

Article

Characterization and Phylogenetic Analysis of Chloroplast and Mitochondria Genomes from the Antarctic Polytrichaceae Species *Polytrichum juniperinum* and *Polytrichum strictum*

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Abstract: In this study, the organelle genomes of *Polytrichum juniperinum* Hedw. and *Polytrichum strictum* Menzies ex Brid. (Polytrichaceae, Bryophyta) from Antarctica were sequenced and compared with the plastomes of the model moss species *Physcomitrella patens* Brid. The sizes of the cpDNA in *P. juniperinum* and *P. strictum* were estimated to be 55,168 and 20,183 bp, respectively; the sizes of the mtDNA were 88,021 and 58,896 bp, respectively. The genomes are very similar to each other, with the possible loss of petN in the cpDNA, which also showed some gene inversions when compared with the cpDNAs of *P. patens* Brid. In the mtDNA, it is possible that rps10 was lost. In contrast, Antarctic Polytrichaceae species have nad7 and orf187, without the occurrence of rearrangement events. Phylogenomic analyses of the plastid and mitochondria revealed that the majority-rule tree suggests some differences in the plastids ancestry, however, *P. juniperinum* and *P. strictum* were grouped in the same clade in chloroplast, but in mitochondria *P. strictum* was grouped with *Atrichum angustatum* (Brid.) Bruch & Schimp. This study helped us understand the evolution of plastomes and chondriosomes in the family Polytrichaceae, and suggest a hybridization event with relation to the mitochondrial data.

Keywords: phylogenomics; mosses; bipolar species; cryptic species

1. Introduction

Polytrichum is a cosmopolitan genus with a bipolar distribution [1] (from the Arctic lands to the Antarctic Continent). In the Antarctic, three species have been reported, all of which are confined to the maritime Antarctic: *Polytrichum juniperinum* Hedw., *Polytrichum piliferum* Hedw., and *Polytrichum strictum* Menzies ex Brid [2]. They play an important role in the terrestrial vegetation of this biome as essential constituents in various communities of moss turf subformations as well as fruticose lichens [1]. The phylogenetic relationships of Polytrichales are particularly relevant when considering the evolutionary history of mosses, since the group is probably one of the first lineages to diverge from the common ancestor of all mosses [3,4]. A recent development in plastome sequencing is the use of total genomic DNA as the template for next-generation sequencing [5,6]. The outcome of

this new development was huge improvements in our understanding of the phylogenetic relationships among plants, particularly mosses. A previous study had suggested that *P. strictum* arose from a reticulation event and *P. juniperinum* is probably its maternal ancestor [4]. However, the phylogenetic position of *P. strictum* is still unclear. Thus, understanding these relationships is necessary to expand the quantity and quality of phylogenetic molecular data available to evaluate the relationships between *P. juniperinum* and *P. strictum* in the Antarctic.

2. Materials and Methods

Gametophyte samples of *P. juniperinum* (62°12'41.93" S and 58°55'44.61" O) and *P. strictum* (62°12'37.36" S and 58°57'49.87" O) were collected from Ardley Island during the austral summer of 2014–2015, Brazilian Antarctic Expedition XXXIII (2014–2015). Part of the samples were incorporated into the Bruno Irgand Herbarium (HBEI) of UNIPAMPA/São Gabriel, under the voucher HBEI 059 and HBEI 060 to *P. juniperinum* and *P. strictum* respectively, the remaining samples were used for the analyzes foreseen in the present work. Total genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction procedure, as described by Shaw [7]. After the DNA extraction, the samples were evaluated with NanoVue™ Plus Spectrophotometer (GE Healthcare, Chicago, IL, USA) and Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) to ensure the quality and quantity of the samples. This DNA was sheared into fragments averaging approximately 250 bp and then, genome sequencing of the Polytrichaceae DNA samples was performed using the Ion Torrent PGM platform (Life Technologies, Carlsbad, CA, USA). In the same conditions, three genomic-DNA libraries were prepared using the Ion One Touch Template Kit (Life Technologies, Carlsbad, CA, USA) (Table 1). The amplified library was sequenced using Ion PGM™ Hi-Q™ Sequencing Kit within the 318 Chip. The three libraries were concatenate with cat command line and a total of 16,333,496 reads from *P. juniperinum* and 16,679,733 reads from *P. strictum* (maximum length, 389 bp and mean length, 170 bp) from single-end type were sequenced (Table 1). The best cut-off values for low quality reads were estimated using the FastQC quality control tool [8]. Then, the reads were filtered for quality using a standard approximating Phred quality score (Q20) in both species set reads with the tool disponible in FASTX Toolkit in the Galaxy platform (<https://mississippi.snv.jussieu.fr>), decreasing the likelihood of low quality reads in contigs assembly. Assembly of the contigs was performed using the Velvet Assembler for short reads [9] utility cpDNA (NC_005087.1) and mtDNA (NC_007945.1) of *Physcomitrella patens* as reference, and the best Kmer estimated in 25 (*P. juniperinum*) and 27 (*P. strictum*) by Kmergenie [10]. Scaffold assembly for cpDNA and mtDNA was performed using the Scaffold Builder assembler version 2.2 and the chloroplast and mitochondria from *P. patens* as the reference genomes [11]. Annotation of the chloroplast was performed using web-based Dual Organellar Genome Annotator (DOGMA) [12] and same parameters adjusted (percent identity cut-off for protein-coding genes estimated in 25; percent identity cut-off for RNAs estimated in 25; e-value estimated in 1×10^{-5}) and cpGAVAS [13] with e-value estimated in 1×10^{-5} Mitochondrial annotation was performed using Mitofy version 1.3.1 of tRNAscan-SE and version 2.2.28 of National Center for Biotechnology Information-Basic Local Alignment Search Tool (NCBI BLAST) [14]. Annotation of cpDNA and mtDNA genes was manually corrected by comparison with complete chloroplast and mitochondrial genomes of other bryophytes using BLASTn [15]. The species were compared with the reference genomes of *P. patens* for generating chloroplast and mitochondrial circular maps for coverage visualization, gene content, and presence/absence of genes with Blast Ring Image Generator (BRIG) [16] 0.95. For the phylogenetic analyses, individual alignments were performed for each gene by using Molecular Evolutionary Genetics Analysis Software (MEGA 5.05) [17] and all alignments were concatenated with sequence matrix 1.883 to create a super-alignment. The best model for nucleotide substitution, TN93 model, was established using MEGA 5.05 and adjusted in jModelTest [18] for each gene alignment. The tree was created on the basis of Bayesian statistical analysis with 10,000,000 million Monte Carlo Markov chains to avoid errors in the posterior probability support of the BEAST package [7]. The base frequency was estimated, and the dataset was partitioned (e.g., codon positions)

into two partitions (1 + 2), 3 with BEAUti (BEAST package). The majority rule tree was constructed with TreeAnnotator (BEAST package). The support of the nodes was calculated through posterior probability that varies from 0 to 1. Frequency convergence of the trees and 25% burn-in were confirmed with Tracer (BEAST package) and this program was used to estimate when the sampling of the trees was stabilized. The divergence dates were obtained considering the estimated date for bryophyte origin [19].

