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DNA Fingerprinting of three Species of Family Alcedinidae (Aves: Coraciiformes) in Egypt using molecular markers

KEYWORDS: RAPD-PCR, DNA-Fingerprinting, Alcedinidae, Birds, Genetic distance

Lamiaa Elsayed Mokhtar Deef

Department of Zoology, Faculty of Science, Damietta University, New Damietta, Damietta, Egypt.

ABSTRACT *The random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction has been one of the most commonly used molecular techniques to develop DNA markers. This technique was applied using four primers. The experimental birds of the study include three kingfishers. The highest genetic distance was found between European kingfisher and pied kingfishers (G_D = 0.706), and the lowest (G_D = 0.650) between white-breasted with European kingfishers, on contrary, the highest genetic identity was observed (I = 0.537) between white-breasted with European kingfishers and the lowest (I = 0.494) between Pied with European kingfishers. According to these results a dendrogram of phylogenetic relationship, was constructed. It appears that white-breasted Kingfisher is the closest to European Kingfisher whereas white-breasted kingfisher was the most distant from pied Kingfisher.*

INTRODUCTION

Alcedinidae is a family of birds in the order Coraciiformes which contain river Kingfishers. Kingfishers are a group of small to medium sized brightly coloured birds. They have a cosmopolitan distribution, with most species being found in the Old World and Australia [1].

Development of Random Amplified Polymorphic DNA (RAPD) gave an advantage in which molecular preliminary information of the species studied is not necessary and polymorphism pattern obtained usually varies among the species [2, 3]. RAPD-PCR has been successfully used for genetic fingerprinting and molecular typing for many species, including fingerprinting of animals [4, 5].

Morphological studies have generally been successful in defining genera, but it is rare to find studies which present a hypothesis of relationship above the level of species comprising a genus, primarily due to a lack of congruence of characters [6]. The molecular data, and inter- and intrageneric relationships are now being rapidly tested and elucidated. Molecular data are also means used to assess the phylogenetic relationships among populations.

RAPD-PCR technology involves amplification of certain regions of the nuclear genome. The primer randomly anneals to an unknown segment on one of the DNA strands. When two species, strains, or individuals are compared, polymorphism between them will be revealed on agarose electrophoresis gels by presence or absence of an amplification product, this method has been applied to the discovery of genetic markers for mapping studies [7] and to elucidate the phylogenetic relationships between species [2, 8]. In this investigation, RAPD-PCR was used to examine the DNA-fingerprint and the similarity among three kingfishers, white-breasted kingfisher (*Halcyon smyrnensis*), European kingfisher, (*Alcedo atthis*) and pied kingfisher (*Ceryle rudis*).

MATERIAL AND METHODS

Twenty four individuals of three Kingfishers, two individuals for each, were used in this study. These Kingfishers were of three exotic species, European kingfisher (UK), white-breasted kingfisher (WK) and pied kingfisher (PK).

DNA extraction

Fresh tissue samples will be preserved in 100% ethanol for the further molecular studies. DNA will be extracted from these samples using the wizard genomic DNA purification kit.

RAPD analysis

Four RAPD primers with arbitrary sequence were screened using six DNA samples and were found to be suitable for the study of all samples (Table. 1).

RAPD-PCR Reactions

The PCR technique was carried out in 0.2 µl microfuge tubes. The total volume consisting of reaction mixture was 25 µl consisting of 19.8 µl sterile distilled water, 2.5 µl 10x PCR optimize buffer, 0.5 µl 25 mM deoxyribonucleotide phosphate, 10 pmol primers, 0.2 µl of 0.5 units *Taq* DNA polymerase and template DNA 1.0 µl. As the negative control, one of the reaction mixtures without DNA template was used. The solution mixture were placed in the thermal cycler and subjected to 45 cycles. The cycling conditions were as follows; predenaturation at 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, and extension at 72°C for 5 minutes, with a final extension at 72°C for 7 minutes at the end of 45 cycles. The amplified products were electrophoresed in1% agarose gel with 0.5x TBE buffer. After the gel had been stained with ethidiumbromide, the fragment sizes were estimated using a 100-bp ladder and band patterns were visualized with a UV transilluminator.

