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Comparative leaf anatomy of some species of *Abies* and *Picea* (Pinaceae)

Balkrishna Ghimire[1, 2], Chunghee Lee[3], Jongcheol Yang[3] and Kweon Heo[1*]

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ABSTRACT

A number of conifer species are still lacking anatomical data, which is significant because morphological and anatomical data are essential for systematic study. Leaf anatomy was studied in selected species of *Abies* and *Picea* using light and scanning electron microscopy. Both genera were found to have typical coniferous and highly xerophytic leaves with sunken stomata and an epidermis covered by a thick cuticle. In the genus *Abies*, species can be differentiated by the nature of the lignified hypodermis and the number and position of resin ducts. *Abies firma* and *A. holophylla* have a continuous hypodermis whereas in *A. koreana* and *A. nephrolepis* the hypodermis is discontinuous and represented by isolated cells or groups of four or five cells. On the other hand, in *Picea* leaf shape, stomata arrangement, and number, position, and nature of resin ducts are the key features for species differentiation. *Picea jezoensis* has a flattened leaf with stomata distributed on the adaxial surface whereas *P. abies* and *P. koraiensis* have a rectangular leaf with stomata found on surfaces.

Keywords: *Abies*, leaf anatomy, *Picea*, scanning electron microscopy, taxonomy

Introduction

The Pinaceae comprise a total of 225 species in 11 commonly accepted genera (*Abies*, *Cathaya*, *Cedrus*, *Keteleeria*, *Nothotsuga*, *Picea*, *Pinus*, *Pseudolarix*, *Pseudotsuga* and *Tsuga*) distributed throughout the world (Farjon 2001). Hart (1987) divided the family into two groups: the presence of resin canals in the seeds and cleavage polyembryony supported the monophyly of *Abies*, *Cedrus*, *Keteleeria*, *Pseudolarix* and *Tsuga* and the presence of resin canals in the secondary xylem and leaves having an endodermis with thickened Casparian strips supported the monophyly of *Cathaya*, *Larix*, *Picea*, *Pinus* and *Pseudotsuga*. On the other hand, on the basis of the morphological structure of vegetative and reproductive organs, Frankis (1988) distinguished four subfamilies of Pinaceae in a widely accepted classification (Farjon 1990): Pinoideae (*Pinus*), Piceoideae (*Picea*), Laricoideae (*Cathaya*, *Larix* and *Pseudotsuga*), and Abietoideae (*Abies*, *Cedrus*, *Keteleeria*, *Nothotsuga*, *Pseudolarix* and *Tsuga*). We considered two genera, *Abies* and *Picea,* in a study of leaf anatomy. Both genera show dissimilar morphological features and thus are generally classified in separate subfamilies in most Pinaceae classifications (Hart 1987; Frankis 1988; Farjon 1990; 2001).

Abies, with 49 species, is the second largest genus in the family Pinaceae after *Pinus* and the largest genus in the subfamily Abietoideae (Farjon 2010). They are naturally found in temperate and boreal regions of the northern hemisphere, chiefly in mountainous regions in North America, Central America, Europe, North Africa, and Asia. The genus has most recently been revised by Liu (1971) and Rushforth (1987) and a full review of previous classification schemes was provided by Farjon and Rushforth (1989). Farjon (2010) categorized the 49 species of *Abies* into 10 sections and nine subsections. In contrast, the genus *Picea*, which includes 28–56 species depending on the system of classification used (Farjon 1990; Ledig *et al*. 2004), is most closely related to the genus *Pinus*. Farjon (2001) recognized 34 spruce species in his conifer checklist, of which 24 are native to Asia, eight to North America, and two to Europe. It is a very uniform genus and thus difficult to work with from a taxonomic perspective due to species having a relatively narrow range of morphological features and ecological preferences (Wright 1955; Taylor and Patterson 1980; Rehfeldt 1994).

Abies and *Picea* are associated with each other on the basis of some morphological features like spirally arranged leaves, the absence of short shoots, and a similar form of petiole pulvinus. On the basis of these features *Picea* was grouped into subfamily Abietoideae along with *Abies*,

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[1] Department of Applied Plant Science, Kangwon National University, Chuncheon 200-701, Korea
[2] Division of Plant Conservation, Korea National Arboretum, Pocheon 487-829, Korea
[3] Korea National Arboretum, Pocheon 487-829, Korea
[*] Corresponding author: laurus@kangwon.ac.kr

Cathaya, *Keteleeria*, *Pseudotsuga* and *Tsuga* by Pilger (1926) and Melchior & Werdermann (1954); this was later rejected on the basis that a classification based on shoot dimorphism is highly artificial because short shoots are a weak phylogenetic character (Hart 1987; Price 1989). The objective of this study was a systematic comparison of leaf anatomical and surface features of some selected species of *Abies* and *Picea*, followed by a discussion of the relationship between these genera on this basis.

