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FULL PAPER

Chemical Constituents in Hybrids of *Ligularia tongolensis* and *L. cymbulifera*: Chemical Introgression in *L. tongolensis*

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Two samples with morphologies intermediate between *Ligularia tongolensis* and *L. cymbulifera* were collected in Desha, Sichuan Province, and one, in Pachahai, Yunnan Province, P. R. China. The DNA sequencing confirmed that the samples were hybrids of the two species. Tetradymol (**1**), the major compound of *L. cymbulifera* not found in *L. tongolensis*, was isolated from the hybrid samples collected at both locations, while furanoeremophilan-15-oic acid derivative **4**, a compound characteristic to *L. tongolensis*, was found in the Pachahai hybrid but not in the Desha hybrids. Thus, the chemical consequence of hybridization can be variable. In addition, analysis of *L. tongolensis* samples at Pachahai indicated that introgression has been a mechanism of generating chemical diversity in the plant. Eleven compounds including three new ones were isolated.

Keywords: *Ligularia*, Hybrid, Furanoeremophilanes, Internal transcribed spacer, Introgression.

Introduction

The genus *Ligularia* (Asteraceae) is highly diversified in the Hengduan Mountains area of China [1] and provides materials suitable for the study of plant evolution. We have been studying the diversity in root chemicals of *Ligularia* in conjunction with the nucleotide sequences of evolutionarily neutral DNA regions, especially the internal transcribed spacers (ITSs) of the ribosomal RNA gene cluster, and have uncovered intraspecific diversity in many *Ligularia* species of the area [2]. It has been proposed that hybridization is a major pathway in the evolution of *Ligularia* [3] and a few *Ligularia* hybrids have actually been described [4]. Hybridization can be a mechanism of generating chemical diversity [3b][5] and our analyses suggested that the ability to produce terpenoids in some individuals in the *L. duciformis* (C. Winkl.) Hand.-Mazz. – *L. kongkalingensis* Hand.-Mazz. – *L. nelumbifolia* (Bur. et Franch.) Hand.-Mazz. complex might have been acquired by hybridization [6][7]. Thus, examination of the chemical consequences of hybridization should yield valuable information on the chemical diversity in *Ligularia*.

We recently reported the results of chemical and genetic studies on natural hybrids of *L. nelumbifolia* and *L. subspicata* (Bur. et Franch.) Hand.-Mazz. [8][9]. Furanoeremophilanes were detected in *L. subspicata* but not in *L. nelumbifolia*; they were detected in the hybrids. A putative hybrid of *L. cyathiceps* Hand.-Mazz. and *L. subspicata* was collected, and compounds of both parents were detected in the hybrid [10].

In the course of our field research, we found *Ligularia* individuals sharing morphological characters with both *L. tongolensis* (Franch.) Hand.-Mazz. and *L. cymbulifera* (W. W. Smith) Hand.-Mazz. in southwestern Sichuan and northwestern Yunnan [11]. DNA analyses showed that they were indeed hybrids of *L. tongolensis* and *L. cymbulifera*, and that introgression frequently occurs between the two species in the area [11]. Our previous chemical analyses showed that 15-oxygenated furanoeremophilanes were present in both *L. cymbulifera* and *L. tongolensis* collected in Yunnan Province [12]. Related eremophilanolides were also isolated from *L. tongolensis* [13]. The major difference between the two species was that tetradymol (**1**) was present in *L. cymbulifera* but not

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Table 1. Summary of the samples

Sample	Location[a])	Morphology	DNA	Isolated compounds
1	Desha	Intermediate	Hybrid	**1**, **2**, **3**, **6**, **7**, **9**
2	Desha	*L. tongolensis*	*L. tongolensis*	**2**, **4**, **9**
3	Desha	*L. cymbulifera*	Introgressed	**1**, **2**, **3**, **6**
4	Desha	Intermediate	Hybrid	**1**, **2**, **3**
5	Pachahai	*L. tongolensis*	Introgressed	**2**, **4**, **8**, **9**, **11**
6	Pachahai	*L. cymbulifera*	*L. cymbulifera*	**1**, **2**, **3**, **5**, **10**
7	Pachahai	Intermediate	Hybrid	**1**, **2**, **3**, **4**
8[b])	Pachahai	*L. tongolensis*	*L. tongolensis*	

[a]) Desha, in Daocheng County, Sichuan Province; Pachahai, in Shangrila County, Yunnan Province. [b]) Sample 12 in ref. [12].

