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Chemical and genetic diversity of *Ligularia vellerea* in Yunnan, China

Motoo Tori [a,*], Hiromi Nakamizo [a], Kanako Mihara [a], Masahiko Sato [b], Yasuko Okamoto [a], Katsuyuki Nakashima [a], Masami Tanaka [a], Yoshinori Saito [a], Masakazu Sono [a], Xun Gong [c,*], Yuemao Shen [c], Ryo Hanai [b,*], Chiaki Kuroda [d,*]

[a] *Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan*
[b] *Department of Life Science, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan*
[c] *Kunming Institute of Botany, Chinese Academy of Science, Kunming 654204, China*
[d] *Department of Chemistry, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan*

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Abstract

Intra-specific diversity in *Liularia vellerea* growing in the northwestern to central Yunnan province of China was studied by chemical and genetic approaches. Samples collected in the Jianchuan, Lijiang, and Zhongdian areas contained 6,15-dioxygenated furanoeremophilanes as their major components (type A); whereas samples from the Luguhu area accumulated 1,6-dioxygenated furanoeremophilanes (type B); a sample from near Kunming, however, contained 6,15-dioxygenated eremophilanolides (type C). 11βH- and 11αH-6β-angeloyloxy-15-carboxyeremophil-7-en-12,8-olides (eremofarfugins D and E) were also isolated and their structures were determined. A correlation between the composition and the DNA sequence was observed in the ITSs.
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Keywords: *Ligularia vellerea*; Asteraceae; Structure determination; Eremophilane-type sesquiterpenoid; Eremofarfugin; A*tpB-rbcL*; ITS

1. Introduction

Ligularia Cass. (Asteraceae) in the Hengduan Mountains area provides interesting samples for the study of plant diversity, since the genus is highly diversified and their evolution is considered to be still continuing in the area (Liu et al., 1994). Our approach to examine its diversity is to combine two different methods, the analyses of chemical compositions and the determination of DNA sequences (Hanai et al., 2005; Nagano et al., 2006; Tori et al., 2006; Torihata et al., 2007; Kuroda et al., 2007). In the chemical analysis of this species, we have focussed on the furanoeremophilanes and related components in the plant roots, since these compounds are often found in *Ligularia*, and are easy to detect by Ehrlich's test on TLC (Kuroda et al., 2004; Kuroda and Nishio, 2007). For DNA analyses, we determined the sequences of the *atpB-rbcL* intergenic region and the internal transcribed spacers (ITSs) of the ribosomal RNA gene. These regions are non-coding and thus the variations therein are mostly neutral to evolution, rendering them suitable for studies of intra-specific diversity (Savolainen et al., 1994; Soltis and Soltis, 1998). Our first finding with this approach was that *Ligularia tongolensis* (Franch.) Hand.-Mazz. in Yunnan was diverse, whereas *Ligularia cymbulifera* (W.W. Smith) Hand.-Mazz. was uniform (Hanai et al., 2005). We also found that *Ligularia pleurocaulis* (Franch.) Hand.-Mazz. (Nagano et al., 2006), *Ligularia virgaurea* (Maxim.) Mattf. (Tori et al., 2006), and *Ligularia tsangchanensis* (Franch.)

* Corresponding authors. Tel.: +81 88 602 8462; fax: +81 88 655 3051 (M. Tori); Tel.: +86 871 5223625 (G. Xun); Tel./fax: +81 3 3985 2377 (R. Hanai); Tel./fax: +81 3 3985 2396 (C. Kuroda).
E-mail addresses: tori@ph.bunri-u.ac.jp (M. Tori), gongxun@mail.kib.ac.cn (X. Gong), hanai@rikkyo.ne.jp (R. Hanai), chkkuroda@grp.rikkyo.ne.jp (C. Kuroda).

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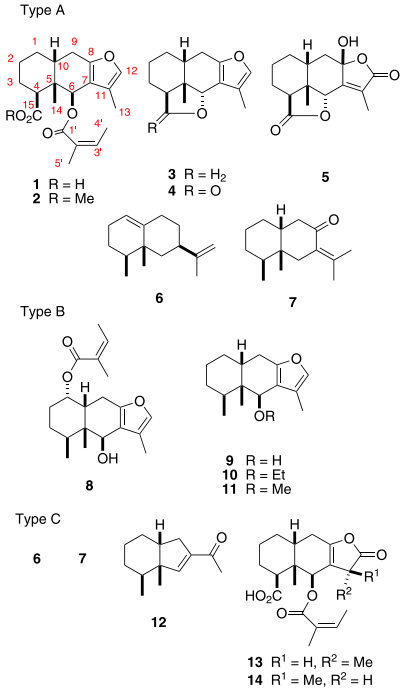
Hand.-Mazz. (Torihata et al., 2007) in the Hengduan Mountains area could be each grouped into two types. In either case, the grouping based on chemical analysis agreed with that based on DNA sequence. Interestingly, the groups in *L. pleurocaulis* and *L. tsangchanensis* were geographically separated (Nagano et al., 2006; Torihata et al., 2007) while those in *L. virgaurea* were not (Tori et al., 2006). These results suggested that there are different modes in generating the intra-specific diversity of *Ligularia*, implying in turn complex mechanisms of evolution.

Our present focus is on *Ligularia vellerea* (Franch.) Hand.-Mazz., which is one of two species belonging to the section Scapicaulis (Liu, 1989). The plant is distributed in northwestern Yunnan and southwestern Sichuan Provinces of China, growing in meadows of various environments at altitudes of about 2500–4000 m. The most distinct morphological feature of the species is the presence of dense white lanate hair at the base of its stem and petioles. Two eremophilan-12,8-olides have been isolated from a sample from Yulongxueshan, Yunnan (Li et al., 2001), and the presence of pyrrolizidine alkaloids has also been reported (Pu et al., 2004). However, to date, the diversity in chemical composition has not been studied. Here we report the diversity of *L. vellerea*, the plants' grouping on the basis of chemical components, the respective DNA sequence, and geography, all of which are in accordance with one another.

