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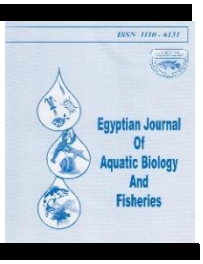

Morphological Traits and Molecular Phylogeny of the Aquatic Bird, *Gallinula chloropus*, Using Mitochondrial *16S* Ribosomal RNA Gene Sequences

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ABSTRACT

Recently, an increasing awareness of the avian environment and the conservation of nature and its species has emerged. The accurate identification of a species is very essential to conserve it and determine suitable management plans. Therefore, the present study aimed to identify the common moorhen, *Gallinula chloropus*, in Egypt as an aquatic bird and determine its phylogeny using mitochondrial *16S rRNA* sequences that have become common in birds' research and can be used to provide both answers to specific research questions and guidance for selecting experimental designs. The sequencing of *16S rRNA* in the understudied species produced a nucleotide length of 556bp and is deposited in GenBank under an accession number (OM943961.1). Representative sequences of *G. chloropus* are inserted within the taxa of *Fulica*, forming a well-supported sister relationship with sequences of *Fulica atra*. Limited morphological distinction and close phylogeny between *G. chloropus* and *F. atra* compared to other taxa of *Fulica* suggest that *F. atra* may be joined to *Gallinula* more than being a member of *Fulica*, as well as warranting a deep taxonomic revision to determine the validity of the separation of *Gallinula* from *Fulica*.

INTRODUCTION

The Rallidae **(Vigors, 1825)** is a large family that constitutes about 85% of the Gruiformes **(Bonaparte, 1854)** and includes most species (around 152), dispersed among 33 to 40 genera **(Garcia-R *et al.*, 2014, 2020)**. The Rallidae has a wide geographic range and a complicated taxonomic structure**(Furo *et al.*, 2021)**. It is found in all insular and continental environments, with the exception of the polar regions, deserts lacking water and mountains above the snow line **(Garcia-R *et al.*, 2020)**.

Phylogenetic relationships in the members of the Rallidae family still show some substantial variations because of the small numbers of species that have been examined viavarious methods. Interest in rail diversity, biogeography and evolution has led to conducting studies on their relationships utilizing morphological and PCR-based Sanger methods **(Slikas *et al.*, 2002; Sangster *et al.*, 2015)**. **Garcia-R *et al.* (2014)** published the

most thorough molecular phylogeny of the rails using about 70% of the recognized extant and the recent extinct rail species diversity.

In their study, **Ruan *et al.* (2018)** identified the common moorhen, *Gallinula chloropus*, (Gruiformes: Rallidae) in China. It is a distinctive aquatic bird that is blackish with a red and yellow beak and long, green legs. In Egypt, *G. chloropus* inhabits fresh and brackish marshes, ponds, lakes and the Nile River. The current study aimed to test the accuracy of the determination of the understudied species, *G.chloropus*, as well as defining its phylogenetic relationships regarding the Rallidae family.

MATERIALS AND METHODS

Morphological investigation

A total of 5 specimens of the common moorhen *Gallinula chloropus* (Gruiformes: Rallidae) were collected from Abu-Rawash (Giza, Egypt) in January 2019, placed in an adequate collection box and transported to the laboratory according to the recommendations of the Assiut University Research Ethics Committee (ethical number: 0620230097). Photomicrographs and measurements were obtained and compared to the standard ones to identify the exact species. Classification and authorities for birds follow the World Bird Database **(Avibase, 2023)**.

DNA extraction and PCR conditions

The genomic DNA was extracted from the preserved sample using the QIAamp DNA mini kit (Qiagen, Hidden, Germany) by following the manufacturer's guidelines. A partial mitochondrial *16S rDNA* gene was amplified using primers 16sar (CGCCTGTTTAACAAAAACAT) and *16sbr* (CCGGTCTGAACTCAGATCACGT) **(Simon *et al.*, 1991)**. PCR analyses were carried out in 50µL reactions containing 25µL PCR master mix; 1µL of each forward and reverse primer, and 1µL of genomic DNA. The PCR cycling conditions were performed with an initial denaturation at 94ºC for 240sec. This step was followed by 30 cycles including denaturation, annealing and an extension for 60sec at 94ºC; for 60sec at 48ºC, and for 60sec at 72°C, respectively. Subsequently, a final extension was conducted at 72ºC for 10min. 1.5% agarose gel containing ethidium bromide was used to separate the amplified products. For the measurement of the amplified PCR fragments, 100bp DNA Ladder RTU (Ready-to-Use), GeneDireX was used.

