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MINI-REVIEW

Comparison and critical evaluation of PCR-mediated methods to walk along the sequence of genomic DNA

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Abstract Although researchers can access information on the entire genomic DNA sequence of typical research organisms, convenient genome walking methods in the laboratory are still needed. For the analysis of microorganisms, these methods are especially useful because the available genetic information is often scarce or limited. Many genomic walking methods are based on the polymerase chain reaction (PCR), and useful methods have been developed. This report reviews the methodologies of PCR-mediated genomic walking methods and evaluates their efficiency and usefulness to help microbiologists to select the appropriate method for each target microorganism. The concept and specific features, such as advantages and disadvantages, of five major PCR-mediated genomic walking methods (random PCR, inverse PCR, panhandle PCR, cassette PCR, and rapid amplification of genomic ends) are briefly described. The improved methods and their characteristics are listed, and a report of experimental comparison of such methods is also introduced briefly. Each of these methods has both advantages and disadvantages, and there is a trade-off between the specificity of target amplification and the ease of the method. The cassette PCR seems to be a standard method, but suitable method should be selected in consideration of the characteristics of the material.

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Introduction

Although we are in the age of high-throughput DNA sequencing (Szybalski 1993) that allows researchers to access information on the entire genomic DNA sequence of typical research organisms, convenient methods of walking the genomic sequence in the laboratory are still needed. For the analysis of microorganisms, genomic walking methods are especially useful in situations in which the available genetic information is relatively scarce or limited. The genomic walking methods available in the laboratory are often based on the polymerase chain reaction (PCR), and since Saiki et al. (1988) reported an in vitro DNA amplification method using a thermostable DNA polymerase, many useful genomic walking methods have been developed.

Recently, the authors reported the "one-base excess adaptor ligation" method, which is a novel PCR-mediated method for genomic walking (Tonooka et al. 2008). In that report, we briefly introduced previous genomic walking methods and listed their names and inventors. Because microbiologists would be aided by a critical review and comparison of the various methods, this report reviews the methodologies of PCR-mediated genomic walking and evaluates their efficiency and usefulness. Improved methods and their characteristics are also introduced briefly.

Methodology

In general, there is a difficulty to apply PCR techniques to genomic walking because PCR is only applicable to the

amplification of the genomic region located between at least two known sequences. Therefore, the aim of PCR-mediated genomic walking is to "find" a determined sequence upstream or downstream from a known sequence. Commonly, nested PCR, using primary PCR products and degenerate primers, is performed for the selective amplification of the target sequence. Also, tagging of the sequence-specific primer (primer designed from a known sequence) by biotinylation is sometimes used to purify the sequence-specific PCR product through streptavidin capture (Jones et al. 1994; Mishra et al. 2002).

PCR with random primers

Researchers have developed methods for the rapid, semi-random amplification of a target unknown region in genomic DNA (Parker et al. 1991). Lin et al. (1995) described a standard protocol that includes PCR with one sequence-specific primer plus primers that have random sequences (Fig. 1). To maximize the efficiency of amplification, the annealing temperature is relatively low, and the random primers are shorter than usual. The advantage of this method is that it is the simplest procedure for amplifying a target region because it does not require the digestion of genomic DNA with restriction enzymes or ligation. One disadvantage of the method is that the target region will be successfully amplified only by chance. The existence of nonspecific PCR products due to the low annealing temperature and random primer sequence is also problematic.

Improvements have been made to random primer methods that allow these drawbacks to be overcome. Liu and Whittier (1995) described the "thermal asymmetric interlaced PCR" method using long sequence-specific primers and a short arbitrary degenerate (AD) primer. The PCR reaction includes high-stringency, low-stringency, and mixed cycles. The low-stringency cycle creates an annealing site adapted for the AD primer. The high-stringency and mixed cycles prevent the amplification of nontarget sequence. Chen and Wu (1997) reported the "uneven PCR" method, in which PCR is performed using a sequence-specific primer and random (arbitrary) primers under the consecutive cycles of two different annealing temperatures. Ge and Charon (1997) developed the "semi-random PCR" method. To select the target product, the primary PCR products amplified using sequence-specific primer and random primers are cloned into plasmid vectors; secondary PCR is performed using sequence-specific and vector-specific primers. Aquino and Figueiredo (2004) reported the "linear amplification followed by single primer PCR" method. In this method, the target unknown region is amplified linearly in the first-step PCR using a single sequence-specific primer with a high annealing temperature, and the second-step PCR is performed

