Bryophytes and other basal land plants: the mitochondrial perspective

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The earliest diversifications of land plants in Ordovician and Silurian times are unclear, mainly due to a lack of macrofossils. The increasing wealth of molecular data, however, converges on the view that bryophytes are paraphyletic with only one of its classes being sister to all other land plants and another bryophyte clade being sister to the tracheophytes. We continue to explore mitochondrial gene regions with respect to their unique modes of gene expression such as RNA editing and trans-splicing and their phylogenetic information potential. Plant mitochondrial genes drift slowly in sequence and mitochondrial introns are mostly stable in position, yet vary in occurrence between clades. These two features make mitochondrial DNA an attractive reservoir of old phylogenetic information. On the other hand, there is striking structural plasticity of mitochondrial DNA (the chondriome) in embryophytes, which grossly contrasts with the conservative evolution of chloroplast DNA (the plastome) in the land plant lineage. We find that mitochondrial intron occurrence strongly adds to the view of the deepest dichotomy separating liverworts and all non-liverwort embryophytes. Other intron occurrences, including the presence of ancestors of trans-splicing group II introns, tend to place hornworts as a sister group to tracheophytes, a grouping that we find corroborated by most multi-gene analyses. Furthermore, conserved intron sequences add phylogenetic resolution within clades. The use of gene spacers for phylogenetic analysis, as commonly investigated in chloroplast DNA, has so far been precluded in plant mitochondria due to rampant recombination of the chondriome, at least in tracheophytes. To investigate whether at least some ancestral gene orders are still conserved among bryophytes and provide useful phylogenetic information, we have recently explored the *nad5-nad4-nad2* gene arrangement. We find it conserved, yet with strikingly different modes of evolution in the two spacers.

KEYWORDS: bryophytes, gene spacers, group II introns, molecular evolution, mtDNA, trans-splicing.

INTRODUCTION

The earliest phylogenetic diversifications of land plants that have occurred up to 500 million years ago in Ordovician times so far remain elusive, mainly due to a lack of paleobotanical evidence. However, it is now generally believed that plants with a bryophyte type of organization were the first true land plants (Kenrick & Crane, 1997) and a liverwort type of organization is at least compatible with the scarce microfossil evidence (Taylor, 1995; Edwards & al., 1998; Wellman & Gray, 2000).

Molecular studies of extant representatives of the three bryophyte classes, hornworts, liverworts and mosses, have confirmed each of them as monophyletic. A recent study using concatenated protein-coding genes from complete chloroplast genomes with one representative each of the hornworts, liverworts and mosses has even identified bryophytes as monophyletic (Nishiyama & al., 2004)—a result that had not been observed in any other recent molecular study and that should be regarded with caution due to taxon under-sampling. Molecular data have also provided valuable insights into the relationships within these clades.

The phylogenetic relationships between bryophytes and their supposed algal ancestors and tracheophyte descendants (Pryer & al., 2002), however, have not been convincingly resolved. The phylogenetic placement of liverworts as the sister group to all other land plants has found strong molecular support from three introns in the mitochondrial DNA that are exclusively present in non-liverwort embryophytes (Qiu & al., 1998). However, subsequent molecular studies (Nickrent & al., 2000) have alternatively proposed hornworts as the earliest diverging land plant lineage. Among the algal ancestors, a strong point has been made for Charales algae as extant representatives of the sister group of land plants and Coleochaetales as the sister group for the joint assembly (Karol & al., 2001). A very early study of land plant evolution based on a short mitochondrial *cox3* gene region had similarly suggested this (Malek & al., 1996), but it also found some support for hornworts as the earliest land plants. Possibly the diversification of the earliest land plants into liverworts, mosses and hornworts took place in a short period of time, helping to explain why the backbone nodes of phylogenetic trees are separated

by only short branches. The number of unequivocal morphological characters is too low to be of significant help in resolving these relationships, and we, therefore, must rely on molecular data.

