

CHLOROPLAST GENOME CHARACTERIZATION OF *LYCOPodium CERNUUM* L.

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Abstract: *The pteridophyte species Lycopodium cernuum is common species found in Indian forests where abundant water is available. It has well studied for its alkaloids and possess many medicinal uses. In present study we extracted chloroplast (cp) DNA from L. cernuum. The quantity and quality of this extracted cp DNA was checked and it's subjected to high throughput sequencing. Obtained reads were assembled based on reference-based assembly and annotated using available servers. Some annotations were manually corrected and this cp genome was deposited in NCBI database with accession number MW538913. It was compared with other available cp genomes of Lycopodiopsida and differences in genome size, GC content, gene numbers, tRNA numbers were observed. We also report microsatellite markers for both Lycopodium species. The Phylogenetic analysis with available 35 Lycopodiopsida species were discussed.*

Keywords: *Lycopodium cernuum*, chloroplast genome, Phylogeny, Pteridophyte

INTRODUCTION

Lycopodium, commonly known as Club moss, Clubfoot Moss, Foxtail, Ground Pine, Sulfer, Wolf's Claw is one of the most widespread species belonging to the family Lycopodiaceae [1]. This spore-bearing vascular plant is used in various traditional systems of medicines. These plants grow best in various areas such as high altitude, in the highlands, in grassy areas. *Lycopodium cernuum* – stem creeping, mostly found on mosses and litter, up to 2.5 mm thick; branches erect from ascending base, branched, up to 30 cm in height. Mature leaves monomorphic, linear, 2.5–5 mm, narrowly acute, with 1.5–4 mm long apical hair tip usually in 12–16 indistinct rows. $2n = 68$. Arctic circumpolar distribution [2]. The *Lycopodium* alkaloids are of great importance due to their

biological activity and unique chemical compounds [3].

Chloroplasts are active metabolic centers that sustain life on earth by converting solar energy to carbohydrates through the process of photosynthesis and oxygen release. Although photosynthesis is often recognized as the key function of plastids, they also play vital roles in other aspects of plant physiology and development, including the synthesis of amino acids, nucleotides, fatty acids, phytohormones, vitamins and a plethora of metabolites, and the assimilation of sulphur and nitrogen [4]. Metabolites that are synthesized in chloroplasts are important for plant interactions with their environment (responses to heat, drought, salt, light, and so on) and their defence against invading pathogens [5].

Chloroplasts are semi-autonomous organelles possessing their own genetic material – the chloroplast genome or plastome. The chloroplast DNA (cpDNA) of higher plants are double-stranded molecules of relatively small size, ranging from 35 to 217 kilobases (kb) with most of the photosynthetic organisms in the range of 115 to 165 kb. Only a proportion of the total cpDNA is circular as opposed to earlier views [6]. They are present in 1,000–10,000 copies per cell. In 1986, the chloroplast genomes of tobacco and the liverwort *Marchantia polymorpha* were sequenced [7,8], making these two the first complete plastome sequences. During the last two decades, plastome sequences have increased at a rapid rate and, currently, many complete plastid genomes are available in NCBI database. Insights gained from complete chloroplast genome sequences have enhanced our understanding of plant biology and diversity; chloroplast genomes have made significant contributions to phylogenetic studies of several plant families and to resolving evolutionary relationships within phylogenetic clades [5].

As the chloroplast genome of pteridophyte are less in number and on the basis of literature this is the first report from India to sequence the chloroplast genome of pteridophyte species. In previous study, we have reported mitochondrial genome of *L. cernuum* [9]. In the present study, we have sequenced chloroplast genome of *L. cernuum* and deposited in NCBI database. We are giving its comparative analysis with the already available cpgenome of *L. clavatum*.

