VILNIUS UNIVERSITY
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Study of plants of the genus *Myriophyllum* using molecular methods

Master's Thesis

Genetics Study Programme

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LIST OF ABBREVIATIONS

ISSR – Inter Simple Sequence Repeat.

ITS – Internal Transcribed Spacer.

*trn*L-*trn*F – region located in the large single-copy region of the chloroplast genome. It consists of the *trn*L gene, a group I intron, and the *trn*L-F intergenic spacer.

SNP – Single Nucleotide Polymorphisms.

AFLP – Amplified Fragment Length Polymorphism.

RAPD – Random Amplified Polymorphic DNA.

CTAB – Cetyltrimethylammonium bromide.

EDTA – Ethylenediaminetetraacetic acid.

TAE – Tris-acetate-EDTA.

TBE – Tris-borate-EDTA.

PCR – Polymerase Chain Reaction.

AMOVA – Analysis of molecular variance.

MSA – Multiple sequence alignment.

MUSCLE – MUltiple Sequence Comparison by Log-Expectation.

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Markas Lukošiūnas

***Myriophyllum* genties augalų tyrimas naudojant molekulinius metodus**

Magistro baigiamasis darbas

SANTRAUKA

Myriophyllum yra gėlavandenių vandens augalų gentis, priklausanti Haloragaceae šeimai, plačiai paplitusi Europoje, Azijoje, Afrikoje, Šiaurės Amerikoje ir Australijoje. Šios genties hibridizacija yra gerai dokumentuota ir vaidina svarbų vaidmenį evoliucijoje, galimai išstumiant tėvines rūšis, didinant genetinę ir morfologinę įvairovę bei prisidedant prie naujų rūšių atsiradimo. Norint tiksliai nustatyti genetinius panašumus ir skirtumus tarp individų ir populiacijų, reikalingi molekuliniai metodai, nes vien morfologiniai žymekliai nėra pakankami. Hidrobiologiniai tyrimai Lietuvoje atskleidė pastebimus fenotipinius skirtumus tarp *Myriophyllum* augalų, kas rodo, kad gali būti kelios rūšys, o ne viena. Tačiau *Myriophyllum* hibridizacija Lietuvoje išlieka mažai tyrinėta. Supratimas apie genetines struktūras ir ryšius genties viduje yra svarbu, siekiant išlaikyti rūšis ir biologinę įvairovę. Šis magistro darbas tiria *Myriophyllum* populiacijų genetinę įvairovę ir santykius, naudojant tokius molekulinius žymeklius kaip ISSR ir ITS. Mėginiai buvo surinkti iš įvairių lokacijų Lietuvoje ir Latvijoje, išskirta DNR analizuota naudojant ISSR-PGR ir ITS sekvenavimą. Rezultatai parodė reikšmingą genetinį skirtumą tarp populiacijų, su didesne genetine įvairove pastebėta tarp populiacijų nei jų viduje.

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Study of plants of the genus *Myriophyllum* using molecular methods

Master thesis

SUMMARY

Myriophyllum is a genus of freshwater aquatic plants in the Haloragaceae family, widely distributed across Europe, Asia, Africa, North America, and Australia. Hybridization within this genus is well-documented and plays a crucial role in evolution by potentially displacing parent species, increasing genetic and morphological variability, and contributing to the emergence of new species. Accurately discerning genetic similarities and differences between individuals and populations requires molecular methods, as morphological markers alone are insufficient. Hydrobiological investigations in Lithuania have revealed discernible phenotypic variations among *Myriophyllum* plants, suggesting the presence of multiple species rather than a single taxonomic entity. However, *Myriophyllum* hybridization in Lithuania remains understudied. Understanding the genetic structures and relationships within the genus is essential for conservation efforts to preserve species and maintain biodiversity. This master thesis investigates the genetic diversity and relationships of *Myriophyllum* populations using molecular markers such as ISSR and ITS. Samples were collected from various locations in Lithuania and Latvia, with DNA extracted and analyzed using ISSR-PCR and ITS sequencing. The results showed significant genetic differentiation among populations, with higher genetic diversity observed between populations than within them.

INTRODUCTION

Myriophyllum is a genus encompassing approximately 69 species of freshwater aquatic plants belonging to the Haloragaceae family. These species are widely distributed across Europe, Asia, Africa, North America, and Australia. *Myriophyllum* plants are often recognized for their elongated stems with air canals and finely divided, pinnate, whorled leaves, although there are notable exceptions to these characteristics. Hybridization within the genus, particularly between *Myriophyllum sibiricum* and *Myriophyllum spicatum*, is well-documented in Eurasia and North America. Hybridization plays a crucial role in evolution by potentially displacing parent species due to the hybrids' enhanced adaptability to altered conditions. Moreover, hybridization increases genetic and morphological variability and can contribute to the emergence of new species, complicating species identification based on morphological markers alone. To accurately discern genetic similarities and differences that are not evident from morphology, molecular methods are essential. Molecular markers such as Inter Simple Sequence Repeat (ISSR) and Internal Transcribed Spacer (ITS) regions have been employed to differentiate *Myriophyllum* species. These markers are also used in population genetics and hybridization studies to detect genetic variations among individuals and populations. In Lithuania, hydrobiological investigations have revealed discernible phenotypic variations among *Myriophyllum* plants, suggesting the presence of multiple species rather than a single taxonomic entity. Species in Lithuania suspected of hybridizing include *Myriophyllum spicatum*, *Myriophyllum verticillatum*, *Myriophyllum sibiricum*, and *Myriophyllum alterniflorum*. Despite the significance of hybridization in water ecosystems, it remains understudied in Lithuania. Studying the genetic structures and relationships within the genus *Myriophyllum* is essential for guiding conservation efforts to preserve species and maintain biodiversity. Therefore, this thesis aims to elucidate the genetic diversity and relationships of *Myriophyllum* populations using molecular methods.

The objectives of this thesis are:

1. To assess primer polymorphisms and select suitable ISSR primers.
2. To evaluate the genetic differentiation and diversity of *Myriophyllum* populations using molecular markers.
3. To sequence the ITS region of *Myriophyllum* individuals, identify their species, and compare their nucleotide sequences.

1. LITERATURE REVIEW

1.1. Morphological variability of *Myriophyllum*

Morphological variability refers to variations in physical traits or outward appearances. Variation in morphology is influenced by environmental effects, phenotypic plasticity, genetic variations, mutations, and speciation.

Morphological plasticity, alternatively known as phenotypic plasticity, refers to an organism's ability to alter its morphological and physiological characteristics in response to environmental stimuli (Fusco & Minelli, 2010). In some cases, different environments can evoke discrete phenotypes, that arise from a single genotype, this is known as polyphenism (Yang & Andrew Pospisilik, 2019). Genetic variation refers to differences in DNA sequences either among individuals or across populations within the same species. Genetic variation within a population, where individuals differ in their genotypes at one or more gene loci, is essential for evolution through natural selection (Ewens, 2013). Another cause for morphological variability is local adaptation. Local adaptation occurs when populations possess traits that enhance their survival and reproduction specifically in their local environment, due to a close match between their genetic variations and the environmental conditions (Blanquart et al., 2013). The difference between genetic variation and phenotypic plasticity is that genetic variation refers to differences in traits among genotypes in a specific environment, while phenotypic plasticity describes how a trait's phenotype can vary across different environments for a given genotype (Klingenberg, 2019).

Aquatic plants are widely acknowledged for their ability to adapt to their environments', with their morphology exhibiting considerable variability across populations and/or seasons. Changes in plant morphology and physiology among populations of the same species are often attributed to physiological or mechanical stresses and are driven by adaptive mechanisms such as phenotypic plasticity or local adaptations (Weyl & Coetzee, 2016). These mechanisms allow them to adapt to the different climatic and environmental stresses to which they are exposed (Weyl & Coetzee, 2016). Many aquatic species have a broad distribution range, with individuals within populations having low genetic variation but high variation between populations, likely due to clonal or vegetative reproduction (Barrett et al., 1993; Grace, 1993; Weyl & Coetzee, 2016). Aquatic plants often possess a "general purpose genotype" with low genetic variability but capable of adapting to diverse environmental conditions through phenotypic plasticity (Barrett et al., 1993; Weyl & Coetzee, 2016).

Phenotypic plasticity and local adaptation play vital roles in the successful invasion of aquatic species. One study, that analyzed invasive submerged macrophytes in New Zealand, came to the conclusion that main adaptive strategy for invasive aquatic species is phenotypic plasticity, given

their low genetic diversity and short residency time in the region (Riis et al., 2010). Local adaptations, driven by natural selection, usually occur over longer timescales and require diverse gene pools within populations (Kawecki & Ebert, 2004; Ward et al., 2008). Multiple introductions from different source populations can enhance genetic diversity, facilitating local adaptation, which is crucial for the successful invasion of species (Parker et al., 2003).

Due to local adaptation and phenotypic plasticity, certain species of *Myriophyllum* may display significant variations in morphology, despite belonging to the same species. For example, *Myriophyllum spicatum* individuals from three different regions in southern Africa exhibited such diverse characteristics that they were initially misidentified as separate species; however, pollen analysis later confirmed them to be a single species (Guillarmod, 1979; Weyl & Coetzee, 2016). *Myriophyllum spicatum* individuals from the Vaal River, Northern Cape, South Africa, are robust with large leaves and thick stems; those from Lake Sibaya, KwaZulu-Natal, have delicate, small leaves and highly branched thin stems; while those from high-altitude regions like the Amathola Mountains and KwaZulu-Natal Midlands have short internodes, resulting in tightly packed leaves resembling a bottlebrush (Figure 1.1.). Early herbarium specimens from these regions display the same morphological traits, indicating the persistence of these characteristics over time (Weyl & Coetzee, 2016). A study aimed to investigate the drivers behind morphological differences among these populations of *Myriophyllum spicatum* in southern Africa concluded that local adaptation is the primary factor influencing the varied morphologies observed in these populations, and that phenotypic plasticity also contributes to these differences. (Weyl & Coetzee, 2016).

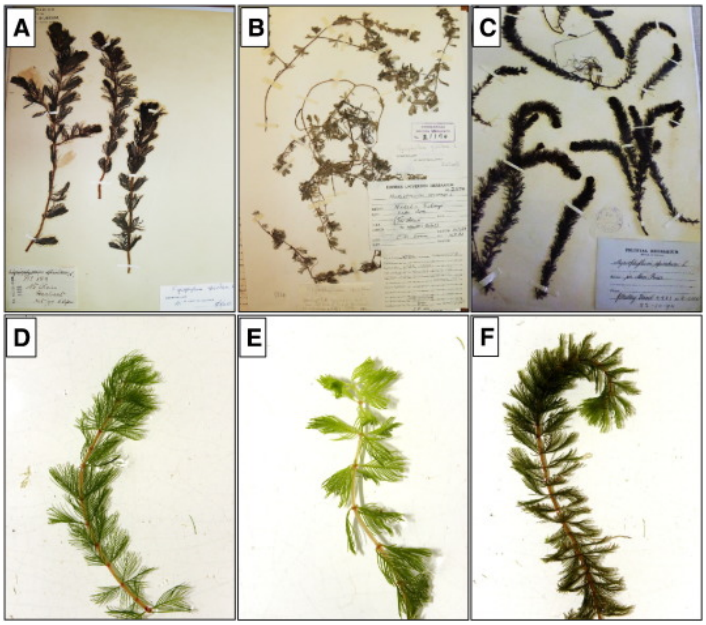

Figure 1.1. The morphological variations of *Myriophyllum spicatum* in southern Africa are documented through herbarium specimens (A–C) and contemporary photographic representations of living specimens (D–F). The first variation (A & D) showcases a robust, large-leaf form retrieved from the Vaal River, Northern Cape. The second variation (B & E) displays a delicate, branched form collected in Lake Sibaya, KwaZulu-Natal. Lastly, the third variation (C & F) exhibits a large growth form characterized by very short internode lengths, resulting in a bottlebrush appearance. The herbarium specimen representing this form was collected in the Mooi River, KwaZulu-Natal Midlands, while the living specimen was obtained from Hogsback, Eastern Cape. Taken from: Weyl & Coetzee, 2016.

