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Research Article

Natural hybridization and introgression in sympatric *Ligularia* species (Asteraceae, Senecioneae)

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Abstract The difficulty in clarifying species of genus *Ligularia* Cass. has been attributed to rapid and continuous allopatric speciation in small and isolated populations, combined with interspecific diploid hybridization in the Qinghai-Tibetan Plateau and adjacent areas. However, no concrete example has been reported to prove this hypothesis. We studied a natural mixed population of six species of *Ligularia* in which some individuals were morphologically intermediate between *L. subspicata* and *L. nelumbifolia*. Based on DNA sequences (*trnC-F, trnL-rpL32, trnQ-5′rps16, trnK-rps16*, and internal transcribed spacer) and inter-simple sequence repeat data, we concluded that putative hybrids are primarily products of hybridization between *L. nelumbifolia* and *L. subspicata*. The other four species or additional, unknown species may also be involved in hybridization. This hybridization is bidirectional but asymmetrical. Hybrid individuals were mostly the first generation, but F_2 and later-generation hybrids were also present. Moreover, the backcrossed individuals detected indicate that natural gene flow occurs among at least three *Ligularia* species. Hybrids may become stabilized to form new species or may function as intermediates in evolutionary diversification.

Key words chloroplast fragments, ISSR markers, *Ligularia*, natural hybridization, nuclear ribosomal ITS.

An increasing number of researchers have focussed on the important role of natural hybridization in plant evolution (Arnold, 1997; Rieseberg, 1997; Rieseberg et al., 2000; Abbott et al., 2008, 2010; Soltis & Soltis, 2009; Chase et al., 2010). The consequences of hybridization may include introgression affecting one or both taxa, the formation of hybrid (especially allopolyploid) species, and the development of reticulate evolutionary patterns within a group (Arnold, 1997).

The genus *Ligularia* Cass. (Asteraceae, Senecioneae) consists of approximately 140 species mainly distributed in eastern Asia, 124 of which occur in China (Liu, 1989; Liu et al., 1994). *Ligularia* species occur in a wide variety of habitats, from forests to high alpine meadows, at elevations ranging from 1000 to 4000 m (Liu, 1989). The majority of *Ligularia* species in the Hengduan Mountains area are endemic (Liu et al., 1994, 2006). The high species diversity of this genus has been attributed to rapid and continuous allopatric speciation in small, isolated populations combined with interspecific diploid hybridization in the Qinghai-Tibetan Plateau and adjacent areas of eastern Asia (Liu et al., 2001, 2006). Because *Ligularia* mostly show rigid outcrossing systems and entomophilous pollination (Liu, 2002; Cao, 2004), there is good reason to believe that species barriers might break down relatively easily within subgenera of *Ligularia*. Natural hybrids are commonly found in certain areas where multiple *Ligularia* species occur in sympatry (Liu et al., 2006). *Ligularia* × *maoniushanensis* X. Gong & Y. Z. Pan has been reported as a natural hybrid species based on a comprehensive study. Its parents are *L. paradoxa* Hand.-Mazz. and *L. duciformis* (C. Winkl.) Hand.-Mazz. (Pan et al., 2008). However, no backcrossed plants have been found, although gene flow between the parental species is not impossible. Furthermore, by comparing chemical and genetic evidence (mainly internal transcribed spacer (ITS) sequences), the present author (Gong) and Japanese researchers have found that at least five species have undergone hybridization in some populations in Yunnan and Sichuan Provinces, China (Nagano et al., 2006; Tori et al., 2006, 2008a, 2008b; Saito et al., 2011).

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These conclusions are mainly conjectural, and they suggest that gene flow and speciation in small, isolated populations may be a vital factor in the diversity of *Ligularia*.

During field collection trips to the Hengduan Mountains in Yunnan, we found a mixed population on Mt. Xiaoxue that included six taxa: *L. subspicata* (Bur. & Franch.) Hand.-Mazz.; *L. nelumbifolia* (Bur. & Franch.) Hand.-Mazz., *L. tongolensis* (Franch.) Hand.-Mazz., *L. cymbulifera* (W. W. Smith) Hand.-Mazz., *L. lingiana* S.W. Liu, *L. vellerea* (Franch.) Hand-Mazz., and a putative natural hybrid. This mixed population grows on a meadow along a stream at an altitude of 3800 m. Morphologically intermediate individuals were considered to be natural hybrids between *L. subspicata* and *L. nelumbifolia* based on their mosaic morphological characters and the partial overlap of the flowering periods. Given this overlap in anthesis, cross-pollination between the two putative parents could occur naturally in sympatry.

In the present study, we used evidence from morphology, cpDNA and nrDNA sequences, and inter-simple sequence repeat (ISSR) markers to resolve the following questions about this particular population: (i) Are the putative natural hybrids actually products of hybridization? (ii) If the mixed population actually includes hybrid individuals, what is the relationship between these hybrids and the six natural species? In other words, what are the parental species of the hybrids? Are all hybrid individuals derived from the same parents, or are they derived from different parents? (iii) What are the statuses of the hybrids? Do they proceed to the F_2 or later generations? Have the parental species been introgressed by backcrossing with hybrids? By answering these questions, we aimed to determine whether interspecific gene flow exists among these taxa and whether hybridization stimulates speciation in this group.

1 Material and methods

1.1 Collection of plant materials and molecular methods

Morphological observations were carried out in the field during the 2009 and 2010 flowering seasons. Leaf form and inflorescence type were the primary diagnostic characters. To use both chloroplast and nuclear sequence data and ISSR markers, we collected young leaves from 51 individuals (including 10 *L. nelumbifolia*, 10 *L. subspicata*, 13 putative hybrids, 5 *L. cymbulifera*, 5 *L. lingiana*, 4 *L. tongolensis*, and 4 *L. vellerea*). Leaves were dried in silica gel in the field. Voucher specimens were deposited in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Total genomic DNA was extracted from the silica-dried leaf tissue using the CTAB method (Doyle & Doyle, 1987) with minor modifications.

