Chemical and Genetic Study of *Ligularia duciformis* and Related Species in Sichuan and Yunnan Provinces of China

by **Hajime Nagano***[a])[1]), **Ryo Hanai***[b])[1]), **Hiroka Yamada**[a]), **Mika Matsushima**[a]), **Yui Miura**[a]), **Takanori Hoya**[c]), **Masaaki Ozawa**[c]), **Miho Fujiwara**[d]), **Hikari Kodama**[d]), **Atsushi Torihata**[e]), **Hiroyuki Onuki**[f]), **Yoko Nezu**[f])[g]), **Satoru Kawai**[g]), **Mizue Yamazaki**[f]), **Hiroshi Hirota***[f])[1])[2]), **Yoshinori Saito**[d]), **Motoo Tori***[d])[1]), **Ayumi Ohsaki***[c])[1]), **Xun Gong***[h])[1]), and **Chiaki Kuroda***[e])[1])

[a]) Department of Chemistry, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan (phone: +81-359785348; fax: +81-359785715; e-mail: nagano.hajime@ocha.ac.jp)

[b]) Department of Life Science and Research Center for Life Science, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan (phone/fax: +81-339852377; e-mail: hanai@rikkyo.ac.jp)

[c]) Institute of Biomaterials & Bioengineering, Tokyo Medical and Dental University, Kanda, Chiyoda-ku, Tokyo 101-0062, Japan (phone/fax: +81-352808153; e-mail: a-ohsaki.fm@tmd.ac.jp)

[d]) Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan (phone: +81-886028462; fax: +81-886553051; e-mail: tori@ph.bunri-u.ac.jp)

[e]) Department of Chemistry and Research Center for Smart Molecules, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan (phone/fax: +81-339852396; e-mail: chkkuroda@grp.rikkyo.ne.jp)

[f]) RIKEN Genomic Sciences Center, Suehiro-cho, Tsurumi-ku, Yokohama 230–0045, Japan (phone: +81-484674839; fax: +81-484621353; e-mail: hhirota@riken.jp)

[g]) Faculty of Science and Engineering, Tokyo Denki University, Hatoyama, Saitama 350-0394, Japan

[h]) Kunming Institute of Botany, Chinese Academy of Science, Kunming 650204, China (phone: +86-8715223625; e-mail: gongxun@mail.kib.ac.cn)

The chemical constituents of the root extracts and the evolutionarily neutral DNA base sequences were studied for 28 samples of *Ligularia duciformis*, *L. kongkalingensis*, and *L. nelumbifolia* collected in Sichuan and Yunnan Provinces of China. The samples could be classified into four chemotypes (*1*–*4*). Sesquiterpenoids having eremophilane and oplopane skeletons were isolated from two (*Chemotype 1*) and three (*Chemotype 2*) samples, respectively. Two new oplopane derivatives were isolated and their structures were determined. In 18 samples, phenylpropenoids were the major components (*Chemotype 3*). In five samples, neither phenylpropenoids nor sesquiterpenoids were found (*Chemotype 4*). Despite this large chemical variety, no correlation was found between the chemotype and the morphological criteria of species identification. The analysis of the evolutionarily neutral DNA regions also indicated that the samples were not separated into distinct clades and that introgression was extensive.

[1]) Corresponding authors: *C. K.* for general information, *X. G.* for taxonomic information, *R. H.* for information on the DNA analysis, *M. T.* for information of the chemical aspects of *Samples 1*, *4*, and *11*, *H. H.* of *Samples 5* and *9*, *A. O.* of *Samples 6*, *10*, *12*, and *13*, and *H. N.* of the other samples, respectively.

[2]) Present address: Chemical Biology Core Facility, RIKEN, Hirosawa, Wako, Saitama 351-0198, Japan.

Introduction. – *Ligularia* (Asteraceae) is a genus consisting of over 100 species. The genus is highly diversified in the Hengduan Mountains of China [1–3], especially in Yunnan and Sichuan Provinces. Our research has aimed at elucidating the ongoing evolution and diversification of the genus by using two independent approaches, *i.e.*, the analysis of the chemical constituents in the root extracts and the analysis of the nucleotide sequences of evolutionarily neutral DNA regions. The DNA data can yield systematics information of the investigated species and also can serve as a measure of diversity, which is independent of the chemical diversity. So far, we have uncovered the presence of intraspecific diversity in major species of *Ligularia* in the provinces of Yunnan and Sichuan, such as *L. pleurocaulis* (Franch.) Hand.-Mazz. [4], *L. virgaurea* (Maxim.) Mattf. [5], *L. vellerea* (Franch.) Hand.-Mazz. [6], and *L. tongolensis* (Franch.) Hand.-Mazz. [7]. Although these species were found to produce furanoeremophilanes, other species, such as *L. lankongensis* (Franch.) Hand.-Mazz. [8] and *L. latihastata* (W. W. Smith) Hand.-Mazz. [9], were not.

The clarification of similarities and differences between morphologically similar species is important not only from a taxonomical, but also from a medicinal point of view, as the roots of some *Ligularia* species are used as Chinese medicine [1]. During the course of our continuing study, we have also found that *i*) *L. latihastata* and *L. villosa* (Hand.-Mazz.) S. W. Liu were chemically and genetically very similar [9], *ii*) *L. lamarum* (Diels) Chang and *L. subspicata* (Bureau & Franch.) Hand.-Mazz. were chemically and genetically indistinguishable [10], *iii*) *L. anoleuca* Hand.-Mazz. and *L. veitchiana* (Hemsl.) Greenm. were chemically and genetically distinct [11], and *iv*) *L. franchetiana* (Lévl.) Hand.-Mazz. and *L. oligonema* Hand.-Mazz. were chemically similar, but genetically distinct [12][13].

In this study, as part of the above mentioned research program, the diversity between *L. duciformis* (C. Winkl.) Hand.-Mazz., *L. nelumbifolia* (Bureau & Franch.) Hand.-Mazz., and *L. kongkalingensis* Hand.-Mazz., collected in the provinces of Sichuan and Yunnan was investigated. These species are morphologically very close to one another, belonging to the section Corymbosae and the series Retusae, and growing at stream banks, in forest understories, in grasslands, and on alpine meadows [1]. *L. duciformis* and *L. nelumbifolia* are distributed in the provinces of Gansu, Hubei, Sichuan, and Yunnan, while *L. kongkalingensis* grows only in Sichuan [1]. The species identification was based on pilose (*L. kongkalingensis*) or glabrous (*L. duciformis* and *L. nelumbifolia*) involucres and shorter (*L. duciformis*) or longer (*L. nelumbifolia*) pappi. However, in our observation, the morphological characteristics were rather continuous.

Several research groups have reported the chemical constituents of the roots of *L. duciformis* collected in Hubei and Gansu Provinces of China, isolating alkaloids [14–17], terpenoids [18][19], and coniferyl and sinapyl alcohols [20–22]. Recently, *Wang et al.* [23] reported the isolation of eremophilane-type sesquiterpene lactones from *L. duciformis* collected in the Meigu County, Sichuan Province. We isolated oplopane-type sesquiterpenoids and 4-*O*-geranylconiferyl alcohol from a sample collected in Yunnan Province (*Sample 3* in *Table 1*) [24]. From *L. nelumbifolia* collected in Hubei and Yunnan Provinces, sinapyl alcohol derivatives have been isolated [25–29], and from a sample collected in Hubei Province, eremophilane and guaiane-type sesqui-

Table 1. *Location of the Collection Site, Chemotype, and Isolated Compounds for the Investigated Samples of* Ligularia duciformis *and Related Species*

Sample[a])	Species[b])	Location	Elevation [m]	Chemotype[c])	Isolated Compounds[d])		
					Sesquiterpenoids (**1**–**7**)	Phenylpropenoids (**8**–**17**)	Others (**18**–**24**)
1	*d*	Dabaoshan	3600	*1*	**1**, **2**	**12**	
2	*d*	Maerkang/Xiaojin	4000	*1*	**3**	**13**, **14**	
3[e])	*d*	Tianchi	3700	*2*	**4**, **5**	**8**	
4	*d*	Shikashan	4300	*2*	**4**, **5**	**8**, **15**–**17**	**18**–**21**
5	*k*	Gonggashan	3500	*2*	**4**–**7**		
6	*d*	Gaoersishan	4000	*3*		**8**–**11**	**22**
7	*d*	Gaoersishan	3700	*3*		**8**–**11**	**23**
8	*k*	Gaoersishan	4300	*3*		**8**–**11**	
9	*k*	Gaoersishan	3700	*3*		**8**–**11**	
10	*d*	Reda	3800	*3*		**8**, **9**, **11**	**22**
11	*d*	Tianchi	3900	*3*		**8**, **11**	**18**
12	*d*	Litang	4100	*3*		(**8**, **11**)	
13	*d*	Yajiang	4400	*3*		(**8**, **11**)	
14	*k*	Daxueshan	4200	*3*		(**9**–**11**)	
15	*k*	Wuminhshan	4600	*3*		**9**–**11**	**23**, **24**
16	*d*	Tuershan	4600	*3*		(**9**–**11**)	
17	*n*	Jiulong/Kangding	4200	*3*		**11**	**23**
18	*d*	Jiulong/Kangding	4100	*3*		(**11**)	(**23**)
19	*d*	Jiawa	4000	*3*		**11**	
20	*d*	Litang	4200	*3*		**11**	**23**
21	*k*	Ganzi/Luhuo	3900	*3*		(**11**)	(**23**)
22	*k*	Laima	3700	*3*		**11**	**23**
23	*k*	Maerkang/Xiaojin	3700	*3*		**11**	**23**
24	*d*	Liziping	3600	*4*			**23**
25	*d*	Daxueshan	4100	*4*			**23**
26	*d*	Kazilashan	4300	*4*			**23**
27	*n*	Xiaojin/Baoxing	4000	*4*			**23**
28	*k*	Zhuqing	4000	*4*			**23**

[a]) *Samples 1* and *3* were collected in 2004, *Samples 5*, *6*, *10*, *12*, *13*, and *19* in 2005, *Samples 7–9*, *14–18*, and *24–26* in 2007, *Samples 4* and *11* in 2008, and *Samples 2*, *20–23*, *27*, and *28* in 2009. [b]) *d* = *L. duciformis*-like, *k* = *L. kongkalingensis*-like, *n* = *L. nelumbifolia*-like; *cf.* text on sample collection. [c]) The characteristic compounds detected in the samples of *Chemotypes 1–3* were eremophilanes, oplopanes, and phenylpropenoids, respectively. Neither sesquiterpenoids nor phenylpropenoids were detected in the samples belonging to *Chemotype 4*. [d]) Compounds detected only on TLC are shown in parenthesis. [e]) Results from [24].

terpenoids have been isolated [30]. To the best of our knowledge, no report on the chemical constituent of *L. kongkalingensis* has been published.

