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Molecular evidence for natural hybridization between *Ligularia nelumbifolia* and *Cremanthodium stenoglossum* (Asteraceae, Senecioneae)

Huai Ning, Yue-Zhi Pan, and Xun Gong

Abstract: Natural hybridization occurred frequently in the sunflower family. To date, however, no study on natural hybridization involving in *Ligularia* and *Cremanthodium* has been reported. Here, we presented the molecular evidence for natural hybridization between *Ligularia nelumbifolia* (Bureau & Franch.) Hand.-Mazz. and *Cremanthodium stenoglossum* Ling & S.W.Liu. Four nuclear DNA regions were sequenced to test the natural hybridization hypothesis, and three chloroplast DNA regions were sequenced to determine the direction of hybridization. Analyses of the investigated DNA data suggested that all of the putative hybrid individuals were derived from hybridization between *L. nelumbifolia* and *C. stenoglossum* and that bidirectional hybridization occurred. Moreover, sympatric *Ligularia tsangchanensis* (Franch.) Hand.-Mazz. and *Ligularia virgaurea* (Maxim.) Mattf. ex Rehder & Kobuski were not apparently involved in the hybridization. Although NewHybrids analysis showed that all the putative hybrid individuals were F_1 class, a low frequency of backcrossing to *C. stenoglossum* might exist in the hybrid swarm. In such a case, hybrids might serve as a bridge facilitating gene flow between *L. nelumbifolia* and *C. stenoglossum*, and hybrid speciation is unlikely to happen for these hybrid individuals without asexual reproduction. Given the poorly resolved phylogenetic relationship between *Ligularia* and *Cremanthodium*, the occurrence of natural hybridization between *L. nelumbifolia* and *C. stenoglossum* might provide new insights into the recircumscription and redelimitation of these two genera.

Key words: natural hybridization, *Ligularia*, *Cremanthodium*, cpDNA, *ITS*.

Résumé : L'hybridation naturelle survient fréquemment chez la famille du tournesol. Jusqu'à présent, toutefois, aucune étude de l'hybridation naturelle chez *Ligularia* et *Cremanthodium* n'a été rapportée. Les auteurs présentent ici la preuve moléculaire d'une hybridation naturelle entre *Ligularia nelumbifolia* (Franch.) Hand.-Mazz. et *Cremanthodium stenoglossum* Ling & S.W.Liu. Quatre régions de l'ADN nucléaire ont été séquencées afin de tester l'hypothèse de l'hybridation naturelle, et trois régions de l'ADN chloroplastique ont été séquencées afin de déterminer la direction de l'hybridation. Les analyses des données d'ADN suggéraient que tous les individus hybrides présumés étaient issus de l'hybridation entre *L. nelumbifolia* et *C. stenoglossum* et qu'une hybridation bidirectionnelle était survenue. De plus, *Ligularia tsangchanensis* (Franch.) Hand.-Mazz. et *Ligularia virgaurea* (Maxim.) Mattf. ex Rehder & Kobuski sympatriques n'étaient apparemment pas impliqués dans l'hybridation. Même si l'analyse à l'aide du logiciel NewHybrids montrait que tous les individus hybrides présumés étaient de classe F_1, une faible fréquence de rétrocroisement vers *C. stenoglossum* pourrait exister dans l'essaim hybride. Dans ce cas, les hybrides pourraient servir de pont pour faciliter le flux de gènes entre *L. nelumbifolia* et *C. stenoglossum*, et la spéciation hybride ne surviendra probablement pas chez ces individus hybrides sans reproduction asexuée. Compte tenu de la relation phylogénétique faiblement résolue entre *Ligularia* et *Cremanthodium*, la survenue d'une hybridation naturelle entre *L. nelumbifolia* et *C. stenoglossum* pourrait fournir un nouvel aperçu de la redélimitation de ces deux genres. [Traduit par la Rédaction]

Mots-clés : hybridation naturelle, *Ligularia*, *Cremanthodium*, cpADN, ITS.

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Introduction

Considering the great number of constructive evolutionary consequences, such as genetic and adaptive variation, functional novelty, and even speciation produced by natural hybridization (Anderson 1949; Arnold 1997; Seehausen 2004; Lexer et al. 2005; Pandit et al. 2011), hybridization has been increasingly recognized as central to our understanding of plant evolution and speciation (Harrison 1990; Arnold 1997; Rieseberg and Carney 1998; Abbott et al. 2013). However, the integrity of parental species may be maintained and the formation of new species of hybrid origin may be hindered by F_1 dominated hybrid swarms or zones in the presence of hybridization (Nolte and Tautz 2010). There are two reasons for this situation. On the one hand, although natural hybridization can produce offspring, these offspring usually have little or no fertility because of various reproductive isolation mechanisms (Ma et al. 2014, 2016; Baack et al. 2015; Xie et al. 2017). On the other hand, even if the fertility of the first generation is high, reproductive isolation between species can result in the inviability of the second and later hybrid generations (Stebbins 1950). In addition, natural hybridization can cause negative consequences for biodiversity conservation, such as outbreeding depression or genetic swamping, the loss of genetic resources for some infrequent species, and even the extinction of rare species, depending on the extent of genetic introgression (Levin et al. 1996; Bleeker et al. 2007; Mameli et al. 2014; Todesco et al. 2016). Moreover, natural hybridization can also cause some taxonomic problems. For example, hybrids may be mistakenly defined as species, subspecies, variants, or races, based on parental, intermediate, and even novel morphology (Rieseberg et al. 1993; Rieseberg 1995; Sun and Lo 2011; Dai et al. 2012). Thus, studying the process of natural hybridization can contribute to elucidating the origin of new adaptive traits as well as new species, developing the conservation strategies for rare and (or) endangered species, and resolving relevant taxonomic problems.

Hybridization within genera is pervasive in the wild and has been observed in a number of taxa within the sunflower family (Asteraceae), mainly in *Senecio* (Kirk et al. 2004; López et al. 2008; Oberprieler et al. 2016), *Helianthus* (Rieseberg et al. 1990), *Tragopogon* (Lipman et al. 2013), *Sphagneticola* (Wu et al. 2013), *Helichrysum* (Galbany-Casals et al. 2012), *Centaurea* (Koutecký et al. 2011; Pisanu et al. 2011), *Bidens* (Knope et al. 2013), *Ainsliaea* (Mitsui et al. 2011), *Taraxacum* (Brock 2009), and *Ligularia* (Pan et al. 2008; Yu et al. 2011, 2014*a*, 2014*b*; Ning et al. 2017; Zhang et al. 2017). In particular, interspecific hybridization occurred more frequently between *Ligularia* species due to incomplete reproductive isolation among closely related species (Pan et al. 2008; Yu et al. 2011, 2014*a*; Ning et al. 2017). Meanwhile, interspecific hybridization between *Ligularia* species has been regarded as a critical driver for diversification, speciation, and evolution of *Ligularia* in the Qinghai–Tibet Plateau region and adjacent areas (Liu et al. 2006). Moreover, studies concerning natural hybridization between genera also have been reported from a few tribes of Asteraceae, such as Astereae (Li 2006), Cichorieae (Fehrer et al. 2007), Liabeae (Soejima et al. 2008), Gnaphalieae (Allan 1939; Mckenzie et al. 2004; Smissen et al. 2007; Smissen et al. 2015), and Senecioneae (Calvo et al. 2013).

Ligularia and *Cremanthodium* are taxonomically placed in the subtribe Tussilagininae (Senecioneae, Asteraceae) and share several morphological traits such as short rhizomes with rosette leaves, palmate or pinnate venation, well-developed basal leaf with broadly sheathed petiole, and radiated or discal capitulum (Liu 1989; Chen et al. 2011). Thus, *Cremanthodium* species were regarded as alpine derivatives of *Ligularia* species (Wulff 1944; Drury 1967). However, *Cremanthodium* was later treated as a separate genus based on its nodding capitula and broadly campanulate or hemispherical involucres (Jeffrey and Chen 1984; Liu 1989; Liu et al. 2001; Chen et al. 2011). Similar to *Ligularia*, most species of *Cremanthodium* are mainly distributed in the alpine meadows of the eastern Qinghai–Tibet Plateau region and adjacent areas (Liu et al. 1994; Chen et al. 2011). Moreover, molecular phylogeny revealed a complicated phylogenetic relationship between *Ligularia* and *Cremanthodium*. Namely, species from these two genera form a complex with other eastern Asian genera of Tussilagininae (Liu et al. 2006; Pelser et al. 2007, 2010; Ren et al. 2017). However, although *Cremanthodium* is closely related to *Ligularia* in morphology and phylogeny, little information is available on natural hybridization involving *Ligularia* and *Cremanthodium*.

During the authors' field investigations to Ganzi County, Sichuan province of China, individuals of *Ligularia nelumbifolia* (Bureau & Franch.) Hand.-Mazz. and *Cremanthodium stenoglossum* Ling & S.W.Liu were found growing together, and an unidentified taxon was discovered. Based on morphological characters of leaf margin, inflorescence type, peduncle and phyllary color, and corolla type, *L. nelumbifolia* and *C. stenoglossum* were easily distinguished in the wild. However, the unidentified taxon typically displayed an intermediate morphology of these two species. Hence, we suspected that these individuals of the unidentified taxon were the offspring of natural hybridization between these two species.

Recently, direct DNA sequencing has been widely applied to natural hybridization studies, resulting in its predominant role in detection and verification of natural hybridization (Pan et al. 2008; Yu et al. 2014*a*; Zhang et al. 2014; Liao et al. 2015; Li et al. 2017). A large number of natural hybrids have been identified based on sequencing the biparentally inherited nuclear DNA (nrDNA) regions and uniparentally inherited chloroplast DNA (cpDNA) regions (Fan et al. 2014; Liu et al. 2014; Yu et al. 2014*a*, 2014*b*; Zhang et al. 2014; Li et al. 2017; Ning et al. 2017; Yan et al. 2017). In the present study, to further

Table 1. Sampling detail and codes of *Ligularia nelumbifolia*, the putative hybrid of *L. nelumbifolia* and *Cremanthodium stenoglossum*, *C. stenoglossum*, *L. tsangchanensis*, and *L. virgaurea*, and an outgroup species (*Senecio vulgaris*) used in this study.

Taxon	Voucher Specimen	Code	Locality (China)	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)
L. nelumbifolia	PG16081213	Ln1–Ln20 (20)	Ganzi County, Sichuan	31.41°	99.96°	4584
Putative hybrid	PG16081217	CL1–CL20 (20)	Ganzi County, Sichuan	31.41°	99.96°	4584
C. stenoglossum	PG16081201	Cs1–Cs20 (20)	Ganzi County, Sichuan	31.41°	99.96°	4584
L. tsangchanensis	PG16081220		Ganzi County, Sichuan	31.41°	99.96°	4584
L. virgaurea	PG16081221		Ganzi County, Sichuan	31.41°	99.96°	4584
S. vulgaris	PG18061301		Kunming Botanical Garden, Yunnan	25.14°	102.74°	1928

examine our hypothesis, four nrDNA regions [*A12*, *B14*, *D30*, and internal transcribed spacer (*ITS*)] and three cpDNA regions (*psb*A–*trn*H, *trn*Q–5′*rps*16 and *trn*K–*rps*16) were sequenced to confirm the natural hybridization between *L. nelumbifolia* and *C. stenoglossum* and to determine the direction of hybridization.

