AFLP-BASED EVALUATION FOR TREE PRODUCTION OF EL-SHEIKH ZEWAIED PEACH CULTIVAR "SINAWI" (*Prunus persica* L.) IN NORTH SINAI, EGYPT

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Peach (*Prunus persica* L., Batsch) a member of the *Rosaceae* family, is one of the most genetically important fruit trees in temperate regions (Arus *et al*., 2012). It is a deciduous tree indigenous to the northwest region of China, today; is commercially grown around the world between 30°C and 45°C latitudes above and below the equator. Peach is one of the three most economically important domesticates in genus *Prunus* globally after apple and pear in the cultivated surface area terms (Velasco *et al*., 2016). Egypt occupies the 11th rank in production amongst the 17th producing countries (FAOSTAT, 2016). Northern Sinai area, a model of the semi-arid region considered one of the focus points of peach cultivation in Egypt. El-Sheikh Zewaied peach cultivar (*P. persica* cv. Sinawi) is a well-defined peach cultivar and considered as a traditional crop of North Sinai, Egypt. Average production in this region declined from 3.14 ton/feddan in 2007 to 1.64 ton/feddan in 2010 (Nagaty *et al*., 2007; El-Kosary *et al*., 2013). In addition to the security issues in that region; genetic and environmental reasons are suspected to the observed inconsistency ones and declination in tree production.

Nowadays, evaluation of the genetic diversity and structure of plant populations is performed using both morphological characters and molecular markers (Zhang *et al*., 2015). Numerous molecular marker systems have been applied to *P. persica* for marker-assisted selection (MAS) programs (Verde *et al*., 2005; Blenda *et al*., 2007), genotyping and genetic diversity analysis (Dirlewanger *et al*., 2002; Yoon *et al*., 2006; Bouhadida *et al*., 2007), linkage maps (Yamamoto *et al*., 2005) and for quantitative trait loci (QTL) localization (Quilot *et al*., 2004; Dirlewanger *et al*., 2006; Cantín *et al*., 2010). Amongst the different markers techniques, Amplified Fragment Length Polymorphism (AFLP) is a molecular technique with high resolution, efficiency, reproducibility, and no prior sequence information for the identification of genetic diversity of plants is needed (Mueller and Wolfenbarger, 1999; Meudt and

Clarke, 2007). AFLP has a broad multi-locus screening that permits for the identification of statistically outlier loci for which represents alleles that are extremely distinguished among populations, and in other studies it was found to be associated with adaptive divergence (Magdy *et al*., 2016). Indeed, F-AFLP outlier loci often occur in non-coding DNA regions; nonetheless, some of the outlier loci may only display the designation of selection because they are associated with the actual target (Schlotterer, 2003). Recently, an increased emphasis has been placed on developing AFLP-based genome scans to reveal outlier loci linked to peach divergence (Li *et al*., 2014), floral divergence (Herrera and Bazaga, 2008), adaptation to altitude (Yang *et al*., 2016 a), climatic gradient (Magdy *et al*., 2016), ecotype divergence (Yang *et al*., 2016 b), host plants (Manel *et al*., 2010), insecticide resistance (Paris *et al*., 2010) or selection during domestication (Rossi *et al*., 2009). This comprehensive assessment gives valuable clues to disentangle the evolutionary effects of forces acting on the whole genome from the effects of forces influencing only particular loci (Bonin *et al*., 2006).

The current study aimed to assess the deterioration and fluctuation of productivity in El-Sheikh Zewaied peach cultivar (*Sinawi)* located in North Sinai, Egypt, and to investigate whether the deterioration is caused by genetic and/or environmental factors using morphological, molecular and biostatistical approaches.

MATERIALS AND METHODS

Survey and sampling collection

Total of 36 trees of El-Sheikh Zewaied peach cultivar "*Sinawi"* were collected as triplicates from six locations (two sites per each location) from three cities (regions), Rafah (2 locations), El-Sheikh Zewaied (3 locations) and El-Arish (1 location) ubicated in North Sinai governorate (Sinai Peninsula, Egypt) between latitude 31° 03' 09.7" to 31° 16' 57.4" N and longitude 34° 12' 48.6" to 34° 12' 48.6" E (Table 1 and Fig. 1).

