

## Validation of Gender Specific CAPS Marker in Turkish Fig (*Ficus carica* L.) Collection and F1 Progenies

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### Abstract

In most dioecious plants, distinguishing male and female progenies is not possible until flowering or fruiting stage. The fig (*Ficus carica* L.) is such a plant where distinguishing male and female plants at the seedling stage can accelerate fig-breeding programs. An orthologue of *RANI* loci was reported to be associated with sex determination in fig (Mori *et al.*, 2017). The objective of this study is to validate this locus on Turkish fig germplasm collection and F1 population obtained from a cross between female genotypes 'Bursa Siyahi' and male genotype 'Ak Ilek'. A total of 144 genotypes from germplasm collection and 115 F1 individuals were tested with CAPS (cleaved amplified polymorphic sequences) marker following the Mori *et al.* (2017). The loci produced a 315bp amplification product from all genotypes. *PciI* digestion of PCR products resulted in 100% concordance between phenotypes and molecular tests. On the other hand, *HpyCH4IV* enzyme digestion of 8 female genotypes resulted in false negatives among the tested materials. Therefore, despite overall results show that the locus is suitable for gender selection of plants at the seedling stage in the breeding programs, care should be taken when *HpyCH4IV* enzyme is to be employed for CAPS assay.

**Keywords:** female plants; fig breeding; male plants; marker assisted selection; sex determination

### Introduction

The fig (*Ficus carica* L.) is an economically important crop in the world especially in countries bordering the Mediterranean Sea, Red Sea, Arabian Sea and U.S.A (Storey, 1975). The world fig production is around 1.13 million tons (FAOSTAT 2013). Turkey ranks the first three among fig producing countries. As in most dioecious plants, only the female fig plants are commercially valued for production and it is not possible to distinguish the male and female trees until they pass through the juvenile period and produce fruits. Furthermore, fig flowers are located inside of the syconia, and produce edible fruits if they are pollinated by special fig wasps (*Blastophaga psenes*) (Condit, 1969). Hence, breeding efforts are hampered by highly outcrossing feature, dioecy, and long juvenile period of plant development. Therefore, distinguishing male and female plants at the seedling stage could accelerate breeding studies by reducing the labor, time and other expenses in breeding programs (Storey, 1975). Different methods have been employed for sex determination in deciduous plant species (Zheng and Zhu, 2006; Mao *et al.*, 2018). In fig, phenolic compounds, leaf properties and number of stomas in male

and female plants were compared and despite presence of higher amounts of phenolic compounds in male genotypes, further detailed studies were recommended (Misirli *et al.*, 1998a, 1998b). Morphological and biochemical markers can vary depending on external factors and may not be fully reliable. On the other hand, molecular markers are easy to practice, more reliable, not effected by environmental conditions and plant growth stage (Francia *et al.*, 2005) and marker assisted selection has become an important tool in selection of progenies carrying the desired characters (Sestras *et al.*, 2009).

The gender specific molecular markers have been developed for some dioecious fruit species such as *Pistacia vera* L. (Hormaza *et al.*, 1994; Yakubov *et al.*, 2005; Kafkas *et al.*, 2015), *Carica papaya* L. (Deputy *et al.*, 2002; Urasaki *et al.*, 2002), *Phoenix dactylifera* L. (Maryam *et al.*, 2016), *Actinidia chinensis* (Zhang *et al.*, 2015), *Simmondsia chinensis* (Agrawal *et al.* (2007); Ince and Karaca (2011); Heikrujam *et al.* (2014) and *Ficus carica* (Mori *et al.*, 2017). In *Ficus fulva*, a male specific AFLP marker has been developed by Parrish *et al.* (2004). After converting this AFLP marker to SCAR marker, the polymorphism between male and female genotypes has been lost (Parrish *et al.*

al., 2004). An association mapping study was carried on for gender specific molecular marker in *Ficus carica* and five markers explained the 77% of the total variation between male and female genotypes (Mutlu et al., 2008). Recently, Mori et al. (2017) found a gender specific locus in *Ficus carica* based on a GWAS analysis of 122 genotypes by using 16,124 SNPs derived from RADseq protocol. The locus was converted to a CAPS assay and PCR products were digested with *PciI* and *HpyCH4IV* enzymes for distinguishing of male and female genotypes. The aim of the present study was to validate previously developed sex-linked CAPS markers by Mori et al. (2017) on the male and female plants obtained from fig genetic resources collection of Turkish origin and further test MAS applicability of locus on F1 population.

