



# Morphological and molecular evidence for natural hybridization between *Sorbus pohuashanensis* and *S. discolor* (Rosaceae)

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**Abstract** In overlapping distribution areas of *Sorbus pohuashanensis* and *S. discolor* in North China (Mount Tuoliang, Mount Xiling and Mount Baihua), *Sorbus* individuals were found with pink fruit, which have never been recorded for the flora of China. Fourteen morphological characters combined with four chloroplast DNA markers and internal transcribed spacer (ITS) were used to analyze the origin of the *Sorbus* individuals with pink fruits and their relationship to *S. pohuashanensis* and *S. discolor*. PCA, SDA and one-way (taxon) ANOVA of morphological characters provided convincing evidence of the hybrid origin of *Sorbus* individuals with pink fruits based on a novel morphological character and many intermediate characters. Haplotype analysis based on four cpDNA markers showed that either *S. pohuashanensis* or *S. discolor* were maternal parents of *Sorbus* individuals with pink fruits. Incongruence of the position of *Sorbus* individuals with pink fruits

between cpDNA and ITS in cluster trees supported by DNA sequence comparative analysis, implying former hybridization events between *S. pohuashanensis* and *S. discolor*. Multiple hybridization events between *S. pohuashanensis* and *S. discolor* might have contributed to the generation of *Sorbus* individuals with pink fruits. This study has provided insights into hybridization between species of the same genus in sympatric areas, which is of great significance for the study of interspecific hybridization.

**Keywords** Hybridization · ITS · Chloroplast DNA · Morphological characters · *Sorbus*

## Introduction

Natural hybridization plays a crucial role in evolution within the plant kingdom and has diverse evolutionary consequences, including the generation of genetic diversity and new ecotypes or species and the breakdown or reinforcement of isolation barriers between species (Emms and Arnold 1997; Coyne and Orr 2004; Wang 2017). Cross-fertilization between closely related taxa in the same region can result in hybrids (Jiggins and Mallet 2000; Rieseberg and Buerkle 2002), including among *Sorbus* L. (Rosaceae) taxa.

Morphological characters (primarily of leaves and fruits) have long been used to classify *Sorbus* hybrid taxa (Németh et al. 2020). Hybrids typically exhibit a mosaic of parent-like, intermediate and transgressive characters (Uhrinová et al. 2017; Yuan et al. 2019; Wang et al. 2021), though novel characters appear frequently in hybrid characters (Zhang et al. 2007). However, morphological characters of natural hybrids are influenced by the environment and expression of genes (Rieseberg et al. 1993) and cannot be used alone to detect putative hybrid individuals.

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DNA sequence analysis is therefore commonly used to detect hybridization. Due to uniparental inheritance, chloroplast DNA (cpDNA) rarely recombines and can thus disclose information about current population structure and previous ancestry (Park et al. 2020; Van et al. 2021), gene flow (seed flow) between and within populations (Aizawa and Iwazumi 2020), and the identity of the plastid-donor parent of the offspring (perhaps maternal in angiosperms) (Triplett and Clark 2021). The nuclear ribosomal internal transcribed spacer (nrITS) region is a universal species-specific marker for plants, which inherits from both parents and contains more information than the cpDNA marker. Accordingly, it is often used to determine the approximate range of the parents to avoid incorrect phylogenetic interpretations caused by uniparental genetic inference (Li et al. 2011; Nobre et al. 2018). Thus, the two types of genetic markers are usually integrated to identify the putative hybrid individuals, infer the maternal contributor, and provide insights into ancient hybridization (Vaezi et al. 2019; Yousefzadeh et al. 2019).

Approximately 100 species belong to *Sorbus*. They are widely distributed in the temperate zone of the Northern Hemisphere throughout Europe, Asia, and northern North America, and the main distribution centers are in the Himalayas, Caucasus, and China (McAllister 2005). From an evolutionary point of view, *Sorbus* is an ideal model for studying the consequences of natural interspecific hybridization, polyploidy, and apomixis, which drive the ongoing genetic diversity in *Sorbus* (Robertson et al. 2010; Ludwig et al. 2013; Németh et al. 2020). In Europe, many *Sorbus* hybrids have been found (Meyer et al. 2014; Sennikov et al. 2016; Hebda et al. 2021) and the resulting taxonomic complexity has made *Sorbus* one of the most taxonomically difficult groups of vascular plants and led to debate over concepts and delimitation of *Sorbus* species (Robertson and Sydes 2006). In China, there are 67 species of *Sorbus* (Yü and Lu 1974). *S. aucuparia* subsp. *pohuashanensis* (Hance) McAll. ( $2n=2x=34$ , subgenus *Sorbus*) and *S. discolor* (Maxim.) Maxim. ( $2n=2x=34$ , subgenus *Albocarmesinae*) are native tree species in China, which are distinct primarily in fruit color; *S. aucuparia* subsp. *pohuashanensis* (Hance) McAll. (hereafter called *S. pohuashanensis*) with red or orange fruits and *S. discolor* with white fruits. Both species grow in mixed broad-leaved forests in montane as an associated species at 500–2000 m a.s.l. in warm temperate zone. Pink-fruited *Sorbus* (hereafter called SH) individuals were found in Hebei Province (Mount Tuoliang, Mount Xiling) and Beijing (Mount Baihua), where the two native *Sorbus* species are commonly distributed. Because eFlora of China has no records of SH individuals, their origins and relationships to *S. pohuashanensis* and *S. discolor* are not clear. Sequence analysis of two cpDNA markers showed that either *S. pohuashanensis* or *S. discolor* was the female parent of SH individuals (Tang et al. 2016).

In this study, 14 morphological characters, combined with four noncoding cpDNA markers (*matK*, *trnL-F*, *trnS-G*, *rpl16*) and ITS were used to analyze the evolution of these SH individuals and determine whether they originated from the hybridization between *S. pohuashanensis* and *S. discolor* and, if so, how this event occurred. We aim to (1) investigate the morphological and molecular evidence for hybridization between *S. pohuashanensis* and *S. discolor*, (2) verify the maternal parent of the SH individuals and (3) explore possible pathways for the crossing.

