INTRASPECIFIC MORPHOLOGICAL AND GENETIC VARIATION IN DYER'S CROTON (*CHROZOPHORA TINCTORIA*) IN MALATYA (TURKEY)

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Abstract. *Chrozophora tinctoria* (L.) A. Juss. (*Euphorbiaceae*) is a ruderal plant found in cultivated areas and known as 'dyer's-croton' since it is used to obtain dye substances. It is native to the Mediterranean, the Middle East, and the Asia. The plant is also present in North America and Australia. In the present study, morphologically divers *C. tinctoria* plants from Malatya province (Turkey) were used as experimental materials to evaluate morphological variation and genetic diversity at the molecular level. The sequence of the internal transcribed spacer ribosomal DNA (ITS rDNA) obtained by polymerase chain reaction (PCR) from morphologically distinct *C. tinctoria* plants were analyzed by molecular cloning and sequencing. The morphological traits such as leaf, fruit, and seed size of the identified genotype were highly dissimilar to those for *C. tinctoria*. However, the sequences of the morphologically distant genotype and of *C. tinctoria* were very similar to each other. The geographical distribution of the genotype is also mapped and illustrated. Results indicated that genetic and morphological traits and the utility of rRNA gene region for systematic inference might allow the characterization of natural dyer's croton diversity.
Keywords: *Chrozophora thinctoira, genotype, morphology, phylogeny, taxonomy, Turkey*

Introduction

A wide range of climatic and soil conditions and its geographical position favors natural growing of many plant species in many regions of Turkey. Because of its rich flora, many studies on the Turkish flora have been carried out.

The members of *Chrozophora* genus (*Euphorbiaceae*) are annual small shrubby monoecious herbaceous plants having simple, alternate, oval to diamond-shaped, hairy leaves. The fruits are capsules (trilobite), covered in scaly warts. They grow wildly and widely in habitats involving sandy soils, stony places, cultivated places, and waste grounds worldwide. They have been reported in Asia, West Africa, and in the Middle East countries (Gupta and Dutta, 1967; Jaradat et al., 2019).

In Euphorbiaceae family, the genus *Chrozophora* Neck. ex A. Juss. involves eight species. Among them, as a widespread weed *Chrozophora tinctoria* has been used as a source for dye substances. Edible red and blue dyes are obtained from different organs such as the flowers, fruit and sap.

During the field survey in Kale and Battalgazi districts of Malatya province, eastern Anatolia, morphologically distinct *C. tinctoira* individuals were observed and collected. Molecular, phylogenetic and morphological studies revealed that the collected specimens differ from all other *Chrozophora* species. Here we present an intraspecific variation that shows some morphological similarities with *C. tinctoria*, an annual summer and warm season weed plant, determined during the field research studies in Malatya province carried out in 2019 and 2020.

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Materials and Methods

Collecting the plant samples

The specimens belonging to *Chrozophora tinctoria* species in Turkey were collected from agricultural fields in Battalgazi (38° 24' 59'' N -38° 26' 06,68'' E) and Kale (38° 23' 43,25'' N - 38° 39' 31'' E) districts of Malatya province in eastern Anatolia (*Fig. 1*). The collected twenty-five specimens were conserved in the herbarium of Malatya Turgut Özal University Department of Plant Protection and compared with other related specimens reported elsewhere in the world and evaluated according to the relevant literature (Davis, 1982). A total of two molecularly studied *C. tinctoria* species samples from surveyed area and thirty-three rDNA accessions were included in this study. Digital images of vegetative and floral parts of the species were prepared using a Cannon camera attached microscope. The measurements of vegetative parts of the new genotype were performed using a ruler with 0.5 mm accuracy.

