

# Whole-genome resequencing of 100 healthy individuals using DNA pooling

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**Abstract.** With the advent of next-generation sequencing technology, the cost of sequencing has significantly decreased. However, sequencing costs remain high for large-scale studies. In the present study, DNA pooling was applied as a cost-effective strategy for sequencing. The sequencing results for 100 healthy individuals obtained via whole-genome resequencing and using DNA pooling are presented in the present study. In order to minimise the likelihood of systematic bias in sampling, paired-end libraries with an insert size of 500 bp were prepared for all samples and then subjected to whole-genome sequencing using four lanes for each library and resulting in at least a 30-fold haploid coverage for each sample. The NCBI human genome build37 (hg19) was used as a reference genome for the present study and the short reads were aligned to the reference genome achieving 99.84% coverage. In addition, the average sequencing depth was 32.76. In total, ~3 million single-nucleotide polymorphisms were identified, of which 99.88% were in the NCBI dbSNP database. Furthermore, ~600,000 small insertion/deletions, 500,000 structure variants, 5,000 copy number variations and 13,000 single nucleotide variants were identified. According to the present study, the whole genome has been sequenced for a small sample subjects from southern China for the first time. Furthermore, new variation sites were identified by comparing with the reference sequence, and new knowledge of the human genome variation was added to the human genomic databases.

Furthermore, the particular distribution regions of variation were illustrated by analyzing various sites of variation, such as single-nucleotide polymorphisms.

## Introduction

The first-generation sequencing technology used in the Human Genome Project is time-consuming and expensive (1). Thus, the advent of next-generation sequencing technology, with higher throughput, time and cost savings, has led to revolutionary changes in the methods used for genomics research (2). Following several years of development, researchers are currently able to combine whole-genome resequencing, exome sequencing, target region sequencing and transcriptomics in order to detect mutations (3-5). Thus far, human genome sequences have been reported for thousands of individuals with ancestry in distinct geographical regions, including Yoruba African people, two individuals of northwest European origin, one individual from each of China and Korea and 44 Caucasians (6-11). In addition, the 1000 Genomes Project Consortium have reported results for Phase 1 of the project (12). However, even next-generation sequencing technology is sufficiently cost-effective for individuals but not for use in large-scale analyses (13). Therefore, a proven effective strategy used to reduce the overall cost is pooling DNA sequences from different individuals and then sequencing the pooled DNA with a high coverage (14,15).

Using this strategy mentioned above, the majority of the whole-genome resequencing performed in human genetics research has focused on identified types of variants, including single-nucleotide polymorphisms (SNPs), copy number variants (CNVs), small insertions/deletions (indels), single nucleotide variants (SNVs) or structure variants (SVs) (16-18). It has been revealed that multiple rare variants may account for only a small proportion of the phenotypic variation in complex diseases (19), and new variants have been detected gradually, which indicates different mutations in different regions (20). This reveals that a considerable number of human genetic

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variants, particularly rare variants, remain to be discovered beyond those currently published in public databases.

In the present study, ~3 million SNPs were identified, as well as ~600,000 indels, 5,000 SVs, 5,000 CNVs and 13,000 SNVs. These variants were subsequently analysed using genomic and bioinformatic methods.

## Materials and methods

**Samples.** The peripheral blood samples examined in the present study (n=100) were collected during a recruitment effort at the health management centre of the Guilin 181st Hospital (Guilin, China). A total of 100 unrelated, healthy ethnic Han Chinese individuals were recruited in the research project. Their age ranged between 40 and 60 years old in the cases examined, and all volunteers were living in Guilin. The present study was approved by the Medical Ethics Committee of People's Liberation Army 181 Hospital (Guilin, China) and written informed consent was obtained from all volunteers before their blood was withdrawn.

**Preparation of DNA pools.** DNA was isolated from peripheral blood samples by the same standard techniques for all volunteers, as previously described (21). The integrity of DNA in every sample was determined by DNA agarose gel electrophoresis, as previously described (22), and the concentration of DNA in every sample was detected by a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Initially DNA was homogenised for 30 min in a thermoshaker at 50°C, and all DNA samples were diluted to ~50 ng/μl as a working solution. Next, each sample was carefully measured using a Qubit fluorometer and diluted further with Tris-ethylenediaminetetraacetic acid buffer (Takara Bio, Inc., Beijing, China) to 20 ng/μl. Finally, 10 μl DNA was extracted from each of the samples, and mixed together with other samples in pools representing 100 individuals.

