Bidirectional natural hybridization between sympatric *Ligularia vellerea* and *L. subspicata*

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ABSTRACT

Natural hybridization has been regarded as a crucial pathway of speciation and provides the raw materials for the evolution of biodiversity. The interspecific natural hybridization of the genus *Ligularia* Cass. is universal and has been considered to be an important factor driving the high diversity of *Ligularia* species in the Hengduan Mountains, China. Although the natural hybridization between *L. vellerea* and *L. subspicata* was reported previously, the direction of hybridization was uncertain due to the limitation of sampling. Thus, in this study, we sampled more individuals and increased two fragments of chloroplast DNA on the basis of the previous study to further verify the natural hybridization between *L. vellerea* and *L. subspicata* and confirm the direction of hybridization. Based on DNA sequences (*atp*B-*rbc*L, *trn*L-*rpl*32, *trn*Q-5'*rps*16, and nuclear ribosomal internal transcribed spacer region) data, we concluded that putative hybrids were primary products of hybridization between *L. vellerea* and *L. subspicata* and the hybridization was bidirectional. Moreover, sympatric *L. tongolensis* was not apparently involved in the hybridization. Surprisingly, some pure *L. subspicata* individuals showed the disaccordance between morphology and DNA data, which might indicate that introgression occurs between *L. vellerea* and *L. subspicata*.

1. Introduction

Natural hybridization has been regarded as a crucial pathway of speciation and provides the raw materials for the evolution of biodiversity (Harrison, 1990; Arnold, 1997). To date, more and more botanists have turned their attention to natural hybridization due to the importance of hybridization in plant evolution (Rieseberg, 1997; Rieseberg et al., 2000; Abbott et al., 2008, 2010; Soltis and Soltis, 2009; Chase et al., 2010). Hybrids can occupy new ecological niches that could not be occupied by the parental species and form new species eventually by accumulation of genetic variation and/or ecological factors (Arnold, 1997; Burke and Arnold, 2001). Homoploid hybrid speciation (Mallet, 2007; Abbott et al., 2010) and allopolyploid speciation (Tate et al., 2005; Arnold, 2006) are two forms of hybrid speciation. Allopolyploid speciation can lead to rapidly reproductive isolation from parental lineages by doubled chromosome and produce new species (Rieseberg and Willis, 2007; Soltis and Soltis, 1999), whereas homoploid hybrid speciation occurs without changes in chromosome numbers (Mallet, 2007; Abbott et al., 2010). Thus, allopolyploid speciation was considered more common than homoploid hybrid speciation in flowering plant (Arnold, 1992; Abbott et al., 2010). However, hybridization events do not always lead to the origin of new species (Arnold, 1997). Oppositely, some hybridization events may form hybrid swarms or hybrid zones depending on the extent of introgression (Nolte and Tautz, 2010). Introgression is a fairly widespread and significant consequence of hybridization (Buerkle et al., 2000; Rieseberg et al., 2003; Martin et al., 2006). If introgression is strong enough, gene flow will occur across two parental species through hybrids and hybrid populations as bridges, thus introgression can play a key role in adaptive evolution (Arnold, 1992; Rieseberg and Wendel, 1993).

Natural hybridization is common in *Ligularia* species and has accelerated the evolutionary process of *Ligularia* species (Pan et al., 2008; Saito et al., 2011; Yu et al., 2011, 2014a, b). Pan et al. (2008) confirmed a hybrid species between *L. paradoxa* Hand.-Mazz. and

