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Molecular markers based on PCR methods: a guideline for mosses

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Abstract – In recent years, the number of studies concerning population genetics and phylogenetics in mosses using molecular markers has remarkably grown. This review summarizes the main features of the most used molecular techniques based on PCR and their application in bryology, with particular attention to mosses. This work also provides a bibliographic guide to 108 molecular studies focused on mosses to assist in choosing the most suitable markers according to the main aims of a specific research.

Population ecology / phylogeny / phylogeography / molecular markers / taxonomy / mosses

INTRODUCTION

The genome of organisms holds very useful information about taxonomy, phylogenies, biogeography, and population dynamics, with great impact in both the theoretical and practical fields of biology. Based on genetic variation, even between closely related individuals, it is possible to characterize organisms or even varieties within a species. Molecular characterization may provide a reliable and replicable tool, leading to the identification of an organism, like barcoding, and/or to the description of the evolutionary relationships among individuals/taxa. Molecular markers provide access to the enormous amount of information contained in genetic material. DNA molecular markers, or genetic markers, are identifiable DNA sequences found at specific locations of the genome, and transmitted to descendants (Semagn *et al*., 2006). As DNA molecular markers may reveal genetic polymorphism, genome provides a new source of traits to test in systematics. Before the introduction of molecular techniques, relationships among organisms relied on morphological traits. Phylogenetic relationships and their associated hierarchy were constructed on the basis of the evolution of homologous morphological characters, whereas in the last decades evolutionary patterns have been regarded, or even redesigned, in the light of the results obtained by DNA molecular markers.

The first DNA sequence obtained for a bryophyte was the 5S gene of *Marchantia polymorpha*, as part of a study concerning the evolutionary history of plants (Hori *et al*., 1985). The first studies specifically focused on bryophyte evolution, however, date to 1992 (Mishler *et al*., 1992; Waters *et al*., 1992), based on genes encoding ribosomal RNAs. The protein coding gene *rbc*L was first targeted by Lewis *et al*. (1997), while Wood *et al*. (1999) sequenced a broad array

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of expressed sequence tag (EST) in *Syntrichia (Tortula) ruralis*. Other techniques requiring sequence information, like microsatellites (simple sequence repeats, SSRs) and sequence characterized amplified region (SCAR) were developed for bryophytes later (Van der Velde *et al*., 2000; Shaw *et al*., 2003). Moreover, although any paper specifically devoted to single nucleotide polymorphisms (SNPs) in bryophyte has not been found in the present investigation, all the papers dealing with sequence data may reveal the presence of SNPs.

With reference to DNA molecular markers providing banding patterns (i.e., multilocus DNA molecular markers based on the size of anonymous loci), random amplified polymorphic DNA (RAPD) were first used in the liverwort *Porella* (Boisselier-Dubayle & Bischler, 1994), whereas first amplifications by inter-simple sequence repeat (ISSR) primers involving bryophytes were carried out in two moss species, *Pogonatum dentatum* and *Sphagnum angermanicum*, in 2003 (Hassel & Gunnarsson, 2003), and intron-exon splice junction (ISJ) amplifications have been performed since 2005 (Polok *et al*., 2005). Since 2000, some techniques based on the combination of PCR amplification and digestion by restriction enzymes has also been applied in bryophytes. Among these techniques, amplified fragment length polymorphism (AFLP) was used at first in a population study on *Amblystegium tenax* (Vanderpoorten & Tignon, 2000), and PCR – restriction fragment length polymorphism (PCR-RFLP), was applied in a taxonomic study within *Rhytidiadelphus* (Vanderpoorten *et al*., 2003). Detection of RFLP using southern blotting has been used on a broad range of organisms, but this method is time consuming and requires large amounts of DNA, rarely available in small bryophytes. Additionally, RFLP may lack sufficient variation for population studies, but it has been useful in testing phylogenetic hypotheses (Boisselier-Dubayle *et al*., 1995).

Here we provide a first review of DNA based investigations in bryophyte systematics, and assessment of the suitability of various methods for distinct research objectives. We grouped the techniques into two main categories according to the key difference of their experimental protocol, those based on actual sequence information (sequencing, SSR, SCAR), and those making inferences based on banding patterns (RAPD, ISSR, ISJ, AFLP, PCR-RFLP).

