Methodologies for soil extraction and conservation analysis of ferns and lycophytes with belowground gametophytes

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This article is part of the special issue “Methodologies in Gametophyte Biology.”

Abstract
Premise: Studies of fern and lycophyte gametophyte biology in natural settings can be challenging, but such analyses are critical to understanding the dispersal, ecology, and conservation of these species. It is especially challenging to study species whose gametophytes and early sporophyte stages develop belowground, as is the case for species of the Ophioglossaceae, Psilotaceae, some species of the Schizeaceae (Actinostachys), and some species of the Lycopodiaceae. To study these taxa, gametophytes and young sporophytes must be extracted from the soil.

Methods: In 1989, Mason and Farrar described a methodology for accomplishing the collection of belowground gametophytes and sporophytes using soil centrifugation. Here, we refine this procedure based on subsequent years of experience.

Results: We found that many more sporophytes exist belowground than are represented by aboveground leaves, and that belowground sporophytes can survive indefinitely without production of aboveground leaves. Belowground gametophytes are common in areas where spore-releasing sporophyte leaves are present. Gametophytes are bisexual with male and female gametangia intermixed. Some species of Botrychium also reproduce asexually belowground through production of gemmae.

Discussion: We conclude that in Botrychium, assessments of population health and structure must include analyses of the belowground plants and their habitat. Conservation management strategies must also include potential changes in the belowground habitat.

KEYWORDS
belowground sexual reproduction, Botrychium, fern and lycophyte gametophytes, methodology, Ophioglossaceae

The study of the independent haploid stages (spores and gametophytes) of ferns and lycophytes is critical to understanding their patterns of dispersal and habitat selection. Conducting these studies in situ is particularly beneficial because spores and gametophytes determine where sporophyte plants are established. For species whose gametophytes and early sporophyte stages develop belowground, as is the case for species of the Ophioglossaceae, Psilotaceae, some species of the Schizeaceae (Actinostachys Wall.) (Bierhorst, 1971), and Lycopodium L., the study of gametophyte biology requires the extraction of these stages from the soil. Mason and Farrar (1989) described methodology for extracting belowground structures, and Johnson-Groh et al. (2002) presented a methodology for belowground ecological sampling. Here, we present revisions to these procedures and use them to explore the biology of the Botrychium Sw. genus.

The morphology and anatomy of belowground fern and lycophyte gametophytes are well documented (e.g., Bierhorst, 1971). These observations are based primarily on the excavation of young sporophyte plants and an examination of the surrounding soil (Mesler, 1976; Bruce and Beitel, 1979). Additionally, gametophytes of a limited...
number of taxa of Psilotales and Ophioglossales have been grown in culture (Whittier, 1972, 1973, 1976, 1981, 1984; Whittier and Thomas, 1993), providing data on their germination, growth, development, and nutritive requirements. Despite these insights, studies of cultured gametophytes provide little information about the population structure and ecological dynamics of reproduction in species with belowground reproductive stages. For these purposes, systematic substrate sampling is necessary.

Our method for the extraction of belowground reproductive structures is based on techniques developed to separate nematodes from soil (Jenkins, 1964). This method takes advantage of the relative weights of dead versus live organic material and organic versus mineral soil particles. When stirred and centrifuged, dead organic material floats in pure water and live organic material floats in a 50% sucrose solution, whereas mineral soil particles sink. We have used the methodology presented below to successfully extract gametophytes at various stages of development, as well as juvenile sporophytes and vegetative reproductive structures (gemmae), from the soil in the vicinity of aboveground leaves from several species of Botrychium and related taxa (Farrar and Johnson-Groh, 1990; Johnson-Groh, 1998; Johnson-Groh et al., 2002). We include photographic and quantitative examples of the results of this process and briefly discuss its application in the study of the biology of Botrychium, as well as potential applications for all species with belowground gametophytes.

METHODS

Our methods for gametophyte collection through soil centrifugation were published previously (Mason and Farrar, 1989), but have been refined here through experience of its use with additional species and different soil types. We recommend first assembling the following list of equipment:

1. A soil-sampling tool capable of delivering a fixed amount of soil. We use a bulb planter 5 cm in diameter and 10 cm deep, extracting approximately 200 cm² of soil.
2. Two three-gallon buckets for the initial soil fragmentation and separation from coarse vegetative material.
3. Hardware cloth cut and folded to cover a bucket and support soil sieves.
4. Three 8-inch diameter U.S. standard soil sieves #10, #35, and #60, with sieve openings of 2 mm, 0.5 mm, and 0.25 mm, respectively.
5. One 3-inch diameter U.S. standard soil sieve #400, with sieve openings of 0.038 mm.
6. Clear plastic 50-mL centrifuge tubes and centrifuge capable of spinning tubes filled with soil and water at 2000 rpm.
7. Table sugar for making 50% sucrose water.

