Plastid genomes reveal evolutionary shifts in elevational range and flowering time of *Osmanthus* (Oleaceae)

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**Abstract**
Species of *Osmanthus* are economically important ornamental trees, yet information regarding their plastid genomes (plastomes) have rarely been reported, thus hindering taxonomic and evolutionary studies of this small but enigmatic genus. Here, we performed comparative genomics and evolutionary analyses on plastomes of 16 of the 28 currently accepted species, with 11 plastomes newly sequenced. Phylogenetic studies identified four main lineages within the genus that are here designated the: "Caucasian Osmanthus" (corresponding to *O. decorus*), "Siphosmanthus" (corresponding to *O. sect. Siphosmanthus*), "*O. serrulatus* + *O. yunnanensis*," and "Core Osmanthus: (corresponding to *O. sect. Osmanthus* + *O. sect. Linocieroides*). Molecular clock analysis suggested that *Osmanthus* split from its sister clade c. 15.83 Ma. The estimated crown ages of the lineages were the following: genus *Osmanthus* at 12.66 Ma; "Siphosmanthus" clade at 5.85 Ma; "*O. serrulatus* + *O. yunnanensis*” at 4.89 Ma; and "Core Osmanthus: clade at 6.2 Ma. Ancestral state reconstructions and trait mapping showed that ancestors of *Osmanthus* were spring flowering and originated at lower elevations. Phylogenetic principal component analysis clearly distinguished spring-flowering species from autumn-flowering species, suggesting that flowering time differentiation is related to the difference in ecological niches. Nucleotide substitution rates of 80 common genes showed slow evolutionary pace and low nucleotide variations, all genes being subjected to purifying selection.

**KEYWORDS**
ancestral state reconstruction, ancestral trait reconstruction, molecular clock, plastomes, purifying selections

**TAXONOMY CLASSIFICATION**
Phylogenetics
Plastosomes play an important role in plant phylogeny and evolutionary studies due to them being structurally stable, generally maternal inherited, and with low levels of recombinant DNA (Gu et al., 2018; Wu et al., 2017). Genes of plastosomes primarily encode core components of the photosynthetic machinery and factors involved in their expression and assembly, as well as those of self-replication. (Gao et al., 2018; Mohanta et al., 2020; Pilot et al., 2018). Thus, plastosomes are generally considered to be conserved, in terms of genomic structures and substitution rates, among the majority of Angiosperms. Recently, however, increasing studies have detected positive selection signals in plastid genes. For example, psaA genes in the 22 closely related Oryza species were found to be undergoing positive selection, which could be related to the adaptation of rice plants to habitats with different light conditions (Gao et al., 2019). The evolution rates of some genes (psaA, rpl16, ndhA, and ndhH) in Rhodiola plants were also found to be accelerating, which possibly allowed Rhodiola species to adapt to the harsh environment of the Qinghai–Tibet Plateau, such as low carbon dioxide concentration and high solar irradiation (Zhao, Yang, et al., 2020; Zhao, Ren, et al., 2020). Furthermore, the evolutionary rates of matK genes in some low-altitude and recently derived lineages of Dysosma were found to be significantly accelerated, which may reflect the adaptability of these species to new environments (Ye et al., 2018). Thus, genetic content held in plastomes can provide useful information to enhance our understanding regarding adaptive evolution in plants. Herein, we assembled plastomes of 16 of the 28 Osmanthus species that are currently accepted (POWO, 2021), with 11 plastomes newly sequenced, which allowed us to (1) provide the most well-sampled phylogenetic and molecular clock analyses of Osmanthus to date; (2) study the evolutionary patterns of elevational shifts and flowering time within Osmanthus based on ancestral traits reconstruction; and (3) calculate the substitution rate of plastid genes and explore whether the differentiation of flowering time and elevational range is related to the environmental pressures on plastid genes.

2 | MATERIALS AND METHODS

2.1 | Plastid genome sequencing, assembly, and annotation

We newly sequenced and assembled the whole plastomes of 11 species of Osmanthus. They are as follows: O. armatus Diels, O. cooperi Hems., O. decorus, O. delavayi, O. enervius Masam. & K. Mori, O. fordii Hems., O. × fortunei Carr., O. heterophyllus (G. Don) P.S. Green, O. serrulatus Rehder, O. suavis King ex C.B. Clarke, and O. yunnanensis. In addition, plastomes of O. austrocaledonicus (Vieill.) Knobl., O. didymopetalus P.S. Green, O. fragrans (Thunb.) Loureiro, O. insularis Koidz., and O. urceolatus P.S. Green were extracted from GenBank (https://www.ncbi.nlm.nih.gov/) that had been sequenced as part of previous publications (Duan, Li, Zheng, et al., 2019; Duan, Li, Zhang, et al., 2019; Olofsson et al., 2019; Zhao, Yang, et al., 2020; Zhao, Ren, et al., 2020). The selection of the outgroups was guided by...

