Molecular phylogenetic analyses based on the complete plastid genomes and nuclear sequences reveal *Daphne* (Thymelaeaceae) to be non-monophyletic as current circumscription

Shiou Yih Lee a,*, Ke-Wang Xu b, Cui-Ying Huang a, Jung-Hyun Lee c, Wen-Bo Liao a, Yong-Hong Zhang d,**, Qiang Fan a,*

a State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, 510275, Guangzhou, China
b Co-Innovation Center for Sustainable Forestry in Southern China, College of Biology and the Environment, Key Laboratory of State Forestry and Grassland Administration on Subtropical Forest Biodiversity Conservation, Nanjing Forestry University, 210037, Nanjing, China
c Department of Biology Education, Chonnam National University, 61186, Gwangju, Republic of Korea
d School of Life Sciences, Yunnan Normal University, 650001, Kunming, China

* Corresponding author. ** Corresponding author.
E-mail addresses: daphnecn@aliyun.com (Y.-H. Zhang), fanqiang@mail.sysu.edu.cn (Q. Fan).

Article history:
Received 29 April 2021
Received in revised form 27 October 2021
Accepted 2 November 2021
Available online 11 November 2021

Keywords:
Daphne
Comparative plastome analysis
Internal transcribed spacer region
Polyphyletic relationship
Wikstroemia

**Abstract**

The diverse members of the genus *Daphne* are prized for their fragrant flowers. Despite being promising ornamental plants in many countries, genetic information of *Daphne* is scarce. In this study, the plastomes of four species and one variety of *Daphne* were sequenced and analyzed. The plastomes were typical and contained a pair of inverted repeat (IR) regions that separated the large single-copy (LSC) region from the small single-copy (SSC) region. With a length ranging from 132,869 bp (*D. genkwa*) to 174,773 bp (*D. championii*), 106 to 141 genes were predicted. Comparative plastome analysis of the newly sequenced plastomes with four publicly available *Daphne* plastomes identified an expansion of the IRs, sequence variations, and mutational hotspots. Phylogenetic analyses indicated that the genus *Daphne* in its current circumscription is polyphyletic. *Daphne genkwa* was nested within the genus *Wikstroemia*, while *D. championii* was well resolved as sister to *Edgeworthia*. These findings concurred with results from our study that used nuclear ribosomal internal transcribed spacer sequence data. The conflicts on the molecular placement of *D. championii* and *D. genkwa* and the present taxonomic classification in *Daphne* suggest that a new intergeneric classification system of *Daphneae* warrants consideration.

Copyright © 2021 Kunming Institute of Botany, Chinese Academy of Sciences. Publishing services by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

The family Thymelaeaceae comprises 45 genera and about 800 species widely distributed in both temperate and tropical regions (Herber, 2003). Recent taxonomic work on Thymelaeaceae has divided the family into two subfamilies: Octolepidoideae and Thymelaeoideae (Herber, 2003). The latter includes the tribes Aquilarieae, Daphneae, and Synandrodaphneae. The tribe Daphneae, synonym Thymelaeoideae *sensu Domke* (1934), is further subdivided into four groups: the *Daphne*, Gnidia, Linos- toma, and Phaleria groups. Members of the Daphne group can be easily distinguished by their lack of interxylary phloem; also, no cladograms on *Daphne* have been recorded in the Daphne group: *Daphne* L., *Daphnopsis* Mart. & Zucc., *Diarthron* Turcz., *Dirca* L., *Edgeworthia* Meisn., *Funifera* Leandro ex C.A. Mey., *Goodallia* Benth., *Lagetta* Juss., *Ovidia* Meisn., *Rhamnnoneuron* Gilg., *Schoenobibulus* Mart. & Zucc., *Stellera* L., *Thymelaea* Mill., and *Wikstroemia* Endl. (Herber, 2003).

The genus *Daphne* is one of the largest genera in the Daphne group, comprising ca. 95 species of well-known ornamental plants distributed in Eurasian and North African (Herber, 2003; Wang et al., 2007a). Thus far, the International Union for Conservation...
of Nature (IUCN) has only addressed the conservation status of six Daphne species, classifying Daphne rodriguezii Texidor and D. sophia Kolenicz. as “Endangered”, D. ludlowii D.G. Long & Rae and D. petraea L. as “Least Concern”, and D. altaica Pall. and D. arbuscular Celak. as “Data Deficient” in the IUCN Red List (IUCN, 2020). Because Daphne spp. are commonly cultivated in parks and gardens (Brickell and Mathew, 1998) and are known to have medicinal properties (Sovrlić and Manojlović, 2017), most studies of this genus have focused on either horticultural or medicinal properties. Although genetic studies have been conducted on Daphne jezoensis Maxim., D. laureola L., and D. rodriguezi (Alonso and Herrera, 2011; Castilla et al., 2012; Kameyama and Hiaro, 2014; García-Verdugo et al., 2019), species demarcation in Daphne is based solely on floral characteristics (Wang et al., 2007a). However, Daphne species are morphologically similar, suggesting that molecular approaches are required to elucidate their taxonomy.