Material and methods

Altogether 18 plant samples were considered for the study, comprising four species of *Abies* Mill. and three species of *Picea* A. Dietr (Tab. 1). The collected leaf materials were fixed in FAA (formalin: glacial acetic acid: 50% ethanol, 5:5:90, by vol.) before being stored in 50% ethanol. Mature, healthy leaves were selected, cut into small pieces of about 5–6 mm, and dehydrated using an ethanol series. Fully dehydrated leaf materials were passed through a mixture made with alcohol/Technovit 7100 resin combinations (3:1, 1:1, 1:3) and 100% Technovit and then embedded in Technovit resin 7100 (Technovit, Germany). Serial sections of 4–5 µm thickness were cut using disposable knives, stuck onto slide glass, and dried on an electric slide warmer for 12 hours. Dried slides were stained with 0.1% Toludine blue O (C.I. 52040) for 60–90 seconds, rinsed with running water, and again dried by electric slide warmer for more than 6 hours to remove water. The stained slides were then mounted with Entellan (Merck Co., Germany) and observed under a BX-50 light microscope (Olympus Co., Japan). Photographs were taken with a digital camera system attached to the microscope and multiple image alignment was done with Photoshop CS6 for Windows 2007.

For scanning electron microscopy, mature leaves were cut into small pieces and dehydrated by ethanol series, samples were dried using a Hitachi II critical point dryer (Hitachi Co., Japan) and coated with platinum using an E-1010 ion-sputter (Hitachi Co., Japan). Micrographs were taken under 15 kV using an S-3500N scanning electron microscope (Hitachi Co., Japan).

Results

Leaf surface

Leaf surface and stomatal features are quite similar in both genera. In all species studied the stomata are highly sunken and develop in longitudinal rows forming the stomatal bands (Figs.1A-H; 2A-F). In *Abies* species the stomata are found only on the abaxial leaf surface, arranged in two bands (Fig.1A, C, E, G) and in *Picea* species the distribution of stomata varies according to the leaf shape. *Picea abies* and *P. koraiensis*, with rectangular leaves, have only one stomatal band on all four sides (Fig. 2A-B, E-F)and *P. jezoensis*, which has flattened bifacial leaves, stomata are found only on the adaxial surface and arranged into two bands (Fig. 2C-D) except at the tip of the needle (not shown).

In all species marginal cells, including subsidiary and accessory cells, are papillose (Figs.1B, D, F, H; 2B, D, F). Two sunken elongated guard cells are surrounded externally by four subsidiary cells. Two lateral and two polar subsidiary cells are poorly differentiated externally, which might be due to the presence of a thick cuticle layer. In *Picea* species, the marginal walls of epidermal cells have numerous small infoldings and that project into the cavities of the cells (Fig. 2B, D, F).

Anatomical features

Abies firma and *Abies holophylla*

These two species belong to section *Momi*. Within section *Momi*, *A. firma* represents the subsection *Firmae* and *A. holophylla* represents *Holophyllae*. Both species leaf cross section clearly reveals a bifacial structure (Fig. 3A-D). The epidermis is single-layered and composed of small elongated or oval-shaped, highly lignified cells (Fig. 3E-F). Numerous stomata are observed on the abaxial leaf surface (Fig. 3G-H). The hypodermis is single-layered with oval-shaped lignified cells (Fig. 3E-G), which is a continuous layer except in the stomatal region of the abaxial surface. Only in a few places particularly in the margin and mid-vein region, the hypodermis comprise more than one cell layer. The mesophyll is composed of parenchyma cells, differentiated into two regions: palisade and spongy (Fig. 3G). The palisade parenchyma is more developed on the adaxial side and consists of radially elongated cells in two or three layers. The spongy parenchyma is organized with loosely arranged polygonal cells, in about 70–80% of the mesophyll (Fig. 3G). Resin ducts pass through the mesophyll, and some of them are attached to the abaxial epidermis or hypodermis (Fig. 3A-F). The Korean samples of *A. firma* had four resin ducts, two large ones in the mesophyll and two small ones attached to the lower epidermis, (Fig. 3A) while the Japanese sample had only two ducts, both attached to the epidermis (Fig. 3B). In contrast, all three samples of *A. holophylla* had two resin ducts, either attached to the epidermis in the Japanese sample (Fig. 3C) or located in the mesophyll in the Korean samples (Fig. 3D).