The ITS region of the nuclear ribosomal DNA of all sampled individuals was amplified using primers ITS1 and ITS4 (White et al., 1990). Polymerase chain reaction (PCR) was carried out in a total reaction volume of 20 μL containing 10 ng template DNA, 2.0 μL 10 $\times$ PCR buffer with $(NH_4)_2SO_4$, 1.0 μL $MgCl_2$ (25 mmol/L), 0.5 μL dNTPs (2.5 mmol/L each), 0.2 mmol/L each primer, and 0.75 unit of *Taq* polymerase (Takara, Shiga, Japan). Amplification was performed in a T1 thermocycler (Biometra, Göttingen, Germany) using the following conditions: 1 cycle, 94 °C, 5 min; 30 cycles, 94 °C, 15 s, 53 °C, 15 s, and 72 °C, 40 s; and 1 cycle, 72 °C, 5 min. The PCR products were purified by electrophoresis on a 1.2% agarose gel and extracted using an EZNA Gel Extraction Kit (Omega, Guangzhou, China). All accessions sequenced using the amplification primers in an ABI 3700 automated DNA sequencer with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequences were aligned and compared in SeqMan (DNASTAR, Beijing, China) (Burland, 1999). All sequences have been deposited in GenBank (Accession Nos JF767223–JF767276). Direct sequencing was successful for *L. nelumbifolia*, *L. subspicata* (except two individuals), and the other four *Ligularia* species, but it produced chimeric or unreadable peaks in the chromatograms for the putative hybrids. Therefore, cloning was carried out for all putative hybrids and for the two *L. subspicata* individuals that also yielded chimeric or unreadable peaks. Purified PCR products were cloned into plasmids using the pUM-T vector system (BioTeke, Beijing, China). Between 4 and 20 positive clones were selected for each amplification product and cultured to isolate their 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.

Four cpDNA fragments were amplified using the following universal primers: *trnC-F* (Taberlet et al., 1991); *trnL-rpL32*, *trnQ-5′rps16*, and *trnK-rps16* (Shaw et al., 2007). Polymerase chain reaction was carried out in a reaction volume of 20 μL containing 10 ng template DNA, 2.0 μL 10$\times$ PCR buffer with $(NH_4)_2SO_4$, 1.0 μL $MgCl_2$ (25 mmol/L), 1.0 μL dNTPs (2.5 mmol/L each), 0.3 mmol/L each primer, and 1.5 unit of *Taq* polymerase (Takara). The amplification conditions were as follows: 1 cycle, 94 °C, 4 min; 30 cycles, 94 °C, 45 s, 53 °C, 45 s, and 72 °C, 1 min or 50 s; and

1 cycle, 72 °C, 7 min. The PCR products were purified and directly sequenced in both directions using the methods described above.

A set of 100 ISSR primers were screened for representative individuals of the putative parental species and hybrids, and 12 primers (University of British Columbia; UBC808, UBC809, UBC811, UBC818, UBC823, UBC834, UBC835, UBC840, UBC845, UBC849, UBC857, and UBC881) were selected for use for further amplification. Final PCRs were carried out in a reaction volume of 20 μL containing 10 ng template DNA, 2.0 μL 10× PCR buffer with $(NH_4)_2SO_4$, 1.6 μL $MgCl_2$ (25 mmol/L), 1.2 μL dNTPs (2.5 mmol/L each), 0.4 mmol/L primer, and 6 units of *Taq* polymerase (Takara). The PCR amplification conditions were as follows: 1 cycle, 94 °C, 5 min; 35 cycles, 94 °C, 1 min, 52.5 °C, 1 min, 72 °C, 1.5 min; and 1 cycle, 72 °C, 7 min. Because the sequence data had excluded the possibility that the other four *Ligularia* species were involved in the origin of the putative hybrids' nucleus, only 40 individuals (including 10 *L. nelumbifolia*, 10 *L. subspicata*, and 11 putative hybrids that yielded high quality DNA) were amplified in each PCR run for each primer (to avoid variation between runs). If no amplification was obtained for more than five individuals in a given run, all results from that run were discarded, and the reaction was repeated for all individuals. The ISSR bands were scored manually as binary characters (0 for the absence and 1 for the presence of a band). Co-migrating bands between different individuals within a gel were considered to be homologous. Only polymorphic bands were used in subsequent analyses because the inclusion of monomorphic bands did not affect the overall relationships between individuals.

1.2 Phylogenetic analysis

Minor variation (usually one or two nucleotide sites) between some clones may have resulted from PCR error caused by the Taq DNA polymerase. Putative PCR-mediated recombinants were excluded before further phylogenetic analysis. The four cpDNA sequences from all individuals were aligned using ClustalX version 1.81 (Thompson et al., 1997). Information about variable sites was obtained using the program DnaSP 4.0 (Rozas et al., 2003). The aligned sequences were used to infer the phylogeny of the sampled individuals using the criterion of maximum parsimony (MP), implemented in PAUP∗ version 4.0b (Swofford, 2002). Parsimony analyses were carried out using a heuristic search with tree bisection and reconnection branch swapping, the Multrees option, ACCTRAN optimisation, and 1000 random addition replicates for the nuclear and chloroplast datasets.

