Complete Chloroplast Genome of a Valuable Medicinal Plant, Huperzia serrata (Lycopodiaceae), and Comparison with Its Congener

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Source: Applications in Plant Sciences, 4(11)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1600071

Applications in Plant Sciences 2016 4(11): 1600071

Genomic Resources Note

Complete chloroplast genome of a valuable medicinal plant, *Huperzia serrata* (Lycopodiaceae), and comparison with its congener[1]

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- *Premise of the study:* Here we report the complete chloroplast genome of the important medicinal species *Huperzia serrata* (Lycopodiaceae) and compare it to the chloroplast genome of the congeneric species *H. lucidula*.
- *Methods and Results:* The whole chloroplast genome of *H. serrata* was sequenced using an Illumina platform and assembled with Geneious version R9.0.5. The genome size of *H. serrata* was 154,176 bp, with 36.3% GC content. The complete chloroplast genome contained 120 unique genes, including 86 coding genes, four rRNA genes, and 30 tRNA genes. Comparison with the chloroplast genome of *H. lucidula* revealed three highly variable regions (*rps16-chlB*, *ycf12-trnR*, and *ycf1*) between these two species and 252 mutation events including 27 insertion/deletion polymorphisms and 225 single-nucleotide polymorphisms (SNPs). Ninety-two SNPs were identified in the gene-coding regions. In addition, 18 microsatellite sites were found, which can potentially be used in phylogeographic studies.
- *Conclusions:* The complete chloroplast genome of *H. serrata* is reported here, and will be a valuable genome resource for further phylogenetic, evolutionary, and medical studies of medicinal plants in the genus *Huperzia*.

Key words: *Huperzia serrata*; lycophytes; Lycopodiaceae; mutation; next-generation sequencing.

The structure of chloroplast genomes in land plants is generally highly conserved in terms of gene order, organization, and content, which makes them suitable for characterizing genetic relationships among species (Bock, 2007). Portions of these genomes have also been widely used by many plant taxonomists as effective DNA barcoding tools. Most of the chloroplast genomes of land plants have a pair of inverted repeats (IRs), separated by one large single copy region (LSC) and one small single copy region (SSC) (Jansen et al., 2005). However, variations occur in certain lineages, and these variations have been proven to be useful in identifying some critical events during the evolution of land plants (Dong et al., 2014; Song et al., 2015). One typical example is the 30-Kb inversion (from *trnC* to *ycf2*) detected in the LSC region from bryophytes and lycophytes to other land plants, supporting the hypothesis that lycophytes are a sister clade to all other extant vascular plants (Raubeson and Jansen, 1992).

[1]Manuscript received 17 June 2016; revision accepted 11 October 2016.

The authors thank Yanlei Feng and Xiao Ma for help with genome assembly and annotation; Wenpan Dong and Changhao Li for help with data analysis; and Li Wang, Ran Wei, and two anonymous reviewers for comments on an earlier version of the manuscript. This research was supported by the Natural Science Foundation of Guizhou Province ([2014]2156) and the National Natural Science Foundation of China (31470317).

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doi:10.3732/apps.1600071

Compared with those on seed plants, studies on chloroplast genomes of ferns and lycophytes have been relatively sparse (Lu et al., 2015). The North American firmoss *Huperzia lucidula* (Michx.) Trevis. (Lycopodiaceae) was the first lycophyte species with a complete chloroplast genome sequence (Wolf et al., 2005; GenBank accession no. NC_006861). Because *H. lucidula* belongs to a significant sister clade of all extant vascular plants, sequencing its complete chloroplast genome facilitates the exploration of the relationships between lycophytes and other vascular plants. Both the rearrangement structure of the chloroplast genome and the phylogenomic analyses of 73 protein-coding genes supported the hypothesis that lycophytes were a sister to both extant fern and seed plant lineages (Wolf et al., 2005).

