CHAPTER III

RESULTS

3.1 DNA extraction

Total DNA was extracted from the adductor muscle tissue of each oysters using a proteinase K-phenol-chloroform extraction method. As can be seen in Fig. 3.1, extracted DNA migrated slower than a λ / *Hin*dIII marker indicating that high molecular weight total DNA (> 23.1 kb) was consistently obtained. DNA concentrations were spectrophotometrically determined by measuring the optical density at 260 nm (1 OD_{260} unit was equivalent to 50 μg DNA/ml). The ratio of OD_{260} / OD_{280} was 2.0 - 2.5 indicating a possible contamination of RNA in the extracted DNA samples. Nevertheless, this contaminant did not interfere subsequent PCR amplification. Approximately 50 μg - 100 μg of nucleic acids were obtained from 100 mg starting adductor muscle tissue.

3.2 Primer screening

A representative of *C. belcheri* and *S. forskali* was tested for the amplification success against 103 decanucleotide primers using RAPD conditions described by Tassanakajorn et al. 1997 (Table 3.1). Eighty-three RAPD primers generated RAPD profiles distinguishing these oyster species clearly. Some of these were further tested against larger number of individuals of all oyster species. Finally, five primers (OPA09, OPB01, OPB08, UBC210 and UBC220) that gave reproducible RAPD patterns and easy scoring results were selected for analysis of genetic diversity and for identification of species-specific markers of oysters in Thailand.

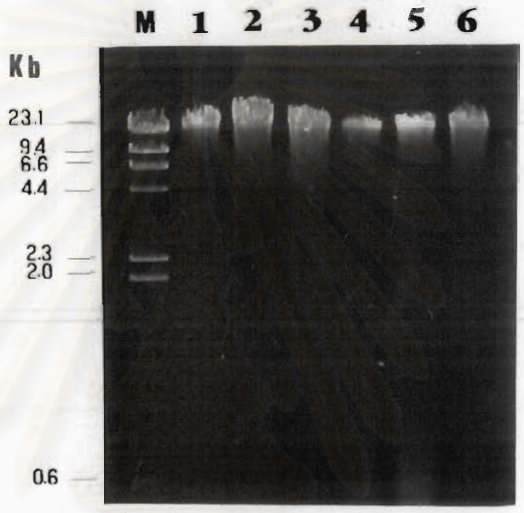


Figure 3.1 A 0.8 % ethidium bromide stained - agarose gel showing the quality of total DNA extracted from the adductor muscle of oysters

lane M = λ / *Hin*dIII

lanes 1-6 = Total DNA extracted from six individuals of oysters

Table 3.1 The amplification success of RAPD primers and their sequences initially screened by this study

Primer	Sequence	Amplification success*	
		C. belcheri	*S. forskali*
OPA01	CAGGCCCTTC	+	+
OPA02	TGCCGAGCTG	+	+
OPA03	AGTCAGCCAC	+	+
OPA04	AATCGGGCTG	+	+
OPA05	AGGGGTCTTG	+	+
OPA06	GGTCCCTGAC	+	+
OPA07	GAAACGGGTG	+	+
OPA08	GTGACGTAGG	+	+
OPA09	GGGTAACGCC	+	+
OPA10	GTGATCGCAG	+	+
OPA11	CAATCGCCGT	+	+
OPA12	TCGGCGATAG	+	+
OPA13	CAGCACCCAC	+	+
OPA14	TCTGTGCTGG	+	+
OPA15	TTCCGAACCC	+	+
OPA16	AGCCAGCGAA	+	+
OPA17	GACCGCTTGT	+	+
OPA18	AGGTGACCGT	+	+
OPA19	CAAACGTCGG	+	+
OPA20	GTTGCGATCC	+	+
OPB01	GTTTCGCTCC	+	+
OPB02	TGATCCCTGG	+	+
OPB03	CATCCCCCTG	+	+
OPB04	GGACTGGAGT	+	+
OPB05	TGCGCCCTTC	+	+
OPB06	TGCTCTGCCC	+	+
OPB07	GGTGACGCAG	+	+
OPB08	GTCCACACGG	+	+
OPB09	TGGGGGACTC	-	-
OPB10	CTGCTGGGAC	+	+
OPB11	GTAGACCCGT	+	+
OPB12	CCTTGACGCA	+	+
OPB13	TTCCCCCGCT	+	+
OPB14	TCCGCTCTGG	+	+
OPB15	GGAGGGTGTT	+	+
OPB16	TTTGCCCGGA	+	+
OPB17	AGGGAACGAG	+	+
OPB18	CCACAGCAGT	+	+
OPB19	ACCCCCGAAG	+	+
OPB20	GGACCCTTAC	+	+
OPM09	GTCTTGCGGA	+	+
OPZ09	CACCCCAGTC	+	+

Table 3.1 (continued)

Primer	Sequence	Amplification success*	
		C. belcheri	*S. forskali*
UBC101	GCGCCTGGAG	+	+
UBC102	GGTGGGGACT	+	+
UBC104	GGGCAATGAT	+	+
UBC105	CTCGGGTGGG	+	+
UBC106	CGTCTGCCCG	+	+
UBC107	CTGTCCCTTT	-	-
UBC108	GTATTGCCCT	-	+
UBC110	TAGCCCGCTT	-	+
UBC112	GCTTGTGAAC	+	-
UBC116	TACGATGACG	+	+
UBC120	GAATTTCCCC	+	+
UBC129	GCGGTATAGT	+	+
UBC133	GGAAACCTCT	-	-
UBC134	AAGCTGCGAG	+	+
UBC137	GGTCTCTCCC	+	+
UBC141	ATCCTGTTCG	-	+
UBC145	TGTCGGTTGC	+	+
UBC149	AGCAGCGTGG	+	+
UBC150	GAAGGCTCTG	+	+
UBC174	AACGGGCAGC	+	+
UBC201	CTGGGGATTT	-	-
UBC202	GAGCACTTAC	+	+
UBC203	CACGGCGAGT	+	+
UBC204	TTCGGGCCGT	+	+
UBC205	CGGTTTGGAA	-	+
UBC206	GAGGACGTCC	-	-
UBC207	CATATCAGGG	-	+
UBC208	ACGGCCGACC	-	+
UBC210	GCACCGAGAG	+	+
UBC214	CATGTGCTTG	+	+
UBC215	TCACACTTGC	+	+
UBC216	CATAGACTCC	-	-
UBC220	GTCGATGTCG	+	+
UBC222	AAGCCTCCCC	+	+
UBC224	TCTCCGGTAT	-	-
UBC226	GGGCCTCTAT	+	+
UBC229	CCACCCAGAG	+	+
UBC231	AGGGAGTTCC	+	-
UBC232	CGGTGACATC	+	+
UBC234	TCCACGGACG	+	+
UBC236	ATCGTACGTG	+	+
UBC238	CTGTCCAGCA	+	+
UBC240	ATGTTCCAGG	+	+

Table 3.1 (continued)

Primer	Sequence	Amplification success*	
		C. belcheri	*S. forskali*
UBC242	CACTCTTTGC	+	+
UBC265	CAGCTGTTCA	+	+
UBC268	AGGCCGCTTA	+	+
UBC421	ACGGCCCACC	+	–
UBC425	CGTCGGGCCT	+	+
UBC428	GGCTCGGGTA	+	+
UBC431	CTGCGGGTCA	+	+
UBC436	GAGGGGGCCA	+	+
UBC438	AGACGGCCGG	+	+
UBC439	GCCCCTTGAC	–	+
UBC444	GCAGCCCCAT	+	+
UBC456	GCGGAGGTCC	+	+
UBC457	CGACGCCCTG	+	+
UBC461	CCCGTATGTC	–	–
UBC463	AGGCGGAAGC	+	–
UBC474	AGGCGGGAAC	+	–
UBC480	GGAGGGGGGA	+	+
UBC482	CTATAGGCCG	+	+

* + and – indicate the amplification success and no amplification, respectively

3.3 Determination of genetic diversity of Thai oysters using RAPD analysis

High genetic diversity levels of *C. belcheri*, *C. iredalei*, *S. cucullata*, *S. forskali*, *S. mytiloides* and other unidentified oysters were observed based on RAPD analysis using 5 decanucleotide primers (OPA09, OPB01, OPB08, UBC210 and UBC220). A total of 254 RAPD fragments ranging from 200 bp and 2500 bp in length were consistently generated (Table 3.2). The number of consistent and reproducible bands across all investigated samples was 55, 42, 62, 44 and 51 bands for primers OPA09, OPB01, OPB08, UBC210 and UBC220 respectively. All of these were polymorphic indicating extremely high polymorphic levels of these primers in oysters. The percentage of polymorphic bands (found in less than 95% of investigated individuals within a particular species) of *C. belcheri*, *C. iredalei*, *S. cucullata*, *S. forskali*, *S. mytiloides*, *Crassostrea sp.*, *Saccostrea sp.* group 1, *Saccostrea sp.*

group 2, *Saccostrea sp.* group 3 and *S. commercialis* was 53.23, 74.67, 97.69, 99.40, 98.50, 85.92, 90.11, 77.63, 89.55 and 86.20, respectively. All *Saccostrea* oysters exhibited greater levels of polymorphic bands than did *C. belcheri* and *C. iredalei* (Table 3.3).

A total of 193, 192, 174, 192 and 181 RAPD genotypes were generated from each respective primer. Disregarding unidentified oysters, the average number of genotypes per primer of *C. belcheri* was 11.6 followed by 18.0, 25.4, 53.0 and 23.0 in *C. iredalei*, *S. cucullata*, *S. forskali* and *S. mytiloides*, respectively. The mean of a ratio between the number of genotypes per primer and the number of investigated specimens in *C. belcheri*, *C. iredalei*, *S. cucullata*, *S. forskali* and *S. mytiloides* was 0.58, 0.90, 0.98, 1.00 and 1.00, respectively. Shared genotypes between individuals of different oyster species were not observed in any primer.

Examples of RAPD amplification patterns generated by those primers are shown in Figs 3.2-3.12. The RAPD patterns of all specimens tested are shown in an appendix B. Only reproducible bands were scored for presence (1) or absence (0) in each individual. Neither RAPD patterns nor fragments of *S. commercialis* and *P. viridis* were considered to be species-specific because the limitation on both numbers of sampling locations and specimens which may not represent the major genetic part of each species.

Table 3.2 Sequences of RAPD primers, sizes and number of amplified bands, and the percentage of polymorphic bands resulted from RAPD analysis of oysters in Thailand

Primer	Sequence	Size-range (bp)	No. of RAPD bands	Polymorphic bands (%)
OPA09	GGGTAACGCC	200-1750	55	100
OPB01	GTTTCGCTCC	375-2100	42	100
OPB08	GTCCACACGG	275-2500	62	100
UBC210	GCACCGAGAG	235-2000	44	100
UBC220	GTCGATGTCG	280-2300	51	100
Average		200-2500	254	100

Table 3.3 Total number of bands, percentage of polymorphic and monomorphic bands within each oyster species and the green mussel (*P. viridis*) revealed by RAPD analysis using primers OPA09, OPB01, OPB08, UBC210 and UBC220

Primer No.	*C. belcheri* (*N* = 20)			*C. iredalei* (*N* = 20)			*S. cucullata* (*N* = 26)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA09	18	14	4	15	13	2	32	31	1
OPB01	11	5	6	17	14	3	28	28	0
OPB08	5	0	5	12	9	3	19	18	1
UBC210	14	10	4	17	12	5	25	25	0
UBC220	14	4	8	14	8	6	26	25	1
Total	62	33 (53.23%)	27 (43.55%)	75	56 (74.67%)	19 (25.33%)	130	127 (97.69%)	3 (2.31%)

Primer No.	*S. forskali* (*N* = 58)			*S. mytiloides* (*N* = 23)			*Crassostrea sp.* (*N* = 9)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA09	28	28	0	26	26	0	18	16	2
OPB01	31	31	0	27	27	0	17	17	0
OPB08	38	38	0	24	24	0	10	10	0
UBC210	29	29	0	24	22	2	8	2	6
UBC220	40	40	1	32	32	0	18	16	2
Total	166	165 (99.40%)	1 (0.60%)	133	131 (98.50%)	2 (1.50%)	71	61 (85.92%)	10 (14.08%)

Table 3.3 (continued)

Primer No.	*Saccostrea sp.* group 1 ($N = 9$)			*Saccostrea sp.* group 2 ($N = 9$)			*Saccostrea sp.* group 3 ($N = 5$)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA09	16	12	4	12	10	2	14	12	2
OPB01	14	13	1	14	14	0	15	15	0
OPB08	17	15	2	17	13	4	12	11	1
UBC210	20	19	1	13	11	2	13	10	3
UBC220	24	23	1	20	11	9	13	12	1
Total	91	82 (90.11%)	9 (9.89%)	76	59 (77.63%)	17 (22.37%)	67	60 (89.55%)	7 (10.45%)

Primer No.	*S. commercialis* ($N = 12$)			*P. viridis* ($N = 12$)		
	No. of bands	No. of polymorphic bands	No. of monomorphic bands	No. of bands	No. of polymorphic bands	No. of monomorphic bands
OPA09	22	19	3	8	3	5
OPB01	20	18	2	12	6	6
OPB08	12	11	1	12	7	5
UBC210	16	13	3	10	7	3
UBC220	17	14	3	10	2	8
Total	87	75 (86.20%)	12 (13.79%)	52	25 (48.08%)	27 (51.92%)

