Acta Botanica Hungarica 60(3–4), pp. 401–417, 2018
DOI: 10.1556/034.60.2018.3-4.10

SPECIES DIFFERENTIATION IN ANNUAL *PERSICARIA* BASED ON DIFFERENT MARKERS

S. Mosaferi[1], M. Sheidai[2], M. Keshavarzi[1] and Z. Noormohammadi[3]

[1]*Plant Science Department, Faculty of Biological Sciences, Alzahra University, Tehran, Iran*
E-mails: s.mosaferi@alzahra.ac.ir, samanehmosaferi@yahoo.com, m.keshavarzi@alzahra.ac.ir
[2]*Faculty of Biological sciences and Technology, Shahid Beheshti University*
Tehran, Iran; E-mail: msheidai@yahoo.com
[3]*Biology Department, Faculty of Sciences, Islamic Azad University*
Sciences and Research Branch, Tehran, Iran; E-mail: marjannm@yahoo.com

(Received 12 September, 2017; Accepted 19 November, 2017)

Persicaria with 70–100 species in the world is distributed in temperate regions of both hemispheres. It has 11 species in Iran growing in moist areas and margins of rivers. Through hybridisation, plasticity and existence of overlapping habitats, species identification shows difficulty. In this study, we aimed to investigate karyotype characters and morphological features, evaluate genetic variability within and among species studied and examine species relationship using ISSR data. Nine annual taxa of *Persicaria* were gathered from different localities in Iran and used for studies. Our studies showed that combination of karyological, morphological and molecular data can delimit species studied. Based on karyological results, three chromosome counts (*P. hydropiper* ($2n = 2x = 20$), *P. maculosa* ($2n = 2x = 22$), *P. orientalis* ($2n = 2x = 22$)) were the first reports for the Flora of Iran. Analyses of morphological characters showed diagnostic features among taxa. STRUCTURE and AMOVA analyses showed high intraspecific genetic diversity. Our results suggested that phenotypic plasticity and hybridisation may cause genetic diversity within *Persicaria* species.

Key words: ISSR, karyology, *Persicaria*

INTRODUCTION

Persicaria (L.) Mill. (Polygonaceae) contains 70–100 species all over the world (The Plant List 2013); out of which 11 species occur in Iran (Mosaferi and Keshavarzi 2010, Mozaffarian 2012, Rechinger and Schiman-Czeika 1968). Members of the genus are annuals and perennials with erect, prostrate or ascending stems, herbaceous, sometimes woody. Leaves are cauline, alternate with shapes of linear-lanceolate, ovate or elliptic with connate, inflated or cordate at base. Ochrea chartaceous or partially foliaceous, ciliate or eciliate, at the mouth. Inflorescences terminal or terminal/axillary, spike, raceme or capitate. Achenes included or exserted in different shapes of 2–3-gonous, biconvex or discoid. *Persicaria* species are commonly found in temperate regions of both hemispheres (Hinds and Freeman 2005).

Although some species are weeds (*P. maculosa*, *P. glabra*), some others are of ornamental (*P. orientalis*, *P. vivipara*, *P. amphibia*) or medicinal (*P. minor*, *P. hydropiper*, *P. lapathifolia*) importance (Abubakar *et al.* 2015, Kim *et al.* 2000, Moyeenul Huq *et al.* 2014, Qader *et al.* 2012, Smolarz and Potrzebowski 2002). As a member of *Polygonum* s. l., there has been always disagreement on position of this taxon in different literatures (Ronse Decraene and Akeroyd 1988). As a separate genus, the number of species of *Persicaria* has been continuously changed (The Plant List 2013).

Persicaria species are morphologically variable due to hybridisation and phenotypic plasticity (Kim *et al.* 2008, Parnell and Simpson 1988, Stanford 1925, Sultan and Bazzaz 1993). Several studies have been done on *Persicaria* mainly concerned with taxonomy and phylogeny (Kim and Donoghue 2008, Mosaferi and Keshavarzi 2011, Ronse Decraene and Akeroyd 1988, Yasmin *et al.* 2010). Different molecular markers like ISSR (Sheidai *et al.* 2016), SSR (Matesanz *et al.* 2014), AFLP (Yasmin *et al.* 2010) and RAPD (Kim *et al.* 2008) have been used only on few species of this genus and there has been no attempt on genetic diversity of natural populations of *Persicaria* species.

Three basic chromosome numbers (x = 10, 11, 12) and different ploidy levels (tetraploid, hexaploid and octaploid) have been previously reported for this genus. Small number of cytological studies mainly focused on chromosome counting (Khatoon and Ali 1993, Kim *et al.* 2008).

As a result of diversification process and dispersal problems of *Persicaria* species, especially annual ones, and existence of overlapping areas, species delimitation is sometimes difficult. Combination of different markers can help to identify these taxa better (Duminil and Di Michele 2009); therefore we use morphological, karyological and ISSR molecular markers to delimit species and elucidate species inter-relationship.

MATERIALS AND METHODS

Plant materials

Twenty-seven populations containing 72 specimens of 9 annual taxa of *Persicaria* were gathered from different geographical regions of Iran (Table 1). The voucher specimens are deposited in the herbariums of Alzahra University (ALUH), Shahid Beheshti University (HSBU) and Kharazmi University (T).

Morphological and karyological studies

Seventy-two individuals were randomly collected from these populations for morphological studies. Totally 11 qualitative and 14 quantitative

