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ANALYSIS OF PHYLOGENETIC RELATIONSHIPS OF POTAMOGETON AND STUCKENIA (POTAMOGETONACEAE) IN IRAN BASED ON MORPHOLOGICAL, ANATOMICAL AND MOLECULAR DATA

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Potamogeton and *Stuckenia* comprise 90 and 7 respectively accepted species worldwide. Because of a wide range of intraspecific morphological variability including extensive phenotypic plasticity and ecological diversity, *Potamogeton* and *Stuckenia* are considered notoriously difficult taxa. A total of 100 accessions representing 11 taxa of *Potamogeton* and three of *Stuckenia* were collected across Iran. An initial screening based on allele lengths of highly variable cpDNA sequences allowed considering 59 different accessions of one to eight individuals of each species for further morphological, anatomical, and molecular analyses. NrDNA ITS and three plastid regions (rbcL, matK, and trnH-psbA) were employed to reconstruct molecular phylogenies using maximum likelihood and Bayesian inferences. Analyses of nrDNA ITS sequences generated well-resolved tree topology than plastid data. There was some incongruence between nuclear data and concatenated chloroplast marker data (P=0.001). Additional testing of ISSR and SRAP markers for 48 specimens showed higher resolution in species delimitation among linear leaved taxa, though inconclusive. Anatomical features could not separate Iranian species alone and should be used in combination with morphological characters that were highly informative. The results showed that Iranian *Potamogeton* species contain a rich gene pool due to the specific and diverse geographical conditions of Iran.

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تجزیه و تحلیل روابط فیلوژنتیکی Potamogeton و Stuckenia (Potamogetonaceae) در ایران بر اساس داده‌های مورفولوژیکی، تشریحی و مولکولی

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Potamogeton و Stuckenia به ترتیب شامل ۹۰ و ۷ گونه پذیرفته شده در سراسر جهان هستند. به دلیل طیف وسیعی از تنوعات مورفولوژیکی درون گونه‌ای از جمله انعطاف‌پذیری فنوتیپی گسترده و تنوع زیست محیطی، Potamogeton و Stuckenia جنس‌های بسیار دشواری در نظر

گرفته می‌شوند. در مجموع ۱۰۰ نمونه به نمایندگی از ۱۱ گونه از Potamogeton و سه گونه از Stuckenia از سراسر ایران جمع‌آوری شدند. غربالگری اولیه بر اساس طول آلل توالی‌های cpDNA، امکان در نظر گرفتن ۵۹ نمونه مختلف از یک تا هشت فرد از هر گونه را برای آنالیزهای مورفولوژیکی، تشریحی و مولکولی بیشتر فراهم نمود. توالی‌های هسته‌ای و کلروپلاستی برای بازسازی درختان مولکولی با استفاده از RaxML و رویکرد بیزین استفاده شدند. آنالیزهای nrDNA وضوح بالاتری را نسبت به داده‌های کلروپلاستی، در واگرایی گونه‌ها نشان دادند. تعدادی ناهماهنگی بین داده‌های هسته‌ای و کلروپلاستی وجود داشت (P=0.001). آنالیزهای اضافه‌تر نشانگرهای ISSR و SRAP برای ۴۸ نمونه، وضوح بالاتری را در تعیین حدود گونه‌ها در میان گونه‌های برگ خطی نشان داد، اگرچه قطعی نیست. ویژگی‌های تشریحی نمی‌توانند گونه‌های ایرانی Potamogeton را به‌تنهایی تفکیک کند و باید در ترکیب با خصوصیات ریخت‌شناسی که بسیار دربردارنده اطلاعات بودند استفاده شود. نتایج نشان دادند که گونه‌های Potamogeton ایرانی با توجه به شرایط خاص و متنوع جغرافیایی ایران دارای خزانه ژنتیکی غنی هستند.

INTRODUCTION

Aquatic plants make up a relatively small lineage of angiosperms but represent a high morphological diversity (Cook 1990). Potamogetonaceae is one of the most important families in aquatic ecosystems (Haynes 1974). There are 90 accepted species of *Potamogeton*, seven species of *Stuckenia*, and 105 confirmed hybrids (99 in *Potamogeton* and 6 in *Stuckenia*; Kaplan & al. 2013; POWO 2023). *Potamogeton* species were traditionally classified and identified according to leaf shape. In the first instance, either broad or linear leaved morphological groups were distinguished (e.g. Fernald 1932; Ogden 1943). A second morphological grouping distinguished heterophyllous species with floating and sub-concatenated foliage from homophyllous species with sub-concatenated foliage only (e.g. Les & Sheridan 1990). This phenotypic diversity involved a great challenge for phylogenetic inference (Les & Haynes 1995). Moreover, mismatches between molecular and morphological data were detected (Wang & al. 2007; Zhang & al. 2008). Hybridization is another frequently assumed factor affecting the complexity of observed phenotypes. Polyploidy and aneuploidy have been discovered within the genus as well with basic chromosome number (x=13, x=14) (Haynes 1974; Les 1983; Wiegleb 1988; Hollingsworth & al. 1998; Kaplan 2002; Fant & al. 2003).

The genera *Potamogeton* L. and *Stuckenia* Borner have been considered to be taxonomically difficult taxa (Wiegleb 1988). Due to a high amount of phenotypic variation in *Potamogeton*, a complex infrageneric classification with a high number of sections has previously been proposed (Lindquist & al. 2006). Previously, several studies had been carried out to propose informal groupings based on morphological affinities (Raunkiaer 1903; Ascherson & Graebner 1907; Hagstrom 1916; Wiegleb 1988). These groupings have been, however, criticized by Les (1983) and Les & Sheridan (1990). In the latest monograph on filiform-leaved *Potamogeton* (incl. *Stuckenia*; Wiegleb & Kaplan 1998), no infrageneric groupings were treated.

Molecular approaches have been successfully applied to resolve taxonomic problems in Potamogetonaceae (Hettiarachi & Triest 1991; Les & Sheridan 1990; Les & Haynes 1995, 1997; Hollingsworth & al. 1998; Iida & al. 2004). The Internal Transcribed Spacer (ITS) of ribosomal DNA is an informative nuclear DNA marker at the generic and infrageneric levels (Wang & al. 2000, Wang & al. 2007; Du & al. 2011). Due to biparental inheritance, ITS sequences have been successfully used to identify progenitors of hybrids (Kaplan & Fehrer 2004) and to investigate the origin of polyploid species (Sun & al. 2002; Liu & al. 2006). Highly polymorphic nuclear markers can be generally used for species delimitation (Zietkiewicz & al. 1994). Also, dominant ISSR and SRAP markers were used for species delimitation of plants (Safaei & al. 2016; Aghaei & al. 2015).

Recently, several molecular studies based on plastid and nuclear DNA sequence data (Iida & al. 2004; Lindqvist & al. 2006, Zhang & al. 2008; Abbasi & al. 2016); as well as RAPD marker (Moallem 2008) have been conducted on evolutionary history of *Potamogeton*.

Phenotypic plasticity plays a key role in the adaptation of organisms to rapidly changing environmental conditions (Schlichting 1986). Phenotypic plasticity is also considered to be the main source of morphological variation within the species of *Potamogeton* (Kaplan & al. 2002). Phenomena such as hybridization and the existence of cryptic species induce more complexities in the identification of *Potamogeton* species. Cryptic species that are morphologically indistinguishable, somehow reproductively isolated, are frequently found in *Potamogeton* (Whittal & al. 2004). Hybridization, as manifested by the high number of reported hybrids

(Wiegleb & Kaplan 1998; Kaplan & al. 2013) represents an obstacle to the practical identification of specimens and the interpretation of results of genetic analyses.

