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Genetic structure of *Zannichellia* L. populations according to mountain barriers in Iran as revealed by ISSR and SRAP markers

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Abstract: *Zannichellia* L. (Potamogetonaceae) is a widespread aquatic genus that is distributed in temperate and subtropical aquatic ecosystems worldwide. Because the biogeographical location of Iran includes mountain ranges and deserts that may cause different habitat types, it was assumed that these *Zannichellia* populations would be stronger differentiated than observed in *Zannichellia* from temperate regions. The present study aimed to test whether *Z. palustris* would demonstrate high levels of genetic differentiation over long distances and across mountain ranges. The genetic structure of the populations of *Zannichellia* was investigated using inter simple sequence repeats (ISSR) and sequence-related amplified polymorphism (SRAP) as molecular markers. The results demonstrated overall high levels of genetic differentiation among populations (GST = 0.53) and isolation by distance. Bayesian assignment analysis at the population level grouped individuals into eight genetic clusters (K = 8) that largely correspond to the topographic barriers. Alongside the same mountain ridge, the freshwater and saltwater habitat differences corresponded to *Z. palustris* var. *palustris* and *Z. palustris* var. *pedicellata,* respectively. Our results support the hypothesis of isolation by distance. This high level of genetic differentiation argues for a conservation strategy to maintain a diversity of populations of *Zannichellia* in mountainous regions.

Key words: Aquatic plant, genetic structure, geographical barriers, Potamogetonaceae

1. Introduction

Many aquatic plants have mixed the modes of reproduction and different models of dispersal. Sexual reproduction in submerged aquatic plants produces new genotypes (Barrett et al., 1993). Vegetative reproduction can expectedly produce many clones with lower genetic diversity compared to generative reproduction. Despite the low clonal diversity between and within plant populations (Barrett et al., 1993), several studies demonstrated that aquatic plant populations similar to nonclonal plant species (Hamrick and Godt, 1989) often have high genetic diversity (Mader et al., 1998; Lyu and Wang, 2016). According to the stepping stone model, increasing the geographical distance between populations expectedly leads to enriched genetic isolation (Hollingsworth et al., 1996; Hou and Lou, 2011; Wu et al., 2016). Furthermore, biogeographic barriers such as mountains and deserts are effective factors for the genetic differentiation of aquatic plant populations. At the largest geographic distance, freshwater organisms are isolated by their high mountain ranges and vast deserts (Moyle and Cech, 2004).

The biogeography of Iran is quite diverse due to several large mountain ranges and deserts that show different habitat types at a low connectivity rate. The Alborz Mountain range is located in the north while the Zagros Mountain range is expanding from the northwest to southeast of Iran and the Karkas Mountain range is nearly located in the central part of Iran. These geographic features are supposed to create a separation between north and south populations and thus can be considered as a strong barrier to dispersal. In some studies, these barriers were determined as a dominant factor for identifying the structure of regional organisms (e.g., Ricklefs and Schluter, 1993; Abbasi et al., 2016). Moreover, it is proven that along the same side of mountain ranges, these geographic barriers develop the homogenization of aquatic biota (Rahel, 2007). Many topographical features in Iran affect the creation of diverse habitats such as Dasht-e Kavir (a desert region in the center of Iran), Lake Urmia (the world's second largest saline lake located in the north-west

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region of Iran), and many different aquatic ecosystems recorded in the Ramsar Convention[1]. Different treats such as human interferences disturb the ecological functions of aquatic habitats (Daryadel and Talaei, 2014).

In recent decades, drought as a climatic event has been considered an important challenge that occurs due to global warming (Gilbert et al., 2012). It further has been a problematic phenomenon for aquatic habitats in Mediterranean temporary pools (Zacharias et al., 2007) and low rainfall regions such as Central and Southwest Asia (Miyan, 2015). Therefore, identifying the gene pool and genetic diversity of the aquatic plants of these regions is of great importance. According to some studies, gene flow and genetic diversity in aquatic plants are influenced by several important factors such as reproduction mode, migratory birds, habitat discontinuation, geographical distance, and geographical barriers (Barrett et al., 1993; Hollingsworth et al., 1996; Hou and Lou, 2011; Wu et al., 2016).

Zannichellia L., which is now regarded as a member of an enlarged family Potamogetonaceae (The Angiosperm Phylogeny Group IV, 2016), is a cosmopolitan genus that is widely distributed in the aquatic ecosystems of the Northern Hemisphere, including fresh waters, brackish waters, and marine intertidal habitats.

Z. palustris is divided into *Z. pedunculata* Reichenb [synonymous with *Z. palustris* subsb. *pedicellata* (Rosen and Wahlenb.) Hook.f.] and *Z. palustris* L. sensu stricto, based on physiological traits (Van Vierssen, 1982), isozymes (Triest and Vanhecke 1991), and chloroplast sequences (Triest et al., 2007). Furthermore, previously morphological and other molecular (using ITS and cpDNA markers) findings indicated a differentiation between Iranian *Z. palustris* var. *pedicellata* and *Z. palustris* var. *palustris* populations using ITS and chloroplast DNA (cpDNA) markers (Abbasi et al., 2019).

Different molecular markers are more used for studying the genetic diversity of plants. Intersimple sequence repeats (ISSR) are microsatellite-based markers that depend on the generality and high diversity of eukaryotic genomes. Recently, different studies have widely applied these markers in the aquatic population (e.g., Chen et al., 2017; Hu et al., 2016; Butkuvienė et al., 2017) because they are more changeable and require less time and money compared to other molecular markers (Harris, 1999). The detection of the genetic variation in endangered species in nature is considered another application of ISSRs (Smith and Bateman, 2002; Kwiecińska-Poppe et al., 2020; Bagheri et al., 2020). Conversely, the sequence-related amplified polymorphism (SRAP) aims to amplify the coding regions of DNA with primers targeting the ubiquitous motifs of open reading frames (Li and Quiros, 2001). These markers are proven to be robust and highly variable (Robarts and Wolfe, 2014). Although ISSR loci are distributed throughout the entire genome, SRAP amplifies functional regions. Thus, the present study used them to evaluate the genetic diversity of *Zannichellia*. Various studies have focused on the genetic diversity of aquatic plants with these markers such as *Stuckenia pectinata* populations (Abbasi et al., 2016), *Blysmus sinocompressus* (Hu et al., 2016), *Nympha tetrafgona* populations (Chen et al., 2017), *Batrachium* (Butkuvienė et al., 2017), and *Ottelia acuminata* var. *jingxiensis* (Li et al., 2019). However, there is recently no comprehensive study on the genetic diversity of *Zannichellia* in the world. Using isozymes, Triest and Vanhecke (1991) indicated low intrapopulation variability in *Zannichellia* due to the predominance of clonal growth in Belgium. In addition, Triest et al. (2010) studiedthe genetic differentiation of the submerged plant populations of *Ceratophyllum* spp., *Callitriche* spp., *Zannichellia palustris*, *Zannichellia pedunculata*, and *Stuckenia pectinatus* L. in Belgium using ISSR markers. They revealed that most genetic diversity was between two taxa of *Zannichellia* that occurred in either brackish water or freshwater habitats.