Table 1
Information of studied taxa and accessions

Pop no.	Species	Locality	Voucher no.
l1	*P. lapathifolia* ssp. *lapathifolia* (L.) Gray	Guilan, Anzali to Astane-ashrafi-yeh, Tamchal	ALUH 508
l2	*P. lapathifolia* ssp. *lapathifolia*	Kermanshah, Kermanshah, Gharesoo river	ALUH 506
l3	*P. lapathifolia* ssp. *lapathifolia*	Mazandaran, Behshahr, Tirtash	ALUH 546
l4	*P. lapathifolia* ssp. *lapathifolia*	Mazandaran, Zirab	HSBU 2014230
n1	*P. lapathifolia* ssp. *nodosa* (Pers.) Á. Löve	Hamedan, Heydareh village	ALUH 504
n2	*P. lapathifolia* ssp. *nodosa*	Kurdistan, Sanandaj, Bayes-parneh village	ALUH 505
b1	*P. lapathifolia* ssp. *brittingerii* (Opiz) Soják	Mazandaran, Noushahr	ALUH 513
b2	*P. lapathifolia* ssp. *brittingerii*	Ghazvin, Danak village	T 7683
b3	*P. lapathifolia* ssp. *brittingerii*	Khorasan-Razavi, Torbate-jam	T 42680
p1	*P. lapathifolia* ssp. *pallida* (With.) Knutsson	East Azerbaijan, Tabriz, Bagh-misheh	T 26911
ma1	*P. maculosa* Gray	Mazandaran, Zirab, Kachid village	ALUH 502
ma2	*P. maculosa*	Tehran, Amol-Haraz road	ALUH 542
ma3	*P. maculosa*	Golestan, Gorgan, Naharkhoran	ALUH 545
h1	*P. hydropiper* (L.) Delarbre	Mazandaran, Kelardasht, Ghavi-tar village	ALUH 500
h2	*P. hydropiper*	Mazandaran, Behshahr, Tirtash	ALUH 501
mi1	*P. mitis* (Schrank) Holub	Tehran, 4 km of Tehran-Rasht rood	ALUH 534
mi2	*P. mitis*	Mazandaran, Abbas Abad	ALUH 535
mi3	*P. mitis*	Guilan, Lahijan	ALUH 536
mi4	*P. mitis*	Mazandaran, Galugah, Touska Cheshmeh	ALUH 537
mn1	*P. minor* (Huds.) Opiz	Guilan, Chaparpord village	ALUH 519
mn2	*P. minor*	Isfahan, Lushab village	ALUH 521
mn3	*P. minor*	Semnan, Damghan, Cheshm-e-Ali	ALUH 524
mn4	*P. minor*	Mazandaran, Zirab	HSBU 2014213
o1	*P. orientalis* (L.) Spach	Khorasan-Razavi, Neyshaboor	T 30014
o2	*P. orientalis*	Mazandaran, Sari	T 28627
o3	*P. orientalis*	Tehran, Shahre-ray	T 26931
o4	*P. orientalis*	Mazandaran, Noushahr	ALUH 540

Table 2
Morphological characters in *Persicaria* species

Quantitative characters	Qualitative characters
Leaf length (mm)	Colour of ochrea (dark brown 1, khaki with brown veins 2, khaki with green veins 3, green 4)
Leaf width (mm)	Ochrea veins (conspicuous 1, inconspicuous 2)
Leaf length / leaf width (mm)	Shape of inflorescence (dense 1, lax 2)
Ochrea length (mm)	Colour of flower (greenish withe 1, dark pink 2, light pink 3, pinkish white 4, pinkish red 5)
Length of ochrea bristles (mm)	Veins of tepal (anchor-shaped 1, not anchor-shaped 2)
Ocreola length (mm)	Colour of ocreola (green 1, greenish white 2, pinkish white 3, dark pink 4)
Length of ocreola bristles (mm)	Leaf shape (narrow elliptic 1, elliptic with cordate base 2, , linear-lanceolate 3, elliptic-lanceolate 4, narrow ovate 5)
Flower length (mm)	Status of achene to perianth (exserted 1, included 2)
Flower width (mm)	Achene apex (short 1, long 2)
Flower length / flower width (mm)	Colour of achene (light brown 1, dark brown 2, black 3)
Pedicel length (mm)	Achene brilliance (shinny 1, dull 2)
Achene length (mm)	
Achene width (mm)	
Achene length / achene width (mm)	

characters were used for morphometric studies (Table 2). Stereomicroscope and Dino-lite digital microscope were used for measurements.

For karyological studies, 9 populations from 8 taxa were used (5 specimens from each population). Ripen achenes were collected from natural populations. After sterilisation by 75% H_2O_2 and washing, achenes were germinated on moist filter paper in Petri dishes at 24–25 °C. Growing root tips with 0.5–1 cm length were pretreated with 0.002 M 8-hydroxyquinolin for 2–3 h at 4 °C (between 6:30–9:00 a.m.). Fixation was done by use of Carnoy solution (1:3 glacial acetic acid/absolute ethanol) for 24 h at 4 °C and then stored in 70% ethanol at 4 °C until use. Root tips were hydrolysed in 1 N HCl for 20 min at 60 °C and rinsed in tap water for a few minutes. Meristematic regions were stained and squashed on slides with 2% aqueous Aceto-orcein for 10 min. At least 5 metaphase plates were photographed with Olympus BX-51 microscope per population.

Karyotype parameters as chromosomes nomenclature (Levan *et al.* 1964), karyotype symmetry (Stebbins 1971), coefficient of variation of centromeric index (CV_{CI}, Paszko 2006), coefficient of variation of chromosome length (CV_{CL}, Paszko 2006), mean centromeric asymmetry (M_{CA}, Peruzzi and Eroğlu 2013), Total Form percentage (TF%, Huziwara 1962), Coefficient of Variation of the chromosome size (CV) as well as A1 and A2 indices of Romero Zarco (1986) were determined.

DNA extraction and ISSR amplification

Genomic DNA was extracted from leaves of 72 samples dried in silica gel powder using modified CTAB protocol (Križman *et al.* 2006). Ten ISSR primers were screened for polymorphism and finally five ISSR primers with strongest and clearest bands were used. These primers were: UBC 834, $(CA)_7GT$, $(GA)_9C$, UBC 807 and $(GA)_9T$. PCR reaction mixture with total volume of 25 µl contained 10 mM Tris-HCl buffer (pH = 8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of dNTP (Bioron, Germany), 0.2 µM of primer, 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). The amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min at 94 ºC, 40 cycles of 1 min at 94 ºC, 1 min at 51–56 ºC and 1 min at 72 ºC and a final cycle of 7 min at 72 ºC. The amplification products were visualised by running on 1% agarose gel, stained with ethidium bromide. The fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

Data analyses

Morphological and karyological studies – The analysis of variance (ANOVA) was performed to show significant morphological difference between the studied populations. Multidimensional Scaling (MDS) and UPGMA clustering were performed to group studied species. Moreover, 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.* 2012).