The leaves have two collateral vascular bundles surrounded by a single-layered endodermis, and these are separated by several layers of parenchymatous cells (Fig. 3I). These paranchymatous bands are later lignified and distinguished from xylem by their larger size. A transfusion tissue composed of transfusion tracheids and parenchyma was observed in both lateral sides of the vascular bundles.

Abies koreana and *Abies nephrolepis*

Both these species belong to subsection *Medianae* of section *Balsamea*. Both species have almost similar tissue

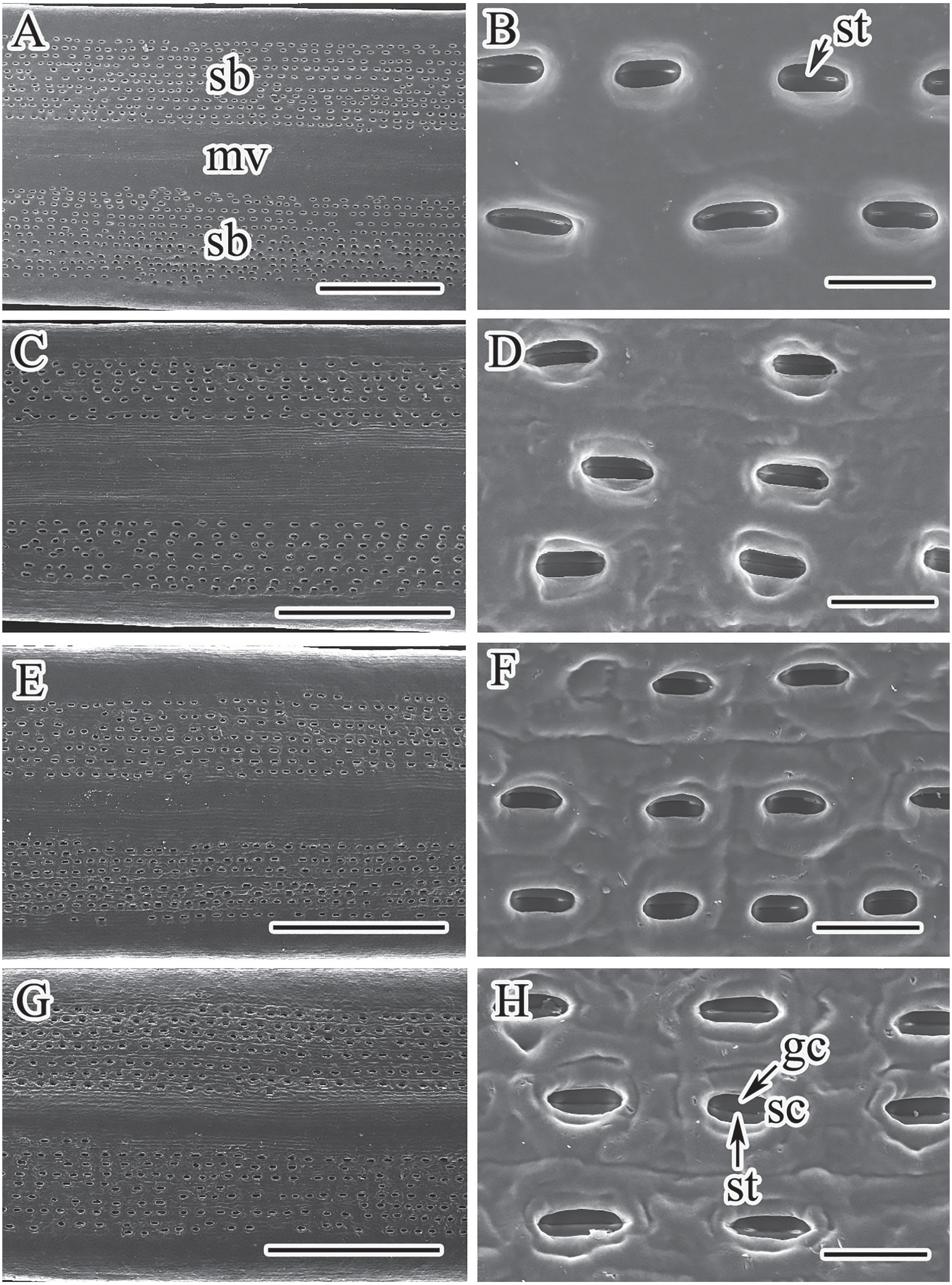

Figure 1. SEM micrograph of abaxial faces from *Abies* species. **A, B** *A. firma*. **A** Stomatal band. **B** Stomata magnified. **C, D** *A. holophylla*. **C** Stomatal band. **D** Stomata magnified. **E, F** *A. koreana*. **E** Stomatal band. **F** Stomata magnified. **G, H** *A. nephrolepis*. **G** Stomatal band. **H** Stomata magnified. *Abbreviations:* gc, guard cell; mv, mid vein; sb, stomatal band; sc subsidiary cells; st, stomata. *Scale bars:* A, C, E, G = 1 mm; B, D, F, H = 50 µm.

