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DNA barcoding and taxonomic identity of the invasive *Bidens pilosa* L. with new records to the Egyptian flora

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Bidens pilosa L. (Asteraceae) is an annual or perennial cosmopolitan noxious *herb.* Populations of this species showed conspicuous pheno-plasticity in Egypt; with taxonomic argument not yet resolved. This study aimed to identify the taxonomic identity of these species to an infra-specific level and deduce the features distinguishing each of the three varieties. The three identified varieties were documented using the "*rbc*L cp DNA marker" region barcoding. This taxonomic revision was mainly based on the morphologic traits, this revision revealed that *B. pilosa L.* is represented in Egyptian flora by three varieties (var. *radiata*, var. *minor* and var. *pilosa*). var. *minor* and var. *pilosa* are new records to the Egyptian flora. The most distinguishing characteristics are the presence of ray florets and their length, it was absent in the var. *pilosa*, while five to eight ray florets/capitulum in both of var. *radiata* and var. *minor*. In addition to the ray length, it is usually longer than 10 mm in var. *radiata* and not exceeding 10 mm in var. *minor.* The three identified varieties were grown in mixed population in all the Egyptian habitats as it well known invasive species. These varieties were documented by DNA-barcoding using the "*rbc*L cp DNA" region and deposited in GenBank for the first time with the accession numbers OR544109; OR544110 and OR544111

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INTRODUCTION

Worldwide Asteraceae, can grow in all habitats, reported that this family includes 13 subfamilies, 1,900 genera and 32,000 species, with many cosmopolitan and invasive species (Haruna et al., 2024). Out of them genus *Bidens* L. comprises about 284 species (Reyes-Ardila et al., 2024). Out of them *Bidens pilosa* L. is an annual or perennial cosmopolitan herb, originates from tropical and central America (Global Invasive Species Database 2018; Amer 2021*)*. Worldwide it is distributed mainly in tropical and subtropical areas extending in some temperate regions (Angelini et al., 2021). It is one of the most noxious weeds in East Africa (Mvere, 2004), and the worst invasion weed in Latin America (Amer, 2021). *Bidens pilosa* has almost 100 vernacular names in different countries, among them black fellows (English), sornet (French), Nadelkurat (German), Bidente (Italian), in Egypt called shoubiet (Arabic) (Amer, 2021; Bartolome et al., 2013; Batanouny, 2017). The species has 78 synonyms (9 homotypic and 69 heterotypic (POWO, 2024).

Bidens pilosa L. is native to tropical and central America (Global Invasive Species Database 2018; Amer 2021*)*, and introduced to the Egyptian land (POWO, 2024; WFO, 2024). *B. pilosa* seems to be introduced to Egyptian land early 1900s, where it was mentioned for the first time by Sickenberger (1901), in Alexandria, Ismailia, Cairo and Giza governorates in Egypt. Later, *Bidens pilosa* L. var. *radiata* was only mentioned by Montasir & Hassib (1956), Täckholm (1956 & 1974). Where, *B. pilosa* L. mentioned without under specific taxa by (Boulos & El-Hadidi 1994; El Hadidi & Fayed (1994/95), Batanouny, 2017; Boulos, 2002 & 2009; Amer, 2021).

Bidens pilosa grows in a wide range of habitats such as fertile, infertile, moist and sandy dry soils (Khamare et al., 2019). In Egypt it is a common weed of cultivations, in newly, old and newly reclaimed lands, disturbed places, pastures, roadsides, streamlines, coastal areas and invasive weed in the field crops (Batanouny, 2017; Amer 2021), in addition to the Nile land, Libyan desert, western Mediterranean costal region, and Sinai (Täckholm 1974; Boulos 2002; Amer 2021).

