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MORPHOLOGICAL AND MOLECULAR DIAGNOSIS OF *RUPPIA MARITIMA* L. IN THE BASRAH GOVERNORATE SOUTHERN OF IRAQ.

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Abstract :

The plants of the family Ruppiaceae are monocotyledons, containing only one genus, which has many species in the world., In Iraq, especially in Basrah Governorate, this study was aimed at morphological and molecular diagnosis to confirm this species, which is the only one registered at the local level in the country and in Basrah in particular. Plant samples of *Ruppia maritima* were isolated from one of the salt marshes(swamps) in a province in the area of five miles and were carried out for phenotypic and then molecular diagnosis for confirmation. The whole plant approximately more than 30 cm (40-65) cm in length and less than 1 mm (0.9 mm) in stem diameter .The plant is monocious and the flowers are bisexual and sessile as groups in the base of leaves ,the length of peduncles more than 5 cm reach to 22-23 cm flexuous ,but not spirally coiled and bearing between 5-11 fruits obliquely ovoid (2-2.5)mm in length. The leaves are distributed alternately across the stem . The PCR amplification of ten samples revealed that most of the samples have a positive band on gel electrophoresis. The partial chloroplast *atpB*-gene was amplified in all positive samples and produced the expected PCR size of 601bp for all samples. The present phylogenetic and genetic tree analysis confirmed that samples in the current study are closest to *R. maritima.* In accordance with the phenotypic classification, this is the first study from a molecular point of view in Iraq to establish this plant species.

Keywords: Morphological , Molecular diagnosis. *Ruppia maritima*, *atp*B gene , PCR

Introduction :

Linnaeus (1753: 127) introduced the genus *Ruppia* to accommodate a single species, *R.maritima.* (1). The cosmopolitan genus *Ruppia L.* (Ruppiaceae) inhabits a wide variety of shallow systems (Coastal lagoons, continental brackish habitats or saltmarsh ponds), where it forms dense and often monospecific meadows that play a key role in the functioning of the ecosystem (-11).

On the basis of morphological features, the genus *Ruppia* has been traditionally included either within the monogeneric family of Ruppiaceae (12-14) or within the family of Potamogetonaceae (15-17). Molecular analysis has highlighted a close phylogenetic relationship of Ruppiaceae with seagrass families such as Cymodoceaceae and Posidoniaceae (14-18-19). At present, several

species are recognized worldwide: *R. cirrhosa, R. maritima, R. megacarpa Mason, R. tuberosa* Davis & Tomlinson and *R. polycarpa* Mason (20 a,b ; 18). The two species *R. maritima* and *R. cirrhosa* are the most widely distributed in the world, whereas the other three are regarded as endemic to Oceans, with reference to European habitats, some authors reported only one species, *R. maritima* (21-23), whereas others recognized two species *R. cirrhosa* and *R.maritima* (24 -26). In the Mediterranean region, three species have been found: *R. maritima, R. cirrhosa and R. drepanensis* Symoens (27-33). In Basrah city, only one species was reported, *R. maritima* (34). In the last study, phenotypic diagnosis was used only, and no molecular previous study was conducted in Iraq to confirm it. Therefore, the current study focused on achieving that goal of diagnosing the genus and species by morphological and molecular methods of this aquatic plant It is located in salt ponds in Basrah Governorate, in southern Iraq.

Material and Methods :

Sample collection :

Ruppia sp. samples were collected by hand from swamps in the 5-mile area in Basrah city southern in Figure (1), on December 29, 2020, they were placed in clean plastic bags. Then samples were brought to the laboratory in the Department of Biology, College of Education for Pure Sciences, University of Basrah. They were placed in large glass Petri dishes. The samples were washed several times with tap water to get rid of the impurities and mud stuck to them, then they were washed with distilled water It is located in salt ponds in Basra Governorate, southern Iraq several times to ensure that they were clean well. (35).

