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20,000 species and five key markers: The status of molecular bryophyte phylogenetics

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Abstract

A number of reviews have accompanied and monitored the progress of molecular phylogenetic research on bryophytes, focusing on the publication record itself, bryophyte phylogeny and systematics in the molecular era, as well as the evolution and phylogenetic utility of markers from different genomes. However, none of the recent reviews include a detailed characterization of all molecular markers used in bryophyte phylogenetics. Here we provide an overview of the history and current state of marker utilization, including coding and non-coding sequence markers from all three genomes as well as fingerprinting approaches. The molecular architecture and evolutionary peculiarities, as well as practical aspects such as amplification and sequencing strategies, are outlined for the DNA sequence markers, with a focus on the most commonly employed regions. Their phylogenetic utility and potential for solving some of the remaining, pressing questions in bryophyte phylogeny, as well as their suitability for molecular species identification by DNA barcoding, are discussed.

Key words: Bryophytes, DNA barcoding, mitochondrial DNA, molecular markers, nuclear DNA, plastid DNA, phylogenetics

Introduction

During the last two decades, analyses of molecular data have had a major impact on our understanding of plant evolution and relationships on all taxonomic levels, from the deep nodes separating the major plant groups to species and populations. Currently, molecular characters are primarily obtained from three different sources: (i) DNA sequences of specific coding or non-coding regions from one of the three plant genomes (plastid, mitochondrial, or nuclear markers), (ii) structural genomic characteristics (e.g. gene order, gain or loss of genes or non-coding regions), and (iii) genetic fingerprints. The largest numbers of publications and sequences are available for angiosperms, the most species-rich extant plant group; however, quite a large amount of molecular data has been accumulated for bryophytes as well. The first DNA sequences of bryophytes were published in the early 1980's (Katoh *et al.* 1983), but it took another decade until molecular phylogenies of bryophytes appeared. For the present overview, 382 papers, published up to the end of 2009, were compiled from different sources (PubMed, Current Contents, manual search of journal contents) that contain original datasets for bryophytes based on DNA sequences and/or DNA fingerprinting. This corresponds to an average of 35 publications per year in the last decade (1999−2009, Fig. 1). Not considered here are technical notes, studies based solely on allozymes, as well as phylogenies including (few) bryophyte representatives but mainly focusing on other plant groups. The earliest studies mainly focused on the relationships between the three major bryophyte groups and other land plants (cf. Fig. 1), an issue only recently resolved with confidence (Qiu *et al.* 2006). In the majority of publications, however, questions at

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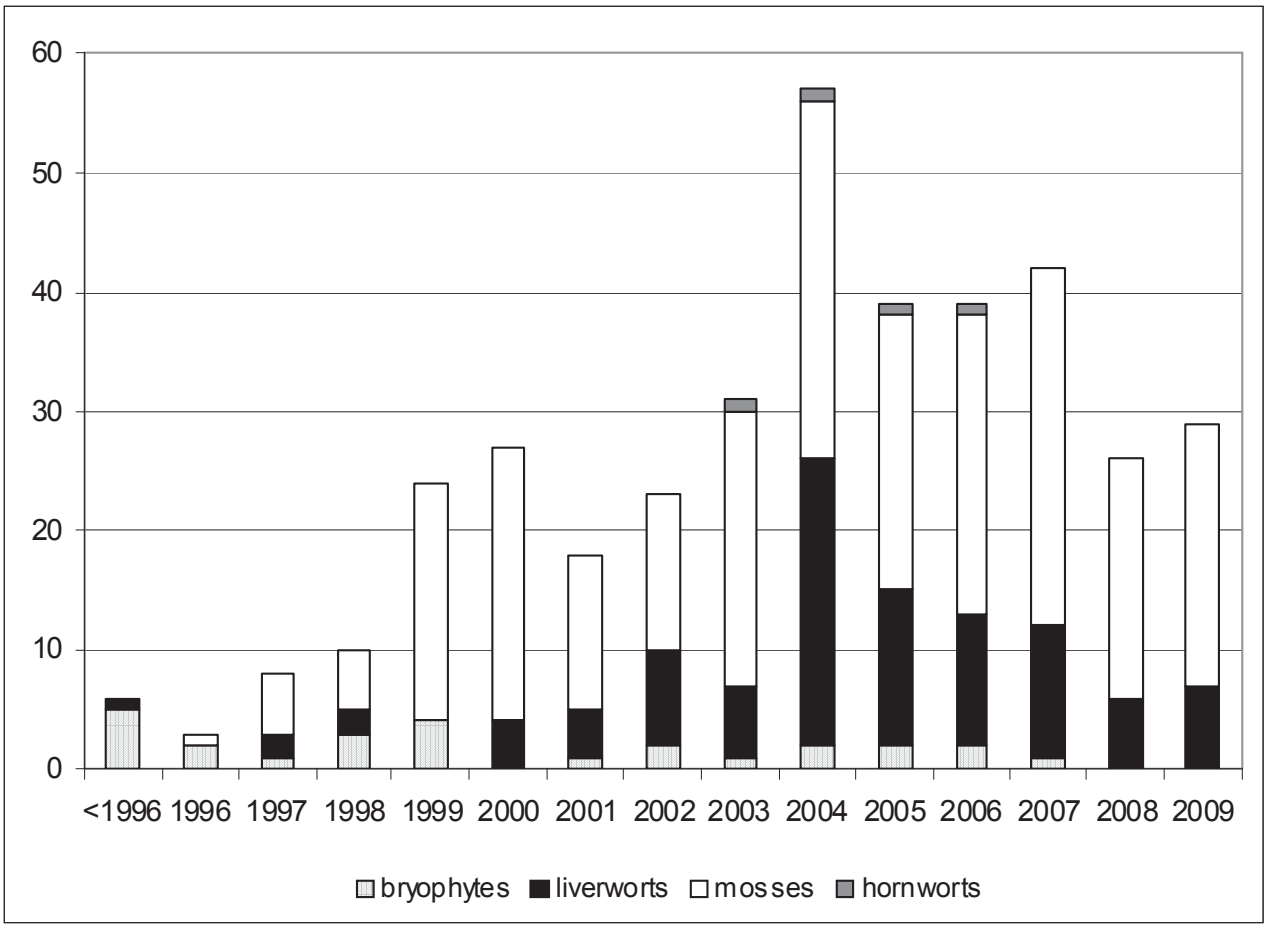

FIGURE 1. Development of the publication record of molecular phylogenetic publications in bryophytes until the end of 2009. Included are 382 publications containing original datasets based on DNA sequences and/or DNA fingerprinting. They are divided into papers dealing with relationships across all bryophytes/land plants, or within the three bryophyte lineages, respectively.

lower taxonomic levels (Fig. 1), such as circumscriptions of taxa, analyses of distribution patterns, intraspecific variation, and increasingly, population structure, are addressed.

A number of reviews or review-like papers have accompanied and monitored the progress of molecular research on bryophytes. These focused on different, partly overlapping aspects, such as the publication record itself (e.g. Goffinet & Hax 2001; Goffinet 2003), bryophyte phylogeny and systematics in the molecular era (partly including notes on marker utility) (e.g. Quandt & Stech 2003; Goffinet & Buck 2004; Shaw & Renzaglia 2004; Troitsky *et al.* 2007; Stech 2009; Vilnet *et al.* 2009), as well as the evolution and phylogenetic utility of markers from different genomes (e.g. Vanderpoorten *et al.* 2006; Borsch & Quandt 2009; Knoop 2010). Furthermore, the results of molecular phylogenetic reconstructions were included in recent (text) books that provide updated systematic treatments (e.g. Frey & Stech 2009; Goffinet & Shaw 2009). However, none of the recent reviews include a detailed characterization of all molecular markers used in bryophyte phylogenetics. We therefore provide an overview of the phylogenetic utility, history and future potential, of the coding and non-coding markers from all three genomes, as well as fingerprinting approaches. Additionally, we highlight several remaining questions in bryophyte phylogeny in the light of future developments in marker use.

Historical development and current state of marker use in bryophyte molecular phylogenetics

Quandt & Stech (2003) provided the first overview of DNA sequence marker use in bryophytes. Their Fig. 10.1 showed that during the years 1990−2001 14 markers were utilized, namely eight plastid regions, the

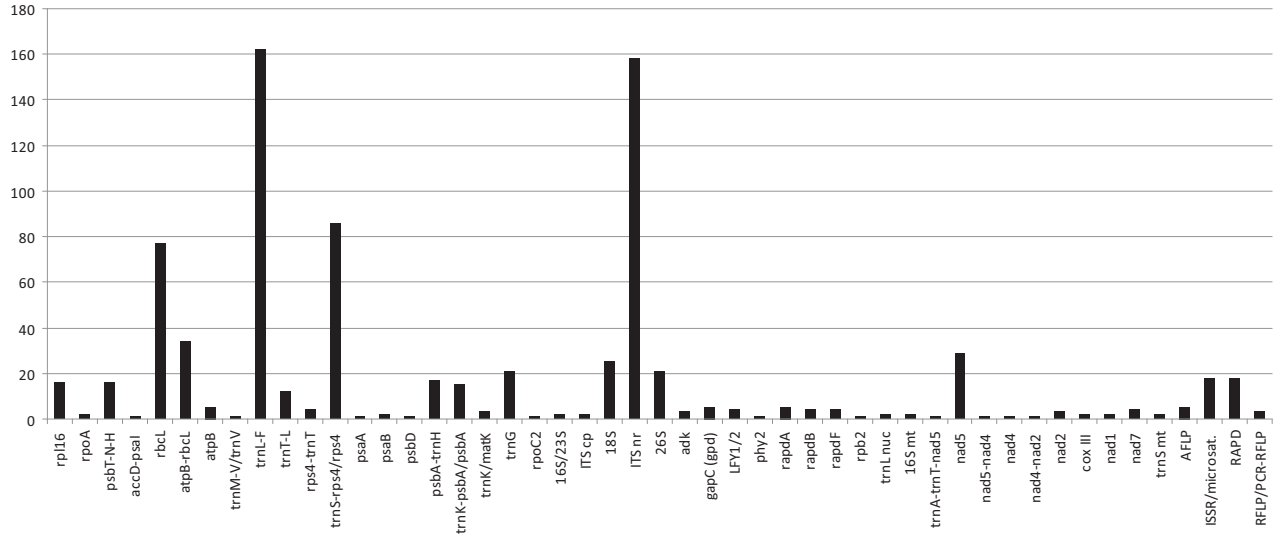

FIGURE 2. Frequency of the use of molecular markers in 382 publications on bryophyte molecular phylogenetics until the end of 2009. Column heights represent the number of publications in which sequences of the specific DNA regions (plastid: *rpl16*−ITS cp, nuclear: 18S−*trnL* nuc, mitochondrial: 16S mt−*trnS* mt) or fingerprinting data, respectively, were used. Gene names: *rpl16* = 50S ribosomal subunit (SU) protein; *rpoA* = RNA polymerase α SU; *psbT-N-H* = photosystem (PS) II proteins; *accD* = acetyl coenzyme A carboxylase β SU; *psaI* = PS I SU; *atpB* = ATP synthase β SU; *rbcL* = ribulose 1,5 bisphosphate carboxylase; *trnM* / *trnV* / *trnT* / *trnL* / *trnF* = tRNAs methionine / valine / threonine / leucine / phenylalanine; *rps4* = 30S ribosomal SU protein S4; *psaA* = PS I P700 apoprotein A1; *psaB* = PS I SU; *psbD* = PS II D2 protein; *psbA* = PS II D1 protein; *trnH* / *trnK* = tRNAs histidine / lysine; *matK* = maturase K; *trnG* = tRNA glycine; *rpoC2* = RNA polymerase β'/β" SU; 16S / 23S = rRNA genes; ITS cp = internal transcribed spacer (ITS) 2 / 3 / 4 of cpDNA; 18S / 26S = rRNA genes; ITS nr = ITS1 / 5.8S rRNA gene / ITS2 of nrDNA; *adk* = adenosine kinase; *gapC* (=*gpd*) = glyceraldehyd 3-phosphate dehydrogenase; LFY1/2 = introns in the *Leafy-Flo* gene (chromatin DNA binding protein, transcription factor); *phy2* = phytochrome 2; *rapdA* / *rapdB* / *rapdF* = anonymous nuclear loci; *rpb2* = RNA polymerase II SU; *trnL* nuc = nuclear *trnL* spacers; 16S mt = rRNA gene (mitochondrial); *trnA* / *trnT* / *trnS* = tRNAs (mitochondrial); *nad1* / *nad2* / *nad4* / *nad5* / *nad7* = NADH:ubiquinone oxidoreductase SUs; *coxIII* = cytochrome *c* oxidase SU III; AFLP = amplified fragment length polymorphism; ISSR/microsat = inter simple sequence repeats / microsatellites; RAPD = random amplified polymorphic DNA; (PCR-)RFLP = (polymerase chain reaction-)restriction fragment length polymorphism.

nuclear ribosomal DNA (18S-ITS1-5.8S-ITS2-26S region), and the mitochondrial *nad2*/*nad5* regions. Of these, only five were of quantitative importance, i.e., more than 85% of all DNA sequence analyses were based on the 'prime markers' *trnL-F*, *rbcL*, *rps4*, 18S, and ITS. When preparing the present review, we expected that in the years following 2001 the picture would have changed in such a way that more markers would have become popular in bryophyte phylogenetics, and that the percentage of studies employing multiple markers would have steadily increased. Together with an increasing average taxon sampling per study (which is not discussed here), such a trend would point towards more reliable and consistent results that overcome the limitations of using single and/or insufficiently variable markers.