PCR sequencing

All DNA sequencing were achieved by Macrogen (Seoul, South Korea). Sequences were subjected to the National Center for Biotechnology Information (GenBank/NCBI) under the accession number of OM943961.1.

One alignment was conducted with the available sequences of taxa within the Rallidae and selected out group taxa for both available and adequate on GenBank.

Resultant alignments were detected by eye using the default parameters of the MUSCLE algorithm **(Edgar, 2004),** as implemented in 7.0 18 **(Kumar *et al.*, 2016)**. Regions of ambiguous alignment were removed and manually trimmed to a point where the majority of sequences had started and finished. The number and percentage of variable sites among sequences of alignment of the partial sequences of *16S rDNA* were calculated based on computing pair-wise distances conducted using the numbers of differences model with the inclusion of (Transitions + transversions) substitutions within the MEGAX v.10.2.6 software package **(Kumar *et al.*, 2018)**.

Phylogenetic analysis

Three phylogenetic analyses were applied to the alignment of the *16S rRNA* data set: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). Maximum parsimony analysis was carried out using MEGAX v.10.2.6 software package **(Kumar *et al.* 2018)**; a heuristic tree searching strategy was applied with a random addition sequence on 1,000 bootstrap replicate data sets, and 50% consensus trees were calculated from the two retained trees. Bayesian inference analysis was performed with Mr. Bayes v.3.2.6 **(Ronquist *et al.* 2012)**. The best nucleotide substitution model was chosen using Mr Modeltest v.2.3 **(Nylander, 2004)**, and the Akaike information criterion (AIC) indicated that SYM+G was the best estimator for this set of data, for BI settings. Nodal support BI was calculated by sampling four simultaneous Markov Chain Monte Carlo (MCMC) chains every 1,000 generations for 1,000,000 generations; the first 3,000 samples were discarded as burn-in, and the average standard deviation of split frequencies was <0.01. BI tree was visualized using Fig. Tree v.1.4.4 **(Rambaut, 2018)**. MEGAX v.10.2.6 software package **(Kumar *et al.*, 2018)** was used to perform the evolutionary analysis by ML of tree construction, with 1000 bootstrap iterations (**Felsenstein, 1985**). The best nucleotide substitution model was chosen using the default parameters implemented in MEGAX v.10.2.6 **(Kumar *et al.*, 2018),** and the AIC indicated that TN9+G was the best estimator for this set of data, for ML settings.

RESULTS

Morphology

The common moorhen, *Gallinula chloropus*, is a fascinating avian species with mainly black and dark brown plumage, a white under-tail, white streaks on the flanks, and green legs with the exception of yellow and red at the tibiotarsus. The bill is red with a yellow tip and a red frontal shield. The common moorhen makes a variety of gargling noises and, if attacked, will hiss loudly. The common moorhen, *Gallinula Chloropus*, may be between medium and large in size, measuring between 30 and 42cm in length and 20 and 35cm across the wings. This species can have a body mass ranging from 190 to 450g (Fig. 1).

Genetic divergence analysis

To identify the hunted bird and confirm its species, DNA analysis was applied by using (*16S rRNA*) sequences. In addition, for the determination of its related species from the GenBank/ NCBI, sequencing of *16S rRNA* produced a nucleotide length of 556bp. and deposited in the GenBank under an accession number (OM943961). The nucleotide frequencies of adenine (A), cytosine (C), guanine (G) and thymine (T) were 32.4%, 25.3%, 20% and 22.3%, respectively. The A+T content is 54.7%, which is higher than the C+G content. The sequence of *16S rRNA* is subjected to BLAST/N at NCBI and aligned with 39 reference sequences (37 representing the most available and appropriate species of related species of the family Rallidae and two out-groups from Gruiformes within *Grus* by an overall average of 38. The available 40 reference sequences are classified into seven sequences representing five species from *Zapornia*; five sequences constituting four species from *Fulica;*four sequences representing three taxa from *Laterallus*; three sequences representing three species from *Porphyrio*; two sequences constituting two species from each *Amaurornis* and *Rallus*; two sequences constituting one species from *Gallinula*, and one sequence from each *Canirallus*, *Eulabeornis*, *Gallirallus*, *Hypotaenidia*, *Rallina*, *Gallicrex*, *Nesotrochis*, *Porzana*, *Tribonyx* and *Coturnicops* (Table 1) The final alignment of the *16S rRNA* datasets is composed of 40 sequences, resulting in an alignment of 466bp, of which 313bp are conserved sites and 152bp (32.6%) are variables, with 123bp (26.4%) prism-information sites, 27bp (5.8%) singleton sites, having a genetic divergence range of 0- 48pb (0– 10.3%).