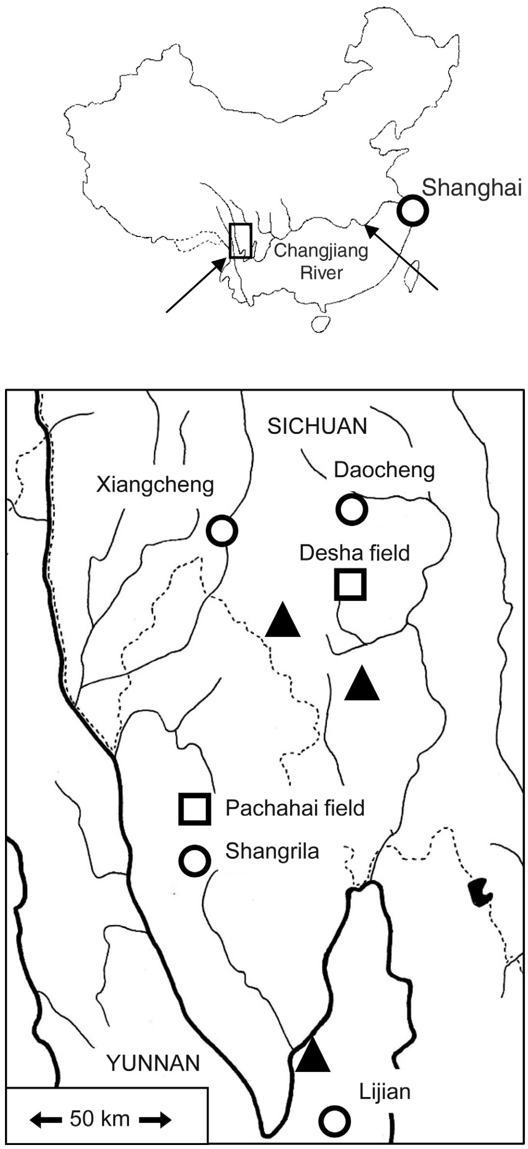

Fig. 1. Locations of sample collection (squares). Circles and filled triangles indicate major cities and peaks, respectively. Solid and dotted lines indicate rivers and province boundary, resp.

Table 2. ITS1-5.8S-ITS2 sequences of the samples[a])

Sample number	ITS1																								5.8S		ITS2											b)
	11	12	21	26	92	104	112	116	122	125	127	129	132	144	164	167	178	193	215	217	231	237	239	246	123	124	35	155	159	136	45	107	115	174	209	220	221	
1	Y	Y	Y	C	K	C	R	Y	S	C	M	G	T	W	Y	M	K	G	Y	G	Y	W	R	C	C	R	C	A	Y	T	R	Y	C	K	Y	Y	T	23
2	T	C	Y	C	T	M	G	T	S	C	A	G	T	T	C	Y	T	G	T	G	T	A	A	C	C	A	C	R	T	T	G	Y	C	G	T	T	T	6
3	Y	Y	C	C	K	C	R	Y	G	S	M	G	T	W	C	M	G	G	Y	G	Y	W	R	C	Y	R	C	A	Y	T	R	C	C	K	Y	Y	T	20
4	Y	Y	Y	C	K	M	R	Y	G	C	M	S	T	W	C	H	K	K	Y	R	Y	W	A	C	C	R	C	R	T	T	R	Y	C	K	Y	Y	T	24
5	C	Y	C	Y	G	C	R	T	G	C	M	G	Y	W	C	M	G	G	Y	G	Y	W	R	Y	C	G	Y	A	Y	W	R	C	Y	G	Y	Y	T	19
6	C	T	C	C	G	C	A	C	G	C	C	G	T	A	C	A	G	G	C	G	C	T	G	C	C	G	C	A	C	T	A	C	C	T	C	C	T	0
7	C	Y	C	Y	G	C	R	Y	G	C	M	G	Y	W	C	M	G	G	Y	G	Y	W	R	C	C	G	Y	A	Y	W	R	C	C	K	Y	Y	T	19
8[c])	C	C	C	Y	G	C	G	T	G	C	A	G	C	T	C	C	G	G	T	G	T	A	A	Y	C	G	T	A	T	A	G	C	Y	G	T	T	T	3
Lc ref[d])	C	T	C	C	G	C	A	C	G	C	C	G	T	A	C	A	G	G	C	G	C	T	G	C	C	G	C	A	C	T	A	C	C	T	C	C	C	0
Lt ref[e])	C	C	C	C	G	C	G	T	G	C	A	G	C	T	C	C	G	G	T	G	T	A	A	C	C	G	T	A	T	A	G	C	T	G	T	C	C	0

[a]) Differences among the sequences are shown. H = A + C + T; K = G + T; M = A + C; R = A + G; S = C + G; W = A + T; Y = C + T. [b]) Number of sites with multiple bases. [c]) Sample 12 in ref. [12]. A sequence with GG in place of GGG at 234 – 236 of ITS1 and a sequence with TTT in place of TT at 157 – 158 of ITS2 were also present with minor abundance. [d]) A *L. cymbulifera* sequence in the database (DQ272330). [e]) A *L. tongolensis* sequence in the database (DQ272337).

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in *L. tongolensis* [12][14]. In northwestern Yunnan, *L. tongolensis* was found to harbor some chemical and genetic diversity, while *L. cymbulifera* was uniform [12]. Here, we examine the chemical outcomes of hybridization between *L. cymbulifera* and *L. tongolensis* against these backdrops.

Results

Locations of Collection and Morphology of the Samples

Samples were collected in August 2011. Two samples with intermediate morphology were collected in Desha, Daocheng County, Sichuan (*Samples 1* and *4*), and one in Pachahai, Shangrila County, Yunnan (*Sample 7*; *Table 1* and *Fig. 1*). *Samples 1* and *4* were morphologically different: leaf-brades of *Samples 1* and *7* were elliptic, like *L. cymbulifera*, while those of *Sample 4* were cordate, like *L. tongolensis*. *L. tongolensis* (*Samples 2* and *5*) and *L. cymbulifera* (*Samples 3* and *6*) were also collected sympatrically. *L. tongolensis* and *L. cymbulifera* were more abundant in the Desha and in the Pachahai locations, respectively. The two locations are separated by about 100 km and high mountains (about 5000 m in altitude).

