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Morphological and Molecular Status of *Daphne wolongensis* C.D.Brickell et B.Mathew as Genetic Resource for Horticulture

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Abstract: *Daphne wolongensis* described on the basis of a few known individuals was investigated in the wild, in Wolong Valley, Sechuan. Its status of valid species was verified morphologically and genetically. Three newly found populations were compared to the closely related species *Daphne retusa, D. tangutica, D. longilobata, D. acutiloba, D. sureil,* to clones available in cultivation and selected cultivars. The high bootstrap values indicate a sufficient level of genetic differentiation between each of the studied species. The hypothesis of whether *D. wolongensis* is a hybridogenous species was rejected, it is a well-defined independent species. Based on morphological and genetic data, it seems possible that another species, *D. limprichtii*, can be a mountain form of *D. tangutica*. Variability of populations in Wolong gives a good opportunity to select genotypes with a higher or better performance of combination of traits. From 67 samples collected from Wolong, it was possible to select the top ten different types as genetic resources for breeding. *Daphne wolongensis,* in the visited sites of Wolong area, occupies less than 1 km^2. Together with isolated finds, the number of individuals is less than 500 and the area surveyed is not larger than 10 km^2, thus it falls into the IUCN category of "Critically Endangered" plants.

Keywords: *Daphne wolongensis*; distribution; morphology; relationships; genetics; AFLP; related species; Rehdera section

1. Introduction

The genus *Daphne* L. (*Thymeleaceae*) comprises approximately 95 species which are distributed primarily in temperate Asia, extending from Japan to Europe [1,2]. The distribution is limited to the northern hemisphere. The Flora of China [2,3] lists 69 species, of which 41 are endemic. *Flora Europaea* lists 18 species [4]. The taxonomy of the genus *Daphne,* with respect to Chinese related species, was seldom studied. In their study, Brickell and Mathew [5] followed the generic classification of [6] adopting five sections. The section *Daphnanthes* C.A.Meyer is subdivided to six subsections. The subsection *Daphnanthoides* comprises 14 related species; *D. acutiloba, D. bholua, D. grueningiana, D. kiusiana, D. longilobata, D. luzonica, D. miyabenana, D. odora, D, papyracea, D. retusa, D. shillong, D. sureil, D. taiwaniana,* and *D. tangutica*. Halda [7] divides the genus *Daphne* into five subgenera and five sections. The section *Rehdera* comprises 14 similar species including *D. acutiloba, D. limprichtii, D. odora,* and *D. tangutica*, while *D. bholua* is separated into an independent subsection.

The genus *Daphne* is very desirable for ornamental horticulture; 34 species are described for horticultural use, 18 interspecific natural and artificial hybrids, and over 100 cultivars and selected forms are in cultivation [5,8]. Halda [9] lists 41 interspecific hybrids comprising both natural and artificial origin.

Daphne wolongensis is a recently described species on the basis of one cultivated plant raised from seed. The initial information on this species was published by Martyn Rix [10] in the description of his journey to Sechuan, China. He published a picture with a legend "*Daphne tangutica*—unusual form" [10]. This original plant was found near the Panda Research Station, Wolong valley (Wenchuan County, Ngawa Prefecture). A British nurseryman, Robin White, raised two plants from collected branches from a Chinese nursery (Kaichen) in 2001 [11]. He selected one plant for propagation in his nursery and named it 'Guardsman' according to its upright habit. He was not able to determine the taxonomic designation at the species level and stated that it is related to *D. retusa* and *D. tangutica* [12]. Another collection of *Daphne wolongensis* came from Mao Lou, Sunang Valley (collected by Ben Wilson), and it was very similar to the Kaichen plant. The third collection came from Kaichen under the name China Pink. The fourth collection was done by Stella and David Rankin from the Wolong region, and it had similar characteristics. David Rankin sent a picture of the plant from the region Wolong to Chris Brickell in 2005. Brickell understood that it is a new species, selected the third one as a type, and described this *Daphne* as a new species [12].

Daphne wolongensis is an erect, evergreen shrub that can grow up to 1.5 m, loosely branched, leaves are subsessile alternate, narrowly elliptic. Inflorescences are axillary, fasciculate flowers that are pink, with cylindrical calyx tubes, and four lobes, the ovaries are ellipsoid and stigma capitate. Fruit is globose to subglobose, fleshy, and red [12]. *Daphne wolongensis* differs from related species *D. retusa* and *D. tangutica* in habit, leaf shape, and with the axillary fasciculate inflorescences. A third related species is *Daphne wilsonii* Rehder, now usually considered a synonym of *D. tangutica* [12]. It was described from western Hubei and western Sichuan. In Rehder's key, *D. wilsonii* is distinguished from *D. retusa*, by its longer, (4–7 cm against 2–3 cm), acute, not retuse leaves [13].

Botanists and horticulturists reported the occurrence of only individual plants along the main road and declared *Daphne wolongensis* as an extremely rare species. However, those fewer than 10 finds remained the only known plants of the species and became the only sources of material for horticulture. They were given the cultivar names: Guardsman, Miya Lou, Kevock Star, and China Pink.

We authors could not believe that *Daphne wolongensis* is limited to only isolated plants along the road in the Wolong, Baoxing, and Sunang valleys (Figure S1), based on possible synanthropic or dirsturbance reasons. To further investigate the taxonomic ambiguity of *D. wolongensis*, we inspected seven herbaria. No collections of *D. wolongensis* were found in Kunming (KMG) and Chengdu (CHG) herbaria. European herbaria accessed online did not yield any *D. wolongensis*. The only specimen was found in RHS Wisley Herbarium (WYS) marked as Stella and David Rankin SDR 2, a holotype from "Wolong, beside the road near Wolong, 2000 m". Its isotype was in Brickell herbarium as well as the original cultivated plant named 'Guardsman'.