Materials and methods

Study species and plant sampling

Plant material was collected from Ganzi County, Sichuan province of China (Table 1), where *Ligularia tsangchanensis* (Franch.) Hand.-Mazz., *Ligularia virgaurea* (Maxim.) Mattf. ex Rehder & Kobuski, *L. nelumbifolia*, the putative hybrid of *L. nelumbifolia* and *C. stenoglossum* (hereinafter, the putative hybrid), and *C. stenoglossum* distribute sympatrically. Based on the morphological descriptions of Liu (1989), the most prominent difference among *L. nelumbifolia*, the putative hybrid, and *C. stenoglossum* was the type of inflorescence. *Ligularia nelumbifolia* displayed a compound corymb consisting of several capitula. In contrast, *C. stenoglossum* displayed a solitary capitulum, and the putative hybrid typically displayed a compound corymb consisting of a small number of capitula. Likewise, the type of corolla for the putative hybrid was intermediate between the two putative parents. The ray florets of *C. stenoglossum* were long but they were absent in *L. nelumbifolia*, whereas the putative hybrid had shorter ray florets than *C. stenoglossum*. In addition, although the leaf shape and the leaf venation were not different among the three taxa, the leaf margin was remarkably different among them. *Ligularia nelumbifolia* had a green and sharply dentate leaf margin, *C. stenoglossum* had a purple and coarsely-angularly dentate leaf margin, and the leaf margin of the putative hybrid was intermediate in morphology between *L. nelumbifolia* and *C. stenoglossum*. Moreover, the color of the peduncle and phyllary also suggested that the putative hybrid had morphologically intermediate characters between *L. nelumbifolia* and *C. stenoglossum*, despite the shape of the involucre of the putative hybrid, which was similar to that of *C. stenoglossum* (Table 2; Fig. 1). At this location, no specific permission was required for scientific research, and no endangered or protected species were involved. For *L. nelumbifolia*, the putative hybrid, and *C. stenoglossum*, 20 individuals of each taxon were randomly selected to collect young leaves. The sampled leaves were dried immediately in silica gel in the field for DNA extraction. Although sympatric *L. tsangchanensis* and *L. virgaurea* have pinnate veins and racemose inflorescences that are different from those of putative parental species, they have flowering periods that overlap with the two putative parental species. Thus, to investigate whether *L. tsangchanensis* and *L. virgaurea* participated in the process of hybridization, *L. tsangchanensis* and *L. virgaurea* were sampled. The sampling detail and codes for each of the samples are listed in Table 1. In addition, *Senecio vulgaris* L. was collected as an outgroup from Kunming Botanical Garden (Table 1). Voucher specimens were deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

PCR amplification and sequencing of seven DNA regions

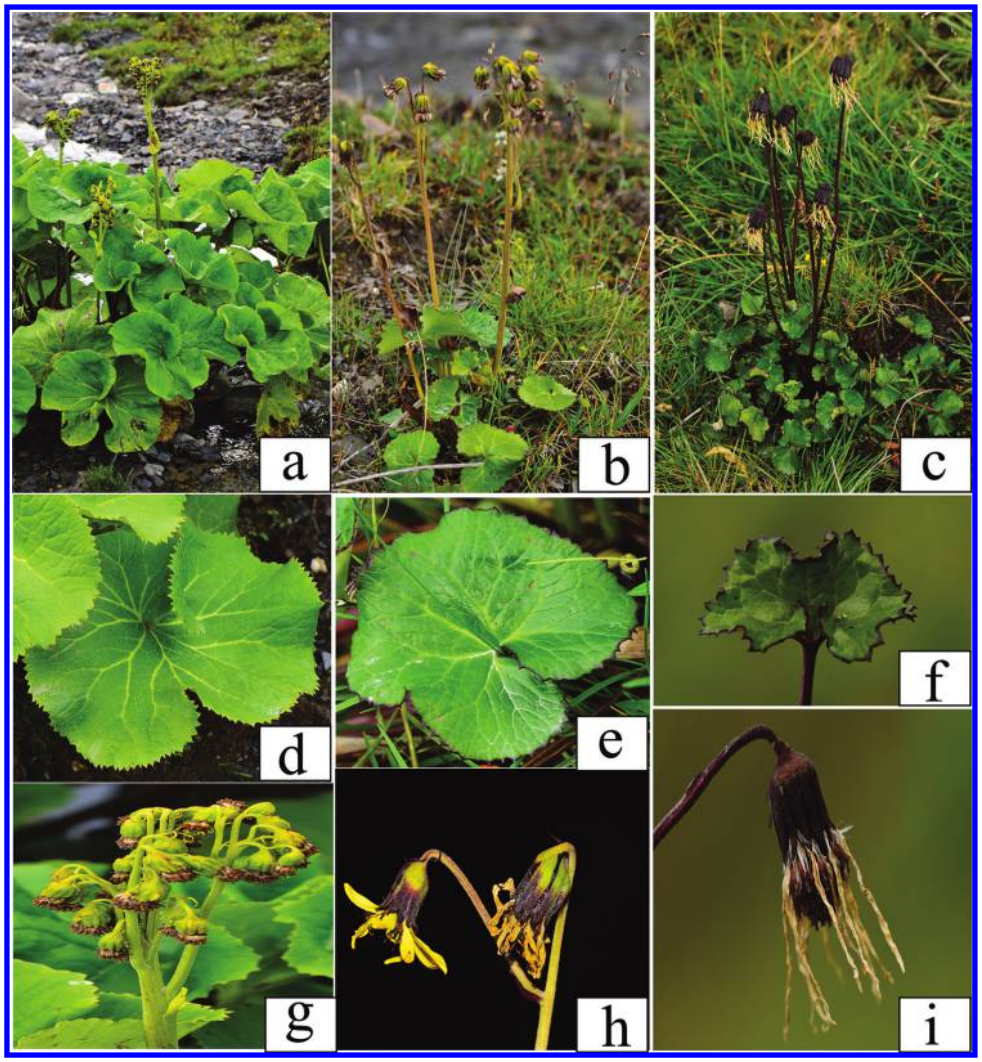
Total genomic DNA was extracted using the cetyltrimethyl ammonium bromide protocol of Doyle and Doyle (1987) with modifications. Three nrDNA regions (*A12*, *B14*, and *D30*) were amplified among all of the sampled individuals using primers developed by Chapman et al. (2007). Polymerase chain reaction (PCR) amplification was performed using a reaction volume of 20 μL containing 7.9 μL ultrapure water, 2.0 μL 10× PCR buffer (Mg^{2+} free), 1.8 μL $MgCl_2$ (25 mmol/L), 1.6 μL dNTP (2.5 mmol/L), 1.6 μL bovine serum albumin (BSA; 20 mg/mL), 0.3 μL *Taq* polymerase (5 U/μL), 0.9 μL each primer (10 μmol/L), and 3.0 μL template DNA (40–120 ng). Amplification reactions were performed at the following temperature profiles: 3 min of initial denaturation at 95 °C; followed by 10 touchdown cycles composed of 30 s at 94 °C; 30 s at decreasing annealing temperatures (1 °C per cycle from 66 °C (*B12* and *D30*) or 60 °C (*A12*) in the first cycle to 56 °C (*B12* and *D30*) or 50 °C (*A12*) in the 10th cycle); 45 s at 72 °C; and 30 cycles of 30 s at 94 °C; 30 s at 56 °C (*B12* and *D30*) or 50 °C (*A12*); 45 s at 72 °C; and 20 min at 72 °C for the final extension step. The amplification products were purified and then directly sequenced by Kunming TSINGKE Biological Technology Co., Ltd. (Kunming, China) using an ABI 3730 automated sequencer.

ITS regions were amplified among all of the sampled individuals using primers ITS4 and ITS5 (White et al. 1990). PCR amplification was performed using a reaction volume of 20 μL containing 12.4 μL ultrapure water,

Table 2. Key morphological comparison of *Ligularia nelumbifolia*, the putative hybrid of *L. nelumbifolia* and *Cremanthodium stenoglossum*, and *C. stenoglossum*.

Taxon	Leaf length (cm)	Leaf width (cm)	Plant height (cm)	Leaf margin	Color of the peduncle and phyllary	Type of corolla	The type of inflorescence
L. nelumbifolia	7–30	13–38	80–100	Green and sharply dentate	Green	Long ray florets	Compound corymb consisting of numerous capitula
Putative hybrid	1.6–13	2.1–17	24–57	Intermediate	Purple–green	Short ray florets	Compound corymb consisting of a small number of capitula
C. stenoglossum	0.7–2	1.5–4	10–32	Purple and coarsely-angularly dentate	Purple	Without ray floret	Solitary capitulum

Fig. 1. Habitats, leaf and flower characteristics of *Ligularia nelumbifolia* (*a*, *d*, and *g*), the putative hybrid of *L. nelumbifolia* and *Cremanthodium stenoglossum* (*b*, *e*, and *h*), and *C. stenoglossum* (*c*, *f*, and *i*) investigated in this study. [Colour online.]



2.0 μL 10× PCR buffer (Mg^{2+} free), 1.0 μL $MgCl_2$ (25 mmol/L), 1.0 μL dNTP (2.5 mmol/L), 0.8 μL BSA (20 mg/mL), 0.3 μL *Taq* polymerase (5 U/μL), 0.5 μL each primer (10 μmol/L), and 1.5 μL template DNA (20–60 ng). Amplification reactions were performed at the following temperature profiles: 5 min of initial denaturation at 94 °C; followed by 36 cycles composed of 45 s at 94 °C; 45 s at 56 °C; 50 s at 72 °C, and 8 min at 72 °C for the final extension step. The amplification products were purified and then directly sequenced using the above-mentioned methods.

Three chloroplast DNA regions were amplified among all of the sampled individuals using universal primer pairs of *psb*A–*trn*H (Sang et al. 1997), *trn*Q–5′*rps*16 (Shaw et al. 2007), and *trn*K–*rps*16 (Shaw et al. 2007). PCR amplification was carried out in a total volume of 20 μL containing 12.5 μL ultrapure water, 2.0 μL 10× PCR buffer

(Mg^{2+} free), 1.0 μL $MgCl_2$ (25 mmol/L), 1.0 μL dNTP (2.5 mmol/L), 1.0 μL DMSO (20 mg/mL), 0.3 μL *Taq* polymerase (5 U/μL), 0.35 μL each primer (10 μmol/L), and 1.5 μL template DNA (20–60 ng). Amplification reactions were performed at the following temperature profiles: 5 min of initial denaturation at 80 °C; followed by 36 cycles composed of 45 s at 94 °C; 45 s at 53 °C; 50 s at 65 °C, and 7 min at 65 °C for the final extension step. The amplification products were purified and then directly sequenced using the above mentioned methods.

Sequence analyses

Sequences of seven DNA regions were initially aligned and compared in SeqMan (DNAstar 7.1, DNASTAR Inc., Madison, Wisconsin, USA). A further alignment of sequences was performed using BioEdit V.7 (Hall 1999) and adjusted manually. DNA sequences have been submitted to the GenBank databases under accession numbers MF688687-MF688758. Two homogeneity tests were performed on three cpDNA regions and four nrDNA regions using PAUP* 4.0b10 (Swofford 2003). The homogeneity test showed no significant incongruence among tree cpDNA regions (P = 1; >0.5), and the homogeneity test showed significant incongruence among four nrDNA regions (P = 0.3; <0.5). Thus, the three cpDNA regions were combined and used in the next analyses. Haplotypes of the combined cpDNA sequences were inferred using DnaSP 5.0 (Rozas et al. 2003). However, four nrDNA regions were analyzed separately. Nuclear DNA regions often generated heterozygous sites in some individuals, which were identified by overlapping peaks in chromatograms. Thus, the nrDNA sequences which contained heterozygous sites were resolved using algorithms of PHASE (Stephens et al. 2001; Stephens and Donnelly 2003) in DnaSP 5.0. After the process of PHASE, one nrDNA sequence was separated into two sequences that did not contain heterozygous sites. The haplotypes of phased nrDNA sequences were inferred by DnaSP 5.0. Haplotype networks for combined cpDNA and each nrDNA region were constructed to resolve the relationships using Network 5.0.0.1 with the median-joining algorithm (Bandelt et al. 1999). Each indel was treated as a single mutational event in the Network analysis. The graph generated from Network 5.0.0.1 was drawn with Adobe Illustrator (Adobe Systems, Mountain View, California, USA). For each nrDNA region and combined cpDNA region, we reconstructed the phylogeny of the haplotypes using Bayesian approach in MrBayes 3.2.1 (Ronquist et al. 2012). The Markov chain Monte Carlo (MCMC) searched 1 000 000 generations, and trees were sampled every 100 generations with the first 25% trees discarded. The optimal substitution model for Bayesian inference was ascertained using jModelTest 2.1.5 with the Akaike information criterion (Darriba et al. 2012), which suggested the GTR model. Moreover, deletions were treated as missing data. Because we excluded the probability that *L. tsangchanensis* and *L. virgaurea* participated in the hybridization in the preliminary experiment (*L. tsangchanensis* and *L. virgaurea* were completely separated with other two species and the putative hybrid based on the *ITS* sequences), the analyses of network, Structure and NewHybrids did not include *L. tsangchanensis* and *L. virgaurea*.

