Molecular structure and phylogenetic analyses of the complete chloroplast genomes of three original species of Pyrrosiae Folium

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[ABSTRACT] Pyrrosia petiolosa, Pyrrosia lingua and Pyrrosia sheareri are recorded as original plants of Pyrrosiae Folium (PF) and commonly used as Chinese herbal medicines. Due to the similar morphological features of PF and its adulterants, common DNA barcodes cannot accurately distinguish PF species. Knowledge of the chloroplast (cp) genome is widely used in species identification, molecular marker and phylogenetic analyses. Herein, we determined the complete cp genomes of three original species of PF via high-throughput sequencing technologies. The three cp genomes exhibited a typical quadripartite structure with sizes ranging from 158 165 to 163 026 bp. The cp genomes of P. petiolosa and P. lingua encoded 130 genes, whilst that of P. sheareri encoded 131 genes. The complete cp genomes were compared, and five highly divergent regions of petA-psbJ, matK-trnM, psbM-psbN and psaC-ndhE were screened as potential DNA barcodes for identification of Pyrrosia genus species. The phylogenetic tree we obtained indicated that P. petiolosa and P. lingua are clustered in a single clade and, thus, share a close relationship. This study provides invaluable information for further studies on the species identification, taxonomy and phylogeny of Pyrrosia genus species.

[KEY WORDS] Complete chloroplast genome; Pyrrosia petiolosa; Pyrrosia lingua; Pyrrosia sheareri; Identification; Phylogenetic relationship


Introduction

Pyrrosia Mirbel, belonging to the family Polypodiaceae, comprises nearly 100 species and is widely found in tropical and subtropical Asia; some Pyrrosia species have also been recorded in Africa and Oceania [1]. Thirty-seven Pyrrosia species are mainly distributed in warm areas in China, such as the Yangtze River Basin and south and southwest China [1]. Most Pyrrosia species have ornamental and medicinal value and grow on trees or rocks, although some species have been observed to grow in the soil. In China, the dried leaves of Pyrrosia sheareri (Bak.) Ching, Pyrrosia lingua (Thunb.) Farwell and Pyrrosia petiolosa (Christ) Ching are used as traditional Chinese medicine to promote haemostasis and diuresis [2]. Modern pharmacological researches have demonstrated that the crude extract and purified active substances of Pyrrosia species can relieve cough, eliminate phlegm, protect kidneys and reduce blood sugar levels [3-10]; moreover, these plants have antibacterial, antiviral, anti-inflammatory, diuretic, anti-oxidation and immunity-strengthening properties. Today, compound preparations of Pyrrosia are widely used in clinical practice [11].

Pyrrosia species have a long history of use as medicine, and their adulteration is a serious concern. Previous studies [12] revealed the availability of counterfeit products in the Chinese medicinal market, which may, at least in part, be due to the incorrect use of closely related species. Such an issue is not conducive to the promotion and safe use of these herbs as medicinal materials. Lepisorus and Microsorum species are often confused with Pyrrosia Folium in the market [13], which
can seriously affect the quality of medicinal materials and clinical efficacy. Traditional identification methods cannot easily differentiate amongst *Pyrrosia* species, especially when they are dried and lose most of their morphological characteristics. With the rapid development of molecular technology in recent years, molecular identification has made a great progress in Chinese medicine, especially DNA barcoding, which is a technique to identify species by analyzing a standard DNA sequence [14], and now it has successfully recognized the identification of medicinal plants, and poisonous medicinal plants [15]. Universal DNA barcodes, such as the *psbA-trnH* sequence, are an ideal means for identifying *P. lingua* and its adulterants but cannot distinguish between *P. petiolosa* and *P. lingua* [16]. Therefore, more scientific and accurate identification methods must be developed.

