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Chemical and Genetic Differentiation of *Ligularia pleurocaulis* in Northwestern Yunnan and Southwestern Sichuan Provinces of China

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Intra-specific diversity of *Ligularia pleurocaulis* was examined from both chemical and genetic aspects, using furanoeremophilanes as the chemical index and the nucleotide sequences in the *atpB-rbcL* and the ITS1 regions as the genetic index. Four samples of *L. pleurocaulis* were collected in northwestern Yunnan, and six in southwestern Sichuan. From the ethanol extracts of the roots of all of the samples, four compounds, 6β-angeloyloxy- and 6β-tigloyloxy-furanoeremophil-1(10)-enes, and 6β-angeloyloxy- and 6β-tigloyloxy-furanoeremophil-1(10)-en-3β-ols were isolated. Further, 3β-angeloyloxyfuranoeremophil-1(10)-en-6β-ol and furanoligularenone were isolated from the Yunnan and the Sichuan samples, respectively. Two variants were found for the *atpB-rbcL* sequence. Base multiplicity in the ITS1 region was observed at only a few positions in the Yunnan samples, whereas it was observed at about 10 positions for the Sichuan samples. These results show that *L. pleurocaulis* is diverse, both chemically and genetically, and that plants in northwestern Yunnan and those in southwestern Sichuan can be distinguished by their chemical and genetic indexes.

The combination of chemical, genetic, and morphological approaches provides a new dimension to the science of plant diversity and ecology. On the basis of this tenet, we investigate inter- and intra-specific diversity of *Ligularia* species in the Hengduan Mountains area. *Ligularia* Cass. (Compositae (or Asteraceae)) is highly diversified in this area, and more than 100 species are extant in the eastern Qinghai-Tibet Plateau and its adjacent areas.[1] It is believed that the evolution and diversification of this genus is still continuing.[2] The ultimate goal of our investigation is to understand the diversity-generating mechanism of *Ligularia* species in the Hengduan Mountains area.

We have chosen the furanoeremophilane type of sesquiterpenes as the chemical index, since many compounds have been isolated from this genus,[3–7] and since intraspecific diversity can be easily described by Ehrlich's test on TLC.[8] As the genetic index, we have chosen the nucleotide sequence of the *atpB-rbcL* intergenic region, because it is non-coding and thus most mutations therein are believed to be neutral to selection.[9] In our previous study, we found that *L. tongolensis* (Franch.) Hand.-Mazz., *L. cymbulifera* (W. W. Smith) Hand.-Mazz., and *L. atroviolacea* (Franch.) Hand.-Mazz., all belonging to the section Corymbosae,[10] are close to one another with respect to the composition of furanoeremophilanes and the nucleotide sequence of the *atpB-rbcL* region.[11] We also found that *L. tongolensis* was diverse in the composition and the sequence, while *L. cymbulifera* was uniform in both.

The present study was focused on *L. pleurocaulis* (Franch.) Hand.-Mazz., which belongs to the section Senecillis.[10] Among various *Ligularia* species growing at an altitude of 2000–4000 m, this species is one of the species well adapted to highlands of around 4000 m. It is distributed widely from northwestern Yunnan to northwestern Sichuan, and sometimes makes a large colony. It is a polymorphous species in characters such as plant height, leaf width, and bract shape.[10] Although forms have been proposed for the species,[12,13] the taxonomic differentiation is debatable, because the morphological variations are continuous.[10] Recently, one of us and co-workers have found that *L. pleurocaulis* in northwestern Yunnan and that in southwestern Sichuan have different karyotypes[14] (see also Ref. 15), demonstrating polymorphism at the cytological level. The present study addresses the diversity in this species at the molecular level, and the results are presented below.