Recently, this species severly invaded the cultivated land as reported by Amer (2021) among the worst invasive species to Egypt and is also a host and vector to noxious parasites as nematodes of root knot and potato spotted wilt virus; and may alter the soil microbial community (Kato-Noguchi & Kurniadie, 2024). It was reported by Hung & Kao (2015) as a common invasive species in subtropical and tropical territories. It retains horrible reproductive potency where each plant can produce three or four generations/year (Arthur et al., 2012; Mtenga & Ripanda, 2022). Kato-Noguchi & Kurniadie (2024), reported that *B. pilosa* species can produce 2000–6000 seeds/individual plant during its life sequences and able to grow in a wide range of soil and pH (4 – 9) with soil salinity up to 100 mM NaCl. It is an annoying weed to almost 30 crops in more than 40 countries as it may cause about 50% reduction in crop yield (Batanouny, 2017). This invasion of food

crops has serious effects in more than 40 countries (Holm et al., 1991; Mitich, 1994; Khanh et al., 2009; Xuan & Khanh, 2016). It also possesses an allelopathic effect (Zungsontiporn, 2007). Furthermore, the global warming drift may favor its spread to both northern and southern hemispheres (Kato-Noguchi & Kurniadie, 2024).

Bidens pilosa is an erect, annual or perennial, glabrous or hairy herb, with serrate opposite lobed or dissected leaves Rocha, 1996; Zhang et al., 2019). Its flowers are white or yellow and the achene fruit is narrow, long, ribbed and black. Achenes are dimorphic in its positions (central and peripheral). Achenes are also heteromorphic in features as achene size, rugosity and the number of barbs even in the same inflorescence (Imbert, 2002). Its heteromorphic achenes support its invasive capacity (Baskin & Baskin, 2014; Yeo et al., 2022); and help the plant to adapt to new and heterogenous ecosystems (Baskin & Baskin, 2014; Yeo et al., 2022).

Globally, the taxonomic complexity of *B. pilosa* in Central and North America were reported by Huang & Kao (2015) as a species complex that holds three species with different chromosome ploidy, namely B. *odorata*, *B. alba* and *B. pilosa* with 2*n*=24, 48 and 72: respectively. While, at the subspecific level *B. pilosa* was distinguished into eight varieties, namely var. *minor*, alba, radiata, odorata, bisetosa, *bimucronata*, *alausensis* and *calcicola* (Holm et al., 1991; Khanh et al., 2009).

Barcoding has been used as a rapid, accurate, and cost-efficient tool for taxa identification using short DNA as ribulose bisphosphate carboxylase large (*rbcL*) (Hebert & Gregory, 2005; Fouad et al., 2022, 2024; EL-Banhawy et al., 2024). Contradictory to morphological identification, DNA barcoding is not tainted by biases and does not impose taxonomist (Bafeel et al., 2012). Moreover, considering sequence recoverability, quality, and the accuracy of species discrimination, the Consortium for the Barcode of Life (CBOL) Plant Working Group recommended engaging barcoding of land plants using *rbcL* (CBOL Plant Working Group, 2009; Fouad et al., 2022). The *rbcL* barcoding will be used to document the identified *B. pilosa* taxa.

Despite the noxious invasiveness of this species to Egypt, to date of issue, the subspecific taxonomy of *B. pilosa* is not yet convincing. This study was carried out to: (1) Identify the taxonomic identity of this species to a sub-specific level. (2) Deduce the morphometric features distinguishing each variety as well as its geographic distribution in Egyptian land, and (3) Document the DNA barcoding of the identified taxa with the plastidial *rbcL* region.

MATERIALS AND METHODS

Plant materials: The fresh plant material was collected from twenty-one populations of *Bidens pilosa* from three localities (Beba, somosta and El fashn; Beni-suef governorate). These localities visited from 2021 to 2023 to collect the different varieties of this species. Representative fresh flowering and fruiting specimens/population were preserved in FAA (50 ml ethyl alcohol, 5 ml glacial acetic acid, 10 ml formaldehyde, and 35 ml dis. water) for further examinations. Macro-morphological characters of stem, leaves, inflorescences, flowers and achenes were examined and recorded for all specimens/population.