The literature mentioned above convinced us of the presence of chemical diversity in *L. duciformis* and the two related species and led us to systematically examine the differences among samples from different localities of the provinces of Sichuan and Yunnan.

Results and Discussion. – *Sample Collection*. In total, 28 samples (*Table 1* and *Fig. 1*) were collected in western Sichuan and northwestern Yunnan Provinces in 2004,

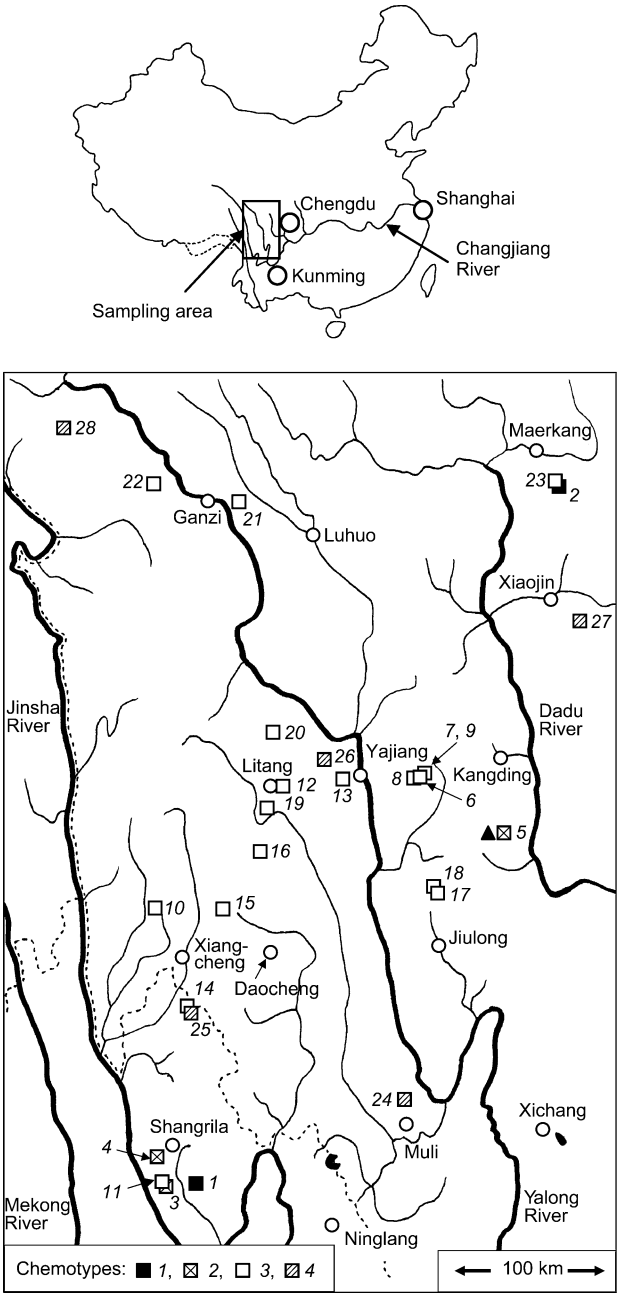


Fig. 1. *Sampling area in Sichuan and Yunnan Provinces and location of the collection sites of the samples* (*1–28*) *of* L. duciformis *and related species.* Rectangles design the collection sites of the samples (sample chemotypes *1–4* are indicated by red, blue, yellow, and green rectangles, resp.), circles the cities, and triangles the major peaks.

2005, and the period from 2007 to 2009. As mentioned above, the morphological characteristics were continuous and, therefore, some samples were difficult to identify unambiguously. Our tentative identification was as follows: *Samples 1–4*, *6–8*, *10–13*, *16*, *18–20*, and *24–26* were identified as *L. duciformis*, *Samples 5*, *9*, *14*, *15*, *21–23*, and *28* as *L. kongkalingensis*, and *Samples 17* and *27* as *L. nelumbifolia*.

Chemical Analysis. For a rough examination of the composition of the root constituents of each sample, extractions with EtOH were carried out without drying the roots, and the compounds therein were analyzed by thin-layer chromatography (TLC). Although furanosesquiterpenoids have been found in many *Ligularia* species [4–7][31–33] (see also references cited in [7]), all the samples, except *Sample 2*, were negative to *Ehrlich*'s test [4], suggesting the absence of furanoeremophilane derivatives. *Sample 2* showed a weak *Ehrlich*-positive spot, however, the identification of the compound was not successful due to its paucity (*vide infra*). The *Ehrlich*-negative compounds were detected on the TLC plate by coloring with $Ce(SO_4)_2/H_2SO_4$ for terpenoids and MoO_3/H_3PO_4 for aromatic compounds.

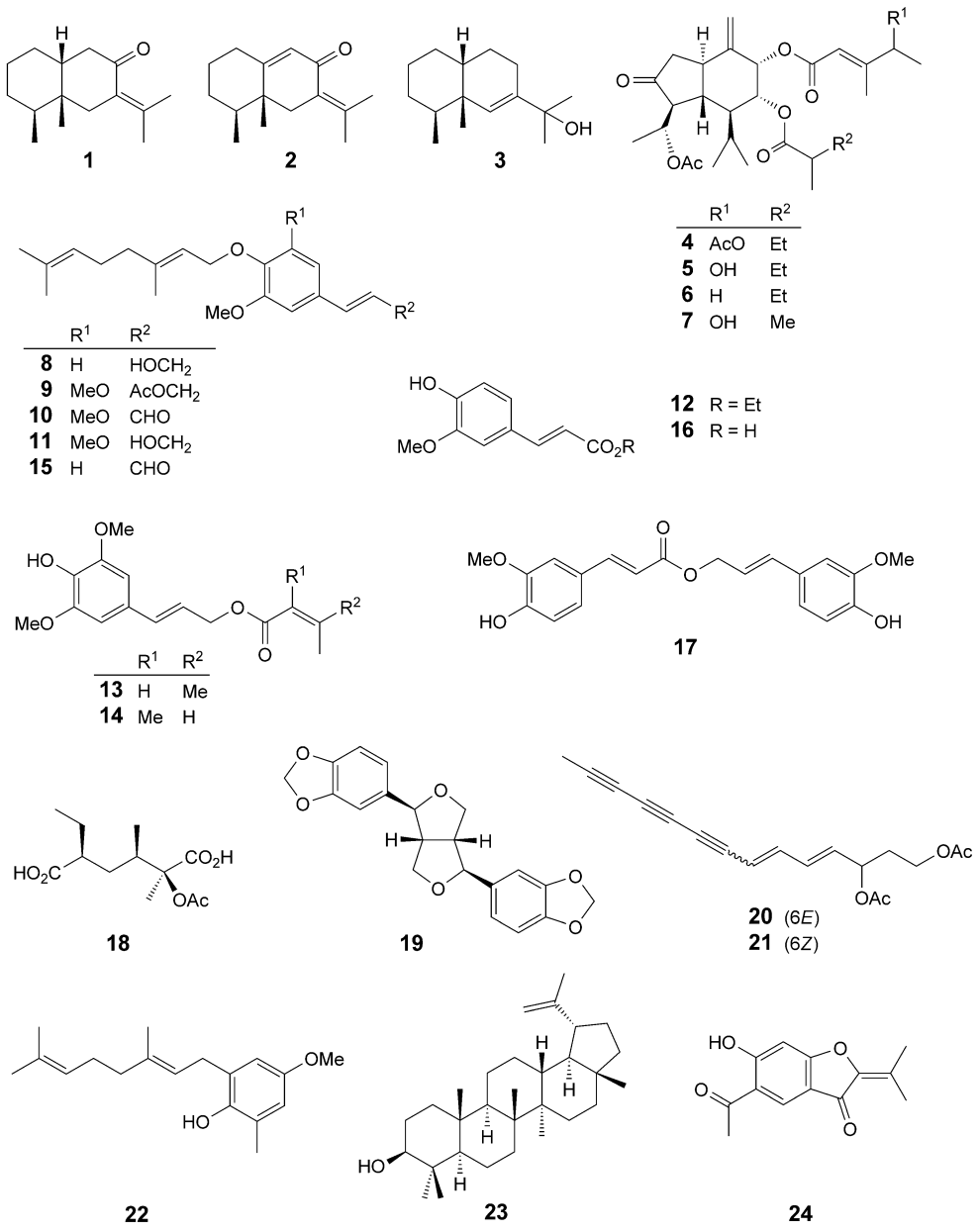
For the analysis of the chemical components, air-dried roots of each sample were extracted with AcOEt or EtOH at room temperature. The extracts were fractionated by silica gel column chromatography and subsequent HPLC or preparative TLC. In total, 24 compounds, **1**–**24**, were isolated. Three of them, **1**–**3**, were eremophilane-type and four of them, **4**–**7**, were oplopane-type sesquiterpenoids, while the majority, **8**–**17**, were phenylpropenoids (cinnamyl alcohol, cinnamaldehyde, and cinnamic acid derivatives; *Table 1*). The samples could be divided into four chemotypes on the basis of the chemical composition, *viz.*, those producing eremophilanes (**1**–**3**; *Chemotype 1*), those producing oplopanes (**4**–**7**; *Chemotype 2*), those producing coniferyl and sinapyl alcohols (**8**–**11**) but no sesquiterpenoids (*Chemotype 3*), and those producing neither sesquiterpenoids nor phenylpropenoids (*Chemotype 4*).

