

Petiole anatomical features in *Salix* and some of its relatives in the family *Salicaceae*

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The classification of the genus *Salix* is troubled by the intra-specific flexibility and diversity of the morphological features. The aim was to prepare a comparative description of petiole anatomy of 23 species of *Salix*, in addition to two species of *Populus* and *Bivinia jalbertii*, as well as to determine the taxonomic importance of petiole features. In order to do so, 10 quantitative and 10 qualitative petiole anatomical features were studied. Moreover, a Bayesian analysis was executed on the relevant ITS sequences obtained from GenBank, included 28 specimens from 17 species. The anatomical features were then mapped on the resulted cladogram and the ancestral states of some prominent petiole anatomical features were reconstructed. Based on the results, no stomata were observed in the petiole's epidermis of any of the species studied; therefore, it could be assumed that, the petiole in the studied species is dependent to the leaf blade for respiration and gas exchange. While the quantitative petiole anatomical features were turn to be variable in the individuals of the same species grown in different habitats, qualitative features showed a desirable consistency. The petiole anatomical features studied were proved to be useful in the differentiation and identification of taxa at sub-generic and specific levels. In addition, the reconstruction of the ancestral state of the studied features revealed that, polygonal cell shape, straight or slightly curved anticlinal wall pattern, horizontal compression of the petiole, the presence of lateral ribs, convex adaxial surface and ribbed petiole outline were primitive features in the species studied.

Keywords: *Amanthaceae*, *Bivinia*, *Malpighiales*, phylogeny, *Populus***صفات تشریحی دمبرگ در بید و برخی از خویشاوندانش در تیره بیدیان***

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خلاصه

رده‌بندی جنس *Salix* L. (تیره بیدیان) به دلیل انعطاف‌پذیری و تنوع درون گونه‌ای در صفات ریخت‌شناختی مشکل‌آفرین است. هدف از این پژوهش، توصیف و مقایسه تشریح دمبرگ ۲۳ گونه از جنس *Salix*، دو گونه از جنس *Populus* L. و گونه *Bivinia jalbertii* Tul. و همچنین تعیین اهمیت سیستماتیکی صفات تشریحی دمبرگ است. به این منظور، ۱۰ صفت کمی و ۱۰ صفت کیفی مرتبط با تشریح دمبرگ بررسی و ارزیابی شدند. علاوه بر این، تحلیل بی‌زین روی توالی ITS موجود از ۲۸ نمونه از ۱۷ گونه مرتبط موجود در GenBank انجام شد، صفات تشریحی روی کلادوگرام به دست آمده مکان‌یابی شدند و حالت اجدادی برخی صفات تشریحی بازسازی شد. براساس نتایج به دست آمده، هیچ یک از ۲۶ گونه بررسی شده دارای روزنه در دمبرگ نبود و بنابراین، می‌توان نتیجه گرفت که دمبرگ برای تنفس و تبادل گاز متکی به پهنک است. با وجود آن که صفات کمی بین افراد مختلف یک گونه که در زیستگاه‌های مختلف رویش کرده بودند متغیر بود، صفات کیفی ثابت خوبی از خود نشان دادند. صفات تشریحی دمبرگ در گونه‌های بررسی شده با موفقیت جهت تمایز بین آرایه‌های زیرجنسی و گونه‌ها به کار برده شدند. به علاوه، بازسازی حالت اجدادی صفات بررسی شده نشان داد که شکل سلولی چندضلعی، الگوی صاف تا اندکی خمیده در دیواره‌های آنتی‌کلینال سلول‌های اپیدرم، فشردگی افقی دمبرگ، حضور پرده‌های جانبی در دمبرگ، سطح فوقانی محدب و حاشیه شیاردار دمبرگ، صفات ابتدایی در گونه‌های بررسی شده بودند.

واژه‌های کلیدی: تبارزایی، شاتون‌داران، صنوبر، مالپیگیال، *Bivinia*

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Introduction

The family *Salicaceae* Mirb. s.l. (including *Flacourtiaceae*, *Samydeaceae*, and *Scyphostegiaceae*) consists of nine tribes, 54 genera and 1250 species (Mabberley 2018). According to the APG IV treatment, *Salicaceae* s.l. is well-placed into the *Malpighiales* clade (Chase *et al.* 2016, Christenhusz 2016). The *Salicaceae* s.s. contains the genus *Salix* L., with 450 species thriving in the temperate regions of the northern hemisphere, and the genus *Populus* L., with 30 species thriving mainly in the cold and temperate regions of the northern hemisphere (Mabberley 2018).

Salix species from Iran were the subject of several studies. In the 4th volume of *Flora Orientalis* (Boissier 1879), 10 *Salix* species were mentioned from Iran. *Flora Iranica* treatment (Skvortsov 1969) recognized the presence of 12 species of *Salix* in Iran. However, according to Maassoumi (2009) and the treatment of the *Flora of Iran* (Maassoumi *et al.* 2011), genus *Salix* has about 32 species and six hybrids in Iran.

The genus *Salix* is originated in the warm temperate regions of the new world and its subsequent speciation occurred in the cold regions of the northern hemisphere (Skvortsov 1968). The most important diversity centers of *Salix* in the old world were found to be in the mountainous regions of China and former Soviet Union (Skvortsov 1968, 1999). This assumption was strengthened by the excavated fossil records of *Salicaceae*, as the earliest known fossil records of *Populus* found in the late Paleocene (ended about 65 MYA), and the fossil records of the genus *Salix* found in the Eocene (55–65 MYA). The oldest fossils belong to the *Salix* subg. *Salix* were found in North American Early Eocene formations in Wyoming and North Dakota, and earliest fossil records of the *Salix* subgen. *Vetrix* were found in Alaska, North America, dated back to Oligocene, 38–55 MYA (Leskinen & Alström-Rapaport 1999).

Salix and *Populus*, the main genera of the family *Salicaceae* s.s., were present in the Linnaeus's *Genera Plantarum* (1737) under the categories of Dioecia

Diandria and Dioecia Octandria, respectively. In the first edition of Linnaeus's *Species Plantarum* (1753), 29 species of *Salix* and five species of *Populus* were described. The first conclusive taxonomic rank which included both *Salix* and *Populus* was made in the family *Amanthaceae* Juss., as described by de Jussieu in 1798. However, this family, was comprised of many distantly-related arboreal catkin-bearing genera, including hermaphrodite genera (*Fothergilla* L., *Ulmus* L., and *Celtis* L.), dioecious genera (*Salix*, *Populus*, and *Myrica* L.), and monoecious genera (*Betula* L., *Carpinus* L., *Fagus* L., *Quercus* L., *Corylus* L., *Liquidambar* L., and *Platanus* L.).

Mirbel (1815), segregated the family *Amanthaceae*, and aggregated the monoecious and dioecious arboreal catkin-bearing taxa of dissolved *Amanthaceae* into the family *Salicaceae* Mirb. (*Salicineae*). The resulting *Salicaceae* included *Salix*, *Populus*, *Betula*, and *Alnus* genera. However, *Salicaceae* s.s. was circumscribed to include only dioecious amentaceous genera, i.e., *Salix* and *Populus*, by Samuel Fredrick Gray in his prominent work entitled: "A natural arrangement of British plants: according to their relations to each other as pointed out by Jussieu, De Candolle, Brown, & c" (1821), and this circumscription was followed by Richard (1828), Lindley (1830), Richard & MacGillivray (1831), Loudon (1838), Hooker & Arnott (1855), Boissier (1879), and Engler (1884).

The circumscription of the family *Salicaceae*, which previously assumed to be highly constant, undergone a major shift in the late 20th. century and early 21st., by the publication of major revisions on the basis of molecular phylogenetic studies. Leskinen & Alström-Rapaport (1999) proposed the inclusion of the family *Flacourtiaceae* into the *Salicaceae*, as the species of the former were placed well into those belong to the latter family. In 2002, Chase *et al.* not only approved the results of Leskinen & Alström-Rapaport (1999) but recommended the recognition of *Salicaceae* *sensu lato*, including tribes *Banareae*, *Bembicidae*, *Scolopieae*, *Samydeae* (syn. *Caseariae*), *Homalieae*, *Flacourtieae*,

Prockieae, and *Saliceae* (*Salicaceae* *sensu stricto*), as well as *Abatieae* and *Scyphostegieae*.

The classification of the genus *Salix* is troublesome due to the tendency of its species for flexibility and diversity in their morphological traits, life forms and habitats, dioecious habit and different flowering times of male and female individuals as well as frequent hybridization and variation in ploidy number (Chen *et al.* 2008a, Khalili *et al.* 2012), resulting in inconclusive arguments about the proper definition of infrageneric taxa and their circumscriptions (Argus 2007, Maassoumi 2009).

The aforementioned uncertainty is clearly reflected in the history of sub-generic classification of *Salix*. Linnaeus in *Species Plantarum* (1753) introduced an undefined sub-generic ranking the classification of the *Salix* species, on the basis of leaf indumentum characteristics. One of the earliest sub-generic systematic classifications of *Salix* was proposed by Dumortier (1862), by the definition of two series (*Caparisalix* and *Vitalisalix*), five subgenera (*Vetrix*, *Vimen*, *Helice*, *Amerina*, and *Lygus*) and eight sections, based on the characteristics of the nectaries and stamens. Andersson (1868) splits the genus *Salix* into 19 sections and three undefined higher taxa, i.e., *Peliandrae*, *Diandrae*, and *Synandrae*, on the basis of the stamen numbers and characteristics. The genus *Salix* was split into two subgenera, *Protitea* and *Euitea*, by Kimura in 1928, considering the bud scale types (Barkalov & Kozyrenko 2014). Rechinger and Akeroyd (1964) classified the species of *Salix* into three subgenera, i.e., *Salix*, *Chamaetia*, and *Caparisalix* on the basis of the plant habit and the characteristics of leaves, catkins, bracts, stamens and nectaries. Skvortsov (1968) included the *Toisusu* Kimura in the genus *Salix* and introduced three subgenera, i.e., *Chamaetia*, *Salix*, and *Vetrix*.

