The chloroplast genomes of four *Bupleurum* (Apiaceae) species endemic to Southwestern China, a diversity center of the genus, as well as their evolutionary implications and phylogenetic inferences

Rong Huang¹, Xuena Xie¹‡, Aimin Chen¹, Fang Li¹, Enwei Tian¹ and Zhi Chao¹,2,3*†

**Abstract**

**Background:** As one of the largest genera in Apiaceae, *Bupleurum* L. is well known for its high medicinal value. The genus has frequently attracted the attention of evolutionary biologist and taxonomist for its distinctive characteristics in the Apiaceae family. Although some chloroplast genomes data have been now available, the changes in the structure of chloroplast genomes and selective pressure in the genus have not been fully understood. In addition, few of the species are endemic to Southwest China, a distribution and diversity center of Chinese *Bupleurum*. Endemic species are key components of biodiversity and ecosystems, and investigation of the chloroplast genomes features of endemic species in *Bupleurum* will be helpful to develop a better understanding of evolutionary process and phylogeny of the genus. In this study, we analyzed the sequences of whole chloroplast genomes of 4 Southwest China endemic *Bupleurum* species in comparison with the published data of 17 *Bupleurum* species to determine the evolutionary characteristics of the genus and the phylogenetic relationships of Asian *Bupleurum*.

**Results:** The complete chloroplast genome sequences of the 4 endemic *Bupleurum* species are 155,025 bp to 155,323 bp in length including a SSC and a LSC region separated by a pair of IRs. Comparative analysis revealed an identical chloroplast gene content across the 21 *Bupleurum* species, including a total of 114 unique genes (30 tRNA genes, 4 rRNA genes and 80 protein-coding genes). Chloroplast genomes of the 21 *Bupleurum* species showed no rearrangements and a high sequence identity (96.4–99.2%). They also shared a similar tendency of SDRs and SSRs, but differed in number (59–83). In spite of their high conservation, they contained some mutational hotspots, which can be potentially exploited as high-resolution DNA barcodes for species discrimination. Selective pressure analysis showed that four genes were under positive selection. Phylogenetic analysis revealed that the 21 *Bupleurum* formed two major clades, which are likely to correspond to their geographical distribution.

* Correspondence: chaozhi@smu.edu.cn
†Rong Huang and Xuena Xie contributed equally to this work.
¹Department of Pharmacy, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, China
²Faculty of Medicinal Plants and Pharmacognosy, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, China
Full list of author information is available at the end of the article

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**Conclusions:** The chloroplast genome data of the four endemic *Bupleurum* species provide important insights into the characteristics and evolution of chloroplast genomes of this genus, and the phylogeny of *Bupleurum*.

**Keywords:** *Bupleurum*, Chloroplast genome, Comparative analysis, Phylogenetic analyses

**Background**

*Bupleurum* L., with more than 180 species, represents one of the largest genera of the family Apiaceae and is distributed in the north temperate zone (mainly in Eurasia, the Mediterranean, North Africa, Asia and North America) [1, 2]. Different from other genera of Apiaceae, life forms in *Bupleurum* vary greatly, ranging from herbs to shrubs [3]. The genus has frequently attracted the attention of evolutionary biologists and taxonomists for its distinctive characteristics in the Apiaceae family [1–6].

*Bupleurum* is easily distinguished from other genera of the family for its simple and entire leaves as well as conspicuous bracts and bracteoles, which are almost unique morphology characteristics in the family [1, 4]. Molecular phylogenetic studies in the recent two decades based on plastid and nuclear markers suggest that it should be considered as an identifiable tribe [7–12]. Interspecific phylogeny of *Bupleurum* presents a long standing problem in the systematics. The genus shows broad intra-specific morphological variations with poorly defined inter-specific boundaries, making the taxonomy based on traditional classification systems extremely difficult as increasing species being discovered [5, 6, 13]. In spite of the efforts at phylogenetic analysis in previous studies, some essential problems concerning the phylogenetic relationship of *Bupleurum* based on nrDNA (ITS) and various plastid sequences (e.g. *rps16*, *trnH–psbA*, and *matK*) still remain to be solved [1, 4, 13, 14].