Samples 1 and *2* belonged to *Chemotype 1*. From the extract of *Sample 1*, two eremophilanes, *i.e.*, fukinone (**1**) [34] and dehydrofukinone (**2**) [35], were isolated in 16 and 0.4% yields, respectively, together with ethyl ferulate (**12**; 1.5%). From the extract of *Sample 2*, eremophil-6-en-11-ol (**3**) [36] and an inseparable mixture of senecioic (**13**) and angelic (**14**) esters (**13**/**14** 3:2) [37] were isolated in 1.1 and 2.0% yields, respectively. Compounds **13** and **14** were isolated for the first time from species of the genus *Ligularia*.

Samples 3–5 belonged to *Chemotype 2*. The chemical constituents isolated from *Sample 3* have already been reported, *viz.*, the oplopane-type sesquiterpenoids **4** and **5** as well as 4-*O*-geranylconiferyl alcohol (**8**) [24]. *Sample 4* also contained the oplopane-type sesquiterpenoids **4** and **5**, as well as alcohol **8**, 4-*O*-geranylconiferyl aldehyde (**15**)[3]), ferulic acid (**16**), coniferyl ferulate (**17**) [38], the dicarboxylic acid **18** [39][3]), sesamin (**19**) [40], and the acetylenic compounds **20** and **21** [41] in yields of 0.8, 0.8, 6.0, 0.4, 0.06, 0.12, 0.9, 0.7, 0.1, and 0.03 %, respectively. Compounds **19**–**21** were isolated from the genus *Ligularia* for the first time. Finally, from the roots of *Sample 5*, the

---

[3]) Spectral data of the known compounds **15** and **18** were given in the *Exper. Part*. Compound **15** is known (CAS 913691-06-1); however, no literature reference has been available. Compound **18** was isolated as a natural product for the first time, although it had been obtained as the acid part of a pyrrolizidine alkaloid.



oplopane-type sesquiterpenoids **4**–**7** were isolated in yields of 3.2, 11, 0.3, and 1%, respectively.

The structures of the new compounds **6** and **7** were determined as follows. The molecular formulae of compounds **6** and **7** were determined to be $C_{28}H_{42}O_7$ (*m*/*z*

491.2985, $[M+H]^+$) and $C_{27}H_{40}O_8$ (*m/z* 493.2811, $[M+H]^+$), respectively, by HR-MS. The structure of **6** was determined by comparing its ^{1}H-NMR spectrum with that of **5**. The signal of the Me(5″) group (δ(H) 0.87) was observed to be a *triplet* in **6**, instead of a *doublet* as in **5**. Moreover, the signal of H–C(4″) observed in the spectrum of **5** was absent in that of **6**, and the presence of signals of a CH_2(4″) group at δ(H) 1.93–1.85 and δ(C) 34.0 in the spectrum of **6** was confirmed by HMQC. Thus, **6** was determined to be the 4″-deoxy derivative of **5**. The HMQC spectra of **5** and **7** were similar. The only prominent difference was that the ^{1}H-NMR spectrum of **7** showed six characteristic Me *doublet* signals at δ(H) 0.9–1.4, of which two Me groups, at δ(H) 1.25 (δ(C) 19) and δ(H) 1.33 (δ(C) 19.5), showed correlation peaks with H–C(2′) at δ(C) 34.5 (δ(H) 2.58) in the COSY and HMBC spectra (*Fig. 2*). The signals of H–C(2′), Me(3′), and Me(4′) were also assigned by HMBC correlations with the C(1′) CO group at δ(C) 175.5. Thus, compound **7** was determined to be a 2-methylpropanoyl analog of **5**. The stereochemistry of compounds **6** and **7** was confirmed by their NOESY spectra (*Fig. 3*).

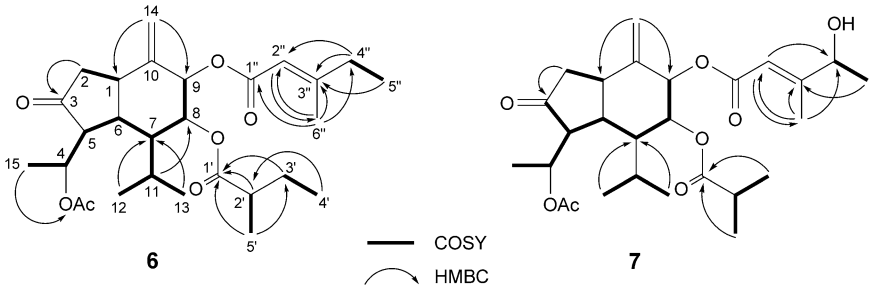


Fig. 2. *Selected HMBC and COSY correlations exhibited by compounds* **6** *and* **7**

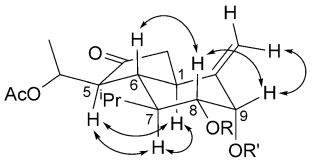


Fig. 3. *Selected NOESY correlations exhibited by compounds* **6** *and* **7**

The majority of the samples (*6–23*) belonged to *Chemotype 3*, which was characterized by four phenylpropenoids as the major components, *i.e.*, 4-*O*-geranylconiferyl alcohol (**8**) [42], 4-*O*-geranylsinapyl acetate (**9**) [27], 4-*O*-geranylsinapyl aldehyde (nelumal A, **10**) [43], and 4-*O*-geranylsinapyl alcohol (nelumol A, **11**) [43]. In addition to the coniferyl and sinapyl alcohol derivatives, the phenol **22** was identified in *Sample 6*. Although this compound had been known to be present in the rhizomes of *Atractylodes lancea* (Asteraceae) [44] and in a brown alga, *Cystophora* sp. [45], it was isolated from the genus *Ligularia* for the first time. Compounds **18**, **23**, and **24** were also found in samples belonging to *Chemotype 3*. The dicarboxylic acid **18** was identified in *Sample 11*, lupeol (**23**), a triterpene alcohol [46], in *Samples 7*, *15*, *17*, *20*, *22*, and *23*, and the benzofuran derivative **24** [27] in *Sample 15*. Compound **23** has been previously obtained from *L. duciformis* collected in Kangding County, Sichuan Province, by *Zhang et al.* [47]. The benzofuran derivative **24** has been obtained from *L. nelumbifolia*

collected in Zhang County, Gansu Province, by *Jia et al.* [27]. In contrast to the samples constituting *Chemotypes 1* and *2*, no sesquiterpenoids were detected in the samples of *Chemotype 3* by the standard analytical methods used such as TLC and HPLC. Compound **11** was common to all samples of *Chemotype 3*. On the other hand, compounds **8**–**10**, observed by TLC (R_f (hexane/AcOEt 7:3) 0.17, 0.45, and 0.25, resp.), could not be detected in all samples. Indeed, among these three compounds, **8**–**10** were present in *Samples 6–9*, **8** and **9** in *Sample 10*, only **8** in *Samples 11–13*, **9** and **10** in *Samples 14–16*, and none of them in *Samples 17–23*.

The chemical composition of *Samples 24–28* (*Chemotype 4*) was different from that of the other samples. Sesquiterpenoids **1**–**7** and phenylpropenoids **8**–**11** were not detected in these samples, while lupeol (**23**) was present.

The four chemotypes observed for the chemical composition of the samples were not correlated with the morphological features of the species (*Table 1*). The production of sesquiterpenoids, detected in *Samples 1–5* and reported in [23][25][30], is probably exceptional in these species, because nearly all the samples lack the ability to produce sesquiterpenoids. A possible mechanism for the production of sesquiterpenes by plants of *Chemotypes 1* and *2* might be that the ability to produce different classes of compounds was brought about by hybridization (*cf. Genetic Analysis*) [48]; namely, the production of sesquiterpenes may have resulted from hybridization with some other plants producing sesquiterpenes. *Samples 1*, *3*, and *4* grew in the southernmost collection areas and *Samples 2* and *5* in the easternmost harvesting areas (*Fig. 1*). These were located on the edge of the distribution area of these plants [1]. In addition, the *L. duciformis* sample reported to produce eremophilane-type sesquiterpene lactones by *Wang et al.* [23] was also collected on the edge of the distribution area in Meigu, *ca.* 100 km east of Xichang (*Fig. 1*). An example has been reported where ecologically marginal populations are composed of individuals resulting from introgressive hybridization [49]. This hybridization mechanism might also explain the production of one or several phenylpropenoids and a triterpene in some of the *Chemotype 3* samples. They may have resulted from hybridization of a population producing phenylpropanoids and a *Chemotype 4* population.