Chloroplasts (cp) are important organelles in plants and play a crucial role in sustaining life on earth [17]. As a unique gene structure of plants, the cp genome is relatively independent of the nuclear genome and has a length of 110–160 kb [18]; moreover, the genome is characterised by a low molecular weight, multiple copies, stable structure and highly conserved order. Knowledge of such genomes can offer sufficient information and variation to solve DNA barcode limitations [19]. Sequence analysis of complete cp genomes is an efficient information and variation to solve DNA barcode limitations. With the rapid development of molecular technology, cp genomes can function as a super-barcode to distinguish species of *Ligularia* [23] and *Dracaena* [24]. High-variation regions in whole cp genomes, such as those of *Ampelopsis humulifolia* and *Ampelopsis japonica*, could be screened and successfully used for species identification [25]. Thus far, over 4000 cp genome sequences have been submitted to the National Centre for Biotechnology Information (NCBI) [26]. However, studies on the phylogenetic position and species diversity of *Pyrrosia* species are limited; indeed, work in this area could be improved by focusing on the cp genomes of these species. Hence, determining the cp genome of PF is necessary to find a specific DNA-barcode suitable for identification of the *Pyrrosia* species.

In this study, the complete cp genomes of three original species of PF were sequenced, and their features were characterised. We detected long repeats and simple sequence repeats (SSRs) and comparatively analysed the complete cp genomes obtained. Furthermore, we constructed a phylogenetic tree based on complete cp genome to evaluate the phylogenetic relationships of *Pyrrosia* species even with pterido-phytes. The data acquired in this study would provide beneficial information supporting further research on the identification and phylogenetic analysis of the Polypodiaceae family, as well as the safe medical applications of *Pyrrosia* herbs.

### Materials and Methods

#### Plant materials and DNA extraction

Fresh leaves of *P. petiolosa*, *P. lingua*, and *P. sheareri* were collected from Xuancheng (Anhui Province), Guangzhou (Guangdong Province), Wuyi Mountain (Fujian Province), in China, respectively. These *Pyrrosia* species were identified by Prof. LIN Yu-Lin and WEI Xue-Ping from Guangzhou, Guangdong Province.

### Table 1  The sample locations and cp genomes characteristics of three *Pyrrosia* species

<table>
<thead>
<tr>
<th>Species</th>
<th><em>P. petiolosa</em></th>
<th><em>P. lingua</em></th>
<th><em>P. sheareri</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection location</td>
<td>Guangzhou, Guangdong Province</td>
<td>Xuancheng, Anhui Province</td>
<td>Wuyi Mountain, Fujian Province</td>
</tr>
<tr>
<td>Latitude &amp;Longitude</td>
<td>N23°18′76.15″, E113°33′46.67″</td>
<td>N30°50′06.6″, E119°14′23.5″</td>
<td>N27°48′27.97″, E117°42′23.74″</td>
</tr>
<tr>
<td>Altitudes (m)</td>
<td>42</td>
<td>73</td>
<td>1034</td>
</tr>
<tr>
<td>Gene size (bp)</td>
<td>163 026</td>
<td>160 568</td>
<td>158 165</td>
</tr>
<tr>
<td>LSC length (bp)</td>
<td>87 355</td>
<td>84 929</td>
<td>82 525</td>
</tr>
<tr>
<td>SSC length (bp)</td>
<td>21 695</td>
<td>21 665</td>
<td>21 726</td>
</tr>
<tr>
<td>IR length (bp)</td>
<td>26 988</td>
<td>26 987</td>
<td>26 957</td>
</tr>
<tr>
<td>Number of genes</td>
<td>130</td>
<td>130</td>
<td>131</td>
</tr>
<tr>
<td>Protein-coding genes</td>
<td>87</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>tRNA genes</td>
<td>35</td>
<td>34</td>
<td>35</td>
</tr>
<tr>
<td>rRNA genes</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Total GC content/%</td>
<td>41.5</td>
<td>41.5</td>
<td>41.6</td>
</tr>
<tr>
<td>GC content of LSC/%</td>
<td>40.2</td>
<td>40.1</td>
<td>40.2</td>
</tr>
<tr>
<td>GC content of SSC/%</td>
<td>36.8</td>
<td>37.1</td>
<td>37.2</td>
</tr>
<tr>
<td>GC content of IR/%</td>
<td>45.6</td>
<td>45.5</td>
<td>45.6</td>
</tr>
</tbody>
</table>
the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). The collection location, latitude, longitude and altitudes of three *Pyrrosia* species are listed in Table 1. The voucher species were deposited in the herbarium of IMPLAD. Total genomic DNA was extracted using a DNeasy Plant Mini Kit following the standard protocol (Qiagen Co., Hilden, Germany). DNA quality and concentration were measured by spectrophotometry and electrophoresis in 1% (W/V) agarose gel.