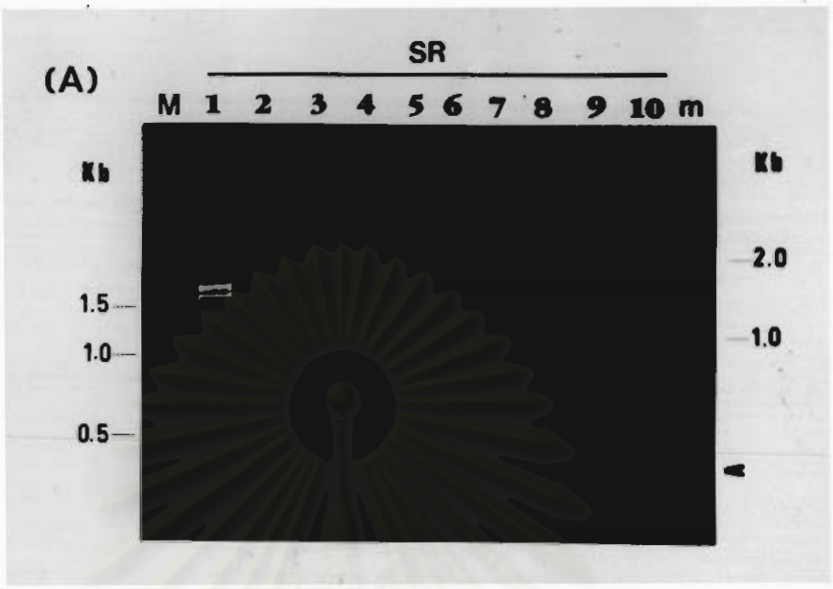
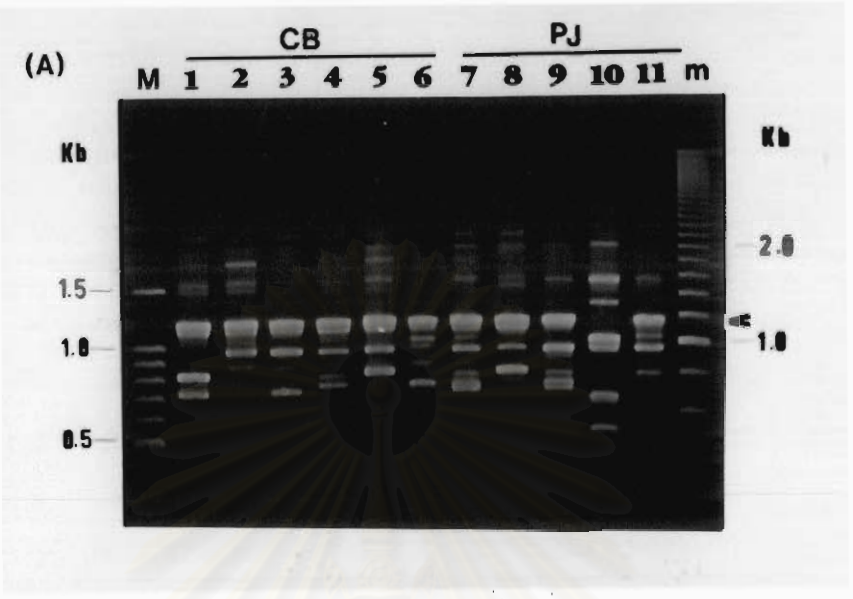


Figure 3.2 RAPD patterns resulted from analysis of *C. belcheri* originating from Suratthani (lanes 1-10, A), Songkhla (lanes 1-4, B), Ranong (lanes 5-7, B), and Krabi (lanes 8-10, B) with the primer OPA09. Lanes M and m are 100 bp and 200 bp ladders, respectively. An arrowhead (<) indicates a species – specific marker (250 bp) found in *C. belcheri*.



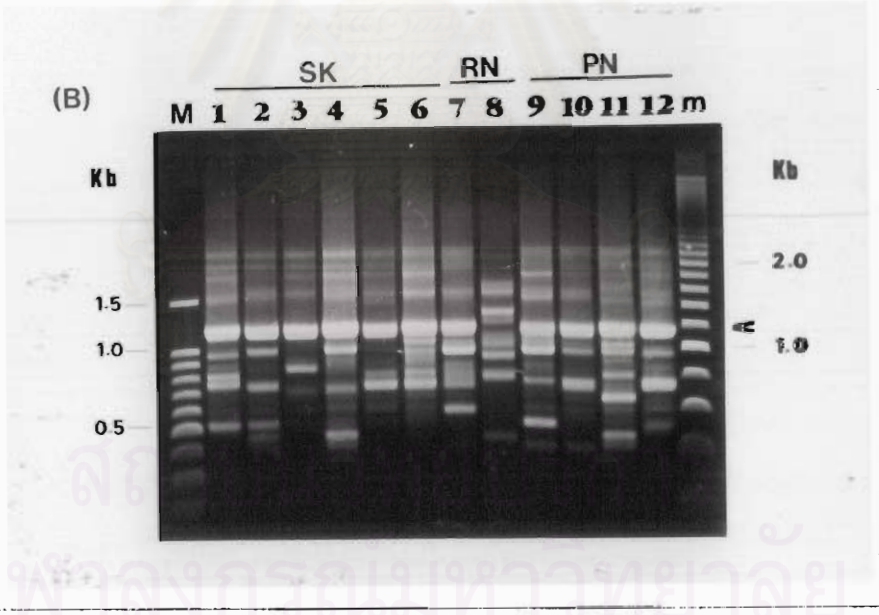


Figure 3.3 RAPD patterns resulted from analysis of *C. iredalei* (and *C. iredalei* – like) originating from Chonburi (lanes 1-6, A), Prachuapkririkhan (lanes 7-9 and 11, A), Songkhla (lanes 1-6, B), Ranong (lane 7, B), and Phangnga (lanes 9-12, B) with the primer OPA09. Arrowheads indicate a species – specific marker (1150 bp) found in this species. Specimens of *C. iredalei* – like (lane 8, B) and *S. forskali*-like oysters (lane 10, A) were included in the analysis. These oysters did not possess a 1150 bp species-specific fragment and were proved to be introgressive hybrids of *C. iredalei* individuals. The 100 bp (lane M) and 200 bp (lane m) ladders were included as DNA markers.

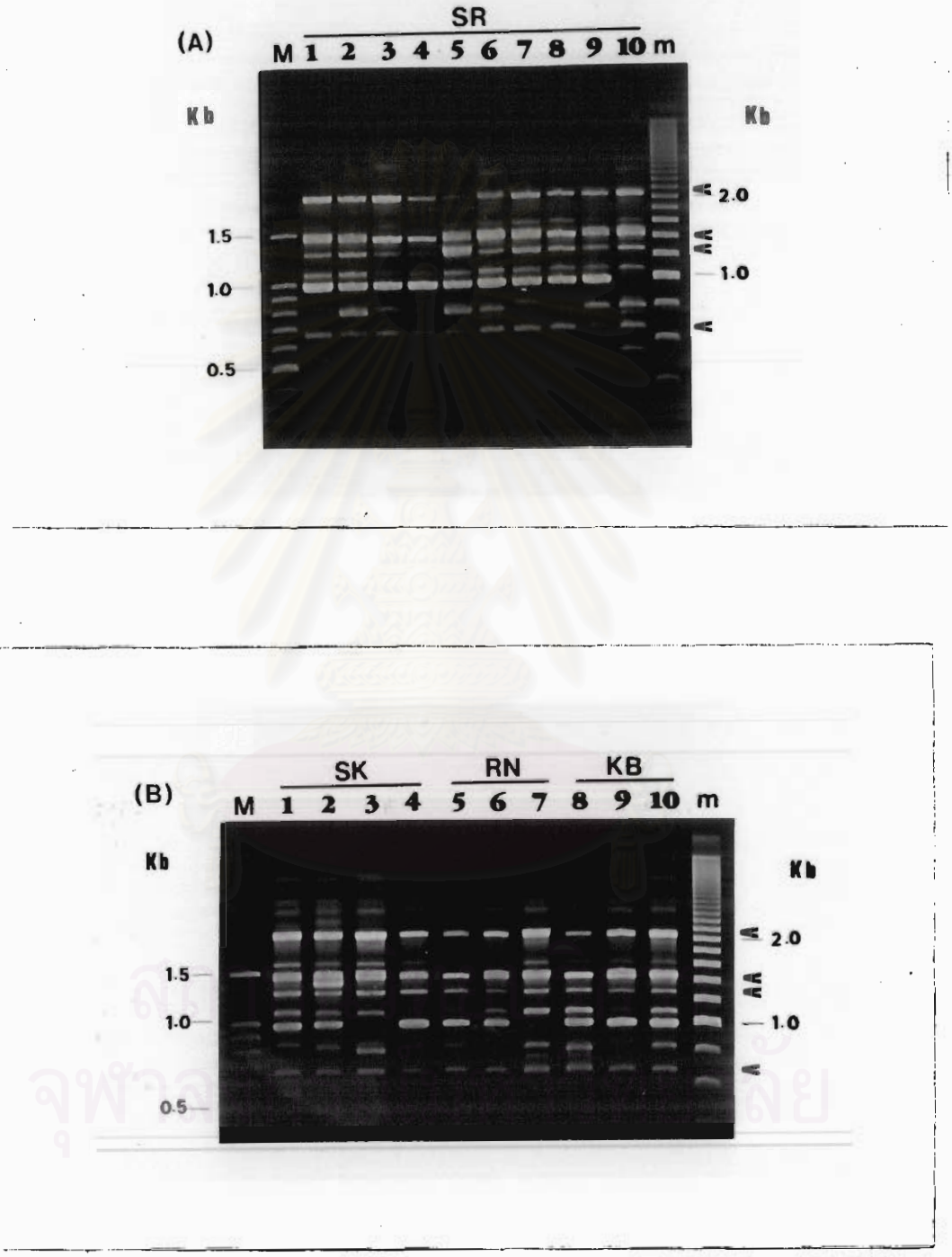


Figure 3.4 RAPD patterns resulted from analysis of *C. belcheri* originating from Suratthani (lanes 1-10, A), Songkhla (lanes 1-4, B), Ranong (lanes 5-7, B), and Krabi (lanes 8-10, B) with the primer OPB01. Lanes M and m are 100 bp and 200 bp ladders, respectively. Arrowheads indicate species – specific markers (2100 bp, 1400 bp, 1250 bp, and 650 bp) found in *C. belcheri*.