The circumscription of Daphne, as well as its phylogenetic relationship with allied genera, has long been controversial. For instance, the boundary between Daphne and Wikstroemia remains in dispute (Hamaya, 1963; Wang et al., 2007a), prompting some to propose transferring Wikstroemia into Daphne and further treating it as a subgenus (Halda, 1999, 2001; Wang et al., 2007a; Zhang et al., 2007). The ambiguity in phylogenetic relationships is largely because the features traditionally used to distinguish species (i.e., the shape of hypogynous disc, the type of fruit, and the leaf arrangement) are exhibited across the genus (Domke, 1934; Hamaya, 1963; Halda, 1999; Wang et al., 2007b), and because of the lack of molecular resources to test phylogenetic inferences. Although molecular studies have been conducted on Thymelaeaceae at the genus-level, these studies have mostly focused on the subfamily Thymelioideae, with particular emphasis on a few genera (Gnidia L., Passerina L. mainly from South Africa, Thymelaea from circum-Mediterranean area, and Pimelea Banks ex Sol. from the Asia—Pacific region) (Van der Bank et al., 2002; Galicia-Herbada, 2006; Beaumont et al., 2009; APG IV, 2016; Foster et al., 2018). Such inadequate taxonomic sampling has failed to yield insights into phylogenetic relationships within Daphne or between Daphne and other related genera (Galicia-Herbada, 2006; Wang et al., 2007b; Beaumont et al., 2009; Zhang et al., 2010).

The plastid genome, or plastome, is an ideal tool for molecular taxonomic studies. Plastomes are small, haploid, inherited unparentally, possess low nucleotide substitution rates, and have highly conserved sequences. Nuclear ribosomal internal transcribed spacer (nrITS) regions, which unlike chloroplast DNA are inherited biparentally, are also known to be useful in assessing genetic variation and reconstructing phylogenetic relationships between closely related species (Neves and Forrest, 2011). For members of Thymelaeaceae, publicly available nrITS sequences far outnumber plastome sequences, likely because nrITS regions are easier to amplify (Alvarez and Wendel, 2003). Used in conjunction, plastid genomes and nrITS sequences promise to advance our understanding of the phylogenetic relationships of Daphne in Thymelaeaceae.

To test the monophyly of Daphne, we first assembled complete plastomes of five Daphne taxa, namely D. championii, D. genkwa Siebold & Zucc., D. kiusiana var. atrocaulis (Rehder) F. Maek., D. odora Thumb., and D. papyracea Wall. ex G. Don. Although these five Daphne species grow in the wild and are domesticated for medicinal or ornamental purposes, their respective genetic identities still remain ambiguous. We analyzed and compared these Daphne plastomes to published plastomes to determine the molecular identities, genetic divergence, and phylogenetic relationships of these species. We also identified highly variable gene regions that may be useful DNA barcodes for Daphne species. The findings of this study will provide future reference for taxonomic studies of Daphne and help elucidate the taxonomy of Thymelaeaceae.

2. Material and methods

2.1. Plant materials and DNA extraction

Fresh leaves from five Daphne taxa were collected from natural populations and arboreta in China. Samples of D. odora were collected from the Germplasm Resource Nursery of Ornamental Plants of Guangzhou Institute of Forestry and Landscape Architecture in the Guangdong Province, while D. kiusiana var. atrocaulis and D. papyracea were collected from their natural habitat in Mount Luoxiao, Hunan Province and Mount Yunkai, Guangdong Province, respectively. D. championii was collected from Lianzhuo, Guangdong Province, and D. genkwa was collected from Mount Tantai, Hubei Province and Anqing, Anhui Province. Leaves were stored with silica gel in aluminum sealed ziplock bags until DNA extraction. The voucher specimens were deposited at the Herbarium of Sun Yat-sen University (SYS) and Herbarium of Yunnan Normal University (YNUB) (Table 1).

Total genomic DNA extraction was carried out using the DN15-Plant DNA Mini Kit (Aidlab, China) according to the manufacturer’s protocol. The quantity and quality of the DNA extracts for next-generation and Sanger sequencing were determined using Qubit™ 4 Fluorometer (Thermo Fisher Scientific, USA) and Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA), respectively.

2.2. Plastid genome sequencing, assembly, and annotation

A ~350-bp insert size genomic library was prepared using a TruSeq DNA Sample Prep Kit (Illumina, USA) and sequencing was conducted on an Illumina NovaSeq platform (Illumina, USA) to obtain 6 Gb of 150-bp pair-end reads. Adapter sequences were removed using NGSToolkit (Patel and Jain, 2012), and the raw reads were fed into the NOVOPlasty 2.7.2 pipeline (Dierckxsens et al., 2017) for de novo assembly. Using the rbcl sequences for Daphne papyracea (LC527404) as the seed sequence, a single contig was obtained at the end of the process for each taxon. The assembled genomes were annotated through the online GeSeq annotation tool (Tillich et al., 2017) and manually checked for errors. The GC content was analysed using MEGA 7 (Kumar et al., 2016) and the circular map of each plastome was created in OGDRAW 1.3.1 (Greiner et al., 2019). The plastome sequences were deposited in the NCBI GenBank database under the accession numbers MT648376 (D. championii), MN563133 (D. genkwa), MT627481 (D. kiusiana var. atrocaulis), MT627479 (D. odora), and MT627480 (D. papyracea).

2.3. Sequence repeats, codon usage, and RNA editing site prediction

Forward, reverse, and palindromic repeat sequences were identified using REPutter (Kurtz et al., 2001), with the Hamming distance set at 3 and the minimum repeat size at 30 bp. The nucleotide sequence of each protein-coding gene in the plastome was extracted for subsequent analyses using FeatureExtract 1.2 Server (Wernersson, 2005). The relative synonymous codon usage (RSCU) for each protein-coding gene was calculated using the Codon Usage Calculator function available in the Sequence Manipulation Suite (Stothard, 2000), and the potential RNA editing sites were predicted using the PREP-Cp function available in the PREP Suite (Mower, 2009) based on default settings.
2.4. Genetic pairwise distance, IR border analysis, and genome comparison