Iran with the mountainous regions represents a specific gene pool diversity. This specific biogeographical situation provides different specific habitat types with presumed low connectivity, influencing the variation of species (Abbasi & al. 2016). The biogeographic situation is leading both to high inter and intraspecific diversity, thus providing a basis for speciation and hybridization simultaneously (Zohary 1973; Wendelbo 1971; Leonard 1988). For accurate identification of *Potamogeton* species, sampling in so far underrepresented 'gap areas' is needed (Du & al. 2011).

Despite being a biogeographically interesting region, no comprehensive research on the species relationships of *Potamogeton s. l.* in Iran has been carried out. In Iran, 14 species of *Potamogeton* (Abbasi & al. 2017; Dinarvand & al. 2022) have been reported so far.

Objectives of the present study are, to evaluate relationships among *Potamogeton* and *Stuckenia* taxa in Iran based on both molecular and non-molecular markers.

MATERIALS AND METHODS

Taxon Sampling

Eleven species of *Potamogeton* and three species of *Stuckenia* were collected during spring and summer from 2012 to 2015 in rivers, wetlands, and other aquatic ecosystems across Iran (Appendix 1). Identification of species was done by using Flora of Iran (Dinarvand 2017).

The anatomical structure of the stem including subepidermal bundles, inter lacunar bundles, and stem shape was studied (Table 1). We also used 29 both vegetative and generative morphological characters in our study (Table 2). For cluster analysis based on morphological and anatomical data, we used NTSYS-Pc software (ver. 2.02e; Rohlf 2000) with the Jaccard coefficient and NJ method.

Table 1. Anatomical characters used for analysis.

Character	Character state
Stele	Proto type:0, Trio type:1, Oblong type:2, Four
Endodermis	O-type:0, U-type:1
Subepidermal bundles	Absent:0, Present:1
Interlacunar bundles	Absent:0
Hypodermis	Absent:0, Present:1

Genomic DNA extraction

In total, 100 individuals were at first tested for differences in allele lengths of ccmp10, ccmp2, and trnH-psbA markers. The individuals with similar genotypes were deleted and only the individuals that were different according to the used markers remained in the analyses. One to eight individuals from each species were retained for further analyses (N=59). Voucher specimens are preserved at HUI. The leaves of *Potamogeton* were dried on silica gel and genomic DNA was extracted from leaf tissue using a modified CTAB method (Abbasi & Afsharzadeh 2016).

PCR amplification and sequencing

For the phylogenetic study, we used four DNA regions including nrDNA ITS, and three plastid sequences (*trn*H-*psb*A, *mat*K, and *rbc*L). The primer pairs used for amplifying each locus were as follows: ITS; ITS1 (Forward) 5′ TCCGTAGGTGAACCTGCGG 3′ and ITS4 (Reverse) 5' TCCTCCGCTTATTGATATGC 3' (White & al. 1990), *trn*H-*psb*A; *trn*Hf (Tate & Simpson. 2003), and psbA (Sang & al. 1997), *mat*K; 390F and 1326R (Cuenoud & al. 2002), *rbc*L; *rbc*L-a-F (Levin & al. 2003) and *rbc*L-a-R (Kress & Erickson 2007). The PCR amplification for nrDNA ITS was performed in a 30 µl reaction mixture containing 3 µl DNA (50 ng), 17.8 µl water, 6 µl PCR buffer 5 mM, 0.6 µl dNTP 10mM, 1.8 µl mgcl2 25 mM, 0.06 µl forward primer 0.1 mM, 0.06 µl reverse primer 0.1 mM, 0.6 µl BSA (10mg/ml) and 0.2 µl Taq (5u/ µl). The PCR amplification for ccmp10, ccmp2, and trnH-psbA was performed in a 12.5 µl reaction mixture containing 2.5 µl water, 6.25 µl MasterMix, 1.25 µl Primer Mix, and 2.5 µl DNA. The PCR amplification for rbcL and matK was performed in a 25 µl reaction mixture containing 5 µl water, 12.5 µl MasterMix, 2.5 µl Primer Mix, and 5 µl DNA. The PCR amplification conditions for the ITS region were as follows: an initial predenaturation step at 95ºC for 4 min, followed by 34 cycles of 1 min at 95ºC, 1 min at 54ºC, and 1 min at 72ºC, with a final extension step of 10 min at 72ºC. Also, another program (multiplex with ccmp2, ccmp10, and trnH-psbA) that was better for four regions was as follows:

Table 2. Morphological characters used for analysis.

Column	Character	Character state
1	LC (Leaf color)	0: green;1: brown
2	LS (Leaf shape)	0: linear; 1: broad; 2: both
3	LS (Leaf diversity)	0: monomorphic; 1: dimorphic
4	LA (Leaf apex)	0: round; 1: acute; 2: acuminate; 3: cuspidate
5	LB (Phyllodial leaves)	0: absent; 1: present
6	LP (Leaf position)	0: subconcatenated; 1: subconcatenated and floating
7	LV (Leaf venation)	0: parallel; 1:reticulate
8	LM (Leaf margin)	0: entire; 1: serrate; 2: denticulate
9	LW (Leaf width)	0:<2 mm; 1:2-5 mm; 2:6-10 mm; 3:>10 mm
10	L/W (Length/Width)	0:<1; 1:>1
11	SS (Stipule shape)	0: connate; 1: convolute
12	SL; (Stipule length)	0:<1 cm; 1:>1 cm
13	P (Petiole)	0: present; 1: absent
14	P/L; (Petiole/Length)	0:<1; 1:1-32:>3
15	SS (Separated stipule)	0: absent; 1: present
16	PW; (Peduncle width)	0:<2; 1:>2
17	FN (Flower number)	0:2-7; 1:7-10; 2:>10
18	FL (Fruit length)	0:2; 1:>2
19	LL (Leaf length)	0:<100; 1:>100
20	K (Fruit keel)	0: absent; 1: present
21	B (Fruit beak)	0: absent; 1: present
22	VN (Vein number)	0:1-3; 1:3-7; 2:>7
23	SC (Stipule color)	0: brown; 1: white
24	LB (Leaf base)	0: acute; 1: round
25	CN (Carpel number)	0:4; 1:1
26	DD (Dichotomous division)	0: absent; 1: Present
27	NS (Nodose stem)	0: absent; 1: Present

An initial predenaturation step at 94ºC for 4 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 57ºC, and 1 min at 72ºC, with a final extension step of 30 min at 72ºC. Amplification of genomic DNA was done in MyCycler TM thermal cycler (Bio-Rad). Amplification products were resolved on 1.5% agarose gels, visualized by ethidium bromide staining under ultraviolet light. The products have been sent to Macrogen Company for sequencing. The PCR amplification for ISSR was performed in a 15 µL volume with 250 nM of each primer (Table. 3), 0.2 mM of each dNTP, 1.5 mM MgCl2, 1 U Taq polymerase, and 50-100 ng of genomic DNA.

After 4 min at 95°C, PCR was followed by 40 cycles of 1 min at 95°C, 1 min at annealing temperature, 2 min at 72°C, followed by a final extension step of 10 min at 72°C. The PCRs for the SRAP marker were performed in 25 µL reaction volumes containing Taq 2× Master Mix Red (Amplicon), 0.1 µM of each forward and reverse primer (Table 4), 50 ng DNA template, and nuclease-free water to 20 µL. The PCR program was conducted with the following cycle profile in an Eppendorf Thermal Cycler (Mastercycler Gradient): 5 min of initial denaturation at 94°C followed by 5 cycles of 1 min denaturing, 1 min annealing at 35°C and 1 min of elongation at 72°C. Then, 35 cycles of 1 min denaturing, 1 min annealing at 50°C ending with an elongation step of 5 min at 72°C.

Table 3. Sequences and annealing temperatures of ISSR primers (Blair & al. 1999).

