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RESEARCH PAPER

HORTICULTURAL STUDIES

Characterization of Citrus × Poncirus Embryo Rescued Hybrids as Rootstock Candidate using Morphological Markers

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Abstract

Generally, there are both nucellar and zygotic embryos in the citrus seeds after cross breeding. Since genetic variations are very important for the success of plant breeding, morphological traits of individuals are evaluated to identify diversity. The present study aimed to characterize of citrus hybrids for new rootstock genotypes based on their morphological characters at the seedling stage. A total of 335 putative interspecific hybrids, derived from 3 crosses [Common sour orange (*Citrus aurantium* L.) × Troyer citrange (*Citrus sinensis* L. × *Poncirus trifoliata* L.), Common mandarin (*Citrus deliciosa* Ten.) × Troyer citrange (*Citrus sinensis* L. × *Poncirus trifoliata* L.) and King mandarin (*Citrus nobilis* L.) × Carrizo citrange (*Citrus sinensis* L. × *Poncirus trifoliata* L.)], were observed by their plant morphology. The eight qualitative and five quantitative characteristics of hybrid plants such as seedling growth, leaf and thorniness characteristics were evaluated. The average plant height of the population was found between 70.2 cm and 133.2 cm. The average stem diameter varied between 5.9 mm and 8.0 mm. Hybrid seedlings were separated on the basis of dominant trifoliate leaf marker. There was wide diversity among the accessions with respect to quantitative leaf characters. In terms of leaf division, 268 genotypes have bifoliate and 67 were trifoliate in all combinations, and many intermediate forms were also observed. In addition 66 of the genotypes were thornless while 269 of the genotypes were thorny. The genotype No. 4, has been assessed as triploid, from Common mandarin × Troyer citrange combination, has the longest and dense spines. Morphological markers data were analyzed by clustering method to compare similarities of hybrids. The dissimilarity index was observed between 0.004 and 17.318 within three hybridization combinations. The hybrids obtained at 110 days after pollination were more distant relative to each other in all hybridization combination.

1. Introduction

The use of rootstock has become widespread due to the damages caused by *Phytophthora* and tristeza diseases, and the evaluation of citrus rootstock studies has accelerated considerably (Castle, 1983). Many different citrus rootstocks have been preferred in the various citrus production regions of the world (Syvertsen and Graham, 1985). Due to the sensitivity of present rootstocks to some diseases and rootstock-scion incompatibility, their use becomes limited. In order to provide uniformity, it is important that therootstock has many nucellar seeds (Soost and Cameron, 1975). Many of the rootstock used for citrus propagation are original species or old natural hybrids. However, intergeneric hybrids (*Citrus* × *Poncirus*) such as citranges, citrumelos, and citrandarins are becoming increasingly important (Ollitrault and Navarro, 2012). *Citrus* genera is the origin of commercial citrus cultivars. *Poncirus* is a sexually compatible relative with *Citrus* in the Rutaceae family. Trifoliate (*P. trifoliata* [L.] Raf.) hybrids are important rootstock in certain countries and widely used as rootstock breeding materials (Swingle and Reece, 1967; Chen et al., 2008). *Poncirus* possesses several disease resistance or stress tolerance genes not found in *Citrus* (Frost, 1925; Krug, 1943). Complete or partial sexual compatibility is one common feature in citrus. Fertile hybrids among *Citrus*, *Poncirus*, and *Fortunella* can be quite easy for the selection of compatible rootstocks or desired scions (Soost and Roose, 1996). For example, Carrizo citrange and Swingle citrumelo, widely used rootstocks, were selected from the hybrids of *C. sinensis* cv. Washington Navel × *P. trifoliata*, and of *C. paradisi* cv. Duncan × *P. trifoliata*, respectively (Castle and Gmitter,1999). Hybridizations between mandarins and *Poncirus* appear promising to tolerance to abiotic and biotic stress conditions both by sexual breeding (Forner et al., 2003) or somatic hybridization (Grosser et al., 2000; Ollitrault et al., 2000).

Poliembryony, the formation of both nusellar and zygotic embryos, is a major problem in citrus breeding. Most of citrus varieties are polyembryonic, and generally produce vigorous and genetically identical nucellar embryos when used as seed parents because of suppressed, underdeveloped zygotic embryos may abort or die (Cameron and Frost,1968). Biotechnology has provided convenience and time savings in breeding and propagation studies. Embryo rescue has been used to isolate immature zygotic embryos at the early stage and *in vitro* to acquire seedlings (Rangan et al., 1969; Gmitter and Ling, 1991). Despite its polyembryonic nature, each seed usually produces one or several vigorous seedlings under *in vivo* conditions (Singh et al., 2020).