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L. duciformis (C. Winkl.) Hand.-Mazz. based on a comprehensive study. Moreover, Saito et al. (2011) presumed that *L. lamarum* (Diels) C.C. Chang and *L. subspicata* (Bur. and Franch.) Hand.-Mazz. hybridize with other *Ligularia* species in certain populations according to the examination of chemical similarities. Yu et al. (2011, 2014a, b) demonstrated the occurrence of natural hybridization between sympatric distributions of *L. vellerea* (Franch.) Hand.-Mazz. and *L. subspicata*, between sympatric distributions of *L. subspicata* and *L. nelumbifolia* (Bur. and Franch.) Hand.-Mazz., and between sympatric distributions of *L. cymbulifera* (W.W. Smith) Hand.-Mazz. and *L. tongolensis* (Franch.) Hand.-Mazz. based on both morphological traits and molecular data (e.g. chloroplast DNA (cpDNA) and internal transcribed spacer (ITS) sequence, ISSR markers, SSR markers). Particularly, most of the current studies have demonstrated that the directions of natural hybridization among most *Ligularia* species are bidirectional (Yu et al., 2011, 2014b). Although the natural hybridization between *L. vellerea* and *L. subspicata* was preliminarily confirmed based on both morphological traits and molecular data, the direction of natural hybridization was uncertain due to the limitation of sampling (only 5 putative hybrids were sampled) (Yu et al., 2014a). In the present study, we sampled 16 putative hybrids from the same mixed population for confirming natural hybridization again and elucidating the pattern of hybridization with the hypothesis of bidirectional hybridization. Moreover, *L. tongolensis* also grows in the mixed population and has overlapped flowering period with the two putative parental species. In order to investigate whether *L. tongolensis* participated in the process of hybridization, the individuals of *L. tongolensis* were also sampled and studied.

Molecular markers have been widely applied for natural hybridization studies (Rieseberg and Ellstrand, 1993). Although multiple molecular markers have been used for identifying plant hybridization since last century (Heiser, 1947; Anderson, 1949; Rieseberg and Brunsfeld, 1992), direct sequencing of DNA have been proven to be the most direct method to detect plant hybridization (Rieseberg and Ellstrand, 1993; Pan et al., 2008; Yu et al., 2011, 2014a, b; Liao et al., 2015). Numerous hybridization events have been verified based on sequences of biparentally inherited nuclear ribosomal DNA (nrDNA), and it has been feasible to confirm the paternal and maternal parents of hybrids according to uniparentally inherited DNA (e.g. Yu et al., 2011, 2014a, b; Yan et al., 2013; Zhang et al., 2014). Here, we investigated the natural hybridization between two morphologically distinct species of *L. vellerea* and *L. subspicata* using ITS region of nrDNA and three cpDNA fragments (*trn*L-*rpl*32, *trn*Q-5'*rps*16 and *atp*B-*rbc*L).

2. Materials and methods

2.1. Experimental materials

Plant materials were collected from Daxue Mountain, Shangri-La County, Yunnan, China, at 99°49.818′E and 28°33.722′N. Leaves of 85 individuals were collected and dried in silica gel in field for DNA extraction and the number of all samples was listed in Table 1. Leaf shape, stem and inflorescence type were primarily diagnostic characters in the field and the main morphological differences among *L. vellerea*, *L. subspicata*, *L. tongolensis* and putative hybrids were listed in Table 2. The voucher specimens were deposited in Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN), with accession numbers PG14081401-PG14081404.

2.2. Experimental methods

Total genomic DNA was extracted from the silica-dried leaf tissues using the CTAB method (Doyle and Doyle, 1987) with minor

Table 1
The number of four taxa.

Taxa	No.
Ligularia vellerea	V1, V2, V3, V4, V5, V6, V7, V8, V9, V10, V11, V12, V13, V14, V15, V16, V17, V18, V19, V20, V21, V22, V23, V24, V25
Putative hybrids	H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16
L. subspicata	S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20, S21, S22, S23, S24
L. tongolensis	T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15, T16, T17, T18, T19, T20