Figure 1. *Location and distribution map of C. tinctoria (▲) and its morphologically distinct genotype (▣)*

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Isolation of plant genomic DNA and PCR amplification of ITS Region

Approximately 100 mg of fresh leaf tissue of *C. tinctoria* and its genotype was used in the isolation of plant DNA using a commercial genomic DNA purification kit (Thermo Scientific, USA) as described by the manufacturer. The preparations were kept at –86 °C until processed. DNA fragments of the partial sequence of the internal transcribed spacer (ITS) region was amplified by polymerase chain reaction (PCR) using universal gene specific primers (ITS4:5'-TCCTCCGCTTATTGATATGC-3'and ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3') in a final volume of 50 µL PCR mixture (White et al., 1990). The PCR amplified DNA fragments were separated on 2% agarose gel then analyzed by electrophoresis and recovered by gel extraction kit (Thermo Scientific, USA).

Molecular cloning, sequencing of ITS region and sequence analysis

The purified PCR products were cloned into TA Cloning vector (Promega, USA) and transformed into *Escherichia coli* (MJ109) by electroporation (Bio-Rad, USA) following manufacturer's instructions. Recombinant plasmids were purified and sequenced by automated DNA sequencer (Applied Biosystems) using universal SP6 and T7 primers. DNA sequencing was performed on both strands. For the alignment, sequence similarity and phylogenetic analysis, DNA sequences of ITS fragments were analyzed using the CLC Main workbench software (Denmark). The sequences studied here are deposited in GenBank database under the accession numbers: (MT894297.1) and (MT894298.1) Sequences for comparison, alignment and phylogenetic were obtained from GenBank. The phylogenetic tree was built under the neighbor joining algorithm using 1000 bootstrap replicates in CLC Main Workbench Version 6.2 software.

Results

The genotype of *Chrozophora tinctoria* (*Figs. 2 and 3*) was identified based on the morphological and molecular characters. The genotype was first collected from harvested wheat fields in Battalgazi/Malatya (38° 24' 59'' N -38° 26' 06,68'' E) in the eastern Anatolian part of Turkey. Based on leaf, seed and flower structure, all *Chrozophora* species were searched in the literature. The studied plant was determined as morphologically a new genotype of *C. tinctoria*. The samples of new genotype had similar shapes and morphological structures to the other *Chrozophora* tinctoria identified in the same ecological niche. The new genotype morphologically differs from *C. tinctoria* with many features. Comparative characters are presented in *Table 1*. The micromorphological characters revealed specific variations significant for the new genotype. The new genotype is bigger, its leaves are light green, short, has soft hairs on both sides, giving velvety appearance, ovate to lanceolate and more often hairy, apex acute, base cordate, repand-dentate, petiole to 6 cm, stipules 3-5 mm. Flowers (female); sepals are linear-lanceolate, 5 mm, extremely stellate-pubescent, 10 units (*Fig. 2*).

Genotype description

Turkey, Malatya: Battalgazi and Kale districts, in apricot orchard 800 m, October 10th, 2019 *Olcay Bozdoğan*. The new genotype is related to *C. tinctoria*, but it differs from *C. tinctoria* by annual habitat, stems 39-110 cm (outside the field) 39-77 cm (in the field) long and 0.50-2.03 cm (outside the field) 0.52-1.40 cm (in the field) thick. The whole

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plant (stem, leaf, flower) is light green in color and more often with stellate hairs. Female flower sepals linear-lanceolate, longer (5 mm), leaves are longer. The seed capsule, flower, stem and leaves of the new genotype is more densely covered with white, wool-like (tomentose) hairs giving an appearance of pale ash-green color. The appearance of hair has been described as a pointed star-shaped (stellate) since groups of hair bristles are arranged as radiating out from a common point (*Figs. 3 and 4*).

