VILNIUS UNIVERSITY

Radvilė
RIMGAILĖ-VOICIK

ORGANIZATION AND FUNCTIONING PATTERNS OF *LYCOPODIUM* L. AND *DIPHASIASTRUM* Holub POPULATIONS WITH AN EMPHASIS ON GAMETOPHYTES AND JUVENILE SPOROPHYTES IN DRY PINE FORESTS

DOCTORAL DISSERTATION
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CONTENTS

ABBREVIATIONS

AFLP – Amplified Fragments Length Polymorphism

AMOVA – Analysis of Molecular Variance

bp – Base Pair

CTAB – hexadecyl-trimethyl-ammonium bromide, $CH_3(CH_2)_{15}N(CH_3)_3Br$, used in the preparation and purification of genomic DNA

DNA – Deoxyribonucleic Acid

GD – Genetic Distances

IPNI – International Plant Names Index

ISSR – Inter-Simple Sequence Repeat

NNI – The Nearest Neighbor Index

NMDS – Nonmetric multidimensional scaling

PCoA – Principal Coordinates Analysis

PCR – Polymerase Chain Reaction

Permutational MANOVA – Permutational Multivariate Analysis of Variance

PPG I – A Community-derived Classification for Extant Lycophytes and Ferns

RM-ANOVA – Repeated Measures Analysis of Variance

UPGMA – Unweighted-Pair Group Method with Arithmetic Means

INTRODUCTION

Archaic monophyletic Lycopodiaceae *s. lat*., comprise approximately 400 living species (Øllgaard and Windisch, 2014; Christenhusz and Byng, 2016). In various temperate forests, homosporous herbaceous lycophytes have an opportunistic, guerrilla-type growth strategy (Harper, 1985) and their evergreen perennial vascular sporophytes form large clones.

Sporophytes of club mosses originate from subterranean, achlorophyllous gametophytes (also called prothallia) that are associated with fungal endophytes (Read et al., 2000), while gametophytes occur from haploid (n) spores. Spores of club mosses only germinate in the dark (Whittier, 1977) and subterranean gametophytes of club mosses do not develop without a specific group of endophytic fungi (Rimington et al., 2014; Pressel et al., 2016). The development of gametophytes from spore germination to fertilization and new sporophyte formation can take five to six or more years (Bruchmann, 1910; Horn et al., 2013). Gametophyte and sporophyte generations are separated. Therefore, juvenile club moss populations are spatially isolated from old clones, have different abiotic and biotic habitat requirements, and emerge in sites with limited habitat disturbance (Bruchmann, 1898; Degener, 1924; Naujalis, 1995). The presence of fire adaptations is also expected (Eames, 1942; Oinonen, 1968).

Subterranean bisexual club moss gametophytes form in the humus horizon at a depth of one to nine centimetres (Bruchmann, 1898; Degener, 1924; Eames, 1942; Naujalis, 1995). The typical shape of mature gametophytes of *Lycopodium* species is an irregular bowl, and is a carrot-beetroot shape for the less common genus *Diphasiastrum* (Bruchmann, 1898; Thomas, 1975). Juvenile sporophytes have a prolonged period of matrotrophy and are dependent on the gametophyte for several years (Renzaglia and Whittier, 2013).

To our knowledge, gametophytes of modern lycophytes in the temperate climate zone were first discovered and described by Fankhauser (1873). Later, Bruchmann (1898) generalized Fankhauser's and other pteridologists' work to describe five structural types of gametophytes, all named by representing species. These types are still used to characterize club moss gametophytes (Bruce 1979a,b; Bruce and Beitel 1979; Naujalis, 1995; Renzaglia and Whittier, 2013). Compared with gametophytes, sporophytes were thought to be much more constant in character (Bower, 1894). The taxonomical value of gametophyte shapes was first acknowledged by Rothmaler (1944), but was later abandoned. Generic classification of the North American lycopsids (Wagner and Beitel, 1992) utilized gametophyte shape among other features. This classification was supported by plastid *rbcL* sequence data (Wikström and Kenrick, 1997, 2000) and implemented in other newest comprehensive classifications of lycophytes and ferns (Øllgaard, 2012; Øllgaard and Windisch, 2014; PPG I, 2016).

Recently, there has been growing interest in gametophyte anatomy and development in lycophytes (Renzaglia and Whittier, 2013; Whittier et al., 2005), but only a few primary investigations have been performed on club moss juvenile sporophytes and gametophytes in nature. Almost all information regarding modern club moss morphology is attributed to Bruchmann (1898) and Treub (1884). Bierhorst (1971) emphasised that embryogenesis is not well understood as research on the topic is limited and lacks better documentation and photographs. Descriptions are often supported with insufficient materials and no studies to support the conclusions.

THE MAIN OBJECTIVE

To study organization and functioning patterns of *Lycopodium* and *Diphasiastrum* populations with an emphasis on gametophytes and juvenile sporophytes emerging in dry pine forests of southern Lithuania.

OBJECTIVES:

1. To investigate spatial structure of club moss subterranean gametophyte and juvenile sporophyte populations.
2. To determine developmental stages and species composition of club moss gametophytes and evaluate subterranean gametophyte population composition.
3. To assess the specificity of habitats required by juvenile club moss populations.
4. To assess vegetation cover change in a community with juvenile club moss sporophytes.
5. To determine relationships of soil properties with juvenile sporophyte and subterranean gametophyte abundance.
6. To assess genetic structure of club moss populations.

SCIENTIFIC AND PRACTICAL SIGNIFICANCE

Our research revealed establishment of numerous and prosperous juvenile club moss populations in dry pine forests of southern Lithuania. Researchers have been unable to find local juvenile club moss populations and discover subterranean gametophytes. The data collected during our research allows for active participation in the scientific debate about club moss reproduction and population development.

Understanding how vegetation and soil characteristics determine the development of a juvenile club moss population is crucial for establishing protection for these archaic plants. Our results might significantly influence future

research on local factors that are crucial for juvenile club moss population establishment. The new knowledge gathered about club moss gametophytes should be used for future botanical studies and in botanical education at universities.

NOVELTY OF THE RESEARCH

This novel research presents the first spatial structure analysis of club moss subterranean gametophyte and juvenile sporophyte populations. Detailed juvenile club moss population characteristics are presented. The results obtained provide the possibility to propose the predominant fertilization pathway. The relationships among juvenile club moss populations, soil parameters and vegetation cover are addressed and primary data on population genetic structure are presented. Additionally, this research presents the first instance of *Diphasiastrum* gametophytes in Lithuania.

Research has never been devoted to the development and function of juvenile club moss populations. The life cycle of club mosses involves an alternation between two generations: asexual sporophyte and sexual gametophyte. However, few studies addressed the development of club moss gametophytes and its habitat specificity. Knowledge about diversity and density of different gametophyte species remains relatively poor as there is no precise methodology for locating gametophytes in a habitat. This research has addressed the above concerns, presenting detailed life cycle data, descriptions of gametophytes, and appropriate methodologies.

STATEMENTS BEING DEFENDED

1. *Lycopodium* gametophytes dominate in juvenile club moss populations of dry pine forests in southern Lithuania.
2. Asynchronous development and heterogenous composition are inherent to subterranean club moss gametophyte populations.
3. Subterranean gametophytes are not randomly distributed in the humus horizon.
4. Juvenile club moss populations in Lithuania are related to increased cover of *Deschampsia flexuosa* (L.) Trin.
5. Low genetic polymorphism is typical for *Lycopodium clavatum* and *L. annotinum* populations

APPROVAL OF THE RESEARCH

Four scientific articles on the dissertation topic have been published by the author. Two of these articles are in scientific journals indexed in the *ISI Web of Science* Database, one is indexed in the *ISI Master Journal List* and one is in a peer-reviewed conference publication. Research results have been presented in six national and international scientific conferences.

VOLUME AND STRUCTURE OF THE THESIS

This dissertation contains 109 pages with supplements. This dissertation consists of an introduction, literature review, research objects and methods, results, discussion, conclusions, references (182), list of the author's publications on the dissertation topic and other publications, supplements. The dissertation is illustrated with 19 figures and 16 tables.

1. *LYCOPODIUM* AND *DIPHASIASTRUM* GAMETOPHYTES AND JUVENILE SPOROPHYTES IN NATURE (review)

Lycophyta represent the oldest extant land plants, with the earliest fossil remains dating back to the Devonian Period (Wikström and Kenrick, 2001; Taylor et al., 2005). Data regarding the life history of Lycopodiaceae accumulates the slowest (Lang, 1899) and remains limited. Club moss nomenclature has been a matter of disagreement for decades. Homosporous lycophytes have traditionally been treated as the single monophyletic family, Lycopodiaceae *s. lat.*, or they were subdivided into two families (Rothmaler, 1944) or up to 16 genera (Holub, 1975, 1983, 1985, 1991; Øllgaard, 1987; Wagner and Beitel, 1992; Haines, 2003). Øllgaard (1987) proposed that the genus *Lycopodium* contains approximately 40 species divided into nine sections (several were previously treated as genera by Holub, 1975, 1983) based on characters of growth habit, leaf differentiation, presence and absence of peduncles, form of sporophylls, sporangium structure, epidermis cell walls, spore ornamentation, shape of gametophyte and chromosome number. The taxonomical value of gametophyte shape was first acknowledged by Rothmaler (1944); spore wall architecture was applied by Wilce (1972); and sporangium wall structure and branching patterns were addressed by Øllgaard (1975, 1979). Generic classification for the North American lycopsids, based on anatomy, chromosomes, spores and gametophytes (Wagner and Beitel, 1992) included three subfamilies: Huperzioideae (genera *Phlegmariurus* Holub and *Huperzia* Bernh.), Lycopodioideae (*Lycopodium s. str.* and *Diphasiastrum*) and Lycopodielloideae (*Pseudolycopodiella* (L.) Holub, *Lycopodiella* Holub and *Palhinhaea* Franco & Vasc.). This classification has been supported by plastid *rbcL* sequence data (Wikström and Kenrick, 1997, 2000) and was accepted by Øllgaard (2012) and Øllgaard and Windisch (2014).

Although homosporous plants could be defined as polyploids based on their absolute chromosome numbers, they appear to be diploids according to isozyme numbers (Haufler and Soltis, 1986). Soltis and Soltis (1987) hypothesized that homosporous plant lineages contain high chromosome numbers, and Haufler (1987) argued that homosporous lineages began with low chromosome numbers and current species arose through episodes of polyploidy, which were followed by genetic diploidization via gene silencing and loss by extinction of progenitor taxa with lower chromosome numbers.

The first findings of gametophytes (Fankhauser, 1873; Treub, 1884, 1887, 1888; Goebel, 1887) provided primary knowledge about their plastic forms. Compared with gametophytes, sporophytes were thought to be much more constant in character (Bower, 1894). Bruchmann (1898) generalized his and other pteridologists' work and described five structural types of gametophytes, all named by a representative species: Type I, *Lycopodium clavatum*; Type II, *Diphasiastrum complanatum* (*L. complanatum*); Type III, *Huperzia selago* (*L. selago*); Type IV, *Lycopodiella inundata* (*L. inundatum*); Type V, *Phlegmariurus phlegmaria* (*L. phlegmaria*). Bruchmann's classification is widely used to characterize gametophytes (Bruce, 1976; Bruce, 1979; Bruce and Beitel, 1979; Whittier, 2003; Whittier et. al., 2005; Renzaglia and Whittier, 2013; Rimgailė-Voicik et al., 2015). The most common gametophyte type in the temperate zone genus *Lycopodium* are subterranean, holomycotrophic (Øllgaard, 1990) fleshy, disk to irregular bowl shape (Type I, according to Bruchmann). Genus *Diphasiastrum* (Type II) gametophytes of carrot, beetroot or teardrop shape are much rarer. Typically, gametophytic meristems tend to be terminal or confined to a central position, but in *Lycopodium* and *Diphasiastrum*, meristems form a subterminal ring that determines the shape of the developing gametophyte (Wagner and Beitel, 1992). Considerable size variation and developmental stages

of gametophyte populations have been discovered (Bruchmann, 1898; Degener, 1924; Eames, 1942; Bruce, 1979b; Naujalis, 1995; Rimgailė-Voicik et al., 2015). The genetic history of homosporous lineages remains undefined: episodes of polyploidy and diploidization via gene silencing is expected as homosporous plants appear to be diploids according to isozyme numbers (Haufler and Soltis, 1986; Haufler, 1987). The knowledge of ecology and population function of lycopods is limited and many fundamental questions remain unanswered.

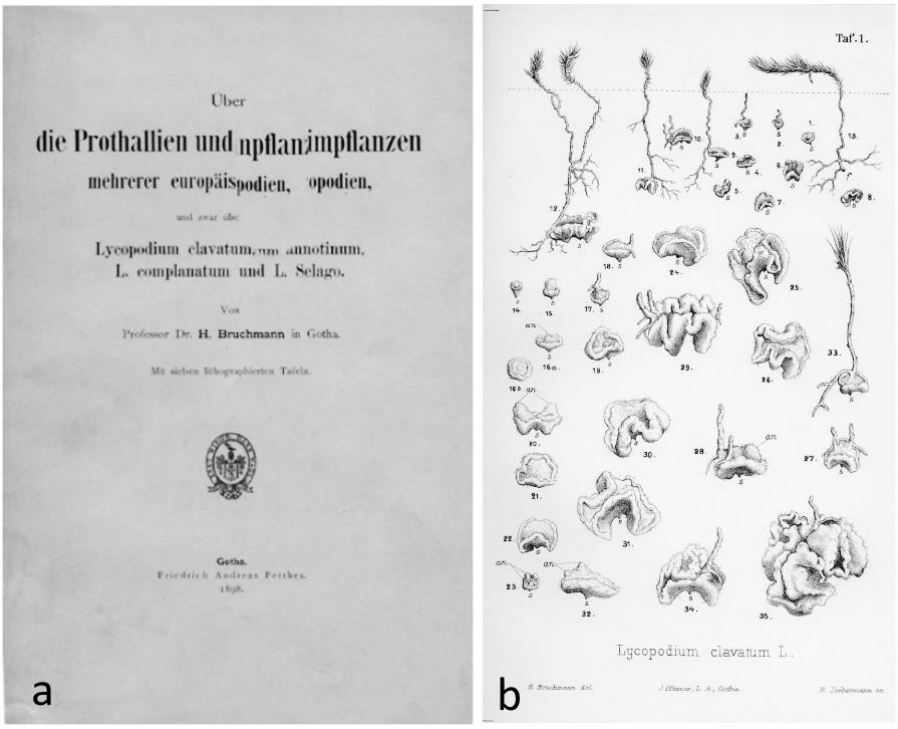
1.1. First findings of subterranean long-lived gametophytes

In 1873 Fankhauser found and described gametophytes and juvenile sporophytes of *Lycopodium annotinum* in Switzerland. During a botanical expedition on a slope of a dark forest, he accidentally noticed 13 juvenile sporophytes growing among mosses. Fankhauser dug out the young club mosses, and upon inspection made several notable observations: 1) juvenile sporophytes developed unevenly; their length varied from three to 18 cm; 2) several juvenile sporophytes had a pin shaped tubercle close to the foot; 3) few juvenile sporophytes had remains of obscure bodies; and 4) one juvenile sporophyte had a perfectly shaped brownish body near the foot. After digging more soil alongside the first field site, Fankhauser found a few irregular shape fleshy yellowish-white bodies with sparse rhizoids. He determined all juvenile sporophytes as *L. annotinum* and, according to recommendations of De Bary (1858), tried to, unsuccessfully, germinate spores in the soil from the same forest.

The most data on gametophytes of *L. clavatum*, *L. annotinum* and *D. complanatum* (or *D. tristachyum*, as argued Wilce, 1972) were accumulated by Bruchmann (1898, 1909, 1910) who investigated club mosses in mountain forests of Thuringia, Germany over a period of thirty years. The first time Bruchmann found gametophytes of *L. annotinum* with juvenile sporophytes was in 1884.

Following this finding, he searched for juvenile sporophytes for a few years with the hope that he would find subterranean gametophytes. For some time, Bruchmann was unable to find sporophytes young enough to have healthy gametophytes. In 1892, he found many gametophytes of *L. annotinum* and *L. clavatum* along roadsides or on forest roads – in places where growth of gametophytes seemed to be impossible. Most juvenile sporophytes and gametophytes of *L. annotinum*, *L. clavatum* and *D. complanatum* were found in a young forest, planted after fir logging. Juvenile sporophytes very often occurred in former cleared forests with young trees of eight to 14 years old. Bruchmann collected soil samples of 1 dm^3 in size and disassembled them with tweezers. In one soil sample, he found up to ten or more subterranean gametophytes and juvenile sporophytes. All gametophytes occurred in the humus horizon, at a depth of 0.5 to 1 cm. Overtime, Bruchmann formed a collection of about 500 subterranean gametophytes and analyzed this collection in various aspects (Fig. 1; Bruchmann, 1898). He provided a series of gametophytes and juvenile sporophytes for Prof. Weiss at Manchester Museum, on which Wigglesworth (1907) wrote a detailed anatomical study of the juvenile sporophytes.

In 1899, Lang published an illustrated, detailed study on the internal anatomy and morphology of *L. clavatum* gametophytes and juvenile sporophytes. He unsuccessfully searched for gametophytes in soil samples in Clova, Scotland, where all British species with the exception of *Lycopodiella inundatum* were present. Later, juvenile plants of *L. clavatum* were found among a patch of *Racomitrium lanuginosum* (Hedw.) Brid. on a rock shaded by a few trees in Glen Doll, Scotland. Soil near these plants was carefully examined and gametophytes, some bearing juvenile sporophytes of various ages, were found.



1. Fig. 1. a – Cover of the most comprehensive work on subterranean club moss gametophytes: Bruchmann, H. 1898. Über die Prothallien und die Keimpflanzen mehrerer europäischer Lycopodien, und zwar über die von *Lycopodium clavatum*, *L. annotinum*, *L. complanatum* und *L. selago*. Perthes, Gotha. b – Taf. 1. *Lycopodium clavatum*, det. H. Bruchmann; J. Pfitzner, L. A., Gotha. E. Liebermann sc.

From 1915 and 1920, Holloway published four articles on *Lycopodium* found in New Zealand. In these articles, Holloway analyzed work from previously published articles, providing valuable information about *Lycopodium* (Holloway, 1916a), methods of vegetative propagation (Holloway, 1916b), species plasticity (Holloway, 1919) and structure of gametophytes (Holloway, 1920). Data on the habitat of subterranean *L. fastigiatum* R. Br., *L. volubile* G. Forst. and *L. scariosum* G. Forst. gametophytes and juvenile sporophytes (found in large numbers) and *L. deuterodensum* Herter juvenile sporophytes in New Zealand were also presented. In his works, Holloway emphasized that juvenile sporophytes and gametophytes cannot be found in localities where adult plants are abundant, while

roadside cuttings, damp shaded clay banks or other patches of disturbed soil were favorable habitats for gametophytes. In 1915, Holloway's student, Edgerley (1915), published a detailed study analyzing Holloway's material and reported on *L. volubile* gametophytes and juvenile sporophytes found on the side of a bank.

Spessard (1917) was the first researcher to identify gametophytes in the USA (Table 1). The discovery of gametophytes and juvenile sporophytes in Marquette, Michigan was almost an accident; juvenile sporophytes were present, but no adult plants of *L. clavatum*, *D. complanatum* or *L. annotinum* were growing nearby. All gametophytes were found in an open, exposed, sandy place beside path and the humus layer was very thin. Also, *L. annotinum* sporelings and three gametophytes were documented "in the middle of a trail which is frequently used by hunters". In total, Spessard (1917) found 21 gametophytes and over 50 juvenile sporophytes of five species (Table 1). In the vicinity, species in the genera *Morchella* Dill. ex Pers., *Polytrichum* Hedw., *Acer* L., *Populus* L., *Pteris* L., *Gautheria* Kalm ex L., *Rhus* L. and *Polygala* L. were present. Later, 30 gametophytes and juvenile sporophytes of *L. obscurum* var. *dendroideum* (Michx.) D. C. Eaton were found in Mid–Island Point, near Lake Superior, Michigan (Spessard, 1922). Spesard (1917) noted that juvenile sporophytes and gametophytes often were found on small, bare, exposed elevations that are more receptive for spores.

Stokey and Starr (1924) and Degener (1924) described eleven locations with *D. complanatum*, *L. obscurum* L. and *L. clavatum* juvenile sporophytes and gametophytes in Western Massachusetts. The authors agreed that the chief difficulty was to find the first juvenile sporophyte and it happened more or less by accident although they had been casually looking for more than ten years. According to Stokey and Starr (1924), all localities described as "poor collecting grounds" fall into three main types: 1) groves of a 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 one station a considerable deposit of dry leaves was present. In all localities, 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, mostly north facing slopes and no consistent grouping pattern was observed. Little herbaceous growth, white pines among second growth timber (of the same age as the sporelings) were good indicators of juvenile club mosses. Juvenile sporophytes were only discovered within three meters from old plants in only two cases; in most cases no adult plants were present nearby, as other researchers noted before (Fankhauser, 1873; Bruchmann, 1898; Lang, 1899; Spessard, 1922).

