REVIEW

Next-generation sequencing of nucleic acids: technology and applications

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ABSTRACT

The demand for sequencing of large biomolecules essential for all forms of life, nucleic acids, is constantly increasing, and has led to technological advancements in present day biotechnology, medicine and life sciences. In the last decade, $3billion was spent to sequence the first human genome using Sanger's chain termination method and since then the demand for more economical, scalable, and accessible methods of genome and transcriptome sequencing gained more magnitude. Next generation sequencing (NGS) refers to massive parallel method of sequencing nucleic acids, and its applications in modern day biology are limitless. In the present review, we aim to elucidate diverse NGS technologies presently available in the market and their wide applications and limitations and to provide a broad comparison of technologies.

KEYWORDS: next generation sequencing, DNA sequencing, Illumina sequencing, Ion torrent sequencing, whole exome sequencing

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INTRODUCTION

DNA sequencing refers to the precise determination of the order of four nucleotide bases, namely adenine, guanine, thymine and cytosine, in a DNA molecule. Sequencing of DNA has had a profound impact on human, plant and microbes research as well as in clinical medicine(Bentley et al. 2008) (Margulies et al. 2005; Shendure et al. 2005; Bentley et al. 2008; Drmanac et al. 2010) . The gradual decline in the cost of genome/transcriptome/exome sequencing has enabled several new applications in human genetics (Ng et al.), cancer biology (Ley et al.; Thomas et al.), and infectious diseases (Andries et al.).

In the late 1970s, Frederick Sanger proposed a chain termination method of reading the four bases of DNA, and Walter Gilbert and Allan Maxam proposed a chemical degradation method (Sanger et al. 1975; Maxam et al. 1977). The chain termination method proposed by Sanger *et al* in 1977 remains the benchmark method until today and has been the foundation of numerous human, plant, animal and microbial genome sequencing studies (Eric S. Lander 2001). The classical Sanger method is based on the key principle of using dideoxynucleotide triphosphates (ddNTPs) as DNA chain terminators *(Sanger et al. 1977)*, and requires a single-stranded DNA template, a DNA primer, a DNA polymerase, and modified nucleotides that terminate DNA strand elongation. The DNA sample was divided into four separate sequencing reactions, containing all four of the standard deoxynucleotides (dATP, dGTP, dCTP and dTTP) and the DNA polymerase.

In 1986, Smith *et al (Smith et al. 1986)* first published a dye-terminator variant of Sanger sequencing in which each of the four dideoxynucleotide chain terminators is labeled with fluorescent dye of different color, thus permitting a single reaction rather than four and thereby making the method quite simple. Incorporation of ddNTPs to the growing DNA strand results in chain termination and therefore the template DNA sequence can be read following polyacrylamide gel electrophoresis to separate nucleic acids based on size. Similar approaches using other fluorescent dyes were also reported (Prober et al. 1987; Cohen et al. 1988). Initially, the separation of DNA bases for sequencing was performed using slab gel electrophoresis and over time, capillary gel electrophoresis emerged as preferred separation method due to high electric fields. One of the major limitations, either with slab gel or capillary electrophoresis separation method is the same sequencing limiting rate (Mathies et al. 1992). To overcome this, in 1992, Huang *et al* (Huang et al. 1992) first reported the process called 'capillary array electrophoresis' (CAE) that allows the analysis of multiple samples in a comparable manner. The major breakthrough of this method is to reuse the capillary arrays in multiple electrophoretic runs, which resulted in increased usage of the Sanger sequencing technique to address numerous biological questions. The use of fluorescently labeled ddNTPs with the Sanger sequencing method has been the leading sequencing technique until the introduction of present day next generation sequencing methods.

Next generation sequencing (NGS) technologies

The need to sequence full genomes in less time drove the need to have parallel sequencing methods, which could reduce the cost and time to sequence. This gave birth to the Next Generation Sequencing (NGS) methods that have now revolutionized the field of DNA and RNA sequencing. Several NGS technologies are available in the current market, including Illumina, Ion Torrent (Thermo Fisher Scientific), Pacific Biosciences, and Roche. Although every sequencing technology employs its own unique specific method of sequencing, all generally share three major steps, i.e. library and template preparations and sequencing or imaging followed by data analysis.

