Molecular Insights Into the Genetic Diversity and Population Structure of *Artemisia Annua* L. As Revealed by Insertional Polymorphisms

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Research

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Abstract

Background

Retrotransposons-RTNs, are main source of variations in plant genomes that copying and pasting themselves into different transposon and work by changing RNA back into DNA via reverse transcription. For that reason, they are largely utilized in plants as optimum molecular markers to determine DNA fingerprinting, genetic mapping, and genetic variability.

Results

Inter-retrotransposon amplified polymorphisms (IRAPs) were used to measure genetic variability and structure in a collection of 118 sweet wormwood (*A. annua*) accessions identifying and amplifying 849 loci using 32 IRAP primers, derived from *Rosaceae*, *Gramineae*, and *Solanaceae* retroelements. Single IRAP primer Tnt1.OL16 based on RTN produced the maximum count of markers. Percentage of polymorphic loci (PPL), mean expected heterozygosity (He), number of effective alleles (Ne) and information index (I) of Shannon, in the studied collection were 95.80%, 0.30, 1.48 and 0.46, in the same order. AMOVA analysis showed nonexistence of significant genetic structures during structure analysis, however, the 4 populations had three clusters based on the NJ dendrogram that depicted a relatively higher level of genetic variation within each population. These clusters were approximately congruent with corresponding geographical distributions.

Conclusions

In conclusion, low genetic diversity of Iranian Sweet wormwood was detected that could be reduced through introduction of appropriate exotic or improved germplasm to reduce the effects of inbreeding depression.

Background

Medicinal Sweet wormwood (*Artemisia annua* L., $2n = 18$), Persian vernacular name “Dermaneh” is a fast growing, branched plant species that reaches about 150 cm in height [1]. It is included among 35 species from genus *Artemisia*, family *Asteraceae* that are widely distributed throughout Iran [2–4] and is highly heterozygous [5]. It is also found in Australia, Central Asian, South East Asian and West Asian countries variably [6, 7].

The plant is used as source of obtaining artemisinin, that is an endoperoxide sesquiterpene lactone, with anti-malarial properties at concentrations of 0.01% – 2% leaf dry weight [8]. Dried leaves of the plant are also a source of both essential oil (1.4-4.0%) and other active metabolites like sesquiterpene lactones, flavonoids, polyalkynes, antioxidants and coumarins [9].

The exact prediction of genetic difference in a specific germplasm is important for efficient utilization, survival, evolution, and conservation of crop genetic resources improvement programs [10, 11]. To begin

with strategic crop improvement programs, information about inter and intra genetic variation among existing *A. annua* germplasm is crucial for their management as well as conservation and development of new cultivars with higher quantities of artemisinin and better quality of other secondary metabolites [12, 13]. Genetic diversity among medicinal plants could be understood using molecular (DNA), phenotypic (morphological) and biochemical markers [14–16], but morphological markers are influenced by environmental determinants controlled by epistatic and pleiotropic gene effects and could show a little variation [14]. In contrast, the use of molecular markers are not influenced by the environment and overcome these disadvantages. The use and development of molecular markers for study of backcrosses, phylogenetic, population genetics, marker assisted selections, mapping of desired genes, analysis of genetic relationships, building linkage maps, the exploitation and detection of DNA polymorphism is being carried out in the subjects of molecular biology, biotechnology and genetics. There is need to carefully select any type of molecular markers. Because variations in their methodologies, applications and principles, need careful considerations before selecting them by their searchers. DNA-based molecular markers generate new probabilities of genome genotyping provided by beneficial molecular evidence to evaluate genetic diversity. Several researches have been performed to measure genetic diversity and structure of *Artemisia* species population worldwide during the last ten years, using RAPD [15–18], AFLP [1], ISSR [18–22], SSR [23] and EST-SSR markers [23, 24]. So far, only one study with significantly small native populations of *A. annua* collected from different areas of Northern Iran size has been studied using four AFLP markers, [1] for evaluation of genetic diversity.

