Potential application of RAPD and RAHM markers in genome analysis of scombroid fishes

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Total genomic DNA from 30 different specimens of Indian mackerel *Rastrelliger kanagurta* (Cuvier) and 5 king seer *Scomberomorus commerson* (Lacepede) was extracted by a simple modified phenol–chloroform protocol. PCR amplification conditions using 10-mer arbitrary primers were optimized. Primers with the same nucleotide sequences produced varying Random amplified polymorphic DNA (RAPD) patterns and those with different sequences generated different banding patterns for the same template in the two commercially important scombroid fishes. Primer OPA 07 (GAAACGGGTG) yielded more polymorphic loci with high reproducibility. Better performance of the primers from Kit A (Operon Technologies, Inc) is attributed to their high G + C content. RAPDs were highly sensitive to the concentrations of primer, $MgCl_2$ and the brand of *Taq* DNA polymerase enzyme. Random amplified hybridization microsatellites (RAHM) can overcome the inherent shortcomings of RAPDs.

Random amplified polymorphic DNA (RAPD) is a novel technique of revealing DNA-based arbitrarily primed polymorphisms[1,2]. Compared with multilocus fingerprinting using microsatellite probes, the RAPD markers are more useful in discriminating populations since they are less variable over generations[3]. RAPD was found to be technically easier with low statistical error, whereas multilocus fingerprinting with 33.15 probe detected more genetic differentiation among strains of *Oreochromis niloticus*[4].

RAPDs have been particularly useful in the genetic fingerprinting of plants[5]. They are used for creating linkage maps, locating disease resistance genes and for identifying chromosome-specific markers. This PCR-based fingerprinting has begun to find application in fish and shellfish genetic analyses. RAPDs were used to detect radiation-induced DNA damages in *Oryzias latipes*[6]. Fingerprinting of 12 species of fishes using arbitrary primer PCR was reported[7]. The technique was applied for species and subspecies identification of *Oreochromis*[8]. Seven hundred and twenty one RAPD polymorphisms were identified between two laboratory strains of zebra fish, *Danio*[9] and 401 of them were used to construct a genetic linkage map[10]. Genetic variations in samples of seabass, *Dicentrarchus labrax* before and after acclimation to freshwater were investigated[11]. Inheritance of RAPD markers in the F_1 progeny of single-pair crosses between two guppy (*Poecilia reticulata*) varieties was studied[12]. Three polymorphic markers were observed out of a total of 48 RAPD loci amplified by 14 arbitrary primers in penaeid prawn *Penaeus monodon*[13].

Random amplified hybridization microsatellites (RAHM) is a new system for microsatellite DNA detection and isolation which combines RAPD amplification and oligonucleotide screening[14]. A similar technique known as *R*andom *A*mplified *M*icrosatellite *PO*lymorphisms (RAMPO) is described[15]. The rapid use and acceptance of microsatellites as genetic markers is due primarily to their comparative ease of assay via PCR

