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A meta-analysis of genetic divergence versus phenotypic plasticity in walnut cultivars (*Juglans regia* L.)

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Abstract. Persian walnut (*Juglans regia* L.), is very auspicious plant species of Iran from both economical and food points of views. Both wildly grown as well as cultivated forms of this plant species are scattered in different geographical regions of the country and are a valuable source for edible nut as well as job employment. Scattered published data on genetic diversity of this important plant species are mainly based on different molecular data analyses; therefore a meta-analysis of the same cultivars based on several different molecular markers including DNA-sequences and multi-locus markers was conducted to provide a detailed insight on genetic structure of walnuts. The results indicated a moderated genetic variability of about 40 percent in the studied cultivars; however these cultivars are genetically differentiated as revealed by Fst and AMOVA. HGT analyses revealed that the cultivars phylogeny differs to some degree by different markers and therefore a heat map was constructed to reveal the cultivars relationships based on combined molecular data. A higher Pst value was obtained compared to that of Fst genetic differentiation, therefore, it seems that local adaptation and selection have played role in the walnut cultivars' morphological divergence. LFMM analysis identified some adaptive multi-locus alleles in the studied walnut cultivars.

Keyword: adaptive alleles, HGT, LFMM, Persian walnut, Pst.

INTRODUCTION

Persian walnut (*Juglans regia* L.), is one of the main economically cultivated crop plants in Iran. We have a rich walnut germplasm with extensive cultivar genetic variability (Tabasi *et al.* 2020). Both wildly grown as well as cultivated forms of this valuable plant species are scattered in different geographical regions of the country. Now and then we encounter new and limited reports on genetic features some of these populations by using different molecular markers (See for example, Maghsoodi *et al.* 2018; Tabasi *et al.* 2020).

We therefore carried out the present meta-analysis study based on a set of Persian walnut cultivars which were used in previous investigation to pro-

vide data for: 1- How much genetic diversity is present in these cultivars based on combined data, 2-Determine the phylogenetic relationship of these cultivars based on combined molecular data, 3-Provide data on phenotypic plasticity of these cultivars.

A meta-analysis is a statistical analysis that combines the results of multiple scientific studies addressing the same question. Each individual study reporting measurements that are expected to have some degree of error, and therefore, The aim is to derive a pooled estimate closest to the unknown common truth based on how this error is perceived (Nakaoka *et al.* 2009). Meta-analysis is mainly a method for systematically combining pertinent qualitative and quantitative data from several selected studies to develop a single conclusion that has greater statistical power. This conclusion is statistically stronger than the analysis of any single study, due to increased numbers of subjects, greater diversity among subjects, or accumulated effects and results (Greenland *et al.* 2008; Walker *et al.* 2008). Meta-analysis would be used for the following purposes: To establish statistical significance with studies that have conflicting results, to develop a more correct estimate of effect magnitude, to provide a more complex analysis of harms, safety data, and benefits (Nakaoka *et al.* 2009).

MATERIAL AND METHODS

Plant materials

Morphological, ISSR, IRAP, REMAP, SCoT, trnH-psbA and ITS data of 6 populations used (Table 1) are collected according to following studies: Maghsoodi *et al.* 2018; Aghaei *et al.* 2019 in press; Tabasi *et al.* 2020.

Data analyses

Genetic analyses

Detrended correspondance analysis (DCA) was performed to study the suitability of molecular markers for population genetic study as performed in PAST version 2.17 (Podani 2000). Genetic distance and Fst values were obtained from published data and also determined by us for DNA sequences. We used DNA sequence comparison non-parametric Kruskal–Wallis test as performed in PAST, and also by DNAsp program (Rozas *et al.* 2019).

Horizontal gene transfer (HGT) was used to reveal the phylogenetic trees discordance as performed in TREX online (Boc *et al.* 2012). Similarly, the Mantel test was used to indicate association between genetic distance and geographical distance in the studied cultivars. PAST program was used for this purpose. A heat map was constructed based on combined genetic data by R-Package. 4.2.

We used LFMM program (Frichot. *et al.* 2013) to identify multi-locus alleles with potential adaptive value. This program includes an integrated framework based on population genetics, ecological modeling and statistical techniques using latent factor mixed model.

Quantitative morphological characters

For Pst (Phenotypic plasticity) analysis, we used four quantitative morphological characters in Persian walnut cultivars. These characters are: 1-Nut length (mm), 2-Nut diameter (mm), 3-Nut length/diameter ratio, and, 4-Thickness of shell (mm). Pst values were determined by R-Package 4.2.

