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Review Article

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Allele Mining for Crop Improvement

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ABSTRACT

Identification and access to allelic variation that affects the plant phenotype is of the utmost importance for the efficient and purposeful utilization of genetic resources in plant variety development. Considering the huge numbers of accessions that are held collectively by gene-banks, genetic resource collections are deemed to harbour a wealth of undisclosed allelic variants. The challenge is how to unlock this variation. Allele mining is a research field aimed at identifying allelic variation of relevant traits within genetic resource collections. It can be defined as an approach that is used to dissect naturally occurring allelic variations or to suitable alleles of a candidate gene controlling key agronomic traits which have potential in crop improvement. It basically helps in tracing the evolution of alleles, identification of new haplotypes and development of allele-specific molecular markers for use in marker-assisted selection (MAS). True allele mining includes the analysis of non-coding and regulatory regions of the candidate gene(s), in addition to analyzing sequence variations in the coding regions of the agronomically important genes so as to cover most of the functional variations of relevance in the genes. Three major approaches such as Eco-tilling, sequencing and association mapping are available for the identification of sequence polymorphism for a given gene in the naturally occurring populations. Allele mining requires various sophisticated bio-informatic tools which are useful for sequence alignment in order to compare the new sequence to reference genome i.e, sequenced genome. Recently, the approaches and applications of allele mining along with the challenges associated have generated a lot of interest among scientists with emphasize on the need for more refined 'mining' strategies for accelerating the process of allele discovery and its utilization in molecular breeding.

Keywords: *Allele mining, Tilling & Eco-Tilling, Association mapping, MAS.*

INTRODUCTION

Progress in plant breeding in terms of development of superior and high yielding varieties of agricultural crops is possible by accumulation of beneficial alleles from vast plant genetic resources existing worldwide[1]. In other words, identification and access to allelic variation that affects the plant phenotype is of the utmost importance for the utilization of genetic resources, such as in plant variety development.

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Considering the huge numbers of accessions that are held collectively by genebanks, genetic resource collections are deemed to harbour a wealth of undisclosed allelic variants. The challenge is how to unlock this variation. Allele mining is a research field aimed at identifying allelic variation of relevant traits within genetic resource collections. For identified genes of known function and basic DNA sequence, genetic resource collections may be screened for allelic variation by allele mining approaches such as eco-tilling, sequencing and association mapping. Allele mining requires various sophisticated bio-informatic tools viz., PLACE, plantCARE, TRANSFAC, JASPAR, MEME, Plantprom DB, DCPD, SCPD, BioEdit, ClustalW[2] etc. These tools are useful for sequence alignment in order to compare the new sequence to reference genome i.e, sequenced genome. Realizing the importance of allele mining in traits like resistance to biotic and abiotic stresses, greater nutrient efficiency, enhanced yield and improved quality[1] for crop improvement, we discuss the concept of allele mining and its approaches along with challenges.

ALLELE MINING AND TRUE ALLELE MINING

Before undergoing in detail about allele mining, we should know the allele first, it is a alternative forms of a gene found at a given locus on a chromosome. Mining is nothing but searching the new alleles in the wild germplasm. So, allele mining means, searching of useful alleles of genes from a wide range of cultivars, related species and even across species. In other words, it is a research field aimed at identifying allelic variation of relevant traits within genetic resource collections[1].

Mutation is considered as an evolutionary driving force which underlies existing allelic diversity in any crop species. For creation of new alleles or causing variations in the existing allele and allelic combinations, mutations in the genic regions of the genome either as single nucleotide polymorphism (SNP) or as insertion and deletion (InDel) are important. The mutations in coding regions and/or regulatory regions may have tremendous effect on the phenotype by altering the encoded protein structure and function. True allele mining includes analysis of non-coding and regulatory regions of the candidate genes in addition to analyzing sequence variations in the coding regions of genes. Example for a mutation in 5' splice site of the1st intron of the waxy (Wx) gene had resulted in tenfold increase in the gene activity in rice[1, 3].

IMPORTANCE OF ALLELE MINING:-

It helps in tracing the evolution of alleles.

Also helps in identification of new haplotypes and development of allele-specific markers for use in marker-assisted selection (MAS).

