Comparative Chloroplast Genome Analyses of Ilex (Aquifoliaceae): Insights Into Evolutionary Dynamics and Phylogenetic Relationships

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Abstract

Background

Despite many species of *Ilex* (Aquifoliaceae) are of horticultural importance and are widely grown in parks and gardens throughout the world for their foliage and decorative berries, limited genetic information has greatly hampered our understanding of the chloroplast genome evolution and phylogenetic relationships within the genus. This study attempted to address these problems by comparing the chloroplast genomes and analyzing phylogenetic relationships within the genus.

Results

In this study, analyses of chloroplast genome structure, codon usage, GC content, gene rearrangement, nucleotide diversity, inverted repeats (IR) boundary, repeat sequence, and SSR component were conducted by comparing 41 chloroplast genomes of *Ilex*. The results showed that these *Ilex* chloroplast genomes were evolutionary conserved at the genome level and no rearrangement of the complete cp genome in the 41 *Ilex* genomes was recorded. On the contrary, there were still a few mutational hotspots identified from these chloroplast genomes, which were considered as hypervariable regions useful for future phylogenetic studies and DNA barcoding. Using the complete chloroplast genome sequences, we reconstructed a highly supported phylogeny of *Ilex* and well-resolved the complicated relationships among the different lineages within *Ilex*.

Conclusion

The present study increased our understanding of the chloroplast genome evolution and phylogenetic relationships within *Ilex*. The availability of these genetic resources will be helpful for the future studies in DNA barcoding, species delimitation, phylogenetic reconstruction as well as the hybridization and introgression events between distantly related lineages within *Ilex*.

Introduction

Ilex L., comprises of ca. 600 evergreen or deciduous tree and shrub species, is the only genus in the family Aquifoliaceae [1]. Members of the genus are mostly distributed in the tropics, but also extend into some temperate regions, making its center of species diversity to be located at the tropical America and the south-east Asia regions [2, 3]. Most species of *Ilex* such as, *I. cornuta* Lindl. et Paxt., *I. purpurea* Hassk., *I. paraguariensis* A. St.-Hil., and *I. rotunda* Thunb., contain important economic values [4–6], while many of them contain high endemism and are limited to certain geographic distributions. To date, as much as 250 species of *Ilex* have been classified as endangered species based on the International Union for Conservation of Nature (IUCN) red list [7].

In the past two decades, the advances in sequencing technology and analytical methods have contributed to the phylogenetic reconstruction of the genus *Ilex* using several plastid DNA fragments, including *rbcl*, *trnL-trnF*, and *atpB-rbcl* as well as the nuclear ribosomal DNA internal transcribed spacers (nrITS) and chloroplast glutamine synthetase gene (*nepGS*) sequences [8–14]. Although a large taxon sampling of *Ilex* has been achieved, the phylogeny of *Ilex* is still not completely resolved [11, 14]. Substantial incongruence between the nuclear phylogeny and the plastid phylogeny of the genus *Ilex* has been found in recent phylogeny studies, but only a few plastid or nuclear gene fragments were used in these studies and the resolution was generally poor due to their relatively conserved features in some plastid genes [8, 11–13]. Furthermore, the findings obtained from molecular phylogenetic analysis did not complement to those previously proposed traditional classification of *Ilex* based on morphological features [15, 16]. At present, the phylogenetic relationships among lineages in genus *Ilex* remain uncertain, thus, further investigations are requisite to decipher the status and evolution pattern of this genus.

In addition to the commonly used short plastid gene sequences that have been useful in analyzing the phylogenetic relationship in most land plants, the complete chloroplast genome sequences have been tested to be more promising in resolving the relationships of many genera at different taxonomic levels [17–19]. In general, the chloroplast genome is relatively stable, and usually contains four extremely evolutionarily conserved parts: a pair of inverted repeat regions (IRa and IRb), a large single-copy region (LSC), and a small single-copy region (SSC) in most land plants [20]. At the same time, it contains a large amount of effective information with a suitable mutation rate that are sufficient for phylogeny reconstruction and species delimitation [21].

To date, the complete chloroplast genome sequences of a total of 34 *Ilex* species have been made available so far in the NCBI GenBank database (accessed on 1 August 2021). Yet, this number only accounts for ca. 5.7% of the total number of species recorded in the genus. Despite the limited number of the complete chloroplast genome reported, phylogenetic analysis of *Ilex* based on the complete chloroplast genome sequence could still provide some insights to the relationship between species of the genus. In the effort to expand the current genetic resource of *Ilex* at chloroplast genome-scale, herein, we newly sequenced the chloroplast genomes of seven species of *Ilex*, including *I. dasyphylla*, *I. fukienensis*, *I. lohfauiensis*, *I. venusta*, *I. viridis*, *I. yunnanensis*, and *I. zhejiangensis*. Three of them, *Ilex fukienensis*, *I. venusta*, and *I. zhejiangensis*, were known to have a very narrow distribution in China [13, 22], while the other four species are widely distributed in China and adjacent regions. We aimed: (i) to investigate the structural and compositional variations of the chloroplast genomes in genus *Ilex* by involving more lineages; (ii) to identify highly variable regions in the chloroplast genomes that could be used as potential markers in resolving the interspecific relationship and species delimitation of *Ilex*; and (iii) to reconstruct a high resolution phylogenetic tree based on the complete chloroplast genome sequences that is further tested for its cyto-nuclear discordance by comparing to existing nuclear-based phylogenetic tree of the genus *Ilex*.