modifications. Because we excluded the probability that *L. tongolensis* participated in the hybridization in the preliminary experiment (i.e., *L. tongolensis* was completely separated with other two species and putative hybrids based on the ITS sequences), we only selected 8 individuals (Table 1) of *L. tongolensis* in subsequent sequencing experiment. The ITS region of all sampled individuals was amplified using primers ITS4 and ITS5 (White et al., 1990). PCR was conducted in a total reaction volume of 20 μL, containing 12.4 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), 0.8 μL BSA (20 mg/mL), 0.3 μL Taq polymerase (5 units/μL), 0.5 μL each primer (10 μmol/L) and 1.5 μL template DNA (20−60 ng). The amplification was performed as following conditions: 1 cycle, 94 °C, 5 min; 36 cycles, 94 °C, 45 s; 56 °C, 45 s; 72 °C, 50 s, and a final extension of 1 cycle, 72 °C, 8 min. The purified PCR products were sequenced by sequencing company with an ABI 3730 automated sequencer. Direct sequencing was successful for *L. vellerea* and *L. tongolensis*, but it produced chimeric or unreadable peaks in the chromatograms for putative hybrids and *L. subspicata*. Therefore, cloning was carried out for all putative hybrids and all *L. subspicata* individuals. Purified PCR products were cloned into plasmids using the pUM-T vector system (Bioteke Corporation, Beijing, China). Four to ten positive clones were selected for each amplification product and cultured to isolate plasmids. Positive clones with inserts of the correct size were confirmed by colony PCR. Plasmids with correct inserts were sequenced using universal M13F/M13R primers. Two cpDNA fragments were amplified using the following universal primers: *trn*L-*rpl*32, *trn*Q-5'*rps*16 (Shaw et al., 2007). PCR was conducted in a reaction 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 units/μL), 0.35 μL each primer (10 μmol/L) and 1.5 μL template DNA (20−60 ng). The amplification was performed as following conditions: 1 cycle, 80 °C, 5 min; 30 cycles, 94 °C, 45 s; 53 °C, 45 s; 65 °C, 50 s, and a final extension of 1 cycle, 65 °C, 7 min. The third cpDNA fragment was amplified using universal primers *atp*B-*rbc*L (Chiang et al., 1998). PCR was conducted in a reaction volume of 20 μL, containing 10.8 μL ultrapure water, 2.0 μL 10 × PCR Buffer (Mg^{2+} free), 2.0 μL MgCl$_2$ (25 mmol/L), 1.6 μL dNTP (2.5 mmol/L), 1 μL DMSO (20 mg/mL), 0.3 μL Taq polymerase (5 units/μL), 0.4 μL each primer (10 μmol/L) and 1.5 μL template DNA (20−60 ng). The amplification was performed as following conditions: 1 cycle, 94 °C, 3 min; 36 cycles, 94 °C, 45 s; 53 °C, 1 min, 65 °C, 90 s, and a final extension of 1 cycle, 65 °C, 7 min. The PCR products of *trn*L-*rpl*32, *trn*Q-5'*rps*16 and *atp*B-*rbc*L fragments were purified and then directly sequenced using an ABI 3770 automated sequencer.

2.3. Data analysis

The sequences were aligned and compared in SeqMan (DNA Star package; DNAStar Inc., Madison, WI, USA, Burland, 1999), then

Table 2
Main morphological differences among four taxa.

Taxa	Morphological traits			
	Inflorescence	Stem	Leaf shape	Florets
Ligularia vellerea	Racemiform	Densely long white puberulent	Lanceolate	Numerous tubular florets, with several ray florets
L. subspicata	Racemiform	Proximally glabrous	Ovate-cordate hastate arrow-shaped	Tubular florets
Putative hybrids	Racemiform	Some glabrous Some sparsely Some densely	Between *L. vellerea* and *L. subspicata*	Numerous tubular florets, some several ray florets, some without ray florets
L. tongolensis	Corymbose	Spider filiform pilose	Ovate-cordate ovate-oblong	Numerous tubular florets, with several ray florets

sequences were edited using BioEdit V.7 (Hall, 1999) and adjusted manually. Three fragments of cpDNA were combined using PAUP*version 4.0b (Swofford, 2002). The base additivity of *L. vellerea* and *L. tongolensis* were disposed using DnaSP5.0 (Rozas et al., 2003). Haplotypes for all sampled individuals were obtained using DnaSP5.0. The all haplotypes of three cpDNA fragments and ITS region examined in this study were archived in NCBI GenBank with accession numbers KY788676 to KY788755. The obtained haplotypes were used to infer the relationships for all sampled individuals using the criterion of neighbor-joining (NJ) tree, which was implemented in MEGA 7.0 (Tamura et al., 2007) and tested by bootstrap method. Bootstrap values were calculated with 1000 replications. It is generally believed that when some haplotypes get together into a cluster with the supporting rate $\geq$50%, these haplotypes have closer relationships (Tripathi et al., 2013).

