Natural Hybridization between Two Butterfly Bushes in Tibet: Dominance of F_1 Hybrids Promotes Strong Reproductive Isolation

Rongli Liao
Kunming Institute of Botany Chinese Academy of Sciences https://orcid.org/0000-0002-6695-2739
Weibang Sun (wbsun@mail.kib.ac.cn)
Yongpeng Ma
Kunming Institute of Botany Chinese Academy of Sciences

Research article

Keywords: Butterfly bushes, Buddleja, Hybridization, F1-dominated hybrids, Reproductive isolation

Posted Date: July 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-42245/v1

Version of Record: A version of this preprint was published at BMC Plant Biology on March 10th, 2021.
See the published version at https://doi.org/10.1186/s12870-021-02909-7.

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_1s. 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_1s[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_1s 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_1s[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 *Buddleja crispa* Benth. and *Buddleja alternifolia* Maxim., because of its morphology, which lies between the morphologies of these two. 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 (Fig. 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).

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

Characters	*B. alternifolia*	*B. × wardii*	*B. crispa*	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 lobe 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 T2 test

The two 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) (Fig. 2a).

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* (Fig. 2 b).

Sequence analyses of the four nuclear genes in the BH population

NrETS: The total length of the nrETS region alignment was 380 bp in all individuals, including 20 nucleotide substitutions (for variation sites, see Additional file 1: Table S1). A total of 19 haplotypes were observed from these loci, among them five, six and thirteen haplotypes from *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. Haplotype network analysis identified two major clusters separated by eight nucleotide substitutions. One cluster comprised five haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and seven haplotypes of *B. × wardii*. The other cluster comprised five haplotypes of *B. crispa* and six haplotypes of *B. × wardii* (Fig. 3 a1).

Only one haplotype from *B. crispa* nested with the *B. alternifolia* cluster, and was found in BHCR2 (H10/H11) and BHCR7 (H10/H12), which show different haplotypes from both clusters. Of the putative hybrid individuals, all but one individual (BHWI9) had two divergent haplotypes nested with each of the two divergent clusters. The individual BHWI9 was homozygous for a *B. crispa* haplotype at this locus (Fig. 3 a1).

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 (for variation sites, see Additional file 2: Table S2). A total of 13 haplotypes were observed for these loci, among them one, nine and nine haplotypes belonging to *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. In the haplotype network analysis, this region was divided into two clusters by twelve nucleotide substitutions. One cluster contained the only one haplotype of *B. alternifolia*, one haplotype of *B. crispa* and two haplotypes of *B. × wardii*. The other cluster contained eight haplotypes of *B. crispa* and seven haplotypes of *B. × wardii*. 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. 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 (H5/H5) (Fig. 3 b1).

PPR24: The total length of the PPR24 region alignment was 647 bp for all individuals, including 43 nucleotide substitutions (for variation sites, see Additional file 3: Table S3). A total of 20 haplotypes were observed for these loci, among them four, ten and fourteen haplotypes from *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. In the haplotypes network analysis, this region was divided into two clusters by twenty-one nucleotide substitutions. One cluster contained four haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and seven haplotypes of *B. × wardii*. The other cluster contained nine haplotypes of *B. crispa* and seven haplotypes of *B. × wardii*. One *B. crispa* individual (BHCR7) showed different haplotypes from both clusters (H1/H14). All *B. × wardii* individuals showed two divergent haplotypes originating from both *B. alternifolia* and *B. crispa* clusters (Fig. 3 c1).

PPR123: After sequence alignment, the PPR123 region was 809 bp in length, and included 23 nucleotide substitutions (for variation sites, see Additional file 4: Table S4). A total of nine haplotypes were observed for these loci, among them two, four and eight haplotypes from *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. Haplotype network analysis identified two major clusters separated by eight nucleotide substitutions. One cluster comprised two haplotypes of *B. alternifolia*, one haplotype of *B. crispa* and five

haplotypes of *B. × wardii*. The other cluster comprised three haplotypes of *B. crispa* and three haplotypes of *B. × wardii* (Fig. 3 d1).

The only one haplotype from *B. crispa* nested within the *B. alternifolia* cluster derived from BHCR7 (H1/H7). Of the putative hybrid individuals, all but one individual (BHWI9) had two divergent haplotypes nested with each of the two divergent clusters. The individual BHWI9 had two haplotypes found in the *B. crispa* cluster (H7/H8) (Fig. 3 d1).

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 bp, and contained 10 nucleotide substitutions (for variation sites, see Additional file 5: Table S5). Seven haplotypes were inferred in total, and each taxon had three haplotypes. Haplotype network analysis indicated that most putative hybrid individuals (75%) shared two haplotypes with *B. crispa,* while they did not share any haplotypes with *B. alternifolia*. The remaining four *B. × wardii* individuals had a unique haplotype (H7), which was one mutational step away from the predominant haplotype of *B. crispa* (H4). Even though *B. alternifolia* and *B. crispa* did not share any haplotypes, the differences between their haplotypes were only one or two mutational steps. The haplotype network of cpDNA could not be divided into two clades. The haplotype H3 from *B. alternifolia* (BHAL13) was closer to the haplotype H6 from *B. crispa* and *B. × wardii* (two mutation sites away) than the haplotype H2 from the closest *B. alternifolia* (six mutation sites away) (Fig. 3 e1).

