Chemical and Genetic Studies on Hybrid of *Ligularia subspicata* and *Ligularia cyathiceps* Collected in Yunnan Province of China

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Two morphologically ambiguous *Ligularia* samples (samples *A* and *B*), and samples with morphology of *Ligularia subspicata* (sample *C*), *Ligularia lamarum* (sample *D*), or *Ligularia cyathiceps* (sample *E*), were collected at Tianchi Pond, Shangrila County, Yunnan Province, China. Analysis of the nucleotide sequence of the internal transcribed spacers (ITSs) in the nuclear ribosomal RNA gene cluster indicated that not only sample *B* but also sample *D* was a hybrid of *L. cyathiceps* and *L. lamarum*/*L. subspicata*. Although the morphology of sample *A* suggested that it was also a hybrid, the ITS sequence of sample *A* was that of *L. cyathiceps*. Twenty compounds were isolated from the five samples, and the structures of two new compounds **7** and **14** were determined. Furanoeremophilanes typical of *L. lamarum*/*L. subspicata* were detected not only in samples *C* and *D*, but also in samples *A* and *B*. These results indicate that the ability to produce root chemicals can spread through introgression.

Introduction. – The genus *Ligularia* (Asteraceae) is highly diversified in the Hengduan Mountains area of China [1]. We have been studying the chemical diversity of *Ligularia* by combining two different approaches: analyses of the chemical composition in the root and the nucleotide sequences of evolutionarily neutral DNA regions. To date, we have reported that many *Ligularia* species harbor intraspecific diversity at various levels [2]. For example, *Ligularia virgaurea* (MAXIM.) MATTF. [3][4] contains five distinct chemotypes, *Ligularia dictyoneura* (FRANCH.) HAND.-MAZZ. [5] and *Ligularia kanaitzensis* (FRANCH.) HAND.-MAZZ. [6][7] harbor complex diversity, while *Ligularia cymbulifera* [8] and *Ligularia cyathiceps* [9] are uniform. Sesquiterpenoids with the eremophilane skeleton have been isolated from most of the *Ligularia* species.

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In the evolution of *Ligularia*, hybridization appears to be a major pathway, resulting in reticulate evolution within and among *Ligularia* and related genera [10]. Some natural hybrids of *Ligularia* have actually been found in the Hengduan Mountains area [11]. A study on *Ligularia duciformis* and related species suggested that the ability to produce sesquiterpenoids was acquired through hybridization [12]. We recently reported that three hybrid individuals of *Ligularia nelumbifolia* and *Ligularia subspicata* produced furanoeremophilanes [13], which were produced by the latter species but not by the former.

In the course of our search in the field, we found morphologically ambiguous *Ligularia* samples at Tianchi Pond in Shangrila County of Yunnan Province of China. Around the pond, *Ligularia lamarum*, *L. subspicata*, and *L. cyathiceps* were growing, the last of which was the most abundant. *L. lamarum* and *L. subspicata* are morphologically almost identical [1]. The major diagnostic difference between the two species is the presence (*L. lamarum*) or the lack (*L. subspicata*) of ligulate florets. However, the length of the florets is variable in *L. lamarum*, and, therefore, the two taxa appear to constitute a complex. In addition, we have found that *L. subspicata* and *L. lamarum* are indistinguishable in terms of both the chemical composition in the root and the nucleotide sequence of the internal transcribed spacers (ITSs) of the ribosomal RNA gene cluster [14][15]. They produce furanoeremophilanes and/or eremophilan-8-ones [14]. Isolation of alkaloids from *L. subspicata* has also been reported [16]. *L. cyathiceps* produces cacalol as the major component of the root chemicals [9]. Several 9-oxofuranoeremophilanes, biosynthetic precursors of cacalol [17], have also been isolated from this species. *L. cyathiceps* has been found to be distinct from *L. lamarum*/*L. subspicata* with respect to the DNA sequence and the chemical composition [9][14][15]. These results suggested that hybrids of the *L. lamarum*/*L. subspicata* complex and *L. cyathiceps* would be identifiable, and that chemical outcomes of hybridization could be studied, leading us to analyze the ambiguous samples.

