

Natural hybridization between two butterfly bushes in Tibet: dominance of F₁ hybrids promotes strong reproductive isolation

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Abstract

Background: F_1 hybrids acting as a bridgehead for producing later generation hybrids can have evolutionary significance through strengthening reproductive isolation or facilitating gene flow between parental species, depending on whether backcrossing can occur. It had been suggested that the Tibetan plant *Buddleja wardii* was a hybrid species between *B. alternifolia* and *B. crispa* based on their sympatric distributions and the morphological characters in last century. Till now however, we still have limited evidence to prove key issues to *B. wardii*, like if it is of hybrid origin indeed and whether it is currently a true hybrid species already.

Results: In the present study, two sympatric populations of these three taxa were examined and compared using four nuclear genes and three chloroplast intergenic spacers, as well as with 10 morphological characters. Our results suggest that at both sites *B. × wardii* was likely to be a hybrids between *B. alternifolia* and *B. crispa*, and moreover, most of the hybrids present were confirmed to be F_1 s. This was further supported by morphology as no transgressive characters were detected. *B. crispa* was found to be the maternal parent in one population (BH), while in the second population (TJ), it was difficult to distinguish the hybridization direction due to shared haplotypes of cpDNA between *B. alternifolia* and *B. crispa*.

Conclusions: These results provide evidence that the natural hybrids between *B. alternifolia* and *B. crispa* mainly comprise F_1 hybrids, which have subsequently been given the name *B. wardii*. The F_1 hybrids have also contributed to strong reproductive isolation between parental species.

Background

Natural hybridization in plants is a common phenomenon, and is thought to play an important role in angiosperm diversity and evolution^[1-4]. If backcrossing occurs, natural hybridization provides a potential way for germplasm to flow between species^[5-7]; however, gene flow can be prevented if, due to intrinsic incompatibilities and/or strong extrinsic selection against later generation hybrids, the only hybrids seen are F_1 s^[8]. Intrinsic incompatibilities include F_1 hybrid sterility and/or hybrid inviability^[4,7,9], whereas extrinsic selection generally leads to the dominance of fertile F_1 individuals in the hybrid zone, with other genotypes of hybrids being outcompeted due to strong habitat selection^[7-8,10-11].

Unlike later generation hybrids, that generally exhibit wide morphological variance because of genetic segregation, F_1 hybrids from particular parental species tend to have similar morphologies due to the complete combination of parental genomes^[12]. Because of this, F_1 s have been often inaccurately described as new species, especially by taxonomists concerned chiefly with morphology. An example of this is *Rhododendron agastum* from Yunnan, China, which has long be treated as a good species^[13]. Recently, however, its hybrid origin has been confirmed, and population studies at the type locality suggest that most hybrids are F_1 s^[7].

The genus *Buddleja* L. (Scrophulariaceae), comprises more than 100 species and is widely distributed throughout tropical, subtropical and temperate regions of the Americas, Africa and Asia^[14-15]. The Sino-Himalayan region is a center of diversity for this genus, with over 75% of the Asian *Buddleja* species occurring in this area^[16-17]. Moreover, most *Buddleja* species prefer to grow in disturbed habitats (e.g. sides of roads and on riverbanks), which is typical of pioneer plants^[18-20], and some species (e.g. *B. davidii*) can become invasive if introduced to

new environments^[21-23]. Due to substantial overlaps in distribution and flowering periods, as well as shared pollinators, interspecific hybridization is assumed to be relatively common in the genus^[14-15,18]. However, to date, only a single case of natural hybridization has been confirmed using molecular data^[24].

Buddleja wardii C. Marquand was originally described as a new species from Tibet by C. Marquand in 1929^[25]. Leeuwenberg^[18] subsequently considered *B. wardii* to be a hybrid between the sympatric species *B. crispa* and *B. alternifolia* based on the morphological characteristics of the type specimen and Ludlow c.s. 4098 (BM, E, K), as well as these two species are the only one otherwise represented in the area. An important trait when determining *B. × wardii* plants is that the leaves are sometimes alternate and sometimes opposite on the same plant, even on the same stem (Figure 1).

In this study, we aim to test the hybrid origin hypothesis of *B. × wardii* in two sites in Tibet. Specifically, we used three chloroplast DNA region, the nuclear ribosomal external transcribed spacer (nrETS) plus three low-copy nuclear genes, to answer the following questions: 1) Are the *B. wardii* plants in fact hybrids between *B. crispa* and *B. alternifolia* at these two sites? 2) If yes, are there any differences between the genetic patterns at these two hybrid zones and 3) does the genetic structure of hybrid zones such as these provide clues to the mechanism of reproductive isolation between parental species?

Results

Morphological analysis

Of the three leaf characters (leaf length, leaf width and ratio of leaf length to width), putative hybrid individuals consistently had two morphological characters, leaf length and leaf width, intermediate in value between *B. alternifolia* and *B. crispa* (Table 1). Ratios of leaf length to leaf width were significantly larger in *B. alternifolia* than the other two taxa (Table 1). Of the seven floral characters, corolla tube width and anther height of *B. × wardii* were intermediate between the values of the two assumed parental species, whereas herkogamy did not differ significantly between the three taxa (Table 1). The remaining four floral characters, corolla tube length (TL), corolla lobe length (CLL), corolla lobe width (CLW) and style length (SL) all showed a similar pattern, with the characters in *B. alternifolia* having significantly lower values than those measured in *B. crispa* or *B. × wardii* (Table 1).

The two putative parental species are morphologically clearly distinct. In the PCA of 10 morphological characteristics, the first and second principal components explained 52.17% and 12.98% of the total variation, respectively. The two-dimensional scatter diagram based on PC1 and PC2 showed clearly the separation of *B. alternifolia* and *B. crispa*. Individuals of *B. × wardii* fell between the two parent species, with a slight overlap with *B. alternifolia* and a large overlap with *B. crispa*. Apart from the character HE, there is little difference in the correlation coefficients between the other nine traits (0.29-0.38) (Figure 2 a).

Petal color reflectance

The reflectance spectrum of the petals showed some variation between the different species. In *B. crispa* and *B. × wardii* there was a clearly marked peak in the reflectance spectrum at 485 nm, with extremely low variation. However, there was no obvious peak in the reflectance spectrum in *B. alternifolia* (Figure 2 b).

Sequence analyses of the four nuclear genes

NrETS: The total length of the nrETS region alignment was 380 bp in all individuals, including 20 and 17 nucleotide substitutions in BH and TJ population, respectively (for variation sites, see Additional file 1: Table S1). These variable sites generated 28 haplotypes, among them five, six and thirteen haplotypes belong to *B. alternifolia*, *B. crispa* and *B. × wardii* in BH population, whereas six, three and seven haplotypes in TJ populations, respectively. Three haplotypes were shared by the two populations. Haplotype network analysis identified two major clusters by six nucleotide substitutions. One cluster comprised five haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and seven haplotypes of *B. × wardii* from BH population, and six haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and three haplotypes of *B. × wardii* from TJ population. The other cluster comprised five haplotypes of *B. crispa* and six haplotypes of *B. × wardii* from BH population and two haplotypes of *B. crispa* and four haplotypes of *B. × wardii* from TJ population (Figure 3 a). Haplotypes of *B. crispa* from allopatric population were nested in this cluster and forming a small group with three step mutations from the main haplotype (Additional file 9: Figure S2).

Two haplotypes from *B. crispa* nested with the *B. alternifolia* cluster, and was found in BHCR2, BHCR7 and TJCR13, which show different haplotypes from both clusters. Of the putative hybrid individuals, all but two individuals (BHWI9 and TJWI1) had two divergent haplotypes nested with each of the two divergent clusters. The remaining two individuals were homozygous for a *B. crispa* haplotype at this locus (Figure 3 a).