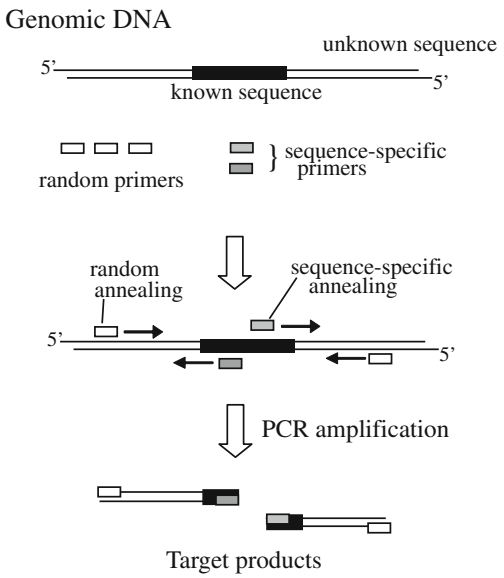

Fig. 1 Scheme of the random PCR method. Sequence-specific primers (*gray boxes*) are designed from known sequence (*black box*), and random primers (*white boxes*) anneal to genomic DNA (*lines*) randomly under the relatively low annealing temperature. Target products are amplified successfully when random primers anneal an appropriate location in the genomic DNA. The *5′* shows the 5′-end of DNA chain (modified from Lin et al. 1995)

using the same primer with a low annealing temperature, such that the primer anneals randomly to the first PCR product. Based on the same concept, Pilhofer et al. (2007) developed the "two-step gene walking" method. In the first step, single-stranded walking PCR products are amplified linearly by a sequence-specific primer followed by complementary double-strand formation by random primers. The second step involves direct sequencing using a sequence-specific nested primer. For high-throughput genome walking, Reddy et al. (2008) reported the hyper-branching of strand-displacement DNA synthesis using a walker adaptor primer and Phi 29 DNA polymerase to create overlapping fragments covering the entire genome; the target region is then amplified using sequence-specific primer.

The products amplified using random PCR can be up to 4-kbp long (Aquino and Figueiredo 2004; Pilhofer et al. 2007); this method also has been applied to gene isolation from polyploid wheat using pairs of random primers (Huang and Cloutier 2007).

Inverse PCR

Ochman et al. (1988) reported the "inverse PCR" method, which uses three steps to amplify the upstream and downstream regions of a known sequence (Fig. 2). This method consists of (a) DNA digestion with a restriction enzyme that generates sticky ends, (b) self-ligation between

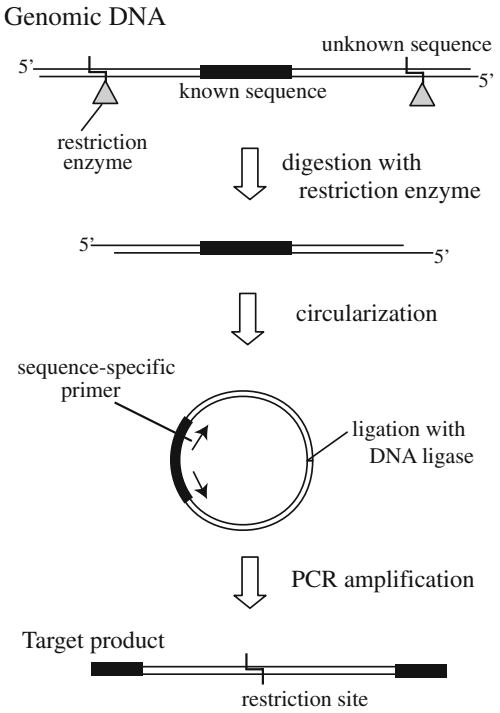

Fig. 2 Scheme of the inverse PCR method. Genomic DNA is digested with a restriction enzyme (a *triangle* with *right-angled lines*), and the two ends of the digested DNA fragment are ligated with DNA ligase (circularization). PCR is performed using a pair of sequence-specific primers (inverse primer) directed to a region outside of the known region. Successful PCR product includes both the upstream and downstream region of known sequence with a restriction site (modified from Ochman et al. 1988)

