Molecular evidence for asymmetric hybridization in three closely related sympatric species

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Received: 23 January 2017  Editorial decision: 5 February 2018  Accepted: 7 February 2018  Published: 9 February 2018

Associate Editor: Silvia Castro

Citation: Zhang N-N, Yu J-J, Wang Y-H, Gong X. 2018. Molecular evidence for asymmetric hybridization in three closely related sympatric species. AoB PLANTS 10: ply011; doi: 10.1093/aobpla/ply011

Abstract. Natural hybridization is common in plants and results in different evolutionary consequences to hybridizing species. Pre- and post-zygotic reproductive isolating barriers can impede hybridization between closely related species to maintain their species integrity. In Northwest Yunnan, three Ligularia species (Ligularia cyathiceps, L. duciformis and L. yunnanensis) and four types of morphologically intermediate individuals were discovered growing together in an area subject to human disturbance. In this study, we used three low-copy nuclear loci to test the natural hybridization hypothesis and the hybridization direction was ascertained by three chloroplast DNA fragments. The results indicated there were two hybridization groups at the study site, L. cyathiceps × L. duciformis and L. duciformis × L. yunnanensis, and two types of morphologically intermediate individuals were produced by L. cyathiceps and L. duciformis, and another two types were produced by L. duciformis and L. yunnanensis, while no hybrids between L. cyathiceps and L. yunnanensis were observed. Both hybridizing groups showed bidirectional but asymmetric hybridization and the factors influencing the symmetry are discussed. Most hybrids produced by the two hybridization groups seemed to be F1 generation. Hybrids with different morphologies within the same hybridization group showed similar genetic components. The results suggest that although human disturbance may promote natural hybridization among the three Ligularia species bringing them together, hybrids are limited to F1s and therefore species boundaries might be maintained.

Keywords: Asymmetric hybridization; chloroplast DNA; F1 generation; Ligularia; nuclear loci; two hybridization groups.

Introduction

Natural hybridization is common across plants, particularly in rapidly radiating groups (Mallet 2005), and can result in both positive and negative evolutionary outcomes (Barton 2001). Hybridization can generate new taxa through homoploid or allopolyploid hybrid speciation; however, it can also reduce the species diversity by blurring species boundaries, especially if introgression occurs (Wang et al. 2001; Ramsey and Schemske 2002; Mallet 2007; Schneider et al. 2011; Beatty et al. 2014). Species integrity is maintained by pre- and post-zygotic reproductive isolating barriers preventing...
hybridization (Dickinson et al. 2012). However, human disturbance is regarded as an important promoter of hybridization (Anderson 1948; Bleeker and Hurka 2001), and previously isolated species may come into contact and hybridize due to human alterations to the environment (Rhymer and Simberloff 1996; Seehausen et al. 2008; Crispo et al. 2011; Bohling et al. 2016). Human disturbance has been proved to increase hybridization rates in some plants, such as breaking geographical barriers and promoting biological invasions (Thompson et al. 2010), changing fire regimes (Ortego et al. 2017) or even changing biological attributes as phenology (Lamont et al. 2003). When hybridization happens, the direction of hybridization is affected by both pre- and post-zygotic barriers and asymmetric hybridization frequently occurs in plants as a result of differences in the strength of reproductive barriers between hybridizing species (Bacilieri et al. 1996; Arnold 1997; Ma et al. 2014; Zhang et al. 2016).

*Ligularia*, a highly diversified genus belonging to Senecioneae (Asteraceae), is comprised of about 140 species distributed in Asia and Europe (Liu and Illarionova 2011) and its major distribution centre is located in Hengduan Mountains (Liu et al. 1994). Natural hybridization is frequent in *Ligularia* and natural hybrids are commonly found in areas of sympatry (Liu et al. 2006). Pan et al. (2008) firstly reported *Ligularia × maoniushanensis* was a natural hybrid produced by *Ligularia paradoxa* and *Ligularia duciformis* in Yunnan, China. Yu et al. (2011, 2014a, b) proved natural hybridization of *Ligularia nelumbifolia* and *Ligularia subspicata*, *Ligularia vellerea* and *L. subspicata* and between *Ligularia cymbulifera* and *Ligularia tongolensis* by using the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA and chloroplast DNA (cpDNA) fragments. Moreover, studies on chemical compounds combined with nuclear ribose ITS sequence also confirmed natural hybridization of *L. nelumbifolia* and *L. subspicata*, and of *L. cymbulifera* and *L. tongolensis* (Hanai et al. 2012; Shimizu et al. 2016). In all the cases described above, natural hybridization usually forms complex hybrid swarms and gene introgression between parental species, which may blur species boundaries between hybridizing species.

During the field investigation in Tianchi (Shangri-La, Yunnan), a place severely disturbed by farming, deforestation and tourism, three *Ligularia* species (*Ligularia cyathiceps*, *L. duciformis* and *L. yunnanensis*) were found growing together and four types of morphologically intermediate individuals (Type A, B, C and D) were discovered. According to morphological distinction, it is assumed that there are two hybridization groups, i.e. Type A and B individuals are presumed to be hybrids of *L. cyathiceps* and *L. duciformis*, while Type C and D individuals are supposed to be hybrids of *L. duciformis* and *L. yunnanensis*. In spite of frequently reported studies on natural hybridization of *Ligularia* in recent years, there is no report on the complicated relationships at the morphologically diverse hybrid zone described above involving three putative parents.

Natural hybrids can show various morphological characteristics, such as parental-like, intermediate or novel traits, and morphological evidence alone is inadequate in the identification of hybrids (Schneider et al. 2011). Molecular techniques can provide more powerful evidence for natural hybridization, and low-copy nuclear genes have been proved to be efficient in solving problems of natural hybridization (Zhang et al. 2013; Fan et al. 2014; Liao et al. 2015). Particularly, the utility of nuclear genes in previous studies is limited to nuclear ribosome ITS region, which have showed disadvantages such as not always tracking both parents’ genomes in hybrids (Jupe and Zimmer 1993) and amplifying pseudogenes or fungal ITS spacers (Buckler and Holtsford 1996; Muir et al. 2001; Song et al. 2012). In this study, three low-copy nuclear loci (A12, B14 and D30) and three chloroplast intergenic spacers (psbA-trnH, trnL-rpl32 and trnQ-5′-rps16) were used to explore the relationships among *L. cyathiceps*, *L. duciformis*, *L. yunnanensis* and all the morphologically intermediate individuals observed in the contact zone. Our aims were to (i) identify if morphologically intermediate individuals are produced by hybridization between the three coexisting species and decouple the occurrence of two natural hybridization groups suggested by the morphologically intermediate individuals between *L. cyathiceps* and *L. duciformis* by one side and between *L. duciformis* and *L. yunnanensis* by the other side; (ii) if hybridization is confirmed, assess the direction of natural hybridization; and (iii) compare the consequences of two putative hybridization groups and their influence to three putative parental species.

