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Article

Comparitive Chloroplast Genomics of Nine Endangered Habenaria Species and Phylogenetic Relationship of Orchidaceae

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Abstract: *Habenaria*, a member of Orchidaceae family is the cosmopolitan distributions, which has significant medicinal and ornamental values. Regardless of morphology and molecular data that have been studied in recent times, the phylogenetic relationship is still under debate. Here, we sequenced, assembled, and annotated the whole chloroplast (cp) genome of two species (*Habenaria aitchisonii* Rchb.f. and *Habenaria tibetica* Schltr.ex Limpricht) of *Habenaria* grown on the Qinghai-Tibetan Plateau (QTP), and combined with seven already published cp genomes which may assist to uncover their genomic profiling. The two genomes ranged from 155259-155269 bp in length and both encoded 132 genes, including 86 protein, 38 tRNA and 8 rRNA. In the cp genomes, the tandem repeats (797), SSRs (2195) and diverse loci (3214) were identified. Comparative analyses of codon usage, amino frequency, microsatellite, oligo repeats and transition and transversion substitutions showed similarities among the species. Moreover, we identified 16 highly polymorphic regions with nucleotide diversity above 0.02, which may be suitable for robust authentic barcoding and inferring in the phylogeny of *Habenaria* species. Among the polymorphic regions, positive selection was significantly exerted on the several genes such as *cemA*, *petA*, and *ycf1*. This may suggest that the important adaptation stratagem for two *Habenaria* species on the QTP. The phylogenetic relationship displayed that *H.aitchisonii* and *H. tibetica* have closer relationship than others and the rest seven species clustered in the other three groups. Our findings also supported the idea that *Habenaria* could be divided into different sections. This study enriched the genomics resources of *Habenaria*, which may be helpful for the conservation efforts of these endangered species.

Keywords: *Habenaria*; chloroplast genome; molecular marker; positive selection; Orchidaceae; phylogenomic

1. Background

Habenarai (Orchidinae, Orchidoidea, Orchidaceae) is a large genus and has more than 891 species [1], which dispersed widely across the tropical, subtropical, temperate, and alpine regions in the world [2,3]. The genus had great ornamental value, but its tubers also had significant therapeutic properties that could be used to cure diuretics, swelling, waist strength and kidney, treating lumbago, and hernia [4–6]. Additionally, people frequently use it to cook meals to cure HIV/AIDs in several regions of Africa [7]. However, as the variation in the morphology, such as tuber, spurred lip, long column, U-shaped wide anther, long caudide, naked viscidium and free stigma [2,8], the new

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descriptions of *Habenaria* species are still going on [9–12], and the phylogenetics relationship in the genus is still on controversy [13–15].

Plastome occupied the quadripartite structure, which consisted of two inverted repeats (IRa and IRb), a large single-copy (LSC) and small single-copy (SSC) regions [16,17]. The size of their genomes ranged from 107kb to 218kb [17]. Like the nuclear genome, there also existed variations such as deletion, insertion, loss, and single nucleotide mutation in certain regions [18,19]. The polymorphism of the chloroplast genome displayed the evolution profiling of the paternal inheritance of the species and could be applied in population genetics, phylogenetics and barcoding for plants [20]. Due to the polymorphism of plastome, several studies have been performed to resolve taxonomic problems and their phylogenetic relationships with high resolution in Orchidaceae [19,21,22]. These studies could not only provide the information to resolve the taxonomic discrepancies of plant lineages, but also supply in-depth insight into the evolution of the plastomes [18,19,23].

Adaptive evolution reflects the adaptability of the species during the evolution process in which natural selection always acts on genetic variation, genetic recombination, and gene flow [24,25]. Therefore, exploring the selection character that species suffer in their evolutionary process is another hot spot in the chloroplast genome analyses. Yang et al (2002) showed that heterogeneity for the plastid *matK* and *rbcL* genes in different species of the family of mangrove genus [26]. In recent years, many studies detected positively selected chloroplast genes through checking Ka (non-synonymous substitution) and Ks (synonymous substitutions) [23,27,28].

Habenaria aitchisonii Rchb.f. and *Habenaria tibetica* Schltr.ex Limpricht belongs to *Habenaria* genus and grew on the QTP or adjacent region [3,11], especially the latter, which was the endemic endangered species only distributed in shrub meadow or alpine meadow. Ecological and biogeographical forces have a great impact on the chloroplast genome rate heterogeneity [26]. In general, substitution rates of cp genomes displayed minimal evolution [28]. Previous studies discovered that the positive selection could accelerate the Ka value, yet it does not affect the Ks value [29]. However, there is little known about the evolution and adaptation profiling of *Habenaria* species.

In this study, the two *Habenaria* species (*H. aitchisonii* and *H. tibetica*) grown on the QTP were sequenced through the next-generation Illumina platform. Combined with the 40 species of cp genomes of Orchidaceae, we aimed to: (1) compare the cp genome structure of species within the *Habenaria* genus; (2) construct the phylogeny of *Habenaria* species in Orchidaceae; (3) investigate selective or adaptive evolution in the cp genomes of *Habenaria* species.

2. Results

2.1. Chloroplast Genome of Habenaria aitchisonii and Habenaria tibetica

Habenaria aitchisonii and *Habenaria tibetica* chloroplast genomes were sequenced with the Illumina sequencing platform and the two genomes were obtained 155, 259 bp (GenBank accession number: OQ701055) and 155, 269 bp (GenBank accession number: OQ701056) in length, respectively. Both species occupied the typical quadripartite structures (Figure 1a and 1b), in which the IRs were separated by the SSC and LSC regions. The LSC regions had lengths of 84,234bp and 84,143bp in *H.aitchisonii* and *H.tibetica* respectively and displayed 91bp constriction in the *H.tibetica*, furthermore the SSC regions were 17,643bp and 17,646bp in *H.aitchisonii* and *H.tibetica* respectively and expended 3bp in the later. Similarly, the IR regions appeared 49bp expansion in the *H.tibetica* as well. The cp genomes of both species encoded 132 genes, including 86 proteins, 38 tRNA and 8 rRNA. Among them, we found 20 genes duplicated in IR regions, including 4RNA gene (*rrn16*, *rrn23*, *rrn4.5* and *rrn5*), 7 tRNA gene (*trnA-UGC*, *trnH-GUG*, *trnI-CAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG* and *trnV-GAC*) and 8 protein-coding genes (*ndhB*, *rpl2*, *rpl23*, *rps12*, *rps19*, *rps7*, *ycf1* and *ycf2*). In addition, and 17 genes had one intron and 1 had two introns (*Clp P*) (Table S1 and Table 1). The ycf1 gene was also observed truncated at the junction of IR/SSC with a function copy (Figure 1). The GC content was quite similar between the two species (36.83 % and 36.84%). However, the IR region had the highest GC content (42.91% to 42.86%), the SSC regions had the least GC content (29.38% to 29.40%).