Illumina sequencing technology

Illumina or solexa dye based sequencing technique (Bennett 2004; Bennett et al. 2005; Bentley 2006) starts with fragmentation of template DNA or cDNA followed by adapter ligation, size selection and amplification. Template library molecules are then loaded into a proprietary glass slide with lanes (flow cell) and each lane contains two types of oligos. Each fragment is then amplified into distinct clonal clusters by a process called bridge amplification (Mitra et al. 1999). The reverse template strands are cleaved and washed after bridge amplification, thereby leaving forward strand. Once the cluster generation is completed the templates are ready for sequencing. Illumina sequencing by synthesis (SBS) technology uses four fluorescently labeled nucleotides to sequence millions of templates in parallel on the clusters on flow cell surfaces (Berglund et al. 2011; Quail et al. 2012). During each sequencing cycle, a single labeled dNTP is added to the nucleic acid chain. The labeled nucleotide serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and thereby allows the incorporation of the next nucleotide by cleavage of enzyme. Incorporation of base bias can be minimized by the natural competition of separate and single molecules of four reversible terminator bound dNTPs. This method of reversible terminator chemistry was first discovered by (Canard et al. 1994a; Canard et al. 1994b). Platforms currently offered by Illumina are listed in Table 1.

Ion Torrent (Thermo Fisher Scientific)

Ion Torrent sequencing technology is the non-optical sequencing technique that employs commonly available CMOS integrated circuits (Rothberg et al. 2011) for sequencing of nucleic acids and has been widely used for a variety of human and plant applications both in industry and research areas. The workflow of this technique in-brief starts with a genomic DNA sample, followed by fragmentation, ligation of adaptors and then amplification. Library molecules are then amplified on bead or sphere by emulsion PCR approach (Dressman et al. 2003; Nakano et al. 2003), followed by enrichment using magnetic beads. Sequencing polymerase and primer are added into the chip loading port along with enriched template positive beads. The sequencing reaction starts with a single stranded DNA template, to which sequencing primer is annealed. The chip is then exposed to four dNTPs or nucleotides individually, enabling the second strand to grow based on the single strand template. Sequencing will be carried out based on the detection of change in the hydrogen ion concentration, which is caused by the release of hydrogen ion from the hydroxide group. The hydrogen ions are detected by the sensing layer on the bottom of each well. Ion Torrent offers three different systems, namely ION PGM, ION PROTON and ION S5XL systems that differ mainly in the type of semiconductor chips used. Table 2 provides an overview of the Ion platforms along with their supported applications.

Pacific Biosciences (Pacbio RS and Sequel systems)

Single molecule real time technology is the principle Pacbio employs for their sequencing methods, which is quite different from existing NGS approaches currently available in the market (Coupland et al. 2012). In order to achieve single-molecule DNA sequencing, Pacbio combines sensitive fluorescence detection with molecular biology and nanotechnology. Library preparation is very similar to the fragment library preparation used for other sequencing technologies. In short, the experimental process begins with fragmentation of the genomic DNA depending on the required size, followed by end repair and blunt end adapter ligation. After the ligation step, the double stranded DNA fragments become circular due to the hairpin structure of adapters (SMRT

Table 1: Currently available Illumina sequencing platforms			
S.No.	Instrument type	Supported Applications	website/reference
1	Miseq Series	Amplicon, targeted gene panel and small genome sequencing	www.illumina.com
2	NextSeq series	Genome, exome and transcriptome	www.illumina.com
3	HiSeq Series	Exome, transcriptome and production scale genome	www.illumina.com

Table 2: Ion sequencing instruments offered by Thermo Fisher			
S.No.	Instrument type	Supported Applications	website/reference
1	ION PGM	Bacterial, amplicon and targeted gene panel sequencing	www.thermofisher.com
2	ION PROTON	Exome and transcriptome sequencing	www.thermofisher.com
3	ION S5XL	Bacterial, amplicon, targeted gene panel, exome and transcriptome	www.thermofisher.com

Table 3: Sequencing platforms offered by Pacific Biosystems			
S.No.	Instrument type	Supported Applications	website/reference
1	RSII	Small genome, targeted gene panel sequencing	www.pacb.com
2	Sequel	Small genome, targeted gene panel sequencing	www.pacb.com

LOOP adapters). Prepared library molecules do not require any template preparation or amplification on beads but proceed directly to the sequencing reaction using library molecule as template.