3. Results

3.1. Sequence Data

A set of 16,333,496 single-end reads of *Polytrichum juniperinum* and 16,679,733 single-end reads of *Polytrichum strictum* was generated with a mean length of 170 bp from a 3/3 run on an Ion Torrent sequencer in PGM platform. Read quality was satisfactory, with a low ratio of duplicates (2%). The representation of the two plant cell genomes in the data were as follows: 103,776 reads were mapped to the chloroplast genome of *P. juniperinum* with a coverage level of 10083, and 32,931 reads to the mitochondria of *P. juniperinum* with coverage level of 2988; 58,624 reads to the chloroplast of *P. strictum* with a coverage level of 1890, and 29,620 reads to the mitochondria genome of *P. strictum* with a coverage level of 26,564. Details from reads and libraries can be visualized in Table 1. *Physcomitrella patens* (Funariaceae) was chosen as a reference species for the assembly of the genome of chloroplasts and mitochondria due mainly to the availability of complete and updated genomic data, as well as to its relatively proximate phylogenetic position with the Polytrichaceae family. The obvious choice as a reference would be of a genome from a same family species, but the lack of complete genomic data for species closer to *Polytrichum* makes this strategy impracticable. *Atrichum angustatum* (Brid.) Bruch & Schimp. (Polytrichaceae) has its mitochondrial genome sequenced, but we can not include it only in the assembly of the mitochondrial genome, this could raise misinterpretations about our inference between presence/absence genes in *Polytrichum* and other species of this family.

3.2. Genomic Organization and Gene Content

After assembly, the *Polytrichum juniperinum* plastid genome (cpDNA) obtained was 55,168 bp in length and had a G + C content of 44.9%, including 51 putative coding genes, 31 tRNAs, and 4 rRNAs. Furthermore, the cpDNA revealed 19 putative protein-coding genes related to photosynthesis, such as putative photosystem I and II proteins. The *Polytrichum strictum* cpDNA assembly generated a size genome of 20,183 bp with a G + C content of 46.8% (similar to *P. juniperinum*). The cpDNA of *P. strictum* also showed 44 putative coding genes, 14 tRNAs, and 4 rRNAs, and 18 putative protein-coding genes related to photosynthesis, such as putative photosystem I and II proteins.

Our data suggests a possible absence of *rpoA* in the cpDNA *P. juniperinum* and *P. strictum* (Table 2). *petN* was absent in *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr [20] and *Tetraphis pellucida* Hedw. [21] and was probably translocated to the nucleus in *P. juniperinum* and *P. strictum*. The BLAST analyses showed that *ycf66* had 90% identity among the Antarctic Polytrichaceae species. In *P. juniperinum*, this gene presented 100% identity with the homologous gene in *Sanionia uncinata* (Hedw.) Loeske, and in *P. strictum*, 94.3% identity with the homologous gene in *S. ruralis*. Some gene regions were found to have an identity lower than 90% when compared with the reference, for example, *psaB*, *trnV*, and *ndhD* (Figure 1). We also observed one inversion event in *ndhA* and *ycf2* between *Physcomitrella patens* and both *Polytrichum* samples from Antarctica (Figure 1) and (Figures 2 and 3).

With these results we have so far, it is possible, but not confirmed yet, that the genes *psaJ*, *psaM*, *atpE*, *rpl36*, and *rps14* may be absent in the two assembled genomes, but they have been reported in *P. patens* [22]. Other genes were analyzed separately, as they were not found with the tools used for annotation in this study. For example, *psaM* and *ccsA* cpDNA genes in *P. juniperinum* and *psaI*, *rpl23*, *rpl32*, *rps7*, *ycf4*, *ccsA*, *matK*, and certain tRNAs in *P. strictum* were found only with BLAST [15] by using the total genome of Polytrichaceae and a lower e-value (10^{-5}). It is possible that these regions

were not sequenced and therefore not included in the percentage of genome coverage or these genes probably have a high degree of rearrangement (deletions, tandem duplications, and inversions) and substitutions. Scaffold Builder assembler version 2.2 is not effective when the sequences have a high degree of rearrangements [23], and the sequences of these genes and sequences of the genome have at least 80% identity. These genes were not accounted for during our analysis, and further studies on the presence/absence of these genes in Polytrichaceae mosses are required.

