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# BARCODING OF *CHENOPODIUM MURALE* L. WITH NEW RECORD TO EGYPTIAN FLORA

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**Abstract**

*Chenopodium* L. is a cosmopolitan genus (family: Amaranthaceae). Hybridization and phenotypic plasticity of its species, complicate the species delimitation. The presence of observed notable phenotypic plasticity among the *C. murale* populations and the newly recorded (suspected *C. hybridum*), makes it challenging to identify these taxa's taxonomic identity. The morphological investigations revealed distinctive features at inter-specific level for *C. murale* populations mainly in stem color, leaf shape, margin dentation, number of stamens and tepals, and the percentages of both hermaphrodite flowers and the 5-staminode flowers. The morphological correlation between distinguished Forms; ranges between 0.070-0.518. While the infra-specific correlation between the *C. murale* forms and the suspected *C. hybridum* ranges from 0.033-0.340. RbcL and matK barcoding were applied to confirm the taxonomic identity as well as, their phylogenetic affinities of the studied taxa. The retrieved phylogenetic affinity between *C. murale* forms showed very low genetic divergence to the *C. hybridum*. The sequences of the confirmed *C. murale* forms and the new record to Egyptian flora *C. hybridum* were deposited in the world database under the accession numbers OR373113, OR394977, OR394978, OR394979, OR405949, OR405950, OR405951, and OR405952 for the *C. murale* and OR394980 and OR405953 for the *C. hybridum.* We investigated the inter-specific relationships among the *C. murale* forms, but the intra-specific relationships between *C. murale* and *C. hybridum* required further investigation to explore and fully understand the origin of this new record.

**Key words:** *Chenopodium murale*, *Chenopodium hybridum*, Phenoplasticity, Gynomonoecy, rbcL, matK, New-record, Egyptian Flora.

## Introduction

Genus *Chenopodium* L., is a cosmopolitan genus of the monophyletic subfamily Chenopodioideae of family Amaranthaceae (Anon., 2023). It includes at least 150 annual and perennial species (Fuentes-Bazan *et al*., 2012), this number recently updated to 280 species, merely of weed in cultivations and waste-land influenced by anthropogenic activities (Anon., 2023). *Chenopodium* species are native to much of Europe, Asia, India, China, and both North and S. America (Morteza-Semnani, 2015). However, the center of origin for the genus *Chenopodium* is difficult to determine due to the long history of human use and cultivation of these plants. Nonetheless, molecular studies have provided evidence that supports the Andean region of S. America as a center of diversity and origin for many *Chenopodium* species (Kadereit *et al*., 2006).

The significance of *Chenopodium* species is due to their wide range of medicinal properties. Plants of this genus are reported to be widely used in folk medicines (Watt & Breyer-Brandwijk, 1962; Vasishita, 1989). It also includes the famous pseudocereal crop known as Quinoa *"Chenopodium quinoa* Willd.*"*.

The taxonomy of the genus *Chenopodium* has been an important point of considerable debate. Phenotypic plasticity is the primary cause of taxonomic ambiguity (Zhou *et al*., 2005), in addition to, the close morphologically similarity between species, the presence of polymorphisms within many individual species, and the occurrence of hybrids complicate the species identification (Cole, 1961). For example, the close similarity between *C. murale* and *C. hybridum* in morphological characteristics, including leaf shape and flower structure, makes it difficult to distinguish between these two species (Mosyakin & Robertson, 2003).

Genus *Chenopodium* possess hermaphrodite or gynomonoecious flowers, perianth green of 5-segments (2-4 in some species). Gynomonoecy is the presence of hermaphrodite and female flowers on the same plant (Mamut *et al*., 2017). This genus is represented in Egyptian flora by nine species namely, *Chenopodium album* L., *C. ambrosoides* L., *C. botrys* L., *C. ficifolium* Sm., *C. glaucum* L., *C. giganteum* D. Don, *C. murale* L., *C. opulifolium* Schrad. ex Koch & Ziz, *C. vulvaria* L. (Boulos, 2009). *Chenopodium murale* L. (Syn. *Chenopodiastrum murale*, Fuentes-Bazan *et al*., 2012) is one of them, it is commonly known as nettle-leaved goosefoot (Kütz, 2007), salt-green, Australian-spinach, and sowbane (Ahmed *et al*., 2017). It is native to Europe, Northern Africa, and Asia, but it has spread throughout the world, especially in tropical and subtropical environments, due to its ease of introduction (Ahmed *et al*., 2017).

DNA barcodes are short gene sequences extracted from a specified section of the target species genome. The ultimate purpose of a DNA barcode is to quickly and accurately identify microorganism, plant and animal species (Kress & Erickson, 2008). Based on evaluations of recoverability, levels of species discrimination, and sequence quality, the Consortium for the Barcode of Life (CBOL) plant working group recommended maturase K (matK) and ribulose-1,5-bisphosphate carboxylase oxygenase large subunit (rbcL) as 2-locus combination for standard plant barcode (Anon., 2009). RbcL and matK sequences have recently been used by a number of researchers for barcoding and species identification (Starr *et al*., 2009, Asahina *et al*., 2010) as well as for phylogenetic analysis (Manen *et al*., 2004, Tamura *et al*., 2004, Kuo *et al*., 2011).

During the field study of *C. murale*, a great morphological variation in leaf, plant color and size within the different population were observed. This phenotypic diversity promoted the authors to examine variation range of *C. murale* populations using morphological and molecular tools and check the identity of the closely related specimens (the suspected *C. hybridum;* this species to date not recorded in Egypt). Finally, to estimate the taxonomic and molecular relationships of the studied *C. murale* populations and the suspected *C. hybridum* traced as coexisting during the field survey.