Variations in *Myriophyllum* morphology extend beyond southern Africa and are observed worldwide. A study examining *Myriophyllum sibiricum*, *Myriophyllum spicatum* and their hybrids across northern Eurasia revealed variations in leaf segment count and variation in the occurrence of trichomes. *Myriophyllum sibiricum* generally exhibits fewer than 25 leaf segments, typically ranging from 13 to 19, while individuals from the Commander Islands and hybrids from the southern Kurils usually feature 15 to 22 segments, and hybrids from the Ivanovo Region and Iturup Island may bear up to 30 to 32 segments (Figure 1.2.) (Volkova et al., 2024). Most *Myriophyllum spicatum* specimens from different regions typically display 17 to 27 leaf segments, but there are exceptions: some from western North America and the southern Russian far east have over 30 segments, while approximately 10 specimens from various regions have fewer than 20 segments (Figure 1.2.) (Volkova et al., 2024). Trichomes were observed in the axils of leaf segments of upper leaves in all *Myriophyllum sibiricum* specimens, whereas the majority of *Myriophyllum spicatum* specimens lacked such trichomes, except for three specimens from the southern Kurils (Volkova et al., 2024). Most *Myriophyllum sibiricum* and *Myriophyllum spicatum* hybrids did not have trichomes, only one hybrid from the Iturup Island had trichomes (Volkova et al., 2024).

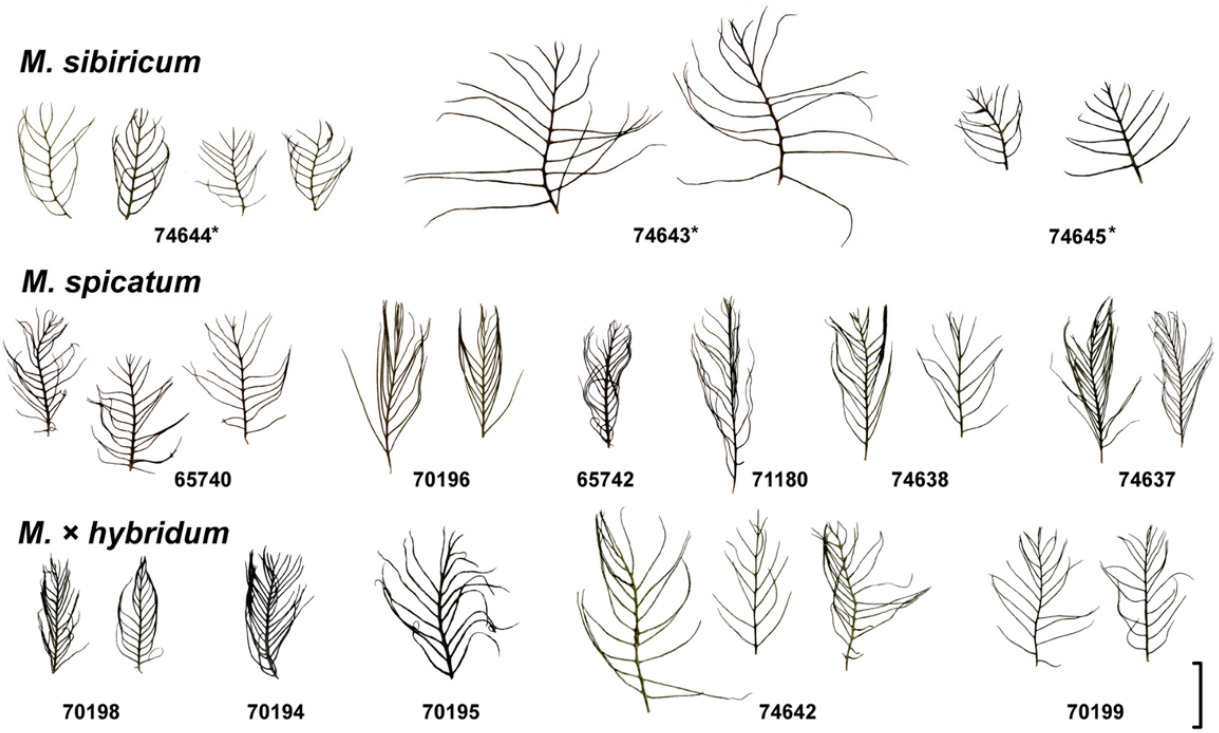

Figure 1.2. Leaf variability of *Myriophyllum sibiricum*, *Myriophyllum spicatum* and their hybrids. Scale bar is 1 cm. Taken from: Volkova et al., 2024.

Another study, conducted in Hungary, revealed a significant correlation between sediment nitrogen concentration and the morphological properties of *Myriophyllum spicatum* (Tóth et al., 2017). Locations characterized by high sediment nitrogen concentrations exhibited *Myriophyllum spicatum* plants that were 5 to 10 times larger but displayed low morphological variability, whereas

sites with lower sediment nitrogen levels featured smaller plants that were four times more variable in their morphology (Tóth et al., 2017). After genetic analysis they also concluded that adverse environmental conditions appeared to have little impact on the genetic composition of the sampled plants, therefore, the significant morphological variability observed, particularly in nitrogen-poor sites, primarily stemmed from the plastic responses of *Myriophyllum spicatum* to its environmental conditions (Tóth et al., 2017). Other studies conducted have also found correlations between sediment nutrient levels and *Myriophyllum spicatum* morphology (Xie & Yu, 2011; J. Cao et al., 2012; Xie et al., 2013). It is also hypothesized that the reduction in morphological variability in *Myriophyllum spicatum* may be a response to nitrogen enrichment, indicating a metabolic shift towards a less conservative, more resource-intensive strategy and that this change could represent the initial stage of plants adapting to specific environments in a manner reminiscent of ecotype-like specialization (Tóth et al., 2017).

Speciation entails the process through which one biological species undergoes divergence, resulting in the emergence of two or more species (Moehring, 2013). Geographical and genetic factors, like polyploidization and hybridization, can affect this process (Moehring, 2013; Lü et al., 2017). It has been proven that hybridization and polyploidization are significant factors in the speciation of the genus *Myriophyllum* (Lü et al., 2017). These divergent species of *Myriophyllum* acquire new characteristics and features, thereby enhancing biodiversity and contributing to morphological diversity. Although closely related species can still be quite similar, sharing many characteristics. The key differences in morphology between *Myriophyllum spicatum, Myriophyllum verticillatum, Myriophyllum sibiricu*m and *Myriophyllum alterniflorum* are summarized in the table below (Table 1.1.) (Grace & Wetzel, 1978; Aiken et al., 1979; Aiken, 1981; Dan Yu et al., 2002; Jacobs & Mangold, 2009; Moody & Les, 2010; Scribailo & Alix, 2023).

Table 1.1. Key morphological characteristics of *Myriophyllum spicatum*, *Myriophyllum verticillatum*, *Myriophyllum sibiricum* and *Myriophyllum alterniflorum*.

Characteristic	***Myriophyllum spicatum***	***Myriophyllum verticillatum***	***Myriophyllum sibiricum***	***Myriophyllum alterniflorum***
Stem Color	Light green, yellowish, reddish	Pale green to bright red or brown	Yellowish to reddish	Pale green to bright red
Stem Length	Up to 6 meters	Up to 3 meters	Up to 6 meters	Up to 2.5 meters
Stem Branching	Few branched on the lower stem and extensive towards the top	Usually branched	Unbranched	Branched

Turions	Absent	Present, transitioning in color, club-shaped, formed in late summer	Present, dark green, cylindric to club-shaped, formed in autumn	Absent
Leaf Arrangement	Whorls of 3 or 4, sometimes 5	Whorls of 3 or 4, rarely opposite	Whorls of 3 or 4	Mostly whorls of 3 or 4, rarely opposite
Inflorescence Length	Up to 15 centimeters	Up to 25 centimeters	Up to 15 centimeters	Up to 12 centimeters
Flower Characteristics	Tiny flowers in whorls, male flowers at tip with 8 stamens, female flowers below males with 4-parted style	Tiny flowers in whorls, male flowers at tip with 8 stamens, female flowers below males with 4-parted style	Tiny flowers in whorls, male flowers at tip with 8 stamens, female flowers below males with 4-parted style	Tiny flowers in whorls, male flowers alternately arranged with 4 or 8 stamens, female flowers below males with 4-parted style
Petal Color and Shape (Male)	Cream, red, dark purple, oblong, elliptical, obovate	Cream-colored, occasionally with purple at the tips, elliptic to obovate	Cream, red, dark purple, oblong, elliptical, obovate	Cream-colored, elliptic, obovate
Petal Color and shape (Female)	Cream-colored, and widely ovate	Cream, purple, elliptic, obovate	Cream-colored and broadly ovate	Cream-colored and broadly ovate
Stamen Color	Greenish cream, yellow, purple	Yellow	Greenish cream, yellow, purple	Cream, purple
Stigma Color	White, red, purple	Red, purple	White, red, somewhat purple	Red, somewhat purple
Fruit Shape and Color	Spherical, four-lobed, olive-brown	Spherical, four-lobed, olive green to brown	Spherical, four-lobed, brown	Ovoid, four-lobed, brown

Individual Mericarp Size	1.5–2.2 mm in length, 0.8–1.3 mm in width	1.5–2.7 mm in length, 0.3–0.4 mm in width	1.5–2.7 mm in length, 0.9–1.7 mm in width	1.3–1.6 mm in length, 0.3–0.4 mm in width

1.2. Genetic variability of genus *Myriophyllum*

Genetic variability encompasses the existence or emergence of genetic differences within a population. It refers to the formation of individuals with distinct genotypes or the coexistence of genetically diverse individuals, as opposed to environmentally induced variations, which typically induce temporary, non-heritable changes in phenotype (Löve, 1969). Genetic variability within a population is vital for biodiversity, as it guarantees that each living organism is unique (Sousa et al., 2011). Genetic variability is important for evolutionary processes and the emergence of new species (Chung et al., 2023).

Many processes can contribute to genetic variability, one of them being homologous recombination. During meiosis, two homologous chromosomes cross over one another and exchange genetic material, this random exchange of genetic material increases variability and is called homologous recombination (Stapley et al., 2017). Another important driver for variability is polyploidy – a condition in which a normally diploid cell or organism acquires one or more additional sets of chromosomes, this enables increased recombination during meiosis, resulting in greater genetic variability among offspring (Zhang et al., 2019). Polyploidization plays a crucial role in the plant speciation and diversification (D. E. Soltis et al., 2009).Genetic mutations are a key factor in generating genetic diversity within a population, with potential outcomes ranging from beneficial to detrimental or having no discernible effect on an organism's fitness. Beneficial mutations are favored by natural selection, enhancing an individual's fitness and spreading throughout the population and harmful mutations may be masked if they do not severely impact survival or reproduction. Mutations are a fundamental driver of evolution, as they introduce variability within populations, and enable evolutionary change (Loewe & Hill, 2010). Another important process for genetic variability is hybridization, where organisms from two different species mix, or breed together. The existence of such hybrids increases genetic diversity (P. S. Soltis & Soltis, 2009; Zalapa et al., 2010). Natural hybridization is common among plants and is recognized as a significant evolutionary mechanism. It can result in several outcomes: enhancing intraspecific genetic diversity within populations, giving rise to new species, potentially leading to species extinction via genetic assimilation, and producing highly invasive genotypes (López-Caamal & Tovar-Sánchez, 2014).

