Genetic divergence and phylogenetic relationship among five sparid species from the coastal waters of Egypt based on protein profiling and RAPD molecular markers

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Abstract: Genetic variations between five sparid species from the northern coastal waters of Egypt; namely *Sparus aurata*, *Diplodus vulgaris*, *Diplodus sargus*, *Diplodus annularis* and *Lithognathus mormyrus*; were analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of sarcoplasmic proteins and rondom amplified polymorphic DNA (RAPD) analyses. SDS-PAGE profiles of cellular sarcoplasmic proteins were diagnostic at the species level; clear cut interspecific variations were observed between the different species. The obtained results revealed a total number of 11 bands at relative front (RF) range from 0.17-0.87. There were four monomorphic bands, six polymorphic bands and one specific band in *L. mormyrus* at RF 0.83 and molecular weight between 17-30 kDa. The average of polymorphism among the different species was 54.5%. RAPD assays were performed using a 20 arbitrary decamer primer panel; however, only 16 primers yielded a total of 191 scorable bands, of which 139 (73%) were polymorphic bands and 31 were specific markers. In addition, distinct bands (n = 5, 1 and 2) for *D. sargus, L. mormyrus* and *S. aurata* were obtained by using different primers, respectively. Similarity values ranged from 0.67 between *L. mormyrus* and *D. annularis* to 0.50 between *D. sargus* and *S. aurata.* The dendrogram showed that the five sparid species are separated from each other into two clusters: the first cluster included *D. vulgaris* and *D. sargus*, whereas the second cluster was divided into two groups, one included *L. mormyrus and D. annularis,* the other group *S. aurata*. In conclusion, these data serve as a useful tool for genetic identification and differentiation of these species in coastal water of Egypt and in the production of their interspecific hybrids.

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1. Introduction

Species are the basic unit of fish biodiversity, since much of evolutionary biology focuses on species limits. An accurate estimate of species limits is a key factor in improving the accuracy and validity of biodiversity assessment **(Puorto et al., 2001).**

Family Sparidae, or sea breams, are demersal fishes distributed worldwide from temperate to tropical waters; many of them live in South African waters. They are chiefly marine, usually inshore, but some periodically enter estuaries **(Hanel and Tsigenopoulos, 2011)**. Hermaphroditism, both protandrous and protogynous, is common in this family **(Smith and Heemstra, 2003)**. There are about 112 species belonging to 35 genera **(Fishbase, 2010).** Most species of the family Sparidae, and especially those species native to the North Eastern Atlantic and Mediterranean coastal waters, are of high commercial importance as food and for recreational fishing **(De la Herrán et al., 2001).** The classification and the phylogenetic relationships of these species, however, remain controversial because traditionally based mainly on the dentition and diet.

The family Sparidae is represented in the Mediterranean Sea by 11 genera) *Dentex, Sparus, Diplodus, Pagellus, Pagrus, Lithognatus, Spondyliosoma, Oblada, Crenidens, Boops* and *Sarpa* (and 24 species that usually inhabit coastal areas and produce pelagic eggs and larvae **(Bauchot and Hureau, 1986)**. There are about 64 fish families present in Mediterranean waters of Egypt, which are represented by 202 species; of these, the family Sparidae is the most dominant, comprising 21 species **(Ibrahim and Soliman, 1996)**. There are about 12 species of this family which are most frequent in the landed catch from Alexandria waters, on the northern coast of Egypt. Those with the highest economic value are *Diplodus annularis, Diplodus sargus, Diplodus vulgaris, Lithognathus mormyrus and Sparus aurata.* Seabreams represented about 15% of the landed catch

in 2011 from Egyptian Mediterranean waters **(GAFRD, 2008)**.

Genetic analysis of electrophoretically detectable variation is a useful mean of inferring the genetic structures of natural and farmed populations and for delineating taxonomic relationships **(Hauser et al., 1998)**. There are a number of different molecular marker techniques which have been applied to study genetic variation and fingerprinting in fish populations. The use of biochemical methods, such as isozymes and protein banding patterns for species identification has been frequently applied in fish **(Na-Nakorn et al., 1998; Na-Nakorn, 2001)**. Furthermore, the use of biomolecular methods, such as the RAPD technique **(Qiubai et al., 2006)**, AFLP technique (Simmones et al., 2006), and microsatellite DNA analysis **(Wachirachaikam and Na-Nakron, 2007)** have been adopted to analyze the genetic divergence and phylogenetic relationship among fish species.

Considering the high economic value of the Egyptian Mediterranean sparid species and the uncertainties surrounding their phylogenetic relationships owing to limited research, the present study was carried out to estimate the genetic structure and diversity among the most important five species of the family Sparidae in Egypt and to construct their phylogenetic relationships, using SDS-PAGE and RAPD-PCR markers. Studies on the genetic diversification and evolutionary relationships of these species are limited. So, the objective of this study was, therefore, to resolve the taxonomic ambiguities and document inter-specific genetic variability among these species.

