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Genetic characterization of almond (*Prunus dulcis*) cultivars and natural resources

J. Halász[1,*], I. Skola[2], O. Kodad[3], S. Ercisli[4], C.A. Ledbetter[5] and A. Hegedüs[1]

[1]Department of Genetics and Plant Breeding, Corvinus University of Budapest, P.O. Box 53, H-1518 Budapest (Hungary)
[2]National Agricultural Research and Innovation Centre, Fruitculture Research Institute, Cegléd (Hungary)
[3]Département d'Arboriculture, École Nationale d'Agriculture de Meknès, BP S/40 (Morocco)
[4]Department of Horticulture, Ataturk University, 25240, Erzurum (Turkey)
[5]United States Department of Agriculture, Agricultural Research Service, Crop Diseases, Pests & Genetics Research Unit, 9611 S. Riverbend Avenue, Parlier, CA 93648-9757
*e-mail: julia.halasz@uni-corvinus.hu

Abstract. This study was conducted to analyse the structure of molecular variation among Hungarian almond genotypes and to estimate the level of genetic diversity. A total of eighty-six almond samples originated from different geographical regions from Central Asia to California were evaluated using a set of 8 SSRs and 5 EST-SSR primer pairs. Amplification of DNA was successful for 11 of the 13 SSR loci. A mean value of 15.45 alleles per locus was found. Our data revealed a high level of genetic variability in most groups of samples. The highest number of alleles was detected in the Moroccan genotypes while the lowest number of alleles occurred in the group of self-compatible cultivars. All Hungarian cultivars and accessions were characterized by an average allele number of above 5. The correspondences between the genetic diversity and mating strategy highlight the role of the self-(in)compatibility system in shaping the genome of a fruit tree species.

Keywords. Almond – *Prunus dulcis* – Genetic diversity – SSR – Microsatellites.

***Caractérisation génétique de cultivars et de ressources naturelles d'amandier* (Prunus dulcis)**

Résumé. *Cette étude a été menée pour analyser la structure de la variation moléculaire entre génotypes hongrois d'amandier et pour estimer le niveau de diversité génétique. Un total de quatre-vingt-six échantillons d'amandiers provenant de différentes régions géographiques, depuis l'Asie centrale jusqu'en Californie, ont été évalués à l'aide d'un ensemble de 8 SSR et 5 primer pairs EST-SSR. L'amplification de l'ADN a été réussie pour 11 des 13 loci SSR. On a trouvé une valeur moyenne de 15,45 allèles par locus. Nos données révèlent un fort niveau de variabilité génétique dans la plupart des groupes d'échantillons. On a détecté le plus grand nombre d'allèles dans les génotypes marocains tandis que le plus petit nombre d'allèles se trouvait dans le groupe de cultivars autocompatibles. Tous les cultivars et accessions hongrois étaient caractérisés par un nombre moyen d'allèles supérieur à 5. Les correspondances entre la diversité génétique et la stratégie de fécondation mettent en relief le rôle du système d'auto-(in)compatibilité pour la configuration du génome d'une espèce fruitière arboricole.*

Mots-clés. *Amandier* – Prunus dulcis – *Diversité génétique – SSR – Microsatellites.*

I – Introduction

The cultivated almond [*Prunus dulcis* (Mill.) D.A. Webb] is thought to have originated in the arid mountainous regions of Central Asia (Grasselly 1976). Molecular results evidenced dissemination of the cultivated almond from Iran to the Eastern Mediterranean and subsequently to the Western Mediterranean regions, to North America and finally to the southern hemisphere including South America and Australia. Almond is a self-incompatible species which is governed by the highly polymorphic, multiallelic *S*-locus (Dicenta and García, 1993). Due to the genetically controlled self-incompatibility system, almond is one of the most polymorphic cultivated fruit species.

Simple sequence repeats (SSR) or microsatellites have been widely used to study tree species (Höhn *et al*., 2010; Lendvay *et al*., 2014). Since first SSR markers were described in peach (Cipriani *et al*., 1999), they have been developed in many other *Rosaceae* species, such as apricot, Japanese plum and cherry (Dirlewanger *et al*., 2002; Messina *et al*., 2004; Mnejja *et al*., 2004). Transferability (being able to use an SSR developed in one species in other species) has been frequently reported, particularly for peach SSRs. No significant differences were detected in transferability and the ability to detect variability between microsatellites of EST and genomic origin (Mnejja *et al.*, 2010). The first set of almond SSRs was published by Testolin *et al*. (2004).