Genetic variations have been observed among various *Myriophyllum* species and populations. Polyploids are frequently encountered in *Myriophyllum*, although the precise ploidy level remains

unknown for most species (Moody & Les, 2010). The base chromosome number of *Myriophyllum* is seven (Lü et al., 2017). Chromosome counts have been determined for several *Myriophyllum* species: *Myriophyllum alterniflorum* [2n = 14], *Myriophyllum verticillatum* [2n = 28], *Myriophyllum sibiricum* [2n = 42] and *Myriophyllum spicatum* [2n = 42] (Aiken, 1981; Harris et al., 1992; Robin W. Scribailo & Mitchell S. Alix, 2023). Knowing the base chromosome number, this suggests that *Myriophyllum sibiricum* and *Myriophyllum spicatum* are hexaploids (42/7 = 6), *Myriophyllum verticillatum* is a tetraploid (28/7 = 4) and *Myriophyllum alterniflorum* is a diploid (14/7 = 2) (Aiken, 1981; Lü et al., 2017). Phylogenetic analysis of *Myriophyllum* suggests that *Myriophyllum sibiricum* and *Myriophyllum spicatum* are closely related and that *Myriophyllum alterniflorum* could be one of the diploid progenitors (Moody & Les, 2007, 2010). Another study confirmed that both *Myriophyllum sibiricum* and *Myriophyllum spicatum* are closely related allohexaploids, likely evolving from ancestors through hybridization and polyploidization involving different parental lineages, however they disproved that *Myriophyllum alterniflorum* could be a diploid ancestor (Lü et al., 2017).

In one study that analyzes ribotypes and haplotypes of *Myriophyllum sibiricum, Myriophyllum spicatum* and their hybrids from Eurasia found that: *Myriophyllum sibiricum* exhibits only one ITS (Internal transcribed spacer) ribotype (designated as A), while all samples of *Myriophyllum spicatum* bear ITS ribotype C, differing from A by five point mutations. Additionally, populations resembling *Myriophyllum sibiricum* from Mednyi Island were characterized by ribotype B, which differs from A by two point mutations and one 1 bp deletion. In two populations from Bering Island resembling *Myriophyllum sibiricum*, a ribotype designated as A × B was found, showing polymorphisms in two sites discriminating A and B, along with a chromatogram "shift" at the site of an indel. Furthermore, several plants with unusual morphology from the southern Kuril islands (Kunashir and Iturup) exhibited a ribotype designated as A × C, showing polymorphisms in all sites discriminating A and C. Plants combining ribotypes of *Myriophyllum sibiricum* and *Myriophyllum spicatum* (A × C), represent their hybrids This same hybrid ribotype was also found in plants resembling *Myriophyllum sibiricum* from the drying canal in Ivanovo Region (Volkova et al., 2024).

Most *Myriophyllum sibiricum* samples from North America and Eurasia exhibited haplotype (a) in the *trn*L-*trn*F region (Lü et al., 2017; Volkova et al., 2024). This haplotype was also found in plants with ribotypes A × B from Bering Island and ribotypes A × C from Kunashir Island. Additionally, some *Myriophyllum sibiricum* samples from the Enisei River Basin had haplotype (b) differing from (a) by one point mutation. *Myriophyllum spicatum* samples exhibited haplotype (c), differing from both (a) and (b) by one point mutation. Haplotype (c) was also found in samples with ribotype A × C from Ivanovo Region and Iturup Island, as well as in samples with ribotype B from

Mednyi Island. One sample with ribotype A × C from Kunashir Island had haplotype (a) (Volkova et al., 2024).

1.3. *Myriophyllum* hybridization

As previously mentioned before hybridization is when individuals from two different species breed together and form a hybrid organism. Natural hybridization is widespread and recognized as a significant catalyst for evolutionary processes and the formation of new species (Wu et al., 2015). Hybridization is prevalent among aquatic plants (e.g. *Myriophyllum*), especially in dynamic environments and marginal populations where it occurs more frequently (Bobrov et al., 2022; Volkova et al., 2024).

The hybridization of *Myriophyllum sibiricum* and *Myriophyllum spicatum* has been well documented across the world (Moody & Les, 2010; Zuellig & Thum, 2012; Wu et al., 2015; Lü et al., 2017; Thum et al., 2020; Madsen et al., 2021; Volkova et al., 2024). Such hybrids can reproduce asexually via fragmentation and are capable of sexual reproduction as well (LaRue et al., 2013). Hybrids of *Myriophyllum sibiricum* and *Myriophyllum spicatum* exhibit traits from both parent species (Madsen et al., 2021). Hybridization is widespread, leading to hybrids with a broad range of characteristics distinct from their parents. These hybrids often display intermediate features, such as leaflet numbers falling within the overlapping range of the parent species (Volkova et al., 2024). Additionally, some hybrids may produce turions, while others may not (Volkova et al., 2024). Moreover, second-generation hybrids have been identified, wherein the initial hybrid crosses with one of the parent species, resulting in new or more variable morphological characteristics (Zuellig & Thum, 2012). Not only are morphological features quite variable but genetic as well. Hybrids are abundant in North America and carry a considerable amount of genetic diversity, indicating frequent hybridization between *Myriophyllum sibiricum* and *Myriophyllum spicatum* (Zuellig & Thum, 2012).Consequently, relying solely on morphology for hybrid identification can be challenging due to the variability in traits and similarity to the parent species (Madsen et al., 2021; Volkova et al., 2024). For this reason, any plant suspected to be a hybrid needs to be confirmed using molecular tools.

The described hybrid, arising from the crossing of *Myriophyllum sibiricum* and *Myriophyllum spicatum*, exhibits a range of morphological distinctions from its parent species (Figure 1.3.). Notably, it lacks the reduced and thickened storage leaves found on the stem base characteristic of *Myriophyllum sibiricum*, and its shoots emerge either from the root crown or the basal part of died-back shoots, unlike the typical basal shoot formation of *Myriophyllum sibiricum*. Stem coloration is green, as opposed to the reddish hue seen in *Myriophyllum spicatum*. Shoot apices are obtuse, contrasting with the rounded or truncated apices of *Myriophyllum sibiricum* and *Myriophyllum*

spicatum, respectively. Leaf characteristics also differ, with the hybrid possessing a greater number of leaf segments arranged at a more acute angle relative to the central axis compared to either parent species. Furthermore, leaf segments tend to lie in a single plane, and the leaf apex is typically obtuse, in contrast to the rounded or truncate apex observed in the parent species. Trichomes in the axils of upper leaf segments are almost absent in the hybrid. Additionally, the absence of turions sets the hybrid apart. These detailed morphological disparities complicate the identification of hybrids based on morphology alone. While the hybrid is fertile and produces globose fruits, its distribution varies, with it being relatively scarce in Eurasia but common in North America, particularly as an invasive species (Volkova et al., 2024).

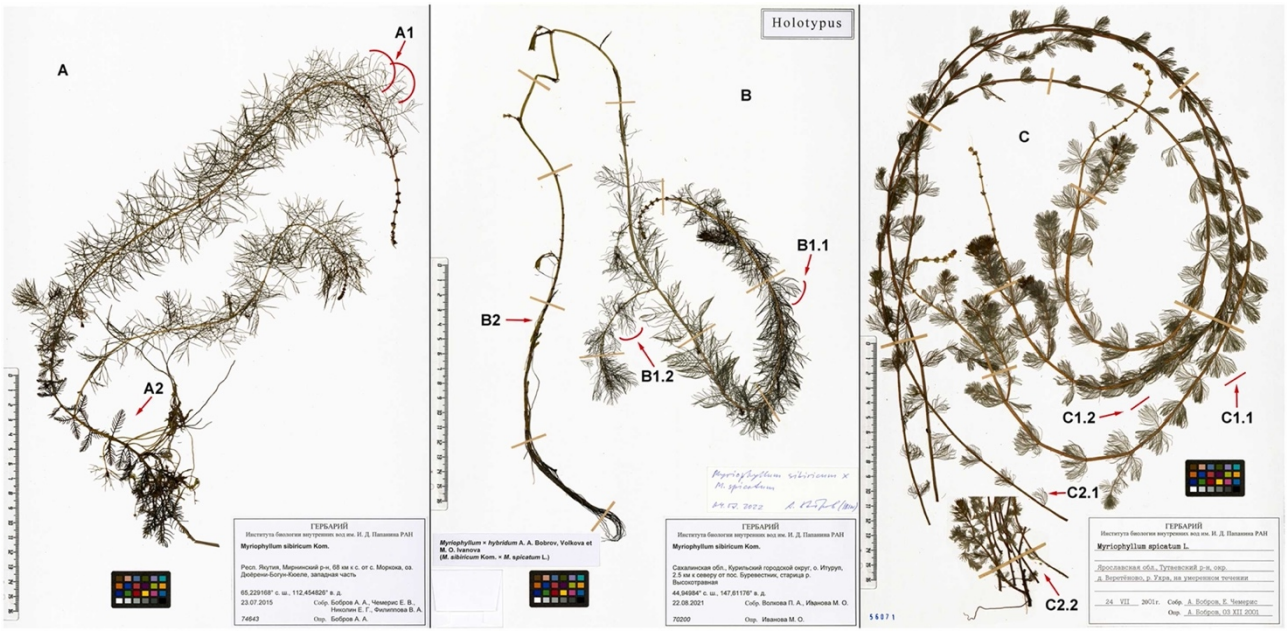

Figure 1.3. Comparison of *Myriophyllum sibiricum, Myriophyllum spicatum* and their hybrid.

Myriophyllum sibiricum (A): Rounded leaf apexes; Basal part of shoot with reduced leaves and thickened segments, originating from turion.

Hybrid from Iturup Island (B): Obtuse leaf apexes; Basal part of shoot originating from root crown.

Myriophyllum spicatum (C): Truncate leaf apexes; Basal part of shoot with normal leaves, originating from remained basal part of died-back shoot.

Taken from: Volkova et al., 2024.

The key features of *Myriophyllum sibiricum* and *Myriophyllum spicatum* hybrids from Eurasia are summarized in the table below (Table 1.2.) (Volkova et al., 2024).

Table 1.2. Morphological characteristics of *Myriophyllum sibiricum* and *Myriophyllum spicatum* hybrids from Eurasia.

Characteristic	Description
Growth	Perennial, shoots die back over winter, leaving roots, rhizomes, and basal part of shoot

Leaf Shape	Capillary leaves only (myriophyllid) with obtuse apex
Stem Length	50–100 cm long, green, glabrous, branched distally
Turions	Mostly absent
Leaf Arrangement	Whorled, 4 leaves in one node
Leaf Size	Length: 12–35 mm, Width: 10–30 mm
Leaf Segments	15–27 linear-filiform segments, forming angles 45–60° with central axis, laying in one plane
Leaf Apex	Obtuse
Inflorescence	Unbranched, erect, up to 15 cm long
Flower Characteristics	Pistillate (female) flowers proximally, staminate (male) flowers distally, with transitional zone of bisexual flowers
Flower Type	Unisexual or bisexual, sessile
Bracteoles	Cream or purple with reddish or brown margin, usually ovate to obovate
Sepals	Cream to stramineous, ovate to triangular
Petals	Cream to red or dark purple, oblong to elliptic or obovate
Stamens	8 filaments up to 1.3 mm long, anthers greenish cream to yellow or purple
Pistils	1–1.5 mm long, stigmas white to red
Fruit	Globose, 4-lobed, olive-green to brown, cylindrical to narrowly ovoid
Mericarps	1.5–2.5 mm long, 1–1.5 mm wide, transversely widely obovate

While studying *Myriophyllum sibiricum* and *Myriophyllum spicatum* hybrids in Eurasia scientists concluded that the hybridization between these species on the Commander and the southern Kuril Islands likely occurred due to climate oscillations during the Pleistocene, enabling contact between parental species. Additionally, ongoing climate warming may expand the range of *Myriophyllum spicatum* northward, increasing the likelihood of hybridization with *Myriophyllum sibiricum*. The discovery of hybrids in central East Europe indicates ecological isolation break due to rapid artificial habitat change. Human-mediated hybridization between the two species could be

widespread in transformed habitats of densely inhabited West and Central Europe (Volkova et al., 2024).

Another study investigated how climate niche influences hybridization between closely related plant species, *Myriophyllum sibiricum* and *Myriophyllum spicatum*. In Eurasia, except for the Qinghai-Tibet Plateau (QTP), *Myriophyllum sibiricum* is typically found farther north than *Myriophyllum spicatum*, resulting in limited overlap between their ranges. This pattern is consistent in the Northeast China (NEC) region as well. Hybrid presence was mainly observed in transition zones where competitive pressures from parental species were strong. In the QTP, where the two species' ranges extensively overlap, hybridization was more common, with hybrid populations scattered across different regions. Notably, climate niche overlap between parental species and hybrids significantly promoted hybridization, especially in the QTP. However, hybrid fitness wasn't solely determined by climate factors; their niche resembled that of either parental species more than exhibiting transgressive traits, suggesting other environmental factors also influenced hybrid fitness. In North America, where *Myriophyllum spicatum* has invaded, hybridization with *Myriophyllum sibiricum* was also widespread, likely facilitated by overlapping climatic niches of the two species (Wu et al., 2015).

