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Determination of salt tolerance levels and genetic relationships of *Vicia sativa* cultivars using gene targeted functional markers

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Abstract – The objectives of the present study were to determine salt tolerance levels of 12 different common vetch (*Vicia sativa* L.) cultivars at germination stage in the presence of 250 mM NaCl and to reveal genetic relationships based on gene targeted functional markers (GTFMs) associated with salt tolerance. The results revealed the presence of a significant genetic variation among the cultivars although salt stress significantly reduced all germination parameters tested. The cultivar Ozveren was the most salt tolerant with 20.1% reduction in final germination percentage compared to control seeds while cultivars Alınoglu, Ayaz and Bakir did not germinate. The maximum delays in germination rate (G_{50} = 3.78 days) and synchrony ($G_{10\text{-}90}$ = 3.45 days) were obtained from the cultivars Urkmez and Ozveren, respectively. The GTFMs provided a total of 53.1% polymorphism. The primers of *MtSOS2* gene gave the highest numbers of alleles per primer pair while the highest polymorphism rate (77.8%) was obtained from the *MtP5CS* gene. The first three components of principal component analysis explained 57.63% of total variation. This study concluded that the cultivars determined to be salt tolerant and sensitive at germination stage distributed into three main clades determined by UPGMA analysis while the GTFMs associated with salt tolerance successfully determined the genetic relationships of common vetch cultivars.

Keywords: common vetch, germination, gene, markers, salt tolerance

Introduction

Soil salinity is one of the most important abiotic stress factors directly limiting plant yield in agricultural production areas of all over the world (Shokat and Großkinsky 2019). Plants respond to salts in soil or in irrigation water at different levels by tuning complex physiological and molecular mechanisms (Mel et al. 2019). High salt prevents the water uptake and creates a physiological drought (Khayamim et al. 2014) or ion toxicity to the embryo of seeds, which directly limits fast and even seed germination (Farooq et al. 2017). Salt tolerance level of a plant can vary based on plant developmental stage (Bu et al. 2015). Seed germination and seedling stages of plants are generally less tolerant than mature plants (Hussain et al. 2010). Therefore, determination of salt tolerance levels of cultivars at germination stage is one of the most important tasks to promote successful plant development for sustainable agricultural production.

The common vetch (*Vicia sativa* L.) is considered one of the most important annual, self-pollinated, diploid forage crop species and has plasticity not only for adaptability to different soil and climate conditions but also its diverse use as grain, straw, hay, silage, and green manure along with soil improvement ability with nitrogen fixation (Sherasia et al. 2017). The common vetch also provides valuable protein and mineral sources for cattle and poultry in Turkey, Australia, New Zealand, China and Eastern Europe (Firincioglu et al. 2007, Sherasia et al. 2017). Like many other legume crops, common vetch has, however, reduced growth and yield under saline condition and is considered more vulnerable to salt stress than some other major crop species such as cereals (Hussain et al. 2010).

To discriminate individuals from various breeding sources, PCR-based molecular markers are the best tool for genetic characterization and the estimation of genetic diversity in various organisms including plants (Poczai et al.

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2013a). Molecular markers are also more reliable than pedigree data for parental selection to estimate genetic diversity in plants (Babic et al. 2016). However, the power of a molecular marker is mainly determined by the level of polymorphism detected (Wan et al. 2004). As a complex taxon, *V. sativa* represents a diverse phylogenetic relationship (Kartal et al. 2020). Various types of molecular markers have been previously used to resolve inter- and intra-specific diversity in common vetch by using SRAP and ISSR (Cil and Tiryaki 2016), SSRs (Raveendar et al. 2015), EST-SSR, (Liu et al. 2014), SCoT (Chai et al. 2017) and SNPs (De la Rosa et al. 2020). The recent sequencing advances in plant biotechnology resulted in an avalanche of information in terms of DNA sequences and functional gene determination in various plant species (Chai et al. 2017). Therefore, not only has the transferability of molecular markers among the species been studied (Raveendar et al. 2015) but new alternative molecular marker techniques have also been developed in diverse plant species (Poczai et al. 2013b). Of those, the molecular markers which are generated based on untranslated regions of expressed sequence tags (ESTs) are called gene-targeted markers (GTMs) (Poczai et al. 2013b) while the gene markers involved in the variation of phenotypic traits due to their functional gene sequences are called gene-targeted functional markers (GTFMs) (Arnholdt-Schmitt 2005).