Compared with nuclear and mitochondrial genome, gene density of chloroplast genome is larger and the evolution rate is moderate, and the segments with different evolution rates may serve for different research purposes [15–19]. The evolution of chloroplast genomes has long fascinated and puzzled evolutionary biologists. The understanding of the evolutionary connection among the plant species, the features they shared, and their differences from other taxonomic groups [20] all benefit from comparative analysis of whole chloroplast genomes that provide insights into the phylogenetic relationships and species evolution in different taxa [21–28]. In general, chloroplast genome has long been considered to be conserved and affected little by adaptive evolution in many genera [18–20]. However, rearrangements, differences in structure, size, gene content and order, and positively selected genes have been documented in some genera, such as *Amphilophium* [29], *Amorphophallus* [30] and the apiaceous genus *Ligusticum* [31]. For such a diversified and wide-distributed genus like *Bupleurum*, we can not assert that there is absolutely no variation in chloroplast genome in a certain group without detailed study. It is important to investigate chloroplast genomes of the taxonomically significant genera, for understanding how infrageneric species are linked, what features are shared among them, and how they are different from other taxonomic groups [20, 29]. The advancement of the high-throughput sequencing technologies has drastically lowered the cost of analysis of the whole chloroplast genome sequences. Previous studies have reported the sequence data of chloroplast genomes for some species of *Bupleurum* [32–40], but unfortunately the changes in the structure of chloroplast genomes or the selective pressure in the genus were seldom addressed. As a result, the evolution of chloroplast of the genus is poorly understood. In the study by Li et al. [37], the chloroplast genomes of only five *Bupleurum* species were reported, but few of the species are endemic to Southwest China. Southwest China, which harbors an extremely high species diversity [41, 42] and is a distribution and diversity center of Chinese *Bupleurum* (ca. 21 species), including 12 species and 8 varieties endemic to China, 11 species and 5 varieties endemic to Southwest China. As key components of biodiversity and ecosystems, endemic species has long attracted the interest of ecologists and evolutionary biologist [43–47]. Investigation of the chloroplast genome features of endemic *Bupleurum* species may provide important insights into the evolution and phylogeny of the genus, especially the endemic *Bupleurum* species, thus helping to clarify the phylogenetic relationships and evolutionary aspects in the genus.

*Bupleurum shanianum*, *B. yunnanense*, *B. kweichowense* and *B. rockii* are endemic to Southwest China [2, 6]. *B. shanianum*, *B. yunnanense* and *B. kweichowense* are 3 perennial herbs with height ca. 6–35(–58) cm, 12–35 cm, 20–40 cm, respectively, while *B. rockii* has a relatively higher height ca. 60–100 cm. *B. shanianum* and *B. yunnanense* grow among grassy places, bushes, or under forests at altitudes of 3200–4400 m and 2500–5000 m, respectively, distributed in the alpine area of eastern Himalayas (Southeast Tibet), Sichuan and Yunnan Provinces of China. *B. rockii* grows in open forests and grasses on mountain slopes at altitudes of 1900–4200 m, occurring in Sichuan and northwest Yunnan Provinces of China. The documentation of *B. kweichowense* is...
extremely poor and only a few collections from northeast Guizhou Province (Fanjing Shan) of China are available. It grows on gravelly slopes in sunny places at altitudes ca. 2100 m.

In this study, we used high-throughput sequencing technologies to sequence the chloroplast genomes of four *Bupleurum* species (B. shanianum, B. yunnanense, B. kweichowense and B. rockii) and assembled their whole chloroplast genomes. Previously published chloroplast genomes of 17 *Bupleurum* species [32–40], including herbs and one shrub (*B. dracanoides*), were downloaded for comparative analysis, and the 21 species are distributed roughly in 3 regions (Southwest China; Northeast China, Korea and Japan; Additional file 1: Fig. S1 and Additional file 2: Table S1), which vary greatly in geomorphology and climate. Environmental heterogeneity plays an important role in evolutionary trajectories and ecological adaption of species. It has been reported that the positive selection on some plastid genes (e.g. *clpP*, *ndhF* and *matK*) were observed on some plastid genes in some genera, which indicate that these genes might be subject to adaptive evolution in response to distinct ecological selective pressures [29–31]. However, to date, adaptive evolution of chloroplast genes in *Bupleurum* has not been fully understand. The sequence data of the chloroplast genomes provide a clue to the evolution and phylogeny of *Bupleurum*. Here, we attempted to answer the following questions: (1) Are there differences in the gene and structure of chloroplast genomes among *Bupleurum* species with different life forms and distributions? (2) Do the genes of *Bupleurum* species suffer positive selection under different habitats? We also constructed a phylogeny using 80 protein-coding genes (PCGs) of 21 *Bupleurum* species and 2 outgroups to explore the interspecific phylogenetic relationship of *Bupleurum* species in these regions.

### Results

#### Chloroplast genome features of four *Bupleurum* species endemic to Southwest China

The sequences of the 4 *Bupleurum* chloroplast genomes ranged from 154,925 bp (B. kweichowense) to 155,323 bp (*B. yunnanense*), all having the typical quadripartite structure, comprising a SSC (17,478–17,575 bp) and a LSC (84,920–85,228 bp) region separated by a pair of IRs (52,572–52,649 bp) (Table 1, Fig. 1). The LSC regions exhibited the greatest standard deviation in sequence length (cv = 0.3%), followed by the IR regions (cv = 0.1%) and SSC regions (cv = 0.08%). The overall GC content was highly similar across the 4 chloroplast sequences (37.7–37.8%) (Table 1).