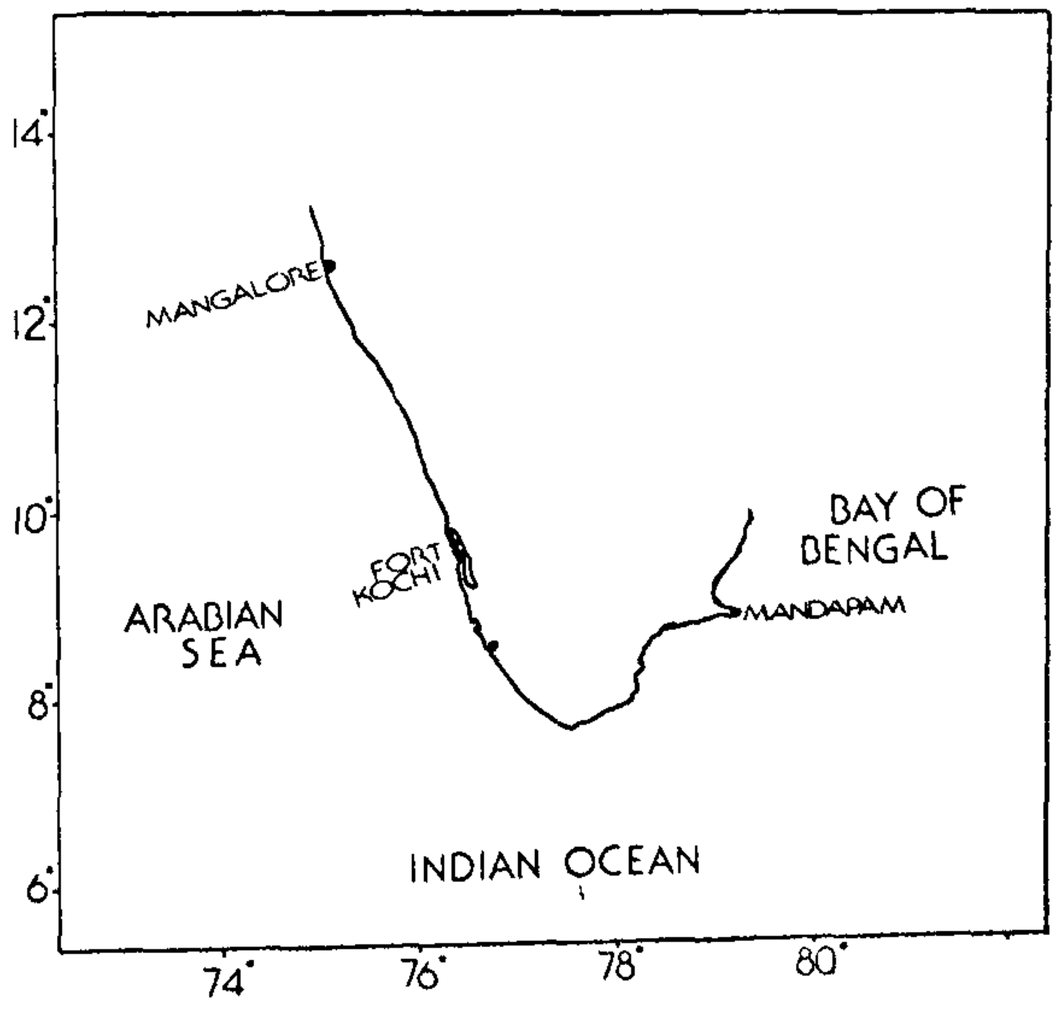

Figure 1. Map showing sampling localities.

Kit A OPA 03 (AGTCAGCCAC), OPA 07 (GAAACGGGTG), OPA 10 (GTGATCGCAG), OPA 13 (CAGCACCCAC), OPA 16 (AGCCAGCGAA), and OPA 20 (GTTGCGATCC).	OPA 01 (CAGGCCCTTC), OPA 04 (AATCGGGCTG), OPA 08 (GTGACGTAGG), OPA 11 (CAATCGCCGT), OPA 14 (TCTGTGCTGG), OPA 18 (AGGTGACCGT),	OPA 02 (TGCCGAGCTC) OPA 06 (GGTCCCTGAC) OPA 09 (GGGTAACGCC) OPA 12 (TCGGCGATAG) OPA 15 (TTCCGAACCC) OPA 19 (CAAACGTCGG)
Kit F: OPF 03 (CCTGATCACC), OPF 07 (CCGATATCCC), OPF 10 (GGAAGCTTGG), OPF 19 (CCTCTAGACC) and	OPF 01 (ACGGATCCTG), OPF 04 (GGTGATCAGG), OPF 08 (GGGATATCGG), OPF 13 (GGCTGCAGAA), OPF 20 (GGTCTAGAGG).	OPF 02 (GAGGATCCCT) OPF 06 (GGGAATTCGG) OPF 09 (CCAAGCTTTC) OPF 18 (TTCCCGGGTT)
Kit G: OPG 18 (GGCTCATGTG) and	OPG 05 (CTGAGACGGA), OPG 20 (TCTCCCTCAG).	OPG 13 (CTCTCCGCCA)

Box 1.

and accuracy of scoring allelic types[16]. In the present study, we have optimized conditions for generating RAPD markers of two commercially important scombroid fishes and attempted RAHM technique potentially useful to discriminate fish populations. DNA samples from 30 different specimens of Indian mackerel *Rastrelliger kanagurta* (Cuvier) and 5 specimens of king seer *Scomberomorus commerson* (Lacepede) were analysed.

Materials and methods

Fish and tissue sampling

Specimens of Indian mackerel of total length range 140–250 mm were collected from commercial gill net landings at Mandapam, Mangalore and Fort Kochi and those of king seer from Mandapam during 1995–96 (Figure 1).

At Mandapam, either liver or muscle was dissected out from the fishes soon after their landings and transported to the laboratory in one of the three following media: (a) crushed ice, (b) lysate buffer (1 M Tris HCl pH 8.0; 0.4 M EDTA pH 8.0; 2% SDS), (c) 95% ethanol. Muscle samples of Indian mackerel from Fort Kochi and Mangalore fish landing centres were preserved and transported in 95% ethanol. In the laboratory all tissues were immediately stored at –70°C until DNA extraction. Total genomic DNA was extracted from 11, 11 and 8 different individual mackerels from Mandapam, Mangalore and Fort Kochi, respectively and from 5 specimens of king seer landed at Mandapam.

