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Research article/Araştırma makalesi

Distribution patterns and ISSR PCR optimisation of invasive plant *Eichhornia crassipes* in Asi River/Turkey

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Abstract

Eichhornia crassipes (Mart.) Solms (Pontederiaceae) is a perennial aquatic invasive weed throughout the tropical and subtropical regions of the world. Recently, this species was detected in Asi River, Hatay. Therefore, knowledge on its population structure, size and density is very important to understand the current status of this species in Turkey. In this study, we have determined the distribution pattern of *E. crassipes*. After monitoring Asi River, spanning Syrian Border to Mediterranian Sea, we have detected three main population located at Reyhanlı, Antakya and Samandağ. Population size of *E. crassipes* varied between 4900-13600. ISSR-PCR protocol was also optimized for *E. crassipes* based on concentrations of $MgCl_2$, primer, dNTP and template DNA. Among 45 ISSR primers tested, 20 of them have produced satisfactory results. Study results suggested that distribution of *E. crassipes* in Turkey is not negligible and populations must be monitored periodically. In addition, the optimized ISSR-PCR procedure can be used to study the genetic structure of *E. crassipes* populations naturally grown in Turkey.

Key words: Asi River, *Eichhornia crassipes*, ınvasive species, ISSR, Turkey

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İstilacı *Eichhornia crassipes* bitkisinin Türkiye'de Asi Nehrinde dağılımı ve ISSR PCR optimizasyonu

Özet

Eichhornia crassipes (Mart.) Solms (Pontederiaceae) dünyada tropik ve sub-tropik bölgelerde yayılış gösteren çok yıllık istilacı bir sulak alan bitkisidir. Bu bitki türü yakın zamanlarda Hatay yöresinde Asi Nehrinde tespit edilmiştir. Bu nedenle bu türün popülasyon yapısı, büyüklüğü, yoğunluğunun belirlenmesi Türkiye'deki güncel durumunun anlaşılması açısından oldukça önemlidir. Bu çalışmada *E. crassipes*'in Asi Nehri üzerindeki dağılım alanları tespit edilmiştir. Nehrin Suriye sınırı ve Akdeniz arasında kalan kısmı tarandığında Reyhanlı, Antakya ve Samandağ'da üç ana popülasyon tespit edilmiştir. Popülasyon büyüklükleri 4900-13600 arasında değişmektedir. Bunun yanında, *E. crassipes* için farklı $MgCl_2$, primer, dNTP ve kalıp DNA konsantrasyonları temel alınarak ISSR-PCR protokolü optimize edilmiştir. Denenen 45 ISSR primerden 20'si kabul edilebilir sonuçlar vermiştir. Çalışma sonuçlarımız, *E. crassippes'*in Türkiye'deki dağılımının göz ardı edilemez seviyede olduğunu ve populasyonların düzenli olarak monitörlenmesi gerektiğini göstermektedir. Ek olarak, optimize edilen ISSR-PCR prosedürü Türkiye'de doğal yayılış gösteren *E. crassipes* populasyonlarının genetik yapılarının çalışılmasında kullanılabilecektir.

Anahtar kelimeler: *Eichhornia crassipes*, istilacı tür, Asi Nehri, ISSR, Türkiye

1. Introduction

Eichhornia crassipes (Mart.) Solms (Pontederiaceae), water hyacinth, is a perennial aquatic invasive plant that is native of the Amazon basin. This colonial wetland plants is defined as one of the most aggressive invaders (Barrett, 1989; Zhang et al., 2010). The species was first discovered in 1823 by the German naturalist C. von Martius who was studying the flora of Brazil. He named it as *Pontederia crassipes*. Sixty years later, Solms included it in the *Eichhornia* genus as described by Kuntz in 1829. At the present time *E. crassipes* is distributed across the tropics and subtropics between 39°N and 39°S (Tellez et al.,2008).

