

# Techniques for locating and analyzing subterranean *Lycopodium* and *Diphasiastrum* gametophytes in the field

Radvilė Rimgailė-Voicik | Jonas Remigijus Naujalis

Institute of Biosciences, Vilnius University,  
Saulėtekio Ave. 7, Vilnius LT-10257, Lithuania

## Correspondence

Radvilė Rimgailė-Voicik, Institute of Biosciences,  
Vilnius University, Saulėtekio Ave. 7, Vilnius  
LT-10257, Lithuania.  
Email: radvile.rimgailaite@gmail.com

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## Abstract

Homosporous club mosses have an archaic life cycle, alternating two locationally, nutritionally, and physiologically independent generations. The sexual generation of club mosses—the gametophytes (or prothallia)—are among the least researched botanical subjects. The gametophytes are responsible for not only sexual reproduction, but also the determination of recruitment of the new sporophyte generation, species habitat selection, migration, and evolution. Researchers often fail to find juvenile club moss populations and thus do not discover subterranean long-lived achlorophyllous gametophytes. To date, the gametophytes of most club moss species remain undiscovered in nature and are not scientifically documented. Almost all researchers who have previously located subterranean club moss gametophytes declared that their first find was due to luck and that subsequently the researcher's intuition plays the most important role; however, intuition and good luck are not scientific methods. In our review, we combine our knowledge with data available in the literature and discuss the following questions using a methodical approach: (1) How can we locate a subterranean club moss gametophyte population? (2) How can we extract the gametophytes? and (3) What new knowledge about club moss population development can be gained by analyzing juvenile club moss populations?

## KEYWORDS

lycophytes, nearest neighbor analysis, prothallia, timed-meander survey, Voronoi polygon

Lycophyta represent the oldest extant land plants, with the earliest globally distributed fossils dating back to the Devonian Period (Wikström and Kenrick, 2001). Their life cycle consists of two separate generations: gametophytes ( $n$ ) and sporophytes ( $2n$ ). Free-living gametophytes are known from Devonian Period terrestrial habitats preserved in the Rhynie chert (Taylor et al., 2005). The first discoveries of gametophytes (Fankhauser, 1873; Goebel, 1887) provided us with primary knowledge about their plastic forms, which contrasts with the much more constant characteristics of the club moss sporophytes (Bower, 1894). Bruchmann (1898) described five structural types of club moss gametophytes, all named by a representative species: Type I, *Lycopodium clavatum* L.; Type II, *Diphasiastrum complanatum* (L.) Holub (*Lycopodium complanatum* L.); Type III, *Huperzia selago* (L.) Bernh. ex Schrank & Mart. (*Lycopodium selago* L.); Type IV,

*Lycopodiella inundata* (L.) Holub (*Lycopodium inundatum* L.); Type V, *Phlegmariurus phlegmaria* (L.) Holub (*Lycopodium phlegmaria* L.). Bruchmann's classification is still widely used to characterize gametophytes (Bruce, 1976; Bruce and Beitel, 1979; Whittier, 2003; Whittier et al., 2005; Renzaglia and Whittier, 2013; Rimgailė-Voicik et al., 2015). Considerable variation in the size and developmental stages of gametophyte populations has been reported (Bruchmann, 1898; Degener, 1924; Eames, 1942; Naujalis, 1995; Rimgailė-Voicik et al., 2015). The taxonomical value of gametophyte morphology was first acknowledged by Rothmaler (1944), but was not taken into consideration in later classifications. The generic classification for the North American lycopsids, based on anatomy, chromosomes, spores, and gametophytes (Wagner and Beitel, 1992), resolved three subfamilies, supported by plastid *rbcL* sequence data (Wikström and

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Kenrick, 1997, 2000), and was implemented in the newest comprehensive classifications of the lycophytes and ferns (Øllgaard, 2012; Øllgaard and Windisch, 2014; PPG, 2016); however, the role of morphological and physiological diversity among gametophytes remains unclear.

Most club moss gametophyte observations are based on rather low numbers and are descriptive; systemic and quantitative investigations of the spatial population structure, age, size, and habitat requirements of club moss gametophytes are still lacking. Little is known about subterranean gametophyte longevity, dominating reproduction type, or repetitive fertilization events.

The absence of ecophysiological data on the gametophyte phase of most spore-bearing plants has left science with misconceptions regarding the critical phase of the life cycle of these archaic plants. The gametophyte phase is thought to be primitive and rare, involving plants that have an uncomplicated anatomy and are, compared with the vegetative expansion, unimportant for conquering new territories. A broader scientific discussion is needed to encourage the elucidation of club moss gametophyte development, as systematic long-term club moss gametophyte research could provide new knowledge about changing species ecologies and adaptations. In this review, we summarized the available knowledge about subterranean long-lived gametophytes of the genera *Lycopodium* L. and *Diphasiastrum* Holub and their habitat features. We searched for scientific articles on Google Scholar and JSTOR using the following combinations of terms: ‘*Lycopodium* gametophytes’, ‘*Lycopodium* prothallia’, ‘*Diphasiastrum* gametophytes’, ‘*Diphasiastrum* prothallia’, ‘subterranean gametophytes’, and ‘subterranean

prothallia’. Additional literature was obtained through conventional searches of the bibliographies of related papers and reports. We did not limit the review to a certain territory or time frame. Scientific works in English, German, French, Russian, Finnish, and Lithuanian were included. Finally, we also discuss the available methods for study of gametophyte and juvenile sporophyte populations and formulate hypotheses for future research.

