BMC Genomics

RESEARCH Open Access

Complete chloroplast genome of two *Brachycorythis* (Orchidaceae) species from China: comparative analysis and phylogenetic implications

Dong Ma[1,2†], Kaifeng Tao[1,2†], Yanqiong Xia[1,2], Yan Luo[3*] and Lu Li[1,2*]

Abstract

Background *Brachycorythis* Lindl. (Orchidinae, Orchidaceae) was characterized by a large and densely leafy stem throughout its length, comprising about 35 species with African-Asian disjunct distribution. However, the intergeneric and infrageneric phylogeny of *Brachycorythis* has been debated based on morphological and molecular data. The chloroplast (cp.) genomes of *B. henryi* and *B. menglianensis* were investigated in detail and compared with those of related taxa for providing new evidence for the phylogenetics of *Brachycorythis*.

Results The cp. genomes of *B. henryi* and *B. menglianensis* exhibited conserved quadripartite structures, measured 153,006 bp and 152,932 bp in length, with an identical GC content of 37.2%. Gene annotations identified 133 genes, 20 duplicated in the inverted repeat regions. Comparative analysis across their related species confirmed stable sequence identity and higher variation in single-copy regions. Phylogenetic analysis based on complete cp. genomic data revealed that these two *Brachycorythis* species formed an independent clade exhibiting distinct branch lengths, representing a sister lineage to their related taxa in Orchidinae *s. s.* It was also supported that six species of *Brachycorythis* formed a monophyletic clade based on *matK*+*rbcL* sequences. Among them, three species from Asia and other three species from Africa, respectively, formed two independent clades. Notably, it was also showed that *B. macowaniana* was not clustered with other six *Brachycorythis* species.

Conclusions This is the first report on complete cp. genomic data of *Brachycorythis*, providing some phylogenetic implications. Some varied cp. genomic features for phylogenetic implications were discussed, including overall genome structure, codon usage, repeat sequences, IR boundaries, DNA polymorphisms, and phylogenetic reconstruction. Based on chloroplast genomic data and *matK*+*rbcL* sequence analysis, phylogenetic results revealed that *Brachycorythis* was not closely related to any of the five genera assumed to be related. Three species from Asia

†Dong Ma and Kaifeng Tao contributed equally to this work and should be considered as co-first authors.

*Correspondence:
Yan Luo
luoyan@xtbg.org.cn
Lu Li
lilu@swfu.edu.cn

Full list of author information is available at the end of the article

and other three species from Africa, respectively, formed two independent clades, which was consistent well with the African-Asian disjunct distribution of *Brachycorythis*. And, it was supported that *Gyaladenia* should be re-established, considering the uncertain species of *B. macowaniana*. In addition, it was revealed that *B. menglianensis* was not a synonym of *B. henryi*, and each of them was a distinct species based on cp. genomic and morphological data. Therefore, the intergeneric and infrageneric relationship of *Brachycorythis* should be understood better by more cp. genomic data.

Keywords *Brachycorythis*, African-Asian disjunct distribution, Orchidinae, The intergeneric and infrageneric relationship, Phylogenetics

Background

Brachycorythis Lindl. (Orchidinae, Orchidaceae) consisted of approximately 35 species, distributed across tropical Africa and Madagascar, with some species extending into tropical and subtropical Asia [1–3]. Three species were recorded in China, including one endemic species (*B. menglianensis* Qian) occurred in Southwestern Yunnan [4]. Their plants are herbaceous, with a large, densely leafy stem along its entire length. The lip is differentiated into a boat or spur-shaped hypochile and a flattened, forward-projecting epichile [4]. Some *Brachycorythis* members possess certain medicinal and edible values. For example, plants of *B. ovata* Lindl. have been used as a charm in Southern Africa [5]. Wild populations of *Brachycorythis* have been declining due to habitat fragmentation and overharvesting, which needed further research for conservation [6, 7].

The intergeneric relationship and species delimitation of *Brachycorythis* have been debated greatly and needed more morphological and molecular evidence [1, 3, 6, 8–14]. This genus, originally placed in the tribe Ophrydeae by Lindley [9], but was moved into currently classified within the subtribe Orchidinae [1, 3, 6]. It was suggested that two species of *Schizochilus* Sond were merged into *Brachycorythis* [8]. The concept was not widely accepted, because of the different floral morphology and habitat requirements between these two genera. It was shown that *Brachycorythis* should be closely related to *Platanthera* Rich., *Gymnadenia* R. Br. and *Neobolusia* Schltr. based on morphological similarities [3, 10]. It was indicated that *Brachycorythis* (only represented by *B. henryi*, *B. macrantha*, and *B. neglecta*) and *Hemipilia s. l.*., were sister taxa based on six DNA markers [11]. It was also proposed that *Brachycorythis* (represented by four Asian species) should be monophyletic and a sister to some Orchidinae taxa (superclades I + II) based on seven DNA markers [12]. It was supported that *Brachycorythis* (only represented by five African species) as a sister lineage to the superclades I + II as above, using *matK*, *rbc*L, and nrITS markers [12, 14]. However, *Brachycorythis* rendered to be paraphyletic since *B. macowaniana* from Africa did not group with the other African members of the genus [14]. It was surprisingly that the systematic position of *B. macowaniana* has been so debated that it changed several times. This species was previously removed from *Brachycorythis* into *Habenaria*, and then back, and into *Gymnadenia*, or *Platanthera* [15–19]. And later, it was treated as one species of a new genus of *Gyaladenia* Schltr [19].

It was noteworthy that *Brachycorythis* is one of the few genera in Orchidinae exhibiting a transoceanic disjunction between Africa (including Madagascar) and subtropical Asia [1, 2, 4]. However, in the molecular phylogenetics of Orchidinae, sampling limitations precluded a detailed exploration of the relationships between *Brachycorythis* species from the two major centers of diversity, namely Africa and Asia [11, 12, 14]. In addition, six species from Asia formed a well-defined group referred to as the '*B. helferi* complex', including *B. helferi* (Rchb.f.) Summerh., *B. henryi* (Schltr.) Summerh., *B. laotica* (Gagnep.) Summerh., *B. obovalis* Summerh., and *B. menglianensis* [20]. Species identification in this complex seemed to be difficult because of their overlapping morphological features and data limited [20]. It was ever proposed that *B. menglianensis* might be synonymous with *B. henryi* [20, 21].

The chloroplast (cp.) genome has been increasingly employed in taxonomy and phylogeny of Orchidaceae [22–26]. To date, the complete cp. genomes of taxa related to *Brachycorythis* were reported, such as *Vietorchis* Aver. & Averyanova, *Platanthera*, and *Dactylorhiza* [13, 24, 27], but there were few genomic data on this genus. Here, the structure of cp. genomes of *B. henryi* and *B. menglianensis* were firstly characterized in detail and compared with those of eight related taxa from Orchidinae. It was aimed to: (1) characterize structures of the complete cp. genome of *B. henryi* and *B. menglianensis* in detail, (2) reconstruct the phylogenetic trees to provide some new evidence for the intergeneric and infrageneric phylogeny of this genus. It was the first report on the complete cp. genome of *Brachycorythis*, which would provide some valuable cp. genomic information for the species identification and phylogenetics of this genus.

Results

Structural features of chloroplast genome in *Brachycorythis henryi* and *B. menglianensis*

The structures of cp. genome of these two species were highly similar. The total size of two cp. genomes was 153,006 bp (*B. henryi*) and 152,932 bp (*B. menglianensis*) in length (Fig. 1). Same as most angiosperms, their cp. genome structure displayed a typical quadripartite structure with a large single-copy (LSC) region (82,818 bp, 82,681 bp), a small single-copy (SSC) region (17,652 bp, 17827 bp), and two inverted repeats (IR) regions (26,268 bp, 26,212 bp) (Table S1).

The GC content of the overall and three positions of the two cp. genomes from two species were consistent (Table S1). The two cp. genomes were all AT-rich, and overall GC content was 37.2%. The GC content in IR regions (43.3%) was higher than in LSC (34.9%) and SSC regions (30%). The GC content of the three codon positions of the two cp. genomes was very similar. Furthermore, the third codon position was related to codon bias and mRNA stability. However, there was some difference on GC content between the two species (Table S1). In *B. henryi*, the third codon position GC content (37.03%) was lower than the second codon position GC content (37.63%), but higher than the first codon position GC content (36.96%). In contrast, in *B. menglianensis*, the third codon position GC content (36.92%) was lower than the first codon position GC content (38.02%), but higher than the second codon position GC content (36.72%).

Both cp. genomes contained 133 genes, including 87 CDS genes, eight rRNAs genes, and 38 tRNAs genes (Table S2). Among them, 93 genes were unique, and 20 genes were duplicated in the IR regions (4 rRNA genes, 8 tRNA genes and 8 CDS genes). The 11 *ndh* (NADH dehydrogenase) genes (*ndh A*/*B*/*C*/*D*/*E*/*F*/*G*/*H*/*I*/*J*/*K*) were identified in the cp. genomes of *B. henryi* and *B. menglianensis* (Table S2).

There were 18 intron-containing genes including 12 protein-coding genes, 6 tRNA genes, and 3 genes (*rps12*, *ycf3* and *clpP*) possessed two introns (Table S3). The length variations among the 12 intron-containing genes were primarily attributed to differences in intron lengths. In particular, 5′-end and 3′-end exons of *rps12* gene were, respectively, located in the LSC and IR regions. Only four of the 12 intron-containing genes were in the IR regions, while the other genes spread across the LSC region (Table S3).

Repetitive sequence variations

Comparative analysis of the cp. genomes between two *Brachycorythis* species and eight closely related species from Orchidinae was conducted in order to elucidate their intergeneric and infrageneric relationship, namely, *Dactylorhiza viridis* (L.) R. M. Bateman, Pridgeon & M.