## Material and Methods

**Plant material:** The present research is based on the examination of herbarium specimens deposited in Cairo University Herbarium (CAI) representing 67 localities in addition to, 25 fresh populations were collected during 2021 to 2023, from localities representing the geographic range of the species phenoplasticity. Ten individuals / population were examined for phenotypic diversity in the distribution range of this species. The collected 25 populations were morphologically grouped to four *C. murale* forms and some specimens suspected to be *C. hybridum*, that was growing in mixed population with *C. murale* in the western Mediterranean stripe in two locations (31°17'40" & 30°16'45" and 31°17'22" & 30°17'35"). Reference herbarium specimens stored at other herbaria available online [New York Botanical Garden (NYBG), Harvard University Herbaria & libraries (HUH), the JSTOR Global Plants Database, acronyms follow Index Herbariorum (*http://sweetgum.nybg.org/ih/*) were also checked. Abbreviations followed IPNI (*http://www.ipni.org/*). Morphological characters of stem, leaves, inflorescence, flower and fruit were examined (50 different morphological characters were checked). Voucher specimens were deposited in CAI.

**Data analysis**: The correlation among the samples of *Chenopodium murale* and *C. hybridum* was done by the analysis of 50 morphological traits. The traits were measured and recorded for all samples and the analysis was carried out using the software (R-4.3.1 for windows) to construct the heat map. A heatmap is a graphic representation of numerical data in which each value is represented as dark squares denote large numerical values while lighter squares denote smaller numerical values (Tiessen *et al*., 2017).

**Molecular study:** The genomic DNA was extracted from one-gram juvenile leaves, ground under liquid nitrogen, for the selected samples (the suspected *Chenopodium hybridum* and four forms *C. murale*) using CTAB (Cetyl-trimethyl ammonium bromide) extraction buffer procedure described by Doyle & Doyle (1990) and modified by Allen *et al*., (2006).

**rbcL and matK barcoding analysis:** PCR Reactions: the reaction mixture for rbcL and matK PCR amplification included 1x buffer (Promega), 15 mM MgCl2, 0.2 mM dNTPs, 20 pmol of each primer, 1µ of Taq DNA polymerase (GoTaq, Promega), 40 ng DNA, and ultra-pure water to a final volume of 50 µL. The sequences for the used primers were: rbcL-F: 5'ATGTCACCACAAA CAGAGA CTAAAGC-3' & rbcL-R: 5'-TCGCAT GTACC TGCAGTAGC-3'; with 600bp/each and matk-F: 5'-CGATCTATTCATTCAATATTTC-3' & matk-R: 5'-TCTAGCACACGAAAGTCGAAGT-3'; with 900bp/each.

**PCR amplification and product detection:** PCR amplification was carried out in a Perkin Elmer/ GeneAmp® PCR System 9700 (PE Applied Biosystems) designed to complete 40 cycles after initial denaturation cycle for 5 min at 94°C. Each cycle consisted of denaturation step was lasting 30 seconds at 94ºC, an annealing step lasting 30 seconds at 50ºC, and an elongation stage lasting 1 min at 72ºC. In the last cycle, the primer extension phase was prolonged to 7 min at 72ºC. Detection of the PCR products was achieved by electrophoresis in a 1.5% agarose gel with ethidium bromide (0.5µg/ml) in 1X TBE buffer at 95 volts (El-Sayed, 2022). The amplification products were separated. A molecular size reference was a 100 bp DNA ladder. PCR products were observed under UV light and photographed using a Gel Documentation System (BIO-RAD, 2000).

**Purification of PCR products:** Three volumes of binding buffer 1 were added after the PCR reaction mixture was transferred to a 1.5 ml microfuge tube. The mixture solution was let to stand for 2 minutes in the EZ-10 spin column at room temperature then centrifuged. After that 750 µl of wash solution was added to the column and centrifuged at 10.000 rpm for two minutes. After repeating the washing process, the column was spun at 10.000 rpm for one more minute to remove any remaining wash solution. The column was put into a clean 1.5 ml microfuge tube, 50 µl of elution buffer was added, the mixture was incubated at room temperature for 2 minutes, and then purified DNA was then stored at -20°C (Elian *et al*., 2021). Using Big Dye TM Terminator Cycle Sequencing Kits and the manufacturer's recommended protocols, the PCR product was sequenced in an automatic sequencer ABI PRISM 3730XL Analyzer (Mohdly *et al*., 2023).

**Computational analysis:** The BLAST (Basic Local Alignment Search Tool) program was used to align the retrieved sequences under accession number OR394980 and OR405953 for *C. hybridum* and under accession numbers OR373113, OR394977, OR394978, OR394979, OR405949, OR405950, OR405951, and OR405952 for the *C. murale* (http://www.ncbi.nlm.nih.gov/BLAST). The reference sequences available online in the Gene bank for *C. hybridum* and *C. murale* were retained to construct a phylogenetic tree and calculate pairwise distances using Maximum Likelihood (ML) method and Clustal W in MEGA5.

## Results

***Chenopodium murale* L.**, Sp. Pl., ed. 1, 219 (1753).

Common name: nettle-leaved goosefoot.

Annual glabrous to sparingly mealy herb; stem ascending-erect, branched, angular and more or less thickened at base, 20-80 cm tall; leaves simple, alternate, petiolate, rhombic-ovate to rhombic-oblong or deltoid, 4-6.5 x 3-4.5 cm, petiole 2-2.5 cm; inflorescences glomerules in terminal and lateral panicles (Fig. 1); the pericarp adherent to the seed coat, and is hardly to separate completely from the seed which is black-brown, 1-2 mm in diameter, ovoid to circular outline and somewhat depressed.