**Methods**

**Study species and plant sampling**

The study site is located in Tianchi, Shangri-La, Yunnan, China (27°37.339′N, 99°38.151′E, 3901 m a.s.l.), where *L. cyathiceps*, *L. duciformis* and *L. duciformis* distribute sympatrically. *Ligularia cyathiceps* and *Ligularia yunnanensis* are two alpine species endemic to Northwest Yunnan with altitudes from 3000 m to 4000 m a.s.l. (Liu and Illarionova 2011). *Ligularia duciformis* distributes widely in West China and grows at altitudes varying from 1900 m to 4300 m a.s.l. (Liu and Illarionova 2011). *Ligularia duciformis* and *L. yunnanensis* belong to the series Retuseae, section Corymbosae, and *L. cyathiceps* is a member of series Ligularia, section Ligularia (Liu 1989). These three species are all diploids with somatic chromosome number 2n = 58 (Pan et al. 2004, 2008).
According to the morphological descriptions in Flora of China (Liu and Illarionova 2011), L. cyathiceps and L. duciformis mainly differ in leaf size, dentate leaf margin, capitula arrangement and presence or absence of ray florets, while L. duciformis and L. yunnanensis have major differences in leaf size, dentate leaf margin, inflorescence branches and indumentum. The diagnostic morphological traits used to identify the species are presented in Table 1 and illustrated in Fig. 1, including the four morphologically intermediate types (Type A, B, C and D) that do not fit in the description of any of the species. Moreover, L. cyathiceps, L. duciformis and all putative hybrids prefer open and sunny habitats and occupy disturbed hillsides and roadsides, whereas L. yunnanensis likes shady and humid environment and is found at intact habitats below the canopy of trees.

A total of 148 individuals were sampled for molecular analysis. For three species L. cyathiceps, L. duciformis and L. yunnanensis, each of 20 individuals were collected. For four morphologically intermediate types, we sampled all the individuals with intermediate morphology and number of sampled individuals was detailed in Table 1. Healthy leaves from each individual were collected and stored in plastic bags with silica gel until DNA extraction. Voucher specimens were deposited in the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN) and voucher numbers are detailed in Table 1.

**DNA extraction, PCR amplification and sequencing of DNA sequences**

Total genomic DNA was extracted from dried leaves using the modified CTAB method (Doyle 1991). We amplified nearly all the universal markers applied to Asteraceae family (Chapman et al. 2007) and finally obtained three low-copy nuclear loci A12, B14 and D30, which could be amplified and sequenced successfully with variable sites in the investigated individuals. Primers for A12, B14 and D30 developed by Chapman et al. (2007) were used in the present study. For B14 and D30 loci, internal primers were designed to obtain complete sequences of some individuals. Designed primers for B14 and D30 were LB14F: 5′ AACGCRTACCTTTCCAACG 3′, LB14R: 5′ TCYGTCGCATTCTCCCTTC 3′ and LD30F: 5′ AATGTTCAGATTTTGGTTAT 3′, LD30R: 5′ CTAGGTGAAATCTGTGTGC 3′, respectively. PCR conditions followed Chapman et al. (2007). Some individuals, especially from putative hybrids, had superimposed chromatograms at multiple sites and cloning sequencing was used to phase the haplotypes. Ligations were conducted using the

| Table 1. Sampling details and morphologically diagnostic characteristics for L. cyathiceps, L. duciformis, L. yunnanensis and putative hybrids. |
|---|---|---|---|---|
| Taxon | No. of individuals (ID) | Voucher | Leaf size | Leaf blade margin | Inflorescence hybrids |
| L. cyathiceps (Lc) | 20 (C1–20) | PG140805 | 8.5–13 × 10.5–22 cm | Coarsely dentate, apex rounded | Racemose, tubular florets, with several ray florets |
| Type A | 15 (F1–15) | PG140816 | Intermediate between Lc and Ld | Coarsely dentate, apex rounded | Compound corymb, tubular florets, with several ray florets |
| Type B | 9 (T1–9) | PG140808 | Intermediate between Lc and Ld | Coarsely dentate, apex rounded | Compound corymb, all tubular florets |
| L. duciformis (Ld) | 20 (D1–20) | PG140813 | 5–16 × 7–50 cm | Denticulate, apex retuse | Compound corymb, all tubular florets, branches spreading, pubescent |
| Type C | 30 (H1–30) | PG140814 | Intermediate between Ld and Ly | Denticulate, apex retuse | Compound corymb, branches spreading relatively, shortly brown pilose |
| Type D | 34 (S1–34) | PG140819 | Intermediate between Ld and Ly | Denticulate, apex retuse | Compound corymb, branches spreading relatively, pubescent |
| L. yunnanensis (Ly) | 20 (Y1–20) | PG140802 | 3–6.5 × 7–11 cm | Coarsely triangular-dentate, apex rounded | Corymb, branches shorter, fasciated, shortly brown pilose |
pMD19-T Vector cloning kit (Takara, Dalian, China). Two to eight positive clones for each individual were selected for sequencing.

Three chloroplast intergenic spacers psbA–trnH, trnL–rpl32 and trnQ–5′rps16 were amplified using universal primers (Song et al. 1997; Shaw et al. 2007). The PCR amplification was carried out in 20 μL reaction volume, containing 20 ng genomic DNA, 2.0 μL 10× PCR buffer, 1.0 μL MgCl₂ (25 mM), 1.0 μL dNTPs (10 mM), 1.0 μL BSA (20 g/L), 0.2 μL Taq DNA polymerase (5 U/μL) (Takara, Shiga, Japan), 0.5 μL of each primer and 12.3 μL double-distilled water. PCR was conducted in a thermocycler with the following conditions: an initial 5 min denaturation at 80 °C, followed by 30 cycles of 45 s at 94 °C, 45 s annealing at 53 °C, 50 s extension at 72 °C and a final extension for 7 min at 72 °C. All PCR products were purified by electrophoresis with a 1.2 % agarose gel and then a Pearl Gel Extraction Kit (Pearl Biotech, Guangzhou, China) was used. Then, they were sequenced in both directions with the amplification primers using an ABI 3730 DNA automated sequencer with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA).