GapC2: The total length of the gapC2 region alignment was 606 bp for all individuals, including 21 nucleotide substitutions, one 2-bp insertion/deletion and one 1-bp insertion/deletion in BH population and 22 nucleotide substitutions and one 1-bp insertion/deletion in TJ population (for variation sites, see Additional file 1: Table S1). A total 21 haplotypes were observed from these loci with three haplotypes were shared between these two populations, among which one, nine and nine haplotypes belong to *B. alternifolia*, *B. crispa* and *B. × wardii* in BH population, whereas three, six and ten haplotypes in TJ populations, respectively. The haplotype network fall into two major clusters separated by 11 nucleotide substitutions. One cluster contained the only one haplotype of *B. alternifolia*, one haplotype of *B. crispa* and two haplotypes of *B. × wardii* from BH population, and two haplotypes of *B. alternifolia* and three haplotypes of *B. × wardii* from TJ population. The other cluster contained eight haplotypes of *B. crispa* and seven haplotypes of *B. × wardii* from BH population, and six haplotypes of *B. crispa* and seven haplotypes of *B. × wardii* from TJ population. Haplotypes of *B. crispa* from allopatric population were nested in this cluster and shared one haplotype with *B. crispa* and *B. × wardii* from BH and TJ (Additional file 9: Figure S2).

Two individuals of *B. crispa*, BHCR2 and BHCR7, had a haplotype found in the *B. alternifolia* cluster but all their other haplotypes were found in the *B. crispa* cluster. In *B. alternifolia*, there was an exception of two individuals, TJAL6 and TJAL12, which had a haplotype found in the *B. crispa* cluster, but all the other haplotypes were found in *B. alternifolia* cluster. All *B. × wardii* individuals but one (BHWI9) showed two divergent haplotypes originating from both clusters, and the individual BHWI9 was homozygous for a haplotype from *B. crispa* cluster (Figure 3 b).

PPR24: The total length of the PPR24 region alignment was 647 bp for all individuals, including 43 and 46 nucleotide substitutions in BH and TJ population, respectively (for variation sites, see Additional file 1: Table S1). This gene showed a high level of genetic variation, with 40 haplotypes formed and two haplotypes were shared between the two populations. *B. alternifolia*, *B. crispa* and *B. × wardii* in BH populations had four, ten and fourteen haplotypes, whereas there were one, eleven and fifteen haplotypes in TJ population, respectively. In the haplotypes

network analysis, this region was divided into two major clusters separated by 21 nucleotide substitutions. One cluster contained four haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and seven haplotypes of *B. × wardii* from BH population, and the only haplotype of *B. alternifolia* and eight haplotypes of *B. × wardii* from TJ population. The other cluster contained nine haplotypes of *B. crispa* and seven haplotypes of *B. × wardii* from BH population, and all ten haplotypes of *B. crispa* and seven haplotypes of *B. × wardii* from TJ population. One *B. crispa* individual (BHCR7) and all *B. × wardii* individuals showed two divergent haplotypes originating from both *B. alternifolia* and *B. crispa* clusters (Figure 3 c).

PPR123: After sequence alignment, the PPR123 region was 735 bp in length, and included 23 and 45 nucleotide substitutions in BH and TJ population, respectively (for variation sites, see Additional file 1: Table S1). These variable sites generated 26 haplotypes, among which two, four and eight belong to *B. alternifolia*, *B. crispa* and *B. × wardii* in BH population, whereas two, ten and ten haplotypes in TJ population, respectively. Haplotype network analysis could be divided into three major clades with seven, eight or nine mutational steps between them. One clade comprised two haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and five haplotypes of *B. × wardii* from BH population, and two haplotypes of *B. alternifolia* and one haplotype of *B. × wardii* from TJ population. The second clade comprised three haplotypes of *B. crispa* and three haplotypes of *B. × wardii* from BH population, and five haplotypes of *B. crispa* and seven haplotypes of *B. × wardii* from TJ population. The third clade comprised five haplotypes of *B. crispa* and two haplotypes of *B. × wardii* from TJ. (Figure 3 d).

All haplotypes from *B. alternifolia* individuals fell into the first cluster. For the *B. crispa* individuals, only one from BH population nested with the first clade derived from BHCR7, ten individuals (TJCR3/5/6/7/9/10/11/12/13/15) had two divergent haplotypes, falling into the second and third clusters, four individuals (TJCR1/4/8/16) had haplotypes nested in the second clade and two individuals (TJCR2/14) had haplotypes nested in the third clade. Of the putative hybrid individuals, all but one individual (BHWI9) had two divergent haplotypes, one in the first cluster and the other in either the second or the third cluster. The remaining BHWI9 had two haplotypes found in the second clade (Figure 3 d).

Sequence analyses for the combined chloroplast regions

The combined length of the cpDNA fragment alignment (including *rpl16*, *trnD-trnT*, *trnS-trnfM*) from the three taxa was 2054 and 2065 bp, and contained ten and five nucleotide substitutions in BH and TJ population, respectively (for variation sites, see Additional file 1: Table S1). Eight haplotypes were inferred in total, and each taxon had three haplotypes in BH population. Haplotype network analysis indicated that most putative hybrid individuals in BH (75%) shared two haplotypes with *B. crispa*, while they did not share any haplotypes with *B. alternifolia*. The remaining four *B. × wardii* individuals (25%) had a unique haplotype with one mutational step away from the predominant haplotype of *B. crispa*. The haplotype network of cpDNA could not be divided into two clades. Even though *B. alternifolia* and *B. crispa* did not share any haplotypes, the differences between their haplotypes were only one or two mutational steps. All individuals in TJ population had only two haplotypes, of them one was derived from six *B. alternifolia* individuals (TJAL2/3/4/5/8/11), and the remaining 42 individuals shared another haplotype consistent with the haplotype only in individual BHAL13 (Figure 3 e).

NewHybrids analysis

Analysis of the four studied nuclear genes using NewHybrids among the three taxa showed that all individuals with *B. alternifolia* morphology were assigned to a pure parental species with high posterior probabilities (>0.988).

Of the individuals morphologically identified as *B. crispa*, all but three (BHCR2, BHCR7 and TJCR13) could be identified as *B. crispa* with high posterior probabilities (>0.996). The individuals BHCR2 and TJCR13 were assigned to *B. crispa* with lower probability of 0.775 and 0.840, and the individual BHCR7 was assigned to F₁ with probabilities of 0.785. Of the individuals morphologically identified as *B. × wardii*, all but two individuals were assigned to the F₁ class with high posterior probabilities (>0.974). The remaining individual BHWI2 was assigned to the F₁ class with probability of 0.778 whereas BHWI9 was assigned to be *B. crispa* with a high posterior probability (0.997) (Figure 4 a).

Population structure analysis

The Bayesian clustering-based structure analysis of all individuals based on all variation loci showed that the highest ΔK value was found with K=2, indicating that the individuals sampled in this study were divided into two genetic clusters. When K=2, the genetic component of almost all individuals morphologically identified as *B. alternifolia* mostly formed one cluster and almost all individuals identified as *B. crispa* formed another cluster. The remaining two individuals of *B. alternifolia* (TJAL6 and TJAL12) showed low genetic admixture from *B. crispa*, moreover, one of the remaining three individuals of *B. crispa* (BHCR7) as well as most *B. × wardii* individuals showed equal proportions from both clusters. The remaining *B. × wardii* (BHWI9) and *B. crispa* (BHCR2 and TJCR 13) individuals had large genetic component belonging to *B. crispa* (BHWI9: 0.840; BHCR2: 0.755; TJCR13: 0.830), indicating a backcrossed generation (Figure 4 b).