## Materials and methods

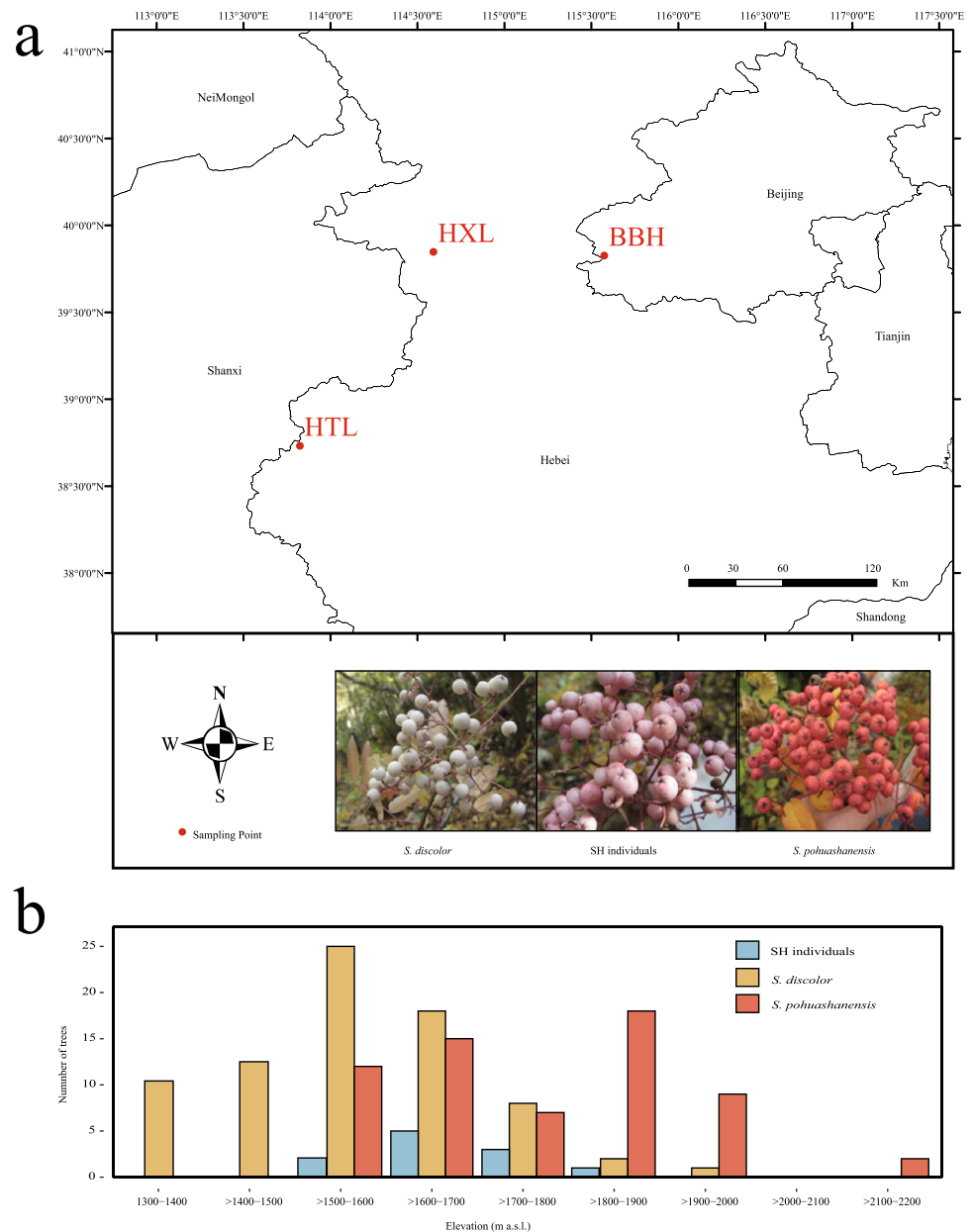
### Sampling of plant material

Several field surveys were conducted at Mount Tuoliang (HTL), Mount Xiling (HXL) and Mount Baihua (BBH) in North China, where *S. pohuashanensis* and *S. discolor* grow together with SH individuals at the same area (Fig. 1a). Mature, undamaged complete compound leaves and fruits without disease and insects were collected from individuals more than 10 m apart. Besides the collected individuals, the geographical coordinates of other *Sorbus* individuals for each site were determined using GPS. The altitudinal distribution pattern was analyzed after field surveys. A total of 85 individuals with fruits were included in morphological analyses, and the leaf material of 65 individuals was dried on silica gel and stored at  $-80^{\circ}\text{C}$  for molecular analyses (Table 1).

In addition to fruit color, the presence of white hairs on the lower side of leaflets, winter buds, and young branches distinguished *S. pohuashanensis* from the glabrous *S. discolor*.

Morphological characters were measured and scored for 10–30 compound leaves and fruits sampled within the middle crown of individual trees from the three *Sorbus* groups at the three sites. For one leaflet chosen from the middle of each compound leaf, 12 quantitative characters were measured with 10–30 replications: length of compound leaf, width of compound leaf, ratio of length to width of compound leaf, length of leaflet, width of leaflet, ratio of length to width of leaflet, length of petiole, internal length between leaflets, pairs of leaflets, vertical length of fruit, horizontal length of fruit, ratio of vertical length/horizontal length of fruit were measured and statistically analyzed after the field investigation. One-way (taxon group) ANOVA and the stepwise discriminant analysis (SDA) were carried out using SPASS 22 (Yockey and Pearson 2010). Principal component analysis (PCA) was conducted using the R package FactoMineR (Lê et al. 2008) implemented in R v. 3.6 (R Core Team 2020).

**Fig. 1** Locations of sampled populations and images of fruits from two *Sorbus* species and SH individuals in North China. **a**, Locations of sampled populations and fruits. **b**, Elevation distribution of two *Sorbus* species and SH individuals at HTL. BBH, Mount Baihua; HTL, Mount Tuoliang; HXL, Mount Xiling



**Table 1** Geographical information and number of trees sampled (*N*) for morphological and molecular analysis of two *Sorbus* species and SH individuals

Sampling site	Geographic coordinates		Mean altitude (m a.s.l.)	<i>N</i> for molecular analysis ( <i>N</i> for morphological analysis)			
	N	E		<i>S. pohuashanensis</i>	<i>S. discolor</i>	SH individuals	Total
HTL	38°44.0′ – 38°45.0′	113°44.0′ – 113°49.0′	640 – 2200	17 (17)	8 (26)	10 (10)	35 (53)
HXL	39°55.5′ – 39°55.6′	114°57.8′ – 114°58.1′	1357 – 1578	6 (8)	5 (8)	5 (7)	16 (23)
BBH	39°50.8′ – 39°51.7′	115°35.6′ – 115°37.0′	1386 – 1652	6 (5)	4 (1)	4 (3)	14 (9)
Totals				29 (30)	17 (35)	19 (20)	65 (85)

BBH, Mount Baihua; HTL, Mount Tuoliang; HXL, Mount Xiling

## DNA extraction, amplification and sequencing

Total genomic DNA was extracted from 20 mg of dried leaf tissue of each sample using Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China) according to the manufacturer's instructions.