1.3 NewHybrids analysis of ISSR data

For each individual, the posterior probability that it belonged to one of the parental species or to an early generation hybrid class (F_1, F_2, or backcross) was estimated using a Bayesian method designed to analyse polymorphic ISSR markers. This procedure used a Markov Chain Monte Carlo method and was implemented in the program NewHybrids version 1.1 beta 3 (Anderson, 2003). This program distinguishes between F_1 hybrids, different types of backcrosses and later generation hybrids, and it does not require the parental populations to be sampled separately (Anderson & Thompson, 2002; Anderson, 2008). The posterior distributions were evaluated after 10^5 iterations of the Monte Carlo Markov Chains without using any individual or allele frequency prior information and independently of whether "Jeffreys-like" or "uniform" priors were implemented for both mixing proportions and allele frequencies (the posterior probabilities were not affected by these priors). Individuals were assigned to one of the six genotypic classes if $P \geq 0.90$ or to two or more genotypic classes if $0.90 \geq P \geq 0.10$.

2 Results

2.1 Morphology

Ligularia subspicata and *L. nelumbifolia* belong to series *Retusae* S. W. Liu and series *Ligularia* Cass., respectively (Liu, 1989). The main morphological differences between the two species are their leaf shapes and inflorescence types (Table 1, Fig. 1). The leaves of *L. nelumbifolia* are reniform (Fig. 1), whereas those of *L. subspicata* are cordate or hastate (Fig. 1). The inflorescence of *L. nelumbifolia* is a compound corymb (Fig. 1), whereas that of *L. subspicata* is racemiform (Fig. 1). The morphological characters of the putative natural hybrids are intermediate between those of the putative parental species, with hastate or cordate leaves and compound corymb inflorescences (Fig. 1). The other four sympatric species can be easily distinguished from the putative parental species by their leaf shapes, venation patterns, and inflorescence types (Table 1).

2.2 Phylogenetic analysis based on nrDNA ITS4–5 sequences

The aligned matrix of ITS sequences was 714 or 710 bp long (because of two indels). All *L. nelumbifolia* individuals had identical ITS sequences (Table 2). Additionally, 8 out of 10 *L. subspicata* individuals shared the same sequence. There were 25 sites that varied between these two putative parental species. However, the two remaining individuals of *L. subspicata* (Table 2,

Table 1 Main morphological differences characterizing *Ligularia subspicata*, *L. nelumbifolia*, putative hybrids, and four other species

Taxon	Characters		
	Venation	Leaf blade	Inflorescence made up of capitula
Ligularia subspicata	Palmate	Ovate-cordate, hastate, or sagittate	Racemose, proximally branched
Putative hybrids	Palmate	Ovate-cordate, hastate, or sagittate	Compound corymb
L. nelumbifolia	Palmate	Reniform	Compound corymb
L. tongolensis	Pinnate	Ovate-cordate, ovate-oblong	Corymb, solitary
L. cymbulifera	Pinnate	Elliptic, ovate-oblong	Compound corymb, much branched
L. lingiana	Pinnate venation, midvein strong, with conspicuous prominent reticulate veins on both surfaces	Elliptic, oblong	Racemiform inflorescence
L. vellerea	Pinnate	Ovate, elliptic, or suborbicular	Racemiform inflorescence

individuals S1 and S3) had directly sequenced ITS chromatograms resembling those of the putative natural hybrids. The putative natural hybrids exhibited more than 10 double peaks, and the cloned sequences (including those of S1 and S3) included two sequence types that were identical to those of *L. nelumbifolia* and *L. subspicata*, respectively, clearly showing that all double copies of the ITS region came from the putative parental species. The other four *Ligularia* species showed 32 variable sites that clearly distinguished them from the putative parental species and hybrids.

A strict consensus tree from the MP analysis of 51 individuals was generated to show their phylogenetic relationships (Fig. 2). The MP tree indicates that the putative parental species form clusters with high bootstrap values. Among the cloned sequences, each cloned individual possessed one copy that clustered with the sequences from each parental species. The other four *Ligularia* species formed separate clusters from the parental species. Hence, all putative hybrid accessions are products of hybridization between *L. nelumbifolia* and *L. subspicata* (Koch et al., 2003). Thus, the other four species are apparently not involved in hybridization at this site.

2.3 Phylogenetic analysis based on concatenated sequences of four cpDNA intergenic spacer regions

Four cpDNA intergenic spacer regions from 51 individuals contained a total of 20 variable sites, including five large indels treated as five single variations (Table 3). Among these sites, there were no polymorphisms within individuals of the putative parental species. Most substitutions were shared among several species, except for five sites: in *trnK-rps16*, one substitution was unique to *L. vellerea* and one to *L. lingiana*; in *trnL-rpL32*, one substitution was unique to A6 and A11 and one to *L. lingiana*; and in *trnQ-5′rps16*, one substitution was unique to *L. lingiana* (L1–L4) and *L. cymbulifera*. Because other sites showed no obvious patterns when examined by eye, we carried out a parsimony analysis of these sequences.

The MP tree obtained from the four concatenated cpDNA regions is shown in Fig. 3. The putative parental species formed two separate clusters. Among the putative hybrids, 8 out of 13 individuals (A1–5, A7, A10, A12) clustered together with *L. subspicata*; 2 out of 13 (A8 and A13) clustered with *L. nelumbifolia*; and 2 out of 13 (A6 and A11) formed a branch of their own. The other four *Ligularia* species were clearly separated from the putative parental species and hybrids. Because chloroplast DNA is maternally inherited in *Ligularia* (Zhang et al., 2003), most putative hybrids had *L. subspicata* as the plastid donor but two accessions had *L. nelumbifolia* as plastid donor. The remaining two had additional, unknown species as plastid donor. Hence, bidirectional but asymmetrical hybridization occurred between *L. subspicata* and *L. nelumbifolia*. Moreover, hybridization happened not only between the two species.

2.4 ISSR data for putative parental species and natural hybrids

A total of 109 scorable polymorphic ISSR markers were generated during the analysis (Table S1). Additional polymorphic markers were present but could not be scored either because of faint, inconsistent amplification or the inability to differentiate two or more fragments of a similar molecular mass.

