Distribution and Phylogenetic Significance of the 71-kb Inversion in the Plastid Genome in Funariidae (Bryophyta)

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INTRODUCTION

The structure and size of the plastid genome has been greatly altered since its endosymbiotic origin (Dyall et al., 2004; Hackett et al., 2004; Timmis et al., 2004). Many more genes have been transferred to the nucleus (Martin et al., 1998, 2002; Korpelainen, 2004) or more rarely to the mitochondrion (Nakazano and Hirai, 1993; Zheng et al., 1997) than gained (Goremykin et al., 2002; Hackett et al., 2004). Some gene transfers to the nucleus are rare and hence phylogenetically highly informative (Kelch et al., 2004), whereas others have occurred repeatedly and hence carry a more complex phylogenetic signal (e.g. Lavin et al., 1990; Doyle et al., 1995; Millen et al., 2001).

Other structural rearrangements relate to the size of the inverted repeat, a duplicated region that separates the large and small single copy units (e.g. Plunkett and Downie, 2000), and to the actual order of genes (e.g. Raubeson and Jansen, 1992). Unlike gene losses and changes in the composition of the inverted repeat, inversions of segments comprising several genes appear to be rather rare events (Soltis and Soltis, 1998) and hence compose a class of powerful phylogenetic markers (Rokas and Holland, 2000). For example, a 22.8-kb and a 3.3-kb inversion mark the split between Barnadesioideae and the remainder of Asteraceae, which all share these genomic changes (Kim et al., 2005). Similarly the phylogenetic distribution of a 50-kb inversion in the plastid genomes of Fabaceae (Sasaki et al., 2005) supports the paraphyly of certain suprageneric taxa and suggests that this extensive rearrangement occurred only once during the evolutionary history of Fabaceae (Doyle et al., 1996).

Less is known about the potential systematic significance of genome structural changes in mosses, but early

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indications suggest that this is a fruitful avenue of research. The plastid genome of Physcomitrella patens (Hedw.) Bruch and Schimp. (Sugiura et al., 2003) differs from typical plant genomes in the loss of the DNA-directed RNA polymerase alpha chain gene (rpoA) to the nuclear genome and from genomes of other early land plants such as liverworts and hornworts by a 71-kb inversion in the large single copy (LSC). Although the gene loss was initially considered diagnostic of mosses (Sugiura et al., 2003), Sugita et al. (2004) and Goffinet et al. (2005) demonstrated that the gene was present in the plastid genome of early diverging lineages, such as the peatmosses (Sphagnum), and lost twice during the evolutionary history of Bryophyta. The 71-kb long inverted fragment comprises 57% of the genome and is the largest inversion reported to date in the plastid genome of plants. Sugiura et al. (2003) considered the inversion to be diagnostic of Physcomitrella since a survey of several arthrodontous mosses (i.e. Bryopsis) revealed that the order of genes in their plastid genomes followed a sequence similar to that found in liverworts (Ohyama et al., 1986) and hornworts (Kugita et al., 2003).

Physcomitrella belongs to Funariaceae, a family of terro-cious mosses defined by rather small gametophytes bearing unicostate leaves composed of smooth, lax rectangular cells, and a unique peristomial architecture (Vitt, 1982; Fife, 1985; Shaw et al., 1989). Funariaceae comprise 16 genera (Goffinet and Buck, 2004; Werner et al., 2007), of which three accommodate approx. 90% of the species diversity (Crosby et al., 1999). The family is considered to be closely allied to Disceliaceae (Goffinet and Cox, 2000), which accommodates a single species, the gametophore of which is highly reduced and arises from a persistent proto- nema (Vitt, 1982). Gigaspermaceae, a family of highly specialized mosses, with short foliate branches developing from underground rhizomes and immersed capsules holding large spores, have traditionally been associated with the former families within Funariaceae (e.g. Vitt, 1982, 1984; Thouvenot, 2000). Phylogenetic inferences suggest that Funariales are most closely related to Encalyptales, although their shared ancestry is only weakly to moderately supported (e.g. Goffinet and Cox, 2000; Cox et al., 2004). The phylogenetic affinities within this clade, and in particular those of Gigaspermaceae, are also ambiguous. Indeed, inferences from plastid and nuclear data, resolved with low bootstrap support, Gigaspermaceae as the sister-group to the remainder of Funariales and Encalyptales (Goffinet and Cox, 2000; Goffinet et al., 2001), rather than a member of Funariales. Timmiaceae, which comprise a single genus, Timmia Hedw., may share a unique common ancestor with Funariales and Encalyptales (Cox et al., 2004), but their inclusion in Funariidae (Goffinet and Buck, 2004) also remains unsettled.