Retrotransposons (RTNs) marker systems have not been previously used for evaluation of any sweet wormwood accessions in Iran. It is important to evaluate population structure and genetic diversity for these exceptionally rare resources. Therefore, this study was carried out to efficiently facilitate utilization and conservation of Iranian Sweet wormwood genotypes. To determine their genetic diversity and population structure along with determining their relationships at species level. Many features of RTNs, such as their ubiquity, activity, abundance and general dispersion in the plant genome, offer a tremendous foundation for the improvement of molecular marker systems [25, 26]. Different types of DNA molecular markers were developed from RTNs that most of RTNs are among the main sources of genetic diversity. Among them, inter RTN amplified polymorphism (IRAP) illuminates polymorphisms that amplifies segments between two close by long terminal repeats (LTRs) using primers complementary to the 3' end of a LTR sequence [25]. IRAP as a RTN based markers system with a high level of polymorphism are rarely used to investigate genetic diversity in medicinal plants such as *Crocus* [27], *Leonurus cardiac* L. [28], and Flax (*Linum usitatissimum* L.) [29, 30], *Hypericum perforatum* L. [31] taro (*Colocasia esculenta*), cocoyam (*Xanthosoma sagittifolium*) [32] and wild almond [33].

We used IRAP system marker to find activity of LTR RTN families in the Iranian Sweet wormwood after isolating them from barley, tobacco and apple. The aim of the research was to find desired RTN markers for checking diversity and characterize them for polymorphism of integration events through combing of the markers among 118 Sweet wormwood accessions that originated from four populations originating from different eco-geographical locations of Iran.

Results

Retrotransposon insertional polymorphism in sweet wormwood genome and IRAP analysis

The study made use of 32 IRAP primers tested on 118 sweet wormwood accessions (Table 1) for initial screening. Among these primers, 12 single primers (Ty1-Copia, LTR6149, LTR6150, LTR1, Nikita, Sukkula, Tnt1.OL16, ToRTL1, 3'LTR, Tnt-1, 5'LTR1, 5'LTR2) and 20 primer combinations (3'LTR/LTR1, 3'LTR/Tnt-1, Bare1/LTR2, Nikita/LTR1, Nikita/Sukkula, Nikita/Ty1-Copia, Tnt-1/Sukkula, Tnt-1/5'LTR2, Tnt1.OL16/Ty1-Copia, Tnt1.OL16/Nikita, Tnt1.OL16/LTR6150, Tnt1.OL16/5'LTR1, Tnt1.OL16/5'LTR2, Tnt1.OL16/Sukkula, ToRTL1/Sukkula, ToRTL1/5'LTR2, LTR6150/5'LTR1, LTR6150/5'LTR2, 5'LTR1/3'LTR, 5'LTR1/5'LTR2) produced 849 distinguished and scored loci, out of 819 loci (95.80%) that were polymorphic and that 14 primers, LTR6149, LTR1, Sukkula, Tnt1.OL16, 3'LTR, Tnt-1, 3'LTR/LTR1, 3'LTR/Tnt-1, Bare1/LTR2, Nikita/LTR1, Nikita/Sukkula, Tnt-1/Sukkula, Tnt1.OL16/Ty1-Copia and ToRTL1/Sukkula, represented 100% polymorphism. The length of the amplified fragments differed in range of 100–3,000 bp, most of the band sizes had range of 500bp -2000bp. Single Primer Tnt1.OL16 generated the maximum amplified and polymorphic loci and showed the highest Ne, He and I (Table 1, Supplementary Fig. 2). The lowest values of Ne, He and I parameters were achieved for the primer combination ToRTL1/Sukkula. The number of scored loci for these primers ranged from 60 to 12 with a mean of 26.53 loci for each primer. Further detail of the primers is shown in Table 1. Supplementary Fig. 2 shows the banding pattern of Tnt1.OL16 and LTR1 primer in some sweet wormwood accessions. RTN activity and comparison of these families are shown in Table 2.

