

First record and molecular identification of *Cynomorium songaricum* Rupr (Cynomoriaceae) and its host *Nitraria billardierei* DC. (Nitrariaceae) from Iraq and neighbors

Rafid M. mikhlef¹ Talib O. AL-Khesraji²

¹: Department of biotechnology, college of applied science, university of samarra

²: Department of Biology, College of Education for Pure Sciences, Tikrit University, Iraq

Corresponding author: rafid.magid@uosamarra.edu.iq

Abstract

This study was carried out on parasitic plants in southwest desert of Iraq during March- April 2019. Two plant species, *Cynomorium songaricum* Rupr (Cynomoriaceae) and *Nitraria billardierei* DC. (Nitrariaceae) were collected and identified as new records to Iraq and bordering countries, based on morphological features and molecular analysis. The study also reported *N. billardierei* as a new host to *C. songaricum*. This is the first molecular report on *Cynomorium* from Iraq. Description of the two species is given for the first time from Iraq. Photographs, notes on uses by locals are also provided.

Key words: holoparasite, *Cynomorium*, *Nitraria*, molecular analysis, Iraq

1 Introduction

Cynomorium (tarthuth in Arabic) is a genus of achlorophyllous holoparasitic plants in the family Cynomoriaceae (Saxifragales) [1-3]. This genus contains two species, *C. coccineum* L. of northern Africa and Mediterranean region including Iraq and its neighboring countries [1,4,5,6] and *C. songaricum* Rupr (formerly = *C. coccineum* subspecies *songaricum*) of central and western Asia [1,2,7,8,9]. According to the available floras and checklists, the genus is represented by only one species, *C. coccineum* in Iraq and bordering countries [1,10-18]. *Cynomorium* grows on roots of various desert species like those belong to Amaranthaceae, Nitrariaceae and Zygophyllaceae occurred in dry sandy soil or in salty habitats [1,3,4,19]. Its species have several uses in traditional medicine in China, Arab countries and other parts of the world [1,9,20,21,22]. However, recent studies showed that the extracts from *C. coccineum* (Maltese mushroom in western countries) and *C. songaricum* (Suo Yang in Chinese) exhibited several biological activities including antioxidant, anticancer, antifungal, anti-diabetic, anti-fatigue and anti-hypoxia activities. [6,9,21,23,24]. In this study *C. songaricum* and its host *Nitraria billardierei* were collected and identified as new confirmed records to Floras of Iraq and its neighbors, based on morphological features and molecular analysis. The study also reported *N. billardierei* as a new host to *C. songaricum*.

2 Materials and methods

Host and parasite plant samples were collected from Al-Ukhaydir desert (50 Km southwest) in Karbala Governorate (192 Km southwest Baghdad) as part of southern desert of Iraq during March-April 2019. Morphological characteristics, habit, habitats, collection sites, flowering period of the plants and their Gen Bank accession numbers were reported. Samples were photographed in nature and in laboratory. IKI stain was used for microscopy. Identification of the plant samples was performed according to relevant sources [7,25-31] and confirmed by Macrogen Inc / South Korea. Identified samples were deposited in Biology Department, College of Education for Pure Sciences, Tikrit University, Iraq.

molecular identification**2.1.1 DNA Extraction**

Samples were dried and powdered to extraction DNA by The Wizard® Genomic DNA Purification Kit / USA according to the manufacturer's instructions.

The ribosomal ITS region

The internal transcribed spacer *ITS* (Integrated DNA Technologies company, Canada). Region of rDNA was amplified by PCR by two universal primers ITS1 5'-TCCGTAGGTGAACCTGCGG -3' as forward and ITS4 (5' TCCTCCGCTTATTGATATGC-3') as reverse [32]. Maxime™ PCR PreMix Kit (i-Taq) (USA) was used for PCR reactions following the manufacturer's instructions. PCR amplification optimum conditions were as: 94 °C for 3min initiating denaturation, 35 cycles comprising 94 °C for 45s, 52 °C for 1min and 72 °C for 1min, and a final extension at 72 °C for 7min. Products of amplification were sent for sequencing in (Macrogen Inc/ south Korea).

Bioinformatics analysis

For identification, the nucleotide sequence data in comparison with sequences available at the National Center for Biotechnology Information (NCBI) internet database (GenBank). The ribosomal *ITS* sequence of *Cynomorium* and *Nitraria* were used for the Basic Local Alignment Search Tool (BLAST) algorithm analysis at the NCBI website. Multiple sequence alignments of the samples. Sequences of the species, got from the GenBank (Table 1,2) were performed with molecular evolutionary genetics analysis Mega-X Program version 10.0.5 [33], were align by Clustal W provided in the Phylogeny.fr software [34]. The identification of sequences was determined as a percentage of species sequence.

