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# Chemical Composition of Intergeneric Hybrids Between *Ligularia* and *Cremanthodium* Collected in Sichuan Province of China

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**Abstract**

Two intergeneric hybrids between *Ligularia nelumbifolia* and *Cremanthodium stenoglossum* were examined with respect to the chemical composition of root extracts and the sequences of neutral DNA regions. The DNA data showed that the direction of hybridization was different between the individuals. Eremophilane sesquiterpenes were found in both hybrids and deduced to have come from their *Cremanthodium* parents, because sesquiterpenes were detected in *C. stenoglossum* but not in *L. nelumbifolia*.

**Keywords**

*Ligularia nelumbifolia*, *Cremanthodium stenoglossum*, hybridization, eremophilane, internal transcribed spacer, *trnL-rpl32*

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More than 100 species of *Ligularia* (Asteraceae) exist in the Hengduan Mountains area of China, providing us with interesting materials for studies of chemical evolution of plants. We have been examining chemical and genetic diversity in *Ligularia* by analyzing root chemicals and the DNA sequence of evolutionarily neutral regions.[1,2] Eremophilane-type sesquiterpenes have been isolated from most of the major species of *Ligularia*. Many species have been found to harbor intraspecific diversity.

Hybridization is an important pathway in plant evolution.[3] Over the course of our *Ligularia* research, spanning nearly 20 years, we have had opportunities to collect hybrids involving *Ligularia*. Detailed morphological and genetic characterization has been performed for some hybrids.[4-8] Chemical characterization has revealed a variety of outcomes of hybridization.[9-15] In some cases, both parents contributed compounds to their offspring; in some, the chemical contribution was only from one parent; in others, hybridization appeared to have engendered compounds that were absent in their parents.[9,12-14] In addition, introgression has been revealed by DNA analysis[10,12,15,16] and inferred to be behind the presence of the compounds that are detected only in some populations within a species.[12,17] These results show that hybridization is important in the chemical evolution of *Ligularia* in the Hengduan Mountains area.

We have found hybrids of *Ligularia nelumbifolia* (Bureau & Franchett.) Handel-Mazzetti and *Cremanthodium stenoglossum* Y. Ling & S.W. Liu in Sichuan Province, China.[8] The genus *Cremanthodium* is taxonomically close to *Ligularia* and found at altitudes of circa 4000 m in the Hengduan Mountains area.[18,19] Sesquiterpenes such as eremophilanes,[20-22] bisabolanes,[23-26] and other compounds[27,28] have been isolated from *Cremanthodium*. These compounds are found in many *Ligularia* species as well. In this report, we describe the chemical composition of the root extracts of 2 hybrid individuals (samples 1 and 2) as well as their parents, *L. nelumbifolia* (sample 3) and *C. stenoglossum* (sample 4), collected at the same location (Table 1). To the best of our knowledge, this is the first chemical study on an intergeneric hybrid between *Ligularia* and *Cremanthodium* and is also the first on *C. stenoglossum*.

DNA sequencing was carried out for the internal transcribed spacer (ITS)1-5.8S-ITS2 region of the nuclear rRNA gene and the plastid *rpl32-trnL* intergenic region. The results

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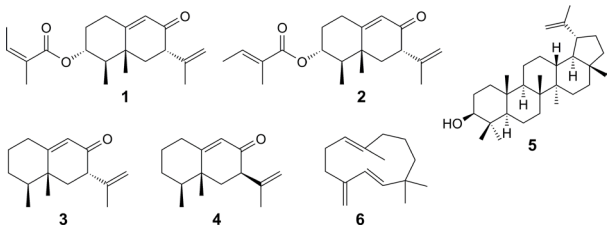
**Table 1.** Compounds in *Ligularia nelumbifolia*, *Cremanthodium stenoglossum*, and Their Hybrids.

| Sample no. | Specimen no. | Species | Compounds[a] |
|---|---|---|---|
| 1 | 2016-38 | *L. nelumbifolia* × *C. stenoglossum* | **1**, **3**, (**2**, **4**) |
| 2 | 2016-39 | *L. nelumbifolia* × *C. stenoglossum* | **1**, (**2**, **3**) |
| 3 | 2016-40 | *L. nelumbifolia* | **5** |
| 4 | 2016-41 | *C. stenoglossum* | (**1**), **2**, **5**, **6** |