Results and Discussion. – *Sample Collection and Morphology.* Five samples were collected at Tianchi Pond in 2011 (*Table 1*). Samples *A* and *D* were collected at a small hollow (a few meters in diameter) *ca.* 100 m from the pond, and sample *C*, at another hollow. Samples *B* and *E* were collected by the pond. Only a few individuals were collected in order not to disturb the populations. Samples *A* and *B* lacked ligulate florets and appeared to be *L. subspicata*. However, other morphological characters did not conform to those of *L. subspicata*. Sample *C* showed morphological characters of *L. subspicata*; sample *D*, of *L. lamarum*; and sample *E*, of *L. cyathiceps.*

Genetic Analysis. The nucleotide sequence of the ITS1-5.8S-ITS2 region of the ribosomal gene cluster was determined for the five samples. The results are compiled in *Table 2*. Comparison of the sequences with those reported earlier for *L. subspicata* [14][15] and *L. cyathiceps* [9] showed that the sequences of samples *A* and *E* were very similar to that of *L. cyathiceps*; the sequence of sample *C* was very similar to that of *L. subspicata*; the sequences of samples *B* and *D* were superpositions of the two types of sequences, indicating that the samples were hybrids of *L. cyathiceps* and *L. lamarum*/*L. subspicata*. These results and the morphological features mentioned above led to the following conclusions. *i*) Sample *A* lacked ligulate florets and was not a genuine *L. cyathiceps*, and therefore, was very likely to be a hybrid. One explanation for the

Table 1. *Morphology, ITS Sequences, and Chemical Compositions of the Samples*

Sample	Morphology	ITS1-5.8S-ITS2 Sequence[a])	Chemical composition[a])
A	Ambiguous	C	LS + C
B	Ambiguous	LS + C	(LS)[b]) + C
C	*L. subspicata*	LS	LS
D	*L. lamarum*	LS + C	LS
E	*L. cyathiceps*	C	C

[a]) LS, Compounds or sequences typical of the *L. lamarum*/*L. subspicata* complex; C, compounds or sequences typical of *L. cyathiceps.* [b]) LS Compounds were detected only on TLC. See text.

sequence is that backcrossing with *L. cyathiceps* has resulted in reversion of the ribosomal gene cluster to that of *L. cyathiceps. ii*) Sample *B* was a hybrid of *L. subspicata* and *L. cyathiceps. iii*) Sample *C* was *L. subspicata*. *iv*) Sample *D* was actually a hybrid, although morphologically indistinguishable from *L. lamarum*. Back-crossing is a possible explanation for the reversion of morphology. *v*) Sample *E* was *L. cyathiceps.*

Table 2. *Differences among the ITS1–5.8S-ITS2 Sequences of the Present Samples and those in the Database*[a])

Sample	ITS1														5.8S			ITS2								
					1	1	1	1	1	1	2	2	2	2		1	1						1	1	1	2
	1	1	4	9	1	2	2	2	8	9	0	1	2	3	4	0	2		1	1	2	3	0	0	5	2
	1	3	6	4	3	5	6	7	3	6	6	7	3	6	8	3	4	3	5	1	5	7	0	7	9	9	0
A	Y	G	C	C	A	C	T	A	C	G	T	–	C	A	G	G	C	C	C	C	–	C	T	T	G	A	T
B	C	R	Y	C	R	Y	Y	M	Y	G	Y	[d])	Y	R	G	G	C	C	C	Y	[e])	C	Y	Y	G	A	Y
C	C	A	T	C	G	T	C	C	Y	S	C	C	T	G	G	G	Y	C	Y	T	A	Y	Y	C	G	C	C
D	C	R	Y	C	R	Y	Y	M	Y	G	Y	[d])	Y	R	G	G	C	Y	C	Y	[e])	C	Y	Y	G	M	Y
E	C	G	C	C	A	C	T	A	C	G	T	–	C	A	R	K	C	C	C	C	–	C	T	T	G	A	C
L. subspicata[b])	C	A	T	T	G	T	C	C	T	G	C	C	T	G	G	G	C	C	C	T	A	C	T	C	T	C	C
L. cyathiceps[c])	C	G	C	C	A	C	T	A	C	G	T	–	C	A	G	G	C	C	C	C	–	C	T	T	G	A	C

[a]) K, C + T; M, A + C; R, A + G; S, C + G; Y, T + C. The base numbering is according to that of the *L. subspicata* sequence. [b]) DQ272338. [c]) DQ272328. [d]) Two sequences with and without a C were present. [e]) Two sequences with and without an A were present.

Chemical Analysis. Prior to isolation and characterization of the root compounds of the samples, *Ehrlich*'s test was carried out by TLC [18][19] using EtOH extracts of fresh roots. Samples *A* and *B* showed many pink spots, suggesting the presence of various furanoeremophilanes. Samples *C* and *D* also showed several pink spots, among which the spot with R_f (hexane/AcOEt 7:3) 0.65 was the largest. Sample *E* showed a large spot with the same R_f, but its color was dark blue, suggesting the presence of cacalol [19].