Trees morphology and production

Tree height, circumference, trunk girth (at 30 cm above the ground) and branch length, were measured using meter scale. The number of flowers, side shoots, tree yield, fruit number, fruit weight, seed weight, flesh weight, flesh thickness and fruit volume were determined as indices for fruit characteristics of each of the twelve sites under investigation. While the age of the tree was calculated based on the farm-owners' information. Whereas, the Total Soluble Solids (T.S.S.) (%) in flesh juice was measured by a table refractometer (Model N-50E; Atago, Tokyo, Japan) and expressed as °Brix. The refractive index was recorded and converted to °Brix. Measurements were performed at 25±0.5°C.

F-AFLP assay

DNA extraction

Genomic DNA was extracted using Gene JET™ Plant Genomic DNA Purifi-

cation Kit (#K0791, Thermo Scientific, Lithuania). The quality and concentration of the DNA samples were checked in a Quawell Q5000 UV-Vis spectrophotometer (V2.1.4, USA). A portion of the DNA was diluted to 50 ng/µl for use in F-AFLP analysis. Both the stock and diluted portions were kept at -20°C.

Testing DNA for contamination

Testing DNA for possible Eukaryotic DNA contamination was performed by amplifying the internal transcribed spacer ITS region using:
ITS1 5´-TCCGTAGAACCTGCGG-3´and ITS4 5´-TCCTCCGCTTATTGATATGC-3´ (White *et al*., 1990). A master mix adjusted and distributed depending on the PCR reactions number (sample/locus). MyTaq Master Mix (Bioline, England) was used in 25 µl reactions mixture of 50 ng of genomic DNA, 0.2 µM of each primer, 5µl of MyTaq Master Mix and deionized water up to 25 µl total volume. PCR cycling parameters included 2 min of initial denaturing at 95°C, 35 cycles of three steps: 30 sec of denaturing at 95°C, 1 min of annealing at 55°C and 1 min of elongation at 72°C. For extension, one cycle of 5min at 72°C. PCR products were separated by 1.5% (1.5 g/100 ml) agarose gel electrophoresis and visualized with 1 X Ethidium Bromide. Samples showed only a single band at the expected length (~600 bp) which was considered for further analysis.

AFLP protocol

The original protocol of Vos *et al*. (1995) was applied, the primers were fluorescently rather than radioactively labeled as a modification (Table 2). Six different selective PCR combinations were successfully applied to 18 samples (3 per location). PCR amplification was performed using the original protocol without modifications. Private Service was contracted to visualize the amplified products using ABI3730 DNA analyzer (Applied Biosystems, USA) with a size standard GS500-LIZ (Macrogen Fragment Analysis Service, Korea).

Data analysis

Morphological and biometrical analysis

To evaluate the tree's production under the local conditions and surrounding factors, series of statistical analysis were performed using Xlstat excel add-on tool (Addinsoft, USA). The tree and fruit morphological traits were transformed and standardized using the z-score method. Based on morphological data, correlation analysis and modeling prediction were performed, to evaluate agro-morpho traits and their relation to tree production. Samples were clustered based on plant production using the Agglomerative Hierarchical Clustering (AHC) method to distinguish the high and low productive trees to be used as contrasting genotypes to facilitate the finding of tree production-related genetic loci.

Molecular data

Loci scoring

Automated fluorescent AFLP scoring was performed using two programs;

Peak Scanner™ (Applied Biosystems, USA) for peak calling and Rawgeno V2 for automated scoring, according to the software's manuals. Loci with rare frequency were cut-off (i.e., a frequency below 10% and/or above 90%). To estimate general genetic parameters, the scored binary data for all genotypes was calculated by FAMD software (Schlueter and Harris, 2006), with the following parameters of variability, the percentage of polymorphism (P%), number of different alleles (*Na*), number of effective alleles (*Ne*), Shannon's index (*I*) and unbiased genetic diversity (*uH*). The dissimilarity matrix and Mantel test were applied by using GeneAlex and Xlstat programs, respectively.