**Morphological diversity within *C. murale* forms**

High degree of phenotypic diversity was observed in 50 morphological characters in four *C. murale* forms. The main distinctive features were the stem color, leaf shape, margin dentation, number of stamens and tepals and the percentages of both hermaphrodite flowers and flowers with 5-staminode, flowers have 4-5 tepals. The number of stamens vary in different hermaphrodite flowers. Flower may have 5 stamens all have the same length, or 2 long stamens and 3 staminodes, or one-stamen and 4 staminodes, or the flower may have only one stamen. Filament of the long stamen (0.8-1.8 x 0.15 mm), anther 0.5 x 0.6-0.8 mm, staminode 0.2-0.7 x 0.1 mm. Based on these morphological features the main distinguishing characters between the detected four forms are: Form 1: possesses up to 25% flowers with 5 staminodes. While the other three forms have no - few flowers with staminodes. Form 2 showed green-red striped stem, red leaf margins, and red-tinged tepals. Form 3: characterized by up to 75% hermaphrodite flowers (less than 50 % in other forms) with deltoid leaf, and brown seeds. Form 4: the shape of style and stigma showed very high diversity in both female and hermaphrodite flowers, where the style present or absent; stigma 1-3 filiform (Fig. 2). The following key can distinguish all the 4 forms:

1. Up to 25% flowers with 5-staminodes ……..……..……..…….. Form 1
 - Flowers with staminodes are very few ……..…….. 2
2. Stem green-red striped, red-tinged tepals ……..……..……..... Form 2
 - Stem not so ……..……..……..…… 3
3. Hermaphrodite flowers up to 75%, leaf deltoid, stigma 1-2 filiform ..……..……..…….. Form 3
 - Hermaphrodite flowers less than 50 %, leaf not deltoid, stigma 1-3 filiform ……...…..... Form 4

***Chenopodium hybridum* L.,** Sp. Pl.: 219 (1753)

This species is recorded from Egypt for the first time, its specimens were quite close to *C. murale*. It is an annual herb; stem ascending to erect, sparsely branched above, 20-80 cm tall, light yellow to purple ribbed; leaves rhombic-ovate to deltoid, base rounded, truncate, margin with few teeth to subentire, apex acute to acuminate, 4-6.5 x 2-3 cm, petiole up to 2.5 cm; inflorescence glomerules in terminal and lateral panicles; flowers hermaphrodite and female; perianth five segments, ovate, apex obtuse, 0.7-1.5 x 0.5-1 mm; stamens five, with membranous margin, stamen filament 1.9-2.1 x 0.2 mm, anther 0.5 x 0.6 mm; gynoecium has ovary 0.3-0.9 x 0.3-1 mm, style 0.1-0.3 x 0.2 mm, 2 stigmatic arms 0.2-0.3 x 0.1 mm; pericarp adherent to the seed coat, seeds black, 2-3 mm in diameter (Fig. 3).

**Table 1. Correlation coefficients between the studied 4 Forms of *C. murale* and *C. hybridum*, based on the investigated 50-morphological characters.**

| | | ***C. murale*** | | | | ***C. hybridum*** |
|---|---|---|---|---|---|---|
| | | **Form 1** | **Form 2** | **Form 3** | **Form 4** | |
| ***C. murale*** | Form 1 | 1.000 | | | | |
| | Form 2 | 0.226 | 1.000 | | | |
| | Form 3 | 0.070 | 0.120 | 1.000 | | |
| | Form 4 | 0.335 | 0.518 | 0.313 | 1.000 | |
| ***C. hybridum*** | | 0.033 | 0.085 | 0.340 | 0.191 | 1.000 |

**Morphological correlation between *C. murale* forms and *C. hybridum***

The correlation between the four forms of *C. murale* and *C. hybridum* was constructed based on the investigated 50-morphological characters (Fig. 4 & Table 1), showed that the highest correlation (0.518) was between Form 2 and Form 4 of *C. murale*, followed by Form 3 and *C. hybridum* with correlation 0.340, and the lowest correlation of 0.033 was between *C. hybridum* and *C. murale* Form 1. According to the constructed heat map the stronger correlation, showed the darker color. The heat map clustering separated two groups. The first group comprised of *C. hybridum* and Form 3 of *C. murale*. The second group included the rest forms (Form 1, 2 & 4) of *C. murale*.

**Genetic correlation between *C. murale* forms and *C. hybridum***

The two nucleotide sequences of both matK and rbcL genes were implemented for tracing the homologous identification of the studied taxa. Only sequences of 96% minimum homogeneity were considered. BLAST searches using both matK and rbcL gene sequences showed the resemblance of the *C. murale* forms sequences with 96-100% similarity to the sequences of *C. murale* available online. These forms deposited with the following accessions: Form 1: OR394977 and OR405949, Form 2: OR394978 and OR405950, Form 3: OR394979 and OR405951 and Form 4: OR373113 and OR405952. While the morphologically identified specimens as *C. hybridum* (accession no OR394980 and OR405953), were of 99% similarity to the *C. hybridum* based on available online data.

The constructed phylogenetic dendrogram based on matk sequences (Fig. 5), showed that all accessions of *C. murale* and *C. hybridum* generate a major cluster splitting into two main groups. The first main group is divided into two subgroups, the first subgroup included all studied Egyptian specimens of *C. murale* and *C. hybridum*. The *C. murale* forms showed very low genetic divergence (less than 0.3%) between them. As specimens 3 & 4 had no genetic divergence (0.0%). While, the *C. hybridum* showed very high resemblance (99.8%) with *C. murale* Form 3. The second subgroup included specimens of *C. murale* from UK, S. Africa, and *C. hybridum* from Romania, with very low genetic divergence between them. The second main group is divided into two subgroups, the first subgroup included *C. hybridum* specimens from UK and China and *C. murale* specimens from Bahrain and Portugal with genetic distance is variable in this subgroup ranges from low distance of 0.2 between specimens of United Kingdom and Portugal to very high distance of 1.2 between specimens of United Kingdom and China. The second subgroup included the specimens of *C. murale* from Egypt, Saudi Arabia, Canada and Portugal, with high genetic distance between specimens.