Extraction of genomic DNA

We have developed a simple and efficient protocol to extract total genomic DNA from field-collected fish. The preparation was found suitable equally for PCR and hybridization-based fingerprinting applications. Briefly, 0.4–1.0 g of tissue is placed in a mortar and kept at –70°C for 1–2 h. Use of liquid nitrogen to flash freeze tissue is obviated in our protocol. The frozen tissue is well ground and immediately mixed with homogenizing buffer (100 mM Tris HCl pH 8.0; 10 mM EDTA pH 8.0; 1.4 M NaCl; 1% CTAB; 0.2% mercaptethanol) and incubated with 150 µg/ml proteinase K (Sigma) at 37°C for 4–12 h. This is followed by phenol–chloroform extraction, precipitation of DNA with cold absolute alcohol, vacuum drying and resuspension in TE. RNA is removed by RNase (Sigma) at 250 µg/ml. DNA is then reextracted and resuspended in TE. Quality and quantity of the preparation were checked by agarose gel electrophoresis and spectrophotometry.

RAPD primers

A total of 35 arbitrary primers from A, F and G kits of Operon Technologies, Inc were tried to amplify DNA by PCR. The nucleotide sequences of the primers used in the present work are given in Box 1.

Optimization of primer and $MgCl_2$ concentrations

Initially four different concentrations each of arbitrary primer (0.32, 0.64, 1.28 and 2.56 µM) and $MgCl_2$ (2.0, 2.2, 2.4 and 2.6 mM) were tested to determine the optimum levels of their requirements for DNA amplification. Better resolution was obtained at 0.64 µM of primer and 2.4 mM of $MgCl_2$.

