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The First Sex-Specific Molecular Marker Discovered in the Moss *Pseudocalliergon trifarium*

Helena Korpelainen, Irene Bisang, Lars Hedenäs, and Johanna Kolehmainen

From the Department of Applied Biology, University of Helsinki, PO Box 27, FI-00014 Helsinki, Finland (Korpelainen and Kolehmainen); and the Swedish Museum of Natural History, Department of Cryptogamic Botany, Box 50007, SE-104 05 Stockholm, Sweden (Bisang and Hedenäs).

Address correspondence to H. Korpelainen at the address above, or e-mail: helena.korpelainen@helsinki.fi.

Abstract

Most dioecious plants do not exhibit discernible sexual dimorphism before sexual maturity. Therefore, it is impossible to address any sex-related questions during the prereproductive phase unless a genetic sex marker is available for gender determination. The aim of the present study was to develop a genetic sex marker for the moss *Pseudocalliergon trifarium* to allow gender and sex ratio determination at any stage in the life cycle. A high proportion of *P. trifarium* populations do not express sex. The screening of genomic DNA with inter simple sequence repeat (ISSR) primers was used to discover sex-specific polymerase chain reaction (PCR) amplification products. A presumably female-specific band was found, excised from the gel, cloned, and sequenced. A sequence-walking method was used to characterize the same region in males. A primer pair was designed to allow the amplification of a 159-bp portion of the female-specific DNA region. All tested material, up to 16-year-old herbarium specimens, provided unambiguous amplification products. This study successfully provides, for the first time in a moss, a sex-specific DNA marker. It allows reliable determination of gender and sex ratios. The short length of the amplification product is an advantage as satisfactory PCR products are more likely when the targeted sequence is short. The amount of variation in the DNA region shared by both sexes was relatively high. If the male sequence can be better characterized, the sex-specific regions could possibly be used to evaluate sex-specific phylogeographic patterns.

The genetic basis of sex determination in dioecious plants appears diverse (for "dioicy" vs. "dioecy," see e.g., Wyatt 1985), including heteromorphic sex chromosomes and also one or several autosomal nuclear sex-determining loci, possibly influenced by cytoplasmic genes and environmental cues (Korpelainen 1998; Ainsworth 2000). Previously, sex markers have been primarily developed for plant species that have been shown or proposed to possess sex chromosomes. They included sex-linked randomly amplified polymorphic DNA (RAPD)-, amplified fragment length polymorphism (AFLP)-, and RAPD-derived SCAR markers and simple sequence repeats in *Actinidia chinensis* (Harvey et al. 1997), *Asparagus officinalis* (Jiang and Sink 1997), *Cannabis sativa* (e.g., Sakamoto et al. 1995; Törjék et al. 2002; Rode et al. 2005), *Carica papaya* (Urasaki et al. 2002), *Dioscorea tokoro* (Terauchi and Kahl 1999), *Ginkgo biloba* (Jiang et al. 2003), *Hippophae rhamnoides* (Persson and Nybom 1998), *Humulus lupulus* (Polley et al. 1997), and *Pistacia vera* (Hormaza et al. 1994; Yakubov et al. 2005). Also, sex chromosome–specific DNA sequences have been characterized further and used as sex markers in *Rumex acetosa* (Shibata et al. 1999; Korpelainen 2002), *Rumex nivalis* (Stehlik and Blattner 2004), and *Silene latifolia* (e.g., Mulcahy et al. 1992; Lyons et al. 1995; Zhang et al. 1998). In bryophytes, characterized by a haploid-dominant life cycle, sex-specific markers have so far been discovered in the liverworts *Marchantia polymorpha* (Okada et al. 2000, 2001; Yamato et al. 2007) and *Sphaerocarpos texanus* (McLetchie and Collins 2001), both of which possess heteromorphic sex chromosomes. As to our knowledge, mosses have not been studied with respect to molecular sexual markers to date. However, Newton (1971) used cytological evidence for gender identification in *Plagiomnium undulatum*.