Species identification: The species was identified based on the floristic references (Täckholm (1974); Boulos & El-Hadidi (1994); Boulos, 2002 & 2009). Addition investigation was added including the herbarium specimens deposited in Cairo University Herbarium (CAI). As well as the digital sources namely Kew herbarium (https://www.kew.org); the plants of the world online (POWO, 2024; WFO, 2024). Reference specimens were deposited in both CAI.

DNA barcoding analysis using plastidial rbcL region: Genomic DNA from juvenile leaf samples was extracted and purified for the identification of the three *B. pilosa* varieties using DNeasy plant mini kit (Qiagen). For PCR amplification and sequencing of *rbcL*, the amplification reaction was carried out in 50 µl reaction volume containing 25 µl Master Mix (sigma), 2µl primer of 600 bp Forward (*rbcL*-F: 5'-ATGTCACCACAAACAGAGACTAAAGC-3'), and 2µl primer Reverse (*rbcL-R*: 5'-TCGCATGTACCTGCAGTAGC-3'), 3 µl template DNA (10ng) and 18 µl dH_2O, according to Ibrahim et al. (2019).

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 30 sec., an annealing step at 50ºC for 30 sec. and an elongation step at 72ºC for 1 min. The primer segment was extended to 7 min at 72ºC in the final cycle (Fouad et al., 2022).

a) Detection of the PCR products and rbcL sequencing: The purified amplification products using EZ-10 spin column PCR products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. A 100 bp DNA ladder was used as a molecular size standard. PCR products were visualized on UV light and photographed using a Gel Documentation System (BIO-RAD 2000). The sequencing of the product PCR was carried out using an automatic sequencer (ABI PRISM 3730XL Analyzer) using Big Dye TM Terminator Cycle Sequencing Kits. Single-pass sequencing was performed on each template using *rbcL* Forward primer. The fluorescent-labeled fragments were purified and subjected to electrophoresis in an ABI 3730xl sequencer (Microgen Company). The sequences were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) Sequences were aligned using Align Sequences Nucleotide BLAST.

RESULTS

Taxonomic treatment

***Biden pilosa* L.** Sp. Pl.: 832 (1753)

Homotypic Synonyms: *Bidens leucanthema* var. *pilosa* (L.) Griseb.; *B. pilosa* sub var. *discoidea* Pit.; *B. pilosa* f. *discoidea* Sch.Bip.; *B. pilosa* var. *Discoidea* J.A. Schmidt; *Ceratocephalus pilosus* (L.) Rich.; *Cosmea pilosa* (L.) Spreng. *Kerneria pilosa* (L.) Lowe; *K. pilosa* var. *discoidea* Lowe; and *K. Tetragona* Moench. Annual or perennial erect herbs, 20-140 cm, stem branched. Leaves are opposite, simple-pinnate (pinnatifid-pinnatisect) 3.5-19 x 1.0-12, petiolate, with 1-3 (-7) leaflets, leaflet is 2.0-6.5 (-10.5) x 0.5-2 (-3) cm, lanceolate - ovate, serrate, acute-acuminate, the terminal leaflet is larger with longer petiole than the laterals. Inflorescence is terminal heads, solitary or in lax paniculate raceme; capitulum 6-17 mm radius with 25-70 flowers, ray florets are white and disc florets yellow, each capitulum subtended by 12-16 biseriate phyllaries, the outer 3.5-7 x 0.5-1.5 mm, spathulate, acuminate, connate at base; inner phyllaries 4.5-6 x 2 mm, deltoid, acute, scarious margin. Ray florets pistillate, 0-5 (-8), ligulate, petal 5-10 x 3-5 mm, stamens 5-connate, 3.5-4 mm, ovary 1.5-2.5x0.5. Disc florets hermaphrodite, 20-65 per capitulum, tubular, petal 3.5-4 x 0.5-0.8 mm, ovary 1.0 x 0.5 mm. Fruit achene 4.5-13 x 0.3-1.0 mm, blackish, linear-tetragonal, ribbed, surface papillate with bristles or smooth. Marginal achenes shorter 2-3.5 mm, surface papillate with stiff bristles, awns 2-5, brownish yellow. Central achenes 2.5-4 mm, longer than marginally, with 2-4 awns. Flowering from February – April and fruiting from March–May.