The molecular identity and accession ID of *C. songaricum* used in this study. **Table 1 :**

No	Species	Country	Identity %	Accession ID
1	<i>Cynomoriumsongaricum</i>	Iraq	---	---
2	<i>Cynomoriumsongaricum</i>	Germany	99 %	ID: HQ222981.1
3	<i>Cynomoriumsongaricum</i>	China	99 %	ID: KX268515.1
4	<i>Cynomoriumsongaricum</i>	China	98 %	ID: MF096217.1
5	<i>Cynomoriumsongaricum</i>	China	98 %	ID: JF915368.1
6	<i>Cynomoriumsongaricum</i>	China	98 %	ID: KC463820.1
7	<i>Cynomoriumsongaricum</i>	China	98 %	ID: MF096214.1

. The molecular identity and accession ID of *N. billardierei* used in this study. : **Table 2**

No	Species	Country	Identity %	Accession ID
1	<i>Nitraria billardierei</i>	Iraq	---	---
2	<i>Nitraria billardierei</i>	China	99 %	ID: KP087775.1
3	<i>Nitraria sibirica</i>	China	98 %	ID: JF977164.1
4	<i>Nitraria roborowskii</i>	China	98 %	ID: JF977162.1
5	<i>Nitraria praevisa</i>	China	98 %	ID: DQ267179.1
6	<i>Nitraria tangutorum</i>	China	98 %	ID: DQ267176.1
7	<i>Nitraria schoberi</i>	China	97 %	ID: KP087771.1
8	<i>Nitraria retusa</i>	China	93 %	ID: KP087773.1
9	<i>Nitrariasphaerocarpa</i>	China	88 %	ID: DQ267177.1

3 Results and discussion

Cynomorium songaricum Rupr :

Identification :Stem subterranean 15-70 cm in length \times 2-4 cm in width, slightly curved, cylindrical nearly equal, aerial part scarlet, reddish brown or pink , subterranean part black with adventitious roots. Leaves scaly triangular up to 1cm tall \times 0.5-1 cm broad. Inflorescence, spadix, 6-8 cm \times 2-5 cm, club shaped to nearly cylindrical, sometimes tapered at apex, reddish brown, densely flowered. Flowers, male, female and bisexual. Male flower 3-6 mm, perianth whitish, purplish at apex, anthers 1-1.5 \times 1 mm, two lobes, open by Longitudinal dehiscence, filaments 2-4 \times 0.5 mm purplish , dorsifixed, suborbicular. Female flower, perianth purplish, one pistil, style 2 mm tall, purplish, stigma flat, ovary inferior, 1.5-2.5 \times ca. 1 mm, subglobose to globose. Bisexual flower rare, stamen and pistil as in male and female flowers. Stem, rich in starch grains and open collateral vascular bundles with irregular arrangement (Fig.1 A-M) . Flowering March-May. Parasitic only on the roots of

N. billardierei in desert area near Al-Ukhaydir fort 50 Km Southwest Karbala, 192 Km Southwest Baghdad. Fresh plant is eaten by locals as food and astringent medicine . *Nitraria billardierei* DC. :

Identification :Woody desert shrub , perennial, up to 1.5 m in length, up to 3.0m in diameter, spiny, young branches hairy, glabrous at age. Leaves 1.5-3.5 \times 0.5-1 cm, sessile, deciduous, succulent , thick , obovate , glabrous , verticillate of 3-6 . Stipules triangular. Flowers , white , bisexual. Sepals , 5 united at base, ovate, up to 2 mm in length, acute. Petals , 5-6 , white, 2-3 mm long. Stamens, 11-15, filaments 2.5-3.5 mm long , above petals and stigma. Ovary white. Fruits, drupe , ovoid , up to 2cm long, sometimes hairy , green becoming yellow , purple or red at maturity (Fig.1N). Flowering , March- May. 50 Km Southwest Karbala near Al-Ukhaydir fort, 192 Km Southwest Baghdad, parasitized by *C. songaricum* .