The main purposes of this work are to: i) provide an overview of the molecular techniques based on PCR and utilized in mosses, specifying their advantages and drawbacks and ii) report on a large body of recent papers that illustrate their applications. About 100 papers were examined and referenced in an extensive table focusing on the main subjects, the taxa studied, and the type of molecular marker. For an easier reading, the referenced papers were grouped into six categories, according to the main field of application: biogeography, gene expression, phylogeny, population ecology, taxonomy and technical. This table may represent a guideline for the choice of a suitable molecular technique in mosses.

MOLECULAR TECHNIQUES REQUIRING SEQUENCE INFORMATION

Sequencing

Apart from paralogous genes, sequencing gives the certainty to compare homologous loci, therefore it is more reliable than markers based on banding pattern. The nuclear ribosomal DNA is widely used to reconstruct phylogenetic relationships (Samigullin *et al*., 1998; Olsson *et al*., 2009; Merget & Wolf, 2010),

within families (Shaw & Allen, 2000), genera (Grundmann *et al*., 2006; Shaw *et al*., 2010; Carter, 2012) or species (Skotnicki *et al*., 2004; Vanderpoorten *et al*., 2004; Grundmann *et al*., 2006; Draper *et al*., 2007; Korpelainen *et al*., 2008b; Draper *et al*., 2011). Particularly, ITS sequences were used as markers in many studies in mosses (e.g. Korpelainen *et al*., 2008b; Spagnuolo *et al*., 2008; Carter, 2012; Terracciano *et al*., 2012b).

The chloroplast (cp) *trn*T-F region and especially the *trn*L_{UAA} intron are the most widely targeted loci, not only in bryophytes, but also in other plants (Quandt & Stech, 2005). The locus has been complemented by the *rps*4 gene in many studies aiming at resolving relationships within classes and families (Buck *et al*., 2000 and 2005; Goffinet *et al*., 2001; Shaw & Allen, 2000; Pedersen *et al*., 2007; Bell & Hyvönen, 2010), genera (Shaw *et al*., 2010; Carter, 2012) and even among populations (Vanderpoorten *et al*., 2004; Werner & Guerra, 2004; Draper *et al*., 2011). The *atp*B-*rbc*L intergenic spacer has also been frequently used in moss phylogeography (e.g., Grundmann *et al*., 2006; Pedersen *et al*., 2007).

The idea that the mitochondrial DNA offers few loci routinely targeted for moss phylogenetic studies is primarily due to the presumably lower variability compared with the cpDNA (Stech & Quandt, 2010). However, the *nad*5 gene, recently sequenced for many moss species (Liu *et al*., 2012), has been used for reconstructing bryophyte phylogenies since 1999 (Beckert *et al*., 1999), and so have other mitochondrial DNA regions (Cox *et al*., 2010; Wahrmund *et al*., 2009 and 2010). Nonetheless, the use of single copy genes, like all chloroplast and mitochondrial genes, in moss systematics, is strongly affected by the available knowledge about the flanking regions of the gene itself to develop suitable primers (Wall, 2002).

SSR (simple sequence repeats)

The microsatellites (also known as simple sequence repeats, SSRs) are tandem repeats of short DNA sequence motifs. These markers have several advantages: i) each locus is well-defined and codominant; ii) they are frequently polymorphic at population level, due to differences in the number of repeats; and iii) they are easily tested by PCR. However, sequence information is required in order to design species-specific primers into the flanking regions of the repeat motif.

The polymorphism of a locus is based on the number of repeats, that increases or decreases the length of the locus, and it is commonly evidenced by comparing PCR-fragment length by capillary electrophoresis. SSRs are highly abundant in eukaryotic genomes (Korpelainen *et al*., 2007). Furthermore, the levels of heterozygosity can be exceptionally high, even in species in which the level of genetic variation detected by other methods may be low. In this laborious technique, the markers act as a single-locus trait, and their development is expensive (Korpelainen *et al*., 2007). These disadvantages are compensated when the protocol optimized to amplify SSRs for a given species can be extended to closely related taxa, as in *Sphagnum* (Provan & Wilson, 2006). Microsatellites have been used to evaluate the genetic diversity (e.g., Liu *et al*., 2010a), to test species delimitation (Shaw *et al*., 2009), to circumscribe geographic distribution of intraspecific genets (Szövényi *et al*., 2008), to identify parental taxa in alloploids (Ricca & Shaw, 2010), or to evaluate degrees of inbreeding and its consequences (Szövényi *et al*., 2009b; Van der Velde *et al*., 2001 a and b). Given the versatility of these markers their use in mosses is expected to increase.