Genetic Analysis

DNA sequencing was carried out for the ITS1-5.8S-ITS2 region of the ribosomal RNA gene cluster. Hybrids will have multiple bases at sites where the sequences of the parents are different. For instance, haplotype H1 of *L. cymbulifera* and haplotype H12 of *L. tongolensis* are different at 16 positions in the region [11]; directly sequencing the PCR product of a hybrid of H1 and H12 should yield a sequence with multiple bases at the 16 positions. The results of direct sequencing are summarized in Table *2*. *Sample 6* had no multiple-base site and was considered to be 'pure' *L. cymbulifera*. *Sample 2* had six multiple-base sites, however, was considered to be 'pure' *L. tongolensis*, because such a number of intraspecific variations is expected. For instance, haplotypes H8 and H12 of *L. tongolensis* had eight differences in the region [11]. Morphologically intermediate individuals (*Samples 1*, *4*, and *7*) had many more multiple-base sites and their sequences were mostly superposition of the sequences of *L. cymbulifera* and *L. tongolensis*, indicating that the individuals were hybrids. In addition, the sequences of *Samples 3* and *5* were also superposition of the sequences of the two species, indicating that they have undergone hybridization and backcrossing, *i.e.*, introgression (*Table 1*).

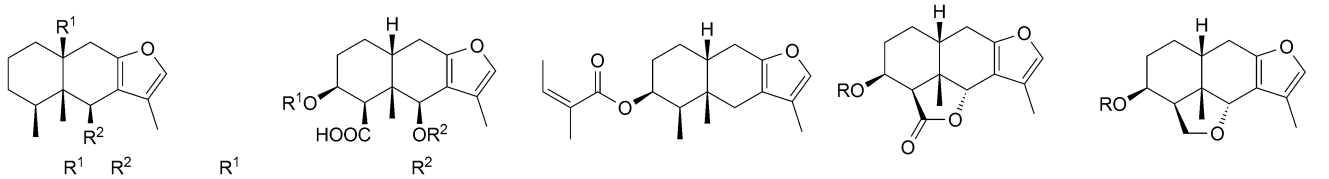
Chemical Analysis

Before isolation of the root compounds of the samples, *Ehrlich*'s test was carried out on a TLC [15] using EtOH extracts of fresh roots. All hybrids (*Samples 1*, *4*, and *7*) showed TLC patterns similar to that of *L. cymbulifera* [12], suggesting the presence of tetradymol (**1**) as the major component.

The chemical composition was also analyzed by LC/MS for the same EtOH extract of each sample. The total ion chromatogram (TIC) is shown in *Fig. 2a*. Many peaks were observed between t_R 8 and 20 min. Some showed similar MS (m/z = 245), indicating that they were structurally related. The chromatograms of the two hybrid samples in Desha (*Samples 1* and *4*) were similar to that of *L. cymbulifera* from the same locations (*Sample 3*). The hybrid in Pachahai (*Sample 7*) also showed a TIC pattern similar to those of the hybrids in Desha (*Samples 1* and *4*).

Isolation and structure determination of major constituents in each sample were carried out for EtOH extracts of air-dried roots. The components were purified by standard silica-gel column chromatography and HPLC methods. Eleven compounds, **1** – **11**, were isolated. All of them were furanoeremophilanes, among which **2** [16][17], **4** [17], **8** [12], **9** [12][18], **10** [12][18], and three new compounds, **3**, **5**, and **11** (see below for structure determination) were 15-oxygenated ones. Tetradymol (**1**) [19], 3-angeloyloxyfuranoeremophilane (**6**) [20], and ligularol (**7**) [21] were also isolated. The compounds isolated from the samples are summarized in *Table 1*.

The structures of the three new compounds **3**, **5**, and **11** were determined as follows. Compound **3** showed a molecular ion peak at m/z 430.1974 in MS and its formula was deduced to be $C_{24}H_{30}O_7$. The IR spectrum indicated the presence of C=O group(s) (1712 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **3** were very similar to those of a



	R^1	R^2
1	OH	H
7	H	OH

	R^1	R^2
2	MeCH=C(Me)CO (*Z*)	MeCH=C(Me)CO (*Z*)
3	CH_2=C(Me)CO	MeCH=C(Me)CO (*Z*)
4	MeCH=C(Me)CO (*Z*)	EtCH(Me)CH_2CO
5	CH_2=C(Me)CO	CH_2=C(Me)CO