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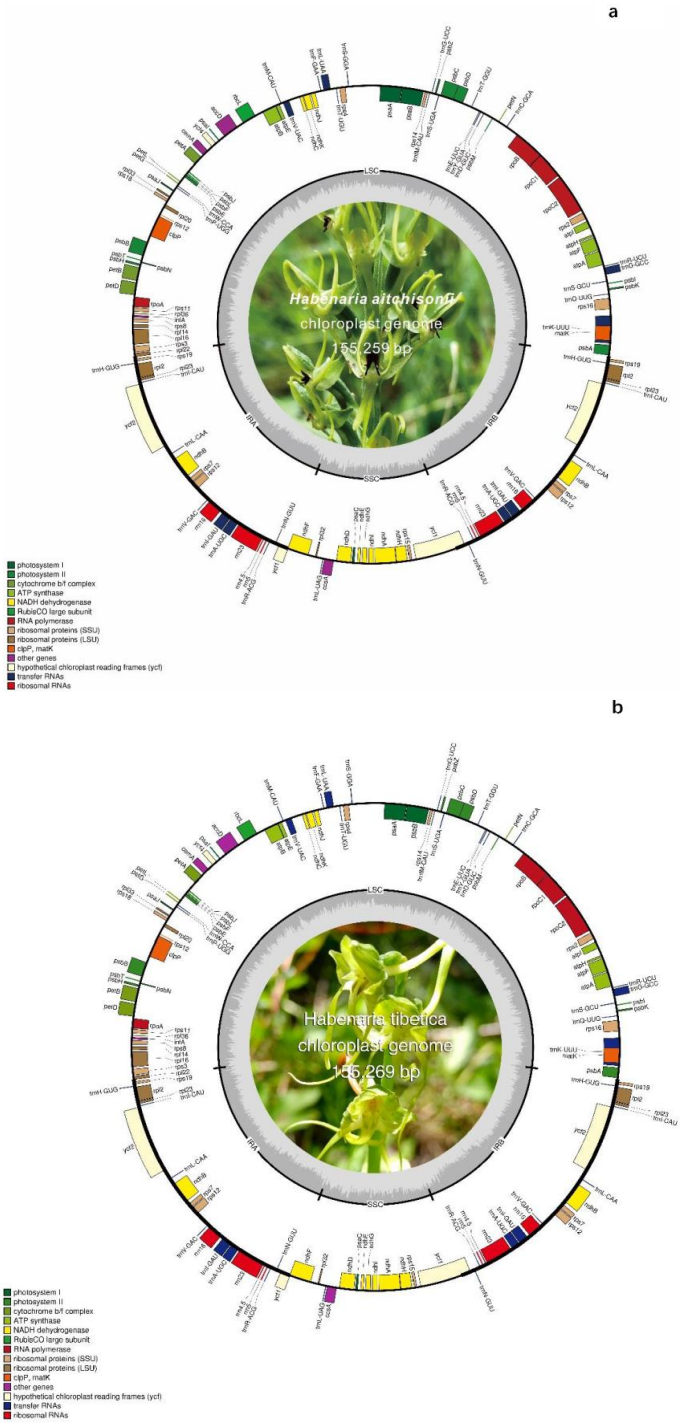

Figure 1. Chloroplast genome maps of *Habenaria aitchisonii* Rchb.f.(a) and *Habenaria tibetica* Schltr.ex Limpricht H. (b). Genes of different functional groups are displayed in color-coded. The darker gray color in the inner correspond to the GC content, regions of the large single-copy (LSC), small single copy (SSC) and inverted repeat (IRA and IRB) are indicated.

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Table 1. General characteristics of the chloroplast genomes of the nine *Habenaria* species. Comparison analyses of *H.aitchisonii* and *H. tibetica* with other close species.

Genome feature	*H. aitchisonii*	*H.tibetica*	*H.dentata*	*H.cruciformis*	*H.ciliolaris*	*H.radiata*	*H.flagellifera*	*H.checiformis*	*H.pantlingiana*
Genome Size(bp)	155,259	155,269	153,682	155,708	154,544	155, 353	155,298	153,896	153,951
LSC (bp)	84,234	84,143	83,963	85,131	84,032	84, 833	85,749	83,732	83,641
SSC (bp)	17,643	17,646	17,041	17,659	19,602	17, 718	18,373	17,026	17,370
IR (bp)	26,691	26,740	26,339	26,459	25,455	26,401	25,595	26,569	26,470
GC content (%)	36.83	36.84	36.62	36.60	37.90	36.60	37.90	36.70	36.60
Total number of genes	132	132	133	131	132	113	130	131	133
Protein-coding gene	86	86	87	79	86	79	81	85	87
tRNA	38	38	37	30	38	30	37	38	38
rRNA	8	8	8	4	8	4	8	8	8

2.2. Comparation of Plastome Features of the Habenaria Genus

To know about the cp genome feature of the *Habenaria* genus, we compared the *H. aitchisonii* and *H. tibetica* with the other seven species. It appeared that the plastome structure of all the species was very conserved (Figure 2; Table S1). Complete plastome sizes ranged from 151,210bp (*H. flagellifera*) to 155,708bp (*H. cruciformis*), the LSC length from 81,072bp (*H. flagellifera*) to 85,131bp (*H. cruciformis*), IR region length from 26,399bp (*H. dentata*) to 26,856bp (*H. ciliolaris*); SSC length from 17,026bp (*H. chejuensis*) to 17,718bp (*H. radiata*) (Figure 2, Table 1). Moreover, the GC content of all plastome ranged from 36.60% to 37.90%. ‘

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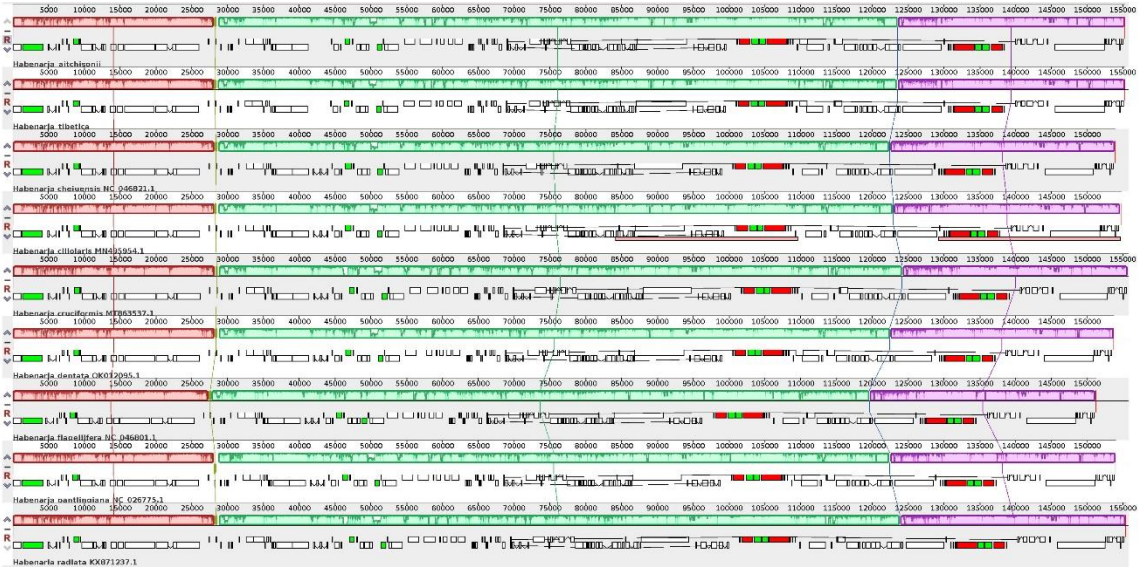

Figure 2. Mauve alignment of organization of the plastomes of nine *Habenaria* species based on collinear blocks. The green part is inversion of single copy and the small blocks of various color represents genes (the black is tRNA, the red is rRNA, the white is protein-coding and the green is intron-containing tRNA).