2. Materials and Methods

Protein Isolation and SDS-PAGE

Five species of the family Sparidae: *S. aurata*, *D. sargus*, *D. vulgaris*, *D. annularis* and *L. mormyrus* were used in the present study. Hundred specimens (20 for each species) were collected from the Eastern Harbour of Alexandria, Egypt and samples of white skeletal muscles were dissected out and frozen. Samples were then homogenized for 2 min in appropriate volume of extraction solution (50 mM Tris-HCl, pH 7.6).The homogenate was centrifuged at 8000 rpm /15 min. The obtained supernatant for all samples was used to determine the protein concentration according to **Bradford (1976),** then placed at -20^{o} C until use. Protein samples were prepared by mixing 100 mg proteins with 5X sample application buffer (0.6 M Tris-HCl, pH 6.8, 1% (w/v) SDS, 10% B-mercaptoethanol, 10% sucrose and 0.05% bromophenol blue) to give a final concentration of 1X. Protein samples (100 mg) were then boiled in water bath for 4 min at 95^{o} C and then subjected to 10% SDS-PAGE gel, according to **Laemmli (1970).** The control well was loaded with an appropriate volume of standard protein markers with molecular weights (MW) of 80, 30 and 17 kDa.

RAPD-PCR analysis

DNA extraction

Whole blood samples were collected from 50 fish individuals (10 for each species). It was withdrawn from heart and then transferred to separate 5-ml tubes containing K_3-EDTA as anticoagulant. DNA was isolated from samples samples with the Thermo Scientific Gene Jet Blood Genomic DNA Purification Mini Kit (Fermentas, EU) according manufacturer's instructions. The purified DNA was stored at -20^{o}C until use. DNA concentration (µg/µl) was evaluated using a spectrophotometer. DNA purity was measured at 260/280 nm, and a reading greater than 1.8 was taken to indicate a high degree of purity **(Sambrook and Russel, 2001)**. Additionally, the quality and quantity of sample DNA was determined by electrophoresis on a 1.5% agarose gel containing a 1kb DNA Ladder (Fermentas, EU) as a standard.

Primer selection

DNA samples from each species were screened using a panel of 20 decamer arbitrary primers synthesized by Operon Technologies (Operon Technologies Inc., Alameda, USA). Primer codes and sequences are shown in Table 1. Sixteen primers were selected, based on their distinct polymorphisms revealing patterns of identifiable amplified bands.

RAPD-PCR conditions and electrophoresis

RAPD-PCR was performed with the selected primers on pooled DNA samples to assess genetic diversity and DNA polymorphisms among the five species of the family Sparidae, and the results were used to construct a dendrogram. PCR was carried out in 25-µl reaction volume, containing 12.5µl of PCR master mix (Fermentas, EU), 2.5µl of decamer primer (10 pmol ml^{-1}), 2.5µl of genomic DNA (25ng/µl), and 7.5µl of sterile distilled water. DNA amplifications were performed using a Biometra thermal cycler (Biometra, Germany) under the following cycling conditions: 40 cycles of 94^{o}C for 30 s, 38^{o}C for 30 s, and 72^{o}C for 1 min. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide (0.5µg/µl) following electrophoresis in 1X TBE buffer for 2 h at 100V. A 1kb DNA ladder (O´ Gene Ruler™ 1kb; Fermentas, EU) was used as a molecular size marker. Gels with clear banding patterns were photographed under UV light for documentation and further analysis.

Statistical analysis

Gels of protein electrophoresis were analyzed using gel analyzer software program (ver. 3) while gel photographs of DNA electrophoresis were analyzed using Total Lab (ver. 2.01) and SPSS (ver.15)

software. The profiles were scored as binary data (1 if the band present and 0 if absent). Similarity indices between species were calculated using the formula: $S_{xy} = 2N_{xy} / (Nx + Ny)$ where N_{xy} is the number of bands shared by x and y species, Nx and Ny are the number of bands scored for each species, and S_{xy} is the similarity index between x and y species. SPSS program was used to construct a dendrogram of the combined binary data derived from all DNA patterns to determine the phylogenetic relationships between the studied species, according to the methods of **Sneath and Sokal, (1973)**; **Bardakci and Skibinski, (1994),** using the unweighted pair group method of analysis (UPGMA).

3. Results and Discussion

The family Sparidae is of significant importance for marine aquaculture, due to their wide geographic distribution, favourable growth rates and high market demand **(Oliva-Teles, 2000; Mylonas et al., 2004).** In the northern coastal waters of Egypt, there are five species of this family with high economic value, namely *S. aurata, D. sargus, D. vulgaris, D. annularis* and *L. mormyrus*.

Cellular protein profile

Electrophoretic analysis (SDS–PAGE) was carried out on sarcoplasmic proteins, whose patterns were diagnostic at the species level (Figure 1). The presence or absence of different bands in each species was illustrated as (1) for presence and (0) for absence. The total numbers of bands were 11 and ranged at relative front (RF) from 0.17-0.87 (Table 2). The total number of bands were: 10 in *L. mormyrus* and *D. annularis*, 8 in *D. vulgaris* and *S. aurata*, and 7 in *D. sargus*. There were 4 monomorphic bands, 6 polymorphic bands and a single specific band in *L. mormyrus* (at RF 0.83 and MW 17-30 kDa) which can be used as diagnostic genetic marker for species identification. The percentage of polymorphism among the different species was 54.5%.