6

8 R = EtCH(Me)CH_2CO
9 R = MeCH=C(Me)CO (*Z*)
10 R = CH_2=C(Me)CO

11 R = EtCH(Me)CH_2CO
12 R = H
13 R = MeCH=C(Me)CO (*Z*)

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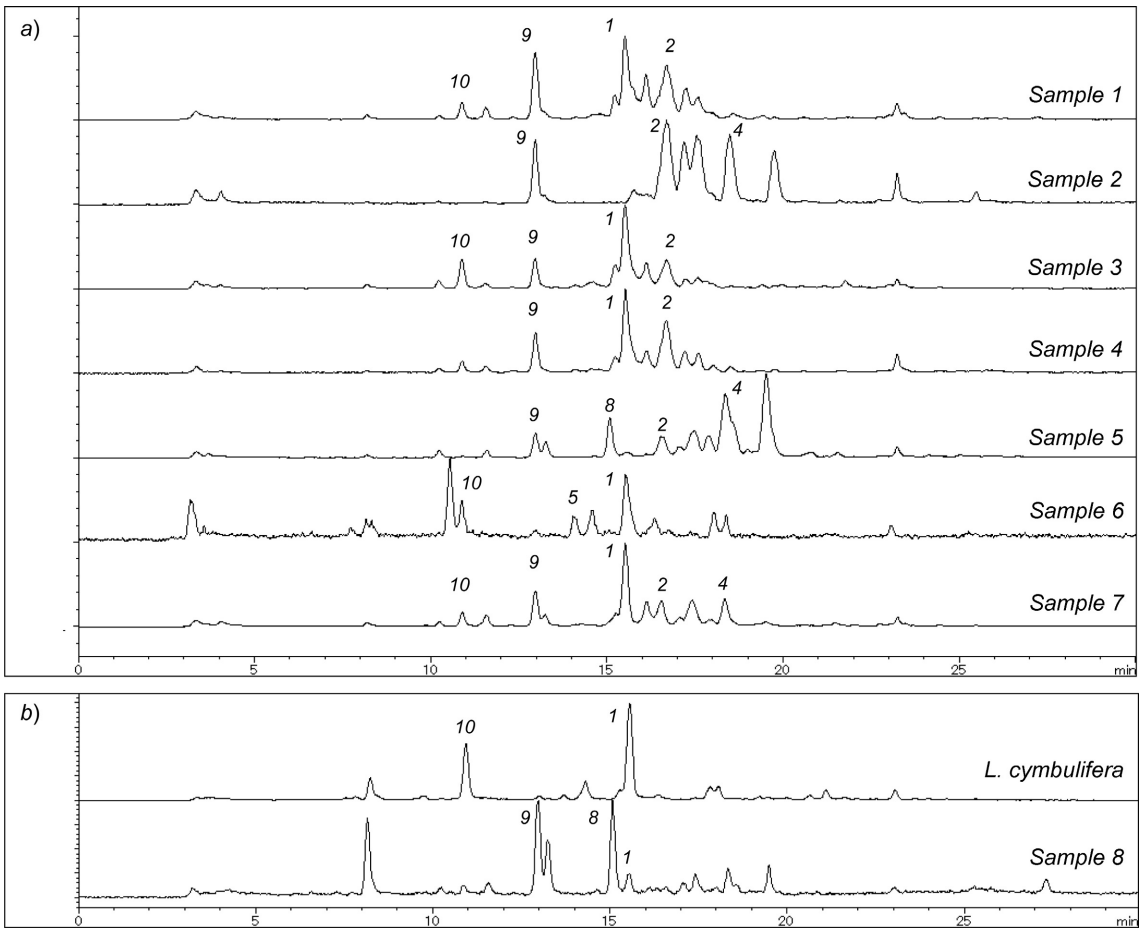

Fig. 2. *a*) LC profiles (total ion chromatogram) for *Samples 1 – 7* and *b*) *Sample 8*. EtOH extracts of fresh roots were used for *Samples 1 – 5* and *7*; an EtOH extract of dried roots was used for *Sample 6*. The profile labeled with '*L. cymbulifera*' is for a sample collected near Shangrila city in 2008. See text and *Table 1* for *Sample 8*. Compounds **1** – **11** were observed at t_R = 15.6, 16.7, 15.7, 18.5, 14.3, 23.4, 15.3, 15.1, 13.0, 11.0, and 19.6 min, resp.

known compound **2**, which was also isolated from the same sample. The difference was in the absence/presence of a methylidene group (δ(H) 5.16 and 6.16; δ(C) 125.4) (*Table 3*), suggesting that one of the two angeloyloxy groups in **2** was replaced by a methacryloyloxy group. The planar structure was established by COSY and HMBC spectra, as shown in *Fig. 3*. The methacryloyloxy and the angeloyloxy group were determined to be attached at C(3) and C(6), respectively, by HMBCs between H–C(3) and C (1′) and between H–C(6) and C(1″). The configuration was determined to be the same as in **2** by the *J* value of each H-atom and the NOE correlations shown in *Fig. 3*, establishing the structure as depicted in the figure.

Compound **5** showed a molecular ion peak at *m*/*z* 416.1829 in MS and its formula was deduced to be $C_{23}H_{28}O_7$. The ^{1}H- and ^{13}C-NMR spectra of **5** were very similar to those of **2** and **3**. The signals of an angeloyloxy group(s) observed in **2** and **3** were absent and signals from two methylidenes were observed (δ(H) 5.14, 5.15, 6.11, and 6.15; δ(C) 125.3 and 126.1; *Table 3*). These data indicated that **5** was a 3,6-bismethacryloyloxy derivative. COSY, HMBC, and NOESY spectra were very similar to those of **3**, establishing the structure as depicted.

