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## Combined data from chloroplast and mitochondrial genome sequences showed paraphyly of bryophytes

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**Abstract:** The present study was conducted using chloroplast and mitochondrial genome sequences of plants to gain insight on evolutionary relationship of bryophytes. The representative taxa were selected considering the availability of both organelle genome sequences. Phylogenetic analysis using combined data from chloroplast and mitochondrial sequences infer paraphyly of bryophytes.

### Introduction

The three extant bryophyte lineages (liverworts, mosses and hornworts) are the earliest land plants sharing ancestry with charophycean algae (Kenrick and Crane 1997, Renzaglia et al. 2000, Qiu et al. 2006, Qiu 2008, Finet et al. 2010) and supposed to diverge before the lineage ancestral to existing tracheophytes (Ligrone et al. 2012). To resolve the relationship among bryophytes lineages several phylogenetic studies were conducted (Lewis et al. 1997, Nickrent et al. 2000, Nishiyama et al. 2004, Gao et al. 2010, Shanker et al. 2011, Chang and Graham 2011, Shanker and Sharma 2012, Shanker 2013, Shanker 2013a). However these studies provide conflicting results.

Among bryophytes the basal position of liverworts in the phylogeny of land plants was inferred by cladistic analysis of morphological characters (Mishler and Churchill 1984) combined with rRNA sequences (Mishler et al. 1994), *rbcL* gene analysis (Lewis et al. 1997), the presence/absence of three mitochondrial introns (Qiu et al. 1998), analysis of three different complementary data sets, a multigene supermatrix, a genomic structural character matrix and a chloroplast genome sequence matrix (Qiu et al. 2006) and plastid protein coding genes (Gao et al. 2010). Mosses show the sister relationship to a clade formed by hornworts and tracheophytes (Qiu et al. 2006, 2007, Qiu 2008, Chang and Graham 2011). Contrary to this mosses appear to jointly form a clade with liverworts (Nickrent et al. 2000, Nishiyama et al. 2004, Shanker et al. 2011) and also appeared as sister group to vascular plants (Boivin et al. 1996).

Hornworts was supported as early diverging bryophyte lineage based on spermatozoid ultrastructure and spermatogenesis data (Renzaglia and Duckett 1991), cladistic analysis of ultrastructural, biochemical and developmental characters (Garbary and Renzaglia 1998, Renzaglia

et al. 2000), DNA sequence analysis of multiple genes (Nishiyama and Kato 1999), nuclear small subunit rDNA (Hedderson et al. 1996), mitochondrial gene *cox3* (Malek et al. 1996), *nad5* (Beckert et al. 1999), mitochondrial small subunit rDNA (Duff and Nickrent 1999) and combined dataset of chloroplast *rbcL* and small subunit rDNA from nuclear, mitochondrial and chloroplast genomes of plants (Nickrent et al. 2000). Furthermore, less supported alternative topologies resolve the hornworts as basal bryophytes (Qiu et al. 2006). Contrary to this, sister relationship of the hornworts with tracheophytes was determined by several studies (Malek and Knoop 1998, Samigullin et al. 2002, Dombrowska and Qiu 2004, Kelch et al. 2004, Groth-Malonek et al. 2005, Wolf et al. 2005, Qiu et al. 2006, Gao et al. 2010).

Comparative immunocytochemical analysis of tissue and cell distribution of xylans (Carafa et al. 2005) and structural homology in the xyloglucans (Pena et al. 2008) also suggests hornworts as sister to tracheophytes. Moreover a recent comparative analysis of chloroplast genomes identified hornworts as transition link between bryophytes and pteridophytes (Shanker and Sharma 2012). Apart from these dilemmas of earliest diverging bryophyte lineage several studies showed monophyly of bryophytes (Garbary et al. 1993, Nishiyama et al. 2004, Goremykin and Hellwig 2005, Shanker et al. 2011).

The availability of complete chloroplast and mitochondrial genome sequences in public databases allow reconstruction of organelle genome based phylogenies of land plants. Therefore the present phylogenetic analysis was designed to gain insight on evolutionary relationship of bryophytes using combined data from chloroplast and mitochondrial genome sequences.

## Materials and methods

### Taxa

A total of 15 organisms were selected with both mitochondrial and chloroplast genomes sequenced. Since none of the representative of hornworts has both organelle genomes sequenced therefore to represent hornworts in combined chloroplast-mitochondria data set chloroplast genome sequence of *Anthoceros* and mitochondrial genome sequences of *Phaeoceros* and *Megaceros* were selected (Table 1). Coding sequences (CDS) and corresponding protein sequences of these organelle genomes were downloaded from National Center for Biotechnology Information (NCBI).

