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Evaluation of transcprition factors affecting some agronomic traits of stone fruit species

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Name of Doctoral school:	**Doctoral Shool of Horticultural Sciences**
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Head of the doctoral school:	**Dr. Éva Németh Zámboriné** professor, DSc SZIU, Faculty of Horticultural Sciences, Department of Medical and Aromatic Plants
Supervisor:	**Dr. Attila Hegedűs** professor, DSc SZIU, Faculty of Horticultural Sciences, Department of Genetics and Plant Breeding

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Dr. Éva Németh Zámboriné	**Dr. Attila Hegedűs**
Head of school	**Supervisor**

1. INTRODUCTION

Spring frost injury has become a critical issue for Hungarian stone fruit production in the past few decades (Pedryc et al., 1999; Szalay et al., 1999). The annual growing cycle of temperate woody plants forms an integrated system with subsequent phases of active growth and dormancy (Hanninen and Tanino, 2011). Endodormancy is a genetically controlled mechanism that is triggered in early autumn by external factors and inhibits bud development even under growth-promoting conditions. Plants require a certain amount of chill for endodormancy-release to enter ecodormancy phase when bud growth is only prevented by unfavourable climatic conditions (Rohde és Bhalerao, 2007; Hanninen és Tanino, 2011).

The genetic factors responsible for the cold- and frost stress responses first have been identified in *Arabidopsis thaliana.* CBF transcription factors belong to the DREB1 subfamily within the AP2/ERF protein family. They are able to bind to the CRT/DRE (C-repeat/dehydration responsive element) sequence motif in the promoter region of the regulated gene, which contains a conserved CCGAC sequence as a binding site for the DNA-binding domain of CBF proteins On low temperature, the *Arabidopsis CBF* genes can be induced within a short time and they activate the 'CBF-regulon', i.e. the cold-regulated genes which contain the CRT/DRE regulatory-element (Stockinger et al., 1997; Reichmann and Meyerowitz, 1998).

Several CBF-coding genes have been described in some species within the *Prunus* genus such as peach (Wisniewski et al., 2011), almond (Barros et al., 2012) and Japanese apricot (Zhang et al., 2013). A full-length cDNA of a peach *CBF* gene (*PpCBF1*) has been isolated by Wisniewski et al. (2011) and its ectopic expression in apple resulted in induced. In case of deciduous woody plants such as Rosaceae species, a special type of MADS-box proteins, the so-called DAM (Dormancy Associated MADS-box) transcription factors are

responsible for dormancy regulation. The six *DAM* genes were first described in the '*evergrowing*' (*evg*) peach mutant that is incapable of forming terminal vegetative buds in response to dormancy-inducing conditions (Bielenberg et al. 2008). The expression of *DAM* genes seems to be tissue-specific, moreover, each of them has a clear seasonal expression-pattern presumably regulated by photoperiod (Li et al., 2009). The seasonal expression level of *DAM5-6* genes shows correlation with bud dormancy induction and break in case of peach. Specific period of low temperature exposure is necessary to reduce the expression of *PmDAM5-6* and to break this inhibition; therefore, they possibly work as dose-dependent growth inhibitors in dormant buds (Yamane et al., 2011).

Apricot (*P. armeniaca L.*) productivity is much affected by its early-flowering time since unfavourable environmental conditions in late winter/early spring frequently result in a considerable yield loss in many producing countries The aim of the present study was to identify *P. armeniaca CBF* and *DAM* gene sequences that may contribute to bud dormancy regulation since there is no data available in case of this species despite of its great agronomic importance.

Besides agronomic aspects, local breeders also need to consider about consumer preferences. One of the most important aims in cultivar improvement is the extension of the ripening season. A main breeding problem in case of woody plants is the long juvenile period. The selection of the desirable genotypes could be facilitating by the application of molecular markers.

