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ANIMAL BREEDING AND REPRODUCTION

Genetic and taxonomic relationships of five species of Rallidae (Aves: Gruiformes) based on mitochondrial cytochrome oxidase subunit I sequences

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ABSTRACT. This is the first study to detection the genetic relationship between *Porphyrio alleni* and four Rallidae species: *Fulica atra*, *Gallinulla angulata*, *Gallinulla chloropus* and *Porphyrio porphyrio*. Mitochondrial cytochrome oxidase subunit I (COI) sequences were used as an effective marker in this study. DNA of Rallidae species were extracted, amplified using polymerase chain reaction (PCR) and then sequenced. The results obtained from information based on COI sequences revealed that *Gallinulla angulata* and *Gallinulla chloropus* fall into two separate clades and they are not monophyletic. This suggests that, two moorhens could not be laid into the same genus. In addition, *Porphyrio porphyrio* was included in the same genus with *Porphyrio alleni* but they were situated in two different clades. *Porphyrio alleni* was more related to *Gallinulla angulate*, *Gallinulla chloropus* and *Fulica atra* than *Porphyrio porphyrio*. It was concluded that, the mitochondrial gene COI can aid in the differentiation of studied species and finding genetic relationships between them.

Keywords: birds; Gruiformes; PCR; phylogeny; sequencing; species.

Relações genéticas e taxonômicas de cinco espécies de Rallidae (Aves: Gruiformes) com base em sequências de subunidade I da citocromo oxidase mitochondrial

RESUMO. Este é o primeiro estudo para a detecção da relação genética entre *Porphyrio alleni* e quatro espécies de Rallidae: *Fulica atra*, *Gallinulla angulata*, *Gallinulla chloropus* e *Porphyrio porphyrio*. As seqüências de subunidade i (COI) do citocromo oxidase foram usadas como marcador efetivo neste estudo. O DNA das espécies Rallidae foi extraído, amplificado e usado na reação de cadeia da polimerase (*polymerase chain reaction*- PCR) e sequenciado então. Os resultados obtidos a partir de informações baseadas em sequências COI revelaram que *Gallinulla angulata* e *Gallinulla chloropus* caem em dois *clades* separados e eles não são monofiléticos. Isto sugere que duas galinhas d'água não puderam ser colocadas no mesmo gênero. Além disso, *Porphyrio porphyrio* incluído no mesmo gênero que *Porphyrio alleni*, mas eles estavam situados em dois *clades* diferentes. *Porphyrio alleni* estava mais relacionado com *Gallinulla angulata*, *Gallinulla chloropus* e *Fulica atra* do que com *Porphyrio porphyrio*. Concluiu-se que o gene mitocondrial COI pode ajudar na diferenciação de espécies estudadas e encontrar relações genéticas entre elas.

Palavras-chave: pássaros; Gruiformes; PCA; filogenia; sequenciamento; espécies.

Introduction

Rallidae was greater in number of species other than Gruiformes families. This diverse Rallidae includes between 135 and 148 known species, comprising nearly 1.3% of recognized birds and 85% of order Gruiform variety, within 33 – 40 genera (Taylor, 1998; Houde, 2009; Clements et al., 2012). The phylogenetic history of one of the most distribution family of birds, the universal family Rallidae, is not fully known. Also, it was characterized by its taxonomic complexity and geographical distribution. Several groups of birds included in this large family such as coots, rails and gallinules.

Fulica and several species of *Gallinula* including *Gallinula angulata* could be combined in a single genus for which *Fulica* has nomenclatural preference (Garcia-R et al., 2014a). However, this design requires additional modulation to correspond the systematic state of the Gallinules, which are not a natural group. These relationships are well-supported and propose that taxonomic revision is ensured between these Rallidae (Olson, 1973; Ripley et al., 1977; Taylor, 1998). So, it was important to perform this study to clarify the phylogenetic relationship between these studied species.

Cytochrome oxidase subunit I (COI) has been popular for estimating relationships among closely allied taxa and efficient explanation of biological diversity, especially the resolution of resemblance below taxonomic levels for that reason; it was used in the present work as first study for detecting genetic and taxonomic relationships between *Porphyrio alleni* and four Rallidae.

