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Species delimitation and population structure analysis in *Polygonum* species (section *Polygonum*)

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Summary: The genus *Polygonum* L. (Polygonaceae) contains about 230 species that are distributed worldwide. These herbaceous annuals and shrubby perennials are usually invasive in nature and occupy different geographical regions. They show morphological and genetic plasticity and they are difficult to deal with taxonomically. In spite of many *Polygonum* species that grow in Iran, we do not have any report on their population genetic structure, mode of divergence and dispersal. Therefore, we planned molecular (ISSR markers) and morphological studies of 37 accessions from 17 species of *Polygonum* (section *Polygonum*) that were collected from different regions in Iran. We try to address the following questions: 1) can ISSR markers delimit *Polygonum* species, 2) what is the genetic structure of these taxa in Iran, and 3) what evolutionary forces are acting for *Polygonum* species divergence? MDS plot of morphological characters separated annual species from perennials. ISSR molecular markers revealed a low degree of genetic variability in some of the studied species but showed high genetic diversity in some others. AMOVA showed strong genetic differentiation among the studied species. STRUCTURE analysis and assignment test revealed some degree of genetic admixture among studied species / populations. The present study show that invasion followed by genetic divergence due to genetic drift and inbreeding, local adaptation and occasional interspecific cross-pollination are the major evolutionary forces in *Polygonum* species and populations divergence.

Keywords: *Polygonum*, ISSR, genetic divergence, morphological variability

Polygonum L. with about 230 species in the world is the largest genus of Polygonaceae (Galasso et al. 2009; Voylokova et al. 2009). The genus as a whole has a wide distribution ranging from the tropics to the polar region and from sea level to the highest altitudes.

There has always been disagreement on classification of *Polygonum* s.l. and different subdivisions on section and genus level have been reported (Meissner 1826; Bentham & Hooker 1880; Gross 1913; Hedberg 1946; Haraldson 1978; Ronse Decraene & Akeroyd 1988). According to Lamb-Frye & Kron (2003), molecular analysis on *Polygonum* species of North America showed that *Polygonum* is paraphyletic. Phylogenetic studies on European *Polygonum* confirm that Polygonaceae are monophyletic and the subfamily Polygonoideae seems to be paraphyletic (Galasso et al. 2009). In this study, *Polygonum* s.str. (tribe Polygoneae) has been adopted. According to previous studies, 29 taxa of *Polygonum* s.str. belonging to 4 sections (*Tiniaria* Meisn., *Aconogonon* Meisn., *Pleuropterus* (Turcz.) Bentham & Hooker., *Polygonum* Tourn.) occur in Iran (Rechinger & Schiman-Czeika 1968; Mozaffarian 2012).

Polygonum s.str. species are annual and perennial herbs, subshrubs or shrubs with woody stocks (Li et al. 2003). Their stems are erect, prostrate or ascending, usually with conspicuously swollen nodes. All of them have membranous tubular ochreas. Their leaves are linear, lanceolate, elliptic, ovate or oblong with or without petioles. Inflorescences are axillary with solitary flowers or few flowered fascicles or lax spikes. Some of them are weeds, while some others have ornamental and medicinal importance across the world (Rechinger & Schiman-Czeika 1968; Qaiser 2001).

Polygonum species, particularly annual taxa, show a wide range of ecological adaptations and occupy different habitats due to their adaptability and plasticity (Sultan 2003; Griffith & Sultan 2006). Although *Polygonum* plants are mainly autogamous, hybridization can occur between annual species (Löve & Löve 1956; Yurtseva 2001). Based on Yurtseva (2001) and Voylokova et al. (2009), possible hybrids between *P. aviculare* and *P. arenastrum* were found in Russia and supported by morphological and molecular studies. Morphological complexity caused by adaptation, plasticity and hybridization made identification of *Polygonum* species difficult. Extensive studies have been performed on *Polygonum*. These were mainly dealing with their taxonomy and molecular phylogenetics (Meerts et al. 1990; Ronse Decraene et al. 2000; Lamb-Frye & Kron 2003; Galasso et al. 2009; Keshavarzi et al. 2012), but there has been no attempt to study genetic diversity, ecological adaptation and intra- and interspecific differentiation along with morphometric studies on *Polygonum* s.str. of Iran.

In this study, we examine the morphology, genetic diversity and population structure of 17 species of section *Polygonum* in Iran. We try to answer the following questions: 1) Is there infra- and interspecific genetic diversity among studied species? 2) Is genetic distance among these species correlated with their geographical distance? 3) What is the genetic structure of populations and taxa? 4) Is there any gene exchange between *Polygonum* species in Iran?

Materials and methods

Plant materials. Hundred and five plants were randomly collected from 37 populations of 17 *Polygonum* species (section *Polygonum*). These species include both annuals (numbers 1–9 in Table 1) and perennials (numbers 10–17 in Table 1). Details of localities of the studied species and populations are given in Table 1. The voucher specimens are deposited in the herbarium of Shahid Beheshti University [HSBU], Alzahra University Herbarium [ALUH], Ferdowsi University Mashhad Herbarium [FUMH], Kharazmi University Herbarium [T] and in the herbarium of Shahid Bahonar University of Kerman.