two ends of the digested DNA fragment (circularization) using DNA ligase, and (c) PCR amplification of target regions using a pair of primers that are directed to a region outside of the known region (inverse primer). The benefit of this method is that both the upstream and downstream regions of the known sequence will be amplified by one PCR reaction if the restriction site is in an appropriate location within the genomic DNA. However, the circularization of target DNA fragment is sometimes unreliable, and this circularization needs to take precedence over ligation between the target DNA fragment and other fragments. In addition, the success of PCR amplification depends on the distance between the restriction enzyme sites and the known region, and so the digested DNA fragments are sometimes too large for standard PCR amplification.

Benkel and Fong (1996) developed an improved inverse PCR method combining inverse PCR and long-range PCR, which utilizes six-cutter restriction fragments (which, as described above, are sometimes too large for standard PCR amplification). This method is suitable for long-range walking in the upstream and downstream regions and produces an approximately 6 kbp. Kohda and Taira (2000) reported the "bridged inverse PCR" method, in which the digested genomic DNA fragment is circularized with a short artificial DNA fragment, or bridge. The first-step PCR is the same as in standard inverse PCR using the inverse primer, and the second-step PCR uses one inverse primer and one bridge-specific primer. This second-step PCR enables selective, effective amplification of the target sequence from a complex mixture.

Panhandle PCR

Jones and Winistorfer (1992, 1993) reported the "panhandle PCR" method, which is named for the shape of the template single-stranded DNA (ssDNA) in the protocol (Fig. 3). Panhandle PCR involves (a) DNA digestion with a restriction enzyme that generates 5′-sticky ends; (b) ligation of the 3′-end of the digested DNA with a single-strand, 5′-phosphorylated oligonucleotide (oligo) that is designed to be reverse complementary to the known sequence adjacent to the target region; (c) panhandle formation (intrastrand annealing) of the denatured ssDNA and polymerase extension of the recessed 3′-region, which generates an annealing site for a sequence specific-primer at the unknown end; and (d) PCR amplification with sequence-specific primers (the 3′-end of the target region now has the reverse complementary sequence of the 5′-region). This method is suitable for both short- and long-range genomic walking up the length of the denatured target DNA fragments forming the panhandle structure. Ironically, the panhandle formation step itself is the weak point of this method; it is complex and rather unreliable.

To address the shortcomings of the panhandle PCR method, Myrick and Gelbart (2002) developed the "universal fast walking (UFW)" method. This method requires neither restriction enzymes nor DNA ligase, and all of the processes can be performed in one tube: (a′) amplification by a sequence-specific primer followed by the destruction of unused primers by exonuclease I (ExoI); (b′) random but stringent annealing of the first amplified chain to a conversion primer that has a random 10-mer sequence at its 3′-end and a known sequence at the 5′-end; (c′) digestion of the 3′-branching of the primer-bound first chain using ExoI; and (d′) sequence conversion of the 3′-end of the first chain using polymerase fill-in, which creates the reverse complementary sequence to the known region for panhandle formation. Combining this UFW method with touchdown PCR (or step down PCR, see below), Walser et al. (2006) reported an improved method for a reliable high-throughput assay to screen length polymorphism. Park (2005) reported the "lariat-dependent nested PCR for rapid amplification of genomic DNA ends" method. In this method, the initial DNA synthesis is primed with a special hybrid primer consisting of a gene-specific 5′-sequence