1 | INTRODUCTION

Attractive fragrance and elegant flowers make species of Osmanthus (Oleaceae) widely used in horticulture, food, and medicine throughout temperate to subtropical regions of the northern hemisphere (Xiang & Liu, 2008). Except for Osmanthus decorus (Boiss. & Balansa), Kasapligil, which is found in the Caucasus Mountains (Green, 1972), the other 27 currently accepted species are found in eastern Asia, from the high-elevation Hengduan Mountains to the low elevations of southeast China, Japan, and Korea (Chang et al., 1996; Green, 1958; POWO, 2021). Osmanthus is characterized by its cymose inflorescences, corolla lobes united in pairs at their base and usually forming a tube, and an androecious breeding system (Chang et al., 1996; Duan, Li, Zheng, et al., 2019; Green, 1958, 1972; Li et al., 2020). Within the genus, previous taxonomic studies divided the 28 species into three sections: O. sect. Osmanthus; O. sect. Siphosmanthus Franch., and O. sect. Linocieroides P.S. Green based on floral characteristics (Chang et al., 1996; Green, 1958; Li et al., 2020).

Genetic studies have discussed the delimitation of Osmanthus using a number of gene fragments (Guo et al., 2011; Li et al., 2020; Lu et al., 2011; Yuan et al., 2010) or a few plastomes (Duan, Li, Zhang, et al., 2019; Olofsson et al., 2019; Zhao, Yang, et al., 2020; Zhao, Ren, et al., 2020). They found that Osmanthus lies in a generic complex in the subtribe Oleinae (Oleaceae), very close to four genera: Nestegis Raf., Notelaea Vent., Phillyrea L., and Picconia DC. (referred to as NNPP here). Within the genus, only sect. Siphosmanthus and sect. Osmanthus were supported (Guo et al., 2011; Li et al., 2020), although detailed studies on intra-generic relationships, as well as the divergence time of lineages, are still lacking. Therefore, a more widely sampled and robust phylogenetic study is urgently needed to guide downstream evolutionary analysis such as molecular dating and morphological trait evolution. In particular, plastomes have been successfully used to construct the phylogenetic framework of Oleaceae (Dupin et al., 2020; Ha et al., 2018; Olofsson et al., 2019), laying the foundation for our follow-up evolutionary analysis.

Flowering time is considered as a crucial life cycle trait that contributes to fitness in plants (Gaudinier & Blackman, 2020). Changes in flowering time between species may reflect the environmental constraints which limit the direction of evolution in certain species (Wadgymar et al., 2018). The variations in light, temperature, and precipitation caused by changes in elevation are also considered one of the main reasons for the high species richness in eastern Asia (Shimono et al., 2010; Sun et al., 2017). In Osmanthus, significant variations in elevational range and flowering time between species have been noted by various morphological and ecological surveys (Li et al., 2019, 2020). For example, high-elevation species such as O. yunnanensis (Franchet) P.S. Green and O. delavayi Franchet tended to bloom in spring (March to April), while low-elevation species such as O. cooperi Hems. tended to bloom in autumn (September to October). Exploring the evolutionary patterns of flowering time and elevational shifts within these species can provide insights into how Osmanthus species adapted to different environments (Gaudinier & Blackman, 2020).
previous research (Niu et al., 2020; Olofsson et al., 2019), and we selected 12 species: *Nestegis apetala* (Vahl) L.A.S. Johnson, *Nestegis cunninghamii* (Hook. f.) L.A.S. Johnson, *Nestegis lanceolata* (Hook. f.) L.A.S. Johnson, *Nestegis sandwicensis* (A. Gray) O. Deg., I. Deg. & L.A.S. Johnson, *Notelaea longifolia* Vent., *Notelaea microcarpa* R. Br., *Notelaea venosa* F. Muell., *Olea europaea* L., *Phillyrae angustifolia* L., *Phillyrae latifolia* L., *Picconia azorica* (Tutin) Knobl., and *Picconia excelsa* (Sol.) A. DC.

Total genomic DNA was isolated from fresh leaves of a single individual using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA), and was used to prepare the shotgun library following the manufacturer’s protocol for HiSeq 4000 Sequencing System (Illumina, CA, USA). The library was sequenced by Nanjing GenePioneer Biotechnologies Inc. (Nanjing, China). Raw reads were obtained and trimmed using CLC Genomics Workbench v9 (CLC Bio, Aarhus, Denmark) with default parameters. The resultant clean reads were then employed to assemble the plastome using the program NOVOPlast (Dierckxsens et al., 2017) with O. *fragrans* (GenBank: MGB20121) (Duan, Li, Zheng, et al., 2019; Duan, Li, Zhang, et al., 2019) as the reference. The resultant genome was annotated by PGA (Qu et al., 2019). The plastomes generated in the present study are available in the NCBI GenBank database. The accession numbers of *Osmanthus* and outgroups are presented in Table 1.