Fig.1. *C. songaricum* . A,B: in nature , C: excavated , D: C enlarged , note,scales(black arrow)and adventitious roots(white arrow),E: in lab. ,F: inflorescence ,G:scales(sc) in lab. ,H: male flower(m), female flower(f) and bisexual (b)flowers,I: male and female flowers ,note ,anthers(an),dorsifixed filament, style and stigma(st)and ovary (o),j: male flower ,note dorsifixed filament and perianth,k: large number of stamens,l: stem c.s.,note ,starch grains(g) and open collateral vascular bundle(vs) with vascular cambium(c),M: sliced,note, colored external surface and white internal tissues ,N: its host, *N. billardierei*.

Identification of the two studied species (host and parasite) was confirmed on the basis of morphological features and molecular analysis. The primers create DNA segments 650 bp (Figure 2). The rDNA-ITS sequences of samples gave high similarity (99 %) with available sequences alignment from BLAST analysis at NCBI GenBank with identical samples ID: HQ222981.1 and ID: KP087775.1 for *C. songaricum* and *N. billardierei* respectively. According to literature used ITS region because it has high efficiency in diagnosing and separating between species. ITS region was used to separate members of parasitic plants such as *Orobanchaceae*, *Cistanche* and *Cuscuta* [35-38] [Selvaraji et al.,] mentioned that DNA barcode provides rapid identification of species without using phenotype characters [39]. This is the first confirmed record of *C. songaricum* from Iraq and from *N. billardierei*. To the best of our knowledge, *N. billardierei* has not been previously reported as a host to any *Cynomorium* species, so the present study recorded it as a new host to *Cynomorium*. According to available sources the present study reports *N. billardierei* as a new addition to the flora of Iraq and its bordering countries [1,10-18,40]. In Iraq, *Nitraria* is represented by two species, *N. retusa* and *N. schoberi* [1,40]. *C. songaricum* was not previously reported from our region (Iraq and neighboring states) but it has been reported from north African as well as from west and central Asia (as in China) [9,21]. In addition to *N. billardierei* reported here, *C. songaricum* was also found on other Nitrariaceae species such as, its preferred hosts, *N. tangutorum* and

N.sibirica[20,21].*C.songaricum* and its host *N.billardierei* among the well-known medicinal plants that exhibit various bioactivities [21,28,41]. Both *C.songaricum* and *N.billardierei*. contain male, female and bisexual flowers and depend on insect pollination [29-31], thus the two partners may share similar pollination vectors. This is supported by the facts: 1- close association between the two plants (Fig. 1A,B), 2- parasitism with large number of flowers (Fig. 1K), 3- pollination in the host mediated by different insect species [42]. However, information on the pollination biology of Cynomoriaceae is very limited [29]. Furthermore, little information is available on the ecological and physiological aspects of the interactions between *C.songaricum* and its hosts [8,21]. This study represents a new addition to the flora of Iraq as well as to our knowledge on parasitic plants in desert areas of the region and thus further studies in this regard are required. The phylogenetic analysis of Iraqi sample *C.songaricum* with accession number (MT587667.1) depending on the phylogenetic tree (Figure 3), all samples 1,2,3,4,5 and 6 were identified as *C.songaricum* with accession numbers, HQ222981.1, KX268515.1, MF096217.1, JF915368.1, KC463820.1 and MF096214.1 respectively. The phylogenetic tree of *C.songaricum* shows high identical percentage (99%) with Germany sample in GenBank. Whereas phylogenetic analysis of Iraqi sample *N.billardierei* with accession number (MW165482.1) depending on the phylogenetic tree (figure 4) one sample in GenBank was identified as *N.billardierei*. China sample in GenBank with accession number (KP087775.1) reveals high identical (99%) with Iraqi sample. While other species in GenBank identified as different species with different identical percentages.

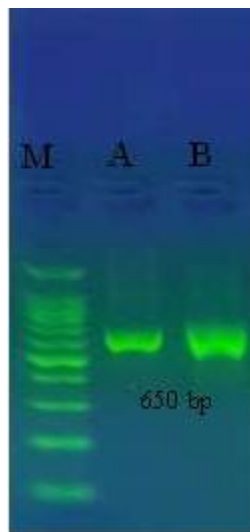


Figure 2 : PCR product the band size 650 bp. The product was electrophoresis on 2% agarose at 5 volt/cm². 1x TBE buffer for 1:30 hours. M: DNA ladder (100) lane .A :*C.songaricum* B: *N.billardierei*.

