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Diversity of Furanoeremophilanes in Major *Ligularia* Species in the Hengduan Mountains

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The chemical and genetic diversity of *Ligularia* species in the Hengduan Mountains area of China is reviewed. A hypothesis that the production of furanoeremophilanes is ecologically advantageous is proposed.

Keywords: *Ligularia*, Hengduan Mountains, Furanoeremophilanes, Sesquiterpenoids, ITS.

1. Introduction

Understanding the diversification and evolution of higher plants is a fundamental theme in natural science. For the past decade, we have been studying the diversity in *Ligularia* species growing in the northwestern Yunnan and the southwestern Sichuan Provinces of China, i.e., a part of the Hengduan Mountains. *Ligularia* Cass. belongs to the family Asteraceae (Compositae), tribe Senecioneae, and is a highly diversified genus, containing six sections and over 100 species [1]. The species of *Ligularia* in the Hengduan Mountains are diverse in appearance and occupy a great variety of habitats from streams to alpine meadows, ranging from 1,000 to 5,000 m in altitude. The Hengduan Mountains are considered to be the main center of their on-going evolution and diversification [2].

Ligularia species have also been studied with respect to secondary metabolites and many sesquiterpenoids have been isolated from them. The major class of sesquiterpenoids in *Ligularia* is the eremophilane type and some of them have a furan ring (furanoeremophilanes). During the 1960s and 1970s, many furanoeremophilanes were isolated from roots of Japanese *Ligularia* species by Takahashi's [3] and Minato's groups [4], and from European species by Bohlmann's group [5]. Following these reports, especially around 2000 and later, many related compounds were isolated from Chinese species as well (examples are listed in [6]; see also ref. [7]).

To understand plant diversity, it is essential to employ multiple disciplines, since the subject is complex. In addition to species identification based on morphology, we have chosen to combine analysis of DNA of evolutionarily neutral regions with analysis of the chemical composition of the root. Observation in the field gives us some information on ecology, which is also an important factor to understand diversity.

Furanoeremophilanes are a good index to describe chemical compositions of *Ligularia*. The presence of furano-sesquiterpenoids can be easily examined by Ehrlich's test on TLC [8], as trisubstituted furan compounds react with *p*-dimethylamino-benzaldehyde under acidic condition to show either a pink or purple color. Most furanoeremophilanes are positive to the test and their color depends on the substituent(s) [9]. By this method, a primary description of the chemical compounds can be made without isolation, and thus, the method is suitable for expeditious survey of chemical diversity. Since the test on TLC gives us only limited structural information, conventional chemical and spectral analyses are also carried out, when necessary.

Evolutionarily neutral DNA regions give us systematics information independent of the chemicals. We initially determined the nucleotide sequence of the *atpB-rbcL* intergenic region in the plastid genome. Although intra-specific sequence variations are observed in this region to varying degrees, the number of variations is limited. Therefore, we added the ITS1-5.8S-ITS2 (ITS) region to the analysis. The region consists of two internal transcribed sequences and the 5.8S RNA of the ribosomal RNA gene in the nuclear genome [10]. Because the nuclear genes are inherited from both parents, whereas the plastid genome is maternally inherited, and because the rRNA gene is present as tandem copies in the order of thousands in higher plants, copy variations are often observed within an individual. Base changes are detected as base multiplicity at the varied base sites when the DNA is amplified with polymerase chain reaction (PCR) and directly sequenced. Insertions/deletions (indels) are detected as a superposition of sequences with a register shift after the indel.

This review describes our recent results, focusing on the furanoeremophilane-producing species, since many of the major species of *Ligularia* produce furanoeremophilanes as the major constituents. We also propose a hypothesis that furanoeremophilane-producing plants have an ecological advantage over those of the same or related species that produce eremophilan-8-one derivatives, which compounds are considered to be the precursors of furanoeremophilanes [11].

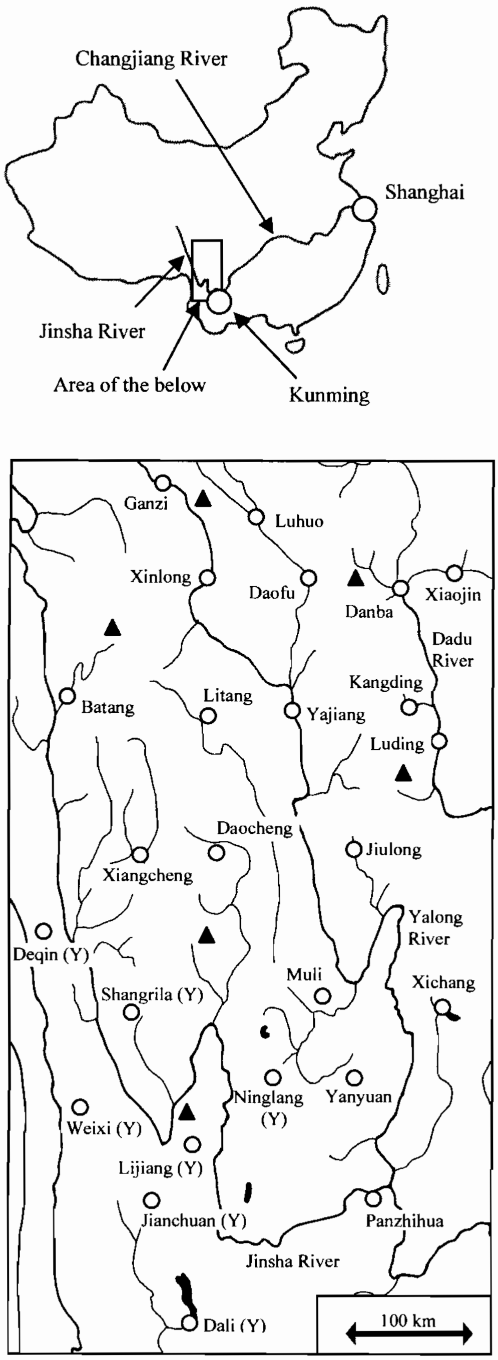


Figure 1: The area where samples of *Ligularia* species are collected. Lines indicate rivers. Open circles and filled triangles indicate major cities (Y = Yunnan Province, other cities belong to Sichuan Province) and peaks, respectively.