SCAR (sequence characterized amplified region)

The SCAR markers are locus-specific markers, which are detected through specific primers designed from identified sequences obtained by other methods. For example, Shen *et al.* (2011) developed the SCAR primers from specific RAPD bands; Albani *et al.* (2004) used instead ISSR bands. Once the sequence of the locus is known, a pair of specific 15-30 bp primers is designed, making this technique suitable for diagnostic purposes, like selecting a commercially valuable crop or variety (e.g. Yang *et al.*, 2013). These markers were used in bryophytes to delimitate *Calypogeia* species (Buczkowska & Dabert, 2011) and to target three anonymous DNA regions in *Sphagnum* (Shaw *et al.*, 2003). SCAR technique provides a useful tool in order to develop new sequence markers starting from any technique based on banding pattern. However, several cloning-sequencing cycles may be needed before obtaining a suitable anonymous region to be characterized.

MOLECULAR TECHNIQUES PRODUCING BANDING PATTERNS

RAPD (random amplified polymorphic DNA)

This technique is a random targeting PCR approach based on short (10 bp) primers. A possible mutation across one or more annealing sites along DNA template will result in the absence of the relative band, and subsequently in a different banding profile. This technique allows fast, easy and cheap comparison between a big set of samples when prior sequence information is not available (Kjolner *et al.*, 2004). Due to these advantages, this technique seemed suitable for populations study (Selkirk *et al.*, 1997; Skotnicki *et al.*, 2004). However, the typically low stringency conditions may lead to a low reproducibility of bands (Kjolner *et al.*, 2004). Other limitations of RAPD technique are the dominant behaviour, the lack of knowledge on amplification products, and the co-migration of fragments that are not homologous but of similar size. This technique thus calls for particular caution in applying the method and interpreting its results. Furthermore, the targeted DNA must be highly purified, because, contaminating (endophytic or epiphytic) fungi may be amplified and lead to artefactual results (Stevens *et al.*, 2007). This technique has been applied to investigate genetic variability and structure among populations, in several moss species (*Hennediella heimii*, *Sarconeurum glaciale* and *Ceratodon purpureus*), and in particular Antarctic mosses (Selkirk *et al.*, 1997; Skotnicki *et al.*, 1998; Skotnicki *et al.*, 1999; Dale *et al.*, 1999; Skotnicki *et al.*, 2004). It was also used to study the species delimitation in *Sphagnum recurvum* (Sastad *et al.*, 1999). However, recent application of RAPD technique in moss populations, compared to SSR, showed that the first lead to an overestimation of the gene diversity due to sample contamination (Clarke *et al.*, 2009). For this reason, the RAPD technique is now used to study somatic mutation in *in vitro* cultured axenic plants (Enan, 2006).

ISSR (inter-simple sequence repeat)

ISSR technique is nearly identical to RAPD technique except that ISSR primers are designed from microsatellite regions and are longer than RAPD

primers. The first ISSR studies were published in 1994, focused on cultivated species (see Wolfe, 2005 for a review). These markers are based on the amplification of DNA segments present between two identical microsatellite repeat regions, usually a dinucleotide or a trinucleotide motif, and oriented in opposite directions. Therefore, the technique uses microsatellites as primers in a single PCR reaction targeting multiple genomic loci to amplify ISSRs of different sizes. The primers can be either unanchored or, more frequently, anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Weising *et al*., 2005). ISSRs have high reproducibility, due to the use of longer primers (16-25 bp), which allow a higher stringency and more reliable and reproducible bands than RAPD (Vanderpoorten *et al*., 2003). Several studies targeted ISSR in mosses, to estimate genetic diversity and clonal structure in populations, in relation to bottleneck/genetic drift processes (Vanderpoorten *et al*., 2003; Gunnarsson *et al*., 2005; Spagnuolo *et al*., 2007a and 2009a), to habitat disturbance and fragmentation (Spagnuolo *et al*., 2007b; Spagnuolo *et al*., 2009b; Patiño *et al*., 2010), and for conservation purposes (Terracciano *et al*., 2012a). In some cases this technique was applied to resolve taxonomic problems (Hassel & Gunnarsson, 2003; Spagnuolo *et al*., 2008), or distinguish sexes (Korpelainen, 2008a). Furthermore, ISSR markers, similarly to RAPD, can be the basis for the development of other markers, i.e. microsatellites (Korpelainen *et al*., 2007; Provan & Wilson, 2006), or SCARs (Buczkowska & Dabert, 2011).

