MOLECULAR PHYLOGENY OF THE GENUS SALIX (SALICACEAE) WITH AN EMPHASIZE TO ITS SPECIES IN IRAN

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This study represents phylogenetic analyses of nrDNA ITS for 62 accessions of 55 *Salix* species and two *Populus* species as outgroups using maximum parsimony and Bayesian methods. A subset of 14 species of *Salix* sampled for nrDNA ITS was included in a phylogenetic analysis using *trn*L-F region. The resulting nrDNA ITS phylogeny revealed that all five currently recognized *Salix* subgenera except the monotypic subgenus *Longifoliae* are not monophyletic. Likewise, most of *Salix* sections are not monophyletic. The analysis showed that *Salix humboldtiana*, native to South America and Mexico, positioned at the base of the tree as sister to the remaining *Salix* species. The Iranian *Salix* species are scattered across the tree. Several polymorphic nucleotide sites of nrDNA ITS were detected for *Salix zygostemon, S. acmophylla* and *S. elymaitica*. This indicates that these taxa may have a hybrid origin. In the case of *Salix zygostemon*, *trn*L-F data showed that it was nested a polytomy containing *S. cinerea* and *S. elbursensis.* While on the nrDNA tree, its position is unclear. Meanwhile, the data suggested that *Salix* may have been originated in warm temperate regions of the new world and then diversified in both warm and cold temperate regions of northern hemisphere.

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Key words. *trn*L-F, phylogeny, *Salix, populus,* nrDNA ITS, *Salicaceae.*

فیلوژنی ملکولی جنس بید (Salicaceae) با تاکید بر گونه‌های ایران

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این مطالعه آنالیز فیلوژنتیکی داده‌های توالی‌های nrDNA ITS برای ٦٢ تاکسون شامل ٥٥ گونه *Salix* و دو گونه ی *Populus* به عنوان برون گروه و توالی‌های *trn*L-F کلروپلاستی برای ١٤ گونه *Salix* استفاده شد. آنالیز فیلوژنتیکی با استفاده از روشهای بیشینه‌ی صرفه جوئی (Maximum Parsimony) و Bayesian انجام گرفت. فیلوژنی حاصل از توالی‌های nrDNA ITS نشان داد که همه‌ی پنج زیرجنس رایج *Salix* به استثنای زیرجنس مونوتیپیک *Longifoliae* تک‌تبار نمی‌باشند. همچنین اکثر بخشهای *Salix* تک‌تبار نمی‌باشند. آنالیزها نشان داد که *Salix humboldtiana*، بومی آمریکای جنوبی و مکزیک، در قاعده درختان به عنوان خواهر بقیه گونه های *Salix* قرار گرفته است. گونه‌های ایرانی *Salix* در سرتاسر درخت پراکنده هستند. چندین جایگاه پلی مورفی نوکلئوتیدی در nrDNA ITS در *S. zygostemon*، *S. acmophylla* و *S. elymaitica* شناسایی شد، که نشان می‌دهد این تاکسون‌ها احتمالا منشا هیبریدی دارند. در مورد *S. zygostemon* داده های *trn*L-F نشان دادند که این گونه با *S. cinerea* و *S. elbursensis* بصورت پلی‌تومی هستند. در حالیکه در درخت nrDNA ITS جایگاه آن نامعلوم است. داده‌های حاضر پیشنهاد می‌کنند که ممکن است منشا *Salix* مناطق معتدله گرم در دنیای جدید و گونه‌زائی بعدی آن در مناطق سرد نیمکره‌ی شمالی باشد.