**Genomic DNA library construction and genome resequencing.** In order to minimise the likelihood of systematic bias and potential sequencing errors in sampling, the DNA library was constructed twice for each sample and every library was sequenced twice. Thus, each sample would be sequenced four times. Genomic DNA was extracted from the blood using standard phenol/chloroform extraction methods (23). The DNA library was prepared using a paired-end DNA sample prep kit (Illumina, Inc., San Diego, CA, USA) and following the manufacturer's instructions. In brief, 2 μg genomic DNA was randomly fragmented by nebulisation, as previously described (24), which generated double-stranded DNA fragments comprised of 3' or 5' overhangs. The overhangs that resulted from fragmentation were converted into blunt ends using T4 DNA and Klenow polymerases (Tiangen Biotech Co., Ltd., Beijing, China). Furthermore, the 3' to 5' exonuclease activity of these enzymes removed 3' overhangs and the polymerase activity filled in the 5' overhangs. The next step was to add an 'A' base to the 3' end of the DNA fragments using the polymerase activity of the Klenow fragment (3' to 5' exo minus). Next, DNA adaptors were ligated to the DNA fragments, and the DNA fragments were purified on a 2% agarose gel to remove all unligated adaptors, adaptors that may have ligated to one another, and select a 500 bp template

to go on the cluster generation platform. The adapter-modified DNA fragments were enriched by 12 cycles of the polymerase chain reaction (PCR), as previously described (25). For quality control, the concentration of the libraries was measured by the absorbance at 260 nm, and the 260/280 ratio was 1.8. Furthermore, an Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA) was used to detect the fragment size and yield, and the results of the library revealed that it contained the expected size and yield. Following quality control, the library generated was used in the cBot System for cluster generation and the samples were then analysed using the Solexa sequencing system (HiSeq 2000 platform; Illumina, Inc.), which is based on sequencing-by-synthesis technology (7,8,26).

**Public data.** The human reference genome was downloaded from the University of California Santa Cruz Genome Browser (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/refGene.txt.gz>) and the human SNP database (dbSNP; [ftp://ftp.ncbi.nih.gov/snp/organisms/human\\_9606](ftp://ftp.ncbi.nih.gov/snp/organisms/human_9606)) was used for comparison of the putative SNPs identified.

**Bioinformatics analysis.** The bioinformatics analysis began with the sequencing data (raw data) generated from the Illumina pipeline. Initially, the adapter sequence in the raw data was removed, and low quality reads with too many Ns or low quality bases were discarded. This step produced 'clean data'. Secondly, the Burrows-Wheeler Aligner (BWA) was used to align reads to the reference sequence (27). The alignment information was stored in BAM format files to be further processed during the following steps: Fixing mate-pairing information, adding read group information and marking duplicate reads caused by PCR. Following these procedures, the final BAM files were ready for variant calling. SNPs were detected using SOAPsnp (28); small insertion/deletions (indels) were detected using SAMtools (29) GATK; CNVs were detected using CNVnator and SNVs were detected using Varscan (30). Additionally, SVs were identified using BreakDancer and a self-method based on the Segseq algorithm (31,32). The pipeline also included purity estimation. Filters were then applied to obtain higher confidence results for the identified variants. Next, ANNOVAR ([www.openbioinformatics.org/annovar/](http://www.openbioinformatics.org/annovar/)) was used to annotate the variants, based on which advanced analysis can subsequently be conducted (33). Quality control (QC) was required at each stage of the analysis pipeline to ensure clean data and to verify the alignment and the called variants.

**Data quality control.** For cases of low-quality sequencing, resequencing was required. The QC steps were conducted as follows: i) Removal of the adapter reads (an adapter read was defined as a read that included the adapter bases, and those adapter reads were removed from the raw FASTQ data); ii) removal of low-quality reads, (if more than half of the bases in a read were low-quality bases that were defined as base quality ≤5, they were treated as low-quality reads and were removed from the raw FASTQ data); and iii) removal of reads in which unknown bases were >10%.