In general, sex markers have been developed only rarely for plants with presumably autosomal sex determination, including *Atriplex garrettii* (Ruas et al. 1998), *Salix viminalis* (Alström-Rapaport et al. 1998; Gunter et al. 2003), and *Uapaca kirkiana* (Mwase et al. 2007). It is clearly more likely to find sex-linked markers in cases where a sex chromosome system operates. Also, most of the known sex-linked markers are male associated, which reflects males being the heterogametic sex (Ainsworth 2000). Mainly, the genetic

methods to identify gender have been developed for the purpose of genetic mapping or breeding work (Ainsworth 2000), and only rarely have such methods been applied to investigations on the sex ratios of natural populations (Lyons et al. 1995; Korpelainen 2002).

Most dioecious plants are not known to be sexually dimorphic before sexual maturity. Therefore, it is impossible to address any sex-related questions during the prereproductive phase unless a genetic sex marker is available for gender determination. Examples of investigations based on the utilization of genetic sex markers include a study by Korpelainen (2002), which showed that the sex ratios of *R. acetosa* populations are about 1:1 in the whole seed pool but that a significant female bias develops by the time of flowering. Comparably, Mulcahy et al. (1992) have examined sex-specific germination patterns in *S. latifolia*. Dioecious species with infrequent sex expression are even more challenging: The gender of most individuals cannot be determined at any stage in the life cycle, and sex-related issues are not possible to investigate in-depth in species with rare sexuality without a genetic sex marker.

A dioecious breeding system is widespread among bryophytes: More than half of all moss taxa and roughly two-thirds of the liverworts worldwide are unisexual (Wyatt 1982) Among these, there is a considerable number of species and many populations which consist of nonexpressing gametophytes only, bearing neither male nor female inflorescences (Longton and Schuster 1983; Bisang and Hedenäs 2005 and references therein). Biased expressed sex ratios are common, and females outnumber males in most species studied so far (reviewed by Bisang and Hedenäs 2005). The underlying causes for this, however, are poorly understood (Bisang et al. 2006).

The pleurocarpous *P. trifarium* (Web. & Mohr) Loeske is a dioecious moss of the order Hypnales. It is relatively common in the northern temperate zone and occurs also in the mountains of South America. The species mainly grows in deep fens or in sloping fens with slowly moving mineral-rich water (Hedenäs 1992; Hedenäs et al. 2003). A high proportion of *P. trifarium* populations are nonexpressing (67%), sporophyte production is rare (4.2%), and the expressed sex ratio is skewed toward the female gender (F:M = 2:7; $N = 215$; Bisang and Hedenäs, 2005; Bisang I, Hedenäs L, unpublished data). It is therefore a well-suited target species for the development of a molecular method to determine sex in nonexpressing moss individuals. Although detailed mechanisms for sex determination are not fully understood in many bryophyte species, there is evidence of sex chromosomes in a number of taxa (e.g., Ramsay and Berry 1982; McDaniel et al. 2007). *Pseudocalliergon trifarium*, however, has not been studied in this respect, and even its chromosome number is unknown (Fritsch 1991).

The aim of the present study is to develop a genetic sex marker for *P. trifarium* to allow gender and sex ratio determination at any stage in the life cycle and, eventually, the understanding of sex ratio dynamics in natural moss populations. In addition, we attempt to provide insights into the genetic basis of sex determination in *P. trifarium*.