Compound **11** showed a molecular ion peak at *m*/*z* 346.2136 in MS and its formula was deduced to be $C_{21}H_{30}O_4$. The IR spectra showed the presence of a saturated ester C=O group at 1731 cm^{-1}. The ^{1}H- and ^{13}C-NMR spectra showed signals characteristic of furanoeremophilanes such as a furan H-atom (δ(H) 6.98; H–C(12)), a *singlet* Me group (δ(H) 1.29; Me(14)) and an olefinic Me group (δ(H) 2.13; Me(13)) (*Table 3*). A pair of O-bearing CH_2 H-atoms were observed at δ(H) 3.81 and 3.89 (δ(C) 67.2), as in **12** [22][23] and **13** [17] [24], suggesting that the compound had an ether linkage between C(15) and C(6). The COSY spectrum revealed connections from H–C(1) to H–C(15), as indicated in *Fig. 4*. The presence of an acyloxy group at C(3) was deduced from signals of an O-bearing CH group (δ(H) 5.04; δ(C) 67.6). The molecular formula suggested that the acyloxy group was $C_5H_{11}COO$, and the COSY and HMBC spectra determined it to be 3-methylpentanoyloxy (*Fig. 4*). Since no useful NOESY signals were

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Table 3. ^{1}H- and ^{13}C-NMR data of compounds **3**, **5**, and **11** in C_6D_6[a])

Position	**3**		**5**		**11**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	0.95 – 1.05 (*m*, 2 H)	26.3	0.90 – 1.02 (*m*, 2 H)	26.3	0.90 – 0.98 (*m*) 1.80 – 1.91 (*m*)	22.7
2	1.80 – 1.91 (*m*) 2.11 – 2.26 (*m*)	26.7	1.81 – 1.92 (*m*) 2.13 – 2.26 (*m*)	26.7	1.22 – 1.34 (*m*) 1.51 – 1.60 (*m*)	25.6
3	5.76 (*dt*, *J* = 11.9, 5.8)	70.4	5.75 (*dt*, *J* = 11.4, 5.8)	70.3	5.04 (br. *s*)[e])	67.6
4	3.37 (*d*, *J* = 5.8)	49.8	3.33 (*d*, *J* = 5.6)	50[d])	1.63 (*ddd*, *J* = 11.4, 7.2, 2.5)	41.6
5	–	41.6	–	41.7	–	42.1
6	6.57 (*s*)	67.9	6.53 (*s*)	68.8	4.55 (*s*)	81.2
7	–	114.8	–	114.7	–	119.9
8	–	150.1	–	150.2	–	148.4
9α	2.00 (*d*, *J* = 17.2)[b])	26.3	1.98 (*d*, *J* = 17.0)	26.3	2.11 – 2.20 (*m*)	23.2
9β	2.64 (br. *dd*, *J* = 17.2, 5.4)		2.63 (br. *dd*, *J* = 17.0, 5.8)		2.27 (br. *dd*, *J* = 16.9, 7.2)	
10	2.37 – 2.48 (*m*)	36.8	2.41 – 2.50 (*m*)	36.8	1.81 – 1.91 (*m*)	37.9
11	–	119.8	–	119.9	–	120.8
12	6.91 (*s*)	139.1	6.89 (*s*)	139.1	6.98 (*s*)	138.1
13	1.86 (*d*, *J* = 0.8)	8.8	1.83 (*s*)	8.7	2.14 (*d*, *J* = 1.1)	8.7
14	1.14 (*s*)	19.5	1.12 (*s*)	19.3	1.29 (*s*)	22.6
15α	–	178.3	–	177[d])	3.89 (*t*, *J* = 7.2)	67.2
15β					3.81 (*dd*, *J* = 7.2, 11.4)	
1′	–	165.7	–	165.7	–	171.9
2′	–	136.7	–	136.7	1.87 – 1.96 (*m*) 2.08 – 2.15 (*m*)	41.8
3′	5.16 (*quint*, *J* = 1.3) 6.16 (br. *s*)	125.4	5.15 (*quint*, *J* = 1.2) 6.15 (br. *s*)	125.3	1.79 – 1.95 (*m*)	32.0
4′	1.79 (*s*)	18.2	1.78 (*s*)	18.2[c])	1.05 (*dquint*, *J* = 13.5, 7.4) 1.22 (*ddq*, *J* = 13.5, 5.4, 7.5)	29.4
5′	–	–	–	–	0.76 (*t*, *J* = 7.5)	11.4
6′	–	–	–	–	0.83 (*d*, *J* = 6.5)	19.3
1″	–	167.6	–	167.3	–	–
2″	–	127.4	–	136.3	–	–
3″	5.67 (*qq*, *J* = 7.2, 1.3)	139.8	5.14 (*quint*, *J* = 1.2) 6.11 (br. *s*)	126.1	– –	– –
4″	1.97 (*dq*, *J* = 7.2, 1.3)	16.0	1.78 (*s*)	18.4[c])	–	–
5″	1.83 (*quint*, *J* = 1.4)	20.8	–	–	–	–

[a]) *J*-value is shown in Hz. The solvent signal (δ(H) = 7.16, δ(C) = 128.0) was used as the reference. [b]) The *J*-value was deduced from the coupled H-atom because of overlapping. [c]) The assignment may be interchanged. [d]) Broad signal. [e]) Half-band width is *ca.* 8.0 Hz, see text.

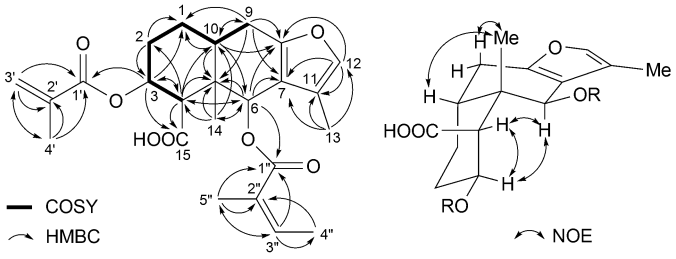

Fig. 3. Selected COSY, HMBC, and NOESY correlations detected for compound **3**.

observed except for correlations between H_β–C(6) and Me(14), and between H–C(10) and Me(14), the configuration was determined from the *J*-values, *i.e.*, $J_{4,15\alpha}$ = 7.2 Hz, $J_{4,15\beta}$ = 11.4 Hz, and $J_{15\alpha,15\beta}$ = 7.2 Hz. These data agreed with those observed for compounds **12** [22] and **13** [17], indicating that **11** has the same configuration. The half-band width of H–C(3) (*ca.* 8 Hz) was also similar to that in **13** (*ca.* 7 Hz) [17], supporting that H–C(3) was equatorial. Thus, the configuration was established as depicted in *Fig. 4*.