Tree production-related loci (Outlier loci)

Based on the production traits tree, the binary scored data of the highest and lowest productive trees were exported to MCHEZA software. Parameters were set to 1,000,000 simulation runs at confidence level of 99.5% to detect outlier loci that differentiate between both categories. Whereas, the identified loci were used STRUCTURE and Mantel test to confirm the marker detection effect on samples clusters. Mantel test was performed between Euclidean distance matrix based on tree production trait to genetic distance matrix based on AFLP all data and again to genetic distance matrix based on the AFLP detected outlier loci at 999,999 premutation steps.

RESULTS AND DISCUSSION

Morphological characterization

Mean, maximum, minimum and the Standard Deviation (SD) of several important morphological characters that may result in the phenotypic variation of *P. persica*, cv. Sinawi were described (Table 3). A considerable morphological diversity in the characters was observed using seven morphological traits for the sampled peach trees (tree production, age, trunk, circumference, height, main branch length and number of side shoots) and eight fruit quality traits (number of flowers, fruits number/fruit limb, fruit volume, fruit weight, flesh weight, flesh thickness, total soluble solids and seed weight) indicating a high level of variation in the studied plant materials. The minimum tree production was 4 kg, while the maximum was 45 kg, with a mean value of 18.64±12.92, the maximum production was exclusively found in El-Arish region (location 6), with a unique occurrence in El-Sheikh Zewaied (location 2). Traits that describe tree morphology; the tree age, trunk, circumference and tree height were found to be uncorrelated (p-value > 0.05) to tree production; branch length and the number of sides shoots significantly correlated to tree production being r-value (p-value $\leq$ 0.0001) are 0.60 and 0.66, respectively. Traits related to fruit quality were found significantly correlated to tree yield ($0.33 \leq$ r-value ≤ 0.77; p-value $\leq$ 0.05) with the exception to seed weight. Regression analysis was performed to define the dependency of tree production on other correlated variables. The beta

regression coefficients of the studied traits were found significant based on t-test (Pr > [t]) for the number of side shoots (beta-value = 0.616; p-value = 0.008), fruit number (beta-value = 1.126; p-value = 0.013) and fruit weight (beta-value = 3.416; p-value = 0.012, respectively (Table 3 and Fig. 2).

Despite the vast number of existing peach cultivars used for the fresh market, there is continuing need to develop new varieties with resistant tolerances to further expand into subtropical climates change; increased fruit quality and appearance are being targeted by breeders worldwide. Some traits are being focused as tree habit, canopy architecture and adaptability have also been among high priority in breeding programs (Hancock *et al*., 2008). Our findings demonstrated that tree production considered the most economically important trait in *P. persica* (Cao *et al*., 2016), that can be used directly to evaluate the tree overall the tree performance. Signification correlation between morphological variable and production which exhibit variation in productivity in location and between regions. Extensive statistical of allelic diversity can predict for that they may cover information concerning the evolutionary potential for adaptation to expected morphological changes (Caballero and Dorado, 2013). Based on tree production traits, the 36 samples were clustered using AHC method (data not shown), three class centroids were defined, class centroid 1 clustered 9 samples with a centric value of 4.44 kg/tree, class centroid 2 clustered 19 samples with a centric value of 16.47 kg/tree, and class centroid 3 clustered 8 samples with a centric value of 40 kg/tree. A variance decomposition for optimal classification scored 6.80% within-classes and 93.20% between-classes. Each class centroid was defined as low productive trees (Class 1), moderate productive trees (Class 2) and high productive trees (Class 3). Regression linear model defined the dependence of tree production on the other correlated variables, the number of side branches and a number of flowers as a direct indicator of tree production. This finding agrees with numerous previous reports such as (Herrera and Bazaga, 2008; Meyer *et al*., 2009; Poncet *et al*., 2010; Li *et al*., 2014; Yang *et al*., 2016 a & b).

Molecular characterization

Internal transcribed spacer (ITS)

The universal primers ITS1 (forward) and ITS4 (reverse) amplified the complete ITS region (ITS1, 5.8S rRNA gene, and ITS2) of the ITS regions for 18 samples with a single amplicon size of ~ 600 bp.