Materials and Methods

Marker Development

The process for developing a sex-specific molecular marker was initiated with the screening of genomic DNA, extracted from dry material representing 6 female and 3 male samples of *P. trifarium* originating from Sweden (PT1–PT9; Table 1), with a set of ISSR primers (tandem arrays of simple nucleotide motifs interrupted in one end by out-of-phase bases). Due to a very small amount of sex-expressing plant material and no possibility to obtain sex-expressing progeny, there was no possibility to conduct a bulk or bulk segregant screening. The used screening method involved amplification by polymerase chain reaction (PCR), in separate reactions (no pooling of DNA or primers) with arbitrary ISSR primers, which had been assembled by the Nucleic Acid–Protein Service Unit, University of British Columbia, Canada. The primers used were the following: 807–812, 814–817, 819–826, 828, 834, 836, 843, 848–853, 858–862, 866, 881, 884, 887–889, and 891–892. The reaction mixture contained about 10–20 ng of genomic DNA, 1.2 units of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland), 1× PCR buffer, 0.4 μl of 10 mM dNTP mix, and 1 μl of a single 5 μM primer. The thermocyler (MJ Research, Inc., model PTC-200) was programed for 4-min denaturation at 94 °C, followed by 45 cycles of denaturation at 94 °C for 45 s, annealing at 50–60 °C for 45 s, and elongation at 72 °C for 90 s. An additional 8-min elongation followed the last cycle. All amplification products were electrophoretically separated on 1.4% agarose gels. The only presumably sex-specific band resulting from the amplification with primer 807 was excised from the gel. The band was present in all females but in none of the males. The excised fragment representing sample PT1 was purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-tek Inc., Norcross, GA). Then, the purified DNA was concentrated to 3–5 μl and cloned using the TOPO TA Cloning kit by Invitrogen (Invitrogen, Carlsbad, CA). Positive clones were selected from the plates and transferred into 50 μl of ddH_2O. DNA amplification was conducted in 20-μl reaction volumes containing 4 μl clone solution, 0.5 units of Phusion High-Fidelity DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 μl of 10 mM dNTP mix, and 1 μl each of primers M13-f and M13-r (at the concentration of 5 μM) included in the cloning kit. The thermocyler was programed for 30 s denaturation at 98 °C, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 20 s, and elongation at 72 °C for 50 s. An additional 8-min elongation followed the last cycle. The amplification product was separated electrophoretically, excised from the gel and purified. The following sequencing was conducted using the M13-f primer. The size of the sequenced female product was 670 bp (GenBank accession number EU368956; Figure 1). Specific primers were developed based on the cloned female sequence. Three primer pairs were tested, PT-1f (5′-TTC CTA GTG GGG AAC AGA AAA A-3′) and PT-1r (5′-GAA TGA TGT TCT TGC ACG TCT C-3′), PT-1f

Table 1. Voucher information for the 33 sex-expressing collections of *Pseudocalliergon trifarium* included in the development and testing of the sex-specific marker

Sample	Sex	Herb.; herb./collection number	Country, province, parish/commune	Year of collection
PT1	Female	S; B104736	Sweden, Jämtland, Åre	2005
PT2	Male	S; B104736	Sweden, Jämtland, Åre	2005
PT3	Female	S; B104736	Sweden, Jämtland, Åre	2005
PT4	Female	S; B104752	Sweden, Jämtland, Kall	2005
PT5	Male	S; B104751	Sweden, Jämtland, Kall	2005
PT6	Female	S; B104188	Sweden, Härjedalen, Tännäs	2005
PT7	Male	S; B93270	Sweden, Jämtland, Frostviken	1988
PT8	Female	S; B104843	Sweden, Jämtland, Åre	1989
PT9	Female	S; B104844	Sweden, Närke, Kil	2005
PT10	Female	Z	Switzerland, Graubünden, Samnaun	2000
PT11	Female	Z	Switzerland, Graubünden, Trun	2001
PT12	Male	Z	Switzerland, Graubünden, Trun	1988
PT13	Female	Herb. Schnyder, 2005194	Switzerland, St Gallen, Lochwis	2005
PT14	Female	Herb. Schnyder, HM87:7	Switzerland, Valais, Orsières	1992
PT15	Female	S; B16963	United States, Minnesota, Becker Co.	1999
PT16	Female	S; B110749	Sweden, Pite Lappmark, Arvidjaur	2000
PT17	Male	S; B112892	Sweden, Jämtland, Åre	2006
PT18	Male	S; B112893	Sweden, Jämtland, Åre	2006
PT19	Male	S; B112894	Sweden, Jämtland, Åre	2006
PT20	Female	S; B112895	Sweden, Jämtland, Åre	2006
PT21	Female	S; B112897	Sweden, Jämtland, Åre	2006
PT22	Male	S; B112899	Sweden, Jämtland, Åre	2006
PT23	Female	S; B121274	Sweden, Härjedalen, Linsell	2007
PT24	Male	S; B121967	Sweden, Jämtland, Åre	2007
PT25	Male	S; B121968	Sweden, Jämtland, Åre	2007
PT26	Male	S; B121969	Sweden, Jämtland, Åre	2007
PT27	Male	S; B121970	Sweden, Jämtland, Åre	2007
PT28	Female	S; B121910	Sweden, Jämtland, Åre	2007
PT29	Female	S; B121911	Sweden, Jämtland, Åre	2007
PT30	Female	S; B121912	Sweden, Jämtland, Åre	2007
PT31	Female	S; B121913	Sweden, Jämtland, Åre	2007
PT32	Female	S; B121971	Norway, Nord-Trøndelag, Meråker	2007
PT33	Female	S; B121917	Sweden, Jämtland, Åre	2007