INTRUDUCTION

Salix L. is the largest genus of *Salicaceae* with about 450 species worldwide (Mabberley, 1990; Argus, 1997), occurring mainly in the Northern Hemisphere. China with over 270 species (Fang et al. 1999), former Soviet Union with ca. 120 species (Skvortsov, 1999), North America with 130 species (Argus, 1997) and Europe with 65 species (Rechinger, 1964, 1992), have been considered as *Salix* centers biodiversity. About 36 *Salix* species (30 species and six hybrids) have been reported in Iran (Maassoumi, 2009). Infrageneric classification of *Salix* has been elusive depending on various authors' treatment. Skvortsov (1999) divided willows of the former USSR into three subgenera, *Salix*, *Chamaetia* and *Vetrix*, which altogether are further divided into several sections. Likewise, Argus (2007) divided willows of North America and North of Mexico into five subgenera (*Protitea*, *Salix*, *Longifoliae*, *Chamaetia* and *Vetrix*) and 34 sections. Ohashi (2000) classified Japanese *Salix* into four subgenera (*Salix*, *Chamaetia, Vetrix* and *Urbanianae*) and 17 sections. He established *Urbanianae* as a new subgenus for accommodating the segregate genera *Chosenia* and *Toisusu* as well as *Salix* subgenera *Protitea* and *Pleuradenia*. Several molecular works using nrDNA ITS (Leskinen and Alstrom-Rapaport, 1999), *rbc*L (Azuma et al. 2000), nrDNA ITS and *mat*K (Brunsfeld and Anttila, 2004; Hardig et al. 2010) and *rbc*L, *trn*D-*trn*T and *atp*B-*rbc*L (Chen et al. 2010) sequence data conducted to test the monophyly of *Salix* and its subgeneric divisions as well as the status *Chosenia* and *Toisusu*. All suggested that *Salix,* with the inclusion of these two genera, is monophyletic, but did not support its subgeneric divisions. Chen et al. (2010) proposed a new subgeneric classification for the genus with splitting traditionally recognized subgenus *Salix* into three subgenera *Salix*, *Chosenia* and *Triandrae* and combining subgenera *Chamaetia* and *Vetrix* as subgenus *Vetrix*. However, their sampling was not adequate to test phylogenetic status of the most diverse and distinct taxa such as *Salix humboldtiana* of South America and Subgenus/section *Longifoliae* of North America.

We here report molecular phylogeny of *Salix* with the broad taxon sampling using nrDNA ITS. And for a subset 15 taxa, the nrDNA ITS was supplemented with less variable chloroplast DNA *trn*L intron, *trn*L-*trn*F intergenic spacer. Both DNA regions have been widely used data source in molecular systematic studies of plants at lower taxonomic levels (e.g., Balwin, 1995, Kazempour Osaloo et al., 2003, 2005, Shaw et al. 2005). The goals of the present work are to: 1) evaluate the monophyly of subgenera and, in particular, sections of *Salix*, 2) determine the phylogenetic placement of the Iranian *Salix* in relation to other *Salix* species, 3) recognize probable hybrid species of the Iranian *Salix*, and 4) assess biogeography pattern of *Salix* species.

MATERIALS AND METHODS

Taxon sampling

The leaf material was taken mostly from herbarium specimens deposited at the herbarium of the Research Institute of Forests and Rangelands (TARI). In some cases, the materials were collected from the Botanical Garden of Munich or field. A total of 64 accessions representing 58 species of *Salix* plus two *Populus* species as outgroups, according to Leskinen & Alström-Rapaport, (1999), were included in phylogenetic analyses using nrDNA ITS region. Thirty-five species were sequenced newly in this study. The remaining 29 sequences were obtained from GenBank. A subset of 14 species of *Salix* sampled for nrDNA ITS was included in a phylogenetic analysis using *trn*L-F region (see Table 1).

DNA isolation, amplification, and sequencing

Total genomic DNA was extracted from leaf tissue following the modified 2×CTAB (Cetyltrimethylammonium bromide) procedure of Doyle and Doyle (1987). The nrDNA ITS region was amplified using primers ITSa and ITSd (Leskinen and Alström-Rapaport 1999). In the case of *Salix australior*, primers AB101 and AB102 of Douzery et al. (1999) were used. The *trn*L-F region was amplified using the primers c and f of Taberlet et al. (1991). Total volume of the amplification reaction was 25 µl including 2.5 µl of 10X Taq polymerase buffer, 2.5 µl (2.5mmol/l) of dNTP, 2µl (50mmol/l) of $MgCl_2$, 0.2 µl (5U/µl) of Taq polymerase (Cinnagen, Iran), 0.5 µl of each primer (5pmol/l), 5-20 ng DNA, 0.2 µl of DMSO 5%, and an appropriate amount of Deionized water. In some cases, we employed the Polymerase Master Mix Red (Amplicon, Cat. No. 180301, Germany). The reaction condition was 5 min at 94 °C for denaturation followed by 35 cycles of 1 min 10 s at 94°C, 50 s at 54°C for annealing and 1 min at 72°C for primer extension, then followed by an additional 10 min extension at 72°C. For *trn*L-F region, the PCR condition was 2 min 30 s at 94°C followed by 35 cycles of 50 s at 94°C, 50 s at 55°C and 1 min 10s at 72°C. A final extension of 5 min at 72 ^{o}C was performed. The ensuring PCR fragments were separated by electrophoresis in 1% agarose gels in 1×TAE ($_{P}$H=8) buffer, stained with ethidium bromide The regions were then sequenced using the 'Big dye terminator cycle

Table 1. Taxa included in nrDNA ITS and cpDNA *trn*L-F phylogenetic analyses.