However, the phylogenetic relationships within this family and particularly within the genus *Huperzia* Bernh. (ca. 55 species) are still unclear because of insufficient phylogenetic data (Zhang and Iwatsuki, 2013). Here we describe the complete chloroplast genome sequence of a valuable species (*H. serrata* (Thunb.) Trevis.) within this genus and compare it to existing chloroplast genome data of *H. lucidula* to better understand the mutation patterns in chloroplast genomes of *Huperzia*. Both *H. lucidula* and *H. serrata* belong to *Huperzia* sect. *Serratae* (Rothm.) Holub and form a clade based on *matK* sequences showing a close phylogenetic relationship (Zhang, 2004; Ji et al., 2007). Furthermore, *H. serrata* is an important medicinal plant containing huperzine A, which several studies have found to be effective in the treatment of Alzheimer's disease (Tang, 1996; Wang et al., 1998; Guo et al., 2005). Thus,

TABLE 1. Summary of *Huperzia serrata* and *H. lucidula* chloroplast features.

Feature	*H. lucidula*	*H. serrata*
Total cpDNA size	154,373	154,176
LSC	104,088	104,080
SSC	19,657	19,658
IR	30,628	30,438
Total GC content (%)	36.3	36.3
LSC	34.4	34.4
SSC	32.8	32.8
IR	44.9	45.0
Total no. of genes	119	120
Protein encoding	86	86
tRNA	29	30
rRNA	4	4

Note: IR = inverted repeat; LSC = large single copy; SSC = small single copy.

this draft genome may not only facilitate investigations into genetic variation but also elucidate relationships within the genus to guide further exploration of compounds in closely related species.

METHODS AND RESULTS

Specimens of *H. serrata* were collected from Helong, Jilin Province, northeastern China. A voucher specimen (*X. C. Zhang 6972*) has been deposited in the Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE). Total DNA was extracted with a modified cetyltrimethylammonium bromide (CTAB) method (Li et al., 2013). The DNAs were sheared into ~350-bp fragments using the Covaris M220 focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA). The NEBNext DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) was used for library construction. Paired-end reads of 2 × 150 bp then were generated using an Illumina HiSeq PE150 (Illumina, San Diego, California, USA). A total of 9,391,796 paired-end sequence reads of 150 bp were generated, of which 406,164 reads belong to the chloroplast genome. The chloroplast genome data were extracted using *H. lucidula* as a reference and assembled de novo with Geneious version R9.0.5 (Kearse et al., 2012). The first de novo assembly generated eight contigs, and the eight contigs were then extended by mapping raw reads to the contigs several times until all contigs were merged into one whole sequence of 138,841 bp. The four ends of IR regions were located through BLAST with the whole sequence itself to assemble into the complete chloroplast genome sequence. The annotation of all the genes encoding proteins, tRNAs, and rRNAs was constructed with Dual Organellar GenoMe Annotator (DOGMA; Wyman et al., 2004) and was uploaded to GenBank. The tRNAs were further verified using tRNAscan-SE version 1.21 (Lowe and Eddy, 1997; Schattner et al., 2005). The genome map was drawn with OGDraw version 1.2 (Lohse et al., 2007).

The chloroplast genome sequence of *H. lucidula* was downloaded from GenBank and aligned with *H. serrata* using MAFFT version 7 (Katoh and Standley, 2013). A sliding window analysis was conducted with DnaSP version 5.1 (Librado and Rozas, 2009) to evaluate the genetic diversity (π) across whole genomes within the genus *Huperzia*. The window length was set to 600 bp with a 200-bp step size based on the proposed length of DNA barcoding regions (Song et al., 2015). DnaSP was also used for identifying insertion/deletion polymorphisms (indels) with the chloroplast genome of *H. lucidula* as a reference. A custom Python script (https://www.biostars.org/p/119214/) based on single-nucleotide polymorphism (SNP) definition (a variation in a single nucleotide that occurs at a specific position in the genome) was employed to call SNPs. The SNPs in coding regions were classified in two ways: synonymous and nonsynonymous; transition and transversion. The simple sequence repeats (SSRs) in *H. serrata* were detected using NWISRL-Imperfect SSR Finder version 1.0 (Stieneke and Eujayl, 2007). The repeats unit length was set to two to nine base pairs with at least five copies for dinucleotide and four copies for other multinucleotide repeats.