[a]Those in parentheses were only detected by LC-MS; the others were isolated.

are summarized in Tables 2 and 3. Most of the multiple-base sites in ITS1-5.8S-ITS2 in samples 1 and 2 are the superposition of the bases in samples 3 and 4 (Table 2). This result confirms that samples 1 and 2 are hybrids of *L. nelumbifolia* and *C. stenoglossum*. The sequence of the *rpl32-trnL* region was quite different between sample 3 (*L. nelumbifolia*) and sample 4 (*C. stenoglossum*), especially by 2 indels (Table 3). The sequence of sample 1 was almost the same as that of sample 3; the sequence of sample 2 was identical to that of sample 4. Because plastids are maternally inherited, the *rpl32-trnL* sequences show that the maternal parent of sample 1 was *L. nelumbifolia* and that of sample 2, *C. stenoglossum*.

Root samples were extracted with EtOH and the extracts were subjected to LC-MS (MeOH/$H_2O$). Their total ion chromatograms are shown in Figure 1. Four peaks were detected for sample 1 in the region of typical terpenoids ($t_R$ = 10-20 minutes). The peaks at $t_R$ = 16.1 ($m/z$ 317), 15.5 ($m/z$ 317), 14.5 ($m/z$ 219), and 14.1 ($m/z$ 219) minutes were identified as **1**, **2**, **3**, and **4**, respectively, by use of the compounds isolated from the present samples (vide infra) and those isolated previously from other *Cremanthodium* species.[20-22] Compounds **1** to **3** were also detected for sample 2, although their quantities were small. A peak was observed at $t_R$ = 15.3 minutes for sample 3. The mass spectrum for this peak was complex, indicating that it contained a mixture. However, the spectrum suggested that the major constituent was likely to be *O*-geranylsinapyl alcohol, which has been isolated from *L. nelumbifolia* and related species.[29] Compounds **1** and **2** were detected in sample 4.




Compounds in the extracts were isolated by use of silica-gel column chromatography and HPLC. From sample 1, eremophilane compounds **1**[30,31] and **3**[31,32] were isolated. From sample 2, compound **1** was isolated. From sample 3, lupeol (**5**) alone was obtained. From sample 4, compounds **2**,[33] **5**, and γ-humulene[34] were isolated.

The results above indicate that the eremophilanes in the hybrid samples must have originated from *C. stenoglossum*, since no eremophilane was detected in the parental *L. nelumbifolia* (sample 3). The plants belonging to what we call the *dkn*-complex, comprising *L. duciformis*, *L. kongkalingensis*, and *L. nelumbifolia*, can be grouped into 4 chemotypes. The typical compounds in each chemotype are eremophilane

**Table 2.** Differences in ITS1-5.8S-ITS2 Among the Samples and Reference Sequences.[a]

| | ITS1 | | | | | | | | | | | | | | 5.8S | | | | | | | ITS2 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | | | 3 | | | | | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| | 1 | 1 | 5 | 0 | 0 | 1 | 1 | 2 | 3 | 8 | 1 | 2 | 2 | 5 | 3 | 3 | 3 | 1 | 1 | 2 | 3 | 6 | 0 | 1 | 2 | 4 | 5 | 7 | 9 | 2 |
| Sample no. | 5 | 8 | 7 | 0 | 8 | 0 | 3 | 6 | 2 | 4 | 4 | 4 | 5 | 0 | 8 | 9 | 3 | 0 | 1 | 8 | 3 | 9 | 4 | 2 | 1 | 9 | 1 | 3 | 9 | 1 |
| 1 | G | R | G | S | R | W | G | Y | Y | Y | C | [b] | [b] | T | T | C | Y | Y | Y | G | R | Y | Y | G | G | R | Y | S | Y | C |
| 2 | G | G | G | S | R | W | G | Y | Y | Y | Y | [b] | [b] | T | T | C | Y | Y | C | K | R | Y | Y | R | G | R | Y | S | Y | Y |
| 3 | G | G | G | G | A | T | G | T | T | C | C | C | G | T | T | C | T | C | Y | G | A | C | C | G | G | A | T | C | T | C |
| Ref[c] | G | G | G | G | A | T | G | T | T | C | C | C | G | T | T | C | T | C | C | G | A | C | C | G | R | A | T | C | C | C |
| 4 | G | G | S | S | G | W | R | Y | C | T | C | - | - | Y | T | C | Y | Y | C | G | G | C | C | G | G | G | C | G | C | C |
| Ref[d] | C | G | G | C | G | A | G | C | C | T | C | - | - | T | G | T | T | T | C | G | G | C | C | G | G | G | C | G | C | C |

