BlastDigester – a web-based program for efficient CAPS marker design

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Single nucleotide polymorphisms (SNPs) and insertion and deletions (InDels), the most common type of intra-specific sequence polymorphism, are rich resources for creating DNA markers for genotyping. In this article, we present a web-based program for generating cleaved amplified polymorphic sequence (CAPS) markers, PCR-based markers that use polymorphism in restriction endonuclease digestion patterns (snip-SNPs). Blast-Digester computationally ‘digests’ the sequence alignments that are returned in a Blast search, identifies snip-SNPs and simplifies PCR-primer generation through dynamic links to Primer3, a primer design program.

DNA sequences from different varieties or accessions of a given species are becoming available through several sequencing projects. Single nucleotide polymorphisms (SNPs) together with insertion and deletions (InDels) are the most common type of polymorphism in the genomes studied to date. Large sets of predicted SNPs are publicly available for the human genome (SNP consortium, http://snp.cshl.org) and for some genetic model organisms including Caenorhabditis elegans [1], Drosophila melanogaster [2] and Arabidopsis thaliana [3]. Approximately 30–40% of SNPs alter restriction endonuclease recognition sites and these are commonly referred to as snip-SNPs. Restriction enzyme digestion patterns that are polymorphic might be used to create cleaved amplified polymorphic sequence (CAPS) markers, which are codominant molecular markers that amplify a short genomic sequence around the polymorphic endonuclease restriction site [4]. They are detected easily by agarose gel electrophoresis. The use of CAPS is thus affordable and practical for genotyping in positional or map-based cloning projects [4–6].

With the increasing availability of parallel genomic sequences from different varieties or accessions of genetic model species, there is a need for a web-based, user-friendly program that facilitates snip-SNP-based CAPS marker design. Currently, there is no free software tool available for the rapid detection of the restriction site polymorphisms in aligned sequences. Some related applications are used for conceptually different markers (SNAPER [7]) or are not freely available for bench scientists through a web interface (autoSNP [8] and SNP2CAPS [9]), have a limited sequence size input and no sequence alignment option (dCAPS Finder [10,11]) or do not integrate primer design (autoSNP [8] and SNP2CAPS [9]).

To overcome these limitations we created Blast-Digester, a web-based program intended for bench scientists, which identifies differential endonuclease restriction sites in the pairwise sequence alignments of a standard Blast output. Dynamic links to Primer3 enable rapid CAPS marker design with the ease of a
mouse click. Using a sample dataset, we show that the software tool is experimentally robust, even with lower quality sequence at 1× coverage.

Creating CAPS markers with BlastDigester

Using BlastDigester, CAPS markers can be generated in a simple, three-step procedure (Figure 1).

The input for the BlastDigester is the local sequence alignments found in the output of Blastn [12] or Blast2Seq [13]. There are two parts for each sequence alignment in the Blast output: (i) a table with the restriction enzymes listed for both sequences; and (ii) the aligned sequence with the exact position of the differential restriction sites (Figure 1b). Clicking on a selected polymorphic restriction site activates the Primer3 program hosted by the Whitehead Institute (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and primers flanking a specified snip-SNP site are automatically generated (Figure 1c). Several parameters for primer design can be defined on the input page of BlastDigester (i.e. primer T_m, GC content,...
**Box 1. The pipeline for CAPS marker generation using BlastDigester**

- Selected coding or non-coding, non-repetitive sequences from two accessions are aligned with Blast. Selection depends on the level of polymorphism between the two sequences. The Blastn or Blast2Seq algorithms can be used for alignment. It is important to use a web-based Blast server that offers the option of saving or displaying the Blast output as plain text, although copied output from NCBI’s Blast2Seq server will also work with the BlastDigester program.
- Each Blast output in plain text format is uploaded in its entirety to the BlastDigester website and the parameters for primer design are defined (i.e. primer Tm, GC content, primer size and product size range). For reasons relating to PCR parameters and gel electrophoresis, we recommend that the PCR product size be between 400–1000 bp. It is also possible to define an exclusion interval (set as default to 50 bp). Potential snip-SNP restriction enzymes (SNPs that alter restriction endonuclease recognition sites) that also cut elsewhere in the sequence within this interval will be flagged in gray in the output.
- The outputs of the BlastDigester program are examined for appropriate snip-SNPs. BlastDigester counts the number of times a potential snip-SNP restriction enzyme cuts elsewhere in the sequence and displays this information, both in tabular format and in an alignment view, therefore, restriction enzymes that cut less frequently or cut uniquely can be chosen. In addition, if the potential snip-SNP restriction enzyme also cuts within the exclusion interval defined on the input page, it is grayed-out. Otherwise, potential snip-SNPs are coloured red or teal and are hyperlinked to Primer3. Clicking on a snip-SNP transfers the appropriate information for primer design to Primer3.
- After clicking on an appropriate snip-SNP restriction enzyme to activate Primer3, the cleaved amplified polymorphic sequence (CAPS) primers are obtained from the Primer3 output page. The dynamic link from BlastDigester contains information that restricts CAPS primer design to regions that are identical in both sequences (i.e. primer design is prohibited in regions of mismatch).

