

Comparative Morphology of the Leaf Epidermis in *Ligusticum* (Apiaceae) from China

Jing Zhou^{1*}, Zhenwen Liu²

¹School of Pharmaceutical Sciences & Yunnan Key Laboratory of Pharmacology for Nature Products, Kunming Medical University, Kunming, China

²Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China

Email: *zhoujing_apiaceae@163.com

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Abstract

To investigate the taxonomic value of leaf epidermal characters in developing a classification for *Ligusticum* (Apiaceae), thirty-eight species (including a cultivar) of the genus were examined using both light and scanning electron microscopy. Our results show that the infrageneric classification of *Ligusticum* based on the characters of involucre bracteoles, mericarps, and pollen grains was not supported as few epidermal characters were relatively constant at section level. Evidence from leaf epidermis, similar to that from molecular and morphological data, supports the separation of *Tilingia* (*i.e.* *L. ajanense* and *L. tachiroei*) from *Ligusticum*. *Ligusticopsis* was found to be indistinguishable from *Ligusticum* in epidermal characters. Furthermore, results from epidermal analysis, together with that from cytological evidence, fruit morphology as well as molecular phylogenetics suggest that *L. delavayi* should be transferred out of *Ligusticum*.

Keywords

Leaf Epidermis, *Ligusticum*, Taxonomy, Umbelliferae (Apiaceae)

1. Introduction

The genus *Ligusticum* L., referred to the family Apiaceae, comprises 60 species widely distributed throughout Eurasia and North America. Of them, 40 species are known from China (of which 35 are being endemic), and most of these are distributed throughout the alpine belt of southwestern China (sub-alpine coniferous forests and forest edges, alpine scrub or meadows), with only a few species extending into northern China [1] [2]. *Ligusticum* exhibits a great diversity in

the macro-morphological characters of leaf blades, bracteoles and mericarps, and is therefore one of the most difficult genera in the family Apiaceae for species delimitation, and the circumscription of *Ligusticum* has varied considerably [3] [4].

Drude [3] established three subgenera of *Haloscia*, *Euligusticum* and *Pachypleurum* in *Ligusticum*; Subsequently, Thellung [5], Schischkin [6] and Leute [7] added subgenus *Mutellina*, *Pachypleuroides* and *Coristospermum* successively. Even now, its relationships with putatively allied genera *Cnidium* Cuss., *Hymenidium* DC., *Pachypleurum* Ledeb., *Paraligusticum* Tichom., *Rupiphila* Pimenov & Lavrova, *Selinum* L., *Tilingia* Regel & Tiling, and *Ligusticopsis* Leute are still not fully resolved [2]. The inclusion of *Tilingia* and *Ligusticopsis* within *Ligusticum* is particularly controversial [8] [9] [10]. Pu [1] arranged the species of Chinese *Ligusticum* into two sections: *L.* section *Ligusticum* L. and *L.* section *Pinnatibracteola* Pu based on the characters of involucre bracteoles, mericarps, the shape and aperture types of pollen grains. Doubt has already been cast on the rationality of this sectional system by our previous cytological study [11]. Many phylogenetic analyses on the Apiaceae family level have suggested that *Ligusticum* is a heterogeneous group as currently circumscribed [12]-[17], and that the Chinese *Ligusticum* species fall into three distant clades [18] [19]. Therefore, more morphological evidence is necessary before there can be further taxonomical revision of *Ligusticum*.

Leaf epidermal characteristics are of potential taxonomical importance [20] [21], of which features such as epidermal cells, types and arrangement of stomata, and surface ornamentation have been repeatedly proved well for taxonomy [22] [23] [24]. Sun *et al.* [25] have investigated the leaf epidermis morphology of Chinese species of *Ligusticum*, but this investigation was not comprehensive, with only 15 species examined. Thus, a more detailed observation of leaf epidermal morphology in the Chinese species of *Ligusticum*, through extensive sampling of taxa, is necessary. As part of a systemic study of this genus in China, we aim to investigate the epidermal features of 38 Chinese *Ligusticum* species, assess whether these epidermal features can provide information of taxonomic value and determine whether such information supports a sectional subdivision system in Chinese *Ligusticum*.

2. Materials and Methods

Plant Materials

For this study, thirty-six species of *Ligusticum* recognized in Flora of China (FOC) were investigated. Furthermore, two formally published species, *L. moniliforme* Peng & Zhang and *L. pseudodaucooides* Peng & Yin, which are not included by FOC were also considered to see if their epidermal characters are consistent with the genus (Table 1). Except fresh material collected from the field, other samples were taken from herbarium specimens in the Kunming Institute of Botany (KUN) and the Chengdu Institute of Biology (CDBI) (Table 1). All

Table 1. Species, localities and vouchers for the leaf epidermal studies of *Ligusticum* (All specimens are deposited at KUN except for those labeled with CDBI).

Taxon	Locality	Voucher
Sect. <i>Ligusticum</i>		
* <i>L. acuminatum</i> Franch.	Maerkang, Sichuan	LZ1141
<i>L. ajanense</i> (Regel & Tiling) Koso-Poljansky	Mt. Shirane, Japan	Miyoshi Fususe 45568
* <i>L. angelicifolium</i> Franch.	Bomi, Xizang	Z15120
* <i>L. brachylobum</i> Franch.	Lijiang, Yunnan	Z1526
<i>L. delavayi</i> Franch.	Heqing, Yunnan	LZ151006
<i>L. discolor</i> Ledeb.	Yili, Xinjiang	Zhangdy371
<i>L. elatum</i> (Edgew.) C. B. Clarke	Zuogong, Xizang	SunH-07ZX-1135
<i>L. jeholense</i> (Nakai & Kitagawa) Nakai & Kitagawa	Wanping, Hebei	YangCG 1691
<i>L. kingdon-wardii</i> H. Wolff	Xiangcheng, Sichuan	Group of Qingzang 4022 (CDBI)
<i>L. litangense</i> Pu	Litang, Sichuan	Z15157
<i>L. littledalei</i> Fedde ex H. Wolff	Chayu, Xizang	Z15109
<i>L. nematophyllum</i> (Pimenov & Kljuykov) Pu & Watson	Songpan, Sichuan	LZ1196
* <i>L. pteridophyllum</i> Franch.	Xiangcheng, Sichuan	Z15177
<i>L. reptans</i> (Diels) H. Wolff	Luding, Sichuan	XuGH 26178 (CDBI)
<i>L. sinense</i> Oliv.	Maerkang, Sichuan	LZ1169
<i>L. sinense</i> cv. <i>Chuanxiong</i> Qiu <i>et al.</i>	Guangnan, Yunnan	LZ20160607
<i>L. tachiroei</i> (Franch. & Savat.) Hiroe & Constance	Mt. Baihua, Beijing	L04
* <i>L. tenuisectum</i> H. de Boissieu	Mt. Jinfo, Chongqing	ZW20090917
<i>L. tenuissimum</i> H. de Boissieu	Neiqiu, Hebei	LiuXY 747
<i>L. thomsonii</i> C. B. Clarke	Yushu, Qinghai	LiuJq-QLS-TXM-089
<i>L. yanyuanense</i> Pu	Yanyuan, Sichuan	Group of Qingzang 12179
Sect. <i>Pinnatibracteola</i>		
* <i>L. capillaceum</i> H. Wolff	Xiangcheng, Sichuan	Z15182
* <i>L. daucoides</i> (Franch.) Franch.	Shangrila, Yunnan	Z15194
* <i>L. franchetii</i> H. de Boissieu	Muli, Sichuan	CaoYL & YaoJ 284 (CDBI)
* <i>L. hispidum</i> (Franch.) H. Wolff	Lijiang, Yunnan	Z1508
<i>L. involucreatum</i> Franch.	Lijiang, Yunnan	Z1509
* <i>L. likiangense</i> (H. Wolff) Pu & Watson	Heqing, Yunnan	LZ151005
* <i>L. multivittatum</i> Franch.	Xiangcheng, Sichuan	Z15172
* <i>L. oliverianum</i> (H. de Boissieu) Shan	Kangding, Sichuan	LZ1107
* <i>L. rechingerianum</i> (Leute) Shan & Pu	Deqin, Yunnan	ZhouZK-07ZX-0424
* <i>L. scapiforme</i> H. Wolff	Muli, Sichuan	LZ1036
<i>L. sikiangense</i> Hiroe	Lugu Lake, Yunnan	LZ1022
Species not included in Pu's division		
<i>L. mairei</i> Hiroe	Qiaojia, Yunnan	HP5121
<i>L. moniliforme</i> Peng & Zhang	Gannan, Gansu	LiuJQ-GN-2011-269
<i>L. mucronatum</i> (Schrenk) Leute	Zhaosu, Xinjiang	Shengtian 957
<i>L. nullivittatum</i> (Fu) Pu & Watson	Zhouzhi, Shanxi	ZhangXM 135
<i>L. pseudodaucoides</i> Peng & Yin	Jingdong, Yunnan	HP2579
<i>L. weberbauerianum</i> Fedde ex H. Wolff	Yajiang, Sichuan	FeiY & LiLQ 2969