The analysis of variance (ANOVA) and the least significant difference test (LSD) was performed to reveal significant difference in Karyotypic characters such as size of chromosomes, size of the long arms and size of the short arms as well as arm ratio among the studied species and populations (Sheidai and Jalilian 2008). In order to group the species and populations, WARD was performed (Podani 2000). PAST ver. 3.01 was used for these analyses (Hammer *et al.* 2012).

Genetic diversity and STRUCTURE analyses – ISSR profiles were scored as binary characters (presence = 1, absence = 0). Genetic diversity parameters as Nei's gene diversity (H), Shannon information index (I), number of effective alleles and percentage of polymorphism were determined for each species using PopGene ver. 1.32 (Freeland *et al.* 2011, Weising *et al.* 2005).

Nei's genetic distance was used for clustering (Weising *et al.* 2005). NJ and UPGMA methods were used for grouping after 100 times bootstrapping/permutations. PAST ver. 3.01 and DARwin ver. 5 (2012) programs were used for these analyses (Freeland *et al.* 2011, Hammer *et al.* 2012, Huson and Bryant 2006). To determine species genetic differentiation, AMOVA (analysis of molecular variance) with 1,000 permutations and Nei's GST analysis were done using GenAlEx ver. 6.4 and GenoDive ver. 2 programs, respectively (Meirmans and Van Tienderen 2004, Peakall and Smouse 2006).

Bayesian model based clustering method implemented in STRUCTURE ver. 2.3.3 was used to study the genetic structure at population level (Falush *et al.* 2007, Pritchard *et al.* 2000). Gene flow was determined by two methods: firstly Nm, an estimate of gene flow from GST using PopGene ver. 1.32 (Nm = 0.5 (1−GST)/GST) and secondly STRUCTURE analysis based on the admixture model (Pritchard *et al.* 2000).

RESULTS

Morphological studies

ANOVA showed significant differences ($P < 0.01$) in quantitative morphological characters. PCA analysis of morphological characters revealed that the first three PCA components comprised about 56.78% of total variation. Morphological characters like leaf shape, shape of inflorescence and veins of tepal with 27.11% of total variation, showed the highest correlation in the first PCA axis. Leaf length and width, achene length and width, flower length and width, ocreola length and petiole length influenced two other PCA axes. Our results showed that these are of diagnostic values in species delimitation.

UPGMA dendrogram separated species into distinct groups. Specimens of *P. orientalis* were placed in a distinct cluster. *P. lapathifolia* ssp. *lapathifolia* and *P. lapathifolia* ssp. *nodosa* showed morphological similarities. Two other subspecies were placed close to each other. *P. mitis*, *P. minor* and *P. hydropiper* with more similarity were placed in the second subcluster while in this subcluster, *P. maculosa* was placed with some distance from others (Fig. 1). MDS plot of morphological characters supported the results of dendrogram (Fig. 2).

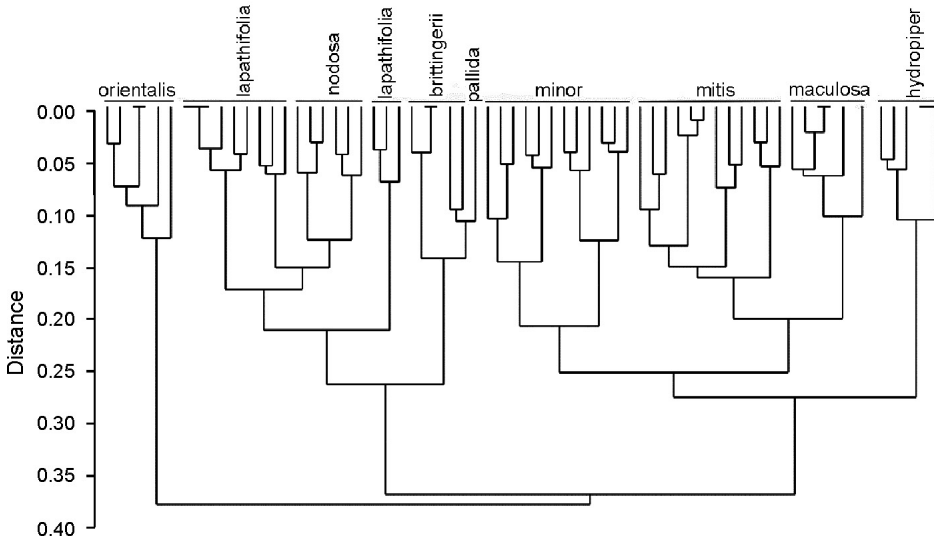

Fig. 1. UPGMA dendrogram of morphological data

Karyological studies

In studied accessions of *P. minor* and *P. mitis*, chromosome counts were $2n = 4x = 40$. Based on basic chromosome number of $x = 10$, these species were tetraploid. *P. hydropiper* showed diploid level with chromosome number of $2n = 2x = 20$ ($x = 10$).

Three subspecies of *P. lapathifolia*, and species *P. maculosa* and *P. orientalis* had chromosome number $2n = 2x = 22$. With basic chromosome numbers of $x = 11$, these taxa were diploid (Fig. 3).

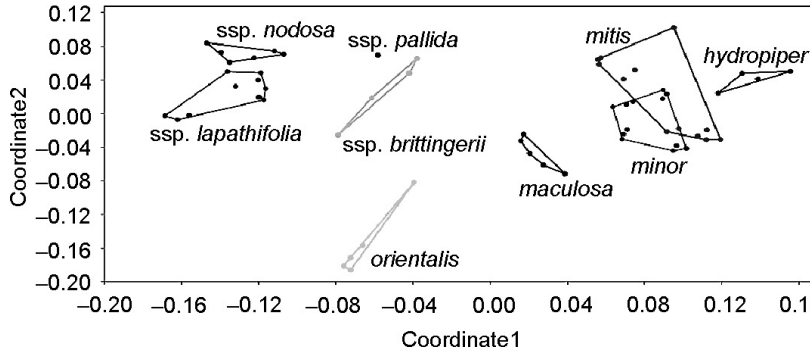

Fig. 2. MDS plot of morphological data

Among the studied taxa, the highest value of total haploid chromatin length (68.67) occurred in Lushab population of *P. minor* while the lowest value (26.96) occurred in *P. lapathifolia* ssp. *nodosa*. The highest and the lowest value of coefficient of variation (CV) were observed in *P. lapathifolia* ssp. *brittingerii* and *P. lapathifolia* ssp. *lapathifolia,* respectively. In higher CV value, variation in chromosome sizes is more and karyotype is more asymmetric. Therefore, *P. lapathifolia* ssp. *brittingerii* indicated the highest values in chromosome size variation. Total form percentage value (TF%) varied from 46.60 in *P. lapathifolia* ssp. *brittingerii* to 41.37 in *P. hydropiper* (Table 3). The studied