Genetic Analysis. The possibilities outlined above can be genetically examined by analyzing the DNA. Therefore, the DNA sequences of the ITS1-5.8S-ITS2 region of the nuclear ribosomal RNA gene and of the *atpB-rbcL* intergenic region of the plastid genome were determined. The results are summarized in *Tables 2* and *4*, respectively. The most noteworthy finding on the ITS1-5.8S-ITS2 region (*Table 2*) is the presence of multiple variant copies of the rRNA gene cluster. Sites with multiple bases were quite common and numerous in some samples. In addition, variants with different lengths were present in most of the samples (*Table 3*). These observations suggest that hybridization is indeed extensive in these plants.

However, no correlation was observed between the sequences and the chemotypes. Actually, when the sequences shown in *Table 2* were subjected to standard neighbor-joining phylogenetic analysis using the program PAUP* [50] with the sequences of *L. franchetiana* [12] and *L. stenoglossa* (AB523365), both closely related to *L. duciformis* taxonomically, the present samples were not separated from one another or from *L. franchetiana*, with the exception of *Sample 5*. This result suggests that the plant species form a complex and that *L. franchetiana* is close to them. Therefore, no conclusion

Table 2. *DNA Base Sequences of the ITS1-5.8S-ITS2 Region of the Nuclear Ribosomal RNA Gene of* Ligularia duciformis *and Related Species*[a])[b])

Sample[c])	Species[d])	ITS1																																							
		1	13	25	33	44	46	54	64	65	68	83	94	95	101	104	108	110	117	125	126	127	132	137	142	148	154	166	184	190	197	204	205	214	216	222	224	225	237	238	240
1	*d*	T	T	C	A	T	T	C	G	G	C	G	C	C	Y	C	A	T	T	C	T	A	T	C	G	C	C	R	C	T	G	A	C	C	C	C	–	–	G	G	T
2	*d*	T	K	C	A	T	T	C	R	K	C	G	C	G	C	C	R	Y	W	C	T	A	Y	C	G	C	C	G	Y	T	G	R	C	C	C	C	C	G	G	G	T
3	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
4	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	R	T	T	C	T	A	Y	C	S	C	C	G	Y	T	G	A	S	C	C	C	C	G	G	G	T
5	*k*	T	K	C	W	W	Y	C	G	G	C	G	C	G	C	C	G	T	T	C	T	A	C	C	G	C	M	G	T	T	G	A	C	C	C	C	–	–	G	G	Y
6	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	Y	G	A	C	C	C	C	C	G	G	G	T
7	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	Y	C	C	C	G	G	G	T
8	*k*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	Y	C	C	C	G	G	G	T
9	*k*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	Y	C	C	C	G	G	G	T
10	*d*	T	T	Y	A	T	T	C	G	G	Y	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
11[e])	*d*	W	K	c	a	t	t	c	g	g	c	g	c	g	c	c	a	t	t	c	t	a	t	c	g	c	c	g	c	t	g	a	c	c	c	c	c	g	g	K	T
12	*d*	T	G	C	A	T	Y	C	G	G	C	G	C	G	C	C	G	T	T	C	T	A	C	Y	G	C	C	G	T	T	G	A	C	C	C	C	–	–	G	G	T
13	*d*	T	T	C	A	T	T	C	G	G	C	G	Y	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	Y	C	C	C	G	G	G	T
14	*k*	T	T	C	A	T	T	Y	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	S	C	G	G	G	T
15	*k*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
16	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	R	C	T	G	A	C	C	C	C	C	G	G	G	T
17	*n*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	Y	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
18	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	R	G	T
19	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	R	C	T	G	A	C	Y	C	C	C	G	G	G	T
20	*d*	T	T	C	A	T	T	C	G	G	C	G	Y	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	Y	C	C	C	G	G	G	T
21	*k*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	Y	C	C	G	G	G	T
22	*k*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
23	*k*	T	T	C	A	T	T	C	G	G	C	G	C	R	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
24	*d*	T	K	C	A	T	T	C	G	G	C	G	C	G	C	C	G	T	T	C	T	A	C	C	G	C	C	G	T	T	G	A	C	C	C	C	–	–	G	G	T
25	*d*	T	T	C	A	T	T	Y	G	G	C	R	C	G	C	C	A	T	T	C	T	A	T	C	G	M	C	G	C	T	G	A	C	C	C	S	C	G	G	G	T
26	*d*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	G	A	C	C	C	C	C	G	G	G	T
27	*n*	T	W	C	A	W	T	Y	G	G	Y	R	C	G	C	C	R	T	T	Y	Y	M	Y	C	G	C	M	G	Y	T	G	A	C	C	C	C	C	G	G	G	Y
28	*k*	T	T	C	A	T	T	C	G	G	C	G	C	G	C	C	A	T	T	C	T	A	T	C	G	C	C	G	C	T	R	A	C	C	C	C	C	G	G	K	T

Table 2 (cont.)

Sample[c])	Species[d])	5.8S		ITS2																																		
		135	140	13	30	34	35	43	48	54	61	71	72	74	78	79	94	99	101	102	103	106	109	113	118	123	151	152	153	158	166	176	188	190	193	201	205	207	209	219
1	*d*	T	G	C	G	G	G	G	C	G	G	C	C	A	G	G	A	W	C	T	C	C	C	T	G	G	A	T	T	G	G	C	S	C	C	C	T	G	G	C
2	*d*	T	G	C	A	G	R	G	C	G	G	C	C	A	G	G	A	T	C	T	Y	C	C	C	G	K	A	T	Y	G	G	C	G	C	C	C	T	G	G	C
3	*d*	T	K	C	G	G	G	K	C	G	G	Y	C	W	G	G	A	T	C	T	C	Y	C	C	G	G	A	T	T	G	G	C	G	C	C	C	T	G	G	C
4	*d*	T	G	C	R	G	G	G	C	G	G	C	C	A	G	G	A	T	C	T	C	Y	C	C	G	G	A	T	Y	G	G	C	G	C	C	C	T	G	G	Y
5	*k*	T	G	C	R	G	G	G	Y	G	K	C	C	A	K	G	A	T	C	Y	C	Y	C	Y	G	G	R	T	Y	G	R	Y	G	C	Y	C	T	G	G	Y
6	*d*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	A	T	C	T	C	C	Y	C	G	G	A	T	Y	G	G	C	G	C	C	Y	T	G	G	C
7	*d*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	W	T	C	T	C	C	Y	C	G	G	A	T	Y	G	G	C	G	C	C	Y	T	G	G	C
8	*k*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	W	T	C	T	C	C	Y	C	G	G	A	T	Y	G	G	C	G	C	C	Y	T	G	G	C
9	*k*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	W	T	C	T	C	C	Y	C	G	G	A	T	Y	G	G	C	G	C	C	Y	T	G	G	C
10	*d*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	W	T	Y	T	C	C	C	C	G	G	A	T	T	G	G	C	G	Y	C	Y	T	G	G	C
11	*d*	T	G	C	R	G	G	K	C	G	G	C	C	A	G	G	A	T	C	T	C	Y	C	C	G	G	A	T	Y	S	G	C	G	C	C	C	T	S	G	Y
12	*d*	T	G	C	A	G	G	G	C	G	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	C	G	G	C	G	C	C	C	T	G	G	T
13	*d*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	Y	T	G	R	C
14	*k*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	R	C	M	Y	T	G	G	C
15	*k*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	W	T	Y	T	C	C	Y	C	R	G	A	T	Y	G	G	C	G	C	Y	Y	T	G	G	C
16	*d*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	W	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	Y	T	G	G	C
17	*n*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	A	T	C	T	C	C	Y	C	G	G	A	T	Y	G	G	C	G	C	C	Y	T	G	G	C
18	*d*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	A	T	Y	T	C	C	C	C	G	G	A	T	Y	G	G	C	G	C	C	Y	T	G	G	C
19	*d*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	W	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	Y	T	G	G	C
20	*d*	T	G	C	G	G	A	G	C	G	G	C	Y	A	G	G	W	T	C	T	C	C	C	C	R	G	A	Y	T	G	G	C	G	C	C	Y	T	G	G	C
21	*k*	T	G	C	G	G	A	G	C	R	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	T	T	G	G	C
22	*k*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	T	T	G	G	C
23	*k*	T	G	C	G	R	A	G	C	G	G	C	C	A	G	K	A	T	C	T	C	Y	C	C	G	G	A	T	T	G	G	C	G	C	C	T	T	G	G	C
24	*d*	T	G	C	R	G	G	K	C	G	G	C	C	A	G	G	A	T	C	T	C	Y	C	C	G	G	A	T	Y	G	G	C	G	C	C	C	T	G	G	Y
25	*d*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	R	C	M	Y	T	G	G	C
26	*d*	T	G	C	G	G	A	G	C	G	G	C	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	Y	T	G	G	C
27	*n*	Y	G	Y	G	G	R	G	Y	G	G	C	C	A	K	G	A	T	C	Y	C	Y	C	C	G	G	R	T	T	G	G	Y	R	C	M	Y	Y	G	G	C
28	*k*	T	G	C	G	G	A	G	C	G	G	Y	C	A	G	G	A	T	C	T	C	C	C	C	G	G	A	T	T	G	G	C	G	C	C	T	T	G	G	C

[a]) Only the base positions with variation are shown; the sequences were otherwise the same. The sequence of *Sample 24* has been deposited in GenBank database (accession AB568479). [b]) K = G + T; M = A + C; R = A + G; S = C + G; W = A + T; Y = C + T; – = deletion. [c]) For samples with multiple sequence variants with different lengths, see *Table 3*. [d]) For the species abbreviations, see *Table 1*. [e]) For lower case letters, see *Table 3*.