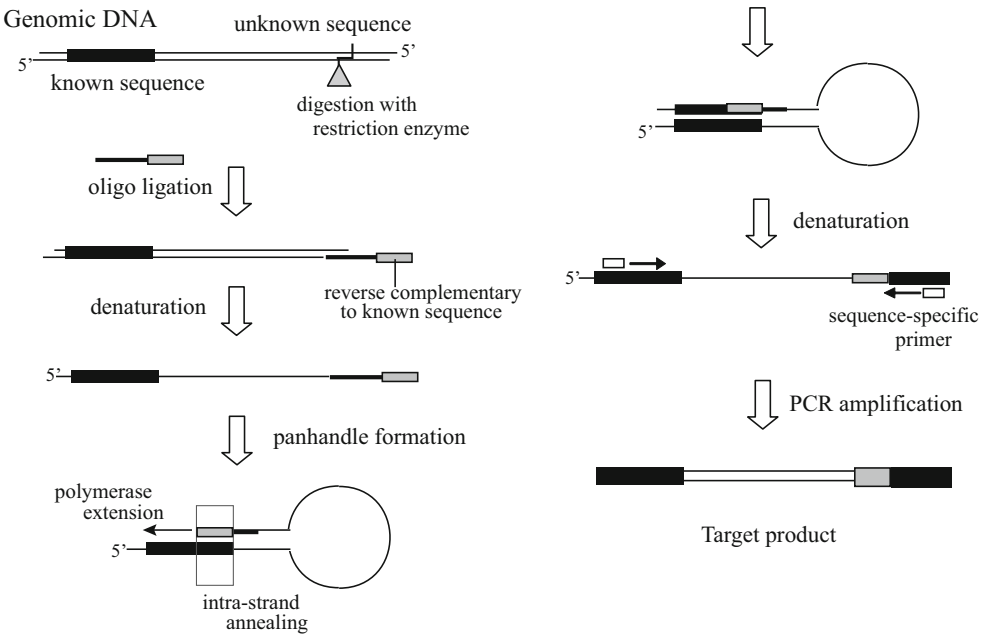

Fig. 3 Schematic description of the panhandle PCR method. Genomic DNA is digested with a restriction enzyme (a *gray triangle* and *right-angled lines*), and the 3′-end of unknown region is ligated to an oligo DNA (*gray box* with a *bold line*), which has a reverse complementary sequence to known region. Ligated DNA is denatured and then forms panhandle structure between known sequence and oligo DNA sequence. DNA polymerase extends the recessed 3′-region, creating an annealing site for a sequence-specific primer (*small white box*). PCR amplification is performed using sequence-specific primers. Known sequence is shown by a *black box* (modified from Jones and Winistorfer 1992)

adjacent to the 3′-terminal degenerate, partially defined sequence. The second strand synthesis fills the 3′-end recession of the first strand to form the reverse complementary sequence for panhandle formation. Wang et al. (2007) reported the "self-formed adaptor PCR" method, which includes one cycle of DNA synthesis at a low annealing temperature (35 °C) with a sequence partially complementary to a known sequence, followed by linear amplification at high temperature with a sequence-specific primer to pool and select the target product for the next panhandle formation.

Cassette PCR

This method involves attaching artificial deoxynucleotides (a cassette or adaptor) to the end of an unknown region, to allow later PCR amplification of the target sequence between the known region and adaptor. This method requires restriction enzyme(s) to digest genomic DNA and DNA ligase to ligate the adaptor and digested DNA fragment. The typical steps involved in this method are: (a) digestion of genomic DNA with restriction enzyme(s); (b) ligation of the digested DNA with the adaptor using DNA ligase; and (c) PCR amplification using a sequence-specific primer and an adaptor primer (a primer designed using the adaptor sequence). Shyamala and Ames (1989) called the principle behind this method "single-specific primer PCR", in which digested genomic DNA fragments are ligated to a plasmid vector; Mueller and Wold (1989) described it as "ligation-mediated PCR", in which chemically cleaved genomic DNA fragments are ligated to a double-stranded oligo linker. Rosenthal and Jones (1990) established the standard procedure: a scheme of the method is shown in Fig. 4. In cassette PCR, the adaptors can be designed specifically, and samples are easy to prepare. However, this method is hampered by the low specificity of PCR amplification caused by short DNA fragments that have adaptor sequences at both ends or by the self-ligation product of the adaptors. More complicated procedures have been designed to increase the efficiency of the method; for example, PCR amplification with a biotin-labeled primer followed by purification of the products using streptavidin, or nested PCR with many steps using different degenerate primers.

