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Full Length Article

Genetic Structure of Invasive Weed *Parthenium hysterophorus* in Australia and Pakistan

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Abstract

Understanding the patterns of genetic structure in the introduced range of invasive species can help elucidate invasion histories and levels of gene flow among populations. Parthenium weed (*Parthenium hysterophorus* L.; PW) is native to the Gulf of Mexico and central South America but has become globally invasive during the last three decades and little is known about the genetics of this species in its invasive range. The present study was conducted to determine the genetic structure of 95 individual samples from 11 populations (9 from Pakistan and 2 from Australia) of PW using ISSR fingerprinting. A total of 30 ISSR primers were screened; of which eight were selected due to their high polymorphism and reproducibility. *In toto* 147 bands were amplified, which ranged in size from 200-2000 bp; among which 97 were polymorphic. Genetic diversity within the populations both from Pakistan and Australia ranged between 0.193-0.278. Approximately 18% of genetic variation occurred among and 82% within populations. Principal Coordinate Analysis showed that within the 95 samples two groups were present: one contained samples collected mainly from Pakistan and the second group included the Australian samples along with two populations from Pakistan. Overall, there was limited gene flow among PW populations in Pakistan, although the genetic diversity within populations was high. The degree of genetic variation inferred from various population diversity measures can predict different events of founding populations, which have passed through complicated processes of invasion, experiencing genetic bottlenecks. Taken together, results showed that PW in Pakistan is genetically heterogeneous and may have been the result of multiple introductions. © 2015 Friends Science Publishers

Keywords: Genetic diversity; Invasion histories; Genetic bottleneck; Australia; Pakistan

Introduction

Biological invasions of weed are worldwide common phenomenon and are associated with biodiversity loss, decreased productivity in agricultural settings and cause large economic losses (Meyerson and Reaser, 2003). As trade and travel between continents continues to increase so does the introduction of problematic weed species into new areas persists. This has resulted in large-scale movement of problem plants to many parts of the world from where they have become globally invasive. In order to better manage problem weeds, it is important to understand their genetic structure and elucidate information about the introduction history of weeds species.

Understanding the patterns of genetic diversity and genetic structure within and among populations of invasive plant species is critical to the management of invasive species and is a major focus of research in biological control programs. The molecular characterization of PW can furnish various control strategies designed and subsequently opted along with the modifications of prevailing management options. The reduced genetic variance can be a consequence of serious genetic bottleneck after introduction into novel habitat, where the population size of the invader is narrowed and hence genetic drift decrease genetic variation. The genetic bottleneck severity is somewhat lessened by multiple introductions (Novak and Mack, 1995) because when two or more different genotypes that vary at some loci in the genome appear in an area there are more chances of genetic recombination and genetic variation. Therefore the study of origin of invasive populations is crucial for determining their beginnings as a result of single or multiple introductions in a particular environment.

Inter-simple sequence repeat (ISSR) markers (developed first by Zietkiewicz *et al.,* 1994) are ideal to examine these patterns because they are more reliable and polymorphic than Randomly Amplified Polymorphic DNA markers (Lima *et al*., 2011). In addition to this, they are reusable and inexpensive to develop and score. ISSR markers are derived from microsatellites/SSRs that are scattered throughout the eukaryotic genome and the primers (16-18 base pair) anchor within the SSRs rather than the

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flanking regions (Korpelainen *et al*., 2007). The amplified DNA band represents the sequence in between two inverted microsatellites, the allelic polymorphism arose due to absence of repeated microsatellite sequence or addition/deletion of the sequence modifies the distance between repeats which can be judged through ISSR in a single reaction.

Parthenium weed (*Parthenium hysterophorus* L.; PW), belonging to Asteraceae family, is an aggressive, annual or ephemeral herbaceous weed of tropical and subtropical regions. It has invaded the natural ecosystems throughout the world (pan-tropical distributions) and endangered the agricultural lands and productivity thereform. In Pakistan, it was first reported in the Gujrat district of Punjab in 1980 (Razaq *et al*., 1994; Shabbir *et al*., 2012). It has strong potential to spread because studies showed that it is extremely allelopathic due to water soluble phenolics and sesquiterpene lactones and constitutes area of its own where it grows. Though it is a weed of wastelands, It has now become a serious environmental and agricultural problem. It has caused loss of about $A16.5 million per annum to cattle industry in beef producing country like Australia (Chippendale and Panetta, 1994) by causing toxicity to the livestock and tainting milk and meat. Furthermore it is a serious health hazard to human beings causing hay fever, contact dermatitis and severe asthma. It has adverse effects on soil as well (Kohli and Rani, 1992).

