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# Organellar Inheritance in Liverworts: An Example of *Pellia borealis*

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**Abstract.** Liverwort *Pellia borealis* is an allopolyploid species that originated after the hybridization and chromosome doubling of two cryptic species; *Pellia epiphylla* species N and *Pellia epiphylla* species S. A sequence comparison of chloroplast $tRNA^{Gly}_{UCC}$, $tRNA^{Lys}_{UUU}$ gene introns, the mitochondrial $tRNA^{Ser}_{GCU}$ gene intron, and the first intron of the coxIII gene in the case of three liverwort species studied revealed that the chloroplast and mitochondrial sequences are identical in *P. borealis* and *P. epiphylla* species N but different from homologous *P. epiphylla* species S sequences. Both mitochondria and chloroplasts of *P. borealis* were thus inherited from one parent—*P. epiphylla* species N. Studies on 14 different populations of *P. borealis* gave the same result. These are the first data on organellar transmission in liverworts, the earliest land plants. Moreover, we show that the intron sequences of some organellar genes, until now not used in any systematic studies, could be very good markers in studying taxonomic relationships in closely related species and reconstructing historical events.

**Key words:** Liverworts — Allopolyploid — Chloroplast genome — Mitochondrial genome — Organellar inheritance — Nucleotide substitutions — Indels

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## Introduction

Organellar inheritance has been studied using cytological and genetic approaches in many plants. Depending on the plant species studied, different patterns of organelle transmission have been reported. Transmission of chloroplasts and mitochondria in angiosperms appears to be generally uniparental and maternal (Rajora 1992; Gillham 1994; Small and Wendel 1999), however, in some genera (*Oenothera*, *Pelargonium*, *Medicago*) biparental inheritance of plastids and maternal inheritance of mitochondria have been observed (Mogensen 1996). In gymnosperms, genera such as *Ephedra* and *Gingko* exhibit mainly maternal uniparental inheritance of both types of organelles. However, in the case of conifers, transmission of plastids and mitochondria from the male parent has been reported as exclusive or predominant, except for the Pinaceae and Taxaceae families, where maternal inheritance of mitochondria has been shown (Dong and Wagner 1994; Mogensen 1996). In the case of the ferns *Pteris vittata* and *Marsilea*, maternal, inheritance of organelles has been documented (Kuroiwa 1991). Data concerning organellar transmission in algae show that in algae that produce isogametes the process is uniparental, and in the case of anisogamous and oogamous algae the process is maternal uniparental. Thus the major type of organellar inheritance in plants seems to be uniparental and, as postulated, is the oldest character state for organelle genomes (Law and Hutson 1992; Gillham 1994; Birky 1995, 2001).

*Pellia borealis* (*Hepaticae*) is a tetraploid species ($n = 18$ in gametophyte) belonging to the subdivision

*Bryophyta*. This species originated, as shown in earlier studies, via hybridization and duplication of chromosome sets of two cryptic liverwort species: *Pellia epiphylla* species N ($n = 9$) and *Pellia epiphylla* species S ($n = 9$) (Odrzykoski et al. 1996; Fiedorow and Szweykowska-Kulińska 1998a; Pacak et al. 1998; Fiedorow et al. 2001). It is important to mention that the hybridization of both *P. epiphylla* species could have taken place only in the past: Nowadays, both species are allopatric. *P. epiphylla* species N is present only in the north of Poland, while *P. epiphylla* species S is present only in the southern part of Poland. *Pellia borealis* occurs frequently in all parts of Poland and seems to be much more restricted in other parts of Europe (Szweykowski et al. 1995). The aim of this study was to solve the problem of mitochondrion and chloroplast origin in this allopolyploid species. Do the organelles originate from *P. epiphylla* species N or *P. epiphylla* species S? It is possible to answer this question using nucleotide sequences from chloroplast and mitochondrial genomes of *P. borealis*, *P. epiphylla* species N, and *P. epiphylla* species S. The answer to this question is quite important since there is no information concerning chloroplast and mitochondrion inheritance in *Bryophyta*. We present the first such evidence and show that, at least in some liverwort species, organellar transmission is uniparental. These data are interesting since liverworts are thought to be the earliest land plants (Qiu 1998).