Results

Chloroplast genome structure of *Ilex* species

All the seven newly sequenced chloroplast genomes obtained through this study possess a typical quadripartite structure of a vascular plant, which consisted of two single-copy regions (LSC and SSC) that are separated by a pair of inverted repeats (IRa and IRb) (Figure 1). By taking account of the 34 chloroplast genomes downloaded from the GenBank, the length of the chloroplast genome of *Ilex* ranged from 157,119 bp (*I. graciliflora*) to 158,020 bp (*I. kwangtungensis*). The length of the LSC region was ranged from 86,506 bp (*I. graciliflora*) to 87,414 bp (*I. crenata*), the SSC regions was ranged from 18,228 bp (*I. yunnanensis*) to 18,447 bp (*I. lohfauiensis*), and the IR regions ranged from 26,005 bp (*I. vomitoria*) to 26,121 bp (*I. rotunda*) (Table 1). The GC content was from 37.60–37.69% (Table 1). All the chloroplast genomes obtained in this study contained 116 genes, including 82 protein-coding, 31 tRNA, and four rRNA genes, except for *I. dasyphylla*, which had 83 protein-coding genes (Table 2 and 3). All chloroplast genomes had the same gene order and gene arrangement.

Table 1

Summary of complete chloroplast genomes of *Ilex* species included in the present study. PCG indicates protein-coding gene.

Taxon	Accession number	Gene number			Length (bp)					GC(%)	AT(%)
		PCG	tRNA	rRNA	Full	Plastome	LSC	IRA/IRB	SSC		
<i>Ilex asprella</i>	NC_045274	94	37	8	139	157,856	87,265	26,075	18,441	37.62	62.38
<i>I. asprella</i> var. <i>tapuensis</i>	MT767004	87	37	8	132	157,671	87,161	26,065	18,380	37.65	62.35
<i>I. championii</i>	MT764248	87	37	8	132	157,468	86,878	26,074	18,442	37.64	62.36
<i>I. chapaensis</i>	MT764251	87	37	8	132	157,665	87,155	26,065	18,380	37.65	62.35
<i>I. cinerea</i>	MT764247	87	37	8	132	157,215	86,601	26,094	18,426	37.69	62.31
<i>I. cornuta</i>	MT764252	87	37	8	132	157,216	86,607	26,091	18,427	37.69	62.31
<i>I. crenata</i>	MW528027	88	38	8	134	157,988	87,414	26,076	18,422	37.65	62.35
<i>I. dabieshanensis</i>	MT435529	90	37	8	135	157,218	86,723	26,034	18,427	37.69	62.31
<i>I. dasyphylla</i>	This study	92	40	8	140	158,009	87,388	26,105	18,411	37.62	62.38
<i>I. dasyphylla</i>	MT764253	87	37	8	132	158,009	87,388	26,105	18,411	37.62	62.38
<i>I. delavayi</i>	KX426470	95	40	8	143	157,671	87,077	26,078	18,438	37.65	62.35
<i>I. dumosa</i>	KP016927	86	37	8	131	157,732	87,033	26,087	18,415	37.62	62.27
<i>I. ficoidea</i>	MT764243	87	37	8	132	157,536	86,922	26,094	18,426	37.64	62.36
<i>I. fukienensis</i>	This study	91	40	8	139	157,474	86,886	26,074	18,440	37.64	62.36
<i>I. graciliflora</i>	MT764249	87	37	8	132	157,119	86,506	26,093	18,427	37.61	62.39
<i>I. hanceana</i>	MT764246	87	37	8	132	157,478	86,889	26,074	18,441	37.63	62.37
<i>I. integra</i>	NC_044417	86	37	8	131	157,548	86,936	26,093	18,426	37.64	62.36
<i>I. intermedia</i>	MT471320	89	37	8	134	157,577	87,083	26,034	18,426	37.63	62.37
<i>I. kwangtungensis</i>	MT764241	87	37	8	132	158,020	87,400	26,104	18,412	37.62	62.38
<i>I. lancilimba</i>	MT767005	87	37	8	132	157,998	87,382	26,105	18,406	37.62	62.38
<i>I. latifolia</i>	NC_047291	95	40	8	143	157,601	87,020	26,077	18,427	37.63	62.37
<i>I. lohfauiensis</i>	This study	91	40	8	139	157,464	86,875	26,071	18,447	37.64	62.36
<i>I. lohfauiensis</i>	MT764240	87	37	8	132	157,470	86,873	26,078	18,441	37.64	62.36
<i>I. memecylifolia</i>	MT764250	87	37	8	132	157,842	87,249	26,076	18,441	37.62	62.38
<i>I. micrococca</i>	MN830251	89	37	8	134	157,782	87,200	26,074	18,434	37.64	62.36
<i>I. paraguariensis</i>	NC_031207	86	37	8	131	157,614	87,154	26,076	18,308	37.63	62.35
<i>I. polyneura</i>	KX426468	95	40	8	143	157,621	87,140	26,061, 25,980	18,434	37.60	62.40
<i>I. pubescens</i>	KX426467	95	40	8	143	157,741	87,143	26,081	18,436	37.61	62.39
<i>I. purpurea</i>	MT471318	89	37	8	134	157,885	87,289	26,104	18,388	37.62	62.38
<i>I. rotunda</i>	MW292559	88	37	8	133	157,743	87,069	26,121	18,432	37.62	62.38