Parameters and Statistical analyses

The gel photographs were then developed and used to estimate the following parameters:

1. Band sharing (BS): All possible pairwise comparisons of amplified bands of any two individual or pooled samples on the same gel were made. The following formula of Jeffreys and Morton [9] and Lynch [10] was used:

$$BS = \frac{2\,(B_{ab})}{B_a + B_b}$$

here, BS indicates band sharing level, B_{ab} indicates

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number of bands shared by samples a and b, Ba and B_b are the total numbers of bands of samples a and b.

2. RAPD genetic distance: RAPD bands were scored as 1 for presence or 0 for absence of the band. Genetic distance (G_D) was calculated using Nei and Li coefficient [11]. It was computed as follows:
$G_D = 1 - BS$

Where BS was the Band sharing.

3. Genetic identity (I): Genetic identity computed according to the equation [12]:

$$I = 1/N \sum_{I=1}^{N} 2V_i (1). V_i^{(2)} /(V_i)^2+(V_i)^2$$

where, *N* is the number of different bands in two given breeds and $Vi^{(1)}$ and $Vi^{(2)}$ are the frequencies of band *i* in the two breeds, respectively.

4. Data analysis:
RAPD data matrix was used in order to compute the genetic distances of the specimens according to Nei and Li coefficient. The MVSP software package version 3.1 [13] was used to calculate Nei and Li [11] similarity coefficients. According to these coefficients a dendogram was constructed by unweighted pair-group method of arithmetic average (UPGMA).

RESULTS

In the present work, four oligonucleotid primers of random sequences were used to amplify the DNA from 24 specimens and a total 108 scorable RAPD bands were obtained. The DNA fragments amplified in 24 specimens using primer 13-16, 27-16, 29-16 and 30-16 have been presented in (Fig.1-4). All primers showed successful amplification of DNA fragments in all individual genomes. The number of bands varied among species from 24 to 32 bands. The number size ranged between 210 bp and 1407 bp length. The majority of RAPD primers gave distinctly reproducible bands in all species, the entire primers produced highly polymorphic pattern. There was not much difference in the number of bands scored in all species using all the primers, whereas individual primer showed more variability. Primer 29-16 gave maximum number of bands 32, while minimum number of bands 24 was recorded with primer 27-16 in all the species (Table 1).

The band sharing (BS) indices between species using different primers are presented in table (2). In this study it was observed that the levels of band sharing were in general low to moderate, significantly differed and ranged between 0.294 in European and Pied Kingfishers to 0.350 in European and White-breasted Kingfishers.

The genetic distance (G_D) and genetic identity (I) between different studied species using Kuhnlein equation and are presented in tables (3). The highest genetic distance was found between European kingfisher and pied kingfishers (G_D = 0.706), and the lowest (G_D = 0.650) between white-breasted with European kingfishers, on contrary, the highest genetic identity was observed (I = 0.537) between white-breasted with European kingfishers and the lowest (I = 0.494) between Pied with European kingfishers.

The RAPD data showed that the different studied species were divided into two main clusters; first cluster formed by European, white-breasted kingfisher, while the Pied kingfisher formed second cluster (Fig. 5).

DISCUSSION

The results of this study show that RAPD can be used to reliably identify bird species and that banding data for incorporation into data matrices, are suitable for phylogenetic reconstructions. In more details, it was observed in the present study that the genetic similarity between species of family Alcedinidae was low to moderate, hence the three species fall in two separate clades.

The data of genetic identity confirm these results where it was also low between three species. On the other hand, it was found that the genetic distance was high between European kingfisher and pied kingfisher. In addition, the genetic distance was also lowest in the European kingfisher and white kingfisher. In contrast, a higher level of band sharing and genetic identity was observed between European kingfisher and pied kingfisher.

