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Full Length Research Paper

Genetic diversity and relationships detected by inter simple sequence repeat (ISSR) and randomly amplified polymorphic DNA (RAPD) analysis among *Polygonum* species growing in North of Iran

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The phylogenic relationship within four species of *Polygonum* (including *Polygonum hyrcanicum* Rech. f. (three samples)*, Polygonum persicaria* Boiss & Bushe Boiss*, Polygonum avicular* L.*,* and *Polygonum hydropiper* L. subsp. *Hydropiper*) was investigated by randomly amplified polymorphic DNA analysis (RAPD) and inter simple sequence repeat (ISSR) markers. All the species were clearly identified using 20 RAPD primers (10-mer ZO_1- ZO_{20}) and 15 ISSR primers (S_1-S_{15}). The total number of amplification products produced with 20 primers of both markers was 315. Genetic distance was calculated in order to construct phylogenic dendrogram or phylogram of closely related samples. Results indicate that the genetic distance between two samples of *P. hyrcanicum* originated from Sari and Goharbaran was considered to be short (0.622) and their RAPD and ISSR banding patterns were quite similar to each other also there is a close relationship between these two samples of *Polygonum* with *P. avicular* (0.848). The species *P. hydropiper* is not only far from two samples of *P. hyrcanicum* but also, has different patterns of RAPD and especially ISSR profiles compared to other samples. It is also interesting that *P. hyrcanicum,* gathered from Veresk, has no close relationship with other pairs of *P. hyrcanicum.* More chemotaxonomic investigation is needed to support the phylogenetic classification trees.

Key words: *Polygonum,* Polygonaceae, molecular markers, *P. hyrcanicum, P. persicaria, P. avicular, P.hydropiper*

INTRODUCTION

The genus *Polygonum* L. (Polygonaceae), well-known as persicaria or knotweed, is an important group of medicinal plants used for various purposes. *Polygonum* is an annual herb, with usual smooth elongated dark green leaves, that grows wildly throughout Iran during the spring and summer. This genus is named Alafe-Haftband or Bandvash in Persian language and including nine endemic species which are exclusively growing in Iran (Mozaffarian, 1996; Rechinger, 1986). The rhizome of *Polygonum* species has been reported in Far-East folk medicine for the treatment of atherosclerosis, hypertension, cough, suppurative dermatitis and gonorrhea (Yi et al., 2007). Decoction of *Polygonum hyrcanicun* is used in north of Iran (Turkmen Sahra) for traditional treatment of anemia, kidney stone, haemorrhoids and liver problems (Ghorbani, 2005). Literature review shows that

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Abbreviations: ISSR, Inter simple sequence repeat; **RAPD,** randomly amplified polymorphic DNA analysis.

Table 1. *Polygonum* samples used for RAPD and ISSR analysis.

Plant name	Origin (Province)	Herbarium specimen number
P. persicaria Boiss & Bushe Boiss	Firuzkuh (Tehran)	6733-TEH
P. hyrcanicum Rech. f.	Veresk village (Mazandaran) Goharbaran (Mazandaran) Sari (Mazandaran)	6729-THE
P. avicular L.	Sari (Mazandaran)	6731-TEH
P. hydropiper L.subsp. *hydropiper*	Sari (Mazandaran)	6735-TEH

the genus *Polygonum* is introducing as a source of numerous phenolic compounds, flavonoids, anthraquinones, stilbenes, and tannins (Lin et al., 2003). Also, some anthraquinones from stilbene class (rhein, emodin, aloe-emodin, chrysophanol and physcion) have been reported (Yi et al., 2007). Among them, flavonoids are the most common compounds in *Polygonum* species and have previously been used as chemotaxonomic markers in the systematics of Polygonaceae plants (Datta et al., 2000). There are a few articles around the phytochemical analysis of the volatile oils of these plants. The components of the essential oil of *Polygonum hydropiper* L. were analyzed by GC-MS and fifty-three components were identified, representing 91.6% of the total oil with a high content of sesquiterpenoids. The main constituents of the essential oil were (E)-β -farnesene (44.1%), phytol (10.8%), (E)-caryophyllene (9.3%), (E)-nerolidol (6.9%), (Miyazawa and Tamura, 2007). Furthermore, volatile compounds were extracted from *P. hydropiper* by three isolation techniques-dynamic headspace sampling, simultaneous distillation and extraction and liquid-liquid extraction with dichloromethane. In the GC-MS analysis of the volatile extracts, a total of 46 compounds were identified. Aldehydes, ketones and alcohols were the predominant classes of volatile compounds. The major compounds were identified as dodecanal (3–40%), (E)-2-hexenal (20–35%), decanal (4–22%), (Z)-3-hexen-1-ol (4–31%), hexanal (1.7–5.1%) and β -caryophyllene (1.7–2.3%) (Jiang, 2005).