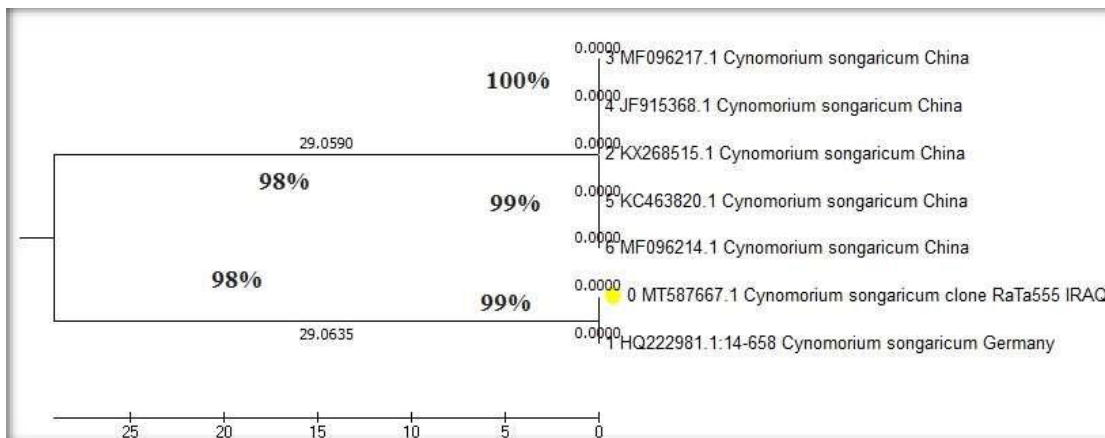


Figure 3 :Phylogenetic tree and relationship of Iraqi *C. songaricum* with six other sequences from GenBank.

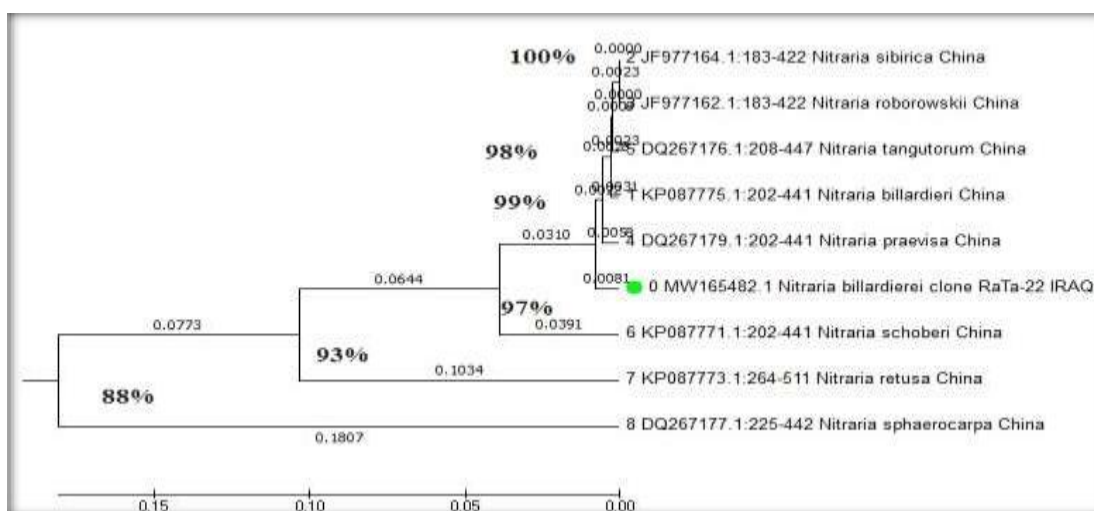


Figure 4 :Phylogenetic tree and relationship of Iraqi *N. billardierei* with eight other sequences from GenBank.

4 Conclusion

In this study *C. songaricum* and *N. billardierei*. are reported as new records for Iraq and bordering countries. The study also reports *N. billardierei*. as a new host to this root parasitic plant . Thus further studies on this new host- parasite association and the distribution of *Cynomorium* species in Iraq and its neighbors are required .

5 References

- [1] Townsed, D.C. and Guest, E.(1980).Cornaceae to Rubicaceae. Flora of Iraq vol.4 part one ministry of agriculture and agrarian reform Iraq.
- [2]Bellot, S., Cusimano, N., Luo, S., Sun, G., Zarre, S., Gröger, A., ... & Renner, S. S. (2016). Assembled plastid and mitochondrial genomes, as well as nuclear genes, place the parasite family Cynomoriaceae in the Saxifragales. *Genome Biology and Evolution*, 8(7), 2214-2230.
- [3] Patočka, J., & Navrátilová, Z. (2020). CYNOMORIUM PLANTS: BIOACTIVE COMPOUNDS AND PHARMACOLOGIC ACTIONS.
- [4] Al-Khesraji, T. O., Wahib, A., & Annon, M. R. (1987). Insect pests attacking parasitic flowering plants in the southern desert of Iraq. *Al-Majalat Al-3ira: qiyyat li-l-3ulu: m Al-zira: 3iyyat*. Zanco (Iraq).