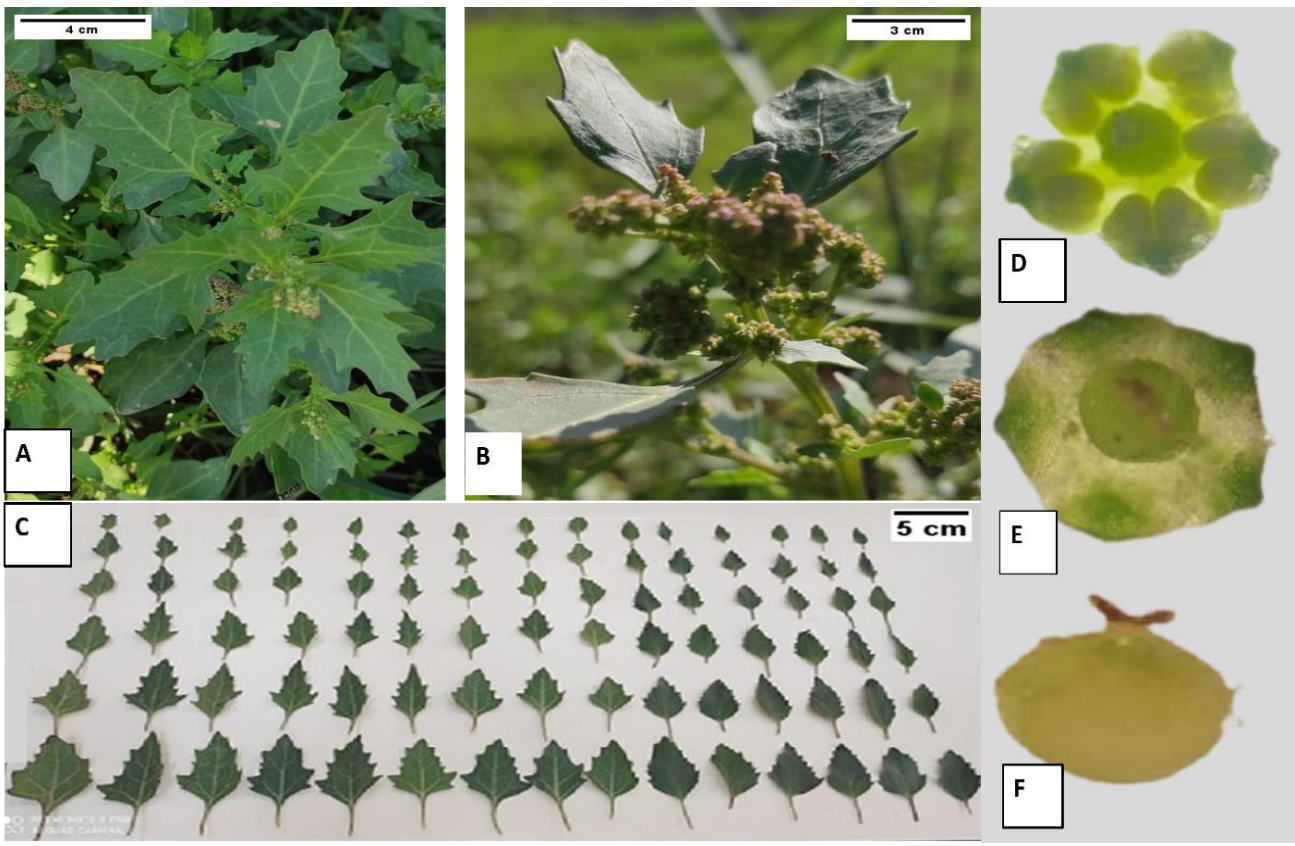


Fig. 1. Morphological variation in *C. murale*, A, B: field pictures showing the variation in leaf shape and inflorescence color; C: Leaf diversity in the shape, and size; D: Hermaphrodite flower; E: Female flower & F: Gynoecium, (D & E x 10; F x 20).

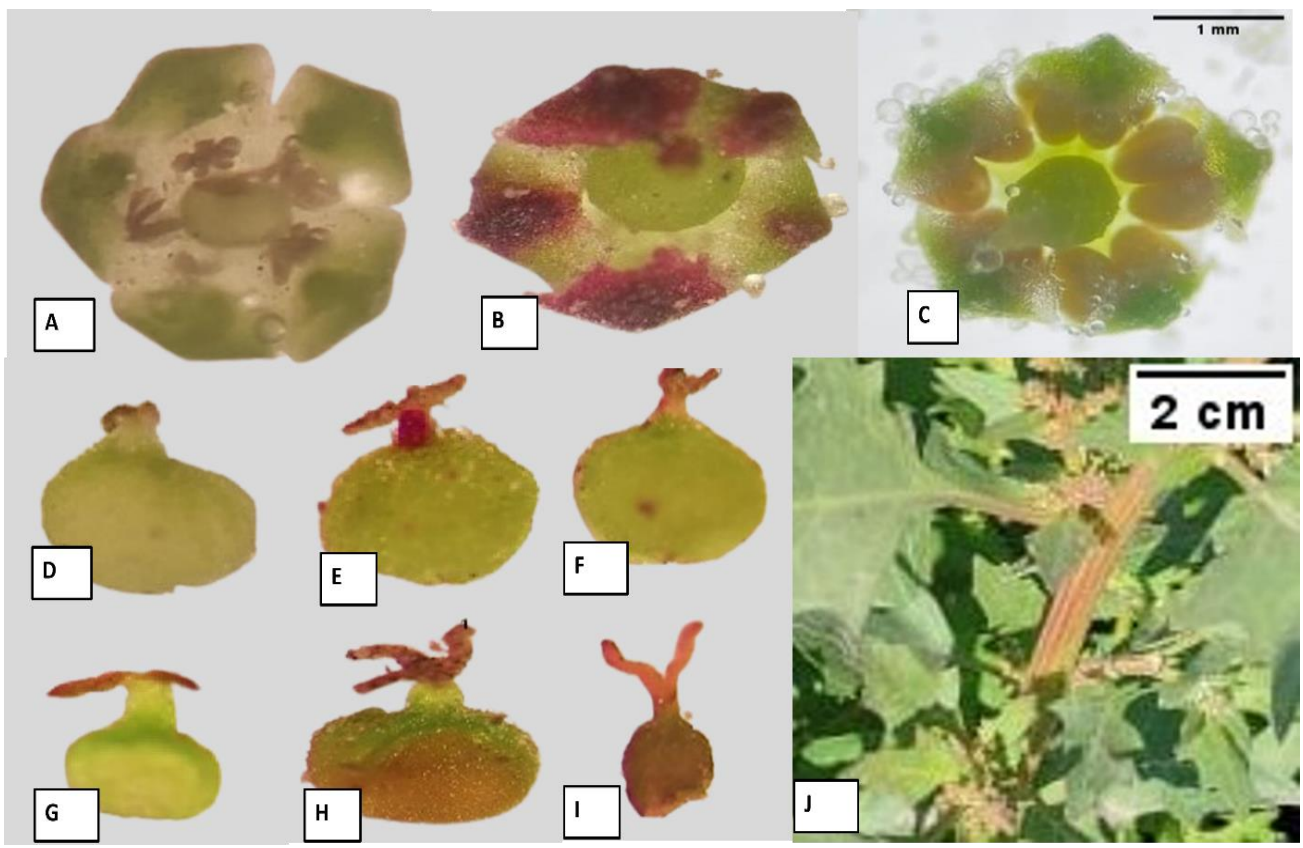


Fig. 2. Diversity in the detected *Chenopodium murale* Forms. (A): Flower with 5 staminodes in Form 1; (B): Flower with red-tinged tepals in Form 2; (C): Hermaphrodite flowers in Form 3; D-I: diversity in style and stigmas in Form 4; J: Green–red striped stem in Form 2, (A, B & I x 10; D-H x 20).