The plastome sequences of four *Daphne* species (*D. kiussiana* var. *kiussiana* (KY991380), *D. giralddi* (MN0080709), *D. laureola* (MN201546), and *D. tangutica* (MK455900)) were downloaded from NCBI GenBank and were included in subsequent analyses. All nine plastomes were aligned using MAFFT (Katoh et al., 2019) and genetic pairwise distance was calculated based on the Kimura 2-parameter DNA substitution model with 1000 bootstrap replicates, using MEGA7 (Kumar et al., 2016). Gaps and missing data in the alignment were not included in the analyses (complete deletion). The borders of the four different regions in the plastomes (large single-copy (LSC), small single-copy (SSC), and inverted repeat A and B (IRA and IRB)) of the nine *Daphne* species were plotted using IScopeto analyse the exact IR border positions and identify adjacent genes (Amiryousefi et al., 2018). The nine plastomes were also aligned and visualised using mVISTA program (Frazer et al., 2004) in Shuffle-LAGAN mode, using *D. laureola* (MN201546) as the reference genome.

2.5. Polymerase chain reaction (PCR) and Sanger sequencing

Three to four biological replicates from each species were selected for Sanger sequencing to obtain their nrITS sequences. PCR amplification of the nrITS region was carried out using primers ITS-p5: 5′-CCT TAT TAT CAY TTA GAA GAA GGA G-3′ (Cheng et al., 2016) and ITS-S3R: 5′-GAC CGT TCT CCA GAC TAC AAT-3′ (Chiou et al., 2007). PCR was conducted in a final reaction volume of 20 μL, containing 10 μL of 2× Taq PCR Starmix with loading dye (Genstar Biosolutions, China), 0.4 μM of each primer, and 20 ng of genomic DNA as template. PCR amplification was conducted in a T100™ Thermal Cycler (Bio-Rad, USA), with thermal settings programmed as follows: initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. PCR products were sent for direct sequencing for both ends on an ABI 3730 DNA Analyser (Applied Biosystems, USA).

2.6. Phylogenetic inference

Prior to phylogenetic analyses, all nine *Daphne* plastome sequences and the nrITS sequences of 39 accessions representing 17 *Daphne* taxa were aligned using MAFFT (Katoh et al., 2019), separately. Based on the findings of Lee et al. (2018), *Eucalyptus grandis* (Myrtaceae; HM347959 and AF390472) and *Gossypium hirsutum* (Malvaceae; DQ345959 and KC404827) were included as outgroups. Both alignments were trimmed using trimAl v1.2 (Capella-Gutiérrez et al., 2009) with the gappput method to reduce the systematic errors produced due to poor alignment. Phylogenetic analyses of plastome sequences were carried out using maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood (ML) tree analysis was conducted with RAxML (Stamatakis, 2014), available in the CIPRES Science Gateway (Miller et al., 2010), and using the general time-reversible (GTR) with gamma distribution (+G)(=GTR+G) nucleotide substitution model and 1000 bootstrap replicates for each branch node. Bayesian inference (BI) tree analysis was conducted using MrBayes (Ronquist et al., 2012), available in the CIPRES Science Gateway (Miller et al., 2010), based on default parameters, with minor adjustments: a mixed substitution type (Nst) was selected for the likelihood model and 2,000,000 generations were set for the Markov chain Monte Carlo (MCMC), with data sampling collected every 100 generations. The final tree results from both analyses were visualized using FigTree (Rambaut, 2018). Phylogenetic analyses of nrITS sequences was carried out using ML and maximum parsimony (MP) methods that are embedded in MEGA7 (Kumar et al., 2016). The optimum DNA substitution model for the ML tree was calculated using the “Find Best DNA/Protein Model (ML)” function available in MEGA 7 and ML tree was constructed using the GTR + G with invariant included (+I)(GTR + G + I) nucleotide substitution model and 1000 bootstrap replicates for each branch node. The MP tree was constructed by means of 1000 bootstrap replicates, under the subtree-pruning-regrafting search method. Gaps and missing data were included in the construction of both trees.

2.7. Identification of divergence hotspot and potential DNA barcoding regions

To detect the nucleotide variability (Pi) in the *Daphne* plastomes, the plastome sequences were aligned using MAFFT (Katoh et al., 2019). Analyses on singleton variable sites and sliding windows, with 500-bp step size and 1000-bp window length, were performed on DnaSP v5.1 (Librado and Rozas, 2009).

3. Results

3.1. Plastid genome features of five *Daphne* species

The total plastome sizes for the five *Daphne* taxa ranged from 132,869 (D. genkwa) to 174,773 bp (D. championii) (Fig. 1). All five plastomes exhibit a typical quadripartite structure consisting of a

| Species (Sample number) | Collector name and voucher record | Number of individuals examined | Sampling location | GenBank accession numbers | Complete chloroplast genome | nrITS |
|-------------------------|-----------------------------------|-------------------------------|------------------|--------------------------|-----------------------------|------|
| *Daphne championii* (1–4) | Shiou Yih Lee & Xinjian Zhang, LSY-THY-4001 | 4 | Lianzhou, Guangdong, China | MT648376 | MT623676-MT623679 |
| *Daphne genkwa* | Yonghong Zhang, SBK12 | 1 | Mount Tiantai, Hubei, China | MN563133 | – |
| *Daphne genkwa* | Shiou Yih Lee, LSY-THY-4013 | 4 | Anqing, Anhui, China | – | MT623680-MT623683 |
| *Daphne kiussiana* var. *kiussiana* (1–4) | Jung-Hyun Lee, LJH-GU01 | 4 | Ryushosan, Kumamoto, Japan | – | MT623684-MT683687 |
| *Daphne kiussiana* var. *kiussiana* (5–8) | Sinpyeong goj-jawal, Jeju-do, Korea | 2 | | – | |
| *Daphne odora* (1–4) | Wenbo Liao, LPX6488 | 3 | Mount Luoxiao, Hunan, China | MT627481 | MT623692-MT623694 |
| *Daphne papyracea* (1–4) | Shiou Yih Lee & Zhihu Chen, LSY-THY-4005 | 4 | Guangzhou Institute of Forestry and Landscape Architecture, Guangdong, China | MT627497 | MT623695-MT623698 |