**DNA sequencing and genome assembly**

The high-quality DNA were used to build shotgun libraries with insert sizes of 500 bp and sequenced using the Illumina HiSeq X. Approximately 5.9 Gb of raw data from *P. petiolosa*, 5.9 Gb from *P. lingua*, and 6.6 Gb from *P. sheareri*. Firstly, the low-quality reads were filtered from the raw reads using Trimmomatic (V0.36, Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany) [27]. The cp sequences of all plants downloaded from the NCBI constructed the reference database. Subsequently, the clean sequences were mapped to the database, and the mapped reads were extracted on the basis of coverage and similarity. The extracted reads were assembled into contigs with the software SOAPdenovo (V2, BGI HK Research Institute, Hong Kong, China) [28]. The scaffold of the cp genome was constructed via SSPACE [29]. Finally, the local gaps were filled by GapFiller [30]. The tRNAs were confirmed with tRNAscan-SE (V2.0, University of California, Santa Cruz, CA, USA) [31]. Circular maps of the three *Pyrrosia* cp genomes were obtained using OGDRAW (V1.2, Max Planck Institute of Molecular Plant Physiology, Potsdam, Germany) [32]. Four boundaries of single copy (SCs) and inverted repeats (IRs) regions of completed cp genomes were validated using PCR-based sequencing and the primers were listed in Table S1. The assembled complete cp genome sequences of the three species were submitted to NCBI with the accession numbers MN885667 (*P. lingua*), MN885668 (*P. sheareri*), and MN885669 (*P. petiolosa*).

**Genome structure analysis**

The software of MEGA (V6.0, Tokyo Metropolitan University, Tokyo, Japan) [33] was used to calculate GC content and relative synonymous codon usages (RSCU). SSRs were detected by the microsatellite search tool MISA [34] with the parameters set in common with those described in Li et al. [35]. We utilized REPuter (University of Bielefeld, Bielefeld, Germany) [36] to identify the size and location of repeat sequences, including forward, palindromic, reverse, and complementary repeats. For all repeat types, the minimal size was 30 bp and the two repeat copies had at least 90% similarity.

**Genome comparison and phylogenetic analysis**

The mVISTA program [37] was applied to compare the cp genomes of PF species and the two published *Pyrrosia* species with *P. lingua* as a reference. A total of 20 cp genomes, including 7 from Polypodiaceae, were used for phylogenetic analyses, along with *Alsophila spinulosa* as an outgroup. Of these, 17 cp genome sequences were downloaded from the NCBI GenBank (Table S2). The sequences were aligned using MAFFT [38] and were manually adjusted. Phylogenetic trees were constructed based on the complete chloroplast genome by Maximum Likelihood (ML) methods with a bootstrap of 1000 repetitions. Modeltest 3.7 [39] was used to determine the best-fitting model for each dataset. Maximum parsimony (MP) analysis was performed with PAUP × 4.0 b10 [40]. Bootstrap analysis was performed with 1000 replicates. Bayesian inference was performed using the program MrBayes v3.1.2 [41]. Markov chain Monte Carlo simulations were independently run twice for 2 million generations, and sampling trees every 100 generations.