Pacbio sequencing technology is predominantly built on phospho-linked nucleotides and zero-mode waveguides (ZMWs). Each SMRT cell contains thousands of ZMWs that are small reaction wells (Levene et al. 2003). Each ZMW is a metallic chamber approximately measuring 17nm wide that

is illuminated with glass support creating a small detection volume of zepto liters. ZMWs also enable the observation of individual molecules against the required background of fluorescently labeled phospho-linked nucleotides while maintaining high signal to noise ratio. The complex containing the template molecule to be sequenced with sequencing primer and DNA polymerase is immobilized on the bottom of the ZMW (Korlach et al. 2008).

Phospho-linked nucleotides are introduced into the ZMW chamber and each nucleotide is labeled with a different colored fluorophore. As base is incorporated into the detection volume, a light pulse is produced and after the incorporation of nucleotide the phosphate chain is cleaved, thereby releasing the fluorophore (Lundquist et al. 2008). This process occurs in parallel in thousands of ZMWs in a SMRT cell. Pacific Biosciences offer two systems that are listed in Table 3 along with the supporting applications for each system. A detailed comparison of NGS technologies is shown in Table 4 and a description of various applications is depicted in Figure 1.

Table 4: Comparison of different sequencing platforms currently available for different sequencing applications

Platform/Instrument type	Read Length	Data output Per run	Run time	Principle involved	Company website/ Reference
Sanger sequencing (3730XL)	1000 bp	96 KB	1 hr	Synthesis using fluorescently labelled ddNTPS	www.thermofisher.com
Illumina (Nextseq series Mid output kit)	2X150 bp	32-29 GB	26 hrs	Sequencing by synthesis with reversible terminators	www.illumina.com
Ion torrent (ION PROTON)	200 bp	10-15 GB	2 hrs	Sequencing by synthesis	www.thermofisher.com
Pacific biosciences (Sequel)	10-15 kb	5-10 GB	Up to 4 hrs	SMRT technology	www.pacb.com
ROCHE 454 GS FLX+	700 bp	0.7 GB	23 hrs	Pyrosequencing	www.roche.com

Application of NGS technologies

The technologies described above have been used to address various biological questions through the sequencing of genome, exome, transcriptome, microbial and plant genome.

Whole exome sequencing (WES)

The exome represents only 1-3% of the genome, but 85% of human disease-causing variants are sheltered in exonic regions (Choi et al. 2009). Today, exome sequencing is the most widely used targeted enrichment technique that enables researchers to focus on specific regions of interest rather on the whole genome and is therefore quite an economical method of choice. The impact of WES

in medical genetics, including the detection of mutations in Mendelian disorders, and in cancer genomics continues to increase and the function or role of 150 genes in various diseases has been identified by this approach (Rabbani et al. 2014). Exome sequencing using the Illumina sequencing platform has been used to reveal the mutational spectrum of testicular germ cell tumors, the most prevalent cancer in young men (Litchfield et al. 2015), for molecular diagnosis for a significant proportion of patients with epilepsy (Helbig et al. 2016), and for definitive diagnosis in child with intractable inflammatory bowel disease (Worthey et al. 2011). Exome sequencing using the Ion Proton platform has been used in children with Autism spectrum disorder to characterize a family with congenital disorder of glycosylation (Tammimies et al. 2015) and to reveal a splice site mutation that causes a rare form of congenital muscular dystrophy (Oliveira et al. 2015). Genes associated with various types of cancer identified by either genome-wide association studies or by WES are shown in Table 5.