Parthenium has achieved major weed status in most of the countries due to its profuse spread in a relatively short period of time. It is the weed of national significance in Australia and since 1976 and the Department of National Resources has an ongoing campaign to reduce the impact and prolific rise of PW from central Queensland to northern New South Wales and the coastal invasions. Although a lot of literature is available on the biology, spread and invasive potential of PW, little is known about its genetic structure and invasion histories. Therefore, ISSR fingerprinting was employed as a preliminary and of course crucial study for determining its genetic structure. In the present study, we are explicitly tackling following key questions; (1) do ISSR markers prove to be efficient in describing ample genetic structure of the sampled 95 accessions of PW populations? (2) what is the extent of the genetic variation within and among PW populations? and (3) based on the information on the genetic structure, do the invasion histories regarding multiple introductions can be inferred?

Materials and Methods

Sample Collection

Different samples of PW were collected in such a way that 10 samples were taken from two separate PW populations in Australia (Toogoolawah and Clermont) in June 2011 from the school of Land and Food Sciences, University of Queensland, Brisbane Australia. Five Toogoolawah population samples were collected from Kilcoy, whilst the other five Clermont population samples were from Emerald. Twenty PW samples were collected from Peshawar valley (including Charsadda, Mardan, Swabi, Peshawar and Attock, Pakistan) in Feb-March 2012; the rest of the samples were taken from regions in the Punjab Province of Pakistan in March-May 2012. A complete list of the sampling sites is contained in Table 1, along with their respective latitudes, longitudes (in degrees) and elevations in Fig. 1. Fresh leaf samples were collected in zip-lock plastic bags containing silica gel where they remained until used for DNA extraction. The minimum distance between sampling points during collections was approximately 50 m.

DNA Extraction

Total Genomic DNA was extracted by using the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol of Doyle (1991) with some modifications. DNA of some samples that showed some phenolic contaminations of the weed and in addition have shown merely smear on the Agarose gel were then extracted by using plant DNA extraction kit (Favorgen). The concentration and quality of extracted DNA was done by 1.0% Agarose gel electrophoresis and comparing it with the known quantities of *Hin*dIII λ DNA ladder (Fermentas) and through spectrophotometer (Eppendorf, Optima Rating100-240VAC and 50-60Hz 1A) as well. The extracted DNA was properly labeled according to the codes from where PW samples were collected (Table 1) and stored at -20ºC for ISSR analysis.

ISSR Primers and PCR

Thirty primers were tested for their ISSR polymorphism (Biopharmaceuticals Company. Inc. CA, USA Table 2). As a preliminary step, optimal ISSR-PCR reaction and cycling conditions were determined by varying various PCR reaction parameters such as the concentrations of template genomic DNA, $MgCl_2$ concentrations and PCR cycling programs. Optimized PCR reaction and temperature cycling conditions were then used in subsequent ISSR experiments for the study of genetic diversity within PW populations from a range of geographical locations. After primary screening, out of 30 primers only eight potential primers were selected that produced reproducible, polymorphic ISSR fragments in all PW samples. Each 25 µL reaction mixtures contained 25-30 ng DNA, 10 p mole primer, 2 mM dNTP mix, 10 mM Tris-HCl, pH 8.2, 50 mM KCl, 25 mM $MgCl_2$ and 1-1.2 U Taq DNA Polymerase (Fermentas). The temperature profile consisted of an initial denaturation of 94ºC for 4 min, followed by 40 cycles of denaturation at 94ºC for 45s, annealing at 54ºC for 1 min and extension (1 min) with a final extension at 72ºC for 10 min. All reactions were performed in Eppendorf mastercycler, Hamburg, Germany. ISSR amplification products were separated by 1.2% Agarose gel electrophoresis and visualized under UV using gel documentation (Wise UV, M-20).