It should be noted that, in addition to the plant habit, leaf characteristics and other features, Skvortsov applied the petiole characteristics in the delimitation of the subgenera and sections. Argus (1997) carried

out a phenetic analysis on the genera *Salix* and *Chosenia* Nakai, and divided the genus *Salix* into four subgenera of *Salix*, *Longifolia*, *Chamaetia*, and *Vetrix*. Ohashi (2000) divided this genus into four subgenera of *Salix*, *Urbanianae*, *Chamaetia*, and *Vetrix* whereas Argus (2007) recognized five subgenera in the genus *Salix*, i.e., *Salix*, *Longifolia*, *Chamaetia*, *Vetrix*, and *Protitea*.

In recent years, numerous molecular studies were performed on *Salicaceae* in general and the genus *Salix* in particular, some of which were those executed by Leskinen & Alström-Rapaport (1999), Chen *et al.* (2010), Hardig *et al.* (2010), Abdollahzadeh *et al.* (2011), Angaji *et al.* (2012), Barkalov & Kozyrenko (2014a,b), Lauron-Moreau *et al.* (2015), Wu *et al.* (2015), Diffey (2017), and Li *et al.* (2019).

In addition to those conventional morphological traits utilized in the sub-generic classification of the genus *Salix*, some anatomical features, e.g., epidermal properties of leaf and stomata, were proved to be useful in the taxonomy of *Salix* (Binns & Blunden 1980, Chen *et al.* 2008b, Ghahremaninejad *et al.* 2012, Khalili *et al.* 2010). Moreover, it should be noted that, the leaf and petiole features were among the subject of interest in the literature of other genera of the *Salicaceae* as well. Thadeo *et al.* (2014) applied foliar anatomical features as well as petiole characteristics to their taxonomic study on some neotropical taxa of *Salicaceae*, and found them suitable for diagnostic purposes. Petiole features were found to be useful in the differentiation and diagnosis of *Populus* species (Klimov & Proshkin 2019). Proshkin & Klimov (2019) successfully applied the petiole features to distinguish the inter-sectional hybrids in *Populus* as well as diagnosis at specific level. Paiero *et al.* (1992) proposed that, the presence of trichomes and the thickness of the wax layer in the leaves of *Salix* species reflect the adaptation strategies of the species studied according to the climatic conditions.

The present study, examined the anatomical features of petiole in 23 species of the genus *Salix*. In addition, two species of the genus *Populus* species and a Madagascan species, *Bivinia jalbertii* Tul. which was previously placed in the *Flacourtiaceae* and subsequently merged into *Salicaceae*, were included. The purpose of this study was to evaluate the value of some anatomical and morphological traits of petiole in the identification and classification of *Salix* species and to compare their features in the genus *Salix* with those in *Populus* and *Bivinia jalbertii*.

Materials and Methods

- Anatomical study

In order to achieve the research objectives, mature leaf petioles were collected from 27 herbarium specimens stored in the herbaria of Natural History Museum of Vienna (W), Kharazmi University (T) and Natural History Museum of Paris (P), the taxon names and the attributed voucher specimens of the sources were presented in table 1. The herbarium specimens were identified using relevant literature, i.e., Flora Iranica (Skvortsov, 1969), Flora of Iran (Maassoumi *et al.* 2011), Flore de Madagascar et des Comores: plantes vasculaires (Humbert & Leroy 1946), Flora of North America (Argus 2010), Flora of Ecuador (Persson *et al.* 2014), and Flora Europaea (Rechinger & Akeroyd, 1964). The petiole specimens were then rehydrated and placed in FAA (formaldehyde-acetic acid-ethanol) fixative solution for 24–48 hours. The petiole specimens were then transferred to 70% alcohol for long-term preservation. The hand-sectioning was performed on the middle region of each petiole, in order to obtain comparable results. The sections were double-stained with Methyl Green and Bismarck Brown. The epidermis of each

petiole specimen was separated and stained by Fuchsin. The prepared samples were investigated by an optical microscope (Zeiss Standard 20) and the images were taken by means of a digital camera.

- Phylogenetic analyses

The ITS sequences of 28 relevant specimens (17 species) were downloaded from GenBank, the taxon name, their original localities and accession numbers were presented in table 2. Sequences were assembled using Geneious Prime2020.1.2 created by Biomatters (<http://www.geneious.com>). The DNA sequences were aligned using Clustal X2 (Larkin *et al.* 2007), followed by manual correction. The Bayesian (BA) analysis was performed to determine the phylogenetic relationships on the ITS dataset, while to select the best model of sequence evolution, MrModeltest Ver. 2.3 software (Nylander, 2004) was utilized.

Bayesian analyses were performed using MrBayes Ver. 3.2.7 (Ronquist & Huelsenbeck 2003). Two independent runs were performed, each consisting of two parallel Markov chain Monte Carlo (MCMC) of 10 million generations (the average standard deviation of split frequencies being lower than 0.01). Trees were sampled every 10,000 generations. The evolution models as selected by MrModeltest were GTR+G, with NST equal to 6. Sumt burn-in was set to 25 percent. The final consensus tree and associated posterior probabilities were graphically visualized by Figtree 1.4.4 (Rambaut 2012).

In order to trace characters over the obtained phylogenetic tree, Delta-Editor software (Dallwitz *et al.* 2007) was utilized to construct the taxon-characteristics matrix, and Mesquite Ver. 3.61 (Maddison & Maddison 2019) was performed to combine the consensus tree with the taxon-characteristics matrix and to analyse the character evolution.

Table 1. Specimens studied in the anatomical survey along with their related data

Taxon	Subgenus	Location	Voucher specimens
<i>Salix aegyptica</i> L.	<i>Vetrix</i>	Iran	T: Azadbakht 7488
<i>S. alba</i> L.	<i>Salix</i>	Iran	T: Azadbakht 23441
<i>S. amygdaloides</i> Andersson	<i>Protitea</i>	United States	W: Beattie 11607
<i>S. appendiculata</i> Vill.	<i>Vetrix</i>	Austria	W: Polatschek s.n. 1969-05-10
<i>S. atrocinerea</i> Brot.	<i>Vetrix</i>	United Kingdom	W: Rechinger 65422
<i>S. babylonica</i> L.	<i>Salix</i>	Afghanistan	W: Rechinger 37197
<i>S. calliantha</i> Jos. Kern.	<i>Vetrix</i>	Austria	W: Woloszczak 1462
<i>S. calodendron</i> Wimm.	<i>Vetrix</i>	Latvia	W: Lackschewitz 105
<i>S. caprea</i> L.	<i>Vetrix</i>	Finland	W: Laaksonen & Kyosti 663
<i>S. cordifolia</i> Pursh.	<i>Chamaetia</i>	France	W: Boivin & Blain 495
<i>S. discolor</i> Muhl.	<i>Vetrix</i>	Canada	W: Rechinger 1223
<i>S. elaeagnos</i> Scop.	<i>Vetrix</i>	Austria	W: Walter 8861
<i>S. excelsa</i> S.G.Gmel.	<i>Salix</i>	Afghanistan	W: Lindberg 1065
<i>S. foetida</i> Schleich.	<i>Vetrix</i>	Austria	W: Polatschek s.n. 1988-09-07
<i>S. glabra</i> Scop.	<i>Vetrix</i>	Austria	W: Strudl s.n. 2013-06-29
<i>S. humboldtiana</i> Willd.	<i>Protitea</i>	Bolivia	W: Ritter, Garrett & Ritter 2135
<i>S. × persica</i> L.	<i>Salix</i>	Iran	T: Azadbakht 7059
<i>S. pycnostachya</i> Andersson	<i>Vetrix</i>	Afghanistan	W: Roemer 32
<i>S. reticulata</i> L.	<i>Chamaetia</i>	Slovakia	W: Mikolas 9722
<i>S. silesiaca</i> Willd.	<i>Vetrix</i>	Slovakia, Bukovské Vrchy Mts.	W: Mikolas 15353
<i>S. silesiaca</i> Willd.	<i>Vetrix</i>	Slovakia, Vysoké Tatry Mts.	W: Mikolas 4690a
<i>S. viminalis</i> L.	<i>Vetrix</i>	Austria	W: Walter 6545b
<i>S. waldsteiniana</i> Willd.	<i>Vetrix</i>	Italy	W: Thomas & Manuel 16974
<i>Salix xanthicola</i> K.I.Chr.	<i>Vetrix</i>	Greece	W: Christensen & Bruhn Moller 3330
<i>Populus alba</i> L.	-	Iran	T: Bahari 10981
<i>Populus nigra</i> L.	-	Iran	T: Azadbakht 7067
<i>Bivinia jalbertii</i> Tul.	-	Madagascar	P: Bardot-Vaucoulon 950

Table 2. Specimens studied in the phylogenetic analyses along with their related data