Most fruit quality traits including maturity date (MD) are quantitatively inherited and controlled by quantitative trait loci (QTL). In peach, QTLs controlling fruit ripening have been mapped on different chromosomes, with a major QTL (*qMD4.1*) located on linkage group 4 (G4) (Dirlewanger et al., 2004). Within *qMD4.1* a NAC-transcription factor coding sequence (*ppa008301m*) has been identified (Eduardo et al. 2011). This *NAC*-type gene indicated as *PpNAC1* was found to be a strong candidate gene for controlling

MD in peach. The Sanger re-sequencing of this candidate gene uncovered an in-frame 9 bp insertion in early MD cultivars, resulting in a tandem duplication of three amino acids in the last exon of the C-terminal domain. The allelic variants co-segregated with the MD of all individuals in the F2 populations: the early ripening individuals had the 9 bp insertion while the late ripening genotypes carried the reference allele. This insertion resulted in the duplication of a threonine – aspartic-acid – proline stretch with possible impact on protein structure and function (Pirona et al., 2013). This allele variant was suggested to be used as a functional marker to determine MD in peach breeding programs. Our aim was to further support this correlation between the *NAC*-genotype and MD in a wide range of peach cultivars with different ripening time.

2. OBJECTIVES

The **aims** of this study were:

1. To identify apricot (*Prunus armeniaca* L.) *CBF (C-repeat biding factor)* and *DAM* (dormancy associated MADS-box) gene sequences that may contribute to bud dormancy regulation.

2. To identify apricot (*Prunus armeniaca* L.) *DAM* (dormancy associated MADS-box) gene sequences that may contribute to bud dormancy regulation.

3. The expressional analysis of the newly identified *CBF*- and *DAM* sequences in two early and two late flowering apricot cultivars, comparing two consecutive dormant seasons.
4. The determination of chilling requirements and flowering dates of the apricot cultivars used in this study, and the evaluation of the pollen development (microsporogenesis) and compare these results with the gene expression studies.

5. Detecting the allele variants of a NAC1 (NAM, ATAF1,2, CUC2 1) transcription factor coding gene in set of peach (*Prunus persica* L.) cultivars with different ripening time, and testing the applicability of the allele variants as a functional marker with statistical analysis based on the correlation between maturity date and *NAC*-genotypes.

6. The identification of *PpNAC1* sequence homologues in case of related *Prunus* species and sequence differences similar to the peach *PpNAC1* allelic variants.

3. MATERIALS AND METHODS

3.1. Plant material

For genomic DNA isolation vegetative buds have been collected in case of all *Prunus* species from three different locations between November 2015 and February 2016. All together 125 peach cultivars were examined showing considerable variations in their origin, phenotypic traits and MDs ranging from very early (the middle of June) to very late (the beginning of October). Besides we have chosen some cultivars with different MD in case of related species (apricot, almond, European plum and sour cherry). For RNA-isolation and gene expression analysis, four apricot (*Prunus armeniaca* L.) cultivars, the early-flowering 'Aurora' and 'Goldrich' (USA) and the late-flowering 'Stella' (USA) and 'Zard' (Central Asia) were sampled for the studies with *CBF*- and *DAM5-6* genes. Flower buds of the four tested apricot cultivars were collected 8 and 7 times in two consecutive dormant periods, 2015/16 and 2016/17, respectively, from leaf fall (November) to the beginning of bloom (mid of March).

3.2. DNA-based methods

The total genomic DNA was extracted from vegetative buds using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). For each sample, PCR amplification was performed and specific published primers (Bielenberg et al, 2008; Wisniewski et al., 2011; Barros et al., 2012; Pirona et al., 2013), designed for related *Prunus* species were applied for the amplification of target sequences. The PCR products were cloned into pTZ57R/T plasmid vector using (Thermo Scientific, Waltham, MA, USA) and sequenced by ABI 3500 XL Genetic Analyzer (Baygen Institute, Szeged, Hungary). For fragment-length analysis of *Prunus NAC* sequences, the PCR products amplified by fluorescently labeled forward primer (NAC-INDELF, 5'6-FAM) were run in an automated sequencer ABI PRISM 3100 Genetic Analyzer (Biomi Ltd, Gödöllő., Hungary).

For data analysis, ABI Peak Scanner 1.0 software and GS500 LIZ size standard were used.