Discussion

In the present study, *B. wardii* has been confirmed to be an F₁ hybrid using both morphological and molecular evidence. *B. × wardii* displayed intermediate morphological characters in the four quantitative characters assessed, whereas the remaining five characters were similar to those of *B. crispa* but different from those of *B. alternifolia*. No transparent traits were observed, indicating very early generation hybrids with limited genetic segregation. Chromatogram additivity of *B. × wardii* at each of the differentiated nuclear genes of the parental species (Table 2), as well as estimation of genotypes using NewHybrids further confirmed that most *B. × wardii* individuals should be considered to be F₁s. Thus evidence from both morphology and molecular markers allowed us to reject the hypothesis that *B. × wardii* had undergone sufficient genetic recombination, generally assumed to be vital for establishing a diploid hybrid species^[26].

The present hybrid populations were found at very high elevation (both hybrid zones > 3400m) in Tibet, and most traditionally considered prerequisites were fulfilled in *B. × wardii*^[1]. Sympatric distribution, overlapping flowering periods (*B. alternifolia* ranging from April to June, while *B. crispa* from March to August)^[27-28] (personal observation) and shared pollinators (bees, bumblebees and butterflies)^[29] (personal observation) all facilitate natural hybridization between the two parental species. Another factor favoring natural hybridization between *B. alternifolia* and *B. crispa* was that they are both diploid with 2n = 38^[16], which may facilitate hybridization^[30-31]. In addition, *Buddleja* in Asia is a young clade that began diversifying approximately 10 Ma during the uplift of the Himalayas, and reproductive isolation between them is still incomplete^[16,32]. Furthermore, *Buddleja* generally grow in disturbed areas, such as road cuts in new development sites. Anthropogenic activities altering the original habitats might result in hybridization and provide suitable habitat for hybrids^[1,33].

Notably, we never found isolated populations *B. × wardii*, and in the only two populations, it grows together with both of the putative parents, *B. × wardii* is therefore not likely to be a full species because it does not appear to be self sustainable^[34-35]. Schumer et al.^[36] suggested three strict criteria that a putative hybrid species (i.e. a species of hybrid origin) should satisfy, including (1) a strong RI mechanism between the putative parental and new hybrid species; (2) genetic evidence of hybridization; and (3) isolating mechanisms derived from hybridization itself. Although Feliner et al.^[35] argue that focusing exclusively on RI and its origin directly from hybridization would shift the interest away from other crucial elements (e.g. ecological dimensions of the process and the production of novel diversity), *B. × wardii* would never be considered as a hybrid species due to the fact that it is not self sustainable, and that most of the hybrids in the study areas have F₁ characteristics. We therefore recommended that the literature in the future should avoid the name *B. wardii*.

Although F1 dominant hybrid zones were confirmed in both study areas, heritable patterns of cpDNA were different. In most angiosperms, chloroplast DNA is maternally inherited^[37]. The sequencing of cpDNA in the BH population showed that *B. crispa* and *B. alternifolia* each have their own specific chloroplast DNA haplotypes, and most *B. × wardii* individuals have the same haplotypes as *B. crispa*. The unique haplotypes seen in the remaining four *B. × wardii* individuals (BHWI8/11/15/16) may due to unsampled polymorphism of the parents. Additionally, interspecific pollinations show that the fruit set was 6.45% (2/31) for *B. alternifolia* flowers and 65.38% (17/26) for *B. crispa* flowers (Liao RL, unpublished data), suggesting strongly asymmetric hybridization or unidirectional incompatibility and *B. crispa* has a strong affinity for alien pollen. Several hypotheses have already been suggested to interpret asymmetric heritage of cpDNA in hybrids, including differences of breeding systems, flowering time, pollinator behavior and the local abundance of parental taxa in contact zone^[38-41]. For the present study, we have no evidence of pollinator behavior data to relate the asymmetry hybridization. It has been hypothesized that it should be more common for the self-compatible species to be the maternal parent^[7]. Previous studies have confirmed self-compatibility in *B. crispa* from the substantial seed set attributed to geitonogamous pollination, similar to that following out-crossing when flowers^[29], whereas no seeds were obtained in self-pollination treatments in *B. alternifolia* (Liao RL, unpublished data). Additionally, the peak flowering time of *B. crispa* is earlier than *B. alternifolia* with only very limited overlap. Based on our observation of flowering time towards the two parental species that had been introduced into Kunming Botanical Garden from Tibet, *B. crispa* was nearly finished flowering when *B. alternifolia* started flowering in 2019 and 2020. It is possible that differences in flowering time could lead to an earlier flowering species tending to receive mainly heterospecific pollen onto its stigmas towards the end of its flowering period, favoring it as maternal parent for hybrids^[7,42]. In both populations, *B. alternifolia* outnumber *B. crispa*, and therefore our results support the prediction that the minority species usually acts as the maternal parent^[41].

Additionally, in the TJ hybrid zone, the maternal parent could not be identified due to the cpDNA results (only 6 individuals of *B. alternifolia* had a haplotype different from other individuals, with all other individuals sharing the same haplotype). Due to the fact that almost all hybrids were F₁s and only a very limited number backcrossed to the parents (NewHybrids, *B. alternifolia*: 0; *B. crispa*: 1; Structure: *B. alternifolia*: 2; *B. crispa*: 1), the explanation that repeated backcrossing contributed to the pattern of cpDNA in this hybrid zone was rejected. However, it is possible that other haplotypes of cpDNA in *B. crispa* existed historically but have disappeared, as we found that > 500 mature *B. alternifolia* and < 100 *B. crispa* individuals were currently distributed in TJ. This may also be due to the slow evolution of chloroplast gene^[43-44], which makes it impossible to identify the maternal parent of hybrids

in some species pair with close relatives. Therefore, more chloroplast gene fragments will be needed in future studies.

Conclusions

We investigated patterns of hybridization in two butterfly bush species in two areas in Tibet, both with elevations above 3400 m. Both morphological and molecular analyses supported our hypothesis that the putative hybrid plants are F_1 s, without evidence of transparental traits or genetic recombination. This system is further effectively promoting nearly complete reproductive isolation between the parental plants. The self compatible breeding system, earlier peak flowering and small number of *B. crispa* individuals suggest that *B. crispa* is the maternal parent, which is supported by the cpDNA analysis of hybrids. Overall, the present study provides mechanisml insight into the maintenance of reproductive isolation, in particular for sympatrically growing pioneer plants in disturbed habitats, which have to date been largely ignored in natural hybridization studies.

Methods

Species and plant material in this study

Both *B. crispa* and *B. alternifolia* are vigorous deciduous shrubs or small trees to 2-4 m high. *B. crispa* is a widespread species distributed in the hot/warm-dry valleys, growing on slopes with boulders, on exposed cliffs, and in thickets at elevations of 1400-4300m, across the Himalaya-Hengduan area^[15,45]. *B. alternifolia* is distributed in northwest of China throughout Tibet to Loess Plateau, where it is naturally found growing along river banks or dried up streams in thickets at an altitude of 1500-4000m^[15,46]. Ecologically, *B. crispa* and *B. alternifolia* are highly susceptible to habitat disturbance^[18,45-46]. The two species occupy similar habitats and often occur sympatrically where their altitudinal ranges overlap^[27,45-46]. Both species are diploid with a chromosome number of $2n=38$ ^[16,18,47-48], and start flowering in spring (*B. crispa*: March to August; *B. alternifolia*: April to June)^[27-28].