Table 1: Ninety five accessions of Parthenium populations analyzed their place of collection in Pakistan and Australia, latitudes, longitudes, altitudes and their respective sample size (N)

Accessions	Sites	Latitude (degrees)	Longitude (degrees)	Elevation (ft)	N
phchar pop1	Charsadda	34.15	71.73	990	5
Phswa	Swabi	34.11	72.46	1094	5
Phmar	Mardan	34.19	72.06	1002	4
Phpes	Peshawar (Grand trunk Road)	34.26	71.54	1158	1
Phpes	Peshawar cantonment	34	71.47	1235	1
Phpes	Kabul River	34.09	71.7	953	2
Phpes	Attock	33.8	71.72	2003	2
phisl pop2	Islamabad (I-8)	33.67	73.07	1715	2
Phisl	Chakshehzad	33.16	73.14	1636	2
Phisl	KaralChowk	33.6	73.13	1602	2
Phisl	Daman-e-Koh	33.74	73.05	2391	2
Phisl	Rawalpindi	33.66	73.16	1658	2
phchk pop4	Chakwal	32.93	72.85	1628	3
Phdin	Dina	33.02	73.58	854	2
phjhl pop5	Jhelum Grand trunk road	32.93	73.71	762	3
Phjhl	Jhelum Cantonment	32.91	72.1	768	2
phshk pop6	Shakargarh	32.26	75.15	879	5
phsia pop7	Sialkot	32.49	74.53	837	5
phlhr pop3	Gujranwala	32.15	74.18	748	5
Phlhr	Lahore	31.96	74.54	715	2
Phlhr	Walton	31.48	74.35	707	3
Phlhr	Changamanga	31.09	73.96	660	4
Phlhr	Barqi road	31.49	74.48	709	3
Phlhr	Ittefaq hospital	31.51	74.32	714	2
Phlhr	Minare Pakistan	31.59	74.32	696	1
phchi pop8	Chiniot	31.72	72.97	597	2
Phfsd	University of Agriculture Fsd	31.44	73.07	606	2
Phfsd	Ayub agriculture Research Institute	31.41	73.06	618 ft	2
Phjhg	Jhang sadder	31.27	72.32	514 ft	2
Phjhg	jhang city	31.3	72.35	507 ft	2
phokr pop9	Okara	30.8	73.44	604 ft	3
Phshw	Sahiwal	30.66	73.1	568 ft	2
phtogw pop10	Kilcoy	26.94	152.56	12259 ft	5
phclm pop11	Emerald	23.5	148.16	589 ft	5

Table 2: ISSR primers used for PCR amplification of Parthenium weed samples and amplified fragments generated from 95 individuals

Sr.no	Primer sequence (5´-3´) R= purins, Y= pyrimidines	Annealing Temperature (ºC)	Total bands	polymorphic bands	% polymorphic bands
53-1	TCTCTCTCTCTCTCTCC	54	22	13	59.1%
53-4	CTCTCTCTCTCTCTCTRC	54	16	10	62.5%
53-5	ACACACACACACACACYT	53	17	11	64.7%
53-6	TGTGTGTGTGTGTGTGA	52	22	18	81.8%
53-12	CTCTCTCTCTCTCTCTRG	54	20	12	60.0%
53-16	ACACACACACACACACYA	53	21	15	71.4%
53-23	GAGAGAGAGAGAGAGAYC	52	15	09	64.2%
53-30	CTCTCTCTCTCTCTCTRG	52.5	14	09	64.2%
Total			147	97	

Statistical Analysis

The ISSR data were converted into the binary data matrix in discrete characters such that 1 represented the presence of the amplified bands and 0 represented the absence of the bands, which was used in all subsequent statistical analyses of both genetic diversity and genetic differentiation. Genetic diversity measures were calculated for each population including (i) number of observed alleles (Na), (ii) number of effective alleles (Ne), (iii) Shannon's information index (I) of phenotypic diversity, (iv) Nei's (1973) expected heterozygosity or gene diversity (He) and (v) unbiased expected heterozygosity (UHe) using the software GenAlEx 6.0 (Peakall and Smouse, 2006). Analyzed samples were grouped into two regions based on geographical origin (Pakistan and Australia). A data matrix of Euclidian distance was calculated between all individuals in the dataset and used in all subsequent analyses. We used a hierarchical model with two regions and populations within regions we implemented an analysis of molecular variance (AMOVA). Specifically, AMOVA was used to partition the amount of genetic variation distributed within and among groups within the native range of both species using GenAlEx 6.0 (Peakall and Smouse, 2006).