## Materials and Methods

### *In Vitro Culture of Liverworts*

Apical parts of thalli were sterilized in 0.5% sodium hypochlorite solution for 15 min. Sterilized sections of thalli were put on a special modified liverwort medium containing, per 1 liter: $NH_4NO_3$, 0.12 g; $KH_2PO_4$, 0.7 g; $MgSO_4 \times 7H_2O$, 0.246 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.03 g; sucrose, 10 g and agar, 10 g. The agar medium was adjusted to pH 5.0 (Lukavsky et al. 1991). We also used 0.5 × Murashige and Skoog medium, pH 5.64 (Murashige and Skoog 1962; Fiedorow and Szweykowska-Kulińska 1998b) of *in vitro* liverwort culture.

### *Total Genomic DNA Isolation*

Total DNA was isolated from 100 mg of plant tissue from *in vitro* culture. Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) procedure. Usually 1.5–3 μg of DNA was obtained. DNA was also extracted from herbarium material (dried) of *Pellia* species using a modified DNeasy Plant Mini Kit (QIAGEN) procedure. The major modification refers to the initial preparation of the dried material: 20 mg of liverwort thalli was ground in a preheated mortar (65°C) until a fine powder was obtained. Further steps of DNA isolation were recommended in the QIAGEN protocol. The yield varied around 0.5 μg.

### *Polymerase Chain Reaction (PCR)*

The PCR mixture contained the following components for 10-μl reactions: 7.5 ng of total DNA, a 0.5 μ*M* concentration of primer A, a 0.5 μ*M* concentration of primer B, 1 μl of 10 × PCR buffer supplied by QIAGEN, 1 m*M* spermidine (Fiedorow and Szweykowska-Kulińska 1997), a 200 μ*M* concentration of each dNTP, and 0.25 U of Taq DNA plolymerase (QIAGEN). PCR was initiated by denaturation at 95°C for 5 min and followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 1 min. The reactions were ended by elongation at 72°C for 5 min. Primers used for PCR reactions were based on complete mitochondrial genome (accession No. M68929) and chloroplast genome (accession Nos. X04465, Y00686) sequences from the liverwort *Marchantia polymorpha* (Ohyama 1996) and represent exon fragments of studied genes.

Primers for PCR amplification were as follows:

For the intron sequence of the chloroplast $tRNA^{Leu}_{(UAA)}$ gene:
A primer, 5′ GGG GGT ATG GCG AAA TTG G 3′;
B primer, 5′ TGG GGG TAG AGG GAC TTG 3′.

For the intron sequence of the chloroplast $tRNA^{Gly}_{(UCC)}$ gene:
A primer, 5′ CGG GTA CGG GAA TCG AAC 3′;
B primer, 5′ GCG GGT ATA GTT TAG TGG 3′.

For the intron sequence of the chloroplast $tRNA^{Lys}_{(UUU)}$ gene:
A primer, 5′ AAC TCA ATG GTA GAG TAC TC 3′;
B primer, 5′ GGC TCG AAC CCG GAA CTC 3′.

For the intron sequence of the mitochondrial $tRNA^{Ser}_{(GCU)}$ gene:
A primer, 5′ GGA GGT ATG GCT GAG TGG 3′;
B primer, 5′ GAG GAA ATG GGA TTT GAA CC 3′.

For the first intron sequence of the mitochondrial coxIII gene:
A primer, 5′ GGA GGC GGA ACA CTT CTT TG 3′;
B primer, 5′ GGA CCA CAA ATG TAT GAT GTC 3′.