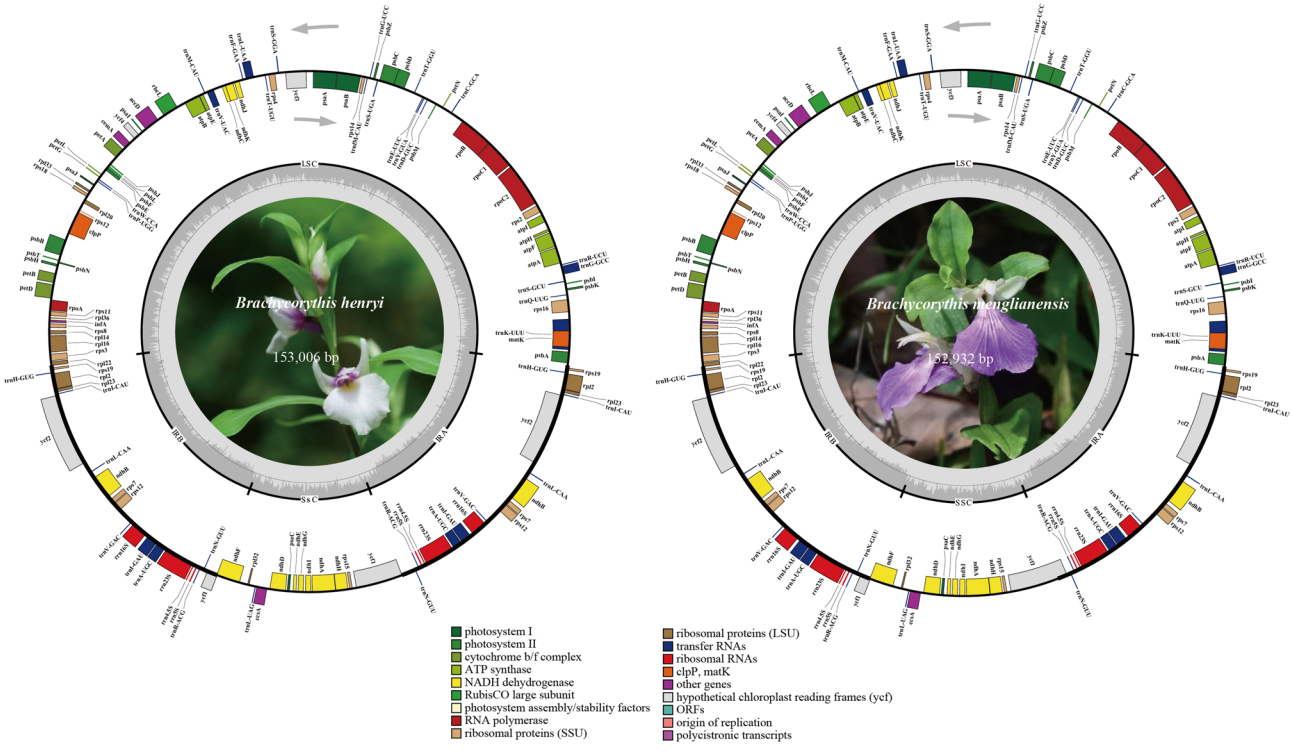

Fig. 1 The cp. genome map of Brachycorythis henryi (left) and B. menglianensis (right). Internal genes were clockwise transcribed, while external genes were counterclockwise transcribed. The inside circle bright and dark gray coloring indicated the genome guanine-cytosine (GC) content

W. Chase, *D. majalis* (Rchb.) P. F. Hunt & Summerh., *Gymnadenia crassinervis* Finet, *G. conopsea* (L.) R. Br., *Hemipilia gracilis* (Blume) Y. Tang, H. Peng & T. Yukawa, *H. yajiangensis* G. W. Hu, Jia X. Yang & Q. F. Wang, *Platanthera chlorantha* Cust. ex Rchb., *P. japonica* (Thunb. ex (A) Murray) Lindl.).

The number of SSRs (simple sequence repeats) identified in the ten cp. genomes from Orchidinae ranged from 68 (*Brachycorythis menglianensis* and *Platanthera chlorantha*) to 92 (*Hemipilia gracilis*) (Table S4, S5). Eight types of SSRs were screened in the ten species (Table S4, S5. Among them, five types of SSRs were consistently identified in all the ten species, including compound (c), mononucleotide (P1), dinucleotide (P2), trinucleotide (P3), and tetranucleotide (P4). *Platanthera chlorantha* exhibited a lower abundance of P1 than the other nine species (Fig. 2A, Table S5). However, the other three types were characterized in different species (Fig. 2A, Table S5). Compound* (c*) was present in the six species, but absent in two *Brachycorythis* species, and *Dactylorhiza viridis* and *Gymnadenia conopsea*. Pentanucleotide (P5) was found in two *Gymnadenia* species, and *D. viridis* and *Hemipilia yajiangensis*, while hexanucleotides (P6) occurred in two *Hemipilia* species and *(B)*

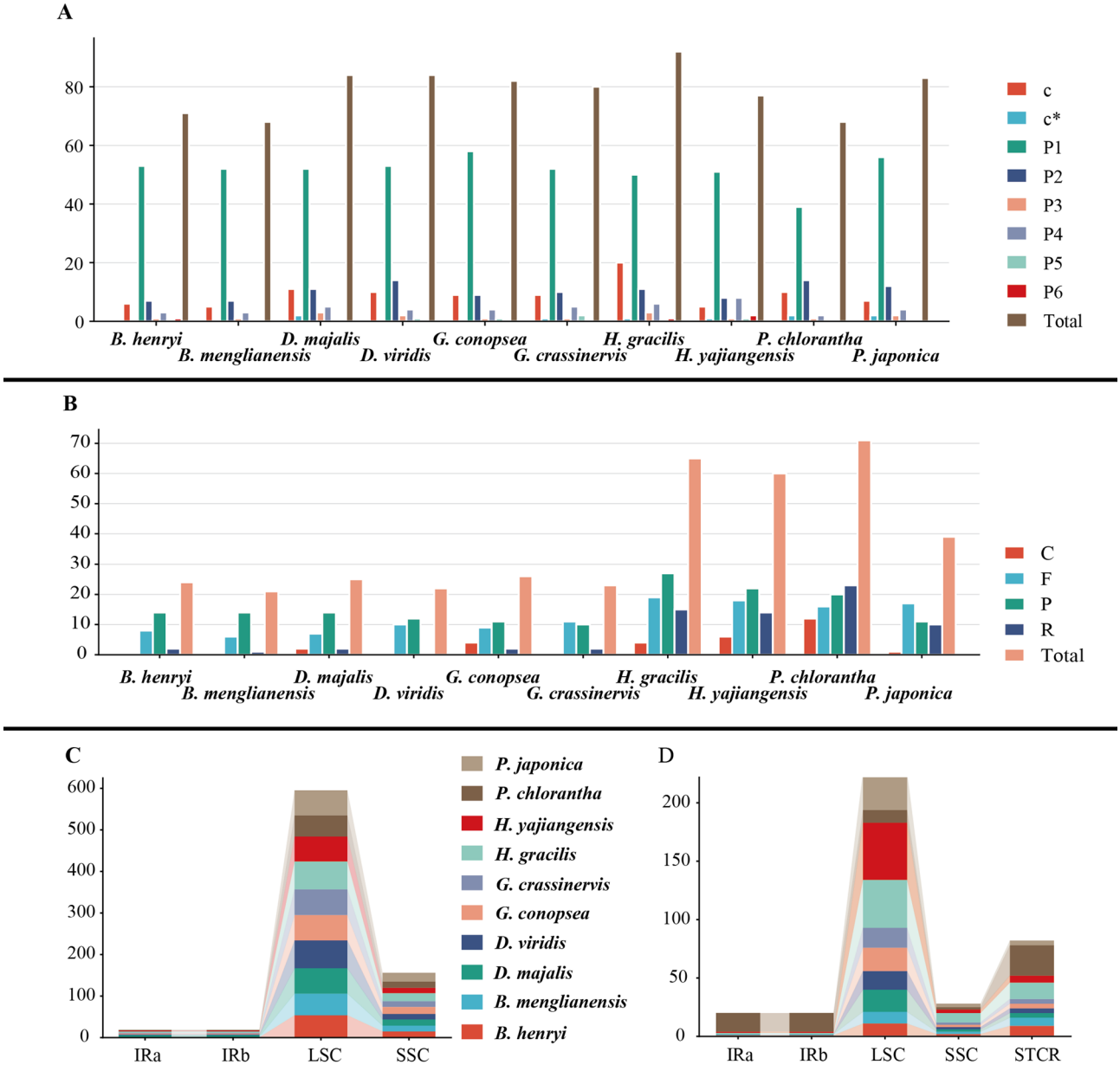

Fig. 2 Repeat sequence analyses of cp. genomes in the ten species from Orchidinae. **A** The number of eight types of SSRs. **B** The number of four types of LSRs. **C** Distribution of SSRs in four regions. **D** Distribution of LSRs in five regions

henryi (Fig. 2A, Table S5). In addition, these ten species had a certain A-T base preference for the base composition of SSRs (Table S4), which were primarily distributed in LSC and SSC region (Fig. 2C, Table S6).

Four types of LSRs (long sequence repeats) were also identified based on the cp. genome sequences, including complement (C), forward (F), palindromic (P), and reverse (R) (Table S7). There were 21 (*Brachycorythis menglianensis*) to 71 (*Platanthera chlorantha*) long repeats detected in the ten cp. genomes (Table S8). F-type and P-type were identified in ten species (Fig. 2B, Table S8). R-type occurred in nine species, except for *Dactylorhiza viridis*. C-type was absent in two *Brachycorythis* species, and *D. viridis* and *Gymnadenia crassinervis*, but present in the other six species (Fig. 2B, Table S8). LSRs were primarily distributed in LSC region, but some LSRs spanned two connected regions (STCR) (Fig. 2D, Table S9).