Based on the sequences of four nrDNA regions, genomic admixture proportions of each hybrid individual derived from the parental genome were calculated using the program Structure version 2.3.4 (Hubisz et al. 2009) with the Bayesian-model-based clustering algorithm. The variable sites of nrDNA sequences for each individual were converted into a digital matrix for the input file format of Structure by the program xmfa2struct.bat and adjusted manually. The admixture model with correlated allele frequencies was adopted and the default settings were used. Run parameters were set to 100 000 iterations of MCMC, preceded by a burn-in of 100 000. No prior knowledge of the species was included, and no popflags were set. To determine the optimal value of K, ΔK was calculated by performing 10 runs for each K ranging from 1 to10 (Evanno et al. 2005).

The program of NewHybrids was used to verify the generations (parents, F_1, F_2, or backcrosses) of all individuals using the default settings (Anderson and Thompson 2002). This program does not require the parental populations to be sampled separately under the presumption that only two generations of crossing have occurred (Anderson and Thompson 2002). We assumed that these nrDNA regions were unlinked and were at linkage equilibrium in the parent species before hybridization (Milne and Abbott 2008). Moreover, we assumed that these nrDNA regions were not subjected to natural selection. Then, each haplotype was treated as an allele and denoted as two digits and each nrDNA region was treated as a locus respectively. The analysis was run for 100 000 rounds after a burn-in of 10 000 MCMC iterations.

Results

Sequence analyses of four nrDNA regions

The aligned length of four nrDNA regions *A12*, *B14*, *D30*, and *ITS* were 268 bp, 430 bp, 465 bp, and 625 bp in three taxa, respectively. After the process of PHASE, four nrDNA regions *A12*, *B14*, *D30*, and *ITS* got 93 sequences, 110 sequences, 101 sequences, 115 sequences in three taxa, respectively. There was a total of 18 fixed nucleotide substitutions and one fixed 2 bp indel between *L. nelumbifolia* and *C. stenoglossum* at four nrDNA regions (Table 3). Among 20 putative hybrid individuals, 17 individuals displayed chromatogram additivity between *L. nelumbifolia* and *C. stenoglossum* at all fixed sites, and the remaining three individuals (CL1, CL8, and CL19) displayed chromatogram additivity at 17 fixed sites but identical nucleotide base with *L. nelumbifolia* at the site of the *B14* region (Table 3). Interestingly, two individuals (Cs6 and Cs7)

Table 3. Variable sites and indels of *Ligularia nelumbifolia*, the putative hybrid of *L. nelumbifolia* and *Cremanthodium stenoglossum*, and *C. stenoglossum* at four nuclear DNA regions.

	Polymorphic sites																							
	A12						*B14*	*D30*	*ITS*															
Individual	109	118	133	143	206	232	261	77	172	175	91	99	101	117	123	175	215	216	382	423	446	562	564	587	612
Ln1–20	G	A	T	G	A	A	T	T	C	G	G	A	T	T	T	C	C	G	T	C	A	A	T	C	T
CL9–CL11, CL14–CL18, CL20	K	M	K	K	M	W	Y	Y	M	S	S	R	W	Y	Y	Y	–	–	Y	Y	R	R	Y	S	Y
CL2–CL7, CL12–CL13	K	M	K	K	M	W	Y	Y	M	S	S	R	W	Y	Y	Y	C	G	Y	Y	R	R	Y	S	Y
CL1, CL19	K	M	K	K	M	W	Y	T	M	S	S	R	W	Y	Y	Y	–	–	Y	Y	R	R	Y	S	Y
CL8	K	M	K	K	M	W	Y	T	M	S	S	R	W	Y	Y	Y	C	G	Y	Y	R	R	Y	S	Y
Cs1–Cs5, Cs8–Cs20	T	C	G	T	C	T	C	C	A	C	C	G	A	C	C	T	–	–	C	T	G	G	C	G	C
Cs6, Cs7	T	C	G	T	C	T	C	C	A	C	S	G	W	Y	C	T	–	–	Y	Y	G	G	C	G	C

Note: –, deletions; G + T = K; A + C = M; A + T = W; T + C = Y; G + C = S; A+G = R.

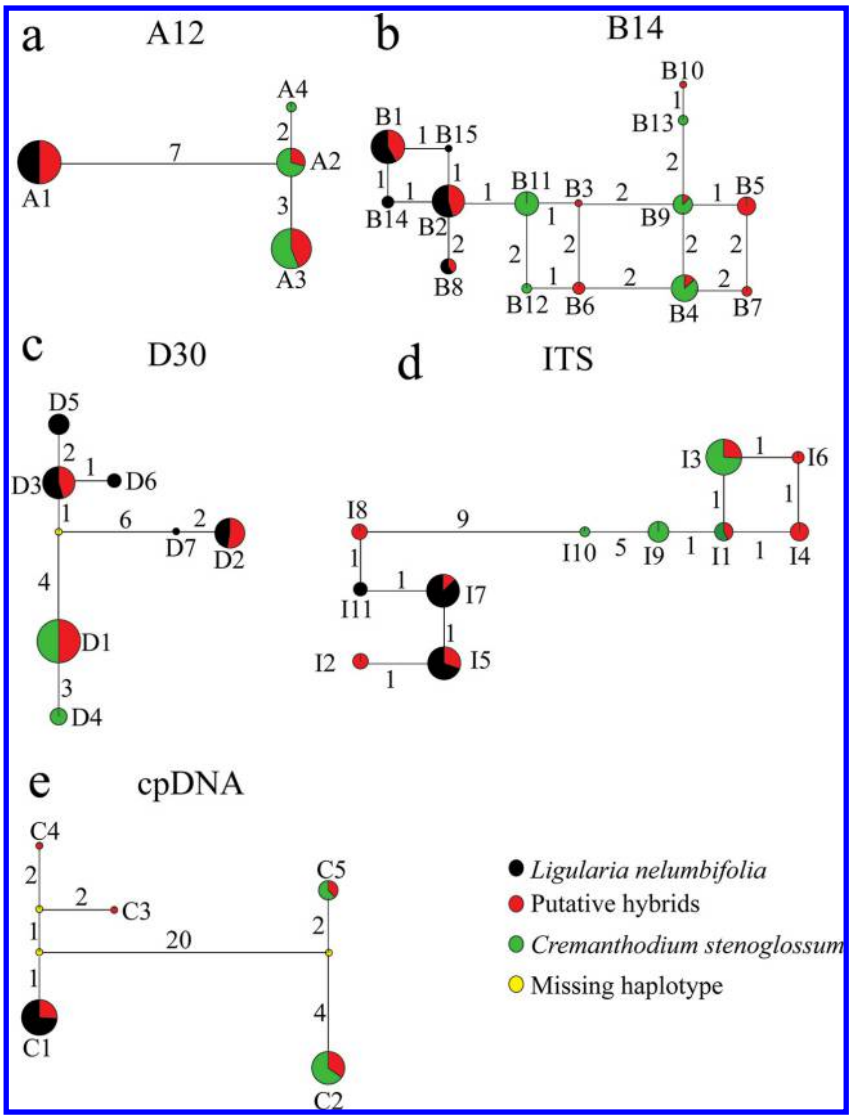
identified as *C. stenoglossum* also showed chromatogram additivity at five sites in the *ITS* region (Table 3).

Haplotype network analysis of *A12* identified two highly divergent clusters (Fig. 2*a*). Only one haplotype was present in *L. nelumbifolia* individuals, which represented one of the clusters, whereas the three haplotypes present in *C. stenoglossum* generally conformed to the other. Each putative hybrid individual had two haplotypes: one was shared with *L. nelumbifolia* and the other was shared with *C. stenoglossum*. (Table 4). Moreover, haplotype network analysis of the *ITS* region also identified two highly divergent clusters (Fig. 2*d*). Namely, three haplotypes of *L. nelumbifolia* formed one cluster, and four haplotypes of *C. stenoglossum* formed another cluster. Each putative hybrid individual had two haplotypes, each originating from one of the divergent clusters (Table 4; Fig. 2*d*). However, for the other two nrDNA regions (*B14* and *D30*), the haplotypes of the two putative parents did not form two highly divergent clusters (Figs. 2*b* and 2*c*) because of the fewer fixed sites between *L. nelumbifolia* and *C. stenoglossum*. Even so, no haplotype was shared between the two species.

Bayesian inference was used to construct phylogenetic relationships among haplotypes of each nrDNA region. For four Bayesian trees, all haplotypes of *L. nelumbifolia* and those of *C. stenoglossum* completely segregated from sympatric *L. tsangchanensis* and *L. virgaurea* in each Bayesian tree (Figs. 3–6). For two nrDNA regions (*A12* and *D30*), the haplotypes of *L. nelumbifolia* and those of *C. stenoglossum* formed two well-separated clades in each Bayesian tree, respectively (Figs. 3 and 5). For two haplotypes of each putative hybrid individual, one shared a clade with *L. nelumbifolia* and the other shared another clade with *C. stenoglossum* in each Bayesian tree (Table 4; Figs. 3 and 5). However, for region *B14*, all haplotypes of *L. nelumbifolia* formed one clade, and all haplotypes but one haplotype (B11) of *C. stenoglossum* formed another clade (Fig. 4). For two phased haplotypes of each putative hybrid individual (except CL1, CL8, and CL19), one shared a clade with *L. nelumbifolia* and the other shared another clade with *C. stenoglossum* (Table 4; Fig. 4), whereas both two-phased haplotypes of individual CL1 and CL8 were nested within the *L. nelumbifolia* clade. Moreover, individual CL1 only had one haplotype (B2) which was shared with *L. nelumbifolia* (Table 4; Fig. 4). For the Bayesian tree of *ITS* region, all haplotypes of *L. nelumbifolia* and one haplotype (I10) of *C. stenoglossum* formed one clade, whereas the remaining haplotypes of *C. stenoglossum* formed another clade (Fig. 6). For two haplotypes of each putative hybrid individual, one nested within each of the two clades (Table 4; Fig. 6).

Structure analysis for the three taxa based on the sequences of four nrDNA regions yielded an optimal ΔK value for $K = 2$ (Fig. 7*a*) and revealed two highly distinct genetic groups (Fig. 7*b*). All but two individuals (Cs6 and Cs7) morphologically identified as *C. stenoglossum* were

Fig. 2. Haplotype network of haplotype data for four nrDNA regions *A12* (*a*), *B14* (*b*), *D30* (*c*), and *ITS* (*d*) and combined three cpDNA regions (*e*) for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as *L. nelumbifolia*, and *C. stenoglossum*. Mutation steps are shown by the numerical value, each indel was treated as a single mutational event, and node size was proportional to the number of haplotypes possessed by all investigated individuals. [Colour online.]



assigned to the Group 1 with high proportion (>99%), whereas all individuals morphologically identified as *L. nelumbifolia* were assigned to the Group 2 with high proportion (>99%). However, two individuals (Cs6 and Cs7) of *C. stenoglossum* contained a mixed genetic component (less than 10%) of Group 2 (Fig. 7*b*). As expected, all putative hybrid individuals presented the genetic admixture of both putative parental groups with nearly equal proportion (Fig. 7*b*).