[a]The base numbering is based on the sequences of *L. nelumbifolia* (sample 3 and a reference sequence; see note b). R = A + G; S = C + G; W = A + C; Y = C + T; - = deletion.
[b]Two sequences with and without CG were present.
[c]The sequence of *L. nelumbifolia* in the database (ID = JF976821).
[d]The sequence of *C. stenoglossum* in the database (ID = AY176136).

**Table 3.** Differences in *rpl32-trnL* Among the Samples.[a]

| | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 7 | | 7 | 7 | | 8 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 0 | 4 | 6 | 6 | 6 | 6 | 6 | 8 | 4 | 6 | 5 | - | 6 | 9 | - | 5 |
| Sample no. | 1 | 4 | 2 | 0 | 1 | 2 | 3 | 4 | 8 | 8 | 4 | 5 | | 4 | 3 | | 5 |
| 1 | T | T | C | A | A | A | A | A | T | T | G | | - | | | - | |
| 2 | A | T | A | T | T | T | T | T | G | T | T | | [b] | | | [b] | |
| 3[c] | T | G | C | A | A | A | A | A | G | A | G | | - | | | - | |
| 4[d] | A | T | A | T | T | T | T | T | G | T | T | | [b] | | | [b] | |

[a]The base numbering is based on the database entry of sample 4 (see note d), which includes a partial sequence of *rpl32*. - = deletion.
[b]The same as in the database entry of sample 4 (see note d).
[c]Deposited in the database with ID = LC457553.
[d]Deposited in the database with ID = LC457552.

sesquiterpenes (type 1), oplopane sesquiterpenes (type 2), phenylpropanoids (type 3), and no sesquiterpenes or phenylpropanoids (type 4), although the entire chemical spectrum is somewhat continuous.[29,35,36] Since lupeol (**5**) was isolated from sample 3 (*L. nelumbifolia*), the sample belongs to chemotype 4. We previously inferred that the ability to produce sesquiterpenes in types 1 and 2 had introgressed from some other *Ligularia* species.[35] The present result suggests that a *Cremanthodium* species can also be the source of the eremophilane-producing ability.

Eremophilanes have been isolated from other *Cremanthodium* species: **1** to **4** from *C. helianthus*[21] ; **1**, **3**, and **4** from *C. lineare*[22]; **3** and **4** from *C. stenactinium*.[20] Eremophilan-8-ones are considered to be the precursors of furanoeremophilanes.[37] We have proposed a hypothesis that the species and the intraspecific groups that produce furanoeremophilanes are ecologically advantageous over those producing eremophilan-8-ones.[1] For example, *L. cymbulifera*, with 10-hydroxyfuranoeremophilane (tetradymol) as the most major compound,[38] is highly abundant in northwestern Yunnan Province. Although most *Cremanthodium* species, studied so far, only produce eremophilan-8-ones, *C. lineare* produces furanoeremophilanes.[22] We are tempted to speculate that more *Cremanthodium* species may acquire an ability to produce furanoeremophilanes in the future.

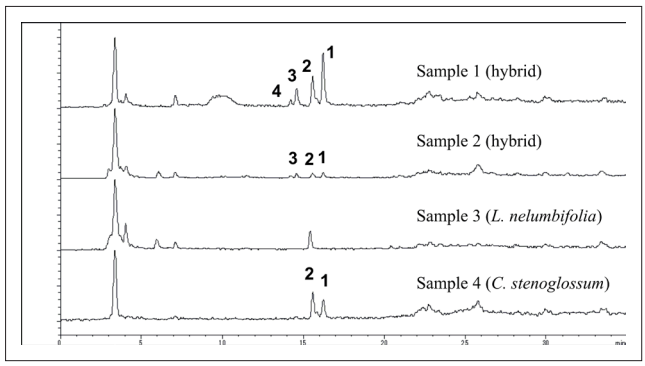



**Figure 1.** Total ion chromatograms of samples 1 to 4.

In conclusion, the present results indicate ongoing chemical exchange between *Ligularia* and *Cremanthodium*, which are undergoing reticulate evolution in the *Ligularia-Cremanthodium-Parasenecio* complex.[19]

