PARALOG-SPECIFIC GENE COPY NUMBER DISCOVERY WITHIN SEGMENTAL DUPLICATIONS

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By

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

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September 2019

With the advancing technology in genome sequencing and analysis, it has become evident that the structural variations are the main source of alteration in human genome. Despite their significance in understanding disease susceptibility, there is no algorithm yet to find all types and sizes of structural variations at once. Structural variation discovery remained problematic since they often overlap with the segmental duplications, nearly identical segments of DNA that appear more than once in the genome. Researchers often excluded these regions that made up ∼5% of the genome because of the complexity it brings to their studies. Only few of them are working in these regions, however, they require a special sequence alignment file where reads are mapped to multiple locations.

Here, we present ParaCoND to discover paralog specific gene copy number within segmental duplications using a sequence alignment file with unique mapping. We utilize the singly unique nucleotides (SUN) that distinguish paralogs from each other in the sequence alignment of the duplicated regions. Our method is based on read depth and is limited to detect only duplications and deletions. We computed the absolute copy numbers of genes using only read depth of SUN. Furthermore, we also computed the paralog specific absolute copy numbers for genes residing in the same segmental duplication.

Keywords: Structural Variation, Segmental Duplication, Copy Number Variation, Singly Unique Nucleotide.

ÖZET

SEGMENTAL DUPLİKASYONLARDA PARALOG-ÖZGÜ KOPYA SAYISI VARYASYONU KEŞFİ

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Genom dizileme ve analizinde gelişen teknolojiyle birlikte, insan genomundaki değişimlerin ana kaynağinin yapısal varyasyonlar olduğu belirginleşti. Çeşitli hastalıklara yol açması bakımından önemli olmalarına rağmen, halen bütün tip ve uzunluktaki yapısal varyasyonları bulan bir algoritma bulunmamaktadır. Yapısal varyasyon keşfi problemli bir alan olarak kalmıştır çünkü sıklıkla segmental duplikasyonların, DNA üzerinde birden fazla bulunan yüksek oranda benzerlik gösteren segmentler, üzerinde bulunmaktadır. Araştırmacılar bütün genomun ∼%5'ine karşılık gelen bu bölgeleri araştırmalarda karmaşıklık getireceği için kapsam dışında bırakmıştır. Sadece birazı bu alanlarda çalışma yapmıştır, ancak onlar da okumaların birden fazla lokasyonla eşleştirildiği özel bir dizi hizalama dosyası kullanmıştır.

Bu çalışmada okumaların tek bir lokasyonla eşleştirildiği dizi hizalama dosyası kullanarak segmental duplikasyonlarda paralog-özgü kopya sayısı varyasyonu keşfi yapan ParaCoND algoritmasını sunuyoruz. Tekrarlı bölgelerdeki paralogları birbirinden ayıran tekli özel nükleotidleri (TÖN) kullanıyoruz. Metodumuz okuma derinliğine dayanmaktadır ve sadece duplikasyon ve silme işlemlerini tespit etmekle sınırlıdır. Genlerin mutlak kopya sayılarını sadece TÖN okuma derinliğini kullanarak hesapladık. Ayrıca, aynı segmental kopyada bulunan genler için paraloga özgü mutlak kopya numaralarını da hesapladık.

Anahtar sözcükler: Yapısal Varyasyon, Segmental Duplikasyon, Kopya Sayısı Varyasyonu, Tekli Özel Nükleotid.

To my family and my dearest friends...

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Chapter 1

Introduction

It all started with Darwin's book of The Origins of Species, which he published in 1859. Since then, the field of genomics have been paid a great deal of attention. With the advancing power of computers, the genomic studies had to adapt itself into this new circumstances. The Human Genome Project was started in 1990 with the goal of determining all the bases in human genome. This was not only exciting but also difficult goal to reach since it could take years or decades to sequence the whole genome with the existing technology. As expected, after 13 years of hard work, the results were published in 2003, and it cost $2.7 billion [3].

The main sequencing method that was used in Human Genome Project was Sanger's chain termination method, or commonly referred to as Sanger sequencing. Sanger sequencing, named after the inventor Frederick Sanger, was good in terms of accuracy, however, it was time-consuming and expensive. In the early 2000s, methods that were better at time and money were finally emerging. Next-generation or high-throughput methods have revolutionized the sequencing business by providing millions of "reads", short sequences, in a massively parallel fashion at a lower cost. As high-throughput sequencing (HTS) methods have become widespread, an effort to make available as many human genomes as possible was set out. For instance, started in 2008, 1000 Genome Project aimed to sequence approximately 2500 individual coming from different ethnic backgrounds

[4]. Several countries, among them are Turkey, have started their own genome projects. In addition to human genome, genomes belonging to other species have also been sequenced [5].

1.1 DNA Sequencing

Before 1950s, the term "gene" was referred to as the smallest unit of genetic information [6]. Scientists at that time were identifying the DNA as the carrier of genetic information, however, they knew very little about how a trait passes down to younger generations. In 1953, there was a breakthrough in the field of genomics with the discovery of double helix structure of DNA by James Watson and Francis Crick. Watson and Crick had answered the biggest question till then in genomics that could lead to solving the mystery about genetic heredity. In a series of articles published in several journals, Watson et al., apart from exposing the structure of DNA, have put their findings together with those of other researchers and come up with a mechanism for DNA replication along with three pieces of evidence supporting the complementary model which suggests that adenine has thymine and cytosine has guanine on the opposite strand of DNA, or vice versa [7].

1.1.1 Early Sequencing Methods

The complementary model opens the door of DNA sequencing since it allows the DNA to be reconstructed from one strand. There were some initial thoughts and methods on how to sequence the DNA, however, none of them were practical in terms of speed to sequence the whole genome. Frederick Sanger and Alan Coulson were the first who have developed the "plus and minus" method, which was soon replaced by chain-termination method, for rapidly determining the order of smallest genetic units in DNA[8]. In an article published in 1970, Sanger et al. have set the gold standard in sequencing. They used DNA polymerase, an enzyme that is essential for DNA replication, to generate copy DNA sequences from the

template DNA strand, all of which should start in the same exact position and end in normally one base larger (and heavier) than the previous. Sanger was able to stop the polymerase by removing an oxygen from the nucleotide at which he wants to stop. Sanger made these DNA sequences move at a speed such that the fewer nucleotides they have, the faster they are. Nucleotides will emit a light in different wavelengths, hence, from a fixed point of view, a light sensor will be able to detect the sequence of nucleotides.

1.1.1.1 Human Genome Project

As the technology in DNA sequencing advances, it has become a must to sequence the whole human genome. After two years of preparation and planning, Human Genome Project (HGP), an international research project, was officially started in 1990 with two simple goals. Along with the purpose of determining all the bases in human DNA, they had also wanted to find out the mapping of the genes in human genome. In 1998, a private company called Celera Genomics have announced that they entered the race to sequence human genome. The competitor have made HGP researchers to speed up their efforts. After eleven years from the start, 90% of human DNA sequence was published in February 2001. The project was officially over in 2003 with 99% of the genome have been published with an error rate of 0.01%.

The results were gathered from the genome of 7-8 individuals to provide researchers with a good approximation of human DNA, which is called the "reference genome". The reference genome is regularly updated by Genome Reference Consortium and useful in genome assembly.

1.1.2 High-Throughput Sequencing

For about 40 years since the invention, Sanger sequencing have been the dominant sequencing method with slight modifications. It was the main sequencing method used in HGP. The experience acquired from HGP have made clear that, despite

its accuracy and provision of long reads, Sanger sequencing was costly and slow.

High-throughput sequencing, on the other hand, have addressed these issues by processing millions of DNA fragments in parallel. Thanks to HTS, cost and time of sequencing a human genome decreased enormously. In 2001, the cost of sequencing a genome was $100 million and it took 13 years. With today's technology, a human genome could be sequenced for around $1300 [3] in a couple of days [9].

Despite its success in terms of time and money over Sanger sequencing, higher sequencing error rates, shorter read lengths and bias against GC-rich regions [10] are among HTS platforms' drawbacks researchers had to deal with.

1.2 Genomic Variation

With the hundreds of thousands human genome have become accessible, researchers have become interested more in the question of what makes one individual different than the other. Early research results have suggested that 99.9% of human genome is identical. Among 3 billion bases making up the human genome, it was only 3 million bases where all the differences between humans were attributed to. Figure 1.1 shows the inverse correlation between the size of genetic variants and their frequencies in the genome. As the genomic variant is getting larger, it can rarely be seen. It has become evident that genetic variants are the reason for many disease including color blindness [11], psoriasis [12], HIV susceptibility [13], Crohn's disease [14] and many more [15].