The primers used to amplify ITS and cpDNA are listed in Table 2 (Taberlet et al. 1991; Hamilton 1999; Parisod and Besnard 2007). The PCR reaction mixtures for ITS and cpDNA consisted of 20  $\mu$ L containing 15  $\mu$ L/mol DNA, 10  $\mu$ L of 2 $\times$  Taq PCR Master Mix (Aidlab, Beijing, China), 0.25 mM of each primer (Majorbio, Shanghai, China), 7  $\mu$ L of ddH<sub>2</sub>O. The thermocycling conditions (PCT-200, Bio-Rad, USA) were initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 45 s, a suitable annealing temperature for each primer combination of between 52 and 55 °C for 45 s; then a final extension step at 72 °C for 10 min. PCR products were sequenced by the Majorbio Company (Shanghai, China) on an ABI 3730xl DNA Analyzer.

## Molecular data analysis

Sequences were edited and assembled using BIOEDIT v7.0.5.3 (Hall 1999), and aligned using Clustal X (Thompson et al. 1997). Sequences of the outgroup species *Pyrus pyrifolia* (Burm. fil.) Nakai, *Malus  $\times$  micromalus* Makino and *Photinia serratifolia* (Desf.) Kalkman were obtained from the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov/>).

Chloroplast DNA haplotypes were identified by treating the four cpDNA markers as a whole without recombination, and nuclear haplotypes were identified using ITS sequences. Haplotype diversity (Hd) and nucleotide diversity ( $\pi$ ) were calculated using DnaSP 5.0 (Librado and Rozas 2009). Haplotype networks for cpDNA and ITS were constructed separately using the software NETWORK V4.5.1.6 and the

median-joining method (Bandelt et al. 1999) to ascertain the haplotype distribution in sampled populations and to identify putative hybridization events in particular.

ITS and the four combined cpDNA regions were phylogenetically analyzed using maximum-parsimony (MP), neighbor-joining (NJ), and Bayesian analysis. The best-fit model of molecular evolution required for Bayesian estimations was selected using the software MrModeltest 2.4 (Nylander 2004). GTR + I was the best model for ITS and GTR + I + G was the best for cpDNA. Bayesian posterior probabilities were estimated using MrBayes 3.2.7a (Ronquist et al. 2012). Bayesian analysis was started with random starting trees and maintained until the standard deviation of split sequences was below 0.05 as a convergence diagnostic value. Two independent Metropolis-coupled Markov chain Monte Carlo (MCMC) runs were done simultaneously, with one cold chain and three incrementally heated chains for each run; all runs were initiated with random starting trees and run for  $10 \times 10^5$  generations for ITS and cpDNA. The fraction of the sampled values discarded as burn-in was set at 0.25. Neighbor-joining (NJ) and maximum-parsimony (MP) analyses were done using MEGA 7 (Kumar et al. 2016). The bootstrap analysis was performed to assess topological robustness with 1000 replicates using simple taxon addition (Felsenstein 1985). Bootstrap support values  $\geq 70\%$  were considered significant.

## Results

### Altitudinal distribution pattern of three *Sorbus* groups

SH individuals were found in HTL, HXL and BBH (Fig. 1a), which are the sympatric distribution areas of *S. pohnuashanensis* and *S. discolor*. In HTL, *S. discolor* is mainly distributed at 1300–2000 m a.s.l., whereas *S. pohnuashanensis* was mainly distributed at 1500–2200 m a.s.l. The SH individuals from HTL were distributed from 1500–1900 m a.s.l. (Fig. 1b), with *S. discolor* at the lower end of the range and *S. pohnuashanensis* at the higher. *S. pohnuashanensis* and *S. discolor* were distributed at 1386–1678 m a.s.l. at BBH and 1357–1578 m a.s.l. at HXL. At both sites, several SH individuals were found in areas with the two species.

### Morphological evidence for hybridization

The SH individuals had pink fruit, a novel character that distinguishes it easily from the two local species. In terms of hairs on the surface of leaflet and young branch, SH individuals with few hairs were intermediate between *S. pohnuashanensis* (dense) and *S. discolor* (glabrous). Regarding hairs on the outer surface of scales of winter bud, the SH individuals had sparse whitish-grey hairs only at the tip

**Table 2** Information of cpDNA and ITS primers

Marker	Primer sequences 5'–3'	Size (bp)	$T_a$ (°C)
<i>TrnL-F</i>	GGTTCAAGTCCCTCTATCCC	650	55
	ATTGAACTGGTGACACGAG		
<i>TrnS-G</i>	GTCCACTCAGCCATCTCTCC	1200	52
	TCGAACAAAGTAATCGGGAGTG		
<i>MatK</i>	CCCRTYCATCTGGAAATCTTGGTTC	720	55
	GCTRTRATAATGAGAAAGATTTCTGC		
<i>Rpl16</i>	GCTATGCTTAGTGTGTGACTCGTTG	1100	55
	CGTACCCATATTTTCCACCACGAC		
ITS 4/5	GGAAGTAAAAGTCGTAACAAGG	750	55
	TCCTCCGCTTATTGATATGC		

$T_a$  is annealing temperature

and margins, while *S. pohuashanensis* was densely and fully covered by hairs, and *S. discolor* had no hairs.

The ANOVA of the 12 quantitative morphological characters showed that nine characters (length of compound leaf, width of compound leaf, length of leaflet, width of leaflet, length of petiole, internal length between leaflets, pairs of leaflets and vertical length of fruit) had extremely significant differences among two *Sorbus* species and SH individuals ( $P < 0.01$ ) (Table 3). The ratio of vertical length/horizontal

length of fruit also differed significantly ( $P < 0.05$ ). Most qualitative leaf and fruit characters of SH individuals were intermediate between *S. pohuashanensis* and *S. discolor*. The vertical length of fruit of SH individuals was significantly longer than that of *S. pohuashanensis* and *S. discolor*, but horizontal length of fruit among the two *Sorbus* species and SH individuals did not differ significantly.