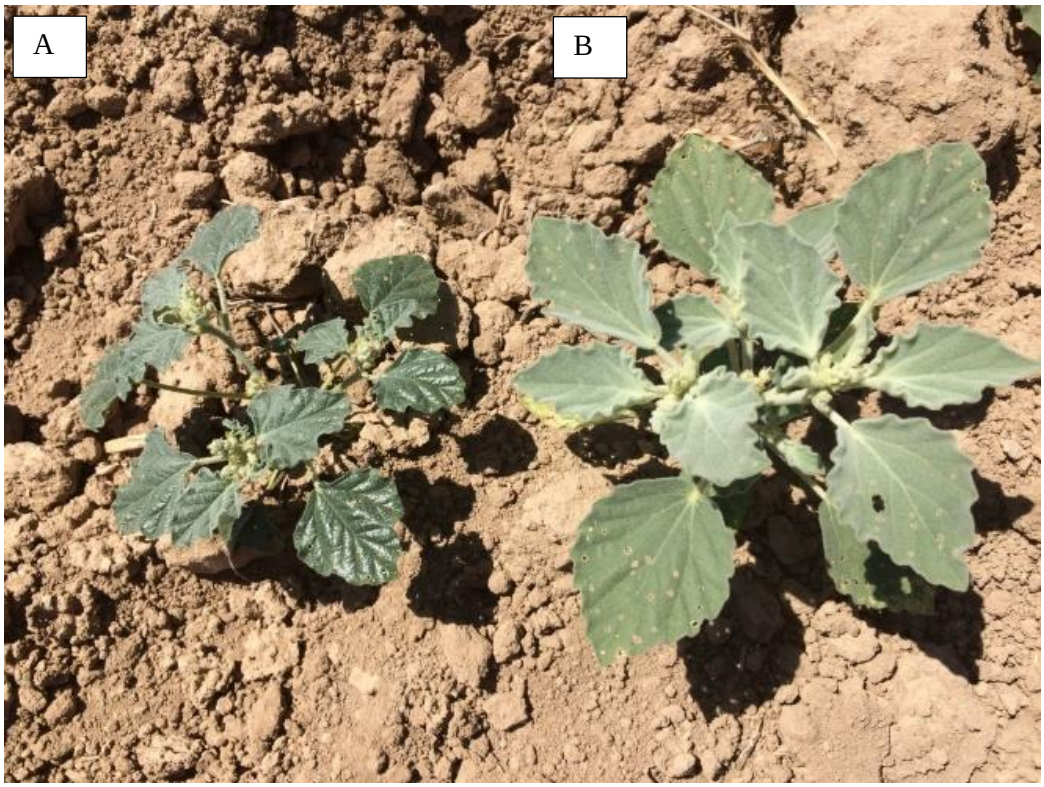

Figure 2. *(A) Chrozophora tinctoria (left), (B) The new genotype (right) of C. tinctoria (Euphorbiaceae)*

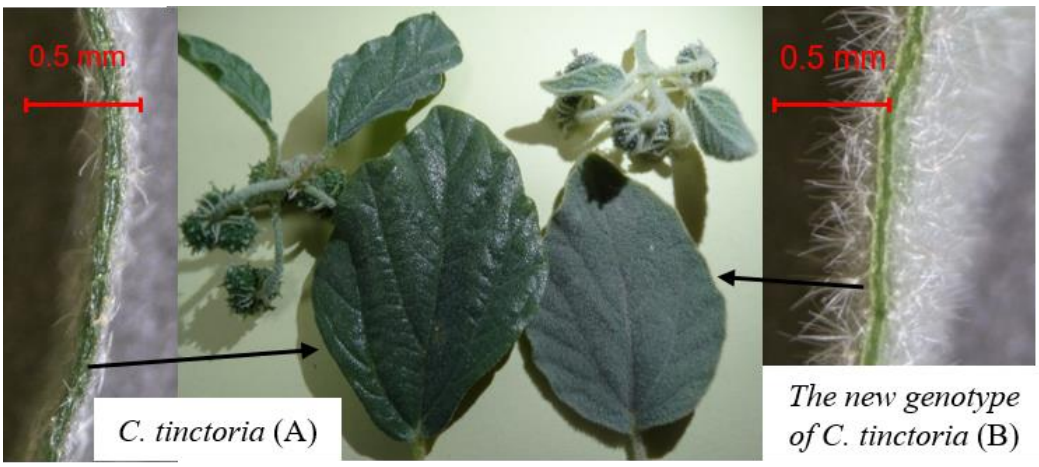

Figure 3. *Mature plant basal leaves and cross sections fruit, left C. tinctoria (A) viewed at 20X magnification, right the new genotype of C. tinctoria (B) viewed at 20X magnification*

