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PLANT GENETICS

Phylogenetic Relationships of *Salix* L. subg. *Salix* Species (Salicaceae) according to Sequencing Data of Intergenic Spacers of the Chloroplast Genome and ITS rDNA

V. Yu. Barkalov and M. M. Kozyrenko

Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, 690022 Russia

e-mail: barkalov@ibss.dvo.ru

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Abstract—A phylogenetic analysis based on a comparison of nucleotide sequences of six regions (*petN–psbM, trnD–trnT, trnC–petN, psaA–ycf3, petG–trnP*, and *rpoB–trnC*) of cpDNA and ITS rDNA allowed for elucidating the relationship among species and sections belonging to the *Salix* subgenus and, more generally, to the *Salix* genus, as well as revealing the relations of the *Chosenia* genus. The definition of the subgenera *Pleuradenia* (including the *Urbanianae* section and the *Chosenia* genus), *Salix* (without the *Triandrae* section), *Triandrae*, and *Longifoliae* is essentially consistent with current classification schemes of the *Salix* genus. The previously defined genera of *Chosenia* and *Toisusu* (*Urbanianae*) are not only merged with the *Salix* genus but are also closely related between themselves. The *Protitea* subgenus only corresponds to the American species of the *Humboldtianae* section (*S. humboldtiana, S. amygdaloides, S. gooddingii*). The relationship of *S. chaenomeloides*, which is a nomenclatural type of this subgenus, as well as the relationship of the *Wilsonia* section, remains unresolved. The *Humboldtianae* section should be interpreted more narrowly, apparently, separating *Acmophyllae* and *Tetraspermae* sections from it. The monotypic American *Floridanae* section is related to the *Salix, Salicaster, Tetraspermae*, and *Wilsonia* sections.

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INTRODUCTION

The genus of *Salix* L. includes 300 to 450 species, which are especially widespread in areas of Eurasia and North America [1–4] with cold and temperate climates and diversified in the mountains of southwest China [1, 5, 6]. The genus represents one of the most complex taxonomical groups of flowering plants, with its intrinsic broad range of variability and interspecific hybridization. The classification schemes of the *Salix* genus, as well as the entire Salicaceae family in the narrow meaning, remain contradictory. Researchers in Russia traditionally consider the genus *Chosenia* Nakai as a separate one [1, 2, 7–9] or accept the genus of *Toisusu* Kimura too [2, 10, 11]. A.K. Skvortsov [1–5] subdivided the Eurasian species of the *Salix* genus into three subgenera: *Salix*, *Chamaetia* (Dumort.) Nasarov, and *Vetrix* (Dumort.) Dumort. Ohashi [4] assumed a broad concept of the *Salix* classification and subdivided the willows of Japan into six subgenera: *Pleuradenia* Kimura, *Chosenia* (Nakai) H. Ohashi, *Protitea* Kimura, *Chamaetia, Salix*, and *Vetrix*. Argus, while studying the New World *Salix* species [3], assumed the American *Longifolae* section to be a separate subgenus rank—*Salix* L. subgen. *Longifoliae* (Andersson) Argus, and placed the sections *Humboldtianae* and *Floridanae* in the *Protitea* subgenus. Chen et al. [12], based on a cladistics analysis, proposed a subdivision of the *Salix* genus into three subgenera: *Chosenia, Salix*, and *Vetrix*.

Molecular genetic studies with the use of nuclear and chloroplast markers revealed a number of discrepancies in the traditional classification of *Salix* based mainly on morphological characteristics (e. g., [1, 3, 13, 14]). For the first time, the suggested phylogenetic relationships among 18 taxons of Salicaceae family, including *Chosenia* and a few representatives of *Salix*, were reconstructed based on the sequences of two internal transcribed spacers (ITS1, ITS2)[1–2] and the 5.8S rRNA gene (ITS rDNA) from the nuclear ribosomal operon [15]. Samples of *Chosenia* and *Salix* constituted a monophyletic group with high significance; however, the results give no clear support to any specific affiliation of *Chosenia arbutifolia* (Pall.) A. K. Skvortsov (reffered to as *C. bracteosa* (Turcz. ex Trautv.) Nakai). Based on the analysis of the rbcL gene of the chloroplast DNA (cpDNA) were shown that species from three recognized genera *Chosenia, Salix* and *Toisusu* form a monophilitic group [16]. However, the data did not clarify the relationship of this group of constituents. According to the results of the analysis of the three markers (*rbcL, trnD–trnT*, and *atpB–rbcL*) of cpDNA [17], *Chosenia* was included into *Salix* as the separate subgenus rank. Also, it was suggested to subdivide *Salix* s.1. into four subgenera: *Triandrae,*

Salix (excluding the sections *Triandrae* and *Urbanianae*), *Chosenia* (including the *Urbanianae* section), and *Vetrix* (includes species from the previously defined subgenera *Vetrix* and *Chamaetia*). This did not confirm the view of Chao et al., who recognized the *Pleiarina* Raf. genus [18]. Based on a comparison of the ITS rDNA and *matK* gene sequences [19] partly supported the most-recent intragenus classification of New World willows [20]. Also, the relationships of Iranian and Chinese *Salix* species were studied with the use of sequencing of ITS rDNA and *trnL–F* cpDNA [21], and ITS rDNA [22], respectively.