To evaluate the success of our extraction process, we “seed” the soil sample with root segments of the species under study cut to the approximate size of young gametophytes (~1 mm). These control root segments are stained red using propyl carmine (or another common stain) for easy detection in estimating the success of the extraction.

The method is performed as follows:

1. Place the soil sample in the first bucket with about two gallons of water and gently wash the soil from the roots and coarse debris of aboveground vegetation, being careful to avoid sloshing water out of the bucket.
2. Place the hardware cloth on the second bucket topped with the #10 sieve and pour the contents of the first bucket through the sieve into the second bucket. Use additional water in a squeeze bottle to wash all of the material out of the first bucket. Rocks and large pieces of plant material will be retained in the sieve and may be examined under a dissecting microscope for larger above- and belowground plants to detect stems and roots of the study species; they can then be discarded.
3. Add 10 stained root segments to the second bucket (containing the sieved soil).
4. Transfer the hardware cloth onto the first bucket. Stir the retained soil and water in the second bucket and pour through stacked #35 and #60 sieves (supported by the hardware cloth) back into the now-empty first bucket. This divides the organic material into coarse and fine fractions.
5. Partition the soil fractions from each sieve into centrifuge tubes, being careful to wash all the retained material from the sieves. Place 10 to 20 mL of soil in each tube using a spatula and add an equal amount of clean water.
6. Stir the tube contents, place them in the centrifuge, and spin at 2000 rpm for 5 min. Decant the water and floating material into a labeled Petri dish for examination under a dissecting microscope. This stage will primarily include dead organic material.
7. Add a solution of 50% sucrose* to the tubes (still containing the soil samples) and repeat step 6. Decant the sucrose solution and floating material into the #400 sieve.** Rinse the sieved material with water, then wash into a Petri dish and examine under a dissection microscope. The contents should contain live organic material, including gametophytes, juvenile sporophytes, roots, and the stained root segments. Count the red-stained root segments to estimate a percentage of recovery.

*The % sucrose can be adjusted to suit particular soils.
**Depending on the soil texture, it may be necessary to very gently wash the sides of the tube, being careful not to dislodge soil at the base of the tube.

RESULTS

To demonstrate the efficacy of this technique, we extracted and analyzed the belowground gametophytes and young sporophytes of the Botrychium genus (Figure 1). The early
stage of Botrychium seasonal growth occurs in the aerial leaves, when the trophophore (bearing pinnae) and sporophore (bearing immature sporangia) have just begun to spread apart, as is depicted for the representative species Botrychium campestre W. H. Wagner & Farrar in Figure 1A. In most species of Botrychium, the stalk of the sporophore elongates just before spore release. Many structures can be detected belowground, including the roots, stem, apical bud, and the leaves that will emerge aboveground the following year (Figure 1B). The stem shows two stages of node elongation between successive leaves: rapid elongation low on the stem (generating long internodes) until the stem apex reaches a level belowground that allows leaves to emerge aboveground with the subsequent minimal stem elongation (resulting in short internodes). Figure 1C shows a gametophyte of Botrychium minganense Vict. with an attached juvenile sporophyte differentiated into a stem and root.

In our soil extractions, the soil and live organic matter separate following the centrifugation in a 50% sucrose solution (Figure 2A), with the soil particles at the bottom of the tube and live organic material floating at the top of the sucrose solution. The live organic material is decanted into the #400 soil sieve, rinsed with water, and then washed into a Petri dish for observation (Figure 2B). In addition to non-subject material (often including invertebrates), the contents in the present study included many Botrychium root fragments, Botrychium gametophytes, and the red-stained root segments (Figure 2C). Gametophytes are distinguished from the root segments by their lighter color, globular appearance, and by the presence of short rhizoids (Figure 2D). The gametophyte is the only stage of the Botrychium life cycle to produce rhizoids or root hairs.

During the early stages of gametophyte sexual expression, either archegonia (Figure 3A) or antheridia (Figure 3B) can develop first. Soon after, both sexes are closely intermixed (Figure 3C).