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E. crassipes has broad, thick, glossy, ovate leaves and may rise above the surface of the water as much as 1 meter in height. Its leaves are 10–20 cm and float above the water surface. It has long, spongy, bulbous stalks and purple-black feathery, freely hanging roots. It also produces large quantities of seeds, and these are viable up to thirty years (Adegunloye et al., 2013).

According to GEIB (Biological Invasion Specialist Group) water hyacinth is in TOP20 most dangerous invasive species. Furthermore, Recommendation no. 133 (2008) of the Bern Convention Standing Committee, adopted on 27 November 2008, provides recommendations on the control of *E. crassipes* (Brundu et al., 2013). However, there are ongoing research to determine its value and economic exploitation strategies. It has been found that *E. crassipes* could be used as a renewable energy resource for biogas production (Harley, 1990). Indeed, it was proposed that it can be used as animal fodder, fertilizer, in the manufacture of paper and furniture, in waste water treatment, and in water quality management (Julien et al., 1999). Moreover, *E. crassipes* is a relatively cheap and environmentally friendly tool for the clarification of contaminated water because of its ability to absorb heavy metals (common pollutants) and its ability to grow rapidly (Muramoto and Oki, 1983; Zhu et al., 1999).

This invasive alien plant was recently seen in Asi River (Orontes), Turkey, and included to the list of quarantine pests of Turkey in 2010 (Anonymous, 2010). This species seems to create populations at riverbed of Asi River in the last 3 years (Figure 1). Although this species is assumed to be transited to Turkey from Syria, there is no accurate and clear information about it. On the other hand, distribution patterns and genetic variation of its population need to be clarified. Moreover, because of high reproducibility and adaptability to various environment, this species rapidly occupies new habitats and causes potential risk to ecosystem. Therefore, knowledge on its population area, size and density as well as genetic structure will provide valuable data about the status of this species in Asi River, Turkey.

Figure 1. General view of *Eichhornia crassipes* (Pontederiaceae)

The aims of our study were to determine distributional patterns and population size of the *E. crassipes* in Asi River flowing in Turkey and also to find suitable ISSR primers to study its clonal genetic diversity. These results will be beneficial for both development of a suitable management programme and specific strategies for utilization of this plant species as a raw material for biogas, fodder, fertilizer, paper and furniture productions in Turkey.

2. Materials and methods

2.1. Study site-Asi River

Asi River is flowing northward transboundary in Lebanon through Syria and Turkey. In Turkey, Asi River spans 88 km and a total area of ~5700 km^2 before entering the Mediterranean Sea (UNESCO-IHE, 2002). According to grid system the research area falls within C6 squares in southern of Turkey (Güngör et al., 2016) We determined localities of *Eichornia crassipes* populations during field work in Hatay, Turkey (Figure 2). The plant specimens are collected and stored (No: 17185) in ANES (Anadolu University Faculty of Science Herbarium).

1.2. Data collection and analyses

The sites were sampled along quadrat established colonia to the riverbed between June and November 2015. Studies were conducted on large rectangular macroplots within 10m x 10m (100 m^2) that were set up in the center of the study sites. The sampling unit in this case is the 1m x 1m quadrat (Şişli, 1996). Based upon these data, the estimated population size (N) of the population studied in the total areas was calculated by the formula: N = mean number of individuals x area size (km^2)

The quadrat was randomly placed and repeated three times at each population. The location and elevation of each plot were recorded by global positioning system (GPS). Based on GPS data, geographic distances between populations were calculated as the shortest distance from the margins of the river following connecting plot sites using ArcMap 10.01 (ESRI, USA). Then, the average of 3 quadrats per population was achieved in order to have an ultimate estimation of population size depending on the total area.

Figure 2. Distrubition patterns of *E. crassipes* on Asi River

1.3. ISSR-PCR

Total genomic DNA was extracted from silica dried leaf materials by a modified hexadecyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). The quantity and quality of DNA were determined by using a Nanodrop® ND-1000 spectrophotometer (Wilmington, DE, USA) and agarose gel electrophoresis. DNA samples were diluted to 2.5 ng/µL. One individual from each population are randomly selected to ISSR-PCR optimisation. A set of 45 ISSR primers (UBC set no. 9) was screened (Zietkiewicz et al., 1994).