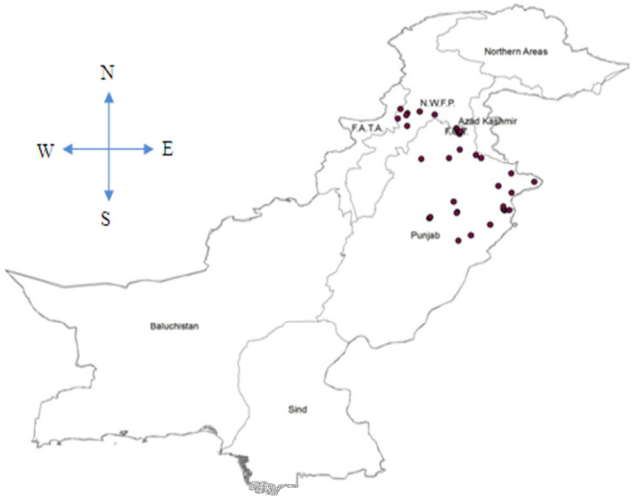

Fig. 1: Map of Pakistan identifying the Parthenium weed sample collection sites. The map was created using ArcGIS 9.3.1

Principal coordinates analysis (PCA) was used to determine the patterns of individual clustering using pair wise Euclidian distance in the above software.

Results

Within 95 individuals (11 populations) of PW sampled from Pakistan and Australia, the eight selected ISSR primers generated total of 147 bands in almost all samples, 97 of which were polymorphic. The amplified DNA bands ranged in size from 250-2000 bp, with an average of 18.3 fragments produced per primer. ISSR primer (S. No. 53-6) showed highest number of polymorphic band in all accessions of PW showing 81.8% polymorphic bands. The figures presenting the banding patterns of all 95 individuals of PW by ISSR primer 53-6 on the 1.2% agarose gels stained by ethidium bromide could be produced on demand. In addition, primer 53-5 and 53-16 also presented higher polymorphisms i.e., >65% (Table 2). While sampling, we have only examined occasional PW in the Southern Punjab comprising geographical regions of Multan and Bahawalpur during the visits in May 2012, although Pakistan has alluvial plains and the flow of rivers is towards the south. Although PW seeds can spread through the water streams to the southern regions but it is speculated that due to high temperature (above 50ºC) in summers, the weed spread is not vivid and only the intermittent plants may be witnessed.

The calculation of the population statistical measures (Table 3) of all the populations i.e. number of effective alleles (Ne), Shannon's information index (I) and expected heterozygosity (He) showed that PW shows high variability at the intra specific level. This finding supports that PW collected from wide geographical regions attributes to the different biotypes of the same species rather than

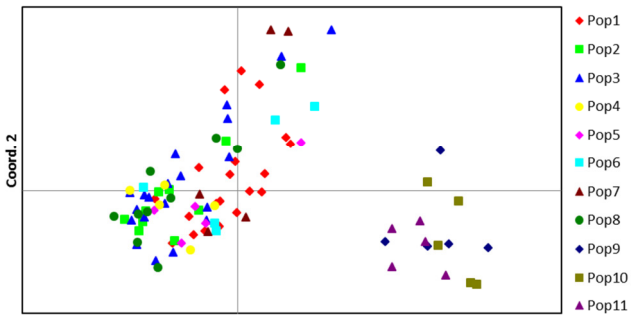

Fig. 2: The principal coordinate analysis of 11 populations of Parthenium weed

different species. Population 1 from Peshawar valley showed highest levels of variability among all the populations with I = 0.399 and He = 0.269. The effective number of alleles (Ne), another measure of genetic diversity, value of which was 1.47 for population 1. In contrast population 5 from Jhelum showed the lowest level of genetic variation (Ne = 1.537, I = 0.266 and He = 0.174). All populations showed similar levels of genetic diversity.