Up to date, several studies were carried out to characterize the SSR diversity of almond cultivars and genotypes originated in specific geographical regions. Xu *et al*. (2004) developed SSR markers for the phylogenetic analysis of almond trees from China and the Mediterranean region. Genetic diversity of the Spanish national almond collection was characterized by Fernández i Martí *et al*. (2009). Twelve highly polymorphic SSR loci were selected to uniquely identify cultivars commonly grown in California, and to allow an accurate assessment of parent/offspring relationships among them (Dangl *et al*., 2009). Zeinalabedini *et al*. (2010) characterized Spanish, French, Italian, American, Iranian, Tunisian, Australian, Ukrainian, Portuguese and Slovakian almond cultivars by chloroplast and nuclear SSRs. Their results established the value of SSR markers for distinguishing different genetic lineages and characterize an extensive gene pool available to almond genetic improvement.

However, until now, there is no molecular information about the genetic diversity and relationship of the Hungarian almonds. Hence, this study was conducted to analyse, for the first time, the structure of molecular variation among Hungarian genotypes and to estimate the level of genetic diversity. In addition, the data are evaluated in comparison with a range of genotypes coming from different geographical areas, from Central Asia to California.

II – Materials and methods

A total of eighty-six almond samples originated from different geographical regions were evaluated. The Hungarian, Ukrainian and Italian cultivars are kept in the collection of the Corvinus University of Budapest, Department of Genetics and Plant Breeding. Other Hungarian samples were collected from abandoned orchards in Gellérthegy, Tétényi-fennsík, Cegléd and Monor. Leaf samples of the Moroccan accessions were collected in wild growing populations. Turkish wild almond genotypes originated in Bademli region and Akdamar Island (Lake Van). Some accessions were collected in Kyrgyzstan (near the city Osh). Californian cultivars and one accession of wild almonds (*Prunus tenella*, *P. arabica* and *P. webbii*) were sampled in the experimental orchard of Agricultural Research Service (United States Department of Agriculture, Fresno, CA).

Genomic DNA was extracted from fully expanded young leaves using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). A set of 8 SSRs and 5 EST-SSR primer pairs were selected on the basis of previous reports on different *Prunus* species, and included 6 for peach, 6 for almond and 1 for plum (Table 1), covering eight linkage groups (G1 to G8). The forward primers were labelled with 6-FAM fluorescent dye for detection in a capillary genetic analyzer. PCR reactions were carried out in a PTC 200 thermocycler (MJ Research, Budapest, Hungary) using the program described for the primers Approximately 20-80 ng of genomic DNA was used for PCR amplification in a 25 µl reaction volume containing 10 × Dream*Taq*™ Green buffer (Fermentas, Szeged, Hungary) as well as KCl and $(NH_4)_2SO_4$ at a ratio optimized for robust performance of Dream*Taq*™ DNA Polymerase in PCR with final concentrations of 4.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.2 µM of the adequate primers and 0.75 U of Dream*Taq*™ DNA polymerase (Fermentas).

Fragment lengths were estimated by comparison with the 1-kb DNA ladder (Promega, Madison, USA). To determine the exact size of the fragments, the fluorescently labelled products were run on an automated sequencer ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary).

For determination of fragment sizes (genotyping), GENOTYPER 3.7 software and the GS500 LIZ size standard (Applied Biosystems) were used. PopGene 1.32 (http://www.ualberta.ca/_fyeh/) software also was used for calculation of observed heterozygosity (*Ho*), expected heterozygosity (*He*), observed number of alleles (*Na*), Shannon's information index (*I*) and gene flow (*Nm* = 0.25(1 – FST)/FST.

III – Results and discussion

In the 86 almond genotypes, amplification of genomic DNA was successful for 11 of the 13 SSR loci including 7 genomic and four EST-SSR primers developed from different *Prunus* species (peach, almond and plum). The primer BPPCT 001 designed from peach genomic sequences provided unclear amplification; while the ASSR27 locus proved to be monomorphic and hence neither of them was included in the analysis. Altogether, 11 primer pairs produced a total of 170 alleles ranging from 4 to 21 per locus (Table 1). All primer pairs produced a maximum of two alleles per genotype in accordance with the diploid state of the species. Genotypes showing a single band were considered homozygous for that particular locus. A mean value of 15.45 alleles per locus was found. SSR primers derived from the non-coding DNA region had more alleles (an average of 17.85 per locus) than EST-SSR primers, originating from coding DNA regions (an average of 11.25 alleles per locus). Among EST-SSR loci, EPDCU 3083 had the highest number of alleles (20), while EPDCU5100 amplified the lowest number of alleles, only four. Observed heterozygosity ranged between 0.62 for CPPCT 044 and 0.81 for BPPCT 025, with an average of 0.73 across the SSR markers, while in case of EST-SSRs the average was much lower (0.53) since one of the loci (the EPDCU 5100) revealed an exceptionally low level of heterozygosity (0.19).