ISSR assays and genetic analyses. Fresh leaves were randomly collected and dried in silica gel powder. For some specimens, we used herbarium materials. Genomic DNA was extracted using CTAB activated charcoal protocol (Križman et al. 2006). 5 ISSR primers, UBC 834, (CA)7GT, (GA)9C, UBC 807 and (GA)9T commercialized by UBC (University of British Columbia), were used. PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Bioron, Germany), 0.2 µM of a single primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplification reactions were performed in a Techne thermocycler (Germany) with the following program: 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 51–56°C and 1 min at 72°C and a final cycle of 7 min at 72°C. The amplification products were visualized by running on 1% agarose gel, followed by ethidium bromide staining. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Morphological study

For morphometric studies, 15 qualitative and 17 quantitative characters were used (Table 2). The analysis of variance (ANOVA) test was performed to show significant morphological differences

Table 1. *Polygonum* species and populations, their localities and voucher numbers.

Species	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1. *P. arenastrum* Boreau	East Azerbaijan, Sar village	38° 20' 03" N	46° 11' 37" E	1946	ALUH 800
	Tehran, Darakeh	35° 48' 34" N	51° 22' 52" E	1756	ALUH 863
	Guilan, Bandar-e Anzali	37° 28' 21" N	49° 27' 31" E	–27	ALUH 801
	Kermanshah, Kermanshah	34° 18' 51" N	47° 03' 54" E	1392	ALUH 802
2. *P. patulum* M. Bieb.	Tehran, Darakeh	35° 48' 34" N	51° 22' 52" E	1756	HSBU 2014219
	Golestan, Gorgan	36° 50' 44" N	54° 26' 22" E	125	HSBU 2014212
3. *P. olivascens* Rech.f. & Schiman-Czeika	Sistan & Baluchestan, Hirmand, Dust Mohammad village	31° 08' 41" N	61° 47' 33" E	470	HSBU 2014213
	East Azerbaijan, Khosrowshahr	37° 56' 59" N	46° 03' 10" E	1356	ALUH 804
	East Azerbaijan, Khvor Khvor village	37° 51' 55" N	45° 46' 58" E	1278	ALUH 805
	Tehran, Nasim Shahr	35° 33' 49" N	51° 09' 31" E	1073	ALUH 906
	Markazi, Saveh, Yalabad village	34° 57' 14" N	50° 18' 24" E	1022	ALUH 905
4. *P. argyrocoleon* Steud. ex Kunze	Khuzestan, Behbahan	30° 35' 45" N	50° 14' 30" E	325	HSBU 2014214
	West Azerbaijan, Dash Band village	36° 38' 45" N	46° 10' 32" E	1336	ALUH 869
	Razavi Khorasan, Mashhad, Kuh sangi	36° 17' 16" N	59° 34' 12" E	1008	ALUH 872
	Kurdistan, 5 km to Divandarreh	35° 54' 50" N	47° 01' 26" E	1838	ALUH 874
	Guilan, Rasht	37° 16' 51" N	49° 34' 59" E	–2	ALUH 866
5. *P. molliiforme* Boiss.	Khorasan Razavi, Saroogh mountain	36° 20' 33" N	57° 18' 41" E	1660	FUMH- 42438
	Khorasan Razavi, Afchang mountains	36° 24' 29" N	57° 38' 18" E	1750	FUMH- 39101
6. *P. polycnemoides* Jaub. & Spach	Tehran, Abali	35° 45' 45" N	51° 57' 45" E	2085	ALUH 872
	Mazandaran, Savadkuh, Kachid village	36° 12' 20" N	53° 00' 38" E	1704	ALUH 803
	Alborz, Asara	36° 02' 11" N	51° 11' 39" E	1877	HSBU- 2014215
	Kerman, Kerman	30° 11' 18" N	57° 08' 11" E	1784	HSBU- 2014216
	Khorasan Razavi, Mashhad	36° 17' 16" N	59° 34' 12" E	1006	HSBU- 2014217
7. *P. kitaibelianum* Sadler.	Khuzestan, Shushtar	32° 02' 44" N	48° 51' 24" E	61	T- 42861
8. *P. aviculare* L.	Mazandaran, Gadook	35° 50' 04" N	52° 55' 47" E	220	HSBU- 2014200
	Kurdistan, Kamyaran	34° 47' 44" N	46° 56' 08" E	1466	HSBU- 2014201
	Lorestan, Khorramabad	33° 30' 49" N	48° 21' 89" E	1257	HSBU- 2014202
	Khorasan Razavi, Torbate heydarieh	35° 17' 11" N	59° 13' 17" E	1372	HSBU- 2014203
9. *P. rottboellioides* Jaub. & Spach	Alborz, Shahrestanak	35° 58' 12" N	51° 21' 13" E	2186	HSBU- 2014218
10. *P. thymifolium* Jaub. & Spach	Khorasan Razavi, North west of Torbate heydarieh- Kadkan mountain	32° 24' 00" N	59° 19' 00" E	1850	FUMH-17529
11. *P. paronychioides* C.A. Mey. ex Hohen	Khorasan Razavi, Gonabad, between Disfan & Khanik village	34° 09' 33" N	58° 24' 39" E	1500	FUMH-12815
12. *P. salicornioides* Jaub. & Spach	Kerman,Gonabad, Hraran village	29° 30' 25" N	56° 42' 36" E	2900	Shahid Bahonar University of Kerman 291
13. *P. luzuloides* Jaub. & Spach	Kermanshah, 40 km Sonqor to Gorveh	35° 03' 36" N	47° 34' 40" E	2259	Shahid Bahonar University of Kerman 292
14. *P. spinosum* H. Gross	Fars, Kazerun	29° 37' 10" N	51° 39' 15" E	850	ALUH 1000
15. *P. hyrcanicum* Rech.f.	Khorasan Razavi, Neyshaboor, Mirabad village	36° 06' 37" N	58° 46' 47" E	1750	FUMH-26061
16. *P. dumosum* Boiss.	Kerman, Sirjan	29° 27' 31" N	55° 40' 17" E	1742	Shahid Bahonar University of Kerman 293
17. *P. alpestre* C.A. Mey.	Tehran, Damavand, Tar lake	35° 43' 48" N	52° 13' 39" E	2900	HSBU-2014220