## Materials and Methods

### Plant material

Fifty-four male and 90 female genotypes from Turkish fig germplasm core collection (Supplementary Table 1) and 115 F1 plants obtained from a cross between female parent 'Bursa Siyahi' and male parent 'Ak Ilek' were used as study material. All study material was kindly provided by Ministry of Food, Agriculture and Livestock, Fig Research Institute, Aydın, Turkey. Sex phenotype data for germplasm collection has been collected by the same Institute since 1970s.

### DNA extraction and molecular diagnostic assay

DNA was extracted from fresh or frozen leaves by following the modified CTAB protocol (Doyle and Doyle, 1990). After DNA extraction, the samples were loaded and run on 1% TAE agarose gel for quality control. A primer pair (Fig. 1) (FM\_f: 5'-CAATACCAAAATGATATGCACGA-3', and Fig. 1) (FM\_r: 5'-TGGCATATACAGTGAGAT-GGATG-3') developed by Mori et al. (2017) were utilized for amplification of sex associated CAPS loci: PCR reaction was optimized for a total volume of 15 µl PCR mix containing 1.5µl 10x reaction buffer, 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 10 pmol of each primer pair, 0.8 unit of Taq DNA polymerase, and 25-30 ng DNA. Thermocycler condition was as follows: 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The program was preceded by a denaturing step at 94 °C for 3 min. and ended with an extension step at 72 °C for 5 min. After amplification, half of each PCR products were digested with *PciI* and *HpyCH4IV* restriction enzymes in separate tubes. In each digestion reaction, a total volume of 10 µl digestion mix set up included 1µl 10x Buffer, 7 µl PCR product, 1U enzyme and 1 µl H<sub>2</sub>O. Both digestion reactions incubated at 37 °C for 2 hours before loading and running on 2% TAE agarose gel for visualization and documentation.

## Results and Discussion

An orthologue of *RANI* locus was reported to be associated with sex determination in fig (Mori et al., 2017).

In current study, this locus was tested on the sex- types known 54 male and 90 female plants from Turkish fig germplasm collection as well as a segregating F1 population. Locus specific primer pair amplified a 315 bp fragments in both female and male plants from whole collection.

### Digestion with *PciI* enzyme

After the digestion of PCR product with *PciI* enzyme, three fragments were clearly visible (242, 186 and 73bp products) as revealed by Mori et al. (2017). One of the digested fragments (242 bp) representing female allele (CGT and TGT) was present in all female plants. On the other hand, 186 bp fragment (CAT allele) appeared in all 54 male plants, but it was absent in all female genotypes. Furthermore, male genotypes had 242 bp fragments (CGT and TGT) as they are all heterozygous for gender as reported by Storey (1975). Digestion product of 73 bp fragment was present in both male and female genotypes and not relevant to sex determination (Fig. 1). Of the 144 genotypes tested from collection, marker predictions were accurate for both male and female genotypes. The results of the molecular analysis with *PciI* enzyme were entirely correlated with phenotypic observations (Supplementary Table 1) and parallel to the results from Mori et al. (2017).