1.2.1 Single Nucleotide Polymorphism

Single nucleotide polymorphism, SNP for short, is a substitution at a specific position in the DNA sequence. A substitution can be called a SNP if and only if it is the case for more than 1% of the population. The implications of a SNP

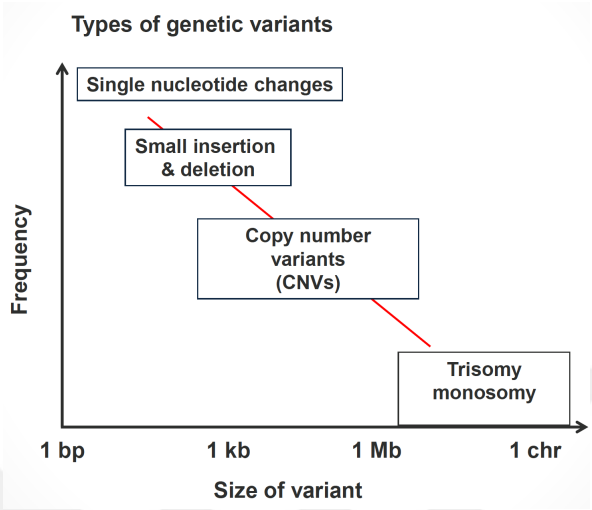


Figure 1.1: Size of different genomic variants with respect to their frequency.

can be significant especially if it exists in a coding region of the DNA. A SNP can change the translation of a codon into an amino acid, what is called a non-synonymous mutation, causing a distortion in the structure of the protein.

1.2.2 Structural Variation

Structural variations (SV) are rearrangements in human genome affecting more than 50 bp that could reach up to several megabases. Structural variations can be categorized according to whether they alter the amount of DNA (copy number variations) or not (balanced rearrangements).

1.2.2.1 Copy Number Variation (CNV)

Copy number variation, also known as unbalanced rearrangement, is a structural variation that changes the amount of DNA. CNVs are also seperated into two:

- Insertions and Deletions: As the name suggests, these are insertions or deletions affecting more than 50 bp.

- Segmental Duplications: They are segments in the DNA that are more than 1000 basepair and 90% or more identical. It can be intra-chromosomal as well as inter-chromosomal. Although segmental duplications account for ∼5% of the genome, they are significant because (1) they often exist in gene-rich chromosomes [16] and (2) they are the most active regions in human genome triggering instabilities and new variations in the chromosome [17, 2].

1.2.2.2 Balanced Rearrangements

Balanced rearrangements are structural variations that do not change the amount of DNA. Balanced rearrangements can be categorized into two;

- Inversions: They are rearrangements where a segment of the DNA is simply inverted.
- Translocations: They are rearrangements where two segments of the DNA exchange their locations.

1.2.3 Chromosomal Changes

Chromosomal changes are variations in the number of chromosome-pairs. They are such major variations that they can be detected by a microscope. It can be categorized into three:

- Monosomy: It is a chromosomal change where there is only one copy of a chromosome.
- Trisomy: It is a chromosomal change where there is an extra copy of a chromosome.
- Uniparental Disomy: It is a chromosomal change where both pairs of chromosome come from the same parent.

1.3 Our Contribution

In this thesis, we present ParaCoND to discover paralog specific copy numbers for genes residing in segmental duplications. This remains an open problem until mrCaNaVaR [18] came out, due to the difficult nature of duplicated regions in genome. However, mrCaNaVaR was not good at differentiating the paralogs. Instead it computes aggregate copy number and assigns it to different copies of genes. Accurate prediction of paralog gene copy number could pave the way of finding the main causes of genetic diseases. For example; opsin is a gene family responsible for color vision. It has three copies that regulate color vision according to wavelengths of different colors. Therefore a variation on a paralog could have an effect on viewing colors of specific wavelengths.

The main improvement of ParaCoND over mrCaNaVaR is that our method utilizes single nucleotide differences among paralogs which helps us to differentiate paralogs from each other. In addition to that, mrCaNaVaR requires a multiple read mapping sequence alignment file, which is not as widespread as unique read mapping. Our method compute the absolute copy numbers of genes residing in segmental duplications using a unique mapping sequence alignment file.

The organization of the thesis is as follows: We briefly introduced the problem in Chapter 1. We further explain the problem and present our method in Chapter 2. We show the experimental results in Chapter 3. We conclude the thesis with final remarks in Chapter 4.

Chapter 2

ParaCoND: Paralog-specific Gene Copy Number Discovery

In this chapter, each step that we followed in our method will be explained in detail.

2.1 Structural Variation Discovery with HTS Data

Structural variations are believed to be highly associated with diseases [19, 14, 15, 13]. Therefore their discovery will be of critical importance in reducing the genetic disease susceptibility. Despite their significance in understanding disease susceptibility, there is no algorithm yet to find all types and sizes of structural variations at once. Before high-throughput sequencing technologies, microarrays were mainly used in structural variation discovery especially for copy number variation [20]. As reviewed by Alkan et al., microarrays are not good at discovering balanced rearrangements and also are not able to locate the copy number variations [20].

Although some are originally designed for old sequencing technologies, high-throughput sequencing platforms have brought novel methods for structural variation discovery.

2.1.1 Read Pair

Paired-end sequencing is a sequencing method where reads are generated from both sides of a DNA fragment whose distance is known. The distance information will be used later by the aligner while aligning the paired-end reads to the reference genome. Read pair method utilizes the distance information between paired-end reads [1]. As shown in the Figure 2.1, paired-end reads that align too distant from each other indicates a deletion, those align too close to each other indicates an insertion.

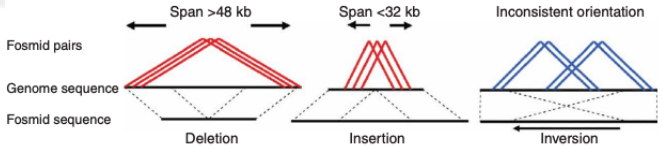


Figure 2.1: Structural variation discovery with read pair. Paired-end reads that are mapped too distant from each other indicates deletion, too close to each other indicates insertion and ahead of what should be behind indicates inversion (adapted from [1]).

Structural variation discovery tools based on read-pair can be categorized as follows:

- Unique Mapping: PEMer [21], BreakDancer [22], GenomeSTRIP [23],
- Multiple Mapping: VariationHunter [24], CommonLAW [25], MoDIL [26], MoGUL [27], HYDRA [28]

2.1.2 Read Depth

Read depth is a simple method to detect duplications and deletions. Based on the assumption that regions are randomly sequenced, for reads that are mapped to the reference genome, the depth of duplicated regions will be higher and the depth of deleted regions will be lower than average [16] as shown in Figure 2.2. Multiple read-mapping is an important aspect of read depth to work effectively. Otherwise the difference of depth between duplicated regions and deleted regions will be insignificant. However known sequencing bias against GC-rich and GC-poor regions [10] should be handled carefully.

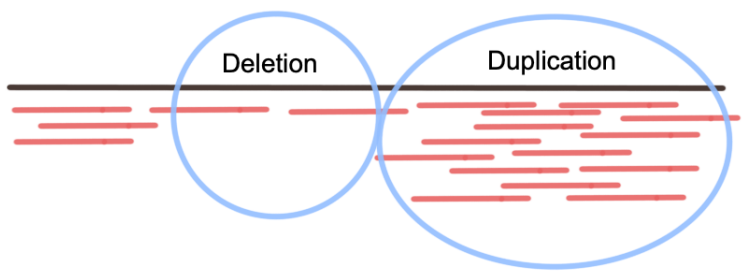


Figure 2.2: Structural variation discovery with read depth.The depth of regions that are duplicated will be higher and the depth of regions that are deleted will be lower than expected depth.

Structural variation discovery tools based on read depth can be categorized as follows:

- Unique Mapping: CNVnator [29], Event-Wise Testing [30]
- Multiple Mapping: Whole Genome Shotgun Sequence [16], mrCaNaVaR [18]

2.1.3 Split Read

Originally designed for longer reads (Sanger sequence etc.), split read based methods were capable of structural variation discovery at one base pair resolution by

trying to map the reads by splitting. Similar to read pair, the location of split reads will tell the class of structural variation. For instance, the distance between split reads shows an insertion or first split coming after second split indicates an inversion.

Structural variation discovery methods based on split read can be categorized as follows:

- Unique Mapping: Pindel [31], SRiC [32]
- Multiple Mapping: Splitread [33]

2.1.4 Sequence Assembly

Although it is in its early stages, sequence assembly methods are, in theory, powerful and requires less computational power. Assuming the whole genome is assembled without using the reference genome (*de novo* assembly), we can easily detect structural variation by comparing the individual's genome with the reference genome. NovelSeq [34], PopIns [35] can be examples of this type.