Analysis of sarcoplasmic protein banding pattern had a value in fish characterization, since fish sarcoplasmic proteins are not denatured immediately after death unlike other proteins (Kjaersgård and Jessen, 2003). Furthermore, 25 to 35% of muscle protein is formed by sarcoplasmic proteins which include low MW (40-60 kDa) proteins, like myoalbumin, globulins and enzymes, that can be extracted from water and natural salt solutions and separated by electrophoresis methods **(Huss, 1995 and Love, 1997**).

On the other hand **Basagalia, (1989)** detected species specific electrophoretic and isoelectric focusing patterns in his study on the soluble proteins of the eye lens and white muscle of 4 species of the genus Diplodus (*D. annularis, D. sargus, D. vulgaris,* and *D. Puntazzo*). Comparison of the protein profiles proved that *D. annularis* and *D. sargus* are closely related and distantly related to *D.vulgaris*. In addition, **Basagalia and Marchetli (1991)** examined the soluble proteins of white skeletal muscle tissue of 15 species of family Sparidae and detected species specific electrophoretic and isoelectric focusing patterns. They also found considerable similarity among species of the genera *Sparus*, *Pagellus* and *Diplodus.*

RAPD profile

A panel of 20 decamer arbitrary primers was used to screen the DNA samples of each species. Only 16 primers: OPC20, OPA18, OPC08, OPC05, OPA7, OPA10, OPA14, OPB5, OPA15, OPAI, OPB12, OPC06, OPA1, OPB08, OPB10 and OPA16 produced distinct polymorphism with patterns of identifiable amplified bands. The results of RAPD profiles showed strongly differentiated fingerprints of the five sparid species, so their discrimination was easy. This coincides with the reports by **Welsh and McClelland (1990); Hadreys et al., (1992).** The RAPD-PCR results are shown in Figs. (2–9). DNA polymorphisms detected by RAPD were identified by two traits: first, by the presence (or absence) of one or more RAPD fragments of a particular size and second, by changes in the intensity of fragments of the same size. The RAPD technique has been widely used to estimate genetic variations within and among fish populations of *D. sargus* and *D.vulgaris* **(Pereira et al., 2010)** as well as *Oreochromis niloticus* **(Rashed et al., 2008).**

DNA fragments amplified with the 16 primers yielded a total of 191 scorable bands, out of which 139 (73%) were polymorphic bands, while 31 were specific markers and the rest common bands. The number of polymorphic bands was relatively high among the studied species and was acquired without any previous knowledge of the sparid genomes. All primers, except OPC20, OPA18, OPB12 and OPA1, generated species–specific markers as summarized in Table (3).

The RAPD-PCR technique, developed by **Williams et al., (1990),** was successfully used for the analysis of genetic similarities and for phylogeny reconstruction (**Abdul Muneer et al., 2011; Bhati et al., 2012**). Nevertheless, the effectiveness of RAPD markers in the detection of genetic polymorphisms within and among different species (random, un-characterized multiple genome loci; dominant nature of markers; and possibility of co-migrating, nonhomologus bands) can lead to limitations. Despite these limitations, RAPD analysis can be used effectively for the initial assessment of genetic variation among fish species **(Barman et al., 2003).**