ISJ (intron-exon splice junction)

ISJs are semi-specific dominant markers being present in the majority of plant genes (Gang *et al*., 2011). The longer primers used in this technique allow a higher annealing temperature, obtaining more reproducible bands. ISJ markers were used in plants (e.g. in cotton, Gang *et al*., 2011) but very few references were found in mosses. Sawiki and Szczecinska (2007) optimized the technique in mosses, evaluating its suitability for *Sphagnum* spp. and *Orthotrichum speciosum*, whereas Sawiki *et al*. (2012) evaluated the distinction between the subgenera *Orthotrichm* and *Pulchella* of *Orthotrichum*. Even if some sequence information was required for their initial development (Weining & Langridge 1991), all primers can be designed based on the constant oligonucleotide sequence located at the junction between intron and exon, with the addition of a short random sequence. In theory, by using a given primer pair, a researcher might also decide whether to amplify exonic or intronic regions, but in practice each primer can also act as a forward and a reverse, so the technique is comparable to the RAPD or the ISSR.

AFLP (amplified fragment length polymorphism)

This DNA fingerprinting technique was developed by Zabeau and Vos (1993) (but see also Vos *et al*., 1995 and Vos & Kuiper, 1997) and is based on PCR amplification of selected restriction fragments of a total digested genomic DNA. AFLP markers reveal a "restriction site" polymorphism, usually made of small DNA fragments of few base pairs (up to 500) that must be amplified by PCR to be visualized. The originality of the AFLP method was to design and synthesize arbitrary primers first, and then to legate them to target DNA fragments. The AFLP arbitrary primers are called "adapters" and consist of a known sequence of about 20 nucleotides. The target DNA sequences are DNA fragments generated

by restriction enzymes. Then, adapters are ligated at each end of a restriction fragment by enzyme ligase. Finally, adapters are used in a PCR as annealing sites to amplify the restriction fragments. Amplified products are separated by electrophoresis on acrylamide gel or by capillary electrophoresis.

AFLP provide reliable dominant markers, which are highly reproducible due to the specificity of restriction enzymes. The great advantage of using AFLPs in population studies is that the number of loci scored and polymorphisms identified in each reaction is high (Blears *et al*., 1998). Moreover, these markers give an estimate of variation across the entire genome, which in turn gives a good general picture of the level of genetic variation of the investigated organism. In mosses, AFLP technique has been used to perform studies having different aims. For example, linkage maps at species level were developed (McDaniel *et al*., 2007 and Kamisugi *et al*., 2008), but always combined with other techniques (like SSRs) to ensure the accuracy of the results. Fernández *et al.* (2006) inferred the presence of cryptic species in *Grimmia laevigata* and Pfeiffer (2006) described the molecular diversity and clonal structure in *Rhytidium rugosum* based on AFLP data.

PCR-RFLP (PCR-Restriction Fragment Length Polymorphism)

The technique consists of a digestion by a restriction endonuclease of a specific amplified DNA segment; the presence or the absence of a given restriction site in the sequence will provide polymorphism between individuals. The technique requires specific primers for locus amplification and may be applied both to known sequences and to anonymous DNA regions; in the latter case, SCAR primers must be designed in order to obtain the amplification product. PCR-RFLP can be performed by single or double digestions; this choice is generally related to the expected polymorphism of a specific DNA segment. If expected divergence is low, then the double or triple digestions may yield more characters.

PCR-RFLP is a fast and reliable technique if applied to known sequences (e.g. ribosomal genes and spacers) for which universal primers are available; it proved useful in the resolution of taxonomic problems (Vanderpoorten *et al*., 2003). It could also provide a first information level in taxonomic studies, before sequencing, but it gives too little information, compared to sequencing, to be used in phylogeny or in biogeography; moreover, the evolution of these traits cannot be modelled properly. Due to their theoretical high level of polymorphism, RFLP of anonymous DNA regions may also be applied in population studies, but the development of specific primers may be expensive and time consuming.

CONCLUSIONS

The table enclosed in this overview may provide a guideline in the choice of suitable molecular markers to address specific questions in moss systematics. Molecular phylogenetic inferences have revealed extremely powerful for testing traditional taxonomic hypotheses, and this is especially true in taxa with rather simple morphologies like bryophytes (Vanderpoorten & Shaw, 2010), particularly when reduction in morphological complexity may obscure the phylogenetic affinities among taxa (e.g. Goffinet *et al*., 2011). Of course, sequences and related