Many improvements have been made to the cassette PCR method to increase its efficacy. With respect to the design of the adaptor, Arnold and Hodgson (1991) reported the "vectorette PCR" method, which is the application of the linker-specific amplifying technique for the yeast artificial chromosome (Riley et al. 1990). The vectorette is a double-stranded oligo adaptor that is partially complementary and contains a central mismatch region. This mismatch only allows amplification by a sequence-specific primer at the first PCR. The newly synthesized chain becomes the template for the adaptor-specific primer after the second PCR, and this amplifies the target region near the known sequence specifically. Vectorette PCR is applicable to genomic walking when information on only one conserved amino acid sequence is available (Laging et al. 2001). Siebert et al. (1995) reported the "suppression PCR" method, in which the nonspecific PCR product containing adaptor sequences at both ends forms a panhandle structure when denatured, due to the presence of an inverted terminal repeat in the adaptor, and panhandle formation suppresses

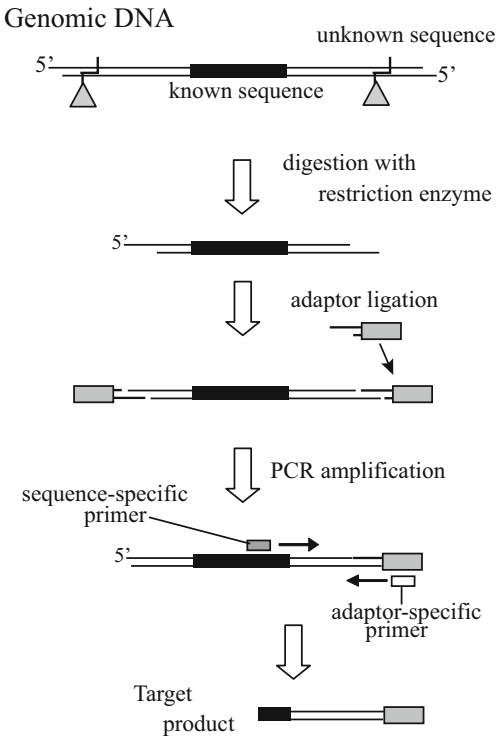

Fig. 4 The basic procedures of the cassette PCR method. Genomic DNA is digested with a restriction enzyme (a *gray triangle* with *right-angled lines*), and the double-stranded oligo adaptor (*gray box* with *short lines*) is ligated to the digested genomic end. PCR is performed using a sequence-specific primer (*small gray box*) and an adaptor-specific primer (*small white box*). Known sequence is shown by a *black box* (modified from Rosenthal and Jones 1990)

the exponential amplification of nonspecific PCR product. This led to the development of "SiteFinding PCR" by Tan et al. (2005), in which the adaptor-specific primer itself contains excess 5′-bases to make nonspecific PCR products that form the panhandle structure. Combining vectorette and suppression PCR, Cottage et al. (2001) reported a modified method using a "vectorette" without an adaptor primer binding site. The primer binding site is created in the first PCR extension by a sequence-specific primer, and the amplification of a sequence with a full-length adaptor at both ends is suppressed by panhandle formation between the terminal repeats in the adaptors. Padegimas and Reichert (1998) achieved efficient-specific PCR amplification using adaptors that have parts forming a hairpin structure, while removing unligated genomic DNA via exonuclease III digestion. The method reported by Tonooka et al. (2008), the "one-base excess adaptor ligation" method, also uses adaptor modification. In this method, the genomic DNA is digested by a restriction enzyme that produces a 5′-sticky end and is ligated to an adaptor with the same restriction site but with one excess base on the 5′-protruding end. This excess base prevents adaptor self-ligation, and the relatively low amounts of ligation products due to the requirement of sequence-specific exonuclease activity by DNA ligase enable specific amplification of the target region. PCR procedures can also be improved. For instance, Zhang and Gurr (2000) developed the "step down PCR" in which the annealing temperature is decreased gradually from 72 to 68 °C in succeeding cycles. This "step down" helps the amplification by favoring gene-specific primer annealing over nonspecific annealing in the earlier cycles. Regarding the utility of the adaptor, Morris et al. (1995) developed a double-stranded oligo adaptor consisting of a universal lower strand and a restriction site-specific upper strand. Each contains a common central core, and the end bases of the core are designed to prevent regeneration of the restriction site once it is ligated to the genomic DNA. Nthangeni et al. (2005) developed a "versatile cassette" that is available in unlimited quantities with variable sticky- and blunt-end restriction enzyme recognition sites. It has a 200-bp sequence using a variety of cassette-specific universal primers. Yuanxin et al. (2003) reported "T-linker PCR", in which a single adenine tail is added by Taq polymerase before the adaptor with a single 5′-protruding thymine is ligated, as in the T–A cloning.