**Data analysis**

All sequences were edited and assembled in SeqMan™ (DNASTAR, Madison, WI, USA). Multiple alignments were performed manually with Geneious Pro version 4.8.2 ( Biomatters Ltd, Auckland, New Zealand). For three low-copy nuclear loci, haplotype inference was implemented with PHASE in DnaSP version 5.0 (Rozas et al. 2003). A congruency test for three combined cpDNA intergenic spacers showed a significant rate of homogeneity ($P > 0.5$) by PAUP*4.0b10 (Swofford 2002), indicating a high degree of homogeneity. Haplotypes of the combined chloroplast sequences were inferred using DnaSP 5.0. Haplotype network was constructed for each nuclear locus and combined cpDNA region using Network version 5.0.0.0 (Forster et al. 2007) with the median-joining algorithm (Bandelt et al. 1999). Indels were treated as single mutational events in network analysis.

**Results**

**Sequence analysis of nuclear loci—A12 locus**

The aligned A12 region was 257 bp in length for all the investigated individuals (for variation sites, see Supporting Information—Table S1). A total of six haplotypes were observed, and low levels of haplotype polymorphism were observed in *L. cyathiceps*, *L. duciformis* and *L. yunnanensis* which had two (cA1–2), two (dA1–2) and two (yA1) haplotypes, respectively (Table 2; Fig. 2A). Haplotypes of *L. cyathiceps*, *L. duciformis* and *L. yunnanensis* generated three clusters (cluster I, II and III) in haplotype network analysis, in which *L. cyathiceps* (cluster I) and *L. duciformis* (cluster II) were separated by six nucleotide substitutions and *L. duciformis* (cluster II) and *L. yunnanensis* (cluster III) were separated by five nucleotide substitutions (Fig. 2A).

For the putative hybrids of *L. cyathiceps* and *L. duciformis* (Type A and B), all individuals but one (T7) showed two divergent haplotypes (cA1/dA1 and cA2/dA1) originated from *L. cyathiceps* and *L. duciformis*, and the haplotype of individual T7 was a combination of two haplotypes (cA1/UN1) found in *L. cyathiceps* cluster (Table 3). For the putative hybrids of *L. duciformis* and *L. yunnanensis* (Type C and D), all individuals but six (H12, S1, S4, S8, S16 and S32) had combined haplotypes (dA1/yA1 and dA2/yA1) nested in clusters of *L. duciformis* and *L. yunnanensis* (Table 3). Individuals S8 and S16 were homozygous for a *L. duciformis* haplotype (dA1/dA1), while the other four individuals H12, S1, S4 and S32 were homozygous for a *L. yunnanensis* haplotype (yA1/yA1).

**Sequence analysis of nuclear loci—B14 locus**

The length of aligned B14 fragment was 466 bp with one 2-bp indel distinguishing *L. duciformis* from *L. yunnanensis* (for variation sites, see Supporting Information—Table S2). There were 21 haplotypes detected in total, of which 7 (cB1–7), 1 (dB1) and 4 (yB1–4) haplotypes were from *L. cyathiceps*, *L. duciformis* and *L. yunnanensis*, respectively (Table 2; Fig. 2B). Three clusters (cluster I, II and III) formed by haplotypes of *L. cyathiceps*, *L. duciformis* and *L. yunnanensis* were identified in haplotype network analysis, in which *L. cyathiceps* (cluster I) and *L. duciformis* (cluster II) were separated by six nucleotide substitutions and *L. duciformis* (cluster II) and *L. yunnanensis* (cluster III) were separated by three nucleotide substitutions (Fig. 2B). For the putative hybrids of *L. cyathiceps* and *L. duciformis* (Type A and B), all individuals but one (F14) had two divergent haplotypes (cB1/dB1, cB2/dB1, cB3/dB1, cB4/dB1, dB1/UN2 and cB3/UN1) identified from *L. duciformis* and *L. cyathiceps* clusters, respectively (Table 3). Individual F14 is homozygous with the same haplotype of *L. duciformis* (dB1/dB1). Haplotypes for the putative hybrids of *L. duciformis* and *L. yunnanensis* (Type C and D) showed higher polymorphism with four types of haplotype composition as follows (Table 3): (i) The majority of individuals possessed two divergent haplotypes (dB1/yB1, dB1/yB2, dB1/UN7, dB1/UN8 and dB1/UN9), each nested in clusters of *L. duciformis* and *L. yunnanensis*, respectively. (ii) Eight individuals (H1, H4, H11, H13, H18, S16, S31 and S34) were homozygous for a *L. duciformis* haplotype (dB1/dB1). (iii) Six individuals (H16, H17, S1, S4, S21 and S32) were homozygous...
for one of L. yunnanensis haplotypes (yB1/yB1 and yB2/yB2). (iv) Nine individuals (H8, H12, H28, H30, S8, S11, S12, S14 and S33) showed mixed haplotypes (yB1/yB2, yB1/UN3, yB1/UN4, yB1/UN5 and UN5/UN6) originated from L. yunnanensis cluster.

Sequence analysis of nuclear loci—D30 locus

The fragment D30 was 504 bp long after sequence alignment with one 1-bp indel distinguishing L. cyathiceps from L. duciformis and one 2-bp indel differing L. duciformis from L. yunnanensis (for variation sites, see Supporting Information—Table S3). There were nine haplotypes identified in all individuals, of which four (cD1–4), one (dD1) and one (yD1) haplotypes were from L. cyathiceps, L. duciformis and L. yunnanensis, respectively (Table 2; Fig. 2C). In haplotype network analysis, three clusters (cluster I, II and III) were generated evidently by haplotypes of L. cyathiceps, L. duciformis and L. yunnanensis, respectively, in which L. cyathiceps (cluster I) and L. duciformis (cluster II) were separated by seven nucleotide substitutions and L. duciformis (cluster II) and L. yunnanensis (cluster III) were separated by 13 nucleotide substitutions (Fig. 2C).

For the putative hybrids of L. cyathiceps and L. duciformis (Type A and B), all individuals had two divergent haplotypes (cD1/dD1, cD2/dD1, cD3/dD1, cD4/dD1, dD1/UN1, dD1/UN2 and dD1/UN3) identified from L. cyathiceps and L. duciformis clusters, respectively (Table 3). For the putative hybrids of L. duciformis and L. yunnanensis (Type C and D), all individuals but seven (H12, H13, H26, S1, S8, S16 and S32) possessed combined haplotypes of L. duciformis and L. yunnanensis (dD1/yD1) (Table 3). Individuals H26, S8 and S16 were homozygous for the L. duciformis haplotype (dD1/dD1), whereas individuals H12, H13, S1 and S32 were homozygous for the L. yunnanensis haplotype (yD1/yD1).