Codon usage analysis

The relative synonymous codon usage (RSCU) of ten cp. genomes from Orchidinae was calculated based on all protein-coding genes. It was revealed that cp. genomes of the ten species contained 64 types of codons encoding 20 amino acids (Fig. 3, Table S10). Among ten species, leucine (Leu) had the highest number of codons, whereas tryptophan (Trp) had the lowest number of codons (Fig. 3, Table S10). According to the RSCU value, ten cp. genomes could be divided into four groups (Table S10), including 29 codons (RSCU < 1) and 35 codons (RSCU ≥ 1) in *Brachycorythis menglianensis*; 30 codons (RSCU < 1) and 34 codons (RSCU ≥ 1) in *Platanthera chlorantha*; 32 codons (RSCU < 1) and 32 codons (RSCU ≥ 1) in *Gymnadenia conopsea* and *Hemipilia yajiangensis*; 31 codons (RSCU < 1) and 33 codons (RSCU ≥ 1) in the other six species. Almost all CDS had the standard ATG start codon in ten species (Fig. 3, Table S10). Among three

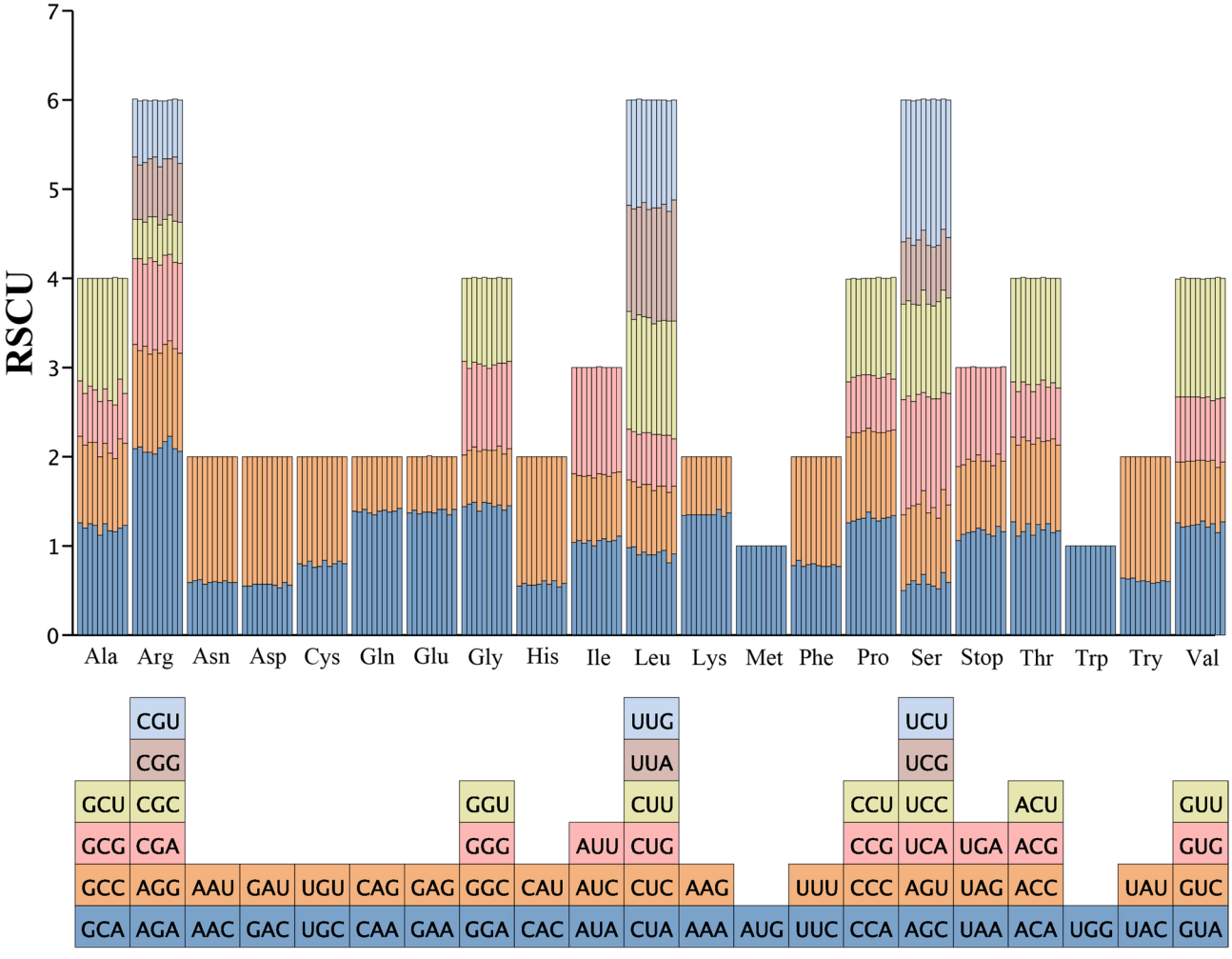

Fig. 3 Codon content of 20 amino acids and stop codons in all protein-coding genes of the cp. genomes of ten species from Orchidinae. The histogram above each amino acid indicating codon usage. Colors in the column graph reflected codons in the same colors showed below the figure. RSCU: relative synonymous codon usage. Ten species were arranged from left to right in the same order as in Fig. 2

stop codons in ten species, TAA was the most common, while TGA and TAG were also found (Fig. 3, Table S10).

IR expansion and contraction

The border regions and adjacent genes of cp. genomes of two *Brachycorythis* species were compared with those of related eight species, in order to analyze the variation in expansion and contraction variation in the junction region. It was observed that the junction positions of IR/SC were conserved in the ten cp. genomes (Fig. 4). For example, these ten cp. genomes exhibited similar SSC/IR boundaries in the coding regions of the *ndhF* (IRb/SSC) and *ycf1* (SSC/IRa) genes, respectively, where *ycf1* gene had a copy in IRb/SSC and overlapped with *ndhF* gene

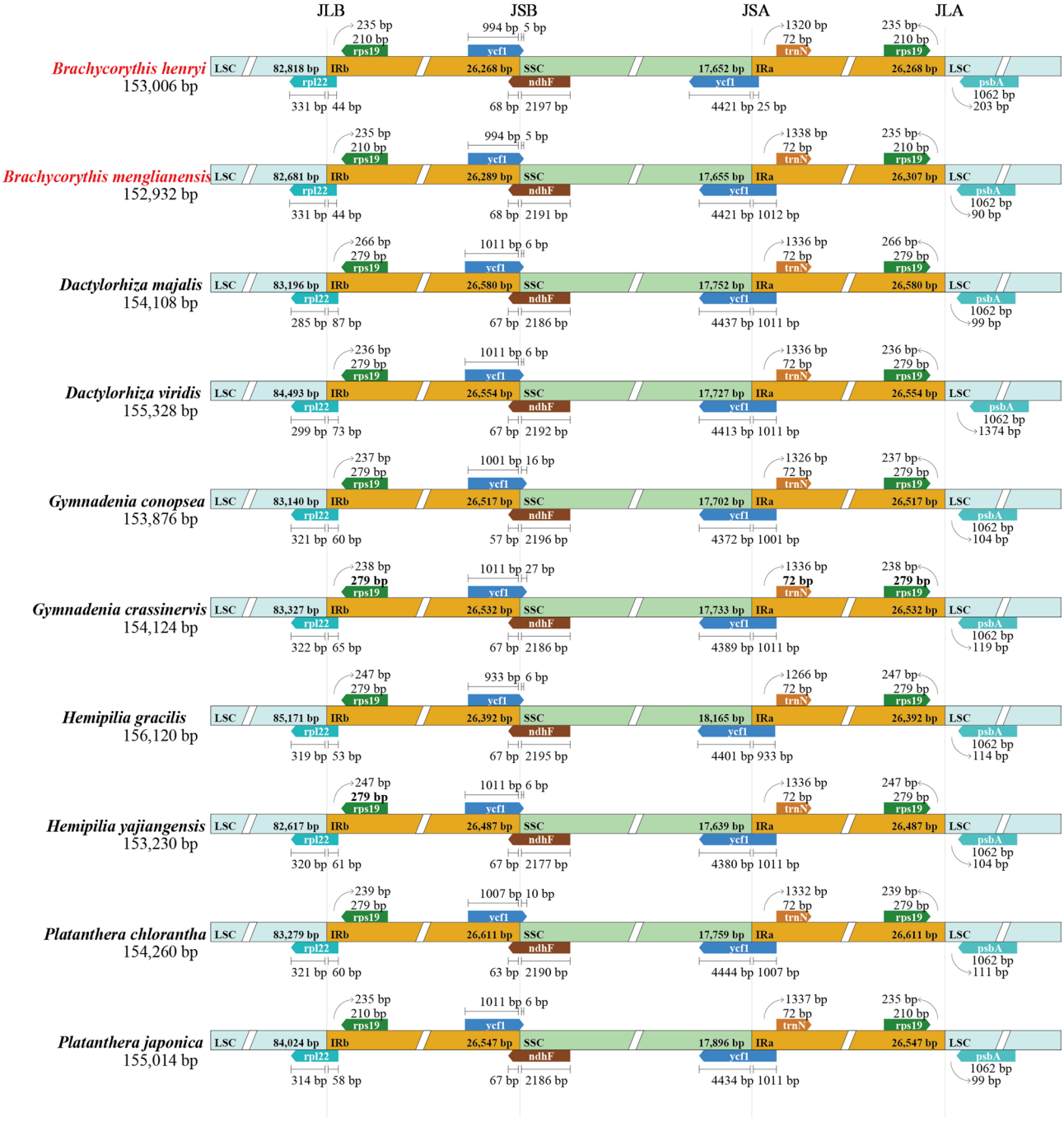

Fig. 4 Comparison of the borders of LSC, SSC and IR regions among ten cp. genomes from Orchidinae. The arrow indicated the number of bp representing genes that were distant from a particular region of the cp. genome. JLB (LSC/IRb), JSB (IRb/SSC), JSA (SSC/IRa), and JLA (IRa/LSC) indicated the junction site between each corresponding two regions on the cp. genome

(Fig. 4). The *IRb* region extended into *rpl22* gene, with the length ranging from 44 to 87 bp (Fig. 4). The *trnN* gene and *rps19* gene were identified in the IR region in ten species (Fig. 4). The *psbA* gene was included in the LSC region in ten species, which was distant from IRa/LSC border with 1374 bp in *Dactylorhiza viridis*, but away from the IRa/LSC border with 97–203 bp in the other nine species (Fig. 4). It is noteworthy that the border region of the ten cp. genomes is relatively stable within the genus (Fig. 4).