LC/MS Analysis was carried out for the same extracts. The direct atmospheric-pressure CI-MS (AP-CI-MS) and the total ion chromatogram (TIC) of the samples are shown in *Figs. 1* and *2*, respectively. Samples *C* and *D* displayed the same spectrum in both AP-CI-MS and TIC, indicating that the two samples were identical in their

chemical composition. Two major peaks were observed at t_R 16.7 (*m*/*z* 347) and 15.3 min (*m*/*z* 235). Sample *B* showed a similar TIC (t_R 16.8 and 15.2 min), however, the compounds were different (*m*/*z* 231 for both peaks). Sample *E* showed a major peak at t_R 10.9 min (*m*/*z* 333), while sample *A* showed many peaks.

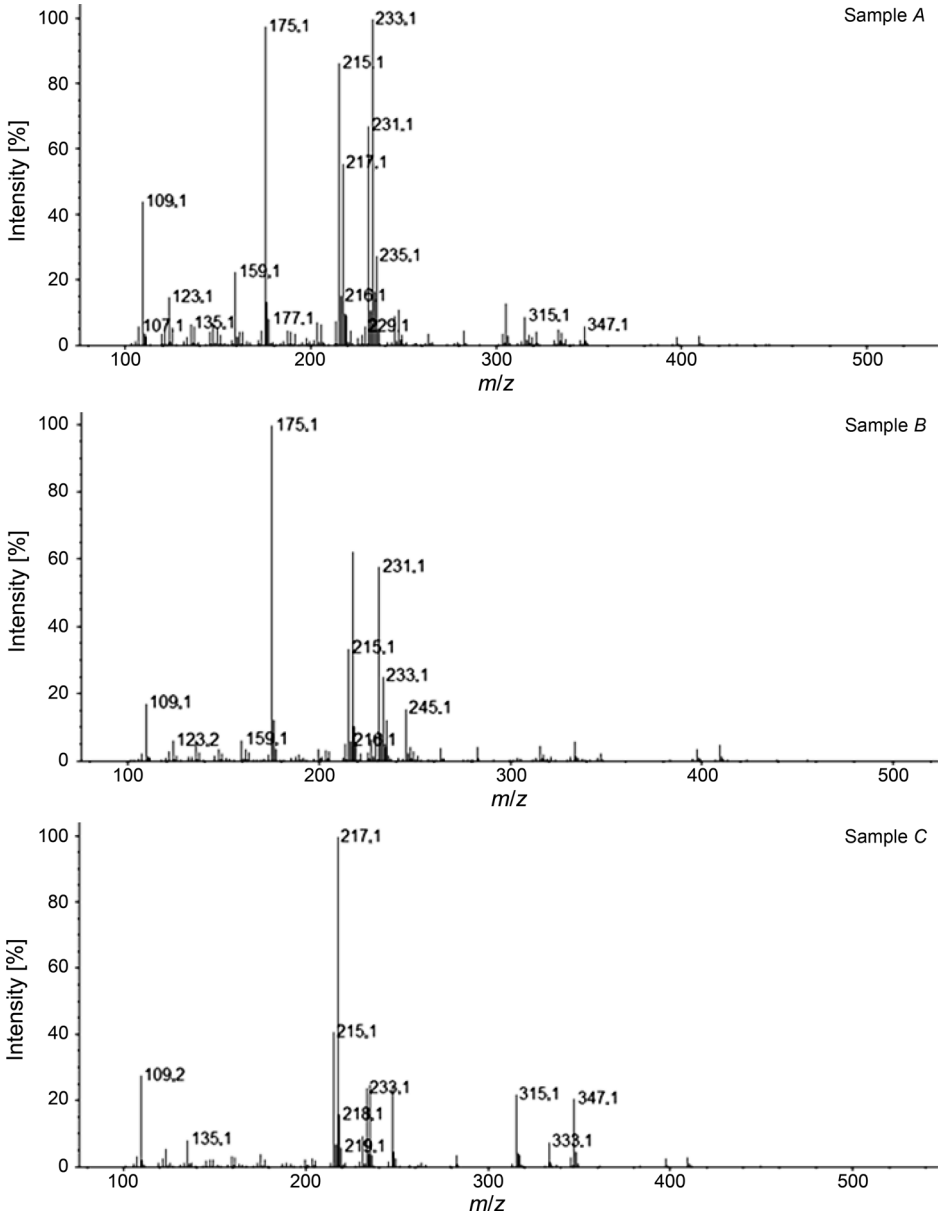


Fig. 1. *Direct AP-CI-MS for samples* A–E (from the top to the bottom)