## LOCATING SUBTERRANEAN CLUB MOSS GAMETOPHYTES IN NATURE

According to the literature, there are 10 main habitats that one should explore when searching for subterranean club moss gametophytes (Figure 1): deep canyon slopes covered with damp dark forest; steep fir or mixed hardwood forest slopes; ski tracks; banks of water bodies; forest roads or paths; dry pine forests or pine plantations; abandoned fields containing *Hamamelis* and *Rhus*; open, exposed, sandy places; under boulders; and on top of rocky extensions.

We propose that these habitats have similar light quality, quantity, and directional patterns, and that they induce chemical club moss signaling responses, which have not yet been adequately studied.

In all forest types, juvenile sporophyte and subterranean gametophyte populations are most commonly found in areas that have undergone small-scale forest floor disturbances. Quite often, subterranean gametophytes were found in areas where surface forest fires occurred 15 to 25 years earlier (Bruchmann, 1898; Eames, 1942; Oinonen,



**FIGURE 1** Ten types of sites where subterranean club moss gametophytes were previously found. (1) Deep canyon slopes covered with damp dark forest (Fankhauser, 1873); fir forest slopes (Bruchmann, 1898); steep forest slope (Degener, 1924); northern slope of the summit (Horn et al., 2013). (2) North- or west-facing slopes with mixed hardwood (Stokey and Starr, 1924). (3) Skiing track dominated by *Calluna vulgaris* (Horn et al., 2013). (4) On the bank of a lake (Stokey and Starr, 1924); on the side of a bank (Edgerley, 1915; Holloway, 1920); between a lake and a marsh (Spessard, 1922); on the slope of the forest road (Schmid and Oberwinkler, 1993). (5) On the forest road, near the path (Bruchmann, 1898; Spessard, 1917). (6) Dry pine forests (Naujalis, 1995; Rimgailė-Voicik et al., 2015). (7) Near a rock shaded by few trees (Lang, 1899); sheltered by a boulder (Degener, 1924); at the top of a rocky extension (Gauthier and Dumais, 1938). (8) Abandoned fields containing *Hamamelis* and *Rhus* (Eames, 1942); forming a mixed forest grove (Gauthier and Dumais, 1938); young mixed deciduous growth (Stokey and Starr, 1924). (9) Open, exposed, sandy places (Spessard, 1917); gravel pit (Øllgaard, 1985); open gravelly knolls (Eames, 1942). (10) Pine plantation (Bruce and Beitel, 1979)

1968; Naujalis, 1995). After a forest fire, the community opens up drastically and spores can easily enter the soil. However, the true viability of club moss spores in the soil has never been determined. If the spores remain viable for many years, the asynchronous development of the gametophytes might be related to the age of the spores, not the nutrient availability. Bioactive compounds that are released during the fire could also enhance spore germination, although this has never been demonstrated for club mosses. One example in seed plants is the key bioactive compound karrikinolide (KAR1), which is known to promote germination (Flematti et al., 2004).

Stokey and Starr (1924) grouped all localities described as “poor collecting grounds” into three main categories: (1) groves of mixed hardwoods on a slope or near a slope, above a body of water; (2) relatively dry depressions in a grove of mixed hardwoods; and (3) groves of hemlock. In all these localities, the soil consisted of sandy loam with a considerable amount of humus. In contrast to what was found by Spessard (1922), all locations were in well-shaded regions on mostly north-facing slopes, and no consistent grouping pattern was observed. Eames (1942) published data on a successful search for *Lycopodium obscurum* L., *Lycopodium clavatum*, *Diphasiastrum complanatum*, and *Lycopodium annotinum* L. gametophytes and juvenile sporophytes at the Cayuga Lake Basin in west-central New York, USA, where the species occurred in young upland deciduous forests, open gravelly knolls, and abandoned fields alongside *Hamamelis* L. and *Rhus* L. species.

Subterranean club moss gametophytes and juvenile sporophytes were found on and near forest roads, tracks, and lines separating forest blocks (Bruchmann, 1898; Degener, 1924; Stokey and Starr, 1924; Oinonen, 1968; Naujalis, 1995; Muller et al., 2003). The high number of juvenile club mosses in Varėna District, Lithuania, can also be related to the traditional activities of the local people, such as mushroom gathering and collecting moss for buildings and farm animals.