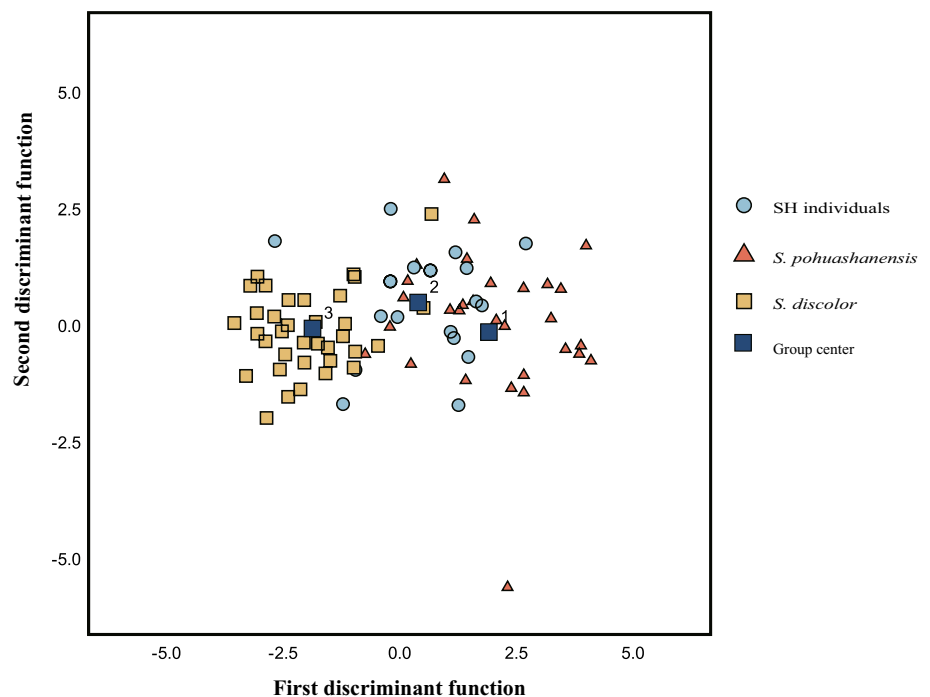
The stepwise discriminant analysis showed that individuals of the three groups clustered into three overlapping

**Table 3** Values for morphological characters of two *Sorbus* species and SH individuals

Character (unit)	Group			<i>P</i>	<i>F</i> among group
	<i>S. pohuashanensis</i>	SH individuals	<i>S. discolor</i>		
Length of compound leaf (cm)	21.05 ± 2.82	18.41 ± 1.92	14.57 ± 1.94	65.945	0.000**
Width of compound leaf (cm)	12.59 ± 1.82	10.57 ± 1.20	8.27 ± 1.80	53.187	0.000**
Ratio of length/width of compound leaf	1.68 ± 0.16	1.75 ± 0.18	1.81 ± 0.26	2.864	0.063
Length of leaflet (cm)	6.57 ± 1.02	5.52 ± 0.62	4.27 ± 0.78	61.146	0.000**
Width of leaflet (cm)	2.15 ± 0.33	1.77 ± 0.23	1.43 ± 0.57	22.308	0.000**
Ratio of length/width of leaflet	3.08 ± 0.35	3.13 ± 0.25	3.15 ± 0.54	0.219	0.804
Length of petiole (cm)	3.85 ± 0.72	3.63 ± 0.47	3.17 ± 0.53	11.070	0.000**
Internal length between leaflets (cm)	1.97 ± 0.27	1.74 ± 0.24	1.37 ± 0.29	40.516	0.000**
Pairs of leaflets	5.98 ± 0.41	5.75 ± 0.29	5.55 ± 0.50	8.584	0.000**
Vertical length of fruit (mm)	7.90 ± 0.99	8.04 ± 0.81	7.06 ± 0.65	12.411	0.000**
Horizontal length of fruit (mm)	7.59 ± 1.16	7.62 ± 0.98	7.17 ± 0.62	2.175	0.120
Ratio of vertical length/ horizontal length of fruit	1.06 ± 0.16	1.06 ± 0.08	0.99 ± 0.06	4.846	0.010*
Fruit color	Red, orange	Pink	White		
Pubescence of bud and leaf	Densely hairy	Sparsely hairy	Glabrous		

Significant differences among the three groups: \* $P < 0.05$ , \*\*  $P < 0.01$

**Fig. 2** Distribution of the individual scores for the first two discriminant functions based on stepwise discriminant analysis of 12 morphological traits





groups (Fig. 2). The first group consisted of *S. pohuashanensis* and five SH individuals with a discriminant accuracy of 70%. The second group included 12 SH individuals, five *S. pohuashanensis* and three *S. discolor* (discriminant accuracy: 60%), and the third group consisted of *S. discolor* and two SH individuals (discriminant accuracy: 94.3%). Length of compound leaf, length of petiole and vertical length of fruit were the main characters that contributed to group separation. The majority of individuals of each *Sorbus* taxon surrounded their respective group centroid. In the discriminant function plot, *S. pohuashanensis* showed the most intraspecific variation. Morphological characters of SH individuals were closer to those of *S. pohuashanensis* than *S. discolor*. The Fisher discriminant functions for *S. discolor*, SH individuals and *S. pohuashanensis* were as follows:

$$S.pohuashanensis : f_1 = 5.729x_1 - 10.040x_2 + 10.719x_3 - 84.438 \quad (1)$$

$$\text{SH individuals} : f_2 = 4.426x_1 - 6.468x_2 + 10.997x_3 - 74.294 \quad (2)$$

$$S.discolor : f_3 = 2.852x_1 - 2.368x_2 + 9.669x_3 - 52.263 \quad (3)$$

where,  $x_1$ ,  $x_2$ , and  $x_3$  represent length of compound leaf, length of petiole and vertical length of fruit, respectively.

Based on the principal component analysis (PCA) of 12 quantitative characters (Fig. 3), two principal components were extracted. The first principal component (PC1) explained 89.50% of the total variability and included length of compound leaf, width of compound leaf, length of leaflet and length of petiole. The second principal component (PC2) explained 7.10% of the total variation in morphological characters. There were also interspecific morphological

variations in the two *Sorbus* species and SH individuals. SH individuals were intermediate between *S. pohuashanensis* and *S. discolor*.

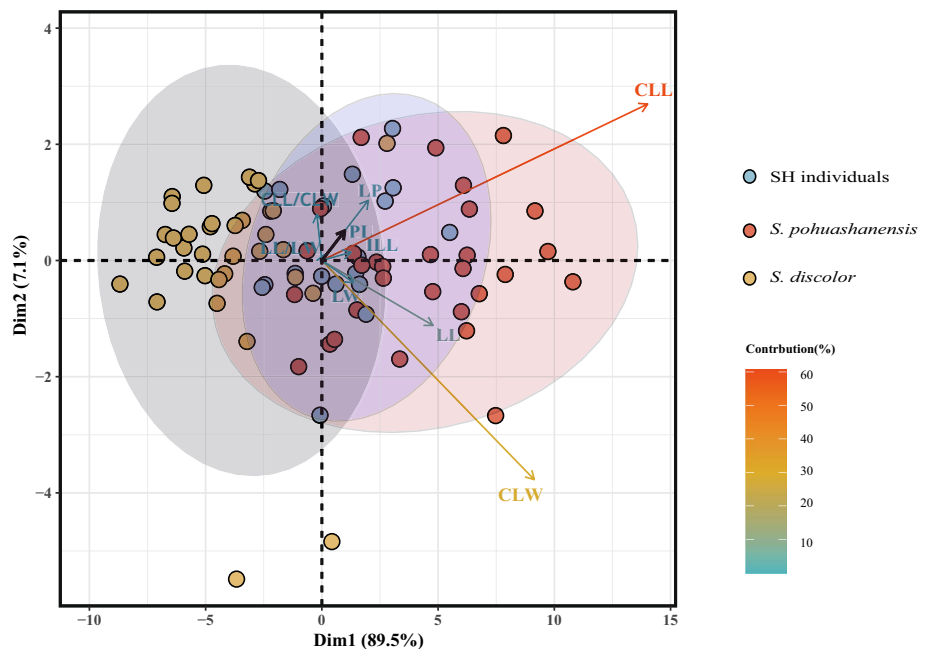
### Sequence analysis of cpDNA region

The length of the aligned cpDNA fragments (*matK*, *trnL-F*, *trnS-G*, *rpl16*) was 3465 bp with 21 polymorphic sites. The cpDNA sequences of all *S. discolor* were identical, while the cpDNA sequences of *S. pohuashanensis* and the SH individuals were diverse, and the variation was mainly concentrated in *trnS-G* (Table 4).