Taxon	Accession number	Gene number				Length (bp)				GC(%)	AT(%)
		PCG	tRNA	rRNA	Full	Plastome	LSC	IRA/IRB	SSC		
<i>I. sp.</i>	KX426469	95	40	8	143	157,611	87,137	26,020	18,434	37.62	62.38
<i>I. suaveolens</i>	MN830249	89	37	8	134	157,857	87,255	26,102	18,398	37.65	62.35
<i>I. szechwanensis</i>	KX426466	95	40	8	143	157,822	87,281	26,053	18,435	37.65	62.35
<i>I. triflora</i>	MT764242	87	36	8	131	157,706	87,183	26,065	18,393	37.67	62.33
<i>I. venusta</i>	This study	91	40	8	139	157,860	87,290	26,079	18,412	37.66	62.34
<i>I. viridis</i>	This study	91	40	8	139	157,673	87,150	26,065	18,393	37.68	62.32
<i>I. viridis</i>	MN830250	89	37	8	134	157,701	87,177	26,065	18,394	37.67	62.33
<i>I. vomitoria</i>	MT471319	90	36	8	134	157,328	86,920	26,005	18,398	37.66	62.34
<i>I. wilsonii</i>	KX426471	95	40	8	143	157,918	87,341	26,073	18,431	37.62	62.38
<i>I. yunnanensis</i>	This study	91	40	8	139	157,833	87,389	26,108	18,228	37.65	62.35
<i>I. zhejiangensis</i>	This study	91	40	8	139	157,182	86,575	26,092	18,423	37.69	62.31

Table 2
List of annotated genes in the chloroplast genomes of the *Ilex* species.

Function of Genes	Group of Genes	Gene Name
Protein synthesis and DNA-replication	Transfer RNAs	<i>trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnI-M-CAU, trnG-GCC*, trnG-UCC, trnH-GUG, trnK-UUU*, trnL-UAA*, trnM-CAU, trnQ-UUG, trnP-GGG, trnP-UGG, trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU (× 2), trnT-UGU, trnV-UAC*, trnW-CCA, trnY-GUA, trnA-UGC* (× 2), trnI-CAU (× 2), trnI-GAU* (× 2), trnL-CAA (× 2), trnL-UAG, trnN-GUU (× 2), trnR-ACG (× 2), trnV-GAC (× 2), trnM-CAU</i>
	Ribosomal RNAs	<i>rrn4.5 (× 2), rrn5 (× 2), rrn16 (× 2), rrn23 (× 2)</i>
	Ribosomal protein large subunit	<i>rpl33, rpl20, rpl36, rpl14, rpl16, rpl22, rpl32, rpl2* (× 2), rpl23 (× 2)</i>
	Ribosomal protein small subunit	<i>rps2, rps14, rps4, rps18, rps11, rps8, rps3, rps19, rps12* (× 2), rps7 (× 2)</i>
	Subunits of RNA polymerase	<i>rpoA, rpoB, rpoC1*, rpoC2</i>
Photosynthesis	photosystem I	<i>psaA, psaB, psaC, psal, psaJ</i>
	Photosystem II	<i>psbA, psbB, psbC, psbD, psbE, psbF, psbG, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, lhba</i>
	ATP synthase	<i>atpA, atpB, atpE, atpF*, atpH, atpI</i>
	Large subunit Rubisco	<i>rbcL</i>
	Cytochrome b/f complex	<i>petA, petB*, petD, petG, petL, petN</i>
	NADH-dehydrogenase	<i>ndhA*, ndhB* (× 2), ndhC, ndhD, ndhE, ndhG, ndhH, ndhI, ndhJ, ndhK</i>
Other genes	Translation initiation factor	<i>infA</i>
	Cytochrome c biogenesis	<i>ccsA</i>
	ATP-dependent protease	<i>clpP**</i>
	Maturase	<i>matK</i>
	Inner membrane protein	<i>cemA</i>
	Acetyl-CoA carboxylase	<i>accD</i>
Genes of unknown function	Conserved hypothetical gene	<i>orf42 (× 2), orf56 (× 2), orf188, ycf3**, ycf4, ycf1, ycf2 (× 2), ycf15 (× 2), ycf68 (× 2)</i>
Note: (× 2) indicates the number of repeat units is 2; *Gene contains a single intron; **Gene contains two introns		

Table 3
Genes with introns in the chloroplast genome of *Ilex* species.