The chloroplast gene contents of the 4 *Bupleurum* species were identical (Table 1). Each *Bupleurum* chloroplast genome encoded a total of 114 unique genes, consisting of 30 tRNA genes, 4 rRNA genes and 80 protein-coding genes (PCGs) with the same gene order (Table 1). The SSC region contained 11 PCGs (*ndhF*, *rpl32*, *ccsA*, *ndhD*, *psaC*, *ndhE*, *ndhG*, *ndhH*, *ndhA*, *rpl15*) and 1 tRNA (*trnL-UAG*) while the LSC region contained 60 PCGs and 22 tRNAs (Table 2). A total of 20 genes were duplicated in the IR regions, including 8 tRNAs (*trnA-UGC*, *trnG-UCC*, *trnL-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG*, and *trnV-GAC*), 8 PCGs (*rps7*, *rpl2*, *rpl23*, *ndhB*, *rps7*, *rps12*, *ycf2* and *ycf15*), and 4 rRNAs (*rrn4.5*, *rrn5*, *rrn16* and *rrn23*) (Table 2). Eighteen genes contain introns, 15 of which contain a single intron, whereas the other 3 (*clpP*, *ycf3* and *rps12*) harbored 2 introns (Table 2). In this study, the incomplete copy of *ycf1* and *rps19* in the IR regions were regarded as pseudogenes.

#### Comparative analysis of chloroplast genome structure of *Bupleurum*

The Mauve alignment analysis revealed that there was no rearrangement in coding and non-coding regions of the 21 *Bupleurum* chloroplast genomes.
indicating that the chloroplast genomes were conserved. Among the 21 Bupleurum species, the genes rpl22 and rpl2 flanked the LSC/IRb junction, and gene rps19 traversed the LSC and the IRb region (JLB line), with 50–82 bp occurring in the IR region (Fig. 3). On the other side of the IRb/SSC was the gene ndhF, which was 15–39 bp away from the IRb/SSC junction. The ycf1 gene traversed the SSC and IRa region, with 1797–2140 bp occurring in the IR region (Fig. 3).

Simple sequence repeats (SSRs) analysis showed that total number of SSR loci ranged from 59 (B. draceanoides) to 83 (B. thianschanicum). The patterns of SSRs distribution were similar among the 21 Bupleurum, as shown in Additional file 1: Fig. S2. Mono-nucleotides were the most frequent in the SSRs (61.5–71.2%), followed by di-nucleotides (10.1–19.7%). Tri-nucleotides and tetra-nucleotides were more frequent than penta- and hexa-nucleotides (Additional file 1: Fig. S2). Short dispersed repeats (SDRs) analysis showed that total
number of SDRs ranged from 34 (B. candollei and B. shaniaanum) to 49 (B. longiradiatum). The species of group I showed less SDRs than those of group II and III (Additional file 1: Fig. S3). The 21 Bupleurum species tended to generate more forward and palindromic repeats rather than reverse repeats, and lacked complement repeats (Additional file 1: Fig. S3).

### Genome divergent hotspot regions in Bupleurum species

Comparative sequence analysis of the 21 Bupleurum species using mVISTA revealed a high sequence identity across the 21 species (Additional file 1: Fig. S4), with identity ranged from 96.4 to 99.2%, suggesting that Bupleurum chloroplast genomes were fairly conserved. Overall, the coding regions (identity = 99.7 ± 0.8%) were less divergent than non-coding regions (identity = 96.7 ± 4.1%), and the IR regions (identity = 99.5 ± 1.1%) were more conserved than LSC (identity = 97.1 ± 3.1%) and SSC (identity = 98.2 ± 2.4%) regions. Variations were observed in some intergenic spacers, including trnK-rps16, rps16-psbK, trnG-trnR, atp1-rps2, trnC-trnT, petA-psbI, psaC-ndhG and ycf1-trnR. A few divergent regions were also observed in some coding regions including psbD, atpB, ndhD and ycf1.