Leaf traits such as trifoliate character from the pollen parent will be exhibited in zygotic hybrids to assist early discrimination of zygotic from nucellar seedlings. The trifoliate of *Poncirus* versus the unifoliate of *Citrus* and others have been used the most widely in crosses between them (Ruiz and Asins, 2003; Gmitter et al., 2007). Morphological evaluation is the basic element for biodiversity and classification. Morphological characters have been used to identify and characterize a species (Susandarini et al., 2013; Sharma et al., 2016). Some studies have been conducted on the taxonomic definition of genotypes based on leaf shape, which is a morphological marker (Teich and Spiegel-Roy, 1972; Hearn, 1977; Chikaizumi and Matsumoto, 1978). Leaf shape has wide variations in genus *Citrus* and has been used as a marker of taxonomic character in classifying citrus species and varieties (Swingle and Reece, 1967; Tanaka, 1969; Handa and Oogaki, 1985). Although importance of leaf shape in taxonomy and breeding, knowledge on its inheritance is limited (Iwata et al., 2002). *Citrus* hybrids from crosses with *Poncirus* generally display multifoliate traits at early stages of growth (Chen et al., 2008; Caruso et al., 2014). In addition, leaf morphology is an important factor affecting fruit quality and quantity in citrus cultivation.

Phenotype of the hybrids is identified by observing specific morphological markers such as plant height, thorn status, leaf size and shapes (Dorji and Yapwattanaphun, 2011a; Roy et al., 2014). Individuals derived from a zygotic embryo in each hybrid seeds could be identified by the examination of their morphological characteristics but the selection could be made after the first fruit set (three to five years after planting) unless they have a distinctive character such as trifoliate (Rodriguez et al., 2004). Seedling height of citrus hybrid populations has been considered as an important criteria for distinguishing hybrid seedlings according to whether the seedlings are larger or smaller than normal (Moore and Castle, 1988).

Breeders are looking for new rootstocks that will solve the problems faced by citrus growers. However, it is imperative that new rootstocks or cultivars are properly selected and identified before introduced to the market. This information will be useful to breeders and geneticists working on citrus rootstock breeding programs. In addition to its convenience the morphological marker is useful for evaluating agronomic traits of research. Further, the technique is relatively cheaper and easier to conduct. The objectives of this study were to determine of leaf morphological characters and plant growth traits to observe developmental differences in hybrid populations generated via embryo rescue for rootstock breeding.

2. Materials and Methods

2.1. Plant materials

This research was conducted at the genetic resource collection parcels and greenhouse of Batı Akdeniz Agricultural Research Institute (BATEM) (36°55′32.40″ N and 35°00′35.75″ E), in Antalya, Türkiye. A total of 1215-controlled crosses were performed in all three hybridization combinations according to Batchelor (1943). The embryo rescue is the most important part of this study. Embryos were cultured at different developmental stages (110, 120, and 130 days after pollination ‘DAP’) with modified Murashige and Tucker (MT) (1969) in *in vitro.* Embryos germinated in MT medium culture were transferred to culture tubes containing Murashige and Skoog (MS) (1962), and survival rates and trifoliate rates were also determined. The peat and perlite (3:1) growth medium were used for these hybrids which were transferred to the greenhouse in late October-Early November. The irrigation and fertilization were applied with octopus drip irrigation system. Morphological observations on the 335 survived individuals obtained for breeding new citrus rootstocks were evaluated in the greenhouse (Figure 1). Hybridization combinations are presented in Table 1.

2.2. Morphological characteristics of hybrids

Plant height and stem diameter were measured during a year to observe the development of hybrid plants transferred to the greenhouse after embryo recovery and then survived. Plant height measurement was made in every month, plant stem diameter was carried out in two periods. The only part is the vegetative shoot that could be morphologically identified at seedling stage. Hybrid seedlings were classified on the dominant trifoliate leaf marker. The dominant trifoliate property over the recessive unifoliate trait makes it easy to determine the first-generation hybrid rootstock seedlings in crosses between *Citrus* and *Poncirus* male parents. Visual observations were also made with regard to leaf morphology, particularly leaf shape and colour (Figure 2). Hybrid rootstock populations were separated by size and other morphological criteria, such as abnormal growth habits or leaf characteristics. Using the morphological descriptors recommended by the International Plant Genetic Resources Institute (IPGRI, 1999) for citrus, features such as leaf type, color or shape, and thorn status were defined (Table 2).