Table 3. *Samples with Multiple Sequence Variants with Different Lengths of the ITS1-5.8S-ITS2 Region of the Nuclear Ribosomal RNA Gene of* Ligularia duciformis *and Related Species*

Sample	Variant
2	Variant with GCG in place of GCGCG at position 221–223 of ITS1 was superimposed with a slightly weaker intensity
3	Variant with A in place of AGA at position 16–18 of ITS1 was superimposed with a weaker intensity; variant with GCG in place of GCGCG around position 221–223 in ITS1 was superimposed with a much weaker intensity
4	Variant with deletion of TA at position 13–14 of ITS1 was superimposed with a much weaker intensity; variant with GCG in place of GCGCG at position 221–223 of ITS1 was superimposed with a slightly weaker intensity
6	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
7	Variant with insertion of T after position 26 of ITS2 was superimposed with a weaker intensity
8	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
9	Variant with insertion of T after position 26 of ITS2 was superimposed with a weaker intensity
10	Variant with insertion of T after position 26 of ITS2 was superimposed with a slightly weaker intensity
11	Variant with A in place of AGA at position 16–18 of ITS1 was superimposed with an equal intensity; variant with GCG in place of GCGCG at position 221–223 of ITS1 with an equal intensity. The majority sequence is listed for the segment between the two insertion/deletion sites, as shown in lower case letters in *Table 2*.
13	Variant with C5 in place of C6 at position 154–159 of ITS1 was superimposed with a weaker intensity; variant with insertion of T after position 26 of ITS2 with a stronger intensity
14	Variant with insertion of T after position 26 of ITS2 was superimposed with a much weaker intensity; variant with deletion of G at position 116 of ITS2 was superimposed with a weaker intensity
15	Variant with insertion of T after position 26 of ITS2 was superimposed with a much weaker intensity
16	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
17	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
18	Variant with insertion of T after position 26 of ITS2 was superimposed with a stronger intensity
19	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
20	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
21	Variant with C5 in place of C6 at position 154–159 of ITS1 was superimposed with an equal intensity
22	Variant with A in place of AGA at position 16–18 of ITS1 was superimposed with a weaker intensity; variant with GCGCG in place of GCG around position 220 of ITS1 was superimposed with a weaker intensity
25	Variant with insertion of T after position 26 of ITS2 was superimposed with a much weaker intensity; variant with deletion of G at position 116 of ITS2 was superimposed with a much weaker intensity
26	Variant with insertion of T after position 26 of ITS2 was superimposed with an equal intensity
27	Variant with C5 in place of C4 around position 213–216 of ITS1 was superimposed with a weaker intensity; variant with GCG in place of GCGCG around position 221–223 of ITS1 was superimposed with a slightly weaker intensity

could be drawn on the mechanism of generation of chemical diversity from the present sequence data; if hybridization has happened, the information on the hybridization partner has already been lost by repeated back crossing or the hybridization partner had a similar sequence.

Table 4. *DNA Base Sequences of the* atpB-rbcL *Intergenic Region of* Ligularia duciformis *and Related Species*

Sample	Base position[a])							Ts[b])	As[c])
	28	65	117	322	344	469	606		
1	G	A	C	C	T	A	C	9	9
2	G	T	C	C	T	C	C	9	10
3	A	T	C	T	T	C	C	9	9
4	G	T	C	C	T	C	T	8	11
5	A	T	C	T	T	C	C	9	9
6	A	T	C	C	G	A	C	9	9
7	A	T	C	C	G	A	C	9	9
8	A	T	C	C	G	A	C	9	9
9	A	T	C	C	G	A	C	9	9
10	G	T	C	C	T	A	C	9	10
11	A	T	C	C	T	C	C	9	9
12	A	T	C	C	T	C	C	9	9
13	A	T	C	C	G	A	C	9	9
14	G	T	T	C	T	C	C	9	9
15	A	T	C	C	G	A	C	9	9
16	G	T	C	C	T	A	C	9	10
17	A	T	C	C	G	A	C	9	9
18	A	T	C	C	G	A	C	9	9
19	G	T	C	C	T	A	C	9	10
20	G	T	C	C	T	A	C	9	10
21	A	T	C	C	G	A	C	9	10
22	A	T	C	C	G	A	C	9	10
23	A	T	C	C	G	A	C	9	9
24	A	T	C	C	G	A	C	9	9
25	A	T	C	C	T	C	C	9	9
26	A	T	C	C	G	A	C	9	9
27	A	T	C	C	T	C	C	8	9
28	A	T	C	C	G	A	C	9	10

[a]) The base numbering is according to that of *L. tongolensis* (GenBank database accession AB126994). The sequences were otherwise the same as the *L. tongolensis* sequence. [b]) Number of Ts in a stretch around the 390th base. [c]) Number of As in a stretch around the 510th base.

One exception was *Sample 5*, which was separated from the other samples and *L. franchetiana* and was placed closer to *L. stenoglossa* with a bootstrap value of 94%. To reveal the cause of this separation, a putative sequence of the hybridization partner was reconstructed by subtracting the majority consensus sequence from the sequence of *Sample 5*. For example, at the 13th position of ITS1, subtracting T (majority) from K (= C+T) would yield C. When the reconstructed sequence was included in the PAUP* analysis, it was found to be placed even closer to the sequence of *L. stenoglossa*. In addition, when the DNA database was searched with Blast [51], the sequence most similar to the reconstructed sequence was found to be that of *L. stenoglossa*. Thus, *Sample 5* appears to have experienced hybridization with a plant that had an ITS1-5.8S-ITS2 sequence resembling that of *L. stenoglossa*. It would be interesting to examine the chemical composition in *L. stenoglossa* and related species in the area.

Conclusions. – The morphological observations and DNA data suggested that *L. duciformis*, *L. kongkalingensis*, and *L. nelumbifolia* constitute a continuous complex in northwestern Yunnan to western Sichuan Provinces of China. The plants were found to harbor chemical diversity with no correlation to their morphology or evolutionarily neutral DNA sequence. The majority of the samples produced phenylpropanoids, whereas the sesquiterpenoid-producing samples were a minority. The DNA data showed that introgression is extensive in these plants, which suggested that the ability to produce sesquiterpenoids may have been acquired by hybridization. Examination of the chemical consequences of hybridization of *L. duciformis* should be actually feasible, as a natural hybrid of *L. duciformis* and *L. paradoxa* HAND.-MAZZ. has been found in Ninglang County, Yunnan (*Fig. 1*) [52].

The authors wish to thank Dr. *Xiaojiang Hao* and Mrs. *Guowen Hu* (Kunming Institute of Botany) for the research coordination. This work was partly supported by the *JSPS* (Grant-in-Aid for Scientific Research, No. 21404009).

Experimental Part

General. Column chromatography (CC): *Wakogel C-300* silica gel (SiO_2; 70–230 mesh; *Fuji Sylisia*), *Merck silica gel 60* (60–230 mesh), and *Kanto silica gel 60 N* (spherical neutral). Anal. TLC: *Merck Kieselgel 60 F_{254}* (layer thickness 0.25 mm). HPLC: *JASCO* pump system with *RI-930* detector or *Jasco GULLIVER* system with Borwin-PDA program and *MD-1510* detector equipped with either a *Chemcopak Nucleosil 50–5* (4.6 × 250 mm) silica-gel column, *YMC Pack SIL A-023* (10 × 250 mm) silica-gel column, or *Cosmosil 5C18-AR-II* (10 × 250 mm) octadecylsilan (ODS) column. Optical rotations and CD Spectra: *Horiba SEPA-300*, *JASCO DIP-1000*, *JASCO J-725*, *JASCO DPI-181*, or *JASCO P-2200* digital polarimeter. IR Spectra: *Horiba FT-720*, *JASCO FT/IR-5300*, or *Shimadzu FTIR-8700* spectrometer. ^{1}H- and ^{13}C-NMR Spectra: *Bruker DRX-500* (500 and 125 MHz, resp.), *Varian Unity 600* (600 and 150 MHz, resp.), *JEOL ECP 400*, or *JEOL AL 400* (400 and 100 MHz, resp.) NMR spectrometers, in $CDCl_3$ or C_6D_6; δ in ppm rel. to Me_4Si, J in Hz. HR-MS: *JEOL JMS-GCmateII* (*JMS-BU25*) or *JEOL JMS-700 MStation*; in *m/z* (rel. %). DNA Sequencing: *HotStarTaq® Plus Master Mix Kit* (*QIAGEN*), *Master Cycler ep Gradient S* (*Eppendorf*), *BigDye Terminator Kit v3.1* (*Applied Biosystems*), and *3130xl Genetic Analyzer* (*Applied Biosystems*).

Plant Material. In total, 28 samples of *Ligularia duciformis*, *L. kongkalingensis*, and *L. nelumbifolia* were collected in August 2004, 2005, and the period from 2007 to 2009 in the localities of western Sichuan and northwestern Yunnan Provinces shown in *Table 1* and *Fig. 1*.

Extraction for Ehrlich*'s Test*. The roots of each plant (2–10 g) were harvested and immediately extracted with EtOH without drying the roots. The solid plant material was removed after several days, and the soln. was subjected to TLC without concentration. See our previous studies for further details and the procedure of the test [4][7].

Compound Isolation and Structure Determination. The roots were dried for *ca*. one week and extracted at r.t. with EtOH or AcOEt. The oily extracts were obtained by standard methods.