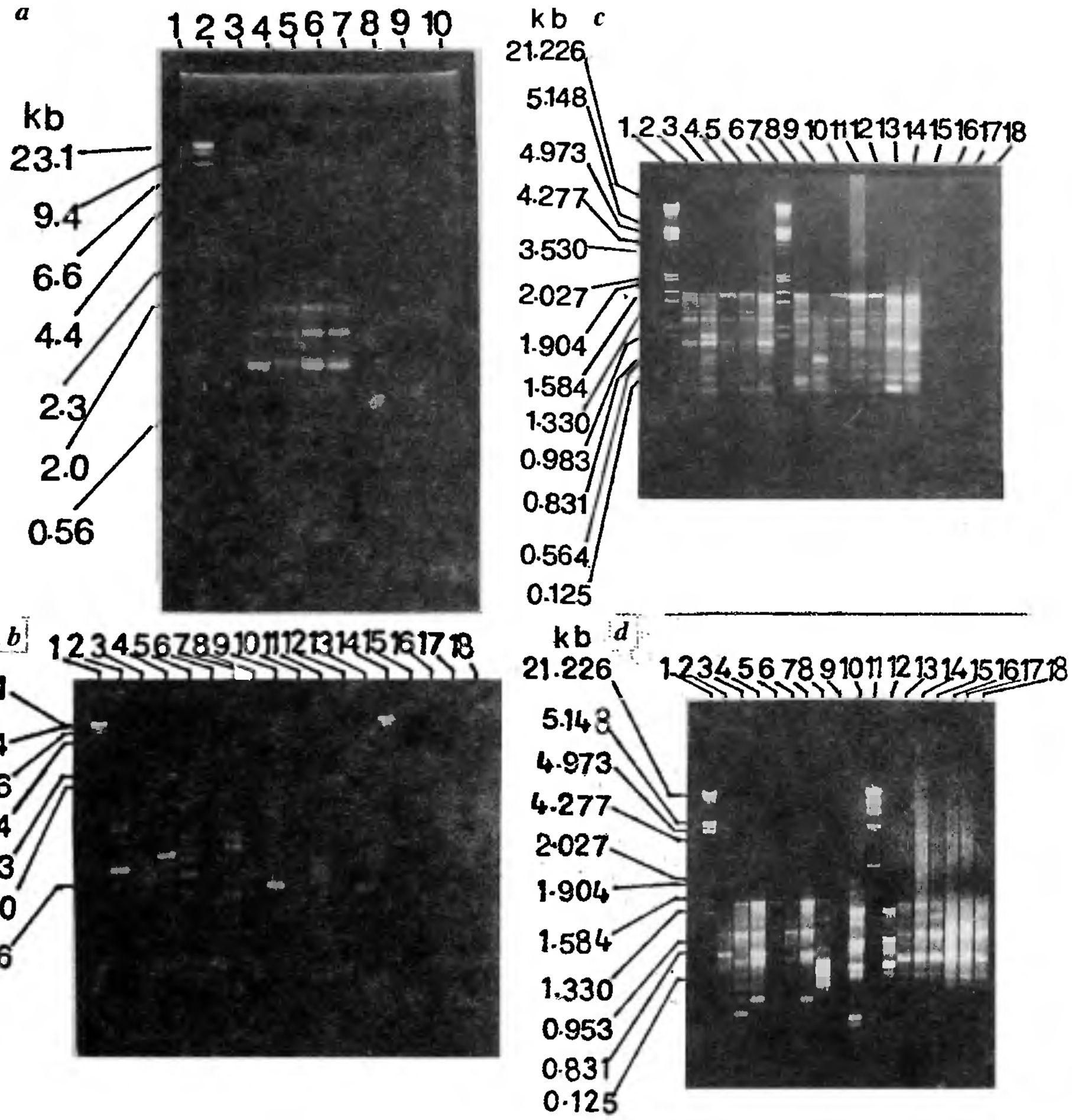

Figure 2. ***a***, Effect of varying concentrations of $MgCl_2$ on RAPD patterns in Indian mackerel (*Taq* 1). Lane 1, *λ Hin*dIII; Lanes 2 and 7–10, Blank; Lane 3, 2.0 μm; Lane 4, 2.2 μm; Lane 5, 2.4 μm; Lane 6, 2.6 μm. ***b***, RAPDs with OPA, OPF and OPG primers (*Taq* 1). Lanes 1 and 14, *λ Hin*dIII; Lanes 2 and 3, Indian mackerel and king seer DNAs (OPF 01); Lanes 4 and 5, Indian mackerel and king seer DNAs (OPA 07); Lanes 6, 8, 10 and 12, one Indian mackerel specimen (OPG 05, 13, 18 and 20, respectively); Lanes 7, 9, 11 and 13, one king seer specimen (OPG 05, 13, 18 and 20, respectively); Lanes 15–18, Blank. ***c***, RAPDs in Indian mackerel generated by OPA 07 (*Taq* 1). Lanes 1 and 7, *λ Hin*dIII + *Eco*RI; Lanes 2 and 3, two specimens from Mandapam; Lanes 4–6 and 8–14, ten specimens from Fort Kochi; Lanes 15–18, Blank, ***d***, RAPDs in Indian mackerel generated by OPA and OPF primers (*Taq* 1 except for lanes 16–18). Lanes 1 and 11, *λ Hin*dIII + *Eco*RI; Lanes 2, 3 and 5–7, five specimens from Mandapam (OPA 07); Lanes 8 and 9, two specimens from Fort Kochi (OPA 08); Lanes 13–15, two specimens from Fort Kochi and one from Mandapam (OPF 01); Lanes 16–18, same as 13–15, but used a *Taq* DNA polymerase prepared in our laboratory. The individual DNAs amplified with OPA 07 are different from those amplified with the same primer in Figure 4.

DNA amplification by PCR

One to two hundred nanograms of DNA samples were amplified in 25 μl reaction mixtures containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 0.001% gelatin, 2.4 mM $MgCl_2$, 0.03 mM each of dATP, dTTP, dGTP and dCTP, 0.64 μM random primer and 1U *Taq* DNA polymerase (Amresco and Rama Biotechnologies). A total of 23 different Indian mackerel DNA templates from three locations under study were amplified with Amresco *Taq* polymerase (hereafter referred to as *Taq* 1) while a total of 30 templates (including the earlier 23) were amplified with Rama Biotechnologies *Taq* (hereafter referred to as *Taq* 2). To test the effect of primer sequences, random selection was made from the pool of DNA samples. King seer DNA was amplified

Table 1. Percentage of templates amplified by OPA-series primers (*Taq* DNA polymerase-Amresco)

Primer	No. of individual templates tested	No. of templates amplified (at least 1 polymorphic band present)	%	Remarks
OPA 01	5	0	0	
OPA 02	5	2	40.0	Bands unscorable
OPA 03	3	2	66.7	Bands unscorable
OPA 04	3	1	33.3	
OPA 06	3	3	100.0	Several scorable bands
OPA 07	23	14	60.9	Several polymorphic bands High reproducibility
OPA 08	9	2	22.2	
OPA 09	3	3	100.0	Several scorable bands
OPA 10	3	3	100.0	Several scorable bands
OPA 11	3	3	100.0	Only a single band scorable
OPA 12	3	0	0	
OPA 13	5	1	20.0	Bands unscorable
OPA 14	3	1	33.3	
OPA 15	3	0	0	
OPA 16	3	3	100.0	Few polymorphic bands
OPA 18	3	3	100.0	One template had a single scorable band
OPA 19	9	4	44.4	Two templates have only a single scorable band each
OPA 20	3	1	33.3	Only a single scorable band

Table 2. Percentage of templates amplified by OPA-series primers (*Taq* DNA polymerase-Rama Biotechnologies)

Primer	No. of individual templates tested	No. of templates amplified (at least 1 polymorphic band present)	%	Remarks
OPA 06	3	3	100.0	Several scorable bands
OPA 07	30	23	76.7	Several scorable bands which are reproducible
OPA 09	3	1	33.3	Several scorable bands
OPA 10	3	2	66.7	1-4 markers
OPA 11	3	2	66.7	2 markers
OPA 16	3	2	66.7	1-4 markers

with *Taq* 1 only. The total reaction mixture was overlaid with 25 µl of mineral oil (Sigma).