To acquire long PCR products, Rishi et al. (2004) developed a method that involves partial (incomplete) genomic DNA digestion with a restriction enzyme before the ligation of the adaptor. They succeeded in amplifying 4–5 kbp products with this method; in comparison, 0.5–1 kbp products are produced by the complete digestion of genomic DNA using the same restriction enzyme. The cassette PCR method is also useful for the microsatellite marker identification (Korpelainen et al. 2007).

Application of rapid amplification of complementary DNA ends

The method used to determine the flanking region of transcripts is also applicable to genomic walking. Based on the rapid amplification of complementary DNA ends (RACE) method (Loh et al. 1989; Ohara et al. 1989), Cormack and Somssich (1997) reported the "rapid amplification of genomic ends (RAGE)" method, in which the restriction enzyme-digested genomic DNA fragments are denatured and polyadenylated using terminal transferase (poly-A tail). PCR is performed with a sequence-specific primer and a special primer including a polythymine (poly-T) sequence at 3′-end. Leoni et al. (2008) reported a similar method that adds a polycytosine (poly-C) 3′-tail to the single-stranded, primarily amplified product using terminal deoxynucleotidyl transferase after the synthesis of the first strand with a sequence-specific primer. PCR amplification uses a sequence-specific primer and an anchor primer including polyguanine (poly-G) sequence. The merits of these "tailing" methods are their rapid amplification of the

target sequence and their simple protocols. However, as in the RACE method, these methods produce nonspecific products because of annealing between the poly-A and poly-T (or poly-C and poly-G) sequences. Nested PCR, therefore, may be indispensable.

Experimental evaluation of the PCR-mediated methods

In a comparative evaluation of various PCR-mediated genomic walking methods, Satyanarayana et al. (2006) isolated the 5′-promoter fragment of the *N*-methyltransferase gene in the coffee plant, *Coffea canephora*, using cassette PCR, RAGE, and uneven PCR using a gene-specific primer and random 10-mer primers. They found that cassette PCR successfully amplified specific products of 800 (*Eco*RV digestion) or 1.5 kbp (digestion by *Dra*I or *Ssp*I). Nevertheless, even after nested PCR, nonspecific products with an adaptor sequence at both ends were observed frequently, despite the specialized adaptor structure that was used for suppression PCR. The RAGE method could not amplify the target sequence; a variety of nonspecific products were amplified; and smears were observed on gel electrophoresis. Uneven PCR generated specific products of about 600 and 400 bp, but also generated a nonspecific 800-bp product.

A brief guide for the genomic walking

Considering the advantages and disadvantages of each genomic walking method described above, a brief index for each method is shown below. Nested PCR and/or use of biotinylated sequence-specific primer are the common options for all methods.

Random PCR This method is suitable for the exploratory research because the total cost (time and material) for the walking PCR seems to be lowest of all five methods. However, if the single PCR product is not acquired, the other methods should be considered. The problem of nonspecific PCR products cannot be bypassed.

Inverse PCR This method is appropriate for the case that both the upstream and the downstream region of the known sequence are to be discovered. DNA polymerase available for the long-range PCR is needed. If not, the bridged inverse PCR method is recommended even if the PCR reaction itself becomes twice.

Panhandle PCR Concerning the practicality, it is difficult to cite the superiority of this method over the cassette PCR method. Both two methods contain the step of oligo ligation to the digested genomic DNA, but the panhandle PCR method has more two steps (panhandle formation and extension) before the walking PCR. The advantage of this method may be limited for the long-range genomic walking.

Cassette PCR This method seems to be most convenient and appropriate method because of its easiness and design ability. The specificity of the walking PCR balances with its cost, and many improvements are available. From these features, this method would be a standard method in many cases. As the adaptor-specific primer, a universal primer is endorsed to avoid the nonspecific amplification.