Following filtering, the remaining reads were referred to as 'clean reads' and were used for downstream bioinformatics analysis. Finally, a statistical analysis was performed in order

Table I. Quality statistics of clean data.

Type	Raw data	Clean data
Number of reads	1,273,028,056	1,210,244,348
Data size	114,572,525,040	108,921,991,320
N of fq1	23,591,083	1,889,209
N of fq2	61,780,180	1,483,604
GC (%) of fq1	39.61-40.1	39.43-40.01
GC (%) of fq2	39.8-40.17	39.55-40.05
Q20 (%) of fq1	94.58-97.09	95.79-97.85
Q20 (%) of fq2	88.51-93.66	92.07-96.12
Q30 (%) of fq1	86.69-92.40	88.20-93.46
Q30 (%) of fq2	78.99-88.05	82.37-90.50
Discard reads related to N	2,264,798	
Discard reads related to low qual	59,735,130	
Discard reads related to adapter	783,780	
Clean data/raw data	95.07%	

Before doing any further analysis, quality control is required in order to detect whether the data is qualified. In addition, filtering of raw data is needed to decrease data noise.

to get the data production for the raw FASTQ data and the clean data.

## Results

**Data production and quality control.** The genomic DNA pool was sequenced using a HiSeq 2000 platform (Illumina, Inc.). A total of 127.3 Gb of raw sequence data were generated, resulting in a sequencing depth of ~30-fold (Table I). Sequence data has been submitted to the NCBI Sequence Read Archive under accession number SRA185897 (<http://www.ncbi.nlm.nih.gov/sra>).

Before performing any further analysis, QC was required in order to detect whether the data was qualified. Raw reads were defined as reads containing the adapter sequence, a high content of unknown bases and low-quality reads, which were removed prior to the data analysis. For instance, Fig. 1A demonstrates an example of an unbalanced base composition percentage, which is unqualified because the T curve is not in accordance with the A curve, whereas Fig. 1B presents a satisfactory base composition. Regarding the base quality, the sequencing quality depicted in Fig. 1C is poor. By contrast, Fig. 1D presents good quality sequences whose base ratios are mostly >20. The quality of the clean data is presented in Table I.

**Alignment/mapping of reads to a reference sequence.** Sequencing reads were aligned to the reference genome sequence using the BWA software. The human genome build37 (Hg19) was used as the reference for this project. The whole-genome size of hg19 was 3,137,161,264 bp, while the effective size is 2,861,327,131 (excluding N bases, random and hap regions and chromosomes Un and M in the reference). Next, Picard was used to mark duplicate reads (redundant information produced by PCR). The alignment results are

shown in Table II. The distribution of the per-base sequencing depth and the cumulative depth distribution in the non-N region of the whole genome were also plotted in Fig. 2. The distribution of the per-base sequencing depth approximately followed a Poisson distribution, demonstrating that the non-N region of the whole genome was evenly sampled (Fig. 2).

**SNP identification and annotation.** An SNP is a DNA sequence variation occurring when a single nucleotide A, T, C or G differs between samples or individuals. SOAPSnp was employed to detect SNPs. Using the consensus sequence, the polymorphic loci between the identified genotype and the reference could be filtered and highlighted, which would then constitute the high confidence SNP dataset. After the SNPs were identified, ANNOVAR was used to perform annotation and classification.

In total, 3,830,314 SNPs were identified. Among the SNPs in the DNA pool, 479,258 were homozygous, while 3,351,056 were heterozygous. Furthermore, 20,616 sites were located in exonic regions whereas 1,330,526 were within intronic regions. In addition, in the dataset of the present study, 93,679 and 2,316,322 SNPs were detected using NcSNPs and Intergenic, respectively. The SNPs located in gene regions in the DNA pool were annotated using ANNOVAR. In total 24,880 SNPs in the untranslated regions, 143 SNPs at splicing sites, and 11,267 SNPs corresponding to synonymous mutations were identified. Detailed statistics are provided to demonstrate the distribution of SNPs in different gene regions (Table III).