markers are very reliable because they compare homologous DNA loci. By contrast, a certain caution is needed while analysing molecular results from markers grounded on banding patterns; assuming that bands having the same bp length are homologous loci is the rationale of these techniques, but it is not necessarily true. In addition, the co-migration of different amplification products having the same length is a further source of artefacts. However, the critical evaluation of a large body of literature highlights that sequence-related markers are not applicable to all research fields. Although they result useful in phylogenies and population studies over wide geographic ranges, in general they may not reveal polymorphisms at small geographic scales and at low taxonomic ranks (i.e., between and within species). This makes it necessary to fall back on molecular techniques based on banding pattern. Among these, the ones requiring capillary electrophoresis, like AFLP, should be preferred because of the high reproducibility and the precision of the detection system. But also ISSR and ISJ may yield robust results if an accurate choice of reproducible bands is made, and high resolution systems for band evaluation are applied. The use of labelled primers, for example, allows for an accurate fragment separation by capillary electrophoresis.

Differences in principles, methodologies, and applications of various types of molecular markers require careful consideration in choosing one or more of such methods, according to the kind of study to be undertaken. Since a literature examination is always a good starting point, in this paper we provide an extensive compilation of molecular studies, specifically devoted to mosses, in the form of a table (Table 1) in which the papers are grouped under different main subjects, specifying the techniques and the taxa studied. This could be a useful tool to choose the most suitable technique and methodology according to the specific research purposes.

Biogeography			
Key words	*Molecular Technique*	*Taxa*	*Reference*
ITS, *trn*L-F, phylogeographic relationships, glacial refugia	cp/nr DNA	*Neckera* and *Exsertotheca*	Draper *et al.*, 2011
Bioindication, floristic province	AFLP	*Grimmia laevigata*	Fernández *et al.*, 2006
Bottleneck, clonal plant, gene flow, invasive species, recombination	ISSR	*Pogonatum*	Hassel *et al.*, 2005
Allopolyploidy, interploidal hybridization, phenotypic variation	SSR	*Sphagnum cuspidatum*	Karlin *et al.*, 2011b
ITS, neighbor-joining analysis	nrDNA	*Hilpertia velenovskyi*	Sabovljevic *et al.*, 2006
Cryptic speciation, DNA sequence variation, disjunctions, liverworts	Isozymes, DNAseq	Bryophyta	Shaw 2001
Antarctica, colonization, genetic diversity	RAPD	*Sarconeurum glaciale*	Skotnicki *et al.*, 1999
Clonal diversity and structure, geographic pattern, vegetative growth	cpDNA, ISSR	*Pleurochaete squarrosa*	Spagnuolo *et al.*, 2009a
Allopolyploid, bayesian analysis, hybridization, *trn*G	cpDNA, SSR	*Sphagnum troendelagicum*	Stenoien *et al.*, 2011
Dispersal, glacial refugia, phylodemography, cp non-coding regions	cpDNA	*Sphagnum fimbriatum, S. squarrosum*	Szövényi *et al.*, 2006
*Gap*C, ITS, sequence variability, multilocus dataset	nrDNA	*Sphagnum fimbriatum, S. squarrosum*	Szövényi *et al.*, 2007