### Digestion with *HpyCH4IV* enzyme

Digestion of PCR amplicons with *HpyCH4IV* enzyme resulted in up to three fragments depending on gender as described by Mori et al. (2017). PCR amplicons from female allele CGT were completely cut with *HpyCH4IV* enzyme producing 186 and 129 bp fragments (Fig. 2), whereas male allele CAT was not cut with *HpyCH4IV* enzyme remaining as intact 315bp PCR amplicon. Therefore, gel profiles from male genotypes appeared as three fragments (315, 186 and 129 bp) since they are all in heterozygous state (Storey, 1975). Nonetheless, digestion of PCR products from 8 female genotypes with *HpyCH4IV* enzyme resulted in 315, 186 and 129 bp products, hence appeared as male genotypes. As a result, gel profiles for 8 female genotypes conflicts with gender expectation. In fact, similar contradictory results were also reported for some female genotypes with *HpyCH4IV* enzyme digestion (Mori et al., 2017) and most probably results from the fact these female genotypes had TGT female allele along with CGT female allele. Since, enzyme *HpyCH4IV* cannot digest TGT female allele, gel profiles from these 8 female genotypes appeared as males.

### Screening of F1 plants at juvenile stage

One hundred fifteen F1 fig plants along with their parents (female parent, 'Bursa Siyahi' and male parent, 'Ak Ilek') were screened at *RANI* loci. PCR reaction produced a 315 bp fragment from all F1 plants as their parents. Digestion with *PciI* enzyme showed expected polymorphism profile between male and female parents as explained above. Of the 115 F1 plant tested, 60 of them produced digestion products of 186 bp fragments (CAT allele) and were predicted as male whereas 55 of the genotypes had a digestion product of 242 bp fragments representing female allele (Fig. 3). Storey (1975) indicated

that a cross between caprifig and fig results in a segregation ratio of 1 male: 1 female in their offspring. Here, we had a similar ratio of 1 male: 0.92 female supporting the reports of Storey (1975). Furthermore, the marker showed 100% reliable sex diagnosis in male and female plants as indicated by concordance between phenotypic and molecular results.

In fig breeding, one-half of the progeny is useless since the fruits of male genotypes are commercially not valuable (Storey, 1975). Fig seedlings takes five to seven years before bearing fruits, hence breeders had to double the resources for their fig breeding program in early years. For example, Doyle and Ferguson (1998) had to eliminate 50% of the seedlings, 6 to 7 years after planting. Early sex determination methods make it possible to select male and female plants at the seedling stage (Uragami *et al.*, 2016). Developing reliable and simple molecular markers with high effectiveness is a long and difficult process. For example, a gender specific molecular marker in pistachio developed by Hormaza *et al.* (1994) from RAPD primers then converted to SCAR by Yakubov *et al.* (2005) and tested by Kafkas *et al.* (2015) in a segregating population, and germplasm

collection of pistachio. The marker generated false negatives in some female individuals and false positives in some male individuals. Hence, testing and validating the markers developed in other populations increase their reliability for MAS in breeding programs (Ejaz *et al.*, 2015; Javid *et al.*, 2015). Although, we confirmed the high efficiency of *RANI* locus to identify gender in 144 Turkish fig germplasm (Supplementary Table 1) and segregating 115 F1 plants, there is a consideration with the use of *HpyCH4IV* enzyme. Since a few female genotypes in collection bear TGT alleles in their background, erroneous elimination of offspring is possibility if those females involved in breeding programs. Therefore, molecular test should be performed on parents before beginning crossing efforts if *HpyCH4IV* enzyme is to be utilized. Nonetheless, *PciI* enzyme does not seem to pose any risk for reliable gender determination hence should be preferred. Overall, the study confirms that CAPS assay on *RANI* locus is a reliable way of determining gender in fig offspring and suitable for employing within fig breeding programs.