### Data sets

Two data sets, one of chloroplast orthologous protein sequences and another of mitochondrial orthologous protein sequence was constructed. Each set of orthologous protein sequences was aligned using MUSCLE (Edgar 2004). The multiple sequence alignment of protein sequences was used to generate corresponding CDS alignment with the help of PAL2NAL (Suyama et al. 2006). CDS alignments of chloroplast and mitochondrial data sets were assembled into combined chloroplast-mitochondria data set. Among hornworts *Anthoceros* has only chloroplast genome sequence while *Phaeoceros* and *Megaceros* have only mitochondrial genome sequences. Therefore to represent hornworts in combined chloroplast-mitochondria data set, concatenated CDS from chloroplast genome of *Anthoceros* was combined with concatenated CDS from mitochondrial genome of *Megaceros/ Phaeoceros*, thus creating a chimerical data set of combined 77 genes. Overall following data sets were used for tree construction: (i) Chimerical chloroplast-mitochondria data set with chloroplastic CDS sequences of *Anthoceros* concatenated with mitochondrial CDS sequences of *Megaceros* (16 taxa, 77 genes, 59331 characters with 13.07% missing data), and (ii) Chimerical chloroplast-mitochondria data set with chloroplastic CDS

sequences of *Anthoceros* concatenated with mitochondrial CDS sequences of *Phaeoceros* (16 taxa, 77 genes, and 59331 characters with 13.15% missing data). Additionally corresponding data sets of protein sequences were generated.

Table 1. Information of chloroplast and mitochondrial genomes.

S. No.	Organisms	Chloroplast genome		Mitochondrial genome	
		Accession No.	Seq*	Accession No.	Seq*
	<b>Charophyta</b>				
1.	<i>Chaetosphaeridium globosum</i>	NC_004115	98	NC_004118	46
2.	<i>Chara vulgaris</i>	NC_008097	105	NC_005255	46
3.	<i>Chlorokybus atmophyticus</i>	NC_008822	114	NC_009630	58
4.	<i>Mesostigma viride</i>	NC_002186	105	NC_008240	41
	<b>Chlorophyta</b>				
5.	<i>Micromonas</i> sp. RCC299	NC_012575	57	NC_012643	39
6.	<i>Ostreococcus tauri</i>	NC_008289	61	NC_008290	43
	<b>Bryophytes</b>				
7.	<i>Anthoceros formosae</i>	NC_004543	90	-	-
8.	<i>Phaeoceros laevis</i>	-	-	NC_013765	38
9.	<i>Megaceros aenigmaticus</i>	-	-	NC_012651	48
10.	<i>Marchantia polymorpha</i>	NC_001319	89	NC_001660	76
11.	<i>Physcomitrella patens</i> subsp. <i>patens</i>	NC_005087	85	NC_007945	42
	<b>Gymnosperms</b>				
12.	<i>Cycas taitungensis</i>	NC_009618	122	NC_010303	39
	<b>Angiosperms</b>				
13.	<i>Arabidopsis thaliana</i>	NC_000932	85	NC_001284	117
14.	<i>Carica papaya</i>	NC_010323	84	NC_012116	39
15.	<i>Triticum aestivum</i>	NC_002762	83	NC_007579	39
16.	<i>Sorghum bicolor</i>	NC_008602	84	NC_008360	32
17.	<i>Oryza sativa</i> Indica Group	NC_008155	64	NC_007886	54
18.	<i>Vitis vinifera</i>	NC_007957	84	NC_012119	74