Considering geographically distant sites with *Z. palustris* populations over a vast range of diverse habitats located at different latitudes, the present study aimed to test whether *Z. palustris* would demonstrate high levels of genetic differentiation over long distances and across mountain ranges.

The findings will be discussed in a context of isolation by distance, geographical mountain barriers, rivers, and their habitat. This will allow understanding the importance of topographic features in the genetic differentiation of *Zannichellia*.

2. Materials and methods

2.1. Sampling and DNA extraction

In total, 240 samples from 24 populations of *Z. palustris* (*Z. palustris* var. *pedicellata* and *Z. palustris* var. *palustris*) were collected from the wetlands, lakes, and rivers of Iran during spring and summer 2019 (Table 1). The geographic positions of populations in Iran are indicated in Table 2. It should be noted that 10 individual shoots were collected at 2–3 m intervals in each population because the populations of this species are still decreasing due to human activities and the recent drought in Iran. The design attempted to cover the environmental range of habitats and the geographical distribution of species in Iran as well as possible.

The geographic distance between the pairs of populations ranged from 20 to 1200 km. Further, the sampling corresponding to the distribution of *Zannichellia* occurs in the northern, central, western, and southern parts of Iran.

[1] Ramsar (2014). The List of Wetlands of International Importance [online]. Website https://www.ramsar.wetlands.org [accessed 21 March 2020].

Table 1. Location details and features of *Z. palustris* populations in Iran (Population codes of 3, 4, 10, 11, 14, 15, 17 and20 are *Z. palustris* var. *pedicellata* and other codes are *Z. palustris* var. *palustris*).

Pop. code	Voucher no.	GenBank code* ITS, PHYB, trnH-psbA, rpl32-trnL	Locality	Habitat	Long (E)	Lat (N)	Type of water
1	22666	LC479793/LC479817/LC479841/LC479865	17 km to Delijan, Neizar	River	50.554	34.307	Freshwater
2	22669	LC479794/LC479818/LC479842/LC479866	Kordistan, Ghorveh	River	47.804	35.168	Freshwater
3	22668	LC479795/LC479819/LC479843/LC479867	West Azerbayjan, Miandoab	Wetland	46.105	36.961	Freshwater
4	22667	LC479796/LC479820/LC479844/LC479868	Borujen, Gandoman	Wetland	51.156	31.864	Freshwater
5	20212	LC479797/LC479821/LC479845/LC479869	Chahar mahal and Bakhtiari, Shalamzar	River	50.817	32.045	Freshwater
6	15498	LC479798/LC479822/LC479846/LC479870	Isfahan, Lenjan, Chamaseman	Channel	51.225	32.372	Freshwater
7	12799	LC479799/LC479823/LC479847/LC479871	Isfahan, Falavarjan	River	51.513	32.552	Freshwater
8	8155	LC479800/LC479824/LC479848/LC479872	Gilan, Astaneh	River	49.533	37.113	Freshwater
9	8241	LC479801/LC479825/LC479849/LC479873	Bakhtiari, Dehno village	River	50.718	31.955	Freshwater
10	8661	LC479802/LC479826/LC479850/LC479874	Yasouj, 15 km to Yasouj	River	51.601	30.657	Brackish water
11	8355	LC479803/LC479827/LC479851/LC479875	Khuzestan, Karun	River	48.932	31.456	Brackish water
12	8326	LC479804/LC479828/LC479852/LC479876	Hamedan, Shirinsoo	Wetland	48.452	35.492	Freshwater
13	8687	LC479805/LC479829/LC479853/LC479877	Fars, Haftbarm	Wetland	52.043	29.828	Freshwater
14	8433	LC479806/LC479830/LC479854/LC479878	Khuzestan, Shoshmazrae	Channel	48.255	32.195	Brackish water
15	8697	LC479807/LC479831/LC479855/LC479879	Fars, Dashte Arjan	River	51.983	29.661	Brackish water
16	8603	LC479808/LC479832/LC479856/LC479880	Khuzestan, Dezful	River	48.410	32.382	Freshwater
17	8549	LC479809/LC479833/LC479857/LC479881	Khuzestan, Andika	Wetland	49.445	32.206	Brackish water
18	8070	LC479810/LC479834/LC479858/LC479882	Khuzestan, Hamidieh	Channel	48.420	31.476	Freshwater
19	8054	LC479811/LC479835/LC479859/LC479883	Khuzestan, Bostan	River	47.991	31.719	Freshwater
20	8840	LC479812/LC479836/LC479860/LC479884	Ahwaz to Shush, Alhaei	River	48.824	31.673	Brackish water
21	8304	LC479813/LC479837/LC479861/LC479885	Kordistan, Marivan	Dam	46.177	35.520	Freshwater
22	8693	LC479814/LC479838/LC479862/LC479886	Fars, between khanezenian and chehelcheshmeh	Wetland	52.054	29.696	Freshwater
23	8321	LC479815/LC479839/LC479863/LC479887	East Azerbaijan, Sarab to Ardebil	River	47.528	37.947	Freshwater
24	22670	LC479816/LC479840/LC479864/LC479888	Khorasan, SW of Mashhad, Binalood, Kordineh	Wetland	59.253	36.268	Freshwater

* GenBank sequences from multiple gene regions as an identifier of the variety in each population (Abbasi et al., 2019).

Voucher specimens were deposited at the Herbarium of the University of Isfahan (HUI) and Herbarium of the Research Center of the Agriculture and Natural Resources of Khuzestan. The leaves of *Zannichellia* were dried on silica gel and then genomic DNA was extracted from the leaf tissue using the modified cetyltrimethylammonium bromide (CTAB) method (Abbasi and Afsharzadeh, 2016).