## Experimental

### *General*

NMR, JEOL ECX-400 (400 MHz for $^{1}H$; 100 MHz for $^{13}C$) spectrometer; MS, JEOL JMS-700 MStation or CMATE II. Column chromatography (CC), silica gel (Wakosil C-200 or C-300). Analytical TLC, Merck Kieselgel 60 F254, 0.2 mm thickness, using *p*-anisaldehyde/AcOH/$H_2SO_4$ as visualizing agents. HPLC, GL Sciences GL-7410 pump with a GL-7450 UV detector, and a Hitachi D-2500 Chromato-Integrator, with a Kanto Mightysil Si60 (10 × 250 mm) column. LC-MS, Agilent 1100 series LC/MSD mass spectrometer with 5C18-MS-II using a gradient system (MeOH/$H_2O$) as eluent. See Ref. 17 for details of LC-MS.

### *Plant Materials*

The samples were collected in August 2016 on a border between Ganze and Xinlong Counties (elevation: 4700 m), Sichuan Province, China, and were identified by X. G. (author). Voucher specimen numbers are 2016-38 (hybrid), 2016-39 (hybrid), 2016-40 (*L. nelumbifolia*), and 2016-41 (*C. stenoglossum*) for samples 1 to 4, respectively (Kunming Institute of Botany).

### *DNA Analyses*

DNA was purified from dried leaves with a commercial kit (DNeasy Plant Minikit, QIAGEN). The amplification of the ITS1-5.8S-ITS2 region by polymerase chain reaction and the sequencing reactions of the region were carried out with the primers described previously.[39] The *rpl32-trnL* region was amplified and sequenced with a primer rpl32 (5'-CAGTTCCA AAAAAACGTACTTC-3') and a primer trnL

(5'-CTGCTTCCTAAGAGCAGCGT-3').[40] The sequences were determined by a Model 3500 Genetic Analyzer (Applied Biosystems).

## *Isolation of Compounds*

Dried roots of sample 1 (hybrid) (10.2 g) were extracted with EtOH. CC (*n*-hexane-EtOAc, gradient) of the resulting extract (556.1 mg) afforded 6 fractions. Fraction 3 (eluted with hexane/EtOAc at 8:2) was subjected to CC (*n*-hexane-EtOAc) and HPLC (hexane/EtOAc 9:1) and yielded **1** (5.0 mg) and triglycerides. Compounds **2** and **4**, detected by LC-MS, was inseparable from the triglycerides. Fraction 4 (eluted with hexane/EtOAc 7:3) was subjected to CC (*n*-hexane-EtOAc) and HPLC (hexane/EtOAc 8:2) and yielded **1** (0.9 mg) and **3** (0.5 mg).

Dried roots of sample 2 (hybrid) (20.4 g) were extracted with EtOH. CC (*n*-hexane-EtOAc, gradient) of the extract (797.7 mg) afforded 6 fractions. Fraction 4 (eluted with hexane/EtOAc 7:3) was subjected to CC (*n*-hexane-EtOAc) and HPLC (hexane/EtOAc 7:3) and **1** (2.8 mg) was obtained.

Dried roots of sample 3 (*L. nelumbifolia*) (9.9 g) were extracted with EtOH. CC (*n*-hexane-EtOAc, gradient) of the extract (797.7 mg) afforded 6 fractions. Fraction 3 (eluted with hexane/EtOAc 8:2) was further subjected to CC and **5** (0.6 mg) was obtained.

Dried roots of sample 4 (*C. stenoglossum*) (18.6 g) were extracted with EtOH. The extract (1093.9 mg) was subjected to CC (*n*-hexane-EtOAc, gradient). From the least polar fraction (eluted with *n*-hexane), **6** (1.2 mg) and **5** (0.5 mg) were obtained by separation using CC (*n*-hexane-EtOAc, gradient) and HPLC (*n*-hexane-EtOAc 98:2 [for **6**] and 80:20 [for **5**]). The other fractions were combined and subjected to CC (*n*-hexane-EtOAc, gradient) and HPLC (*n*-hexane-EtOAc 8:2) and **2** (1.4 mg) was obtained.

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