Primer ID	Sequence (5'→ 3')	T°C
ISSR 807	AGAGAGAGAGAGAGAGT	50
UBC 872	GATAGATAGATAGATA	38
ISSR 823	TCTCTCTCTCTCTCTCC	52
ISSR 826	ACACACACACACACACC	52
ISSR 811	GAGAGAGAGAGAGAGAC	52
ISSR 812	GAGAGAGAGAGAGAGAA	50
UBC 873	GACAGACAGACAGACA	48
ISSR 2	AGAGAGAGAGAGAGAGG	52.6
ISSR 4	CTCTCTCTCTCTCTCTGG	45.7
ISSR 810	GAGAGAGAGAGAGAGAT	54.3
ISSR 3	AGCAGCAGCAGCAGCAGCG	52.6

Table 4. Sequences of used SRAP primers (Li & Quiros 2001).

Primer ID	Sequence (5'→ 3')
Me 1	TGAGTCCAAACCGGATA
Me 2	TGAGTCCAAACCGGATA
Me 3	TGAGTCCAAACCGGAAT
Me 4	TGAGTCCAAACCGGACC
Me 5	TGAGTCCAAACCGGAA
Me 6	TGAGTCCAAACCGGACA
Em 2	GACTGCGTACGAATTTGC
Em 3	GACTGCGTACGAATTGAC
Em 4	GACTGCGTACGAATTTGA
Em 17	GACTGCGTACGAATTCCA

Molecular analysis

The sequences were edited using ChromasPro version 1.7.7 and were aligned using CLUSTAL X (Thompson & al. 1994) and Muscle (Edgar 2004). The alignments were then checked manually. The inter-and intra-specific variation of each region was characterized by calculating Kimura 2-parameter (K2P) distance (Kimura 1980) with 2000 bootstrapping replicates in MEGA6 (Tamura & al. 2011). Phylogenetic relationships were analyzed by maximum parsimony (MP), maximum likelihood (ML), molecular trees based on the unweighted pair group method with arithmetic mean (UPGMA), neighbor-joining (NJ), and Bayesian analysis. *Ruppia maritima* L. was used as an outgroup. For both methods, the best models of sequence evolution were selected using MrModeltest 2.3 (Nylander 2008) based on the Akaike information criterion (AIC) (Posada & Buckley 2004). The ML was done using RAxML (randomized accelerated maximum likelihood, version 7.0.4; Stamatakis 2006) with the GTR GAMMA model. Bayesian inference was performed using Mrbayes 3.1 software (Ronquist & Huelsenbeck 2003) for 50,000,000 generations using the Markov chain Monte Carlo method (MCMC) with the (GTR+I+G) model and trees sampled at every 1000 generations. Each analysis was performed with this model.

The first 25% of trees were discarded as the burn-in. The remaining trees were then used to build a 50% majority rule consensus tree accompanied by posterior probability (PP) values. All trees were viewed with the TreeView 1.5 (Page 1996) program. Bootstrap values for ML were calculated in RaxMl based on 500 replicates.

The ISSR and SRAP data were analyzed using NTSYSpc version 2.02 based on neighbor-joining and Nei's genetic distance method (Rohlf 2000).

In the trees, the most of species are populations of *S. pectinata* because they have a larger distribution than other species in Iran and present identification issues due to large phenotypic variability. A Wilcoxon signed-rank test was used to compare the sequences (Woolson 2007).

RESULTS

According to the morphological analysis (Fig. 1), all of the species are divided into two groups (I & II). Group I (non-filiform species) is separated from each other relatively well. Four clusters (*P. nodosus*+*P. natans*); (*P. lucens*+*P. gramineus*+*P. schweinfurthii*); (*P. crispus*); (*P. Pusillus*+*P. friesii*+*P. berchtoldii*+*P. perfoliatus*+*P. trichoides*) were found for this group.

Filiform-leaved species of *Potamogeton* (*S. pectinata*; *S. diliformis*, and *S. amblyophylla*) are located in group II. This group is divided into two subgroups (IIa & IIb). Subgroup IIa (*S. pectinata*) is also divided into two groups. Subgroup IIb includes *S. filiformis* and *S. amblyophylla.*

Cluster analysis according to anatomical features (Fig. 2) includes five clusters. This analysis couldn't separate species from each other well. Group I includes *P. nodosus* and *P. perfoliatus*, group II includes *P. crispus*, group III includes *P. friesii*, *P. trichoides*, *P. pusillus*, and *P. berchtoldii*, group IV includes *P. natans*, *P. lucens*, *P. gramineus*, and group V includes filiform-leaved *Potamogeton* (*S. pectinata*; *S. diliformis*, and *S. amblyophylla*).

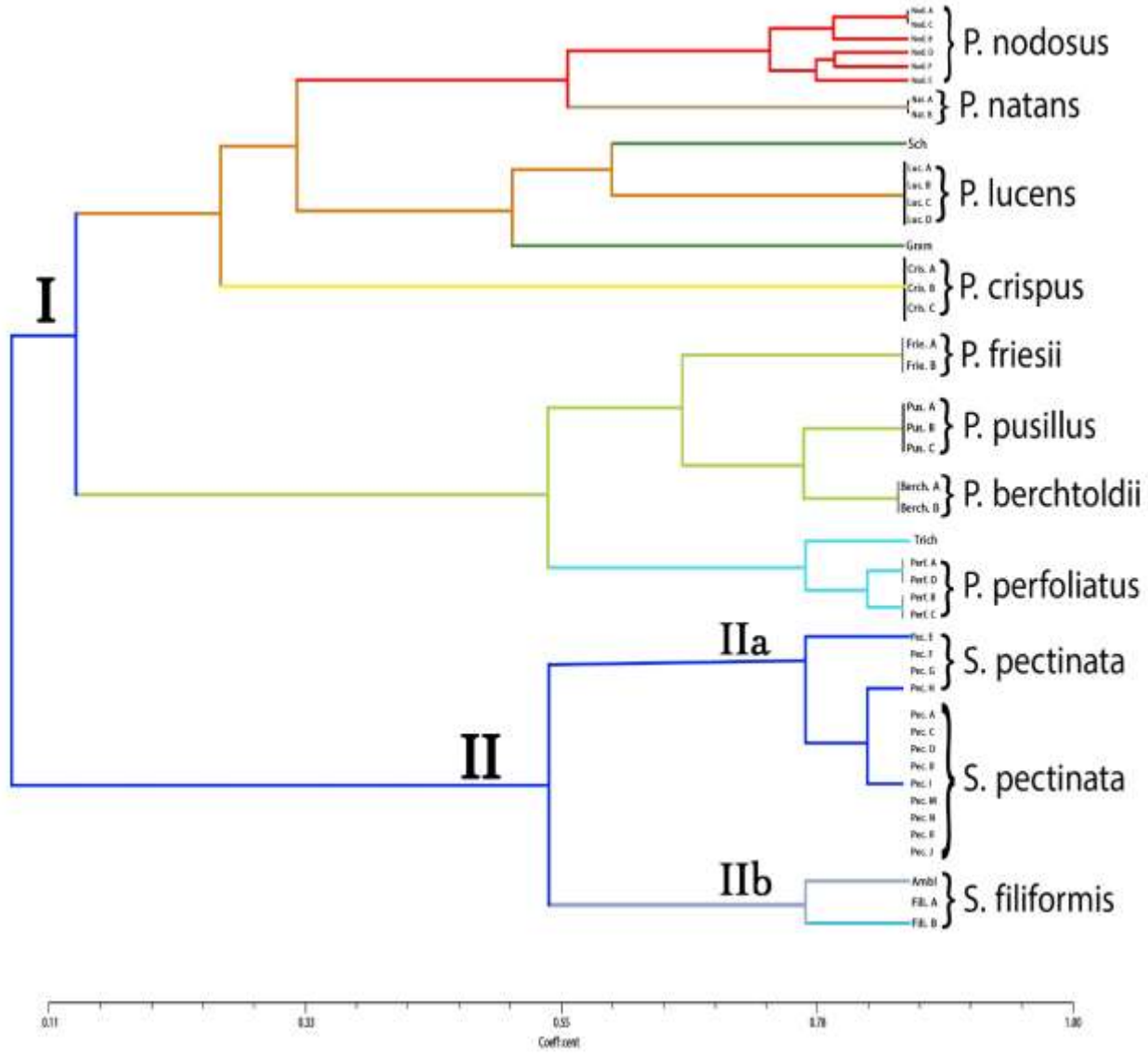

Fig. 1. Similarity dendrogram of 11 Iranian *Potamogeton* species and 3 *Stuckenia* species based on 27 morphological traits (*Potamogeton nodosus* in red; *P.* natans in brown; *P. lucens* in orange; *P. crispus* in yellow; *P. friesii*, *P. pusillus*, *P. berchtoldii*, *P. gramineus*, *P. schweinfurthii* in green; *P. perfoliatus*, *P. trichoides* in blue; *Stuckenia pectinata*, *S. amblyophylla*, *S. filiformis* in dark blue). Abbreviation of species are presented in the figure.