Sequence analysis of cpDNA fragments

The combined length of aligned cpDNA fragments (psbA–trnH, trnl–rpl32 and trnQ–5′ rps16) was 2214 bp with 27 polymorphic sites and seven indels [see Supporting Information—Table S4]. Nine haplotypes were inferred in total, of which three (cP1–3), one (dP1) and three (yP1–3) haplotypes were from L. cyathiceps, L. duciformis and L. yunnanensis, respectively (Table 2; Fig. 2D). Haplotype network analysis indicated all three L. cyathiceps haplotypes (cP1–3) grouped into one cluster (cluster I), whereas two L. yunnanensis haplotypes (yP2–3) and the L. duciformis haplotype (dP1) formed into another cluster (cluster II) and another L. yunnanensis haplotype (yP1) was in the third cluster (cluster III) (Fig. 2D). Clusters I and II were separated by 23 nucleotide substitutions and clusters II and III were separated by 15 nucleotide substitutions.

For the putative hybrids of L. cyathiceps and L. duciformis (Type A and B), most (17 of 24) individuals had the same L. duciformis haplotype (dP1), and six individuals (F3, F11, T2, T3, T4 and T7) had haplotypes consistent with L. cyathiceps (cP1, cP2 and cP3) (Table 3). Individual F15 had a unique haplotype (UN1) with two nucleotide substitutions differed from the common L. cyathiceps haplotypes (cP1 and cP2). For the putative hybrids of L. duciformis and L. yunnanensis (Type C and D), most (53 of 64) individuals possessed haplotypes of L. yunnanensis (yP1) and five individuals (H3, H15, S6, S16 and S26) had haplotypes of L. duciformis (dP1) (Table 3). The other six individuals (H13, H22, H26, S24, S25 and S30) had a unique haplotype (UN2) differed from the haplotype of L. yunnanensis (yP1) with one mutation step. Genotypes at three low-copy nuclear loci and combined cpDNA fragments for all the investigated individuals are listed in Supporting Information—Table S5.

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### Table 2. Haplotypes for three Ligularia species and putative hybrids of three nuclear loci and combined cpDNA fragments.

| Taxon                  | A12  | B14  | D30              | cpDNA     |
|------------------------|------|------|------------------|-----------|
| L. cyathiceps          | cA1, cA2 | cB1, cB2, cB3, cB4, cB5, cB6, cB7 | cD1, cD2, cD3, cD4 | cp1, cp2, cp3 |
| Type A                 | cA1, cA2, dA1 | cB1, cB2, cB3, cB4, dB1 | cD1, cD2, cD3, cD4, dD1, UN1, UN2, UN3 | cp2, dp1, UN1 |
| Type B                 | cA1, dA1, UN  | cB1, cB2, cB3, cB4, dB1, UN1, UN2 | cD1, cD3, dD1, UN2 | cp1, cp2, cp3, dp1 |
| L. duciformis          | dA1, da2 | dB1  | dD1              | dP1       |
| Type C                 | dA1, da2, yA1 | dB1, yB1, yB2, UN3, UN5, UN9 | dD1, yD1 | yP1, UN2 |
| Type D                 | dA1, da2, yA1 | dB1, yB1, yB2, UN4, UN5, UN6, UN7, UN8 | dD1, yD1 | dP1, yP1, UN2 |
| L. yunnanensis         | yA1  | yB1, yB2, yB3, yB4 | yD1  | yP1, yP2, yP3 |
| Total haplotype number | 6    | 21   | 9                | 9         |

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Discussion

Natural hybridization among *L. cyathiceps*, *L. duciformis* and *L. yunnanensis* from molecular evidence

In this study, we sequenced three low-copy nuclear loci and three cpDNA fragments to assess natural hybridization between *L. cyathiceps*, *L. duciformis* and *L. yunnanensis* in an area of contact in Tianchi, Shangri-La, Yunnan where the three species occur together. Our results suggested that the endemic *L. cyathiceps* showed relatively higher haplotype diversity than widely distributing *L. duciformis*, indicating high genetic diversity of *L. cyathiceps* at the study site. In addition, *L. duciformis* and *L. yunnanensis* showed closer genetic distance in two nuclear loci (A12 and B14) and combined cpDNA data, particularly in cpDNA data where two *L. yunnanensis* haplotypes grouped with the *L. duciformis* haplotype. These observations are consistent with the

Table 3. Haplotype combination for the putative hybrids of three nuclear loci and combined chloroplast fragments. UN means unique haplotype to the putative hybrids and haplotype names is in concordance with those in Fig. 2.

| Locus | Haplotype combination | Number of individuals of the putative hybrids | Haplotype combination | Number of individuals of the putative hybrids |
|-------|------------------------|-----------------------------------------------|------------------------|-----------------------------------------------|
|       |                        | Type A | Type B |                        | Type A | Type B |
| A12   | cA1/dA1                | 14     | 8      | dA1/yA1                | 26     | 27     |
|       | cA2/dA1                | 1      | 0      | dA2/yA1                | 3      | 2      |
|       | cA1/UN1                | 0      | 1      | dA1/dA1                | 0      | 2      |
|       | –                      | –      | –      | yA1/yA1                | 1      | 3      |
| B14   | cB1/dB1                | 1      | 3      | dB1/yB1                | 18     | 19     |
|       | cB2/dB1                | 2      | 1      | dB1/yB2                | 0      | 1      |
|       | cB3/dB1                | 6      | 2      | dB1/UN7                | 0      | 1      |
|       | cB4/dB1                | 5      | 1      | dB1/UN8                | 0      | 1      |
|       | dB1/UN2                | 0      | 1      | dB1/UN9                | 1      | 0      |
|       | dB1/UN1                | 0      | 1      | yB1/UN3                | 1      | 0      |
|       | –                      | –      | –      | yB1/UN4                | 0      | 1      |
|       | –                      | –      | –      | yB1/UN5                | 2      | 3      |
|       | –                      | –      | –      | UN5/UN6                | 0      | 1      |
|       | –                      | –      | –      | dB1/dB1                | 5      | 3      |
|       | –                      | –      | –      | yB1/yB1                | 1      | 4      |
|       | –                      | –      | –      | yB1/yB2                | 1      | 0      |
|       | –                      | –      | –      | yB2/yB2                | 1      | 0      |
| D30   | cD1/dD1                | 9      | 3      | dD1/yD1                | 27     | 30     |
|       | cD2/dD1                | 1      | 0      | dD1/dD1                | 1      | 2      |
|       | cD3/dD1                | 1      | 4      | yD1/yD1                | 2      | 2      |
|       | cD4/dD1                | 1      | 0      | –                      | –      | –      |
|       | dD1/UN1                | 1      | 0      | –                      | –      | –      |
|       | dD1/UN2                | 1      | 2      | –                      | –      | –      |
|       | dD1/UN3                | 1      | 0      | –                      | –      | –      |
| cpDNA | dP1                    | 12     | 5      | yP1                    | 25     | 28     |
|       | cP1                    | 0      | 2      | dP1                    | 2      | 3      |
|       | cP2                    | 2      | 1      | UN2                    | 3      | 3      |
|       | cP3                    | 0      | 1      | –                      | –      | –      |
|       | UN1                    | 1      | 0      | –                      | –      | –      |