Amplification was performed in Coy II Tempcycler programmed for one initial cycle of 30 S denaturation (94°C), 30 S annealing (36°C) and 1 min extension (72°C) followed by 45 cycles of 30 S denaturation (94°C), 30 S annealing (36°C) and 2 min extension (72°C). At the end, a final extension for 7 min was performed at 72°C. Amplified products were resolved in 1.4% agarose (Sigma) gel electrophoresis in 1 × TAE. They were stained in ethidium bromide solution and under UV illumination either polaroid photographs or gel documentation pictures were taken. The RAPD bands are referred to as loci and markers interchangingly.

RAHM

RAPDs generated by OPA 07 in Indian mackerel were transferred to nylon membrane (Hybond-N, Amersham) by vacuum blotting. Thirty pmols of $(GT)_8$ (Rama Biotechnologies) were 5′ end labelled with 50 µCi of γ ^{32}PdATP. The unincorporated nucleotides were separated from the probe using Nuc Trap probe purification push column as per manufacturer's (Strategene) instructions. The purified probe had a radioactivity of 2.4×10^6 cpm. The nylon membrane containing immobilized RAPDs was prehybridized at 45°C for 3 h in a solution containing 0.1% SDS, 5 × each of SSPE and Denhardt's solution and approximately 64 µg of sonicated *E. coli* DNA. This was followed by hybridization at 45°C for 12 h in a solution containing 0.1% SDS, 5 × each of SSPE and Denhardt's solution, 64 µg of sonicated *E. coli* DNA, 1.5 g of Dextran sulphate and 120 µl of purified probe. The filter was given three final washes each for 20 min at 37°C in 3 × SSC and 0.3% SDS, dried, wrapped in cling film and developed in a phosphorimager (Molecular Dynamics Inc).