Generations (parents, F_1, F_2, or backcrosses) of all individuals were confirmed using the program NewHybrids based on the haplotypes of four nrDNA regions. All individuals previously identified as *C. stenoglossum* based on morphology were all assigned to pure *C. stenoglossum* with >99% posterior probabilities (Fig. 7*c*). All individuals previously identified as *L. nelumbifolia* based on morphology were all assigned to pure *L. nelumbifolia* with >99% posterior probabilities. All individuals morphologically identified as the putative hybrid were all assigned to F_1 class with high posterior probabilities (>96%).

Sequence analyses of combined cpDNA

The aligned length of *psb*A–*trn*H, *trn*Q–5′*rps*16, and *trn*K–*rps*16 were 454 bp, 957 bp, and 939 bp respectively. The combined length of the three cpDNA regions was 2350 bp. All individuals of *L. nelumbifolia* had identical sequences and did not contain any intraspecific polymorphism (Table 5). However, sequences variations were detected in five individuals (Cs2, Cs3, Cs4, Cs19, and Cs20) of *C. stenoglossum* at four sites of *trn*Q–5′*rps*16 region. These five individuals exhibited identical nucleotide bases with *L. nelumbifolia* at these four sites. A total of

Table 4. Haplotypes for all individuals of the three taxa (*Ligularia*, *Cremanthodium*, and *Senecio*) in each nuclear DNA region and the combined cpDNA.

Individual	Haplotypes of *A12*	Haplotypes of *B14*	Haplotypes of *D30*	Haplotypes of *ITS*	Haplotypes of combined cpDNA
Cs1	A3	B11, B12	D1	I3, I9	C2
Cs2	A2, A3	B9, B13	D1, D4	I3, I9	C5
Cs3	A2	B4, B11	D1	I3, I9	C5
Cs4	A3	B4, B9	D1	I3, I9	C5
Cs5	A2, A3	B4, B9	D1	I3, I9	C2
Cs6	A3	B4, B11	D1, D4	I3, I10	C2
Cs7	A2, A3	B9, B11	D1, D4	I3, I10	C2
Cs8	A2, A3	B4, B9	D1	I3, I10	C2
Cs9	A3	B4, B11	D1	I3	C2
Cs10	A2, A3	B11, B13	D1	I3, I9	C2
Cs11	A3, A4	B11	D1, D4	I3	C2
Cs12	A2, A3	B4, B9	D1	I1, I3	C2
Cs13	A2, A3	B4, B9	D1	I1, I3	C2
Cs14	A2, A3	B11	D1, D4	I1, I3	C2
Cs15	A2, A3	B4, B11	D1	I3	C2
Cs16	A2, A3	B4, B11	D1	I1, I3	C2
Cs17	A3, A4	B4, B11	D1	I3	C2
Cs18	A3	B4, B11	D1, D4	I3, I9	C2
Cs19	A3	B4, B11	D1	I3	C5
Cs20	A2, A3	B11, B12	D1	I3, I9	C5
CL1	A1, A2	B1, B2	D1, D2	I1, I2	C1
CL2	A1, A3	B1, B5	D1, D2	I4, I5	C5
CL3	A1, A3	B2, B6	D1, D2	I4, I5	C2
CL4	A1, A3	B2, B4	D1, D3	I4, I5	C2
CL5	A1, A3	B5, B8	D1, D3	I4, I7	C5
CL6	A1, A3	B1, B7	D1, D2	I6, I7	C1
CL7	A1, A3	B2, B9	D1, D3	I4, I5	C1
CL8	A1, A3	B1, B2	D1, D3	I5, I6	C2
CL9	A1, A3	B8, B10	D1, D3	I3, I8	C2
CL10	A1, A2	B1, B3	D1, D2	I2, I3	C2
CL11	A1, A3	B2, B4	D1, D3	I2, I3	C3
CL12	A1, A2	B2, B5	D1, D3	I4, I5	C4
CL13	A1, A3	B1, B5	D1, D2	I6, I7	C1
CL14	A1, A3	B1, B6	D1, D2	I1, I2	C2
CL15	A1, A3	B2, B7	D1, D3	I3, I8	C1
CL16	A1, A2	B1, B6	D1, D3	I3, I8	C1
CL17	A1, A2	B1, B5	D1, D2	I1, I2	C1
CL18	A1, A3	B1, B5	D1, D3	I4, I5	C5
CL19	A1, A3	B2	D1, D2	I3, I8	C2
CL20	A1, A3	B2, B5	D1, D2	I3, I8	C2
Ln1	A1	B1	D3, D5	I5, I7	C1
Ln2	A1	B1, B15	D5, D6	I5, I7	C1
Ln3	A1	B1	D2, D5	I7, I11	C1
Ln4	A1	B1, B2	D6, D7	I5, I7	C1
Ln5	A1	B2	D2, D5	I7, I11	C1
Ln6	A1	B2, B8	D3, D6	I5, I7	C1
Ln7	A1	B1, B2	D2, D3	I5, I7	C1
Ln8	A1	B2	D3, D5	I5, I7	C1
Ln9	A1	B2	D2, D3	I7, I11	C1
Ln10	A1	B2	D2	I5, I7	C1
Ln11	A1	B1, B2	D2, D3	I5, I7	C1
Ln12	A1	B8	D3, D5	I5, I7	C1
Ln13	A1	B1, B2	D3	I5, I7	C1
Ln14	A1	B1, B2	D3, D6	I5, I7	C1
Ln15	A1	B2, B14	D3, D5	I5, I7	C1
Ln16	A1	B1, B8	D3, D5	I5, I7	C1
Ln17	A1	B1, B2	D5	I5, I7	C1
Ln18	A1	B2, B14	D2	I7, I11	C1
Ln19	A1	B1, B2	D2	I5, I7	C1
Ln20	A1	B1, B14	D2, D3	I5, I7	C1

Fig. 3. Phylogenetic analyses of haplotype data for nrDNA region *A12* for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as *L. nelumbifoli*, and *C. stenoglossum*. "*_L. nelumbifolia*_PH" represents the haplotype that included the individuals of *L. nelumbifolia* and the putative hybrid. "*_C. stenoglossum*_PH" represents the haplotype that included the individuals of *C. stenoglossum* and the putative hybrid. "*_L. nelumbifolia*" represents the haplotype that only included the individuals of *L. nelumbifolia*. "*_C. stenoglossum*" represents the haplotype that only included the individuals of *C. stenoglossum*. "_PH" represents the haplotype that only included individuals of the putative hybrid. Numbers above the branches indicate the supporting rate (>50%).

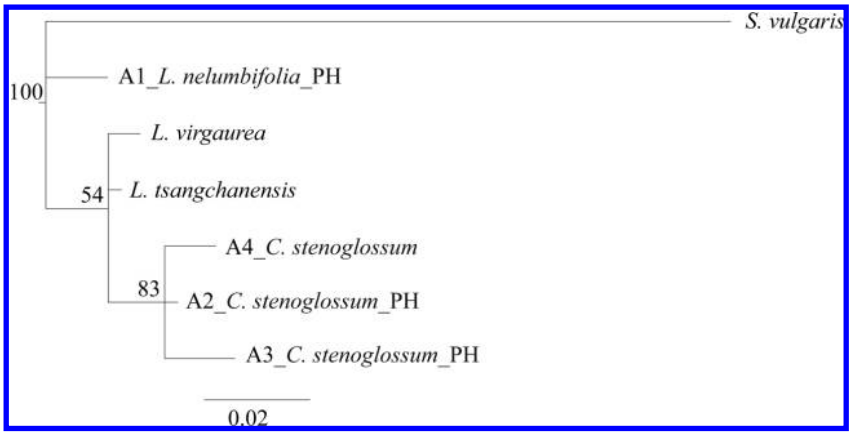
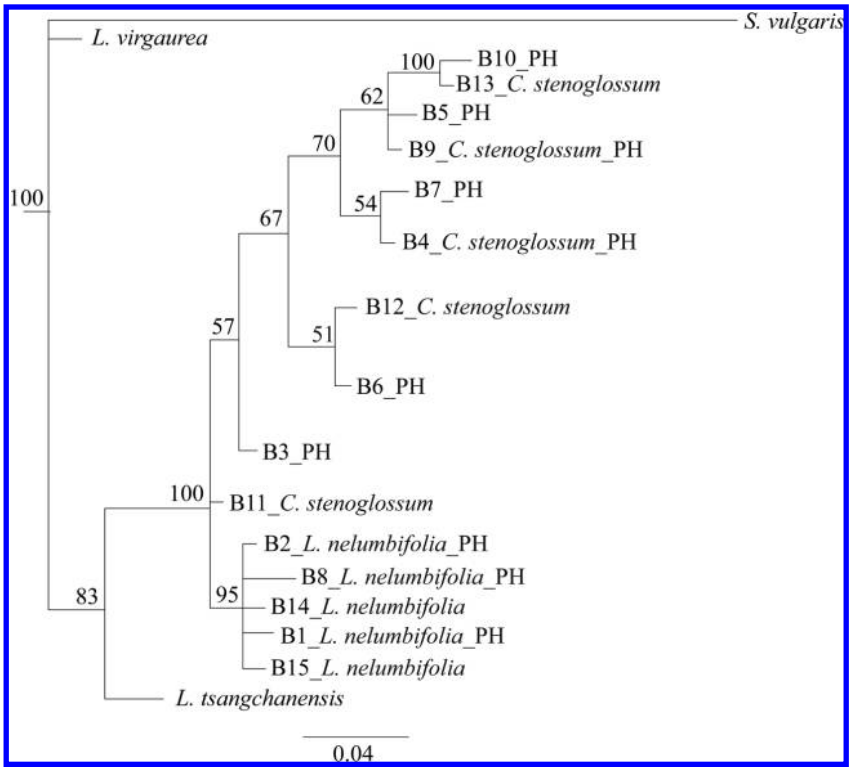


Fig. 4. Phylogenetic analyses of haplotype data for nrDNA region *B14* for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as *L. nelumbifolia* and *C. stenoglossum*. "*_L. nelumbifolia*_PH" represents the haplotype that included the individuals of *L. nelumbifolia* and the putative hybrid. "*_C. stenoglossum*_PH" represents the haplotype that included the individuals of *C. stenoglossum* and the putative hybrid. "*_L. nelumbifolia*" represents the haplotype that only included the individuals of *L. nelumbifolia*. "*_C. stenoglossum*" represents the haplotype that only included the individuals of *C. stenoglossum*. "_PH" represents the haplotype that only included individuals of the putative hybrid. Numbers above the branches indicate the supporting rate (>50%).



15 fixed nucleotide substitutions and eight indels were identified between *L. nelumbifolia* and *C. stenoglossum* at three cpDNA regions. For the putative hybrid, seven individuals displayed identical sequences with *L. nelumbifolia* at all fixed sites and indels, and 11 individuals exhibited identical sequences with *C. stenoglossum* at all fixed sites and indels. However, the remaining two individuals (CL11 and CL12) displayed identical nucleotide bases with

Fig. 5. Phylogenetic analyses of haplotype data for nrDNA region *D30* for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as *L. nelumbifolia,* and *C. stenoglossum*. "*_L. nelumbifolia*_PH" represents the haplotype that included the individuals of *L. nelumbifolia* and the putative hybrid. "*_C. stenoglossum*_PH" represents the haplotype that included the individuals of *C. stenoglossum* and the putative hybrid. "*_L. nelumbifolia*" represents the haplotype that only included the individuals of *L. nelumbifolia*. "*_C. stenoglossum*" represents the haplotype that only included the individuals of *C. stenoglossum*. "_PH" represents the haplotype that only included individuals of the putative hybrid. Numbers above the branches indicate the supporting rate (>50%).