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 (>0.999). Of the 16 individuals morphologically identified as *B. crispa*, all but two (BHCR2 and BHCR7) could be identified as *B. crispa* with high posterior probabilities (>0.996). The individuals BHCR2 and BHCR7 were assigned to *B. crispa* and F_1 with the lower probabilities of 0.775 and 0.785, respectively. Of the 15 individuals morphologically identified as *B. × wardii*, 13 individuals were assigned to the F_1 class with high posterior probabilities (>0.974). The remaining individual BHWI2 was assigned to the F_1 class with probability of 0.778 whereas BHWI9 was assigned to be *B. crispa* with a high posterior probability (0.997) (Fig. 4 a1).

Population structure analysis

The Bayesian clustering-based structure analysis of all 48 individuals based on all variation loci showed that the highest $\triangle$K 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 the individuals morphologically identified as *B. alternifolia* in the BH population mostly formed one cluster and 14 of the 16 individuals identified as *B. crispa* formed another cluster. One of the remaining two individuals of *B. crispa* (BHCR7) as well as 15 *B. × wardii* individuals showed equal proportions from both clusters. The remaining *B. ×*

wardii (BHWI9) and *B. crispa* (BHCR2) individuals had large genetic component belonging to *B. crispa* (BHWI9: 0.840; BHCR2: 0.755), indicating a backcrossed generation (Fig. 4 b1).

Sequence analyses of the four nuclear genes in the TJ population

NrETS: The total length of the nrETS region alignment was 380 bp in all individuals, including 17 nucleotide substitutions (for variation sites, see Additional file 1: Table S1). A total of 12 haplotypes were observed from these loci, among them six, three and seven haplotypes from *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. Haplotype network analysis identified two major clusters separated by eight nucleotide substitutions. One cluster comprised six haplotypes from *B. alternifolia*, one haplotype from *B. crispa* and three haplotypes from *B. × wardii*. The other cluster comprised two haplotypes from *B. crispa* and four haplotypes from *B. × wardii* (Fig. 3 a2).

Only one haplotype from *B. crispa,* derived from TJCR13 (H4/H11), fell into the *B. alternifolia* cluster, and shows different haplotypes from both clusters. Of the putative hybrid individuals, all but one (TJWI1) had two divergent haplotypes from each of the two divergent clusters. The remaining one individual (TJWI1) was homozygous for a *B. crispa* haplotype at this locus (H12/H12) (Fig. 3 a2).

GapC2: The total length of the gapC2 region alignment was 606 bp for all individuals, including 22 nucleotide substitutions and one 1-bp insertion/deletion (for variation sites, see Additional file 2: Table S2). A total of 11 haplotypes were observed for these loci, among them three, six and ten haplotypes from to *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. In the haplotype network analysis, this region was divided into two clusters by twelve nucleotide substitutions. One cluster contained two haplotypes from *B. alternifolia* and three haplotypes from *B. × wardii*. The other cluster contained six haplotypes from *B. crispa* and seven haplotypes from *B. × wardii*. In *B. alternifolia*, there was an exception of two individuals, TJAL6 and TJAL12, which had a haplotype (H9) found in the *B. crispa* cluster, but all the other haplotypes were found in *B. alternifolia* cluster. All *B. × wardii* individuals showed two divergent haplotypes originating from both clusters (Fig. 3 b2).

PPR24: The total length of the PPR24 region alignment was 647 bp for all individuals, including 46 nucleotide substitutions (for variation sites, see Additional file 3: Table S3). A total of 22 haplotypes were observed for these loci, among them one, eleven and fifteen haplotypes belong to *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. In the haplotypes network analysis, this region was divided into two clusters by twenty-one nucleotide substitutions. One cluster contained the only haplotype from *B. alternifolia* and eight haplotypes from *B. × wardii*. The other cluster contained all ten haplotypes from *B. crispa* and seven haplotypes from *B. × wardii*. All *B. × wardii* individuals showed two divergent haplotypes originating from both the *B. alternifolia* and *B. crispa* clusters (Fig. 3 c2).

PPR123: After sequence alignment, the total length of the PPR123 region was 735 bp, including 45 nucleotide substitutions (for variation sites, see Additional file 4: Table S4). A total of 17 haplotypes were observed for these loci, among them two, ten and ten haplotypes from *B. alternifolia*, *B. crispa* and *B. × wardii,* respectively. The haplotype network analysis identified three major clusters separated by seven or

eight nucleotide substitutions. One cluster comprised two haplotypes from *B. alternifolia* and one haplotype from *B. × wardii*. The second cluster comprised five haplotypes from *B. crispa* and two haplotypes from *B. × wardii*. The third cluster comprised five haplotypes rom *B. crispa* and seven haplotypes from *B. × wardii* (Fig. 3 d2).

All haplotypes from *B. alternifolia* individuals fell into the first cluster. For the *B. crispa* individuals, ten individuals (TJCR3/5/6/7/9/10/11/12/13/15) had two divergent haplotypes, falling into the second and third clusters, two individuals (TJCR2/14) had haplotypes nested in the second cluster and four individuals (TJCR1/4/8/16) had haplotypes nested in the third cluster. Of the putative hybrid individuals, all had two divergent haplotypes, one in the first cluster and the other in either the second or the third cluster (Fig. 3 d2).