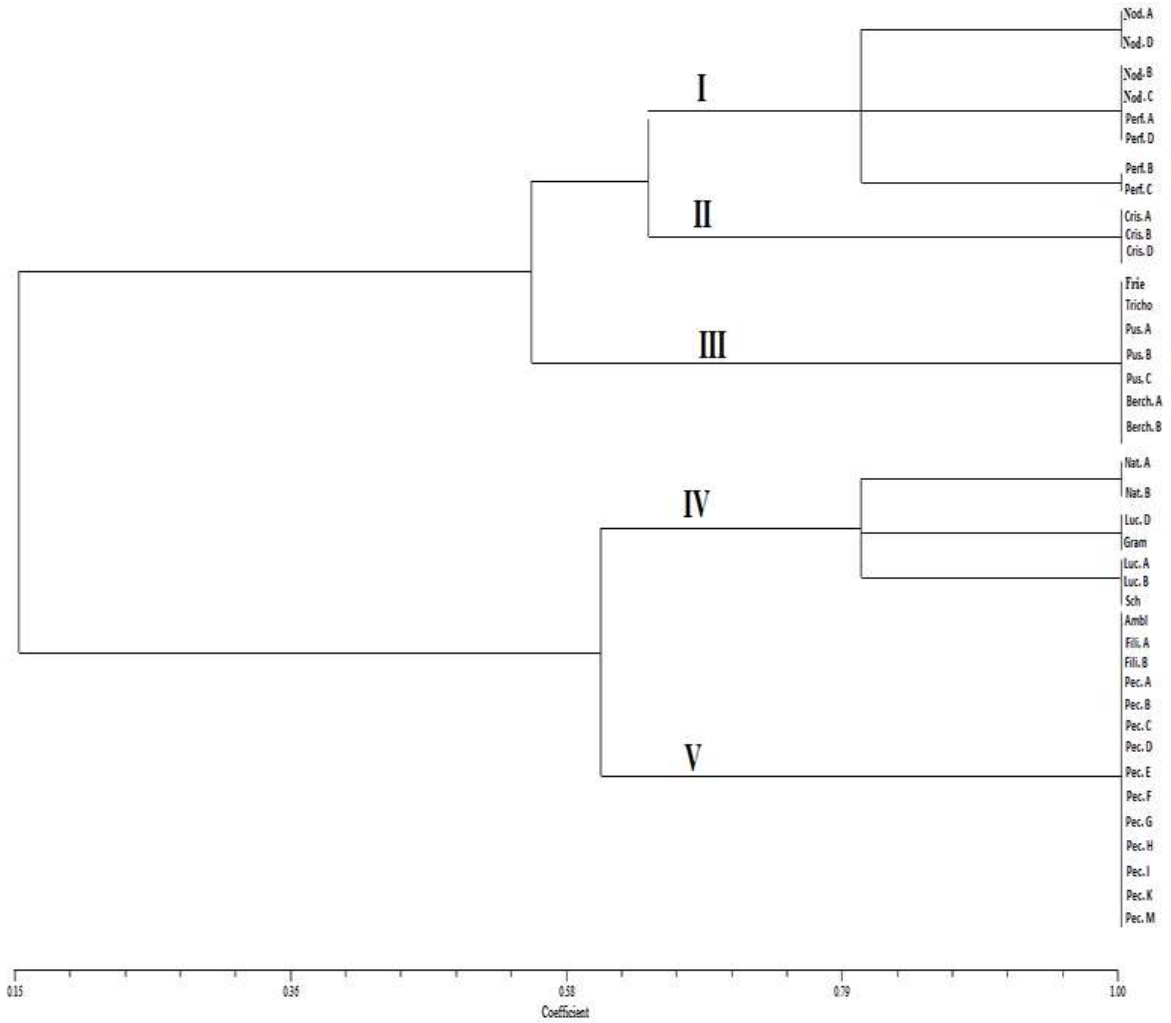

Fig. 2. Similarity dendrogram of *Potamogeton* species based on five anatomical traits. Abbreviations of species are presented in the figure.

The nrDNA ITS, *rbc*L, *trn*H-*psb*A, and *mat*K regions showed almost 100% amplification and sequencing success of the tested species. For individual regions, fragment lengths were from 396bp for trnH-psbA to 936bp for matK. The non-coding regions (ITS and trnH-psbA) demonstrated greater interspecific divergence than the coding regions (matK and rbcL). ITS showed the highest inter-specific divergence, followed by trnH-psbA and matK. The rbcL region had the lowest interspecific variation.

A Wilcoxon signed rank test on the inter-specific divergence data showed an inter-specific variation of the four loci and concatenated plastid sequence data that can be ranked as follows: ITS> TP> concatenated = *mat*K> *rbc*L. ITS (Fig. 3) and *mat*K (Table 5) were the most successful in resolving species in distinct lineages.

The molecular trees obtained from nrDNA ITS and concatenated cpDNA indicated the following incongruences; *P. schweinfurthii*, *P. filiformis*, and *S. pectinata* (Fig. 3 & 4).

According to nrDNA ITS (Fig. 3), different species of *Potamogeton* separated well based on their morphological groups (broad-leaved *Potamogeton*, linear-leaved *Potamogeton*, and filiform-leaved *Potamogeton*). In this dendrogram, filiform-leaved species made a separate clade, but in the *Stuckenia* clade, the species are not well resolved. *Stuckenia filiformis* is located among the *S. pectinata* and *S. amblyophylla*.