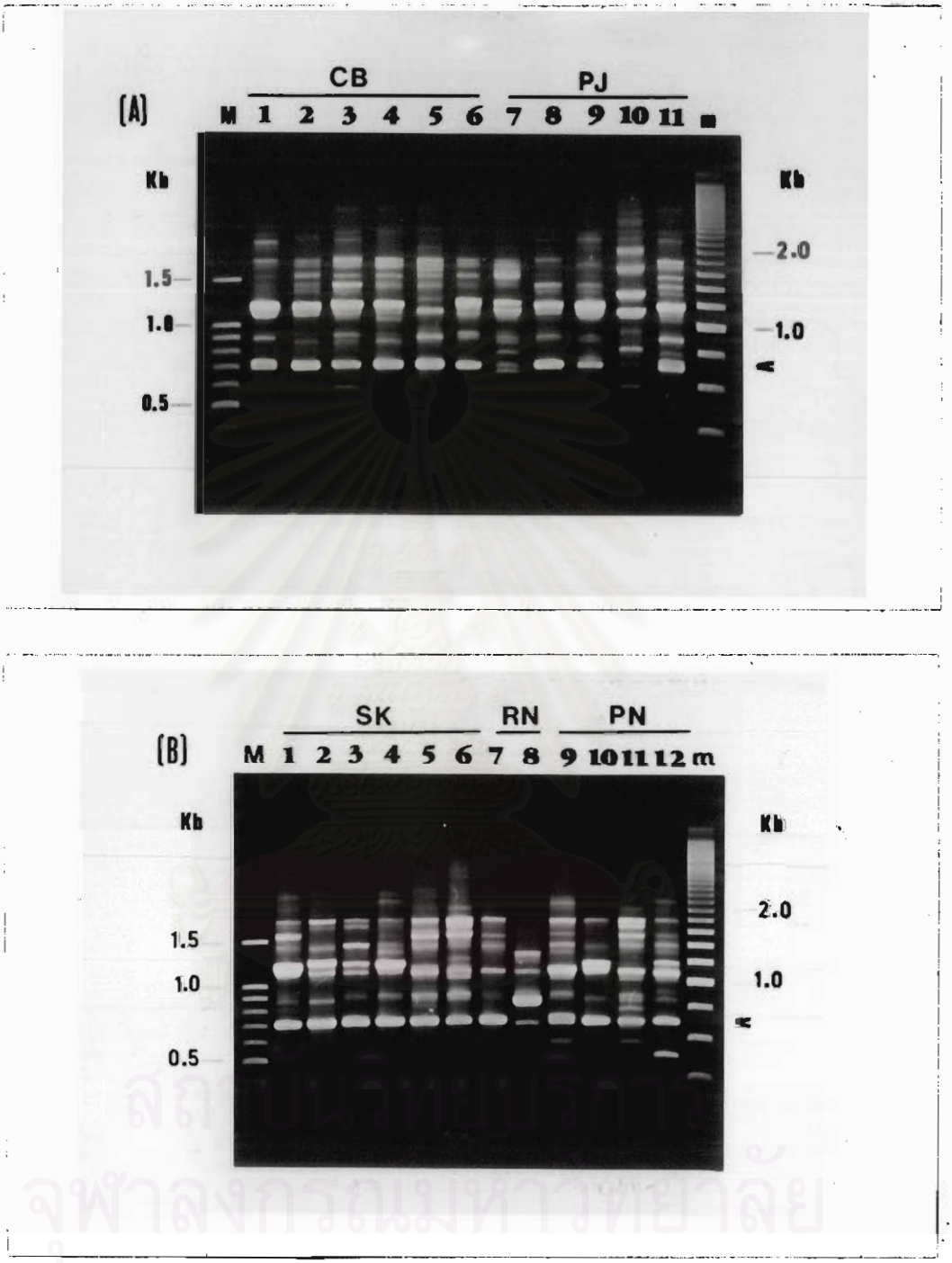


Figure 3.5 RAPD patterns resulted from analysis of *C. iredalei* (and *C. iredalei* – like) originating from Chonburi (lanes 1-6, A), Prachuapkririkhan (lanes 7-9 and 11, A), Songkhla (lanes 1-6, B), Ranong (lane 7, B), and Phangnga (lanes 9-12, B) with the primer OPB01. Arrowheads indicate a species – specific marker (700 bp) found in this species. Specimens of *C. iredalei* – like (lane 8, B) and *S. forskali*-like oysters (lane 10, A) were included in the analysis. The latter did not possess a 700 bp species-specific fragment. These oysters were proved to be introgressive hybrids of *C. iredalei* individuals. The 100bp (lane M) and 200 bp (lane m) ladders were included as DNA markers.

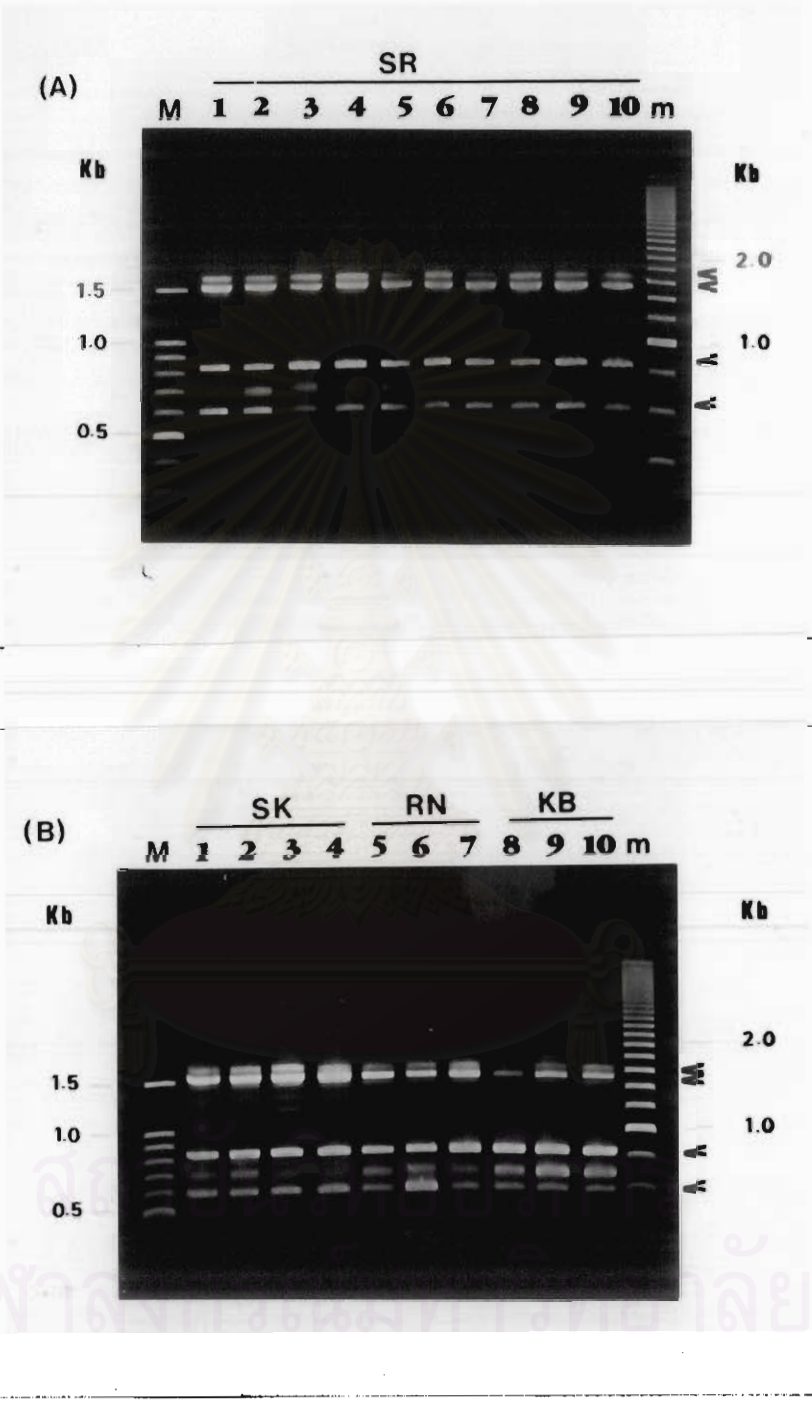


Figure 3.6 RAPD patterns resulted from analysis of *C. belcheri* originating from Suratthani (lanes 1-10, A), Songkhla (lanes 1-4, B), Ranong (lanes 5-7, B), and Krabi (lanes 8-10, B) with the primer OPB08. Lanes M and m are 100 bp and 200 bp ladders, respectively. Arrowheads indicate species – specific markers (1650 bp, 1550 bp, 835 bp and 600 bp) found in *C. belcheri*.

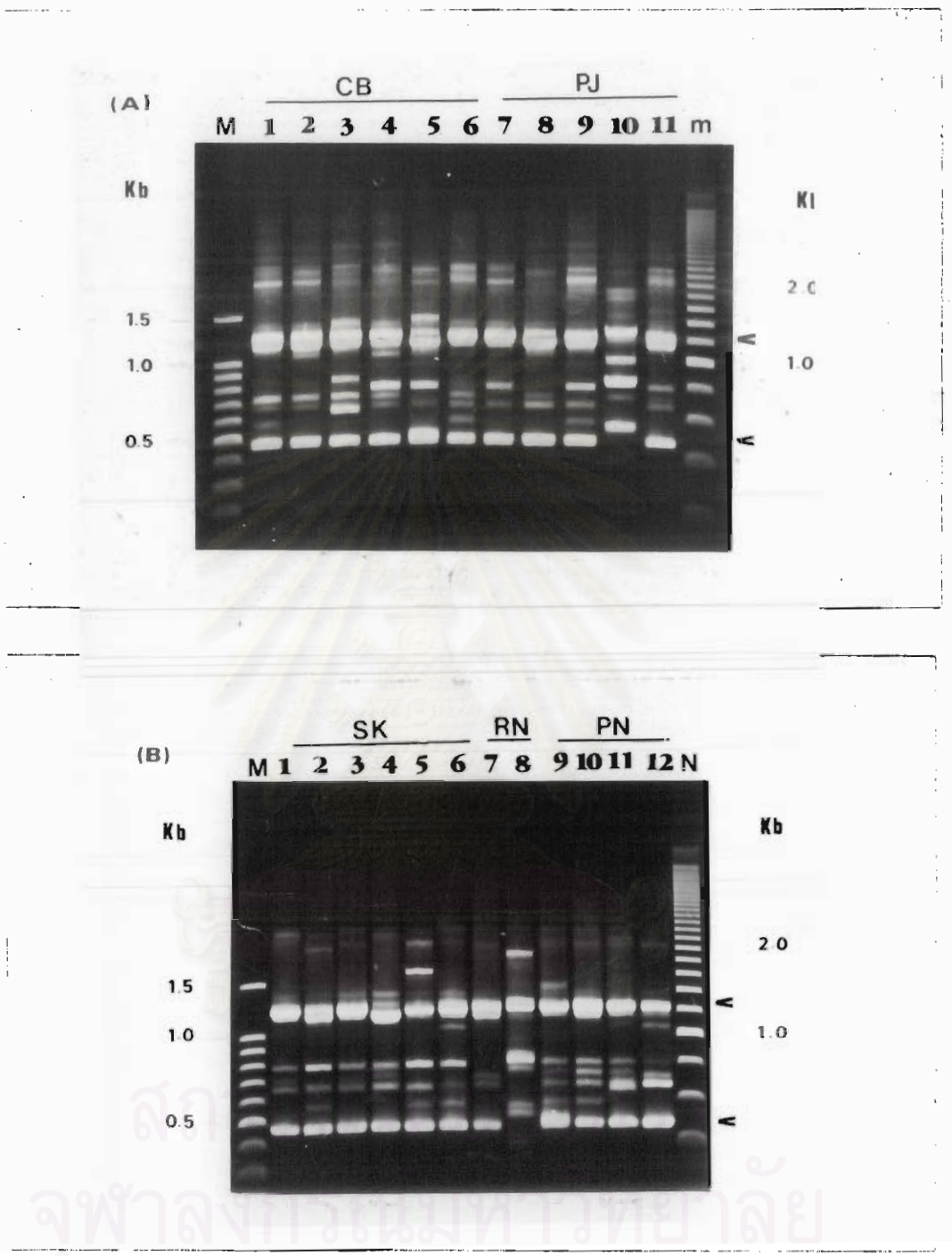


Figure 3.7 RAPD patterns resulted from analysis of *C. iredalei* (and *C. iredalei* – like) originating from Chonburi (lanes 1-6, A), Prachuapkririkhan (lanes 7-9 and 11, A), Songkhla (lanes 1-6, B), Ranong (lane 7, B), and Phangnga (lanes 9-12, B) with the primer OPB08. Arrowheads indicate species – specific markers (1250 bp and 450 bp) found in this species. Specimens of *C. iredalei* – like (lane 8, B) and *S. forskali*-like oysters (lane 10, A) were included in the analysis. These oysters did not possess a 450 bp species-specific fragment and were proved to be introgressive hybrids of *C. iredalei* individuals. The 100 bp (lane M) and 200 bp (lane m and N) ladders were included as DNA markers.

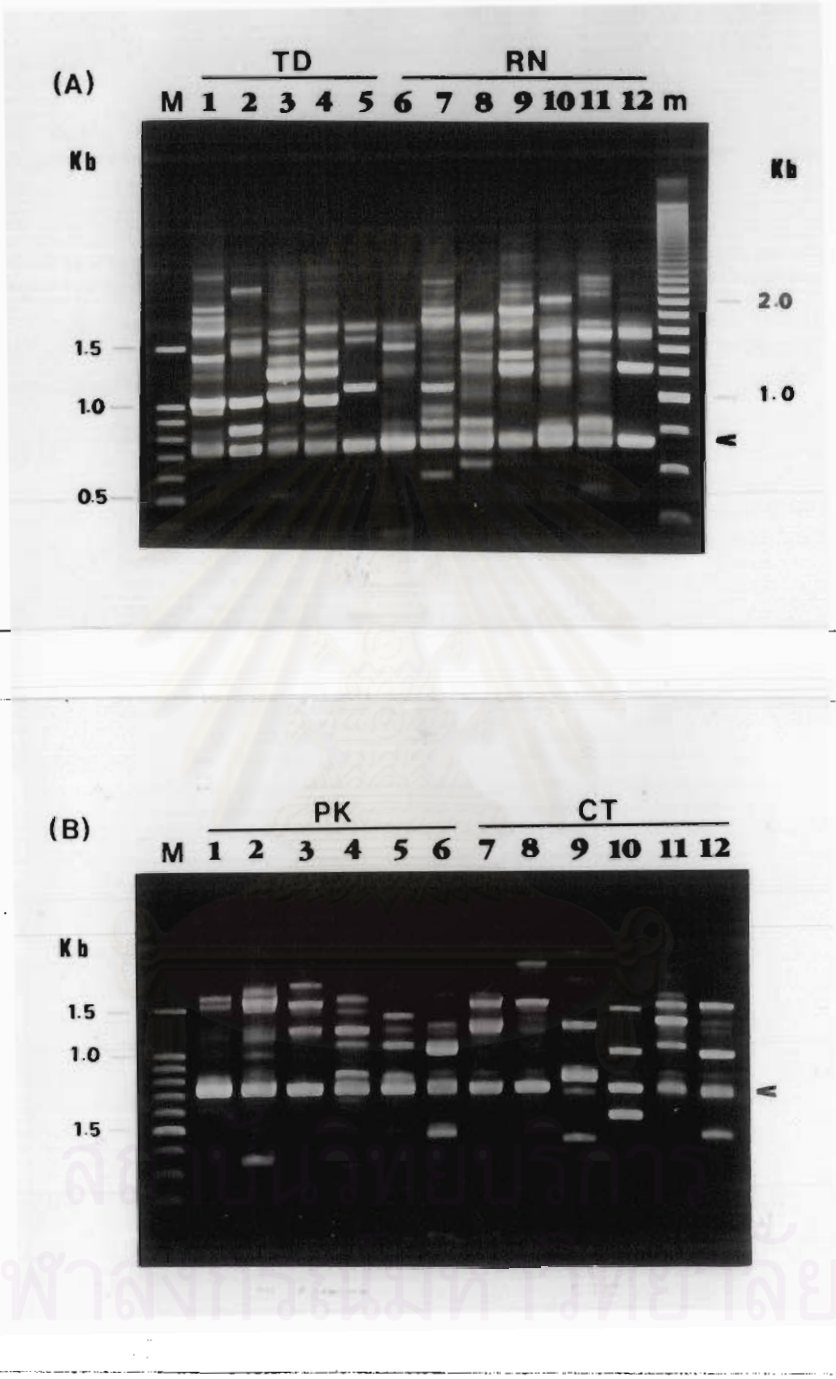


Figure 3.8 RAPD patterns resulted from analysis of *S. cucullata* originating from Trad (lanes 1-5, A), Ranong (lanes 6-12, A), Phuket (lanes 1-6, B), and Chantraburi (lanes 7-8 and 10-12, B) with the primer OPB08. Lane 9, panel B is a *S. forskali* individual collected from Chantraburi. The 100 bp (lane M) and 200 bp (lane m) ladders were included as DNA markers. Arrowhead indicates a species – specific marker (750 bp) found in *S. cucullata*.