In fact, Fig. 2 shows that the number of markers used in bryophyte phylogenetics has increased dramatically since 2001, with an increase of about 250% in the plastid, 470% in the nuclear, and 550% in the mitochondrial genome. These numbers are approximations because we treat a few markers separately here that in Quandt & Stech (2003) were comprised as one (e.g. the *trnT-trnL* spacer separately from the rest of the *trnT-F* region), to achieve a more precise picture in the discussion of DNA regions below (for full names of genes see legend of Fig. 2). Almost all nuclear markers, apart from the nuclear ribosomal DNA, and almost all mitochondrial markers were newly employed for bryophytes in the last eight years. However, the majority of all analyses still rely on the traditional markers. Four of the 'top five' markers mentioned above, namely *trnL-F*, *rbcL*, *rps4*, and ITS, are still the most frequently used regions, and rather few of the newly employed markers have become significantly widespread. Among the latter are the *atpB-rbcL* spacer, *trnG* intron, *trnK-psbA-trnH* region, and the mitochondrial *nad5* group I (G1) intron.

A clear trend from single-marker studies towards phylogenetic reconstructions based on two or more markers can be observed for the last few years only (Fig. 3). Until 2006, 40−100% of all publications per year

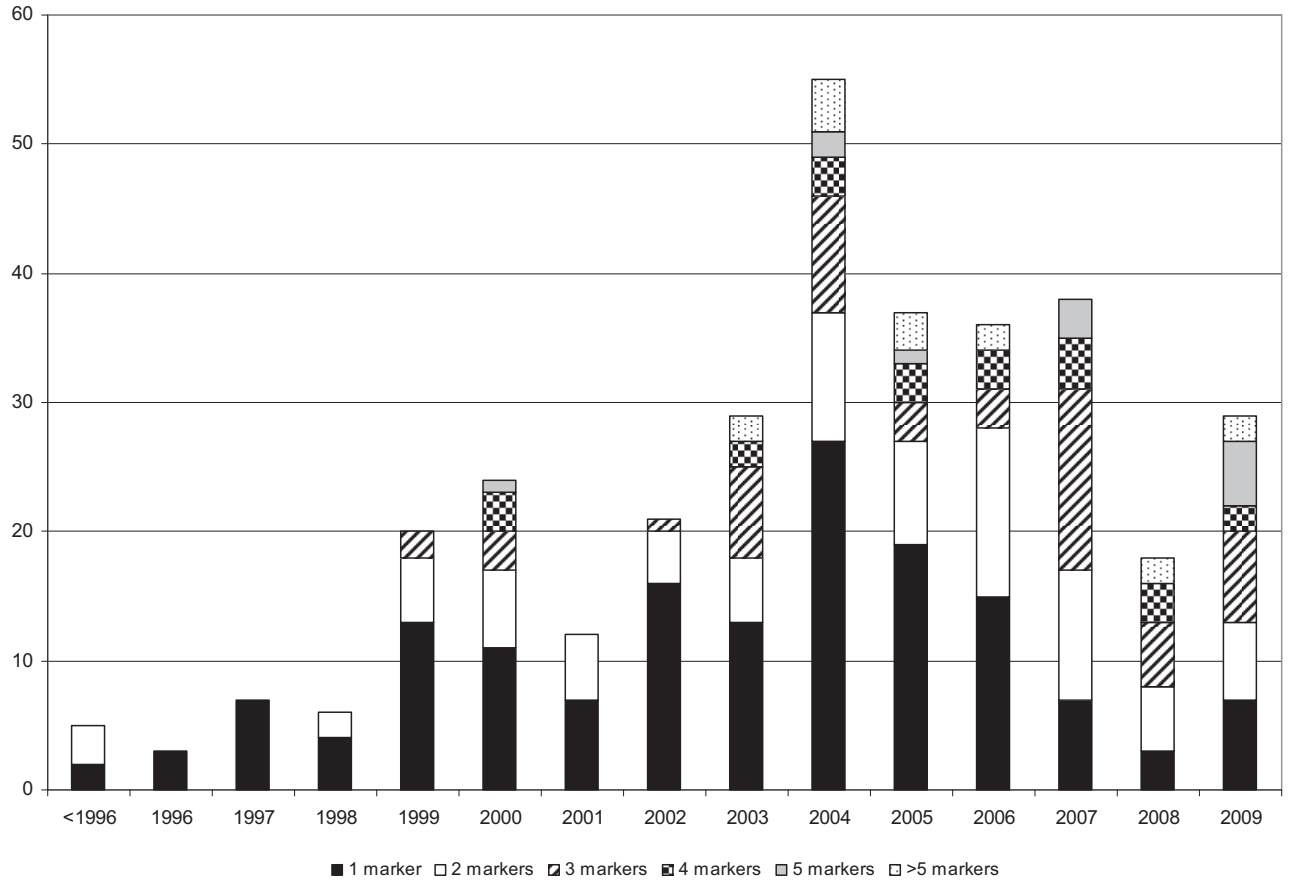

FIGURE 3. Development of the number of molecular markers used per phylogenetic study of bryophytes until the end of 2009.

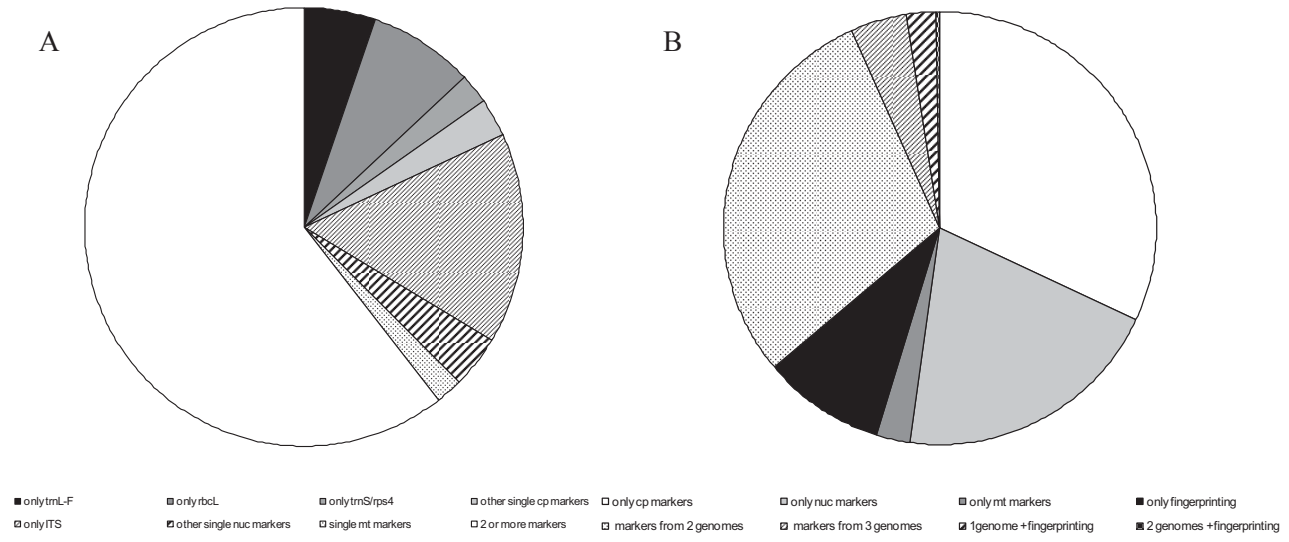

FIGURE 4. A. Proportions of phylogenetic studies of bryophytes based on one versus two or more molecular markers. Single-marker studies are discriminated into the most frequently used markers *trnL-F*, *rbcL*, *rps4* and nrITS, as well as studies based on other single markers from the three genomes. B. Proportions of phylogenetic studies of bryophytes based on one or more markers from single genomes versus combinations of markers from two or three genomes and/or DNA fingerprinting.

were based on a single marker, whereas in the last years this percentage dropped to 18−24%. One or two markers were used in 62−100% of all publications per year until 2007, and only in the last two years studies with more than two markers became the majority.

Although multiple-marker approaches employing more than five markers (in total 15 studies) remain the exception, it is a good sign that the majority of all publications involve two or more markers (Fig. 4A). While

in angiosperms 34% of all published phylogenetic hypotheses have been based exclusively on ITS sequences (at least until 2003; Álvarez & Wendel 2003), this percentage is much lower (16%) for bryophytes (Fig. 4A). Inferences in some groups, e.g. *Campylopus*, are of necessity based mainly on ITS only, as no plastid marker is available until now that provides a resolution comparable with the high level of divergence exhibited by ITS (cf. Stech *et al.* 2010a). On the other hand, comparisons of different markers within one study are mostly limited to the plastid genome in bryophytes, and usually concern *trnL-F* versus other plastid regions. The availability of 'standard' markers from all three genomes (plastid regions plus ITS plus *nad5*) is a promising foundation for this type of analysis, but studies that compare markers from different genomes are still the minority (Fig. 4B).

Finally, a rough count of Genbank entries (April 2010) for the most prominent markers further highlights the major importance of *trnL-F* and ITS both in liverworts and mosses (Fig. 5). The majority of papers sampled here deal with mosses rather than liverworts, a fact that is congruent with the larger amount of publically available *trnL-F* and ITS sequences for mosses. Although liverworts are still certainly underrepresented in both the publication record and DNA sequence databases, the number of published phylogenetic analyses for this group increased significantly in the last few years (Fig. 1; see also Stech 2009). For most other 'standard' markers, the bias between moss and liverwort sequence entries in Genbank is more or less equal or even stronger (Fig. 5), with the exception of *rbcL*. Thus phylogenetic inferences in liverworts seem to rely more strongly on a few markers, in contrast to similar studies in mosses. Furthermore, the small number of hornwort entries almost completely comprises sequences of *rbcL*, 18S, and *nad5* only.

Plastid genome markers

Locations of markers in the circular plastid genome that have been used in bryophyte phylogenetics are indicated in Fig. 6. At present, five complete plastid genomes of bryophytes have been sequenced: the liverworts *Marchantia polymorpha* Linnaeus (1753: 1137) (Ohyama *et al.* 1986) and *Aneura mirabilis* (Malmborg 1933: 122) Wickett & Goffinet (2008: 1-12) (Wickett *et al.* 2008), the hornwort *Anthoceros formosae* Stephani (1916: 1002) (Kugita *et al.* 2003), and the mosses *Physcomitrella patens* (Hedwig 1801: 20) Bruch & Schimper (1849: 13) (Sugiura *et al.* 2003) and *Tortula ruralis* (Hedwig 1801: 121) Gärtner, Meyer & Scherbius (1802: 91) (Oliver *et al.* 2010). The general structure of the plastid genome, namely its division into a large single-copy region (LSC) and a short single-copy region (SSC), separated by two inverted repeats (IR_A, IR_B), is the same in all five bryophytes and all land plants in general. Additionally the gene content and order is largely similar between all bryophyte plastid genomes, although all three bryophyte lineages share a symplesiomorphic approx. 31 kb inversion from *ycf2* to *psbM* compared to most tracheophyte plastid genomes (Wicke at al. submitted, Quandt & Stech 2003). However, the *Physcomitrella patens* genome shows an additional 71 kb inversion from *petD* to *rpoB* (that includes the former), which seems to be restricted to Funariaceae, Disceliaceae, and Encalyptaceae (Goffinet *et al.* 2007), and lacks the *rpoA* gene, which is characteristic for all peristomate-arthrodontous mosses (Bryopsida, except Buxbaumiidae) and the nematodontous Tetraphidopsida (Goffinet *et al.* 2005). The gene order in Fig. 6 follows the genomes without the lineage specific 71 kb inversion. Except for the plastid ribosomal genes and spacers located in the inverted repeats, all markers originate from the LSC. They comprise both protein-coding genes as well as non-coding regions, namely introns and intergenic spacers. Among the former, the $trnL_{UAA}$ intron is the only group I (G1) intron, whereas all other plastid introns, among them the other ones used in bryophyte phylogenetics (in *rpl16*, $trnV_{UAC}$, $trnK_{UUU}$, and $trnG_{UCC}$), are group II (G2) introns. Here, we provide an overview of the features and phylogenetic utility of the plastid markers shown in Fig. 6, with the more often used markers being characterized in more detail.