materials were identified by the first author using published keys. Mature leaves were investigated under light microscopy (LM) and scanning electron microscopy (SEM). To check the constancy of epidermal structure, at least five slides were made from different parts of a single leaf for each species. Materials for LM study were boiled in distilled water for 20 minutes before being macerated in 75% NaClO. Pieces of leaf epidermis were stained with Safranin solution (1%), and then dehydrated in an ethanol series (from 75% to 100%) before being mounted in Canada balsam. Materials for SEM were directly attached to stubs without any treatment. After gold sputtering, the specimens were examined and photographed under a Hitachi S-4800. Stomatal terminology was based on the classification proposed by Baranova [21] [26] and Wilkinson [27].

3. Results

Leaf epidermal features under LM and SEM listed in **Table 2** & **Table 3**, corresponding to **Figures 1-6**. Other figures are provided as Supplementary material (**Figures S1-S3**).

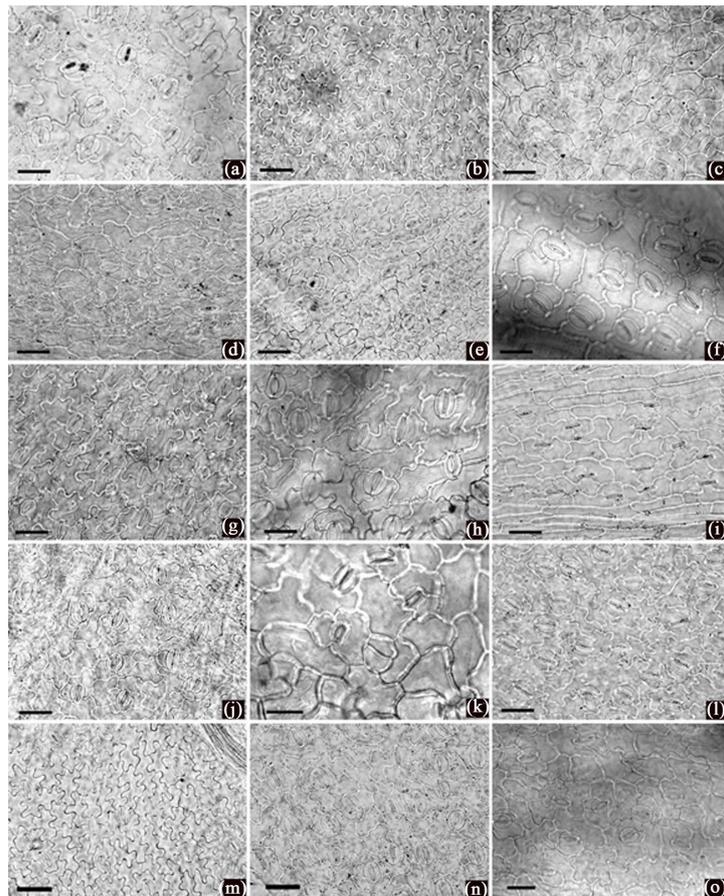


Figure 1. Characteristics of epidermal cells from *Ligusticum* species (light microscopy), all abaxial. (a) *L. daucooides*; (b) *L. pteridophyllum*; (c) *L. litangense*; (d) *L. tachiroei*; (e) *L. hispidum*; (f) *L. nematophyllum*; (g) *L. sinense*; (h) *L. involucreatum*; (i) *L. delavayi*; (j) *L. oliverianum*; (k) *L. likiangense*; (l) *L. littledalei*; (m) *L. acuminatum*; (n) *L. angelicifolium*; (o) *L. scapiforme*. Scale bar 50 μm .

Table 2. Leaf epidermal characters of *Ligusticum* under LM (surface view).

Taxon	Adaxial Epidermis			Abaxial Epidermis			Figure	
	Stomatal apparatus	Pattern of anticlinal walls	Shape of ordinary cells	Pattern of anticlinal walls	Stomatotype	Stomata index (%)		Size of stomata (μm^2)
Sect. <i>Ligusticum</i>								
<i>L. acuminatum</i>	A	Sinuuous	Irr	Sinuuous	Ano	18.6	13.37×7.47	1(m), S1(m)
<i>L. ajanense</i>	A	Str-Arc	Irr	Repand	Ani	25.8	24.18×12.39	2(o), S2(o)
<i>L. angelicifolium</i>	A	Repand	Irr	Repand	Ani	24.4	22.96×11.69	1(n), S1(n)
<i>L. brachylobum</i>	P	Repand	Irr	Repand	Ani	28.6	21.49×10.95	2(f), S2(f)
<i>L. delavayi</i>	P	Repand	Irr + rectangular	Repand	Ani	18.0	15.37×9.26	1(i), S1(i)
<i>L. discolor</i>	P	Sinuuous	Irr	Sinuuous	Ano	23.1	21.19×10.00	2(l), S2(l)
<i>L. elatum</i>	P	Str-Arc	Pol	Str-Arc	Ani	17.9	16.13×10.67	3(b), S3(b)
<i>L. jeholense</i>	A	Sinuuous	Irr	Sinuuous	Ano	20.6	17.29×9.65	2(m), S2(m)
<i>L. kingdom-wardii</i>	A	Str-Arc	Irr	Sinuuous	Ano	18.2	25.09×14.15	3(d), S3(d)
<i>L. litangense</i>	P	Str-Arc	Pol	Str-Arc	Ani	23.6	19.85×10.15	1(c), S1(c)
<i>L. littledalei</i>	A	Repand	Irr	Sinuuous	Ano	25.0	16.82×10.71	1(l), S1(l)
<i>L. nematophyllum</i>	A	Repand	Irr	Repand	Ani	21.1	24.00×12.91	1(f), S1(f)
<i>L. pteridophyllum</i>	A	Sinuuous	Irr	Sinuuous	Ano	23.5	18.46×12.69	1(b), S1(b)
<i>L. reptans</i>	A	Str-Arc	Pol	Str-Arc	Ani	30.1	18.90×9.59	3(c), S3(c)
<i>L. sinense</i>	A	Sinuuous	Irr	Sinuuous	Ano	28.4	15.24×5.95	1(g), S1(g)
<i>L. sinense</i> cv. <i>Chuanxiong</i>	A	Repand	Irr	Sinuuous	Ano	19.3	23.35×14.65	3(h), S3(h)
<i>L. tachiroei</i>	A	Str-Arc	Irr	Repand	Ani	15.4	15.92×7.50	1(d), S1(d)
<i>L. tenuisectum</i>	A	Repand	Irr	Sinuuous	Ani	18.9	29.09×9.48	2(e), S2(e)
<i>L. tenuissimum</i>	A	Str-Arc	Pol	Str-Arc	Ani	15.9	18.82×8.97	2(g), S2(g)
<i>L. thomsonii</i>	P	Str-Arc	Irr	Repand	Ani	19.8	17.05×10.21	2(j), S2(j)
<i>L. yanyuanense</i>	P	Str-Arc	Pol	Str-Arc	Ani	23.4	22.46×13.28	2(h), S2(h)
Sect. <i>Pinnatibracteola</i>								
<i>L. capillaceum</i>	P	Repand	Irr	Repand	Ani	20.6	14.25×10.38	2(c), S2(c)
<i>L. daucooides</i>	P	Repand	Irr	Repand	Ani	17.6	17.41×11.53	1(a), S1(a)
<i>L. franchetii</i>	A	Repand	Irr	Sinuuous	Ano	25.2	15.51×9.31	3(e), S3(e)
<i>L. hispidum</i>	P	Repand	Irr	Repand	Ani	11.0	13.81×8.21	1(e), S1(e)
<i>L. involucreatum</i>	P	Str-Arc	Irr	Repand	Ani	17.7	20.76×13.03	1(h), S1(h)
<i>L. likiangense</i>	P	Str-Arc	Irr	Repand	Ani	19.0	21.22×12.44	1(k), S1(k)
<i>L. multivittatum</i>	P	Repand	Irr	Sinuuous	Ano	17.0	13.25×9.67	2(d), S2(d)
<i>L. oliverianum</i>	P	Sinuuous	Irr	Sinuuous	Ani	17.3	15.90×10.86	1(j), S1(j)
<i>L. rechingerianum</i>	P	Str-Arc	Pol	Str-Arc	Ani	12.8	13.91×10.14	3(g), S3(g)
<i>L. scapiforme</i>	P	Repand	Irr	Repand	Ani	24.4	14.59×9.42	1(o), S1(o)
<i>L. sikiangense</i>	P	Repand	Irr	Repand	Ani	18.5	19.74×10.81	2(a), S2(a)
Species not included in Pu's division								
<i>L. mairei</i>	P	Str-Arc	Pol	Str-Arc	Ani	19.4	25.17×15.67	2(b), S2(b)
<i>L. moniliforme</i>	A	Sinuuous	Irr	Sinuuous	Ano	28.3	41.22×20.49	3(f), S3(f)
<i>L. mucronatum</i>	P	Str-Arc	Irr	Repand	Ani	14.9	18.82×13.16	2(i), S2(i)
<i>L. nullivittatum</i>	A	Sinuuous	Irr	Sinuuous	Ano	27.4	19.75×9.88	2(k), S2(k)
<i>L. pseudodaucooides</i>	A	Str-Arc	Pol	Str-Arc	Ani	25.6	21.32×13.09	3(a), S3(a)
<i>L. weberbauerianum</i>	A	Str-Arc	Pol	Str-Arc	Ani	11.7	18.89×12.36	2(n), S2(n)