When selecting sites to search for club moss gametophytes in temperate regions, we propose that researchers first analyze the geological data, then move to more recent times and look through archival aerial images of the region of interest, as well as gather information about forest floor fires and engineering activities such as power lines or gas pipelines. For example, the continental sand dunes on the surface of the Varėna District in Lithuania were formed during the last ice age, approximately 12,000 years ago. The area has sandy soils with dry Scots pine (*Pinus sylvestris* L.) forests, which is the most promising habitat for the establishment of juvenile club moss populations; however, gametophytes have also been found in wet alder (*Alnus* Mill.) forests (Naujalis, 1995). We believe that the history of the site (up to 70 years) might play an important role for club moss spore germination and juvenile club moss population establishment. When comparing aerial photos from the 1950s with images from today, we can clearly see that the sites where juvenile club moss populations were discovered

had open sand or were near sites with open sand, but the territories that were open in the 1950s have either naturally overgrown with forest or trees were planted in those areas. In the approximately 240-ha forested territory in southern Lithuania, club moss outgrowths were more commonly discovered near forest roads, tracks, and the edges of forest blocks, or in abandoned fields and near electricity lines (Naujalis, 1995). These gametophyte-rich sites may represent a specific forest successional stage. The adult club moss clones were always spatially separated from the juvenile populations (Naujalis, 1995).

Various authors (Bruchmann, 1898; Spessard, 1922; Stokey and Starr, 1924) suggested looking for gametophyte populations in less extreme conditions than those in which the parent plants grow. Holloway published articles on *Lycopodium* found in New Zealand (Holloway, 1916a, 1916b, 1919), in which he emphasized that juvenile sporophytes and gametophytes cannot be found in localities where adult plants are abundant, noting that roadside cuttings, damp shaded clay banks, or other patches of disturbed soil were favorable habitats for gametophytes (Holloway, 1920). Spessard (1917), who was first to collect gametophytes in North America (Marquette, Michigan, USA), found juvenile sporophytes and gametophytes on small, bare, exposed elevations that were more receptive for spores and where no adult plants of *Lycopodium clavatum*, *D. complanatum*, or *Lycopodium annotinum* were growing nearby.

Because juvenile club mosses are evergreen, it is possible to search for them in suitable habitats (Figure 1) nearly year-round. Other spore-bearing plants with long-lived subterranean gametophytes, such as members of the genus *Botrychium* Sw., have a short vegetation time frame and their populations are even more challenging to locate. Traditional plant population-monitoring methods, such as transects with various modifications or a modified-Whittaker nested vegetation sampling method (Stohlgren et al., 1995), are labor intensive, but insufficient when the research objects are rare or semi-rare or distributed heterogeneously (Barnett and Stohlgren, 2003; Huebner, 2007).

In order to improve the efficiency of juvenile club moss and subterranean gametophyte research, some alternative approaches should be tested, such as strip adaptive cluster sampling (Abrahamson et al., 2011), adaptive line transects (Pollard et al., 2002), or timed-meander surveys (Goff et al., 1982). The strip adaptive cluster sampling method could improve the detection of species that are low in abundance. The first phase of this method is the creation of a transect of 1 × 1-m quadrats. When the target species is detected within a quadrat, additional quadrats are sampled surrounding the initial quadrat. The successive quadrats are sampled using the same neighborhood rules, until the target species are not detected. The adaptive line transects method is valuable for detecting and evaluating clusters within the population; the most common zigzag pattern creates the trackline, which is easy to repeat and does not contain any gaps or transect intersections. The timed-meander survey is often selected for endangered and rare species monitoring because it is not

dependent on the prior identification of the territory size, geographic features, and even the experience of the researcher (Bourdaghs, 2014; Bohnen and Galatowitsch, 2016), and it allows the use of intuition for quantitative data collection (Goff et al., 1982).

The timed-meander survey procedure begins by defining the study site (this could be a potential site known from historical sources, or a habitat where a viable population is monitored), after which the starting point is selected. The researcher walks for a defined period (usually 5 or 10 min), registering all the species in all vegetation layers or looking for one specific plant (in our case, for juvenile club mosses). The researcher stops after the defined time period, and the results are calculated (e.g., total number of species, number of individuals or groups of individuals). The researcher then proceeds to search for the same time interval again, continuing from where they last stopped. The trajectory is intuitively chosen, and the goal is to survey the selected area; the search may end when no new plant species are found, or after a certain amount of time (30–60 min depending on the size of territory) if no rare species are found. For a general estimate, about 30 min would be needed to survey a 0.5-ha study site for club moss species, not including time needed for species identification. The method can be easily adapted to best suit the researcher's needs; for example, one can include fixation of coordinates or specimen collection. Juvenile club moss sporophytes can be marked with flags, and the territory can later be checked in more detail by taking soil samples for a gametophyte search. This method was successfully adapted for a *Botrychium simplex* E. Hitchc. population analysis in the United States (Johnson-Groh et al., 2002). The collected data allows the generation of a species-effort curve, which can be interpreted as the species-area curve (Goff et al., 1982) to allow the data to be analyzed as a statistical model (Moore et al., 2011).