PHYLOGENETIC INFORMATION IN STRUCTURAL ALTERATIONS OF ORGANELLE DNAS

Plant mitochondrial DNA contrasts in nearly every aspect with evolution of the quickly evolving sequences in the compact and structurally conserved mitochondrial genome of most animals (Knoop, 2004). Initially aimed at shedding light on the evolution of peculiar phenomena such as trans-splicing group II introns (Malek & al., 1997) and RNA editing (Malek & al., 1996), the investigation of mitochondrial sequences through their generally very slow sequence drift additionally appeared to provide phylogenetic information on the diversification of old land plant clades (Beckert & al., 1999, 2001; Vangerow & al., 1999). In fact, the mitochondrial genome in the plant cell is generally the most slowly evolving of its three genomes (Wolfe & al., 1987). However, this statement must not be oversimplified. Whereas sequence drift in exons and introns is, in fact, on average significantly slower than in the chloroplast or nuclear DNA, the structural plasticity of plant mitochondrial DNA is significantly larger. Whereas the circular structure of cpDNA with large and small single copy regions separated by inverted repeat sequences is a common denominator of photosynthetic life, no such structural conservation exists in mtDNA of plants. Simple circular mtDNAs are present in algae and the liverwort *Marchantia polymorpha* (Oda & al., 1992), but among angiosperms mtDNA differences are evident even between isolates of a single species (Ullrich & al., 1997). In most angiosperms mitochondrial DNA is a pool of recombining sub-genomic molecules that only together can represent something like a "master circle", whose actually physical presence in the mitochondrion is doubtful (Bendich, 1993; Oldenburg & Bendich, 2001).

Rare structural changes in chloroplast DNA may convey significant phylogenetic information such as defining the origin of embryophytes from charophycean algae (Manhart & Palmer, 1990), or the split of lycophytes from other tracheophytes, shown more than 10 years ago (Raubeson & Jansen, 1992). The recently available completed chloroplast genomes of the moss *Physcomitrella patens* (Sugiura & al., 2003), the hornwort *Anthoceros formosae* (Kugita & al., 2003) and the fern *Adiantum capillus-veneris* (Wolf & al., 2003) in comparison to that of the liverwort *Marchantia polymorpha* (Fukuzawa & al., 1988), however, allow for alterna-

tive parsimonious explanations of bryophyte phylogeny and for explaining the few genomic structural changes that are observed. Unfortunately, some highly interesting changes, such as the transfer of the *rpoA* gene into the nucleus in *Physcomitrella* are so far unique observations and cladistically uninformative [but see Goffinet & al., this volume, pp. 353–360; eds.].

PLANT MITOCHONDRIAL INTRONS IN EVOLUTION

In mitochondrial DNA, the appearance (and possibly the disappearance) of introns in early land plant evolution appears to provide informative genomic characters. The split of liverworts and non-liverwort embryophytes (NLE), initially suggested by three mitochondrial introns in *cox2* and *nad1* (Qiu & al., 1998), is now corroborated by observations in other genes such as *nad4* and *nad7* (Pruchner & al., 2001), which suggest invasion of genes in the liverwort lineage and the NLE lineage by different introns in different positions. Clade-specific introns are also present in the mitochondrial genes *nad1* and *nad2* (Pruchner & al., 2002; Dombrovska & Qiu, 2004) and confirm monophyly of several land plant clades but fail to provide conclusive evidence on the phylogenetic backbone of early land plant evolution. In *nad1*, for example, the now well established monilophyte clade, which comprises ferns, whisk ferns and horsetails, is strongly confirmed through presence of intron *nad1i258*, which is absent in other land plant clades (Dombrovska & Qiu, 2004). In *nad2*, mosses share their single *nad2* intron with angiosperms, hornworts share two different *nad2* introns with angiosperms, and only one of the latter is the single intervening sequence in *nad2* of liverworts (Pruchner & al., 2002).