MATERIALS AND METHODS

Lyophilised leaf powder was used for cpDNA extraction. The Chloroplast DNA was extracted from *L. cernuum* using a standard protocol of Bhatt and Thaker [10]. The Chloroplast DNA quality and quantity were assessed based on spectrophotometry and purity was checked on 1% agarose gel electrophoresis. This DNA was subjected to high throughput sequencing using an ion-torrent genome machine and reads were obtained by torrent suit software version v3.0

Data collection: From NCBI organelle database complete Lycopodiopsida chloroplast sequences retrieved are *Huperzia lucidula* (NC_006861), *Huperzia serrata* (NC_033874), *Isoetes butleri* (NC_038071), *Isoetes cangae* (NC_039392), *Isoetes*

engelmannii (NC_038080), *Isoetes flaccida* (NC_014675), *Isoetes graniticola* (NC_039821), *Isoetes mattaponica* (NC_039703), *Isoetes melanospora* (NC_038072), *Isoetes nuttallii* (NC_038073), *Isoetes serracarajensis* (NC_039393), *Isoetes valida* (NC_038074), *Isoetes yunguiensis* (NC_041146), *Phlegmariurus carinatus* (NC_050663), *Selaginella bisulcate* (NC_041640), *Selaginella doederleinii* (NC_041641), *Selaginella hainanensis* (NC_041642), *Selaginella indica* (NC_041098), *Selaginella lyallii* (NC_041556), *Selaginella pennata* (NC_041643), *Selaginella remotifolia* (NC_041644), *Selaginella sanguinolenta* (NC_041645), *Selaginella tamariscina* (NC_041646), *Selaginella vardei* (NC_041099), *Selaginella uncinata* (MG272483), *Dendrolycopodium obscurum* (NC_040923), *Diphasiastrum digitatum* (NC_040993), *Isoetes malinverniana* (NC_040924), *Isoetes piedmontana* (NC_040925), *Lycopodium clavatum* (NC_040994), *Selaginella kraussiana* (NC_040926), *Selaginella lepidophylla* (MK0895-31) and *Selaginella moellendorffii* (NC_013086).

Genome Structure Analyses: Geneious was used in gene size, regions comparison, GC content calculation from the fifteen cpgenome. ChloroMitoSSRDB [11] was used to detect microsatellites (mono-, di-, tri-, tetra-, penta-, hexanucleotide repeats) with the default settings of MISA and without primer synthesis model was used. Gene which are varied in size were further subjected for analysis of variable sites, parsimony informative sites, and nucleotide diversity. Multiple sequence alignment of these genes was performed in Clustal X2 [12], this alignment was exported in MEGA X [13].

Phylogenetic analyses: To estimate phylogenetic relationships within the Lycopodiaceae. Plastomes of *L. cernuum* and 34 lycopodiopsida species were selected. Common protein-coding genes were identified and concatenated protein-coding sequences were prepared. Multiple sequence alignment of these concatenated sequences was performed using CLC genomics workbench v12. This alignment was exported to MEGA X and a phylogenetic tree was prepared using the maximum likelihood method using 1000 bootstrap replicates.

RESULT AND DISCUSSION

In NCBI organelle database total 5197 chloroplast genomes and 375 mitochondrial genomes are

