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Full Length Article

Assessing Genetic Diversity of Invasive Plant *Nicotiana glauca* in Abha Region, Saudi Arabia by RAPD, ISSR and Mixed Biomarkers

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Abstract

Abha area, Saudi Arabia has unique environmental conditions that may support the expansion of *Nicotiana glauca* and many other weed plants. In this study, *Nicotiana glauca* samples were collected from seven populations having various elevations namely King Abdullah Road- altitude 6.955 feet, Alsamer- altitude 7.272 feet, Lasan- altitude 7.000 feet, Aseer Mall- altitude 6.845 feet, Almahalah- altitude 6.762 feet, Al-sarhan- altitude 6.888 feet and Almoadafeen- altitude 7.015 feet in Abha area, Saudi Arabia. The DNA was extracted from fresh leaves of *Nicotiana glauca* and ten random amplified polymorphic DNA (RAPD) markers, eight inter-simple sequence repeat (ISSR) markers and nine mixed RAPD primers were used to investigate the genetic variations. RAPD markers produced a total of 151 scorable bands and the highest genetic similarity (58.95%) was found between Lasan (7.000 feet) and Aseer Mall (6.845 feet) populations, while the lowest similarity (26.89%) between Alsamer (7.272 feet) and Al-sarahan (6.888 feet) populations. ISSR markers produced 187 bands and revealed that the highest similarity (60.52%) was between Al-sarhan (6.888 feet) and Almoadafeen (7.015 feet) populations, while the lowest similarity value (32.62%) between King Abdullah Road (6.955 feet) and Aseer Mall (6.845 feet) populations. Mixed RAPD primers provided 135 bands and the highest similarity value (59.76%) was between Lasan (7.000 feet) and Aseer Mall (6.845 feet), while the lowest similarity value (24.42%) was between King Abdullah Road (6.955 feet) and Al-sarhan (6.888 feet). Total RAPD, ISSR and mixed RAPD data showed that the highest similarity value (51.12%) was between Lasan (7.000 feet) and Aseer Mall (6.845 feet), while the lowest similarity value (33.05%) between King Abdullah Road (6.955 feet) and Al-sarhan (6.888 feet). In conclusion, investigated biomarkers including RAPD, ISSR and mixed RAPD showed varied ability to distinguish among the genotypes of *N. glauca* plant growing in Abha region, Saudi Arabia and explain why the plant invasive to many localities. © 2018 Friends Science Publishers

Keywords: DNA; Similarity value; Biomarkers; PCR; Polymorphic bands

Introduction

Nicotiana glauca Graham (wild tobacco) (Family: *Solanaceae*) considers as a new invasive introduced plant to Abha region, Saudi Arabia. It is small erect plants grown in dense patches in dry and in neglected areas such as river side's and in road sides in height between 0.000 to 3700 meters above the sea level (González *et al*., 2012). Its high fruiting rates and high seeds viability leads to that the plant always seeing in monodominant stands and forming dominant populations competing or vanishing the native species (Ollerton *et al*., 2012). It was reported that the adaptation of *N. glauca* plants to drought and salinity are due to water conservation by closing the stomata and the ability to adjust the process of osmotic pressure due to the presence of a waxy layer on leaves (González *et al*., 2012). *N. glauca* first cultivated in many countries for decorations and later the species dispersed in many countries either nationally or internationally (DiTomaso *et al*., 2013).

The molecular characterization and identification is considered as an important key to determine, analyze and estimate the genetic diversity in plant species. The molecular markers techniques include inter simple sequence repeat (ISSR), the randomly amplified polymorphic DNA (RAPD) and/or mixed biomarkers either RAPDs or ISSRs. RAPD, ISSR considered as an easy tool for characterization and identification the genetic variation among many plant at the species or varieties (Williams *et al*., 1990). Those types of primers can be used to amplify specific fragments of genomic DNA and clearly specify the fingerprinting of investigated plants (Bielawski *et al*., 1996). For example, PCR-based RAPD and ISSR or mixed type had been used successively to distinguish among the plant such as *euphorbia* species (Moustafa *et al*., 2016a), among *Ziziphus spina-christi* L. populations in Abha area (Moustafa *et al*., 2016b) and for *Pittosporum eriocarpum* (Thakur *et al*., 2016) and many else. As no report available about genetic diversity of *N. glauca* plants, therefore, this study was conducted to investigate the genetic identification and diversification of *N. glauca* plants invasive to Abha region,

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KSA by using numbers of randomly amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) markers and mixed RAPD primers.