During the three-year period of study (in 2011, 2012, and 2013) within the Wolong valley, we sought *D. wolongensis* along the Yuzi River and road G350 and explored varied plant communities. No focal plants were found along the Yuzi River. Plants previously reported along roads were mostly damaged by an earthquake in 2008 being covered by huge landslides, or also by the construction of a new road between 2010–2012, mostly on the other side of the River.

The main aim of the study was to test whether *D. wolongensis* can be a well-defined species on both phenotype and genotype levels and what affinities can be found to related species. Another aim was to look for variation useful for breeding and ornamental horticulture. Genotypic assessments utilized Amplified Fragment Length Polymorphism (AFLP) method, described further in Section 2.

2. Materials and Methods

2.1. Sampling

Herbarium specimens were viewed/examined at the Kunming (KMG) and Chengdu (CHG) herbaria, China. European herbaria in Kew, the British Museum, Edinbourgh (K, BM, E, respectively, United Kingdom), and Vienna (Austria, W) were accessed online for available related species (*D. retusa, D. tangutica,* and *D. longilobata*). The distribution area of *D. wolongensis,* the Wolong Valley

around Wolong village, was visited in 2011, 2012, and 2013 (area marked W, Figure S1). *Daphne wolongensis* specimens were collected for herbarium, biometrics, and DNA analyses. Spare branches were grafted for cultivation in order to have living voucher specimens. Other related *Daphne* species, mainly from the section *Rehdera,* were collected for comparison. Morphological characters were measured on fresh plants in situ, in cultivation, and on herbarium specimens. Measurements of floral parts and indumentum were taken under a stereomicroscope in the laboratory.

Fresh leaves were collected for DNA analyses. After returning to the laboratory, samples were frozen and stored at −80 °C until DNA extraction. Closely related species *D. tangutica*, *D. retusa,* and *D. limprichtii* were added for comparison as well as other more distantly related species *D. calcicola, D. sureil,* and *D. acutiloba*.

Daphne wolongensis samples were collected from three populations in the Wolong Valley, China, and marked W1, W2, and W3 (Table S1, Figure S1). The plants were numbered for measurement and further study (W1: 1 to 23, W2: 1 to 22 and W3: 1 to 22). In total, 67 individuals were analyzed in situ morphologically and 38 samples were used for DNA extraction in laboratory.

Related species, *D. retusa, D. tangutica,* and *D. limprichtii* were investigated in wild populations in Yunnan and Sechuan regions and *D. longilobata* in Eastern Tibet, China (Table 1). The *Daphne calcicola, of Dielsia* section was used as a check in DNA evaluation. All *Daphne* spp. collections made by Vojtěch Holubec are currently in his herbarium for reference and will be deposited to the Herbarium of the Botanical Institute, Prague, Czech Republic (PR) immediately after publication of this contribution. For conservation assessments, the International Union for Conservation of Nature red list categories and criteria [14] have been applied.

Total genomic DNA was extracted from leaves according to the optimized protocol using Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer (2 M NaCl, 0.2 M Tris, 50 mM Ethylene Diamine Tetraacetic Acid (EDTA), 2% CTAB, pH 7.5) and 10 mg Polyvinylpyrrolidon (PVP) per each sample [15]. DNA was precipitated by one volume of absolute ethanol and diluted in an appropriate volume of TE (Tris and EDTA) buffer. DNA was run in 0.8% agarose gels to verify the quality and the concentration. λ HindIII (Fermentas, Vilnius, Lithuania) was used to determine the size and the concentration of DNA.

Table 1. Investigated species and localities/sources of living material of *Daphne* section *Rehdera*.

Species	Locality/*Source of Living Material	Altitude	Habitat Description	No. of Samples
D. wolongensis	W1, Wolong Valley, Sechuan	2060	open forest along rivulet	23
D. wolongensis	W2, Wolong Valley, Sechuan	2084	shady gorge along fast flowing stream	22
D. wolongensis	W3, Wolong Valley, Sechuan	2100	forest along small river	22
D. wolongensis	Coll Martyn Rix, ex United Kingdom*			1
D. wolongensis	Miya—Low, ex United Kingdom*			1
D. wolongensis	China Pink ex ChenYi, ex United Kingdom*			1
D. wolongensis	Kevock Star, ex United Kingdom*			
D. acutiloba	CDC 626, ex United Kingdom*			1
D. retusa	Zheduo Shan, Kanding, Sechuan	4100	subalpine shrubland	1
D. retusa	Huanglong, Min Shan, Sechuan	4300	alpine shrubland	1
D. retusa	ex United Kingdom*			1
D. tangutica	Zhongdian, Yunnan	3600	open forest and shrubland	1
D. tangutica	form A, ex United Kingdom*			1
D. tangutica	form B, ex United Kingdom*			1
D. tangutica	Coll B4, Keith Rushforth, ex United Kingdom*			1
D. limprichtii	Jiuzhai, Min Shan, Sechuan	4100	alpine shrubland	1
D. longilobata	Baimucun, Lunang, Tibet	3600	deciduous forest	10
D. sureil	ex United Kingdom*			1
D. calcicola	Zhongdian, NapaHai, Yunnan	3644	evergreen oak forest (*Q. aquifolioides*)	1

* Cuttings for DNA extraction were provided by Mr. Robin White, Blackthorn, United Kingdom.