PCR products were separated on 0.8% agarose gel in 1 × TBE and purified using the QIAquick Gel Extraction Kit (QIAGEN).

### *Restriction Fragment Length Analysis*

PCR products of chosen intron sequences were digested using the Tsp509I enzyme. Digestion reactions were carried out according to the recommended protocols (New England BioLabs; Tsp509I) and the digestion products were separated electrophoretically on an 8% polyacrylamide and an 0.8% agarose gel, stained with ethidium bromide, and analyzed under an UV lamp.

### *Cloning, Sequencing, and Sequence Analysis*

PCR products were cloned using the pGEM-T Easy Vector System (Promega). DNA sequencing of direct PCR products and PCR products cloned in plasmid was carried out using the *fmol* DNA Cycle Sequencing System (Promega) and the CEQ 2000 Dye Terminator Cycle Sequencing Kit from Beckman Coulter using CEQ Dye Terminators and a CEQ 2000XL sequencer. PCR products of all genes studied were sequenced, both directly and after cloning in pGEM-T Easy Vector. Sequencing was always for two different populations of each liverwort species.

Alignments of nucleic acid sequences were constructed using the Clustal X program (version 1.64b and 1.81) (Thompson et al. 1997) with default setting of parameters, and then irregular insertion of gaps was modified manually. The alignments for phylogenetic study have been deposited and are available in the Popset (GenBank Database).

## Results

To study the problem of organellar inheritance in the allopolyploid species *Pellia borealis*, we decided to sequence and analyze intron nucleotide sequences from genes in the chloroplast and mitochondrial ge-

**Table 1.** Intron lengths of the organellar genes studied from *P. epiphylla* species S, *P. epiphylla* species N (sequences identical to that in *P. borealis*), and *M. polymorpha*; number of nucleotide substitutions, deletions, and insertions (indels) in intron sequences of the chloroplast $tRNA^{Gly}_{UCC}$ and $tRNA^{Lys}_{UUU}$ genes, mitochondrial $tRNA^{Ser}_{GCU}$ gene, and first intron of the coxIII gene of the two parental species, *P. epiphylla* species N and *P. epiphylla species S;* and number of nucleotide substitutions per nucleotide site (Nei 1987) in sequenced chloroplast and mitochondrial genes of *P epiphylla species S* and *P. epiphylla* species N

| | Organellar gene | | | | |
|---|---|---|---|---|---|
| | cp-$tRNA^{Leu}_{UAA}$ | cp-$tRNA^{Gly}_{UCC}$ | cp-$tRNA^{Lys}_{UUU}$ | cp-$tRNA^{Ser}_{GCU}$ | Intron 1 from coxIII gene |
| | Intron length (nucleotides) | | | | |
| *M. polymorpha* | 315 | 593 | 2111 | 991 | 907 |
| *P. epiphylla* species S | 310 | 741 | 2152 | 1315 | 1036 |
| *P. epiphylla* species N | 310 | 738 | 2156 | 1316 | 1035 |
| Nucleotide sequence differences in *P. epiphylla* species S compared to the other parental species *P. epiphylla species N* | | | | | |
| Deletions | 0 | 1 | 1 | 3 | 5 |
| Insertions | 0 | 2 | 0 | 3 | 6 |
| Indels | 0 | 3 | 1 | 6 | 11 |
| Substitutions | 0 | 6 | 9 | 0 | 1 |
| | Number of nucleotide substitutions per nucleotide site | | | | |
| | 0 | $8.11 \times 10^{-3}$ $\approx 6.14 \times 10^{-3}$ | $4.18 \times 10^{-3}$ | 0 | $0.96 \times 10^{-3}$ |