Table 1. Published reports on *Lycopodium* and *Diphasiastrum* subterranean gametophytes found

Habitat	Location	Depth (cm)	Reference
In deep canyon slope, covered with damp and dark forest	Tobel, Langnau am Albis, Kanton Zürich, Switzerland	–	Fankhauser, 1873
On fir forest slopes and roads	Thuringia, Germany	0.5–10	Bruchmann, 1898
On a rock shaded by few trees, among a patch of *Racomitrium lanuginosum*	Clova, Glen Doll, Scotland, United Kingdom (UK)	–	Lang, 1899
On the side of a bank	New Zealand	0.5–1.3	Edgerley, 1915
By a path, in open, exposed, sandy places; in the middle of a trail	Marquette and Munising, Michigan (MI), United States of America (USA)	1–7	Spessard, 1917, 1918, 1922
On a damp shaded clay bank; roadside cutting; disturbed soil	New Zealand	4–6	Holloway, 1920
In an alder clump between the lake and the marsh to the east	Mid–Island Point, MI, USA	1–2	Spessard, 1922
–	Rhinelander and Pembine, Wisconsin (WI), USA	–	Spessard, 1922
On a NE facing slope, mixed hardwoods with a few pines	South Hadley, Massachusetts (MA), USA	0.5–3.5	Stokey and Starr, 1924
On an island, NW facing slopes, deciduous forest	Forge Pond, Granby, MA, USA	2–5	Stokey and Starr, 1924
On a W facing slope, mixed hardwoods	Mount Toby, Sunderland, MA, USA	2–6	Stokey and Starr, 1924
On a N facing slope, dense grove of young hemlocks	Aldrich Mills, Granby, MA, USA	–	Stokey and Starr, 1924

(continued on next page)

Habitat	Location	Depth (cm)	Reference
In a well defined depression, open deciduous grove	Dark Woods, Granby, MA, USA	0.7–1.5	Stokey and Starr, 1924
Young mixed deciduous growth	Smith Ferry Woods, South Hadley, MA, USA	1–3, 2.5–4	Stokey and Starr, 1924
On a bank of a lake with few deciduous trees	Upper Lake Woods, South Hadley, MA, USA	1–3	Stokey and Starr, 1924
Shelter of a boulder, among the stumps and old laurel bushes.	East Rock Mountain, Great Barrington, MA, USA	0.1–3	Degener, 1924
Hemlock grove	Orient Springs, MA, USA	–	Degener, 1924
Steep slope covered with large hemlocks	Amherst, MA, USA	2.5–7 9 1–5	Degener, 1924
Open growth–the first of white pines, and the second of oaks	Wheaton College, Norton, Massachussets	–	Ames, 1926
Towards the top of a rocky extension with a fine layer of humus, in a very slight depression, under forming mixed forest grove	Lac–des–Sables, Quebec, Canada	–	Gauthier and Dumais, 1938
Light wood forest	Quebec zoo, Charlesbourg, Quebec, Canada	–	Gauthier and Dumais, 1938
Herbarium samples	Ithaca, Dryden and Junius, New York (NY), USA	–	Gauthier and Dumais, 1938
Young upland deciduous forest; White pine forest; Open gravelly knolls; abandoned fields with *Hamamelis* and *Rhus*; low red–maple forest near peat bogs	Cayuga Lake Basin, NY, USA	3–10	Eames, 1942
–	North Wales, UK	–	Thomas, 1975
Towards the top of a rocky extension with a fine layer of humus, in a very slight depression, under forming mixed forest grove	Lac–des–Sables, Quebec, Canada	–	Gauthier and Dumais, 1938
Towards the top of a rocky extension with a fine layer of humus, in a very slight depression, under forming mixed forest grove	Lac–des–Sables, Quebec, Canada	–	Gauthier and Dumais, 1938
Light wood forest	Quebec zoo, Charlesbourg, Quebec, Canada	–	Gauthier and Dumais, 1938
Herbarium samples	Ithaca, Dryden and Junius, New York (NY), USA	–	Gauthier and Dumais, 1938
Young upland deciduous forest; White pine forest; Open gravelly knolls; abandoned fields	Cayuga Lake Basin, NY, USA	3–10	Eames, 1942
Jack Pine plantation	Michigan, USA	–	Bruce and Beitel, 1979

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Habitat	Location	Depth (cm)	Reference
Gravel pit	Viborg, Central Jutland, Denmark	–	Øllgaard, 1985
Slope of a forest road, a spruce forest	Black Forest, Baiersbronn, Germany	2–3	Schmid and Oberwinkler, 1993
Pine forests, dry alder forests, in little deppresions or on humps	Varėna Distr., Švenčionys Distr., Kretuonas Lake; Kupiškis Distr., Kepurinė village, Lithuania		Naujalis, 1995
Northen slope of the summit, mountain heathland	Mountain Grosser Arber; Bavaria, Germany;	1–2	Horn et al., 2013
Southern bank of a drinking water reservoir	Kubova Hut', Jihoceský Kraj, Czech Republic,		
–	Storrs, Connecticut, USA	–	Renzaglia and Whittier, 2013
Dry pine forest	Varėna District, Lithuania	0.2–1.8	Rimgailė-Voicik and Naujalis, 2015

Degener (1924) accidentally found juvenile sporophytes and gametophytes at East Rock Mountain, Great Barrington, Massachusetts. A slope had been stripped of timber several years before and Rosaceae Juss. species had grown in their place. Young sporophytes were growing in the shelter of a boulder. Seven juvenile sporophytes of *D. complanatum*, four with carrot shape gametophytes attached, were found. As previously noted by Spessard (1922), not all gametophytes were oriented vertically. Near Amherst, MA, Degener (1924) also found a few *L. obscurum* var. *dendroideum* juvenile sporophytes incipient with gametophytes. Some were growing from inverted gametophytes on a steep slope, covered with hemlocks. One sporophyte was found entirely surrounded by a *Tremellodendron* G. F. Atk. fungus. In the same locality over a few afternoons in an area of less than one meter in diameter, 200 to 300 specimens of *L. annotinum* and *L. clavatum* were collected. Degener emphasized that the number of sporophytes living in an area can be up to thousands.

Ames (1926) published a short note about finding *L. obscurum* var. *dendroideum* gametophytes and juvenile sporophytes in two places: open growth

of white pines and open growth of oaks near Wheaton College, Norton, Massachusetts. Gauthier and Dumais (1938) published an article about *L. obscurum* and *L. clavatum* gametophytes found in Quebec County, Canada and presented information about herbarium samples of *L. obscurum* and *D. digitatum* (Dillenius ex A. Braun) Holub gametophyte samples that were collected in New York, USA by various researchers. Gametophytes with juvenile sporophytes were detected in small groups in open slight depressions (Gauthier and Dumais, 1938). Eames (1942) published data on a successful search for *L. obscurum*, *L. clavatum*, *D. complanatum* and *L. annotinum* gametophytes and juvenile sporophytes at Cayuga Lake Basin in west-central New York. All listed species occurred in young upland deciduous forests, open gravelly knolls and abandoned fields with *Hamamelis* Gronov. ex L. and *Rhus* species.

To our knowledge, publications on subterranean long-lived club moss gametophytes found in nature stopped for more than thirty years (Table 2; Thomas, 1975; Bruce and Beitel, 1979; Schmid and Oberwinkler, 1993; Naujalis, 1995).

Table 2. Data on *Lycopodium* and *Diphasiastrum* gametophytes and juvenile sporophytes found

Species	Juvenile sporophytes	Gametophytes	References
L. annotinum	+	+	Fankhauser, 1873
L. annotinum; L. clavatum	+	+	Bruchmann, 1898
L. clavatum	+	+	Lang, 1899
L. volubile	+	+	Edgerley, 1915
L. fastigiatum, L. volubile, L. scariosum	+	+	Holloway, 1916a
L. deuterodensum	+	–	Holloway, 1916a
L. clavatum; D. complanatum, L. annotinum	+	+	Spessard, 1917, 1922
L. obscurum var. dendroideum(?)	+	+	Spessard, 1918, 1922
D. complanatum, L. obscurum, L. clavatum	+	+	Stokey and Starr, 1924
D. complanatum, L. annotinum, L. clavatum, L. cernuum	+	+	Degener, 1924

(continued on next page)

Species	Juvenile sporophytes	Gametophytes	References
L. obscurum var. dendroideum	+	+	Ames, 1926
L. clavatum	+		Saint–Marc–sur–Richelieu, 1932
L. obscurum, L. fastigiatum, Diphasiastrum digitatum		+	Gauthier and Dumais, 1938
L. clavatum, L. obscurum	+	+	Gauthier and Dumais, 1938
L. obscurum, L. annotinum, D. complanatum	+	+	Eames, 1942
D. alpinum		+	Thomas, 1975
L. clavatum	+		Thomas, 1975
L. annotinum, L. clavatum, L. digitatum, L. lucidulum, L. obscurum, L. dendroideum (?)	+	+	Bruce and Beitel, 1979
L. clavatum, L. alpinum	+		Øllgaard, 1985
L. clavatum		+	Øllgaard, 1985
L. clavatum	+	+	Schmid and Oberwinkler, 1993
L. clavatum, L. annotinum	+	+	Naujalis, 1995
D. alpinum	+	+	Horn et al., 2013
L. obscurum		+	Renzaglia and Whittier, 2013
L. clavatum, L. annotinum, Diphasiastrum complanatum	+	+	Rimgailė-Voicik et al., 2015
Diphasiastrum complanatum	+	+	Rimgailė-Voicik and Naujalis, 2015
L. clavatum, L. annotinum	+	+	Rimgailė-Voicik and Naujalis, 2016

1.2. Habitats of club moss juvenile sporophytes and gametophytes

In all forest types the most common feature of juvenile sporophyte and subterranean gametophyte populations is connected with small scale forest floor disturbances, which occur for various reasons (Burchmann, 1898; Naujalis, 1995). Quite often, subterranean gametophytes were found in areas where surface forest fires occurred 15 to 25 years earlier (Bruchmann, 1898; Eames, 1942; Oinonen, 1968; Naujalis, 1995). Eames (1942) noted that a prosperous juvenile club moss population was discovered in a forest with trees and shrubs of five to 25 years old, grown after a forest fire following an earlier timber cutting. It is possible that microbiological and biochemical processes initiated by fire together with

mechanical disturbances are responsible for the occurrence of juvenile club moss populations. After a forest fire, closure of the community decreases drastically and spores can easily enter the soil and reach their necessary depth. A key bioactive compound in smoke, known as karrikinolide or KAR1 (Flematti et al., 2004), is known to promote germination in seed plants and may be responsible for triggering spore germination as well. Yet no data has been presented about the influence of karrikins on moss spore germination (Waters et al. 2011), but KAI2 orthologs are present in basal land plants: D14 was detected in *Selaginella moellendorffii* (Waters et al 2015).

Forest fire sites are not the only localities with subterranean gametophytes. Subterranean gametophytes and juvenile sporophytes were found on and near forest roads, tracks and near lines separating forest blocks (Bruchmann, 1898; Degener, 1924; Stokey and Starr, 1924; Oinonen, 1968; Soltis and Soltis, 1988; Naujalis, 1995; Muller et al., 2003). In a 242 ha territory in southern Lithuania, club moss clones were more often discovered near forest roads, tracks and edges of forest blocks or in abandoned fields and near electricity lines (Naujalis, 1995).

Theoretically, gametophytes and juvenile sporophytes could emerge in any forest site. Still, even being part of the same community, subterranean gametophytes and juvenile sporophytes are completely separated from adult clones. Stokey and Starr (1924) and Degener (1924) provided numerous examples on spatial links among juvenile sporophytes, gametophytes and adult clones in Western Massachusetts. In a northeastern facing slope covered with young growth of mixed hardwoods (*Tilia americana* L., *Carpinus caroliniana* Walter and *Fraxinus americana* L.), about 100 juvenile sporophytes of *D. complanatum* were found, and the nearest sporulating *D. complanatum* was about 25 m away. In an island with mixed growth of hardwoods (*Betula populifolia* Marsh., *Acer rubrum* L. and *Viburnum dentatum* L.), several patches of *L. obscurum* sporelings with

numerous gametophytes and *D. complanatum* gametophytes were found 2–6 m away from sporulating *L. obscurum* and non–sporulating *L. clavatum*. On the mainland, up to 250 m away, abundant growth of sporulating *L. obscurum* and *D. complanatum* were present. Few *L. obscurum* sporelings were found in a defined depression 0.4–1 m below the surrounding level in a rather open grove with *A. rubrum* and *B. populifolia*. In Canada, gametophytes of *L. obscurum* were found in a light wood forest of *Acer sacharrum* Marshall and *Abies balsamea* (L.) Mill. in grass layer where *Pyrola* L. and *Vaccinium* L. species were present (Gauthier and Dumais, 1938).

In a stable forest community in Lithuania, club moss populations of different developmental stages were found in areas with forest canopy openings (Naujalis, 1995; Rimgailė-Voicik et al., 2015). In *Fagus sylvatica* L. dominated forests in Germany, research on *L. annotinum* clones showed (Wittig et al., 2007) that in sites without disturbance, juvenile populations do not emerge. Former forest logging areas that have been replanted are favourable for juvenile club moss populations. For example, massive amounts of three species of juvenile club mosses were found in a *Picea abies* (L.) H. Karst. plantation that was eight to 14 years old in Germany (Bruchmann, 1898). In Michigan, USA, in a *Pinus banksiana* Lamb. plantation, a juvenile populations of up to six club moss species were discovered (Bruce and Beitel, 1979).

Club moss spores germinate and gametophytes form in small, localized plots (Fankhauser, 1873; Bruchmann, 1898; Lang, 1899; Spessard, 1922; Degener, 1924; Bruce and Beitel, 1979; Naujalis, 1995). Microrelief might have an impact on club moss spore germination. In wet alder and birch forests, *L. annotinum* gametophytes were growing on mounds and in slight depressions in dry pine forests (Naujalis, 1995). Juvenile club moss sporophytes appeared in forest

planting beds as such areas could lead to a more favorable moisture regime and large amount of spores may have been washed in.

1.3. Diagnosing subterranean gametophyte populations

Subterranean achlorophyllous holomycotrophic gametophytes develop in the soil after spore germination and are rarely found, but in suitable habitats they usually form numerous prospering populations with individuals from one up to six species (Fankhauser, 1873; Bruchmann, 1898; Lang, 1899; Spessard, 1922; Stokey and Starr, 1924). Eames (1942) stated that staff members at Cornell University had been searching and collecting club moss gametophytes for 15 years with marked success. Yet today, more than seventy years later, no specific methodology or habitat patterns for localization of club moss gametophyte populations are available.

Juvenile sporophytes are important indicators of subterranean club moss gametophytes. Young sporophytes, occurring among mosses and lichens can be easily noticed by experienced pteridologists or can be found accidentally, for example, while picking mushrooms. Juvenile sporophytes at early developmental stages may look similar to *Polytrichum* mosses as they have isophyllous leaves and only orthotropic growth, and may be incipient with gametophytes (Bruchmann, 1898; Spessard, 1922; Eames, 1942; Wagner and Beitel, 1992; Naujalis, 1995). All authors agree that the highest chance to find gametophytes is by gradually disassembling soil with tweezers near juvenile sporophytes. Sieving or washing soil samples is an unsatisfactory method as gametophytes become damaged (Spessard, 1922; Degener, 1924; Naujalis, 1995) and data for spatial analyses are lost.

1.4. Spores and gametophytes of club mosses

According to the time required for spore germination in nature, Lycopodiaceae can be divided into two groups (Bierhorst, 1971): spores of holomycothropic gametophytes that germinate slowly, and those that photosynthesize and germinate quickly (for example, *Lycopodiella* Holub; Ollgaard, 1987, 1989). Bruchmann (1910) found that *L. clavatum* and *L. annotinum* spores needed from six to seven years to germinate while *Huperzia selago* needed from three to five years. *Lycopodium inundatum* L. (De Bary, 1858), *L. cernuum* L. and *L. salakense* Treub (Treub, 1884; 1888) spores were able to germinate within a week. Spores of *L. obscurum* (Whittier, 1977) and *D. digitatum* (Whittier, 1981) required one year to germinate in culture, and *L. clavatum* spores germinated more efficiently when they were kept in the dark for three months and treated with cold for the same period of time (Whittier, 1998).

Species composition of juvenile club moss populations depends on the composition of colonizing spores. Even though wind can carry spores from many different regions in a given forest, the largest portion of spores in the soil should be from the native populations of club mosses. Among *Diphasiastrum* species, the production of unreduced viable diplospores is known (Wagner et al., 1986; Øllgaard and Tind, 1993; Bennert et al., 2011). The production of unreduced spores during meiosis may cause the transformation of sterile hybrids to fertile polyploids (Harlan and deWet, 1975).

Lycopodium clavatum is widely used as a medical herb. Clones must be carefuly exploited with special protection during forestry works (Budriūnienė, 1972). Unfavourable effects on stands were noted in Poland (Piękoś-Mirkowa, and Mirek, 2003) and the USA (Nauertz and Zasada, 1999). In Lithuania, harvesting of club moss herb is restricted. In the Varėna District, Lithuania, medical herb shops purchase approximately 200−220 kg of club moss spores

every season from local villagers (Rimgailė-Voicik et al., 2015). From 1961–1971 a total of 49.1 tons of spores with strobili (average of 4.9 t/year) were collected in Lithuania (Budriūnienė, 1972).

Usually in Lithuania, club moss sporulation begins in August and continues until October (Minkevičius, 1959; Naujalis, 1995), but was noted in early December as well (unpublished data). In Germany and Poland, winter sporulation of *L. annotinum* from February till March has also been recorded (Sonnberger et al., 2008). The variation of sporulation intensity of *L. clavatum* was noted in Lithuania; during a twelve-year cycle, nine years had different levels of fertility and three years were infertile (Šimkūnaitė, 1969).

Club moss sporophytes produce trilete reticulate spores ranging from 20 to 40 μm in diameter (Paw, 1983; Li et al., 2000) that germinate in the dark (Whittier, 2008). Previously shown in unrelated families (*L. clavatum* and *Ophioglossum crotalophoroides* Walter; Whittier, 2006; 2008), the active form of phytochrome (Pfr) inhibits spore germination in seedless vascular plants with subterranean mycorrhizal gametophytes. Studies performed in northern Sweden (Callaghan et al., 1986a) showed that during the vegetation growing season, 1.88×10^6 club moss spores colonize an area of 100 cm^2. Spores can travel long distances; for example, in India (Pandey, 1985) spores of common European club mosses were discovered at heights of 2000 to 3200 m above sea level, but the largest amount of spores concentrate close to the clones and form spore banks.

Strobili comprise about 5% of total club moss biomass and their viability might be about 4% (Callaghan et al., 1986b), thus the number of spores produced every year is enormous. Plotnikov (1977) in South Ural, Russia discovered that one strobil of *L. annotinum* produces about 0.4×10^6 spores. Research on pteridophyte spore banks is common (Milberg, 1991; Ramirez-Trejo et al., 2004), but data on

club moss spore viability is lacking and it remains unknown if spore viability is the main limiting factor of subterranean gametophyte emergence.

It is possible that spores can be moved into deeper layers of the soil while adhered to ants or microarthropods. Invertebrates can spread huge numbers of spores; in 1 m^2 of pine forest soil, more than 110 individuals belonging to 54 species were found (Eitminavičiūtė, 2001).

Orthotropic sporulating shoots of *Lycopodium* and *Diphasiastrum* grow 20 to 30 cm above the moss layer. As noted by Bruchmann (1898), spores should stay viable for at least three to eight years, but slow and localized germination of spores might be due to specific obligative relationships with soil mycota. Fungal hyphae enter gametophyte cells during primary developmental stages and infected cells form separate layers in the tissues, probably ensuring normal development (Bruchmann, 1898; Horn et al., 2013).

1.5. Club moss gametophyte and juvenile sporophyte mortality

No comprehensive data on mortality rates of gametophytes and juvenile sporophytes has been published. Death of juvenile sporophytes may be caused by phytogenic, zoogenic, mycogenic and abiotic factors. Tree canopy overshading (Bruchmann, 1898) or competition for light, water and nutrients with other plants might be possible phytogenic factors. For example, it was noted (Naujalis, 1995) that after two to three years juvenile *L. annotinum* was completely overgrown by *D. complanatum* branches. A more common potential phytogenic factor causing death was due to moss growth. In the Vosges Mountains, France, *D. tristachyum* juvenile plants become dominated or overgrown by *Calluna vulgaris* L. (Muller et al., 2003). As noted Bruchmann (1898), massive death of juvenile *L. annotinum*, *L. clavatum* and *D. complanatum* sporophytes in fir plantations occured if the number of juvenile sporophytes was high and overshading caused by thick thorn

cover on the forest floor increased. 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%) *L. annotinum* juvenile sporophytes died (Naujalis, 1995). During one year, anywhere from one to seven *L. annotinum* sporophytes died. Loss was not as massive as among pteridophyte sporophytes (Hamilton and Lloyd, 1991). This can be partly explained by the long maintained connection with the gametophyte (Bruchman, 1898, Horn et al., 2013; Renzaglia and Whittier, 2013).

No data on zoogenic reasons for juvenile sporophyte mortality has been previously presented. Presumably, ants do have an impact on juvenile sporophyte and gametophyte population formation, but direct impact has not been researched.

Mycogenic factors of the mortality of juvenile club moss sporophytes might be connected with ineffective mycorrhizal or other relationships with endophytic fungi. The association of subterranean or partly subterranean gametophytes with fungi has been known since the late 1800s within Lycopodiaceae (Treub, 1884; Bruchmann, 1898; Goebel, 1887; Lang, 1899; Holloway, 1920, Spessard, 1922; Bruce, 1979; Schmid and Oberwinkler, 1993). Spessard (1922) suggested that various gametophytes may be associated with different fungi, Stokey and Starr (1924) agreed, but also noted that the low rate of successful development may be connected to the length of infancy. Knowledge on the ultrastructure of such mycorrhiza–like associations is remarkably low. Schmid and Oberwinkler (1993) found that aseptate fungus occupies a distinct zone in the lower gametophyte region and forms very regular intracellular coils, while the central tissue and sex organs remain uninfected. Thus, Schmid and Oberwinkler (1993) proposed the term ‘Lycopodioid mycothallus interaction’ to describe relationships between fungi and *L. clavatum* gametophytes. Most 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 of both groups were found in Devonian plants (Strullu-Derrien et al., 2014).

No direct transfer of mycorrhizal infection through a gametophyte-sporophyte junction was detected in *L. clavatum* (Lang, 1899), *L. cernuum* (Duckett and Ligrone, 1992) or *H. hypogaea* B. Øllg. (Winther and Friedman, 2007a). Infection *de novo* gives a possibility that symbionts in sporophytes differ from those in gametophytes primarily because of physiological differences (Leake et al., 2008). The alternating generations of *Botrychium* Sw. and *Huperzia* Bernh. have been shown to share five to nine phylotypes of Glomus A group AM fungi in sporophytes (Winther and Friedman, 2007a,b; Kovács et al., 2007). *Botrychium* and *Huperzia* gametophytes seem to have high fungal specificity (Bidartondo et al., 2002). Later, Winther and Friedman (2007a) confirmed that Glomerian fungi are symbionts in *L. clavatum* and *Huperzia* spp. Horn et al. (2013) hypothesized that *D. alpinum* mycoheterotrophic gametophytes participate in the mycorrhizal symbiosis and obtain nutrients from *Calluna vulgaris* via the fungus. Arbuscular mycorrhizal (AM) mycelial networks could connect between generations and allow autotrophic sporophytes (Winther and Friedman, 2007a, b) or other species through epiparasitism to supply carbohydrates to heterotrophic gametophytes, but no experimental evidence is present (Leake et al., 2008).