All material for morphological characters and molecular analysis were field-collected. In Lhasa and Nyingchi, Tibet, China, individuals of intermediate morphologies between *B. alternifolia* and *B. crispa* were found co-occurring with sympatric populations of the two species along two branches of Brahmaputra river: the Ni-yang River and the Lhasa River (Figure 5). In this study, we sampled 17, 15 and 16 individuals of *B. alternifolia*, *B. crispa* and *B. × wardii* from Bahe town (BH) in Nyingchi, and 15, 16 and 17 individuals of *B. alternifolia*, *B. crispa* and *B. × wardii* from Taji county (TJ) in Lhasa (Table 3) for molecular analysis. No specific permissions were required for the relevant locations/activities and these species have not been included in the list of national key protected plants. The formal identification of the plant material was undertaken by Dr. Yongpeng Ma following the Flora of China (FOC)^[27]. Voucher specimens were deposited at the Yunnan Key Laboratory for Integrative Conservation of Plant Species with Extremely Small Populations, Kunming Institute of Botany, Chinese Academy of Sciences (Additional file 2: Table S2). In both sampled populations, many (more than 500) *B. alternifolia* individuals were found. However, at both BH and TJ, population size estimates of both *B. crispa* and the putative hybrid were fewer than 100 plants per population.

Measurements and data analysis of morphological traits

Three leaf morphological characters were measured from 30 mature leaves without any apparent damage, randomly selected from 30 individuals per taxon (one leaf per individual). Seven morphological flower characters were measured from 30 randomly selected healthy flowers during the flowering period from 30 plants per taxon (one flower per individual). These 10 characteristics were measured using digital calipers to the nearest 0.01 mm as follows: 1) leaf length (L, from the tip of the leaf to the point where the petiole joins the lamina); 2) leaf width (W, width at widest point); 3) ratio of leaf length to leaf width (L/W); 4) corolla tube length (TL); 5) corolla tube width (TW, diameter of the top of corolla tube); 6) corolla lobe length (CLL); 7) corolla lobe width (CLW); 8) anther height (AH, from the top of stamen to the start of ovary); 9) style length (SL, from the base of ovary to the top of stigma); 10) herkogamy (HE, separation between tip of style and base of stamen) (Additional file 3: Table S3).

In order to reduce the impact of extreme values, a trimmed means approach was used to calculate the means for every 30 individuals of each taxon^[49]. One-way ANOVA was used to analyze these traits among the three taxa in SPSS Statistics 16.0^[50], and a standard F statistic was used to determine the significance of differences between means. A Welch statistic was employed where the data did not satisfy the criterion of homogeneity of variance. And post hoc Tamhan's test was performed in pairwise comparison. The data were then subjected to two-dimensional Procrustes fitting in PAST (PALaeontology STATistical) software ver. 3.26^[51] to standardize landmark coordinates, followed by a shape principal components analysis (PCA) to perform multivariate analyses of the measured morphological characters (leaf and floral traits)^[52].

Petal color analysis

To assess patterns of light reflection in these three taxa at different wavelengths, we obtained spectral data from petals using a S2000 miniature fiber optic spectrometer with a PX-2 pulsed xenon lamp (Ocean Optics, Dunedin, FL, USA). All measurements were carried out in the range from 250 nm to 750 nm, using 0.30 nm increments^[53]. We choose 30 petals from 30 individuals per taxon (one flower per individual) and took one measurement per petal (Additional file 4: Table S4).

DNA extraction, PCR amplification and DNA sequencing

Total DNA was extracted from approximately 50 mg dried shoot tissues using the modified cetyl trimethyl ammonium (CTAB) method following Doyle and Doyle^[54], and samples were stored at -20°C before further analyses. PCR was conducted using 2×Taq PCR Master-mix (Tiangen) with the following conditions: initial denaturation at 94°C for 4 min; followed by 32 cycles of 94°C for 30s, corresponding annealing temperature at 52°C for 40s, and 72°C for 50s; and finally an extension step at 72°C for 10 min. The PCR products were purified by running them on a 1.2% agarose gel and were then directly sent for DNA sequencing on an ABI 3730 DNA analyzer (Applied Biosystems).

We generated sequence data from the nuclear ribosomal locus external transcribed spacer (nrETS), two low-copy nuclear genes from the pentatricopeptide repeat (PPR) gene family (PPR24 and PPR123)^[55], one low-copy nuclear gene from the gapC gene family (gapC2)^[24], and three plastid regions (rpl16, trnD-trnT, trnS-trnfM). The nrETS region was amplified using the universal primers ETS-B and 18S-IGS. For the locus PPR24, primers PPR24-140F and PPR24-1354R were used. For the locus PPR123, primers PPR123-550F and PPR123-1890R were used^[50]. The gapC2 locus was amplified following Liao et al.^[24]. The trnD-trnT region was amplified using the primers trnD^{GUC}F and trnT^{GGU}, the trnS-trnfM region with primers trnS^{UGA} and trnfM^{CAU} from Chau et al.^[55], and the rpl16

region with rpl16-F71 and rpl16-R1516 from Borg et al. [56]. Sequences of all the primers used are listed in Additional file 5: Table S5. Sequences in this study have been deposited in GenBank with accession numbers MT7333350-MT733514. In addition, published data of 12 individuals of *B. crispa* from an allopatric population in Xishan Mountain of Kunming in Yunnan of China as reference, and analyses in two nuclear genes of ETS and gapC2 [24].

Data analysis

DNA sequences were aligned, assembled and compared using SeqMan™ (DNASTAR, Madison, Wisconsin, USA) (<https://www.dnastar.com/>) [4,24]. PHASE in DNASP ver. 5.10.01 was used to infer the haplotypes, and to calculate the number of nucleotide polymorphisms and haplotypes [57-59]. For the few haplotypes estimates with uncertain phases, we choose the haplotype with the highest probability for analysis. The haplotype network of each locus was constructed using the median-joining algorithm in Network ver. 5.0.1.1 (<http://www.fluxus-engineering.com>) [52]. The program NewHybrids ver. 1.1 was used to assign each individual to a genotype category: the two parental species, F₁ and F₂ generations, backcross to each parental species. Using this program does not require pure samples of parental species and we identified the hybrid classes of samples by using the default settings with 100,000 burn-in iterations and 100,000 subsequent Markov Chain Monte Carlo (MCMC) [60] (Additional file 6: Table S6).

Genomic admixture proportions were determined using a Bayesian approach implemented in Structure ver. 2.3.4 (<https://web.stanford.edu/group/pritchardlab/structure.html>) using the default settings [61-62]. Analyses were run with numbers of distinct clusters (K) varying from 1 to 10, with ten iterations performed for each K and a burn-in of 100000 and a Markov chain Monte Carlo (MCMC) of 100000 iterations (Additional file 7: Table S7). The optimal K of distinct groups was obtained using ΔK statistics through Structure Harvester ver. 0.6.94 (http://taylor0.biology.ucla.edu/struct_harvest/) [63-64] (Additional file 8: Figure S1). Membership coefficients at each of the suggested numbers of clusters for each individual were then estimated across the 10 independent runs using CLUMPP ver. 1.1.2 [65]. Finally, Distruct ver. 1.1 [66] was used to generate a graphical display of the population structuring.

Abbreviations

ANOVA: Analysis of Variance; DNA: DeoxyriboNucleic Acid; cpDNA: chloroplast DNA; PCR: Polymerase Chain Reaction.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data sets supporting the results of the present study are included within this article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YM and RL collected the plant materials and wrote the manuscript; RL performed the experiments and analyzed the data; YM and WS designed the experiment and revised the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Morphological traits used to distinguish between *B. alternifolia*, *B. crispa* and *B. × wardii*.