Mauve-based analysis showed similarity in gene arrangement and gene content in the *Habenaria* genus (Figure 2). The rearrangement of genes did not happen in the nine plastomes and the differences appeared in the intron-contained RNA regions. All plastomes displayed similarity among the LSC/IRb and SSC/IRa junctions (Figure 3). *rpl22* and *ycf1* existed in the junction region, respectively. However, at the IRb and SSC junctions exhibited a bit of difference, In *H. pantling* plastome *ycf1* clearly covered the junction's region and others close to the junction regions. Interestingly, the *ycf1* gene displayed inverted in the plastome in *H. chejuensis* (Figure 3).

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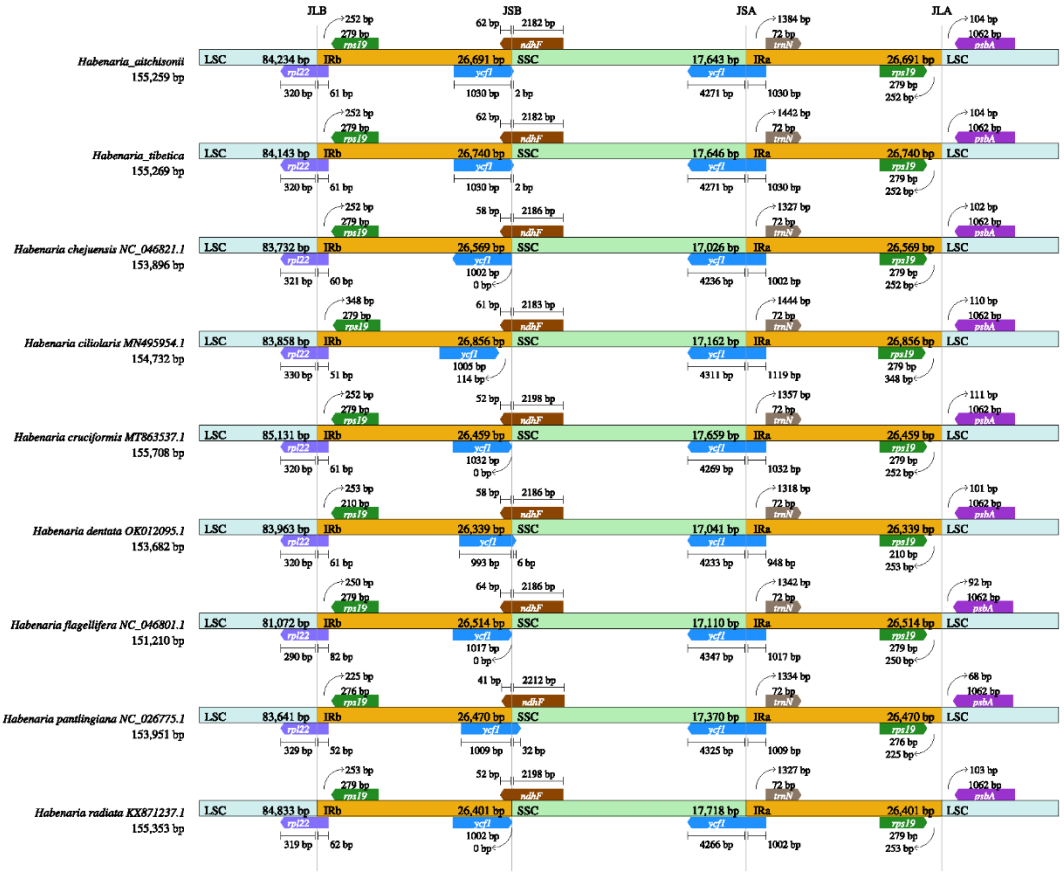

Figure 3. Analyses of expansion and contraction of inverted repeats in the nine *Habenaria* chloroplast genomes.

2.3. Relative Synonymous Codon usage and Amino Acid Frequency

RSCU frequency plays an important role in reflecting mutation bias during evolution. To know the codon usage and amino acid frequency, RSCU and amino acid frequency were analyzed in the plastomes. *H. aitchisonii* and *H. tibetica* had 79,767bp and 79,749bp protein-coding genes, respectively. The two plastomes had similar RSCU frequencies (Figure S1 and Table S2). Among the protein-coding genes, Leucine was the most abundant amino acid (10.35% and 10.34%). The Serine (7.91% and 7.09%) and Arginine (5.96% and 5.95%) were the second and third places. Whereas only 461 (1.73%) encoded tryptophan, which is the least frequent amino acid (Figure S1 and Table S2). There were 29 and 28 codons displayed clearly biased usage (RSCU>1) in *H. aitchisonii* and *H. tibetica*, respectively. However, the tryptophan seems no biased usage in both species (RSCU=1) (Table S2). Further, codons usage appeared bias in *H. aitchisonii* and *H. tibetica* compared with other species (Figure 4; Table S3). The results displayed that the four parameters involved in codon usage bias were a bit higher than other seven *Habenaria* species, *Goodyera*, *Anoetochilus* and *Vanilla*, while the CAI and CBI appeared the lower (Figure 4).

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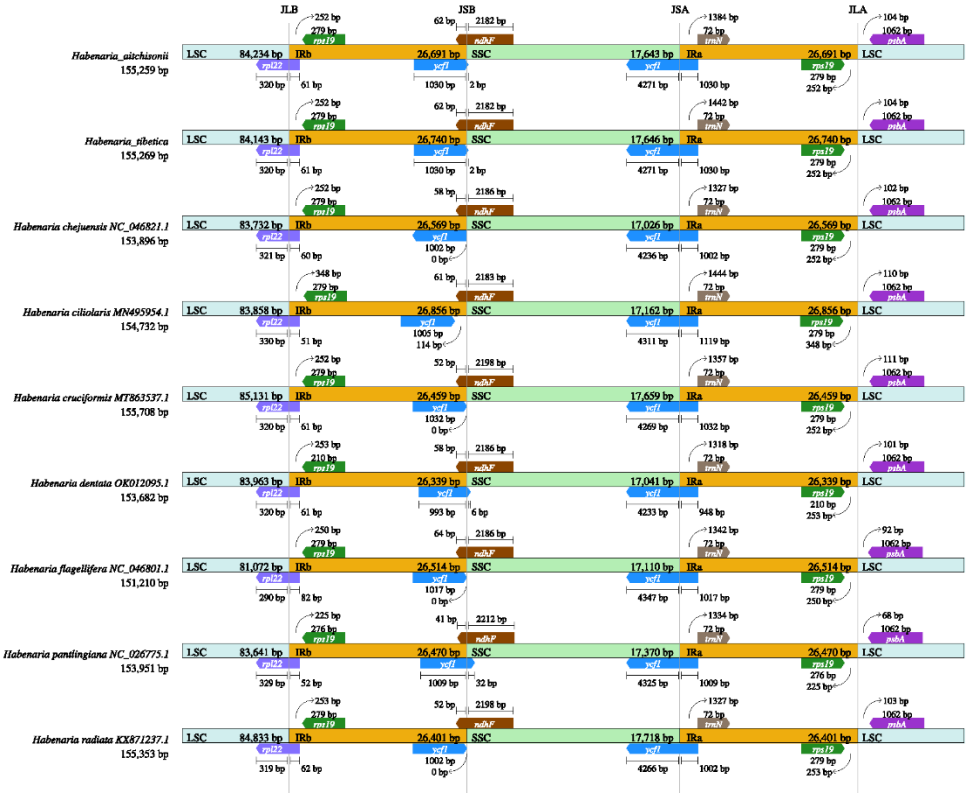

Figure 4. The comparative analysis of codon usage bias in *Habenaria* and other species. (*Habenaria* 1: seven other *Habenaria* species; *Habenaria* 2: *H.tibetica* and *H.aitchisonii*).(A) ENC effective number of codons;(B) GC3s GC of synonymous codons in 3rd position;(C) G3s GC of synonymous codons in 3rd position;(D) CBI codon bias index;(E) CAI codon adaptation index;(F) Fop Frequency of optimal codons index.