Table 1
General information and NCBI GenBank accession numbers of eight *Daphne* taxa used in this study.
pair of IRs (9372–43,948 bp) that separate the LSC (84,681–85,728 bp) and SSC (1557–28,397 bp) regions. Total plastome gene number ranged from 106 (D. genkwa) to 141 (D. championii). Plastomes included from 71 (D. genkwa) to 95 (D. championii) protein-coding genes, 31 (D. genkwa) to 38 (D. championii, D. odora, and D. papyracea) tRNA genes, and four (D. genkwa) or eight (D. genkwa, D. kiusiana var. atrocaulis, D. odora, and D. papyracea) rRNA genes. Of these genes, four are duplicated in the plastome of D. genkwa, while 28 are duplicated in the plastomes of D. kiusiana var. atrocaulis, D. odora, and D. papyracea, and 31 are duplicated in the plastome of D. championii, all in the IR regions. The GC content of the plastomes ranged from 36.3% (D. genkwa) to 36.8% (D. papyracea).

3.2. Large repeats analyses

Large repeats were detected in the plastomes of all five Daphne taxa, including between 24 (D. genkwa) and 26 (D. championii and D. kiusiana) forward repeats and between 16 (D. genkwa) and 25 (D. odora) palindrome repeats (Fig. S1). Only four reverse repeats were recorded, all in the plastome of D. genkwa, and no complementary repeats were detected.

3.3. Relative synonymous codon usage and predicted RNA editing sites

The protein-coding sequences of Daphne plastomes had between 24,654 (D. genkwa) and 34,003 (D. championii) codons (Table S1). Among the encoded amino acids, leucine was most frequent in the plastomes of D. genkwa, D. kiusiana var. atrocaulis, D. odora, and D. papyracea, and serine was most frequent in plastomes of D. championii and D. papyracea. While methionine was least frequent in the plastomes of D. championii, D. odora, and D. papyracea, tryptophan was the least frequent in the plastomes of D. genkwa and D. kiusiana var. atrocaulis. Between 56 (D. genkwa) and 66 (D. kiusiana) potential RNA editing sites were predicted from the protein-coding genes of Daphne plastomes (Fig. 2). The most frequent amino acid conversion in all five Daphne species was serine-to-leucine (S-L). The least frequent amino acid conversions (i.e., occurred only once) differed among Daphne species. For instance, the least frequent amino acid conversion in D. championii was arginine-to-cysteine (R-C); in D. genkwa it was arginine-to-tryptophan (R-W). D. kiusiana var. atrocaulis had three one-time amino acid conversions (alanine-to-valine (A-V), R-C, and R-W), whereas D. odora (R-C and R-W) and D. papyracea (A-V and R-C) each had two.

3.4. Plastome variations

Genetic pairwise distance based on plastome sequences of nine Daphne taxa was highest between D. championii and D. genkwa (0.0338), whereas the genetic distance was lowest between D. kiusiana var. kiusiana and D. kiusiana var. atrocaulis (0.0003) (Table 2). IR border analysis indicated that in all Daphne species except D. championii, rps19 and rpl2 genes were adjacent to the LSC/
IRb junction (JLB); in *D. championii* rpl16 is located at the JLB (Fig. 3). *NdhF* is located at the SSC/IRb junction (JSB) in all *Daphne* species except in *T. kiusiana* var. *atrocaulis* and *D. papracea*, the *ndhF* gene is located adjacent to the JSB; in *D. genkwa* the gene adjacent to the JSB is ycF2, which is located in the IRb region. In the other *Daphne* species the ycF2 gene is adjacent to the SSC/IRA junction (JSA) in the IRa region, whereas the rpl32 gene is adjacent to the JSA within the SSC. In all *Daphne* taxa, the trnH gene is located in the LSC region adjacent to the LSC/IRA junction (JLA). In *D. championii* the rpl2 gene located in the IRA region adjacent to the JLA is not present; instead the rps3 gene is located adjacent to the JLA.

The plastome alignment of nine *Daphne* taxa, with the plastome sequence of *D. laureola* as the reference genome, revealed high sequence conservatism across the plastomes of six *Daphne* taxa, including *D. giralldii, D. kiusiana* var. *atrocaulis, D. kiusiana* var. *kiusiana, D. odora, D. papracea*, and *D. tangutica* (Fig. 4). Hypervariable regions in the form of continuous distinct small gaps were detected in the LSC region of *D. championii* and *D. genkwa*, while three large gaps were detected in the SSC region of *D. genkwa*, when compared to *D. laureola*.

### 3.6. Divergence hotspots

Because our phylogenetic analysis placed *Daphne championii* and *D. genkwa* in unexpected positions, we excluded these species from our efforts to identify divergence hotspots. Genome alignment of seven species of *Daphne* indicated that Pi-values ranged from 0 to 0.02376 and had an average Pi-value of 0.00379. Two gene regions recorded Pi-values greater than our cut-off point (>0.015; Fig. S2). The two highly divergent regions were located at the psal gene region (61,755 to 62,934 bp) of the LSC and the *ndhF*-rpl32 gene region (130,710 to 133,470 bp) of the SSC.