Table 1
Characteristics of the IRAP primers used in this study

Primer	TNB	NPB	PPB	Ne	I	He	Annealing temp. (C°)	Band size (bp)
Ty1-Copia	42	41	97.6	1.50	0.49	0.32	58	100–3000
LTR6149	18	18	100	1.56	0.48	0.32	65	150–2000
LTR6150	14	13	92.8	1.45	0.43	0.29	59	250–2000
LTR1	35	35	100	1.50	0.47	0.31	64	150–3000
Nikita	20	19	95	1.44	0.43	0.27	65	200–2000
Sukkula	23	23	100	1.52	0.48	0.31	64	100–3000
Tnt1.OL16	60	60	100	1.68	0.58	0.39	61	100–3000
ToRTL1	43	41	95.3	1.45	0.44	0.29	61	100–3000
3'LTR	30	30	100	1.64	0.54	0.37	64	200–3000
Tnt-1	12	12	100	1.36	0.39	0.24	65	200–1500
5'LTR1	15	13	86.7	1.41	0.40	0.26	61	200–2000
5'LTR2	17	14	82.3	1.51	0.48	0.31	62	150–2000
3'LTR/LTR1	24	24	100	1.55	0.49	0.32	64	200–3000
3'LTR/Tnt-1	33	33	100	1.51	0.48	0.31	64	100–2500
Bare 1/LTR2	28	28	100	1.64	0.55	0.37	61	150–2500
Nikita/LTR1	21	21	100	1.55	0.49	0.32	64	150–2000

TNB total number bands, *NPB* number of polymorphic bands, *PPB* percentage of polymorphic bands, *Ne* effective number of alleles, *I* Shannon's information index, *He* expected heterozygosity

Primer	TNB	NPB	PPB	Ne	I	He	Annealing temp. (C°)	Band size (bp)
Nikita/Sukkula	33	33	100	1.42	0.41	0.26	58	100–3000
Nikita/Ty1-Copia	21	20	95.2	1.50	0.48	0.31	59	200–2500
Tnt-1/Sukkula	25	25	100	1.39	0.40	0.25	65	150–2000
Tnt-1/5'LTR2	23	20	87	1.50	0.47	0.30	62	200–2000
Tnt1.OL16/Ty1-Copia	42	42	100	1.31	0.36	0.21	58	100–2500
Tnt1.OL16/Nikita	24	22	91.7	1.46	0.41	0.27	65	100–3000
Tnt1.OL16/LTR6150	15	14	93.3	1.43	0.41	0.29	65	100–1500
Tnt1.OL16/5'LTR1	21	20	95.2	1.49	0.46	0.30	60	100–2000
Tnt1.OL16/5'LTR2	26	24	92.3	1.51	0.48	0.31	59	100–2500
Tnt1.OL16/Sukkula	17	16	94.1	1.41	0.44	0.31	60	200–2500
ToRTL1/Sukkula	25	25	100	1.23	0.30	0.17	62	150–2500
ToRTL1/5'LTR2	31	30	96.8	1.55	0.49	0.31	63	100–2500
LTR6150/5'LTR1	34	32	94.1	1.56	0.49	0.31	60	100–3000
LTR6150/5'LTR2	25	23	92	1.48	0.49	0.30	64	100–2500
5'LTR1/3'LTR	30	28	93.3	1.49	0.49	0.31	61	100–3000
5'LTR1/5'LTR2	22	20	90.9	1.48	0.47	0.30	61	200–2000
Total	849	819	-	-	-	-	-	-
Average	26.53	25.59	95.80	1.48	0.46	0.30	-	-
TNB total number bands, NPB number of polymorphic bands, PPB percentage of polymorphic bands, Ne effective number of alleles, I Shannon's information index, He expected heterozygosity								

Table 2
Comparison of RTN family in studied sweet wormwood (*A. annua*) accessions from Iran

RTN family	TL	PL	PPL	He	Ne	I
Ty1-Copia	105	103	98	0.28	1.44	0.44
LTR6149	18	18	100	0.32	1.56	0.48
LTR6150	88	82	93	0.30	1.48	0.45
LTR1	80	80	100	0.32	1.53	0.48
Nikita	119	115	97	0.28	1.47	0.44
Sukkula	123	122	99	0.26	1.39	0.41
Tnt1.OL16	205	198	97	0.30	1.47	0.45
ToRTL1	99	96	97	0.26	1.41	0.41
3'LTR	117	115	98	0.33	1.55	0.50
Tnt-1	93	90	97	0.27	1.44	0.43
5'LTR1	122	113	93	0.30	1.49	0.46
5'LTR2	144	131	91	0.30	1.50	0.48
LTR2	28	28	100	0.37	1.64	0.55
Bare 1	28	28	100	0.37	1.64	0.55
<i>TL</i> Total loci, <i>PL</i> Polymorphic loci, <i>PPL</i> Percentage of polymorphic loci, <i>He</i> Mean of expected heterozygosity, <i>Ne</i> Number of effective alleles, <i>I</i> Shannon's information index						