primer size and product size range; Figure 1a). To generate a sufficient fragment size difference between candidate markers, an exclusion window can be defined: if the potential snip-SNP restriction enzyme also cuts within the exclusion zone, it is ‘grayed-out’ in the output, eliminating ‘imperfect’ CAPS markers (Box 1). In cases where two sequences have a higher rate of polymorphism, the BlastDigester is designed to restrict primer selection by Primer3 to regions that do not have nucleotide mismatch (Figure 1c).

BlastDigester was implemented in Perl using several of the packages available in the Bioperl distribution (http://www.bioperl.org) [14]. The program is offered freely to non-commercial users through the Internet (http://bbc.botany.utoronto.ca:88). The source code for BlastDigester is also available upon request for local installation and supports a command line option.

**Generating CAPS markers in Arabidopsis with BlastDigester**

To test the efficacy of BlastDigester on a sample dataset, ten candidate CAPS markers were identified using Blast-Digester for the Arabidopsis accessions Columbia-0 (Col-0) and Landsberg erecta (Ler), which are found between the markers nga129 and m558A on chromosome 5. The Col-0 sequence is complete and is >99.99% accurate [15], whereas the Cereon Arabidopsis Ler sequence collection consists of ~80 000 short contigs and sequences at ~1 x coverage [16]. The DNA sequences of Col-0 and Ler are available at the TAIR website (http://www.arabidopsis.org) [17]. Selected 3’ untranslated regions (UTRs) and intergenic regions up to 5 kb in length of the Col-0 sequence were used to search for homologous Ler contigs using Blast. A given Blast output was then analyzed with BlastDigester, which identified polymorphic restriction enzyme sites in the aligned sequences. PCR primers flanking selected sites were generated by linking to Primer3. The candidate CAPS:PCR products were digested with the appropriate enzymes and the results are shown in Figure 1d (the positive controls for restriction enzyme digestions are not shown). Eight out of the ten markers tested yielded differential restriction enzyme digestion patterns. The failed markers were due to errors in the Ler sequence and were confirmed by sequencing the PCR products (data not shown). Primer sequences for the ten CAPS markers used were deposited in the TAIR database and a list of the CAPS markers with TAIR accession numbers is available in Table 1 of the supplementary data on the BlastDigester website (http://bbc.botany.utoronto.ca:88).

The specific example of CAPS marker design using Arabidopsis genomic sequence data that we present here demonstrates the usefulness and intuitiveness of the BlastDigester program. However, this tool is applicable to snip-SNP analysis in any organism where parallel sequence data of two varieties (or accessions) are available.

**General considerations for generating CAPS markers with BlastDigester**

BlastDigester integrates efficiently the three main components that are necessary for CAPS marker generation: (i) a local sequence alignment; (ii) the search for existing differential restriction endonuclease enzyme cutesis; and (iii) primer design.

The input size of the aligned sequence is not limited in the current version of BlastDigester. However, the program is not designed for whole-genome high-throughput snip-SNP analysis because of the nature of the Blast algorithm itself. Instead, appropriately selected, shorter DNA sequence segments (up to several kilobases) are recommended for generating Blast alignments. The identification of polymorphic restriction enzyme cutesis by BlastDigester depends on the frequency, distribution and the types of existing intra-specific sequence variation. Because SNPs and InDels are not distributed evenly throughout a given genome, the selection of the appropriate genomic segment of the reference sequence to be used for marker development is important. For instance, in the Arabidopsis Col-0 and Ler accessions, exons have low levels of polymorphism; therefore, 3’ UTRs and intergenic regions are ideal locations to create CAPS markers. Logically, exon regions might also be used if the necessary polymorphisms are present. For genomes such as maize, where intergenic regions contain blocks of repetitive DNA, untranslated genic regions (introns) could represent potential spots for snip-SNP mining and CAPS marker design.

The success of BlastDigester depends on the accuracy of the sequences used for the alignments. As seen in the Arabidopsis example, sequence errors in the Ler ecotype
Conservation of sequence and function in the **Pax6** regulatory elements

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**Genome Analysis**

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**References**