2.2. Polymerase chain reaction (PCR) amplification and sequencing

The PCR amplification for 13 ISSR primers 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, and the final extension step of 10 min at 72 ˚C. The PCRs for 15 combined SRAP primers (Table 4) were performed in 25 µL reaction volumes containing Taq 2× Master Mix Red (Ampliqon), 0.1 µM of each forward and reverse primer, 50 ng DNA template, and nuclease-free water to 20 µL. Furthermore, the PCR program was conducted in an Eppendorf Thermal Cycler (Mastercycler Gradient) with the cycle profile of 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. It was then followed by 35 cycles of 1 min denaturing, and 1 min annealing at 50 ˚C ending with an elongation step of 5 min at 72 ˚C. Following PCR, samples were loaded onto a 1.5% agarose gel in the TBE1X buffer and stained with ethidium bromide. Additionally, a 100 bp ladder (Thermo), along with negative and positive controls was loaded and run at a constant voltage (100 V) for 2 h. After running, the gels were UV visualized and recorded using the gel documentation system (GeneFlash). Finally, each DNA extraction, PCR amplification, and gel running was repeated twice to verify the repeatability of the obtained data.

2.3. Molecular analysis

To perform multilocus analysis, fifty combinations of ISSR and SRAP primers were tested and repeated, from which primers amplifying detectable and polymorphic

Table 2. Populations of Iranian *Zannichellia* according to their geographic locations.

Population code	Geographic location
- 1, 2, 12, 21 (*Z. palustris* var. *palustris*).	West
- 9, 13, 16, 18, 19, 22 (*Z. palustris* var. *palustris*). - 4, 10, 11, 14, 15, 17, 20 (*Z. palustris* var. *pedicellata*).	South
- 5, 6, 7 (*Z. palustris* var. *palustris*).	Center
- 3 (*Z. palustris* var. *pedicellata*), 8, 23, 24 (*Z. palustris* var. *palustris*).	North

Southern populations are divided into two varieties.

Table 3. Sequences of ISSR primers (Blair et al., 1999).

Primer code	Sequence (5'→ 3')	BR (bp)	PB	MB	PPB	PIC
UBC 872	GATAGATAGATAGATA	350–1500	10	0	100	0.30
ISSR 823	TCTCTCTCTCTCTCTCC	300–1100	13	0	100	0.25
ISSR 811	GAGAGAGAGAGAGAGAC	250–1000	8	0	100	0.20
ISSR 812	GAGAGAGAGAGAGAGAA	150–800	9	0	100	0.25
UBC 873	GACAGACAGACAGACA	200–700	7	0	100	0.33
ISSR 2	AGAGAGAGAGAGAGAGG	200–2000	16	0	100	0.15
ISSR 4	CTCTCTCTCTCTCTCTGG	150–700	11	0	100	0.50
ISSR 810	GAGAGAGAGAGAGAGAT	200–1500	12	0	100	0.46
ISSR 3	AGCAGCAGCAGCAGCAGCG	100–1000	15	0	100	0.25
ISSR 1	CAGCAGCAGCAGCAG	150–3000	15	0	100	0.20
ISSR 808	AGAGAGAGAGAGAGAGC	140–1500	11	0	100	0.20
UBC 818	CACACACACACACACAG	250–1500	13	1	92	0.50
ISSR 880	GGAGAGGAGAGGAGA	300–1000	6	1	85	0.35
Total			146	2		
Average			11.23	0.15	98.23	0.30

BR: Band range, PB: No. of polymorphic bands, MB: No. of monomorphic bands, PPB: Percentage of polymorphic bands, PIC: Polymorphic information content.

Table 4. Sequences of used SRAP primers (Li et al., 2015).

Primer ID	Sequence (5'→ 3')	BR (bp)	PB	MB	PPB	PIC
Me 1 Em 3	TGAGTCCAAACCGGATA GACTGCGTACGAATTGAC	100–1200	12	2	85.71	0.25
Me 2 Em4	TGAGTCCAAACCGGATA GACTGCGTACGAATTTGA	150–1200	12	1	92.30	0.35
Me 4 Em4	TGAGTCCAAACCGGACC GACTGCGTACGAATTTGA	150–1200	13	0	100	0.50
Me 1 Em4	TGAGTCCAAACCGGATA GACTGCGTACGAATTTGA	100–1000	11	0	100	0.20
Me 2 Em 3	TGAGTCCAAACCGGATA GACTGCGTACGAATTGAC	100–1200	13	0	100	0.25
Me 4 Em 3	TGAGTCCAAACCGGACC GACTGCGTACGAATTGAC	100–1000	11	1	91.66	0.30
Me 6 Em 2	TGAGTCCAAACCGGACA GACTGCGTACGAATTGC	150–1000	14	0	100	0.35
Me3 Em 3	TGAGTCCAAACCGGAAT GACTGCGTACGAATTGAC	100–1200	16	0	100	0.25
Me1 Em 2	TGAGTCCAAACCGGATA GACTGCGTACGAATTGC	100–1000	10	1	90.9	0.44
Me 6 Em 4	TGAGTCCAAACCGGACA GACTGCGTACGAATTTGA	100–150	12	0	100	0.25
Me 2 Em 2	TGAGTCCAAACCGGATA GACTGCGTACGAATTGC	100–800	12	0	100	0.15
Me 4 Em 2	TGAGTCCAAACCGGACC GACTGCGTACGAATTGC	100–1000	14	0	100	0.15
Me 6 Em 3	TGAGTCCAAACCGGACA GACTGCGTACGAATTGAC	100–1200	12	0	100	0.35
Me 4 Em 17	TGAGTCCAAACCGGACC GACTGCGTACGAATTCCA	100–1200	13	0	100	0.27
Me5 Em 17	TGAGTCCAAACCGGAA GACTGCGTACGAATTCCA	100–1200	13	0	100	0.15
Total			188	5		
Average			12.53	0.33	97.37	0.28