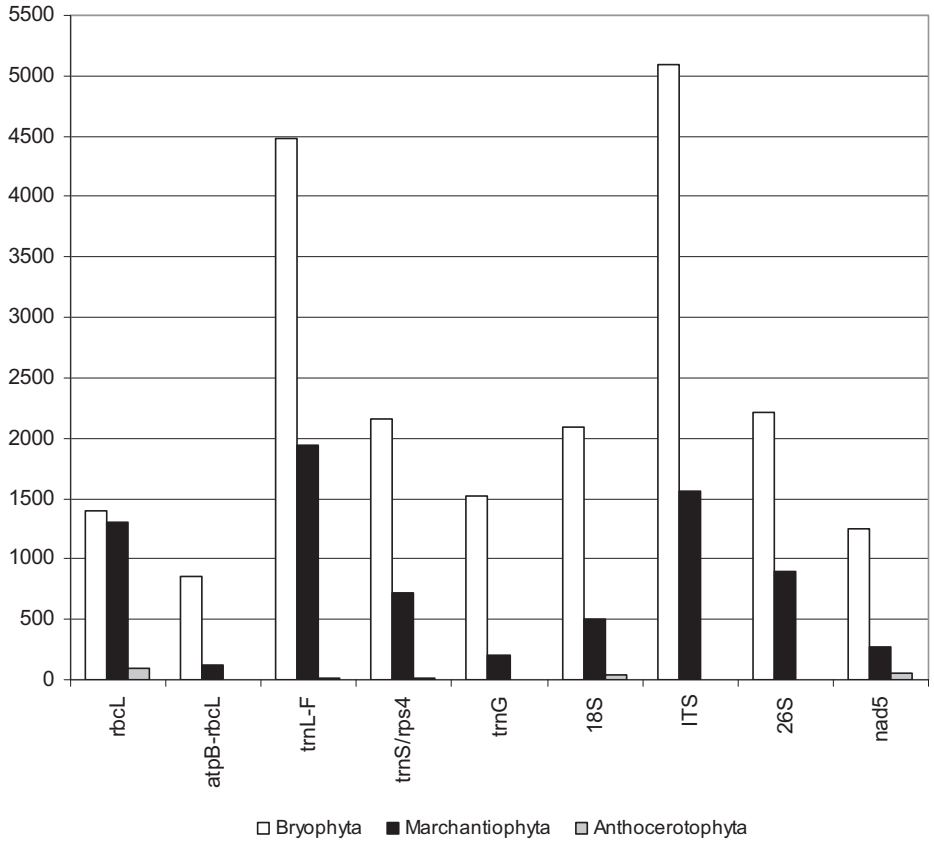

FIGURE 5. Genbank entries (April 2010) of liverwort and moss sequences for the most prominent DNA markers.

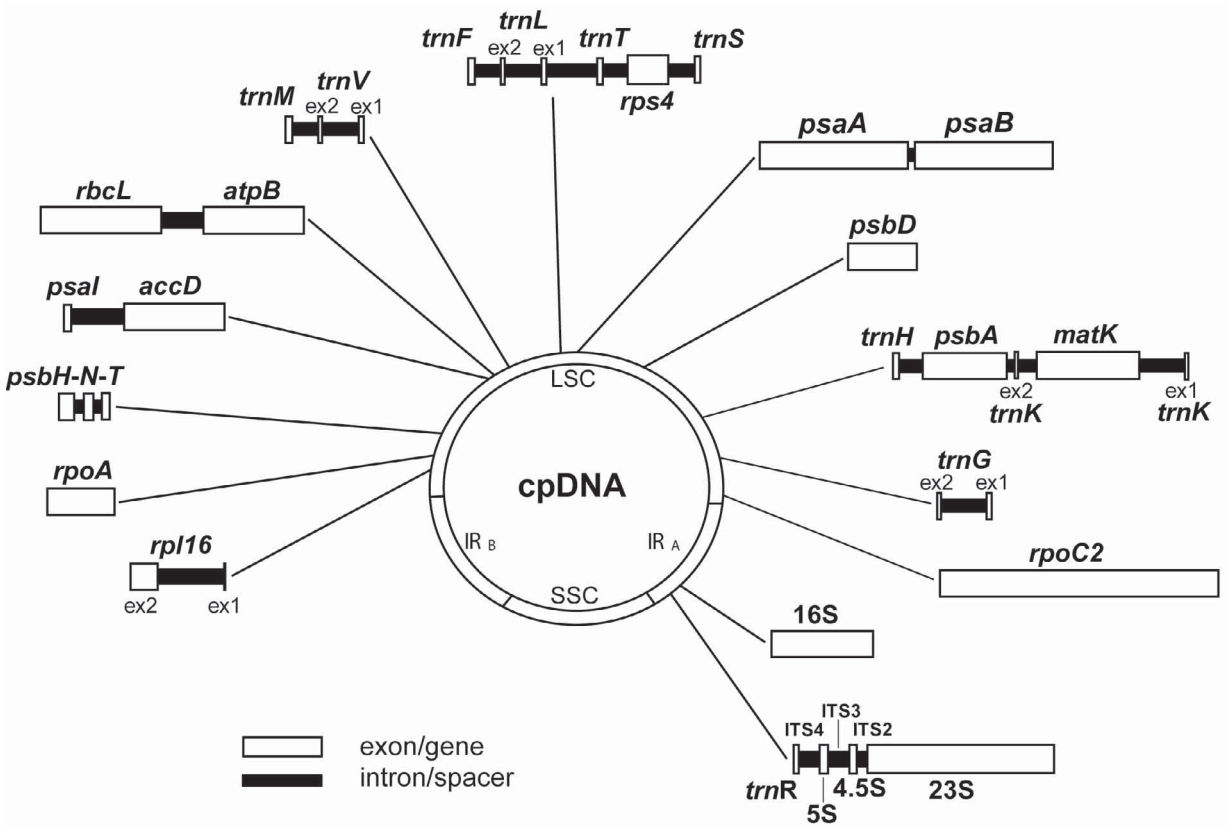

FIGURE 6. Overview of the cpDNA indicating the positions of coding and non-coding regions which have so far been used in bryophyte phylogenetics. Abbreviations: IR_A/IR_B = inverted repeats; SSC/LSC = small/large single copy region; for gene names see Fig. 2.

Main plastid marker regions

***trnS-F* region (*trnS*$_{GGA}$-*rps4*-*trnT*$_{UGU}$-*trnL*$_{UAA}$-*trnF*$_{GAA}$).** The *trnS-F* region comprises a tRNA gene cluster, including 4 tRNA genes (*trnS*$_{GGA}$, *trnT*$_{UGU}$, *trnL*$_{UAA}$, and *trnF*$_{GAA}$), a fast evolving protein-coding gene (*rps4*), four intergenic spacers (IGS) separating the coding regions, as well as the only plastid group I intron (G1), which resides in *trnL*. The region is situated in the LSC flanked by *ycf3* and *ndhJ*. In a historical as well as technical perspective, the region can be divided into three parts that include the two most frequently used plastid markers, the *trnL-F* and *trnS-rps4* regions (thereafter referred to as *trnL-F* and *rps4*, respectively), as well as the spacers separating *rps4*, *trnT*, and *trnL*. Since the introduction of universal primers situated in *trnT* (primer A), the *trnL* exons (B, C, D, E), and *trnF* (F) by Taberlet *et al*. (1991), *trnL-F* and, especially, the *trnL* intron became the most prominent marker for studying relationships among different land plant lineages (see Quandt & Stech 2003; Borsch & Quandt 2009). In contrast, the *trnT-L* spacer was employed only in a minority of cases in bryophytes (Fig. 2) and other land plant lineages (Borsch & Quandt 2009). Bryophyte sequences spanning the 'Taberlet region' from *trnT* to *trnF* were published more than ten years ago (Meißner *et al.* 1998; Frey *et al.* 1999), and *trnS-rps4* has also been continuously employed from 1999 onwards, starting with Cox & Hedderson (1999). The full length region from *trnS* to *trnF*, in contrast, has been applied only recently (e.g. Hernández-Maqueda *et al*. 2008a,b; Olsson *et al.* 2009a,b; Sotiaux *et al*. 2009). The following presents the different markers of the *trnS-F* region based on typical amplification strategies.

***rps4* (*trnS*$_{GGA}$-*rps4* spacer, *rps4* gene).** The original protocol to amplify the *rps4* gene positioned the amplification primers almost directly at both ends of the gene (Nadot *et al*. 1994). Whereas the forward primer (rps5') started with the start codon ATG of *rps4*, the reverse primer (rps3') was designed to align with a region 18 nt upstream of the stop codon (Nadot *et al.* 1994). Later, Souza-Chies *et al*. (1997) exchanged the reverse primer with a newly designed primer (trnS) situated in *trnS*, thus including the *trnS-rps4* spacer in the amplicon and yielding almost full length *rps4* sequences (i.e. minus the 5' primer sequence). This amplification and sequencing strategy was maintained by most researchers sequencing *rps4*, and to our knowledge works well for all bryophyte lineages. Perhaps due to the summer 2000 issue of The Bryologist, which comprised a number of molecular studies that were at least partially based on *trnS-rps4* sequences, *rps4* became one of the most frequently used markers in bryophyte phylogenetics, whereas it remained a minor player in angiosperms. In addition, *rps4* provides a higher relative amount of parsimony informative positions compared to, e.g. *rbcL*, and is therefore nowadays often favoured over *rbcL* (see O'Brien 2007; Quandt *et al*. 2007). Alignment of the gene (609 nt) is straightforward, as length mutations in the *rps4* CDS, apart from a 3 nt deletion characteristic for Funariaceae, have not been reported. The co-amplified, short (approx. 30−130 nt) *trnS-rps4* spacer requires a little more attention, as simple sequence repeats are rather frequent. Traditionally, *rps4* was mainly used to resolve ordinal level relationships among mosses (e.g. Cox & Hedderson 1999; Hyvönen *et al.* 1999; Buck *et al.* 2000; Goffinet *et al.* 2001; Magombo 2003; Bell *et al.* 2007) and later also among liverworts (e.g. Davis 2004; Crandall-Stotler *et al.* 2005; Forrest & Crandall-Stotler 2005; Forrest *et al*. 2006; He-Nygrén *et al.* 2006). The marker is also widely used to infer relationships within families and genera (e.g. Pedersen & Hedenäs 2003; Shaw *et al.* 2003; Goffinet *et al.* 2004; Groth *et al.* 2004; Werner *et al*. 2004; Forrest *et al.* 2005; De Roo *et al*. 2007; Harris 2008; Stech *et al.* 2010b). Few studies employed the region at the species level, either as single marker (Werner & Guerra 2004) or in a multi-locus approach (e.g. McDaniel & Shaw 2003; Grundmann *et al*. 2006). However, in our experience the region is less suitable at the species level and most certainly not as a stand-alone DNA barcoding marker for molecular species identification in bryophytes.

***trnL*$_{UAA}$ G1 intron.** The *trnL*$_{UAA}$ G1 intron (*trnL* intron) entered eukaryotes through cyanobacterial endosymbiosis and represents one of the most ancient plastid introns (Kuhsel *et al.* 1990; Paquin *et al.* 1997). It still resides at the same position in chloroplasts and cyanobacteria, interrupting the anticodon loop of the

tRNA^{Leu}. The intron is easily amplified in streptophytes using the original Taberlet *et al.* (1991) primers C and D that are situated in the conserved *trnL* 5' and 3' exons. In most bryological studies, however, the *trnL* intron is co-amplified with the rather short *trnL-F* spacer (see below), an approach mostly followed in seed plants as well (see Borsch & Quandt 2009). As with all other G1 introns, the *trnL* intron can be characterized by a mosaic structure of highly conserved elements called P, Q, R, and S, which are essential for correct splicing (Davies *et al.* 1982; Cech 1990) and alternate with less constrained stem-loop regions P1−P9 of variable size (see Borsch *et al.* 2003; Stech *et al.* 2003a, Quandt *et al.* 2004; Quandt & Stech 2005; Borsch & Quandt 2009). Length variability is mostly confined to P6 and P8, which display a high degree of microstructural changes including inversions or deletions (e.g. Stech *et al.* 2003a; Quandt *et al.* 2004; Quandt & Stech 2005; Hernández-Maqueda *et al.* 2008b). As the *trnL* intron represents one of the first widely used phylogenetic markers and can be sequenced easily, extensive sequence data is available for all three bryophyte lineages (see Stech *et al*. 2003a; Quandt & Stech 2005). Considerable differences exist concerning the variability of the intron in the three bryophyte lineages. Whereas the intron is rather short in mosses, i.e., generally below 350 nt (except *Sphagnum*) and only about 265 nt in the pleurocarpous orders Hypnales and Hookeriales (Quandt & Stech 2005), it can exceed 800 nt in hornworts (Stech *et al*. 2003a). In correlation with the observed length differences, comparably low sequence variability is reported, resulting in unresolved or poorly supported trees at the ordinal and familial levels (e.g. Buck *et al.* 2000). Nevertheless, the *trnL* intron has been used at all taxonomic levels and is considered as a prime candidate for DNA barcoding of plants in general (e.g. Taberlet *et al.* 2007), including bryophytes (Liu *et al.* 2010). Advantages of this region include: easy amplification with universal primers, a vast amount of knowledge regarding its molecular evolution, and the abundance of publically available sequences for comparison. Taberlet *et al.* (2007) tested not only the whole intron for barcoding purposes, but also stem-loop region P6 only (amplified with new primers G and H situated in the conserved elements Q and R), which resulted in species identification percentages of 67−85 or 19−77 %, respectively, for different datasets. For bryophytes, however, such a short barcode is certainly not enough for species identification in all lineages.