Genetic divergence in the 466bp long *16S rRNA* sequences between understudied *G. chloropus* and constituted sequences of *G. chloropus* were three and six pb, with accessions DQ485864 and NC015236, respectively. Differences between understudied *G. chloropus* and the available taxa of *Fulica* ranged from 1.72% (8 bp) with *Fulica armillata* (KP313718 and NC025500) to 2.79% (13 bp) with *Fulica armillata* (KC613995).

Phylogenetic analysis

Phylogenetic analysis of the *16S rRNA* dataset resulted in the taxa of the Rallidae forming a monophyletic clade to the exclusion of the outgroup taxa. Taxa of *Gallinula* were represented by one species, *G. chloropus* was resolved as a monophyletic clade with strong support (BI=1; ML=89; MP=95 which in turn are inserted within a highly supported larger cluster, *Fulica*/*Gallinula* clade (1/97/99). Within this clade, *G. chloropus* formed a sister relationship with sequences of *Fulica atra* in a clade of a well-suppert values (92/54/74) (Figs. 2- 4).

Fig. 1: Morphological features of the common moorhen, *Gallinula chloropus*

Table 1. Genetic divergence analysis of the understudied *Gallinula chloropus* with its related species from the GenBank/ NCBI based on (*16S rRNA*) sequences

No.	Species	Accession no.	Bp differences	Similarity %	Location
1	*Amaurornis phoenicurus*	NC024593.1	38	91.84	USA, China
2	*Amaurornis phoenicurus phoenicurus*	KF289829.1	38	91.84	India
3	*Canirallus oculeus*	MK434261.1	27	94.20	Australia
4	*Coturnicops noveboracensis*	NC063128.1	40	91.41	USA
5	*Eulabeornis castaneoventris*	NC025501.1	35	92.49	USA, New Zealand
6	*Fulica americana*	DQ485863.1	10	97.85	USA
7	*Fulica armillata*	KC613995.1	13	97.21	New Zealand
8	*Fulica atra*	KP313718.1	8	98.28	China
9	*Fulica atra*	NC025500.1	8	98.28	USA, New Zealand
10	*Fulica leucoptera*	KC613993.1	9	98.07	New Zealand
11	*Gallicrex cinerea*	NC028408.1	35	92.49	USA, Korea
12	*Gallinula chloropus*	NC015236.1	6	98.71	USA, China
13	*Gallinula chloropus*	DQ485864.1	3	99.35	USA
14	*Gallirallus austral is australis*	KF425525.1	34	92.70	New Zealand
15	*Grus Americana*	NC020576.1	52	88.84	USA
16	*Grus japonensis*	NC020575.1	53	88.62	USA
17	*Hypotaenidia philippensis*	DQ485860.1	36	92.27	USA
18	*Laterallus jamaicensis jamaicensis*	OM677841.1	39	91.63	USA
19	*Laterallus melanophaius*	DQ485859.1	38	91.84	USA
20	*Laterallus rogersi*	NC039814.1	40	91.41	USA, Sweden
21	*Laterallus rogersi*	MN356443.1	39	91.63	China
22	*Lewinia muelleri*	NC025502.1	38	91.84	USA, New Zealand
23	*Lewinia striata*	NC041577.1	36	92.27	USA, China
24	*Nesotrochis steganinos*	NC054210.1	45	90.34	USA
25	*Porphyrio hochstetteri*	NC010092.1	47	89.91	USA, New Zealand
26	*Porphyrio porphyrio*	DQ485858.1	48	89.70	USA
27	*Porphyriops melanops*	KC613998.1	24	94.85	New Zealand
28	*Porzana Carolina*	DQ485862.1	35	92.49	USA
29	*Rallina eurizonoides sepiaria*	NC012142.1	39	91.63	USA, Japan
30	*Rallus aquaticus*	NC041578.1	35	92.49	USA, China
31	*Rallus indicus*	NC068741.1	35	92.49	USA, Korea
32	*Tribonyx mortierii*	KC613999.1	25	94.63	New Zealand
33	*Zapornia akool*	NC023982.1	40	91.41	USA, China
34	*Zapornia atra*	NC052808.1	41	91.20	USA, China
35	*Zapornia fusca*	KY009736.1	28	93.99	China
36	*Zapornia fusca erythrothorax*	LC541456.1	28	93.99	Japan
37	*Zapornia paykullii*	NC037406.1	33	92.92	USA, China
38	*Zapornia pusilla*	NC053843.1	32	93.13	USA, China
39	*Zapornia pusilla*	KY009737.1	31	93.35	China