Results

Ten samples of *L. pleurocaulis* (Table 1 and Fig. 1) were collected in northwestern Yunnan and southwestern Sichuan Provinces in 2003–2005. Extraction was made in ethanol for the roots of each plant sample, compounds therein were subjected to TLC, and furanoeremophilanes were visualized by Ehrlich's reaction on the TLC plates.[8] No Ehrlich-negative compounds were observed when $Ce(SO_4)_2/H_2SO_4$ was used as a coloring reagent. All ten samples showed three common Ehrlich-positive spots ($R_f = 0.85$, 0.43, and 0.38, hexane/AcOEt 7:3), which were dark blue. Besides these spots, a spot of a light blue color (after several hours of coloring procedure) was observed at $R_f = 0.63$ for samples 5–10, which were collected in Sichuan. Samples 1–4, collected in Yunnan, also contained an Ehrlich-positive component at the same R_f (=0.63), however, its color was dark purple and not light blue.

The Ehrlich-positive compounds in samples 2, 4, and 5 were isolated and their structures were determined. The common

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Table 1. Collection Locality and the *atpB-rcbL* Genetic Type of *L. pleurocaulis* Samples

Sample[a)]	Locality[b)]	Elevation/m	Type of *atpB-rbcL*	Components[c)]
1	Shuduhu (Y)	3500	A-A9	A[d)]
2	Qianhushan (Y)	3500	G-A10	A[e)]
3	Xiaozhongdian (Y)	3500	A-A9	A[d)]
4	Gezan (Y)	4000	A-A9	A[e)]
5	Wumingshan (S)	3800	G-A10	B[e)]
6	Haizishan (S)	4300	G-A10	B[d)]
7	Daocheng (S)	4100	G-A10	B[d)]
8	Xiangcheng (S)	4100	A-A9	B[d)]
9	South of Litang (S)	4000	A-A9	B[d)]
10	Honglong (S)	4300	A-A9	B[d)]

a) Samples 1 and 8–10 were collected in 2005. Samples 2 and 5–7 were collected in 2003. Samples 3 and 4 were collected in 2004. b) Y = Yunnan Province, S = Sichuan Province. c) Component at $R_f = 0.63$ (hexane/AcOEt 7:3): A = 3β-angeloyloxyfuranoeremophil-1(10)-en-6β-ol, B = furanoligularenone. d) Determined by TLC. e) Isolated.

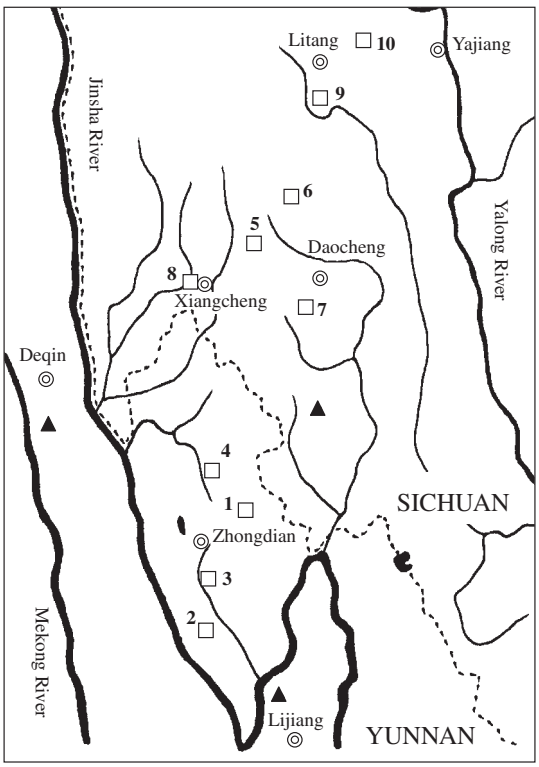
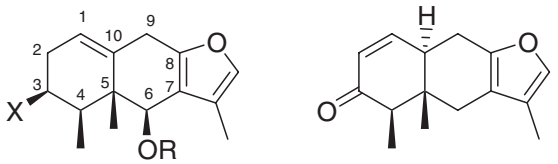


Fig. 1. Locations where samples of *L. pleurocaulis* (open squares) were collected. Filled triangles and double circles indicate major peaks and major cities, respectively.