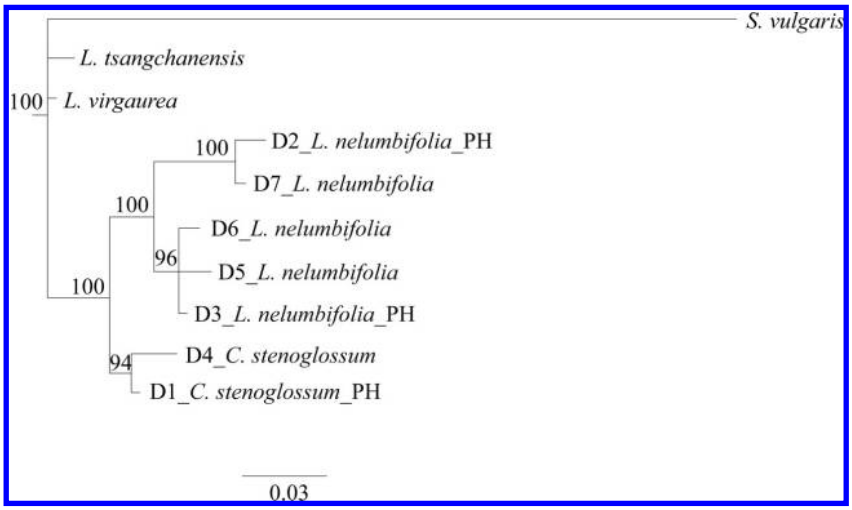
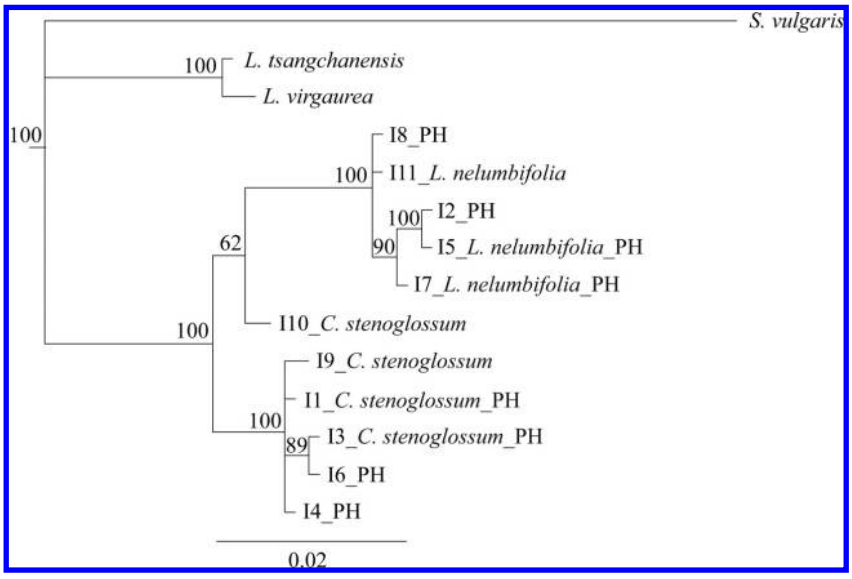


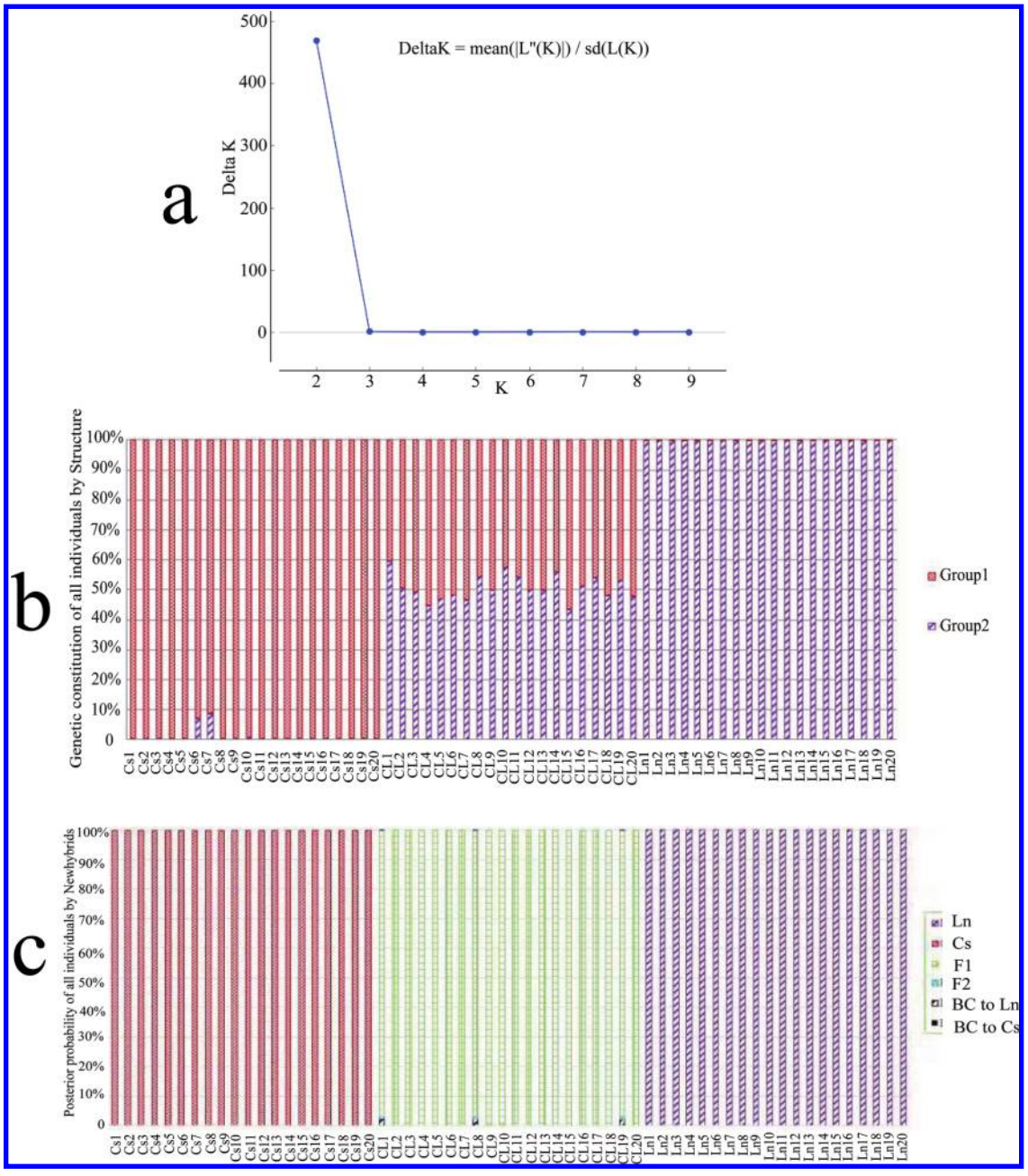
Fig. 6. Phylogenetic analyses of haplotype data for nrDNA region *ITS* for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as *L. nelumbifolia,* and *C. stenoglossum*. "*_L. nelumbifolia*_PH" represents the haplotype that included the individuals of *L. nelumbifolia* and the putative hybrid. "*_C. stenoglossum*_PH" represents the haplotype that included the individuals of *C. stenoglossum* and the putative hybrid. "*_L. nelumbifolia*" represents the haplotype that only included the individuals of *L. nelumbifolia*. "*_C. stenoglossum*" represents the haplotype that only included the individuals of *C. stenoglossum*. "_PH" represents the haplotype that only included individuals of the putative hybrid. Numbers above the branches indicate the supporting rate (>50%).



L. nelumbifolia at only 13 fixed sites and all indels, whereas these two individuals displayed identical nucleotide bases with *C. stenoglossum* at the remaining two fixed sites (Table 5).

Haplotype network analysis produced two highly divergent clusters, each comprising one *L. nelumbifolia* haplotype and two *C. stenoglossum* haplotypes, respectively (Fig. 2*e*). For the putative hybrid, nine individuals nested within the *L. nelumbifolia* cluster, whereas the remaining 11 individuals nested within the *C. stenoglossum* cluster (Table 4; Fig. 2*e*). The Bayesian tree of combined cpDNA suggested that haplotypes (C2 and C5) of *C. stenoglossum* formed one well-separated clade, whereas *Ligularia* species formed another well-separated clade (Fig. 8). Moreover, the private haplotype (C3) of the putative hybrid was closer to *L. nelumbifolia*, whereas the private

Fig. 7. The results of Δ*K* by performing 10 runs for each *K* ranging from 1 to10 (*a*). Genetic constitution analysis by Structure (*b*) and posterior probability of each individual belonging to parental or hybrid class by NewHybrids (*c*) for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as individuals of *L. nelumbifolia*, and *C. stenoglossum*, based on the sequence data from four nuclear regions. [Colour online.]



haplotype (C4) of the putative hybrid was closer to *L. tsangchanensis* and *L. virgaurea* (Table 4; Fig. 8).

Discussion

Molecular identification of natural hybridization between *L. nelumbifolia* and *C. stenoglossum*

No study on natural hybridization involving *Ligularia* and *Cremanthodium* has been reported to date. In this study, we validated the natural hybridization between *L. nelumbifolia* and *C. stenoglossum* based on morphological and molecular data. Each of the parental species, *L. nelumbifolia* and *C. stenoglossum*, had a distinct morphological appearance (Table 1; Fig. 1), and the putative hybrid displayed intermediate characters suggesting natural hybridization between these two species (Grant 1981). However, morphological evidence alone is limited for identifying natural hybrid and sometimes may impede the determination of hybrid status (Morrell and Rieseberg 1998; Marhold et al. 2002; Park et al. 2003). Morphological intermediacy may result from continuous variation and the high plasticity of many traits in

Table 5. Variable sites and indels among the putative hybrid *Ligularia nelumbifolia* and *Cremanthodium stenoglossum* at combined cpDNA.

	Polymorphic sites																								
	*psbA–trn*H				*trn*Q–5′*rps*16														*trn*K–*rps*16						
Individual	113	169	234	300	59	87	147	229	421	424	428–429	481–487	540	548	761	793	809–816	856–867	948	167–170	31	213	313	379	416–423	834	853
Ln1–20	–	A	T	C	C	A	A	C	T	T	▲	–	C	C	T	C	–	–	A	G	–	C	G	A	–	G	C
CL1, CL6–CL7, CL13, CL15–CL17	–	A	T	C	C	A	A	C	T	T	▲	–	C	C	T	C	–	–	A	G	–	C	G	A	–	G	C
CL11, CL12	–	T	T	C	C	A	A	C	T	T	▲	–	C	C	T	C	–	–	A	G	–	T	G	A	–	G	C
CL3–4, CL8–CL10, CL14, CL19–CL20	A	T	G	A	G	T	C	T	A	G	–	●	A	G	–	T	▶	◆	T	A	■	T	A	G	★	A	T
CL2, CL5, CL18	A	T	G	A	G	A	A	C	A	G	–	●	C	G	–	T	▶	◆	T	A	■	T	A	G	★	A	T
Cs2–Cs4, Cs19–Cs20	A	T	G	A	G	A	A	C	A	G	–	●	C	G	–	T	▶	◆	T	A	■	T	A	G	★	A	T
Cs1, Cs5–Cs18	A	T	G	A	G	T	C	T	A	G	–	●	A	G	–	T	▶	◆	T	A	■	T	A	G	★	A	T

