



Phylogenetic Analysis of the Far Eastern *Salix* (Salicaceae) Based on Sequence Data from Chloroplast DNA Regions and ITS of Nuclear Ribosomal DNA

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ABSTRACT

In order to further elucidate the relationships in the *Salix* genus, phylogenetic analysis was conducted based on comparison of the nucleotide sequences of the chloroplast DNA regions (*petG-trnP*, *petN-psbM*, *psaA-ycf3*, *psbM-trnD*, *rpoB-trnC*, *trnC-petN*, *trnD-trnT*, *trnS-psbZ*, *rbcL*, *matK*) and the ITS region (ITS1–5.8S rRNA–ITS2) of nuclear ribosomal DNA of 86 *Salix* species from three subgenera: *Salix*, *Chamaetia*, *Vetrix*, 27 sections and *Chosenia arbutifolia*. The previously recognized genera *Chosenia* and *Toisusu* merged with *Salix*. The divergence of species in the *Salix* genus (according to ITS and cpDNA data) was very low. In most species from *Chamaetia* and *Vetrix* subgenera, the sequences differed by several substitutions or were identical. Molecular differences were identified mainly in species belonging to the subgenus *Salix* in its previous broad meaning. ITS-phylogeny generally agrees with the most-recent infrageneric classification of *Salix*: the isolation of subgenera *Pleurodenia*, *Chosenia*, *Salix* (for exception sect. *Triandrae*), *Longifoliae* has been well supported, but the isolation of subgenera *Protitea* and *Chamaetia* is not supported. Relationships between the subgenera *Chamaetia* and *Vetrix* remain unresolved. High similarity of the plastid and nuclear genomes revealed in the majority of *Salix* species included in the analysis may indicate that they have relatively recently diverged from a common ancestor or there is a result of lineage sorting through hybridization.

Keywords

chloroplast DNA, ITS, phylogenetic analysis, Salicaceae, *Salix*

РЕЗЮМЕ

Баркалов В.Ю., Козыренко М.М. Филогенетический анализ дальневосточных *Salix* (Salicaceae), основанный на данных секвенирования регионов хлоропластной ДНК и ITS ядерной рибосомной ДНК

С целью дальнейшего выяснения взаимоотношений в роде *Salix* проведен филогенетический анализ на основе сравнения нуклеотидных последовательностей регионов хлоропластной ДНК (*petG-trnP*, *petN-psbM*, *psaA-ycf3*, *psbM-trnD*, *rpoB-trnC*, *trnC-petN*, *trnD-trnT*, *trnS-psbZ*, *rbcL*, *matK*) и ITS региона (ITS1–5.8S rRNA–ITS2) ядерной рибосомной ДНК 86 видов *Salix* из трёх подродов: *Salix*, *Chamaetia*, *Vetrix* и 27 секций, а также *Chosenia arbutifolia*. Дивергенция видов внутри рода *Salix* по данным ITS и хпДНК была очень низкой, у большинства видов из подродов *Chamaetia* и *Vetrix* последовательности различались несколькими заменами или были идентичны. Молекулярные различия выявлены главным образом у видов, принадлежащих подроду *Salix* в прежнем широком его понимании. ITS-филогения согласуется, в основном, с современными классификационными схемами рода *Salix* на уровне подродов: хорошо поддерживает выделение подродов *Pleurodenia*, *Chosenia*, *Salix* (за исключением сект. *Triandrae*), *Longifoliae*, но не поддерживает выделение подродов *Protitea* и *Chamaetia*. Взаимоотношения подродов *Chamaetia* и *Vetrix* остаются не разрешенными. Высокое сходство пластидного и ядерного геномов, выявленное у большинства включенных в анализ видов *Salix*, может указывать на их относительно недавнюю дивергенцию от общего предка или на пересечение генеалогических линий через гибридизацию.

Ключевые слова

хлоропластная ДНК, ITS, филогенетический анализ, Salicaceae, *Salix*

The genus *Salix* L., the largest one in the family Salicaceae, comprises 300–450 species widespread mainly in cold and temperate regions of the Northern Hemisphere (Skvortsov 1968, 1999, Nedoluzhko 1995a, Argus 1997, Ohashi 2001). Most species occur in the northern regions

of Eurasia and North America, as well as in the mountains of China (Skvortsov 1968, Fang et al. 1999). Only a few members of this genus reach South America and Africa. The Bering Strait region, the mountains of Southern Siberia and Northeast Asia are centers of taxonomic diversity of

shrubby and dwarf willows that were included in the subgenus *Chamaetia*. One hundred and thirty-five species are indicated for the former Soviet Union (Skvortsov 1968). According to our sources, more than 70 species, including *Salix arbutifolia* Pall. and *S. cardiophylla* Trautv. et C.A. Mey., are found in the Russian Far East. In a monographic treatment of the Far East willows (Nedoluzhko 1995a), the latter two species were assigned to the genus *Chosenia* Nakai and genus *Toisusu* Kimura, respectively. Four new species have recently been described by Barkalov (2012): *Salix woroschilovii* Barkalov (Southern Sikhote-Alin), *S. moczalovae* Barkalov and *S. olaënsis* Barkalov (Kolyma Upland), *S. schmidtiana* Barkalov (Northern Sakhalin).

Classification schemes pertaining to the genus *Salix* characterized by dioeciousness, considerable variability in the morphology, diversity of life forms and habitats, still remain controversial at both generic and infrageneric levels (Chen et al. 2008). Skvortsov (1966, 1968) justifies the position of recognizing *Chosenia* as a separate genus; however, he also assumes that it can be classified as a subgenus of the genus *Salix*, taking into account its significant similarity with willows, primarily with the East Asian *S. cardiophylla* from the section *Urbanianae* (in terms of the structure of buds and reproductive organs) and members of the American section *Longifoliae* (in terms of similar anatomical structure of the leaf). The main differences between *Chosenia* and willows are identified in the anatomy of wood (characterized by homogeneous rays) and wind-pollination, which is a feature of poplars, but these features in *Chosenia* are a secondary acquisition (Skvortsov 1966). Reduction of nectaries (glands) in *Chosenia* can probably be attributed to the transition to wind pollination. The East Asian oligotypic genus *Toisusu* was assigned by Skvortsov (1968) to the genus *Salix* and was placed into the section *Urbanianae* of the subgenus *Salix*. Kimura (1931) described *Salix kamikotica* Kimura (= *x Toisochosenia kamikotica* (Kimura) Kimura: Kimura 1937), which is a natural intergeneric hybrid of *Toisusu* and *Chosenia*. He subsequently identified rudimentary nectaries in fertile catkins in the *Chosenia* specimen from northeast China and proposed the name of *C. macrolepis* Turcz. mstr. *adenantha* Kimura for this anomalous form (Kimura 1938). Subsequently Kimura (1988) made a combination at the form level, but in the *Salix* genus – *S. arbutifolia* Pall. f. *adenantha* (Kimura) Kimura, which he regarded as an intermediate between *Chosenia* and *Salix*. Based on the existence of a natural hybrid and the specified form, Kimura (1988) reduced the status of *Chosenia* to the rank of a section with the identical name (sect. *Chosenia*) and placed it in the subgenus *Pleuradenia* in the genus *Salix*, which had previously been identified by him.

Skvortsov (1968) subdivided the genus *Salix* within the boundaries of the Eurasia region into three subgenera: *Salix*, *Chamaetia*, and *Vetrix*, which, in turn, were divided into a number of sections. Chao et al. (1998, 2010), in addition to *Chosenia* and *Salix*, recognized *Pleiarina* Raf. (= *Opodix* Raf., Lim & Carvalho 2013) to be a separate genus comprising all *Salix* species with multistaminate flowers. In the “Flora of China” Fang et al. (1999) subdivided the genus *Salix* into 37 sections without distinguishing subgenera and

considered *Chosenia* to be a genus in the family Salicaceae. Based on the results of the cladistic analysis, Chen et al. (2008) proposed to split the genus *Salix* into three subgenera, namely, *Chosenia*, *Salix*, and *Vetrix*.

The recent molecular-genetic studies have revealed a number of incongruities with respect to the conventional classification of *Salix* at the infrageneric level, which is based primarily on morphological features (e.g., Skvortsov 1968, 1999, Dorn 1976, Kimura 1988, Argus 1997). There are very few works focused on phylogeny of *Salix*, among them: Leskinen & Alström-Rapoport (1999), Azuma et al. (2000), Chen et al. (2010), Hardig et al. (2010), Abdollahzadeh et al. (2011). It should be noted that in comparison to other groups of flowering plants the number of species belonging to this genus included in the studies is insignificant.

Based on variability of sequences in the ITS region of nuclear DNA ribosomal operon, Leskinen & Alström-Rapoport (1999) reconstructed for the first time the tentative phylogenetic relationships of 18 taxa of the *Salicaceae* family, including *Chosenia* and a few members of *Salix* subgenera. The study by Leskinen & Alström-Rapoport demonstrated that sequence divergence in the ITS region and the number of phylogenetically informative sites were very low in *Salicaceae* family, especially among *Salix* and *Chosenia* species. Specimens of *Chosenia* and *Salix* formed a well-supported monophyletic group; however, their ITS sequences give no clear support to any specific affiliation of *Chosenia arbutifolia* (referred to as *C. bracteosa* (Turcz. ex Trautv.) Nakai) within *Salix*. It is necessary to clarify the definitions of voucher specimens of “*Chosenia*” because when attempts to use ITS sequences of *Chosenia* from the GenBank (Leskinen & Alström-Rapoport data) in our analysis were made, both specimens in the MP-tree grouped with *S. rorida* Laksch. (BP 62 %) rather than with *S. arbutifolia*. Based on the MP-analysis of nucleotide sequences of the cpDNA *rbcL* gene, Azuma et al. (2000) demonstrated that species from three typically recognized genera – *Chosenia*, *Salix* and *Toisusu* – form a monophyletic group. Based on the results of analysis of three cpDNA markers (*rbcL*, *trnD-trnT*, *atpB-rbcL*), Chen et al. (2010) included *Chosenia* in *Salix* at the rank of subgeneric level and proposed to subdivide the genus *Salix* s.l. into four subgenera: *Triandrae*, *Salix* (without sections *Triandrae* and *Urbanianae*), *Chosenia* (including section *Urbanianae*), and *Vetrix*, which combines the species from the previously established subgenera *Vetrix* and *Chamaetia*. The data obtained by Chen et al. (2010) did not support the opinion of Chao et al. (1998) concerning the recognition of *Pleiarina* as a separate genus. The results of the study conducted by Hardig et al. (2010) based on the comparison of the sequences of the cpDNA *matK* gene and the ITS region partly supported the most-recent infrageneric classification proposed by Argus (2010). The research by Abdollahzadeh et al. (2011) was mainly focused on establishing the relationships between the Iranian species of *Salix* by using sequences of the ITS and *trnL-F* region of cpDNA.

Today, there are two extensive regional monographic treatments based on contemporary approaches to infrageneric classification of *Salix*: by willows from Japan (Ohashi 2001) and North America (Argus 1997, 2010). Oha-

shi accepts the broad concept with respect to the genus *Salix*, relying on the data on pollen morphology (Sohma 1993), the results of molecular analysis (Leskinen & Alström-Rapaport 1999, Azuma et al. 2000), and the novel evaluation of morphological features. He divided *Salix* in Japan into six subgenera: *Pleuradenia*, *Chosenia*, *Protitea*, *Chamaetia*, *Salix*, and *Vetrix*. Ohashi included the section *Urbaniana* in the subgenus *Pleuradenia*, and sections *Floridanae*, *Humboldtianae*, *Tetraspermae*, *Wilsonia* from the subgenus *Salix* in the subgenus *Protitea*. Based on the phenetic study of species widespread in the New World, Argus (1997) proposed a new classification of *Salix* at the subgeneric and sectional levels, which differed from the scheme previously proposed by Skvortsov (1968) in terms of details. For the sake of completeness of the data, this author included a number of species representing close European or Asian sections of *Salix*, as well as the genus *Chosenia*, in the study. He regarded the American section *Longifoliae* as an independent subgenus: *Salix* subgen. *Longifoliae* (Andersson) Argus. The sections *Humboldtianae* and *Floridanae* were placed by Argus (2010) in the subgenus *Protitea*, which had previously been included in the subgenus *Salix* by Skvortsov (1968).

Thus, the molecular studies have demonstrated the monophyly for the genera *Toisusu*, *Salix*, and *Chosenia*. However, the evolutionary relationships within the *Salix* s.l. still remain highly controversial. High degree of individual variability and broad development of hybridization processes are the typical features of willows. Apparently, this fact is a major obstacle on the way to establishing phylogenetic relationships in the genus at both morphological and molecular levels. The aim of our study was to reveal the relationships between the Far Eastern *Salix* species and their position in the modern system of genera based on a comparative analysis of sequences of ten regions of chloroplast DNA (cpDNA; *petG-trnP*, *petN-psbM*, *psaA-ycf3*, *psbM-trnD*, *rpoB-trnC*, *trnC-petN*, *trnD-trnT*, *trnS-psbZ*, *rbcL*, *matK*) and the ITS region (ITS1–5.8S rRNA gene–ITS) of the nuclear ribosomal DNA (nrDNA), and to develop the infrageneric classification of *Salix*.