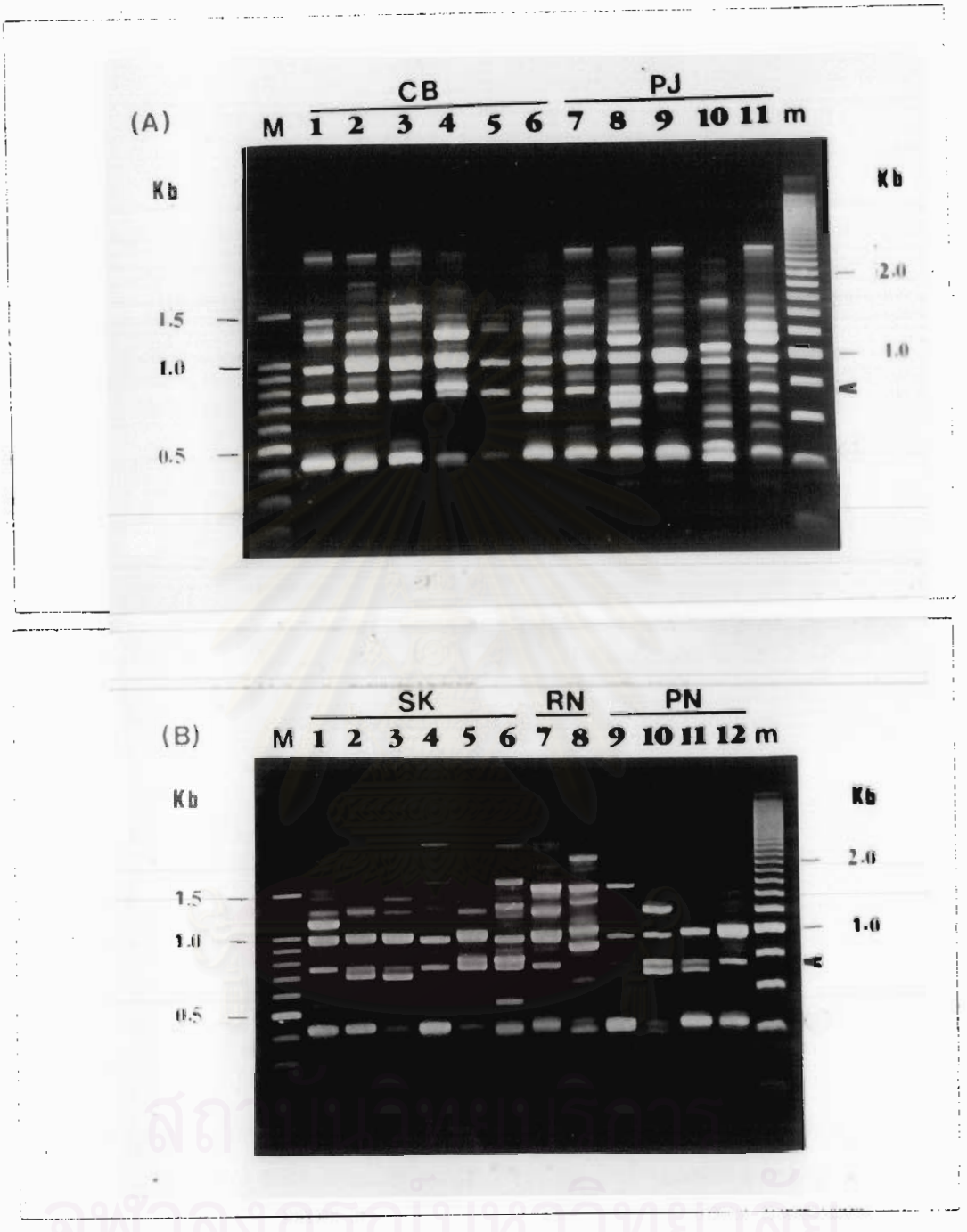
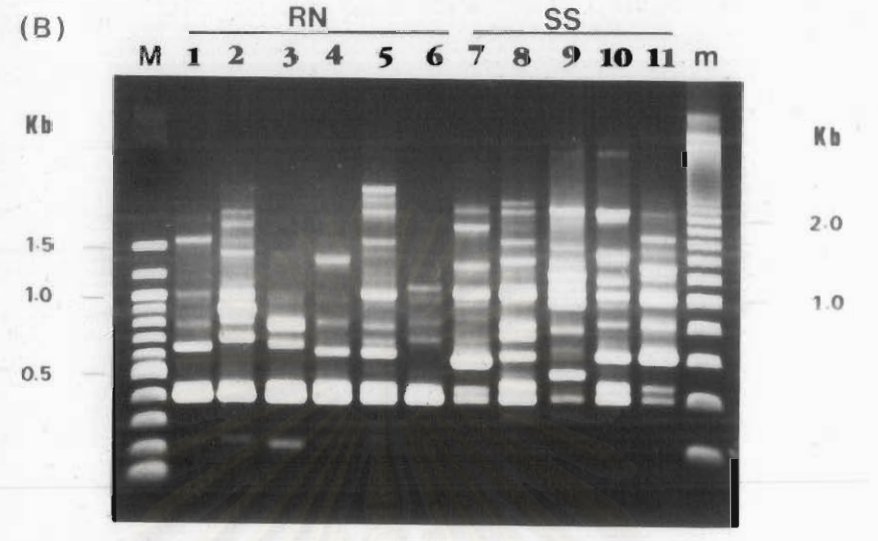


Figure 3.9 RAPD patterns resulted from analysis of *C. iredalei* (and *C. iredalei* – like) originating from Chonburi (lanes 1-6, A), Prachuapkririkhan (lanes 7-9 and 11, A), Songkhla (lanes 1-6, B), Ranong (lane 7, B), and Phangnga (lanes 9-12, B) with the primer UBC210. Arrowheads indicate a species – specific marker (745 bp) found in this species. Specimens of *C. iredalei* – like (lane 8, B) and *S. forskali*-like oysters (lane 10, A) were included in the analysis. The former did not possess 745 bp species fragments. These specimens were proved to be introgressive hybrids of *C. iredalei* individuals. The 100 bp (lane M) and 200 bp (lane m) ladders were included as DNA markers.



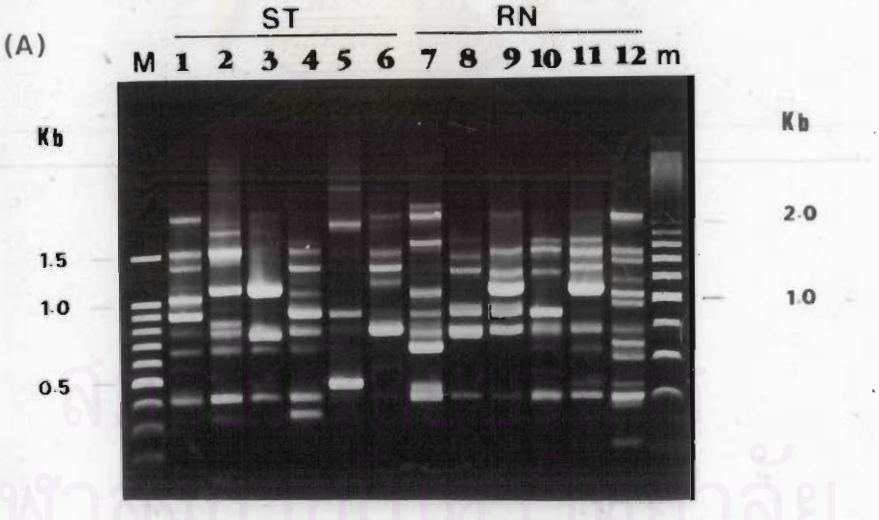


Figure 3.10 RAPD patterns resulted from analysis of *S. forskali* (A) originating from Satun (lanes1-6) and Ranong (lanes 7-12) and *S. mytiloides* (B) originating from Ranong (lanes 1-6) and Samut Sakhon (lanes 7-12) with the primer UBC210. Lanes M and m are a 100 bp and 200 bp ladders, respectively.

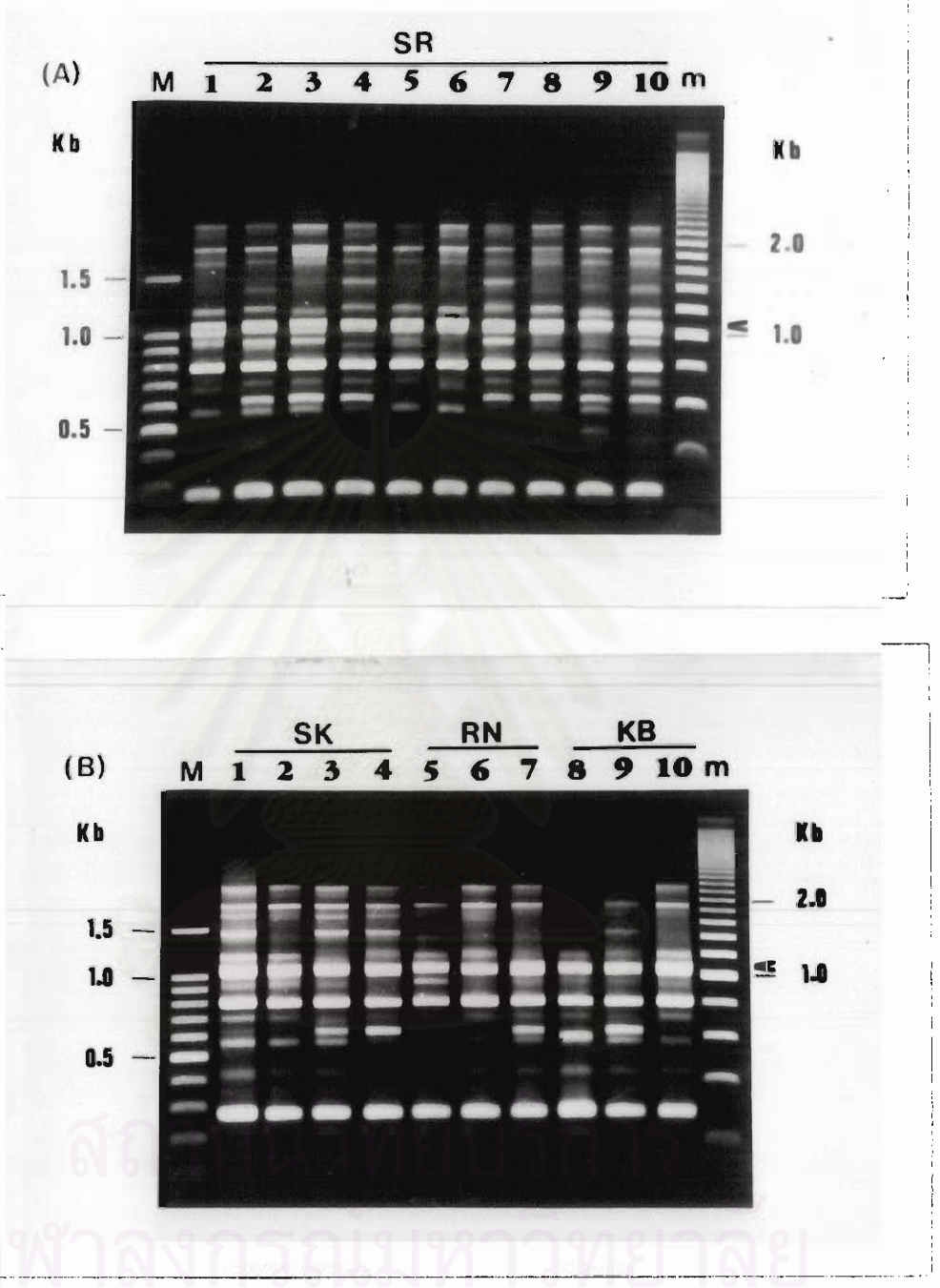


Figure 3.11 RAPD patterns resulted from analysis of *C. belcheri* originating from Suratthani (lanes 1-10, A), Songkhla (lanes 1-4, B), Ranong (lanes 5-7, B), and Krabi (lanes 8-10, B) with the primer UBC220. Lanes M and m are 100 bp and 200 bp ladders, respectively. Arrowheads indicate a species – specific marker (1050 bp) found in *C. belcheri*.