The nucleotide diversity (Pi) of the chloroplast genomes in the 21 Bupleurum species was also calculated to assess the sequence divergence level of these species. In the LSC region, Pi values averaged 0.01087 (range 0.00063–0.03092), and in the SSC region, the average value was 0.01527 (range 0.00394–0.00963) (Additional file 1: Fig. S5). Most of the sequences with high Pi values were spacer regions between genes. Among these spacer regions, trnK-rps16, rps16-psbK, trnG-trnR, atp1-rps2, trnC-trnT, petA-psbI, psaC-ndhG and trnR-ycf1 were the 8 regions having Pi values > 0.02000. Only 4 coding regions (psbD, atpD, ndhD and ycf1) had high Pi values over 0.02000.

| Table 2 List of genes encoded in four Bupleurum plastomes |
|----------------------------------------------------------|
| **Gene Category** | **Genes** | **Number** |
| Ribosomal RNAs | rrn4,5; rrn5; rrn16; rrn23 | 4 |
| Transfer RNAs | trnA-UGG; trnC-GAC; trnD-GUC; trnE-UUC; trnF-GAA; trnM-CAU; trnG-GCC; trnG-UCC; trnH-GUG; trnI-CAU; trnM-GAU; trnM-UUU; trnL-CAA; trnL-UAA; trnL-UAG; trnL-MAU; trnM-GUU; trnQ-UUG; trnR-ACG; trnR-UCC; trnS-GCU; trnS-UGA; trnT-GGU; trnT-UGC; trnV-GAC; trnV-UAC; trnW-CCA; trnY-GUA | 37 |
| Subunits of photosystem I | psaA; psbA; psaC; psaI; ycf3; ycf4 | 7 |
| Subunits of photosystem II | psbB; psbE; psbD; psbE; psbF; psbH; psbI; psbK; psbL; psbM; psbN; psbT; psbZ | 15 |
| ATP-dependent protease subunit P | cdpD | 1 |
| Large subunit of rubisco | rbcL | 1 |
| NADH dehydrogenase | ndhA; ndhB; ndhC; ndhD; ndhE; ndhF; ndhG; ndhH; ndhI; ndhK | 12 |
| Ribosomal protein (large subunit) | rpl2; rpl14; rpl16; rpl20; rpl22; rpl23; rpl33; rpl34; rpl36 | 11 |
| Small subunit of ribosomal proteins | rps2; rps3; rps4; rps7; rps8; rps11; rps12; rps14; rps15; rps16; rps18; rps19 | 14 |
| DNA-dependent RNA polymerase | rpoA; rpoB; rpoC1; rpoC2 | 4 |
| Subunits of ATP synthase | atpA; atpB; atpE; atpF; atpH; atpI | 6 |
| C-type cytochrome synthesis gene | ccsA | 1 |
| Subunits of cytochrome b/f complex | petN; petA; petL; petG; petB; petD | 6 |
| Envelop membrane protein | cemA | 1 |
| Maturase | matK | 1 |
| Hypothetical chloroplast reading frames | ycf1; ycf2 | 5 |
| Subunits of Acetyl-CoA-carboxylase | accD | 1 |
| Pseudogenes | infA; rps19; ycf1; ycf15 | 4 |
| Total | 114 single-copy genes, 132 in total. | |

(x2): Two gene copies in the IRs; 1 Gene containing one intron; 2: Gene containing two introns; * means the incomplete copy located in the IR of the gene straddling the IR and LSC/SSC regions.
Selective pressure analysis

The rate of synonymous substitutions and non-synonymous substitutions (Ka/Ks) of 80 protein-coding genes were calculated to assess the selection pressure between *Bupleurum* species. At the species level, by concatenating all of the 80 genes into a super-matrix, the Ka/Ks ratios ranged from 0.50 (*B. commelinoideum* vs *B. pusillum*) to 5.0 (*B. longiradiatum* vs *B. boissieuanum*), with an average ratio of 0.92 (Fig. 4). The Ka/Ks ratios between group II and group III were the highest (averaging 1.10) (Additional file 2: Table S2). The Ka/Ks ratios within group II (averaging 0.98) and group III (averaging 0.92) were higher than those between group I and group II, (averaging 0.83), between group I and group III (averaging 0.71), and within group I (averaging 0.69) (Additional file 2: Table S2).

The Ka/Ks ratios were also calculated for all the 80 protein-coding genes in the chloroplast genomes of the 21 *Bupleurum* species separately (Additional file 2: Fig. S6 and Fig. S7). Among the genes, *matK* had highest Ka/Ks ratios (around 1.0, especially in group II), following by *ycf2, accD*, and *clpP*. The remainder had Ka/Ks ratios ranged from 0 to 0.6. The mean Ka/Ks ratios of protein-coding genes in LSC (0.10) and SSC regions (0.15) were
lower than those in the IR regions (0.19). After sorting
the genes into functional categories and groups, Ka/Ks
of the photosynthetic genes (0.0412 ± 0.0683) were lower
than those of genes related to self-replication (0.2507 ±
0.2197) as well as other genes (0.2065 ± 0.1812).