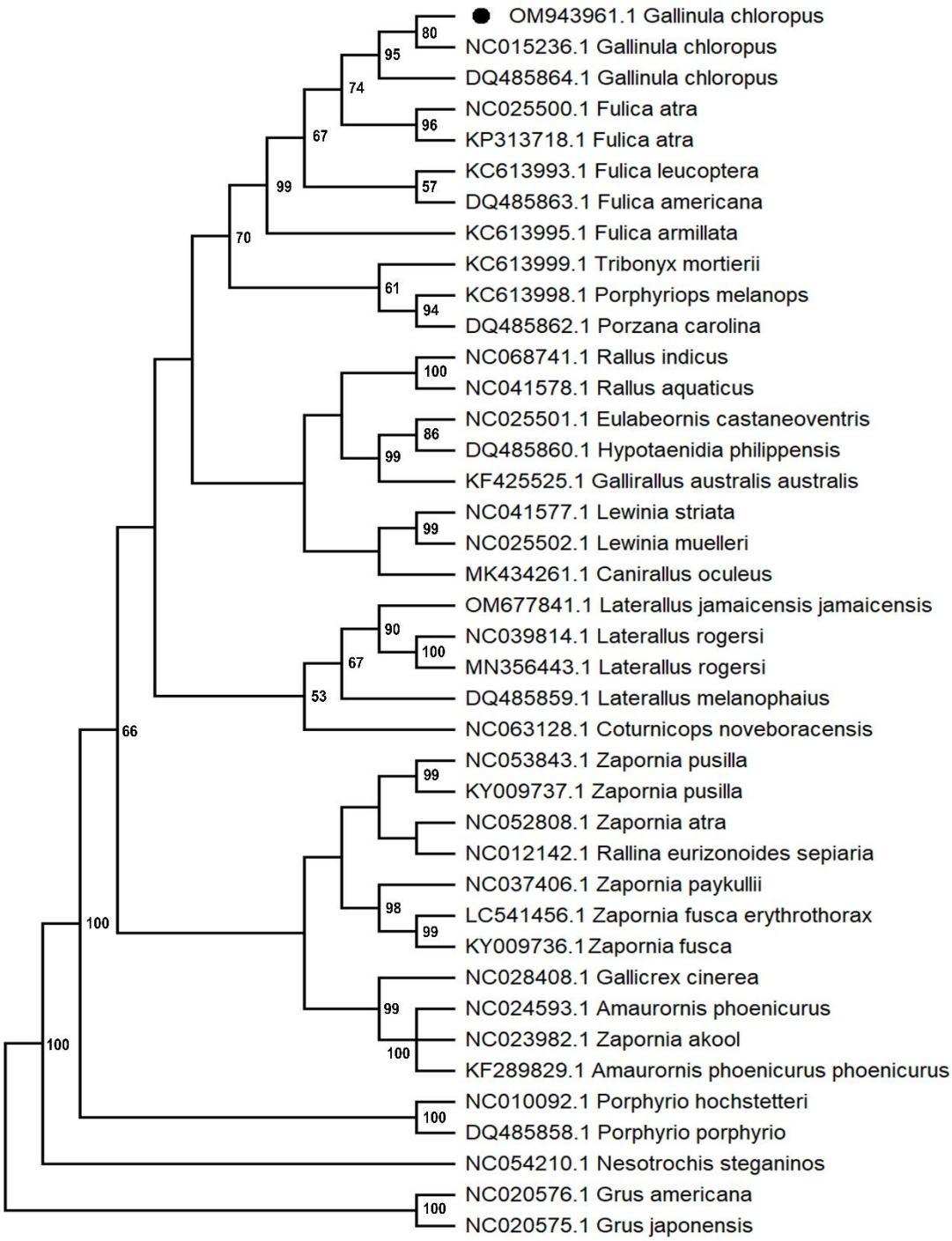

Fig. 2. Maximum parsimony phylogenetic tree illustrating phylogenetic relationships of the common moorhen, *Gallinula chloropus* with retrieved taxa of the Rallidae from the GenBank/ NCBI based on (*16S rRNA*) sequence