MATERIALS AND METHODS

Taxon sampling

The specimens were taken from natural populations in different regions of the Russian Far East. The list of species used in this study is outlined in Appendix I. All voucher specimens are deposited at the Regional Herbarium of the Institute of Biology and Soil Science, Vladivostok (VLA). The authors of taxa were indicated on the International Plant Names Index (IPNI) database (<http://www.ipni.org/ipni/authorsearchpage.do>).

DNA isolation, amplification, and sequencing

Total genomic DNA was extracted from silica-dried leaves using DNeasy Plant Mini Kits (Qiagen, Hilden, Germany), following the manufacturer's instructions.

The ITS region (ITS1–5.8S rRNA gene–ITS2) of the nrDNA and ten plastid regions (*petG-trnP*, *petN-psbM*,

psaA-ycf3, *psbM-trnD*, *rpoB-trnC*, *trnC-petN*, *trnD-trnT*, *trnS-psbZ*, *rbcL* and *matK*) were amplified using the polymerase chain reaction (PCR). The previously published primer pairs (Table 1) and recommended PCR parameters (Leskinen & Alström-Rapaport 1999, Mir et al. 2010, Hamza-Babiker et al. 2009, Huang et al. 2002, Shaw et al. 2005) were used to amplify these regions. Amplification and sequencing reactions were carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA). Product size and concentration were estimated by agarose gel electrophoresis.

The PCR products were sequenced using a BigDye terminator v.3.1 sequencing standard kit (Applied Biosystems, USA). Sequencing was carried out in both directions under cyclic sequencing conditions described by Leskinen & Alström-Rapaport (1999) for the ITS region of nrDNA and Shaw et al. (2005) for cpDNA regions using the same primer pairs that were used for amplification. In addition, internal primers were used for sequencing of the *trnD-trnT* and the *rpoB-trnC* IGS (Table 1). The sequences were analyzed on an ABI 3130 sequencer (Applied Biosystems, USA).

Sequence alignment and phylogenetic analysis

All sequences were assembled using the Staden Package v. 1.4 (Bonfield et al. 1995) and aligned manually using the SeaView program (Galtier et al. 1996). Three datasets were generated: matrix containing data from all ten cpDNA regions of Far Eastern *Salix* species; matrix containing data from the ITS and cpDNA of Far Eastern *Salix* species; and matrix containing data from ITS region of Far Eastern *Salix* species and thirty accessions from the EMBL/GenBank database. The alignments were imported into PAUP 4.0b10 (Swofford 2003) and analyzed using maximum parsimony as the optimality criterion (Swofford et al. 1996).

Indels in all datasets were coded as binary characters based on the Simmons & Ochorena (2000) – simple coding option, in FastGap 1.2 (Borchsenius 2009). A heuristic search employing 1,000 random addition sequence replicates with tree bisection and reconnection branch swapping was implemented and the strict consensus tree was then calculated from the most parsimonious trees. The robustness of the trees was estimated by bootstrap percentages (BP; Felsenstein 1985) using 1,000 replications. BP less than 50 % were not considered and are not shown in the figures 1–3.

RESULTS

The *petG-trnP*, the *petN-psbM*, the *psaA-ycf3*, the *psbM-trnD*, the *rpoB-trnC*, the *trnC-petN*, the *trnD-trnT*, the *trnS-psbZ*, the *rbcL*, the *matK* regions of cpDNA and the ITS region of nrDNA were successfully amplified and sequenced for all taxa. Sequences were deposited in the European Molecular Biology Laboratory (EMBL-Bank) and accession numbers for all sequences produced are given in Appendices I and II.

Phylogenetic relationships inferred from combining ten regions containing cpDNA sequences

The sequence characteristics of ten cpDNA regions are summarized in Table 2. Informativity of cpDNA regions is

Table 1. Primers used for amplification and direct sequencing of the ITS nrDNA and the ten cpDNA regions in this study

Region	Code	Sequence (5'g 3')	Author
ITS1–ITS2	ITS1	TCCGTAGGTGAACCTGCGG	Mir et al. 2010
	ITS4	TCCTCCGCTTATTGATATGC	Mir et al. 2010
	a	TCGTAACAAGGTTTCCGTAGG	Leskinen & Alström-Rapaport 1999
	d	TTCCCTCCGCTTATTGATATGC	Leskinen & Alström-Rapaport 1999
<i>petG–trnP</i>	petG	GGTCTAATTTCCTATAAATTGGC	Huang et al. 2002
	trnP	GGGATGTGGCGCAGCTTGG	Huang et al. 2002
<i>petN–psbM</i>	trnC400F	TAGTAAGTCTCGCTTGGGCT	Hamza-Babiker et al. 2009
	trnC1000R	CACTATGTCTATTTTATTTCCTCCG	Hamza-Babiker et al. 2009
<i>psaA–ycf3</i>	psaAF	ACTTCTGGTTCCGGCGAACGAA	Demesure et al. 1995
	ycf3-3R	GCRGARGCTTGGTTYGATCAAGC	Heinze 2007
<i>psbM–trnD</i>	trnD450F	TTGTGTATGTGCTACCGGGA	Hamza-Babiker et al. 2009
	trnDM	GGGATTGTAGTTCAATTGGT	Demesure et al. 1995
<i>rpoB–trnC</i>	rpoB	CACCCRGATTYGAACCTGGGG	Shaw et al. 2005
	trnC ^{GCA}	CKACAAAAYCCYTCAAATG	Shaw et al. 2005
	rpoBF1	ATTTGCAACAGATACAACTGG	self-designed primer
	rpoBR1	TTCAGGACAAAATTGGAACCG	self-designed primer
<i>trnC–petN</i>	trnCF	CCAGTTCAAAATCTGGGTGTC	Demesure et al. 1995
	trnC400R	AGCCCAAGCGAGACTTACTA	Heinze 2007
<i>trnD–trnT</i>	trnD ^{GUCF}	ACCAAATTGAACTACAATCCC	Shaw et al. 2005
	trnT ^{GGUR}	CTACCACTGAGTTAAAAGGG	Shaw et al. 2005
	trnE ^{UUCR}	AGGACATCTCTCTTTCAAGGAG	Shaw et al. 2005
	trnY ^{GUA} F	CCGAGCTGGATTGAAACCA	Shaw et al. 2005
<i>trnS–psbZ</i>	trnSP	GAGAGAGAGGGATTTCGAACC	Demesure et al. 1995
	ycf9m	CAAAMACAGCCAATTGGAAAGC	Heinze 2007
<i>rbcL</i>	carbcLF	TCCTCAACCTGGAGTTCCTCGC	Hamza-Babiker et al. 2009
	carbcLR	TCGCATGTACCTGCAGTAGC	Hamza-Babiker et al. 2009
<i>matK</i>	trnK710F	GTATCGCACTATGTWTCATTTGA	Brunsfeld et al. 2007
	matK1556R	GAGGCTGTCCCCCAATCC	Brunsfeld et al. 2007

low. The alignment of the combined ten cpDNA regions in 68 specimens of Far Eastern *Salix* species and *Populus suaveolens* Fisch. as an outgroup, was 6957 characters in length. The data matrix contained 410 (6 %) variable sites, of which 149 (2 %) were potentially parsimony informative (PI). The combined parsimony analysis recovered a single-most parsimonious tree with the tree length = 539, consistency index (CI) = 0.7755, and the retention index (RI) = 0.7551. The strict consensus tree is shown in Fig. 1. The MP analysis produced two unresolved clades. Clade 1 was composed of three accessions of two species: *S. pseudopentandra* (Flod.) Flod. and *S. pierotii* Miq. from the subgenus *Salix* (BP 95 %), and clade 2 was composed of 65 accessions of 56 species from different subgenera (BP 88 %). MP analysis was not able to resolve the positions of species from subgenera *Chamaetia* and *Vetrix*, including *S. cardiophylla* (*Toisusu*) from the subgenus *Salix*, in clade 2. The topology demonstrates a strongly supported sister group relationship between *S. arbutifolia* (*Chosenia*) and the remaining taxa (BP 77 %), and *S. nipponica* Franch. et Sav. was sister to aggregate of *S. arbutifolia* and all other taxa with high support (BP 88 %).

Phylogenetic relationships inferred from combining ten cpDNA regions and ITS sequences

The length of the ITS region for *Salix* species with the exception of *S. nipponica* and *S. reticulata* L. (621 bp) was 620 bp, and for *Populus suaveolens* it was 619 bp. The level of divergence among *Salix* species was very low and ranged from 0 to 2.7 %. The ITS sequences for the species

of subgenera *Chamaetia* and *Vetrix* were either identical or differed only by one or two substitutions (overall mean distance 0.004).

The alignment dataset combining ten cpDNA regions and ITS sequences of Far Eastern *Salix* species was 7527 characters in length. Among the total characters, 465 (6 %) were variable and 180 of the variable characters were PI. The combined parsimony analysis recovered a single-most parsimonious tree with the tree length = 657, CI = 0.7260, and RI = 0.7015. The strict consensus tree is shown in Fig. 2. The topology of this tree was nearly identical to that of the tree constructed on the basis of MP analysis of the dataset of ten regions of cpDNA sequences. After examining these trees, we noted that topological differences were mostly due to the position of *S. cardiophylla*. This specimen of *S. cardiophylla* is sister to a group species from subgenus *Chamaetia* and subgenus *Vetrix* (BP 66 %).

Phylogenetic relationships inferred from ITS sequences

The sequences of the ITS region of 72 Far Eastern specimens and 29 accessions from the EMBL/GenBank database of 86 *Salix* species from three subgenera (*Salix*, *Chamaetia*, *Vetrix*), 27 sections and one *Chosenia* (*S. arbutifolia*) species were used for phylogenetic analysis. The subgenus *Salix* consisted of species from *Salix*, *Salicaster* (*Pentandrae*), *Humboldtianae*, *Urbanianae*, *Subalbae*, *Longifoliae*, *Triandrae* (*Amygdalinae*), *Magnificae*, and *Wilsonia* (*Glandulosae*) sections. Here we use the infrageneric classification of *Salix* that was proposed by Skvortsov (1968) and was developed on the basis of morphological characteristics.

Table 2. Sequence information for plastid loci and ITS of *Salix* species

Region	Length of sequences (bp)	Number of constant sites (excluding gaps)	Number of variable sites (excluding gaps)	Number of parsimony informative sites (excluding gaps)
<i>petG-trnP</i>	566–621	561	18	11
<i>petN-psbM</i>	545–568	513	11	7
<i>psaA-ycf3</i>	744–798	720	13	5
<i>psbM-trnD</i>	409–459	386	13	6
<i>rpoB-trnC</i>	675–697	619	27	13
<i>trnC-petN</i>	407–442	397	9	7
<i>trnD-trnT</i>	935–989	883	28	12
<i>trnS-psbZ</i>	455–475	445	8	4
<i>rbcL</i>	597	588	9	5
<i>matK</i>	835	823	12	6
ITS1–ITS2	620–621	588	32	22

The data matrix with a length of 634 characters contained 101 variable sites, of which 70 were PI. The combined parsimony analysis recovered a single-most parsimonious tree with the tree length = 152, CI = 0.7171, and RI = 0.8365. The strict consensus tree is shown in Fig. 3. The topology of the MP phylogenetic tree demonstrates the monophyly of the genus *Salix* with all *Salix* species forming two clades with a strong statistical support (BP 100 %).

Clade 1 is composed of the accessions of three species from the subgenus *Salix* (BP 100 %), and clade 2 with moderate support is composed of 96 accessions of 82 species (BP 75 %). Separate branches within clade 2 with a high statistical support are formed by *S. pierotii* and *S. miyabeana* Seemen (BP 85 %); *S. dasyclados* Wimmer and *S. viminalis* L. (BP 90 %); *S. floridana* Chapm., *S. fragilis* L., *S. lucida* Muhl., *S. acmophylla* Boissieu, *S. alba* L., *S. pentandra* L. and *S. pseudopentandra* (BI 79 %); the two accessions of *S. exigua* Nutt., *S. interior* Rowlee, *S. melanopsis* Nutt. and *S. taxifolia* Kunth (BP 77 %). The other small groups with a moderate support are formed by two specimens of *S. alaxensis* (Andersson) Coville (BP 57 %); *S. cardiophylla* and *S. arbutifolia* (BP 57 %); *S. nipponica* and the two accessions of *S. triandra* L. (BP 55 %); *S. krylovii* E.L. Wolf, *S. integra* Thunb. ex Murray, *S. dasyclados* and *S. viminalis* (BP 61 %). The relationships between species from the subgenera *Chamaetia* and *Vetrix* and the sections *Wilsonia* and *Magnificae* of the subgenus *Salix* remain unresolved.