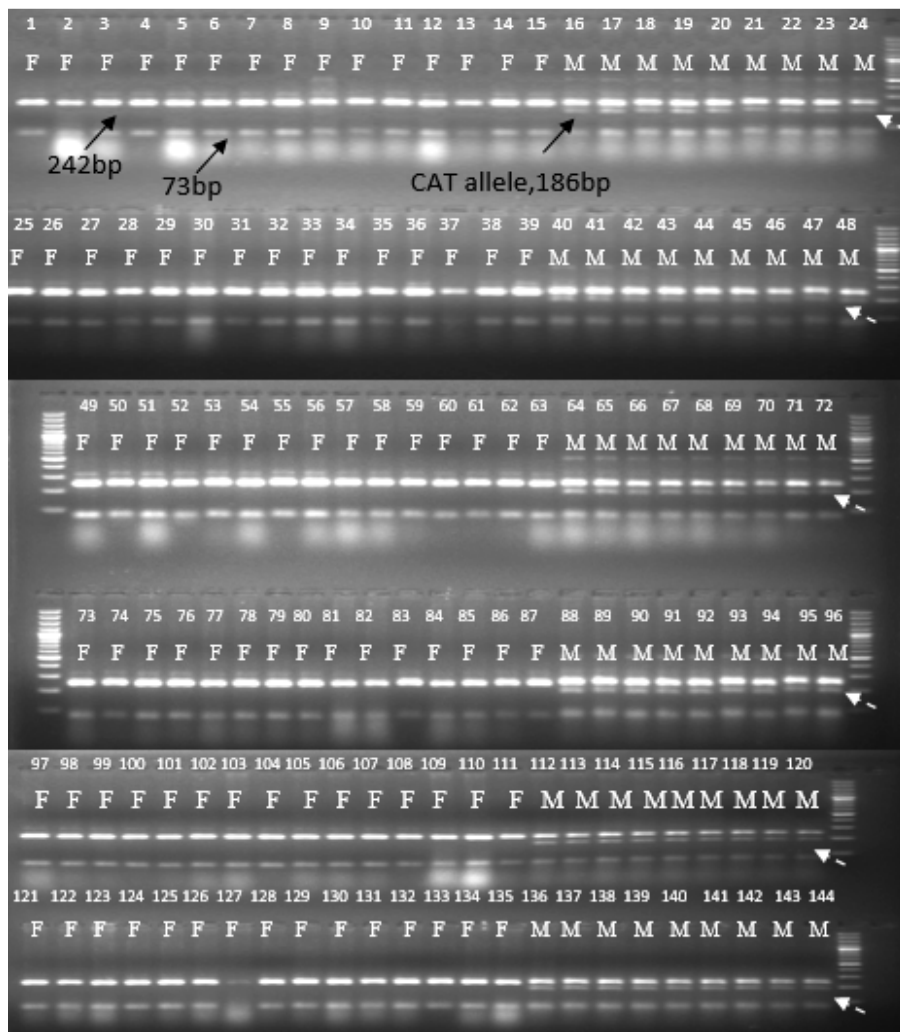


Fig. 1. Analysis of 144 genotypes detecting male and female genotypes using developed CAPS marker by Mori *et al.* (2017) (digestion with *PciI* enzyme)