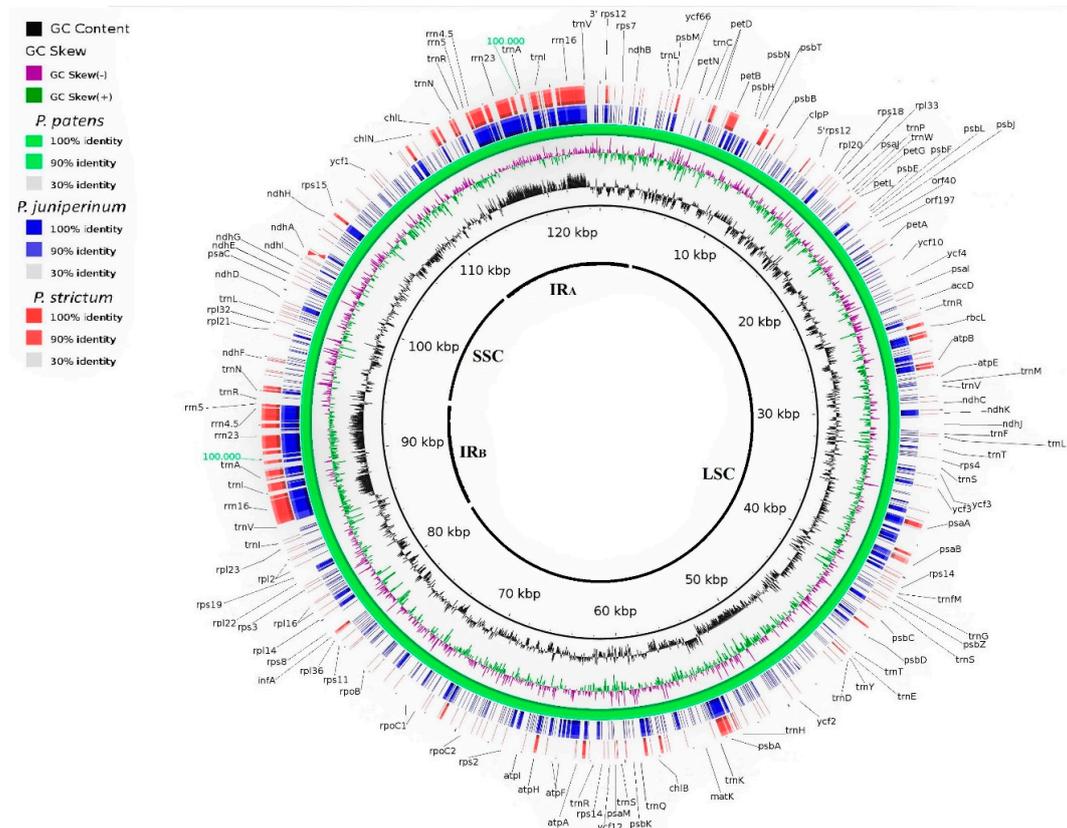


Figure 1. Blast Ring Image Generator output image of the chloroplast genome comparing *Physcomitrella patens* and Antarctic *Polytrichum* species. The internal ring represents the *P. patens* chloroplast genome (green). BLAST match of *Polytrichum juniperinum* and *Polytrichum strictum* are in blue and red gradient, respectively. The legend showing color gradient for percentage similarity between the reference and *Polytrichum* species. The innermost rings show the GC skew (purple/green) and GC content (black). The highlighted blocks show the inversions observed between *P. strictum* and *P. juniperinum* with the reference genome. The inverted repeat region (IRs), long single copy section (LSC), and short single copy section (SSC) are indicated.

The *P. juniperinum* mitochondrial genome (mtDNA) has a total of 88,021 bp and 41.4% GC content. In total, this genome contains 67 genes, including 2 rRNA genes (1 rnl and 1 rns), 19 tRNAs, 3 rRNAs, 3 open reading frames (ORFs; ORF533, ORF622, and ORF187), and 12 protein-coding genes related to mitochondrial oxidative metabolism. Among these, 12 ribosomal proteins (4 rpl and 8 rps) with absence of rps10. The *P. strictum* mtDNA has a total of 58,896 bp and 41.1% GC content. The genome contains a total of 62 genes, including 2 rRNA genes as in *P. juniperinum* (1 rnl and 1 rns), 19 tRNAs, 3 rRNAs, 3 ORFs (ORF533, ORF622, and ORF187), 13 protein-coding genes related to mitochondrial oxidative metabolism, and 13 ribosomal proteins (4 rpl and 9 rps). The rps10 gene that encoded a protein from the 40S subunit of the ribosome was not found in Antarctic Polytrichaceae, apparently that region was lost before the split of mosses lineage since this absence is also evident in

Tetraphidaceae [21] and Funariaceae [24]. The *nad7* pseudogene in *Marchantia polymorpha* and ORF187 is frequently observed in the mtDNA of *M. polymorpha* [25] and contradictorily does not occur in *Tetraphis pellucida* but seems to be present in the *Polytrichum* species studied (Table 2). The BLAST analyses showed that *nad7* and ORF187 has 55.3% and 43.8% of similarity, respectively, in both species studied by us. The *nad7* gene from *P. juniperinum* showed 98% identity with its homologue in *Sanionia uncinata*, and *P. strictum* showed 97.4% identity with its homologue in *Atrichum angustatum*. ORF187 in *P. juniperinum* showed 96.8% identity with ORF187 in *Marchantia paleacea*, and *P. strictum* showed 98.6% identity with its homologue in *A. angustatum*. This suggests that, in relation to the analysis of *ycf66*, *nad7*, and ORF187, for both *Polytrichum* samples, seems that these genes evolved independently, presenting significant mismatch and gaps in the sequence between the homologues in the different species. This can occur due to different types of RNA editing events during the evolution in the two species. Other potential causes of these differences include a gene duplication event leading paralogous genes in the *Polytrichum* species, although we did not find obvious signatures for these affirmations in our data. However, complementary analyses on the substitution rates and the type of selection pressure on each gene reported are necessary to verify the hypothesis of different type RNA editing event or paralogy between the homologues. The genes *rps19*, *nad3*, *sdh3*, *atp8*, and *atp9*, so far, were not found in the mtDNA of *P. strictum*, similar to the cpDNA.

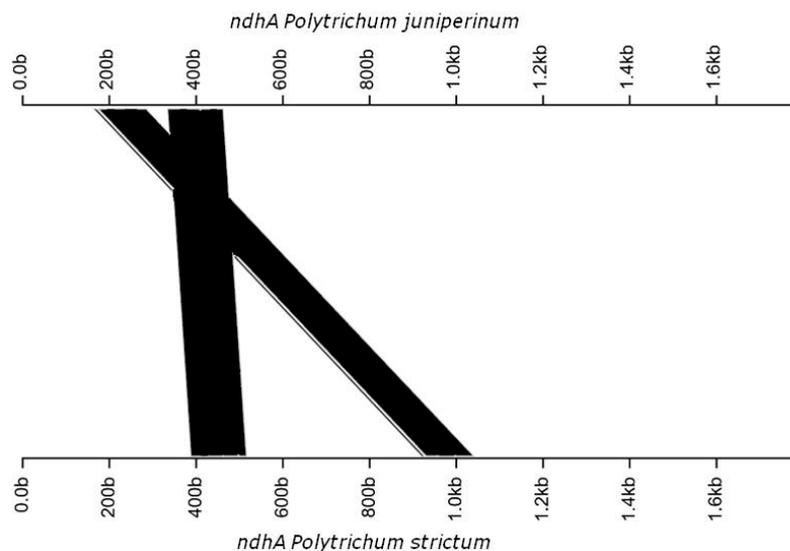


Figure 2. Linear model of *ndhA* gene inversion in *Polytrichum strictum*.

With respect to mtDNA it is evident that *P. juniperinum* and *P. strictum* share many blocks with *P. patens* (Funariaceae) (Figure 4). Rearrangements were not observed in the mitochondrial genome.

The differences in the gene content of the cpDNAs and mtDNAs of the three classes of bryophytes, including the representatives of the family Polytrichaceae and seed plants, are summarized in Table 2. In the chloroplast and mitochondrial genomes, the two algal lineages diverge with respect to the content of the preserved genes and those that have been lost; this shows how the algal lineage varies with respect to both size and gene content because of the various rearrangements that occurred during evolution [26]. *Marchantia polymorpha* with its large-sized cpDNA [27] and mtDNA [28] genomes remains with some unknown genes compared with other species, including many ORFs predicted as possible genes, as example the ORF187 that is shared with *Polytrichum* but not *T. pellucida*. *Marchantia polymorpha* share the lack of *petN* in the cpDNA with some mosses, exceptly with *P. patens*. This species has the mitochondrial *nad7* as a pseudogene; *Anthoceros formosae* Steph [29] has maturase K and *rps15* as pseudogenes, characterizing these two pseudogenes in cpDNA of Anthocerotophyta [30]. In addition, *nad7* from *T. pellucida* is considered a pseudogene [8]. The mosses share practically the

same lack of gene among their representatives; only *T. pellucida* shows a lack of *rps10* and ORF187 in the mtDNA. Seed plants usually have gene contents that apparently do not differ substantially, and previous studies showed that gene loss in plastids is associated with an increase in parasitism [31,32].