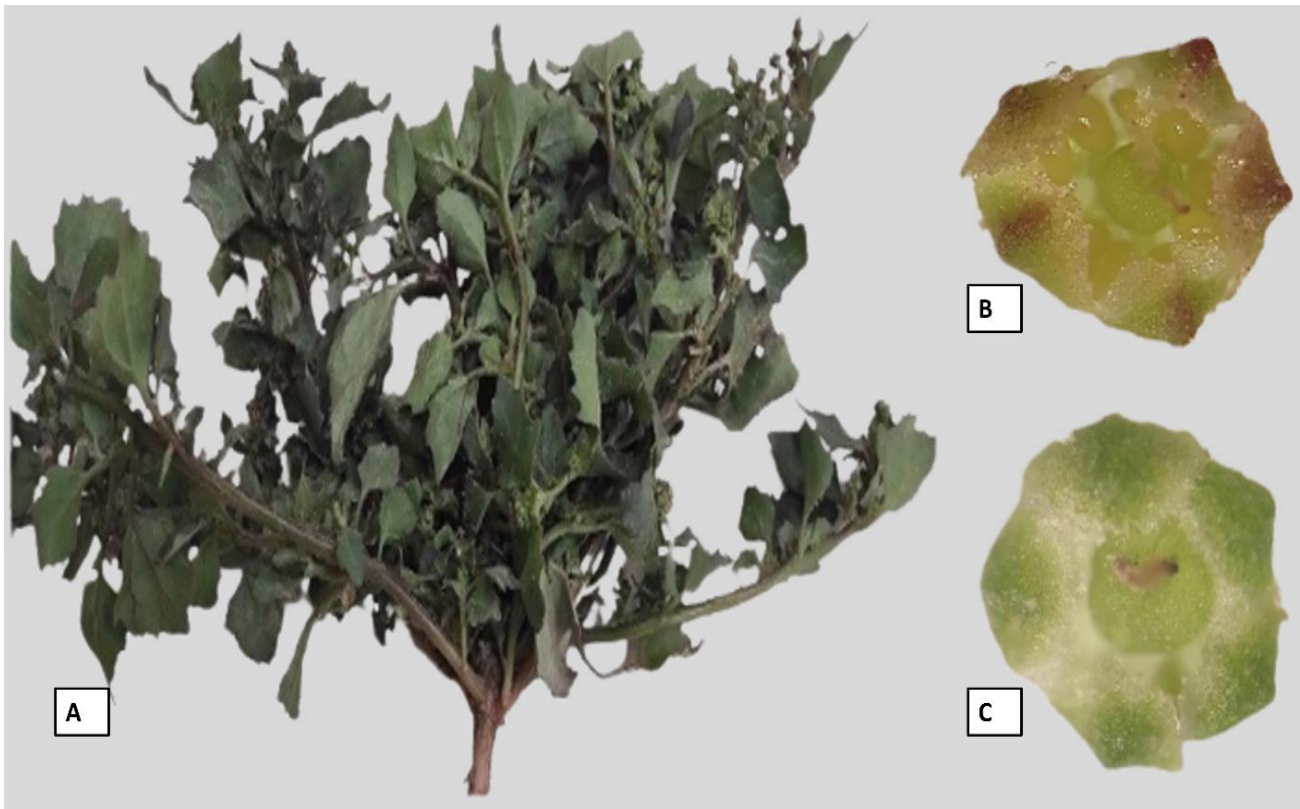


Fig. 3. Morphological features of *C. hybridum* (A x 3; B & C x 10).