Table 1. *Juglans regia* populations.

No	Type	Name of population	Province	Locality	Longitude	Latitude	Altitude
1	Wild	Nahavand	Hamadan	Nahavand	23°48'	23°48'	1627
2	Cultivated	Soozani	Markazi	Tafresh	59°49'	42°34'	1838
3	Cultivated	Basloghi	Markazi	Tafresh	59°49'	42°34'	1837
4	Wild	Astara	Gilan	Astara	52°48'	20°38'	-26
5	Cultivated	Kaghazi	Markazi	Tafresh	59°49'	42°34'	1838
6	Wild	Khoy	West Azarbaijan	Khoy	57°44'	33°38'	1135

RESULTS

Genetic divergence

DCA (Detrended correspondence analysis) plot (Fig. 1) of combined molecular markers (ISSR, IRAP, REMAP, SCot, cp-DNA, and ITS data), produced a well scattered diagram, indicating that these molecular markers in combination can differentiate walnut populations well-enough.

In Maghsoodi *et al.* (2018) study on walnut populations, the mean Nei' genetic distance for ISSR data was 0.3, while the mean genetic distance based on chloroplast-DNA as well as nuclear ITS (Maghsoodi *et al.* 2018) sequence analysis were 0.1.

The Fst analysis for all genetic markers produced a significant difference among walnut cultivars ($P < 0.05$). For example, the Kruskal-Wallis test for equal medians in nuclear ITS DNA sequences as well as chloroplast DNA, produced a significant difference. ($P = 0.03$, and $P < 0.001$, respectively). Similarly, dnaSP population' genetic differentiation test produced significant Fst value for most of the pair-wise population compared (See for example Table 2).

The Mantel test produced significant correlation ($r = 0.14$, $P = 0.04$) between genetic distance and geographical distance of the studied populations. These results

indicate that an extensive genetic changes have occurred during walnut cultivar divergence.

Cultivar phylogeny

Cultivar grouping obtained by different molecular markers differed in some degree from each other. The HGT (Horizontal gene transfer) analysis between chloroplast DNA and combined multi locus data (ISSR, REMAP, and SCot), revealed in total two HGT events due to phylogenetic dis-agreement (Fig. 2).

Similarly, HGT analysis revealed that three HGT events occurred between cp-DNA and nuclear ITS-DNA sequences (Fig. 3).

The Mantel test performed among chloroplast and nuclear ITS-DNA sequences as well as combined multi-locus molecular markers, after 10000 permutation, did not produce significant association between phylogenetic trees obtained from these (Partial correlation $r = 0.50$, $P = 0.14$). Moreover, Robinson and Foulds distance between phylogenetic trees produced $RF = 6$. These results are in agreement with HGT results, and indicate that phylogenetic relationship of walnut cultivars based on different molecular markers differ to some extent, and care should be taken for drawing concrete conclusions in these types of investigations.