Goffinet et al. (2005) reported that the 71-kb inversion characterized not only the genome of Physcomitrella but also that of two other taxa within Funariaceae [i.e. Funaria hygrometrica Hedw. and Entosthodon laevis (Mitt.) Fife] and Encalypta ciliata Hedw. (Encalyptaceae). By contrast, the plastid genome of Timmia lacked the rearrangement. Whether the inversion characterizes all remaining members of Funariidae (i.e. all Funariales and Encalyptales) remained ambiguous. Here, DNA sequences spanning the putative end points of the inversion in the LSC unit are surveyed in various members of Funariidae to assess the distribution and the phylogenetic significance of the inversion in this lineage of mosses.

MATERIALS AND METHODS

Taxon sampling

Exemplars of 13 genera of Funariales (eight Funariaceae, one Disceliaceae and four Gigaspermaceae) and all three genera of Encalyptales (sensu Goffinet and Buck, 2004) were studied for the organization of their plastid genome. The species sampled were: Discelium nudum (Smith 47505; NYSM-Disceliaceae), Aphanorrhigma serrata (Goffinet s.n), Entosthodon bonplandii (Goffinet 6326), E. laevis (Goffinet 5601), E. serratum (Bowers 13109), Funaria flavicans (Goffinet 4798), F. hygrometrica 1 (Goffinet 5576), F. hygrometrica 2 (Goffinet 9278), Funariella curviseta (Ros and Werner 15/1/2006), Goniomitrium acuminatum (Curnow 6532), G. serol (Puche 26/1/2004), Physcomitrella patens (Culture WTC, University of Geneva, Switzerland), Physcomitrium lori- entzii (Goffinet 5348), P. pyriforme 1 (Goffinet 4737), P. pyriforme 2 (Goffinet 9276), Pyramidalula tetragona (Ros et al. 15/3/1997) – all Funariales; Chamaebyrium pottioide (vanRooy 974200 1), Gigaspermum repens 1 (Schofield 90527) Gigaspermum repens 2 (Tyshing s.n.), Lorentziella imbricata (Schinini 24785, NY), Oedipodiella australis (Thouvenot s.n) – all Gigaspermaceae; Bryobartramia nova-alesiae (Magill and Schelpe 3218A), Bryobrittonia longipes (Ignatov 1997, NY), Encalypta armata (Goffinet 5613, DUKE) – all Encalyptales. Catascopiaceseae, which were resolved with poorly supported affinities to Funariales by Goffinet et al. (2001) have now been shown to share a common ancestry with Dicraniiidae (Quandt et al., 2007) and hence are not sampled here. All vouchers are deposited in the herbarium of Duke University (DUKE), unless otherwise indicated. Material adequate for DNA extraction could not be obtained for several exotic and monospecific genera of Funariaceae or for Costesia Théér. (Gigaspermaceae).

DNA extraction, PCR amplification and sequencing

DNA was extracted using the NucleoSpin® Plant kit from Macherey Nagel (Düren, Germany) following the manufacturer’s protocol. The inversion breaks the sequence between the RNA polymerase β chain gene (rpoB) and the gene encoding the tRNA-Cys (trnC_GCA) at the 5’ end, and between the ribosomal protein S11 (rps11) and the cytochrome b6/f complex subunit IV (petD) genes at the 3’ end. To test whether the 71-kb inversion in the gene order that characterized the Physcomitrella genome is present in other taxa, the region spanning both sides of the breakpoint point at the ends of the inversion was targeted with primers originally designed by Sugiura et al. (2003) (rps11F: TTTTGTTCGTAGTGAATCTCATGT; rpoBR:
CTACCATAGCATCCTCAGTAGATT) and several newly designed primers (petN-F2: CCAATTTAACACCCAAOGC; Giga-petD-R2: GTTATCTTGGAGACGC; petD-FunF: CTTTTCGTCGCCAGTAG; rps11-Fun: CATATGGGRTGRGRCCTCC; rpoB-Fun: GGAATACTTCAAATATATAAG GAAACTCTCC and trnC-Fun: GCAATCTCTGCTCCAC). The primers were used in various combinations reflecting the gene arrangements at the ends of the potentially inverted region and the gene order at the 3′ end of the plesiomorphic gene order (see Fig. 1A). The amplification was performed in 25 μL with one unit of Hot Master Taq polymerase (Eppendorf AG, Westbury, NY, USA), 1 μL each of a 10 mM solution of each primer, 1 μL of a 10 mM solution of dNTPs, and a 99.9% pure solution of dimethyl sulfoxide (only with rps11-F-Giga-petD-R2). The annealing temperature was optimized for individual combinations as follow: 52°C for rps11-Fun with rpoB-Fun, rps11F with Giga-petD-R2 and trnC-Fun with petD-FunF; 55°C for petNF2 with petD-FunF and trnC-Fun with petD-FunF; 56°C for rps11F with rpoBR. The amplification followed the same profile in each case: 95°C for 1 min followed by 30 cycles of denaturation (1 min at 95°C), annealing (1 min) extended (1 min at 72°C), and a final extension at 72°C for 7 min. Amplicons were purified using the NucleoSpin® ExtractII kit from Macherey Nagel following the manufacturer’s instructions.