This study aimed to determine the salt tolerance levels of 12 common vetch cultivars at germination stage by using seed germination parameters and to reveal genetic relationships based on salt tolerance-related gene primers. The aim of the study was also to reveal whether or not GTFMs can also distinguish the salt tolerance and sensitivity levels of common vetch cultivars at germination stage in the presence of 250 mM NaCl.

Materials and methods

Seed material

All available 12 common vetch cultivars were kindly provided from the Ankara Field Crops Central Research Institute (Alınoglu, Ayaz, Ankaramoru, Bakir, Farukbey, Zemheri), Izmir Aegean Agricultural Research Institute (Alper, Cumhuriyet, Selcuk, Urkmez) and Adana Eastern Mediterranean Agricultural Research Institute in Turkey (Ozveren, Yucel). A range of NaCl concentrations were tested in pre-experimental trials and 250 mM NaCl was chosen to determine the salt tolerance levels of the 12 common vetch cultivars.

Germination experiment

A single layer of 50 seeds of each cultivar was placed in covered petri dishes (80 × 15 mm) on double layers of filter papers saturated with 4 mL of 250 mM NaCl. A temperature-controlled incubator kept at 20 ± 0.5 °C in darkness was used for germination test. Four replications of 50 seeds were arranged in a completely randomized block design. Seeds showing a radicle exceeding 2 mm in length emerging from the testa were recorded as germinated. The germinated seeds were daily removed from the petri dishes until the germination count was unchanged for 3 subsequent days (total 15 days). The final germination percentage (FGP), germination rate and span of germination parameters were determined as described previously (Tiryaki and Kaplan 2019). Germination rate was used to estimate days to 50% of FGP (G_{50}) and the span of germination ($G_{10\text{-}90}$) was used to estimate the time from 10% to 90% of FGP by using methods described previously (Tiryaki and Kaplan 2019). The control seeds of each cultivar were treated with 4 mL of dH_2O and were germinated under the same germination conditions used for the salt experiment as described above.

DNA extraction and quality control

The seeds of each cultivar were planted in rows with 10 cm raw space in plastic boxes (10 × 35 × 45 cm) filled with peat with 3 cm planting depth and were incubated in a plant growth chamber with a 12 h light (350 μmol m^{-2} s^{-1})/dark cycle at 20 °C. The young leaves of five seedlings from each cultivar were bulked and were used for DNA extraction by using a plant genomic DNA extraction kit (Favorgen, Pingtung, Taiwan) following the manufacturer's instruction. The DNA concentrations were estimated by comparing known concentration of λ DNA on 1% agarose-gel electrophoresis and were used in PCR analysis after concentration equalization.

Gene Targeted Functional Markers and PCR amplification

In all, nine gene specific primer pairs (Tab. 1) were used after PCR amplifications of each primer pair were optimized in a gradient PCR (Thermo Fisher Scientific, Inc., USA) to determine the best annealing temperature in common vetch genome. Eight of nine gene specific primer pairs were either directly or indirectly associated with salt tolerance in plants while *MtActin* (*Medicago truncatula* Actin) gene was used to reveal the level of variation of *MtActin* gene in the common vetch genome. The reaction mixtures of PCR were set in a 20.5 μL reaction mixture containing 100 μM each of dATP, dGTP, dCTP, and dTTP, 10 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 1 unit of *Taq* DNA polymerase (invitrogen), and 20 ng of genomic DNA (Kang et al. 2002). The PCR cycles were set as follows: 5 pre-cycles of 1 min at 95 °C for denaturing, 45 seconds at 35 °C for annealing and 30 seconds at 72 °C for extension. Annealing temperature (Ta) of each primer was raised to the temperature given in Table 1 for 1 min for another 35 cycles along with 15 seconds at 95 °C for denaturing, 1 min at 72 °C for extension and 7 min at 72 °C for final cycle extension. Amplicons of PCR products were separated on a 2% (w/v) agarose gel in 1×TBE buffer at constant 80 V for 3 h, stained with ethidium bromide (2 μL/100 mL) and visualized under UV light source.

Tab. 1. Salt tolerance related genes used as gene targeted functional markers in the study. The name of genes, references, forward (F) and reverse (R) primer sequences, annealing temperature (Ta), the number of amplicons, the number of polymorphic amplicons, polymorphism rate (%) and polymorphism information content (PIC) values. *Medicago truncatula* salt overly sensitive gene 1 (*MtSOS1*), *M. truncatula* salt overly sensitive gene 2 (*MtSOS2*), *M. truncatula* salt overly sensitive gene 3 (*MtSOS3*), *Arabidopsis thaliana* dehydration responsive element binding factor 1B (*AtDREB1B*), A. thaliana delta1 pyroline-5-carboxylate synthase (*AtP5CS*), *M. truncatula* delta1 pyroline-5-carboxylate synthase (*MtP5CS*), *M. truncatula* proline dehydrogenase (*MtProDH*), A. thaliana vacuolar Na^+/H^+ exchanger gene 1 (*AtNHX1*) and *M. truncatula* Actin (*MtActin*).