3.3. RNA isolation and gene expression studies

Total RNA from approx. 100 mg of bud tissue was extracted using the protocol of Jaakola et al. (2001). After DNase I treatment and reverse-transcription, the expression level of *P. armeniaca CBF* and *DAM5-6* genes was determined by means of real-time PCR (Bio-Rad CFX96 Touch real-time PCR instrument, Bio-Rad Laboratories, Hercules, CA, USA). The quantitative PCR primers were designed in this study from specific regions of the newly identified *ParCBF1*, *ParDAM5* and *ParDAM6* genes and actin was used as reference.

3.4. Bioinformatics and statistics

The homology test of *Prunus NAC, CBF* and *DAM5-6* sequences were obtained with the NCBI MegaBLAST (Morgulis et al., 2008) algorithm. An alignment of sequences was carried out using MEGA6 (Tamura et al., 2011) To demonstrate the molecular structure of the typical protein-domains of CBF and DAM transcription factors, sequence homology was used in the SWISS-MODEL server (Arnold et al., 2006). The phylogenetic analysis of *CBF* and *DAM* sequences was inferred using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992), using MEGA5.1 (Tamura et al., 2011). For data analysis of *Prunus NAC* fragment length polymorphism, ABI Peak Scanner 1.0 software and GS500 LIZ size standard were used. The correlation between the *NAC*-genotype and MD has been evaluated by Cramer's V-test.

In case of gene expression studies the relative fold change (FC) values were calculated with the ΔΔCt method (Bookout and Mangelsdorf, 2003). Real-time qPCR data presented for each sample represent the mean values determined for three independent replicates and one-way analysis of variance (ANOVA)

and Duncan's multiple range test with $P < 0.05$ was carried out to determine significant differences.

3.5. Evaluation of chilling requirement and microsporogenesis of apricot cultivars used in this study

The amount of cold received by the plants was quantified using the chill units of the Utah model (Richardson et al., 1974) and chill portions of the Dynamic model (Fishman et al., 1987). The date of breaking endodormancy was determined by forcing apricot branches with approximately 100 buds/cultivar according to the method described by Ruiz et al. (2007). Flowering date was recorded when 50% of 500 tested flower buds were open.

The male gametophyte development (microsporogenesis) was studied in 2016 and 2017 from January to March using anther-preparations via Zeiss Axio Imager 2 (Carl Zeiss, Thornwood, New York, USA) optical microscope at 400× magnification. Four stages of were distinguished in both years: archesporium – undifferentiated sporogenic tissue; premeiotic conditions (development and separation of pollen mother cells); tetrad (after meiosis) and microspores (development of pollen grains).

4. RESULTS

4.1. Identification and characterization of *ParCBF1* and *ParDAM5-6* genes

An apricot *CBF*-like sequence (756 bp) has been isolated from the genomic DNA of *P. armenica* 'Korai zamatos' using CBF-F forward (Barros et al., 2012) and CBF1 R reverse (Wisniewski et al., 2011) primer pair designed for peach and almond. Moreover a *DAM5*- (729 bp) and *DAM6*-like (230 bp) sequences were determined from cDNA obtained from flower buds of *P. armenica* 'Zard' using *Pp*DAM5/3-F (Bielenberg et al., 2008) *Pp*DAM5/2-R (Yamane et al., 2011), and *Pp*DAM6/3 (Bielenberg et al., 2008) primers designed for peach. The sequences were deposited into GenBank under the accession number of MH464453, MH464454 and MH464455, respectively.

Homology test revealed high similarity with sequences from related species. *ParCBF1* was most likely homologous to *Prunus mume CBF1* while *ParDAM5* show close relation to peach and Japanese apricot homologous sequences (*PpDAM5* and *PmDAM5*). *ParDAM6* was highly similar also to the peach, Japanese apricot and *P. pseudocerasus DAM6* sequences. All highly conserved structural motifs and the 3D model of the DNA-binding domain indicate an unimpaired DNA-binding ability of the AP2 domain of *Par*CBF1. *Par*DAM5 also contained all characteristic domains of the type II ($MIKC^C$) subfamily of MADS-box transcription factors (M-, I and K- domains). The amino acid positions or small motifs that are diagnostic characteristics of DAM5 and DAM6 were determined.