Table 1: Comparative features of all Lycopodiopsida chloroplast genomes

Sr.no.	Organism Name	Size (bp)	GC%	CDS	Genes	Pseudogene	rRNA	tRNAs	Country	Accession no.
1	<i>Lycopodium cernuum</i>	106709	37.8	66	88	3	7	15	India	MW538913
2	<i>Huperzia lucidula</i>	154373	36.25	87	129	1	8	33	USA	NC_006861
3	<i>Selaginella moellendorffii</i>	14378	51	70	101	3	8	15	Canada	NC_013086
4	<i>Isoetes flaccida</i>	145303	37.94	84	136	7	8	37	USA	NC_014675
5	<i>Huperzia serrata</i>	0.154176	36.28	87	131	1	8	35	China	NC_033874
6	<i>Isoetes butleri</i>	0.144912	38.02	84	136	7	8	37	USA	NC_038071
7	<i>Isoetes cangae</i>	0.14338	37.71	83	119	0	7	29	Brazil	NC_039392
8	<i>Isoetes engelmannii</i>	0.144817	38.05	84	136	7	8	37	USA	NC_038080
9	<i>Isoetes graniticola</i>	0.145118	37.99	84	136	7	8	37	USA	NC_039821
10	<i>Isoetes mattaponica</i>	0.145065	37.98	84	136	7	8	37	USA	NC_039703
11	<i>Isoetes melanospora</i>	0.145045	38	84	136	7	8	37	USA	NC_038072
12	<i>Isoetes nuttallii</i>	0.14468	38.18	84	136	7	8	37	USA	NC_038073
13	<i>Isoetes serracarajensis</i>	0.14338	37.71	83	119	0	7	29	Brazil	NC_039393
14	<i>Isoetes valida</i>	0.145132	37.99	84	136	7	8	37	USA	NC_038074
15	<i>Selaginella uncinata</i>	0.144161	54.88	81	107	3	8	15	China	MG272483
16	<i>Selaginella vardei</i>	0.121254	53.21	64	85	1	8	12	China	NC_041099
17	<i>Dendrolycopodium obscurum</i>	0.160877	34.99	88	133	1	8	36	USA	NC_040923
18	<i>Diphasiastrum digitatum</i>	0.159614	35.72	88	133	1	8	36	USA	NC_040993
19	<i>Isoetes malinverniana</i>	0.145535	38	84	130	0	8	38	USA	NC_040924
20	<i>Isoetes piedmontana</i>	0.14503	37.99	84	136	7	8	37	USA	NC_040925
21	<i>Lycopodium clavatum</i>	0.151819	34.49	88	133	1	8	36	USA	NC_040994
22	<i>Selaginella bisulcata</i>	0.140509	52.77	62	104	19	8	15	China	NC_041640
23	<i>Selaginella doederleinii</i>	0.142752	51.13	76	103	3	8	16	China	NC_041641
24	<i>Selaginella hainanensis</i>	0.144201	54.83	80	107	4	8	15	China	NC_041642
25	<i>Selaginella kraussiana</i>	0.129971	52.33	74	94	1	8	11	USA	NC_040926
26	<i>Selaginella pennata</i>	0.138024	52.92	74	99	6	8	11	China	NC_041643
27	<i>Selaginella remotifolia</i>	0.131867	56.5	73	96	1	8	14	China	NC_041644
28	<i>Selaginella sanguinolenta</i>	0.147148	50.78	69	113	11	8	25	China	NC_041645
29	<i>Selaginella tam ariscina</i>	0.1267	54.06	62	80	1	8	9	China	NC_041646
30	<i>Isoetes yunguiensis</i>	0.145355	37.99	84	135	7	8	36	China	NC_041146
31	<i>Selaginella indica</i>	0.12246	53.55	64	87	1	8	14	China	NC_041098
32	<i>Selaginella lepidophylla</i>	0.114693	51.94	64	87	2	8	13	USA	MK089531
33	<i>Selaginella lyallii</i>	0.110411	50.75	61	84	0	8	15	China	NC_041556
34	<i>Phlegmariurus carinatus</i>	0.150349	33.98	79	120	0	8	33	China	NC_050663
35	<i>Phlegmariurus phlegmaria</i>	0.149711	33.8	53	90	1	6	30	China	NC_052703

Table 2: Gene identified in the Chloroplast genome of *L. cernuum*

Category of genes	Group of genes	Name of genes
Genes for photosynthesis	Subunits of ATP synthase	<i>atpB, atpE, atpF, atpH, atpI</i>
Genes for photosynthesis	Subunits of photosystem II	<i>psbA, psbB, psbC, psbD, psbF, psbI, psbL, psbN, psbT, psbZ, ycf3</i>
Genes for photosynthesis	Subunits of NADH-dehydrogenase	<i>ndhA, ndhB, ndhC, ndhD, ndhE, ndhG, ndhH, ndhI, ndhJ, ndhK</i>
Genes for photosynthesis	Subunits of cytochrome b/f complex	<i>petA, petD</i>
Genes for photosynthesis	Subunits of photosystem I	<i>psaA, psaA, psaB, psaB, psaC</i>
Genes for photosynthesis	Subunit of rubisco	<i>rbcL</i>
Self-replication	Large subunit of ribosome	<i>rpl14, rpl2, rpl20, rpl33</i>
Self-replication	Small subunit of ribosome	<i>rps11, rps14, rps15, rps18, rps19, rps2, rps4, rps8</i>
Other genes	Subunit of Acetyl-CoA-carboxylase	<i>accD</i>
Other genes	c-type cytochrome synthesis gene	<i>ccsA</i>
Other genes	Envelope membrane protein	<i>cemA</i>
Other genes	Protease	<i>clpP</i>
Unkown	Conserved open reading frames	<i>ycf1, ycf2, ycf4</i>