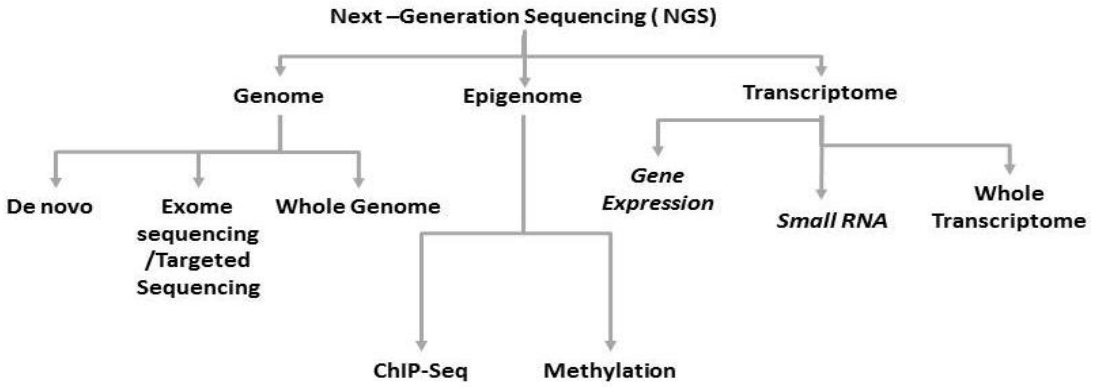

Figure 1. Description of various applications of NGS

Table 5: Genes associated with various types of cancer identified by either genome-wide association studies or by whole exome sequencing (WES)

Type of cancer	Gene(s) Identified/involved	Method	Reference
Triple negative breast cancer	FANCM	Exome sequencing	*(Kiiski et al. 2014)*
Bladder cancer (TCC)	*STAG2, ESPL*	Genome and Exome sequencing	(Guo et al. 2013)
Prostate cancer (advanced and lethal)	TP53, DLK2, GPC6, and SDF4.	Exome sequencing	(Kumar et al. 2011)
Bladder cancer	*CDKN1A*and *FAT1*	Genome Sequencing	(Cazier et al. 2014)

Esophageal squamous cell carcinoma (ESCC)	*TP53*, *RB1*, *CDKN2A*, *PIK3CA*, *NOTCH1*,*NFE2L2*, *ADAM29 and FAM135B*	Genome and Exome sequencing	(Song et al. 2014)
Hepatocellular carcinoma	TP53, CTNNB1, KEAP1, C16orf62, MLL4, and RAC2	Exome sequencing	(Cleary et al. 2013)
Gingivo-buccal oral squamous cell carcinoma (OSCC-GB)	*USP9X*, *MLL4*, *ARID2*, *UNC13C* and *TRPM3*),	Exome sequencing	(India Project Team of the International Cancer Genome 2013)
Cervical cancer	*MAPK1*, *HLA-B EP300*,*FBXW7*,,*NFE2L2*,*TP53*, *ERBB2*, *ELF3*, *CBFB*	Exome sequencing	(Ojesina et al. 2014)
Chronic lymphocytic leukemia (CLL)	*SF3B1*	Exome sequencing	(Quesada et al. 2013)
Colorectal cancer	APC	Exome sequencing	(Ashktorab et al. 2015)
Familial cutaneous malignant melanoma	POT1	Exome sequencing	(Shi et al. 2014)
Endometrial cancer	*ARID1A*, *INHBA*,*KMO*, *TTLL5*, *GRM8*, *IGFBP3*, *AKTIP*, *PHKA2*, *TRPS1*, *WNT11*, *ERBB3*, *RPS6KC1*)	Exome sequencing	(Liang et al. 2012)

RNA sequencing (RNA-Seq)

RNA-Seq or transcriptome sequencing is the method of choice for transcriptome profiling that provides a complete picture of the whole transcriptome rather than a subset of genes. RNA-Seq technique and RNA-based measurements using NGS enable researchers and clinicians to understand the vast diversity of RNA biomolecules that play important roles in various biological processes, diagnostic marker-based disease diagnosis and varied areas of human and animal life (Byron et al. 2016). RNA-Seq standard library preparation starts with isolation of total RNA from the desired species followed by enrichment of RNA type to be profiled in the experiment. Different enrichment methods are available depending upon the application (Table 6). The enriched RNA molecules are then converted into cDNA fragments with or without adaptors attached to one or both ends of the cDNA molecule, amplified and subsequently sequenced using high end technology of choice to obtain single or paired end reads. The read length varies from 30-400bp

depending on the technology method used. After the end of the sequencing procedure, the obtained paired or single end reads are aligned to reference genome or to reference transcripts or simply assembled *de novo* without any genomic reference to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression of each gene.