Structural comparison and divergence hotspot identification analysis

Based on the annotation of *Brachycorythis henryi* as the reference, the cp. genome sequences of *B. menglianensis* and other eight species from Orchidinae were compared by mVISTA. The IR regions were more stable than the LSC and the SSC regions, and the rRNA genes were highly conserved (Fig. 5). Meanwhile, the non-coding regions (CNS) were more diverse than the coding regions (Fig. 5). Some divergence hotspot regions were identified in the ten cp. genomes (Fig. 6, Table S11, S12). It was demonstrated that Pi value in the coding regions ranged from 0.0017 to 0.0812, with an average of 0.0131. There were 12 coding regions with Pi value exceeding 0.02, including *psbT*, *rpl20*, *atpF*, *accD*, *psbK*, *ndhF*, *rpl32*, *matK*, *ndhE*, *rps15*, *ccsA*, and *ndhD* (Fig. 6A, Table S11). However, Pi value in the non-coding regions ranged from 0.0649 to 0.4128, with an average of 0.2928. And there were 9 intergenic regions, where Pi value exceeded 0.35, namely, *atpH-atpI*, *psaI-ycf4*, *accD-psaI*, *rps2-rpoC2*, *matK-rps16*, *clpP-psbB*, *cemA-petA*, *rbcL-accD*, and *petA-psbJ* (Fig. 6B, Table S12).

Phylogenetic analysis

A maximum likelihood (ML) phylogenetic tree was constructed based on 62 single-copy CDS sequences from 31 species as ingroup, containing two *Brachycorythis* species and 29 species reported from Orchidinae, with four *Goodyera* R. Br. species as outgroup (Fig. 7, Table S13, S14). It was shown that these two *Brachycorythis* species were clustered as an independent clade, but with different branch lengths, and as a sister to their related taxa in Orchidinae *s. s.* with strong support (UFBoot: 100%) (Fig. 7). It was noted that species from the three related genera assumed with *Brachycorythis* were clustered with some members of other genera with strong support. For example, *Ophrys*, *Orchis*, and *Galearis*. In addition, given the taxonomic problems of *Brachycorythis*, a phylogenetic tree was constructed based on *matK*+*rbcL* sequences available of 40 species from Orchidinae as ingroup, including seven *Brachycorythis* species, 14 species from the five genera related, and 19 other Orchidinae species, together with four species from *Goodyera* as

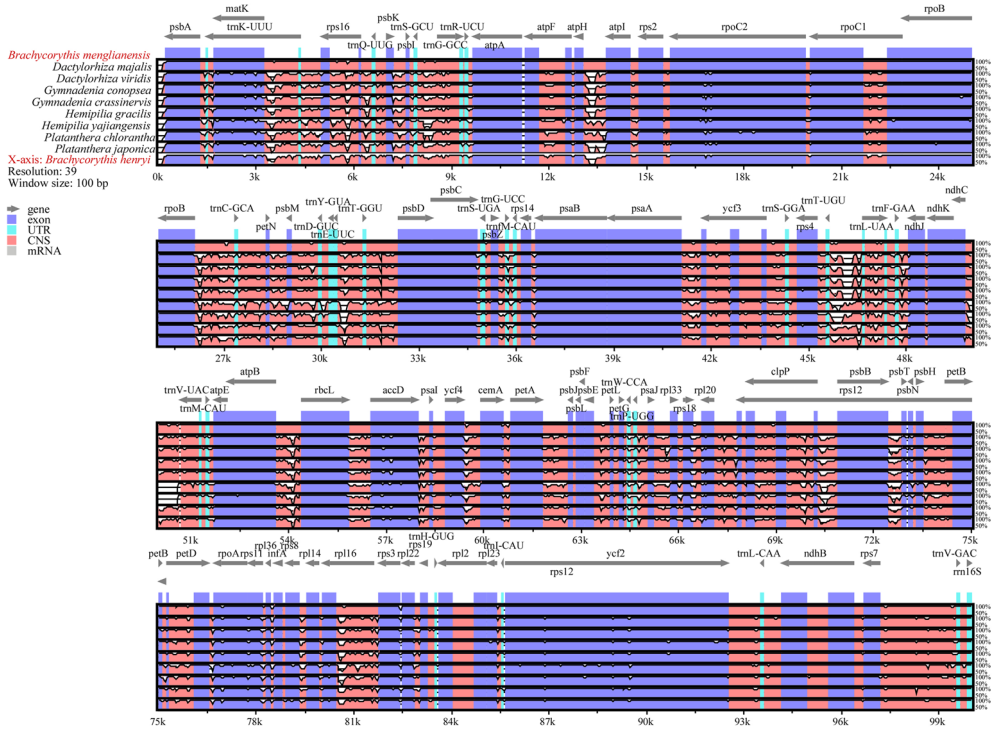

Fig. 5 Sequence alignment of cp. genomes of the ten species from Orchidinae using mVISTA. The vertical scale indicated the percentage of identity, ranging from 50 to 100%. The horizontal axis indicated the coordinates within the cp. genome. Genome regions were color coded as exon, intron, and conserved non-coding sequences (CNS) and mRNA

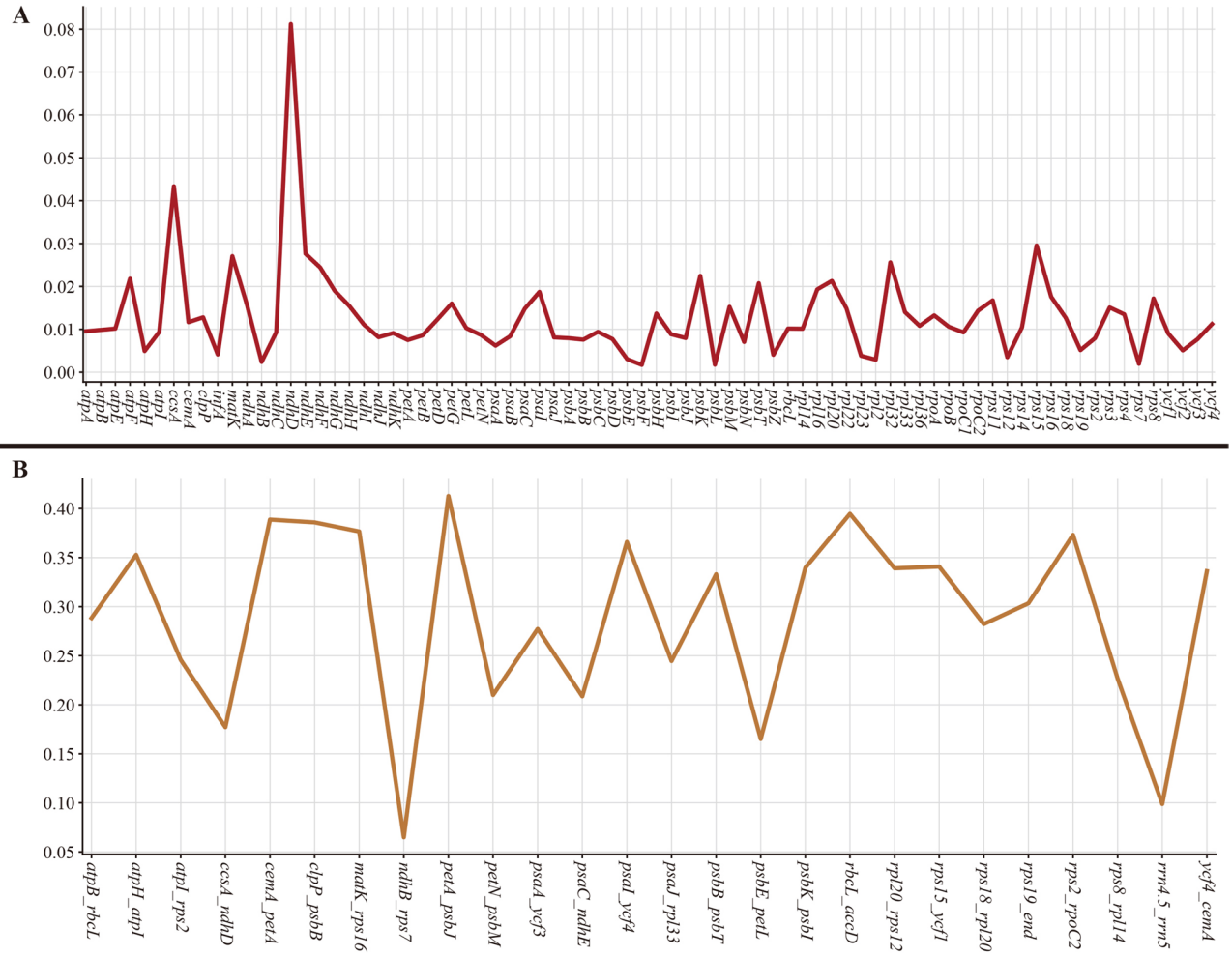

Fig. 6 Pi value in homologous regions in ten cp. genomes from Orchidinae. **A** Coding regions. **B** Non-coding regions

outgroup using ML method (Fig. 8, Table S13). This tree topology was similar to that based on cp. genomic data above (Figs. 7 and 8). It was confirmed that six species of *Brachycorythis* formed a monophyletic clade (UFBoot: 99%), which was not only clustered with the five genera related, but also close to other members from Orchidinae, including *Ophrys*, *Orchis*, and *Galearis* (Fig. 8). Among them, three species from Asia (*B. henryi*, *B. menglianensis*, and *B. obcordata* (Lindl. ex Wall.) Summerh.) and other three species from Africa (*B. buchananii* (Schltr.) Rolfe, *B. pubescens* Harv. and *B. congoensis* Kraenzl.), respectively, formed two independent clades with strong supports (UFBoot: 99%) which were sister (Fig. 8). It was observed that *B. macowaniana* from Africa seemed to be a sister to some species from Orchidinae *s. s.*, which was not clustered with other six *Brachycorythis* species (UFBoot: 96%) (Fig. 8).