**Results and Discussion**

**Characteristics of chloroplast genomes of three *Pyrrosia* species**

Approximately 40 000 000, 40 000 000, and 44 378 182 raw reads of *P. petiolosa*, *P. lingua*, and *P. sheareri* derived from NGS, respectively. The respective cp genomes of *P. petiolosa*, *P. lingua* and *P. sheareri* were 163 026, 160 568 and 158 165 bp in length. These genomes displayed a typical quadripartite structure containing a large single-copy (LSC) region, and a small single-copy (SSC) region separated by two inverted repeat (IR) regions (Fig. 1). The four regions from the three species had similar lengths. LSC lengths ranged from 82 525 to 87 355 bp, whilst IR lengths ranged from 26 957 to 26 988 bp. SSC lengths ranged from 21 665 to 21 726 bp are, thus, larger than those of the pteridophyte *Psilotum nudum* (16 304 bp) [42]. The overall GC contents of *P. petiolosa*, *P. lingua* and *P. sheareri* were 41.5%, 41.5% and 41.6%, respectively. As shown in Table 1, the GC contents of LSC (40.2%, 40.1% and 40.2% for *P. petiolosa*, *P. lingua* and *P. sheareri*, respectively) and SSC (36.8%, 37.1% and 37.3% for *P. petiolosa*, *P. lingua* and *P. sheareri*, respectively) regions were lower than those of IR regions (45.6%, 45.5% and 45.6% for *P. petiolosa*, *P. lingua* and *P. sheareri*, respectively). The sizes of these four regions and the GC contents of the three *Pyrrosia* species are similar to those of other *Pyrrosia* species, such as *P. bonii* [43] and *P. calvata* [44].

The cp genomes of *P. petiolosa* and *P. lingua* encoded 130 genes, including 86 protein-coding genes, 35 tRNA genes, 8 rRNA genes, and one pseudogene (rps16) in *P. petiolosa* and 88 protein-coding genes, 34 tRNA genes and 8 rRNA genes in *P. lingua*. A total of 131 genes were annotated in the *P. sheareri* cp genome, including 88 protein-coding genes, 35 tRNA genes, and 8 rRNA genes (Table 2). The number of genes contained in *Pyrrosia* is comparable with that in giant tree ferns, e.g. *Alsophila gigantea* (133 genes) [45] and *Alsophila costularis* (133 genes) [46]. The analysis results showed that *trnV-UCAC gene* deletion was found in the cp genome of *P. lingua*, and a pseudogene *rps16* was observed in the *P. petiolosa* cp genome, while neither gene deletion nor pseudogene existed in the *P. sheareri*. Amongst the genes
found, fourteen genes, including six tRNAs, four rRNAs and four protein-coding genes, presented as duplicates. Fifteen genes ([atpF], [ndhA], [ndhB], [petB], [petD], [rpl16], [rpoC1], [rpl2], [rps12], [trnG-UCC], [trnV-UCG], [trnA-UGC], [trnL-UA4], and [trnT-UGU]) contained one intron, whilst two genes ([ycf3] and [clpP]) possessed two introns (Table S3/S4/S5). In addition, fifteen genes were duplicated in two IR regions, including five PCGs ([ndhB], [rps12], [psbA] and [ycf2]), six tRNA genes ([trnN-GUU], [trnH-GUG], [trnL-GAU], [trnA-UGC], [trnT-UGU] and [trnR-ACG]) and four rRNA genes ([rrn4.5], [rrn5], [rrn16] and [rrn23]), similar to the results of *P. calvata* [44] and *Leptochilus hemionitidoides* [47].

**Codon usage analysis**

Relative synonymous codon usage (RSCU), a measure of the preference for the use of a synonymous codon, is defined as the ratio of the observed number of synonymous codons used to the expected value of the codon occurrence frequency [48]. Analysing codon usage is essential to evaluate the evolution of the cp genome. All of the protein-coding genes of *P. petiolosa*, *P. lingua* and *P. sheareri* were composed of 53 522, 54 342 and 52 721 codons, respectively (Table S6). The contents of 20 amino acid and stop codons in all protein-coding genes of the cp genomes of PF are shown in Fig. 2. Codons for leucine, serine and arginine were the most abundant (n = 6), whilst those for methionine and tryptophan numbered the least (n = 1). Whereas most of the amino acid codons showed some preference for certain codons, methionine and tryptophan appeared to have no such bias (RSCU = 1.00). The amino acid codons in the cp genome of *Pyrossia* preferentially ended with A or U, which is a crucial factor in genome organization and stability [49]. This codon usage pattern occurs in the majority of plant cp genomes, such as those of *Aristolochia contorta* [50], *Zingiber officinale* [51] and *Taxillus* [52] species.