A: Absent; P: Present; Pol: Polygonal; Irr: Irregular; Str-Arc: Straight to arched; Ani: Anisocytic; Ano: Anomocytic.

Table 3. Leaf epidermal characters of *Ligusticum* under SEM (surface view).

Taxon	Abaxial epidermis				Figure
	Outline of the pair of guard cells	Inner margin of outer stomatal rim	Cuticular membrane ornamentation	Wax ornamentation	
Sect. <i>Ligusticum</i>					
<i>L. acuminatum</i>	NE	Smooth	Striate	Invisible	4(m)
<i>L. ajanense</i>	Ell	Smooth	Wrinkled & Striate	Scales	5(o)
<i>L. angelicifolium</i>	Ell	Smooth	Striate	Invisible	4(n)
<i>L. brachylobum</i>	NE	Smooth	Striate	Invisible	5(f)
<i>L. delavayi</i>	Ell	Smooth	Smooth	Invisible	4(i)
<i>L. discolor</i>	Ell	Erose	Striate	Flackes	5(l)
<i>L. elatum</i>	Ell	Smooth	Striate	Invisible	6(b)
<i>L. jeholense</i>	Ell	Smooth	Sparse radiating Striate	Invisible	5(m)
<i>L. kingdon-wardii</i>	NE	Smooth	Striate	Invisible	6(d)
<i>L. litangense</i>	Ell	Smooth	Striate	Invisible	4(c)
<i>L. littledalei</i>	Ell	Smooth	Striate	Invisible	4(l)
<i>L. nematophyllum</i>	Ell	Smooth	Striate	Invisible	4(f)
<i>L. pteridophyllum</i>	WE	Nearly Smooth	Striate	Invisible	4(b)
<i>L. reptans</i>	NE	Smooth	Striate	Invisible	6(c)
<i>L. sinense</i>	NE	Erose	Striate	Invisible	4(g)
<i>L. sinense</i> cv. <i>Chuanxiong</i>	NE	Smooth	Sparse radiating Striate	Invisible	6(h)
<i>L. tachiroei</i>	NE	Erose	Striate	Invisible	4(d)
<i>L. tenuisectum</i>	Ell	Smooth	Striate	Invisible	5(e)
<i>L. tenuissimum</i>	NE	Erose	Striate	Invisible	5(g)
<i>L. thomsonii</i>	Ell	Erose	Striate	Invisible	5(j)
<i>L. yanyuanense</i>	Ell	Erose	Sparse radiating Striate	Parcels	5(h)
Sect. <i>Pinnatibracteola</i>					
<i>L. capillaceum</i>	Ell	Smooth	Striate	Invisible	5(c)
<i>L. daucooides</i>	Ell	Erose	Wrinkled	Scales	4(a)
<i>L. franchetii</i>	NE	Erose	Striate	Invisible	6(e)
<i>L. hispidum</i>	Ell	Smooth	Wrinkled	Flacks	4(e)
<i>L. involucreatum</i>	Ell	Smooth	Radiating Striate	Invisible	4(h)
<i>L. likiangense</i>	Ell	Smooth	Sparse radiating Striate	Invisible	4(k)
<i>L. multivittatum</i>	Ell	Smooth	Striate	Invisible	5(d)
<i>L. oliverianum</i>	Ell	Erose	Striate	Invisible	4(j)
<i>L. rechingerianum</i>	Rou	Erose	Smooth	Scales	6(g)
<i>L. scapiforme</i>	Ell	Erose	Wrinkled & Striate	Invisible	4(o)
<i>L. sikiangense</i>	Ell	Smooth	Striate	Invisible	5(a)
Species not included in Pu's division					
<i>L. mairei</i>	Ell	Smooth	Striate	Invisible	5(b)
<i>L. moniliforme</i>	Ell	Smooth	Striate	Invisible	6(f)
<i>L. mucronatum</i>	Ell	Erose	Striate	Invisible	5(i)
<i>L. nullivittatum</i>	Ell	Smooth	Concentric Striate	Invisible	5(k)
<i>L. pseudodaucooides</i>	Ell	Smooth	Wrinkled & Striate	Invisible	6(a)
<i>L. weberbauerianum</i>	Ell	Smooth	Concentric Striate	Invisible	5(n)

Ell: Elliptical; NE: Narrowly Elliptical; WE: Widely Elliptical; Rou: Roundish.