Characters	<i>B. alternifolia</i>	<i>B. × wardii</i>	<i>B. crispa</i>	F	Welch	P value
L (mm)	16.00±3.15 ^a	27.31±10.02 ^b	64.65±25.47 ^c		62.971	<0.001
W (mm)	3.62±0.66 ^a	14.00±3.26 ^b	36.61±11.72 ^c		235.112	<0.001
L/W	4.52±0.99 ^a	1.94±0.47 ^b	1.76±0.31 ^b		97.410	<0.001
TL (mm)	7.10±0.80 ^a	9.77±1.04 ^b	10.25±1.51 ^b		79.584	0.017
TW (mm)	0.93±0.17 ^a	1.09±0.15 ^b	1.20±0.18 ^c	17.584		0.641
CLL (mm)	1.43±0.27 ^a	2.29±0.37 ^b	2.17±0.40 ^b	49.695		0.073
CLW (mm)	1.60±0.20 ^a	2.41±0.35 ^b	2.45±0.46 ^b		80.282	0.004
AH (mm)	5.22±0.76 ^a	5.97±0.93 ^b	6.66±0.96 ^c	18.266		0.539
SL (mm)	2.89±0.47 ^a	4.14±0.91 ^b	4.41±1.05 ^b		46.621	0.001
HE (mm)	1.08±0.67	0.75±0.72	0.95±0.87	1.261		0.588

1 mean ± standard deviation are shown for the three traits.

2 L: leaf length, W: leaf width, L/W: ratio of leaf length to leaf width, TL: corolla tube length, TW: corolla tube width, CLL: corolla length, CLW: corolla lobe width, AH: anther height, SL: style length, HE: herkogamy.

3 ^{a, b, c}: the means with different superscripts are significantly different from each other at the 0.05 level and based on Tamhane's

Table 2 Haplotypes and genotypes of *B. × wardii*(Bw/Tw) between *B. alternifolia* (Ba/Ta) and *B. crispa* (Bc/Tc) at fo nuclear genes and the combined chloroplast regions (cpDNA). The code of each haplotype is the same as that in Fig

Equals ETS-B	GapC2	PPR24	PPR123	cpDNA
1 Ba1(Bw1)	Ba1(Bw1)	Bw6	Ba1(Bw1, Bc1)	Bc3(Bw2)
Bc3(Bw8)	Bc4(Bw5)	Bc2(Bw9)	Bc3(Bw7)	
2 Bw2	Ta1(Tw1, Bw2, Bc1)	Bw2	Bw5	Bc1(Bw1)
Bc3(Bw8)	Bc6(Bw6, Tw2, Tc1)	Bw13	Bc3(Bw7)	
3 Bw5	Ta1(Tw1, Bw2, Bc1)	Bw6	Ba1(Bw1, Bc1)	Bc1(Bw1)
Bc5(Bw10)	Bc9(Bw8)	Bc5(Bw10)	Bc2(Bw6)	
4 Bw5	Ta1(Tw1, Bw2, Bc1)	Ba2(Bw4)	Ba1(Bw1, Bc1)	Bc1(Bw1)
Bc3(Bw8)	Bc6(Bw6, Tw2, Tc1)	Bc5(Bw10)	Bc3(Bw7)	
5 Ba1(Bw1)	Ba1(Bw1)	Bw6	Ba1(Bw1, Bc1)	Bc3(Bw2)
Bc3(Bw8)	Bc4(Bw5)	Bc6(Bw11)	Bc3(Bw7)	
6 Ba3(Bw3)	Ba1(Bw1)	Bw5	Ba1(Bw1, Bc1)	Bc3(Bw2)
Bc3(Bw8)	Bc6(Bw6, Tw2, Tc1)	Bw8	Bw8	
7 Ba1(Bw1)	Ta1(Tw1, Bw2, Bc1)	Bw6	Ba1(Bw1, Bc1)	Bc1(Bw1)
Bw11	Bc6(Bw6, Tw2, Tc1)	Bc2(Bw9)	Bc2(Bw6)	
8 Ba1(Bw1)	Ba1(Bw1)	Bw2	Ba2(Bw4)	Bw3
Bw13	Bw7	Bc5(Bw10)	Bc3(Bw7)	
9 Bc3(Bw8)	Bw4	Bw3	Bc3(Bw7)	Bc3(Bw2)
Bc3(Bw8)	Bw4	Bc2(Bw9)	Bw8	
10 Ba1(Bw1)	Ta1(Tw1, Bw2, Bc1)	Bw2	Ba1(Bw1, Bc1)	Bc1(Bw1)
Bw12	Bc3(Bw3)	Bw8	Bc2(Bw6)	
11 Ba1(Bw1)	Ta1(Tw1, Bw2, Bc1)	Ba4(Bw7)	Ba2(Bw4)	Bw3
Bc3(Bw8)	Bc6(Bw6, Tw2, Tc1)	Bc5(Bw10)	Bc3(Bw7)	
12 Ta1(Bw4)	Ta1(Tw1, Bw2, Bc1)	Ta1(Tw2, Ba1, Bw2)	Bw2	Bc1(Bw1)
Bc3(Bw8)	Bc6(Bw6, Tw2, Tc1)	Bc7(Bw12)	Bc3(Bw7)	
13 Ta2(Tw1, Tc1, Ba5, Bw7)	Ta1(Tw1, Bw2, Bc1)	Ta1(Tw2, Ba1, Bw2)	Ba1(Bw1, Bc1)	Bc1(Bw1)
Bc3(Bw8)	Bc9(Bw8)	Bc9(Bw14)	Bc3(Bw7)	
14 Ta2(Tw1, Tc1, Ba5, Bw7)	Ba1(Bw1)	Ta1(Tw2, Ba1, Bw2)	Bw3	Bc1(Bw1)
Bc3(Bw8)	Bw9	Bc2(Bw9)	Bc3(Bw7)	
15 Bw6	Ta1(Tw1, Bw2, Bc1)	Ta1(Tw2, Ba1, Bw2)	Ba1(Bw1, Bc1)	Bw3
Bc3(Bw8)	Bc6(Bw6, Tw2, Tc1)	Bc5(Bw10)	Bc3(Bw7)	
16 Ba1(Bw1)	Ta1(Tw1, Bw2, Bc1)	Ta1(Tw2, Ba1, Bw2)	Ba1(Bw1, Bc1)	Bw3
Bw9	Bc6(Bw6, Tw2, Tc1)	Bc2(Bw9)	Bc2(Bw6)	
Tw3	Ta2(Tw3)	Bc1(Tw1)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Tw3	Tc3(Tw5)	Tc2(Tw11)	Tc7(Tw5)	
Tc2(Tw2, Bc4)	Ta2(Tw3)	Bc1(Tw1)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tc3(Tw5)	Tc1(Tw9)	Tw4	
Tc2(Tw2, Bc4)	Ta1(Tw1, Bw2, Bc1)	Bc1(Tw1)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tw7	Tc2(Tw11)	Tw8	
Tc2(Tw2, Bc4)	Ta1(Tw1, Bw2, Bc1)	Tw8	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tw6	Tc2(Tw11)	Tw7	
Tc2(Tw2, Bc4)	Ta2(Tw3)	Bc1(Tw1)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tc4(Tw8, Ta3)	Tc8(Tw15)	Tw10	
Tc2(Tw2, Bc4)	Ta1(Tw1, Bw2, Bc1)	Bc1(Tw1)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tc4(Tw8, Ta3)	Tc1(Tw9)	Tc4(Tw3)	
Tc2(Tw2, Bc4)	Ta1(Tw1, Bw2, Bc1)	Tw7	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tc6(Tw10)	Tc8(Tw15)	Tc7(Tw5)	
Tc2(Tw2, Bc4)	Tw4	Tw7	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Bc6(Bw6, Tw2, Tc1)	Tc5(Tw14)	Tc7(Tw5)	
Tc2(Tw2, Bc4)	Ta1(Tw1, Bw2, Bc1)	Bc1(Tw1)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
Ta2(Tw1, Tc1, Ba5, Bw7)	Tc4(Tw8, Ta3)	Tw13	Tc7(Tw5)	
0 Tc2(Tw2, Bc4)	Ta1(Tw1, Bw2, Bc1)	Tw4	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)