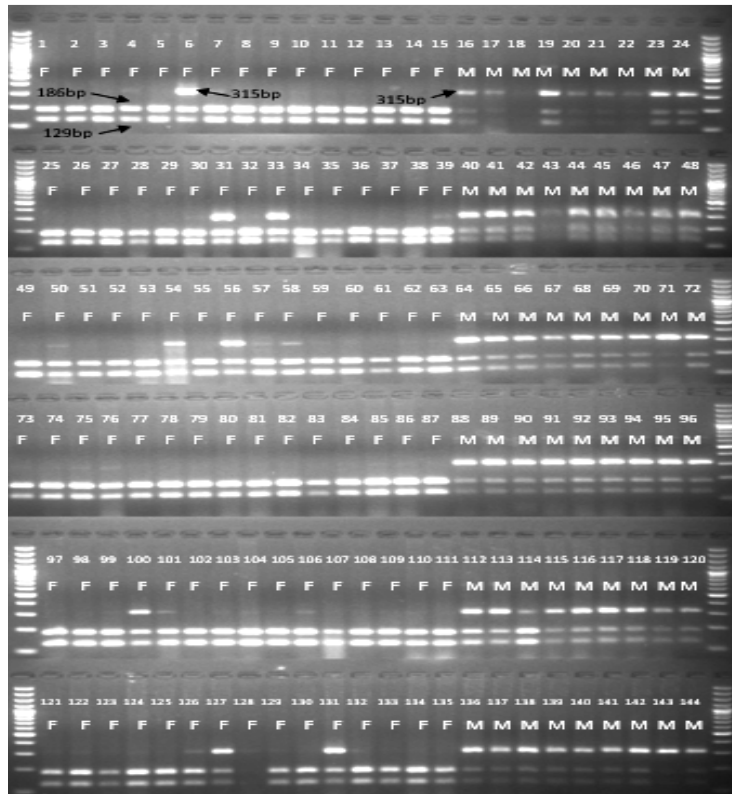


Fig. 2. Analysis of 144 genotypes detecting male and female genotypes using developed CAPS marker by Mori *et al.* (2017) (digestion with *HpyCH4IV*)

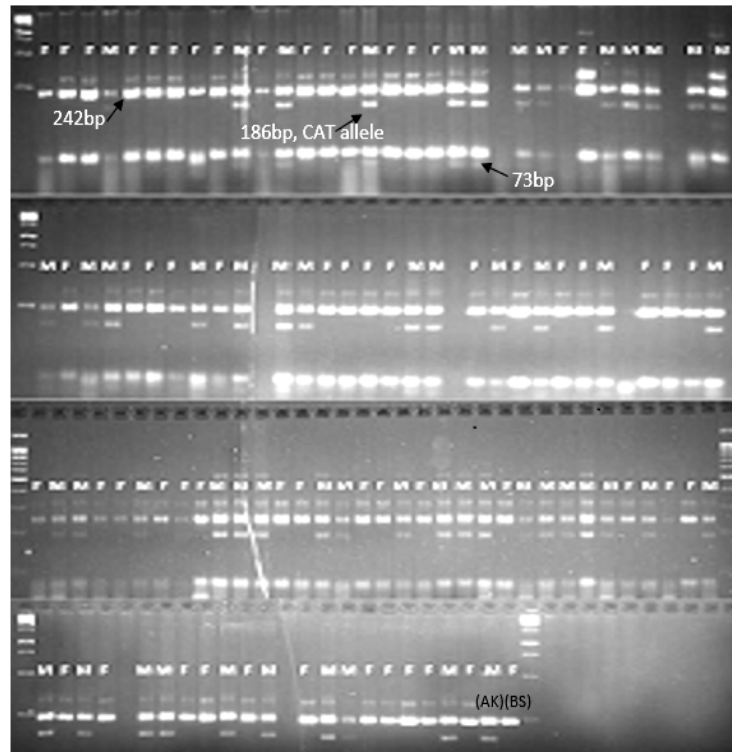


Fig. 3. Analysis of 115 F1 individuals as well as the parental genotypes ('Bursa Siyahi', BS and 'Ak Ilek', AK) detecting male and female genotypes using developed CAPS marker by Mori *et al.* (2017) (digestion with *PciI* enzyme)

## Conclusions

In this study, we screened the fig genotypes for gender determination and report the validation of already developed sex-linked DNA marker in fig genotypes from genetic resources collection plot and F1 population. The marker offers potential to be used for detection of female and male plants in the F1 population, making this marker suitable for employing within the fig-breeding program.

## Acknowledgements

We thank to Akdeniz University Scientific Research Projects Coordination Unit for financial support (grant number: FYL-2017-2633) and Fig Research Institute-Aydin, Turkey for research material.

## Conflicts of interest

The authors declare that there are no conflicts of interest related to this article.