In total, seven haplotypes were identified from 65 individuals with a haplotype diversity of  $H_d = 0.771$  (Table 5). Haplotype diversity varied across populations, ranging from 0.000 (SDHXL, SDBBH, SDHTL) to 1 (SHBBH). Nucleotide diversity was estimated within the species as a whole ( $\pi = 0.00087$ ) and within populations, ranging from 0.0000 (SDHXL, SDBBH, SDHTL) to 0.00114 (SHBBH). The haplotype frequencies of H1–H7 are 15.4%, 6.2%, 6.2%, 23.0%, 9.2%, 38.5%, 1.5%, respectively.

All samples of *S. discolor* had haplotype H6, while *S. pohuashanensis* had five haplotypes (H1–H5) (Table 5). *S. pohuashanensis* from HXL and BBH had two haplotypes (H4 and H5). *S. pohuashanensis* from HTL was diverse with H1–H3. SH individuals (HTL2, 3, 7–10; BBH1; HXL5) shared H6 with *S. discolor*, and the other SH individuals shared H2 (HTL6, H4 [BBH2; HTL5; HXL3, 4], and H5 (HTL1; HXL1, 2) with *S. pohuashanensis*. In addition, one individual (SH [HTL] 4) had a unique haplotype (H7; Fig. 4a).

**Fig. 3** First two principal component analysis of three *Sorbus* groups using 12 morphological traits. CLL, length of compound leaf; CLW, width of compound leaf; CLL/CLW, ratio of length/width of compound leaf; LL, length of leaflet; LW, width of leaflet; LL/LW, ratio of length/width of leaflet; LP, length of petiole; ILL, internal length between leaflets; PL, pairs of leaflets



**Table 4** Informative variant sites on cpDNA haplotypes of *Sorbus* samples

Haplotype	Variable sites																							
	<i>matK</i>												<i>trnS-G</i>								<i>trnL-F</i>		<i>rpl16</i>	
	72	103	275	293	294	295	299	300	301	302	303	304	305	306	307	695	1116	106	838	839	840			
H1	T	T	T	G	A	A	A	-	-	-	-	-	-	-	T	T	G	A	A	-				
H2	T	C	T	C	T	T	T	C	C	T	T	T	T	A	T	T	G	A	A	-				
H3	T	C	-	C	T	T	T	C	C	T	T	T	T	A	C	T	G	A	A	-				
H4	T	T	T	G	A	A	A	-	-	-	-	-	-	-	T	T	T	A	A	-				
H5	G	T	T	G	A	A	A	-	-	-	-	-	-	-	T	T	T	A	A	-				
H6	T	T	T	G	A	A	A	A	A	T	T	-	-	T	A	T	G	T	T	T				
H7	T	T	T	G	A	A	A	A	A	T	T	-	-	T	A	T	G	A	A	-				

The upper part of the table with numbers gives information for the 21 variable sites in the four cpDNA sequences. The dash indicates a missing base. H1, H3, *S. pohuashanensis*; H2, H4, H5, *S. pohuashanensis* and SH individuals; H6, *S. discolor* and SH individuals; H7, *S. discolor*

**Table 5** Chloroplast DNA haplotypes for *Sorbus* groups in three locations

Location	Group	Haplotype (N)	Hd	$\pi$
BBH	<i>S. pohuashanensis</i>	H4 (7), H5 (1)	0.250	0.00007
	SH individuals	H4 (1), H6 (1)	1.000	0.00114
	<i>S. discolor</i>	H6 (4)	0.000	0.00000
HTL	<i>S. pohuashanensis</i>	H1 (10), H2 (3), H3 (4)	0.603	0.00069
	SH individuals	H2 (1), H4 (1), H5 (1), H6 (6), H7 (1)	0.667	0.00082
	<i>S. discolor</i>	H6 (8)	0.000	0.00000
HXL	<i>S. pohuashanensis</i>	H4 (4), H5 (2)	0.533	0.00015
	SH individuals	H4(2), H5(2), H6(1)	0.800	0.00063
	<i>S. discolor</i>	H6(5)	0.000	0.00000
Total			0.771	0.00087

BBH, Mount Baihua; HTL, Mount Tuoliang; HXL, Mount Xiling; Hd, haplotype diversity;  $\pi$ , nucleotide diversity

Both the MP tree and NJ tree based on chloroplast sequences were consistent with the Bayesian tree, so only the Bayesian consensus tree is displayed (Fig. 4b). Phylogenetic analysis showed that nine SH individuals, including SH (HTL) 1, 4–6, SH (BBH) 2, SH (HXL) 1–4 clustered with *S. pohuashanensis*, and the others clustered with *S. discolor* (Fig. 4b). The phylogenetic analysis also showed that H5 was most closely related to H4, and they formed a monophyletic group together with H1–H3. *S. pohuashanensis* and some of the SH individuals shared haplotypes H1–H5.