## COLLECTING DATA ON SUBTERRANEAN GAMETOPHYTE AND JUVENILE SPOROPHYTE PHENOLOGY

Bisexual club moss gametophytes develop slowly and reach maturity after five to 10 years (Bruchmann, 1910; Horn et al., 2013). Juvenile sporophytes have a prolonged period of matrotrophy and are dependent on their associated gametophytes for several years (Renzaglia and Whittier, 2013). No comprehensive data on the mortality rates of gametophytes and juvenile sporophytes have been published, and little is known about the dynamics and viability of juvenile club moss populations; however, the long-maintained juvenile sporophyte connection with the gametophyte is thought to increase its survival rate (Bruchman, 1898; Horn et al., 2013; Renzaglia and Whittier, 2013). Only extremely young sporophytes indicate the presence of gametophytes in the soil. During the earliest developmental stages, sporophytes are achlorophyllous and receive nutrition via the

gametophyte placenta region. Later, when the sporophyte shoot reaches the surface of the soil or moss layer, the gametophyte decomposes. During early development, young photosynthesizing sporophytes may look similar to *Polytrichum* Hedw. mosses as they have isophyllous leaves and only orthotropic growth, and may be incipient with the gametophytes (Bruchmann, 1898; Spessard, 1922; Eames, 1942; Wagner and Beitel, 1992; Naujalis, 1995; Rimgailė-Voicik et al., 2015).

The viability of young club moss sporophytes has not been monitored in the field, but available observations give us knowledge about their low competitiveness. For example, as noted by Bruchmann (1898), juvenile *Lycopodium annotinum*, *Lycopodium clavatum*, and *D. complanatum* sporophytes in fir plantations are likely to die if the number of juvenile sporophytes was high and overshadowed by a thick fir needle cover on the forest floor. Gradually, only club mosses on the edges of forest stands and in forest rifts remained. During seven years (1985–1991) of observations in dry pine forests, it was discovered that 19 out of 35 (54.3%) *Lycopodium annotinum* juvenile sporophytes died (Naujalis, 1995). In the Vosges Mountains, France, *Diphasiastrum tristachyum* (Pursh) Holub juvenile plants were observed to become overgrown by *Calluna vulgaris* (L.) Hull (Muller et al., 2003).

Relationships with endophytic fungi are crucial for successful gametophyte development, yet we have limited knowledge of club moss mycorrhiza and nutrient sharing among other species in the community. Recent cytological and molecular analyses showed that both Mucoromycotina and Glomeromycota fungi associate with lycopods, sometimes in the same plants (Rimington et al., 2014). Fungi with similar characteristics to both groups were found associated with Devonian fossil plants (Strullu-Derrien et al., 2014). No direct transfer of mycorrhizal infection through a gametophyte–sporophyte junction was detected in *Lycopodium clavatum* (Lang, 1899), *Lycopodium cernuum* L. (Duckett and Ligrone, 1992), or *Huperzia hypogaea* B. Øllg. (Winther and Friedman, 2007). The suspected de novo infection pathway means it is possible that symbionts in sporophytes differ from those in the gametophytes primarily because of physiological differences (Leake et al., 2008).

Abiotic factors can cause juvenile sporophyte mortality as well. For example, the death of four *Lycopodium clavatum* juvenile sporophytes was observed near a high-voltage power line due to bare soil overheating (Naujalis, 1995). Similarly, Holloway (1916a) indicated that juvenile *Lycopodium fastigiatum* R. Br. sporophytes and gametophytes perished during dry summers in New Zealand. Juvenile sporophytes are more likely to occur after 10–12 years without a drought (Eames, 1942).

We recommend that club moss researchers establish permanent monitoring sites to enable data collection on juvenile club moss competition. We evaluated a pilot site in Maskauka, Lithuania, every August from 2012 to 2015, recording the vegetation coverage and the location of juvenile sporophytes (Rimgailė-Voicik and Naujalis, 2016). The size

**TABLE 1** Geographical coverage of cited references on subterranean club moss gametophytes

Author	Year	Country	Continent
Fankhauser	1873	Switzerland	Europe
Bruchmann	1898	Germany	Europe
Lang	1899	Scotland, UK	Europe
Edgerley	1915	New Zealand	Oceania
Holloway	1920	New Zealand	Oceania
Spessard	1917	Michigan, USA	North America
Stokey and Starr	1924	Massachusetts, USA	North America
Degener	1924	Massachusetts, USA	North America
Ames	1926	Massachusetts, USA	North America
Gauthier and Dumais	1938	Quebec, Canada	North America
Eames	1942	New York, USA	North America
Thomas	1975	North Wales, UK	Europe
Bruce and Beitel	1979	Michigan, USA	North America
Øllgaard	1985	Denmark	Europe
Schmid and Oberwinkler	1993	Germany	Europe
Naujalis	1995	Lithuania	Europe
Horn et al.	2013	Germany	Europe
Horn et al.	2013	Czech Republic	Europe
Rimgailė-Voicik et al.	2015	Lithuania	Europe