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## Annexes

Supplementary Table 1. List of female and male fig genotypes

Species	Genotype No	Variety/accession name and code	Sexuality	Sampling location
	1	3107	Female	Fig Research Institute, Aydın, Turkey
	2	712 Siyah incir	Female	Fig Research Institute, Aydın, Turkey
	3	705 K.formu	Female	Fig Research Institute, Aydın, Turkey
	4	708 Darpak	Female	Fig Research Institute, Aydın, Turkey
	5	710 Ekşi incir	Female	Fig Research Institute, Aydın, Turkey
	6	251 Dereköy	Female	Fig Research Institute, Aydın, Turkey
	7	215 Midilli	Female	Fig Research Institute, Aydın, Turkey
	8	252 Löp inciri	Female	Fig Research Institute, Aydın, Turkey
	9	219	Female	Fig Research Institute, Aydın, Turkey
	10	228 İpek inciri	Female	Fig Research Institute, Aydın, Turkey
	11	245 Sarı Yemiş	Female	Fig Research Institute, Aydın, Turkey
	12	401 Mor özer	Female	Fig Research Institute, Aydın, Turkey
	13	538 Kabak inciri	Female	Fig Research Institute, Aydın, Turkey
	14	221 Yeşil incir	Female	Fig Research Institute, Aydın, Turkey
	15	537 Kara incir	Female	Fig Research Institute, Aydın, Turkey
	16	Kızılay2	Male	Fig Research Institute, Aydın, Turkey
	17	Körpe ilek	Male	Fig Research Institute, Aydın, Turkey
	18	Mor ilek	Male	Fig Research Institute, Aydın, Turkey
	19	Afyoncu	Male	Fig Research Institute, Aydın, Turkey
	20	Adalı	Male	Fig Research Institute, Aydın, Turkey
	21	Gabalı	Male	Fig Research Institute, Aydın, Turkey
	22	Ayardolduran	Male	Fig Research Institute, Aydın, Turkey
	23	Siyah ilek	Male	Fig Research Institute, Aydın, Turkey
	24	Omerbeylikaba	Male	Fig Research Institute, Aydın, Turkey
	25	230 Siyah	Female	Fig Research Institute, Aydın, Turkey
	26	525 Ak incir-1	Female	Fig Research Institute, Aydın, Turkey
	27	505 Kara sultani	Female	Fig Research Institute, Aydın, Turkey
	28	241 Gök bardak	Female	Fig Research Institute, Aydın, Turkey
	29	3304	Female	Fig Research Institute, Aydın, Turkey
	30	704 Yabani f.	Female	Fig Research Institute, Aydın, Turkey
	31	227 Yediveren	Female	Fig Research Institute, Aydın, Turkey
	32	515 Tabak inciri	Female	Fig Research Institute, Aydın, Turkey
	33	232 Kocayemiş	Female	Fig Research Institute, Aydın, Turkey
	34	533 Agarsak	Female	Fig Research Institute, Aydın, Turkey
	35	701 Yabani-Mor	Female	Fig Research Institute, Aydın, Turkey