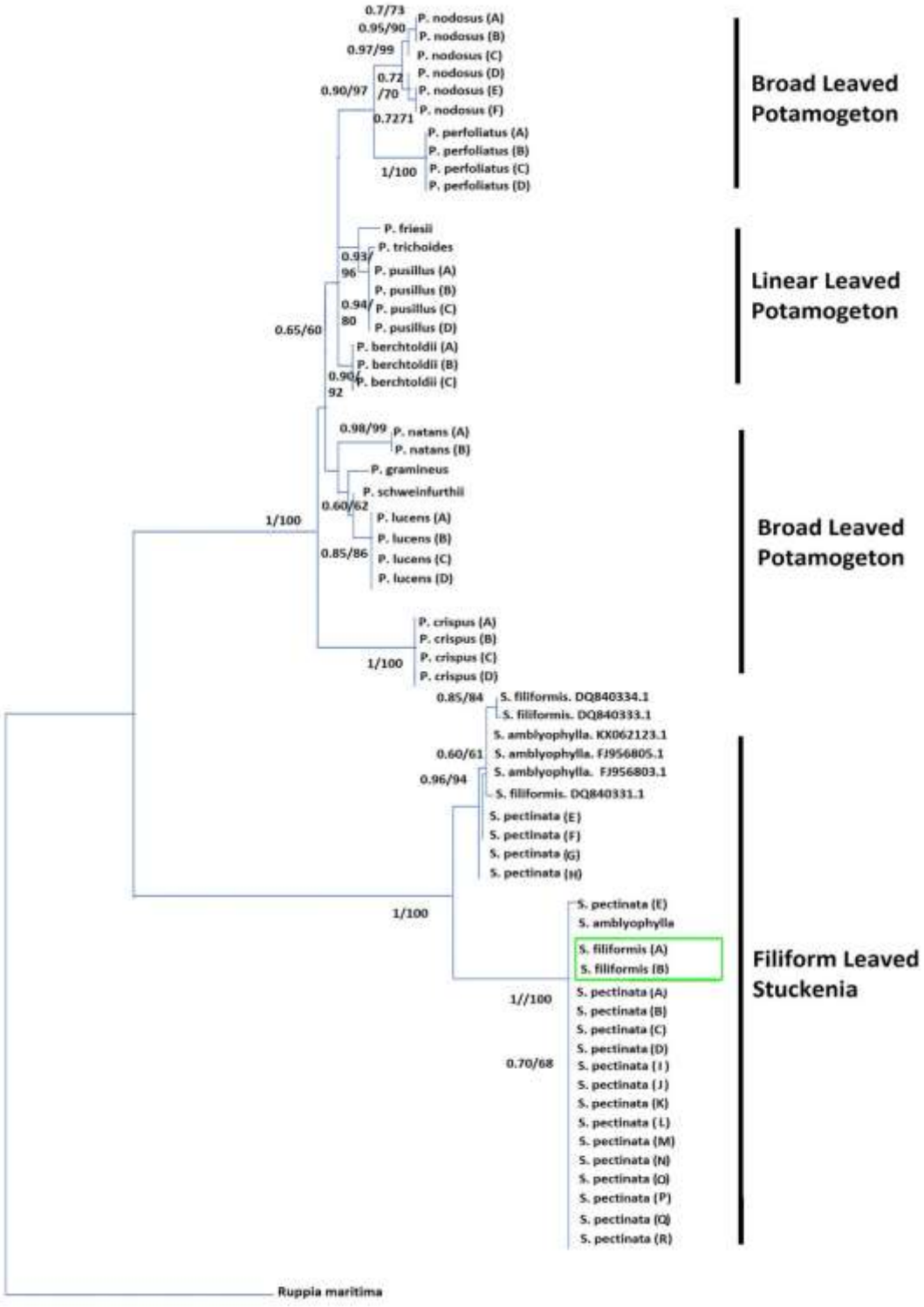

Fig. 3. ML (maximum likelihood) tree based on ITS (numbers at nodes are Posterior Probability/bootstrap values).

Cp-DNA tree (Fig. 4), couldn't separate species of *Potamogeton* as well as ITS tree. *P. schweinfurthii* is located among *P. lucens* and *P. gramineus*. In this dendrogram, filiform-leaved species make a separate clade. *Stuckenia amblyophylla* is among some of *S. pectinata*. and *S. filiformis* is located in a separate clade.

ISSR and SRAP markers could separate different species of *Potamogeton* and *Stuckenia* well. Each species is presented with a color. In both trees, filiform-leaved species separated from each other (Figs. 5 & 6). According to Fig. 6, *P. crispus* made a separate clade.

The range of genetic identity in ISSR was between 0.73-1.00 (around 1.7) but it was between 0.11-1.00 (around 1.1) for SRAP data. ISSR has a large distribution in the whole genome, while SRAP amplifies the functional region of the genome. So, the use of ISSR is more informative than SRAP.

Table 5. Wilcoxon signed-rank test of interspecific divergence among loci (concatenated is combined chloroplast data including matK, trnH-psbA, rbcL).

W+	W-	Relative rank	n	p-value	Result
matK	ITS	W+=1, W-=25.5	52	≤ 0.001	ITS > matK
concatenated	ITS	W+=14.82, W-=43.32	78	≤ 0.001	ITS > Concatenated
rbcL	ITS	W+=0.0, W-=38.5	78	≤ 0.001	ITS > rbcL
trnH-psbA	ITS	W+=15.76, W-=45.01	78	≤ 0.001	ITS > TP
concatenated	matK	W+=29.03, W-=16.37	52	0.948	Concatenated > matK
rbcL	matK	W+=9.5, W-=21.39	52	≤ 0.001	matK > rbcL
trnH-psbA	matK	W+=23.61, W-=19.54	52	≤ 0.001	TP > matK
rbcL	Concatenated	W+=14.07, W-=32.19	78	≤ 0.001	Concatenated > rbcL
trnH-psbA	Concatenated	W+=37.97, W-=35.02	78	≤ 0.001	TP > Concatenated
trnH-psbA	rbcL	W+=38.08, W-=8.5	78	≤ 0.001	TP > rbcL

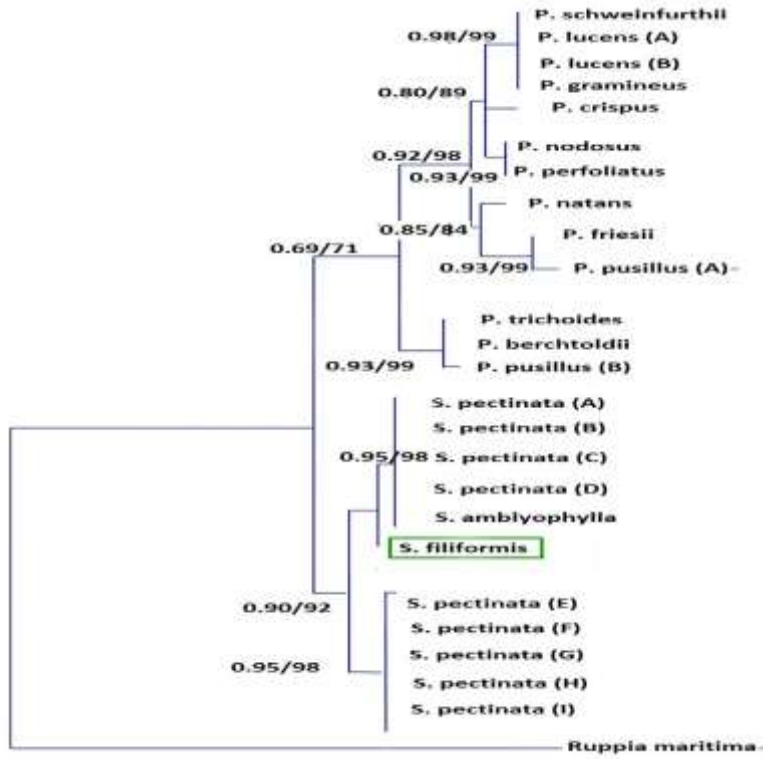

Fig. 4. Concatenated cpDNA dendrogram (numbers at nodes are Posterior Probability/bootstrap values).