This method is accepted as a standard for identifying DNA of organisms and is now being suggested as a method for cataloguing life of many species (Marshall, 2005).

Material and methods

Bird samples and DNA extraction

The study was carried out on a twenty five bird samples included in the Family Rallidae (water birds) and Order Gruiformes. These are: *Fulica atra* (Coot); *Gallinulla angulata* (Lesser moorhen); *Gallinulla chloropus* (moorhen); *Porphyrio porphyrio* (purple swamphen); *Porphyrio alleni* (Allen's gallinule). These species are belonging to three different genera of family Rallidae termed as, *Fulica, Gallinulla* and *Porphyrio*. Five samples from each species were collected from different aquatic regions of Damietta Governorate, Egypt from December 2014 to February 2015 except Allen's gallinule which collected from Salloum, Matruh Governorate (Figure 1).

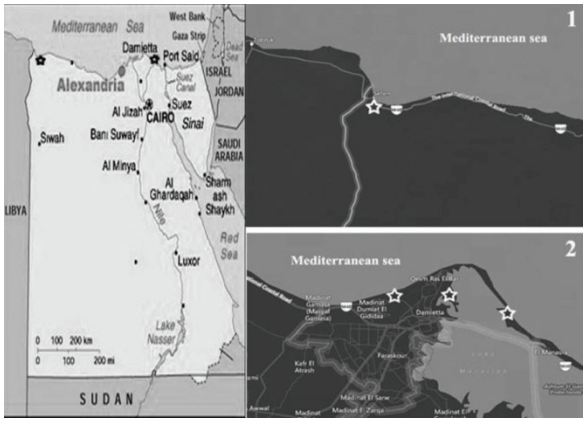

Figure 1. The map of Egypt set on the left of figure and locations of Damietta Governorate and Salloum, Matruh Governorate are shown with black stars, but on the right is the map of Salloum, Matruh Governorate No. 1, which is the site of catching of *Porphyrio alleni* (Allen's gallinule) is shown in white star and below it is the map No. 2 of Damietta Governorate, which is showing sites of catching of *Fulica atra* (Coot), *Gallinulla angulata* (Lesser moorhen), *Gallinulla chloropus* (moorhen) and *Porphyrio porphyrio* (purple swamphen) with white stars.

All the studied Rallidae were resident birds except Coot and Allen's gallinule (migratory species). These birds were taken to the laboratory for dissection. Samples of liver tissues from all studied species were taken immediately and frozen at - 80ºC. A GeneJET™ kit Genomic DNA Kit K0721 was used to extracting of DNA.

PCR amplification

Amplification of the COI gene fragments was carried out using the primer, BF1 (5' TTC TCC AAC CAC AAA GAC ATT GGC AC 3') and BR1 (5'ACG TGG GAG ATA ATT CCA AAT CCT G 3'). The 20 µL PCR reaction mix included 50 ng of genomic DNA template; 13.44 µL sterile ultrapure water, 2.0 µL of 10X buffer, 1.0 µL of MgCl, 0.8 units of Taq DNA polymerase, 0.4 µL of each forward and reverse primer and 2.0 µL of DNA template. The PCR amplification program consisted of 3 min at 94°C followed by five cycles of 35 s at 94°C, 40 s at 56°C and 35 s at 72°C, followed by another 30 cycles of 35 s at 94°C, 40 s at 58°C, and 35 s at 72°C, and finally 7 min at 72°C. The PCR products were visualized in a 1.0% agarose gels and staining with ethidium bromide to visualize bands and viewed with an ultraviolet light source. A GeneJET™ kit (Thermo K0701) used to purification the amplified PCR products according to the manufacturer's protocol. An ABI 3730xl DNA sequencer was used to performance sequencing of amplified PCR products.

Alignment and sequence properties

All mtDNA nucleotide sequences obtained in this work were aligned by using the Clustal W software and identical sequences were considered as the same haplotype. Maximum Likelihood phylogenetic tree was constructed by calculating distance matrix of different studied species through MEGA v.5 software (Tamura et al., 2011). Bootstrap values were used to estimating the support for tree nodes with 500 replicates.