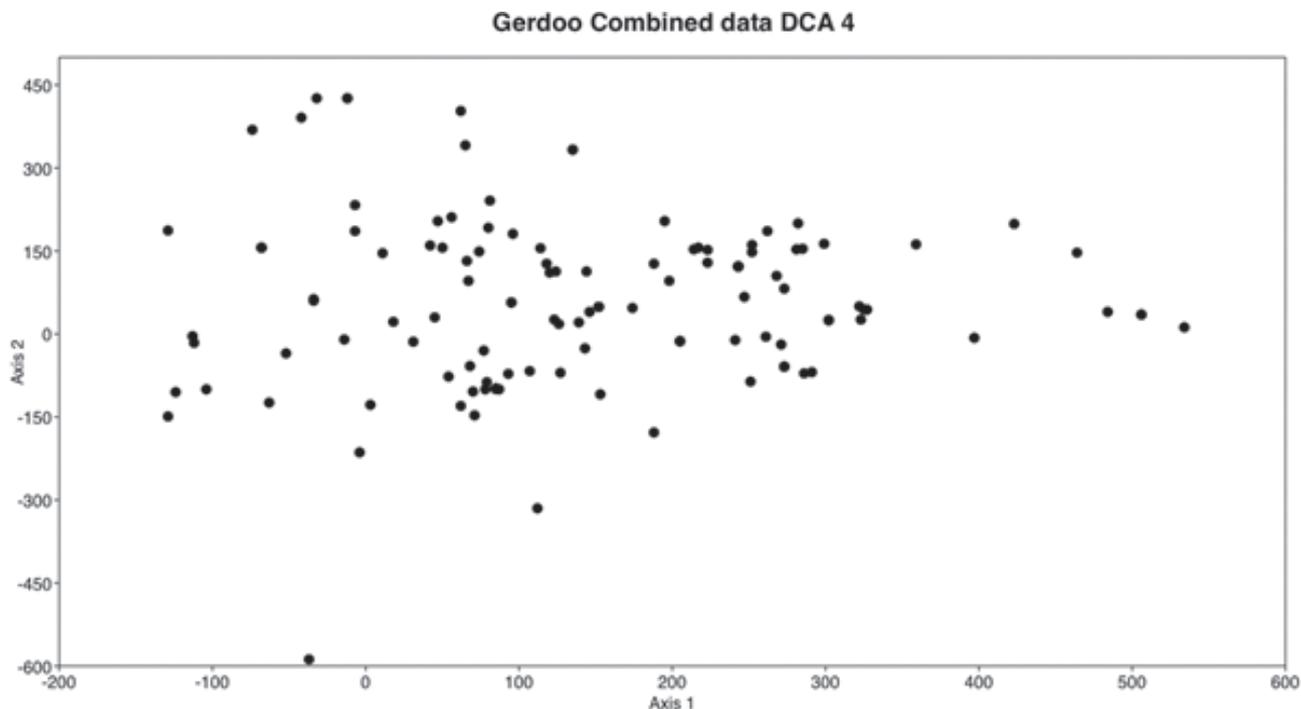


Figure 1. DCA plot of walnut cultivars based on combined sequence and multi-locus data.

Table 2. Pair-wise Fst value for ITS DNA sequences among walnut cultivars (the number of cultivars are according to Table1).

	1	2	3	4	5	6
1	—					
2	0.47	—				
3	0.05	0.29	—			
4	0.05	0.29	1	—		
5	0.24	0.30	0.43	0.43	—	
6	0.04	0.08	0.09	0.09	0.04	—

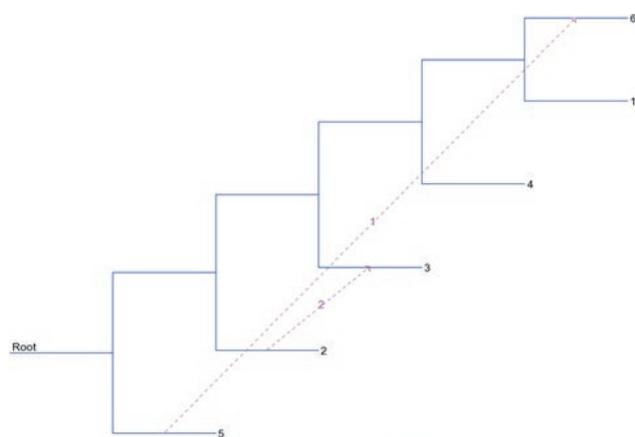


Figure 2. HGT between cp DNA and combined multi locus markers in walnut cultivars.

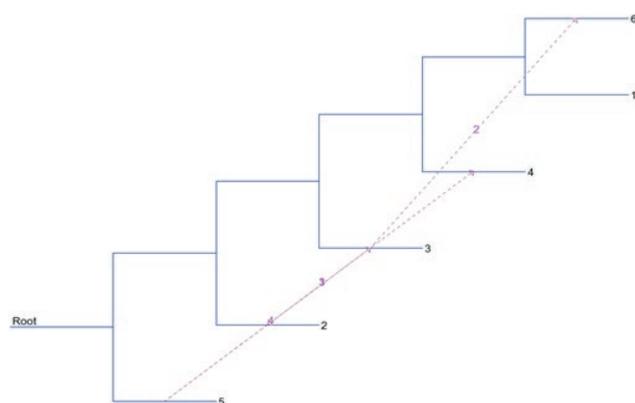


Figure 3. HGT tree between chloroplast and ITS-DNA sequences in walnut cultivars.

A phylogenetic heat map constructed based on combined molecular data (Fig. 4), revealed that the cultivar 1 is genetically stands far from the others due to genetic difference.