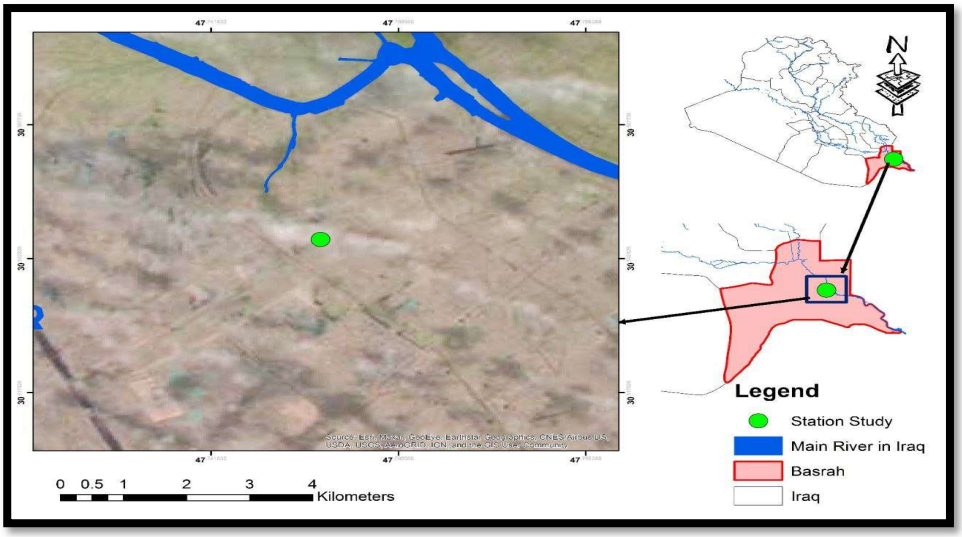

Fig.(1) : Showing the study area in the province of Basrah

Ecological factors

Several environmental factors were measured directly and other factors were measured laboratory, air temperature, water temperature, express the result in degrees Celezia (ºC) and pH was measured and recorded in the study stations directly using a device Multi-meter model 340i,

as for the other factors phosphorous, nitrite and nitrate was measured according to (36,37), express the result in degrees µg/L.

Morphological examination and Identification of *Ruppia* sp.

Aquatic plant samples were exanimated after cleaning from mud and other attached organisms by using a dissecting microscope type Zeiss under different magnification powers and a digital camera type (Sony). The identification of this species was dependent on the flora of Iraq (38,39).

Purification, drying and preservation of *Ruppia* sp.

The method of (40) was used to purify aquatic plants, which involved washing the plants with tap water several times to get rid of the mud, algae, and other materials attached to the aquatic plants, then washing with distilled water afterwards. Ultrasonicator Telesonic type for three minutes. To get rid of small algae sticking to the plant, as well as bacteria and fungi, if any, these samples were washed with distilled water 12 times (35) and then placed on Wattman N0.1 filter paper to dry at laboratory temperature. Biomass was dried using a (Topt 10 D freez dryer), then crushed and kept in clean and airtight plastic bottles in a refrigerator at -18°C.

Molecular study :

Extraction of DNA :

The DNA was extracted from *Ruppia* sp. using the DNA extraction Genomic DNA Mini kit® according to manufacturer protocol. PCR amplification was applied to each DNA extract using the primer according to (41) and tables 1, 2, and 3.

Table (1) The primers used for amplifications and sequencing of the *atp*-B genes in the present study, (41).

Designations	Sequence (5' to 3')
CH-atpB-F5	CAGGTGCTCGCATGAGAGTTGG
CH-atpB-R7	CCAGTAAAGACTTCTGCTACAA

PCR . polymerase chain reaction

The materials for the polymerase chain reaction (PCR) were prepared as in Table 3 .

Table (2): Components and volume of the polymerase chain reaction (PCR)

Component	Volume
GoTaq® Green Master Mix, X 2	12.5µl
upstream primer, 10µM	2 µl
downstream primer, 10µM	2 µl
DNA template	5µl
Nuclease-Free Water	3.5µl
Total volume	25µ

PCR procedure :

1- Take 12.5µl of GoTaq® (Master Mix, 2X) and placed in a PCR tube.
2- Add to it 2µl of primer upstream, 10 µM and 2µl of downstream primer, 10µM
3- Then 5µl of DNA template was added.
4- Add 3.5 µl of Water Free-Nuclease to get the required volume which is 25µl, The reactant was mixed with the Vortex for 30 seconds, and it was ready to be applied in the PCR machine.

Table (3): Shows the steps involved for the P.C.R. program.

Stage	**Temperature**	**Time (min)**
Initial denaturation	95	5
No. of cycles = 35 cycle		
Denaturation	95	45
Anneling	61	1
Extention	72	2
Final extension	72	5

Results

Morphological study:

Ecological factors

Table (4) Showed some values of environmental factors in the salt marshes (Swamps) of *R.martima* during the sampling process, in December 2020.

Table (4): Some environmental factors that were recorded in the salt marshes(Swamps) of *R.martima*.

Environmental factors	Values
Air temperature	14◦C
Water temperature	19◦C
Phosphorous	0.013 µg/L
Nitrite	0.247 µg/L

Nitrate	1.652 µg/L
pH	7.11

Identification of *Ruppia*:

This genus was classified according to the following taxa: Kingdom :Plantae ; Clade :Monocots; Clade :Angrosperms; Clade: Tracheophytes; Order : Alismataley; Family : Ruppiaceae; Genus : *Ruppia* sp; species: *maritima* L., which is characterized by some morphological characteristics represented by its presence in an environment consisting of small ponds in Basrah governorate in winter months, especially in December 2020 .It was morphologically classified according to (42) as bearing the following features: The length of the plant approximately 30cm (40-65) cm, with an internode 5-7cm in length and 0.9 cm in diameter. The main branch is usually branched dichotomously. These aquatic plants are monoicous. The fruits are born on special branchless 22–23 cm in length with clusters of fruits between 6–11 (2.25-2.5) mm in length and (0.5–0.75)mm in diameter. While flowers are found as sessile clusters between branches (leaf branches) with 5-10 mm in diameter Figure 2 (A-D).