This capability will be important for giving breeders direct access to key alleles conferring:

- resistance to biotic stresses
- tolerance to abiotic stresses
- greater nutrient use efficiency
- enhanced yield
- improved quality

It can also provide insight into molecular basis of novel trait variations and identify the nucleotide sequence changes associated with superior alleles.

APPROACHES FOR ALLELE MINING:-

There are three approaches for allele mining such as modified tilling procedure called eco-tilling-based allele mining, sequencing-based allele mining and association mapping-based allele mining.

Modified Tilling procedures called Eco-Tilling

TILLING (Targeting Induced Local Lesions IN Genomes) is a technique that can identify single base-pair allelic variation in target gene (more specifically induced point mutations) while Eco-Tilling technique detects natural mutation. It allows the rapid detection of variation in many individuals and is cost effective because only one individual for each haplotype need to be sequenced.

Tilling consists of several major steps: Development of a mutagenized population, DNA preparation and pooling, and mutation

discovery (Figure 1). At first, random mutations are induced in genomes by using chemical mutagens. Seeds are mutagenized by treatment with ethylmethanesulfonate (EMS) etc. The resulting M1 plants are self-fertilized, and M2 individuals are used to prepare DNA samples for mutational screening. DNA is extracted from test samples. The DNA samples are pooled and arrayed into 96 wells containing microtiter plates. Screening for mutations begins with PCR amplification of a target fragment using gene-specific infrared dye-labeled primers. The forward primer is 5'-end labeled with a fluorescent dye that is detected at 700 nm (IRDye 700) and the reverse primer is labeled with the IRDye 800 nm[4]. These PCR products are denatured and re-annealed to allow the formation of mismatches or heteroduplexes, which represent naturally occurring single nucleotide polymorphisms (SNPs) and induced SNPs. Samples, are then incubated with a single-strand specific nuclease to digest mismatched base pairs. For mismatch-specific cleavage, several enzymes, including S1 nuclease[5], T4 endonuclease VII[6] and Cel-1[7] have been used. Cleaved bands representing mutations or polymorphisms are visualized using denaturing polyacrylamide gel.