Table 3. Haplotype combination for the putative hybrids of three nuclear loci and combined chloroplast fragments. UN means unique haplotype to the putative hybrids and haplotype names is in concordance with those in Fig. 2.
morphological classification (Liu 1989) and preliminary molecular phylogenetic results (W.-Y. He and Y.-Z. Pan, Kunming Institute of Botany, Chinese Academy of Sciences, unpubl. data). Nevertheless, L. cyathiceps, L. duciformis and L. yunnanensis remained well separated in the haplotype network analysis, indicating their clear divergence from each other. In general, morphologically intermediate individuals Type A and B showed chromatogram additivity for L. cyathiceps and L. duciformis at most nuclear loci, while most Type C and D individuals for L. duciformis and L. yunnanensis, providing strong evidence for natural hybridization hypotheses above and for lack of hybridization between L. cyathiceps and L. yunnanensis. Additionally, the occurrence of unique haplotypes in putative hybrids of two hybridization groups may be intragenic recombination between haplotypes or caused by unsampled polymorphisms in parental individuals.

Pre- and post-zygotic barriers among L. cyathiceps, L. duciformis and L. yunnanensis

Different pre- and post-zygotic barriers can reduce potential cross-breeding and result in reproductive isolation between species pairs (Dobzhansky 1937; Grant 1981; Stace 1991; Field et al. 2011; Ma et al. 2016). Meanwhile, natural hybridization may occur between closely related species with incomplete pre- and post-zygotic barriers. Natural hybridization is often associated with disturbed habitats as human disturbance can disrupt ecological barriers and promote natural hybridization (Anderson 1948; Rieseberg and Carney 1998; Ma et al. 2010). Human disturbance can create intermediate habitat suitable for hybrids, promoting the maintenance of hybrid swarms in these habitats (Anderson 1948; Eilstrand and Schierenbeck 2000). In previous hybridization studies of Ligularia, hybrid zones often locate at roadsides, mountain slopes destroyed by fire and other areas subjected to human disturbance, suggesting that hybridization in Ligularia may be promoted by human activities (Pan et al. 2008; Yu et al. 2011, 2014a, b). In this study, once again, the hybrid zone is located in an area severely disturbed by human activities such as tree felling and grazing, supporting the observation of previous studies.

But, could pre- and post-zygotic barriers explain the hybridization patterns observed in this contact zone? The overlap of blooming periods provides the first condition for hybridization since it enables pollen movement by pollinator vectors. In the present study, both L. duciformis and L. cyathiceps flower from July to August, while L. yunnanensis flowers from May to August (Liu and Illarionova 2011). Moreover, Ligularia plants have generalized pollination system and about 10 insects belonging to three orders (Diptera, Lepidoptera and Hymenoptera) are the major pollinators (Liu 2002; Cao et al. 2008). Generalized pollinators shared between species offer opportunities for the pollen transfer. Thus, incomplete pre-zygotic barriers such as the overlap of blooming periods and generalized pollinators will largely contribute for natural hybridization of L. cyathiceps × L. duciformis and L. duciformis × L. yunnanensis.

Similar inflorescence arrangement may be another factor significantly contributing for the hybridization between L. duciformis and L. yunnanensis. These two species present similar arrangement of the capitula in corymb inflorescences. The generalized pollinators may tend to visit inflorescences with similar morphologies, thus promoting pollen transfer between these two species. Indeed, pollen transfer between species with similar inflorescence arrangement has been observed in natural hybrid zones of Ligularia (Pan et al. 2008; Yu et al. 2014b).

In addition, close relationship between L. duciformis and L. yunnanensis may also work as a less effective post-zygotic barrier to hybridization in closely related species. In previous studies, hybridization has been detected in Ligularia species pairs that are closely related, such as L. paradoxa and L. duciformis (Pan et al. 2008) and L. cymbulifera and L. tongolensis (Yu et al. 2014b). In the present study, L. duciformis and L. yunnanensis both belong to Series Retusae, Section Corymbosae (Liu 1989) and are closely related according to haplotype analysis (Results section in this study) and preliminary molecular phylogenetic study (W.-Y. He and Y.-Z. Pan, Kunming Institute of Botany, Chinese Academy of Sciences, unpubl. data).

Since L. cyathiceps and L. yunnanensis also possess the overlapping blooming periods and generalized pollinators, it seems that there are no pre-zygotic barriers reducing pollen transfer between them. The lack of hybrids between L. cyathiceps and L. yunnanensis may be attributed to post-zygotic barriers. Actually, sympatric species in Ligularia could coexist without hybridization, if they have undergone long isolation and accumulated enough mutations, as indicated by species in the Ligularia-Cremanthodium-Parasenecio (L–C–P) complex (Liu et al. 2006). In the network analysis of three low-copy nuclear loci and combined cpDNA fragments, L. cyathiceps and L. yunnanensis showed relatively higher genetic distance than each of them with L. duciformis. Although the genetic difference shown in the network analysis is limited, it may be caused by the restricted loci used in this study. Therefore, the accumulation of mutations between L. cyathiceps and L. yunnanensis may drive the species divergence, reduce interspecies crossability and/ or lower fitness of possible hybrids. Post-zygotic barriers...
Asymmetric hybridization of *L. cyathiceps* × *L. duciformis* and *L. duciformis* × *L. yunnanensis*

As *Ligularia* was proved to be chloroplast maternally inherited (Zhang et al. 2003), combined cpDNA fragments would predict the direction of natural hybridization. For the hybridization *L. cyathiceps* × *L. duciformis*, cpDNA data indicate *L. duciformis* is the maternal parent of most (70.83 %) putative hybrids, thus natural hybridization between *L. cyathiceps* and *L. duciformis* is bidirectional but asymmetric, and *L. duciformis* is the primary maternal parent. However, for the hybridization *L. duciformis* × *L. yunnanensis*, cpDNA results suggest that *L. yunnanensis* is the maternal parent of most (82.81 %) putative hybrids. Two hybridization groups show distinctive asymmetry in natural hybridization and different factors may be responsible for their asymmetry.

