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Complex Diversity in *Ligularia kanaitzensis*

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Two samples of *Ligularia kanaitzensis* collected in the Shizhongshan Mountain region of Yunnan Province, as well as one sample collected in Sichuan Province, were analyzed with regard to root constituents and neutral DNA sequences. The two Shizhongshan samples were found to be different from each other and from other samples analyzed previously, indicating that the intra-specific diversity in the species was highly complex.

Keywords: *Ligularia kanaitzensis*, Asteraceae, Furanoeremophilanes, Sesquiterpenoids, ITS.

The genus *Ligularia* (Asteraceae) in the Hengduan Mountains area of China [1,2] provides us with samples interesting to study because of their chemical diversity. We have observed intra-specific diversity in many *Ligularia* species (for example, see refs. [3-6]) and proposed a hypothesis that the production of furanoeremophilane is ecologically advantageous over that of eremophilan-8-one derivatives [7].

In the course of our continuing study on *Ligularia*, we recently reported that *L. kanaitzensis* Hand.-Mazz. collected in Yunnan Province of China was highly diverse in comparison with other typical *Ligularia* species in the area [8]. Fifteen samples collected in the northwestern Yunnan Province (Figure 1, except for samples 1-3) were grouped into two (types A and B) on the basis of the DNA sequence of the ITS1-5.8S-ITS2 region. This region is part of the ribosomal RNA gene in the nuclear genome and has been used to extract systematics information, since the internal transcribed spacers (ITSs) are non-coding and evolutionarily neutral. The samples were also divided into a furanoeremophilane-producing group and an eremophilan-8-one-producing group. However, the genetic types (types A and B) and the chemical types (the furano type, **F**, and the non-furano type, **N**) did not coincide (Table 1 and Figure 1). Two taxa, var. *kanaitzensis* and var. *subnudicaulis*, have been proposed for the genus on the basis of a difference in the shape of foliose bracts [1]. In our observation, however, the difference is not distinct [8].

Further search in the field provided us with three additional samples (Table 1), two of which, samples 1 and 2, were collected on the east and west sides of Shizhongshan Mountain in Jianchuan County, Yunnan Province, respectively (Figure 1). Although the two collection sites were separated by only about 2.4 km, the two samples were clearly different in morphology. Sample 1 had narrow bracts and was identified as var. *kanaitzensis*, while sample 2 had rhombic bracts and was identified as var. *subnudicaulis*. Sample 3, collected at Yanyuan County, Sichuan Province, is the only *L. kanaitzensis* sample that we have collected so far in the Province.

Table 1: Collection locality, chemical composition, and genetic type of *L. kanaitzensis* samples.

Sample	Locality	Chemical composition [a]	ITS sequence
1	Shizhongshan (east)	**F**	see text
2	Shizhongshan (west)	**F**	see text
3	Pingchuan	**N**	B
Area 1 (4 samples) [b]	Lidiping/Labadi	**F**	B
Area 2 (2 samples) [b]	Jianchuan	**N**	B
Area 3 (4 samples) [b]	Annan/Jiulong	**F**	A

a Major components: **F**, furanoeremophilanes; **N**, non-furano eremophilanes.
b Ref. [8]. See Figure 1 for Areas 1-3.

Parts of the roots of each sample were extracted with ethanol immediately after collection, and the extracts were subjected to Ehrlich's test on TLC plates [9]. Sample 2 showed many pink spots, indicating the presence of many furanoeremophilanes. In contrast, only one light-blue spot was detected for sample 1, suggesting that the substituent(s) was different from typical furanoeremophilanes [10]. Sample 3 was negative to the test.

For detailed chemical analyses, dried roots of each sample were extracted with EtOAc, and the extracts were separated by silica-gel column chromatography and HPLC. From sample 1, only furanoeremophil-9-ene (**1**) [11] was isolated. Although TLC analysis of the extract showed that **1** was the major component, the compound was so labile that the yield was only 6.5% of the extract. From sample 2, eight eremophilanes (**2-9**) were isolated. These compounds, except for **6** [12] and **8** [13], had been isolated from the same species [8]. A new coniferyl alcohol derivative (**10**) was also isolated. From sample 3, compound **9** was isolated as the major component, together with minor components (**11**, **12** [14], and **13** [4]). Compound **11** had been isolated from the species [8].