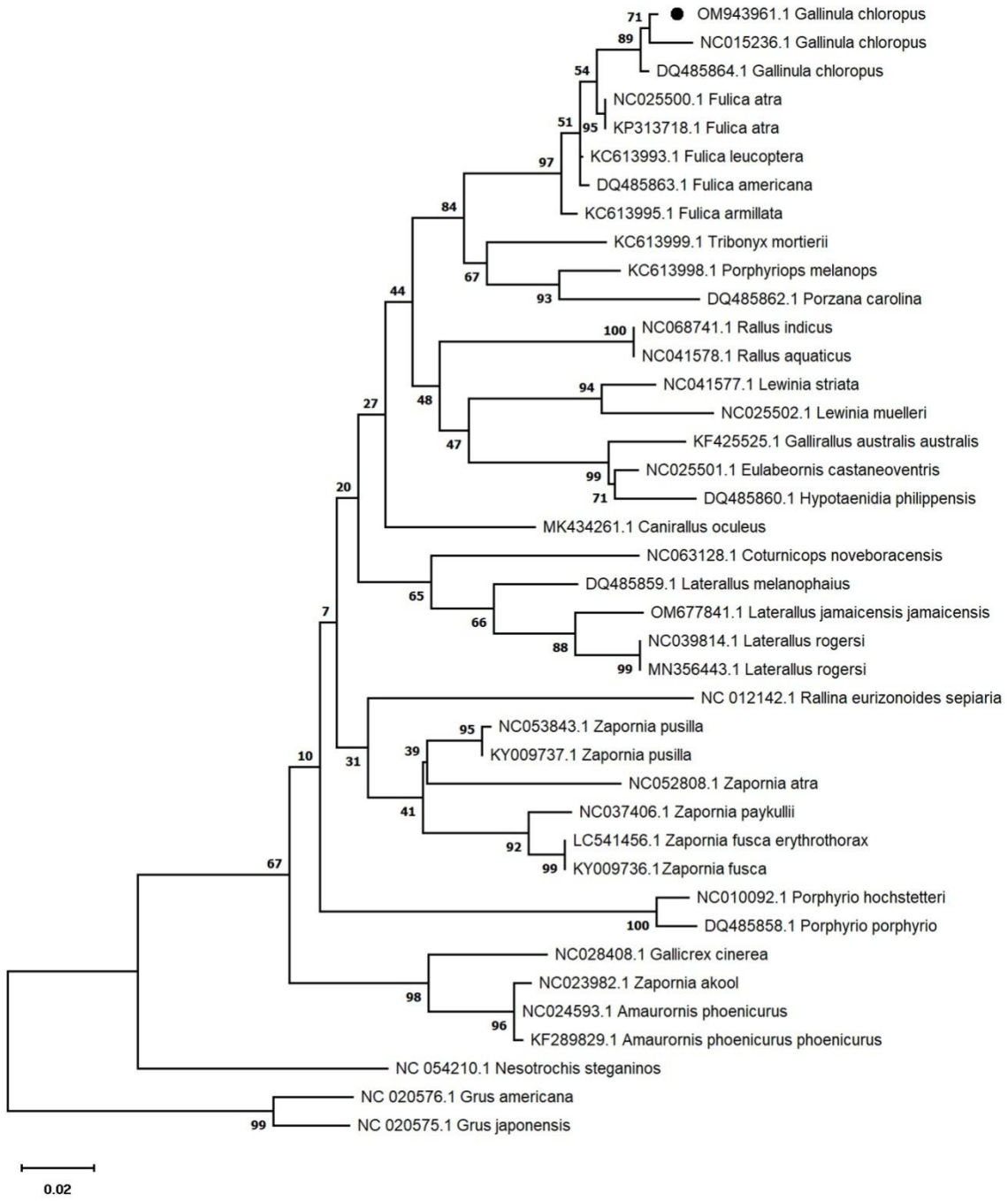

Fig. 3. Maximum-likelihood phylogenetic tree illustrating phylogenetic relationships of the common moorhen, *Gallinula chloropus* with retrieved taxa of the Rallidae from the GenBank/ NCBI based on (*16S rRNA*) sequence