Abiotic factors may cause juvenile sporophyte mortality as well. The death of four *L. clavatum* juvenile sporophytes near a high-voltage power line caused by bare soil overheating was observed (Naujalis, 1995). Holloway (1916a) indicated that in New Zealand during dry summers, juvenile *L. fastigiatum* sporophytes and gametophytes perished. According to Stokey and Starr (1924), juvenile sporophytes would be more likely to occur after 10–12 years without a drought,

and many gametophytes of *L. clavatum* and *L. obscurum* were killed by a drought in 1930 and 1940 (Eames, 1942).

As noted by Haufler (2002), there also appear to be genetic and chemical mechanisms to promote outcrossing in homosporous species, because gametophyte self-fertilization would lead to total homozygosity and expression of all recessive genes. Gametophytes that carry a single recessive lethal gene could not form sporophytes. Several archegonia may get fertilized simultaneously establishing competition among developing embryos (Klekowski, 1982), and archegonia may form before antheridia (Lloyd, 1974).

1.6. Diversity of *Diphasiastrum* and *Lycopodium* subterranean gametophytes

When club moss spores are dispersed by wind and enter the forest floor litter layer, subterranean gametophytes form in the humus horizon at a depth of 1–9 cm (Bruchmann, 1898; Spessard, 1922; Eames, 1942; Naujalis, 1995; Horn et al., 2013). The gametophyte of *Lycopodium* species is a white, grey or grey-brown irregular bowl; the gametophyte of *Diphasiastrum* species is a orange–brown carrot–beetroot shape (Bruchmann, 1898; Bruce, 1979; Renzaglia and Whittier, 2013). Bisexual gametophytes develop slowly and reach maturity after five to ten years (Bruchmann, 1910; Horn et al., 2013). Developmental stages of gametophytes found vary because of repeated spore germination and different gametophyte growth rate (Eames, 1942). Juvenile sporophytes have a prolonged period of matrotrophy and are dependent for several years on associated gametophytes (Renzaglia and Whittier, 2013). Mycoheterotrophy in gametophytes of non-vascular plants allows minimal maternal investment (Leake et al., 2008).

Subterranean club moss gametophytes develop in the soil humus horizon among disintegrating mosses, bark parts or pine cones (Naujalis, 1995) usually up

to 2.8 cm in depth, the deepest in 9–10 cm (Table 1). Sometimes gametophytes appeared primarily in a line, marked with charcoal from a forest fire and between the humus and the soil below (Eames, 1942). Gametophyte populations detected in forests were formed from one (Thomas, 1975, Naujalis, 1995, Horn et al., 2013) up to six species (Bruce and Beitel, 1979). Bruce and Beitel (1979) found 476 subterranean gametophytes belonging to up to six species in a *P. banksiana* plantation in Michigan, USA. The prothallia of five club moss species have been found within a few centimeters of each other (Spessard, 1922) and juvenile sporophytes of *L. clavatum* and *L. obscurum* were growing within 2 cm of each other (Spessard, 1917). No generalization about juvenile sporophyte groupings have been made as many isolated (Bruchmann, 1898; Stokey and Starr, 1924; Horn et al., 2013) or grouped individuals were discovered (Stokey and Starr, 1924; Eames, 1942). Usually, groups of two to five gametophytes at different stages of development were found. It has been proposed that these groups form gradually (Eames, 1942; Bruce and Beitel, 1979; Naujalis, 1995).

Klekowski (1979) developed terms to describe the breeding systems possible for homosporous plants: intergametophytic crossing – true outcrossing (sporophytic outcrossing as proposed Haufler et al., 2016); intergametophytic selfing (sporophytic selfing) – selfing between two gametophytes; intragametophytic selfing (gametophytic selfing) – selfing in the same gametophyte when sporophytes produced are 100% homozygous. Klekowski (1973) reasoned that if bisexual gametophytes of homosporous plants frequently fertilized themselves, lineages should rapidly become highly homozygous and lose the capacity to store and release genetic variability and become polyploid as an adaptive reaction. Then intragametophytic selfing would yield polyploid sporophytes in a single step and new allopolyploids would be fixed because of homoelogous heterozygosity (Haufler, 2002). The true selfing mechanism has

never been directly shown. Flagelated club moss spermatozoids are small (8-10 × 4-5 μm in *L. cernuum* and *D. complanatum*; Robbins and Carothers, 1978) and show chemotaxis caused by organic acids. For example, Bruchmann (1909) found that sperm of *L. clavatum* reacts positively to citrate and boiled prothallial homogenate, but the experiments were not repeated.

In Michigan, Spessard (1917) found 5 × 3–6.5 mm gametophytes, 17 out of 21 gametophytes found were within an area of 10 m^2 and only about ¼ of that area was dug up. In Massachusetts, Degener (1924) collected between 200 and 300 *L. annotinum* and *L. clavatum* gametophytes and their size varied from 1.5 to 10 mm or more, the largest gametophyte had a formed sporophyte. The largest portion of gametophytes were found at a depth of 2.5–7 cm, few at 1–5 cm and one at 9 cm. From around 140 gametophytes measured, about 60 gametophytes were 4–5 mm in diameter and 30 gametohytes were 6–7 mm. The smallest gametophyte with a sporophyte was 4 mm and the largest was 10 mm in diameter. Additionally, four sporophytes were developed from inverted gametophytes, and all sporelings were growing in close proximity in a circular area of 1 m in diameter.

Stokey and Starr (1924) found more than 100 juvenile *D. complanatum* sporophytes, some of them with gametophytes and some gametophytes without juvenile sporophytes were found at a depth of 0.5–3.5 cm. At one site, gametophytes were less symmetrical than a typical form, but in the other, gametophytes were symmetrical and large, 11 × 2.5 mm in size and located at a depth of 2.5–4 cm. Later, Gauthier and Dumais (1938) reported *L. clavatum* gametophytes of 3–5 × 4–6.5 mm in size, juvenile sporophytes 2–7.5 cm in length and *L. obscurum* gametophytes in various sites were found 0.7–5 cm below the surface. In a circle of 0.6 m^2 ten *L. obscurum* juvenile sporophytes with gametophytes were found, six gametophytes were starting to decay, four others

were well developed, the largest 14 × 17 mm and the length of juvenile sporophyte was 3.5–4.5 cm.

Schmid and Oberwinkler (1993) indicated that *L. clavatum* gametophytes were circular bulges of 5–10 mm in diameter and 1–3 cm in thickness. Eames (1942) grouped gametophytes as follows: smallest of 2–3 mm; largest of 1–2 cm in diameter. The largest gametophytes found by Spessard (1917) were those of *L. annotinum* at 10 × 7 mm in diameter; gametophytes of *D. complanatum* were found the deepest, at a depth of up to 7 cm. Bruce and Beitel (1979) measured 48 *L. clavatum* gametophytes and they were 6–22 mm in diameter; the diameter of one *L. annotinum* gametophyte found together with *L. clavatum* gametophytes was 19 mm.

As noted by Haufler et al. (2016), homosporous vascular plants should not be depicted as extreme inbreeders; in reality, ferns and lycophytes have a wide range of mating systems. Currently, no comprehensive data on subterranean club moss gametophyte population structure and function has been published. In many aspects our knowledge mainly refers to works of Bruchmann (1898, 1909, 1910), which have never repeated at the same scale. The amount of research published between the 1910s and 1930s in New Zealand and the USA (Spessard, 1917; Holloway, 1920; Stokey and Starr, 1924; Degener, 1924; Eames, 1942) showed possibilities to answer questions of club moss sexual propagation, but for unknown reasons, interest in gametophyte research in nature diminished for more than thirty years.

While research on pteridophyte spore banks is common (Milberg, 1991; Ramirez-Trejo et al., 2004), data on club moss spore viability is lacking. In all forest types, the emergence of juvenile club moss populations is connected with small scale forest floor disturbances: subterranean gametophytes and juvenile sporophytes were found on and near forest roads, tracks and near lines separating

forest blocks or sites where forest fires occurred up to 25 years earlier (Bruchmann, 1898; Degener, 1924; Stokey and Starr, 1924; Oinonen, 1968; Soltis and Soltis, 1988; Naujalis, 1995; Muller et al., 2003). Various authors (Bruchmann, 1898; Degener, 1924; Gauthier and Dumais, 1938; Naujalis, 1995) emphasized that gametophytes can only be diagnosed by juvenile sporophytes. There is currently no non-invasive method to determine boundaries of subterranean gametophyte populations, thus we can only address juvenile club moss sporophyte distribution.

Eames (1942) mentioned a very successful search and collection of club moss gametophytes over the span of 15 years. Yet today, more than seventy years later, no specific methodology or habitat patterns for localization of club moss gametophyte populations are available. Considerable size variation and developmental stages of gametophyte populations were discovered (Bruchmann, 1898; Degener, 1924; Eames, 1942; Bruce, 1979b; Naujalis, 1995; Rimgailė-Voicik et al., 2015), but no biological explanation for this phenomenon was ever formulated. Little is known about dynamics and effectiveness of populations. Having 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.

There is current and renewed interest in the assessment of diversity in ecology and population genetics (Ricotta and Szeidl, 2006; Jost, 2007). Plant biologists still await fully sequenced genomes for most major lineages (Friedman, 2011). The first complete chloroplast genome of a lycophyte was *Huperzia lucidula* (Wolf et. al, 2005), later heterosporous *Isoetes flaccida* chloroplast genome was sequenced (Karol et al., 2010). The first lycophyte (*Selaginella*) genome (Banks et al., 2011) to be fully sequenced opened opportunities to gain insights into the history of the land plant lineages.

2. RESEARCH OBJECTS AND METHODOLOGY

2.1. Study area

Field research for this dissertation began in June 2012 and lasted till August 2015. Sampling plots in forest blocks were selected when juvenile sporophyte populations were discovered. All sampling sites were selected in dry pine forests of Varėna District, Lithuania. In total, the frequency of sporophytes in various developmental stages and vegetation cover were evaluated and samples to conduct a gametophyte search were collected in nine sites (Fig. 2). The coordinates of the sites are: Maskauka I (N54.28547, E024.60547, WGS), Maskauka II (N54.28029, E024.59781), Varėnė I (N54.26412, E024.53109), Varėnė II (N54.26451, E024.53276), Puvočiai (N54.11251, E024.31869), Bingeliai (N54.17616, E024.26510), Beržupis (N54.23603, E024.59468), Žilinėliai (N54.32018, E024.64004) and Glėbas (N54.24304, E024.46539).

Fig. 2. Map of the study area in Varėna District, Lithuania. The samples for a gametophyte search were collected from nine different sites (Maskauka I, Maskauka II, Varėnė I, Varėnė II, Puvočiai, Bingeliai, Beržupis, Žilinėliai and Glėbas) and the Maskauka site was selected for repeated assessment of vegetation cover and juvenile sporophytes. Because of the close vicinity of the sites, Maskauka I and Maskauka II, and Varėnė I and Varėnė II are represented as one dot.

The Maskauka site (N54.28202, E024.59892) had the largest cover of juvenile sporophytes and was selected for repeated assessment of vegetation cover and juvenile sporophytes. Around Maskauka I, Varėnė I, Puvočiai, Žilinėliai and Glėbas sampling sites separate clones of *L. annotinum* and *L. clavatum* were sampled for DNA extraction in rectangular polygons.

Forests occupy about 68.9% of the Varėna District (Anonymous, 2015). Pure pine forests with *P. sylvestris* or mixed pine forests with *Betula* L., *Quercus robur* L. and *Picea abies* dominate. Forests where research was conducted can be attributed to ass. *Peucedano-Pinetum* W.Mat. (1962) 1973 (Matuszkiewicz, 2001). Forests between Varėna City and Druskininkai City occupy the largest territory in Lithuania, about 106,000 ha. The age structure of Druskininkai forest is uneven. Stand stocking level averaged 0.71 in 50 years old stands. The majority of stands (61%) are coniferous (Karazija, 1988; Balevičienė ir Vaičys, 2001). According to Balevičienė and Vaičys (2001), 53% of forest litter is pine needles. Pine forests lack abundant soil bacteria (6.8-10 mln. cells/1g dry soil) because soil is formed from sand and the pH is acidic and structural functional ratio is shifted towards humificator's activity (1/1.41). Microorganisms that dominate are micromycetes (Raguotis, 2001). Only a small part of forests in the Varėna District are natural; the majority were planted around 1950.

Mean absolute temperature in January is −4.2 °C and +17 °C in July. Mean annual precipitation is 670 mm; snow cover lasts for 90 days. Hilly moraine relief soils are mostly podzolic, light (sandy or sandy loam; Anonymous, 2015). Six species of club mosses were present in the researched forests: *Lycopodium annotinum, L. clavatum, Diphasiastrum complanatum, D. tristachyum* and *D. × zeilleri, Huperzia selago*.

Nomenclature follows The International Plant Names Index (2012) for vascular plants, Jukonienė (2003) for mosses and Index Fungorum (2017) for lichens and fungi.

2.2. Research objects

Juvenile club moss populations include spores, subterranean gametophytes, sporophyte sprouts (still incipient with the gametophyte) and juvenile sporophytes (already independent from the gametophyte). The present study focuses on subterranean gametophytes, incipient sporophytes and juvenile sporophytes and the vegetation in the surrounding community. In the alternation of generations, diploid vascular sporophytes (asexual generation) alternate with haploid multicellular gametophytes (sexual generation; Fig. 3). Both generations are critical for survival of the species and from a genetic perspective, sporophytes could be considered the less important generation (Haufler, 2002).

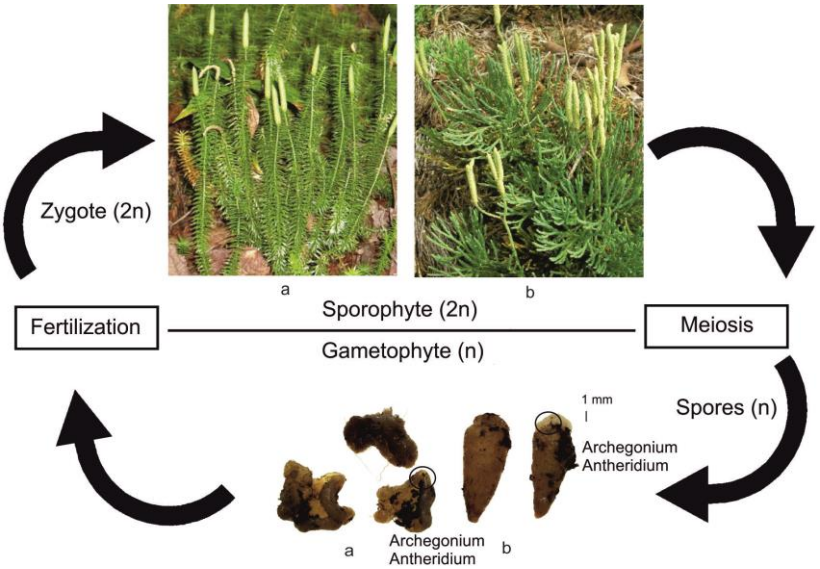

Fig. 3. Life cycle of model lycophyte: a – *Lycopodium annotinum* sporophyte and gametophyte, b – *Diphasiastrum* sp. sporophyte and gametophyte (Rimgailė-Voicik et al., 2015)

Photosynthesizing sporophytes that were no longer connected to gametophytes but had gametophyte remains and developing creeping growth were called juvenile sporophytes. Sporophytes that had an attached gametophyte were called sporophyte sprouts regardless their size or photosynthesizing abilities. Usually sporophyte sprouts had three or fewer vertical stems (Fig.4).

Fig. 4. Stages of club moss sporophyte development: A – subterranean gametophyte; B – sporophyte sprouts; C – annual constriction on juvenile sporophyte (Rimgailė-Voicik and Naujalis, 2016)

There are seven species in Lycopodiaceae in Lithuania: *L. annotinum*, *L. clavatum*, *D. complanatum*, *D. tristachyum* (Gudžinskas, 1999), *D.* × *zeilleri* (Rouy) Holub (Tupčiauskaitė and Žemgulytė, 2012), *Lycopodiella inundata* and *H. selago*. The latter two species are included in the Red Book of Lithuania (Tupčiauskaitė, 2007a,b). In this study we examined occurrence rates of adult and juvenile *L. annotinum*, *L. clavatum* and *D. complanatum s. lat.*, and their subterranean gametophytes at different developmental stages.

Lycopodium annotinum – main shoots creeping on the ground or in moss or the litter layer, up to 1.5 mm thick. Branches erect, up to 20 cm in height, unbranched or sparsely branched, yearly growth 1–6 cm. Leaves form 8–10 indistinct rows, patent or appressed, linear-lanceolate, ending in a short, stiff point, 2.5–10 mm in length (free part), 2n = 68. Boreal circumpolar distribution (Jonsell, 2000). In Lithuania, the growth rate during one season was estimated to be up to 31.2 cm for creeping and 11.2 cm for vertical shoots (Naujalis, 1995). Common in all of Lithuania, growing in wet dark coniferous forests, near bogs and water bodies; sporulate from June till September. Strobili solitary, sessile up to 4 cm in length.

Lycopodium clavatum – stem creeping, mostly found on mosses and litter, up to 2.5 mm thick; branches erect from ascending base, branched, up to 30 cm in height. Mature leaves monomorphic, linear, 2.5–5 mm, narrowly acute, with 1.5–4 mm long apical hair tip usually in 12–16 indistinct rows. 2n = 68. Arctic circumpolar distribution (Jonsell 2000). In Lithuania, the growth rate during one season was estimated up to 45.1 cm for creeping and 9.8 cm for vertical shoots (Naujalis, 1995). Common in all of Lithuania, growing in dry pine forests, dry areas in raised bogs; sporulate from July till September. Up to 4 strobili together, with peduncle up to 12 cm.

Diphasiastrum complanatum – main shoots creeping on the ground or within the litter layer, up to 2.5 mm thick. Orthotropic branchlets up to 40 cm tall and 4 mm wide, unequal in length, not forming regular conical fascicles; lateral leaves revolute and ventral leaves appressed; free part 0.8–1.8 mm. Internodes of branches relatively long. 2n = 46. Boreal circumpolar distribution (Jonsell, 2004). In Lithuania, the growth rate during one season was estimated to be up to 21.3 cm for creeping and 5.3 cm for vertical shoots (Naujalis, 1995). Common in the eastern part of Lithuania, known in the west and middle part of Lithuania.

Sporulates from June till September. Up to 4 strobili on each peduncle, up to 3.5 cm in length.

Diphasiastrum tristachyum – stem creeping up to 20 cm below ground, orthotropic branches forming conical fascicles along the horizontal shoots; up to 5 mm thick. Orthotropic branches up to 30 cm tall, branchlets up to 2 mm wide, dorsal and ventral sides almost equal. Lateral leaves not revolute. Internodes of branches relatively short. 2n = 46 Distributed in central and eastern Europe and eastern North America (Jonsell, 2000). Common in southeastern Lithuania. Sporulate from July till October. Up to 6 strobili on one peduncle, up to 2.5 cm in length.

Diphasiastrum × *zeilleri* – stem creeping, usually subterranean, found up to 10 cm deep, up to 4 mm thick. Orthotropic branches up to 30 cm in height, branchlets up to 2.5 mm wide, unequal in length, not forming regular conical fascicles. Dorsal and ventral side of the branch unequal. Internodes of branches relatively long. Rare, total distribution uncertain (Jonsell, 2000). Common in southeastern Lithuania. Sporulate from July till December (unpublished data). Up to 4 strobili on one peduncle, 3 cm in length.

All species of genus *Diphasiastrum* form large clones, sometimes fairy rings. Clones of *L. annotinum* can exceed two hectares (Wittig, 2007) while *L. clavatum* rarely exceeds 13% coverage. The amount of spores produced fluctuates greatly and repeats cyclically: during a 12 year cycle, nine years have different levels of fertility and three are infertile. During one cycle, 43–45 kg of not quite ripe strobili with spores could be gathered from 1 ha of forest, where *L. clavatum* coverage is 10% (Šimkūnaitė, 1969). In Lithuania, *L. clavatum* is a restricted resource herb that must be valuated as deficit and exploited wisely with special protection during forestry works (Budriūnienė, 1972).

2.3. Sampling design

In 2012, juvenile club moss populations were searched for in dry pine forests near Senoji Varėna Village. Nine sites were selected for further analysis. In twenty one sites with adult club moss populations, geobotanical descriptions were made using percentage scale. In every study site, juvenile sporophyte populations were detected using standardized routine method. Species coverage was determined using percentage scale in a 10 × 10 m^2 field, 1 m^2 plot (permanent research square) or 0.25 m^2 plot (samples for gametophyte search). Using the spot route method (Korchagin, 1964; Rimgailė-Voicik et al., 2015) three species of club mosses were evaluated. In every research site, three 0.25 m^2 soil samples with intact forest floor were collected for gametophyte search.

2.3.1. Assessment of club moss occurrence in dry pine forests

Occurrence of club moss sporophytes was evaluated in nine research sites in August 2013 and 2014. An adapted spot route method (Korchagin, 1964; Rimgailė-Voicik et al., 2015) was used to evaluate the occurrence rate of sporophytes. Occurrence of club moss sporophytes was evaluated in a rectangular field of 4,590 m^2 in nine research sites. The number of investigated rows was 17. The distance between rows was three meters and the distance between spots in a row was three meters. In every row, the occurrence of club moss sporophytes was evaluated 30 times with a total of 510 spots in each study site (Fig. 5). Three species of club mosses were evaluated: *L. annotinum*, *L. clavatum* and *D. complanatum s. lat*. We determined the occurrence frequency for three different sporophyte developmental stages: 1) adult sporophytes with strobili, 2) adult sporophytes without strobili and 3) juvenile sporophytes. Sites with the highest occurrence rate of juvenile sporophytes were selected for further research as subterranean gametophytes were more likely to be present. Within a rectangular

field, vegetation coverage was evaluated with a percentage scale in a 10 m^2 square. Signs of former forest fires (charred trunks) were recorded. The square was divided into transects, and three soil samples (0.25 m^2) with intact forest floor were collected for to search for gametophytes. In every site, soil samples were taken in a transect. The bare soil in the sampling site was then covered with forest floor litter.