2. Results

Nineteen samples of *L. vellerea* (Table 1 and Figs. 1 and 2) were collected in the northwestern Yunnan Province. Most of the samples were obtained from the Zhongdian area (samples 4–15), together with one sample from the Jiangchuan area (sample 1), two from the Lijiang area (samples 2, 3), three from the Luguhu area (samples 16–18), and one from the Kunming area (sample 19). The respective ethanol extracts of the fresh root samples were subjected to Ehrlich's reaction on TLC plates. The twelve samples collected near the city of Zhongdian (samples 4–15) each exhibited a strong Ehrlich-positive spot ($R_f = 0.58$, hexane/AcOEt 7:3) and some small spots (type A in Table 1). The two samples collected near the city of Lijiang (samples 2 and 3), and one sample from the Jianchuan area (sample 1), gave almost the same sample features, except that a small additional spot (orange) was also detected in the latter. In contrast, the constituents of the three samples collected near Luguhu (samples 16–18) were completely different (type B). Namely, these samples exhibited two major Ehrlich-positive compounds (the most abundant one: $R_f = 0.62$). Finally, the only sample collected near Kunming (sample 19) showed a third type of pattern, which consisted of many Ehrlich-positive spots (type C).

The structure of the chemicals from typical samples were determined. From the type-A samples (samples 1, 2, 3, 7, and 12), four Ehrlich-positive compounds were isolated. They were identified as 6β-angeloyloxyfuranoeremophilan-15-oic acid (**1**) (Bohlmann and Zdero, 1980), methyl 6β-angeloyloxyfuranoeremophilan-15-oate (**2**) (Bohlmann and Grenz, 1979), 6α,15-epoxyfuranoeremophilane (**3**) (Ishizaki et al., 1979), and furanoeremophilan-15,6α-olide (**4**) (Ishizaki et al., 1979). The three Ehrlich-negative compounds, 8β-hydroxyeremophil-7(11)-ene-15,6α:12,8-diolide (**5**) (Moriyama and Takahashi, 1977), eremophila-1(10),11-diene (**6**) (Krepinsky et al., 1968; Zhao et al., 2004) and eremophil-7(11)-en-8-one (= fukinone, **7**) (Naya et al., 1968) were also obtained. From the type-B samples (samples 17 and 18), the two Ehrlich-positive compounds, 1α-angeloyloxyfuranoeremophilan-6β-ol (**8**) (Bohlmann et al., 1986) and ligularol (= petasalbin, **9**) (Ishii et al., 1965; Yamakawa and Satoh, 1979) were isolated as the major components, together with ligularol ethyl (**10**) (Kuroda et al., 2004; Nagano et al., 1982) and methyl (**11**) (Naya et al., 1971) ethers. Although TLC of the extracts of fresh roots detected Ehrlich-positive compounds in the type-C sample (sample 19), the extract of dried roots of the same sample contained no Ehrlich-positive compounds. The furano-components in the sample were presumably labile enough to decompose on drying. From the sample, the three known compounds, **6** (Krepinsky et al., 1968; Zhao et al., 2004), **7** (Naya et al., 1968), and 1-[(3a*R*,4*S*,7a*R*)-3a,4,5,6,7a-hexahydro-3a,4-dimethyl-1*H*-inden-2-yl]ethanone (**12**) (Tori et al., 2006), and the two new compounds, **13** and **14**, were isolated. The structures of the new compounds were determined as follows.



Compound **1** is a carboxylic acid, as characterized from the presence of a broad absorption around 3500–3000 cm^{-1}, as well as 1700 cm^{-1}, in the IR spectrum. Its

Table 1
Collection locality and *atpB-rbcL* genetic type of *L. vellerea* samples

Sample	Locality	Elevation (m)	Genetic type[a,b]	Chemical type[c]
Jianchuan aera				
1	Jiushijiulongtan	3300	A-A11	A
Lijiang area				
2	Yulongxueshan	3300	G-A10	A
3	Yulongxueshan	3600	G-A10	A
Zhongdian area				
4	Qianhushan	3800	G-A10	A
5	Xiaozhongdian	3200	G-A10	A
6	Xiaozhongdian	3100	G-A10	A
7	Dabaoshan	3300	G-A10, 409T	A
8	Dabaoshan	3300	A-A9	A
9	Tianshengqiao	3200	G-A10	A
10	Shuduhu (Shudu lake)	3500	G-A10	A
11	Shikashan	3400	G-A10	A
12	North of Napahai	3600	G-A10	A
13	Geza	3800	G-A10	A
14	Daxueshan	3500	A-A9	A
15	Daxueshan	3600	G-A10	A
Luguhu area				
16	Luguhu	3000	G-A10	B
17	Yongning	2700	G-A10, 409T	B
18	Mudiqing	3000	G-A10	B
Kunming area85				
19	Liangwangshan	2700	G-A10	C

[a] See our previous report (Hanai et al., 2005) for the designation.
[b] The 409th base was A except for samples 7 and 17, in which it was T.
[c] See text. The major components were 6,15-dioxygenated furanoeremophilanes (type A), 1,6-dioxygenated furanoeremophilanes (type B), and 6,15-dioxygenated eremophilanolides (type C).

