

## Molecular identification of sex in dioecious moss *Nyholmiella obtusifolia* (Orthotrichaceae) on the basis of ISSR markers

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**Abstract:** As far as an unisexuality in the animal kingdom is typical, in higher plants there most species are bisexual. Whereas in haploid bryophyte it can be noticed that dioecy in this group prevails. In the dioecious bryophyte a huge problem makes diagnostics of gender. Most dioecious mosses do not exhibit any discernible sexual dimorphism. The aim of present research was a finding genetic differences between two gender in the dioecious moss *Nyholmiella obtusifolia* with use of ISSR markers. This study was conducted on 16 individuals: 6 females (without sporophytes) and 10 randomly selected (unknown gender), what was dedicated by a very small number of sex-expressing plant material (rare sporophytes). Out of 12 ISSR primes using to this research, only 4 of them gave clear band patterns. There were: IS807, IS825, IS835 and IS840. The following results are based only on the amplification with ISSR 807 primer. It revealed the products of amplification, which seem to be typical for males and females, which sizes were approximately 600 bp for the former and 500 bp for the latter. This study without doubt is the first trial of finding of genetic differences between females and males in dioecious moss *Nyholmiella obtusifolia*. This is the first step in development of sex-specific molecular marker for *Nyholmiella obtusifolia*. It is insomuch important, because it would be just the second moss, for which a molecular sex marker would be devised.

**Key words:** *Nyholmiella obtusifolia*, dioecious moss, DNA markers, sexual dimorphism, sterile

### Introduction

As far as an unisexuality in the animal kingdom is typical, in plants there most species are bisexual. Only about 6% of Angiospermae has got separated gender (Renner and Ricklefs 1995). Whereas in haploid bryophyte it can be noticed that dioecy in this group prevails. About 57% of moss species and 68% of liverworts are dioecious (Shaw and Goffinet 2000). An occurrence of gender became a starting point to the research on a sex ratio in natural populations and also induced to search for causes of such a gender distribution. A sex ratio in populations where a sexual dimorphism can be noticeable is quite easy to determine. A diagnostics of sex at an nearly stage of growth, when there are no flowers, can be some problem. However, a sexual dimorphism in higher plants is generally quite well discernible.

A completely different situation is in the dioecious bryophyte, where a huge problem makes here a diagnostics of gender. A sexual dimorphism is not in mosses well visible. Only a few species have got smaller males in comparison with females. This property is met in the following families: Dicranaceae, Leucobryaceae, Brachythecaceae and Orthotrichaceae (Shaw and Goffinet 2000). It sometimes happens that the differences between sizes of gametophyte are subtle (Shaw and Gaughan 1993), for instance in *Orthotrichum lyellii* (Vitt 1968) and then it is very hard to identify a sex in such a population.

Another helpful parameter in diagnostics of moss gender seems to be a size of spores. In literature there are some mentions that many dioecious mosses phenotypically express sex in the form of anisospory. This relationship is especially noticeable in family *Orthotrichaceae*. In this group, anisospory appears to be correlated with a reduction in the Y chromosome and also with reduction in size of the male gametophores. It is supposed that the smaller spores (microspores) are produced by males, and the bigger ones (macrospores) – by females. However, not always dioecy of bryophyte is dictated by a production of anisospores. For example, the dioecious *Eurhynchium pulchellum* produces only homospores (Vitt 1968).

A good and probably the best determinant among the morphological markers, because giving a hundred per cent certainty of correct identification of gender, could be a presence of the sex organs: male- antheridia and female- archegonia. However, this index not always can be used to a sex identification, because not all of the individuals form them. It sometimes happens that the dioecious species of mosses reproduce vegetatively with the use of gemmae and it is unnecessary for them a production of the sex organs. These individuals are called sterile. Anderson i Lemmon (1974) showed that it is possible that just only 10% of individuals in the same population produces the sex organs. Mishler i Oliver (1991) suggest that the females show more often a sex expression than the males. So all in all, determination of sex in the populations of mosses with the use of classical methods based on the observation can be impossible or a miscalculation. It is connected with the occurrence of steriles.