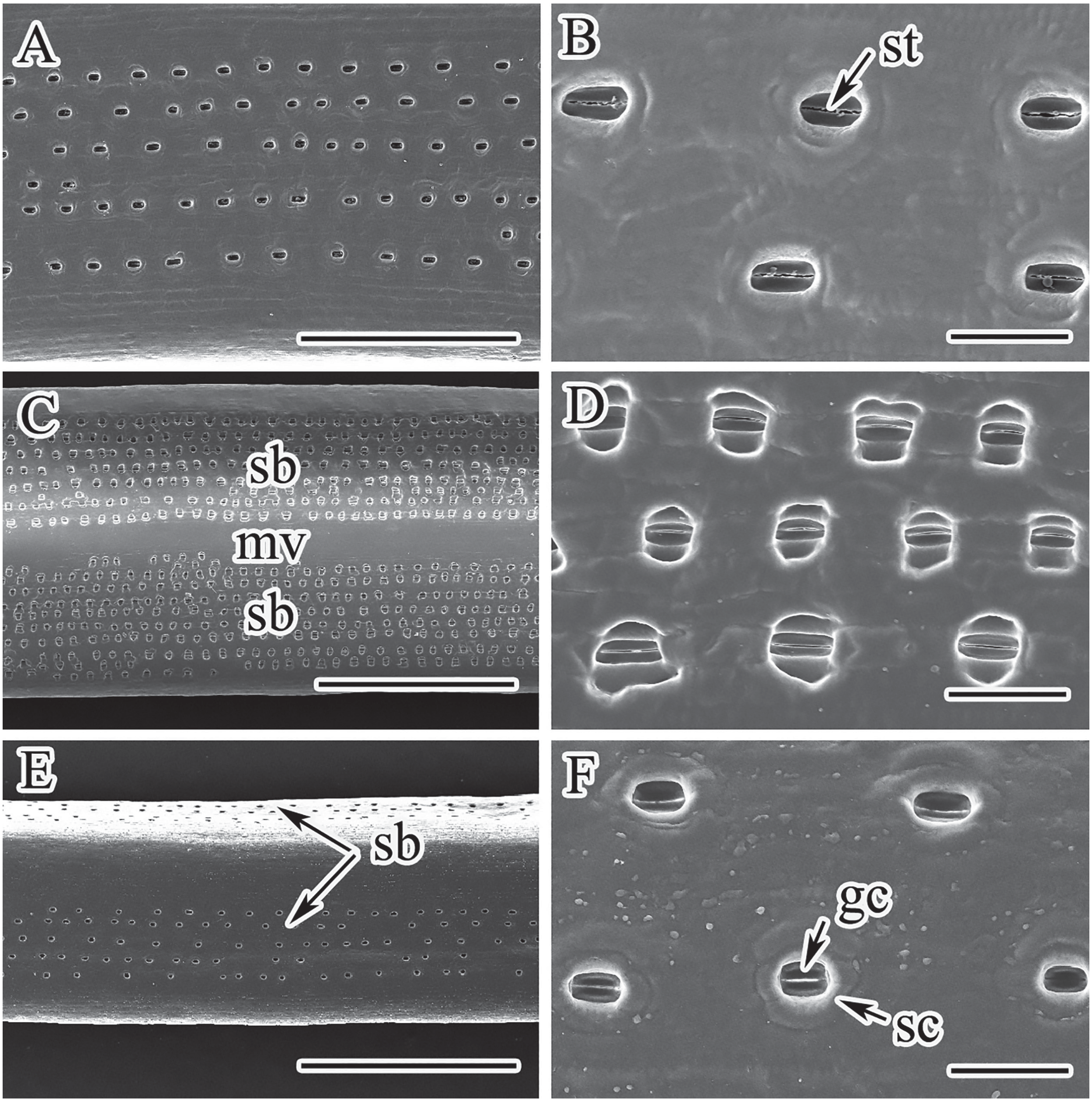

Figure 2. SEM micrograph of abaxial faces from *Picea* species. **A, B** *P. abies*. **A** Stomatal band. **B** Stomata magnified. **C, D** *P. jezoensis*. **C** Stomatal band. **D** Stomata magnified. **E, F** *P. koraiensis*. **E** Stomatal band. **F** Stomata magnified. *Abbreviations:* gc, guard cell; mv, mid vein; sb, stomatal band; sc subsidiary cells; st, stomata. *Scale bars:* A = 500 µm; B, D, F = 50 µm; C, E = 1 mm.