Table 2. Morphological characters in studied species.

1. Leaf length (mm)	17. Status of achene to perianth (exserted 1, included 2)
2. Leaf width (mm)	18. Color of flower (green with whitish margin 1,green with pinkish margin 2,dark pink 3, white 4, green 5)
3. Leaf length / leaf width (mm)	19. Shape of base of ochrea (tubular 1, funnel form 2, inflated 3)
4. Ochrea length (mm)	20. Shape of apex of ochrea (lacerate 1, acuminate 2, flat 3)
5. Flower length (mm)	21. Ochrea veins (conspicuous 1, inconspicuous 2)
6. Flower width (mm)	22. Achene apex (short 1, long 2)
7. Flower length / flower width (mm)	23. Color of leaf (light green 1, dark green 2)
8. Achene length (mm)	24. Stem branches (branched from the base 1, erect with branches 2, dichotomously branched 3)
9. Achene width (mm)	25. vegetative form (prostrate 1, decumbent 2, erect 3, procumbent 4, fragile and drooping 5, shrubby 6, shrubby-caespitose 6)
10. Achene length / achene width (mm)	26. Pedicel (pedicellate 1, subsessile 2)
11. Length of longest internode (mm)	27. Petiole (petiolate 1, sessile 2)
12. Petiole length (mm)	28. Leaf shape (linear 1, lanceolate 2, oblong 3, elliptic 4, ovate-lanceolate 5, linear-lanceolate 6, oblong-elliptic 7, oblong-linear 8, oblong-lanceolate 9)
13. Pedicel length (mm)	29. Shape of inflorescence (axillary with solitary flowers 1, flowers in fascicle 2)
14. Ratio of tube to perianth length (mm)	30. Achene shape (ovoid 1, digonous 2, trigonous 3)
15. Ocreola length (mm)	31. Color of achene (light brown 1, dark brown 2, black 3)
16. Color of ochrea (light brown 1, hyaline 2, dark brown 3)	32. Plant life form (annual 1, perennial 2)

between the studied populations. Multidimensional Scaling (MDS) was performed to group studied species and Principle Component Analysis (PCA) was used to identify the most variable morphological characters among these species (Podani 2000). Data analyses were performed using PAST ver. 2.17 (Hammer et al. 2001).

Genetic diversity and population structure

ISSR bands were coded as binary characters (presence = 1, absence = 0). Genetic diversity parameters like Nei's gene diversity (H), Shannon information index (I), number of effective alleles and percentage of polymorphism were determined for each species (Weising et al. 2005; Freeland et al. 2011). PopGene ver. 1.32 was used for these parameters.

Ward, NJ clustering and UPGMA methods (PopGene ver. 1.32 & PAST ver. 2.17) were used for grouping after 100 times bootstrapping replicates. Jaccard and Nei's genetic distance was used for clustering (Freeland et al. 2011; Huson & Bryant 2006).

The Mantel test was performed to investigate the correlation between geographical and genetic distances of studied populations by GenAIEx ver. 6.4.

Pearson coefficient of correlation using PAST ver. 2.17 was determined between geographical features (altitude, longitude and latitude) and genetic diversity parameters.

AMOVA (Analysis of Molecular Variance) test (with 100 permutations) as implemented in GenAlEx ver. 6.4 (Peakall & Smouse 2006) and Nei's G_{ST} analysis of GenoDive ver. 2 (2013) (Meirmans & VanTienderen 2004), were used to reveal significant genetic differences among the studied species (Ijbari et al. 2014).