Species	DNA source (voucher information)	GenBank accession number (nrDNA ITS/*trn*L-F)
Salix acmophylla Boiss.	Maassoumi & Safavi 90115 (TARI)	AB685275/AB685313
Salix acmophylla Boiss.[a]	Buechler Acmo1 (ID)	EF060388/-
Salix aegyptiaca L.	Maassoumi & Safavi 90425 (TARI)	AB685276/-
Salix alaxensis Cov.[a]	Furniss 2956 (ID)	EF060390/-
Salix alba L.	Kazempour Osaloo, 2007-1 (TMUH)	AB685277/-
Salix alba L.[a]	-[b]	-/AJ849556
Salix alba L.	Maassoumi, Safavi & Alizadeh 90238(TARI)	AB685278/-
Salix alba L. f. *alba*	Maassoumi 90569 (TARI)	AB685279/-
Salix atrocinerea Brotero	Maassoumi & Safavi 90438 (TARI)	AB685280/-
Salix australior Andersson	Hemati & Ghasemi 84237 (TARI)	AB685281/-
Salix amygdaloides Andersson[a]	Leskinen & Alström-Rapaport S-2 (UPS)	AJ006424/-
Salix bebbiana Sargent[a]	Brunsfeld 5018 (ID)	EF060369/-
Salix babylonica L.	Abdollahzadeh, 2007-2 (TMUH)	AB685282/-
Salix babylonica L.[a]	-[b]	-/AJ849558
Salix baladehensis Maassoumi, Moeeni & Rahiminejad	Maassoumi 90547(TARI)	AB685283/-
Salix caprea L.	Maassoumi & Safavi 89975 (TARI)	AB685284/-
Salix caspica Pall.	Bozorgmehr 85/15 (TARI)	AB685285/-
Salix carmanica Bornm. ex Gröez	Maassoumi 89639 (TARI)	AB685286/-
Salix cinerea L.	Maassoumi, Safavi &Alizadeh 90204 (TARI)	AB685287/AB685314
Salix cordata Michaux[a]	Brunsfeld 5039a (ID)	EF060393/-
Salix arbutifolia Pall.[a] [=*Chosenia arbutifolia* (Pall.) Skvortsov]	Leskinen & Alström-Rapaport S-14(UPS)	AJ006436/-
Salix chaenomeloides Kimura[a]	Buechler chae 2 (ID)	EF060386/-
Salix dasyclados Wimm.[a]	Leskinen & Alström-Rapaport S-3(UPS)	AJ006425/-
Salix daviesii Boiss.	Hatami et al. 83398 (TARI)	AB685288/-
Salix elbursensis Boiss.	Maassoumi & Sadati 90490 (TARI)	AB685289/AB685315
Salix elymaitica Maassoumi	Hatami 2203 (TARI)	AB685290
Salix eriocephala Michaux[a]	Brunsfeld 17 (ID)	EF060367/-

Table 1. (continued).

Species	DNA source (voucher information)	GenBank accession number (nrDNA ITS/*trn*L-F)
Salix excelsa Gmelin	Maassoumi & Safavi 90463 (TARI)	AB685291/AB685316
Salix exigua Nutt.[a]	Sytsma, no voucher	AJ006426/-
Salix firouzkuhensis Maassoumi	Maassoumi 90595(TARI)	AB685292/-
Salix floridana Chapman[a]	Miller 6016 (ID)	EF060380/-
Salix fragilis L.[a]	Leskinen & Alström-Rapaport S-4(UPS)	AJ006427/-
Salix fragilis L.[a]	-[b]	-/AJ849557
Salix herbacea L.[a]	Leskinen & Alström-Rapaport S-5(UPS)	AJ006428/-
Salix helvetica Vill.	Cultivated in the Munich Botanical Garden	AB685293/-
Salix humboldtiana Andersson[a a]	Brunsfeld 3004-mx (ID)	EF060372/-
Salix issatissensis Maassoumi, Moeeni & Rahiminejad	Jamzad et al. 69529 (TARI)	AB685294/-
Salix lacus-tari Maassoumi & Kazempour	Maassoumi 90571(TARI)	AB685295/-
Salix lacus-tari Maassoumi & Kazempour	Maassoumi 90573(TARI)	AB685296/-
Salix lucida Muhlenberg[a]	Brunsfeld PA5025 (ID)	EF060371/-
Salix moupinensis Franch.	Cultivated in the Munich Botanical Garden	AB685297/-
Salix magnifica Hemsl.[a]	Buechler Mag1 (ID)	EF060379/-
Salix matsudana Koidz.[a]	-[b]	DQ217771/-
Salix melanopsis Nutt.[a]	Brunsfeld 5075MT (ID)	EF060375/-
Salix pedicellata Desf.	Maassoumi & Safavi 89973 (TARI)	AB685298/-
Salix pycnostachya Andersson	Hemati & Safavi 85917 (TARI)	AB685299/-
Salix pentandra L.[a]	Leskinen & Alström-Rapaport S-6 (UPS)	AJ006429/-
Salix pentandra L.[a]	-[b]	-/AJ849559
Salix purpurea L.[a]	Leskinen & Alström-Rapaport S-7(UPS)	AJ006430/-
Salix purpurea L.[a]	-[b]	-/AJ849584
Salix rosmarinifolia L.	Cultivated in the Munich Botanical Garden	AB685300/-
Salix reinii Franch. & Sav. ex Seemen[a]	-[b]	AB096873/-
Salix retusa L.[a]	Leskinen & Alström-Rapaport S-8(UPS)	AJ006431/-
Salix reticulate L.[a]	Argus13928c (ID)	EF060383/-