Materials and Methods

The Study Area

Sample of *N. glauca* were collected from seven various populations that located near from each other at altitudinal range between N 18° 19' 15.4194" to N 18° 11' 7.3284" and at longitudinal range between E 42° 39' 27.8022" to E 42° 30' 41.9544" and at height between 6.762 to 7.272 feet above the sea level. These seven sites namely; King Abdullah Road (N 18° 11' 7.3284", E 42° 38' 27.7506" and altitude 6.955 feet), Alsamer (N 18° 13' 29.5284", E 42° 30' 41.9544" and altitude 7.272 feet), Lasan (N 18° 14' 13.2432", E 42° 35' 24.6516" and altitude 7.000 feet), Aseer Mall (N 18° 14' 35.4048", E 42° 36' 43.7076" and altitude 6.845 feet), Almahalah (N 18° 19' 15.4194", E 42° 35' 23.1678" and altitude 6.762 feet), Al-sarhan (N 18° 11' 13.3038", E 42° 39' 27.8022" and altitude 6.888 feet) and Almoadafeen (N 18° 14' 12.3144", E 42° 35' 46.7484" and altitude 7.015 feet) as shown in (Fig. 1).

Plant Materials

Three grams from fresh young leaves were collected from healthy plants from each population in July 2016 and kept in the refrigerator at -4^{o}C for extraction the genomic DNA.

Extraction Genomic DNA

The genomic DNA was extracted from frozen young leaves of *N. glauca* by using commercial kit, "DNeasy plant mini kit provided by QIAGEN-USA COMPANY". All the steps were indicated in the Kit.

Estimation DNA Concentration by Spectrophotometer

Estimation the genomic DNA from each sample was accomplished by using a Thermo Scientific™ BioMate 3S UV-Visible (260 nm). The purity of genomic DNA was estimated by calculating the absorbance ratio at OD_{260}/OD_{280}. As described previously by Maniatis *et al*. (1982), Rawashdeh (2011), the quality and integrity of obtained DNA from the fresh leaves of invasive *N. glauca* were analysed by 1% electrophoresis agarose gel.

Primers

Ten RAPD markers, eight ISSR markers and nine of mix RAPD primers had been used to investigate is any change in DNA finger prints pattern of *N. glauca* plants grown close to each other.

PCR Conditions

Polymerase chain reaction (PCR) for RAPD, ISSR and mixed RAPD markers were performed by using thermal cycler Bio-Rad. In a final volume of 25 μL composed of 25 ng of genomic DNA template from *N. glauca* plants, 12.5 μL of Taq DNA polymerase (2.5 m*M* $MgCl_2$, 2.5 m*M* of each dNTPs) and 7 μL from each primer and the total volume adjusted with water free nuclease (Sushant *et al*., 2013). Amplification process as follows: Initial denaturation of genomic DNA at 94°C for 5 min., followed by repeated 49 cycles of denaturation at 94°C for 1 min., annealing temperature 30°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min.

Agarose Gel Electrophoresis

Agarose gel (1%) was prepared for visualization of resulted genomic DNA from each *N. glauca* plants. 1.43% agarose gel had been applied to visualize the amplified fragments of DNA resulted from RAPD-PCR, ISSR-PCR and mix RAPD-PCR products. Agarose gels had been strained with ethidium bromide in final concentration 0.5 μg/mL. Gels were run horizontally in 0.5X Tris-borate-EDTA (TBE) buffer. Electrophoresis buffer was added to cover the gel and run for 60 min at 90 voltages and visualized by a gel documentation system (ProXima AQ-4) (Marsafari and Mehrabi, 2013).