DNA fragments were repeated and selected for further analysis from genomic DNA of *Zannichellia*. The electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and thus were not scored. Additionally, the DNA fragment profile was scored as 0 and 1 indicating the absence and presence of the band, respectively, and then genetic similarity was calculated based on Jaccard (1908) similarity coefficients. Then, data were analyzed using NTSYSpc (version 2.1; Rohlf, 1998) based on the neighbour-joining and similarity coefficient of Jaccard method. The correlation between genetic distances and geographic distances (r) was measured using Mantel test statistics (Mantel, 1967) with 999

permutations implemented in GenAlEx software (version 6.5; Peakall and Smouse, 2006). To assess genetic diversity, basic parameters including the Nei's gene diversity index (h), Shannon index (I), the percentage of polymorphic loci (PPL), polymorphic information content (PIC), and genetic differentiation coefficient (GST) were calculated from the data using POPGENE software (version 1.32; Yeh et al., 1999). Then, the analysis of molecular variance (AMOVA) was performed to calculate the proportion of intrapopulation and interpopulation genetic diversity using GenAlEx software, version 6.5. Further, a principal coordinate analysis (PCoA) was performed using GenAlEx 6.5. To infer the genetic population structure, the Bayesian clustering method was conducted using STRUCTURE software (version 2.3; Pritchard et al., 2000) at the population level. Furthermore, this method utilizes a Markov chain Monte Carlo (MCMC) algorithm to cluster individuals into populations relying on multi-loci genotype data (Falush et al., 2003). 10 independent runs of K = 1–27 were conducted to ensure consistent findings. The highest value for K was calculated with STRUCTURE HARVESTER (Earl and VonHoldt, 2012) by predicting from the plots of the ad hoc posterior probability models of K. The K statistic was more appropriate than the highest LnPr (X/K) method for inferring the population number (Evanno et al., 2005).

3. Result

The present study examined the genetic diversity and genetic structure of *Z. palustris* in Iran based on 13 ISSR and 15 SRAP primer combinations, respectively (Tables 3 and 4).

In total, the production of combined primers (ISSR and SRAP primers) was 341 multiple DNA fragments from the genomic DNA of all 24 populations of *Z. palustris* of which 97.77% were polymorphic. The number of bands per primer ranged from seven to 16 per primer combination.

The overall percentage of polymorphism at the population level and the mean observed heterozygosity were 54% and 0.309, respectively (Table 5).

In the Mantel tests for the correlation of genetic with geographic distances, the ISSR data revealed a high correlation (r = 0.56, p = 0.001) while SRAP data demonstrated a low correlation between genetic and geographic distance (r = 0.110, p = 0.001). The corresponding value was r = 0.50 and p = 0.001 for the combined dataset.

Genetic differentiation coefficient (GST) among populations was 0.53.

The findings of the analysis of molecular variance (AMOVA) demonstrated that 69% and 31% of genetic variation was between and within population, respectively (Table 6).

Table 5. Genetic diversity of the populations of *Zannichellia* in Iran.

Population code	P	Ae	Ho	He	h
1	50	1.4	0.130	0.136	0.135
2	61	1.2	0.218	0.135	0.110
3	65	1.2	0.660	0.117	0.111
4	71	1.1	0.520	0.120	0.110
5	71	1.1	0.510	0.125	0.110
6	71	1.1	0.620	0.220	0.130
7	71	1.1	0.650	0.224	0.110
8	63	1.1	0.330	0.117	0.135
9	41	1.1	0.230	0.123	0.133
10	66	1.2	0.218	0.134	0.132
11	41	1.1	0.193	0.135	0.125
12	50	1.1	0.228	0.117	0.120
13	43	1.1	0.230	0.134	0.130
14	41	1.2	0.218	0.137	0.130
15	46	1.2	0.193	0.134	0.110
16	50	1.1	0.221	0.117	0.150
17	46	1.1	0.230	0.134	0.125
18	36	1.3	0.218	0.137	0.150
19	42	1.2	0.193	0.126	0.130
20	45	1.2	0.221	0.117	0.130
21	50	1.1	0.230	0.134	0.110
22	49	1.1	0.218	0.177	0.110
23	52	1.2	0.193	0.134	0.110
24	67	1.2	0.550	0.125	0.130
Average	54	1.2	0.309	0.137	0.124

P is the percentage of polymorphic loci at the population level; Ae represents the mean effective number of alleles. Moreover, Ho indicates the mean observed heterozygosity (Shannon index) and He denotes the mean expected heterozygosity (unbiased), respectively. Finally, h is Nei's (1973) gene diversity.

According to the cluster analysis of combined markers (ISSR and SRAP) in Figure 1, the populations of *Zannichellia* are divided into three main clusters corresponding to their geographic locations in Table 2. Cluster 1 includes populations from the west (*Z. palustris* var. *palustris*), south (*Z. palustris* var. *pedicellata*), and north of the country (*Z. palustris* var. *palustris*). Moreover, clusters II and III encompass populations from the south (*Z. palustris* var. *palustris*) and center of Iran (*Z. palustris* var. *palustris*), respectively.

Table 6. AMOVA results of *Zannichellia* populations.

Source of variance	d.f.	SSD	MSD	Est. Var.	% Total
Among pops	23	2169.466	94.324	17.284	69%
Within pops	96	758.4	7.9	7.9	31%
Total	119	2927.866		25.184	100%

AMOVA: Analysis of molecular variance; SSD: Sum of squared deviation; MSD: Mean squared deviation.

