It can also indicate whether the collection was made from a calcareous habitat because, if it is calcareous and bits are present with the sample, it will produce bubbles.

Ultrasound

It is uggested using a sonicator to clean bryophytes before making slides, especially when they are used for photography. The bryophyte can be suspended by forceps into the vibrating bath. These are available for cleaning jewelry and watches in small sizes at relatively inexpensive prices. It might be helpful to add a cleaning agent (Tween 80 as a detergent) to the water to facilitate removal.

Aquatic Bryophytes

Aquatic bryophytes can be particularly challenging. They typically are covered with epiphytes, some of which (*e.g.*, the diatom). It is found that household bleach, diluted to 0.5%, causes no chlorophyll bleaching. These methods did not appear to remove the epiphytes. Ultimately, 5 minutes cleaning with 3% peroxide (H_2O_2) and agitation seemed to remove approximately 85-90% of the epiphytes.

Dealing with old specimens

Old samples can be brittle and fragile. Placing them in water to soak can further degrade them so that they fall apart when cut. Takes from an old sample, a single branch and puts it on a slide in a drop of water without soaking. Do not soak the parts as they get very soft. There is no need of using forceps or anything else to hold the moss. After cutting you can heat the sections carefully for swelling for a very short time with the help of a very small amount of KOH under the cover slip.

Sorting the Plants

A classic mistake in identifying bryophytes is looking at the sporophyte of one species and the leaves of another. Sporophytes often originate deep in the clump and may actually belong to a species that achieved sufficient dominance in a previous year to produce a capsule. But another species can easily encroach or simply intermix enough to confuse the unwary. Be sure to track the sporophyte down and locate its attached gametophyte. This sorting should be done with bryophytes that are moist enough to be soft, but not soaked. Dry mosses are likely to break before you can pull the gametophyte out from among its trappings.

Wetting Agents

Assuming your specimens have not been collected in the same day and have gotten dry and brittle, the first step is to re-wet them before attempting to make a slide or even examine them with the dissecting microscope.

Most bryophytes will wet up adequately by dipping them in water or dropping water or misting on the desired portion of the sample. But some mosses simply don't wet well. In fact, some bryophytes repel water and may even trap large air bubbles that further keep them from getting wet. Wetting agents help to avoid the air bubbles trapped in leaf folds by reducing or eliminating the surface tension of the water. Warm water can sometimes actually increase the bubbles. Soap is a wetting agent, and it doesn't take much. One drop in your dropper bottle is likely to be more than needed. But beware, soap and the other wetting agents, as well as heating, will usually kill the bryophytes and destroy the cell contents. A traditional wetting agent is one known by the German word *Pohlstoffe*. This is a non-technical name for a wetting agent (di-octyl sodium sulfosuccinate) available from Fisher Scientific, known as ***Aerosol* OT.**

Also uses **Agral 600** (horticultural wetting agent). The latter kills the animal life that often accompanies the bryophytes but does not seem to affect the plants. This is useful to avoid introducing dermestids and other hungry creatures into the herbarium.

Rehydrating Capsules

1. Put the capsules in a drop of 2% KOH on a glass slide

2. Puncture one side of each capsule (I do this with the tip of very fine forceps)

3. Gently heat the KOH solution with a naked flame beneath the slide

This appears to rehydrate most capsules to their original Dimensions.

Clearing Leaves

Lactic Acid

The lactic acid clears all the gunk from the cells, making the walls much easier to see. The lactic acid may also be added under the cover glass of stems and leaves that have been mounted moist, but not flooded. Gently warm the slide using heat from an incandescent desk lamp.

KOH or NaOH

Usually these methods will only require a few minutes to clear the specimens. However, for especially dirty ones, you may need to leave the specimen overnight to clear. Potassium hydroxide (KOH) or sodium hydroxide (NaOH) will also clear tissues – particularly if the material is in contact with the air.

Dehydration

Usually specimens are air dried and this is adequate for most species. Some thallose liverworts require preservation, but mosses rarely do. For higher quality specimens, cleaned specimens can be dehydrated with a series of ETOH (70, 90, 100%). Following the dehydration series, specimens are placed in a 1:1 ethyl alcohol:xylene solution, then transferred to 100% xylene.