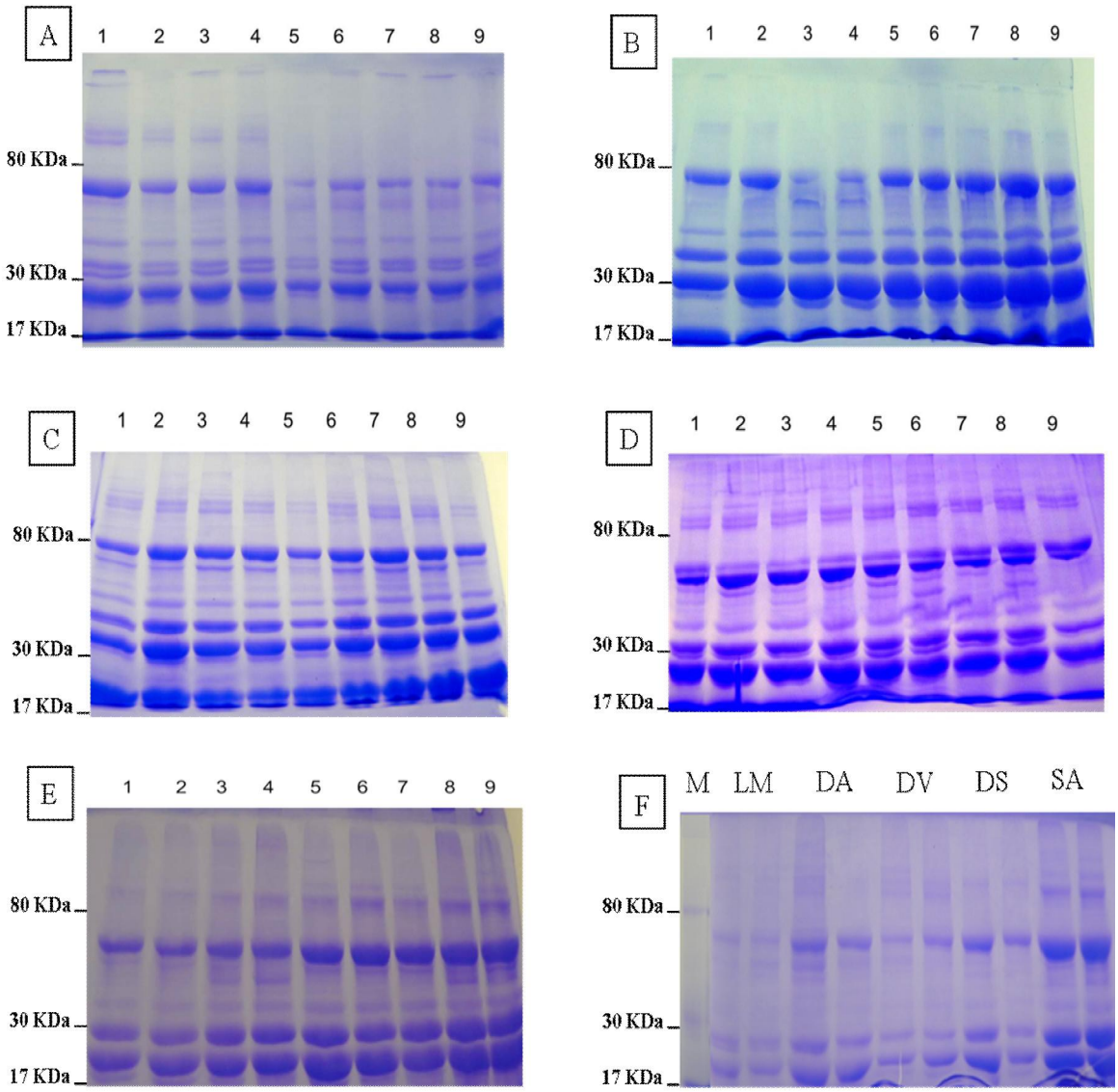

Figure 1. Protein bands for sarcoplasmic proteins of five sparid species obtained by SDS- PAGE and stained with coomassie blue: A= *Lithognathus mormyrus* (LM), B= *Diplodus annularis* (DA), C= *Diplodus vulgaris* (DV), D= *Diplodus sargus* (DS), E= *Sparus aurata* (SA), and E= bulked protein samples from each population. M= Protein Marker.

A number of distinguished bands, 5, 1 and 2, for the species *D. sargus, L. mormyrus* and *S. aurata)*, respectively were obtained with different primers (OPA14 at MW 1134bp; OPA15 at MW 1426, 645, 514, 357 bp), (OPB10 at MW 213bp), and (OPB5 at MW 174 bp, OPC06 at MW 5621 bp) respectively. These bands are true genetic markers.

The similarity index among the sparid species was calculated according to band sharing. As shown in Table (4) and it is ranged from 0.672 between *L. mormyrus* and *D. annularis* to 0.502 between *D. sargus* and *S. aurata*.

The analysis of RAPD data by the UPGMA cluster program divided the studied species into two clusters, the first cluster included two closely related species (*D. vulgaris* and *D. sargus*), whereas the second cluster was divided to two groups, one group included another two closely related species (*L. mormyrus and D. annularis*), the other group contained *S. aurata* (Figure 10). Similar results were obtained by by **De La Herrán et al., (2001),** who studied the phylogenetic relationships of 16 sparid species by using two satellite DNA families. They found two major lineages: one including the species of the genera *Diplodus, Sparus, Lithognathus, Spondilyosoma, Boops, Sarpa* and *Pagellus (P. bogaraveo)*, the other comprising the species of *Dentex, Pagrus* and *Pagellus (P. erythrinus).*

Actually, **Dujakovic and Glamuzina (1990)** performed interspecific hybridization between *Sparus aurata* x *Diplodus vulgaris* and they obtained a successful hybrid.

In conclusion, our results suggest that, since *Diplodus vulgaris* is related to *Diplodus sargus* and *Lithognathus mormyrus* to *Diplodus annularis,* these pairs might be crossed to generate successful hybrids. In addition, these data are valuable for both species characterization and breeding programs and we prospect the need for future research.

Table 1. Primer codes and sequences of the twenty arbitrary 10-mer RAPD markers employed for the genetic characterization of five species of the family Sparidae

Primer code	Nucleotide sequence (5'-3')	Primer code	Nucleotide sequence (5'-3')
OPA16	AAG CGA CCG A	OPB3	CAT CCC CCT G
OPB10	CAG GGT CGT C	OPA1	ACA GGT GCT G
OPC11	GGC GTC GAA A	OPA18	AGG TGA CCG T
OPC06	CTC AGG CAA G	OPA10	GTG ATC GCA G
OPC20	CAC CGC TTC A	OPA I	CAG GCC CTT C
OPC08	GTG GCC AGG T	OPA8	GTG ACG TAG G
OPB12	ACG CAG TTC C	OPA14	TTC GAG CCA G
OPC05	CCG CCA GTA G	OPA7	TGG CGC AGT G
OPA20	CCT AGC GTT G	OPA15	TTC GAG CCA G
OPB08	GGC ACA CCT G	OPB5	TGC GCC CTT C

Table 2. Results of SDS-PAGE of sarcoplasmic proteins among the five sparid species*