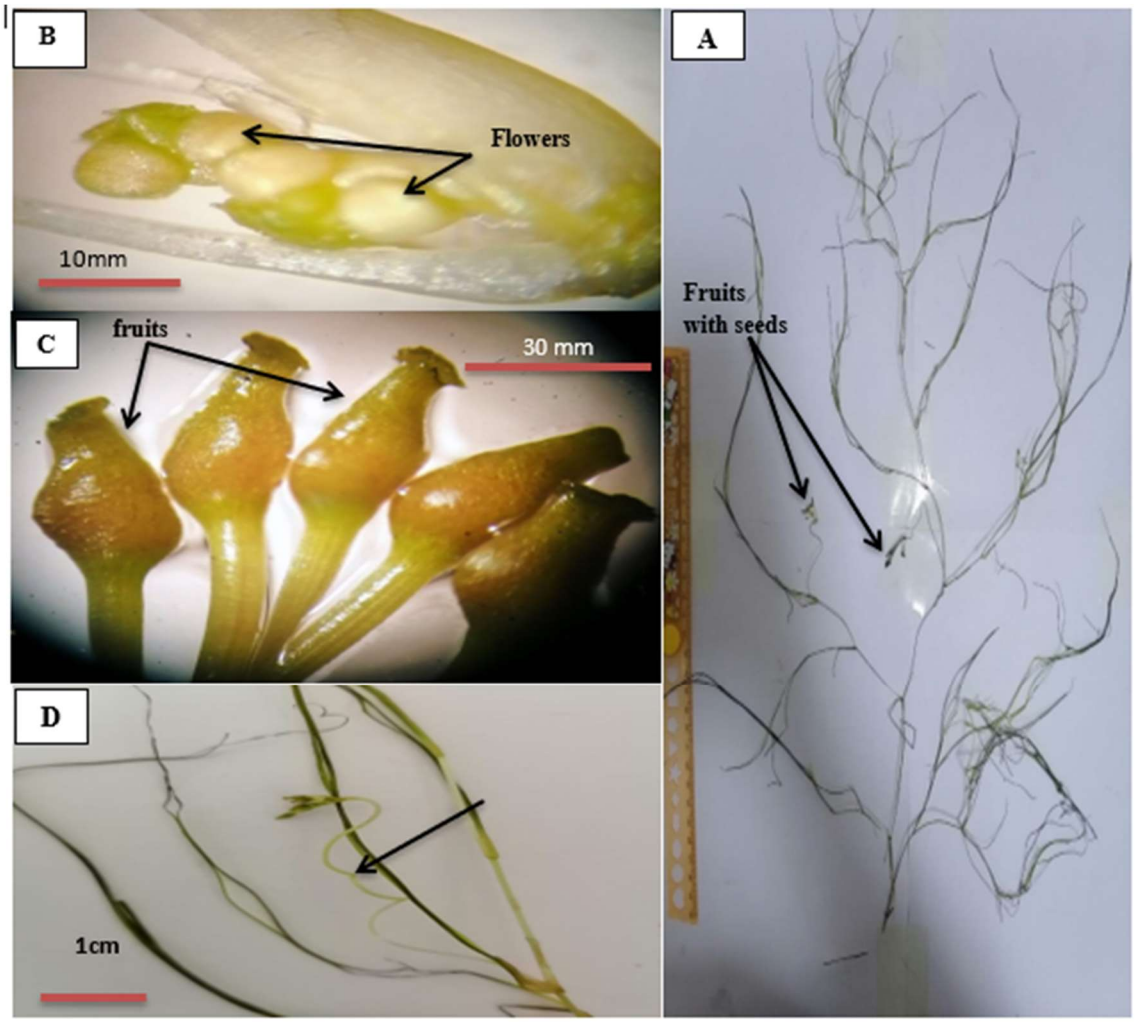

Figure 2: Morphological characteristic of Ruppia sp. A: Whole plants B: Clusters of flowers C: fruits. D: Branchless bearing fruits(Arrow) .

Molecular study

Ten sample of current study were used for PCR amplification by using the partial *atp*B gene. The PCR amplification of sample isolates with different molecular sizes showed that all isolates It has a positive range on gel electrophoresis. The partial *atp*B gene was amplified in all positive and samples produced the expected size of the PCR of 601bp (Fig. 3).