Population genetic differentiation was studied by G'_{ST}_est = standardized measure of genetic differentiation (Hedrick 2005), and D_est = Jost measure of differentiation (Jost 2008). In order to overcome potential problems caused by the dominance of ISSR markers, a Bayesian program, Hickory ver. 1.0 (Holsinger & Lewis 2003), was used to estimate parameters related to genetic structure (theta B value) (Tero et al. 2003).

Bayesian based model STRUCTURE analysis was used to study the genetic structure of populations (Pritchard et al. 2000). For this analysis, data were scored as dominant markers (Falush et al. 2007). The Evanno test was used to identify optimum number of genetic groups (K; Evanno et al. 2005).

Gene flow

Gene flow was determined by different approaches: 1) Calculating Nm: an estimate of gene flow from G_{ST} by PopGene ver. 1.32 as: $Nm = 0.5 (1 - G_{ST})/G_{ST}$. This approach considers equal amounts of gene flow among all populations. 2) STRUCTURE analysis based on the admixture model (Pritchard et al. 2000) and 3) Population assignment test based on maximum likelihood as performed in GenoDive ver. 2. To check if ISSR markers show correlation with environmental features of the studied populations, LFMM analysis (Latent Factor Mixed Models) was done. This method tests correlations between environmental and genetic variation while estimating the effects of hidden factors that represent background residual levels of population structure (Frichot et al. 2013).

Results

Morphometry

ANOVA showed significant differences ($P < 0.01$) in quantitative morphological characters among the species studied. In order to determine the most variable characters among the taxa studied, PCA analysis has been performed. It revealed that the first three factors comprised over 61.54% of the total variation. In the first PCA axis with 41.03% of total variation, such characters as leaf length, leaf width, ochrea length, length of longest internode, length of petiole and pedicel have shown the highest correlation (>0.7). Ratio of leaf length to width, shape of leaf, shape of stems branches and vegetative form and plant life form were characters influencing PCA axis 2 and 3 respectively.

The MDS plot of morphological characters (Fig. 1) separated the species into distinct groups. The plants of *P. olivascens* showed more similarity to *P. patulum* and *P. argyrocoleon*. Similarly, plants of *P. aviculare* and *P. arenastrum* were placed close to each other due to morphological similarity. Three perennial species of *P. spinosum*, *P. salicornioides* and *P. dumosum* (No. 12, 14 & 16) were placed close to each other and were separated from the annuals.

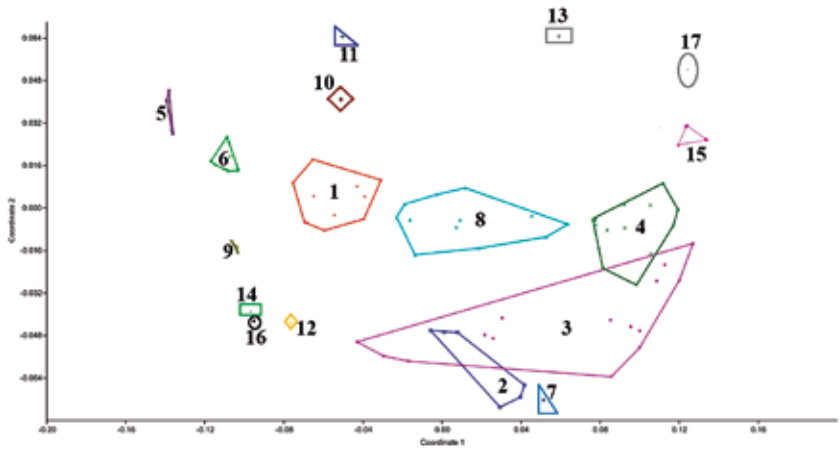

Figure 1. MDS plot of *Polygonum* species based on morphological characters (Species numbers according to Table 1).

Species delimitation and genetic diversity

All ISSR primers produced polymorphic bands. Genetic diversity parameters determined in the studied species are provided in Table 3. We used few plants for genetic diversity analysis in some of the species as we could obtain only few samples from different herbaria. Therefore, we believe that with a higher number of samples, we could get more precise information on the genetic variation within each species. However, in some cases, even with only three plants, high degree of genetic polymorphism was obtained. For instance, *P. rottboellioides* with three plants had a high degree (21.43%) of genetic polymorphism. Similarly, other species studied had only three samples included in the analysis but differed in genetic diversity parameters and also differed significantly in AMOVA test (see below). Moreover, irrespective of plant samples used in each species, they showed a very similar level of effective number of alleles (Table 3). Therefore, we can compare these values and draw some conclusions.

Polygonum olivascens and *P. argyrocoleon* had the highest value for genetic polymorphism (68.57% and 64.29% respectively). These two species also had high value for Nei gene diversity (0.18). However, *P. arenastrum* had the highest value for this parameter (0.19).