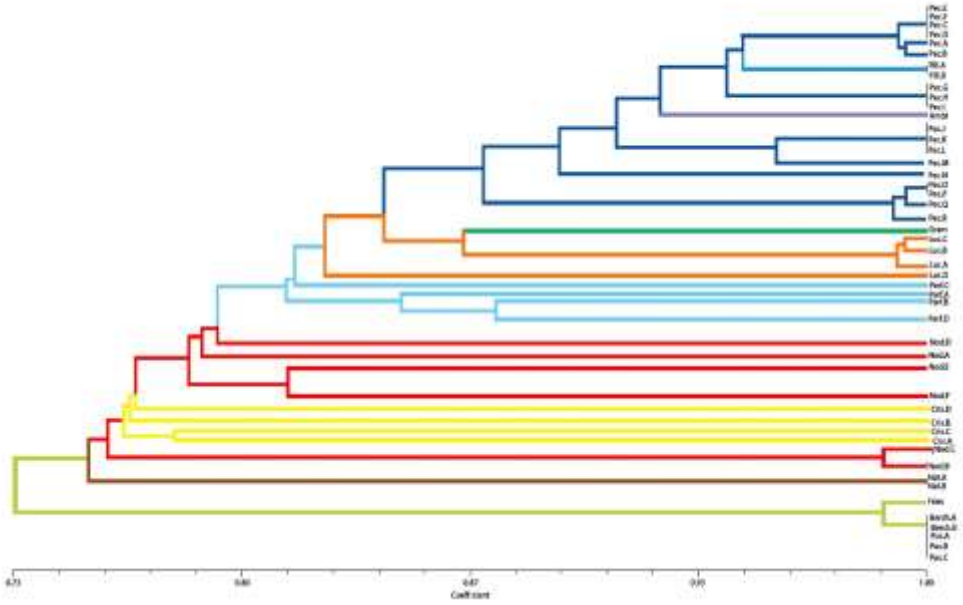

Fig. 5. *Potamogeton* species relationships with ISSR marker (*Potamogeton nodosus* in red; *P.* natans in brown; *P. lucens* in orange; *P. crispus* in yellow; *P. gramineus* in dark green*; P. friesii*, *P. pusillus*, *P. berchtoldii* in green; *P. perfoliatus*, *P. trichoides* in blue; *Stuckenia pectinate in* dark blue, *S. amblyophylla* in purple, *S. filiformis* in light green). Abbreviation of species are presented in the figure.

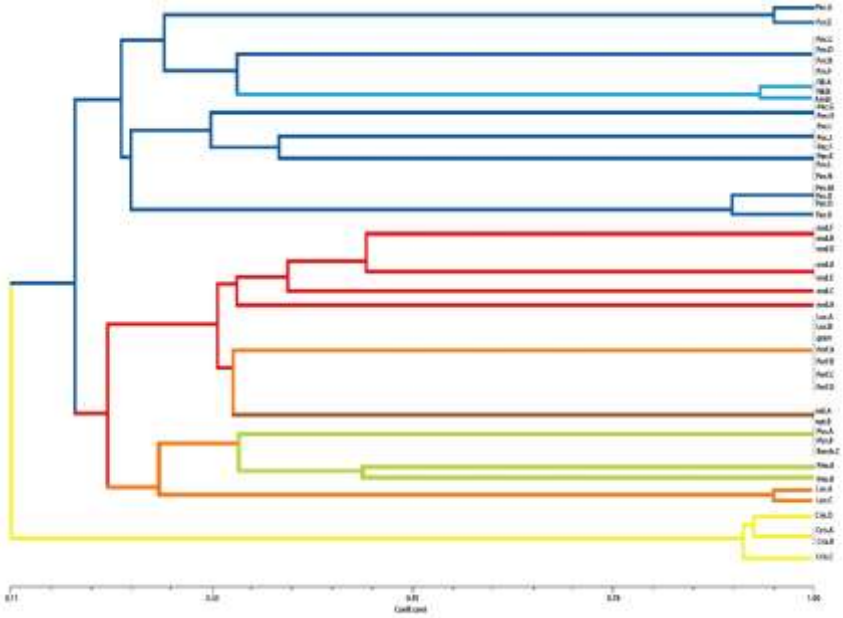

Fig. 6. Species relationships of *Potamogeton* with SRAP marker (*P. nodosus* in red; P. natans in brown; *P. lucens* in orange; *P. crispus* in yellow; *P. gramineus* in orange; *P. friesii*, *P. pusillus*, *P. berchtoldii* in green; *P. perfoliatus* in orange, *P. trichoides* in blue; *Stuckenia pectinate* in dark blue, *S. amblyophylla* in purple, *S. filiformis* in light green). Abbreviation of species are presented in the figure.

DISCUSSION

Recent molecular phylogenetic studies suggested that the former subgenus *Coleogeton* should be elevated to the rank of a genus, *Stuckenia* (Les & Haynes 1995; Holub 1997; Haynes & al. 1998; Lindqvist & al. 2006; Kaplan 2008). This is well consistent with our findings, in which *Potamogeton* species are distinct from *S. pectinata* assemblage.

Species with linear leaves such as *S. pectinata* are more primitive than the species with broad and heterophyllous leaves (Zhang & al. 2008). Our trees indicate that the ancestral species of the genus *Potamogeton* should be a homophyllous species with linear leaves, as assumed by Zhang & al. (2008). It has been proposed by using the nrDNA ITS marker that homophylly was the ancestral state in *Potamogeton* and heterophylly has evolved several times in different lineages in the genus by parallel evolution (Wang & al. 2007; Zhang & al. 2008). Broader leaves appeared more than once and one reversal back to narrower leaves has occurred (Lindqvist & al. 2006). It was also assumed that both species with linear leaves and heterophyllous species have evolved from broad-leaved homophyllous species to adapting to different aquatic living conditions (Raunkiær 1903). Reticulate venation is an advanced character that leads to the adaptation and success of plants in warm and dry climate conditions (Sack & Scoffoni 2013).

Both nrDNA ITS and matK markers are the best candidate regions for the separation of the species in *Potamogeton,* even for some closely related species. The species of the *P. pusillus* complex, a difficult taxonomic group (including *P. pusillus* & *P. berchtoldii*; Haynes 1974), and broad-leaved species such as *P. nodosus* and *P. natans* were successfully discriminated. Previous studies demonstrated that the *rbc*L region has a low diversity (Kress & al. 2005), especially in closely related species, which is consistent with our conclusion. Despite the high divergence of *trn*H-*psb*A this region is not resolving as well as ITS or matK because of the wide overlap between intra- and intergeneric diversity for this marker (Meier & al. 2006). Some studies have pointed out the unique position of *P. crispus* in *Potamogeton*. Iida & al. (2004) showed that this species is distinct by having a long deletion and several autapomorphic substitutions in the *trn*T-*trn*L sequence. Also, allozyme analyses have shown this taxon as distinct from other species in the genus (Hettiarachchi & Triest 1991). In our trees, this species is also easily distinguished with a 100% bootstrap value. Anatomical characters in the genus *Potamogeton* (Hagstrom 1916; Tur 1982; Wiegleb 1988) are useful for the separation of some species (such as *P. nodosus* and *P. natans*). Recently, Aykurt & Deniz (2016) showed that the variability of stem anatomical characters in three *Stuckenia* species is higher than expected so far and similar anatomical patterns may occur in unrelated species. In most floras, *P. friesii* is considered a distinct species close to the *P. pusillus* complex. Our molecular data tree also confirmed that this species with a high bootstrap (96%) is closely related to the *P. pusillus* complex. Isozymes (Hetiarachchi & Triest 1991), and cytotaxonomical studies (Kaplan & al. 2013) also demonstrated a close relationship between *P. friesii* and *P. pusillus*.

According to our morphological dendrogram, *P. perfoliatus* is located at a separate position. Other studies demonstrated high morphological variability including the shape of the leaf apex in this species (Vari & al. 2010). In the results of Moallem (2008) based on a morphological study of *Potamogeton*, *S. pectinata* (=*P. pectinatus*) showed the basal position in the tree. Our molecular trees showed that there is a distinction within *S. pectinata* specimens. This separation may be considered as an evolutionary unit (EU) in its Iranian germplasm because this EU is separated from other species such as *S. amblyophylla* from GenBank with a high bootstrap value.

The position of some species in molecular trees in this study might indicate the occurrence of cryptic taxa. Regarding our molecular trees, there are some incongruences between nuclear and chloroplast trees. This variation in the tree topologies may be caused by chloroplast capture, the introgression of chloroplasts from one species into another through hybridization (Rieseberg & Soltis 1991; Fehrer & al. 2007; Wang & al. 2007; Ito & al. 2016). *Stuckenia filiformis* was separated in the ISSR, SRAP, and concatenated chloroplast trees, but were nested among *S. pectinata* in both nuclear and chloroplast trees (an incomplete lineage sorting).