Species of the *Salix* subgenus substantially prevail in worm-temperate and partly in tropical climates [1], and only a few of them are capable of inhabiting cold areas. In the Russian Far East, this group numbers six species, including two introduced species *S. alba* L. and *S. babylonica* L. *Chosenia* is widespread in northeast Asia, penetrating deep into the permafrost areas of Sakha-Yakutia and Chukotka.

Since the *Salix* subgenus is characterized by different sets of ancestral characters, it is particularly rich in taxonomic problems [19]. As was fairly noted by Skvortsov [1, page 76], "a significant discrepancy between the individual types therein can be identified, and it is difficult to single out any particular type that would be the most primitive in all respects: certain sections are more primitive with respect to some features, others – with respect to other features". According to his view, this subgenus probably should be considered as a natural (not polyphyletic) entity. Figure 1 shows the author's attempt to reconstruct the phylogenetic connections within the *Salix* subgenus [1].

Unlike Skvortsov, Argus [3] noted that the *Salix* subgenus, on the modern treatments [1, 13, 23], is morphologically heterogeneous based on the combination of primitive and progressive characters and he suggested its polyphyletic. The volume of the *Salix* subgenus, as well as the *Salix* genus itself, is controversial because it results from the set of characters of different taxonomic importance. Moreover, some authors attempt to divide this subgenus using a single character, for instance, a bud scale type [24, 25] or the number of stamens [26]. Kimura [25] divided it into two subgenera, *Protitea* and *Euitea*, taking into account the bud scale type, whereas Chao and Gong [26] referred all multistamen willows (3–15 stamens) to the genus of *Pleiarina* Raf. As is known, Skvortsov [1] did not agree with the attempts to split the subgenus Salix into smaller subgenera, but noted the most isolated position of two sections *Urbanianae* and *Longifoliae* and discussed their possible definition as separate subgenera. Because of the similarity between *Salix* and *Chosenia*, this author also assumed considering *Chosenia* as a subgenus [7].

In this study, we use Skvortsov's classification of *Salix* [1, 7]. In the Northern Hemisphere, the *Salix* subgenus is subdivided into the sections *Salix*, *Salicaster* Dumort. (*Pentadrae* (Borrer) C.K. Schneid.), *Humboldtianae* Pax, *Floridanae* Dorn, *Urbanianae* (Seemen) C.K. Schneid, *Subalbae* Koidz., *Longifoliae* Andersson, *Triandrae* Dumort. (*Amigdalinae* W.D.J. Koch), and *Wilsonia* K.S. Hao ex C.F. Fang et A.K. Skvortsov (*Glandulosae* Kimura). The goal of this study is to clarify the phylogenetic relationship of species and sections belonging to the *Salix* subgenus, as well as to identify the phylogenetic connections of *Chosenia* based on a comparison of the nucleotide sequences of the intergenic spacers of *petN–psbM*, *trnD–trnT, trnC–petN, psaA–ycf3, petG–trnP, and rpoB–trnC* of the chloroplast genome, and also ITS rDNA.

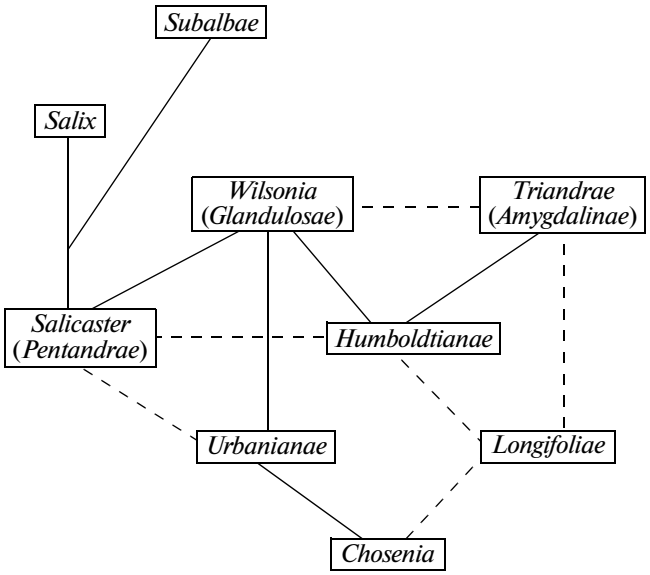

Fig. 1. Phylogenetic scheme of *Salix* L. (Skvortsov [1]).