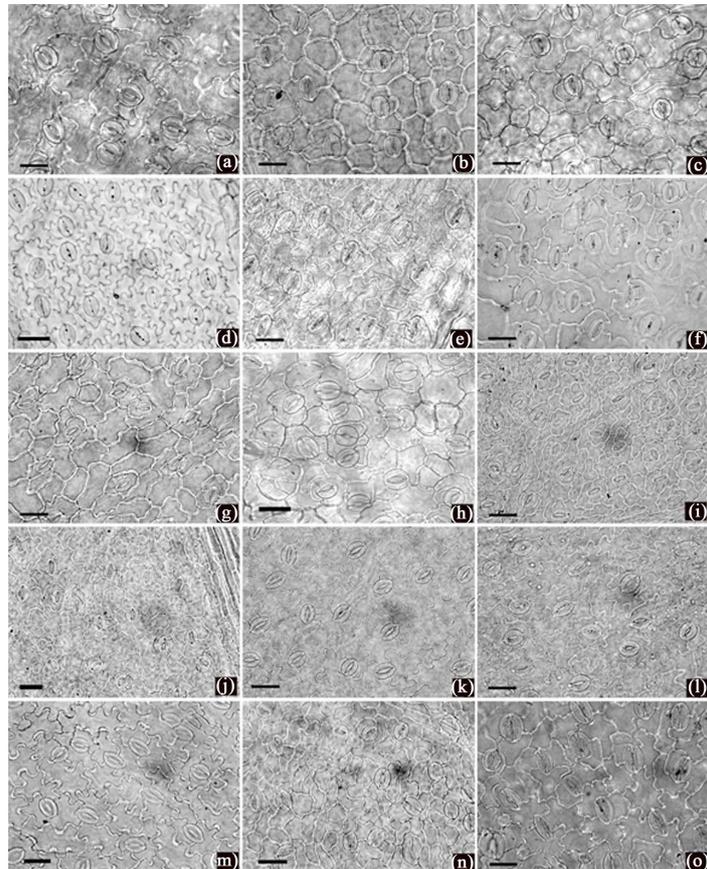


Figure 2. Characteristics of epidermal cells from *Ligusticum* species (light microscopy), all abaxial. (a) *L. sikiangense*; (b) *L. mairei*; (c) *L. capillaceum*; (d) *L. multivittatum*; (e) *L. tenuisectum*; (f) *L. brachylobum*; (g) *L. tenuissimum*; (h) *L. yanyuanense*; (i) *L. mucronatum*; (j) *L. thomsonii*; (k) *L. nullivittatum*; (l) *L. discolor*; (m) *L. jeholense*; (n) *L. webbauerianum*; (o) *L. ajanense*. Scale bar 50 μ m.

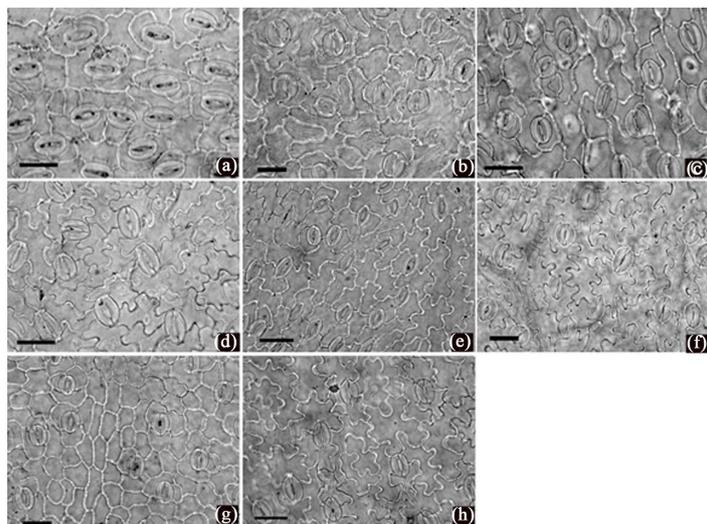


Figure 3. Characteristics of epidermal cells from *Ligusticum* species (light microscopy), all abaxial. (a) *L. pseudodaucoides*; (b) *L. elatum*; (c) *L. reptans*; (d) *L. kingdon-wardii*; (e) *L. franchetii*; (f) *L. moniliforme*; (g) *L. rechingerianum*; (h) *L. sinense* cv. *Chuanxiong*. Scale bar 50 μ m.

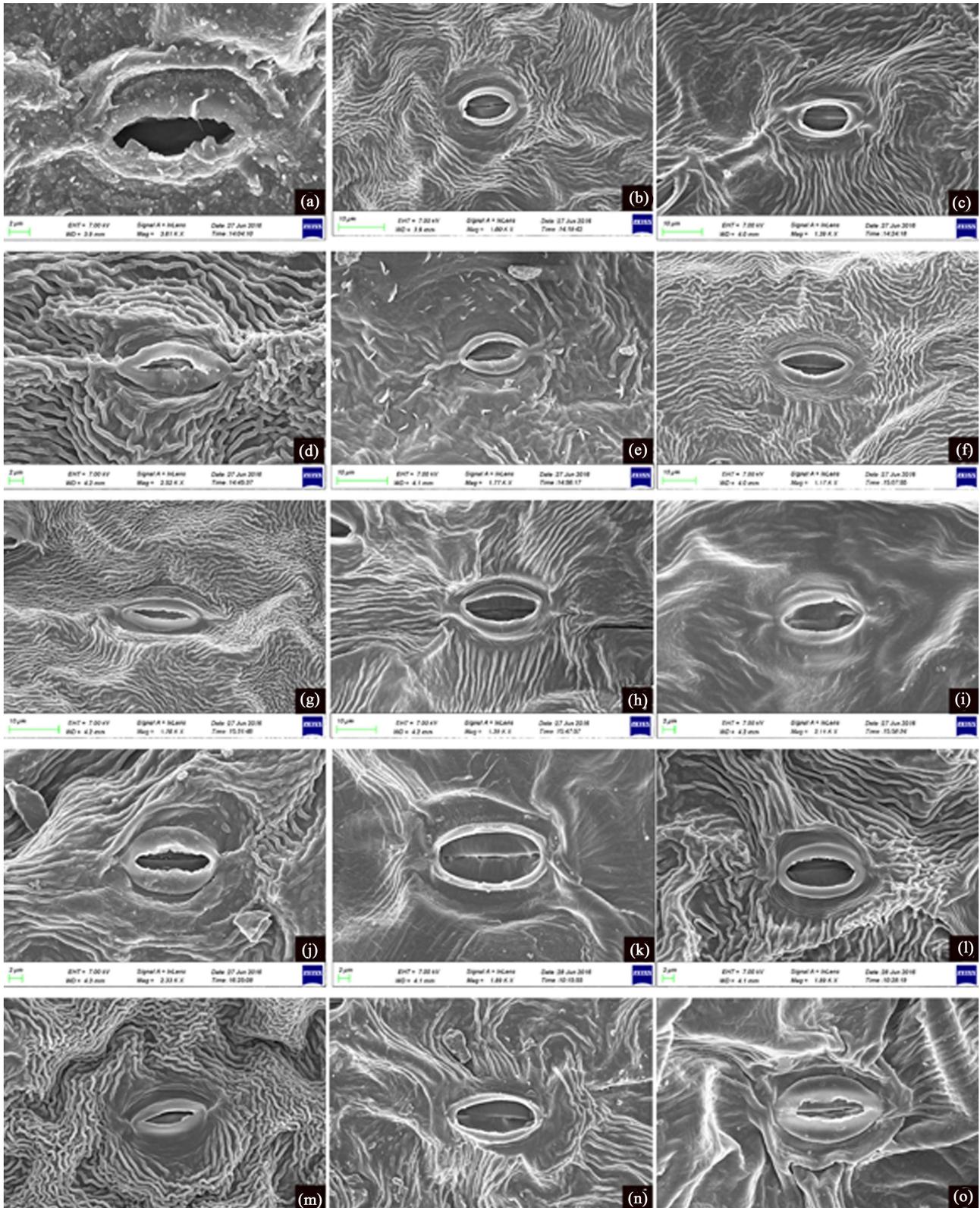


Figure 4. Characteristics of epidermal cells from *Ligusticum* species (scanning electron microscopy), all abaxial. (a) *L. daucooides*; (b) *L. pteridophyllum*; (c) *L. litangense*; (d) *L. tachiroëi*; (e) *L. hispidum*; (f) *L. nematophyllum*; (g) *L. sinense*; (h) *L. involucreatum*; (i) *L. delavayi*; (j) *L. oliverianum*; (k) *L. likiangense*; (l) *L. littledalei*; (m) *L. acuminatum*; (n) *L. angelicifolium*; (o) *L. scapiforme*. Scale bar 20 μm .