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Table 1. *Morphological features of Chrozophora tinctoria and the new genotype of C. tinctoria*

	Chorozophora tinctoria*	**The new genotype of *Chorozophora tinctoria*****
Stem	30-100 cm long, dark green	39-77 cm (in the field), 39-110 cm (outside the field) long and 0,52-1,40 cm (in the field), 0,50-2,03 cm (outside the field) thick, light green often more hairs
Leaves	Dark green, 2-9 x 1-7 cm rhombic-ovate to ovate-lanceolate, shiny	Light green, 3,6- 4,3 x 5,6-7,2 cm light green, ovate to lanceolate, often more hairs, short, soft hairs on both sides, giving velvety appearance
Flower	male flower petal yellowish. female flower sepal (4 mm)	Male flower petal yellowish, female flower longer sepal (5 mm)
Fruit	Fruit sparingly to evenly lepidote	Fruit sparingly to evenly lepidote and often more hairs. 93-973 (in the field), 152-2522 number / per plant (outside the field)

* Davis, (1982), ** Values from 25 plant measurements

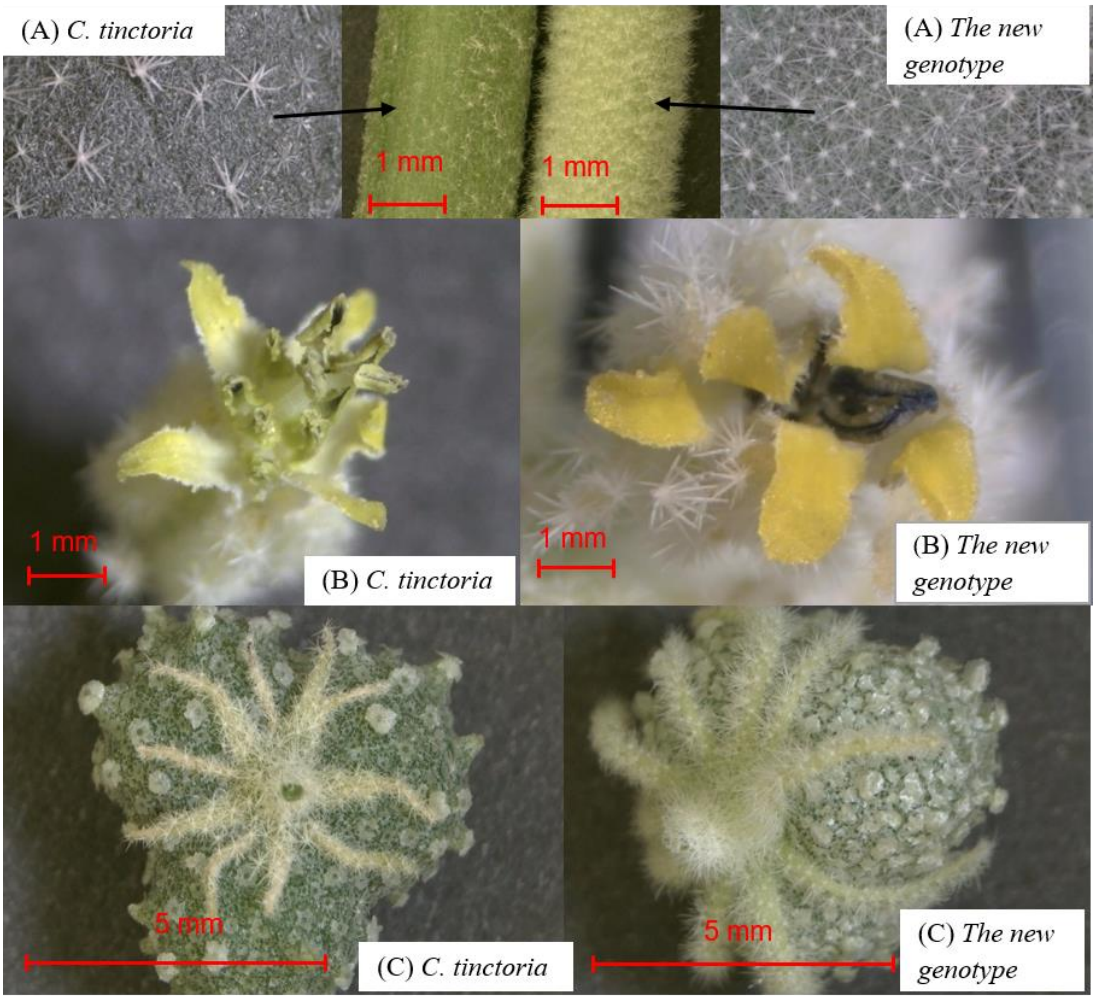

Figure 4. *Close up picture of stem (A) (viewed at 6.7X magnification) in general and closer view of male flower (B) (viewed at 10X magnification) and fruit (C) of C. tinctoria and the new genotype of C. tinctoria (viewed at 6.7X magnification)*

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Ascending annual herb, often becoming woody, extremely pubescent at base, 39-77 cm (field), 39-110 cm (roadside), leaves light green, ovate to lanceolate, 3.6-4.3 x 5.6-7.2 cm, apex acute, base cordate, repand-dentate, petiole to 6 cm, stipules 3-5 mm. Flowers; sepals linear-lanceolate, 5 mm, extremely stellate-pubescent, 10 units, petals yellow obcordate, 3 units, fruit lepidote. Seeds 4 mm, grey. Fl. 7-10. Roadsides, apricot orchard, non-agricultural areas, fallow fields, harvested cereal fields, 700-800 m (*Figs. 3 and 4*) (O. Bozdogan, personal observation).