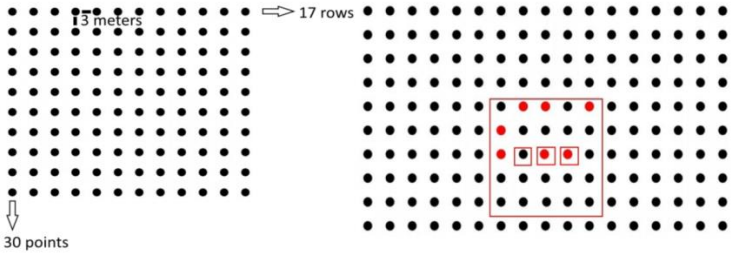

Fig. 5. Scheme of the adapted spot-route method and site selection for soil sampling for the search for gametophytes. Red dots represent spots with juvenile club mosses

2.3.2. Assessment of subterranean gametophyte and juvenile sporophyte population structure

Methodology was tested in 2012 using two soil samples of 50 × 50 × 10 cm in size with intact forest floor, which were collected from the Maskauka site. In the laboratory, soil samples were carefully disassembled with tweezers to look for gametophytes. A modified method for quantifying soil invertebrates (Ghilarov and Striganova, 1987; Rimgailė-Voicik and Naujalis, 2015) was used. The number of juvenile sporophytes and the number of gametophytes were recorded and their developmental stage was evaluated. During 2013–2014, in nine sites with previously identified juvenile sporophytes, 50 × 50 × 10 cm soil samples were collected (Fig. 6) and analysed using the previously described scheme. In total, 29 samples were used for statistical analyses. Vegetation coverage of the samples collected was evaluated.

In the laboratory, the moss layer of every soil sample was removed. Then, using pins, every sample was divided into 10 × 10 cm plots to increase accuracy. We looked for gametophytes by gradually disassembling soil samples with tweezers. The coordinates (x, y) and soil depth of every gametophyte located in the 10 × 10 cm subfields were registered and recounted in a 0.25 m^2 sample. Depth was determined by comparison with the closest intact sample part that had the moss layer removed.

Fig. 6. Extraction of the soil samples for club moss gametophyte search

The diameter of every gametophyte was determined. Using external macroscopic features, we divided the gametophytes into six groups: globular, disk shape, irregular bowl shape, irregular bowl shape with a sprout, carrot shape and carrot shape with a sprout (Fig. 7). Terminology outlined by Bruce (1979) was employed to describe external features of gametophytes.

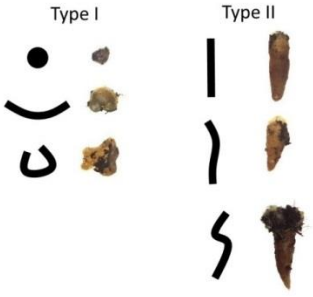

Fig. 7. Development of Type I (*Lycopodium*) and Type II (*Diphasiastrum*) gametophytes, according to Bruce (1979)

2.3.3. Repeated vegetation analysis in a dry pine forest community with a juvenile *Lycopodium* population

At the permanent Maskauka site, vegetation coverage and the location of juvenile sporophytes were recorded at one-year intervals, from 2012 to 2015, in August. For visual cover evaluation, a plot of 100 m^2 was marked and divided into 1 m^2 subplots with wooden dowels (Fig. 8). Vegetation coverage was evaluated using percentage scale in 100 1 m^2 subplots. In 2013 and 2014, the development of juvenile sporophytes was evaluated by counting the number of shoots seen above the moss layer in 100 subplots. In August 2015, tree canopy projection was performed and the diameter at breast height (DBH) was measured.

At the end of August 2015, all juvenile sporophytes at the Maskauka site were excavated with 10 × 10 × 10 cm soil samples. In the lab, soil samples were gradually disassembled with tweezers to search for club moss gametophytes and extract juvenile sporophytes. The size of the identified juvenile sporophytes was described and their age was determined according to the number of annual constrictions (Primack, 1973; Naujalis, 1986, 1995), the number of shoots and their growth pattern (orthotropic or plagiotropic).

2.4. Chemical soil analysis

In the permanent Maskauka research site, a humus horizon soil sample for chemical analysis was collected from three subsamples taken in the permanent research field in 2015. Soil analyses were performed at the National Public Health Surveillance Laboratory in Vilnius, Lithuania. Soil pH was determined in an aqueous suspension (ISO 15933), total amount of nitrogen was determined using a Kjeldahl digestion (ISO 11261) and spectrometry was used to determine total phosphorus (ISO 11263).

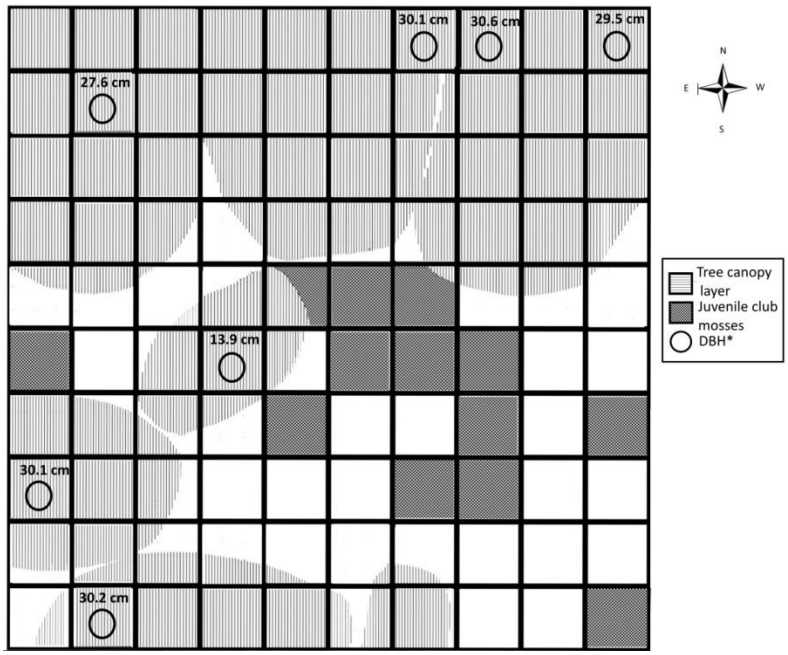

Fig. 8. Scheme of the permanent 10 × 10 m^2 Maskauka site of with a juvenile club moss population in 2015 (Rimgailė-Voicik and Naujalis, 2016).
* tree diameter at breast height

Soil samples for chemical analyses were collected from the nine field sites and prior to analyses, samples were dried and sifted through a plastic sieve. Soil analyses were performed at the Agrochemical Research Laboratory at the Lithuanian Research Centre for Agriculture and Forestry in Kaunas, Lithuania. Soil pH was determined in a 1 mol/l KCl suspension (ISO 10390:2005) and organic carbon content was measured (ISO 10694:1995). Using a spectrometric flow injection analysis (FIA) method developed by the laboratory, nitrogen content (sum of $N\text{-}NO_3$ and $N\text{-}NO_2$; LVP D-05:2016) and ammonia nitrogen content (NH_4-N; LVP D-05:2016) were determined. Mineral nitrogen value was calculated by adding the value of nitrogen content to the value of ammonia nitrogen content. Plant available phosphorus (P_2O_5) content and plant available potassium (K_2O) content were determined using the Egner-Riehm-Domingo (A-L) method developed by the laboratory (LVP D-07:2016).

2.5. Genomic DNA extraction and PCR amplification

In order to avoid sampling members of the same interconnected clone, collections for DNA extraction were made from club moss aggregations which were at least 20 m apart. DNR was purified from fresh apical branches and gametophytes of *L. annotinum* and *L. clavatum* using the modified CTAB method (Doyle and Doyle, 1990; Table 3).

Up to 100 mg of plant tissue was ground to a fine powder in liquid nitrogen. Powdered samples were then placed in 2 mL microtubes containing 0.8 mL 2% CTAB extraction buffer [for 0.4 ml 0.5M EDTA (pH 8), 1 mL 1M TRIS (pH 8), 2.75 5M NaCl, 2 ml 10% CTAB, 0.02 ml mercaptoethanol, 100g PVP-40]. The solution was incubated at 65 ºC while centrifuged at 300 rpm for 25 min using an Eppendorf Thermomixer Comfort. Then samples were cooled to room temperature and 1 mL of chloroform was added to the tubes and gently mixed for 1 min. Samples were centrifuged using a centrifuge Eppendorf 5415R for 3 min. at 10,000 rpm. Up to 700 µL of the supernatant was then transferred to fresh 2 mL tubes following the addition of 1 mL chloroform, gently mixed and centrifuged for 3 min at 10,000 rpm. Up to 300 µL of the supernatant was then transferred to fresh 1.5 mL tubes, and 150 µL 5M NaCl and 600 µL 95% ethanol (-20 ºC) were added. Samples were mixed gently and kept at +4 ºC for 30 min. Centrifuge was cooled off till +9 ºC and samples were centrifuged for 5 min at 10000 rpm, it was possible to visualize the DNA adhered to the bottom of the tube. Supernatant was then poured off and 200 µL of 70% ethanol was added. Samples were centrifuged again for 5 min at 10,000 rpm, and the supernatant was poured off. Samples were centrifuged for 20 s and the remaining supernatant was removed. DNA samples were left for 30 min in a fume hood to dry and then 50 µL of sterile deionized H_2O was added and samples were stored at +4 ºC overnight.

The next day, samples were gently mixed and centrifuged for 5 min at 10,000 rpm. DNA concentrations were measured using a BioPhotometer, then samples were frozen and stored at -20 ºC. The A_{260}/A_{280} ratio of a good quality DNA sample must be around 1.7 and 1.9.

Table 3. Chemical compounds used for DNA extraction and ISSR-PCR reactions

Compound	Formula	Manufacturer, country
	For DNA extraction	
EDTA	$C_{10}H_{14}N2Na_2O_8•2H_2O$	ROTH, Germany
TRIS	$C_4H_{11}NO_3$ / 1M	ROTH, Germany
Sodium chloride	NaCl	ROTH, Germany
CTAB	$CH_3(CH_2)_{15}N(CH_3)_3Br$	ROTH, Germany
2-Mercaptoethanol	C_2H_6OS	Sigma, Germany
Polyvinylpyrrolidone, PVP-40	$(C_6H_9NO)_n$	Sigma, Germany
Chloroform	$CHCl_3$	ROTH, Germany
Ethyl alcohol	C_2H_5OH	
	ISSR-PCR reaction	
10x bufer *Taq* $MgCl_2$	+KCl	Thermo Scientific, Lithuania
2 mM dNTP Mix	dATP, dGTP, dCTP, dTTP	Thermo Scientific, Lithuania
25 mM $MgCl_2$		Thermo Scientific, Lithuania
Primer		
Taq DNA polymerase		Thermo Scientific, Lithuania
DNA		
Deionized water	H_2O	
Bromophenol Blue	$C_{19}H_9Br_4O_5SNa$	ROTH, Germany
	Agarosis gel	
Agarosis	$(C_{12}H_{18}O_9)n$	Thermo Scientific, Lithuania
Ethidium bromide	$C_{21}H_{20}N_3Br$	ROTH, Germany
10 × TBE buffer [890 mM TRIS, 890 mM Boric acid, 20 mM EDTA, distilled H_2O]		
	For measuring length of DNA fragments	
	GeneRuler™ DNA Ladder Mix 10000-100 bp	Thermo Scientific, Lithuania
	MassRuler™ DNA Ladder Mix 10000-250 bp	Thermo Scientific, Lithuania

ISSR-PCR analyses were performed according to Patamsytė et al. (2011). For PCR we used 5 ng/μL DNA samples. Each PCR amplification was performed in a

10 μL reaction mixture [1 μL 10 × Taq $MgCl_2$ buffer, 1.2 μL 25 mM $MgCl_2$, 1 μL 2 mM dNTPs, 0.4 μL primer, 4.32 μL deionized H_2O, 0.08 μL Taq DNA polymerase and 2 μL of club moss DNA]. ISSR reactions were carried out in an Eppendorf thermo cycler. The reaction program was set to: 94 °C for 7 min, 32 cycles of 94 °C for 30 s, 55/39/46 °C for 5 min, 72 °C 2 min), and a final extension of 72 °C for 7 min. Four primers were used.

The amplification products were analysed using electrophoresis in a 1.5% TBE agarose gel. The gel was run for about 3.5 h. Gel results were registered using a BioDocAnalyse documentation system (Biometra, Germany). GeneRulerTM DNA Ladder Mix (10000-100 bp) and MassRulerTM DNA Ladder Mix (10000-250 bp) were used as standards.

2.6. Data analysis

Statistical analyses were performed in R using 'BiodiversityR' Vegan (Oksanen et al., 2016). Nonmetric multidimensional scaling (NMDS) was performed to test for differences in vegetation and soil properties among samples collected for gametophyte search and to explore probable patterns of subterranean gametophyte emergence in relation to vegetation composition. Distances were calculated using the 'vegdist' function for Bray–Curtis.

Permutational MANOVA was applied to test for significant differences among forest sites and number of juvenile sporophytes and gametophytes, and generated Bray-Curtis and Hellinger dissimilarity measures. Results were calculated with the function 'adonis' (Oksanen et al., 2016) in the package 'Vegan'. For the permanent research field, permutational MANOVA was used to test if there were no differences in squares with and without tree canopies.

Shannon and Simpson indexes were subjected to repeated measures ANOVA (RM-ANOVA) with years as within-subject factors. Significance level was

adjusted using the Greenhouse-Geisser correction. Significant main effects and interaction effects were followed up with post hoc pairwise comparisons adjusted using a Bonferroni correction. Calculations were performed with the function 'ezANOVA' in the package 'ez' (Lawrence, 2011).

Species richness accumulation curves were generated and the Chao species richness estimator was calculated. For evaluation and comparison of diversity, Renyi diversity profiles among the nine gametophyte search sites and in the four year vegetation composition in the permanent research field (2012-2015) were generated. Coverage data per species was transformed from percentage to relative coverage. The Renyi diversity was suggested (Renyi, 1961) in the form

$$H_\alpha = \frac{1}{1-\alpha} \ln\left(\sum_{i=1}^{S} p_i^\alpha\right)$$

where p_i is relative abundance of *i-th* species and *S* is a total number of species in the sample. An equation is interpreted for the range of scale parameter α: $\alpha \geq 0$, $\alpha \neq 0$. This method considers several diversity indices and binds them into coherent framework. Renyi's entropy is related to a wide range of diversity indices by a power law—the Hill number (Na = eHa) and many diversity indices are special cases of the Hill number (Hill, 1973). The Renyi index estimates total richness for α = 0, Shannon-Weiner index for α = 1, the inverse Simpson's-Yule index for α = 2 and 1/Berger-Parker or dominance index for α = Inf. More extended use of this method is suitable for conservation biology and interpretation of diversity by placing various weights on different abundance categories (Lövei et al., 2013).

Correlation matrices were generated to test for significant correlations among different species coverage over the four year period in the permanent Maskauka research site and among the nine forest sites where a search for gametophytes was

performed. Also, a Pearson correlation coefficient was calculated between the number of gametophytes and number of juvenile sporophytes detected.

In the spatial analysis, each individual gametophyte and sporophyte was represented with its coordinates in the overall sample. To evaluate the distribution of gametophytes and juvenile sporophytes, we used the extended method, which was originally created by Clark and Evans in 1954 (Levine, 2010). This method calculates an expected mean nearest neighbor distance (r_E) using the overall density of the population (p) given a Poisson distribution:

$$\overline{r_E} = \frac{1}{2\sqrt{\rho}} \quad 1)$$

The nearest neighbor index (NNI) was then derived from the ratio of r_E to the observed mean nearest neighbor distance (r_O).

$$NNI = \frac{\overline{r_O}}{\overline{r_E}} \quad 2)$$

The properties of the index are: NNI = 1, spatial randomness; NNI < 1, spatial aggregation; NNI > 1 regular spatial distribution. Nearest neighbor analysis (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 (NNI). The higher-order nearest neighbor analysis expected distance values for the K^{th} nearest neighbor were calculated as follows:

$$\overline{r_{KE}} = \frac{K(2K)!}{(2^K K)!\sqrt{\rho}} \quad 3)$$

Where K is the order of the neighbor being examined.

Spatial patterning was quantified based on the distribution of first and higher order nearest neighbors at each sample in which juvenile sporophytes or gametophytes were found. Only samples where the number of gametophytes or juvenile sporophytes found was higher than eight were included in this analysis. We measured the spatial aggregation of gametophytes and juvenile sporophytes

separately, but this was not exclusive to genus (*Diphasiastrum* or *Lycopodium*). Spatial analyses were conducted with CrimeStatsIII (Levine, 2010). This program was used to evaluate relationships among individuals in a population in other similar research (Bosiacka et al., 2008; Webster and Jenkins, 2008).

Only well-defined and reproducible DNA bands were included in the binary data matrix. The percentage of polymorphic loci, Shannon's information index, population genetic differentiation coefficient (G_{ST}), expected heterozigosity were calculated using program POPGENE, version v. 1.31. PCoA was conducted using GenAlEx v. 6.5 (Peakall and Smouse, 2006). An unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on pairwise Nei's (Nei, 1978) unbiased genetic distances (GD) with 1000 Bootstrap permutation was used to draw dendrograms with TREECON v. 1.3 (Van de Peer and De Watcher, 1994) program. The analysis of molecular variance (AMOVA) (Excoffier et al., 1992) within and among *L. annotinum*, *L. clavatum* and all club moss populations with permutation of 999 was performed using GenAlEx v. 6.5.

Nei's G_{ST} is typically used for describing the average amount of differentiation observed over multiple loci (Ryman and Leymar, 2009). G_{ST} is defined as

$$G_{ST} = \frac{H_T - H_S}{H_T}$$

where H_S and H_T are the expected heterozygosities within subpopulations and for the total population, respectively (Nei et al., 1975). H_T and H_S are both smaller than unity and $H_T \geq H_S$, which implies that G_{ST} goes towards zero when H_S approaches unity, and that G_{ST} cannot exceed $1 - H_S$ (Ryman and Leymar, 2009).

Genetic distance was generated according to Nei and Li's (1979) formula:

$$GD_{xy} = \frac{1 - 2N_{xy}}{N_x + N_y}$$

where N_{xy} – mutual DNA bands of plants x and y, N_x – DNA bands specific to plant x and N_y – DNA bands specific to plant y.

3. RESULTS

3.1. Habitats of subterranean gametophyte and juvenile sporophyte populations

During four years of field research, juvenile club moss sporophytes were discovered in 21 localities. In total 41 geobotanical descriptions of sites were performed: 21 with juvenile club moss populations (Supplement 1), ten descriptions with adult *L. clavatum* and ten with adult *D. complanatum*. The total species richness determined in sites with adult club moss sporophytes was 82 (Chao index estimated 92) and in sites with juvenile club mosses, total species richness was 55 (Chao index estimated 68). Species accumulation curve reached an asymptote after 15 samples of adult *L. clavatum* and *D. complanatum*, and in samples with juvenile club moss individuals, a true asymptote was never reached.

The Renyi diversity profile indicated that species richness and overall diversity (Shannon-Weiner index for $\alpha = 1$) was highest in sites with adult *L. clavatum* (Fig. 9). Sites with adult *D. complanatum* and juvenile club mosses had similar diversity and total species richness, but according to the Simpson similarity index ($\alpha = 2$), sites with juvenile club mosses were more diverse.

All three groups were plotted to NMDS with a Bray-Curtis dissimilarity index (Fig. 10). Sites with juvenile club mosses were very similar to each other while sites with adult club mosses were more diverse. Sites with adult *D. complanatum* were more similar to sites with juvenile club mosses.

The dry pine forests with juvenile club moss population in the Varėna District of Lithuania were not species rich and had no shrub layer (Fig. 11). In sites dominated by the grass *Deschampsia flexuosa*, no adult club moss clones were present in close proximity.

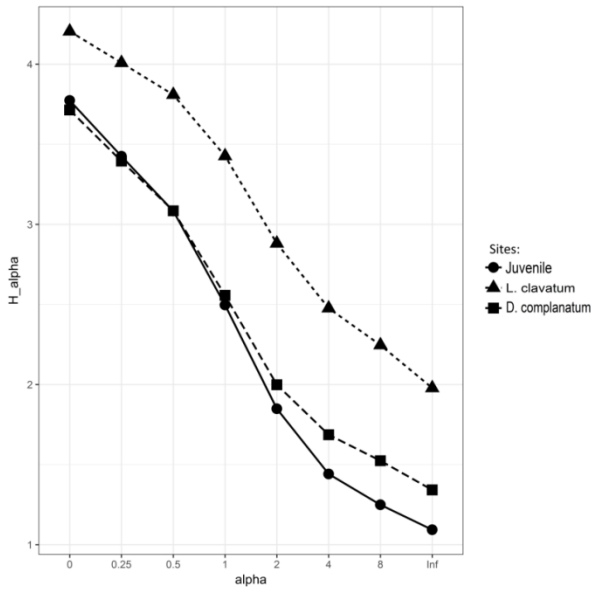

Fig. 9. Renyi diversity profiles of three groups of club moss sites in dry pine forests: adult *L. clavatum*, adult *D. complanatum* and juvenile club mosses. The scale parameter (α) gives the order of Renyi diversity; α = 0 is the logarithm of species richness, α = 1 is the Shannon diversity index, α = 2 is the logarithm of the reciprocal Simpson's diversity index, α = Inf refers to the proportion of the most abundant species. The x and y-axis show, respectively, the alpha value of the Renyi formula and their associated Renyi diversity profile values

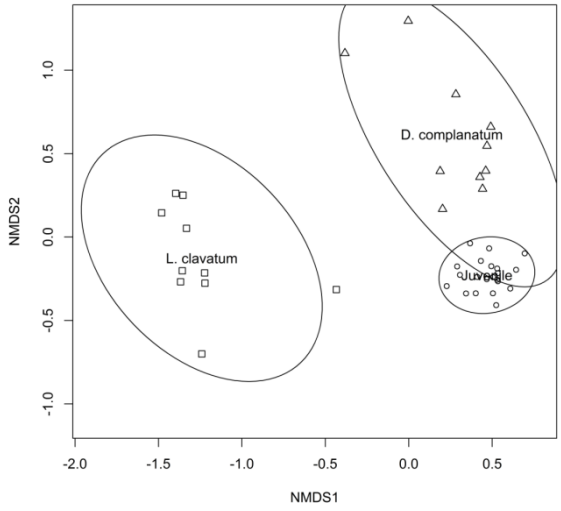

Fig. 10. Multivariate structure of the dry pine forest communities with juvenile and adult *L. clavatum* and *D. complanatum* club moss populations based on Bray-Curtis distances plotted with nonmetric multidimentional scaling (NMDS)

The Renyi diversity index (Lövei et al., 2013) indicated that forest sites with juvenile slub mosses were slightly stressed and disturbed. We propose that all of the above listed characteristics made the researched forests suitable to support juvenile club moss populations.