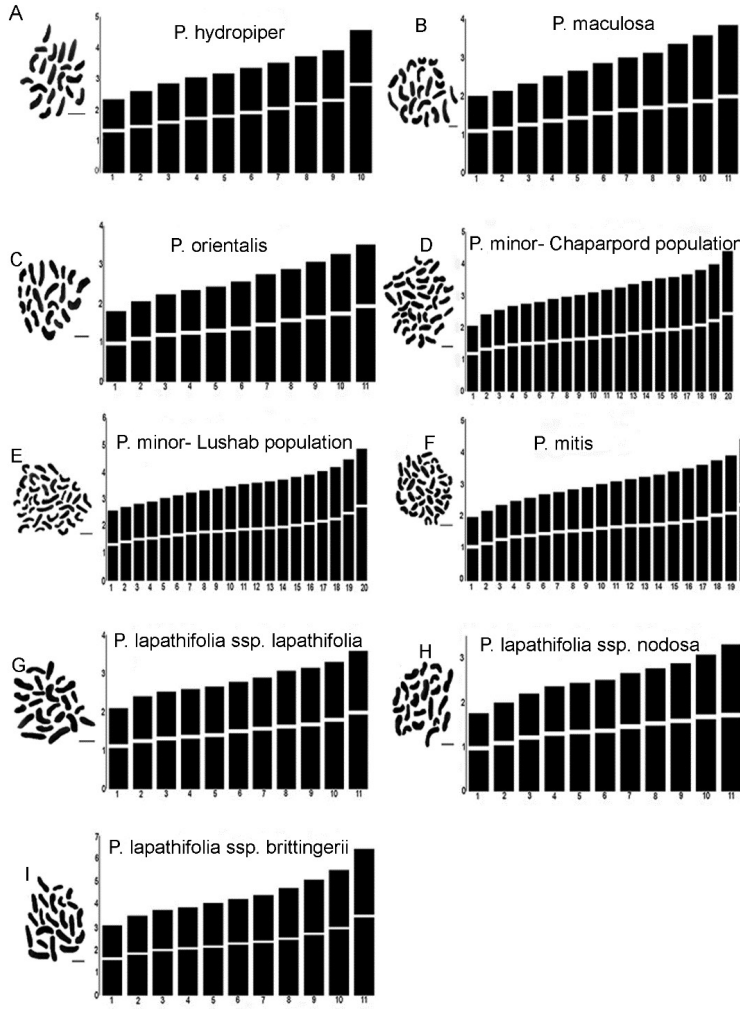

Fig. 3. Representative somatic cells of studied taxa and populations (scale bar = 10 μm)

Table 3
Karyotype features of *Persicaria* species

Species	code	Ch no.	Range S–L	TL	L/S	A1	A2	CV_{CI}	CV_{CL}	M_{CA}	TF%	CV	ST	KF
P. minor	mn1	40	1.97–4.31	61.66	2.19	0.96	0.18	1.81	18	9.65	45.22	18.51	3B	20 m
P. minor	mn2	40	2.49–4.77	68.67	1.92	0.96	0.17	2.25	17	7.6	46.08	17.20	3A	20 m
P. mitis	mi1	40	1.88–4.29	58.98	2.28	0.96	0.20	1.60	20	9	45.80	20.34	3B	20 m
P. hydropiper	h1	20	2.26–4.49	32.32	1.99	0.93	0.20	3.91	20	16.70	41.37	20.43	3A	10 m
P. maculosa	ma1	22	1.92–3.75	30.40	1.95	0.92	0.21	2.42	21	8.27	46.05	21.38	3A	11 m
P. lapathifolia ssp. *lapathifolia*	l1	22	2.01–3.49	30.03	1.74	0.92	0.16	2.38	16	6.81	46	15.75	3A	11 m
P. lapathifolia ssp. *nodosa*	n2	22	1.66–3.22	26.96	1.94	0.92	0.18	2.14	18	9.36	45.44	18.11	3A	11 m
P. lapathifolia ssp. *brittingerii*	b1	22	2.99–6.35	47.66	2.12	0.92	0.22	1.13	22	6.64	46.60	22.40	3B	11 m
P. orientalis	o4	22	1.72–3.54	27.98	2.06	0.92	0.20	1.30	20	7.64	46.14	20.47	3A	11 m

Abbreviations: Ch no. = chromosome number, L = size of the longest chromosome pair (μm), S = size of the shortest chromosome pair (μm), TL = total chromatin length (μm), L/S = ratio of the longest to shortest chromosome (μm); A1 = intrachromosomal asymmetry indices (Romero Zarco); A2 = interchromosomal asymmetry indices (Romero Zarco); CV_{CI} = heterogeneity of the centromeric index; CV_{CL} = coefficient of variation of chromosome length; M_{CA} = mean centromeric asymmetry; TF = total form percentage; CV = coefficient of variation; ST = Stebbins' symmetry class; KF = karyotypic formula

taxa had metacentric chromosomes (m) and occupied classes 3A and 3B of Stebbins (1971). *P. hydropiper* showed the highest value of CV_{CI} and M_{CA}, while *P. lapathifolia* ssp. *brittingerii* had the lowest value of both indices.

The ANOVA and LSD tests revealed a significant difference ($p < 0.05$) in total size of the chromosomes, the size of the short arms, the long arms and L/S among the species and populations studied.

In WARD dendrogram (Fig. 4), two major clusters were formed. *P. minor* and *P. mitis* showed more karyotype similarities and placed in the first major cluster. In the second major cluster, *P. hydropiper* was placed in a distinct position based on different karyotype features.

In spite of being in a separate subcluster, *P. lapathifolia* ssp. *brittingerii* was grouped near to two other subspecies of *P. lapathifolia*. *P. maculosa* and *P. orientalis* showed more similarities and were placed near each other in WARD dendrogram.