AMOVA determined that the populations show significant structure with approximately 18% of genetic variation among populations while most of the variation resided within populations of PW sampled extensively from wide geographical range i.e., 82% of diversity found within populations (Table 4). Two dimensional PCA found that the populations consisted of two groups (group 1 populations 1-8; group 2 populations 9-11). Overall, principal poordinate 1 (axis 1) explained 33.8% of the variation; while principal coordinate 2 (axis 2) explained 22.4% of the variation (Fig. 2). The Okara and Sahiwal samples (southern part of the Punjab province) form distinct group with the Australian populations (Clermont and Toogoolawah).

Discussion

Genetic variation is extremely significant for the survival of a species, and each individual of a species, except the genome of monozygotic twins, possesses a unique DNA sequence. PW represented high genetic variability as shown in the statistical analysis and for this reason it has high invasive potential (Navie *et al.*, 1996) along with strong adaptive characters that assured high fitness and competitive success in any environment. Biologists evidenced that PW reproduces generally by intra specific hybridization (cross pollination) and PW pollens spread by amphiphilous mode of pollination i.e., partly by insects and partly by the wind (Pandey *et al.*, 2003), which is the source of increased heterogeneity. In contrast, low genetic diversity results in reduced adaptability finally leading to the species extinction (Chen *et al.*, 2010).

The dominant markers such as ISSR are very efficient alternative to the isozymes etc. due to their hyper variable nature, large number of polymorphic loci analyzed and the

Table 3: Population genetic diversity measures calculated for 11 Parthenium weed populations along with their respective means and standard errors (SE), N is the sample size in the respective populations, Na is the observed no. of alleles; Ne is the effective no. of alleles, I is the Shannon's information index and He is the expected heterozygosity whereas UHe is the Unbiased effective heterozygosity= 2N (2N-1) ×He

Population		N	Na	Ne	I	He	Uhe
Pop1	Mean	20.000	1.746	1.471	0.399	0.269	0.276
	SE	0.000	0.054	0.048	0.034	0.024	0.025
Pop2	Mean	10.000	1.746	1.362	0.341	0.221	0.232
	SE	0.000	0.054	0.042	0.031	0.022	0.023
Pop3	Mean	20.000	1.672	1.321	0.319	0.204	0.210
	SE	0.000	0.058	0.037	0.031	0.021	0.021
Pop4	Mean	5.000	1.328	1.335	0.277	0.188	0.209
	SE	0.000	0.091	0.049	0.037	0.026	0.029
Pop5	Mean	5.000	1.537	1.287	0.266	0.174	0.193
	SE	0.000	0.061	0.043	0.033	0.023	0.026
Pop6	Mean	5.000	1.493	1.352	0.302	0.205	0.228
	SE	0.000	0.068	0.046	0.036	0.025	0.028
Pop7	Mean	5.000	1.657	1.449	0.375	0.255	0.284
	SE	0.000	0.058	0.048	0.036	0.025	0.028
Pop8	Mean	10.000	1.657	1.296	0.294	0.188	0.198
	SE	0.000	0.058	0.038	0.031	0.021	0.022
Pop9	Mean	5.000	1.612	1.409	0.352	0.237	0.263
	SE	0.000	0.067	0.047	0.035	0.025	0.028
Pop10	Mean	5.000	1.388	1.285	0.269	0.176	0.195
	SE	0.000	0.090	0.041	0.033	0.023	0.025
Pop11	Mean	5.000	1.537	1.361	0.321	0.213	0.237
	SE	0.000	0.078	0.045	0.034	0.024	0.027

Table 4: Summary of AMOVA showing degree of freedom (df), sum of squares (SS), Mean Sum of squares (MS), estimated variance and percentage variation

Source	df	SS	MS	Estimated. Variance	% variation
Among Pops	10	259.966	25.997	2.023	18%
Within Pops	84	779.550	9.280	9.280	82%
Total	94	1039.516		11.303	100%

use of small amount of fresh/dried sample (Zhang *et al*., 2006). However, the accuracy of the estimation is reduced in dominant data because the frequency of dominant allele is hard to be calculated directly from the gels. This is due to the fact that the presence of the band can denote either a dominant homozygote (AA) or a dominant heterozygote (Aa). ISSR fingerprinting protocols were chosen as the preliminary studies on the genome of PW as ISSR fingerprinting has not been applied to PW populations in Pakistan and Australia, where no prior knowledge of sequence information was present.