Discussion

Comparative analysis of cp. genome between *Brachycorythis* and its alliance in Orchidinae

Brachycorythis henryi and *B. menglianensis* exhibited some conserved structure of cp. genomes, including a typical quadripartite circular structure with the LSC and SSC regions partitioned by the IR regions, which was also common in most angiosperms [28–30]. And the GC contents in IR region were remarkably higher than that in other two regions, which was consistent with other orchids [22–24, 27]. The reason for this phenomenon was generally believed to be the existence of rRNA gene and tRNA gene within the region [22–24]. In addition, it was shown that presence of a complete functioning set of *ndh* genes (*ndh A*/*B*/*C*/*D*/*E*/*F*/*G*/*H*/*I*/*J*/*K*) in the two cp. genomes, which was characterized as *ndh*-complete [31, 32]. The *ndh* complex was considered unnecessary in certain photoautotrophic plants [31, 32], which was varied at subfamily levels of Orchidaceae, such as *ndh*-complete in Apostasioideae, *ndh*-deleted in Vanilloideae, both *ndh*-complete and *ndh*-deleted in Cypripedioideae,

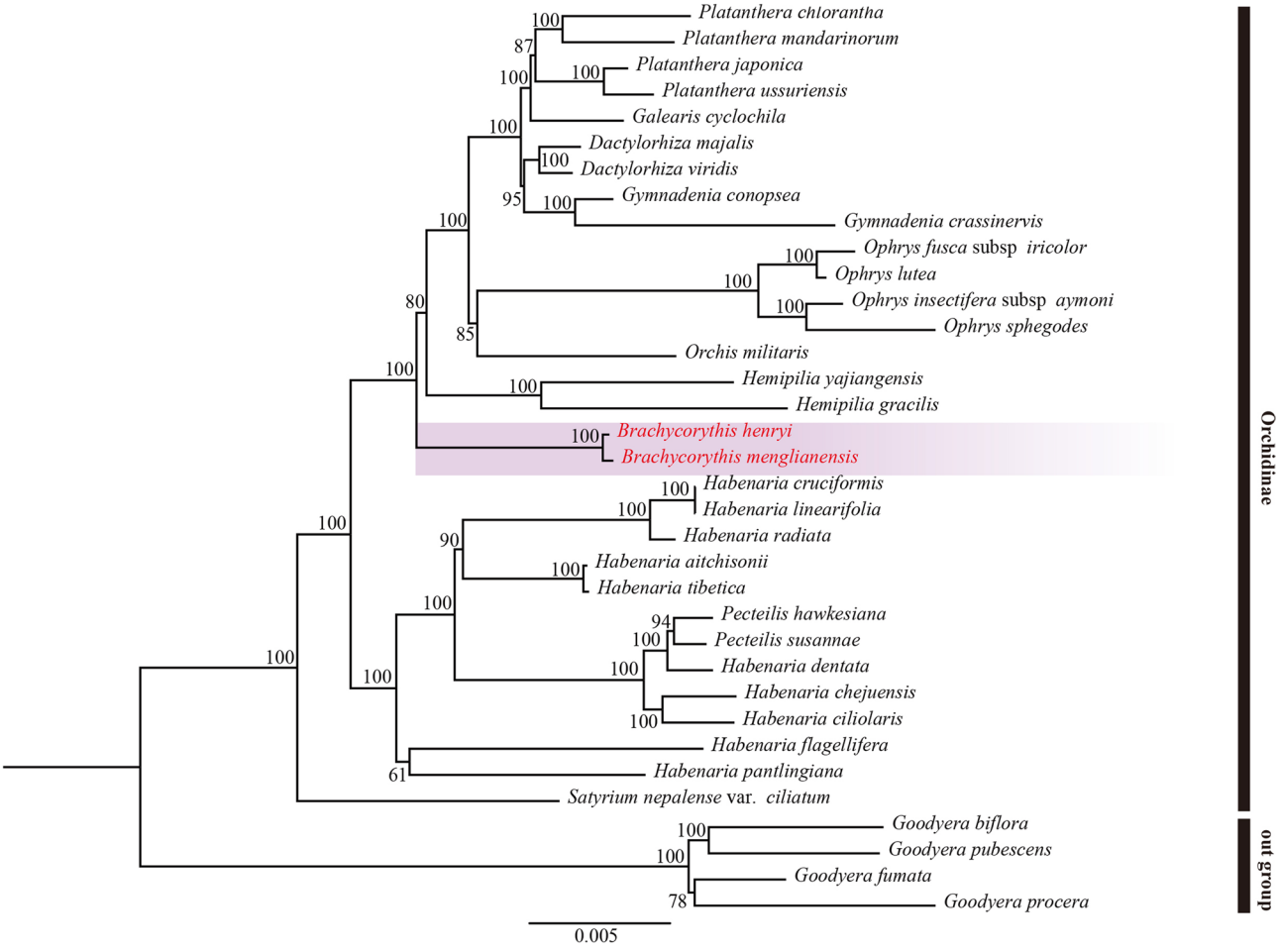

Fig. 7 A maximum likelihood phylogenetic tree reconstructed based on 62 single-copy CDS sequences from 31 species in Orchidinae available, with four *Goodyera* species as outgroup. Numbers at nodes indicating ultrafast bootstrap values

Orchidoideae, and Epidendroideae [31, 32]. It was suggested that a complete set of functional *ndh* genes existed in the common ancestor of orchids [31]. In order to further explore the chloroplast genomic information *B. henryi* and *B. menglianensis*, the following studies were done.

In order to get some implications of the phylogenetic affinities of *Brachycorythis*, the obtained cp. genome data of both species were searched against the NCBI database using the BLAST tool (https://www.ncbi.nlm.nih.gov/). It was shown that *Brachycorythis* possessed high similarity with *Dactylorhiza* Neck. ex Nevski (97–98%) and the related genera from Orchidinae based on cp. genomic features. Meanwhile, it was proposed that *Brachycorythis* should be closely related to the five related genera, namely, *Platanthera*, *Gymnadenia*, *Hemipilia s. l.*, *Schizochilus* and *Neobolusia* based on morphological and DNA markers [3, 8, 10, 11]. It was revealed that there were some highly conserved structures of cp. genome from ten species representing related taxon, excluding *Schizochilus* and *Neobolusia*, that lacking data. However, there were some different features of cp. genome among these ten species, which possess some phylogenetic implications as below.

Firstly, simple sequence repeats (SSRs), which are short tandem repeats consisting of 1 to 6 bp units, possess distinct features that make them efficient genetic markers, including abundance in number, highly repetitive, a simple structure, maternal inheritance of cp. genomes, and relatively conserved [33–36]. A total of 68 to 92 SSRs were identified in the six cp. genomes. Consistent with patterns observed in other orchids, SSRs of cp. genomes were predominantly comprised of short poly-A or poly-T repeats, and the mononucleotide repeats were the most commonly encountered forms [23–25]. It was observed that eight types of SSRs were identified in the ten species, which varied from species to species. Compound* (c*) was present in six species, but absent in two *Brachycorythis* species, *Dactylorhiza viridis*, and *Gymnadenia conopsea*. Pentanucleotide (P5) was found in two *Gymnadenia* species, and *D. viridis* and *Hemipilia yajiangensis*, while hexanucleotides (P6) was identified in two *Hemipilia* species and *B. henryi.* In addition,

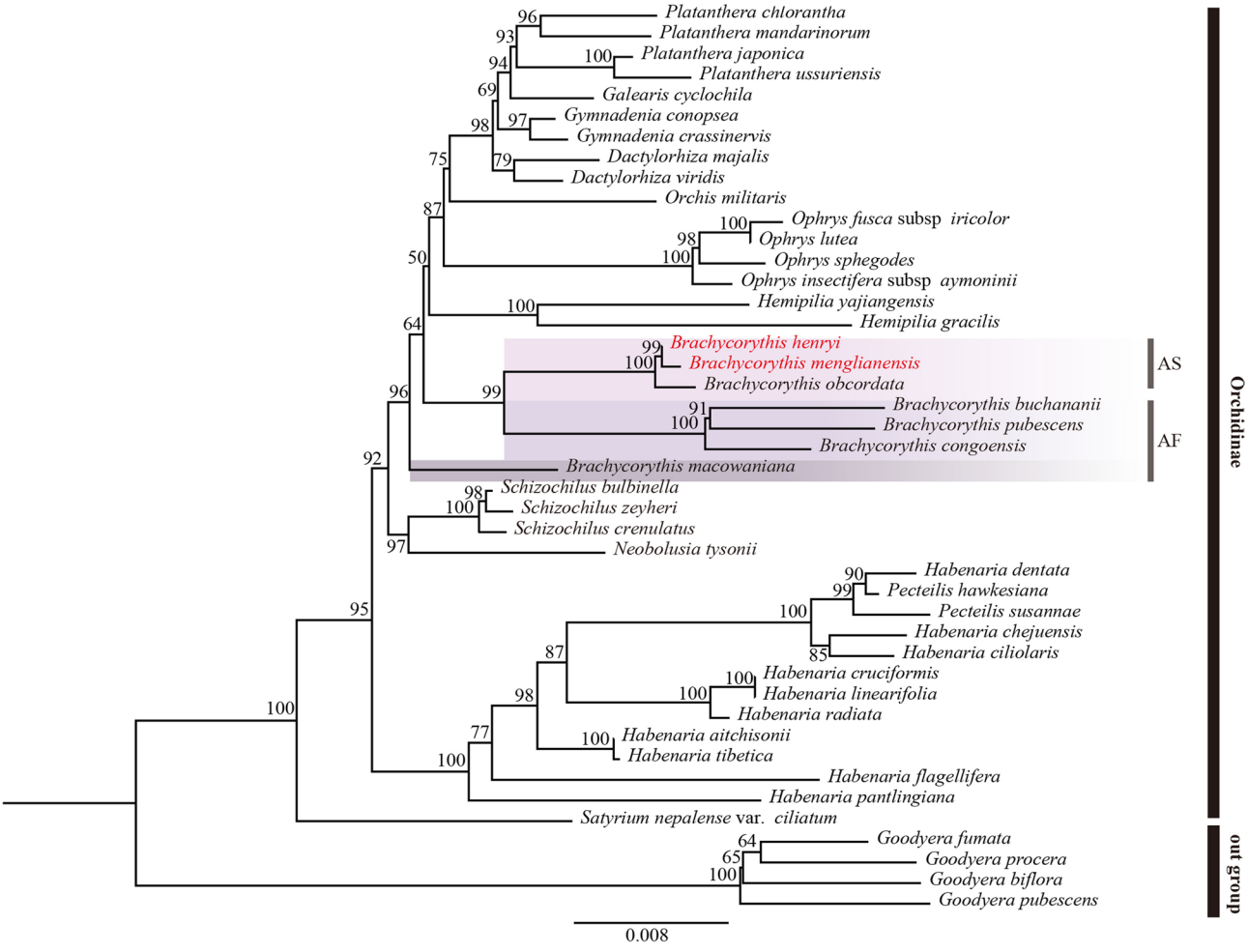

Fig. 8 A maximum likelihood phylogenetic tree based on *matK*+*rbcL* sequence from 40 related species in Orchidinae as ingroup, together with four *Goodyera* species as outgroup. Numbers at nodes indicating ultrafast bootstrap values. AS, Asia; AF, Africa