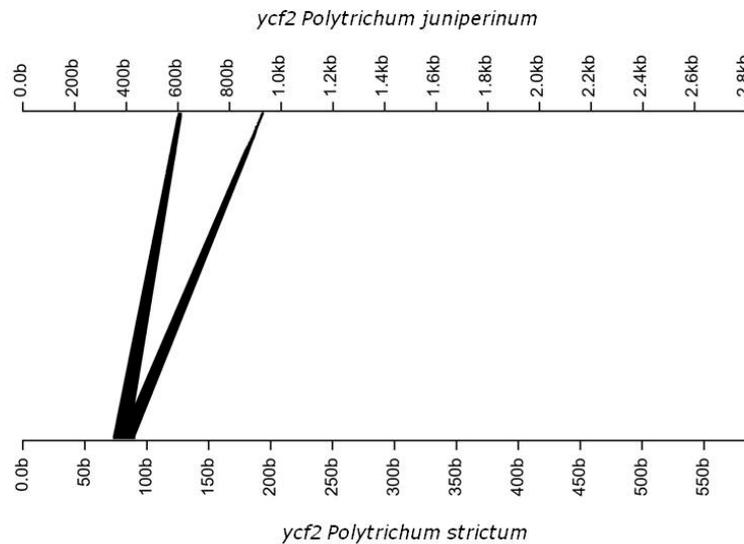


Figure 3. Linear model of *ycf2* gene inversion in *Polytrichum juniperinum*.

3.3. Phylogenetic Analysis: Chloroplast and Mitochondria

The phylogenetic analysis of the chloroplast included all assembly sequences from both species genomes. The plastid genomes of eight species, four species from the division Marchantiophyta, two species from Anthocerophyta, and the two *Polytrichum* species, were included in the analysis. *Marchantia polymorpha* L. were included as the outgroup, due to be the sister group of all land plants groups, as mosses and hornworts [33,34]. The majority-rule tree constructed with the plastid genomes showed branching into three clades, which is consistent with the taxonomic classification of the division Bryophyta sensu lato and *Marchantia polymorpha* as the sister group (Figure 4). Peristomate mosses [35] were resolved as a clade supported by 0.99 Bayesian posterior probability (pP), with the representatives of *Sphagnum* as the basal lineage. A sister relationship between *P. juniperinum* and *P. strictum* received high support (pP = 1). Furthermore, these species were grouped with *T. pellucida*, indicating *Polytrichum* as an apparent basal lineage; thus, the grouping characterizes the nematodontous mosses [8,36], supported by a pP of 1. This positioning is corroborated by other studies [37–41]. However, other authors have reported Tetraphidopsida as the basal group for Polytrichopsida [26]. The remaining species of the division Marchantiophyta formed a clade with little support (Pp = 0.60); however, this result is consistent with the phylogenomic study of Qiu et al [34]. Finally, Anthocerophyta species *Nothoceros aenigmaticus* J.C. Villarreal & K.D. McFarland and *Anthoceros angustus* Steph. formed a supported group (Pp = 0.93), and this is consistent with the findings of Qiu et al [34]. The tree topology showed no conflicting clade, corroborating the results of other authors [40–42].

The phylogenetic relationship, resulting from the majority rule tree inferred from assembly of mitochondrial genome from *Polytrichum* species selected for the present study—including 31 moss species, 2 hornwort species, and 4 liverwort species—are shown in Figure 5. The same outgroup species used for chloroplast phylogenomic analysis was chosen for mitochondrial phylogenomics (*Marchantia polymorpha*). In the present study, the analysed species were placed in three separate clades, one corresponding to Bryophyta; one, Marchantiophyta; and one mixed with Anthocerophyta and the moss species *Ptychomnion cygnisetum* (Müll. Hal.) Kindb. The two representative species of Antarctic *Polytrichum* are closely related moss species, forming a supported clade (Pp = 1) but not grouped in the same branch. The other Polytrichaceae species included in the present analysis, *Atrichum angustatum*,

was grouped with *P. strictum* to form a close clade. The topology of the tree not according to that proposed by Liu et al. [38,43,44].