### 2.2 | Phylogenomic analyses

Two different kinds of plastid data were adopted for the phylogenetic analyses of 16 *Osmanthus* species and 12 outgroups. The first dataset was made by the concatenation of 80 common protein-coding genes (File S1). The second dataset consisted of whole plastomes, which was designed to be compared with the results of the concatenation sequences. In order to reduce information bias caused by duplicate sequences in the plastomes, one of the two IR regions was removed (File S2). The sequences were aligned using MAFFT (Katoh & Standley, 2013). The poorly aligned sequences were trimmed by trimAL software (Capella-Gutiérrez et al., 2009). The best-fitting nucleotide substitution models for each gene and for the whole plastomes were calculated by ModelFinder (Kalyaanamoorthy et al., 2017) (Files S1 and S3). Phylogenetic trees were constructed by maximum likelihood (ML), Bayesian inference (BI), and maximum parsimony (MP). ML analyses were performed in IQ-TREE v1.6.12 (Nguyen et al., 2015), with 1 million ultrafast bootstrap analyses. BI analyses were conducted using MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003). The search started from a random tree and used two independent Markov chain Monte Carlo (MCMC) chains run for 1 million generations with sampling of trees every 100 generations. The first 25% of the trees were discarded as burn-in, and the remaining trees were used to generate the 50% majority rule consensus tree. The average standard deviation of split frequencies reached to 0.01, implying convergence of the two runs. MP analyses were performed in MEGA 7 (Kumar et al., 2016) using the search method of tree-bisection and reconnection (TBR) with 1000 random addition replicates. Branch support was calculated based on the bootstrap method with 1000 replications.

### 2.3 | Molecular clock estimation

MCMCtree in the PAML 4.9j package (Yang, 2007) was used to estimate divergence time with an approximate likelihood calculation. The BI phylogenetic tree based on full-length plastomes from 16 *Osmanthus* and 12 outgroups in subtribe Oleinae was used for divergence time estimation. Estimation of the overall substitution rate was conducted using the BaseML program in PAML. The overall substitution rate (\(\text{rate}_{\text{gene}}\)) and rate drift parameter (\(\text{sigma}_2\)) were set as G (1, 128) and G (1, 4.5), respectively. The gradient and the Hessian matrix were estimated using the MCMCtree program in PAML under the GTR substitution model. The MCMC process of MCMCtree was run to sample 20,000 times, with sample frequency set at 500, after a burn-in of 10 million iterations. In total, the MCMC ran for 20,000,000 iterations. Two independent runs were performed to ensure convergence. Distributions of the parameter from MCMC samples were checked by effective sample sizes (ESS) > 200 using Tracer v1.7 (Rambaut et al., 2018). The results of molecular dating are influenced by many factors, such as the differences between genes, the selection of evolutionary models, and the setting of calibration points. The calibration data for plants are usually derived from limited fossil evidence or secondary calibrations based on previous molecular dating analyses. Therefore, the final results tend to float with the change in calibration schemes (Sauquet et al., 2012). Due to the current lack of fossil evidence in the genus *Osmanthus*, a secondary calibration approach was adopted. The time calibrations were selected based on previous molecular clock studies of Oleaceae. First, based on age estimations from Besnard et al. (2009), the split of genus Olea and the NNPP + *Osmanthus* clade occurred at 32.6 (28.50–37.80) Ma. Second, the divergence between genus *Osmanthus* and the NNPP clade occurred at 15.99 (12.38–19.60) Ma (Olofsson et al., 2019).