Sequence analyses for the combined chloroplast regions

The combined length of the aligned cpDNA fragments (rpl16, trnD-trnT, trnS-trnfM) from the three taxa was 2065 bp, containing 5 nucleotide substitutions (for variation sites, see Additional file 5: Table S5). All individuals had only two haplotypes, of them one was derived from six individuals (TJAL2/3/4/5/8/11), and the remaining 42 individuals shared another haplotype consistent with the haplotype only in individual BHAL13 (H3) from the BH population (Fig. 3 e2).

NewHybrids analysis

Analysis of four nuclear genes using NewHybrids on 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 16 individuals morphologically identified as *B. crispa*, all but one (TJCR13) were identified as *B. crispa* with high posterior probabilities (>0.997). The individual TJCR13 was assigned to *B. crispa* with lower probability (0.840). Of the 17 individuals morphologically identified as *B. × wardii*, all were assigned to the F_1 class with high posterior probabilities (>0.974) (Fig. 4 a2).

Population structure analysis

The Bayesian clustering-based structure analysis of all 48 individuals sampled from the TJ population based on all variable loci showed that the highest $\triangle$K value was obtained for K=2, indicating that the individuals sampled in this study could be divided into two genetic clusters. When K=2, the genetic component of 13 of the 15 individuals morphologically identified as *B. alternifolia* from the TJ population mostly formed one cluster, whereas 15 of 16 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, the remaining single *B. crispa* (TJCR13) contained a large genetic proportion similar to that of *B. crispa* (0.830), indicating a backcross. All individuals morphologically identified as *B. × wardii* displayed an approximately equal proportion of genetic material from both clusters (Fig. 4 b2).

Discussion

It has been suggested that *B. × wardii* is a perfect intermediate between *B. alternifolia* and *B. crispa*[18] based on the type specimen and Ludlow c.s. 4098 (BM, E, K), as well as their overlapping distribution, but details of the hybridization have not been substantiated. In the present study, *B. wardii* has been confirmed to be an F_1 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 F_1s. 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].

Table 2

Haplotypes and genotypes of the 33 individuals (BH:16, TJ:17) of *B. × wardii* at four nuclear genes and cpDNA. Haplotypes with wavy, single and double underlines are identical to those from only *B. crispa*, only *B. alternifolia* and both parents, respectively. The code of each haplotype is the same as that in Fig. 3.

Individuals	GapC2	ETS-B	PPR24	PPR123	cpDNA
BHWI1	H1H6	H1H12	H8H11	H1H7	H6
BHWI2	H2H8	H2H12	H2H17	H5H7	H4
BHWI3	H2H12	H7H15	H8H14	H1H6	H4
BHWI4	H2H8	H7H12	H5H14	H1H7	H4
BHWI5	H1H6	H1H12	H8H15	H1H7	H6
BHWI6	H1H8	H4H12	H7H10	H1H8	H6
BHWI7	H2H8	H1H17	H8H11	H1H6	H4
BHWI8	H1H11	H1H19	H2H14	H4H7	H7
BHWI9	H5H5	H12H12	H4H11	H7H8	H6
BHWI10	H2H4	H1H18	H2H10	H1H6	H4
BHWI11	H2H8	H1H12	H9H14	H4H7	H7
BHWI12	H2H8	H6H12	H3H16	H2H7	H4
BHWI13	H2H12	H9H12	H3H19	H1H7	H4
BHWI14	H1H13	H9H12	H3H11	H3H7	H4
BHWI15	H2H8	H8H12	H3H14	H1H7	H7
BHWI16	H2H8	H1H13	H3H11	H1H6	H7
TJWI1	H2H4	H1H1	H3H11	H1H10	H2
TJWI2	H2H4	H2H11	H3H9	H1H8	H2
TJWI3	H1H6	H2H11	H3H11	H1H14	H2
TJWI4	H1H5	H2H11	H8H11	H1H13	H2
TJWI5	H2H9	H2H11	H3H19	H1H17	H2
TJWI6	H1H9	H2H11	H3H9	H1H6	H2
TJWI7	H1H11	H2H11	H7H19	H1H10	H2
TJWI8	H3H8	H2H11	H7H16	H1H10	H2
TJWI9	H1H9	H2H11	H3H14	H1H10	H2

Individuals	GapC2	ETS-B	PPR24	PPR123	cpDNA
TJWI10	H1H4	H2H11	H2H9	H1H6	H2
TJWI11	H1H8	H2H10	H2H9	H1H6	H2
TJWI12	H1H10	H5H11	H2H9	H1H3	H2
TJWI13	H1H8	H2H10	H6H19	H1H3	H2
TJWI15	H1H9	H2H12	H5H19	H1H10	H2
TJWI16	H1H9	H2H11	H1H13	H1H10	H2
TJWI17	H1H9	H2H11	H5H10	H1H16	H2
TJWI18	H1H8	H3H11	H4H19	H1H11	H2

The present hybrid populations were found at very high elevation (both hybrid zones > 3400 m) 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. 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, 30]. This is supported by the plastid genes in our study, where, in the TJ population, *B. alternifolia* and *B. crispa* can not be distinguished using cpDNA. 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, 31].