**SSRs and long repeats analysis**

SSRs were made up of 1–6 nucleotide repeat units and also called microsatellites [53]. SSRs represent potentially useful molecular markers and have been widely used in species identification, phylogenetic investigations and population genetics owing to their high levels of polymorphisms [54-60]. Five types of SSRs with mononucleotide, dinucleotide, trinucleotide, tetranucleotide and pentanucleotide repeats were found in the cp genomes of the three *Pyrossia* species, and these were mainly distributed in the non-coding segments of
A total of 67, 64, and 58 SSRs were detected in the cp genomes of *P. lingua*, *P. petiolosa* and *P. sheareri*, respectively (Table S7). Of these SSRs, mononucleotide repeats were the most abundant, and pentanucleotide repeats numbered the least. Analysis of the three original species of PF cp genomes revealed that most mononucleotides and dinucleotides were composed of A and T, similar to the A/T richness of the gymnosperm *Pinus taeda* [57]. However, more C/G mononucleotide repeats (18, 56.2%) than A/T mononucleotide repeats (14, 43.8%) were found in the *P. sheareri* cp genome. The repeats identified could provide valuable resources for species identification and population studies of *Pyrrosia*.

Structures longer than 30 bp are known as long repeats, which are mostly distributed in the intergenic spacer (IGS) and intron regions, play a significant role in genomic structur-

### Table 2 Gene contents in the cp genomes of three *Pyrrosia* species

<table>
<thead>
<tr>
<th>No.</th>
<th>Group of Genes</th>
<th>Gene names</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Photosystem I</td>
<td>psaA, psaB, psaC, psaI, psaJ</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Cytochrome b/f complex</td>
<td>petA, petB', petD', petG, petL, petN</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>ATP synthase</td>
<td>atpA, atpB, atpE, atpF', atpH, atpI</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>Rubisco large subunit</td>
<td>rbcL</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>RNA polymerase</td>
<td>rpoA, rpoB, rpoC1', rpoC2</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>Ribosomal proteins (SSU)</td>
<td>rps2, rps3, rps4, rps7 (× 2), rps8, rps11, rps12' (× 2), rps14, rps15, rps16', rps18, rps19</td>
<td>14</td>
</tr>
<tr>
<td>9</td>
<td>Ribosomal proteins (LSU)</td>
<td>rpl2', rpl14, rpl16, rpl20, rpl21, rpl22, rpl23, rpl32, rpl33, rpl36</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>Proteins of unknown function</td>
<td>ycf1, ycf2 (× 2), ycf3'*, ycf4</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>Transfer RNAs</td>
<td>35 tRNAs (6 contain an intron, 6 in the IRs)</td>
<td>35</td>
</tr>
<tr>
<td>12</td>
<td>Ribosomal RNAs</td>
<td>rnr4.5 (× 2), rnr5 (× 2), rnr16 (× 2), rnr23 (× 2)</td>
<td>8</td>
</tr>
<tr>
<td>13</td>
<td>Other genes</td>
<td>accD, clpP', matK, ccsA, cemA, infA, chlB, chlN, chlL</td>
<td>9</td>
</tr>
</tbody>
</table>

*Gene contains one intron; **the gene contains two introns; (× 2) indicates the number of the repeat unit is 2

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*Fig. 2 Codon content of 20 amino acid and stop codons in all protein-coding genes of the cp genomes of three *Pyrrosia* species. The order of every three columns is *P. petiolosa*, *P. lingua*, and *P. sheareri*, respectively*
al variations, expansions, and rearrangements in the cp genome [58]. Four types of long repeats, namely, forward (F), palindromic (P), reverse (R) and complementary (C), were observed in the cp genomes of the three Pyrrosia species (Fig. 3). Long repeats observed were mainly of the F and P types and ranged from 30 to 50 bp in length. The cp genome of P. lingua possessed 21 F, 30 P, 7 R and no C repeats. By comparison, P. petiolosa contained 14 F, 29 P, 7 R and 7 C repeats. Furthermore, the P. sheareri cp genome revealed 15 F, 25 P, 4 R and 3 C repeats.