Table 1. (continued).

Species	DNA source (voucher information)	GenBank accession number (nrDNA ITS/*trn*L-F)
Salix songarica Andersson	Hojjati & Zanguii 32854 (TARI)	AB685301/-
Salix schwerinii E. Wolf[a]	**Alström-Rapaport** S-9(UPS)	AJ006433/-
Salix serpyllifolia Scop.[a]	**Leskinen & Alström-Rapaport** S-10(UPS)	AJ006432/-
Salix sericea Marshall[a]	Brunsfeld 5061i (ID)	EF060387/-
Salix taxifolia Kunth[a]	Brunsfeld 3008 (ID)	EF060373/-
Salix triandra L.	Maassoumi, Safavi & Alizadeh 90236 (TARI)	AB685302/-
Salix triandra L.	Maassoumi & Safavi 90450 (TARI)	AB685303/-
Salix triandra L.	Fattahi et al. 2329 (TARI)	AB685304/-
Salix triandra L.[a]	-[b]	-/AJ849560
Salix viridiformis Maassoumi	Maassoumi &Safavi 90437 (TARI)	AB685305/-
Salix viminalis L.[a]	**Leskinen & Alström-Rapaport** S-12 (UPS)	AJ006435/-
Salix viminalis L.[a]	-[b]	-/AJ849562
Salix vitellina L.[a]	-[b]	-/AJ849563
Salix wilhelmsiana M. B.	Maassoumi 90576 (TARI)	AB685306/AB685317
Salix wolfii Bebb[a]	Brunsfeld 5092 (ID)	EF060389/-
Salix zygostemon Boiss.	Jahanbazi & Talebi 84253 (TARI)	AB685307/AB685318
Salix zygostemon Boiss.	Maassoumi 90563 (TARI)	AB685308/-
Salix zygostemon Boiss.	Maassoumi & Safavi 90110 (TARI)	AB685309/-
Salix sp.	Maassoumi & Jalili 83520 (TARI)	AB685310/-
Populus caspica Bornm.	Wendelbo & Foroughi 12761 (TARI)	AB685311/-
Populus euphratica Olivier	Kazempour Osaloo 2006 (TMUH)	AB685312/-
Populus nigra L.[a]	-[b]	-/AF327591

Abbreviations used in voucher information: ID, University of Idaho Stillinger Herbarium; TARI, Herbarium of the Research Institute of Forests and Rangelands, Tehran; TMUH, Tarbiat Modares University Herbarium, Tehran; UPS, Botanical Museum, Uppsala University. [a] Sequences were obtained from GenBank. [b] Voucher information for these taxa is not available.

sequencing ready reaction kit' with the same c and f primers in an ABI Prism 377 DNA sequencer.

Sequence alignment

Sequences were edited using BioEdit ver. 7.0.9.0 (Hall 1999) and aligned using ClustalX (Larkin et al. 2007) followed by manual adjustment. Alignment of the datasets required the introduction of several single and multiple-base indels (insertions/deletions). Positions of indels were treated as missing data for all datasets.

Phylogenetic analyses

PARSIMONY METHOD

Parsimony analyses were conducted using the PAUP* version 4.0b10 (Swofford 2002) for phylogenetic analyses. The heuristic search option was employed for each of the datasets, using tree bisection-reconnection (TBR) branch swapping, with simple addition sequence and Maxtree set to 50000 (only nrDNA ITS). Uninformative characters were excluded from the analyses. Branch support was assessed by bootstrap values (BS, Felsenstein 1985) calculated from 20000 replicates of a heuristic search strategy with TBR branch swapping and the MulTrees option off.