2.2. Molecular Analysis

AFLP markers were designed based on the publication by [16]. All oligos, adapters, and fluorescently signed primers were synthesized by Generi-Biotech Company (Hradec Kralove, Czech Republic). DNA digestion was carried out using the restriction enzymes *Eco*RI and *Mse*I [16]. Eighty combinations of primers were tested, and eighteen pairs were selected for further analyses. These combinations of primer pairs were chosen because they generated a high number of scorable fragments with a range of sizes (100–500 bp). The selective amplification, with *Mse*I primers and fluorescently marked *Eco*RI primers, was performed as a multiplex PCR in a Labcycler (SensoGuest GmbH, Göttingen, Germany) with a reaction mixture of 10 μL containing the following: 0.2 mM dNTP, 1 μM MseI primer, 3 × 0.5 μM *Eco*RI primers, 1 U *Taq* polymerase (Qiagen GmbH, Hilden, Germany), 1× buffer with 10 mM $MgCl_2$ and 1 μl diluted (1:20) preselective amplification reaction. Amplification products of 38 individuals per each of six multiplex amplifications were separated by capillary electrophoresis in an ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA) and analyzed using GeneScan (v. 1.5) and Genotyper (v. 3.1) software (Applied Biosystems). Based on the presence or absence of AFLP amplification products at specific loci, a binary matrix was built and used for data analysis.

2.3. Data Analysis

A matrix of distances between all genotypes was calculated using the Jaccard dissimilarity coefficient in the DARwin software [17]. For clustering, an unweighted neighbor-joining method was used. The support for the phenogram branches was obtained using 2000 bootstrap re-samplings. This software was used also for Principal Coordinate Analysis (PCoA). The matrix of dissimilarities was transformed to the matrix of Euclidean distances between individuals. Ten axes or better latent variables were set to be computed. The graph of PCoA was done in the software Origin (v. 2018b; OriginLab Corporation, Northampton, MA, USA).

To find a population structure among analyzed individuals, we used Bayesian statistics implemented in the software Structure version 2.3.4 [18]. Ten independent runs of 1–20 clusters (K = 1–20) were performed using 100,000 Markov chain iterations after a burn-in period of 10,000 iterations. The number (K) of clusters into which the sample data (X) were fitted with posterior probability Pr (X|K) was estimated using a model with admixture and correlated allele frequency [19]. The most probable K-value was determined using the log probability of the data [LnP(D)] and delta K (ΔK) based on the rate of change in [LnP(D)] between successive K-values [20].

The diversity statistics for each population included the percentage of polymorphic loci, the average diversity of the loci using Nei's unbiased gene diversity $\hat{h}$ [21], and the Shannon Information index [22,23]. All of these statistics were calculated using the POPGENE software, version 1.32 [24]. An exact test for population differentiation was calculated using the Tools for Population Genetic Analyses (TFPGA; version 1.3; [25]) with 10,000 permutation steps.

Principal Component Analysis (PCA) and Discrimination Analysis of morphological data were computed in the software Statistica v 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Only individuals with the whole set of variables (leaf length, leaf width, leaf index, leaf thickness, flower tube length, lobe length, lobe width, bract length, bract width, bud scale length, bud scale width) were included in the analysis. A stepwise procedure was used to test the discrimination between group 1 (*D. wolongensis* wild), 2 (*Daphne* spp. without *D. wolongensis*), and 3 (*D. wolongensis*—varieties) was carried out with all of the variables. The number of parameters was then reduced by the following methods: 1) the forward introduction of significant variables, one at a time; and 2) the backward elimination of non-significant parameters (deleting one variable at a time). The discriminant power of the proposed model was then tested by Wilks' criterion λ. The classification of the samples into groups was performed on the basis of their Mahalanobis distance and posterior probabilities.

3. Results

3.1. Distribution and Habitat of D. wolongensis

Visiting tributaries of the river revealed three strong populations of the species in 2013 (Plate 1). Therefore, the previously found isolated plants along the road were just sporadic seedlings. All three populations were located mainly on riverbanks, in coarse sandy deposits, in stony soil, among boulders, amongst dense willow shrubs, and in the surrounding open leafy forest. The site marked Wolong 1 (W1, Table S1), occurring along a rivulet, had a population of *Daphne wolongensis* over a distance of about 300 m, with about 100–150 plants of different ages. The site Wolong 2 (W2) was found in a deep, steep and shady gorge alongside a fast-flowing stream. The site was less than 100 m long and the number of plants there was approximately 100. The site marked Wolong 3 (W3) was situated in a shallower valley in forest, over a distance of about 1 km and the number of individuals was 150–300. All three plant populations were associated with the valley bottom, not climbing the rocky sides (Figure S1).

Closely related species of the section *Rehdera* differ ecologically. While *D. wolongensis* is a typical species for marginal communities of water gullies mainly in wet habitats, *D. retusa,* and *D. tangutica* grow in subalpine shrublands and pastures, usually on slopes with good drainage. *D. limprichtii* is a high alpine species growing in grasslands. *D. longilobata* is a typical forest species occupying rather wet habitats.

3.2. Morphological Diversity of D. wolongensis

The plants within found populations were considerably variable in morphological characters (Table S3). The shrub height was in the range (50–) 100–180 cm. The mean leaf length in W1 plants was 47 (27 to 70) mm long, in W2 plants was 48.8 (30 to 90) mm long and in W3 plants was 54.3 (40 to 72) mm long. The largest leaves were noticed within the population W3, and the most variable were in the population W2. The leaf width was between 8 and 16 mm for W1 (mean 12.6 mm), 8–22 mm for W2 (mean 13.02 mm) and 8–18 mm for W3 (mean 11.78 mm). The length/width index was 2.2 to 8 for W1, 2.2 to 7.8 for W2, and 2.6 to 7 for W3. The leaf shape was linear lanceolate, oblanceolate to oblong with acute to acuminate apex, the latter in longer leaves. The base is cuneate. The margin is more or less revolute, more so in those of smaller leaves. The plants with larger leaves usually had thinner lamina than smaller leaves that had thicker dark green lamina and a more leathery structure. The smaller leaf forms resemble the related *D. retusa*. The flowers are borne in rich fascicules in axillar inflorescences and even richer in terminal inflorescences, equally in all three populations. The bracts varied in shape from ovate to lanceolate, 8–12 mm long, glabrous, occasionally ciliate on margin. The flower color is pink for all three populations, there are plants with darker flowers, and some nearly white. The perianth tube formed by the calyx is narrowly cylindrical, 9–12 mm long, glabrous, lobes 4, ovate, 6–9 mm long, and 4–8 mm wide, with acuminate apex. The variation in flower parts is analogic in all three populations. Fruits are very juicy, nearly globose 8–12 mm long, the largest in population W2. Seeds are subglobose 5–7 × 4–6 mm, smaller in population W1.