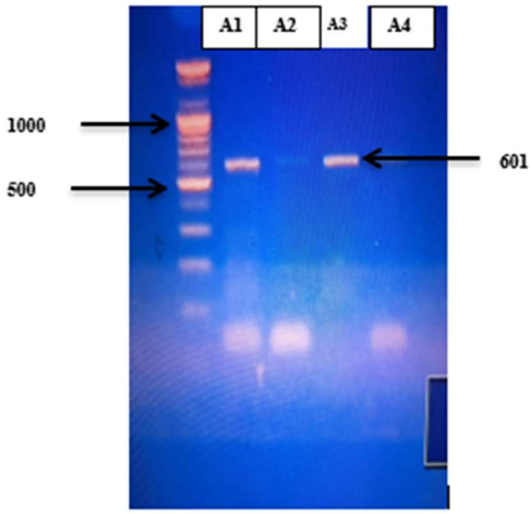

Figure (3) PCR products of partial *atp*B gene of *Ruppia maritima* appears in current study , presented bands at 601 bp on 1% agarose gel electrophoresis. M: 100 bp DNA Ladder. Lanes are Positive to the (A1 and A3) while Lanes are Negative to the (A2 and A4) as replicate.

Sequencing analysis and Phylogenetic tree :

Sanger sequencing was performed to identify *R. maritima* by using forward and reverse primers for two samples. The present findings proved that all samples belong to *R. maritime* according to the accession no. (HQ901526.1) comparison with the nucleotide sequences obtained in our findings with the same sequence identity between *R. maritima* isolate from Basra city and the sample that is recorded in GenBank. The results of the genetic tree were shown (Fig. 4) using the *atp*B gene of the genus *Ruppia* as it showed that all isolates of the genus *Ruppia* when they are identified with Gen Bank, the presence of a strain of type *R. maritima* located in Basrah Governorate, and that this strain previously Gen Bank deposited sequence with accession number HQ901526.1 from recorded in Canada percentage of 99.13% and 98.28 respectively for samples (A1 & A3), as shown in Figure (5), which explains the sequence alignment of the current study with the sequence of samples of similar studies.

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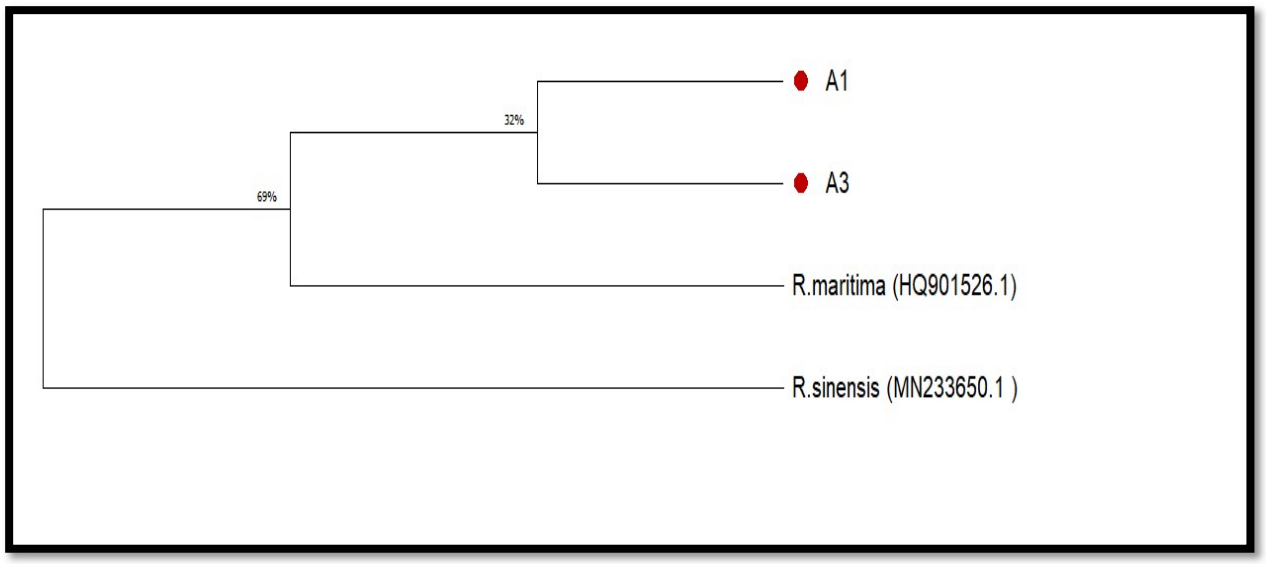

Figure (4) : Phylogenetic tree of selected *Ruppia maritima* by the program MEGA11 using maximum likelihood-based. The present study isolates represent as Sample A1 and Sample A3 .