The sex identification with cytological level, which consists in a searching the morphologically differential sex chromosomes in the whole of chromosomal set, can also not be reliable. So far an existence of the heteromorphic sex chromosomes has only been reported among the liverworts: *Sphaerocarpos donnellii* (Allen 1917), *Sphaerocarpos texanus* (Korpelainen 2008), *Marchantia polymorpha* (Bischler 1986) and in the mosses, genus *Macromitrium* (Ernst-Schwarzenbach 1944; Ramsay 1966). Therefore it appears that an effective method of sex identification in bryophyte has to be investigated on the genetic plane. The aim of present research was a finding genetic differences between two gender in the dioecious moss *Nyholmiella obtusifolia* with use of ISSR markers.

## Material and Methods

The research were carry out on dioecious moss *Nyholmiella obtusifolia*. This is the most often occurred species in the family *Orthotrichaceae*, which has belonged to the genus *Orthotrichum*, subgenus *Orthophyllum* for long time. However, molecular studies showed evidently that two species: *Orthotrichum obtusifolium* and *Orthotrichum gymnostomum* are clearly distinct from other species of the genus *Orthotrichum* (Goffinet et al. 2004, Sawicki et al. 2009). These phylogenetic analysis clearly supported previous concepts (Hagen 1908) postulating that species of the subgenus *Orthophyllum* should be recognized as the separate genus *Nyholmiella* (Sawicki et al. 2010). The current name of genus *Nyholmiella* was proposed by Holmen and Warncke (Damsholt et al. 1969) and includes two species: *Nyholmiella gymnostoma* and *Nyholmiella obtusifolia*.

*Nyholmiella obtusifolia* and *Nyholmiella gymnostoma* may be distinguished from each other on the basis of the peristome, which is double in the former and lacking in the latter. Gametophytes of the two species are easily separated by leaf margins and cell papillosity: *Nyholmiella gymnostoma* has inrolled margins and two papillae per leaf cell surface while *Nyholmiella obtusifolia* has erect margins and unipapillose cell surfaces (Sawicki, unpublished).

*Nyholmiella obtusifolia* is widespread in the European continent (north to Svalbard), Greenland, the Caucasus, the Himalayas, Turkey, central and eastern Asia and North America. Populations of *Nyholmiella obtusifolia* occur in large number on the urbanized regions as well as in the natural forest ecosystems. This species is found in Poland very often, but in the herbarium collections there are only steriles. A population included in this research came from the part of Olsztyn city named Kortowo. A specificity of this population is an appearance of sporophytes (Sawicki, Plášek & Szczecińska 2011), what was very important and useful in this study.

Total genomic DNA was extracted from fresh material representing 16 individuals: 6 females (without sporophytes) and 10 randomly selected (unknown gender). In order to avoid DNA contamination by fungi and other microorganisms, only fresh green parts of a gametophyte were taken, rinsed in distilled water and drained on an absorbent paper. DNA was isolated with ZR Plant/Seed DNA Kit <sup>TM</sup> (ZymoResearch) according to the manufacturer's instruction. Extracted DNA samples were stored at -20 °C.

In order to investigate genetic differences between females and males ISSR (Inter Simple Sequence Repeat) markers were used. The markers of this class show polymorphism of DNA fragments located between microsatellitary regions (Ziętkiewicz et al. 1994). The target sequences of ISSR markers does not require prior identification, which makes such markers suitable for species where species-specific primers amplifying microsatellite loci (Simple Sequence Repeat, SSR) have not been developed yet (Buczowska et al. 2010). The ISSR primers used were the following: 807, 810, 813, 816, 819, 822, 825, 831, 834, 835, 840, 846 (see Table 1).

The PCR reactions for ISSR markers were conducted in 20 µl of reaction mixture containing 0.5µl of the genomic DNA solution (40ng), 1µM primer, 1µl of BSA solution (0.25 mg/ml), 0.3µl Ultrapure dNTPs Set (EURx), 2µl 10 x Pol Buffer B (EURx) and 1U YellowTaq polymerase (EURx).

PCR-ISSR reactions were performed as follows: (1) initial denaturation- 4 min. at 94 °C, (2) denaturation- 45 s. at 94 °C, (3) annealing- 50 s. at 52 °C, (4) elongation- 1 min. 30 s. at 72 °C, (5) final elongation- 7 min. at 72 °C. Stages 2-4 were repeated 40 times. The products of the ISSR- PCR reactions were separated on a 2% agarosegel, followed by DNA staining with ethidium bromide. After rinsing in deionized water, the agarose gel was analysed in transilluminator under UV light and photographed.