The complete genome sequence of *H. serrata* (GenBank accession no. KX426071) was 154,176 bp long, 197 bp shorter than that of *H. lucidula* (154,373 bp; GenBank accession no. NC_006861). Both genomes had GC content of 36.3% (Table 1). A pair of IRs of 30,438 bp was separated by an LSC and a SSC of 104,080 bp and 19,658 bp, respectively, in *H. serrata*. The complete chloroplast genome contained 120 putative unique genes, including 86 coding genes, four rRNA genes, and 30 tRNA genes. The gene map of *H. serrata* is shown in Fig. 1. Based on our preliminary analysis, we found that 15 genes have one predicted intron (10 coding genes and five tRNA genes) and two coding genes have two introns (*clpP* and *ycf3*). Compared with *H. lucidula*, we found that the gene order and features are almost identical in genomes of *H. serrata*. Because comparisons between *H. lucidula* and other land plants have already been conducted in previous studies (Wolf et al., 2005), we did not repeat the work again. However, some unusual features also existed: an extra tRNA *trnI*-GAU between *rrn16* and *trnA*-UGC in the IR region, and an intron within *ycf66* in the LSC region were first annotated in *H. serrata*. These three genes were also annotated in the chloroplast genome sequence of another lycophyte plant, *Isoetes flaccida* A. Braun (GenBank accession no. NC_014675) (Karol et al., 2010). Similar to *H. lucidula*, nine predicted protein-coding genes (*ndhJ*, *atpI*, *chlL*, *ndhH*, *ccsA*, *rpl36*, *ycf1*, *rps15*, *ndhD*) lack their canonical start codons and/or stop codons at the expected positions. A triplet ACG, which is changed into a start codon by C to U RNA editing, and another triplet CAA, which is changed into a stop codon by C to U RNA editing, appear in the position of the expected start codon and stop codon, respectively (Tsuji et al., 2007). Furthermore, *rps16* has two internal stop codons in the chloroplast genome and is therefore considered to be a pseudogene, but the chloroplast transcriptome evidence is needed to prove this hypothesis (Oldenkott et al., 2014).

The nucleotide variability (π) of the aligned genome sequences of *H. lucidula* and *H. serrata* was calculated with DnaSP version 5.1 to explore the level of sequence divergence. The value varied from 0 to 0.1 with an average of 0.00143, showing that divergence between the genomes of these closely related species is small. However, three highly variable regions—*rps16-chlB*, *ycf12-trnR*, and *ycf1*—were located (Fig. 2). Only *ycf1* is in the SSC region; the other two loci were in the LSC region. None of these highly variable regions have been employed in previous phylogenetic analyses of ferns and lycophytes (Kuo et al., 2011; Li et al., 2011). Based on these results, we infer that *ycf1*, *ycf12-trnR*, and *rps16-chlB* ($\pi > 0.008$) could be suitable for phylogenetic analyses at the species level.

Eighteen potential SSR motifs were found, and most were located in the intergenic regions of the LSC region (Table 2). Only three types were identified, with the majority belonging to di- and trinucleotide motifs. ACT/TCT and AAT/TAA/TAG/TAT motifs were found among trinucleotide SSRs while only AT/TA motifs were identified for the dinucleotide motifs. Twenty-seven indels were revealed in the comparison between the chloroplast genome sequences of *H. serrata* and *H. lucidula*. Most indels ranged from one to nine base pairs in size and were located in noncoding regions, while three indels occurred within the coding region of the *rpoC2* gene, with lengths of 24 bp, 30 bp, and 126 bp, respectively (Table 3). The three indels are all deletions in *rpoC2* of *H. serrata*. Ninety-two SNPs, including 75 transitions and 17 transversions in gene-coding regions, and 133 SNPs, including 88 transitions and 45 transversions in noncoding regions, were detected (Table 4). Among gene-coding regions, 36 synonymous and 56 nonsynonymous substitution sites existed in the whole genomes. Thirty-seven out of 86 coding genes have nonsynonymous substitution sites. Among these genes, *rpoC2*, *ycf1*, and *ycf2* have the most nonsynonymous substitution sites, showing that these three genes may have relatively fast rates of evolution and can be used in phylogenetic analyses.

CONCLUSIONS

Here we report the complete chloroplast genome sequence of *H. serrata*, an important and widely distributed medicinal plant. Availability of this chloroplast genome sequence and the existing *H. lucidula* chloroplast genome sequence enable us to evaluate the genome-wide mutational events within the genus *Huperzia*. The genome arrangement, gene order, gene size, and GC content of *H. serrata* and *H. lucidula* are almost identical.