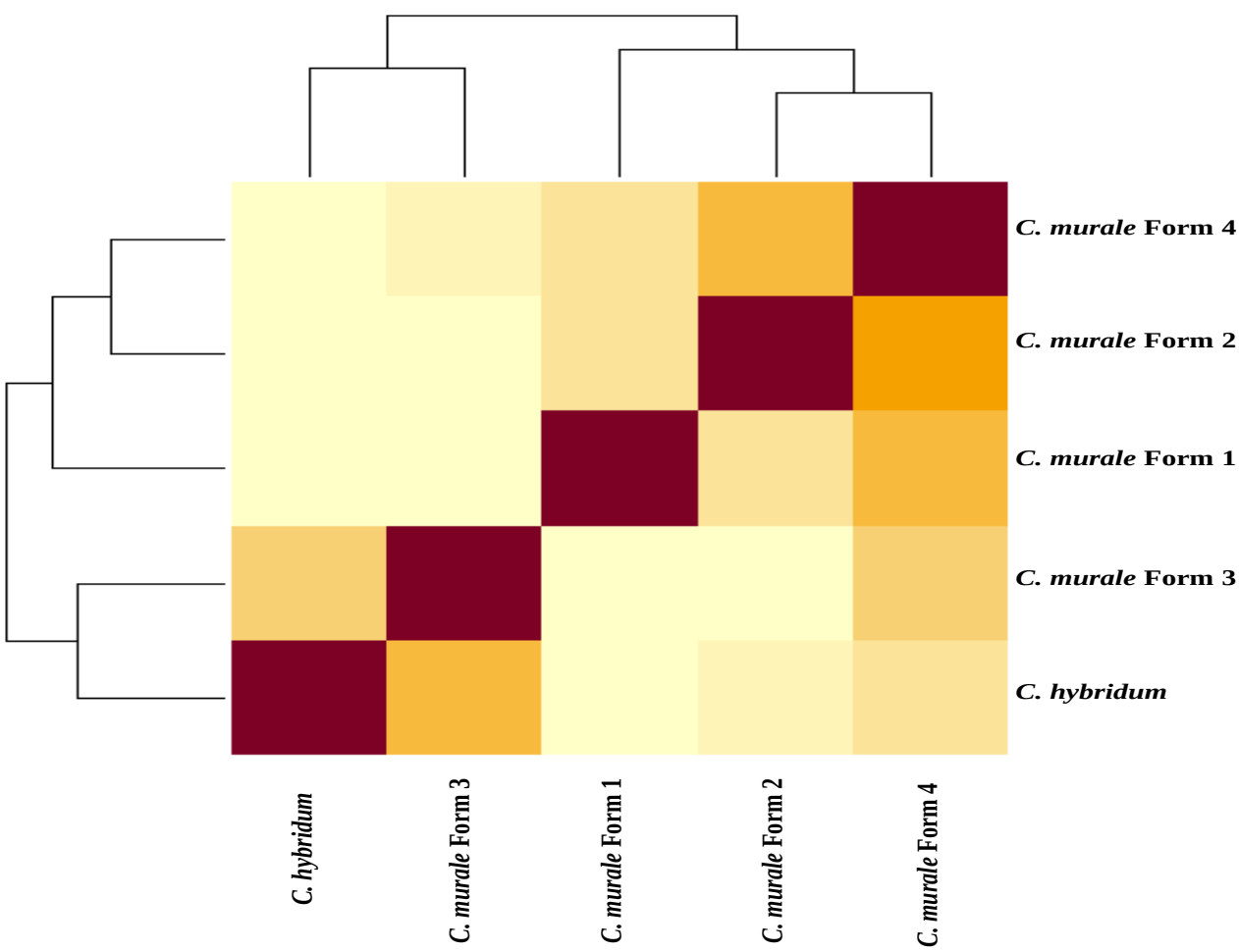


Fig. 4. Heat map for the correlation coefficients between morphological characters of *C. murale* (Forms 1, 2, 3 & 4*)* and *C. hybridum*.

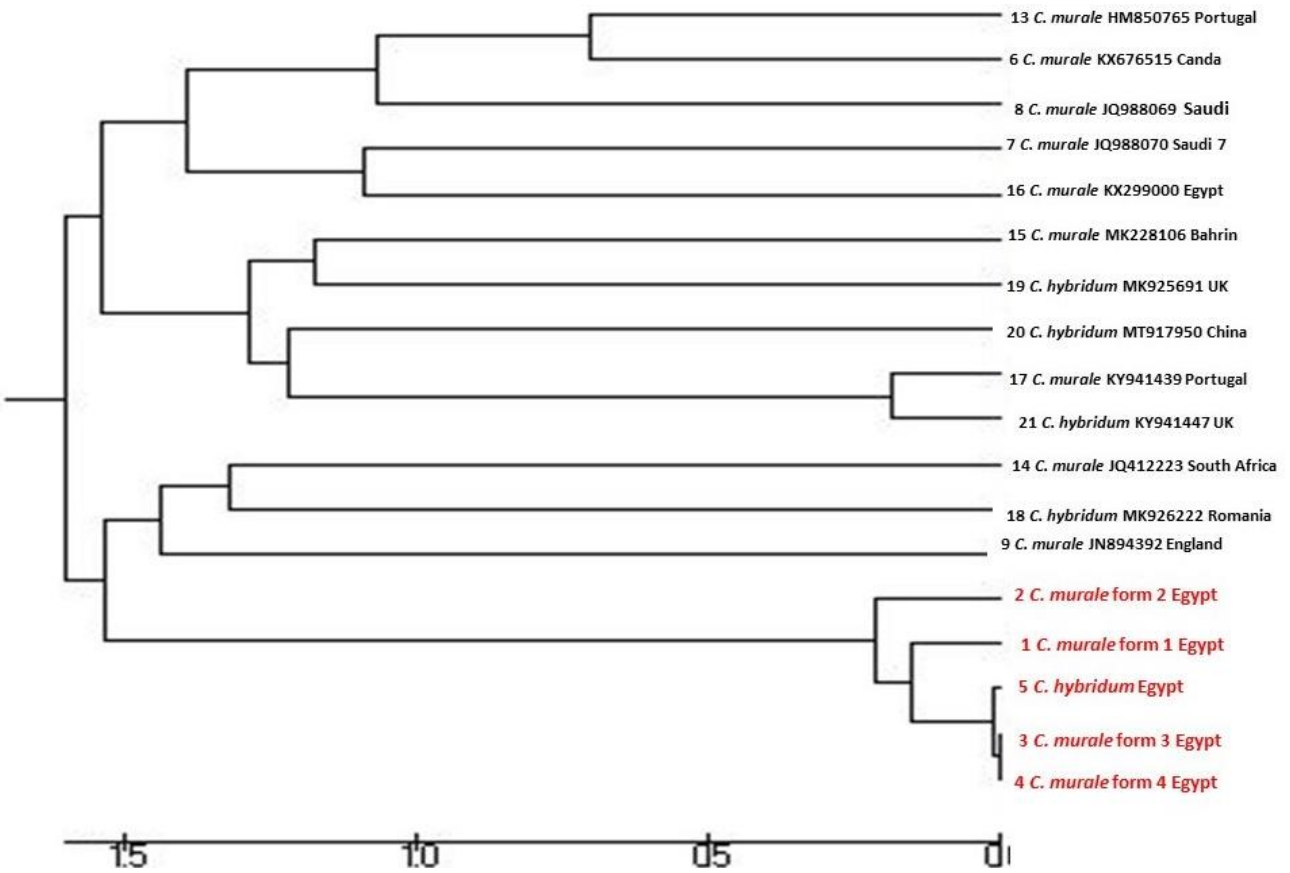


Fig. 5. Phylogenetic dendrogram based on the combination of matK data, constructed by UPGMA based on values of genetic dissimilarity distance between the studied *C. murale* forms and *C. hybridum*.