2.4. Analysis of Microsatellites and Oligonucleotide Repeats

A total of 233 SSRs and 232 SSRs were identified in the plastome of *H. aitchisonii* and *H. tibetica*, respectively. Among them, 136 SSRs (58.40%) existed in the LSC region, 52 SSRs (22.30%) in the IR region and 45 SSRs (19.30%) in the SSC region in the former. Similarly, there were 134 SSRs (57.80%), 46 SSRs (19.80%) and 52 SSRs (22.40%) in LSC, IR and SSC regions in the latter, respectively (Table S4a). In both species, 99 SSRs appeared in the intergenic region.

To know about the SSRs in the *Habenaria* genus, we compared all 2195 SSRs in the nine species.The mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide of these SSRs accounted for 67.26, 5.65, 27.43, 1.82, 0.68 and 0.23%, respectively. In all 27 types of SSRs, the A/T was the largest group, AAG/CTT types and AAT/ATT were the second and third group (Figure 5a). In the nine species, *H. pantlingiana* had more unique types (4), *H. flagellifera* had 3, *H. chejenensis* and *H. radiata* had one. The rest of the species appeared no unique SSR type.

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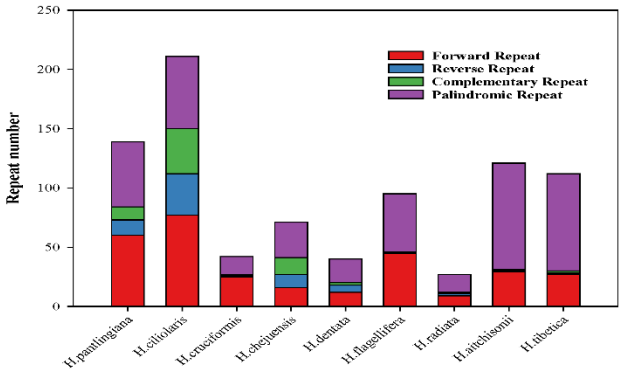

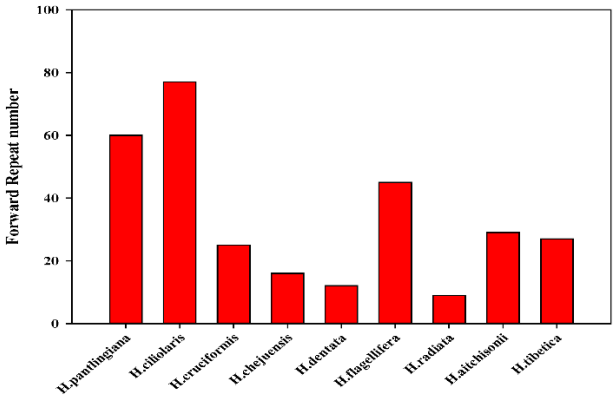

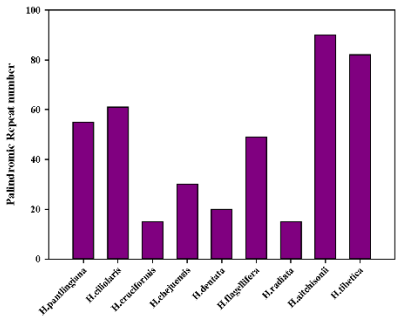

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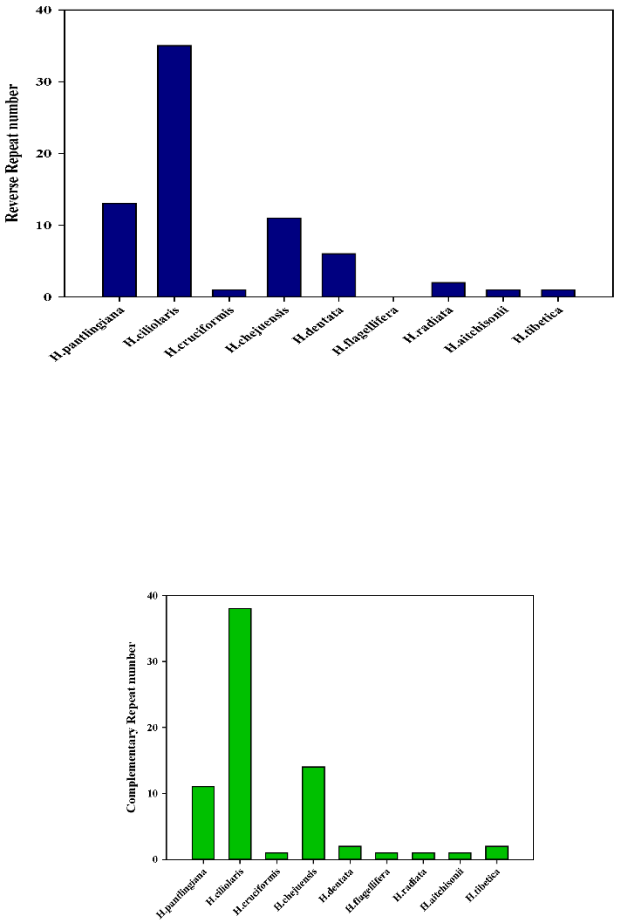

Figure 5. Comparison of SSR profiling and the oligonucleotide repeats in the nine *Habenaria* species. (a. SSR profiling; b. oligonucleotide repeats).

To compare the oligonucleotide repeats in all nine species, we applied the REPuter software. As Figure 5b shows, the total number showed clearly different among the nine plastomes (Table S4b). *H.ciliolaris* had the most repeats(211) than others while the *H. radiata* had the least (26) (Figure 5b-a). At the same time, *H. ciliolaris* had the most forward repeats (77) while the *H. radiata* had the least (9) (Figure 5b-b). In the reverse repeat(5b-c), *H. ciliolaris* had the largest number (35) while *H. flagellifera* had none. In the palindromic repeat, *H. aitchisonii* and *H. tibetica* are the first and second largest, respectively. While the *H. cruciformis* and *H. radiata* had the least (Figure 5b-d). In the complementary repeat, four species (*H. cruciformis*, *H. flagellifera*, *H. radiata* and *H. aitchisonii*) had one and *H. ciliolaris* had the most (38) (Figure 5b-e).

2.5. Identification of Polymorphic Loci

To understand the nucleotide polymorphic profiling, the cp genomes of nine *Habenaria* species were analyzed with DnaSP software. As Figure 6 showed, LSC, SSC and IR regions exhibited clearly differences in polymorphic loci. In the IR region, the Pi value changed a bit and the value ranged from 0-0.0074, with an average 0.0001431. The highest polymorphism appeared in SSC regions with an average value 0.021857. The highest Pi value was 0.04527(*ycf1*). The LSC region had the highest Pi value larger than 0.0001431, despite the average value being 0.010343(Figure 6; Table S5).