AMOVA test showed significant genetic difference (P = 0.01) among studied species. It revealed that 40% of total variation was among species and 60% was within species. Pair-wise F_{ST} values showed significant difference among all studied species (Table 4). Moreover, genetic differentiation of these species was demonstrated by significant Nei's G_{ST} (0.54, P = 0.01) and D_est values (0.174, P = 0.01).

UPGMA, Ward and NJ clustering of the studied species produced similar results. Therefore, the Ward dendrogram is presented and discussed (Fig. 2). Most of the plants of each species were grouped together in a separate cluster. This indicates that ISSR molecular markers can be used in *Polygonum* species delimitation.

The perennial and annual species were separated in different clusters. The perennial cluster contained two sub-clusters. *P. dumosum* and *P. spinosum* showed genetic affinity to each other and formed the first sub-cluster. The other studied perennial species, i.e. *P. salicornioides*, *P. alpestre*, *P. thymifolium*, *P. paronychioides*, *P. luzuloides* and *P. hyrcanicum* comprised the other sub-cluster.

Table 3. Genetic diversity parameters in *Polygonum* species.

Species	N	Na	Ne	I	He	UHe	%P	Hs
P. arenastrum	12	1.21	1.33	0.29	0.19	0.20	60	0.22
P. patulum	6	0.97	1.26	0.24	0.16	0.17	48.57	0.24
P. olivascens	15	1.39	1.28	0.29	0.18	0.19	68.57	0.26
P. argyrocoleon	15	1.29	1.29	0.28	0.18	0.19	64.29	0.23
P. molliiforme	6	0.53	1.05	0.04	0.03	0.03	8.57	0.04
P. polycnemoides	10	1.26	1.33	0.30	0.20	0.21	62.86	0.24
P. kitaibelianum	2	0.29	1	0	0	0	0	0
P. aviculare	12	1.17	1.25	0.25	0.16	0.17	58.57	0.23
P. rottboellioides	3	0.46	1.14	0.12	0.08	0.10	21.43	0.14
P. thymifolium	3	0.19	1.07	0.05	0.03	0.04	7.14	0.04
P. paronychioides	3	0.11	1.03	0.02	0.01	0.02	2.86	0.02
P. salicornioides	3	0.13	1.05	0.04	0.03	0.03	5.71	0.01
P. luzuloides	3	0.16	1.05	0.04	0.03	0.03	5.71	0.03
P. spinosum	3	0.09	1	0	0	0	0	0.00
P. hyrcanicum	3	0.06	1.03	0.02	0.01	0.02	2.86	0.01
P. dumosum	3	0.37	1.08	0.06	0.04	0.05	8.57	0.05
P. alpestre	3	0.30	1.06	0.05	0.03	0.04	7.14	0.04

N: number of samples, Na: mean number of alleles, Ne: number of effective alleles, I: Shannon's information index, He: gene diversity, UHe: unbiased genetic diversity, %P: Percentage of polymorphic loci, Hs: Genetic diversity due to population.

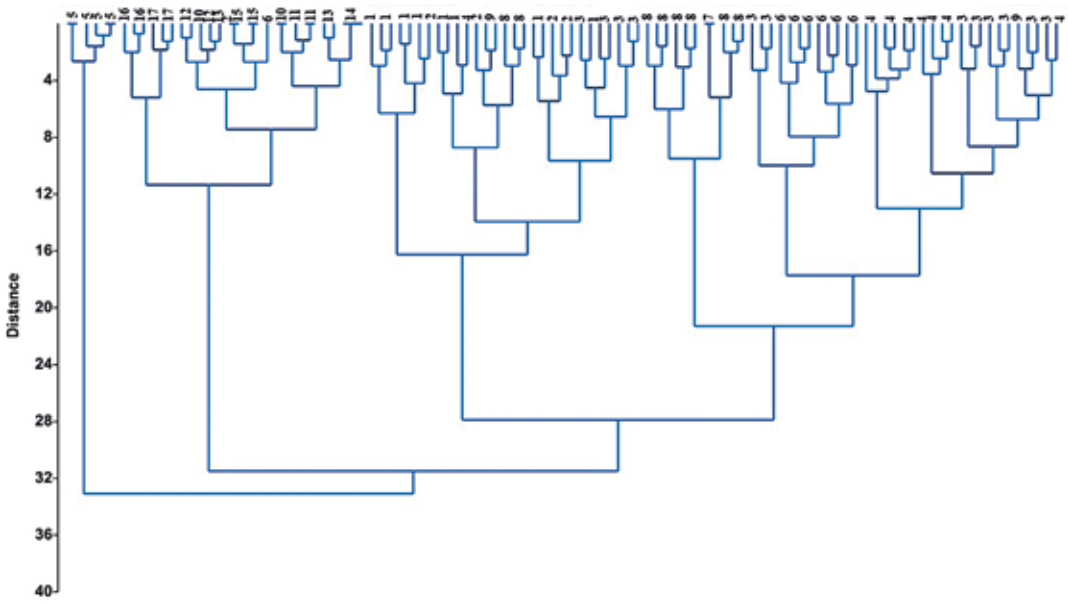

Figure 2. Ward clustering of *Polygonum* species based on ISSR data.