Genetic diversity and population structure of Iranian sweet wormwood accessions

The properties of amplified IRAP loci utilizing 32 IRAP primers are shown in the Table 3. The means (%) of IRAP polymorphic loci in the population ranged 26.02 (East Azerbaijan) to 95.35 (Mazandaran) averaging 76.25. Number of private marker varied from zero in population East Azerbaijan to 6 in population Mazandaran with an average of 3.5. Population Golestan had the maximum genetic diversity ($He = 0.26$, $I = 0.40$), followed by Gilan ($He = 0.24$, $I = 0.38$), East Azerbaijan that noted the minimum genetic diversity indices ($He = 0.11$, $I = 0.16$). To partition and evaluate total genetic variation within and among the populations, AMOVA was carried out that was based on four populations using 849 IRAP loci. Significant variations were detected within populations ($\Phi_{IPT} = 0.05$, $P \leq 0.010$). The level of genetic variation was higher within each population (94%) in comparison to variations across populations (6%) (Data not shown). Gene flow, as estimated by Φ_{IPT} [$N_m = 0.25 (1/\Phi_{IPT}) - 1$], was 3.987, that indicated that genetic variation does not exist among the natural *A. annua* populations.

Table 3
Characteristics of amplified IRAP loci in the studied sweet wormwood (*A. annua*) populations

Parameters	Azerbaijan	Gilan	Golestan	Mazandaran
Number of loci	566	828	815	830
Percentage of polymorphic loci	26.02	93.31	90.33	95.35
No of loci with frequency $\geq 5\%$	566	794	779	775
Number of private loci	0	3	5	6
No of less common loci ($\leq 25\%$)	0	0	0	0
No of less common loci ($\leq 50\%$)	0	58	51	57
Mean of heterozygosity	0.11	0.24	0.26	0.22
Shannon's information index (I)	0.16	0.38	0.40	0.36
Number of effective alleles (N_e)	1.18	1.42	1.38	1.35

To further elucidate and explain concepts the population structure, distance- and model-based cluster analyses we performed. Neighbor-Joining (NJ) clustering to check pattern of differences among 118 accessions. The un-weighted Neighbor-joining algorithm and Number of differences evolutionary distance coefficient sourced IRAP data that grouped 118 accessions of the four populations into three major clusters (Fig. 1). In the resulting tree, the accessions were mainly grouped based on their geographical origins with minor mixture. Cluster I was dominated by the accessions originated from Mazandaran population and consisted of the maximum number of accessions. In addition, three accessions from Gilan population namely Gilan-Raz Kan, Gilan-Rahim Abad and Gilan-Rahim Abad-Polam were classified together in cluster I. Cluster II comprised the accessions originated from Gilan population along with the two accessions from East Azerbaijan. In addition, five accessions from Mazandaran population namely Mazandaran-Mzarostaq, Mazandaran-Shirag, Mazandaran-Ncka-Chaleh Pola, Mazandaran-Ramsar-Ezaki, Mazandaran-Ramsar-Garesmasar and two accessions from Golestan population namely Golestan-Tangrah2, Golestan-Jokleh Bandan were included in this cluster. The third cluster included most of the accessions from Golestan population along with the three accessions from Mazandaran population and three accessions from Gilan population.

To settle or find a solution to the pattern of variation, principal coordinate analysis (PCoA) was used to further elucidate relationships among the selected accessions and assess the population subdivisions. The PCoA bi-plot showed no distinguished cluster pattern for 118 sweet wormwood accessions studied (Fig. 2). The first three axes accounted for 27.00, 19.13 and 15.65% genetic variation in same order, explaining altogether 61.78% of the total variation. These results showed that Mazandaran population was distanced from Golestan population on axis 1. The accessions of Gilan population were scattered over a large area. The second coordinate could not well separate the accessions of Mazandaran, Gilan,

Golestan and East Azerbaijan. Coordinates 1 and 2 enabled a discrimination of all three clusters in the phylogenetic dendrogram in combination. Color-codes of all accessions in 2-dimensional PCoA plot was in accordance with these population groups noted from the “structure” analyses (Fig. 3). Because only 61.78% of the differences in this study was added in the 1st three coordinate components, the sweet wormwood germplasm was also analyzed utilizing the model-based method implemented in the software “structure”. The accessions within a cluster were represented by unique colour while accessions with two different colours indicated admixed forms. The results detected the maximal ΔK at $K = 2$ followed by $K = 7$. The ΔK value reduced with increased K , without showing any peak of ΔK at $K > 7$ (Fig. 3A). The guessed delta K value was 222.23 for 118 sweet wormwood accessions, which represented two subpopulations. More subgroups were noted at the value of $K = 7$ (Fig. 3C), that showed that genotypes admixture confirmed the PCA. Most of sweet wormwood accessions expressed populations admixture of four populations as shown in the structure analysis at $K = 7$ (Fig. 3C).