of the plot was 100 m<sup>2</sup> and the evaluation was performed on 1-m<sup>2</sup> subplots marked with wooden dowels. At the end of August 2015, all juvenile sporophytes at the Maskauka site were excavated in 10 × 10 × 10-cm soil samples. In the lab, the soil samples were gradually disassembled with tweezers to search for club moss gametophytes and to extract juvenile sporophytes. The age of the identified juvenile sporophytes was determined according to the number of annual constrictions (Primack, 1973). From this study, we could summarize that a four-year period is too short for generating data on longevity, but the size of the selected plot was suitable. Using smaller soil sample sizes reduced the amount of labor invested, but other important spatial data may have been collected if larger sample volumes had been used (Table 1).

## METHODS FOR SUBTERRANEAN GAMETOPHYTE EXTRACTION

In general, there are three ways to collect the underground gametophytes or other propagules: you can either sift the soil samples through sieves, centrifuge them, or disassemble soil samples by hand. All of the literature analyzed for this

review agreed that gradually disassembling the soil with tweezers near juvenile sporophytes provides the best results. We learned that disassembling samples with tweezers is very slow but rewarding, yielding not only undamaged gametophytes with various stages of sporophyte growth, but also enabling the collection of data on the distribution of the gametophytes in the soil sample. Moreover, disassembling soil samples gradually can provide data regarding gametophyte cluster structure and their relationship with microhabitat conditions, such as aboveground vegetation. While researchers have agreed that this type of soil sample analysis is difficult and extremely tiring (Spessard, 1922; Rimgailė-Voicik et al., 2015), it has also been noted that sieving or washing soil samples is an unsatisfactory method (Spessard, 1922; Degener, 1924; Naujalis, 1995), although it can be useful for primary research when gametophyte presence merely needs to be confirmed.

During our research (Rimgailė-Voicik et al., 2015), 50 × 50 × 10-cm soil samples were collected (Figure 2). In the laboratory, the moss layer of every soil sample was removed. Using pins, every sample was then divided into 10 × 10-cm plots to increase the accuracy of the gametophyte and juvenile sporophyte coordinates registered. We then looked for gametophytes by gradually disassembling the soil samples with tweezers. The coordinates ( $x$ ,  $y$ ) of every gametophyte and juvenile sporophyte located in the 10 × 10-cm subfields were registered and recounted in a 0.25-m<sup>2</sup> sample. The developmental stage of each gametophyte was recorded, and its size was measured. It would also be useful to measure the dry weight of gametophytes, although this was not done in the current study. The sporophytes were herbarized, while the gametophytes were fixed in 50% ethanol and stored at 4°C for future analysis (e.g., number of antheridia, archeogonia, endophytic fungi hyphae).

Mason and Farrar (1989) proposed centrifuging as an effective method for discovering underground *Botrychium* propagules. The samples are first washed and sifted a few times to remove large debris and root parts. The resulting mass of soil containing the remaining organic structures was first suspended in water and centrifuged, after which the sample was resuspended in 30% sucrose solution and centrifuged again. The first centrifugation resulted in sediments of *Botrychium* underground structures, while centrifuging the samples with 30% sucrose allowed the extraction of particles of interest from the upper fraction and the preparation of the material for examination under a microscope (Johnson-Groh et al., 2002; Farrar and Johnson, 2022).

## ANALYZING SUBTERRANEAN GAMETOPHYTE POPULATION STRUCTURES

There is currently no non-invasive method for determining the boundaries of subterranean gametophyte populations; thus, we can only address juvenile club moss sporophyte



**FIGURE 2** Extraction of the soil samples ( $0.25 \text{ m}^2$ ) for the detection of club moss gametophytes. (A) In the field, the sample size can be marked with branches (recommended length: 50 cm). (B) The perimeter of the sample must first be cut out with a shovel. (C) When removing the shovel from the ground, move it back and forth gently to loosen the sample. (D) If the cut-out sample contains many grasses, it can be removed and placed in a box for transportation. If mosses and lichens dominate, we recommend placing plywood or a similar material beneath the sample before removing it. (E) The depth of the sample should be up to 10 cm. (F) The same branches can be used to mark the next sample. It is important not to oversample. One person needs around three working days to survey one sample. The samples should be kept in a moist environment

distribution. No long-term research has dealt with the details of subterranean club moss gametophyte population structure and function. In the Northern Hemisphere, Type I gametophytes of *Lycopodium clavatum* (according to Bruchmann, 1898) seem to develop more often than those of *Diphasiastrum complanatum* (Type II). The gametophytes of *Lycopodium* species are white, gray, or gray-brown irregular bowls, while the gametophytes of *Diphasiastrum* species are orange-brown carrot/beetroot shapes (Bruchmann, 1898; Rimgailė-Voicik et al., 2015). In suitable habitats, subterranean achlorophyllous gametophytes typically form numerous prospering populations comprising individuals from up to six different species (Fankhauser, 1873; Bruchmann, 1898; Lang, 1899; Spessard, 1922; Stokey and Starr, 1924; Rimgailė-Voicik et al., 2015).