available as of April 2021, from these 34 of chloroplast and only 1 of mitochondrial belong to Lycopodiopsida. This is the first report of chloroplast genome sequencing from the Indian lab for Lycopodiopsida.

Chloroplast genome structure: The chloroplast genome of *L. cernuum* was sequenced and obtained reads were of good quality with no adapter contamination. The chloroplast genome of *Lycopodium cernuum* (Fig.1) was compared with the already available chloroplast genome of *L. clavatum* and three other species of family Lycopodiaceae was taken for comparison. All the species of Lycopodiopsida had a typical quadripartite structure, with a circular molecule of 106709 bp to 160877 bp in length. A number of genes ranges from 88 to 136 and the number of proteins from 53 to 88. However, the number of tRNAs are lowest in *L. cernuum* but still no such big difference is there in protein-coding genes. Overall GC content for these genomes varies from 34 to 37 (Table 1). The highest GC content from all four regions is for IR region and about 84 protein-coding genes primarily participating in photosynthesis, transcription and translation. Their number size does not directly correlate with plastome size [5]. The chloroplast genomes of land plants have highly conserved structures and organization of content; they comprise a single circular molecule with a quadripartite structure that includes two copies of an IR region that separate large and small single-copy (LSC and SSC) regions. The chloroplast genome includes 120–130 genes, primarily participating in photosynthesis, transcription, and translation [5]. Recent studies have identified significant diversity within non-coding intergenic spacer regions, which often include chief regulatory sequences [14]. Certain lineages of land-plant chloroplast genomes also show

Table 3: Comparative analysis of introns in cpDNA of two *Lycopodium* species

Genes	<i>L. Cernuum</i>	<i>L. Clavatum</i>
<i>rpl2</i>	1	1
<i>rpl19</i>	1	-
<i>clpP</i>	2	2
<i>trnK-UUU</i>	2	2
<i>trnL-UAA</i>	-	2
<i>ycf3</i>	2	2
<i>trnK-UUU</i>	-	2
<i>trnL-CAA</i>	-	2
<i>trnW-CCA</i>	-	2
<i>atpF</i>	1	1
<i>rpoc1</i>	-	1
<i>ndhB</i>	1	1
<i>trnE-UUC</i>	2	2
<i>trnA-UGC</i>	2	2
<i>ycf1</i>	-	1
<i>ndhA</i>	1	1
<i>trnA-UGC</i>	2	2
<i>trnE-UUC</i>	2	2

significant structural rearrangements, with evidence of the loss of IR regions or entire gene families [15].

Gene identified in the chloroplast genome of *L. cernuum*: These can be divided into three broad categories: The first category comprises of genes for the photosynthetic apparatus. This category includes photosystem I (*psaA*, *psaB*, etc.), photosystem II (*psbA*, *psbB*, etc.), cytochrome b6f (*petA*, *petB*, etc.), ATP synthase (*atpA*, *atpB*, etc.), RuBisCo (*rbcL*) and NAD(P)H dehydrogenase genes (*ndhA*, *ndhB*, etc.). The second category comprises RNA genes and genes for the genetic apparatus. This includes transfer RNA (*trnH*, *trnK*, etc.), ribosomal RNA (*rrn16*, *rrn5*, etc.), RNA polymerase (*rpoA*, *rpoB*, etc.) and ribosomal subunit genes (*rps2*, *rps3*, *rpl2*, *rpl16*, etc.). The third category comprises of conserved ORFs called and potential protein-coding genes like *matK* and *cemA* (Table 2). In comparative intron content study intron loss in *rpl19* gene in *L. clavatum* is observed, while intron