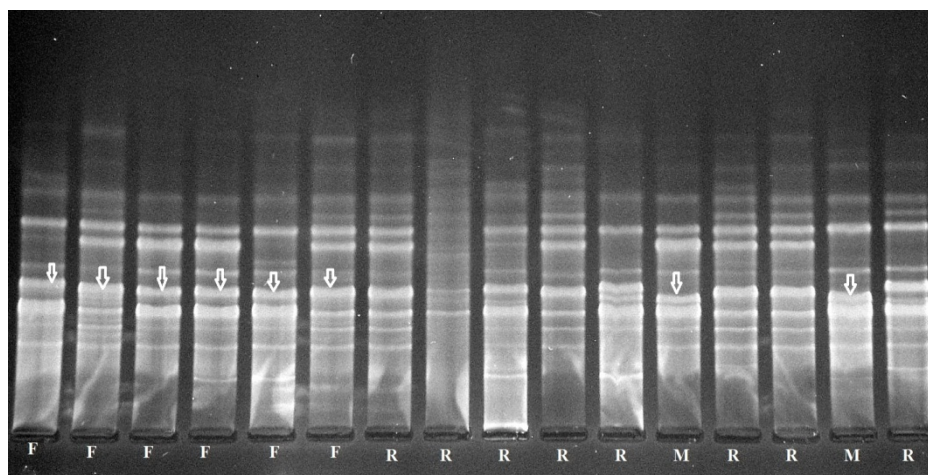
**Table 1:** Sequences of ISSR primers used in studies on *N. obtusifolia* with number of amplified loci.

Nr.	primer	sequence (5'- 3')	bp	number of ampl. loci
1	IS807	AGAGAGAGAGAGAGAGT	17	19
2	IS810	GAGAGAGAGAGAGAGAT	17	smear
3	IS813	CTCTCTCTCTCTCTTT	17	smear
4	IS816	CACACACACACACAT	17	smear
5	IS819	GTGTGTGTGTGTGTGTA	17	smear
6	IS822	TCTCTCTCTCTCTCTCA	17	smear
7	IS825	ACACACACACACACT	17	15
8	IS831	ACCACCACCACCACCACC	18	smear
9	IS834	ATGATGATGATGATGATG	18	smear
10	IS835	AGAGAGAGAGAGAGAGYC	18	12
11	IS840	ACTTCCCCACAGGTAAACACA	21	25
12	IS846	GGGTGGGGTGGGGTG	15	smear

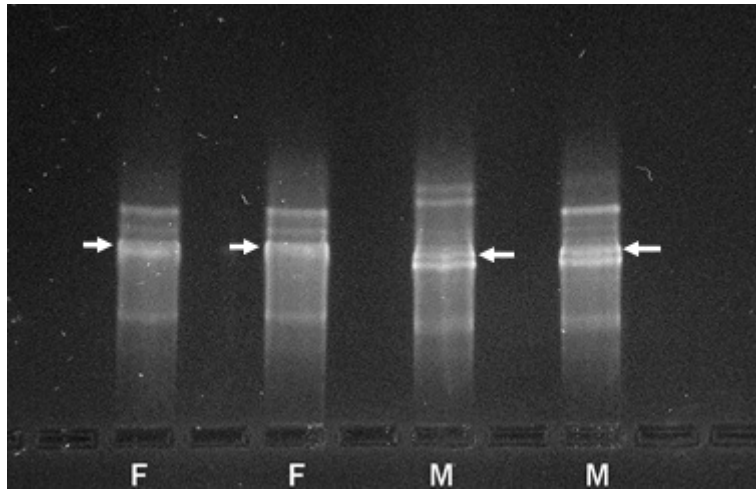
## Results

From among 12 ISSR primers tested in this research only 4 of them gave clear band patterns, without smears. There were the following primers: IS807, IS825, IS835 and IS840 (tab.1.). These successful primers allowed for amplification of 71 loci (17.75 loci per one primer on average). It was decided that PCR reactions with these primers would be repeated and the products of these amplifications would be electrophoretically separated for longer time and on the longer gel. In this way more clearly band patterns could be obtained. The present results are based only on the amplification with ISSR 807 primer. It is suggested to repeat PCR reactions with the rest of three ISSR primers and observe the received band patterns in search of genetic variability between females and males.