BN	**RF**	**LM**	**DA**	**DV**	**DS**	**SA**	**BF**
1	0.17	0	1	0	0	1	0.40
2	0.26	1	1	1	1	0	0.80
3	0.30	1	1	1	0	1	0.80
4	0.41	1	1	1	0	0	0.60
5	0.46	1	1	1	1	1	1
6	0.51	1	1	1	1	1	1
7	0.58	1	1	0	1	1	0.80
8	0.70	1	1	1	1	1	1
9	0.79	1	1	1	1	1	1
10	0.83	1	0	0	0	0	0.20
11	0.87	1	1	1	1	1	1

*(1) indicates the presence of a band, (0) indicates the absence of a band. BN=Band number, RF= Relative front, BF= Band frequency, *LM = Lithognathus mormyrus, DA= Diplodus annularis, DV = Diplodus vulgaris, DS = Diplodus sargus, SA = Sparus aurata*

Table 3. Number of amplified fragments and specific markers for five species of the family Sparidae, based on RAPD-PCR analysis*

Primer code	Species										TAF	PB	TSM
	DS		DV		LM		DA		SA				
	AF	SM	AF	SM	AF	SM	AF	SM	AF	SM			
OPC20	9	0	8	0	9	0	7	0	10	0	14	13	0
OPA18	6	0	7	0	7	0	6	0	6	0	9	7	0
OPC08	10	0	10	1	11	1	13	1	8	0	16	6	3
OPC05	9	2	4	0	4	0	11	1	8	2	15	10	5
OPA7	7	0	3	0	11	2	8	0	11	1	14	11	3
OPA10	5	1	7	1	7	1	3	0	3	0	10	6	3
OPA14	11	1	12	0	9	0	10	0	9	0	14	10	1
OPB5	6	0	5	0	4	0	6	0	2	1	7	5	1
OPA15	11	4	6	0	7	0	1	0	4	0	12	8	4
OPAI	6	2	5	1	0	0	0	0	2	1	8	4	4
OPB12	5	0	1	0	2	0	8	0	6	0	9	9	0
OPC06	5	0	7	0	12	0	11	0	9	1	15	13	1
OPA1	9	0	8	0	10	0	7	0	8	0	11	8	0
OPB08	0	0	0	0	5	0	9	1	10	1	11	9	2
OPB10	6	0	8	0	10	1	8	0	9	0	12	9	1
OPA16	0	0	13	2	4	0	9	0	9	1	14	11	3

*List of abbreviations: AF = Amplified Fragments, SM = Specific Marker including either the presence or absence of a band in a specific species, TAF = Total Amplified Fragments, PB = Polymorphic Bands, TSM = Total no. of Specific Markers across species.

Table 4. Similarity indices showing of five species of the family Sparidae, as determined by RAPD analysis with 16 different primers

Species	DS	DV	LM	DA	SA
DS	1.000				
DV	0.612	1.000			
LM	0.608	0.602	1.000		
DA	0.568	0.624	0.672	1.000	
SA	0.502	0.532	0.637	0.641	1.000