For addressing the problem of dominant data, AMOVA was determined, which is actually based on genetic distances and the observed variation is partitioned into within and among population components. AMOVA results in our study are in close accordance to the study done on Chinese PW populations (Tang *et al*., 2009). Similarly, the Shannon's Information index is also calculated which actually measures the diversity estimates based on band phenotypes (DNA band present/absent). Thus it is according to the band matching similarity coefficients like Jaccard's coefficient (1908), Dice coefficient (1945) and Nei and Li coefficients (1979). The population diversity measures, PCA and AMOVA indicated that PW population was highly heterogonous and contain high degree of genetic variation, which is also evident by the varying morphological characters in the populations.

In two dimensional PCA, Okara and Sahiwal samples form a distinct group along the Australian PW samples may be due to the cultivation of imported wheat in the fields at Okara and other parts of southern Punjab and PW grows as the contaminant of food grains. Other Pakistani populations formed another distinct group, which showed that the climate change and environment at distinct locations are responsible for posing range of diversity among the PW populations. However, further studies are required on this. It is noted that during mid1960's short statured Maxi-Pak wheat variety was introduced from Mexico in Pakistan, and it is likely that PW invaded along Maxi-Pak and spread throughout in north eastern part of Pakistan.

It was observed that the PW was scarcely found on the way on newly opened Peshawar Motorway (M1) while at the Grand Trunk (GT) Road and National Highway it was present in majority with diverse morphological characters. These roads are mostly used by the heavy transport for transfer of materials, goods and imported food grains etc.

from Karachi (Major sea port of Pakistan) to all over in Pakistan. The spread of the weeds through the vehicles and mechanized farming is in accordance with the work done at South east Queensland, Australia by Khan *et al*. (2012).

The role of multiple introductions poses a favorable environment for the founder population where genetic variation increase due to the variable influxes of the gene pools that interact to form novel genetic combinations. Thus the gene flow increases and hence in successful invasions multiple introductions are mandatory and seem to be common (Novak and Mack, 2005). Similarly PW exhibit immense phenotypic plasticity and presumed to spread in the same described way. PW exhibits strong adaptive morphological and reproductive characters such as strong allelopathy, rapid growth rate, consist of strong tap root, drought resistance, efficient seed spread mechanisms due to light and small seeds/cypsella and large and long lived seed banks (Adkins and Navie, 2006). The occasional presence of PW in the southern Punjab region (Multan and Bahawalpur) of Pakistan and the satellite PW populations in the central Queensland around the core distribution of Clermont is hypothesized to be suffering from the genetic bottleneck in the recently invaded area. At this situation these founder populations have reduced number of individuals and thus lack of the genetic variation (due to reduced gene pool) might be easier to manage through biological control measures, however more studies are required for understanding the relationship between the genetic diversities and control realization. The heterogonous PW population collected from geographically vast areas in Pakistan with varying ecological conditions is the indicator that multiple introductions have occurred, which has led to the successful invasion along with the environmental effects. Further studies in this regard will indicate the invasion routes and the number of multiple introductions.

Conclusion

Results of this study strongly suggest that high genetic diversity within and among populations based on ISSR markers is correlated with the invasive potential of PW in Pakistan and Australia. Increased genetic diversity i.e., presence of different genotypes at different loci attributes to its intra specific hybridization and hence increased genetic pool assisting PW to adapt novel habitats in a sparse time. PW has invaded Pakistan approximately two decades ago and in the present status PW is not only the weed of wastelands but now possess strong potential to spread in the crop fields reducing the produce/yields and ultimately affecting the GDP of agricultural countries like Pakistan and Australia. Therefore, strict quarantine measures should be opted for the import of different food materials/crops so that the spread of such noxious weeds could be halted. In addition, rigorous integrated control programs are necessary to be launched.

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