The dried roots (20 g) of *Sample 1* (*Chemotype 1*) were extracted with AcOEt to give a crude extract (700 mg), which was separated by CC (SiO_2, hexane/AcOEt gradient) to afford *fukinone* (**1**; 111 mg, 16%), *dehydrofukinone* (**2**; 2.8 mg, 0.4%), and *ethyl ferulate* (**12**; 10.5 mg, 1.5%).

The dried roots (29.5 g) of *Sample 2* (*Chemotype 1*) were extracted with EtOH to give a crude extract (865 mg). Part of the extract (394 mg) was submitted to CC (SiO_2, hexane/AcOEt 20 : 1) to give fractions (57 mg) containing *eremophil-6-en-11-ol* (**3**), and more polar fractions (21 mg) containing *sinapyl senecioate* (**13**) and *sinapyl angelate* (**14**). Alcohol **3** (4.5 mg, 1.1% yield) was isolated by CC (SiO_2; hexane/AcOEt 4 : 1) in pure form. The more polar product was submitted to CC (SiO_2; hexane/AcOEt 5 : 1) to give an inseparable mixture of sinapyl esters **13** and **14** (7.7 mg, 2.0% yield).

The dried roots (67.0 g) of *Sample 4* (*Chemotype 2*) were extracted with AcOEt to give a crude extract (1.15 g), which was subjected to CC (SiO_2; hexane/AcOEt gradient) to afford acetylenic

compounds **20** (1.5 mg, 0.1%) and **21** (0.3 mg, 0.03%), *4*-O-*geranylconiferyl alcohol* (**8**; 70.8 mg, 6%), *(+)-sesamin* (**19**; 7.5 mg, 0.7%), oplopane-type sesquiterpenoids **4** (9.3 mg, 0.8%) and **5** (9.3 mg, 0.8%), dicarboxylic acid **18** (10.6 mg, 0.9%), *4*-O-*geranylconiferyl aldehyde* (**15**; 4.7 mg, 0.4%), *ferulic acid* (**16**; 0.7 mg, 0.06%), and ester **17** (1.4 mg, 0.12%).

The dried roots (9 g) of *Sample 5* (*Chemotype 2*) were extracted with AcOEt. A half portion of the AcOEt extract was concentrated under reduced pressure to give a residue (58.6 mg), which was subjected to CC (SiO_2; hexane/AcOEt gradient) to afford oplopane-type sesquiterpenoids **6** (0.2 mg, 0.3%), **4** (1.9 mg, 3.2%), and **5** (6.1 mg, 10%) and a mixture containing **5** and **7**. The mixture was separated by ODS HPLC (*Cosmosil AR2*, 75 % MeCN) to afford **5** (0.5 mg, 0.8%) and **7** (0.7 mg, 1%).

The dried roots (37 g) of *Sample 6* (*Chemotype 3*) were extracted with AcOEt. The crude extract (1.39 g) was subjected to CC (SiO_2, hexane/$CHCl_3$ gradient) to give a fraction, which was then purified by HPLC (*Nucleosil 50-5*, hexane/AcOEt 97:3) to give phenol **22** (0.44 mg, 0.032%). A more polar fraction containing phenylpropenoids **8**–**11** was subjected to CC (hexane/AcOEt gradient) to give **8** (6.4 mg, 0.46%) and *4*-O-*geranylsinapyl alcohol* (**11**; 12.7 mg, 0.91%) together with a mixture of the less polar compounds **9** and **10**, which was submitted to HPLC (*Nucleosil 50-5*, hexane/AcOEt 98:2 and 90:10) to give *4*-O-*geranylsinapyl alcohol acetate* (**9**; 4.8 mg, 0.34%) and *4*-O-*geranylsinapyl aldehyde* (**10**; 1.0 mg, 0.072%).

The dried roots (51.1 g) of *Sample 7* (*Chemotype 3*) were extracted with AcOEt to give an oil (791 mg). Part of the extract (124 mg) was subjected to CC (SiO_2, hexane/AcOEt 20:1) to give *lupeol* (**23**) (11 mg, 8.9%), crude products **9**, **10**, and **8**, and pure alcohol **11** (35.5 mg, 28.7%). The purification of **9** and **10** was performed by prep. TLC (hexane/AcOEt 7:3) to give acetate **9** (1.7 mg, 1.4%) and aldehyde **10** (1.9 mg, 1.5%). The crude product containing **8** was further purified by prep. TLC (hexane/AcOEt 1:1) to give **8** (3.4 mg, 2.7%).

The dried roots (37 g) of *Sample 8* (*Chemotype 3*) were extracted with EtOH to give an oil (431 mg). Part of the extract (237 mg) was subjected to CC (SiO_2, hexane/AcOEt 20:1) to afford compounds **8** (6.7 mg, 2.8%), **9** (11 mg, 4.7%), **10** (2.0 mg, 0.8%), and **11** (24.4 mg, 10%).

The dried roots (45 g) of *Sample 9* (*Chemotype 3*) were extracted with EtOH to give an oil (330 mg). Part of the EtOH extract (198 mg) was subjected to CC (SiO_2; hexane/AcOEt 40:1) to give alcohols **8** (13 mg, 6.6%) and **11** (13.5 mg, 6.8%). The separation of the minor components **9** and **10** was attempted, but in vain.

The dried roots (22 g) of *Sample 10* (*Chemotype 3*) were extracted with AcOEt. The crude extract (1.11 g) was subjected to CC (SiO_2, hexane/$CHCl_3$ gradient) to give a less polar fraction, which was purified by HPLC (*Nucleosil 50-5*, hexane/AcOEt 97:3) to give phenol **22** (0.14 mg, 0.013%). A more polar fraction containing major products was subjected again to CC (hexane/AcOEt gradient). A less polar fraction was further purified by HPLC (*Nucleosil 50-5*, hexane/AcOEt 9:1) to give acetate **9** (8.7 mg, 0.78%), and a more polar fraction was also purified by HPLC (*Nucleosil 50-5*, hexane/AcOEt 7:3) to give alcohols **8** (4.9 mg, 0.44%) and **11** (4.0 mg, 0.36%).

The dried roots (10.3 g) of *Sample 11* (*Chemotype 3*) were extracted with EtOH to give a crude extract (210 mg), which was separated by CC (SiO_2; hexane/AcOEt gradient) to afford **8** (2.6 mg, 1.2%), **11** (1.0 mg, 0.5%), and dicarboxylic acid **18** (1.4 mg, 6.6%).

The dried roots (39.8 g) of *Sample 15* (*Chemotype 3*) were extracted with AcOEt to give an oil (1.08 g). Part of the extract (98 mg) was subjected to CC (SiO_2, hexane/AcOEt 20:1) to give four crude products, *i.e.*, **23**, *5-acetyl-6-hydroxy-2-isopropylidenebenzodihydrofuran-3-one* (**24**), **9**, and **10**, as well as pure **11** (21 mg, 22%). The crude products **23**, **24**, and **9** were further purified by prep. TLC (hexane/AcOEt 7:3; developed twice) to give **23** (7 mg, 7%), **24** (1 mg, 1%), and **9** (2.3 mg, 2.4%). The mixture containing **10** was further purified by prep. TLC (hexane/AcOEt 1:1) to give aldehyde **10** (1.5 mg, 1.5%).

The isolation of alcohols **11** and **23** from the EtOH extract of *Sample 17* (*Chemotype 3*) was performed following the chemical analysis procedures of *Sample 19*.

The dried roots (31 g) of *Sample 19* (*Chemotype 3*) were extracted with AcOEt. One third of the extract was concentrated under reduced pressure to give a residue (1.9 g), which was chromatographed on SiO_2. The fractions eluted by hexane/AcOEt (3:1 to 1:1) gave alcohol **11** (15.7 mg, 0.82%).

The dried roots (21 g) of *Sample 20* (*Chemotype 3*) were extracted with EtOH to give a crude extract (642 mg). From the extract (83 mg), **23** (4.8 mg, 5.8 %) and alcohol **11** (4.4 mg, 5.3%) were isolated following the chemical analysis procedures of *Sample 19*.

The dried roots (30 g) of sample *Sample 22* (*Chemotype 3*) were extracted with EtOH to give a crude extract (781 mg), which was then submitted to CC (SiO_2, hexane/AcOEt gradient) to give **23** (27 mg) and alcohol **11** (17 mg, 2.2%). The TLC patterns of *Samples 21* and *22* were similar one to the other.

The dried roots (23.5 g) of *Sample 23* (*Chemotype 3*) were extracted with EtOH to give a crude extract (286 mg). Part of the extract (89.6 mg) was submitted to CC (SiO_2; hexane/AcOEt gradient) to give alcohol **11** (9.1 mg, 10%) together with a mixture containing **23** (3.6 mg). The purification of the mixture was attempted again (SiO_2, hexane/AcOEt 40:1), but compound **23** was not obtained in pure form.

From the EtOH extracts of *Samples 24–26* (*Chemotype 4*), **23** was isolated following the chemical analysis procedures of *Sample 27*.

The dried roots (48.6 g) of sample *Sample 27* (*Chemotype 4*) were extracted with EtOH to give a crude extract (1.12 g). Part of the extract (326 mg) was submitted to CC (SiO_2; hexane/AcOEt 20:1) to give a crude product containing **23**, which was then submitted to CC (SiO_2, hexane/AcOEt gradient) to afford pure **23** (12 mg, 1.1%).

The dried roots (36 g) of *Sample 28* (*Chemotype 4*) were extracted with EtOH to give a crude extract (480 mg). From the extract (119 mg), **23** (17 mg, 14 %) was isolated using the procedures described for the chemical analysis of *Sample 27*.