Discussion

RTN activity and insertional polymorphism in Iranian *A. annua* genome

The main reason of the study was to build a base for the breeding of *A. annua* and find exact level of variation in Iranian populations of sweet wormwood due to the deficiency of enough available data on the subject. Transposable elements may affect the adaptation due to their ability to spread into a genome by self-duplication, and evolution potential of hosts using events like insertion mutations, gene interruption, increment of chromosomal rearrangements and gene expression [34]. Therefore, transposable element-based marker systems could provide authentic and reliable information regarding genotype identification and performances. Recently, Sorkheh et al. [33] exhibited that the molecular genetic diversities measured by RTNs were more than the values measured by amplified fragment length polymorphism (AFLP), of 18 wild almond species.

To date, the IRAP markers are not utilized to measure genetic diversity researches in Iranian *A. annua* accessions. In this study, 32 IRAP primers amplified polymorphic and clear separate banding patterns were utilized to study genetic diversity among 118 sweet wormwood accessions. Single IRAP primers Ty1-Copia, LTR6149, LTR6150, LTR1, Nikita, Sukkula, Tnt1.OL16, ToRTL1, 3'LTR, Tnt1, 5'LTR1, 5'LTR2 generated scoreable banding patterns, that indicated the presence and insertional activities of these elements in *A. annua* genome (Table 1). The multiplicity of IRAP primers bands Tnt1.OL16 favored the opinion that the LTR families, have tendency to induce clusters in *A. annua* genome [35]. Primers Bare1 and LTR2 were monomorphic or did not generate any banding presenting infrequent insertion or absence of these retroelements in the *A. annua* genome, but Bare1 generated much more polymorphism in a combination with LTR2, showing the insertion of these two RTNs near or into each other in the *A. annua* genome. The insertion of Bare1 near the LTR2 and other RTNs has been reported in wild almond genome [33]. The application of the barley RTNs for genome analysis among closely related genera, across species lines, and even sometimes between plant families has been previously demonstrated (27, 31, 33, 36, 37). Sorkheh et al. [33] indicated that LTR primers designed based on barley produced a high

level of insertional polymorphism in wild almond and was active throughout evolution of *Prunus* species. Presence and insertional polymorphism of the Nikita, Sukkula, and Bare-1 elements in *Hypericum perforatum* has also been documented [31]. Du et al. [38] used RTN primers successfully. These were derived from *Gramineae* to identify and characterize 13 fruit crops and described that RTN sequences were isolated from one species can be utilized in another plant species.

The high percentage (%) of polymorphism noted by IRAP (95%) markers proposed activities of the RTN families used in the Iranian sweet wormwood genome in our study. Several studies describe the highly distinguished variations in RTN contents under several abiotic and biotic stress conditions that increased genome size [39, 40]. Sorkheh et al. [33] observed same primers to describe relationships and genetic diversity across wild almond species obtained from west, north-west and central Iran that described that barley RTN based primers showed more distinguished participation to express genetic diversity among the Iranian species.

Genetic characterization and relationship of sweet wormwood accessions

The mean value of expected heterozygosity (H_e), Shannon's Information index (I) and number of effective alleles (N_e), (0.21, 0.32 and 1.33 respectively) in the current study can be compared to that reported with Gaafar et al. [22], Huang et al. [20] and Huang et al. [41]. Huang et al. [20] in a study of genetic diversity among 290 China *A. halodendron* genotypes reported the high level of genetic variation ($I=0.323$, $N_e=1.39$, and Nei's gene diversity (h) =0.22). In another study by Huang et al. [41], reported average values of $I=0.32$, $H_e=0.21$, $N_e=1.33$ in Chinese materials. Studying the genetic diversity of Egyptian *A. judaica* by ISSR markers [22], the Nei gene diversity values were reported from 0.000 to 0.189 with an average value of 0.139 and the I values were varied between 0.000 to 0.278 with an average value of 0.199. These relatively low values indicate moderate insertional activity of the used RTNs, significant inbreeding in the populations studied and nature of floral biology of *A. annua*, induce deadlock throughout process of *A. annua* evaluation and selection. In this study as regards four populations studied from any geographic region tend to have very similar genetic relationship indicating limited germplasm variation or inter-population hybridization regionally. Another reason of low level of variability could be due to human practices such as trading of seeds. However, the strict selection pressure imposed due to genetic improvement results in eroding genetic diversity fully or partially.