Data Analysis

Resulted DNA fragments from RAPD, ISSR and mix RAPD were manually scored as present (1) or absent (0) from the photographs to calculate the amount of similarity of the tested *N. glauca* plants. The matrix of similarity, based on Rogers and Tanimoto similarity coefficient and squared euclidean distance was used to calculate the distances and to generate a dendrogram (Chikkaswamy and Prasad, 2012).

RAPD Analysis

The reproducible bands from seven populations of *N. glauca* genotypes using ten RAPD primers are shown in (Fig. 2). The genetic diversity among *N. glauca* populations was calculated by using Rogers and Tanimoto's similarity coefficient and the data are shown in (Table 1). The highest similarity was 58.95% between Lasan and Aseer Mall populations. The lowest similarity was 26.89% between Alsamer and Al-sarahan populations. The dendrogram that gained from *N. glauca* based on Wards Euclidean methods showed three main clusters (Fig. 5A). The first cluster consisted of two subclusters. King Abdullah Road population represented the first subcluster with similarity values ranged from 37.9 to 50.25% with other populations.
The second subcluster includes Lasan and Aseer Mall populations which showed high relationship to each other

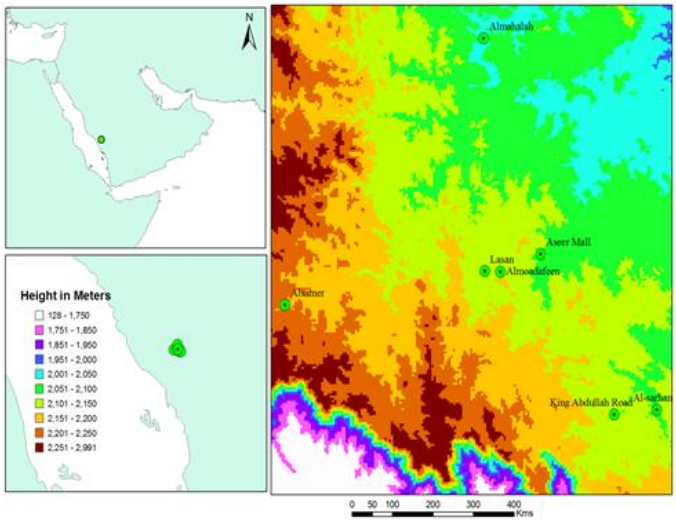

Fig. 1: Sampling sites in Abha region, KSA. Seven sites including King Abdullah Road, Alsamer, Lasan, Aseer Mall, Almahalah, Al-sarhan, Almoadafeen

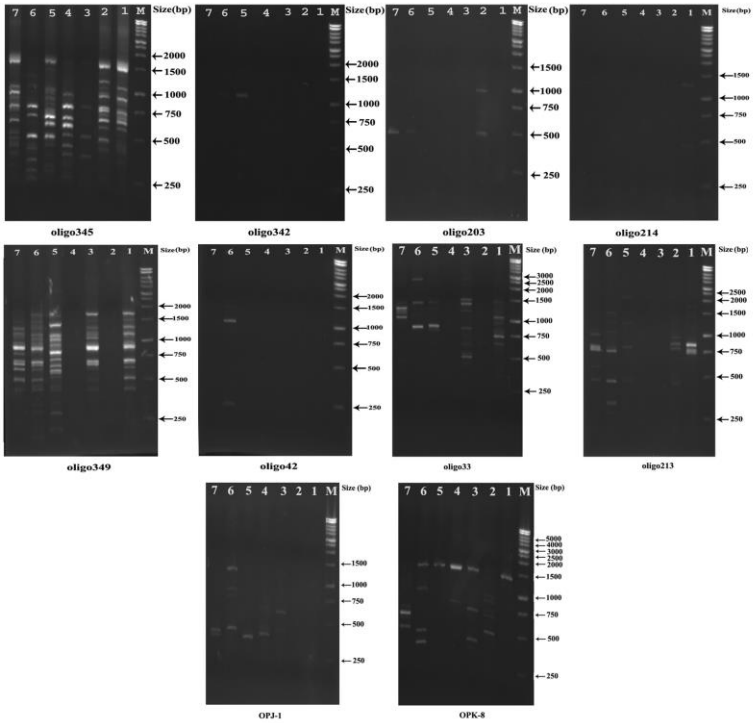

Fig. 2: Amplified bands gained from RAPD primers applied to 7 populations of *N. glauca*. Lane (1) King Abdullah Road, Lane (2) Alsamer, Lane (3) Lasan, Lane (4) Aseer Mall, Lane (5) Almahalah, Lane (6) Al-sarhan, Lane (7) Almoadafeen; M, Molecular weight marker (1 kb DNA Ladder in right side)

