Chemical and Genetic Differentiation ofLigularia tsangchanensis in Yunnan and Sichuan Provinces of China

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Chemical and Genetic Differentiation of *Ligularia tsangchanensis* in Yunnan and Sichuan Provinces of China

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The intraspecific diversity in *L. tsangchanensis* collected in the Chinese Provinces Yunnan and southwestern Sichuan was studied by chemical and genetic approaches. The samples collected in Yunnan were found to contain cacalol (**1**) as the sole major component, while samples from Sichuan contained 7α- and 7β-eremophila-9,11-dien-8-one (**5** and **6**) as well as the 3α-angeloyloxy derivative **7** as major components. In addition, the sequences of the internal transcribed spacers (ITSs) of the ribosomal RNA gene indicated that the Yunnan and the Sichuan samples constitute separate clades. These results demonstrate that *L. tsangchanensis* in Yunnan and Sichuan are distinct.

Introduction. – *Ligularia* CASS. (Asteraceae, tribe Senecioneae) is highly diversified in the Hengduan Mountains in mainland China [1], and its evolution in the region is considered to be continuing [2]. We have been studying the intraspecific diversity of some *Ligularia* species in this area by chemical and genetic analyses. We have chosen terpenoid composition as a chemical index, and DNA base sequences of the *atpB-rbcL* intergenic region and/or the internal transcribed spacers (ITSs) of the ribosomal RNA gene as a genetic index [3–5]. As for the chemical index, the composition of furanoeremophilanes and related compounds is particularly useful in outlining diversity, since these compounds are often found in *Ligularia* species [6] and can be detected easily by *Ehrlich*'s test on thin-layer chromatography (TLC) plates [7]. The *atpB-rbcL* region in the plastid genome and the ITSs in the nuclear genome are non-coding, and variations therein are considered to be neutral to evolution [8]. Previously, we came to the following three conclusions: *1*) the species *L. pleurocaulis* (FRANCH.) HAND.-MAZZ. of northwestern Yunnan is distinct, both chemically and genetically, from the same species found in southwestern Sichuan [3]; *2*) the species *L. virgaurea* (MAXIM.) MATTF. of southwestern Sichuan can be divided into two groups, which, however, are not geographically separated [4]; and *3*) no diversity has been observed in *L. cymbulifera*, and the diversity in *L. tongolensis* is limited [5].

In this report, we describe the results of chemical and genetic analyses of *L. tsangchanensis* (FRANCH.) HAND.-MAZZ., which belongs to the section Ligularia, series Racemiferae [9]. The species is widely distributed in southeastern Tibet,

[1]) Corresponding author for general information and chemical aspects of the work; the genetic and taxonomic works were performed under the supervisions of *R. H.* and *X. G.*, resp.

southwestern Sichuan, and in northwestern to northeastern Yunnan Province, growing in grasslands, alpine meadows, and forest understories of 3000–4000 m in altitude. Although some pyrrolizidine alkaloids have been isolated from *L. tsangchanensis* [10], terpenoids in the plant have not been studied. Here, we show that the plant produces terpenoids, and that the plant in Yunnan and that in Sichuan are distinct with respect to both terpenoid composition and DNA sequence.

Results. – Fifteen samples (*1–15*) of *L. tsangchanensis* were collected in Yunnan and southwestern Sichuan areas (see *Fig.* and *Table 1*). Thereof, samples *4–11* were collected near Zhongdian (Shangrila) city. The roots of each sample were extracted