AMOVA analysis revealed 94% variation to be within-populations, and 6% among-populations, it was concluded that the analyzed Sweet wormwood germplasm are genetically structured. In *A. halodendron* Huang et al. [20] reported vast genetic differences within-populations (90%) than among populations (10%). Similarly Huang et al. [41], reported 9% differences among populations compared to 91% variations within populations. Low level of variability among population can be explained that these populations share nearly the same gene pool and experience high connectivity, showing that genetic exchange within the population was higher, resulting in wide variation. Therefore, genetic variation between and within populations of a medicinal plant species can be very important for breeding and conservation.

The PCoA (Fig. 2) and Bayesian analysis (Fig. 3) results also support weak divergence between these accessions. But clustering analysis based on NJ algorithm (Fig. 1) clearly discriminated the accessions in three main groups in concordance with geographical distribution of the genotypes. Most of the accessions from Mazandaran, Gilan and Golestan populations aggregated together in cluster I, II and III respectively. In addition two accessions from East Azerbaijan located in cluster II. In an analysis of genetic diversity of Judean wormwood populations collected from Egypt by Gaafar et al. [22] using ISSR markers, clustering according to geographical origin was evident. Principal coordinate analysis (PCoA) demonstrated spatially the relative genetic distances among the individual accessions. The plane of the first three PCoA axes accounted for 61.78 % of the total variation (first axis = 27.00%, second = 19.13 %, third = 15.65%). Most of accessions from Mazandarn population along with a number of accessions from Gilan population clustered separately on the left plane. The total accessions from Golestan population along with a number of accessions from Gilan population clustered together on the left plane. Population structure analysis showed genotypes grouped into 3 subpopulation at $K = 2$ and more division took place at $K = 7$ indicating that all accessions were genetically taken from 7 subpopulations. Any association or relationship with place of origin was not detected among the accessions despite the accessions were collected from separate and distinguished geographical regions of Iran. Population genetic structures were affected by many factors, including fragmentation of habitat, breeding system, gene flow and mechanism of seed dispersal [42]. If gene flow (Nm) < 1 , the population/s is/are highly susceptible to genetic drift [43]. The average Nm value of natural *A. annua* is 3.987, which indicated that genetic variation of *A. annua* is due to gene drift among inter-populations, and the influence of the gene flow within the populations was non-significant.

Conclusion

This study evaluated the population structure, genetic diversity, and relationships of *A. annua* germplasm across Iran using 18 IRAP primers. RTN-based markers are being used newly and are yet to be exploited fully. This approach can provide comprehensive information about level of genetic diversity and population structure of *A. annua* which could be useful for conservation and management of *A. annua* germplasm genetic resource. Single IRAP primer Tnt1.OL16 showed more distinguished contribution in defining the genetic diversity of the studied taxon's or species and their segregation. These results described that the genetic diversity of Iranian Sweet wormwood is low and that makes it a distinct population for extraction of artemisinin, uniformly. However, low genetic diversity in the plant could lead to genetic depression in the plant and lead to number of genetic depression related problems. Therefore, it is important to increase the genetic base of Sweet wormwood germplasm in Iran from a place with significant genetic variation to carry out extended breeding studies or through introduction of exotic germplasm from a place with appropriate eco-geographical background or source population; as the plant is highly heterozygous, cross-pollinating plant with very self-pollination. This will conserve the plant and reduce loss of genetic diversity to improve the effects of inbreeding depression on these genotypes.