- [5] Govaerts R1999*World Checklist of Seed Plants* 3(1, 2a & 2b): 1-1532. MIM, Deurne.
- [6] Li, X., Sdiri, M., Peng, J., Xie, Y., & Yang, B. B. (2020). Identification and characterization of chemical components in the bioactive fractions of *Cynomorium coccineum* that possess anticancer activity. *International journal of biological sciences*, 16(1), 61.
- [7] Chen J& Funston A2007*Cynomoriaceae*. *Flora of China*, 13, 434.
- [8] Yang, Y., Yi, X., Peng, M., & Zhou, Y. (2012). Stable carbon and nitrogen isotope signatures of root-holoparasitic *Cynomorium songaricum* and its hosts at the Tibetan plateau and the surrounding Gobi desert in China. *Isotopes in Environmental and Health Studies*, 48(4), 483-493.
- [9] Al-Abbasi, S. H. A., Al-Majmaei, A. A. M., Al-Naqib, A. T. H., Hameed, A. M., AL-Samarraie, M. Q., & H Altaef, A. (2021). Isolation and identification of some fungi from rhizospheric soils of some wild plants at Samarra University, Iraq. *Caspian Journal of Environmental Sciences*, 19(5), 829-839.
- [10] Migahid AM, El Shiekh AM. 1977. Types of desert habitat and their vegetation in central and Eastern Arabia. *Proc. Saudi Biol. Soc.*, 1: 5-13.
- [11] Migahid A1978*Flora of Saudi Arabia* 2nd Ed Riyadh.
- [12] Daoud H 1985*Dicotyledons*, pp.Flora of Kuwait Vol. I 190–194. (Kuwait: KPI in association with Kuwait University).
- [13] Al-Abbasi, S. H. A., H Altaef, A., Q AL-Samarraie, M., Al-Naqib, A. T. H., & Al-Majmaei, A. A. M. (2022). Collection and diagnosis of some wild plants in the vicinity of Samarra University, Iraq. *Caspian Journal of Environmental Sciences*, 20(2), 437-440.
- [14] Musselman L2011*Checklist of the plants of Lebanon and Syria*. <http://ww2.odu.edu/~lmusselm/plant/lebsyria/Checklist%20of%20Lebanon%20Plants.pdf>.
- [15] Yusuf, M., Al-Oqail, M. M., Al-Sheddr, E. S., Al-Rehaily, A. J., & Rahman, M. A. (2014). Diversity of medicinal plants in the flora of Saudi Arabia 3: An inventory of 15 plant families and their conservation management. *International Journal of Environment*, 3(3), 312-320.
- [16] Sürmen, B., Kutbay, H. G., & Yilmaz, H. (2015). Parasitic angiosperm plants of Turkey. *Journal of the Institute of Science & Technology*, 5, 17-24..
- [17] Uludag, A., Aksoy, N., Yazlık, A., Arslan, Z. F., Yazmış, E., Uremis, I., ... & Brundu, G. (2017). Alien flora of Turkey: checklist, taxonomic composition and ecological attributes. *NeoBiota*, 35, 61
- [18] TaifourH El-Oqlah A& Ghazanfar S2017*The plants of Jordan: an annotated checklist*(Kew Publishing).
- [19] AL-Khesraji T 1994 *Journal of college education for Girls / Baghdad University* 5 (2) 37.
- [20] Dharmananda S 2011 *Cynomorium* Parasitic plant widely used in traditional medicine [<http://www.itmonline.org/arts/cynomorium.htm>].
- [21] Cui, J. L., Vijayakumar, V., & Zhang, G. (2018). Partitioning of fungal endophyte assemblages in root-parasitic plant *Cynomorium songaricum* and its host *Nitrariotangutorum*. *Frontiers in microbiology*, 9, 666.
- [22] Leonti, M., Bellot, S., Zucca, P., & Rescigno, A. (2020). Astringent drugs for bleedings and diarrhoea: The history of *Cynomorium coccineum* (Maltese Mushroom). *Journal of ethnopharmacology*, 249, 112368.