Overall, the study underscores the role of climate niche overlap in driving hybridization between closely related plant species. While climate factors influence hybridization patterns, other environmental factors also play a crucial role in determining hybrid fitness and adaptation. Further research is needed to unravel the complex interactions between environmental factors and hybridization dynamics in these *Myriophyllum* species.

1.4. Genetic markers

A genetic marker refers to a gene or DNA sequence located on a chromosome whose known position can help identify individuals or species. Essentially, it's a variation that can be observed and may arise from mutations or alterations in genomic loci. Genetic markers can vary in length, ranging from short sequences surrounding a single base-pair change, known as single nucleotide polymorphisms (SNPs), to longer ones like minisatellites and microsatellites. Microsatellites consist of short units arranged in tandem, typically less than 10 base pairs, whereas minisatellites consist of longer units, usually between 10 and 100 base pairs, arranged in tandem (Richard & Pâques, 2000). Microsatellites, also known as simple sequence repeat (SSR) markers, consist of multiple copies of a repeated short sequence. They are valuable for mapping due to their high informativeness, being polymorphic and possessing numerous different alleles spread throughout populations.

Genetic markers are often used in plant sciences (Amiteye, 2021). They can help identify species and confirm the existence of hybrids (Mandal, 2018; Zhu et al., 2022; Chakraborty &

Rannala, 2023). This can be useful when morphology alone is not enough to identify a species or hybrid. Other applications of genetic markers include: Genetic Diversity Analysis, Genetic Linkage Map Construction, Mapping of quantitative trait loci (QTLs), Map based cloning of genes, Mapping of mutations, Marker-assisted selection (MAS), Marker assisted backcross breeding (MAB), Marker-assisted pyramiding, Mapping major genes, Characterization of transformants, etc. (Mandal, 2018). Because of their vast applications and ease of use genetic markers are often used in systematics, population genetics, evolutionary biology and conservation genetics (Mandal, 2018).

Genetic markers have also been used when studying and analyzing *Myriophyllum* (Moody & Les, 2010; Thum et al., 2011; Zuellig & Thum, 2012; LaRue et al., 2013; Wu et al., 2015; Lü et al., 2017; Tóth et al., 2017; Thum et al., 2020; Madsen et al., 2021; Wu et al., 2022; Volkova et al., 2024). In a study employing AFLP (Amplified Fragment Length Polymorphism) molecular markers to investigate genetic variations in *Myriophyllum spicatum*, *Myriophyllum sibiricum*, and hybrids, researchers compared taxonomic identifications using ITS and AFLP markers. They found that ITS generally offers more reliable identifications. However, they also noted a low error rate (1.7%), indicating that ITS does not always accurately distinguish some genetically distinct groups among *Myriophyllum spicatum*, *Myriophyllum sibiricu*m, and their hybrids (Zuellig & Thum, 2012). Another study concluded that using SNP markers instead of microsatellites for lineage identification and tracking is expected to improve accuracy and efficiency. Additionally, finding diploidized SNP markers would help analyze allele frequencies, unlike the current method that treats polyploid markers as dominant data and identifies unique multilocus genotypes (Thum et al., 2020). Short DNA regions, namely nuclear ITS2 and plastid DNA *trn*L-*trn*F, were found to effectively discriminate between *Myriophyllum spicatum*, *Myriophyllum sibiricum*, and their hybrids in Eurasia (Volkova et al., 2024).

1.4.1. Inter Simple Sequence Repeats (ISSR)

ISSRs are sections of the genome flanked by simple sequence repeat or microsatellite sequences, distributed randomly throughout the genome (Amiteye, 2021). This marker system relies on PCR amplification of genomic regions flanked by identical microsatellites in opposite orientations, generating multilocus markers (Amiteye, 2021). It offers a straightforward and rapid method combining the advantages of microsatellites (SSRs) and Amplified fragment Length Polymorphism (AFLP) with the universality of Random Amplified Polymorphic DNA (RAPD) (Marwal & Gaur, 2020). Highly polymorphic ISSR markers are valuable in genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology studies (Marwal & Gaur, 2020; Amiteye, 2021). One major benefit is the independence from prior DNA sequence knowledge to design primers (Amiteye, 2021). Additionally, ISSR analysis requires only a small amount of

DNA, typically 10–50 ng per reaction, overcoming practical limitations of SSR, AFLP, and RAPD analyses (Amiteye, 2021). ISSRs have also been used to identify closely related species (Sorof Uddin & Cheng, 2015). The ISSR marker system can also detects polymorphisms in inter-microsatellite DNA regions without any prior sequence knowledge (Sorof Uddin & Cheng, 2015). ISSR markers were utilized in a study of *Myriophyllum* conducted in China (Q. Cao et al., 2017).

1.4.2. Internal transcribed spacer (ITS)

The Internal Transcribed Spacer (ITS) is a nonfunctional RNA segment situated between structural ribosomal RNAs (rRNA) within a shared precursor transcript, serving as a valuable tool in discerning relationships among species within the same genus or closely related genera (Hao et al., 2015). Sequence comparisons of eukaryotic ITS regions are widely employed in taxonomy and molecular phylogeny due to several advantageous properties (Baldwin et al., 1995). ITS is routinely amplified thanks to its small size and the presence of highly conserved flanking sequences, making it easy to detect even in small DNA quantities due to the high copy number of rRNA clusters (Baldwin et al., 1995). Rapid concerted evolution occurs via processes like unequal crossing-over and gene conversion, promoting intra-genomic homogeneity of repeat units (Ganley, 2013). However, high-throughput sequencing has revealed frequent variations within plant species (Song et al., 2012). There is a notable degree of variation even among closely related species, attributed to the relatively low evolutionary pressure acting on non-coding spacer sequences like ITS (Kovarik et al., 2008). Many studies have used ITSs when studying *Myriophyllum* (Moody & Les, 2010; Thum et al., 2011; Zuellig & Thum, 2012; Lü et al., 2017; Madsen et al., 2021; Volkova et al., 2024).

2. MATERIALS AND METHODS

2.1. Materials

A list of materials that were used during this study is listed below (Table 2.1.).

Table 2.1. Materials that were used during the study and their manufacturer.

Material	Manufacturer
Sodium Chloride (NaCl)	Thermo Fisher Scientific, Lithuania
β-Mercaptoethanol	Merck KGaA; Germany
CTAB	Merck KGaA; Germany
Polyvinylpyrrolidone (PVP)	Merck KGaA; Germany
EDTA	Merck KGaA; Germany
Tris-Cl	Fisher Scientific, Belgium
RNase	Thermo Fisher Scientific, Lithuania
Chloroform	ROTH, Germany
Ethanol	AB "Stumbras", Lithuania
Water, nuclease-free	Thermo Fisher Scientific, Lithuania
DreamTaq Green PCR Master Mix (2×)	Thermo Fisher Scientific, Lithuania
Various primers	—
TopVision Agarose	Thermo Fisher Scientific, Lithuania
TAE Buffer (50×)	Thermo Fisher Scientific, Lithuania
Ethidium Bromide	ROTH, Germany
GeneRuler DNA Ladder Mix	Thermo Fisher Scientific, Lithuania
GeneJET Gel Extraction Kit	Thermo Fisher Scientific, Lithuania

Several different primers were employed for PCR amplification, the primers used are listed below (Table 2.2.).

Table 2.2. Primers that were utilized during the study.

Where **B** = G, T or C; **D** = G, A or T; **H** =A, T or C; **V** = G, A or C.

Primer	Sequence, 5'→3'	T_m, °C
ISSR-I-39a	$(AGC)_4AC$	42
ISSR-D	$(AG)_9$	43
ISSR-F	$(AC)_8$	43
ISSR-Ward-2	$(AC)_8G$	47
ISSR FR02	**BDB**$(CA)_7$	37

ISSR FR01	**VBV**$(AC)_7$	37
UBC 808	$(AG)_8C$	47
UBC809	$(AG)_8G$	47
UBC 878	$(GGAT)_4$	43
UBC 890	**VHV**$(GT)_7$	47
ITS1	GGAAGTAAAAGTCGTAACAAGG	54
ITS4	TCCTCCGCTTATTGATATGC	54

2.2. Methods

2.2.1. *Myriophyllum* sample collection.

90 *Myriophyllum* samples were collected from 6 different locations in Lithuania and Latvia (Figure 2.1.):

1. Švento lake – 24 samples,
2. Kuršėnų pond – 12 samples,
3. Balžio lake – 24 samples,
4. Verniejaus lake – 6 samples,
5. Širvintos river – 12 samples,
6. Stirns lake (Latvia) – 12 samples.

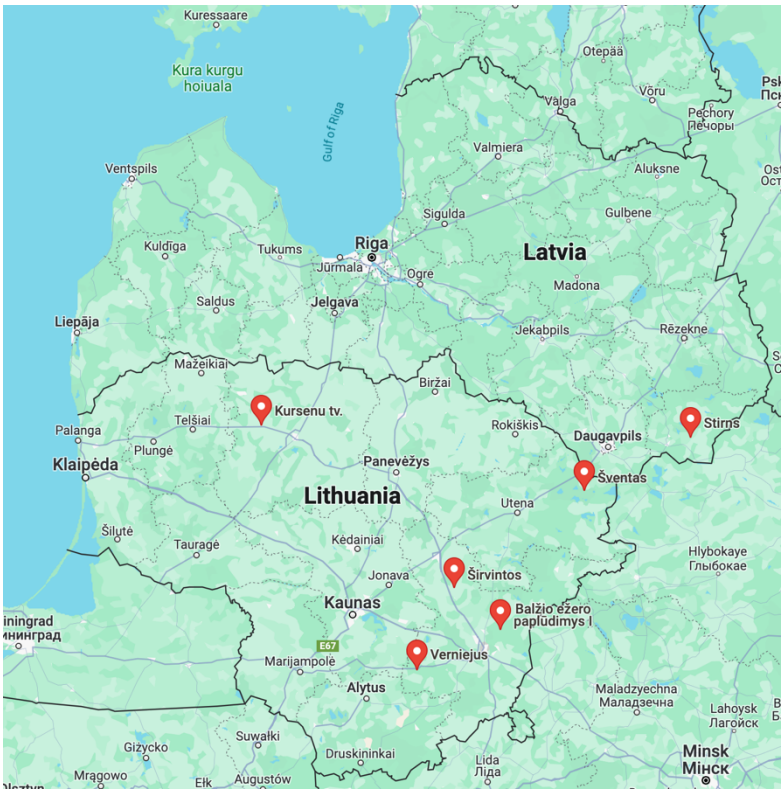

Figure 2.1. The geographical sites where *Myriophyllum* samples were collected. Photo taken from Google Maps.

Individuals were collected for herbarium specimen preparation, to use in morphological studies, and molecular analysis. Each sample was placed in a Ziploc bag and transported to the laboratory for further analysis. The collected samples are believed to belong to the following species: *Myriophyllum spicatum*, *Myriophyllum verticillatum*, *Myriophyllum sibiricum*, and *Myriophyllum alterniflorum*. The individuals were identified based on morphology (Table 1.1.).

2.2.2. CTAB DNA extraction

The collected *Myriophyllum* sample DNA was extracted using a standard CTAB DNA extraction protocol.