2. Field and plant materials

The field of our search is shown in Figure 1. We collect samples from northwestern Yunnan to southwestern Sichuan Provinces at about 2500-4500 m in elevation. Our field work was started in 2002. Fifty to 100 samples are collected every year, and their root chemicals and DNA sequences are analyzed. For Ehrlich's test, fresh root of each sample (2-10 g) is extracted with EtOH immediately without drying to avoid decomposition during handling. For structure determination, 10-200 g of dried root is extracted with either AcOEt or EtOH.

From a decade of observation in the field, we have found that most of the ecologically major *Ligularia* species produce furanoeremophilanes. Intra-specific diversity has been revealed in many species, to various degrees. For example, samples of some species, such as *L. pleurocaulis*, *L. virgaurea*, *L. vellerea*, and *L. tsangchanensis*, were grouped into two (or more) distinct subgroups; some species, such as *L. dictyoneura*, *L. kanaitzensis*, and *L. subspicata*, showed complex diversity; in contrast, *L. cymbulifera* and *L. cyathiceps* were homogeneous, both chemically and genetically. Results on the diversity in each species are described in the following.

3. Species consisting of distinct subgroups

3-1. *L. pleurocaulis* (Franch.) Hand.-Mazz. [12]

This species belongs to the section *Senecillis* and is well adapted to highlands of around 4000 m. It is distributed widely from northwestern Yunnan to northwestern Sichuan Provinces and sometimes makes a large colony. It is a polymorphous species in such characters as plant height, leaf width, and bract shape, but the morphological variations are continuous [1].

Ten samples were collected in northwestern Yunnan and southwestern Sichuan Provinces in 2003-2005 (Figure 2; squares without asterisk). TLC analysis showed that all samples were Ehrlich-positive, indicating the presence of furanoeremophilanes. Four compounds, **1-4**, were included as the common components in all samples. In addition, compound **5** and furanoligularenone **(6)** were obtained as the characteristic compounds of the Yunnan and Sichuan samples, respectively (Chart 1). This indicates that *L. pleurocaulis* can be classified into two groups on the basis of its chemical composition.

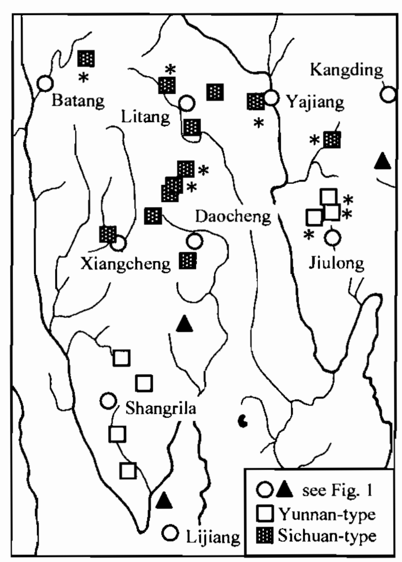


Figure 2: Locations where samples of *L. pleurocaulis* were collected. Asterisks indicate samples collected after publication of [12].

Base multiplicity in the ITS sequence was observed only at a few base sites in the samples collected in Yunnan, whereas it was observed at about 10 sites in those collected in Sichuan, suggesting that the plants in Sichuan are likely to be a hybrid of populations.

We obtained nine additional samples in Sichuan Province in 2007 after the publication of these results (Figure 2; asterisked samples). The samples collected in areas from Batang to Kangding Counties were of the Sichuan type, but those collected in Jiulong County

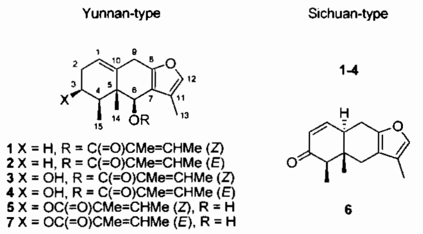


Chart 1: Isolated compounds from *L. pleurocaulis*.

were of the Yunnan type. In the latter, an additional furanoeremophilane, 3β-tigloyloxyfuranoeremophil-6β-ol (**7**), was present [13].

It has been reported that furanoligularenone (**6**) was obtained from *L. pleurocaulis* in Kangding County [6a], suggesting that the sample was of the Sichuan type. Therefore, it is likely that the Yunnan type is distributed in Yunnan Province and southern Sichuan Province, while the Sichuan type is distributed in the northern part of the distribution area of the species.

3-2. *L. virgaurea* (Maxim.) Mattf. [14]

This species is widely distributed in the Hengduan Mountains, including Yunnan, Sichuan, Qinghai, and Gansu Provinces in China, in Nepal, and in Bhutan. The plant belongs to section *Senecillis* and inhabits alpine meadows of more than 4000 m in altitude [1]. As far as we have observed in the field, this is the most abundant *Ligularia* species in western and northern Sichuan Province. Three varieties, *virgaurea*, *pilosa*, and *oligocephala*, have been proposed on the basis of morphological variations. In studies prior to ours, eremophilanolides and related compounds had been isolated [15].

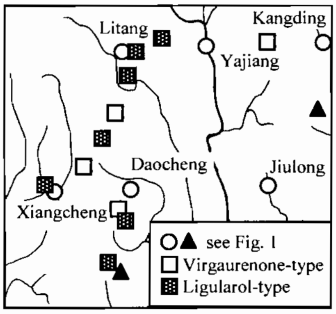


Figure 3: Locations where samples of *L. virgaurea* var. *virgaurea* were collected.