nomes and, additionally, from the two cryptic species from which it originated, *Pellia epiphylla* species N and *Pellia epiphylla* species S. Intron sequences are known to exhibit the nucleotide substitution rate slightly lower than fourfold degenerate sites which show the highest rate within the gene (Li 1997). We first searched the complete chloroplast and mitochondrial genome sequences of *Marchantia polymorpha* for genes containing introns (Oda et al. 1992; Ohyama 1996). We anticipated that the same genes should contain introns in *Pellia* species chloroplast and mitochondrial genomes too. Based on our search, we chose the following five organellar genes for sequencing: chloroplast $tRNA^{Leu}$ (anticodon UAA), $tRNA^{Gly}$ (UCC), and $tRNA^{Lys}$ (UUU) genes, the mitochondrial $tRNA^{Ser}$ (GCU) gene, and the first intron of the coxIII gene. According to the GenBank Database search transfer RNA genes mentioned above and the coxIII gene are present in single copies in organellar genomes in *M. polymorpha* and all higher plants studied. All tRNA genes contain a single intron, while the coxIII gene contains two introns. Table 1 compares the length of homologous introns in *M. polymorpha*, *P. borealis*, *P. epiphylla* species N, and *P. epiphylla* species S. In the case of the chloroplast $tRNA^{Leu}$ and $tRNA^{Lys}$ genes, the *M. polymorpha* and *Pellia* introns are of similar length, while the $tRNA^{Gly}$ and $tRNA^{Ser}$ genes and the first intron of the coxIII gene (one chloroplast and two mitochondrial) are notably longer in the *Pellia* species than in *M. polymorpha*. The intron positions in the homologous genes studied are always the same: in the chloroplast $tRNA^{Leu}$ gene, the intron (which is a group I intron) interrupts the first and the second position of the anticodon; in the $tRNA^{Gly}$ gene, the intron (a group II intron) interrupts the DHU stem; in the $tRNA^{Lys}$ gene, the intron (group II) is localized at the 3′ boundary of the anticodon loop and stem; and in the case the mitochondrial $tRNA^{Ser}$ gene, the intron (group II) is localized in the anticodon stem, between position 43 and position 44. Figure 1 shows the intron positions in the cloverleaf structures of liverwort pre-tRNAs derived from tRNA genes studied and exon–intron structure of the coxIII gene. Inspection of the GenBank nucleotide sequences shows a conervative intron position in $tRNA^{Leu}$, $tRNA^{Gly}$, and $tRNA^{Lys}$ genes in all available plant chloroplast genomes. Moreover, comparing sequences in the GenBank database with our data revealed that the presence of an intron in the mitochondrial $tRNA^{Ser}$ gene is unique to *Bryophyta* [it has been found in liverworts from genus *Pellia* and *Porella* and peatmosses *Sphagnum rubellum* and *Sphagnum subsecundum* (unpublished data)].

The comparison of the $tRNA^{Leu}$ intron nucleotide sequence for *P. borealis*, *P. epiphylla* species N, and *P. epiphylla* species S showed these sequences to be identical. The $tRNA^{Gly}$ and $tRNA^{Lys}$ intron sequences of *P. epiphylla* species S differed from those of *P. borealis* and *P. epiphylla* species N (which are identical). This supports the idea that the chloroplast of *P. borealis* originated from *P. epiphylla* species N. As for the chloroplast genes, mitochondrial intron sequences from the $tRNA^{Ser}$ and coxIII genes of *P. epiphylla* species S showed differences in nucleotide sequences compared with those of *P. borealis* and *P. epiphylla* species N. This suggests that *P. borealis* mitochondria also derive from *P. epiphylla species N*

Table 1 summarizes the numbers of nucleotide substitutions, deletions, and insertions (indels) in the