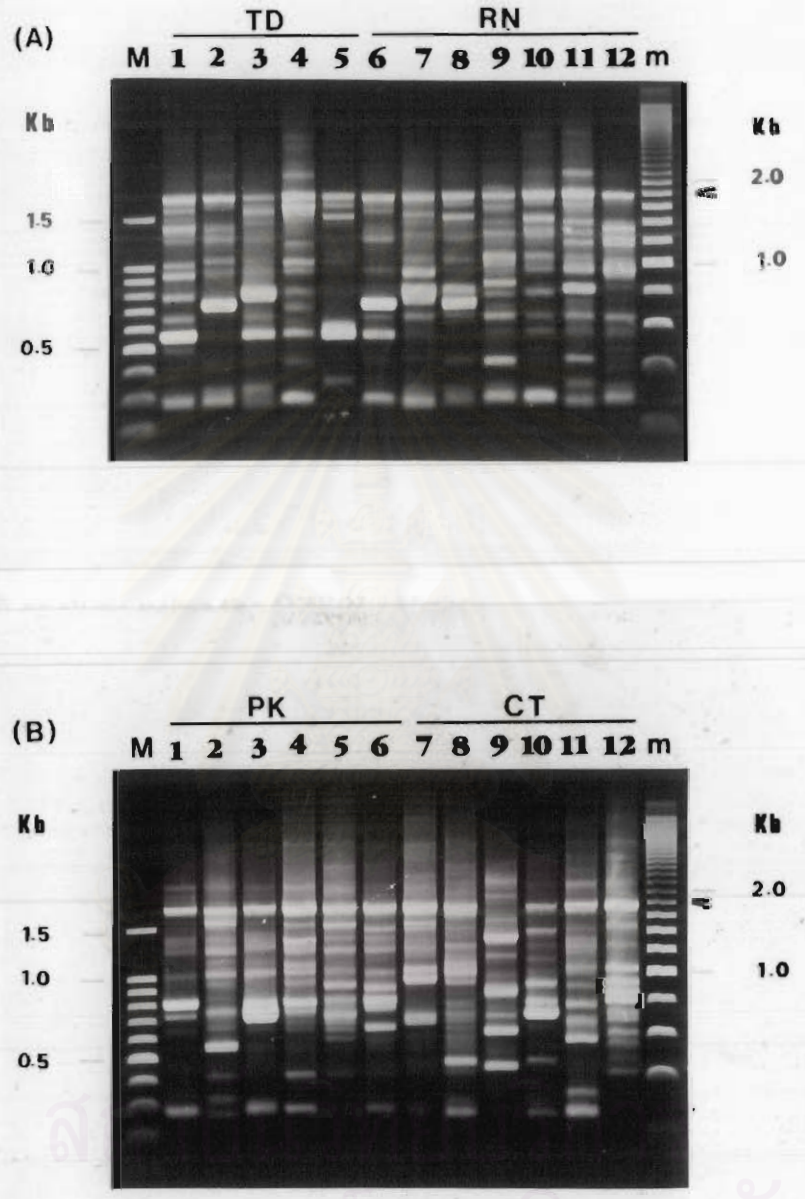


Figure 3.12 RAPD patterns resulted from analysis of *S. cucullata* originating from Trad (lanes 1-5, A), Ranong (lanes 6-12, A), Phuket (lanes 1-6, B), and Chantraburi (lanes 7-8 and 10-12, B) with the primer UBC220. Lane 9, panel B is a *S. forskali* individual collected from Chantraburi. The 100 bp (lane M) and 200 bp (lane m) ladders were included as DNA markers. Arrowheads indicate a species – specific marker (1800 bp) found in *S. cucullata*.

3.3.1 RAPD analysis using the OPA09 primer

A total of 55 RAPD fragments were generated when analyzed overall specimens with this primer. One hundred and ninety-three genotypes were observed. Although high polymorphic levels of oysters were observed with this primer, species-specific markers were found in both *C. belcheri* (250 bp), and *C. iredalei* (1150 bp) but not in other oyster species. A 500 bp bands was found in all *S. cucullata* individuals but was not considered to be a species-diagnostic marker because it was also found in *C. belcheri* (55%) and *C. iredalei* (35%), respectively. Three fixed bands (1225 bp, 735 bp and 575 bp) were found in *P.viridis*. These bands were not found in any oyster species (Appendix C.1).

3.3.2 RAPD analysis using the OPB01 primer

Fourty-two reproducible RAPD fragments were generated by the OPB01. These resulted in a total of 192 RAPD genotypes. Four RAPD fragments (2100 bp, 1400 bp, 1250 bp and 650 bp) were found in all *C. belcheri* individuals. These bands can be used as species-diagnostic markers in this species. An RAPD analysis using this primer also exhibited a *C. iredalei*-specific fragment at approximately 700 bp. A 1800 bp band was found in all *C. iredalei* individuals but was not regarded as a species-diagnostic markers because it was faint and may not be reliably identified if the sample size is increased. Four fixed bands (at 2000 bp, 1815 bp, 875 bp and 465 bp) were found in *P. viridis* but disappeared in any oyster species (Appendix C.2).

3.3.3 RAPD analysis using the OPB08 primer

Sixty – two reproducible bands were generated by amplification of overall specimens used in this study with the primer OPB08. A total of 174 genotypes were found. Like primers OPA09 and OPB01, the OPB08 also yielded species-diagnostic markers in *Crassostrea* oysters. Four *C. belcheri* - specific fragments 1650 bp, 1550 bp, 835 bp and 600 bp were found whereas two specific fragments at 1250 bp and 450 bp were observed in *C. iredalei*. The 700 bp band was intensely present in most

C. belcheri individuals. Nevertheless, some individuals exhibited this band faintly, therefore it was not regarded as a species-specific marker in *C. belcheri*. A 750 bp band was specifically observed in *S. cucullata* and used as a diagnostic marker in this species. The 1075 bp and 1350 bp fragments were found in 100% and 91.67% of *S. commercialis*, respectively. These fragments were not found in other species. A 2500 bp band was specifically found in all *P. viridis* individuals (Appendix C.3)

Comparing with other RAPD markers, the primer OPB08 generated the highest number of diagnostic bands in oysters than did others. These markers were potential for differentiation of three oyster species; *C. belcheri C. iredalei* and *S. cucullata* at adults. Moreover, they may be utilized for species-diagnosis at different larval stages of those species.

3.3.4 RAPD analysis using the UBC210 primer

A total of 44 fragments was generated from analysis of experimental specimens with this primer. One hundred and ninety-two genotypes were generated by this useful primer. Using the UBC210 for amplification analysis, species – specific markers were found in *C. iredalei* (745 bp). A band at 1415 bp was fixed in *C. belcheri*, *C. iredalei* and *Crassostrea sp.* but was not considered to be a marker for the genus *Crassostrea* because it was very faint. A 1390 bp band was found in *Crassostrea sp.* (originating from Klong Bo Tho, Krabi; west of peninsular Thailand) but did not observed in any species. Both 435 bp and 400 bp bands were monomorphic in *S. mytiloides* (found at 95.65% of overall *S. mytiloides* specimens). Disregarding unidentified oysters, and an ingroup and an outgroup references, they were also found at high frequencies (30.00%-86.21%) in other species. A 1550 bp band was found in all *S. commercailis* individuals. Two fixed frequency bands (785 bp and 500 bp) were found in *P. viridis* (Appendix C.4).

3.3.5 RAPD analysis using the UBC220 primer

A total of fifty-one RAPD fragments were scored. One hundred and eighty-one RAPD patterns were observed when 203 individuals were investigated with this primer. A 1050 bp band was found in all *C. belcheri* individuals. This band can be used as a species – diagnostic marker in that species. Likewise, a 1800 bp band in *S. cucullata* can be used as a diagnostic marker for this species. The *Crassostrea sp.* individuals exhibited a fixed 2100 bp band. A 620 bp band was common (98.28%) in *S. forskali* but also found in 55- 86.96% of described oyster species (disregarding unidentified oysters and an ingroup and an outgroup reference). A 1325 bp fragment was found in all individuals of *P. viridis* but was not found in any other species (Appendix C.5).

3.4 Genetic relationships of similarities and distances in Thai oysters

All investigated taxa were divided to 29 operational taxonomic units (OTUs), simply called hereafter as 29 geographic samples. The similarity indices between and within these samples analyzed by 5 RAPD primers (OPA09, OPB01, OPB08, UBC210 and UBC220) are illustrated by Table 3.4.

The average similarity index across all samples resulted from primers OPA09, OPB01, OPB08, UBC210 and UBC220 were 0.759, 0.716, 0.792, 0.790 and 0.790, respectively (Table 3.4). The mean similarity within each geographic sample averaged overall primers ranged from 0.487 (SfCT) to 0.925 (CbRN). Basically, *C. belcheri* exhibited the highest level of the similarity index within a species (0.903) whereas *S. forskali* showed the lowest similarity level (0.577) within a species. The results suggested that *Crassostea* oysters were genetically closely related within the genus than those of *Saccostrea* oysters. The average similarity index within samples of *Crassostrea* species were 0.779-0.925 but those of *Saccostrea* samples were 0.487-0.688. The unidentified species (including CsKB, S1SR, S2RN, and S3SS) had the similarity index between 0.604-0.693.

Table 3.4 Estimated similarity indices (S) within geographic samples of Thai oysters, *S. commercialis* and *P. viridis* using 5 selected RAPD primers

Sample	Primer					Average similarity within a sample across all primers
	OPA09	OPB01	OPB08	UBC210	UBC220	
CbSR	0.850	0.896	1.000	0.807	0.927	0.896
CbSK	0.857	0.900	1.000	0.817	0.920	0.899
CbRN	0.819	0.896	1.000	0.968	0.939	0.925
CbKB	0.698	0.932	1.000	0.891	0.944	0.893
CiCB	0.849	0.835	0.818	0.856	0.882	0.848
CiPJ	0.761	0.811	0.872	0.806	0.863	0.823
CiSK	0.667	0.833	0.871	0.784	0.885	0.808
CiPN	0.700	0.746	0.817	0.758	0.875	0.779
ScTD	0.695	0.466	0.605	0.510	0.636	0.582
ScRN	0.598	0.523	0.739	0.582	0.760	0.640
ScPK	0.682	0.377	0.803	0.696	0.825	0.676
ScCT	0.475	0.492	0.639	0.585	0.512	0.541
SfCT	0.606	0.431	0.417	0.541	0.442	0.487
SfSR	0.657	0.729	0.721	0.673	0.662	0.688
SfCBA	0.592	0.447	0.570	0.671	0.555	0.567
SfPJ	0.641	0.496	0.491	0.659	0.612	0.580
SfSK	0.617	0.428	0.571	0.676	0.643	0.587
SfST	0.560	0.573	0.716	0.600	0.542	0.598
SfRN	0.430	0.554	0.629	0.601	0.513	0.546
SfCBS	0.621	0.535	0.593	0.514	0.548	0.562
SmPK	0.592	0.536	0.452	0.676	0.536	0.558
SmRN	0.751	0.711	0.500	0.716	0.684	0.672
SmSS	0.647	0.472	0.519	0.592	0.568	0.560
CsKB	0.590	0.635	0.641	0.950	0.652	0.693
S1SR	0.736	0.623	0.668	0.629	0.558	0.643
S2RN	0.760	0.703	0.764	0.759	0.833	0.764
S3SS	0.632	0.492	0.623	0.729	0.541	0.604
Scom	0.682	0.683	0.705	0.700	0.731	0.700
Pevi	0.960	0.851	0.858	0.792	0.946	0.882
Average similarity of each primer across all samples	0.759	0.716	0.792	0.790	0.790	

The similarity between samples ($\bar{S}_{ij}$) of each primer is calculated as the average of all possible comparisons of individuals between pairs of samples. Genetic distances ($\bar{D}_{ij}$) were converted from the index of similarity between samples ($\bar{D}_{ij}$= 1- $\bar{S}_{ij}$). The results from each primer are shown in Appendix D. The average genetic distance between geographic samples within species across all primers of *C. belcheri* was 0.1262 which was much lower than those of *C. iredalei* (0.2657), *S. cucullata* (0.4460), *S. forskali* (0.5475) and *S. mytiloides* (0.6069), respectively. Genetic differences within species were lower than those between different oyster species (Table 3.5).