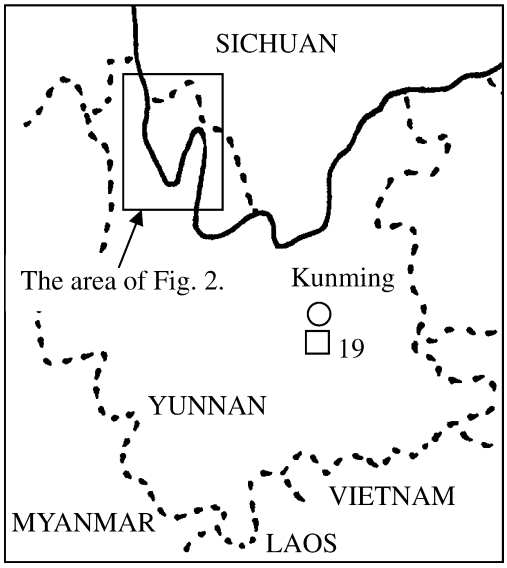


Fig. 1. Locations where samples of *L. vellerea* (open squares) were collected. Open circles indicate major cities.

molecular formula is $C_{20}H_{26}O_5$ according to HRMS, whereas the ^1H and ^{13}C NMR spectra show only broad signals. Such a phenomenon is frequently encountered in some cases of enol-lactones and epoxy-lactones due to the slow rotation in the medium (Tori et al., 1998, 2000, 2005, 2006). Nevertheless, a proton signal from a furan at C-12 and resonances from an angelate moiety

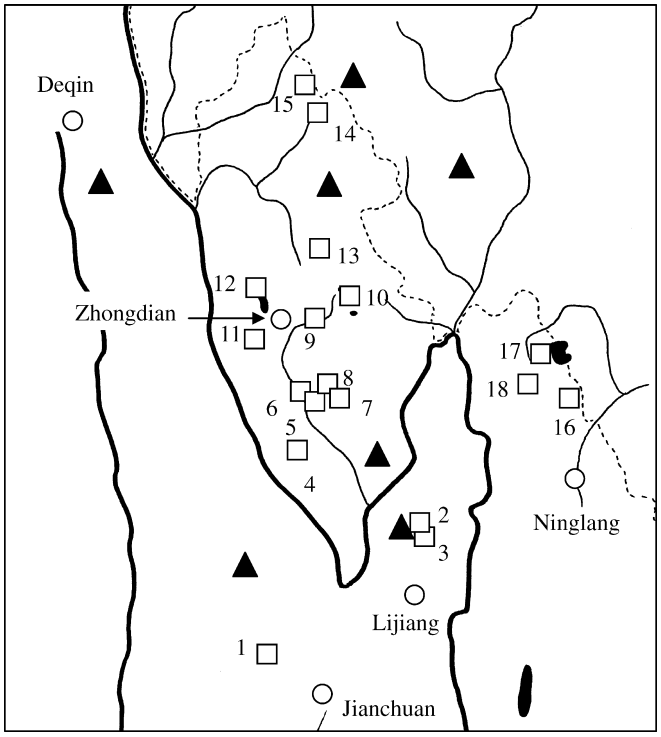


Fig. 2. Locations where samples of *L. vellerea* (open squares) were collected. Filled triangles and open circles indicate the major peaks and major cities, respectively.

(Tori et al., 1998) are observable. Even when 2D NMR spectra were obtained in C_6D_6 at 65 °C, some carbon signals were still unobservable. Therefore, pyridine-d_5 was used as the solvent and the 2D NMR was measured at 100 °C. Analysis of the HMBC spectrum established the presence of an angelate moiety attached to C-6 and a carboxylate at C-15. The relative stereochemistry as depicted in the formula was established by analysis of the NOESY spectrum. Although this compound was already reported by Bohlmann (Bohlmann and Zdero, 1980), the structure was erroneously drawn, in that article. This constitutes the reconfirmation of their work (see Fig. 3).

The IR spectra of compounds **13** and **14** indicate the presence of an enol-lactone at 1800 cm^{-1} for both compounds (Tori et al., 1998, 2000, 2005, 2006). Their molecular formula is $C_{20}H_{26}O_6$ (HRMS). The ^1H NMR spectra taken at 60 °C shows the presence of a doublet methyl, a singlet methyl, and two methyl groups attached to the olefinic carbons. Although only poor correlations were obtainable for either compound, we were still able to assign their structures as shown in Fig. 4 from HMBC correlations and by a comparison with the spectroscopic data of the previously isolated enol-lactones (Tori et al., 2000, 2006). Since the NOESY spectra show no significant correlation, the stereochemistry is only speculative (Tori et al., 1998). The configuration at the C-11 position was deduced by comparing the chemical shifts of both compounds: H-6 of **13** appears at δ 5.98, while that of **14** at δ 5.78. Since H-13 (the methyl group) is close to H-6 in compound **13**, the signal of H-6 is shifted to a lower field than that of H-6 of compound **14** (Bhacca and Williams, 1964). The ^{13}C NMR chemical shift of C-13 of compound **14** resonates at δ 14.5, which is higher field than that of compound **13** (δ 15.5), due to steric compression by the angelate moiety. Because NMR measurements at 60 °C deteriorated the samples significantly, it was not feasible to repeat the measurement at 100 °C. Compounds **13** and **14** were named as eremofarfugins D and E, respectively (Okamoto et al., 2006).

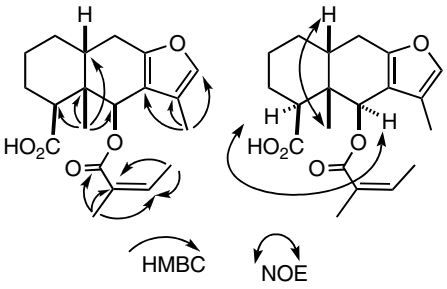


Fig. 3. Selected HMBC and NOESY for compound **1**.