a) Infra specific varieties

The detailed investigation using 34 macro- and micro-morphological traits of the studied populations revealed the identification of three morphologically distinct varieties (Table 1), namely: *B. pilosa* var. *radiat*a (Sch.Bip.) Sherff, *B. pilosa* var. *minor* (Blume) Sherff, *B. pilosa* L. var. *pilosa*. The taxonomic key to distinguishing the identified varieties was developed as follows:

- Ray floret absent, leaves pinnatisect 3-7 leaflets ... **var. *pilosa***
- Ray floret present, leaves pinnatisect 1-5 leaflets ...2
- Ray floret's length more than 10 mm, leaf length exceeds 10 cm … **var. *radiata***
- Ray floret's length less than 8 mm, leaf length doesn't exceed 10 cm … **var. *minor***

B. pilosa* L. var. *pilosa

Plant height 100-140 cm, the stem is heavily branched, 8-22 branches/plant. Leaves pinnatisect, with 3-7 leaflets (Figure 1). Inflorescence in terminal heads, 183-287 heads/plant, head peduncle 4-6 cm, capitulum 6-7 mm radius, ray florets absent (Figure 2), each capitulum subtended by 13-14 biseriate phyllaries. Disc florets 4.0-5.5 mm, marginal and central achenes are 6-9 x 0.5-0.8 mm and 6.5-13 x 0.4-0.8: respectively (Figure 3).

***Bidens pilosa* var. *minor* (Blume) Sherff**

Plant height 20-130 cm, the stem is poorly branched, 2-6 branches/plant. Leaves simple to pinnatisect, with 1-5 leaflets (Figure 1). Inflorescence terminal heads: 73-138 heads/plant, head peduncle 3.5-11 cm, capitulum 9-16 mm radius, with 5-8 ray florets (Figure 2), each capitulum subtended by 12-16 biseriate phyllaries. Disc florets 5.0-8.0 mm. Marginal and central achenes are 6-9 x 0.7-1.1 mm and 7.5-11 x 0.5-0.8: respectively (Figure 3).

***Bidens pilosa* var. *radiata* (Sch.Bip.) Sherff**

Plant height 108-130 cm, the stem is moderately branched, 8-10 branches/ plant. Leaves simple to pinnatisect, 7.5-20 x 4.5-12, petiolate, with 1-5 leaflets (Figure 1). Inflorescence terminal heads, 31-142 heads/plant, head peduncle 5.5-12 cm, capitulum 15-17 mm radius, with 5-8 ray florets (Figure 2), each

Table 1. Morphological characters of the identified B. *pilosa* varieties: (mean value in the middle; upper limit to the right and lower limit to left). C: Central, Inv.: Involucre, and M: Marginal.