Young sporophyte plants are initially attached to mature gametophytes (Figure 4). Typically, we found one sporophyte per gametophyte (see Figure 1C), but in some species a single gametophyte can produce more than one sporophyte; for example, three sporophytes were produced by a single (1 cm long) Botrychium mormo W. H. Wagner gametophyte (Figure 4A), while a larger (3 cm long) gametophyte of Botrypus virginianus (L.) Michx. (formerly Botrychium virginianum (L.) Sw.) was observed with two attached sporophyte plants (Figure 4B).

Vegetative belowground reproduction was detected for Botrychium campestre (Figure 5). In addition to sexual reproduction, this and many other species of Botrychium reproduce vegetatively by dispersible gemmae formed on belowground stems (Farrar and Johnson-Groh, 1990).

The numbers of aboveground leaves were found to fluctuate in the Botrychium populations (Figure 6), which may or may not accurately reflect the health of the belowground population, as shown in Table 1.

Finally, we estimated the density of the gametophytes, gemmae (if present), and belowground juvenile sporophytes extracted from soil samples taken in an area (approximately 800 m²) within populations of emergent sporophyte leaves (Table 1), as determined using the systematic sampling design of Johnson-Groh et al. (2002).
DISCUSSION

Here, we present brief discussions of the development of our methodology and the studies in which we have applied it to the belowground analysis of the *Botrychium* life cycle. We also suggest potential additional applications for our procedure in the study of the life histories and conservation concerns for *Botrychium*, which may be relevant to other ferns and lycophytes that undergo belowground sexual reproduction.

History of the methodology

The discovery of a new species, *Botrychium campestre*, in the dry prairies of western Iowa, USA (Wagner and Wagner, 1986), prompted us to investigate this unusual habitat for ferns. In monitoring populations of marked individuals annually, we found that individual plants did not produce aboveground leaves every year (Johnson-Groh, 1998). Excavating non-emergent plants, using the methodology described herein, revealed the presence of healthy

FIGURE 2  Process and products of soil centrifugation using the method presented here. (A) Soil is centrifuged in 50% sucrose, separating the soil particles (at the base) and the live organic material (floating). (B) The live organic fraction containing many *Botrychium* root segments and gametophytes (G). (C) Stained *Botrychium* root sections (SR) and gametophytes (G). (D) Five *Botrychium* gametophytes, distinguishable from sporophyte parts by the presence of rhizoids (R). Gametophyte and root diameters range from 1 to 2 mm.

FIGURE 3  Sexual maturation of the young gametophytes of *Botrychium campestre*. (A) First sexual expression as an archegonium (AR). (B) First sexual expression as an antheridium (AN). (C) Early bisexual expression showing little separation between the multiple archegonia and antheridia. Gametophyte diameters range from 1 to 2 mm. Photos reproduced with permission from Dauphin et al. (2020).
belonging to the genera Botrychium and Botrypus. To determine the distribution and abundance of the belowground structures, it was necessary to sample repeatedly across a population of aboveground plants using a systematic sampling design that facilitates estimates of the densities of the gametophytes, gemmae (if present), and belowground juvenile sporophytes extracted from soil samples taken from an area with populations of emergent sporophyte leaves. Our sampling design (Johnson-Groh et al., 2002) facilitated the collection of data on the abundance and distribution of belowground propagules (gametophytes, gemmae, and belowground juvenile sporophytes) for several species (Table 1). We determined that, in the populations sampled, the belowground propagules greatly exceeded the number of aboveground plants, although there was significant variability among the different Botrychium species.

Belowground breeding behavior

Here, we found that Botrychium gametophytes in the earliest stages of sexual differentiation produce either an antheridium or an archegonium as the first gametangium, but become bisexual thereafter (Figure 3). This early and continuous bisexuality is consistent with reports from studies of a number of Ophioglossaceae species (e.g., Soltis and Soltis, 1986; Farrar, 1998; Hauk and Hauffer, 1999; Stensvold and Farrar, 2016), which were proposed to undergo gametophytic selfing as evidenced by their extremely low allelic variation. While low allelic variability is characteristic of most populations of all species of Botrychium (Stensvold and Farrar, 2016), we identified high levels of genetic variability in high-mountain populations of two species, Botrychium campestre (Farrar and Gilman, 2017) and Botrychium lunaria (L.) Sw. (Dauphin et al., 2020), indicating that they are outcrossing populations. Bisexual gametophytes were extracted from the soil at the outcrossing sites of Botrychium campestre, indicating that outcrossing can be maintained despite the prevalence of belowground bisexual gametophytes. These results support the suggestion of Dauphin et al. (2020) that melting snow seepage on steep slopes at high elevations may promote outbreeding through sperm transport between gametophytes by the mass flow of belowground water.
Asexual belowground reproduction

Asexual gametophytic reproduction by gemmae is well known in epiphytic species with superficial gametophytes (Farrar, 1967; Emigh and Farrar, 1977; Dassler and Farrar, 1997). Gemma production by gametophytes of *Psilotum* (Bierhorst, 1971) remains the only known example of asexual reproduction by belowground gametophytes. We have not documented this for belowground gametophytes of *Botrychium*, but note that the gametophytes of *Botrychium montanum* W. H. Wagner appear to be friable, disintegrating into fragments that appear to be healthy and capable of further growth. Whether this observation is anecdotal or common in *Botrychium* warrants further investigation.