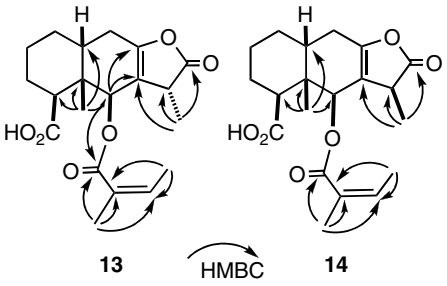


Fig. 4. Selected HMBC for compounds **13** and **14**.

DNA sequencing was carried out for the *atpB-rbcL* region in the plastid genome and the ITSs of the ribosomal RNA gene on the nuclear genome (Hanai et al., 2005). The results for the *atpB-rbcL* region are summarized in Table 1. It has been observed that the 28th base and the number of adenines in a stretch around the 510th base were variable within a species of *Ligularia* (Hanai et al., 2005; Torihata et al., 2007). The majority of the samples had guanine at the 28th position and 10 adenines in the stretch [G-A10 under our designation (Hanai et al., 2005)]. In addition to variants A-A9 and A-A11, samples 7 and 17 were found to have one base change from adenine to thymine at the 409th position.

The results of ITS sequencing are given in Table 2. For samples 16–19, part of the sequencing data of ITS2 was a superposition of two signals, which made the ITS2 sequence ambiguous. Analysis of data carried out for both strands of the DNA indicates that an additional sequence of a different length was present within the sequenced individuals. In samples 16–18, the additional sequence had an extra GT doublet after the 97th position. In sample 19, the additional one had an extra A after the 173rd. Such additional sequences had been observed in some samples of *L. pleurocaulis* (Nagano et al., 2006) and *L. tsangchanensis* (Torihata et al., 2007) as well.

3. Discussion

In the present study, *L. vellerea* was examined with respect to the composition of furanoeremophilanes and related sesquiterpenes and the nucleotide sequences of the *atpB-rbcL* region and the ITSs of the ribosomal RNA gene. Furanoeremophilanes were isolated from samples 1–18. Although no furano-compound was isolated from sample 19, several spots positive to Ehrlich's test were detected on a TLC plate when an extract of fresh root was used. Such discrepancy between TLC results and the isolated compounds was seen in other samples as well. While all the samples from the Zhongdian area (type A) showed one strong Ehrlich-positive spot of lactone **4** (Ishizaki et al., 1979) on TLC plates, 6β-angeloyloxyfuranoeremophilan-15-oic acid (**1**) and/or its ester **2** (Bohlmann and Grenz, 1979) were isolated instead of the lactone from some samples (see Section 5). Presumably, compound **4** (Ishizaki et al., 1979) was generated from **1** or **2** (Bohlmann and Grenz, 1979) in the presence of other components as catalyst, because

Table 2
Sequence variations in ITSs[a,b]

Sample No.	ITS1													ITS2				
	3	24	40	83	92	135	182	188	200	216	222	230	238	3	27	92	167	220
1	G	C	A	G	G	Y	A	C	T	Y	C	G	C	Y	C	A	T	C
2	G	C	A	G	G	Y	A	S	T	Y	C	G	C	C	C	A	Y	Y
3	G	C	A	G	G	Y	A	C	T	Y	Y	G	C	C	C	A	T	Y
4	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
5	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
6	G	C	A	K	K	C	A	C	T	C	C	G	C	C	C	A	T	C
7	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
8	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
9	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
10	G	C	A	G	G	C	A	C	T	C	C	G	C	C	Y	A	T	C
11	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
12	G	C	A	G	G	C	A	C	Y	C	C	G	C	C	C	A	T	C
13	G	C	A	G	G	C	A	C	T	C	C	G	C	C	C	A	T	C
14	G	C	A	K	K	C	A	C	T	C	C	K	C	C	C	M	T	C
15	G	C	A	G	G	C	A	C	T	C	C	K	C	C	Y	M	Y	C
16	G	Y	A	G	G	C	R	C	T	Y	C	K	Y	–[c]	–[c]	–[c]	–[c]	–[c]
17	R	C	A	G	G	C	A	C	T	C	C	G	C	–[c]	–[c]	–[c]	–[c]	–[c]
18	G	C	A	G	G	C	A	C	T	C	C	K	C	–[c]	–[c]	–[c]	–[c]	–[c]
19	G	C	R	G	G	C	A	C	T	C	C	G	C	–[d]	–[d]	–[d]	–[d]	–[d]

[a] K = G + T; M = A + C; R = A + G; Y = C + T.
[b] The majority sequence with no additional base was exactly the same as those in the database (accessions DQ272336 and AY458840).
[c] An additional minor sequence with an extra GT double after the 97th position of ITS2 was present. Hence, the ITS2 sequence could not be determined unambiguously.
[d] An additional sequence with an extra A after the 173rd position was present.

elimination of a hydroxy or an acyloxy group at C-6 can easily occur (Kuroda et al., 2004) with an ensuing attack of the carboxyl group at C-15. Lactonization from 6β-acyloxyfuranoeremophilan-15-oic acid to a 6α,15-olide in the presence of acetic acid has been reported (Kuroda et al., 1982). Compound **4** (Ishizaki et al., 1979) was obtained as the major component (30% of extract) from the ethanol extract of a *L. vellerea* sample collected in 2001 at the same place as sample 2. Li and co-workers also stated that **4** was a major component of *L. vellerea* (Li et al., 2006) although its isolation had not been reported in their early work (Li et al., 2001).

One of the major findings of the present study is that the variety in the chemical composition is related to the geographical distribution of the samples. On the basis of the composition, the samples could be divided into the three groups, type A (from the Jianchuan, the Lijian, and the Zhongdian areas), type B (from the Luguhu area), and type C (from the Kunming area). The major components isolated from types A, B, and C were 6, 15-dioxygenated furanoeremophilanes, 1,6-dioxygenated furanoeremophilanes, and 6,15-dioxygenated eremophilanolides, respectively. TLC results of sample 1 showed a small orange-colored spot indicative of a 6-oxofuranoeremophilane derivative (Kuroda and Nishio, 2007), although it could not be isolated. Among the fifteen type-A samples, this sample (from the Jianchuan area) is geographically isolated (Fig. 2). Types A and C are somewhat similar, as compound **1** is a furano-derivative of lactone **13/14** with the same substituents at C-6 and C-15. In contrast, the type B is clearly different from the types A and C, with no common compound.