The spacers *rps4-trnT*$_{UGU}$, *trnT*$_{UGU}$*-trnL*$_{UAA}$, and *trnL*$_{UAA}$*-trnF*$_{GAA}$. The second short spacer of the *trnS-F* region, the *trnL*$_{UAA}$*-trnF*$_{GAA}$ spacer (*trnL-F* spacer), is usually co-amplified with the *trnL* intron. Alternatively, the original primer E (Taberlet *et al.* 1991) can be used in combination with primer F to amplify the *trnL-F* spacer alone. Reported sequence lengths of the *trnL-F* spacer differ among the three bryophyte lineages. The longest sequences are reported from hornworts (393 nt, mean ~ 366) whereas the average sequence lengths of liverworts (~ 70 nt) and mosses (~ 82) are much smaller (Stech *et al*. 2003a; Quandt & Stech 2004). In contrast, the *trnT*$_{UGU}$*-trnL*$_{UAA}$ spacer (*trnT-L* spacer) yields much longer sequences in all bryophyte lineages (Quandt & Stech 2004) and is traditionally amplified using primers A and B (Taberlet *et al*. 1991). In order to obtain overlapping sequencing products across the *trnL* 5' exon, internal primers were developed that anneal to the highly conserved R element in the *trnL* intron, namely trnL_P6/7 (Quandt *et al.* 2004), D$_x$ (Schaumann *et al.* 2004), and D$_i$ (Stech 2004). The combination of primers trnL_P6/7 (reverse) and rps4_166F (Hernandez-Maqueda *et al*. 2008b) in *rps4* substitutes the traditional Taberlet (1991) amplification strategy by producing an amplicon that includes the spacers *trnT-L* and *rps4-trnT*$_{UGU}$ (*rps4-trnT* spacer) and thus closes the gap between *trnT* and *rps4* (e.g. Hernandez-Maqueda *et al*. 2008a,b; Olsson *et al*. 2009a,b,c, 2010).

One factor responsible for a mosaic pattern of (hyper-)variable and more conserved parts within intergenic spacers is the presence of regulatory elements, namely promoter elements. In the *trnS-F* region, three bacterial type promoters are present. Two promotors are located in the *trnT-L* spacer, one for the transcription of *trnL-F* and the other for *trnT-rps4*, while the third promoter is located in the *trnL-F* spacer upstream of *trnF*. As reported by Quandt & Stech (2004) the *trnL-F* spacer harbours a hairpin-associated inversion that flips at the population level which may severely affect phylogenetic reconstructions if undetected (see Quandt *et al.* 2003 and Borsch & Quandt 2009 for a more detailed discussion).

rbcL. As in most other land plant lineages, *rbcL* is consistently present in the LSC of bryophyte plastid genomes next to *atpB* and *accD* on the other side, the latter being frequently lost in various angiosperm lineages (e.g. Lee *et al.* 2007, Jansen *et al*. 2007). In addition, a $trnR_{CCG}$ gene has been reported between *rbcL* and *accD* for charophytes and bryophytes (Ohyama *et al.* 1986; Turmel *et al.* 2002; Kugita *et al.* 2003; Sugiura *et al*. 2003), lycophytes (Wolf *et al*. 2005), ferns (Wolf *et al.* 2003; Roper *et al*. 2007), and gymnosperms (including Gnetales; Wakasugi *et al.* 1994; Wu *et al.* 2007; McCoy *et al.* 2008). Being one of the first target genes for phylogenetic studies, an impressive number of primers have been published that anneal to similar sites. One of the first studies using *rbcL* sequences from a variety of green algae and embryophytes was Manhart (1994), who proposed four primers, the first situated in the *atpB* gene 175 nt downstream of the start codon (atpB-175R), the second spanning the first 26 nt of *rbcL* (rbcL-RH1), the third being placed at the 3' end of *rbcL* (rbcL-1385R) and finally trnR situated in $trnR_{CCG}$. The Manhart (1994) approach thus yielded the *atpB-rbcL* and *rbcL-trnR* spacer sequences as well, which were largely overlooked in the following years, with the exception of Tsubota *et al.* (2001) who, in addition, designed several internal amplification and sequencing primers. Maeda *et al.* (2000) utilized the rbcL-RH1 primer and combined it with a newly designed reverse primer, R1351 matching the *Nicotiana* sequence, thus including several polymorphic sites compared to *Marchantia* or *Physcomitrella* that perhaps necessitated the described nested PCR approaches. A different approach targeting only at the *rbcL* ORF with primers situated near the 5' and 3' end was proposed by Lewis *et al.* (1997) when analysing phylogenetic relationships among liverworts. The Lewis *et al.* (1997) primers M34 and M1390, or modifications thereof, are perhaps the most frequently used primers to obtain almost full length *rbcL* products of 1428 nt. Caution should be taken though, as the reverse primers are only listed as complement sequences. Modifications of primer M34 are rather frequent in the literature and concern lineage-specific base modifications or a size increase, such as NM34 published by Cox *et al.* (2000). A third approach (Goffinet *et al.* 1998) reduced the amplicon to 679 nt by placing the primers well inside the ORF at positions 427 (forward) and 1081 (reverse). However, as primers and sequences for new loci are often shared and communicated personally, these modifications mainly appear as personal communications in the publications (e.g. Wolf *et al.* 1994; Goffinet *et al.* 1998; Cox *et al.* 2000).

The plastid *rbcL* gene is certainly the most sequenced locus among land plants and has also been extensively sequenced for bryophytes. However, in bryophyte molecular systematics *rbcL* seems to be less popular than in ferns or angiosperms. One reason is the rather low sequence variation at family level and below that soon became evident in early studies and indicated that multi-gene analyses are required to corroborate the findings (e.g. Goffinet *et al.* 1998; De Luna *et al.* 2000; Maeda *et al.* 2000; Tsubota *et al.* 2001). Another culprit is the introduction of the above mentioned regions *rps4* and *trnL-F* that became available to bryophyte phylogenetics roughly at the same time and gained popularity. Due to its conserved nature, *rbcL* has only been employed at the family level and above in recent publications (e.g. Shaw *et al.* 2003; Forrest *et al.* 2006; Bell *et al.* 2007; Heinrichs *et al.* 2005; 2007; Wahrmund *et al.* 2010) and is not suitable for species and population level analysis or barcoding approaches in bryophytes. This contrasts with Newmaster *et al.* (2006) and Liu *et al.* (2010), who considered *rbcL* as the marker with the best performance as DNA barcode in bryophytes. However, as Liu *et al.* (2010) themselves state, their taxon sampling, comprising divergent species across all mosses but only one or very few species per genus, may lead to overestimation of the discrimination power of *rbcL* and other markers tested.

***atpB-rbcL* spacer.** The *atpB-rbcL* spacer is one of the three most frequently utilized plastid spacers (Fig. 2). In contrast to the *trnL-F* and *trnS-rps4* spacers, which are usually amplified together with the *trnL* intron or *rps4*, respectively, the *atpB-rbcL* spacer has often been sequenced independently from its adjacent genes in bryophytes, in particular from the likewise commonly used *rbcL* (but see comments on *rbcL* and *atpB*), using primers atpB-1 and rbcL-1 by Chiang *et al.* (1998). As the reverse primer (rbcL-1) residing in *rbcL* basically represents the M34 primer by Lewis *et al.* (1997) (see above), just in reverse order, it is unfortunately not possible to join the spacer sequence with the *rbcL* ORF without a gap when the standard Lewis *et al.* (1997) approach for sequencing of *rbcL* is applied.

Compared to reported sequence lengths from seed plants (approx. 800−1,000 nt), the *atpB-rbcL* spacer is considerably shorter in bryophytes, ranging only between approx. 370−700 nt (Chiang *et al.* 1998; Stech & Quandt 2006; Stech & Frey 2008). However, the *atpB-rbcL* spacer is longer and provides more information in terms of informative sites than the other commonly used plastid spacers *trnL-F*, *psbA-trnH* and *rps4-trnS*. The molecular evolution and phylogenetic utility of the *atpB-rbcL* spacer in bryophytes has recently been reviewed by Stech & Quandt (2006). Due to its length and variability, it has been applied at different taxonomic levels, from inferring relationships across the Bryopsida (e.g. Stech & Frey 2008) to phylogeographic studies at the intraspecific level (e.g. McDaniel & Shaw 2003). Most studies, however, deal with relationships at the familiy and genus level in both liverworts and mosses (e.g. Stech 2004; Pedersen *et al.* 2007; Aigoin *et al.* 2009; Devos & Vanderpoorten 2009). Like the other spacers mentioned above, the *atpB-rbcL* spacer has only been used in combination with other markers. Similar to the stem-loop region P8 of the *trnL* intron, the middle part of the *atpB-rbcL* spacer shows high length variability with the frequent occurrence of indels, usually smaller repeats of 2−8 nt (cf. Fig. 4 in Stech & Quandt 2006). A wide variety of putative hairpin secondary structures have been observed, with different stem lengths and loop sizes of 4−31 nt (e.g. Stech 2004; Stech & Quandt 2006). Intraspecific variation seems to be a common phenomenon in the *atpB-rbcL* spacer in bryophytes. This does not necessarily exclude its suitability for molecular species identification, but as the *atpB-rbcL* spacer is also not always reliably amplified and internal primers are difficult to design due to the high sequence variability, it is not a prime candidate for DNA barcoding of bryophytes.

***trnG*$_{UCC}$ G2 intron.** The *trnG*$_{UCC}$ gene is situated in the LSC relatively close to the large *rpoC1-rpoC2* gene cluster, between *trnR*$_{UCU}$ and – in bryophytes and non-angiosperm vascular plants – *ycf12* (*trnS*$_{GCU}$ in angiosperms, cf. Borsch & Quandt 2009). It harbours a G2 intron that is the target for phylogenetic analysis in bryophytes. For tracheophytes primer sets were developed to co-amplify the adjacent spacers together with the *trnG* intron (*trnS-trnG* region, cf. Borsch & Quandt 2009; *trnG-trnR* spacer plus *trnG* intron, Hennequin *et al.* 2010). The primers used in bryophytes, in contrast, are both situated in the *trnG* exons and thus only amplify the *trnG* intron itself. The original primers trnGF and trnGR (Pacak & Szweykowska-Kulińska 2000) were designed for liverworts and used at different taxonomic levels in this group, from organellar inheritance within species (e.g. Pacak & Szweykowska-Kulińska 2003), phylogeography of species (Fuselier *et al.* 2009) to phylogenetic reconstructions at the family level (De Roo *et al.* 2006). The same primers were used in different groups of mosses, especially *Sphagnum* (e.g. Shaw *et al.* 2003, 2010), from family and genus level (e.g. Pedersen & Hedenäs 2003; Shaw *et al.* 2008a) to species and intraspecific levels (e.g. Hedenäs 2008, 2009) in Bryopsida. Only recently, Werner *et al.* (2009) presented an alternative forward primer, also named trnGF, for *Fissidens*, the first haplolepideous moss analyzed with *trnG.* As discussed in Borsch & Quandt (2009), no comparative studies and no exact secondary structures for the *trnG* G2 intron are available at present, and sequence lengths (ca. 500−800 nt in bryophytes) are not indicated in most bryophyte studies. Detailed comparison and estimation of lengths, sequence variation, molecular evolution and lineage-specific mutational hotspots should be done based on the available Genbank entries; however, this is beyond the scope of this review. Nevertheless, the *trnG* intron shows a relatively high level of variability in bryophytes (e.g. Werner *et al.* 2009), in some cases seemingly higher compared to the *trnL-F* region (Pacak & Szweykowska-Kulińska 2000; Pedersen & Hedenäs 2003), and seems to be much easier amplified using universal primers than the most popular (at least in angiosperms) G2 intron residing in *trnK* (see below). Its increasing utilization at different taxonomic levels indicates that the *trnG* intron has the potential to become an even more popular marker in bryophyte phylogenetics. Similar to the *rpl16* G2 intron that is discussed in the following section, the *trnG* intron may even provide useful information at the species level within the derived pleurocarpous order Hypnales (e.g. Hedenäs 2009), which is known for its notorious paucity of molecular variability.

***rpl16* G2 intron.** The *rpl16* gene is usually flanked by *rpl14* and *rps3* in the LSC near the IR border of streptophyte plastid genomes. Amplification and sequencing strategies for the *rpl16* G2 intron have recently been reviewed by Borsch & Quandt (2009) for land plants. Accordingly we concentrate here on specific aspects for bryophytes. Most bryological studies using *rpl16* generally follow the original set up by Jordan *et al.* (1996). The primers employed are F71 (forward), situated in the rather short *rpl16* 5'exon (9 nt) and reaching into the adjacent *rps3-rpl16* spacer, combined with R1661 (reverse) situated well inside the *rpl16* 3'exon, approx. 100 nt from the intron/exon junction. However, due to amplification and sequencing problems Olsson *et al.* (2009b) proposed to substitute R1661 with a new primer rpl16R to yield better amplification and sequencing results. Future studies might want to position the forward primer inside *rps3* instead of using F71, as the latter does not provide the full G2 intron length (see Tesfaye *et al.* 2007) and thus limits molecular evolutionary studies. As reviewed by Borsch & Quandt (2009), the highly variable *rpl16* intron represents one of the largest plastid introns that offers a comparatively high phylogenetic structure at least in angiosperms (Löhne *et al.* 2007; Sánchez del-Pino *et al.* 2009). However, Kelchner (2002) reported mutational heterogeneity, especially with respect to microstructural mutations. This has been further exemplified by Sotiaux *et al.* (2009) who showed that the presence or absence of a 20 nt simple sequence repeat (SSR) can be of independent origin, even at species level. This example, however, also illustrates the potential of the region and its current application. Although most studies used the *rpl16* G2 intron at the genus or higher taxonomic levels (e.g. Shaw 2000; Pedersen & Hedenäs 2003; Pedersen *et al.* 2003; Harris 2008; Huttunen *et al.* 2008; Olsson *et al.* 2009a,b, 2010), it is increasingly becoming a marker for systematic and phylogeographic analyses of single or closely related species, even among the Hypnales (e.g. Hedenäs & Eldenäs 2007; Hedenäs 2008, 2009; Olsson *et al.* 2009c; Sotiaux *et al.* 2009). Overall comparisons for bryophytes are not yet possible as the *rpl16* intron has been sparingly used, by researchers that are either concentrating on peat mosses, Bryaceae, or pleurocarps.