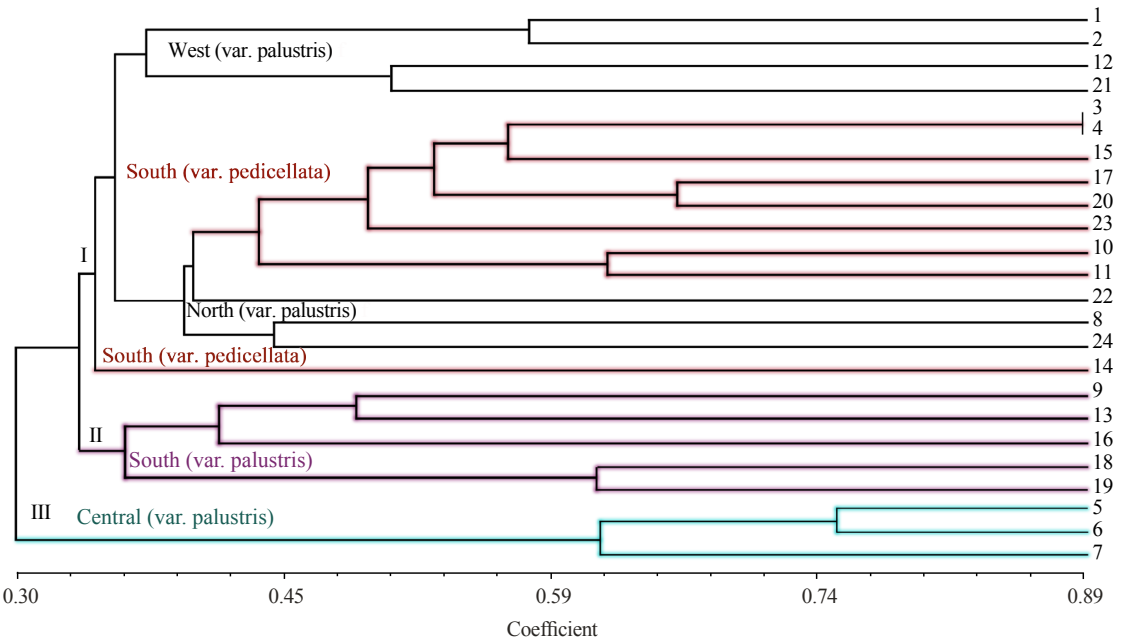

Figure 1. Neighbour-joining dendrogram generated from combined ISSR and SRAP data and the similarity coefficient of Jaccard (1908) showing relationships between the populations of *Zannichellia* in Iran.

According to the PCoA (Figure 2), grouping followed geographic origin and confirmed the grouping of cluster analysis (The percentage of variations explained by the first three axes in PCoA at 24 populations is 26% and 20% for the first and second axes, respectively). In this analysis, similar to cluster analysis, the populations of *Zannichellia* are divided into three main clusters corresponding to their geographic locations in Table 2 and central and southern populations (*Z. palustris*. var. *palustris*) are separated from other populations.

The genetic structure of 24 populations of *Zannichellia* in Iran showed eight genetic clusters (K = 8) and higher levels of genetic differentiation, which are displayed in Figure 3. In this figure, populations are demonstrated corresponding to topographic barriers (Alborz Mountains, Zagros Mountains, and Karkas Mountains) in the map of Iran. Similar to cluster analysis and PCoA, the populations from the central part (5, 6, and 7: yellow dots) are located separately.

4. Discussion

To the best of our knowledge, this study was the first comprehensive study on *Zannichellia* specimens based on intersimple sequence repeats (ISSR) and sequence-related amplified polymorphism (SRAP) markers.

According to Triest et al. (2010), the highest ISSR variation was observed between two *Zannichellia* taxa. In the present study, the highest variation was reported between the populations of two varieties of Iranian *Zannichellia* based on ISSR and SRAP markers.

Triest et al. (2010) observed 38 ISSR loci (27 polymorphic bands) in *Zannichellia* and seven MLGs (Multilocus Genotypes) in three brackish water *Z. pedunculata* and one MLG in freshwater *Z. palustris*. In this research, 146 and 188 polymorphic loci were obtained for ISSR and SRAP in *Zannichellia*, respectively. The smaller number of samples per site allowed obtaining many more alleles, compared to temperate regions, and detecting relevant genetic structures in agreement

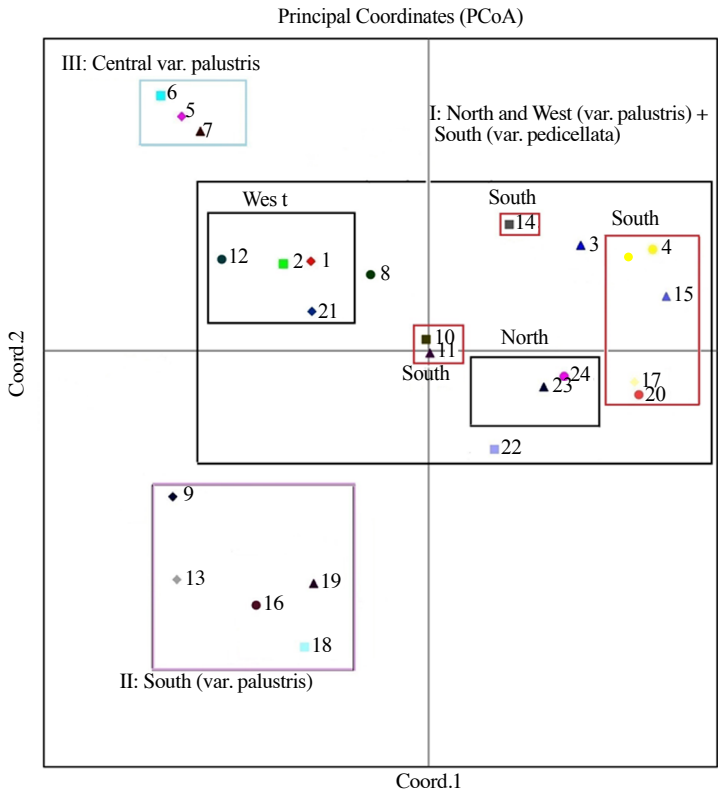

Figure 2. Principal coordinate analysis 2D plot based on combined data (ISSR and SRAP). Cluster I includes populations from the west, south (*Z. palustris* var. *pedicellata*), and north of the country. In addition, cluster II contains populations from south (*Z. palustris* var. *palustris*), and cluster III includes populations from the central part of Iran (*Z. palustris* var. *palustris*).

with biogeography and mountain ranges. However, the sample size did not prevent us from observing the strong differentiation since alleles would remain different across sites and regions.

Mountains and river valleys act as a genetic barrier and a corridor for gene flow, respectively. Small and isolated populations are affected by founder effects or genetic drifts, which increase genetic differentiation (Wei et al., 2013; Wu et al., 2019; Xia et al., 2020). Strong genetic differentiation was observed among the Lithuanian river populations of *Nuphar lutea* (Vyšniauskienė et al., 2020), the invasive Eurasian *Myriophyllum* populations (Thum et al., 2020), the *Batrachium bungei* populations growing in the rivers of the Hengduan Mountains in China (Wang et al., 2010), and those of Iranian *Stuckenia* (Abbasi et al., 2016). In this research, the populations of *Zannichellia* are differentiated and structured, corresponding to topographic barriers (Alborz, Zagros, and Karkas Mountains) and ecological features. Many groups were obtained according to the mountain position, including NE Alborz (24), NW Alborz (8), across the site of Lake Urmia (3, 23), SW Zagros (11, 14, 16-20), SE Zagros (13, 15, 22), the central regions of Zagros (4, 9, 10), N Zagros or Iran's central regions (5, 6, 7), and NW Karkas Mountains (2, 12, 21). The findings further demonstrated that *Z. palustris* var. *pedicellata* was distributed in the north and south of Iran. Although this variety was well-separated from *Z. palustris* var. *palustris*, there additionally was a large genetic differentiation between the populations of each variety. Barriers such as Alborz, Zagros, and Karkas Mountains made these strong diversifications and differentiation.