BAYESIAN METHOD

Model of sequence evolution for the datasets was selected using the program MrModeltest version 2.3 (Nylander 2004) as implemented in MrMTgui (Nuin 2005) based on the Akaike information criterion (AIC) (Posada and Buckley 2004). The nrDNA ITS dataset was analyzed with GTR+G model using the program MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Posteriors on the model parameters were estimated from the data, using the default priors. The analysis was done with 2 million generations, using Markov chain Monte Carlo search. MrBayes performed two simultaneous analyses starting from different random trees (Nruns=2) each with four Markov chains and trees sampled at every 100 generations. The trees sampled after reaching stationary phase were collected and used to build a 50% majority rule consensus tree accompanied with posterior probability (PP) values. Tree visualization was carried out using Tree View version1.6.6 (Page 2001).

RESULTS

The aligned nrDNA ITS dataset is 608 nucleotide sites long, of which 49 were phylogenetically informative. Parsimony analyses of the dataset excluding uninformative sites resulted 50000 most-parsimonious trees (length = 81 steps, consistency index (CI) = 0.716, retention index (RI) = 0.889, trees not shown). A 50% majority rule consensus tree resulting from Bayesian analyses along with PP and BS values are shown in Fig. 1. This three is topologically is almost the same as the strict consensus tree from parsimony analysis. At the base of these trees *Salix humboldtiana* was the first branch with strong support and sister to a large polytomy. In this assemblage, several subclades comprising two through 14 species (16 accessions) with low to high support are present.

DISCUSSION

Infrageneric relationships within *Salix*

The present nrDNA ITS data show that all five currently recognized *Salix* subgenera except the North American *Longifoliae*, appear to be non-monophyletic. The previous works based on nrDNA ITS, *rbc*L, and the combined *atp*B-*rbc*L-*trn*D-T sequences data (Leskinen and Alstrom-Rapaport 1999; Azuma et al. 2000; Chen et al. 2010; Hardig et al. 2010) reached the same conclusion that the traditionally recognized subgenera *Salix*, *Vetix* and *Chamaetia* are not monophyletic. The subgenus *Salix* is the largest and morphologically divergent taxon of the genus encompasses species distributing from South America through North America to Eurasia. Based on the combined cpDNA sequence data, Chen et al. (2010) split traditionally recognized subgen. *Salix* into three subgenera *Salix*, *Chosenia* and *Triandrae*. Argus (2007) transferred members of the two New World sections *Floridanae* (*S. floridana*) and *Humboldtianae* (including seven species such as, S. *humboldtiana* and *S. amygdaloides* studied herein) from the subgen. *Salix* to the already established subgen. *Protitea* Kimura (Kimura 1928) mainly based on the free and imbricate bud scale margin and staminate flowers with 3-12 stamens. Our nrDNA ITS phylogeny and Chen et al.'s cpDNA phylogenies (2010) indicated that both S. *floridana* and *S. amygdaloides* (as well as their allies) belong to a well supported large clade of mostly Old World species of the subgen. *Salix*. Therefore, with the classification of these two species and allies under the subgen. *Salix* sensu Chen et al. (2010), the subgen. *Protitea* might be the monotypic taxon including *Salix humboldtiana* solely (but see Hardig et al. 2010). This species, native to South America and Mexico, is positioned at the base of nrDNA ITS tree sister to an assemblage of the other *Salix* species. Among eight sections of subgen. *Salix* analyzed here, three sections *Acmophyllae*, *Salix*, and *Triandrae* appear not to be monophyletic. (See Fig. 1). As noted above, Chen et al. (2010) split sect. *Triandrae*, including two accession of *S. triandra*, from the subgen. *Salix* and treated it as subgen *Triandrae*. In our nrDNA ITS tree, the three accessions of the species also formed a clade with a high PP support. Another species of the section is