Notably, we never found isolated populations *B. × wardii*, and *B. × wardii* grows together with either both or one of the putative parents, *B. × wardii* is therefore not likely to be a full species because it does not appear to be self sustainable[32–33]. Schumer et al.[34] 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.[33] 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 F_1 characteristics. We therefore recommended that the literature in the future should avoid the name *B. wardii*.

Although F_1 dominant hybrid zones were confirmed in both study areas, heritable patterns of cpDNA were different. In most angiosperms, chloroplast DNA is maternally inherited[35]. 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. Several hypotheses have already been suggested to interpret asymmetric heritage of cpDNA in hybrids, including differences of breeding systems, general matching between lengths of style and pollen tubes, flowering time, pollinator behavior and the local abundance of parental taxa in contact zone[36–39]. For the present study, we have no evidence of pollinator behavior data to relate the asymmetry hybridization. The significantly longer style of *B. crispa* than *B. alternafolia* also allowed us to reject the former as the maternal parent, because the plant with the shorter style is most commonly observed to be the maternal species[38]. Other reasons why *B. crispa* is likely to be maternal parent include the breeding system, phonology, and the number of individuals of the parental species. 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, 40]. 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[39].

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 F_1s 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.

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_1s, 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 mechanismal 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, 41]. *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, 42]. Ecologically, *B. crispa* and *B. alternifolia* are highly susceptible to habitat disturbance[18,41–42]. The two species occupy similar habitats and often occur sympatrically where their altitudinal ranges overlap[27,41–42]. Both species are diploid with a chromosome number of 2n = 38[16,18,43–44], 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. 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 6: Table S6). 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.

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 cumbers of the individual samples are given in brackets.

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

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 0.01mm 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 7: Table S7).

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[45]. One-way ANOVA was used to analyze these traits among the three taxa in SPSS Statistics 16.0[46], 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 Tamhane'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[47] 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)[48].

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 [49]. We choose 30 petals from 30 individuals per taxon (one flower per individual) and took one measurement per petal (Additional file 8: Table S8).

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[50], 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)[51], 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 [51]. 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.[51], and the rpl16 region with rpl16-F71 and rpl16-R1516 from Borg et al.[52]. Sequences of all the primers used are listed in Additional file 9: Table S9.

Data analysis

DNA sequences were aligned, assembled and compared using SeqMan^{TM} (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[53-55]. 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)[48]. The program NewHybrids ver. 1.1 was used to assign each individual to a genotype category: the two parental species, F_1 and F_2 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)[56] (Additional file 10: Table S10).

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[57-58]. 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 11: Table S11). 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/)[59-60] (Additional file 12: 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[61]. Finally, Distruct ver. 1.1[62] 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.

Funding

This work was supported by the National Natural Science Foundation of China (NSFC) (Grant No. 31770240), the Youth Innovation Promotion Association, Chinese Academy of Sciences (Grant No. 2018428) and the Reserve Talents for Academic and Technical Leaders of Middle-aged and Young People in Yunnan Province (Grant No. 2018HB066). The founder only provided the financial support and did not involve in the design of the experiment, collection, analysis and interpretation of data, decision to publish, or the preparation of the manuscript.

Authors' contributions

WS designed the research; YM and RL collected the plant materials; RL performed the experiments, analyzed the data and write the manuscript; YM and WS revised the manuscript. All authors read and

approved the final manuscript.

Acknowledgments

We thank Ms. Lidan Tao for her help in the field investigations.

References

1. Arnold ML. Natural hybridization and evolution. Demand: Oxford University Press. 1997.
2. Rieseberg LH. The role of hybridization in evolution-old wine in new skins. Am J Bot. 1995;82(7):944-953.
3. Soltis PS, Soltis DE. The role of hybridization in plant speciation. Annu Rev Plant Biol. 2009;60:561-588.
4. Zhang RS, Liu T, Wu W, Li YQ, Chao LF, Huang LS, Shi SH, Zhou RC. Molecular evidence for natural hybridization in the mangrove fern genus *Acrostichum*. BMC Plant Biol. 2013;13:74.
5. Grant V. Plant speciation. New York: Columbia University Press. 1981.
6. Wu CI. The genic view of the process of speciation. J Evolution Biol. 2001;14:851-865.
7. Zha HG, Milne RI, Sun H. Asymmetric hybridization in *Rhododendron agastum*: A hybrid taxon comprising mainly F_1s in Yunnan, China. Ann Bot. 2010;105:89-100.
8. Milne RI, Terzioglu S, Abbott R. A hybrid zone dominated by fertile F1s: Maintenance of species barriers in *Rhododendron*. Mol Ecol. 2003;12(10):2719-2729.
9. Zhang NN, Yu JJ, Wang YH, Gong X. Molecular evidence for asymmetric hybridization in three closely related sympatric species. AoB Plants. 2018;10:ply011.
10. Kyhos DW, Clark C, Thompson WC. The hybrid nature of Encelia-laciniata (Compositae, Heliantheae) and control of population composition by post-dispersal selection. Syst Bot. 1981;6(4):399-411.
11. Nagano Y, Hirao AS, Itino T. Genetic structure of a hybrid zone between two violets, *Viola rossii* Hemsl. and *V. bissetii* Maxim.: Dominance of F_1 individuals in a narrow contact range. Plant Spec Biol. 2015;30:237-243.
12. Milne RI, Abbott RJ. Reproductive isolation among two interfertile *Rhododendron* species: low frequency of post-F_1 hybrid genotypes in alpine hybrid zones. Mol Ecol. 2008;17:1108-1121.
13. Fang MY, Fang RZ, He MY, Hu LZ, Yang HB, Chamberlain DF. *Rhododendron*. In: Wu ZY, Raven PH, editors. Flora of China, Vol. 14. Beijing and St Louis: Science Press and Missouri Botanical Garden, 2005. p. 260-455.
14. Norman EM. Buddlejaceae. New York: The New York Botanical Garden Press. 2000.
15. Stuart DD. Buddlejas. Portland: Timber Press. 2006.
16. Chen G, Sun WB, Sun H. Ploidy variation in *Buddleja* (Buddlejaceae) in the sino-himalayan region and its biogeographical implications. Bot J Linn Soc. 2007;154:305-312.