2.3. Data analysis

The research was established in a randomized plot design. Data were subjected to analysis of variance with mean separation by least significant difference (LSD) test. These 8 qualitative and 5 quantitative characteristics were evaluated on the hybrid seedlings. The quantitative data obtained from morphological characterization studies were presented with descriptive as minimum, maximum and average values. The data on the difference of leaf lenght, leaf width, leaf length/width, plant height and plant stem diameter each combination were compared by the ANOVA in the SAS package program (SAS Institute, Cary, NC, USA). In addition, the LSD multiple comparison test (P<0.05)

Figure 1. Morphological observations on the hybrid plants in greenhouse.

Figure 2. Differences in leaves in terms of color, size and shape and thorn state of hybrid plants.

Table 1. Combinations of hybridization.

Female genotypes	Male genotypes
Common sour orange (CSO) (*Citrus aurantium* L.)	Troyer citrange (TC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)
Common mandarin (CM) (*Citrus reticulata* Blanco)	Troyer citrange (TC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)
King mandarin (KM) (*Citrus nobilis* L.)	Carrizo citrange (CC) (*Citrus sinensis* L. × *Poncirus trifoliata* L.)

was used to compare the averages. Data obtained by morphological characterization were subjected to the Ward Hierarchical Clustering Method in the MINITAB package program to demonstrate the overall phenotypic relationships among these genotypes.

3. Results and Discussion

3.1. Hybrid plant height and stem diameter

The hybrid plants showed linear growth in general as a result of plant height and stem diameter measurements made for 1 year after transfer to the greenhouse (Table 3). There was a high variation within the population in terms of growth parameters. The end of the growth season, the height of the plants of hybrid population varied between 8.0 cm and 205.0 cm. The average plant height of the population was found between 70.2 cm and 133.2 cm depending on the hybridization combination. The genotypes stem diameter varied between 2.4 mm and 12.7 mm. Viloria et al. (2005) observed the plant height of seedlings obtained from embryos at different stages of development in hybridization combinations. They reported that seedlings from smaller embryos grew more slowly. The effect of embryo development stage on the stem diameter was less in combinations that the female parent was mandarin. It is thought that the variation seen in plant heights is due to the fact that hybrid individuals have different genetic backgrounds. The selection of the hybrid population

Table 2. Traits identified in hybrid plants for morphological evaluation.

Traits	Classes
Plant height	From the root collar of the plant by a meter (cm)
Plant stem diameter	Measured above the root collar by a digital caliper (mm)
Leaf division (LD)	Simple (S), Bifoliate (B), Trifoliate (T)
Leaf color (LC)	Light Green (LG), Green (G), Dark Green (DG)
Leaf length (LE)	By digital caliper (mm)
Leaf width (LW)	By digital caliper (mm)
Ratio length/width (RTW)	By calculation (mm)
Leaf stalk (LS)	Absent (A), Short (Sh), Long (Lo)
Petiole wings state (PW)	Absent (A), Narrow (N), Large (L)
Petiole wings shape (PWS)	Obcordate (Oc), Obdeltate (Od), Obovate (Oo), Linear (L)
Leaf lamina shape (LLS)	Elliptic (E), Ovate(O), Obovate (Oo), Lanceolate (L), Orbicular (Or), Obcordate (Oc)
Shape of the leaf edges (SE)	Crenate (C), Dentate (D), Entire (E), Sinuate (Si)
Thorn status (TS)	Short (Sh), Medium (M), Long (Lo)

Table 3. Hybrid plants height (cm) and stem diameter (mm) obtained from embryos at different stage of combinations.