Gene	Reference	F/ R primer 5'-3'	Annealing temperature (Ta. °C)	No. of total amplicons	No. of polymorphic amplicons	Polymorphism rate (%)	PIC
MtSOS1	(Liu et al., 2015)	GCTGACTTTCCCGTATG TGGCACCCAGTTCTTTC	48	8	6	75.0	0.50
MtSOS2	(Liu et al., 2015)	CCGTGGTATCTTCTGTT CAAGGGTTAGGTGTATT	48	11	5	45.5	0.29
MtSOS3	(Liu et al., 2015)	TCTGAGGCAAACAGGGTA CTGGGAAATGCTAAGGTAAT	48	1	0	0.0	0.00
AtDREB1B	(Wang et al., 2006)	GGATCCTGATCAATGAACTACATTTTC AGCTCCCATTCTAAAAAGGAAC	50	4	3	75.0	0.44
AtP5CS	(Yamada et al., 2005)	TCAGAGGACTACGTGTTGGA ATGAGTACTAAGCAGAGAGG	55	8	6	75.0	0.32
MtP5CS	(Quan et al., 2016)	GAGAGGGAACGGCCAAGTG CAGATCCTTGTGTGTATA	52	9	7	77.8	0.45
MtProDH	(Planchet et al., 2014)	CCAACGTCCACGCTGATAAGA ACAGGTCCTATAGCCGTTGCA	57	3	1	33.3	0.19
AtNHX1	(Yokoi et al., 2002)	CAACACCCCAAAATCCATAC ATATCCCTTTGTTGGACCAA	52	7	5	71.4	0.25
MtActin	(Wang et al., 2017)	ACGAGCGTTTCAGATG ACCTCCGATCCAGACA	50	4	1	25.0	0.23
Mean				6.1	4.3	53.1	0.30
Total				55	34	–	–

Data analysis

An angular transformation (arcsine√FGP) was applied to the final germination percentage (FGP) data and was used in statistical analysis (Tiryaki and Kaplan 2019). To eliminate differences among the cultivars that might be due to the differences in percentages of live seeds in the seed stocks, an adjustment was performed for the FGP values of each cultivar determined under salt stress germination conditions as described before (Tiryaki and Andrews 2001) using the following equation: adjusted FGP value of cultivar n = [(FGP value of control seeds of cultivar n / FGP of salt stress seeds of cultivar n)] × 100. These values were then used to calculate the FGP reduction percentage due to salt stress for each cultivar.

Analysis of variance was used to calculate statistically significant differences of germination rate, span of germination and transformed FGP data using SAS software (SAS 1997). The mean separation was done using Fisher's least significant difference (LSD) test if the *F-test* was significant at $P < 0.05$. The presence (1) or absence (0) of DNA bands with high intensity was used for allele scoring. The polymorphism information content (PIC) of each primer was calculated as described before (Powell et al. 1996):

$$PIC = 1 - \sum(Pij)^2$$

where Pij is the frequency of the i^{th} band revealed by the j^{th} primer, P(ij) is summed for all the bands of each primer. To determine the genetic similarity of the genotypes, numerical taxonomy multivariate analysis system (NTSYSpc-2.1) was used by using the Dice coefficient (Dice, 1945). The unweighted pair-group with arithmetic mean (UPGMA) method was used to construct the dendrogram. The EIGEN and PROJ modules of NTSYSpc-2.1 program were used for PCA (the principal component analysis) analysis.

Results

Effects of salt stress on germination performance of the cultivars

Salt stress significantly reduced the FGPs of all cultivars while the same cultivars had high final germination percentages (FGPs) in nonstress (control) conditions with a few exceptions (Fig. 1). The cultivars Ayaz, Alınoglu and Bakir had very low levels of FGPs under salt (0.4%, 0.7% and 0.6% of FGPs, respectively) stress conditions and the germination data of those cultivars were, therefore, excluded from further analysis. The seeds of cultivar Ozveren had the highest FGP in the presence of 250 mM NaCl and were determined to be the most salt tolerant cultivar at germination stage (Fig. 1). In contrast, the cultivars Zemheri and Ankaramoru were statistically the most sensitive cultivars to salt stress since they had the lowest FGPs (5.8% and 6.0%, respectively). The reduction rates in FGPs due to salt stress were 94.0% and 93.5% for the cultivars Zemheri and Ankaramoru, respectively while the cultivar Ozveren had the lowest reduction rate (20.1%) (Fig. 1).