The PCR amplifications were conducted in an Applied Biosystems Veriti gradient thermocycler using the following thermal profile: 3 min of predenaturation at 94°C, 40 cycles of 45 s at 94 °C, 45 s at the annealing temperature of 50°C to 64°C depending on the ISSR primers, 1 min at 72°C, with a final extension at 72°C for 5 min. The amplification products were separated on 1.4 % agarose gel containing ethidium bromide at 90V for 70 min and digitally photographed.

Among 45 ISSR primers tested, 20 of them ($GAG(CAA)_5$, $(CAG)_5$, $(GA)_8YC$, $(GA)_8T$, $(AC)_8YT$, $(GA)_8A$, $(AG)_8T$, $(AG)_8C$, $(AC)_8C$, $(AGC)_6G$, $BDB(ACA)_5$, $DBD(AC)_7$, $DD(CGA)_5$, $(AG)_8YT$, $(CT)_8G$, $(TC)_8C$, $(CA)_8RC$, $(AGT)_6$, $BDB(CA)_7$ and $(CCG)_6$) have produced satisfactory results (Table 1).

Table 1. List of ISSR primers amplifying clear products

Primer No	Primer name	Sequence (5'-3')	Tm (^OC)	Length (bp)	% G/C
1	GAG(CAA)5	GAG CAA CAA CAA CAA CAA	50	18	38.89
2	(CAG)5	CAG CAG CAG CAG CAG	50	15	66.67
3	(GA)8YC	GAG AGA GAG AGAGAG AYC	55	18	50.00
4	(GA)8T	GAG AGA GAG AGA GAG AT	50	17	44.44
5	(AC)8YT	ACA CAC ACA CAC ACA CYT	50	18	44.44
6	(GA)8A	GAG AGA GAG AGA GAG AA	50	17	47.06
7	(AG)8T	AGA GAG AGA GAG AGA GT	50	17	47.06
8	(AG)8C	AGA GAG AGA GAG AGA GC	52	17	52.94
9	(AC)8C	ACA CAC ACA CAC ACA CC	52	17	52.94
10	(AGC)6G	AGC AGC AGC AGC AGC AGC G	64	19	68.42
11	BDB(ACA)5	BDB ACA ACA ACA ACA ACA	52	18	27.78
12	DBD(AC)7	DBD ACA CAC ACA CAC AC	52	17	41.18
13	DD(CGA)5	DDC GAC GAC GAC GAC GA	55	17	58.82
14	(AG)8YT	AGA GAG AGA GAG AGA GYT	52	18	44.44
15	$(CT)_8G$	CTC TCT CTC TCT CTC TG	52.8	17	52.9
16	$(TG)_8C$	TGT GTG TGT GTG TGT GC	52.8	17	52.9
17	$(CA)_8RC$	CAC ACA CAC ACA CAC ARC	53.7	18	50.0
18	$(AGT)_6$	AGT AGT AGT AGT AGT AGT	46.9	18	33.3
19	$BDB(CA)_7$	BDB CAC ACA CAC ACA CA	47.9	17	41.2
20	$(CCG)_6$	CCG CCG CCG CCG CCG CCG	47.9	17	41.2

D = (A, G, T); Y = (C, T); R = (A, G). Tm: Annealing temprature.

ISSR PCR protocol was optimized based on different concentration of $MgCl_2$ (1-3 mM), template DNA (2-10 ng), dNTP (2.5-10 mM) and primers concentration (2.5-10 µM).

3. Results

After monitoring 9 sites in following regions: Hacıpaşa, Bohşin, Demirköprü, Güzelburç, Küçükdalyan, Antakya, Sutaşı, Tekebaşı, and Meydan along Asi River; we found that *E. crassipes* was represented with three main populations (Reyhanlı, Antakya, Samandağ) (Figure 2). It was observed that first population was located immediately after Syrian border near Hacıpaşa and after that other populations were seen along the river until entering the Mediterranian Sea. There were also numerous small plant colonies between these main populations (Figure 3).