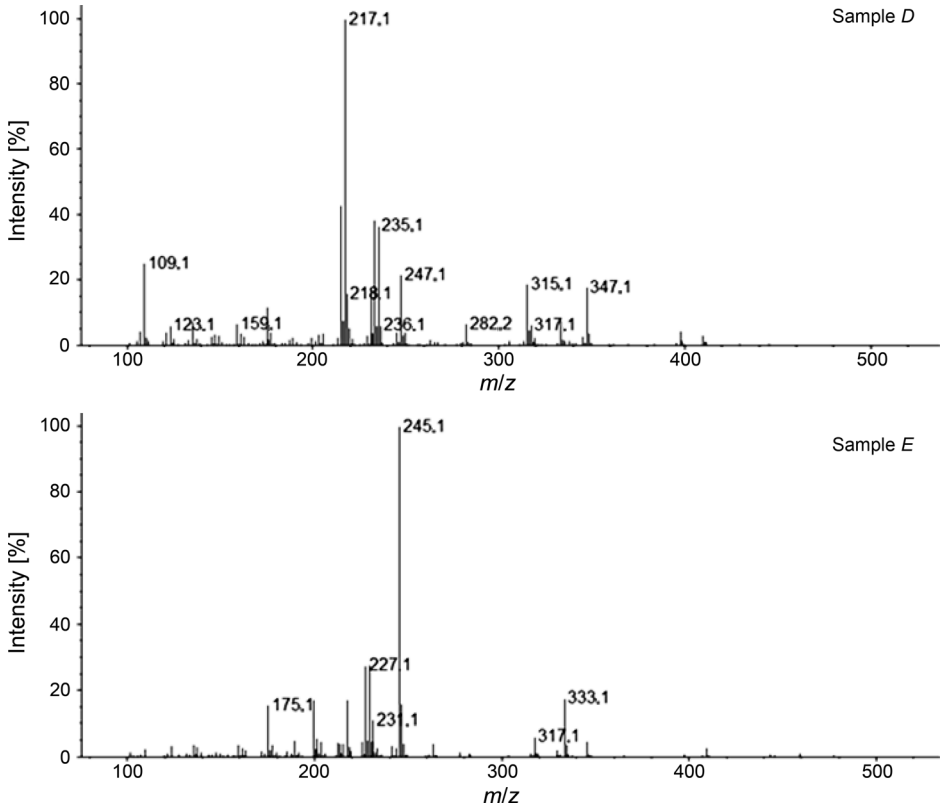
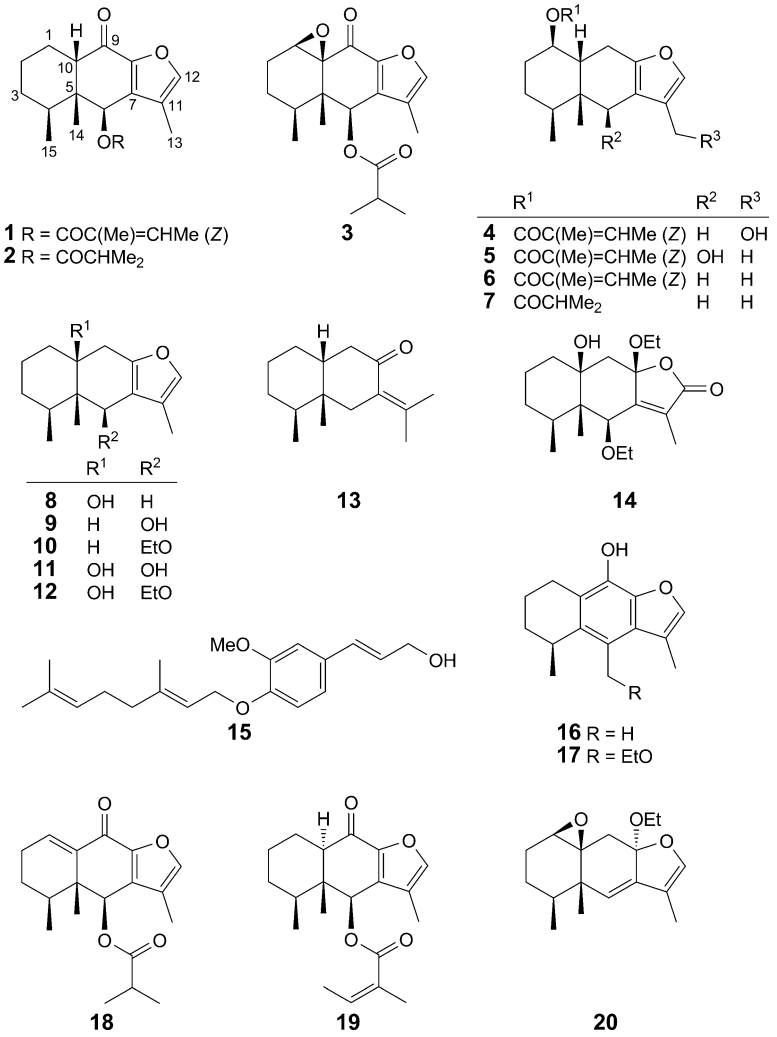


Fig. 1. (cont).