Salt stress significantly increased the time required for 50% of FGP in all cultivars (Fig. 2). The germination rates ranged from 1.07 days (Urkmez) to 2.21 days (Zemheri) under no salt stress conditions and from 4.0 days (Zemheri) to 5.62 days (Ankaramoru) (Fig. 2) at 250 mM NaCl. Due to salt stress, the highest delay in the time required for 50% of FGP was determined in the cultivar Urkmez with 3.78 days

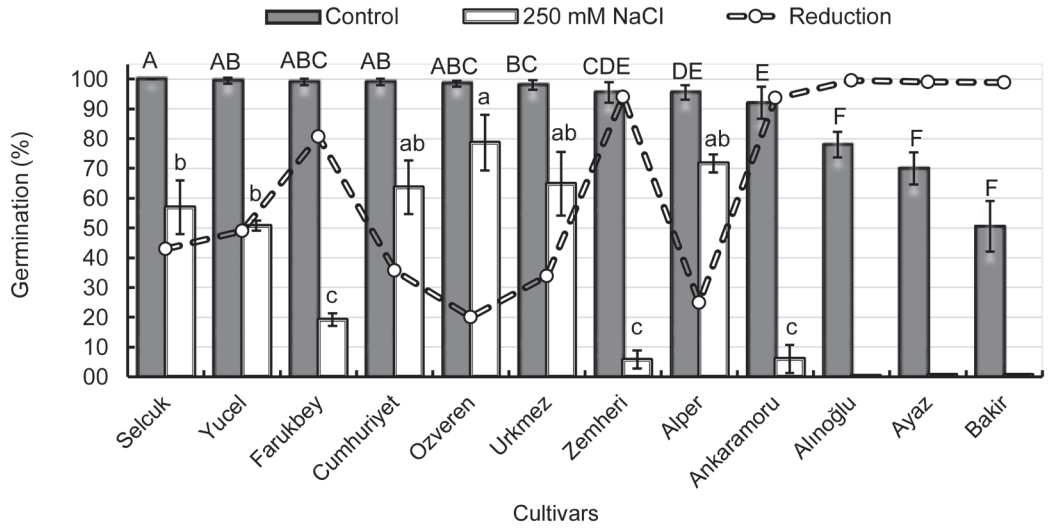

Fig. 1. The final germination percentage (FGP) of *Vicia sativa* cultivars germinated in the presence of 250 mM NaCl and no salt conditions (control) at 20 ± 0.5 °C in darkness, and reduction rate (%) in FGP due to salt tolerance. Decline in FGP of each cultivar due to salt stress was presented as reduction percentage. The bars indicate standard deviation (n = 4). The means that differ significantly (Alpha = 0.05) are indicated by different letters.