We collected eleven samples of *L. virgaurea* var. *virgaurea* in southwestern Sichuan Province (Figure 3). Ehrlich's test on TLC plates showed the presence of two chemo-types. Four samples showed two major components with yellow Ehrlich coloring. The compounds were identified as virgaurenones A (**8**) and B (**9**). Related enones, virgaurenones C (**10**) and D (**11**), as well as lactones **12** and **13**, were isolated as minor constituents (type 1; hereafter called virgaurenone type). The other seven samples showed several pink spots on TLC. The major components were ligularol (= petasalbin, **14**) and its derivatives such as its methyl ether **15**, ethyl ether **16**, and furanoeremophilane-6β,10β-diol (**17**) (type 2; hereafter called this ligularol type). Eight non-furan types of compounds **18-25** were also isolated from these samples, among which **18** and **20** had only 13 (dinor-eremophilane) and 14 carbons (nor-eremophilane), respectively. Virgaurenones A-D (**8-11**) were not found in the ligularol-type samples (Chart 2). Thus, although both types produce furanoeremophilanes, they are clearly different from each other.

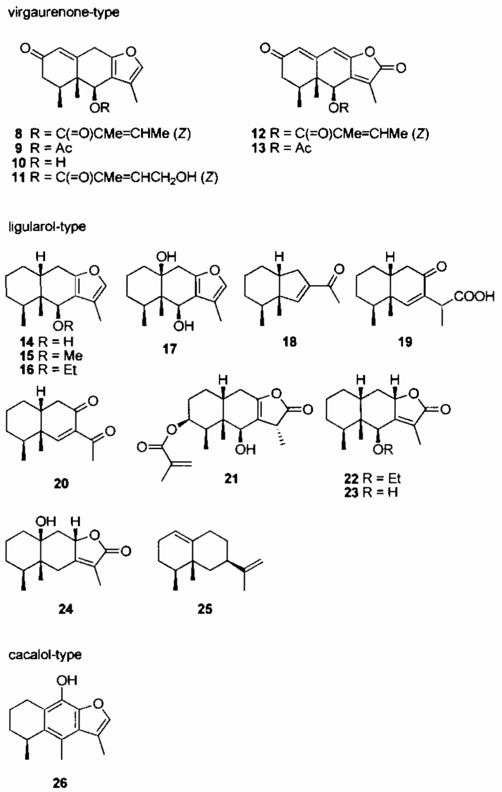


Chart 2: Isolated compounds from *L. virgaurea*.

The samples could also be grouped into two on the basis of the ITS sequences. The grouping agreed with that based on the chemical composition (the virgaurenone and the ligularol types). Thus, the chemical diversity in *L. virgaurea* appears to result from a difference(s) of a genetic character, but not from an environmental difference(s).

In contrast to *L. pleurocaulis*, the molecular characters of the *L. virgaurea* samples showed no geographic correlation, suggesting that geographic separation is not the direct cause of the differentiation.

Additional samples of *L. virgaurea* were collected in northwestern and southwestern Sichuan Province after the publication of the above results. Although most samples were of either the virgaurenone or ligularol type, a sample of a third type was found near Ganzi City (see Figure 1). From the sample, cacalol (**26**) was isolated as the major component (a cacalol type). Since cacalol had previously been isolated from *L. virgaurea* in Gansu [15b,c] and Qinghai [15d], this type may be distributed from northern Sichuan to Gansu/Qinghai. Details of our new results will be published elsewhere.

3-3. *L. vellerea* (Franch.) Hand.-Mazz. [16]

This species is one of the two belonging to the section *Scapicaulis*. The most distinct morphological feature of the species is the presence of dense white lanate hairs at the base of its stem and petioles. The plant is distributed in northwestern Yunnan and southwestern Sichuan Provinces of China, growing in meadows of various environments at relatively lower altitudes (2500-4000 m) [1]. In our observation in the field, the species is one of the most abundant *Ligularia* species near Shangrila (Zhongdian) City.

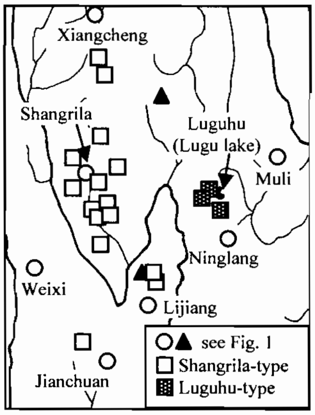


Figure 4: Locations where samples of *L. vellerea* were collected. One sample collected near Kunming (Kunming type) is not shown.

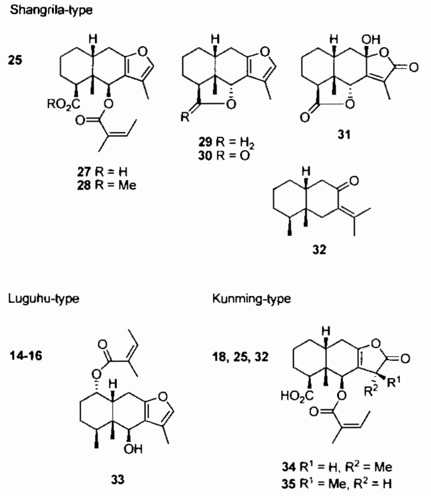


Chart 3: Isolated compounds from *L. vellerea*.

Nineteen samples of *L. vellerea* were collected in northwestern Yunnan Province and one, near Kunming, central Yunnan (Figure 4). On the basis of the chemical composition, the samples could be divided into three groups, a Shangrila type (Shangrila, Jianchuan, and Lijian areas), a Luguhu type (Luguhu area), and a Kunming type (Kunming area). These three types were described as types A-C in our original paper [16]. From the Shangrila-type samples, furanoeremophilanes **27-30** were isolated as the major components, together with three Ehrlich-negative compounds **25**, **31**, and **32**. From the Luguhu-type samples, furanoeremophilanes **33** and **14** were obtained as the major components, together with **15** and **16**. Although TLC of the extracts of fresh roots detected Ehrlich-positive compounds in the Kunming-type sample, only Ehrlich-negative compounds, **18**, **25**, **32**, **34**, and **35**, were obtained (Chart 3). A sample collected in the Jianchuan area showed a small orange-colored spot indicative of a 6-oxofuranoeremophilane derivative [9], although it could not be isolated. The location of this sample was geographically separated from the other Shangrila-type samples. While the Shangrila and the Kunming types were somewhat similar in their chemical composition, the Luguhu type was clearly different from the other two types, with no common compound.