Gene	Location	Exon I(bp)	Intron I (bp)	Exon II (bp)	Intron II (bp)	Exon III (bp)
<i>rpl2</i>	IRa+IRb	393	661	435		
<i>rps12</i>	LSC+IRs	114	543	232		26
<i>clpP</i>	LSC	69	819	291	602	78
<i>atpF</i>	LSC	159	681	408		
<i>rpoC1</i>	LSC	456	756	1,635		
<i>ndhA</i>	SSC	552	1,140	540		
<i>ndhB</i>	IRA	777	679	756		
<i>petB</i>	LSC	6	745	657		
<i>trnA-UGC</i>	IRa+IRb	38	807	35		
<i>trnI-GAU</i>	IRa+IRb	42	934	35		
<i>trnL-UAA</i>	IRa+IRb	37	490	50		
<i>trnV-UAC</i>	IRa+IRb	39	579	37		
<i>trnG-GCC</i>	LSC	23	703	48		
<i>trnK-UUU</i>	LSC	37	2,562	35		
<i>ycf3</i>	LSC	126	727	228	749	153

Comparative analysis of genomic divergence and genome rearrangement

The diversity of nucleotide variability (Pi) for the 41 chloroplast genomes was between 0.000 and 0.01266, while the average Pi value was 0.00286. Based on the cutoff value of $Pi \geq 0.009$, eight highly variable regions (807 bp+*trnR*^{UCU}+384 bp, 579 bp+*psaC*+382 bp, *ycf1* (3378 bp–4798 bp), 136 bp+*trnT*^{GCU}+801 bp, *rbcL* (335 bp–1134 bp), *ndhC-trnV*^{JAC}, 1449 bp+*trnQ*^{UUG}+24 bp and *petN-psbM*) were identified, in which six of them (*rbcL*, *trnQ*, *trnR*, *trnT*, *ndhC-trnV*, and *petN-psbM*) were located at the LSC region, while two of them (*psaC* and *ycf1*) were from the SSC region (Figure 2, Additional files 1: Table S1). The Pi value of the eight hypervariable loci ranged from 0.00754 (807 bp+*trnR*^{UCU}+384 bp) to 0.00955 (*petN-psbM*) (Table 4). At least four distinct gaps were observed in the chloroplast genome alignment, in which all of them were located in the LSC region (Figure 3). The gaps were located at the intergenic spacer regions, including *cemA-ycf4*, *petA-psbJ*, *rpoB-trnC*, and *trnL-trnT*. Species that have gap at the *rpoB-trnC* region were *I. championii*, *I. fukienensis*, *I. hanceana*, and *I. lohfaensis*; while three species, *I. polyneura*, *I. pubescens*, and *I. rotunda*, had gap at the *petA-psbJ* region. Species that contained gaps at the *cemA-ycf4* region also contained gaps at the *trnL-trnT* region, which include *I. cinerea*, *I. cornuta*, *I. dabieshanensis*, *I. ficoidea*, *I. graciliflora*, *I. intermedia*, *I. latifolia*, *I. zhejiangensis*, and *Ilex* sp. However, two species, *I. delavayi*, and *I. integra* only had one of these gaps, which was the gap located at the *cemA-ycf4* region. Upon manual checking, these variations were in forms of indels, ranging from about 210 bp (*petA-psbJ*) to 379 bp (*rpoB-trnC*) in length. Genome synteny of the 41 chloroplast genomes revealed no presence of large gene rearrangement event (Figure 4). In addition, a total of 2,947 polymorphic, 1,630 singleton variable, and 1,317 parsimony-informative sites were detected in the 41 chloroplast genome sequences.

Table 4
Variable site analyses in the chloroplast genomes of *Ilex* species.

Region	Total number of sites	Polymorphic sites	Singleton variable sites	Parsimony informative sites	Nucleotide diversity
LSC	88,362	2182	1200	982	0.00384
IRa	26162	94	57	37	0.00055
SSC	18460	582	319	263	0.00498
IRb	26167	89	54	35	0.00050
Plastome	159151	2947	1630	1317	0.00286

Expansion and contraction of the IR regions

Comparative sequence analysis of the *Ilex* species showed that chloroplast genome structure and the number and sequence of genes were highly conserved. However, some structure and size variations at the IR boundaries of those chloroplast genomes in *Ilex* were detected. The length of IRs among all *Ilex* species included for analysis was relatively consistent, in which the IRs of *I. vomitoria* were the shortest (26,005 bp), while those of *I. rotunda* were the longest (26,121 bp). For a total of 41 genomes analyzed in this study, the LSC/IRa junction of 22 species were located in the *rps19* gene, with 4 to 5 bp spanning into the IRa region, indicating an expansion event of the IR in these species (Figure 5). The IRa/SSC junctions of majority species were located adjacent to genes *ycf1* and *ndhF*. Overlap with 22 to 61 bp between the *ndhF* and *ycf1* genes was detected in 26 *Ilex* species. However, in *I. dasyphylla*, *I. fukienensis*, *I. lohfauiensis*, *I. venusta*, *I. viridis*, *I. yunnanensis*, and *I. zhejiangensis*, *ndhF* and *ycf1* genes were absent from the IRa/SSC junction. In all *Ilex* chloroplast genomes, the SSC/IRb junction is located in the *ycf1* gene, and the extension in sequence length into the IRb region ranged from 1,047 bp (*I. lohfauiensis*) to 1,166 bp (*I. dumosa*) (Figure 5).