In agreement with the result of Wei et al., [14] who study the band sharing and similarity between different bird species, the results indicate the presence of high band sharing between species of family Alcedinidae.

According to these results a dendrogram of phylogenetic relationships, was constructed. It appears that white breasted kingfisher is the closest to European kingfisher whereas pied kingfisher was the most distant from European kingfisher. The results of genetic distance showed that there is a close relationship between European kingfisher and white breasted kingfisher, both of them clustered together in one clade and one of them is a sister group to the others, while pied kingfisher fall in separate clade. The resultant dendrogram also showed close proximity of European and white-breasted kingfishers, followed by pied kingfisher although they are clustered in one family Alcedinidae. In the present study, results showed RAPD analysis was a rapid and useful approach for distinguishing closely related species in the family Alcedinidae as well as for estimating genetic distance among the species.

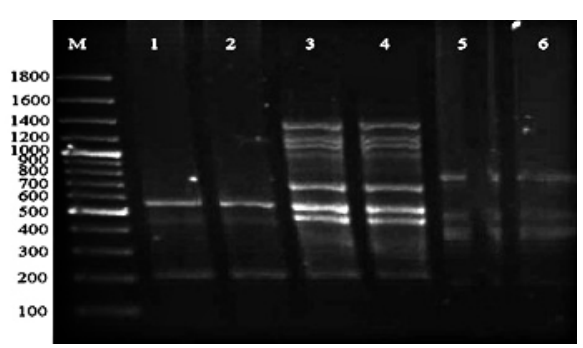

Figure (1): RAPD amplification products of primer 13-16. Lane M: DNA marker, lanes 1 and 2 are European kingfisher (*Alcedo atthis*), lanes 3 and 4 are White-breasted kingfisher (*Halcyon smymenis*) and lanes 5 and 6 are pied kingfisher (*Ceryle rudis*).

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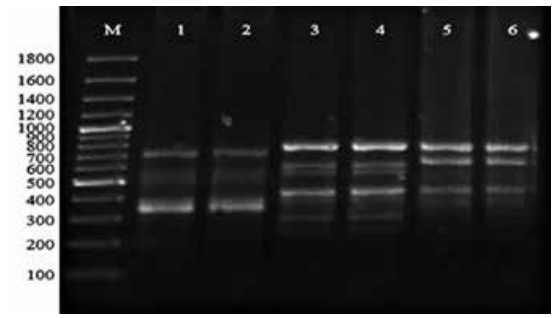

Figure (2): RAPD amplification products of primer27-16. Lane M: DNA marker, lanes 1 and 2 are European kingfisher (*Alcedo atthis*), lanes 3 and 4 are White-breasted kingfisher (*Halcyon smymenis*) and lanes 5 and 6 are pied kingfisher (*Ceryle rudis*).

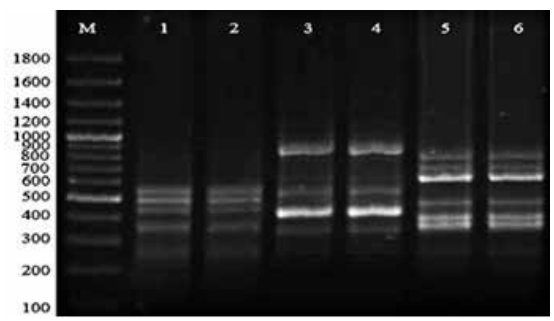

Figure (3): RAPD amplification products of primer29-16. Lane M: DNA marker, lanes 1 and 2 are European kingfisher (*Alcedo atthis*), lanes 3 and 4 are White-breasted kingfisher (*Halcyon smymenis*) and lanes 5 and 6 are pied kingfisher (*Ceryle rudis*).