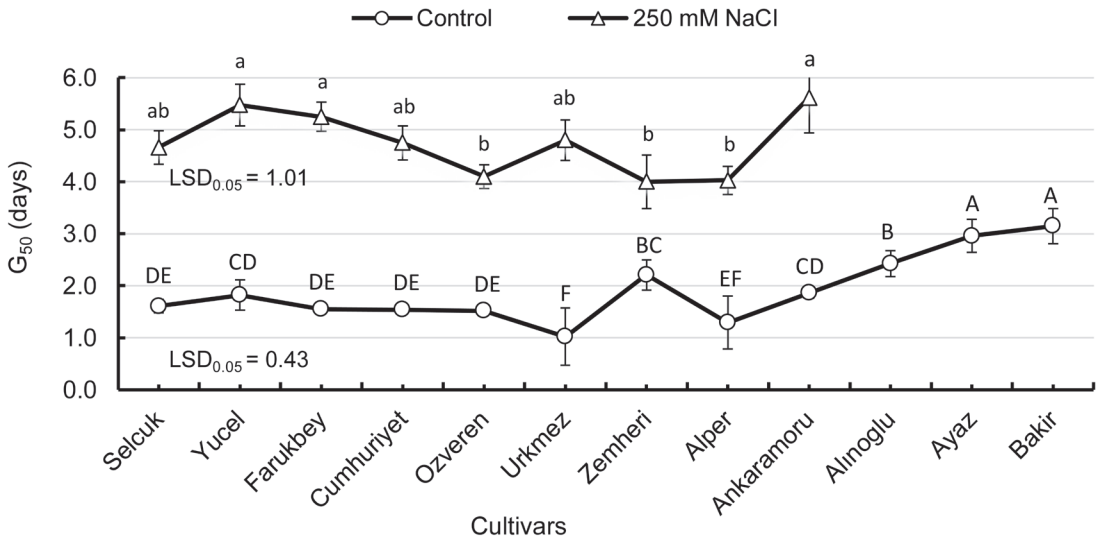

Fig. 2. The time to 50% of final germination percentage (G_{50}) of *Vicia sativa* cultivars germinated in the presence of 250 mM NaCl and no salt conditions (control) at 20 ± 0.5 °C in darkness. The bars indicate standard deviation (n = 4). The means that differ significantly (Alpha = 0.05) are indicated by different letters.

in comparison to control seeds while the cultivar Zemheri had the least delay (1.79 days) (Fig. 2).

The salt stress significantly extended the time required from 10% to 90% of FGP for all cultivars tested in comparison to control seeds (Fig. 3). The span of germination ranged from 2.02 days (Ankaramoru) to 4.45 days (Selcuk) and from 0.6 days (Ozveren) to 2.35 days (Zemheri) for stress and no stress conditions, respectively. The cultivars Zemheri and Ankaramoru had about the same span of germination in both salt stress (G_{10-90} = 2.37 days, G_{10-90} = 2.02 days) and no stress conditions (G_{10-90} = 2.35 days, G_{10-90} = 1.94), respectively (Fig. 3). Because of salt stress, the highest delay (G_{10-90} = 3.45 days) in the germination synchrony was determined with the cultivar Ozveren with 3.45 days (Fig. 3).

Genetic diversity based on GTFMs

Nine loci specific markers produced a total of 55 alleles 34 of which were polymorphic (Tab. 1). The *MtSOS3* (*M. truncatula* salt overly sensitive gene 3) gene marker provided one monomorphic band only. The polymorphism rate of the markers used in the study ranged from 25.0% to 77.8% with an average of 53.1% per locus. The numbers of amplicons per marker changed from 1 to 11, an average of 6.1 alleles. The marker *MtSOS2* had the highest number of amplicons (11 bands) and 5 of them were polymorphic while the polymorphism rate of *MtP5CS* (*M. truncatula* Delta1 pyroline-5-carboxylate synthase) gene marker produced the highest polymorphism rate (77.8%). The average PIC values

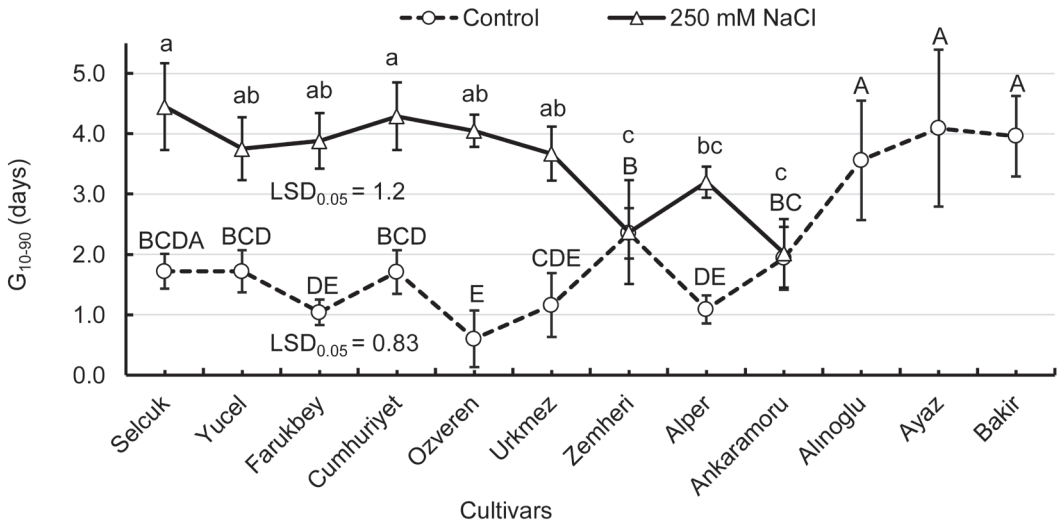

Fig. 3. The time from 10% to 90% of final germination percentage (G_{10-90}) of *Vicia sativa* cultivars germinated in the presence of 250 mM NaCl and no salt conditions (control) at 20 ± 0.5 °C in darkness. The bars indicate standard deviation (n = 4). The means that differ significantly (Alpha = 0.05) are indicated by different letters.