All samples, except PT1 and PT2, originate from different populations. Further locality details available from the authors. Herb., herbarium.

and PT-2r (5′-TAA GCG GAA AAA TGG GAT TAG A-3′), and PT-3f (5′-GGA TTG ATA TTG GCA TTG AGT T-3′) and PT-3r (5′-TGG AAT GTC ACA TTG TTT AGG A-3′) with expected product sizes of 468 bp, 182 bp and 159 bp, respectively.

Marker Testing

The plant material used for testing the functioning of the sex marker included all available samples of sex-expressing *P. trifarium* specimen: 9 Swedish samples (6 females, 3 males) used in the screening for a sex-specific marker and 24 additional samples (PT10–PT33; 15 females, 9 males) originating from Sweden, Norway, Switzerland, and the United States (Table 1). When testing the functioning of the primer pairs, the following PCR conditions were used: The 20-μl reaction volumes contained about 10–20 ng of genomic DNA, 1.2 units of DyNAzyme II DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 μl of 10 mM dNTP mix, and 1 μl of both 5 μM primers. The thermocyler was programed for denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 53–55 °C 45 s, and elongation at 72 °C for 60 s. An additional 8-min elongation followed the last cycle. All amplification products were electrophoretically separated on 1.4% agarose gels.

Characterization of Marker Region

To further characterize the sex-specific DNA region, genomic DNA of a female (sample PT23) and a male (sample PT24) were subjected to sequence walking using a method modified from Korpelainen et al. (2007) (Figure 2). Concentrated genomic DNA (about 15–20 ng/μl) was treated with two 4-cutter restriction enzymes leaving blunt ends after the protocol: restriction for 4 h at +37 °C utilizing *Hae*III and *Rsa*I restriction enzymes (both 4-base cutters) in separate reactions each in a volume of 8 μl containing 6 μl of genomic DNA, 4 units of either restriction enzyme, 1× restriction buffer, and 8 μg BSA. Then, a 2-stranded adaptor, composed of a 44-bp sequence (5′-GAA CTA GTC TCG ACT CCA GTC AGA GAT TCC ACC GCC GTG ACC GC-3′) and an 8-bp sequence with 5′-phosphorylation and an amino modifier at the

AGAGAGAGAGAGAGTGATAGTGGTGAAGGGTTTAAGTACCCGACATTCTGCCAATGG
►►►►► PT-1f ►►►►►
TTCCTAGTGGGGAACAGAAAAAGAGCCATTCCTGAAACGGACCCCTAGTTTCCTAGGTC