1. Prepare CTAB extraction buffer (amount for 10 mL):
    - CTAB 10% – 3 mL;
    - 5 M NaCl – 2.8 mL;
    - 0.5 M EDTA (pH 8.0) – 0.4 mL;
    - 1 M Tris-Cl (pH 8.0) – 1 mL;
    - Polyvinylpyrrolidone (PVP) – 0.3 g;
    - β-Mercaptoethanol – 0.02 mL;
    - H_2O – 2.48 mL.
2. Add 5 µL of RNase to the microcentrifuge tubes.
3. Add 800 µL of the prepared CTAB extraction buffer directly to the dry *Myriophyllum* sample and grind the samples using a mortar and pestle.
4. Transfer the liquid to microcentrifuge tubes (with the RNase), then incubate at 60°C while shaking at 650 rpm for 1 hour using a thermoshaker.
5. After incubation, add 1 mL of chloroform.
6. Centrifuge the samples at high speed (12,000 x g) for 5 – 10 minutes at room temperature.
7. Carefully transfer the upper aqueous phase (containing DNA) to a fresh microcentrifuge tube, leaving behind the interphase and organic phase.
8. Add 0.5 volumes of NaCl and centrifuge again.
9. Add 2 volumes of 96% ethanol and mix gently by inversion.
10. Incubate the samples at -20°C for 30 minutes to allow DNA precipitation.
11. Centrifuge the samples at high speed (12,000 x g) for 5 – 10 minutes.
12. Carefully decant the supernatant without disturbing the DNA pellet.
13. Add 200 µL of 70% ethanol and wash the DNA pellet by gently vortexing.
14. Centrifuge the samples at high speed (12,000 x g) for 5 minutes.
15. Carefully remove the ethanol and air-dry the DNA pellet for 10-15 minutes.

16. Add 50 µL of H_2O and resuspend the DNA pellet.
17. Store the purified DNA at -20°C or proceed with downstream applications.

2.2.3. Spectrophotometric measurement of DNA concentration and purity.

The concentration of *Myriophyllum* sample DNA was assessed by measuring the light absorbance at 260 nm using the NanoDrop™ 2000/2000c Spectrophotometer. Absorbance at 280 and 230 nm was also measured to determine the purity of the DNA.

Nucleic acids absorb light at 260 nm and proteins absorb at 280 nm. EDTA, carbohydrates and phenol all have absorbance near 230 nm. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A 260/280 ratio of ~1.8 is generally accepted as "pure" for DNA and a 260/280 ratio of ~2.0 is generally accepted as "pure" for RNA. If the ratio is lower in either case, it may indicate the presence of protein other contaminants that absorb strongly at or near 280 nm. Good-quality DNA will have a 260/280 ratio of 1.7 – 2.0. 260/230 ratio is used as a secondary measure of nucleic acid purity. Good-quality DNA will have a 260/230 ratio of 2.0 – 2.2. If the ratio is lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

After evaluating the concentration and quality, 60 samples were chosen for subsequent analysis, and their DNA was diluted to 5 ng/µL.

The samples were from four different locations:

1. *M. sibiricum*, Švento lake (samples 1 – 12)
2. *M. alterniflorum*, Švento lake (samples 13 – 24)
3. *Myriophyllum* sp., Kuršėnų pond (samples 25 – 36)
4. *Myriophyllum* sp., Širvintos river (samples 37 – 48)
5. *M. sibiricum*, Balžio lake (samples 49 – 60)

2.2.4. ISSR-PCR

To amplify ISSRs from *Myriophyllum* samples, PCR was conducted using a set of 10 different ISSR and UBC primers. The primers used for ISSR-PCR: ISSR-I-39a; ISSR-D; ISSR-F; ISSR-Ward-2; ISSR FR02; ISSR FR01; UBC 808; UBC809; UBC 878; UBC 890 (Table 2.2).

The PCR reaction mix for 1 reaction is listed below (Table 2.3).

Table 2.3. PCR reaction mix.

Component	Volume
Water, nuclease-free	2 µL
DreamTaq Green PCR Master Mix (2×)	5 µL
Primer	1 µL

2 µL of the extracted genomic DNA is then added to the reaction mix. 8 µL rection mix + 2 µL DNA = 10 µL total volume.

The PCR conditions utilized are detailed below (Table 2.4.). The PCR reactions were conducted using the Eppendorf Mastercycler ® PCR Thermal Cycler.

Table 2.4. PCR protocol.

Step	Temperature, °C	Time	Number of Cycles
Initial Denaturation	94	7 min	1
Denaturation	94	30 s	32
Primer Annealing	T_m-5	45 s	
Extension	72	2 min	
Final Extension	72	7 min	1
HOLD	4	∞	1

2.2.5. ISSR fragment electrophoresis

To see the amplified ISSR fragments agarose gel electrophoresis was performed. A 200 mL TBE agarose gel with a concentration of 1.2% was prepared.

Procedure:

1. Prepare the agarose gel by dissolving 2.4 g of agarose powder in 200 mL of TBE buffer (40 mL TBE (5×) + 160 mL H_2O).
2. Heat the mixture in a microwave or on a hot plate until the agarose is completely dissolved. Allow it to cool to about 50-60°C.
3. Add 2 – 5 µL of ethidium bromide or another DNA staining dye to the gel mixture if desired, and swirl gently to mix.
4. Pour the agarose solution into the gel tray, ensuring no air bubbles are trapped, and insert the comb to create wells for loading samples.
5. Allow the gel to solidify for about 30 minutes to 1 hour.
6. Carefully remove the comb and place the gel into the electrophoresis chamber. Fill the chamber with enough TBE buffer to cover the gel.
7. Mix the DNA samples with gel loading dye (if using) and load them into the wells of the gel using a micropipette.
8. Connect the electrodes to the power supply and run the gel at an appropriate voltage (typically 80-120 volts) until the dye reaches the desired position (usually about 30 minutes to 2 hours).

9. After electrophoresis, turn off the power supply and carefully remove the gel from the chamber.
10. Visualize the DNA bands by placing the gel on a UV transilluminator. Take a photograph of the gel for documentation if needed.
11. Dispose of the gel and buffer properly.

2.2.6. Statistics analysis of ISSR markers

GenAlEx was employed to analyze the ISSR fragments from the *Myriophyllum* samples. It is a versatile software package for population genetic analyses that operates within Microsoft Excel. GenAlEx facilitates the analysis of diploid codominant, haploid, and binary genetic loci, as well as DNA sequences.

Additional genetic analysis was preformed using POPGENE. POPGENE is a collaborative project developed by Francis Yeh, Rongcai Yang, and Timothy Boyle. It is a user-friendly freeware tool designed for analyzing genetic variation both among and within populations using co-dominant and dominant markers.

The use of ISSR markers and statistical programs like GenAlEx and POPGENE provide an effective way of assessing the genetic diversity and structure of *Myriophyllum* populations. These tools can help in identifying genetic similarities and differences that are not evident from morphological data alone.

2.2.7. ITS PCR

To amplify the ITS region from *Myriophyllum* samples, PCR was conducted using the ITS 1 and ITS4 primers (Table 2.2).

The PCR reaction mix for 1 reaction is listed below (Table 2.5).

Table 2.5. PCR reaction mix.

Component	Volume
Water, nuclease-free	21 µL
ITS1	1 µL
ITS4	1 µL

25 µL of DreamTaq Green PCR Master Mix (2×) and 2 µL of the extracted genomic DNA is then added to the reaction mix. 23 µL rection mix + 25 µL DreamTaq + 2 µL DNA = 50 µL total volume.

The PCR conditions utilized are detailed below (Table 2.6.). The PCR reactions were conducted using the Eppendorf Mastercycler ® PCR Thermal Cycler.

Table 2.6. PCR protocol.

Step	Temperature, °C	Time	Number of Cycles
Initial Denaturation	94	2 min	1
Denaturation	94	1 min	30
Primer Annealing	54	30 s	
Extension	72	2 min	
Final Extension	72	5 min	1
HOLD	4	∞	1

2.2.8. ITS electrophoresis

To see the amplified ITS fragments agarose gel electrophoresis was performed. A 150 mL TAE agarose gel with a concentration of 0.8% was prepared.

The subsequent procedures mirror those outlined in section 2.2.5., with the exception that the gel volume is reduced to 150 mL, the gel concentration is adjusted to 0.8%, and TAE buffer is substituted for TBE buffer.

2.2.9. DNA Gel extraction

Since we want to sequence the amplified ITS region, we need to extract it from the agarose gel. For this we utilized the GeneJET Gel Extraction Kit.

DNA gel extraction protocol:

1. Prepare the Gel: Run the DNA sample on an agarose gel according to standard protocols until the desired DNA bands are clearly separated.
2. Visualize DNA Bands: After electrophoresis, visualize the DNA bands under UV light using a gel documentation system or a UV transilluminator. Mark the positions of the DNA bands of interest on the gel using a clean, fine-tip marker.
3. Excise DNA Bands: Using a clean scalpel or razor blade, carefully excise the gel slice containing the DNA band of interest. Take care to minimize contamination from adjacent gel regions.
4. Weigh Gel Slice: Weigh the gel slice containing the DNA band to determine the amount of agarose present. Record this weight for later reference.
5. Minimize Gel Contamination: Transfer the gel slice to a clean, sterile microcentrifuge tube. Minimize the amount of agarose transferred to avoid contamination and interference with downstream applications.

6. Gel Extraction: Perform gel extraction according to the provided GeneJET Gel Extraction Kit instructions. Briefly, this involves crushing the gel slice, solubilizing the DNA, and binding it to a silica-based membrane in a spin column.
7. Wash and Elute DNA: Wash the DNA-bound membrane to remove contaminants and then elute the purified DNA from the membrane using an appropriate elution buffer or water.
8. Store DNA: Store the purified DNA at -20°C or -80°C for long-term storage or proceed directly to downstream applications such as PCR, cloning, or sequencing.

2.2.10 DNA sequencing and ITS region analysis

The extracted DNA is subsequently prepared and sent for Sanger sequencing at the BaseClear Sequencing Center in the Netherlands. Following sequencing, the obtained sequences undergo analysis using MEGA X. This includes performing multiple sequence alignments (MSA), creating a distance matrix, constructing phylogenetic trees, and other relevant analyses.

The *Myriophyllum* samples were labeled: MY01 – MY36, MY being short for *Myriophyllum*.

3. RESULTS AND DISCUSSION

3.1. ISSR amplification and electrophoresis

DNA from 60 *Myriophyllum* samples, collected from four different locations in Lithuania (Švento lake, Kuršėnų pond, Balžio lake and the Širvintos river), was extracted and amplified using 10 selected ISSR and UBC primers via PCR (section 2.2.4.). The amplified DNA products were visualized on an electrophoresis gel, the resulting bands were quantified, and their statistical analysis was conducted.

The primers used for ISSR-PCR: ISSR-I-39a; ISSR-D; ISSR-F; ISSR-Ward-2; ISSR FR02; ISSR FR01; UBC 808; UBC809; UBC 878; UBC 890. The primer sequences and melting temperature can be found in Table 2.2.

ISSR amplification was successful, and bands were clearly visible in the electrophoresis gel (Figure 3.1.).