The scored markers were as follows: 14 present only in *L. subspicata*; 12 present only in *L. nelumbifolia*; 6 present only in the putative hybrids; 27 shared by *L. subspicata* and the putative hybrids but not by *L. nelumbifolia*; 26 shared by *L. nelumbifolia* and the putative hybrids but not by *L. subspicata*; and 6 markers shared by the putative parental species but not present in the putative hybrids. All of these markers show that the three taxa are closely related.

2.5 NewHybrids analysis

The NewHybrids analysis (Table S2, Fig. 4) confirmed that all individuals of *L. nelumbifolia* and most

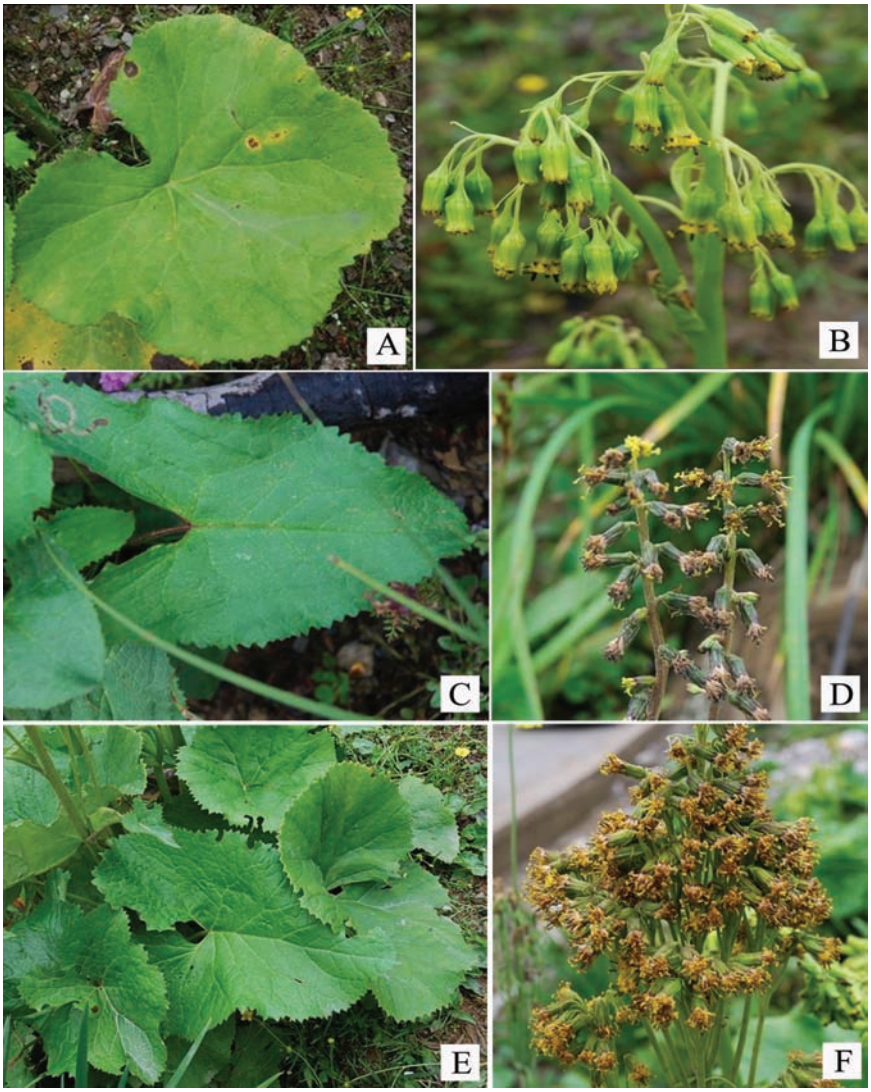

Fig. 1. Laminas and inflorescences of a putative hybrid and its putative parents. **A, B,** Reniform leaf and compound corymb of *Ligularia nelumbifolia*. **C, D,** Hastate leaf and racemiform inflorescence of *L. subspicata*. **E, F,** Hastate or cordate leaves and compound corymb of the putative hybrid. Photographs not to scale.

individuals of *L. subspicata* belonged to the pure parental species with >95% probability. Among 11 putative hybrid individuals, 7 were determined to be F_1s with >95% probability. The remaining hybrid individuals could not be confidently assigned to a class with greater than 91% probability. Of these four, one individual (A11) had a 39.79% probability of being F_1, a 35.47% probability of being F_2, and a 24.74% probability of being a backcross to one parent (*L. subspicata*). The other three had a probability 86.38%–91.52% of being F_1, a probability of 8.45% to 12.49% of being F_2 and a low probability (0.05% to 1.13%) of being backcrosses. Therefore, the hybrids are mainly maintained in the form of F_1 plants. However, hybridization probably proceeds beyond the F_1 level; at least one of the sampled individuals was the progeny of a backcross to a parental species.

3 Discussion

3.1 Habitat and probability of occurrence of natural hybrids

The putative parental species, *L. nelumbifolia* and *L. subspicata*, often coexist in southeastern Yunnan (X. Gong, personal observation). The putative hybrids

Table 2 Variable sites and indels in internal transcribed spacer 4–5 sequences from related individuals in sympatric *Ligularia* species