Fankhauser (1873) found and described gametophytes and juvenile sporophytes of *Lycopodium annotinum* in Switzerland. During a botanical expedition on a slope in a dark forest, he fortuitously noticed 13 juvenile sporophytes growing among the mosses. Fankhauser dug out the young club mosses and made several observations: (1) juvenile sporophytes developed unevenly, with their lengths ranging from 3 to 18 cm; (2) several juvenile sporophytes had a pinhead-shaped tubercle close to the foot; (3) few juvenile sporophytes had the remains of obscure bodies; and (4) one juvenile sporophyte had a brownish body near the foot. Fankhauser collected additional soil from alongside the first

field site, where he found a few irregularly shaped fleshy yellowish-white bodies with sparse rhizoids, which were suspected to be *L. annotinum* gametophytes.

The most data on the gametophytes of *Lycopodium clavatum*, *Lycopodium annotinum*, and *D. complanatum* were accumulated by Bruchmann (1898, 1909, 1910), who investigated club mosses in the mountain forests of Thuringia, Germany, over a period of 30 years. Most juvenile sporophytes and gametophytes were found in a young forest that was planted after fir logging. The juvenile sporophytes often occurred in former cleared forests comprising young trees eight to 14 years old. Bruchmann collected  $1\text{-dm}^3$  soil samples and disassembled them with tweezers. In these samples, he found up to 10 (or sometimes even more) subterranean gametophytes and juvenile sporophytes. Over time, Bruchmann formed a collection of about 500 subterranean gametophytes of different developmental stages (Bruchmann, 1898). Near Amherst, Massachusetts, USA, Degener (1924) collected 200 to 300 specimens of *Lycopodium annotinum* and *Lycopodium clavatum* gametophytes over a few afternoons in an area less than 1 m in diameter and subsequently emphasized that thousands of sporophytes can be present in a given area. Our research (Naujalis, 1995; Rimgailė-Voicik and Naujalis, 2016) also showed that the development of gametophyte populations was asynchronous, and the size of gametophyte clusters might exceed 500 individuals in a  $1\text{-m}^2$  sample area.

Similarly, the data from research on *Botrychium* ferns showed that the number and density of subterranean individuals might substantially exceed the number of above-ground individuals, in a ratio of 700:1 (Johnson-Groh et al., 2002).

It was proposed that the presence of varying club moss gametophyte developmental stages within a given area is the result of repeated spore germination and different gametophyte growth rates (Eames, 1942). In Massachusetts, Degener (1924) collected *Lycopodium annotinum* and *Lycopodium clavatum* gametophytes with sizes ranging from 1.5 to over 10 mm. Stokey and Starr (1924) found more than 100 juvenile *D. complanatum* sporophytes up to  $11 \times 2.5$  mm in size, some of which were found with gametophytes. The gametophytes we found previously also varied in size (Naujalis, 1995; Rimgailė-Voicik et al., 2015), although the reasons for this have never been experimentally explained. The considerable variation observed in the size of the gametophytes could be related to their developmental stages, but their size could also be influenced by moisture, topographic and edaphic factors, and the presence of appropriate endophytic fungi.

The traditional methods used for spatial analysis of club moss gametophyte aggregations are destructive, point-based, and labor-intensive. To overcome these challenges, future researchers could use ground-penetrating radar (GPR) to locate and evaluate subterranean gametophytes. GPR is a non-invasive on-site measurement technique providing aerial and repeatable underground measurements that is widely used in civil engineering, geophysical investigations (Carrière et al., 2013), archaeological research (Conyers, 2013), and coarse tree root (>0.2 cm) detection (Guo et al., 2013). The size of gametophytes is suitable for detection with GPR, as demonstrated in research by Spessard (1917), who found five *Lycopodium clavatum* gametophytes 3–6.5 mm in size, and Gauthier and Dumais (1938), who reported *Lycopodium clavatum* gametophytes 4–6.5 mm diameter; therefore, GPR is able to detect objects of an appropriate size for identifying gametophytes. GPR has also been used to estimate root diameter (Cui et al., 2011), root biomass (Zhu et al., 2014), and root zone area (Lorenzo et al., 2010), as well as for root mapping (Hruska et al., 1999). Some advanced GPR methods have recently been adopted for agricultural soil research focused on the evaluation of field management via root sensing (Liu et al., 2016), but its use in detecting plant biomass remains limited (Thompson, 2014). Other methods for belowground measurement that could be potentially useful in subterranean club moss gametophyte microniches are soil moisture sensors (Baker and Allmaras, 1990), soil conductivity (Rhoades and Corwin, 1981), the mini-rhizotron technique (Sharma et al., 2014), and digital root imaging (Clark et al., 2011).