Table 4: Type and size of SSRs in the *L. cernuum* and *L. clavatum* chloroplast genome

SSR <i>L. cernuum</i>	Size	Gene Name	Start	End
(T)10	10	<i>petD- petB</i>	6080	6089
(A)10	10	<i>psbD- clpP</i>	10506	10515
(T)11	11	<i>rps12</i>	13087	13097
(T)10	10	<i>rps12, petA- cemA</i>	19055	19064
(A)11ttgtttaa(T)12	32	<i>rps12, ycf3</i>	31535	31566
(G)10	10	<i>rps12</i>	32732	32741
(T)10	10	<i>rps12</i>	44286	44295
(A)10	10	<i>rps12, psbD- psbA</i>	44516	44525
(TA)6	12	<i>rps12, psbD- psbA</i>	48236	48247
(A)11	11	<i>rps12, atpF</i>	56796	56806
(AT)7tc(T)10	26	<i>rps12, ndhB</i>	71541	71566
(A)10	10	<i>rps12, ndhB</i>	71937	71946
(T)11	11	<i>rps12, ndhB</i>	72087	72097
(GA)6	12	<i>ndhH</i>	88824	88835
(A)10ggaaaaaac(T)11	31	<i>ndhA</i>	90695	90725

Table 5: Chloroplast genome sequences used for phylogenetic tree construction with their accession numbers and classification

Species Name	GenBank ID	Classification
<i>Lycopodium cernuum</i>	MW538913	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Lycopodiaceae; <i>Lycopodium</i>
<i>Huperzia lucidula</i>	NC_006861	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Huperziaceae; <i>Huperzia</i>
<i>Huperzia serrata</i>	NC_033874	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Huperziaceae; <i>Huperzia</i>
<i>Isoetes butleri</i>	NC_038071	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes cangae</i>	NC_039392	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes engelmannii</i>	NC_038080	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes flaccida</i>	NC_014675	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes granitcola</i>	NC_039821	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes mattaponica</i>	NC_039703	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes melanospora</i>	NC_038072	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes nuttallii</i>	NC_038073	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes serracarajensis</i>	NC_039393	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes valida</i>	NC_038074	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes yunguensis</i>	NC_041146	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Phlegmariurus carinatus</i>	NC_050663	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Huperziaceae; <i>Phlegmariurus</i>
<i>Selaginella bisulcata</i>	NC_041640	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella doederleinii</i>	NC_041641	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella hainanensis</i>	NC_041642	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella indica</i>	NC_041098	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella lyallii</i>	NC_041556	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella pennata</i>	NC_041643	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella remotifolia</i>	NC_041644	Eukaryota; Diaphoretikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>

Continue table 5

<i>Selaginella sanguinolenta</i>	NC_041645	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella tamariscina</i>	NC_041646	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella vardei</i>	NC_041099	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella uncinata</i>	MG272483	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Dendrolycopodium obscurum</i>	NC_040923	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Lycopodioidae; <i>Dendrolycopodium</i>
<i>Diphasiastrum digitatum</i>	NC_040993	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Lycopodioidae; <i>Diphasiastrum</i>
<i>Isoetes malinverniana</i>	NC_040924	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Isoetes piedmontana</i>	NC_040925	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Isoetales; Isoetaceae; <i>Isoetes</i>
<i>Lycopodium clavatum</i>	NC_040994	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Lycopodiales; Lycopodiaceae; Lycopodioidae; <i>Lycopodium</i>
<i>Selaginella kraussiana</i>	NC_040926	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella lepidophylla</i>	MK089531	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginella; Selaginella; Selaginellaceae; <i>Selaginella</i>
<i>Selaginella moellendorffii</i>	NC_013086	Eukaryota; Diaphoreticikes; Archaeplastida; Plantae; Streptophyta; Embryophytes; Polysporangiophytes; Tracheophytes; Lycophytes; Lycopodiopsida; Selaginellales; Selaginellaceae; <i>Selaginella</i>

loss in *L. cernuum* is found in *trnL-UAA*, *trnK-UUU*, *trnL-CAA*, *trnW-CCA*, *rpoC1* and *ycf1* (Table 3).