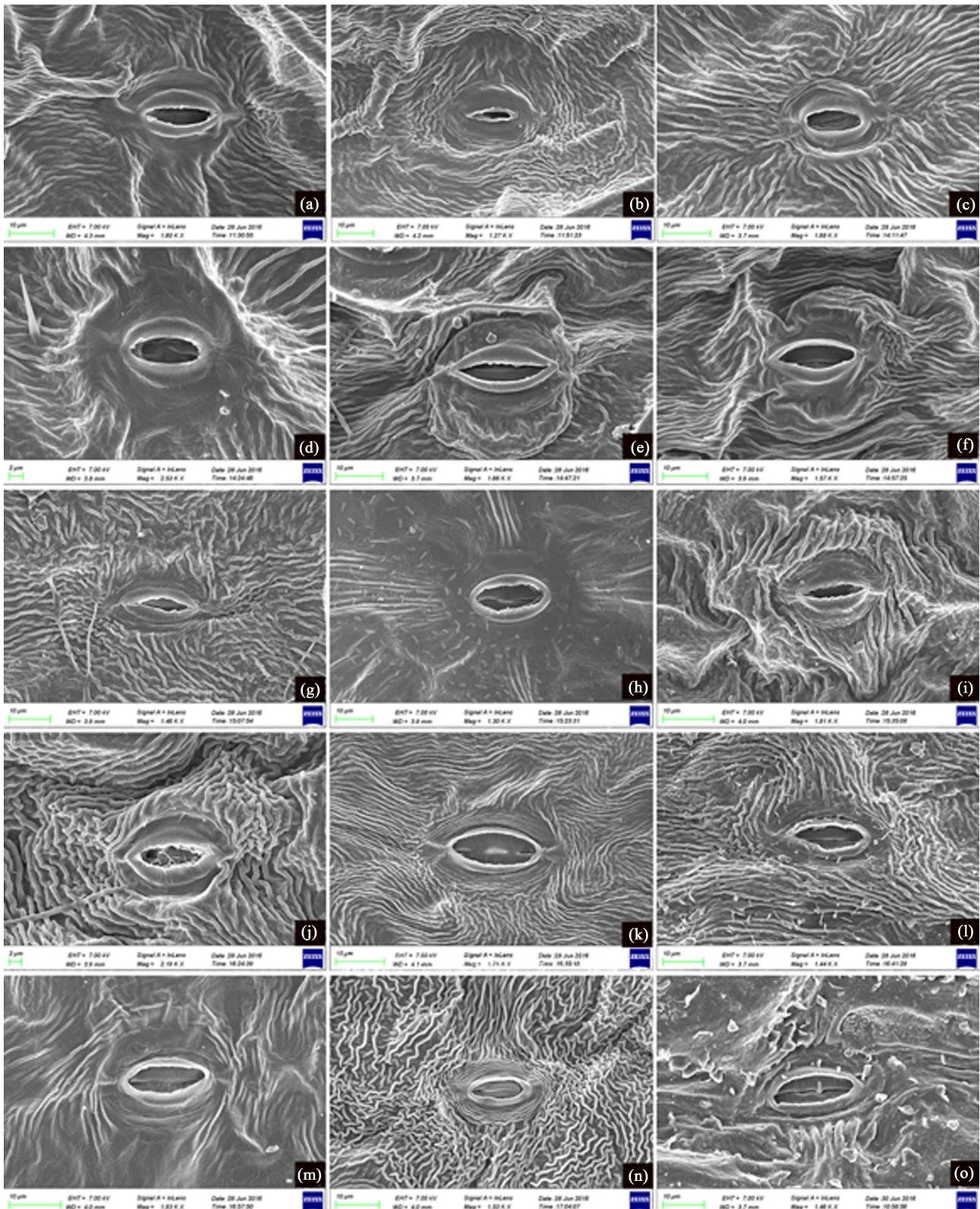


Figure 5. Characteristics of epidermal cells from *Ligusticum* species (scanning electron microscopy), all abaxial. (a) *L. sikiangense*; (b) *L. mairei*; (c) *L. capillaceum*; (d) *L. multivittatum*; (e) *L. tenuisectum*; (f) *L. brachylobum*; (g) *L. tenuissimum*; (h) *L. yanyuanense*; (i) *L. mucronatum*; (j) *L. thomsonii*; (k) *L. nullivittatum*; (l) *L. discolor*; (m) *L. jeholense*; (n) *L. weberbauerianum*; (o) *L. ajanense*. Scale bar 20 μm .

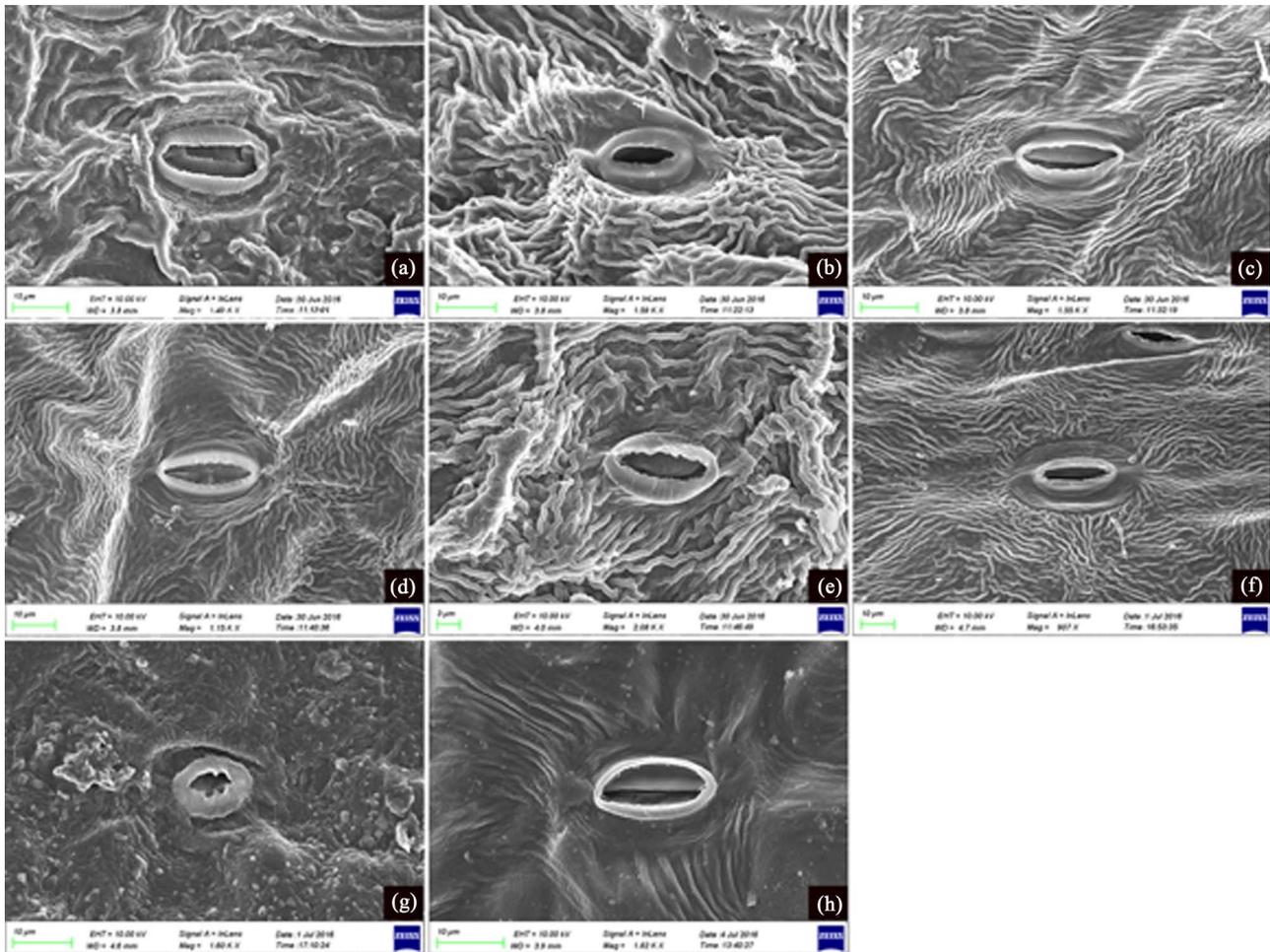


Figure 6. Characteristics of epidermal cells from *Ligusticum* species (scanning electron microscopy), all abaxial. (a) *L. pseudo-daucooides*; (b) *L. elatum*; (c) *L. reptans*; (d) *L. kingdon-wardii*; (e) *L. franchetii*; (f) *L. moniliforme*; (g) *L. rechingerianum*; (h) *L. sinense* cv. *Chuanxiong*. Scale bar 20 μm .