**Identification and annotation of indels.** Pair-end reads for gap alignment were used in order to detect indels using the program mpileup in SAMtools. After the indels were identified, ANNOVAR was employed in order to perform annotation and classification (Table IV). Among the indels in the DNA pool, 361,730 (60%) were located in intergenic regions, 403 in

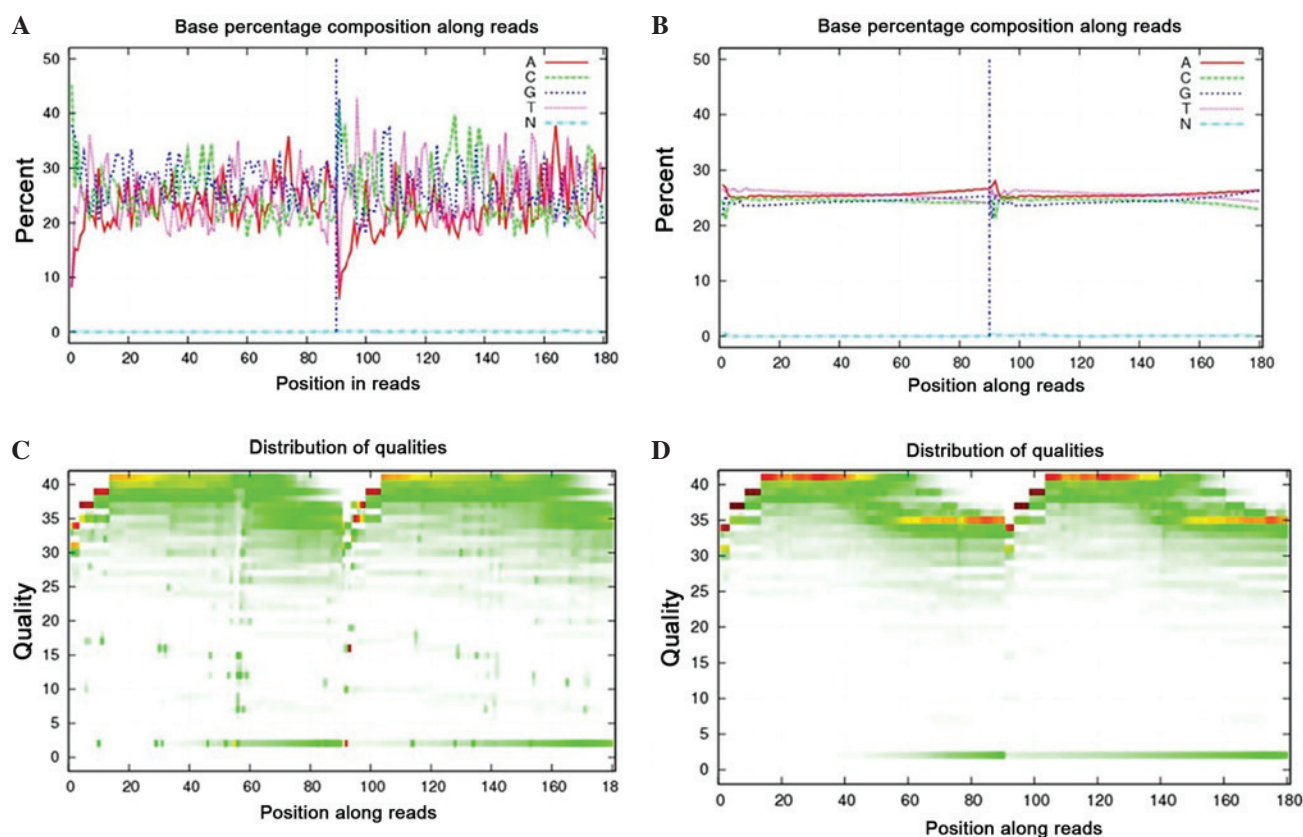


Figure 1. Analysis of base composition and quality. (A) Unbalanced base composition of raw reads. (B) Balanced base composition of raw reads. (C) Low quality distribution of bases along reads. Each dot in the image represents the quality value of the corresponding position along reads. If the percentage of the bases with low quality (<20) was considered very high, then the sequencing quality of this lane was considered bad. (D) High quality distribution of bases along reads. Each dot in the image represents the quality value of the corresponding position along reads. If the percentage of the bases with low quality (<20) was considered low, then the sequencing quality of this lane was considered good.