1 X = H, R = C(=O)CMe=CHMe(*Z*)
2 X = H, R = C(=O)CMe=CHMe(*E*)
3 X = OH, R = C(=O)CMe=CHMe(*Z*)
4 X = OH, R = C(=O)CMe=CHMe(*E*)
5 X = OC(=O)CMe=CHMe(Z), R = H

6

Chart 1.

spot at $R_f = 0.85$ was found to consist of two compounds, 6β-angeloyloxy- and 6β-tigloyloxy-furanoeremophil-1(10)-enes (**1**[16–18] and **2**;[19] minor components). Major components were assigned to be 6β-angeloyloxyfuranoeremophil-1(10)-en-3β-ol (**3**, $R_f = 0.43$)[19] and 6β-tigloyloxyfuranoeremophil-1(10)-en-3β-ol (**4**, $R_f = 0.38$).[19] In addition to these compounds, the samples collected in Yunnan contained 3β-angeloyloxy-furanoeremophil-1(10)-en-6β-ol (**5**)[20] in 2.3 and 15% yields for samples 2 and 4, respectively, showing a dark purple spot on TLC at $R_f = 0.63$. The compound characteristic of the Sichuan samples was assigned to furanoligularenone (**6**)[4,21–26] (8.6% yield for sample 5), the light blue spot. This fact indicates that *L. pleurocaulis* can be classified into two groups on the basis of the Ehrlich-positive components at $R_f = 0.63$ as the chemical index. Differences between the Yunnan and the Sichuan samples were also detected in the ratio of the angelate **3** and the tiglate **4**. Namely, samples 2 and 4, collected in Yunnan, gave compound **3** as the only major component in 52 and 45% yields, respectively, whereas the extract of sample 5, collected in Sichuan, gave **3** and **4** in a 1:1.3 ratio (Chart 1) (see Experimental section for the details).

DNA was purified from leaves and the base sequence of the *atpB-rbcL* region was determined. The sequence was the same as that previously reported for *L. tongolensis*, *L. cymbulifera*, and *L. atroviolacea*, with sequence variations at the same positions: Adenine or guanine was at the 28th nucleotide position and the number of bases in the adenine stretch around the 510th position varied.[11] The base at the 28th position and the number of adenines in each sample are shown in Table 1. Two types, A-A9 and G-A10, were found. However, no correlation was seen between the genetic type and the furanoeremophilane composition.

We also analyzed the sequence of internal transcribed spacers (ITSs) of the gene for 18S, 5.8S, and 26S ribosomal RNAs (rRNAs). The three rRNAs are generated from a single transcript. The ITS1 is a segment between the 18S and the 5.8S rRNAs, and the ITS2, between the 5.8S and the 26S. They are nonfunctional, and consequently most mutations therein should be neutral. Because the rRNA gene is present as tandem