***psbT-H* region (*psbT-psbN-psbH*).** The *psbT-psbN-psbH* region is part of the *psbB* operon in the LSC, which contains the genes *psbB*, *psbT*, *psbH*, *petB* and *petD*. Although *psbN* is located between *psbT* and *psbH*, it does not belong to the operon as it is encoded on the complementary strand. One of the first phylogenies that included sequence data from the *psbT-H* region was published by Graham & Olmstead (2000), who compared the utility of 17 different molecular markers using basal angiosperms as a model group. At the same time, this region was introduced to bryophyte molecular phylogenetics by Shaw (2000) in an analysis of peat mosses. In contrast, the medium-sized group II intron residing in the upstream part of *petD*, which has been promoted and successfully used for phylogenetic reconstructions among angiosperms (e.g. Löhne & Borsch 2005; Worberg *et al.* 2007), has not yet been employed for bryophytes. Whereas Graham & Olmstead (2000) used the primer combination 69F/71R, Shaw (2000) employed the protocol that was later published by Krellwitz *et al.* (2001) in the frame of a phylogeographic study on the chlorophyte genus *Bryopsis*. Krellwitz *et al.* (2001) substituted the forward primer 69F with the original Hong *et al.* (1995) primer psbT and combined it with a newly designed reverse primer, psbH. This primer combination (psbT/psbH) was used in a series of bryological studies (e.g. He-Nygrén & Piippo 2003; Davis 2004; Fuselier *et al.* 2009). An alternative primer combination (psbT/71R), with both primers being slightly modified for bryophytes, was used by Quandt *et al.* (2003) and subsequently employed in several other studies, mainly among diplolepideous-alternate mosses (e.g. Stech *et al.* 2003b, Huttunen & Ignatov 2004; Quandt & Huttunen 2004). These primers yield an amplicon of approx. 500 nt. For equivalent taxa sets, the reported sequence variation as well as the percentage of informative sites of the *psbT-H* region ranges generally below the *trnL-F* region (see Stech *et al.* 2003b; Quandt *et al.* 2009). Similar to the *trnL-F* spacer, a hairpin associated inversion has been reported from the *psbT-N* spacer that occurs randomly in different unrelated families, orders and classes, and even switches between closely related species (Quandt *et al.* 2003). If undetected during the alignment process, this minute inversion significantly reduces the robustness of the phylogenetic hypothesis inferred from the data (Quandt *et al.* 2003). This is also true for any other minute inversion (compare Hernandez-Maqueda *et al.* 2008b; Lehtonen *et al.* 2009) and therefore a careful inspection of the alignment with regards to inversions should be

performed prior the phylogenetic analyses. For a more detailed summary of microstructural mutations and their treatment in phylogenetics we refer to Borsch & Quandt (2009).

***trnK/matK* region.** In the five sequenced bryophyte plastid genomes this region is consistently present in the LSC, flanked by *psbA* and *rps16*. In contrast to tracheophytes, however, *trn*K/*mat*K is not found near the inverted repeat boundary, due to a ~31 kb inversion in bryophyte plastid genomes (cf. Quandt & Stech 2003; Wicke *et al.* submitted). The region is composed of a G2 intron that hosts the only plastid maturase gene (*matK*) and is easily accessible with standard PCR primers annealing to the conserved *trnK* exons. The reverse primer (e.g. psbA-R; K. Steele in Johnson & Soltis 1995) can be situated in the *psbA* gene and thus the *trnK-psbA* spacer is included in the amplicon. Whereas this marker is already well established for flowering plants, mainly using the primers designed by Johnson & Soltis (1995) and Hilu & Liang (1997), the region been has been employed only rarely in bryological studies (see Borsch & Quandt 2009; Wicke & Quandt 2009). Long *et al*. (2000) optimized the Johnson & Soltis (1995) primers based on the *Marchantia polymorpha* plastid genome and provided the first phylogenetic study among bryophytes using *trnK/matK* in an attempt to resolve the relationships of *Asterella*. However, as reviewed by Wicke & Quandt (2009), amplification success with the above mentioned primers is rather limited among early diverging land plants, which forced Hausner *et al.* (2006) as well as Wicke & Quandt (2009) to develop new primer sets and protocols that turned out to be universal for land plants with high amplification efficiency. An amplification approach that places the primers in the *trnK* exons, or even in *psbA,* requires several generally lineage-specific internal amplification and sequencing primers. To design them, a primer walking strategy based on a few full length amplicons, which are best obtained from fresh material, is needed. Internal amplification primers can then be designed that allow amplification of the region in two overlapping halves, which is particularly important for difficult templates (e.g. DNA isolates from herbarium specimens).

Both published studies using *trnK/matK* in bryophytes indicate that the region is quite promising for bryophyte phylogenetics, especially at the genus level and above. Although sequence divergence in *trnL-F* is roughly 1.3−1.5 times higher compared to *matK*, the relative amount of parsimony-informative sites is higher in *matK* compared to *trnL-F* (Long *et al.* 2000; Hernandez-Maqueda *et al*. 2008a). Unfortunately, the values provided by Long *et al.* (2000) are only based on a fraction of the *matK* CDS that was used in the phylogenetic analyses, despite the whole region being amplified. However, the same is true for the full length *matK* as well as *trnK* G2 intron as shown in a phylogenetic study of Grimmiaceae (Hernandez-Maqueda *et al*. 2008a). So far, data concerning species or population level variability is rather limited, but the few available sequences question the use of the region for biogeographic analyses or barcoding in bryophytes (cf. also Liu *et al.* 2010), whereas a portion of *matK* was advocated as barcode marker for angiosperms (Lahaye *et al.* 2008).

trnK-psbA-trnH. The *psbA* gene is situated downstream of *trnK*/*matK,* followed by the $trnH_{GUG}$ gene. The spacer between *psbA* and *trnH* has been the subject of much attention, because it is one of the most (length-) variable plastid spacers in angiosperms and shows complex molecular evolutionary patterns (cf. Borsch & Quandt 2009). It has been used frequently in several groups of angiosperms, with different success concerning its phylogenetic utility (cf. Borsch & Quandt 2009), and was also proposed as a universal barcode for land plants by Kress *et al.* (2005) (see discussion below). Sang *et al.* (1997) first designed primers situated in the *psbA* and *trnH* genes; later Hamilton (1999) published universal primers (H and PSBA) that have been successfully used across angiosperms. In bryophytes, there are studies employing either only the *psbA-trnH* spacer, or parts of the *psbA* gene and/or the *psbA-trnK* spacer, which is not always easy to infer from the names of the markers given in respective publications. Shaw *et al.* (2003) were the first to include '*psbA*' in their multigene study of *Sphagnum*. Their newly designed primers in fact covered the *trnK-psbA* spacer plus part of *psbA* ('*psbA1*' with primers trnK2F and psbA576R), and the *psbA-trnH* spacer plus the other part of *psbA* ('*psbA2*' with primers psbA501F and trnHR). Thereafter, the region was frequently used in liverwort phylogenetics (Davis 2004; Forrest & Crandall-Stotler 2004; Crandall-Stotler *et al.* 2005; Forrest *et al.* 2006; Heinrichs *et al.* 2007), at the family and genus level (e.g. Forrest *et al.* 2005; Wilson *et al*. 2007; Hentschel *et*

al. 2009). In mosses, two studies investigated the utility of the *psbA* region (Cox *et al.* 2004), or the *psbA-trnH* spacer only (Stech & Frey 2008), at suprafamilial level, whereas few other studies used the region for inferring relationships within families and genera (e.g. Hedderson *et al.* 2007; Vanderpoorten *et al.* 2008; Aigoin *et al.* 2009; Stech 2009). Again, some of these studies employed the *psbA-trnH* spacer together with parts of the *psbA* gene, using the Shaw *et al.* (2003) primers (e.g. Vanderpoorten *et al.* 2008: '*psbA2* gene'), while others were based on the spacer only, which was then sequenced with the Sang *et al.* (1997) primers or a modified version of the reverse primer (trnH-R2, Stech & Frey 2008; Stech 2009).

As observed for other plastid spacers, the *psbA-trnH* spacer is much shorter in bryophytes than in angiosperms. With approx. 70−200 nt it is similar in length to the *trnS-rps4* and *trnL-F* spacers in bryophytes, but shorter than the *atpB-rbcL* spacer. In addition to its short length, relatively large parts of the *psbA-trnH* spacer are attributed to putative hairpin loops or otherwise hypervariable regions (Stech & Frey 2008), which further hampers its use at higher taxonomic levels. Although the *psbA-trnH* spacer can contribute to solving relationships at the species level, it can surely not serve as a stand-alone barcoding marker for species identification in bryophytes, especially not in the derived pleurocarps, in contrast to its potential as barcoding marker in angiosperms (e.g. Kress & Erickson 2007; Layahe *et al.* 2008). However, its short length and easy amplification can be advantageous when degraded DNA forms the template, e.g. in older herbarium specimens. In these cases, *psbA-trnH* could be used, in combination with other 'prime' barcoding markers, to infer the identity of difficult material (cf. also Liu *et al.* 2010).

Other plastid markers

rpoA. The *rpoA* gene was used only in two publications as a phylogenomic marker to analyse differences in plastid genome organization in mosses, without actual phylogenetic analyses of *rpoA* sequences. In contrast to the previously sequenced plastid genomes of *Marchantia polymorpha*, *Aneura mirabilis* and *Anthoceros formosae*, the plastid genome of *Physcomitrella patens* (Sugiura *et al.* 2003) lacks the *rpoA* gene. A subsequent study (Sugita *et al.* 2004) confirmed the absence for four other 'true' mosses (compare *Tortula ruralis*, Oliver *et al.* 2010), whereas in the basalmost lineages (Sphagnophytina, Takakiophytina) it was present, situated between *rps11* and *petD* in the LSC. Goffinet *et al.* (2005) again expanded the survey of presence/absence of *rpoA* in mosses, and concluded that the absence of *rpoA* is characteristic for all peristomate-arthrodontous mosses (Bryopsida), except Buxbaumiidae, as well as the nematodontous Tetraphidopsida.

accD-psaI. The *accD-psaI* spacer is situated in the LSC close to *rbcL* (*accD* and *rbcL* are separated by the $trnR_{CCG}$ gene; cf. discussion of *rbcL* above). It was introduced in bryophyte systematics by Korpelainen *et al.* (2004) to study intraspecific variation in the liverwort *Trichocolea tomentella* (Ehrh.) Dumortier (1861: 67), and so far only used in that study. The universal primers accD-769f and psa-75r of Small *et al.* (1998) were used for amplification and sequencing. The spacer had a length of 156 nt, with no intraspecific variation in contrast to the findings by Small *et al.* (1998) for angiosperms.

atpB. In contrast to *rbcL* and the *atpB-rbcL* spacer (see above), the *atpB* gene has rarely been used in bryophytes, mainly in phylogenetic reconstructions of land plants (e.g. Qiu *et al.* 2006), liverworts (e.g. Davis 2004; Forrest & Crandall-Stotler 2004, 2005; Crandall-Stotler *et al.* 2005), and pleurocarpous mosses (O'Brien *et al.* 2007). Sequencing was performed with a number of different primers, even between comparable studies of liverworts. For example, Davis (2004) used five primers (atpB672F, atpE384R, atpB493F, atpB1163F, atpB910R) originally designed for ferns (Pryer *et al.* 2001), while Forrest & Crandall-Stotler (2004) used the same primers except atpB493F, with the addition of two newly designed primers (LVWT 3F and LVWT 3R). These studies obtained the entire *atpB* gene of 1124 nt (Forrest & Crandall-Stotler 2004), whereas O'Brien *et al.* (2007) only sequenced 400 nt of *atpB* plus the adjacent *atpB-rbcL* spacer, using

primers matpB601F and M007R designed by J. Wheeler. This study reported that the *atpB* region was more variable than *rbcL*, *rps4* and *trnL*, but this is obviously due to the fact that the spacer was included, whereas in liverworts *rps4* and *trnL* were clearly more variable than *atpB* (Forrest & Crandall-Stotler 2004).