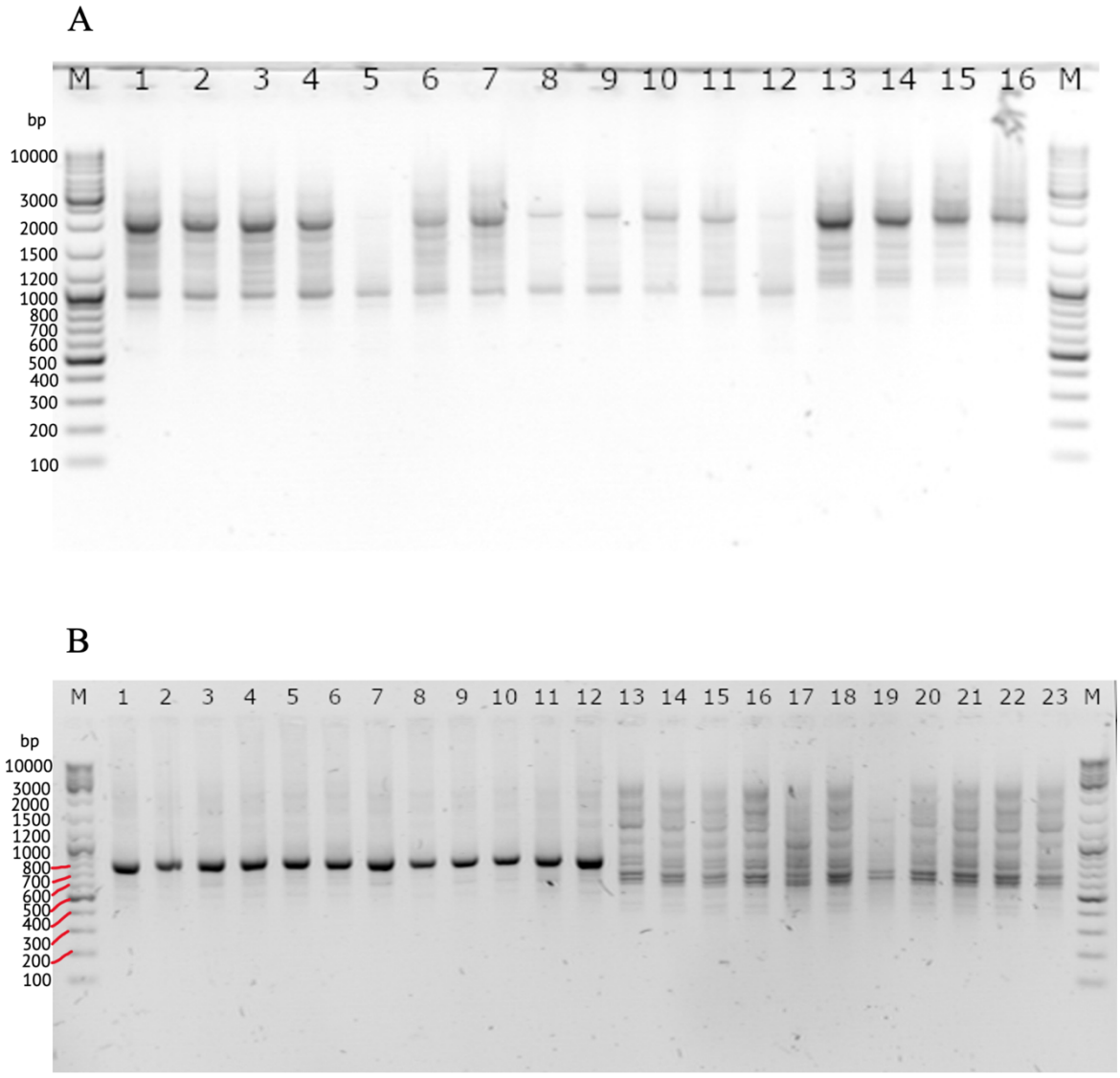

Figure 2.1. Electrophoresis results after ISSR-PCR. A – results from ISSR-D primer; B – results from UBC 890 primer; 1-23 – *Myriophyllum* samples; M – GeneRuler DNA Ladder Mix; bp – Base Pair.

Despite the success of ISSR amplification and electrophoresis, it is important to note that not all samples were consistently amplified in most PCR reactions (Figure 3.2.). The majority of primers amplified approximately 54 samples. Notably, only the UBC 890 primer successfully amplified all 60 *Myriophyllum* samples, whereas primer UBC 878 exhibited the lowest amplification success, with only 49 samples being amplified (Table 3.1.).

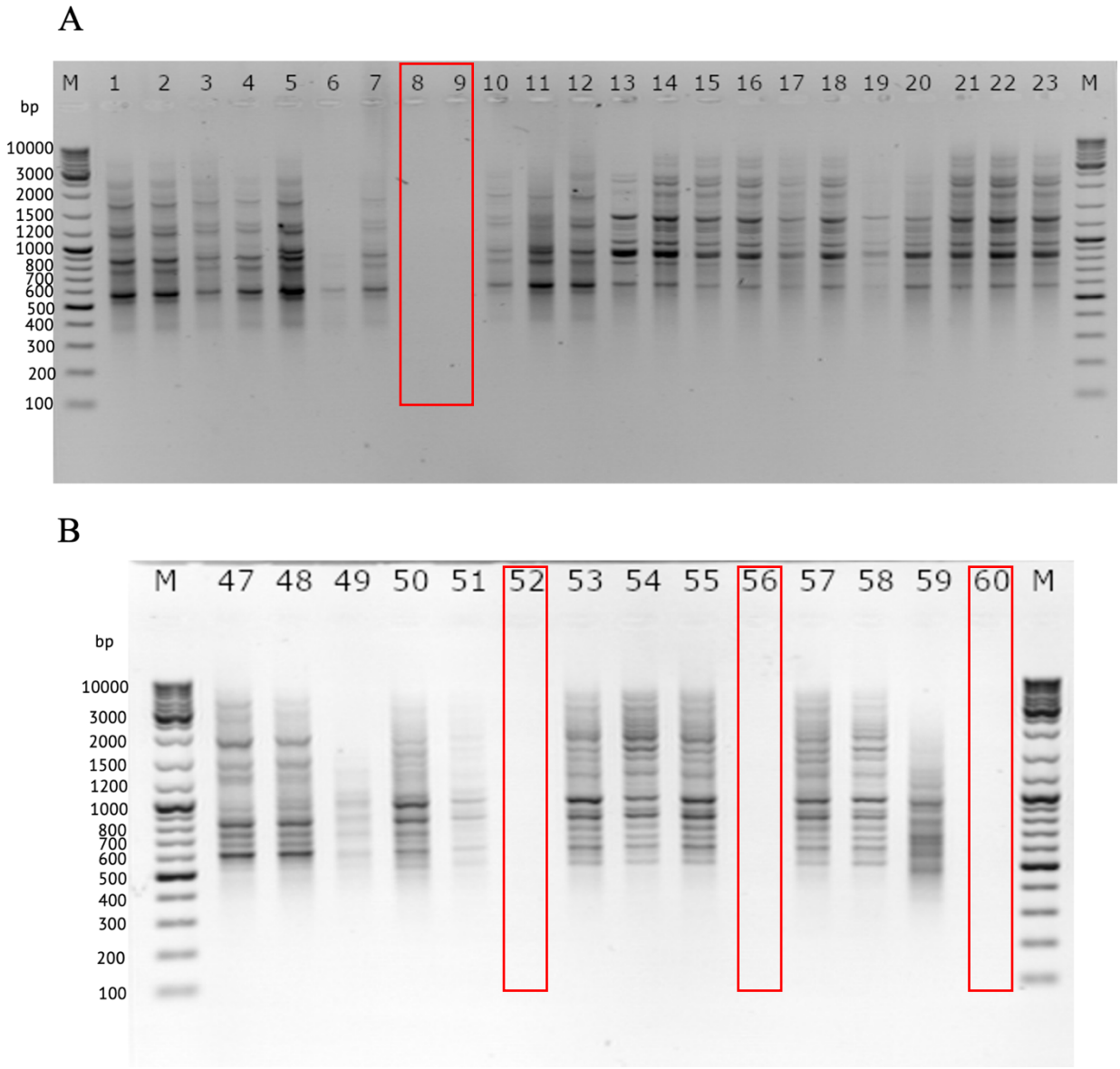

Figure 3.2. Electrophoresis results after ISSR-PCR when some of the samples do not amplify during PCR. Samples that failed to amplify are outlined in red. A – results from ISSR-F primer; B – results from ISSR-Ward 2 primer; 1-23, 47-60 – *Myriophyllum* samples; M – GeneRuler DNA Ladder Mix; bp – Base Pair.

Table 3.1. Summary of ISSR primer amplification success. The table summarizes the number of *Myriophyllum* samples successfully amplified by each of the 10 ISSR primers. The percentage column indicates the proportion of samples amplified for each primer.

Primer	Number of amplified samples	Number of samples that failed to amplify	Amplification success
ISSR-I-39a	56	4	93.3%
ISSR-D	54	6	90%
ISSR-F	53	7	88.3%
ISSR-Ward-2	52	8	86.6%
ISSR FR02	57	3	95%
ISSR FR01	56	4	93.3%
UBC 808	53	7	88.3%
UBC 809	58	2	96.6%
UBC 878	49	11	81.6%
UBC 890	60	0	100%

The amplified ISSR DNA fragments used for further genetic analysis ranged in size from 400 bp to 3000 bp. The primer ISSR-D produced the widest range of fragment lengths, spanning from 700 to 3000 bp, while primer ISSR-I-39a yielded the smallest range, from 400 to 1300 bp. A total of 87 loci were identified across all primers, with ISSR-D detecting the highest number of loci (12) and both UBC 878 and UBC 890 identifying the fewest (6). Each primer exhibited high polymorphism, between 85.71% and 100%. Seven primers had no monomorphic loci and three primers (ISSR-I-39a, ISSR-F, ISSR FR01) had only one monomorphic locus. ISSR-I-39a had the lowest level of polymorphism with 85.71% (Table 3.2).

Table 3.2. Characteristics of amplified ISSR DNA fragments. The table below presents the range of fragment sizes and the number of monomorphic, polymorphic and total loci identified for each primer used in the genetic analysis of *Myriophyllum* samples.

Primer	Fragment size, bp	Number of monomorphic loci	Number of polymorphic loci	Total loci number
ISSR-I-39a	400 – 1300	1	6	7
ISSR-D	700 – 3000	0	12	12
ISSR-F	550 – 2250	1	8	9
ISSR-Ward-2	600 – 2000	0	11	11
ISSR FR02	400 – 2000	0	8	8
ISSR FR01	400 – 2200	1	9	10

UBC 808	600 – 2000	0	10	10
UBC 809	500 – 2000	0	8	8
UBC 878	700 – 2500	0	6	6
UBC 890	600 – 1700	0	6	6

All ISSR primers demonstrated polymorphism, making them suitable for further analysis of *Myriophyllum* populations.

3.2. Genetic analysis of ISSR markers using GenAlEx

Five populations, each consisting of 12 individuals (totaling 60 samples), were analyzed. A dominant (binary) loci matrix was created based on the 87 identified loci. This matrix was subsequently used for analysis in GeneAlEx.

The populations that were used in the analysis:

1. *M. sibiricum*, Švento lake (samples 1 – 12)
2. *M. alterniflorum*, Švento lake (samples 13 – 24)
3. *Myriophyllum* sp., Kuršėnų pond (samples 25 – 36)
4. *Myriophyllum* sp, Širvintos river (samples 37 – 48)
5. *M. sibiricum*, Balžio lake (samples 49 – 60)

AMOVA analysis revealed that genetic diversity between the studied populations was higher than within the populations. Specifically, molecular variance between populations accounted for approximately 63%, while diversity within populations constituted 37%. (Figure 3.3, Table 3.3).

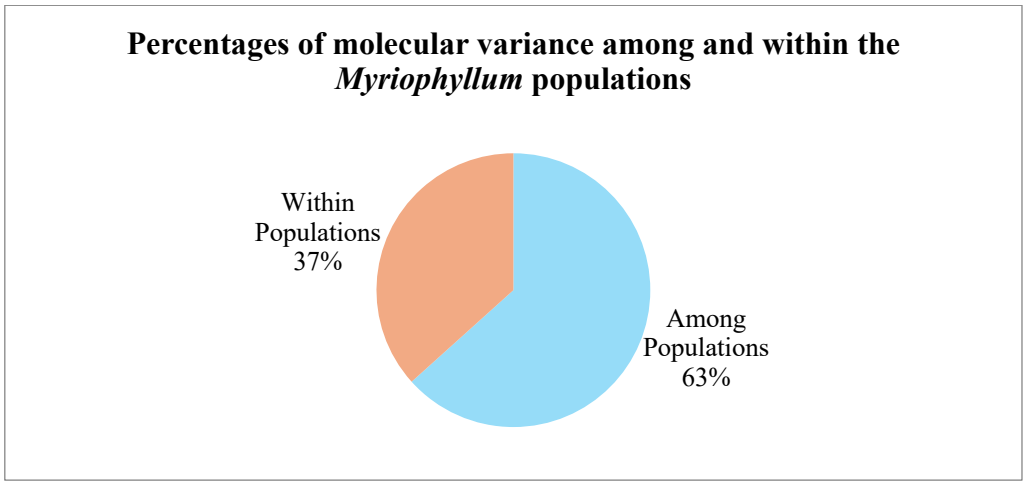

Figure 3.3. AMOVA analysis of the *Myriohyllum* sp. populations.