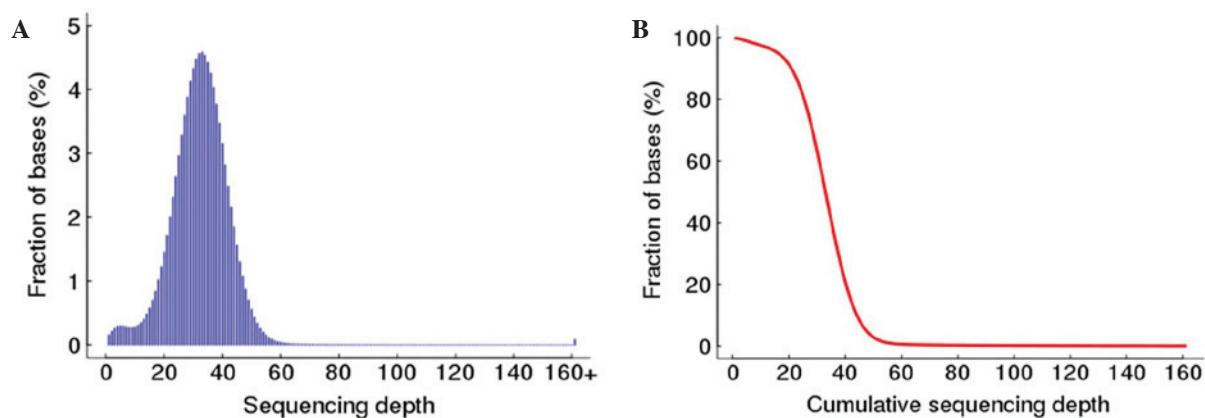


Figure 2. Depth distribution. (A) X-axis denotes the sequencing depth, while the y-axis indicates the percentage of the non-N region of the whole genome under a given sequencing depth. (B) Plot of cumulative depth distribution in the non-N region of the whole genome, the x-axis denotes sequencing depth while the y-axis indicates the fraction of bases that achieves at or above a given sequencing depth.

exonic regions and 211,208 (35%) in intronic regions. There were 101,236 homozygous and 499,888 heterozygous indels identified in the DNA pool. The length distributions of the indels within the whole genome and the coding region were plotted in Fig. 3.

**Identification and annotation of SVs.** Paired-end sequencing provides a powerful tool for detecting genome-wide structural variation. BreakDancer/CREST was used to detect SVs. When

aligning the paired-end reads, if an SV existed between the sequencing results and the reference it would not have met the requirements for pair-end alignment, and therefore these anomalous read pairs and soft-clipped reads would have been used to detect SVs. Using this method a catalogue of 5,412 SVs was generated, including 4,834 deletions and 352 insertions, and 1,823 SVs were found in intronic regions, 6 SVs in exonic regions and 3,409 SVs in intergenic regions. The result is a list of SVs detected at the whole-genome level (Table V).



Table II. Alignment results.

Item	Value	Item	Value
Clean reads	1,210,244,348	Duplicate rate	8.51%
Clean bases (bp)	108,921,991,320	Mismatch bases	425,479,678
Mapped reads	1,173,317,876	Mismatch rate	0.41%
Mapped bases (bp)	103,953,154,126	Average sequencing depth	32.76
Mapping rate	96.95%	Coverage	99.84%
Uniq reads	1,125,241,695	Coverage at least 4X	99.21%
Uniq bases (bp)	99,700,359,408	Coverage at least 10X	97.48%
Unique rate	95.90%	Coverage at least 20X	91.30%
Duplicate reads	99,867,211		

Bp, base pairs.

Table III. SNPs summary of annotation.

Categories	Value	Categories	Value
Total	3,830,314	Splicing	143
1000 genome and dbsnp135	3,768,967	NcRNA	93,679
1000 genome specific	1572	UTR5	3,747
dbSNP135 specific	56,946	UTR5 and UTR3	12
dbSNP rate	99.89%	UTR3	24,880
Novel	2,829	Intronic	1,330,526
Hom	479,258	Upstream	18,144
Het	3,351,056	Upstream and downstream	580
Synonymous	11,267	Downstream	21,376
Missense	9,534	Intergenic	2,316,322
Stopgain	71	SIFT	1,138
Stoploss	33	Ti/Tv	2.1055
Exonic	20,616	dbSNP Ti/Tv	2.1068
Exonic and splicing	289	Novel Ti/Tv	1.1191

SNP, single-nucleotide polymorphism; UTR, untranslated region; SIFT, sorting intolerant from tolerant; Ti, transition; Tv, transversion.