Amplification scoring and readability

PCR amplification was succeeded for six pairs of primers combinations. The rate of amplification was up to 173 peaks per primer pair. Peak analyses and automated band scoring were successful, and quality tests showed adequate quality. A total of 801 polymorphic amplicons were scored with band size between 150-480

bp. The mean amplicon size was ~251 bp with a standard deviation of ~89 bp per primer. After cut-off, the 801 bands retained for further analysis. As illustrated in Table (4), the maximum and minimum values of polymorphism percentage per location (P%) was 50.05% from location 6 (El-Arish), and 28.02% from location 5 (El-Shiekh Zewaied). Overall, location 6 (El-Arish), recorded the maximum value for the total number of different alleles (*Na*), Shannon's information index (*I*), the total number of effective alleles (*Ne*) and unbiased diversities (*uHe*) were 1.123±0.030; 0.319±0.013; 1.400±0.013; and 0.334±0.011, respectively. In contrast, location 5 (El-Shiekh Zewaied) exhibited the minority values of the same parameters; 0.599±0.29; 0.178±0.012; 1.224±0.012 and 187±0.010, respectively. In our results, the total unbiased diversity (*uHe*) was 0.258 which reflects low diversity level of Sinawi cv. genetic pool from the sampled within locations. The genetic structure revealed by F-AFLPs (F_{st} = 0.123) is also concordant with the pattern exposed by earlier reported (Yang *et al*., 2016b). Identification of locus-specific under divergent selection is a critical step to understanding the evolutionary process for the population genetic variations that affect fitness in different environments (Beaumont and Balding, 2004).

Loci under selection related to tree production

Detection of positive selection signatures (outliers)

The F-AFLP all dataset of *P. persica*, cv. Sinawi was analyzed for outlier loci detection by using MCHEZA program on nine samples divided into two groups; high productive trees (5 samples from cluster class 1) and low productive trees (4 samples from cluster class 3) the other retained nine samples were discarded as they belong to moderate productive trees (cluster class 2). The pairwise comparison based on F_{st} (Wright's fixation index) (Fixation indexes can be determined for differentiated hierarchical levels of a population structure, to indicate, for example, the degree of differentiation within a population among groups of demes (F_{SG}), within groups among demes (F_{GT}), and within a population among demes (F_{ST}) (Hartl and Clark 1997). Values of each locus between the two groups were plotted against their heterozygosity values (Fig. 4). Only three loci out of the 801 loci were identified as outlier loci under selection due to genetic variation between two productive groups; at 99.5% confidence level (He = 0.52; F_{st} > 0.59); one of the three loci showed the highest F_{st} value of 0.84.

Validation and influence of outlier loci

Outliers tested loci were compared to AFLP all loci dataset on the 18 samples using non-spatial Bayesian clustering implemented in Structure software. Outliers loci are expected to reduce within locations variation and increase variation percentage among locations, while principle coordinate analysis should reveal major differences among the three detected clas-

ses that a clear divergence of each group should be explained clearly when AFLP outlier loci were used.

Analysis of molecular variance (AMOVA)

AMOVA test was performed to measure the changes in the pairwise differentiation of the F_{st} for the AFLP all dataset in comparison with the F_{st} of AFLP outlier loci dataset. F_{st} increased from 0.256 in F-AFLP neutral loci to 0.723 in AFLP outlier loci. The proportion of variation due to differences within locations dramatically decreased from 74% in AFLP all data to 26% in AFLP outlier loci, in contrast, the percentage of variation increased among locations from 23% (all dataset) to 74% (AFLP outlier loci). Based on outlier loci, more genetic differentiation among the locations was confirmed. Variation reflected within geographical location is in accordance with the high within variation estimated by AMOVA. It has demonstrated that the three loci had highly genetic differentiation among locations than the putative neutral loci (74% for the outlier loci as compared to 23% for AFLP neutral loci) and decreased diversity within locations (from 74% for AFLP neutral loci to 26% AFLP three outlier loci). F_{st} outlier approach specifically developed for AFLP markers, which considers the intensity of AFLP bands instead of mere presence/absence based on estimates of allele frequencies. In our results a significant F_{st} value of the neutral markers was 0.84, which is higher than earlier reports by Magdy *et al*., (2016) (F_{st} =0.23) and Yang *et al*., (2016a) (F_{st} =0.571). Loci that demonstrate significantly higher or lower among-population genetic differentiation than expected under neutrality are identified as outliers (Feng *et al*., 2015). Interestingly, outlier loci which can potentially be detected by a high correlation between allele frequencies and environmental parameters (Roesti *et al*., 2012).