MATERIALS AND METHODS

Total genomic DNA of six species: *Salix cardiophylla* Trautv. et C. A. Mey. (*Toisusu cardiophylla* (Trautv. et C. A. Mey.) Kimura), *S. nipponica* Franch. et Sav., *S. pierotii* Miq., *S. pseudopentandra* (Flod.) Flod., *S. arbutifolia* (*Chosenia arbutifolia*), and *Populus* suaveolens Fisch., were isolated from leaf tissue dried in silica gel using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The samples of this species were collected in natural populations (Table 1) and deposited in the collection of the Institute of Biology and Soil Sciences of the Far Eastern Branch of the Russian Academy of Sciences (VLA). The authors of the taxon names were taken from the database of International Plant Names Index (IPNI; http://www.ipni.org/ipni/authorssearch-page.do).

Amplification of ITS rDNA and the *petN–psbM, trnD–trnT, trnC–petN, psaA–ycf3, petG–trnP*, and

Table 1. Samples and EMBL/GenBank accession numbers of nucleotide sequences of ITS, *petN–psbM*, *trnD–trnT*, *trnC–petN*, *psaA–ycf3*, *petG–trhP*, and *rpoB–trnC*

Species	Habitat	EMBL/GenBank accession number ITS/*petN–psbM*/*trnD–trnT*/*trnC–petN*/*psaA–ycf3*/*petG–trnP*/*rpoB–trnC*
Populus suaveolens Fisch.	Kamchatka, Tolbachik volcano	HE800886/HE614650/HE614679/HE612016/HE613156/HE820948/HE820965
Salix arbutifolia Pall. (*Chosenia arbutifolia* (Pall.) A.K. Skvortsov)	Kamchatka, Alney Mt., upper coarse of Kirevna River	HE800885/HE614649/HE614678/HE612015/HE613155/HE820949/HE820964
Salix nipponica Franch. et Sav.	Primorskii Krai, valey of Knevichanka River, tract Solovey Kljuch	HE800864/HE611313/HE611972/HE611995/HE613135/HE613211/HE613244
Salix cardiophylla Trautv. et C.A. Mey. (*Toisusu cardiophylla* (Trautv. et C.A. Mey.) Kimura)	Sakhalin, middle coarse of Pilenga River	HE800841/FR694589/FR694558/FR694798/FR695024/FR695506/FR744742
Salix pseudopentandra Flod.	(1) Kamchatka, Tolbachik volcano	FR693646/FR694616/FR694584/FR694829/FR695053/FR695537/FR715080
	(2) Kamchatka, village Esso	FR693647/FR694617/FR694585/FR694830/FR695054/FR695538/FR715081
Salix pierotii Miq.	Primorskii Krai, vicinity of village Terehkovka	HE800865/HE614627/HE614657/HE611996/HE613136/HE613212/HE820956

rpoB–trnC regions of cpDNA was carried out with the use of the universal primers and conventional reaction parameters as recommended [15, 27–30]. Cyclic sequencing of both strands of DNA fragments was performed with the use of a set of fluorescently labeled nucleotides (Big Dye Terminator v.3.1 Applied Biosystems). The nucleotide sequences were read with the use of a genetic analyzer ABI 3130 (Applied Biosystems, Foster City, United States) and assembled with the use of Staden Package v. 1.5 software [31]. The obtained sequences of seven regions were deposited in the EMBL/GenBank database (Table 1).

The polymorphism of each region was evaluated with the use of the DNASP v. 5.10 computer program [32]. Two matrices were composed for phylogenetic analysis: (1) sequences of six cpDNA regions were joined for each sample; (2) The ITS sequences obtained in this study were joined with sequences of 18 *Salix* species (27 samples) and one species of *Populus* from the EMBL/GenBank database (sequence IDs are represented in Fig. 3). Sequences of *P. suaveolens* and *P. trichocarpa* were used as an outgroup. The neighbor-joining (NJ) method and the PAUP v. 4 computer program were used for phylogenetic tree building [33]. The evolutionary model was chosen with the help of the Modeltest v. 3 [34]. The branch support was evaluated by the bootstrap method using 1000 pseudoreplicates. Bootstrap percentage values (BP) less 50 % are not considered and are excluded in the Fig. 3.

RESULTS

The evolution of intergenic spacers in cpDNA includes three mutational events: a nucleotide change, an insertion/deletion (indel), and an inversion. The first two events were found in the sequences of six regions concerning the species studied. The sequence length of the same region in different species varied because of the presence of indels. For instance, the *petG–trnP* length ranged from 595 to 621 bp in the *Salix* and the *Chosenia* species, while in *P. suaveolens* it accounted for 566 bp with low informativeness (Table 2). The overall sequence length of the six joined cpDNA regions, including indels, constituted 4368 bp for each sample. From 243 polymorphic sites found, 77 were informative, according to the maximum parsimony method. The samples were distributed in two unresolved clusters following NJ analysis (Fig. 2). The species *S. pseudopentandra* and *S. pierotii* forming one cluster with a strong statistical support (BP = 87%).