**CNV identification and annotation.** CNVs, a form of structural variations, are alterations of the DNA of a genome that result in the cell having an abnormal number of copies of one or more sections of a DNA sequence (34). CNVs correspond to relatively large regions of the genome that have been deleted (fewer copies than the normal number) or duplicated (more copies than the normal number) on certain chromosomes. The CNVs in each sample were detected with a CNVnator. After the CNVs were identified, ANNOVAR was also used to perform the annotation and classification (Table VI).

## Discussion

In the present study, a whole-genome resequencing protocol combined with DNA-pooling technology was used to identify this type of genetic variation across populations. This is a proven and effective strategy for sequencing (35). Despite the rapid development of genetic technology and the routine

performance of whole genome human sequencing, we believe that the data of the present study will provide basic information for such studies and enrich the analysis of human genomic variation across different ethnic groups and regions (36,37).

The present study focused on the assessment of genome coverage, sequencing depth, detection of variations, validation, annotation and classification. Bioinformatic techniques were used to analyse gene sequence data. The preliminary results were obtained by comparing with a reference genome sequence. Furthermore, a total of 127.3 Gb of raw sequence data were generated in a short period of time, and ~3.83 million SNPs were identified in the sample genome obtained via DNA-pooling, among which 2,829 SNPs were recognised to be novel. The trends of novel SNP depth analysis should be the same as what is already known (Fig. 4) (38). Additionally, the total number of transition SNPs to the total number of transversion SNPs ratio was 2.10 (Table III). The number of transition SNPs that have been published in the dbSNP database to the

Table IV. Insertion/deletion summary of annotation.

Categories	Value	Categories	Value
Total	601,124	Stopgain	1
1000 genome and dbsnp135	301,621	Stoploss	1
1000 genome specific	73,292	Exonic	403
dbSNP135 specific	119,018	Exonic and splicing	6
dbSNP rate	69.98%	Splicing	77
Novel	107,193	NcRNA	15,081
Hom	101,236	UTR5	438
Het	499,888	UTR5 and UTR3	3
Frameshift insertion	123	UTR3	4,954
Non-frameshift insertion	85	Intronic	211,208
Frameshift deletion	100	Upstream	3,074
Non-frameshift deletion	99	Upstream and downstream	99
Frameshift block substitution	0	Downstream	4,051
Non-frameshift block substitution	0	Intergenic	361,730

SNP, single-nucleotide polymorphism; UTR, untranslated region.

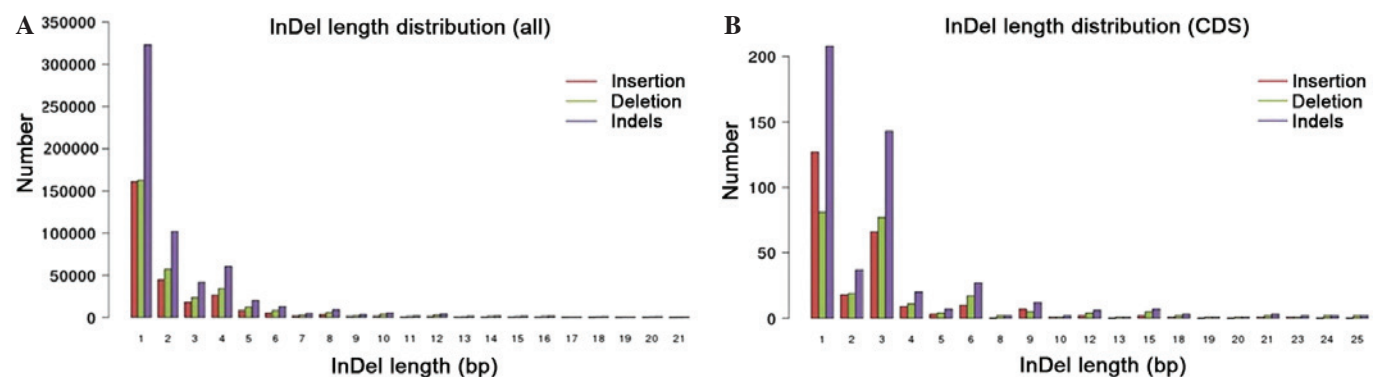


Figure 3. InDel length distribution. Length distribution of the InDels in (A) whole genome and (B) CDS were also plotted below. The length distribution of InDels in coding region shows that peaks are present in length (bp). The InDels with this periodicity are non-frameshift InDels, they have relatively small effect on the genome comparing with the frameshift InDels. InDel, insertion/deletion; CDS, coding sequence.