All amplicons were sequenced using the PCR primers and these reactions were performed using the ABI PRISM® BigDye™ Terminators ver. 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) optimized for half- or quarter-size reactions. Sequencing products were purified using Sephadex G-50 (Amersham, Piscataway, NJ, USA) gel filters, and then separated by capillary electrophoresis using an ABI PrismTM 3100 Genetic Analyser. Nucleotide sequences were edited using Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA), entered in PAUP* version 4.0b10 for Macintosh-PPC (Swofford, 2002) and manually aligned to those published for Physcomitrella (Sugiura et al., 2003).

### Table 1. Length of the spacer regions spanning both break points of the 71-kb inversion in Funariidae and their homologous regions in non-inverted genomes

<table>
<thead>
<tr>
<th>FUNARIALES</th>
<th>rps11-rpoB, 5′ end</th>
<th>petD-petN, 3′ end*</th>
<th>trnC-rpoB, 5′ end</th>
<th>rps11-petD, 3′ end</th>
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<td>698 (EF173158)</td>
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<td>Entosthodon bonplandii (Hook.) Mitt.</td>
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<td>685 (EF173153)</td>
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<td>Funaria hygrometrica Hedw. 1</td>
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<td>716 (EF173150)</td>
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<td>Bryobrittonia novae-calediae (Broth. ex G. Roth) I.G. Stone and G.A.M. Scott</td>
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<td>293</td>
<td>(EF173140)</td>
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<td>OUTGROUP TAXA</td>
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<td>526</td>
<td>(AY911401)</td>
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<td>Brachythecium salebrosum (Hoffm. ex F. Weber and D. Mohr) Schimp.</td>
<td>290 (EF173149)</td>
<td>309 (AY911404)</td>
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</table>

All sequences were deposited in GenBank.

—, Amplicon obtained but no sequence available.

* Unless otherwise noted, the petN-petD region includes the petN-trnC spacer, trnC (70 bp) and the trnC-petD spacer.

1 trim-petD spacer only.
or other mosses representing a range of lineages lacking the inversion and the rpoA gene (Goffinet et al., 2005), in order to define genes and intergenic spacers. All sequences were submitted to GenBank (Table 1).

RESULTS

Amplification using primer pairs compatible with the gene order at both ends of the inverted fragment described for Physcomitrella yield products for species of seven additional genera of Funariaceae (Aphanorrhegma, Entosthodon, Funaria, Funariella, Goniomitrium, Physcomitrium and Pyramidula), Disceliaceae (Discelium) and for exemplars of the three genera of Encalyptaceae (Fig. 1B). The length of the rps11-rpoB intergenic spacer varied between 220 and 239 nucleotides. The amplicon obtained for Discelium could only be sequenced in the reverse direction. This sequencing reaction yielded a sequence that included a portion of the petD gene and must but not all of the rps11-rpoB intergenic spacer. The sequence of the intergenic spacer in these taxa could be unambiguously aligned to the published sequence of Physcomitrella. The two exemplars of Physcomitrium pyriforme differ by a single additional T in a poly-T region. No size variation was observed between two accessions of Funaria hygrometrica. The 5′ end of the inversion was targeted using distinct primer pairs that span only the petD-trnC region or the longer petN-petD region, which includes the petN-trnC spacer, the trnC gene (70 bp) and the trnC-petD spacer. An amplicon was obtained from Goniomitrium seroi and Bryobrittonia longipes, but forward and reverse reactions failed to join in the trnC-petD spacer region. Ambiguous base calls seem to be caused by difficulties in sequencing through poly nucleotide or short dinucleotide repeat regions. All other newly generated sequences are complete and align unambiguously with those of Physcomitrella. For none of the exemplars of Funariaceae, Disceliaceae and Encalyptaceae could a PCR product, compatible with a non-inverted architecture, be obtained. Conspecific samples of Funaria hygrometrica differ by two additional adenosines in a poly-A region in the trnC-petD intergenic spacer. Physcomitrium pyriforme I differs from the other exemplar of this species by the insertion of three nucleotides and the deletion of one in the petN-trnC intergenic spacers and the insertion of five adenosines in a poly-A region in the trnC-petD spacer.