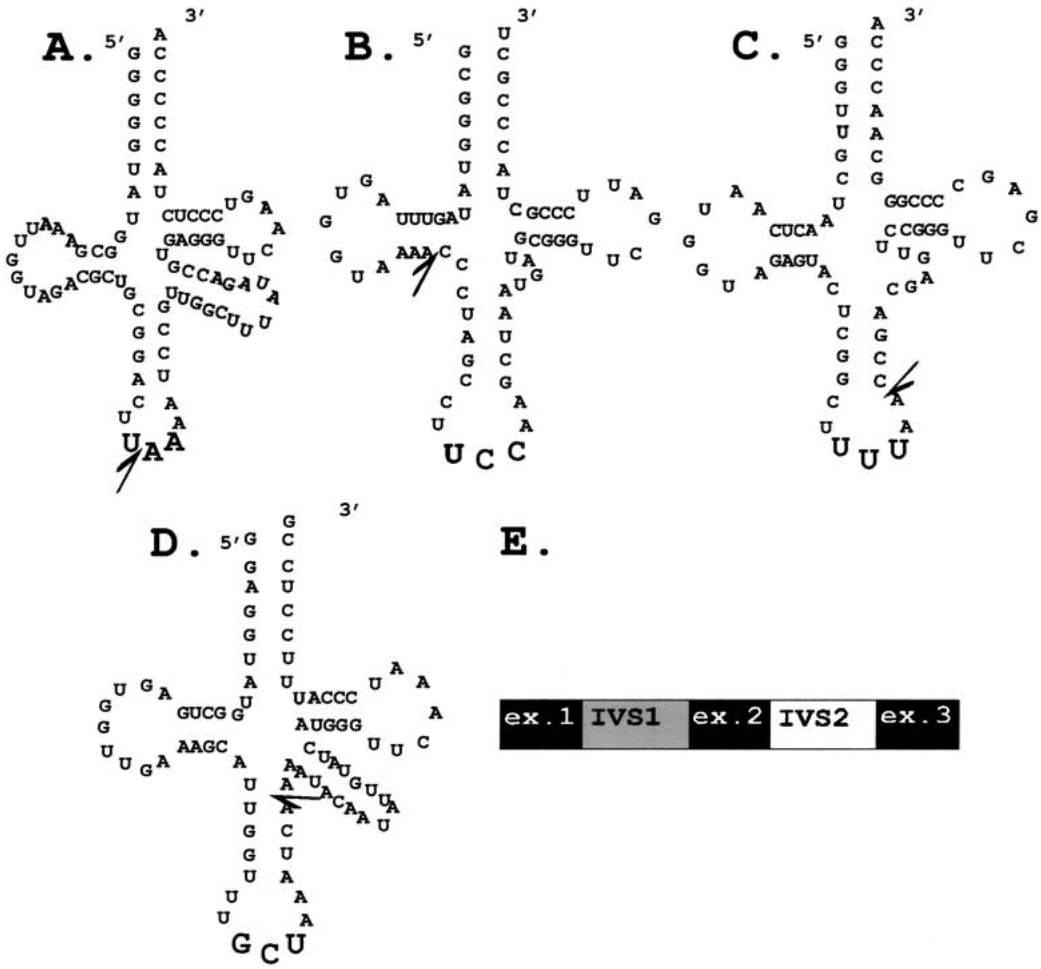


**Fig. 1.** Intron positions in the cloverleaf structures of pre-tRNAs from *Pellia* species studied, derived from (**A**) the chloroplast $tRNA^{Ieu}_{UAA}$ gene, (**B**) the chloroplast $tRNA^{Gly}_{UCC}$ gene, (**C**) the chloroplast $tRNA^{Lys}_{UUU}$ gene, and (**D**) the mitochondrial $tRNA^{Ser}_{GCU}$ gene. (**E**) The exon–intron structure of the mitochondrial coxIII gene. The shadebox IVS 1 represents the intron studied.