RNA sequencing using the Illumina platform has been used to understand diseases and complex traits as well as identify novel transcript isoforms in neurological diseases and rheumatoid arthritis (Heruth et al. 2012; Costa et al. 2013; Wang et al. 2014). The Ion Torrent Proton platform has been used for RNA-Seq of various applications, including gene expression profiling of an induced pluripotent stem cell model for schizophrenia that provided molecular insight into how the *DISC1* mutation causes synaptic defects (Wen et al. 2014). RNA and DNA sequencing on the Ion PGM System was used to identify RNA and DNA differences (RDDs) in the gene encoding interleukin-12 receptor β1 (Turner et al. 2015).

Microbial sequencing

Studying the DNA or RNA sequence of microbes and viruses is important in better understanding human infectious diseases and their treatment options, and in monitoring and surveillance of sudden disease outbreaks etc. In the past several decades, studies of 16s ribosomal RNA sequencing using the capillary electrophoresis method have established this as widely followed and inexpensive technique to study bacterial communities, population and taxonomic analysis, and specifies identification. Although 16srRNA sequencing using the Sanger sequencing method is widely utilized, it has several limitations including scalability, cost, and throughput. Recent innovations in the arena of NGS have greatly facilitated deeper understanding of microbial genetics, and have opened a new area of study of metagenomics using NGS. Metagenomics is the study of metagenomes to understand the biological diversity of a microbial population (microbiome) in an environmental sample. Two approaches are followed to study metagenomics, namely whole genome and targeted approaches, which are outlined in Figure 2 and Table 7 along with the advantages and disadvantages of these methods. In recent years, several NGS technologies have been successfully used to study and characterize bacterial communities significant for human health using archived samples from diverse locations including the mouth (Jünemann et al. 2012), the hands of healthcare workers (Rosenthal et al. 2014), airway (Blainey et al. 2012; Salipante et al. 2013), diabetic myeloma (or "Madura foot), and the human and murine intestinal tract (Milani et al. 2013; Mir et al. 2013; Petrof et al. 2013). NGS has also been used to study the oral ecology of periodontal disease (Ly et al. 2014), to assess how early life antibiotic exposure alters gut microbiome and increases diabetes in a mouse model (Candon et al. 2015), and to profile meconium to identify bacteria correlated with premature birth (Ardissone et al. 2014).

Table 6: Enrichment methods available for various applications of RNA sequencing

Preferred Enrichment Method	Target Selection Approach	Reads required in Number	Target Application

Small RNA & miRNA Sequencing	microRNA selection	1-2M for expression	Gene expression quantitation and gene level detection
		2-5M for discovery	
mRNA Sequencing	Poly-A selection	15-25M	Gene level detection and gene expression quantitation
			Equal level of information also observed in microarray research
Whole Transcriptome	rRNA depletion	30-40M	Enables detection of fusion transcripts, novel events, alternative splicing
			Detect ncRNA
Deep Whole Transcriptome	rRNA depletion	80-200M	Detection of low abundance transcripts