3. Results

3.1. ITS analysis

The aligned length of all the ITS sequences was 612 bp. Clearly, there were twelve nucleotide substitutions and one insertion/ deletion (indel) were detected between two putative parents (Table 3). For putative hybrids, all individuals showed twelve chromatogram additivity sites between *L. vellerea* and *L. subspicata* and one deletion. Although at these fixed sites, sympatric *L. tongolensis* showed identical informative sites with either *L. vellerea* or *L. subspicata*, *Ligularia tongolensis* had twenty-three fixed informative sites separately. For *L. subspicata* and putative hybrids, direct sequencing of the nrITS region generated many chimeric and unreadable peaks in the chromatogram. Thus, in the subsequent cloning sequencing, we obtained more haplotypes than *L. vellerea*. Interestingly, one exception was detected among four *L. subspicata* individuals (S1, S2, S3 and S15). The four individuals had the accordant variable sites with putative hybrids. Namely, the four individuals also showed chromatogram additivity at fixed sites between *L. vellerea* and *L. subspicata*.

A strict consensus tree from nrDNA haplotypes was generated to show the relationships among three species and putative hybrids. The NJ tree from the nrDNA suggested that the haplotypes of putative parental species and putative hybrids formed two respective branches with high supporting values (92 and 88). However, the haplotypes of sympatric *L. tongolensis* formed the third branch (Fig. 1). Hence, all putative hybrids were products of hybridization between *L. vellerea* and *L. subspicata* (Koch et al., 2003) and sympatric *L. tongolensis* was not apparently involved in the hybridization between *L. vellerea* and *L. subspicata*.

3.2. Combined cpDNA intergenic spacer regions analysis

The aligned sequences of *atp*B-*rbc*L, *trn*L-*rpl*32 and *trn*Q-5'*rps*16 fragments were 746 bp, 866 bp and 945 bp, respectively. The combined length was 2557 bp. *Ligularia vellerea* and *L. subspicata*

differed in twenty nucleotide substitutions and four fixed indels (Table 4). All individuals of putative hybrids had the same cpDNA sequences with either *L. vellerea* or *L. subspicata*. *Ligularia vellerea* and *L. subspicata* both had two haplotypes respectively. Sympatric *L. tongolensis* had one haplotype and differed from the putative parents by two nucleotide substitutions and two fixed indels in the chloroplast sequences. Similarly, one exception was also detected among three *L. subspicata* individuals (S1, S8 and S10). Namely, the three individuals all showed the identical cpDNA sequences with *L. vellerea*.

The NJ tree from combined three cpDNA fragments showed clearly relationships of all samples. The haplotypes of two putative parental species and putative hybrids formed two respective branches with high supporting values (100 and 99), and the haplotypes of sympatric *L. tongolensis* shared a branch with the *L. vellerea* (Fig. 2). Because chloroplast DNA is maternally inherited in *Ligularia* species (Zhang et al., 2003), the hybridization between *L. vellerea* and *L. subspicata* is bidirectional. Namely, one of the two parents of *L. vellerea* and *L. subspicata* can not only act the maternal parent, but also act the paternal parent.

4. Discussion

4.1. The occurrence and direction of natural hybridization

In sympatric two closely related species, interspecific natural hybridization may arise if the flowering periods overlap partly or totally (Nishiwaki et al., 2011). In the case of our study system, the flowering periods of two putative parents are somewhat overlap because they all bloom in late June to August (Liu, 1989). Moreover, pollinator observations in the highlands have shown that pollinators do not strictly discriminate among *Ligularia* species (Liu, 2002; Cao et al., 2008). Thus, when different species are flowering at the same place and time, nectar-collecting insects may transfer pollen from one *Ligularia* species to another *Ligularia* species by chance. Hence, these conditions provide the probability for the natural hybridization between *L. vellerea* and *L. subspicata*. Based on the morphological comparisons, we preliminary judged that morphologically intermediate individuals are produced by the hybridization between *L. vellerea* and *L. subspicata* in the field. The leaf shape, stem and inflorescence type are easily distinguished and the putative hybrids have the intermediate morphology of these characteristics between *L. vellerea* and *L. subspicata* (Table 2). In other words, the possibility of hybridization between them has been implied by the morphological characteristics, sympatric distribution and overlapped flowering periods. Furthermore, the results of ITS data provided another evidence for the occurrence of hybridization between *L. vellerea* and *L. subspicata*, which is exactly consistent with that of Yu et al. (2014a). In addition, the results of cpDNA data clearly show that the direction of natural hybridization between *L. vellerea* and *L. subspicata* is bidirectional, which supports our previous hypothesis. Interestingly, the result is inconsistent with that of Yu et al. (2014a), which shows the evidence of

Table 3
Variable sites and indels in internal transcribed spacer 4−5 sequences from related individuals in four taxa and the haplotype distribution of all individuals.