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TCAAAACCTGCATAGCAGAACGACCCGTGAACATGTAACAACAATCGGGTGTCCTTGGTA  60
TCGGGCTCTTGTTCGATTAATTGGATGCCTTGTCGATGTGCGTCTTTGGTCAGCCCTTTG 120
GGTCCTAAGGACGTCACATTGGCGCAACAACAAACCCCCGGCACGGCATGTGCCAAGGAA 180
AATTAAACTTAAGAAGGGCCTGTACCATGCTTCCCCGTTTGCGGGGTTTGCATGTGACGT 240
GGCTTCTTTATAATCA                                              256
```

Fig. 2. The sequence of the major variant of the ITS1 in sample 3. The sequence has been deposited to the DDBJ/EMBL/GenBank database (accession AB212735) together with the sequences of the 5.8S ribosomal RNA gene, the ITS2, and parts of the 18S and the 25S ribosomal RNA genes flanking the ITSs.

Table 2. Base Multiplicity[a),b)] in ITS1 in *L. pleurocaulis*

	Base position as in Fig. 2												
Sample	3	18	32	64	69	108	115	134	196	200	215	225	235
1	A	G	C	G	T	G	C	T	G	C	C	R	T
2	R	G	C	G	T	G	C	T	G	C	C	R	T
3	A	G	C	G	T	G	C	T	G	C	C	R	T
4	A	K	C	K	T	G	C	T	K	C	C	R	T
5	R	K	Y	G	W	G	Y	W	K	Y	S	G	K
6	R	K	Y	G	W	G	Y	W	K	Y	S	G	K
7	R	K	Y	G	W	K	Y	W	K	Y	S	G	K
8	R	K	Y	G	W	G	Y	W	K	Y	S	G	K
9	R	K	Y	G	W	G	Y	W	K	Y	S	G	K
10	R	K	Y	G	W	G	Y	W	K	Y	S	G	K

a) Y = C + T, R = A + G, S = G + C, W = A + T, and K = G + T. b) All the observed multiplicities are listed regardless of the ratio of the bases.

copies in the order of thousands in higher plants, inter-copy variation often exists.[9] Direct sequencing of the rRNA gene amplified by the polymerase chain reaction (PCR) results in superposition of multiple sequences, if an alternative variant(s) is present at a detectable frequency. Sequencing results indicated the presence of multiple variants of ITS1 in every sample. The sequence of the major variant of the ITS1 in sample 3 is shown in Fig. 2. The sample had a small additional signal of adenine at the 225th position. At several base positions in some samples, signals for an additional base were observed of a substantial strength. A summary of the multiplicity is given in Table 2. Base multiplicity was observed only at a few positions in the samples collected in Yunnan, whereas it was observed at about 10 positions in the samples collected in Sichuan. Repeated experiments reproduced the results and ruled out the possibility of an artifact due to PCR, in which DNA can be amplified from a very small number of molecules. The base sequence of ITS2 was also determined for the Yunnan samples and were included in the database entry. However, no unambiguous determination of the sequence was possible for the Sichuan samples because of the presence of an alternative variant(s) that had a base deletion.

Discussion

In the present study, several samples of *L. pleurocaulis* were examined with respect to the composition of furanoeremophilanes and the nucleotide sequences of the *atpB-rbcL* and the ITS1 regions. Furanoeremophilanes were found to be present in all of the samples, and their compositions detected by Ehrlich's reaction on TLC were essentially the same, with the one exception of a compound at $R_f = 0.63$. At the R_f, 3β-angeloyloxyfuranoeremophil-1(10)-en-6β-ol (**5**) was in the samples collected in Yunnan, and furanoligularenone (**6**) in the samples collected in Sichuan. Recently, Zhang et al. reported that furanoligularenone was present in *L. pleurocaulis* in the Kangding prefecture, Sichuan.[27] Although the exact locality was not reported, their result agrees with our finding that *L. pleurocaulis* in Sichuan produces furanoligularenone.

The most interesting finding in the present study was that the furanoeremophilane composition and the multiplicity in ITS1 were correlated and that these two indexes agreed with the geographic distribution of the samples. The samples from Sichuan contained larger multiplicity in the ITS1 sequence than those from Yunnan (Table 2). Such a difference in multiplicity was observed in the ITS2 as well. These results suggest that the plants in Sichuan are likely to be a hybrid of populations with different ITS variants (see Ref. 28 for the evolution of the rDNA gene copies). This inference is supported by the chemical results. The structure of furanoligularenone (**6**), obtained only from the Sichuan samples, is relatively different from the other furanoeremophilanes **1**–**5**, suggesting that a genetic element(s) for furanoligularenone formation is present only in the plants in Sichuan.