with similarity value 58.95%. The second cluster (out-group) included Alsamer population with similarity values ranged from 26.89 to 51% with other populations. The third cluster contained three genotypes of *N. glauca* which bifurcate into two subclusters; the first one included Almahalah population and Almoadafeen population with similarity value of 49.5%, while the second subcluster contained one genotype of *N. glauca* from Al-sarhan population with similarity values ranged from 26.89 to 45.19% with other populations. The ten RAPD primers produced a total of 151 scorable bands from the seven studied populations of *N. glauca* with an average 15.1 per marker. The polymorphic bands were 72 with an average of 7.2 polymorphic fragments per primer. The polymorphism percentage ranged from 0.00 (*Oligo214*) to

Table 1: Genetic similarity among *N. glauca* populations based on RAPD markers

Population	King Abdullah Road	Al-samer	Lasan	Aseer Mall	Al-mahalah	Al-sarhan	Al-moadafeen
King Abdullah Road	100						
Alsamer	37.9	100					
Lasan	50.25	36.65	100				
Aseer Mall	45.19	51.00	58.95	100			
Almahalah	41.78	39.17	44.50	51.00	100		
Al-sarhan	37.27	26.89	38.53	36.65	45.19	100	
Almoadafeen	43.81	41.12	46.60	48.77	49.50	44.50	100

Table 2: The statistical data from ten RAPD primers

Primer ID	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism %
Oligo203	7.00	1.00	0.00	6.00	14.29
Oligo342	5.00	1.00	0.00	4.00	20.00
Oligo345	31.00	23.00	0.00	8.00	74.19
Oligo42	5.00	1.00	0.00	4.00	20.00
Oligo349	24.00	17.00	0.00	7.00	70.83
Oligo214	2.00	0.00	0.00	2.00	0.00
Oligo213	19.00	8.00	0.00	11.00	42.11
Oligo33	26.00	11.00	0.00	15.00	42.31
OPK-8	20.00	7.000	0.00	13.00	35.00
OPJ-1	12.00	3.000	0.00	9.00	25.00
Total	151	72.00	0.00	79.00	34.373

Table 3: Genetic similarity among *N. glauca* populations based on ISSR markers

Population	King Abdullah Road	Al-samer	Lasan	Aseer Mall	Al-mahalah	Al-sarhan	Al-moadafeen
King Abdullah Road	100						
Alsamer	55.83	100					
Lasan	53.28	53.91	100				
Aseer Mall	32.62	33.10	40.07	100			
Almahalah	36.00	34.53	47.24	39.55	100		
Al-sarhan	36.50	38.01	47.83	38.01	59.83	100	
Almoadafeen	33.57	33.10	46.67	38.01	52.03	60.52	100

74.19% (*Oligo345)* with an average of 34.37% polymorphisms. No any monomorphic bands and 79 unique bands with an average of 7.9 unique bands per primer were obtained as shown in Table (2).