To further support the homology and functional integrity of the new apricot sequences, a phylogenetic analysis was made using homologous CBF- and DAM sequences of several monocot and dicot species. In both cases, members of the Prunoideae and Maloideae subfamilies formed two well distinguishable sister clades within the group of Rosaceae species. The

*Par*CBF1 was found within a complex sub-structured cluster including *P. dulcis*, *P. persica* and *P. mume* CBF sequences. The sequences did not form species-specific clusters but were scattered among statistically supported sub-clusters and mixed with sequences from other species. *ParCBF1* was most likely related to *Prunus mume* and *Prunus dulcis CBF1*. In case of DAM sequences the *Prunus* clade was divided into six sub-clades according to the six different (*DAM1* to *DAM6*) genes. The *Par*DAM5 sequence showed the closest relationship with *P. mume* DAM5, while *Par*DAM6 clustered with the corresponding *P. persica* sequence. Our analysis confirmed that *ParCBF1*, *ParDAM5* and *ParDAM6* are evolutionarily related to *CBF*, *DAM5* and *DAM6* genes in other *Prunus* species, pointing to a putatively identical function in *P. armeniaca*.

4.2. Functional studies of *ParCBF1* and *ParDAM5-6* genes

For functional studies of the newly identified *P. armeniaca* CBF- and DAM genes, we calculated the chilling requirement (CR) of apricot cultivars used in this experiment in chill-units (Utah model) and chill portions (Dynamic model) in both seasons between October and March. The two early-flowering cultivars, 'Aurora' and 'Goldrich' had lower CR in both seasons compared to the late-flowering 'Stella' and 'Zard'. The flowering of 'Aurora' and 'Goldrich' occurred on the same day in both seasons, while the flowering of 'Stella' and 'Zard' was delayed by one day in 2016/17 compared to 2015/16.

The expression of *ParCBF1* was highly upregulated during the first part of the dormant season (December – first two weeks of January). The increase of expression in coincided with temperatures falling below the freezing point in both seasons. In addition, statistically significant differences were observed in *ParCBF1* expression of 'Aurora' and both 'Stella' and 'Zard' in each of the seasons. The expression of *ParCBF1* was downregulated in all cultivars to the end of January in both seasons and remained almost zero until the time of

budbreak. A characteristic seasonal pattern was observed in the expression rate of *ParDAM5-6* genes, which were quite similar in both seasons. Transcript levels were high at the beginning of the dormant period in both years. The highest gene expression levels were detected in December 2015 and in November 2016 for both *ParDAM5* and *ParDAM6*. Then, the expression levels of both genes started to decrease gradually in accordance with the accumulated chill units.

Correspondences among the expression rates of *ParCBF1* and *ParDAM5-6* were evident in December 2015 and January 2017 when cultivars showing higher expression rates of *ParCBF1* also had higher transcript levels for both *ParDAM5* and *ParDAM6*. The expression of all three genes were definitely downregulated once the chilling requirements of the cultivars had been fulfilled. It is clearly shown by the significantly higher *ParDAM6* expression rates in 'Stella' and 'Zard' on 8 February 2016, which resulted in a delay of 10–14 days in dormancy breaking time compared to early-flowering cultivars.

4.3. Correspondence between maturity date and *NAC* genotype of peach

The fragment-length analysis of 125 peach cultivars resulted in polymorphic chromatograms representing three distinct genotypes: 1) homozygous for the 192 bp reference allele, 2) homozygous for the 201 bp allele and 3) heterozygous cultivars carrying both the 192 bp and 201 bp alleles. For statistical support, we conducted a 3 × 3 contingency table analysis for independence according to the three categories in MD (early, mid-season and late) and three genotypes (192/192 bp, 192/201 bp and 201/201 bp) and used the chi-square test for significance analysis. The association of *NAC* genotype and MD was significant and difference between the observed and expected frequencies indicated considerable distortions as 192/192 homozygotes were less frequent in the early and mid-season MD categories and 201/201

homozygotes were underrepresented in mid- and late season categories. The frequency of the heterozygous cultivars peaked in the mid-season category.