2.1.5 Hybrid Methods

There are also some methods using two or more of these techniques in order to increase their accuracy. Examples of these type of methods can be DELLY [36] in which read pair and split read were used, LUMPY [37] in which read pair, read depth and split read were used, TARDIS [38] in which read pair, read depth and split read were used.

2.2 Dataset

We have the data showing the genome-wide segmental duplications (SD). The data is coming from Segmental Duplication Database of UW Genome Sciences Human Paralogy Laboratory [39, 16]. The data consist of 51,599 SD pairs with their exact locations. Furthermore, we have global alignments of SD pairs in files generated using a sequence alignment tool called ClustalW [40].

2.3 Data Preprocessing

Since entries in our data show the duplications in pairs, we had to turn these pairs into a multiple sequence alignment in order to locate singly unique nucleotide positions. However, our data lack some pairs. For example, we have SD pairs of A-B and B-C, therefore A-C should also be in our data whereas there is not in some cases. Using the Algorithm 1, we have identified 21,023 new SD pairs using existing pairs and have them aligned.

Algorithm 1 An algorithm to find missing SD pairs

```
procedure FIND_MISSING_PAIRS
    M ← map of Segmental Duplications as read from data
    for each key-value pair in M do
        Let temp_set be an empty set
        for values in M[key] do
            temp_set ← temp_set ∪ M[value]
            clear M[value]
        while true do
            flag ← true
            for each key-value pair in M do
                Find intersection of temp_set and value
                if intersection is not empty then
                    temp_set ← temp_set ∪ M[value]
                    flag ← false
            if flag is true then
                break
```

2.4 Singly Unique Nucleotides

Structural variation discovery has always been problematic since they tend to overlap with segmental duplications [2]. Researchers that were unable to discriminate slight differences in these regions which prevents copy number variation from being accurately predicted have excluded these highly active regions from their studies. They have instead focused on unique regions of the genome.

In nearly identical regions, nucleotide difference information can be useful in discriminating segmental duplications from each other. Singly unique nucleotide (SUN) is a nucleotide difference between segmental duplications as shown red in Figure 2.4. A nucleotide difference is called a SUN if and only if they reside in only one paralog. Otherwise, as shown blue in the Figure 2.4, they are called paralogous sequence variant (PSV).

2.4.1 Extracting Singly Unique Nucleotides

In order for paralogous sequences to be accurately identified, we need a database of positions and letters of all SUNs that reside in a segmental duplication. Instead of aligning paralogs using a multiple sequence alignment tool, which requires quite some time and computational power, it could be done using pairwise alignments of paralogs which we have in our hand (Figure 2.5). Let A, B and C are three paralogs and S_{ab} denotes the singly unique nucleotides of A relative to B. The singly unique nucleotides of A will be $S_{ab} \cap S_{ac}$. The same equation is valid for other paralogs respectively. Therefore, we could figure out all SUN positions as $(S_{ab} \cap S_{ac}) \cup (S_{ba} \cap S_{bc}) \cup (S_{ca} \cap S_{cb})$.

Algorithm 2 uses an alignment file to find the pairwise SUN positions and letters. As shown in Figure 2.5, an alignment file contains the DNA sequences of two segments and an alignment results between them. The symbols $*$, | and space denote a match, a mismatch and an indel respectively. It is important to state that, if there is a deletion in one sequence, every nucleotide position opposite of

the deletion will be counted as a SUN. On the other hand, the deletion will be counted as one SUN. Another thing to pay attention is the strand of the sequences. Segmental duplications can be found in opposite strand of the DNA. In that case, the nucleotide in the alignment file will be replaced by its complement before getting saved to SUN database.

In algorithm 3, we use algorithm 2 to create a SUN database of segmental duplications by intersecting relative SUN sets. First we find SUNs of A relative to B and SUNs of B relative to A. And then, if A or B come out for the first time, we save the relative SUNs directly to the *sun_map* since there is no set of SUNs yet to intersect. On the other hand, if A or B is found in *sun_map*, we intersect the old set of SUNs with the relative SUNs. We store the database in a map where its keys are segmental duplications and its values are SUN locations and letters.

As a result, we have found 15,352,106 unique SUN locations together with their bases on a positive strand of the DNA.

2.5 Depth of Singly Unique Nucleotides

The depth or coverage, a term that entered the literature thanks to the next generation sequencing, is defined as the number of 'reads' that include a particular nucleotide. In next generation sequencing, the DNA is separated into little and overlapping portions, called 'reads', to be assembled later. When the reads are aligned with respect to their mapping locations, nucleotides are sequenced in varying numbers as shown in Figure 2.6. Therefore the number the nucleotide gets sequenced is called the depth of that position.

As explained in Section 2.4, we have created a SUN database showing all the SUN locations. In Algorithm 4, we basically compute the depth of SUN locations if base in 'read' matches with the base of SUN. In order to use this function, we need a BAM file as a parameter. BAM [41] is a file type for storing sequence

Algorithm 2 An algorithm to find pairwise SUN locations and letters

```
procedure GET_RELATIVE_SUN(alignment_file_path, strand)
    Open alignment file
    if file is open then
        Let S_ab and S_ba empty sets
        while getline(file) ≠ EOF do
            seq1 ← getline(file)
            aligment ← getline(file)
            seq2 ← getline(file)
            for i ← 0 to size of alignment do
                if strand is −1 then
                    seq2[i] ← GET_OPPOSITE_PAIR(seq2[i]) /* It
                    returns the complement of the input nucleotide, for example
                    if input is A, T is returned etc.*/
                if alignment[i] is '*' then
                    S_ab ← S_ab ∪ seq1[i]
                    S_ba ← S_ba ∪ seq2[i]
                if alignment[i] is ' ' then
                    if seq1[i] is '-' then
                        S_ba ← S_ba ∪ seq2[i]
                    if seq2[i] is '-' then
                        S_ab ← S_ab ∪ seq1[i]
```

Algorithm 3 An algorithm to find all SUN locations and letters

```
procedure GET_ALL_SUN(duplication_file_path)
    Open duplication file
    if file is open then
        Let sun_map an empty map showing segmental duplication and
        its SUN locations as key-value pair
        for each entry in duplication file do
            Let A, B segmental duplications as parsed from duplication file
            alignment_file_path ← parsed from duplication file
            strand ← parsed from duplication file
            S_ab, S_ba ← GET_RELATIVE_SUN(alignment_file_path, strand)
            if A exists in sun_map then
                sun_map[A] ← sun_map[A] ∩ S_ab
            else
                sun_map[A] ← S_ab
            if B exists in sun_map then
                sun_map[B] ← sun_map[B] ∩ S_ba
            else
                sun_map[B] ← S_ba
```

alignment data in binary format. For each alignment it contains read name, read sequence, read quality, alignment information, and custom tags. We used HTSLib [42], a C library for reading/writing high-throughput sequencing data, to efficiently read a BAM file.

2.5.1 GC Correction

The next generation sequencing platforms are known to be biased against regions that are rich or poor in terms of GC content [10]. This has been a known issue of HTS platforms that lead to uneven or even no coverage of reads across the genome. Unless it was handled carefully, structural variation discovery methods that rely on coverage data would not be applicable. Fortunately, there are statistical methods out there to fix the uneven distribution of reads. We used multiplicative version of locally weighted scatter plot smooth [43], LOWESS or LOESS in short, to smooth our GC content vs read depth curve. The method

Algorithm 4 An algorithm to find depth of SUN locations

```
procedure FIND_DEPTH(bam_file, sun_set)
    Open bam_file
    if file is open then
        for every read in bam_file do
            chr ← parsed from bam_file
            pos ← parsed from bam_file
            len ← parsed from bam_file
            seq[len] ← CONVERT_ID_TO_NUCLEOTIDE // In a bam file,
            each base is encoded in 4 bits: 0001: A, 0010: C, 0100: G,
            1000: T, 1111: N. This procedure converts the binary-coded
            nucleotide to its letter representation.
            for each SUN falling into the 'reads' range do
                if seq[SUN_index] is equal to SUN nucleotide then
                    Increment depth of SUN by 1
                else
                    Remove from sun_set
```

starts with computing the average read depth of non-overlapping sequence windows of size 1 kilobase. Then the sequence windows are binned according to their GC percentage. There is 0.1% difference between two consecutive bins. And again we compute the average read depth for each bin.

The Figure 2.7 shows how the average read depth is impacted by GC content. The average read depth are getting lower with increasing GC content. We need to adjust the red line so that it comes closer to the green line. In multiplicative version of LOESS method, we define a correction factor, k_{gc}, which is equal to

$$k_{gc} = \frac{\mu_{total}}{\mu_{gc}}$$

where μ_{total} is the average read depth of all sequence windows (i.e. green line) and μ_{gc} is the fit curve smoothed by LOESS (i.e. red line). Having computed k_{gc} for all GC percentages and known the old value for read depth d, new value for read depth, d', is calculated as;

$$d' = d * k_{gc}$$

.