Taxon	GenBank accession number	Location
<i>Populus alba</i>	JQ898621	China
<i>P. nigra</i>	JQ898631	China
<i>Salix acmophylla</i> (syn.: <i>S. persica</i>)	AB685275	W. Asia (Iran)
<i>S. acmophylla</i> (syn.: <i>S. persica</i>)	EF060388	N. Africa
<i>S. aegyptiaca</i>	AB685276	W. Asia (Iran)
<i>S. alba</i>	AB685277	W. Asia (Iran)
<i>S. alba</i>	AB685278	W. Asia (Iran)
<i>S. amygdaloides</i>	EU784080	Canada
<i>S. amygdaloides</i>	KC415493	Canada
<i>S. atrocinerea</i>	AB685280	W. Asia (Iran)
<i>S. babylonica</i>	AB685282	W. Asia (Iran)
<i>S. babylonica</i>	KC415496	China
<i>S. caprea</i>	AB685284	W. Asia (Iran)
<i>S. caprea</i>	MG772956	China
<i>S. discolor</i>	KX008814.1	Canada
<i>S. discolor</i>	KX008815.1	Canada
<i>S. elaeagnos</i>	KX008819.1	Europe
<i>S. elaeagnos</i>	KX008820.1	Europe
<i>S. excelsa</i>	AB685291	W. Asia (Iran)
<i>S. humboldtiana</i>	EF060372	C.S. America
<i>S. humboldtiana</i>	KX008848	C.S. America
<i>S. purpurea</i> (syn.: <i>S. calliantha</i>)	AJ006430	Europe
<i>S. purpurea</i> (syn.: <i>S. calliantha</i>)	LN734818	Europe
<i>S. pycnostachya</i>	AB685299	W. Asia (Iran)
<i>S. reticulata</i>	FR693637	E. Russia
<i>S. reticulata</i>	KX008918	Canada
<i>S. viminalis</i>	KX008956	Europe
<i>S. viminalis</i>	MH711555	China

Results

Notable anatomical features in studied *Salix* species are summarized in tables 3 & 4. These features were obtained by the examination of the prepared specimens of petiole epidermis and petiole cross sections, and the micrographs of which were depicted in figures 1–3.

The studied species were found to have polygonal (e.g., in *Salix aegyptica* and *S. alba*), polygonal to spherical (e.g., in *Bivinia jalbertii*), rectangular to slightly polygonal (e.g., in *S. discolor* and *S. pycnostachya*) or long spindle-shaped (e.g., in *S. amygdaliodes*) epidermal cells; straight (e.g., in *S. aegyptica* and *S. atrocinerea*), straight-slightly curved (e.g., in *S. calliantha* and *S. excelsa*) or straight-curved (e.g., in *S. caprea* and *B. jalbertii*) anticlinal cell-walls in epidermis; glabrous (e.g., in *P. nigra* and *S. glabra*) or pubescent (e.g., in *B. jalbertii*, *P. alba* and *S. discolor*) indumentum; (if pubescent) sparse (less than 10 trichomes in 1 mm², e.g., in *S. cordifolia* and *S. discolor*), intermediate (10–100 trichomes in 1 mm², e.g., in *S. foetida* and *S. persica*) or dense (more than 100 trichomes in 1 mm², e.g., in *S. aegyptica* and *S. viminalis*) trichome density; trichomes distributed on abaxial-adaxial (e.g., in *S. calodendron* and *S. eleagnos*) or adaxial (e.g., in *S. persica* and *S. reticulata*) surface; circular (e.g., in *S. aegyptica* and *S. alba*), oval (e.g., in *S. caprea* and *S. cordifolia*) or triangular-shaped (e.g., in *S. discolor* and *S. foetida*) petiole; smooth (e.g., in *B. jalbertii* and *S. humboldtiana*), smooth-ribbed (e.g., in *S. pycnostachya* and *S. viminalis*) or ribbed (e.g., in *S. eleagnos* and *S. reticulata*) petiole outline; vascular bundles with continuously oval (e.g., in *S. aegyptica* and *S. alba*), interrupted oval (e.g., in *S. calliantha* and *S. cordifolia*), continuously circular (e.g., in *S. appendiculata* and *S. persica*) or interrupted circular (e.g., in *P. nigra* and *S. glabra*) in shape; petioles compressed either horizontally (e.g., in *S. caprea* and *S. excelsa*) or vertically (e.g., in *Populus alba* and *P. nigra*); convex (e.g., in *S. discolor* and *S. alba*), straight (e.g., in *S. appendiculata* and *S. calodendron*) or

concave (e.g., in *S. calliantha* and *S. cordifolia*) adaxial surface curvature; with or without lateral ribs; and 1 central (e.g., in *S. aegyptica* and *S. alba*), 1 central and two marginal (e.g., in *S. calliantha* and *S. amygdaliodes*) or 1 central and 1 marginal (e.g., in *S. excelsa* and *S. waldsteniana*) vascular bundles in the middle region of the petiole.

The petiole thickness varied between 301.55 to 1126.93 µm, with the lowest amounts were belonged to *S. amygdaliodes* and *S. discolor*, while the highest amount of which was belonged to *S. aegyptica*. The phloem layer thickness, as measured in the species studied, varied between 27.19 µm in *S. calliantha* to 101.88 µm in *S. aegyptica*. The xylem layer thickness ranged between 28.44 µm in *S. amygdaliodes* to 117.39 µm in *S. aegyptica*. The thickness of upper parenchyma layer ranged between 41.82 µm in *S. amygdaliodes* to 258.92 µm in *S. aegyptica*, while the thickness of lower parenchyma layer varied between 53.98 µm in *S. humboldtiana* to 255.30 µm in *S. calodendron*. The thickness of upper collenchyma layer ranged between 43.19 µm in *S. discolor* to 186.70 µm in *S. aegyptica*, while the thickness of lower collenchyma layer varied between 53.30 µm in *S. amygdaliodes* to 178.17 µm in *S. aegyptica*. The thickness of upper sclerenchyma layer ranged between 8.95 µm in *S. amygdaliodes* to 49.86 µm in *S. viminalis*, while the thickness of lower sclerenchyma layer varied between 12.75 µm in *S. amygdaliodes* to 53.83 µm in *S. excelsa*.

The study on the petiole dermis revealed different values in the size of the collenchyma and parenchyma layers which caused an imbalance in the thickness of the upper and lower dermis, for instance, in *S. amygdaloides* and *S. glabra*.

The vascular bundles in the *Salix* species studied were found to be arrangement horizontally, however, the vascular bundles were found to be arranged vertically in *Populus alba* and *P. nigra*.

No stomata were found in the epidermis of any species studied. The indumentum of petiole specimens was found to be glabrous (e.g., in *S. amygdaloides* and

S. babylonica) or pubescent. The pubescent species could be further divided into those with dense trichomes (e.g., *S. elaeagnos* and *S. viminalis*), intermediate density trichomes (e.g., *S. purpurea* and *S. acmophylla*) and those with sparse trichomes (e.g., *S. reticulata* and *S. discolor*).

It should be noted that, the species with pubescent petiole and concave adaxial surface have their trichomes dispersed only on their upper surface of the petioles, some examples of which were *S. reticulata*, *S. cordifolia*, *S. × persica*, and *S. calliantha*.

The 50% majority rule consensus tree from the Bayesian analysis was depicted as a phylogram in figure

4A and a cladogram in figure 4B, while its branch support values were attributed as Bayesian posterior probabilities. The sub-generic status and the selected petiole anatomical features, i.e., the indumentum of epidermis, adaxial surface curvature, petiole outline, as well as the presence of absence of lateral ribs, were traced on the Bayesian-inferred phylogenetic tree as depicted in figure 5. The clade a in the cladogram of figure 4B contained the species belong to the subgenus *Protitea*, while the partially unresolved clade b had two subsets, group c with members of subgenera *Vetrix* and *Chamaetia*, and clade d with species belong to the subgenus *Salix*.

Table 3. Anatomical traits of epidermis in the studied species of *Salix*, *Populus*, and *Bivinia*

Taxon	Epidermal cell shape	Anticlinal wall pattern	Indumentum	Trichome density	Trichome distribution
<i>Salix aegyptica</i>	Polygonal	Straight	Pubescent	Dense	Adaxial-abaxial
<i>S. alba</i>	Polygonal	Straight-slightly curved	Glabrous	-	-
<i>S. amygdaliodes</i>	Long spindle-shaped	Straight-slightly curved	Glabrous	-	-
<i>S. appendiculata</i>	Polygonal	Straight-slightly curved	Pubescent	Intermediate	Adaxial-abaxial
<i>S. atrocinerea</i>	Polygonal	Straight	Pubescent	Dense	Adaxial-abaxial
<i>S. babylonica</i>	Polygonal	Straight-slightly curved	Glabrous	-	-
<i>S. calliantha</i>	Polygonal	Straight-slightly curved	Pubescent	Intermediate	Adaxial
<i>S. calodendron</i>	Polygonal	Straight-slightly curved	Pubescent	Dense	Adaxial-abaxial
<i>S. caprea</i>	Polygonal	Straight-curved	Pubescent	Dense	Adaxial-abaxial
<i>S. cordifolia</i>	Polygonal	Straight-slightly curved	Pubescent	Sparse	Adaxial
<i>S. discolor</i>	Rectangular to slightly polygonal	Straight-slightly curved	Pubescent	Sparse	Adaxial-abaxial
<i>S. eleagnos</i>	Polygonal	Straight-slightly curved	Pubescent	Dense	Adaxial-abaxial
<i>S. excelsa</i>	Polygonal	Straight-slightly curved	Glabrous	-	-
<i>S. foetida</i>	Polygonal	Straight-slightly curved	Pubescent	Intermediate	Adaxial-abaxial
<i>S. glabra</i>	Polygonal	Straight-slightly curved	Glabrous	-	-
<i>S. humboldtiana</i>	Polygonal	Straight-curved	Glabrous	-	-
<i>S. × persica</i>	Polygonal	Straight-slightly curved	Pubescent	Intermediate	Adaxial
<i>S. pycnostachya</i>	Rectangular to slightly polygonal	Straight-slightly curved	Pubescent	Dense	Adaxial-abaxial
<i>S. reticulata</i>	Polygonal	Straight	Pubescent	Sparse	Adaxial
<i>S. silecisa</i> (Tatry Mts.)	Polygonal	Straight-slightly curved	Pubescent	Intermediate	Adaxial-abaxial
<i>S. silecisa</i> (Vrchy Mts.)	Polygonal	Straight-slightly curved	Pubescent	Intermediate	Adaxial-abaxial
<i>S. viminalis</i>	Polygonal	Straight-slightly curved	Pubescent	Dense	Adaxial-abaxial
<i>S. waldsteniana</i>	Polygonal	Straight-slightly curved	Glabrous	-	-
<i>S. xanthicola</i>	Polygonal	Straight-slightly curved	Pubescent	Dense	Adaxial-abaxial
<i>Populus alba</i>	Polygonal	Straight-slightly curved	Pubescent	Dense	Adaxial-abaxial
<i>P. nigra</i>	Rectangular to slightly polygonal	Straight	Glabrous	-	-
<i>Bivinia jalbertii</i>	Polygonal to spherical	Straight-curved	Pubescent	Intermediate	Adaxial-abaxial