	36	542 Siyah Bakele	Female	Fig Research Institute, Aydın, Turkey
<i>Ficus carica</i>	37	236 Bardak	Female	Fig Research Institute, Aydın, Turkey
	38	254 Midilli	Female	Fig Research Institute, Aydın, Turkey
	39	Dilbi	Female	Fig Research Institute, Aydın, Turkey
	40	Bozdoğanaba	Male	Fig Research Institute, Aydın, Turkey
	41	Elma ilek	Male	Fig Research Institute, Aydın, Turkey
	42	Bostanlı	Male	Fig Research Institute, Aydın, Turkey
	43	Kara ilek	Male	Fig Research Institute, Aydın, Turkey
	44	Şişek ileği	Male	Fig Research Institute, Aydın, Turkey
	45	Kuyucak	Male	Fig Research Institute, Aydın, Turkey
	46	Damarlı	Male	Fig Research Institute, Aydın, Turkey
	47	Hacı mestan	Male	Fig Research Institute, Aydın, Turkey
	48	Kızılay1	Male	Fig Research Institute, Aydın, Turkey
	49	235 Yediveren	Female	Fig Research Institute, Aydın, Turkey
	50	512 İstanbul inciri	Female	Fig Research Institute, Aydın, Turkey
	51	404 Kış Hayri	Female	Fig Research Institute, Aydın, Turkey
	52	255 Siyah	Female	Fig Research Institute, Aydın, Turkey
	53	250 Yediveren	Female	Fig Research Institute, Aydın, Turkey
	54	706 Çilci	Female	Fig Research Institute, Aydın, Turkey
	55	403 Sultani	Female	Fig Research Institute, Aydın, Turkey
	56	534 Sarı incir	Female	Fig Research Institute, Aydın, Turkey
	57	3111	Female	Fig Research Institute, Aydın, Turkey
	58	702 Yabani f.	Female	Fig Research Institute, Aydın, Turkey
	59	Zapı	Female	Fig Research Institute, Aydın, Turkey
	60	201 İstanbul inciri	Female	Fig Research Institute, Aydın, Turkey
	61	209 Mor incir	Female	Fig Research Institute, Aydın, Turkey
	62	3110	Female	Fig Research Institute, Aydın, Turkey
	63	239 İsyemez	Female	Fig Research Institute, Aydın, Turkey
	64	Bardakçı	Male	Fig Research Institute, Aydın, Turkey
	65	Kıbrıslı	Male	Fig Research Institute, Aydın, Turkey
	66	Hacı Abdullah	Male	Fig Research Institute, Aydın, Turkey
	67	Ak ilek	Male	Fig Research Institute, Aydın, Turkey
	68	Şeytan1	Male	Fig Research Institute, Aydın, Turkey
	69	Ak erkek1	Male	Fig Research Institute, Aydın, Turkey
	70	Yanako1	Male	Fig Research Institute, Aydın, Turkey
	71	Freng	Male	Fig Research Institute, Aydın, Turkey
	72	Mıstık ilek	Male	Fig Research Institute, Aydın, Turkey
	73	519 Şeker inciri	Female	Fig Research Institute, Aydın, Turkey