Correlation was seen between the chemical composition and the ITS sequence. The ITS2 of the Luguhu-type and the Kunming-type samples contained indels, whereas those of the Shangrila-type samples did not. The presence of multiple sequences is considered to indicate hybridization in the recent past [17].

The number of sites with multiple bases in ITSs was not the same among the samples from the Shangrila area. All the samples but one from the central region (Xiaozhongdian area) did not contain such a site, while the samples from the outer regions contained some. This suggested that introgression has occurred more frequently in the outer regions. In our observation in the field, *L. vellerea* is very abundant in the Xiaozhongdian area, the distribution center of the species. Samples collected from this place were almost uniform, both in the chemical composition and in the DNA sequence, with few exceptions. This observation may indicate that the area offers an environment more suitable to *L. vellerea*.

3-4. *L. tsangchanensis* (Franch.) Hand.-Mazz. [18]

This species belongs to the section *Ligularia*, series *Racemiferae*, and is widely distributed in southeastern Tibet, southwestern Sichuan, and northwestern to northeastern Yunnan Provinces, growing in grasslands, alpine meadows, and forest understories of 3000-4000 m in altitude [1].

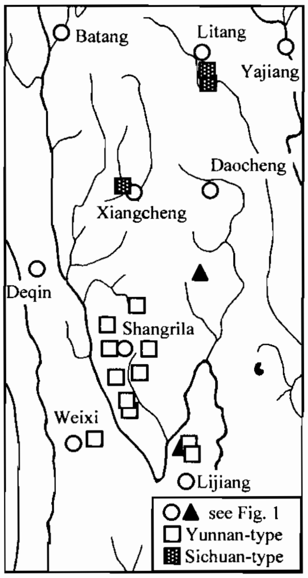


Figure 5: Locations where samples of *L. tsangchanensis* were collected. One sample collected near Kunming is not shown.

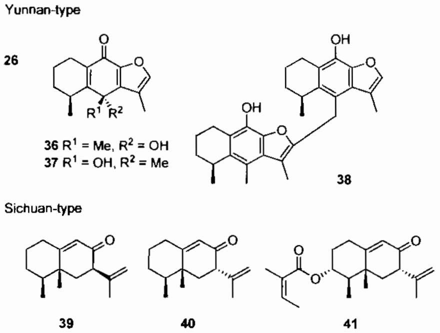


Chart 4: Isolated compounds from *L. tsangchanensis*.

Fifteen samples were collected in the northwestern Yunnan and southwestern Sichuan areas (Figure 5). Eight of these samples were collected near Shangrila City. All the samples collected in Yunnan Province showed one strong Ehrlich-positive spot with a dark blue color. The compound was found to be cacalol (**26**), which constituted about 10% of the extract. Related compounds, cacalone (**36**), epicacalone (**37**), and a cacalol dimer (= adenostin A, **38**) were also obtained. In contrast, the three samples collected in Sichuan were negative to Ehrlich's test. From them, eremophila-9,11-dien-8-one derivatives **39-41** were isolated as the major components (Chart 4). These results suggested that the *L. tsangchanensis* samples could be grouped into two types, a Yunnan type and a Sichuan type.

The analysis of the ITS sequence indicated that the Yunnan and the Sichuan samples were unambiguously separated. These chemical and genetic results indicate that our *L. tsangchanensis* samples can be grouped into two. The grouping agreed with geographical separation.

It is noteworthy that all the Yunnan samples of *L. tsangchanensis* produced cacalol (**26**) as the sole major component. Cacalol has a carbon skeleton rearranged from furanoeremophilane [19], while the eremophilan-8-one derivatives, the major components of the Sichuan samples, are a precursor of furanoeremophilane [11]. This point will be discussed in section 8.

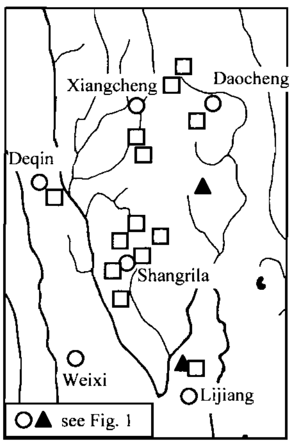
4. Homogeneous species

4-1. *L. cymbulifera* (W. W. Smith) Hand.-Mazz. [20]

This species belongs to the section *Corymbosae* and occupies a great variety of habitats ranging from 2000 to 4000 m in altitude in Yunnan and Sichuan Provinces [1]. As far as we have observed in the field, *L. cymbulifera* is the most ecologically advantageous species near Shangrila City, forming large colonies.

Thirteen samples were collected in northwestern Yunnan and southwestern Sichuan Provinces (Figure 6). All the samples contained the same Ehrlich-positive compounds. Four furanoeremophilanes **42-45** were isolated, together with **46** (Chart 5). Tetradymol (**45**) was the most abundant component, consisting of about one third of the extract [21].

Figure 6: Locations where samples of *L. cymbulifera* were collected.



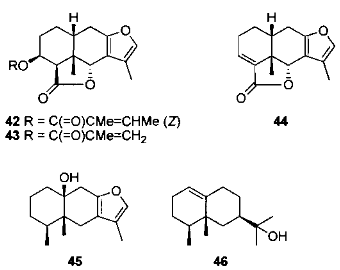


Chart 5: Isolated compounds from *L. cymbulifera*.