The most common methods for the analysis of spatial population structure in botany are quadrat analyses and nearest neighbor analyses (NNAs). We believe that NNA should be especially favorable for gathering data on subterranean gametophyte populations because it enables the

modeling of juvenile club moss populations. The method allows the determination of whether the distribution of individuals is random or not (Perry et al., 2006). For the successful application of NNA, each individual gametophyte and juvenile sporophyte must be represented by its coordinates in the overall sample (Rimgailė-Voicik et al., 2015). This method calculates an expected mean nearest neighbor distance ( $r_E$ ) using the overall density of the population ( $p$ ) given a Poisson distribution:

$$\bar{r}_E = \frac{1}{2\sqrt{p}}$$

The nearest neighbor index (NNI) was then derived from the ratio of  $r_E$  to the observed mean nearest neighbor distance ( $r_o$ ):

$$\text{NNI} = \frac{\bar{r}_o}{\bar{r}_E}$$

The properties of the index are: NNI = 1, spatial randomness; NNI < 1, spatial aggregation; NNI > 1, regular spatial distribution. The NNA measures the distance of each point to its nearest neighbor, determines the mean distance between neighbors, and compares the mean distance to what would have been expected in a random nearest neighbor distribution. The higher-order NNA expected distance values for the  $k$ -th nearest neighbor can help to determine the size of the clumped groups more precisely (Perry et al., 2006). The NNA does have disadvantages, however; the results depend on the size of the study and the aggregation intensity within the analyzed metapopulations (Hutchings and Discombe, 1986).

Another method for spatial analysis that is less dependent on the study scale is Voronoi diagrams (also called Thiessen diagrams or Dirichlet tessellation), which was first suggested for use in ecological research by Mead (1966). The analysis includes the creation of an individual polygon of every point by characterizing the plot size, form, and relationships among the plots (Kenkel, 1990; Palaghianu, 2012), and can therefore be used to model population structure with an emphasis on individual adaptability and survival processes. The plot size and shape determined by Voronoi diagrams represents the area potentially available (APA) to an individual plant. In addition, Voronoi diagrams can be applied on different scales and modified according to the parameters that interest the researcher most (e.g., phenology or morphological criteria). Voronoi plates are formed to every point in a point set during the territory acquisition, so that every site in the plate is closer to the central point than to any other point. The boundaries of the polygons are drawn as perpendicular lines through the middle of the straight lines connecting the nearest distances (i.e., the Delaunay triangulation lines). In this way, the polygons fill all the researched territory, do not overlap, and create a classical Voronoi diagram (Yamada, 2017). The Voronoi diagrams allow the prediction of how an individual

area is related to interspecies competition and resource acquisition, and how individual physiomorphological patterns are related to the number of neighbors present (Bauer et al., 2004). Methods that allow the estimation of plant species abundance according to presence/absence usually do not include spatial information (McCaffrey et al., 2014), so this has potential to enhance the analysis of club moss species.

More than one sporophyte can grow from a single gametophyte. According to Eames (1942), two sporophytes are common, three to five occasional, and as many as seven well-formed sporophytes have been found on one large gametophyte. Bruce and Beitel (1979) found 26 gametophytes with one sporophyte each, 25 that had produced more than one, and notably, one gametophyte with 13 emerging sporophytes. During recent (Rimgailė-Voicik et al., 2015) and earlier (Naujalis, 1995) research in Lithuania, a few gametophytes with two or three sporophyte sprouts were discovered. All of these examples support the hypothesis that fertilization events in a single gametophyte can be repetitive. How repetitive fertilization events are related to the spatial population structure is not clear, however, as representative data for this analysis have not been collected.

The study of natural populations of both sporophytes and gametophytes would provide a much more complete picture of the genetic diversity of lycophyte species. The gametophytes of homosporous lycophytes are usually bisexual, which allows for three reproductive options: true outcrossing, sporophytic (=intergametophytic) selfing, and gametophytic (=intragametophytic) selfing (Haufler et al., 2016). Soltis and Soltis (1988) estimated low rates of intragametophytic selfing using enzyme electrophoresis to determine the sporophytic genotype frequencies for natural populations of three lycopod species (*Lycopodium clavatum*, *Lycopodium annotinum*, and *Huperzia miyoshiana* (Makino) Ching). To the best of our knowledge, the genetic variability of subterranean club moss gametophytes has never been analyzed. In fact, flow cytometry has only been used to demonstrate the haploid state of gametophytes of *Diphasiastrum complanatum* (Schnittler et al., 2019). Only a few researchers have analyzed the development and anatomy of club moss gametophytes (Bruce, 1979; Renzaglia and Whittier, 2013), and our limited knowledge of the development of gametangia or sperm chemotaxis mostly dates back to the works of Bruchmann (1898, 1909, 1910). No molecular markers have yet been developed to test whether the gametophytes in one population are produced by the same club moss stand.