Table 3.3. AMOVA summary. df – degreed of freedom; SS – sum of squares; MS – mean squares; Est. Var. – estimate of variance.

Source	df	SS	MS	Est. Var.	Percentage of total variation
Among Populations	4	516.167	129.042	10.259	63%
Within Populations	55	326.583	5.938	5.938	37%
Total	59	842.750		16.197	100%

The higher percentage of molecular variance between populations indicates significant genetic differentiation among the five populations of *Myriophyllum* sp. studied. This suggests that each population has unique genetic characteristics, likely influenced by their different geographic locations and possibly environmental conditions. The within-population diversity, while present, is less pronounced compared to the variation observed between the populations.

When analyzing band patterns across populations, we observed that the number of DNA bands varied between 47 and 61 across the five populations. *Myriophyllum alterniflorum* samples from Švento lake exhibited the highest number of bands at 61, while samples from Kuršėnų pond and Balžio lake had the lowest at 47. All bands had a frequency of ≥5%. Additionally, bands with a frequency of ≤50% were present in all populations (Figure 3.4. and Table 3.4.).

Unique bands were identified in all populations except for *M. sibiricum* from Balžio lake, which had no unique bands. *M. alterniflorum* from Švento lake exhibited the highest number of unique bands, totaling 22. In contrast, the remaining populations displayed only 1 to 2 unique bands each (Figure 3.4. and Table 3.4.).

The mean heterozygosity across all populations ranged from 0.093 to 0.233. *M. sibiricum* from Balžio lake exhibited the highest heterozygosity at 0.233, while *Myriophyllum* sp. from Širvintos river had the lowest at 0.093. The heterozygosity in the remaining populations was similar, ranging from 0.151 to 0.197 (Figure 3.4. and Table 3.4.).

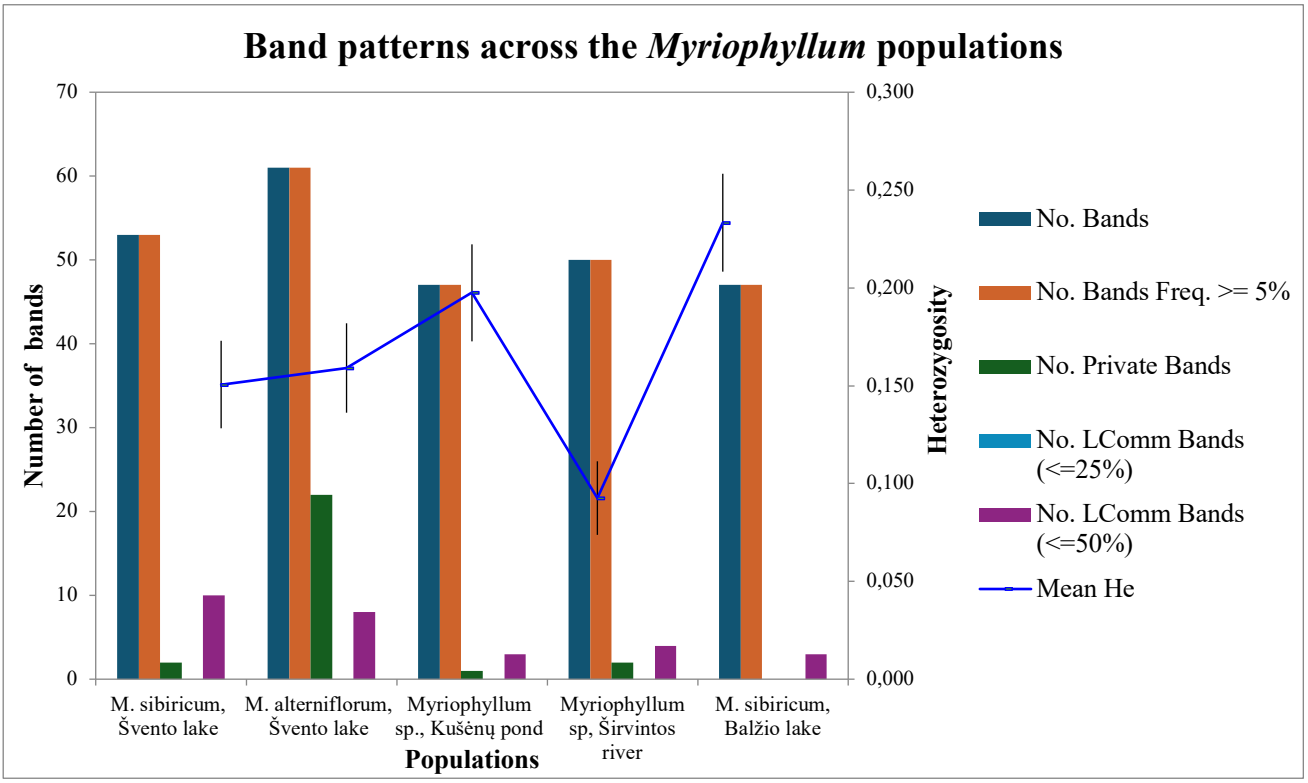

Figure 3.4. Distribution of bands observed in electrophoresis across the studied *Myriophyllum* populations. **No. Bands** = Number of Different Bands; **No. Bands Freq. >= 5%** = Number of Different Bands with a Frequency >= 5%; **No. Private Bands** = Number of Bands Unique to a Single Population; **No. LComm Bands (<=25%)** = Number of Locally Common Bands (Freq. >= 5%) Found in 25% or Fewer Populations; **No. LComm Bands (<=50%)** = Number of Locally Common Bands (Freq. >= 5%) Found in 50% or Fewer Populations; **He** = Expected Heterozygosity = 2 * p * q.

Table 3.4. Band patterns of the five *Myriophyllum* sp. populations.**No. Bands** = Number of Different Bands; **No. Bands Freq. >= 5%** = Number of Different Bands with a Frequency >= 5%; **No. Private Bands** = Number of Bands Unique to a Single Population; **No. LComm Bands (<=25%)** = Number of Locally Common Bands (Freq. >= 5%) Found in 25% or Fewer Populations; **No. LComm Bands (<=50%)** = Number of Locally Common Bands (Freq. >= 5%) Found in 50% or Fewer Populations; **He** = Expected Heterozygosity = 2 * p * q.

Population	*M. sibiricum*, Švento lake	*M. alterniflorum*, Švento lake	*Myriophyllum* sp., Kušėnų pond	*Myriophyllum* sp., Širvintos river	*M. sibiricum*, Balžio lake
No. Bands	53	61	47	50	47
No. Bands Freq. >= 5%	53	61	47	50	47
No. Private Bands	2	22	1	2	0
No. LComm Bands (<=25%)	0	0	0	0	0

No. LComm Bands (<=50%)	10	8	3	4	3
Mean He	0.151	0.159	0.197	0.093	0.233

It can be inferred that there is significant variation in genetic diversity and band patterns among the five *Myriophyllum* sp. populations. *M. alterniflorum* from Švento lake stands out with the highest number of bands and unique bands, suggesting high genetic diversity, enhanced ability to adapt to its environment and significant genetic differentiation from the other populations. Conversely, *M. sibiricum* from Balžio lake shows high heterozygosity, suggesting a high level of genetic variation, but no unique bands, meaning it lacks unique genetic markers that distinguish it from other populations. The lower heterozygosity in *Myriophyllum* sp. from Širvintos river suggests less genetic diversity within this population. The presence of bands with frequencies ≤50% across all populations suggests shared genetic markers, indicating some common ancestry or gene flow between these populations. The variations in band patterns and heterozygosity highlight the complex genetic structure within and between these *Myriophyllum* sp. populations, which could be influenced by factors such as geographic location, ecological conditions, and evolutionary history.

When analyzing the amount of polymorphic and monomorphic loci, it was found that *M. sibiricum* from Balžio lake exhibits the highest percentage of polymorphic loci at 52.87%. In contrast, *Myriophyllum* sp. from Širvintos river shows the lowest polymorphism at 25.29%. Both *M. sibiricum* and *M. alterniflorum* from Švento lake have an identical percentage of polymorphic loci, each at 40.23%. This indicates considerable genetic variation among these populations, with the Balžio lake population displaying the greatest genetic diversity and the Širvintos river population showing the least (Table 3.5.).

Table 3.5. Percentage of polymorphic loci among the studied *Myriophyllum* populations.

Population	Precent of polymorphic loci
M. sibiricum, Švento lake	40.23%
M. alterniflorum, Švento lake	40.23%
Myriophyllum sp., Kuršėnų pond	43.68%
Myriophyllum sp., Širvintos river	25.29%
M. sibiricum, Balžio lake	52.87%

Lastly, Principal Coordinates Analysis (PCoA) was performed to gain insights into the genetic relationships among the five studied *Myriophyllum* sp. populations. The PCoA plot illustrates the genetic distances and relationships among the populations, with closer proximity on the plot indicating greater genetic similarity and further distance indicating greater genetic distinctness.

The first principal coordinate (Coord. 1) is the most significant, explaining over half of the genetic variation (53.60%), suggesting that it captures a major source of genetic differentiation across the populations. The second principal coordinate (Coord. 2) explained 16.15% of the genetic diversity (Figure 3.5.).

The plot reveals two distinct genetic clusters among the populations (outlined in red). *Myriophyllum sibiricum* individuals from Švento lake and Balžio lake, along with *Myriophyllum* sp. individuals from Kuršėnų pond and Širvintos river, clustered closely together, forming one group. This indicates that populations in this group are more genetically similar. *M. alterniflorum* individuals from Švento lake formed the second group, which was more genetically distinct and less similar to the other populations (Figure 3.5.).

Outliers were also identified among three populations. One *M. alterniflorum* individual was significantly apart from the rest of its population. While two *Myriophyllum sibiricum* individuals from Balžio lake and one *Myriophyllum* sp. individual from Kuršėnų pond were also further away from their respective group on the second principal coordinate axis. These four outlier individuals could be potential hybrid organisms (Figure 3.5.).

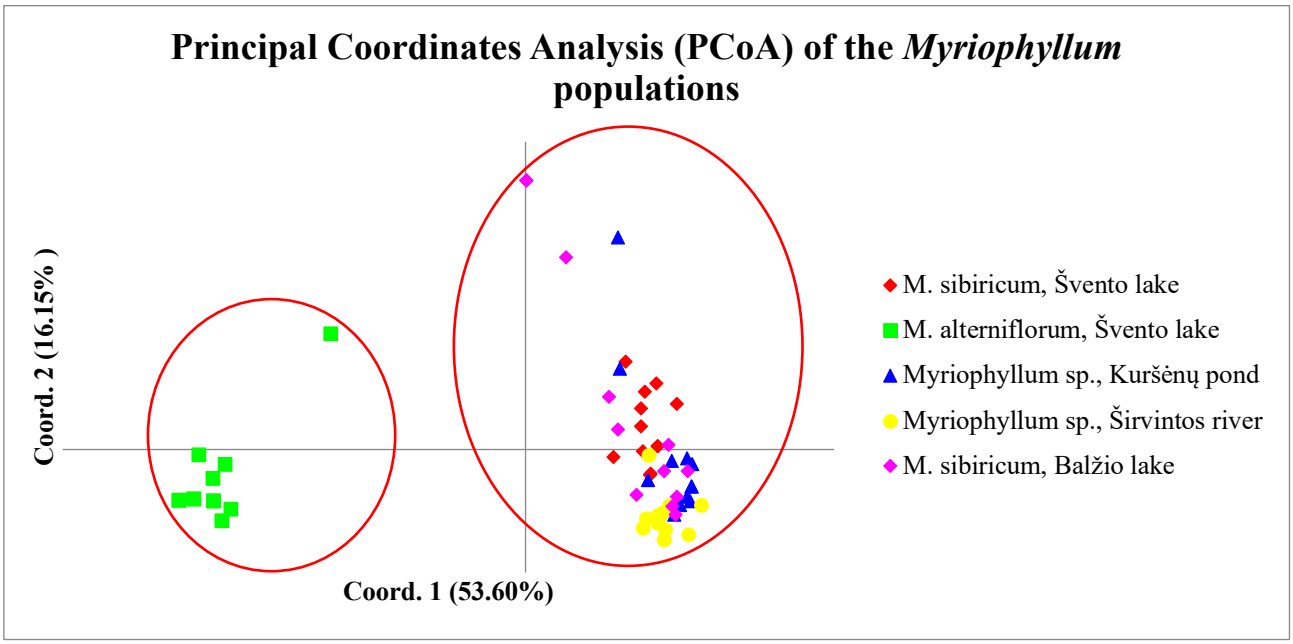

Figure 3.5. Distribution of the studied *Myriophyllum* populations based on Principal Coordinates Analysis (PCoA) results.