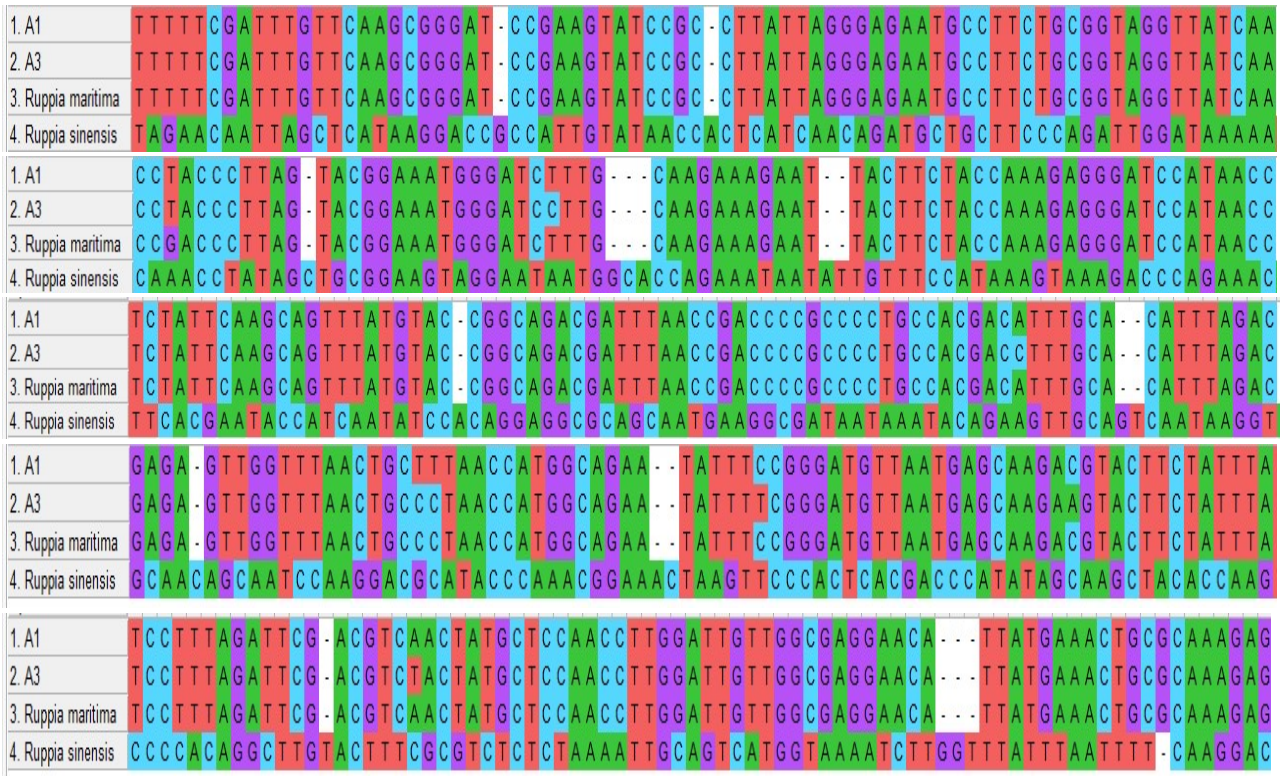

Figure (5) : Sequence alignment of the current study samples with the sequence of samples of similar studies.

Discussion :

The reason for choosing these plants in the current study is the lack of studies on them, especially in Basrah, southern Iraq, as we mentioned earlier, and their widespread growth and reproduction in the environment, which gives a large live mass without the need to culture them in the laboratory. This study is considered the first molecular to identify and confirm the aquatic plant *R. maritima* in Iraq.

The growth cycle of *R. maritima* is also significantly affected by environmental conditions (43). In the present study, some environmental factors surrounding *R. maritima* were measured, and it was shown through the results that this genus prefers to live in brackish water. This result agrees with the findings of (44), who mentioned the genus *Ruppia*, a cosmopolitan aquatic plant complex, is generally restricted to shallow waters such as coastal lagoons and brackish habitats characterized by fine sediments . The water temperature was 19◦C. This result agrees with the findings (2 , 5 , 6 , 45, and 46). In particular, *Ruppia* plants tolerate a wide range of water temperatures (mainly between 5 and 30 ◦C) and their optimum range is narrower (0.3–15 psu) . In the present study, phosphorus was 0.013 µg/L and pH was 7.11. This result agrees with (47) showed *Ruppia maritima* for pH recorded (7.7-9.4). The study of (48) mentioned that the high production of *Ruppia* can lead to carbon limitation (pH > 9) during the summer productivity peaks.

(49) also found R.maritima in waters with higher than average nitrate levels (0.9-6.8 mg/L) in interior Canada. This result is in agreement with the current study, where it was recorded for nitrate (1.652 µg/L), (50) mentioned Culture experiments show that *R. maritima* leaves and roots take up ammonia and phosphate, but that root-to-shoot translocation predominates.

The resultant nitrification around the root zone probably is not an important source of nitrogen. However, as the roots seem best adapted to take up ammonia rather than nitrates or nitrites (51). This agrees with the present study, which recorded a significant decrease in nitrite (0.247 µg/L) .