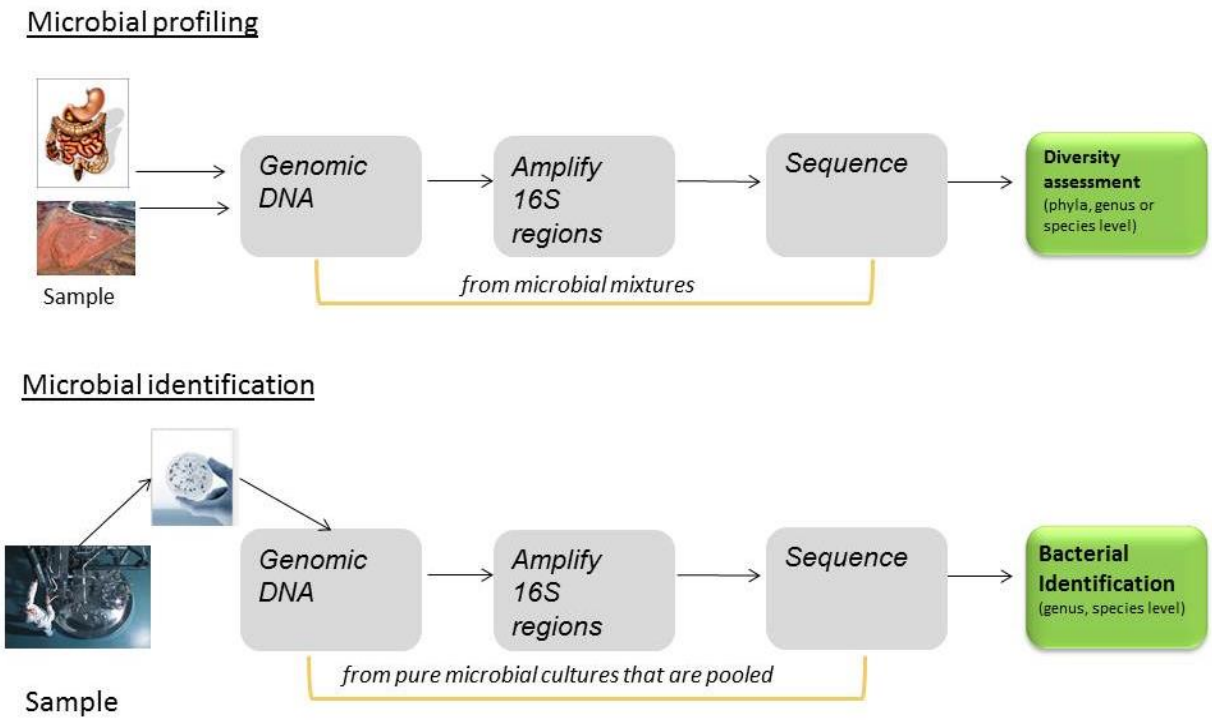

Figure 2. Applications of 16S based sequencing.

Table 7: Metagenomics approaches

	Whole Genome	Targeted Approach
Work flow	a. Extract DNA and digest b. Sequence c. Analyze d. Assemble e. Annotate	a. Extract DNA and amplify 16S variable regions b. Sequence c. Analyze d. Map to Reference database
Merits	Complete picture of the sample enables discovery	Much more sensitive to species present at low levels, easy data analysis
Demerits	Inefficient use of sequencing reads, complicated analysis, not sensitive to species present at low levels	Restricted to what is known

Viral sequencing

In developed and developing countries, infections caused by viruses are of major concern and contribute to significant human morbidity and mortality. According to the World Health Organization, viral infections caused 15 million deaths across the globe in 2002. Over the past several decades, a number of viral pandemics have been reported, of which influenza (1918, 1957 and 1963), HIV/AIDS (1970s), and severe acute respiratory syndrome (2003) resulted in huge human loss (Tatem et al. 2006). Hence, the identification, characterization and diagnosis of viral infections in humans plays a pivotal role in treatment, control and eradication of the viral diseases. Modern globalization and trade contributes significantly to the spread of viral infections (Tatem et al. 2006) and as a consequence human isolation is condensed. In the past, various methods have been employed in the detection and identification of viruses that includes (but is not limited to) inoculation techniques, PCR, cell culture technique etc. According to Anthony *et al* (Anthony et al. 2013), 32,000 mammalian viruses are yet be revealed. With the advent of NGS technologies, the identification and characterization of viruses is faster than ever before and NGS has proven to be a sensitive and powerful tool for viral detection (Anthony et al. 2013) that allows detection even of viral particles present at very low frequencies. One of the major advantages of NGS technologies is that sequencing can be performed at greater depth which results in spontaneous sequencing of viral DNA or RNA, thereby enabling the discovery of new or unknown viruses. These high throughput sequencing technologies have been used in identifying the causative agents of viral outbreaks such as the influenza (H1N1) outbreak in 2009 (Greninger et al. 2010; Yongfeng et al. 2011), HPyV9 (human polyomavirus 9) associated with diarrhea (Sauvage et al. 2011), Ebola virus protection in non-human primates (Thi et al. 2015), the involvement of camels in human infections of MERS (Briese et al. 2014), and SAdV-C (simian adenovirus C) associated with acute respiratory illness in humans (Chiu et al. 2013). NGS studies have been conducted on DNA viruses (Conway et al. 2012; Chiu et al. 2013), RNA viruses (Chandriani et al. 2013), animal viruses (Biek et al. 2012; Rao et al. 2013), and plant viruses (Rowe et al. 2013; Roy et al. 2013) to identify causative agents involved in disease.