Taxa	Polymorphic sites																														
	54	55	76	84	86	88	10	124	128	136	150	155	158	162	167	168	169	174	186	190	196	209	226	238	250	251	257	282	283	284	286
S2, S4–S10	A	A	T	C	A	T	G	C	T	Y	G	A	C	G	T	C	C	C	G	C	A	C	T	G	T	G	C	A	C	G	G
S1, S3	A	W	T	C	W	T	G	Y	T	C	R	A	C	G	Y	Y	M	Y	G	C	M	C	Y	G	T	G	C	A	Y	G	G
A1–A13	A	W	T	C	W	T	G	Y	T	C	R	A	C	G	Y	Y	M	Y	G	C	M	C	Y	G	T	G	C	A	Y	G	G
N1–N10	A	T	T	C	T	T	G	Y	T	C	A	A	C	G	C	T	A	T	G	C	C	C	C	G	T	G	C	A	T	G	G
C1–C4	T	T	T	T	A	C	G	C	Y	C	G	A	C	G	C	T	C	T	A	C	C	A	T	A	T	T	C	T	C	G	G
T1–T4	T	T	T	T	A	C	G	C	T	C	G	R	Y	G	C	T	M	Y	W	C	C	M	T	A	T	T	Y	W	C	R	G
L1–L4	A	T	C	C	A	C	T	C	T	T	G	A	C	G	C	T	A	C	G	C	A	C	T	G	C	G	C	A	C	G	A
L5	A	T	C	C	A	C	T	C	T	T	G	A	C	G	C	T	A	C	G	C	A	C	T	G	C	G	C	A	C	G	A
V1–V4	A	T	T	C	A	T	G	C	T	C	G	A	C	A	C	T	A	C	G	T	A	C	T	G	T	G	C	A	C	G	A

Taxa	Polymorphic sites																														
	284	286	344	348	468	484	497	498	501	509	510	511	513	541	565	570	576	579	596	614	621	633	649	654	664	674	684	686			
S2, S4–S10	G	G	C	C	C	C	G	G	T	G	G	T	T	T	C	C	C	G	C	G	G	A	T	G	C	C	C	C			
S1, S3	G	G	Y	C	C	C	G	R	T	R	G	Y	T	K	Y	C	C	G	C	R	G	A	Y	R	M	Y	Y	C			
A1–A13	G	G	Y	C	C	C	G	R	T	R	G	Y	T	K	Y	C	C	R	C	R	G	A	Y	R	M	Y	Y	C			
N1–N10	G	G	T	C	C	C	G	A	T	R	G	C	T	G	T	C	C	R	C	A	G	A	C	R	M	Y	T	C			
C1–C4	G	G	T	T	C	C	G	G	T	G	A	T	A	T	C	C	C	G	A	G	A	A	T	G	C	T	T	T			
T1–T4	R	G	T	T	Y	Y	G	G	W	G	R	T	A	T	C	C	C	G	A	G	A	A	K	G	C	T	T	T			
L1–L4	G	A	C	C	C	C	A	G	T	G	G	T	T	T	C	T	C	G	C	G	G	T	T	G	C	C	T	C			
L5	G	A	C	C	C	C	A	G	T	G	G	T	T	T	C	T	C	G	C	G	G	T	T	G	C	Y	T	C			
V1–V4	G	A	C	C	C	C	A	G	T	G	G	T	T	T	C	T	T	G	C	G	G	A	T	G	C	C	T	C			

Note: C + T = Y, A + C = M, A + T = W, A + G = R, G + T = K, C + G = S.
Taxa: A, putative hybrid; C, *Ligularia cymbulifera*; L, *L. lingiana*; N, *L. nelumbifolia*; S, *L. subspicata*; T, *L. tongolensis*; V, *L. vellerea.* Numbers following taxon initials are sample numbers.

grow in a forest that was previously dominated by fir. However, the trees were destroyed by a fire during the 1980s, and a meadow subsequently developed. A total of six *Ligularia* species occur sympatrically as dominant species, especially *L. nelumbifolia*. This abrupt change of habitat may have promoted hybridization between these closely related species. New habitats can bring into sympatry species that were formerly isolated by biogeographic barriers. Such special circumstances are also more likely to give rise to new species of hybrid origin (Buerkle et al., 2000; Rieseberg et al., 2003; Gompert et al., 2006).

Our sampling site was located at an altitude 3800 m, and the wintry highland climate requires species to evolve certain common adaptive mechanisms. *Ligularia* species often share the same pollinators in the highlands (Liu, 2002; Cao et al., 2008). When different species are flowering at the same place and time, nectar-collecting insects may transfer one species' pollen to another species' flowers by chance. The flowering times of the sympatric *Ligularia* species overlap, according to previous researchers (Liu, 1989; Liu et al., 1994) and our three-year field observations. All six *Ligularia* species flower in July to August, but their flowering times differ somewhat: *L. subspicata*, *L. lingiana*, and *L. vellerea* reach anthesis slightly earlier than the other species. Because the species have similar flowering times, it is difficult to reject any of them as possible participants in interspecific hybridization.

3.2 Pre-existing natural hybridization and introgression

Our ITS data definitely support the hypothesis that the morphologically intermediate individuals are the products of hybridization. According to cpDNA analysis, we can also conclude that the hybridization is bidirectional but predominantly in the form of *L. subspicata*♀ × *L. nelumbifolia*♂ (A1–A5, A7, A10, A12). These results differ from those of Pan et al. concerning natural hybrids between *L. paradoxa* and *L. duciformis*, in which hybridization was found to be unidirectional (Pan et al., 2008). This bidirectional hybridization suggests that the two parental species (*L. subspicata* and *L. nelumbifolia*) are equally cross-compatible. The approximate number of individuals of *L. nelumbifolia* was more than five times that of *L. subspicata* in the hybrid zone. Furthermore, with their