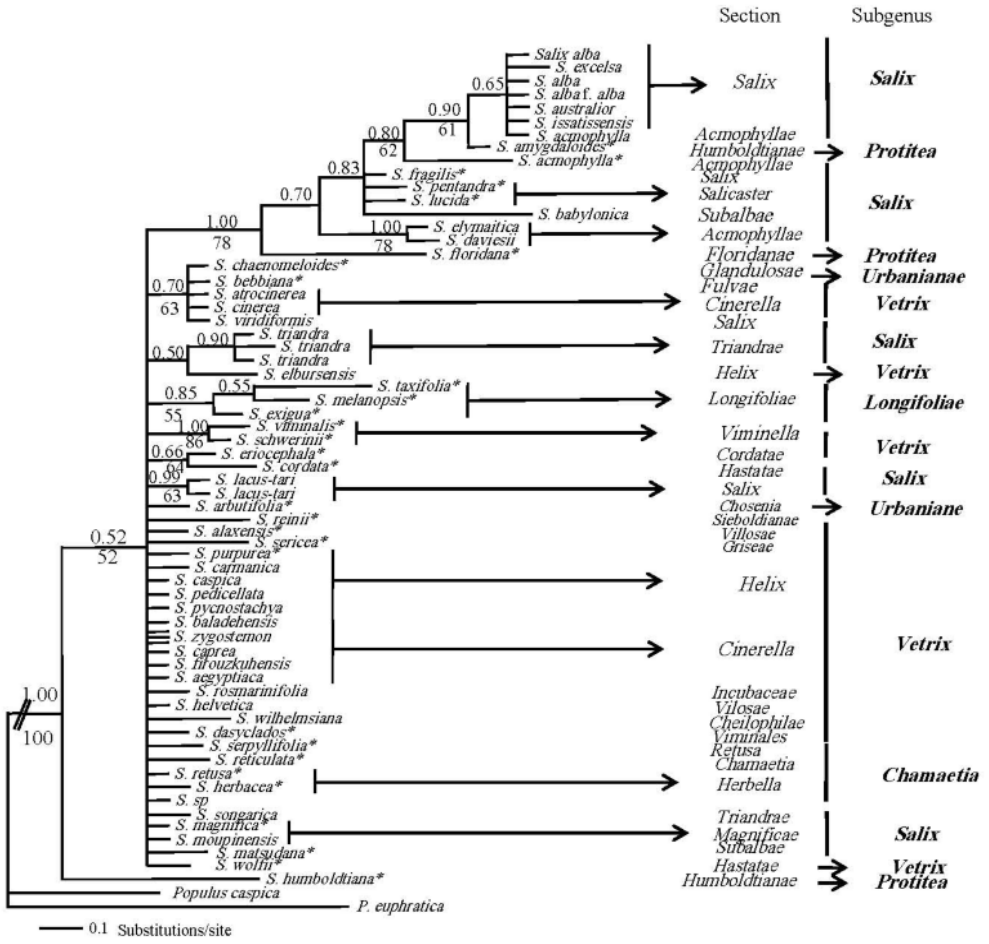

Fig. 1. Fifty percent majority rule consensus tree resulting from Bayesian analysis of the nrDNA ITS data set. Numbers above branches are posterior probabilities and the numbers below them indicate MP bootstrap values. Values < 50% were not shown. * Sequences were obtained from GenBank.

S. songarica which is not allied with *S. triandra*, instead, nested among *Vetix*/*Chamaetia* species. In agreement with Chen et al. 'study (2010), some members of sect. *Salix* and other sections of subgen. *Salix* such as *Helix*, *Eriostachyae, Hastatae* and *Subalbae* should move to the subgen. *Vetrix*. Some members of the subgen. *Vetrix* form single clades and the other sections are unresolved branches. In contrast to cpDNA phylogeny of Chen et al. (2010), the present nr DNA ITS phylogeny did not resolve the status of *S. arbutifolia* (*Urbaniane* sect. *Chosenia*) within the genus, that may be due to low sequence divergence. Similarly, *S. chaenomeloides* (*Urbaniane* sect. *Glandulosae*) was nested in a clade with some members of *Vetrix*/*Salix*, indicating that the subgenus *Urbaniane* is no longer tenable.

Phylogenetic status of the Iranian *Salix* species

According to the recent treatment by Maassoumi (2009), 36 *Salix* species are growing in Iran. Twenty-six species analyzed herein are scattered throughout the nrDNA ITS tree. Of which, 11 species (*Salix songarica*, S. sp., *S. aegyptiaca*, *S. firuzkuhensis*, *S.caprea*, *S. zygostemon*, *S. pycnostachya*, *S. pedicellata*, *S. caspica*, *S. carmanica*, and *S. lacus-tari*) are unresolved branches and the remainder are gathered in three clades within the large assemblage (see Fig. 1). *Salix triandra* with three accessions form a well supported clade and weakly allied with *S. elbursensis*. *S. cinerea*, *S. atrocinera* and *S. viridiformis* are nested in a clade with *S. bebbiana* (from North America) and *S. chaenomeloides* (from China, Japan and Korea). Third clade contains 10 species from Iran plus three from North America. Within this clade, *S. elymaitica*