CH	Hybrid plant height (cm)								
	110 DAP			120 DAP			130 DAP		
	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
CSO×TC	8.0	194.0	79.0 ± 10.95 b*	45.0	199.0	127.7 ± 11.07 a	41.0	205.0	118.4 ± 10.29 ab
CM×TC	81.0	171.0	133.2 ± 6.59 NS	60.5	172.0	133.0 ± 6.60	99.0	196.0	130.1 ± 6.29
KM×CC	27.0	143.0	70.2 ± 7.08	29.5	129.0	77.5 ± 5.90	9.0	129.0	73.5 ± 7.26
CH	Hybrid plant stem diameter (mm)								
CSO×TC	2.4	9.8	5.9 ± 0.47	4.5	12.7	8.0 ± 0.56	2.4	10.0	7.0 ± 0.43
CM×TC	5.6	9.5	8.0 ± 0.26	4.0	9.4	7.7 ± 0.32	5.8	9.2	7.6 ± 0.20
KM×CC	3.4	8.5	6.0 ± 0.32	4.6	12.1	6.6 ± 0.41	3.5	11.9	6.5 ± 0.43

CH: Combinations of hybridization, DAP: Days after pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CS: Carrizo citrange; * Different letters indicate significant differences (P<0.05) according to the Least Significant Difference test, LSD: (hybrid plant height: 40.612), NS; Non significant.

Table 4. Quantitative leaf traits of genotypes obtained from embryos at different stage of three hybridization combinations.

CH	Leaf characteristics									
		Leaf length (mm)			Leaf width (mm)			Leaf length / width		
	DAP	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
CSO×TC	110	40.3	161.7	92.1 ± 5.6 a *	13.6	54.6	36.4 ± 2.0 b	0.33	0.50	0.41 ± 0.02
	120	54.7	176.8	107.9 ± 5.0 ab	17.1	77.8	45.3 ± 1.8 a	0.24	0.62	0.43 ± 0.01
	130	26.0	165.9	120.0 ± 7.03 b	18.1	65.1	48.0 ± 2.3 a	0.32	1.04	0.42 ± 0.02
CM×TC	110	45.5	114.8	84.9 ± 3.2 NS	20.1	56.9	37.1 ± 1.3	0.33	0.63	0.44 ± 0.01
	120	67.4	117.7	90.8 ± 2.3	23.1	57.9	39.0 ± 1.5	0.32	0.57	0.43 ± 0.01
	130	54.4	124.9	82.4 ± 2.3	19.3	51.1	35.2 ± 1.1	0.33	0.60	0.43 ± 0.01
KM×CC	110	63.7	151.5	122.8 ± 4.8	29.1	66.4	53.1 ± 2.0	0.37	0.54	0.43 ± 0.01 b
	120	46.7	160.1	128.4 ± 4.0	18.8	74.7	58.9 ± 1.9	0.40	0.58	0.46 ± 0.01 a
	130	27.1	166.2	119.8 ± 4.9	12.2	73.5	54.6 ± 2.2	0.40	0.54	0.46 ± 0.01 ab

CH: Combinations of hybridization, DAP: Days after pollination, CSO: Common sour orange, TC: Troyer citrange, CM: Common mandarin, KM: King mandarin, CS: Carrizo citrange; * Different letters indicate significant differences (P<0.05) according to the Least Significant Difference test, LSD: (leaf length: 22.294), (leaf width: 7.856), (leaf lenght/ width: 0.023), NS; Non significant.

is possible by examining the morphological characteristics.

3.2. Morphological analysis of hybrid populations

Leaf length, width, and index were evaluated to hybrid plants (Table 4). The leaf morphological characters were observed among the progenies either all three hybridizations or each hybridization combination. As a result of observations, leaf division was detected from completely unifoliate to completely trifoliate, also many intermediate forms were also observed. This situation showed that the leaf division character is expressed non-uniformly in hybrid seedlings. In citrus, various methods have been used to assess of diversity and genetic relationships among the genotypes. Researchers assessed morphological analysis as a tool to study variation between Kinnow mandarin and Rough lemon (Jaskani et al., 2006; Altaf and Khan, 2008). In the current study, leaf length, width and index among the combinations was found to be similar to each other, contrary to previous reports (Teich and Spiegel-Roy,1972; Stitou et al., 2020). Different morphological markers have successfully been utilized for separating nucellar and zygotic citrus seedlings among polyploid parents including characters like shape of the leaf edges (Jaskani and Khan, 2000), ratio leaf length and width, petiole size (Donadio, 1981), petiole wing (Ballve et al., 1997) and stem diameter (Khan et al., 1992).