4.4. *NAC* sequence variations in *Prunus* species

The successful amplification indicated the presence of the NAC-domain containing sequence in other stone fruit species, as well. Tthe fluorescently labeled NAC-INDEL-specific primer pair was used to amplify DNA from such samples to look for intraspecific small-scale size variations. However, the majority of the analyzed accessions amplified only one fragment with almond cultivars showing the 192 bp peach reference allele size, apricot and European plum cultivars amplified a single 188 bp fragment, sour cherry) had a 189 bp sized amplicon. Only ‘Kántorjánosi 3’, a sour cherry cultivar had two differently sized fragments of 189 and 192 bp. In contrast to peach, cultivars of almond, apricot and plum with considerably differing MD did not show consequent size polymorphism in the C terminal part of the NAC gene.

Sequence alignment of other *Prunus* species showed high level of similarity and some characteristic alterations compared to the peach reference allele. Although, the 9 bp insertion of the early-ripening peach cultivars were not present in sequences from other *Prunus* accessions, several variations were detected. A total of 74 single nucleotide polymorphisms (SNP) were detected in the coding region of the partial *NAC* domain sequences with 25 synonymous and 49 non-synonymous and one nonsense single base substitutions. Only three mutations induced non-conservative AA replacements in specific apricot and sour cherry alleles. A nonsense mutation was also detected in a sour cherry cultivar, ‘Korai pipacsmeggy’. These sequence variations might affect the functional integrity of the protein, although we need further studies to reveal their specificity.

4.5. New results

1. A 756 bp length partial sequence of a *C-repeat Binding Factor (CBF)* protein coding gene has been identified from *P. armenica* 'Korai zamatos' genomic DNA. The sequence was submitted into the NCBI GenBank database under the accession number of MH464453, MH464454 and MH464455, respectively. For identification of the *ParCBF1* sequence a homology test and phylogenetic analysis was made using homologous sequences of related species.
2. The molecular structure of the AP2-domain of the *ParCBF1* was modelled using a protein-modeling software.
3. *Dormancy Associated MADS-box 5* (729 bp) and *6*-like (230 bp) sequences were determined from cDNA obtained from flower buds of *P. armenica* 'Zard'. The sequences were submitted into the NCBI GenBank database under the accession number of MH464454 and MH464455, respectively. For the identification of the *ParDAM5* and *ParDAM6* sequences a homology test and phylogenetic analysis was made using homologous sequences of related species. According to the alignment of deduced amino acid sequences the characteristic structural elements of DAM-proteins were identified.
4. In case of *Par*DAM5 molecular structure of the M and K -domains was modelled using a protein-modeling software.
5. Gene expression analysis proved that the activity *ParCBF1* was controlled by low temperatures during the dormant period. We suggest that expression rate of *ParDAM5-6* is defined by the activity of *Par*CBF1 transcription factor and also by the fulfillment of CR of the cultivars used in this study.

6. The difference in timing of the anther development of early- and late flowering cultivars coincided with the significant decrease of *ParDAM5-6* expression rate and the fulfilment of the chilling requirements of apricot cultivars. All cultivars reached the tetrad stage after their CR was fulfilled.
7. The *NAC*-genotype of 125 peach cultivars with different pomological traits has been identified. Statistical analyses revealed significant correlation between *NAC*-genotype and MD in case of peach cultivars used in this study. These results prove the applicability of *PpNAC1* allelic variations as a functional marker during selection for MD.
8. The presence of the homologous *NAC* sequence has proved in the genomic DNA samples from all of the related *Prunus* species used in this study. We determined 15 new homologous sequences of *PpNAC1* from apricot (*P. armeniaca*), almond (*P. dulcis*), sour cherry (*P. cerasus*) and European plum (*P. domestica*). A total of 74 single nucleotide polymorphisms were detected in the coding region of the partial *NAC* domain sequences. The further evaluation of these mutations may provide information about the role of these genes.

5. CONCLUSIONS

5.1. Structural and functional verification of *ParCBF1* and *ParDAM5-6* genes

The 3D model of the AP2 domain of *Par*CBF1 was reliable and declared the typical AP2 fold with a three-stranded beta-sheet and an alpha helix almost parallel to the beta-sheet. This topology was found to be associated with contacting eight consecutive base pairs in the major groove of DNA and hence it indicates an unimpaired DNA-binding ability of *Par*CBF1. Since CBF transcription factors may bind specifically to the C-repeat/dehydration-responsive (CRT/DRE) element in the promoters of *DAM* genes, the intact AP2 domain structure indicates a possible connection between CBF and dormancy-associated MADS-box proteins.