Phylogenetic analysis
Results of Bayesian and ML analyses of the 21 Bur-
pleurum and 2 outgroup chloroplast genomes are pre-
sented in Fig. 5. The phylogenetic trees estimated from
the Bayesian and ML analyses showed congruence in
their topologies, high bootstrap support values (BS >
90%) and strong posterior probabilities (PP = 1) for most
of the nodes. The two phylogenetic trees highlighted
two clades (clade I and II), containing 4 (PP = 100, BS =
100%) and 17 species (PP = 100, BS = 100%), respectively.

Discussion
Differences in gene and structure of chloroplast genomes
among Bupleurum species
We for the first time described the chloroplast genomes
of the 4 Bupleurum species endemic to Southwest

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| Species            | LSC | SSC | IRa | LSC | SSC | IRa |
|--------------------|-----|-----|-----|-----|-----|-----|
| B. draccaenoides   | 85.32kb | 76.30kb | 185.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. candollei       | 86.23kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. yunnanense      | 86.22kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. shanianum       | 84.92kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. rockii          | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. commeloinoidum  | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. kwewichowense   | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. tenue           | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. marginatum      | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. triradiatum     | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. pusillum        | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. densilorum      | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. thyanschanicum  | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. boissieuanum    | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. chinense        | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. scorzeronfolium | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. yinchowense     | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. sibiricum       | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. longiradiatum   | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. latissimus      | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |
| B. falcatus        | 84.90kb | 76.30kb | 174.30kb | 214.30kb | 363.30kb | 185.30kb |

Fig. 3 Comparison of the LSC, SSC and IR junction among the 21 Bupleurum cp genomes
China, which provide important insights into the characteristics of plastid genomes of the members of this genus. Previous studies have suggested that the *Bupleurum* is a monophyletic group based on morphology and molecular (nrDNA and chloroplast fragments) evident. In present study, we provide new insight into the evolution of *Bupleurum* in term of chloroplast genomes [1, 4, 7–14]. Our results showed that the chloroplast genomes of *Bupleurum* species are extremely similar, indicating their high level of conservation especially in terms of chloroplast genome organization, tendency of SDRs and SSRs, gene content. The results support that *Bupleurum* is a monophyletic group in the aspect of chloroplast genomes as they are remarkably conserved.

The chloroplast genomes of the 21 *Bupleurum* species displayed the typical quadripartite structure, comprising a pair of IR regions which were separated by a LSC and a SSC region. The chloroplast genomes size of *Bupleurum*, ranging from 154,925 to 156,108 bp, is larger than those of the other genera in the Apiaceae family (e. g. *Angelica*, *Arracacia*, *Coriandrum*, *Glehnia*, *Heraclaeum*, *Ligusticum*, *Ostericum*, *Pastinaca*, *Pimpinella*, *Saposhnikovia*, *Semenovia*, *Seseli*, *Tetrataenium* [45–48]). The chloroplast genomes of the *Bupleurum* species showed only minor differences (~1 kb) in sizes. Previous studies suggested that the size variations of the chloroplast genomes resulted from expansion and contraction of the IR regions [20, 24, 48–50]. The IR boundary comparative analysis revealed that gene distributions at SC/IR junctions of *Bupleurum* chloroplast genomes were almost identical, and only minor differences were found in length of these genes (rps19 and ycf1) and SC/IR borders. However, the LSC/IR borders showed differences among *Bupleurum* species and some Apiaceae taxa. For instance, while the LSC/IRb junction was located within the rps19 gene in *Bupleurum* species as well as some taxa in Apiaceae (*Anthriscus*, *Daucus*, *Tiedemannia*, [48]), it resided within the rps12 gene in *Anethum* [48], *Foeniculum* [48], *Prangos* [51, 52], *Petroselinum* [48, 52]. Our repetitive sequences analysis in addition to previous studies [32–40] showed that the tendency of SDRs and SSRs were similar among *Bupleurum* species, while the certain diversity in numbers varied.

The gene/intron content and relative gene positions were highly conserved in *Bupleurum* species and almost identical to those in other members of the Apiaceae family [48, 51–56]. Two genes were found to be pseudogenes in *Bupleurum* species. The genes ycf1 and rps19, located in the IRa/SSC and LSC/IRb boundaries,
respectively, were identified as pseudogenes on account of an incomplete duplication of the normal functional copy.

**High variable regions for potential molecular markers**

Most *Bupleurum* species have important pharmaceutical values and their accurate identification is crucial to their utilization. However, the morphological similarities of the *Bupleurum* species make their authentication extremely difficult. With the development of the molecular technology, many of the chloroplast genome regions, especially the mutational hotspots (e.g. *ndhF*, *matK*, *trnS-trnG*), have been widely implemented [21, 22, 57], while none of the commonly used region in chloroplast genome were effective for identification for different plant taxa.

In a comparative analysis of the complete plastid genome of 5 *Bupleurum* species, only 8 highly variable regions (Pi > 0.01) (e.g. *ndhF*, *matK*, *trnS-trnG*), have been widely implemented [21, 22, 57], while none of the commonly used region in chloroplast genome were effective for identification for different plant taxa.