Biogeography			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Shared polymorphism, intercontinental migration, isolation with migration	SSR	*Sphagnum*	Szövényi *et al.*, 2008
Gametophytic selection, molecular adaptation, GDPH, *Gap*C, *Rpb*2	DNAseq	*Sphagnum fimbriatum*	Szövényi *et al.*, 2009a
Genetic structure, inverse isolation hypothesis, island colonization, long-distance dispersal	SSR	*Sphagnum*	Szövényi *et al.*, 2012
Phylogeography, coalescent theory, gene flow, molecular evolution	SSR	*Sphagnum angermanicum*	Stenoien *et al.*, 2010
Gene sequences, *rps*4, evolution, molecular systematics	cpDNA	*Tortula muralis*	Werner & Guerra, 2004
Gene expression			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Evolution, gene expression, molecular modeling, nonsymbiotic hemoglobins	DNAseq	*Ceratodon purpureus*	Garrocho-V.& Arredondo-P., 2008
Plastid DNA, transcript profiling	Microarray	*Physcomitrella patens*	Nakamura *et al.*, 2005
Chloroplast genome sequence, gene content, polymorphism, *Physcomitrella patens*	cpDNA	*Tortula ruralis*	Oliver *et al.*, 2010
Molecular evolution, *trn*T-F, *trn*L, groupI intron, inversion, hairpins	cpDNA	Bryophyta	Quandt & Stech, 2004
Molecular evolution, *trn*L_{UAA} intron, secondary structure	cpDNA	Bryophyta	Quandt & Stech, 2005
Salt tolerance, transcription factors, NCED, abscisic acid	Microarray	*Physcomitrella patens*	Richardt *et al.*, 2010
Moss, MADS-box gene, MICK*-type, evolution	RT-PCR, DNA seq	*Physcomitrella patens*	Riese *et al.*, 2005
Phylogeny			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Bayes factors, incongruent gene trees, phytogeography, reticulate evolution, 18S	cp/nrDNA	Polytrichopsida	Bell & Hyvönen, 2010
Morphological concepts, *rps*4, *trn*L-F, sequences	cpDNA	Pleurocarpous mosses	Buck *et al.*, 2000
Hookeriales, *nad*5, *rps*4, *trn*L-F, 26S	cp/mit/nrDNA	Pleurocarpous mosses	Buck *et al.*, 2005
Systematics, *Timmia*, *atp*B-*rbc*L, *trn*L-*trn*F, 26S	cp/nrDNA	Timmiaceae	Budke & Goffinet, 2006
ITS, *rps*4, *psb*A2, *trn*G, monophyly, speciation	cp/nrDNA	*Scleropodium*	Carter, 2012
mosses, Bryopsida, phylogeny, phylogenetic diversity, Bayesian phylogenetic analysis	cp/mit/nrDNA	Mosses (655 genera)	Cox *et al.*, 2010
Hybridization, ITS, *trn*G, molecular and morphological incongruence	cp/nrDNA	*Isothecium*	Draper *et al.*, 2007
Evolution, systematics, *rbc*L	cpDNA	Orthotrichaceae	Goffinet *et al.*, 1998
Phylogenetic relationships, cryptic species, transatlantic distributions, ITS	cp/nrDNA	*Pleurochaete*	Grundmann *et al.*, 2006
Genetic diversity and structure, postglacial, dispersal history, rare species	ISSR	*Sphagnum angermanicum*	Gunnarsson *et al.*, 2005
Chloroplast sequences, systematics, *trn*L-F, *trn*S-*rps*4	cpDNA	Grimmiaceae and Ptycho-mitriaceae	Hernández-M. *et al.*, 2007
Inversions, *trn*L, Group I intron, microstructural changes	cpDNA	Grimmiaceae and Ptycho-mitriaceae	Hernández-M. *et al.*, 2008

Phylogeny			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Linnean shortfall, Macaronesia, morphology	cp/nrDNA, SSR	*Rhynchostegium riparioides, R. alopecuroides,* and *Gradsteinia torrenticola*	Hutsemékers *et al.*, 2012
*rbc*L, *rps*4, *trn*L-F, *nad*5, 18S rDNA, parsimony analyses	cp/mit/nrDNA	Polytrichales	Hyvönen *et al.*, 2004
Allopolyploidy, allotriploid gametophytes, speciation	SSR	*Sphagnum*	Karlin *et al.*, 2009
Founder effect, intragametophytic selfing	SSR	*Sphagnum*	Karlin *et al.*, 2011a
Data partitioning, monophyly test, conflict visualization, sequence	mitDNA	Funariaceae	Liu *et al.*, 2012
Linkage map, *adk* and *phy*2 genes, interpopulation cross	AFLP	*Ceratodon purpureus*	McDaniel *et al.*, 2007
Genealogical conflict, hybrid speciation, reproductive isolation, adk, apr, papr	cp/nrDNA	*Physcomitrium*	McDaniel *et al.*, 2009
Cytoplasmic inheritance, maternal inheritance	cp/mitDNA	*Sphagnum*	Natcheva & Cronberg, 2007
Asymmetric recombination, hybridization, introgression	ISSR	*Sphagnum capillifolium, Sphagnum quinquefarium*	Natcheva & Cronberg, 2007
Glossadelphus, *Taxiphyllum*, taxonomy, evolution, Miyabeaceae, Hypnales, phylogeny, *Homaliadelphus*, *Miyabea*, *Bissetia*	cp/mit/nrDNA	Hypnales	Olsson *et al.*, 2009
Evolution, ancestral character states, maximum parsimony, bayesian inference	cpDNA	Bryaceae	Pedersen *et al.*, 2007
Cytotypes, peat moss, polyploidy, allopolyploidy, homoploid hybridization	cpDNA, SSR	*Sphagnum subsecundum* complex	Ricca and Shaw, 2010
Sphagnum subsecundum complex, allopolyploidy	SSR	*Sphagnum lescurii*	Ricca *et al.*, 2011
ITS, sequence	cp/nrDNA	Bryophyta	Samigullin *et al.*, 1998
ITS, molecular taxonomy, *trn*H-*psb*A, genetic relationships	cp/nrDNA, ISJ, ISSR	*Orthotrichum*	Sawiki *et al.*, 2012
ITS, 26S, *psb*T, *trn*L, evolution, monophyly, phylogenetics	cp/nr DNA	*Sphagnopsida*	Shaw, 2000
Morphological incongruence, geographic speciation, *trn*L-*trn*F, ITS	cp/nrDNA	Fontinalaceae	Shaw & Allen, 2000
Bayesian inference, bryophytes, peatmoss, phylogenetic reconstruction	DNA seq	Sphagnopsida	Shaw *et al.*, 2003
Species delimitation, recombination, gene flow, monophyly	cp/nrDNA	*Sphagnum* section *Acutifolia*	Shaw *et al.*, 2005
Long-distance dispersal, northern hemisphere biogeography, anonymous loci	ITS, LFY1/2, *trn*L/G	*Sphagnum subsecundum* complex	Shaw *et al.*, 2008a
Ascertainment bias, DNA fingerprinting	SSR	*Sphagnum*	Shaw *et al.*, 2008b
Allopolyploidy, PCR recombination, species delimitation	SSR	*Sphagnum subsecundum*	Shaw *et al.*, 2008c
Peat mosses, species delimitation	SSR	*Sphagnum atlanticum, S. bergianum*	Shaw *et al.*, 2009