Fig. 11. Typical research site with juvenile club moss population: a – *Lycopodium annotinum*, b – *Diphasiastrum complanatum*

3.2. Frequency of *Lycopodium* and *Diphasiastrum* club mosses

The highest rate of total club moss occurrence was recorded in Varėnė II (133 points of 510 assessed, 26.07%). The lowest rate of total club moss occurrence was recorded in Bingeliai (two points, 0.39%). The highest rate of juvenile club moss individuals in most cases did not coincided with the total occurrences of club moss detected. The highest rate of juvenile club moss occurrence was recorded in Maskauka I (35 points, 6.86%) and the lowest rate of juvenile club moss occurrence was recorded in Bingeliai (two points, 0.39%). When the percentage of juvenile club moss occurrence and total occurrence in all nine research sites were compared, four groups were noticed: 1) localities where juvenile club mosses dominated; 2) localities where juvenile club mosses comprised around 50%; 3) localities where juvenile club mosses comprised around 30%; 4) localities where juvenile club mosses comprised less than 7%.

In two sites, more than 80% of all club moss occurrences were juvenile club mosses: Bingeliai (100%) and Maskauka I (81%). In three sites, more than 50% of all club moss occurrences were juvenile club mosses: Beržupis (57%), Varėnė I (51%) and Glėbas (51%). In one site, more than 30% of club moss occurrences were juvenile club mosses: Žilinėliai (32%). In three other sites, less than 7% of all club moss occurrences were juvenile club mosses: Varėnė II (6%), Maskauka II (5%) and Puvočiai (5%).

The occurrence rates of different club moss species evaluated varied among the sites (Table 4). In five study sites, *L. annotinum* had the highest total occurrence rates and in the other four sites, *L. clavatum* had the highest total occurrence. When the occurrence rates of juvenile club mosses were compared over the nine sites, the highest occurrence rates were of juvenile *L. annotinum*, varying from two to 33 points out of 510 assessed. Juvenile *L. clavatum* occurred only in three sites and *D. complanatum* occurred in four sites; the occurrence rates varied from one to three points. In three sites, juvenile club mosses of all three species assessed were found growing in close vicinity: Maskauka I, Varėnė I and Glėbas. When the occurrence rates of sporulating club mosses were compared, we noticed that in zero out of nine sites all three species were sporulating together. Adult club mosses of the three species were detected growing together in one site (Puvočiai). Sporulating *L. annotinum* occurred only in one site, while sporulating *L. clavatum* occurred in five sites with occurrence rates varying from five (Žilinėliai) to 87 points (Varėnė II). Sporulating *D. complanatum* occurred in three sites; in the Varėnė site it was dominant with occurrence at eleven points. In six sites, *L. clavatum* adult club mosses dominated with occurrence rates varying from three to 36 points. In three sites, *L. annotinum* adult club mosses dominated with occurrence rates varying from two to 21 points.

Pine forests of the Varėna District showed unique, mosaic club moss population structure of populations composed from different species and developmental stages located close to each other. While Bingeliai and Maskauka I populations seemed to be the youngest, Varėnė II, Maskauka II and Puvočiai might have reached their final maturity stage. Cluster analysis (Fig. 12) grouped all nine research sites into two large groups. The first group (previously referred to as the oldest) was composed of Glėbas, Puvočiai, Masauka II and Varėnė II and the second group (previously reffered to as the youngest) comprised Beržupis, Žilinėliai, Varėnė I, Maskauka I and Bingeliai. The most closely related sites were Maskauka II and Varėnė II; and Maskauka I and Bingeliai. In all nine research sites, the highest occurrence rates were of juvenile *L. annotinum*. The most common sporulating club moss was *L. clavatum*. In three sites, Maskauka, Varėnė and Glėbas, juvenile club mosses of all three species were found growing in close vicinity, but sporulating individuals were never found together in one site.

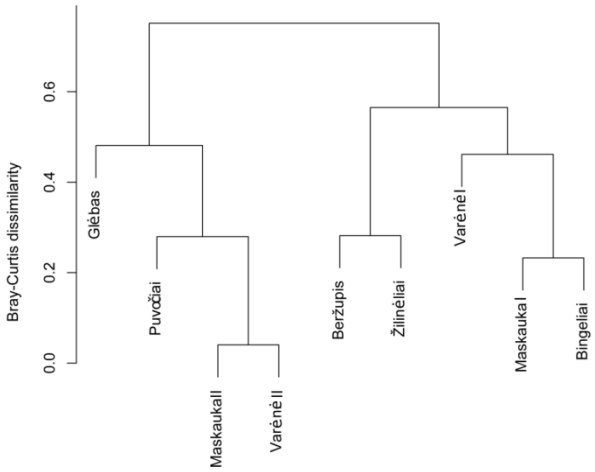

Fig. 12. Cluster diagram presenting the similarity of nine research sites with occurrence rates of club moss developmental stages: juvenile, adult not sporulating, sporulating. Bray-Curtis dissimilarity matrix was generated using an average-linkage algorithm.

Table 4. Occurrence rates of club mosses in research sites

Developmental stages /study sites	Maskauka I N54.28547 E24.60457		Maskauka II N54.28029 E24.59781		Varėnė I N54.26412 E24.53109		Varėnė II N54.26451 E24.53276		Puvočiai N54.11251 E24.31869		Bingeliai N54.17616 E24.26510		Beržupis N54.23603 E24.59468		Žilinėliai N54.32018 E24.64004		Glėbas N54.24304 E24.46539	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	N	%
Lycopodium annotinum																		
Adult with strobili	0	0	1	0.19	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Adult without strobili	2	0.39	3	0.58	0	0	0	0	8	1.56	0	0	3	0.58	21	4.11	2	0.39
Juvenile sporophytes	33	6.47	6	1.17	15	2.94	9	1.76	2	0.39	2	0.39	4	0.78	11	2.15	13	2.54
In total:	**35**	**6.86**	**10**	**1.96**	**15**	**2.94**	**9**	**1.76**	**10**	**1.96**	**2**	**0.39**	**7**	**1.37**	**32**	**6.27**	**15**	**2.94**
Lycopodium clavatum																		
Adult with strobili	0	0	71	13.9	0	0	87	17.05	15	2.94	0	0	0	0	5	0.98	13	2.54
Adult without strobili	6	1.17	27	5.29	4	0.78	36	7.05	9	1.76	0	0	0	0	0	0	3	0.58
Juvenile sporophytes	1	0.19	0	0	2	0.39	0	0	0	0	0	0	0	0	0	0	3	0.58
In total:	**7**	**1.37**	**98**	**19.2**	**6**	**1.17**	**123**	**24.11**	**24**	**4.70**	**0**	**0**	**0**	**0**	**5**	**0.98**	**19**	**3.72**
Diphasiastrum complanatum																		
Adult with strobili	0	0	0	0	11	2.15	1	0.19	1	0.19	0	0	0	0	0	0	0	0
Adult without strobili	0	0	0	0	0	0	0	0	2	0.39	0	0	0	0	0	0	0	0
Juvenile sporophytes	1	0.19	0	0	1	0.19	0	0	0	0	0	0	0	0	1	0.19	3	0.58
In total:	**1**	**0.19**	**0**	**0**	**12**	**2.35**	**1**	**0.19**	**3**	**0.58**	**0**	**0**	**0**	**0**	**1**	**0.19**	**3**	**0.58**
***Lycopodiaceae* in total:**	**43**	**8.43**	**108**	**21.2**	**33**	**6.47**	**133**	**26.07**	**37**	**7.25**	**2**	**0.39**	**7**	**1.37**	**37**	**7.25**	**37**	**7.25**

*n – number of spots in which lycophyta occurred. Occurrence percentage share of the 510 spots is also given. In every row, occurrence of club moss sporophytes was evaluated 30 times, with a total of 510 spots in each study site. The chosen number of rows was 17. Distance between rows was 3 m, distance between spots in a row 3 m.

3.3. Diversity of subterranean gametophyte and juvenile sporophyte populations

In total, 595 club moss gametophytes were found in 31 soil samples (Table 5). We found 253, 122 and 220 gametophytes in 2013, 2014 and 2012, respectively. In 2014, all gametophytes found were from *Lycopodium* sp. (Type I according to Bruchmann, 1898). In 2013 we found 38 *Diphasiastrum* sp. gametophytes (Type II), the rest of the gametophytes were *Lycopodium* sp. We identified 109, 27 and 26 juvenile sporophytes in 2014, 2013 and 2012, respectively.

Table 5. Number of club moss gametophytes found in soil samples

Year of research	Number of samples	Number of gametophytes found
2012	5	220
2013	6	253
2014	20	122
In total:	**31**	**595**

The gametophyte distribution in soil samples was uneven. The largest number of gametophytes found in one soil sample was 133 (Varėnė I) and the lowest was two (Žilinėliai). The ratio of aboveground and belowground structures was also uneven. In two samples from Beržupis, three and five juvenile sporophytes were present, but no gametophytes were present in the soil. In 12 samples out of 31, the number of aboveground sporophytes was lower than the number of subterranean gametophytes found in the soil. In ten samples out of 31, the number of aboveground sporophytes was higher than the number of subterranean gametophytes found in the soil. In two samples, the ratio of aboveground and belowground structures found was equal. In four samples, no juvenile sporophytes and no subterranean gametophytes were found. In one sample from Žilinėliai no juvenile sporophytes were present, but two gametophytes were found. In the soil samples at the Puvočiai and Beržupis sites, gametophytes were not found, only decaying pieces were present. There were no gametophytes found in one sample from Bingeliai and Glėbas.

We split the diversity of Type I gametophytes into four groups (globular, disk shape, irregular bowl shape and irregular bowl shape with sprout). Type II gametophytes were categorized into two groups (carrot shape and carrot shape with sprout). These groups represent different developmental stages of gametophytes (Fig. 13).

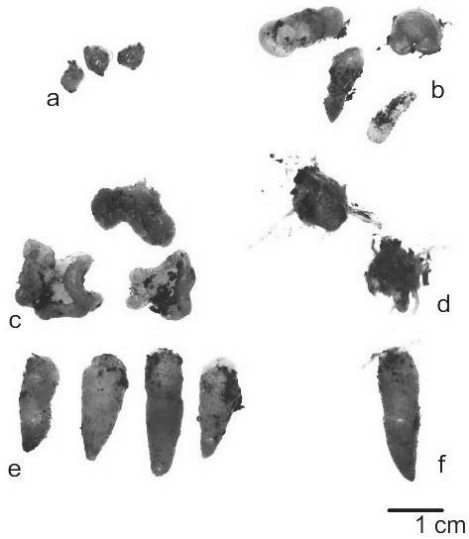

Fig. 13. Diversity of club moss gametophytes found in research sites
Type I (*Lycopodium* sp.): six groups: a – globular, b – disk shape, c – irregular bowl shape, d – irregular bowl shape with a sprout; Type II (*Diphasiastrum* sp.): e – carrot shape and f – carrot shape with a sprout (Rimgailė-Voicik et al., 2015)

In 31 soil samples collected in nine localities, we found only 16 globular *Lycopodium* gametophytes (Table 6) and their length did not exceeded 3 mm. All gametophytes of this developmental stage were yellowish gray, without rhizoids and with a smooth surface or with sporadically situated tubercles. We also registered 80 disk shape gametophytes. In most cases their length was 4 mm, and a maximum length of 9 mm and minimum length of 2 mm were recorded. These 80 gametophytes were plain with thickened edges and were sparsely covered with rhizoids. The largest group recorded was of the irregular bowl shape gametophytes (372). Their average length was 5 mm, the maximum length was 22 mm and the minimum length was 2 mm. Fifty-four irregular bowl shaped gametophytes had sprouts of sporophytes, occurring from fertilized ovum. The

average length of gametophytes with sporophyte sprouts was 4 mm, and the maximum length was 18 mm and minimum length was 3 mm. All gametophytes in this group were covered with rhizoids and had sprouts of sporophytes of different developmental stages (from achlorophyllous to photosynthesizing).

Table 6. The number of different developmental stage club moss gametophytes and juvenile sporophytes found in soil samples

Gametophytes and sporophytes	2014	2013	2012	**In total**
Globular	5	3	8	**16**
Disk shape	33	7	40	**80**
Bowl shape	69	183	120	**372**
Bowl shape with sprout	12	22	20	**54**
Carrot shape	3	33	31	**67**
Carrot shape with sprout	0	5	2	**7**
Gametophytes in total	**122**	**253**	**221**	**595**
L. annotinum juvenile sporophytes	108	26	21	**155**
L. clavatum sprouts	0	1	2	**3**
D. complanatum sprouts	1	0	3	**4**
Juvenile sporophytes in total	**109**	**27**	**26**	**162**

Regarding Type II gametophytes, 67 brownish-orange individuals were considered tear drop to carrot-beetroot shape. In many cases gametangial caps were not expressed; also, rhizoids were absent or occurred singly. Type II gametophytes were of different stages of maturity as gametangial caps were unevenly developed. Gametophyte lower parts were angled off to the right or left. The possible reason for flexion was obstacles in the soil. The average length of gametophytes was 9.4 ± 2.3 mm, the average width was 3.7 ± 0.9 mm, the maximum length determined was 13.4 mm and the maximum width was 5.9 mm. Seven Type II gametophytes were found with juvenile sporophytes; their average length was 15 mm, the maximum length was 20 mm and the minimum length was 4 mm. In most cases, rhizoids were present on the top of the gametophyte. Sporophyte sprouts were achlorophyllous and in different stages of development.

Voucher specimens of *D. complanatum* and *Lycopodium* sp. gametophytes and juvenile sporophytes were deposited at the Vilnius University Herbarium (WI).

3.4. Spatial analysis of subterranean gametophyte and juvenile sporophyte populations

Higher order nearest neighbor analysis of Maskauka, Varėnė and Bingeliai samples (Fig. 14) showed that gametophytes and juvenile sporophytes tend to cluster and their distribution in the soil is not absolutely random. Juvenile sporophytes tend to cluster more.

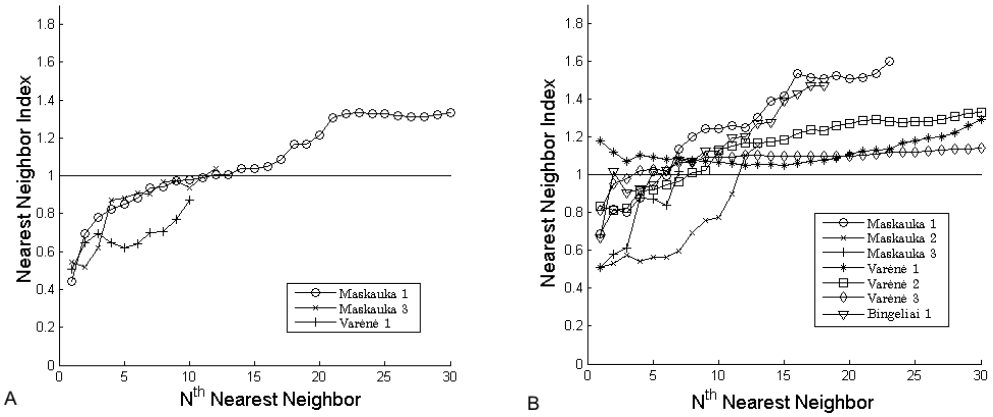

Fig. 14. Spatial neighborhood structure of club moss populations within sample plots (1-3) in southern Lithuania: Maskauka, Varėnė and Bingeliai research sites

A – juvenile sporophytes, B – gametophytes. Values < 1 indicate aggregation, = 1 random distribution, > 1 regular distribution (Rimgailė-Voicik et al., 2015)

The nearest neighbor index (NNI) in Maskauka and Varėnė samples were in the rage of 0.44–0.55 with an average distance (r_A) between juvenile club mosses of 1.65–3.78 cm (Table 6). In all cases, these distances were smaller than expected in a random distribution. Clasterization of gametophytes was not that apparent; the NNI in all research samples was 0.51–0.84. The highest NNI was in the Varėnė samples. In one Varėnė sample, the NNI was above one: 1.18. This shows a larger distance than expected in a random distribution between the gametophyte groups, and the r_A here was larger than expected: 5.05 cm (Table 7). In the other Varėnė

sample, 133 gametophytes were found and the NNI was 0.81 and the distribution was not absolutely random.

Table 7. Neighborhood analysis of juvenile *Lycopodium* populations.

Site/Stage	N	r_A	σr_A	r_E	NNI	P-value*
Maskauka (plot 1)						
Gametophytes	24	3.52	3.54	5.10	0.69	0.01
Juvenile sporophytes	45	1.65	1.1	3.72	0.44	0.0001
Maskauka (plot 2)						
Gametophytes	14	3.28	3.64	6.68	0.51	0.001
Maskauka (plot 3)						
Gametophytes	8	4.49	5.37	8.84	0.51	0.01
Juvenile sporophytes	13	3.78	4.445	6.93	0.55	0.01
Varėnė (plot 1)						
Gametophytes	34	5.05	4.03	4.29	1.18	0.05
Juvenile sporophytes	11	3.83	2.24	7.54	0.51	0.01
Varėnė (plot 2)						
Gametophytes	40	3.3	3.2	3.95	0.84	0.05
Varėnė (plot 3)						
Gametophytes	133	1.76	1.05	2.17	0.81	0.0001
Bingeliai (plot 1)						
Gametophytes	19	3.83	2.71	5.74	0.67	0.01

Notes: N – sample size; r_A – observed mean nearest neighbor distance (cm); σr_A – standard deviation of r_A; r_E – mean expected nearest neighbor distance (cm); NNI – nearest neighbor index for first-order nearest neighbors (values < 1 indicate aggregation, = 1 random distribution, > 1 regular distribution) *Test if the NNI differs significantly from 1 (a random distribution).

All gametophytes found were located in the humus layer, in 0.1–2 cm depth. In most cases, globular, irregular bowl shape and irregular bowl shape with sprout gametophytes were found at a depth of 0.2–0.3 cm. Disk shape gametophytes were found at 0.2–0.4 cm in depth. Several disk shaped gametophytes occurred at a depth of 1 cm, irregular bowl gametophytes with sprouts were found at 1.7 cm and irregular bowl shape gametophytes were found at 2 cm in depth. In most cases gametophytes occurred at a depth of 0.2–0.4 cm. The upper part of Type II gametophytes were located at a depth of 0.2 cm, but few were found in the depth of 1.2–1.8 cm. No correlation was found between the length of gametophytes and the depth in which they were found (r_{xy} = 0.113, p = 0.06). Further analysis between different gametophyte shapes and their origin depth did not provide additional insight.

3.5. Relationships between subterranean gametophyte and juvenile sporophyte abundance and soil properties

The reasons for local occurrence and development of juvenile club moss populations remain unclear. We determined the main soil components that might influence club moss population development. Adonis function in R revealed that only the amount of mineral nitrogen differed significantly among sites ($R2 = 0.135$, $p < 0.05$). The pH was acidic in all sites (3.1–3.7) and all other parameters (Table 8) were similar. The amount of organic carbon varied (0.84–3.95) and was highest in the Puvočiai site where pH was the lowest (3.1) and no gametophytes were present. Žilinėliai had the highest quantities of all other parameters measured (P_2O_5, K_2O, Ca, Mg, N total etc.), but Žilinėliai was not rich with gametophytes: only six gametophytes and nine juvenile sporophytes were found in total. Maskauka I had the lowest amounts of P_2O_5, K_2O and total C, while the Bingeliai site had the lowest amounts of Ca, Mg and second lowest amounts of P_2O_5 and K_2O. Bingeliai and Maskauka I were quite similar according to the occurence rate of different developmental stage club mosses and the number of gametophytes found were similar (45 in Maskauka I and 38 in Bingeliai). The Varėnė I site outstanded with 207 gametophytes present, but the soil parameters determined did not greatly differ from the other eight sites.

We can conclude that soil in sites with abundant juvenile club moss populations was extremely acidic and outstanded as poor, with low level of humus and nutrients.

We plotted soil parameters on an NMDS plot generated with Bray-Curtis dissimilarity on floristic data from nine research sites. The number of gametophytes found in sites correlated positively with increasing pH value while the amount of mineral nitrogen decreased (Fig. 15).

Table 8. Soil characteristics determined in nine sites with juvenile club moss populations.

Site/parameter	pH, 1mol/l KCl	P_2O_5, mg/kg	K_2O, mg/kg	C total, %	Ca, mg/kg	Mg, mg/kg	N total, %	$N\text{-}NO_3$ and $N\text{-}NO_2$, mg/kg	NH_4-N, mg/kg	$N\text{-}NO_3$, $N\text{-}NO_2$ and NH_4-N, mg/kg
Beržupis	3.5	28	82	3.18	392	86	0.131	0.2	22.84	23.04
Bingeliai	3.4	20	50	1.99	137	37	0.094	0.11	8.42	8.53
Glėbas	3.7	24	68	2.55	279	61	0.131	0.17	6.12	6.29
Maskauka I	3.5	17	50	0.84	250	60	0.036	0.11	7.17	7.18
Maskauka II	3.4	24	86	2.85	299	61	0.151	0.19	11.99	12.18
Puvočiai	3.1	33	87	3.95	235	77	0.166	0.23	17.71	17.94
Varėnė I	3.5	22	67	2.53	207	59	0.123	0.19	11.94	12.13
Varėnė II	3.6	31	78	2.57	343	87	0.126	0.17	10.77	10.94
Žilinėliai	3.2	44	110	3.8	494	92	0.194	0.18	15.49	15.67

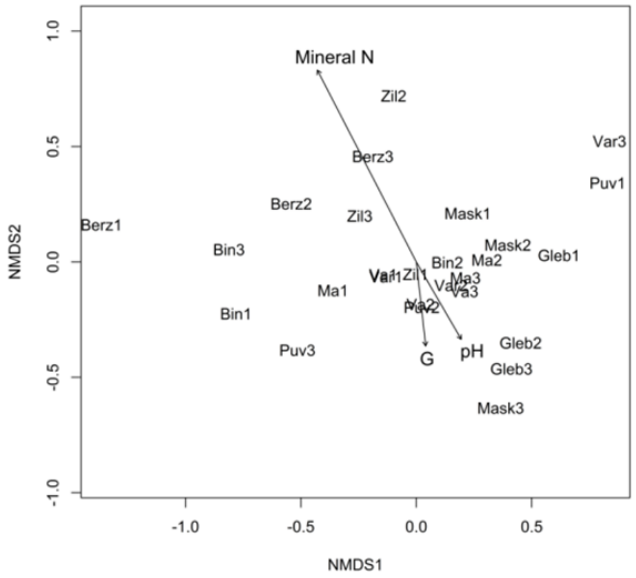

Fig. 15. Multivariate structure of the dry pine forest communities with juvenile club moss populations based on Bray-Curtis distances. pH and G vectors represent a positive relationship between the increasing number of gametophytes and pH value in a sample, while Mineral N shows a negative relationship with nitrogen in the two other vectors. Bin1, Bin2, Bin3 – Bingeliai samples; Gleb1, Gleb2, Gleb3 – Glėbas samples; Berz1, Berz2, Berz3 – Beržupis samples; Zil1, Zil2, Zil3 – Žilinėliai samples; Var1, Var2, Var3 – Varėnė II samples; Va1, Va2, Va3 – Varėnė I samples; Mas1, Mas2, Mas3 – Maskauka I samples; Mask1, Mask2, Mask3 – Maskauka II samples; Puv1, Puv2, Puv3 – Puvočiai samples.