Stains

For most observations, stains are not necessary. But some things are simply too transparent or lack contrast. Stains can be used for a variety of purposes. They can distinguish cell types, make pores visible, clarify cell walls, make starch visible, and solve other problems in distinguishing special structures. Most stains are readily available, such as one drop of red or blue food coloring in 30 mL of water, or for greater detail and contrast, a mix of one or two drops each of red and blue food coloring, five drops water, two to three drops white vinegar, and three to five drops rubbing alcohol.

Staining Stems

Stems usually have specialized cells, including the epidermis, the cortex, and often a central strand. Others may have hydroids and leptoids and a second distinguishable layer inside the epidermis. In some species, natural colors distinguish the layers, but other specializations may not be easily recognizable.

Etzold Stain

Dissolve in 1L water:

Acetic acid (100 %): 20 ml

Fuchsin (bas.) 0.1 g

Chrysoidin 0.143 g

Astralblue 1.25 g

Color Results

Non-ligneous cell walls:	blue
Ligneous cell walls, sclerenchym and xylem:	red
Phloem:	blue

Acid Fuchsin

Acid fuchsin has been used to stain a variety of plant, animal, and fungal tissues. Acid fuchsin, along with I2KI and gentian violet to distinguishes the internal anatomy of stems.

Eosin

Eosin is a red dye that stains cytoplasm. It is water soluble and thus can be used to follow water movement through plants.

Fast Green

Fast green is the green dye used in food coloring, but it is known to have tumorogenic effects. It is a protein stain.

Fuchsin

The dye fuchsin is a biological stain that is produced by oxidation of a mixture of aniline and toluidine, producing a brilliant bluish red.

Leaves

KOH in concentrations from 2% to saturated will stain cell walls of many mosses. It can be used on whole leaf mounts and on sections. The KOH should not be kept in glass dropper bottles because it reacts with the glass to form a precipitate. If the specimen will later be mounted with an acidic mountant, add a drop or two of dilute HCl to the specimen.

Safranin O / Fast Green

Applies this directly to the leaves then washes them in water, Safranin normally dyes lignin red.

Reproductive Structures

Iron Haematoxylon / Fast Green

This stain works very well to show archegonia and spermatogenous cells in antheridia

Bulbils and Spores

Fluorescence and Fluorescent Dyes

Gisela Nordhorn-Richter discovered the fluorescence of bulbils in *Pohlia.* In the dried condition, fluorescing substances of bryophytes are very stable, with rhizoid bulbils of *Pohlia* that are more than 100 years old still exhibiting brilliant fluorescence. Chlorophyll, on the other hand, loses its fluorescent ability upon drying. To hide the fluorescence of chlorophyll, which can interfere with fluorescence of other substances, a suppression filter of 650 nm can absorb its red fluorescence. Alternatively, the chlorophyll can be extracted by 80% acetone or DMSO without interfering with other fluorescent substances. The fluorescence technique for bryophytes permits one to find rhizoid gemmae. Live spores exhibit red fluorescence, permitting estimation of vitality that can be quantified with a fluorescence spectrophotometer.

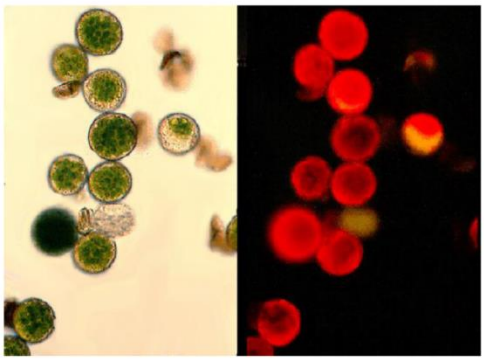

Figure 85. Spores of ***Fontinalis squamosa*** showing spores in white light on left and fluorescing under UV light on right. Note that the living spores show up as red under fluorescence, whereas dying and dead spores are yellow or invisible. Photos by Janice Glime.

Stains can provide one with the ability to see structures using fluorescence microscopy. Both fresh and fossil pollen could be detected with fluorochromes, with better results if acridine orange was added to enhance detail. Stains can provide one with the ability to see structures using fluorescence microscopy. Brandes (1967) explained the use of acridine orange as a vital stain for use with fluorescence microscopy of protonemal pro-buds and buds. Fluorochrome 3,3'Dihexyloxacarbocyanine iodine [DiOC6(3)] can be used to locate selectively the fungal hyphae among the rhizoids of bryophytes.