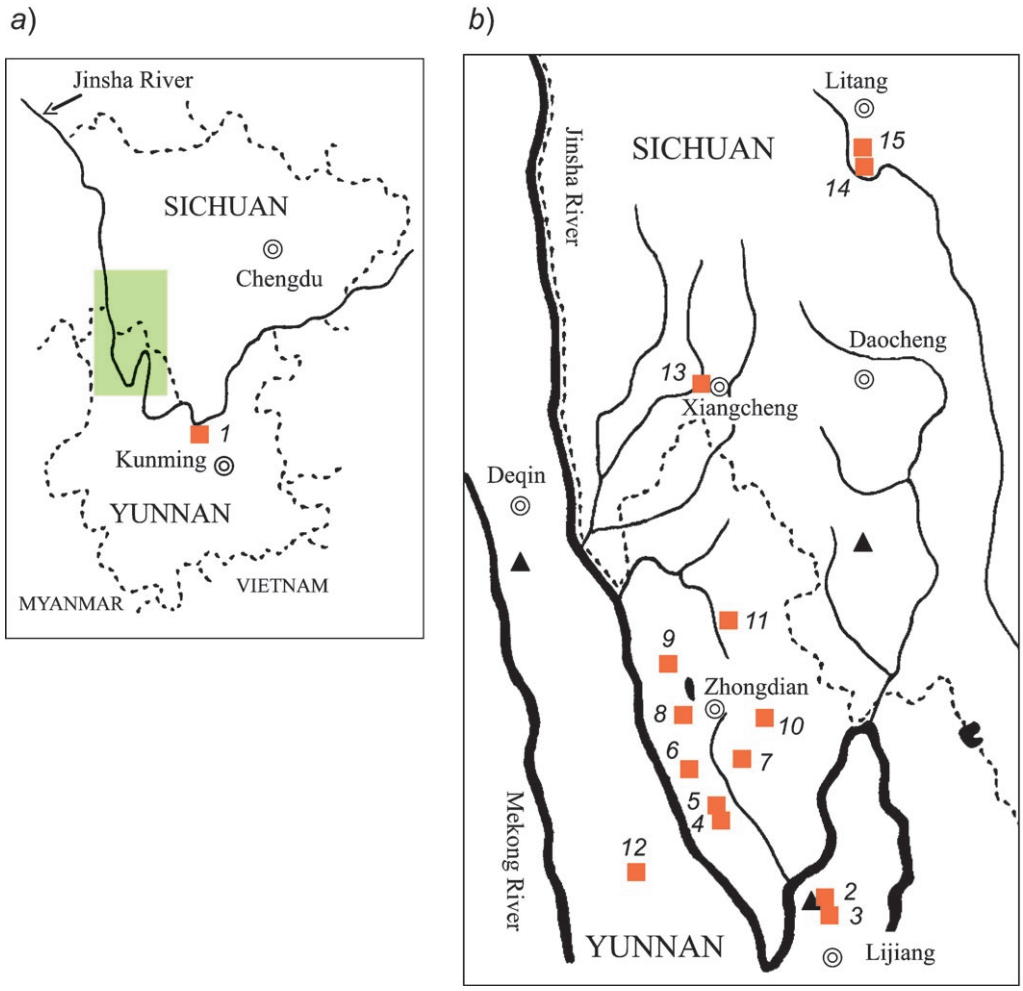


Figure. *Maps of Yunnan and Sichuan Provinces with collection sites of samples of* L. tsangchanensis (red boxes). Most samples (*2–15*) were collected in the green-shaded area (*a*), a close-up of which is given (*b*). Double circles and filled triangles indicate major cities and peaks, resp.

Table 1. *Collection Details, Genetic Type, and Major Terpenoid Component of Samples of* L. tsangchanensis

Sample[a])	Location[b])	Altitude [m]	*atbB-rbcL*[c])					Major terpenoid
			28	344	409	T[d])	A[e])	
1	Jiaozishan (Y)	3,600	G	T	A	9	10	**1**
2	Yulongxueshan (Y)	3,500	G	T	A	8	10	**1**
3	Yulongxueshan (Y)	3,400	G	T	A	8	10	**1**
4	Qianhushan (Y)	3,900	G	T	A	9	10	**1**
5	Qianhushan (Y)	3,200	G	T	A	8	10	**1**
6	Tianchi (Y)	3,800	G	T	T	9	9	**1**
7	Xiaozhongdian (Y)	3,500	G	T	A	9	10	**1**
8	Shikashan (Y)	3,500	G	T	T	9	9	**1**
9	Nixi (Y)	3,700	G	T	A	9	9	**1**
10	Tianshengqiao (Y)	3,400	A	G	A	9	10	**1**
11	Geza (Y)	4,000	G	T	A	9	9	**1**
12	Lidiping (Y)	3,300	G	T	A	8	9	**1**
13	Xiangcheng (S)	4,100	G	T	A	9	11	**7**
14	South of Litang (S)	3,800	G	T	A	9	9	**5**
15	South of Litang (S)	4,000	G	T	A	9	10	**7**

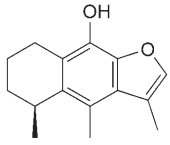
[a]) Sample *5* was collected in 2003; samples *1–4*, *6–8*, and *11* were collected in 2004; samples *9* and *13–15* were collected in 2005; samples *10* and *12* were collected in 2006. [b]) Y = Yunnan Province, S = Sichuan Province. [c]) Base numbering according to [5]; other bases were the same as those given in [5]. [d]) Number of T residues in a stretch around the 390th base. [e]) Number of A residues in a stretch around the 510th base.