The chloroplast-encoded *atp*-B gene is one of the photosynthetic genes and has been used in the phylogeny of certain plants and algae (52-58). The two studies (57) and (58) showed that the divergences of *atp*B genes among the fern genera and the colonial Volvocales, respectively, are similar to those of the *rbc*L genes. Therefore, combined data from *atp*B and *rbc*L gene sequences seems to be efficient for resolving detailed phylogenetic relationships .

In the current study, the partial *atp*B gene was used However, PCR amplification of ten samples showed that most of the samples had a positive range on gel electrophoresis. The partial *atp*-B gene was present in all positive samples and yielded a predicted PCR size of 601 base pairs for all samples. This result agrees with (59) that the genomic DNA was extracted from *R. maritima* and optimized for PCR reactions. Thereafter, PCR amplification successfully produced 606 bp.

The present phylogenetic analysis was successfully performed where the samples were inserted into the genetic tree and compared with the accession number registered at NCBI The genetic tree

analysis revealed the alignment of samples in two main groups, sample A1 Closely related with A3, These samples were the closest to the second major group, *R. maritima*, compared to *R. sinensis*, which moved away from them in the third major group. In the end, the current study showed the confirmation of the diagnosis of the *R. martima* in Iraq, specifically in Basra, by molecular methods, which confirmed its phenotypic diagnosis.

Conclusion :

The result concludes this is the first study to isolate, characterize and classified the aquatic plants *R.maritima* in Iraq (Morphology and molecular study).In addition, the current study is the first record in Iraq of two strains of *R.maritima*

References :

1. Linnaeus, C. 1753. Species plantarum, vol. 1. Holmiae [Stockholm]: impensis Laurentii Salvii. https://doi.org/10.5962/bhl.title.669
2. Verhoeven, J.T.A., 1979. The ecology of *Ruppia*-dominated communities in western Europe. I. Distribution of *Ruppia* representatives in relation to their autecology. Aquat. Bot. 6, 197–268.
3. Calado, G., Duarte, P., 2000. Modelling growth of R. cirrhosa. Aquat. Bot. 68, 29–44. Capiomont, A., Sandmeier, M., Caye, G., Meinesz, A., 1996. Enzyme polymorphism in Posidonia oceanica, a seagrass endemic to the Mediterranean. Aquat. Bot. 54, 265–277.
4. Teixeira da Silva, E., Asmus, M.L., 2001. A dynamic simulation model of the widgeon grass *Ruppia maritima* and its epiphytes in the estuary of the Patos Lagoon, RS, Brazil. Ecol. Model. 137, 161–179.
5. Menéndez, M., Hernández, O., Comín, F.A., 2002. Spatial distribution and ecophysiological characteristics of macrophytes in a Mediterranean coastal lagoon. Estuarine Coastal Shelf Sci. 55, 403–413.
6. Malea, P., Kevrekidis, T., Mogias, A., 2004. Annual versus perennial growth cycle in *Ruppia maritima* L.: temporal variation in population characteristics in Mediterranean lagoons (Monolimni and Drana lagoons, Northern Aegean sea). Bot. Mar. 47, 357–366.
7. Den Hartog, C., Kuo, J., 2006. Taxonomy and biogeography of seagrasses. In: Larkum, A.W.D., Orth, R.J., Duarte, C. (Eds.), Seagrasses: Biology, Ecology and Conservation. Springer, Dordrecht, pp. 1–23.
8. Mannino, A.M., Sarà, G., 2006. The effect of *Ruppia cirrhosa* features on macroalgae and suspended matter in a Mediterranean shallow system. Mar. Ecol. 27, 350–360.
9. Pergent, G., Pasqualini, V., Pergent-Martini, C., Ferrat, L., Fernandez, C., 2006. Variability of *Ruppia cirrhosa* in two coastal lagoons with differing anthropogenic stresses. Bot. Mar. 49, 103–110.
10. Rodrigues, R.S., Savant, S.B., Jagtap, T.G., 2009. A note on *Ruppia* species (Ruppiaceae) from abandoned saltpan in the Gulf of Kutch, India. Ind. J. Mar. Sci. 38, 457–460.
11. Obrador, B., Pretus, J.L., 2012. Budgets of organic and inorganic carbon in a Mediterranean coastal lagoon dominated by submerged vegetation. Hydrobiologia 699, 35–54