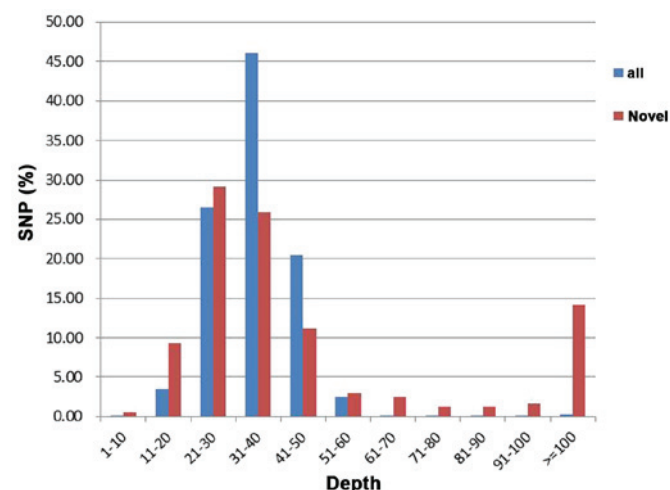


Figure 4. SNP depth distribution. X-axis denotes different sequencing depth, while y-axis indicates the percentage of SNP number. The trends of novel SNP depth analysis should be same like known. SNP, single-nucleotide polymorphism.

number of transversion SNPs that have been published in the dbSNP database was 2.10 (Table III). Furthermore, the number of novel transition SNPs to the number of novel transversion SNPs was 1.19 (Table III). All of these results were consistent with a previous report (10). Regarding indels, 107,193 indels were found to be novel, and the remaining 69.98% were found in the dbSNP database, with the result of indel annotation being consistent with a previous report (10).

For different ethnic groups and regions, the data of the present study constitutes an important supplement to the current gene bank. A sizeable number of unreported SNVs, short indels, SVs and CNVs were revealed in the analysis. Ultimately, with the decreasing cost of genetic sequencing technology, there will be increasing numbers of people who will be sequenced. Therefore, personal genome sequencing may eventually become an essential tool for the diagnosis, prevention and treatment of human diseases.

To the best of our knowledge the present study resequenced the whole genome sequence through a small sample of southern

Table V. Structure variants summary of annotation.

Categories	Value	Categories	Value
Total	5,412	NcRNA	114
Insertion	352	UTR5	3
Deletion	4,834	UTR5 and UTR3	0
Inversion	14	UTR3	8
ITX	120	Intronic	1,823
CTX	92	Upstream	11
Exonic	6	Upstream and downstream	2
Exonic and splicing	1	Downstream	29
Splicing	6	Intergenic	3,409

ITX, intra-chromosomal translocation; CTX, inter-chromosomal translocation; SNP, single-nucleotide polymorphism; UTR, untranslated region.

Table VI. Copy number variations summary of annotation.

Categories	Value	Categories	Value
Total	5,201	UTR3	7
Exonic	954	Intronic	1,174
Exonic and splicing	0	Upstream	59
Splicing	274	Upstream and downstream	3
NcRNA	196	Downstream	35
UTR5	0	Intergenic	2,499
UTR5 and UTR3	0	Amplification size	12,106,400
		Deletion size	85,672,600

UTR, untranslated region.

China. A total of 127.3 Gb of raw sequence data were generated, new variation sites were revealed by comparing with reference sequence, and new knowledge of human genome variation was added to the Human genomic databases. A total of 107,193 novel variations were identified by comparing with a known database. In addition, the particular distribution regions of variation were illustrated by analyzing its sites. In conclusion, in the present research whole genome sequencing was adopted to detect genome variation at a populational level, and summarized that the uploaded sequence data in NCBI is sufficient to provide a research foundation for future researchers.

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