## Annexes

74	Keten göynüğü	Female	Fig Research Institute, Aydın,Turkey
75	B. incir	Female	Fig Research Institute, Aydın,Turkey
76	3105	Female	Fig Research Institute, Aydın,Turkey
77	507 Balduzdın	Female	Fig Research Institute, Aydın,Turkey
78	3303	Female	Fig Research Institute, Aydın,Turkey
79	3106	Female	Fig Research Institute, Aydın,Turkey
80	3104	Female	Fig Research Institute, Aydın,Turkey
81	246 Gelin yanağı	Female	Fig Research Institute, Aydın,Turkey
82	705 K. formu	Female	Fig Research Institute, Aydın,Turkey
83	204	Female	Fig Research Institute, Aydın,Turkey
84	3108	Female	Fig Research Institute, Aydın,Turkey
85	210 Mor incir	Female	Fig Research Institute, Aydın,Turkey
86	240 Lop	Female	Fig Research Institute, Aydın,Turkey
87	528 Kara incir	Female	Fig Research Institute, Aydın,Turkey
88	Yanako2	Male	Fig Research Institute, Aydın,Turkey
89	Küçükkonkur	Male	Fig Research Institute, Aydın,Turkey
90	Çaçaron	Male	Fig Research Institute, Aydın,Turkey
91	Armut ilek	Male	Fig Research Institute, Aydın,Turkey
92	Kavun ilek	Male	Fig Research Institute, Aydın,Turkey
93	Çakır1	Male	Fig Research Institute, Aydın,Turkey
94	Kara erkek2	Male	Fig Research Institute, Aydın,Turkey
95	12no ilek	Male	Fig Research Institute, Aydın,Turkey
96	Karabulut	Male	Fig Research Institute, Aydın,Turkey
97	234 Siyah	Female	Fig Research Institute, Aydın,Turkey
98	529 Köniş inciri	Female	Fig Research Institute, Aydın,Turkey
99	704 Yabani f.	Female	Fig Research Institute, Aydın,Turkey
100	711 Beyaz incir	Female	Fig Research Institute, Aydın,Turkey
101	Benati Siirt	Female	Fig Research Institute, Aydın,Turkey
102	222 Kavak yediveren	Female	Fig Research Institute, Aydın,Turkey
103	3301	Female	Fig Research Institute, Aydın,Turkey
104	213 Kara yemiş	Female	Fig Research Institute, Aydın,Turkey
105	244 Patlıcan	Female	Fig Research Institute, Aydın,Turkey
106	206 Bogmalı dizilik	Female	Fig Research Institute, Aydın,Turkey
107	Hazreki	Female	Fig Research Institute, Aydın,Turkey
108	521Siyah Patlıcan	Female	Fig Research Institute, Aydın,Turkey
109	248 Siyah incir	Female	Fig Research Institute, Aydın,Turkey
110	504 Siyah incir	Female	Fig Research Institute, Aydın,Turkey
111	522 Turnaboyu	Female	Fig Research Institute, Aydın,Turkey
112	Çiçekli2	Male	Fig Research Institute, Aydın,Turkey
113	Mehmet	Male	Fig Research Institute, Aydın,Turkey
114	26 no ilek	Male	Fig Research Institute, Aydın,Turkey
115	Kara erkek	Male	Fig Research Institute, Aydın,Turkey
116	Esref2	Male	Fig Research Institute, Aydın,Turkey
117	İsmail Barbaros	Male	Fig Research Institute, Aydın,Turkey
118	Esref1	Male	Fig Research Institute, Aydın,Turkey
119	15 no ilek	Male	Fig Research Institute, Aydın,Turkey
120	6 no ilek	Male	Fig Research Institute, Aydın,Turkey
121	523 Dilaver	Female	Fig Research Institute, Aydın,Turkey
122	513 Filestos	Female	Fig Research Institute, Aydın,Turkey
123	225 Kabak yemişi	Female	Fig Research Institute, Aydın,Turkey
124	506 Beyaz sultani	Female	Fig Research Institute, Aydın,Turkey
125	Fethiye	Female	Fig Research Institute, Aydın,Turkey
126	223 Yediveren	Female	Fig Research Institute, Aydın,Turkey
127	202 Siyah	Female	Fig Research Institute, Aydın,Turkey
128	214	Female	Fig Research Institute, Aydın,Turkey
129	238 Beyaz Bardak	Female	Fig Research Institute, Aydın,Turkey
130	502 İstanbul inciri	Female	Fig Research Institute, Aydın,Turkey
131	517 Mor incir	Female	Fig Research Institute, Aydın,Turkey
132	531 Değirmen inciri	Female	Fig Research Institute, Aydın,Turkey
133	242 Sarı bardak	Female	Fig Research Institute, Aydın,Turkey
134	233	Female	Fig Research Institute, Aydın,Turkey
135	212 Çiçek inciri	Female	Fig Research Institute, Aydın,Turkey
136	Ak erkek2	Male	Fig Research Institute, Aydın,Turkey
137	Aydın2	Male	Fig Research Institute, Aydın,Turkey
138	Kara erkek	Male	Fig Research Institute, Aydın,Turkey
139	Hacı Yusuf	Male	Fig Research Institute, Aydın,Turkey
140	Derviş Ali	Male	Fig Research Institute, Aydın,Turkey
141	Büyük konkur	Male	Fig Research Institute, Aydın,Turkey
142	Şeytan2	Male	Fig Research Institute, Aydın,Turkey
143	Çankurt	Male	Fig Research Institute, Aydın,Turkey
144	Kızılburun	Male	Fig Research Institute, Aydın,Turkey