Literature review also revealed that the phylogenetic (molecular markers, ISSR and RAPD) and chemotaxonomic (used the volatile constituents as the chemical markers) investigation of the *Polygonum* species have not been documented until now. Here, we focused on some Iranian and endemic species of this genus to compare and align with genetic markers.

MATERIALS AND METHODS

Sample collection

Plant samples of four *Polygonum* species including *P. hyrcanicum* Rech. f. (three samples), *Polygonum persicaria* Boiss & Bushe Boiss, *Polygonum avicular* L., and *P. hydropiper* L. subsp. *Hydropiper* were collected at flowering stage from different regions in the north and central parts of Iran. Plant materials were dried in shadow and the leaves of plant were separated from the stem, and ground in a grinder. Then, the powdered plant material was used for DNA extraction. The voucher specimens have been deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The locations of plants' growing together with herbarium numbers and their morphological characteristics are shown in Table 1.

DNA extraction

Genomic DNA(s) were extracted from plant materials using a modified method described by Lin et al. (Doyle and Doyle, 1990). Approximately 100 mg of each plant sample was frozen in liquid nitrogen (in 2-ml Eppendorf tubes). 500 ml of DNA extraction buffer (contains, 2% CTAB, 100 mM TrisHCl (pH=8), 20 mM EDTA, 1.4 M NaCl, 0.2% 2-mercaptoethanol and 4% PVP) was added to each Eppendorf tube and mixed well. The mixture was incubated at 65˚C in a water bath for 40 min with intermittent shaking at 5 to 10 min intervals. The mixture was mixed with equal volume of phenol: cloroform: isoamylalchol (25:24:1), and centrifuged at 13000 × g for 10 min at 24°C. The supernatant was transferred into a new 1.5-ml tube and 800 µl cold isopropanol (from freezer) was added and inverting until thoroughly mixed and placed in the freezer (-20°C) for 20 min. The mixture centrifuged at 13000 × g for 5 min at 4°C. The supernatant was removed and the precipitate was kept at room temperature for 15 min and then, mixed gently with 300 µl ammonium acetate (7.5 M) for 20 min at room temperature. After centrifugation at 13000 × g for 10 min at 4°C, the supernatant was removed and 600 µl of ethanol (70%) was added then centrifuged at 6000 × g for 10 min at 4°C. The DNA was pelleted by centrifugation and the ethanol was poured off, the DNA was allowed to air-dry before being dissolved in 200 µl of TE buffer.

Primers

Twenty RAPD (10-mer) primers (Operon Technologies, Alameda, California, USA) and fifteen synthesized ISSR primer (Cinnagen, Tehran, Iran) were initially tested using one species of *Polygonum* to determine the suitability of primer sets for the study. Primers were chosen for further analysis (Table 2) based on their ability to produce distinct and polymorphic amplified products within the samples (Saeidnia et al., 2005).

RAPD and ISSR assay

Polymerase chain reactions (PCR) with single primer were carried

Table 2. Total number of amplified fragments and number of polymorphic fragments generated using selected RAPD and ISSR primers.