Although analysis of the ITS sequence remains to be carried out, the *atpB-rbcL* sequence was the same in all the *L. cymbulifera* samples, indicating little genetic diversity in this species.

4-2. *L. cyathiceps* Hand.-Mazz. [22]

This species, belonging to the section *Ligularia*, grows in a variety of habitats including stream banks, valleys, and grassy slopes in northwestern Yunnan Province, China [1].

Eleven samples were collected (Figure 7). From the samples, cacalol (**26**) was isolated as the major component, together with cacalone (**36**), epicacalone (**37**), eremophilanes **47-59**, **25**, and a eudesmane **60** (Chart 6). Cinnamyl alcohol derivatives were also included. Compounds **36**, **37**, and **47-53** are presumed to be highly related to cacalol (**26**) with respect to their structures, as a biosynthetic relation between cacalol and a 6-acyloxy-9-oxofuranoeremophilane derivative has been suggested [19]. Coincidental isolation of cacalol and 9-oxygenated furanoeremophilane(s) is not rare. Intra-specific variation was limited to the composition of minor components.

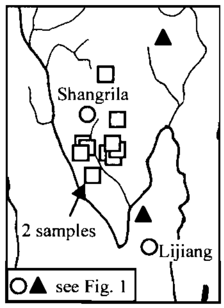


Figure 7: Locations where samples of *L. cyathiceps* were collected.

Variation in the ITS sequence was found to be limited. These similarities in the chemical composition and in the DNA sequences among the samples may be a result of limitation of our collection locality to Shangrila County. The plant has also been found in Deqin and Gongshan Counties of Yunnan Province [1].

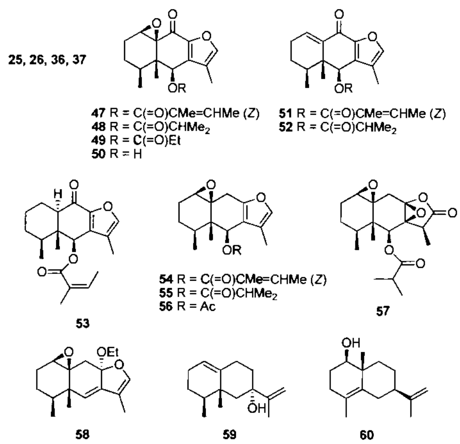


Chart 6: Isolated compounds from *L. cyathiceps*.

5. Highly diversified species

5-1. *L. dictyoneura* (Franch.) Hand.-Mazz. [23]

This species belongs to the section *Senecillis*, growing on grassy slopes and in forest understories of 2000-3500 m in altitude in northwestern Yunnan and southwestern Sichuan [1].

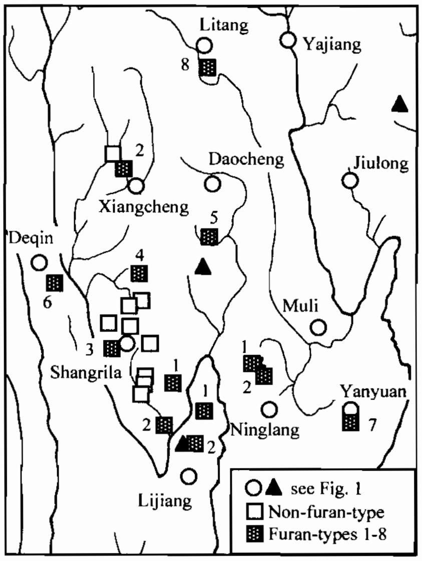


Figure 8: Locations where samples of *L. dictyoneura* were collected.

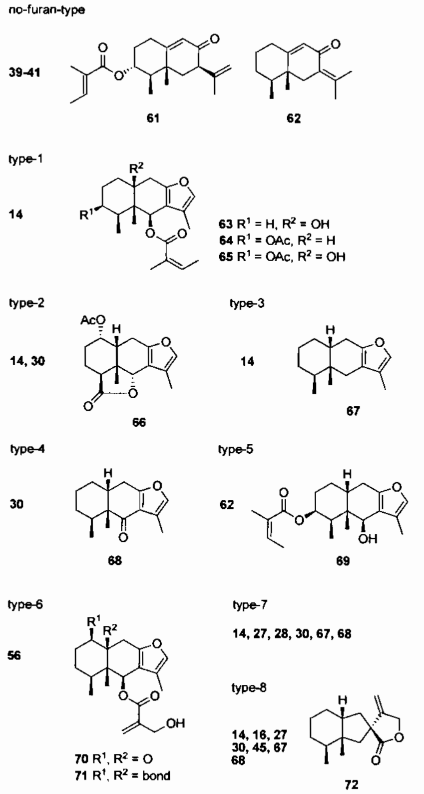


Chart 7: Isolated compounds from *L. dictyoneura*.

Twenty samples were collected in 2003-2006 (Figure 8, samples except for types 7 and 8, see below). About half of the samples were Ehrlich-positive. Most samples collected near Shangrila City were Ehrlich-negative, with one exception (type 3), while the samples obtained from the other places were mostly Ehrlich-positive. From Ehrlich-negative samples, eremophilan-8-one derivatives **39-41**, **61**, and **62** were isolated (Chart 7). The Ehrlich-positive samples were grouped into six (types 1-6) on the basis of the chemical composition. Furanoeremophilanes were obtained from them, namely, **14** and **63-65** from type 1 samples; **14**, **30**, and **66** from type 2; **14** and **67** from type 3; **30** and **68** from type 4; **62** and **69** from type 5; and **56**, **70**, and **71** from type 6 (Chart 7 and Figure 8).