trnM-V. The *trnM-V* region was recently introduced to bryophyte phylogenetics by Werner *et al.* (2009), who analyzed relationships within a group of closely related *Fissidens* species. It comprises the $trnM_{CAU}$-$trnV_{UAC}$ spacer as well as the $trnV_{UAC}$ G2 intron and is situated between *ndhC* and *atpE* in the LSC, close to *rbcL*/*atpB* (Fig. 6). Primers (trnMF/trnVR) were newly designed for the study of Werner *et al.* (2009). Sequence variability of the *trnM-V* region was reported as low in *Fissidens*, but comparable to that of *trnG* intron or *atpB-rbcL* spacer. Although too few data are available to assess its phylogenetic utility, the *trnV-M* region may serve as an additional useful marker in addition to the *rpl16* and *trnG* introns.

psaA*, *psaB*, *psbD. These three genes were used together in an early study of phylogenetic relationships of land plants (Nishiyama & Kato 1999). Thereafter, only the phylogenetic utility of *psaB* has been further tested, namely as an alternative to *rbcL* for elucidating relationships of pleurocarpous mosses (Arikawa & Higuchi 2003). The latter study employed the same primers as Nishiyama & Kato (1999) for sequencing of *psaB* (psaB-F, PSAB2, psaB-1R, PSAB3, PSAB2R, PSAB4, psaB-4R2, psaB-R), which were published there for the first time, as Nishiyama & Kato (1999) provided the primer sequences on request. These primers yielded a sequence of 1,722 nt (compared to the total length of *psaB* of 2205 nt in *Marchantia polymorpha*), of which 1512 nt were used for the phylogenetic reconstructions. Compared with *rbcL*, *psaB* in fact turned out to be more variable, and a combined *rbcL*/*psaB* analysis improved the resolution of phylogenetic reconstructions of pleurocarpous mosses in Arikawa & Higuchi (2003). However, taxon sampling was limited, and the solution to resolving the backbone and family relationships within Hypnales may require the combination of further non-coding plastid markers and single-copy nuclear genes, rather than expanded sequencing of plastid genes.

rpoC2. The *rpoC2* gene has also been used by Nishiyama & Kato (1999) for elucidating phylogenetic relationships of land plants. This gene is one of the longest plastid genes, but has not been further evaluated within the major bryophyte lineages.

Plastid ribosomal DNA (16S, 23S, cpITS). The plastid ribosomal DNA (rDNA) comprises the only plastid markers employed in bryophytes which are not situated in the LSC, but in the inverted repeats of the plastid genome. It includes an operon of the 23S, 4.5S, and 5S rRNA genes plus in most plastid genomes a co-transcribed $trnR_{ACG}$ gene downstream of 5S (e.g. Samigullin *et al.* 1998), as well as the 16S rRNA gene separated from this operon.

Partial 16S and 23S sequences of seven bryophyte species were used in one of the first molecular phylogenetic analyses of bryophytes by Mishler *et al.* (1992), which appeared in the same year as the first phylogenetic inference in bryophytes based on the nuclear ribosomal 18S and 26S genes (Waters *et al.* 1992). Only about 250 nt of each of the two genes was amplified and sequenced by Mishler *et al.* (1992), using newly designed primers (16S1/16S2R, 23S1/23S2R). The limited data set restricted the conclusions that could be drawn concerning the evolutionary significance of the plastid ribosomal genes. While the nuclear ribosomal 18S, and to a lesser extent also 26S, were further employed in a number of studies (see below), 16S and 23S were only used once again, in the most comprehensive phylogenetic analysis of land plant relationships to date by Qiu *et al.* (2006, 2007). Their sequencing strategy included the combined analysis of selected genes with slow evolutionary rates (rDNA genes from all three genomes) and those with moderate or fast evolutionary rates (protein-coding plastid and mitochondrial genes).

The difference in utilization for phylogenetic reconstructions between the plastid and nuclear rDNA is even more pronounced concerning the respective intergenic spacers. Whereas the ITS1 and 2 of the nuclear

rDNA became one of the most popular markers (Fig. 2; see below), the respective plastid counterparts cpITS2 and 3 plus the 5S-*trnR* spacer ('cpITS4') were only used by Samigullin *et al.* (1998, 2002). They amplified the respective region in two parts, using two unnamed primers published by Goremykin *et al.* (1996) for cpITS2−3 and newly designed primers (cpITS4F/cpITS4R) for cpITS4. In total, ca. 700 nt of non-coding DNA plus the 4.5S, 5S, and *trnR* genes were spanned by the two amplicons. Length variation of the three spacers ranged between 62 and 238 nt in bryophytes (Samigullin *et al.* 1998, no sequence lengths mentioned in Samigullin *et al.* 2002). In contrast to the conserved 16 and 23S genes, the cpITS showed considerable length variability across bryophytes (Samigullin *et al.* 2002) and vascular plants (Goremykin *et al.* 1996), including several rather long but yet phylogenetically informative indels. However, findings such as the complete absence of cpITS2 in *Riella* and a markedly different cpITS2−4 sequence in *Takakia* than in all other bryophytes (Samigullin *et al.* 2002) indicate that the phylogenetic utility of this region at higher taxonomic levels in bryophytes is limited. Besides, the published alignments indicate that most informative characters in bryophytes can be ascribed to indels rather than to subsitutions, which may coincide with the low substitution rate in comparison with *rbcL* as inferred for vascular plants (Goremykin *et al.* 1996). Despite the potential difficulty of homology assessment in length-variable regions, the high length variability cpITS2−4 in liverworts may be of interest for phylogenetic analyses at lower taxonomic levels, whereas in mosses the spacers may again be too conserved to further employ this region as a molecular marker.

Mitochondrial genome markers

In contrast to the steadily increasing number of fully sequenced streptophyte plastid genomes, only 26 streptophyte mitochondrial genomes have been fully sequenced so far. One reason certainly is the multipartite organization of mitochondrial DNA, especially in tracheophytes, displaying a high degree of recombination (e.g. Ogihara *et al*. 2005; Grewe *et al*. 2009), in contrast to the rather compact and structurally conserved 'circular' organization of the plastid genome (but see Lilly *et al*. 2001). The high degree of reorganisation, in combination with rather frequent intron gains and losses (see, e.g., Knoop 2010) and the supposedly lower variability compared to the plastid genome, hampered developing and promoting mitochondrial markers in plant phylogenetics, especially with respect to non-coding spacers and introns. However, during the last decade the group of Volker Knoop proposed several mitochondrial markers, mainly introns, for phylogenetic reconstructions, of which the *nad5* G1 intron became one of the most prominent molecular markers in bryophyte systematics.

The *nad* cluster (*nad5-nad4-nad2*). The *nad5-nad4-nad2* gene arrangement represents a conserved gene cluster among all three bryophyte lineages (Groth-Malonek *et al*. 2007). It harbors two spacers as well as various introns in the three genes (see below). Whereas the *nad4-nad2* IGS is surprisingly conserved at a size of only 26 bp in all three bryophyte lineages, the *nad5-nad4* IGS reaches ~ 600 bp in mosses, 1,000–1,300 bp in liverworts, and exceeds 3,000 bp in hornworts (Groth-Malonek *et al*. 2007). Thus only the *nad5-nad4* IGS might qualify as a promising molecular marker, as underscored by the study of Wahrmund *et al*. (2009). The three conserved genes have mainly been targets of studies dealing with gains and losses of introns among early diverging embryophytes in a phylogenetic context (e.g. Pruchner *et al*. 2001, 2002; Beckert *et al*. 1999, 2001) or aimed at resolving the backbone phylogeny of mosses (e.g. Beckert *et al*. 1999, 2001). Of greater attraction for phylogenetic reconstructions, however, are the intervening non-coding sequences, i.e. the G1 and G2 introns that are often incorrectly referred to only by the gene abbreviations. For example, most studies using '*nad5*' or *'nad2*' as molecular markers actually aim at the G1 intron nad5i753g1, or the G2 intron nad2i156g2, respectively (for nomenclature see Dombrovska & Qiu 2004; Knoop 2004).

***nad*5 G1 intron (nad5i753g1).** This G1 intron has received most of the attention of all mitochondrial markers in bryophyte systematics, as it is conserved between mosses and liverworts and is easily accessible using

standard primers. Two primer sets can be found in the literature, K/L (Beckert *et al.* 1999) and nad5F4/nad5R3 (Shaw *et al*. 2003). Whereas the original K/L primer set amplifies the G1 intron plus large parts of both exons, the second set aims at full length sequences of the intron only. In order to optimize amplification success for herbarium samples with lower DNA quality, a nested approach was recently suggested by replacing nad5R3 in the initial PCR with nad5_2220R (Buchbender & Quandt accepted) and using nad5F4/nad5R3 in the nested PCR.

Use of the *nad5* G1 intron for inferring higher level phylogenetic relationships of mosses (e.g. Beckert *et al.* 1999, 2001; Cox *et al*. 2004; Bell *et al*. 2007; Quandt *et al*. 2007; Wahrmund *et al*. 2009, 2010) and liverworts (e.g. Davis 2004; Crandall-Stotler *et al*. 2005; Forrest & Crandall-Stotler 2005; Forrest *et al*. 2006; Heslewood & Brown 2007) has been underscored in several single, as well as multigene, analyses. The intron has been frequently used in large-scale analyses addressing relationships, e.g. within Sphagnaceae (Shaw *et al*. 2003), Polytrichaceae (Hyvönen *et al*. 2004), Ptychomniaceae (Pedersen & Newton 2007), and the pleurocarpous, large crown group of mosses, i.e. Hookeriales/Hypnales (e.g. Buck *et al*. 2005; Shaw *et al*. 2008a; Olsson *et al*. 2009a,b). However, although the intron provides an unexpectedly high level of variation among early diverging pleurocarps (see Bell *et al*. 2007), sequence variation within the derived pleurocarpous lineages is rather limited, resulting in largely unresolved and unsupported family level phylogenetic reconstructions (unpublished data by PERG - Pleurocarp Evolutionary Research Group).

Wahrmund *et al*. (2009) provided a comparison of support values that showed slightly increased support for the phylogenic reconstructions based on the mitochondrial genes *nad5* or *nad2* (including the introns) compared to *rbcL* or *rps4*. However, it should be kept in mind that the amount of sequence data for each of the mitochondrial regions is two to four times larger than each of the plastid regions analyzed.

Other mitochondrial markers. Coding regions such as *cox3* or '19S' (mtSSU) were among the first sequenced mitochondrial markers and mainly used to infer backbone relationships of land plants (Hiesel *et al*. 1994; Malek *et al*. 1996; Duff & Nickrent 1999; Nickrent *et al*. 2000, Qiu *et al*. 2006). Pacak & Szweykowska-Kulińska (2003) as well as Jankowiak & Szweykowska-Kulińska (2004) successfully used the *cox3* locus to trace organeller inheritance in liverworts. Later phylogenetic studies addressing relationships within the bryophyte grade favoured the more variable non-coding regions, such as introns (see above) and lately spacers, both in the *nad* cluster (see above) and the *trnA-trnT-nad7* region (e.g. Groth-Malonek *et al*. 2007; Wahrmund *et al*. 2008, 2009, 2010; see also Knoop 2010). Recently, two additional mitochondrial group I introns, cox1i624 and cobi420, were established as promising markers in bryophyte phylogenetics (Wahrmund *et al*. 2010; Volkmar & Knoop 2010).

Nuclear genome markers

In contrast to the unipaternially inherited organellar genomes the evolutionary history of the nuclear genome is more complex as it represents a mixture of both paternal lineages with a high degree of recombination. In addition, the organisation of the nuclear genome differs considerably from the organellar chromosomes. Whereas, organellar chromosomes generally encode only one gene copy (single-copy), apart from for instance the plastid inverted repeats (IR), nuclear genes are organised in gene families and generally present in multiple copies entailing the problem of specifically amplifying orthologous loci. Due to the rather conserved nature of suitable amplification primer sites, paralogous copies are usually co-amplified and thus cloning is required prior sequencing in order to obtain high quality sequence reads. Later, the desired orthologous copy can be extracted from the obtained sequence pool and perhaps more lineage specific amplification primers may be designed (compare Vanderpoorten *et al*. 2004). However, direct sequencing of PCR products may not be achieved. Thus, working with nuclear regions is less straight forward, laborious, and a more costly procedure compared to working with, for example, plastid regions. Yet, this is different for the well established nuclear ribosomal DNA, due to a process commonly referred to as concerted evolution that

homogenises the multiple copies of the transcription unit. Thus, although multiple nrDNA copies (up to 22,000; compare Rogers & Bendich 1987) exist, amplification and sequencing is generally straightforward. Therefore, efforts to include nuclear markers in bryophyte phylogenetics have concentrated almost completely on the nuclear ribosomal DNA, especially the internal transcribed spacer ITS1 and ITS2, and to a lesser extent the 18S and 26S rRNA genes (Fig. 2). The molecular organization and phylogenetic utility of the nuclear rDNA, in particular of the ITS, has been extensively reviewed by, e.g., Hershkovitz *et al.* (1999), Álvarez & Wendel (2003), Quandt & Stech (2003), Vanderpoorten *et al.* (2006) and Calonje *et al.* (2009). Nevertheless, since Wall (2002) introduced the *gpd* (*gapC*) gene for phylogenetic inference in *Mitthyridium* (Calymperaceae), the list of available nuclear markers used in bryophyte phylogenetics has been steadily increasing (Fig. 2), mainly due to the efforts of Shaw and colleagues (Shaw *et al*. 2003, 2004, 2005a, 2008b; McDaniel & Shaw 2005; Fuselier *et al.* 2009). At present, five single-copy or low-copy nuclear loci (partial genes or introns within the genes, respectively) have been employed in bryophytes, namely *adk*, *gapC* (*gpd*), *LFY*, *phy2*, and *rpb2*, as outlined below. The large majority of publically available nuclear sequence data from byrophytes is attributable to *Sphagnum*, from ITS, 26S, *gpd*, *LFY*, and three anonymous nuclear loci (*rapdA*, *rapdB*, *rapdF*) (e.g. Shaw *et al.* 2003; Szövényi *et al.* 2007; Shaw *et al.* 2008b). The anonymous loci are not further discussed here. Unfortunately, none of the nuclear markers outside the rDNA have been widely used in other studies, as amplification and sequencing is usually not straightforward and requires taxon-specific primer design and cloning after initial PCR to identify orthologous sequences. Therefore, several labs currently search the nuclear genome for easily accessible single- or low-copy loci suitable for bryophyte phylogenetics. Easily accessible and highly phylogenetically structured nuclear loci would finally open the door to confidently test phylogenetic hypotheses inferred from organellar DNA. Moreover, parallel and combined inferences using sequence data from all three genomes will lead to better approximations of the complex evolutionary species histories.