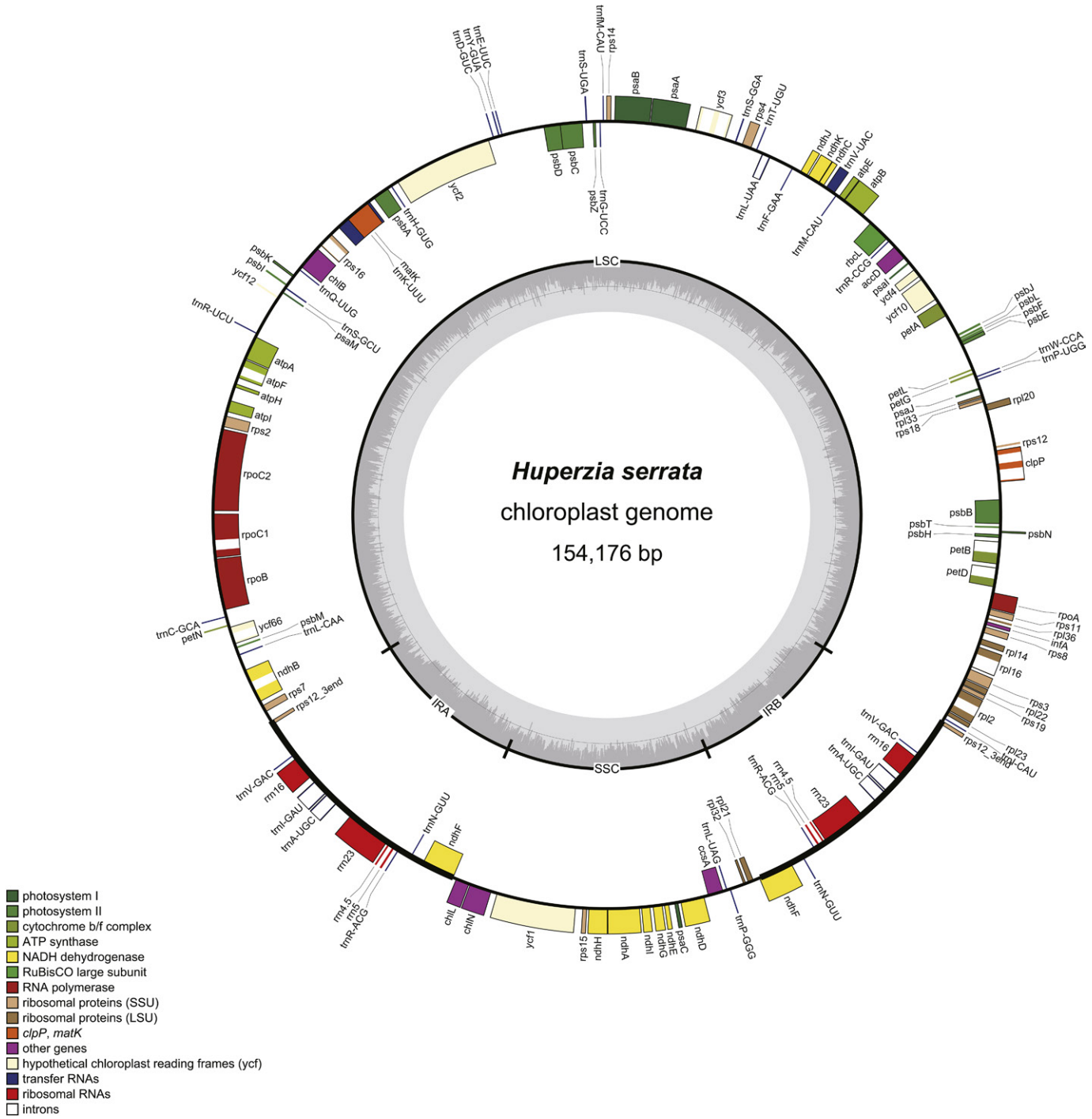

Fig. 1. Gene map of the *Huperzia serrata* chloroplast reference genome. Genes outside of the outer circle are transcribed clockwise, whereas genes inside the outer circle are transcribed counterclockwise. The colored bars indicate different functional groups. The dashed darker gray area in the inner circle denotes GC content while the lighter gray area shows the AT content of the genome. IR = inverted repeat; LSC = large single copy; SSC = small single copy.