The fruit of *C. tinctoria* and the new genotype are like a capsule in the shape of three spheres attached to a rounded-triangular structure. Fruits of *C. tinctoria* and the new genotype have three angular seeds. The new genotype of *C. tinctoria* is approximately 40-110 cm in height, monoecious producing tiny and inconspicuous male and female flowers separately. The male flowers have a 5-sepal calyx, 5 yellow petals and a cluster of approx. 5-10 central stamens which have dark center and yellowish margins. The female flowers have a 10-sepals around a spherical ovary, no petals and 3 styles which each subdivide into 2 stigmata. The male and female flowers outgrow as a raceme at the top of the branch, but they are so densely packed that appear to be a spike. Male flowers are above the basal female flowers in the spike-like raceme.

The fruit of the new genotype has the appearance of capsule with the shape of 3 spherical bodies fused in a rather rounded-triangular structure. The fruits of both species have perpendicular stubby projections and white scales contrasting with the dark green wall of the fruit. However, in the new genotype, these structures are much denser creating lighter ash-gray green appearance (*Fig. 4*). Each fruit holds 3 seeds. The seeds of both species are oval with a rough texture, 4mm in size and grey to light brown color.

The leaves of *C. tinctoria* and the new genotype are not found in large numbers per plant and usually grow alternately along the stem. The mature leaves of the new genotype are longer than the leaves of *C. tinctoria* and much hairier. The leaf margins of both species are sinusoidal (wavy). Unlike the *C. tinctoria*, the new genotype has a shorter petiole than the leaf length, having a rhombic to ovate shape.

Molecular phylogeny of the ITS region

Approximately a 715 bp long DNA fragments of ITS region of nuclear ribosomal DNA (nrDNA) (ITS1-5.8S-ITS2) amplified using the universal ITS4 and ITS5 primers and templates of total genomic DNA of *C. tinctoria* and its new genotype (*Fig. 5*) and molecularly cloned and sequenced.