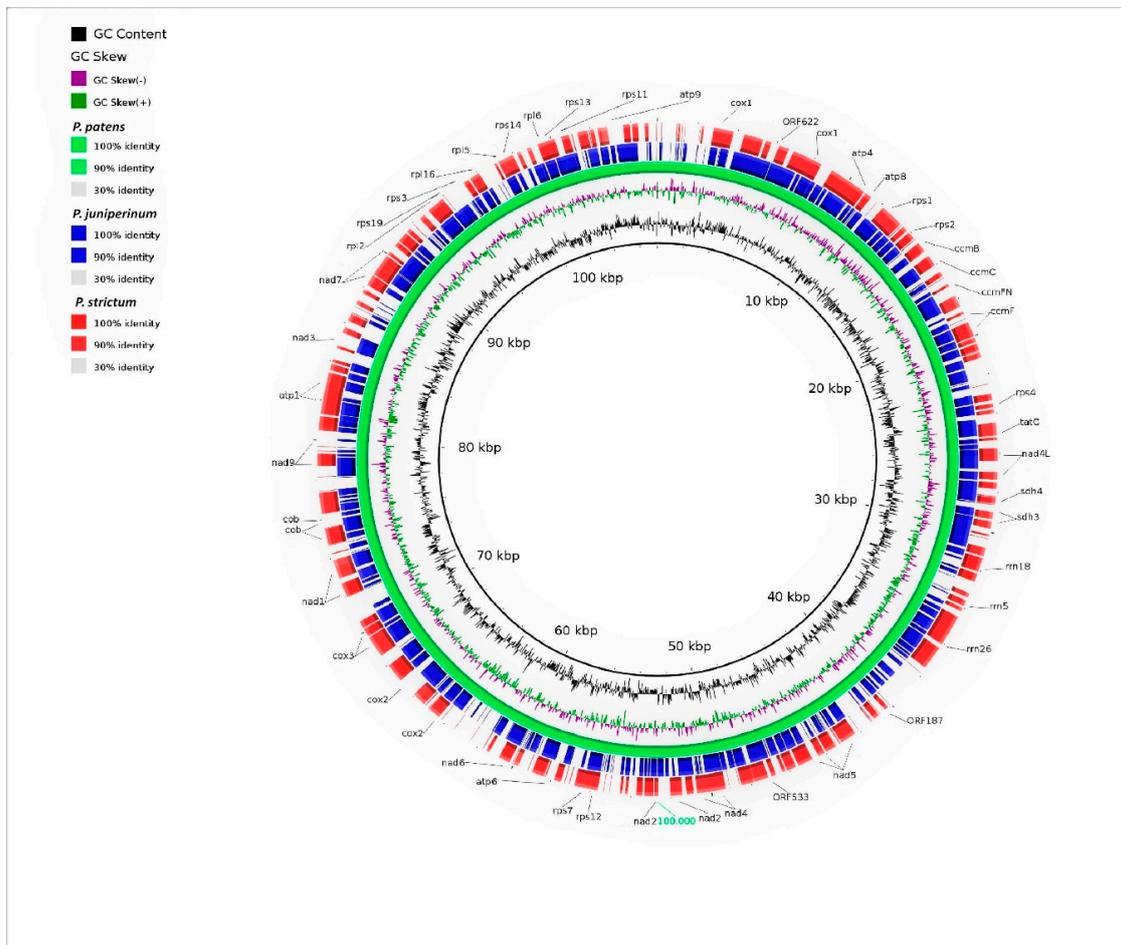


Figure 4. Blast Ring Image Generator output image of the draft mitochondrial genome. The internal rings represent the *Physcomitrella patens* genome (green). Basic Local Alignment Search Tool match of *Polytrichum juniperinum* and *Polytrichum strictum* are in blue and red gradient, respectively. The legend showing color gradient for percentage similarity between the reference and *Polytrichum* species. The referred mitochondrial genes are indicated around the map. The innermost rings show the GC skew (purple/green) and GC content (black).

Table 1. Selected statistics for chloroplast and mitochondria genomes of *Polytrichum* species.

Reads Count	<i>Polytrichum juniperinum</i>		<i>Polytrichum strictum</i>	
Total reads library I	4,215,441		4,992,524	
Total reads library II	8,693,811		8,443,552	
Total reads library III	3,424,244		3,243,657	
Total reads in concatenate library	16,333,496		16,679,733	
Sequence length	25–383		25–389	
High quality reads	9,164,328		9,563,865	
	Chloroplast	Mitochondria	Chloroplast	Mitochondria
Mapped reads	103.776 (1.13%)	32.931 (0.35%)	58.624 (0.61%)	29.620 (0.30%)
N50	10,083	2988	1890	26,564

Table 2. Gene content of cpDNA and mtDNA from algae, bryophytes, and higher plants.

Species	CpDNA Ac. Num.	Chloroplast Genes					MtDNA Ac. Num.	Mitochondrial Genes		
		<i>rpoA</i>	<i>ycf66</i>	<i>petN</i>	<i>matK</i>	<i>rps15</i>		<i>rps10</i>	<i>nad7</i>	ORF187
<i>Chlorella sp.</i>	(NC_001865.1) (NC_023835.1)	+	-	-	-	-	(NC_024626.1) (NC_025413.1)	+	+	-
<i>Chaetosphaeridium globosum</i>	NC_004115.1	+	+	+	+	+	NC_004118.1	+	+	-
<i>Marchantia polymorpha</i>	NC_001319.1	+	+	-	+	+	NC_001660.1	+	Ψ	-
<i>Anthoceros angustus</i>	NC_004543.1	+	-	+	Ψ	Ψ	0	0	0	0
<i>Physcomitrella patens</i>	NC_005087.1	-	+	+	+	+	NC_007945.1	-	+	+
<i>Polytrichum juniperinum</i>	KY795004	-	+	-	-	-	KY795005	-	+	+
<i>Polytrichum strictum</i>	KY795006	-	+	-	-	-	KY795007	-	+	+
<i>Tetraphis pellucida</i>	NC_024291.1	-	+	-	+	+	NC_024290.1	-	-	-
<i>Arabidopsis thaliana</i>	NC_000932.1	+	-	-	+	+	NC_001284.2	-	+	+
<i>Oryza sativa Indica Group</i>	NC_027678.1	+	-	+	+	+	NC_007886.1	-	+	+
<i>Triticum aestivum</i>	NC_002762.1	+	-	-	+	+	NC_007579.1	-	+	-

The presence (+) or absence (-) of each molecular character, absence of sequenced genome and molecular data dispoible in GenBank (0) and pseudogene (Ψ) are shown. The data comes from [11].