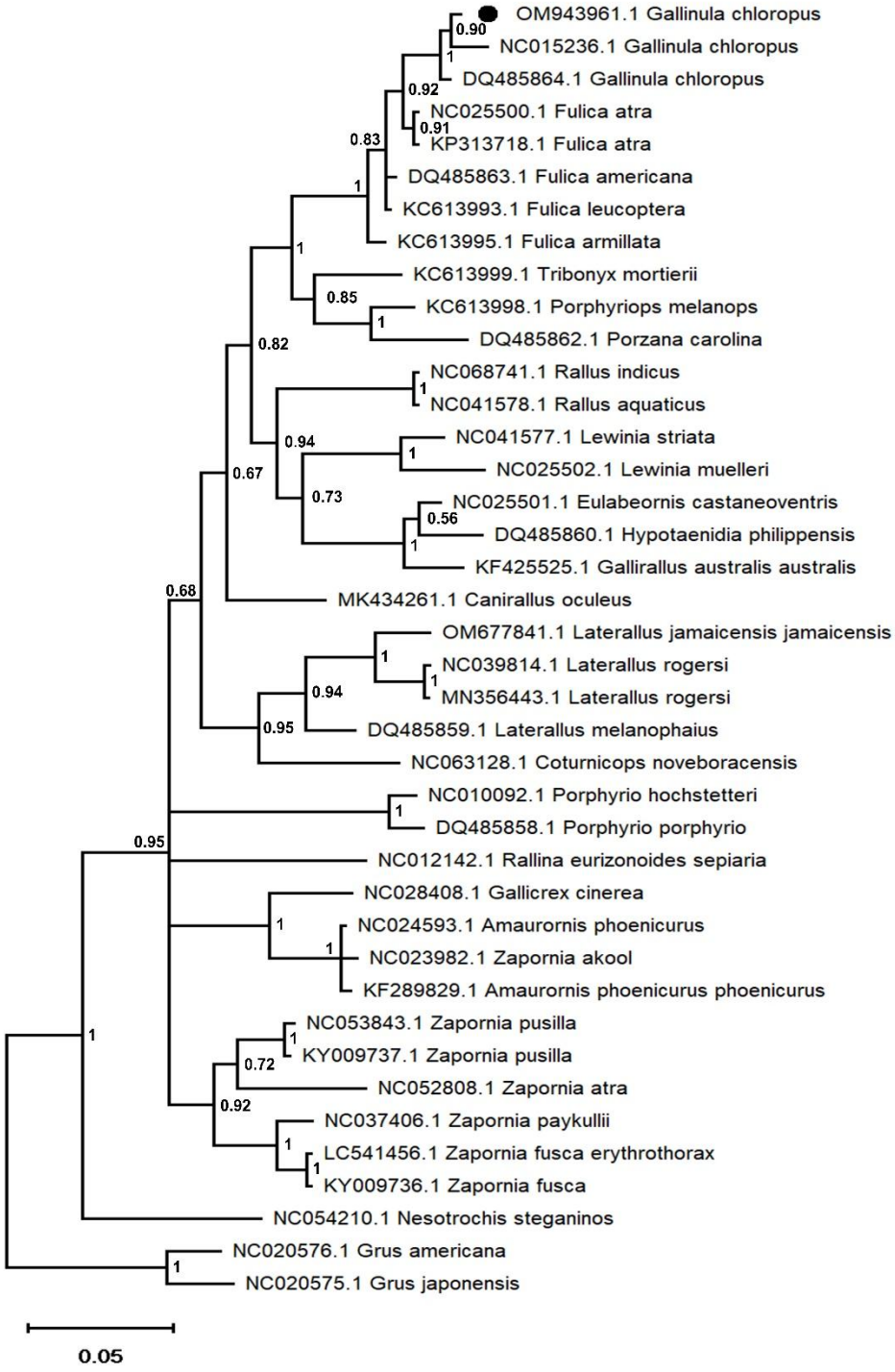

Fig. 4. Bayesian phylogenetic tree illustrating phylogenetic relationships of the common moorhen, *Gallinula chloropus* with retrieved taxa of the Rallidaefrom the GenBank/ NCBI based on (*16S rRNA*) sequence.

DISCUSSION

Morphological traits between closely related species and congeners are very limited and not sufficient to define organisms and determine the relationships between them **(Nagaratti & Nagaraja, 1968, 1971; Pinto & Stouthomer, 1994)**. This was observed in the specimens understudy, where it overlaps and confuses with other *Gallinula* taxa and some *Fulica* spp. Thus, through combined morphological and molecular approaches, the present study revealed that the understudied species is identified as *G. chloropus*. Based on identical morphological features and strongly supported phylogenetic analysis, slight observed genetic differences (3– 6bp) between the specimens under investigation and those previously registered sequences of *G. chloropus* can be attributed to the change in the geographical distributions, where the specimens are recorded from the Soharo-Arabian realm (Egypt), whereas previously registered sequences are from other two distant realms, the Nearctic realm (USA) and the Palearctic one (China). This, in turn, corresponds to the fact that *G.chloropus* is worldwide distributed and is as abundant as its vernacular name implies in many parts of its range **(Taylor & van Perlo, 1998; Wang *et al.*, 2006)**; it is normally a non-migratory species but is forced to migrate in the northern parts of its range **(Taylor & van Perlo, 1998; Ruan *et al.*, 2018)**.

In the current study, DNA sequences of the *16S rRNA* gene were applied following the method used in the study of **Spicer and Dunipace (2004)** on songbirds (Passeriformes) and stepping the guidelines of **Fain *et al.* (2007)** for *G. chloropus*. However, some previous studies on birds used a different ribosomal gene such as the *12S rRNA* **(Houde *et al.*, 1997)**.