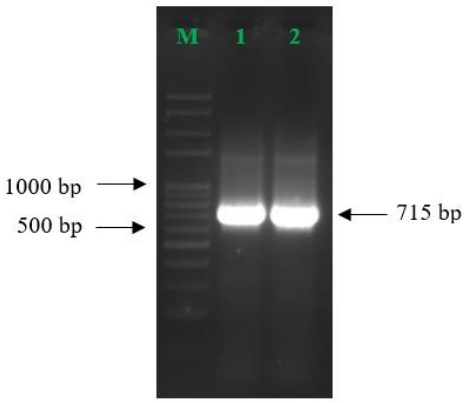

Figure 5. *Agarose gel image of amplified ITS region of Chrozophora tinctoria (row 1) and the new genotype (row 2), M: 3.000 bp molecular markers*

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In *Figure 6*, the neighbor joining phylogram displays the relationships among species belonging to *Chrozophora* genus and 34 additional species belonging to Euphorbiaceae reported elsewhere. The sequences of *C. tinctoria* collected from Malatya province was grouped with the members belonging to *Chrozophora* genus. Likewise, the sequence of the new genotype formed a single clade with other *C. tinctoria* indicating its high level of genetic relatedness with this species (*Fig. 6*).

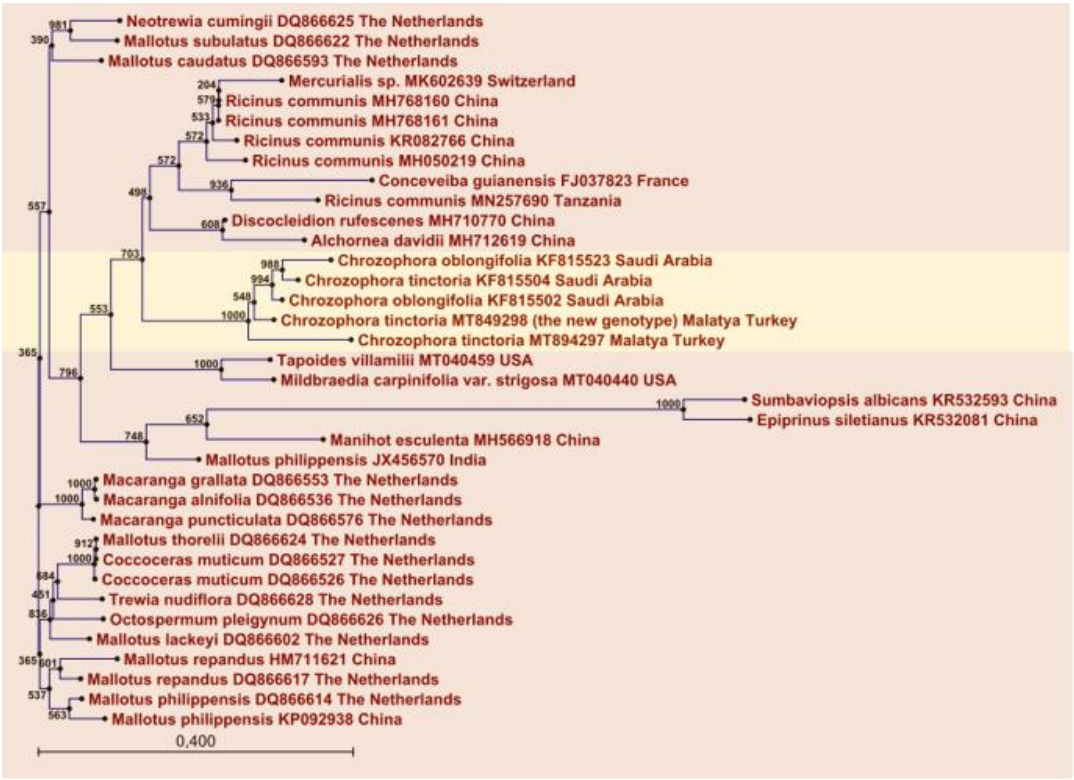

Figure 6. *Molecular phylogeny of Chrozophora tinctoria (MT894297.1) and its new genotype (MT894298.1). The phylogenetic tree was built under the neighbor joining algorithm using 1000 bootstrap replicates and are indicated at key nodes*

The new genotype grows frequently at an altitude of 700-800 m in dry fields, waste ground, fallow fields, harvested wheat fields, the apricot orchards and non-agricultural lands of Kale and Battalgazi districts. It has been thought to be endemic to Malatya province.