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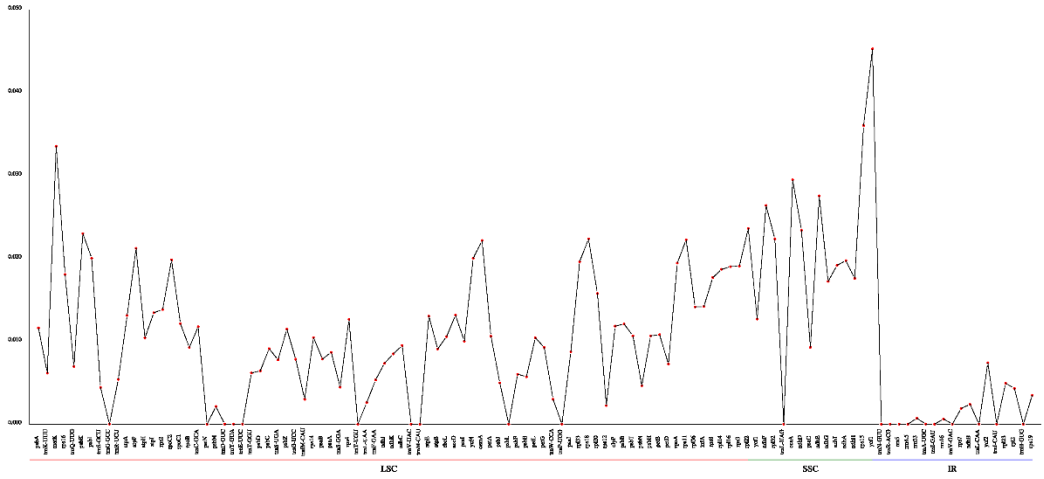

Figure 6. Nucleotide variability values compared between the nine chloroplast genomes of *Habenaria* species using the window sliding analysis.

2.6. Molecular Evolution Analysis

The pairwise Ka/Ks were analyzed in nine species of *Habenaria* (Figure 7), which reflected the selected pressure on the sequences. *H.aitchisonii* and *H.tibetica* species displayed no positive sites (Ka/Ks>1) or neutral sites (Ka/Ks=1). Only six genes exhibited purified pressure and most genes had no changes in Ka or Ks (Table S6). However, either *H. aitchisonii* and *H. tibetica* and other species in *Habenaria* could calculate 3, 4 or 5 genes suffering the positive selection. These genes included *cemA*, *petA*, *rps11*, *rpl14*, *ycf1*, *psbK*, *rpl22*, *ycf2*, *ycf2-2*, *psbH*, and *ndh Ⅰ* (Table S6). To check the positive selection genes of *Habenaria*, the *cemA*, *petA*, *rps11*, *rpl14*, *ycf1*, *psbK*, *rpl22*, *ycf2*, *ycf2-2*, *psbH*, and *ndh Ⅰ* were analyzed in the *H. aitchisonii* and *H.tibetica* branch and other close related species(Table S7), all the genes had no significant posterior probabilities on the *H. aitchisonii* and *H. tibetica* branch(Table S4).However, six genes *petA*, *rps11*, *ndh Ⅰ*, *rpl22*, *ycf1* and *ycf2* displayed the sites with positive selection in the BEB test. Among them *petA*, *ndh Ⅰ*, *rpl22*, *ycf1* and *ycf2* had more than one positive selective site. Moreover, two sites appeared ndh Ⅰ, rpl22, ycf1 and ycf2 genes on the *H. aitchisonii* and *H. tibetica* branch (Table 2; Table S7). These results suggested that the two species had suffering the adaptation evolution on the QTP.

Table 2. The potential positive selection test based on the branch-site model.

Gene	Null hypothesis			Alternative hypothesis			Significant test	
name	InL	df	ω	InL	df	ω	BEB	*p*-value
cemA	-1967.69	1	1	-1971.76	3	0.547	104K	$p<0.05$
petA	-2147.03	1	1	-2147.03	3	1.000	none	$p>0.05$
rps11	-1126.26	1	1	-1124.08	3	999.0	38V,82A,88T	$p<0.05$
ndhi	-1371.40	1	1	-1369.59	3	147.6	38I,95F	$p<0.05$
psbH	-493.70	1	1	-492.37	3	999.0	10S	$p<0.05$
psbK	-462.73	1	1	-462.73	3	2.140	none	$p>0.05$
rpl14	-851.21	1	1	-850.77	3	52.192	17Q,119P	$p>0.05$
rpl22	-1130.19	1	1	-1130.11	3	1.643	120V	$p>0.05$
ycf1	-23972.75	1	1	-23972.75	3	1.000	none	$p<0.05$
ycf2	-16608.99	1	1	-16608.99	3	1.000	None	$p<0.05$

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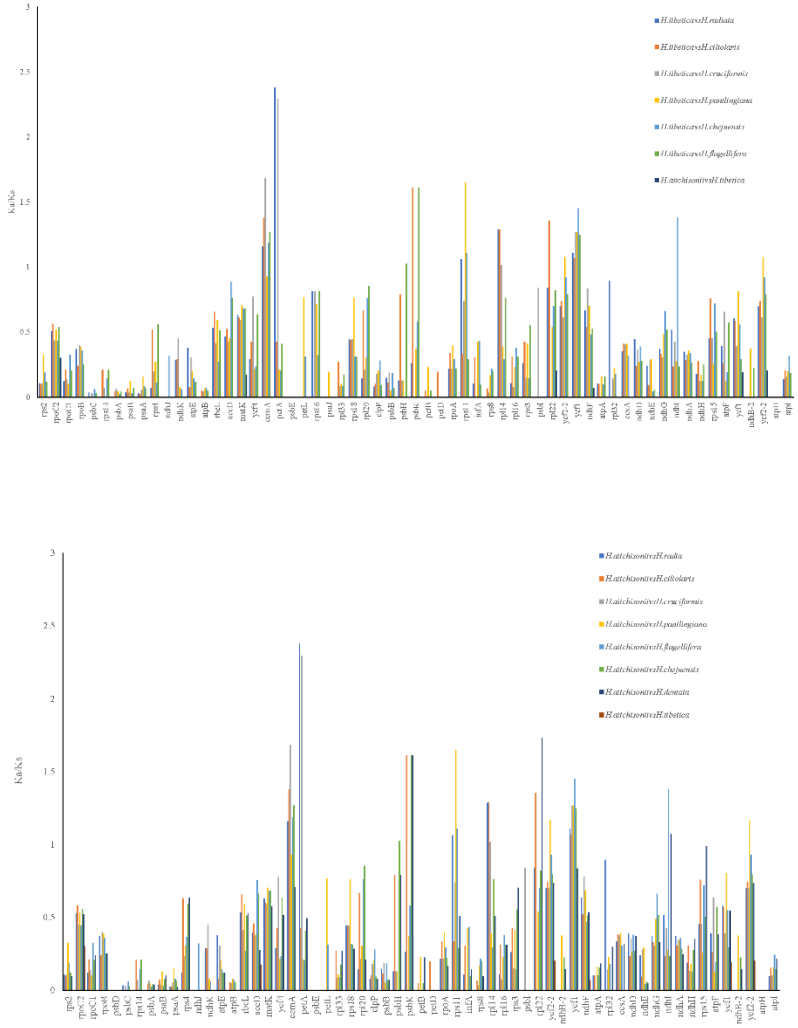

Figure 7. The pairwise Ka/Ks were analyzed in nine species of *Habenaria*.