with EtOH, and the alcoholic extracts were subjected to *Ehrlich*'s test on TLC plates. All the samples collected in Yunnan Province showed one strong *Ehrlich*-positive spot, the color of which was not pink (in contrast to those of most furanoeremophilanes), but dark-blue. Interestingly, samples *13–15*, collected in Sichuan, showed no *Ehrlich*-positive spots. These results suggested that the samples of *L. tsangchanensis* could be grouped into two types: a Yunnan type and a Sichuan type.

The *Ehrlich*-positive compound was isolated from sample *2*, and was found to be cacalol (**1**) [11][12], which constituted 10% of the extract. As minor components, an inseparable 1:1 mixture of cacalone (**2**) [12] and epicacalone (**3**) [13], and the cacalol dimer adenostin A (**4**) [14] were identified. GC/MS Analyses led to the detection of cacalol (**1**) as the sole major constituent for all samples collected in Yunnan, *i.e.*, samples *1–12*.

Since we could collect only limited amounts (1–3 g) of the samples in Sichuan (*13–15*), only the major compounds were examined. From sample *14*, a 5:1 epimeric mixture of eremophila-9,11-dien-8-one (**5**/**6**) [15] was obtained, together with petasin (**7**), the corresponding 3α-angeloyloxy derivative [16]. Petasin (**7**) was also isolated as a major component from samples *13* and *15*. The chemical composition of the Sichuan samples *13–15* were analyzed by GC/MS, and the results are summarized in *Table 2*. In contrast to the Yunnan samples, many compounds were detected for these three samples. Notably, the chemical compositions of samples *13* and *15* were almost identical, while that of sample *14* was slightly different.

Yunnan samples:



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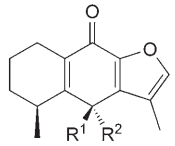
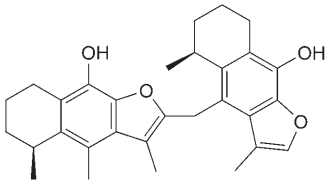


2 R^1 = Me, R^2 = OH
3 R^1 = OH, R^2 = Me



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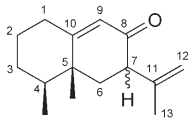
Sichuan samples:



5 7α-H
6 7β-H

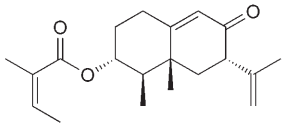


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Table 2. *GC/MS Analyses of the Samples from Sichuan Province*

Sample	t_R [min][a])	Intensity[b])	*m/z*	Compound
13	12.3	7	207	n.d.[c])
	15.0	4	218	**5**
	20.1	9	216	n.d.
	23.3	100	216	**7**
	24.3	11	216	n.d.
	24.6	16	216	n.d.
14	15.0	885	218	**5**
	23.1	100	216	**7**
15	14.1	14	204	n.d.
	15.0	14	218	**5**
	20.2	36	216	n.d.
	23.2	100	216	**7**
	24.3	25	216	n.d.

[a]) Retention time for the following GC conditions: *DBI* capillary column (0.25 mm × 30 m, 0.25 μm); 1.7 ml He as carrier gas; temp. gradient 50° (2 min) to 200° (15 min). [b]) Values rel. to those for **7** (= 100%). [c]) Structure not determined.

The DNA base sequences of both the *atpB-rbcL* and the ITS regions were determined. The *atpB-rbcL* sequence was essentially the same as that in *L. tongolensis* [5]. Seven variants of the *atpB-rbcL* sequence were found (*Table 1*). Variations were observed at the 409th base (A or T), a T stretch around the 390th base, and an A stretch around the 510th base in most of the samples. The 28th and the 344th bases were also different in sample *10*. No correlation with the terpenoid composition was apparent.

Variations were observed in the ITS1, the 5.8S, and the ITS2 sequences of the ribosomal RNA gene (*Table 3*). Most notably, the three Sichuan samples contained two alleles, in one of which the 125th base in the ITS1 was missing. Base variations in the 5.8S and ITS2 regions also appear to suggest that the Yunnan and Sichuan samples form separate clades. This observation was supported by PHYLIP analysis [17]. The result of bootstrap-NJ analysis using the 5.8S and ITS2 data indicated that the Yunnan and Sichuan sequences can be unambiguously separated, with a bootstrap value of 98%. Although sample *3* from Yunnan also contained two alleles of different ITS1 lengths, the missing base was different from that in the Sichuan samples. It is not clear how much the presence of an additional allele contributes to the possible separation of this sample from the other Yunnan samples.