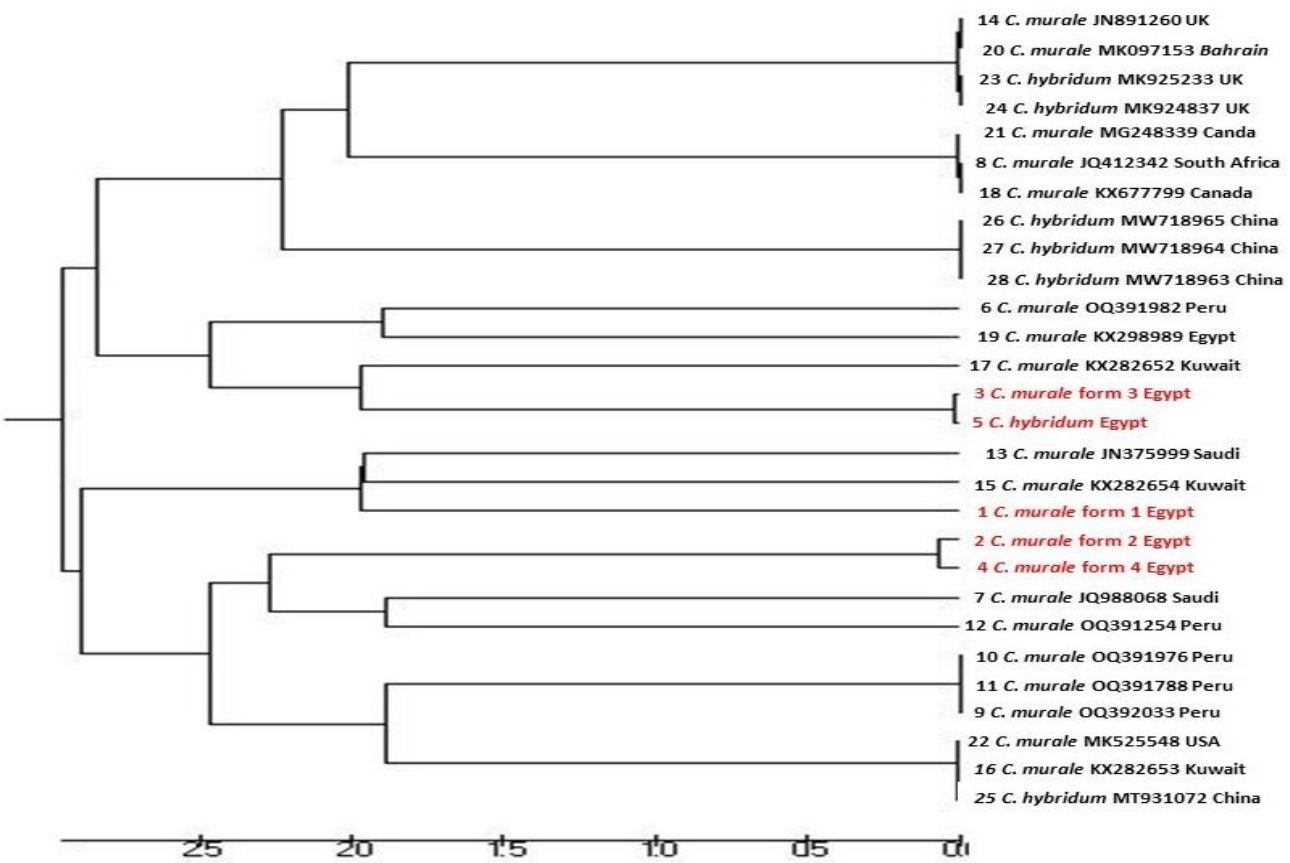


Fig. 6. Phylogenetic dendrogram based on the combination of rbcl data, constructed by UPGMA based on values of genetic dissimilarity distance between the studied *C. murale* forms and *C. hybridum*.

The phylogenetic dendrogram based on rbcl sequences (Fig. 6), showed that *C. murale* and *C. hybridum* all accessions generated a major cluster splitting into two main groups. The first main group is divided into two groups. Generally, the *C. hybridum* showed close affinity with low divergence distance with *C. murale* from different geographic regions (Fig. 6). The first group of them included the specimens of *C. murale* from Egypt (specimen 1), Kuwait, Saudi Arabia with high genetic distance between specimens. The second group is divided into two sub-groups. The first sub-group included the specimens of *C. murale* from Egypt: specimens 2 & 4 with very high similarity (99.9%) and this result agreed and reflected on the morphological features and resemblance of these taxa.

The second main group is also divided into two sub-groups. The first sub-group included specimen no. 3 of *C. murale* and specimen no. 5 of *C. hybridum* from Egypt with very low distance of 0.01 between, reflecting the morphological resemblance, and also included *C. murale* specimens from Kuwait, Egypt and Peru with high distance between them. Overall, the constructed phylogenetic dendrograms (matk and rbcl), revealed that *C. murale* and *C. hybridum* were very closely genetically related species.

**Discussion**

The current taxonomic study of *C. murale* in Egypt revealed the presence of notable morphological inconsistency at the inter-specific level. This phenotypic plasticity is the main cause of taxonomic problems (Kurashige & Agarwal, 2005, Zhou *et al*., 2005). As well as, at the generic level, it is a complex that is little understood (Basset & Crompton, 1982), and makes its taxonomy a source of contention for a long time (Singh, 2010).

The inter-specific divergences in morphology (distinguished as 50 characters) of the studied *C. murale* populations in Egypt enabled us grouping *C. murale* variations to 4 distinct forms (1-4). The main distinctive characters of these forms were: red leaf margin and stem (Form 2); high percentage of hermaphrodite flowers up to 75%, deltoid leaf, and brown seeds (Form 3); up to 25% flowers with 5 staminodes (Form 1); diversity in style and stigmatic arms (Form 4). Relevant morphological features including differences in plant height, leaf shape and size, inflorescence structure, and seed morphology were recorded and distinguished the *C. murale* populations in India (Nandagopal & Sivarajan, 1990) and in Cyperus (Teophanides & Constantinidou, 2012). While, the seed coat micro-sculpturing distinguished the different populations in Pakistan and Kashmir (Hameed *et al*., 2006). The identified *C. murale* Forms (1-4) were also, characterized, by gynomonoecous flowers and the flowers having 4-5 perianth segments. These features were reported earlier in this species (Boulos, 1999, Fuentes-Bazan *et al*., 2012, Anon., 2023).