Samples collected near the city of Zhongdian (type A) are almost uniform in their furanoeremophilane composition. From the same area, we previously sampled *L. tongolensis* and *L. cymbulifera* and found that the former was diverse in its chemical composition, but the latter was not (Hanai et al., 2005). The furanoeremophilane compounds obtained from *L. vellerea* in the Zhongdian area were 15-oxygenated forms, which had been seen in the three Corymbosae species, *L. cymbulifera*, *L. tongolensis*, and *L. atroviolacea* (Hanai et al., 2005) from the same area. However, the oxidation level at C-3 was different. 15-Oxygenated furanoeremophilanes produced by the three Corymbosae species are all oxidized at C-3 as well, while the furanoeremophilanes **1**, **2**, **3** (Ishizaki et al., 1979), **4**, and **5** (Moriyama and Takahashi, 1977), in *L. vellerea* from the area are not.

A correlation is also seen between the chemical composition and the ITS sequence. The type A samples do not contain any additional ITS sequence of different lengths while types B and C contain an additional sequence with different insertions. Such a correlation had been found in *L. pleurocaulis* and *L. virgaurea* var. *virgaurea* (Nagano et al., 2006; Tori et al., 2006). The presence of multiple sequences is considered to indicate hybridization in the recent past (Koch et al., 2003). Further studies are necessary to discern whether the chemical difference has been brought about by hybridization or by genetic drift due to geographical isolation.

The number of sites with multiple bases in ITSs is worth mentioning. The number was not the same among the samples from the Zhongdian area (Table 2): samples from the central region (Xiaozhongdian area; samples 4–8) do not contain such a site, except for sample 6, while the samples from the outer regions contained some. This suggests that introgression, *i.e.* inflow of genetic information, has occurred more frequently in the outer regions. The implication of introgression to adaptation has been studied in a European sedge, *Carex curvula* (Choler et al., 2004). In ecologically marginal niches, populations of each of *C. curvula* subsp. *curvula* and *C. curvula* subsp. *rosae* were found to be mainly composed of individuals with genotypes resulting from introgressive hybridization. In contrast, no hybrids were found in typical habitats. In our experience, *L. vellerea* is easier to find in the Xiaozhongdian area. This observation may indicate that the central region offers an environment more suitable to *L. vellerea*. It would then follow that the plants in the outer regions have adapted to less suitable environments with introgression.

4. Conclusion

Intra-specific diversity in *L. vellerea* of northwestern to central Yunnan province of China was found to be large in the root constituents. On the basis of the eremophilane composition, nineteen collected samples could be grouped into three types (types A–C). The classification agreed with the geographic distribution and the classification based on the ITS sequences. The major components of Jianchuan, Lijiang, and Zhongdian samples (type A), Luguhu samples (type B), and a Kunming sample (type C) are 6,15-dioxygenated furanoeremophilanes, 1,6-dioxygenated furanoeremophilanes, and 6,15-dioxygenated eremophilanolides, respectively. The two new compounds, 11βH- and 11αH-6β-angeloyloxyeremophil-7-en-8,12-olides (eremofarfugins D and E) were isolated and their structures have been established.

5. Experimental

5.1. General procedures

See our previous report (Hanai et al., 2005) for general procedures and apparatus. DNA sequencing was also carried out using BigDye Terminator Kit (Applied Biosystems) and 3130xl Genetic Analyzer (Applied Biosystems).

5.2. Plant material

The samples of *L. vellerea* were collected in August, 2002–2006. Each plant was identified by Xun Gong, one of the authors, and voucher specimen were deposited in the herbarium of the Kunming Institute of Botany (KUN), sample 1:K-G-0306, 2:K-G-0410, 3:K-G-0415, 4:K-G-0452, 5:K-G-0207, 6:K-G-0313, 7:H-G-06-79, 8:K-G-0430, 9:K-G-0224, 10:K-G-0222, 11:K-G-0459, 12:K-G-0468, 13:K-G-0440, 14:K-G-0336, 15:K-G-0346, 16:K-G-0483, 17:K-G-0477, 18:K-G-0474, 19:H-G-06-01 (Table 1 and Fig. 1).

5.3. Ehrlich's test

The roots of each plant (2–10 g) were harvested, and extraction with ethanol was started immediately without drying. Solid plant material was removed after several days. See our previous report (Hanai et al., 2005) for the procedures of Ehrlich's test on TLC.

5.4. Extraction for structure determination

For the samples collected in 2003, the roots were cut into small pieces without drying, and immediately extracted with EtOH at room temperature. Each extract was filtered and concentrated to afford an oily residue with an aqueous phase. EtOAc was added to this oil/aqueous mixture and the organic layer was recovered. Evaporation of the solvent afforded an oily residue, to which water soluble starch was added for handling purpose. For the samples collected in 2004–2006, the roots were dried and extracted with EtOH or AcOEt at room temperature. Oily extracts were obtained by the standard method.