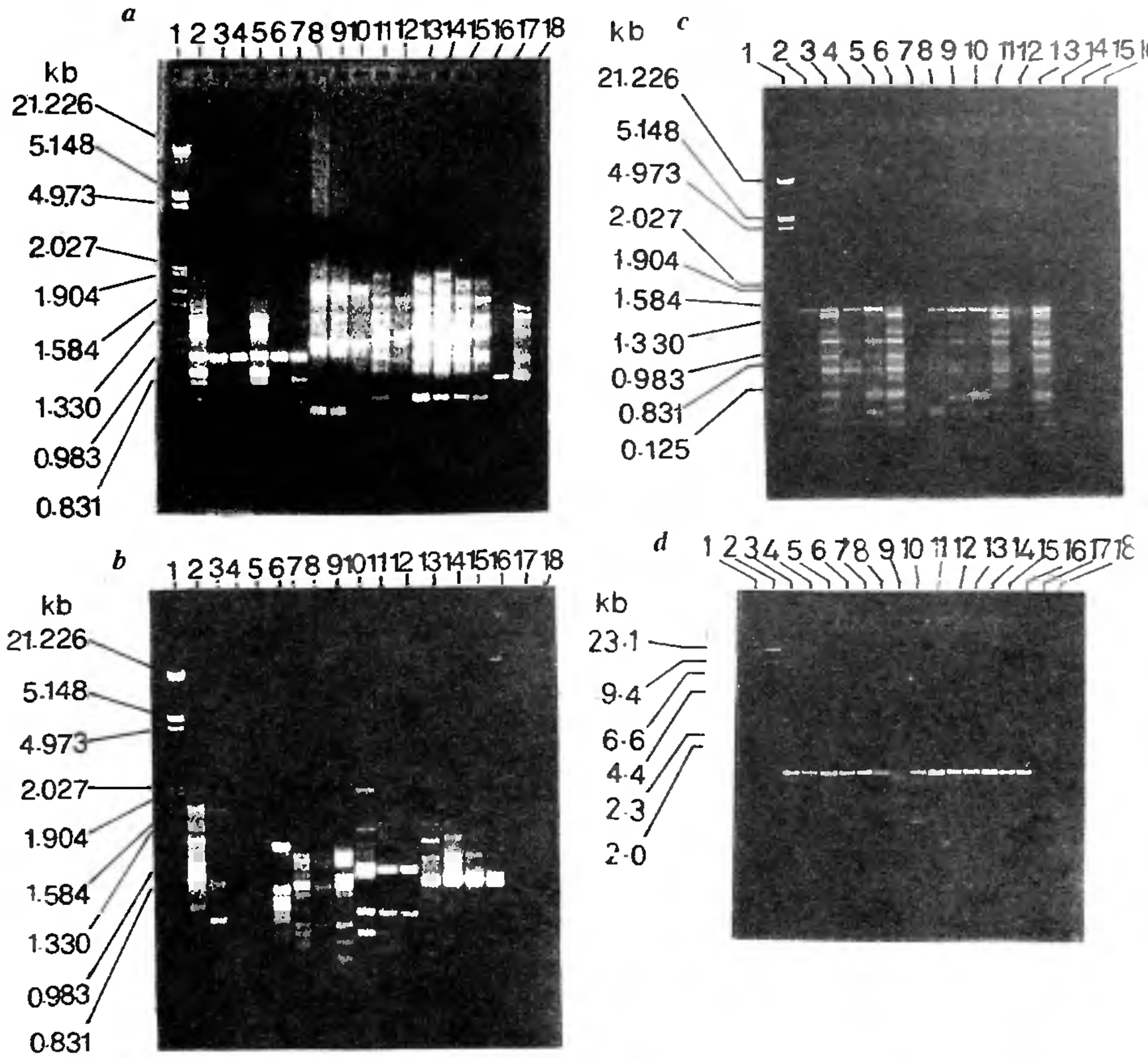

Figure 3. *a*, RAPDs in Indian mackerel generated by OPA and OPF primers (*Taq* 1), Lane 1, λ *Hin*dIII + *Eco*RI; Lanes 2, 5, 12, 15 and 18, a specimen from Fort Kochi (OPA 18, 19, 06, 10 and OPF 04, respectively); Lanes 3, 6, 11, 14 and 17, a specimen from Mandapam (OPA 18, 19, 06, 10 OPF 04, respectively); Lanes 4 and 7, a specimen from Mangalore (OPA 18 and 19, respectively); Lanes 8–10, 13 and 16, a specimen from Mangalore (OPF 18, 19, 20, OPA 06 and 10, respectively). *b*, RAPDs in Indian mackerel generated by OPA and OPF primers (*Taq* 1). Lane 1, λ *Hin*dIII + *Eco*RI; Lanes 2, 7, 10, 13 and 14, a specimen from Mandapam (OPF 03, OPA 09, 11, 14 and 16, respectively); Lanes 3, 5, 6, 9, 12 and 16, a specimen from Mangalore (OPF 06, 09, OPA 04, 09, 11 and 16, respectively); Lanes 4, 8, 11 and 15, a specimen from Fort Kochi (OPF 09, OPA 09, 11 and 16, respectively); Lanes 17 and 18, Blank. *c*, RAPDs in Indian mackerel generated by OPA 07 (*Taq* 1). Lane 1, λ *Hin*dIII + *Eco*RI; Lanes 2 and 11–13, four specimens from Fort Kochi; Lanes 5, 7, 9 and 10, four specimens from Mangalore; Lanes 14–16, Blank. The individuals are different from ones whose DNAs were amplified with OPA 07 as in Figure 2 *c*, *d*. *d*, RAPDs in Indian mackerel generated by OPA 07 (*Taq* 2). Lanes 1 and 2, λ *Hin*dIII; Lanes 3–7, five specimens from Mandapam; Lanes 8–11, four specimens from Fort Kochi; Lanes 12–16, five specimens from Mangalore. Lanes 17–18, Blank.

Results

Relatively better resolution of RAPD loci was obtained when 2.4 mM $MgCl_2$ was used (Figure 2 *a*, lane 5). With random primers from Kit G, 100 ng of genomic DNA each from king seer and Indian mackerel were amplified. While comparing lanes 6, 8, 10 and 12 with 7, 9, 11 and 13 in Figure 2 *b* it is apparent that the OPG random primers amplify DNA of king seer better than that of Indian mackerel. Further, lanes 2–13 show that difference in primer sequence would produce different RAPD patterns for the same template in both these scombroid fishes.

Considering the large number of templates tested, high percentage of amplification (amplification refers to the presence of at least one polymorphic band per gel) as well as better reproducibility, OPA 07 was found to be the ideal choice for producing RAPDs in Indian mackerel (Table 1). Figures 2 *c*, *d* and 3 *a–c* show RAPDs using *Taq* 1.

Performance of OPA 07 was even better in amplification of Indian mackerel DNA with *Taq* 2 (Table 2). Number as well as patterns of markers for the same templates generated with the same primers and two *Taq* enzymes were different. Figures 3 *d*, 4 *a–c* depict RAPD patterns using *Taq* 2.