We obtained two additional Ehrlich-positive samples in 2007 in Sichuan Province after the publication of the above results. From a sample collected in Yanyuan County, furanoeremophilanes **14**, **27**, **28**, **30**, **67**, and **68** were isolated (type 7) [13]. The other sample, collected near Litang, contained **16**, tetradymol (**45**), and bakkenolide A (**72**) in addition to the compounds obtained from the Yanyuan sample (type 8) (Chart 7 and Figure 8). The presence of two additional types indicated that the species is even more diverse.

Variations were also observed in the ITS sequence, although the whole sequence could be only partially determined in many samples because of indels. There were two types of the *atpB-rbcL* sequence. These data indicated that the intraspecific diversity in *L. dictyoneura* was extremely high in comparison with the other *Ligularia* species described above. Eight samples collected in the Shangrila area contained no furano-compounds and the samples had one type of the *atpB-rbcL* sequence, and thus, they appeared to be similar both chemically and genetically. In contrast, the distribution of furanoeremophilane-producing samples was complex. For instance, type-1 and type-2 samples were found in distant areas, but in a mixed manner. The ITS sequence was dissimilar among the type-1 samples. It is also noteworthy that a sample collected near Deqin (type 6) had not only its own chemical composition, but as many as 8 base substitutions in the ITS1-5.8S-ITS2 region. The sample was collected at an isolated location at very high altitude.

The presence of multiple ITS sequences within individuals suggests past hybridization. The difference between the two types of the *atpB-rbcL* sequence concerned four base sites and no intermediate type was observed. Thus, the high diversity in *L. dictyoneura* may have been brought about by hybridization of two differentiated populations.

5-2. *L. kanaitzensis* (Franch.) Hand.-Mazz. [24]

This species belongs to the section *Ligularia*, series *Racemiferae*, and inhabits grassy slopes, swamps, and scrub lands at around 3000 m in altitude. The species is divided into two varieties, var. *kanaitzensis* and var. *subnudicaulis* (Hand.-Mazz.) S. W. Liu, based on morphological differences such as the shape of the foliose bract [1]. However, in our observation, the difference was not distinct but continuous. Therefore, we did not distinguish the two varieties.

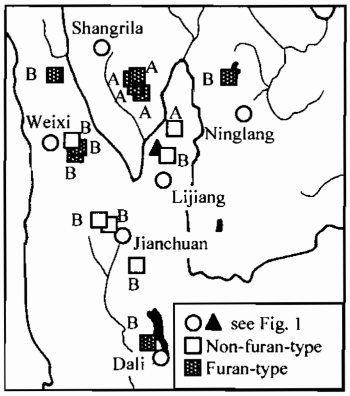


Figure 9: Locations where samples of *L. kanaitzensis* were collected. A and B indicate the sequence types of the ITS region.

Fifteen samples were collected in northwestern Yunnan Province. Ehrlich's test on TLC showed that the samples could be grouped into two on the basis of the presence/absence of furanoeremophilanes (Figure 9). Thirty-one compounds were isolated from the samples (Chart 8, a cinnamyl alcohol derivative is

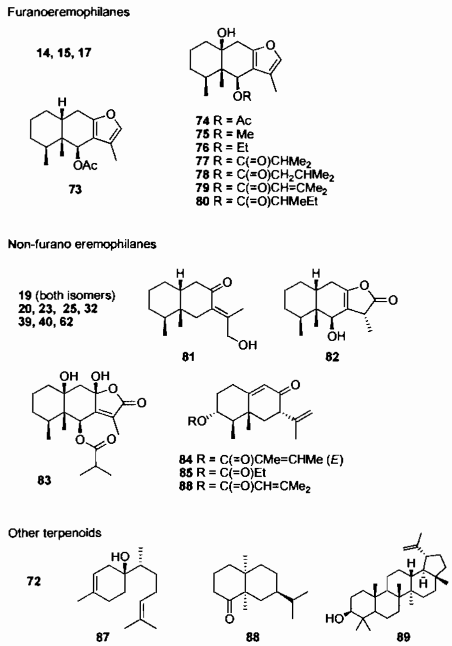


Chart 8: Isolated compounds from *L. kanaitzensis*.

not shown). Eleven of them, **14**, **15**, **17**, and **73-80**, were furanoeremophilanes. Compounds **19**, **20**, **23**, **25**, **32**, **39**, **40**, **62**, and **81-86** were eremophilanes with no furan ring. Sesquiterpenes with other carbon skeletons, including **87**, **88**, and bakkenolide A (**72**), were also isolated, as well as a triterpene (lupeol; **89**). The chemical spectrum of eremophilane-type sesquiterpenoids in the samples appeared more or less continuous. In four samples, all the isolated compounds were non-furano eremophilanes. Two other samples contained similar compounds, together with a small amount of furanoeremophilane(s). These six samples are designated as the non-furan type in Figure 9. In contrast, nine samples contained furanoeremophilanes as major components (the furan type).

The ITS sequences clearly separated the samples into two groups. One consisted of five samples (type A) and the other, the rest of the samples (type B) (Figure 9). Multiple sequences were present in many samples, suggesting past hybridization. In addition, the sequence variation appeared as large as would be seen between species.

Among the isolated compounds, kanaitzensol (**81**), isolated from a sample near Lijiang City, is especially noteworthy. Compound **81** is a geometrical isomer of the proposed intermediate from eremophil-7(11)-en-8-one to furanoeremophilane with respect to the 7(11)-double bond [11]. Therefore, **81** would not form a furanoeremophilane because of geometric mismatch, and in fact, no furan was isolated from the sample.

5-3. *L. subspicata* (Bureau & Franch.) Hand.-Mazz. and *L. lamarum* (Diels) C. C. Chang [25][26]

These species belongs to the section *Ligularia*, series *Ligularia*. The difference between the two is only in the presence (*L. lamarum*) or absence (*L. subspicata*) of ligulate florets and in the color of pappi [1]. The two species occupy similar habitats, such as swamps, scrub, and forest understories at around 3000-5000 m in altitude, and we have found them intermingling at some locations.