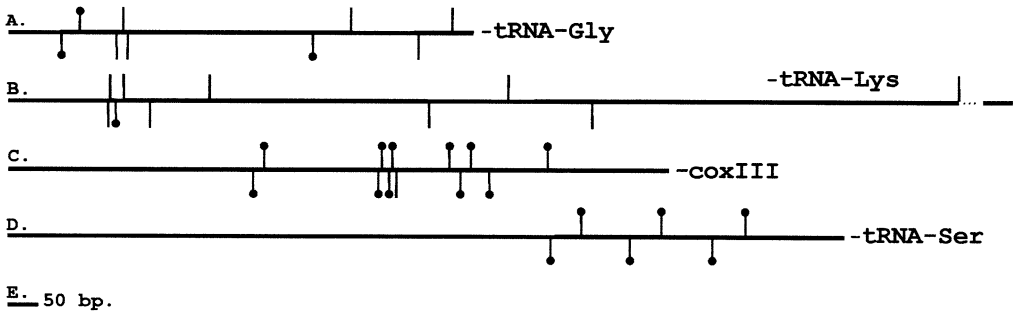


**Fig. 2.** Diagram showing the distribution of the differences in the intron nucleotide sequences of the chloroplast $tRNA^{Gly}_{UCC}$ gene (**A**), $tRNA^{Lys}_{UUU}$ gene (**B**), mitochondrial coxIII gene, first intron (**C**), and $tRNA^{Ser}_{GCU}$ gene (**D**) from *P. epiphylla* species S and *P. epiphylla* species N. The drawing is approximately to scale, with nucleotide substitutions shown as *vertical lines* and indels shown as *vertical lines with end dots*. Accession numbers in GenBank Database: cp-$tRNA^{Gly}_{UCC}$—*P. epiphylla* species S, AF240161; *P. borealis*, AF240473; and *P. epiphylla* species N, AF217210; cp-$tRNA^{Lys}_{UUU}$—*P. epiphylla* species S, AF238497; *P. borealis*, AF238498; and *P. epiphylla* species N, AF238496; mt-coxIII, intron 1—*P. epipylla* species S, AF443198; *P. borealis*, AF441788; and *P. epiphylla* species N, AF443199; and mt-$tRNA^{Ser}_{GCU}$—*P. epiphylla* species S, AF242357; *P. borealis*, AF244576; and *P. epiphylla* species N, AF242358.

intron sequences from the mitochondrial and chloroplast genes of the parental liverwort species studied. In the case of chloroplast genes, the number of nucleotide substitutions is much higher than the number of indels. In contrast, in mitochondrial genes studied we observed only one substitution and

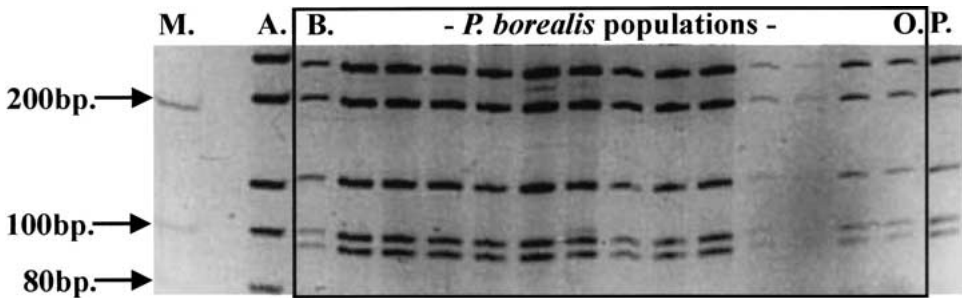


**Fig. 3.** Electrophoretic separation on an 8% polyacrylamide gel of chloroplast $tRNA^{Gly}$ genes digested with Tsp5091. M., DNA molecular weight marker GeneRuler 100-bp DNA ladder, MBI Fermentas. (**A**) *P. epiphylla* species S 38446; (**B–O**) different populations of *P. borealis* [numbers 3–16 in Table A1 (Appendix)]; (**P**) *P. epiphylla* species N 37729