Table 4. Pair-wise F_{ST} values among the studied *Polygonum* species. (Above diagonal = F_{ST} value, bellow diagonal = P value).

	sp1	sp2	sp3	sp4	sp5	sp6	sp7	sp8	sp9	sp10	sp11	sp12	sp13	sp14	sp15	sp16	sp17
sp1	—	0.173	0.272	0.359	0.639	0.325	0.519	0.335	0.297	0.457	0.47	0.436	0.485	0.506	0.435	0.541	0.523
sp2	0.001	—	0.112	0.25	0.645	0.153	0.443	0.24	0.14	0.204	0.255	0.225	0.278	0.364	0.25	0.445	0.412
sp3	0.001	0.014	—	0.124	0.54	0.16	0.369	0.248	0.101	0.238	0.303	0.256	0.239	0.269	0.246	0.393	0.406
sp4	0.001	0.001	0.001	—	0.532	0.266	0.454	0.277	0.25	0.297	0.33	0.329	0.293	0.328	0.314	0.475	0.458
sp5	0.001	0.005	0.001	0.001	—	0.585	0.922	0.618	0.828	0.895	0.917	0.897	0.905	0.919	0.918	0.894	0.911
sp6	0.001	0.003	0.001	0.001	0.001	—	0.399	0.282	0.271	0.382	0.424	0.403	0.389	0.43	0.403	0.433	0.45
sp7	0.006	0.041	0.008	0.012	0.029	0.018	—	0.392	0.725	0.884	0.956	0.916	0.915	1	0.956	0.908	0.913
sp8	0.001	0.001	0.001	0.001	0.001	0.001	0.01	—	0.212	0.282	0.278	0.226	0.311	0.362	0.185	0.488	0.419
sp9	0.003	0.078	0.083	0.003	0.012	0.004	0.1	0.014	—	0.545	0.585	0.482	0.544	0.651	0.452	0.677	0.686
sp10	0.004	0.047	0.007	0.003	0.017	0.003	0.084	0.008	0.102	—	0.323	0.289	0.372	0.762	0.696	0.827	0.799
sp11	0.002	0.03	0.004	0.002	0.012	0.003	0.094	0.008	0.092	0.097	—	0.561	0.679	0.889	0.8	0.867	0.841
sp12	0.003	0.045	0.004	0.004	0.01	0.005	0.101	0.022	0.1	0.187	0.104	—	0.529	0.789	0.6	0.836	0.816
sp13	0.002	0.013	0.006	0.003	0.008	0.004	0.09	0.009	0.098	0.222	0.097	0.094	—	0.778	0.7	0.831	0.815
sp14	0.003	0.029	0.003	0.001	0.013	0.006	0.111	0.003	0.102	0.09	0.091	0.099	0.111	—	0.909	0.885	0.891
sp15	0.002	0.081	0.006	0.003	0.014	0.004	0.113	0.023	0.101	0.105	0.094	0.103	0.098	0.101	—	0.844	0.838
sp16	0.004	0.02	0.004	0.002	0.01	0.002	0.102	0.004	0.105	0.081	0.115	0.1	0.101	0.099	0.104	—	0.764
sp17	0.003	0.011	0.005	0.003	0.012	0.005	0.116	0.003	0.099	0.104	0.095	0.091	0.106	0.114	0.087	0.101	—

The annual species revealed a higher variation and were scattered in different sub-clusters. In general, *P. olivascens* and *P. argyrocoleon* (coded 3 and 4 respectively), showed genetic affinity and were placed close to each other. *P. kitaibelianum* and *P. aviculare* (coded 7 and 8 respectively), showed closer genetic affinity and formed a separate sub-cluster. *P. molliiforme* (coded 5) was genetically different from the other annual species and was placed far from the other annual taxa studied. In general, species relationships obtained from ISSR data agrees well with species relationship obtained from morphological characters.

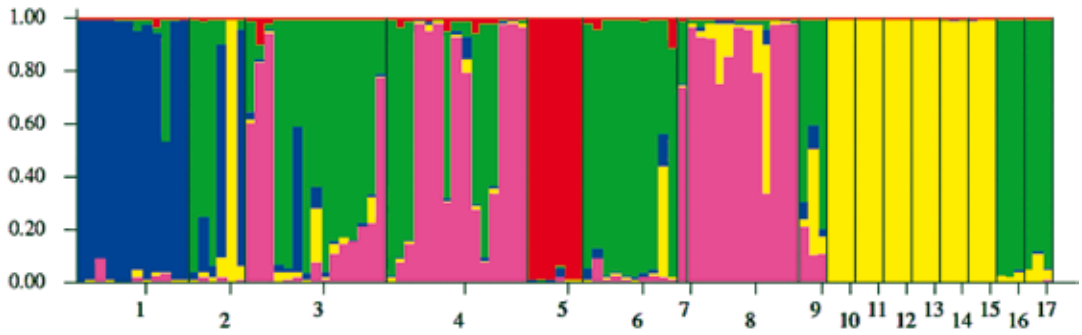

Figure 3. STRUCTURE plot of *Polygonum* species based on ISSR data.