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**Do the genes of Bupleurum suffer positive selection under different habitats?**

Plants are subjected to different selection pressures due to different types of stresses in varied habitats, and genes related to a specific environment are usually assumed to be under positive selection [59]. The *Bupleurum* species growing at different latitudes and different altitudes are exposed to different light intensities and temperature, and positive selection is likely to occur among these species in different regions.

Our results indicated that *Bupleurum* species under different habitats suffer positive selection, especially in Northeast China and Northwest China. We found that some genes, including *matK*, *ycf2*, *accD*, and *clpP*, were subjected to positive selection, suggesting that adaptive changes may have occurred more frequently in response
to the highly selective conditions in different habitats. These genes have also previously been found under positive selection [60–62]. The clpP gene is essential for plant cells and encodes clpP proteases that degradants polypeptides [63]. The matK gene is one of the fastest evolutionary genes, which functions in light-regulated activities and plant development [64, 65]. The accD gene encodes the β-carboxyl transferase subunit of Acetyl-CoA carboxylase that is essential for the synthesis of products required for the extraplastidic processes needed for leaf development [66]. Gene ycf2 encodes products that are essential for cell survival [67] and chloroplast protein import [68]. These positively selected genes may contribute to the adaptation of species in Bupleurum to various environments. However, our analyses also showed strong purifying selection on most of the genes. Previous studies suggest that purifying selection acting on the genes generally leads to low synonymous and non-synonymous DNA substitution rates in chloroplast genomes of angiosperms, such as Araceae [69, 70] and Liliaceae [71, 72]. Purifying selection, one of the most prevalent mechanisms in natural selection, constantly eliminates deleterious mutations [73]. Most of the genes in these chloroplast genomes were thus subjected to extensive purifying selection to retain conserved functions in Bupleurum. The distribution of Ka/Ks indicated that most of the genes in the SSC region have experienced higher selection pressures than those in other chloroplast genome regions, whereas the IR region is more conserved. In addition, the genes involved in photosynthesis tend to have lower rates of evolution than genes related to self-replication and other functions. These differences are likely the results of variations in generation time, gene expression level, gene function, lengths of the encode protein products, and relaxed selection [74–77].

Phylogenetic inference from plastid genomes of the genus Bupleurum in East Asia

Currently there is no widely acceptable infrageneric classification system of Bupleurum [78–80]. East Asian, especially China, is one of the diversity center for the genus Bupleurum. Bupleurum species in these regions are diverse, exhibiting various life forms (including herbs and shrubs) and pollen types (e. g. subrhomboid, sub-spheroid and subellipsoid) [40, 81]. However, there are gaps in phylogenetic relationships of East Asian Bupleurum, only a few efforts have been made based on morphology [81] and several DNA fragments [4, 13, 14]. Shu et al. proposed to divide Chinese Bupleurum into monotypic subgenus Longifolia (Wolff) Yuan and subgenus Eubupleura (including section Falcata and section Ranunculoidea) [81], while Wang et al. [4] did not support this treatment and proposed to divide the genus Bupleurum into two clades corresponding to the two subgenera (subg. Penninervia, subg. Bupleurum) of Neves and Watson [1]. The 21 Bupleurum species here have various life forms and widely distribute throughout East Asia (Southwest China; Northeast China, Korea and Japan), providing an opportunity to study the phylogenomic relationship in East Asian Bupleurum species. Based on the results of phylogenetic analysis, we propose to divide the 21 Bupleurum species, which were all from Asia, into two clades (clade I and clade II). The phylogenetic relationships within Bupleurum based on chloroplast genomes we presented herein are largely congruent to that of Wang et al. [4, 13] with only a few differences. B. rockii occurred in clade I in our study, but in another clade by Wang et al. [3, 13]. The two clades are likely to correspond to their geographical distribution, not the characteristics of bracteoles. The clade I consists of 4 species from Southwest China, with two endemic species to Southwest China (B. shanianum and B. yunnanense) and two species (B. marginatum and B. candollei) with extended distribution in such Himalayan countries as Bhutan, India, Kashmir, Myanmar, Nepal, Pakistan and Sikkim. The species in the clade II, except for B. dracaenoides, B. rockii and B. kweichowense, occur at higher latitudes of China such as Northwest and North China, Japan and Korea. Our results do not fully support Shu et al.’s treatment on Bupleurum [81], which divided the subg. Eubupleura into sect. Ranunculoidea and sect. Falcata based on the difference of bracteoles characteristic. However, the subgenus subdivision of the genus Bupleurum remains unresolved in this present study because of the lack of chloroplast genome data from subg. Penninervia, most species of which are distributed in the Mediterranean region. To access a complete reassessment of the interspecific relationship of Bupleurum, more complete plastid genomes information is required, especially the data of subg. Penninervia.