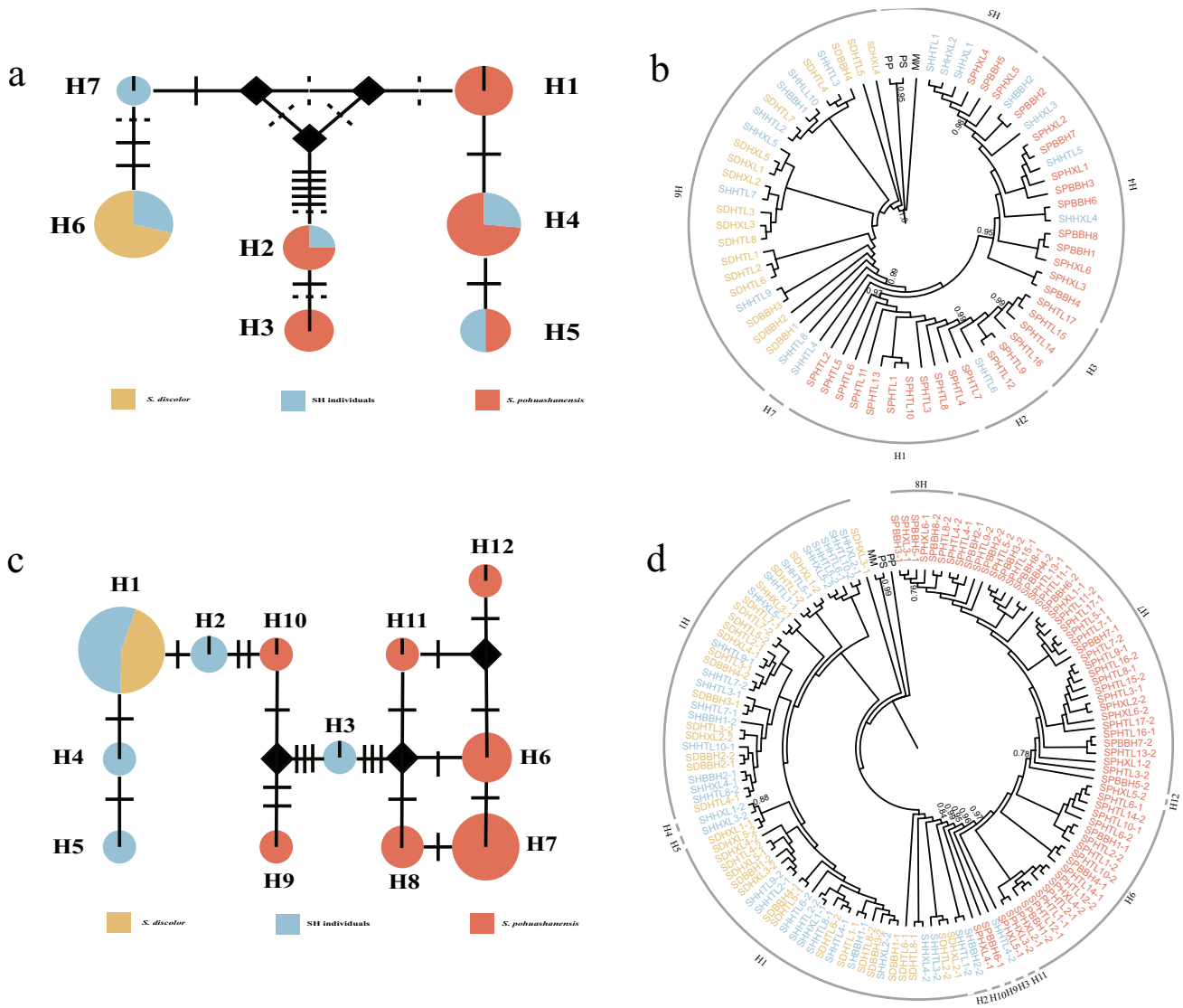
#### Analysis of ITS sequences of two *Sorbus* species and SH individuals

A total of 12 variable sites were found in the 585-bp ITS sequences (Table 6). No variations in ITS or cpDNA sequences were detected in samples of *S. discolor*, while there were abundant and diverse variations among

individuals of *S. pohuashanensis*. There were three types of ITS sequences of *S. pohuashanensis*. The first type of ITS sequences of *S. pohuashanensis* (SP [BBH] 2, 7, 8; SP [HTL] 3–5, 7–9, 11, 13, 15–16; SP [HXL] 1) is homozygous, which are completely different from the sequence of *S. discolor*. The second type of ITS sequences of *S. pohuashanensis* (SP [BBH] 1; SP [HTL] 1, 2, 6, 10, 12, 14) is also homozygous, which had a mutation at locus 130 and the bases at the other 11 loci were completely different from the sequence of *S. discolor*. The third type is heterozygous with six genotypes: (1) SP (BBH) 3, 5, SP (HXL) 6; (2) SP (BBH) 4, SP (HXL) 2; (3) SP (BBH) 6; (4) SP (HXL) 3; (5) SP (HXL) 4 and; (6) SP (HXL) 5. The ITS sequences of some SH (BBH1; HTL2, 3, 5–10; HXL2, 4, 5) were as same as *S. discolor*, while the rest had heterozygous loci, which had four genotypes: (1) SH (BBH) 2 and SH (HTL) 1; (2) SH (HXL) 1; (3) SH (HXL) 3; (4) SH (HTL) 4. Those SH individuals showed chromatogram additivity compared with *S. discolor* and *S. pohuashanensis* at most nuclear loci, providing support for a natural hybridization hypothesis between *S. discolor* and *S. pohuashanensis*.

A total of 12 ITS haplotypes were identified from ITS (Table 7 and Fig. 4c). There was only one haplotype H1 in the samples from three sites of *S. discolor*, but seven haplotypes (H6–H12) in the samples of *S. pohuashanensis*. In addition to the unique haplotypes H2–H5, the SH individuals shared H1 with *S. discolor* and H6, H7 with *S. pohuashanensis*. In all populations, mean nucleotide diversity ( $\pi$ ) was 0.01024 (from 0 to 0.00443) and haplotype diversity (Hd) was 0.673 (0 to 0.848) as inferred from ITS (Table 7).

In the Bayesian phylogenetic tree (Fig. 4d), *S. pohuashanensis* and *S. discolor* clustered into separate branches. The majority of the SH individuals clustered with *S. discolor*, but only SH (HTL) 4 with heterozygous ITS sequences clustered with *S. pohuashanensis* and *S. discolor*. Moreover, this maternal parent of the individual with unique haplotype H7 for cpDNA could not be detected.



**Fig. 4** Chloroplast and nuclear ITS haplotype networks (a, c) and phylogenetic trees (b, d) for two *Sorbus* species and SH individuals. a, Chloroplast haplotype network. b, Phylogenetic tree for cpDNA sequences using Bayesian approach. c, ITS haplotype network. d, Phylogenetic tree for ITS using Bayesian approach. SD, *S. discolor*; SP, *S. pohuashanensis*; SH, SH individuals; BBH, Mount Baihua; HTL, Mount Tuoliang; HXL, Mount Xiling; MM, *Malus × micro-malus* Makino; PP, *P. pyrifolia*; PS, *P. serratifolia*. Circle sizes in networks correspond to the haplotype frequency of haplotype; pie charts

indicate proportions. Black rhombus in network represents missing haplotypes. Mutational steps of network are indicated by black bars. Dashed lines in network represent gaps and missing data. Number behind “location” (i.e., HTL) are the sample codes. Support values were estimated with posterior probabilities (in percentages). The “-1” and “-2” after the name of each represents the two homologous sequences of the ITS sequence split degenerate bases. Number behind “H” (i.e., H1) outside the phylogenetic tree indicate haplotypes