5.5. Purification and identification of chemical components

The isolation work was carried out for samples 1, 2, 3, 7, 12, 18, and 19. The EtOH extract of sample 1 (1.16 g with starch, extracted from 55 g of fresh root) was separated by silica-gel OCC (Hexane-EtOAc, 0–100%), followed by HPLC (Hexane-EtOAc, Nucleosil 50–5) to afford **4** (3.2 mg); The EtOH extract of sample 2 (559 mg) was separated by silica-gel OCC (Hexane-EtOAc, 0–100%), followed by HPLC (Hexane-AcOEt, Nucleosil 50–5) to afford **2** (7.4 mg), **3** (2.6 mg), **5** (74.4 mg), **6** (5.1 mg), and **7** (1.6 mg). The EtOH extract of sample 3 (1.8 g) was separated by silica-gel OCC (Hexane-EtOAc, 0–100%) to afford **1** (296.1 mg), **2** (75.3 mg), and **6** (7.9 mg). The EtOH extract of sample 7 (331 mg) was separated by silica-gel OCC (Hexane-AcOEt, 0–100%) to afford **1** (80.4 mg) and **4** (17.4 mg). The EtOH extract of sample 12 (222 mg) was separated by silica-gel OCC (Hexane-EtOAc, 0–100%), followed by HPLC (Hexane-AcOEt, Nucleosil 50–5) to afford **1** (56.5 mg), **2** (2.5 mg), and **3** (3.0 mg). The EtOH extract of sample 17 (229 mg) was separated by silica-gel OCC (Hexane-EtOAc, 0–100%), followed by HPLC (Hexane-AcOEt, Nucleosil 50–5) to afford **8** (43.3 mg), **9** (28.2 mg), and **11** (19.7 mg). The EtOH extract of sample 18 (336 mg) was separated by silica-gel OCC (Hexane-AcOEt, 0–100%), followed by HPLC (Hexane-AcOEt, Nucleosil 50–5) to afford **8** (103.1 mg), **9** (86.1 mg), and **10** (8.4 mg). The EtOAc extract of sample 19 (3.0 g) was separated by silica-gel OCC (Hexane-EtOAc, 0–100%), followed by HPLC

(Hexane-EtOAc, Nucleosil 50–5) to afford **6** (9.3 mg), **7** (1.6 mg), **12** (1.2 mg), **13** (4.8 mg), and **14** (5.6 mg).

5.6. 6β-Angeloyloxyfuranoeremophilan-15-oic acid (*1*)

Colorless gum; $[\alpha]_D^{18}$ −23.9 (*c* 1.03, EtOH); MS (CI) *m/z* 347 $[M+H]^+$, 287, 263, 247 (base); HRMS (CI) Obs. *m/z* 347.1855 $[M+H]^+$. Calcd. for $C_{20}H_{27}O_5$ 347.1858; FT-IR (KBr): 3500–2500, 1730, 1700, 1640 cm^{-1}; ^{13}C NMR (100 MHz, C_6D_6, 65 °C) δ 8.5 (C-13), 15.7 (C-4′), 19.1 (C-14), 20.7×2 (C-3, C-5′), 24.5 (C-2), 26.4 (C-1), 27.2 (C-9), 37.2 (C-10), 40.0 (C-5), 44.6 (C-4), 69.0 (C-6), 115.9 (C-7), 120.0 (C-11), 128.5 (C-2′), 138.2 (C-3′), 138.8 (C-12), 151.5 (C-8), 167.7 (C-1′), 180.2 (C-15); ^1H NMR (400 MHz, C_6D_6, 65 °C) δ 1.11 (2H, *m*, H-1), 1.21 (3H, *s*, H-14), 1.28 (2H, *m*, H-2), 1.75 (2H, *m*, H-3), 1.83 (3H, *s*, H-5′), 1.93 (3H, *d*, *J* = 7.0 Hz, H-4′), 1.98 (3H, *s*, H-13), 2.22 (1H, *dd*, *J* = 17.0, 4.0 Hz, H-9), 2.45 (1H, *m*, H-10), 2.57 (1H, *brs*, H-4), 2.66 (1H, *dd*, *J* = 17.0, 5.9 Hz, H-9), 5.75 (1H, *qq*, *J* = 7.0, 1.5 Hz, H-3′), 6.55 (1H, *s*, H-6), 6.94 (1H, *s*, H-12); ^{13}C NMR (100 MHz, C_6D_5N, 100 °C) δ 7.8 (C-13), 15.0 (C-4′), 18.3 (C-14), 19.9 (C-5′), 20.1 (C-3), 24.4 (C-2), 25.6 (C-1), 26.5 (C-9), 36.3 (C-10), 39.5 (C-5), 44.2 (C-4), 69.2 (C-6), 115.9 (C-7), 119.5 (C-11), 128.1 (C-2′), 136.7 (C-3′), 138.2 (C-12), 151.4 (C-8), 167.3 (C-1′), 175.7 (C-15); ^1H NMR (400 MHz, C_6D_5N, 100 °C) δ 1.21 (2H, *m*, H-1), 1.46 (3H, *s*, H-14), 1.68 (2H, *m*, H-2), 1.92 (2H, *m*, H-3), 1.99 (3H, *s*, H-5′), 2.05 (3H, *d*, *J* = 7.0 Hz, H-4′), 2.11 (3H, *s*, H-13), 2.56 (1H, *dd*, *J* = 17.2, 5.5 Hz, H-9), 2.75 (1H, *m*, H-10), 2.78 (1H, *m*, H-4), 2.90 (1H, *dd*, *J* = 17.2, 7.0 Hz, H-9), 6.03 (1H, *q*, *J* = 7.0 Hz, H-3′), 6.75 (1H, *s*, H-6), 7.29 (1H, *s*, H-12).