A neighbor-joining (NJ) tree constructed from average genetic distance between paired geographic samples indicated phylogenetically clear separation between investigated oyster species (Fig. 3.13). The NJ tree from each RAPD primer is shown by Appendix E. As can be seen from Fig. 3.13, clear differences between *Crassostrea* and *Saccostrea* species were observed. All oysters except those of *S. forskali* (SfSR and SfCBS) showed monophyletic relationships within a particular species. At the intraspecific level, phylogeographic differentiation between oysters from different coastal areas was found in *C. belcheri.* The phylogenetic relationship of *S. forskali* divieded this taxa to two different groups that (SfPJ, SfSK, SfCBA, SfCT, SfRN and SfST) showing closed relationship intraspecifically and that (SfSR and SfCBS) clustering with *S. mytiloides* samples (SmPK, SmRN and SmSS). The unknown *Saccostrea sp.* group 1 (S1SR) from Suratthani were allocated into the latter group of *S. forskali*. The unknown *Saccostrea sp.* group 2 (S2RN) from Ranong was allocated as a new branch indicating that this might be a possible unidentified new species. The *Saccostreaa sp.* group 3 is *S. mytiloides*-like oysters based primarily on morphological identification. This group was phylogenetically to be *S. mytiloides.*

RAPD analysis using OPA09, OPB01, OPB08, UBC210 and UBC220 can be unambiguously identified *C. belcheri*, *C. iredalei* and *S. cucullata* using molecular markers developed in this studies. Nevertheless, *S. forskali* and *S. mytiloidis* are closely related. These two species cannot be differentiated precisely using either

morphology or RAPD analysis (due to a lack of species-specific marker in their species).

3.5 Species-diagnostic markers of Thai oysters

All primers (OPA09, OPB01, OPB08, UBC210 and UBC220) generated RAPD fragments exhibiting fixed frequencies in at least one species (Table 3.6, Figs. 3.2 – 3.9 and 3.11 -3.12). The primer OPB08 yielded species-specific markers in all commercially cultured oyster species in Thailand (1650 bp, 1550 bp, 835 bp and 600 bp in *C. belcheri*, 1250 bp and 450 bp in *C. iredalei* and 750 bp in *S. cucullata*). Primers OPA09 and OPB01 specifically provided RAPD markers in *C. belcheri* (250 bp from OPA09 and 2100 bp, 1400 bp 1250 and 650 bp from OPB01, respectively), and *C. iredalei* (1150 bp and 700 bp from OPA09 and OPB01, respectively). The primer UBC210 showed species-specific nature in *C. iredalei* (745 bp). Additional RAPD markers in *C. belcheri* (1050 bp) and *S. cucullata* (1800 bp) were identified by the primer UBC220. No species-specific RAPD fragment in *S. mytiloides* and S. forskali were found. Population-specific markers were not observed in any oyster species.

3.6 Cloning of *C. belcheri*-specific RAPD fragments

Three RAPD bands constituting of a 600 bp and a 835 bp fragments from OPB08 and a 650 bp fragment from OPB01 were cloned using either a *Bam*HI- sticky end adapter method (Vincent et al., 1993) or a T-A cloning approach (Hoelzel and Green, 1992). After electrophoresed, a 600 bp and a 835 bp fragments were separately excised and recovered from the gel using a Qiaquick gel extraction kit (Qiagen Ltd.). The purified DNA was reamplified by the primer OPB08 containing a *Bam*HI restriction site (CGGGATCCCGGTCCACACGG) (Fig. 3.14). Each reamplified fragment was eluted and purified by a proteinase K / phenol / chloroform extraction method as described in Chapter 2 (section 2.8.2 and 2.8.3). These and a 650 bp fragment were cloned using the procedures described in section 2.9. To determine sizes of DNA insert, recombinant clones were digested with *Bam*HI (for 600 bp and

835 bp inserts) and *Eco*RI (for a 650 bp insert) and analyzed with 1.0% agarose gels. The results are shown in Fig 3.15. As expected, insert fragment sizes of pPACB1, pPACB2 and pPACB3 were 650 bp, 835 bp and 600 bp fragments, respectively.

3.7 DNA sequence analysis and primer design

Three recombinant clones (pPACB1 for a 650 bp insert, pPACB2 for a 835 bp insert and pPACB3 for a 600 bp insert) were sequenced for both direction using an automatic sequencer (ABI377, PE Applied Biosystems). Results from sequencing are shown in Fig 3.16. The actual molecular length of pPACB1, pPACB2 and pPACB3 was 637 bp, 811 bp and 563 bp, respectively. Comparisons of these sequences with those previously deposited in GenBank did not reveal significant similarity with any sequence. A pair of primers from each insert was designed (Table 3.7) and used to amplify *C. belcheri* total DNA. Sizes of PCR products were identical to those expected from their DNA sequences (536 bp, 600 bp and 506 bp, respectively). Heterozygotes were not observed in any investigated *C. belcheri* individual when analysed with these primer sets implying the retention of dominant segregated fashion of the original RAPD species-specific markers.

Table 3.5 The average genetic distances (below diagonal) and similarity indices (S_{ij}, above diagonal) between and within species of Thai oysters, the Australian oyster, *S. commercialis* and the mussel, *P. viridis*

	CbSR	CbSK	CbRN	CbKB	CiCB	CiPJ	CiSK	CiPN	CsKB	ScTD	ScRN	ScPK	ScCT	SfCT	SfSR
CbSR	-	0.8950	0.8720	0.8449	0.3226	0.3011	0.2997	0.3270	0.2860	0.2204	0.2303	0.2161	0.2340	0.2450	0.2310
CbSK	0.1050	-	0.8764	0.8497	0.2950	0.3054	0.3100	0.3245	0.2817	0.2291	0.2348	0.2221	0.2360	0.2614	0.2519
CbRN	0.1280	0.1236	-	0.9046	0.3012	0.3136	0.3012	0.3197	0.3161	0.2199	0.2085	0.1981	0.2243	0.2457	0.2420
CbKB	0.1551	0.1503	0.0954	-	0.3303	0.3379	0.3229	0.3430	0.3075	0.2062	0.2102	0.1890	0.2157	0.2545	0.2624
CiCB	0.6774	0.7050	0.6988	0.6697	-	0.8507	0.3950	0.7953	0.2674	0.2244	0.2468	0.2227	0.2167	0.2618	0.2629
CiPJ	0.6989	0.6946	0.6861	0.6621	0.1493	-	0.7987	0.7837	0.2768	0.2247	0.2439	0.2074	0.2192	0.2670	0.2611
CiSK	0.7003	0.6900	0.6988	0.6771	0.6050	0.2013	-	0.7825	0.2645	0.2405	0.2625	0.2330	0.2173	0.2596	0.2559
CiPN	0.6730	0.6755	0.6803	0.6570	0.2047	0.2163	0.2175	-	0.2735	0.2332	0.2535	0.2293	0.2299	0.2595	0.2784
CsKB	0.7140	0.7183	0.6839	0.6925	0.7326	0.7232	0.7355	0.7265	-	0.2686	0.2254	0.2202	0.2028	0.2658	0.2186
ScTD	0.7796	0.7709	0.7801	0.7938	0.7756	0.7753	0.7595	0.7668	0.7314	-	0.5820	0.5850	0.4939	0.2898	0.3067
ScRN	0.7697	0.7652	0.7915	0.7898	0.7532	0.7561	0.7375	0.7465	0.7746	0.4180	-	0.5962	0.5050	0.3057	0.3249
ScPK	0.7839	0.7779	0.8019	0.8110	0.7773	0.7926	0.7670	0.7707	0.7798	0.4150	0.4038	-	0.5620	0.3122	0.3174
ScCT	0.7660	0.7640	0.7757	0.7843	0.7833	0.7808	0.7827	0.7701	0.7972	0.5061	0.4950	0.4380	-	0.3180	0.2980
SfCT	0.7550	0.7386	0.7543	0.7455	0.7382	0.7330	0.7404	0.7405	0.7342	0.7102	0.6943	0.6878	0.6820	-	0.4540
SfSR	0.7690	0.7481	0.7580	0.7376	0.7371	0.7389	0.7441	0.7216	0.7814	0.6933	0.6751	0.6826	0.7020	0.5460	-
SfCBA	0.7520	0.7360	0.7490	0.7388	0.7756	0.7675	0.7816	0.7757	0.7609	0.7344	0.6870	0.6903	0.6647	0.5172	0.5399
SfPJ	0.7843	0.7645	0.7919	0.7784	0.7463	0.7385	0.7430	0.7527	0.7717	0.6970	0.6390	0.6385	0.6454	0.5136	0.5619
SfSK	0.7654	0.7546	0.7671	0.7520	0.7597	0.7351	0.7486	0.7436	0.7744	0.7034	0.6441	0.6731	0.6597	0.5261	0.6031
SfST	0.7646	0.7564	0.7783	0.7694	0.7665	0.7576	0.7024	0.7562	0.7619	0.6858	0.6761	0.6259	0.6320	0.5837	0.6158
SfRN	0.7560	0.7523	0.7692	0.7601	0.7655	0.7634	0.7592	0.7581	0.7571	0.6999	0.6793	0.6387	0.6408	0.5774	0.6289
SfCBS	0.7413	0.7420	0.7675	0.7568	0.7597	0.7659	0.7821	0.7525	0.7477	0.7188	0.6726	0.6660	0.6712	0.5948	0.5548
SmPK	0.7285	0.7160	0.7250	0.7280	0.7474	0.7363	0.7556	0.7294	0.7030	0.6961	0.6721	0.6662	0.6655	0.5353	0.5338
SmRN	0.7567	0.7467	0.7718	0.7623	0.7870	0.7931	0.8085	0.7821	0.7708	0.5647	0.7568	0.7331	0.7028	0.7102	0.6786
SmSS	0.7555	0.7454	0.7646	0.7570	0.7548	0.7493	0.7516	0.7472	0.7179	0.5657	0.7156	0.7006	0.6850	0.6913	0.5924
S1SR	0.7551	0.7512	0.7779	0.7584	0.7454	0.7528	0.7517	0.7218	0.7919	0.5337	0.7058	0.7028	0.6868	0.7028	0.5614
S2RN	0.6967	0.6996	0.6931	0.7200	0.7248	0.7098	0.7305	0.7477	0.7451	0.6628	0.6934	0.6875	0.7455	0.7036	0.6758
S3SS	0.7748	0.7648	0.7873	0.7701	0.7487	0.7588	0.7656	0.7516	0.7673	0.5733	0.7086	0.6633	0.6683	0.6763	0.6010
Scom	0.7642	0.7499	0.7665	0.7477	0.7738	0.7533	0.7068	0.7633	0.7447	0.6513	0.7398	0.7312	0.7135	0.7095	0.6505
Pevi	0.7496	0.7535	0.7645	0.7791	0.7412	0.7500	0.7456	0.7431	0.7597	0.7518	0.7757	0.7627	0.7467	0.7482	0.7750

Table 3.5 (continued)