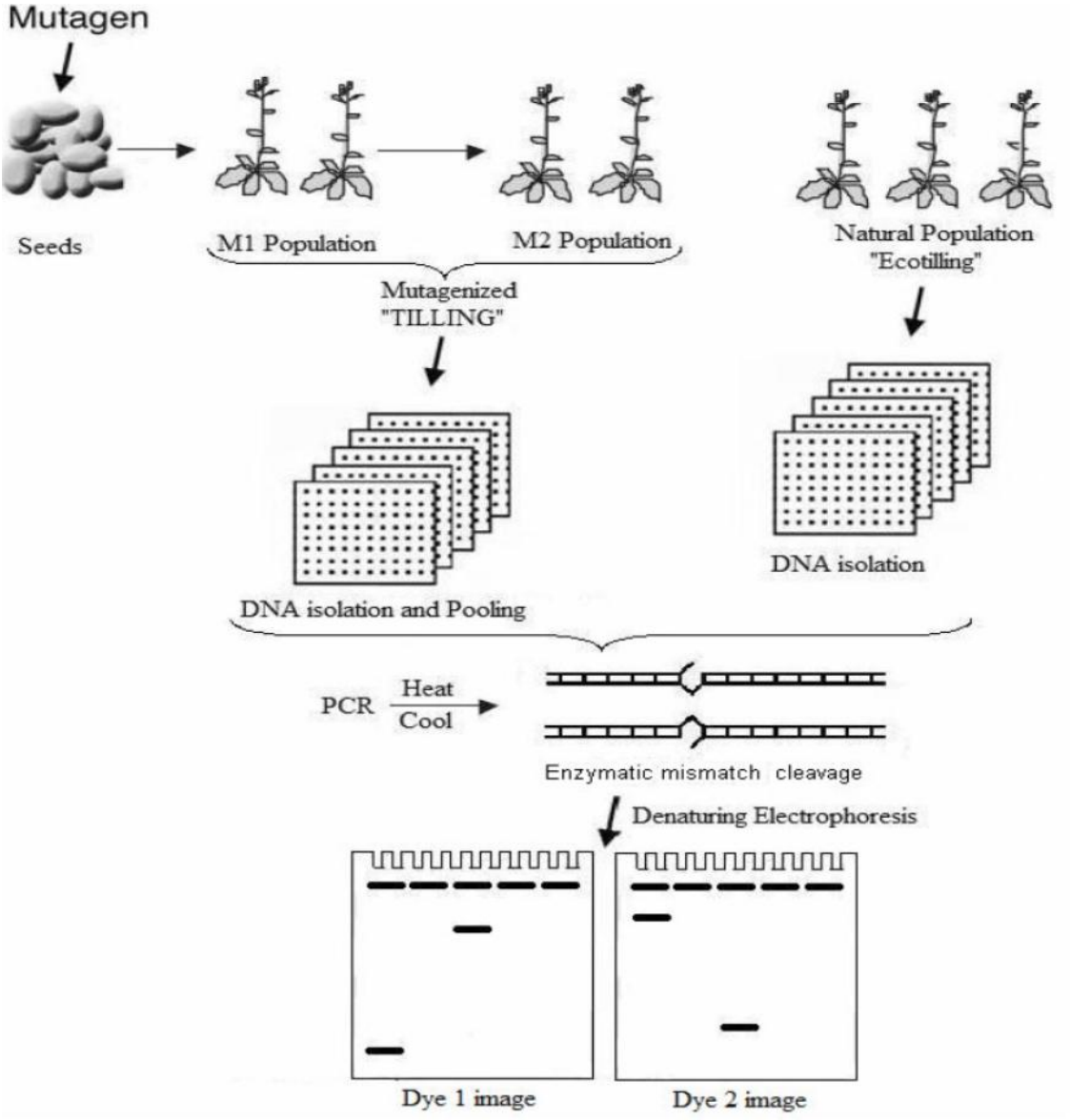

Fig.1. Outline of the basic steps for typical TILLING and Eco-Tilling assays

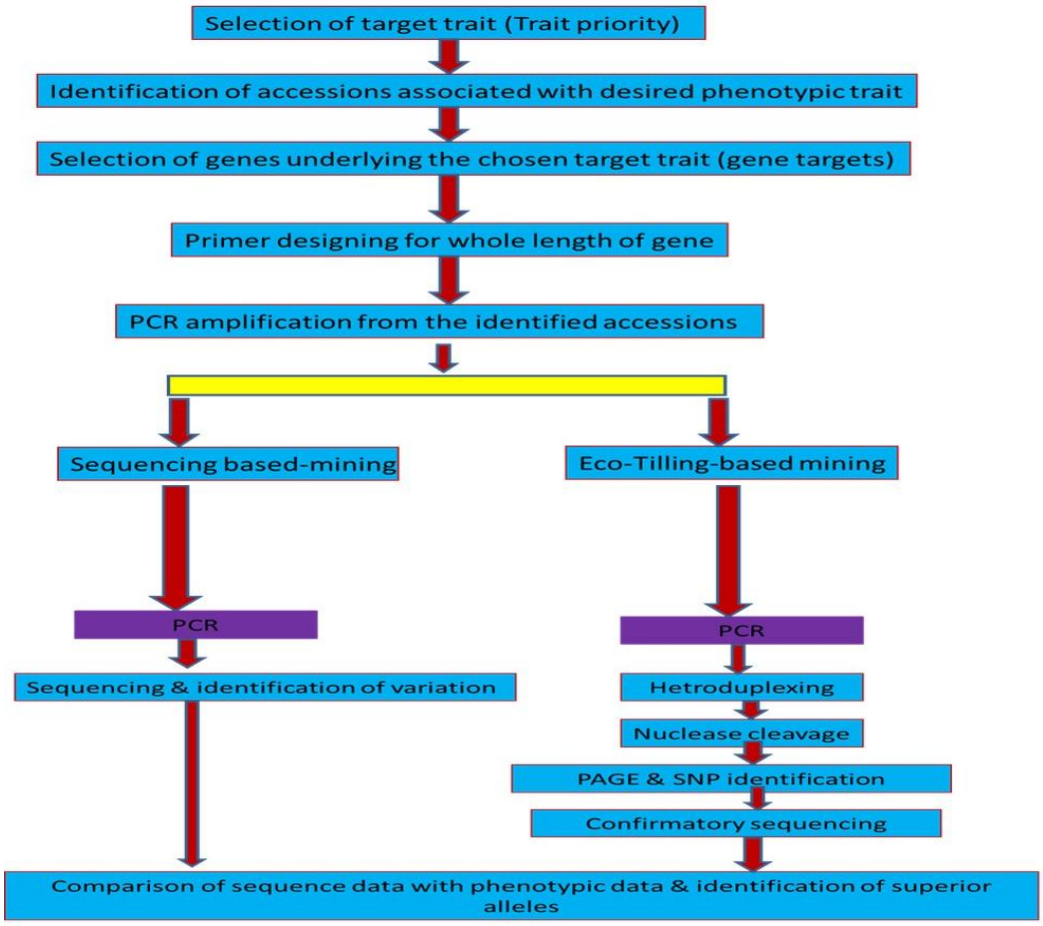

Fig. 2. Different steps involved in two approaches (Eco-Tilling and sequencing) of allele mining

Table 1: Comparison between Eco-Tilling and Sequencing-based allele mining

Sl.No.	Parameters	Eco-TILLING	Sequencing-based allele mining
1.	DNA pooling	Yes	No
2.	PCR	Yes	Yes
3.	Heteroduplex formation	Yes	No
4.	Primer labeling by fluorescent dyes	Yes	No
5.	Primer cost	High	Low
6.	Enzymatic mismatch cleavage	Yes	No
7.	Electrophoresis	Fragments are denatured and separated typically on a LI-COR DNA analyser	Simple Agarose Gel
8.	Sequencing	Yes	Yes
9.	Cost of Sequencing	Medium-to-high	High
10.	Samples size for sequencing	Less	More
11.	Time required	More (especially for sample preparation)	Less
12.	Technical expertise	High	Low
13.	Lab facilities	High sophisticated	Low sophisticated
14.	Nucleotide detection	Effective in detection of SNPs rather than InDels	Effective in detection of any type of nucleotide
15.	Throughput	Medium-to-high	High
16.	Complexity	More	Less

Sequencing-based allele mining

Sequencing based allele mining involves amplification of alleles in diverse genotypes through PCR followed by identification of nucleotide variation by DNA sequencing techniques[6]. In other words, multiplication of specific segment of deoxy-ribose nucleotides as well as, we can identify various allele among the cultivars through sequence based allele mining[2].

Next generation sequencing for allele mining