ISSR Analysis

Seven genotypes of *N. glauca* plant collected from Abha region were amplified by using eight ISSR primers. The resulted bands from ISSR primers were shown in (Fig. 3). ISSR primers yielded a total of 187 scorable bands with average 23.375, out of which 121 bands were found to be polymorphic bands, 9.00 monomorphic band, 57.00 unique bands and the percentage of polymorphism was 65.04% as shown in (Table 4). The highest similarity value (60.52%) was between Al-sarhan and Almoadafeen populations while the lowest similarity value (32.62%) between King Abdullah Road and Aseer Mall as shown in (Table 3). The dendrogram showed three clusters (Fig. 5B). The first cluster included two subclusters, the first sub cluster between King Abdullah Road and Alsamer with similarity index value 55.83%. The second sub cluster contained one *N. glauca* genotype grown in Lasan population with similarity values ranged from 40.07 to 53.91% with other populations. The second cluster (out-group) contained genotype *N. glauca* from Aseer Mall population which formed a separate cluster with similarity index values ranged from 32.62 to 40.07% with other populations. The third cluster included three genotypes of *N. glauca* from Almahalah population, Al-sarhan population and Almoadafeen population which showed highest relationship between Al-sarhan population and Almoadafeen population with similarity index value 60.52% to a moderate relationship between Almahalah population and to the other two locations Al-sarhan and Almoadafeen with similarity index values between 52.03 to 59.83%.

MIX Analysis

Nine mixed RAPD primers were used for amplification the seven populations of *N. glauca* genotypes (Fig. 4). The DNA bands profile generated by the mixed RAPD primers were 135 as a total number of bands that yielded 7.78 polymorphic bands per primer.

The mixed RAPD primers generated 15 as an average of the total bands of *N. glauca* growing in seven studied populations, out of which 70.00 bands were found to be polymorphic, 2.00 monomorphic bands with an average of 0.22 band per primer and 63.00 unique bands with an average of 7 unique bands per primer (Table 6). The

Table 4: The statistical data from eight ISSR primers

Primer ID	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism %
Primer3	29.00	17.00	0.00	12.00	58.62
Primer4	21.00	16.00	2.00	3.00	76.19
UBC823	26.00	21.00	0.00	5.00	80.77
UBC824	20.00	14.00	0.00	6.00	70.00
UBC826	26.00	22.00	2.00	2.00	84.62
Primer1	16.00	11.00	5.00	0.00	68.75
MB11	20.00	8.00	0.00	12.00	40.00
MB14	29.00	12.00	0.00	17.00	41.38
Total	187	121	9.00	57.00	65.04

Table 5: Genetic similarity among *N. glauca* populations based on mixed RAPD markers

Population	King Abdullah Road	Al-samer	Lasan	Aseer Mall	Al-mahalah	Al-sarhan	Al-moadafeen
King Abdullah Road	100						
Alsamer	38.46	100					
Lasan	39.90	45.95	100				
Aseer Mall	45.95	52.54	59.76	100			
Almahalah	31.71	39.90	38.46	45.95	100		
Al-sarhan	24.42	37.06	37.06	45.95	37.06	100	
Almoadafeen	37.76	48.35	53.41	58.82	45.16	40.63	100

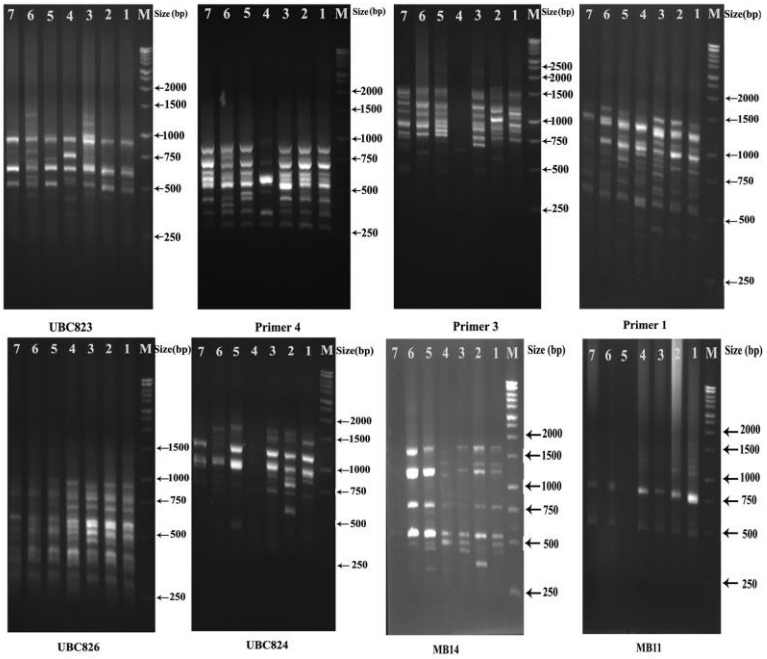

Fig. 3: Amplified bands gained from ISSR primers applied to 7 populations of *N. glauca*. Lane (1) King Abdullah Road, Lane (2) Alsamer, Lane (3) Lasan, Lane (4) Aseer Mall, Lane (5) Almahalah, Lane (6) Al-sarhan, Lane (7) Almoadafeen; M, Molecular weight marker (1 kb DNA Ladder in right side)