SSR Polymorphisms and Long Repeat Sequence Analysis

A total of 2,146 simple sequence repeat (SSR) loci were detected among the 41 *Ilex* chloroplast genomes, with a length variation from 10 to 168 bp (Figure 6, Additional files 2: Table S2). The mononucleotide repeats were most abundant (1,771), while tetranucleotide repeats had the least occurrence (49). In contrast, the number of di-, trinucleotide and compound repeats were 109, 79, and 138, respectively. For mononucleotide repeats, the A/T repeats occurred the most (1769), while C/G repeats were only detected from two taxa, including *I. asprella* var. *tapuensis* and *I. micrococca*. For dinucleotide repeats were represented with only the AT/TA motif; while trinucleotides and tetranucleotides contained motifs AAT/ATT, CAG/CTG, and TTC/GAA, as well as AAAG/CTTT, ATAA/TTAT, ATTT/AAAT, TATT/AATA, and TCTT/AAGA repeats, respectively. Most of the SSRs were primarily located at the LSC region (1649), followed by the IR regions (275), and the SSC region (222).

A total of 2843 large repeats were detected (Figure 7, Additional files 3: Table S3), in which *I. crenata* contained the highest number of large repeats (79), while *I. latifolia* had the least (62). All species involved in the long repeat sequence analysis had forward, palindromic, and tandem repeats. While 11 chloroplast genomes contained all four types of large repeats, 30 of them did not come with any complementary and/or reverse repeats.

Codon usage bias

From the total number of 23,092 (*I. asprella* and *I. dumosa*) to 23,137 (*I. dabieshanensis*) encoded codons analyzed in this study, the codon encoded amino acid that recorded the highest frequency was leucine (vary from 10.51–10.57% in different species), while the stop codon had the least occurrence (77 codons, 0.33% in each species) (Additional files 4: Table S4). The alanine-encoded GCU had the highest relative synonymous codon usage (RSCU) value (1.83–1.86), while both the serine-encoded AGC and alanine-encoded GCG were recorded with the lowest RSCU value (0.34–0.36) (Figure 8). Codon bias was not detected in methionine-encoded AUG or tryptophan-encoded UGG and was detected in other amino acid encoded codons (Figure 8, Additional files 4: Table S4). For stop codon, the amino acid UAA was like preferred when compared to UAG. Overall, codons with A/U-ends were more preferred than those with G/C-ends among the amino acids in *Ilex* species (Figure 8, Additional files 4: Table S4).

Phylogenomic analyses

The phylogenetic trees of both the maximum likelihood (ML) and Bayesian inference (BI) were reconstructed based on the 52 complete chloroplast genomes. The closely related species *Helwingia himalaica* (NC031370) was selected as outgroup [23]. The total alignment length of the dataset used was 157,836 bp after trimmed. A total of 8,869 variable sites and 1,735 parsimony informative sites were detected in the alignment. The phylogenetic trees based on the ML and BI displayed similar topologies; thus, the posterior probability (PP) values were merged into the ML tree for clearer presentation (Figure 9). The monophyly of *Ilex* was well supported by the two analyses (Fig. 8; ML BS: 100%; BI PP: 1.00).

Based on our phylogenetic analysis and with consideration of macro-morphological and distribution information, the genus *Ilex* was categorized into five highly supported clades (Figure 9; ML BS: 100%; BI PP: 1.00). The relationships among the five major clades were well resolved with high resolution (ML BS: 100%; BI PP: 1.00) in the present phylogenetic study. Members of clade D and clade E were sister to the rest, followed by the clade C, which is sister to a clade containing clade A and clade B. The relationships within each clade were also well resolved with high support values.

Discussion

Comparison of the chloroplast genome in genus *Ilex*

The chloroplast genomes of *Ilex* were found to possess the typical quadripartite structure with similar genome size of most land plant genomes [20]. At genus level, all 41 chloroplast genomes showed high conservatism in term of genome structure, but still exhibited moderate variations among different species. The genome length of the chloroplast genes of *Ilex* was similar among different species with a maximum 901 bp difference in length. Expansion and contraction events at the SC/IR boundaries usually give rise to the change of the chloroplast genome length [24]. Although small variations around the IR junctions were detected in the present study, the IR regions of the chloroplast genomes of these *Ilex* species showed a modest expansion or contraction and the length of IR regions varied from the longest 26,121 bp to the shortest 25,080 bp.

In addition, variation in gene spacer region, the loss or gain of genes and introns also play an important role in determining the chloroplast genome size of most plants [20, 25]. In the seven newly sequenced chloroplast genomes, except for *I. dasyphylla*, all species lacked the gene *psbI*. The event of gene loss in genus *Ilex* (e.g., deletions in the *trnT-trnL* and *ycf4-cemA* spacers of *I. graciliflora*) has been identified in an earlier report [26], which suggested that the gene loss in *Ilex* could be a common phenomenon. The length of the SSC and the IR regions were very similar, while that of LSC region showed a difference of about 900 bp (Table 1).