However, the newly recorded *C. hybridum* was collected from the same habitat with *C. murale* in the western Mediterranean stripe in Egypt. Mosyakin & Robertson (2003), reported that *C. murale* being more common in warmer regions and can tolerate saline soil, while *C. hybridum* being more common in cooler regions and prefers well-drained soils.

Mosyakin & Robertson (2003), reported that the distribution ranges of *C. murale* and *C. hybridum* do not completely overlap. These species seemed to overlap as both species were recorded from in Iran (Amini *et al*., 2021), where *C. hybridum* was able to tolerate a range of soil types, salinity levels, and low rainfall (Yavari *et al*., 2019). Similarly, in Algeria it was recorded in wide range of habitats in both natural and anthropogenic habitats (Boudjelal *et al*., 2020).

However, the newly recorded *C. hybridum* showed close similarity with *C. murale*, in several morphological features among them the gynoecium parts and presences of few flowers with 5-staminodes. It is distinguished from *C. murale* by yellow-purple ribbed stem; leaves rhombic-deltoid leaf, and margin with few teeth to subentire. Fig. (4) and (Table 1), showed the correlation between the *C. hybridum* and *C. murale* Form 3 was 0.340. This close similarity between *C. murale* and *C. hybridum* make it difficult to distinguish between these two species (Mosyakin & Robertson, 2003). Taxonomic problems are attributed to the parallel evolution (Duke & Crawford, 1979), and hybridization (Cole, 1961, Rahiminejad & Gornall, 2004).

Using morphological and molecular markers, the genetic diversity of populations or individuals can be identified. Since morphological characteristics have limitations as they controlled by environmental conditions and the stage of plant development (El-Domyati *et al*., 2011). Contrarily, molecular markers were utilized during this study to explore the interspecific similarity between the distinguished morphological forms of *C. murale* as well as the intra-specific divergence between *C. murale* and *C. hybridum*. The molecular markers have been used in fields like taxonomy, genetics, physiology, and embryology (El-Domyati *et al*., 2011).

**Genetic similarity between *C. murale* forms**

The constructed matK dendrogram (Fig. 5), grouped all the *C. murale* in one sub-group with inter-specific genetic similarity between the four forms was up to 99.97% and it was 98.2% using rbcL (Fig. 6). However, the forms were grouped into two sub-groups. While, the inter-specific genetic similarity was up to 98.2 % with the *C. murale* from the arid- region (Kuwait and Saudi Arabia; Fig. 6). The grouping of Form 3 in separate sub-group using the rbcL (Fig. 6), denoting that genetic divergence of this form from the other forms. The inter-specific relationship among the *C. murale* populations showed 95.2% of genetic similarity in Pakistan using ISSR markers (Ihsanullah *et al*., 2021). An average of 65.5% of the variation within populations in Italy using microsatellite markers attributing these variations to the geographic factors (Maggioni *et al*., 2017). Using ISSR markers in Italy, the genetic diversity within populations ranged from 0.13 to 0.28, and this might be due to the high level of gene flow facilitated by the seed dispersal mechanisms (Fricano *et al*., 2009). Genetic similarity showed an average of 89% in Bulgaria using AFLP markers (Popova *et al*., 2018). In Iran using RAPD markers the genetic diversity within populations ranged from 0.28 to 0.45, this was due to geographical barriers (Ghasemi *et al*., 2013). In Greece

using AFLP markers, the genetic diversity among populations was 0.32, this diversity might be due to the plant's ability to adapt to a wide range of environmental conditions and the frequent disturbances caused by human activities (Tani *et al*., 2013). In Northwest China Huang *et al*., (2018), found high levels of genetic diversity, and low levels of genetic differentiation among populations, related this to the gene flow facilitated by human activities. Huang *et al*., (2018), reported that the genetic diversity of *C. murale* populations was influenced by environmental factors, such as soil type and altitude. Where, the populations growing in sandy soils and at higher elevations had lower levels of genetic diversity compared to populations growing in loamy soils and at lower elevations.

**Similarity between *C. murale* and *C. hybridum***

The intra-specific genetic similarity observed by matK (Fig. 5) was confirmed by rbcL (Fig. 6) where the genetic similarity between *C. murale* and *C. hybridum* was 99.98% and 99.99%; respectively. This molecular result was supported by the observed morphological relationship (Fig. 4). This close genetic similarity was observed using matK in other sub-group, between *C. mural* from Portugal and *C. hybridum* from UK, both from Europe, similarly from different geographic regions, the *C. murale* from Bahrain with *C. hybridum* from UK (Fig. 5). Similar results were also obtained with samples related to different geographic regions using rbcL (Fig. 6). The current detected close genetic relationship between *C. murale* and *C. hybridum* was based on the barcodes (matK & rbcL) is congruent with that achieved in India, using ITS2 and the matK gene (Kaur *et al*., 2019); and in Italy, Maggioni *et al*., (2017), using a set of microsatellite markers. The ability of molecular markers to distinguish *C. hybridum* from *C. murale* may attributed to their different genome sizes and chromosome number, accordingly they may have undergone different evolutionary trajectories (Franzke *et al*., 2020).

The current study revealed the grouping of *C. murale* with *C. hybridum* based on both molecular and morphological data in one sub-group, this data is congruent to that achieved by Manzanilla *et al*., (2018). However, the significant morphological and genetic variation of *Chenopodium* species indicate that this group is a complex (Rahiminejad & Gornall, 2004).

**Conclusion**

The inter-specific relationships between *C. murale* forms were explored and found to be supported with similar studies in other countries. While, the intra-specific relationships between *C. murale* and *C. hybridum* is a complex and requires further investigation to explore and fully understand the relationships between these two species; especially when it found in mixed population as the current status in Egypt.

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