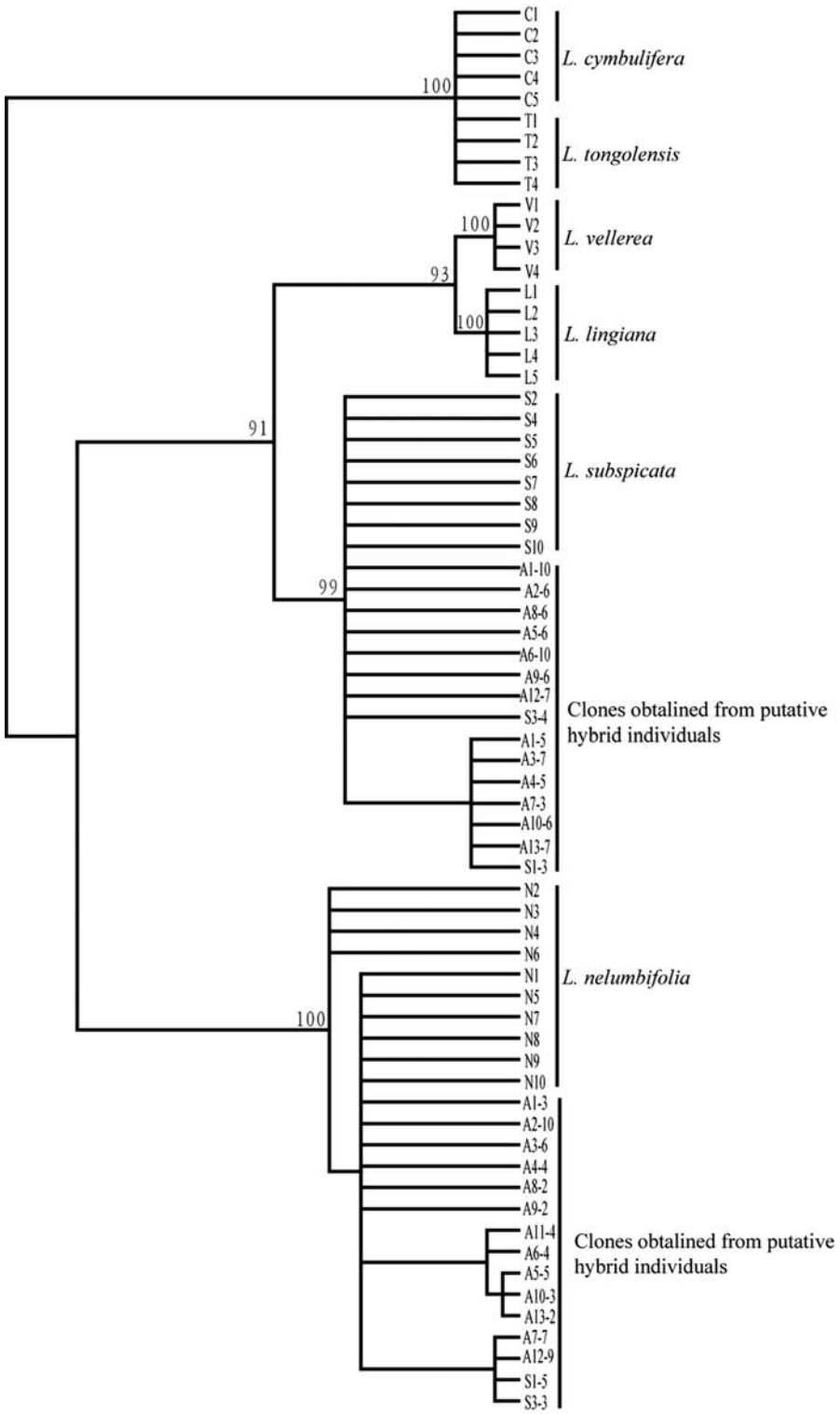

Fig. 2. Strict consensus tree from a parsimony analysis of internal transcribed spacer 4–5 sequences from putative hybrid individuals and their putative parental species. Taxa: A, putative hybrid; C, *Ligularia cymbulifera*; L, *L. lingiana*; N, *L. nelumbifolia*; S, *L. subspicata*; T, *L. tongolensis*; V, *L. vellerea.* Numbers following taxon initials are sample numbers and clone numbers (if any). Bootstrap percentages >50% are shown above the branches.

Table 3 Variable sites and indels in four chloroplast sequences from related individuals in sympatric *Ligularia* species

Taxa	Polymorphic sites																			
	trnK-rps16						*trnL-rpL32*								*trnC-F*		*trnQ- 5′rps16*			
	233	2	271	5	7	855	2	2	3	4	881	6	7	782	3	6	1	2	419	5
	–	4	–	2	1	–	0	6	4	2	–	5	3	–	6	4	7	8	–	9
	238	9	397	4	1	863	3	3	7	7	891	6	6	884	5	2	9	8	420	8
S1–S10	–	G	–	A	A	–	T	A	T	A	+4	C	T	+5	T	G	G	A	TT	A
A1–A5, A7, A10, A12	–	G	–	A	A	–	T	A	T	A	+4	C	T	+5	T	G	G	A	TT	A
A6, A11	+1	G	+2	A	A	+3	T	A	T	C	+4	A	T	–	T	G	T	A	AG	C
A9	+1	G	+2	A	G	+3	G	A	A	A	+4	A	T	–	T	A	T	A	TT	C
A8, A13	+1	G	+2	A	G	+3	G	T	A	A	–	A	T	–	G	A	T	A	TT	C
N1–N10	+1	G	+2	A	G	+3	G	T	A	A	–	A	T	–	G	A	T	A	TT	C
C1, C5	+1	G	+2	A	A	+3	T	A	T	A	+4	C	T	–	T	G	T	C	TT	C
C2–C3	+1	G	+2	A	A	+3	T	A	T	A	+4	C	T	–	T	G	T	A	TT	C
T1–T4	+1	G	+2	A	A	+3	T	A	T	A	+4	C	T	–	T	G	T	A	TT	C
L1–L4	+1	G	+2	C	A	+3	T	A	A	A	+4	A	A	–	T	G	T	C	AG	C
L5	+1	G	+2	A	A	+3	T	A	A	A	+4	A	A	–	T	G	T	A	AG	C
V1–V4	+1	A	+2	A	A	+3	T	A	T	A	+4	A	T	–	T	G	T	A	AG	C

–, Deletions; +1 to +5, presence of insertion; + 1, ATATTC (6 bp); +2, TGGAAAACCTTATTTGATTGATCCTC ACAAAATAAAAAATTATGGCATTACCTGCAATCGCGTGGTTTTCATTCGAACTCTAATACCAAGTAAGAAACAAGCAATCGAAGTTCTTTA TTCGACTTAT (127 bp); +3, TTTTATTTC (9 bp); +4, GTATTTAACAT (11 bp); + 5, TTGAACTAGTTTTTTTATCAATTTGAATCTTTAC-TAAAAAACTTATTGAATTAACTT (57 bp).