Table 5. The population assignment test.

Individual	Current	Inferred	Lik_max	Lik_home	Lik_ratio
10	Pop1	Pop3	-37.296	-42.281	9.971
16	Pop2	Pop3	-32.485	-39.157	13.344
17	Pop2	Pop8	-18.506	-31.885	26.758
18	Pop2	Pop1	-28.084	-35.181	14.194
24	Pop3	Pop4	-39.388	-42.327	5.878
27	Pop3	Pop2	-37.287	-37.303	0.033
34	Pop4	Pop6	-26.177	-38.972	25.589
44	Pop4	Pop3	-38.096	-38.596	0.999
63	Pop6	Pop2	-18.183	-46.952	57.539
64	Pop6	Pop3	-40.926	-53.259	24.667
65	Pop7	Pop6	-45.856	—	—
66	Pop7	Pop6	-45.856	—	—
79	Pop9	Pop3	-28.717	—	—
80	Pop9	Pop3	-31.621	—	—
81	Pop9	Pop3	-26.513	—	—
82	Pop10	Pop2	-30.156	—	—
83	Pop10	Pop2	-30.156	—	—
84	Pop10	Pop2	-18.183	—	—
85	Pop11	Pop2	-24.862	—	—
86	Pop11	Pop2	-24.862	—	—
87	Pop11	Pop2	-26.472	—	—
88	Pop12	Pop8	-25.745	—	—
89	Pop12	Pop2	-25.085	—	—
90	Pop12	Pop8	-27.806	—	—
91	Pop13	Pop2	-31.765	—	—
92	Pop13	Pop3	-29.956	—	—
93	Pop13	Pop3	-29.323	—	—
94	Pop14	Pop4	-30.386	—	—
95	Pop14	Pop4	-30.386	—	—
96	Pop14	Pop4	-30.386	—	—
97	Pop15	Pop8	-23.799	—	—
98	Pop15	Pop8	-18.506	—	—
99	Pop15	Pop8	-23.799	—	—
100	Pop16	Pop6	-59.693	—	—
101	Pop16	Pop6	-59.693	—	—
102	Pop16	Pop3	-53.156	—	—
103	Pop17	Pop2	-51.557	—	—
104	Pop17	Pop8	-50.993	—	—
105	Pop17	Pop2	-48.561	—	—

Mantel test with 5000 permutations showed a significant correlation ($r = 0.13$, $p < 0.01$) between genetic distance and geographical distance, so isolation by distance (IBD) occurred among the *Polygonum* species studied. Altitude was negatively correlated with genetic polymorphism ($r = -0.516$, $P = 0.03$) and gene diversity ($r = -0.40$, $P = 0.11$). Similarly, latitude was negatively (but not significantly) correlated and the longitude was positively (but not significantly) correlated with these genetic diversity parameters.

The species genetic STRUCTURE

We performed STRUCTURE analysis followed by the Evanno test to identify the optimal number of genetic groups. We used the admixture model to illustrate interspecific gene flow or / and ancestrally shared alleles in the species studied.

STRUCTURE analysis followed by Evanno test produced $\Delta K = 5$. The STRUCTURE plot (Fig. 3) produced more detailed information about the genetic structure of the species studied as well as shared ancestral alleles and / or gene flow among *Polygonum* species. This plot revealed that the perennial species (10–15) are genetically different from the studied annual *Polygonum* species (1–9), supporting results from the Ward dendrogram. However, the species 16 and 17 had shared alleles (green colored segment) with some of the annual species (2, 3, 4 and 6). The STRUCTURE plot also revealed that the annual species 1 and 5 differed genetically from the other studied species. This plot revealed a higher degree of genetic admixture among the annual *Polygonum* species compared to the perennials.

The low Nm value (0.28) indicates limited gene flow or ancestrally shared alleles between the species studied. The population assignment test (Table 5) also supported gene flow between species, particularly between species 2, 3 and 4 and also revealed the occurrence of shared alleles between species 2 and 17, 2 and 8, 8 and 15, 8 and 16, etc.

The LFMM analysis identified 8 ISSR loci out of 70 as adaptive loci. These are ISSR loci 22, 30, 41, 55, 58, 65 and 68 (Table 6). Except ISSR locus 58 that had Nm value > 1.0, the other 7 loci had low Nm values (< 1.0) and are private alleles. These loci had strong G_{ST} values (differentiating power) and low Hs values (diversity value) (Table 7).

Discussion

Taxonomy of *Polygonum* is complicated due to species morphological similarities (Hong et al. 1998, Meerts et al. 1990). For instance, morphological characters between *P. aviculare* and *P. arenastrum* are not clearly expressed and show some degree of overlap in Flora Iranica (Rechinger & Schiman-Czeika 1968; Mozaffarian 2012). *Polygonum aviculare* s.l. is a polyploid complex of selfing annual weeds colonizing various man-disturbed habitats. Members of this complex share extensive phenotypic plasticity, autogamy and polyploidy. Genetic variation in morphometric and life history traits is related to the ecological specialization in members of this complex (Meerts et al. 1998).