Simple sequence repeat (SSR) in *L. cernuum* and *L. clavatum*: Repeats in two cp genomes were analyzed using MISA server including two species of *L. cernuum* and *L. clavatum*. The total numbers of SSRs were also identified in the cp genomes of the two species. From analyzed types of Mononucleotide repeats founds 10 SSRs. Microsatellite repeats 57 were found in *L. clavatum* while the number of repeats 15 were found in *L. cernuum* (Table 4). The longest repeats, with a length of 32 bp (*rps12* & *ycf3*) in *lycopodium cernuum* and the longest repeats, with a length of 122 bp (*atpF-atpH*) in *Lycopodium clavatum*. The total numbers of SSRs were also identified in the cp genomes of the two species. From repeat Mononucleotide repeats found in *Lycopodium cernuum* with 10 and *Lycopodium clavatum* 122 repeat mononucleotides. *Lycopodium cernuum* is 3 hexanucleotide and *L. clavatum* is 9 hexanucleotide. Simple sequence repeat (SSR), are widely dispersed over the genome and have a high degree of polymorphism [16]. As a

result, SSRs are widely used as a molecular marker for breeding [17], population genetics [18], genetic linkage map construction and gene mapping [19].

Phylogenetic analyses: Thirty-four conserved plastid protein-coding genes were used to reconstruct the phylogenetic framework using 34 species from previously published plastomes of land plants (from pteridophyte (Table 5)). We downloaded the raw reads from GenBank, assembled plastid contigs and extracted the 32 gene sequences since the complete plastome of *L. lycopodium* has not been released on GenBank. A total of bp sequences were aligned at the protein level by CLC using the translation-aligned function in Geneious v. 9.1.4. The phylogenetic relationship among these species were presented in the figure 2 and *L. cernuum* shows the closest match with *L. clavatum*.

REFERENCES

- [1] Baytop, T.: J. Pharm. Istanbul Univ., **2**: 312 (1999).
- [2] Saenz, A. Jonsell, S. and Froelich, P.: Physical review letters, 84(2): 242 (2000).