Population and genetic structure

The principal coordinate analysis reflected the differentiation among the geographic location of the samples. RF-1 and Ar regions; RF-2, SZ-2 and SZ-3; SZ-1 formed three relatively clustered groups, respectively. Rafah locations, belonged to two different groups, as same as El-Sheikh Zewaied regions; multiple introductions of different genotypes to the same region can be observed (Fig. 4). To ensure the exact relationship between calculated genetic markers and tree productivity mantel test was performed between the genetic distance of all dataset and the Euclidean distance matrices of tree production was found insignificant (r-value = -0.0208; p-value = 0.7997). While, a positive significant seen with the outlier's loci (r-value = 0.2215; p-value =0.0019) against tree productive, which proved their potential to be used as a molecular genetic marker to tree production in *P. persica* L. cv. Sinawi.

In subsequent studies, AFLP-based genome scans had been used successfully to detect outlier loci linked to adaptation with the invasive plant (Wang *et al*., 2012). Another successful implementation

of AFLP genome scan with the morphological trait has been reported among six natural populations of Rosa species (Yang *et al*., 2016b). Such a result refers to outlier AFLP loci are likely not the target of natural selection, but the neighboring genes of these loci might involve in tree production. According to (Bonin *et al*., 2006; Yang *et al*., 2016b) such test proved the association (correlation based on Mantel test and AMOVA) between the outlier's loci to the tree production trait, consequently prove their potential to utilized as a molecular genetic marker linkage to tree production in Sinawi cv. of *P. persica* L. Currently, molecular markers (locus-specific) linked to traits of interest are vital for both MAS and improvement of selection efficiency in the conventional breeding program, especially for economic trials that are difficult to select by phenotype early in the plant life cycle (Byrne *et al*., 2012). In particular, selection based on genomic predictions of breeding values approaches like next-generation sequencing (NGS) technologies (Yamamoto and Terakami, 2016), and genome-wide association studies (GWAS), are another alternative for MAS which are now emerging as powerful tools in peach (Byrne *et al*., 2012). Newly developed the availability of whole genome sequences and expressed sequence tag (EST) databases for peach genome *via* the recent introduction of NGS technologies (Badenes *et al*., 2016), represents a significant revolution in implementing unique tools for discovering the locus-specific and genes /or genomic intervals monitoring significant traits for selection in breeding programs.

SUMMARY

Tree production considered the most economically important trait in *Prunus persica* that can be used directly to evaluate the overall tree performance. The genetic variability was assessed using fluorescently labelled AFLP-based genome scans to capture the loci under selection that are correlated to the production limitation beyond the environmental reasons within the cultivar Sinawi of *P. persica* L. Regression linear model defined the dependence of tree production on the number of side branches and a number of flowers as a direct indicator of tree production. A total of 801 F-AFLP polymorphic amplicons were scored with unbiased diversity (*uHe*) of 0.258, which reflects a low genetic pool from the sampled within locations. PCoA (Principal Coordinate Analysis) and STRUCTURE analysis showed a tendency to three sub-populations. Out of 801 F-AFLP loci, three outlier loci were identified as a putatively positive outlier to tree production trait. The statistical correlation of the Euclidean distance matrix between the three outlier's loci to the tree production trait found to exhibit increased differentiation among locations along with a decreased diversity within locations, prove their potential to be utilized as molecular genetic markers linkage to tree production in Sinawi cv.

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Table (1): Sampling details of six locations in North Sinai region, Egypt.