Three divergence hotspots (*rps16-chlB*, *ycf12-trnR*, and *ycf1*), 18 SSRs, 27 indels, and 225 SNPs across the whole genome were identified and could provide useful phylogenetic and phylogeographic information for closely related species. Moreover, conserved primers could be designed for the highly variable regions in *Huperzia* based on these two complete chloroplast genomes.

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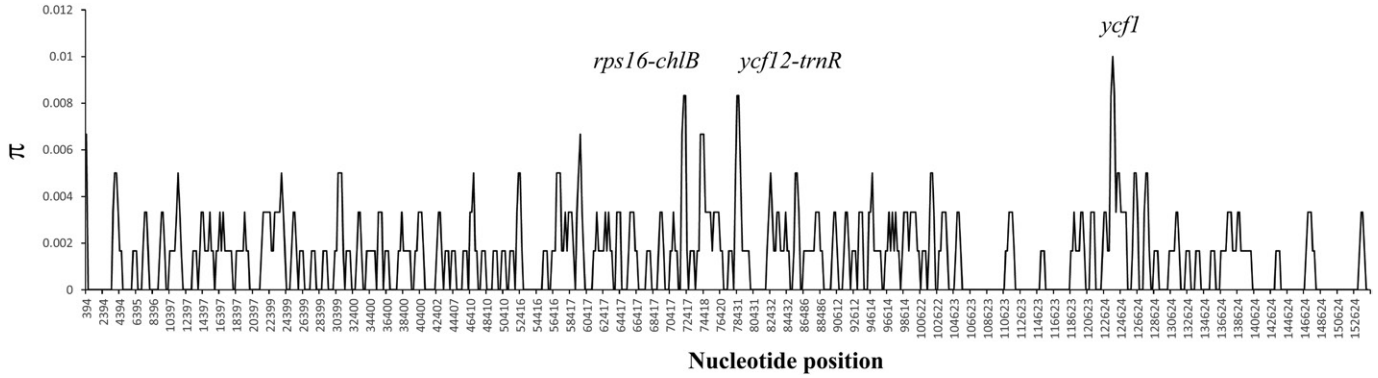

Fig. 2. Sliding window analysis of the whole chloroplast genomes of *Huperzia serrata* and *H. lucidula*. Window length = 600 bp; step size = 200 bp; *x*-axis = position of the midpoint of a window; *y*-axis = value of π of each window.

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Table 2. Location of simple sequence repeats in *Huperzia serrata.*

No.	Start	End	Location	Region	Motif	No. of repeats
1	11,242	11,255	*petB*	Intron	TA	7
2	40,488	40,497	*trnF-trnL*	Intergenic	TA	5
3	43,483	43,492	*rps4-trnS*	Intergenic	AT	5
4	56,437	56,454	*psbD-trnE*	Intergenic	AT	9
5	70,509	70,522	*trnK-rps16*	Intergenic	AT	7
6	74,093	74,104	*trnQ-psbK*	Intergenic	TA	6
7	84,161	84,178	*atpI-rps2*	Intergenic	TA	9
8	85,147	85,161	*rps2-rpoC2*	Intergenic	TAG	5
9	87,843	87,878	*rpoC2*	CDS	TGCTTCATC	4
10	93,017	93,031	*rpoC1*	CDS	TCT	5
11	97,444	97,457	*trnC-petN*	Intergenic	TA	7
12	99,074	99,097	*psbM-trnL*	Intergenic	TAT	8
13	99,938	99,947	*trnL-ndhB*	Intergenic	TA	5
14	100,289	100,298	*trnL-ndhB*	Intergenic	AT	5
15	121,906	121,917	*chlN-ycf1*	Intergenic	TAA	4
16	127,339	127,350	*ycf1-rps15*	Intergenic	ACT	4
17	130,250	130,261	*ndhA*	Intron	AAT	4
18	138,671	138,685	*rpl21-ndhF*	Intergenic	TAA	5

Note: CDS = coding DNA sequence.