RAGE This method is convenient if there are the materials for RACE method already. The protocol itself is very simple. However, it should be noted that the specificity of primary walking PCR is inferior to the cassette PCR or panhandle PCR method, because the stringency of the poly-T (or poly-G) primer is lower than that of the adaptor-specific primers designed appropriately.

Conclusion

As described above, each of the PCR-mediated genomic walking methods has both advantages and disadvantages. In these methods, we concluded that the cassette PCR method is a standard method applicable to various situations. However, the indication by Satyanarayana et al. (2006), based on a previous comparison of these methods, should be noticed that a single method cannot be employed universally, and the adoption of different methods is advisable. There is often a trade-off between the specificity and the ease of a method: efforts to increase the efficiency of the amplification of the target tend to increase the complexity of the procedures used. In addition, the length and efficiency of walking to an unknown region depend on the materials and methods, such as the target organism, enzymes, oligos, experimental conditions, PCR procedures, and the structure of the genomic DNA that is involved. Authors hope that this minireview becomes an index for microbiologists to use to select an appropriate method for genomic walking in target microorganisms and that it provides clues for the future improvement of these methods.

References

Aquino VH, Figueiredo LTM (2004) Linear amplification followed by single primer polymerase chain reaction to amplify unknown DNA fragments: complete nucleotide sequence of Oropouche virus M RNA segment. J Virol Methods 115:51–57

Arnold C, Hodgson IJ (1991) Vectorette PCR: a novel approach to genomic walking. PCR Methods Appl 1:39–42

Benkel BF, Fong Y (1996) Long range-inverse PCR (LR-IPCR): extending the useful range of inverse PCR. Genet Anal 13:123–127

Chen X, Wu R (1997) Direct amplification of unknown genes and fragments by uneven polymerase chain reaction. Gene 185:195–199

Cormack RS, Somssich IE (1997) Rapid amplification of genomic ends (RAGE) as a simple method to clone flanking genomic DNA. Gene 194:273–276

Cottage A, Yang A, Maunders H, de Lacy RC, Ramsay NA (2001) Identification of DNA sequences flanking T-DNA insertions by PCR-walking. Plant Mol Biol Rep 19:321–327

Ge Y, Charon NW (1997) Identification of a large motility operon in *Borrelia burgdorferi* by semi-random PCR chromosome walking. Gene 189:195–201

Huang X-Q, Cloutier S (2007) Hemi-nested touchdown PCR combined with primer-template mismatch PCR for rapid isolation and sequencing of low molecular weight glutenin subunit gene family from a hexaploid wheat BAC library. BMC Genet 8:18

Jones DH, Winistorfer SC (1992) Sequence specific generation of a DNA panhandle permits PCR amplification of unknown flanking DNA. Nucleic Acids Res 20(3):595–600

Jones DH, Winistorfer SC (1993) Genome walking with 2- to 4-kb steps using panhandle PCR. PCR Methods Appl 2:197–203

Jones DSC, Platzer M, Rosenthal A (1994) Extension of incomplete cDNAs (ESTs) by biotin/streptavidin-mediated walking using the polymerase chain reaction. J Biotechnol 35:205–215

Kohda T, Taira K (2000) A simple and efficient method to determine the terminal sequences of restriction fragments containing known sequences. DNA Res 7:151–155

Korpelainen H, Kostamo K, Virtanen V (2007) Microsatellite marker identification using genome screening and restriction-ligation. BioTechniques 42:479–486

Laging M, Fartmann B, Kramer W (2001) Isolation of segments of homologous genes with only one conserved amino acid region via PCR. Nucleic Acids Res 29(2):e8

Leoni C, Gallerani R, Ceci LR (2008) A genome walking strategy for the identification of eukaryotic nucleotide sequences adjacent to known regions. BioTechniques 44:229–235

Lin X, Kelemen DW, Miller ES, Shih JCH (1995) Nucleotide sequence and expression of *kerA*, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. Appl Environ Microbiol 61 (4):1469–1474

Liu Y-G, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25:674–681

Loh EY, Elliott JF, Cwirla S, Lanier LL, Davis MM (1989) Polymerase chain reaction with single-sided specificity: analysis of T cell receptor δ chain. Science 243:217–220