3.3. Differences among Related Species

Morphological diversity of *D. wolongensis* is much higher than in any of the related investigated species. Generally, most of the characters stay between *D. retusa* and *D. tangutica*. *D. wolongensis* differs from D*. retusa, D. tangutica, D. limprichtii,* and *D. longilobata* by a combination of morphological traits (Table S3, Plates 1 and 2). Biometric values of those traits partly overlap (Figure S2). In direct observation, four differentiating traits for *D. wolongensis* were identified: presence of axillar inflorescences together with terminal, young branch and peduncle indumentum being densely hairy to villous, shape, and size of bracts and leaf thickness. The bracts are concave and large compared with other listed species, where the bud scales are long acuminate. The leaf thickness ranges between *D. longilobata* and other species and make the leaf appearance thinly leathery, but not papery. Outer winter bud scales are ovate and deeply concave compared with other species.

The species differ also in habitat and altitude (Table 1 and S2). *D. wolongensis* is nearly always attached to running water along streams, or at least to periodically flooded valley bottoms in primary habitats except occasional seedlings found along the road. All other investigated species have other habitats. *D. wolongensis* grows in the mountain forest zone in a limited altitude amplitude 2000–2200 (–2800) m. *D. retusa* and *D. limprichtii* are upper montane/subalpine to alpine species. *D. tangutica* and *D. longilobata* have a wide altitude habitat range from a lower montane forest to the subalpine zone.

Principal Component Analysis (PCA) confirms a high level of morphological diversity between *D. wolongensis* individuals (Figure 1). The first three components represent 79.1 % genetic variability. In particular, component 1 discriminates between *D. wolongensis* and other *Daphne* spp. These results are supported by variance analysis (Figure S2).

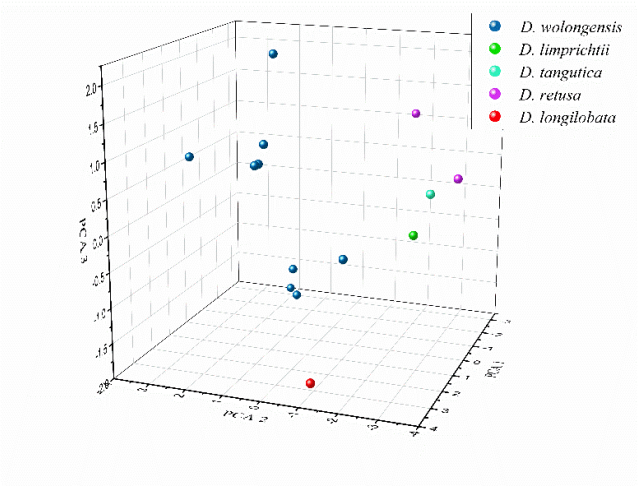

Figure 1. Principal Coordinate Analysis (PCoA) analysis of morphological data of *Daphne* spp.

Using a forward stepwise method within Discriminant analysis, the following three variables were added to the model: bract width, bud scale length, and lobe width. The Wilks′ λ value of 0.00007 indicates the good discriminatory power of the model. The least value of the partial Wilks′ λ indicated that the variable bract width contributes most to the overall discrimination. Discriminant functions (roots) computed by canonical analysis were considered statistically significant (Root 1: $\chi^2 = 68.59$; $p < 0.01$; Root 2: $\chi^2 = 23.89$; $p < 0.01$). These results were visualized as a scatterplot of canonical scores (Figure 2). The first discriminant function (root) discriminates between *D. wolongensis* and other *Daphne* species, and the second function provides discrimination between wild individuals of *D. wolongensis* and its varieties. These results were confirmed by further classification based on the Mahalanobis distances and posterior probabilities (Table S4). All individuals with a full set of morphological data were correctly classified.

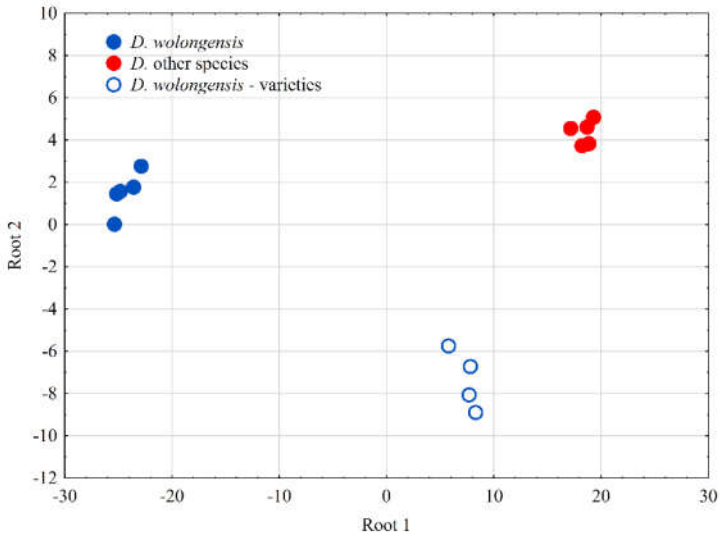

Figure 2. A scatterplot of canonical scores of the three groups of *Daphne* spp.