there were 21 to 71 long repeats detected in the ten cp. genomes, which were recognized as four types. The large number of LSRs likely maintained the stability of the cp. genome and was effective in increasing the length of the plastid [37, 38]. F-type and P-type were more abundant than R-type and C-type, which was consistent with other angiosperms [22, 23, 39]. Meanwhile, the presence of R-type and C-type was varied among species, which should be of phylogenetic importance as a cp. genomic character. R-type occurred in nine species, except for *Dactylorhiza viridis*, but C-type was present in six species but absent in two *Brachycorythis* species, and *D. viridis*, and *Gymnadenia crassinervis*. The results of the above study may provide a data basis for further population genetics studies.

Secondly, codon usage bias is useful for investigating the evolutionary history of organisms, predict expression level, and understand the evolutionary processes acting on genome at the molecular level [40, 41]. It was referred to the phenomenon, in which synonymous codons in protein transcripts were not uniformly used to encode all amino acids in the protein, except for methionine and tryptophan [37]. It was shown that cp. genomes of the ten species contained 64 types of codons encoding 20 amino acids. Among ten species, leucine (Leu) had the highest number of codons, whereas tryptophan (Trp) had the lowest number of codons. And, almost all CDS had the standard ATG start codon. According to the RSCU value, ten cp. genomes could be divided into four groups. It was shown that the codon usage bias was observed between different species. Given the differences in the degree of variation and selection pressure, the variation in compositional constraints among different genomes was a key factor in the formation of codon usage bias [42, 43].

Thirdly, the boundaries of IR region demonstrated frequent contractions and expansions, associated with the evolution of the cp. genome, representing the primary driver of variations in cp. genome length [44, 45]. The boundaries of IR region were the most conservative section within the ten cp. genomes. It was also showed that

the junction positions of IR/SC were conserved in the ten species from Orchidinae. Unlike basal angiosperms and eudicots, most monocots typically harbored *trnH-rps19* clusters in each IR region [46]. The *trnH-rps19* clusters were also located within each IR region in two *Brachycorythis* species, and *ycf1* gene spanned the SSC and IR regions which was consistent with other eight species. It was noteworthy that the border region is relatively stable within the genus.

Finally, divergent regions, serving as valuable sources of data for DNA barcoding and phylogenetic research, were frequently employed as molecular markers in phylogenetic reconstruction [39]. In this study, the nucleotide sequence of non-coding regions was more varied than the coding regions, which was generally consistent with other cp. genomes in Orchid family [22–24]. Meanwhile, the analysis of coding sequence regions revealed that the genes (*psbT*, *rpl20*, *atpF*, *accD*, *psbK*, *ndhF*, *rpl32*, *matK*, *ndhE*, *rps15*, *ccsA*, and *ndhD*) had significantly higher Pi values. Notably, *ycf1*, akin to *matK*, has been utilized as DNA markers for phylogenetic reconstruction [39]. Here, *atpH-atpI*, *psaI-ycf4*, *accD-psaI*, *rps2-rpoC2*, *matK-rps16*, *clpP-psbB*, *cemA-petA*, *rbcL-accD*, and *petA-psbJ* also possessed the higher degree of variability. Simultaneously, regions including *trnS-trnG*, *psaC-ndhE*, *clpP-psbB*, etc., possessed the highest degree of variability in *Phalaenopsis* Blume [22], while *psbB-psbT*, *psbE-petL*, and others showed the highest degree of variability in *Aerides* Lour [23]. It was indicated cp. genomes in Orchidaceae exhibited a diversity array of highly variable sequences [22–24, 26].

In summary, the cp. genomes of the ten species were highly similar, but also had some differences, such as repeats sequence, codon usage bias, contractions and expansions in the IR region. The results of the above study might provide new data for population genetics.

The intergeneric relationship of *Brachycorythis*

The intergeneric relationship of *Brachycorythis* between the five related genera has been disputed, including *Schizochilus*, *Neobolusia*, *Platanthera*, *Gymnadenia*, and *Hemipilia*. *Brachycorythis* was presumed previously to be related with *Schizochilus* [8], and then with *Platanthera* Rich., *Gymnadenia* R. Br. and *Neobolusia* Schltr [3, 10]. based on morphological data. However, it seemed to be close to *Hemipilia s. l.* based on six sequences genes (*matK*, *psaB*, *rbcL*, *trnL-F*, *trnH-psbA*, nrITS, and *Xdh*) [11], but to be monophyletic and a sister to some species from Orchidinae based on more molecular data (*matK*, *psaB*, *rbcL*, *trnL-F*, *trnH-psbA*, nrITS, and *Xdh*) [12]. In this research, a phylogenetic analysis, based on 62 single-copy CDS from 40 complete cp. genomes, showed that two *Brachycorythis* species formed a monophyletic clade, a sister to some members of Orchidiniae *s.s.*, including eight species from three related genera (*Platanthera*, *Gymnadenia*, and *Hemipilia*) and eight species from other four genera (*Ophyris*, *Orchis*, *Galearis*, and *Dactylorhiza*). It seemed that *Brachycorythis* was not close to three of the five genera mentioned above, which might be involved with a lack of cp. genomic data on other two genera (*Schizochilus* and *Neobolusia*). Therefore, another phylogenetic tree was reconstructed based on *matK* + *rbcL* sequence from an expanded ingroup, including 12 species from all the five related genera assumed. The tree topology was similar to that obtained by cp. genomic above, which indicated that the intergeneric relationship of *Brachycorythis* could be clarified. It was confirmed that six *Brachycorythis* species were clustered into an independent clade, while *B. macowaniana* from Africa was distant from them. It was suggested that *Brachycorythis* should not be related to each of the five genera mentioned.

The intrageneric taxonomy of *Brachycorythis*

The intrageneric taxonomy of *Brachycorythis* has been challenging [20, 21]. It is one of the few genera in Orchidinae exhibiting a transoceanic disjunction between Africa (including Madagascar) and subtropical Asia [1, 2, 4]. The African-Asian disjunct distribution was well supported by *matK* + *rbcL* sequence data here. Six *Brachycorythis* species formed an independent clade, containing two sister subclades, namely, three species from Africa, and the other three species from Asia. In addition, *B. menglianensis* was ever treated as a synonym of *B. henryi* [20, 21]. Two phylogenetic trees, based on 62 single-copy CDS sequences and *matK* + *rbcL* sequence data, respectively, indicated that *B. menglianensis* and *B. henryi* should be separated species, since they were clustered into a clade with different branch lengths. Meanwhile, *B. menglianensis* could be distinguished from *B. henryi* by some cp. genomic features, such as the length of cp. genome, the number and type of repeated sequence, and codon GC content and RSCU value. And what's more, there were some different morphological features between *B. menglianensis* and *B. henryi* [4], which were also observed here (Fig. 1). *B. menglianensis* was characterized by small plant with leaves ovate, lip suborbicular with apex emarginate, and a spur conic and curved. In contrast, *B. henryi* was diagnosed by large plant with leaves oblong-elliptic, lip suborbicular with apex entire, and a spur subcylindric-conic with apex 2-lobed and often hooked. Therefore, it was supported that *B. menglianensis* should be treated as an independent species from *B. henryi*.

The systematic position of *Brachycorythis macowaniana*

Here, it also showed that *B. macowaniana* was not grouped with the other six *Brachycorythis* species, indicating that *Brachycorythis* was rendered to be paraphyletic. In fact, the systematic position of *B. macowaniana* has been uncertain and changed several times. It was firstly described as one species in *Brachycorythis* [15], and then moved into *Habenaria* Willd [16]. Interestingly, Schlechter thought at first that it was better placed in *Gymnadenia* [17], but moved it into *Platanthera* after he had seen living materials [18]. And later, it was treated as one species of a new genus of *Gyaladenia* Schltr [19]. However, *Gyaladenia* is currently as a synonym of *Brachycorythis* [47], but could be resurrected if a better sampled study could confirm that *B. macowaniana* should not be close to the rest members of *Brachycorythis* [14]. Fortunately, our current sampling included three Asian species and three African species, and therefore, it was shown that *B. macowaniana* was not clustered with the other six species of *Brachycorythis*. Therefore, it was proposed that *Gyaladenia* be re-established to accommodate *B. macowaniana*, based on its unique morphological characters and systematic position [19].