AAAAGATAGATAGCATAGGCTCTCTCTTTCCGTCCCGATTTCGGATTCTGGATCTGGTT
◄◄◄◄◄ PT-2r ◄◄
GAGATCCATCCTACTGAATTGATTAGTTTCCGAAAAGTTGGG***TCTAATCCCATTTTTCC***
◄◄◄
GCTTAACTGATTCTAGTATTTATTTGACTAACTAAAAATTTGAAAAATTCTACTCTATT
← Males and females | Females →
GTAATACTCATACCTACTAGCTCCGTGCATTCTGAGAATTCAGATTCCGACAGGAACTA

GATCTACTAACTAGACTTGGCCCTCTCCTCTCAAACTTGTATCGAGTTGAATCACAATT
►►►►► PT-3f ►►►►►►
GCGATCTACTA***GGATTGATATTGGCATTGAGTT***GAAGTGCAATCAAGATAAATTAGAGG
◄◄◄◄◄ PT-1r ◄◄◄◄◄
GAGTAGACAAATCATTGCACCTTCGAACAAATA***GAGACGTGCAAGAACATCATTC***AATC
◄◄◄◄◄ PT-3r ◄◄◄◄◄
AAGAAAGGAACTAAAGCCAATTTCTGTGAA***TCCTAAACAATGTGACATTCCA***AGGGAAA

AGCAAGGGATCCTTCATCAATTCATACATTGCATAGTTTGAATTGATAATAGAATTTAT

AAACACTCTCTCTCTCTCT

Figure 1. The sequence of the cloned female-specific marker (sample PT1). The sites of the tested primers, and the site where the sequence shared by both sexes becomes female specific are shown.

3′ end (5′-pGCG GTC AC-NH_2-3′), was ligated to the restricted DNA. The ligation reaction was conducted for 2 h at +22 °C in a volume of 20 μl containing the previous 8-μl mix of restricted DNA, 0.5 μl of double-stranded 50 μM adaptor, 5 units of T4 ligase, 1× ligation buffer, 2 μl of 50%

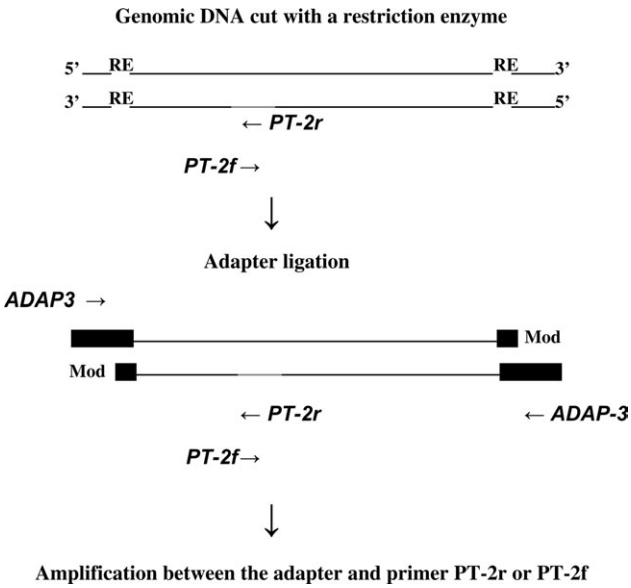

Figure 2. General outline (modified from Figure 2 in Korpelainen et al. 2007) of the sequence-walking technique used to discover the DNA sequence flanking the primer region PT-2r/PT-2f in males and females. The 2-stranded adaptor contains an amino modification at the 3′ end of the short strand to prevent amplification between 2 adaptors. RE, cutting site for a restriction enzyme; ADAP3, adaptor sequence specific primer.

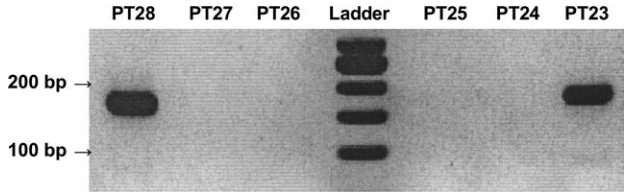

Figure 3. The results of the sex-marker testing in females (PT23 and PT28) and males (PT24–PT27) using the primers PT-3f and PT-3r. Females showed a clear amplification product.