2.7. Phylogenetic Relationship Analysis

To understand the phylogenetic relationship between *H. aitchisonii* and *H. tibetica* and other species of Orchid, we constructed the phylogenetic trees with 51 cp genomes (Table S8). The phylogenetic trees showed that the *H. aitchisonii* and *H. tibetica* were clustered together with 100% bootstrap support (Figure 8). The nine species of *Habenaria* were clustered in one clade and had closely relationship with Goodyeras. In the *Habenaria* clades the *H. pantingiana* and *H. flagellifera* were grouped together, *H. dentata*, *H. ciliolaris*, and *H. chejuensis* were clustered in other branches. While the *H. crucicifomis* and *H. radiata* appeared as two parallel branches.

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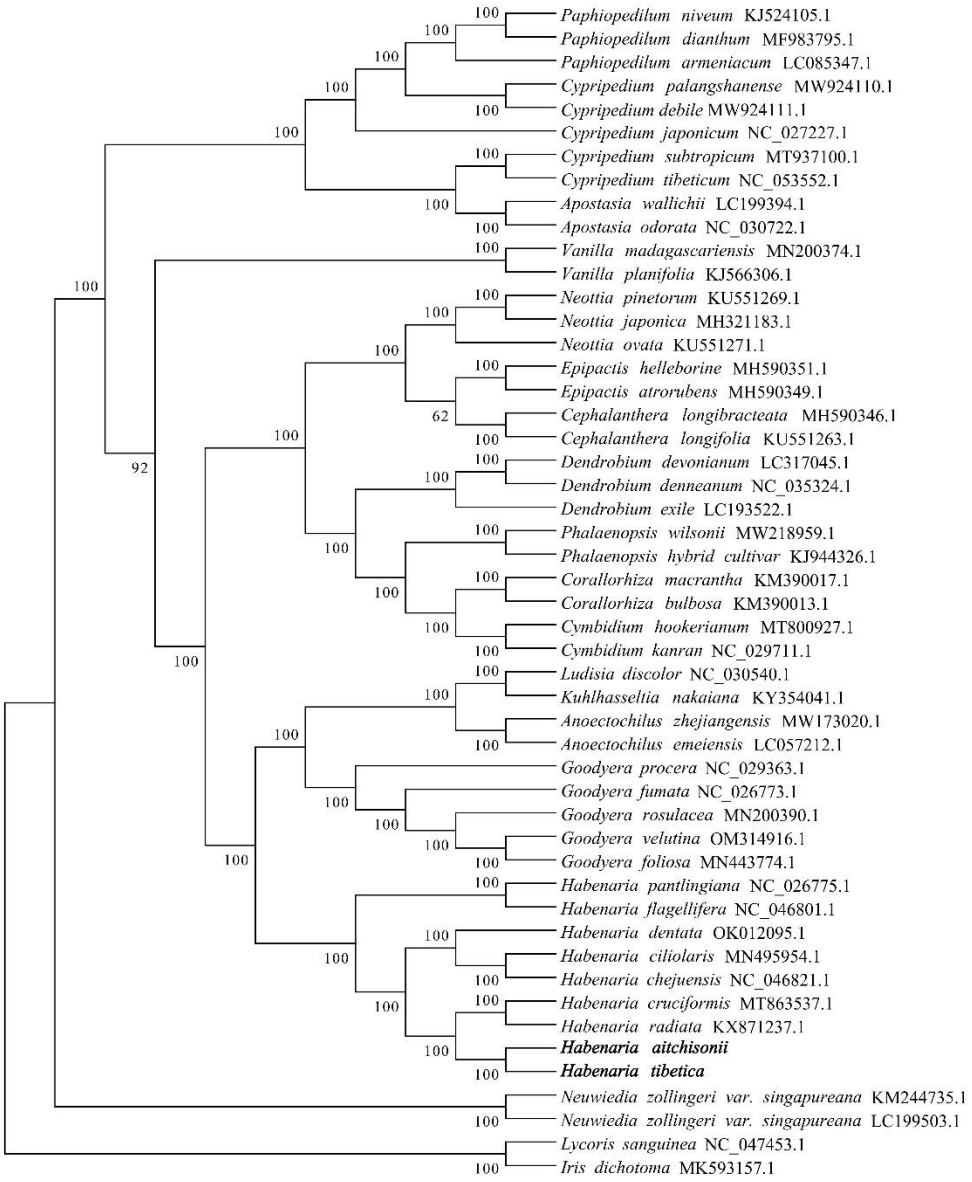

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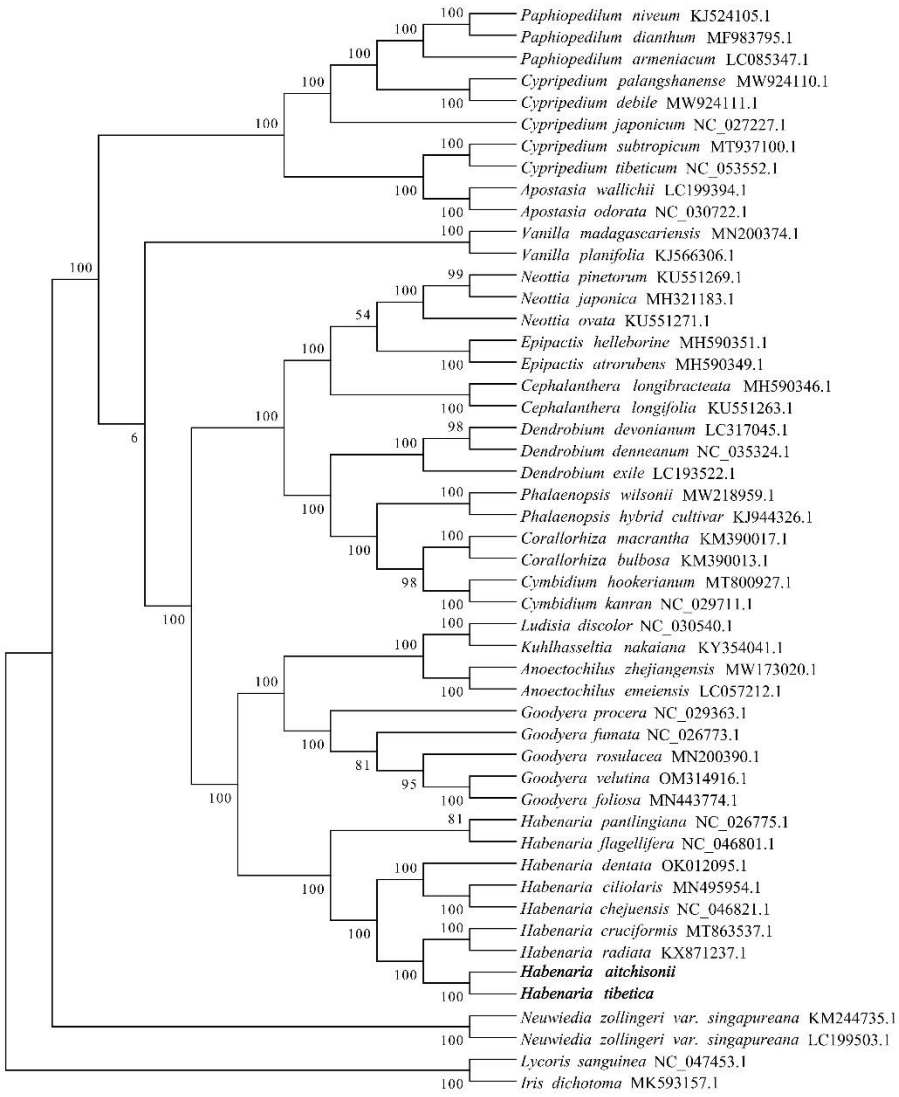

Figure 8. The Phylogenetic relationships of two new *Habenaria* species and related species of Orchidaceae using ML method. Bootstrap values were shown at the nodes.