Conclusion

It was the first report on the cp. genomic data on *Brachycorythis*, providing some phylogenetic implications. The complete cp. genome of *Brachycorythis henryi* and *B. menglianensis* were sequenced and analyzed in detail, including the general genome structure, codon usage, repeat sequences, boundaries within the inverted repeats, DNA polymorphism, and phylogenetic implications. A comparative analysis of cp. genome between these two species and other eight Orchidinae species indicated that their cp. genomic feature was almost congruent and highly conserved, but there were some varied features from taxa sampled, which could be used to clarify evolutionary relationships of *Brachycorythis*. It was indicated that *Brachycorythis* was not related to each of the five related genera based on cp. genomic and *matK* + *rbcL* sequence data. Meanwhile, three species from Asia and other three species from Africa respectively, formed two independent clades, which was consistent well with the African-Asian disjunct distribution of *Brachycorythis*. What's more, it was proposed that *Gyaladenia* be re-established to accommodate *B. macowaniana*. In addition, it was revealed that *B. menglianensis* was not a synonym of *B. henryi*, and each of them was a separated species based on cp. genomic and morphological data.

Materials and methods

Ethical statement

No specific permits were required for the collection of specimens for study, and all procedures were conducted in compliance with relevant Chinese laws.

Plant materials and chloroplast genome sequencing

Plants of *Brachycorythis henryi* and *B. menglianensis* were cultivated in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Yunnan, China. Prof. Luo Yan (luoyan@xtbg.org.cn) identified these two species sampled by the leaf and flower morphology. Leaf samples were obtained from these plants, and the specimens were deposited in the Herbarium of Southwest Forestry University (HSFU, Lilu2019001 and Lilu2019002, lilu@swfu.edu.cn). Genomic DNA of each sample was extracted from the silica gel-dried leaf tissues using the modified CTAB method with the TiangenDNA kit (TIANGEN, China) [48]. Paired-end libraries with an average insert size of approximately 400 bp were prepared using a TruSeq DNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions. The libraries were sequenced on the Illumina HiSeq 2500 platform at Personalbio (two times 150 bp; Illumina, Shanghai, China). Raw data were filtered using Fastp v0.23.1 (https://github.com/OpenGene/fastp) to obtain high-quality reads by the sliding window method to drop the low-quality bases of each read's head and tail [49].

Chloroplast genome assembly and annotation

The two complete cp genomes from the clean reads were assembled by the GetOrganelle version 1.7.7.1 (https://github.com/Kinggerm/GetOrganelle) [50]. The assembled cp genomes were annotated using the online program CPGAVAS2 (http://47.96.249.172:16019/analyzer/home), and further manually adjusted and confirmed using Geneious Prime version 2020.0.4 (https://www.geneious.com/features/prime) [51]. The complete cp genomes sequences of *Brachycorythis henryi* and *B. menglianensis* were submitted to GenBank (Accession numbers: PQ149858 and PQ149859). The circular genome maps were drawn by the OGDRAW program (https://chlorobox.mpimp-golm.mpg.de/OGDraw) [52].

Comparative analysis

The cp genomes of two *Brachycorythis* species and eight related species from Orchidinae (Table S13) were selected for a comparative analysis.

By setting the minimum number of repeats to 10, 5, 4, 3, 3, and 3 for mononucleotide (P1), dinucleotide (P2), trinucleotide (P3), tetranucleotide (P4), pentanucleotide (P5), and hexanucleotide (P6), respectively, simple sequence repeats (SSR), a tract of repetitive DNA that

typically ranges in length from 1 to 6 nucleotides, were detected via MISA (https://webblast.ipk-gatersleben.de/misa/index.php?action=1) [53, 54]. The repetitive structures, repeat sizes, and locations of forward match (F), reverse match (R), palindromic match (P), and complementary match (C) nucleotide repeat sequences were identified by REPuter v2.74 (https://bibiserv.cebitec.uni-bielefeld.de/reputer/) [55], with minimal repeat size set to 30 bp, hamming distance set to 3 and maximum computed repeats set to 90 [24].

Condon usage was analyzed by MEGA11 software [56], and the relative synonymous codon usage (RSCU) and amino acid frequencies were calculated with default settings [57]. The RSCU analysis was performed using JSHYCloud (http://cloud.genepioneer.com:9929). In addition, the GC content of the three position was analyzed by CUSP on EMBOSS program (http://emboss.toulouse.inra.fr/cgi-bin/emboss/cusp) [58]. Using the web tool IRSCOPE (https://irscope.shinyapps.io/irapp/), the contraction and extension of the IR borders between the four major areas (LSC/IRa/SSC/IRb) of the eight cp genome sequences were performed [59]. The pairwise alignments and sequence divergence using the mVISTA with Shuffle-LAGAN mode (https://genome.lbl.gov/cgi-bin/VistaInput?num_seqs=2) [60].

Phylogenetic analysis

For phylogenetic analysis, the cp genomes of 35 species from Orchidinae were selected (Table S13). The ingroup contained the cp genomes of 31 species from Orchidinae, of which 29 species were downloaded from the NCBI database. Four species of *Goodyera* were selected as outgroup [11–14]. The 62 common single-copy CDS sequences in the cp genomes were used for phylogenetic analyses (Table S14). These 62 single-copy CDS sequences were extracted by PhyloSuite version 1.2.2 (https://github.com/dongzhang0725/PhyloSuite) [58, 60], aligned by MAFFT version 7 [61], trimmed by Gblocks [62], and concatenated by plugins in PhyloSuite version 1.2.2 [58, 60]. All possible nucleotide substitution models (including GTR, HKY, etc.) under the AIC criterion using ModelFinder (IQ-TREE 2 built-in module, -MFP command call) [63, 64], while accounting for gamma-distributed rate heterogeneity (+G) and the proportion of invariant sites (+I). The Maximum-Likelihood (ML) tree was performed in GTR+F+I+G4 mode based on CDS sequences by IQ-TREE 2 (https://github.com/iqtree/iqtree2) with 5000 ultrafast bootstraps (UFBoot) [63–65]. The 42 *matK* and *rbcL* gene sequences, marked complete CDS, were downloaded from the NCBI database (Table S13), extracted by Geneious Prime version 2020.0.4 [51], and trimmed by Gblocks [62], and concatenated by plugins in PhyloSuite version 1.2.2 [58, 60]. The phylogenetic tree, based on *matK*+*rbcL* gene sequences from 40 species involved,was constructed by IQ-TREE 2 in GTR+F+I+G4 mode, with 5000 ultrafast bootstraps (UFBoot) [63–65]. The ingroup contained seven *Brachycrorythis*, and 33 other Orchidinae species. And four *Goodyera* species were used as outgroup.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11791-8.

Supplementary Material 1.

Acknowledgements

We thank Dr. Lei Tao for suggestions on data analysis, and Associate Professor Yuxiao Zhang for providing the computer server.

Authors' contributions

LL. and YL. conceived and designed the experiment, and improved the manuscript. DM, KFT, and YQX finished the data analysis and wrote the draft. All authors reviewed and approved the final manuscript.

Funding

This research was funded by National Nature Science Foundation of China (NSFC 32060049 and NSFC 32270225), and Yunnan Fundamental Research Project (No. 202501BD070001-018).

Data availability

The datasets generated or analyzed during the current study are available in the NCBI BioProject (PRJNA1143595 and PRJNA1143686, SRA: SRR30118422 and SRR30171006).

Declarations

Ethics approval and consent to participate

The plant materials used in this research comply with relevant institutional, national, and international guidelines and legislation. Plants of *Brachycorythis henryi* and *B. menglianensis* were cultivated in Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]College of Forestry, Southwest Forestry University, Kunming, Yunnan 650224, China
[2]Yunnan Academy of Biodiversity, Southwest Forestry University, Kunming, Yunnan 650224, China
[3]Southeast Asian Biodiversity Research Institute, Chinese Academy of Sciences & Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China

Received: 20 November 2024 / Accepted: 5 June 2025
Published online: 02 July 2025

References

1. Seidenfaden G. *Brachycorythis* Lindl. Orchid genera in Thailand V. Copenhagen: Dansk Botanisk Arkiv; 1997. pp. 9–15.
2. Pridgeon AM, Cribb PJ, Chase MW. Brachycorythis. Genera Orchidacearum. Oxford: Oxford University Press; 2001. pp. 265–9.
3. Summerhayes VS. A revision of the genus *Brachycorythis*. Kew Bull. 1955;10:221–64.