Most recently we have investigated the complete structure of *nad5*, the largest protein encoding gene in mitochondria (Groth-Malonek & al., 2005). We observe a gradual gain of group II introns *nad5i230*, *nad5i1455*, *nad5i1477* and *nad5i1872* in the NLE (i.e., the moss-hornwort-tracheophyte) lineage (Fig. 1). Introns *nad5i1455* and *nad5i1477* are conserved in at least some early tracheophytes (Malek & Knoop, 1998) and later get disrupted as trans-splicing introns in the angiosperms, whereas their status in the gymnosperm classes is as yet unclear. Intron *nad5i753* under that phylogenetic scenario would be a very early emergence in the embryophytes that disappears in the common ancestor of hornworts and tracheophytes. Three introns from the total of eight so far identified in the *nad5* genes of embryophytes are of restricted occurrence and thus lower phylogenetic value: *nad5i1242* is present in spore-bearing tracheophytes (lycophytes and monilophytes) and appears to be
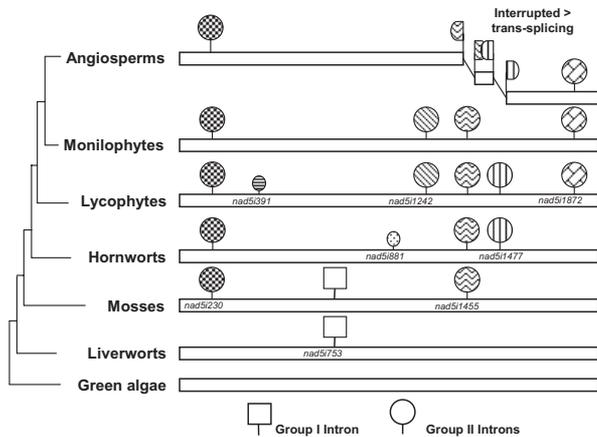
Fig. 1. Structure of the mitochondrial *nad5* gene in land plant evolution. Overview of *nad5* introns in the major land plant clades. Square pinheads represent the single group I intron shared between liverworts and mosses, circular pinheads represent group II introns. Intron nomenclature is according to positions in the uninterrupted reading frame of *Marchantia polymorpha*, as proposed recently (Dombrovska & Qiu, 2004). Differential shadings indicate homologous introns at identical positions. Introns *nad5i1242* and *nad5i1477* are shown to be secondarily lost. Introns *nad5i391* and *nad5i881* are so far identified in single taxa only, *Huperzia selago* and *Anthoceros agrestis*, respectively.

secondarily lost in the eusporangiate fern *Ophioglossum* and the horsetail *Equisetum*. Intron *nad5i881* is so far unique to the hornwort *Anthoceros agrestis* and intron *nad5i391* to the lycophyte *Huperzia selago*. Convincing phylogenetic reliability from sequence-based trees is lacking so far, but a parsimonious intron gain and loss scenario is easily mapped on our phylogenetic tree construction to suggest hornworts as sister to tracheophytes. Notably, this phylogenetic topology conforms to other recent molecular studies (Samigullin & al., 2002; Jankowiak & al., 2004).

■ PLANT MITOCHONDRIAL SPACERS IN EVOLUTION: PRELIMINARY INSIGHTS

A striking observation concerning chondriome evolution in the land plant lineage is the tremendous gain in size, which can exceed 2000 kb in the angiosperm family Cucurbitaceae (Ward & al., 1981). Almost certainly, such size increases of plant mtDNA are neither due to gain of further introns later in angiosperm diversification nor the extension of coding capacity. Only one example of a group I intron frequently and independently inserting into the *cox1* gene is known, which most likely has a fungal origin (Cho & Palmer, 1999). Whether horizontal

gene transfer into plant mitochondrial DNA as recently claimed will play a major role remains to be seen (Bergthorsson & al., 2003; Won & Renner, 2003; Davis & Wurdack, 2004). More likely, the most significant contributions come from foreign DNA originating from the chloroplast or the nucleus. For example, all types of nuclear retrotransposon sequences can still be detected by scattered yet significant sequence homologies even in the moderately sized 368 kb mtDNA of *Arabidopsis thaliana* (Knoop & al., 1996). Frequent recombination in non-coding intergenic regions of plant chondriomes then results in lack of conservation of gene orders among angiosperms. However, some gene orders are conserved between algae close to the land plant lineage, such as *Chara vulgaris*, the likely closest ancestor of the land plant lineage (Turmel & al., 2003), *Chaetosphaeridium globosum*, a Coleochaetales alga (Turmel & al., 2002), and the liverwort *Marchantia polymorpha*. The approximately three-fold size increase in chondriomes from the algae to the liverworts (as a likely extant representative of the early embryophytes) is partly due to the acquisition of introns but also the enlargement of some spacer regions between genes without destroying their order.