An overall isolation by distance was found across the distribution range of *Zannichellia* in Iran. However, the genetic differentiation was larger for populations separated by the Alborz Mountains than those separated by the Zagros Mountains. Despite a short distance between some populations, a high level of differentiation occurred because of dispersal barriers such as the Alborz Mountains for separation of populations (8, 24) from (3, 23) or Karkas Mountains for separation of population (1) from (5, 6, 7). When compared to the study on genetic diversity and structure of *Stuckenia pectinata*, the role of mountains for the genetic differentiation of the populations of *Zannichellia* was higher. It can be due

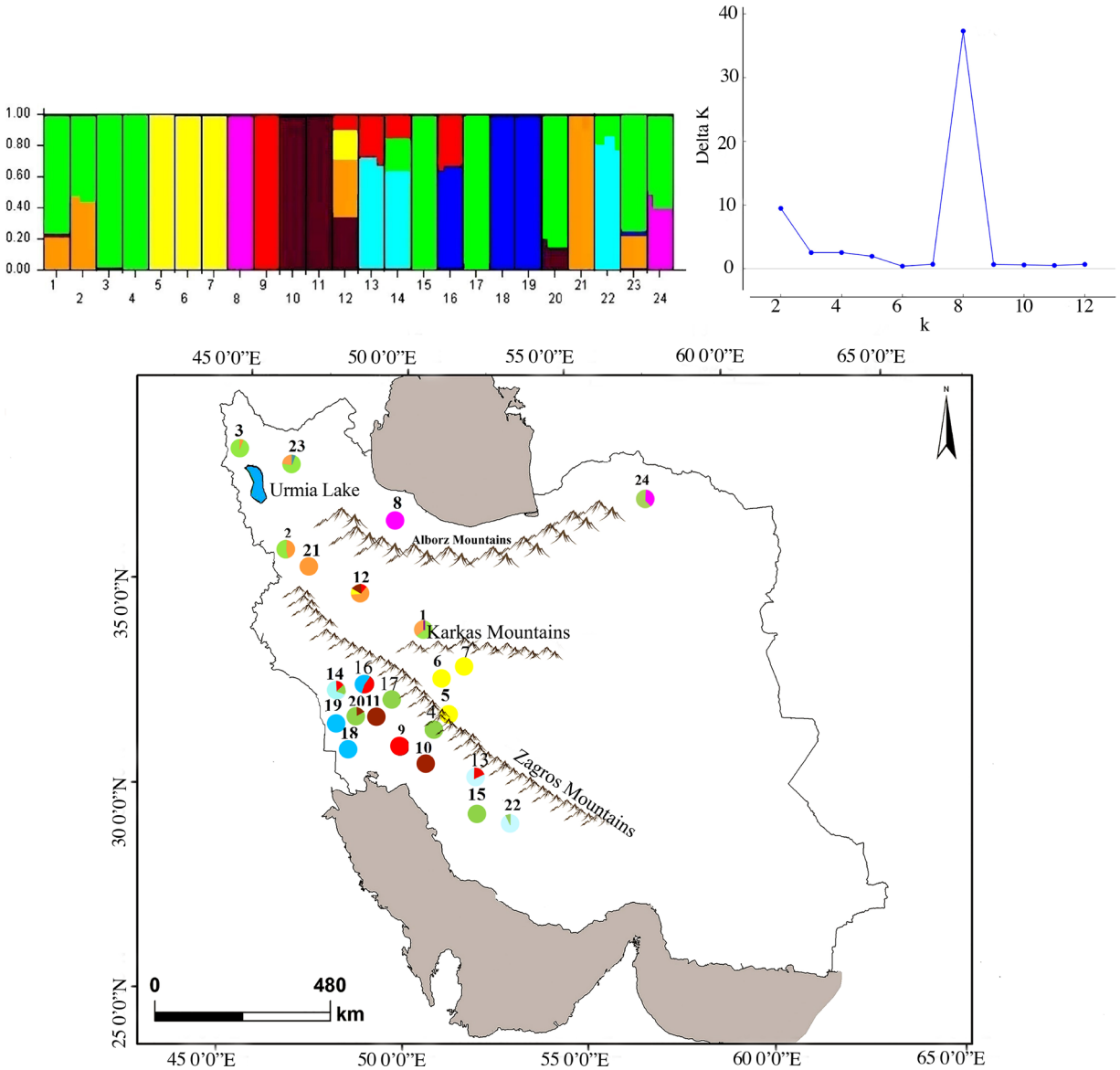

Figure 3. Map of the collection site and population structure of 24 populations of *Zannichellia* grouped into different geographic regions and analyzed using ISSR and SRAP markers. The bar plot shows a genetic relationship among the populations of *Z. palustris* var. *palustris* and *Z. palustris* var. *pedicellata* generated using STRUCTURE software at the population level (The colour of each population corresponds to the genetic cluster in the bar plot).

to two *Zannichellia* varieties (although *S. pectinata* also intrinsically shows taxonomic variations) and a higher number of genetic markers used for *Zannichellia*.

There was some connectivity between both sides of the Zagros Mountains, namely, between SE Zagros (13, 22) and SW Zagros (14). This area in Iran was reported as a route for bird migration (Sehhatisabet and Khaleghizadeh, 2013). Connectivity between populations (3, 4, 15, 17, 20: over the longer distance) and connectivity between (14, 15, 17: a corridor on the same side of the mountainsides) are probably better explained from PHYB and cpDNA sequences (Abbasi et al., 2019), together with the bird migration evidence. The connectivity along the same side of the Zagros Mountains showed low differentiation between SW Zagros and SE Zagros, and this was confirmed from the STRUCTURE results in this study.