types and arrangement as describe for first two species. In *Abies koreana* and *A. nephrolepis* there are only two resin ducts, located in the spongy mesophyll region (Fig. 3J-K). They differ from members of *Momi* by their hypodermis. Both species lack a continuous hypodermal layer, which is found only in a few places as either isolated cells or groups of four or five cells. In the mid vein region, the hypodermis appears as group of more than five cells (Fig. 3L-M). Stomata are restricted to the abaxial surface of the leaf (Fig. 3N). Two vascular bundles separated by layers of parenchyma cells are embedded in a single layered endodermis (Fig. 3O-P).

Picea abies, *Picea jezoensis,* and *Picea koraiensis*

In cross section, leaves of *Picea abies* and *P. koraiensis* are rectangular in shape whereas leaves of *P. jezoensis* are flattened and bifacial (Fig. 4A-E) but tissue types and their arrangement are similar in all species. The epidermis is unstratified and comprises lignified and elongated or oval-shaped cells. The stomata are highly sunken and present on both sides of *P. abies* and *P. koraiensis* leaves (Fig. 4A, C, F-G) and only on the adaxial surface of *P. jezoensis* (Fig. 4D-E) leaf. The hypodermis is well developed in the

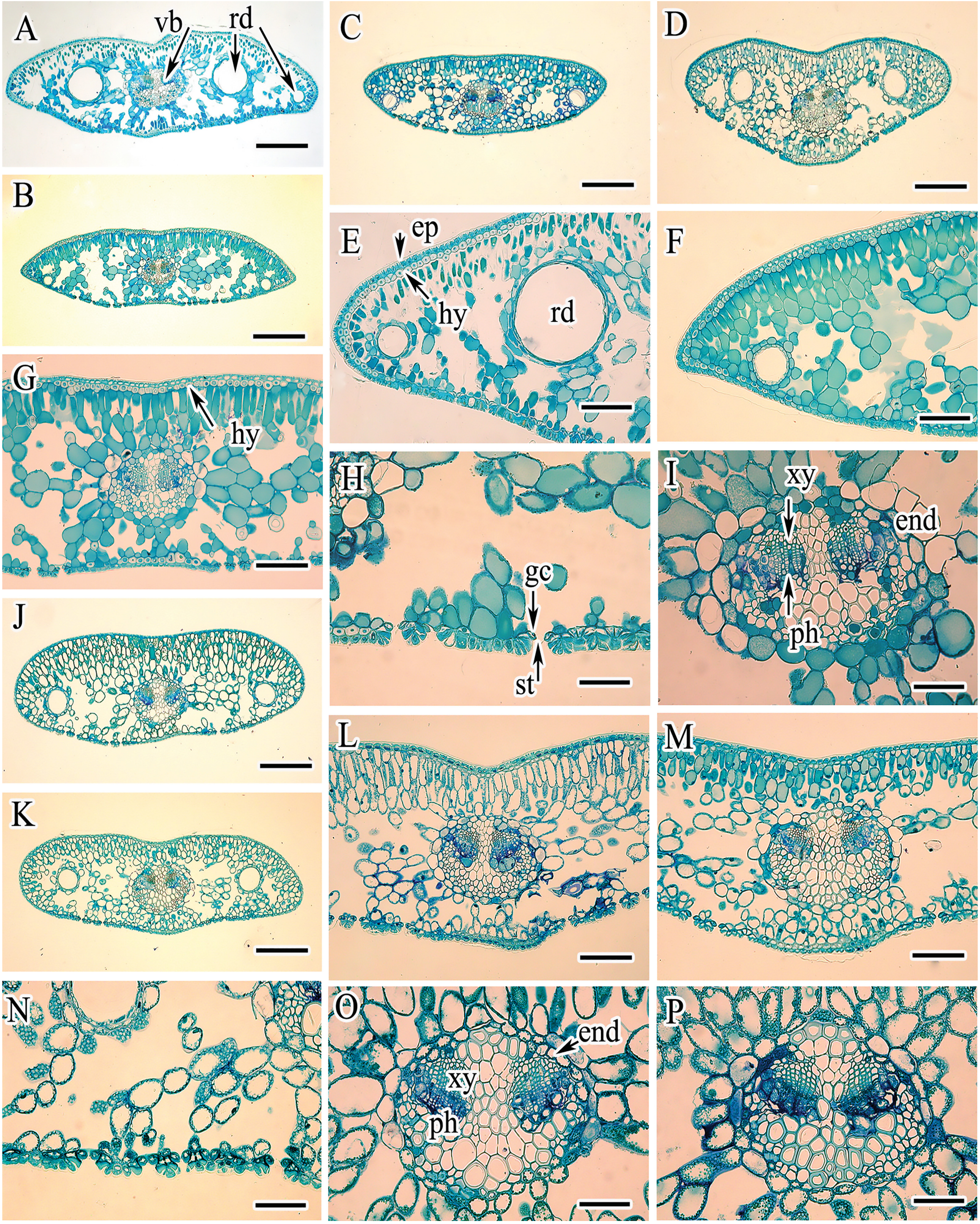

Figure 3. Cross section (CS) of *Abies* species. **A, B** *A. firma*. **C, D** *A. holophylla*. **E, F** Resin ducts of *A. firma*. **G** Mid-vein portion of *A. firma*. **H** Abaxial epidermis and stomata of *A. firma*. **I** Vascular bundle of *A. firma* leaf. **J** *A. koreana* leaf. **K** *A. nephrolepis*. **L** Portion through mid-vein of *A. koreana*. **M** Mid-vein