2.6 Gene Copy Number

Copy number variation is a structural variation that changes the amount of DNA. The simple yet powerful method for finding absolute copy number of a region is read depth. However, without multiple read mapping, applying read depth will give poor results. What we compute here is a copy number that will be useful later while computing the paralog specific copy number.

Algorithm 5 An algorithm to find gene copy number

```
procedure FIND_GENE_COPY_NUMBER(gene_file_path, sun_set)
    Let Genes be a vector of genes as read from gene_file_path
    for each gene in Genes do
        Let temp be empty vector
        for each SUN in sun_set do
            if SUN is in gene then
                Add read depth of SUN to temp
        if temp is not empty then
            Compute mean and median of temp
```

In algorithm 5, we try to find average gene copy numbers using only the depth information of SUNs residing in that gene. The procedure takes two parameters. One is the *gene_file_path*, the file that contains gene location information of reference genome version GRCh37 (hg19). The other is *sun_set* produced by previous algorithms with read depth information.

As a result of the algorithm 5, we found 4279 genes that has at least one SUN on them and therefore an average copy number can be calculated for them. These genes are residing in a segmental duplication which makes them paralog genes.

2.7 Paralog Gene Copy Number

Paralog genes are genes that are related as a result of duplication [44]. Unlike orthologs, paralog genes do not have to have the same functionality, however, it can be related since they are coming from a common ancestor. Paralog genes are

important factors of genomic evolution [45].

Computing paralog specific copy number is important since they can be associated with disease or phenotypes. For instance opsin is a gene family that is responsible for color vision with 3 genes, namely *OPN1LW* (long wave), *OPN1MW* (medium wave), and *OPN1SW* (short wave). Mutations in this gene family can cause color blindness. More specifically, genomic variations in *OPN1LW* can cause reduction in color vision for colors whose wavelength is long in the visible spectrum such as red or orange. Likewise, a variation in *OPN1MW* can cause yellow-green blindness, and a variation in *OPN1SW* can cause blue-violet blindness.

2.7.1 Paralog Specific Copy Number

Paralog genes show more variation in copy number than those that are non-paralog since they exist in duplicated regions. We determine paralog genes according to whether they reside in a segmental duplications or not. If a gene is in a segmental duplication, it means it has a paralog on other copies.

Algorithm 6 An algorithm to find paralog specific copy number

```
1: procedure FIND_PARALOG_SPECIFIC_COPY_NUMBER(gene_file_path, segmental_duplication_file_path)
2:     SegmentalDuplications ← parsed from segmental_duplication_file_path
3:     Genes ← parsed from gene_file_path
4:     for each segDup in SegmentalDuplications do
5:         totalCNV ← 0
6:         for each gene in Genes do
7:             if gene is on segDup then
8:                 totalCNV ← totalCNV + gene.getCNV()
```

In algorithm 6, we try to compute paralog specific copy number of paralog genes. Since we use a sequence alignment file in which the reads were mapped to a single location by the aligner, the average copy number of genes are expected to be lower. Therefore we sum the average copy numbers of genes that are on the same segmental duplication (i.e. paralog). The result will be the absolute copy

number of those genes.

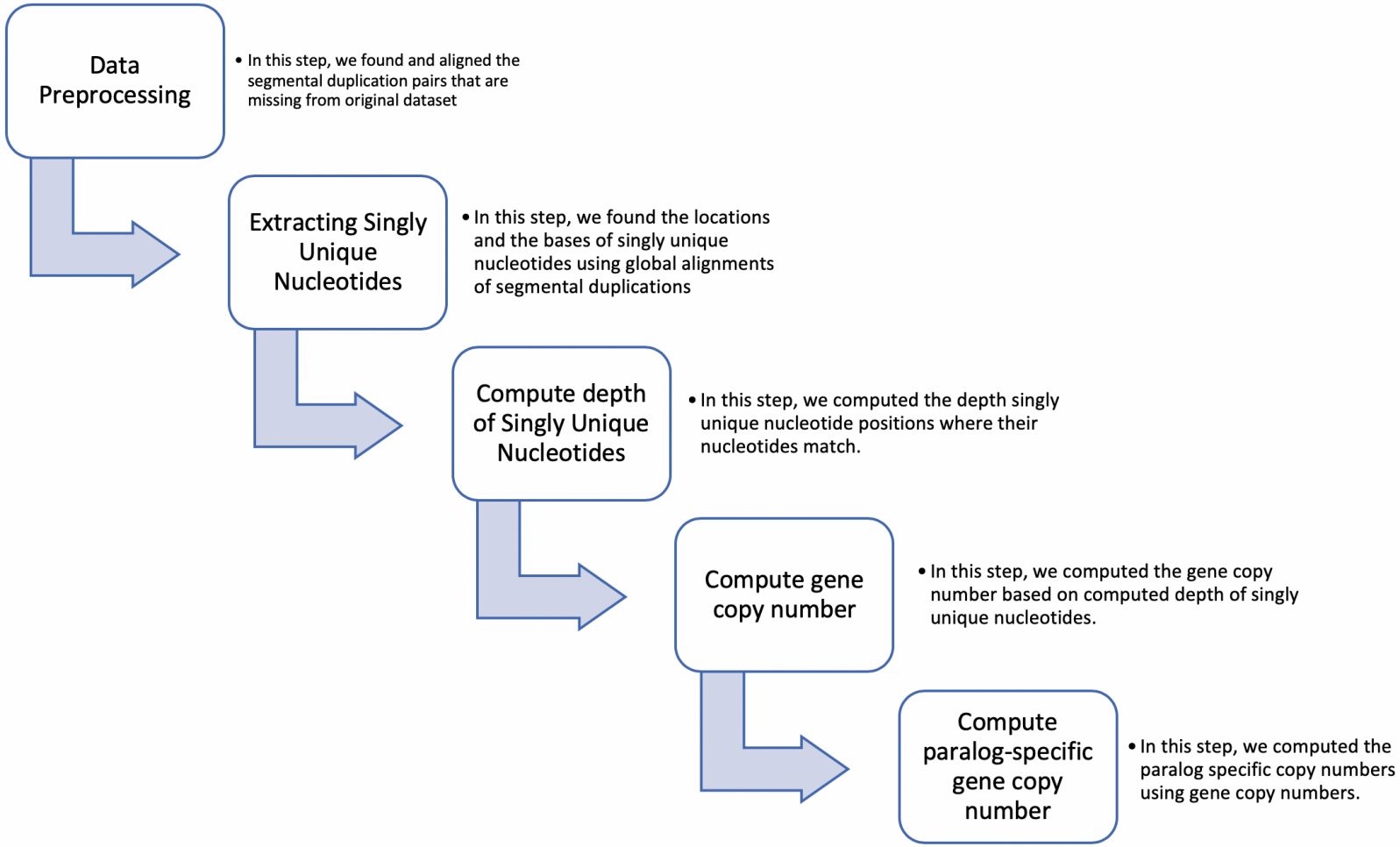


Figure 2.3: Workflow figure of ParaCoND.

```
Paralog        SUN positions          Non-SUN PSVs
1  GTAGCTATCCTGGTGTACGCCATGGCCGGCGGCATCGAGACGATTG
2  GTACCTATCCTTGTGTACGCCATGGCCGGCGGCATCGAGACGACTG
3  GTAGCTATCCTTGTGTACGCCA-GGCCGGCAGCATCGAGACGACTG
4  GTAGCTATCCTTGTGTAAGCCATGGCCGGCAGCATCGAGACGATTG
```

Figure 2.4: Singly unique nucleotides and paralog specific variants. Singly unique nucleotides are single nucleotide differences among segmental duplications. If there are more than one paralog that contains a nucleotide difference, it will be called a paralog specific variant (adopted from [2]).

```
          76848940  76848950  76848960  76848970  76848980  76848990
chr10      TGGAAGGATGCTCTCCTGAGTTACAGAAGGAATGGTCTGGAGGTTAAGATAAAACACAAG
1          ||||*||||*|||||*||||*|||||||*|||**||*|||*||*|||||||||||||||||
chr14      TGGAGGGATACTCTCTTGAGCTACAGAAAGAACAGTGTGGTGGCTAAGATAAAACACAAG
            59648720  59648710  59648700  59648690  59648680  59648670

          76849000  76849010  76849020  76849030  76849040  76849050
chr10      TCAAACTTATTAGAGTTGTCCACAGTCAGCAATGGTGATCTTCTTGCTGGTCTTGCCATT
61         *||||*||||||||||||||||||||*||||||||*||||||||||*||||||||||||||
chr14      CCAAATTTATTAGAGTTGTCCACAGTTAGCAATGGCGATCTTCTTGTTGGTCTTGCCATT
            59648660  59648650  59648640  59648630  59648620  59648610

          76849060          76849070  76849080  76849090  76849100
chr10      CCTGGACCC-------TCCATGGCCTCCACAATATTCATGCCTTCTTTCACCTTGCCAAA
121        *||||||||       ||**||*|*||||**||||||*|*|**|||||**|*|**|*|||
chr14      TCTGGACCCAAAGCACTCTGTGCCTTCCAAGATATTCCTTCTGTCTTTACCTTGCCAAAA
            59648600  59648590  59648580  59648570  59648560  59648550
```

Figure 2.5: A sample screenshot from an alignment file.

```
Read 1: CGTGCGCGTAGTCGATC
Read 2:    GCGCGTAGTCGATCGATCACGTACGAACTG
Read 3:         TAGTCGATCGATCACGTACGAACTGACGTTCAT
Read 4:                 TCGATCACGTACGAACTGACGTTCATA

Depth : 1112222233333334433333333333333333222222221
```

Figure 2.6: Sequencing coverage (depth) computation.