Sequencing is a technique which enables us to understand arrangement of this nucleotide in DNA molecules. In the last few years, 'massively parallel' methods have also emerged and lead to the development of 'next generation' sequencing platforms with increased throughput and accuracy. These methods used for resequencing, alignment of the sequence data and their comparison with reference genome. The first of this type was commercialized by 454 Life Sciences and this technique relied on pyrosequencing while eliminating the need for cloning.With this 454 sequencing platform, it is possible to produce 100 Mb of sequence with 99.5% accuracy and increase read length averaging over 250 bases[8]. Another massively parallel sequencer Illumina/Solexa genome analyzer has been developed and this is capable of sequencing one billion bases (1 Gb) of 30-40 base sequence reads in a single run in a short timer period of 3-4 days.

Association mapping-based allele mining

According to Zhu and coworkers[9] the strategy is used to establish regions of the genome associated with critical phenotypes by association or linkage-disequilibrium mapping. The approach relies on the assumption that alleles responsible for a phenotype, along with the markers which flank the locus, are inherited as a block (Figure 3). Using DNA markers has been suggested as a means to identify useful alleles in the vast reservoirs of genetic diversity.

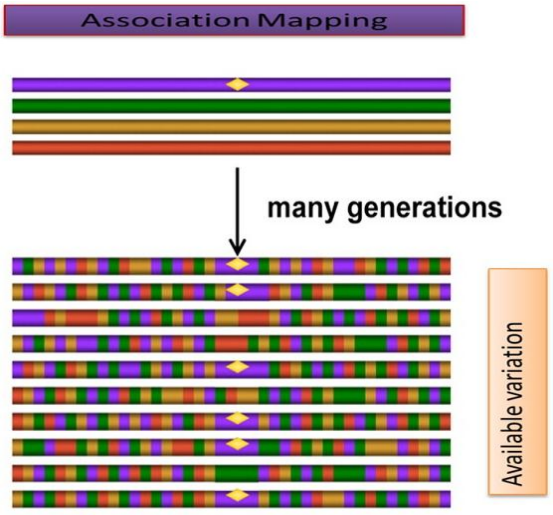

Fig. 3. Association mapping (Zhu et al., 2008)

BIOINFORMATIC TOOLS REQUIRED FOR ALLELE MINING

Allele mining requires various sophisticated bioinformatic tools *viz.*, PLACE, plantCARE, TRANSFAC, JASPAR, MEME, Plantprom DB, DCPD, SCPD, BioEdit, ClustalW etc. These tools useful for sequence alignment in order to compare new genome sequence to reference genome i.e, sequenced genome data[2].