3.6. Relationships between subterranean gametophyte and juvenile sporophyte abundance and vegetation cover

The number of gametophytes found in one assessed soil sample varied from two to 133. The Pearson correlation coefficient showed moderate positive association between the number of gametophytes and juvenile sporophytes (r_{xy} = 0.57; $p < 0.05$). We used species cover data to test for a correlation among different species. A weak positive correlation was found between *Deschampsia flexuosa* and *L. annotinum* (r_{xy} = 0.39; $p < 0.05$). *Deschampsia flexuosa* and *Vaccinium myrtillus* L. (r_{xy} = -0.41; $p < 0.05$), *L. annotinum* and *V. myrtillus* (r_{xy} = -0.37; $p < 0.05$) and *D. flexuosa* and *Dicranum polysetum* (r_{xy} = -0.45; $p < 0.05$) had moderately negative Pearson correlations.

NMDS (Fig. 16) showed that samples with a larger number of gametophytes found were more similar and that increasing number of juvenile club moss sporophytes was related with a larger subterranean gametophyte population in a sample.

All Beržupis samples, two Bingeliai samples and two Žilinėliai samples were more scattered in the NMDS plot, with a Kendall's stress value of 7%. Vegetation data from other samples tended to cluster. No gametophytes were found in one out of three samples in the Maskauka II, Varėnė II, Bingeliai, Žilinėliai and Glėbas sites. Puvočiai samples were scattered in all plots and seemed to be very different from each other. According to the Renyi diversity profiles (Fig. 17), the eight field sites cannot be ranked, as curves cross each other or intersect at least once. The Žilinėliai site had the highest species richness and lowest coverage of the most abundant species. The same results were seen in the NMDS plot.

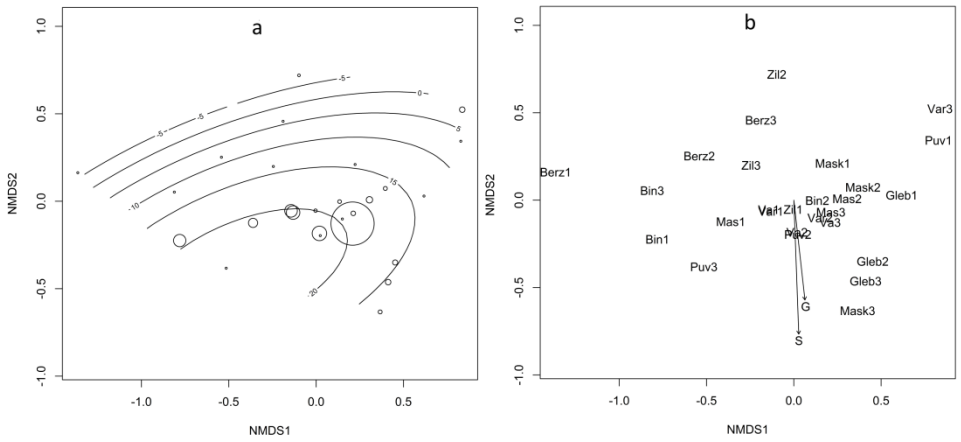

Fig. 16. Multivariate structure of the dry pine forest communities with juvenile club moss populations based on Bray-Curtis distances: a) samples plotted with nonmetric multidimentional scaling (NMDS); b) contour lines representing the average number of gametophytes found, fit after the ordination. S and G vectors represent positive relationship between the increasing number of juvenile sporophytes and gametophytes in a sample.

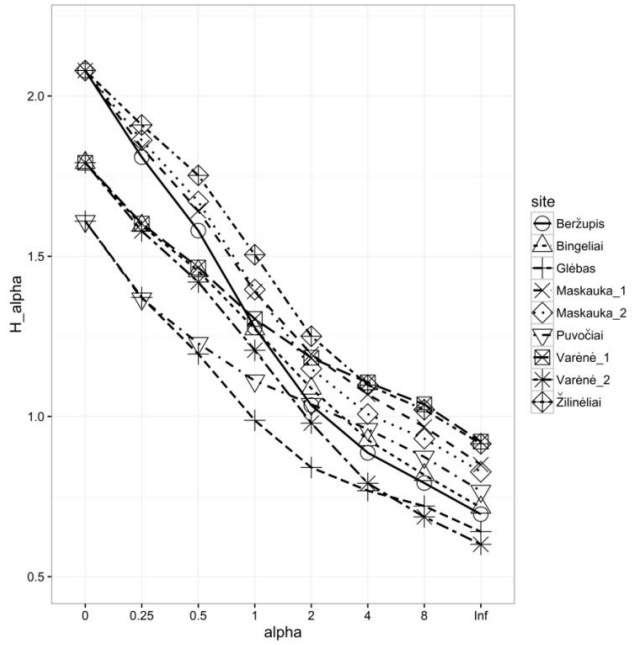

Fig. 17. Renyi diversity profiles of nine sites with juvenile club moss populations. The scale parameter (α) gives the order of Renyi diversity; α = 0 is the logarithm of species richness, α = 1 is the Shannon diversity index, α = 2 is the logarithm of the reciprocal Simpson's diversity index, α = Inf refers to the proportion of the most abundant species. The x and y-axis show, respectively, the alpha value of Renyi's formula and their associated Renyi diversity profile values (Rimgailė-Voicik and Naujalis, 2016)

3.7. Vegetation cover change in a dry pine forest community with a juvenile *Lycopodium* population

The flora in the permanent Maskauka site was not particularly species-rich, and over four years, species richness varied slightly (Table 9). The species accumulation curve based on data from the Maskauka site reached an asymptote in 2012 after less than 50 subplots out of 100 were sampled. In all other years, an asymptote was never reached. The Renyi diversity profile indicated that the evaluated years cannot be ranked. The highest diversity according to species richness was in 2015, but when common species had more weight (Simpson's-Yule index for $\alpha = 2$), all years were similar.

Table 9. Characteristics of the vegetation in a 100 m^2 research plot with juvenile Lycopodiaceae sporophytes and gametophytes in the Varėna District (54.28202, 24.59892 WGS; Rimgailė-Voick and Naujalis, 2016).

Characteristics	2012	2013	2014	2015
Number of quadrats* with juvenile club moss individuals in 2012	12	11	13	13
Average ± SD of herb-subshrub layer cover (%),	34 ± 23	35 ± 23	34 ± 23	36.3 ± 15
Average ± SD cover of moss-lichen layer (%)	95 ± 14	95 ± 13	95 ± 14	98 ± 9
Absolute abundance of the most abundant species:				
Pleurozium schreberi	52.1	51.7	54.4	52.9
Deschampsia flexuosa	20.6	21.3	20.3	20.4
Dicranum polysetum	17.5	17.5	15.1	14.0
Vaccinium myrtillus	1.1	1.1	1.4	2.7
Vaccinium vitis-idaea	0.7	0.7	0.8	1.5
Number of vascular plant species	7	8	10	13
Number of moss-lichen species	6	4	6	5
Total estimated species richness	13	12	16	18

Total number of quadrats was 100. SD – standard deviation

The average coverage of grass-subshrubs in subplots with juvenile sporophytes was 46%. *Deschampsia flexuosa* was identified in 96 subplots. *Deschampsia flexuosa* appeared together with *Lycopodium annotinum* in all subplots (average cover of 35%). The correlation matrix showed a weak, but significantly positive correlation between *D. flexuosa* and *L. annotinum* cover ($r_{xy} = 0.22$; $p<0.05$) using average cover over four years in the calculation. No significant correlation was detected between *L. annotinum* and any other species.

Statistical analysis revealed that subplots not shaded by the tree canopy were more stable in regards to species diversity and abundance, and did not change significantly during the research period. Juvenile lycopods were identified in subplots that were not shaded. The subplots with and without tree canopy shading differed significantly from 2012 to 2014 (permutational MANOVA on Bray-Curtis distances; $R2 = 0.05$; $p < 0.01$), but not in 2015. The Shannon index among subplots in different years differed significantly (repeated measures ANOVA with a Greenhouse-Geisser correction; $F = 19.1649$; $p < 0.0001$). To detect where the difference emerged, post hoc tests using the Bonferroni correction were applied and results revealed that only 2015 significantly differed from all other years ($p < 0.01$). Further analysis showed that the Shannon index significantly differed between time points (repeated measures ANOVA; $F = 18.08$; $p < 0.0001$) and when subplots with only tree canopy cover were included in the analysis. Simpson's index also differed significantly between time points (repeated measures ANOVA; $F = 18.17$; $p < 0.0001$) and post hoc tests using the Bonferroni correction revealed that only 2015 significantly differed from all other years ($p < 0.01$). Also, Simpson's index differed significantly between years ($F = 18.08$; $p < 0.0001$) when only subplots with tree canopy cover were included.

In the Maskauka permanent research site, the tree canopy layer was around 40%, and the shrub layer was absent. In subplots with juvenile club moss sporophytes, no pine trunks were present and the canopy did not shade club moss sporophytes. For most subplots, the grass-subshrub layer covered more than 50% and remained unaffected. Club mosses were determined to cover 10% of the study site; the average cover increased from 2% in 2012 to 3% in 2015.

Every year in the Maskauka subplots, seedlings of *Frangula alnus* Mill., *Betula pendula* Roth, *B. pubescens* Ehrh., *P. sylvestris*, *Juniperus communis* L. and *Quercus robur* L. were observed. One *Q. robur* seedling remained throughout the study, but the other seedlings perished. Only *P. sylvestris* seedlings occurred in subplots with juvenile club mosses, but they did not overwinter.

The total cover of mosses and lichens was close to 100%. Only *Pleurozium schreberi* (Brid.) Mitt. occurred in all subplots together with juvenile sporophytes and the average coverage of *P. schreberi* was 88%. *Dicranum polysetum* Sw. had an average coverage of 10% in nine out of 14 subplots with juvenile club mosses present. In one subplot, juvenile sporophytes occurred together with *Dicranum scoparium* Hedw. (coverage: 10%). Even though statistical analysis showed that subplots with juvenile club mosses had stable vegetation, in these subplots new moss species were detected that did not overwinter or were replaced by other species. In 2012 in one subplot, *Hylocomium splendens* (Hedw.) Schimp. (coverage 5%) occurred, and in 2014 in one subplot, *Polytrichum piliferum* Hedw occurred.

Throughout our research, juvenile club moss sporophytes were detected in 14 out of the 100 assessed subplots (Table 9). Juvenile *L. annotinum* dominated the subplots; *L. clavatum* growing with *L. annotinum* was only observed in one subplot. *Diphasiastrum* species did not occur. In 2012, we observed 35 shoots above the moss layer in twelve subplots. Three of the 35 observed shoots had both orthotropic and plagiotropic growth in 2012, but no drastic changes were observed in other years.

In 2015, 20 soil samples were analyzed and 23 juvenile sporophytes were found: 22 *L. annotinum* and one *L. clavatum*. Additionally, three juvenile *L. annotinum* sporophytes were found dead and covered with mosses. In the humus horizon of one sample, two irregular bowl shape gametophytes (Type I, *Lycopodium clavatum* type, according to Bruchmann, 1898: *Lycopodium* sp.; 1.5 × 1.8 cm and 0.4 × 1.2 cm) were found at a depth of 0.2 cm. Nine out of 23 juvenile sporophytes had orthotropic growth, and the remaining 14 had both orthotropic and plagiotropic growth. The length, branching pattern and other growth traits were uneven (Table 10). According to shoot branching, the population of juvenile sporophytes was attributed to three groups that resembled different developmental stages: 1) juvenile sporophytes with orthotropic

branchless shoots; 2) juvenile sporophytes with orthotropic shoots and short branches; and 3) juvenile sporophytes with plagiotropic and orthotropic growth. The chemical analysis of the top 0–10 cm of soil in the permanent research site showed that soil was acidic (pH 5), total nitrogen was 1.3 ± 0.11 g/kg and total phosphorus was 10 ± 1 mg/kg.

Table 10. Data on club moss juvenile sporophytes from the Maskauka permanent research site in 2015 (Rimgailė-Voick and Naujalis, 2016).

Characteristics	Average and SD	Max	Min
Moss layer thickness, cm	4.65 ± 1.87	9	2
Humus layer, cm	2.36 ± 0.97	4	0.8
Number of juvenile sporophytes per sample*	1.21 ± 0.71	4	1
Number of annual constrictions	3.68 ± 1.55	8	2
Number of roots	2.52 ± 1.16	6	1
Number of branches	4.04 ± 3.5	13	1
Distance between branches, cm	2.43 ± 1.77	17	0.8
Length of branches, cm	3.27 ± 2.97	12.3	0.2
Length of juvenile sporophytes, cm	16.39 ± 7.87	39.9	7.2

*A total of 23 juvenile sporophytes at different developmental stages were found.

3.8. Assessment of genetic structure in club moss populations by ISSR polymorphism

During the genetic analysis 103 individuals from ten Varėna District populations were examined: 50 *L. annotinum* and 53 *L. clavatum* plants. The level of polymorphism within and among five *L. clavatum* and five *L. annotinum* populations was analysed using four ISSR primers. In *L. clavatum* populations the total DNA band number was 129 and in *L. annotinum* the total DNA band number was 127. The size of the amplified fragments in both species ranged from 280 to 1,800, six of them were monomorphic (Table 11).

The highest number of bands was analysed from samples collected from the Maskauka LA and Maskauka LC populations. The UPGMA method revealed that all populations analysed fell into two large groups that represent species *L.*

clavatum and *L. annotinum* (Supplement 2). All plants were more similar within the populations (Supplement 3 and 4).

All populations had low DNA polymorphisms. Average polymorphism was lower in *L. clavatum* (18.37%) populations than in *L. annotinum* (22.97%) populations. The highest polymorphism was measured within *L. annotinum* collected from the Maskauka population (35.14%; Table 12).

Table 11. DNA polymorphism of club moss species determined by ISSR analysis

Primer	Sequence5'→3'	Analysed bans	Monomorfic/ bands	Polymorfic	DNA band length (bp)	Polymorphism, %
			L. annotinum			
ISSR B	$(AG)_8CG$	23	0	23	320-1800	100
ISSR C	$(AG)_8TG$	34	1	33	280-1800	97
ISSR I-28	$(GT)_6CG$	34	1	33	320-1800	97
ISSR I-50a	$CCA(GCT)_4$	36	0	36	380-1800	100
	In total	127	2	125	280-1800	98
			L. clavatum			
ISSR B	$(AG)_8CG$	24	2	22	280-1200	91
ISSR C	$(AG)_8TG$	31	0	31	320-1800	100
ISSR I-28	$(GT)_6CG$	35	1	34	320-1600	97
ISSR I-50a	$CCA(GCT)_4$	39	1	38	320-1800	97
	In total	129	4	125	280-1800	96

The highest Nei's distance, calculated as 0.36, was found among *L. annotinum* genotypes in Žilinėliai. The highest Nei's distance among *L. clavatum* genotypes was in Varėnė and was calculated as 0.26 (Table 13).

The coefficient of genetic differentiation between populations (G_{ST}) based on ISSR polymorphism analysis was 0.74 for *L. clavatum* populations and 0.69 for *L. annotinum* (Table 14). The gene flow parameter N_m, that represents the rate of migration among populations, was approximately 0.2 in all cases. The number of observed alleles exceeded the number of effective alleles in all populations studied.

Table 12. Genetic characteristics of 10 club moss populations based on ISSR polymorphism analysis

Population	No. of individuals	No. of bands	No. of unique bands	No. of polymorphic loci	Polymorphism, %	Na[1] ±SD	Ne[2] ± SD	h[3] ± SD	I[4] ± SD
Puvočiai LC	10	60	1	20	13.51	1.135 ± 0.343	1.080 ± 0.232	0.047 ± 0.129	0.070 ± 0.188
Žilinėliai LC	10	55	1	22	14.86	1.149 ± 0.357	1.112 ± 0.282	0.062 ± 0.154	0.090 ± 0.220
Varėnė LC	12	60	1	29	19.59	1.196 ± 0.398	1.134 ± 0.309	0.074 ± 0.163	0.109 ± 0.233
Maskauka LC	10	82	8	34	22.97	1.230 ± 0.422	1.159 ± 0.313	0.091 ± 0.173	0.133 ± 0.250
Glėbas LC	11	66	3	31	20.95	1.210 ± 0.408	1.126 ± 0.286	0.073 ± 0.157	0.108 ± 0.226
Puvočiai LA	10	49	0	23	15.54	1.155 ± 0.364	1.101 ± 0.258	0.059 ± 0.144	0.087 ± 0.209
Žilinėliai LA	10	58	0	36	24.32	1.243 ± 0.431	1.131 ± 0.265	0.081 ± 0.154	0.123 ± 0.229
Varėnė LA	10	56	0	27	18.24	1.182 ± 0.388	1.099 ± 0.245	0.060 ± 0.138	0.091 ± 0.204
Maskauka LA	10	95	11	52	35.14	1.351 ± 0.479	1.220 ± 0.335	0.130 ± 0.187	0.193 ± 0.273
Glėbas LA	10	62	2	32	21.62	1.216 ± 0.413	1.123 ± 0.275	0.073 ± 0.153	0.110 ± 0.224

1 – observed number of alleles, 2 – effective number of alleles (Kimura and Crow, 1964) 3 – Nei's (1973) genetic diversity (average expected heterozygosity), 4 – Shannon index

Table 13. Parameters of Nei and Li's genetic distances determined among ten club moss populations

	L. annotinum				
	Puvočiai	Žilinėliai	Varėnė	Maskauka	Glėbas
GDpop[1]	0.16	0.26	0.15	0.18	0.15
GDmin[2]	0.04	0.2	0.04	0.07	0.04
GDmax[3]	0.29	0.36	0.29	0.27	0.28
	L. clavatum				
	Puvočiai	Žilinėliai	Varėnė	Maskauka	Glėbas
GDpop	0.08	0.11	0.15	0.01	0.12
GDmin	0.03	0.03	0.01	0.02	0.04
GDmax	0.15	0.20	0.26	0.16	0.23

1 – mean genetic distance; 2 – minimum genetic distance; 3 – maximum genetic distance

Dendrograms based on UPGMA analysis of ISSR data (Fig. 18) indicated that Maskauka populations of *L. clavatum* and *L. annotinum* were the most divergent. PCoA plots generated separately for *L. clavatum* and *L. annotinum* populations showed that *L. annotinum* populations from Žilinėliai, Glėbas and Puvočiai were closely related, while plants collected from Varėnė and Maskauka were more genetically divergent. *Lycopodium clavatum* populations of Žilinėliai, Glėbas and

Varėnė were closely distributed in the PCoA plot while Maskauka and Puvočiai showed distinct parameters.

Table 14. Among-population diversity parameters established using ISSR markers

Diversity parameter/sample size	*L. clavatum*	*L. annotinum*
No. of plants	53	50
G_{ST}[1]	0.74	0.69
N_m[2]	0.18	0.22
Na[3] ±SD	1.818 ± 0.388	1.851 ± 0.357
Ne[4] ± SD	1.437 ± 0.345	1.442 ± 0.371
h[5] ± SD	0.262 ± 0.179	0.260 ± 0.186
I[6] ± SD	0.398 ± 0.247	0.395 ± 0.250

1 – mean genetic differentiation between populations; 2 – estimated gene flow between populations; 3 – observed number of effective alleles; 4 – estimated number of effective alleles; 5 – Nei's genetic diversity; 6 – Shannon's information index

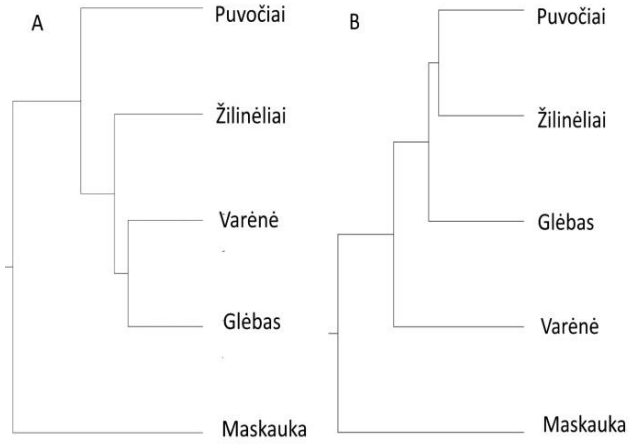

Fig. 18. Dendrograms based on Nei's genetic distance measures using UPGMA. A – *L. clavatum* populations, B – *L. annotinum* populations

Analysis of molecular variance (AMOVA), calculated on genetic distance matrix (Table 15), showed that 69% of genetic variability was among *L. annotinum* populations while within populations the variance was 31%. Genetic differentiation among *L. clavatum* populations was higher (78%) while the variance within the population was only 22%.