Table 4. Anatomical traits of petiole in the studied species of *Salix*, *Populus*, and *Bivinia*

Taxon	Petiole shape and outline	Vascular bundle shape	Petiole compression	Adaxial surface curvature	The Presence of Lateral ribs	Number of vascular bundles	Petiole thickness (µm)	Phloem thickness (µm)	Xylem thickness (µm)	Upper parenchyma thickness (µm)	Lower parenchyma thickness (µm)	Upper collenchyma thickness (µm)	Lower collenchyma thickness (µm)	Upper sclerenchyma thickness (µm)	Lower sclerenchyma thickness (µm)
<i>Salix aegyptica</i>	Circular, ribbed	Continuously oval	Horizontal	Convex	Absent	1	1126/93	101/88	117/39	258/92	250/90	186/70	178/17	35/91	35/03
<i>S. alba</i>	Circular, smooth	Continuously oval	Horizontal	Convex	Absent	1	845/60	60/70	78/81	109/48	104/29	86/54	74/80	49/54	27/61
<i>S. amygdaliodes</i>	Oval, ribbed	Interrupted oval	Horizontal	Concave	Present	1 (2)	301/55	39/48	28/44	41/82	71/92	62/29	53/30	8/95	12/75
<i>S. appendiculata</i>	Triangular, ribbed	Continuously circular	Horizontal	Straight	Absent	1 (2)	670/33	38/47	53/04	166/02	137/24	124/86	101/47	24/95	30/06
<i>S. atrocinerea</i>	Circular, ribbed	Continuously oval	Horizontal	Convex	Absent	1	1098/33	89/00	84/57	195/40	200/74	132/06	86/93	32/44	29/50
<i>S. babylonica</i>	Oval, ribbed	Interrupted circular	Horizontal	Straight	Absent	1 (2)	762/57	38/15	62/36	92/19	109/87	110/60	109/44	24/49	37/46
<i>S. calliantha</i>	Triangular, ribbed	Interrupted oval	Horizontal	Concave	Present	1 (2)	472/36	27/19	54/71	105/10	146/82	94/13	111/11	29/55	35/75
<i>S. calodendron</i>	Oval, ribbed	Continuously oval	Horizontal	Straight	Present	1	940/36	48/40	51/41	206/03	255/30	108/79	137/64	28/41	34/90
<i>S. caprea</i>	Oval, ribbed	Continuously oval	Horizontal	Convex	Present	1	539/78	60/40	41/97	85/97	84/06	86/57	81/91	13/87	21/39
<i>S. cordifolia</i>	Oval, ribbed	Interrupted oval	Horizontal	Concave	Present	1	359/21	30/52	40/78	73/18	74/88	57/60	61/64	14/87	17/13
<i>S. discolor</i>	Triangular, ribbed	Continuously oval	Horizontal	Convex	Present	1	316/18	52/43	37/69	52/14	58/95	43/19	45/05	12/19	16/87
<i>S. eleagnos</i>	Circular, ribbed	Interrupted oval	Horizontal	Straight	Present	1	877/82	79/88	72/11	157/16	174/87	116/30	116/16	11/35	26/35
<i>S. excelsa</i>	Oval, ribbed	Interrupted oval	Horizontal	Concave	Absent	1 (2)	893/52	68/19	93/44	183/11	250/74	96/90	102/18	40/36	53/83
<i>S. foetida</i>	Triangular, ribbed	Continuously oval	Horizontal	Straight	Present	1	730/01	89/21	75/45	204/74	149/74	58/80	64/29	29/37	28/44
<i>S. glabra</i>	Oval, smooth	Interrupted circular	Horizontal	Concave	Absent	1 (2)	736/75	44/44	62/98	130/53	174/29	84/54	105/99	30/34	36/30
<i>S. humboldtiana</i>	Oval, smooth	Interrupted oval	Horizontal	Concave	Absent	1	302/16	45/13	52/12	53/44	53/98	102/21	80/89	14/48	27/04
<i>S. × persica</i>	Oval, ribbed	Continuously circular	Horizontal	Concave	Absent	1 (2)	895/49	52/24	83/56	179/04	185/85	120/64	115/23	40/23	35/67
<i>S. pycnostachya</i>	Oval, smooth-ribbed	Continuously oval	Horizontal	Straight	Present	1	974/37	68/95	75/46	183/45	202/85	104/13	100/00	45/16	40/43
<i>S. reticulata</i>	Oval, ribbed	Interrupted oval	Horizontal	Concave	Present	1	518/17	49/77	55/50	142/10	91/20	55/03	56/94	20/60	25/90
<i>S. silecisa</i> (Vrchy Mts.)	Circular, smooth-ribbed	Continuously oval	Horizontal	Straight	Present	1	716/78	57/91	63/19	102/15	125/23	69/92	59/12	23/90	24/79
<i>S. silecisa</i> (Tatry Mts.)	Circular, smooth-ribbed	Continuously oval	Horizontal	Straight	Present	1	863/19	63/40	70/92	133/29	142/58	105/53	98/56	24/62	29/10
<i>S. viminalis</i>	Circular, smooth-ribbed	Continuously oval	Vertical	Convex	Present	1	1014/07	72/75	68/57	190/91	158/87	103/21	112/88	49/86	49/36
<i>S. waldsteniana</i>	Oval, smooth-ribbed	Continuously oval	Horizontal	Concave	Present	1 (1)	815/47	88/24	51/64	195/18	194/58	88/69	103/48	31/90	32/67
<i>S. xanthicola</i>	Circular, smooth-ribbed	Interrupted oval	Horizontal	Convex	Present	1	746/85	30/92	30/82	208/26	171/49	96/48	71/82	26/56	28/80
<i>Populus alba</i>	Circular, ribbed	Continuously oval	Vertical	Convex	Absent	1 (2)	1263/54	56/90	101/07	122/40	130/23	68/65	77/78	38/94	49/46
<i>P. nigra</i>	Oval, smooth	Interrupted circular	Vertical	Convex	Absent	1 (2)	765/04	31/89	44/35	77/25	99/19	61/78	73/14	20/59	24/51
<i>Bivinia jalbertii</i>	Circular, smooth-ribbed	Interrupted oval	Horizontal	Convex	Absent	1	573/48	68/13	76/31	87/82	140/57	69/22	89/72	18/98	26/61