APPLICATIONS OF ALLELE MINING

Allele mining can be effectively used for gene prediction, expression study, evolutionary study, discovery of superior alleles, identification of new haplotypes, similarity analysis-inter and intra species and functional molecular marker development for MAS[1]. While general applications of allele mining are described in figure 4.

Fig. 4: Applications of allele mining

SOME EXAMPLES OF ALLELE MINING FOR CROP IMPROVEMENT

Allele mining for blast resistance genes in finger millet and rice

Finger millet is a nutritionally rich crop and its major limitation is susceptible to blast fungus *Magnaporthe grisea* which is also the causative agent of blast in rice. Blast disease causes about 90% yield losses in rice and upto 50% losses in finger millet[10]. Hence, serious efforts are required to understand molecular mechanisms disease resistance & identify genes responsible for the blast disease. Though, finger millet genome sequence is not yet available but rice genome is completely sequenced. Comparative genomics plays a very important role in finger millet to dissect

the highly useful and agronomically important traits like blast resistance. Recently, Srinivasachary and coworkers[11] made a comparative analysis of finger millet chromosomes with rice chromosomes and showed most of the chromosomes were highly collinear with 85% synteny (Table 2).

By using syntenic relationships between rice and finger millet[12] recently mapped genes for blast resistance through association mapping approach by using the EST sequences of NBS-LRR, *M. grisea* and Pi genes of rice. They found QTLs of finger blast and neck blast were linked to genes like Pi5, Pi21, Pi-d(t), NBS-LRR and *M. grisea.* Hence, blast resistance genes like Pi5, Pi21, Pi-d(t), NBS-LRR and *M. grisea* may be targeted for allele mining in finger millet.

Table2: The syntenic relationship observed between rice and finger millet chromosomes[10].

Finger millet chromosome	Rice chromosome	% synteny
IA & 1B	1	85
1A & 2B	2 , 10	
3A	3	91
4	4	48
5A & 5B	5, 12	93 & 54 respectively
6A, 6B	6,9	85-100
7A, 7B	7	85
8A, 8B	8	90
9	11	86

Association mapping-based allele mining for node of first fruiting and its height in Upland cotton (*Gossypium hirsutum* L.)

Node of first fruiting/sympodial branch (NFFB) and its height (HNFFB) are two important indicators to measure cotton (Gossypium spp.) early maturity. A total of 172 upland cotton cultivars & 331 SSR markers were selected to construct an association mapping[13]. They found a total of 18 markers (JESPR101, BNL4108, DPL0124, MGHES70, MGHES73, NAU0490, JESPR201, BNL0598, NAU2251, NAU3561, NAU3398, DPL0281, TMB1268, NAU1102, DPL0522, NAU1025, NAU5195 and DPL0702) associated with NFFB were detected in at least two environments, and the range of explained phenotypic variation by markers in different environments was 2.28-11.36 % with a mean value of 5.20 % and a total of 19 markers (NAU5077, DC30046, DC40182, DPL0852, MGHES70, MGHES73, NAU0490, JESPR201, CGR6772, BNL2651, NAU2165, NAU3337, NAU3736, NAU2443, DPL0215, NAU5046, NAU5189, NAU2687 and SHIN1339) associated with HNFFB were detected in at least two environments, and the range of explained phenotypic variation by markers in different environments was 2.83-16.96 % with a mean value of 7.92 %. These markers detected in multiple environments are of good stability and can be used for marker-assisted selection (MAS) of target traits.