Myriophyllum sp. specimens from Kuršėnų Pond and the Širvintos River could not be conclusively identified based on morphology alone and could be potential hybrids. However, the results from Principal Coordinates Analysis (PCoA) suggest that these individuals are likely *M. sibiricum*.

3.3. Genetic analysis of ISSR markers using POPGENE

The genetic diversity of the studied *Myriophyllum* sp. populations was calculated using the POPGENE statistical program, utilizing the same binary matrix as before. Nei's genetic diversity (h)

values ranged from 0.0926 to 0.2333, while Shannon's index (I) values ranged from 0.1366 to 0.3319. The number of polymorphic loci varied between 22 and 46. Notably, both populations from Švento lake had 35 polymorphic loci. *M. sibiricum* from Balžio lake exhibited the highest genetic diversity, with Nei's genetic diversity at 0.2333, Shannon's index at 0.3319, and the highest percentage of polymorphic loci at 52.87%. Conversely, *Myriophyllum* sp. from Širvintos river had the lowest genetic diversity, with Nei's genetic diversity at 0.0926, Shannon's index at 0.1366, and the lowest percentage of polymorphic loci at 25.29%. Populations from Švento lake (both *M. sibiricum* and *M. alterniflorum*) and Kuršėnų pond displayed moderate levels of genetic diversity, with similar values for Nei's genetic diversity and Shannon's index (Table 3.6.).

Table 3.6. Indicators of genetic diversity in each *Myriophyllum* sp. population: h – Nei's genetic diversity; I – Shannon's index; SD - standard deviation

Population	Nei's gene diversity (h ± SD)	Shannon's index (I ± SD)	Number of polymorphic loci	Percentage of polymorphic loci
M. sibiricum, Švento lake	0.1506±0.2086	0.2201±0.2949	35	40.23%
M. alterniflorum, Švento lake	0.1591±0.2131	0.2305±0.3022	35	40.23%
Myriophyllum sp., Kuršėnų pond	0.1975±0.2312	0.2801±0.3256	38	43.68%
Myriophyllum sp., Širvintos river	0.0926±0.1753	0.1366±0.2516	22	25.29%
M. sibiricum, Balžio lake	0.2333±0.2333	0.3319±0.3272	46	52.87%

M. sibiricum from Balžio lake has the highest genetic diversity among the studied populations. This suggests a high level of genetic variation within this population, which could be beneficial for its adaptability and resilience. *Myriophyllum* sp. from Širvintos river has the lowest genetic diversity, indicating a more genetically homogeneous population. This could imply a higher susceptibility to environmental changes and potential inbreeding. The genetic diversity metrics for *M. sibiricum* and *M. alterniflorum* from Švento lake are quite similar.

Populations with higher genetic diversity, such as *M. sibiricum* from Balžio lake, should be prioritized for conservation efforts to maintain their genetic variability. While populations with lower genetic diversity, like from Širvintos river, may require genetic monitoring and management to prevent further genetic erosion and to enhance their resilience to environmental changes.

3.4. ITS region analysis

For ITS region analysis, DNA from 14 *Myriophyllum* sp. individuals was selected. Their sequences were prepared as described in Sections 2.2.7 to 2.2.10.

After Sanger sequencing and performing multiple sequence alignment (MSA) using the MUSCLE algorithm, it was revealed that the ITS regions ranged in length from 422 to 659 nucleotides (nt). The aligned sequence length was 665 nt, with 256 conserved sites (38.5%) and 405 variable sites (60.9%).

When comparing the ITS region sequences of the studied *Myriophyllum* sp. individuals with those in the NCBI GenBank® database, 12 sequences exhibited similarities to the ITS regions of *Myriophyllum sibiricum*, while one individual (*Myriophyllum* 17 or MY17) matched *Myriophyllum spicatum,* highlighting its genetic distinctiveness within the analyzed samples. Notably, one individual (MY32) had an ITS region similar to *Cirsium occidentale* (accession number: AF443702.1). Additionally, three individuals (MY19, MY20, and MY22), which were excluded from the final analysis, did not exhibit significant similarities to any known sequences.

Using the Maximum Likelihood (ML) method, a phylogenetic tree was constructed from the sequenced data, distinguishing two clear clades (outlined in red and blue). One major cluster included individuals MY01, MY15, MY13, MY26, MY28, and MY14 (outlined in red). This cluster suggests a close genetic relationship among these samples. Another significant cluster comprised MY06, MY35, MY03, MY16, and MY32 (outlined in blue). This cluster indicates another group of closely related individuals. MY02 formed a separate branch, indicating divergence from other groups. MY36 and MY17 also formed distinct branches (Figure 3.6.).

Individual MY32, identified as having an ITS region similar to *Cirsium occidentale*, appeared in a separate sub-cluster within the major group containing MY16, suggesting a unique evolutionary pathway. This also indicates some genetic similarity between MY32 and MY16 despite the very unique ITS region of MY32. (Figure 3.6.).

The bootstrap values on the branches (e.g., 63, 60, 45) indicate the reliability of the branching patterns, with higher values suggesting greater confidence in the depicted phylogenetic relationships. Typically, bootstrap values above 70% are considered to indicate moderate to strong support, while values below 50% suggest weak support. All the branches in the ML phylogenetic tree had a bootstrap value lower than 70%. The highest bootstrap value was 63% and the lowest – 14% (Figure 3.6.).

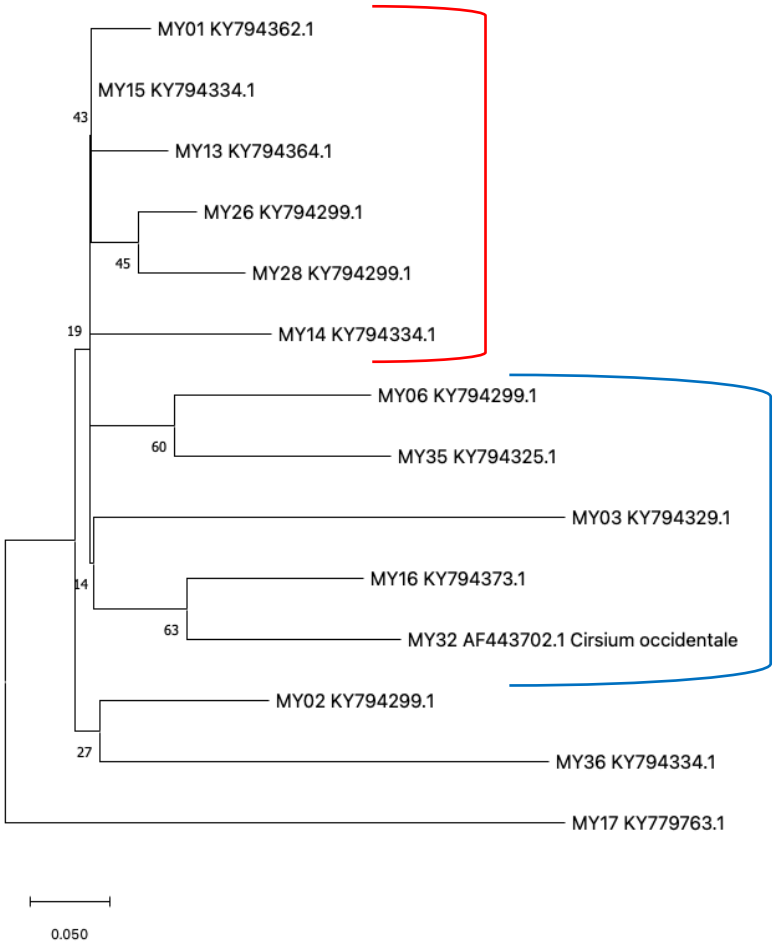

Figure 3.6. The phylogenetic tree was constructed using the Maximum Likelihood (ML) method from the ITS region sequences of the studied *Myriophyllum* individuals. The samples are labeled MY01 to MY36. The accession numbers indicate the sequences that showed the most similarities with the studied *Myriophyllum* samples.

When interpreting a phylogenetic tree, nodes with low bootstrap values should be viewed with caution. Low bootstrap values in a ML phylogenetic tree indicate lower confidence in the robustness and reliability of the branching patterns for specific nodes or branches. These values suggest that the relationships depicted by these branches are not consistently supported by the data, leading to uncertainty about the exact evolutionary relationships among the taxa connected by these branches. This lack of strong support may result from insufficient data, high levels of noise, or conflicting signals within the dataset. In the context of evolutionary processes, low bootstrap values may reflect complex scenarios such as rapid radiations, hybridization, or horizontal gene transfer, which create patterns that are difficult to resolve with standard phylogenetic methods.

Both the ISSR PCoA and ITS analyses identified two major clusters among the *Myriophyllum* sp. individuals, indicating consistent patterns of genetic differentiation. Both analyses also identified certain individuals as outliers. MY32 was identified as having a unique ITS region similar to *Cirsium occidentale*, while MY02, MY36, and MY17 formed separate branches in the ML phylogenetic tree, indicating divergence, and 4 outliers were also noted in the PCoA results. The

high genetic diversity observed in the ITS analysis (60.9% variable sites), aligns with the high molecular variance between populations (63%) found in the AMOVA results.

3.5. Discussion of results

The genetic diversity between the studied *Myriophyllum* sp. populations was higher than within the populations. Similar results were also achieved by Ryan A. Thum and colleagues, where they analyzed the genetic diversity and differentiation of *Myriophyllum spicatum, Myriophyllum sibiricum* and their hybrids in different lake populations in Minnesota and Michigan (Thum et al., 2020). They found that within-lake (within population) genetic diversity was low, and among-lake (among population) genetic diversity was high (Thum et al., 2020). When genetic diversity between *Myriophyllum* sp. populations is higher than within populations, it indicates that the populations are genetically distinct and have limited gene flow between them.

Both the ISSR PCoA and ITS analyses identified two major clusters among the *Myriophyllum* sp. individuals, indicating consistent patterns of genetic differentiation. This is consistent with findings by Tóth and colleagues, where RAPD marker analysis of *Myriophyllum spicatum* in different shallow water bodies of Hungary also revealed two distinct clusters (Tóth et al., 2017). The genetic analysis also demonstrated large differences between the populations and significant similarities within them, which also indicates limited gene flow between populations (Tóth et al., 2017).

Our study did not definitively identify hybrids. This is in contrast with findings from studies in North America where hybridization between *Myriophyllum spicatum* and *Myriophyllum sibiricum* was prevalent, as observed by Wu, who highlighted extensive hybridization facilitated by overlapping climatic niches of the two species (Wu et al., 2015). Hybridization between *Myriophyllum spicatum* and *Myriophyllum sibiricum* in Eurasia was also described by Volkova (Volkova et al., 2024).

CONCLUSIONS

1. All 10 tested ISSR primers showed polymorphisms, making them suitable for *Myriophyllum* sp. population and hybridization studies.
2. The genetic differentiation among the *Myriophyllum* sp. populations was high, demonstrated by the significant molecular variance observed between populations (63%). *Myriophyllum sibiricum* individuals from Balžio Lake exhibited the highest genetic diversity, whereas *Myriophyllum* sp. individuals from the Širvintos River showed the lowest genetic diversity.
3. Based on ITS region sequences, 12 out of the 14 *Myriophyllum* sp. individuals were most similar to *Myriophyllum sibiricum*, 1 was similar to *Myriophyllum spicatum*. The sequenced ITS regions, ranging in length from 422 to 659 nucleotides, exhibited a high percentage of variable sites (60.9%), indicating substantial genetic diversity among the 14 *Myriophyllum* individuals.

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