Six *DAM* genes were identified in peach and Japanese apricot (Bielenberg et al., 2008) but only two of the six (*DAM5* and *DAM6*) genes were reported to be associated with endodormancy release in peach generative buds (Yamane et al., 2011b). Using primers specifically amplifying *DAM5* and *DAM6*, we determined the partial sequences of their *P. armeniaca* homologs. For *PpDAM5*, the homology was confirmed by MegaBlast and phylogenetic analyses, indicating that *ParDAM5* belongs to the super-clade of *SVP*/*AGL24* *Arabidopsis* sequences as was previously reported for *P. mume DAM6* (Yamane et al., 2011). The identification of protein domains and the homology modelling of characteristic domains suggest its functional integrity.

The expression of *CBF* coding genes was associated with decreasing ambient temperatures in case of *Arabidposis* (Stockinger et al., 1997), and woody plants (Wisniewski et al., 2011; Zhang et al., 2013). This tendency was also observed in case of *ParCBF1* and validates its physiological role as an important element of the low temperature signalling cascade leading to dormancy induction and release in apricot.

The presence of a conserved core sequence motif (CCGAC) in the promoter region of *DAM5* and *DAM6* genes in peach and Japanese apricot suggests their participation in the CBF regulon (Yamane et al., 2011). *ParDAM5* and *ParDAM6* had similar expression patterns as their peach and Japanese apricot homologs (Yamane et al., 2011). Significant differences between the expression levels of *ParDAM6* in early- and late-flowering cultivars could be also detected 12–14 days later than in case of *ParDAM5*, which indicates differences in their regulation. The expression levels of *ParDAM5* and *ParDAM6* changed according to low temperature induced *CBF1* expression rates and the fulfilment of cultivar chilling requirements. The expression levels of *ParDAM5* and *ParDAM6* were related to the daily minimum temperatures and *CBF1* expression rates: the sampling day in December 2015 was colder than in 2016 and *ParCBF1* and *ParDAM5* and *6* had higher expression rates for all cultivars in 2015.

In Hungary, annual temperature has increased 0.8°C over the past century (Lakatos et al., 2011) and this warming tendency resulted in a shift of endodormancy release time by 19–23 days over 24 years while flowering time occurred three days earlier (Szalay et al., 2019). Hence, the identification and characterization of the apricot homolog of three genes that have been associated with the onset and release of flower bud dormancy in other *Prunus* species are of crucial importance. *ParCBF1*, *ParDAM5* and *ParDAM6* show all typical structural features and genetically and environmentally controlled expression levels over the endodormancy stages to be important elements of the molecular network behind bud dormancy of apricot trees.

5.2. The applicability of *PpNAC1* as a functional marker

A 9 bp insertion in the C terminal domain of a peach *NAC* gene was suggested to be associated with early maturity date in two segregating populations (Pirona et al., 2013). We wanted to clarify the influence of *PpNAC1* gene by genotyping of 125 peach cultivars characterized by different MD and validate its application as a reliable molecular marker. Significance analysis and Cramér's V test indicated that the two variables (*NAC* genotype and maturity category) are probably measuring the same concept. This extremely strong correlation was further supported by the Goodman-Kruskal index (λ) indicating substantial increase in the probability of correct prediction of MD when *NAC* genotype is considered. These data support the use of this marker in marker assisted selection for MD.

However, there were some outlier cultivars in both the early and the late MD group. The early ripening 'Favorita Moretti' (first (decade of June) and 'Chinese 8' (second decade of June) were homozygous to the 192 bp allele, while late ripening 'Royal Pride' (end of August), 'Harken' and 'Orion' (first decade of September) were homozygous to the 201 bp. These results indicate that other loci may also contribute to the MD determination. This is reasonable since NAC proteins are part of a complex network.

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PUBLICATIONS RELATED TO THE PRESENT STUDY

Reseach papers with impact factor:

Balogh E., Halász J., Szani Zs., Hegedűs, A. (2018): Correspondance between maturity date and molecular variations in a *NAC* transcription factor of diploid and polyploid *Prunus* species. Turkish Journal of Agriculture and Forestry 48(1**). IF: 1,731** (doi:10.3906/tar-1711-19)

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