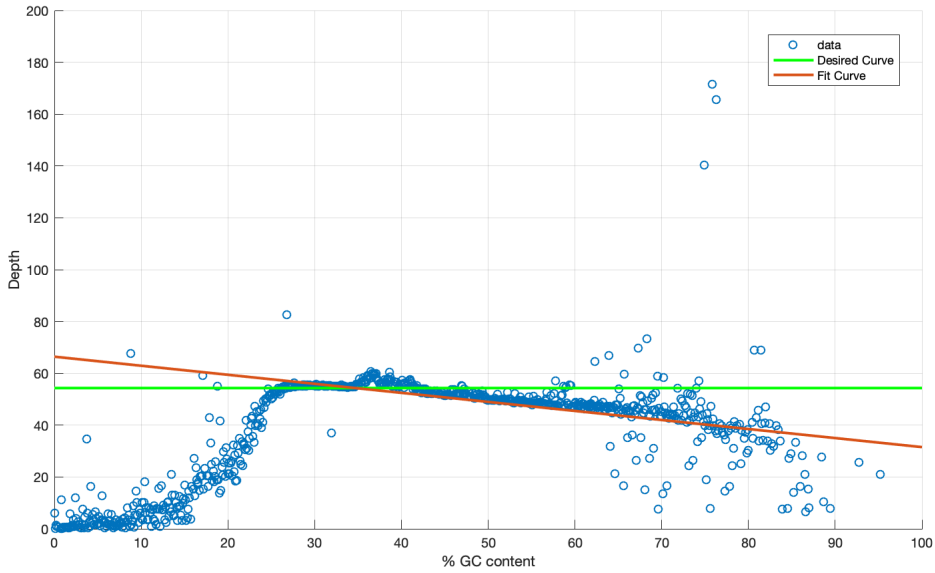


Figure 2.7: The average depth of 1 kb windows with respect to their GC content for NA12878.

Chapter 3

Experimental Results

In this chapter, we present the results that we acquired while testing our method. We tested our method in three different ways. First, we used experimentally validated copy numbers from [18]. They are validated by the fluorescent in situ hybridization (FISH) method, which is commonly used to test whether a specific chromosome region is present or not. Second, we used the tool ART [46] to generate synthetic next-generation sequencing reads from the reference genome. Since reference genome is known to be free of copy number variation, we expect our method to compute copy numbers around 2. And lastly we tested our method with real datasets collected from 1000 Genome Project[47] and Turkish Genome Project [48]. Since there is no complete validated results for paralog genes, we compare our results with the results of mrCaNaVaR [18], a tool to discover structural variations in duplicated regions. mrCaNaVaR, like our method, was based on read depth and it was designed to determine absolute copy numbers in duplicated regions [49].

3.1 Fluorescent in situ hybridization (FISH) Validation

Fluorescent in situ hybridization (FISH) is a technique developed in 1982 by Langer-Safer et. al. to detect the presence or absence of specific DNA sequence [50]. Alkan et. al. performed FISH analysis on selected regions of genome NA18507 from 1000 Genome Project [18, 47]. Table 3.1 shows the performance of ParaCoND over mrCaNaVaR with FISH validated data.

Table 3.1: Comparison of mrCaNaVaR and ParaCoND with validated results obtained from FISH analysis.

Region	**FISH**	**mrCaNaVaR**	**ParaCoND**
chr7:143,471,773-143,513,118	4	4.0	5.31
chr8:7,697,239-7,734,464	3	3.04	1.27
chr15:20,384,910-20,425,229	1	0.8	2.32
chr16:68,708,494-68,749,105	5	5.1	3.43
chr17:41,870,497-41,906,290	2	1.77	3.36
chr17:42,986,667-43,025,934	12	12.98	5.67

FISH analysis results was taken from [18].

3.2 Reference Genome Simulation with ART

Reference genome is a good approximation of human genome without any variation. We used ART [46], a tool that generates synthetic next-generation reads from assembled genome, to generate random reads from reference genome. Then we realigned them to reference genome using BWA-MEM [51] in order to create a variation-free sequence alignment file.

Figure 3.1 shows the results gathered from both methods using the synthetic data generated from reference genome. Since mrCaNaVaR computes aggregate copy number for paralogs, it can be expected mrCaNaVaR to compute higher than 2. On the other hand, ParaCoND outperforms mrCaNaVaR in this dataset by computing gene copy numbers around 2.

3.3 Real Datasets

We tested ParaCoND with two human genomes: NA12878 from 1000 Genome Project and 42S291210 from Turkish Genome project. In general, 30X sequencing coverage is adequate for clinical-level research [52]. Therefore we picked one genome (NA12878) [47] as an example of a human genome sequence with a high coverage (43 times) and another genome (42S291210) [48] as an example of relatively low coverage genome (20 times).

3.4 Results

In this section, the results of mrCaNaVaR and ParaCoND are compared via several plots. The results include absolute copy numbers of paralog genes (i.e. genes overlapping with segmental duplications). It is not expected the results to match perfectly, however, they should show some level of consistency. We measure the consistency as neighborhood percentages. The neighborhood percentage is the ratio of absolute copy numbers. For example, we say 90% or more consistent if the following inequality holds;

$$\frac{90}{100} \leq \frac{CNV_{ParaCoND}}{CNV_{mrCaNaVaR}} \leq \frac{100}{90}$$

.

Table 3.2 shows the statistics on the level of consistency between ParaCoND and mrCaNaVaR. For NA12878, among 1205 genes for which an absolute copy number is computed by both method, 98 genes show 90% or more in their neighborhood and for 42S291210, among 1052 genes, 48 genes show 90% or more in their neighborhood. The reason behind the small difference between two genomes is most likely caused from their sequencing coverage. NA12878 is sequenced 2.15 times more than 42S291210. High sequencing coverage means high quality reads and low sequencing error.

Table 3.3 shows how the results are impacted from the value of absolute copy

Table 3.2: Comparison of ParaCoND and mrCaNaVaR

Neighborhood Percentage	**NA12878**	**42S291210**
90% or more	103	76
75% or more	250	194
50% or more	561	420
25% or more	923	733
Total	1205	1052

Table entries show the number of genes that are in their neighborhood according to copy number computation by both methods.

numbers. As the absolute copy numbers grows, their neighborhood percentages decreases. This is mainly because the sensitivity of the methods are getting lower when absolute copy number is high.

Table 3.3: Comparison of methods according to copy number values

		<10	**10-20**	**20+**
90% neighborhood	NA12878	54	27	22
	42S291210	41	14	21
75% neighborhood	NA12878	127	59	64
	42S291210	105	34	55
50% neighborhood	NA12878	280	157	124
	42S291210	211	105	104
25% neighborhood	NA12878	488	243	192
	42S291210	377	149	207

The entries in the table indicates the number of genes. First column indicates the genes having absolute copy numbers less than 10, second column indicates the genes having absolute copy numbers between 10 and 20 and the third column indicates the genes having absolute copy numbers more than 20 according to mrCaNaVaR.

We compare the results of two methods via several plots. The first three plots show the results with respect to their neighborhood percentages. We also plot the results according to their absolute copy numbers computed by mrCaNaVaR.

The computed absolute copy numbers for paralog genes by both method can be seen in Figure 3.2. In Figure 3.3, we filter in the genes that are 90% or more in the neighborhood. As can be seen in the figure, most of them have an absolute

copy number less than 10. Lastly the Figure 3.4 shows the absolute copy numbers computed by both methods in which they are 50% or more in their neighborhood.