Materials And Methods

Plant materials and DNA isolation

This study included 118 Iranian sweet wormwood (*A. annua*) accessions as plant material. Names of accessions, information about collection, and their geographical locations are presented in Supplementary Table 1 - Fig. 1. The seeds were sown in pots having 1-3 cm diameter filled with a mixture of peat and vermiculite in the greenhouse at temperature of $25\pm 1^{\circ}\text{C}$. Above ground leaves and stems were collected after about 4 weeks of growth followed by their instant freezing in 2 mL tubes using liquid nitrogen. Total genomic DNA from each sample was extracted using CTAB method [44]. The quality and quantity of DNAs was measured utilizing spectrophotometer (Bio- Photometer 6131, Eppendorf, Germany). The DNA samples of each plant were diluted using sterilized deionized H_2O to $25\text{ ng }\mu\text{L}^{-1}$ for use in PCR amplification.

IRAP analysis

IRAP analysis was carried out using 14 LTR primers specifically designed and based on *Rosaceae*, *Gramineae*, and *Solanaceae* RTNs [45-48] (Supplementary Table 2) and their pair-wise combinations. PCR amplification was done in a 10 μL reaction mixture with 2 μL of genomic DNA, 4 μL of Master Mix, 3 μL of dd H_2O , and 1 μL of each primer. Optimization of annealing temperature was carried out using gradient PCR. The cycling profile is described as; initial denaturation temperature 94°C for 300 sec; 40 cycles of 94°C for 60 sec, $50\text{-}65^{\circ}\text{C}$ (annealing temperature according to primers; (Table 1) for 60 sec; 72°C for 120 sec; and final extension at 72°C for 420sec. The PCR products were analyzed by 4% ultra-thin (0.2 mm) non-denatured polyacrylamide gel and checked using EtBr stain, utilizing a Gel-Scan 3000 electrophoresis system (Corbett, Sydney, Australia). Thermo Scientific GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp was utilized to estimate size of amplified fragments.

Data analysis

The absence (0) or presence (1) of clear and distinguishable amplified fragments were scored for IRAP analysis. Only well separated and bright bands were scored to compare the extent of the activity, variability and discriminating power of each RTN family, mean of expect heterozygosity (H_e), number of effective alleles (N_e), Shannon's information index (I) were calculated for each RTN family, and percentage (%) of polymorphic bands (PPB), number of bands with $\geq 5\%$ frequency number of private bands, number of less common bands with $\leq 25\%$ and 50% frequency were calculated. AMOVA (Analysis of molecular variance) was carried out to separate the total genetic variation within and among origins based on IRAP data. Principal coordinate analysis (PCoA) was computed making use of genetic distances utilizing binary data described above implemented with GenAlEx 6.4 [49]. A cluster analysis helped to generate a dendrograms utilizing Neighbor Joining (NJ) algorithm, Number of variations, evolutionary distance coefficient with MEGA 4.0 [50].

Pattern of the selected population structure in the selected accessions was analyzed with Bayesian cluster approach shown in Structure 2.3.1 [51]. Ten independent replications were used to set the number of subpopulations (k) from 1 to 10, burn in period and MCMC iterations, both to 100,000, an admixture

model and correlated allele frequencies. The optimal K value (as true cluster number) was calculated by the posterior probability [$\ln P(D)$] and an ad hoc statistic ΔK based on the rate of change in [$\ln P(D)$] between successive K [52] using the software Structure Harvester. Inferred ancestry estimates of genotypes (Q-matrix) were taken out for the selected subpopulations [51].

Declarations

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Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to publication.

Availability of data and materials

The data are available on request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Concept and design of the experiment: NV, HAH, and SAM, Data analysis: HAH, NV and SAM, Writing the manuscript: HAH, NV, SAM and KMK.

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Figures

Figure 1

Grouping of 118 Sweet wormwood (*A. annua*) accessions based on IRAP data using Neighbor Joining clustering algorithm along with number of difference evolutionary distance coefficient.

Figure 2

Principal coordinate analysis (PCoA) of 118 Sweet wormwood (*A. annua*) accessions based IRAP markers. Each individual in the study is represented by one dot, with a symbol color identical to the specified subgroup classification.

Figure 3

The pattern of population structure of the 118 Sweet wormwood (*A. annua*) accessions inferred using IRAP data. (A) Estimated ΔK of possible clusters (K) from 1 to 10; (B) ΔK based on the rate of change of $\text{LnP}(D)$ between successive K and (C) Population structure based on K = 2, 3, 4, 5, 6 and 7.

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