	Ta2(Tw1, Tc1, Ba5, Bw7)	Tc3(Tw5)	Tc1(Tw9)	Tc4(Tw3)	
1	Tc2(Tw2, Bc4) Tw6	Ta1(Tw1, Bw2, Bc1)	Tw4	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
2	Tw5	Bc6(Bw6, Tw2, Tc1)	Tc1(Tw9)	Tc4(Tw3)	
	Ta2(Tw1, Tc1, Ba5, Bw7)	Ta1(Tw1, Bw2, Bc1)	Tw4	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
	Tc2(Tw2, Bc4) Tw6	Tc5(Tw9)	Tc1(Tw9)	Tc2(Tw2)	
3	Ta1(Tw1, Bw2, Bc1)	Tw6	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)	
	Bc6(Bw6, Tw2, Tc1)	Tc8(Tw15)	Tc2(Tw2)		
5	Tc2(Tw2, Bc4) Ta6(Tw7)	Ta1(Tw1, Bw2, Bc1)	Ta1(Tw2, Ba1, Bw2)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
	Tc2(Tw2, Bc4)	Tc4(Tw8, Ta3)	Tc8(Tw15)	Tc7(Tw5)	
6	Ta1(Tw1, Bw2, Bc1)	Tw3	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)	
	Ta2(Tw1, Tc1, Ba5, Bw7)	Tc4(Tw8, Ta3)	Tw12	Tc7(Tw5)	
7	Tc2(Tw2, Bc4) Ta2(Tw1, Tc1, Ba5, Bw7)	Ta1(Tw1, Bw2, Bc1)	Ta1(Tw2, Ba1, Bw2)	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)
	Tw4	Tc4(Tw8, Ta3)	Tw10	Tw9	
8	Ta1(Tw1, Bw2, Bc1)	Tw5	Ta1(Tw1)	Ba3(Ta1, Tc1, Tw1)	
	Ta2(Tw1, Tc1, Ba5, Bw7)	Bc6(Bw6, Tw2, Tc1)	Tc8(Tw15)	Tc8(Tw6)	

Note. BHWI1-BHWI16 and TJWI1-TJWI16 in the first column are 33 individuals of the *B. × wardii* from BH and TJ population, respectively. “Ba”, “Bc” and “Bw” refer to *B. alternifolia*, *B. crispa*, and *B. × wardii* from BH population, respectively; while “Ta”, “Tc” and “Tw” refer to *B. alternifolia*, *B. crispa*, and *B. × wardii* from TJ population, respectively.

Table 3 Details of the sampling of the three *Buddleja* taxa examined in this study, including the altitude, latitude and longitude of the sampled populations. The collection codes of the individual samples are given in brackets.

Sampling location	Taxon	Number of individuals	Altitude	Longitude	Latitude
Bahe (BH)	<i>B. alternifolia</i>	17 (BHAL1-17)	3434 m	93°14'0.50"	29°52'58.54"
	<i>B. crispa</i>	15 (BHCR1-15)			
	<i>B. × wardii</i>	16 (BHWI1-16)			
Taji (TJ)	<i>B. alternifolia</i>	15 (TJAL1-15)	3725 m	91°26'56.97"	29°44'56.15"
	<i>B. crispa</i>	16 (TJCR1-16)			
	<i>B. × wardii</i>	17 (TJWI1-13,15-18)			

Figures

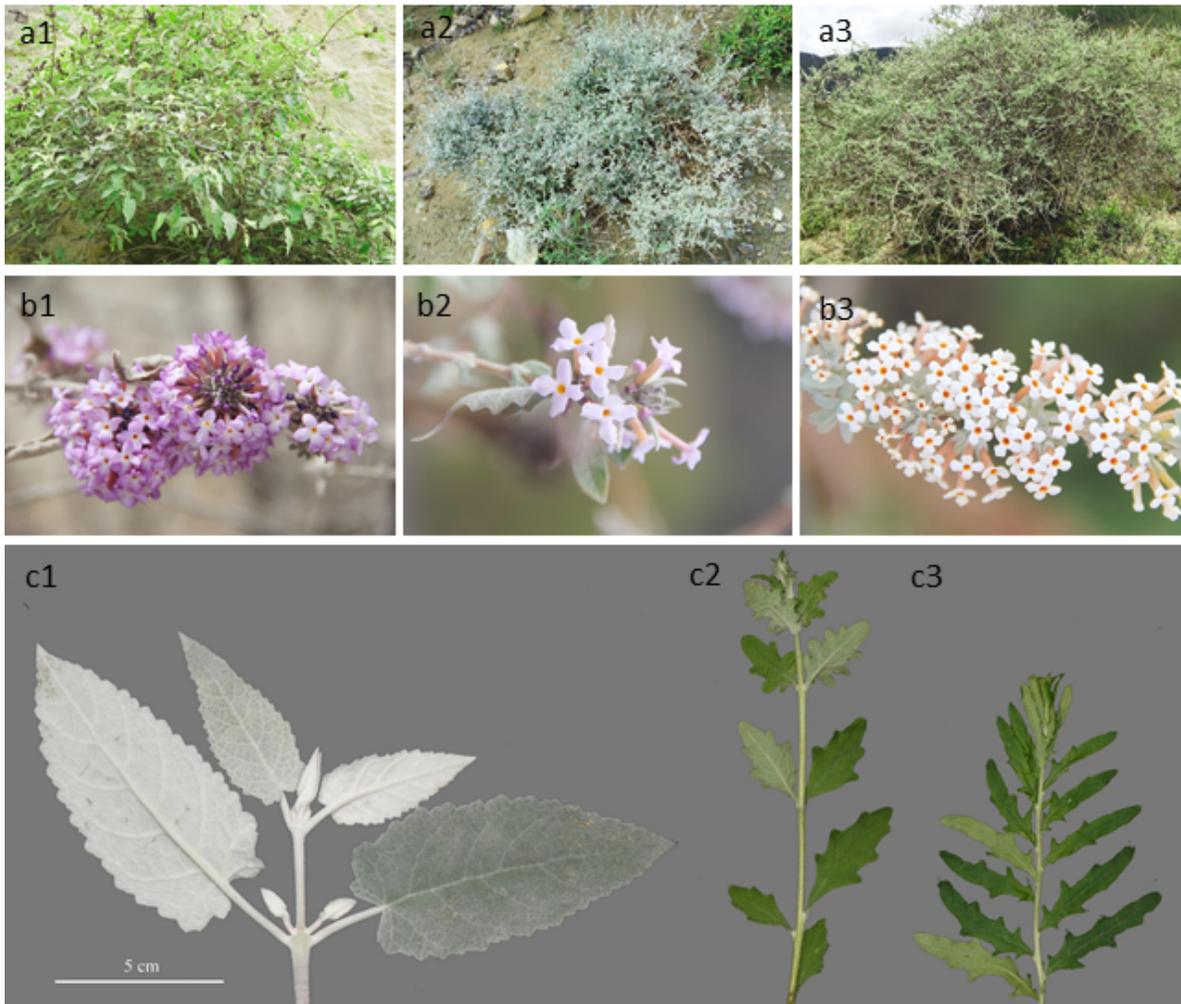


Figure 1

Morphological details of *B. crispa* (the left column), *B. × wardii* (the middle column) and *B. alternifolia* (the right column),.