Table 15. Analysis of molecular variance (AMOVA) for ISSR data of 103 individuals in ten populations of club mosses with a p-value of 0.001

Source of variation	Df[1]	SS[2]	MS[3]	Est. var.[4]	%[5]
L. annotinum					
Among populations	4	687.48	171.87	16.4	69%
Within populations	45	339	7.53	7.53	31%
L. clavatum					
Among populations	4	885.18	221.29	20.36	78%
Within populations	48	278.05	5.79	5.79	22%

1 – degrees of freedom; 2 – sum of squares, 3 – mean of squares, 4 – variance components, 5 – percentage of variation

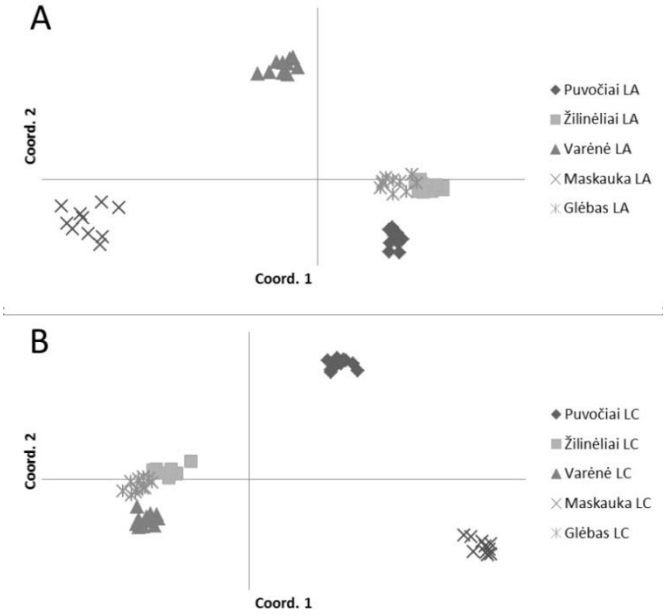

Fig. 19. Principal coordinate analysis (PCoA) plot based on ISSR polymorphism showing similarity and genetic variation among A – five populations of *L. annotinum* and B – five populations of *L. clavatum*

According to Nei's genetic identity, the populations from Glėbas LC and Varėnė LC (84.4%), and Žilinėliai LA and Puvočiai LA (84.1%) were similar. According to Nei's genetic distance, the populations from Maskauka LC and Puvočiai LC (46.3%), and Žilinėliai LA and Maskauka LA (42%) had the least similarities (Table 16).

Table 16. Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

L. clavatum					
	Puvočiai LC	Žilinėliai LC	Varėnė LC	Maskauka LC	Glėbas LC
Puvočiai LC	****	0.784	0.728	0.629	0.770
Žilinėliai LC	0.244	****	0.819	0.643	0.819
Varėnė LC	0.318	0.200	****	0.645	**0.844**
Maskauka LC	**0.463**	0.442	0.438	****	0.694
Glėbas LC	0.262	0.201	0.170	0.365	****
L. annotinum					
	Puvočiai LA	Žilinėliai LA	Varėnė LA	Maskauka LA	Glėbas LA
Puvočiai LA	****	**0.841**	0.752	0.684	0.816
Žilinėliai LA	0.173	****	0.783	0.657	0.834
Varėnė LA	0.285	0.245	****	0.736	0.775
Maskauka LA	0.375	**0.420**	0.306	****	0.676
Glėbas LA	0.204	0.182	0.255	0.391	****

4. DISCUSSION

The stability of lycopods external and internal features manifested since the Devonian period may be explained in terms of a long-lasting relationship between the organism and genetic homeostasis or the type habitats (Levin and Crepet, 1973). Few studies have addressed genetic diversity and population structure of club mosses. Scientific data regarding the ecology and population function of lycopods is also limited and many fundamental questions remain unanswered. Research on juvenile club moss populations in nature and descriptions of habitats are especially rare. The sexual generation of club mosses — gametophytes (or prothallia) — are the least researched, especially subterranean long-lived achlorophyllous gametophytes. From 1873 to present, fewer than 25 scientific articles on juvenile club mosses and subterranean gametophytes found in nature have been published.

To our knowledge, there are no articles published on the dynamics of subterranean club moss gametophyte populations. Our research in the pine forests of southern Lithuania showed that in certain forest sites there are abundant populations of club moss gametophytes (potentially up to 500 in 1 m^2). Other researchers also described abundant subterranean club moss gametophyte populations. For example, in Massachusetts, Degener (1924) collected around 300 *Lycopodium annotinum* and *L. clavatum* gametophytes, and Stokey and Starr (1924) found more than 100 juvenile *Diphasiastrum complanatum* sporophytes. From 31 samples taken from nine juvenile sporophyte growth sites in the Varėna District, 20 samples held subterranean gametophytes. Moderate correlation between the number of juvenile sporophytes and the number of gametophytes found in soil samples was observed. Various authors (Bruchmann, 1898; Degener, 1924; Gauthier and Dumais, 1938) emphasized that gametophytes can only be diagnosed by juvenile sporophytes, which usually can be found in places where club mosses do not grow. Spessard (1922), Stokey and Starr (1924) suggested to

look for gametophytes in conditions less extreme than those in which parent plants grow.

There is currently no non-invasive method to determine boundaries of subterranean gametophyte populations, thus we can only address juvenile club moss sporophyte distribution. The ratio of belowground gametophytes and aboveground juvenile sporophytes was uneven and varied. In 12 samples out of 31, the number of aboveground sporophytes was lower than the number of subterranean gametophytes found in the soil. In ten samples out of 31, the number of aboveground sporophytes was higher than the number of subterranean gametophytes found in the soil. When comparing belowground density of gametophytes to the aboveground density of sporophytes, there was a remarkable lack of ferns present (Milberg, 1991; Rydgren and Hestmark, 1997). When *Botrychium* was observed, belowground density of gametophytes exceeded the aboveground density of sporophytes several times (Johnson-Groh et al., 2002).

Juvenile sporophytes at early developmental stages may look similar to *Polytrichum* mosses as they have isophyllous leaves and only orthotropic growth, and may be incipient with gametophytes (Bruchmann, 1898; Spessard, 1922; Eames, 1942; Wagner and Beitel, 1992; Naujalis, 1995). A successful search depends on the size of the sample. It is better to collect a smaller soil sample in the field, because larger samples that are used for spatial analysis (25 × 25 or 50 × 50 cm) may take two to four days to analyze depending on the size and the number of gametophytes present (Naujalis, 1995; Rimgailė-Voicik et al., 2015). Spessard (1922) analyzed square soil samples of 4–5 cm in depth and recalled that the analysis was difficult and extremely tiring.

The height of the moss layer must also be taken into consideration. A well-developed moss layer might be up to 20 cm in height and then only the tops of juvenile sporophytes might be seen; thus, there is a better chance to find subterranean club moss gametophytes in forest sites with low moss cover. After a few years, young sporophyte shoots outgrow mosses, but by then underground

gametophytes might die and decompose. The annual growth rate of juvenile club moss sporophytes was about 1–3 cm (Naujalis, 1995), while in temperate forests the annual growth rate of mosses *Hylocomium splendens* and *Pleurozium schreberi* was about 2.6 cm and 2.1 cm, respectively (Zechmeister, 1995).

4.1. Structure and diversity of juvenile club moss populations

No 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 different development stages of juvenile club mosses found in the permanent Maskauka research site approved a hypothesis of asynchronous development within a club moss population. Our results support findings reported by Eames (1942): either the germination of spores occurs at different times, perhaps even years apart, or that the growth rate of gametophytes varies greatly as plants of different sizes and ages can sometimes be found together. The overall results indicated that juvenile club moss populations were formed from sporophytes of different ages that emerged from gametophytes and therefore are not likely to be genetically identical. It has generally been concluded that seedling establishment is infrequent in clonal plant populations and that such populations are numerically maintained mainly by vegetative propagation (Harper 1977; Cook, 1985). However, the degree of variation in rates of seedling recruitment between plant species and environments, and the consequences for clonal life histories of such a variation are far from clear (Eriksson, 1989). The pattern of recruitment will have impact on the overall dynamics of genets in populations, and of the effects of natural selection and thereby on life history evolution. In the Varėna District, *L. annotinum* and *L. clavatum* form two to five m^2 stands. They might appear after vegetative or generative propagation. In Poland, previously investigated stand size varied from five to 67 m^2 (Bogdanowicz and Sliwinska-

Wyrzychowska, 2008) while in Lithuania, sporulating stands were 30 to 32 m^2 (Naujalis, 1995).

We found more juvenile sporophytes than gametophytes in samples collected from Maskauka I. It is possible that in other research sites, the survival rate of sporophytes or the fertilization rate in gametophytes was lower than in this research site. Genetic variability within clones is rarely reported (Major and Ódor, 1999; Wittig et al., 2007). If a clone is one individual, after a certain time in a juvenile population only one individual should remain alive, but no evidence of this appeared during our four years of observation at the permanent Maskauka site. The juvenile club moss population in the permanent Maskauka site may have developed approximately ten to 15 years ago and the development remains continuous, as we found living gametophytes in 2015.

The main function of juvenile sporophyte populations is to occupy new forest sites. The question arose as to whether each stand represents unique individuals or whether one stand consists of one individual that was separated from the main clone during forest management. ISSR-PCR generated genetic distances were used for PCoA analysis and revealed that at the Maskauka site, *L. annotinum* and *L. clavatum* populations were the most divergent. We propose that the genetic polymorphism of the *L. annotinum* population at the Maskauka site was the largest because of ongoing sexual propagation and recruitment of juvenile sporophytes.

The determined gametophyte abundance in samples collected from sites 0.25 m^2 in size varied and represented different developmental stages of gametophytes. The largest group recorded was of irregular bowl shape gametophytes (372). No generalization about juvenile sporophyte groupings have been made, as many isolated (Bruchmann, 1898; Stokey and Starr, 1924; Horn et al., 2013) or grouped individuals were discovered (Stokey and Starr, 1924; Eames, 1942). The development of gametophyte populations was asynchronous. Our results suggest that in the soil humus horizon club moss gametophytes: 1) can grow separately, 2)

can come close without direct contact and 3) can come into direct contact. Usually, groups are formed from two to five gametophytes of different development stages. It has been proposed that these groups form gradually (Naujalis, 1995). Bisexual gametophytes can be considered an optimal reproductive system because the chance of fertilization is much higher when archegonia and antheridia are much closer; however, homozygosity increases potentially causing inbreeding depression (Charnov, 1982). Wittig et al. (2007) revealed unexpected genetic uniformity in large *L. annotinum* stands by using DAF analysis and concluded that microsatellites could give deeper insight into population structure. Our results were similar. We determined a G_{ST} value of 0.69 for *L. annotinum* populations while Wittig et al. (2007) reported 0.7. Additionally, 0.26 was calculated as the average Nei's genetic diversity for *L. annotinum* using ISSR markers for populations and that for *L. annotinum* populations in Germany was 0.3 (Wittig et al., 2007). Little interpopulation differentiation was observed and coupled with regional differentiation in *Huperzia lucidulum* populations (Levin and Crepet, 1973). AFLP markers revealed a low level of differentiation among ten *Huperzia serrata* populations with 86.5% of bands being polymorphic (Huang and He, 2010). In seven populations of *Isoetes sinensis* the average percentage of polymorphic bands was 35.2% when AFLP markers were used (Kang et al., 2005).

Most club moss gametophytes were found in the humus layer from 0.2 to 0.4 cm. Gametophytes of the same shape, i.e. the same stage of development, were found in the upper, middle and lower layers of the humus horizon. The nearest neighbor index (NNI) showed that club moss juvenile sporophytes tend to group more than gametophytes. Data suggest that occurring sporophytes may result from intergametophytic selfing, but in most cases the distances between gametophytes were too large and intragametophytic fertilization was plausible. It is possible that a closer arrangement in the population of juvenile sporophytes contributes to higher survival rates and increased competitiveness against specific biotic and

abiotic factors. Presumably, distances among gametophytes and sporophytes occurred as an effect of uneven distribution of nutrients. It seems plausible that intraspecific competition among gametophytes was stronger than among juvenile sporophytes. The processes of emergence and development of subterranean club moss gametophyte populations remains insufficiently investigated.

During recent (Rimgailė-Voicik et al., 2015) and former (Naujalis, 1995) research in Lithuania, few gametophytes with two or three sporophyte sprouts were discovered. Sporophytes were at different stages of development including photosynthesizing, above ground or those that were achlorophyllous and situated below ground without leaves. This supports the hypothesis that fertilization events in a single gametophyte can be repeated. More than one sporophyte growing from a gametophyte has been reported. According to Eames (1942), two are common, three to five occasional and even seven well-formed sporophytes have been found on one large gametophyte. Bruce and Beitel (1979) found 26 gametophytes with one sporophyte while 25 had more than one; one gametophyte had 13 emerging sporophytes!

4.2. Suitable habitats for juvenile club moss populations

The occurrence of club moss juvenile sporophytes in pine forests is highly determined by specific environmental conditions. Soil heterogeneity is influenced by variations in microtopography, microclimate, parent material, mycorrhizae and microorganisms (Stark, 1994). It is not clear if juvenile club mosses emerge from gametophytes every year or whether photosynthesis or mycorrhizae play the most important role for successful juvenile sporophyte recruitment. For example, during twenty years of field research on *Diphasiastrum alpinum*, gametophytes were only found in three sites (Horn et al., 2013). In suitable habitats, subterranean gametophytes and juvenile sporophytes usually form numerous prospering populations with individuals from one up to six species (Fankhauser,

1873; Bruchmann, 1898; Lang, 1899; Spessard, 1922; Stokey and Starr, 1924; Rimgailė-Voicik et al., 2015).

In the Varėna District of Lithuania, sites with adult *D. complanatum* were less diverse than sites with *L. clavatum*. Sites with adult *D. complanatum* were more similar to sites with juvenile club mosses: they had similar diversity and total species richness. According to the NMDS analysis, sites with juvenile club mosses were quite similar. Sites with juvenile club mosses were dominated by the grass *Deschampsia flexuosa*. The Renyi diversity index (Lövei et al., 2013) indicated that forest sites with juvenile club moss populations were slightly stressed and disturbed. Other research showed (Strengbom et al., 2004; Ruotsalainen et al., 2007) that decreased competition for light increases the size of areas dominated by *D. flexuosa* and this species can become dominant in disturbed forests. Various researchers noted (Fankhauser, 1873; Bruchmann, 1898; Lang, 1899; Spessard, 1922; Stokey and Starr, 1924; Degener, 1924; Gauthier and Dumais, 1938; Eames, 1942; Bruce and Beitel, 1979) that juvenile sporophytes and gametophytes do not appear near adult clones. Our results support these findings (Rimgailė-Voicik and Naujalis, 2015, 2016).

Forest humus chemistry is related to understory species and dominant conifers (Bradshaw and Zackrisso, 1990; Mallik, 2003). When comparing chemical soil characteristics with long-term research performed in nearby forests of the Varėna District (Armolaitis et al., 2011) and Lithuanian forest soil monitoring data (Beniušis, 2008), research sites can be attributed to pine forests never used as arable soil or fertilized. We propose that dry pine forest sites in the Varėna District that were not species rich and had no shrub layer and no adult club moss clones nearby are suitable to support juvenile club moss populations.

It can be assumed that in the same locality populations of gametophytes may exist for up to decades. The main factor for this stability might be a constant enrichment with new gametophytes and the longevity of gametophytes. This leads

us to conclude that environmental conditions necessary for gametophyte populations to occur are not temporary.

Among emerging juvenile sporophytes in the Varėna District, *L. annotinum* individuals dominated and gametophytes of *L. annotinum* comprised more than 80% of the total gametophytes found (Rimgailė-Voicik et al., 2015). The same proportion was discovered in 1986–1989 during research near Glėbas Lake, Lithuania (Naujalis, 1995). While Bingeliai and Maskauka I populations seemed to be the youngest, Varėnė II, Maskauka II and Puvočiai might have reached their final maturity stage.

In some locations where juvenile sporophyte populations were identified, the stems of *Pinus sylvestris* were charred. Presumably, repeated forest fires created preferable habitats for club moss populations. Previously, subterranean gametophytes and juvenile sporophytes were found on and near forest roads, tracks, near lines separating forest blocks and skiing tracks (Bruchmann, 1898; Degener, 1924; Horn et al., 2013; Muller et al., 2003; Stokey and Starr, 1924). In Lithuania, juvenile club moss populations also occurred in previously disturbed sites (Naujalis, 1995; Rimgailė-Voicik et al., 2015). Stokey and Starr (1924) described all localities with juvenile club moss populations as poor collecting grounds. Eames (1942) noted that a prosperous juvenile club moss population was discovered in a forest with trees and shrubs of 5–25 years old, grown after a forest fire following timber cutting. Stokey and Starr (1924) suggested that juvenile sporophytes would be more likely to occur after 10–12 years without a drought. During observations over seven years (1985–1991) in dry pine forests, more than 50% of *L. annotinum* juvenile sporophytes died (Naujalis, 1995). During four years research in the permanent Maskauka site, the main reason for juvenile sporophyte mortality appeared to be competition with the moss layer.

Development of juvenile club moss populations depend on other nearby species in the community. We found a weak significantly positive correlation between *D. flexuosa* and *L. annotinum* cover in the permanent Maskauka site, but no

significant correlations with other species were detected. Ódor (1996) argued that *L. clavatum* and *D. complanatum* can easily coexist with *V. myrtillus*. Our study showed a weak negative correlation between *L. annotinum* and *V. myrtillus*, but slight positive correlation between *L. annotinum* and *D. flexuosa*. In Varėna District study sites *D. flexuosa* predominated over the subshrubs *V. myrtillus* and *Vaccinium vitis-idaea* L. However, 40 years ago, *D. flexuosa* was considered a rare species in Lithuania (Natkevičaitė-Ivanauskienė, 1963).

Species composition in the permanent Maskauka site did not change over our four-year study, but reappearing tree seedlings showed the presence of open microniches. Cousens et al. (1985) suggested that sites for gametophyte development were rare and lead to clustering of spores of many different species in a small territory. This hypothesis was supported by the present study, as we found diverse developmental stages of gametophytes of different species in close proximity to each other.

4.3. Viable spore banks for juvenile club moss population recruitment

Club moss species composition depends on spores colonizing forest soil. Even though wind can carry spores from many different regions, the largest portion of spores in the soil should be from native club moss populations. Pine forests in the Varėna District showed a unique mosaic structure of the club moss populations: in a close vicinity, populations composed of different species and developmental stages were found. Club moss spore banks likely remain viable for long periods of time, as was estimated for ferns (Miller, 1968; Windham et al., 1986).

Lycopodium clavatum dominated among the sporulating plants in the Varėna District. A possible reason for this phenomenon might be an abnormality of *L. clavatum* spores resulting in unviable spore banks. The reversion phenomenon, when the apex resumes its vegetative growth after the production of generative structures, was only discovered in *L. annotinum* (Gola et al., 2015) and was connected with *LAMB1* gene expression in the sporogenous tissue (Svensson et

al., 2000). In three (Maskauka I, Varėnė I and Glėbas) sites, juvenile club mosses of all three species assessed were found growing in close vicinity, but sporulating individuals were never found together. Only a few juvenile sporophytes of *Diphasiastrum complanatum* were found (determination was approved by Karsten Horn).

In dry pine forests in southern Lithuania, club mosses of five different species sporulate: *L. annotinum*, *L. clavatum*, *D. complanatum*, *D. tristachyum* and *D.* × *zeilleri*. Based on previous research (Naujalis, 1995) the sporulation frequency rate was *L. clavatum* > *D. complanatum* > *L. annotinum* > *D. tristachyum*. Most gametophytes were irregular bowl shape and belong to the genus *Lycopodium*. This suggested that in the pine forests studied, the relative abundance proportion of different genera, including both sporophytes and gametophytes, was similar.

CONCLUSIONS

1. Among the juvenile club moss sporophytes discovered, *Lycopodium annotinum* dominated while among sporulating club mosses, *Lycopodium clavatum* was the most common at nine research sites in dry pine forests of the Varėna District.
2. Among the gametophytes discovered, the genus *Lycopodium* dominated. Type I (*Lycopodium clavatum* type) gametophytes developed more often than Type II (*Diphasiastrum complanatum* type) in dry pine forests of the Varėna District. Five-hundred and twenty-two *Lycopodium* and 74 *Diphasiastrum* gametophytes were found; more than one sporophyte sprout was discovered emerging from a gametophyte.
3. Research conducted in the permanent Maskauka site showed that the development of the juvenile club moss populations is a long-term process, during which the unsynchronized occurrence of juvenile sporophytes from subterranean gametophytes takes place and partial elimination of juvenile sporophytes occurs.
4. There was a moderately positive correlation ($r_{xy} = 0.57$; $p < 0.05$) between the number of club moss gametophytes and juvenile sporophytes present in the soil sample, supporting the hypothesis that the best place to look for gametophytes is where juvenile sporophytes are present.
5. Juvenile sporophytes tend to cluster more than the gametophytes. The Nearest Neighbor Index (NNI) for juvenile sporophytes varied from 0.44–0.55 with an average distance (r_A) between sproutlings of 1.65–3.78 cm. Clasterization of gametophytes was not that apparent; the NNI for gametophytes varied from 0.51–1.18.
6. Asynchronous development was discovered within juvenile club moss populations. Gametophytes varied in size and shape, and were distributed only in the soil humus layer.

7. In the permanent Maskauka research site, the correlation matrix showed a weak, but positive correlation between *Deschampsia flexuosa* and *L. annotinum* cover (r_{xy} = 0.22; $p < 0.05$). However, when comparing the nine research sites, a weak positive correlation was found between *D. flexuosa* and *L. annotinum* (r_{xy} = 0.39; $p < 0.05$) and a weak negative correlation was determined between *D. flexuosa* and *Vaccinium myrtillus* L. (r_{xy} = -0.41; $p < 0.05$), *L. annotinum* and *V. myrtillus* (r_{xy} = -0.37; $p < 0.05$).
8. Club moss populations in Varėna District were characterised by low ISSR polymorphism: average percentage of polymorphic loci in *L. clavatum* populations was lower than in *L. annotinum* populations (18.37 % and 22.97 %, respectively).
9. *L. clavatum* and *L. annotinum* populations were highly differentiated (78% and 69% respectively), which imply that gene flow among populations was very limited.

LIST OF SCIENTIFIC WORKS

Publications on the dissertation topic

Scientific papers published in journals, listed by ISI Web of Science (WoS):

Rimgailė-Voicik R., Naujalis J. R. 2016. Presence of juvenile club moss (Lycopodiaceae) sporophytes and gametophytes in relation to vegetation cover in dry pine forests. Am. Fern J. 106(4):242–257.

Rimgailė-Voicik R., Naujalis, J. R., Voicikas A. 2015. Organization of club moss gametophytes and juvenile sporophyte populations in pine forests. Pol. J. Ecol. 66:311–324.

Scientific papers published in journals, listed by ISI Master List:

Rimgailė-Voicik R., Naujalis J. R. 2015. Reporting on first genus *Diphasiastrum* subterranean gametophyte findings in Lithuania. Bot. Lith. 21:133–135.

Scientific papers in other reviewed periodicals:

Rimgailė-Voicik R., 2015: Localized events of juvenile club moss populations occurrence: a field study. IDK 2015 conference book: 535–544.

Conference thesis:

Rimgailė-Voicik R. 2016: Initial club moss populations: locating and evaluating – Current Issues of Plant Conservation, 16 p., Kaunas.

Rimgailė-Voicik R., Naujalis J. R. 2015: Initial application of morphological features in club moss (*Lycopodiaceae*) population studies. – The 8th Baltic Morphology scientific conference: Interdisciplinary Nature of Contemporary Morphology: 61 p., Vilnius.