Portion of *A. nephrolepis*. **N** Abaxial epidermis and stomata of *A. koreana*. **O** Vascular bundle of *A. koreana*. **P** Vascular bundle of *A. nephrolepis*. *Abbreviations:* ep, epidermis; end, endodermis; gc, guard cell; hy, hypodermis; ph, phloem; rd, resin duct, st, stomata; vb, vascular bundle; xy, xylem. *Scale bars*: A, B = 500µm; E, F, J, K = 400µm; G, L, M = 200µm; C, D, H, I, N, O, P =100µm.

three species and generally composed of a single-layer of lignified cells (Fig. 4A-B, E-H). The mesophyll is composed of elongated to polygon-shaped parenchyma cells with the palisade mesophyll poorly distinguished in the *P. abies* and *P. koraiensis* leaves and well developed in the adaxial surface of the *P. jezoensis* (Fig. 4A-E) leaf. The mesophyll cell walls have numerous infoldings projected into the cells with mesophyll of *Picea* species (Fig. 4F, J-K). In *P. koraiensis* there are one or two resin ducts are usually attached to the epidermis (Fig. 4B-C) and in *P. jezoensis* there are two resin ducts attached to abaxial epidermis (Fig. 4D-E, H). In all three species the vascular bundle is single or poorly divided in two by a layer of parenchyma cells and surrounded by the well-defined parenchymatous endodermis (Fig. 4J-L). A number of parenchyma cells inside the endodermis become lignified and are distinguished from the xylem cells by their larger size (Fig. 4J-L).

Discussion

Abies and *Picea* belong to two different main clades of Pinaceae, one comprising *Cathaya*, *Picea*, *Pinus*, *Larix* and *Pseudotsuga* and the other comprising *Abies*, *Cedrus*, *Keteleeria*, *Nothotsuga*, *Pseudolarix* and *Tsuga*. However,