Samples of *L. lamarum* and *L. subspicata* were collected in Yunnan and Sichuan Provinces (Figure 10). Some samples of the two species were sympatric at five locations.

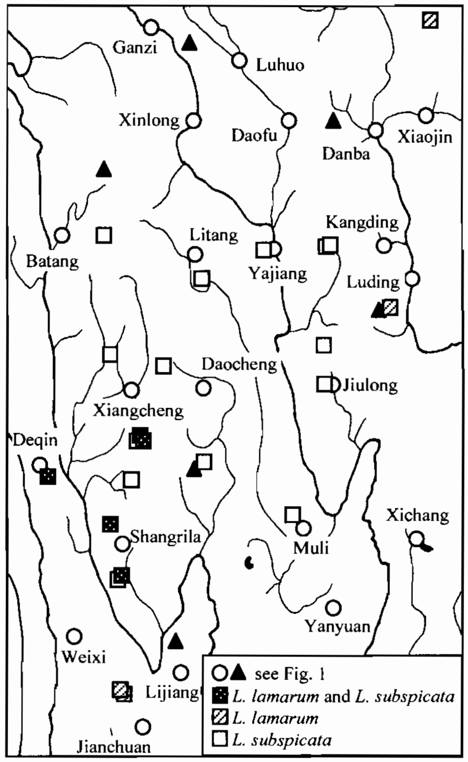


Figure 10: Locations where samples of *L. lamarum* and *L. subspicata* were collected.

Furanoeremophilanes were isolated from Ehrlich-positive samples, and eremophilan-8-one derivatives from the Ehrlich-negative ones. Many compounds were isolated (Chart 9), and the compounds were of four categories and mostly eremophilanes: (1) subspicatins (1-acyloxyfuranoeremophilane derivatives) **90-96**; (2) 6,10-dihydroxyfuranoeremophilane derivatives **14-17**, **45**, **73-77**, **80**, **97-101** and their oxidized compounds **23**, **82**, **102**, and **103**; (3) eremophilan-8-one derivatives **19**, **20**, **25**, **32**, **39-41**, **62**, **84**, **86**, and **104-116**; (4) compounds having other carbon skeletons, **72** and **117-119**. Subspicatins were new compounds characteristic of *L. lamarum* and *L. subspicata*, but related compounds were subsequently isolated from *Parasenecio petasitoides* [27].

Each species was found to harbor chemical diversity with a continuous chemical spectrum. The chemical spectra of the two species were overlapping. The ITS sequence failed to distinguish the two species. Thus, the two species were indistinguishable either chemically or genetically.

6. Other furanoeremophilane-producing species

L. hodgsonii Hook. is distributed not only in China, but also in Japan. We collected several samples in Japan and found that they were uniform both in the root chemicals and in the ITS sequence. In contrast, one sample collected in Yunnan, China, was chemically and genetically different from the Japanese samples. Furanoeremophilanes were isolated from both the Japanese and the Chinese samples; namely, compounds **27** and **30** were isolated from the Japanese samples, and **120** and **121**, from the Chinese sample [28]. Compound **121** was also isolated from a sample of *L. oligonema* Hand.-Mazz., together with its derivative **122** [29] (Chart 10).

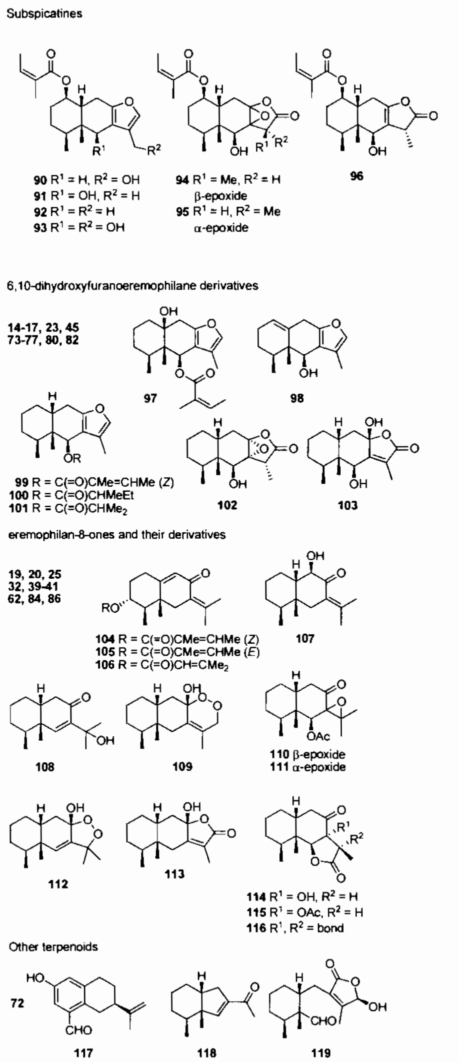


Chart 9: Isolated compounds from *L. lamarum* and *L. subspicata*.

From two samples of *L. anoleuca* Hand.-Mazz., three furanoeremophilanes **70**, **71**, and **123** were obtained [30]. From *L. franchetiana* (Lével.) Hand.-Mass., **25** and **124** were isolated together with a bakkane-type spirolactone **125** [31]. (Chart 10).

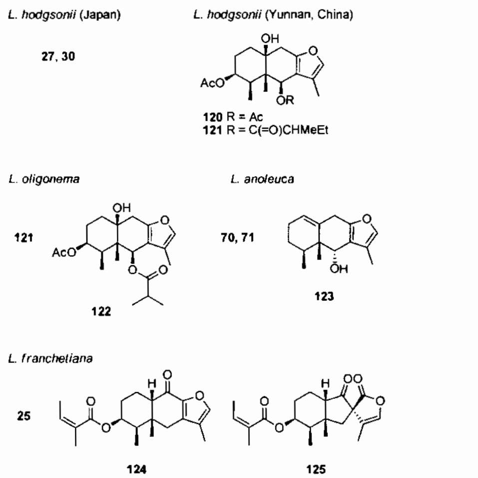


Chart 10: Isolated compounds from *L. hodgsonii*, *L. oligonema*, *L. franchetiana*, and *L. anoleuca*.