3.4. *Genetic Diversity of* D. wolongensis *and Related Species*

From 18 unique primer combinations (Table S6), we found 522 unique polymorphic loci from AFLP analysis of 38 *Daphne* spp. samples that were at our disposal. This corresponds to an average of 34.8 polymorphic bands per primer combination. Gene diversity ranged from 0 to 0.507, with an average of 0.222 (Table S7). The level of genetic diversity was the lowest in *Daphne tangutica* population ($\hat{h}$ = 0.024; I = 0.034). The level of gene diversity of *D. wolongensis* is of moderate level (Table 2). A higher diversity was found in *D. wolongensis* varieties ($\hat{h}$ = 0.108; I = 0.181). The highest genetic distance was found between *D. calcicola* and other *Daphne* species and the lowest between populations W1, W2, and W3 of *D. wolongensis*. The lowest genetic distance between *D. wolongensis* and other species is those between *D. wolongensis* and *D. retusa* (0.26), and then between *D. wolongensis* and *D. limprichtii* (0.28) (Table 3).

Table 2. Diversity characteristics of three *Daphne wolongensis* local populations, *Daphne wolongensis* varieties, and another *Daphne* species based on AFLP analysis**.**

Species/Populations	n[a]	P (%)[b]	$\hat{h}$[c]	I[d]	G_{ST}[e]	Nm[f]
D. wolongensis—W1	7	24.7	0.088	0.133		
D. wolongensis—W2	12	25.1	0.087	0.131		
D. wolongensis—W3	6	15.5	0.061	0.091		
D. wolongensis—varieties	3	28.4	0.126	0.181		
D. wolongensis—total	28	43.87	0.108	0.171	0.237	1.614
D. retusa	3	20.3	0.09	0.129		
D. tangutica	3	5.4	0.024	0.034		
D. sureil, calcicola, acutiloba	4	66.1	0.294	0.421		
D. spp.	10	67.05	0.284	0.434	0.521	0.460
Total	38	99.43	0.215	0.351	0.301	1.164

[a] number of plants analyzed; [b] percentage of polymorphic loci; [c] Nei´s [26] unbiased heterozygosity; [d] Shannon Information index as a measure of gene diversity [23]; [e] F-statistics [21]; [f] Nm = estimate of gene flow from Gst., Nm = 0.5(1−Gst)/Gst.

Table 3. Matrix of genetic distances between *Daphne* species/populations based on Nei´s unbiased distances [26].

	D. wolongensis—W1	**D. wolongensis—W2**	**D. wolongensis—W3**	**D. wolongensis—varieties**	**D. acutiloba**	**D. retusa**	**D. tangutica**	**D. limprichtii**	**D. sureil**	**D. calcicola**
D. wolongensis—W1	0.000									
D. wolongensis—W2	0.012	0.000								
D. wolongensis—W3	0.028	0.032	0.000							
D. wolongensis—varieties	0.030	0.028	0.053	0.000						
D. acutiloba	0.444	0.441	0.450	0.361	0.000					
D. retusa	0.274	0.265	0.287	0.206	0.370	0.000				
D. tangutica	0.361	0.352	0.376	0.285	0.424	0.146	0.000			
D. limprichtii	0.292	0.284	0.317	0.234	0.459	0.134	0.265	0.000		
D. sureil	0.463	0.461	0.484	0.398	0.408	0.424	0.470	0.450	0.000	
D. calcicola	0.884	0.881	0.880	0.794	0.752	0.741	0.955	0.697	0.627	0.000

Cluster analysis (Figure 3) showed three main clusters. The first one involves genotypes of *D. acutiloba*, *D. calcicola*, and *D. sureil*. The second cluster contains items of *D. retusa*, *D. tangutica*, and *D. limprichtii*. The largest cluster is made of genotypes of *D. wolongensis* including varieties derived from this species. The high bootstrap values indicate a good level of genetic differentiation between each of the studied species. This fact was supported by structure analysis (Figure 4). Four genetic populations (K = 4) were identified within the data representing the species: *D. wolongensis* (K3), *D. retusa* (K1), *D. tangutica* (K4), and *D. acutiloba* with *D. calcicola* (K2). According to the membership proportion of individuals into each of the clusters K1–K4, *D. limprichtii* is likely to be a mixture of *D. tangutica* and *D. retusa* gene pools (Table 3). Based on an exact test of population differentiation (χ^2= 4248.3, df = 1044, $p < 0.01$), a null hypothesis about the identity of *D. wolongensis* and other analyzed species was rejected. *D. wolongensis*, on genotype level, is a well-defined separate species.