Taxa	21	25	47	62	71	72	80	91	95	99	104–106	123	127	133	175	188	192	195	201–202	204	211	214	219	221	260	266
V1−V4, V6−V7, V13−V25	C	T	C	G	G	C	C	A	C	A	CTA	G	T	A	G	G	–	C	AC	G	C	G	G	A	T	C
V5, V8−V12	C	T	C	K	K	C	C	A	C	A	CTA	G	T	A	G	G	–	C	AC	G	C	G	G	A	T	C
S6−1, S10−1, S12−1, S17−1, S21−1, S21−3, S22−3	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	G	G	G	T	C
S7−7, S17−5	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	G	G	G	T	C
S13−1, S13−2, S14−5, S18−2, S23−2	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	G	G	G	T	T
S14−4, S22−2	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	G	G	G	T	C
S4−3, S6−2, S8−6, S8−7, S9−3, S10−4, S16−2, S18−3, S19−1, S19−2, S21−2, S22−6, S24−1, S24−2	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	G	G	G	T	C
S5−1, S7−5, S7−6, S8−3, S11−2, S11−3, S16−5, S17−2, S20−4, S20−6	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	T	C	GT	T	C	G	G	G	T	C
S4−1, S4−2	C	T	T	G	G	C	T	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	C	G	G	T	C
S10−2	C	T	C	G	G	C	C	A	C	G	TCC	G	C	A	G	G	C	C	GT	G	C	G	G	G	T	C
S20−5	C	T	C	G	G	T	C	A	C	G	TCC	G	C	A	G	G	–	C	GT	G	C	G	G	G	A	C
S1	C	T	C	G	G	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	G	G	R	T	C
S2, S3, S15	C	T	C	G	G	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	C	C	G	G	R	T	C
H6, H10, H13	C	T	C	K	K	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	C	G	R	T	C
H4, H8, H14	C	T	C	K	K	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	G	G	R	T	C
H3, H16	C	T	C	G	G	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	G	G	R	T	C
H1, H12, H15	C	T	C	G	G	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	G	G	R	T	C
H7, H11	C	T	C	G	G	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	G	G	R	T	C
H2, H5, H9	C	T	C	G	G	C	C	A	C	R	YYM	G	Y	A	G	G	–	C	RY	G	C	G	G	R	T	C
T1−T8	T	C	C	G	G	C	C	G	T	G	CTA	T	C	C	A	T	–	T	GC	G	T	G	A	G	T	C

Taxa	369	373	403	411	418	419	421–422	427	430	432	436	468	504	511	515	532	556	574	599	603	609	Haplotype
V1−V4, V6−V7, V13−V25	C	C	C	C	A	C	CC	C	T	A	T	T	T	T	C	C	G	T	C	T	T	Hap3
V5, V8−V12	C	C	C	C	A	C	CC	C	T	A	T	T	T	T	C	C	G	T	C	T	T	Hap3, Hap15
S6−1, S10−1, S12−1, S17−1, S21−1, S21−3, S22−3	C	C	C	T	A	C	CC	C	C	G	T	T	C	C	C	C	G	T	C	C	T	Hap1
S7−7, S17−5	C	C	C	T	A	C	CC	C	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap2
S13−1, S13−2, S14−5, S18−2, S23−2	C	C	C	T	G	C	–	C	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap4
S14−4, S22−2	C	C	C	T	G	C	–	C	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap8
S4−3, S6−2, S8−6, S8−7, S9−3, S10−4, S16−2, S18−3, S19−1, S19−2, S21−2, S22−6, S24−1, S24−2	C	C	C	T	A	C	CC	T	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap9
S5−1, S7−5, S7−6, S8−3, S11−2, S11−3, S16−5, S17−2, S20−4, S20−6	C	C	C	T	A	C	CC	C	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap10
S4−1, S4−2	C	C	C	T	A	C	CC	C	C	G	T	T	C	C	C	C	G	T	C	C	T	Hap19
S10−2	C	C	C	T	A	C	CC	C	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap28
S20−5	C	C	C	T	G	C	–	C	T	G	T	T	C	C	C	C	G	T	C	C	T	Hap30
S1	C	C	C	Y	A	C	CC	C	T	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap3, Hap10, Hap12
S2, S3, S15	C	C	C	Y	A	C	CC	Y	Y	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap10, Hap11, Hap26, Hap27, Hap33
H6, H10, H13	C	C	C	Y	A	C	CC	C	T	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap3, Hap10, Hap15, Hap20−Hap21, Hap31, Hap32
H4, H8, H14	C	C	C	Y	A	C	CC	Y	T	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap1, Hap12−Hap13, Hap15, Hap22−Hap24, Hap29
H3, H16	C	C	C	Y	A	C	CC	C	Y	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap1, Hap3, Hap14, Hap19
H1, H12, H15	C	C	C	Y	A	C	CC	Y	Y	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap1−Hap3, Hap7−Hap8, Hap15−Hap16
H7, H11	C	C	C	Y	A	C	CC	C	T	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap3, Hap15, Hap25, Hap28, Hap34
H2, H5, H9	C	C	C	Y	A	C	CC	Y	T	R	T	T	Y	Y	C	C	G	T	C	Y	T	Hap3−Hap6, Hap9, Hap17−Hap18, Hap35
T1−T8	T	T	T	C	A	T	CC	C	T	G	A	A	C	C	Y	A	A	G	T	T	C	Hap36−Hap37