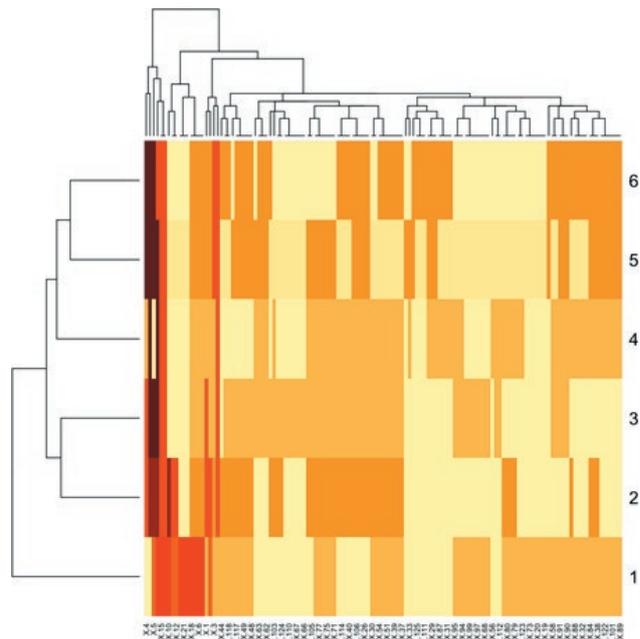


Figure 4. Heat map of walnut cultivars based on combined molecular data.

Genetic diversity and local adaptation

The LFMM did not produce a significant association between IRAP and REMAP loci with geographical distribution of the studied walnut cultivars. However, a significant association was obtained between three ISSR loci as well as two SCoT loci with geographical features (Longitude, latitude and altitude) in walnut populations (Table 3, Fig. 5).

Phenotypic plasticity versus genetic divergence

We obtained a significant correlation ($P < 0.01$) between morphological characters studied (Table 4). Moreover, ANOVA revealed that the studied cultivars differ significantly in these morphological features ($P = 0.01$).

The Pst values obtained for the studied quantitative morphological characters were much higher than genetic Fst value (Mean = 0.25). For example, representative Pst values for the nut height and width are provided in Tables 4 and 5. The higher Pst values compared to that of Fst indicate that the studied morphological characters diverged among walnut cultivars under influence of either local environmental conditions, or due to cultivating and selection practice available in the region (Table 6).

Table 3. LFMM results for ISSR data in walnut populations.

ISSR loci	Zscore	-log ₁₀ (p-value)	p-value
SNP_1	0.215472	0.0812363	0.829399
SNP_2	1.07686	0.550458	0.281541
SNP_3	0.257655	0.0987196	0.796673
SNP_4	0.640301	0.282349	0.521977
SNP_5	0.090856	0.032636	0.927607
SNP_6	1.69642	1.04669	0.0898066
SNP_7	0.309536	0.120954	0.756914
SNP_8	3.24327	2.92751	0.001181
SNP_9	0.369296	0.147577	0.711907
SNP_10	0.014174	0.00493936	0.988691
SNP_11	0.00968666	0.00336958	0.992271
SNP_12	0.651046	0.288179	0.515017
SNP_13	0.162106	0.0598711	0.871222
SNP_14	0.502927	0.211114	0.615015
SNP_15	0.538672	0.229065	0.590113
SNP_16	3.17389	2.82271	0.0015041
SNP_17	0.551459	0.235586	0.581319
SNP_18	2.34492	1.72054	0.019031
SNP_19	0.63401	0.278953	0.526074
SNP_20	0.477576	0.198629	0.632952
SNP_21	2.72343	2.18971	0.00646084
SNP_22	0.498368	0.208854	0.618224
SNP_23	0.383171	0.153915	0.701593
SNP_24	0.590575	0.255859	0.554805
SNP_25	0.798828	0.372235	0.42439
SNP_26	0.449796	0.185181	0.652858
SNP_27	0.083641	0.0299592	0.933342
SNP_28	1.82734	1.16974	0.0676484
SNP_29	0.838597	0.396103	0.401695
SNP_30	0.0258046	0.00903406	0.979413
SNP_31	1.89246	1.23337	0.0584291
SNP_32	1.44793	0.830805	0.147637
SNP_33	1.45811	0.839203	0.144809
SNP_34	1.38228	0.777582	0.166885
SNP_35	0.0207987	0.00726703	0.983406
SNP_36	0.451128	0.185821	0.651898
SNP_37	1.03731	0.523472	0.299591
SNP_38	1.55968	0.925052	0.118836
SNP_39	0.576949	0.248741	0.563974
SNP_40	1.16006	0.609022	0.246024
SNP_41	0.0331206	0.0116291	0.973578
SNP_42	1.06123	0.539726	0.288585
SNP_43	1.23962	0.667327	0.215116
SNP_44	0.928131	0.451808	0.35334
SNP_45	0.906725	0.43824	0.364552
SNP_46	0.908105	0.43911	0.363823
SNP_47	0.643651	0.284162	0.519802