Location	City	Latitude(N)	Longitude(E)
1	Rafah (RF-1)	31°16'57.4"	34°12'48.6"
2	Rafah (RF-2)	31°14'12.8"	34°13'37.4"
3	El-Sheikh Zewaied (SZ-1)	31°12'12.7"	34°04'14.1"
4	El-Sheikh Zewaied (SZ-2)	31°11'26.1"	34°02'42.5"
5	El-Sheikh Zewaied (SZ-3)	31°06'48.3"	34°09'20.8"
6	El-Arish (Ar)	31°03'09.7"	33°50'39.6"

Table (2): List of oligonucleotides (primer and/or adaptors) used for F-AFLP amplification. Selective nucleotides are written in bold at 3´ end; and fluorochrome molecules used for labelling selective primers are indicated in italic at 5´ end.

Primer/Adaptor	5´ ⟶ 3´
EcoRI - A1	CTCGTAGACTGCGTACC
EcoRI - A2	AATTGGTACGCAGTC
Eco + A	GACTGCGTACCAATTCA
Eco + ACA	*FAM*-GACTGCGTACCAATTCACA
Eco + AGG	*HEX*-GACTGCGTACCAATTCAGG
Eco + ATA	*CY3*-GACTGCGTACCAATTCATA
Mse I - A1	GACGATGAGTCCTGAG
Mse I - A2	TACTCAGGACTCAT
Mse + C	GATGAGTCCTGAGTAAC
Mse + CAA	GATGAGTCCTGAGTAACAA
Mse + CTC	GATGAGTCCTGAGTAACTC

Table (3): Morphological traits description summery based on 36 peach tree samples. Minimum, maximum and average with standard deviation are shown for each trait. Correlation (r-value) and its significant level, and regression coefficient (beta-value) and its significance t-test were estimated to tree production trait. Significant values are written in bold.

Category	Variable	Min	Max	Mean	Std. deviation	r-value	p-value	Beta - value	Pr > |t|
Tree morphology	Tree yield	4.00	45.00	18.64	12.92	**1.00**	**0.00**	N/A	-
	Age	5.00	10.00	6.53	1.68	0.14	0.43	0.003	0.988
	Trunk girth	20.00	60.00	31.97	10.52	0.24	0.15	0.091	0.477
	Tree circumference	1.30	10.00	5.36	2.41	0.05	0.76	-0.020	0.874
	Height (cm)	100.00	295.00	180.31	53.75	0.32	0.06	-0.106	0.413
	Branch length (cm)	33.25	785.00	237.65	153.56	**0.60**	**0.00**	0.040	0.832
	Number of side shoots	3.25	42.00	17.67	8.64	**0.66**	**< 0.0001**	**0.616**	**0.008**
Fruit quality	Number of flowers	14.50	599.00	124.25	153.38	**0.68**	**< 0.0001**	-1.002	0.082
	Fruit number	4.00	528.00	103.14	145.89	**0.70**	**< 0.0001**	**1.126**	0.062
	Fruit volume (cm^3)	23.00	63.33	40.46	11.80	**0.44**	**0.01**	0.169	0.220
	Fruit weight (gm)	20.00	59.00	36.83	10.80	**0.51**	**0.00**	**3.416**	**0.013**
	Flesh weight (gm)	15.70	55.00	32.95	10.76	**0.48**	**0.00**	-3.523	**0.012**
	Flesh thickness (cm)	1.00	1.80	1.22	0.16	**0.33**	**0.05**	-0.189	0.158
	T.S.S. °Brix (%)	10.00	12.00	11.00	0.83	**0.77**	**< 0.0001**	0.353	0.12
	Seed weight (gm)	2.50	6.00	3.88	0.85	0.31	0.07	0.000	N/A

