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RGF–PCR: A technique to isolate different copies of a multi-copy gene

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Isolation of different copies of a multi-copy gene from the genome is a difficult process. To overcome the difficulty, a novel method known as 'Restricted Genomic Fraction–Polymerase Chain Reaction' (RGF–PCR) has been developed. RGF–PCR is a combination of fractionation of the restricted genome and PCR methods. The transposon Mariner-Like Elements (MLEs) occur as multi-copy genes in the silkworm, *Bombyx mori* genome. Using RGF–PCR method, isolation of different copies of the multi-copy MLE from different loci of the silkworm genome has been demonstrated. Polymorphic copies of MLEs have been isolated from restricted genomic fractions of silkworm, and the nature of polymorphism has been analysed and presented in this paper.

IT is often difficult to isolate different copies of multi-copy genes from the genome[1]. To understand the micro-heterogeneities and minor polymorphism of the multi-copy genes and gene families, it is essential to isolate and clone different copies of the genes. Using conventional method of PCR, multiple copies may be amplified from the genomic DNA in a single reaction. However, the amplified copies may not be distinguished as different copies of the gene[2]. Similarly, degenerate primers can also amplify multiple copies of the gene from the genome; however, the amplified copies may not be differentiated from one another as from different loci. To pick up different copies of a multi-copy gene, genomic or subgenomic libraries can be screened. However, it is a laborious and time-consuming process[3]. Following 'Restricted Genomic Fraction–Polymerase Chain Reaction' (RGF–PCR) strategy, different copies of a multi-copy gene can be easily isolated from different loci of the genome and the method is described in this article.

The transposons, Mariner-Like Elements (MLEs) are present in *Bombyx mori* genome as multi-copy genes. Four different types of MLEs, namely BmSMmar[4], Bmmar1[5], BmMLE[6] and BmoMar[7] are present in the silkworm genome. Among these four types, the copy number of Bmmar1 and BmMLE is about 2400 and 100/haploid genome, respectively[5,6], while BmSMmar

Table 1. Details of different primers used and the expected size of PCR products

Primer	Amplifying region	Expected PCR product size (bp)	Reference
BmSMmar124F BmSMmar265R	Internal region of BmSMmar transposon	444	4
MAR124F MAR276R	Internal region of all types of mariner transposons	470	4
Bmmar1-ITR	Full length Bmmar1 transposon	1300	5

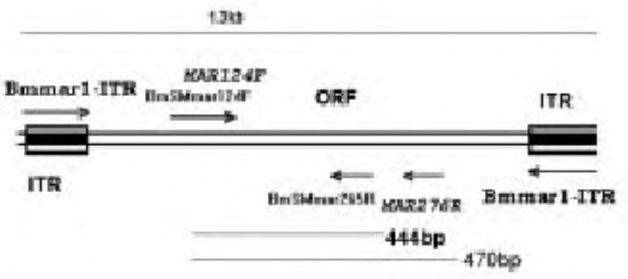

Figure 1. Schematic representation of the different primers used in RGF–PCR and their respective positions on a typical mariner-like transposable element. PCR amplifying regions and the fragment sizes are indicated. ITR, Inverted Terminal Repeat; ORF, Open Reading Frame. The primer sequences are as follows. MAR124F–5′TGGGTNCCNCAYGARYT 3′; MAR276R–5′ GGNGCNNARR TCNGG 3′; BmSMmar124F–5′ TGGGTGCCGCACCGAGTT 3′; BmSMmar265R–5′ GCCTAGCTCTGCGGCTTTC 3′; Bmmar1-ITR–5′ TCCTTACATATGAAATTAGCGTTTTGT 3′.