Hundreds of detached gemmae can be extracted from soil samples in the vicinity of mature plants of gemma-producing species, as shown in Figure 5. These spherical structures are produced on sporophyte stems and, at maturity, contain an apical meristem cell from which they elongate and differentiate roots and a stem with an apical leaf bud (Farrar and Johnson-Groh, 1990). At a very early stage of differentiation, the growing gemmae also produce additional gemmae, as shown in Figure 5B. In early development, the gemmae appear to be randomly placed on the stem, but on older plants with distinct leaf internodes they appear to be associated with leaf scars.

Population establishment

Little is known about how *Botrychium* spores percolate into the soil or the length of time or the depth needed for germination. In some soils (beach or volcanic), the germination depth, as estimated by the stem depth of the sporophytes, can be fairly deep (5–50 cm), but in rich organic soils with periodic high water tables, germination can be quite shallow (1–5 cm) (personal observation by the authors).

The length of time required from spore germination to the appearance of the first leaf aboveground is also poorly understood. Whittier (1972, 1973) stated that the germination of *Botrychium* spores is triggered by moist darkness, but the gametophyte then remains at the one- or few-cell stage until infected by a mycorrhizal fungus, upon which the belowground *Botrychium* plants depend for water, minerals, and photosynthates derived from other host plants of the fungus (Winther and Friedman, 2007). Although determining the length of the development phase to sexual maturity may be difficult, the appearance of gametangia on very small gametophytes and the relative absence of asexual gametophytes in our extractions implies a short time until sexual maturity (Figure 3). Bierhorst (1971), noting the presence of seven leaf primordia in belowground apical buds, estimated the time to the appearance of the first aboveground leaf to be at least eight years after the initiation of the apical bud. Earlier studies by Bruchmann (1906) on *Botrychium lunaria* and by Campbell (1922) on *Botrychium simplex* E. Hitchc. reached similar conclusions, as have our previous studies (Johnson-Groh, 1998).

The minimal age of individual sporophytes of *Botrychium* can be estimated by counting the number of roots and leaf scars present on the belowground stem. Monitoring studies of aboveground leaves have shown that no more than one leaf is produced per year. Johnson (unpublished data) found that one root is produced belowground annually. Older roots are often necrotic, but it is still possible to estimate age using roots and leaf scars. Counting only leaf scars, we have estimated ages of up to 30 years for individual *Botrychium lunaria* plants, and up to 43 years for *Botrychium pumicola* Coville ex Underw. plants. Because we do not know the fate

| Species                  | Density (per m²) | Aboveground leaves | Gametophytes | Belowground juvenile sporophytes | Gemmae | All belowground structures | % Belowground/aboveground* |
|--------------------------|-----------------|--------------------|--------------|---------------------------------|--------|---------------------------|---------------------------|
| *Botrychium campestre*   | 6.7             | 21                 | 198          | 5907                            | 6126   | 914                       |                           |
| *Botrychium hesperium*   | 0.4             | 478                | 281          | 21                              | 780    | 1950                      |                           |
| *Botrychium gallicomontanum* | 16.1          | 10                 | 0            | 4170                            | 4180   | 260                       |                           |
| *Botrychium lanceolatum* | 3.1             | 135                | 10           | *                               | 145    | 47                        |                           |
| *Botrychium montanum*    | 1.2             | 738                | 0            | *                               | 738    | 615                       |                           |
| *Botrychium mormo*       | 12.8            | 728                | 104          | *                               | 832    | 65                        |                           |
| *Botrychium yaaxudakeit* | 1.4             | 281                | 42           | *                               | 321    | 229                       |                           |
| *Botrychium virginianus* | 0.5             | 42                 | 0            | *                               | 42     | 84                        |                           |
| **Average**              | 5.27            | 304                | 79           | 1262                            | 1645   | 312                       |                           |

*Species not known to produce gemmae.

*The total number of belowground structures divided by the number of leaves, expressed as a percentage.