Phylogeny			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Bryophyte evolution, Miocene, peatlands	cp/mit/nrDNA	*Sphagnum*	Shaw *et al.*, 2010
ITS 1 phylogeny, ribosomal DNA	ITS sequences	*Pottiaceae*	Spagnuolo *et al.*, 1999
DNA barcoding, mitochondrial DNA, nuclear DNA, plastid DNA, phylogenetics	Review	*Bryophyta*	Stech & Quandt, 2010
Haplolepideous mosses, non-coding plastid markers, *trn*L-F, *rps*4	cpDNA	*Dicranidae*	Stech *et al.*, 2012
Gene trees, adenosine kinase gene, gene duplication, paralogy, polyploids	cp/nrDNA	*Hypnum*	Terracciano *et al.*, 2012b
Species concept, paraphyly, budding speciation, monophyly	Review	Bryophyta	Vanderpoorten & Shaw, 2010
Sequence, *trn*L-*trn*F, *atp*B-*rbc*L, 18S-5.8S-26S, adk gene	cp/nrDNA	*Hygroamblystegium*	Vanderpoorten *et al.*, 2004
Group I and II intron, indels, intergenic region, RNA editing	cp/mitDNA	Bryophyta	Wahrmund *et al.*, 2009
Group I intron cobi420, inversions.	mitDNA	Bryophyta	Wahrmund *et al.*, 2010
Population ecology			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Genetic diversity, Antarctica	RAPD	*Hennediella heimii*	Dale *et al.*, 1999
ISSR protocol, population genetics, PCR techniques	ISSR	*Pogonatum* and *Sphagnum*	Hassel & Gunnarsson, 2003
Genetic structure, aquatic moss	SSR	*Platyhypnidium riparioides*	Hutsemékers *et al.*, 2008
Clonality, morphological shift, population structure, species delimitation	SSR	*Sphagnum cribosum*	Johnson *et al.*, 2012
Allopolyploid, clone, genetic diversity, long-distance dispersal, vegetative propagation	SSR	*Sphagnum cuspidatum*	Karlin *et al.*, 2011c
Clonal growth, genetic isolation, genetic richness, forest disturbance	SSR	*Acanthorrhynchium papillatum*	Leonardi *et al.*, 2012
Epiphytic bryophyte, exotic tree plantation, forest disturbance, habitat suitability	ISSR	*Isothecium myosuroides*	Patiño *et al.*, 2010
Clonal reproduction, dispersal, habitat, vegetative reproduction	AFLP	*Rhytidium rugosum*	Pfeiffer *et al.*, 2006
Genetic variation, Antarctic populations	RAPD	*Sarconeurum glaciale*	Selkirk *et al.*, 1997
Genetic variation, RAPD profiling of genetic diversity, Antarctica	RAPD	*Ceratodon purpureus*	Skotnicki *et al.*, 1998
Clonal moss, genetic diversity, *trn*L intron	cpDNA, ISSR	*Pleurochaete squarrosa*	Spagnuolo *et al.*, 2007a
Epiphytic moss, genetic structure, genetic impoverishment, habitat disturbance	ISSR	*Leptodon smithii*	Spagnuolo *et al.*, 2007b
Inbreeding, mating system, reproductive skew, selective embryo abortion	SSR	*Sphagnum lescurii*	Szövényi *et al.*, 2009b
Conservation genetics, fragmentation, habitat disturbance	ISSR	*Sphagnum palustre*	Terracciano *et al.*, 2012a
AFLP, *Amblistegium*, polymorphism, genetic differenziation	AFLP	*Amblystegium tenax*	Vanderpoorten & Tignon, 2000
Clonal structure, genotypic diversity, male fertilization success, paternity analysis	SSR	*Polytrichum formosum*	Van der Velde *et al.*, 2001