### 2.4 | Elevational range shifts and flowering time evolution

In order to investigate the evolutionary patterns of flowering time and elevational shifts, we reconstructed the ancestral states for 16 *Osmanthus* and 12 outgroups based on the time tree obtained in the last step. Data on the elevational range and flowering time were obtained based on field observations, literature studies (Chang et al., 1996; Green, 1958, 1972, 2004; Li et al., 2020; Xiang & Liu, 2008), and herbarium information stored in the Chinese Virtual Herbarium (http://www.cvh.ac.cn), the Global Biodiversity Information Facility (http://www.gbif.org/), and JSTOR (https://www.jstor.org). For the 16 *Osmanthus* species, we collected a total of 431 specimens as well as data on their geographical distribution, of which *O. fragrans* had the most locations (183), while
| Species                        | GenBank       | IRa (bp) | IRb (bp) | LSC (bp) | SSC (bp) | Total (bp) | Gene | PCGs | tRNA | rRNA | GC (%) | References          |
|-------------------------------|---------------|----------|----------|----------|----------|------------|------|------|------|------|--------|---------------------|
| Nestegis apetala             | NC_036983     | 25723    | 25723    | 85898    | 17505    | 154,849    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Nestegis cunninghamii        | NC_042455     | 25723    | 25723    | 85941    | 17520    | 154,907    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Nestegis lanceolata          | NC_042456     | 25706    | 25706    | 86009    | 17556    | 154,977    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Nestegis sandwichensis      | NC_042457     | 25717    | 25717    | 86464    | 17489    | 155,565    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Notelaea longifolia          | NC_042458     | 25713    | 25713    | 86117    | 17494    | 155,037    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Notelaea microcarpa          | NC_042459     | 25713    | 25713    | 86119    | 17474    | 155,019    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Notelaea venosa              | NC_042427     | 25713    | 25713    | 86098    | 17480    | 155,012    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Olea europaea                | MT182986      | 25742    | 25742    | 86611    | 17791    | 155,806    | 133  | 87   | 37   | 8    | 37.8   | Niu et al. (2020)     |
| Osmanthus armatus            | MW648024      | 25689    | 25689    | 86676    | 17338    | 155,292    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus austrocaledonicus  | MK299397      | 25713    | 25713    | 86538    | 17496    | 155,460    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Osmanthus cooperi            | MW727458      | 25691    | 25691    | 86496    | 17366    | 155,243    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus decorus            | MW727459      | 25708    | 25709    | 86544    | 17501    | 155,455    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus delavayi           | MW727460      | 25715    | 25715    | 86474    | 17460    | 155,364    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus didymopetalus      | MT362090      | 25707    | 25707    | 86384    | 17360    | 155,155    | 133  | 87   | 37   | 8    | 37.8   | Zhao, Yang, et al. (2020) |
| Osmanthus enervius           | MW727461      | 25611    | 25611    | 86639    | 17166    | 155,225    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus fordii             | MW727462      | 25692    | 25692    | 86543    | 17368    | 155,292    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus xfortunei          | MW727463      | 25702    | 25702    | 86512    | 17367    | 155,280    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus fragrans           | MH687871      | 25687    | 25690    | 86547    | 17372    | 155,296    | 133  | 87   | 37   | 8    | 37.8   | Duan, Li, Zhang, et al. (2019) |
| Osmanthus heterophyllus      | MW727464      | 25702    | 25702    | 86512    | 17367    | 155,280    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus insularis          | NC_042264     | 25702    | 25702    | 86526    | 17368    | 155,298    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Osmanthus serrulatus         | MW727466      | 25707    | 25707    | 86536    | 17464    | 155,411    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus suavis             | MW727467      | 25686    | 25686    | 86477    | 17454    | 155,303    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Osmanthus urceolatus         | MH229859      | 25691    | 25691    | 86491    | 17385    | 155,258    | 133  | 87   | 37   | 8    | 37.8   | Directly submission   |
| Osmanthus yunnanensis        | MW727465      | 25707    | 25707    | 86584    | 17490    | 155,485    | 133  | 87   | 37   | 8    | 37.8   | This study            |
| Phillyrea angustifolia       | NC_042464     | 25709    | 25709    | 86416    | 17501    | 155,335    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Phillyrea latifolia          | NC_042465     | 25706    | 25705    | 86400    | 17489    | 155,300    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Picconia azorica             | NC_042428     | 25706    | 25715    | 86419    | 17510    | 155,350    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
| Picconia excelsa             | NC_042466     | 25705    | 25705    | 86449    | 17496    | 155,355    | 133  | 87   | 37   | 8    | 37.8   | Olofsson et al. (2019) |
2.5 | Environmental analysis

To investigate the environmental differences between spring- and autumn-flowering plants in the genus *Osmanthus*, we downloaded high-resolution (30 arc sec) climatic data from CHELSA (https://chelsa-climate.org/), and extracted 19 variables for the 431 coordinates of the 15 *Osmanthus* species (except for *O. austrocaledonicus*) using "extract" function in the R package "raster" (R Core Team, 2021). The climate information of all sampled points is shown in File S4.

Phylogenetic principal component analysis (pPCA) was employed based on the “BM” method using the “phyl.pca” function in the R package "phytools" to dimensionally reduce the 19 high-resolution environmental variables to two principal component axes, with a consideration of phylogenetic relationships between species, and to detect correlations among them. The data scaling and centralizing were conducted before the pPCA analysis in the R (R Core Team, 2021) platform.

2.6 | Substitution rates and nucleotide diversity of plastid genes

To recognize the mutation hotspots for 80 common protein-coding genes across the 16 *Osmanthus* species, nucleotide diversity (Pi) values were calculated by DnaSP V. 5.10 software (Rozas & Rozas, 1995). To evaluate whether certain genes in specific lineages within *Osmanthus* (e.g., species with spring- vs. autumn-flowering time or high vs. low elevation) are undergoing positive selection, we performed pairwise Ka/Ks calculations on 80 orthologous genes from the 16 *Osmanthus* species using KaKs_calculator 2.0 (Wang et al., 2010) with the settings genetic code table 11 (bacterial and plant plastid code) and the “YN” method of calculation. If a particular gene in a particular lineage is positively selected for, then we will see that its paired Ka/Ks will be greater than 1, and if it is being subjected to neutral or purifying selection then we will get a value of 1 or less than 1. In our results, some genes had Ka/Ks values equal to “NA,” indicating that no synonymous substitutions (Ks) occurred in these genes.

3 | RESULTS

3.1 | Phylogenetic analyses

Phylogenetic trees constructed from two datasets (whole plastomes and concatenation of 80 protein-coding genes) using three different tree building methods (BI, ML, and MP) are shown in File S6. The topologies of all trees were highly consistent and similar, but the BI tree constructed from whole plastomes exhibited the highest branch support, and so was used for display and downstream analyses.