Figure 3. Populations of *Eichhornia crassipes* on Asi River, Hatay

All 9 sites were located between 36º 4′ E, 35º 59′ N and 36º 14′ E, 36º 21′ N, 22-120m (Table 2). Population size of *E. crassipes* in the study area ranged from 4.900 to 13.600 plants (median= ~9000 plants).

Table 2. Distribution of *E. crassipes* on Asi River

Pop code*	Sampling locality	Longitude/Latitude Altitude	Population size
Pop 1 (Reyhanlı)	1) Hacıpaşa	36º14′37″ E, 36º21′4″ N, 115 m	2500
	2) Bohşin	36º14′54″ E, 36º21′15 ″ N, 120 m	3200
	3) Demirköprü	36º13′22″ E, 36º22′35 ″ N, 122 m	2900
Pop 2 (Antakya)	4) Güzelburç	36º14′33″ E, 36º11′19″ N, 83 m	5300
	5) Küçükdalyan	36º14′35″ E, 36º11′35 ″ N, 81 m	3600
	6) Antakya	36º14′34″ E, 36º11′34 ″ N, 85 m	4700
Pop 3 (Samandağ)	7) Sutaşı	36º4′57″ E, 35º58′48″ N, 24 m	1800
	8) Tekebaşı	36º4′16″ E, 35º59′19 ″ N, 22 m	1500
	9) Meydan	36º5′5″ E, 35º58′44″ N, 28 m	1600

As a first step in analysing genetic diversity PCR optimisation and detection of suitable primers are very crucial. To achieve this, we screened 45 ISSR primers by using representative *E. crassipes* DNA samples from each population detected in Asi River. Clear and reproducible amplifications were obtained by using 2.2 mM $MgCl_2$, 2 µM primer, 2.5 mM dNTPs and 8 ng template DNA (Table 3). Representative gel pictures of ISSR PCR results were given in Figure 4.

Table 3. Optimization of the ISSR-PCR reaction parameters for *E. crassipes*

PCR parameter	Tested range	Optimum conditions
DNA template concentration (ng)	2, 5, 8, 15, 20, 30	8 ng
Magnesium chloride (mM)	1.0, 1.5, 2.2, 2.5, 3.0	2.2 mM
Deoxynucleotide triphosphates (dNTPs) (mM)	4, 8, 10, 12, 16	10 mM
Primer concentration (µM)	0.5, 1, 2, 2.5, 3	2.0 µM
Annealing temperature (°C)	50-64	50, 52 ,55 ,64 etc. for different primer