Differences in floral traits could drive differences in floral preferences and floral constancy of pollinators, which may affect the levels and direction of hybridization (Aldridge and Campbell 2007; Castro et al. 2011). This could be occurring, for example, between *L. duciformis* and *L. cyathiceps*. *Ligularia duciformis* have larger compound corymb inflorescences than the racemose inflorescences of *L. cyathiceps*; therefore, it would be likely that *L. duciformis* is more attractive to pollinators and acts as the maternal parent to accept pollen transferred from *L. cyathiceps*.

Contrarily, for *L. duciformis* and *L. yunnanensis*, having similar inflorescence traits, the asymmetric hybridization may be associated to the relative abundance of parental species. The prediction that the rare species, undergoing ‘pollen swamping’ by more abundant congeners, usually acts as the maternal parent, is confirmed by many examples in plants and animals (Arnold et al. 1993; Rieseberg 1995; Levin et al. 1996; Wirtz 1999; Lepais et al. 2009). At the present study site, *L. duciformis* occupies more habitat than *L. yunnanensis* occurring in intact habitat, and *L. duciformis* plants greatly outnumber *L. yunnanensis* plants, thus *L. yunnanensis* would be more likely the maternal parent.

Consequences of natural hybridization among *L. cyathiceps*, *L. duciformis* and *L. yunnanensis*

In the present study, most putative hybrid individuals in two hybridization groups show chromatogram additivity at all of three randomly selected nuclear loci, suggesting they might be F₁ s. Hybrids restricted to F₁ generation can impede gene flow between species and keep hybridizing species pairs reproductively isolated from each other (Milne et al. 2003; Milne and Abbott 2008; Twyford et al. 2015). Four morphologically intermediate individuals S16 and H12, S1, S32 are pure with haplotypes of *L. duciformis* and *L. yunnanensis*, respectively, at three nuclear loci. It might be unlikely that these homozygous individuals are caused by repetitive backcrossing with corresponding parents, since there is no occurrence of later-generation individuals. They may result from sampling confusion mistakes between hybrids and pure parents, indicating further morphological studies are needed in this area of contact. There are two morphology-differential types of hybrids produced by two hybridization groups, especially in the *L. cyathiceps* and *L. duciformis* hybridization group where Type A and Type B differ in the presence/absence of ray floret. Nevertheless, it is noteworthy that different types in these two hybridization groups show similar intra-group nuclear and chloroplast haplotype composition.

In previous reports on natural hybridization of *Ligularia*, hybrid swarms are common and introgression occurs between parental species (Yu et al. 2011, 2014a, b). Being a genus with high species diversity formed by rapid radiation (Liu et al. 2006), species in *Ligularia* may not be completely isolated reproductively and sympatric hybridization is expected to be frequent. However, the existence of F₁ hybrids without later-generation individuals prevents introgression and facilitates reproductive isolation among sympatric species. Moreover, the lack of later-generation hybrids seems to be the result of a fitness disadvantage of the hybrids produced by *L. cyathiceps* × *L. duciformis* and *L. duciformis* × *L. yunnanensis*, which is also a barrier to hybridization at a more advanced stage. Unlike the F₁-dominated hybrid zone in *Rhododendron* described by Milne et al. (2003), hybridization in this study might be promoted by human disturbance such as tree felling and grazing; however, post-zygotic barriers such as the sterility of F₁ s may contribute for no later-generation hybrids. Human disturbance can bring species into contact and trigger natural hybridization (Anderson 1948), and can furtherly promote hybridization through increasing opportunities for gene flow (Lamont et al. 2003; Zha et al. 2009; Thompson et al. 2010; Ortego et al. 2017). In the present study, although human disturbance might influence or promote the hybridization among the three *Ligularia* species studied, they may still keep their species distinctiveness and maintain reproductive isolation under the circumstance that hybridization takes place. Nevertheless, in the future studies, experiments such as controlled pollination crosses, seed germination...
and hybrid fitness examination need to be conducted to furtherly reveal the asymmetric hybridization and pre- and post-zygotic isolating barriers among three *Ligularia* species in this hybrid zone in the hotspot area of Northwest Yunnan.

**Conclusions**

The natural hybridization of *L. cyathiceps* × *L. duciformis* and *L. duciformis* × *L. yunnanensis* was confirmed based on three low-copy nuclear loci and three cpDNA fragments. In the two hybridization groups, most hybrids seem to be *F*₁, which suggests the maintenance of species boundaries between hybridizing species. There were no hybrids between *L. cyathiceps* and *L. yunnanensis*, which may be attributed to post-zygotic reproductive barriers such as hybrid inviability and sterility. Chloroplast DNA data indicated asymmetric hybridization, with *L. duciformis* as primary maternal parent in the *L. cyathiceps* × *L. duciformis* hybridization group, and *L. yunnanensis* for the *L. duciformis* × *L. yunnanensis* hybridization group. Pollinator preferences and the relative abundance...
of parental species may lead to asymmetric hybridization. Still, the three *Ligularia* species seem to maintain the species integrity in the studied sympatric area.

**Accession Numbers**

The data set of DNA sequencing data have been deposited in GenBank under accession numbers KX779147–KX779271.

**Sources of Funding**

This research was supported by the National Natural Science Foundation of China (grant no. 31600178 to J.-J.Y.).

**Contributions by the Authors**

X.G. and Y.-H.W. conceived and designed the experiments. X.G. and J.-J.Y. collected plant materials. N.-N.Z. performed the experiments, analysed the data and wrote the manuscript. X.G. and J.-J.Y. revised the manuscript. All authors read and approved the final manuscript.

**Conflict of Interest**

None declared.

**Acknowledgements**

We thank R. Zhang and Y. Zheng for their help in the plant materials collection. We thank Y.-P. Ma for his valuable comments on the manuscript. We thank editors and the anonymous reviewer for their helpful comments to improve the manuscript.

**Supporting Information**

The following additional information is available in the online version of this article—

*Table S1.* Variation sites of A12 locus for six haplotypes in all investigated individuals.

*Table S2.* Variation sites and indels of B14 locus for 21 haplotypes in all investigated individuals.

*Table S3.* Variation sites and indels of D30 locus for nine haplotypes in all investigated individuals.