- [23] Cui, Z., Guo, Z., Miao, J., Wang, Z., Li, Q., Chai, X., & Li, M. (2013). The genus *Cynomorium* in China: an ethnopharmacological and phytochemical review. *Journal of Ethnopharmacology*, 147(1), 1-15
- [24] Hao-Cong, M. E. N. G., Shuo, W. A. N. G., Ying, L. I., Kuang, Y. Y., & Chao-Mei, M. A. (2013). Chemical constituents and pharmacologic actions of *Cynomorium* plants. *Chinese Journal of Natural Medicines*, 11(4), 321-329.
- [25] Watson L&Dallwitz M1992*The grass genera of the world* CAB international.
- [26] Wilson, K. L.2007 "Nitrariabillardierei DC". Royal Botanic Gardens & Domain Trust, Sydney Australia. Retrieved 14 November.
- [27] Jeanes J 1999*Zygophyllaceae*. In: Walsh, N.G.; Entwisle, T.J. (eds), *Flora of Victoria* Vol. 4, Cornaceae to Asteraceae. Inkata Press, Melbourne.
- [28] Du, Q., Xin, H., & Peng, C. (2015). Pharmacology and phytochemistry of the *Nitraria* genus. *Molecular Medicine Reports*, 11(1), 11-20
- [29] Wang, D., Yu, H. and Chen, G. (2020), Scent chemistry and pollinators in the holoparasitic plant *Cynomorium songaricum* (Cynomoriaceae). *Plant Biol J*. doi:10.1111/plb.13180
- [30] Messina A2020*Nitrariaceae in Phillip G. Kodela* (ed.), *Flora of Australia*. Australian Biological Resources Study, Department of Agriculture, Water and the Environment.
- [31] Barker, R. M (2020). *Nitrariabillardierei*, in (ed.), *Flora of Australia*. Australian Biological Resources Study, Department of Agriculture, Water and the Environment: Canberra.
- [32] White T Bruns T Lee STaylor J 1990*Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) *PCR Protocols: a Guide to Methods and Applications*, pp. 315-322(Academic Press, New York).
- [33] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, 28(10), 2731-2739
- [34] Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., ... & Claverie, J. M. (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic acids research*, 36(suppl_2), W465-W469.
- [35] Sun, Z., Song, J., Yao, H., & Han, J. (2012). Molecular identification of *Cistanche herba* and its adulterants based on nrITS2 sequence. *Journal of Medicinal Plants Research*, 6(6), 1041-1045
- [36] Wu C-S, Wang T-J, Wu C-W, Wang Y-N, Chaw S-M.(2017) Plastome evolution in the sole hemiparasitic genus laurel dodder (*Cassytha*) and insights into the plastid phylogenomics of Lauraceae. *Genome Biol Evol.*; 9(10): 2604±2614.
- [37] Fu, W. Liu, X. Zhang, N. Song, Z. Zhang,W. Yang, J. Wang,Y.(2017). Testing the Hypothesis of Multiple Origins of Holoparasitism in Orobanchaceae: Phylogenetic Evidence from the Last Two Unplaced Holoparasitic Genera, *Gleadovia* and *Phacellanthus*. *Journal frontiers in plant science* . Volume 8 | Article 1380.p: 1-9.
- [38].Al-Gburi, B. Al-sahafF,H. Al-Fadhil F,A. Del Monte J,P.(2018).Morphological and molecular diagnosis of *Cuscuta* spp. Parasitizing solanaceae plants in the middle of Iraqi provences. *Journal of Research in Ecology* (2018) 6(2): 2415-2433.
- [39] Selvaraj, D., Park, J. I., Chung, M. Y., Cho, Y. G., Ramalingam, S., & Nou, I. S. (2013). Utility of DNA barcoding for plant biodiversity conservation. *Plant Breeding and Biotechnology*, 1(4), 320-332.

- [40] AL Rawi A1964 *Wild plants of Iraq with their distribution* (Government Press , Third print Baghdad Iraq).
- [41] Wei, F., He, Q., Wang, W., Pei, D., & Zhang, B. (2019). Toxicity assessment of Chinese herbal medicine *Cynomorium songaricum* Rupr. *Evidence-Based Complementary and Alternative Medicine*, 2019.
- [42] Dixon K2020 *Coastal Plants A Guide to the Identification and Restoration of Plants of the Greater Perth Coast* (Csiro Publishing , Melbourne ,Australia .345pp).