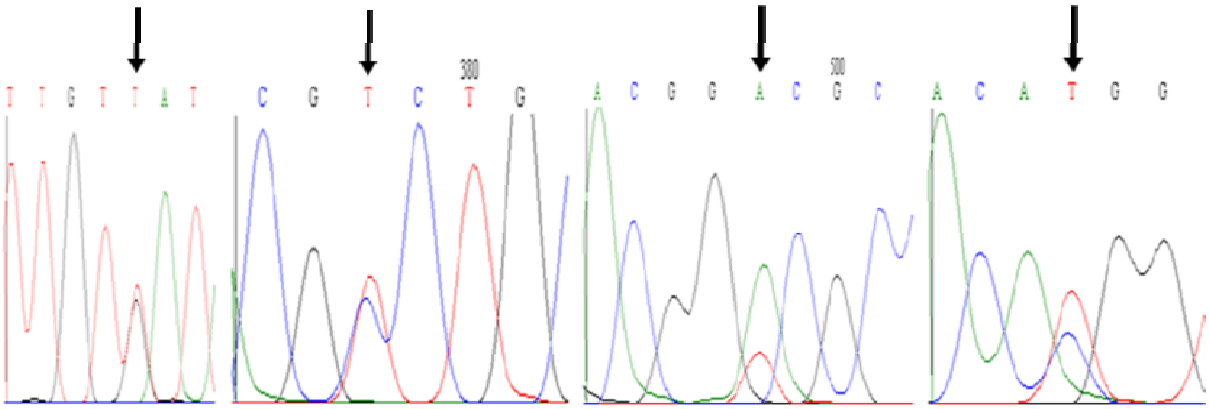

Fig. 2. Portion of nrDNA ITS sequence chromatogram from the hybrid species *Salix zygostemon* showing four polymorphic sites T/G, T/C, A/T and T/C as indicated by arrows.

and *S. daviesii* are closely related species and along with *S. floridana* formed successive grades. *S. elymaitica* was recently described as a new species (Maassoumi 2009). *S. daviesii* was previously treated as a synonymy of *S. acmophylla* (Skvortsov 1969). It is distinguished from *S. acmophylla* by four erected stamens not by five deflexed stamens (Maassoumi 2009). In our nrDNA ITS tree, *S. acmophylla* has no relationship with *S. daviesii*. *Salix alba* and related species including *S. excelsa*, *S. australior*, *S. acmophylla* and the newly described *S. issatissensis* (Maassoumi et al. 2008) formed a weakly supported clade, as well united with S. *amygdaloides* of North America. Another accession of *S. acmophylla* (retrieved from GenBank) is weakly sister to this clade. Finally, *S. fragilis*, *S. pentandra* (nrDNA ITS of both from GenBank) and *S. babylonica* are unresolved branches.

Hybridization

High frequency of hybrids has been reported in many *Salix* species, and natural hybridization along with polyploidy is thought to have played an important role in *Salix* evolution (Skvortsov 1969; Brunsfeld et al. 1992; Skvortsov 1999; Argus 1997, 1999, 2004, 2007; Ohashi 2000; Decker 2006). The importance of hybridization as a source of variability in willows is well known too (Rechinger 1992; Argus 1997; Skvortsov 1999; Maassoumi 2009).

In the present study, several polymorphic nucleotide sites of nrDNA ITS were detected for *Salix zygostemon, S. elymaitica* and *S. acmophylla* (from Iran). The sequences for three accessions of *S. zygostemon* were polymorphic at the same nucleotide sites (Fig. 2). This indicates that *S. zygostemon* has a hybrid origin resulting from cross between *S. elbursensis* and *S. cinerea*. Our *trn*L-F tree showed that *S. zygostemon*, was nested in a clade containing *S. cinerea* and *S. elbursensis* (Fig. 3). Whereas, in nrDNA tree, it was an unresolved branch (Fig. 1). Furthermore, treating the polymorphic sites as unambiguous nucleotides like that of its putative parents, this species was allied either with *S. elbursensis* or *S. cinerea* (trees not shown). Skvortsov (1969) postulated that *S. zygostemon* is a hybrid between *S. aegyptiaca* and *S. elbursensis*. This is partly concordant with our analyses as Maassoumi (2009) reached the same conclusion as ours. Moreover, the recent leaf anatomical study also confirmed that *S. zygostemon* is an interspecific hybrid of *S. elbursensis* and *S. cinerea* (Khalili et al., 2010). At the present, the putative parents of both *S. acmophylla* and *S. elymaitica* are undetectable. Nevertheless, the one parent of *S. acmophylla* may be *S. alba*, as the species was allied with it. *Salix daviesii* can be a putative parent of *S. elymaitica*, since this species is well allied with it.

Salix biogeography

It seems that *Salix* were originated in warm temperate regions of Southern Hemisphere and southern United States and then expanded to cold temperate regions of Northern Hemisphere (especially Eurasia) (e.g., Skvortsov 1999; Ohashi 2000) *Salix homboldtiana* was mainly occurring in the subtropical New World (Argus 1997) and it is Native to South America and Mexico. Our nrDNA ITS analyses showed that *Salix humboldtiana* was placed at the base of the tree as the sister taxon to the remaining *Salix* species. This indicates that the origin and early diversification of willow is in South America and subsequently have been extending into warm/cold temperate regions in North America and Eurasia. Another notable species is *S. floridana*, native to the warm temperate region of southeastern USA, is well allied to a clade of mostly Eurasian willows.