### 3.5. Phylogenetic Inferences

ML and BI analyses based on the complete plastome sequences of nine *Daphne* taxa strongly suggested a paraphyletic relationship within *Daphne*, with three well-supported clades (BS > 95%; PP > 0.90) (Fig. 5). *Daphne championii* formed an independent clade and was sister to a species of another genus, *Edgeworthia chrysantha* Lindl. *D. genkwa* clustered with the *Wikstroemia* clade, sister to *Wikstroemia indica* (L.) C.A. Mey. *D. kiusiana* var. *atrocaulis, D. odora*, and *D. papracea*, along with the four other *Daphne* species, formed a monophyletic group. ML and MP analyses based on nrITS sequences also revealed a paraphyletic relationship in *Daphne* (Fig. 6). The ML tree had a reliable backbone that was well-supported (BS > 75%) for its major clades (Fig. 6a), but the major clades on the backbone of the MP tree was only partially supported (BS > 75%) (Fig. 6b). Both ML and MP trees revealed similar clustering patterns for the five *Daphne* species. *D. championii* was sister to *E. chrysantha; D. genkwa* was nested within the *Wikstroemia* clade and was sister to *W. monnula* Hance; and *D. kiusiana* var. *atrocaulis, D. odora*, and *D. papracea* formed a monophyletic clade with the other 12 *Daphne* species.

### 4. Discussion

#### 4.1. Gene Variations Due to IR Contraction and Expansion

This study is the first to describe plastome sequences of *Daphne championii, D. genkwa, D. kiusiana* var. *atrocaulis, D. odora*, and *D. papracea*. The total number of genes present in the plastomes ranges between 135 and 141 except for in *D. genkwa*, which has only 106. In addition, *D. genkwa* has an IR 4.5 times shorter than that of the average IR length in *Daphne* species. Furthermore, *D. genkwa* has an SSC region approximately 12 times longer than that of the average SSC of other *Daphne* taxa. Contraction and expansion at the IR borders are common during evolution and may cause variations in the size of each region or the plastome as a whole (Knox and Palmer, 1999). Analysis of gene order at the IR border allowed us to categorize the plastomes of the nine *Daphne* taxa examined into three types (Fig. 3) that we have named Type I, II, and III. Type I is the most common type and is found in the plastomes of *D. giralldii, D. kiusiana* var. *kiusiana, D. kiusiana* var. *atrocaulis, D. laureola, D. odora, D. papracea*, and *D. tangutica*. Type II is exclusive to *D. genkwa*, whereas Type III is exclusive to *D. championii*. Type I and Type III plastomes have only two genes in the SSC region. In contrast, Type II plastomes have 20 genes in the SSC region. The plastomes of other members of Thymelaeaceae (e.g., *Aquilaria malaccensis* Lam., *E. chrysantha*, *Stellera chamaejasme* L., and *Wikstroemia chamaeaphne* (Bunge) Mei.) exhibit gene content near the IR boundaries similar to that of Type I (Lee et al., 2021).

| Table 2: Interspecific pairwise distances of complete plastid genome sequences between nine *Daphne* taxa used in this study. |
|-------------------------------------------------------------|
| **Species**       | **DC** | **DGi** | **DKk** | **DKa** | **DP** | **DO** | **DT** | **DL** |
|-------------------|--------|---------|---------|---------|--------|--------|--------|--------|
| *Daphne championii* (DC) | 0.0295 |         |         |         |        |        |        |        |
| *Daphne giralldii* (DGi) | 0.0288 | 0.0051  |         |         |        |        |        |        |
| *Daphne kiusiana* var. *kiusiana* (DKk) | 0.0289 | 0.0052  | 0.0003  |         |        |        |        |        |
| *Daphne kiusiana* var. *atrocaulis* (DKa) | 0.0289 |         |         | 0.0017  | 0.0018 |        |        |        |
| *Daphne papracea* (DP) | 0.0286 | 0.0049  | 0.0016  | 0.0017  | 0.0016 |        |        |        |
| *Daphne odora* (DO) | 0.0285 | 0.0049  | 0.0015  | 0.0016  | 0.0014 | 0.0012 |        |        |
| *Daphne laureola* (DL) | 0.0286 | 0.0075  | 0.0069  | 0.0070  | 0.0068 | 0.0067 | 0.0066 |        |
| *Daphne genkwa* (DGc) | 0.0338 | 0.0229  | 0.0226  | 0.0226  | 0.0224 | 0.0222 | 0.0220 |        |
D. papyracea has since been proposed that in the plastome sequences of two accessions of the same species were higher than those reported in the plastome sequences of the inter- and intraspecific analysis have indicated that D. kiusiana and D. odora (Keissler, 1898; Rehder, 1916). However, it has since been proposed that D. kiusiana var. atrocaulis is closer to D. papyracea and D. bholua Buch.-Ham. ex D. Don than to D. odora and D. kiusiana var. kiusiana (Mathew, 1989). Taxonomic revisions of the D. kiusiana complex based on extensive morphological analysis have indicated that D. kiusiana var. kiusiana and D. kiusiana var. atrocaulis are distinct taxa (Wang et al., 2007a). We found that the inter- and intraspecific pairwise distance between the plastome sequences of D. odora and D. kiusiana complex were 0.0016 and 0.0017, respectively (Table 2). However, intraspecific pairwise analysis indicated that the genetic distance between D. kiusiana var. kiusiana and D. kiusiana var. atrocaulis was much smaller (0.0003). Although 64 singleton variable sites were detected between D. kiusiana var. kiusiana and D. kiusiana var. atrocaulis plastome sequences, the genetic distance between the two D. kiusiana varieties was much lower than the genetic distance between other species, e.g., Cycas debaoensis Y.C. Zhong & C.J. Chen (Cycadaceae; 0.0056) (Jiang et al., 2016). Moreover, the singletons found across two accessions of the same species were higher than those reported in the plastome sequences of Camellia japonica L. (Theaceae; 25 singletons) and Dysphania pumilio (R.Br.) Mosyakin & Clemants (Amaranthaceae; 25 singletons) (Park and Kim, 2019; Park et al., 2019). This low nucleotide variation and small genetic distance are consistent with our observation that D. kiusiana var. atrocaulis is homogeneous with its original.