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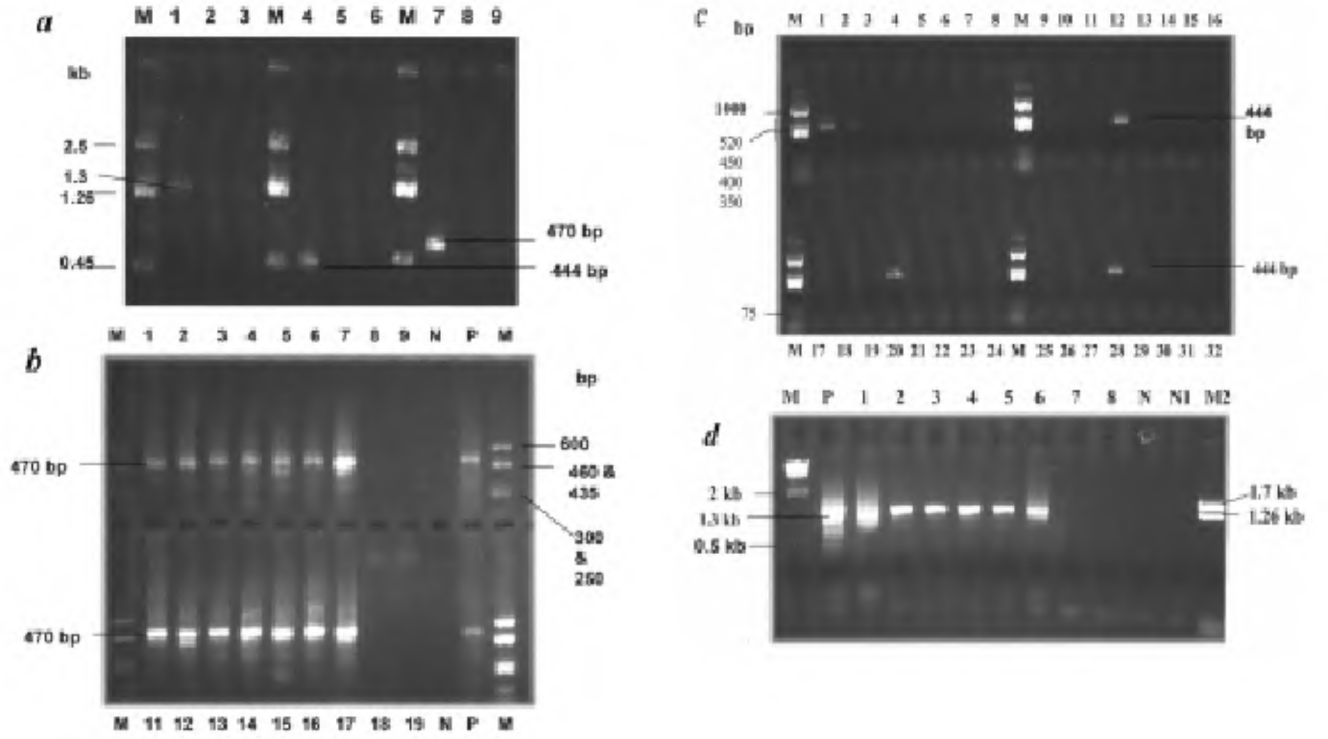