Triest et al. (2010) observed the influence from brackish or freshwater habitats on genetic differentiation, referring to varieties of *Zannichellia*. A region with the highest distribution of *Zannichellia* populations in the south of Iran also showed a high genetic differentiation between the two varieties. Some southern populations

(10, 11, 14, 15, 17, and 20: *Z. palustris* var. *pedicellata*) are differentiated from other southern ones (9, 16, 18, and 19: *Z. palustris* var. *palustris*). Additionally, the southern populations of *Z. palustris* var. *pedicellata* and *Z. palustris* var. *palustris* were in the lower reaches of the Karun and Karkheh rivers, respectively. These rivers are different in their salinity. Karun River originates from the mountains of Chaharmahal via Bakhtiari Province, characterized by relatively high pollution levels due to urban and industrial wastewater and a high amount of plaster. Furthermore, the Gotvand dam, located on the Karun River, is one of the largest dams in Iran, filled with salt. In addition, River Karkheh originates from the mountains of Kermanshah Province and has a lower pollution rate (UN-ESCWA and BGR, 2013). Thus, *Z. palustris* var. *pedicellata* and *Z. palustris* var. *palustris* are further growing in saltwater and freshwater habitats, respectively.

The high level of genetic differentiation and the low level of gene flow between the *Zannichellia* populations in Iran indicate that conservation should consider both varieties and habitat preservation, allowing further genetic differentiation of *Zannichellia*.

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References

Abbasi S, Afsharzadeh S (2016). An efficient and simple CTAB based method for total genomic DNA isolation from low amounts of aquatic plants with a high level of secondary metabolites. Progress in Biological Sciences 6 (1): 1-12. doi: 10.22059/pbs.2016.59012

Abbasi S, Afsharzadeh S, Saeidi H, Triest L (2016). Strong genetic differentiation of submerged plant populations across mountain ranges: evidence from *Stuckenia pectinatus* in Iran. PLoS ONE 11 (8): e0161889. doi: 10.1371/journal.pone.0161889

Abbasi Sh, Afsharzadeh S, Dinarvand M (2019). A synopsis of *Zannichellia* L. (Potamogetonaceae) in Iran. Iranian Journal of Botany 25 (2): 103-114. doi: 10.22092/ijb.2019.128488.1263

Bagheri A, Abbasi S, Mahmoodi M, Akhavan Roofigar A, Blattner FR (2020). Genetic structure and conservation status of *Astragalus subrecognitus* (Fabaceae): a very rare and narrow endemic species. Plant Ecology and Evolution 153 (1): 101-107. doi:10.5091/plecevo.2020.1648

Barrett SCH, Eckert CG, Husband BC (1993). Evolutionary processes in aquatic plant populations. Aquatic Botany 44 (2-3): 105-145. doi: 10.1016/0304-3770(93)90068-8

Blair MW, Panaud O, McCouch SR (1999). Inter simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and finger printing in rice (*Oryza sativa* L.). Theoretical and Applied Genetics 98: 780-792. doi: 10.1007/s001220051135

Butkuvienė, J, Sinkevičienė Z, Naugžemys D, Patamsytė J, Žvingila D (2017). Genetic diversity of *Batrachium* (Ranunculaceae) species reveals the necessity of their protection in Lithuanian rivers. Aquatic Botany 142: 61-70.

doi: 10.1016/j.aquabot.2017.06.009

Chen YY, Fan XR, Li Z, Li W, Huang WM (2017). Low level of genetic variation and restricted gene flow in water lily *Nymphae tetragona* populations from the Amur River. Aquatic Botany 140: 55-61. doi: 10.1016/j.aquabot.2016.10.003

Daryadel E, Talaei F (2014). Analytical study on threats to wetland ecosystems and their solutions in the framework of the Ramsar convention. World Academy of Science, Engineering and Technology 8 (7): 2100-2110. doi:10.1016/j.aquabot.2016.10.003

Earl DA, VonHoldt BM (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359-361. doi: 10.1007/s12686-011-9548-7

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14 (8): 2611-2620. doi: 10.1111/j.1365-294X.2005.02553.x

Falush D, Stephens M, Pritchard JK (2003). Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164 (4): 1567-1587.

Gilbert S, Lackstrom K, Tufford D (2012). The Impact of Drought on Coastal Wetlands in the Carolinas. Columbia, South Carolina, South-eastern United States: Carolinas Integrated Sciences and Assessments Press.

Hamrick JL, Godt MJW (1989). Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS (editors). Plant Population Genetics, Breeding, and Genetic Resources. Sunderland, UK: Sinauer Associates Press, pp. 43-63.

Harris J (1999). RAPDs in systematics – a useful methodology? In: Hollingsworth PM, Bateman RM, Gornall RJ (editors). Molecular Systematics and Plant Evolution. London, UK: Systematics Association Press, pp. 211-228.

Hollingsworth PM, Preston CD, Gornall RJ (1996). Genetic variability in two hydrophilous species of *Stuckenia*, *P. pectinatus* and *P. filiformis* (Potamogetonaceae). Plant Systematics and Evolution 202: 233-254.

Hou Y, Lou Y (2011). Population genetic diversity and structure of a naturally isolated plant species, *Rhodiola dumulosa* (Crassulaceae). PLoS ONE 6 (9): e24497. doi: 10.1371/journal.pone.0024497

Hu YP, Bao R, Shi L, Wang JK, Wang J et al. (2016). Analysis of population structure of *Blysmus sinocomressus* in the Qilin Mountains by ISSR markers. Aquatic Botany 134: 54-60.

Kwiecińska-PoppeE, Haliniarz M, Sowa S, Paczos-Grzęda E (2020). Genetic diversity and population structure of endangered plant species *Anagallis foemina* Mill. Physiology and Molecular Biology of Plants 26 (8): 1675-1683. doi: 10.1007/s12298-020-00839-6

Li G, Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theoretical and Applied Genetics 103: 455-461.

doi: 10.1007/s001220100570

Li P, Zhan X, Que Q, Que W, Liu M et al. (2015). Genetic diversity and population structure of *Toona ciliata* Roem. based on sequence-related amplified polymorphism (SRAP) markers. Forests 6 (4): 1094-1106. doi: 10.3390/f6041094

Li ZZ, Lu MX, Gichira AW, Islam MR, Wang QF et al. (2019). Genetic diversity and population structure of *Ottelia acuminata* var. *jingxiensis*, an endangered endemic aquatic plant from southwest China. Aquatic Botany 152: 20-26. doi: 10.1016/j.aquabot.2018.09.004

Lyu N, Wang XF (2016). A comparative study of clonal structure in two *Sagittaria* species with the same clonal growth modes: *Sagittaria trifolia* and *S. pygmaea*. Aquatic Botany 128: 41-47. doi: 10.1016/j.aquabot.2015.09.007

Mader E, Van Viersen W, Schwenk K (1998). Clonal diversity in the submerged macrophyte *Stuckenia pectinata* L. inferred from nuclear and cytoplasmic variation. Aquatic Botany 62: 147-160.