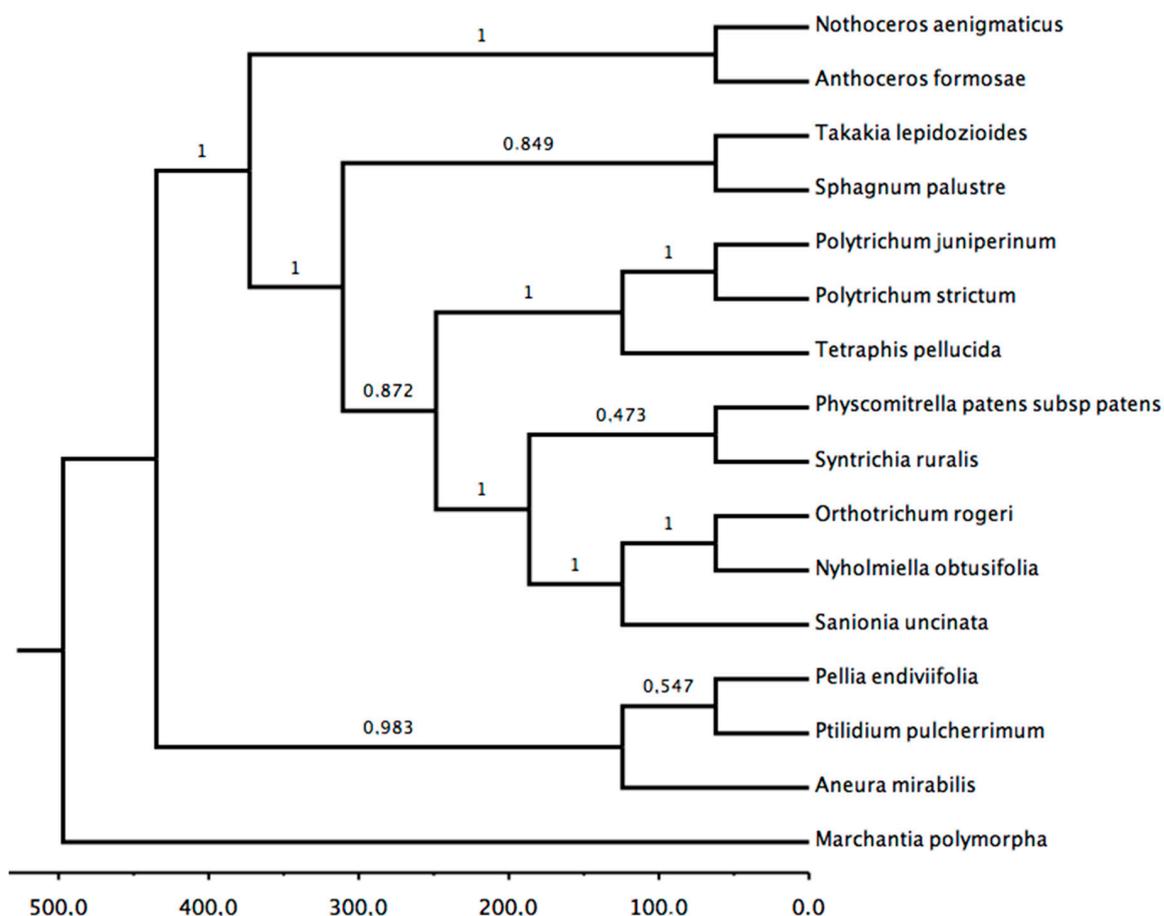


Figure 5. Maximum clade credibility tree created using Bayesian analysis of the chloroplast gene dataset. The robustness of each node is represented by a posterior probability value (Pp) that varies between 0 and 1 and was obtained after 10,000,000 Monte Carlo Markov chains (MCMC). The tree was re-rooted using *M. polymorpha* as outgroup, due to be the sister group of all land plants groups. Time scale root age estimated in 497 ma [19].

4. Discussion

The chloroplast genome of *P. juniperinum* and *P. strictum* had a smaller cpDNA than those of some moss, liverwort, and hornwort species deposited in NCBI database [32,45]. The smaller size of *Polytrichum* genomes, obtained until now in the present study, might reflect the life history [46], evolutionary affiliation [47], and geographical distribution [48] of the species. The latter is more important here, as the Antarctic is geographically isolated continent and their hardly conditions contributing to a selective force [49] that can affect genome size variation, but further studies, including samples from other climatic region become necessary to assess whether those conditions are really affecting the size of the genomes of these species. However, this small size does not interfere with the G + C content, which is high. The plastid genomes of the closest *Polytrichum* species have a GC percentage of 28% to 33% [21,22] and those of seed plants range between 34% and 40% [50]. Cai et al., observed high G + C contents in the chloroplast coding regions, and certain regions had higher percentages than others, such as the IR region with its four genes with high levels of G and C [51]. Thus, the distribution of GC content in the chloroplast is unequal, and, perhaps, the higher content presented by the *Polytrichaceae* species refers to the significant number of coding regions scaffolded. The mitochondria of *Polytrichaceae* are smaller size than those of other mosses and similar to the mtDNA of *Buxbaumia aphylla* Hedw.; the size of the mitochondrial genome of mosses is between 100,000 and

141,000 bp, and Anthoceroophyta species have the largest mitochondrial genome (209,482 bp). However, we must take into account that our results refer to only one draft of mt genomes, which further studies can contribute to whole picture about the size of these genomes. Despite the small size of the mitochondrial genome, % GC is consistent with the mitochondrial GC content of other moss species [21,24,52].

The gene content of cpDNA in the two Polytrichaceae genomes was similar to that of *Tetraphis pellucida*, however, for Polytrichaceae species, so far, no maturase K have been found in their genome; further studies based in overall Polytrichum species both from Antarctica and other regions are required to evaluate this loss. The rpoA gene seems to have been lost in Polytrichaceae. The absence of this gene had been reported in *T. pellucida* as well as in all arthroodontous groups [53,54]. Goffinet et al. [55] showed that rpoA does not seem to have been lost in *Polytrichum pallidisetum* Funck. However, we did not identify this gene, and it is possible that this gene has been lost or translocated in the Antarctic Polytrichaceae species, but only complementary data could confirm or not this hypothesis. According to Sheveleva et al. [56], the presence of rpoA gene is quite variable from species to species. The membrane thylakoid gene ycf66 is absent in *A. formosae* [30,57,58] but remains more stable in Polytrichaceae than in ferns [59]. Only two species of bryophytes are currently known to lack the petN gene [20,21], part of the photosynthetic cytochrome b6/f complex in the chloroplast, and it is possible, according Oliver et al., that another nuclear-encoded gene product performs the same function as a subunit of the complex [20].

The overall gene content of the mitochondrial genome from the two Polytrichaceae species is close to that observed in *P. patens* (Figure 4). The rps10 gene seems be absent in the Polytrichaceae mitochondrial genome but is present in *P. patens*. Adams et al., reported that rps10 has been frequently lost (26 times) and transferred to the nucleus of 277 diverse angiosperms, and they suggest that the gene loss is a frequent event [60]. The mitochondrial genes that seemed to remain in the Polytrichaceae species are nad7 and ORF187; these were identified initially in *M. polymorpha* [28] and later in *P. patens* mtDNA [24]. Absence of the nad7 mitochondrial gene in the *Nicotiana sylvestris* Speg. CMSII mutant caused an abnormal phenotype, poor growth, and male sterility [61]. In the Antarctic Polytrichaceae species, the nad7 gene perhaps has a key role in sustaining the phenotype.

Some genes seem to have been lost multiple times in the chloroplast and mitochondrial genomes during evolution [50], and other genes appear to be present or absent only in particular clades. For example, diverse genes are lacking in mosses and liverworts, such as rps16; however, the gene is present in hornworts and some vascular plants. The gene psaM is absent in three polyploid ferns (*Adiantum capillus-veneris* L., *Cheilanthes lindheimeri* Hook., and *Pteridium aquilinum* (L.) Kuhn.) as well as two *Selaginella* plastomes, and most of the seed plant plastomes. Seed plant plastomes as well as two *Selaginella* plastomes lack rpl21. In angiosperms, most of the gene transfer to the nuclear genome affects the subunits of the ribosomal proteins as rps and rpl [60]. In contrast, some genes remain present, such as the plastid gene ycf66, that seems to be an independent loss in multiple clades of land plants, including hornworts, ferns, and seed plants [62]. Gene transfer is a continuous event in plant evolution, and this is promoted possibly by high-frequency translocation of gene-rich organelle DNA into the nucleus and the relatively rare, or entirely absent, transfer of DNA encoding complete genes from the nucleus to the organelles [63].