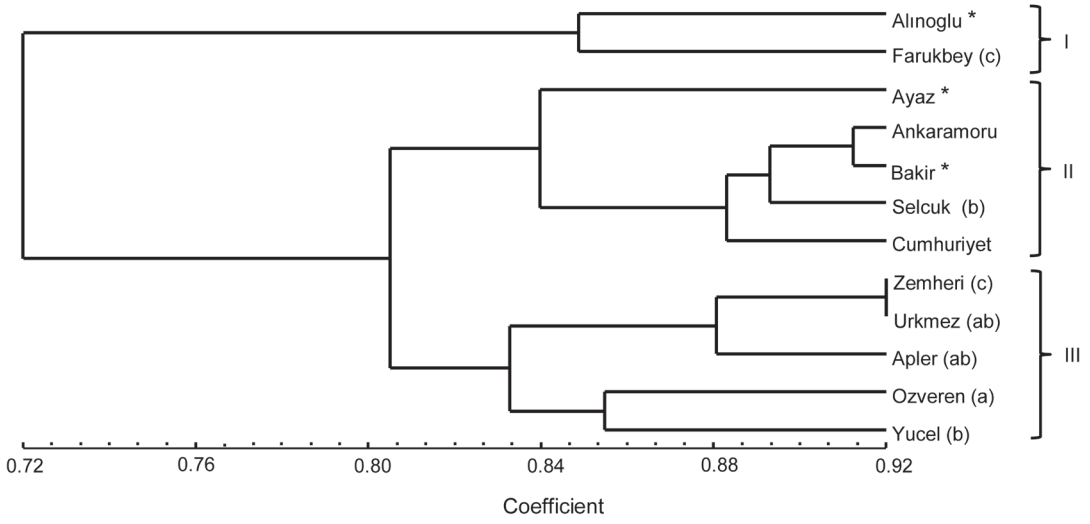

Fig. 4. Genetic similarity dendrogram based on Nei (1972) for 12 *Vicia sativa* cultivars created according to the UPGMA method using 9 salt tolerance-related gene targeted functional markers. Asterisk (*), indicates the cultivars which did not germinate at 250 mM NaCl. The different letters given in the parentheses indicate statistical mean differences (Alpha = 0.05) of final germination percentage for the cultivars germinated at 250 mM NaCI.

of nine markers changed from none to 0.50 with an average of 0.30 while the marker *MtSOS1* (*M. truncatula* salt overly sensitive gene 1) had the highest (0.50) (Tab. 1).

Twelve common vetch cultivars were divided into three main clades based on UPGMA analysis generated by using 55 alleles (Fig. 4). The cluster analysis revealed that the cultivars Alınoglu and Farukbey were grouped in clade I and distinctly separated from the others (Fig. 4). Clades II and III shared the remaining cultivars equally. The Eigen values of the C1, C2 C3 components of PCA analysis explained 57.63% of total variation. The two- and three-dimensional plots of PCA analysis also showed that the cultivars Ayaz and Yucel were cultivars the most distinct from each other (Fig. 5).

Discussion

The results of this study showed that final germination percentage, germination rate and span of germination parameters of 12 common vetch cultivars were significantly reduced at 250 mM NaCl and that the adverse effects of salt stress varied according to the cultivar used. These results revealed the presence of genetic variation among the common vetch cultivars for salt tolerance level at germination stage. Cultivar Ozveren was the most salt tolerant with 20.1% reduction rate in FGP at 250 mM NaCl while cultivars Alınoglu, Ayaz and Bakir did not germinate (Fig. 1). High salt concentration also significantly delayed the time required for 50% of FGP in all cultivars (Fig. 2) while span of germination was not changed for cultivars Zemheri and Ankaramoru (Fig. 3). These findings suggested that FGP and germination rate are better parameters to determine salt tolerance levels of the common vetch cultivars than span of germination. Similarly, a greater salt susceptibility and a better detection of genotypic variability in germination rate under salt stress were also reported for other plant species including *Vicia faba* L. (El-Bok et al. 2015) and *Oryza sativa* L. (Ologundudu et al. 2014). The results of this study also showed that the salt concentration used in this study can successfully be used to determine salt tolerance levels of common vetch cultivars at germination stage. Akhtar and Hussain (2009) reported that germination percentage of common vetch was significantly reduced at 150 mM NaCl which was used as the highest NaCl concentration tested. However, our pre-experiment trials showed that lower concentrations of NaCl were not able to discriminate the high salt tolerant common vetch cultivars from the moderate salt tolerant ones. Therefore, the right salt concentration is one of the most important prerequisites for the determination of salt tolerance levels of cultivars. Salt concentrations that are too high fully inhibit seed germination while relatively low salt concentrations are not able to distinguish salt tolerant genotypes from nontolerant or moderately tolerant. Previous reports indicated that *Vicia sativa* can tolerate moderate salt levels at germination stage (Akhtar and Hussain 2009) and the level of salt tolerance is less in comparison to *Pisum sativum* L. (Bilgili et al. 2011). However, this study revealed that the cultivars Ozveren, Alper, Urkmez and Cumhuriyet can be used as salt tolerant cultivars and should be compared with other important seed legume crops since they had relatively high FGPs at 250 mM NaCl which was generally considered a high salt concentration for legume plants at germination stage (Farooq et al. 2017). Adverse effects of salt stress at lower salt concentrations than in the current study were reported for other vetch species including *Vicia pannonica* Crantz. (Ertekin et al. 2018), *V. faba* and *V. villosa* Roth. (Lee et al. 2014) at germination stages.