Members of the four genera of Gigaspermaceae tested negative for the inversion and positive for the non-inverted
genome architecture (Fig. 1B). Their gene arrangements in regions homologous, in terms of their position, to the end points of the inverted region in Physcomitrella are compatible with the non-inverted type characteristic of other mosses (Fig. 1B). The fragment of the tmnC-rpoB intergenic spacer in Gigaspermaceae varies in length between 264 and 285 bp, which is similar to the range found among the three outgroup taxa screened here (Fig. 1B and Table 1). The sequence of the spacer aligns well across these taxa. Amplification spanning the end point at the 3' end yielded a single band for Chamaebyrum, Lorenziella and Oedipodiella but two for Gigaspermum. The amplicon spanning the rps11 to petD region is much longer than that of most other members of Bryopsida that lack the rpoA gene (results not shown). The rps11-petD amplicon could only be sequenced for Chamaebyrum for which it is 969 bp long compared with 197 bp in Tetraploodon minioides or 720 bp in Tetraphis pellucida (Goffinet et al., 2005). A Blast search for this sequence yielded no match.

DISCUSSION
Gene order in the plastid genome of embryophytes is considered rather conserved (Raubeson and Jansen, 2005). Alterations in the sequence of genes result either from gene losses due to the transfer to the nuclear genome or small permutations. The inversion of 71 kb of the LSC of Physcomitrella (Sugiura et al., 2003) is the largest inversion documented in plants to date. Initially considered diagnostic of Physcomitrella, and then shown to occur in other members of Funariidae (Goffinet et al., 2005), it is here revealed to characterize the genome of all members of Funariales, Disceliaceae and Encalyptales screened in this study. By contrast, species of four genera of Gigaspermaceae, a family traditionally considered closely related to Funariales and Disceliaceae with which they compose Funariales, lack the inversion. Recent phylogenetic inferences suggested that Funariales and Disceliaceae share a most recent common ancestry with Encalyptales rather than Gigaspermaceae (Goffinet et al., 2000; Goffinet et al., 2001). Considered dubious because of the lack of support from nucleotide sequence data alone, this hypothesis was ignored in the most recent classification of mosses (Goffinet and Buck, 2004).

Genomic rearrangements are considered rare and thus phylogenetically highly informative events (Rokas and Holland, 2000). Although this view may be biased due to the paucity of taxa sampled for genomic reconstructions (Goffinet et al., 2005), it may hold true especially for alterations involving large portions of the genome, such as the inversion of a fragment spanning more than half the plastid genome. The inverted order of genes in the genome of Funariales, Disceliaceae and Encalyptales is thus likely to be inherited from a common ancestor that did not give rise to Gigaspermaceae, which lack the inversion. The distribution of the inversion is thus compatible with the hypothesis of Encalyptales being closely related to Funariales and Disceliaceae and of Funariales (including Gigaspermaceae) being paraphyletic (Goffinet and Cox, 2000).