−, deletions; K = G + T; Y = C + T; R = A + G; M = A + C.

unidirectional hybridization between the two species. And notably, we notice that the flowering periods of *L. tongolensis* also partly overlap with the *L. vellerea* and *L. subspicata,* which implies *L. tongolensis* may participate in natural hybridization. However, this can be strongly excluded by the results of ITS data analyses, because *L. tongolensis* is completely separated from other two species and putative hybrids based on ITS data (see Table 3 and Fig. 1). Although *L. vellerea*, *L. subspicata* and *L. tongolensis* grow
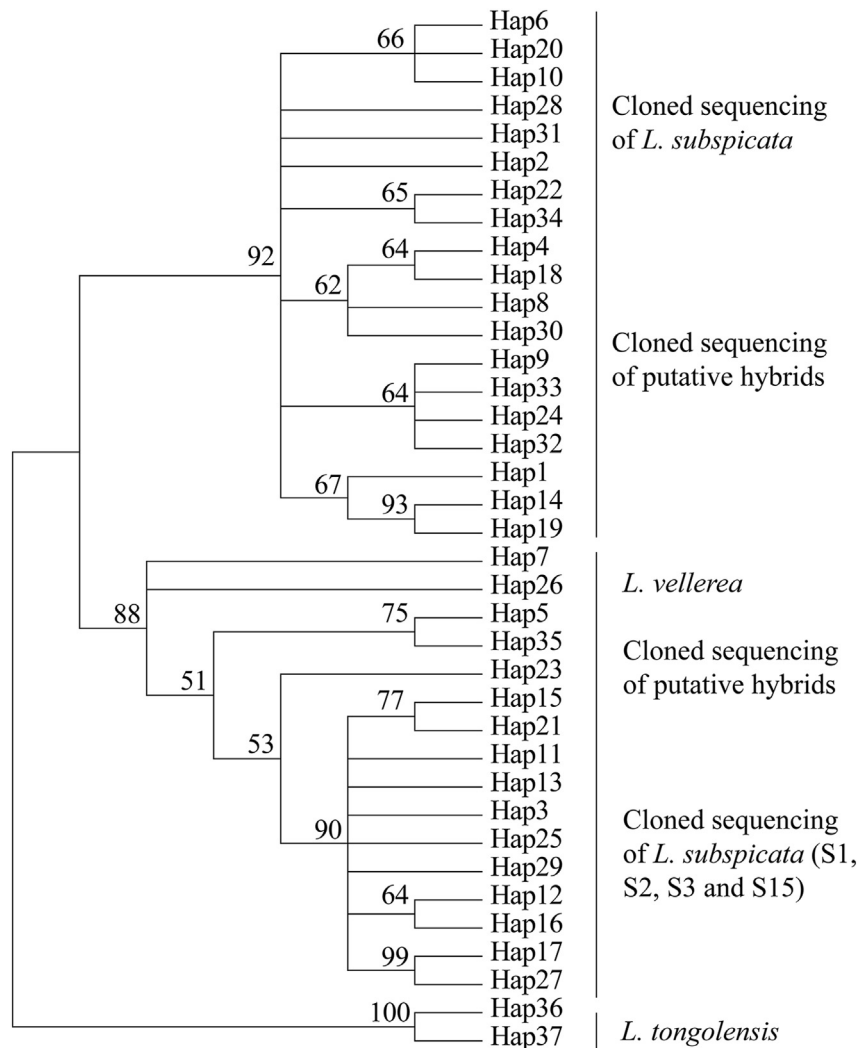
Fig. 1. Phylogenetic relationships of the nrITS haplotypes of all *Ligularia* species distributed within the mixed population. Supporting rate >50% is shown above branches.