Thus, parsimony analyses of the ITS and cpDNA regions as separate or combined datasets are indistinguishable for many closely related *Salix* species, and sometimes are not very closely related (from different subgenera, for example, *S. pierotii* and *S. miyabeana*). A high level of similarity of the nuclear and plastid genomes of most species from the genus *Salix* may be regarded as an indication of their relatively recent divergence from a common ancestor, high level of interspecific hybridization, and the reticulate pattern of evolution typical of the genus (Argus 1997).

DISCUSSION

The subgenus *Salix* in the Northern Hemisphere is divided into the following sections: *Humboldtianae*, *Floridanae*, *Longifoliae*, *Salix*, *Salicaster*, *Subalbae*, *Triandrae*, *Urbanianae*, and *Wilsonia* (Skvortsov 1968, Dorn 1976, Argus 1997). Skvortsov (1968) viewed the section *Humboldtianae* rather broadly and included the Asian sections *Tetraspermae* Andersson and *Acrophyllae* Andersson therein. This section is distributed over the Old and the New World, over tropical, subtropical and partly over warm-temperate regions. Argus (1986) has earlier placed *S. floridana* from the monotypical American section *Floridanae* in the section *Humboldtianae* under an assumption that its immediate relatives may belong to the *Tetraspermae* section from the Old World. In our study, the section *Humboldtianae* s.l. is represented by five species: *S. amygdaloides* Andersson, *S. gooddingii* C.R. Ball, *S. humboldtiana* Willd., *S. acmophylla*, and *S. floridana*. According to the results of the ITS data analysis (Fig. 3), this section in the sense adopted here is not monophyletic. In the phylogenetic tree, species belonging to the section *Humboldtianae* are appeared in two sister clades: *S. amygdaloides*, *S. gooddingii*, and *S. humboldtiana* form a well-supported first clade (BP 100 %), while *S. acmophylla* and *S. floridana* are placed in the second clade (BP 75 %) along with the other *Salix* species included in the analysis, and are grouped into a single cluster (BP 79 %) with species from the sections *Salix* and *Salicaster*. Results of the *matK*-based phylogeny (Hardig et al. 2010) do not provide strong support for placing *S. floridana* in the section *Humboldtianae* too. The position of this species remains unresolved in their phylogenetic tree. Argus (1997) has previously revealed phenetic similarity of *S. floridana* and *S. tetrasperma* Roxb., but he considered that it was rather distant. Following Dorn (1976), he left the sections *Floridanae* and *Humboldtianae* in the subgenus *Salix*.

In his later work, Argus (2010) relocated the sections *Floridanae* and *Humboldtianae* from the subgenus *Salix* to the subgenus *Protitea*. This subgenus is characterized by bud-scales with distinct, overlapping margins and flowers with multiple stamens. Ohashi (2001) placed sections *Humboldtianae*, *Tetraspermae* and the East Asian section *Wilsonia*, including *S. chaenomeloides* Kimura, in the sub-

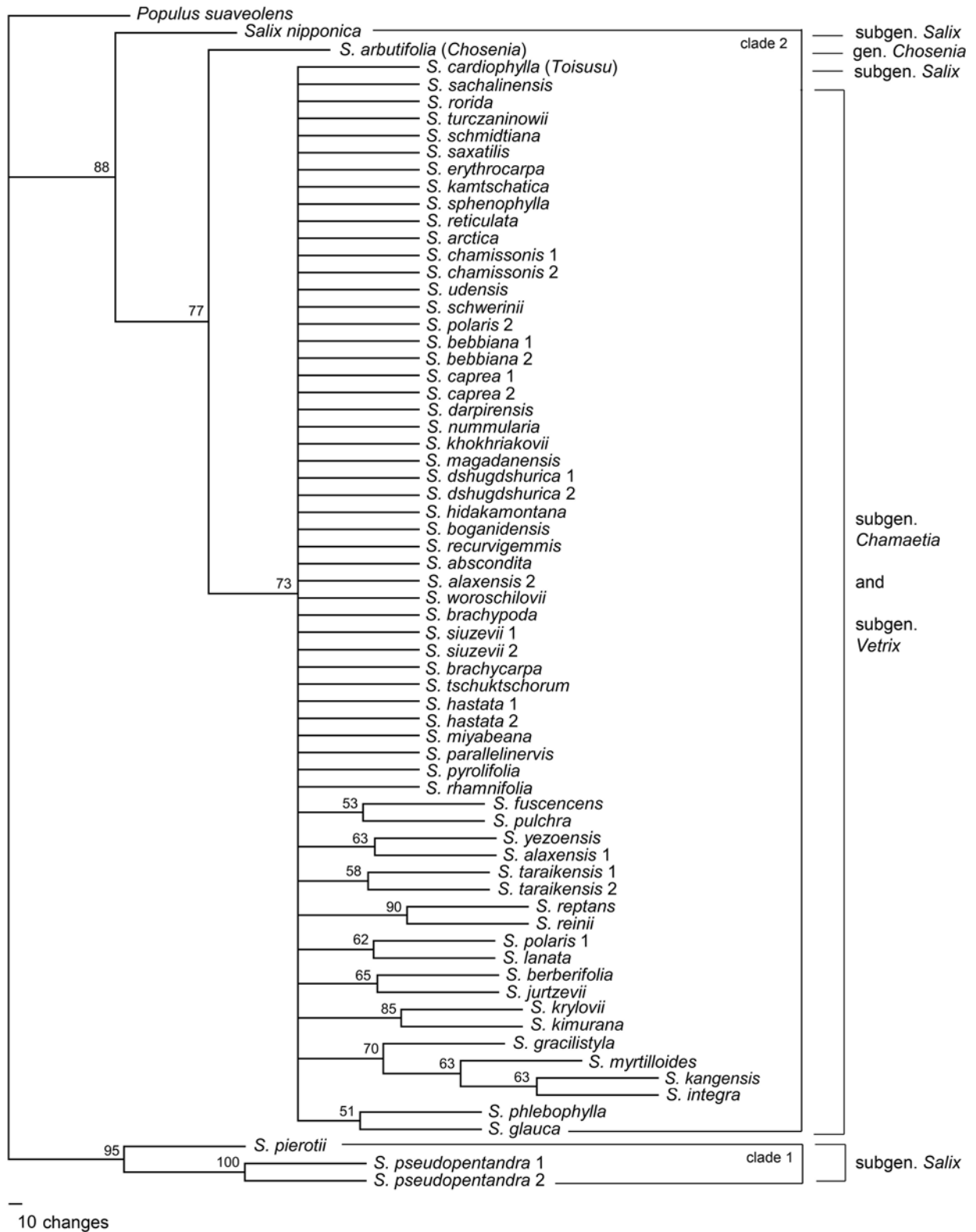


Figure 1 Strict consensus tree constructed from MP analysis of the ten cpDNA regions sequences *Salix* species with *Populus* species as outgroups. Bootstrap percentages greater than 50 % are displayed on the branches. The subgenera are given according to Skvortsov (1968)

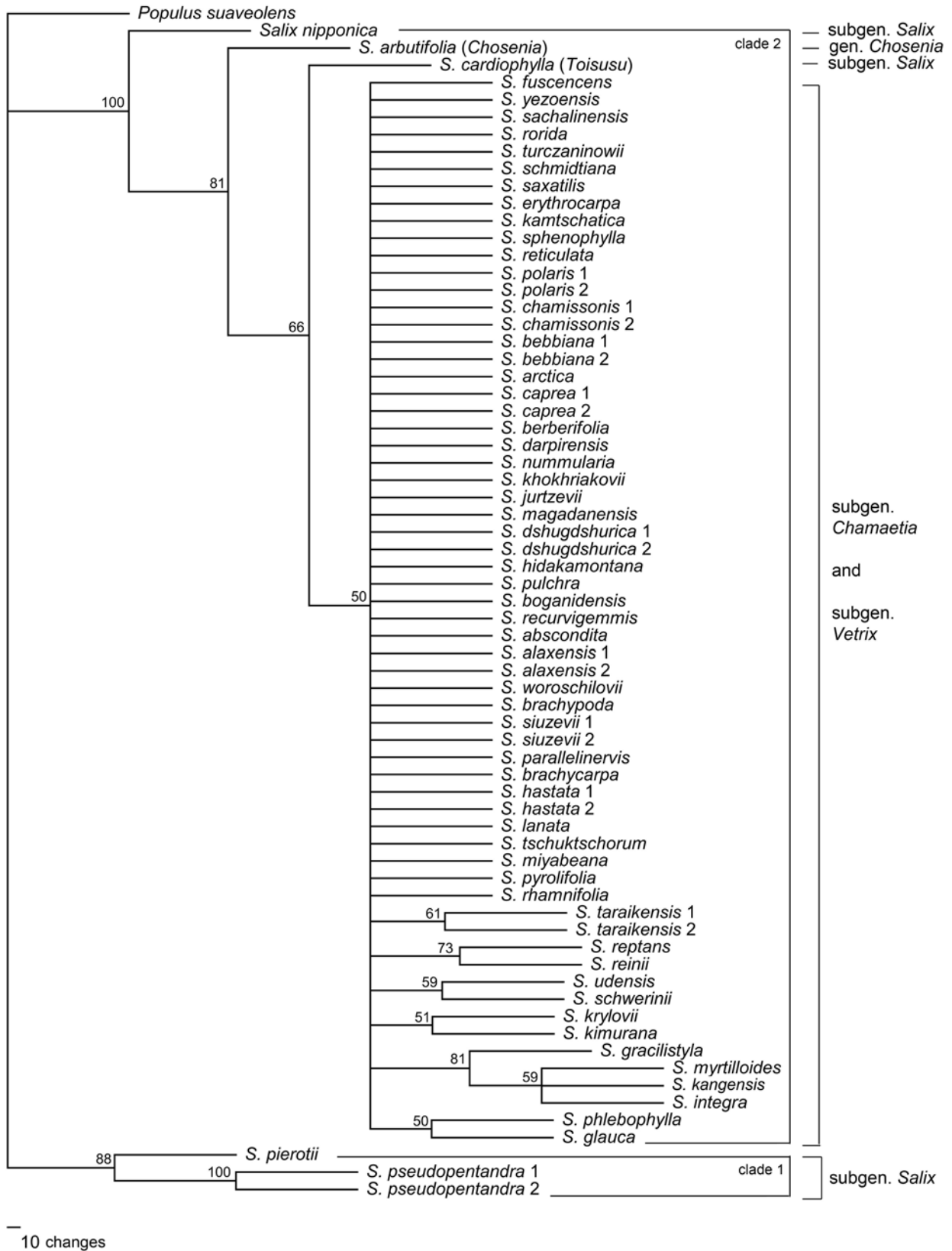


Figure 2 Strict consensus tree constructed from MP analysis of combined the ten of cpDNA regions and ITS of nrDNA sequences *Salix* species with *Populus* species as outgroups. Bootstrap percentages greater than 50% are displayed on the branches. The subgenera are given according to Skvortsov (1968)