Results

In the current study a 720 pb segment was amplified from DNA of five species by employing the COI primer pairs. The resulted COI sequences of Rallidae were submitted to the Gen Bank (NCBI) and the accession numbers were represented in table 1. Forty four sequences collected from NCBI were belonging to various studied birds (Table 1) were used for constriction of phylogram and genetic distance detection.

Sequences of five species of Rallidae were compared with records placed in Gen Bank, the results with a COI sequence similar with a record

submitted to European Nucleotide Archive (ENA); for *Fulica atra*; *Gallinulla angulata*; *Gallinulla chloropus*; *Porphyrio porphyrio*; *Porphyrio alleni* revealed 12, 1, 25, 5 and 1 COI sequences similar with each one respectively (Table 1).

Table 1. List of Rallidae members sequenced at mitochondrial DNA loci (COI).

Species	No. sequences	Accession number
Fulica atra (Coot)	12	JQ342125; JF499133; GU571901; KC439318; KP313718; GU571900; GU571405; GU571406; JQ342126; KP252184; GQ481938; KF644582
Gallinulla angulata (Lesser moorhen)	1	KC614041
Gallinulla chloropus (moorhen)	25	DQ433656; DQ432936; FJ027609; DQ433657; FJ027608; DQ433654; DQ433655; DQ43600; FJ027610; AB843519; EF515779; GQ481956; AB842793; AB843518; GU571906; JQ342116; AB843520; AB842794; KF946699; GU571907; GU571413; JQ342115; HQ896036; JF499135; KC614041
Porphyrio porphyrio (purple swamphen)	5	JQ175970; JQ175971; kF701062; KC439332; KP252237
Porphyrio alleni (Allen's gallinule)	1	KC614052

Moreover, the average nucleotide frequencies are 26.7% (A), 25.1% (T/U), 32. 0% (C) and 16.2% (G). The percent composition of nucleotide varied from 25. 6 to 28.2% (A), 24.1 to 25.8% (T), 30.1 to 33.3% (C), and 15.3 to 16.5% (G), which infers that studied birds, are C rich and poor in G, A and T (Table 2).

Table 2. Percentage composition of nucleotides A, T, G, C, AT and GC in Rallidae species.

Family: Rallidae	A%	T%	G%	C%	AT%	CG%
Fulica atra	25.8	25.8	15.3	33.1	51.6	48.4
Gallinulla chloropus	25.6	24.8	16.3	33.3	50.4	49.6
Gallinulla angulata	26.8	25.3	16.4	31.5	52.1	47.9
Porphyrio alleni	28.2	25.3	16.5	30.1	53.5	46.6
Porphyrio porphyrio	26.8	24.1	16.4	32.7	50.1	49.1
Mean	26.7	25.1	16.2	32.0	51.5	48.3

The transition/transversion rate ratios are k_1 = 2.291 (purines) and k_2 = 5.764 (pyrimidines). The content of pyrimidine was higher than that of purine. These values showed a strong A + T (51.5%) to G + C (48.3%) asymmetry in nucleotide composition (Table 2). The maximum AT content was found in *Porphyrio alleni* (53.5%) and the minimum in *Porphyrio porphyrio* (50. 1%). The maximum and minimum GC contents were observed in *Gallinulla chloropus* (49.6%) and *Porphyrio alleni* (46.6%) respectively.

Genetic distance was calculated between the species belonging to three genera of Rallidae. Distances calculated between species pairs showed that the smallest differences (0.281) existed between *Fulica atra* and *Gallinulla chloropus* whereas the highest genetic distance detected between *Fulica atra* and *Porphyrio porphyrio* amounted to 0.659. *Porphyrio porphyrio* showed more genetic distance from the other members of family Rallidae (Table 3).

Table 3. Total genetic distance between the studied species.

	Fulica atra	*Gallinulla chloropus*	*Gallinulla angulata*	*Porphyrio alleni*	*Porphyrio porphyrio*
Fulica atra	0				
Gallinulla chloropus	0.281	0			
Gallinulla angulata	0.392	0.364	0		
Porphyrio alleni	0.479	0.474	0.464	0	
Porphyrio porphyrio	0.659	0.657	0.643	0.642	0

The phylogenetic tree of Rallidae, was constructed using the Maximum Likelihood method, based on COI sequences (Figure 2).