percentage of polymorphism was 47.17%, whereas primers (*oligo203+oligo349*) yielded the highest percentage value of polymorphism of 69.23% while the lowest percentage value of polymorphism of 20.00% was obtained from primers (*oligo203+oligo42*) (Table 6). Similarity coefficient for the seven populations of *N. glauca* genotypes based on mixed RAPD primers was ranged from 59.76% between Lasan and Aseer Mall to 24.42% between King Abdullah Road and Al-sarhan (Table 5). The dendrogram pattern (Fig. 5C) generated by nine mixed RAPD primers had three clusters. The first cluster (out-group) included *N. glauca* from King Abdullah Road population with least similarity index values ranged from 24.42 to 45.95% with other populations' genotype. The second cluster (in-group) contained four genotypes of *N. glauca* grown in Alsamer population, Lasan population, Aseer Mall population and Almoadafeen population. Alsamer population formed a separate sub cluster from other three populations with similarity index

Table 6: The statistical data for nine mixed primers

Primer ID	Total of bands	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism (%)
oligo203+oligo342	17.00	8.00	0.00	9.00	47.06
oligo203+oligo345	7.00	2.00	0.00	5.00	28.57
oligo203+oligo42	5.00	1.00	0.00	4.00	20.00
oligo203+oligo349	26.00	18.00	0.00	8.00	69.23
oligo203+oligo214	12.00	6.00	0.00	6.00	50.00
oligo203+oligo213	7.00	4.00	1.00	2.00	57.14
oligo203+oligo33	27.00	14.00	0.00	13.00	51.85
oligo203+OPJ-1	18.00	8.00	1.00	9.00	44.44
oligo203+oligo342+oligo345	16.00	9.00	0.00	7.00	56.25
Total	135	70.00	2.00	63.00	47.17

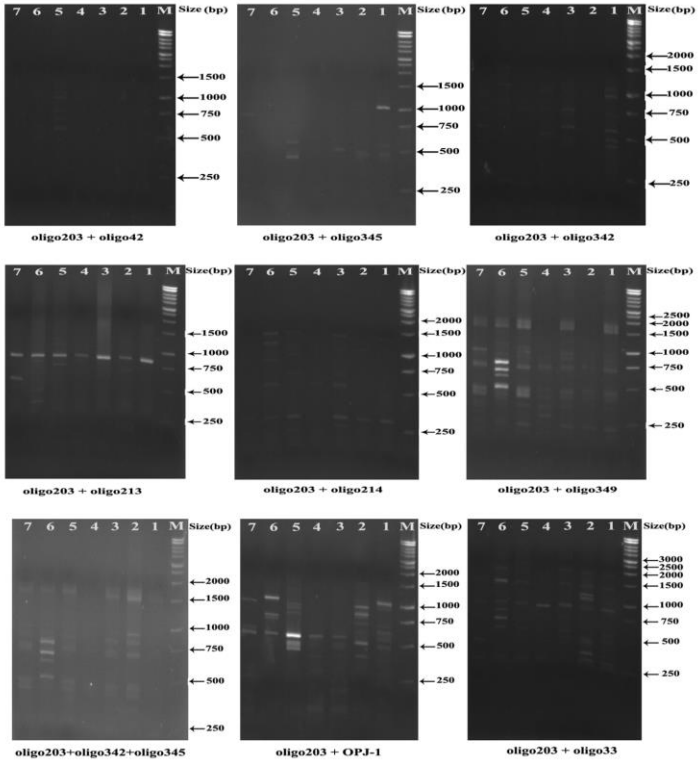

Fig. 4: Amplified bands gained from mixed RAPD primers applied to 7 populations of *N. glauca*. Lane (1) King Abdullah Road, Lane (2) Alsamer, Lane (3) Lasan, Lane (4) Aseer Mall, Lane (5) Almahalah, Lane (6) Al-sarhan, Lane (7) Almoadafeen; M, Molecular weight marker (1 kb DNA Ladder in right side)