The structure of compound **10** was determined as follows. The high-resolution mass spectrum showed a molecular ion peak at *m/z* 359.2200 ($M^+ + H$) and its formula was deduced to be $C_{22}H_{31}O_4$. The IR spectrum indicated the presence of an ester carbonyl group

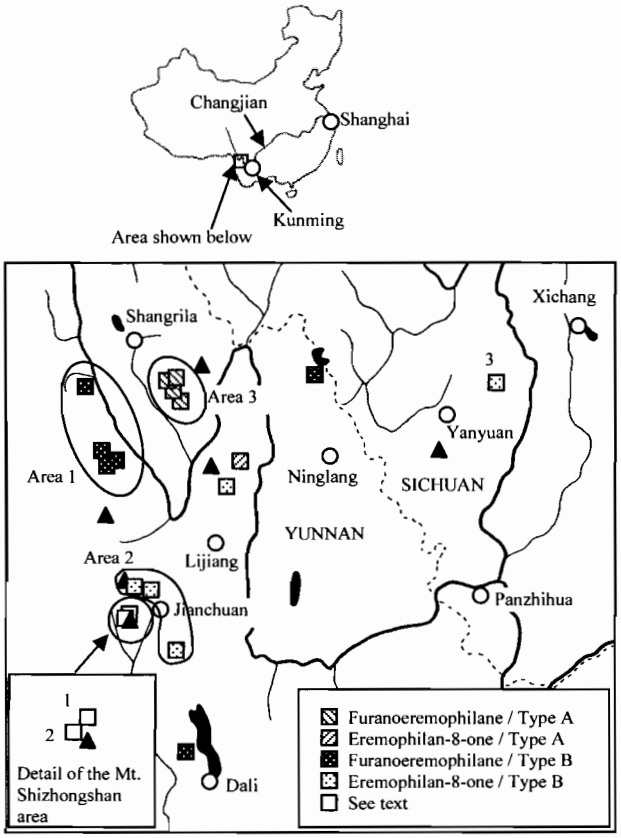
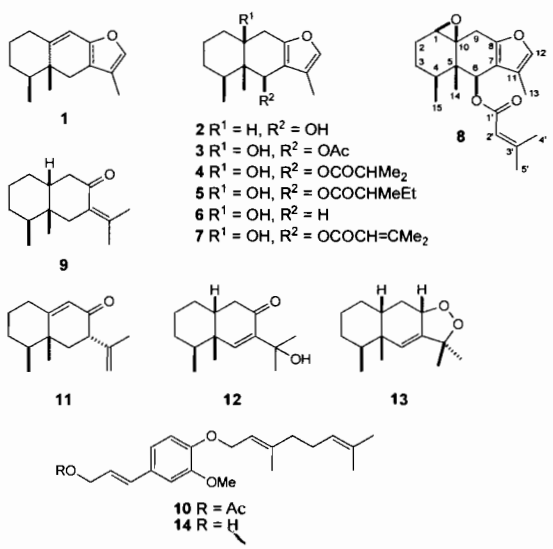


Figure 1: Locations where samples of *L. kanaitzensis* (squares) were collected. Solid and dotted lines indicate rivers and the border between Yunnan and Sichuan Provinces, respectively. Samples 1-3 were analyzed in the present study; all the others had been reported [8]. Open circles and filled triangles indicate major cities and peaks, respectively.



at 1736 cm^{-1}. The ^1H NMR spectrum of **10** was very similar to that of a known compound **14** [15]. Differences were observed in the presence of an acetyl group (δ 2.01) and the low-field shift of H_2-1 (δ 4.71) in **10**. The ^{13}C NMR spectrum was also similar to that of **14**, except for the presence of an acetyl group (δ 170.9 and 21.1). These data strongly suggested that **10** is the acetate of **14**. The structure was finally confirmed by COSY and HMBC correlations

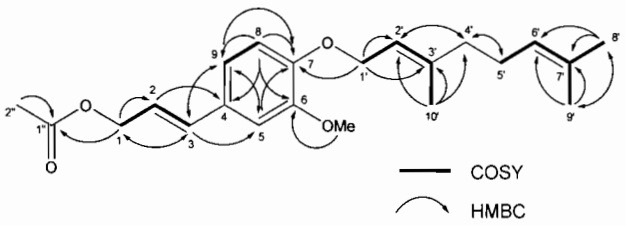


Figure 2: COSY and HMBC correlation detected for **10**.