Mishra RN, Singla-Pareek SL, Nair S, Sopory SK, Reddy MK (2002) Directional genome walking using PCR. BioTechniques 33 (4):830–834

Morris DD, Reeves RA, Gibbs MD, Saul DJ, Bergquist PL (1995) Correction of the β-mannanase domain of the *cel*C pseudogene from *Caldocellulosiruptor saccharolyticus* and activity of the gene product on Kraft pulp. Appl Environ Microbiol 61(6):2262–2269

Mueller PR, Wold B (1989) In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. Science 246:780–786

Myrick KV, Gelbart WM (2002) Universal fast walking for direct and versatile determination of flanking sequence. Gene 284:125–131

Nthangeni MB, Ramagoma F, Tlou MG, Litthauer D (2005) Development of a versatile cassette for directional genome walking using cassette ligation-mediated PCR and its application in the cloning of complete lipolytic genes from *Bacillus* species. J Microbiol Methods 61:225–234

Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623

Ohara O, Dorit RL, Gilbert W (1989) One-sided polymerase chain reaction: the amplification of cDNA. Proc Natl Acad Sci USA 86:5673–5677

Padegimas LS, Reichert NA (1998) Adaptor ligation-based polymerase chain reaction-mediated walking. Anal Biochem 260:149–153

Park DJ (2005) LaNe RAGE: a new tool for genomic DNA flanking sequence determination. Electron J Biotechnol 8(2):218–225

Parker JD, Rabinovitch PS, Burmer GC (1991) Targeted gene walking polymerase chain reaction. Nucleic Acids Res 19(11):3055–3060

Pilhofer M, Bauer AP, Schrallhammer M, Richter L, Ludwig W, Schleifer K-H, Petroni G (2007) Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel two-step gene walking method. Nucleic Acids Res 35(20): e135

Reddy PS, Mahanty S, Kaul T, Nair S, Sopory SK, Reddy MK (2008) A high-throughput genome-walking method and its use for cloning unknown flanking sequences. Anal Biochem 381:248–253

Riley J, Butler R, Ogilvie D, Finniear R, Jenner D, Powell S, Anand R, Smith JC, Markham AF (1990) A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. Nucleic Acids Res 18(10):2887–2890

Rishi AS, Nelson ND, Goyal A (2004) Genome walking of large fragments: an improved method. J Biotechnol 111:9–15

Rosenthal A, Jones DSC (1990) Genomic walking and sequencing by oligo-cassette mediated polymerase chain reaction. Nucleic Acids Res 18(10):3095–3096

Saiki RK, Gelfand DH, Stofeel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–491

Satyanarayana KV, Chandrashekar A, Ravishankar GA (2006) Evaluation of PCR-based methods for isolating flanking regions of genes. Mol Biotechnol 32:111–116

Shyamala V, Ames GFL (1989) Genome walking by single-specific-primer polymerase chain reaction: SSP-PCR. Gene 84:1–8

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res 23(6):1087–1088

Szybalski W (1993) From the double-helix to novel approaches to the sequencing of large genomes. Gene 135:279–290

Tan G, Gao Y, Shi M, Zhang X, He S, Chen Z, An C (2005) SiteFinding-PCR: a simple and efficient PCR method for chromosome walking. Nucleic Acids Res 33(13):e122

Tonooka Y, Mizukami Y, Fujishima M (2008) One-base excess adaptor ligation method for walking uncloned genomic DNA. Appl Microbiol Biotechnol 78(1):173–180

Walser J-C, Evgen'ev MB, Feder ME (2006) A genomic walking method for screening sequence length polymorphism. Mol Ecol Notes 6:563–567

Wang S, He J, Cui Z, Li S (2007) Self-formed adaptor PCR: a simple and efficient method for chromosome walking. Appl Environ Microbiol 73(15):5048–5051

Yuanxin Y, Chengcai A, Li L, Jiayu G, Guihong T, Zhangliang C (2003) T-linker-specific ligation PCR (T-linker PCR): an advanced PCR technique for chromosome walking or for isolation of tagged DNA ends. Nucleic Acids Res 31(12):e68

Zhang Z, Gurr SJ (2000) Walking into the unknown: a 'step down' PCR-based technique leading to the direct sequence analysis of flanking genomic DNA. Gene 253:145–150