3.1. Characteristics of the Leaf Epidermis under LM

The form of epidermal cells in *Ligusticum* as seen under LM was usually irregular or polygonal (rarely rectangular in *L. delavayi*, **Figure 1(i)** and **Figure S1(i)**), with anticlinal walls straight to arched, repand or sinuous (**Table 2**). The pattern of anticlinal cells may vary between different species and also between the adaxial and abaxial epidermis of a given species. In about 2/3 of the species studied, the anticlinal cells of both sides were consistent with straight to arched walls or repand and sinuous walls, while in the other 12 species, the anticlinal walls varied between the adaxial and abaxial surfaces (**Table 2**). Stomata were present on both surfaces in 19 of the observed species (**Table 2**). The patterns of the stomata were anisocytic or anomocytic, inconsistent with Sun *et al.* [25] in which four types of stomata were found. The stomatal index ranged from 11.0% in *L. hispidum* (**Figure 1(e)** and **Figure S1(e)**), up to 30.1% in *L. reptans* (**Figure 3(c)** and **Figure S3(c)**). Particularly, stomatas observed were usually closed, indicating that *Ligusticum* is relatively suitable to arid habitats.

3.2. Characteristics of the Leaf Epidermis under SEM

The outlines of the pair of guard cells were elliptical, narrowly elliptical, and roundish. The inner margins of the outer stomatal rim were observed to be of two types of smooth or nearly smooth, and erose (**Table 3**). The cuticular membrane ornamentations were striate, wrinkled and smooth. The striate type was found in most species investigated, with some variations discovered in a few taxa: a sparse radiating striate cuticular membrane occurred in *L. jeholense* (**Figure 5(m)**), *L. sinense* cv. *Chuanxiong* (**Figure 6(h)**), *L. yanyuanense* (**Figure 5(h)**) and *L. likiangense* (**Figure 4(k)**); the membrane of *L. involucratum* (**Figure 4(h)**) was of a radiating striate type; and a concentric striate membrane was found in both *L. weberbauerianum* (**Figure 5(n)**) and *L. nullivittatum* (**Figure 5(k)**). A wrinkled type was present in both *L. daucooides* (**Figure 4(a)**) and *L. hispidum* (**Figure 4(e)**). *L. delavayi* (**Figure 4(i)**) and *L. rechingerianum* (**Figure 6(g)**) had smooth ornamentations. The cuticular membranes of the remaining species were of a mixture of two types. Both wrinkled and striate membranes were present in *L. ajanense* (**Figure 5(o)**), *L. scapiforme* (**Figure 4(o)**) and *L. pseudodaucooides* (**Figure 6(a)**).

Wax ornamentation was absent in 32 species, while the remaining six had wax scales or other ornamentations. Scales occurred in three species: *Ligusticum ajanense* (**Figure 5(o)**), *L. daucooides* (**Figure 4(a)**) and *L. rechingerianum* (**Figure 6(g)**). *L. discolor* (**Figure 5(l)**) and *L. hispidum* (**Figure 4(e)**) had flacks, while parcels appeared in *L. yanyuanense* (**Figure 5(h)**).

4. Discussion

It was observed that most species has its own unique combination of leaf epidermal features, making the characters taxonomically useful. In our analysis, epidermal characters with potential systematic values are compared with previous phylogenetic results, and morphological traits such as fruit are also considered. Pu [1] (1991) arranged the species of Chinese *Ligusticum* into two sections: section *Ligusticum* with bracteoles linear or lanceolate, entire, and section *Pinnatibracteola* with bracteoles 1 - 3-pinnatisect or 2 - 3-lobed at the apex. In our study, 21 species of sect. *Ligusticum* and 11 species of sect. *Pinnatibracteola* were included. As indicated in **Table 2** & **Table 3**, no differences were detected in epidermal characters under LM and SEM between these two sections, both sections having irregular and polygonal epidermal cells, straight to arched, sinuous and repand anticlinal walls, anisocytic and anomocytic stomata, elliptical guard cells, and smooth or erose stomatal rims. This was in consensus with a previous karyological study on *Ligusticum*, in which that no chromosomal differences detected between the two sections [11]. Furthermore, previous molecular analysis also showed that the species with entire bracteoles fell into two distant clades, and those with pinnate ones did not constitute a monophyletic group [18] [19]. Thus, this infrageneric subdivision provides a useful key for species identification, but is not a natural classification and is in need of further

revision. Previous studies indicated that the pattern of anticlinal walls was associated with environmental factors [22] [28] [29] [30]. In our analysis, anticlinal walls in *Ligusticum* seems to be not related with their geographic distribution and environmental conditions where the species grow. For example, *L. tenuissimum* from northern China (growing on with rocky slopes) have straight to arched walls on both surfaces, while *L. rechingianum* from southwest (on alpine meadows) also possess the same type.

4.1. Relationships with *Tilingia* and *Ligusticopsis*

The genus *Ligusticopsis* Leute has 14 species confined to China, all of which have been included in present study (note with * in **Table 1**). *Ligusticopsis* was separated from *Ligusticum* based on the prominent calyx teeth of the former [4]. However, these prominent calyx teeth, actually occur in certain species of *Ligusticum*, e.g. *L. involucreatum* Franch., which has conspicuous, triangular calyx teeth. Molecular analysis indicated that *Ligusticopsis* is not monophyletic, as species referred to *Ligusticopsis* fell into two distant clades, Selineae and *Sinodielisia* [18] [19]. In present study, epidermal characters including stomatal types, anticlinal walls and the cuticular membrane, could not differentiate *Ligusticopsis* from *Ligusticum*, as well as karyological evidence [11] and pollen morphology [31].

Tilingia Regel & Tiling was established by Regel and Tiling [8], on the basis of distinct calyx teeth and the carpels bearing a solitary vitta in each furrow (*T. ajanensis* Regel & Tiling is the type species). However, this treatment was not accepted by Kozo-Poljansky [9] and Hiroe & Constance [10], also following the pollen morphology experiments of Wang *et al.* [31]. In the present study, three species previously referred to *Tilingia*, *L. ajanense* (Regel & Tiling) Kozo-Poljansky, *L. tachiroei* (Franch. & Savat.) Hiroe & Constance and *L. jeholense* (Nakai & Kitagawa) Nakai & Kitagawa were included. *L. ajanense* and *L. tachiroei* are characterized by their anticlinal walls of adaxial and abaxial epidermis straight to arched and repand respectively, and the adaxial sides with no stomata. Morphologically, ribs on mericarps of these two species are prominent and subequal, which is differed from other species of the genus with winged lateral ribs. Previous molecular analysis showed that *T. ajanensis* allied with *Pleurospermum hookeri* C.B. Clarke, *P. yunnanense* Franch. and *L. delavayi* Franch. of the *Acronema* Clade, distantly related to other *Ligusticum* species [19]. Therefore, in accordance with the molecular and morphological results, epidermal evidence also supported the segregation of *L. ajanense* and *L. tachiroei* from *Ligusticum*.