We have used gene-specific primers as GTFMs which were directly or indirectly related to salt tolerance in plants (Tab. 1). All the primers of GTFMs amplified in common vetch genome and provided a total of 53.1% polymorphism rate (Tab. 1), suggesting a high degree of transferability for the primers of salt tolerance-related genes among distantly related species. The highest polymorphism rate was obtained from *MtP5CS* gene primers with 77.8% while *MtSOS2* gene primers had the highest numbers of alleles per primer pair (11 bands). The *MtSOS1* gene primers produced more polymorphic bands (75.0%) and gave the highest PIC value (0.50), higher than *MtSOS2* (45.5%) and *MtSOS3* (single monomorphic band only) genes. The *MtSOS1* gene is known to be among the most important loci and provides a better salt tolerance to plants than other members of *SOS* genes (Shi et al. 2000). This study confirmed that *MtSOS1* gene has more allelic variation in common vetch plants than the other members of the gene family and may play a more critical role in the control of salt tolerance in this genus. As a most abundant member of the sodium/proton antiporter gene family, *AtNHX1* (Arabidopsis thaliana (L.) Heynh. vacuolar Na^+/H^+ exchanger gene 1) mediates salt tolerance in plants due to regulation of Na^+ homeostasis in the cell (Shi and Zhu 2002). The *AtNHX1* gene specific primers used in this study provided a 71.4% polymorphism rate, indicating the presence of a high level of allelic variation for *AtNHX1* gene in common vetch. Transcriptional factor *MtDREB1B* (*Arabidopsis thaliana* Dehydration Responsive Element Binding factor 1B) gene encodes dehydration responsive element binding protein and is involved in plant responses to several abiotic stresses including salt tolerance (Donde et al. 2019). Up regulation of such transcription factors in plants increases proline content, which is one of the key molecules protecting plant cells in their responses to various abiotic stresses including salt (Dar et al. 2016). The results of this study revealed that *AtDREB1B* gene primers have a higher polymorphism rate (75%) along with the genes related to proline biosynthesis, namely delta1 pyroline-5-carboxylate synthase genes *AtP5CS* (75%) and *MtP5CS* (77.8%) than *MtProDH* (*M. truncatula* proline dehydrogenase) gene primers which had 33.3% polymorphism rate (Bouazzi et al. 2019). On the other hand, *MtActin* gene is generally used as control in transcription analysis due to its lower level of variation than other constitutive genes under various stress conditions including salt (Zhang et al. 2019). The results of this study showed that *MtActin* gene primers