Table 3. Percentage of templates amplified by OPF-series primers (*Taq* DNA polymerase-Amresco)

Primer	No. of individual templates tested	No. of templates amplified (at least 1 polymorphic band present)	%	Remarks
OPF 01	5	3	60.0	
OPF 02	8	2	25.0	
OPF 03	3	1	33.3	
OPF 04	3	2	66.7	One template had a single scorable band
OPF 06	3	1	33.3	
OPF 07	3	1	33.3	Bands unscorable
OPF 08	3	1	33.3	Bands unscorable
OPF 09	3	2	66.7	One template had a single scorable band
OPF 10	3	0	0	
OPF 13	3	2	66.7	One template had a single band Others had all unscorable bands
OPF 18	3	2	66.7	One template had a single scorable band
OPF 19	3	1	33.3	
OPF 20	3	1	33.3	

None of the random primers from Kit F produced loci in 100% of templates tested (Table 3). Further, the number of loci was less and reproducibility low. Figures 2 *d*, 3 *a* and *b* depict RAPD markers in different specimens of Indian mackerel sampled from Mandapam, Mangalore and Fort Kochi amplified with OPF primers.

RAHM markers of Indian mackerel are shown in Figure 4 *d*. Lanes 3, 4, 7, 8, 10 and 13 contain a total of 9 bands having complementary sequence of $(GT)_8$. This gives 25% of positive fragments after hybridization with the dinucleotide probe. Signal intensity of bands depends both on the microsatellite motifs and the abundance of the particular loci.

Discussion

RAPD could prove to be a useful tool to estimate genetic variability, relatedness, inbreeding levels, species/strain verification, pedigree analysis, detection of economic traits and in other marker-based studies in fish and shellfish. RAPDs that are diagnostic at different taxonomic levels can be generated by employing different primers and they can be used to determine the relatedness between taxa for which diagnostic RAPD fingerprints have been established[3]. However, for this PCR-based fingerprinting to provide supporting evidence for taxonomic relationships in fishes, data should be generated using adequate sample size for each species[7]. RAPD loci produced by the same random primer showed markedly different patterns between Indian mackerel and king seer in our work. Similarly, arbitrary primers of different sequences yielded different banding patterns in the same template of both scombroid fishes. This indicates utility of RAPDs by manipulation of primer sequence to generate amplification products of desired complexity to suit different purposes like genetic mapping or genotyping[7].

In a work on RAPD analysis of 12 species of fishes, there was no clear relation observed between the length of random primers and the number of fragments amplified. However, there were relatively more amplified products with primers having higher G + C content[7]. The number of products may be related to the G + C content of primers and template DNA sequence rather than to the primer length[17]. We have tested primers having G + C content 60–70% (Kit A), 50–60% (Kit F) and 50–70% (Kit G). Better performance of primers from Kit A in terms of greater percentage of templates amplified and more number of loci generated, could be attributed to their higher G + C content.

Our study clearly shows high sensitivity of RAPDs to PCR conditions. For example, the same DNA templates amplified with two different commercial brands of *Taq* DNA polymerases generated different fingerprints. This is in conformity with earlier observations[5]. Concentrations of primer and $MgCl_2$ were critical *vis-à-vis* resolution and reproducibility of RAPD loci. However, we did not find the DNA template concentration to be a decisive factor within a wide range of 1–200 ng. Total genomic DNA extracted from frozen, buffered and frozen or alcohol-preserved and frozen tissues yielded uniform results in RAPD-PCR.

Relative ease of development, low expense and potential usefulness for any kind of organism make RAPDs attractive molecular markers. However, criticisms are levelled at them for their genetic dominance (inability to differentiate homozygotes from heterozygotes), low degree of polymorphisms and exquisite sensitivity to amplification conditions. Hybridizing RAPDs with