DISCUSSION

Genetic diversity and cultivars phylogeny

We obtained almost close value for genetic diversity of Persian walnut cultivars by both DNA-sequences analysis as well as multi-locus molecular markers data. A significant genetic difference observed in both kinds of data indicates genetic differentiation of the studied cultivars which can be utilized in future breeding and hybridization projects.

Detailed data on genetic structure and phylogenetic relationship of economically crop plants like the Persian walnut is of immediate importance for genetic conservation and breeding programs. Moreover, knowledge about the effect of environmental/geographical features on genetic diversity as well as agronomic features of crop plants can improve above mentioned tasks.

Meta-analysis is an approach which either increase the number of observation in a particular study, or combine scattered data from several sources on a similar subject. Both cases can improve insight about a particular problem or task (Heidari *et al.* 2018).

The present study revealed that a combined data analysis based on different molecular markers may improve our insight on both genetic structure as well as phylogenetic relationship of important crop plants like Persian walnut. We also noticed that walnut cultivar agronomic features may be affected by both artificial as well as natural selection. A proper QTL (Quantitative Trait Locus) study can also reveal association of important agronomic characters.

The present study identified some of multi-locus molecular markers are either adaptive or are the genomic sites in the vicinity of adaptive genes. This is an important finding for QTL investigation of Persian walnut.

Meta-analysis concerned with plant genetic studies have been performed with regard to genetic correlations between plant resistances to multiple natural enemies (Leimu *et al.* 2006). These studies are considered important to determine the mode of selection that natural enemies impose on a host plant, the structure of herbivore and pathogen communities, all of which determine the success of plant breeding for resistance to multiple diseases and pests (Leimu *et al.* 2006). Similarly, a meta-analysis was performed to understand the genetic control of flavor in tomato cultivars (Zhao *et al.* 2019). These authors used data of genome-wide association studies (GWAS) using 775 tomato accessions and 2,316,117 SNPs from three GWAS panels and reported several significant associations for the contents of sugars, acids, amino acids, and flavor-related volatiles. They also concluded that fruit citrate and malate contents were affected by selection during domestication and improvement.