Conclusion

In this study, we for the first time described 7 full chloroplast genomes of 4 Bupleurum species endemic to Southwestern China. Comparative analysis of chloroplast genomes of the 4 species against the published data of 17 Bupleurum species revealed that the chloroplast genomes of Bupleurum species were extremely conserved with similarities in terms of chloroplast genome organization, tendency of SDRs and SSRs, and gene content. In spite of the highly conservation of the chloroplast genomes of the Bupleurum species, some mutational hotspots have been detected, which can be potentially used as high-resolution DNA barcodes in discrimination of Bupleurum taxa. Bupleurum species under different habitats suffer positive selection, and some genes (matK, ycf2, accD, and clpP) are also
subjected to positive selection. Phylogenetic analysis revealed that the 21 *Bupleurum* formed two clades, which are likely to correspond to their geographical distribution. The chloroplast genomes information reported herein and the comparative analysis of *Bupleurum* chloroplast genomes provide important insights into the evolution of the chloroplast genomes and phylogeny of *Bupleurum*.

**Methods**

**Sample collection**

Leaf from a total of 7 individuals of the 4 *Bupleurum* species (two individuals for each of *B. shanianum*, *B. yunnanense*, *B. rockii*, while one for *B. kweichowense*) were collected from the wild and were dried with silica gel, and stored at −80 °C until required for DNA extraction. Voucher specimens were collected for each samples and deposited at the herbarium of Southern Medical University. All species were authenticated by prof. Zhi Chao (Southern Medical University). Details information of the samples and voucher numbers of the specimens were shown in Additional file 2: Table S3.

**DNA extraction, library construction and sequencing**

Total DNA was extracted from silica-dried leaf material using a modified extraction method described by Yang et al. [82]. The quality and concentration of the extracted DNA were detected by 1.0% agarose gel electrophoresis and by a NanoDrop 2000C spectrophotometry (Thermo Fisher, US). The extraction genomic DNA (approximately 1 μg) was subjected to random degradation by Covaris (E210), and then fragments with a size of 200–400 bp were selected by using Agencourt AMPure XP-Medium kit. The selected fragments were amplified after suffering from end repair, 3′-adenylation and adaptor ligation. Then, they were heat denatured to single strand after purification. The single strands were circularized, and single strand circle DNA was obtained as the final library. The final library was sequenced by BGISEQ-500 (BGI, Shenzhen, China) to generate raw reads. The details of the quantity and quality of raw reads, and coverage depth of the assembled genomes were provided in Additional file 2: Table S4.

**Genome assembly and annotation**

The generated raw sequencing data was filtered using program SOAPnuker [83] with default parameters to remove adapters, low-quality reads with quality value ≤10, to final obtain high-quality reads. Then, the high-quality reads were aligned to the published *B. commelinoideum* chloroplast genome (NCBI accession MT162552) using Geneious v 10.2.2 [84] with default settings. Subsequently, the matched reads were selected for de novo assembled with SPAdes v3.11.1 [85]. The accuracy of assembly was evaluated by detecting the sequence coverage and the reading segment coverage at the contig connection.

The assembled chloroplast genome annotations were annotated using GeSeq [86] with the reference chloroplast genome of *B. commelinoideum* (NCBI accession MT162552). All the tRNAs were scanned with tRNAscan-SE [87] and ARAGORN [88]. The visual presentations of the physical circular maps of the genomes were generated using OGDRAW [89]. Finally, the annotated chloroplast genomes of the 4 *Bupleurum* species were submitted to the National Center for Biotechnology Information database (NCBI) under the accession numbers MW135450-MW135456, which were listed in Additional file 2: Table S3.

**Genome structure and comparative analysis**

First, the chloroplast genome characteristics of the 4 *Bupleurum* species were described. Therein, Geneious R8.1 [90] was employed to conduct the GC content. MISA-web (https://webblast.ipk-gatersleben.de/misa/) was implemented to search simple sequence repeats (SSRs) with minimum numbers of 10, 5, 4, 3, 3, 3 repeat units for mono-, di-, tri- tetra-, penta-, and hexa-nucleotide SSRs, respectively. Short dispersed repeats (SDRs) analysis was implemented in REPtuer [91] with the following parameters: a minimal repeat size of 30 bp, and sequence identity ≥90% (hamming distance of 3 kb). To determine the IR expansion/contraction, genes distributed in and beside the borders of LSC, SSC and IR regions were compared.