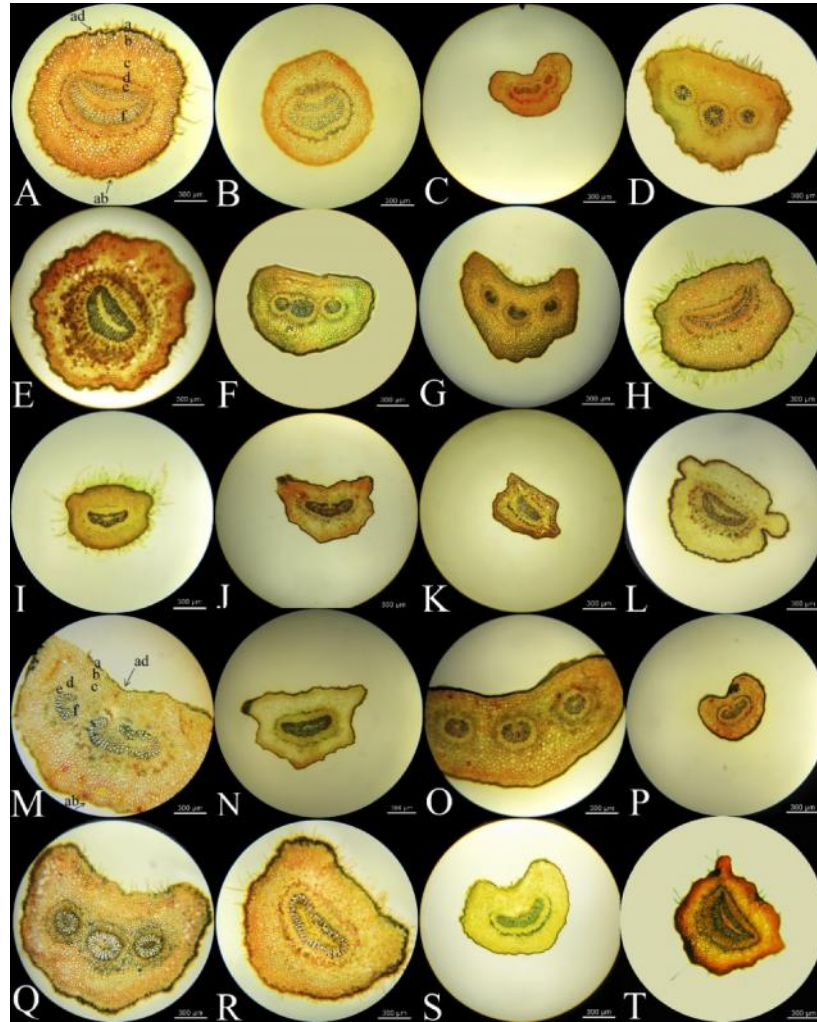


Fig. 1 (A-T). Cross section of petiole under optical microscope with a magnification of 10x: A. *Salix aegyptica*, B. *S. alba*, C. *S. amygdaliodes*, D. *S. appendiculata*, E. *S. atrocinerea*, F. *S. babylonica*, G. *S. calliantha*, H. *S. calodendron*, I. *S. caprea*, J. *S. cordifolia*, K. *S. discolor*, L. *S. eleagnos*, M. *S. excelsa*, N. *S. foetida*, O. *S. glabra*, P. *S. humboldtiana*, Q. *S. × persica*, R. *S. pycnostachya*, S. *S. reticulata*, T. *S. silecisa* (Tatry Mts.). ab: abaxial surface, ad: adaxial surface, a: epidermis, b: collenchyma, c: parenchyma, d: sclerenchyma, e: xylem, f: phloem (Bars = 300 µm).

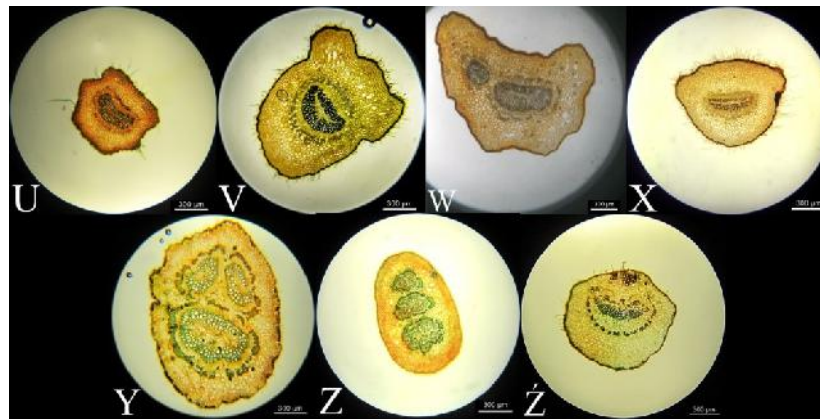


Fig. 1 (U-Z). Cross section of petiole under optical microscope with a magnification of 10x: U. *Salix silecisa* (Vrchy Mts.), V. *S. viminalis*, W. *S. waldsteniana*, X. *S. xanthicola*, Y. *Populus alba*, Z. *P. nigra*, . *Bivinia jalbertii* (Bars = 300 µm).

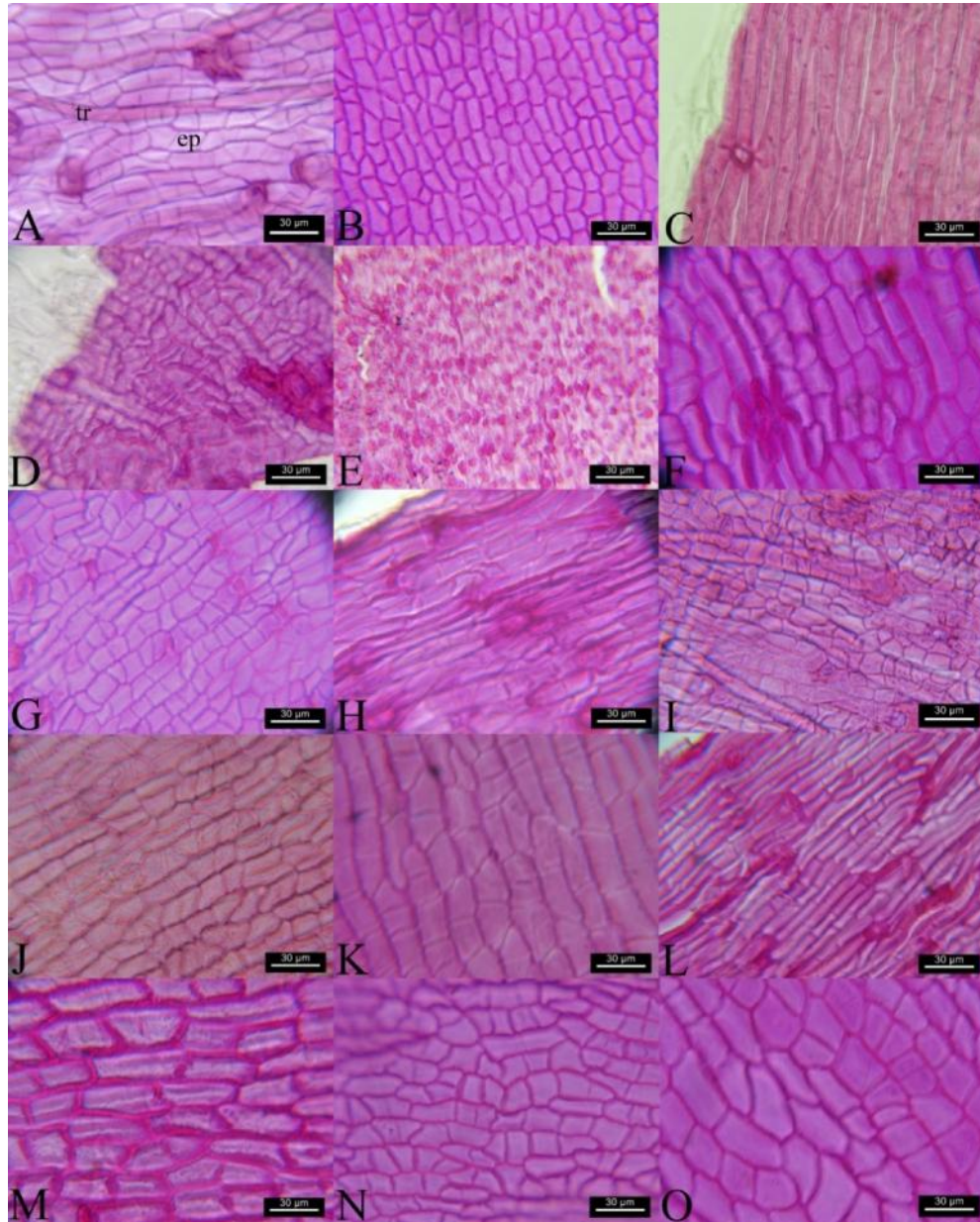


Fig. 2 (A-O). Petiole epidermis under optical microscope with a magnification of 10x: A. *Salix aegyptica*, B. *S. alba*, C. *S. amygdaliodes*, D. *S. appendiculata*, E. *S. atrocinerea*, F. *S. babylonica*, G. *S. calliantha*, H. *S. calodendron*, I. *S. caprea*, J. *S. cordifolia*, K. *S. discolor*, L. *S. eleagnos*, M. *S. excelsa*, N. *S. foetida*, O. *S. glabra*. tr: trichome, ep: epidermis (Bars = 30 µm).