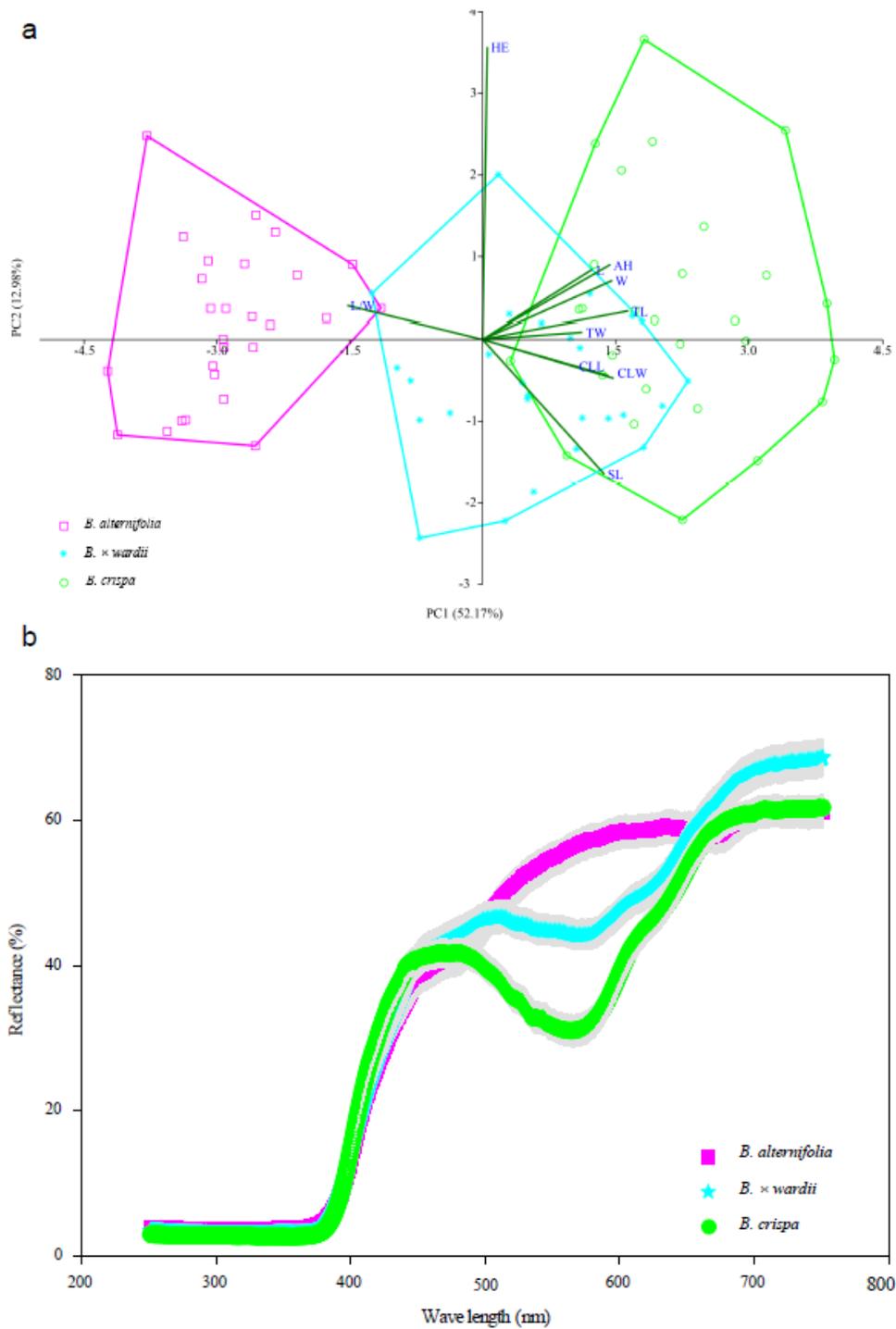


Figure 2

Two-dimensional scatter diagram of the first and second components from the PCA using 10 morphological characteristics (a), and petal reflectance spectra (b) in *B. alternifolia*, *B. crispa*, and *B. x wardii*. Pink square, green circle and calamine blue star represent *B. alternifolia*, *B. crispa* and *B. x wardii*, respectively.

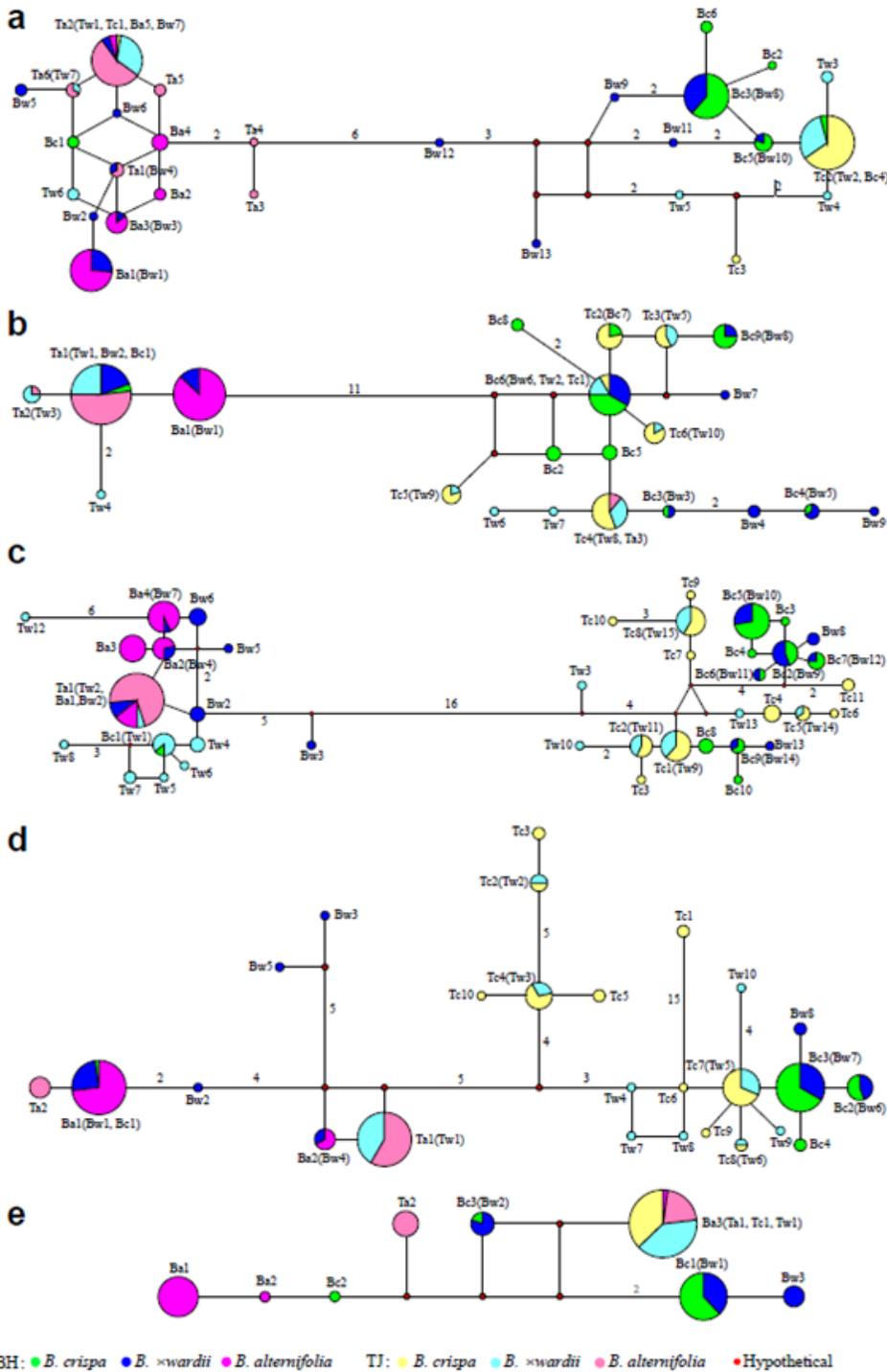


Figure 3

Haplotype network for ETS (a), gapC2 (b), PPR24 (c), PPR123 (d), cpDNA (e). Haplotypes of each taxon are denoted using the first letter of its population and species name (“Ba”, “Bc” and “Bw” refer to *B. alternifolia*, *B. crispa*, and *B. × wardii* from BH population, respectively; while “Ta”, “Tc” and “Tw” refer to *B. alternifolia*, *B. crispa*, and *B. × wardii* from TJ population, respectively). The number of mutations separating two haplotypes is indicated by the number shown on the connecting lines, the number is omitted for those with only one mutational step, and node size is proportional to the frequency of each haplotype. Color circles represent haplotypes of different species as follows: green and yellow represent *B. crispa*; pink and peach represent *B. alternifolia*; blue and calamine blue represent *B. × wardii*. Small red circles represent hypothetical or unsampled haplotypes.