4.2. The relationship between *Hymenidium* and *Ligusticum delavayi*

As mentioned above, *L. delavayi* comprises a well-supported clade with *Pleurospermum hookeri* and *P. yunnanense* of the *Acronema* Clade. These taxa have similar fruit structures and membranous-margined bracts and all have been referred to genus *Hymenidium* Lindl. [32]. However, the other species referred to

Hymenidium fell within tribe Pleurospermeae near the base of the trees, distant from *Acronema* Clade [19]. Therefore, *Hymenidium* as currently circumscribed is not monophyletic. In this study, *L. delavayi* was quite different from other *Ligusticum* species. For example, its epidermal cells were both irregular and rectangular, while cuticular membrane ornamentation was smooth. Furthermore, according to a previous study, the karyotype asymmetry of *L. delavayi* was the largest among species examined (T.C. % = 58.53%) [11]. In fact, with ultimate segments setuliform and membranous margined bracts, *L. delavayi* is distinct and easily differentiated from other *Ligusticum* species. Therefore, epidermal evidence, combined with molecular and karyological results, support that *L. delavayi* should be separated from *Ligusticum*.

4.3. The Placement of *L. pseudodaucooides* and *L. moniliforme*

Ligusticum moniliforme and *L. pseudodaucooides* are two recently published taxa, which are not included by FOC. *L. pseudodaucooides* was recorded only from the type locality, with its trinacriiform bracteoles as the diagnostic character, and it most closely resembles *L. daucooides* (Franch.) Franch. [33]. However, its epidermal characters were different from that of *L. daucooides* (Table 2 & Table 3). *L. pseudodaucooides* distributed in Wuliangshan range of Yunnan Province, the southernmost geographical limit for the genus in China, while *L. daucooides* occurred in northern Yunnan and other provinces [2] [33]. *L. moniliforme* was described as a new species for its moniliform rhizome [34], however, *L. nematophyllum* (Pimenov & Kljuykov) Pu & Watson also has clustered fusiform or tuberous roots. Other characters, e.g. leaf blade, bracteoles and petals are also very similar between the two species, as is the epidermal analysis under SEM. *L. nematophyllum* and *L. moniliforme* occur in western Sichuan and Gansu Province, respectively. Although our present analyses supply some leaf features for these two poorly known species (*L. pseudodaucooides* and *L. moniliforme*), their relationships with each morphologically similar *L. daucooides* and *L. nematophyllum* needs more evidence.

5. Conclusion

In this study, a detailed observation of epidermal characters from across the genus *Ligusticum* is presented. Although not all species can be distinguished from each other by its own combinations of epidermal features, many characters seem to be important for distinguishing some closely related taxa. Furthermore, the previous infrageneric classification of Pu [1] is not supported by our present study, but the data do provide evidences for taxonomic treatment of some controversial taxa. In general, the results presented here will promote the future revisionary studies of this poorly known genus.

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Supplements

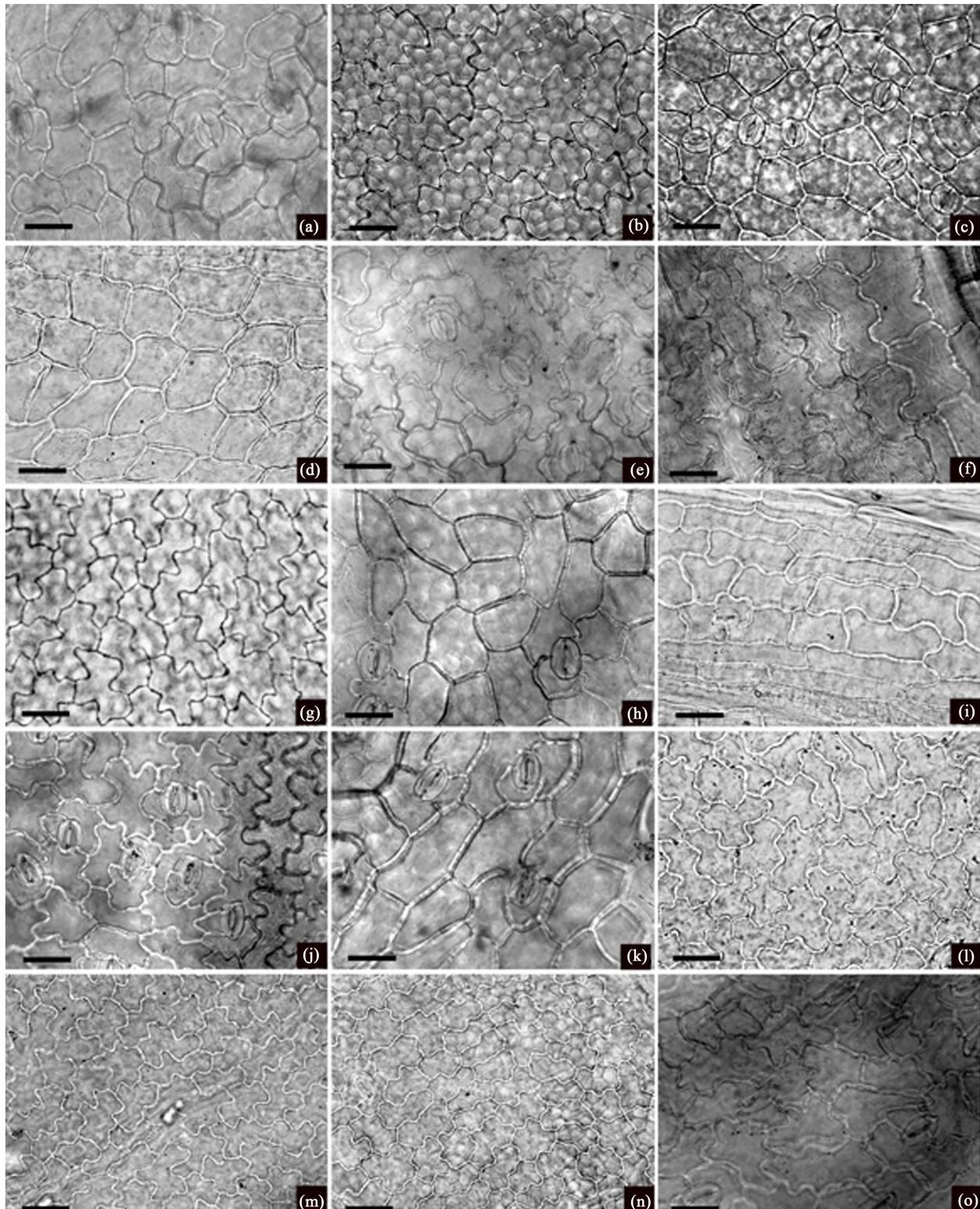


Figure S1. Characteristics of epidermal cells from *Ligusticum* species (light microscopy), all adaxial. (a) *L. daucooides*; (b) *L. pteridophyllum*; (c) *L. litangense*; (d) *L. tachiroei*; (e) *L. hispidum*. (f) *L. nematophyllum*; (g) *L. sinense*; (h) *L. involucreatum*; (i) *L. delavayi*; (j) *L. oliverianum*; (k) *L. likiangense*; (l) *L. littledalei*; (m) *L. acuminatum*; (n) *L. angelicifolium*; (o) *L. scapiforme*. Scale bar 50 μm .