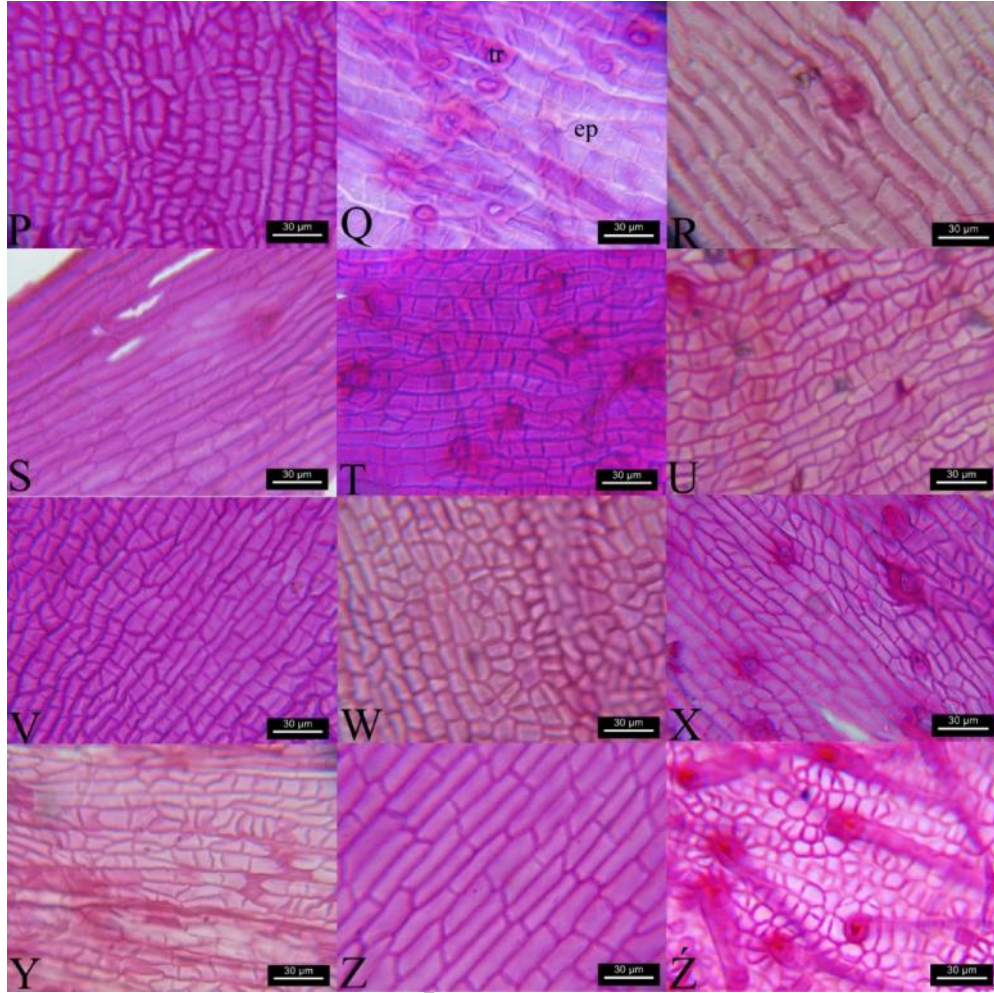


Fig. 2 (P-). Petiole epidermis under optical microscope with a magnification of 10x: P. *Salix humboldtiana*, Q. *S. × persica*, R. *S. pycnostachya*, S. *S. reticulata*, T. *S. silecisa* (Tatry Mts.), U. *S. silecisa* (Vrchy Mts.), V. *S. viminalis*, W. *S. waldsteniana*, X. *S. xanthicola*, Y. *Populus alba*, Z. *P. nigra*, . *Bivinia jalbertii*. tr: trichom, ep: epidermis (Bars = 30 µm).

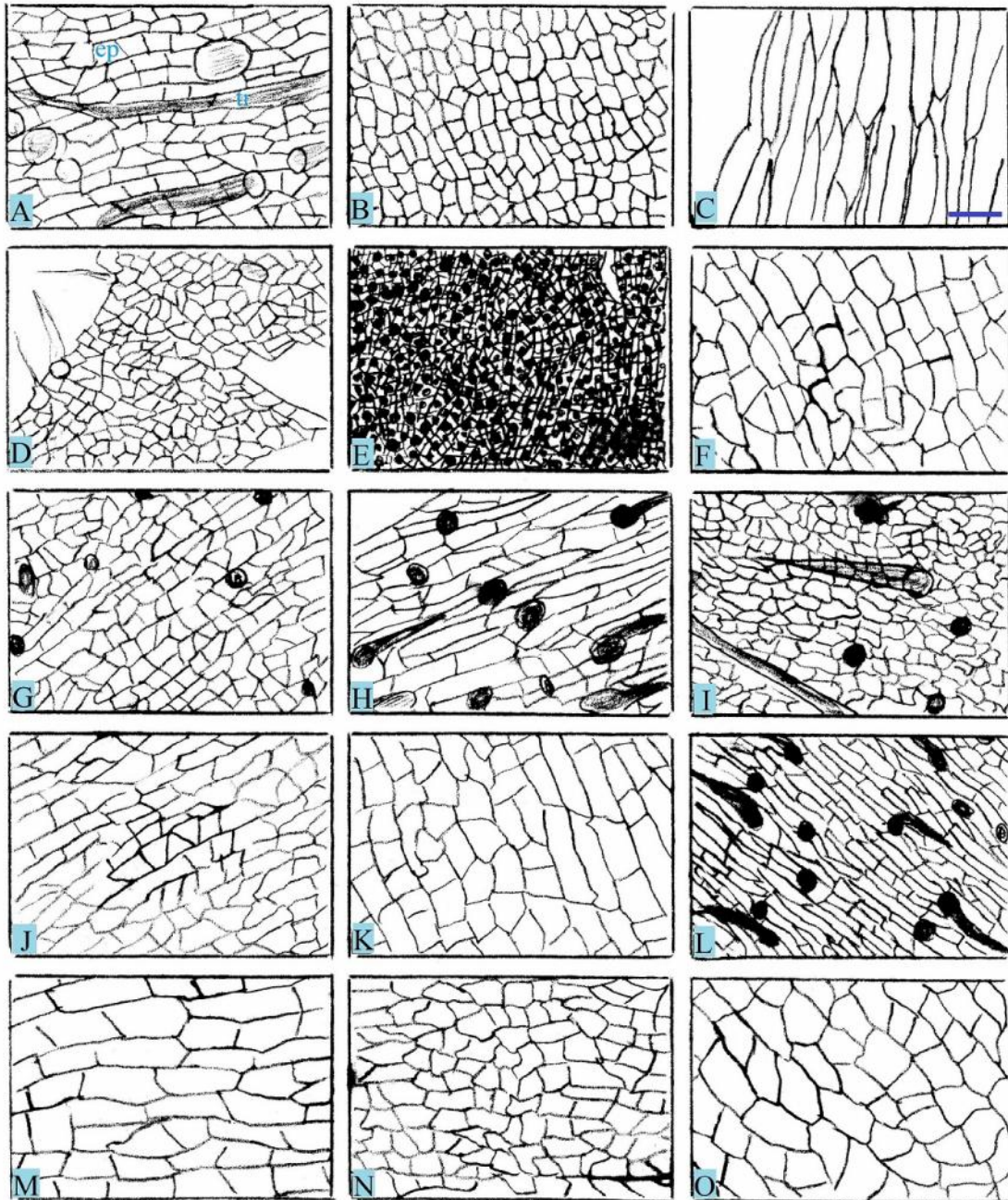


Fig. 3 (A-O). Petiole epidermis hand-drawings: A. *Salix aegyptica*, B. *S. alba*, C. *S. amygdaliodes*, D. *S. appendiculata*, E. *S. atrocinerea*, F. *S. babylonica*, G. *S. calliantha*, H. *S. calodendron*, I. *S. caprea*, J. *S. cordifolia*, K. *S. discolor*, L. *S. eleagnos*, M. *S. excelsa*, N. *S. foetida*, O. *S. glabra*. tr: trichom, ep: epidermis (Bars = 30 μ m).

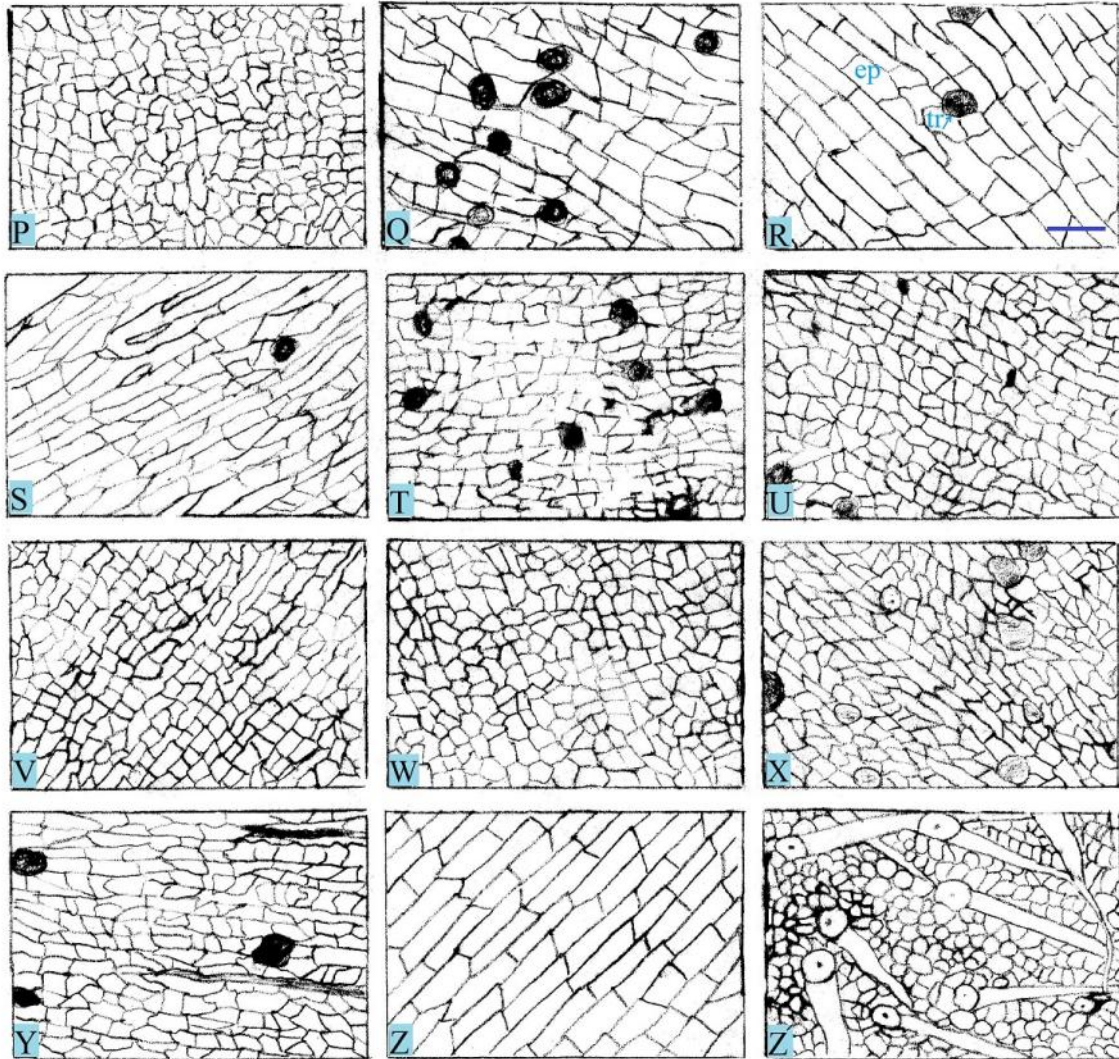


Fig. 3 (P-). Petiole epidermis paintings: P. *Salix humboldtiana*, Q. *S. × persica*, R. *S. pycnostachya*, S. *S. reticulata*, T. *S. silecisa* (Tatry Mts.), U. *S. silecisa* (Vrchy Mts.), V. *S. viminalis*, W. *S. waldsteniana*, X. *S. xanthicola*, Y. *Populus alba*, Z. *P. nigra*, . *Bivinia jalbertii*. tr: trichom, ep: epidermis (Bars = 30 μm).