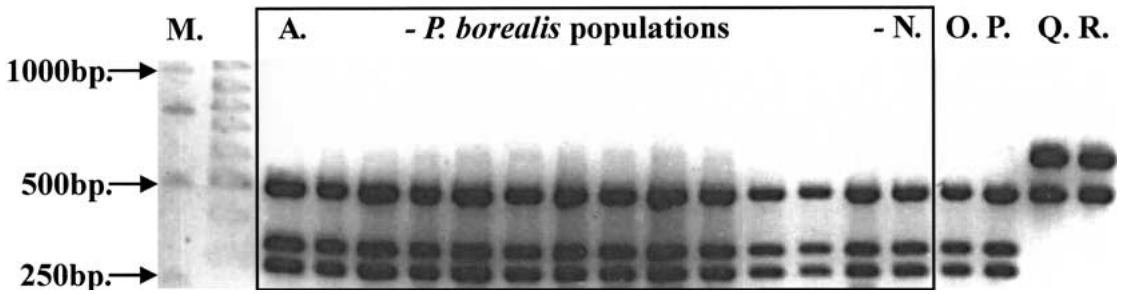


**Fig. 4.** Electrophoretic separation on an 0.8% agarose gel of the part of the mitochondrial coxIII gene containing intron 1 digested with Tsp509I. M. marker GeneRuler1-kb DNA ladder, MBI Fermentas. (**A–N**) Different populations of *P. borealis* [numbers 3–16 in Table A1 (Appendix)]; (**O,P**) *P. epiphylla* species N (numbers 17 and 18 in Table A1); (**Q,R**) *P. epiphylla* species S (numbers 1 and 2 in Table A1).

17 indels. Figure 2 shows the distribution of the nucleotide sequence differences in the introns studied from *P. epiphylla* species S and *P. epiphylla* species N. Comparison of the number of nucleotide substitutions per nucleotide site (Nei 1987) in the case of chloroplast and mitochondrial intron sequences (Table 1) shows that its value is six times higher in chloroplast than in mitochondrial genome. In angiosperms mtDNA evolves at least three to four times slower than cpDNA (Wolfe 1987; Palmer and Herbon 1988; Palmer 1990). Based on the nucleotide sequence differences in the introns of the genes investigated, restriction sites for enzyme Tsp509I have been found that generate DNA fragments of different length from PCR products derived from *P. epiphylla* species S from one site and *P. borealis* and *P. epiphylla* species N from the other site. To find out whether different *P. borealis* populations show the same pattern of organellar inheritance, we collected 14 populations of *P. borealis* from various regions, covering the whole of Poland. Figures 3 and 4 show examples of restriction fragment patterns derived from the $tRNA^{Gly}$ and coxIII introns, respectively. *P. borealis* populations studied have *P. epiphylla* species N as the donor of their mitochondrial and chloroplast genomes. The same results were achieved when the introns form $tRNA^{Lys}$ and $tRNA^{Ser}$ were studied using restriction endonucleases (data not shown).

## Discussion

Our data show that both mitochondria and chloroplasts of *P. borealis* are derived from one parent—*P. epiphylla* species N, and that organellar inheritance is uniparental. However, our studies do not show whether this inheritance occurred via a maternal or a parental line. Either is possible, based on current knowledge of bryophyte gametes. Typically male gametes posses only five organelles: a cylindrical nucleus, an anterior mitochondrion, a posterior mitochondrion, a plastid, and a locomotory assemblage. In liverworts the anterior mitochondrion is associated with the "multilayered structure" and the posterior mitochondrion is situated between the plastid and the nucleus, near the cell posterior. In at least some liverworts, the posterior part of a male gamete is discarded before fertilization. However, it is possible that the anterior mitochondrion gets into the egg. Also we cannot exclude the possibility that male gamete plastids enter the female gamete (Duckett et al. 1983; Renzaglia and Garbary 2001).