17. Ge J, Cai L, Bi GQ, Chen G, Sun WB. Characterization of the complete chloroplast genomes of *Buddleja colvilei* and *B. sessilifolia*: Implications for the taxonomy of *Buddleja*. Molecules. 2018;23:1248.
18. Leeuwenberg AJM. The Loganiaceae of Aafrice . *Buddleja* . Revision of the African and Asiatic species. Wageninge: Mededelingen Landbouwhogeschool. 1979.
19. Gong WC, Chen G, Liu CQ, Dunn BL, Sun WB. Comparison of floral scent between and within *Buddleja fallowiana* and *Buddleja officinalis* (Scrophulariaceae). Biochem Syst Ecol. 2014;55:322-328.
20. Raja S, Ramya I. A review on ethnopharmacology, phytochemistry and pharmacology of *Buddleja asiatica*. Int J Pharm Sci Res. 2016;7(12):4697-4709.
21. Sheppard AW, Shaw RH, Sforza R. Top 20 environmental weeds for classical biological control in Europe: A review of opportunities, regulations and other barriers to adoption. Weed Res. 2006;46(2):93-117.
22. Tallent-Halsell NG, Watt MS. 2009. The invasive *Buddleja davidii* (butterfly bush). Bot Rev. 2009;75:292-325.
23. Wittig R. 2012. Frequency of *Buddleja davidii* Franch. (Buddlejaceae) in Germany along ecological gradients. Flora. 207:133-140.
24. Liao RL, Ma YP, Gong WC, Chen G, Sun WB, Zhou RC, Marczewski T. Natural hybridization and asymmetric introgression at the distribution margin of two *Buddleja* species with a large overlap. BMC Plant Biol. 2015:15:146.
25. Marquand CVB. The botanical collection made by Captain F. Kingdon Ward in the eastern Himalaya and Tibet in 1924-25. Bot J Linn Soc. 1929;48:149-229.
26. Ma YP, Zhang CQ, Zhang JL, Yang JB. Natural hybridization between *Rhododendron delavayi* and *R. cyanocarpum* (Ericaceae), from morphological, molecular and reproductive evidence. J Integr Plant Biol. 2010;52(9):844-851.
27. Li PT, Leeuwenberg AJM. Loganiaceae. In: Wu ZY, Raven PH, editors, Flora of China, Vol. 15. Beijing: Science Press. 1996. p. 320-338.
28. Wang HY, Zhang XS, Li XQ, Zhao HH, Shi XB. Rapid propagation of *Buddleja alternifolia*. Guizhou Agri Sci. 2010;38(6):30-31.
29. Gong WC, Chen G, Vereecken NJ, Dunn BL, Ma YP, Sun WB. Floral Scent Composition Predicts Bee Pollination System in Five Butterfly Bush (*Buddleja*, Scrophulariaceae) Species. Plant Biol. 2015;17:245-255.
30. Chau JH. Systematics of *Buddleja* (Scrophulariaceae): Phylogenetic relationships, historical biogeography, and phylogenomics. Washington: University of Washington. 2017.
31. Grant PR, Grant BR. Speciation undone. Nature. 2014;507:178-179.
32. Abbott RJ. Sex, Sunflowers, and Speciation. Science. 2003;301:1189-1190.