We reasoned that such gene arrangements may have been conserved in the NLE lineage as well. The evolution of intergenic mitochondrial regions could independently corroborate or question the phylogenetic topology of early land plants as suggested in Fig. 1. Hence, we have chosen to investigate the gene orders *nad5-nad4-nad2* and *trnA-trnT-nad7*, both of which are conserved between *Chara* and *Marchantia*. Design of primers for PCR to amplify across the total of four intergenic regions in the two clusters was comparatively straightforward. So far our results show that the *nad5-nad4-nad2* gene order is indeed also conserved in mosses (Fig. 2). Only the *nad5-nad4* spacer seems to experience a significant gain of size in early embryophyte evolution, whereas the *nad4-nad2* spacer is even somewhat smaller (26 bp) in bryophytes than in algae. Partial sequencing has already confirmed the correct identity of the PCR products so far obtained (unpubl.). Our preliminary observations suggest that the *nad5-nad4* spacer size is in the range of 300–600 bp in the mosses, around 1000 bp in the liverworts, and still significantly larger in the hornworts.

The situation appears to be quite similar for the *trnA-trnT-nad7* region (Fig. 3). Again, the gene arrangement is not only conserved in liverworts but also in mosses and, again, the liverworts tend to show a stronger increase of spacer sizes. However, in the *trnA-trnT-nad7* case one intergenic region is increased simultaneously in mosses and liverworts, whereas the other one is elongated in liverworts only.

Conclusions about the evolution of the two chondriome regions must await their sequencing for a reason-
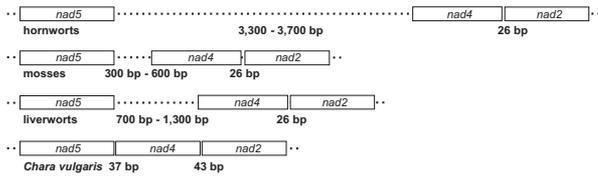
Fig. 2. The mitochondrial *nad5-nad4-nad2* gene arrangement. The gene order in the alga *Chara vulgaris* L. (accession NC_005255), where spacer size is increased in the *nad5-nad4* spacer, appears to be conserved in all bryophytes, whereas the *nad4-nad2* spacer is slightly reduced in size. Size of the spacers is inferred from PCR products, the identity of which is verified by partial sequencing. The exact sizes of the spacers in *Marchantia polymorpha* deduced from database accession NC_001660 are 1,333 bp and 26 bp.

ably wide spectrum of liverwort, moss and hornwort taxa (Groth-Malonek, Rein & Knoop, in prep.). An interesting aspect of the *trnA-trnT-nad7* region is that *nad7* itself is a pseudogene in *Marchantia*. The *nad7* reading frame is rendered non-functional through the introduction of six stop codons, and the functional *nad7* copy of *Marchantia* resides in the nucleus (Kobayashi & al., 1997). The degeneration of a plant mitochondrial gene through point mutations, instead of disintegration through recombination events (as usually observed in angiosperms) after the functional copy is established in nuclear DNA, is peculiar. The *nad7* gene is functional in mosses where it has gained two of the four group II introns that are present and highly conserved in the mitochondrial *nad7* gene of angiosperms (Pruchner & al., 2001). In that regard it will be interesting to obtain insights into mitochondrial *nad7* copy in other liverworts; our preliminary data suggest that *nad7* is usually degenerated into a pseudogene by point mutations introducing stops.

Taken together, the data suggest that the increase of chondriome sizes was a very early process in the establishment of embryophytes. The origin of many new intergenic sequences may not be traceable any more due to
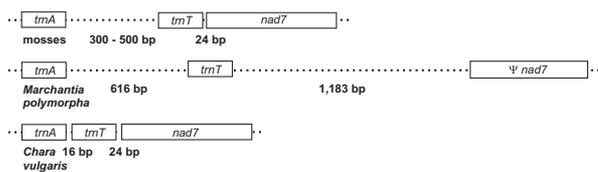

Fig. 3. The mitochondrial *trnA-trnT-nad7* gene arrangement. The gene order is conserved in mosses and liverworts. Size is increased in the *trnA-trnT* spacer in both bryophyte classes, whereas the *trnT-nad7* spacer is enlarged only in liverworts. Spacer sizes are inferred from PCR product length and partial sequencing and taken from the database in the case of *Marchantia polymorpha* (NC_001660) and *Chara vulgaris* (NC_ 005255).

more than 400 million years of sequence drift. The recombinational activity in mitochondrial DNA that is so vast in angiosperms may be a secondary process gained in the NLE lineage, possibly with the emergence of tracheophytes. Plant mitochondrial sequences themselves, but maybe even more the genomic structural changes in land plant chondriomes, could possibly reveal more of early embryophyte diversification than evolution of chloroplast DNA.

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