SSRs and simple sequence repeat analyses

SSRs derived from the plastid region were commonly known to be the markers of choice by population genetics and evolutionary studies owing to their high polymorphism rate and abundant variation at the species level [27]. In the present study, a total of 2146 SSR loci were identified from the 41 *Ilex* chloroplast genomes (Figure 6, Additional files 2: Table S2). Few studies have involved in the population genetics using data of SSRs in genus *Ilex*, and these SSR loci will contribute to further studies of genetic diversity and polymorphisms at the population, intraspecific and cultivar levels, as well as phylogeography for a certain species in *Ilex*.

Long repeat sequences with length longer than 30 bp are important because they might play important roles in the insertion/deletion mismatches and rearrangements that led to genomic variation [28–30] and the increase in the genetic diversity of populations [31]. We found that the number of long repeat sequences in *Ilex* is comparably higher to some other angiosperm species (e.g., 364 long repeats in Oxalidaceae [32]; 403 in *Veratrum* [33]; 32 in *Oresitrophe rupifraga*, and 34 in *Mukdenia rossii* and [34]). Among these long repeats, the forward, palindromic and tandem repeats were rather common, accounting for 33.84%, 30.81%, and 34.44% of the total number of repeats, respectively, while the complementary and reverse repeats were quite rare, only accounting for 0.42% and 0.49%, respectively.

Hypervariable regions for future studies of phylogeny and DNA barcoding

Hypervariable regions are usually used as effective evidence for delimitation of closely related taxa as well as to provide a wealth of phylogenetic information [35, 36]. In general, sequence divergence in IR regions is more conserved than that in the SSC and the LSC regions [37]. In the present study, the eight hypervariable regions including four genes and four gene with their adjacent and overlapped regions were detected and selected as the most divergent loci (Table 4). All the hypervariable loci were distributed in the SC regions, and also showed lower levels of variation in the IR regions, which was consistent with most angiosperm genera [32, 33].

Previous phylogenetic analyses of *Ilex* that were based on a handful of plastid markers (mainly including *atpB-rbcL*, *psbA-trnH*, *rbcL*, and *trnL-trnF*) did not generate well-resolved interspecific relationships within genus *Ilex* [1, 2, 8, 11, 13, 38–40]. When comparing these markers to those of highly variable regions identified from this study, only one of them, which is the *rbcL* region, was present. Therefore, we proposed the use of the eight highly variable regions reported in this study to be used as potential markers for reconstruction of the phylogenetic tree based on short plastid regions. Furthermore, with the high resolution at species level, a powerful DNA barcode can be obtained for *Ilex* that is suitable for species identification and delimitation. However, further studies are required to evaluate the delimitation strength in these regions prior to conducting studies on the DNA barcoding of *Ilex*.

Phylogenetic inference

Researchers have attempted to resolve the phylogenetic relationships among major lineage of *Ilex* and to test the consistency between the molecular phylogeny and the traditional taxonomic systems based on morphology evidence [8–13, 23, 38]. However, these studies failed to elucidate the phylogenetic relationships effectively, especially at the species level. The presences of poor species resolution and weak support at most branch nodes were due to lack of sufficient variable information sites when using short DNA markers [8, 10–12, 23, 38]. Undoubtedly, this limitation can be addressed by using longer DNA sequences that contain an abundant of variable sites [41]. The complete chloroplast genome sequences containing sufficient informative sites and can obviously resolve the phylogenetic relationships at almost any taxonomic level [18]. Earlier phylogenetic analyses using the complete chloroplast genome sequences based on a small quantity of samples achieved the same conclusion [14, 26, 42].

At present, the relationships within the genus *Ilex* were well resolved based on the current phylogeny, and five major clades (lineages), clade A to clade E, were identified with consideration of macro-morphological and distribution information (Figure 9). The main lineages were largely consistent with the previous plastid phylogeny, while the relationships among these major lineages were largely discrepant [8, 11, 13]. Our results showed that the American groups (clade E) and the Eurasia groups (clade F) were sister to each other, while both of them formed the basal clade of the genus. These two basal lineages were sister to the rest (clade A–C), which were mostly from the Asia region. This finding was not consistent to the previous results produced by Manen [11], of which the American groups (group 3) and the Eurasia groups (group 4) were the most recent diversified groups. The discordance between our results and the previous conclusions mainly owed to the fact that lacking genes from hypervariable regions by their phylogenetic studies and the low resolution among major clades [8, 11].

The inconsistency between the plastid phylogeny and the traditional taxonomic systems was also revealed by the present studies (Figure 9). Almost all the subgenera, sections and series included in the analysis were support as non-monophyly (both the two subgenera, five out of the six sections, and six out of eleven series). We did not rule out the possibility that more non-monophyly groups will be present in this plastid-based phylogenetic tree when more taxa are involved. In addition, though the resolution of phylogenetic tree in early studies was quite low, they indicated that the nuclear and plastid phylogeny were highly incongruent and the nuclear phylogeny was more consistent with traditional morphological classification than the plastid one [11]. We here confirmed their conclusions by improving the resolution of plastid phylogenetic tree using the complete chloroplast genome data.