## DISCUSSION

The study of subterranean club moss gametophytes requires substantial time and financial resources. We propose that, before going into the field, researchers should first perform

a comprehensive analysis of available cartographic sources (e.g., Google Earth).

The striking stability of the external and internal features of lycopods manifested since the Devonian period may be explained in terms of a long-lasting relationship between the organism and its habitat, or by genetic homeostasis (Levin and Crepet, 1973). The study of key habitat features (e.g., light quality, water availability, and nutritional status of the soil) could elucidate the relationships of lycopod gametophytes with local vegetation and soil fungi, fauna, and flora; however, the field investigation of gametophyte biology remains minimal on many levels. The number and frequency of recruits (i.e., emerging juvenile sporophytes forming new populations), as well as the time frame of gametophyte development (maturation, sexual differentiation, and sporophyte production), all remain unexplained.

Currently, no comprehensive data on subterranean club moss gametophyte population structure and function are available. The majority of our knowledge in this area comes from the works of Bruchmann (1898, 1909, 1910), which have never been repeated at the same scale. A large amount of club moss research was published between the 1910s and 1930s in New Zealand and the United States (Spessard, 1917; Holloway, 1920; Degener, 1924; Stokey and Starr, 1924; Eames, 1942; Figure 3) and made significant progress in answering questions surrounding club moss sexual propagation, but for unknown reasons, interest in gametophyte research in nature subsequently diminished.

Today, no specific methodology or habitat patterns are available for the detection of club moss gametophyte populations. Several questions desperately need answering: (1) Are generations of sporophytes and gametophytes spatially separated, and therefore established in habitats representing different stages of succession? No experimental data have been published on the average distance between adult club moss clones and juvenile populations. We do not know whether gametophytes would be discovered by sampling the soil in transects starting within a club moss stand. The information about the vegetation in sites containing gametophyte and sporophyte populations is insufficient; therefore, we cannot adequately evaluate the possibilities of club moss habitat succession at different developmental stages or their use of ecological micro-niches. (2) Do club moss secondary compounds influence population structure and development? Biotic conditions created by fern sporophytes influence the development of sporophytes and gametophytes of the same or different fern species (Munther and Fairbrothers, 1980); thus, it is possible that juvenile club moss population structures are influenced by similar mechanisms. More than 500 known secondary metabolites from 46 species belonging to the genus *Lycopodium* have anti-inflammatory, anti-microbial, and anti-viral effects (Wang et al., 2021), yet their true function in natural habitats, for example, allelopathic activity, has not been determined. (3) Does the variation in gametophyte size reflect their age and developmental stage? Fern gametophytes can be male, female, male then





**FIGURE 3** Variety of subterranean gametophyte forms. (A) *Lycopodium annotinum* gametophytes and juvenile sporophytes (Fankhauser, 1873). (B) *Lycopodium* gametophyte in the soil sample, image by R. Rimgaile-Voicik. (C) *Lycopodium clavatum* gametophytes and juvenile sporophytes (Bruchmann, 1898). (D) *Lycopodium* gametophytes (Degener, 1924). (E) *Diphasiastrum* gametophytes and juvenile sporophytes (reprinted with permission from Eames, 1942)

female, female then male, hermaphroditic, or asexual (Atallah and Banks, 2015). The long-lived subterranean gametophytes of club mosses are thought to be hermaphroditic (Bruchmann, 1898; Schnittler et al., 2019), but it remains unclear whether and how secondary compounds could influence the maturation of antheridia or archegonia. It is also not clear how the maturation of the gametangia is related to the size and age of the gametophyte, and similarly, club moss spore viability has not been investigated. Spore germination is critical for gametophyte establishment, but most of our knowledge comes from the observation of club moss spore germination under laboratory conditions (Whittier et al., 2005). Variation in spore germination duration or repetitive germination events may also influence the size of gametophytes, as can microhabitat qualities (e.g., light, moisture, topographic, and edaphic factors), although the relative importance of

these factors has not been tested. (4) Is outcrossing a dominant type of reproduction in subterranean club moss gametophyte populations? Levin and Crepet (1973) proposed that even though hermaphroditic gametophytes may produce sporophytes through self-fertilization, it is unlikely that a single gametophyte would act as colonizer. Our research (Rimgaile-Voicik et al., 2015) showed that closely spaced *Lycopodium* gametophytes are more likely to produce viable sporophytes. No large-scale studies have explored the spatial structure of the club moss gametophyte populations.

Bearing in mind all the contemporary research tools that are now available to researchers, a major breakthrough in the research of club moss sexual reproduction must be near. With this review, we want to encourage researchers to choose club moss subterranean gametophytes as research subjects.

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## AUTHOR CONTRIBUTIONS

R.R.V. and J.R.N. planned and designed the research. Both authors wrote and edited the first draft of the manuscript. All authors approved the final version of the manuscript.

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