Taxonomy			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Genomic relationship, molecular and morphological congruence	RAPD	*Thuidium tamariscinum, Hyophyla comosa*	Alam *et al.*, 2012
ITS, molecular systematics, *Tortula ruralis* complex	nrDNA	*Tortula densa*	Frahm & Sabovljevic, 2006
New Zealand, Australia, alloploid	SSR	*Sphagnum* sect. *Sphagnum*	Karlin *et al.*, 2008
Allopolyploidy, eastern North America, peat mosses, species concept	SSR	*Sphagnum*	Karlin *et al.*, 2010
Allopolyploidy, long-distance dispersal, *Sphagnum*, taxonomy	SSR	*Sphagnum falcatulum* species complex	Karlin *et al.*, 2013
ISSR, ITS, PCR-RFLP, species concept, species delimitation	nrDNA, SSR	*Rhytidiadelphus*	Korpelainen *et al.*, 2008b
Ex situ conservation, threatened bryophytes, barcoding, *trn*L-F intron	AFLP, cpDNA	*Orthodontium gracile*	Rowntree *et al.*, 2010
Sphagnum balticum, S. lindbergii, population analysis	Isozymes, RAPD	*Sphagnum recurvum* complex	Sastad *et al.*, 1999
Genetic similarity, *kat*G, ITS, species identification	nrDNA, ISJ, ISSR, RAPD	*Sphagnum* sect. *Acutifolia*	Sawiki & Szczecinska, 2011
DNA sequence analysis, ITS, within- and among-colony variation	nrDNA, RAPD	*Ceratodon purpureus*	Skotnicki *et al.*, 2004
Corsica, endemism, species concept, incongruence, morphology	cp/nr DNA	*Leptodon corsicus, Neckera besseri, Homalia webbiana*	Sotiaux *et al.*, 2009
Genetic variation, ITS, *trn*L, species concept	cp/nr DNA, ISSR	*Hypnum cupressiforme* complex	Spagnuolo *et al.*, 2008
Species-level systematics, sibling species	nrDNA, ISSR, RFLP	*Rhytidiadelphus*	Vanderpoorten *et al.*, 2003
ITS sequence data, Canary Islands	nrDNA	*Platyhypnidium torrenticola, P. riparioides*	Werner *et al.*, 2007
Haplolepidous mosses, *rps*4, *trn*L-F	cpDNA	*Hymenoloma mulahaceni*	Werner *et al.*, 2013
Technical			
Key words	*Molecular Technique*	*Taxa*	*Reference*
Linkage map, genome sequence	AFLP, SSR	*Physcomitrella patens*	Kamisugi *et al.*, 2008
Marker identification, genome screening, cloning, SSR development	ISSR, SSR	Bryophyta, algae	Korpelainen *et al.*, 2007
Gender ratio determination, sex ratio determination, ISSR sequence	ISSR	*Pseudocalliergon*	Korpelainen *et al.*, 2008a
DNA barcoding, *rbc*L, *rpo*C1, *rps*4, *trn*H-*psb*A, *trn*L-*trn*F, sequence	cp/mit/nr DNA	Bryophyta	Liu *et al.*, 2010b
Development of microsatellite markers, population genetics	SSR	*Ptychomitrium gardneri*	Liu *et al.*, 2010a
AFLP reproducibility, genotyping error rate	AFLP	*Campylopus introflexus*	Mikulášková *et al.*, 2012
Peat bogs, ISSR cloning, conservation	ISSR, SSR	*Sphagnum capillifolium*	Provan & Wilson, 2007
AFLP, ISSR, RAPD, bryophytes	ISJ	*Sphagnum* and *Orthotrichum*	Sawiki & Szczecinska, 2007
Gene targeting efficiency	Genetic transform	*Physcomitrella patens*	Schaefer, 2001
Bioinformatics, EST database, plant genome	SSR	*Physcomitrella patens*	von Stackelberg *et al.*, 2006

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