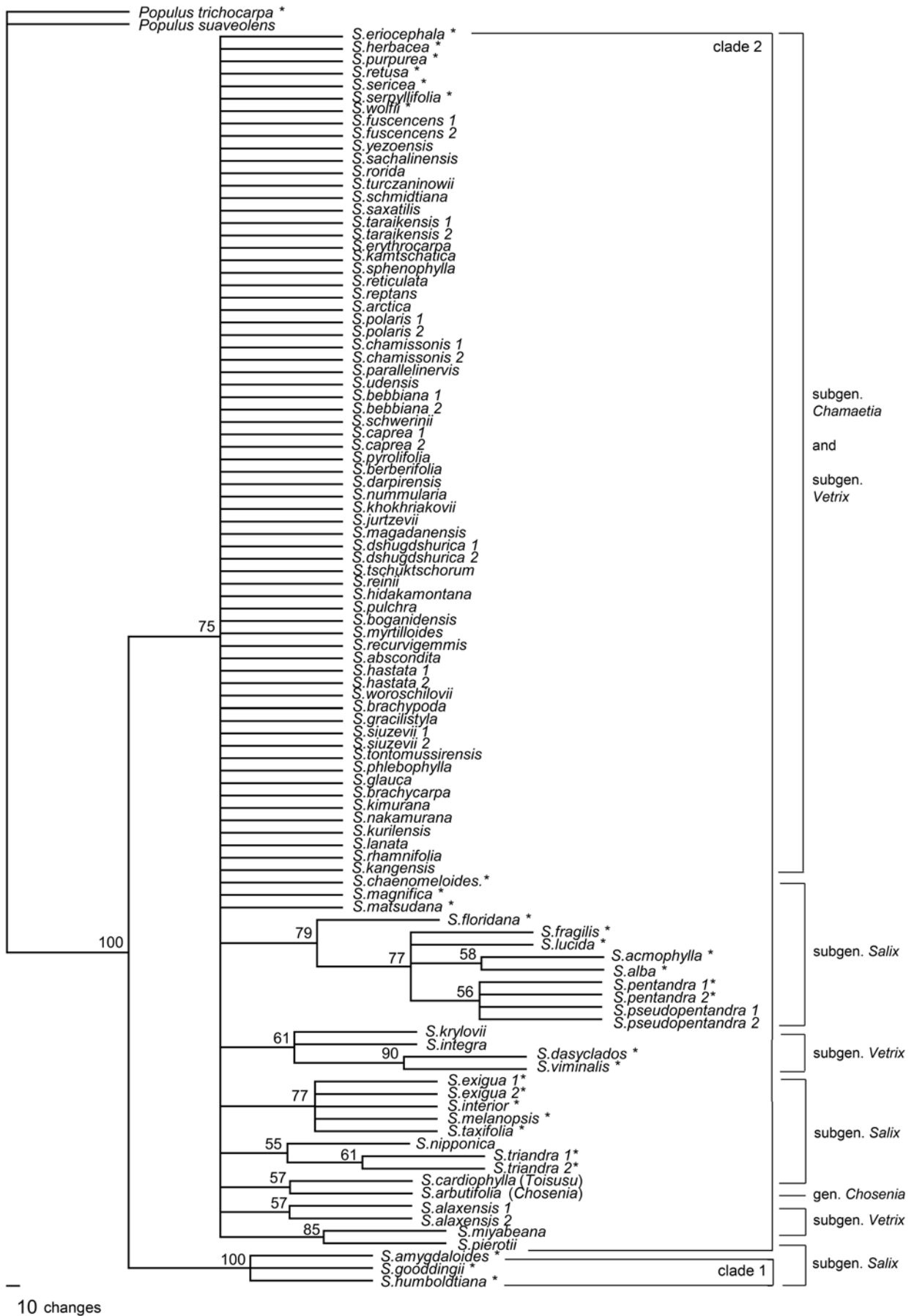


Figure 3 Strict consensus tree of 1000 equally parsimonious trees constructed from MP analysis of 101 ITS sequences *Salix* species with *Populus* species as outgroups. Bootstrap percentages greater than 50 % are displayed on the branches. Asterisks denote the sequences obtained from GenBank. The subgenera are given according to Skvortsov (1968)

genus *Protitea*. Earlier, Skvortsov (1968) considered the section *Wilsonia* (referred to as sect. *Glandulosae*) in the subgenus *Salix* and highlighted its clear connection with the sections *Humboldtianae* and *Salicaster* (referred to as sect. *Pentandrae*). The relationship between these sections was confirmed using the analysis based on *rbcL* sequencing (Azuma et al. 2000, Chen et al. 2010). According to the results of molecular studies (Azuma et al. 2000, Hardig et al. 2010, Chen et al. 2010, our data), species assigned to the subgenus *Protitea* (*S. amygdaloides*, *S. gooddingii*, *S. humboldtiana*, *S. floridana*, *S. tetrasperma*, *S. acmophylla*, and *S. chaenomeloides*) (Ohashi 2001, Argus 2010), appear in various clusters or clades, attesting to its polyphyletic nature. According to Ohashi (2000, 2001), Asian *S. glandulosa* Seemen (= *S. chaenomeloides* Kimura), whose genetic relationships remained unresolved in our study, is the type species of this subgenus. In the *rbcL*-phylogeny (Azuma et al. 2000, Chen et al. 2010), the *S. chaenomeloides* is placed in the same cluster along the species of the subgenus *Salix* s.l., demonstrating the resemblance to the Asian *S. tetrasperma* (sect. *Humboldtianae*) and African *S. mucronata* Thunb. (sect. *Octandrae* Andersson). In our phylogenetic tree, which is based on the ITS data, the subgenus *Protitea* corresponds to a small group of American species from the section *Humboldtianae* found in the first clade (Fig. 3). The further studies are to be conducted for the taxonomic status of the subgenus *Protitea* using a larger number of species and/or molecular markers other than ITS.

The natural habitat of *S. acmophylla* is entirely confined to the arid continental areas of Asia, from the Middle East to northern India, including Turkey, Iran, Afghanistan, and Tajikistan (Skvortsov 1968). Nasarow (1936) regarded *S. acmophylla* as a part of the special section *Acrophyllae* and noted that it hybridizes with *S. alba*. Abdollahzadeh et al. (2011) found several polymorphic sites in the ITS region of nuclear DNA in *S. acmophylla* and suggested that it was of hybrid origin, one of its parental species could have been *S. alba*. In the phylogenetic tree obtained by us (Fig. 3), both of these species form a moderately supported branch (BP 58 %), which could support this opinion. In the *matK*-based phylogeny (Hardig et al. 2010), *S. acmophylla* is grouped in the same cluster with species of the subgenera *Vetrix* and *Chamaetia*, whose relationships remain unresolved.

According to certain morphological characteristics (shape and internal structure of the buds, shape of stipules, the pale color of floral bracts deciduous after flowering in female flowers), *S. fragilis* belonging to a small section of *Salix* is close to *S. pentandra* from the section *Salicaster*, which attests to the relationship between the two sections (Skvortsov 1968). Male flowers of *S. fragilis* and its close relative *S. alba* normally contain two stamens, but as noted by Skvortsov, there occasionally occur specimens with multistaminate flowers (up to four or eight stamens). According to his opinion, multistaminal characteristic should probably be regarded as a genuine atavism, which also speaks in favor of the close relationship between the sections *Salix* and *Salicaster*. In the phylogenetic tree obtained by us (Fig. 3), the species of both sections are grouped in the same cluster with a relatively high statistical support (BP



Figure 4 Male aments of *Salix abscondita* (Primorsky Territory, vic. of Ussuriysk city)

77 %), which also confirms their relationship at the genetic level. The same results were obtained in studies by Leskinen & Alström-Rapoport (1999), which were based on the ITS data, and in studies by Abdollahzadeh et al. (2011), which were based on the *trnL-F* data.

The section *Salicaster* contains two well-distinguished groups that can be regarded as subsections (Skvortsov 1968). In addition to *S. pentandra* and *S. pseudopentandra*, the first group, includes species that are closely related to them, namely, Chinese *S. paraplesia* C.K. Schneid. and the North American *S. serissima* (L.H. Bailey) Fernald. The second group comprises two or three North American boreal species: *S. lucida*, *S. lasiandra* Benth., and *S. caudata* Heller. Argus (2010) considered the latter species to have the variant rank: *S. lasiandra* var. *caudata* (Nutt.) Sudw. In our analysis, *S. lucida* occupies a special position in the cluster as compared to two other species in the section, *S. pentandra* and *S. pseudopentandra* (Fig. 3). This fact to some extent supports the view of intrasectional division of *Salicaster* proposed by Skvortsov. In the analysis based on the *rbcL* data (Chen et al. 2010), species of the section *Salicaster* appear in two sister clades: *S. lucida* is grouped in the clade with species from subgenus *Salix* of the New World, including section *Longifoliae*; *S. pentandra* and *S. paraplesia* are grouped in the clade with the species of the subgenus *Salix* of the Old World. In the *matK*-based phylogeny (Hardig et al. 2010), two samples of *S. lucida* appear in different clades. The authors attributed this to the chloroplast capture effect during hybridization with other *Salix* species.

Skvortsov (1968) did not agree with the attempts to split the subgenus *Salix* into smaller subgenera, but noted the most isolated position of two sections there: the East Asian *Urbaniana* and American *Longifoliae*. The section *Urbaniana* with its extremely primitive structure of the flower (stamens or stipe of ovary fused with floral bract, resembling the flowers of *Populus*) was often regarded as a separate genus *Toisusu* different from the genus *Salix* (Kimura 1928, 1989, Ohwi 1965, Zhang 1994, Nedoluzhko 1995a, 1995b) or was included in the subgenus *Pleuradenia* of the genus *Salix* (Kimura 1988, Ohashi 2001). In addition, the

species from this section are characterized by the following features: bud-scales with distinct, overlapping margins, pendulous flowering catkins with a thin flexible rachis, 5–10 stamens, two transversally located adaxial nectaries and one abaxial nectary, floral bracts deciduous in the pistillate flowers after flowering, branches of the style together with stigmas deciduous prior to the period of fruiting. According to the analysis of the ITS data, *S. cardiophylla* belonging to the section *Urbanianae*, is placed on the same branch with *S. arbutifolia* (*Chosenia*) in the second clade (Fig. 3) with a moderate statistical support (BP 57 %), which to a certain extent attests to their genetic relationship. The *Urbanianae* section has not been previously represented in the study based on the ITS data.

In terms of flower structure, style deciduous to the period of capsule maturation, bud structure, anatomical structure of the leaf, and bark features, the former genus *Chosenia* demonstrates similarities with *Salix* (the sections *Longifoliae*, *Triandrae*, *Urbanianae*). According to the data obtained by Nedoluzhko (1995b), fruits of *S. cardiophylla* (*Toisusu*) over the entire area of its distribution in Russia contain four seeds. This feature is also characteristic of *S. arbutifolia* (*Chosenia*) and species belonging to the sections *Tetraspermae* and *Floridanae* from the subgenus *Salix*, which may be indicative of close genetic relationships between the above mentioned taxa. Ohashi (2001) lowered the genus *Chosenia* to the rank of subgenus: *Salix* subgen. *Chosenia*, and the former genus *Toisusu*, followed by Kimura (1988), included in the subgenus *Pleuradenia* of the *Salix* genus. He believed that the subgenus *Chosenia* was closer to the subgenus *Salix* than the subgenus *Pleuradenia* as the abnormal form – *S. arbutifolia* f. *adenantha* – may be indicative of recent divergence of *Chosenia* from *Salix* with the loss of nectaries. According to Skvortsov (1966), *Chosenia* became isolated after the separation of willows and poplars and has evolved from a certain trunk part of primitive willows; wind pollination is the secondary acquired characteristic. There is an opinion based on the data from *matK* and ITS sequencing that *Chosenia* is a morphologically divergent part of the *Salix* subgen. *Vetrix* (Brunsfield & Antilla 2004). Perhaps there is some truth to this notion; however, further evidence is required to adopt or refute this hypothesis. Narrow confinement to the alluvial deposits of the middle flow rate of the mountain rivers and wind pollination enabled *Chosenia* to spread far to the north, to the permafrost areas up to Chukotka.

Accepting the subgenus *Pleuradenia* with a single polymorphic species *S. cardiophylla*, Ohashi (2001) relied exclusively on the general morphology and considered this subgenus to be more primitive in the genus *Salix* than the subgenus *Chosenia*. Based on the data obtained by analyzing cpDNA, Chen et al. (2010) also considered that *Chosenia* had a rank of subgenus, but as opposed to Ohashi, they placed the section *Urbanianae* containing *S. cardiophylla* in it. Chao (1996) included three very similar species, *S. cardiophylla*, *S. maximowiczii* Kom. and *S. urbaniana* Seemen, in the genus *Chosenia* and proposed new nomenclatural combinations for them. However, this was not adopted in the 'Flora of China' (Fang et al. 1999). As noted above, *S. cardiophylla* and *S. arbutifolia* representing the previously

recognized genera (*Toisusu* and *Chosenia*, respectively) are grouped on the same branch of the MP-tree, but with a moderate statistical support. Taking into account the significant differences in morphology between *Toisusu* and *Chosenia* as well as with other *Salix* species, they should be recognized as the subgenera – *Salix* subgen. *Pleuradenia* and *Salix* subgen. *Chosenia*, respectively, as it was adopted in the classification scheme proposed by Ohashi (2001).

The American section *Longifoliae* adapted to the arid climate occupies a special position in the subgenus *Salix*. It is the only section that retained the primitive structure of the flower (but the number of stamens is reduced to two), bilateral, chlorophyll-deficient, one-layer hypodermis in structure of the leaf similar to that in *Chosenia* and *Populus* subgen. *Turanga*, and the ability to form root shoots resemble to poplars (Skvortsov 1968). Such an important feature as branching catkins that has been noted as a characteristic feature exclusively for the aforementioned section (Argus 1997) was also observed by Barkalov in male plants of Asian species belonging to the subgenus *Vetrix* – *S. abscondita* Laksch. (Fig. 4), *S. caprea* L. and *S. integra*. In this case, it should presumably be regarded as an atavism.