Separation of the chemicals was carried out for EtOH extracts of air-dried roots by standard techniques such as silica-gel column chromatography and HPLC.

From sample *A*, **1** [20], **2** [21], **3** [22], subspicatins A [15], B [15], C [15] (**4**–**6**, resp.), a new furanoeremophilane **7**, tetradymol (**8**) [23], ligularol (= petasalbin; **9**) [24], **10** [15][25], **11** [26], **12** [6], and fukinone (**13**) [27] were isolated. Among them, **4**–**6** (subspicatins) had previously been isolated from *L. subspicata* and *L. lamarum* [14][15] as characteristic compounds of these species. Compound **7** is a derivative of a subspicatin. Ligularol (**11**), tetradymol (**8**), and their derivatives **9**, **10**, and **12** have been found in various *Ligularia* species [2] including *L. subspicata* [14][15], while these compounds have not been found in *L. cyathiceps*. In contrast, 9-oxofuranoeremophilanes **1**–**3** are characteristic of *L. cyathiceps* [9] and have not been found in *L. subspicata*. The major peaks in LC/MS were identified as **12** (t_R 17.4 min; *m*/*z* 233), **1** (t_R 16.8 min; *m*/*z* 347), **8** (t_R 15.6 min; *m*/*z* 235), **2** (t_R 15.2 min; *m*/*z* 231), and **11** (t_R 11.5 min; *m*/*z* 233) by comparison with the t_R and the *m*/*z* values of pure compounds.

From sample *B*, only 9-oxofuranoeremophilanes **1** and **3** were isolated. In addition, **2** was detected in LC/MS (t_R 15.2 min), together with **1** (t_R 16.8 min). While these compounds are known to be negative to *Ehrlich*'s test [19], many pink spots were



detected on TLC of the extract of fresh root, as described above. The *Ehrlich*-positive compounds were probably too small in quantity to be isolated. Since compounds isolated from *L. cyathiceps* are mostly *Ehrlich*-negative [9] (also see below), the compounds of the pink spots are likely to be furanoeremophilanes originating from *L. subspicata*.

From sample *C*, **4**, **5**, **9**, **10**, and **12** were isolated. All of them are characteristic of *L. lamarum*/*L. subspicata*. Two major peaks were observed in LC/MS and identified as subspicatin A (**4**; t_R 16.7 min; *m*/*z* 347) and ligularol (**9**; t_R 15.3 min; *m*/*z* 235).

LC/MS Analysis indicated that the chemical composition of sample *D* was identical to that of sample *C* (*Figs. 1* and *2*), as described above. The two major peaks at t_R 16.7 and 15.3 min were attributed to **4** and **9**, respectively. However, these compounds were not isolated, as most of the components in the sample decomposed during handling.

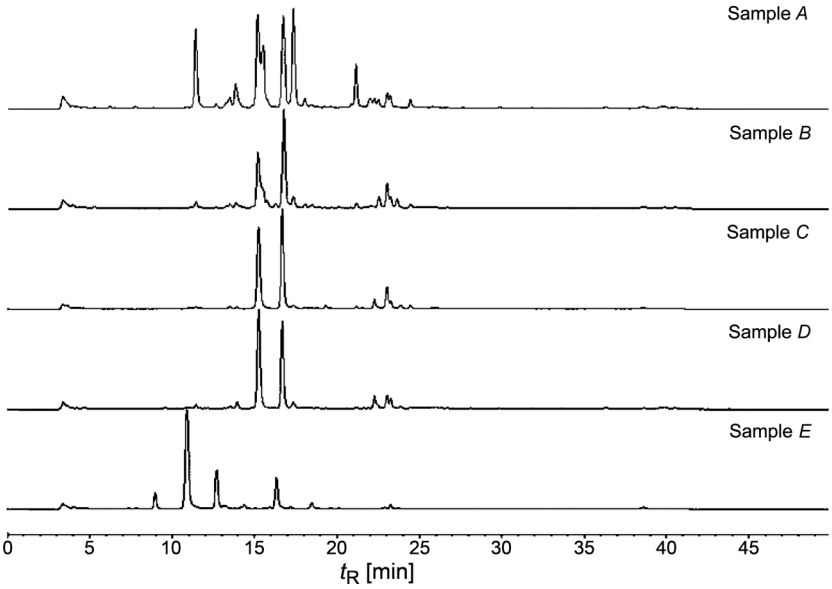


Fig. 2. *LC Profiles for samples* A–E *(from the top to the bottom)*

Only, a new eremophilanolide, **14**, and an *O*-geranylconiferyl alcohol, **15** [28], alone were isolated.