Figure 2. Electrophorograms showing the PCR amplifications of MLEs from different restricted genomic fractions (RGF–PCR) of *B. mori*. The template DNA used in different PCRs are specified as follows: (*a*), *Positive and negative controls*. M, pBSSK + /*Pvu* II and pBSSK + /*Bgl* I marker. *Bmmar1-ITR primer:* Lane 1, Positive control – *B. mori* whole genomic DNA; lane 2, Negative control – without template; lane 3, Negative control – *Pseudomonas* sps, genomic DNA. *BmSMmar124F and BmSMmar265R primers:* Lane 4, Positive control – *B. mori* whole genomic DNA; lane 5, Negative control – without template; lane 6, Negative control – *Pseudomonas* sps, genomic DNA. *MAR124F and MAR176R* primers: lane 7, Positive control – *B. mori* whole genomic DNA; lane 8, Negative control – without template; lane 9, Negative control – *Pseudomonas* sps, genomic DNA. ***b***, *MAR124F and MAR276R – Degenerate primers*. M, pBSSK + *Hae*III marker, lanes 1 and 11, > 10 kb fraction; lanes 2 and 12, 10–7 kb fraction; lanes 3 and 13, 7–5 kb fraction; lanes 4 and 14, 5–3 kb fraction; lanes 5 and 15, 3–2 kb fraction; lanes 6 and 16, 2–1 kb fraction; lanes 7 and 17, 1–0.5 kb fraction; lanes 8 and 18, 0.5–0.3 kb fraction; lanes 9 and 19, < 0.3 kb fraction; lanes, 1–9, *Sac*I fractions and lanes, 11–19, *Eco*RI fractions. N, Negative control (without template) P, Positive control – whole genomic DNA (*B. mori*). (***c***) *BmSMmar primers* M, pBSSK + /*Hinf*I marker; lanes 1, 9, 17 and 25, > 10 kb fraction; lanes 2, 10, 18 and 26, 10–7 kb fraction; lanes, 3, 11, 19 and 27, 7–5 kb fraction; lanes 4, 12, 20 and 28, 5–3 kb fraction; lanes 5, 13, 21 and 29, 3–2 kb fraction; lanes 6, 14, 22 and 30, 2–1 kb fraction; lanes, 7, 15, 23 and 31, 1–0.5 kb fraction; lanes 8, 16, 24 and 32, 0.5–0.3 kb fraction; lanes 1–8. *Eco*RI fractions; lanes 9–16, *Hae*II fractions, lanes 17–24, *Hin*dIII fractions and lanes 25–32, *Sac*I fractions. (***d***) Bmmar1-ITR primer. M, λ-*Hin*dIII marker; M2-pBSSK + /*Bgl*I marker; P, Positive control – whole genomic DNA (*B. mori*). N, Negative control (without template); N1, *Pseudomonas* sps genomic DNA (no MLE in the genome); lane 1, *Sac*I, > 10 kb fraction; lane 2, *Sac*I, 10–7 kb fraction; lane 3, *Sac*I, 7–5 kb fraction; lane 4, *Sac*I, 5–3 kb fraction; lane 5, *Sac* I, 3–2 kb fraction; lane 6, *Sac*I, 2–1 kb fraction; lane 7, *Sac*I, 1–0.5 kb fraction; lane 8, *Sac*I 0.5–0.3 kb fraction.

Table 2. Amplification pattern of mariner transposons from different restricted genomic fractions. Primers used are specified. Expected size of the PCR product is given in parenthesis

Primer →	BmSMmar 124F and 265R (444 bp)				MAR124F and MAR276R (470 bp)				Bmmar1-ITR (1300 bp)
Enzyme → DNA fraction ↓	*Hae*II	*Sac*I	*Eco*RI	*Hin*dIII	*Hae*II	*Sac*I	*Eco*RI	*Hin*dIII	*Sac*I
> 10 kb	–	–	+	–	+	+	+	+	+
10–7 kb	–	–	+/–	–	+	+	+	+	+
7–5 kb	–	–	–	–	+	+	+	+	+
5–3 kb	+	+	–	+	+	+	+	+	+
3–2 kb	–	+/–	–	–	+	+	+	+	+
2–1 kb	–	–	–	–	+	+	+	+	+
1–0.5 kb	–	–	–	–	+	+	+	+	–
0.5–0.3 kb	–	–	–	–	–	–	–	–	–
< 0.3 kb	–	–	–	–	–	–	–	–	–
Without template negative control	–	–	–	–	–	–	–	–	–
MLE –ve genome Negative control			–				–		–

Table 3. Details of the MLEs amplified by MAR124F and MAR276R primers from various genomic fractions. Sequence similarities between the RGF clones and the close MLEs are illustrated