In the *rbcL*-phylogeny (Azuma et al. 2000, Chen et al. 2010), the species from the section *Longifoliae* were placed in the same cluster with the American representatives of the sections *Humboldtianae*, *Floridanae*, *Maccalianae*, and *Salicaster* from the subgenus *Salix*, but on a separate branch. Some biological (Mosseler 1989, 1990) and biochemical (Chong et al. 1995) features are indicative of genetic relationship between the section *Longifoliae* and the subgenus *Vetrix*. Species from the section *Longifoliae* (*S. exigua*, *S. interior*, *S. melanopsis*, *S. taxifolia*) included in our analysis form an unresolved group (Fig. 3) with high statistical support (BP 77 %). The results of the ITS-phylogeny (Leskinen & Alström-Rapoport 1999, Hardig et al. 2010, our data) indicate the naturalness of this group of willows and are consistent with the opinion of Argus (1997), who raised section *Longifoliae* to subgenus level – *Salix* subgen. *Longifoliae*.

Salix triandra and *S. nipponica* included in the analysis in this study belong to the small Eurasian section *Triandrae*. Skvortsov (1968) assigned the rank of subspecies to *S. nipponica* – *S. triandra* subsp. *nipponica* (Franch.) A.K. Skvortsov; while Ohashi (2001) included it in a number of synonyms of *S. triandra*. In our study, the ITS sequences of these species differ from each other in terms of two C↔T substitutions in the ITS1 region. Furthermore, only the diploid chromosome number $2n = 38$ has been identified for *S. nipponica* (Suda 1958 as *S. subfragilis* Andersson, Probatova et al. 2011), whereas the tetraploid number with $2n = 76$ has been identified for *S. triandra*. Chen et al. (2010) proposed to isolate the section *Triandrae* and proclaim it to be a special subgenus based on the comparative analysis of the sequences of three cpDNA regions and the available data obtained in morphological, biochemical and genetic studies of others authors. In our MP-tree, *S. nipponica* and two samples of *S. triandra* form a separate branch in the clade 2 among most species included in the analysis (Fig. 3), but with a relatively low statistical support (BP 55 %). The po-

sition of *S. triandra* in the genus *Salix* has not been resolved in the genetic research conducted by Leskinen & Alström-Rapoport (1999) and Hamza-Babiker et al. (2009). In the study performed by Azuma et al. (2000), *S. nipponica* (referred to as *S. subfragilis*) was grouped in the same clade along with *Chosenia*, *Toisusu* and species from the subgenera *Chamaetia* and *Vetrix*, but in the sister position. The same situation can be observed for *S. triandra* in the results obtained by Chen et al. (2010). The study of relationships in the genus *Salix* using AFLP (amplified fragment length polymorphism) (Trybush et al. 2008) demonstrated that the genetic similarity between *S. triandra* and the subgenera *Salix* and *Vetrix* was identical.

Species belonging to the section *Triandrae* differ from other willows mainly by the bark of old trees, which resembles the bark of *Chosenia*. Furthermore, they have special structure of anthers: both anther bags face forward rather than sideward (Skvortsov 1968). Sometimes short ferruginous hair are involved in indumentums of young shoots, stems, leaves and bracts in *S. nipponica*, which was observed in *S. pierotii* from the subgenus *Salix*, as well as in *S. caprea*, *S. divaricata* Pall., *S. fuscescens* Andersson, *S. hastata* L., *S. integra*, *S. vulpina* Andersson from the subgenus *Vetrix*. In *S. triandra* and *S. nipponica*, flowers typically have three stamens, but there are also specimens with two, four or five stamens (Fang et al. 1999, Ohashi 2001). The above-mentioned characteristics presumably result from hybridization with species belonging to the other *Salix* groups during the evolution of the group. Molecular studies have demonstrated that based on the requirements of monophyly, the section *Triandrae* representing the xeromorphic line of *Salix* evolution should nominally be made regarded as a separate subgenus. However, further research with larger amount of data is required.

Certain representatives of the sections belonging to the subgenus *Vetrix* form separate branches in the MP-tree; less frequently they are grouped with species from another subgenus. Thus, *S. miyabeana* from the section *Helix* of the subgenus *Vetrix* and *S. pierotii* from the section *Subalbae* of the subgenus *Salix* form a sister pair (BP 85 %, Fig. 3). The East Asian section *Subalbae*, in addition to *S. pierotii*, includes *S. jessoënsis* Seemen, *S. eriocarpa* Franch. et Sav., *S. matsudana* Koidz., and *S. babylonica* L. (Ohashi 2001); among which two species – *S. pierotii* and *S. matsudana* (= *S. babylonica* L.: Liu et al. 2012) – were included in our analysis. Likewise, the results of phenetic analysis (Argus 1997) support neither the isolation of the section *Subalbae* (e.g., Skvortsov 1968, Dorn 1976, Zhang 1994, Ohashi 2001) nor its inclusion in the section *Salix* (e.g., Wang & Fang 1984, Fang et al. 1999). According to *rbcL* sequencing data, *S. babylonica* belonging to the section *Subalbae* is located in the same cluster along with *S. alba* from the section *Salix* and *S. pentandra* from the section *Salicaster* (Azuma et al. 2000, Chen et al. 2010). In our study, which was based on the ITS data, genetic relationships between *S. pierotii* and *S. matsudana* belonging to the section *Subalbae* remained unresolved. Therefore, molecular studies provide a clear picture neither for the taxonomic status of the section *Subalbae* nor for its position in the subgenus *Salix*.

Skvortsov (1968) has noted that the genetic relationships of predominantly Eurasian section *Helix* are very problematic. According to his opinion, this section has a number of primitive (for willows) characteristics: flat crenulated leaves, short stamens, small bracts covered with short hair from the inside, and the presence of large trees among a number of species, and the southern habitat in general. Based on these facts, direct relationship with the subgenus *Salix* can be assumed (e.g., with the section *Subalbae*); however, most *Helix* species are characterized by many advanced features as well (lack of hypodermis in leaves, black color of bract scales, presence of one nectary only, frequently observed bright yellow color of the phloem). According to the results of the comparative sequence analysis of ten cpDNA regions, *S. pierotii* (sect. *Subalbae*) and two specimens of *S. pseudopentandra* (sect. *Salicaster*) form clade 1 with high statistical support (BI 95 %), and *S. miyabeana* is grouped in clade 2 along with species belonging to the subgenera *Chamaetia* and *Vetrix* (Fig. 1). These data do not support the relationship between the sections *Subalbae* and *Helix*.

According to the results of the chloroplast and the ITS analysis, two species from the subgenus *Vetrix* – *S. alaxensis* and *S. krylovii*, which are placed in the section *Villosae* (Skvortsov 1968, Bolshakov 1992, Nedoluzhko 1995a), – are not grouped together (Figs. 1–3). According to the ITS data (Fig. 3), two specimens of *S. alaxensis* form an independent branch, while *S. krylovii* is grouped with species belonging to the section *Viminella*, *S. dasyclados* and *S. viminalis*, and section *Helix* – *S. integra*, but with a relatively low statistical support (BP 61%). Skvortsov (1968) highlights the undeniable genetic relationship of the section *Villosae* with the section *Viminella* (referred to as sect. *Vimen*) and the tentative relationships with the sections *Canae* and *Lanatae*. He placed North American species *S. candida* Flüggé ex Willd., which is closely related to the Asian *S. krylovii*, in the section *Villosae*. Argus (1997) considered *S. candida* and *S. krylovii* to belong to the section *Candidae*. He left *S. alaxensis* in the section *Villosae*. Phenetic analysis conducted by this author demonstrated close resemblance of the section *Villosae* with the sections *Lanatae* and *Candidae* and only distantly with *Viminella* or *Canae*. However, in addition to a long thin style, *S. alaxensis* is characterized by such an important morphological feature as extended petioles that are swollen by the end of growing season. These features occur in *S. dasyclados* and *S. schweerinii* E.L. Wolf (sect. *Viminella*), and in *S. gracilistyla* Miq. (sect. *Subviminalis*). The data of the phylogenetic analysis conducted by us support the Argus' view (1997) regarding the isolation of the section *Candidae* and indicate its relationship with the section *Viminella*.

The subgenus *Salix* is particularly rich in taxonomic problems because it is defined by different subset of ancestral characters (Hardig et al. 2010). As it was justly noted by Skvortsov (1968: 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”. The phylogenetic analysis based on the ITS data

largely supports the current classification schemes of the genus *Salix* at the subgeneric level (e.g., Ohashi 2001, Argus 2010), except for the subgenera *Protitea* and *Chamaetia*. Isolation of the subgenera *Pleuradenia*, *Chosenia*, *Salix* (without sect. *Triandrae*), and *Longifoliae* has been supported well. The inclusion of *Chosenia* and *Toisusu* as subgenera into the genus *Salix* renders it more natural. The appearance of the individual branches in the phylogenetic tree with the representatives belonging to the subgenus *Vetrix*, mainly in valley habitats, can be explained by crossing of the breeding lines through hybridization with the species from the subgenus *Salix*. Molecular and genetic studies do not support any of the other subdivisions within *Salix* at the subgenus level. Distinctions of the subgenera *Longifoliae* and *Triandrae* from the subgenus *Salix* only emphasize their features as natural groups of willows corresponding to the conditions of the environment in which they have evolved.

CONCLUSIONS

The comparative analysis of chloroplast DNA and the ITS region of nuclear DNA ribosomal operon enabled revealing the molecular differences mostly in species belonging to the subgenus *Salix* in its earlier broad meaning. The genera *Chosenia* and *Toisusu* exhibit close genetic relationships and are included in the genus *Salix*. High similarity of the plastid and nuclear genomes characteristic of most species belonging to the genus *Salix* included in the analysis can attest to the fact that they have relatively recently diverged from a common ancestor or there is result of lineage sorting through hybridization. None of the molecular and genetic studies performed provided support for the isolation of the subgenus *Chamaetia*. Further research involving larger number of species, especially those from southwest China and the Himalayas, and/or other molecular markers is required to improve the classification of *Salix*.

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LITERATURE CITED

- Abdollahzadeh, A., S. Kazempour Osaloo & A.A. Maassoumi 2011. Molecular phylogeny of the genus *Salix* (Salicaceae) with an emphasize to its species in Iran. *Iranian Journal of Botany* 17: 244–253.
- Argus, G.W. 1997. Infrageneric classification of *Salix* (Salicaceae) in the New World. *Systematic Botany Monographs* 52: 1–121.
- Argus, G.W. 2010. *Salix*. In: *Flora of North America North of Mexico*. (Flora of North America Editorial Committee, eds.). Vol. 7, pp. 23–162. Oxford University Press, New York, NY, and Oxford, UK.
- Azuma, T., T. Kajita, J. Yokoyama & H. Ohashi 2000. Phylogenetic relationships of *Salix* (Salicaceae) based on *rbcL* sequence data. *American Journal of Botany* 87: 67–75.