together, *L. vellerea* and *L. subspicata* have the identical in-florescences, which may make pollinating insects transfer pollen between them other than *L. tongolensis*. On the other hand, in *Ligularia* phylogeny, the relationship between *L. vellerea* and *L. subspicata* is closer (He and Pan, 2015) than *L. tongolensis*, which may suggests that, in comparison with *L. tongolensis*, the reproductive isolation between them may be easier to be broken and the natural hybridization can be promoted.

4.2. The inconsistency between cpDNA and nrDNA for sympatric L. tongolensis

For sympatric *L. tongolensis*, the results of cpDNA and nrDNA are inconsistent. For example, the haplotypes of cpDNA of *L. tongolensis* formed a cluster with *L. vellerea* (Fig. 2), whereas the nrDNA results suggested *L. tongolensis* was separated completely from *L. vellerea* (Fig. 1). The inconsistency between results of cpDNA and nrDNA may be caused by the heterogeneity of evolution rate (Coyne and Orr, 2004), incomplete lineage sorting (Comes and Abbott, 2001), recurrent hybridization (Tsukaya et al., 2003; Yatabe et al., 2009), and convergent evolution (Davis, 1998; Desplanque et al., 2000). Here, we argue that this discordance may be explained by the heterogeneity of evolution rate. The evolutionary rate of nrDNA is faster than cpDNA (Wolfe et al., 1987). Moreover, the ITS region of

nrDNA can better distinguish the species of *Ligularia* than cpDNA (He and Pan, 2015). Hence, in the case of our study, the slower evolution rate of cpDNA results in that the combined cpDNA fragments can not distinguish the *L. tongolensis* from *L. vellerea* explicitly.

4.3. The confirmation of some individuals (S1, S2, S3, S8, S10 and S15) of putative parent L. subspicata

In this study, we notice that some individuals of *L. subspicata* are peculiar. For example, individual S1 had the identical nrITS sequence with putative hybrids and identical cpDNA sequences with *L. vellerea*. Furthermore, individual S2, S3 and S15 had the identical nrITS sequences with putative hybrids and identical cpDNA sequences with *L. subspicata*. Individual S8 and S10 had the identical nrITS sequences with *L. subspicata* and identical cpDNA sequences with *L. vellerea* (Tables 3 and 4). This phenomenon may be attributed to introgression (Harrison, 1986). Introgression can produce hybrids that are similar to one parent and have some particular characters from another parent. Further, introgression can provide the opportunity for gene flow between the putative parents, resulting in more complex relationships among these taxa (Harrison, 1986). In addition, introgressive hybridization can produce considerable numbers of new genotypes and lead to the
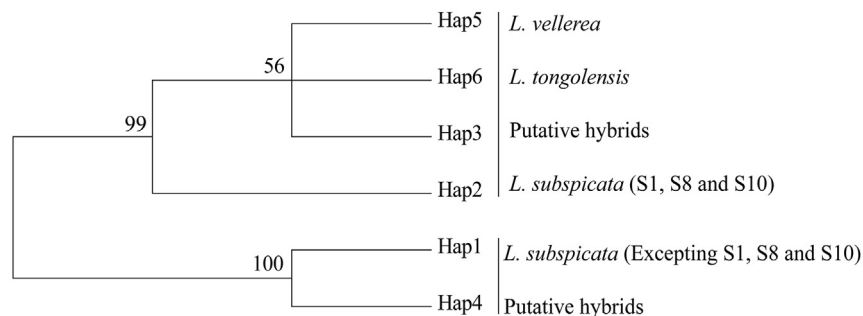
Table 4
Variable sites and indels in three chloroplast sequences from related individuals in four taxa and the haplotype distribution of all individuals.