Although there is a reported hybrid for Iranian *Potamogeton* (Abbasi & al. 2017) but regarding to geographical barriers in aquatic habitats of Iran it is expected that we have potential new hybrids and new species. According to the results, there is high phenotypic variation in some species in different aquatic habitats of Iran for example; *S. pectinata*, *P. nodosus*, *P. natans*, and *P. perfoliatus*. In contrast, other taxa such as *S. filiformis*, *S. pectinata* from Sivand, and *P. schweinfurthii* can be considered as potential hybrids due to incongruences in nuclear and cpDNA trees. Molecular features such as ITS and *mat*K sequences proved to be helpful markers for more exact identification of the species except for Iranian *Stuckenia*. These markers appeared suitable for the identification of ESU and cryptic species in the Iranian gene pool of *Potamogeton*.

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Appendix 1. Alphabetical list of studied taxa voucher information and GeneBank accession numbers.

Nuclear: ***Potamogeton berchtoldii*** Fieber (A), Iran, Gilan, Dinarvand, 8156, LC374660.1; ***P. berchtoldii*** Fieber (B), Iran, Lorestan, Dinarvand, 8713, LC374659.1; ***P. berchtoldii*** Fieber (C), Iran, Mazandaran, Abbasi & Afsharzadeh, 20214, LC374658.1; ***P. crispus*** L. (A), Iran, West Azerbaijan, Bagheri, 2024, MF070538; ***P. crispus*** L. (B), Iran, West Azerbaijan, Bagheri, 2025, MF070539; ***P. crispus*** L. (C), Iran, Kermanshah, Abbasi & Afsharzadeh, 2029, MF070540; ***P. crispus*** L. (D), Iran, Khuzestan, Dinarvand, 8104, MF070541; ***P. friesii*** Rupr., Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 19637, MF070558.1; ***P. gramineus*** L., Iran, Khuzestan, Dinarvand, 8760, LC374663.1; ***P. lucens*** L. (A), Iran, Mazandaran, Abbasi & Afsharzadeh, 20197, LC374668.1; ***P. lucens*** L. (B), Iran, Gilan, Abbasi & Afsharzadeh, 20198, LC374667.1; ***P. lucens*** L. (C), Iran, Lorestan, Abbasi & Afsharzadeh, 20200, LC374666.1; ***P. lucens*** L. (D), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20199, LC374665.1; ***P. natans*** L. (A), Iran, East Azerbaijan, Bagheri, 20209, LC374662.1; ***P. natans*** L. (B), Iran, Lorestan, Dinarvand, 20208, LC374661.1; ***P. nodosus*** Poir. (A), Iran, Kerman, Abbasi & Afsharzadeh, 20191, LC374649.1; ***P. nodosus*** Poir. (B), Iran, Isfahan, Abbasi & Afsharzadeh, 20196, LC374648.1; ***P. nodosus*** Poir. (C), Iran, Mazandaran, Abbasi & Afsharzadeh, 20195, LC374647.1; ***P. nodosus*** Poir. (D), Iran, Mazandaran, Abbasi & Afsharzadeh, 20192b, LC374646.1; ***P. nodosus*** Poir. (E), Iran, Mazandaran, Abbasi & Afsharzadeh, 20192a, LC374645.1; ***P. nodosus*** Poir. (F), Iran, Mazandaran, Abbasi & Afsharzadeh, 20193, LC374644.1; ***P. perfoliatus*** L. (A), Iran, Mazandaran, Abbasi & Afsharzadeh, 20181, LC374653.1; ***P. perfoliatus*** L. (B), Iran, West Azerbaijan, Abbasi & Afsharzadeh, 20186, LC374652.1, ***P. perfoliatus*** L.(C), Iran, West Azerbaijan, Abbasi & Afsharzadeh, 20182, LC374651.1; ***P. perfoliatus*** L. (D), Iran, Khorasan, Basiri, 20184, LC374650.1; ***P. pusillus*** L. (A), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20212, MF070549.1; ***P. pusillus*** L. (B), Iran, Khuzestan, Dinarvand, 8137, MF070548.1; ***P. pusillus*** L. (C), Iran, Hamadan, Dinarvand, 8325, MF070547.1; ***P. pusillus*** L. (D), Iran, Gilan, Dinarvand, 21407, MF070546.1; ***P. schweinfurthii*** A. Benn., Iran, Lorestan, Dinarvand, 8761, LC374664.1; ***P. trichoides*** Cham. et Schltdl., Iran, Azerbaijan, Abbasi & Afsharzadeh, 70520, MF070550.1; ***Stuckenia amblyophylla*** (C.A.Mey) Holub, Iran, Gilan, Abbasi & Afsharzadeh, 20210, LC374677.1; ***S. filiformis*** (Pers.) Borner, (B), Iran, Khuzestan, Dinarvand, 8090, LC374678.1; ***S. filiformis*** (Pers.) Borner, (A), Iran, Khuzestan, Dinarvand, 8090, LC374679.1; ***S. pectinata*** (L.) Boerner (A), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20169, MF070565.1; ***S. pectinata*** (L.) Boerner, (B), Iran, East Azerbaijan, Abbasi & Afsharzadeh, 20145, LC374686.1; ***S. pectinata*** (L.) Boerner, (C), Iran, Isfahan, Abbasi & Afsharzadeh, 20161, LC374692.1; ***S. pectinata*** (L.) Boerner, (D), Iran, Mazandaran, Abbasi & Afsharzadeh, 20154, LC374691.1; ***S. pectinata*** (L.) Boerner, (E), Iran, Fars, Abbasi & Afsharzadeh, 20176, LC374690.1; ***S. pectinata*** (L.) Boerner, (F), Iran, Khuzestan, Abbasi & Afsharzadeh, 20166, LC374689; ***S. pectinata*** (L.) Boerner, (G) Iran, Khuzestan, Abbasi & Afsharzadeh, 20170, LC374688.1; ***S. pectinata*** (L.) Boerner, (H) Iran, Fars, Abbasi & Afsharzadeh, 20150, LC374687.1; ***S. pectinata*** (L.) Boerner, Iran, (I), Mazandaran, Abbasi & Afsharzadeh, 20148, LC374685.1; ***S. pectinata*** (L.) Boerner, (J), Iran, Mazandaran, Abbasi & Afsharzadeh, 20146, LC374685.1; ***S. pectinata*** (L.) Boerner, (K), Iran, Mazandaran, Abbasi & Afsharzadeh, 20152, LC374683.1; ***S. pectinata*** (L.) Boerner, (L), Iran, Khuzestan, Abbasi & Afsharzadeh, 20175, LC374682.1; ***S. pectinata*** (L.) Boerner, (M), Iran, Markazi, Abbasi & Afsharzadeh, 20156, LC374681.1; ***S. pectinata*** (L.) Boerner, (N), Iran, Khuzestan, Abbasi & Afsharzadeh, 20167, LC374680.1; ***S. pectinata*** (L.) Boerner, (O), Iran, Mazandaran, Abbasi & Afsharzadeh, 20155, LC374676.1; ***S. pectinata*** (L.) Boerner, (P), Iran, Kerman, Abbasi & Afsharzadeh, 20180, LC374675.1; ***S. pectinata*** (L.) Boerner, (Q), Iran, Kerman, Abbasi & Afsharzadeh, 20179, LC374674.1; ***S. pectinata*** (L.) Boerner, (R), Iran, Khuzestan, Abbasi & Afsharzadeh, 20157, LC374672.1.
Outgroup: ***Ruppia maritima*** L. from NCBI: JQ034336.
Chloroplast: ***Potamogeton berchtoldii*** Fieber, Iran, Lorestan, Dinarvand, 8713, LC374774.1; ***P. berchtoldii*** Fieber, Iran, Lorestan, Dinarvand, 8713, LC374773.1; ***P. berchtoldii*** Fieber, Iran, Lorestan, Dinarvand, 8713, LC374772.; ***P. crispus*** L., Iran, Mazandaran, Abbasi & Afsharzadeh, 20217, LC374720.1; ***P. crispus*** L., Iran, Mazandaran, Abbasi & Afsharzadeh, 20217, LC374719.1; ***P. crispus*** L., Iran, Mazandaran, Abbasi & Afsharzadeh, 20217, LC374718.1; ***P. friesii*** Rupr., Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 19637, MF070558.1; ***P. friesii*** Rupr., Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 19637, LC374758.1; ***P. friesii*** Rupr., Iran, Chaharmahal and Bakhtiari,