The chloroplast and mitochondrial genomes of *P. juniperinum* and *P. strictum* are identical with respect to overall gene content and structure, as shown in the maps; however, *P. strictum* genomes show lower degree of synteny with the reference. We observed variations in the chloroplast genomes, for example, inversions. Inversions represent a type of rearrangement, and one gene inversions were observed in the cpDNA of *P. juniperinum* (Figure 3), which is not shared with *P. patens*/*P. strictum*, and one inversion between the cpDNAs of *P. strictum* (Figure 2) and *P. patens*/*P. juniperinum*. The gene content and gene arrangement of the chloroplast are highly conserved in land plants [64]. Large inversions and other chloroplast genome rearrangements are relatively uncommon among land plants [65], but small inversions are common and widespread in the plant plastid genomes

and have been reported in a variety of plants, including bryophytes [66–68]. Generally, such small inversions provide a rather interesting phylogenetic marker between species, but also a vision of the relationships among groups. These inversions seem restricted to the species and do not characterize the genera. The mitochondrial genome shows conservation between Antarctic *Polytrichum* species. Previous studies have shown that the structural evolution of the mitochondrial genome is highly conservative not only within each individual lineage but also across mosses; however, this is most evident when compared with more distant orders within the large group Bryophyta that shows some rearrangements that are very conserved [50]. The occurrence of some rearrangements was observed between *Marchantia polymorpha* mtDNA and *P. patens*, as these species diverged more than 375 million years ago [69].

Over the last few decades, single-gene phylogenetic analyses have served as powerful tools for reconstructing the evolutionary history of every major lineage of life on Earth [70]. Indeed, with next-generation sequencing technologies, complete plastome sequences are now being fastest generated [71–73]. We sought to analysis the phylogenetic positions of *P. juniperinum* and *P. strictum* by using the plastid and mitochondrial gene data of representative moss families, hornworts, and liverworts deposited in GenBank. We wanted to form a hypothesis on the origin of the *P. strictum*, since there is oodles debate on its origin and definition as a species or variant of *P. juniperinum*. *Polytrichum strictum* has morphological characteristics similar to those of *P. juniperinum* [74–77] and it differs from *P. juniperinum* in that it occurs in habitats in the north, such as wetlands (North America), and has, among other morphological characteristics, a remarkable coverage of white rhizoids [78]. Bell and Hyvönen conducted a study on the phylogeny of mosses of the class Polytrichopsida and proposed that the origin of *P. strictum* (samples used were from Chile and Finland) could be from a cross-linking event [4]. For these authors, *P. strictum* could be the product of hybridization between the *P. juniperinum* lineage (sample used from Finland) and a basal lineage of another Polytrichaceae representative. According to the topology presented by the study, the samples of *P. strictum* were grouped into the same branch, and *P. juniperinum* appears in a sister branch of *P. strictum*, suggesting this species as maternal ancestor of *P. strictum*. In the present study, despite the low number of samples studied, ours results corroborating this ancestry. Because of the lack of well-supported resolution for the positions of *Polytrichum hyperboreum* R. Br. and *P. piliferum*, one of these species or a related extinct taxon could easily be the paternal progenitor. In another study on Polytrichales, molecular and morphological data suggested the grouping of *P. juniperinum* and *P. piliferum* [40].

The phylogenetic analysis of the partial data from the chloroplast presented a substantial support branch (Figure 5). The Bayesian analysis was selected, as it would be more effective for a large amount of data used in the phylogenetic analysis. The nematodontous mosses are grouped in the same clade, suggesting that *P. strictum* is a sister of *P. juniperinum* and *T. pellucida* appears outside of these grouping. This topology is consistent with that reported by several authors who study nematodontous mosses [21] and that of Cox et al., with respect to the earliest divergence from Tetraphidales and Polytrichales [79]. The Bryopsida clade comprising *Nyholmiella obtusifolia* (Brid.) Holmen & E. Warncke, *Orthotrichum rogeri* Brid, *Syntrichia ruralis*, *S. uncinata*, and *Takakia lepidozoioides* S. Hatt. & Inoue was supported; however, some authors have proposed different topologies for the class Bryopsida [79–82]. Although a smaller number of chloroplast genomes than mitochondrial genomes are available for moss species, the phylogenetic positioning of some branches could be reconstructed with a larger amount of data. Whole-genome phylogeny has been shown to be congruent with respect to most of the topologies already inferred for bryophytes, especially when compared to the approaches where only some regions—such as *psaA*, *atpH*, *atpI*, *chlL*, *rbcL*, and *rpl16* for chloroplast and *atp1*, *ccmB*, *cob*, *nad3*, *nad4*, *rpl5*, *rpl6*, *rps1*, and *rps11* for mitochondria—are used for the resolution of evolutionary relations [83,84].

Analysis of the mitochondrial genes showed a high branch support for most nodes and little support in some branches, such as the node of *Racomitrium* and *Codriophorus* but even so agrees partial with Sawicki et al., that grouping species from Grimmiaceae family [85]. The same occurred with the node for *Climacium americanum* Brid., *S. uncinata*, *Hypnum imponens* Hedw. with Orthotrichaceae. Polytrichaceae representatives appear to form a clade, but *Atrichum angustatum* appears inside this clade, close to *P. strictum*. Although *A. angustatum* is a more basal species in Polytrichaceae phylogeny [4], here, the species seems to be placed incorrectly in the mitochondrial tree. However, this result can generate two interpretations, the first involving a possible misunderstanding due to the reference genome used in assembling the plastids sequenced for the *Polytrichum* species used since the reference was based on the genome of *P. patens*; this may induce the resulting genomes to be more similar to the genomes of *P. patens* than *A. angustatum*. Particularly, recent literature gives assurances of mtDNA conservation among the moss lineage, which lowers the expectations of the results obtained from an assembly error [25,43,52]. A second interpretation would be that mtDNA ancestry is distinct for *Polytrichum juniperinum* and *Polytrichum strictum*, consequently the resolution of this relationship will remain unclear until more genomic data are generated for inclusion in this analysis, although we have obtained good coverage of the mitochondrial genome

Our mitochondrial phylogenomic tree (Figure 6) does not match the reconstructed plastid tree (Figure 5) presenting another topology. Differential inheritance of organelles in the same cytoplasm can break the typically expected linkage equilibrium between the chloroplast and mitochondrion [86–88] and if this happens, then phylogenetic reconstructions of these two organellar genomes can conflict. The uniparental inheritance in *Rhizomnium* moss both for chloroplast as mitochondria genomes has been previously reported [89]. In contrast to higher plants, there have been few studies on organellar inheritance in bryophytes [90]; however, the maternal inheritance of the chloroplast in mosses has been reported [89,91]. Therefore, the stasis on the mitochondria genome evolution in mosses should also be taken into account. Liu et al., reports the stasis for mt genomes in mosses, suggesting that the mt genome structure remained virtually frozen for 350 My [29], which may contribute to the distinct topology in the phylogeny obtained from mitochondrial genome data. Although the various gene losses or the pseudogenizations identified in the mitochondrial genomes of mosses [43] may also influence these ambiguous topologies, such as the approximation of *Ptychmonium cygnisetum* and hornworts in our phylogenomic analysis for mt genomic data. A previous study reported that incongruence can be caused by a very small number of characters that are in conflict with other sources of data and excluding part of the data would be warranted only if we knew a priori which part of our data is unreliable [40]. Potential incongruence between chloroplast DNA and mitochondrial DNA markers has been reported [92,93].