**Discussion**

**Morphological and molecular evidence for hybridization**

SH individuals had novel pink fruit, and most quantitative leaf and fruit characters were intermediate between *S. pohuashanensis* and *S. discolor*, which is consistent with individuals that are products of hybridization featured with

novel traits or intermediate morphological characters in other studies (Robertson et al. 2004; Price and Rich 2007; Hajrudinović et al. 2015; Zbiljić et al. 2021). For instance, the morphology of hybrid offsprings (natural and artificial) of *Rhododendron decorum* Franch. and *R. delavayi* Franch. was intermediate between the parental species (Zheng et al. 2017). Hybrids were shown to be mosaics of parental-like, intermediate, and transgressive phenotypes in the central part of the Western Carpathians (Đurković



**Table 6** Informative nucleotide sites in the ITS sequences

Group	115	127	130	155	162	163	236	242	566	571	578	599
SD	T	T	G	G	G	C	T	G	T	C	A	C
SH	T/Y	T/C/Y	G/C	G/A/R	G/T/K	C/A/M	T/G	G/A/R	T/C/s/Y	C/T/Y	A/C	C/A
SP	C/Y	C/Y	C/G/S	A/R	T/G/K	A/C/M	G/T/K	A/G/R	C/T/Y	T/C/Y	A/C/M	A/C/M
SD; SH (BBH1; HTL2, 3, 5-10; HXL2, 4, 5)	T	T	G	G	G	C	T	G	T	C	A	C
SH (BBH2; HTL1)	T	T	G	G	G	C	T	R	T	C	A	C
SH (HTL4)	T	Y	G	R	K	M	T	R	Y	Y	A	C
SH (HXL1)	T	T	G	R	G	C	T	G	T	C	A	C
SH (HXL3)	T	T	G	R	G	M	T	G	T	C	A	C
SP (BBH1; HTL1, 2, 6, 10, 12, 14)	C	C	G	A	T	A	G	A	C	T	C	A
SP (BBH2, 7, 8; HXL1; HTL3-5, 7-9, 11, 13, 15, 16);	C	C	C	A	T	A	G	A	C	T	C	A
SP (BBH3, 5; HXL6)	Y	C	C	A	T	A	G	A	T	T	C	A
SP (BBH4; HXL2)	C	C	S	A	T	A	G	A	T	T	C	A
SP (BBH6)	Y	Y	C	R	T	A	K	A	T	T	C	M
SP (HXL4)	Y	Y	G	R	T	A	K	A	Y	Y	M	M
SP (HXL3)	Y	C	S	A	T	A	G	A	C	T	C	A
SP (HXL5)	Y	C	G	A	T	A	G	A	Y	C	C	A

SD, *S. discolor*; SP, *S. pohuashanensis*; SH, *Sorbus* hybrids. BBH, Mount Bathua; HTL, Mount Tuoliang; HXL, Mount Xiling. Number behind "location" (i.e., HTL) is the sample code. M = C/A, Y = T/C, R = A/G, K = T/G

**Table 7** ITS haplotypes of *Sorbus* groups at three locations

Location	Group	ITS haplotypes	Hd	$\pi$
BBH	<i>S. pohnuashanensis</i>	H6, H7, H8, H9	0.592	0.00219
	SH individuals	H1, H2	0.500	0.00086
	<i>S. discolor</i>	H1	0.000	0.00000
HTL	<i>S. pohnuashanensis</i>	H6, H7	0.471	0.00081
	SH individuals	H1, H2, H3	0.198	0.00136
	<i>S. discolor</i>	H1	0.000	0.00000
HXL	<i>S. pohnuashanensis</i>	H6, H7, H8, H10	0.848	0.00443
	SH individuals	H1, H4, H5	0.378	0.00096
	<i>S. discolor</i>	H1	0.000	0.00000
Total			0.673	0.01024

BBH, Mount Baihua; HTL, Mount Tuoliang; HXL, Mount Xiling; Hd, haplotype diversity;  $\pi$ , nucleotide diversity

et al. 2012). However, hybrid offsprings with parent-like morphological characters were not found using cpDNA and ITS markers in this study. Further studies using other molecular markers, such as cpDNA combined with SSR or SNP in parent-like individuals that have red- or white-fruited are needed.

In addition to the morphological evidence, our comparative analysis of DNA sequences demonstrated hybridization between *S. discolor* and *S. pohnuashanensis*. Therefore, we infer that the SH individuals are likely hybrid offsprings of *S. discolor* and *S. pohnuashanensis*.

### Parental origin

Sequence analyses of four cpDNA markers showed that either *S. pohnuashanensis* or *S. discolor* is the female parent of SH individuals, which have multiple origins rather than one origin. This result is consistent with those from two cpDNA fragments (Tang et al. 2016).

Multiple origins are common for *Sorbus* hybrids. Hybrids between *S. aucuparia* (diploid/sexual reproduction) and *S. hybrida* L. (tetraploid/facultative parthenogenesis) have several independent origins, and each population originated from multiple crosses between local parents (Levin et al. 2018). Similar results were also found for other hybrid species, such as *Argyranthemum sundingii* L. Borgen, *Helianthus deserticola* Heiser, *Hippophae goniocarpa* Y. S. Lian, X. L. Chen and K. Sun ex Swenson and Bartish (Brochmann et al. 2000; Gross et al. 2003; Jiang et al. 2014).

Except for SH individuals with the same haplotype as the parents, 6% of the SH individuals have a unique haplotype, perhaps as a result of natural variations of these hybrids of *S. pohnuashanensis* and *S. discolor*. In addition, perhaps the small sample size did not fully cover all female parent donors for SH individuals.

### Putative scenarios of hybridization and possible crossing pathways

SH individuals were found to show chromatogram additivity compared with *S. discolor* and *S. pohnuashanensis* at most nuclear loci, implying natural hybridization between two parental species. The cpDNA analyses showed that 42% of the SH individuals share the chloroplast haplotype with *S. discolor*, while 53% of the SH individuals share the chloroplast haplotype with *S. pohnuashanensis*, suggesting that *S. discolor* and *S. pohnuashanensis* may be the maternal donors of SH individuals with reciprocal gene flow between them.

According to the comparative analysis of the cpDNA and ITS data set of the SH individuals, two crossing pathways, SP/SD and SD/SD, were inferred as follows:

(1) SP/SD crossing type: SH(HTL)1, 4–6; SH(BBH)2; SH(HXL)1–4. These individuals belong to clade *S. pohnuashanensis* in the cpDNA phylogenetic tree and shared the cpDNA haplotype with *S. pohnuashanensis*. Furthermore, they were nested within the *S. discolor* clade in the ITS phylogenetic tree and shared the ITS haplotype with *S. discolor*, confirming *S. discolor* as the male parent. Those SH individuals (SP/SD crossing type) with incongruence in their position in the cpDNA and ITS phylogenetic trees are assumed to be interspecific hybrids of *S. pohnuashanensis* and *S. discolor* and have undergone previous hybridization events or backcrosses with their parents in these three sympatric locations. In this case, *S. pohnuashanensis* served as the maternal donor and *S. discolor* as the pollen donor. Using phylogenetic incongruence of different genes is a common and preferred method to identify interspecific hybridization (Wang 2017), such as *Ficus* L. (Huang et al. 2019), *Acer* L. (Areces-Berazain et al. 2020), and *Lachemilla* Rydb. (Morales-Briones et al. 2018).