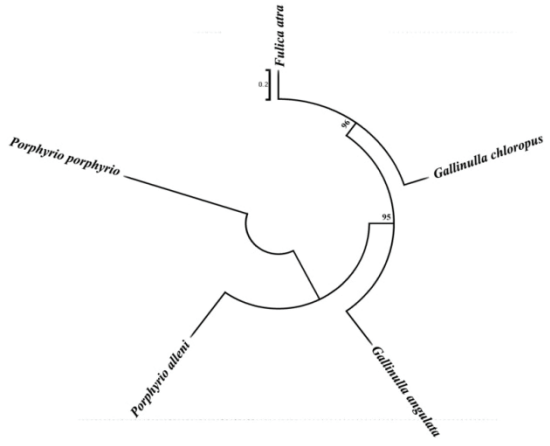

Figure 2. The Maximum Likelihood tree of 5 species belonging to family Rallidae. Numbers on the branches refer to bootstrap values.

The results show that in the topological structures of the tree there are two separate genetic branches, one branch for *Porphyrio porphyrio* and other branches for *Fulica atra*; *Gallinulla angulata*; *Gallinulla chloropus*; *Porphyrio alleni* with strong bootstrap support (99%). At the same time, *Porphyrio porphyrio* is in a separate group, with a node connected to the other studied birds and having the farthest genetic distance with the other bird species. Although *Porphyrio porphyrio* included in the same genus with *Porphyrio alleni* it was situated in two different clades. In addition, *Porphyrio alleni* was more related to *Gallinulla angulata*; *Gallinulla chloropus* and *Fulica atra* than *Porphyrio porphyrio*. Instead, a branched diagram

revealed that, the *Gallinulla chloropus* is more closely related to *Fulica atra*. Thus, they are grouped in the same branch and forms a sister group to each others. *Gallinulla angulata* was laid in another branch. Occurrence of *Gallinulla angulata* in the another clade not with *Gallinulla chloropus* despite they are belong to one genus *Gallinulla* and greatly similar to each other in morphology was an interesting observation which make the wonder of its close genetic relatedness and hence tree based on COI sequences data was constructed.

In case of mitochondrial tree, a total of forty four unique haplotypes were identified in sequences from mitochondrial COI of studied specimens of Rallidae. All haplotypes of *Fulica atra* formed the monophyletic cluster on the phylogenetic tree based on COI dataset. The haplotypes of *Fulica atra* and *Gallinulla chloropus* were deposited together in the phylogram and appeared as sister group. Haplotypes of *Gallinulla angulata, Porphyrio porphyrio* and *Porphyrio alleni* were clustered together with high nodal support (bootstrap value 99%) (Figure 3).

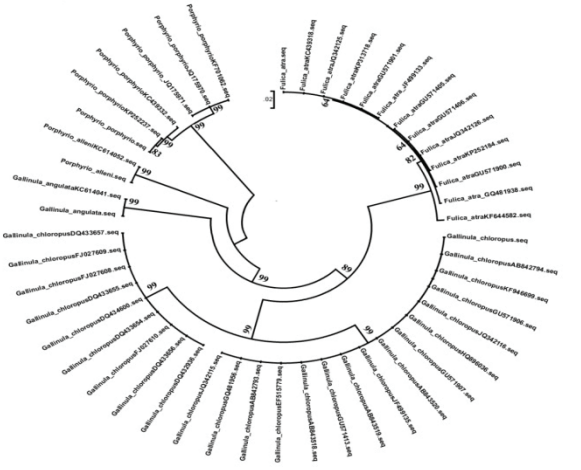

Figure 3. Tamura-Nei distance Maximum Likelihood tree of 44 barcode sequences from 5 species belonging to family Rallidae. Specimens' numbers denote the accession number of NCBI database. Numbers on the branches refer to bootstrap values.

In the tree based on COI sequences data, two main clades were produced: in the first clade, *Fulica atra* was clustered as closest taxa to *Gallinulla chloropus*, being a sister group to each others. In the second branch, *Gallinulla angulata* was clustered together with *Porphyrio alleni* and *Porphyrio porphyrio*. Similarly, *Porphyrio alleni* and *Porphyrio porphyrio* were placed in two different branches in this mitochondrial tree.