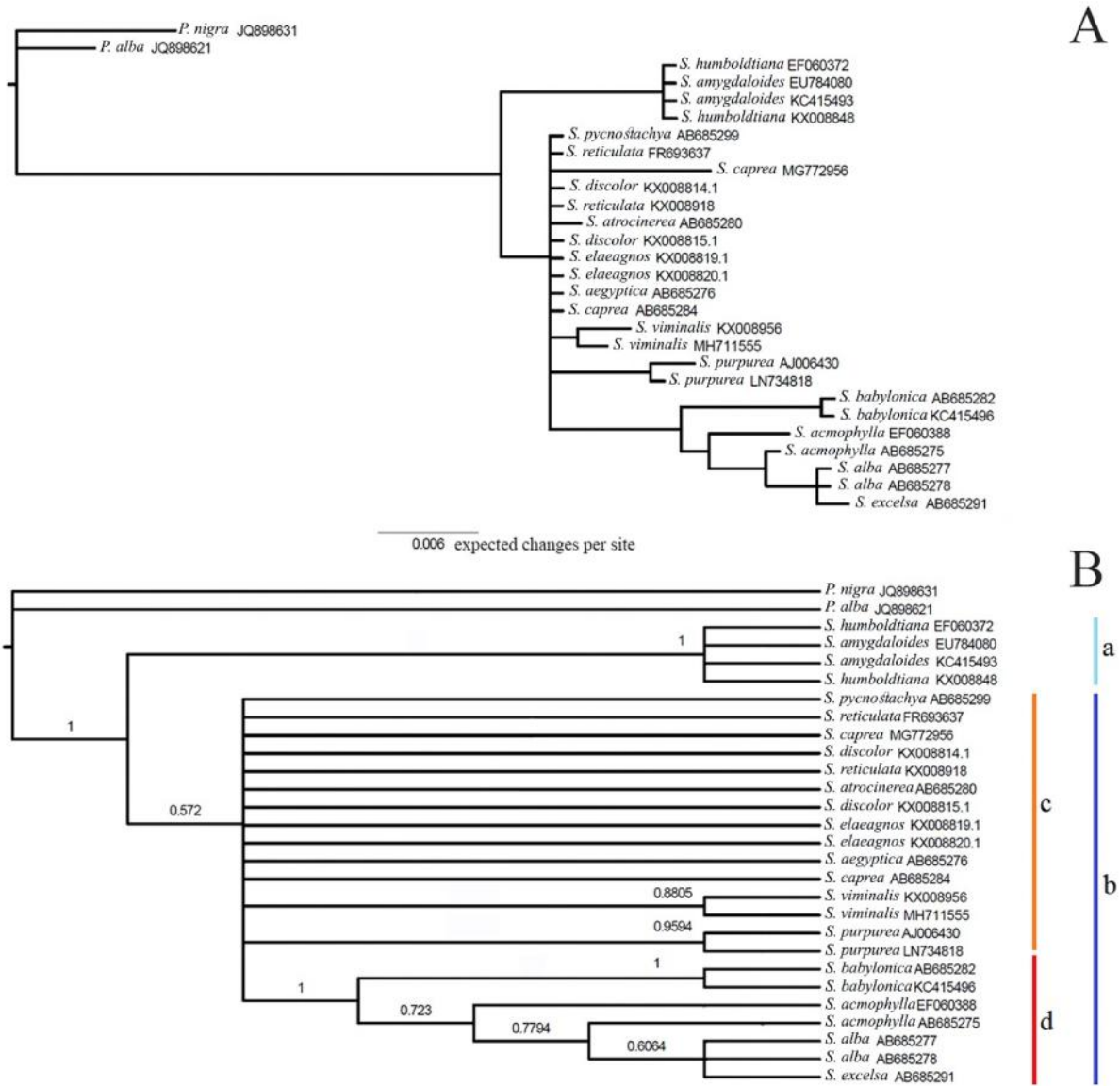


Fig. 4. The phylogram (A) and cladogram (B) of the 50% majority rule consensus tree inferred from the Bayesian analysis of ITS dataset. Branch support values are Bayesian posterior probabilities.

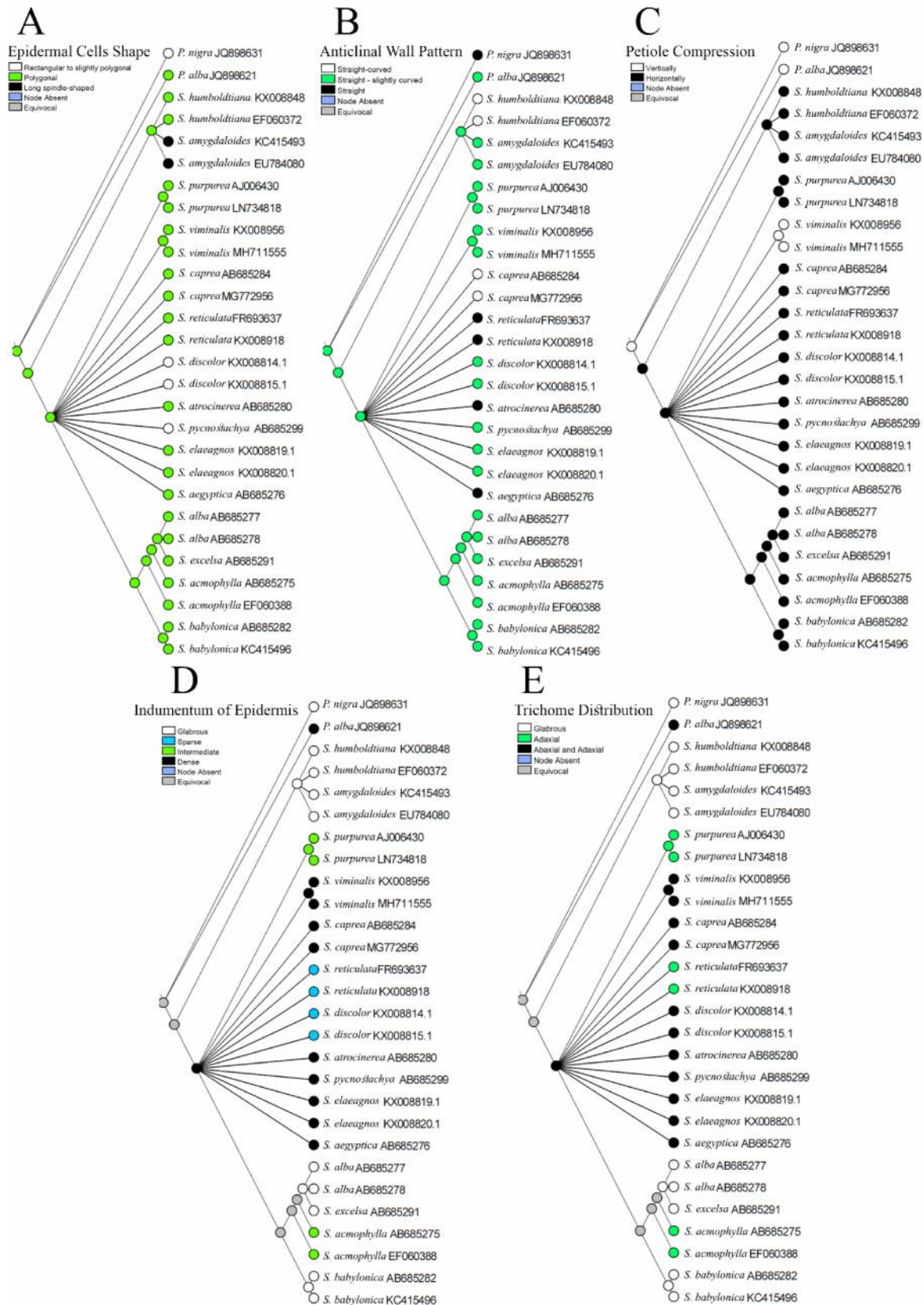


Fig. 5. Ancestral state reconstruction and character state trace of (A) Epidermal cells shape, (B) Anticlinal wall pattern, (C) Petiole compression, (D) Indumentum of epidermis and (E) Trichome distribution, on the phylogenetic tree inferred from the Bayesian analysis of ITS dataset. Equivocal state indicates nodes with major character state shifts.