In the study, leaf characteristics such as leaf length, width, leaf length/width index, leaf division, leaf color, petiole wings state, petiole wings shape, leaf lamina shape of the leaf edges, as well as thorn state were also examined. Width of petiole wing is a morphological marker for screening of hybrids in citrus (Blanco et al., 1998). Similarly, Ballve et al. (1997) found that the broadness of leaf petiole wing is a good indicator for the identification of hybrids of sour orange (*C. aurantium*) and sweet orange (*C. sinensis*) in crosses with very narrow-winged species such as *C. sunki* and *C. limonia*. Furthermore, they reported that 90% of the hybrids were visually identified.

There was significant diversity in terms of leaf traits among hybridization combinations. In CSO × TC combination, 76 bifoliate, 46 trifoliate, 109 thorny and 13 accessions as thornless were observed, while 102 bifoliate, 12 trifoliate genotypes, all of were as thorny in the CM × TC combination. Because of ploidy analysis, one of these genotypes was determined as triploid (Kurt and Koyuncu, 2023). The thorn status of this genotype, which has long and dense spines, is compatible with the article of Padoan et al. (2013). In the KM × CS combination, these traits were assessed as; 90 bifoliates, 9 trifoliates and 53 thornless genotypes. Leaf properties are one of the basic characters within morphological evaluation of *Citrus*. Das et al. (1998) reported that Rangpur lime and Rough lemon were crossed with Troyer citrange and trifoliate orange, leaf characteristics varied from fully unifoliate to fully trifoliate. Similarly, Singh (2006) reported that leaves in both Indian wild orange and sour pummelo have simple leaf characteristics. Furthermore, in the study on the inheritance of agronomic traits in citrus, it was stated that these traits are controlled by multiple genes, which are assessed through morphological evaluation (Liu and Deng, 2007).

3.3. Cluster dendrograms of hybridization combinations

Morphological characterization data were evaluated using cluster analysis to compare similarities between hybrids. The morphological characteristics of the 122 hybrids in the CSO × TC population were analyzed and represented in a dendrogram (Figure 3). The similarities were obtained between 0.023 and 17.318 as two main groups. In the resulting dendogram, No.35 (120 DAP) and No.9 hybrids (130 DAP) were found the most similar to each other, while No.1 (110 DAP) and No.3 hybrids (110 DAP) were the most distant relatives.

The dendrogram, made from morphological characteristics of 114 hybrids obtained crossbreeding Common mandarin with Troyer citrange, a genetic difference between 0.004-12.861 was obtained (Figure 4). According to dendrogram, No.4 (130 DAP) and No.25 hybrids (130 DAP) were similar to each other, while No.1 (110 DAP) and No.7 hybrids (110 DAP) were the most distant relatives.

The dendrogram obtained by analyzing the morphological characters of the KM × CC hybrid populations is presented in Figure 5. Among the 99 hybrids, No.11 (110 DAP) and No.14 hybrids (120 DAP) were similar to each other in terms of morphological features, with a similarity ratio of 0.015. With a similarity ratio of 16.862, No.1 (110 DAP) and No.5 hybrids (110 DAP) were found as the most distant relatives.

The citrus hybrids of could selected through morphological identification (Oliveira et al., 2002; Koehler-Santos et al., 2003; Malik et al., 2012). Moreover, this method is relatively simple, easy, cost-and time-saving (Dorji and Yapwattanaphun, 2011b). Reece (1969) stated that Tanaka (1969) accepted 35 species within mandarins and the key to distinguishing them was the differences in leaf and fruit sizes. Many previous authors (Koehler-Santos et al., 2003; Campos et al., 2005) reported that molecular and morphological diversity is independent and rather complementary to genetic diversity in citrus. Budiarto et al. (2021) stated that morphological observations on the 21 citrus genotypes at the seedling stage confirmed the similar grouping pattern because of both cluster analysis and principal component analysis (PCA). Traband et al. (2023) reported, the analysis of the