T.S.S. = Total Soluble Solids

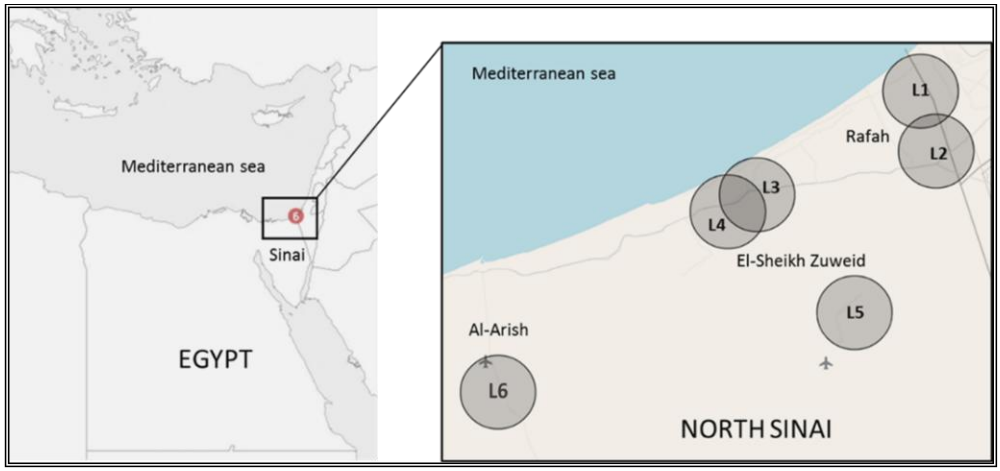

Fig. (1): Geographical map illustrates sampling locations of the current study along the Northern coast of Sinai Peninsula, Egypt.

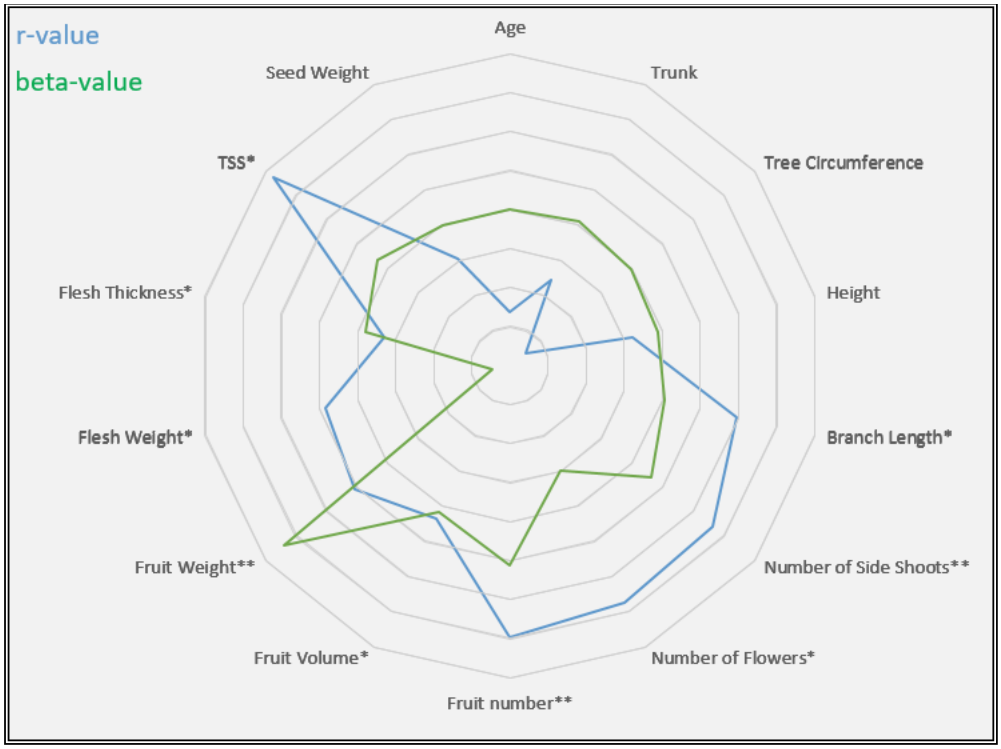

Fig. (2): Radar chart shows the correlation values (r-value) and beta regression coefficient values (beta-value) of all measured morphological traits to tree production. * Traits showed significant r-value; ** traits showed significant r-value and beta-value.