From sample *E*, cacalol (**16**) [17][29], 14-ethoxycacalol (**17**) [4], and three 9-oxofuranoeremophilanes, *i.e.*, **3**, adenostylone (**18**) [30], and **19** [31], were isolated. These compounds are typical components of *L. cyathiceps* [9]. The major component was **3** (t_R 10.9 min, *m*/*z* 333), which constituted about one fourth of the extract (see the *Exper. Part*). Compounds **20** [9] and **15** were also isolated.

The structures of the new compounds **7** and **14** were determined by spectroscopic analysis as follows.

The molecular formula of **7** was determined as $C_{19}H_{28}O_3$ by HR-CI-MS. Absorption at 1723 cm^{-1} in the IR spectrum indicated the presence of an ester C=O group. The ^{1}H- and ^{13}C-NMR spectra were very similar to those of the known compound **4**, except that the signals of an ^{i}Pr group (one H-atom *septuplet* at δ(H) 2.34 and two Me *doublet*s at 1.04) were observed instead of those of an angeloyl group, suggesting that **7** was an isobutyrate. The planar structure was established by COSY and HMBC correlations, and the configuration was determined by the NOESY spectrum (*Fig. 3*). The α-axial orientation of H–C(1) was supported by the coupling pattern (*td*, $J = 10.8, 4.8$), which was very similar to that of H–C(1) of **4**. Therefore, the structure of **7** was established as depicted.

The molecular formula of **14** was deduced as $C_{19}H_{30}O_5$ from HR-CI-MS. The IR spectrum evidenced the presence of an OH and an ester C=O group. The ^{1}H- and ^{13}C-NMR spectra indicated the presence of two EtO groups, in addition to three Me groups of eremophilanolides. The planar structure was established by COSY and

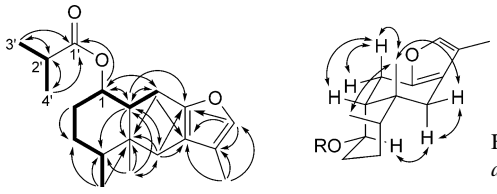


Fig. 3. *Key* ^{1}*H,*^{1}*H-COSY* (—), *HMBC* (H → C), *and NOESY* (H ↔ H) *correlations of* **7**

HMBC correlations (*Fig. 4*). The configuration was established by NOE correlations as depicted in *Fig. 4*. This compound may be an artifact generated from **11** or **12** during EtOH extraction [6][13]. Compounds **10**, **12**, and **17** may also be artifacts.

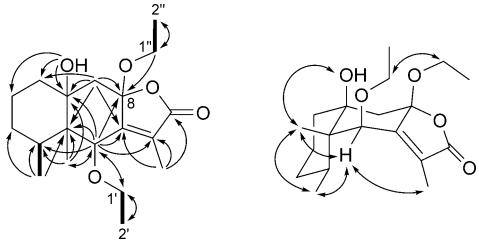


Fig. 4. *Key* ^{1}*H,*^{1}*H-COSY* (—), *HMBC* (H → C), *and NOESY* (H ↔ H) *correlations of* **14**

Conclusions. – Five *Ligularia* samples, including two morphologically ambiguous ones, collected at Tianchi, Yunnan Province, were analyzed. Morphology and DNA sequences indicated that three of them (samples *A*, *B*, and *D*) were hybrids, while samples *C* and *E* were *L. subspicata* and *L. cyathiceps*, respectively. In the hybrid samples, furanoeremophilanes, presumably originating from *L. lamarum*/*L. subspicata*, were detected, although their quantities were small in sample *B*. We recently reported that hybrids of *L. subspicata* and *L. nelumbifolia* also produced furanoeremophilanes of *L. subspicata* origin [13]. Signs of past hybridization have been observed in a number of *Ligularia* specimens in the present and the previous studies [4][12][13][32]. Thus, it seems likely that some *Ligularia* species have acquired the ability to produce a variety of compounds through crossing and backcrossing, namely introgression.

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Experimental Part

General. Anal. TLC: silica gel *60* F_{254} (SiO_2; layer thickness, 0.2 or 0.25 mm; *Merck*); visualized by either *Ehrlich*'s reagent (*p*-(dimethylamino)benzaldehyde and HCl) [18][19] or *p*-anisaldehyde/AcOH/H_2SO_4. Column chromatography (CC): SiO_2 (*Wakogel C-200* or *C-300*, or *Kanto 60 N* (spherical neutral)). HPLC: *Shimadzu LC-20AT* pump, *SPD-20A* UV/VIS detector, *Kanto Mightysil Si60* (10 × 250 mm) ODS column. Optical rotations: *JASCO DIP-370* digital polarimeter. IR Spectra: *JASCO FT/IR-230* spectrometer; $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR: *JEOL ECX-400* or *JEOL AL-400* (400 and 100 MHz, resp.) spectrometers with $CDCl_3$ or C_6D_6 as solvents; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. AP-CI- and HR-CI-MS: *JEOL JMS-700 Mstation*; in *m*/*z* (rel. %). LC/MS: *Agilent 1100 series LC/MSD*