Polygonum species studied are predominantly self-pollinated and are expected to show low genetic diversity. In fact, some of the species showed low genetic polymorphism, but some others had a high degree of genetic variation (up to 68% genetic polymorphism).

Polygonum olivascens and *P. argyrocoleon* exhibited intraspecific genetic variation and genetic admixture in STRUCTURE plot. Intraspecific genetic variation has been reported in some other

Table 6. LFMM result showing ISSR loci with significant association with geographical parameters studied.

Name	Z score	-log 10 (p-value)	P-value
ISSR 22	3.46859	3.28134	0.000523192
ISSR 30	2.11301	1.46092	0.0346001
ISSR 41	3.54403	3.40443	0.000394064
ISSR 55	1.89369	1.23458	0.058266
ISSR 58	2.98875	2.55266	0.00280118
ISSR 65	2.05601	1.40032	0.0397817
ISSR 68	3.80138	3.84196	0.000143893

Polygonum species, too. For example, allozyme analysis of three alpine populations of *Polygonum viviparum*, a common herbaceous perennial with no observed sexual reproduction, revealed surprising levels of genetic diversity (Diggle et al. 1998). Similarly, high genetic variation was reported within and among selfing mating *Polygonum cespitosum* populations (Matesanz et al. 2014).

Different reasons can be considered for intraspecific genetic variation of the species studied.

1) Occasional cross-pollination: Most authors have considered *P. aviculare* and related taxa to be self-pollinated (Scholz 1958; Styles 1962; Meerts et al. 1998). Indeed, the inconspicuous inflorescences with flowers partially hidden in the ochrea and the reduction of floral nectaries seem to indicate an evolutionary tendency in this direction. Yurtseva (1998) also reported that emasculated chasmogamous flowers did not produce fruits. However, the floral dimorphism present in *P. aviculare* complex is difficult to explain in view of the autogamy reported for these plants. Structures that make cross-pollination possible in other *Polygonum* s.l. are preserved in *P. aviculare* and there are numerous reports of flowers visited by insects (Löve & Löve 1956; Schmid 1983; Karlsson 2000). The possibility of limited ongoing gene flow among some of the species studied is supported by the fact that morphological similarities and overlapping characters do occur in these species. This is also supported by population assignment test presented before.

2) Phenotypic plasticity: Phenotypic plasticity is a well-known phenomenon in *Polygonum* (Zangerl & Bazzaz 1983; Sultan 2003; Matesanz et al. 2012). This plasticity can lead to genome expression differences and morphological differences.

Table 7. Nm analysis of ISSR loci showing loci identified as adaptive loci in LFMM analysis.

Locus	Hs	Gst	Nm
Locus 22	0.086	0.8061	0.1203
Locus 30	0.12	0.6315	0.2918
Locus 41	0.0929	0.6966	0.2178
Locus 55	0.1249	0.3576	0.8980
Locus 58	0.1532	0.2670	1.3727
Locus 65	0	1	0
Locus 68	0.1606	0.3380	0.9795

STRUCTURE analysis and AMOVA indicated high interspecific genetic differentiation among the studied *Polygonum* species. These species are invasive and invasive plants form new colonies and occupy new places by few founder individuals. Therefore, genetic drift and inbreeding become important evolutionary forces in the species history. The low level of within species genetic variation observed in some of the species studied might be due to genetic drift (DeWalt & Hamrick 2004; Novak & Mack 2005). This is supported by LFMM analysis that identified 8 ISSR loci out of 70 as adaptive loci. Seven loci had low Nm value (< 1.0) and were private alleles. These private alleles may be fixed by genetic drift and inbreeding in new founders during invasion in new habitats (Matesanz et al. 2012; Matesanz et al. 2014).

To conclude, the present study revealed the use of ISSR molecular markers along with morphological characters in *Polygonum* species delimitation. This molecular marker revealed genetic distinctness of annuals versus perennials. Some degrees of interspecific genetic admixture occur in *Polygonum*, but the studied species are strongly differentiated during the speciation process and invasion in new habitats. Genetic drift, strong inbreeding and local adaptation are effective evolutionary forces operating in *Polygonum* species and population divergence and adaptation.

Plant species delimitation is of central importance in phylogenetic systematics, evolution, biogeography and biodiversity. It is significant to infer patterns and mechanisms of speciation and hybridization, the evolutionary process by which new biological species arise and gene flow between closely related phylogenetic species can occur (Schluter 2001; Duminil & Di Michele 2009). Isolation by distance, local adaptation and gene flow are different mechanisms responsible for species differentiation and genetic diversity (Freeland et al. 2011; Frichot et al. 2013). Genetic diversity of *Polygonum* species can be due to these factors. However, further investigations can help to interpret this diversification better.

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