*Table S4.* Variation sites and indels of three chloroplast fragments for nine haplotypes in all investigated individuals.
Table S5. Genotypes at three low-copy nuclear loci and combined cpDNA fragments for all the investigated individuals.

Literature Cited

Aldridge G, Campbell DR. 2007. Variation in pollinator preference between two Ipomopsis contact sites that differ in hybridization rate. Evolution 61:99–110.

Anderson E. 1948. Hybridization of the habitat. Evolution 2:1–9.

Arnold ML. 1997. Natural hybridization and evolution. Oxford: Oxford University Press.

Arnold ML, Hamrick JL, Bennett BD. 1993. Interspecific pollen competition and reproductive isolation in Iris. Journal of Heredity 84:13–16.

Bacilieri R, Ducouso A, Petit RJ, Kremer A. 1996. Mating system and asymmetric hybridization in a mixed-genotype European oaks. Evolution 50:900–908.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16:37–48.

Barton NH. 2001. The role of hybridization in evolution. Molecular Ecology 10:551–568.

Beatty GE, Barker L, Chen PP, Kelleher CT, Provan J. 2014. Cryptic introgression into the kidney saxifrage (Saxifraga hirsuta) from its more abundant sympatric congener Saxifraga spathularis, and the potential risk of genetic assimilation. Annals of Botany 115:179–186.

Bleeker W, Hurka H. 2001. Introgressive hybridization in Rorippa (Brassicaceae): gene flow and its consequences in natural and anthropogenic habitats. Molecular Ecology 10:2013–2022.

Bohling JH, Dellinger J, McVey JM, Cobb DT, Moorman CE, Waits LP. 2016. Describing a developing hybrid zone between red wolves and coyotes in Eastern North Carolina, USA. Evolutionary Applications 9:791–804.

Buckler ES, Holtsford TP. 1996. Zea ribosomal repeat evolution and substitution patterns. Molecular Biology and Evolution 13:623–632.

Cao Y, Ma RJ, Wang GX. 2008. The breeding system of three species of genus Ligularia in the east of Qinghai-Tibet Plateau. Guihua 28:302–306.

Castro S, Münzbergová Z, Raabová J, Loureiro J. 2011. Breeding barriers at a diploid–hexaploid contact zone in Aster amellus. Evolutionary Ecology 25:795–814.

Chapman MA, Chang J, Weisman D, Kesseli RV, Burke JM. 2007. Universal markers for comparative mapping and phylogenetic analysis in the Asteraceae (Compositae). Theoretical and Applied Genetics 115:747–755.

Costa CB, Lambert SM, Borda EL, de Queiroz LP. 2007. Postzygotic reproductive isolation between sympatric taxa in the Chamaecrista desvauxii complex (Leguminosae-Caesalpinioideae). Annals of Botany 99:625–635.

Crispo E, Moore JS, Lee-Yaw JA, Gray SM, Haller BC. 2011. Broken barriers: human-induced changes to gene flow and introgression in animals. BioEssays 33:508–518.

Dickinson GR, Lee DJ, Wallace HM. 2012. The influence of pre- and post-zygotic barriers on interspecific Corymbia hybridization. Annals of Botany 109:1215–1226.

Dobzhansky TH. 1937. Genetics and the origin of species. New York: Columbia University Press.

Doyle J. 1991. DNA protocols for plants-CTAB total DNA isolation. In: Hewitt GM, Johnston A, eds. Molecular techniques in taxonomy. Berlin: Springer, 283–293.

Ellstrand NC, Schierenbeck KA. 2000. Hybridization as a stimulus for the evolution of invasiveness in plants? Proceedings of the National Academy of Sciences of the United States of America 97:7043–7050.

Fan Q, Chen S, Li M, Guo W, Jing H, Wu W, Zhou R, Liao W. 2014. Molecular evidence for natural hybridization between wild loquat (Eriobotrya japonica) and its relative E. Pinoides. BMC Plant Biology 14:275.

Field DL, Ayre DJ, Whelan RJ, Young AG. 2011. The importance of pre-mating barriers and the local demographic context for contemporary mating patterns in hybrid zones of Eucalyptus aggregata and Eucalyptus rubida. Molecular Ecology 20:2367–2379.

Forster M, Forster P, Watson J. 2007. NETWORK (version 4.2.0.1): a software for population genetics data analysis. Clare: Fluxus Technology Ltd.

Grant V. 1981. Plant speciation, 2nd edn. New York: Columbia University Press. xi, 563p.+illus., maps. chrom. nos. 2nd edition. Maps, Chromosome numbers. General (KR, 198300748).

Hanai R, Yamada H, Suzuki Y, Nagano H, Kawahara T, Yu JJ, Gong X, Kuroda C. 2012. Chemical constituents of Ligularia nelumbifolia and L. Subspicata hybrid collected in Shangrila County, Yunnan Province of China. Natural Product Communications 7:1565–1568.

Jupe ER, Zimmer EA. 1993. DNasei-sensitive and undermethylated rDNA is preferentially expressed in a maize hybrid. Plant Molecular Biology 21:805–821.

Lamont BB, He T, Enright NJ, Krauss SL, Miller BP. 2003. Anthropogenic disturbance promotes hybridization between Banksia species by altering their biology. Journal of Evolutionary Biology 16:551–557.

Lepais O, Petit RJ, Guichoux E, Lovabre JE, Alberto F, Kremer A, Gerber S. 2009. Species relative abundance and direction of introgression in oaks. Molecular Ecology 18:2228–2242.

Levin DA, Francisco-Ortega J, Jansen RK. 1996. Hybridization and the extinction of rare plant species. Conservation Biology 10:10–16.

Liao RL, Ma YP, Gong WC, Chen G, Sun WB, Zhou RC, Marczewski T. 2015. Natural hybridization and asymmetric introgression at the distribution margin of two Buddleja species with a large overlap. BMC Plant Biology 15:146.

Liu SW, Deng DS, Liu JQ. 1994. Origin, evolution and distribution of Ligularia Cass. (Compositae). Acta Phytotaxonomica Sinica 32:514–524.

Liu SW, Illariova ID. 2011. Ligularia. Flora of China 20:371–544.

Liu JQ, Wang YJ, Wang AL, Hideoki O, Abbott RJ. 2006. Radiation and diversification within the Ligularia-Cremastium-Parasenecio complex (Asteraceae) triggered by uplift of the Qinghai-Tibetan Plateau. Molecular Phylogenetics and Evolution 38:31–49.