NrITS sequence analysis established that the genetic distance between Daphne odora and D. kiusiana var. kiusiana from Japan, D. kiusiana var. kiusiana from Korea, and D. kiusiana var. atrocaulis were 0.0040, 0.0013, and 0.0013 respectively. The genetic distance between D. kiusiana var. atrocaulis and each the Japanese D. kiusiana var. kiusiana and the Korean D. kiusiana var. kiusiana was 0.0026 for both accessions; the genetic distance between the Korean D. kiusiana var. kiusiana and D. kiusiana var. atrocaulis was zero. Five singleton variable sites were detected in the plastome alignment of three D. kiusiana accessions. All these sites were detected in sequences of two D. kiusiana accessions and the number of sites detected in the sequences for both the Japanese and Korean D. kiusiana var. kiusiana against D. kiusiana var. atrocaulis were four and one, respectively.

Phylogenetic analysis based on plastome sequences indicated that Daphne kiusiana var. atrocaulis is a well-supported sister to D. kiusiana var. kiusiana (Fig. 5); however, phylogenetic trees based on nrITS sequence data indicated that the intraspecific relationships are not well-defined. Our analysis did not consistently recognize Daphne kiusiana var. kiusiana as a monophyletic group, although samples from Korea and Japan were unequivocally established as two distinct monophyletic clades. Furthermore, MP and ML analyses did not establish D. kiusiana var. atrocaulis as monophyletic; also, the relationship between the D. kiusiana complex and D. odora was not uniform in either tree (Fig. 6). Discrepancies between the nrITS trees are likely because the
computational approaches used for each model are influenced by evolutionary factors, including reversals, convergence, and homoplasys (Downie and Katz-Downie, 1996). The taxonomic status of the *D. kiusiana* complex derived from nuclear sequence data requires more extensive sampling and additional nuclear genes. Our phylogenetic analyses based on plastome sequences support morphological evidence that separates *D. kiusiana* from *D. odora*, although this finding is only partly supported by nrITS sequence data.

In our study, complete plastomes showed higher resolution in resolving species relationships than nrITS sequences (Figs. 5 and 6). This implies that super-barcoding (or ultra-barcoding) of *Daphne* species using complete plastome sequences is reliable and effective (Kane et al., 2012; Li et al., 2015). However, although the cost of next-generation sequencing has become affordable in many countries, performing high-throughput DNA sequencing for diverse genera, such as *Daphne*, may be cost-prohibitive. Therefore, a cost-effective alternative such as using selected highly polymorphic and

---

**Fig. 4.** Comparative plastid genome analysis of nine *Daphne* taxa using mVISTA under Shuffle-LAGAN mode. Figure legend describes the direction and types of gene regions using color codes. Probability threshold was set at 50% and the plastid genome of *Daphne laureola* (MN201546) was selected as the reference genome.
and were grouped within the section Genkwa species have long been considered morphologically, closely related to each other, taxonomists have highlighted several morphological features in the species Daphne championii Hooker, 1880, a grouping that is still generally accepted. However, genetic distances between D. championii and D. genkwa have been a topic of debate for quite some time (Dute et al., 1996). Despite being under the same genus, the interspecies discrimination rate should be a member of Genkwa (Bentham and Hooker, 1880), a grouping that is still generally accepted. However, taxonomists have highlighted several morphological features in D. championii and D. genkwa that raised doubts about this treatment. For instance, the presence of long styles and filaments as well as short and upright calyx teeth suggest that D. championii should be grouped in the genus Eriosolena (Domke, 1934). Daphne genkwa exhibit the opposite leaf arrangement, which rarely occurs among Daphne species, and have disks at the base of the floral tube that divide into individual scales or threads, suggesting that the members of this genus should be reassigned to Wikstroemia (Domke, 1934).

The taxonomic delimitation of Daphne and Wikstroemia is challenging, with many Wikstroemia species previously known as members of Daphne and vice versa (Wang and Gilbert, 2007a; Wang et al., 2007a). Although the major morphological features that delimit Daphne from Wikstroemia are the shape of its disk and type of fruit, these features are not consistent across a number of species in either genus (Zhang et al., 2016). Furthermore, anatomical features, such as the presence of tori in the intervascular pit membrane (Dute et al., 1996) and leaf epidermal microfeatures (Zhang et al., 2015), are also unsuitable to delimit the two genera because tori are found in both genera and there is no significant variation in leaf epidermal microfeatures between members of Daphne and Wikstroemia. The Daphne section Eriosolena, which was initially proposed to belong to the genus Daphne (Bentham and Hooker, 1880), is now considered a distinct genus Eriosolena in the family Thymelaeaceae (Wang and Gilber, 2007b). Molecular evidence from our study showed that the strong sister relationship between D. championii and E. chrysanthi has raised doubts about whether D. championii should be a member of Eriosolena. Researchers have proposed that Daphne can be delimited from the genera Eriosolena and Edgeworthia by the absence of bicollateral vascular bundles in their midribs (Domke, 1934).