ISSR – All ISSR primers used in this study produced reproducible bands. The highest and lowest number of bands viewed in Chaparpord population of *P. minor* (25) and Sari population of *P. orientalis* (10), respectively. AMOVA showed significant genetic difference among the studied populations and species ($p = 0.01$). It also revealed that 28% of total genetic variation was due to among species and 72% of this variation was due to within species. As presented in Table 4, genetic diversity parameters in studied species showed *P. minor* with the highest level of genetic polymorphism (85.94%) and *P. lapathifolia* ssp. *pallida* with lowest level of genetic polymorphism (1.56%). Moreover these two taxa had the highest and the lowest degree of unbiased genetic diversity and Shannon's information index parameters, too. Pairwise Fst value showed the highest degree of genetic similarity between *P. hydropiper* and *P. mitis* (0.986) and the lowest one between the *P. lapathifolia* ssp. *nodosa* and *P. lapathifolia* ssp. *pallida* (0.819) (Table 5).

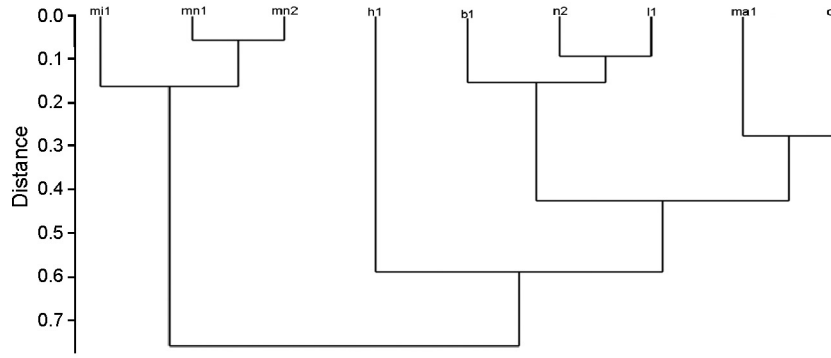

Fig. 4. WARD dendrogram of karyological data

Table 4
Genetic diversity parameters in *Persicaria* species

Taxa no.	Species	N	Na	Ne	I	He	UHe	PPB
1	*P. lapathifolia* ssp. *lapathifolia*	12	10.063	1.298	0.268	0.177	0.185	53.13
2	*P. lapathifolia* ssp. *nodosa*	6	0.75	1.212	0.175	0.119	0.129	32.81
3	*P. lapathifolia* ssp. *brittingerii*	8	1.172	1.325	0.287	0.19	0.203	57.81
4	*P. lapathifolia* ssp. *pallida*	2	0.156	1.011	0.009	0.006	0.009	1.56
5	*P. maculosa*	8	0.922	1.147	0.166	0.10	0.106	45.31
6	*P. hydropiper*	6	0.688	1.101	0.122	0.072	0.079	32.81
7	*P. mitis*	12	1	1.206	0.209	0.132	0.138	50
8	*P. minor*	12	1.719	1.309	0.334	0.205	0.214	85.94
9	*P. orientalis*	10	1.031	1.296	0.260	0.174	0.183	84.44

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; PPB = percentage of polymorphic bands

In UPGMA tree based on Nei genetic distance (Fig. 5), subspecies of *P. lapathifolia* were placed close to each other, supporting their position in morphological and karyological studies. Although placed in a separate subcluster, *P. orientalis* showed close affinity with subspecies of *P. lapathifolia* same as morphological dendrogram. Unlike karyological tree, *P. mitis* and *P. hydropiper* were clustered together, while *P. minor* was joined them with some distance. ISSR tree revealed that *P. maculosa* differed genetically from other

Table 5
Pair-wise Fst values among the studied *Persicaria* species (above diagonal = Fst value; below diagonal = P value; taxa numbers based on Table 4)

Statistic	Taxon 1	Taxon 2	Taxon 3	Taxon 4	Taxon 5	Taxon 6	Taxon 7	Taxon 8	Taxon 9
Taxon 1	–	0.938	0.948	0.907	0.971	0.937	0.966	0.927	0.922
Taxon 2	0.064	–	0.896	0.819	0.912	0.863	0.881	0.861	0.862
Taxon 3	0.054	0.110	–	0.929	0.935	0.925	0.952	0.912	0.954
Taxon 4	0.098	0.199	0.074	–	0.893	0.904	0.930	0.856	0.905
Taxon 5	0.029	0.092	0.067	0.113	–	0.965	0.973	0.938	0.919
Taxon 6	0.065	0.147	0.078	0.100	0.036	–	0.986	0.940	0.914
Taxon 7	0.034	0.126	0.049	0.072	0.027	0.014	–	0.946	0.938
Taxon 8	0.076	0.150	0.092	0.156	0.064	0.062	0.055	–	0.887
Taxon 9	0.081	0.148	0.047	0.100	0.085	0.090	0.064	0.120	–

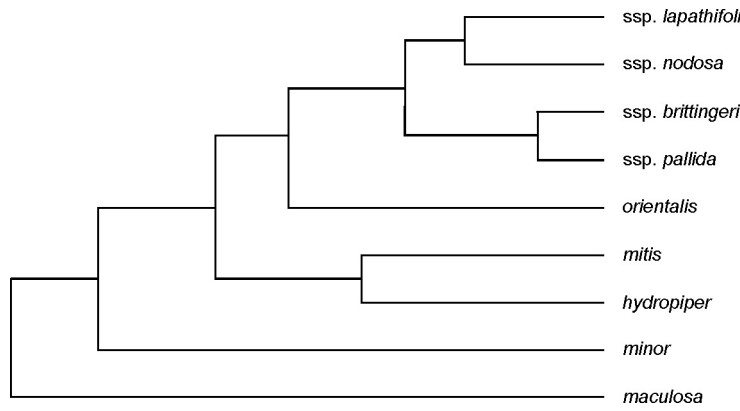

Fig. 5. UPGMA dendrogram of ISSR data

species studied as this species was nested in a discrete subcluster with great distance. Our results showed that species studied are genetically different from each other. Moreover, Nm analysis showed restricted gene flow (Nm = 0.71) among species studied supporting ISSR tree and AMOVA test mentioned before.

