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Preparing bryophyte specimens for DNA extractions for Sanger sequencing

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We use this protocol and it's working

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ABSTRACT

This protocol describes part of the pipeline employed at the Royal Botanic Garden, Edinburgh to generate DNA barcode reference libraries for bryophytes using Sanger sequencing protocols.

Here we describe the steps from field or herbarium sampling to tissue homogenization, prior to starting a specific DNA extraction protocol.

GUIDELINES

Ensure sampling is compliant with the Nagoya Protocol (Buck & Hamilton, 2011), which came into force on the 12th October 2014

MATERIALS

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- Autoclaved, acid-washed sand **Contributed by users**
- Grinding balls 3mm tungsten carbide approx 200 pieces **Wolflabs Catalog #22.455.0006**
- Grinding balls (stainless steel cone balls) for reaction vials and wet/ultra fine grinding 3mm **Wolflabs Catalog #22.455.0006C**

1 **Plant Material.** In contrast to most other land plants, where DNA extractions are usually made from a single individual, bryophyte extractions are frequently from multiple stems or thalli, which can potentially represent several individuals. Thus, single bryophyte DNA extractions can contain DNA from multiple genotypes. We use either herbarium specimens or, preferably, silica-gel dried plant material, depending on availability.

1.1 **Herbarium.** Destructive sampling from within a herbarium (with permission from the herbarium curator) is often successful, with reasonable DNA preservation. Care should be taken picking material, as mixed collections are far more common than for vascular plants. Processing of the specimen is done using a dissecting microscope, and care taken to exclude the almost inevitable fragments of other bryophytes that can be included in the collection.

When extracting from herbarium material, avoid material that has obviously been rewetted at some point for morphological examination. If the plant has to be dampened prior to extraction in order to verify its identification or to remove it from a substrate, proceed with the extraction immediately afterwards rather than allowing it to undergo another drying process, which will lead to further DNA loss and degradation. If there is unused material that has been rehydrated and dried, that is put into a separate subpacket and labelled as such (with information about how it was treated), before it is returned to the herbarium packet.

1.2 **Silica-gel dried.** For new field-collections of bryophytes, we advise collecting living plants into plastic bags, and bringing samples back to the lab for further preparation. Although it is apparent that vascular plant DNA quality declines rapidly after harvesting leaves or stems into bags, the entire bryophyte plant is usually collected, and in most cases survives inside a zip-lock bag for a week or more (although more rapid processing is recommended whenever possible).

Processing of the specimen is done using a dissecting microscope, as soon as possible after collection. Minute fragments of other plants, grit, soil and insects can be removed at this point with forceps and water. Diatoms and other microorganisms can often be seen on bryophyte leaf or thallus surfaces; some labs use protocols that involve cleaning the sample further with sonication (to dislodge particles) or with bleach – however both techniques can rapidly strip the colour out of these plants and so can be presumed harmful. Algae and other contaminants should not be amplified with direct PCR for commonly used plant barcoding loci, which are land-plant or bryophyte specific. With complex thalloid liverworts, the scales and rhizoids on the thallus underside often trap organic matter that may also contain PCR inhibitors, as well as small plant fragments; scales and rhizoids can be stripped off with a razor blade or forceps. Once the bryophyte has been physically cleaned for DNA extraction, it should be put straight into silica gel to dry.

The silica-gel dried plant material can either be stored long-term in ziplock bags containing silica gel, preferably in the dark to avoid light-bleaching and associated DNA degradation, or the dry tissue can be removed from the silica and placed in low-humidity cabinets for long-term storage. Due to the small size and fragility of bryophyte tissue, valuable collections can be housed in small tight-sealing petri dishes (e.g. Gelman sterile Petri Dishes 50x11) within the

low humidity cabinets, rather than in paper envelopes or ziplock bags in which the material may be crushed and lost.

1.3 **Vouchering.** The DNA voucher is either the herbarium specimen that was sampled for DNA, or the herbarium specimen that forms part of the same collection as the silica-gel dried tissue that was sampled for DNA.The packet is annotated to say that it is the DNA voucher. Vouchers are deposited in publically accessible herbaria. (Index Herbariorum (Thiers, 2017; http://sweetgum.nybg.org/science/ih/) provides guidance and detailed information of local and international herbaria.)

Bryophyte specimens are typically preserved in small paper packets that are either stored upright similar to filing cards or (at RBGE) mounted on regular herbarium sheets and stored horizontally. The specimens themselves are usually loose inside the packets, although material of particular note (e.g. fertile parts, or cleaned material left over from DNA work) can be separated into subpackets.

2 We add roughly a microcentrifuge tube lid sized piece of plant tissue to the microcentrifuge tube for DNA extraction. We select the appropriate microcentrifuge tube (1.5 ml or 2 ml) based on the homogenization method that we are going to use. For most material we use a 2 ml tube and a QIAGEN TissueLyser. For higher-throughput processing, we put the dried plant material and beads into 1.2 ml QIAGEN collection microtubes and grind these in plates of 96 samples.

3 **Homogenization**. Homogenization of bryophyte material is quite straightforward, as the leaves or thalli are usually quite thin. Our protocol varies depending on whether we are extracting from fresh or dry tissue:

3.1 **Fresh bryphyte material**: Grind with a pinch of acid-washed sand and a little extraction buffer (choice of buffer depends on subsequent extraction protocol), in a 1.5 ml microcentrifuge tube using a disposable plastic pestle. Liquid nitrogen can be used to freeze the tube and sample (in which case do not add buffer until immediately after grinding), but we do not find that it is necessary.

3.2 **Dry bryophyte material**: Herbarium or silica-gel dried material is usually easily disrupted in a 2 ml microcentrifuge tube or plates of 96 QIAGEN collection microtubes in a Retsch™ Mixer Mill, QIAGEN TissueLyser or equivalent, with the appropriate sized tungsten or stainless steel bead. For individual 2 ml microcentrifuge tubes we usually use 3 mm conical tungsten beads, with 1-2 beads per tube, and grind at 20 Hz for 4 minutes, stopping and rotating the samples after the first 2 minutes. For the 1.2 ml collection microtubes we use 1.5-2 mm ball bearings, again at 20 Hz for 4 mins. Grinding at a higher frequency or for much longer can crack the sample tubes.

4 Proceed to chosen DNA extraction method.