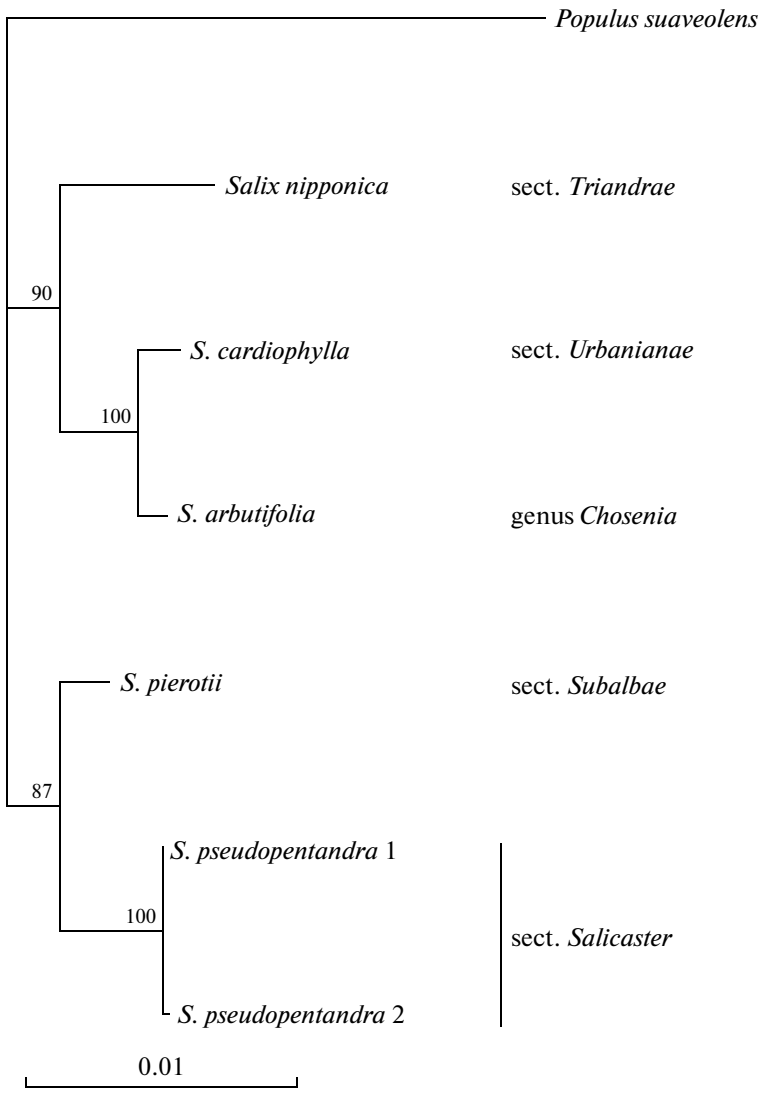

Fig. 2. NJ phylogenetic tree of the subgenus of *Salix* subg. *Salix* and *Chosenia* genus (*Populus* as an outgroup) built on the basis of a comparison of cpDNA sequences: *petN–psbM*, *trnD–trnT*, *trnC–petN*, *psaA–ycf3*, *petG–trhP*, and *rpoB–trnC*. Numbers designate bootstrap index values (%) from 1000 pseudoreplicates. Section names are shown as in [1].

The second cluster included *S. nipponica, S. cardiophylla*, and *S. arbutifolia* (BP = 90%). The two latter species formed a sister group with 100% statistical support.

All samples had ITS rDNA sequences of 616 bp in length with the exception of *S. nipponica* (617 bp) and *P. suaveolens* (615 bp). The phylogenetic analysis matrix with additional ITS sequences from the EMBL/GenBank database was 620 bp. It contained 79 polymorphic sites, of which 52 were informative following the maximum parsimony method. NJ tree topology confirms with high significance (BP = 100%) the monophyly of the *Salix* genus as all its species, including *S. arbutifolia* (*Chosenia*) form two clades (Fig. 3). The first clade joins with a high BP value (99%) *S. amygdaloides* Andersson, *S. gooddingii* C.R. Ball, and *S. humboldtiana* Willd. (*Humboldtianae* section). The second clade includes with medium BP (68%) all other species grouped into two clusters. The species of *S. floridana* Champ., *S. acmophylla* Boissieu, *S. alba* L., *S. tetrasperma* Roxb., *S. wilsonii* Seemen ex Diels, *S. mesnyi* Hance, *S. lucida* Muhl., *S. pentandra* L., and *S. pseudopentandra* are significantly (BP= 80%) joined in one cluster. The other taxa form an unresolved cluster, which includes the following statistically supported groups: *S. exigua* Nutt., *S. interior* Rowlee, *S. melanopsis* Nutt., and *S. taxifolia* Kunth of *Longifoliae* section (BP = 72%); *S. nipponica* and *S. triandra* L. of *Triandrae* section (BP = 51%); *S. matsudana* Koidz. and *S. pierotii* of *Subalbae* section (BP = 60%); *S. cardiophylla* of *Urbanianae* section and *Chosenia* (BP = 64%).