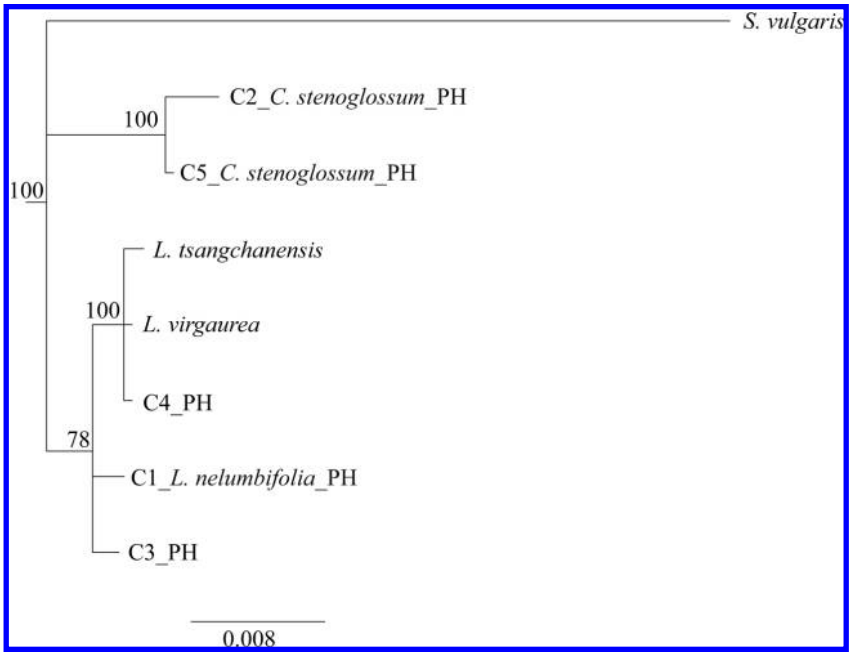
Note: –, deletions; ▲●▼◆■★, presence of insertion; ▲, GT (2bp); ●, TAGAAAA (7bp); ▼, AACAAAAA (8bp); ◆, ATTATCTATTTT (12 bp); ■, TAAA (4 bp); ★, TAAAAAGG (8 bp).

response to various environmental factors other than hybridization (Craft et al. 2002; Zhang et al. 2013). Therefore, a total of seven DNA regions were chosen to further verify the putative natural hybridization. Although two nrDNA regions (*B14* and *D30*) did not differentiate these two species well in the haplotype networks (Figs. 2*b* and 2*c*), all individuals of the putative hybrid displayed perfect chromatogram additivity in at least three nrDNA regions (Table 3), providing powerful evidence for natural hybridization between *L. nelumbifolia* and *C. stenoglossum*. In addition, for the two haplotypes of each putative hybrid individual, one nested within *L. nelumbifolia* clade and the other nested within *C. stenoglossum* clade in at least three nuclear Bayesian trees, providing further confirmation of hybridization (Table 4; Figs. 3, 5, and 6). Meanwhile, the Structure analysis clearly showed that *L. nelumbifolia* and *C. stenoglossum* formed two well-differentiated genetic groups, and all individuals identified as the putative hybrid in the field showed genetic admixture from both putative parental groups (Fig. 7*b*), which further supported the hypothesis of the hybrid origin of morphologically intermediate individuals. Moreover, although the private haplotype (C4) in combined cpDNA of the putative hybrid was closer to *L. tsangchanensis* and *L. virgaurea* (Table 5; Fig. 8), all of the nuclear Bayesian trees suggested that sympatric *L. tsangchanensis* and *L. virgaurea* were not apparently involved in the hybridization between *L. nelumbifolia* and *C. stenoglossum*.

Because cpDNA is maternally inherited in *Ligularia* (Zhang and Liu 2003), analyses of combined cpDNA indicated that the hybridization between *L. nelumbifolia* and *C. stenoglossum* was bidirectional. In other words, both parental species could act either as pollen donor or as pollen receptor during the process of hybridization. Notably, 11 unique haplotypes were observed in the putative hybrid using *B14*, *ITS*, and combined cpDNA (Figs. 2*b*, 2*d*, and 2*e*), which may be due to unidentified polymorphisms from the parental species, or new mutations in the hybrid individuals (Fan et al. 2014). Moreover, for the four variable sites at *trn*Q–5′*rps*16 region among *C. stenoglossum* individuals, the most reasonable explanation was that the four sites were the variation sites within the population of *C. stenoglossum*. Another explanation for this pattern may be the result of chloroplast capture due to hybridization between the two species (Smissen et al. 2004; Soejima et al. 2008).

The classification and phylogenetic relationships of *Ligularia* and *Cremanthodium* are still debated, pending a new circumscription and delimitation of these groups (Wulff 1944; Drury 1967; Jeffrey and Chen 1984; Liu 1989; Liu et al. 2001, 2006; Pelser et al. 2007, 2010; Chen et al. 2011; Ren et al. 2017). *Cremanthodium* was once considered to be the alpine derivative of *Ligularia* (Wulff 1944; Drury 1967), but some researchers accepted the status of *Cremanthodium* based on its nodding capitula and broadly

Fig. 8. Phylogenetic analyses of the haplotype data for combined cpDNA for the putative hybrid of the putative hybrid of *Ligularia nelumbifolia* and *Cremanthodium stenoglossum*, as well as *L. nelumbifolia* and *C. stenoglossum*. "*_L. nelumbifolia*_PH" represents the haplotype that included the individuals of *L. nelumbifolia* and the putative hybrid. "*_C. stenoglossum*_PH" represents the haplotype that included the individuals of *C. stenoglossum* and the putative hybrid. "*_L. nelumbifolia*" represents the haplotype that only included the individuals of *L. nelumbifolia*. "*_C. stenoglossum*" represents the haplotype that only included the individuals of *C. stenoglossum*. "_PH" represents the haplotype that only included individuals of the putative hybrid. Numbers above the branches indicate the supporting rate (>50%).



campanulate or hemispherical involucres (Jeffrey and Chen 1984; Liu 1989; Liu et al. 2001; Chen et al. 2011). However, molecular phylogeny studies revealed that *Ligularia* and *Cremanthodium* were closely related and their species were grouped together (Liu et al. 2006; Pelser et al. 2007, 2010; Ren et al. 2017). The occurrence of natural hybridization between *L. nelumbifolia* and *C. stenoglossum* implied that reproductive barriers may not be well-developed between these two genera and that they may not be highly genetically differentiated (Soltis and Soltis 1986). Thus, the occurrence of natural hybridization between *L. nelumbifolia* and *C. stenoglossum* could contribute to further understanding the relationship between *Ligularia* and *Cremanthodium*.

Factors that facilitate natural hybridization between *L. nelumbifolia* and *C. stenoglossum*

Bidirectional natural hybridization frequently occurs between *Ligularia* species because of geographical sympatry, overlapped flower periods, and common pollinators (Yu et al. 2011, 2014*a*; Ning et al. 2017; Zhang et al. 2017). However, natural hybridization between *Cremanthodium* species has never been reported. In this study, the co-occurrence of *L. nelumbifolia* and *C. stenoglossum* in the same territory allowed the possibility for their hybridization in the field. In addition, *L. nelumbifolia* and *C. stenoglossum* are both diploid with $2n = 58$ (Liu et al. 2001; Ren 2012), which may facilitate hybridization (Kou et al. 2017). Another factor favoring natural hybridization was the overlapping flowering periods. Coincidentally, *C. stenoglossum* bloomed from July to August and *L. nelumbifolia* bloomed from July to September (Liu 1989). Moreover, our field observations showed that nectar-collecting insects had no species preference between *L. nelumbifolia* and *C. stenoglossum* and shuttled frequently among flowers in the study area. Hence, nectar-collecting insects could transfer pollen from each other by chance, providing opportunity for the occurrence of hybridization between them. As a consequence, these factors provided ample opportunities for natural hybridization between *L. nelumbifolia* and *C. stenoglossum*.

Consequences of the hybridization between *L. nelumbifolia* and *C. stenoglossum*

All the putative hybrid individuals were identified as F_1 class with high posterior probabilities (>96%) based on the NewHybrids analysis (Fig. 7*c*), which concurred with the results of a natural intergeneric hybridization study between *Aster ageratoides* var. *scaberulus* and *Kalimeris indica* (Li 2006). However, current analysis of NewHybrids failed to explain some sequence variations in two *C. stenoglossum* individuals (Cs6 and Cs7), which might be the progenies of backcrossing based on *ITS* sequences (Table 2; Fig. 7*c*). Moreover, the results of Structure analysis also showed that these two individuals had a small genetic component of the *L. nelumbifolia* group (less than 10%), supporting the presence of introgression (Fig. 7*b*). On the other hand, in the *ITS* Bayesian tree, the two

individuals were also located in the *L. nelumbifolia* clade *and C. stenoglossum* clade, which also suggested the presence of introgression. Introgression has frequently presented in *Ligularia* and has resulted in complex relationships among *Ligularia* species by serving as a bridge to gene flow (Yu et al. 2011, 2014*a*; Zhang et al. 2017). During the authors' field investigations, hundreds of hybrid individuals were found, indicating relatively weak pre-zygotic barriers between *L. nelumbifolia* and *C. stenoglossum*, and that their zygotes could survive and develop into mature plants. The observed high frequency of F_1s among hybrid individuals could be explained by a strong post-zygotic barrier such as hybrid sterility at the hybrid stage (Dobzhansky 1937; Milne and Abbott 2008; Pellegrino et al. 2009; Guo et al. 2011). In other words, in this hybrid swarm, strong post-zygotic isolation limited the fates of hybrid individuals where later generations of hybrid individuals could not be detected. Under such circumstances, hybrids might represent a bridge for gene flow between *L. nelumbifolia* and *C. stenoglossum* and it might be impossible for the formation of hybrid species without asexual reproduction (Milne et al. 2003; Milne and Abbott 2008; Pellegrino et al. 2009; Guo et al. 2011).

Conclusions

This work presented molecular evidence for a natural hybridization between *L. nelumbifolia* and *C. stenoglossum* and confirmed bidirectional hybridization. The occurrence of natural hybridization between *L. nelumbifolia* and *C. stenoglossum* supported the view that *Ligularia* and *Cremanthodium* are closely related. Moreover, the high frequency of F_1 class and fairly fewer backcrossing individuals might prevent the formation of new species with hybrid origin. However, the mechanism of reproductive isolation in the process of hybridization is still unclear. Thus, future work should focus on the mechanisms of reproductive isolation between these two species.

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References

Abbott, R., Albach, D., Ansell, S., Arntzen, J.W., Baird, S.J.E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C.A., Buggs, R., Butlin, R.K., Dieckmann, U., Eroukhmanoff, F., Grill, A., Cahan, S.H., Hermansen, J.S., Hewitt, G., Hudson, A.G., Jiggins, C., Jones, J., Keller, B., Marczewski, T., Mallet, J., Martinez-Rodriguez, P., Möst, M., Mullen, S., Nichols, R., Nolte, A.W., Parisod, C., Pfennig, K., Rice, A.M., Ritchie, M.G., Seifert, B., Smadja, C.M., Stelkens, R., Szymura, J.M., Väinölä, R., Wolf, J.B.W., and Zinner, D. 2013. Hybridization and speciation. J. Evol. Biol. **26**(2): 229–246. doi:10.1111/j.1420-9101.2012.02599.x. PMID:23323997.

Allan, H.H. 1939. Remarks on × *Leucoraoulia*. Trans. R. Soc. N.Z. **68**: 457–461.

Anderson, E. 1949. Introgressive hybridization. John Wiley & Sons, New York.

Anderson, E.C., and Thompson, E.A. 2002. A model-based method for identifying species hybrids using multilocus genetic data. Genetics, **160**(3): 1217–1229. PMID:11901135.

Arnold, M.L. 1997. Natural hybridization and evolution. Oxford University Press, New York.

Baack, E., Melo, M.C., Rieseberg, L.H., and Ortiz-Barrientos, D. 2015. The origins of reproductive isolation in plants. New Phytol. **207**(4): 968–984. doi:10.1111/nph.13424. PMID:25944305.

Bandelt, H.J., Forster, P., and Röhl, A. 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. **16**(1): 37–48. doi:10.1093/oxfordjournals.molbev.a026036. PMID:10331250.

Bleeker, W., Schmitz, U., and Ristow, M. 2007. Interspecific hybridisation between alien and native plant species in Germany and its consequences for native biodiversity. Biol. Conserv. **137**(2): 248–253. doi:10.1016/j.biocon.2007.02.004.

Brock, M.T. 2009. Prezygotic barriers to gene flow between *Taraxacum ceratophorum* and the invasive *Taraxacum officinale* (Asteraceae). Oecologia, **161**(2): 241–251. doi:10.1007/s00442-009-1383-0. PMID:19504127.