Plant genome sequencing

Despite rapid advances in genome sequencing approaches both in microbial and mammalian systems there seems to be a lag in genomic information among several disciplines of plant biology that includes forestry, horticulture, agronomy etc (Hamilton et al. 2012). The first whole genome sequence of *Arabidopsis thaliana* was announced in 2000. Since then, this study has had a widespread influence that has enhanced the

demand for plant genome information and led to the sequencing of several plant genomes, some of which are detailed in Table 7. One of the foremost motivating forces for the rapid increase in plant genome sequencing studies is the gradual decrease in the cost of the sequencing machinery and the speed at which genome can be sequenced. It would be a daunting task to select the right plant genome to be sequenced in a world with >26000 known plant species (Jackson et al. 2006). Currently, the key decision of which plant genome to sequence is mainly based on the economic importance of a plant species, whether it serves as a model organism, whether there is an effective research community working on the particular species, the genome size, and availability of inbred lines etc. (Michael et al. 2013). Although several plant genome sequences are available, some have been studied more extensively, including economically important plants such as cotton and tobacco, and model organisms like *Arabidopsis thaliana*. Table 8 illustrates the some of the important plant genomes sequenced so far.

Table 8: Important plant genomes sequenced so far

SNO	Species Name	Platform	Genome size (MB)	Reference
1	A. thaliana genomes;	Illumina	357 Mb	(Ossowski et al. 2008)
2	Cucumis sativus L.	Sanger and Illumina	367	(Huang et al. 2009)
3	Malus domestica	Sanger and 454	742	(Velasco et al. 2010)
4	Solanum tuberosum	Sanger, 454, Illumina	844	(Potato Genome Sequencing Consortium 2011)
5	Gossypium raimondii	illumina	880	(Wang et al. 2012)
6	Solanum lycopersicum	Sanger, 454, Illumina, SOLiD	900	(Consortium 2012)
7	Azadirachta indica	Illumina	364	(Krishnan et al. 2012)
8	Cicer arietinum	illumina	738	(Varshney et al. 2013)
9	Hevea brasiliensis	4544, illumina, SOLiD	2150	(Rahman et al. 2013)
10	Nicotiana tabacum	illumina	4500	(Sierro et al. 2014)
11	Eucalyptus grandis	Sanger, Illumina	640	(Myburg et al. 2014)
12	Solanum commersonii	illumina	838	(Aversano et al. 2015)

Future perspectives

Greater understanding of human, plant, or microbial genome, exome, or transcriptome using NGS technologies provides appealing insights into several human diseases including cancer and helps in advancing diagnostic, prevention, and treatment strategies for various applications in human medicine. Despite the rapid maturation of NGS technologies, sequencing the human genome is still expensive and whole exome sequencing provides a temporary alternative to understand human coding regions. Future developments may lead to remarkable technological improvements in our ability to read and analyze the bases of genetic code compared to existing technologies. Vast data obtained from genome, exome, and transcriptome sequencing must be organized so that information can be systematically shared by researchers and clinicians. Continual improvements are required in data analysis and the development of user friendly software for comprehensive sequence data

evaluation. The dearth of databases that bridge the needs of research scientists and clinicians may present challenges in data storage and distribution, creating a need for molecular biologists to interpret the large amount of genetic data generated by these technologies.

Conflict of interest statement

All the authors in this manuscript declare that there is no competing interest.

Authors' contributions

PVB and BPN conceived the idea. Both the authors wrote the manuscript. BPN designed the tables and figures with inputs from PVB.

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