Character	***B. pilosa* varieties**		
	var. *pilosa*	var. *minor*	var. *radiata*
Stem			
Plant height / cm	100 – 140	20 –130	108 - 130
Number of branches/plants	8 - 22	2 - 6	6 -10
Leaf			
Number of leaflets/ Leaf	3 - 7	1 -5	1-5
Length / cm	5 - 15	3 - 10	7 - 20
Width / cm	4 - 10	1 - 7	4 - 12
Leaflet length / cm	2 - 6.5	2 - 6.5	2 - 10.5
Leaflet width / cm	1 - 3	1 - 2	0.5 - 3
Number of teeth /cm	4 - 5.5	3.5 - 5.5	3 - 4.5
Number of teeth / leaf side	7 - 13	11 - 18	7 - 16
Inflorescence			
Peduncle length / cm	4 -6	3.5 - 11	5.5 - 12
Number of infl. / plants	183 - 287	73 - 138	31 - 142
Number of infl. / branch	24 - 75	13 - 37	6 - 25
Disc radius with rays/ mm	6 - 7	9 - 16	15 - 17
Disc radius without rays/ mm	6 - 7	6 - 11	6 - 7
Number of outer inv. Bracts / infl.	8 - 9	7 - 11	7 - 10
Number of inner inv. Bracts / infl.	5	5	5
Number of ray florets / infl.	0	5 - 8	5 – 8
Number of disc florets / infl.	55 - 60	20 - 47	50 - 65
Number of chaffy bracts / infl.	45 - 50	17 - 38	40 - 65
Outer inv. bracts length / mm	4 - 4.5	3.5 - 4.5	4 - 7
Inner inv. bracts length / mm	4.5 - 5.5	4.5 - 5	5 - 6
Length of ray ligule / mm	0	4.5 - 6.5	4 – 6
Width of ray ligule / mm	0	3.5 – 4 - 5	3 - 4.5
length of ray floret / mm	0	5 – 7.6	10-12
Length of the disc floret / mm	4 - 6	5 - 8	5 - 7
Achene			
Achene length (without awns) – M / mm	6 - 9	6 - 9	4.5 - 8.5
Achene length (without awns) – C / mm	6.5 - 13	7.5 - 11	6 - 7 – 9.5
Achene width (widest point) – M / mm	0.5 - 0.8	0.7 - 1.1	0.3 - 0.8
Achene width (widest point) – C / mm	0.4 - 0.8	0.5 - 0.8	0.3 - 0.9
Achene width (narrowest point)–M / mm	0.2 - 0.5	0.4 - 0.7	0.2 - 0.5
Achene width (narrowest point)–C / mm	0.2 - 0.5	0.2 - 0.4	0.2 - 0.5
Number of awns – M / mm	3 - 4	2 - 3	3 - 4 - 5
Number of awns – C / mm	3 - 4	2 - 3	2 - 3 - 4
Length of longest awn – M / mm	2 - 3.5	2 - 3	2 - 3
Length of longest awn – C / mm	2.5 - 4	2.5 - 3.5	2.5 - 3.5

C : Central , Inv. : Involucre, and M : Marginal

capitulum is subtended by 12-15 biseriate phyllaries. Disc florets 5.0-6.5 mm. Marginal and central achenes are 4.5-8.5 x 0.3-0.8 mm and 6.0-9.5 x 0.3-0.9; respectively (Figure 3).

DNA barcoding analysis using plastidial *rbc*L region

The retrieved barcoding sequences for the three varieties showed close similarity. Where the similarities were 0.983; 0.947 and 0.94 for the following pairs var. *pilosa* & var. *minor;* var. *radiata* & var. *minor* and var. *pilosa* & var. *radiata;* respectively. The possible taxonomic perceptions of the current recorded *rbcL* sequences for the *B. pilosa* varieties showed tremendous grouping patterns with the deposited sequences in GenBank. Variety *radiata* was grouped in one cluster with sequences from China, USA, Mexico and India with 56% similarity. Where the second cluster grouped both of var. *minor* and var. *pilosa* with no earlier references deposited in the GenBank (Figure 4). The retrieved *rbc*L of the three *B. pilosa* identified varieties var. *pilosa,* var. *minor* and var. *radiata* were documented and deposited in the GenBank for the first time with the accession numbers OR544109; OR544110 and OR544111*;* respectively.