The comparison of computed absolute copy numbers for genes whose copy number is less than 10 can be seen in Figure 3.5. In Figure 3.6, we compare the absolute copy numbers of genes whose copy numbers vary between 10 and 20. We also compare the absolute copy numbers of genes whose copy number greater than 20 in the Figure 3.7.

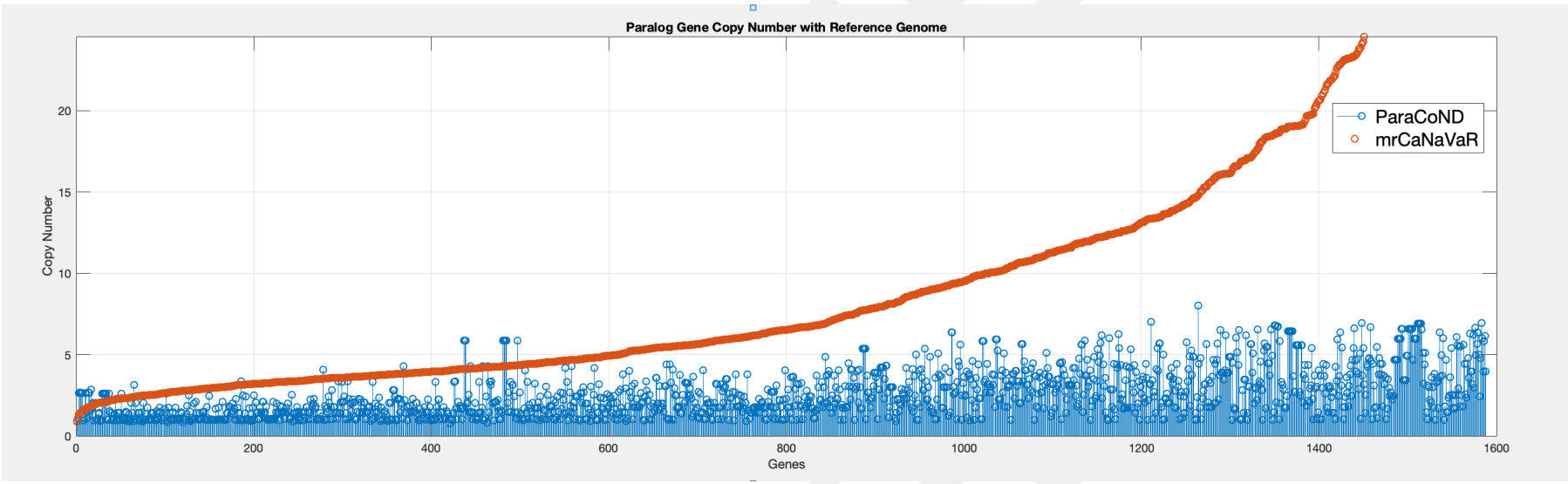
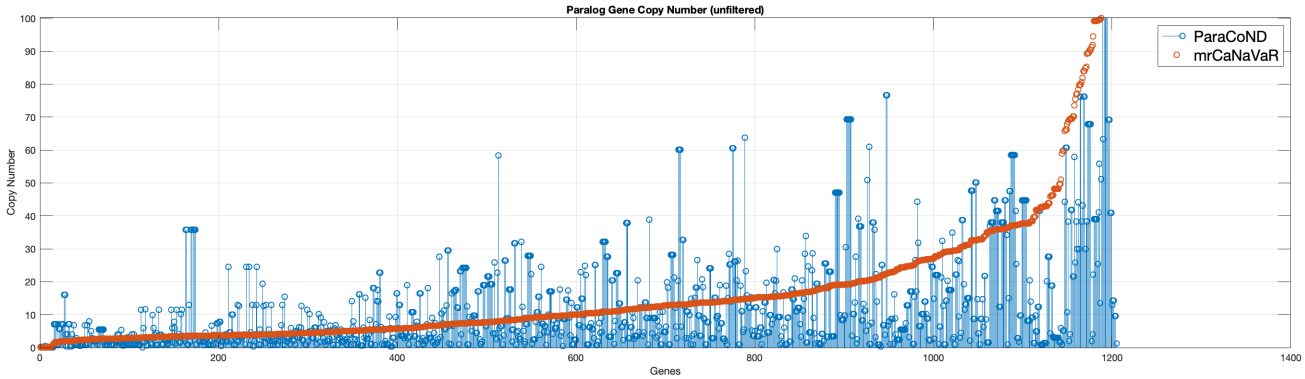
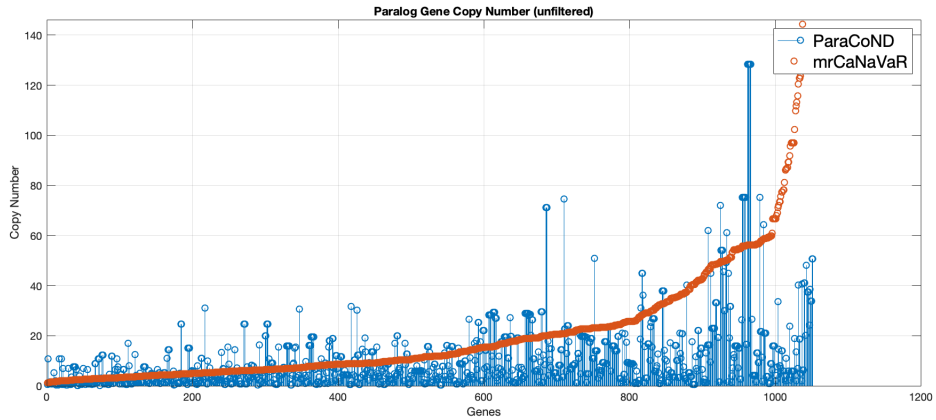


Figure 3.1: Comparison of results with synthetic data generated from reference genome. We used ART, a tool that generates random next-generation sequencing reads from assembled genome. The data was sorted by the results of mrCaNaVaR.

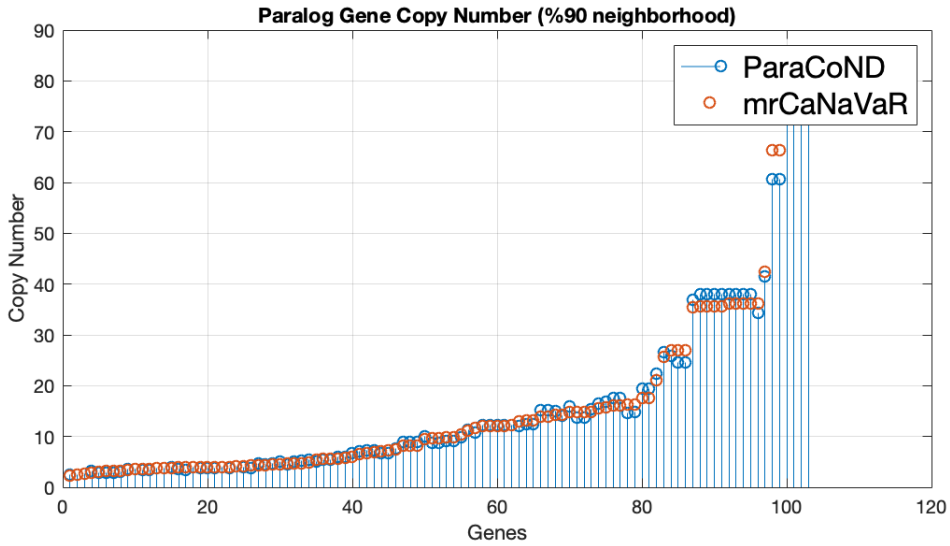


(a) NA12878

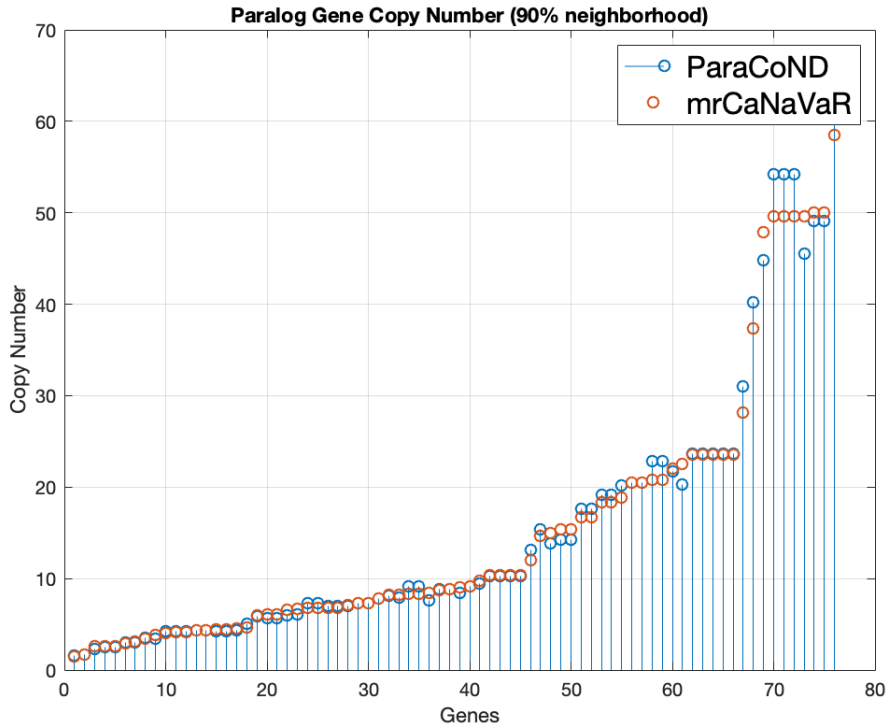


(b) 42S291210

Figure 3.2: Absolute copy numbers of genes overlapping with segmental duplications.