Appendix 1. Continued

Abbasi & Afsharzadeh, 19637, LC374757.1; ***P. lucens*** L. (A), Iran, Mazandaran, Abbasi & Afsharzadeh, 20197, LC374701.1; ***P. lucens*** L. (A), Iran, Mazandaran, Abbasi & Afsharzadeh, 20197, LC374700.1; ***P. lucens*** L. (A), Iran, Mazandaran, Abbasi & Afsharzadeh, 20197, LC374699.1; ***P. lucens*** L. (B), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20199, LC374698.1; ***P. lucens*** L (B)., Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20199, LC374697.1; ***P. lucens*** L. (B), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20199, LC374696.1; ***P. natans*** L., Iran, East Azerbaijan, Bagheri, 20209, LC374756.1; ***P. natans*** L., Iran, East Azerbaijan, Bagheri, 20209, LC374755.1; ***P. natans*** L., Iran, East Azerbaijan, Bagheri, 20209, LC374754.1; ***P. nodosus*** Poir. (A), Iran, Kerman, Abbasi & Afsharzadeh, 20191, LC374738.1; ***P. nodosus*** Poir. (A), Iran, Kerman, Abbasi & Afsharzadeh, 20191, LC374737.1; ***P. nodosus*** Poir. (A), Iran, Kerman, Abbasi & Afsharzadeh, 20191, LC374736.1; ***P. perfoliatus*** L., Iran, Khorasan, Basiri, 20184, LC374750.1; ***P. perfoliatus*** L., Iran, Khorasan, Basiri, 20184, LC374749.1; ***P. perfoliatus*** L., Iran, Khorasan, Basiri, 20184, LC374748.1; ***P. pusillus*** L. (A), Iran, Khuzestan, Dinarvand, 20214, LC374768.1; ***P. pusillus*** L. (A), Iran, Khuzestan, Dinarvand, 20214, LC374767.1; ***P. pusillus*** L. (A), Iran, Khuzestan, Dinarvand, 20214, LC374766.1; ***P. pusillus*** L. (B) Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20164, LC374761.1; ***P. pusillus*** L. (B), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20164, LC374760.1; ***P. pusillus*** L. (B), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20164, LC374759.1; ***P. schweinfurthii*** A. Benn., Iran, Lorestan, Dinarvand, 8761, LC374695.1; ***P. schweinfurthii*** A. Benn., Iran, Lorestan, Dinarvand, 8761, LC374694.1; ***P. schweinfurthii*** A. Benn., Iran, Lorestan, Dinarvand, 8761, LC374693.1; ***S. amblyophylla*** (C.A.Mey) Holub, Iran, Gilan, Abbasi & Afsharzadeh, 20210, LC374792.1; ***S. amblyophylla*** (C.A.Mey) Holub, Iran, Gilan, Abbasi & Afsharzadeh, 20210, LC374791.1; ***S. amblyophylla*** (C.A.Mey) Holub, Iran, Gilan, Abbasi & Afsharzadeh, 20210, LC374790.1; ***S. filiformis*** (Pers.) Borner, Iran, Khuzestan, Dinarvand, 8090, LC374795.1; ***S. filiformis*** (Pers.) Borner, Iran, Khuzestan, Dinarvand, 8090, LC374794.1; ***S. filiformis*** (Pers.) Borner, Iran, Khuzestan, Dinarvand, 8090, LC374793.1; ***S. pectinata*** (L.) Boerner, (A), Iran, Khuzestan, Abbasi & Afsharzadeh, 20166, LC374780.1; ***S. pectinata*** (L.) Boerner, (A), Iran, Khuzestan, Abbasi & Afsharzadeh, 20166, LC374779.1; ***S. pectinata*** (L.) Boerner, (A), Iran, Khuzestan, Abbasi & Afsharzadeh, 20166, LC374778.1; ***S. pectinata*** (L.) Boerner, (B), Iran, Mazandaran, Abbasi & Afsharzadeh, 20152, LC374783.1; ***S. pectinata*** (L.) Boerner, (B), Iran, Mazandaran, Abbasi & Afsharzadeh, 20152, LC374779.1; ***S. pectinata*** (L.) Boerner, (B), Iran, Mazandaran, Abbasi & Afsharzadeh, 20152, LC374778.1; ***S. pectinata*** (L.) Boerner, (C), Iran, Khuzestan, Abbasi & Afsharzadeh, 20170, LC374786.1; ***S. pectinata*** (L.) Boerner, (C), Iran, Khuzestan, Abbasi & Afsharzadeh, 20170, LC374785.1; ***S. pectinata*** (L.) Boerner, (C), Iran, Khuzestan, Abbasi & Afsharzadeh, 20170, LC374784.1; ***S. pectinata*** (L.) Boerner, (D), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20169, LC374789.1; ***S. pectinata*** (L.) Boerner, (D), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20169, LC374788.1; ***S. pectinata*** (L.) Boerner, (D), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20169, LC374787.1; ***S. pectinata*** (L.) Boerner, (E), Iran, Fars, Abbasi & Afsharzadeh, 20176, LC374801.1; ***S. pectinata*** (L.) Boerner, (E), Iran, Fars, Abbasi & Afsharzadeh, 20176, LC374800.1; ***S. pectinata*** (L.) Boerner, (E), Iran, Fars, Abbasi & Afsharzadeh, 20176, LC374799.1; ***S. pectinata*** (L.) Boerner, (F), Iran, Kerman, Abbasi & Afsharzadeh, 20180, LC374804.1; ***S. pectinata*** (L.) Boerner, (F), Iran, Kerman, Abbasi & Afsharzadeh, 20180, LC374803.1; ***S. pectinata*** (L.) Boerner, (F), Iran, Kerman, Abbasi & Afsharzadeh, 20180, LC374802.1; ***S. pectinata*** (L.) Boerner, (G), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20168, LC374807.1; ***S. pectinata*** (L.) Boerner, (G), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20168, LC374806.1; ***S. pectinata*** (L.) Boerner, (G), Iran, Chaharmahal and Bakhtiari, Abbasi & Afsharzadeh, 20168, LC374805.1; ***S. pectinata*** (L.) Boerner, (H), Iran, Kerman, Abbasi & Afsharzadeh, 20179, LC374810.1; ***S. pectinata*** (L.) Boerner, (H), Iran, Kerman, Abbasi & Afsharzadeh, 20179, LC374809.1; ***S. pectinata*** (L.) Boerner, (H), Iran, Kerman, Abbasi & Afsharzadeh, 20179, LC374808.1; ***S. pectinata*** (L.) Boerner, (I), Iran, Khuzestan, Abbasi & Afsharzadeh, 20157, LC374813.1; ***S. pectinata*** (L.) Boerner, (I), Iran, Khuzestan, Abbasi & Afsharzadeh, 20157, LC374812.1; ***S. pectinata*** (L.) Boerner, (I), Iran, Khuzestan, Abbasi & Afsharzadeh, 20157, LC374811.1. **Outgroup:** ***Ruppia maritima*** L. from NCBI: AB728733.1; ***R. maritima*** L. from NCBI: HQ901576; ***R. maritima*** L. from NCBI: JX438642.1.