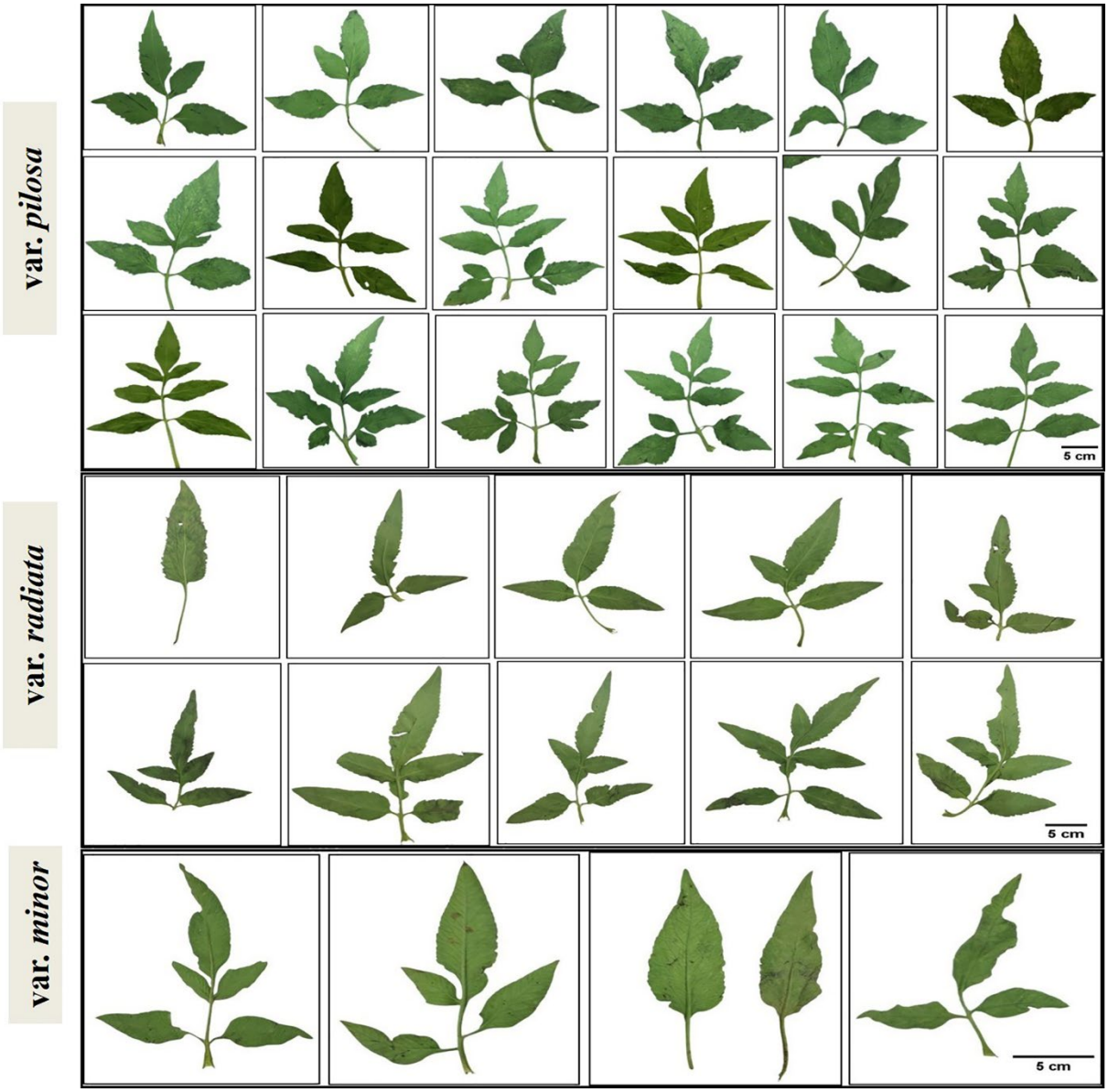

Figure 1. Leaf diversity of the identified *B. pilosa* varieties. var. *pilosa*: Leaves pinnatisect, with 3-7 leaflets; sometimes with pinnatisect lobe/s. var. *radiata*, var. *minor*; Leaves simple to pinnatisect, with 1-5 leaflets.