Taxa: A, putative hybrid; C, *Ligularia cymbulifera*; L, *L. lingiana*; N, *L. nelumbifolia*; S, *L. subspicata*; T, *L. tongolensis*; V, *L. vellerea.* Numbers following taxon initials are sample numbers.

compound corymb inflorescences, *L. nelumbifolia* adults can have more than 100 cephaloids with 6∼8 florets each. In contrast, the racemiform inflorescences of *L. subspicata* have no more than 40 cephaloids with 9–16 florets each. During the overlap in flowering, *L. nelumbifolia* obviously produces much more pollen than *L. subspicata* in this mixed population. This asymmetry is likely to explain why the hybrid form of *L. subspicata*♀× *L. nelumbifolia*♂ is more common than the reciprocal cross.

In the cpDNA data, which is relatively simple compared to the nrDNA sequence data, the other four *Ligularia* species could not be excluded as possible parents because of the two individuals that formed a cluster by themselves (A6, A11). Any of the four species or perhaps another, unknown species may also participate in hybridization. Although the origin of these two individuals (A6, A11) could not be defined, the putative parental species undoubtedly participated in the formation of their nucleoplasm, and at least one other species may have contributed their chloroplast genome. Thus, the mixed population is more complex than we initially believed.

Two accessions of one putative parental species, *L. subspicata* (individuals S1 and S3), also showed double peaks for ITS4–5 and had cloned sequences identical to those of both pure parental species, suggesting that the hybrid population also includes backcrossed progeny. Backcrossing could provide the opportunity for gene flow between the putative parents, producing more complex relationships between these taxa.

3.3 Population structure of putative hybrids: F_1-dominated hybrid zones?

Based on ISSR markers, a Bayesian analysis was carried out using the program NewHybrids, which indicated that all 11 putative hybrid individuals were hybrid progeny. Seven of these individuals were confirmed to be F_1s, and three were more likely to be F_1s but also may have been F_2s. It is reasonable to conclude that this hybrid zone is dominated by F_1 individuals along with some F_2s. In separate seed germination experiments, 3 out of per 1000 seeds of putative hybrid germinated (Yu, 2010, unpublished data). Although we could not determine whether these seeds were F_2s, Fns or backcross progeny, we were able to confirm that the putative hybrids can produce robust offspring. Their germination rate seems extremely low, but with compound corymb inflorescences, each putative hybrid individual can yield more than 1000 seeds and thus can generate several robust offspring. For the continued existence of a new hybrid species, it is vitally important that the hybrids be able to reproduce in their habitat.

This new habitat has existed for no more than two decades, and *Ligularia* species usually require 4–5 years of vegetative growth to reach the reproductive phase (Liu, 2002; Cao et al., 2008; Wu et al., 2010). Considering that the putative parental species occur sympatrically in this habitat, that natural hybridization has occurred, and that the hybrids have reached the flowering stage, 20 years is a relatively short period for a new hybrid species to become established and evolve unique traits. Thus, this mixed population remains an F1 dominated