Clone name	Genomic fraction used for PCR	MLE type	Nearest neighbour MLEs for the clone	DNA similarity (%) with close MLEs
RGF1	*Hae*II, 5–3 kb	BmoMar	*Atteva punctella* (U91342)	91
RGF2	*Hae*II, 2–1 kb	BmoMar	*B. mandariana* (AF212132)	97
RGF3	*Sac*I, > 10 kb	BmoMar	*B. mori* (U91382)	92
RGF4	*Eco*RI, 5–3 kb	BmoMar	*B. mori* (U91382)	93
RGF5	*Hin*dIII, 2–1 kb	Antheraea	*Antheraea yamami* (AB041902)	80
RGF6	*Hae*II, > 10 kb	Antheraea	*Antheraea roylei* (AF125231)	85
RGF7	*Sac*I, 2–1 kb	BmMLE	*B. mori* (D88671)	63
RGF8	*Hae*II, 10–7 kb	BmMLE	*Hyalophora cecropia* (L10446)	80

Table 4. Similarities among the eight copies of MLEs amplified by RGF–PCR. Nucleotide similarity is mentioned in percentage. None of the sequences are identical. Variations in the sequence indicate that all the MLEs amplified are different copies of a multi-copy gene

	RGF1	RGF2	RGF3	RGF4	RGF5	RGF6	RGF7	RGF8
RGF1	100	87.5	87.4	88.6	45.3	41.2	52.6	49.3
RGF2		100	90.7	86.5	49.0	45.5	52.9	48.0
RGF3			100	88.4	45.5	44.6	52.7	42.6
RGF4				100	44.0	43.7	46.8	43.3
RGF5					100	78.8	56.8	49.2
RGF6						100	50.7	40.5
RGF7							100	77.0
RGF8								100

exists in fewer numbers (unpublished observation). Using three different sets of MLE type specific primers, different copies of MLEs were amplified from the restricted fractions of the silkworm genomic DNA and the use of RGF–PCR method was tested.

Genomic DNA was extracted from silkworm following standard protocol[8]. About 15 µg DNA was completely digested (individually) with the following restriction enzymes: *Sac* I, *Hae*II, *Hin*dIII and *Eco*RI. The restricted DNA was resolved in 0.6% agarose gel; a long gel of about 20 cm was used and electrophoresed at low voltage for about 14 h. Restricted DNA was fractionated based on its size. The following DNA fractions were collected by gel elution method (Clean Genei kit, India): 10 kb and above, 10–7 kb, 7–5 kb, 5–3 kb, 3–2 kb, 2–1 kb, 1–0.5 kb, 0.5–0.3 kb and below 0.3 kb. The eluted DNA fragments were dissolved in 100 µl TE buffer and the sizes of the fractions were checked on a gel. About 5 µl (10–50 ng) of the eluted DNA was used for subsequent PCR amplifications. Three different PCRs were carried out from each fraction using different sets of primers (Table 1). The position of the primers used with reference to a transposable element is shown in Figure 1. Since the MLEs occur in high copy numbers, all the primers, except the BmSMmar, are expected to amplify the MLEs from most of the fractions. The expected size of PCR products for each primer is shown in the Table 1. Appropriate positive and negative controls were used to check the false positive amplifications (Figure 2 *a*).