Mantel N (1967). The detection of disease clustering and a generalized regression approach. Cancer Research 27 (2): 209-220.

Miyan MA (2015). Droughts in Asian least developed countries: vulnerability and sustainability. Weather and Climate Extremes 7: 8-23. doi: 10.1016/j.wace.2014.06.003

Moyle PB, Cech JJ (2004). Fishes, an Introduction to Ichthyology. 5th ed. Upper Saddle River, NJ, USA: Pearson Prentice Hall Press.

Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6 (1): 288-295. doi: 10.1111/j.1471-8286.2005.01155.x

Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics 155 (2): 945-959.

Rahel FJ (2007). Biogeographic barriers, connectivity and homogenization of freshwater faunas: it's a small world after all. Freshwater Biology 52 (4): 696-710. doi: 10.1111/j.1365-2427.2006.01708.x

Ricklefs RE, Schluter D (1993). Species diversity: regional and historical influences. In: Ricklefs RE, Schluter D (editors). Species Diversity in Ecological Communities: Historical and Geographical Perspectives. Chicago, IL, USA: The University of Chicago Press, pp. 350-363.

Robarts DW, Wolfe AD (2014). Sequence-related amplified polymorphisms (SRAP) markers: a potential resource for studies in plant molecular biology. Applications in Plant Sciences 2 (7): 1-13. doi: 10.3732/apps.1400017

Rohlf FJ (1998). NTSYS-pc Numerical Taxonomy and Multivariate Analysis System Version 2.1. Setauket, NY, USA: Exeter Publishing.

Sehhatisabet ME, Khaleghizadeh AA (2013). Review of current knowledge of radio-tracking of waterbirds and raptors in Iran. Podoces 8 (2): 22-30.

Smith JF, Bateman TA (2002). Genetic differentiation of rare and common varieties of *Eriogonum shockleyi* (Polygonaceae) in Idaho using ISSR variability. *Western North American Naturalist* 62 (3): 316-326.

The Angiosperm Phylogeny Group (2016). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. Botanical Journal of the Linnean Society 181 (1): 1-20. doi: 10.1111/boj.12385

Thum RA, Chorak GM, Newman RM, Eltawely JA, Latimore J et al. (2020). Genetic diversity and differentiation in populations of invasive Eurasian (*Myriophyllum spicatum*) and hybrid (*Myriophyllum spicatum* × *Myriophyllum sibiricum*) watermilfoil. Invasive Plant Science and Management 13 (2): 59-67. doi: 10.1017/inp.2020.12

Triest L, Vanhecke L (1991). Isozymes in European and Mediterranean *Zannichellia* (Zannichelliaceae) populations: a situation of predominant inbreeders. Opera Botanica Belgica 4: 133-166.

Triest L, Thi VT, Sierens T (2007). Chloroplast microsatellite markers reveal *Zannichellia* haplotypes across Europe using herbarium DNA. Belgian Journal of Botany 140 (1): 128-139.

Triest L, Thi VT, Thi DL, Sierens T, VanGeert A (2010). Genetic differentiation of submerged plant populations and taxa between habitats. Hydrobiologia 656: 15-27. doi: 10.1007/s10750-010-0434-1

UN-ESCWA and BGR (United Nations Economic and Social Commission for Western Asia; Bundesanstalt für Geowissenschaften und Rohstoffe) (2013). Inventory of Shared Water Resources in Western Asia. Beirut, Lebanon: Salim Dabbous Printing Company Press.

Van Vierssen W (1982). The ecology of communities dominated by *Zannichellia* taxa in Western Europe. I. Characterization and autecology of the *Zannichellia* taxa. Aquatic Botany 12: 103-155. doi: 10.1016/0304-3770(82)90010-9

Vyšniauskienė R, Rančelienė V, Naugžemys D, Rudaitytė-Lukošienė E, Patamsytė J et al. (2020). Genetic diversity of *Nuphar lutea* in Lithuanian river populations. Aquatic Botany 161: 103173. doi: 10.1016/j.aquabot.2019.103173

Wang YH, Chen JM, Xu CH, Liu X, Wang QF et al. (2010). Population genetic structure of an aquatic herb *Batrachium bungei* (Ranuculaceae) in the Hengduan Mountains of China. Aquatic Botany 92 (3): 221-225. doi: 10.1016/j.aquabot.2009.12.004

Wei X, Meng H, Jiang M (2013). Landscape genetic structure of a streamside tree species *Euptelea pleiospermum* (Eupteleaceae): contrasting roles of river valley and mountain ridge. PLoS ONE (6): 1-8. doi: 10.1371/journal.pone.0066928

Wu Z, Yu D, Li X, Xu X (2016). Influence of geography and environment on patterns of genetic differentiation in a widespread submerged macrophyte, Eurasian watermilfoil (*Myriophyllum spicatum* L., Haloragaceae). Ecology and Evolution 6 (2): 460-468. doi: 10.1002/ece3.1882

Wu Z, Xu X, Zhang J, Wiegleb G, Hou H (2019). Influence of environmental factors on the genetic variation of the aquatic macrophyte *Ranunculus subrigidus* on the Qinghai-Tibetan Plateau. BMC Evolutionary Biology 19: 228. doi: 10.1186/s12862-019-1559-0

Xia L, Geng Q, An Sh (2020). Rapid genetic divergence of an invasive species, *Spartina alterniflora*, in China. Frontiers in Genetics 11: 284. doi: 10.3389/fgene.2020.00284

Yeh F, Boyle T, Rongcai Y, Ye Z (1999). POPGENE: Microsoft Window-based Freeware for Population Genetic Analysis Version 1.3. Edmonton, AB, Canada: University of Alberta Press.

Zacharias I, Dimitriou E, Dekker A, Dorsman E (2007). Overview of temporary ponds in the Mediterranean region: threats, management and conservation issues. The Journal of Environmental Biology 28 (1): 1-9.