Calvo, J., Álvarez, I., Aedo, C., and Pelser, P.B. 2013. A phylogenetic analysis and new delimitation of *Senecio* sect. *Crociseris* (Compositae: Senecioneae), with evidence of intergeneric hybridization. Taxon, **62**(1): 127–140.

Chapman, M.A., Chang, J.C., Weisman, D., Kesseli, R.V., and Burke, J.M. 2007. Universal markers for comparative mapping and phylogenetic analysis in the Asteraceae (Compositae). Theor. Appl. Genet. **115**(6): 747–755. doi:10.1007/s00122-007-0605-2. PMID:17634914.

Chen, Y.L., Liu, S.W., Liu, Y., Yang, Q.E., Nordenstam, B., Illarionova, I.D., Jeffrey, C., Koyama, H., and Vincent, L. 2011. Asteraceae-senecioneae. *In* Flora of China. *Edited by* Z.Y. Wu, P.H. Raven, and D.Y. Hong. Science Press, and Missouri Botanical Garden Press, St. Louis/Beijing. pp. 371–544.

Craft, K.J., Ashley, M.V., and Koenig, W.D. 2002. Limited hybridization between *Quercus lobata* and *Quercus douglasii* (Fagaceae) in a mixed stand in central coastal California. Am. J. Bot. **89**(11): 1792–1798. doi:10.3732/ajb.89.11.1792. PMID:21665607.

Dai, S.P., Wu, W., Zhang, R.S., Liu, T., Chen, Y.Y., Shi, S.H., and Zhou, R.C. 2012. Molecular evidence for hybrid origin of *Melastoma intermedium*. Biochem. Syst. Ecol. **41**: 136–141. doi:10.1016/j.bse.2011.12.010.

Darriba, D., Taboada, G.L., Doallo, R., and Posada, D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods, **9**(8): 772–772. doi:10.1038/nmeth.2109. PMID:22847109.

Dobzhansky, T.G. 1937. Genetics and the origin of species. Columbia University Press, New York.

Doyle, J.J., and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**: 11–15.

Drury, D.G. 1967. A taxonomic study of Compositae with special reference to *Senecio*. Ph.D. thesis, Southampton University, Southampton, UK.

Evanno, G., Regnaut, S., and Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. **14**(8): 2611–2620. doi:10.1111/j.1365-294X.2005.02553.x. PMID:15969739.

Fan, Q., Chen, S.F., Li, M.W., Guo, W., Jing, H.J., Wu, W., Zhou, R.C., and Liao, W.B. 2014. Molecular evidence for natural hybridization between wild loquat (*Eriobotrya japonica*) and its relative *E. prinoides*. BMC Plant Biol. **14**(1): 275. doi:10.1186/s12870-014-0275-6. PMID:25300306.

Fehrer, J., Gemeinholzer, B., Chrtek, J.J., and Bräutigam, S. 2007. Incongruent plastid and nuclear DNA phylogenies reveal ancient intergeneric hybridization in *Pilosella* hawkweeds (*Hieracium*, Cichorieae, Asteraceae). Mol. Phylogenet. Evol. **42**(2): 347–361. doi:10.1016/j.ympev.2006.07.004. PMID:16949310.

Galbany-Casals, M., Carnicero-Campmany, P., Blanco-Moreno, J.M., and Smissen, R.D. 2012. Morphological and genetic evidence of contemporary intersectional hybridisation in Mediterranean *Helichrysum* (Asteraceae, Gnaphalieae). Plant Biol. **14**(5): 789–800. doi:10.1111/j.1438-8677.2012.00568.x. PMID:22443112.

Grant, V. 1981. Plant speciation. Columbia University Press, New York.

Guo, M.M., Zhou, R.C., Huang, Y.L., Ouyang, J.H., and Shi, S.H. 2011. Molecular confirmation of natural hybridization between *Lumnitzera racemosa* and *Lumnitzera littorea*. Aquat. Bot. **95**(1): 59–64. doi:10.1016/j.aquabot.2011.03.001.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41**(41): 95–98.

Harrison, R.G. 1990. Hybrid zones: windows on evolutionary process. Oxf. Surv. Evol. Biol. **7**: 69–128.

Hubisz, M.J., Falush, D., Stephens, M., and Pritchard, J.K. 2009. Inferring weak population structure with the assistance of sample group information. Mol. Ecol. Resour. **9**(5): 1322–1332. doi:10.1111/j.1755-0998.2009.02591.x. PMID:21564903.

Jeffrey, C., and Chen, Y.L. 1984. Taxonomic studies on the tribe Senecioneae (Compositae) of Eastern Asia. Kew Bull. **39**(2): 205–446. doi:10.2307/4110124.

Kirk, H., Máčel, M., Klinkhamer, P.G., and Vrieling, K. 2004. Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: molecular and chemical evidence. Mol. Ecol. **13**(8): 2267–2274. doi:10.1111/j.1365-294X.2004.02235.x. PMID:15245400.

Knope, M.L., Pender, R.J., Crawford, D.J., and Wieczorek, A.M. 2013. Invasive congeners are unlikely to hybridize with native Hawaiian *Bidens* (Asteraceae). Am. J. Bot. **100**(6): 1221–1226. doi:10.3732/ajb.1300018. PMID:23733530.

Kou, Y.X., Xiao, K., Lai, X.R., Wang, Y.J., and Zhang, Z.Y. 2017. Natural hybridization between *Torreya jackii* and *T. grandis* (Taxaceae) in southeast China. J. Syst. Evol. **55**(1): 25–33. doi:10.1111/jse.12217.

Koutecký, P., Badurová, T., Štech, M., Košnar, J., and Karásek, J. 2011. Hybridization between diploid *Centaurea pseudophrygia* and tetraploid *C. jacea* (Asteraceae): the role of mixed pollination, unreduced gametes, and mentor effects. Biol. J. Linn. Soc. **104**(1): 93–106. doi:10.1111/j.1095-8312.2011.01707.x.

Levin, D.A., FranciscoOrtega, J., and Jansen, R.K. 1996. Hybridization and the extinction of rare plant species. Conserv. Biol. **10**(1): 10–16. doi:10.1046/j.1523-1739.1996.10010010.x.

Lexer, C., Fay, M.F., Joseph, J.A., Nica, M.S., and Heinze, B. 2005. Barrier to gene flow between two ecologically divergent *Populus* species, *P. alba* (white poplar) and *P. tremula* (European aspen): the role of ecology and life history in gene introgression. Mol. Ecol. **14**(4): 1045–1057. doi:10.1111/j.1365-294X.2005.02469.x. PMID:15773935.

Li, M.W., Chen, S.F., Zhou, R.C., Fan, Q., Li, F.F., and Liao, W.B. 2017. Molecular evidence for natural hybridization between *Cotoneaster dielsianus* and *C. glaucophyllus*. Front. Plant Sci. **8**: 704. doi:10.3389/fpls.2017.00704. PMID:28536587.

Li, W.P. 2006. Natural hybridization between *Aster ageratoides* var. *scaberulus* and *Kalimeris indica* (Asteraceae): evidence from morphology, karyotype, and ITS sequences. Bot. Stud. **47**(2): 191–197.

Liao, R.L., Ma, Y.P., Gong, W.C., Chen, G., Sun, W.B., Zhou, R.C., and Marczewski, T. 2015. Natural hybridization and asymmetric introgression at the distribution margin of two *Buddleja* species with a large overlap. BMC Plant Biol. **15**(1): 146. doi:10.1186/s12870-015-0539-9. PMID:26081944.

Lipman, M.J., Chester, M., Soltis, P.S., and Soltis, D.E. 2013. Natural hybrids between *Tragopogon mirus* and *T. miscellus* (Asteraceae): a new perspective on karyotypic changes following hybridization at the polyploid level. Am. J. Bot. **100**(10): 2016–2022. doi:10.3732/ajb.1300036. PMID:24088339.

Liu, J.Q., Liu, S.W., Ho, T.N., and Lu, A.M. 2001. Karyological studies on the Sino-Himalayan genus, *Cremanthodium* (Asteraceae: Senecioneae). Bot. J. Linn. Soc. **135**(2): 107–112. doi:10.1111/j.1095-8339.2001.tb01085.x.

Liu, J.Q., Wang, Y.J., Wang, A.L., Hideaki, O., and Abbott, R.J. 2006. Radiation and diversification within the *Ligularia-Cremanthodium-Parasenecio* complex (Asteraceae) triggered by uplift of the Qinghai–Tibetan Plateau. Mol. Phylogenet. Evol. **38**(1): 31–49. doi:10.1016/j.ympev.2005.09.010. PMID:16290033.

Liu, S.W. 1989. Compositae-Senecioneae. *In* Flora Reipublicae Popularis Sinicae. *Edited by* Y. Ling and S.W. Liu. Science Press, Beijing. pp. 4–115.

Liu, S.W., Deng, D.S., and Liu, J.Q. 1994. Origin, evolution and distribution of *Ligularia* Cass.(Compositae). Acta Phytotaxon. Sin. **32**(6): 514–524.

Liu, T., Chen, Y.Y., Chao, L.F., Wang, S.Q., Wu, W., Dai, S.P., Wang, F., Fan, Q., and Zhou, R.C. 2014. Extensive hybridization and introgression between *Melastoma candidum* and *M. sanguineum*. PLoS ONE, **9**(5): e96680. doi:10.1371/journal.pone.0096680. PMID:24797959.

López, M.G., Wulff, A.F., and Xifreda, C.C. 2008. Natural hybrids in *Senecio* (Asteraceae): new records from Argentina. Plant Biosyst. **142**(1): 186–191. doi:10.1080/11263500701873075.

Ma, Y.P., Xie, W.J., Tian, X.L., Sun, W.B., Wu, Z.K., and Milne, R. 2014. Unidirectional hybridization and reproductive barriers between two heterostylous primrose species in north-west Yunnan, China. Ann. Bot. **113**(5): 763–775. doi:10.1093/aob/mct312. PMID:24492637.

Ma, Y.P., Xie, W.J., Sun, W.B., and Marczewski, T. 2016. Strong reproductive isolation despite occasional hybridization between a widely distributed and a narrow endemic *Rhododendron* species. Sci. Rep. **6**: 19146. doi:10.1038/srep19146. PMID:26751844.

Mameli, G., López-Alvarado, J., Farris, E., Susanna, A., Filigheddu, R., and Garcia-Jacas, N. 2014. The role of parental and hybrid species in multiple introgression events: evidence of homoploid hybrid speciation in *Centaurea* (Cardueae, Asteraceae). Bot. J. Linn. Soc. **175**(3): 453–467. doi:10.1111/boj.12177.

Marhold, K., Lihova, J., Perny, M., Grupe, R., and Neuffer, B. 2002. Natural hybridization in *Cardamine* (Brassicaceae) in the Pyrenees: evidence from morphological and molecular data. Bot. J. Linn. Soc. **139**(3): 275–294. doi:10.1046/j.1095-8339.2002.00066.x.

Mckenzie, R.J., Ward, J.M., Lovis, J.D., and Breitwieser, I. 2004. Morphological evidence for natural intergeneric hybridization in the New Zealand Gnaphalieae (Compositae): *Anaphalioides bellidioides* × *Ewartia sinclairii*. Bot. J. Linn. Soc. **145**(1): 59–75. doi:10.1111/j.1095-8339.2003.00282.x.

Milne, R.I., and Abbott, R.J. 2008. Reproductive isolation among two interfertile *Rhododendron* species: low frequency of post-F_1 hybrid genotypes in alpine hybrid zones. Mol. Ecol. **17**(4): 1108–1121. doi:10.1111/j.1365-294X.2007.03643.x. PMID: 18261051.

Milne, R.I., Terzioglu, S., and Abbott, R.J. 2003. A hybrid zone dominated by fertile F_1s: maintenance of species barriers in *Rhododendron*. Mol. Ecol. **12**(10): 2719–2729. doi:10.1046/j.1365-294X.2003.01942.x. PMID:12969475.