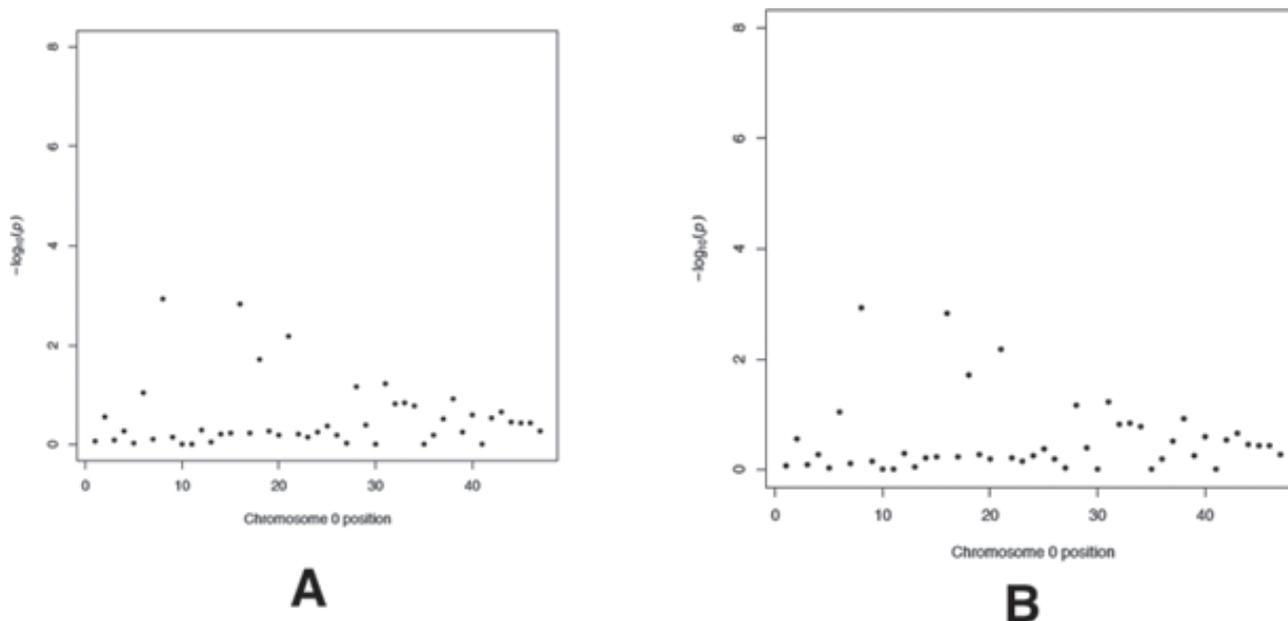


Figure 5. LFMM Manhattan plots showing three ISSR loci. (A) and two SCoT loci (B), with $-\log_{10}(p) > 2$, which indicate association with geographical features in walnut populations.

Table 4. Pearson's coefficient of correlation among morphological characters studied. (Characters A-D are: The nut height, nut width, ration of nut height/nut width, and the nut diameter. All values are in mm).

	A	B	C	D
A	—	2.5062E-05	0.026767	0.31372
B	0.77027	—	1.0055E-10	0.021356
C	-0.53937	-0.92392	—	0.17431
D	-0.38474	-0.55061	0.42827	—

Bellow diagonal = r value, above diagonal = P value.

In a meta-analysis with respect to plant crop cultivars genetic diversity (Van de Wouw *et al.* 2010), it was concluded that in the long run no substantial reduction in the regional diversity of crop varieties which were released by plant breeders has taken place, and a gradual narrowing of the genetic base of the varieties was not observed.

Genetic versus phenotypic differentiation

We obtained a higher value for Pst versus Fst, in quantitative morphological characters of fruit among representative walnut cultivars studied. The Pst is taken as index for morphological local adaptation under the influence of natural selection imposed by environment

Table 5. Pst values for the nut height among studied walnut cultivars. (The mean Fst value = 0.25).

	1	2	3	4	5	6	7	8	9
1	--								
2	0.05	—							
3	0.88	0.70	—						
4	0.73	0.40	0.70	—					
5	0.88	0.70	0.04	0.71	—				
6	0.84	0.65	0.07	0.56	0.16	—			

Table 6. Pst values for the nut width (mm) among studied walnut cultivars. (The mean Fst value = 0.25).

	1	2	3	4	5	6	7	8	9
1	--								
2	0.18	—							
3	0.77	0.77	—						
4	0.76	0.74	0.20	—					
5	0.89	0.87	0.46	0.73	—				
6	0.90	0.86	0.17	0.73	0.38	—			

(Brommer 2011). When Pst = FST, it is believed that morphological divergence is due to genetic drift; but while Pst > Fst, it indicates the role of directional selection among the studied populations. However, a lower

value of Pst in contrast to Fst, indicates that the same phenotypes are favored in different populations due to stabilizing selection. We may therefore, conclude that, due to some local environmental conditions/geographical coordinates, or local practices of cultivation or selection, some adaptive changes have occurred in walnut cultivars. Similar studies have shown that morphological and agronomical traits divergence have been occurred in many taxa (See for example, Leinonen *et al.* 2013; Stojanova *et al.* 2018; Caré *et al.* 2018).

Stojanova *et al.* (2018), found an adaptive differentiation in phenotypic traits across the climatic gradient in different populations of *Festuca rubra*. Similarly, Caré *et al.* (2018), reported morphological differentiation in crown architecture in geographical populations of German Norway spruce. They reported a high Pst value (0.952–0.989) between the neighboring autochthonous and allochthonous stands of similar age in contrast to a very low neutral genetic differentiation (Fst = 0.002–0.007; Gst = 0.002–0.030) probably due to the effect of directional selection on adaptive gene loci involved in phenotypic differentiation.

In conclusion, the meta-analysis based on published data on different molecular markers concerned with the same Persian walnut cultivars, revealed that a combined analysis of molecular data matrix (including chloroplast and nuclear DNA, as well as multi-locus markers, like ISSR, IRAP, REMAP, and SCoT markers), produce more accurate and significantly improved result for genetic diversity analysis as well as finding the cultivars' phylogenetic relationship.