	SfCBA	SfPJ	SfSK	SfST	SfRN	SfCBS	SmPK	SmRN	SmSS	S1SR	S2RN	S3SS	Scom	Pevi
CbSR	0.2480	0.2157	0.2346	0.2354	0.2440	0.2587	0.2715	0.2433	0.2445	0.2449	0.3033	0.2252	0.2358	0.2504
CbSK	0.2640	0.2355	0.2454	0.2436	0.2477	0.2580	0.2840	0.2533	0.2546	0.2488	0.3004	0.2352	0.2501	0.2465
CbRN	0.2510	0.2081	0.2329	0.2217	0.2308	0.2325	0.2750	0.2282	0.2354	0.2221	0.3069	0.2127	0.2335	0.2355
CbKB	0.2612	0.2216	0.2480	0.2306	0.2399	0.2432	0.2720	0.2377	0.2430	0.2416	0.2800	0.2299	0.2523	0.2209
CiCB	0.2244	0.2537	0.2403	0.2335	0.2345	0.2403	0.2526	0.2130	0.2452	0.2546	0.2752	0.2513	0.2262	0.2588
CiPJ	0.2325	0.2615	0.2649	0.2424	0.2366	0.2341	0.2637	0.2069	0.2507	0.2472	0.2902	0.2412	0.2467	0.2500
CiSK	0.2184	0.2570	0.2514	0.2976	0.2408	0.2179	0.2444	0.1915	0.2484	0.2483	0.2695	0.2344	0.2932	0.2544
CiPN	0.2243	0.2473	0.2564	0.2438	0.2419	0.2475	0.2706	0.2179	0.2528	0.2782	0.2523	0.2484	0.2367	0.2569
CsKB	0.2391	0.2283	0.2256	0.2381	0.2429	0.2523	0.2970	0.2292	0.2821	0.2081	0.2549	0.2327	0.2553	0.2403
ScTD	0.2656	0.3030	0.2966	0.3142	0.3001	0.2812	0.3039	0.4353	0.4343	0.4663	0.3372	0.4267	0.3487	0.2482
ScRN	0.3130	0.3610	0.3559	0.3239	0.3207	0.3274	0.3279	0.2432	0.2844	0.2942	0.3066	0.2914	0.2602	0.2243
ScPK	0.3097	0.3615	0.3269	0.3741	0.3613	0.3340	0.3338	0.2669	0.2994	0.2972	0.3125	0.3367	0.2688	0.2373
ScCT	0.3353	0.3546	0.3403	0.3680	0.3592	0.3288	0.3345	0.2972	0.3150	0.3132	0.2545	0.3317	0.2865	0.2533
SfCT	0.4828	0.4864	0.4739	0.4163	0.4226	0.4052	0.4647	0.2898	0.3087	0.2972	0.2964	0.3237	0.2905	0.2518
SfSR	0.4601	0.4381	0.3969	0.3842	0.3711	0.4452	0.4662	0.3214	0.4076	0.4386	0.3242	0.3990	0.3495	0.2250
SfCBA	-	0.5216	0.5244	0.4462	0.4658	0.4144	0.4498	0.3306	0.3442	0.6331	0.3271	0.3566	0.3102	0.2676
SfPJ	0.4784	-	0.5663	0.4795	0.4728	0.3984	0.4694	0.3370	0.3988	0.4351	0.3405	0.3034	0.3631	0.2514
SfSK	0.4756	0.4337	-	0.4700	0.4555	0.4426	0.4621	0.3700	0.4234	0.4156	0.3261	0.4330	0.3603	0.2203
SfST	0.5538	0.5205	0.5300	-	0.5692	0.3962	0.3965	0.3446	0.4270	0.4060	0.3228	0.4250	0.3645	0.2296
SfRN	0.5342	0.5272	0.5445	0.4308	-	0.4109	0.3911	0.3291	0.3743	0.4268	0.3134	0.4125	0.3522	0.2325
SfCBS	0.5856	0.6016	0.5574	0.6038	0.5891	-	0.4398	0.3139	0.3612	0.4090	0.2965	0.3882	0.3718	0.2143
SmPK	0.5502	0.5306	0.5379	0.6035	0.6089	0.5602	-	0.3519	0.3804	0.4641	0.2975	0.4127	0.3462	0.2641
SmRN	0.6694	0.6630	0.6300	0.6554	0.6709	0.6861	0.6481	-	0.4469	0.3672	0.2630	0.4137	0.3549	0.2488
SmSS	0.6558	0.6012	0.5766	0.5730	0.6257	0.6388	0.6196	0.5531	-	0.3797	0.2161	0.2982	0.2760	0.2488
S1SR	0.3669	0.5649	0.5844	0.5940	0.5732	0.5910	0.5359	0.6328	0.6203	-	0.3088	0.4480	0.3192	0.2158
S2RN	0.6729	0.6595	0.6739	0.6772	0.6866	0.7035	0.7025	0.7370	0.7239	0.6912	-	0.5269	0.3536	0.2433
S3SS	0.6434	0.6966	0.5670	0.5750	0.5875	0.6118	0.5873	0.5863	0.7018	0.5520	0.4731	-	0.3517	0.2716
Scom	0.6898	0.6369	0.6397	0.6355	0.6478	0.6282	0.6538	0.6451	0.7240	0.6808	0.6464	0.6483	-	0.1954
Pevi	0.7324	0.7486	0.7797	0.7704	0.7675	0.7853	0.7359	0.7512	0.7512	0.7842	0.7567	0.7284	0.8046	-

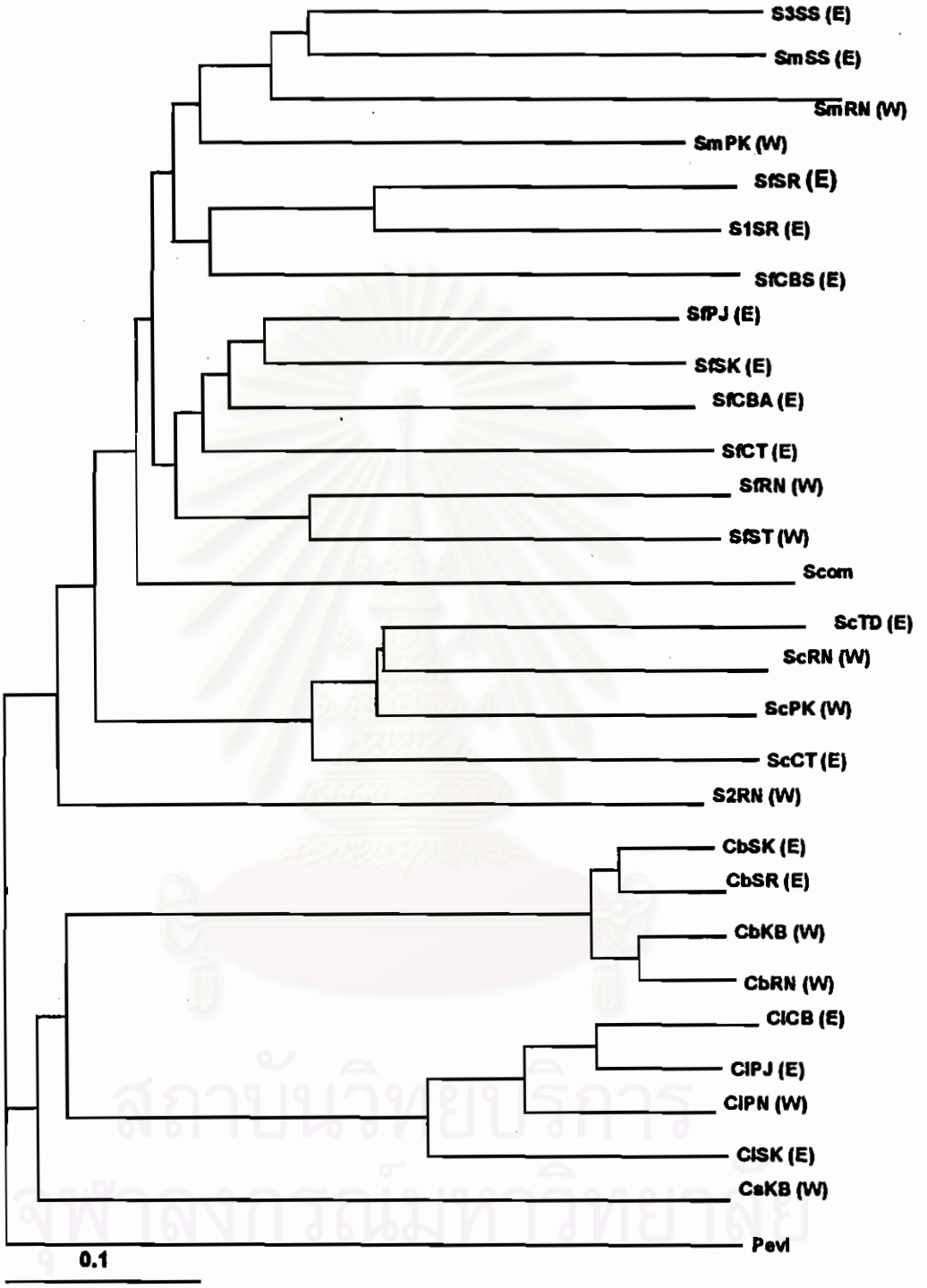


Figure 3.13 A neighbor-joining tree illustrating genetic relationships of oyster species locally found in Thailand based on genetic distances resulted from RAPD analysis using five primers (OPA09, OPB01, OPB08, UBC210 and UBC220). The Australian oyster (*S. commercialis*) and the mussel (*P. viridis*) were included as an ingroup and an outgroup references, respectively. Detailed information and abbreviations of sample sites are shown in Table 2.1.

Table 3.6 Species-specific RAPD markers of commercial oysters in Thailand revealed by RAPD analysis

Species	Primer	RAPD marker
C. belcheri	OPA09	250 bp
	OPB01	2100 bp, 1400 bp, 1250 bp and 650 bp
	OPB08	1650 bp, 1550 bp, 835 bp and 600 bp
	UBC220	1050 bp
C. iredalei	OPA09	1150 bp
	OPB01	700 bp
	OPB08	1250 bp and 450 bp
	UBC210	745 bp
S. cucullata	OPB08	750 bp
	UBC220	1800 bp

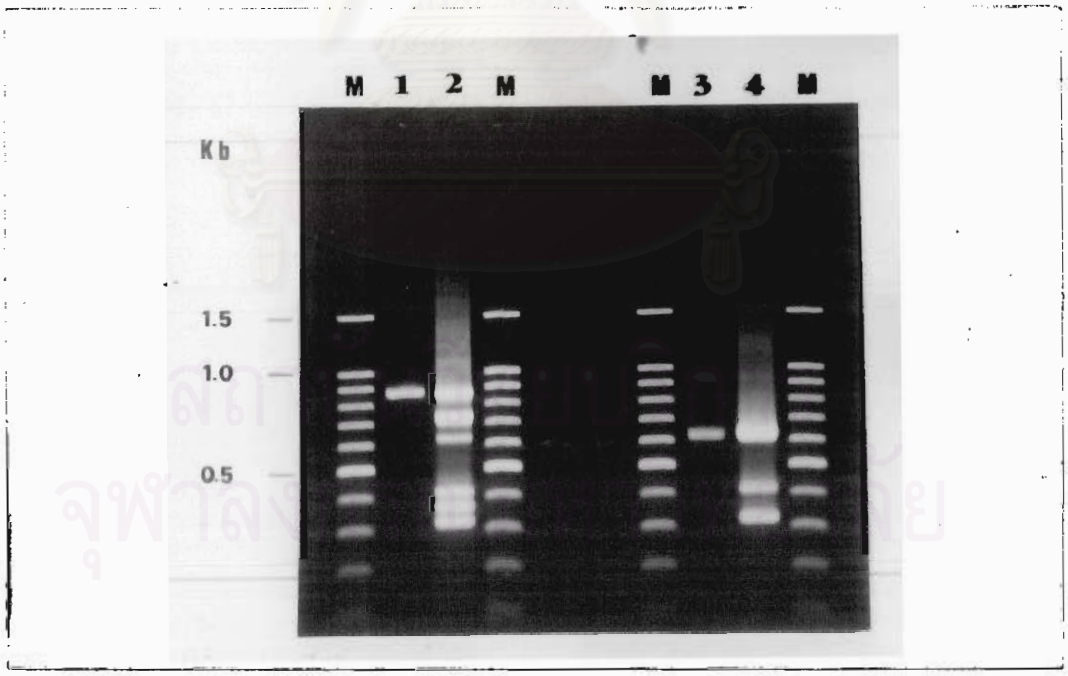


Figure 3.14 Reamplification of 835 bp and 600 bp fragments specifically found in *C. belcheri*. These fragments were eluted from agarose gels and served as the DNA template for reamplification using the primer OPB08 containing a *Bam*HI restriction site. The 835 bp (lane 1) and 600 bp (lane 3) original fragments were electrophoretically analysis compared with reamplified 835 bp (lane 2) and 600 bp (lane 4) products. A 100 bp ladder is used as a marker (lane M).

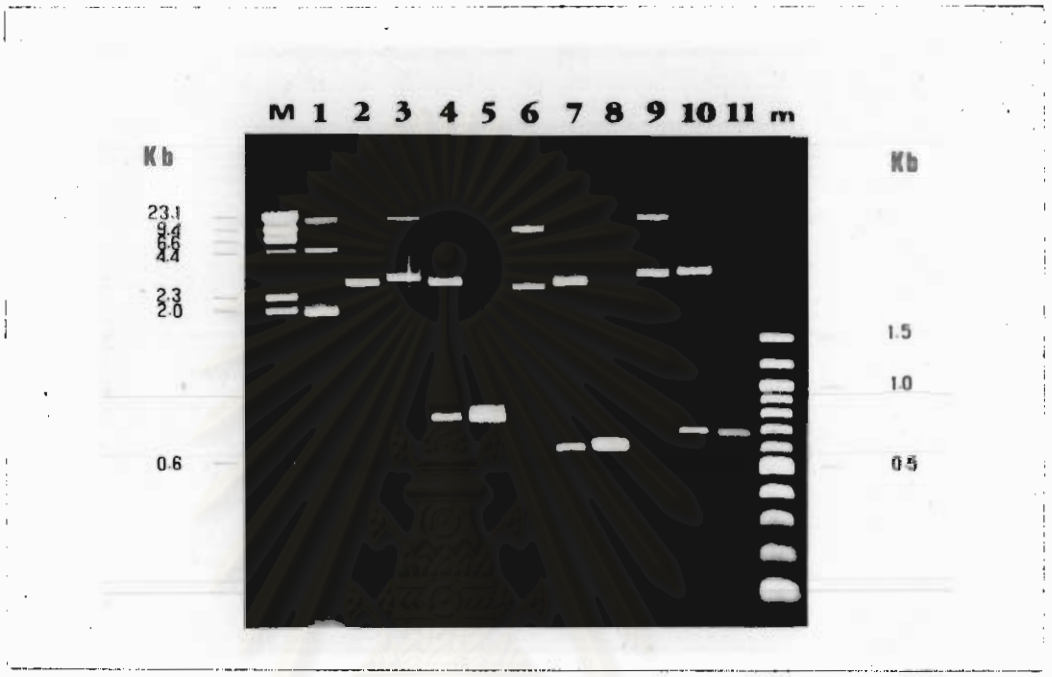


Figure 3.15 Characterization of inserted fragments of three recombinant clones (pPACB1, pPACB2 and pPACB3) on 1.0% agarose gel

Lane M = λ / *Hin*dIII standard marker
Lane 1 = undigested pUC18 vector
Lane 2 = pUC18 vector linearized with *Bam*HI
Lane 3 = undigested pPACB2
Lane 4 = *Bam*HI digested pPACB2
Lane 5 = a 835 bp eluted fragment
Lane 6 = undigested pPACB3
Lane 7 = *Bam*HI digested pPACB3
Lane 8 = a 600 bp eluted fragment
Lane 9 = undigested pPACB1
Lane 10 = *Eco*RI digested pPACB1
Lane 11 = a 650 bp eluted fragment
Lane m = a 100 bp DNA ladder