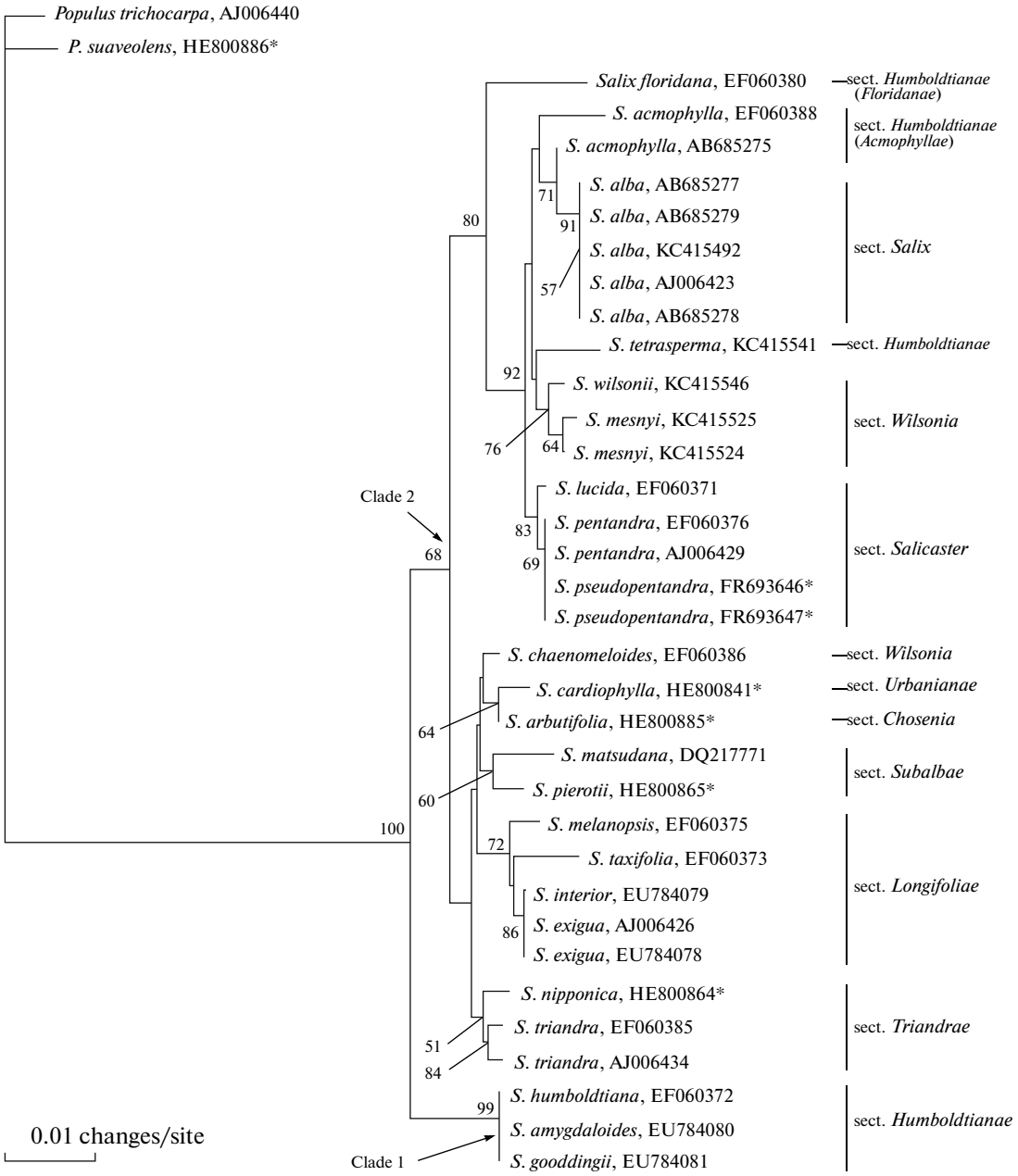

Fig. 3. NJ phylogenetic tree of the subgenus of *Salix* subg. *Salix* and *Chosenia* genus (*Populus* as an outgroup) based on comparison of rDNA ITS sequences. Numbers designate bootstrap index values (%) from 1000 pseudoreplicates. Asterisks designate sequences obtained in this study. Section names are shown as in [1].

DISCUSSION

The *Salix* subgenus consists of a group of considerably divergent sections, of which each one has its own primitive and progressive traits [1]. Skvortsov [1] considered *Humboldtianae* section in a broad meaning and included in it two Asian sections *Tetraspermae* Andersson and *Acmophyllae* Andersson, together with the African section *Madagaskariensis* Kimura. Argus [23] placed also *S. floridana* from the monotypic American section *Floridanae* in *Humboldtianae* section, suggesting that the closest relatives of this species may belong to the Old World section of *Tetraspermae*. This author [3] revealed phenetic similarity of *S. floridana* and *S. tetrasperma*, but considered that it was rather distant. Therefore it would be better to place *S. floridana* in a special section.