Ma YP, Xie WJ, Sun WB, Marczewski T. 2016. Strong reproductive isolation despite occasional hybridization between a widely distributed and a narrow endemic Rhododendron species. Scientific Reports 6:19146.
Ortego J, Gugger PF, Sork VL. 2017. Impacts of human-induced Z
species – Natural hybridization in three Ligularia
Muir G, Fleming CC, Schlötterer C. 2001. Three divergent rDNA clusters
Milne RI, Terzioglu S, Abbott RJ. 2003. A hybrid zone dominated by
Seehausen O, Takimoto G, Roy D, Jokela J. 2008. Speciation reversal
Sang T, Crawford D, Stuessy T. 1997. Chloroplast DNA phyl-
Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R. 2003. Dnasp,
Rieseberg LH. 1995. The role of hybridization in evolution: old wine
Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants.
Pan YZ, Shi SH, Gong X, Kuroda C. 2008. A natural hybrid between
Pan YZ, Gong X, Yang ZY, Yin Q. 2004. Karyological studies on five
Ma YP, Xie WJ, Tian XL, Sun WB, Wu ZK, Milne RI. 2014. Unidirectional
Ma YP, Zhang CQ, Zhang JL, Yang JB. 2010. Natural hybridization
Zhang
Zha HG, Milne RI, Sun H. 2009. Asymmetric hybridization in
Ma YP, Zhang CQ, Zhang JL, Yang JB. 2010. Natural hybridization
between Rhododendron delavayi and R. Cyanocarpum (Ericaceae), from morphological, molecular and reproductive evidence. Journal of Integrative Plant Biology 52:844–851.
Mallet J. 2005. Hybridization as an invasion of the genome. Trends in Ecology & Evolution 20:229–237.
Mallet J. 2007. Hybrid speciation. Nature 446:279–283.
Milne RI, Abbott RJ. 2008. Reproductive isolation among two interfertile Rhododendron species: low frequency of post-F1 hybrid genotypes in alpine hybrid zones. Molecular Ecology 17:1108–1121.
Milne RI, Terzioglu S, Abbott RJ. 2003. A hybrid zone dominated by fertile F1s: maintenance of species barriers in Rhododendron. Molecular Ecology 12:2719–2729.
Muir G, Fleming CC, Schlötterer C. 2001. Three divergent rDNA clusters predate the species divergence in Quercus petraea (Matt.) Liebl. and Quercus robur L. Molecular Ecology 18:112–119.
Ortego J, Gugger PF, Sork VL. 2017. Impacts of human-induced environmental disturbances on hybridization between two eco-
logically differentiated Californian oak species. New Phytologist 213:942–955.
Pan YZ, Gong X, Yang ZY, Yin Q. 2004. Karyological studies on five species of the genus Ligularia (Compositae: Senecioneae). Acta Botanica Yunnanica 26:65–72.
Pan YZ, Shi SH, Gong X, Kuroda C. 2008. A natural hybrid between Ligularia paradoxa and L. duciformis (Asteraceae, Senecioneae) from Yunnan, China. Annals of the Missouri Botanical Garden 95:487–494.
Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants.
Annual Review of Ecology and Systematics 33:589–639.
Rhymer JM, Simberloff D. 1996. Extinction by hybridization and introgression. Annual Review of Ecology and Systematics 27:83–109.
Rieseberg LH. 1995. The role of hybridization in evolution: old wine in new skins. American Journal of Botany 82:944–953.
Rieseberg LH, Corney SE. 1998. Plant hybridization. New Phytologist 140:599–624.
Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R. 2003. Dnasp, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19:2496–2497.
Sang T, Crawford D, Stuessy T. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). American Journal of Botany 84:1120.
Schneider JV, Schulte K, Aguilar JF, Huertas ML. 2011. Molecular evidence for hybridization and introgression in the neotropical coastal desert-endemic Palaua (Malveae, Malvaceae). Molecular Phylogenetics and Evolution 60:373–384.
Seehausen O, Takimoto G, Roy D, Jokela J. 2008. Speciation reversal and biodiversity dynamics with hybridization in changing envi-
Shaw J, Lickey EB, Schilling EE, Small RL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. American Journal of Botany 94:275–288.
Shimizu A, Hanai R, Okamoto Y, Turi M, Yu JJ, Gong X, Kuroda C. 2016. Chemical constituents in hybrids of Ligularia tongolensis and L. cymbullifera: chemical introgression in L. tongolensis. Chemistry & Biodiversity 13:837–844.
SongHX,GaoSP,JiangMY,LiuGL,YuXF,ChenQB.2012. The evolution and utility of ribosomal ITS sequences in Bambusinae and related species: divergence, pseudogenes, and implications for phylogeny. Journal of Genetics 91:129–139.
Stace CA. 1991. Plant taxonomy and biosystematics. Cambridge: Cambridge University Press.
Swafford DL. 2002. PAUP*: phylogenetic analysis using parsimony (* and other methods), version 4.0 b10. Sunderland, MA: Sinauer Associates.
Thompson SL, Lamothe M, Meirmans PG, Péretin P, Isabel N. 2010. Repeated unidirectional introgression towards Populus balsamifera in contact zones of exotic and native poplars. Molecular Ecology 19:132–145.
Twyford AD, Kidner CA, Ennos RA. 2015. Maintenance of species boundaries in a Neotropical radiation of Begonia. Molecular Ecology 24:4982–4993.
Wong XR, Szmidt AE, Savolainen O. 2001. Genetic composition and diploid hybrid speciation of a high mountain pine, Pinus densata, native to the Tibetan plateau. Genetics 159:337–346.
Wirtz P. 1999. Mother species-father species: unidirectional hybridization in animals with female choice. Animal Behaviour 58:1–12.
Yu JJ, Kuroda C, Gong X. 2011. Natural hybridization and introgression in sympatric Ligularia species (Asteraceae, Senecioneae). Journal of Systematics and Evolution 49:438–448.
Yu J, Kuroda C, Gong X. 2014a. Natural hybridization and introgression between Ligularia cymbullifera and L. tongolensis (Asteraceae, Senecioneae) in four different locations. PLoS One 9:e115167.
Yu JJ, Li P, Pan YZ, Xun G. 2014b. Natural hybrids between Ligularia vellerea and L. subspicata (Asteraceae: Senecioneae). Plant Diversity and Resources 36:219–226.
Zhang RS, Liu T, Wu W, Li YQ, Chao LF, Huang LS, Huang YL, Shi GL, Yang JB. 2010. Natural hybridization and introgression despite pre- and post-pollination reproductive barriers between two Silene species. AoB Plants 8:plw032; doi:10.1093/aobpla/plw032.