values ranged from 45.95 to 52.54%. The other subcluster contained three genotypes of *N. glauca* from Lasan population, Aseer Mall population and Almoadafeen population which showed highest relationship between Lasan population and Aseer Mall population with similarity index value 59.76% . The moderate relationship between Almoadafeen population and between Lasan and Aseer Mall with similarity index values ranged from 53.41 to 58.82%. The third cluster composed of two genotypes of *N. glauca* from Almahalah and Al-sarhan with index similarity value 37.06%.

Sum of RAPD, ISSR and Mixed RAPD Data

Combined analysis of RAPD, ISSR and mixed RAPD primers are shown in (Table 7). The highest similarity value (51.12%) was between Lasan and Aseer Mall, while the lowest similarity value (33.05%) between King Abdullah Road and Al-sarhan. The dendrogram depicted the genetic relationship among the clusters from seven populations of *N. glauca* genotypes based on RAPD, ISSR and mixed RAPD data had three clusters (Fig. 5D). The first cluster comined between King Abdullah Road and Alsamer populations with similarity index value 44.65%. The second cluster contained two genotypes of *N. glauca* from Lasan population and Aseer Mall population which showed a high relationship to each other with similarity index value 51.12%. The third cluster contained Almahalah population with similarity values ranged from 33.05 to 49.21% and the sub cluster combined between Al-sarhan and Almoadafeen with similarity index value 49.21%.

Table 7: Genetic similarity among *N. glauca* populations based on sum RAPD, ISSR and mixed RAPD data

Population	King Abdullah Road	Al-samer	Lasan	Aseer Mall	Al-mahalah	Al-sarhan	Al-moadafeen
King Abdullah Road	100						
Alsamer	44.65	100					
Lasan	48.28	45.76	100				
Aseer Mall	40.15	43.77	51.12	100			
Almahalah	36.51	37.50	43.77	44.87	100		
Al-sarhan	33.05	33.99	41.62	39.73	48.04	100	
Almoadafeen	37.90	39.73	48.51	46.89	49.21	49.21	100

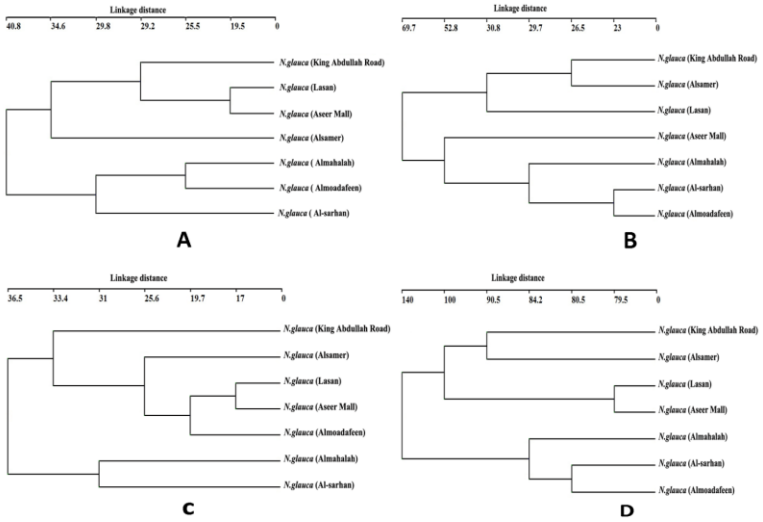

Fig. 5: Genetic similarity relationships among seven populations of *N. glauca* genotypes based on: (A) RAPD, (B) ISSR, (C) mixed RAPD data, (D) Sum of RAPD, ISSR and mixed RAPD data