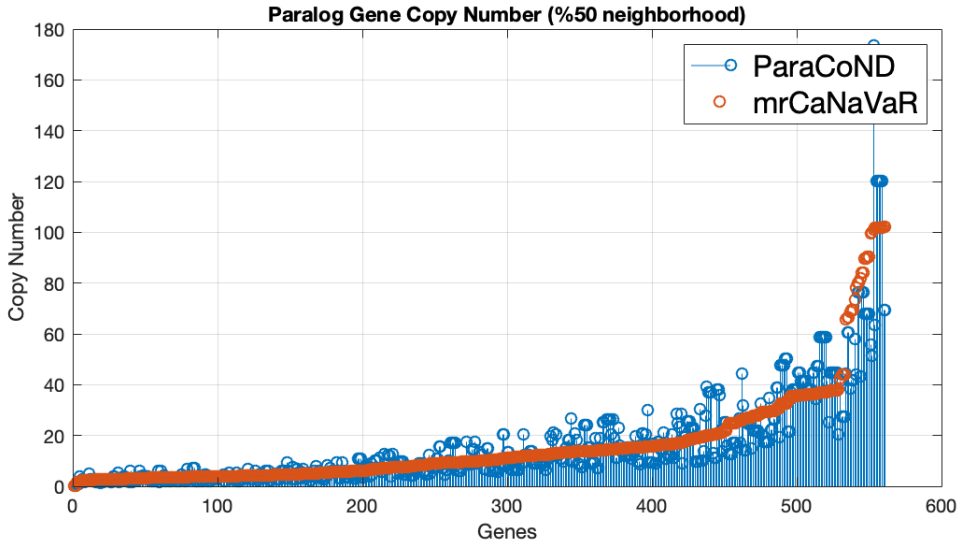


(a) NA12878

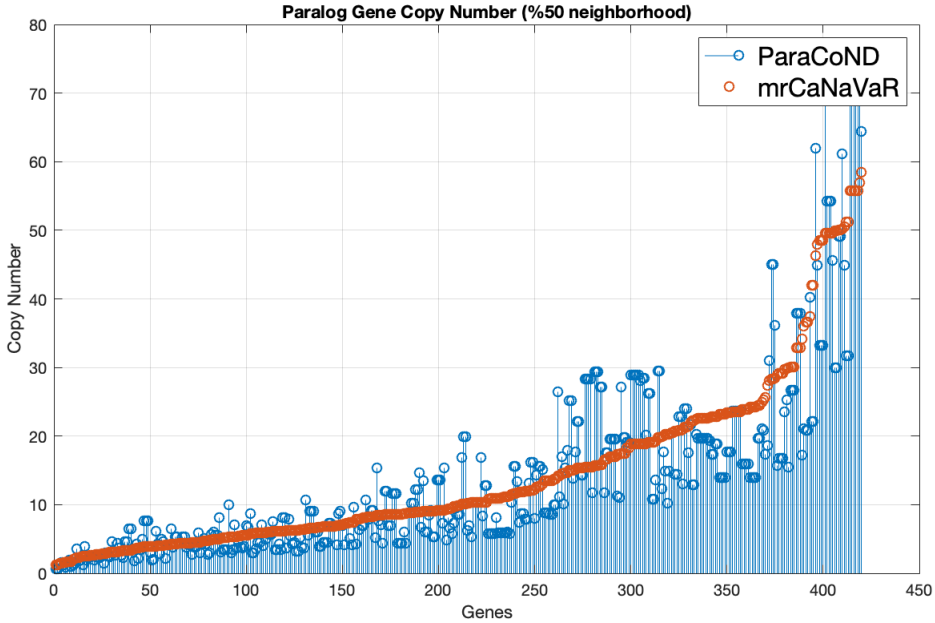


(b) 42S291210

Figure 3.3: Absolute copy numbers of genes that are 90% or more in the neighborhood by both methods.

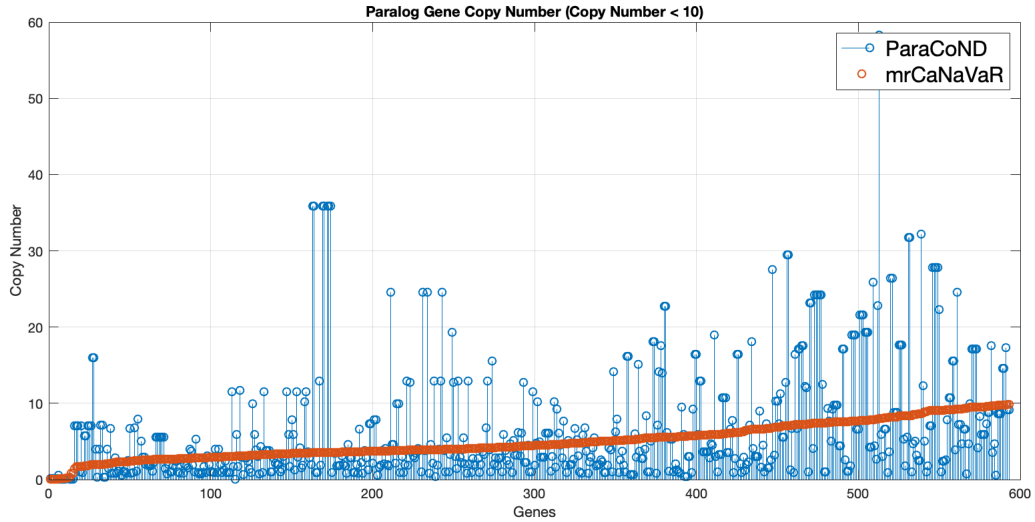


(a) NA12878

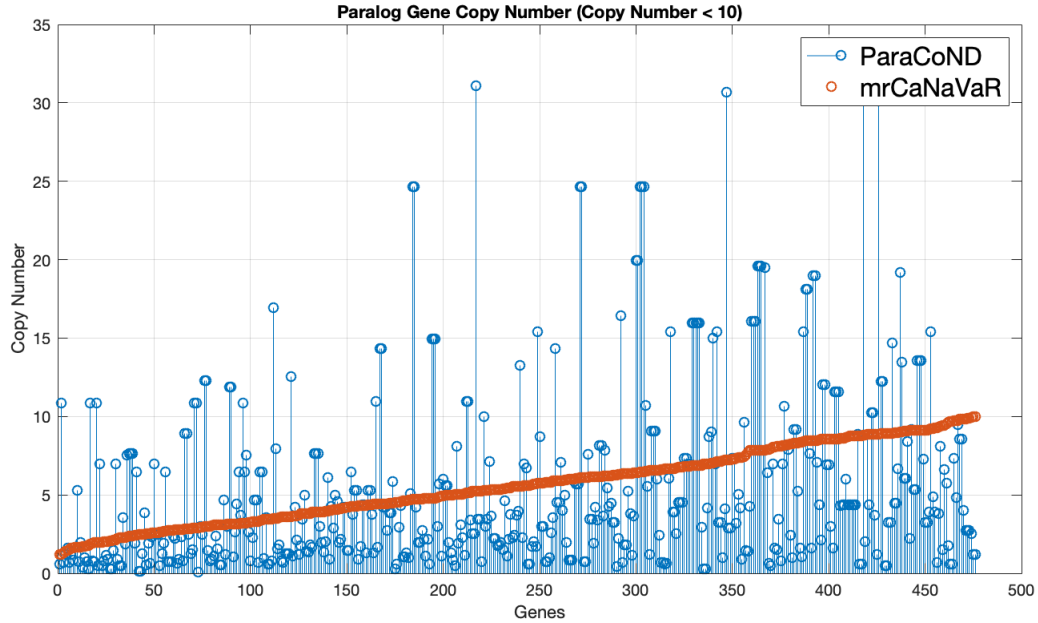


(b) 42S291210

Figure 3.4: Absolute copy numbers of genes that are 50% or more in the neighborhood by both methods.