In general, species of the same origin should be clustered next to each other in the same clade due to them having the same inheritance. This is proven accurate for some of the *Ilex* species present in our study, including *I. cornuta*, *I. dasyphylla*, *I. latifolia*, and *I. integra*. However, the accessions from *I. pubescens* and *I. lohfaensis* displayed otherwise; the two accessions of *I. pubescens* were resolved in two distinct clades (clade A and clade B), and the two accessions of *I. lohfaensis* were separated from each other, with *I. championii* placed in between them. Three samples of *I. viridis* were clustered with morphologically similar species *I. triflora*. Some of the non-monophyly species might be caused by genomic capture or hybridization events, as well as misidentification caused by taxonomic confusion among different taxa. However, our well resolved phylogeny will help to clarify the taxonomic confusion among closely related species in the genus.

Conclusions

In this study, the characterization and phylogenetic analysis of the chloroplast genome of *Ilex* have been carried out, and as a result, an in-depth understanding on the evolution of *Ilex* at genome-scale level was obtained. However, we proposed the future studies on *Ilex* should focus on the phylogenetic reconstruction based on the nuclear region. We propose the capturing of low-copy nuclear genes from the genome-skimming data, which is thought to provide a better resolution for most plant species when compared to short nuclear DNA markers, i.e. ITS, and incorporate it with the phylogenetic tree based on the complete chloroplast genomes as well as morphology analyses, to enhance our understanding of the complex evolutionary history of genus *Ilex*.

Materials And Methods

Taxon sampling, DNA extraction, and sequencing

Seven species of *Ilex*, including *I. dasyphylla*, *I. fukienensis*, *I. lohfauiensis*, *I. venusta*, *I. viridis*, *I. yunnanensis*, *I. zhejiangensis*. *Ilex fukienensis*, *I. venusta*, and *I. zhejiangensis*, were collected from their natural accessions in China. Fresh leaf specimens were collected in the field and stored in silica gels prior to be sent to the laboratory for DNA extraction. Voucher specimens were prepared and deposited at the herbarium of Nanjing Forestry University (NF). In addition, 34 complete chloroplast genomes of *Ilex* species that are publicly available in the NCBI GenBank were downloaded for their sequences in fasta format and kept for subsequent comparative analyses (Additional files 5: Table S5). Based on the classification of *Ilex* that is generally accepted [22], the current dataset comprised of species from six sections and 11 series of the genus *Ilex*.

Total genomic DNA was extracted using the Plant Genomic DNA Kit (Tiangen Biotech, China) following the manufacturer's protocol. The DNA extract was inspected with agarose gels, and quantified using Qubit 2.0 (Life Technologies, Country) for its integrity, purity, and concentration. The qualified DNA (≥ 50 ng) was used to construct a paired-end (2×150 bp) library, and sequencing was conducted on a HiSeq X Ten platform (Illumina, USA). The generated raw data were filtered with fastp v.0.20.0 software [43] to remove low-quality reads.

Chloroplast genome assembly and annotation

The filtered raw data were fed into the NOVOPlasty 2.6.3 [44] pipeline for genome assembly, with the *rbcL* gene sequence of *I. latifolia* (Accession number: KX897017) as the seed sequence and the chloroplast genome sequence of *I. latifolia* (Accession number: MN688228) as reference genome. A contig was obtained at the end of the process, and annotation was conducted using Plann [45], in which the annotated chloroplast genome of *I. latifolia* (Accession number: MN688228) was set as reference. Start and stop codons in the chloroplast genomes were manually corrected using DOGMA [46], while the tRNA genes were verified using tRNA scan-SE v2.0.3 embedded in GeSeq [47] based on default parameters. The circular chloroplast genome maps were visualized using OrganellarGenomeDRAW [48].

Genome comparative analyses

Sequence alignment of the 41 complete chloroplast genomes was carried out using MAFFT v.7 [49] and the alignment was further trimmed using trimAl v1.2 using the “-gappyout” setting [50]. The expansion and contraction of the IR regions were visualized using online IRscope [51] and then was manually checked. The nucleotide diversity (π) was estimated using DnaSP v.5 [52] with a step size of 200 bp and a window length of 800 bp. The genome variability across the 41 species of *Ilex* was assessed using mVISTA [53], under Shuffle-LAGAN mode. Mauve version 2.3.1 [54] plug-in that was available in Geneious version 11.0.3 [55] was used to identify locally collinear blocks presented among the chloroplast genomes based on default parameters.

Repeat sequence identification

The number of large repeats, including forward, palindromic, reverse, and complement repeats were identified using the onlineREPuter program [56] according to the following criteria: sequence identities of 90%, cutoff point at ≥ 30 bp, Hamming distance set at 3, and a minimum repeat size of 30 bp. Tandem Repeat Finder [57] was used to analyze tandem repeat sequences with the default parameters. SSRs were identified using web-MISA [58], with minimum repeat number set at 10, 5, 4, 3, 3, and 3 for mono-, di-, tri-, tetra-, penta-, and hexanucleotides, respectively. Compound SSRs were detected by identifying independent SSRs that are separated by less than 100 nucleotides and were combined into one.