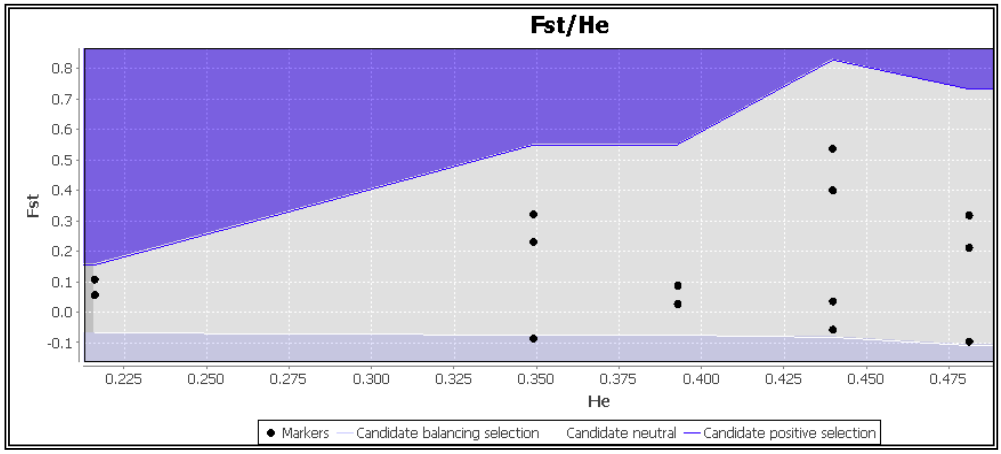

Fig. (3): Mcheza graphical output of each AFLP locus (black dots) plotted based on its Fst value against heterozygosity (He). The outlier zone (Blue) is defined at ≥ 99.5% confidence level, neutral zone (gray) is defined between < 99.5% and > 0.05% and the balancing selection zone (violate) is defined at ≤ 0.05%. Loci with the same Fst/He value are overlapped.

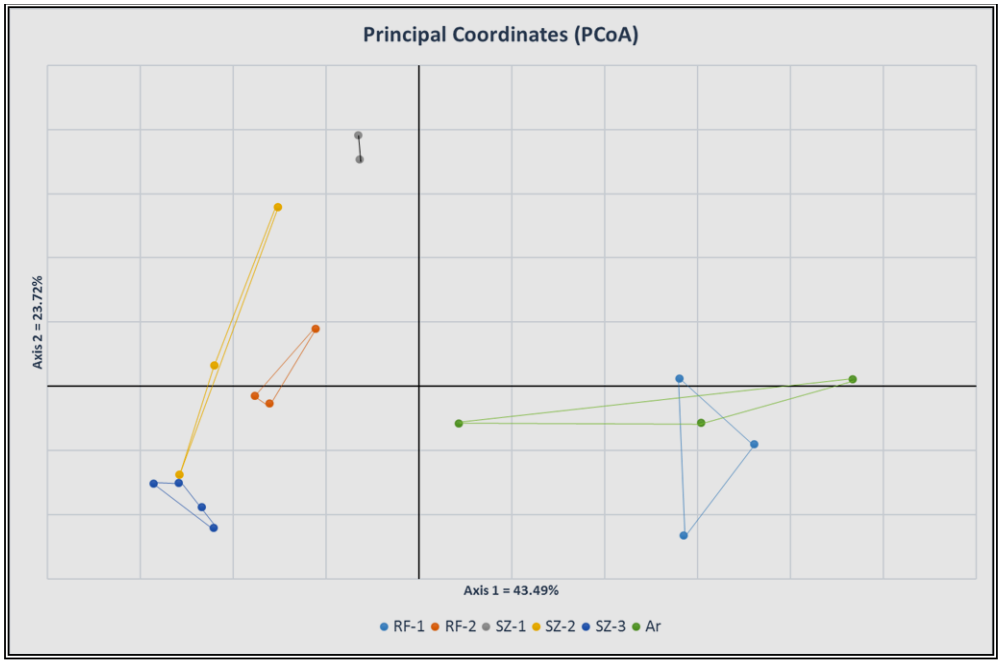

Fig. (4): Principal coordinate analysis (PCoA) of each examined sample coloured by its geographical location is shown. PCoA was revealed by axis 1 and 2, that explained 43.49% and 23.72% of genetic variation. Coloured lines drawn to connect all samples from the same location.