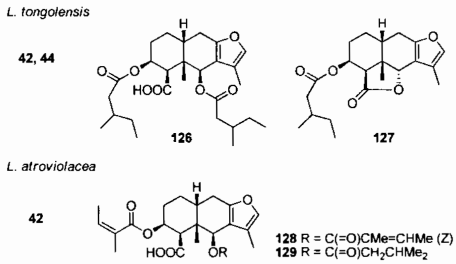


Chart 11: Isolated compounds from *L. tongolensis* and *L. atroviolacea*.

The chemical compositions of *L. tongolensis* (Franch.) Hand.-Mazz. and *L. atroviolacea* (Franch.) Hand.-Mazz. were similar to those of *L. cymbulifera* and *L. vellerea* with 15-oxidized furanoeremophilanes, **42**, **44**, and **126-129**, as the major constituents [20] (Chart 11).

7. *Ligularia* species in the Hengduan Mountains producing different types of compounds

Although many major *Ligularia* species collected in the Hengduan Mountains area were found to produce eremophilane-type sesquiterpenoids, some species were not. *L. latihastata* and *L. villosa* produce euparin-type benzofuran derivatives instead of eremophilanes [32]. Related compounds were isolated from *L. veitchiana* [30]. *L. lankongensis* was found to produce bisabolane-type sesquiterpenoids [33], which were also isolated from a sample of *L. duciformis* [34]. It had been reported that *L. duciformis* and its related species produce cinnamyl alcohol derivatives [35]. We recently found that there were also minor populations producing sesquiterpenoids. These results indicate that some different lineages other than eremophilane-producing species are present.

8. Furanoeremophilanes and ecology

As reviewed above, most of the major *Ligularia* species in the Hengduan Mountains area produce eremophilane-type sesquiterpenoids, among which furanoeremophilanes are the major class of compounds. Although the physiological role(s) of the compounds is not known, the plants producing furanoeremophilanes were more abundant than those of the same species producing only eremophilan-8-one derivatives. For example, the furanoeremophilane-producing type of *L. dictyoneura* near Shangrila city (type 3; Figure 8) was very abundant, growing among scrub on one whole hill. Considering a hypothesis that the furan ring in furanoeremophilanes is derived from an eremophil-7(11)-en-8-one derivative [11], the type-3 population may have evolved by acquiring the ability to produce furanoeremophilane from some other plant. In *L. kanaitzensis*, populations that produce furanoeremophilane derivatives were occupying predominant niches, whereas the other populations of the plant were intermingled with other plants. In particular, four samples collected in the southeast of Shangrila City (Annan area, Figure 9) were homogenous both in their chemical composition (furanoeremophilane derivatives) and in their DNA sequence (type A); the plant was very abundant. These observations in the field allowed us to propose a hypothesis that furanoeremophilane-producing *Ligularia* species and populations have ecological advantages over eremophilan-8-one-producing *Ligularia*. It is likely that *L. kanaitzensis* of the Annan area achieved an advantage by the production of furanoeremophilanes.

In addition, as far as we have observed in the field, most of the abundant *Ligularia* species produce furanoeremophilanes. For example, *L. cymbulifera* was very abundant in northwestern Yunnan

Province, often forming large exclusive colonies in open fields. The major component of the species was tetradymol (**45**). *L. vellerea* was also abundant in the southern part of Shangrila city, producing 15-oxygenated furanoeremophilanes (**27**, **28**, and **30**) as the major components. *L. virgaurea* was the most abundant *Ligularia* species in western Sichuan Province, producing either virgaurenones (**8**, **9**) or ligularol (**14**) as the major component(s). Large colonies were also found both of the Yunnan- and the Sichuan-types of *L. pleurocaulis*. From these species, eremophilan-8-one derivatives were either not obtained or obtained only as minor components.

Cacalol (**26**) has a rearranged carbon skeleton and is considered to be generated from a furanoeremophilane derivative [19]. Cacalol was the major component of the Yunnan type of *L. tsangchanensis* and was also isolated from *L. cyathiceps*. The compound had been obtained not only from *Ligularia* [15], but also from species of other genera of Senecioneae, such as *Cacalia* [19,36], *Senecio* [37], *Psacalium* [38], *Othonna* [39], and *Euryops* [40] suggesting that the compound is a common terpenoid in Senecioneae. Cacalol is probably a compound that has reached an evolutionarily "stable point", although it is not clear whether the compound is more useful than furanoeremophilanes. This premise is consistent with the observation that *L. tsangchanensis* is distributed more widely in Yunnan Province than in Sichuan (Figure 5).

The results of DNA analysis showed past hybridization in many species, suggesting that the hybridization is one of the major pathways for the evolution of *Ligularia*. Reticulate evolution within and among *Ligularia* and related genera has been strongly suggested [41]. The ability to produce furanoeremophilanes may have been acquired by hybridization, which evolved plants from a non-furan to a furan type. Eight different types of furanoeremophilane-producing *L. dictyoneura* may be a result of hybridization. The occurrence of cacalol both in *L. tsangchanensis* and *L. cyathiceps* may not be a mere coincidence.

9. Conclusion

A decade of our continuing study on *Ligularia* in the Hengduan Mountains area has demonstrated the presence of intra-specific diversity in many *Ligularia* species. Production of furanoeremophilanes is proposed to be ecologically advantageous over production of eremophilan-8-ones. Past hybridization was detected in many species, suggesting that hybridization is a major pathway of evolution, including that from a non-furan to a furan type. In order to understand the evolution of the whole *Ligularia* genus, further search in the field of *Ligularia* and closely related genera, i.e. *Cremanthodium*, is necessary.

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