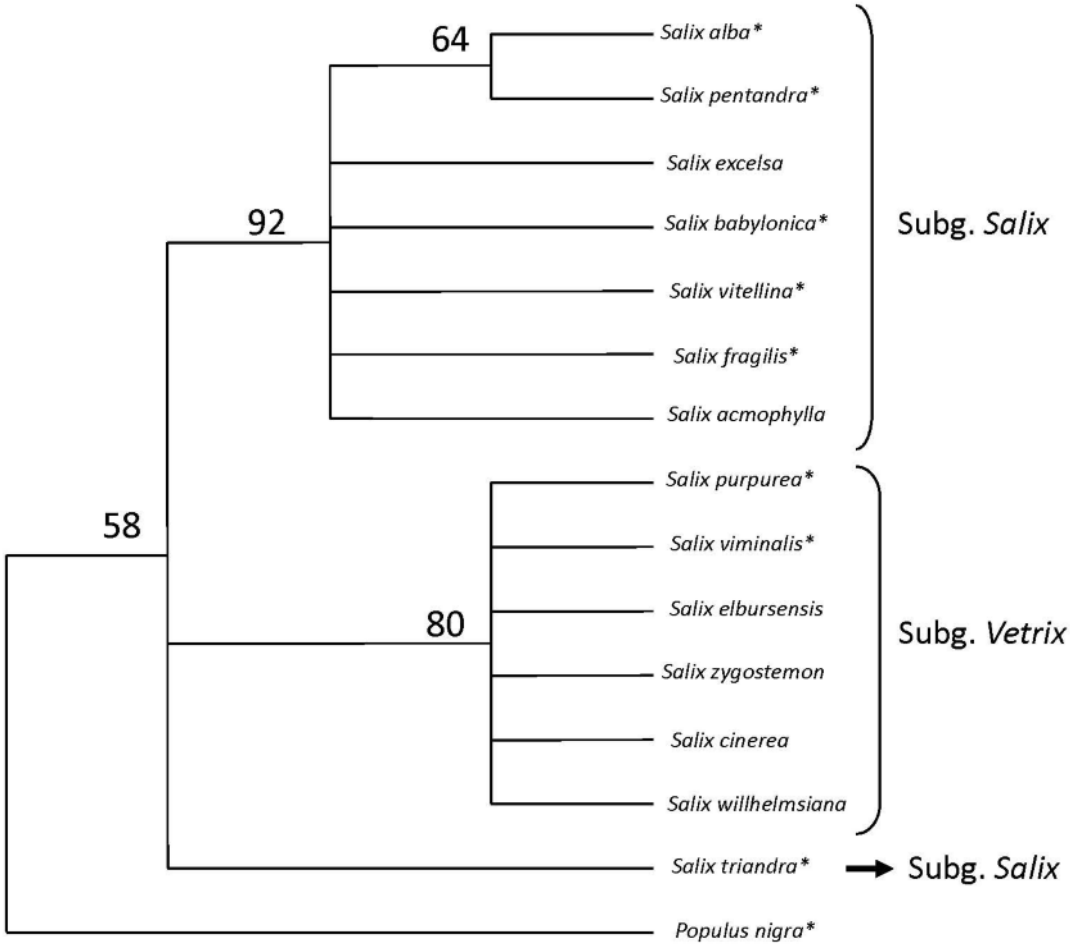

Fig. 3. Strict consensus tree of 33542 shortest trees resulting from Maximum parsimony analysis of *trn*L-F data set. Numbers above branches are bootstrap values. * Sequences were obtained from GenBank.

CONCLUSIONS

The current nrDNA ITS phylogeny in agreement with the previous works (Leskinen and Alstrom-Rapaport 1999; Azuma et al. 2000; Chen et al. 2010; Hardig et al. 2010) showed that all traditionally recognized subgenera of *Salix* except *Longifoliae* are not monophyletic. Likewise, most of *Salix* sections are not monophyletic. The willows distributing in Iran are scattered across nrDNA ITS tree. *Salix zygostemon* and perhaps *S. elymaitica* and *S. acmophylla* are hybrid species. Our analyses revealed that *Salix* originated in South America and then diversified in both North America and Eurasia. To get a clear cut picture of phylogenetic relationships among *Salix* species and delimitation of its infrageneric taxa, more DNA sequences including *trn*D-*trn*T, *trn*H-*psb*A and *trn*L_{UAG}-*ndh*F, are definitely necessary.

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