PEG 4000, and followed by inactivation at +65 °C for 15 min. After ligation, PCR amplifications were conducted with the primer PT-2r (found to target both male and female DNA template) and the adaptor primer ADAP3 (5′-TCG ACT CCA GTC AGA GAT TCC AC-3′) and with the primer PT-2f (complementary to PT-2r) and the adaptor primer ADAP3 in 20-μl reaction volumes containing 2 μl restriction-ligation DNA, 0.5 units of DyNAzyme II Hot Start DNA polymerase (Finnzymes), 1× PCR buffer, 0.4 μl of 10 mM dNTP mix, and 1 μl of both 5 μM primers. The thermocyler was programed for 10-min denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 25 s, and elongation at 72 °C for 40 s. An additional 8-min elongation followed the last cycle. The PCRs were followed by electrophoretic separation of the amplification products, fragment excision and purification, and sequencing with the adaptor primer. The resulting male or female sequence was presumed to represent the sequence either upstream or downstream from the primer region PT-2r, depending whether the primer PT-2f or PT-2r was used in combination with the primer ADAP3 (Figures 1 and 2).

Results

Out of more than 100 clear, scorable bands, only one band produced using the ISSR primer 807 showed a consistent sex-specific pattern present in all females but in none of the males. The fragment was cloned and sequenced, and primer pairs were designed. When testing the suitability of the 3 primer combinations to be used to amplify the female-specific marker, the first primer pair (PT-1f and PT-1r) resulted in a 468-bp amplification product only in females, as expected, but the amplification suffered from an uneven quality, varying from strong to weak. Surprisingly, the second primer pair combination (PT-1f and PT-2r) amplified well in both males and females (product size 182 bp). The third combination (PT-3f and PT-3r) amplified very well in females, but did not amplify in males (size 159 bp, Figure 3). Therefore, PT-3f and PT-3r were chosen to be the adequate primers for the sex marker of *P. trifarium*.

The clearly readable portions of the sequence-walked female (sample PT23) and male (sample PT24) sequences equalled 319 bp and 510 bp, respectively (GenBank

accession numbers EU368957 and EU368958). When the female PT23 sequence was aligned with the cloned female PT1 sequence, the differences between the sequences included 17 substitutions (5.3%). On the other hand, when the male sequence PT24 was aligned with the cloned female sequence PT1, the first 290 bp aligned well, including 33 substitutions (11.4%), whereas the rest of the male sequence (220 bp) could not be aligned with the female sequence (Figure 1). When comparing the sequences PT23 and PT24 within the aligned 290-bp sequences, the number of substitutions equalled 16 (5.5%). All 3 sequenced samples represent different populations, with pairwise geographic distances equalling 24 km (PT1–PT24), 135 km (PT1–PT23), and 150 km (PT23–PT24).

A BLAST search (a nucleotide query vs. nucleotide database) of the cloned female-specific sequence and the sequence-walked male sequence against the sequences in GenBank failed to identify any match with the available sequence resources.

Discussion

This study successfully provides, for the first time in a moss, a sex-specific DNA marker. The marker is specific to females. The marker region was partially characterized in males as well, revealing the beginning of the DNA region unique to each sex. In addition, the ISSR primer 807, which resulted in the amplification of 3–5 clear bands, including the female-specific band (670 bp), can be used as a gender determination tool. However, the longer, specific primers PT-3f and PT-3r provide a single, unambiguous, easy to score amplification product. They are therefore the recommended way to conduct genetic gender determination in *P. trifarium*. When applying the sex marker to determine the gender of *P. trifarium* samples, it is important to use another PCR as an internal control for successful PCRs to avoid false negatives.

When the examined DNA is degraded, the short length (159 bp) of the female-specific amplification product is an advantage as obtaining satisfactory PCR products is more likely when the targeted sequence is short. The uneven quality of the larger female-specific product resulting from the amplification with the primers PT-1f and PT-1r may have been due to problems with degraded DNA in some herbarium specimens. In the tested material, amplifications with the primers PT-3f and PT-3r were very good in all samples, ranging from DNA extracted within weeks after field collection of the material to DNA extracted from herbarium specimens up to 16 years old.