5.7. 11βH-6β-Angeloyloxy-15-carboxyeremophil-7-en-12,8-olide (eremofarfugin D) (*13*)

Colorless gum; $[\alpha]_D^{22}$ +1.4 (*c* 0.48, EtOH); MS (CI) *m/z* 363 $[M+H]^+$, 303, 263 (base), 218, 169, 101; HRMS (CI) Obs. *m/z* 363.1805 $[M+H]^+$. Calcd. for $C_{20}H_{27}O_6$ 363.1808; FT-IR (KBr): 3500–3000 (br), 1800, 1730, 1700, 1640 cm^{-1}; ^{13}C NMR (100 MHz, C_6D_6, 60 °C) δ 15.5 (C-13), 15.8 (C-4′), 18.7 (C-14), 20.5 (C-5′), 24.4, 24.8, 36.0, 39.3, 40.4 (C-11), 43.8, 69.3 (C-6), 112.5 (C-7), 128.2 (C-2′), 139.4 (C-3′), 151.7 (C-8), 167.3 (C-1′), 177.1 (C-15), 178.1 (C-12), two missing; ^1H NMR (400 MHz, C_6D_6, 60 °C) δ 1.09 (3H, *s*, H-14), 1.15 (3H, *d*, *J* = 7.3 Hz, H-13), 1.75 (3H, quintet, *J* = 1.4 Hz, H-5′), 1.91 (3H, *dq*, *J* = 7.3, 1.4 Hz, H-4′), 3.02 (1H, *m*, H-11), 5.72 (1H, *qq*, *J* = 7.3, 1.4 Hz, H-3′), 5.98 (1H, *m*, H-6), assignment of other protons was not feasible.

5.8. 11αH-6β-Angeloyloxy-15-carboxyeremophil-7-en-12,8-olide (eremofarfugin E) (*14*)

Colorless gum; $[\alpha]_D^{22}$ +32.3 (*c* 0.56, EtOH); MS (CI) *m/z* 363 $[M+H]^+$, 305, 263 (base), 223, 169, 101; HRMS (CI) Obs. *m/z* 363.1814 $[M+H]^+$. Calcd. for $C_{20}H_{27}O_6$ 363.1808; FT-IR (KBr): 3500–3000 (br), 1800, 1730, 1700, 1640 cm^{-1}; ^{13}C NMR (100 MHz, C_6D_6, 60 °C) δ 14.5 (C-13), 15.8 (C-4′), 18.7 (C-14), 20.6 (C-5′), 24.4, 35.8, 39.0, 39.6 (C-11), 43.6, 68.8 (C-6), 113.5 (C-7), 128.2 (C-2′), 138.7 (C-3′), 151.6 (C-8), 167.5 (C-1′), 177.1 (C-15), 178.2 (C-12), three missing; ^1H NMR (400 MHz, C_6D_6, 60 °C) δ 1.09 (3H, *s*, H-14), 1.37 (3H, *d*, *J* = 7.3 Hz, H-13), 1.75 (3H, quintet, *J* = 1.4 Hz, H-5′), 1.87 (3H, *dq*, *J* = 7.3, 1.4 Hz, H-4′), 3.04 (1H, *m*, H-11), 5.71 (1H, *qq*, *J* = 7.3, 1.4 Hz, H-3′), 5.78 (1H, *m*, H-6), assignment of other protons was not feasible.

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References

Bhacca, N.S., Williams, D.H., 1964. Applications of NMR Spectroscopy in Organic Chemistry. Illustrations From the Steroid Field. Holden-day, Inc., San Francisco.

Bohlmann, F., Grenz, M., 1979. Ein neues Furanoeremophilan-Derivat aus *Ligularia macrophylla*. Phytochemistry 18, 491–492.

Bohlmann, F., Zdero, C., 1980. New furanoeremophilane derivatives from *Ligularia thyrsoidea*. Phytochemistry 19, 1550–1551.

Bohlmann, F., Jakupovic, J., Warning, U., Grenz, M., Chau-Thi, T.V., King, R.M., Robinson, H., 1986. Sesquiterpenes with new carbon skeletons Furoeremophilanes, secoeremophilanes, and other constituents from Argentinian *Senecio* species. Bull. Soc. Chim. Belg. 95, 707–736.

Choler, P., Erschbamer, B., Tribsch, A., Gielly, L., Taberlet, P., 2004. Genetic introgression as a potential to widen a species' niche: insights from alpine *Carex curvula*. Proc. Natl. Acad. Sci. USA 101, 171–176.

Hanai, R., Gong, X., Tori, M., Kondo, S., Otose, K., Okamoto, Y., Nishihama, T., Murota, A., Shen, Y., Wu, S., Kuroda, C., 2005. Chemical and genetic study of *Ligularia tongolensis*, *Ligularia cymbulifera*, and *Ligularia atroviolacea* in the Hengduan mountains of China. Bull. Chem. Soc. Jpn. 78, 1302–1308.

Ishii, H., Tozyo, T., Minato, H., 1965. Sesquiterpenoids – IX. Structure of ligularol and ligularone from *Ligularia sibirica*. Tetrahedron 21, 2605–2610.

Ishizaki, Y., Tanahashi, Y., Tsuyuki, T., Takahashi, T., Tori, K., 1979. Furanoeremophilan-14β,6α-olide. A new furanosesquiterpene lactone of an eremophilane-type from *Ligularia hodgsoni* Hook, f. Bull. Chem. Soc. Jpn. 52, 1182–1186.

Koch, M.A., Dobeš, C., Mitchell-Olds, T., 2003. Multiple hybrid formation in natural populations: concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). Mol. Biol. Evol. 20, 338–350.

Krepinsky, L., Motl, O., Dolejs, L., Novotny, L., Herout, V., Bates, R.B., 1968. Terpenes. CXCIV. The structure of eremophilene, the sesquiterpenic hydrocarbon from *Petasites* genus. Tetrahedron Lett., 3315–3318.

Kuroda, C., Nishio, E., 2007. Substituent effect in color of Ehrlich's test of tetrahydrobenzofuran. Nat. Prod. Commun. 2, 581–585.

Kuroda, C., Murae, T., Tada, M., Nagano, H., Tsuyuki, T., Takahashi, T., 1982. 3β,6β-Bis(acyloxy)furanoeremophilan-14-als and -14-oic acids from *Syneilesis palmata* (THUNB) MAXIM. Bull. Chem. Soc. Jpn. 55, 1228–1233.