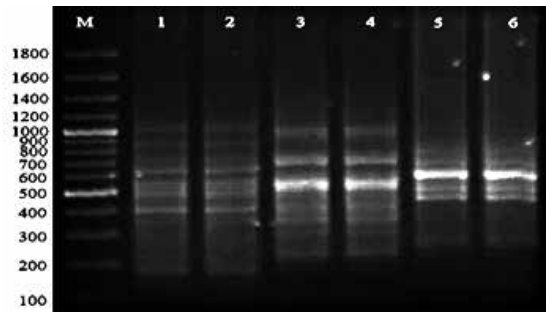

Figure (4): RAPD amplification products of primer30-16. Lane M: DNA marker, lanes 1 and 2 are European kingfisher (*Alcedo atthis*), lanes 3 and 4 are White-breasted kingfisher (*Halcyon smymenis*) and lanes 5 and 6 are pied kingfisher (*Ceryle rudis*).

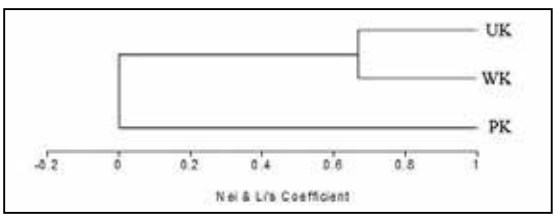

Figure (5): Dendrogram depicting three Kingfishers based on the genetic distance generated by four random primers (based on Nei and Li's index), UK, WK and PK, indicate European kingfisher (*Alcedo atthis*), White-breasted kingfisher (*Halcyon smymenis*) and pied kingfisher (*Ceryle rudis*), respectively.

Table (1): Total and average number of DNA bands, by species and primer (Individual samples).

Primers		**13-16**	**27-16**	**29-16**	**30-16**	**Total**
Sequence (3´ - 5')		CGA GCC CCG AAC AAT	GCA CGC ATG GTT TGC	CCC GCG GCC TAT GAC	CCA GGG TGA GCG GCT	
total bands		30	24	32	28	114
Molecular weight range (bp)		210-1407	309-800	262-855	367-1062	
DNA band numbers	UK	3	3	5	5	16
	WK	9	5	4	5	23
	PK	3	4	7	4	18

UK, WK and PK, indicate European kingfisher (*Alcedo atthis*), White-breasted kingfisher (*Halcyon smymenis*) and pied kingfisher (*Ceryle rudis*), respectively.

Table (2): Band sharing indices between different species, by primer (Individual samples).

Species	**Primer 13-16**	**Primer 27-16**	**Primer 29-16**	**Primer 30-16**	**Mean**
UK↔ WK	0. 333	0.000	0. 667	0. 400	0. 350
WK ↔ PK	0. 167	0.444	0. 182	0. 444	0. 309
UK ↔ PK	0. 333	0. 286	0. 333	0. 222	0. 294

UK, WK and PK, indicate European kingfisher (*Alcedo atthis*), White-breasted kingfisher (*Halcyon smymenis*) and pied kingfisher (*Ceryle rudis*), respectively.

Table (3): Genetic distance and Genetic identity indices between different species, by primer (Individual samples).

Genetic distance					
Species	**Primer 13-16**	**Primer 27-16**	**Primer 29-16**	**Primer 30-16**	**Mean**

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UK↔ WK	0. 667	1.000	0. 333	0. 600	0. 650
WK ↔ PK	0. 833	0.556	0. 818	0. 556	0. 691
UK ↔ PK	0. 667	0. 714	0. 667	0. 778	0. 706
Genetic identity					
Species	**Primer 13-16**	**Primer 27-16**	**Primer 29-16**	**Primer 30-16**	**Mean**
UK↔ WK	0. 513	0.368	0. 717	0. 549	0. 537
WK ↔ PK	0. 434	0.573	0. 414	0. 573	0. 499
UK ↔ PK	0. 513	0. 490	0. 513	0. 459	0. 494

UK, WK and PK, indicate European kingfisher (*Alcedo atthis*), White-breasted kingfisher (*Halcyon smymenis*) and pied kingfisher (*Ceryle rudis*), respectively.

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