Codon Usage Analysis

RSCU is defined as the ratio of the observed frequency of codons to the expected frequency. The sequences of 77 shared protein-coding genes were extracted and concatenated using PhyloSuite v1.2.1 [59]. The value of RSCU was calculated using MEGA X [60]. A dendrogram based on the value of RSCU was constructed using pheatmap, which is available in R package (<https://cran.r-project.org/web/packages/pheatmap/>).

Phylogenetic Analyses

Phylogenetic analysis was conducted based on 51 complete chloroplast genome sequences representing 39 *Ilex* species from six sections and 11 series. Based on the finding from Yao et al. [23], *Helwingia himalaica* (Accession number: NC031370) was selected as the outgroup. Genome alignment was carried out using MAFFT v.7 [49] and then trimmed using trimAl v1.2 with the “-gappout” setting [50].

Maximum likelihood (ML) analyses were conducted using IQ-tree [61] under 10,000 ultrafast bootstrap support values (UFBS) replicates [62]. According to Bayesian information criterion (BIC), the best fitting substitution model that was estimated using ModelFinder [63] was GTR+F+I+G4. Bayesian inference (BI) analysis was carried out using MrBayes version 3.2 [64] that was implemented in CIPRES [65]. The Markov chain Monte Carlo analysis was executed for 20,000,000 generations, with four chains (one cold and three heated), each starting with a random tree, and readings were sampled at every 1000 generations. Convergence of runs was accepted when the average standard deviation of split frequencies achieved <0.01. The first 25% of the trees were discarded as burn-in, and the remaining trees were used to construct majority-rule consensus trees. The final tree from both analyses was visualized using FigTree v.1.4.2 [66].

Declarations

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Authors' contributions

Conceptualization, K.X. and K.M; methodology, K.X. and K.M; formal analysis, K.X. and K.M; investigation, K.X.; resources, K.X.; data curation, K.X. and K.M; writing—original draft preparation, K.X.; writing—review and editing, K.X., S.Y. Lee, K.M and K.M; visualization, K.X. and K.M; supervision, K.X., L.M. and K.M; project administration, K.X. and L.M.; funding acquisition, K.X. and L.M. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analyzed in this study were included in this published article and the Additional files. The complete chloroplast genomes of seven newly sequenced *Ilex* species were submitted to GenBank and the accession numbers could be found in Additional file 5. All raw reads are available in the short sequence archive under accession no. PRJNA768933. All of the complete genome sequences used in this study were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov>), and the accession numbers could also be found in Additional file 5.

Ethics approval and consent to participate

Not applicable. No specific permits were required for the collection of specimens for this study. All materials used in the study were collected in public areas of China in compliance with the relevant laws of China. The formal identification of the plant material was carried out by Kewang Xu. Voucher specimens were prepared and deposited at the herbarium of Nanjing Forestry University (NF) and their collection numbers could be found in Additional file 5.

Consent for publication

Not applicable.

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Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Figure 1

Gene circle maps of seven newly sequenced *Ilex* species. The colored bars indicate different functional groups. Thick lines of the large circle indicate the extent of the inverted repeat regions (IRa and IRb), which separate the genome into small (SSC) and large (LSC) single copy regions. Genes on the inside and outside of the large circle are transcribed clockwise and counterclockwise, respectively. The darker gray columns in the inner circle correspond to the GC content, the light gray to AT content.

Figure 2

Figure 2

Sliding-window analysis showing the nucleotide diversity (Pi) values of the aligned *Ilex* chloroplast genomes.

Figure 3

Figure 3

Sequence alignment of 41 *Ilex* chloroplast genomes using the mVISTA program with *I. szechwanensis* as a reference. The vertical scale indicates the percent identity, ranging from 50% to 100%. The horizontal axis indicates the location within the plastomes. Genome regions are color-coded as exon, intron and untranslated regions (UTRs).

Figure 4

Mauve multiple alignment of 41 *Ilex* chloroplast genomes revealing no interspecific rearrangements.

Figure 5

Figure 5

Comparison of the SC/IR junctions among the 41 *Ilex* chloroplast genomes. JLA, LSC/IRa boundary; JSA, SSC/IRa boundary; JSB, SSC/IRb boundary; JLB, LSC/IRb boundary.

Figure 6

Figure 6

Analysis of simple sequence repeats (SSR) in the 41 chloroplast genomes of *Ilex* species. (A) Number of different SSR types detected in the 41 genomes; (B) Number of different SSR types in LSC, SSC and IR regions.

Figure 7

Figure 7

Analysis of long repeats in 41 chloroplast genomes of *Ilex* showing the number of complement, forward, palindromic, reverse and tandem long repeats.

Figure 8

Figure 9

Figure 9

Phylogenetic trees inferred from maximum likelihood (ML) and Bayesian inference (BI) analyses of 52 accessions based on the complete chloroplast genomes. Numbers near the nodes are ML bootstrap support values (BS, left of the slashes) and Bayesian posterior probabilities (PP, right of the slashes). 100% BS or 1.00 PP were indicated by asterisks. Incongruences between the BI and ML trees were indicated by dashes. Hu's classification was illustrated by color graphic pattern. Recognized groups (major clades) were also marked by the right-hand black bar.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.xls](#)
- [TableS2.xls](#)
- [TableS3.xlsx](#)
- [TableS4.xls](#)

- [TableS5.xlsx](#)