33. Feliner GN, Alvarez I, Fuertes-Aguilar J, Heuertz M, Marques I, Moharrek F, Pineiro R, Riina R, Rossello JA, Soltis PS, Villa-Machio I. Is homoploid hybrid speciation that rare? An empiricist's view. Heredity. 2017;118:513-516.
34. Schumer M, Rosenthal GG, Andolfatto P. How common is homoploid hybrid speciation? Evolution. 2014;68(6):1553–1560.
35. Morgensen HL. The hows and whys of cytoplasmic inheritance in seed plants. Am J Bot. 1996:83(3), 383-404.
36. Ma YP, Milne R, Zhang CQ, Yang JB. Unusual patterns of hybridization involving a narrow endemic *Rhododendron* Species (Ericaceae) in Yunnan, China. Am J Bot. 2010;97(10): 1749-57.
37. Ma YP, Xie WJ, Tian XL, Sun WB, Wu ZK, Milne R. Unidirectional hybridization and reproductive barriers between two heterostylous primrose species in north-west Yunnan, China. Ann Bot. 2014;113:753-761.
38. Tong ZY, Huang SQ. Pre- and post-pollination interaction between six co-flowering Pedicularis species via heterospecific pollen transfer. New Phytol. 2016;211(4):1452-1461.
39. Zhou RC, Gong X, Boufford D, Wu CI, Shi SH. Testing a hypothesis of unidirectional hybridization in plants: observations on Sonneratia, Bruguiera and Ligularia. BMC Evol Biol. 2008;8:149.
40. Zhang JJ, Montgomery BR, Huang SQ. Evidence for asymmetrical hybridization despite pre- and post-pollination reproductive barriers between two Silene species. AoB Plants. 2016;8:plw032.
41. Yue LL, Chen G, Sun WB, Sun H. Phylogeography of *Buddleja crispa* (Buddlejaceae) and its correlation with drainage system evolution in southwestern China. Am J Bot. 2012;99(10):1726-1735.
42. Wang JH, Zhang JG, Xu Y, Wu HW, Sun GP. Study on biology characteristics of drought enduring shrub *Buddleja alternidolia*. Chinese Wild Plant Resour.. 2007;26(6):23-25.
43. Moore RJ. Cytotaxonomic studies in the Loganiaceae Ⅱ. Chromosome numbers and phylogeny in the Loganaceae. Am J Bot. 1947;34(10):527-538.
44. Moore R. Polyploidy, phylogeny, and photoperiodism in Old World *Buddleja*. Evolution. 1961;15(3): 272-280.
45. Marczewski T, Ma YP, Zhang XM, Sun WB, Marczewski AJ. Why is population information crucial for taxonomy? A case study involving a hybrid swarm and related varieties. AoB Plants. 2016;8:plw070.
46. SPSS. SPSS Statistics 16.0 SPSS Inc., Chicago. 2007.
47. Hammer Ø, Harper DAT, Ryan PD. Past: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica. 2001;4(1):9.
48. Bandelt HJ, Forster P, Röhl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol. 1999;16(1):37-48.
49. Ma YP, Wu ZK, Dong K, Sun WB, Marczewski T. Pollination biology of *Rhododendron cyanocarpum* (Ericaceae): An alpine species endemic to NW Yunnan, China. J Syst Evol. 2015;53(1):63-71.

50. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bulletin. 1987;19(1):11-15.
51. Chau JH, O'Leary N, Sun WB, Olmstead RG. Phylogenetic relationships in tribe Buddlejeae (Scrophulariaceae) based on multiple nuclear and plastid markers. Bot J Linn Soc. 2017;184:137-166.
52. Borg AJ, McDade LA, Schönenberger J. Molecular phylogenetics and morphological evolution of Thunbergioideae (Acanthaceae). Taxon. 2008;57(3):811-822.
53. Librado P, Rozas J. Dnasp v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009;25(11):1451-1452.
54. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet. 2001;68:978-989.
55. Stephens M, Scheet P. Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. Am J Hum Genet. 2005;76:449-462.
56. Anderson EC and Thompson EA. A model-based method for identifying species hybrids using multilocus genetic data. Genetics. 2002;160(3):1217-1229.
57. Hubisz MJ, Falush D, Stephens M, Pritchard JK. Inferring weak population structure with the assistance of sample group information. Mol Ecol Resour. 2009;9:1322-1332.
58. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155(2):945-959.
59. Earl DA, Vonholdt BM. Structure harvester: A website and program for visualizing structure output and implementing the evanno method. Conserv Genet Resour. 2012;4(2):359-361.
60. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. Mol Ecol. 2005;14(8):2611-2620.
61. Jakobsson M, Rosenberg NA. Clumpp: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics. 2007;23(14):1801-1806.
62. Rosenberg NA. Distruct: A program for the graphical display of population structure. Mol Ecol Notes. 2004;4(1):137-138.