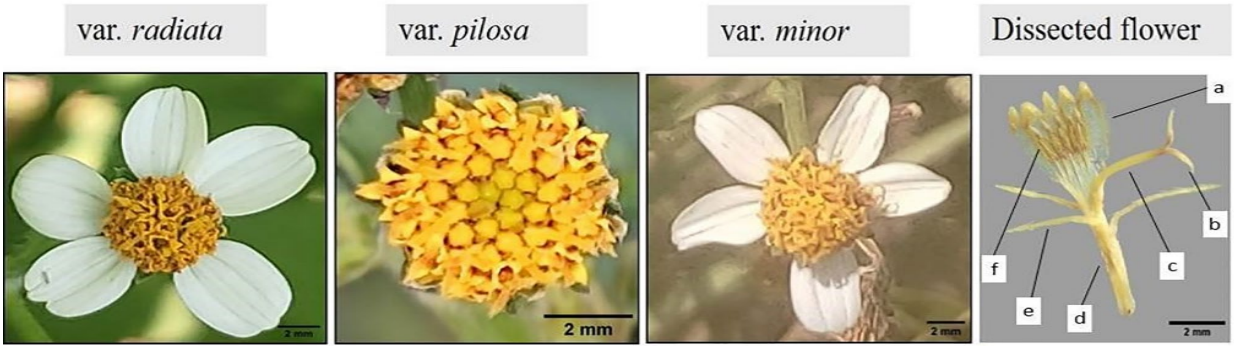

Figure 2. Inflorescence diversity of the identified *B. pilosa* varieties. The dissected flower shows: a: united petals, b: stigma, c: style, d: inferior ovary, e: retrosely barbed awns, f: united stigma.

Figure 3. Achene diversity in the identified *B. pilosa* varieties, showed short thick marginal achenes and long thin compressed central achenes.

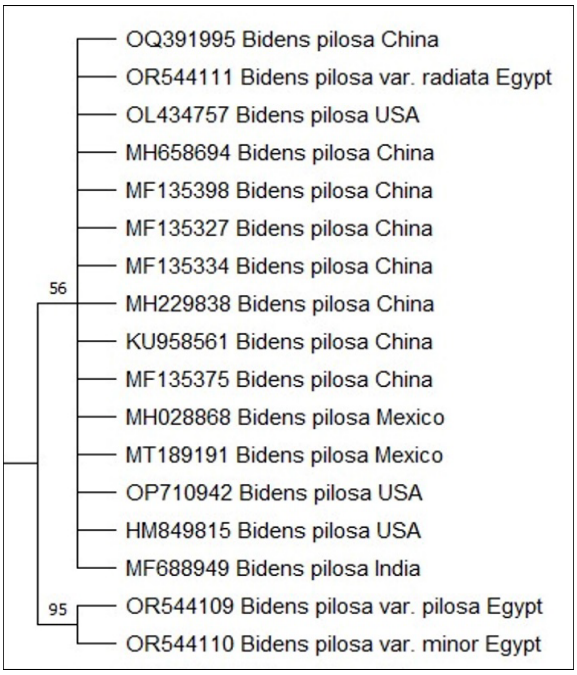

Figure 4. Molecular phylogenetic affinity based on *rbc*L sequence for the identified B. *pilosa* varieties based on rbcL sequence compared to the data available in the ddatabase(http://www.ncbi.nlm.nih.gov).

DISCUSSION

The taxonomy of *B. pilosa* is still under argumentation worldwide. It was considered as a species complex holding three species (Huang & Kao, 2015); or species with eight varieties (Holm et al., 1991; Khanh et al., 2009). The current study resolved this complexity in Egyptian flora, where this species was identified as three varieties var. *minor,* var. *pilosa* and var. *radiata* (Table 1). Remaining that var. *radiata* Sch. Bip. was mentioned earlier by Täckholm (1974) and *B. pilosa* var. *minor* (Bl.) Sherff was mentioned by Boulos (2002 & 2009), as a synonym of this species.