4. Chen X, Gale SWG, Cribb PJ. Brachycorythis. Flora of china: Orchidaceae. Beijing: Science; 2009. pp. 100–1.
5. Teoh ES. Medicinal Orchid usage in rural Africa. Orchids as aphrodisiac, medicine or food. Cham: Springer International Publishing; 2019. pp. 305–62.
6. Klopper R, Winter P, Le Roux M. The South African National plant checklist: maintaining the taxonomic backbone for a megadiverse country. BISS. 2021;5:e73899.
7. Williams VL, Victor JE, Crouch NR. Red listed medicinal plants of South africa: status, trends, and assessment challenges. South Afr J Bot. 2013;86:23–35.
8. Olędrzyńska N, Szlachetko DL. Contribution to the taxonomic revision of *Brachycorythis* -complex (Orchidaceae, Orchidoideae). Biodivers Res Conserv. 2021;62:5–117.
9. Lindley John. The genera and species of orchidaceous plants. London: Ridgways; 1840.
10. Kurzweil H, Weber A. Floral morphology of Southern African orchideae. I. Orchidinae. Nord J Bot. 1991;11:155–78.
11. Tang Y, Yukawa T, Bateman RM, Jiang H, Peng H. Phylogeny and classification of the East Asian *Amitostigma alliance* (Orchidaceae: Orchideae) based on six DNA markers. BMC Evol Biol. 2015;15:96.
12. Jin W-T, Schuiteman A, Chase MW, Li J-W, Chung S-W, Hsu T-C, et al. Phylogenetics of subtribe Orchidinae *s. l.* (Orchidaceae; Orchidoideae) based on seven markers (plastid *matK*, *psaB*, *rbcL*, *trnL-F*, *trnH-psbA*, and nuclear nrITS, *Xdh*): implications for generic delimitation. BMC Plant Biol. 2017;17:222.
13. Samigullin TH, Logacheva MD, Averyanov LV, Zeng S-J, Fu L-F, Nuraliev MS. Phylogenetic position and plastid genome structure of *Vietorchis*, a mycoheterotrophic genus of Orchidaceae (subtribe Orchidinae) endemic to Vietnam. Front Plant Sci. 2024;15:1393225.
14. Ngugi G, Le Péchon T, Martos F, Pailler T, Bellstedt DU, Bytebier B. Phylogenetic relationships amongst the African genera of subtribe Orchidinae s. L. (Orchidaceae; Orchideae): Implications for subtribal and generic delimitations. Mol Phylogenet Evol. 2020;153:106946.
15. Reichenbach HG. Otia botanica Hamburgensia-Fasciculi secundi Pars Prima. Hamburgi: Typis T. T. Meissneri; 1878.
16. Brown NE. New or notheworthy plants, Habenaria Macowaniana. Gard Chron Ser. 1889;3:168.
17. Schlechter R. Beiträge zur kenntnis der Orchidaceen und Asclepiadaceen Südafrikas. 1893;35:44–54.
18. Schlechter R. Beiträge Zur Kenntnis Neuer und kritischer Orchideen Aus Sudafrika. Bot Jahrbiicher. 1895;20:1–46.
19. Schlechter R. Revision von Schizochilus Sond. and Brachycorythis Lindl. Beih Bot Centralbl. 1921;38:80–131.
20. Pedersen HAe. Species delimitation and recognition in the *Brachycorythis Helferi* complex (Orchidaceae) resolved by multivariate morphometric analysis. Bot J Linn Soc. 2010;162:64–76.
21. Qian YY. A new species of *Brachycorythis* lindl. (Orchidaceae) from yunnan, China. J Univ Chin Acad Sci. 2001;39:278–9.
22. Tao L, Duan H, Tao K, Luo Y, Li Q, Li L. Complete Chloroplast genome structural characterization of two *Phalaenopsis* (Orchidaceae) species and comparative analysis with their alliance. BMC Genomics. 2023;24:359.
23. Tao K, Tao L, Huang J, Duan H, Luo Y, Li L. Complete Chloroplast genome structural characterization of two *Aerides* (Orchidaceae) species with a focus on phylogenetic position of *Aerides flabellata*. BMC Genomics. 2024;25:552.
24. Han C, Ding R, Zong X, Zhang L, Chen X, Qu B. Structural characterization of *Platanthera ussuriensis* Chloroplast genome and comparative analyses with other species of Orchidaceae. BMC Genomics. 2022;23:84.
25. Wu Y, Zeng M-Y, Wang H-X, Lan S, Liu Z-J, Zhang S, et al. The complete Chloroplast genomes of *Bulbophyllum* (Orchidaceae) species: insight into genome structure divergence and phylogenetic analysis. IJMS. 2024;25:2665.
26. Tao K, Tang L, Luo Y, Li L. Complete Chloroplast genome of eight *Phaius* (Orchidaceae) species from china: comparative analysis and phylogenetic relationship. BMC Plant Biol. 2025;25:37.
27. May M, Novotná A, Minasiewicz J, Selosse M-A, Jąkalski M. The complete Chloroplast genome sequence of *Dactylorhiza majalis* (Rchb.) P.F. Hunt et summerh. (Orchidaceae). Mitochondrial DNA Part B. 2019;4:2821–3.
28. Gichira AW, Avoga S, Li Z, Hu G, Wang Q, Chen J. Comparative genomics of 11 complete Chloroplast genomes of senecioneae (Asteraceae) species: DNA barcodes and phylogenetics. Bot Stud. 2019;60:17.
29. Le TTN, Vu MT, Do HDK. The complete Chloroplast genome of *Dicliptera tinctoria* (Nees) kostel. And comparative analysis of Chloroplast genomes in Acanthaceae. Genet Mol Biol. 2024;47:e20230297.
30. Li L, Yang M, Qi Y, Yu Y, Gao P, Yang S, et al. Complete Chloroplast genome and phylogenetic analysis of *Amorphophallus paeoniifolius* (Araceae). Mitochondrial DNA Part B. 2024;9:865–70.
31. Lin C-S, Chen JJW, Huang Y-T, Chan M-T, Daniell H, Chang W-J, et al. The location and translocation of *Ndh* genes of Chloroplast origin in the Orchidaceae family. Sci Rep. 2015;5:9040.
32. Lin C, Chen JJW, Chiu C, Hsiao HCW, Yang C, Jin X, et al. Concomitant loss of NDH complex-related genes within Chloroplast and nuclear genomes in some orchids. Plant J. 2017;90:994–1006.
33. Zhang L, Meng Y, Wang D, He G-H, Zhang J-M, Wen J, et al. Plastid genome data provide new insights into the dynamic evolution of the tribe ampelopsideae (Vitaceae). BMC Genomics. 2024;25:247.
34. Zhang S-Y, Yan H-F, Wei L, Liu T-J, Chen L, Hao G, et al. Plastid genome and its phylogenetic implications of *Asiatic spiraea* (Rosaceae). BMC Plant Biol. 2024;24:23.
35. Agrama HA, Tuinstra MR. Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. Afr J Biotechnol. 2003;2(10):334–40.
36. Li X, Zhao Y, Tu X, Li C, Zhu Y, Zhong H, et al. Comparative analysis of plastomes in oxalidaceae: phylogenetic relationships and potential molecular markers. Plant Divers. 2021;43:281–91.
37. Zeb U, Dong W, Zhang T, Wang R, Shahzad K, Ma X, et al. Comparative plastid genomics of *Pinus* species: insights into sequence variations and phylogenetic relationships. J Sytematics Evol. 2020;58:118–32.
38. Li J, Tang J. Comparative plastid genomics of four *Pilea* (Urticaceae) species: insight into interspeci c plastid genome diversity in *Pilea*. BMC Plant Biol. 2021;21:25.
39. Menezes APA, Resende-Moreira LC, Buzatti RSO, Nazareno AG, Carlsen M, Lobo FP, et al. Chloroplast genomes of *Byrsonima* species (Malpighiaceae): comparative analysis and screening of high divergence sequences. Sci Rep. 2018;8:2210.
40. Jia J, Xue Q. Codon usage biases of transposable elements and host nuclear genes in Arabidopsis thaliana and Oryza sativa. Genom Proteom Bioinform. 2009;7:175–84.
41. Leffler EM, Bullaughey K, Matute DR, Meyer WK, Ségurel L, Venkat A, et al. Revisiting an old riddle: what determines genetic diversity levels within species?? PLoS Biol. 2012;10:e1001388.
42. Sharp PM, Li W-H. The codon adaptation index-a measure of directional synonymous codon usage bias, and its potential applications. Nucl Acids Res. 1987;15:1281–95.
43. Paul P, Malakar AK, Chakraborty S. Codon usage and amino acid usage influence genes expression level. Genetica. 2018;146:53–63.
44. Raubeson LA, Peery R, Chumley TW, Dziubek C, Fourcade HM, Boore JL, et al. Comparative Chloroplast genomics: analyses including new sequences from the angiosperms *Nuphar advena* and *Ranunculus macranthus*. BMC Genomics. 2007;8:174.
45. Dugas DV, Hernandez D, Koenen EJM, Schwarz E, Straub S, Hughes CE, et al. Mimosoid legume plastome evolution: IR expansion, tandem repeat expansions and accelerated rate of evolution in ClpP. Sci Rep. 2015;5:16958.
46. Wang R-J, Cheng C-L, Chang C-C, Wu C-L, Su T-M, Chaw S-M. Dynamics and evolution of the inverted repeat-large single copy junctions in the Chloroplast genomes of monocots. BMC Evol Biol. 2008;8:36.
47. *Gyaladenia* Schltr.| Plants of the World Online| Kew Science. Plants of the World Online. http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:29596-1. Accessed 19 Nov 2024.
48. Healey A, Furtado A, Cooper T, Henry RJ. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. Plant Methods. 2014;10:21.
49. Chen S, Zhou Y, Chen Y, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34(17):884–90.
50. Jin J-J, Yu W-B, Yang J-B, Song Y, Depamphilis CW, Yi T-S, et al. GetOrganelle: a fast and versatile toolkit for accurate de Novo assembly of organelle genomes. Genome Biol. 2020;21:241.
51. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9.
52. Greiner S, Lehwark P, Bock R. Organellar genome DRAW (OGDRAW) version 1.3.1: expanded toolkit for the graphical visualization of organellar genomes. Nucleic Acids Res. 2019;47:59–64.
53. Beier S, Thiel T, Münch T, Scholz U, Mascher M. MISA-web: a web server for microsatellite prediction. Bioinformatics. 2017;33:2583–5.

54. Thiel T, Michalek W, Varshney R, Graner A. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L). Theor Appl Genet. 2003;106:411–22.
55. Kurtz S. REPuter: the manifold applications of repeat analysis on a genomic scale. Nucleic Acids Res. 2001;29:4633–42.
56. Kumar S, Nei M, Dudley J, Tamura K. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform. 2008;9:299–306.
57. Indrabalan UB, Suresh KP, Shivamallu C, Patil SS. An extensive evaluation of codon usage pattern and bias of structural proteins p30, p54 and, p72 of the African swine fever virus (ASFV). VirusDis. 2021;32:810–22.
58. Xiang C, Gao F, Jakovlić I, Lei H, Hu Y, Zhang H, et al. Using phylosuite for molecular phylogeny and tree-based analyses. iMeta. 2023;2:e87.
59. Rice P, Longden I, Bleasby A. EMBOSS: the European molecular biology open software suite. Trends Genet. 2000;16:276–7.
60. Zhang D, Gao F, Jakovlić I, Zou H, Zhang J, Li WX, et al. PhyloSuite: an integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. Mol Ecol Resour. 2020;20:348–55.
61. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013;30:772–80.
62. Talavera G, Castresana J. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol. 2007;56:564–77.
63. Kalyaanamoorthy S, Minh BQ, Wong TKF, Von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods. 2017;14:587–9.
64. Hoang DT, Chernomor O, Von Haeseler A, Minh BQ, Vinh LS. UFBoot2: improving the ultrafast bootstrap approximation. Mol Biol Evol. 2018;35:518–22.
65. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A, et al. IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol. 2020;37:1530–4.

Publisher's Note