PCR-amplified mariner elements from different fractions were purified (Qiagen, PCR product purification column), blunted using *Pfu* DNA polymerase[9] and ligated to a blunt-ended plasmid vector (pMos blue, Amersham/pBSSK$^+$, Stratagene). Recombinants were screened and the plasmid DNA isolated from the recombinant clones was sequenced by the dideoxy termination method[10] in automated DNA sequencer (ABI prism, Perkin Elmer) or with ET terminator in MegaBACE1000 (AP Biotech), using universal and custom-made primers. Nucleotide sequences were analysed using the GCG software (version 9.1) (Wisconsin University, Madison, USA) in a Unix-based server.

```
RGF1            TGGGTGCCACATGAGTTCACTGAAAGAAACCTAATGGAAGGTGTATTCATTCGTGATTGT
RGF2            TGGGTGCCACATGAGCTCACTGAAAGAAACCTAATGAACCGTGTACTCATTTGTGATTCT
RGF3            TGGGTTCCGCATGAGCTCACTGAAAGAAACCTAATAAACCGTGTACTCATTTGTGATTCT
RGF4            TGGGTTCCACACGAGCTTACTGAAAGAAACCTA-TGAAC-GTGTATTCATTTGTGATTCT
                ***** ** ** *** * *************** *  *  ***** ***** ****** *

RGF1            TTATTACGACGTAATGAAACCGTACCATTTTTTAAGAAGCTGATAACTGGTGATGAAAAG
RGF2            TTATTACGACGTAATGAAACCGAACCATTTTTGAAGAAGCTGATAACTGGTGATGAAAAG
RGF3            TTATTACGACGTAATGAAACCGAACCATTTTTGAAGAAGCTGATAACTGATGATGAAAAG
RGF4            TTATTACGACGTAATGAA-CCGAACCGTTTTTGAAGAAGCTGATAAGTAATGATGAAAAG
                ****************** *** *** ***** ************* *  **********

RGF1            TGGATCACGTACGACAAGAACATGCGGAAAAG--GTCGTGGTCAAAGGCCGGTCAGGCTT
RGF2            TGGATCACGTACGACAACAACGTGCGAAAGAG--GTCGTGGTCAAAGACCAGTCAGGCTT
RGF3            TAGATCACGTACGACAAGAACGTGCGAACGTGCGGTCGTGGACAAAGGCCGGTCAGGCTT
RGF4            TGGATCACGTACGACAAGAACGTGTGAAAAAT--GCCGTGGTCAAAGGCCGATCAGGCTT
                * *************** *** ** * *       * ***** ***** **  ********

RGF1            CACATACTGTGGCGAAACCCGGGATAACTCGCAACAAGGTGATGCTGCATGTG-------
RGF2            CACAGACTGTGGCGAAACTCGGGCTAACTCGCAAAAAGGTGATGCGAGATGTGATCACAC
RGF3            CACAGACTGTGGCGAAGCCCGGGTTAACTCGCAACAAGGTGATGCTGTGTGTG-------
RGF4            CACAGACTGTGGTGAAACCCGGGTTGACT---------GTG--------TGTG-------
                **** ******* *** * **** * ***          ***         ****

RGF1            ---TGGTGTGATTGGAAAGGCATTATTCATTATGAGCTGTTACCACCAGGCAGGACCATC
RGF2            ACACGGTGGGGTTGGAAGAGCATTATTCATTATGGGCTGTTACCAGCAGGCAGGACCATC
RGF3            -T--GGTGGGATTGGAAGAGCATTATTCGTTATGAGCTGTTACCAACAGGCAGGACCATC
RGF4            ---TGGTGGGATTGGAAGGGCATTATTCATGATGAGCTGTTACCACCAGGCAGGACCATC
                    **** * ******  ********* * *** ********** **************

RGF1            GATTCTGAACTCTACTACGAACAACTAATGAGATTAAAGCAAGAAGCTGAGAGAAAGCGG
RGF2            GATTCTCAGCTCTACTGCGAACAACTGATGAGATTAAAGCAAGAAGTTGGGAGAAAGCGG
RGF3            GATTCTGAACTCTACTGCGAACAACTGATGAAATTAAGGCAAGAAGTTGAGAGAAAGCGG
RGF4            GATTCTGAACTCTACTGCGAACAACTGAT-----TAAAGCAAGAAGTTGAGAGAAAGCGG
                ****** * ******* ********* **     *** ******** ** **********

RGF1            CTGGAATTAATCAACAGAAGGGGTGTAGTTTTTCATCATGATA----------TCACACA
RGF2            CCGGAATTCATCAACAGAAGGG-----GTTTTTCATCATGATAACGCTAGCCCTCACACA
RGF3            CTGGAATTAATCAACAGAAGGGATGTGGTTTTTCATCATGATAACGCTAGACCTCGCACA
RGF4            C-GGAATTAATCAACAGAAGGGGTGTGGTTTTTCATCATGATAACGCTAGACCTCGCACG
                * ****** *************     ****************          ** ***

RGF1            TGTTGAATCACTCAGCAAAAAGTAAGAGAGCTTGGCTGGGAGATGTTAATGCATCCACCG
RGF2            TCTTTAGCCAGTCAGCAAAAATTGAGAGGGCTTGGCTGGGAGGT-TTAATGCATCCGCCG
RGF3            ATTTTAGCCACTCAGCAAAAATTAAGAGAGCTTGGCTGGG-----------CATCTGCCG
RGF4            TCTTTAGCCACTCAGCAAAAATTAAGAGAGCTTAGCTGGAAGGG-TTAATGTATCCGCT-
                  ** *  ** ********** * **** **** *****             ***  *

RGF1            TACACCCCTGGACCTGCCCC