Nuclear ribosomal DNA (18S-ITS1-5.8S-ITS2-25S-5S). The general architecture of the nuclear ribosomal repeat unit and its characteristics in land plants, with a focus on bryophytes, have been extensively described elsewhere (e.g. Quandt & Stech 2003; Vanderpoorten *et al.* 2006; Wicke *et al.* in press). Here we focus on the different elements of the nuclear rDNA region that are traditionally amplified as separate amplicons and thus regarded as separate markers, namely 18S (SSU), ITS1-5.8S-ITS2, 26S (LSU), and 5S, and summarize their history and phylogenetic utility as molecular markers in bryophytes.

18S (SSU). The nuclear ribosomal SSU (18S rRNA gene) was one of the most widely used markers in the 1990s for inferring phylogenetic relationships within land plants, and until 1998 the most frequently sequenced gene in bryophytes. Waters *et al.* (1992) for the first time sequenced partial bryophyte SSUs (ca. 50% of the total gene length) with five primers (18E, G, H, J, L) reported by Hamby *et al.* (1988). Further Hamby primers (18Q, ERC, GRC, KRC) were used by Hedderson *et al.* (1998) to obtain full-length 18S sequences. Various sets of these primers, plus two new amplification primers (NS1/PCRB) by T. Hedderson (in Cox *et al.* 2000), were used in most subsequent studies of bryophytes. In contrast, I. Capesius (e.g. Capesius 1995; Bopp & Capesius 1998) obtained complete 18S sequences by using the 13 primers published by Elwood *et al.* (1985).

Early phylogenetic analyses based on 18S were contradictory with respect to the monophyly (e.g. Hedderson *et al.* 1998) or paraphyly (e.g. Bopp & Capesius 1998) of liverworts. Both topologies were hampered by incomplete taxon sampling and use of 18S alone, as discussed in Stech & Frey (2001). All subsequent molecular analyses, however, supported the monophyly of liverworts. From 1997 onwards, 18S was also used at the subclass and ordinal level in mosses (e.g. Capesius & Stech 1997; Cox & Hedderson 1999; Hyvönen *et al.* 1999; contributions in the summer 2000 issue of The Bryologist; Cox *et al.* 2004). After 2000, the number of studies using 18S in mosses dropped considerably. In contrast, in liverwort phylogenetics the SSU was first employed in 2004 (Davis 2004) and thereafter only used in three studies published in 2005 (e.g. Forrest & Crandall-Stotler 2005). Most studies from 1999 onwards have used SSU together with at least

one or two plastid markers, at times adding 26S sequences. Due to its limited sequence variation below the family level, the SSU has not been used to infer relationships between and within genera, in contrast to the LSU (see below).

ITS1-5.8S-ITS2. The nuclear ribosomal internal transcribed spacers are by far the most widely used nuclear markers for plant phylogenetic inference. They are generally easy to amplify and usually provide high levels of sequence variation for addressing relationships at the genus to population level. There are, of course, inherent disadvantages as well as problems that arise from relying solely on ITS for phylogenetic inference, as discussed, e.g. by Álvarez & Wendel (2003). In bryophytes, ITS has been constantly employed, from the first attempt to infer length variation of ITS1 in Pottiaceae (Spagnuolo *et al.* 1997) to the most recent publications. For a detailed overview of the phylogenetic history and utility of ITS in bryophytes, see Vanderpoorten *et al.* (2006), who also provided a list of amplification and sequencing primers.

26S (LSU). The history of utilization of the nuclear ribosomal LSU (26S rRNA gene) in bryophytes differs completely from that of the SSU, with the exception that early sequencing of this locus was carried out by Waters *et al.* (1992), who sequenced ca. 10% of the LSU with two primers (26C/D) reported by Hamby *et al.* (1988). Thereafter the LSU became frequently used in liverworts (e.g. Boisselier-Dubayle *et al.* 1997; Wheeler 2000; Davis 2004; Forrest & Crandall-Stotler 2005; Forrest *et al.* 2006), whereas in mosses it was first used in *Sphagnum* (e.g. Shaw 2000; Shaw *et al.* 2003). Few studies employed 26S sequences for inferring higher-level phylogenetic relationships of mosses (Cox *et al.* 2004; Buck *et al.* 2005). Furthermore, in contrast to 18S, partial 26S sequences have been used at the family and genus level, especially in *Sphagnum* and various other groups of mosses (e.g. Stech 2004; Budke & Goffinet 2006; Pedersen *et al.* 2007; Shaw *et al.* 2008a), but also in liverworts (e.g. Forrest *et al.* 2005).

All studies employed only partial 26S sequences, mostly ca. 900−1400 nt of the 5' end. Different amplification strategies and primer sets were developed: Wheeler (2000) amplified the ITS2 together with partial 26S, using primer ITS3 of White *et al.* (1990) and a novel reverse primer LR1010 plus two internal sequencing primers (LF47/LR654). This amplicon comprised the first four 26S domains (cf. Kuzoff *et al.* 1998), of which two were conserved and two variable. Shaw (2000) published a set of six primers designed by the Manos lab (Duke), all situated within 26S, to amplify and sequence two separate fragments of the gene. Primers LS0F and LS12R (plus the internal sequence primer LS8R) yielded ca. 1000 nt of the 5' part, i.e., helices B13_1 to D4 of the LSU RNA secondary structure model for *Funaria hygrometrica* Hedwig (1801: 172) in Capesius & van de Peer (1997). These primers were applied in several subsequent studies of mosses (e.g. Shaw *et al.* 2003; Buck *et al.* 2005; Budke & Goffinet 2006) and liverworts (Davis 2004). With the other three primers, LS23F/LS33R for amplification and the internal sequencing primer LS27R, Shaw (2000) obtained another ca. 1000 nt of the 3' end of 26S. Cox *et al.* (2004) used primers LS30F and LS12R (stated as novel although LS12R was already published) plus one further primer by the Manos lab (LS4F) for sequencing. Another set of primers was published by Boisselier-Dubayle *et al.* (1997, 2002), which included universal primers C'_1, $C'_7$2 and D_8 as well as a bryophyte-specific primer D_2B, and extended the region sequenced by Wheeler (2000) by the four subsequent domains (again, two conserved and two variable ones). Forrest & Crandall-Stotler (2005) and Forrest *et al.* (2006) combined primers from these different sources, namely ITS3, D_2, LR1010, and LS0F/LS12R/LS8R. Finally, Stech *et al.* (2004) used the bryophyte ITS primer 5.8F plus primer 950rev (Kuzoff *et al.* 1998) for amplification, plus three internal primers (26S–1R, 26S–2F, and 26S–3F; modified after primers N-nc26S2, N-nc26S3 and 268rev of Kuzoff *et al.* 1998) for sequencing. This approach covered a region intermediate in length between Wheeler (2000) and Boisselier-Dubayle *et al.* (2002), which included the first three conserved and respective variable domains.

Initial comparisons indicated that the LSU has a slightly higher substitution rate than the SSU in land plants (Hamby & Zimmer 1992). Together with its length and mosaic structure of conserved regions and interspersed variable expansion elements (cf. Kuzoff *et al.* 1998), the LSU could have phylogenetic potential at different taxonomic levels, possibly down to genera and species. However, in bryophytes the LSU seems to

be less variable at the family and genus level than commonly used plastid regions, mitochondrial *nad5*, or nrITS (e.g. Shaw *et al.* 2003; Budke & Goffinet 2006; Forrest *et al.* 2006; Shaw *et al.* 2008a). Also at higher taxonomic levels, the LSU provided the second lowest number of informative characters in liverworts, with only the SSU being more conserved (Forrest & Crandall-Stotler 2005). Additionally, alignment of the variable domains can be ambiguous due to weaker evolutionary constraints, at least in the loop regions of the respective secondary structure, as is the case in *Campylopus* (Stech 2004). Although the LSU can contribute characters from the nuclear genome to supplement those from ITS, when no single-copy genes are available, its phylogenetic utility in bryophytes seems relatively limited.

5S. In contrast to angiosperms, a copy of the 5S rRNA gene is encoded within the nuclear ribosomal repeat unit in bryophytes (Sone 1999; Wicke *et al.* in press). Although 5S was the first DNA region which was sequenced in bryophytes in the early 1980's (Katoh *et al.* 1983), it was only used in some of the very first molecular phylogenetic reconstructions of green plants/eukaryotes (e.g. Hori *et al.* 1985; van de Peer *et al.* 1990) and has never played a role for phylogenetic inference within the bryophyte lineages. Its limitations, such as short size (120 nt) and low sequence variation, were attempted to be overcome in early sequencing of the plastid ribosomal 16S/23S (Mishler *et al.* 1992) and nuclear ribosomal 18S/26S (Waters *et al.* 1992) genes. In the light of recent phylogenetic reconstructions such as Qiu *et al.* (2006), the 16S/23S and 18S/26S approaches seemed in fact to be more reliable, for example by resolving liverworts as monophyletic in contrast to the 5S trees. However, the taxon sampling of all early studies was too limited to infer the suitability of ribosomal genes for phylogenetic inference with confidence.

Nuclear *trnL* spacers. The earliest employed nuclear marker outside the nuclear rDNA comprised intergenic spacers between clustered nuclear tRNA genes. Transfer RNA genes in the nuclear genome can be either scattered throughout the genome or arranged in tandem repeats of one or several types of tRNA genes (e.g. Beier *et al.* 1991; for a recent study on nuclear tRNA diversity in photosynthetic eukaryotes see Maruyama *et al.* 2010). Fiedorow & Szweykowska-Kulińska (1998) introduced the intergenic spacers between clustered $trnL_{CAA}$ genes based on a sequence of *Pellia neesiana* (Gottsche 1867: 69) Limpricht (1876: 18), and Fiedorow *et al.* (2001) applied them for phylogenetic inference in *Pellia*. Fiedorow & Szweykowska-Kulińska (1998) used three primer pairs (I/II, A1/A2, B1/B2), situated at different parts of the tRNA genes and within the spacers, to amplify and sequence the spacer as well as the respective upstream and downstream tRNA gene copies. In Fiedorow *et al.* (2001), primer L1 (a part of the original primer I) and an obviously newly designed primer L2 were used. They received a spacer sequence of 78−104 nt in *Pellia*, which resembled plastid intergenic spacers in its AT content of 66% (*P. neesiana*). The analysis by Fiedorow *et al.* (2001) indicated some phylogenetic potential of the intergenic spacer at the species level, but respective studies of other taxa are needed before its phylogenetic utility can be assessed. The considerable length difference of the *trnL* spacer between *Pellia* and *Conocephalum* (355 nt) observed by Fiedorow *et al.* (2001) will probably restrict its use at higher taxonomic levels.

adk. The adenosine kinase gene was introduced by Vanderpoorten *et al.* (2004) to help resolving the phylogeny of the taxonomically complex genus *Hygroamblystegium*. Thereafter, it was also used in *Ceratodon purpureus* (Hedwig 1801: 36) Bridel (1826: 480) (McDaniel & Shaw 2005) and *Acrocladium* (Blöcher *et al.* 2006). Together with *gpd* (see below), *adk* is the only nuclear gene of which the phylogenetic utility at inter- to intraspecific levels has been addressed in more than one bryophyte genus. Vanderpoorten *et al.* (2004) amplified and sequenced ca. 900 nt of *adk* with primers F and R designed by J. Wheeler (Berkeley), which included four of the 11 introns described for *adk* in *Arabidopsis* (cf. Moffatt *et al.* 2000). They further developed specific primer pairs for *Hygroamblystegium* (1F/2R, 4F/3R). The *adk* gene was found to be polymorphic with multiple copies being present within individuals, likely due to repeated events of gene duplication and loss, posing a problem of paralogy in the phylogenetic reconstructions. McDaniel & Shaw (2005) used the same primers F and R for amplification and sequencing of partial *adk* in *Ceratodon purpureus*

and two other Ditrichaceae species as outgroups. Numbers of unique haplotypes and segregating sites in *C. purpureus* were much higher in *adk* than in the plastid *atpB-rbcL* spacer, but phylogeographic structure of both markers was similar. Finally, Blöcher *et al.* (2006) referred to Vanderpoorten *et al.* (2004) concerning the amplification and sequencing stategy of *adk* in *Acrocladium*, although they did not explicitly state that the specific primers developed for *Hygroamblystegium* were also used in that genus. They were only able to obtain partial sequences from one sample each of the two *Acrocladium* species, which showed higher sequence variation than the plastid *trnL* and *rps4* as well as nuclear ITS regions. Summarizing the results of these three studies, the *adk* gene (or its introns, respectively) seems to show variation in evolutionary patterns accross different moss genera, but may be potentially useful for resolving inter- and intraspecific relationships, if the problems of obtaining clear sequences and identifying orthologous copies can be managed.