The new genotype of *Chrozophora tinctoria* is recorded from only 2 localities near Battalgazi and Kale districts of Malatya in an area of occupancy estimated to be more than 1000 km^2; the population size is estimated to be well above 5000 mature individuals. Therefore, it is not classified as endangered species (IUCN, 2001).

Discussion

Genetic and morphological traits are both used in this study to display DNA sequence and morphological variation at the intraspecific level in *C. tinctoria*. During the field work, the new genotype of *C. tinctoira* was found growing in the harvested wheat fields, road sides and apricot orchards of Kale and Battalgazi districts. The new genotype of

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C. tinctoira found in this study was compared with other plant species in the genus *Chrozophora* in the world. As a result of comparisons, *Chrozophora brocchiana* (Vis.) Schweinf., (African Plants - A Photo Guide, 2021*)*, *Chrozophora gangetica* Gand. (POMO, 2021a), *Chrozophora sabulosa* Kar. & Dirt. (Prain, 1918), *Chrozophora mujunkumi* Nasimova (POMO, 2021b), *Chrozophora plicata* (Vahl) A.Juss. ex Spreng. (POMO, 2021c), *Chrozophora oblongifolia* (Delile) A.Juss. ex Spreng. (GBIF, 2021), *Chrozophora rottleri* (Geiseler) Spreng. (India Biodiversity Portal, 2021), and *Chrozophora senegalensis* (Lam.) A.Juss. ex Spreng. (POMO, 2021d) species were not similar to the new genotype of *C. tinctoria* in terms of morphological characters. Flowering and fruiting periods of this new genotype are July and late October. Between the years 2010 and 2014, numerous new species and genotypes have been reported from Malatya province (Yıldırım et al., 2010; Tan et al., 2012; Mutlu and Karakuş, 2012; Koç and Aksoy, 2013; Uzunhisarcıklı et al., 2013; Yıldırım and Erol, 2013). More recently, Yıldırım and Şenol (2014) have been described *Campanula alisan-kilincii* Yıldırım & Şenol (Campanulaceae) as a new species from Malatya, in eastern Anatolia, Turkey.

The new genotype is morphologically similar to the *C. tinchtoria*. The size and the shape of seed capsule of the new genotype are almost the same with *C. tinctoria* except with an excessive hairy capsule surface of the new genotype. More importantly, the results of our research show that the hairier appearance of the new genotype remains when the plant gets older, and those structures cannot be abraded by wind, and the new variation remains distinctive during the vegetation period. Comparative characters are summarized in the *Table 1*. With the present study, it has been revealed that the morphological characteristics of the new genotype seed, leaf and stem surfaces are a useful tool for identifying the species.

The alignment of ITS nucleotide sequences with 34 ITS nucleotide sequences from several species belonging to Euphorbiaceae in databases revealed that the new genotype differed from these species and clustered in *Chrozophora* genus. Combined molecular data and morphological characters support the conclusion that this accession may belong to *C. tinctoria* and showed strong support for the grouping in the *Chrozophora* genus. Molecular techniques such as sequencing of internal transcribed sequence region (ITS) of ribosomal RNA gene, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellites and single nucleotide polymorphisms (SNP) have recently been widely used for plant identification and diversity studies (Arif et al., 2010; Keskin et al., 2017; Kaya et al., 2020; Tursun et al., 2021). Although the new genotype of *C. tinctoria* has been described from two districts of Malatya, the actual dissemination of the genotype is still unknown. Apricot is an economically important strategic crop cultivated in Malatya province. Additional studies are needed to determine the distribution of the new genotype and economic impact on crop plants and especially apricot orchards in Malatya.

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