**Comparative genomic analysis**

The complete cp genomes of P. petiolosa, P. lingua and P. sheareri were compared with that of P. bonii (NC_040226) and P. calvata (NC_047436) using mVISTA (Fig. 4). The results showed the variability of IR regions is less than that of LSC and SSC regions. Overall, the cp genomes showed more variation in their non-coding regions than coding regions. These variations were observed, for example, in the rpoC2 and ycf1 genes and intergenic regions, such as matK-rps16, psbM-petN, ndhC-trnM and psaC-ndhE. In particular, P. sheareri, P. bonii, and P. calvata showed great variations of petA-psbJ in intergenic regions, which was not observed in the two other species. These highly variable regions could be used to develop potential DNA barcodes for identifying Pyrrosia. Four rRNA genes (rrn4.5, rrn5, rrn16, and rrn23) were identified, and the Y-scale represents the percent identity ranging from 50% to 100%.
and \textit{rrn23}) were greatly conserved and showed nearly no difference amongst the five \textit{Pyrrosia} species. The greater conservation of gene-coding regions compared with non-coding regions is consistent with the pattern found in most angiosperms. Seven highly divergent regions were found in mVISTA, including two protein coding genes, namely \textit{rpoC2} and \textit{ycf1}, and five gene spacer regions (\textit{matK-rps16, psbM-petN, ndhC-trnM, petA-psbJ, and psaC-ndhE}), which could be used as potential DNA barcodes for identifying \textit{Pyrrosia} species.

**Phylogenetic analysis**

Observation of the taxonomic and phylogenetic relationships of \textit{Pyrrosia} based on morphology is extremely difficult due to the high similarity of morphological characteristics of species in this genus \cite{59}. In addition, the monophyletic and systematic position of \textit{Pyrrosia} in subfamily Platycerioideae has generated some controversy \cite{60}. The cp genome sequences observed could provide essential data with which to further elucidate and understand phylogenetic relationships amongst \textit{Pyrrosia} species. In this study, 17 complete cp genome sequences were obtained from GenBank, and subjected to construct maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) trees with \textit{Alsophila spinulosa} as an outgroup. The three phylogenetic analyses (MP, ML, and BI) revealed congruent topologies based on the complete cp genomes, and all of the nodes in the phylogenetic trees have high bootstrap support values (Fig. 5).

Polyophyllaceae

Oleandraceae

Tectariaceae

Dryopteridaceae

Hypodematiaceae

Onocleaceae

Athyriaceae

Cyatheaceae (outgroup)

**Fig. 5** Phylogenetic trees constructed by the complete chloroplast genome of 20 species using maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI) methods. Numbers on branches are support values (BS\textsubscript{ML}/BS\textsubscript{MP}/PP\textsubscript{BI})
Conclusions

This study determined the cp genomes of three Pyrrosia species and revealed the basic structures, conservation and variability of these sequences. The three original species of PF typically possessed cp genomes with a quadripartite structure and relatively well-conserved gene content and arrangement. Cp genomes hold substantial information that can be tapped to improve the resolution of phylogenetic relationships at all taxonomic levels [61]. Highly divergent regions were screened, and the sequences obtained could be treated as potential markers to differentiate P. petiolosa, P. lingua and P. shearerii. The ML tree indicated that complete cp genomes of P. petiolosa and P. lingua. Highly divergent regions at all taxonomic levels. Cp genomes hold substantial information that can be used to identify Pyrrosia species. The acquisition of Pyrrosia cp genomes could help reduce the misuse of medicinal materials and lay the foundation for further research on the identification of species and determination of evolutionary-terms within Polypodiaceae.

Supplementary Materials

All the supporting information of this paper can be requested by sending E-mails to the corresponding authors.

References