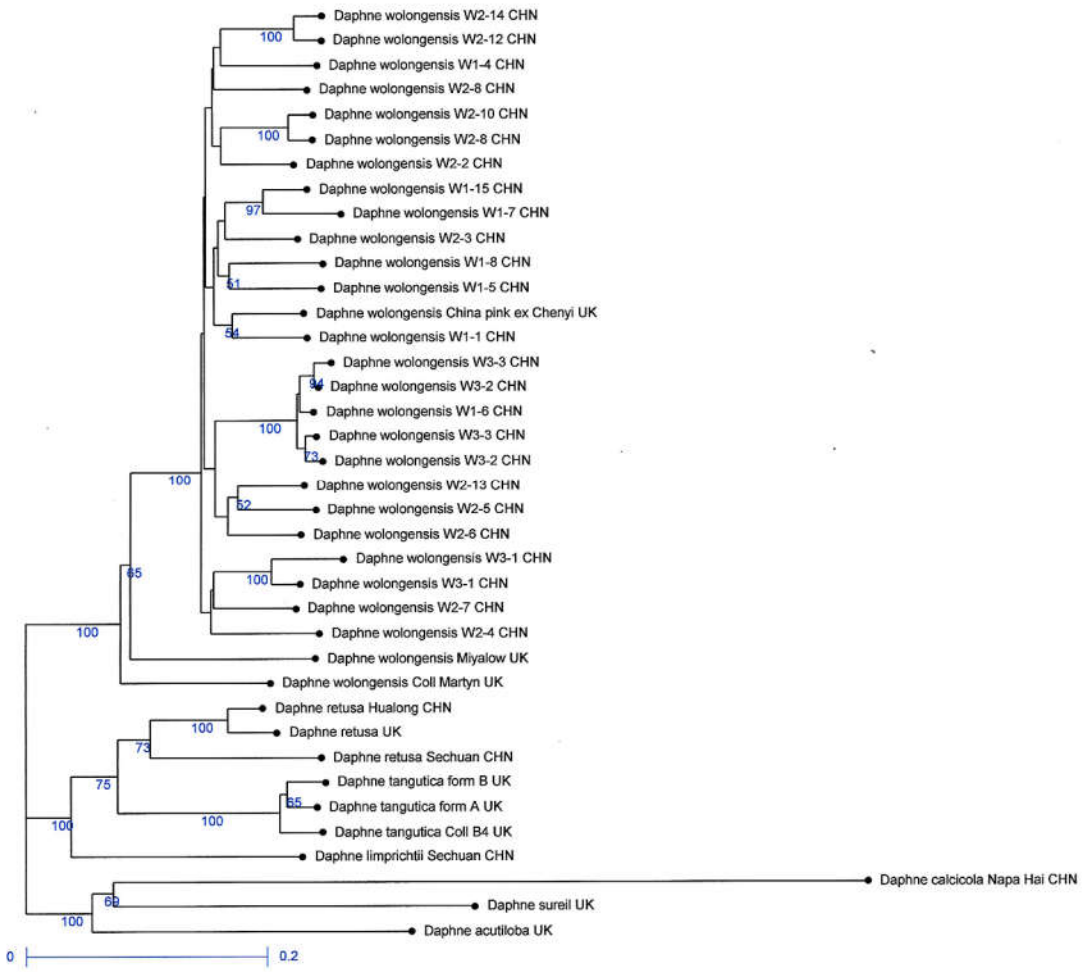

Figure 3. Dendrogram constructed using an unweighted neighbor joining clustering method based on a genetic distance matrix computed by means of Jaccard coefficients with 2000 bootstraps.

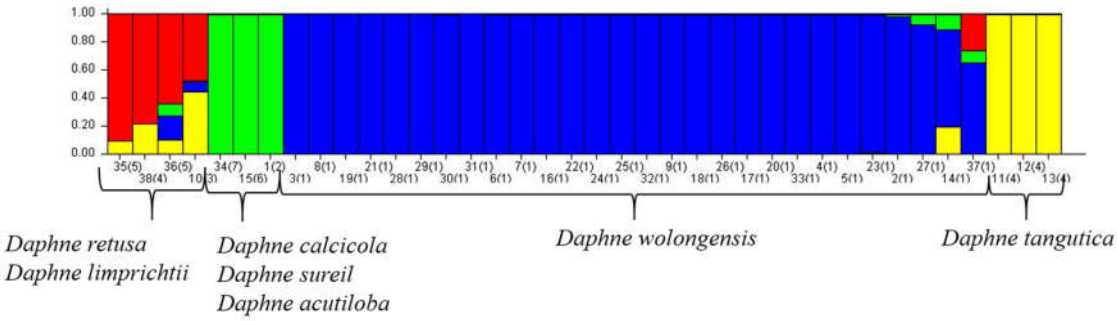

Figure 4. Cluster analysis of *Daphne* spp. based on a Bayesian approach. Each genotype is represented by a bar divided into K colors, where K is the number of clusters assumed in the analysis: K1—red color; K2—green color; K3—blue color; K4—yellow color. The *y*-axis represents posterior probability of individual assignment to genetic populations K1—K4.

There is a low level of genetic diversity within the three tested populations W1, W2, and W3 (Table 2). Only the variety China Pink was identified as pure *D. wolongensis*. The other analyzed varieties were derived from *D. wolongensis* by long-lasting isolation or hybridization with other *Daphne* species (Table 4). PCoA also shows the level of differences between *Daphne* spp. and a low

level of within species variability. The first three dimensions represent 50.5% of total variability detected in 38 *Daphne* spp. samples (Figure 5).

Table 4. Proportions of membership of each analyzed individual in each of the four clusters (K).

Species	Population	Origin	K1	K2	K3	K4	K
Daphne wolongensis	W1	China	0.004	0.001	0.994	0.001	3
Daphne wolongensis	W1	China	0.002	0.001	0.996	0.001	3
Daphne wolongensis	W1	China	0.001	0.001	0.997	0.002	3
Daphne wolongensis	W1	China	0.007	0.014	0.977	0.003	3
Daphne wolongensis	W1	China	0	0	0.999	0	3
Daphne wolongensis	W1	China	0.003	0.002	0.995	0.001	3
Daphne wolongensis	W1	China	0	0	0.999	0	3
Daphne wolongensis	W2	China	0	0	0.999	0	3
Daphne wolongensis	W2	China	0.001	0	0.998	0.001	3
Daphne wolongensis	W2	China	0.002	0.002	0.995	0.001	3
Daphne wolongensis	W2	China	0	0	0.999	0	3
Daphne wolongensis	W2	China	0.001	0.001	0.998	0	3
Daphne wolongensis	W2	China	0.001	0.001	0.986	0.012	3
Daphne wolongensis	W2	China	0.001	0.001	0.998	0	3
Daphne wolongensis	W2	China	0.003	0.001	0.994	0.002	3
Daphne wolongensis	W2	China	0.001	0.001	0.998	0	3
Daphne wolongensis	W2	China	0.001	0	0.997	0.001	3
Daphne wolongensis	W2	China	0.001	0	0.999	0	3
Daphne wolongensis	W3	China	0.001	0.001	0.998	0	3
Daphne wolongensis	W3	China	0	0	0.999	0	3
Daphne wolongensis	W3	China	0.001	0.001	0.997	0	3
Daphne wolongensis	W3	China	0.006	0.071	0.923	0	3
Daphne wolongensis	W3	China	0	0	0.999	0	3
Daphne wolongensis	W3	China	0	0	0.999	0	3
Daphne wolongensis	Coll Martyn	UK	0.001	0.111	0.691	0.196	3
Daphne wolongensis	China pink	UK	0.001	0.001	0.998	0	3
Daphne wolongensis	Miyalow	UK	0.26	0.089	0.65	0.001	3
Daphne tangutica	form A	UK	0.002	0	0.001	0.997	4
Daphne tangutica	form B	UK	0.002	0.001	0	0.997	4
Daphne tangutica	Coll B4	UK	0.001	0.001	0.001	0.997	4
Daphne sureil	White fls	UK	0.001	0.996	0.001	0.001	2
Daphne calcicola	Napa Hai	China	0.001	0.999	0	0	2
Daphne acutiloba		UK	0.003	0.99	0.002	0.005	2
Daphne retusa	Sechuan	China	0.903	0.002	0.002	0.093	1
Daphne retusa	Hualong	China	0.782	0.002	0.001	0.215	1 + 4
Daphne retusa		UK	0.476	0.008	0.067	0.449	1 + 4
Daphne limprichtii	Sechuan	China	0.64	0.083	0.175	0.102	1 + 3 + 4