\*Seq - Number of Coding/protein sequences

#### Phylogenetic analysis

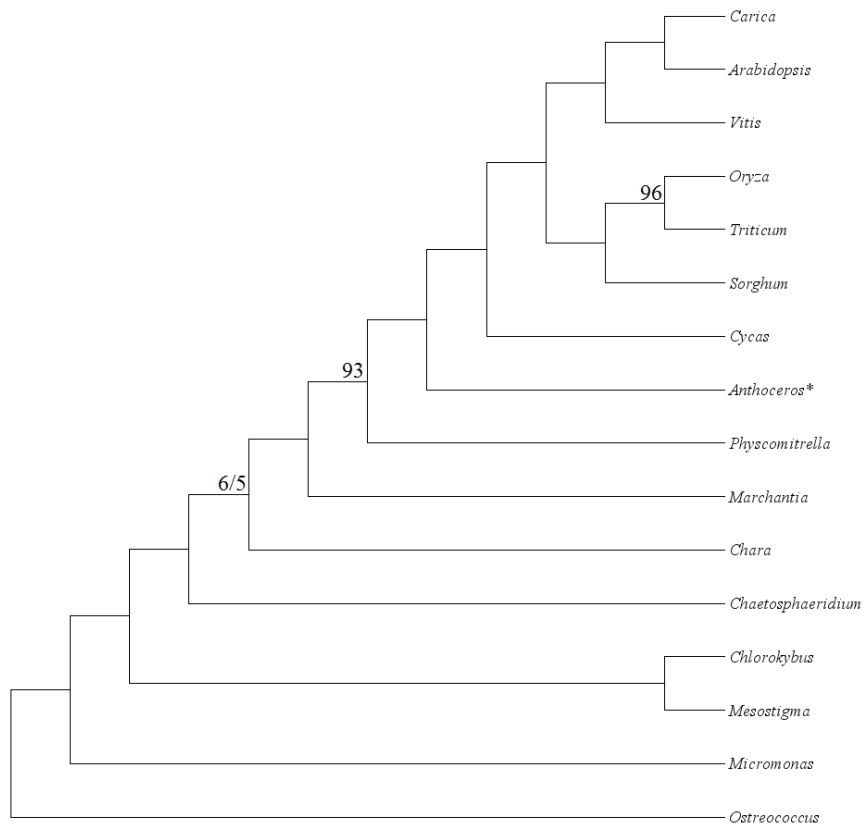
The best fitting model of nucleotide substitution was determined using Mraic.pl (Nylander 2004), a PERL script, which showed GTRIG as the best model considering Akai Information Criterion (AIC). Phylogenetic trees of nucleotide data sets were constructed using PHYML (Guindon and Gascuel 2003) with 500 bootstrap replicates, and MrBayes (Huelsenbeck and Ronquist 2001). The best fitting model of amino acid substitution (CpREV+I+G+F) was determined using ProfTest (Abascal et al. 2005) for combined chloroplast-mitochondria data sets and trees were constructed using PHYML. *Ostreococcus tauri* was used to root all the trees.

#### Results and discussion

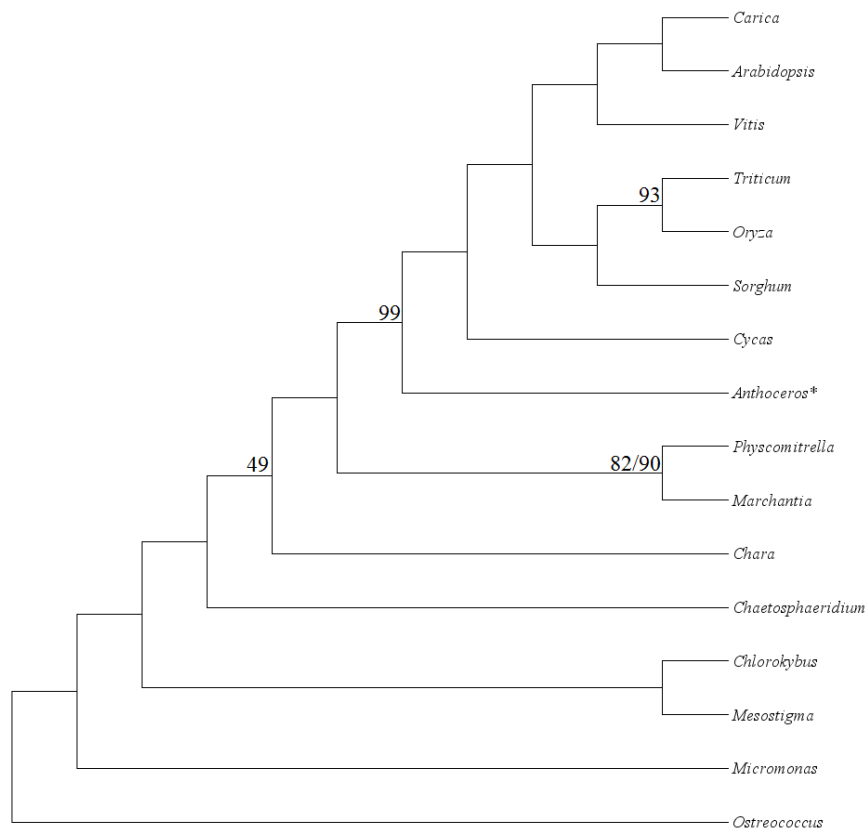
A large number of chloroplast genome sequences are available in NCBI database however most of them belong to angiosperms. Additionally sequences of a small number of mitochondrial genomes of plants are also available. Among these organelle genomes only few sequences belongs to

bryophytes (Shanker 2012, Shanker 2012a). Since none of the representative of pteridophytes were found with both mitochondrial and chloroplast genome sequences deposited in the database therefore they were not included in the analysis. Consequently the present analysis includes taxa from green algae, bryophytes, gymnosperms and angiosperms. Considering the diversity of extant bryophytes the data sets used in this study were not taxon rich. Moreover the data sets differ in taxon and character sampling. However by using sequences from available mitochondrial and chloroplast genomes the data sets compiled were rich in characters.