Discussion

Examination of genetic diversity among the *N. glauca* populations which collected from various localities in close distance in Abha region, KSA, were evaluated by various biomarkers. The present results showed that the PCR amplifications from RAPD primers yielded a high values of polymorphism. This variation in polymorphisms might be due to the deletion, addition or substitution of base within the priming site sequences same as reported by (Williams *et al*., 1990). This also support the research established by Khan and Narayan (2007) who studied the genetic relationships between species of genus *Nicotiana* by using RAPDs analysis and found that species within a genus diverged from a common ancestral genetic stock and there were variations in polymorphism between the investigated plants that resulted from the DNA sequence. The variations in the number of fragments produced by these arbitrary primers may be also attributed to the differences in the binding sites throughout genome of *N. glauca* plant collected from different sites. Other researchers showed that the variation in the number of bands produced from applying various primers was affected by many factors such as primer structure, quantity of genomic DNA and number of annealing sites, types of equipment used in experimental works also could influence the number of amplified band (Devos and Gale, 1992; Kernodle *et al*., 1993).

In this study, ISSR primers yielded a high percentage of polymorphic bands than RAPD primers. This explains that the ISSR markers were efficient than RAPD markers in the ability to reveal more informative bands. It was found that ISSR primers produced more information than RAPD markers in studying the genetic diversity among date palm cultivars and among wheat plants (Nagaoka and Ogihara, 1997; Elmeer *et al*., 2017). However, the obtained values are not in agreement with very recent study by Aladadi *et al*. (2018) who observed that old genomic cultivars of date palm (*Phoenix dactylifera* L.) generated more specific bands from RAPD markers than ISSR markers. Again, this probably explains that the number of repetitive sequences among plant species varies greatly. Therefore, ISSR technology or RAPD markers are sensitive to considerable levels of genetic variation and providing complementary information for studying the genetics on a wide range of plant species, as well as for identifying species, cultivars, or population of the same species because they detect different evolutionary features and polymorphisms. Different markers might reveal different classes of variation and these may correlate with the genome fractions surveyed by each kind of marker and their distribution in various parts of the genome (Loveless and Hamrick, 1984). In the present research, the monomorphic bands appeared with ISSR primers while no any monomorphic bands by using tested RAPD primers. In addition, there were differences among the seven populations

of *N. glauca* in terms of unique bands which explains that *N. glauca* plant possesses high level of genetic diversity. The differences among *N. glauca* populations at the level of genetic may be resulted from the slight change in environmental factors, for example, types of soils, amount of precipitation and many other biotic and abiotic factors. Kooyers *et al.* (2015) clarified that genetic diversity for *Mimulus guttatus* plant had significant variations in case changes in environmental conditions accompanied with variation in altitudinal gradients.

To the best of our knowledge this is the first report indicating the use of mixed RAPD primers in a sequential way, to investigate the plant genetic diversity. However, Aguoru *et al*. (2015) used two types of multiplex primers in studying molecular characterization of some *Solanum* species. In our study, the DNA bands generated by the di-mixed and multiplex primers could be considered as a great step in the genetic analysis whereas in some cases the resulted bands more countable. The obtained results also showed that obtained polymorphic bands from mixed RAPD-PCR more than RAPD-PCR alone and this also indicates the usefulness to study the biological diversity to *N. glauca* plants using such primers. The dendrogram generated from combined RAPD, ISSR and mixed RAPD markers showed high degree of converge among studied populations more than other studied cases. This variation between the obtained results of RAPD-PCR and ISSR-PCR probably due to the fact that PCR profiles from both primers are amplified from different non-repetitive and repetitive regions of the genome of *N. glauca* plants by applying the two marker systems as indicated early (Thormann *et al*., 1994).

Conclusion

The genetic relationships among *N. glauca* growing in close distance in Abha region, KSA, were evaluated by RAPD, ISSR and mixed RAPD primers. These biomarkers showed ability to distinguish genotypes of *N. glauca* plant grown in various localities in Abha region at the DNA level. High genetic diversity among the species of *N. glauca* plant grown in the same area explain why the plant consider as invasive species to many localities of the world.

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