- Barkalov, V.Y. 2012. New *Salix* (Salicaceae) from the Russian Far East. *Botanicheskii Zhurnal* 97(6): 797–806 (in Russian with English summary). [Баркалов В.Ю. Новые виды *Salix* (Salicaceae) с российского Дальнего Востока // Ботанический журнал. 2012. Т. 97, № 6. С. 797–806 (с вклейкой).]
- Bolshakov, N.M. 1992. Salicaceae. In: *Flora of Siberia* (Krasnoborov I.M. & Malyshev L.I. eds.). Vol. 5, pp. 8–59 (in Russian). Nauka, Novosibirsk. [Большаков Н.М. 1992. Salicaceae // Флора Сибири / под ред. Красноборова И.М., Малышева Л.И. Новосибирск: Наука. Т. 5. С. 8–59.]
- Bonfield, J.K., K.F. Smith & R. Staden 1995. A new DNA sequence assembly program. *Nucleic Acids Research* 23: 4992–4999.
- Borchsenius, F. 2009. FastGap 1.2. Department of Biological Sciences, University of Aarhus, Denmark. Published online at http://192.38.46.42/aubot/fb/FastGap_home.htm
- Brunsfeld, S.J., T.R. Miller & B.C. Carstens 2007. Insights into the Biogeography of the Pacific Northwest of North America: Evidence from the Phylogeography of *Salix melanopsis*. *Systematic Botany* 32: 129–139.
- Brunsfeld, S.J., & C.K. Antilla 2004. Complexities in the phylogeny of *Salix* (Salicaceae). Botany. Systematic section / ASPT. Cottonwood B (Snowbird Center). Abstract ID: 791.
- Brunsfeld, S.J., D.E. Soltis & P.S. Soltis 1992. Evolutionary patterns and processes in *Salix* sect. *Longifoliae*: evidence from chloroplast DNA. *Systematic Botany* 17: 239–256.
- Chao, N. 1996. On the problems of the classification of *Toisusu Kimura* (Salicaceae) in Northeastern Asia. *Journal of Sichuan Forestry Science and Technology* 17(2): 12–15.
- Chao, N., G-T. Gong & J. Liu 1998. On the classification and distribution of the family Salicaceae. *Journal of Sichuan Forestry Science and Technology* 19(4): 9–20.
- Chao, N., G-T. Gong & J. Liu 2010. On the subdivision of the genus *Pleiarina* Rafn. [sic] (Salicaceae). *Journal of Sichuan Forestry Science and Technology* 31(5): 8–20.
- Chen, J.H., H. Sun, J. Wen & Y.P. Yang 2008. Cladistic analysis of the genus *Salix* (Salicaceae). *Acta Metallurgica Sinica* 30: 1–7.
- Chen, J.H., J. Wen & Y.P. Yang 2010. Molecular phylogeny of *Salix* L. (Salicaceae) inferred from three chloroplast datasets and its systematic implications. *Taxon* 59: 29–37.
- Clement, M., D. Posada & K.A. Crandall 2000. TCS: A computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.
- Demesure, B., N. Sodzi & R.J. Petit 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Molecular Ecology* 4: 129–131.
- Dorn, R.D. 1976. A synopsis of American *Salix*. *Canadian Journal of Botany* 54: 2769–2789.
- Fang, C.F., S.D. Zhao & A.K. Skvortsov 1999. Salicaceae. In: *Flora of China*. (Wu Z.Y., Raven P.H., eds.). Vol. 4, pp. 139–274. Science Press, Beijing; Missouri Botanical Garden Press, St. Louis.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783–791.
- Galtier, N., M. Gouy & C. Gautier 1996. SeaView and Phylowin, two graphic tools for sequence alignment and molecular phylogeny. *Computer Applications in the Biosciences* 12: 543–548.
- Hamza-Babiker, N., B. Heinze, J. Glössl & C. Arnold 2009. Chloroplast DNA identification of eight closely related European *Salix* species. *Austrian Journal of Forest Science* 126: 175–193.
- Hardig, T.M., C.K. Antilla & S.J. Brunsfeld 2010. A phylogenetic analysis of *Salix* (Salicaceae) based on *matK* and ribosomal DNA sequence data. *Journal of Botany* 2010: 1–12 (Article ID 197696, 12 pages, 2010, doi:10.1155/2010/197696).
- Hardig, T.M., S.J. Brunsfeld, R.S. Fritz, M. Morgan & C.M. Orians 2000. Morphological and molecular evidence for hybridization and introgression in a willow (*Salix*) hybrid zone. *Molecular Ecology* 9: 9–24.
- Heinze, B. 2007. A database of PCR primers for the chloroplast genomes of higher plants. *Plant Methods* 3–4: 1–7.
- Huang, S.S.F., S-Y. Hwang & T-P. Lin 2002. Spatial pattern of chloroplast DNA variation of *Cyclobalanopsis glauca* in Taiwan and East Asia. *Molecular Ecology* 11: 2349–2358.

- Kimura, A. 1928. Über *Toisusu* – eine neue Salicaceen-Gattung und die systematische Stellung derselben. *Botanical Magazine* (Tokyo) 42: 287–290.
- Kimura, A. 1931. The scientific name of Japanese “*Salix viminalis* (non L.)?”. *Botanical Magazine* (Tokyo) 45: 28 (in Japanese).
- Kimura, A. 1937. Symbolae Iteologicae IV. *Science Reports of the Toboku Imperial University*, Series 4, Biology 12: 311–321.
- Kimura, A. 1938. Symbolae Iteologicae VI. *Science Reports of the Toboku Imperial University*, Series 4, Biology 13: 390–394.
- Kimura, A. 1988. De Salicis subgenera *Pleurodenia* commentation. *Science Reports of the Toboku University*, Series 4, Biology 39(3): 143–147.
- Kimura, A. 1989. Salicaceae. In: *Wild flowers of Japan woody plants*. (Satake Y., Hara H., Watari S. & Tominari T. eds.). Vol. 1, pp. 31–51, pls. 39–58. Heibonsha Ltd. Publisher, Tokyo (in Japanese).
- Leskinen, E. & C. Alström-Rapoport 1999. Molecular phylogeny of Salicaceae and closely related Flacourtiaceae: Evidence from 5.8 S, ITS 1 and ITS 2 of the rDNA. *Plant Systematics and Evolution* 215: 209–227.
- Lim G.S. & M.R. Carvalho 2013. A reconsideration of the typification of Rafinesque generic names allied to *Salix* L. *Rhodora* 115(962):170–190, doi: <http://dx.doi.org/10.3119/12-11>.
- Liu M., F. Zhu, Z. Liu, H. Zhang 2012. Phylogenetic relationship among *Salix* species based on ITS sequences. *Guizhou Forestry Science and Technology* 4: 30–35.
- Mir, B.A., S. Koul, A. Kumar, M.K. Kaul, A.S. Soodan & S.N. Raina 2010. Intraspecific variation in the internal transcribed spacer (ITS) regions of rDNA in *Witbania somnifera* (Linn.) Dunal. *Indian Journal of Biotechnology* 9: 325–328.
- Mosseler, A. 1989. Interspecific pollen-pistil incongruity in *Salix*. *Canadian Journal of Forestry Research* 19: 1161–1168.
- Mosseler, A. 1990. Hybrid performance and species crossability relationships in willows (*Salix*). *Canadian Journal of Botany* 68: 2329–2338.
- Nasarow, M.I. 1936. *Salix*. In: *Flora of the USSR*. (Komarov V.L. ed.). Vol. 5, pp. 24–216, 707–713 (in Russian). [Назаров М.И. 1936. *Salix* // Флора СССР / под ред. Комарова В.Л. Т. 5. М., Л.: Издательство АН СССР. С. 24–216, 707–713.]
- Nedoluzhko, V.A. 1995a. Salicaceae. In: *Plantae Vasculares Orientis Extremi Sovietici*. (Kharkevich S.S. ed.). Vol. 7, pp. 145–212. Nauka, Saint-Petersburg (in Russian). [Недолужко В.А. 1995а. Salicaceae // Сосудистые растения советского Дальнего Востока / под ред. Харкевича С.С. Т. 7. Санкт-Петербург: Наука. С. 145–212.]
- Nedoluzhko, V.A. 1995b. *Conspectus of dendroflora of the Russian Far East* (Baranov V.I. ed.). 208 p. Dal'nauka, Vladivostok (in Russian). [Недолужко В.А. 1995б. Конспект дендрофлоры российского Дальнего Востока / под ред. Баранова В.И. 208 с. Владивосток: Дальнаука.]
- Ohashi, H. 2000. A systematic enumeration of Japanese *Salix* (Salicaceae). *Journal of Japanese Botany* 75(1):1–41.
- Ohashi, H. 2001. Salicaceae of Japan. *Science Reports of the Toboku University*, Series 4, Biology 40(4): 269–396.
- Pleines, T., S.S. Jakob & F.R. Blattnerl 2009. Application of non-coding DNA regions in intraspecific analyses. *Plant Systematics and Evolution* 282: 281–294.
- Probatova, N.S., S.G. Kazanovsky, E.G. Rudyka, V.Yu. Barkalov, V.P. Seledets & V.A. Nechaev 2011. In: IAPT/IOPB chromosome data 12 (K. Marhold ed.) *Taxon* 60(6): 1790–1794.
- Rozas, J., J.C. Sanchez-Delbarrio, X. Messeguer & R. Rozas 2003. DnaSP, DNAPolymorphism analyses by the coalescent and other methods. *Bioinformatics* 19: 2496–2497.
- Shaw, J., E.B. Lickey, J.T. Beck, S.B. Farmer, W. Liu, J. Miller, K.C. Siripun, C.T. Winder, E.E. Schilling & R.L. Small 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142–166.
- Simmons, M.P. & H. Ochoterena 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369–381.
- Skvortsov, A.K. 1966. Salicaceae. In: *Flora Arctica URSS*. (Tolmachev A.I., ed.). Vol. 5, pp. 7–118. Nauka Publishers, Moscow, Leningrad (in Russian). [Скворцов А.К. 1966. Salicaceae // Арктическая флора СССР / под ред. А.И. Толмачева. Т. 5. С. 7–118. М., Л.: Наука]
- Skvortsov, A.K. 1999. Willows of Russia and adjacent countries. Taxonomical and geographical revision. *University of Joensuu Report*. Series No 39 Biology. Joensuu, Finland. P. 1–307.
- Skvortsov, A.K. 1968. Willows of the USSR. A taxonomic and geographic revision. In: *Proceedings of the study of the fauna and flora of the USSR, published by Moscow Society of Naturalists* (V.N. Tikhomirov ed.). New ser., Sect. Bot. 15 (XXIII). 263 с. Nauka, Moscow (in Russian). [Скворцов А.К. 1968. Ивы СССР: систематический и географический обзор // Материалы к познанию фауны и флоры СССР, издаваемые Московским обществом испытателей природы / под ред. В.Н. Тихомирова. Новая серия, Отд. ботанический. Вып. 15 (XXIII). 263 с. Москва: Наука.]
- Sohma, K. 1993. Pollen diversity in *Salix* (Salicaceae). *Science Reports of the Toboku University*, ser. 4, Biology 40: 77–178.
- Sourdis, J. & M. Nei 1988. Relative efficiencies of the maximum and distance-matrix methods in obtaining the correct phylogenetic tree. *Molecular Biology and Evolution* 5: 298–311.
- Staden, R., K.F. Beal & J.K. Bonfield 1999. The Staden Package 1998. *Computer Methods in Molecular Biology* 132: 115–130.
- Suda, Y. 1958. The number of chromosomes in Japanese salicaceous plants. *Science Reports of the Toboku University*, ser. 4, Biology 24: 1–4.
- Swofford, D.L., J.L. Olsen, P.J. Waddell & D.M. Hillis 1996. Phylogenetic inference. In: *Molecular systematic*. (Hillis D.M., Moritz C., Mable B.K. eds.). P. 407–514. Sinauer Associates, Sunderland, MA.
- Swofford, D.L. 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4.04. MA: Sinauer Associates, Sunderland.
- Trybush, S., Š. Jahodová, W. Macalpine & A. Karp 2008. A genetic study of a *Salix* germplasm resource reveals new insights into relationships among subgenera, sections and species. *BioEnergy Research* 1: 67–79.
- Wang, C. & C.F. Fang 1984. Salicaceae. In: *Flora Reipublicae Popularis Sinicae*. (Wu Z.Y. ed.). Vol. 20, issue 2. P. 79–403. Science Press, Beijing (in Chinese).
- Zhang, M.L. 1994. A preliminary cladistic study on the multistaminal willows (*Salix*) in China. *Bulletin of Botanical Research* 14: 299–305.

Appendix 1. Species sampled for the study. The classification is given according to Skvortsov (1968)

Outgroup taxa:

Populus suaveolens Fisch., Russia, Kamchatka, volcano Tolbachik, V. Barkalov 82, HE800886; *P. trichocarpa* Torr. et A. Gray, Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006440 (Leskinen & Alström-Rapaport 1999).

Ingroup taxa:

Genus *Chosenia* Nakai

Chosenia arbutifolia (Pall.) A.K. Skvortsov (= *Salix arbutifolia* Pall.), Russia, Kamchatka, River Kirevna, V. Barkalov 83, HE800885.

Genus *Salix*

Subg. *Salix*

Sect. *Humboldtianae* Pax: *S. acmophylla* Boissieu, USA, Idaho, Boise, W. Buehler (cultivated, Botanic Garden, Egypt, Cairo), EF060388 (Hardig et al. 2010); *S. amygdaloides* Andersson, Canada, Ontario, vic. Belleville, S. Brunsfeld, EU784080 (Hardig et al. 2010.); *S. gooddingii* C.R. Ball, USA, New Mexico, S. Brunsfeld, EU784081 (Hardig et al. 2010); *S. humboldtiana* Willd., Mexico, Oaxaca, vic. San Felipe, S. Brunsfeld, EF060372 (Hardig et al. 2010).

Sect. *Floridanae* Dorn: *S. floridana* Chapm., USA, Florida, Ocala, L. Miller, EF060380 (Hardig et al. 2010).

Sect. *Wilsonia* K.S. Hao ex C.F. Fang et A.K. Skvortsov (sect. *Glandulosae* Kimura): *S. chaenomeloides* Kimura, USA, Idaho, Boise, W. Buehler (cultivated, Forest Farms Nursery, Oregon, Williams), EF060386 (Hardig et al. 2010).

Sect. *Triandrae* Dumort. (sect. *Amygdalinae* W.D.J. Koch): *S. nipponica* Franch. et Sav., Russia, Primorye, vic. Solovei Kluch, V. Barkalov 49, HE800864; *Salix triandra* L. # 1, Finland, Oulu, C. Antilla, EF060385 (Hardig et al., 2010); # 2, Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006434 (Leskinen & Alström-Rapaport 1999).

Sect. *Urbaniana* (Seemen) C.K. Schneid.: *Salix cardiophylla* Trautv. et C.A. Mey. (*Toisusu cardiophylla* (Trautv. et C.A. Mey.) Kimura), Russia, Sakhalin, river Pilenga, V. Barkalov 1, HE800841.