The chemical compositions of *Samples 12–14*, *16*, and *18* (*Chemotype 3*) were analyzed by TLC. Comparison of the TLC patterns indicated the following: *Samples 12* and *13* contained compounds **8** and **11**, *Samples 14* and *16* compounds **9**–**11**, and *Sample 18* compounds **11** and **23**, resp.

rel-*(1*R*,3a*S*,5*R*,6*S*,7*R*,7a*S*)-1-[(1*S*)-1-(Acetyloxy)ethyl]octahydro-6-[(2-methylbutanoyl)oxy]-4-methylidene-2-oxo-7-(propan-2-yl)-1*H*-inden-5-yl (2*E*)-3-Methylpent-2-enoate* (**6**). Colorless oil. $[\alpha]_D^{25} = -47$ ($c = 0.02$, EtOH). IR (neat): 2925, 1733, 1718, 1458, 1143. ^{1}H-NMR (500 MHz, C_6D_6): 6.24 (*d*, J = 3.1, H–C(9)); 5.96 (br. *s*, H–C(2″)); 5.39 (*dd*, J = 9.1, 3.1, H–C(8)); 5.20–5.14 (*m*, H–C(4)); 5.14, 4.60 (2br. *s*, 1 H each, CH_2(14)); 2.64–2.54 (*m*, H–C(1)); 2.57–2.42 (*m*, H–C(11)); 2.47–2.42 (*m*, H–C(2′)); 2.42–2.37 (*m*, H–C(5)); 2.33–2.26 (*m*, H–C(7)); 2.26 (br. *s*, Me(6″)); 2.19–2.11, 1.89–1.79 (2*m*, 1 H each, CH_2(2)); 1.96–1.87, 1.56–1.44 (2*m*, 1 H each, CH_2(3′)); 1.93–1.85 (*m*, CH_2(4″)); 1.88 (*s*, AcO); 1.35 (*d*, J = 7.3, Me(12) or Me(13)); 1.38–1.30 (*m*, H–C(6)); 1.33 (*d*, J = 6.9, Me(5′)); 1.09 (*d*, J = 6.6, Me(15)); 0.98 (*t*, J = 7.4, Me(4′)); 0.97 (*d*, J = 7.2, Me(13) or Me(12)); 0.87 (*t*, J = 7.4, Me(5″)). ^{13}C-NMR (125 MHz, C_6D_6): 211.5 (C(3)); 175 (C(1′)); 173 (C(1″)); 170 (AcO); 162 (C(3″)); 114.5 (C(2″)); 112 (C(14)); 73 (C(8)); 72.5 (C(9)); 70 (C(4)); 57 (C(5)); 49 (C(7)); 45.5 (C(6)); 42.5 (C(2)); 42 (C(1)); 42 (C(2′)); 34 (C(4″)); 28.5 (C(11)); 27 (C(3′)); 24 (C(12) or C(13)); 21 (AcO); 19 (C(6″)); 17.5 (C(5′)); 16 (C(15)); 16 (C(13) or C(12)); 12 (C(4′)); 12 (C(5″)); C(10) could not be identified. HR-FAB-MS (*m*-nitrobenzyl alcohol): 491.2985 ($[M + H]^+$, $C_{28}H_{43}O_7^+$; calc. 491.3009).

rel-*(1*R*,3a*S*,5*R*,6*S*,7*R*,7a*S*)-1-[(1*S*)-1-(Acetyloxy)ethyl]octahydro-4-methylidene-6-[(2-methylpropanoyl)oxy]-2-oxo-7-(propan-2-yl)-1*H*-inden-5-yl (2*E*)-4-Hydroxy-3-methylpent-2-enoate* (**7**). Colorless oil. $[\alpha]_D^{25} = +1.9$ ($c = 0.07$, EtOH). IR (neat): 3471, 2924, 1731, 1716, 1456, 1149. ^{1}H-NMR (500 MHz, C_6D_6): 6.34 (br. *s*, H–C(2″)); 6.25 (*d*, J = 3.0, H–C(9)); 5.35 (*dd*, J = 9.5, 3.0, H–C(8)); 5.18–5.14 (*m*, H–C(4)); 5.08, 4.57 (2*d*, J = 2.0, 1 H each, CH_2(14)); 3.77 (*q*, J = 6.5, H–C(4″)); 2.58 (*sept.*, J = 7.0, H–C(2′)); 2.56–2.50 (*m*, H–C(1)); 2.51–2.45 (*m*, H–C(11)); 2.40 (*dd*, J = 10.0, 3.0, H–C(5)); 2.26 (*t*-like, J = 10.0, H–C(7)); 2.17 (*s*, Me(6″)); 2.13, 1.81 (2*dd*, J = 14.0, 5.0; J = 14.0, 12.0, 1 H each, CH_2(2)); 1.89 (*s*, AcO); 1.33 (*d*, J = 7.0, Me(12) or Me(13)); 1.33 (*d*, J = 7.0, Me(3′) or Me(4′)); 1.35–1.29 (*m*, H–C(6)); 1.25 (*d*, J = 7.0, Me(4′) or Me(3′)); 1.09 (*d*, J = 6.5, Me(15)); 1.01 (*d*, J = 6.5, Me(5″)); 0.94 (*t*, J = 7.0, Me(13) or Me(12)). ^{13}C-NMR (125 MHz, C_6D_6): 211 (C(3)); 175.5 (C(1′)); 170 (AcO); 163 (C(3″)); 113.5 (C(2″)); 112 (C(14)); 73.5 (C(8)); 73 (C(9)); 72 (C(4″)); 69.5 (C(4)); 57 (C(5)); 48.5 (C(7)); 46 (C(6)); 42.5 (C(2)); 42 (C(1)); 34.5 (C(2′)); 28 (C(11)); 24 (C(12) or C(13)); 22 (C(5″)); 21 (AcO); 19.5 (C(3′) or C(4′)); 19 (C(4′) or C(3′)); 16.5 (C(13) or C(12)); 16 (C(15)); 15.5 (C(6″)); C(10) and C(1″) could not be identified. HR-FAB-MS (*m*-nitrobenzyl alcohol): 493.2811 ($[M + H]^+$, $C_{27}H_{41}O_8^+$; calc. 493.2801).

*(2*E*)-3-(4-{[(2*E*)-3,7-Dimethylocta-2,6-dien-1-yl]oxy}-3-methoxyphenyl)prop-2-enal* (**15**). Colorless oil. IR (neat): 1672, 1620. ^{1}H-NMR (500 MHz, C_6D_6): 9.59 (*d*, *J* = 7.4, H–C(9)); 6.83 (*d*, *J* = 15.8, H–C(7)); 6.76 (*dd*, *J* = 8.2, 2.1, H–C(6)); 6.67 (*d*, *J* = 2.1, H–C(2)); 6.58 (*d*, *J* = 8.2, H–C(5)); 6.57 (*dd*, *J* = 15.8, 7.4, H–C(8)); 5.54 (*dq*, *J* = 6.5, 1.2, H–C(2′)); 5.12 (br. *t*, *J* = 7.0, H–C(6′)); 4.37 (*d*, *J* = 6.5, CH_2(1′)); 3.30 (*s*, MeO); 2.06 (*td*, *J* = 7.4, 7.0, CH_2(5′)); 1.96 (*t*, *J* = 7.4, CH_2(4′)); 1.65 (*d*, *J* = 1.2, Me(8′)); 1.51 (*d*, *J* = 0.5, Me(9′)); 1.48 (*d*, *J* = 1.2, Me(10′)). ^{13}C-NMR (125 MHz, C_6D_6): 192.4 (C(9)); 152.0 (C(4)); 151.9 (C(7)); 150.5 (C(3)); 140.9 (C(3′)); 131.6 (C(7′)); 127.4 (C(1)); 127.0 (C(8)); 124.3 (C(6′)); 123.1 (C(6)); 120.0 (C(2′)); 113.0 (C(5)); 110.7 (C(2)); 65.7 (C(1′)); 55.3 (MeO); 39.7 (C(4′)); 26.6 (C(5′)); 25.8 (C(8′)); 17.7 (C(9′)); 16.5 (C(10′). CI-MS: 314 (M^+), 178 (base). HR-CI-MS: 314.1875 (M^+, $C_{20}H_{26}O_3^+$; calc. 314.1882).

rel-*(2*R*,3*S*,5*R*)-2-(Acetyloxy)-5-ethyl-2,3-dimethylhexanedioic Acid* (**18**). Colorless oil. $[\alpha]_D^{22} = +31.4$ (*c* = 1.06, EtOH). IR (neat): 3500–2500, 1738, 1730, 1713. ^{1}H-NMR (500 MHz, C_6D_6): 2.29 (*dq*, *J* = 10.0, 6.3, H–C(3)); 2.20–2.13 (*m*, H–C(5)); 1.94 (*dd*, *J* = 12.8, 12.4, 1 H of CH_2(4)); 1.63 (*s*, AcO); 1.61–1.53 (*m*, CH_aH_bMe); 1.48 (*s*, Me–C(2)); 1.17 (*dqd*, *J* = 13.7, 7.3, 6.4, CH_aH_bMe); 0.98–0.92 (*m*, 1 H of CH_2(4)); 0.92 (*d*, *J* = 6.3, Me–C(3)); 0.75 (*t*, *J* = 7.3, CH_2*Me*). ^{13}C-NMR (125 MHz, C_6D_6): 182.7 (C(6)); 179.1 (C(1)); 169.7 (AcO); 83.0 (C(2)); 45.9 (C(5)); 38.8 (C(3)); 34.7 (C(4)); 26.3 (CH_2Me); 20.5 (OAc); 15.5 (*Me*–C(2)); 14.0 (*Me*–C(3)); 11.9 (CH_2*Me*). CI-MS: 261 ($[M+H]^+$), 243, 201, 183, 155 (base). HR-CI-MS: 261.1342 ($[M+H]^+$, $C_{12}H_{21}O_6^+$; calc. 261.1338).