Shoewn in Figure 2. While it was possible that **10** was generated from **14** during extraction with EtOAc, we tend to believe that **10** was a natural product, because **14**, but not **10**, had been isolated from another sample of *L. kanaitzensis* with the same treatment [8].

The DNA sequence of the ITS1-5.8S-ITS2 region was determined for the three samples. The results are shown in Table 2. The sequences and those of our previous samples were subjected to a standard cladistic analysis. The result showed the previous type-A samples formed a clade with a bootstrapping value of 86% and that the previous type-B samples and sample 3 formed a clade with a somewhat small bootstrapping value of 74%. However, samples 1 and 2 belonged to neither clade. They did not form a separate clade, either. Thus, in terms of the ITS1-5.8-ITS2 sequence, samples 1 and 2 appeared different from the others and also from each other.

Table 2: ITS sequence of *L. kanaitzensis* samples[a].

	ITS1																5.8S			ITS2																					
	1	1	1	4	4	9	0	1	1	2	2	4	2	2	4	3	3	5	1	3	3	4	7	8	9	0	0	1	6	7	9	0	0	0	0	1
	2	3	4	6	7	4	0	1	5	6	7	8	1	2	1	8	6	9	1	0	2	4	5	1	8	2	1	5	7	8	9	6	2	3	4	5	6	8
1	T	A	T	C	R	C	G	A	C	T	A	T	Y	C	A	W	Y	Y	C	T	A	G	S	-	G	C	C	T	G	T	T	A	C	C	T	T	G	C
2	A	T	A	Y	G	C	G	C	Y	Y	M	C	G	Y	G	T	C	T	Y	Y	R	R	G	-	A	C	C	C	R	Y	A	R	C	C	C	T	R	Y
3	A	A	A	C	G	Y	A	C	T	C	C	C	G	C	G	T	C	T	T	T	G	G	G	C	A	T	A	C	G	T	A	A	Y	T	C	K	G	T
Ref[b]	A	A	A	C	G	C	A	C	T	C	C	C	G	C	G	T	C	T	T	T	G	G	G	C	A	T	A	C	G	T	A	A	C	T	C	T	G	T

a Only the differences among the sequences of the present samples and the reference sequence are shown.
b Voucher 02269 (Pan and Gong, KIB); accession DQ272335.

These results show that the intra-specific diversity in *L. kanaitzensis* is higher than we reported previously [8]. Sample 3 was similar to some samples of our previous report in that its ITS sequence was of the type B and its major chemical component was an eremophilan-8-one. However, the sequences of samples 1 and 2 were different from both types and the major component in sample 1 was furanoeremophil-9-ene (**1**), which had not been obtained from *L. kanaitzensis* [8, 16]. The chemical composition of sample 2 was similar to the samples collected in the Lidiping/Labadi area (area 1 in Figure 1), but not to the samples of the nearby area (area 2).

We proposed that furanoeremophilane-producing *Ligularia* species have an ecological advantage over eremophilan-8-one-producing species [7]. In the Shizhongshan population, both samples 1 and 2 produced furanoeremophilanes. However, there was a difference between the populations of these samples. The population of sample 2 was making large colonies, while that of sample 1 was not. Ecological predominance was also observed for the *L. kanaitzensis* populations in Annan/Jiulong area (area 3 in Figure 1). Furanoeremophilane derivatives having a hydroxy group at C-10 and/or C-6 were obtained as the major constituent from sample 2 and Annan/Jiulong samples [8], but not from sample 1. Compound **6**, one of the simplest compounds of this category, was the major

constituent of *L. cymbulifera*, which is very abundant in the Shangrila (Zhongdian) area [5, 17]. These data suggest that there may be an ecological difference(s), even among furanoeremophilanes.