Investigations of the sex ratios of plant populations have mostly relied on information obtained at sexual maturity. If uneven sex ratios are observed at maturity, it is of interest to reveal the processes leading to deviations from 1:1, which is the expected primary sex ratio at the end of parental investment (Fisher 1930). Biased expressed adult sex ratios may result from mechanisms acting at different ontogenetic stages, such as differences in life-history traits (e.g., germination rate, mortality, and vegetative vigor), sex-specific adult reproductive costs, or environmental responses (Bisang et al. 2006 and references therein). They may also be due to genetic mechanisms distorting the sex ratios (Taylor 1999). In organisms with infrequent sexuality, as in our study species *P. trifarium*, an observed skewed sex ratio might be the consequence of different degrees of sex expression (i.e., gametangia formation) between genetic sexes. Such species are a major challenge for studies of the sex ratio dynamics as the actual sex ratios cannot be properly revealed at any stage of life unless a molecular, sex-specific marker system is available.

It is not known whether the sequenced male and female regions of *P. trifarium* represent autosomal or partly homologous sex-chromosomal sequences. Because the males and females share a large part of the sequenced 670-bp female-specific product, it is possible that this region does not represent DNA in a heterogametic sex chromosome. However, most other plants, for which sex markers are available, have sex chromosomes, including 2 bryophyte species, *M. polymorpha* (Okada et al. 2000, 2001) and *S. texanus* (McLetchie and Collins 2001). On the other hand, it is conceivable that *P. trifarium* possesses heterogametic sex chromosomes and that the X and Y chromosomes contain partially shared sequences. Yamato et al. (2007) described the gene organization of the Y chromosome in *M. polymorpha* in detail. They identified 64 genes, of which 14 were detected only in the male genome and were expressed in the male reproductive organs suggesting their participation in male reproductive functions. At least 6 of the 40 male genes expressed both in thalli and male sexual organs have divergent X-linked female counterparts that are expressed in the thalli and sexual organs of female plants. These findings suggest that the Y and X chromosomes of *M. polymorpha* share the same ancestral autosome (Yamato et al. 2007).

Because the aligned part of the DNA region sequenced in *P. trifarium* was highly variable, it is likely to represent noncoding DNA which has accumulated mutations in both sexes. Previously, high mutation rates have been detected in Y-chromosomal heterochromatin in the males of *R. acetosa* (Navajas-Pérez et al. 2006; Korpelainen and Kostamo 2007). Evidently, the region sequenced in the moss *P. trifarium*, a part of which is the basis of the female-specific marker, is unlikely to represent a gene region involved in sex determination. Most likely, it is linked to a sex-determining locus, which is either autosomal or represents a partly homologous X- and Y-chromosomal regions. There may be one or, more likely, several loci which are involved in the sex determination of *P. trifarium*.

Future Perspectives

The characterization of a sex marker for *P. trifarium* opens up several fields of potential interest. We can now, for the first time, detect actual sex ratios in this species, from the species or wide geographical levels down to the population level, and compare these with the ratios of expressing plants.

Gametangia formation in *P. trifarium* may not occur in all habitats where one sex grows. The new marker allows the investigation of expression rates in various habitats for each sex separately. Thus, it is possible to study both whether sex-specific habitat requirements occur or whether specific conditions are necessary for sex expression.

Depending on when the sex-specific regions evolved, a smaller or larger monophyletic group, including *P. trifarium*, could be expected to share the same sex marker. This should be tested at first for members of *Pseudocalliergon* and *Drepanocladus* (cf., Vanderpoorten et al. 2002, 2003). The amount of variation among the specimens in the region shared by both sexes was relatively high. If the male sequence can be better characterized, the specific regions could possibly be used to evaluate sex-specific phylogeographic patterns.

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