The phylogenetic positions of D. championii and D. genkwa have been a topic of debate for quite some time (Dute et al., 1996). Despite being under the same genus, the interspecies discrimination rate should be a member of Genkwa (Bentham and Hooker, 1880), a grouping that is still generally accepted. However, taxonomists have highlighted several morphological features in D. championii and D. genkwa that raised doubts about this treatment. For instance, the presence of long styles and filaments as well as short and upright calyx teeth suggest that D. championii should be grouped in the genus Eriosolena (Domke, 1934). Daphne genkwa exhibit the opposite leaf arrangement, which rarely occurs among Daphne species, and have disks at the base of the floral tube that divide into individual scales or threads, suggesting that the members of this genus should be reassigned to Wikstroemia (Domke, 1934).

The phylogenetic relationships of Daphne and its allied genera in family Thymelaeaceae based on the plastid genome sequences of 18 taxa representing seven genera of the family Thymelaeaceae. The phylogenetic tree was constructed using both maximum likelihood (ML) and Bayesian inference (BI). All branch nodes were calculated with 1000 bootstrap replicates and reliable bootstrap supports (ML: BS ≥ 95%; BI: PP ≥ 90%) are indicated with an asterisk (*). Sequences obtained through this study are indicated in bold and two species, Eucalyptus grandis (HM347959) and Gossypium hirsutum (DQ345959), were included as outgroups.

4.3. Current circumscription of the genus Daphne is polyphyletic

Molecular evidence based on both plastome and nrITS sequences clustered Daphne genkwa within the Wikstroemia clade and removed D. championii from the Daphne clade, placing it into an independent clade that has an affinity with Edgeworthia chrysanthi. The phylogenetic positions of D. championii and D. genkwa have been a topic of debate for quite some time (Dute et al., 1996). Despite being under the same genus, the interspecies pairwise genetic distances between D. championii and D. genkwa were greater than those of other Daphne species (Table 2). The two species have long been considered morphologically, closely related and were grouped within the section Genkwa (Bentham and Hooker, 1880), a grouping that is still generally accepted. However, taxonomists have highlighted several morphological features in D. championii and D. genkwa that raised doubts about this treatment. For instance, the presence of long styles and filaments as well as short and upright calyx teeth suggest that D. championii should be grouped in the genus Eriosolena (Domke, 1934). Daphne genkwa exhibit the opposite leaf arrangement, which rarely occurs among Daphne species, and have disks at the base of the floral tube that divide into individual scales or threads, suggesting that the members of this genus should be reassigned to Wikstroemia (Domke, 1934).

The phylogenetic relationships of Daphne and its allied genera in family Thymelaeaceae based on the plastid genome sequences of 18 taxa representing seven genera of the family Thymelaeaceae. The phylogenetic tree was constructed using both maximum likelihood (ML) and Bayesian inference (BI). All branch nodes were calculated with 1000 bootstrap replicates and reliable bootstrap supports (ML: BS ≥ 95%; BI: PP ≥ 90%) are indicated with an asterisk (*). Sequences obtained through this study are indicated in bold and two species, Eucalyptus grandis (HM347959) and Gossypium hirsutum (DQ345959), were included as outgroups.
relationship between Eriosolena and Edgeworthia is supported by the absence of tori in Eriosolena wallichii Meisn., which was once considered a synonym of Eriosolena involucrata (Wall.) Tiegh. (synonym Eriosolena composita (L.f.). Merr.) and is reported to have identical wood features as Daphne pendula Sm. (synonym E. composita) (The Plant List, 2010, 2013) and Edgeworthia papyrifera Siebold & Zucc. (synonym E. chrysantha) (Dute et al., 1996). Unfortunately, studies on the presence of tori in D. championii are not available and molecular information of the monotypic genus Eriosolena is scarce. Therefore, work to verify the presence of tori in D. championii and DNA sequencing of E. composita would be useful in providing insights to the taxonomic status of D. championii.

4.4. Daphne and Wikstroemia are independent genera

The merging of Wikstroemia into Daphne was previously proposed based on traditional distinguishing characteristics (Halda, 2001); however, this treatment has not been universally accepted due to a lack of both morphological and molecular evidence, as well as the vast alteration on taxonomic nomenclature (Wang et al., 2007b; Zhang et al., 2007). Although our sample size was small, our results clearly demonstrate that Daphne and Wikstroemia are each monophyletic. Furthermore, molecular evidence in this study combined with previously identified morphological features
(Domke, 1934; Dute et al., 1996) support the separation of *D. championii* and *D. genkwa* from *Daphne*. Taken together, these findings imply that the taxonomic confusion in *Daphne*, *Wikstroemia* and allied genera may have been caused by long-term misplacement of certain problematic taxa. If *Daphne* and *Wikstroemia* are not sister groups, as shown by both plastome and nrITS trees, the combination of both genera is not suitable and should be excluded. On the contrary, the independent taxonomic status of *Daphne* and *Wikstroemia* should be retained. Reasonable transfer of *Daphne* species to *Wikstroemia*, and vice versa, based on the morphological characteristics and molecular evidence will be appropriate under current genera delimitation.

Finally, we would like to iterate that evolutionary assessments based on limited sampling sizes, either molecular or morphological, can be elucidated when the context of other biological attributes is duly considered. However, further research that includes more taxa from Thymelaeaceae is essential to validate these hypotheses.

**Author contributions**

S.Y.L, W.L and Y.Z conceived and designed this study. S.Y, C.H and J.H.L conducted the experiments. S.Y and K.K analyzed the data. S.Y wrote the manuscript. K.K, J.H.L, W.L and Q.F edited the manuscript. All authors read and approved the manuscript.

**Declaration of competing interest**

The authors declare that they have no conflict of interest.