(2) SD/SD crossing type: SH(HTL)2, 3, 7–10, SH(BBH)1, SH(HXL)5. CpDNA haplotype and ITS sequences of these SH individuals were identical to those of *S. discolor*. Therefore, the molecular evidence is not sufficient to prove that these SH individuals are hybrids, because their female parent is *S. discolor*, but their fruit is pink. So, we speculate that those individuals are hybrids produced by a cross between *S. discolor* (female parent) and *S. pohnuashanensis* (male parent). These individuals may have originated from an ancient hybridization event, following by multiple backcrosses between hybrid offspring and *S. discolor*. This process might provide opportunities for SH individuals to be genetically “restored” to *S. discolor*. Another hypothesis is that those SH individuals were individuals of *S. discolor*, with characters that have not yet been discovered or documented. This hypothesis needs to be tested by expanding or deepening the investigation of *S. discolor*, especially for populations without gene introgression from other *Sorbus* species.

The formation of SH individuals might involve biparental origins and multiple hybridization events. Such reticular evolution also occurred for the subgenus *Cerasus* Mill. group in Jeju Island, Korea (Cho et al. 2014). As the maternal parent, *Prunus serrulata* Lindl./*Prunus sargentii* Rehder crossed with *P. spachiana* f. *ascendens* and produced the hybrid *Prunus yedoensis* Matsum., which is more similar to *P. spachiana* f. *ascendens* due to repeated backcrossing with *P. spachiana* f. *ascendens*. A reverse direction of hybridization events was also detected. The diversity and variation of *Cerasus* plants were attributed to backcrossing and bidirectional hybridization.

ITS sequences of all individuals of *S. discolor* are identical. Some individuals of *S. pohnuashanensis* are heterozygous in ITS sequences, which may be caused by the genetic introgression of *S. discolor* into *S. pohnuashanensis*. However, the amount and direction of gene flow need further studies.

### Possible factors contributing to hybridization

Environmental factors and biological features create opportunities for hybridization between *S. pohnuashanensis* and *S. discolor*. First, the overlapping geographical distributions and similar habitats permit spatial contact for hybridizations that occur for other plant genera such as *Rhododendron* L. (Zhang et al. 2007), *Ostryopsis* Decne. (Wang et al. 2021) and *Quercus* L. (Li et al. 2021). Second, the possibility of interspecific natural hybridization of two closely related species in sympatric regions may be enhanced by the partially or totally overlapping flowering periods and identical pollinators. Phenological observations revealed that the two *Sorbus* species flower in April and May and are commonly pollinated by ants and bees. More importantly, the mating system of *S. pohnuashanensis* and *S. discolor* is mainly outcrossing (McAllister 2005; Zheng et al. 2009). The hybrids can bear fruits and are suspected to reproduce.

The cross-compatibility between the two subgenera was proved by *Sorbus* variety Pink-Ness (pink-fruited), which was bred by crossing *S. discolor* (subgenus *Albocarmesinae*) and *S. aucuparia* (subgenus *Sorbus*) in Europe. Moreover, *S. ×pekinensis* was mentioned as a natural hybrid of *S. pohnuashanensis* and *S. discolor* in the monograph by McAllister (2005) but was not described. It occurs in mountain areas west of Beijing, as do HTL, BBH and HXL. So, we infer that SH individuals are natural hybrids of *S. pohnuashanensis* and *S. discolor*, namely *S. ×pekinensis* (McAllister 2005).

At present, *S. pohnuashanensis* is used for urban greening in Northeast China due to its adaptability to the climate at high altitudes. However, it is difficult to grow at low elevations in North China because leaves can be burned by high temperature in summer. We found that the hybrids had fewer sunburned leaves and were more resistant to high

temperatures than *S. pohnuashanensis* possibly due to the heterosis. Heterosis of hybrids between *S. pohnuashanensis* and *S. discolor* may be one of the ways to cope with this difficulty.

### Description of *S. ×pekinensis*

Trees to 10 m tall, usually in moist habitats such as shady slopes, semi-shady slopes, and valleys. Branchlets brown or purplish brown to grayish brown, tomentose-villous when young, gradually glabrescent, with small grayish-white lenticels; buds conic-ovoid, reddish-brown, with whitish-grey, sparsely tomentose margins, apex acuminate. Ovate-lanceolate compound leaves are 16.49–20.33 cm long and 9.37–11.77 cm wide; petioles 3.16–4.10 cm; stipules broadly ovate or semi-orbicular, margin coarsely sharply serrate; leaflets 5–7 pairs, 4.9–6.14 cm long, 1.54–2.00 cm wide, at intervals of 1.50–1.98 cm, ovate, upper and lower surface with sparse pubescence, basal leaflets usually smaller than others, top 1/3 margin singly or doubly toothed; lateral veins 9–16 pairs, slightly arcuate-anastomosing at margin. Compound corymbs terminal, densely flowered, 8.3–12.6 cm in diameter. Flowers 0.9–1.3 cm in diameter. Petals are broadly ovate or subrotund, sparsely hirsute at the base of upper surface, 6.2–4.2 mm long, 2.9–3.1 mm wide; stigma 3–5 mm. Sepals triangular, acuminate or acute, both surfaces tomentose. Fruit pink, sometimes with red dots, 7.23–8.85 mm long, 6.64–8.60 mm wide. Flowering in April–May, fruiting in September–October.

### Conclusions

Our morphological and molecular results provided evidence that *Sorbus* individuals with pink fruits are natural hybrids of *S. pohnuashanensis* and *S. discolor* in three sympatric areas in North China. We verified that the maternal genome of the putative hybrid individuals came from either *S. pohnuashanensis* or *S. discolor*. The results shed light on the formation and maintenance of genetic diversity in *Sorbus* and also provide an important basis for studying the mechanism and evolutionary process of interspecific hybridization between closely related species in sympatric areas. The results are important information for breeding *Sorbus* cultivars that can adapt to a warming climate. Because of the multiple copies of ITS sequences in nuclear DNA, the assumption of crossing origin of the putative hybrids needs to be verified by further cloning of ITS sequences. In addition, more molecular markers, such as simple sequence repeats or single-copy sequences of nuclear DNA should be deployed to confirm the natural hybridization events between the two *Sorbus* species in North China.

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