mass spectrometer (cap. voltage, 3.5 kV; crona current, 4 μA; cap. exit voltage (fragmentor), 90 V; drying temp., 330°; drying flow, 9 l min^{-1}; nebulizer pressure, 50 psig) with *5C18-MS-II* (*COSMOSIL*; 4.6 × 150 mm; 5 μm octadecyl column); in *m/z*. DNA Sequencing: *3130xl* and *3500 Genetic Analyzers* (*Applied Biosystems*).

Plant Material. Samples were collected at Tianchi Lake (elevation 3900 m), Shangrila County, Yunnan Province of China, in August, 2011. Each sample was identified by *X. G.* Voucher specimen Nos. were 2011–117 (sample *A*), 2011-118 (sample *D*), 2011-119 (sample *E*), 2011-120 (sample *B*), and 2011-121 (sample *C*).

Extraction for Ehrlich*'s Test and LC/MS*. The roots of each plant (2–10 g) were harvested, and the extraction with EtOH was started immediately without drying. Solid plant material was removed after several days, and the soln. was subjected to TLC without concentration (see our previous report for the procedure of the test [8]).

Extraction and Purification. Dried roots of sample *A* (17.7 g) were extracted with EtOH at r.t., and the solvent was evaporated under reduced pressure to give an oily extract (1.16 g), which was subjected to CC (SiO_2 (20 g); hexane/AcOEt 20:1 to 3:2). Fractions eluted with hexane/AcOEt 20:1 were subjected to HPLC to afford **6** (7.0 mg), **7** (13.8 mg), **13** (4.3 mg), **12** (81.4 mg), **1** (11.7 mg), **2** (7.8 mg), **9** (0.6 mg), and **10** (13.4 mg). Compound **8** was also obtained as an inseparable mixture with triglyceride (6.3 mg). From fractions eluted with hexane/AcOEt 4:1, **5** (14.9 mg) was isolated. From fractions eluted with hexane/AcOEt 3:2, **11** (5.3 mg) and **4** (16.5 mg) were isolated.

Dried roots of sample *B* (1.5 g) were extracted with EtOH as described above, and the resultant oily extract (111 mg) was submitted to CC (SiO_2 (11 g); hexane/AcOEt, gradient). Fractions eluted with hexane/AcOEt 20:1 to 10:1 were subjected to HPLC to afford **1** (11.4 mg). From the polar fractions, **3** (11.0 mg) was isolated.

Dried roots of sample *C* (11.4 g) were extracted with EtOH as described above, and the resultant oily extract (569 mg) was subjected to CC (SiO_2 (22 g); hexane/AcOEt, gradient). Fractions eluted with hexane/AcOEt 20:1 were further subjected to HPLC to afford **10** (27.1 mg). From fractions eluted with hexane/AcOEt 10:1 and 5:1, **9** (14.7 mg) and **5** (4.1 mg) were isolated. Compound **12** was also obtained as an inseparable mixture with triglyceride (10.3 mg). From the polar fractions, **4** (63.7 mg) was obtained.

Dried roots of sample *D* (4.0 g) were extracted with EtOH described as above, and the resultant oily extract (355 mg) was subjected to CC (SiO_2 (12 g); hexane/AcOEt, gradient). Fractions eluted with hexane/AcOEt 10:1 were further purified by HPLC to afford **14** (2.1 mg) and **15** (1.6 mg).

Dried roots of sample *E* (7.9 g) were extracted with EtOH as described above, and the resultant oily extract (1.07 g) was subjected to CC (SiO_2 (18 g); hexane/AcOEt, gradient). Compounds eluted with hexane/AcOEt 97:3 were further purified by CC and HPLC to give **20** (4.6 mg), **16** (31.5 mg), and **17** (2.1 mg). From fractions eluted with hexane/AcOEt 95:5, **19** (3.1 mg) and **18** (4.5 mg) were obtained. From fractions eluted with hexane/AcOEt 90:10, **3** (254.3 mg) and **15** (1.8 mg) were obtained.