The highest number of alleles for most of the analysed markers (Table 1) was detected in the Moroccan genotypes (7.09). This huge genetic variability might be explained by previous studies based on nuclear DNA markers and showing that Moroccan genotypes are genetically different from the tested commercial cultivars (El Hamzaoui *et al.*, 2013). The lowest number of alleles occurred in the group of self-compatible cultivars (3.63). All Hungarian cultivars and accessions were characterized by an average allele number of above 5. The highest observed heterozygosity was detected in the Turkish accessions from Akdamar Island (0.76), while self-compatible cultivars showed the lowest value (0.58). The four groups of Hungarian genotypes (cultivars, accessions from Gellért-hegy, accessions from the Tétényi-fennsík and old genotypes from Cegléd and Monor) revealed very similar data for all parameters including Na, I, He, Ho, and N_m; however, two Turkish groups (accessions from Akdamar Island and Bademli region) showed marked differences. It was also reflected by the fact that approximately the same proportion of unique alleles (approx. 70%) was found in those groups as shown for groups more distantly related groups (e.g. Californian cultivars and self-compatible almonds).

Table 1. Number of alleles, Shannon's Information index (I), observed heterozygosity (*Ho*), expected heterozygosity (*He*), and gene flow (N_m) in the tested geographic groups of almond

Geographic groups	Number of alleles (*Na*)	Shannon's Information index (*I*)	Observed (*Ho*) heterozygosity	Expected (*He*) heterozygosity	Gene flow (N_m)
Hungarian cultivars	5.27	1.37	0.67	0.71	0.25
Gellért-hegy, Hungary	5.18	1.41	0.67	0.75	0.23
Tétényi-fennsík, Hungary	5.45	1.45	0.67	0.75	0.23
Old Hungarian genotypes	5.09	1.35	0.61	0.71	0.21
Californian cultivars	4.18	1.12	0.63	0.63	0.30
Akdamar Island, Turkey	4.45	1.16	0.76	0.64	0.41
Bademli region, Turkey	5.09	1.28	0.61	0.66	0.23
Morocco	7.09	1.60	0.66	0.75	0.20
Self-compatible cultivars	3.63	1.06	0.58	0.64	0.25

Our data revealed a high level of genetic variability in most groups of samples, which was very similar to the trend emerging from other studies on almond genetic variability (Szikriszt *et al*., 2011). Many of the genetic diversity parameters were almost identical for most groups with only few exceptions. Californian cultivars, accessions from Akdamar island and self-compatible cultivars showed decreased extent of variability.

Akdamar is a small island in Lake Van, while Bademli is a region where almond trees have been long known to grow around the region. Several trees in Akdamar Island share common or almost identical genotypes pointing to the consequences of a founder effect. It is supposed that only some trees have been brought to the island and all extent accessions are the offspring of a small number of individuals originally reaching the island. However, observed heterozygosity remained high since almond is self-incompatible. In turn, Nm is high, indicating only a moderate genetic differentiation of the population. In contrast, almonds in Bademli show higher genetic diversity since they do not face with such a geographic isolation. However, it is interesting to realize that trees from Bademli village and those from Akdamar Island showed as low genetic relatedness as trees originated in different regions of the world. We have determined self-incompatibility RNase alleles in Bademli almonds and found several *Prunus webbii* alleles in their *S*-locus. It suggests introgressive hybridization between *P. dulcis* and naturally occurring *P. webbi* accessions. This was also confirmed with other species like *P. orientalis* (Delplancke *et al*., 2012; Zeinalabedini *et al*., 2010) and indicates that differences in specific geographic regions might be formed by introgressive hybridization.

The number of alleles and Shannon index also indicated decreased genetic variability for Californian commercial cultivars. The use of a small number of accessions in breeding programs will eventually lead to a marked decrease in genetic variability as was experienced in our study, as well. Self-compatibility was described in some almond cultivars, and a slight decrease in variability parameters could have been shown in the present study. Self-compatibility in almond is a relatively new trait (spanning only some decades in time) and hence it has not been enough time to induce genetic erosion. The correspondences between the genetic diversity and mating strategy highlight the role of the self-(in)compatibility system in shaping the genome of a fruit tree species.

IV – Conclusions

There is no indication of major decrease in genetic variability in almond germplasm from Asia to Europe. Slight losses of genetic diversity are attributable to different reasons including geographic isolation, human selection and especially the relatively recent occurrence of self-compatibility. This information might be taken as a warning signal by almond breeders.

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