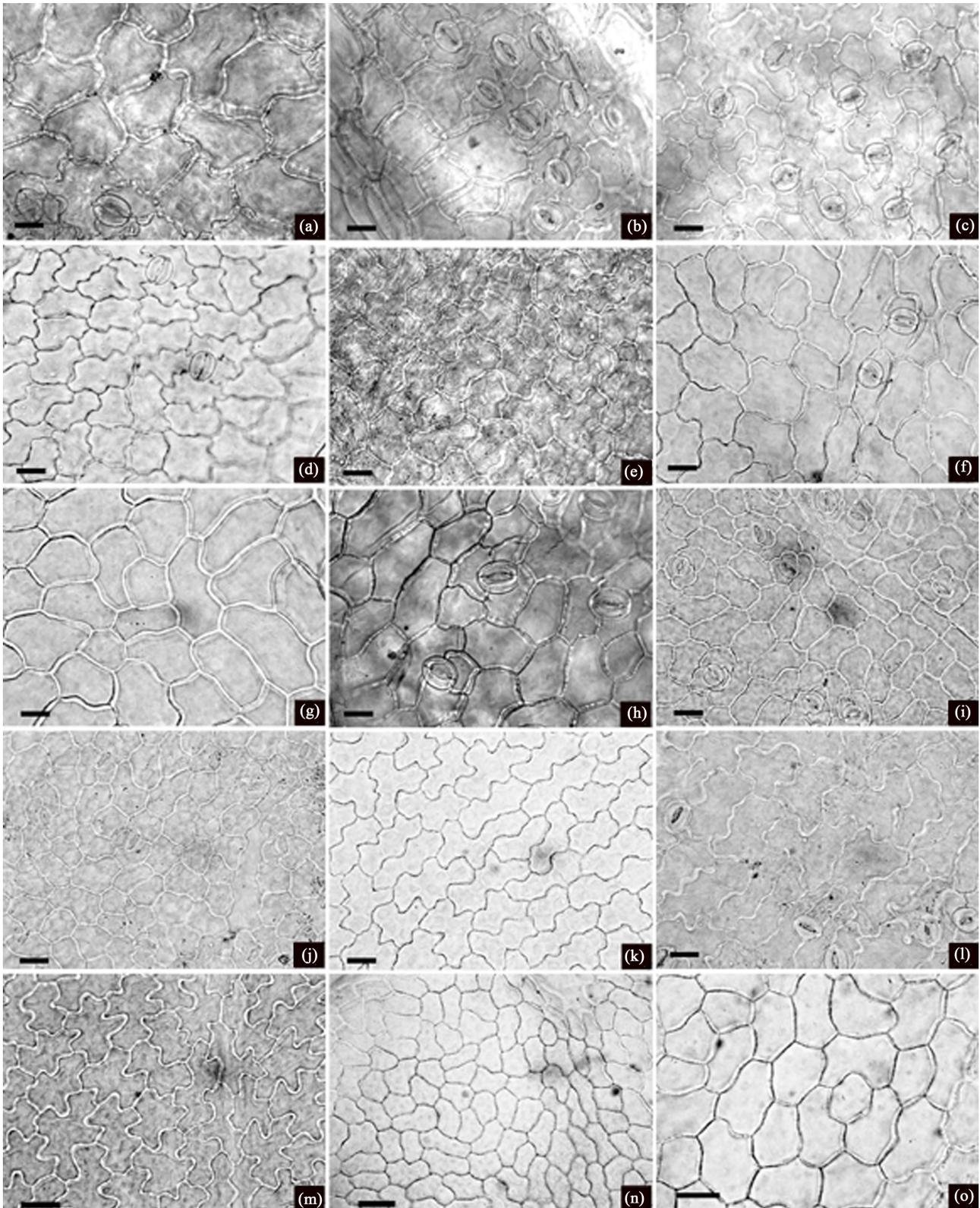


Figure S2. Characteristics of epidermal cells from *Ligusticum* species (light microscopy), all adaxial. (a) *L. sikiangense*; (b) *L. mairei*; (c) *L. capillaceum*; (d) *L. multivittatum*; (e) *L. tenuisectum*; (f) *L. brachylobum*; (g) *L. tenuissimum*; (h) *L. yanyuanense*. I. *L. mucronatum*; (j) *L. thomsonii*; (k) *L. nullivittatum*; (l) *L. discolor*; (m) *L. jeholense*; (n) *L. weberbauerianum*; (o) *L. ajanense*. Scale bar 50 μ m.

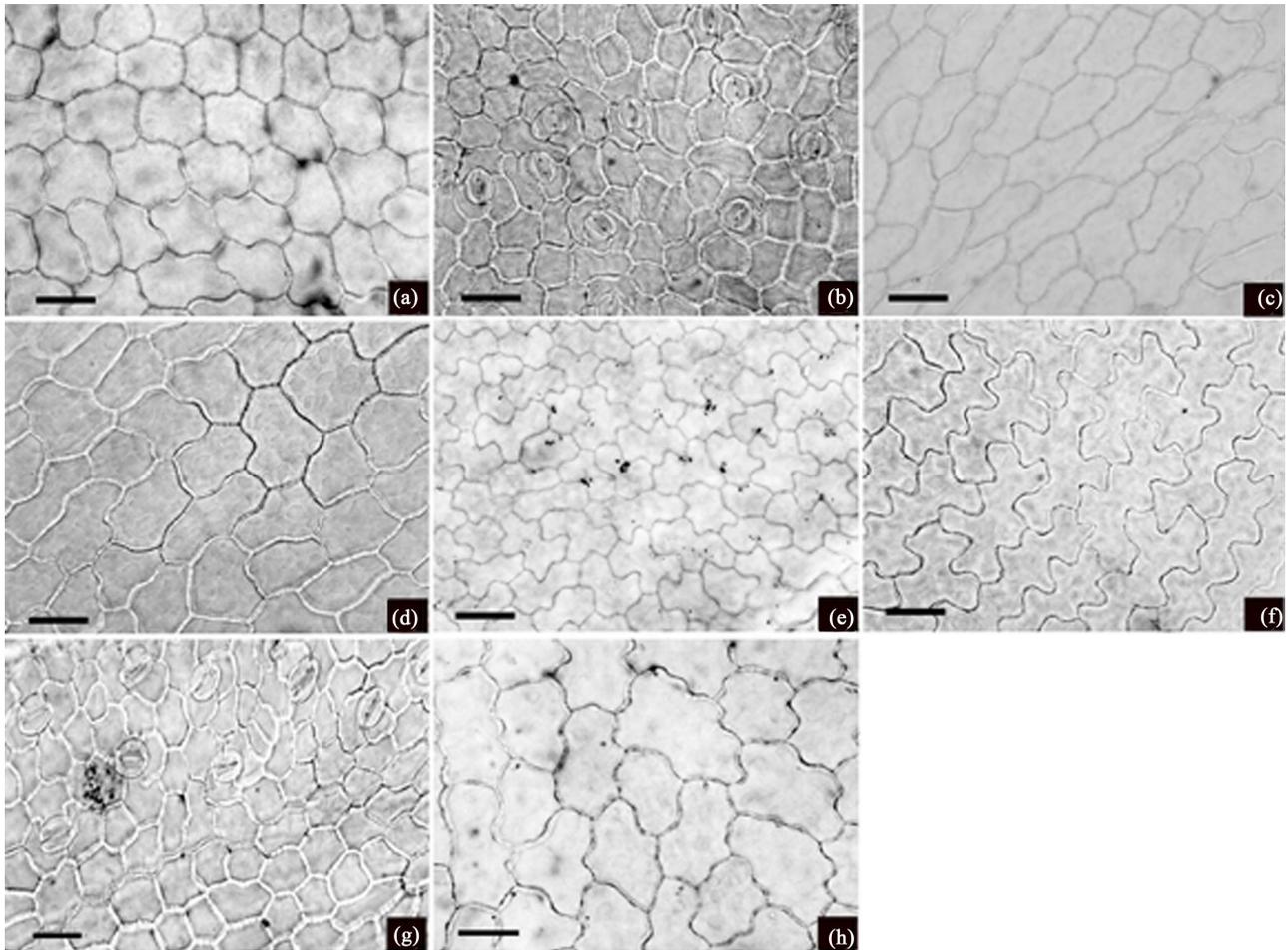


Figure S3. Characteristics of epidermal cells from *Ligusticum* species (light microscopy), all adaxial. (a) *L. pseudodaucooides*; (b) *L. elatum*; (c) *L. reptans*; (d) *L. kingdon-wardii*; (e) *L. franchetii*; (f) *L. moniliforme*; (g) *L. rechingerianum*; (h) *L. sinense* cv. *Chuan-xiong*. Scale bar 50 μm .