**Acknowledgements**

This work was supported by the Fundamental Research Funds for the Central Universities (33000-31611215), the Guangzhou Science and Technology Program (201903010076) as well as the National Natural Science Foundation of China (31700048).

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2021.11.001.

**References**

Alonso, C., Herrera, C.M., 2011. Back-and-forth hermaphroditism: phylogenetic context of reproductive system evolution in subdiploic *Daphne laureola*. Evolution 65, 1680–1692. https://doi.org/10.1111/j.1558-5646.2011.01246.x.

Alvarez, I.F.E., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phyl. Genet. Evol. 21, 417–434. https://doi.org/10.1016/S1055-7903(03)00208-2.

Amourouset, A., Hyvönen, J., Pocial, P., 2018. iScope: an online program to visualize the junction sites of chloroplast genomes. Bioinformatics 34, 3030–3031. https://doi.org/10.1093/bioinformatics/bty220.

APC IV. 2016. An update of the Angiosperm phylogeny group classification for the orders and families of flowering plants: APC IV. Bot. J. Linn. Soc. 181, 1–20. https://doi.org/10.1111/boj.12385.

Beaumont, A.J., Edwards, T.J., Manning, J., et al., 2009. *Amiryouse*. Linn., in *The Genus in the Wild and in Cultivation*. Am. J. Bot. 96, 64–99.

Herber, B.E., 2003. *Thymelaeaceae*. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants, vol. 5. Springer, Berlin, pp. 373–396.

IUCN, 2020. The IUCN Red List of Threatened Species. Version 2020-1. https://www.iucnredlist.org. (Accessed 5 June 2020).

Kurtz, S., Choudhuri, J.V., Ohlebusch, E., et al., 2001. REPuter: the manifold application of repeat maskers for the genomes of organisms from lower eukaryotes to the human genome. Bioinformat. Bioinform. Biocomput. Bioinform. Biot. 3, 166–166. https://doi.org/10.1111/bib.1100570.

Keisser, K.V., 1898. Die Arten der Gattung *Daphne* aus der Section Daphnathes. Bot. Jahrb. Syst. 25, 29–125.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw121.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw121.

Kurtz, S., Choudhuri, J.V., Ohlebusch, E., et al., 2001. REPuter: the manifold application of repeat maskers for the genomes of organisms from lower eukaryotes to the human genome. Bioinformat. Bioinform. Biocomput. Bioinform. Biot. 3, 166–166. https://doi.org/10.1111/bib.1100570.

Keisser, K.V., 1898. Die Arten der Gattung *Daphne* aus der Section Daphnathes. Bot. Jahrb. Syst. 25, 29–125.

Kurtz, S., Choudhuri, J.V., Ohlebusch, E., et al., 2001. REPuter: the manifold application of repeat maskers for the genomes of organisms from lower eukaryotes to the human genome. Bioinformat. Bioinform. Biocomput. Bioinform. Biot. 3, 166–166. https://doi.org/10.1111/bib.1100570.

Keisser, K.V., 1898. Die Arten der Gattung *Daphne* aus der Section Daphnathes. Bot. Jahrb. Syst. 25, 29–125.
Sovrli, A., 2018. FigTree v1.4. http://tree.bio.ed.ac.uk/software/

Rehder, A., 1916. Thymelaeaceae. Pl. Wilson. 2, 530.

Qian, S., Zhang, Y., Li, G., 2020. The complete chloroplast genome of a medicinal plant. Phytochemistry, Biological and Pharmacological Activity. 1312–1313. https://doi.org/10.1016/j.bjpco.2019.10.003.

Stothard, P., 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques 28, 1102–1104. https://doi.org/10.2144/0026i002.

The Plant List, 2010. Version 1 (superseded). http://www.theplantlist.org/1.

Mower, J.P., 2009. The PREP suite: predictive RNA editors for plant mitochondrial genomes. Nucleic Acids Res. 37, W253–W259. https://doi.org/10.1093/nar/gkp337.

Mwanzia, V.M., He, D.X., Gichira, A.W., et al., 2020. The complete plastome sequence of the invasive weed {Dysphania pumilio} (R. Br.) Mosyakin & Clemants (Amaranthaceae): intraspecies variations on common camellia chloroplast genomes. Mitochondrial DNA B 4, 1428–1429. https://doi.org/10.1080/23802359.2019.1591214.

Park, J., Xi, H., et al., 2019. The complete chloroplast genome of common camellia tree in Jeju island, Korea, Camellia japonica L. (Theaceae): intraspecies variations on common camellia chloroplast genomes. Mitochondrial DNA B 4, 1292–1293. https://doi.org/10.1080/23802359.2019.1598807.

Patel, R.K., Jain, M., 2012. NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 7, e30619. https://doi.org/10.1371/journal.pone.0030619.

Tan, S., Zhang, Y., Li, G., et al., 2020. The complete chloroplast genome of a medicinal plant, Wikstroemia chamaedaphne (Thymelaeaceae). Mitochondrial DNA B 5, 648–649. https://doi.org/10.1080/23802359.2019.1711228.

Rambaut, A., 2018. FigTree v1.4. http://tree.bio.ed.ac.uk/software/figtree.

Rehder, A., 1916. Thymelaeaceae. Pl. Wilson. 2, 530–531.

Ronquist, F., Teslenko, M., Van der Mark, P., et al., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542. https://doi.org/10.1093/sysbio/sys029.

Sovrilic, M.M., Manojlovic, N.T., 2017. Plants from the genus Daphne: a review of its traditional uses, phytochemistry, biological and pharmacological activity. Serb J. Exp. Clin. Res. 18, 69–80. https://doi.org/10.1515/sjerc-2016-0024.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.