RGF2            TACACTCCTGACTTAGCCCC
RGF3            TATTCACCTGATTTCGCCCC
RGF4            TATACCCCCGACTTGGCCCC
                **  * ** *     *****
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Figure 3 *a*. Multiple alignment of the BmoMar-type mariner elements, which were amplified by RGF–PCR from the following restricted genomic fractions: RGF1–*Hae*II, 5–3 kb; RGF2–*Hae*II, 2–1 kb; RGF3–*Sac*I, > 10 kb; RGF4–*Eco*RI, 5–3 kb fraction.

MAR124F and MAR276R are degenerate primers and can amplify the internal region (470 bp) of all kinds of MLEs[2]. These primers amplified the MLEs in all the fractions of restricted genomic DNA of 1 kb and above (Figure 2 *b*). BmSMmar primers are specific primers and are expected to amplify 444-bp internal region of BmSMmar; these primers amplified the MLEs in few fractions because the BmSMmar is a low-copy transposon (Figure 2 *c*). Bmmar1-ITR primer amplified the expected 1300 bp fragment from most of the fractions (Figure 2 *d*).

Figure 3 *b*. Alignment showing the diversity of Antheraea-type MLEs (RGF5 and RGF6). RGF5 element was isolated from *Hin*dIII, 2–1 kb fraction and RGF6 element was from *Hae*II > 10 kb fraction. These two elements are 78.8% similar in their nucleotide sequence.

Table 5. MLE types amplified using MAR124F and MAR276R primers from the genome of *B. mori* by various workers

Reference	PCR amplified MLE type	Numbers of MLE types amplified in the study
Mathavan *et al.*[4]	BmSMmar	1
Tomita *et al.*[6]	BmMLE	1
Robertson *et al.*[11]	BmoMar	1
Jinsung Lee[7]	BmoMar	1
Current study (RGF–PCR)	BmMLE, BmoMar and Antheraea	3

As the genomic fragments of varying sizes (generated by different restriction enzymes) are used as templates for the amplification of the MLEs (Table 2), these MLEs must represent different individual copies from different loci of the genome. Most of the PCR fragments amplified from different fractions were cloned into plasmid vectors. Eight clones of MLEs that were amplified by MAR124F and MAR276R primers from different fractions were sequenced. Sequence analysis reveals that all the eight clones represent eight different copies of MLEs (Table 3). These clones comprise three different types of MLEs such as BmoMar, BmMLE and Antheraea. Among these eight clones (RGF1–RGF8),

Figure 3 *c*. Alignment of the BmMLE-type mariner elements, which were amplified by RGF–PCR. RGF7 element was isolated from *Sac*I, 2–1 kb fraction and RGF8 element was from *Hae*II, 10–7 kb fraction. These two elements show 77% similarity.

four clones represent BmoMar-type MLEs, two clones represent the BmMLE-type and the remaining two clones belong to antheraea-type of MLEs. Apart from representing three categories of MLEs, within each category, all the amplified elements are diverging from each other, which obviously means that all the eight clones represent eight different copies of the MLEs (Table 4). Sequences of the different types of MLEs amplified by RGF–PCR were aligned and presented in Figure 3 *a–c*. The multiple alignments show the variations among the individual copies of the multi-copy MLE. Moreover, the clones RGF5 and RGF6 show about 90% similarity with the MLEs of *Antheraea pernyi, A. yamamai* and *A. mylitta*, which are Chinese, Japanese and Indian wild type silkworm, respectively. The notable point is the Antheraea-type MLE, which is amplified by RGF–PCR in the current study, is a report on the occurrence of Antheraea-type of MLE in *B. mori*. With this element, the number of existing mariner subfamilies in *B. mori* is five. Extensive sequencing of more copies of MLEs from each genomic fraction will reveal the complexity of intragenomic polymorphism of the mariner transposon.