TABLE 3. Location of indels in the genomes of *Huperzia serrata* and *H. lucidula.*

No.	Position	Location	Region	Motif	Size (bp)	Direction[a]
1	5862	*rpl14-rps8*	Intergenic	A	1	Insertion
2	7064	*rpl36-rps11*	Intergenic	G	1	Insertion
3	9953	*petD*	Intron	A	1	Insertion
4	20,555–20,556	*rpl20-rps18*	Intergenic	GG	2	Insertion
5	30,794	*psaI-accD*	Intergenic	T	1	Deletion
6	42,082–42,083	*trnL-UAA*	Exon	CC	2	Insertion
7	42,758–42,759	*trnT-rps4*	Intergenic	CC	2	Deletion
8	44,361–44,363	*trnS-ycf3*	Intergenic	GGG	3	Deletion
9	44,986–44,989	*ycf3*	Intron	TTC	3	Insertion
10	51,669–51,673	*psaB-rps14*	Intergenic	GGGGG	5	Deletion
11	51,749	*psaB-rps14*	Intergenic	G	1	Deletion
12	58,365	*psbD-trnE*	Intergenic	T	1	Insertion
13	73,875	*chlB-trnQ*	Intergenic	T	1	Insertion
14	75,019	*psbK-psbI*	Intergenic	A	1	Insertion
15	76,144	*psaM-ycf12*	Intergenic	A	1	Deletion
16	77,128	*ycf12-trnR*	Intergenic	A	1	Insertion
17	77,497–77,505	*ycf12-trnR*	Intergenic	CGTAGTATT	9	Deletion
18	78,117	*ycf12-trnR*	Intergenic	G	1	Deletion
19	81,577	*atpF*	Intron	A	1	Deletion
20	85,338–85,361	*rpoC2*	CDS	TCGGTTGCTTCACCAACAGTTTCC	24	Deletion
21	85,557–85,586	*rpoC2*	CDS	TTATCACTAGTTTCTTCATCACTAGTTTCT	30	Deletion
22	88,781–88,906	*rpoC2*	CDS	TTCAAATTCTGTCTGATCTTCTTCTAAAGAAGAATCAAATGATT CAAATTCTGTCTGATCTTCTTCTAAAGAAGAATCAAATGATT CAAATTCTGTCTGATCTTCTTCTAAAGAAGAATCAAATGA	126	Deletion
23	92,802–92,803	*rpoC1*	Intron	TT	2	Insertion
24	99,278–99,283	*psbM*	CDS	TATTAT	6	Deletion
25	100,234–100,235	*trnL-ndhB*	Intergenic	CC	2	Deletion
26	104,210	*rps7-rps12*	Intergenic	T	1	Deletion
27	122,056	*chlN-ycf1*	Intergenic	A	1	Insertion

Note: CDS = coding DNA sequence.

[a]The plastome of *Huperzia lucidula* was used as a reference.

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TABLE 4. Comparisons of mutations, number of transitions (Ts) and transversions (Tv), and number of synonymous (S) and nonsynonymous (N) substitutions per gene of *Huperzia serrata* and *H. lucidula*.

Gene type	Gene	Ts	Tv	S	N
Photosynthetic apparatus	*petB*	1	0	0	1
	petD	1	0	0	1
	petN	1	0	1	0
	psaA	0	1	1	0
	psaB	3	0	1	2
	psbB	3	0	1	2
	psbD	1	0	1	0
Photosynthetic metabolism	*atpA*	1	0	0	1
	atpB	3	0	1	2
	atpE	1	0	1	0
	atpH	2	0	2	0
	atpI	2	0	0	2
	ndhA	0	1	0	1
	ndhB	1	0	0	1
	ndhC	1	0	1	0
	ndhF	2	2	0	4
	ndhG	1	0	0	1
	ndhH	1	0	1	0
	ndhK	1	0	1	0
	rbcL	2	0	2	0
Gene expression	*rpl21*	1	0	1	0
	rpoB	5	0	2	3
	rpoC1	2	0	2	0
	rpoC2	6	4	4	6
	rps11	1	0	1	0
	rps12	1	0	1	0
	rps7	2	0	0	2
	rps8	1	0	1	0
	accD	2	0	0	2
	clpP	1	0	0	1
	matK	1	1	0	2
Other genes	*chlB*	2	0	2	0
	chlL	2	0	0	2
	chlN	2	0	0	2
	ycf1	13	3	6	10
	ycf10	1	0	0	1
	ycf2	4	5	2	7
	Total	75	17	36	56