Sect. *Salicaster* Dumort. (sect. *Pentandrae* (Borrer) C.K. Schneid.): *S. lucida* Muhl., USA, Pennsylvania, Blakeslee, S. Brunsfeld, EF060371 (Hardig et al. 2010); *S. pentandra* L. # 1, Sweden, Vestbreen, H. Roininen, EF060376 (Hardig et al. 2010); # 2, Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006429 (Leskinen & Alström-Rapaport 1999); *S. pseudopentandra* Flod. # 1, Russia, Kamchatka, volcano Tolbachik, V. Barkalov 19, FR693646; # 2, Russia, Kamchatka, vic. Esso, V. Barkalov 27, FR693647.

Sect. *Salix*: *S. alba* L., Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006423 (Leskinen & Alström-Rapaport 1999); *S. fragilis* L., Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006427* (Leskinen & Alström-Rapaport 1999). * – Sampler belongs to *S. euxina* I. Beljaeva (Argus 2010).

Sect. *Subalbae* Koidz.: *S. matsudana* Koidz., GenBank, DQ217771 (Abdollahzadeh et al. 2011); *S. pierotii* Miq., Russia, Primorye, vic. Terekhovka, V. Barkalov 50, HE800865.

Sect. *Magnificae* C.K. Schneid.: *S. magnifica* Hemsl., USA, Idaho, W. Buehler (cultivated), EF060379 (Hardig et al. 2010)

Sect. *Longifoliae* Andersson: *S. exigua* Nutt. # 1, USA, University of Wisconsin-Madison, Sytsma, AJ006426** (Leskinen & Alström-Rapaport 1999); # 2, USA, Washington, vic. Richland, S. Brunsfeld, EU784078 (Hardig et al. 2010); *S. interior* Rowlee, USA, Wisconsin, vic. Hudson, S. Brunsfeld, EU784079 (Hardig et al. 2010); *S. melanopsis* Nutt., USA, Montana, vic. Saltese, S. Brunsfeld, EF060375 (Hardig et al. 2010); *S. taxifolia* Kunth, Mexico, Vera Cruz, vic. Tlapacoyan, S. Brunsfeld, EF060373 (Hardig et al. 2010). ** – Sampler belongs to *S. interior* (Argus, 2010).

Subg. *Chamaetia* (Dumort.) Nasarow

Sect. *Chamaetia*: *S. reticulata* L., Russia, Kamchatka, Mt. Alnei, V. Barkalov 14, FR693637.

Sect. *Herbella* Ser. (sect. *Retusae* A. Kern.): *S. darpirensis* Jurtzev et A.P. Khokhr., Russia, Magadan, Mt. Zamkovaya, O. Mochalova 30 (VLA), HE800845; *S. herbacea* L., Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006428 (Leskinen & Alström-Rapaport 1999); *S. jurtzevii* A.K. Skvortsov, Russia, Magadan, Ol'skoye plateau, O. Mochalova 33, HE800848; *S. nummularia* Andersson, Russia, Magadan, Ol'skoye plateau, V. Barkalov 31, HE800846; *S. polaris* Wahlenb. # 1, Russia, Kamchatka, Mt. Alnei, V. Barkalov 17, FR693641; # 2, Russia, Kamchatka, Mt. Alnei, V. Barkalov 24, FR693643; *S. retusa* L., Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006431 (Leskinen & Alström-Rapaport 1999); *S. serpyllifolia* Scop., Sweden, Uppsala Botanical Garden, E. Leskinen & C. Alström-Rapaport, AJ006432 (Leskinen & Alström-Rapaport 1999); *S. turczaninowii* Laksch. ex Printz, Russia, Sakhalin, Nabil'ski Ridge, Chamginski Pass, V. Bakalin & O. Pisarenko 6, FR693631.

Sect. *Myrtilloides* Koehne: *S. fuscescens* Andersson # 1, Russia, Sakhalin, vic. Moskal'vo, V. Barkalov 2, FR693627; # 2, FR693628; *S. myrtilloides* L., Russia, Magadan, River Seimchan, V. Barkalov 42, HE800857.

Sect. *Glaucae* Pax: *S. arctica* Pall. subsp. *crassijulis* (Trautv.) A.K. Skvortsov, Russia, Kamchatka, Mt. Alnei, V. Barkalov 16, FR693640; *S. brachycarpa* Nutt., Russia, Chukotka, River Alyarmagty, P. Zhukova & V. Petrovsky 59 (LE), HE800873; *S. glauca* L., Russia, Kamchatka, vic. Lake Nerpich'ye, V. Yakubov 58, HE800872; *S. bidakamontana* H. Hara, Russia, the Kurile Islands, Shikotan Isl., V. Barkalov 38, HE800853; *S. nakamurana* Koidz., Russia, the Kurile Islands, Iturup Isl., V. Barkalov 53, HE800875; *S. reptans* Rupr., Russia, Kamchatka, Mt. Alnei, V. Barkalov 15, FR693638; *S. sphenophylla* A.K. Skvortsov, Russia, Kamchatka, Mt. Alnei, V. Barkalov 13,

FR693636; *S. tontomussirensis* Koidz., Russia, Sakhalin, Moneron Isl., V. Barkalov 56, HE800870.

Sect. *Myrtosalix* A. Kern.: *S. berberifolia* Pall., Russia, Khabarovsk, Atagski Ridge, L. Antonova 29 (VLA), HE800844; *S. chamissonis* Andersson # 1, Russia, Kamchatka, Mt. Alnei, V. Barkalov 18, FR693639; # 2, Russia, Kamchatka, volcano Tolbachik, V. Barkalov 25, FR693644; *S. erythrocarpa* Kom., Russia, Kamchatka, Mt. Alnei, V. Barkalov 11, FR693634; *S. kamtschatica* (A.K. Skvortsov) Vorosch. Russia, Kamchatka, Mt. Alnei, V. Barkalov 12, FR693635; *S. kbokbriakovii* A.K. Skvortsov, Russia, Magadan, Ol'skoye plateau, V. Barkalov 32, HE800847; *S. kimurana* (Miyabe et Tatewaki) Miyabe et Tatewaki, Russia, Sakhalin, Nabil'ski Ridge, Chamginski Pass, V. Barkalov 62, HE800874; *S. magadanensis* Nedol., Russia, Magadan, Zaviyalova Isl., O. Mochalova 34, HE800849; *S. phlebophylla* Andersson, Russia, Khabarovsk, Atagski Ridge, L. Antonova 57 (VLA), HE800871; *S. saxatilis* Turcz. ex Ledeb., Russia, Sakhalin, River Bol'shoi Garomai, V. Barkalov 9, FR693633; *S. schmidtiana* Barkalov, Russia, Sakhalin, Peninsula Shmidta, V. Barkalov 8, FR693632; *S. tshuktschorum* A.K. Skvortsov, Russia, Chukotka, River Dvoynaya, D. Lysenko 36, HE800851.

Subg. *Vetrix* (Dumort.) Dumort.

Sect. *Hastatae* A. Kern.: *S. eriocephala* Michx., Idaho, USA, vic. Spring Valley Reservoir, S. Brunsfeld, EF060391 (Hardig et al. 2010); *S. hastata* L. # 1, Russia, Magadan, Ol'skoye plateau, V. Barkalov 46, HE800861; # 2, Russia, Kamchatka, vic. Lake Nerpich'ye, V. Yakubov 76, HE800881; *S. pyrolifolia* Ledeb., Russia, Magadan, River Stolbovaya, O. Mochalova 28 (VLA), HE800842; *S. wolfii* Bebb ex Rothr., USA, Idaho, Stanley, S. Brunsfeld, EF060389 (Hardig et al. 2010);

Sect. *Glabrella* A.K. Skvortsov: *S. reinii* Seemen, Russia, the Kuril Islands, Shikotan Isl., V. Barkalov 37, HE800852.

Sect. *Vetrix*: *S. abscondita* Laksch., Russia, Magadan, River Seimchan, V. Barkalov 44, HE800859; *S. bebbiana* Sarg. # 1, Russia, Kamchatka, vic. Esso, V. Barkalov 22, FR744739; # 2, Russia, Evreiskaya Autonomy, Ekaterino-Nikol'skoye, V. Yakubov 70 (VLA), HE800877; *S. caprea* L. # 1, Russia, Kamchatka, Kireunskiye Hot Springs, V. Barkalov 26, FR744741; # 2, Russia, Primorye, vic. Terekhovka, V. Barkalov 71, HE800878; *S. taraiensis* Kimura # 1, Russia, Sakhalin, River Bol'shoi Garomai, V. Barkalov 10, HE800843; # 2, Russia, Primorye, vic. Ussuriysk, V. Barkalov 55, HE800869.

Sect. *Arbuscella* Ser. ex Duby: *S. boganidensis* Trautv., Russia, Magadan, River Stolbovaya, D. Lysenko 41 (VLA), HE800856; *S. dsbugdshurica* A.K. Skvortsov # 1, Russia, Khabarovsk, Atagski Ridge, L. Antonova 35 (VLA), HE800850; # 2, Russia, Khabarovsk, Dzhugdzhur Ridge, River El'domu-Makit, S. Dudov 79 (VLA), HE800883; *S. parallelinervis* Flod. (*S. pulchra* Pall. subsp. *parallelinervis* (Flod.) A.K. Skvortsov), Russia, Kamchatka, vic. Esso,

V. Barkalov 20, FR693642; *S. pulchra* Cham., Russia, Magadan, River Seimchan, V. Barkalov 39, HE800854; *S. rhamnifolia* Pall., Russia, Khabarovsk, Geran Ridge, River Ulaya, S. Dudov 80 (VLA), HE800884.

Sect. *Viminella* Ser. (sect. *Vimen* Dumort.): *S. dasyclados* Wimmer, Sweden, Ultuna Experimental field, E. Leskinen & C. Alström-Rapaport, AJ006425 (Leskinen & Alström-Rapaport 1999); *S. sachalinensis* F. Schmidt (*S. udensis* Trautv. et C.A. Mey. s.l.), Russia, Sakhalin, river Pilenga, V. Barkalov 4, FR693630; *S. schwerinii* E.L. Wolf s. str., Russia, Kamchatka, vic. Esso, V. Barkalov 23, FR744740; *S. siuzevii* Seemen (*S. udensis* Trautv. et C.A. Mey. s.l.), # 1, Russia, Primorye, vic. Mnogoudobnoye, V. Barkalov 53, HE800867; # 2, Russia, Primorye, vic. Terekhovka, V. Barkalov 69, HE800876; *S. udensis* Trautv. et C.A. Mey. s. str., Russia, Kamchatka, vic. Esso, V. Barkalov 21, FR693645; *S. viminalis* L., Sweden, Ultuna Experimental field, E. Leskinen & C. Alström-Rapaport, AJ006435 (Leskinen & Alström-Rapaport 1999); *S. jezoënsis* Kimura (*S. schwerinii* E.L. Wolf s.l.), Russia, Sakhalin, river Pilenga, V. Barkalov 3, FR693629.

Sect. *Villosae* Rouy: *S. alaxensis* (Andersson) Coville # 1, Russia, Magadan, Ol'skoye plateau, V. Barkalov 45, HE800860; # 2, Russia, Kamchatka, River Mutnaya, V. Yakubov 74, HE800879; *S. krylovii* E.L. Wolf, Russia, Magadan, vic. Talaya, V. Barkalov 40, HE800855.

Sect. *Lanatae* Koechne: *S. lanata* L. subsp. *richardsonii* (Hook.) A.K. Skvortsov, Russia, Kamchatka, vic. Lake Nerpich'ye, V. Yakubov 75, HE800880; *S. recurvigemmis* A.K. Skvortsov, Russia, Magadan, Ol'skoye plateau, V. Barkalov 43, HE800858; *S. woroschilovii* Barkalov, Russia, Primorye, Mt. Ol'khovaya, V. Barkalov 47, HE800862.

Sect. *Subviminalis* (Seemen) C.K. Schneid.: *S. gracilistyla* Miq., Russia, Primorye, vic. Terekhovka, V. Barkalov 52, HE800866.

Sect. *Griseae* (Borrer) Barratt ex Hooker: *S. sericea* Marshall, USA, New York, vic. Milford, S. Brunsfeld, EF060368 (Hardig et al. 2010).

Sect. *Daphnella* Ser. ex Duby: *S. rorida* Laksch., Russia, Sakhalin, river Pilenga, V. Barkalov 5, FR744735.

Sect. *Nakaianae* (A.K. Skvortsov) Korkina: *S. kangensis* Nakai, Russia, Primorye, vic. Ussuriysk, V. Barkalov 2012, HE856317.

Sect. *Incubatae* A. Kern.: *S. brachypoda* (Trautv. et C.A. Mey.) Kom., Russia, Primorye, vic. Ussuriysk, V. Barkalov 48, HE800863.