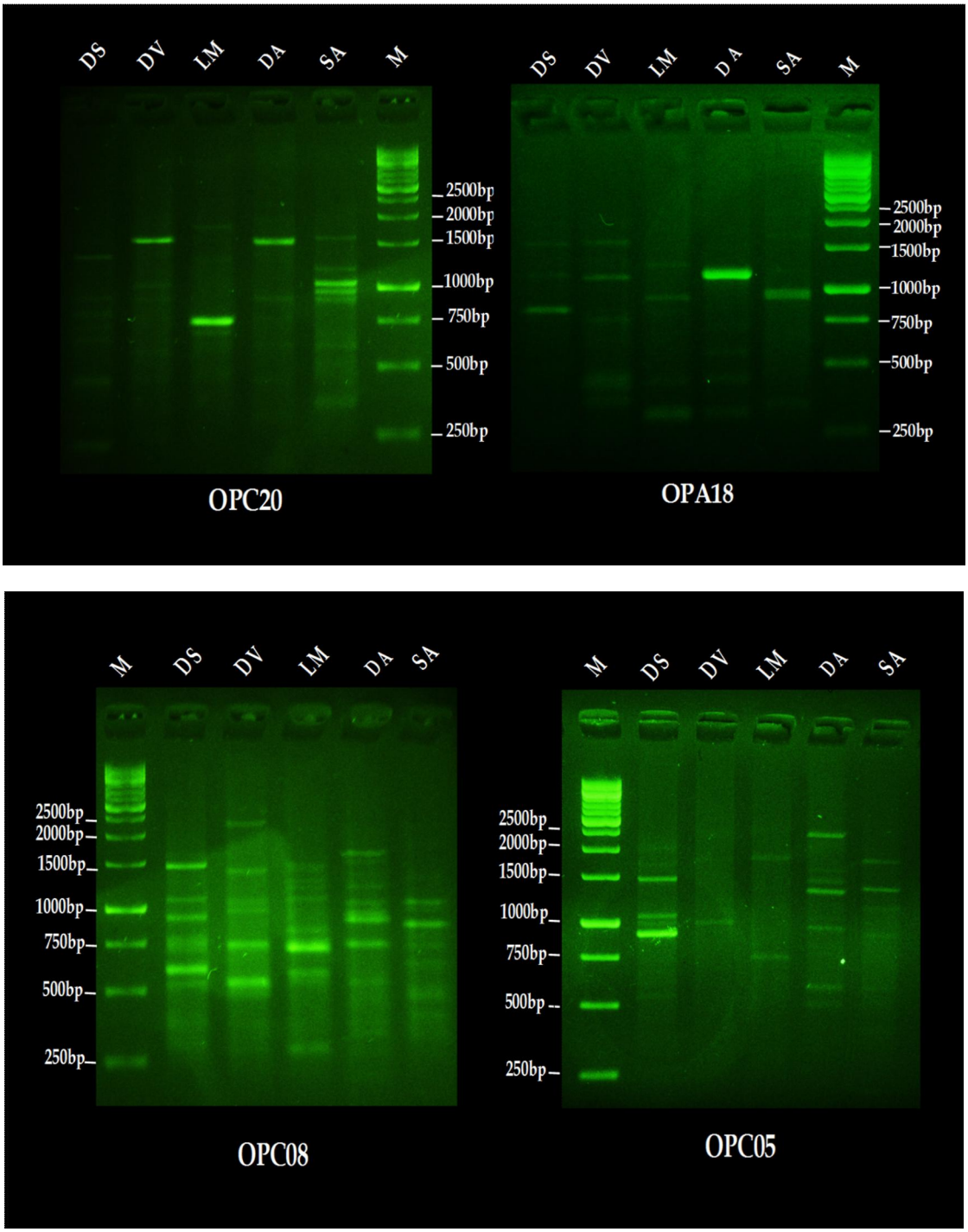

Figure 2. RAPD-PCR profile for five sparid species produced by using OPC20, OPA18, OPC08, and OPC05 primers. M = Molecular marker, *DS = Diplodus sagrus, DV = Diplodus vulgaris, LM = Lithognathus mormyrus, DA = Diplodus annularis, and SA = Sparus aurata*