In our new phylogeny (Figure 1 and File S6), the New Caledonian species *O. austrocaledonicus* separated from all other
Asian Osmanthus species (the Osmanthus clade) as a distinct lineage nested within the Australasian genera Notelaea and Nestegis with strong support (BIPP: 1.00/1.00; MLBS: 100%/100%; and MPBS: 100%/100%). Except for O. austrocaledonicus, the monophyly of the Osmanthus clade is confirmed (BIPP: 1.00/1.00; MLBS: 100%/100%; MPBS: 99%/99%) and is resolved as sister to the NNPP clade. Within the Osmanthus clade, the 15 species were grouped into four major clades: the (here designated) Caucasian Osmanthus clade (BIPP: 1.00/1.00; MLBS: 100%/100%; MPBS: 100%/100%), the (here designated) Siphosmanthus clade (BIPP: 1.00/1.00; MLBS: 100%/100%; MPBS: 100%/100%), the (here designated) O. serrulatus + O. yunnanensis clade (BIPP: 1.00/1.00; MLBS: 100%/94%; MPBS: 99/92%), and the Core Osmanthus clade (BIPP: 1.00/1.00; MLBS: 100%/100%; MPBS: 100%/100%). The Caucasian Osmanthus clade (corresponding to O. decorus) was resolved as sister to the remaining 14 accessions of Osmanthus, followed by the strongly supported Siphosmanthus clade (corresponding to taxa of O. sect. Siphosmanthus: O. suavis and O. delavayi). The Siphosmanthus clade was placed sister to a large clade comprising the O. yunnanensis + O. serrulatus clade and the Core Osmanthus clade, the branch support of this clade being low (BIPP: 0.74/0.79; MLBS: 87%/78%; MPBS: 88%/75%). The Core Osmanthus clade (corresponding to taxa of O. sect. Osmanthus and O. sect. Linocieroides) contained the most species, including O. cooperi, O. heterophyllus, O. × fortunei, O. fragrans, O. fordii, O. enervius, O. armatus, O. urceolatus, and O. didymopetalus.

3.2 Molecular dating

Based on the results of MCMCtree estimation (Figure 2; File S7), the most recent common ancestor (MRCA) of Olea europaea and the remaining 27 species were estimated to have diverged at 32.33 Ma.

![Figure 2](image_url)
(95% HPD: 27.94–36.88 Ma) in the Early Oligocene. The MRCA of the Osmanthus and NNPP clade was estimated to have diverged at 15.83 Ma (95% HPD: 12.23–19.04 Ma) in the Middle Miocene. The crown age for the NNPP clade was estimated as 13.38 Ma (95% HPD: 10.07–16.54 Ma) in the Middle Miocene. The crown age of genus Osmanthus was estimated as 12.66 Ma (95% HPD: 9.00–16.26 Ma) in the Middle Miocene. The crown age of Siphosmanthus clade was estimated as 5.85 Ma (95% HPD: 3.21–8.69 Ma). The MRCA of O. yunnanensis + O. serrulatus clade and the Core Osmanthus clade was estimated to have diverged at 8.02 Ma (95% HPD: 3.21–8.69 Ma) in the Late Miocene. The crown age of O. yunnanensis + O. serrulatus clade was estimated as 4.89 Ma (95% HPD: 1.87–8.23 Ma) in the Late Miocene. The crown age of the Core Osmanthus was estimated as 6.2 Ma (95% HPD: 3.97–8.47 Ma) in the Late Miocene.

3.3 | Ancestral state reconstructions

Ancestral state reconstructions are presented in Figure 3a,b. Flowering in spring was inferred to be the ancestral state in Olea,
**Phillyrea, Picconia, Notelaea, Nestegis, and Osmanthus.** Flowering in autumn was inferred to be a derived character in Nestegis and Osmanthus. Autumn-flowering species evolved independently two times in our analysis, one time in Nestegis and the other time in Osmanthus. The most recent ancestors of autumn-flowering ancestral species in Osmanthus appeared at 6.2 Ma during the Late Miocene. Low elevation (mean elevation ca. 500 m) was reconstructed as the ancestral habitat of Olea, Phillyrea, Picconia, Notelaea, Nestegis, and Osmanthus. High elevation (mean elevation ca. 1500 m) was recognized as a derived state in Osmanthus, having evolved twice in the genus: once with the emergence of O. delavayi and O. suavis at 8.3 Ma, another with the emergence of clade O. serrulatus and O. yunnanensis at 8.02 Ma.