STRUCTURE plot based on admixture model (Fig. 6) showed genetic affinity between subspecies of *P. lapathifolia*, which is in concordance with ISSR tree. *P. maculosa* had allele compositions differed from others supporting UPGMA trees of ISSR and morphological data. *P. mitis* and *P. hydropiper* displayed significant genetic similarity as their positions in ISSR tree. Despite distinct allele composition, some degrees of genetic admixture through shared common alleles can be observed between species, for example between *P. orientalis*, *P. lapathifolia* ssp. *brittingerii* and *P. lapathifolia* ssp. *pallida*, and between *P. minor* and *P. mitis*.

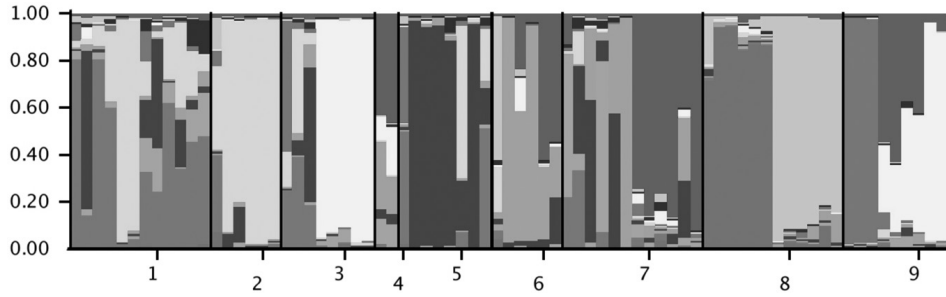

Fig. 6. STRUCTURE plot of ISSR data (taxa numbers based on Table 4)

DISCUSSION

Mitotic chromosome numbers and their relation with genetic diversity parameters

We have previously reported that *P. lapathifolia* subspecies are diploid ($2n = 2x = 22$), and *P. minor* and *P. mitis* are tetraploid ($2n = 4x = 40$) (Keshavarzi and Mosaferi 2013). Despite previous chromosome counts in *P. minor*, *P. mitis* and *P. lapathifolia* subspecies, it should be mentioned here that it was restricted only to chromosome counts and no karyological analyses were done before. In this research, we found that *P. hydropiper* ($2n = 2x = 20$), *P. maculosa* and *P. orientalis* ($2n = 2x = 22$) are diploid supporting previous studies (Al-Bermani *et al.* 1993, Májovský 1978, Probatova 2000). It is the first report of chromosome counts of these taxa for the Flora of Iran.

Genetic diversity parameters showed *P. mitis* with nearly high polymorphism. This species is mostly sympatric with *P. hydropiper*. Despite *P. mitis*, *P. hydropiper* has low genetic polymorphism. As *P. hydropiper* is diploid, its low genetic polymorphism is not unexpected (Soltis *et al.* 2014).

Diploid *P. orientalis* showed nearly high levels of genetic parameters. This species can be found in few habitats in Iran. Maybe this taxon had wider distribution in the past, but as a result of global warming, human activities and climate change, limit distribution and populations decline have been occurred for this taxon in the country. This needs more data to confirm. High genetic diversity in diploids was also reported by Purdy and Bayer (1995) in *Deschampsia*, Ferriol *et al.* (2014) in *Centaurea* and Tabin *et al.* (2016) in *Rheum webbianum*.

With few exceptions, generally our results indicated that tetraploid species possess higher genetic diversity than diploid ones. This can be assumed as a result of higher levels of alleles in polyploids (Meirmans and Van Tienderen 2013).

Infraspecific genetic variation

AMOVA and STRUCTURE analyses displayed genetic differentiation in species studied but showed some degree of common alleles. Nm value also revealed low gene flow between taxa. Based on AMOVA analysis, 72% of genetic variation was due to within taxa, which can be seen in STRUCTURE plot, too. Although different factors can cause infraspecific genetic variation, it seems that two factors; phenotypic plasticity and hybridisation, are the main in *Persicaria*.

Local adaptation to each habitat can cause distinction of species populations. Phenotypic plasticity is the result of environmental changes (Pigliucci 2001, Weinig 2000). This phenomenon not only affects different morphological traits, but may also elicit to differentiated expression of genes. Through plasticity, genotypes can successfully grow in different environment and this can influence patterns of evolutionary diversification at population and species level (Sultan 2003). Phenotypic plasticity has previously been reported in different *Persicaria* species (Griffith and Sultan 2006, Heschel *et al.* 2004, Sheidai *et al.* 2016).

Hybridisation as an evolutionary force may lead to intraspecific genetic diversity, speciation and species extinction (López-Caamal and Tovar-Sánchez 2014). Although self-fertilisation and cleistogamy are commonly found in *Persicaria*, hybridisation is recorded between some species (Kim *et al.* 2008, Simmonds 1945, Stanford 1925). Interspecific hybridisation plays an important role in diversification of *Persicaria* species (Kim and Donoghue 2008, Kim *et al.* 2008).

CONCLUSIONS

Previous studies on annual *Persicaria* taxa using micromorphological and seed protein SDS-PAGE data showed that these features are of taxonomic importance in species delimitation (Mosaferi and Keshavarzi 2011, Mosaferi *et al.* 2011). Results of present study showed that morphological, karyological and molecular data can delimit the studied species. In conclusion, this is the first study of genetic diversity using molecular markers in *Persicaria* species distributed in Iran. With few exceptions, a high level of genetic diversity was recorded for all studies species of *Persicaria*, which indicates the possible cross-breeding events between different species of *Persicaria*.

*

Acknowledgement – The authors thank the curator of Kharazmi University Herbarium [T] for permission to extract DNA from selected specimens.

REFERENCES

Abubakar, M. A., Zulkifli, R. M., Hassan, W. N. A. W., Shariff, A. H. M., Malek, N. A. N. N., Zakaria, Z. and Ahmad, F. (2015): Antibacterial properties of Persicaria minor (Huds.) ethanolic and aqueous-ethanolic leaf extracts. – *J. A. P. S.* **5**: 50–56. https://doi.org/10.7324/JAPS.2015.58.S8

Al-Bermani, A. K. K. A., Al-Shammary, K. I. A., Gornall, R. J. and Bailey, J. P. (1993): Contribution to a cytological catalogue of the British and Irish flora, 3. – *Watsonia* **19**: 169–171.