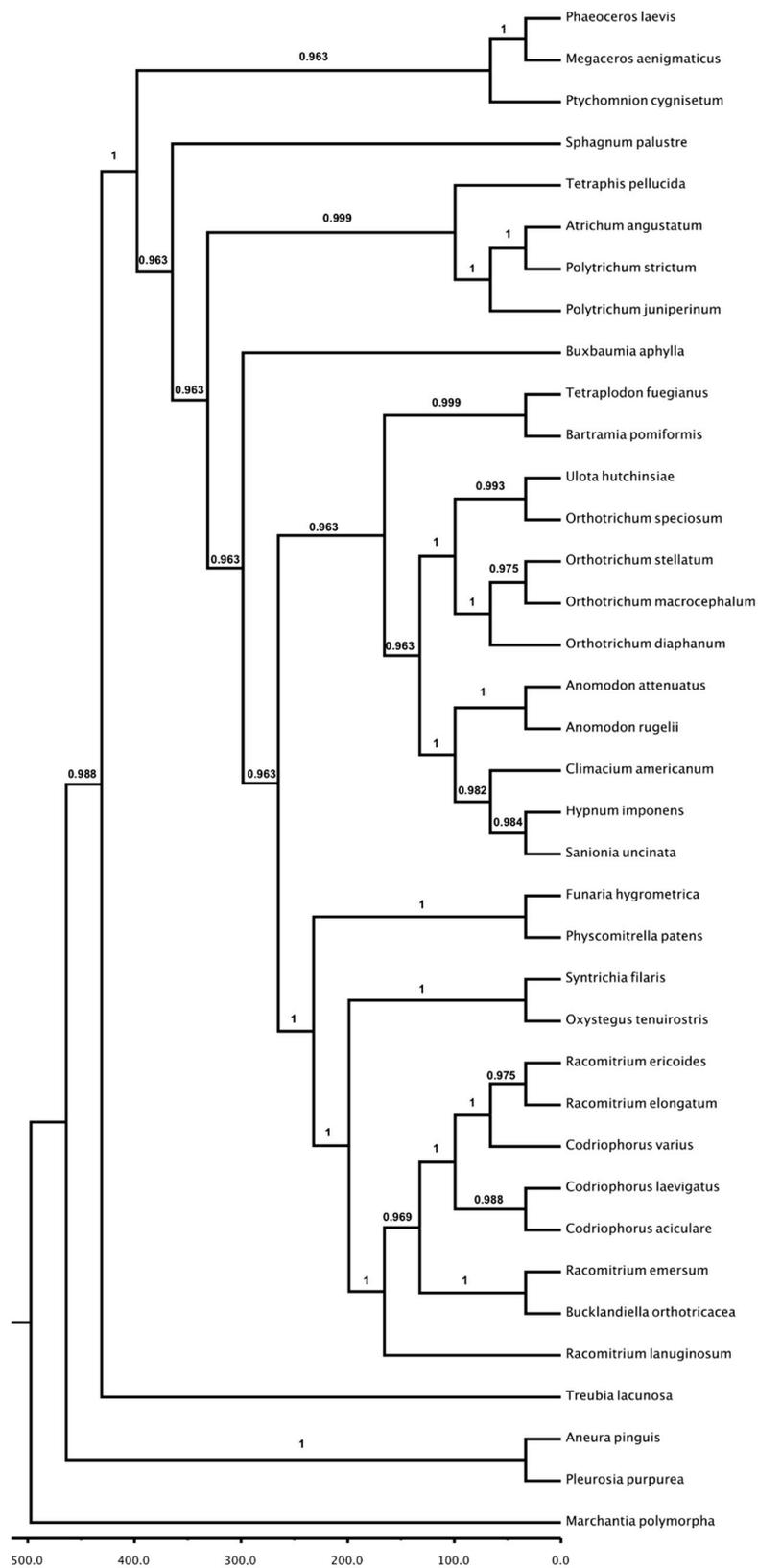


Figure 6. Maximum clade credibility tree obtained using Bayesian analysis of the mitochondrial gene dataset. The robustness of each node is represented by the posterior probability value obtained after 10,000,000 Monte Carlo Markov chains (MCMC). The tree was reroot using *M. polymorpha* as outgroup due to be the sister group of all land plants groups. Time scale root age estimated in 497 ma [19].

Molecular phylogenies derived from plastidial, mitochondrial, and nuclear plant genomes can provide insight into the evolutionary history of plant groups influenced by reticulation events [94]. In this study, the chloroplast phylogeny suggests that *P. juniperinum* is a sister species of *P. strictum*, at least from Antarctic samples studied; however, the mitochondrial phylogeny suggests *P. juniperinum* as a maternal ancestor for *P. strictum*, as well as in study reported by Bell and Hyvönen for both *Polytrichum* species diverse world regions [4]. This can indicate that the structure of mitochondria remained virtually frozen in Antarctic *Polytrichum*. However, to confirm the suggested hypothesis, it is necessary include the other all *Polytrichum* species samples collected from different locations to study the distribution of the species. Currently, constructing a phylogeny for a group of poorly studied organisms requires substantial research. This study present contributes to a preliminary understanding of plastomes and chondriosomes evolution in the family Polytrichaceae, and so far, reveals prelude information that allows the distinction between *P. juniperinum* and *P. strictum* from a molecular scenery.

5. Nucleotide Sequence Accession Numbers

This draft genome BioProject has been deposited at GenBank under accession number SUB2397616. The genome accession numbers are KY795004, KY795005, KY795006, and KY795007 from *Polytrichum juniperinum* cpDNA and mtDNA and *Polytrichum strictum* cpDNA and mtDNA, respectively.

Author Contributions: K.E.J.d.F carried out experiments in both *Polytrichum juniperinum* and *Polytrichum strictum*, carried out the DNA extraction for sequencing analysis, performed the initial genome assembly, annotation of the partial genome assembled and wrote the manuscript with assistance from the co-authors. G.F.M carried out the initial bioinformatic analysis. E.R.P.C. carried out the initial phylogenic analysis. L.F.W.R. designed and carried out the Next Generation Sequencing. A.B.P. co-directed the project and carried. F.C.V. conceived and co-directed the project, designed and carried out the sample collection, the evolutionary analysis, and genome assembly.

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