### 3.4 Environmental analysis

In the pPCA for spring- and autumn-flowering species in Osmanthus, the first two axes explained 38% and 31% of total variation, respectively (Figure 3c,d). The PC1 axis represented the global structure, indicating the variables are more similar in closely related species (species with the same flowering time) than in distant species (species with different flowering time), while the PC2 axis represented the local structure indicating the variables can create dissimilarities among closely related species (species with the same flowering time). Species with different flowering times could be distinguished from each other along the PC1 axis: Autumn-flowering species (O. armatus, O. cooperi, O. didymopetalus, O. fordii, O. × fortunei, O. fragrans, O. heterophyllus, O. insularis, O. enervius, and O. urceolatus) clustered together on the negative side of PC1, while spring-flowering species (O. decorus, O. serrulatus, O. suavis, O. yunnanensis, and O. delavayi) were clustered on the positive side of PC1. For spring-flowering species, all East Asian species were on the positive side of the PC2 axis, while only O. decorus from West Asia was on the negative side of the PC2 axis. For autumn-flowering species, only O. enervius from Taiwan island, China, was on the negative side of the PC2 axis, while all other species were positioned on the positive side of the PC2 axis (Figure 3d).

### 3.5 Genome structures and nucleotide variation

The plastome sizes ranged from 155,155 bp to 155,485 bp (Table 1). The length of IRs ranged from 25,611 bp to 25,715 bp. The length variation for the SSC, ranged from 17,166 bp to 17,501 bp. The length of the LSC varied from 86,384 bp to 86,676 bp. Plastomes of Osmanthus encoded an identical set of 133 predicted functional genes, in which 87 are protein-coding genes (eight duplicated genes), 37 are tRNA genes (seven duplicated genes), and 8 are rRNA genes (four duplicated genes). The overall GC content of the genomes was 37.8%, while the IRA and IRB regions showed higher GC contents. The locally collinear blocks (LCBs) showed all the genes maintain a consistent position and direction, with no rearrangement or inversion events being found (File S8).

Nucleotide variation analyses of the 80 protein-coding genes revealed Pi values in the range 0–0.005 with an average of 0.0006 (Figure 4a,c, File S9). No hypervariable loci (Pi > 0.01) were found. Only the ycf1 gene had the highest nucleotide variation (Pi > 0.003) of all 80 genes. In terms of gene function, genes related to photosynthesis and self-replication showed the lowest values of nucleotide variation (Figure 4a,c). A total of 8322 pairs of Ka/Ks values were calculated for the 80 genes among the 15 Asian Osmanthus species. The value of Ka ranged from 0.0001 to 0.008, with an average of 0.0003. The value of Ks ranged from 0.0006 to 0.05, with an average of 0.001. The value of Ka/Ks ranged from 0 to 0.98, with an average of 0.02 (Figure 4b,d, File S10). The ycf1 gene exhibited the highest Ka/Ks value with an average of 0.35. In terms of gene function, the genes associated with photosynthesis had the least values of Ka/Ks, followed by self-replication genes and other genes (Figure 4b,d).

### 4 DISCUSSION

#### 4.1 Phylogenetic relationships of Osmanthus

Previous molecular studies have recognized close relationships between Osmanthus and the other four genera in the NNPP clade (Guo et al., 2011; Li et al., 2020; Lu et al., 2011; Yuan et al., 2010). Our plastome phylogeny also confirm their results and suggest a polyphyletic status of traditional Osmanthus. Particularly, our plastome data find the New Caledonian species, O. austrocaledonicus, is in a subclade consisting of Nestegis and Notelaea species. Nuclear-based phylogenetic trees also agree with this finding (Dupin et al., 2020; Li et al., 2020; Olofsson et al., 2019). Morphologically, Asian Osmanthus species are different from species of the NNPP clade and O. austrocaledonicus in their inflorescences being cymose, axillary, with corollas imbricate in bud, as well as wood with a torus (Dute et al., 2010; Li et al., 2020). Therefore, based on molecular, morphological, and biogeographical evidence, we recommend exclusion of O. austrocaledonicus from Osmanthus to ensure the monophyly of the genus.