Primer	Sequence (5' to 3')	Total band	Polymorphism percentage
ZO-3	AGACGTCCAC	11	100
ZO-4	GGAAGTCGCC	16	100
ZO-5	AGTCGTCCCC	23	90
ZO-6	CTGCATCGTG	18	100
ZO-7	GAAACACCCC	37	100
ZO-9	ACGCGCATGT	15	100
ZO-10	GACGCCACAC	18	70
ZO-12	AATGGCGCAG	12	90
ZO-13	CACTCTCCTC	10	78
ZO-14	GAATCGGCCA	32	99
ZO-15	CTGACCAGCC	28	100
ZO-16	GGGAGACATC	23	100
ZO-19	GGAGGAGAGG	9	84
S-1	AGA GAG AGA GAG AGA GT	12	100
S-6	AGA GAG AGA GAG AGA GYT	4	76
S-7	AGA GAG AGA GAG AGA GYC	6	70
S-8	AGA GAG AGA GAG AGA GYA	12	100
S-9	CTC TCT CTC TCT CTC TRC	4	95
S-12	ACA CAC ACA CAC ACA CYG	9	98
S-13	ATG ATG ATG ATG ATG ATG	16	98
Total	-	315	-

out in a final volume of 20 µl containing 20 ng template DNA, 20 ng of primer (0.5 to 1 µl), 6 µl of RNase-free water and 10 µl of *Taq* PCR Master Mix kit (includes 1.5 mM $MgCl_2$, 125 units of *Taq* DNA Polymerase, and 200 µM each dNTP), purchased from Qiagen, USA. Amplification was performed in a Primus 25 (Peqlab, Germany) thermal cycler, programmed for a preliminary 3 min denaturation step at 94°C, followed by 33 cycles of denaturation at 94°C for 20 s, annealing at 36 + 4˚C/ 40 s and extension at 72°C for 1 min, finally at 72°C for 10 min for amplification.

PCR products (alongside the negative control and GelPilot DNA Mulecular Weight Marker: 100 bp or 1 kb plus ladder) were separated by 0.8 and 1.5% (w/v) agarose gel electrophoresis for RAPD and ISSR respectively. Ethidium bromide was used to visualize under UV light (Benchtop 3 UV ™ Transilluminator) and photographs were recorded by a Canon digital camera.Data were summarized based on the presence or absence of unique and shared polymorphic bands from the photographs. Each amplification fragment was detected by approximate size in base pairs. A pair-wise difference matrix between samples was determined for the RAPD and ISSR data using simple matching coefficient (Ssm) followed by calculation of genetic distances (d) (Saeidnia et al., 2009). UPGMA (unweighted pair-group method arithmetic average) was used to construct the dendrogram. UPGMA employed a sequential clustering algorithm, in which genetic distances were used in order to show similarity, and the phylogenetic tree was built in a stepwise manner (Nei and Li, 1979).

RESULTS AND DISCUSSION

Both RAPD and ISSR markers, based on PCR techniques have proven to be a reliable, easy to generate, inexpensive and versatile set of markers. Pair-wise comparison of all RAPD and ISSR profiles revealed a similarity matrix. Simple matching coefficients (S_{sm}) and genetic distances (d), derived from RAPD and ISSR banding patterns, are shown in Table 3. The genetic distance between the two samples of *P. hyrcanicum* originated from Sari and Goharbaran was considered to be short (0.622) and their RAPD and ISSR banding patterns were quite similar to each other; also there is a close relationship between these two samples of *Polygonum* with *P. avicular* (0.848). The cladogram constructed base on genetic distances, derived from RAPD and ISSR analysis, shown in Figure 1. Also, slanted and rectangular phylograms are shown in Figure 2. Clustering analysis was on the basis of UPGMA. The indicated cladogram and phylograms were designed by the software Dendroscope which is freely available from www.dendroscope.org (Huson et al., 2007).

As shown in the dendrogram, *P. avicular* represents the closest relationship with two samples of *P. hyrcanicum*. Another important point extracted from phylogenetic distances is that *P. hydropiper* is not only far from two samples of *P. hyrcanicum* but also, has different patterns of RAPD and especially ISSR profiles compare to other samples. It is also interesting to note that *P. hyrcanicum,* gathered from Veresk, has no close relationship to other pairs of *P. hyrcanicum* (Sari and Goharbaran). Bibliography of the main morphological characters of the *Polygonum* species (Ghahreman, 1975-2000) has shown that *P. hydropiper i*s an erect herb with round stems.

Table 3. Simple matching coefficient (S_{sm}, above the diagonal) and genetic distances (d, below the diagonal) between pairs of *Polygonum* samples resulted from RAPD and ISSR.