The present finding of the difference between the Yunnan and the Sichuan plants agrees with the results of a study that found a cytological difference;[14] the previous and the present results are directly related. A sample from Wumingshan, Sichuan, which was found to have a karyotype of 2n = 58 = 24m + 32sm + 3-5B, was actually collected at the same locality as sample 5. A sample from Zhongdian, Yunnan, with 2n = 58 = 36m + 22sm, was collected at the same locality as sample 1.

Intraspecific variation was also observed in the *atpB-rbcL* sequence. The result strengthens our previous finding of corre-

lation between the presence of diversity in the furanoeremophilane composition and that in the *atpB-rbcL* sequence.[11] However, no direct correlation was observed between the *atpB-rbcL* type and the furanoeremophilane composition in *L. pleurocaulis*. Genes involved in terpene synthesis have been reported[29] and they are encoded in the nuclear DNA, while the *atpB-rbcL* region is in the plastid DNA. Thus, a stronger correlation can be expected between the results on the ITSs of the rDNA gene, which is in the nuclear DNA, and the composition results.

In conclusion, *L. pleurocaulis* was found to be diverse with respect to the furanoeremophilane composition and nucleotide sequences. These indexes are independent, since the nucleotide sequences studied here are non-coding and neutral to natural selection. Two independent indexes, furanoeremophilane composition and the ITS1 sequence, lead us to one common conclusion: *L. pleurocaulis* in northwestern Yunnan and that in southwestern Sichuan have differentiated. This is supported by the previous karyotyping results, the third index. Since the composition of secondary metabolites sometimes depends on the environment,[30] it would have been difficult to obtain the present conclusion only from the chemical result. In addition, the presence of further diversity was revealed by analysis of the *atpB-rbcL* sequence. Thus, this study demonstrates that combination of natural product chemistry and molecular biology opens a new dimension in plant science.

Experimental

General. ^{1}H NMR spectra were recorded on a GNM-AL 400 (400 MHz) spectrometer with $CDCl_3$ as the solvent and tetramethylsilane as an internal standard. ^{13}C NMR spectra were recorded on the same instrument operating at 100.5 MHz with $CDCl_3$ as the solvent and the internal standard (δ 77.0). IR spectra were taken on a Shimadzu FTIR-8700 spectrometer. Mass spectra (EI^+) were obtained on a JEOL JMS-700 mass spectrometer. Optical rotations were determined on a JASCO DPI-181 polarimeter. Precoated Merck Kieselgel 60 F_{254} was used for analytical and preparative TLC. Merck Kieselgel 60 and Merck Kieselgel 60 F_{254} (layer thickness 0.2 mm) were used for flash chromatography and preparative TLC, respectively.

A DNeasy Plant Mini Kit (Qiagen) was used to purify DNA from dried leaves and glass milk (Gentra Systems) was used to purify the DNA further. HotStarTaq polymerase (Qiagen) was used for polymerase chain reaction (PCR). A High Pure PCR Product Purification Kit (Roche Diagnostics) was used to purify the PCR product after separation by agarose gel electrophoresis. DNA sequencing reactions were carried out with a DYEnamic ET Sequencing Kit (Amersham) and analyzed on an ABI Prism 310 analyzer or with a CEQ DTCS Kit (Beckman) and on a CEQ 2000 sequencer (Beckman).

Plant Materials. Samples of *L. pleurocaulis* were collected in each August of 2003–2005 at several locations described in Table 1 and Fig. 1. Each plant was identified by Xun Gong, one of the authors.

Ehrlich's Test. The roots of each plant (2–10 g) were harvested, and extraction with ethanol was started immediately without drying. Extraction was continued at room temperature for several days. After filtering and without concentration, plant extracts were subjected to TLC using hexane–ethyl acetate (7:3) as the solvent. The TLC plate was dipped in a 1% solution of *p*-dimethylaminobenzaldehyde in ethanol. The plate was dried and then dipped in a 1 mol dm^{-3} solution of hydrochloric acid in aqueous ethanol, and the resultant coloring was photographed.