The degenerate primers MAR124F and MAR276R were initially used by various groups[4,6,7,11] to scan the *B. mori* genome for the MLEs. In all the cases it amplified only one type of MLE, despite the feature that these primers are capable of amplifying multiple types of mariner elements[2]. But in the current study, RGF–PCR amplified three different types of MLEs (Table 5), which was not possible in the earlier studies. The probable reason for this is that in conventional PCR, while there are numerous copies of a gene, some copies might be amplified in the earlier cycles and those copies will be amplified further and further. Moreover in a given PCR condition, some copies may amplify better than their counterparts of the same genome. But in RGF–PCR, different regions of the template DNA are accessible to primers in different fractions and so all the copies have a chance for amplification from one or the other individual fractions. Further, using different restriction enzymes and different combinations of restriction enzymes, many regions of the genomic DNA can be fractionated and more individual copies can be amplified.

Limitation in this technique may be due to the following: (i) partial or incomplete digestion of the genomic DNA; (ii) shearing of DNA during extraction process, and (iii) molecular trapping of part of the smaller fragments of DNA in large fragments in gel electrophoresis. These factors can result in the amplification of the same copy from different fractions. However, such kind of limitations can be eliminated by careful extraction of the genomic DNA, complete digestion of the DNA with restriction enzyme and by complete fractionation of the fragments. Despite these features, RGF–PCR will be a useful technique to isolate, amplify and analyse the intragenomic polymorphism of different copies of a multi-copy gene. Apart from analysing the multi-copy genes, this technique can be used to (i) analyse the repetitive DNA elements, and (ii) locate and analyse the integration site of the transgene in different regions of the genome of a transgenic animal.

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Live sperm from post-mortem preserved Indian catfish*

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In the 240-day post-mortem preserved (at –20°C) specimens of *Heteropneustes fossilis*, live, fertile sperm are present. Motility and initiation of development were adduced as evidence for survival and fertility of the cadaveric sperm. Of the normal eggs that were fertilized by the cadaveric sperm, 97% underwent cortical reactions, 95% first few cell divisions, 40% blastulation, 3% gastrulation and 2% alone successfully hatched.

THIS communication reports about the discovery of live, fertile sperm from dead but preserved (at –20°C) specimens of the teleostean fish *Heteropneustes fossilis* for 240 days. Investigations on mammalian organ transplantation, especially humans have shown that non-vascularized cornea of the eye can be *in vitro* preserved for the longest duration of 4.6 years[1]; however, transplantation of vascularized organs like kidney and heart is directly made from the donor; the recently developed perfusion techniques have extended the life of cadaver kidney (33 days)[2] or heart (2 days)[3] for a limited duration prior to its transplantation. Hence, the life of vascularized organs can be prolonged under special conditions for a known period of time, even after their removal from live or just-dead donors. However, the need for such transfer of other vascularized organs like testis seems not to have arisen; for a literature search indicated that except for a stray report of Dushkina[4],

*Dedicated to Prof. M. S. Swaminathan, scientist, scholar and humanist, on his 76th birthday.

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