The phylogenetic tree inferred from combined nucleotide sequence data of chloroplast-mitochondria showed paraphyly of bryophytes with liverworts as first diverging lineage and hornworts as sister to vascular plants with 100% BS/1 PP support values at both nodes (Fig. 1). However the trees inferred from corresponding protein sequences show sister relationship between mosses and liverworts with 82% (*Anthoceros* + *Megaceros* sequences; Fig. 2) and 90% (*Anthoceros* + *Phaeoceros* sequences) BS support.



**Figure 1.** Bryophytes phylogeny inferred from combined chloroplast-mitochondria nucleotide data sets. All the internal nodes in trees inferred using Bayesian method have posterior probability 1. Bootstrap support values (<100%) are shown on the internal nodes. \*Chloroplast sequences of *Anthoceros* were concatenated with mitochondrial sequences of *Megaceros/Phaeoceros* and BS values are shown in the same order. Identical BS values are shown only once.



**Figure 2.** Maximum likelihood phylogeny inferred from combined chloroplast-mitochondria protein sequences data sets. Bootstrap support values (<100%) are shown on the internal nodes. \*Chloroplast sequences of *Anthoceros* were concatenated with mitochondrial sequences of *Megaceros/Phaeoceros* and BS values are shown in the same order. Identical BS values are shown only once.

Previously the paraphyly of bryophytes was inferred based on morphological characters (Mishler and Churchill 1984, Kenrick and Crane 1997, Renzaglia et al. 2000). Absence of three mitochondrial group II introns in algae and liverworts was shown to support the bryophytes paraphyly hypothesis (Qiu et al. 1998). Moreover a six gene data set from 193 green algae and land plants, a mitochondrial group II intron insertion site matrix and a chloroplast genome sequence matrix also inferred paraphyly of bryophytes (Qiu et al. 2006). Recent analysis using chloroplast (Shanker 2013) and mitochondrial (Shanker 2013a) genomes also resolved bryophytes as a paraphyletic group. The findings of the present analysis are in strong agreement with all these studies. Contrary to this the monophyly of bryophytes was inferred by other studies (Garbary et al. 1993, Renzaglia et al. 2000, Nishiyama et al. 2004, Goremykin and Hellwig 2005, Shanker et al. 2011). The limitation of taxon or character sampling may contribute to such finding (Qiu 2008).

Considering paraphyly of bryophytes inferred in this study and several other studies discussed above, resolving the conflict in branching of bryophyte lineages is also of utmost importance as it helps to identify early diverging land plant lineage. Additionally it gives insight on bryophytes lineage which act as sister to vascular plants. The basal position of liverworts in land plant phylogeny was inferred by cladistic studies of morphological and biochemical data (Mishler and

Churchill 1984, Bremer 1985, Kenrick and Crane 1997). These results were later supported by analysis of mitochondrial group II introns (Qiu et al. 1998, 2006). A recent chloroplast phylogenomics study also provided additional evidence supporting liverworts as the early diverging lineage in land plants (Gao et al. 2010).

On the other hand, the sister group relationship between mosses and liverworts received support from data derived from sperm ultrastructure (Maden et al. 1997), mtSSU rDNA (Duff and Nickrent 1999), analysis using multiple genes (Nishiyama and Kato 1999, Nickrent et al. 2000) and trees inferred using chloroplast-mitochondria protein sequence data in this study. Moreover maximum likelihood analysis using amino acid residues from chloroplast genomes also showed mosses and liverworts to jointly form a clade (Nishiyama et al. 2004, Shanker et al. 2011). There are a bunch of studies suggesting hornworts as sister to vascular plants (Lewis et al. 1997, Malek and Knoop 1998, Samigullin et al. 2002, Dombrowska and Qiu 2004, Kelch et al. 2004, Groth-Maloney et al. 2005, Wolf et al. 2005, Qiu et al. 2006, Gao et al. 2010). Moreover a bioinformatic analysis based on comparative analysis of chloroplast genomes using newly defined parameters to detect unbiased conservation between genomes (Shanker et al. 2009) identified hornworts as transition link between bryophytes and pteridophytes (Shanker and Sharma 2012).

As a concluding remark the available chloroplast and mitochondrial genome sequences of plants were successfully used to infer paraphyly of bryophytes and resolving early diverging bryophytes lineage. Additional data from more bryophytes taxa will facilitate to further support these findings.

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Online May 2, 2013