Clearing Spores

The ornamentation of the spores can help in identification, but it is not possible to observe it clearly with transmitted light. It is suggested using a combination of transmitted and reflected light.

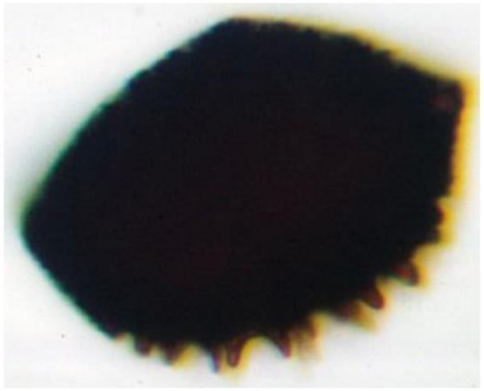

Figure 113. Spore of ***Riccia sorocarpa*** showing its dark color and density, preventing one from seeing spore wall details without special techniques. Photo from EOL through Creative Commons.

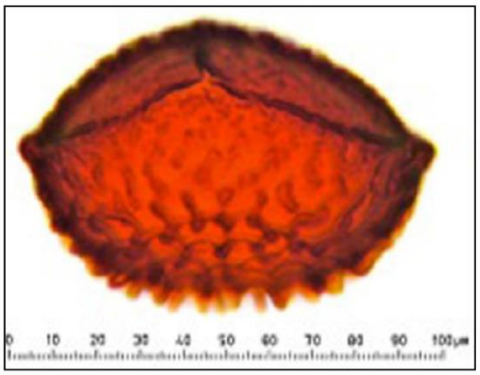

Figure 114. Spore of ***Riccia sorocarpa*** using both transmitted and reflected light plus stacking. Compare the clarity to that of the same species in Figure 113. Photo by David Wagner.

By using 5-10% bleach (NaOCl – 8% of active chlorine) for 1-3 minutes to clear the spores. It is suggested staining spores with malachite green, acid fuchsin, and orange G, a method used for testing pollen. The viable pollen stains deep red-purple, whereas the aborted pollen stains green. This recipe uses chloral hydrate. The solution uses 10 ml ethanol; 1 ml 1% malachite green in 95% ethanol; 50 ml distilled water; 25 ml glycerol; 5 gm phenol; 5 gm chloral hydrate; 5 ml 1% acid fuchsin in water; 0.5 ml 1% orange G in water; and 1-4 ml glacial acetic acid (for very thin to very thick walls). This should work as well for bryophyte spores.

SEM

Scanning Electron Microscopy (SEM) can reveal details not visible with an ordinary light microscope.

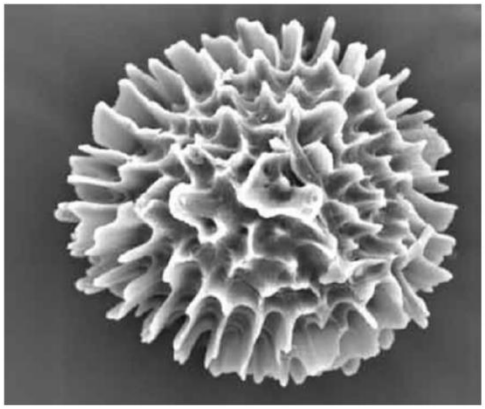

Figure 115. ***Fossombronia longiseta*** spore proximal SEM. Photo courtesy of William T. Doyle.

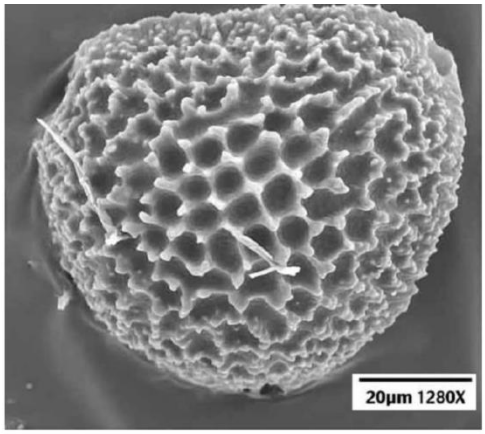

Figure 116. ***Riccia sorocarpa*** distal spore wall SEM. Photo courtesy of William T. Doyle.