Kuroda, C., Ueshino, T., Nagano, H., 2004. Ehrlich's reaction of furanoeremophilanes. Bull. Chem. Soc. Jpn. 77, 1737–1740.

Kuroda, C., Kiuchi, K., Torihata, A., Takeshita, K., Gong, X., Shen, Y., Hirota, H., Onuki, H., Hanai, R., 2007. Chemical and genetic diversity of Ligularia latihastata and Ligularia villosa in Yunnan Province of China. Chem. Biodiv. 4, 2210–2217.

Li, Y., Luo, S., Zhang, M., Chen, J., Wang, Z., 2001. Constituents of *Ligularia vellerea* (Franch.) Hand-Mass. China J. Chin. Mat. Med. 26, 835–837.

Li, Y., Wang, Z., Luo, S., Li, S., Zhu, D., 2006. Sensitized photooxidation of furanoeremophilane with singlet oxygen and their biogenetic relationship. Nat. Prod. Res. Part A 20, 724–730.

Liu, S.-W., 1989. Flora Reipublicae Popularis Sinicae, vol. 77. Science Press, Beijing.

Liu, S.-W., Deng, D.-S., Liu, J.-Q., 1994. The origin, evolution and distribution of *Ligularia* Cass. (Compositae). Acta Phytotaxonom. Sin. 32, 514–524.

Moriyama, Y., Takahashi, T., 1977. Skeletal rearrangement of furanoeremophilane-6β,10β-diol into farfugin A, farfugin B, and 6-[(4R)-chloropentyl]-3,5-dimethylbenzofuran. Bull. Chem. Soc. Jpn. 50, 463–465.

Nagano, H., Kuroda, C., Moriyama, Y., Tsuyuki, T., Takahashi, T., 1982. Mass spectra of furanoeremophilane derivatives. Bull. Chem. Soc. Jpn. 55, 1221–1227.

Nagano, H., Iwazaki, Y., Gong, X., Shen, Y., Kuroda, C., Hanai, R., 2006. Chemical and genetic differentiation of *Ligularia pleurocaulis* in Northwestern Yunnan and Southwestern Sichuan Provinces of China. Bull. Chem. Soc. Jpn. 79, 300–304.

Naya, K., Takagi, I., Kawaguchi, Y., Asada, T., Hirose, Y., Shinoda, N., 1968. The structure of fukinone, a constituent of *Petasites japonicus* maxim. Tetrahedron 24, 5871–5879.

Naya, K., Nakagawa, M., Hayashi, M., Tsuji, K., Naito, M., 1971. Constituents of *Petasites japonicus* rhizomes. Tetrahedron Lett. 31, 2961–2964.

Okamoto, Y., Tachikawa, K., Matsuo, Y., Nakashima, K., Sono, M., Tori, M., Hanai, R., Kuroda, C., Gong, X., Shen, Y., 2006. In: 50th Symposium on the Chemistry of Terpenes, Essential Oils, and Aromatics. Yokohama, November 2006. Chemical Society of Japan, Tokyo. Abstracts, pp. 355–357.

Pu, S.-B., Xu, D.-R., Zhang, M., Zhou, H.-H., Wang, Z.-T., Yu, G.-D., 2004. Detection of hepatotoxic pyrrolizidine alkaloids in *Ligularia* Cass with LC/MSn. Yaoxue Xuebao Bianjiby 39, 831–835.

Savolainen, V., Mane, J.F., Douzery, E., Spichiger, R., 1994. Molecular phylogeny of families related to Celastrales based on rbcL 5′ flanking sequences. Mol. Phylogenet. Evol. 3, 27–37.

Soltis, D.E., Soltis, P.S., 1998. In: Soltis, P.S., Soltis, D.E., Doyle, J.J. (Eds.), Molecular Systematics of Plants, II DNA Sequencing. Kluwer Academic Publishers, Boston.

Tori, M., Kawahara, M., Sono, M., 1998. Eremophilane-type sesquiterpenes from fresh rhizomes of *Petasites japonicus*. Phytochemistry 47, 401–409.

Tori, M., Shiotani, Y., Tanaka, M., Nakashima, K., Sono, M., 2000. Eremofarfugin A and eremopetasitenin B3, two new eremophilanolides from *Farfugium japonicum*. Tetrahedron Lett. 41, 1797–1799.

Tori, M., Kume, M., Nakashima, K., Sono, M., Tanaka, M., 2005. Eremopetasitenin A3 and 8-Oxoeremophil-6-en-12-oic acid from *Petasites japonicus* ssp *giganteus*. Heterocycles 65, 887–891.

Tori, M., Honda, K., Nakamizo, H., Okamoto, Y., Sakaoku, M., Takaoka, S., Gong, X., Shen, Y., Kuroda, C., Hanai, R., 2006. Chemical constituents of *Ligularia virgaurea* and its diversity in southwestern Sichuan of China. Tetrahedron 62, 4988–4995.

Torihata, A., Hanai, R., Gong, X., Shen, Y., Kuroda, C., 2007. Chemical and genetic differentiation of *Ligularia tsangchanensis* in Yunnan and Sichuan Provinces of China. Chem. Biodiv. 4, 500–507.

Yamakawa, K., Satoh, T., 1979. Studies on terpenoids and related alicyclic compounds. XVIII. Stereoselective synthesis of (±)-furanofukinol and (±)-petasalbin. Chem. Pharm. Bull. 27, 1747–1752.

Zhao, Y., Shenk, D.J., Takahashi, S., Chappell, J., Coates, R.M., 2004. Eremophilane Sesquiterpenes from Capsidiol. J. Org. Chem. 69, 7428–7435.