12. Cronquist, A., 1981. An Integrated System of Classification of Flowering Plants. Columbia University Press, New York.
13. Tomlinson, P.B., 1982. Potamogetonaceae. In: Metcalfe, C.R. (Ed.), Anatomy of the Monocotyledons. VII. Helobiae (Alismatidae). Clarendon Press, Oxford, pp. 270–335.
14. Les, D.H., Cleland, M.A., Waycott, M., 1997. Phylogenetic studies in Alismatidae, II: evolution of marine angiosperms (seagrasses) and hydrophily. Syst. Bot. 22, 443–463.
15. Gamerro, J.C., 1968. Observaciones sobre la biologia floral y morfologia de la potamogetonacea *Ruppia cirrhosa* (Petag.) Grande (=R. spiralis L. ex Dum.). Darwiniana 14, 575–607.
16. Jacobs, S.W.L., Brock, M.A., 1982. A revision of the genus *Ruppia* (Potamogetonaceae) in Australia. Aquat. Bot. 14, 325–337.
17. Dahlgren, R.M.T., Clifford, H.T., Yeo, P.F., 1985. The Families of the Monocotyledons. Springer-Verlag, Berlin.
18. Zhao, L.C., Wu, Z.Y., 2008. A review on the taxonomy and evolution of *Ruppia*. J. Syst. Evol. 46, 467–478.
19. Triest, L., Sierens, T., 2014. Seagrass radiation after messinian salinity crisis reflected by strong genetic structuring and out-of-Africa scenario (Ruppiaceae). PLoS ONE 9 (8), e104264, http://dx.doi.org/10.1371/journal.pone.0104264.
20 a. Brock, M.A., 1982a. Biology of the salinity torelant genus *Ruppia* L. in saline lakes in south Australia. I. Morphological variation within and between species and ecophysiology. Aquat. Bot. 13, 219–248.
20 b. Brock, M.A., 1982b. Biology of the salinity torelant genus *Ruppia* L. in saline lakes in south Australia. II. Population ecology and reproductive biology. Aquat. Bot. 13, 249–268.
21. Ascherson, P., Graebner, P., 1907. In: Engler, A. (Ed.), Potamogetonaceae. Das Pflanzenreich Heft 31, Leipzig, pp. 1–184.
22. Tallon, G., 1957. Ruppiacées de Camargue. Terre Vie 11, 103–116. Teixeira da Silva, E., Asmus, M.L., 2001. A dynamic simulation model of the widgeon grass Ruppia maritima and its epiphytes in the estuary of the Patos Lagoon, RS, Brazil. Ecol. Model. 137, 161–179.
23. Van Ooststroom, S.J., Reichgelt, Th.J., 1964. Ruppiaceae. Flora Neerl. 1, 80–83.
24. Reese, G., 1962. Zur intragenerischen taxonomie der gattung *Ruppia* L. ein cytosystematischer beitrag. Z. Bot. 50, 237–264.
25. Den Hartog, C., 1971. De Nederlandse Ruppia-soorten. Gorteria 5, 148–153.
26. Sfriso, A., 2010. Chlorophyta multicellulari e fanerogame acquatiche. Ambienti di transizione italiani e litorali adiacenti. I Quaderni ARPAEmilia-Romagna. Odoya srl, Bologna, pp. 320.
27. Van Vierssen, W., Van Wijk, R.J., Van Der Zee, J.R., 1981. Some additional notes on the cytotaxonomy of *Ruppia* taxa in western Europe. Aquat. Bot. 11, 297–301. Verhoeven, J.T.A., 1975. Ruppia communities in the Camargue, France. Distribution and structure, in relation to salinity and salinity fluctuations. Aquat. Bot. 1, 217–242.
28. Cirujano, S., 1986. El género *Ruppia* L. (Potamogetonaceae) en la Mancha (Espana). ˜ Bolet. da Soc. Brot. Série 2 59, 293–303.