The results of LC/MS (*Fig. 2a*) and compound isolation (*Table 1*) indicated that tetradymol (**1**) and compound **4** were the characteristic compounds of *L. cymbulifera* and *L. tongolensis*, respectively, as

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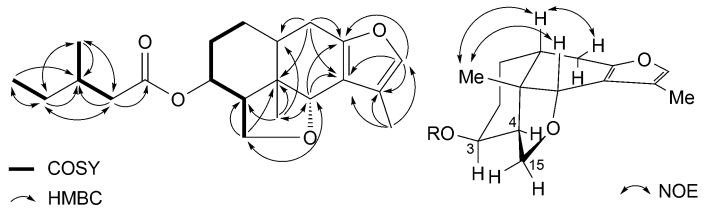

Fig. 4. Selected COSY, HMBC, and NOESY correlations detected for compound **11**.

previously observed [12][14]. Tetradymol (**1**) was detected at t_R = 15.6 min in TIC for *L. cymbulifera* (*Samples 3* and *6*, see also *Fig. 2b*), while it was not detected for *L. tongolensis* (*Samples 2* and *5*); **4** was detected at t_R = 18.5 min (*m*/*z* 345, 245) as a large peak for *L. tongolensis* (*Samples 2* and *5*), while no such peak was detected for *L. cymbulifera* (*Samples 3* and *6*) (*Sample 6* showed a peak at t_R = 18.6 min, however, its MS pattern was different). Tetradymol (**1**), characteristic to *L. cymbulifera*, was detected by LC/MS for all three hybrid samples (*Samples 1*, *4*, and *7*) and also isolated from the samples. Compound **4**, characteristic to *L. tongolensis*, was also detected in the Pachahai hybrid (*Sample 7*), however, its quantity was very small in the Desha hybrids (*Samples 1* and *4*) (*Fig. 2a*). Compound **2**, common to the parents at the collection locations, was also detected at t_R = 16.7 min for the hybrid samples. These results show that the hybrid in Pachahai contained root chemicals of both parents, while the hybrids in Desha were more similar to *L. cymbulifera*, indicating some variability in the chemical composition among hybrids.

Re-Analysis of a Previous Sample

At the Pachahai location, we had collected a sample of *L. tongolensis* (sample 12 in ref. [12]). The sample contained a small amount of a strongly *Ehrlich*-positive compound [12], which was not detected in the present Pachahai sample of *L. tongolensis* (*Sample 5*). Although isolation of the compound was not feasible at the time, its R_f value (0.61; hexane/AcOEt 7:3) and *Ehrlich* color (changing from pink to blue within 1 h) suggested that the compound was tetradymol. We carried out re-analysis of the sample by LC/MS previously not employed. The result is shown as *Sample 8* in *Fig. 2b*. A small peak of tetradymol (t_R = 15.6 min, *m*/*z* 235) was indeed detected. We also determined the sequence of the ITS1-5.8S-ITS2 region, which had not been examined in the previous report. The result, summarized in *Table 2*, shows that the sequence of the sample was typical of *L. tongolensis*, although it contained additional haplotypes with an insertion or a deletion (see footnote c).

Discussion

Several cases have been reported where hybrids inherit compounds from both parents [3b][5]. We also reported that hybrids of *L. nelumbifolia* and *L. subspicata* produced compounds of both parents additively [8][9]. In the present case of hybrids of *L. cymbulifera* and *L. tongolensis*, the chemical outcome was variable. Such chemical variability has been discussed in terms of genetic heterogeneity within the parental species [25].

Repeated backcrossing of a hybrid will result in a plant with morphology of the backcrossing species, however, its genome may retain some of the genes of the first hybridization partner; conversely, genes of another species are transferred. In the present case of *Sample 5*, an individual with morphology of *L. tongolensis*, the ribosomal RNA gene cluster retained copies from *L. cymbulifera*; however, the gene for tetradymol production appears to have been lost in the process of repeated backcrossing. We previously reported similar cases in which DNA analysis indicated introgression while the chemical composition agreed with morphology [10][26]. In contrast, *Sample 8* (= sample 12 of ref. [12]) appears to have retained the gene(s) to produce tetradymol, while the ribosomal RNA gene cluster has been converted to that of *L. tongolensis*. Because hybridization between *L. cymbulifera* and *L. tongolensis* occurs at the same location (Pachahai) and hybrids produce tetradymol, *L. cymbulifera* is likely to be the source of the gene(s) for tetradymol production. Another candidate for the source is *L. lamarum*/*L. subspicata*, as the plant in northern Shangrila is known to produce tetradymol [27]. In either case, hybridization/introgression would be the mechanism behind tetradymol production and thus a mechanism of generating the chemical diversity in *L. tongolensis* that we previously reported [12].