Sect. *Helix* Dumort.: *S. integra* Thunb. ex Murray, Russia, Primorye, vic. Ussuriysk, V. Barkalov 2012, HE856318; *S. miyabeana* Seemen, Russia, Primorye, vic. Solovei Kluch, V. Barkalov 78, HE800882; *S. purpurea* L., Genbank, Canada, Scoles G. University of Saskatchewan, A. Ngantcha, GU556175.

Appendix 2. GenBank accession numbers of specimens used for ITS and chloroplast DNA sequences. Voucher specimens are given in Appendix 1.

Species (specimens)	ITS	petN-psbM	trnD-trnT	trnC-petN	rbcL	matK	psaA-ycf3	petG-trnP	rpoB-trnC	psbM-trnD	trnS-psbZ
<i>Salix cardiophylla</i> 1	HE800841	FR694589	FR694558	FR694798	FR694836	FR694987	FR695024	FR695506	FR744742	FR715086	FR715113
<i>S. fuscescens</i> 2 (1)	FR693627	FR694590	FR694559	FR694799	FR694837	FR694988	FR695025	FR695507	FR715052	FR715087	FR715114
<i>S. fuscescens</i> 2 (2)	FR693628	FR694591	FR694560	FR694800	FR694838	FR717459	FR695026	FR695508	FR715053	FR717428	FR715115
<i>S. yezoensis</i> 3	FR693629	FR694592	FR717455	FR694801	FR694839	FR694989	FR695027	FR695509	FR715054	FR715088	FR715116
<i>S. sachalinensis</i> 4	FR693630	FR694593	FR694561	FR694802	FR694840	FR694990	FR695028	FR695510	FR715055	FR715089	FR715117
<i>S. rostrata</i> 5	FR744735	FR694594	FR717454	FR694803	FR694841	FR694991	FR695029	FR695511	FR715056	FR717429	FR715118
<i>S. turczaninowii</i> 6	FR693631	FR694595	FR694562	FR694804	FR694842	FR694992	FR695030	FR695512	FR715057	FR715090	FR715119
<i>S. schmidiana</i> 8	FR693632	FR694603	FR694569	FR694814	FR694852	FR695002	FR725979	FR695522	FR715065	FR717435	FR715129
<i>S. saxatilis</i> 9	FR693633	FR694604	FR694572	FR694817	FR694855	FR695005	FR695041	FR695525	FR715068	FR715096	FR715132
<i>S. erythrocarpa</i> 11	FR693634	FR694605	FR694573	FR694818	FR694856	FR695006	FR695042	FR695526	FR715069	FR715097	FR715133
<i>S. kamschatica</i> 12	FR693635	FR694606	FR694574	FR694819	FR694857	FR695007	FR695043	FR695527	FR715070	FR715098	FR715134
<i>S. sphenophylla</i> 13	FR693636	FR694607	FR694575	FR694820	FR694858	FR695008	FR695044	FR695528	FR715071	FR717437	FR715135
<i>S. reticulata</i> 14	FR693637	FR694608	FR694576	FR694821	FR694859	FR695009	FR695045	FR695529	FR715072	FR715099	FR715136
<i>S. reptans</i> 15	FR693638	FR694609	FR694577	FR694822	FR694860	FR695010	FR695046	FR695530	FR715073	FR715100	FR715137
<i>S. arctica</i> 16	FR693640	FR694610	FR694578	FR694823	FR694861	FR695011	FR695047	FR695531	FR715074	FR715101	FR715138
<i>S. polaris</i> 17	FR693641	FR694611	FR694579	FR694824	FR694862	FR695012	FR695048	FR695532	FR715075	FR715102	FR715139
<i>S. polaris</i> 24	FR693643	FR694612	FR694580	FR694825	FR694863	FR695013	FR695049	FR695533	FR715076	FR715103	FR715140
<i>S. chamissonis</i> 18	FR693639	FR694613	FR694581	FR694826	FR694864	FR695014	FR695050	FR695534	FR715077	FR715104	FR715141
<i>S. chamissonis</i> 25	FR693644	FR694614	FR694582	FR694827	FR694865	FR695015	FR695051	FR695535	FR715078	FR717438	FR715142
<i>S. pseudopentandra</i> 19	FR693646	FR694616	FR694584	FR694829	FR694872	FR695017	FR695053	FR695537	FR715080	FR715106	FR715144
<i>S. pseudopentandra</i> 27	FR693647	FR694617	FR694585	FR694830	FR694873	FR695018	FR695054	FR695538	FR715081	FR715107	FR715145
<i>S. parallelinervis</i> 20	FR693642	FR694618	FR694586	FR694831	FR694867	FR695019	FR695055	FR695539	FR715082	FR715108	FR715146
<i>S. udensis</i> 21	FR693645	FR694619	FR694587	FR694832	FR694868	FR695020	FR695056	FR695540	FR715083	FR715109	FR715147
<i>S. bebbiana</i> 22	FR744739	FR694620	FR694588	FR694833	FR694869	FR695021	FR695057	FR695541	FR715084	FR715110	FR715148
<i>S. schuerinii</i> 23	FR744740	FR694621	FR717452	FR694834	FR694870	FR695022	FR695058	FR695542	FR715085	FR715111	FR715149
<i>S. caprea</i> 26	FR744741	FR717450	FR717451	FR694835	FR694871	FR695023	FR695059	FR695543	FR725982	FR715112	FR715150
<i>S. pyrolifolia</i> 28	HE800842	HE611298	HE611957	HE611974	HE612018	HE612054	HE613114	HE613190	HE613229	HE613329	HE613923
<i>S. taraiensis</i> 10	HE800843	HE611297	HE611956	HE611973	HE612017	HE612053	HE613113	HE613189	HE613228	HE613328	HE613922
<i>S. berberifolia</i> 29	HE800844	HE611299	HE611958	HE611975	HE612019	HE612055	HE613115	HE613191	HE613230	HE613330	HE613924
<i>S. darpinensis</i> 30	HE800845	HE611300	HE611959	HE611976	HE612020	HE612056	HE613116	HE613192	HE613231	HE613331	HE613925
<i>S. nummularia</i> 31	HE800846	HE611301	HE611960	HE611977	HE612021	HE612057	HE613117	HE613193	HE613232	HE613332	HE613926
<i>S. khokhriakovii</i> 32	HE800847	HE611302	HE611961	HE611978	HE612022	HE612058	HE613118	HE613194	HE613233	HE613333	HE613927
<i>S. jurtzevii</i> 33	HE800848	HE614621	HE614651	HE611979	HE612023	HE612059	HE613119	HE613195	HE620950	HE613334	HE613928
<i>S. magadanensis</i> 34	HE800849	HE611303	HE611962	HE611980	HE612024	HE612060	HE613120	HE613196	HE613234	HE613335	HE613929

Appendix 2. Continued

<i>S. dshudshurica</i> 35	HE800850	HE611304	HE611963	HE611981	HE612025	HE612061	HE613121	HE613197	HE613235	HE613336	HE613930
<i>S. tschuktschorum</i> 36	HE800851	HE611305	HE611964	HE611982	HE612026	HE612062	HE613122	HE613198	HE613236	HE613337	HE613931
<i>S. reinii</i> 37	HE800852	HE614622	HE614652	HE611983	HE612027	HE612063	HE613123	HE613199	HE613236	HE613338	HE613932
<i>S. hidakamoniana</i> 38	HE800853	HE614623	HE614653	HE611984	HE612028	HE612064	HE613124	HE613200	HE613237	HE613339	HE613933
<i>S. pulchra</i> 39	HE800854	HE611306	HE611965	HE611985	HE612029	HE612065	HE613125	HE613201	HE613238	HE613340	HE613934
<i>S. krylovii</i> 40	HE800855	HE611307	HE611966	HE611986	HE612030	HE612066	HE613126	HE613202	HE613239	HE613341	HE613935
<i>S. boganiensis</i> 41	HE800856	HE611308	HE611967	HE611987	HE612031	HE612067	HE613127	HE613203	HE613240	HE613342	HE613936
<i>S. myrtilloides</i> 42	HE800857	HE611309	HE611968	HE611988	HE612032	HE612068	HE613128	HE613204	HE613241	HE613343	HE613937
<i>S. recurvigenensis</i> 43	HE800858	HE611310	HE611969	HE611989	HE612033	HE612069	HE613129	HE613205	HE613242	HE613344	HE613938
<i>S. abscondita</i> 44	HE800859	HE614624	HE614654	HE611990	HE612034	HE612070	HE613130	HE613206	HE613243	HE613345	HE613939
<i>S. alaxensis</i> 45	HE800860	HE611311	HE611970	HE611991	HE612035	HE612071	HE613131	HE613207	HE613244	HE613346	HE613940
<i>S. hastata</i> 46	HE800861	HE611312	HE611971	HE611992	HE612036	HE612072	HE613132	HE613208	HE613245	HE613347	HE613941
<i>S. woroschilovii</i> 47	HE800862	HE614625	HE614655	HE611993	HE612037	HE612073	HE613133	HE613209	HE613246	HE613348	HE613942
<i>S. brachypoda</i> 48	HE800863	HE614626	HE614656	HE611994	HE612038	HE612074	HE613134	HE613210	HE613247	HE613349	HE613943
<i>S. nipponica</i> 49	HE800864	HE611313	HE611972	HE611995	HE612039	HE612075	HE613135	HE613211	HE613248	HE613350	HE613944
<i>S. pirovii</i> 50	HE800865	HE614627	HE614657	HE611996	HE612040	HE612076	HE613136	HE613212	HE613249	HE613351	HE613945
<i>S. gracilistyla</i> 51	HE800866	HE614629	HE614658	HE611997	HE612041	HE612077	HE613137	HE613213	HE613250	HE613352	HE613946
<i>S. stuzevii</i> 53	HE800867	HE614630	HE614659	HE611998	HE612042	HE612078	HE613138	HE613214	HE613251	HE613353	HE613947
<i>S. tarakensis</i> 55	HE800868	HE614632	HE614661	HE612000	HE612043	HE612079	HE613139	HE613215	HE613252	HE613354	HE613948
<i>S. phlebophylla</i> 57	HE800871	HE614634	HE614663	HE612001	HE612044	HE612080	HE613140	HE613216	HE613253	HE613355	HE613949
<i>S. glauca</i> 58	HE800872	HE614635	HE614664	HE612002	HE612045	HE612081	HE613141	HE613217	HE613254	HE613356	HE613950
<i>S. brachycarpa</i> 59	HE800873	HE614636	HE614665	HE612003	HE612046	HE612082	HE613142	HE613218	HE613255	HE613357	HE613951
<i>S. kimurana</i> 62	HE800874	HE820937	HE614666	HE612004	HE612047	HE612083	HE613143	HE613219	HE613256	HE613358	HE613952
<i>S. stuzevii</i> 69	HE800876	HE614640	HE614669	HE612006	HE612048	HE612084	HE613144	HE613220	HE613257	HE613359	HE613953
<i>S. bebbiana</i> 70	HE800877	HE614641	HE614670	HE612007	HE612049	HE612085	HE613145	HE613221	HE613258	HE613360	HE613954
<i>S. caprea</i> 71	HE800878	HE614642	HE614671	HE612008	HE612050	HE612086	HE613146	HE613222	HE613259	HE613361	HE613955
<i>S. alaxensis</i> 74	HE800879	HE614643	HE614672	HE612009	HE612051	HE612087	HE613147	HE613223	HE613260	HE613362	HE613956
<i>S. lanata</i> 75	HE800880	HE614644	HE614673	HE612010	HE612052	HE612088	HE613148	HE613224	HE613261	HE613363	HE613957
<i>S. hastata</i> 76	HE800881	HE614645	HE614674	HE612011	HE612053	HE612089	HE613149	HE613225	HE613262	HE613364	HE613958
<i>S. miyabeana</i> 78	HE800882	HE614646	HE614675	HE612012	HE612054	HE612090	HE613150	HE613226	HE613263	HE613365	HE613959
<i>S. dshugshurica</i> 79	HE800883	HE614647	HE614676	HE612013	HE612055	HE612091	HE613151	HE613227	HE613264	HE613366	HE613960
<i>S. rhamnifolia</i> 80	HE800884	HE614648	HE614677	HE612014	HE612056	HE612092	HE613152	HE613228	HE613265	HE613367	HE613961
<i>S. arbutifolia</i> 83	HE800885	HE614649	HE614678	HE612015	HE612057	HE612093	HE613153	HE613229	HE613266	HE613368	HE613962
<i>Populus suaveolens</i> 82	HE800886	HE614650	HE614679	HE612016	HE612058	HE612094	HE613154	HE613230	HE613267	HE613369	HE613963
<i>S. kangensis</i> 2012	HE856317	HE856325	HE856335	HE856339	HE856321	HE856319	HE856327	HE856329	HE856333	HE856329	HE856337
<i>S. integra</i> 2012	HE856318	HE856326	HE856336	HE856340	HE856322	HE856320	HE856328	HE856324	HE856334	HE856330	HE856338