DATA ARCHIVING STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

AUTHORS' CONTRIBUTIONS

M.T: data collection and lab work, M.Sh and F.K: conceptualization of the project and analyses of data, D.H: providing samples. The authors accept responsibility for releasing this material

REFERENCES

Bitton F, Bauchet G, Liu D, Huang S, Tieman D.M, Klee HJ, Causse M (2019). Meta-analysis of genome-wide

- association studies provides insights into genetic control of tomato flavor. *Nat Commun* 10: 1534.
- Boc A, Diallo A B, Makarenkov V (2012) T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks, *Nucleic Acids Research*, 40(W1), W573-W579.
- Brommer JE (2011) Whither PST? The approximation of QST by PST in evolutionary and conservation biology. *J. Evol. Biol.* 24(6): 1160-1168.
- Caré O, Müller M, Vornam B, Höltnen AM, Kahlert K, Krutovsky KV, Gailing O, Leinemann L (2018) High Morphological Differentiation in Crown Architecture Contrasts with Low Population Genetic Structure of German Norway Spruce Stands. *Forests* 9(12): 752.
- Frichot E, Schoville, SD, Bouchard G, François O (2013) testing for associations between loci and environmental gradients use latent factor mixed models. *Mol Biol Evol* 30(7): 1687–1699.
- Greenland S, O' Rourke K (2008) Meta-Analysis. In: Rothman KJ, Greenland S, Lash T(eds) *Modern Epidemiology*, Lippincott Williams and Wilkins pp.652.
- Heidari Horestani M, Atri Roozbahani G, Sheidai M (2018) The Potential Role of TNF- α (rs361525 and rs1800629) in Hepatocellular Carcinoma: Multivariate Analysis (Meta-Analysis). *J. Gastrointest. Cancer* 50:744–749
- Leimu R, Koricheva J. A (2006) Meta-analysis of genetic correlations between plant resistances to multiple enemies. *Am Nat* 168(1): E15-E37.
- Leinonen T, McCairns RJS, O'Hara RB, Merilä J (2013) QST – FST comparisons: evolutionary and ecological insights from genomic heterogeneity. *Nat Rev Genet.* 14: 179–190.
- Maghsoodi M, Sheidai M, Koohdar F (2018) Population genetic study in *Juglans regia* L. (Persian walnut) and its taxonomic status within the genus *Juglans* L. *Phytotaxa* 376 (4): 154–166.
- Nakaoka H, Inoue I (2009) Meta-analysis of genetic association studies: methodologies, between-study heterogeneity and winner's curse. *J Hum Genet* 54: 615–623.
- Nakaoka H, Inoue I (2009) Meta-analysis of genetic association studies: methodologies, between-study heterogeneity and winner's curse. *J. Hum. Genet.* 54:615–623.
- Podani J (2000). *Introduction to the Exploration of Multivariate Data*. Backhuyes, Leiden.
- Rozas J, Ferrer-Mata A, Sánchez-DelBarrio GC, Guirao-Rico S, Librado P, Ramos-Onsins S, Sánchez-Gracia A (2019) *Universitat de Barcelona Current Beta Version: 6.12.03* (February 26, 2019)
- Stojanova B, Šurinová M, Klápště. J, Kolářiková V, Hadincová V, Adaptive V, Münzbergová, ZB (2018) Dif-

ferentiation of *Festuca rubra* along a climate gradient revealed by molecular markers and quantitative traits. PLOS ONE 13(4): e0194670.

- Tabasi M, Sheidai M, Hassani D, Koohdar F (2020) DNA fingerprinting and genetic diversity analysis with SCoT markers of Persian walnut populations (*Juglans regia* L.) in Iran. Genet Resour Crop Evol. <https://doi.org/10.1007/s10722-020-00914-7>.
- Van de Wouw M, Van Hintum T, Kik C, Kik Ch, van Treuren R, Visser B (2010) Genetic diversity trends in twentieth century crop cultivars: a meta-analysis. Theor Appl Genet 120: 1241–1252.
- Walker E, Hernandez AV, Kattan MW (2008) Meta-analysis: Its strengths and limitations. Cleve Clin J Med 75 (6): 431–9.
- Zhao J, Sauvage C, Zhao J, Bitton F, Bauchet G, Liu D, Huang S, Tieman DM, Klee HJ, Causse M (2019) Meta-analysis of genome-wide association studies provides insights into genetic control of tomato flavor. Nat. commun 10(1): 1-12.