Rimgailė-Voicik R. 2015: Juvenile populations of club mosses: alternative research component for evaluating club moss population vitality. – In:

Proceedings of III(XI) International Botanical Conference of Young Scientists in Saint-Petersburg, 133 p., St. Petersburg, Russia.

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Rimgailaitė R. 2014: Gametophytes of Club Mosses in Pine Forests of Southeastern Lithuania. – International Conference The Coins abstract book: 61–62 pp.

Participation in Lithuanian and international conferences:

Current Issues of Plant Conservation: August 16-18th, 2016, Kaunas.

The 8th Baltic Morphology scientific conference: November 12-14th, 2015, Vilnius.

III (XI) International 8 Botanical Conference of Young Scientists and V Russian Geobotanical School-Conference: October 9-14th, 2015, Saint Petersburg, Russia.

Internatonal Doctoral Conference: May 14-15th, 2015, Pecs, Hungary.

Mokslas Gamtos mokslų universitete: October 3rd, 2014, Vilnius.

International Conference The Coins: March 7th, 2014, Vilnius.

Popular science publications:

Rimgailė-Voicik R., Juzėnas, S. 2016. Ką žinome apie archajiškiausius miškų augalus? [What do we know about the most archaic forest plants?] Mūsų girios 12:24–25. (in Lithuanian).

Other scientific work, not related to the dissertation topic

Scientific papers published in journals, listed by ISI Web of Science (WoS):

Naujalis J. R., **Rimgailė-Voicik R.**, 2016. Plant community associations and complexes of associations in the Lithuanian seashore: retrospective on the studies and tragic fate of the botanist Dr Abromas Kisinas (1899–1945). Israel Journal of Plant Sciences 63(3):167–175. doi.org/10.1080/07929978.2016.1154320

Other scientific papers:

Meldžiukienė A., **Rimgailė-Voicik R.**, Rasimavičius M. 2015. Botanikos rinkiniai Vilniaus senienų muziejuje. pp. 411–423, *in*: Kova dėl istorijos: Vilniaus senienų muziejus (1855-1915) straipsnių rinkinys. Lietuvos nacionalinis muziejus, Vilnius.

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Oral presentations:

2015 09 06: "The European Day of Jewish Culture", excursion in VU herbarium: "What Paneriai hills can tell: prof. Jokūbas Movšovičius and research on Lithuanian flora during interwar period" (in Lithuanian).

2014 09 18: "Spaceship Earth": practical seminar "Dusts of flowers" (in Lithuanian).

2013 12 10: excursion to VU herbarium for the European Medicines Agency's Committee on Herbal Medicinal Products (in English).

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SUPPLEMENTS

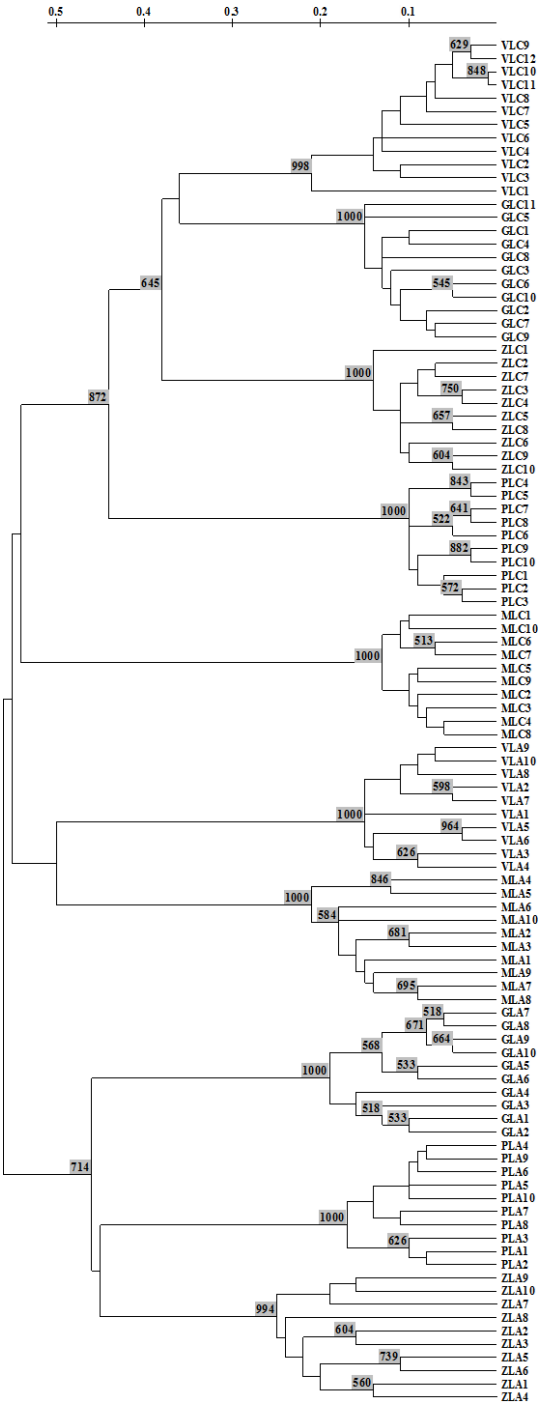
1 supplement. Fragment of geobotanical description (1-15) matrix used for statistical analysis in R

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pinus.sylvestris	0.138889	0.060976	0.057143	0.053763	0.153061	0.178571	0.123153	0.098859	0.07109	0.265957	0.242857	0.076923	0.15	0.177778	0.170455
Picea.abies	0	0	0	0.026882	0	0	0	0	0	0	0	0	0	0	0.005682
Betula.pendula	0	0	0	0.005376	0	0	0.024631	0.019011	0	0.010638	0.004762	0.006993	0.007143	0	0
Betula.pubescens	0	0	0	0	0	0	0	0	0.004739	0	0	0	0	0.022222	0.028409
Sorbus.aucuparia	0	0	0	0	0	0	0	0.003802	0	0	0	0	0	0	0
Populus.tremula	0	0	0	0	0	0	0	0	0	0	0	0.006993	0	0	0
Crataegus.curvisepala	0	0	0	0	0	0	0	0.003802	0	0	0	0	0	0	0
Corylus.avelana	0	0	0.005714	0	0	0	0	0	0	0	0	0	0	0	0
Frangula.alnus	0.005556	0.006098	0	0	0	0	0	0.003802	0.004739	0	0	0	0.007143	0	0
Quercus.robur	0.005556	0.006098	0.028571	0	0.005102	0.005952	0	0.038023	0.004739	0	0.004762	0	0	0.111111	0.028409
Salix.caprea	0	0	0	0	0	0	0	0.019011	0	0	0	0	0	0	0
Juniperus.cummunis	0	0.030488	0.005714	0	0.02551	0.029762	0.024631	0.038023	0.023697	0.010638	0	0	0.178571	0.022222	0
Lycopodium.clavatum	0	0	0	0	0	0	0	0.019011	0	0	0	0.006993	0	0	0
Diphasiastrum.complanatum	0.005556	0	0	0	0	0	0	0	0	0.010638	0	0	0	0	0
Hieracium.umbellatum	0	0	0	0	0	0	0	0	0	0	0.02381	0	0	0	0
Solidago.virgaurea	0	0	0	0	0	0	0	0	0	0.010638	0	0	0	0	0
Melampyrum.pratense	0	0	0	0	0	0	0	0.019011	0.047393	0	0	0	0	0	0
Anthoxanthum.odoratum	0	0	0	0	0	0	0	0.019011	0.004739	0	0	0	0	0	0
Hieracium.pilosela	0	0	0	0	0	0	0	0.003802	0	0	0.02381	0	0	0	0
Chameron.angustifolium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fragaria.vesca	0	0	0	0	0	0	0	0.003802	0	0	0	0	0	0	0
Festuca.ovina	0	0	0	0	0	0	0.024631	0.038023	0.047393	0	0.004762	0.006993	0.007143	0	0
Luzula.pilosa	0	0	0	0	0	0	0	0.038023	0	0	0	0	0	0.022222	0
Thymus.pulegioides	0	0	0	0	0	0	0	0.003802	0	0	0.004762	0.006993	0	0	0

(continued in next page)

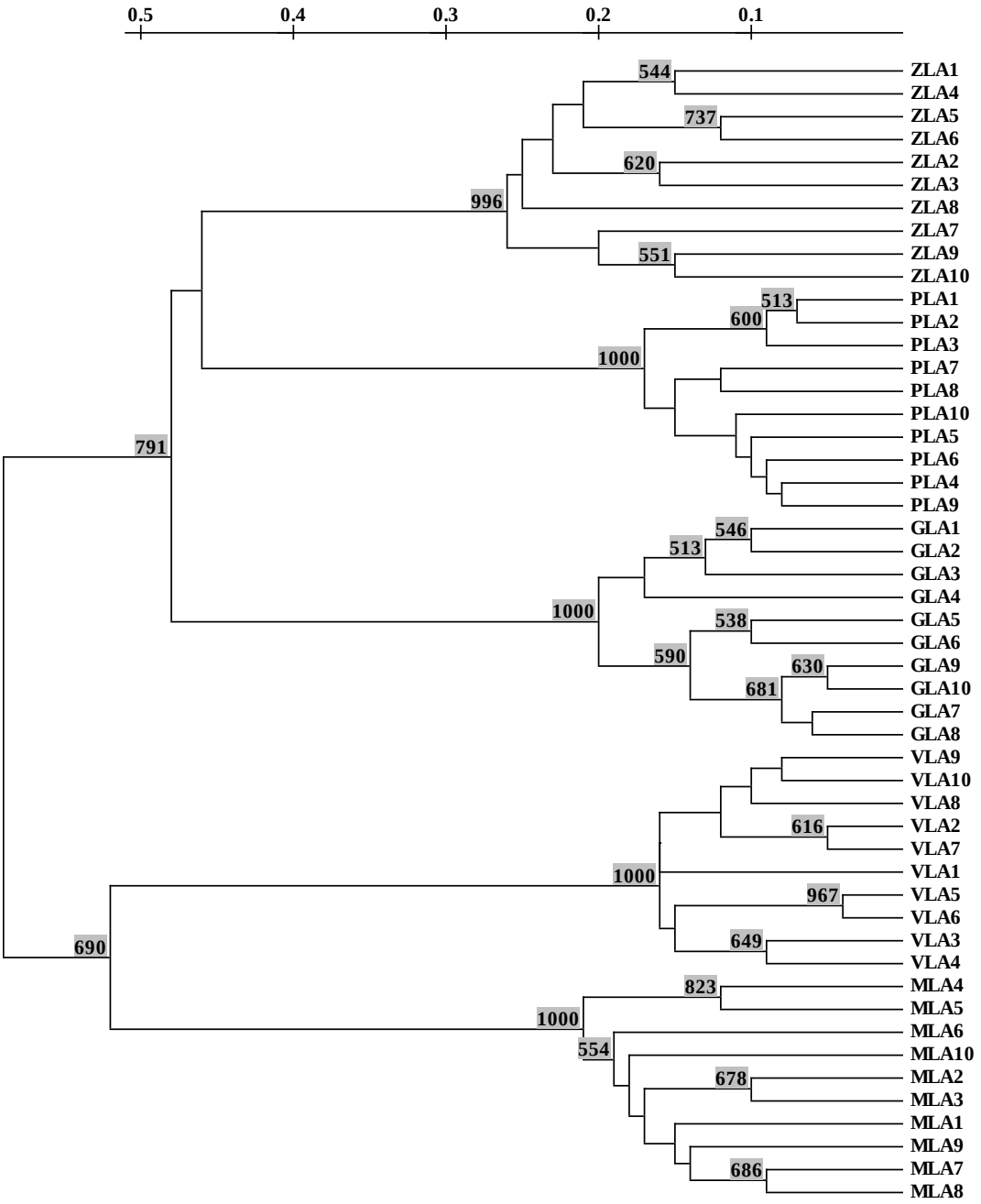
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Vaccinium.vitis.idaea	0.055556	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Koeleria.glauca	0	0	0	0	0	0	0	0	0	0	0.004762	0	0	0	0
Vaccinium.myrtilus	0.055556	0.030488	0.005714	0.053763	0.05102	0.059524	0.024631	0.038023	0.118483	0.010638	0.004762	0.06993	0.007143	0	0.056818
Viola.rhiviniana	0	0	0	0	0	0	0	0	0	0	0.004762	0	0	0	0
Festuca.rubra	0	0	0	0	0	0	0	0.019011	0	0	0	0	0	0	0
Chimaphila.umbellata	0	0	0	0	0.005102	0.029762	0.049261	0.019011	0	0.053191	0.02381	0.006993	0.007143	0	0
Dryopteris.spinulosa	0	0	0	0	0	0	0	0.003802	0	0	0	0	0	0	0
Deschampsia.flexuosa	0.138889	0.152439	0.142857	0.134409	0.127551	0.14881	0.246305	0.038023	0.047393	0.106383	0.02381	0.06993	0.178571	0.111111	0.056818
Arctostaphylos.uva.ursi	0	0	0	0	0	0	0	0	0.047393	0	0	0	0.007143	0	0
Calluna.vulgaris	0	0	0	0	0.05102	0	0	0	0	0	0	0	0	0	0
Lycopodium.annotinum	0.005556	0.006098	0.028571	0.026882	0.005102	0.005952	0.004926	0.019011	0.047393	0.010638	0.004762	0	0.007143	0.022222	0.028409
Pyrola.minor	0	0.060976	0	0.026882	0	0	0	0.019011	0	0	0	0	0	0	0
Gypsophila.fastigiata	0	0	0	0	0	0	0	0.003802	0	0	0	0	0	0	0
Heracium.murorum	0	0	0	0	0.005102	0	0	0.038023	0.023697	0.010638	0.02381	0	0	0	0
Goodyera.repens	0	0	0	0	0.005102	0	0.004926	0.003802	0.023697	0	0	0	0	0	0
Monotropa.hypopitis	0	0	0.005714	0	0	0	0	0	0.004739	0	0	0	0	0	0.028409
Carex.digitata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pleurozium.schreberi	0.416667	0.457317	0.285714	0.403226	0.382653	0.446429	0.369458	0.285171	0.35545	0.265957	0.357143	0.524476	0.178571	0.222222	0.284091
Rhytidiadelphus.triquertus	0	0	0	0	0	0	0	0	0	0	0.02381	0.034965	0	0	0
Brachytecium.sp.	0	0	0	0	0	0	0	0	0	0	0.02381	0.034965	0	0	0
Hylocomium.splendens	0	0.030488	0.057143	0.134409	0	0	0	0.003802	0.023697	0	0	0.034965	0	0	0
Ptilium.crista.castrensis	0	0	0	0	0	0	0	0	0.023697	0	0	0	0	0	0
3olytrichum.commune	0.027778	0	0	0.026882	0	0	0	0.003802	0.004739	0	0	0	0.007143	0.111111	0.056818
Dicranum.polysetum	0.138889	0.152439	0.285714	0.053763	0.127551	0.059524	0.049261	0.095057	0.047393	0.106383	0.119048	0.06993	0.178571	0.044444	0.056818
Dicranum.scoparium	0	0	0.028571	0.053763	0.05102	0.029762	0.024631	0.019011	0.023697	0	0.047619	0.034965	0.071429	0.044444	0.056818
Cladonia.rangiferina	0.005556	0.006098	0.005714	0	0	0	0	0	0	0.010638	0	0	0	0.044444	0.056818
Cladonia.arbuscula	0	0	0.028571	0	0.005102	0.005952	0.004926	0.019011	0	0.106383	0.02381	0.006993	0.007143	0.044444	0.056818
Cetraria.islandica	0	0	0.028571	0	0	0	0.024631	0	0	0.010638	0	0	0	0	0.028409
Leucobryum glaucum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

2 supplement. Dendrogram of the UPGMA cluster analysis based on Nei and Li genetic distances estimated using ISSR markers among ten club moss populations investigated with a 1000 Bootstrap permutations



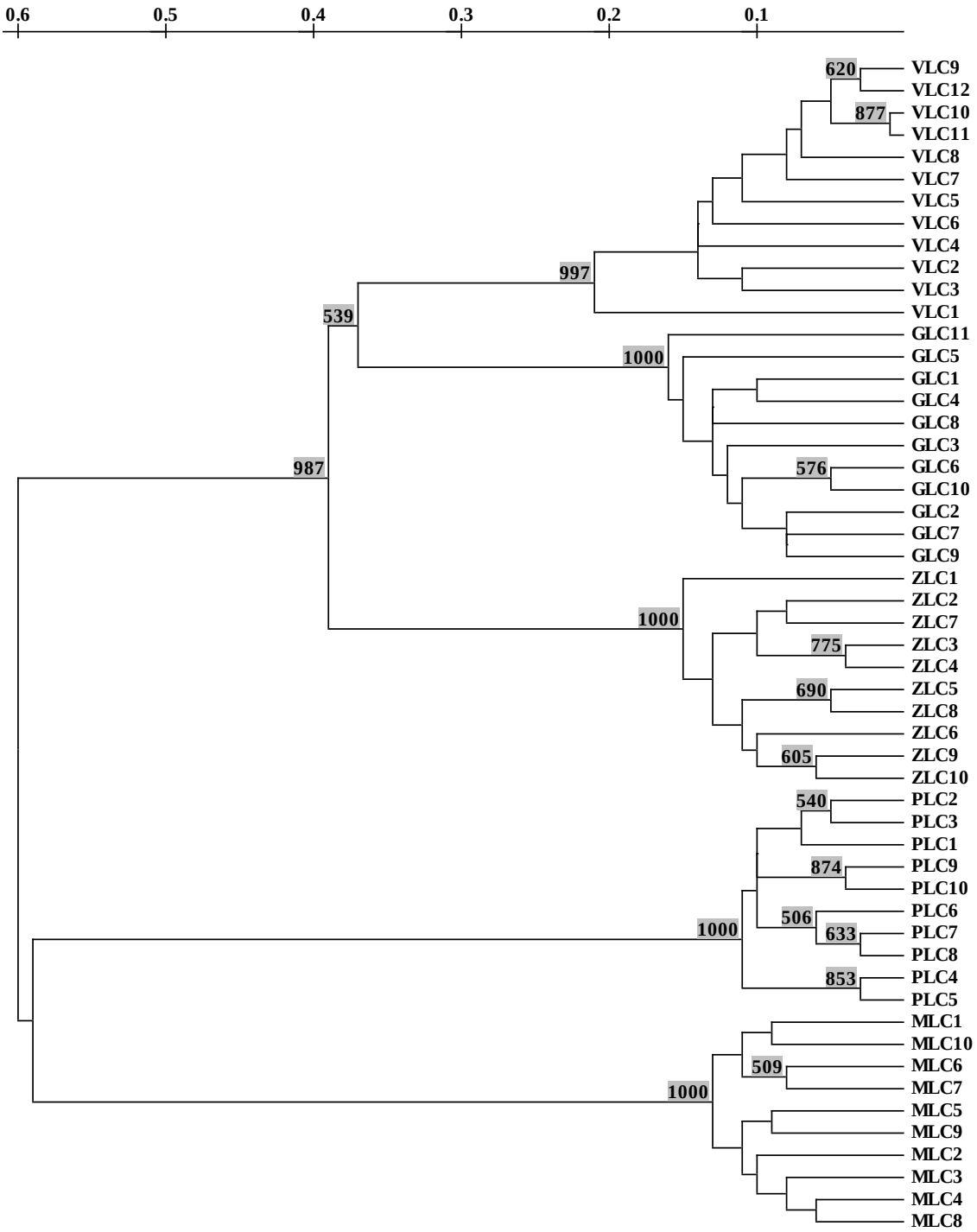
LC – *L. clavatum*, LA – *L. annotinum* populations: V – Varėnė, G – Glėbas, Z – Žilinėliai, P – Puvočiai, M – Maskauka.

3 supplement. Dendrogram of the UPGMA cluster analysis based on Nei and Li genetic distances estimated using ISSR markers among five *L. annotinum* club moss populations investigated with a 1000 Bootstrap permutations



LC – *L. clavatum,* LA – *L. annotinum* populations: V – Varėnė, G – Glėbas, Z – Žilinėliai, P – Puvočiai, M – Maskauka.

4 supplement. Dendrogram of the UPGMA cluster analysis based on Nei and Li genetic distances estimated using ISSR markers among five *L. clavatum* club moss populations investigated with a 1000 Bootstrap permutations



LC – *L. clavatum*, LA – *L. annotinum* populations: V – Varėnė, G – Glėbas, Z – Žilinėliai, P – Puvočiai, M – Maskauka.

5 supplement. Data on research sites in Varėna District

No.	Research site and coordinates (WGS)	Forestry	Directorate	Block	Plot	Stand age	Height, m	Stocking	Habitat	Stand structure	Origin, notes	Aerial photo number
1.	Maskauka I (54.28447; 24.60357)	Glūkas	Varėna	175	5	50 70	16 32	0.7	Nal, cl	8P 2P		317-2229
2.	Maskauka II (54.28029; 24.59781)	Glūkas	Varėna	175.	13	80	26	0.7	Nbl, vm	10P	fire	317-2229
3.	Žilinėliai (54.32018; 24.64004)	Valkininkai	Valkininkai	80	27	40	9 13	0.9	Nal, cl	5P 5B	K	317-2180
4.	Varėnė I (54.26412; 24.53109)	Glūkas	Varėna	217	3	80 55	23 16	0.7	Nal, cl	8P 2P		317-614
5.	Varėnė II (54.26451; 24.53276)	Glūkas	Varėna	217	5	85	26	0.7	Nbl, vm	10P	K	317-614
6.	Beržupis (54.23603; 24,59468)	Dainava	Varėna	537	2	45	15	0.9	Nal, cl	10P Pb B	K	317-621
7.	Bingeliai (54.17616; 24.26510)	Merkinė	Druskininkai	127	13	39	14.2	0.9	Nal, cl	10P Pb B		317-2225
8.	Glėbas (54.24304; 24.46539)	Glūkas	Varėna	239	8	40	16	1.0	Nal, cl	9P 1B	K	317-2178
9.	Puvočiai (54.11251; 24.31869)	Marcinkonys	Varėna	563	31	105 40	28 14	0.6-0.3	Nbl vm	10P 10E		317-2253

Notes: P – *Pinus sylvestris*, B – *Betula*, Pb – *Pinus banksiana*, E – *Picea*; cl – *cladoniosa*, vm – *vaccinio-myrtillosa*; Nbl – normal irrigation infertile light soils; Nal – normal irrigation extremely infertile soils; K – planted forest.