Discussion

In the past, the systematic position of many monotypic genera of birds was dubitable. This is because the genera were not based on phylogenetic studies but on subjectively deduce evolutionary specialty which in turn was based on phenotypic distinctiveness and commonly their discrete location. Indeed, some taxonomists have grouped large numbers of species in monotypic genera but the introduction of molecular phylogenetic methods in bird taxonomy exposed that many of these were nested within other genera (Johnson et al., 2001; Gibson & Baker, 2012; Alström et al., 2015).

Until now, performance of the COI region for genetic identification and classification have been evaluated in many birds (Yoo et al., 2006; Kerr et al., 2007; Chaves et al., 2008; Kerr et al., 2009a; Kerr et al., 2009b). The present study based on COI gene sequencing data of three genera and five species of the family Rallidae revealed different systematic classification of the current classification and question existence of genus *Gallinula*. Different phylogenetic approaches resulted in tree topology and the clades well supported in this study. *Gallinula choloropus* and *Gallinulla angulatus* included in the same genus, greatly resemble to each other morphologically thus it was expected to be placed in the same clade but in this study, *Fulica atra* and *Gallinula choloropus* clustered together and were supported as sister group to each other, they are embedded in the same clade with *Gallinulla angulatus*. *Gallinulla angulatus* could be removed from *Gallinula* and placed in a separate new genus.

The results of COI sequences in the current study agreed with García–R et al. (2014a) who reported that, the position of *Fulica* as the sister of *Gallinula* indicates that, the time of divergence between *Fulica* and *Gallinula* is similar to or transcend that of several widely accepted genera of rails. This further supports that, *Gallinula* and *Fulica* are best kept as separate genera. Furthermore, COI sequencing can be greatly used to differentiate bird species. Topology of phylogeny based COI sequences had a good phylogenetic signal.

On the other hand, *Porphyrio porphyrio* was divergently clustered outside this group. *Porphyrio porphyrio* being highly distance from the other members of studied resident and migratory Rallidae.

However, *Porphyrio alleni* greatly look alike *Porphyrio porphyrio* morphologically, genetic distances calculated between species pairs showed that *Porphyrio alleni* was more related to *Gallinulla angulata*; *Gallinulla chloropus* and *Fulica atra* than *Porphyrio porphyrio*. Indeed, *Porphyrio alleni* and *Porphyrio porphyrio* were placed in two different branches instead of placing two Rallidae in the same clade.

Members of the genus *Gallinula* have been progressively revised by transmitting of phylogenetically divergent species to other genera. Molecular phylogenetic evidence with plumage features displaying a closer relationship to purple swamphens and far removed from moorhens (Trewick, 1997; Garcia-R et al., 2014b). Such detections demonstrate the effect of convergence in body form and coloration of separate rail lineages on taxonomic inference. Reconciliation was using molecular data helps in understanding how phenotypic feature improve in response to ecological status, and in this case shows that the familiar composition of gallinules is not a natural grouping.

Conversely, several species have been shown to occupy a phylogenetic position inconsistent with their traditional generic assignment, resulting in the recognition of new monotypic genera (Chesser et al., 2009; Slager & Klicka, 2014). For this reason, the present results supported phylogenetic position of *Gallinulla angulata* outside the *Gallinula-Fulica* clade and gives new hypothesis that *Gallinulla angulata* and *Gallinulla chloropus* should be considered in two different genuses or new genus should be given for *Gallinulla angulata*.

Conclusion

This is the first study to detection the genetic relationship between *Porphyrio alleni* and four Rallidae; (*Fulica atra*, *Gallinulla angulata*, *Gallinulla chloropus* and *Porphyrio porphyrio*). Furthermore, using mitochondrial cytochrome oxidase subunit I (COI) sequences in this study suggests the separation of *Gallinulla angulatus* into a new genus. Using of COI sequencing identified successfully five bird species. Moreover, the COI sequencing technique developed in this study was proved to be a simple, reliable and rapid method for differentiating closely related taxa and considered a useful source of phylogenetic data.

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