ISSR 807 primer revealed the products of amplification, which seem to be typical for males and females (Fig. 1 and Fig. 2), which sizes were approximately 600 bp for the former and 500 bp for the latter.



**Fig 1:** Amplification patterns of 6 females (F), presumably 2 males (M) and others randomly selected individuals (R) with primer ISSR 807.



**Fig 2:** Fragment of amplification patterns with ISSR 807 primer showing significant differences between females and presumably males.

## Discussion

This study without doubt is the first trial of finding of genetic differences between females and males in dioecious moss *Nyholmiella obtusifolia*. In this research, completely just like in Korpeleinen's studies (2008), only a few individuals were used due to a very small amount of sex-expressing plant material (rare sporophytes). The identification a genetic difference between two gender in this species may be called a big achievement. It results from poor, discernible sexual dimorphism. Vitt (1968) suggests that there is not any morphological dimorphism in this species and in addition to this spores of *Nyholmiella obtusifolia* are homomorphic. Farther a finding of archegonia and antheridia in this species is a rarity, because a vegetative reproduction is here common and an occurrence of sporophytes is an infrequent phenomenon (Sawicki, unpublished).

The obtained data are very optimistic, because they point DNA specific sequences both for females and males. The first genetic method for determination of sex in a moss (*Pseudocalliergon trifarium*) was developed by Korpelainen et al. (2008), but there only one sex-specific DNA marker (one sex-specific band) for females was found, whereas in males there was not any typical PCR- product identified. It is very disadvantageous, because a potential lack of amplification may not only follow an absence of complementary to primer sequence, but also degraded DNA or an existence of PCR- inhibitors. It is worth to notice that the same primer ISSR 807 revealed differences between females and males in *Pseudocalliergon trifarium* (Korpelainen et al. 2008) as well as in *Nyholmiella obtusifolia*, but the primers: IS810, IS816, IS819, IS822 and IS834 were failed in both cases. Korpelainen (2008) did not test the primers IS835 and IS840, whereas in this research screening with these primers is recommended in the further studies as well as primer IS825, which in research of Korpelainen was unsuccessful.

The present studies on identification of sex in mosses are just the second ones where ISSR-PCR analysis was applied. Previously this method was only used in *Pseudocalliergon trifarium* (Korpelainen et al. 2008). In the liverworts: *Marchantia polymorpha* (Fujisawa et al. 2001) and *Sphaerocarpos texanus* (McLetchie and Collins 2001) others methods were applied, namely Representational Difference Analysis (RDA) and Random Amplification of Polymorphic DNA (RAPD), respectively. The RAPD method, developed in 1990 (Williams et al.), was a technique the most often used to identify sex in various dioecious taxa (Ainsworth 2000). From 1994 (Ziętkiewicz et al.) more and more frequently ISSR method is applied. The ISSR technique is similar to RAPD, except that ISSR primers consist of di- or trinucleotide simple sequence repeat with a 5' or 3' anchoring sequence of 1-3 nucleotides.

Compared with RAPD primers, the ISSR primer sequence is usually larger, allowing for a higher primer annealing temperature, which results in greater band reproducibility than RAPD markers (Culley and Wolfe 2000). In our work ISSR- PCR was shown to be effective for searching differences between two gender in *Nyholmiella obtusifolia* despite of low number of used primers. Testing of 12 ISSR primers was enough to find different band patterns. For comparison to detect genetic differences between females and males in *Sphaerocarpos texanus* with RAPD primers it was carried out PCR reactions with 238 different decamer primers (McLetchie and Collins 2001). ISSR technique is also useful in population genetic research, where level of genetic variation is low (Ziętkiewicz et al. 1994). It takes place in analysed population of *Nyholmiella obtusifolia* because of its preference of vegetative reproduction to sexual reproduction.

The presented studies are the first step in development of sex-specific molecular marker for *Nyholmiella obtusifolia*. It is insomuch important, because it would be just the second moss, for which a molecular sex marker would be devised. The sex-specific genetic markers are very desirable and useful tools. They allow not only to identify a plant gender at any stage in the life cycle, but also enable to conduct the research on sex ratio, which among the mosses are poor.

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