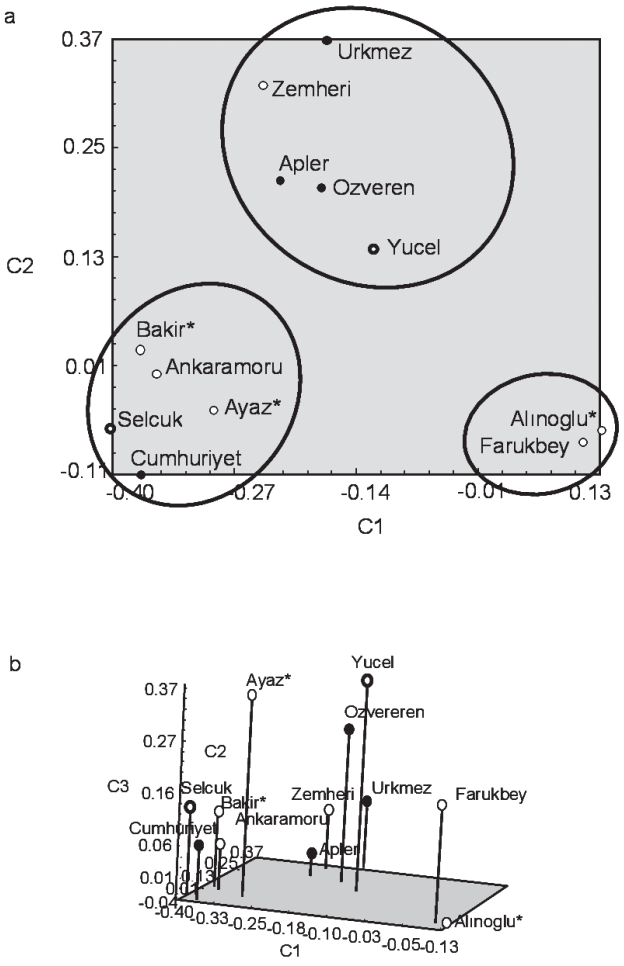

Fig 5. Two (a) and three-dimensional (b) PCA plots of 12 *Vicia sativa* cultivars analyzed by 9 salt tolerance related gene targeted functional markers. The Eigen values of C1, C2 and C3 components were 23.87, 20.39 and 13.35%, respectively. Asterisk (*), indicates the cultivars which did not germinate at 250 mM NaCl. The most and the only moderately salt tolerant cultivars are indicated by filled and open thicker circles, respectively.

had a relatively low polymorphism rate (25%) in common vetch compared to the other gene specific primers tested, suggesting that a low level of variation of *MtActin* gene in common vetch should be considered when this gene is used as a housekeeping gene in RT-qPCR analysis.

The first three components of PCA analysis explained 57.63% of total variation and the cultivars Alınoglu and Farukbey separated from the rest of the cultivars while the cultivars Ayaz and Yucel were determined as the most distant cultivars (Figs 4 and 5). The UPGMA analysis provided three main clades (Figs 4 and 5). Of those, clade III included 5 cultivars and four of them (Urkmez, Alper, Ozveren and Yucel) were determined to be salt tolerant at germination stage while cultivar Zemheri in the same clade was determined to be salt sensitive. Clade II also included both salt tolerant and sensitive cultivars while clade I contained 2 salt sensitive cultivars only (Figs 4 and 5). It was previously hypothesized that alternative oxidase (*AOX*) gene can be used as a functional marker under stress conditions (Arnholdt-Schmitt et al. 2006). Recent advances in current genome sequencing technologies and gene expression studies also resulted in the identification of a large number of functional genes involved in controlling agronomic traits including salt tolerance (Arnholdt-Schmitt 2005) and opened a new era in which those complete or partial gene sequences as molecular markers can be employed. As a result of these efforts, CAAT box derived polymorphism (CBDP) and start codon targeted (SCoT) polymorphism become important types of molecular markers in plant genome analysis (Sharma et al. 2019). In this study, we have used the primers for salt tolerance related genes as GTFMs to reveal genetic relationship as well as to determine the possibility of those gene primers to evaluate salt tolerance levels of the common vetch cultivars. The results revealed that salt tolerance-related GTFMs did not clearly distinguish the salt tolerant and sensitive common vetch cultivars defined in germination stage at 250 mM NaCl although the same markers were successfully able to determine the genetic relationships among the cultivars (Figs. 4, 5). One of the reasons for such discrepancies may be due to gene primers indirectly related to salt tolerance being used in the study such as *DREB1B*, *ProDH* and *P5CS* genes along with *MtActin* gene. Another reason may come from the trait of salt tolerance per se which is a polygenic trait influenced by other environmental stimuli including drought stress, and its signaling cross talk with other important functional genes in various stages of plant growth and development. These findings suggested that a better clustering in terms of salt tolerance levels of the cultivars might be obtained if more specific gene markers associated with salt tolerance are used. However, some other genes which have a cross talk in response to salt tolerance in plants should also be considered to have a better resolution in such analysis. Overall, the results of this study demonstrate that gene specific primers related to salt tolerance in plants could be used to discriminate the salt tolerant and salt sensitive cultivars at germination stage as well as to determine intraspecific genetic diversity if a higher number of salt tolerance related genes are used as GTFMs. However, further studies should be conducted to test the reliability and reproducibility of GTFMs and their relation to not only salt tolerance but also to other abiotic stresses at various plant growth and development stages in various plant species.

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Author Contribution Statements

I.T. conceived the idea, granted financial support, planned the experiments, processed the experimental data, performed the analysis, performed all the numerical calculations, interpreted the data, designed the figures and table, drafted the manuscript. N.I. carried out the experiments.

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