Experimental

General: NMR, JEOL ECX-400 (400 MHz for ^{1}H; 100 MHz for ^{13}C) spectrometer; IR, JASCO FT/IR-230 spectrometer; MS, JEOL JMS-700 MStation. Column chromatography was performed on silica gel (Wakosil C-200 or C-300). Analytical TLC was carried out on Merck Kieselgel 60 F_{254}, 0.2 mm thickness, using either Ehrlich's reagent (*p*-dimethylaminobanzaldehyde and HCl) [9] or *p*-anisaldehyde/AcOH/H_2SO_4 as visualizing agents. HPLC was carried out on a Shimadzu LC-20AT pump, SPD-20A Prominence UV/VIS detector, Kanto Mightysil Si60 (10 × 250 mm) ODS column.

Plant materials: *L. kanaitzensis* samples were collected at three locations shown in Table 1 and Figure 1. Each sample was identified by X. G. (author). Voucher specimens were deposited in the Herbarium of Kunming Institute of Botany (No. 2007-31, 2008-58, and 2008-59 for samples 1-3, respectively).

Extraction and purification: The dried roots of each sample were extracted with EtOAc at room temperature, and the extract was concentrated under reduced pressure to obtain the extract.

About half of the extract of sample 1 (40.2 mg), obtained from 11 g of dried root, was subjected to column chromatography (SiO_2 60N 15 g; eluent: *n*-hexane-Et_2O 9:1) to give crude fractions containing **1**. Purification by further column chromatography (eluent: *n*-hexane-Et_2O 99:1) furnished **1** (2.6 mg).

The dried roots of sample 2 (35 g) were extracted with EtOAc to give a crude extract (3.11 g) which was separated by silica-gel column chromatography (*n*-hexane–EtOAc gradient) to afford terpenoid-containing fractions (1.56 g). Repetition of the column chromatography and HPLC afforded compounds **2** (123.2 mg), **3** (15.5 mg), **4** (55.3 mg), **5** (50.6 mg), **6** (107.4 mg), **7** (36.3 mg), **8** (2.6 mg), **9** (3.2 mg), and **10** (1.6 mg).

The dried roots of sample 3 (20.2 g) were extracted with EtOAc to give a crude extract (706.8 mg), which was separated by silica-gel column chromatography as above (*n*-hexane–EtOAc gradient). Further purification by HPLC furnished **9** (274.4 mg), **10** (15.1 mg), **11** (1.4 mg), and **12** (22.1 mg).

DNA analysis: Purification of DNA, amplification of the ITS1-5.8S-ITS2 region by polymerase chain reaction, and DNA sequencing were carried out as previously described [3]. Cladistic analysis by the N-J method was carried out by MEGA5 [18].

Compound 10

IR (neat/NaCl): 1736, 1458, 1259, 1028 cm^{-1}.
^{1}H NMR ($CDCl_3$): 1.60 (3H, s, H_3-9'), 1.67 (3H, s, H_3-8'), 1.72 (3H, s, H_3-10'), 2.10 (3H, s, H_3-2"), 2.03-2.15 (4H, m, H_2-4' and H_2-5'), 3.89 (3H, s, OMe), 4.62 (2H, d, J = 6.5 Hz, H_2-1'), 4.71 (2H, d, J = 6.5 Hz, H_2-1), 5.08 (1H, t-like, J = 6 Hz, H-6'), 5.51 (1H, t, J = 6.5 Hz, H-2'), 6.16 (1H, dt, J = 16.0, 6.5 Hz, H-2), 6.59 (1H, d, J = 16.0 Hz, H-3), 6.82 (1H, d, J = 8.3 Hz, H-8), 6.90 (1H, dd, J = 1.5, 8.3 Hz, H-9), 6.94 (1H, d, J = 1.5 Hz, H-5).
^{13}C NMR ($CDCl_3$): 16.7 (C-10'), 17.7 (C-9'), 21.1 (C-2"), 25.7 (C-8'), 26.2 (C-5'), 39.5 (C-4'), 55.8 (OMe), 65.3 (C-1), 65.9 (C-1'), 109.0 (C-5), 112.9 (C-8), 119.6 (C-9), 119.9 (C-2'), 121.0 (C-2), 123.8 (C-6'), 129.2 (C-4), 131.8 (C-7'), 134.4 (C-3), 140.7 (C-3'), 148.5 (C-7), 149.5 (C-6), 170.9 (C-1").
CIMS: *m/z* (%) 299 [M^+ - OAc] (4), 222 (100), 163 (61).
HR-CIMS: *m/z* [M + H^+] calcd for $C_{22}H_{31}O_4$ 359.2223; found: 359.2200.

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