Determination of DNA Sequences. The nucleotide sequences of the ITS1-5.8S-ITS2 region and the *atpB-rbcL* intergenic region were determined as described previously [10].

REFERENCES

[1] S.-W. Liu, 'Flora Reipublicae Popularis Sinicae', Science Press, Beijing, 1989, Vol. 77.
[2] T.-L. Min, 'Flora Yunnanica', Science Press, Beijing, 2004, Vol. 13.
[3] S.-W. Liu, D.-S. Deng, J.-Q. Liu, *Acta Phytotaxon. Sin.* **1994**, *32*, 514.
[4] H. Nagano, Y. Iwazaki, X. Gong, Y. Shen, C. Kuroda, R. Hanai, *Bull. Chem. Soc. Jpn.* **2006**, *79*, 300.
[5] M. Tori, K. Honda, H. Nakamizo, Y. Okamoto, M. Sakaoku, S. Takaoka, X. Gong, Y. Shen, C. Kuroda, R. Hanai, *Tetrahedron* **2006**, *62*, 4988.
[6] M. Tori, H. Nakamizo, K. Mihara, M. Sato, Y. Okamoto, K. Nakashima, M. Tanaka, Y. Saito, M. Sono, X. Gong, Y. Shen, R. Hanai, C. Kuroda, *Phytochemistry* **2008**, *69*, 1158.
[7] R. Hanai, X. Gong, M. Tori, S. Kondo, K. Otose, Y. Okamoto, T. Nishihama, A. Murota, Y. Shen, S. Wu, C. Kuroda, *Bull. Chem. Soc. Jpn.* **2005**, *78*, 1302.
[8] H. Onuki, M. Yamazaki, A. Nakamura, R. Hanai, C. Kuroda, X. Gong, Y. Shen, H. Hirota, *J. Nat. Prod.* **2008**, *71*, 520.
[9] C. Kuroda, K. Kiuchi, A. Torihata, K. Takeshita, X. Gong, Y. Shen, H. Hirota, H. Onuki, R. Hanai, *Chem. Biodiversity* **2007**, *4*, 2210.
[10] Y. Saito, M. Hattori, Y. Iwamoto, Y. Takashima, K. Mihara, Y. Sasaki, M. Fujiwara, M. Sakaoku, A. Shimizu, X. Chao, C. Kuroda, X. Gong, R. Hanai, M. Tori, *Tetrahedron* **2011**, *67*, 2220.
[11] H. Nagano, M. Kanda, H. Yamada, R. Hanai, X. Gong, C. Kuroda, *Helv. Chim. Acta* **2010**, *93*, 1945.
[12] M. Tori, Y. Tanio, Y. Okamoto, Y Saito, X. Gong, C. Kuroda, R. Hanai, *Heterocycles* **2008**, *75*, 2029.
[13] H. Nagano, M. Matsushima, H. Yamada, R. Hanai, X. Gong, C. Kuroda, *Nat. Prod. Commun.* **2010**, *5*, 1.
[14] C.-F. Zhang, Q. Wang, M. Zhang, *J. Asian Nat. Prod. Res.* **2009**, *11*, 339.
[15] S. Pu, D. Xu, M. Zhang, H. Zhou, Z. Wang, G. Yu, *Acta Pharm. Sin.* **2004**, *39*, 831.
[16] D.-Z. Zhang, M. Zhang, X.-H. Xu, G.-D. Yu, Z.-T. Wang, *Chin. J. Nat. Med.* **2003**, *1*, 207.
[17] G. Lin, K.-Y. Zhou, X.-G. Zhao, Z.-T. Wang, P. P. H. But, *Rapid Commun. Mass Spectrom.* **1998**, *12*, 1445.
[18] K. Fu, N. Yu, Y. Zhang, Y. Zhao, *Acta Pharm. Sin.* **2007**, *42*, 621.
[19] K. Gao, L. Yang, Z.-J. Jia, *J. Chin. Chem. Soc. (Taipei)* **1999**, *46*, 619.
[20] K. Gao, Z.-J. Jia, *Indian J. Chem., Sect. B* **2000**, *39*, 160.
[21] K. Gao, W.-S. Wang, Z.-J. Jia, *Phytochemistry* **1998**, *47*, 269.

[22] K. Gao, Z. Jia, W. Wang, *Chem. Res. Chin. Univ.* **1997**, *13*, 382.
[23] W.-S. Wang, X. Dai, L.-Y. Hong, P. Lu, J.-C. Feng, Y.-G. Jiao, *Helv. Chim. Acta* **2008**, *91*, 1118.
[24] M. Tori, M. Fujiwara, Y. Okamoto, M. Tanaka, X. Gong, Y. Shen, R. Hanai, C. Kuroda, *Nat. Prod. Commun.* **2007**, *2*, 357.
[25] H. R. Peng, Z. J. Jia, L. Yang, Y. Zhao, *Chin. Chem. Lett*. **1995**, *6*, 583.
[26] Y. Zhao, H. R. Peng, Z. J. Jia, *Chin. Chem. Lett.* **1995**, *6*, 387.
[27] Y. Zhao, Z. Jia, L. Yang, *Phytochemistry* **1994**, *37*, 1149.
[28] Y. Zhao, Z. J. Jia, L. Yang, J. G. Wang, *Chin. Chem. Lett.* **1993**, *4*, 895.
[29] Y. Zhao, X. Hao, W. Lu, J. Cai, H. Yu, T. Sevénet, F. Guéritte, *J. Nat. Prod.* **2002**, *65*, 902.
[30] H.-R. Peng, Y.-P. Shi, Z.-J. Jia, *Planta Med.* **1997**, *63*, 335.
[31] H. Nagano, Y. Iwazaki, M. Matsushima, M. Sato, X. Gong, Y. Shen, H. Hirota, C. Kuroda, R. Hanai, *Chem. Biodiversity* **2007**, *4*, 2874.
[32] M. Tori, A. Watanabe, S. Matsuo, Y. Okamoto, K. Tachikawa, S. Takaoka, X. Gong, C. Kuroda, R. Hanai, *Tetrahedron* **2008**, *64*, 4486.
[33] M. Tori, Y. Okamoto, K. Tachikawa, K. Mihara, A. Watanabe, M. Sakaoku, S. Takaoka, M. Tanaka, X. Gong, C. Kuroda, M. Hattori, R. Hanai, *Tetrahedron* **2008**, *64*, 9136.
[34] K. Naya, I. Takagi, Y. Kawaguchi, Y. Asada, Y. Hirose, N. Shinoda, *Tetrahedron* **1968**, *24*, 5871.
[35] K. Naya, K. Tsuji, U. Haku, *Chem. Lett*. **1972**, *1*, 235.
[36] C.-F. Wang, Y. Zhao, Y.-Z. Liu, Z.-Z. Zhang, *Helv. Chim. Acta* **2008**, *91*, 1712.
[37] F. Bohlmann, R. K. Gupta, J. Jackupovic, *Phytochemistry* **1981**, *20*, 831.
[38] F. Bohlmann, C. Zdero, *Chem. Ber.* **1977**, *110*, 468.
[39] M. Hikichi, T. Furuya, Y. Iitaka, *Tetrahedron Lett.* **1978**, *19*, 767.
[40] W. A. Jones, M. Beroza, E. D. Becker, *J. Org. Chem*. **1962**, *27*, 3232; K. Takahashi, T. Nakagawa, *Chem. Pharm. Bull*. **1966**, *14*, 641.
[41] R. Jente, F. Bohlmann, S. Schöneweiss, *Phytochemistry* **1979**, *18*, 829.
[42] H. Shibuya, Y. Takeda, R. Zhang, R.-X. Tong, I. Kitagawa, *Chem. Pharm. Bull.* **1992**, *40*, 2325.
[43] F. Bohlmann, M. Grenz, R. K. Gupta, A. K. Dhar, M. Ahmed, R. M. King, H. Robinson, *Phytochemistry* **1980**, *19*, 2391.
[44] M. Resch, J. Heilmann, A. Steigel, R. Bauer, *Planta Med.* **2001**, *67*, 437.
[45] R. J. Capon, E. L. Ghisalberti, P. R. Jefferies, *Phytochemistry* **1981**, *20*, 2598.
[46] D. Burns, W. F. Reynolds, G. Buchanan, P. B. Reese, R. G. Enriquez, *Magn. Reson. Chem.* **2000**, *38*, 488.
[47] Q. Wang, C. Zhang, M. Zhang, Z. Wang, *China J. Chin. Mater. Med.* **2008**, *33*, 1018.
[48] J. B. Harborne, B. L. Turner, 'Plant Chemosystematics', Academic Press, London, 1984.
[49] P. Choler, B. Erschbamer, A. Tribsch, L. Gielly, P. Taberlet, *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 171.
[50] D. L. Swofford, 'PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods)', Sinauer Associates, Sunderland, 2000.
[51] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **1990**, *215*, 403.
[52] Y. Pan, S. Shi, X. Gong, C. Kuroda, *Ann. Missouri Bot. Garden* **2008**, *95*, 487.

Received July 10, 2011