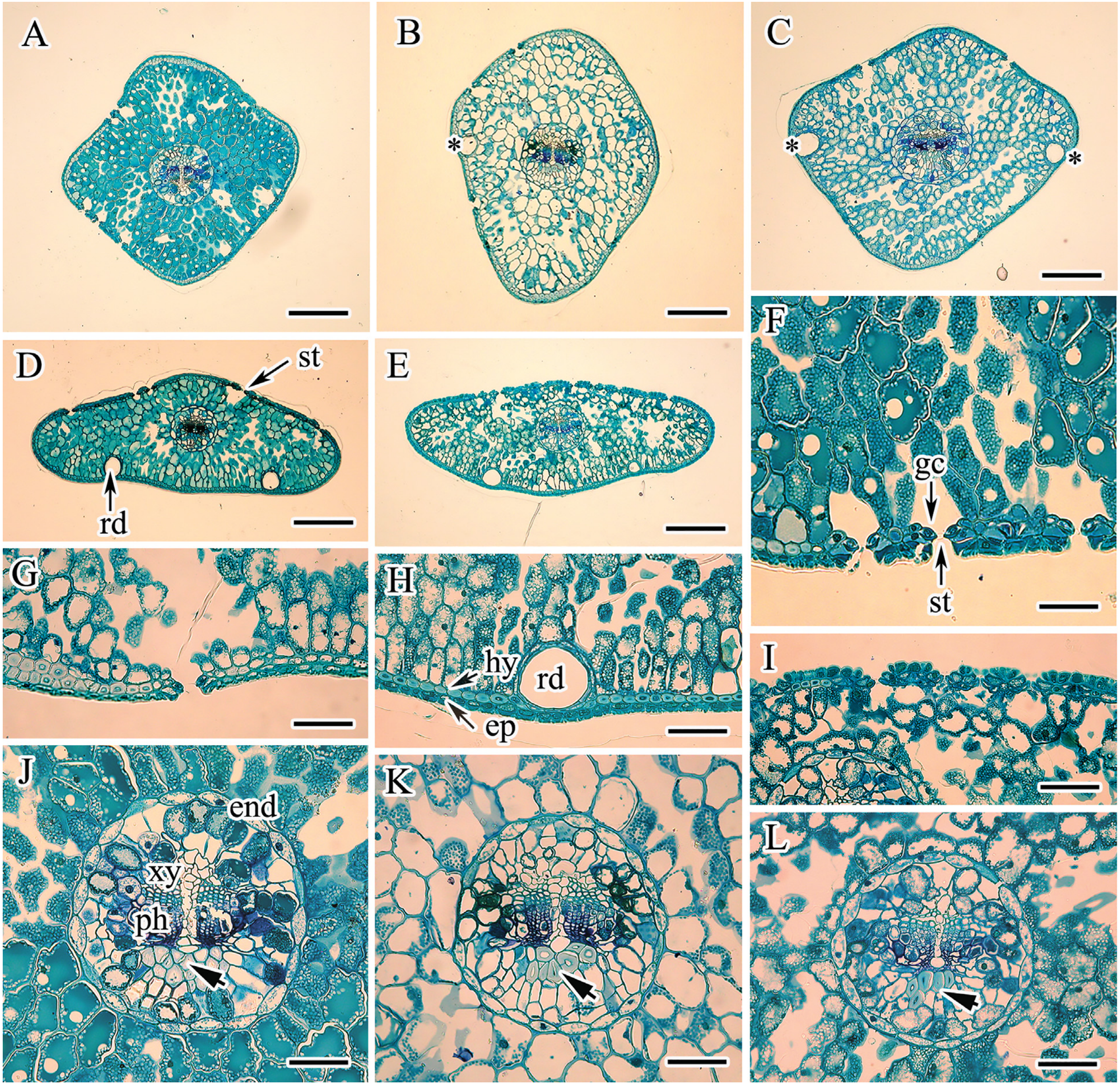

Figure 4. Cross section (CS) of *Picea* species. **A** *P. abies* leaf. **B, C** *P. koraiensis* with one and two resin ducts (asterisk). **D, E** *P. jezoensis* leaf. **F** Adaxial epidermis and stomata of *P. abies*. **G** Epidermis and stomata of *P. koraiensis*. **H** Abaxial epidermis and resin duct of *P. jezoensis*. **I** Adaxial epidermis and stomata of *P. jezoensis*. **J** Vascular bundle of *P. abies*. **K** Vascular bundle of *P. koraiensis*. **L** Vascular bundle of *P. jezoensis*. *Abbreviations:* ep, epidermis; end, endodermis; gc, guard cell; hy, hypodermis; ph, phloem; rd, resin duct; st, stomata; xy, xylem. *Scale bars*: A, D, E, I, J = 300 µm; B, C, F, G, H, K, L = 75 µm.

based on some morphological features a few earlier studies (Pilger 1926; Melchior & Werdermann 1954) placed *Abies* and *Picea* in the same subfamily, Abietoideae. Morphologically, these two genera exhibit similar leaf arrangement, an absence of short shoots, and similar form of the petiole pulvinus. Nevertheless, the grouping of the taxa based on these characters alone is very much artificial and this classification scheme for the Pinaceae was later rejected. By studying leaf anatomy and stomata we can see that these two genera only resemble in leaf surface and stomata features.

Within genus *Abies*, the species of sections *Momi* and *Balsamea* were distinguished by the nature of the hypodermal layer and the position of resin ducts. *Abies firma* and *A. holophylla* both have a continuous single-layered hypodermis whereas *A. koreana* and *A. nephrolepis* lack a continuous hypodermis, having instead isolated cells or groups of four or five lignified cells in the hypodermal region. However, this feature is not efficiently distributed in the *Abies* species. Bercu *et al*. (2010) found a continuous hypodermis in *A. alba* and discrete hypodermis in *A. nordmanniana*, and both species belong to the same section. From this it is clear that the formation of lignified hypodermis is not a significant character to distinguish section level, although it could reasonably be useful for species level. These kind of lignified cells have a protective function and thus formation of such cells might also depend on the environment condition as altitude and CO_2 concentration which have a significant effect on the thickness of hypodermis in some *Pinus* species (Lin *et al*. 2001; Tiwari *et al*. 2013).