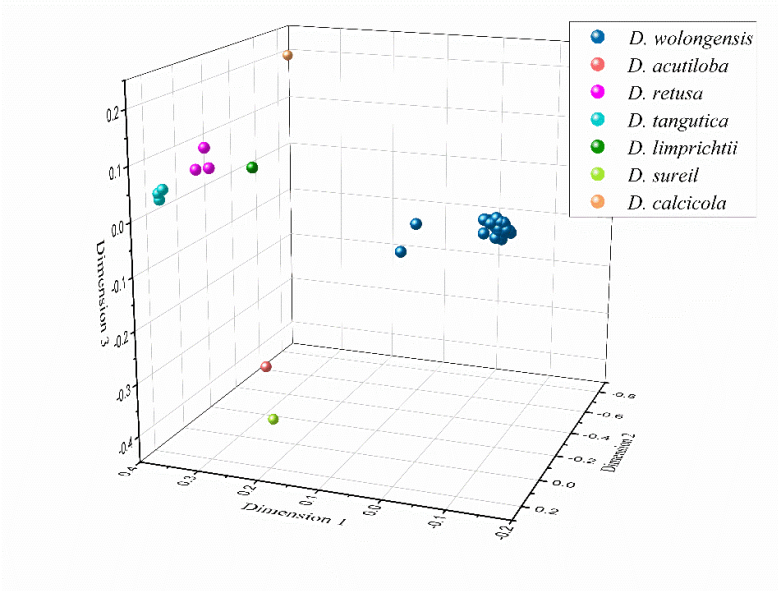

Figure 5. Principal Coordinate Analysis (PCoA) plot of 38 *Daphne* spp. individuals based on AFLP data.

Based on morphological and genetic data, it seems likely that *D. limprichtii* can be a mountain form of *D. tangutica*. On the other hand, *D. wolongensis* is a well-defined and independent species.

4. Discussion

4.1. Status and Diversity of D. wolongensis

D. wolongensis is not listed in Flora of China [3], having been described later, after its publication. Apart from its original description [12], there is no botanical treatment available in literature. A world checklist of *Thymeleaceae* only states: "This species is tentatively accepted and may only be known from three localities in the wild" [27]. There is only one source of horticultural information [10]. The lack of treatment is most likely due to its remote distribution area.

D. wolongensis is highly variable and intermediate in morphological characters between *D. tangutica* and *D. retusa*, but it is genetically clearly independent. There are unique characters that distinguish it from both the above-mentioned and other species. Combinations of traits distinguish related species of the *Rehdera* section satisfactorily (Table S2, Figure 1 and 5). The high bootstrap values indicate a good level of genetic differentiation between each of the studied species. The description of the third related species *Daphne wilsonii* Rehder [13] compares it with *D. odora* and describes as 'Frutex erectus to 1.5 m', although it was not described with axillary inflorescences. A form of *D. odora* called 'Mazelii' has axillary flower clusters as does a variant of *D. tangutica* known as *D. tangutica* 'Alba', which probably derives from Farrer 585 [12].

The hypothesis of whether *D. wolongensis* is a hybridogenous species was rejected, based on detailed morphological evaluation and genetic study. However, the hybridogenous origin can be considered in the evolution within the *Rehdera* section. There are species with a large area of distribution like *D. tangutica* and *D. retusa*, and very localized species like *D. wolongensis* and *D. thanguensis.* Both narrow endemic species are related to *D. tangutica.* The recently described *D. thanguensis* differs from *D. tangutica* by having leaves with revolute margin and a tuft of hairs at the apex, ebracteate inflorescence and flowers, calyx lobes with a tuft of hairs at the apex and annular, slightly undulate hypogynal disk [28]. Both species *D. wolongensis* and *D. thanguensis* evolved in the distribution area of *D. tangutica* and *D. retusa*, but, without their current presence, thus isolated.

The low level of genetic diversity within the three tested populations W1, W2, and W3 and a higher level comparing to cultivars especially cv. Miya Low and especially cv. Guardsman can also