Figures

Figure 1

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

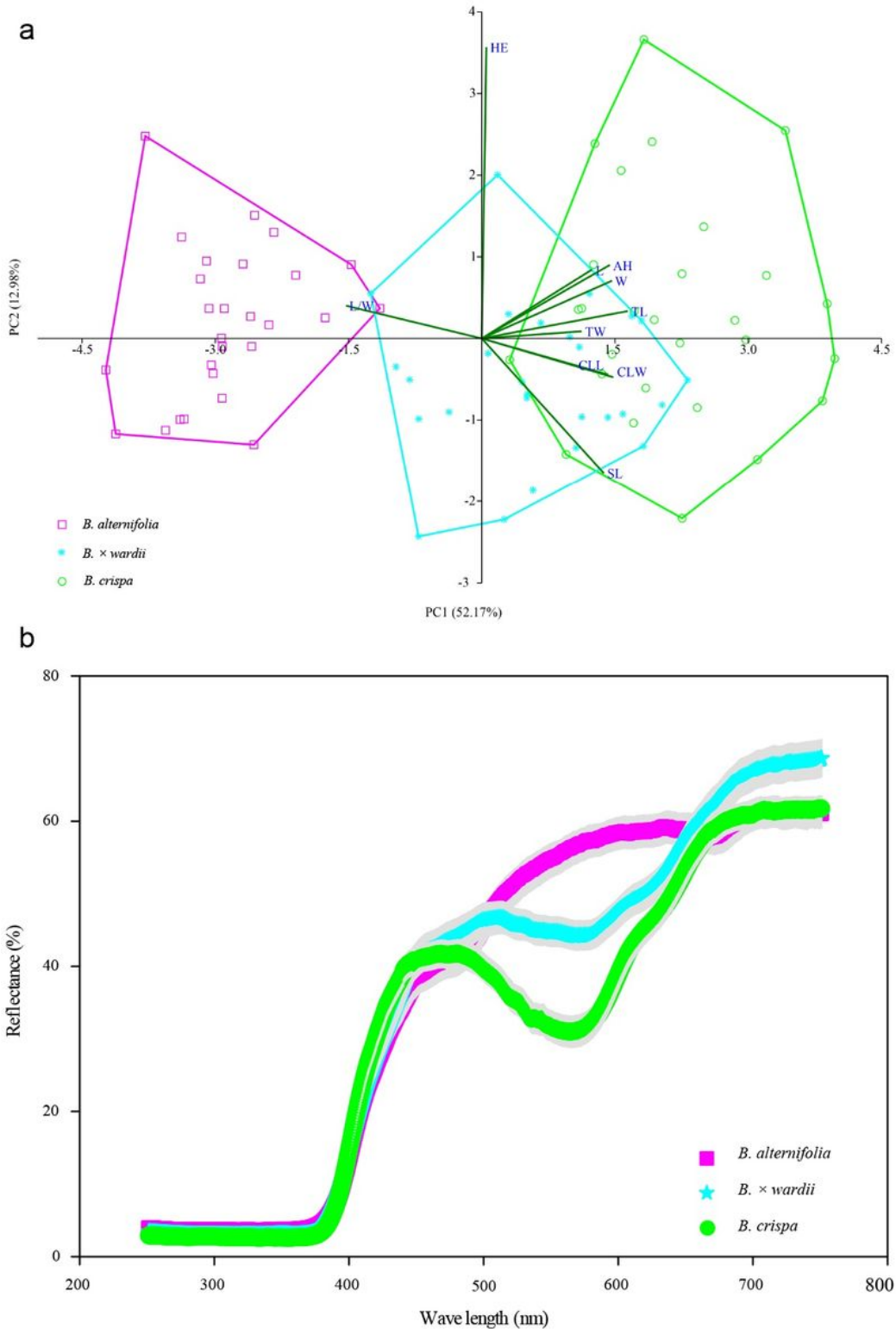

Figure 2

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. × wardii. Pink square, green circle and calamine blue star represent B. alternifolia, B. crispa and B. × wardii, respectively.