Extraction for the Structure Determination. For the samples collected in 2003, the roots of *L. pleurocaulis* were cut into small pieces without drying, and immediately extracted with ethanol at room temperature. The extracted solution was filtered and concentrated to afford an oily residue together with an aqueous phase. Ethyl acetate was added to this oil/aqueous mixture, and the organic layer was separated. Evaporation of the solvent afforded an oily residue, to which water-soluble starch was added for handling purposes. Just before purification of the chemical components, the starch was removed by partitioning between diethyl ether and water. The ethereal solution was dried over anhydrous sodium sulfate, filtrated, and then evaporated to give an oily extract.

For the samples collected in 2004, the roots were dried and then extracted with ethanol at room temperature. Filtration of the extracted solution followed by evaporation of the solvent afforded an oily extract, which was then partitioned between diethyl ether and water. The ethereal solution was dried over anhydrous sodium sulfate, filtrated, and then evaporated to give an oily extract.

Purification and Identification of Chemical Components. The extract of *L. pleurocaulis* (112 mg) collected in Yunnan Province (sample 2) was subjected to preparative TLC using hexane–ethyl acetate (7:3) as the solvent to give a crude mixture of **1** and **2** (8.1 mg) and an inseparable mixture of **3** and **4** [66.4 mg, 59.6% yield; ratio 7:1; $R_f = 0.43$ and 0.38, respectively (hexane–ethyl acetate 7:3)]. The former mixture was subjected to preparative TLC (hexane–ether 20:1) to give an inseparable mixture of **1** and **2** (3.7 mg, 3.3% yield; ratio 2.3:1; $R_f = 0.85$). Column chromatography of the extract of the same sample (249 mg) using hexane–ethyl acetate (8:1) as the eluent gave compound **5** (5.8 mg, 2.3% yield; $R_f = 0.63$). Similar treatment of the extract of *L. pleurocaulis* collected in Yunnan Province (sample 4) gave compounds **1**–**5** in 1.7, 1.7, 45, 14, and 15%, respectively.

The extract of *L. pleurocaulis* (544 mg) collected in Sichuan Province (sample 5) was subjected to column chromatography using hexane–diethyl ether (40:1 then 30:1) as the eluent to give a crude mixture of **1** and **2** (19 mg) and a mixture of **3** and **4** (143 mg, 26% yield; ratio 1:1.3). The former mixture was separated by preparative TLC (hexane–diethyl ether 20:1) to give **1** (5.6 mg, 1% yield) and **2** (5.4 mg, 1% yield). The mixture of **3** and **4** was partly separated by preparative TLC (dichloromethane–ethyl acetate 20:1) to give pure compound **3**. Column chromatography of the extract of the same sample (864 mg) using hexane–ethyl acetate (50:1) as the eluent gave compound **6** (75 mg, 8.6% yield; $R_f = 0.63$).

The structural assignments of **1**–**6** were carried out by comparing their ^{1}H and ^{13}C NMR, IR, and mass spectral data with those reported in literature.[16–26] Compound **6**: mp 90–92 °C (recrystallized from hexane–diethyl ether 9:1) (lit. 25: 91–92 °C); $[\alpha]_D$ +2.4 (*c* 1, $CHCl_3$) (lit. 25: +3.25 (*c* 1, $CHCl_3$).

Determination of DNA Sequence. Purification of DNA from dried leaves, PCR amplification of the *atpB-rbcL* region, and determination of the nucleotide sequence in the region were carried out as reported.[11] PCR amplification and sequence determination of the rDNA region were carried out as follows. Forty cycles of amplification with ITS-5m and ITS-4 primers[31] were run, with each cycle consisting of a 30-s denaturation period at 95 °C, a 30-s annealing period at 40 °C, and a 1-min extension at 72 °C. PCR products were separated by agarose gel electrophoresis, the major

band was cut out, and the DNA was purified with the High Pure PCR Product Purification Kit (Roche Diagnostics). DNA sequencing reactions were carried out with a DYEnamic ET Sequencing Kit (Amersham) and analyzed on an ABI Prism 310 analyzer. The primers used for the sequencing were ITS-2, ITS-3, ITS-4, ITS-5m,[31] and the newly designed ITS-2B (5′ CTCGATGGTTC-ACGGGAT 3′).

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