(A) pPACB1

```
ATGTAATTTC AACACGACAA AATCAGTGTT GATTTAAAAT ATATTGTTTT 50
                          pPACB1-F
TTACGGCGTT GCGCTAATGC TTGGCAGCCT TCTAGCAATC GTCTGCATTG 100
CAAATCGTCC AAAAATTCAT GCTCTGCACC GCCTTCTAAC TGTGCATAAG 150
AAACGAGTTC CATAAATTAT TAAAAATATT TCAAGATTTT TATTCCATTT 200
ATTCAACTTA ATTGTGTACA AAAAATCCAA CTTTGTCTCC CTGATTATTA 250
ACAACTCATT GCATAAGAAT AAATTTGCTT AATCAAGAAA TGGCGGATGA 300
ATACGATAAT GTGTAAAAAA ATTCACCAAA TATTATTTTC GTTTATCGCC 350
TATATTTTAT TTGGAGACCA TGCCCGACGG AAATAAAATT TAACACTTAA 400
ATTCAATTGT TATTGGGCAC GGTCTCCAAA ATAAATGTAA GCGATAAACG 450
TATCTAGTGT TTTTGTTGCA ATAGGTAAAC ATGATATAGA TACGGCAGAT 500
CAATTCATGC ACGTAGTGTG GATTCATACC ATGACTATTA GTATAGAAAT 550
TCAAATTAAT GATATCTATA TAAAAGAAAA TGTCTTGTTA ACGCTGTAAA 600
  pPACB1-R
ACTTTGATGC ATTGTGTATG TGAATATCAT ACCGAGA              637
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(B) pPACB2

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GATAGCAAAA GTTGACCAAG TGTGATACTC GGATACACTT GGATCCCATT 50
ATAAAATTGT TAAAGAGATT TAAAATACTC TGCATTGGAA GCAATTTGCA 100
GGATTTACGT ACATCAAAAA ATTACATCCA ACAGAATTTA GATTGACACA 150
pPACB2-F
CTCGCACACA ACCGTTCCTT GAAAACGTCA TGAATTATTG CTCGCATATT 200
AAGACAGAAA TATTTAACTT AATTGCACAA CACAAATTAA AAGGATGGCC 250
TACTTTATTA TTTCATACCT GGTTTAGAAG TATCGTTCAC CCTGGGCTCA 300
GATACACAGA GTACATGTGG ACACAGCAAG AAGAGAAGAA ATATCGATAA 350
TCGTCCATAA TAATCATAGG CTGGGGCCAT GTCAGGCGAT ATGAGAAGTT 400
GACTTTCAGA CGTTAAATCC AAGAACTCTA GTCATCACTT AAGCACAAGA 450
CACACACCAT AACCATTCTG TAGGTTACAA AATCAAACAC GGGAATATTG 500
ATGGCCGGTG TGTAAAAATG ATCGGACAGT TATATTTCCT CGTCGAATAA 550
TGACCAGTAG TGTTGGCATA TAGTTAGCAT GCGTGTTAAG TTTTGAGTAA 600
CCAAAAGGCA AAGTCGCGTG AAATAGCGTT ATATACAGAA TTTACCTGGA 650
GGCTACAGTA GTCGCCGTGT TACGCTTGGT TGCAAGTAGG TACGGCCAAT 700
                                        pPACB2-R
ACAGTTATAC AGATCTTTTC ACCTGAGGAT TCGCTGTTTT TCACTTTTAA 750
TCAGATGATA TCATTTATAT CGGATCTGAC ACAGGTAAAC TGGTTTCGCC 800
AAGTCACCAG G                                           81
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(C) pPACB3

pPACB3-F

AAATATTCAC ATTATGCTAA ATGAAAAGTC CGCAAAGCAT TTCCAGATTG 50

CAGATAACTG AATAAAAAAG ACTATTCAAA ATAGAATAGC ATGTAAAATA 100

AATAATTTAA TTGAAAACCT TACCGCCGAC ATCATTTTCT GTTACCAAAT 150

CCGTTTTTGT TTTGTTTTGT GAATATACAG AACTCTGAGA ACTGAGAGCA 200

TAAATAATTC CTGTGATTTG TAAATGATCA AGTGTCATAT TGAGTGATCA 250

AAACGTCCCT GTGTCCTGTC AGAATCAATT TAGGACGATG GGTCGGGACG 300

AACACCCATA ACAATCAGGG CCCCGCGTGC ACACCCCCGG GGTGACATTA 350

ATTGTATAAT ATATGATGGG CCTGCTGACT GTAATTCTTT CTTGTGGGTC 400

AATTCAAGGG ACATAATTGT AGCAGGGGAT TAGGGACAAC AGAGCACAAA 450

ATAGAATAAA ATTACAGACA TGAAAAGACA TAAAGATGTT TTTTTATCTG 500

pPACB3-R

TTATGTGTTA AGAAGCGTTG TTAAATTACG TGAATTACAA TGGAATTGAT 550

TTAAAAGTTA AAG 563

Figure 3.16 Nucleotide sequences of pPACB1 (A), pPACB2 (B) and pPACB3 (C) specimens. The locations and sequences of *C. belcheri*-specific forward primers (pPACB1-F, pPACB2-F and pPACB3-F) and those complementary to reverse primers (pPACB1-R, pPACB2-R and pPACB3-R) are labelled in boldface and underlined.

Table 3.7 Sequences of oligonucleotide primers designed from each recombinant clone carrying a species-specific fragment of *C. belcheri*

Clone	Primer sequence
pPACB1 (a 650 bp insert)	Forward primer : 5′- CAG CCT TCT AGC AAT CGT CT -3′ Reverse primer : 5′- GCA TCA AAG TTT TAC AGC GTT -3′
pPACB2 (a 835 bp insert)	Forward primer : 5′- CAC TCG CAC ACA ACC GTT CC -3′ Reverse primer : 5′- AAA GTG AAA AAC AGC GAA TCC-3′
pPACB3 (a 600 bp insert)	Forward primer : 5′- CTA AAT GAA AAC TCC GCA AA-3′ Reverse primer : 5′- AAC AAC GCT TCT TAA CAC ATA-3′

3.8 Sensitivity and species-specific tests

Primer sensitivity was tested by PCR amplification using varying concentrations of homologous DNA template (25 ng, 12.5 ng, 6.25 ng, 3.125 ng, 1 ng, 500 pg, 250 pg, 100 pg, 65 pg, 32.5 pg and 16.25 pg, respectively). Generally, there was good correlation between the amount of DNA template and intensity of a PCR product of all primer set. The sensitivity of detection roughly reached 30 pg of DNA template for each pair of primers (Fig. 3.17). This sensitivity level allows the possibility to use these markers to study dispersal and recruitment processes of *C. belcheri* larvae over vast geographic areas in Thailand.

Only, a pPACB1 primer pair was chosen for the specificity test in all local oyster species, the Australian oysters, *S. commercialis*, and the mussel, *P. viridis*. A positive 536 bp band was found in all *C. belcheri* individuals but was not observed in other species (Fig. 3.18). The results eliminated the possibility that a 650 bp fragment in *C. belcheri* and a 700 bp fragment in *C. iredalei* were homologous.

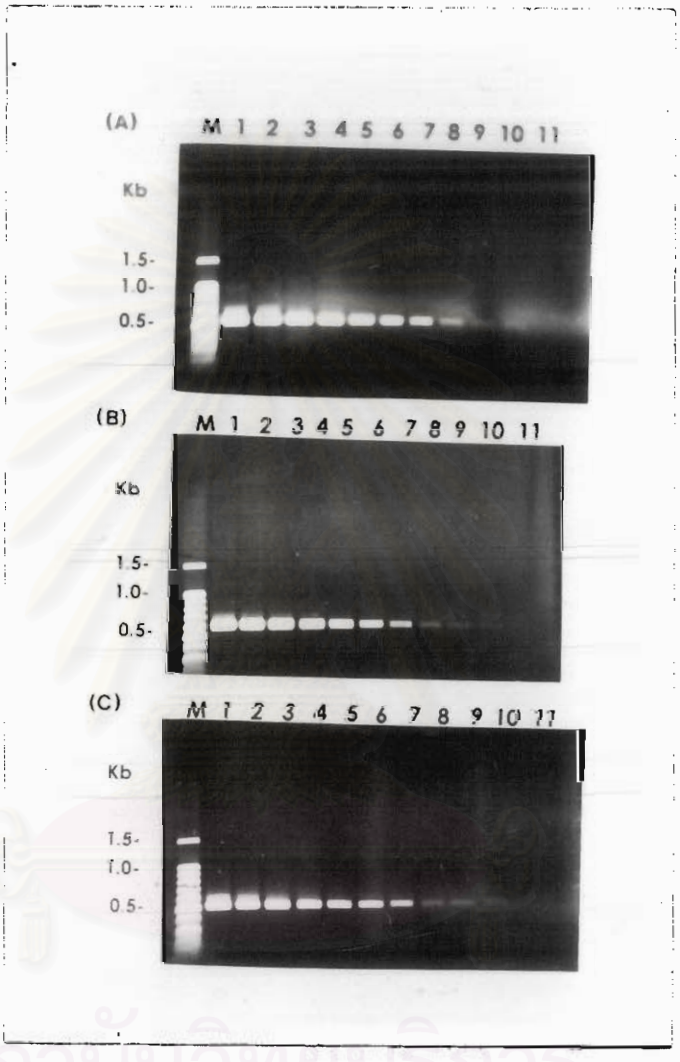

Figure 3.17 Sensitivity of pPACB1 (A), pPACB2 (B) and pPACB3 (C) primer sets was examined. PCR was carried out in the presence of the serial dilution of *C. belcheri* DNA template (25 ng, 12.5 ng, 6.25 ng, 3.125 ng, 1 ng, 500 pg, 250 pg, 100 pg, 65 pg, 32.5 pg and 16.25 pg corresponding to lanes 1-11, respectively) and electrophoretically analysed by ethidium bromide stained 1.0% agarose gels. A 100 bp ladder (lane M) was used as a DNA marker.

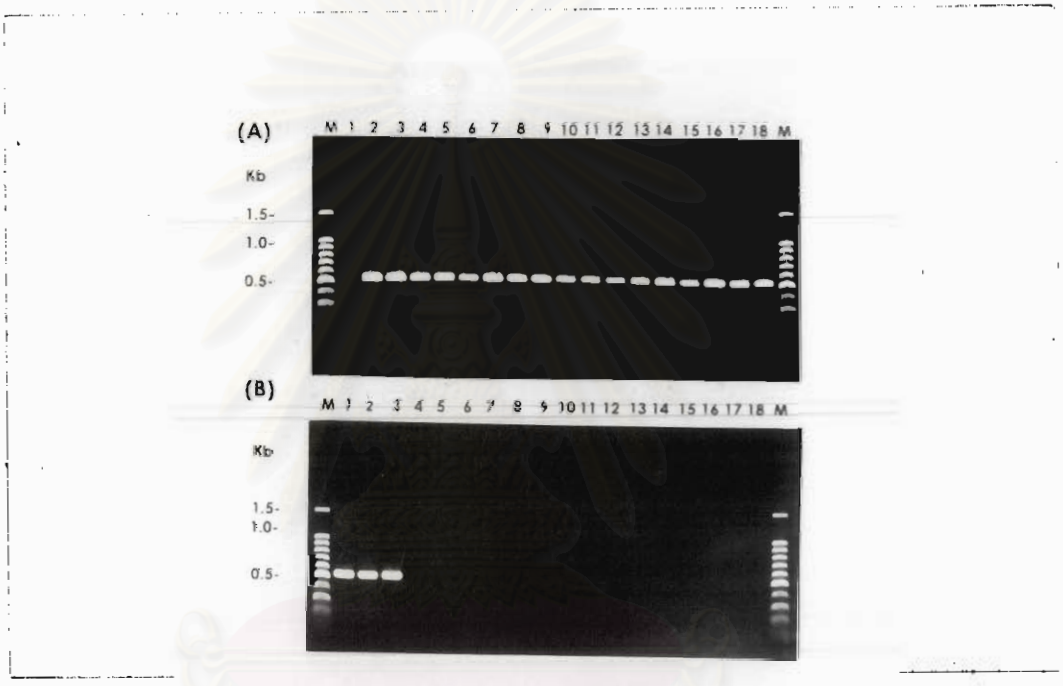

Figure 3.18 Agarose gel electrophoresis illustrating species-specific nature of a 536 bp PCR product of pPACB1 primers in *C. belcheri* (lanes 2-18, A and lanes 1-3, B). This fragment was not found in *C. iredalei* (lanes 4-6, B), *S. cucullata* (lanes 7-9, B), *S. mytiloides* (lanes 10-12, B), *S. forskali* (lanes 13 and 14, B), *S. commercialis* (lanes 15 and 16, B) and *P. viridis* (lanes 17 and 18, B). lane 1 is a negative control (without *C. belcheri* DNA). A 100 bp ladder was used as a DNA marker.