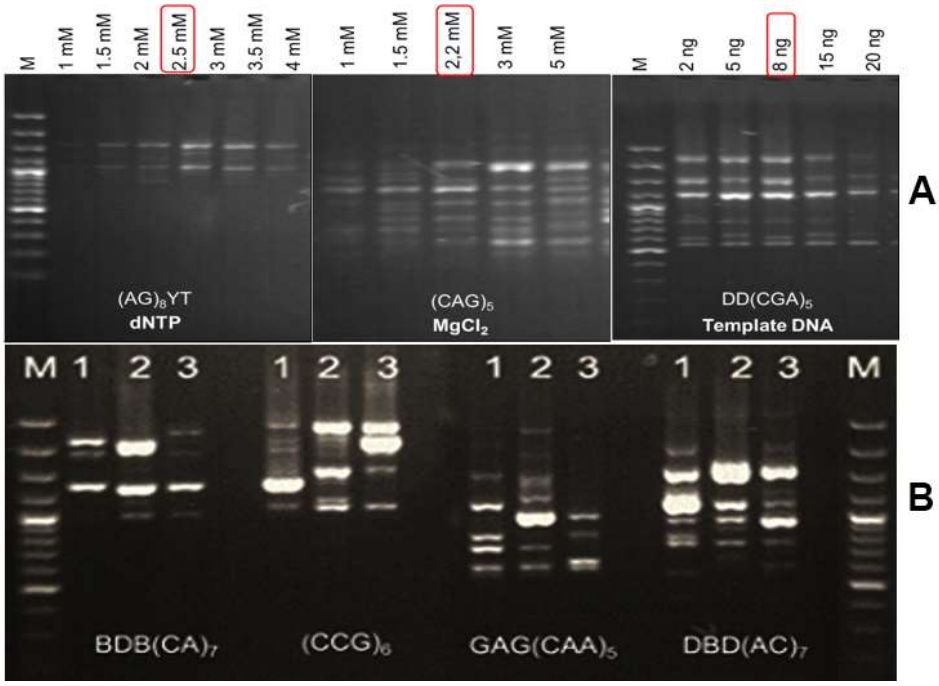

Figure 4. The results of ISSR-PCR optimisation for some primers. (M: 100 bp plus DNA ladder marker, 1: Pop 1, 2: Pop 2, 3: Pop 3)

4. Conclusions and discussion

This work represents first population monitoring and ISSR PCR study on invasive weed *E. crassipes* that was recently reported in Asi River, Turkey. Number of populations and population size for this species were determined in 2015. According to Üremiş et al., (2014) *E. crassipes* became common in Asi River in 2010. Since then, the species has established itself tremendously becoming the most dominant floating plant species in the river. In the literature, it is reported that people play basic role in the spread of *E. crassipes* (Tellez et al., 2008). However, because the species was observed near Syria border and flow direction of Asi River caused to think us *E. crassipes* transited from Syria via water flow rather than introduction by people. Most of the plant is found concentrated along the middle of the river around Güzelburç. In this region, the river is closed with temporary dam in spring for irrigation of agricultural land each year. So, it is also common to find the plant rooted in shallow water and muddy riverside of the river (Figure 3). *E. crassipes* blocks irrigation canals. It was reported that one hectare of this plant may contain more than 2 million individual plants (Center and Spencer, 1981) and causes big environmental impacts in wetlands because of its tolerance to extreme biological and ecological conditions (Center et al., 2002). This species can also tolerate fluctuations of the water level, extreme differences in amounts of nutrients and pollutants, and also salinity values. Our results showed that number and size of *E. crassipes* populations were not very high at present, however it could proliferate and spread further in Asi River and may pose potential risk for this ecosystem (Figure 3). Therefore, periodical monitoring of its populations is necessary.

Studying clonal genetic diversity in any invasive plant species is important to determine whether single or multiple introduction of the species to a new area occurred (Lambertini et al. 2010). Molecular markers have been used as an effective and important tool to provide informative data on the levels of genotypic variation and patterns of invasion (Ahmad et al., 2008, Haddadchi et al., 2013). Until today, clonal genetic diversity of *E. crassipes* were studied by using AFLP, ISSR and RAPD markers (Ren et al., 2005; Li et al., 2006; Ren and Zhang, 2007; Zhang et al., 2010). These studies either focused on global clones of *E. crassipes* including both the native and introduced range distributed in South America and Asia or clones distributed in China. There is no similar study undertaken in the Middle East and

especially in Turkey. Therefore, the optimized ISSR-PCR procedure and selected ISSR primers will be employed to study the genetic structure of Turkish *E.crassipes* populations in future (Table 1, 3).

In conclusion, this study represents first report about the distribution of *E.crassipes* in Asi River in Turkish boarder. Since this plant species is considered as a pest due to its invasive characteristics it poses a potential risk for both ecosystem and farmers using Asi River for irrigation purposes. In order to prevent the problems caused by this plant, monitoring is essential. Foreseeing its invasive characteristic management programs should be developed by local authorities. In addition, the cleaned *E.crassipes* samples with these programs can be considered as raw material for the production of biogas, fodder, organic fertilizer, paper and furniture.

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