### TABLE 1  Comparative densities of the aboveground leaves and belowground structures in seven species of *Botrychium* and *Botrypus virginianus* (modified from Johnson-Groh et al., 2002).
of leaf primordia in years when leaves do not emerge aboveground, nor how long it takes to begin producing leaves, it is fair to say that the actual age is higher than these estimates.

A related finding of our belowground extractions is that gametophytes of the Ophioglossales can continue to grow after the production of their first sporophyte, presumably for more than one season, and can produce multiple sporophytes, as shown in Figure 4. The approximate ages to which these gametophytes survive could possibly be determined by counting sporophyte scars, especially in species with very large gametophytes, such as those of Botrypus virginianus (Figure 4B). Whether such gametophytes produce sporophytes annually or less frequently remains to be determined. Although we have only rarely observed the production of multiple sporophytes, the frequency of its occurrence, and whether it occurs in all taxa, warrants further investigation.

**Population persistence**

Our long-term monitoring of the aboveground populations of many species of *Botrychium* has shown that these populations persist, with significant annual variability, for more than a decade (Figure 6). Similar studies have been published by Ahlenslager and Potash (2007) and Lesica and Ahlenslager (1996). Moreover, revisiting many historical collections at very specific sites has revealed extant populations up to a century old (Farrar, personal collection data).

The aboveground plant numbers of *Botrychium* populations may decline to a few individuals or even disappear for multiple years (Johnson-Groh, 1998). A remarkable case of persistence through drought was established through a belowground analysis of a population of *Botrychium campestrum* first noted in 1990 in a dry native prairie in easternmost Colorado, USA. Over the next 10 years of monitoring, no more than six aboveground leaves were recorded in any one year, and no plants were found in the following 10 years. Fearing the population was destroyed by persistent drought, we conducted a belowground analysis in 2007, revealing the persistence of belowground gemmae and juvenile sporophytes. After two consecutive years of above-normal precipitation, 12 aboveground leaves were again seen in 2010, and in 2011 we recorded more than 50 robust plants at the site. Such perseverance of *Botrychium* populations is likely due to their mycotrophic access to water, minerals, and carbohydrates during periods of environmental stress. Sampling soils in habitats where populations are thought to have been extirpated has thus revealed healthy belowground propagule banks. Sampling in habitats where plants have never been recorded but good habitat parameters exist may also merit investigation.

The decline of populations has also been documented for *Botrychium* using belowground analyses. Aboveground and belowground populations of *Botrychium mormo* in northern Minnesota, USA, were sampled in 2002 and 2012 to ascertain the damage caused by introduced earthworm invasions (Johnson, 2015), revealing that both aboveground and belowground populations had significantly declined.

**Conservation of populations**

Populations of *Botrychium* are often considered species of concern by agencies charged with their protection. The analysis of belowground populations can contribute greatly to assessments of population health and the design of conservation practices; for example, in a viability assessment of the rare species *Botrychium mormo* in the Great Lakes region of North America (Berlin et al., 1998), our data concluded that the abundance of the belowground stages (see Table 1) was not a limiting factor for the viability of the species. Understanding the depth of germination can also be important for conservation, such as determining whether they require protection from grazing or all-terrain vehicles. An example of this is *Botrychium pumicola*, a protected species in Oregon, USA, that inhabits loose volcanic soils. Our preliminary studies indicate that the depth of germination for this species may be greater than 0.5 m, minimizing the impact of aboveground activities on the belowground population.

**Summary**

Additional belowground analyses are critical for the further elucidation of the biology and ecology of plants with subterranean reproductive stages. Documenting the belowground dynamics enables us to better understand their basic biology, longevity, population dynamics, and response to environmental stresses.

Phylogenetic analyses consistently place the Ophioglossales, Psilotales, and Lycopodiales as sister to the remainder of ferns (Pryer et al., 2004; Rothfels et al., 2015), and they share many similarities with the earliest land plants, including their dependence on endomycorrhizal fungi (Taylor et al., 2005; Gerrienne and Gonet, 2011). The continued study of ferns and lycophytes with belowground reproductive biology therefore has great potential for further documenting the adaptations that have allowed these ancient lineages to survive in modern ecosystems.

**AUTHOR CONTRIBUTIONS**

D.R.F. and C.L.J. designed the research, wrote and edited the manuscript, and contributed to the figures. Both authors approved the final version of the manuscript.

**ACKNOWLEDGMENTS**

We express immense gratitude and appreciation for the many students in each of our labs who scrutinized countless dishes of floating organic debris in search of moonwort structures. Open access funding provided by the Iowa State University Library.