Although *L. tongolensis* is chemically diverse in the Shangrila (Zhongdian) County of Yunnan, *L. cymbulifera* is uniform in the area [12]. The previous genetic analyses [11] and the present result showed introgression in *L. cymbulifera* in Sichuan. Although the chemical diversity in *L. cymbulifera* in Sichuan has not been examined, the plant may also harbor chemical diversity, as *Gao* and coworkers [28] reported isolation of bisabolane compounds from *L. cymbulifera* in Muli, Sichuan.

Conclusion

The chemical consequences of hybridization between *L. cymbulifera* and *L. tongolensis* were examined. Production of chemicals was mostly additive but variable. Introgression appears to have produced individuals with

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various genetic/chemical traits, proffering a mechanism of generating intra-specific diversity.

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Experimental Part

General

Column chromatography (CC): silica gel (*Wako-gel C-200* or *C-300* (*Wako*, Osaka, Japan) or *silica gel 60 N* (*spherical neutral*; *Kanto*, Tokyo, Japan)) with hexane/AcOEt as eluent. Analytical TLC: silica gel (*Kieselgel 60 F_{254}*, layer thickness 0.2 or 0.25 mm; *Merck*, Darmstadt, Germany) using either *Ehrlich*'s reagent (*p*-dimethylaminobenzaldehyde and HCl) [15] or *p*-anisaldehyde/AcOH/H_2SO_4 as a visualizing agent. HPLC: *LC-20AT* pump, *SPD-20A* UV/VIS detector (*Shimadzu*, Kyoto, Japan), *Mightysil Si60* (10 × 250 mm; 5 μm; *Kanto*) column. IR: *FT/IR-230* spectrometer (*JASCO*, Tokyo, Japan). ^{1}H- and ^{13}C-NMR: *ECX-400* (400 and 100 MHz, resp.; *JEOL*, Tokyo, Japan) spectrometers with $CDCl_3$ or C_6D_6 as the solvent and TMS as an internal standard. Mass spectra (MS): *CMATE II* or *JMS-700 Mstation (JEOL)*. LC/MS: *1100 series LC/MSD* mass spectrometer (*Agilent*, Santa Clara, CA, USA; capillary voltage 3.5 kV; crona current 4 μA; capillary exit voltage (fragmentor) 90 V; drying temp. 330 °C; drying flow 9 l/min; nebulizer pressure 50 psig) with *5C18-MS-II* (*COSMOSIL*; *Nakarai,* Kyoto, Japan; 4.6 × 150 mm; 5 μm octadecyl column) using gradient system (MeOH/H_2O; 0 min (7:3) – 20 min (10:0) – 35 min (10:0) – 40 min (7:3) – 45 min (7:3); 0.5 ml/min) as eluent. Optical rotations: *DIP-370* digital polarimeter (*JASCO*). DNA Sequencing: *3130xl and 3500 Genetic Analyzers* (*Applied Biosystems*, Waltham, MA, USA). Purification of DNA from dried leaves, amplification of the ITS1-5.8S-ITS2 region by polymerase chain reaction, and DNA sequencing of the regions were carried out as described [27].

Plant Materials

Samples were collected at Desha, Daocheng County, Sichuan Province (*Samples 1 – 4*), and Pachahai, Shangrila (Zhongdian) County, Yunnan Province of China (*Samples 5 – 7*; *Table 1* and *Fig. 1*). Each sample was identified by *X. G.* (author). Voucher specimen numbers are No. 2011-101 (*Sample 1*, hybrid), 2011-102 (*Sample 2*, *L. tongolensis*), 2011-103 (*Sample 3*, *L. cymbulifera*), 2011-104 (*Sample 4*, hybrid), 2011-114 (*Sample 5*, *L. tongolensis*), 2011-115 (*Sample 6*, *L. cymbulifera*), 2011-116 (*Sample 7*, hybrid), and 2003-30 (*Sample 8*, *L. tongolensis*) (Kunming Institute of Botany).

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 solution was subjected to a TLC without concentration. Our previous report describes the procedure of *Ehrlich*'s test [12].

Extraction and Purification

Dried roots of *Sample 1* (hybrid; 6.96 g) was extracted with EtOH (*ca.* 50 ml) for about 6 months. The extract (638 mg) was subjected to a CC (SiO_2 (*60 N*, 27 g), hexane/AcOEt 1:1) to obtain a less polar fraction (496.3 mg), which was further subjected to a CC (SiO_2 (*C-200*, 21 g), hexane/AcOEt gradient) to give three fractions. From the fraction eluted with 5% AcOEt, **6** (1.1 mg) was obtained by a repeated CC and HPLC (*Mightysil*, hexane/Et_2O 97:3). From the fraction eluted with 10% AcOEt, **1** (14.1 mg), **7** (2.0 mg), and **9** (2.4 mg) were obtained by CC and HPLC (hexane/Et_2O 4:1 for **1** and **7**; 7:3 for **9**). From the fraction eluted with 15 – 20% AcOEt, **2** (22.6 mg) and **3** (16.8 mg) were obtained by CC and HPLC (hexane/Et_2O 1:1).

Dried roots of *Sample 2* (*L. tongolensis*; 8.27 g) was extracted with EtOH, as above, to obtain an extract (820 mg), which was separated by a CC (SiO_2 (23 g), hexane/AcOEt gradient). From the fraction eluted with 10% AcOEt, **9** (2.8 mg) was isolated by CC and HPLC (hexane/Et_2O 6:4). From the fraction eluted with 20 – 40% AcOEt, **2** (216.4 mg) and **4** (59.7 mg) were isolated by CC and HPLC (hexane/Et_2O 1:1).