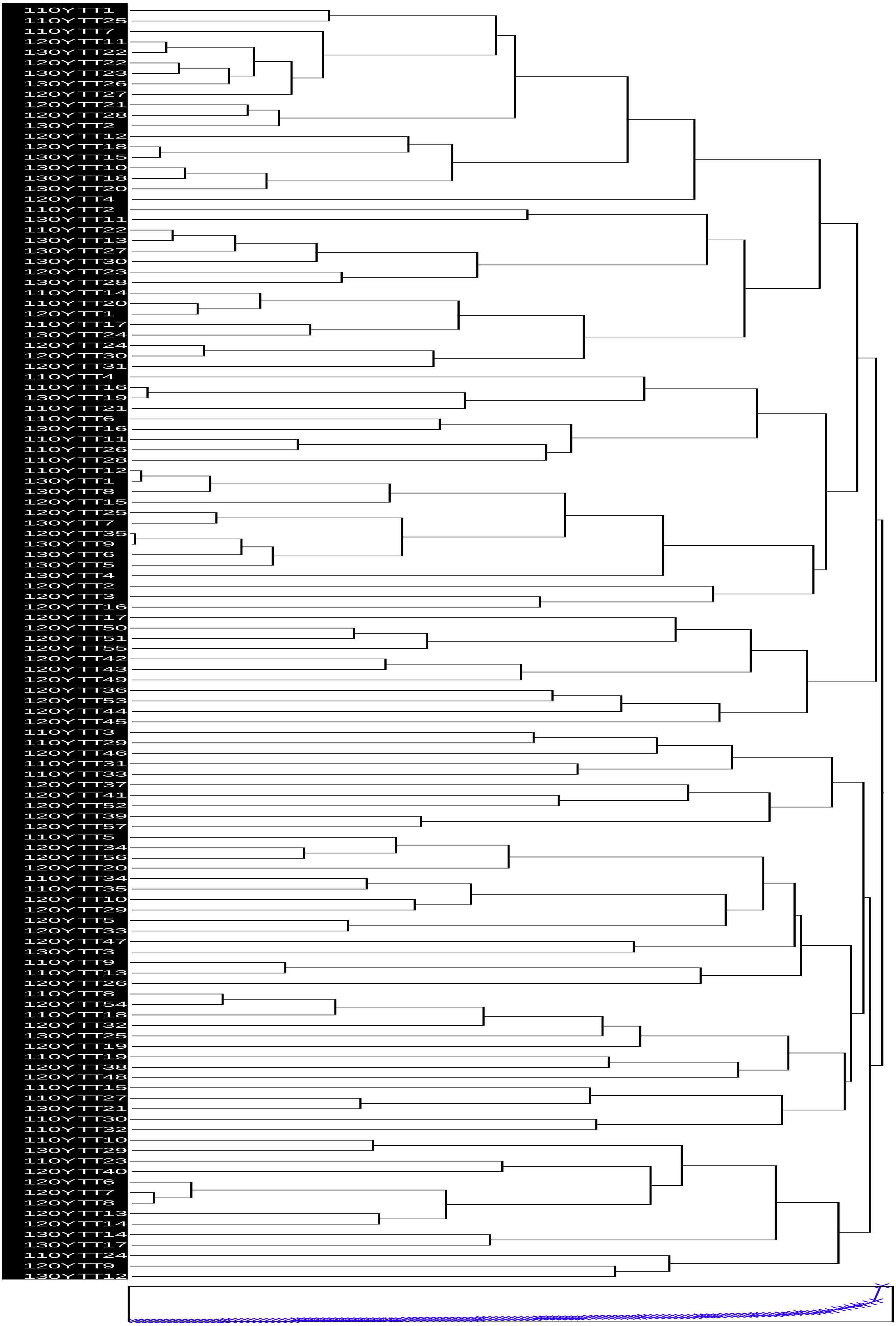

Figure 3. Dendrogram illustrating morphological dissimilarities of 122 hybrids of CSO × TC at seedling stage.