Regarding strong phylogenetic relationships and close genetic convergence between the taxa of *Gallinula* and *Fulica* can be attributed to highly close morphological features. According to **Adams (2016)** who described the common Coot, *F. atra* clarified that *G. chloropus* seems quite similar to *F. atra*. The similarity is represented in both of them having a dark brown-black body and green legs eyes. They share food and feeding habits (feed mainly on aquatic vegetation that they dive underwater to find food) as well as similar habitats and life patterns (diving and flightless birds and life cycle). The only variations observed between them are that *G. chloropus* has a red and yellow bill and redhead shield, while *F. atra* has a white bill and head shield. The iris of eye in *G. chloropus* is dark red, while in *F. atra* it is bright red. The tail of *G. chloropus* is longer than that of *F. atra*. In addition, the toes of *F. atra* have lobes, whereas that of *G. chloropus* has no lobes. Therefore, the present authors showed that the relationship between *G. chloropus* and *F. atra* is morphologically very strong and phylogenetically highly close compared to other taxa of *Fulica* demonstrated in the phylogenetic trees of the present study (Figs. 2– 4). In comparison to other *Fulica* spp., the common Coot, *F. atra* differs in the color of the bill, frontal shield and the legs. In addition, it seems to be smaller in size. Accordingly, the present study suggests that *F. atra* may be joined to

Gallinula more than to be a member of *Fulica*. Thus, this relationship needs more attention and more future study to determine the accurate site of each species.

The current study confirmed that DNA sequences of the *16S rRNA* gene are a good marker to describe and identify *G. chloropus*, as well as demonstrating its relationships with its related species. These relationships are well-supported and suggest that taxonomic revision is a warrant to determine the validity separation of *Gallinula* from *Fulica*. The present findings based on *16S rRNA* are aligned with multiple GenBank sequences deposited by multiple authors through multiple studies **(Sangster *et al.*, 2015; Ruan *et al.*, 2018)**.

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