Table 3. *Base-Sequence Variations at Specific Positions in the ITS1, 5.8S, and ITS2 Regions of the rRNA Gene of* L. tsangchanensis. Abbreviations: M = A + C; R = A + G; S = C + G; W = A + T; Y = C + T. Base multiplicities are listed regardless of the relative abundance of the bases.

Sample	ITS1				5.8S		ITS2															
	16	48	141	205	121	138	11	16	32	93	99	101	104	148	155	167	179	182	185	198	199	203
1	Y	G	G	C	C	G	C	C	A	G	C	C	T	A	C	T	C	C	T	C	C	T
2	C	G	S	C	C	G	C	C	A	R	C	Y	T	A	C	T	C	C	T	Y	C	T
3	[a])	[a])	[a])	[a])	C	G	C	C	A	G	C	C	T	A	C	T	C	C	T	C	C	T
4	Y	G	G	C	C	G	C	C	A	G	C	C	T	A	C	T	C	C	T	C	C	T
5	C	G	G	C	Y	G	C	C	A	G	C	C	T	A	C	T	C	C	T	C	C	T
6	C	G	G	C	C	G	C	C	A	G	C	C	T	A	C	T	C	C	T	C	Y	T
7	C	G	G	M	C	G	C	C	A	G	Y	C	T	A	C	T	C	C	T	C	C	T
8	C	G	G	C	C	G	C	C	A	G	Y	C	T	A	C	T	C	C	T	C	C	T
9	C	G	G	M	C	G	C	C	A	G	Y	C	T	A	C	T	C	C	W	C	Y	T
10	C	G	G	M	C	G	C	C	A	G	Y	C	T	A	C	T	C	C	T	C	Y	T
11	C	G	G	M	C	G	C	C	A	G	Y	C	T	A	C	T	C	C	T	C	Y	T
12[b])	C	R	G	C	C	G	C	C	A	G	C	C	T	A	C	T	C	C	T	C	C	T
13	[c])	[c])	[c])	[c])	C	G	C	Y	G	G	C	C	C	W	Y	C	M	Y	T	C	C	C
14	[c])	[c])	[c])	[c])	C	R	C	Y	G	G	C	C	C	W	C	C	C	Y	T	C	C	T
15	[c])	[c])	[c])	[c])	C	R	Y	Y	R	G	C	C	C	W	C	C	C	Y	T	C	C	T

[a]) Not determined due to the presence of an additional allele in which the 50th base was missing. [b]) The base sequence of sample *12* was deposited at the DDBJ/GenBank/EMBL data-base (accession AB284129). [c]) Not determined due to the presence of an additional allele in which the 125th base was missing.

Discussion. – The results of both the chemical and genetic analyses indicate that our *L. tsangchanensis* samples can be grouped into two. The grouping based on the terpenoid composition agrees with that based on the ITS sequences; besides, it also agrees with geographical division. Similar results have been obtained in our analyses of *L. pleurocaulis* [3], which was also divided into a Yunnan and a Sichuan type.

A remarkable finding from the chemical analysis is that all the Yunnan samples of *L. tsangchanensis* have cacalol (**1**) as the sole major component. Cacalol has a rearranged Me group at C(6), and, therefore, is considered to be derived from the eremophilane skeleton [11]. Conceivably, the Yunnan type diverges from the Sichuan

type, which produces the eremophilane derivatives **5**–**7** lacking a furan ring. Although the biochemical/ecological role of cacalol (**1**) or of related furanoeremophilanes is unknown, our recent chemical results and observations during field survey suggest that *Ligularia* species producing furano compounds are ecologically predominant. For example, large colonies of *L. cymbulifera* are found in the Zhongdian area, and the plants there produce furanoeremophilan-10β-ol as the major component [5]. A similar result has been obtained in our recent study on *L. kanaitzensis*, in which a furanoeremophilane-producing group appears to be gaining dominance [18]. Therefore, the Yunnan type of *L. tsangchanensis* may be better adapted ecologically than the Sichuan type. This is in agreement with our experience that *L. tsangchanensis* is easier to spot in Yunnan than in Sichuan.