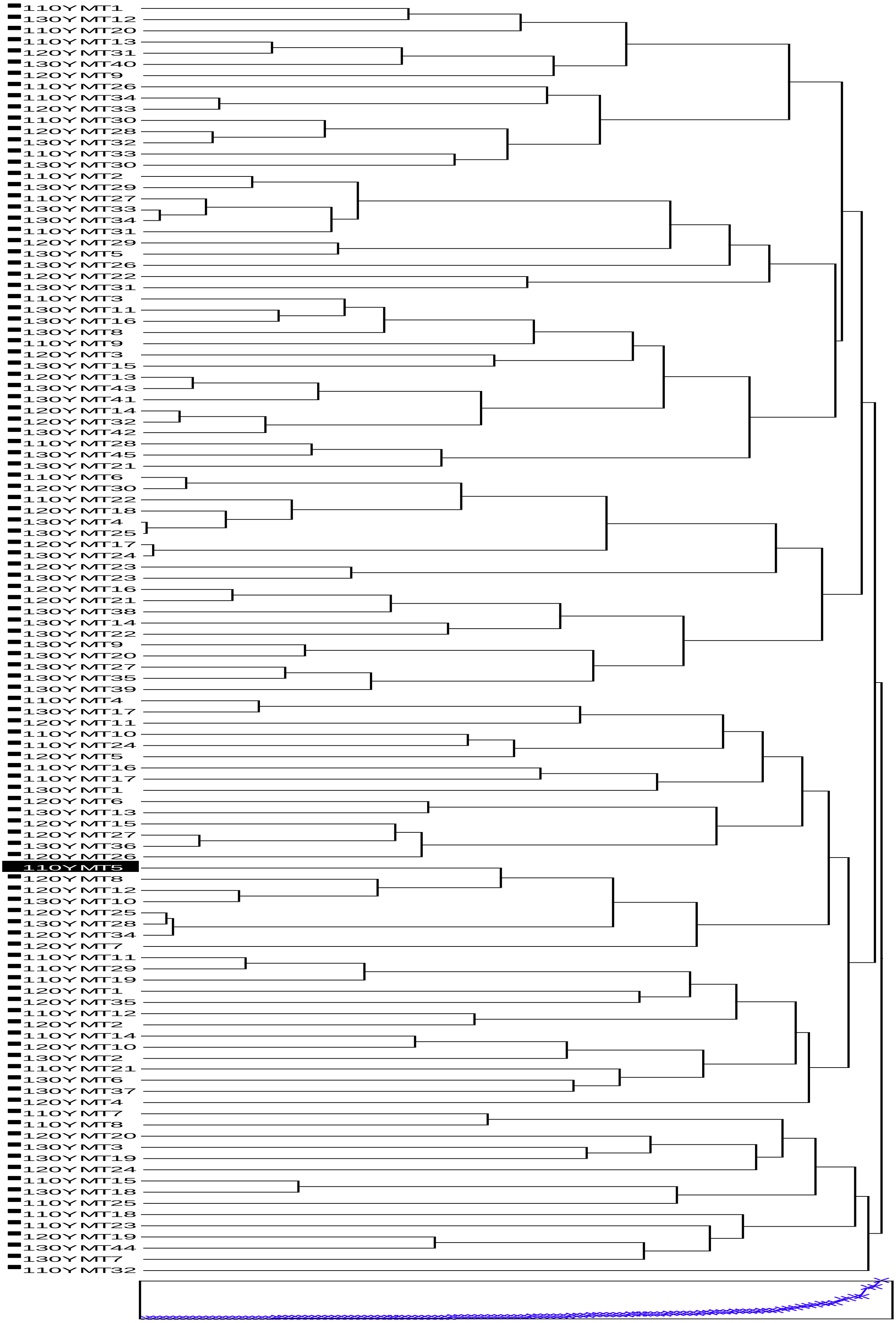

Figure 4. Dendrogram illustrating morphological dissimilarities of 114 hybrids of CM × TC at seedling stage.