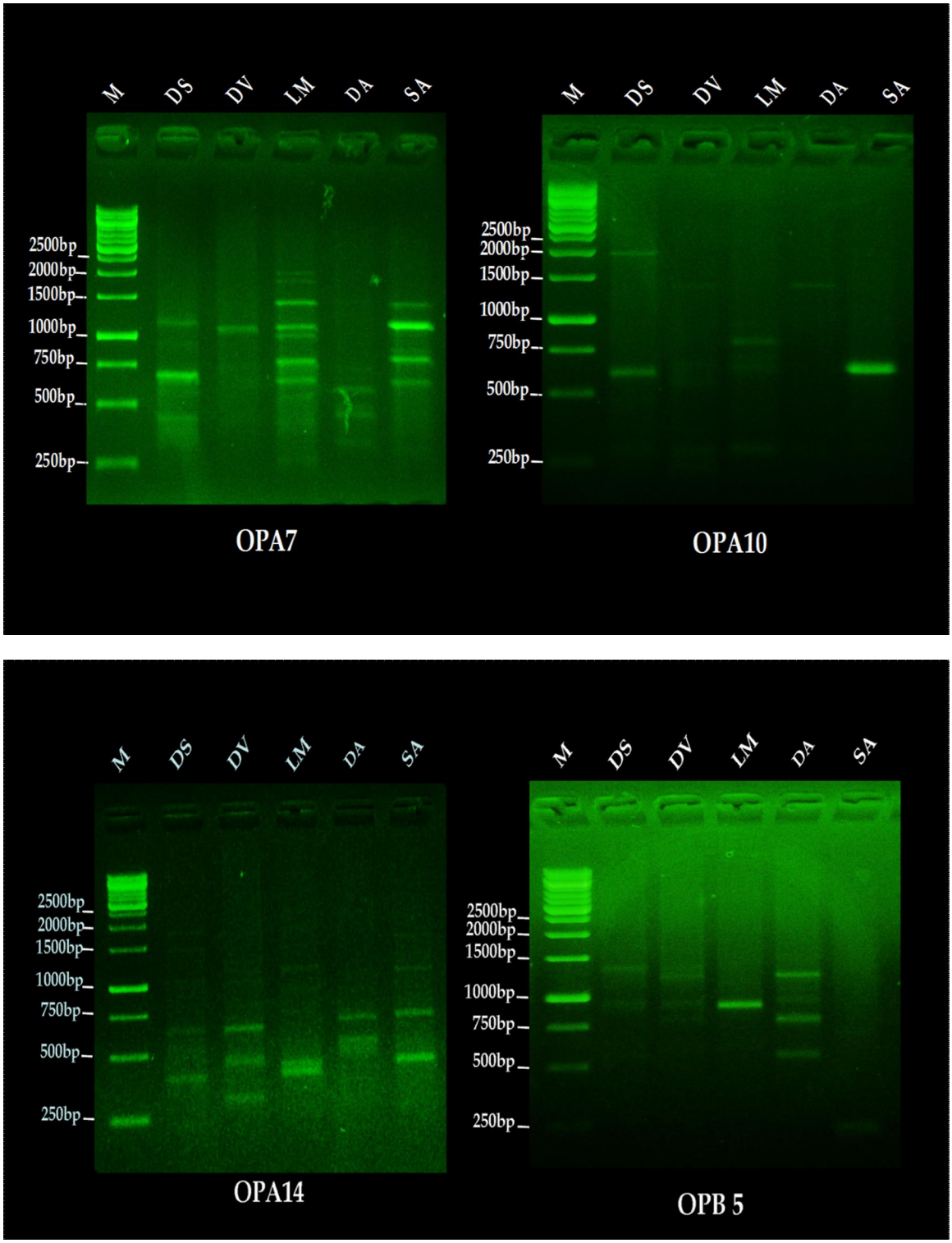

Figure 3. RAPD-PCR profile for five sparid species produced by using OPA7, OPA10, OPA14, and OPB5 primers. M = DNA Marker, *DS = Diplodus sagrus, DV = Diplodus vulgaris, LM = Lithognathus mormyrus, DA = Diplodus annularis, and SA = Sparus aurata*

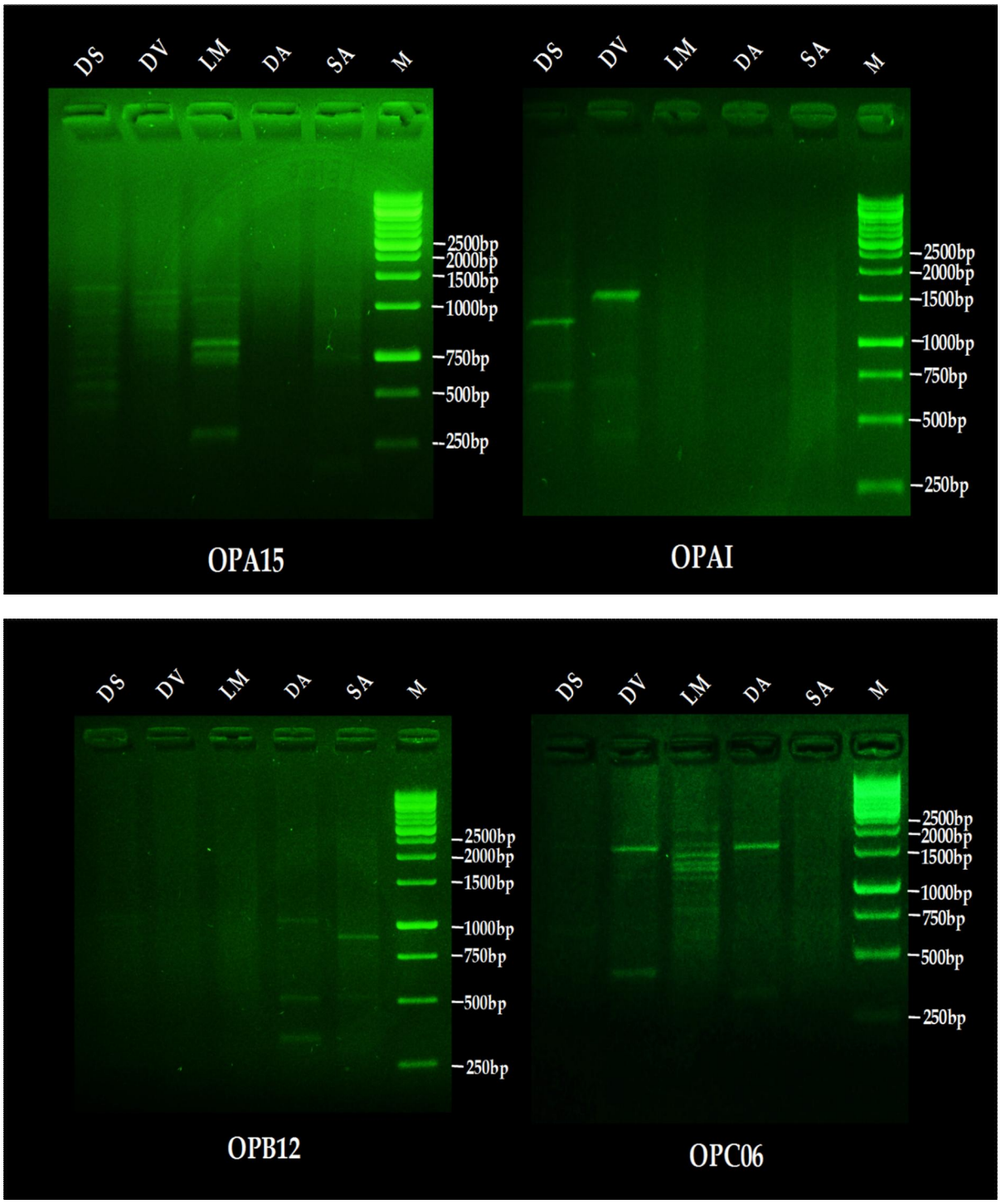

Figure 4. RAPD-PCR profile for five sparid species produced by using OPB12, PC06, OPA15**,** and OPAI primers. M = DNA Marker, *DS = Diplodus sagrus, DV = Diplodus vulgaris, LM = Lithognathus mormyrus, DA = Diplodus annularis, and SA = Sparus aurata* .