Not all researchers [6, 20] agree with Skvortsov [1] in that *Humboldtianae* section was enlarged. According our ITS data (Fig. 3), *Humboldtianae* section is not monophyletic, as six species from this section (*S. amygdaloides, S. gooddingii, S. humboldtiana,*

Table 2. Sequence information for intergenic spacers cpDNA and ITS rDNA of five *Salix* species and *P. suaveolens*

Region	Sequence length, bp	Number of sites (excluding deletions)		
		monomorphic	polymorphic	parsimony informative
petG–trnP	566–621	541	19	8
petN–psbM	545–568	513	14	4
psaA–ycf3	744–798	681	13	4
rpoB–trnC	683–697	587	27	11
trnC–petN	407–442	378	22	5
trnD–trnT	935–984	848	40	7
ITS1–ITS2	615–617	566	47	10

S. tetrasperma, S. acmophylla, and *S. floridana*) are distributed in two sister clades.

As was noted, Kimura [24, 25] subdivided the subgenus *Salix* into two subgenera (*Protitea* and *Eutea*), taking into account the character of the bud scale type. In the current classification schemes [4, 20], the sections *Humboldtianae, Tetraspermae, Wilsonia*, and *Floridanae*, which previously belonged to the *Salix* subgenus [1, 23], were placed in *Protitea* subgenus.

According to the results of ITS analysis (Fig. 3), the group of the American species alone (*S. humboldtiana, S. amygdaloides*, and *S. gooddingii*) of the section of *Humboldtianae* s. str. from the first clade corresponds to *Protitea* subgenus. The relative connections of the East-Asian species *S. chaenomeloides* (*S. glandulosa* Seemen), which is a nomenclatural type of *Protitea* subgenus, remain unresolved. *S. chaenomeloides*, following the *rbcL* phylogeny [16, 17], is placed together with Asian *S. tetrasperma* of *Humboldtianae* section and African *S. mucronata* Thumb. of the section *Octandrae* Andersson, forming one cluster.

Species of the East-Asian *Wilsonia* section, also referring to *Protitea* subgenus [4], are found in different clusters. *S. wilsonii* and two samples of *S. mesnyi* are grouped with species from the sections *Humboldtianae, Salix*, and *Salicaster*, while *S. chaenomeloides* is placed together with *Chosenia* and species from the sections *Urbanianae, Subalbae, Longifoliae*, and *Triandrae*. Thus, the relations of species of *Wilsonii* section remain unresolved in this study. Skvortsov [1] emphasized the close connections of the *Wilsonia* section (referred to as the *Glandulosae* section) with the sections *Salicaster, Humboldtianae*, and *Urbanianae* (Fig. 1), which is confirmed in this study.

High statistical support for joining American *S. floridana* with species from the sections of *Salix* and *Salicaster* (Fig. 3) probably indicates the belonging of *Floridanae* section to *Salix* subgenus [e.g., 3, 13], although *S. floridana* has bud scales with loose and overlapping edges. In *Humboldtianae* section, as Skvortsov noted [1], a transition from the bud scale with loose edges to the calyptiform bud scale took place. For instance, plants of the African *S. subserrata* Willd. and American *S. amygdaloides* sometimes have bud scales with partially coalescent edges. This character does not seem to be used as a principal one in the case of defining the levels of genus and subgenus. In some phenetic analyses [3], *S. floridana* was grouped with species from *Salicaster* section of *Salix* subgenus. To determinate the positions of *Floridanae* and *Wilsonia* sections in the genus system, further investigations are necessary.

Nazarov [35] assumed that *S. acmophyla* belong to *Acmophyllae* section and emphasized that it can be hybridized with *S. alba*. Skvortsov [1] observed similar hybrids both in herbaria and in nature. Abdollahzadeh et al. [21] suggested, following ITS analysis, that *S. acmophylla* is of hybrid origin and that one of the parental species may be *S. alba*. According to our results (Fig. 3), one accession of *S. acmophylla* and five sequences of *S. alba* constitute a well supported group (BP = 71%), which confirms those authors' conclusion. Skvortsov [1] referred *Acmophyllae* section to the section *Humboldtianae*; however, molecular studies showed that the taxonomic status of this section, as well as its position in *Salix* subgenus system, need further investigation.

S. alba, belonging to the small nominal section of *Salix*, is similar to *S. pentandra* (*Salicaster* section) with respect to morphological characters (the shape and internal structure of buds; the shape of stipules; pale floral bracts, which deciduous after the flowering of pestillate catkins), all indicating the relationships between two sections (Fig. 1). Normally, male flowers of *S. alba* have two stamens; however, as Skvortsov [1] noted, sometimes individuals with multistaminate flowers (4–8 stamens) can be observed. In his opinion, multistaminal should be considered as real atavism,

which also favors the close relationship between *Salix* and *Salicaster* sections. The ITS results showed (Fig. 3) that species of both sections were grouped in the same cluster (BP = 92%), which confirms their relationships. Similar results were obtained by other authors [15, 21].

The species *S. lucida, S. pentandra, S. pseudopentandra*, belonging to *Salicaster* section, significantly (BP= 83%) constitute a supported group (Fig. 3). American *S. lucida* occupied a specific place apart from the two Asian species of this section (*S. pentandra* and *S. pseudopentandra*). To some degree, this confirms Skvortsov's suggestion [1] about intrasectional division of the section. In *matK* based phylogeny [19], the two samples of *S. lucida* are located in different clades. The authors explained this by chloroplast capture effect during hybridization with other *Salix* species.