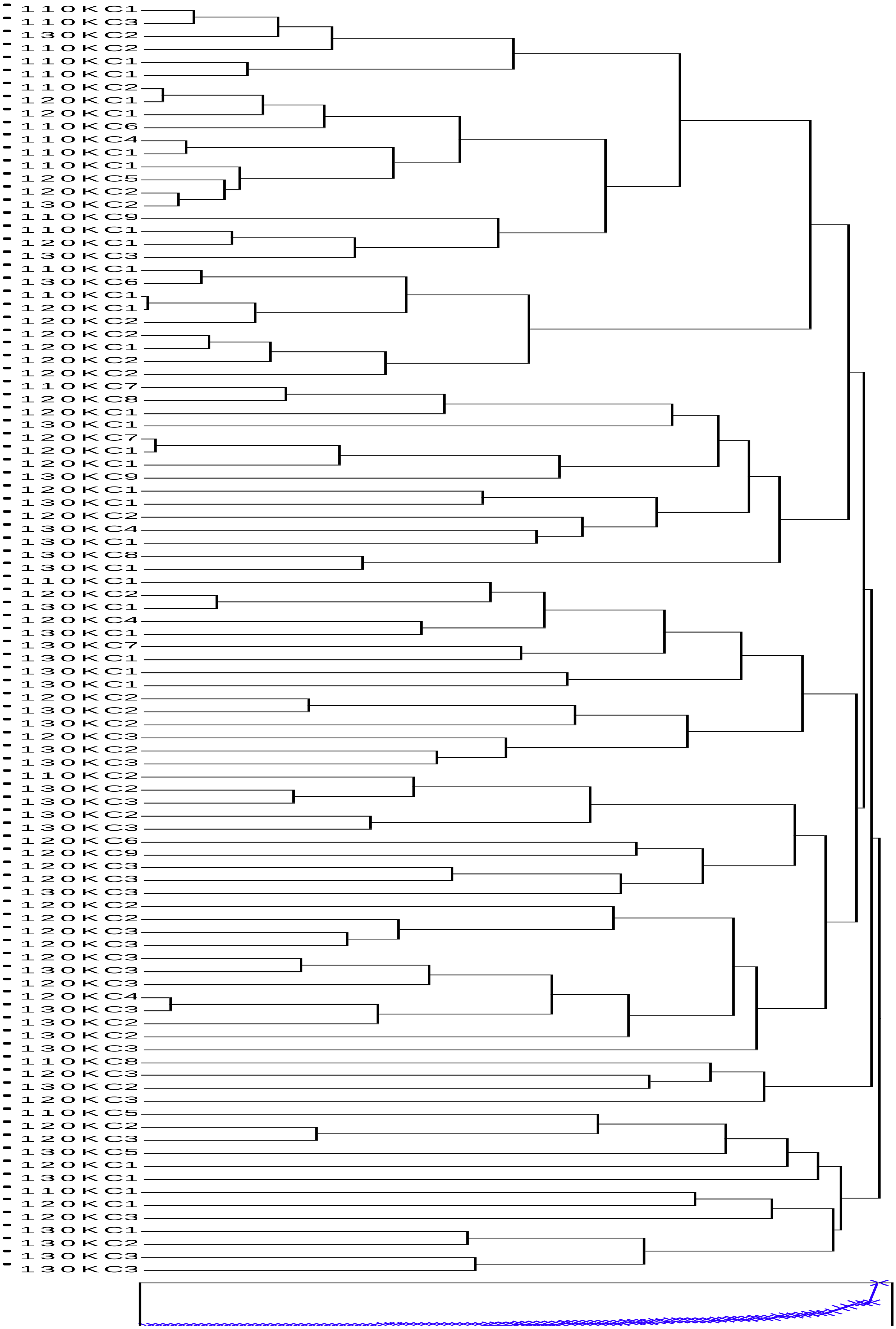

Figure 5. Dendrogram illustrating morphological dissimilarities of 99 hybrids of KM × CC at seedling stage.

morphology of more than 4000 leaves in more than 190 varieties were found significant differences in leaf morphology among the main citrus species groups, and hybrid varieties obtained through breeding exhibited intermediate leaf morphology compared to the parent citrus species. Çimen et al. (2016) evaluated 17 different morphologic characters such as shoot growth, leaf structure and thorniness in their studies.

In a cluster analysis based on morphological traits of 335 hybrids, it was seen that the hybrids obtained at 110 DAP were more distant relative to each other in all hybridization combinations. The diversity among genotypes also varied for all the leaf characters. Morphological analysis showed variation among hybrids. This variability of genotypes could be attributed to cross-pollination and it is very promising for breeders and growers.

4. Conclusions

The present study aimed to identify the diversity of citrus hybrids based on its morphological characters at seedling stage. A total of 335 putative interspecies hybrids, derived from 3 crosses were observed by their leaf morphology. According to our results, it can be concluded that wide diversity existed among the accessions with respect to quantitative leaf characters. In terms of leaf division, 268 genotypes have bifoliate and 67 were trifoliate in all combinations. In addition, 66 of the genotypes were thornless while 269 of the genotypes were thorny. The No. 4 genotype (120 DAP), found as triploid in CM × TC combination, has the longest and dense spines. The dissimilarity index in clustering analysis conclusion was between 0.004 and 17.318 within three hybridization combinations.

The morphological descriptions are very important regarding identification of citrus rootstocks and evaluating their characteristics in breeding programs and germplasm. However, selecting the hybrids only by leaf morphology, size, and growth habit is not always reliable. Because of the difficulty to visuall identification of some hybrids, modern techniques such as isoenzyme analysis and molecular analysis could be reliable.

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