Mitsui, Y., Nomura, N., Isagi, Y., Tobe, H., and Setoguchi, H. 2011. Ecological barriers to gene flow between riparian and forest species of *Ainsliaea* (Asteraceae). Evolution, **65**(2): 335–349. doi:10.1111/j.1558-5646.2010.01129.x. PMID:20840597.

Morrell, P.L., and Rieseberg, L.H. 1998. Molecular tests of the proposed diploid hybrid origin of *Gilia achilleifolia* (Polemoniaceae). Am. J. Bot. **85**(10): 1439–1453. doi:10.2307/2446401. PMID:21684896.

Ning, H., Yu, J.J., and Gong, X. 2017. Bidirectional natural hybridization between sympatric *Ligularia vellerea* and *L. subspicata*. Plant Divers. **39**(4): 214–220. doi:10.1016/j.pld.2017.07.001. PMID:30159514.

Nolte, A.W., and Tautz, D. 2010. Understanding the onset of hybrid speciation. Trends Genet. **26**(2): 54–58. doi:10.1016/j.tig.2009.12.001. PMID:20044166.

Oberprieler, C., Bog, M., and Berchtold, B. 2016. Herbivory and fitness components in an introgressive hybrid swarm of *Senecio hercynicus* and *S. ovatus* (Compositae, Senecioneae). Flora, **220**: 117–124. doi:10.1016/j.flora.2016.03.001.

Pan, Y.Z., Shi, S.H., Gong, X., and Kuroda, C. 2008. A natural hybrid between *Ligularia paradoxa* and *L. duciformis* (Asteraceae, Senecioneae) from Yunnan, China. Ann. Mo. Bot. Gard. **95**(3): 487–494. doi:10.3417/2006034.

Pandit, M.K., Pocock, M.J.O., and Kunin, W.E. 2011. Ploidy influences rarity and invasiveness in plants. J. Ecol. **99**(5): 1108–1115. doi:10.1111/j.1365-2745.2011.01838.x.

Park, K.R., Pak, J.H., and Seo, B.B. 2003. Allozyme variation in *Paraixeris*: a test for the diploid hybrid origin of *Paraixeris koidzumiana* (Compositae). Bot. Bull. Acad. Sin. **44**: 113–122.

Pellegrino, G., Bellusci, F., and Musacchio, A. 2009. Genetic integrity of sympatric hybridising plant species: the case of *Orchis italica* and *O. anthropophora*. Plant Biol. **11**(3): 434–441. doi:10.1111/j.1438-8677.2008.00135.x. PMID:19470114.

Pelser, P.B., Nordenstam, B., Kadereit, J.W., and Watson, L.E. 2007. An ITS phylogeny of tribe Senecioneae (Asteraceae) and a new delimitation of *Senecio* L. Taxon, **56**(4): 1077–1104. doi:10.2307/25065905.

Pelser, P.B., Kennedy, A.H., Tepe, E.J., Shidler, J.B., Nordenstam, B., Kadereit, J.W., and Watson, L.E. 2010. Patterns and causes of incongruence between plastid and nuclear Senecioneae (Asteraceae) phylogenies. Am. J. Bot. **97**(5): 856–873. doi:10.3732/ajb.0900287. PMID:21622451.

Pisanu, S., Mameli, G., Farris, E., Binelli, G., and Filigheddu, R. 2011. A natural homoploid hybrid between *Centaurea horrida* and *Centaurea filiformis* (Asteraceae) as revealed by morphological and genetic traits. Folia Geobot. **46**(1): 69–86. doi:10.1007/s12224-010-9085-2.

Ren, C. 2012. Cytotaxonomy, molecular systematics, and biogeography of the *Ligularia-Cremanthodium-Parasenecio* complex (Asteraceae-Senecioneae). Ph.D. thesis, University of Chinese Academy of Sciences.

Ren, C., Hong, Y., Wang, L., and Yang, Q.E. 2017. Generic recircumscription of *Parasenecio* (Asteraceae: Senecioneae) based on nuclear ribosomal and plastid DNA sequences, with descriptions of two new genera. Bot. J. Linn. Soc. **184**(4): 418–443. doi:10.1093/botlinnean/box034.

Rieseberg, L.H. 1995. The role of hybridization in evolution: old wine in new skins. Am. J. Bot. **82**(7): 944–953. doi:10.1002/j.1537-2197.1995.tb15711.x.

Rieseberg, L.H., and Carney, S.E. 1998. Plant hybridization. New Phytol. **140**(4): 599–624. doi:10.1046/j.1469-8137.1998.00315.x.

Rieseberg, L.H., Carter, R., and Zona, S. 1990. Molecular tests of the hypothesized hybrid origin of two diploid *Helianthus* species (Asteraceae). Evolution, **44**(6): 1498–1511. doi:10.1111/j.1558-5646.1990.tb03841.x. PMID:28564296.

Rieseberg, L.H., Ellstrand, N.C., and Arnold, M. 1993. What can molecular and morphological markers tell us about plant hybridization? Crit. Rev. Plant Sci. **12**(3): 213–241. doi:10.1080/07352689309701902.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., and Huelsenbeck, J.P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. **61**(3): 539–542. doi:10.1093/sysbio/sys029. PMID:22357727.

Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., and Rozas, R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics, **19**(18): 2496–2497. doi:10.1093/bioinformatics/btg359. PMID:14668244.

Sang, T., Crawford, D., and Stuessy, T. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Am. J. Bot. **84**(8): 1120. doi:10.2307/2446155. PMID:21708667.

Seehausen, O. 2004. Hybridization and adaptive radiation. Trends Ecol. Evol. **19**(4): 198–207. doi:10.1016/j.tree.2004.01.003. PMID:16701254.

Shaw, J., Lickey, E.B., Schilling, E.E., and Small, R.L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am. J. Bot. **94**(3): 275–288. doi:10.3732/ajb.94.3.275. PMID:21636401.

Smissen, R.D., Breitwieser, I., and Ward, J.M. 2004. Phylogenetic implications of trans-specific chloroplast DNA sequence polymorphism in New Zealand Gnaphalieae (Asteraceae). Plant Syst. Evol. **249**(1): 37–53. doi:10.1007/s00606-004-0209-0.

Smissen, R.D., Breitwieser, I., and Ward, J.M. 2007. Genetic characterization of hybridization between the New Zealand everlastings *Helichrysum lanceolatum* and *Anaphalioides bellidioides* (Asteraceae: Gnaphalieae). Bot. J. Linn. Soc. **154**(1): 89–98. doi:10.1111/j.1095-8339.2007.00632.x.

Smissen, R.D., Thorsen, M.J., Breitwieser, I., and Ward, J.M. 2015. DNA sequence analysis confirms the identity of the intergeneric hybrid *Argyrotegium mackayi* × *Leucogenes leontopodium* (Asteraceae: Gnaphalieae). N.Z. J. Bot. **53**(4): 210–215. doi:10.1080/0028825X.2015.1093000.

Soejima, A., Wen, J., Zapata, M., and Dillon, M.O. 2008. Phylogeny and putative hybridization in the subtribe Paranepheliinae (Liabeae, Asteraceae), implications for classification, biogeography, and Andean orogeny. J. Syst. Evol. **46**(3): 375–390.

Soltis, D.E., and Soltis, P.S. 1986. Intergeneric hybridization between *Conimitella williamsii* and *Mitella stauropetala* (Saxifragaceae). Syst. Bot. **11**(2): 293–297. doi:10.2307/2419118.

Stebbins, G.L. 1950. Variation and evolution in plants. Columbia University Press, New York.

Stephens, M., and Donnelly, P. 2003. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. Am. J. Hum. Genet. **73**(5): 1162–1169. doi:10.1086/379378. PMID:14574645.

Stephens, M., Smith, N.J., and Donnelly, P. 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. **68**(4): 978–989. doi:10.1086/319501. PMID:11254454.

Sun, M., and Lo, E.Y.Y. 2011. Genomic markers reveal introgressive hybridization in the Indo-West Pacific mangroves: a case study. PLoS ONE, **6**(5): e19671. doi:10.1371/journal.pone.0019671. PMID:21589927.

Swofford, D.L. 2003. PAUP*: phylogenetic analysis using parsimony, version 4.0 b10. Sinauer Associates, Sunderland, Mass.

Todesco, M., Pascual, M.A., Owens, G.L., Ostevik, K.L., Moyers, B.T., Hübner, S., Heredia, S.M., Hahn, M.A., Caseys, C., and Bock, D.G. 2016. Hybridization and extinction. Evol. Appl. **9**(7): 892–908. doi:10.1111/eva.12367. PMID:27468307.

White, T.J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, **18**(1): 315–322.

Wu, W., Zhou, R.C., Ni, G.Y., Shen, H., and Ge, X.J. 2013. Is a new invasive herb emerging? Molecular confirmation and preliminary evaluation of natural hybridization between the invasive *Sphagneticola trilobata* (Asteraceae) and its native congener *S. calendulacea* in South China. Biol. Invasions, **15**(1): 75–88. doi:10.1007/s10530-012-0269-y.

Wulff, E.V. 1944. Historical plant geography: history of the world flora. Chronica Botanica, Waltham, Mass.

Xie, Y.P., Zhu, X.F., Ma, Y.P., Zhao, J.L., Li, L., and Li, Q.J. 2017. Natural hybridization and reproductive isolation between two *Primula* species. J. Integr. Plant Biol. **59**(8): 526–530. doi:10.1111/jipb.12546. PMID:28429397.

Yan, L.J., Burgess, K.S., Milne, R., Fu, C.N., Li, D.Z., and Gao, L.M. 2017. Asymmetrical natural hybridization varies among hybrid swarms between two diploid *Rhododendron* species. Ann. Bot. **120**(1): 51–61. doi:10.1093/aob/mcx039. PMID:28444136.

Yu, J.J., Kuroda, C., and Gong, X. 2011. Natural hybridization and introgression in sympatric *Ligularia* species (Asteraceae, Senecioneae). J. Syst. Evol. **49**(5): 438–448. doi:10.1111/j.1759-6831.2011.00150.x.

Yu, J.J., Kuroda, C., and Gong, X. 2014*a*. Natural hybridization and introgression between *Ligularia cymbulifera* and *L. tongolensis* (Asteraceae, Senecioneae) in four different locations. PLoS ONE, **9**(12): e115167. PMID:25551379.

Yu, J.J., Pan, L., Pan, Y.Z., and Gong, X. 2014*b*. Natural hybrids between *Ligularia vellerea* and *L. subspicata* (Asteraceae: Senecioneae). Plant Divers. Resour. **36**: 219–226.

Zhang, Q., and Liu, Y. 2003. Examination of the cytoplasmic DNA in male reproductive cells to determine the potential for cytoplasmic inheritance in 295 angiosperm species. Plant Cell Physiol. **44**(9): 941–951. doi:10.1093/pcp/pcg121. PMID: 14519776.

Zhang, N.N., Yu, J.J., Wang, Y.H., and Gong, X. 2018. Molecular evidence for asymmetric hybridization in three closely related sympatric species. AoB Plants, **10**(1): ply011–ply011. PMID:29492242.

Zhang, R.S., Liu, T., Wu, W., Li, Y.Q., Chao, L.F., Huang, L.S., Huang, Y.L., Shi, S.H., and Zhou, R.C. 2013. Molecular evidence for natural hybridization in the mangrove fern genus *Acrostichum*. BMC Plant Biol. **13**(1): 74. doi:10.1186/1471-2229-13-74. PMID:23634934.

Zhang, R., Gong, X., and Folk, R. 2017. Evidence for continual hybridization rather than hybrid speciation between *Ligularia duciformis* and *L. paradoxa* (Asteraceae). PeerJ, **5**: e3884. PMID:29038755.

Zhang, W.Y., Kuo, L.Y., Li, F.W., Wang, C.N., and Chiou, W.L. 2014. The hybrid origin of *Adiantum meishanianum* (Pteridaceae): a rare and endemic species in Taiwan. Syst. Bot. **39**(4): 1034–1041. doi:10.1600/036364414X682616.

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