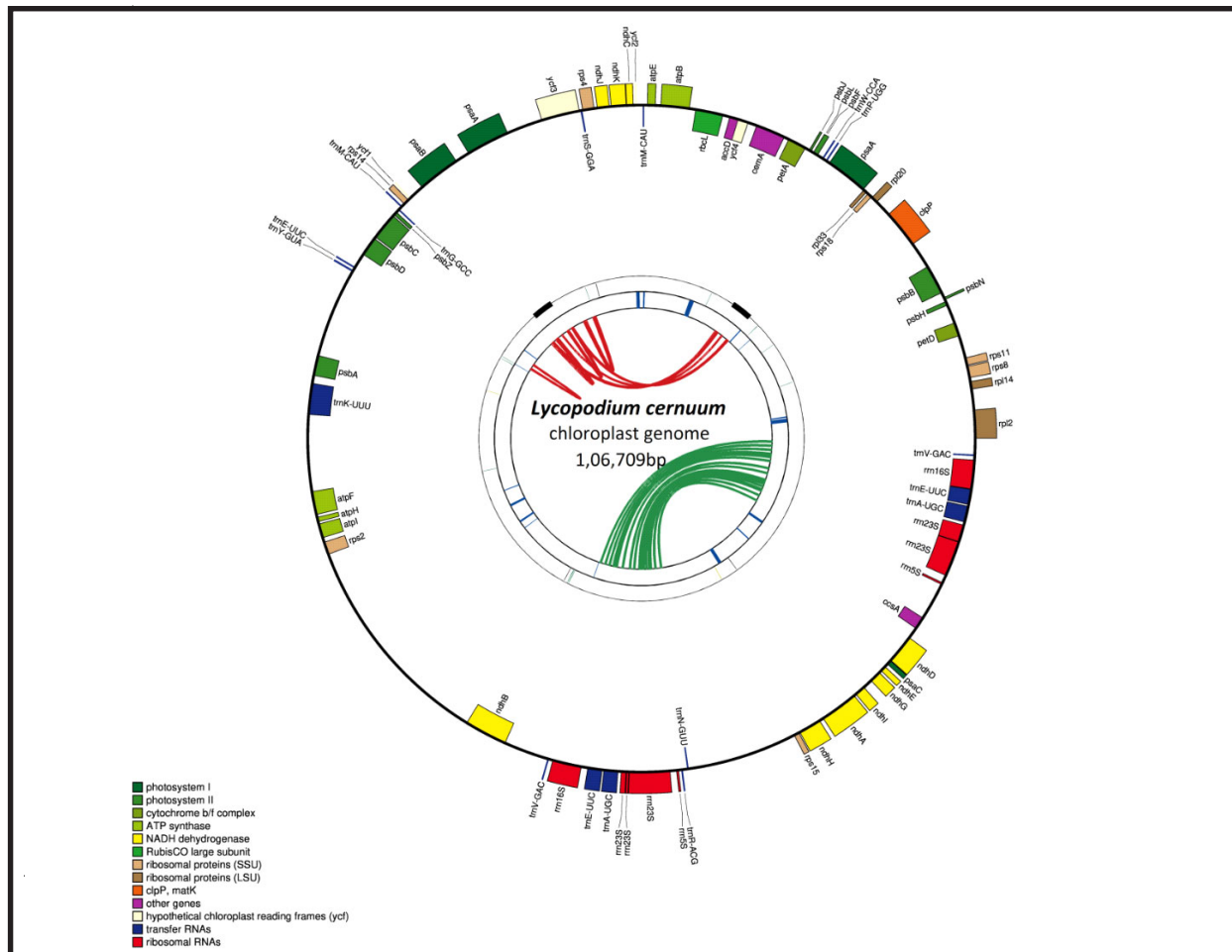


Fig. 1: Genome map of the chloroplast genome of *L. cernuum*. Gene on the inside of the circle are transcribed clockwise, while those outside are transcribed counter clockwise.