The studied *B. pilosa* populations revealed an identification of three varieties that co-exist in the same population in Egypt, these varieties are var. *pilosa*, var. *minor* and var. *radiata*. These three varieties were reported to co-exist in Taiwan by Hung & Kao (2013). This identification was centered on 35 macro- and micromorphological characters (Table 1). The most important distinguishing characters are the presence of ray florets and their length, it was absent in the var. *pilosa*, and both of var. *radiata* and var. *minor* possess five to eight ray florets /capitulum. In addition to the ray length, it is

usually longer than 10 mm and shorter than 8 mm for var. *radiata* and var. *minor*; respectively (Figure 2). Congruent data was reported earlier by Hung & Kao (2013), who claimed that morphological differentiation between the three varieties depends on their ray florets. The differences are also extending to the species hybridization mode, where var. *radiata* is self-incompatible and the var. *minor* and var. *pilosa* are self-fertile varieties, and no hybridization was enlisted between var. *radiata* and the other two varieties (Huang & Kao, 2015).

Achenes are heteromorphic in features inter- and intra-varieties (Table 1 & Figure 3), as number of barbs, achene width, length and rugosity of the identified varieties. The retrieved data are consistent with Imbert (2002) and Yeo et al. (2022) who reported that the achene heteromorphism are styles of phenotypic plasticity where each genotype gives a morpho- physiological form of achenes; and this phenomenon was particularly conspicuous in weed species of the family Asteraceae. This achene heteromorphy strengthens its invasiveness performance (Yeo et al., 2022); and improves its adaptation to wide range of heterogenous environments (Baskin & Baskin, 2014).

The retrieved *rbc*L sequences for variety *radiata* were aligned in one cluster with sequences from China, USA, Mexico and India (Figure 4); indicating the global spread of this variety, and it was recorded as weed in Egyptian flora since 1974 (Täckholm 1974). Analogously, it was recorded in 1909 in Tiwan, as aggressive weed and one of the major invasive species there (Hung & Kao 2013). Contrary, the identified var. *minor* and var. *pilosa* were not matched with any of the database deposited in GenBank. This may reflect the narrow distribution range of these varieties; then this study documents them for the first time. The *rbc*L is regularly used to document endemic species (Amandita et al., 2019; Fouad et al., 2019; Fouad et al., 2022; Shamso & Fouad, 2019), endangered plants (Rajaram et al., 2019), the species richness (Fouad et al., 2022; Worthy et al., 2019); and to identify new species (Gutteridge & Burns, 2013).

The high molecular similarity between var. *pilosa* and var. *minor* was confirmed by the morphological characters amongst them are the outer and inner involucre bracts lengths, the length of the marginal and central achenes (Table 1 & Figure 3). And confirmed by their similarity in chromosome numbers that both are hexaploid (2*n* = 72) and tetraploid (2*n* = 48) in var. *radiata* (Huang & Kao, 2015).

The current identified varieties showed morphological and *rbc*L diversity that distinguished clearly these varieties. Conversely, these varieties are among others that were classified as synonyms (https://powo.science.kew.org), Ballard (1986) recommended upgrading of var. *radiata* Sherff to be *B. odorata* (2*n* = 24, diploid) or *B. alba* (2*n* = 48, tetraploid). In addition to the Taiwan *B. pilosa* var. *radiata* (2*n* = 48) should be upgraded to be *B. alba; B. pilosa* in Central & North America must be reclassified as three species *B. alba, B. odorata* and *B. pilosa* (Huang & Kao, 2015). Accordingly, the taxonomic identity of *B. pilosa* is still disagreeable and this species complex needs global collaborative effort to judge the taxonomic status of this species.

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