In order to examine the divergence hotspots among the *Bupleurum* species, the whole chloroplast sequences of the 4 *Bupleurum* species, together with 17 published chloroplast genomes of *Bupleurum* species downloaded from NCBI database (Additional file 2: Table S5), were aligned using Geneious software. Subsequently, they were compared and visualized using mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml) with the reference chloroplast genome sequence of *B. commelinoideum* (NCBI accession MT162552). DnaSP v. 6.0 was used for sliding window analysis for computing nucleotide diversity (pi) among the chloroplast genome sequences [92], with 600 bp windows size and 200 bp step size.

Selective pressure estimation for the 21 *Bupleurum* species was carried out by calculating the ratio of the non-synonymous substitution (Ka) to the synonymous substitution rate (Ks) for all protein-coding genes sequences in DnaSP v6.

**Phylogenetic analysis**

In order to gain insight into the phylogeny of East Asian *Bupleurum*, a total of 19 available chloroplast complete
logenomes of 17 *Bupleurum* species were downloaded from the NCBI database (Additional file 2: Table S4). The chloroplast data of two *Chamaesium* species (*C. paradoxum*, NCBI accession MK780227; *C. spathuliferum*, NCBI accession MN119371), belonging to subfamily Apioideae, were also downloaded and set as outgroups (Additional file 2: Table S4). Only a dataset of 80 protein-coding genes (PCGs) was used for the phylogenetic analyses. A 23-taxon sequence matrix including two outgroups were aligned using the Geneious software.

Phylogeny was conducted through two approaches, namely the Maximum likelihood (ML) analyses and a Bayesian inference (BI) analyses. ML phylogenetic analysis was performed in RAxML v8.2.4 [93]. First, the best likelihood tree was obtained from 100 starting trees using rapid bootstrap analyses with 1000 replicates. Multiparametric bootstrapping analyses with 1000 replicates was conduct to obtained the bootstrap for each node. Substitution model for the two analyses were GTR+GAMMA model. Bayesian inference (BI) was conducted in MrBayes v3.2.6 [94, 95]. The best-fit nucleotide substitution model (GTR + I + GAMMA) for Bayesian analysis was inferred from jModelTest v. 2.1.10 under the Akaike information criteria (AIC) [96]. Markov chain Monte Carlo (MCMC) analysis was performed with 50 million generations and sampling trees every 5000 generations. The first 10% of trees were discarded as burn fraction, and the remaining trees were combined to synthesized the consistent tree and estimate posterior probabilities. The resulting trees were rooted with *C. paradoxum* and *C. spatuliferum* and visualized with FigTree v 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

**Abbreviations**
ITS: Internal transcribed spacer region; rDNA: Nuclear ribosomal DNA; NGS: Next-generation sequencing; Cp: Chloroplast; LSC: Large single copy; SSC: Small single copy; IR: Inverted repeat; tRNA: Transfer RNA; rRNA: Ribosomal RNA; SSR: Simple sequence repeat; SDR: Short dispersed repeat; Met: Methionine; Trp: Tryptophan; Arg: Arginine; Leu: Leucine; Ser: Serine; RSCU: Synonymous codon usage; GC3: GC content on the third codon position; Ks: Rate of synonymous substitutions; Ka: Rate of non-synonymous substitutions; Pi: Polymorphic information; synonymous codon position; ML: Maximum likelihood; BS: Bootstrap support value; PP: Posterior probability; BI: Bayesian inference; AIC: Akaike information criteria; MCMC: Markov chain Monte Carlo.

**Supplementary Information**
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Additional file 1. Additional file 2.

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**Authors’ contributions**
ZC conceived the study and provided the funding, reviewed and revised the drafts of the paper. RH analyzed data, and wrote and revised the manuscript. XNX performed the genome assembly and annotation, and assisted with the molecular experiments and data analysis. AMC conducted the molecular experiments, performed the genome assembly and annotation. FL assisted with the molecular experiments and the genome assembly. EWT collected field samples and assisted with the molecular experiments. All authors read and approved the final manuscript.

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**Availability of data and materials**
Sequence information of the 7 chloroplast genomes of the four *Bupleurum* species (*B. shanianum, B. yunnanense, B. kweichowense* and *B. rockii*) is available in the National Center for Biotechnology Information database (NCBI) under the accession number MW135450-MW135456 (Additional file 2: Table S3). The accession numbers corresponding to the additional datasets used and analysed in this study can be found in Additional file 2: Table S5. These additional data were retrieved from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/).

**Declarations**

**Ethics approval and consent to participate**
No specific permissions were required for the collection of plant material that was conducted in this study. Our field works and molecular experiments were carried out in compliance with the relevant laws of China.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

**Author details**
1Department of Pharmacy, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, China. 2Faculty of Medicinal Plants and Pharmacognosy, School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, China. 3Guangdong Provincial Key Laboratory of Chinese Medicine Pharmaceutics, Guangzhou 510515, China.

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