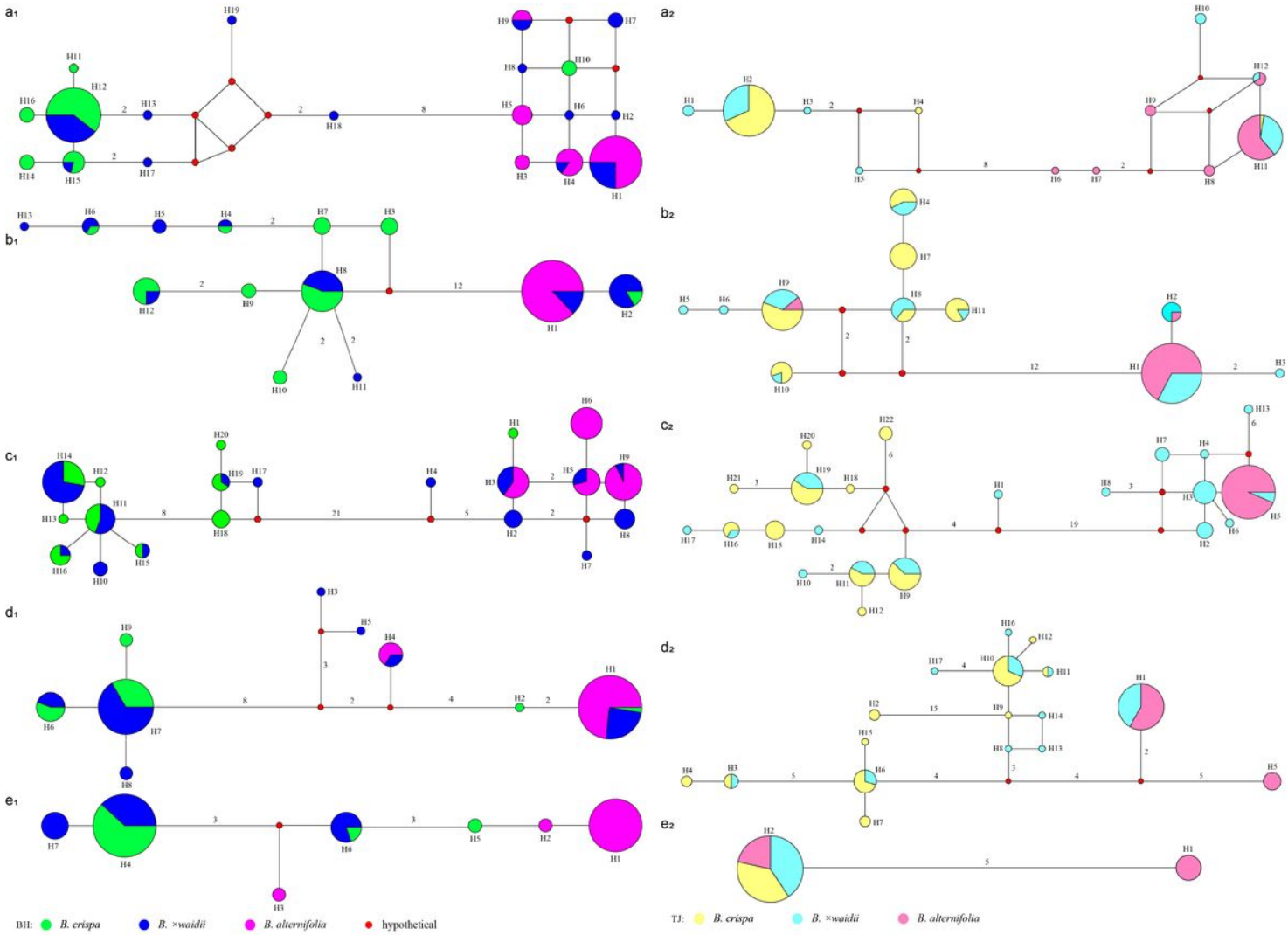

Figure 3

Haplotype network for ETS (a), gapC2 (b), PPR24 (c), PPR123 (d), cpDNA (e) in BH (leaf) and TJ (right) populations. 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.

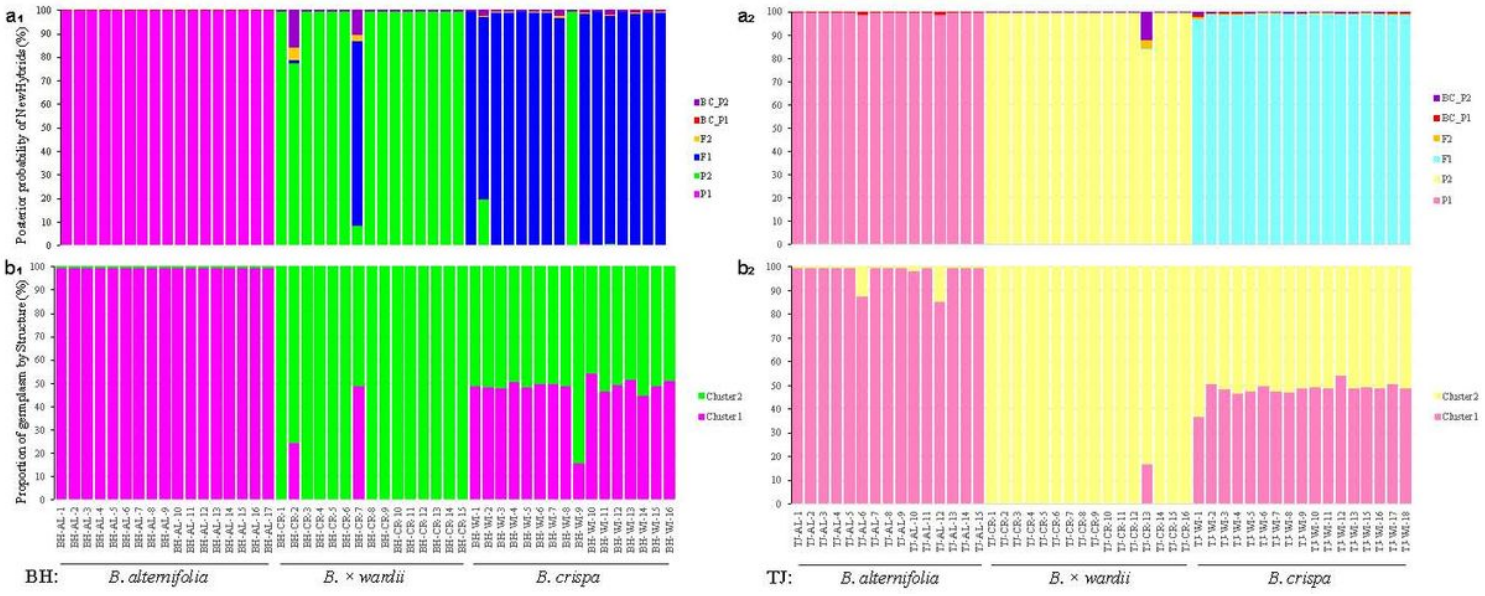

Figure 4

Genotype class assignment by NewHybrids (BH:a1,TJ:a2) and clustering analysis by Structure (BH:b1,TJ:b2) 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.In a1 and a2, bars in different color 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. In b1 and b2, bars in different color represent different clusters: pink and peach represent Cluster 1; green and yellow represent Cluster 2.

Supplementary Files

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

- renamed0edff.png
- TableS11.xlsx
- TableS10.xlsx
- TableS9.xlsx
- TableS8.xlsx
- TableS7.xlsx
- TableS6.xlsx
- TableS5.xlsx
- TableS4.xlsx

- TableS3.xlsx
- TableS2.xlsx
- TableS1.xlsx