Both species of section *Momi* collected from Japan have two marginal resin ducts, and Korean samples of *A. firma* have two marginal and two medial resin ducts and those of *A. holophylla* have two medial resin ducts. On the other hand, both *Balsamea* species included in this study, *A. koreana* and *A. nephrolepis*, had two medial resin ducts. Eo & Hyun (2013) described a similar position to this study for resin ducts in *A. nephrolepis* but found both marginal and medial ducts for *A. koreana*. They also presumed that the movement of resin ducts from the marginal to the medial position is latitude-dependent, with samples of lower latitude having marginal ducts and samples of higher latitude having medial ducts. Surprisingly enough, in this study, *A. koreana*, collected from three different locations including Mt. Hala of Jeju Island, had no marginal resin ducts. Bercu *et al*. (2010) described only marginal resin ducts in two species *A. alba* and *A. nordmanniana* of section *Abies*.

As already stated, the infrageneric classification of the genus remains quite controversial (Liu 1982; Schmidt 1989; Farjon 1990; 2001; Fu *et al*. 1999), and use of morphology alone is not a reliable guide to relationships within *Picea* (Ran *et al*. 2006; Ledig *et al*. 2004). Results from this study were no exception, with no significant differences observed in tissue type or arrangement in three species, except the flattened shape of *P. jezoensis*. *P. abies* and *P. koraiensis* resembled each other in many details, including shape and distribution of stomata, except the number and position of resin ducts. The number and position of resin ducts are also an extremely flexible character in species, as seen for *A. firma* in this study and for many *Pinus* species (Sheue *et al*. 2003; Tiwari *et al*. 2013). Furthermore, needle anatomy of North American species revealed continuous and discontinuous types of resin duct in *Picea* (Weng & Jackson 2000). In this study, we did not observe the *Picea* needle in such a way; however, it is worthwhile mentioning that *P. abies* and *P. koraiensis* might represent the discontinuous-ducts group because different sections exhibited variation in the number of resin ducts. On the other hand, all the sections of *P. jezoensis* had two resin ducts in the same position, and so might represent the continuous-ducts group.

Stomata were distributed on the adaxial surface of *P. jezoensis* leaves, which seems unusual for a flat bifacial leaf. Identical leaf form, stomatal distribution, and equal number and position of resin ducts have been observed in section *Omorika* (Marco 1939). *Picea jezoensis* also differs from *P. abies* and *P. koraiensis* by virtue of its flexible and loosely imbricate seed scale compared to the rigid and closely imbricate seed scale in the latter two species. More importantly, in molecular phylogeny of the genus, *P. abies* and *P. koraiensis* were nested in the same clade and *P. jezoensis* was placed in a separate clade (Ran *et al*. 2006).

The common leaf anatomical features in *Abies* and *Picea* include sunken stomata, lignified single-layered epidermis with lignified hypodermis, mesophyll comprising parenchyma cells full of plastids, presence of resin ducts, and well-developed endodermis encircling either one or two fibrovascular bundles. There are two fibrovascular bundles in *Abies* surrounded by common endodermis and separated by a band of parenchyma tissue which is later lignified. However, in *Picea* the single vascular bundle is weakly divided by one or two layers of parenchyma half way through the bundle. The parenchyma bands in the middle of the vascular bundle are evidence that in an earlier evolutionary period there might have been two bundles (Marco 1939). Lacassagne (1934) stated that in the cortex (sterigmata) the bundles of the leaf trace are distinct but in the leaves they are fused. Weng & Jackson (2000), however, showed only one vascular bundle in North American spruces.

In conclusion, leaf anatomy of both genera clearly displays the typical coniferous and xerophytic features. Some of the anatomical features, such as shape in cross section, arrangement of hypodermis, position, nature and number of resin ducts, and distribution of stomata might be key features for distinguishing species. In *Abies*, the species of the two sections are quite easily distinguished by the nature of the hypodermis and the position of resin ducts. However, we only considered two species from each section, and study of more species from both sections will help to validate this result. In *Picea*, leaf anatomical features are quietly in support of molecular data. As a result, even with geographical disjunction, *P. koraiensis* is more closely related to *P. abies* than *P. jezoensis*. We believe that anatomical data are useful for the systematic study of conifers and this study have provided potential output for systematic and phylogenetic studies of *Abies* and *Picea*. Similar research on other species will help to elucidate phylogenetic relationships both between species in each genus and among genera.

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