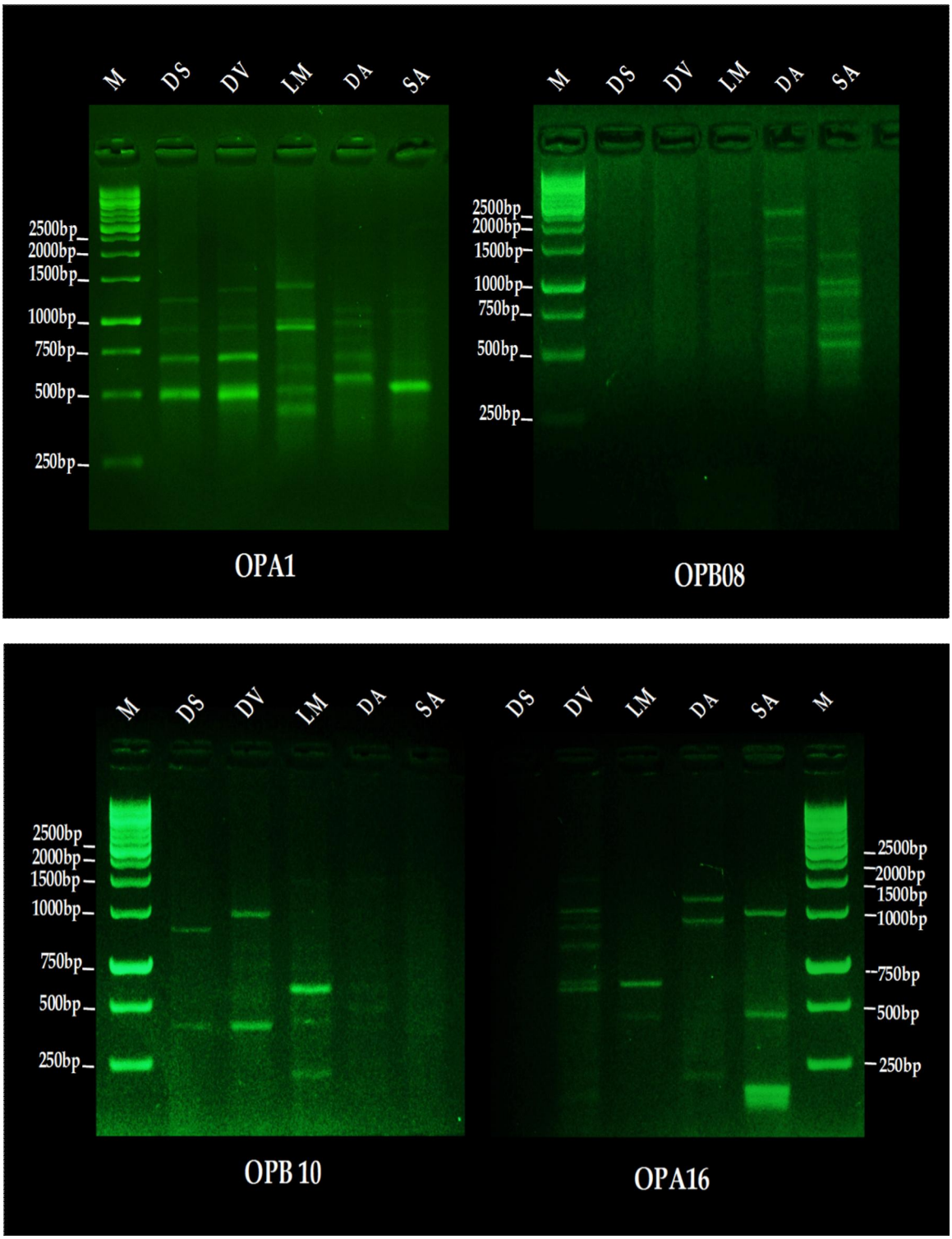

Figure 5. RAPD-PCR profile for five sparid species produced by using OPA1**,** OPB08, OPB10, and OPA16 primers. M = DNA Marker, *DS = Diplodus sagrus, DV = Diplodus vulgaris, LM = Lithognathus mormyrus, DA = Diplodus annularis, and SA = Sparus aurata.*

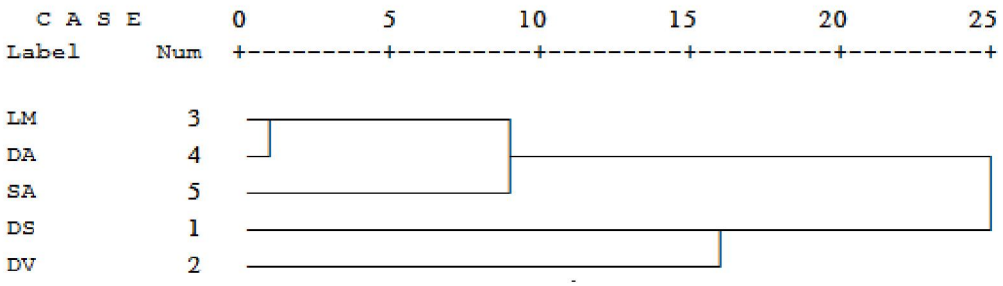

Figure 6. UPGMA Dendrogram revealing the genetic relationships among five species of the family Sparidae as inferred from RAPD-PCR analysis

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