*(4a*R*,5*S*,8*R*,8a*R*)-3,4a,5-Trimethyl-4,4a,5,6,7,8,8a,9-octahydronaphtho[2,3-*b*]furan-8-yl 2-methylpropanoate* (**7**). Oil. $[\alpha]_D^{22} = -50.2$ ($c = 0.24$, $CHCl_3$). FT-IR (neat): 1723 (C=O). ^{1}H-NMR (C_6D_6)[2]): 0.74 (*s*, Me(14)); 0.82 (*d*, $J = 7.2$, Me(15)); 1.04 (*d*, $J = 6.9$, Me(3′), Me(4′)); 1.11–1.18 (*m*, H_β–C(3)); 1.20–1.30 (*m*, H–C(4)); 1.38–1.44 (*m*, H_α–C(2)); 1.66 (br. *d*, $J = 15.8$, H_β–C(6)); 1.70–1.82 (*m*, H_α–C(3), H–C(10), overlapped); 1.75 (*d*, $J = 1.0$, Me(13)); 1.91–1.98 (*m*, H_β–C(2)); 2.34 (*sept.*, $J = 6.9$, H–C(2′)); 2.45–2.52 (*m*, H_β–C(9)); 2.52 (br. *d*, $J = 15.8$, H_α–C(6)); 2.88 (br. *d*, $J = 17.0$, H_α–C(9)); 4.76 (*td*, $J = 11.0$, 4.6, H_α–C(1)); 7.01 (*s*, H–C(12)). ^{13}C-NMR (C_6D_6)[2]): 8.2 (C(13)); 14.7 (C(15)); 19.1 (C(3′)); 19.1 (C(4′)); 21.4 (C(9)); 24.8 (C(14)); 26.7 (C(2)); 27.2 (C(3)); 30.1 (C(6)); 34.4 (C(2′)); 37.4 (C(4)); 37.4 (C(5)); 40.4 (C(10)); 72.3 (C(1)); 115.8 (C(7)); 119.8 (C(11)); 138.2 (C(12)); 147.8 (C(8)); 175.7 (C(1′)). CI-MS: 304 (45, M^+), 217 (100), 41 (46). HR-CI-MS: 304.2042 (M^+, $C_{19}H_{28}O_3^+$; calc. 304.2039).

*(4*S*,4a*S*,5*S*,8a*S*,9a*S*)-4,9a-Diethoxy-8a-hydroxy-3,4a,5-trimethyl-4a,5,6,7,8,8a,9,9a-octahydronaphtho[2,3-*b*]furan-2(4*H*)-one* (**14**). Oil. $[\alpha]_D^{25} = +41.9$ ($c = 0.58$, MeOH). FT-IR (neat): 3527 (OH), 1761 (C=O). ^{1}H-NMR ($CDCl_3$)[2]): 0.82 (*d*, $J = 6.3$, Me(15)); 1.17 (*s*, Me(14)); 1.19 (*t*, $J = 7.0$, Me(2″)); 1.15–1.25 (*m*, H–C(4)); 1.20 (*t*, $J = 7.0$, Me(2′)); 1.23–1.42 (*m*, H–C(1), CH_2(2), CH_2(3), overlapped); 1.63–1.73 (*m*, H–C(1)); 1.92 (*s*, Me(13)); 2.23 (*d*, $J = 14.4$, H–C(9)); 2.25 (*d*, $J = 14.4$, H–C(9)); 3.37 (*dq*, $J = 8.7$, 7.0,

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[2]) Assignments according to the furanoeremophilane atom numbering as indicated in the *Formulae*.

H–C(1′)); 3.43 (*dq*, *J* = 8.7, 7.0, H–C(1″)); 3.55 (*dq*, *J* = 8.7, 7.0, H–C(1′)); 3.68 (*dq*, *J* = 8.7, 7.0, H–C(1″)); 4.14 (*s*, OH); 4.32 (*s*, H–C(6)). ^{13}C-NMR ($CDCl_3$)[2]): 8.8 (C(13)); 10.8 (C(14)); 14.9 (C(2′)); 15.0 (C(2″)); 16.5 (C(15)); 21.8 (C(2)); 29.8 (C(3)); 33.3 (C(4)); 34.2 (C(1)); 41.7 (C(9)); 47.9 (C(5)); 59.2 (C(1″)); 66.8 (C(1′)); 74.2 (C(10)); 78.9 (C(6)); 106.6 (C(8)); 128.9 (C(11)); 153.7 (C(7)); 170.9 (C(12)). CI-MS: 339 (100, $[M+H]^+$), 321 (45, $[M-H_2O+H]^+$), 293 (87, $[M-EtOH+H]^+$), 275 (41), 168 (34). HR-CI-MS: 339.2173 ($[M+H]^+$, $C_{19}H_{31}O_5^+$; calc. 339.2172).

DNA Analysis. DNA Purification from dried leaves, amplification of the ITS1-5.8S-ITS2 region by polymerase chain reaction, and sequencing were carried out as described in [33].

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