| Taxa | Polymorphic sites | | | | | | | | | | | | | | | | | | | |
| | atpB-rbcL | | | | trnQ-5'rps16 | | | | | | | trnL-rpl32 | | | | | | | | |
	43	314	379	695	161	213	263–265	293	404–405	512	580	127	188	189–195	249	332	363	459–461	496	511–515
V1, V19–V20, V22–V25	T	T	A	T	T	A	–	A	TT	C	C	T	T	▼	T	T	G	–	T	–
V2–V18, V21	G	T	A	C	T	A	–	A	AG	C	C	T	–	–	T	T	G	◆	T	–
H6, H11	T	T	A	T	T	A	–	A	TT	C	C	T	T	▼	T	T	G	–	T	–
H5	G	T	C	T	T	–	–	A	TT	C	C	T	G	▼	T	A	G	–	T	–
H10, H13	G	T	A	C	T	A	–	A	AG	C	C	T	–	–	T	T	G	◆	T	–
H2	G	A	A	C	G	A	–	A	TT	–	A	T	T	▼	A	T	T	–	–	–
H1, H3–H4, H7–H9, H12, H14–H16	G	A	A	C	G	A	–	A	TT	–	A	T	T	▼	T	T	G	–	T	–
S1	G	T	A	C	T	A	–	A	AG	C	C	T	–	–	T	T	G	◆	T	–
S8, S10	G	T	C	T	T	–	–	A	TT	C	C	T	G	▼	T	A	G	–	T	–
S3, S5, S6, S9, S11, S14–S18, S23–S24	G	A	A	C	G	A	–	A	TT	–	A	T	T	▼	T	T	G	–	T	–
S2, S4, S7, S12–S13, S19–S22	G	A	A	C	G	A	–	A	TT	–	A	T	T	▼	A	T	T	–	–	–
T1–T8	G	T	A	C	T	A	▲	G	TT	C	C	G	T	▼	T	T	G	–	T	■

| Taxa | Polymorphic sites | | | | | | | | | | | Haplotype |
| | trnL-rpl32 | | | | | | | | | | | |
	774	776–777	779	781–786	787–791	793	797	802	804–805	807–810	811–866	
V1, V19–V20, V22–V25	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap3
V2–V18, V21	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap2
H6, H11	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap3
H5	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap5
H10, H13	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap2
H2	T	GA	C	★	TTATC	A	T	C	TT	CTAA	►	Hap4
H1, H3–H4, H7–H9, H12, H14–H16	T	GA	C	★	TTATC	A	T	C	TT	CTAA	►	Hap1
S1	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap2
S8, S10	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap5
S3, S5, S6, S9, S11, S14–S18, S23–S24	T	GA	C	★	TTATC	A	T	C	TT	CTAA	►	Hap1
S2, S4, S7, S12–S13, S19–S22	T	GA	C	★	TTATC	A	T	C	TT	CTAA	►	Hap4
T1–T8	G	TC	A	–	CTCGA	G	–	A	AA	TAGG	–	Hap6

–, deletions; ▲▼◆■★►, presence of insertion; ▲, TAA(3bp); ▼, AAGATTA(7bp); ◆, ACT(3bp); ■, TTATA(5bp); ★, AGTTTT(6bp); ►, AAAAACTTATTTGATTGAAT-TAACTTGTTCAATCTCGACGATTGAA TATAAATAGG(56bp).

Fig. 2. Phylogenetic relationships of the three cpDNA intergenic spacer regions haplotypes of all *Ligularia* species distributed within the mixed population. Supporting rate >50% is shown above branches.

establishment of new ecotypes which can adapt to particular environments (Arnold and Hodges, 1995; Arnold, 1997). Consequently, the occurrence of introgressive hybridization may increase the difficulties for confirming these individuals of *L. subspicata*. Namely, these individuals may be the progeny which are produced by the backcrossing between hybrids and putative parents. The occurrence of backcrosses can provide the opportunities for gene flow between two putative species and may indicate that introgression occurs between *L. vellerea* and *L. subspicata*.

Declaration of authorship

Xun Gong conceived and designed the research. Jiaojun Yu collected the experimental materials and analyzed the data. Huai Ning conducted the experiment, analyzed the data and wrote the manuscript. And all authors contributed to reviewing the manuscript.

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