Dried roots of *Sample 3* (*L. cymbulifera*; 7.14 g) was extracted with EtOH, as above, to obtain an extract (774 mg), which was separated by a CC (SiO_2 (25 g), hexane/AcOEt gradient). From the fraction eluted with 5% AcOEt, **6** (0.5 mg) was isolated by CC and HPLC (hexane/Et_2O 98:2). From the fraction eluted with 10% AcOEt, **1** (38.9 mg) was isolated by CC and HPLC (hexane/Et_2O 4:1). From the fraction eluted with 20 – 40% AcOEt, **2** (142.3 mg) was isolated by a CC, together with **3** (34.5 mg) which was obtained after purification by a HPLC (hexane/AcOEt 3:1).

Dried roots of *Sample 4* (hybrid; 7.70 g) was extracted with EtOH, as above, to obtain an extract (780 mg), which was separated by a CC (SiO_2 (23 g), hexane/AcOEt gradient). From the fraction eluted with 10% AcOEt, **1** (20 mg) was isolated by a CC. From the fraction eluted with 20% AcOEt, **2** (161.2 mg) and **3** (22.4 mg) were isolated by CC and HPLC (hexane/AcOEt 7:3).

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Dried roots of *Sample 5* (*L. tongolensis*; 0.65 g) was extracted with EtOH (*ca.* 30 ml), as above, to obtain an extract (136 mg), which was separated by a CC (SiO_2 (12 g), hexane/AcOEt gradient). From the fraction eluted with 10% AcOEt, **8** (0.9 mg), **9** (0.9 mg), and **11** (1.3 mg) were isolated by CC and HPLC (hexane/Et_2O 4:1 for **11**; 7:3 for **8** and **9**). From the fraction eluted with 20% AcOEt, **2** (11.9 mg) and **4** (12.6 mg) were isolated by a HPLC (hexane/AcOEt 3:1).

Dried roots of *Sample 6* (*L. cymbulifera*; 2.15 g) was extracted with EtOH (*ca.* 30 ml), as above, to obtain an extract (337 mg), which was separated by a CC (SiO_2 (25 g), hexane/AcOEt gradient). From the fraction eluted with 10% AcOEt, **1** (79.9 mg) was isolated by a CC. From the fraction eluted with 20% AcOEt, **2** (15.4 mg), **3** (16.3 mg), **5** (16.2 mg), and **10** (3.4 mg) were isolated by CC and HPLC (hexane/AcOEt 7:3 for **2**, **3**, and **5**; hexane/Et_2O 65:35 for **10**).

Dried roots of *Sample 7* (hybrid; 6.02 g) was extracted with EtOH, as above, to obtain an extract (600 mg), which was separated by a CC (SiO_2 (25 g), hexane/AcOEt gradient). From the fraction eluted with 10% AcOEt, **1** (40 mg) was isolated by a CC. From the fraction eluted with 20% AcOEt, **2** (108.2 mg), **3** (13.5 mg), and **4** (42.7 mg) were isolated by CC and HPLC (hexane/AcOEt *ca.* 7:3).

(4*S*,4a*S*,5*R*,6*S*,8a*R*)-4,4a,5,6,7,8,8a,9-Octahydro-3,4a-dimethyl-6-[(2-methylacryloyl)oxy]-4-{[(2*E*)-2-methylbut-2-enoyl]oxy}naphtho[2,3-*b*]furan-5-carboxylic Acid (**3**). Oil. $[\alpha]_D^{27}$ = –24.1 (*c* = 0.29, MeOH). FT-IR (neat): 1712 (C=O), 1641 (C=C). ^{1}H- and ^{13}C-NMR: see *Table 3*. CI-MS: 431 (2, $[M + H]^+$), 430 (2, M^+), 331 (41), 245 (100). HR-CI-MS: 430.1974 (M^+, $C_{24}H_{30}O_7^+$; calc. 430.1992).

(4*S*,4a*S*,5*R*,6*S*,8a*R*)-4,4a,5,6,7,8,8a,9-Octahydro-3,4a-dimethyl-4,6-bis[(2-methylacryloyl)oxy]naphtho[2,3-*b*]furan-5-carboxylic Acid (**5**). Oil. $[\alpha]_D^{28}$ = –24.5 (*c* = 0.19, MeOH). FT-IR (neat): 1717 (C=O), 1636 (C=C). ^{1}H- and ^{13}C-NMR: see *Table 3*. CI-MS: 416 (1, M^+), 359 (2), 331 (27), 245 (100). HR-CI-MS: 416.1829 (M^+, $C_{23}H_{28}O_7^+$; 416.1835).

(2a*S*,3*S*,5a*R*,9b*R*,9c*S*)-2a,3,4,5,5a,6,9b,9c-Octahydro-9,9c-dimethyl-2*H*-naphtho[2,3-*b*:4,5-*b'c'*]difuran-3-yl 3-Methylpentanoate (**11**). Oil. $[\alpha]_D^{27}$ = –11.2 (*c* = 0.12, MeOH). FT-IR (neat): 1731 (C=O). ^{1}H- and ^{13}C-NMR: see *Table 3*. CI-MS: 346 (100, M^+), 231 (95), 207 (58). HR-CI-MS: 346.2136 (M^+, $C_{21}H_{30}O_4^+$; 346.2145).

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