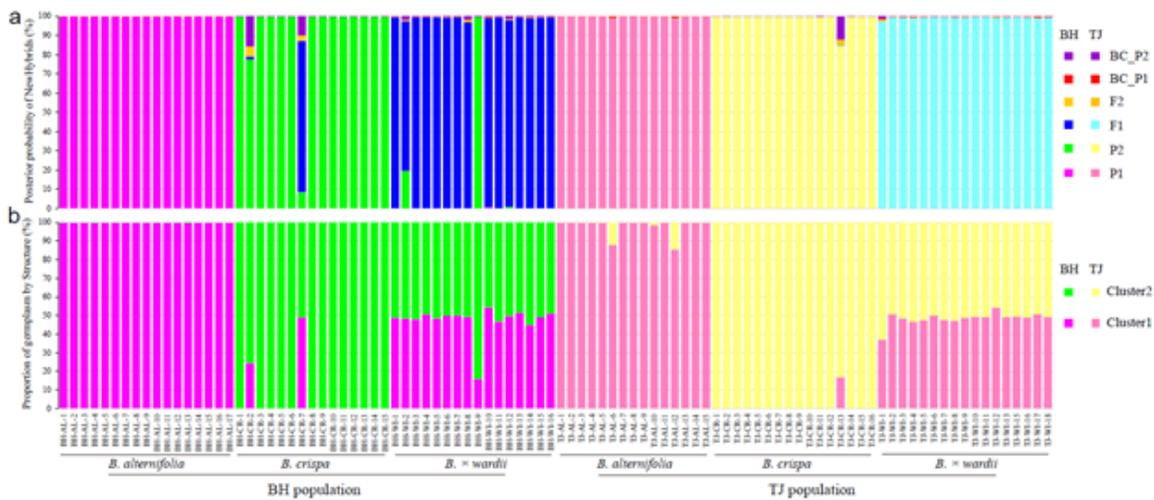


Figure 4

Genotype class assignment by NewHybrids (a) and clustering analysis by Structure (b) for *B. alternifolia*, *B. crispa*, and *B. × wardii*, based on sequence data of nuclear genes. For the label of each accession, “BH” and “TJ” refers to individuals collected in BH population and TJ Population, “AL”, “WI” and “CR” refer to *B. alternifolia*, *B. crispa*, and *B. × wardii*, respectively. Bars in different color in a represent different genotype class: pink and peach represent Parent 1; green and yellow represent Parent 2; blue and calamine blue represent F1 hybrid; orange, red and purple represent F2 hybrid, back cross to Parent 1 and back cross to Parent 2, respectively. Bars in different color in b represent different clusters: pink and peach represent Cluster 1; green and yellow represent Cluster 2.

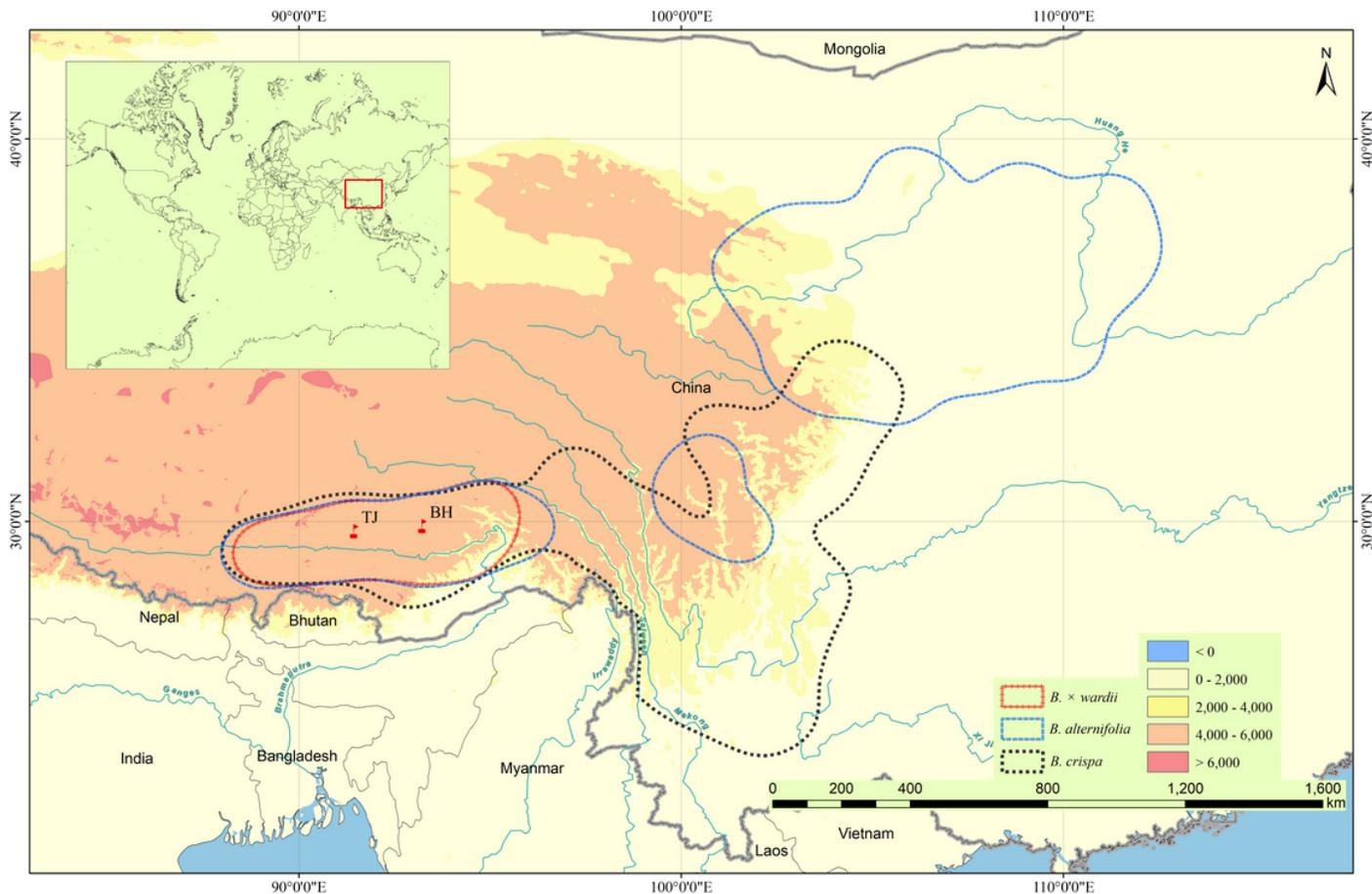


Figure 5

Known geographical distributions of *B. alternifolia*, *B. crispa*, and *B. × wardii* in China. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

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