gpd. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) is involved in two metabolic pathways in plants, the Calvin cycle in the chloroplasts and glycolysis in the cytosol. The Calvin cycle GADPH is encoded by the *GapAB* gene, and the cytosolic enzyme by *GapC*. Of these, *GapC* in part, (referred to as *gpd*) was first used in plant phylogenetics by Martin *et al.* (1993), and for the first time employed in bryophytes by Wall (2002, 2005) in *Mitthyridium*. Later, this marker was also used for the Calymperaceae in general (Fisher *et al.* 2007) and in *Sphagnum* (Szövényi *et al.* 2007). *GapC* was chosen because it is easy to amplify and single-copy in bryophytes (cf. Szövényi *et al.* 2006), although in angiosperms it may comprise multiple loci (e.g. Pérusse & Schoen 2004). In phylogenetic treatments of Calymperaceae, ca. 900 nt of *gpd* were amplified and sequenced with newly designed primers 1790F and 3050R by Wall (2002); the designation '3090F' for the forward primer in Fisher *et al.* (2007), rather than referring to a novel primer, is likely given in error. These primers are situated in exons 5 and 9 and consequently amplify exons 6-8 and introns i5-i8. Szövényi *et al.* (2006) sequenced almost the whole gene from exon 2 to exon 9' with amplification primers Gap2exon2 forw./ Gap2exon9 rev. plus five internal sequencing primers newly designed for *Sphagnum*. Ultimately, however, the same portion of *gdp* as Wall (2002) was used for analysis, as the other portions did not show intraspecific variation. Length of the region including introns i5-i8 is ca. 1500 nt in *Sphagnum*, with low intraspecific variation, similar to ITS.

LFY. FLORICAULA/LEAFY (FLO/LFY) is a single-copy homeotic gene containing two introns, named FLint1/FLint2 (e.g. Grob *et al.* 2004) or LEAFY1/LEAFY2 (e.g. Shaw *et al.* 2003). In bryophytes, both introns were amplified and sequenced in four studies of *Sphagnum* by Shaw and colleagues (2003, 2004, 2005a, 2008b), using primers 1428F/2327R by C.J. Cox (published in Shaw *et al.* 2003). In the data set of Shaw *et al.* (2005a), LEAFY1 was about twice as long as LEAFY2 and comparable in size with nrITS and plastid *trnL-F*. It showed considerably higher variation than these markers and provided a similarly well-resolved tree as ITS, albeit with lower statistical branch support. LEAFY1 may therefore be a promising candidate to complement ITS data in other bryophyte groups.

phy2. The phytochrome 2 gene has so far been employed only once, in *Ceratodon purpureus* (McDaniel & Shaw 2005). At present, this study is unique in that it compares two nuclear gene regions outside the nrDNA (*phy2* and *adk*) in moss genus other than *Sphagnum*. McDaniel & Shaw (2005) amplified and sequenced ca. 700 nt of *phy2* with two newly designed unnamed primers, the amplicon of which comprised introns 2 and 3, parts of exon 2, and complete exon 3. Recombination in *phy2* was lower than in *adk* within *C. purpureus*, although numbers of unique haplotypes and segregating sites were similar. Phylogenetic structure was low in the *phy2* data and differed from that in *adk*. However, McDaniel & Shaw (2005) concluded that a pattern of isolation by distance at a continental scale, as shown by *phy2*, is more likely to be representative for the whole genome in *C. purpureus* than the pattern of high-frequency, derived alleles in *adk*. These results underpin the need of including more than one single-copy nuclear marker in future phylogenetic and phylogeographic studies, to assess differences in molecular evolution within the nuclear genome.

***rpb2*.** The gene for RNA polymerase II subunit 2 (*rbp2*) has recently been introduced in a phylogenetic and phylogeographic study of three *Metzgeria* species (Fuselier *et al.* 2009). Little information was given in that study concerning the amplification and sequencing strategy, with a reference to the earlier study of Denton *et al.* (1998), who sequenced ca. 50% of *rpb2* (1668 nt) with a total of ten primers. The length of *rpb2*, 675 nt, in Fuselier *et al.* (2009) clearly indicates that only partial *rpb2* sequences were used, but the exact location of this stretch, and possible inclusion of one or more of the 24 introns described for *Arabidopsis* (cf. Denton *et al.* 1998), remains unclear. Concerning the number of informative characters and phylogenetic resolution in *Metzgeria*, the partial *rpb2* gene region seems to be of potential for resolving species-level relationships, at least in liverworts.

Fingerprinting methods

DNA fingerprinting has been relatively little employed in bryophytes (Fig. 2, 4B). The first study based not only on isozymes but also DNA fingerprints, was performed by Boisselier-Dubayle *et al.* (1995) to distinguish subspecies within *Marchantia polymorpha*, using RAPD (random amplified polymorphic DNA) and RFLP (restriction fragment length polymorphism) techniques, which were the first DNA fingerprinting techniques available. In particular RAPD became widely used, including in bryophytes, for some time in the 1990s. However, RAPD patterns turned out to be not always reproducible, and new techniques such as AFLP (amplified fragment length polymorphism) and ISSR (inter-simple sequence repeats) were developed to replace them (cf. Mueller & Wolfenbarger 1999). The change from RAPD and RFLP to ISSR/microsatellites and AFLP has also taken place in bryophytes, although a number of analyses of genetic variation in Antarctic mosses based on RAPD were published until 2004 (e.g. Skotnicki *et al.* 2002, 2004) and the most recent RAPD analyses dates from 2008 (Li *et al.* 2008).

The first study using AFLP was performed by Vanderpoorten & Tignon (2000) in *Hygroamblystegium*, followed by further studies on both acrocarpous and pleurocarpous mosses (e.g. Pfeiffer *et al.* 2006; Stech *et al.* 2008). Inferences in these studies were based on patterns deduced from one to four AFLP selective primer combinations (mostly Mse-CTA or Mse-CAT with different EcoRI primers such as EcoRI-AAC or EcoRI-AGG), and were, in part, used in combination with DNA sequence data (e.g. Stech *et al.* 2008). The first description of microsatellite isolation and characterization in mosses (*Polytrichum*) appeared in the same year as the first AFLP analysis (van der Velde *et al.* 2000). Despite the fact that AFLP can be applied more easily without the need of developing taxon-specific primers, ISSR/microsatellites became more popular in bryophytes, and were, in contrast to AFLP, also applied in liverworts (Hock *et al.* 2008). The number of ISSR studies now almost equals that of RAPD analyses (Fig. 2), with markers having been developed for different groups of acrocarpous and pleurocarpous mosses. However, both ISSR and AFLP still play a rather minor role at the intraspecific level in comparison with haplotype analyses based on sequence markers, especially in liverworts.

Outlook

Higher-level phylogenetic relationships were, to a considerable degree, resolved with high confidence within all three bryophyte groups. This is particularly evident in mosses, in which the various published phylogenetic reconstructions (e.g. Cox *et al.* 2004; Tsubota *et al.* 2004; Shaw *et al.* 2005b; Stech & Quandt 2006; Bell *et al.* 2007; Goffinet *et al.* 2007; Quandt *et al.* 2007; Stech & Frey 2008; Wahrmund *et al.* 2010) seem to converge on a common hypothesis of the 'backbone' relationships of the moss phylogeny. Revised classifications taking into account recent molecular data are characterized by a shift towards higher taxonomic levels (Stech & Frey 2008; Goffinet *et al.* 2009). Stech & Frey (2008) divided the division Bryophyta into three subdivisions, of which the largest, Bryophytina, is further subdivided into five classes, and the largest class,

Bryopsida, into eight subclasses. Goffinet *et al.* (2009) continued this trend with separating Andreaeopsida into two further subdivisions, Andreaeophytina and Andreaeobryophytina. Also the hornwort phylogeny seems to be largely resolved with confidence (e.g. Stotler & Crandall-Stotler 2005; Duff *et al.* 2007), with a basic subdivision of Anthocerotophyta into Leiosporocerotopsida and Anthocerotopsida. In liverworts, the major lineages were well-circumscribed in recent, comprehensive phylogenetic reconstructions (e.g. Davis 2004; Forrest & Crandall-Stotler 2005; Forrest *et al.* 2006; He-Nygrén *et al.* 2006), however, different opinions were expressed with respect to their taxonomic ranks in recent classifications (Frey & Stech 2005; He-Nygrén *et al.* 2006; Crandall-Stotler *et al.* 2009). Some higher-level phylogenetic relationships are still partly contradictory or do not receive significant statistical support, both in liverworts and in mosses. The most difficult questions in mosses concern ordinal and familial relationships within the two largest clades, the haplolepideous mosses (Dicranidae) and the pleurocarpous Hypnales. But also the exact relationships of subclasses in Bryopsida, in particular of Timmiidae, and of orders within the diplolepideous-alternate Bryidae need to be tackled again, using more and novel markers (e.g. Wahrmund *et al.* 2010).

Nevertheless, future molecular studies of bryophytes will likely emphasize the circumscription and relationships of families, genera and species. A number of large families (and genera) are still awaiting comprehensive molecular phylogenetic treatments, such as Hypnaceae, and others that have already been tackled (e.g. Lepidoziaceae, Lophoziaceae, Pottiaceae, Sematophyllaceae) should be re-addressed to further clarify generic circumscriptions and relationships. But also at the inter- and intraspecific levels there is a number of challenging questions to be answered. For example, an increasing number of studies indicates considerable genetic divergence in contrast to the morphological uniformity of the taxa, both at the species level ('cryptic speciation', e.g. Shaw 2001; McDaniel & Shaw 2003; Feldberg *et al.* 2004; Stech & Wagner 2005; Fuselier *et al.* 2009; Heinrichs *et al.* 2009; Sotiaux *et al.* 2009) or between genera (e.g. morphological convergence between molecularly unrelated liverwort genera, cf. Crandall-Stotler 2010). Providing exact species circumscriptions and molecular species identification tools, analyzing intraspecific genetic variation, and mapping the geographical distribution of genotypes is a crucial task not only for scientific reasons, such as to clarify constraints of morphological evolution in bryophytes and the influence of contrasting dispersal abilities (cf. the review of Frey *et al.* 2010). These data will also provide a baseline for conservation management plans, inclusion of bryophyte species in ecological and ecosystem studies, and improved uses of bryophytes in biomonitoring and for applied purposes.

The problems described here in relation to different taxonomic levels raise the question of future marker utilization for phylogenetic studies and molecular species identification by DNA barcoding. At the moment, no universal stand-alone DNA barcoding marker for bryophytes is available, and it is unlikely that such a marker will be found. In fact, we consider that, at least for derived groups such as Hypnales, a combination of markers will be necessary, although this contradicts the original idea of DNA barcoding. Even the optimal combination of markers may vary across all bryophytes, although the *trnL* intron may still be regarded as a prime candidate, together with one or two variable other plastid or even nuclear markers (e.g. Liu *et al.* 2010). Concerning bryophyte phylogenetics, it is obvious that there is a trend towards using more markers per phylogenetic or phylogeographic study, and the comparison of markers from different genomes should be continued. The problem, however, is to identify markers that are suitable to overcome the limitations of the traditional markers and provide sufficient phylogenetic structure to address the open systematic questions efficiently. Several markers mentioned here have been limited to use in a small number of studies and/or in a single genus, and assessment of their phylogenetic utility for bryophytes in general is not yet possible. In particular for the pleurocarpous mosses it is difficult to suggest further plastid markers, as virtually all non-coding regions of the plastid genome show relatively little phylogenetic structure in this group. Perhaps one has to combine several suboptimal markers to collect the small amount of synapomorphic sites in each of them until well-resolved phylogenetic trees can be produced. In general, we expect an increase in the utilization of plastid G2 introns (*trnG*, *trnV*, *rpl16*, *trnK*) and fast-evolving plastid genes such as *matK* or *ndhB*, as they are relatively long in contrast to parts of the plastid intergenic spacers, yet exhibit a considerable amount of variation. In addition, low copy nuclear markers are highly desired and expected to be a major new

tool for tackling unresolved phylogenetic relationships. However, we need to be aware of potentially different evolutionary patterns between the nuclear markers, and of inconsistencies between organellar and nuclear markers, which may become more prominent when nuclear DNA will be employed more frequently. Technical advances such as efficient whole-genome sequencing will greatly facilitate the search for new markers of potential phylogenetic utility. We have surely not reached the end of possibilities to solve the remaining questions in bryophyte phylogenetics.

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