29. Aedo, C., Fernández Casado, M.A., 1988. The taxonomic position of *Ruppia* populations along the Cantabrian coast. Aquat. Bot. 32, 187–192.
30. Cirujano, S., García-Murillo, P., 1990. Ruppiaceae. Fontqueria 28, 159–165.
31. Triest, L., 1991. Enzyme polymorphism and its relationships to biological features in aquatic plants (including a comparison with terrestrial plants). In: Triest, L. (Ed.), Isozymes in water plants. Op. Bot. Belg. 4, 201–240.
32. Comín, F.A., Rodó, X., Menéndez, M., 1993. Spatial hetereogeneity of macrophytes in Lake Gallocanta (Aragón, NE Spain). Hydrobiologia 267, 169–178.
33. Talavera, S., García-Murillo, P., Herrera, J., 1993. Chromosome numbers and a new model for karyotype evolution in Ruppia L. (Ruppiaceae). Aquat. Bot. 45, 1–13.
34. Al-Mayah, A. Alwan and Al-Asadi W. M. 2018. The impact of increase Salinity on the Aquatic Plants assemblage in Shatt Al-Arab river, Iraq. . Univ. Basrah, Marsh bulletin 13(2) September 74–86.
35. Weideman , V.E.; Walne, P.R. and Tainor, F.R. (1984). A new technique for obtaining axenic culture of algae. Can. J. Bot.,42: 58-59.
36. Wood, E. D. ; Armstrong, F. A. J. and Richards, F. A. (1967). Determination of nitrate in sea water by cadmium-copper reduction to nitrite. J. Mar. Biol. Ass. U.K., 47:23-31p.
37. APHA (2012). Standard method 2130: turbidity. Standard method for the examination of water and wastewater, 22 nd edition . Washington, DC: American Public Health Association, American water works association and water environment.
38. Guest,E.(1985).Flora of Iraq . Vol.1.Ministry of Agriculture, Iraq .
39. Al- Saadi, S. A. A. (2009). Taxonomical and Ecological study of the wetland Plants of Southern Iraq. Ph. D. thesis , Sci. Coll. Basrah Univ, 69 pp.
40. Giusti, E., Marsili-Libelli, S., 2005. Modelling the interactions between nutrients and the submersed vegetation in the Orbetello lagoon. Ecol. Model. 184, 141–161
41. Sakayama H., Nozaki H., Kasaki H. & Hara Y. 2004. Taxonomic re-examination of six species of *Nitella* (Charales, Charophyceae) from Asia, and phylogenetic relationships within the genus based on rbcL and *atp*B gene sequences. Phycologia 43: 91- 1 04.
42. Dandy, J. E. 1985: Ruppiaceae in C. C. Townsend & E. Guest, Flora of Iraq, vol. 8: 27- 29.- Baghdad.
43. Cho, H.J., Poirrier, M.A., 2005. Seasonal growth and reproduction of *Ruppia maritima* L. s.l. in Lake Pontchartrain, Louisiana, USA. Aquat. Bot. 81, 37–49.
44. Mannino, A.M., Menéndez, M., Obrador, B., Sfriso, A. & Triest, L. 2015. The genus *Ruppia* L. (Ruppiaceae) in the Mediterranean region: An overview. Aquatic Bot. 124: 1–9. https://doi.org/10.1016/j.aquabot.2015.02.005.
45. Obrador, B., Pretus, J.L., 2010. Spatiotemporal dynamics of submerged macrophytes in a Mediterranean coastal lagoon. Estuarine Coastal Shelf Sci. 87, 145–155
46. Mannino, A.M., Graziano, M., 2014. Differences in the growth cycle of *Ruppia cirrhosa* (Petagna) Grande in a Mediterranean shallow system. Plant Biosyst., http://dx.doi.org/10.1080/11263504.2014.906511.

47. Pip, E. 1984. Ecogeographical tolerance range variation in aquatic macrophytes. Hydrobiologia 108:37-48.
48. Obrador, B., 2009. Environmental Shaping and Carbon Cycling in a Macrophyte-dominated Coastal Lagoon. University of Barcelona (Ph.D. Dissertation).
49. Pip, E. 1979. Survey of the ecology of submerged aquatic macrophytes in central Canada. Aquat. Bot. 7:339-357.
50. Thursby, G. B. 1984 . Nutritional requirements of the submerged angiosperm *Ruppia maritima* in algae-free culture. Mar. Ecol. Prog. Ser. 16:45-50.
51. Thursby, G. B. 1983. Culture-nutrient relations in the aquatic angiosperms Zostera marina L. and *Ruppia maritima* L. Ph.D. dissertation, University of Rhode Island, Kingston. 144 pp.
52. LOCKHART P.J., BEANLAND TJ., HOWE c.J. & LARKUM A.W.D. 1992. Sequence of Prochloron didemni *atp*B E and the inference of chloroplast origins. Proceedings of the National Academy of Sciences 89: 2742-2746.
53. HOOT S.B. 1995. Phylogeny of the Ranunculaceae based on preliminary *atp*B, *rbc*L and 1 8S nuclear sequence data. Plant Systematics and Evolution 9, supplement: 24 1 -25 1.
54. HOOT S.B. & CRANE P.R. 1995. Inter familial relationships in the Ranunculaceae based on molecular systemetics. PLant Systematics and Evolution 9, supplement: 1 19-13 I .
55. HOOT S.B., CULHAM A. & CRANE P.R. 1995a. Phylogenetic relationships of the Lardizabalaceae and Sargentodoxaceae: chloroplast and nuclear DNA sequence evidence. Plant Systematics and Evolution 9, supplement: 195- 199.
56. HOOT S.B., CULHAM A. & CRANE P.R. 1995b. The utility of *atp*B gene sequences in resolving phylogenetic relationships: comparison with *rbc*L and 18S ribosomal DNA sequences in the Lardizabalaceae. AnnaLs of the Missouri Botanical Garden 82: 1 94-207.
57. WOLF PG. 1997. Evaluation of *atp*B nucleotide sequences for phylogenetic studies of ferns and other pteridophytes. American JournaL of Botany 84: 1 429- 1440.
58. NOZAKI H., OHTA N., TAKANO H. & WATANABE M.M. 1999. Reexamination of phylogenetic relationships within the colonial Volvocales (Chlorophyta): an analysis of *atp*B and *rbc*L gene sequences. Journal of Phycology 35: 104- 11.
59. Lucas, C., T. Thangaradjou and J. Papenbrock, (2012). Development of a DNA barcoding system for seagrasses: Successful but not simple. Plos ONE, 7, e29987.