3. Discussion

Here, we sequenced and assembled *H.aitchisonii* and *H.tibetica* grown on the QTP (Figure 1), our result displayed that the chloroplast genomes of two species were very similar in structure and cp genome size. Combined with the published cp genomes [22,30], our result also displayed that the species of *Habenaria* was 153,682-155,708bp, which is smaller than that of Cypripedium [30] and larger than that of Vanilla, Cyrtosia, Gastrodia and Epipogium species [22]. Moreover, the GC content of the nine *Habenaria* species ranged from 36.60% to 37.90% (Table 1), and the average GC content was 36.95%, which was a bit larger than the average GC content of orchid plastomes (36.40%)[30] and Cypripedium species (31.8%)[21].GC content might be related to the ancestral feature of monocot genomes[31], secondly, selection and mating system also could drive GC content and GC3 usage[32].We suggest that high GC content of *Habenaria* species may be related to the mutation from these process.

Gene number and gene order are another evolution characterization of the cp genomes. A previous study reported that the average number of 124 orchid species was 113 genes [21]. However, our results displayed that most of the species had more than 130 coding-genes except *H. radiata* (113 genes) (Table 1), which also a bit more than Cypripedium (128-130 genes). That may be caused by the more duplication genes. In the two new species, more than 20 genes were duplicated. *Ndh* genes were

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lost or deleted in Cypripedium and other orchid species [22,30]. Interestingly, our results displayed that the species of *Habenaria* contained all 11 *ndh* genes (Table S6). *Ndh* genes involved in photosynthesis or plastome stability [21]. Therefore, the *ndh* genes would play important roles in *Habenaria* adaptations.

Highly variable regions could be used to design the markers in phylogenetic and biogeographic analysis [33]. In the study, the highest polymorphism appeared in SSC regions had the highest average Pi value of 0.021857 and *ycf1* displayed the high Pi value (Figure 6). This result was consistent with the Blumea species [18]. Except for the conserved IR regions, the LSC regions also displayed high variations (*matK-rps16*, *psbK-psbI*, *atpA-atpF*, *rps2-rpoC2*, *ycf4-cemA*, *rpl33-rps18*, and *rps11-rpl36*) (Figure 6; Table S3). Which may suggest that this region has the potential for markers. Moreover, 16 genes (*atpF*, *matK*, *ycf4*, *cemA*, *psbK*, *rps18*, *psbI*, *rps11*, *rpl22*, *rps15*, *ycf1*, *ndhF*, *rpl32*, *ccsA*, *ndhD* and *ndhE*) were detected with nucleotide diversity more than 0.02 (Figure 7; Table S4). Among these loci, *ndhF*, *rps15*, *ccsA*, and *rpl32* have been detected as highly variable regions in different species [18,21]. Therefore, the high variation information identified in this study has the potential to be exploited as candidate barcode sequences in the phylogenetic analysis of *Habenaria*. Moreover,

233 SSRs and 232 SSRs were identified in the plastome of two new species and more SSRs exited in IGS regions (Figure 5a; Table S4a). Among the six types, more than sixty are mononucleotides, the results were in accord with other species [18,21]. Moreover, our results identified 27 types of SSR in the nine *Habenaria* that is less than that of the Cypripedium [21]. This may be due to the Cyripedium having larger chloroplast genomes and enlarged IR regions. In Cypripedium, the cp genome expansion is associated with the proliferation of IGS regions [21]. Therefore, repeat regions in the study may help for population genetics studies of *Habenaria*.

Habenaria is a large genus and most species of *Habenaria* are terrestrial orchids and nearly cosmopolitan, occurring in the tropical, subtropical, temperate, and alpine regions [2,3]. In the ML tree, our results displayed that the *Habenaria* is not the monophyletic and the nine species could be divided into five different groups (Figure 8). Among them, the two new species (*H. aitchisonii* and *H. tibetica*) clustered together with 100% bootstrap support. Interestingly, the two species were grown on the QTP and showed similar morphological characteristics [3]. The two species both had two basal subopposite leaves, petals slightly 2-lobed, raceme with flower, lip deeply 3-lobed and spur slightly clavate [3]. Our results with whole cp genomes also displayed that the two species had close relationship. The results are also supported by the previous studies [9,10]. Using the *rbcL+matK+ITS*, the *Habeniara* could be divided into eleven clades and the species from tropical and alpine regions could group into different subclades [10]. Subclade I most from the tropical region and Subclade II was the alpine species. Our results showed that *H. chejuensis*, *H. ciliolaris* and *H. dentata* were group into another branches (Figure 8), which may originate in tropical regions [10]. Although the cp genomes data here still quite limited and couldn't clarify the phylogenetic relationship in the *Habenaria* genus, our results still provide the cue that the cp genomes could solve the phylogenetic inference when more cp genome information obtained in the future.

In the previous study, some chloroplast genes were proved to be the target of natural selections [23,26–28,34]. Our results showed that there were no genes subjected to natural selection between two alpine species (Ka/Ks<1). However, Both the new species had more than 3 genes had Ka/Ks>1 compared with others. These genes included cemA, petA, rps11, *rpl14*, *ycf1*, *psbK*, *rpl22*, *ycf2*, *ycf2-2*, *psbH*, and *ndh Ⅰ* (Figure 7). To further understand the positive genes in *Habenaria* genus, codon model was used in ten genes [24,25]. Codon sites with higher posterior were another aspect sign of divergent selective pressure [24,25]. The results also displayed that the six genes may be under positive selection significantly (Table 2; Table S7). These results suggested that the positive selections had been happened in the species of two alpine species. Besides the genes associated with photosynthesis (petA, psbH), NADH-dehyrogenase subunits(ndh Ⅰ), self-replication process gene (rps11, rpl22) and ycf1, ycf2 also displayed positive selection. Photosynthesis system and NADH-dehyrogenase contributed light harvest and electron transport to produce ATP, were suffering to positive selection in *Allium* [34]. Here, our results were consistent with their conclusion. Moreover, our results also discovered that genes related to cp ribosome (rpl22 and rpl11) had significant positive

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selection. This may suggest that protein synthesis play the important roles in two alpine species stress adaptation.