be explained by isolation of source localities (Figure S1). High mountains are continental islands from a biogeographic perspective, each one surrounded by low-altitude environments characterized by unsuitable present-day climatic conditions for cold-adapted mountain plants [29]. Consequently, many mountain plants often show disjunctive geographic distributions. Within each distribution patch, the species colonizes a geographic gradient of environmental conditions and becomes more abundant in localities, where individual survival, reproduction, and hence population growth are the highest [30]. Such a combination of geographic variation in population size and spatial isolation is expected to have important consequences for the genetic structure of plant populations [31,32]. Populations W1, W2, and W3 and cv. Kevock Star (Stella and David Rankin SDR 2) come from the Wolong Valley, cv. Miya Low and cv. Guardsman come from Sunang Valley that is about 100 km north of the Wolong Valley. The genetic similarity of W1, W2, and W3 populations implies that a gene flow between them exists. It is provided by pollinators within the Wolong region, but it is unlikely for the seed dispersal vectors of the species [33]. In particular, results showed that the highly isolated cultivars from the northern population constitute a genetic group strongly differentiated from those in the Wolong Valley, supporting a positive relationship between geographic isolation and genetic differentiation [34,35]. Castilla et al. [36] tested the level of genetic differentiation between populations of *Daphne laureola* across the Baetic Ranges and compared their diversity, and population size with regard to their spatial isolation. They calculated connectivity index per population and used a distance of 25 km that potentially covers the maximum travel distance of *D. laureola*'s pollinators and seed dispersers. For the study, they also used AFLP markers. Western populations proved to be strongly differentiated from the other populations. The westernmost population had the highest number of private fragments, despite its low values for mean gene diversity and percentage of polymorphic loci [36]. Similarly, *D. wolongensis* populations from the Wolong Valley differ considerably from Miya Low and Guardsman collected in northern Sunang Valley. Analyzing genetic structure of populations is useful to understand how the orography of heterogeneous landscapes contributes to genetic isolation of populations promoting intraspecific differentiation [37,38]. Based on morphological data, it seems likely that *D. limprichtii* can be a mountain form of *D. tangutica*, but genetic data do not fully support that. On the other hand, *D. wolongensis* is a well-defined and independent species.

4.2. Conservation Status

Daphne wolongensis, in the visited sites of Wolong area, occupies less than 1 km^2. Together with isolated finds, the area is not larger than 10 km^2. The number of individuals in the visited Wolong area is approximately 500. Rankin [39] estimated the number of plants to about 1000, by counting those visible from a length of road projected to the probable area of distribution. It makes the species fall in the IUCN category of "Critically Endangered" (CE) plants [14] similar to *D. thanguensis* [28]. The small area of distribution of *D. wolongensis* and its occurrence in isolated fragmented populations require appropriate habitat and population botanical monitoring before any conservation action. Secondary habitats along the road are endangered mainly by human activities, while primary habitats seem safe except for natural factors (earthquakes). As crop wild relatives are sources of genes for breeding, it is necessary to conserve the broadest genetic diversity [40], and there is a need for detailed demographic and genetic monitoring [41,42]. The region falls in the Chinese-Japanese Regions of plant diversity according to Vavilov [43] and the Sino-Himalayan diversity hotspot [44]. Currently *D. wolongensis* has no conservation coverage. Therefore, an effort to protect the new species in its habitat is urgent.

4.3. Impact for Horticulture

Many *Daphne* spp. of the section *Rehdera* are widely used in horticulture. *D. odora* cultivars are offered by more than 50 nurseries (e.g., https://portlandnursery.com/shrubs/daphne/; www.countylinenursery.com), *D. retusa, D. tangutica,* and *D. bholua* are similarly offered in several cultivars (e.g., https://www.burncoose.co.uk). *D. wolongensis* is currently offered very rarely (e.g.,

http://www.daphnes.be; http://www.seidelbast.net/). *D. wolongensis* is underrepresented in horticulture. The present cultivars Kevock Star, China Pink, and Miya Low are morphologically similar among themselves, and their morphological characters are within the variation of populations W1, W2 and W3 (Plate 1). The cultivar Guardsman differs from the other cultivars by a denser upright crown, higher number of axillar flowers, and deeper rose color of buds and florets. (Plate S2).

Variability of populations in Wolong gives a good opportunity to select genotypes with a higher or better performance of combination of traits. From 67 samples collected in Wolong, it was possible to select the top ten different types (Table S5). For horticultural use of *D. wolongensis*, the following characters are desirable to select: compact plant habit, shape and color of leaves, richness of flowering, size and color of flowers, nectaria producing pleasant odor, and the production of large fruits. The ten selected genotypes offer good diversity in characters for breeding and to enlarge the cultivated germplasm, which is presently available. The main disadvantage of growing in a continental climate is a low winter hardiness of the species because all three populations are from around 2000 m where frosts are only light and occasional.

This contribution uncovers a real distribution of the species, uncovers variation in characters in the wild, and brings material for breeding. Until recently, only progenies of several collected specimens have been available, and cultivars were made on the basis of occasional finds. Desirable germplasm for hybridisation with other species is now available, enabling the introduction of new characters, colors, and plant shape. It will be an enrichment of cultural plants from crop wild relatives (CWR).

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/10/11/1628/s1, Figure S1. Map of *Daphne wolongensis* sites. Wolong Valley (W), Baoxing Valley (B), Sunang Valley (S), Figure S2: Boxplot by group computed on morphological data: a) lv_length; b) lv_width; c) lv_index; d) lv_thickness; e) fl_tube_length; f) lobe_length; g) lobe with; h) bract_length; i) bract_width; j) bud_scale_length; k) bud_scale_width, Table S1: Morphological characters—differentiation of *D. wolongensis* within species (populations W1–W3, cultivars), Table S2: Morphological characters—differentiation of *D. wolongensis* to related species, Table S3: Morphological trait mean values of *D. wolongensis* populations and related species of the *Rehdera* section, Table S4: Classification of *Daphne* spp. individuals based on Mahalanobis distances and posterior probabilities as a result of Discriminant analysis, Table S5: Selected genotypes of *D. wolongensis* with a good performance for further use in breeding, Table S6: AFLP analysis of 38 *Daphne* spp. individual data, Table S7: AFLP loci gene diversity data. Photographs are combined to plates: Plate 1. Site Wolong 1, Site Wolong 2, *D. wolongensis* fruit, Site Wolong 3, *D. wolongensis* W2_7, W1_9, W2_10, Kevock Star (Photo: David Rankin), Kewock Star (Photo: Dirk Jockel), Miya Lou, W2_5 (Photo: Dirk Jockel), Plate 2. *D. retusa, D. tangutica*, *D. longilobata*. (Photo: Vojtech Holubec except marked in brackets).

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