Other similarities and inconsistencies are apparent when comparing our findings with previous taxonomic research (Chang et al., 1996; Green, 1958) based on purely morphological characters, which divided Osmanthus into three sections (O. sect. Linocieroides [only one species O. didymopetalus]; O. sect. Siphosmanthus [O. delavayi and O. suavis]; and O. sect. Osmanthus [all remaining species]). First, our new phylogeny, as well as previous molecular studies (Li et al., 2020), found that O. sect. Linocieroides should be merged into O. sect. Osmanthus. Second, O. serrulatus and O. yunnanensis were classified into O. sect. Osmanthus in previous morphological studies (Chang et al., 1996; Green, 1958). Now, the sister relationship of this lineage between the O. sect. Osmanthus have received low support in our phylogenetic studies. This suggests that plastomes weakly support the inclusion of this lineage in O. sect. Osmanthus. We also found morphological evidence, such as bracts ciliate, leaf venation reticulate, and spring...
FIGURE 4  (a) Comparison of nucleotide variability (Pi values) in Osmanthus plastomes. (b) Comparison of substitution ratio (Ka/Ks) of 80 common genes in Osmanthus plastomes. (c) Comparisons of divergence of Pi among genes for photosynthesis, self-replication genes, and other genes in Osmanthus. (d) Comparisons of divergence of Ks/Ks among genes for photosynthesis, self-replication genes, and other genes in Osmanthus.
flowering, which are significantly different from other species in O. sect. Osmanthus (Chang et al., 1996). Third, O. decorus, previously considered as belonging to O. sect. Osmanthus, was distinguished as a distinct lineage sister to all other Osmanthus species in the phylogeny, and is here termed the Caucasian Osmanthus clade until a more comprehensive phylogenetic study can be done to determine its subgeneric ranking. The Caucasian Osmanthus clade is noted for its unique geographical distribution: O. decorus is the only representative of Osmanthus in West Asia and has been considered as a tertiary relict species from high elevations of the Caucasus region (Browicz, 1989; Manvelidze et al., 2010; Melia et al., 2012). Fourth, the Core Osmanthus clade, aside from the addition of O. didymopetalus and removal of O. decorus and "O. serrulatus + O. yunnanensis," is largely consistent with that delineated in previous taxonomic research. As delimited here, the Core Osmanthus clade contains the largest number of species and characterized by short corolla tubes (≤5 mm) and long leaf length (7–14 cm), which occupy lower elevations (mean elevation ca. 500 m) and are all endemic to East Asia. Fifth and lastly, our findings did corroborate the previous delimitation of O. delavayi and O. suavis as belonging to a distinct lineage within Osmanthus, which we here termed the "Siphosmanthus clade" until a more comprehensive phylogenetic study has been done to determine the subgeneric ranking of this lineage. The Siphosmanthus clade is resolved in a grade between the Caucasian Osmanthus and the other East Asian species, with strong branch support. This lineage is morphologically distinct from other Osmanthus species in having long corolla tubes (6–10 mm) and short leaf lengths (1–7 cm). In addition, they are the species that occupy the highest elevations (mean elevation ca. 2000 m) (Chang et al., 1996).

4.2 | Molecular dating of Osmanthus

Molecular dating shows that the MRCA of Osmanthus and the NNPP clade occurred at ca. 15.83 Ma (during the Langhian stage in the middle Miocene). The LTT plot (Figure 2b) suggests that NNPP clade speciation occurred slightly earlier (ca. 1 Ma) than that of the Osmanthus clade. Ample evidence suggests that Oleaceae exhibited greater diversity in Europe during the tertiary (Sachse, 2001; Srivastava et al., 2015). After the Oligocene, the Tethys or Tethys Ocean that was found between Gondwana and Laurasia, and influenced global oceanic circulation and climate patterns, climate gradually became cool and arid, with many temperate floral elements increasing in the ancient Mediterranean flora. Analysis of Miocene pollen and leaf fossils in Western Europe has shown that plant diversity in the temperate zone increased significantly compared with that in the Eocene, some modern Mediterranean elements such as Olea and Phillyrea beginning to appear during this period (Sachse, 2001; Sun & Li, 2003; Sun et al., 2017; Torfstein & Steinberg, 2020). The crown age of the genus Osmanthus is here estimated to have appeared ca. 12.66 Ma (during the Langhian stage in the middle Miocene). Despite the lack of direct fossil evidence of Osmanthus, the Tethyan relict of O. decorus is supported by multiple studies (Browicz, 1989; Manvelidze et al., 2010; Melia et al., 2012; Sachse, 2001), and thus agrees with the results of our molecular clock. Within the genus, most species of Osmanthus appeared after the middle Miocene and originated ca. 4 Ma more recently than those of the NNPP clade. Two steps of species accumulation were depicted in the LTT plot (Figure 2b), which occurred at ca. 8 and 6 Ma, respectively, corresponding to the formation of the high- and low-elevation species of Osmanthus. The emergence of the two high-elevation clades (O. delavayi + O. suavis and O. serrulatus + O. yunnanensis), which are distributed in the Hengduan Mountains, mainly occurred in the late Miocene.

4.3 | Evolutionary shifts of elevational range and flowering time

Integrating the results of molecular dating and stochastic character/state mapping, we find that Osmanthus originated at low elevations in the middle Miocene, then higher-elevation species began to appear in the Hengduan Mountains in the late Miocene. According to geological evidence, orogenesis was still occurring on the Qinghai–Tibet Plateau and its adjacent areas such as the Hengduan Mountains during this period. Recent studies have suggested that orogenesis was one of the important driving forces for the increase in species diversity in this region (Xing & Ree, 2017; Ye et al., 2019). Thus, we speculate that the emergence of high-elevation species in Osmanthus is closely related to the uplift of the Tibetan Plateau.