Ordinal affinities of mosses are primarily established based on their peristome architecture (Vitt, 1984; Buck and Goffinet, 2000; Goffinet and Buck, 2004). However, reduction in sporophyte complexity, and hence in peristome differentiation, is rampant in mosses (Vitt, 1981; Zander, 1993; Buck et al., 2000), and consequently the relationships of taxa with reduced morphologies are drawn from other morphological characters, such as those of the gametophyte. Funariales share few apomorphies in the architecture of their vegetative (gametophytic) plants. Vitt (1982) considered not only the lax rectangular cells as diagnostic. The monophyly of Funariales sensu Vitt (1982) and Funariineae sensu Vitt (1984) was first questioned by Goffinet and Cox (2000) who suggested, based on phylogenetic inferences from nuclear and plastid DNA sequences, that Ephemereaceae, a lineage of tiny ephemeral mosses lacking a peristome, should be transferred to Pottiales. Their hypothesis subsequently gained support from ontogenetic studies (Pressel and Duckett, 2005). Gigaspermaceae also share a similar leaf architecture with Funariales (Vitt, 1982), but differ in a suite of putative adaptations to xeric environments. The vegetative gametophyte is stoloniferous, with the creeping stems producing short erect branches. The sporophyte may be dehiscent or not, but in either case, the capsule is gymnostomous (lacking a peristome). Fife (1980) implicitly considered that the two families also differ in the structure of the stoma, with two guard cells defining the pore in Gigaspermaceae, whereas a single, incompletely divided guard cell defines the stoma in Funariales (Vitt, 1980). However, Brotherus (1924) described the stomata of Gigaspermaceae as unicellular, whereas Scott and Stone (1976) and Crum and Anderson (1981) reported the number of guard cells to vary between one and two. In Encalyptales, the stoma are always surrounded by two guard cells (Horton, 1982). The single, so-called doughnut-shaped guard cell could be seen as a synapomorphy for Funarionales sensu Vitt (1982), and hence support the monophyly of the order. However, unicellular stomata occur also in Buxbaumia and Polytrichum (Paton, 1957), and hence are not free of homoplasy. Furthermore, polymorphism in the architecture of the stoma in Gigaspermaceae may leave reconstructions of ancestral states equivocal.

The distribution of the inversion in the plastid genome of Funariales is congruent with the hypothesis that the order is paraphyletic as proposed by Goffinet and Cox (2000), based on phylogenetic inferences from variation in the nucleotide sequence of three loci: Funariales, Disceliaceae (Funariales) and Encalyptaceae (Encalyptales) share a large inversion in their plastid genome that probably occurred in their common ancestor. Gigaspermaceae (Funariales, sensu Goffinet and Buck, 2004), which lack the inversion, are considered to have diverged earlier. For the classification to reflect a phylogenetic scenario wherein Gigaspermaceae comprise the sister group to the remainder of Funariales and Encalyptales, the circumscription of Funariales could be broadened to include Encalyptaceae or, alternatively, Gigaspermaceae could be excluded from Funariales and accommodated in their own order. A third possibility would be to recognize a
paraphyletic Funariales; however, the absence of an unambiguous morphological character uniting Gigaspermaceae to Funariales provides no foundation for such concept. Encalyptales differ from Funariales in virtually all aspects of the vegetative morphology and in the architecture of the peristome. Merging the two orders would obscure the wide morphological divergence between these lineages and hence should be avoided. The exclusion of Gigaspermaceae from Funariales is not significantly incongruent with the phylogenetic signal of any morphological character. Hence we recommend addressing the paraphyly of Funariales sensu Goffinet and Buck (2004) by placing Gigaspermaceae in their own order, Gigaspermales Goffinet, Wickett, O. Werner, Ros, A.J. Shaw and C.J. Cox ord. nov. (Plantae terrestres stoloniferae ramis brevibus erectis, folia unicostata cellulis laxis laevibus, peristomium destitutum; Type genus: Gigaspermum Lindh., Öfversigt af Förhandlingar: Kongl. Svenska Vetenskaps-Akademi 21: 599. 1865).

A hypothesis of a shared ancestry for Gigaspermales, Funariales and Encalyptales emanates, if only with weak support, from various phylogenetic analyses of nucleotide sequences (Goffinet and Cox, 2000; Goffinet et al., 2001; C. J. Cox et al., Natural History Museum, London UK, unpubl. res.). This combined lineage exhibits a wide range of morphology, and no unambiguous morphological synapomorphy has been identified. The shared ancestry may be supported by ontogenetic data and, in particular, patterns of cell division in the inner peristome forming layer (Goffinet et al., 1999) but critical developmental studies of the sporophyte of Gigaspermales would be required to substantiate this hypothesis.

In conclusion, the inversion of an extensive fragment of the LSC of the plastid genome is considered to have occurred once (Fig. 1C) in the ancestor to Funariaceae, Disceliaceae and Encalyptales. This genomic change strengthens the weak phylogenetic signal extracted from sequences of two plastid loci and one nuclear locus, whereby Funariales and Encalyptales sharing a unique common ancestor that did not give rise to Gigaspermaceae. To reflect such evolutionary history, Gigaspermaceae are accommodated in their own order. Funariales continue to emerge from recent phylogenetic reconstructions (Goffinet and Cox, 2000; Goffinet et al., 2001) as a crown group of an early diverging lineage rather than the closest extant relative of the ancestor to the vast majority of true mosses as hypothesized by Vitt (1984).

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LITERATURE CITED