Samples*	1	2	3	4	5	6
1	-------	0.100	0.183	0.107	0.133	0.038
2	0.949	-------	0.242	0.169	0.128	0.067
3	0.903	0.871	-------	0.222	0.337	0.054
4	0.945	0.912	0.882	-------	0.613	0.078
5	0.931	0.934	0.814	0.622	--------	0.079
6	0.981	0.966	0.973	0.960	0.960	--------

*(1) *P. persicaria*, (2) *P. hyrcanicum* from Veresk, (3) *P. avicular*, (4) *P. hyrcanicum* from Goharbaran, (5) *P. hyrcanicum* from Sari, (6) *P. hydropiper.*

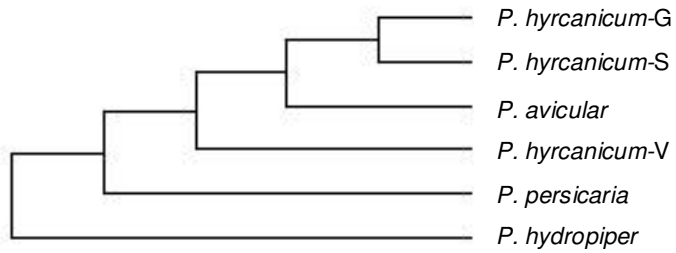

Figure 1. Cladogram of *Polygonum* samples based on the UPGMA analysis.

H0.01

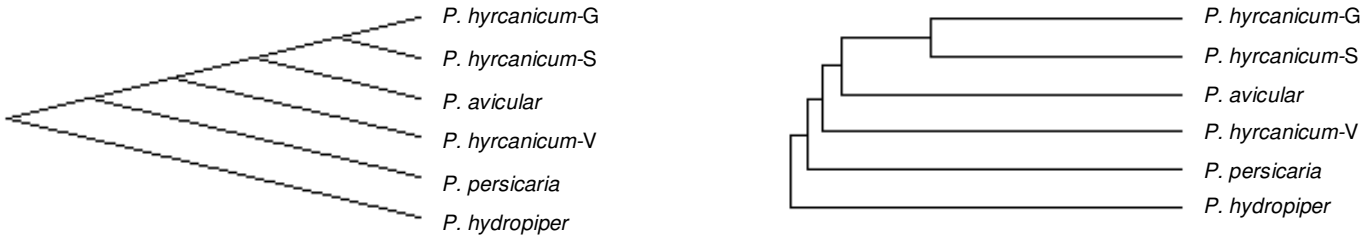

Figure 2. Slanted and rectangular phylograms of *Polygonum* samples based on UPGMA analysis.

The leaves are simple and dark green, with a sheathing stipule (or ochrea) at the petiole base. It is native to Southeast Asia and grows as a weed in some wet places. This plant is morphologically different from *P. hyrcanicum*. Especially, in case of leaves shape and flowers color and size. Furthermore, oval lance shape leaves of *P. avicular* (approximate size 20-25×5-10 cm) and *P. hyrcanicum* (approximate size 20-25×4-7 cm) together with very small axial flowers (Rechinger, 1986) show similarities to each other which are in agreement with the results of genetic diversity. Literature review revealed that there is no report about the phytochemical investigation of two endemic species of Iran, *P. hyrcanicum* and *P. persicaria*. The main concern about the results of this study is that the phytochemical constituents of the mentioned species may be different from each other and also from *P. hydropiper.* The chemical profiles (for both volatiles and non-volatiles) of these species are recommended for comparison in order to find the chemotaxonomic relations between these plants. Williams et al. (1990) mentioned that genetic polymorphism in medicinal plants can help in distinguishing plant samples at inter- or intra-species level (Williams et al., 1990). Regarding this point, phylogenetic

study of *Polygonum* can be very beneficial for conservation and preservation of the genetic variation in populations of ecologically and medicinal food varieties or genotypes and hence preventing the potential extinction.

Conclusion

Molecular biological assays (PCR-based) using RAPD and ISSR primers show that *P. hyrcanicum* and *P. avicular* are very similar to each other and *P. hydropiper* L. subsp. *Hydropiper* has a far genetic distance from other species of *Polygonum*.

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