The organelle inheritance pattern in 14 different populations of *Pellia borealis* collected from different parts of Poland suggests, in all cases, that both mitochondria and chloroplasts originated from the same species *P. epiphylla* species N. This result can be explained in two ways: (i) either all populations of *P. borealis* derived from a single hybridization event

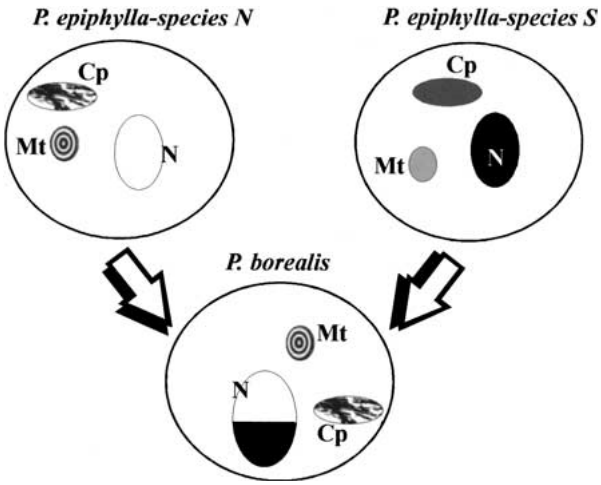


**Fig. 5.** The origin of chloroplast and mitochondria in the allopolyploid species *Pellia borealis*.

or (ii) hybridization took place several times, but in all cases *P. epiphylla* species N was the donor of organelles.

Figure 5 summarizes historical events that led to the creation of *Pellia borealis* species.

Our work presents the first case of organellar transmission in liverworts (that belong to nonflowering plants). Moreover, these studies have been done using genetic data determining the mode of inheritance of organellar genes. We have proven that *Pellia borealis* obtained both types of organelles from one parent. Could it be a general mode of organellar transmission in liverworts? In our laboratory, we have also studied another example of an allopolyploid liverwort species, *Porella baueri*. Using the same approach as presented in this work, we have found out that also in this case both types of organelles were inherited from one parent—namely—*Porella cordaeana* (data under preparation for publication).

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## Appendix

**Table A1.** List of samples, with their collection number (No.) and voucher number (No*.) (in herbarium POZW)

| No. | No.* | Locality[a] | Year | c[b] |
|---|---|---|---|---|
| | | *Pellia epiphylla* species S ($n = 9$) | | |
| 1 | 32816 | SE Poland | 92 | S.J. et al. |
| 2 | 38446 | S Poland | 96 | O.I. |
| | | *Pellia borealis* ($n = 18$) | | |
| 3 | 36928 | N Poland | 94 | S.J. et al. |
| 4 | 35822 | N Poland | 94 | S.J. et al. |
| 5 | 36918 | N Poland | 95 | B.A., B.K. |
| 6 | 39189 | W Poland | 00 | B.K., P.A. |
| 7 | 39190 | W Poland | 00 | B.K., P.A. |
| 8 | 39209 | W Poland | 00 | B.K., P.A., S.J. |
| 9 | 39213 | W Poland | 00 | B.K., P.A., S.J. |
| 10 | 39216 | SW Poland | 00 | L.M. |
| 11 | 39259 | S Poland | 00 | B.K., P.A. |
| 12 | 39549 | S Poland | 01 | C.E. |
| 13 | 34377 | NW Poland | 93 | S.J. |
| 14 | 35287 | W Poland | 93 | B.K., S.J. |
| 15 | 35270 | SW Poland | 93 | B.K., S.J. |
| 16 | 34908 | SE Poland | 93 | B.K., S.J. |
| | | *Pellia epiphylla* species N ($n = 9$) | | |
| 17 | 37729 | N Poland | 96 | B.K. |
| 18 | 37730 | N Poland | 96 | B.K. |

[a] For detailed localization see Pacak et al. (1998) and Fiedorow et al. 2001.

[b] Collector initials: B.K., Buczkowska K.; B.A., Bączkiewicz A.; C.E., Chudzińska E., L.M. Lembicz M.; O.I., Odrzykoski I.; P.A., Pacak A.; S.J., Szweykowski J.

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