The QTP is the largest and highest plateau in the world and one of the important hotspots of biodiversity [35]. Orchid species are extremely sensitive to environmental change [36]. Acharya et al (2011) thought that the precipitation and temperature could affect the abundance and distribution of the orchid species [37]. Similarly, Hu et al (2022) study displayed that annual precipitation, elevation, and top-soil pH(H_2O) had a large important influence on the distribution of the orchid species in the QTP[38]. Ka/Ks rations have been widely used to infer evolutionary dynamics and identify adaptive signatures among species. Ka/Ks ratios suggested that positive selection existed in Allioideae species [32] and Solanaceae species [19,23]. Here, our results also showed that positive selection have existed in two alpine *Habenaria* species. The precipitation, elevation, and top-soil pH (H_2O) might be the potential environmental factors in the QTP.

4. Conclusions

In this study, the two new chloroplast genomes of *Habenaria* in the QTP were sequenced and compared genomic profiling with seven other published species. We revealed similarities in gene arrangement and gene content in the *Habenaria* genus. The rearrangement of genes did not happen in the nine plastomes. Comparative of tandem of codon usage, amino frequency, microsatellite, oligo repeats and transition and transversion substitutions showed similarity in the two new species. Moreover, we identified 16 highly polymorphic regions with nucleotide diversity above 0.02, which may be suitable for robust authentic barcoding and inferring in the phylogeny of *Habenaria* species. Among the polymorphic regions, positive selection was significantly exerted on *cemA*, *petA*, *ndh I*, *rpl22*, *rps11and psbH*. The phylogenetic relationship displayed that *H.aitchisonii* and *H. tibetica* have more close relationship than others and the rest seven species clustered in other three groups. The data-sets of the study also enriched the genomics resources of *Habenaria* in Orchidaceae, which may be helpful for the conservation efforts of these endangered species.

5. Materials and Methods

5.1. Sample Collection and DNA Extraction

In the flowering season in 2021, the fresh leave of *H. aitchisonii and H. tibetica* were collected in Maixiu National Forestry Park (35.2619° N,101° 8861° E, Alt.3200m) and Sanjiang Source National Park (32.9385° N, 100.7436° E, Alt. 3300m), and the vouch specimen(ZDW-2021-024 and ZDW-2021-030) was deposited in the Herbarium of Northwest Institute of Plateau Biology, Chinese Academy of Sciences and voucher specimen was identified by Prof. Pengcheng Lin. The total DNA was extracted using the CTAB method [39].

5.2. Plastome Genome Sequencing, Assembling and Annotation

The high-quality DNA was sequenced with the Illumina NovaSeq 6000 sequencing Platform (Nanjing Genepioneer Biotechnologies Inc.). fastp (version 0.20.0 , https://github.com/OpenGene/fastp) was used to filtrate the raw reads, and the clean reads were mapped to the chloroplast genomes in the GenBank. The contigs were obtained with SOAPdenovo2 v3.10.1（http://cab.spbu.ru/software/spades/） under kmer=55, 87 and 121[40].The scaffold was constructed by SSPACE v2.0 [41], and GapCloser was used to fill the gaps [40]. PCR amplification and Sanger sequencing were also used to confirm the assembly boundaries. The annotation of the complete chloroplast genomes was executed using DOGMA (http://dogma.ccbb.utexas.edu/) [42] and the circular chloroplast genome map was generated by OGDRAW[43].

5.3. SSRs, Codon Usages and Nucleotide Diversity Analysis

Web-based REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer/) was used to detect repeats including forward, palindrome, reverse and complement repeats. The minimal repeat size

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was set to 30 bp, and the sequence identity was>90%. Simple sequence repeats (SSRs) were identified by MISA (Micro Satellite identification tool) [44] with the minimum repeats of mono-, di-, tri-, tetra-, penta- and hexanucleotides set to 8, 5, 4, 3, 3 and 3, respectively.

Codon usage analysis only 53 protein-coding genes with lengths >300bp were chosen for synonymous codon using the tool CodonW1.4.2 to avoid sampling errors (http://codonw.sourceforge.net). The overall codon usage and the relative synonymous codon usage (RSCU) were analyzed. The number of polymorphic sites and nucleotide variability (Pi) were evaluated using a sliding window with a 200bp step size and a 600bp window length implemented in DnaSP v.5.10.1[45].

5.4. Comparative Analysis of cp Genomes

The plastome of *H.aitchisonii* and *H. tibetica* were compared with the cp genomes of other species in *Habenaria* using mauve software to identify evolutionary events such as gene loss, duplication, rearrangement [46].The junction of the cp genomes was analyzed in IRscope [47].

5.5. Molecular Evolution Analysis

To analyze synonymous (Ks) and non-synonymous (Ka) substitution rates, the protein-coding genes of *H.aitchisonii*, *H. tibetica* and other seven closely related species in the *Habenaria* genus were analyzed. The corresponding functional protein-coding gene between compared species was separately aligned using MAFFT [48], and then the Ka/Ks value was calculated by the KaKs_calculator 2.0 [49] with the settings genetic code table 11 (bacterial and plant plastid code) and the NG method of calculation, developed by Nei and Gojobori as implemented in KaKs_calculator. There were genes with Ka/Ks value of "NA" in the results, indicating that were not applicable. When Ks =0, this happed (in cases with no substitutions in the alignment, or 100 percent match). To identify the positively selected genes, the branch-site model was used in PAML software (http://abacus.gene.ucl.ac.uk/software/PAML.html).

5.6. Phylogenetic Analysis

The whole cp genome and coding-proteins of 48 species of the Orchidaceae were retrieved from the GenBank and used to construct the phylogenetic tree. The cp genomes of *Iris dichotoma* (Iridaceae) and *Lycorris sanguinea* (Amaryllidaceae) were used as the outgroup [21]. The two sets of sequences were aligned by MAFFT [46], and then the alignments were adjusted by the Gblocks program [50]. The maximum likelihood (ML) method was employed to construct phylogenetic trees by RAxMLversion 8.0 software using the GTRGAMMA model [51]. Bootstrap analysis for each branch was calculated by 1000 replications.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1. Codon content of 20 amino acid and the stop codon of 81 coding genes of Habenaria aitchisonii (a)and Habenaria tibetica(b). Table S1. Comparitive of genes among Habenaria aitchisonii and Habenaria tibetica with other Habenaria species. Table S2. The codon usage and codon-anoticodon recognition pattern for Habenaria aitchisonii and Habenaria tibetica cp genome. Table S3. a Comparison of repeat sequences on Habenaria aitchisonii , Habenaria tibetica and seven related species. Table S3. b Distribution of SSRs in the Habenaria aitchisonii , Habenaria tibetica cp genome. Table S4. a The comparative analysis of codon usage bias. Table S4. b The oligonucleotide of Habenaria cp genome analysis. Table S5. Comparison of nucleotide variability (Pi) among Habenaria aitchisonii , Habenaria tibetica and related species. Table S6. The Ka/Ks ratios of protein-coding genes of Habenaria aitchisonii , Habenaria tibetica and related species. Table S7. Codon model analysis of ten cp genome genes. Table S8. List of chloroplast genomes belonging to Orchidaceae used for the phylogenetic analysis.

Author Contributions: Z-DW, L-PC, and C-WD conceived and designed the experiments. Z-JK, Z-SQ, W-M, W-H and S-SB analyzed the data. Y-X, M-J, and Z-YW participated in the material collected. Z-DW, F-M, Z-JK, S-SB and W-H prepared the manuscript and revised the manuscript. All authors read and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials: The chloroplast genome sequences of *H.aitchisonii* and *H. tibetica* were submitted to the GenBank and the accession numbers were: OQ701055 and OQ701056, respectively.

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