Skvortsov [1] emphasized the most isolated position of the two sections in *Salix* subgenus: East-Asian *Urbanianae* and American *Longifoliae*. *Urbanianae* section is extremely primitive (stamens or the ovarian pedicle are coalescent with floral bract resembling the flowers of *Populus* and was accepted frequently for the *Toisusu* genus, which is different from *Salix* [2, 10, 24, 36–38]. Otherwise it was included in subgenera of *Pleuradenia* Kimura of *Salix* genus [4, 14]. According to the results on ITS and the six cpDNA regions (Figs. 2 and 3), *S. cardiophylla* (*Urbanianae* section) is joined with *S. arbutifolia* (*Chosenia genus*), BP = 64% and 100%, respectively). These data indicate to the genetic relationship between the previously recognized genera of *Chosenia* and *Toisusu*.

Ohashi [4] assumed the rank of subgenus for *Chosenia* (*Salix* L. subg. *Chosenia* H. Ohashi) and included the former *Toisusu* genus in the subgenus *Pleuradenia* of *Salix* genus followed by Kimura [14]. The subgenus *Chosenia* is similar to *Salix* (the sections *Longifoliae, Triandrae*, and *Urbanianae*) according to the flower structure; the breaking of styles with stigmas prior to the period of capsule maturation; the bud structure; leaf anatomy; and bark characters. The species of these sections *S. arbutifolia* (genus *Chosenia*) are grouped in the same cluster (Fig. 3). According to the results of Kuprijanova [39], the genera *Salix* and *Chosenia* are characterized by a similar morphology of the pollen grains (ellipsoid-like), which is indicative of their relationship. Fruits of *S. cardiophylla* (*Toisusu cardiophylla* (Trautv. et C.A. Mey) Kimura) contain four seeds [10]. This trait is characteristic also for *S. arbutifolia* and species from the sections *Tetraspermae* (=*Humboldtianae*) and *Floridanae* of *Salix* genus. It seems to have emerged independently in different groups, as those taxa are located in different clusters (Fig. 3).

Kimura [40] was the first who noted a similarity between *Chosenia* and *S. cardiophylla* (*Toisusu*), taking into account a number of morphological characters. He revealed rudimental nectaries in fruiting catkins of an individual from northeastern China. Similar to *S. cardiophylla*, we also found rudimentary nectaries located transventral in female catkins of *Chosenia* inhabiting Kolyma Upland (Bolshoi Tuonnakh range, the Verina River). This fact is additional support for the secondary transition of *Chosenia* to wind-pollinating and its designation to *Salix* genus.

Accepting *Pleuradenia* subgenus with a single polymorphic species of *S. cardiophilla*, Ohashi [4] was based only on common morphology and assumed it to be more primitive in *Salix* genus as compared to *Chosenia* subgenus. Other authors, based on cpDNA sequencing data [17], also accepted the rank of subgenus in the case of *Chosenia* and, nevertheless, included in it the section *Urbanianae*, to which *S. cardiophylla* belongs. Our results represent support for the viewpoint of Chen et al. [17]; however, unlike them we placed *Chosenia* and *Urbanianae* section in *Pleuradenia* subgenus. The subgenus *Pleuradenia* is considerably distinct from the subgenera of *Salix* and *Longifoliae* in such important morphological characters as the bud internal structure, the transventral location of nectaries in female flowers, and the coalescence of stamen filaments with floral bracts in male flowers. Moreover, the natural hybrids between *S. arbutifolia* (*Chosenia arbutifolia*) and *S. cardiophylla* are known [41]. The volume of the *Pleuradenia* subgenus is limited by two sections (*Urbanianae* and *Chosenia* (Nakai) Kimura), although take into account the important morphological distinction and biology of *S. arbutifolia*, it possibly can be placed in the separate subgenus *Chosenia*.

According to morphological characters, the American section *Longifoliae* occupies a separate position in *Salix* subgenus [1, 3]. Only species of this section preserved a primitive flower structure: stamens were reduced to the number of two; the bilateral hypoderma with almost no chlorophyll is intrinsic to *S. arbutofolia* leaves (probably because of the arid habitat) in the same way as in the case of *Chosenia* and turanga poplars; and the ability for coppice shoot formation is similar to that of poplars [1]. The genetic connection between *Longifoliae* section and *Vetrix* subgenus follows from several biological [42, 43] and biochemical [44] traits. The species of this section (*S. melanopsis, S. taxifolia, S. interior*, and *S. exigua*) included in ITS analysis (Fig. 3) form a well supported group (BP = 72%), which is located in the same cluster as the sections *Urbanianae, Triandrae*, and *Wilsonia*, together with *Chosenia* subgenus. The results confirm Skvortsov's viewpoint [1] on the possible relationship between *Longifoliae* section, on the one hand, and *Chosenia* subgenus and *Triandrae* section on the other hand (Fig. 1), although the connection between *Longifoliae* section and the sections *Humboldtianae* and *Salicaster*, which is traced in other studies [16, 17], is not confirmed. Thus, our data suggest a natural formation of this *Salix* group and they are consistent

with Argus's study, in which he accepted for the group the subgenus rank [3].