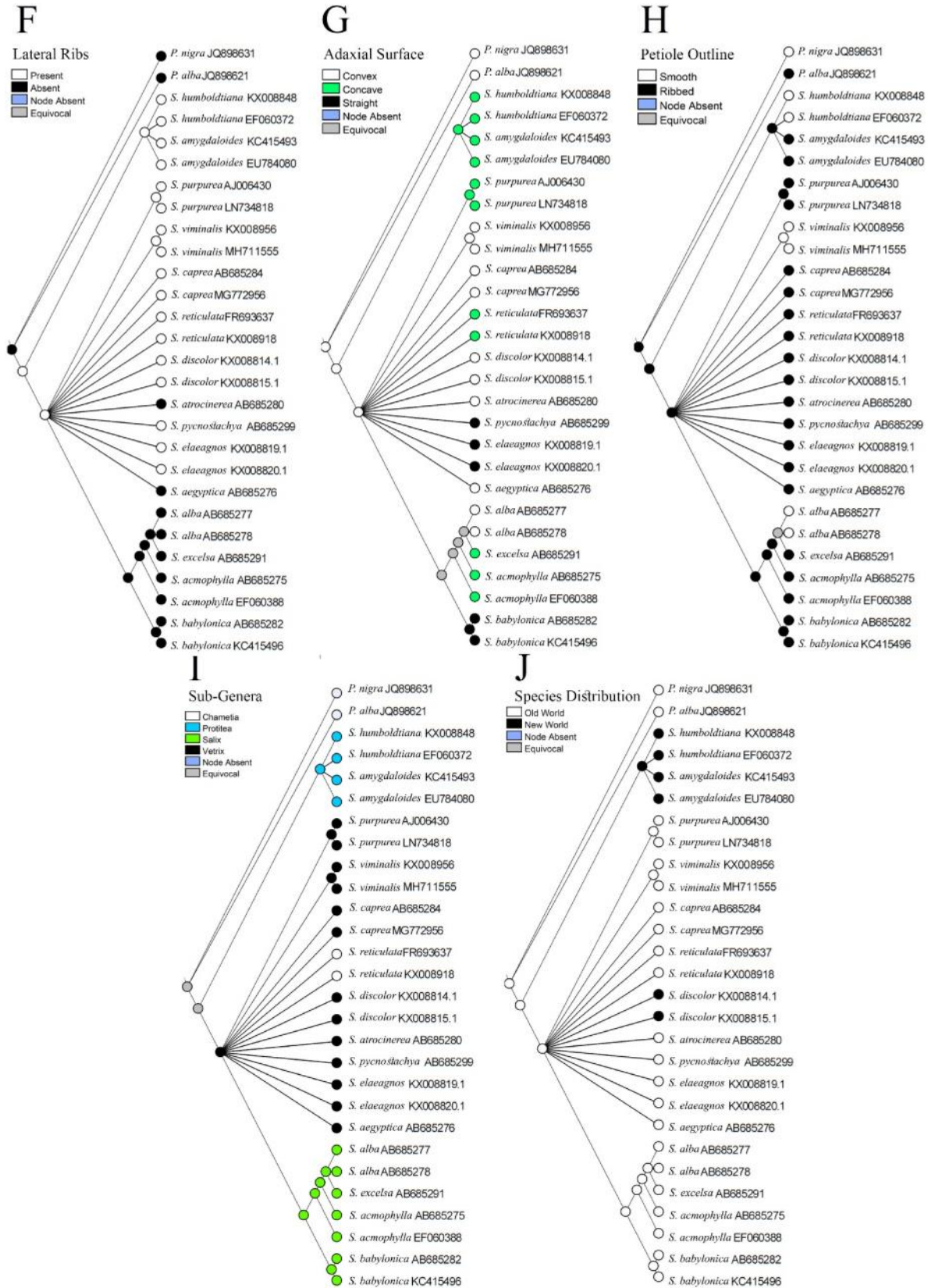


Fig. 5 (contd). Ancestral state reconstruction and character state trace of (F) Presence of lateral ribs, (G) Adaxial surface curvature, (H) Petiole outline, (I) Sub-generic status and (J) Species distribution, on the phylogenetic tree inferred from the Bayesian analysis of ITS dataset. Equivocal state indicates nodes with major character state shifts.

Discussion

Leaf anatomical and morphological features were widely-used in the circumscription, diagnosis and differentiation of the infra-familial taxa of *Salicaceae* in general and the infrageneric, specific and infraspecific taxa of the genus *Salix* in particular (Isebrands & Richardson 2014, Skvortsov 1999). Multiple studies have proved that, usefulness of leaf anatomical traits in the differentiation of *Salix* species. Khalili *et al.* (2010) examined the anatomical structure of *Salix* leaf and confirmed that, leaf anatomical traits can differentiate between problematic *Salix* species, e.g., *S. aegyptica* and *S. caprea*. Ghahremaninejad *et al.* (2012) proved the credibility of leaf epidermis traits in the differentiation of *Salix* sub-generic taxa. For example, subsections *Caesia* and *Kirilowianae* of the section *Helix* (subgenus *Vetrix*) were found to be distinguishable by the type of the stomatal apparatus. In addition, the leaf epidermis traits studied were in agreement with the inclusion of *Chosenia* into the genus *Salix*. Chen *et al.* (2008b) used the leaf epidermis traits to differentiate between the species of sects. *Lindleyanae* and *Retusae* by having anomocytic stomatal apparatus from the rest of *Salix* sections; Moreover, they approved the exclusion of the *Chosenia* from the genus *Salix* by the presence of cyclocytic stomata in the former.

In this study, 10 quantitative and 10 qualitative anatomical features of petiole were examined in the *Salicaceae* species. The primary evaluation of petiole anatomical traits revealed that, the quantitative traits, e.g., the thickness of petiole and the thickness of various layers of petiole tissues, were found to be varied among the different individuals of the same species, i.e., *S. silesiaca*, probably due to the effect of environmental factors such as temperature, which was in agreement with previous studies such as Su *et al.* (2009). However, the qualitative traits studied showed a considerable intra-specific consistency, therefore, they may be assumed as species-specific characteristics.

The provided qualitative petiole anatomical features were capable to differentiate the studied genera

in the family *Salicaceae* in total and sub-generic and specific taxa in the genus *Salix* in particular. The present members of the genus *Populus*, i.e., *P. alba* and *P. nigra*, had vertically compressed petioles in comparison with the horizontally compressed petioles of *Salix* species (except *S. viminalis*), therefore, petiole compression easily differentiated major genera of *Salicaceae* s.s. However, *Bivinia jalbertii* which was previously classified into the *Flacourtiaceae* and recently transferred to the *Salicaceae* on the basis of phylogenetic analyses (Liu *et al.* 2016, Chase *et al.* 2002), showed more similarities to the species of *Salix*, rather than those of *Populus*, especially in having characteristic horizontally compressed petiole. However, *Bivinia jalbertii* differed from the species of the genus *Salix* in the morphology of epidermal cells and the anticlinal cell wall pattern, being polygonal to spherical and straight to curvy, respectively.

The members of *Salix* subgenus *Protitea*, i.e., *S. humboldtiana* and *S. amygdaliodes*, could be easily separated from the rest of *Salix* species studied by their oval petiole shape, glabrous indumentum and their concave petiole adaxial surface. The absence of lateral ribs, straight to slightly curved anticlinal walls and glabrous or intermediate density of trichomes only on the adaxial surface were the features uniting the studied species of the subgenus *Salix*. The studied members of *Salix* subgenus *Chamaetia*, i.e., *S. cordifolia* and *S. reticulata*, were separated from the members of subgenus *Vetrix* by having oval petiole shape, ribbed outline, concave adaxial petiole surface, sparse trichome density and adaxial trichome distribution. In addition, petiole anatomical features could be easily applied to differentiate problematic species, e.g., *S. aegyptica* and *S. caprea*; while the overall morphology of the cross section of the petiole was round and the anticlinal cell wall pattern was straight in *S. aegyptica*, those traits were oval-round and straight-curvy in *S. caprea*, respectively. Moreover, the unique long spindle-shaped epidermal cells of *S. amygdaliodes* easily differentiated the species from the rest of the *Salix* species studied.

It is important to note that, no stomata were observed in the petiole's epidermis of any of the 26 species studied; as a result, it could be assumed that, the petiole is dependent to the leaf blade for respiration and gas exchange.

The overall topology of the obtained phylogenetic tree was in agreement with those in previous studies (Chen *et al.* 2010, Barkalov & Kozyrenko 2014b, Lauron-Moreau *et al.* 2015), in which subgenera *Proitea* and *Salix* formed more or less resolved clades of their own (clades a and d in Fig. 4B, respectively), while subgenera *Vetrix* and *Chamaetia* formed a highly unresolved group (group c in Fig. 4B).

The reconstruction of the ancestral states of the features studied (Fig. 5) suggested that, polygonal cells shape, straight-slightly curved anticlinal wall pattern, horizontal compression of the petiole, the presence of lateral ribs, convex adaxial surface and ribbed petiole

outline were primitive features in the species studied. However, it should be noted that, the frequent reversal events and major character state shifts, as depicted in figure 5, reduces the robustness of further generalization.

While the complicity of relationships among the members of *Salicaceae* in total and the genus *Salix* in particular were yet to be fully resolved, obtaining more taxonomically relevant features could be regarded as an important step forward, in order to obtain a better classification of *Salicaceae* and the genus *Salix*, on the basis of monophyletic groups with distinguishable morphological and anatomical synapomorphies.

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