The ancestors of Osmanthus were presumed to be spring-flowering species, while autumn-flowering species are only found in the Core Osmanthus clade, which are distributed in southeastern China and Japan. Research has found that species flowering times do shift with climate change to optimize reproductive strategies (Bucher & Römermann, 2020; Gaudinier & Blackman, 2020; Janet, 2020). Abiotic factors such as droughts and seasonal precipitation are also considered as potentially selective forces on flowering phenology (Franks et al., 2007; Lesica & Kittelson, 2010). In the pPCA analysis, we found that spring- and autumn-flowering species were clearly distinguished along the PC1 axis. This indicates ecological niche divergence between spring- and autumn-flowering plants. From the perspective of geographical distribution, the spring-flowering lineages of Osmanthus are native to the Caucasus region and Hengduan Mountains, both of which have their lowest precipitation in spring (March to April) (Yang et al., 2009). On the other hand, the autumn-flowering lineages are native to southeastern China and Japan where the lowest precipitation is usually in autumn and winter (September to February) (Yao et al., 2017). Based on this, we hypothesize that low precipitation might create relatively favorable conditions for flowering and pollination. Some cases have shown that increased precipitation may alter the flowering time and increase the risk of pollen degradation and nectar dilution (Lawson & Rands, 2019). Although the relationship between flowering phenology and environmental conditions is systematic and complex, our research provides new insights into the adaptation of species to different environments in eastern Asia.
4.4 | Conserved evolution of *Osmanthus* plastomes

*Osmanthus* species displayed the same genetic composition and high collinearity, with no recombination or inversion being found. Most of the genetic regions exhibited low nucleotide variability (Pi < 0.004) and almost all the nucleotide variations were non-synonymous substitutions. This indicates that genetic variation within *Osmanthus* is very limited. When comparing between genes, we found that the ycf1 gene harbored the most nucleotide variability across *Osmanthus* species. It is worth noting that many recent studies have found ycf1 to be taxonomically informative in phylogenetic research (Dong et al., 2015; Liu et al., 2010), thus making it a good candidate marker as an *Osmanthus* plant barcode.

By calculating the nucleotide substitution rate, we found the Ka/Ks values of 80 common genes were less than 1. This indicates that all the genes are undergoing strong purifying selection, which was expected for genes under strong functional constraints like photosynthesis. Purifying selection usually reduces genetic diversity via selective removal of alleles that are deleterious (Cvijović et al., 2018). More exactly, the functional importance of a protein is a major determinant of its evolutionary rate (Zhang & Yang, 2015). Our Ka/Ks pairwise calculation did not detect the signal of positive gene selection in specific species groups (high elevation vs. low elevation; spring flowering vs. autumn flowering). This indicates that the plastid genes are likely to not be significantly involved in adaptation to altitude or precipitation. Thus, a nuclear genome-wide or transcriptome approach is necessary to study selection pressures on *Osmanthus* species.

5 | CONCLUSION

This is the first well-sampled report on the plastomes of *Osmanthus*. Comparative genomic analysis revealed conserved genome structures and low nucleotide polymorphism, although identifying ycf1 as a phylogenetically informative marker that could be used in future studies to gain a more comprehensive overview of *Osmanthus* systematics and identification. Our phylogenetic analysis partially supports previous morphological classification systems including the distinctiveness of taxa belonging to *O*. sect. Siphosmanthus and *O*. sect. *Osmanthus*. We also provide new insights, such as *O*. decorus found to be an independent lineage and sect. *Linocieroides* was found to belong within sect. *Osmanthus*. The phylogenetic relationships between species are also much clearer than those resolved in previous studies. Molecular dating of each lineage was also estimated for the first time using two secondary calibration points, with the results suggesting that *Osmanthus* is a tertiary relict genus. The origin and diversification of *Osmanthus* species is speculated to be closely related to the climatic changes and the orogenesis of the Qinghai-Tibet Plateau during the Miocene. Analysis of character evolution revealed the ancestral states of spring-flowering time and low-elevation distribution in the genus. Differentiation of spring- and autumn-flowering time in *Osmanthus* was also found to be related to the differences in ecological niche between species. The evolutionary rates of *Osmanthus* plastomes are very conservative, and so are unable to provide effective genetic information for the study of adaptive evolution. Our results provide a framework for the further study of the systematics and historical biogeography of *Osmanthus*, including formalizing a subgeneric classification of *Osmanthus*. A comprehensive molecular sampling of all species should now be focused on in order to achieve these aims.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Yongfu Li: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (lead); Project administration (lead); Resources (lead); Software (lead); Validation (lead); Visualization (lead); Writing – original draft (equal); Writing – review & editing (equal). Xuan Li: Formal analysis (equal); Resources (equal).

Steven Paul Sylvester: Writing – original draft (equal); Writing – review & editing (equal). Min Zhang: Software (supporting). Xianrong Wang: Funding acquisition (lead); Supervision (lead). Yifan Duan: Funding acquisition (supporting); Supervision (supporting).

DATA AVAILABILITY STATEMENT

The *Osmanthus* plastomes generated in this study are available in the NCBI GenBank repository: *O*. armatus (MW648824), *O*. cooperi (MW727458), *O*. fordii (MW727462), *O*. enervius (MW727461), *O*. fortunei (MW727463), *O*. heterophyllus (MW727464), *O*. ser. rutilus (MW727466), *O*. yunnanensis (MW727465), *O*. delavayi (MW727460), *O*. suavis (MW727467), and *O*. decorus (MW727459). Aligned DNA sequences in FASTA format are deposited in the Dryad repository (https://doi.org/10.5061/dryad.bcc2fzqf6).

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