The species *S. nipponica* and *S. triandra*, belonging to the small Eurasian section *Triandrae*, form a separate branch (Fig. 3) with relatively low statistical support (BP = 51%) in the unresolved cluster, which joins the bulk of analyzed species of the sections *Urbanianae*, *Longifoliae, Subalbae, Wilsonia*, as well as *Chosenia*. Previously, the position of *S. triandra* in *Salix* genus was unresolved [15, 29]. Azuma et al. [16] grouped *S. nipponica* (referred to as *S. subfragilis* Andersson) together with *Chosenia* and *Toisusu*, as well as species of the subgenera *Chamaetia* and *Vetrix* but in a sister position. Similar results were obtained by Chinese researchers for *S. triandra* [17]. The relationship of *Triandrae* section with *Urbanianae* (*Toisusu*) section and *Chosenia* genus was confirmed with the results of the analysis of six cpDNA regions (Fig. 2). Species of these taxa are located in the same cluster with high significance (BP= 90%). According to results of molecular studies [21, 45-47], *S. triandra* occupies a separate position among various groups of *Salix* genus as follows from dendrograms.

Chen et al. [17] showed that the genus of *Salix* is not monophyletic and proposed excluding from the genus *Triandrae* section, raising its rank to an independent subgenus. Argus [20] also supported a possible change of the status of the section *Triandrae* to a special subgenus. Our results are consistent with these authors' opinions. Studies on the relations in *Salix* genus with the use of AFLP markers [49] showed the same similarity of *S. triandra* with the subgenera of *Salix* and *Vetrix*. This possibly indicates that *Triandrae* section might have diverged from some primitive *Vetrix* sections that preserved traits of the *Salix* s.l subgenus. Species of *Triandrae* sections differ from other species of *Salix* subgenus in that their bark resembles the bark of *Chosenia* (the bark of old trees is peeled off in thin plates) and their anthers have a specific structure (both anther sacs face ahead, not aside) [1, 7]. Both *S. triandra* and *S. nipponica* flowers normally have three stamens; however, plants with 2, 4, or 5 stamens are also exist [4, 6].

Species of the East-Asian *Subalbae* section (*S. pierotii* and *S. matsudana*) are grouped together with relatively low support (BP = 60%) and located in one unresolved cluster with species of other sections of willows and genus *Chosenia* (Fig. 3). The section *Subalbae* also includes the species *S. jessoënsis* Seemen, *S. eriocarpa* Franch. et Sav., and *S. babylonica* L. [4]. Recent studies on Chinese willows [22] showed that *S. matsudana* is a synonym for *S. babylonica*. Phenetic analysis results [3] support neither an assignment of the rank of section to *Subalbae* [e.g., 1, 4, 5, 13, 38], nor its inclusion into the *Salix* section (for an example, see [6, 49]). According to *rbcL* cpDNA sequencing data [16, 17], *S. babylonica*, a representative of *Subalbae* section, is grouped together with *S. alba* (*Salix* section) and *S. pentandra* (*Salicaster* section), which confirms to some extent the relationship among these sections, as is shown in Skvortsov's scheme (Fig. 1). According to our results (Fig. 2), there is a relationship between *Subalbae* and *Salicaster* sections, because the species *S. pierotii* and *S. pseudopentandra* are joined with high significance (BP = 87%). Thus, a molecular approach also does not give a clear view on either the taxonomic status of *Subalbae* section or its position in *Salix* subgenus.

Phylogenetic analysis based on the variability of six cpDNA regions and ITS allowed elucidation of some phylogenetic relations of species and sections, both in *Salix* subgenus and in the entire *Salix* genus, as well as the identification of the related connections of *Chosenia*. The definitions of the subgenera *Pleuradenia* (including *Urbanianae* section and *Chosenia* genus), *Salix* (without *Triandrae* section), *Triandrae*, and *Longifoliae* are well supported. The previously defined genera *Chosenia* and *Toisusu* are merged with *Salix* genus and are moreover related to each other. The subgenus *Pleuradenia* includes the sections *Urbanianae* and *Chosenia*. A group of the American species *S. humboldtiana, S. amygdaloides*, and *S. gooddingii* of the section Humboldtianae s. str. correspond only to *Protitea* subgenus. However, the relations of the East-Asian *S. chaenomeloides*, which is a nomenclatural type of this subgenus, as well as the relations of *Wilsonia* section and its position in the system of *Salix* genus, remain unresolved. The section *Humboldtianae* can be accepted in a narrower sense, after defining within it the sections *Acmophyllae* and *Tetraspermae*. As can be seen, the monotypic American *Floridanae* section is related to the sections *Salix, Salicaster, Tetraspermae*, and *Wilsonia*. For a more profound understanding, further investigations of a larger number of species, especially from the sections *Tetraspermae, Acmophyllae*, and *Wilsonia*, are needed.

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