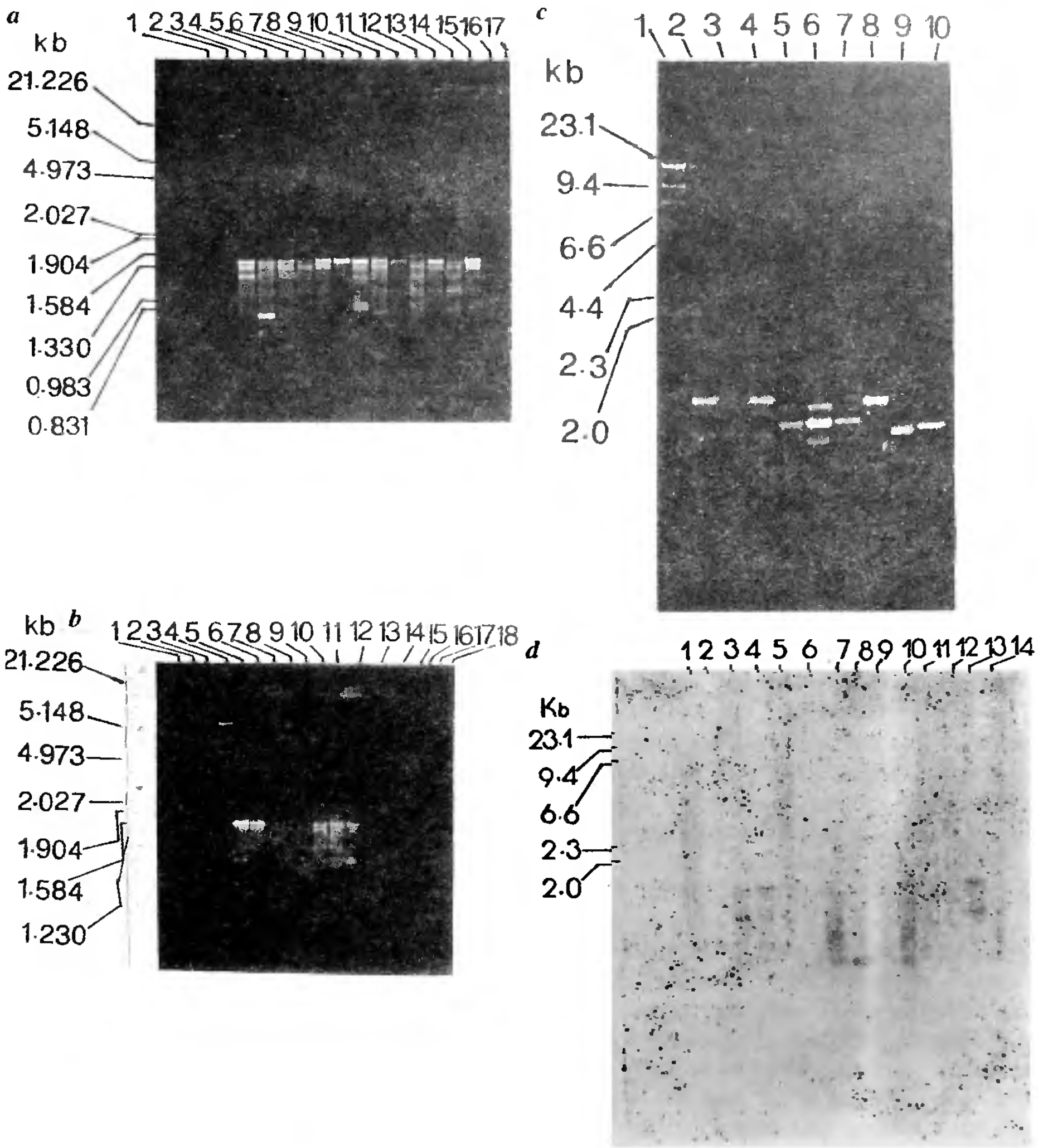

Figure 4. *a*, RAPDs in Indian mackerel generated by OPA 07 (*Taq* 2). Lanes 1, 16 and 17, Blank; Lane 2, λ *Hin*dIII; Lanes 3, 9, 10 and 15, four specimens from Mangalore; Lanes 4, 5, 8 and 11, four specimens from Fort Kochi; Lanes 6, 7 and 12–14, five specimens from Mandapam. The individual DNAs depicted here are different from those in Figure 3 *d*. *b*, RAPDs in Indian mackerel generated by OPA 07 (*Taq* 2). Lanes 1–3 and 13–18, Blank; Lane 4, λ *Hin*dIII + *Eco*RI; Lanes 5 and 12, two specimens from Mandapam, Lanes 6, 9 and 10, three specimens from Mangalore; Lanes 7, 8 and 11, three specimens from Fort Kochi. All individual DNAs depicted here are different from those in Figures 3 *d* and 4 *a*. *c*, RAPDs in Indian mackerel generated by OPA series primers (*Taq* 2). Lane 1, λ *Hin*dIII + *Eco*RI; Lanes 2–4, three specimens from Mandapam (OPA 06); Lanes 5 and 6, two specimens from Mandapam (OPA 10); Lanes 7 and 8, two Mandapam specimens (OPA 11); Lanes 9 and 10, two Mandapam specimens (OPA 16). *d*, RAPDs depicted in Figure 3*d* southern-blotted and hybridized witb 5′ end labelled $(GT)_8$. RAPD loci of less than 2 kb are seen hybridized in lanes 3, 4, 7, 8, 10 and 13.

microsatellite probes can yield novel and highly reproducible fingerprinting profiles. Ethidium bromide staining can detect only the most abundant amplicons whereas majority of minor fragments may remain below the detection level to form a background smear in the gel. The ubiquitous presence of dinucleotide repeats in eukaryotic genomes provides a means of visualizing a subset of such minor amplification products by hybridization[14].

Comparing lanes 13 and 3 of Figure 4 *d* respectively with 15 and 5 of Figure 3 *d* in our paper shows generation of more number of bands when RAPDs were south-

ern-blotted. RAHM technique can provide more information from RAPD gels and also help to reveal microsatellite genomic clones without the time-consuming screening of genomic libraries[13]. This technique offers immense potential for genome analyses in fish and shellfish.

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