Cacalol (**1**) has been isolated from another *Ligularia* species [19], as well as from species of other genera of Senecioneae, such as *Cacalia* [11–13], *Senecio* [20], *Psacalium* [21], *Othonna* [22], and *Euryops* [23]. The results described in these reports indicate that **1** is a common terpenoid component in Senecioneae. Therefore, cacalol may be a compound that has reached an evolutionarily 'stable point'. This premise is consistent with the observation that *L. tsangchanensis* of the Yunnan type is distributed widely from Kunming to Zhongdian in Yunnan Province.

Conclusions. – The species *L. tsangchanensis* was found to be diverse both in terms of sesquiterpene composition and nucleotide sequence. The plant growing in Yunnan and that in southwestern Sichuan are distinct with respect to the two independent indices. The major component in the samples from Yunnan is cacalol (**1**), which has a rearranged eremophilane skeleton and is a common terpenoid in Senecioneae. Instead, the eremophilane derivatives **5**–**7** are the major components in the samples from Sichuan. Considering that the Yunnan type is more abundant than the Sichuan type, furano compounds seem to be of ecological relevance.

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Experimental Part

1. *General.* Column chromatography (CC): silica gel (*Wakogel C-200*), unless otherwise noted. Other purification and anal. methods, including *Ehrlich*'s test on TLC plates and the determination of DNA sequences were performed as described previously [3–5]. The known compounds **1**–**7** were identified by IR, NMR, and MS analyses (data not given) on the following instruments. IR Spectra were recorded on a *Jasco-230 FT-IR* spectrometer. ^{1}H- and ^{13}C-NMR Spectra were recorded on a *JEOL GSX-400* spectrometer at 400 or 100 MHz, resp., in $CDCl_3$ or C_6D_6 soln. Both low- and high-resolution mass spectra were obtained on *JEOL SX-102A* or *CMATE-II* mass spectrometers. GC/MS Analyses were performed on a *Shimadzu QP5050* mass spectrometer using a *DBI* cap. column (0.25 mm $\times$ 30 m, 0.25 μm).

2. *Plant Material.* Samples of *L. tsangchanensis* were collected in August of 2003–2006 in northwestern Yunnan Province, and in southwestern Sichuan Province. All plant samples were identified by *X. G.*

3. *Extraction and Purification.* The root samples collected in 2004 were dried and then extracted with EtOH at r.t. Filtration and evaporation of the solvent afforded an oil, which was partitioned between Et_2O and H_2O. The org. phase was dried (Na_2SO_4), filtered, and concentrated *in vacuo* to afford an oil. The samples of 2005 were extracted with AcOEt and filtered, and evaporation of the solvent afforded an oil.

3.1. *Purification of Sample* 2. The extract of *L. tsangchanensis* (sample *2*; 4.32 g) collected in Yunnan Province was separated by CC (7 g SiO_2; hexane/Et_2O gradient) to afford two fractions: *Fr. A* (eluted with hexane/Et_2O 9:1) and *Fr. B* (eluted with Et_2O). *Fr. A* was further separated by CC (SiO_2; hexane/ether 97:3→7:3) to afford cacalol (**1**; 425 mg) and adenostin A (**4**; 3.2 mg). Repeated purification of *Fr. B* by CC (SiO_2; hexane/Et_2O 7:3→6:4) afforded an inseparable 1:1 mixture of **2** and **3** (80 mg).

3.2. *Purification of Sample* 13. The extract of sample *13* (350 mg) was separated into an AcOEt-soluble and an -insoluble part. The former was subjected to CC (7 g SiO_2; hexane/AcOEt 3:1) to afford a fraction containing the major component, which was further purified by CC (SiO_2; hexane/AcOEt gradient) to afford petasin (**7**; 53.2 mg).

3.3. *Purification of Sample* 15. The same purification procedure was applied as described for sample *13* (*Sect. 3.2*). Thus, from 399 mg of sample *15* were obtained 62 mg of petasin (**7**).

3.4. *Purification of Sample* 14. The extract of sample *14* (370 mg) was separated by CC (SiO_2; AcOEt). The resulting AcOEt-soluble part was subjected to CC (5 g SiO_2; hexane/AcOEt 4:1) to afford two fractions, a less-polar *Fr. C* and a more-polar *Fr. D*. From *Fr. D*, petasin (**7**; 21.8 mg) was isolated after repeated chromatographic purification. From *Fr. C*, an inseparable mixture of **5** and **6** (22.6 mg) was obtained.

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