Duminil, J. and Di Michele, M. (2009): Plant species delimitation: a comparison of morphological and molecular markers. – *Plant Biosyst.* **143**(3): 528–542. https://doi.org/10.1080/11263500902722964

Falush, D., Stephens, M. and Pritchard, J. K. (2007): Inference of population structure using multilocus genotype data: dominant markers and null alleles. – *Mol. Ecol. Notes* **7**(4): 574–578. https://doi.org/10.1111/j.1471-8286.2007.01758.x

Ferriol, M., Merle, H. and Garmendia, A. (2014): Microsatellite evidence for low genetic diversity and reproductive isolation in tetraploid Centaurea seridis (Asteraceae) coexisting with diploid Centaurea aspera and triploid hybrids in contact zones. – *Bot. J. Linn. Soc.* **176**: 82–98. https://doi.org/10.1111/boj.12194

Freeland, J. R., Kirk, H. and Peterson, S. D. (2011): *Molecular ecology*. 2nd ed. – Wiley-Blackwell, Chichester, 449 pp.

Griffith, T. M. and Sultan, S. E. (2006): Plastic and constant developmental traits contribute to adaptive differences in co-occurring Polygonum species. – *Oikos* **114**: 5–14. https://doi.org/10.1111/j.2006.0030-1299.14472.x

Hammer, Ø., Harper, D. A. T. and Ryan, P. D. (2012): PAST: paleontological statistics software package for education and data analysis. – *Palaeontol. Electron*. **4**: 9.

Heschel, M. S., Sultan, S. E., Glover, S. and Sloan, D. (2004): Population differentiation and plastic responses to drought stress in the generalist annual Polygonum persicaria. – *Int. J. Plant Sci* **165**: 817–824. https://doi.org/10.1086/421477

Hinds, H. R. and Freeman, C. C. (2005): *Persicaria*. – In: Editorial Committee (eds): Flora of North America, No. 5. Oxford University Press, New York and Oxford, pp. 574–594.

Huson, D. H. and Bryant, D. (2006): Application of phylogenetic networks in evolutionary studies. – *Mol. Biol. Evol.* **23**(2): 254–267. https://doi.org/10.1093/molbev/msj030

Huziwara, Y. (1962): Karyotype analysis in some genera of Compositae. VIII. Further studies on the chromosomes of Aster. – *Am. J. Bot.* **49**: 116–119. https://doi.org/10.2307/2439026

Keshavarzi, M. and Mosaferi, S. (2013): Chromosome numbers of some Persicaria (Polygonaceae) species from Iran. – *Iranian J. Bot.* **19**: 75–77.

Khatoon, S. and Ali, S. I. (1993): *Chromosome atlas of the angiosperms of Pakistan*. – Department of Botany, University of Karachi, Karachi, 232 pp.

Kim, S. T. and Donoghue, M. J. (2008): Molecular phylogeny of Persicaria (Persicarieae, Polygonaceae). – *Syst. Bot.* **33**: 77–86. https://doi.org/10.1600/036344808783887302

Kim, S. T., Sultan, S. E. and Donoghue, M. J. (2008): Allopolyploid speciation in Persicaria (Polygonaceae): insights from a low-copy nuclear region. – *PNAS* **105**: 12370–12375. https://doi.org/10.1073/pnas.0805141105

Kim, Y., Jang, D. S., Parh, S. H., Yun, J., Min, B. K., Min, K. R. and Lee, H. K. (2000): Flavonol glycoside gallate and ferulate esters from Persicaria lapathifolia as inhibitors of superoxide production in human monocytes stimulated by unopsonized zymosan. – *Planta Med.* **1**: 72–74. https://doi.org/10.1055/s-0029-1243112

Kim, Y. H., Tae, K. H. and Kim, J. H. (2008): Genetic variations and relationships of Persicaria thunbergii (Sieb. & Zucc.) H. Gross ex Nakai (Polygonaceae) by the RAPD analyses. – *Korean J. Plant Resour.* **21**: 66–72.

Križman, M., Jakse, J., Baricevic, D., Javornik, B. and Prosek, M. (2006): Robust CTAB-activated charcoal protocol for plant DNA extraction. – *Acta Agric. Slov.* **87**: 427– 433.

Levan, A. K., Fredga, K. and Sandberg, A. A. (1964): Nomenclature for centromic position on chromosomes. – *Hereditas* **52**: 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

López-Caamal, A. and Tovar-Sánchez, E. (2014): Genetic, morphological, and chemical patterns of plant hybridization. – *Rev. Chil. Hist. Nat.* **87**: 1–14. https://doi.org/10.1186/s40693-014-0016-0

Májovský, J. (1978): Index of chromosome numbers of Slovakian flora (Part 6). – *Acta Fac. Rerum Bot.* **26**: 1–42.

Matesanz, S., Theiss, K. E., Holsinger, K. E. and Sultan, S. E. (2014): Genetic diversity and population structure in Polygonum cespitosum: insights to an ongoing plant invasion. – *PLoS One* **9**: e93217. https://doi.org/10.1371/journal.pone.0093217

Meirmans, P. G. and Van Tienderen, P. H. (2004): GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. – *Mol. Ecol. Notes* **4**(4): 792–794. https://doi.org/10.1111/j.1471-8286.2004.00770.x

Meirmans, P. G. and Van Tienderen, P. H. (2013): Statistical challenges for population genetics of polyploids: the effects of inheritance in tetraploids on genetic diversity and population divergence. – *Heredity* **110**: 131–137. https://doi.org/10.1038/hdy.2012.80

Mosaferi, S. and Keshavarzi, M. (2010): Micromorphology and first record of Persicaria hydropiperoides (Polygonaceae) in Iran. – *TBJ* **1**: 63–72.

Mosaferi, S. and Keshavarzi, M. (2011): Micromorphological study of Polygonaceae tribes in Iran. – *Phytol. Balcan.* **17**: 89–100.

Mosaferi, S., Keshavarzi, M. and Ghadam, P. (2011): Biosystematic study of annual species of Persicaria from Iran using SDS-PAGE. – *Phytol. Balcan.* **17**: 185–190.