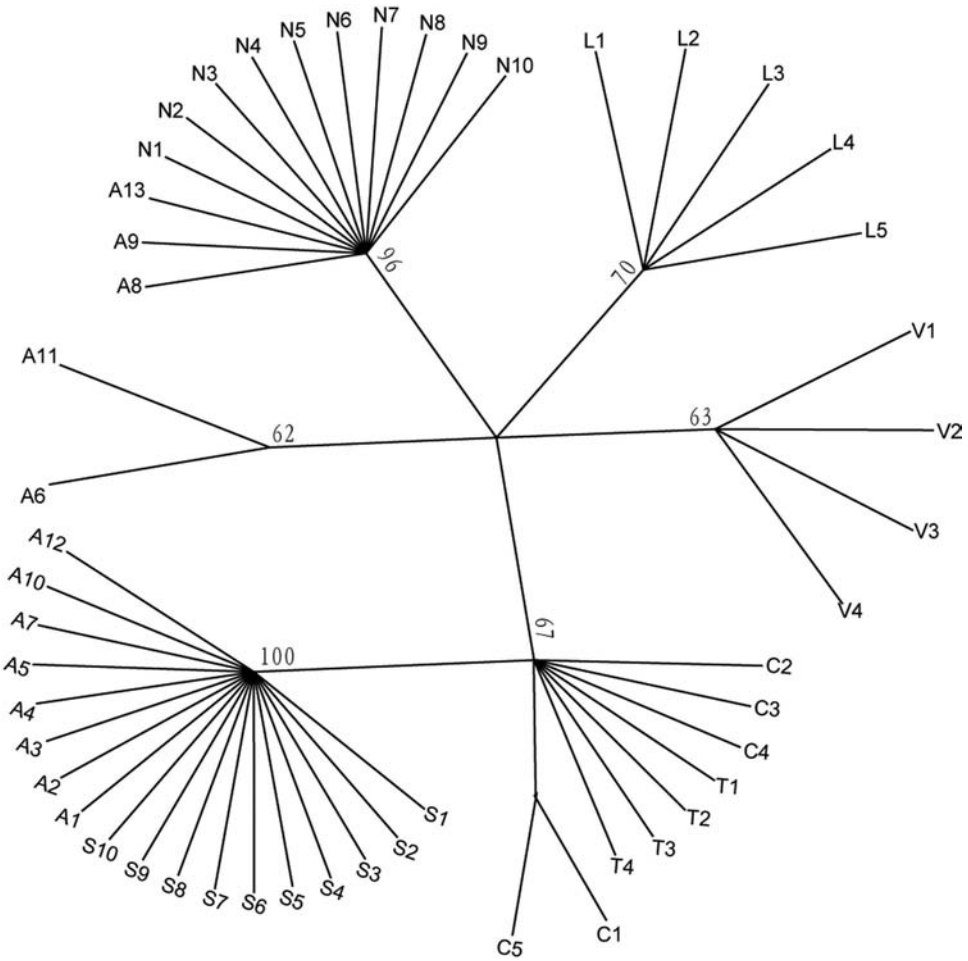

Fig. 3. Maximum parsimony tree inferred from four concatenated cpDNA sequences to show the relationships between the putative hybrids and sympatric *Ligularia* species. Bootstrap percentages >50% are shown above the branches. Taxa: A, putative hybrid; C, *Ligularia cymbulifera*; L, *L. lingiana*; N, *L. nelumbifolia*; S, *L. subspicata*; T, *L. tongolensis*; V, *L. vellerea.* Numbers following taxon initials are sample numbers.

hybrid zone at present. Backcross and F2 individuals probably occur; therefore, we confidently conclude that *Ligularia* species in this population are capable of hybridization and forming complex relationships.

4 Conclusions

Through comprehensive research, we conclude that the putative hybrid individuals found on Mt. Xiaoxue are the products of natural hybridization between *L. nelumbifolia* and *L. subspicata*. Introgression has occurred in this population, indicating that the pollen of hybrids has some fertility. In addition, seeds collected from hybrid plants showed partial germination ability (3‰; Yu, 2010, unpublished data). Like one of the parental species (*L. nelumbifolia*), the hybrids are perennial and capable of clonal propagation (X. Gong, personal observation). If a new genotype more fit for the native habitat emerges, a new species may form, given sufficient time. Thus, this mixed population has the potential to generate new species through hybridization. The occurrence of backcrossing and interspecific hybridization, as shown in this study, corroborates earlier hypotheses (Liu et al., 2006). Gene flow and diploid hybridization (Yu, 2010, unpublished chromosomal data) play an important role in the diversity of this genus at a local scale.

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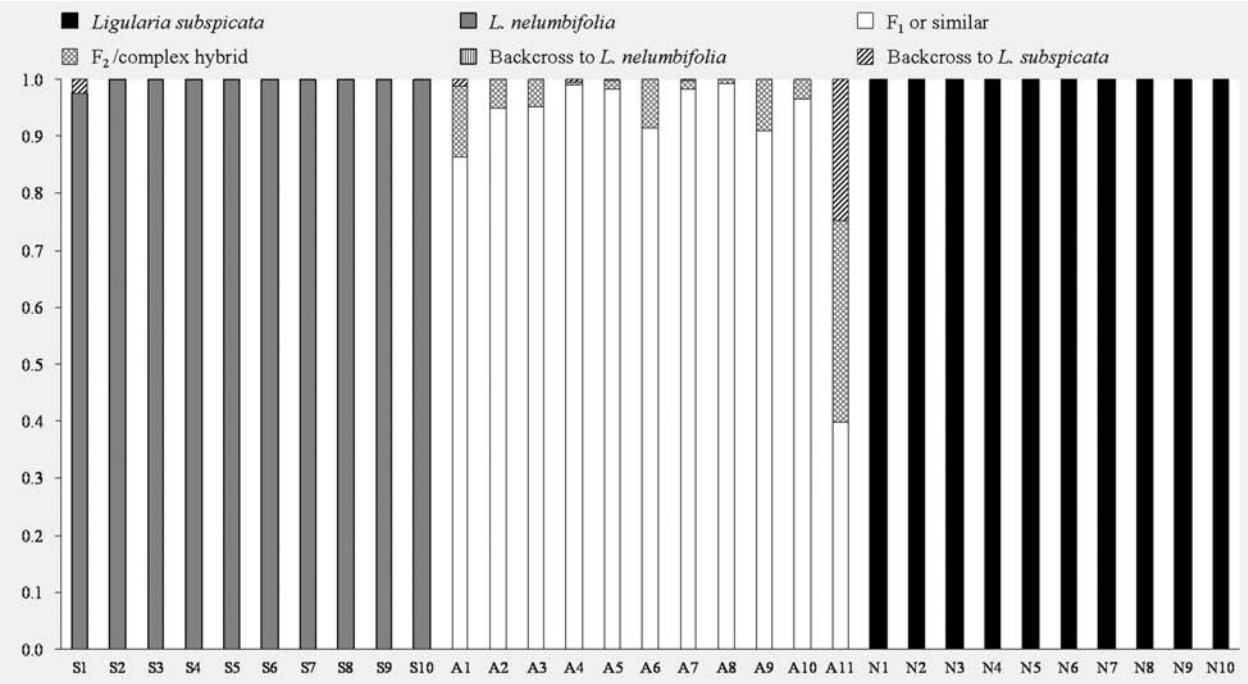

Fig. 4. Output from NewHybrids indicating the individual posterior probability of belonging to each parental or hybrid class for each accession. Taxa: A, putative hybrid; N, *Ligularia nelumbifolia*; S, *L. subspicata.* Numbers following taxon initials are sample numbers.

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Supplementary Material

The following supplementary material is available in the online version of this article:

Table S1 ISSR data for NewHybrids.

Table S2 Result of NewHybrids analysis indicating individual posterior probability of belonging to each parental or hybrid class for each accession.

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