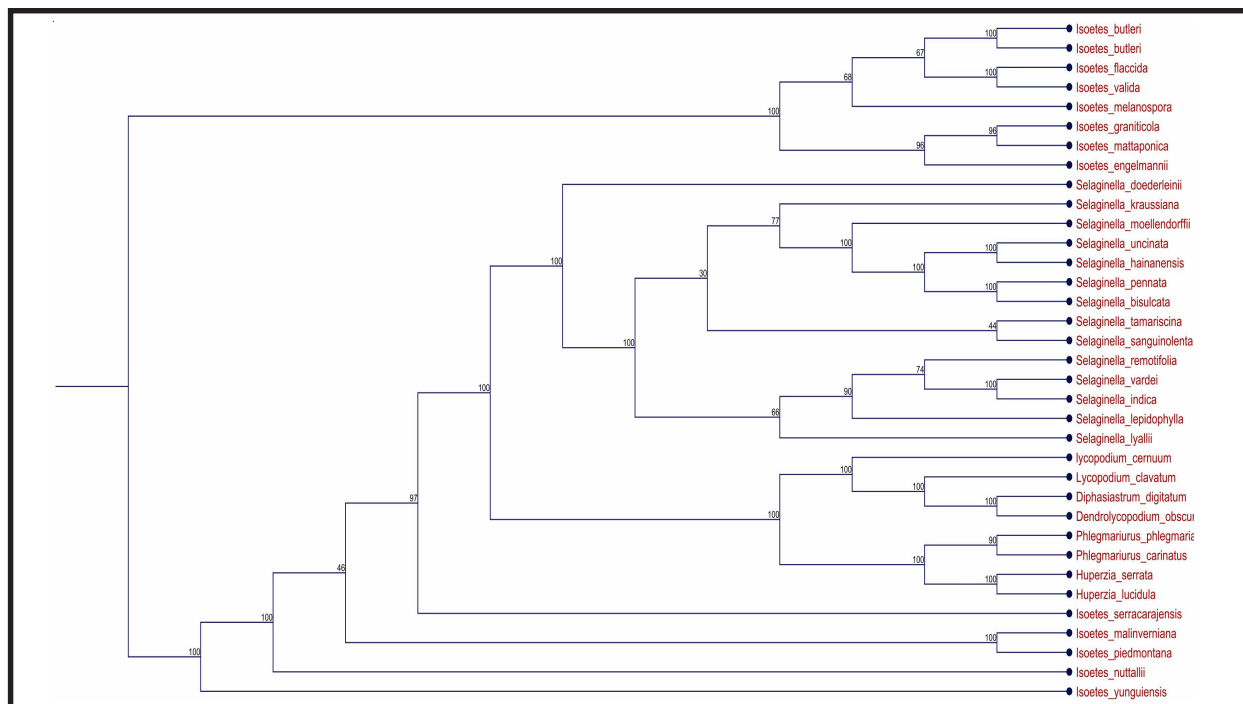


Fig. 2: Phylogenetic tree of *L. cernuum* with other 32 species of Lycopodiopsida constructed using maximum likelihood method by 1000 bootstrap replicates.

- [3] Ma, X. and Gang, D.: *Nat. Prod. Rep.*, 21(6):752-772 (2004).
- [4] Pick, T.R. and Weber, A.P.: *Front. Plant Sci.*, 5: 410 (2014).
- [5] Daniell, H., Lin, C.S., Yu, M. and Chang, W.J.: *Biol.*, 17(1): 1-29 (2016).
- [6] Bendich, A.J.: *The Plant Cell.*, 16 (7): 1661-1666 (2004).
- [7] Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z. and Aota, S.I.: *Nature.*, 322: 572-574 (1986).
- [8] Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi Shinozaki, K. and Ohto, C.: *The EMBO journal*, 5: 2043-2049 (1986).
- [9] Kanagara, K., Kanzariya, R., Bhatt, P., Monpara, J., Chudasama, K., and Thaker, V.: *J. Cell Tissue Res.*, 21(1): 7069-7076 (2021).
- [10] Bhatt, P. and Thaker, V.: *Meta Gene*, 29: 100921 (2021).
- [11] Sablok, A., Batra, A., Thariani, K., Batra, A., Bharti, R. Aggarwal, A.R., Kabi, B.C. and Chellani, H.: *J. Clin. Endocrinol. Metab.*, 83: 536-541 (2015).
- [12] Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R. and Thompson, J.D.: *Bioinformatics*, 23: 2947-2948 (2007).
- [13] Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K.: *Mol. Biol. Evol.*, 35: 1547 (2018).
- [14] Repetti, S.I. Jackson, C.J. Judd, L.M. Wick, R.R. Holt, K.E. and Verbruggen, H.: *Peer J.*, 8: e8273 (2020).
- [15] Chiba, Y.: *Cytologia.*, 16: 259-264 (1951).
- [16] Oldenburg, D. J. and Bendich, A. J.: *Curr. Genet.*, 62: 431-442 (2016).
- [17] Chen, W., Zheng, R., Baade, P.D., Zhang, S., Zeng, H., Bray, F., Jemal, A. Yu, X.Q. and He, J.: *CA: Cancer J. Clin.*, 66: 115-132 (2016).
- [18] Gupta, P.K. and Varshney, R.K.: *Euphytica*, 113: 163-185 (2000).
- [19] Varshney, R.K., Bertoli, D.J., Moretzsohn, M.D., Vadez, V., Krishnamurthy, L., Aruna, R., Nigam, S.N., Moss, B.J., Seetha, K., Ravi, K. and He, G.: *Theor. Appl. Genet.*, 118: 729-739 (2009).