Moyeenul Huq, A. K. M., Jamal, J. A. and Stanslas, J. (2014): Ethnobotanical, phytochemical, pharmacological, and toxicological aspects of Persicaria hydropiper (L.) Delarbre. – *Evid.-based Compl. Altern. Med.* **2014**: 1–14. https://doi.org/10.1155/2014/782830

Mozaffarian, V. (2012): A revision of Polygonum L. sensu lato, (Polygonaceae) in Iran. – *Iranian J. Bot.* **18**: 159–174.

Parnell, A. N. and Simpson, D. A. (1988): Hybridization between Polygonum mite Schrank, P. minus Huds. and P. hydropiper L. in Northern Ireland with comments on their distinction. – *Watsonia* **17**: 265–272.

Paszko, B. (2006): A critical review and a new proposal of karyotype asymmetry indices. – *Plant. Syst. Evol* **258**: 39–48. https://doi.org/10.1007/s00606-005-0389-2

Peakall, R. and Smouse, P. E. (2006): GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. – *Mol. Ecol. Notes* **6**(1): 288–295. https://doi.org/10.1111/j.1471-8286.2005.01155.x

Peruzzi, L. and Eroğlu, H. (2013): Karyotype asymmetry: again, how to measure and what to measure? – *Comp. Cytogen.* **7**: 1–9. https://doi.org/10.3897/compcytogen.v7i1.4431

Pigliucci, M. (2001): *Phenotypic plasticity: beyond nature and nurture*. – John Hopkins University Press, Baltimore, 344 pp.

Podani, J. (2000): *Introduction to the exploration of multivariate data.* – Backhuys Publishers, Leiden, Netherlands, 470 pp.

Pritchard, J. K., Stephens, M. and Donnelly, P. (2000): Inference of population structure using multilocus genotype data. – *Genetics* **155**(2): 945–959.

Probatova, N. S. (2000): Chromosome numbers in some plant species from the Razdolnaya (Suifun) river basin (Primorsky Territory). – *Bot. Zhurn.* **85**: 102–107.

Purdy, B. G. and Bayer, R. J. (1995): Genetic diversity in the tetraploid sand dune endemic Deschampsia mackenzieana and its widespread diploid progenitor D. cespitosa (Poaceae). – *Am. J. Bot.* **82**: 121–130. https://doi.org/10.2307/2445794

Qader, S. W., Abdulla, M. A., Lee, S. C. and Hamdan, S. (2012): Potential bioactive property of Polygonum minus Huds (kesum) review. – *SRE* **7**: 90–93.

Rechinger, K. H. and Schiman-Czeika, H. (1968): *Polygonaceae*. – In: Rechinger, K. H. (ed.): Flora Iranica. No. 56. Akademische Druck- u. Verlagsanstalt, Graz, pp. 46–83.
Romero Zarco, C. (1986): A new method for estimating karyotype asymmetry. – *Taxon* **35**: 526–530. https://doi.org/10.2307/1221906
Ronse Decraene, L. P. and Akeroyd, J. R. (1988): Generic limits in Polygonum and related genera (Polygonaceae) on the basis of floral characters. – *Bot. J. Linn. Soc.* **98**: 321–371. https://doi.org/10.1111/j.1095-8339.1988.tb01706.x
Sheidai, M. and Jalilian, N. (2008): Karyotypic studies in some species and populations of the genus Lotus L. in Iran. – *Acta Bot. Croat.* **67**: 45–52.
Sheidai, M., Mosaferi, S., Keshavarzi, M., Noormohammadi, Z. and Ghasemzadeh-Baraki, S. (2016): Genetic diversity in different populations of Persicaria minor (Polygonaceae), a medicinal plant. – *The Nucleus* **59**: 115–121. https://doi.org/10.1007/s13237-016-0169-0
Simmonds, N. W. (1945): Polygonum persicaria L. – *J. Ecol* **33**: 121–131. https://doi.org/10.2307/2256568
Smolarz, H. D. and Potrzebowski, M. J. (2002): Persilben, a new carboxystilbene from Polygonum persicaria. – *J. Mol. Struct.* **605**: 151–156. https://doi.org/10.1016/s0022-2860(01)00758-x
Soltis, D. E., Visger, C. J. and Soltis, P. S. (2014): The polyploidy revolution then…and now: Stebbins revisited. – *Am. J. Bot.* **101**: 1057–1078. https://doi.org/10.3732/ajb.1400178
Stanford, E. E. (1925): The inflorescence and flower-form in Polygonum, subgenus Persicaria. – *Rhodora* **27**: 41–47.
Stebbins, G. L. (1971): *Chromosomal evolution in higher plants*. – Edward Arnold Press, London, 216 pp.
Sultan, S. E. (2003): Phenotypic plasticity in plants: a case study in ecological development. – *Evol. Develop.* **5**: 25–33. https://doi.org/10.1046/j.1525-142x.2003.03005.x
Sultan, S. E. and Bazzaz, F. A. (1993): Phenotypic plasticity in Polygonum persicaria. III. The evolution of ecological breadth for nutrient environment. – *Evolution* **47**: 1050–1071. https://doi.org/10.2307/2409974
Tabin, S. H., Kamili, A. N., Ganie, Sh. A., Zargar, O., Sharma, V. and Gupta, R. Ch. (2016): Genetic diversity and population structure of Rheum species in Kashmir Himalaya based on ISSR markers. – *Flora* **223**: 121–128. https://doi.org/10.1016/j.flora.2016.05.001
The Plant List (2013): Version 1.1. Published on the internet. – http://www.theplantlist.org/ (accessed 25th January 2017)
Weinig, C. (2000): Plasticity versus canalization: population differences in the timing of shade-avoidance responses. – *Evolution* **54**: 441–451. https://doi.org/10.1111/j.0014-3820.2000.tb00047.x
Weising, K., Nybom, H., Wolff, K. and Kahl, G. (2005): *DNA fingerprinting in plants. Principles, methods, and applications*. 2nd ed. – CRC Press, Boca Raton, 472 pp. https://doi.org/10.1201/9781420040043
Yasmin, G. H., Pearce, S. R., Khan, M. A., Kim, S. T., Shaheen, N. and Hayat, M. Q. (2010): Taxonomic implication of AFLP fingerprinting in selected Polygonaceous species. – *PJB* **42**: 739–750.