Allele mining and haplotype discovery in barley candidate genes for drought tolerance

Cseri and coworkers[14] were examined haplotype and sequence variation (SNP; INDEL) at nine loci (*HvARH1*, *HvSRG6*, *HvDRF1*, *HVA1*, *HvDREB1*, *HvNHX1*, *HVP1*, *HvNud* and *HvPPRPX*) involved in the abiotic stress response pathways in a set of 96 barley cultivars and landraces collected worldwide and containing drought tolerant and sensitive genotypes. The analysis comprised sequence information of approximately 1.5 million base pairs in barley and the identified haplotypes and polymorphisms were recorded in a web-based database named BAHADAS ("BArley HAplotype DAtabaSe for drought-related candidate genes").

Eco-Tilling for Allelic Variation of Salinity Tolerance Genes in Barley cultivars

Adnan Al-Yassin and Raheleh Khademian[15] were used EcoTILLING technique in barley for different purposes including discovery and detection of DNA polymorphisms for salt stress tolerance. It was concluded that it is possible to combine different alleles from wild barley to obtain cultivars with more salt stress tolerance (Table 3). There are several crop plants in which have scientists used allele mining approaches to study a specific trait for crop improvement (Table 4).

Table 3: List of the most important genes related to salinity stress tolerance in barley and their function

Gene names	Function of gene
HvHAK1	K^+ transporter
HvHAKT1	K^+ transporter
HvHVA/68	H^+ ATPase
HvHVP1	H^+ ATPase
HvHNX1	Na^+/H^+ antiporter
HvHNX3	Na^+/H^+ antiporter
HvHNX4	Na^+/H^+ antiporter
HvHKT2	$Na^+$and K^+ transporter
HvCBL4	Na^+/H^+ antiporter
HvHKT1	Na^+ transporter
HVA22	Na^+ transporter

Table 4: Status of allele mining in crop plants

Crop	Allele/locus	Trait/name of the protein	Author
Wheat	Viviparous-1	Pre-harvest sprouting tolerance	16
Rye	*Alt3*	Aluminum tolerance	17
Apple	*Mal d 3*	Allergenicity	18
Apricot	*S*	Self-incompatibility	19
Barley	*Amy32b*	α amylase	20
Barley and wheat	transcription factor -GAMYB	GAMYB-involved in gibberellin signaling	21
Barley	*VRN-H1* and *VRN-H2*	Vernalization requirement	22
Barley	*Bmy1*	β-amylase I- starch break down enzyme	23
Tomato	*Pto*	Disease resistance	24
Barley	*Gpc-B1*	Grain protein content	25
Barley	*rps2*	Ribosomal protein S2	26
Grapevine	*VvmybA1*	Transcriptional regulator of anthocyanin biosynthesis	27
Wheat	*Pm3*	Powdery mildew resistance	28
Pea	*TI1*	Trypsin inhibitors characterization	29
Phaseolus sp.	Lectin locus	Storage and defense proteins	30
Potato	*Rpi-blb1*	Late blight resistance	31
Rice	*Badh2*	Fragrance	32
Rice	*Pi ta*	Blast resistance	33

CHALLENGES IN ALLELE MINING

1. Selection of genotypes:-

Several approaches such as development of core/mini core collections, accurate phenotyping methods and flexible computational tools through which the prioritization of genotypes for allele mining can be done.

2. Handling genomic resources

To keep pace with rapid accumulation of nucleotide and gene expression data, computational tools need to be developed for analyzing the functional nucleotide diversity and to predict specific nucleotide changes responsible for altered function. Exploiting the developments in allele mining, association genetics and comparative genomics by combining expertise from several disciplines, including molecular genetics, statistics and bioinformatics is the suggested way.

3. Demarcation of promoter region

Promoters and regulatory elements are spread across the upstream region and their locations are variable from one gene to another. There is a need to develop specific software tools which can accurately predict the core promoter region based on the representation/over-representation of regulatory motifs.

4. Characterization of regulatory region

In contrast to identifying variation in coding regions of the genome, characterizing the extent of cis-acting regulatory variation presents a much greater challenge, since it is not possible to discern this even in the fully

sequenced genomes. Screening for regulatory variants based on differences in transcript levels between individuals is confounded by potential trans-acting factors or environmental differences[1]. As the promoter regions can function in bidirectional pathway, prediction of the orientation will bedifficult even by the softwares.

5. Higher sequencing costs

One of the important challenges is to minimize the time and efforts required while reducing the cost per data point. These challenges may partly be overcome by resorting to cheaper and faster sequencing platforms for high throughput detection of allelic variations.

Conflict of Interest

The authors declare no conflict of interest.

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