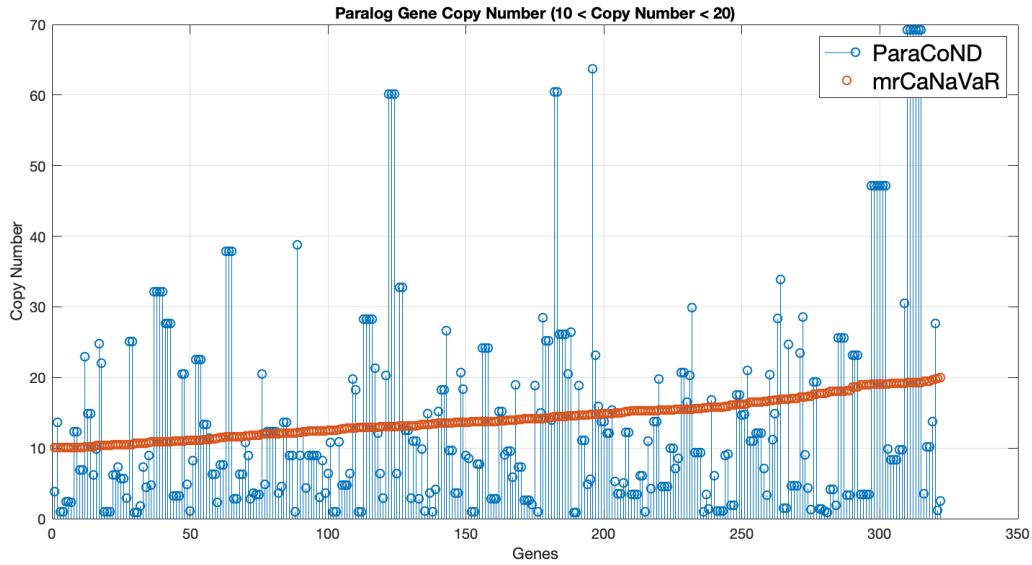


(a) NA12878

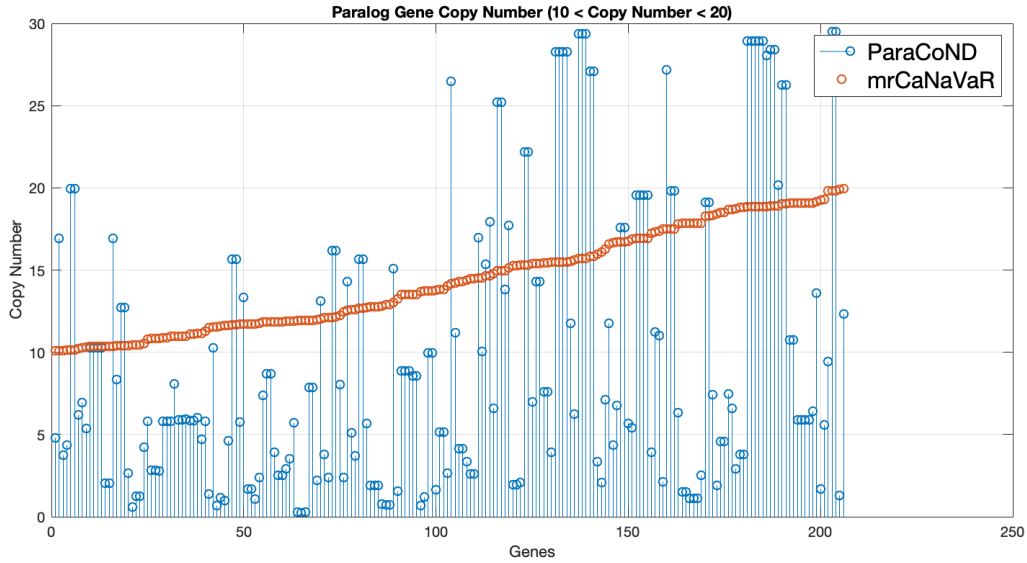


(b) 42S291210

Figure 3.5: Absolute copy numbers of genes whose copy numbers are less than 10 according to mrCaNaVaR.

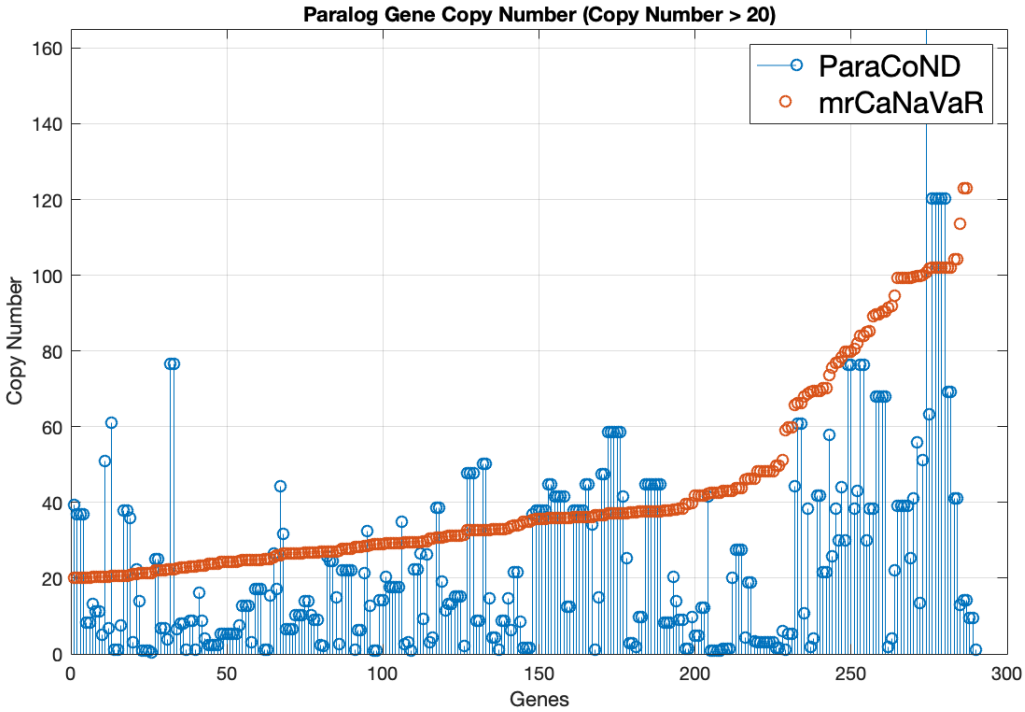


(a) NA12878

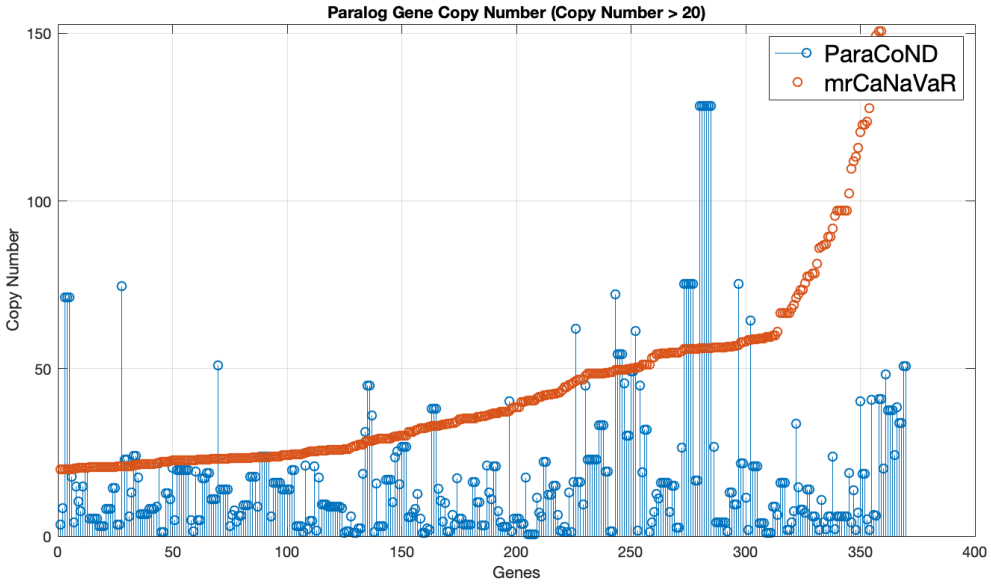


(b) 42S291210

Figure 3.6: Absolute copy numbers of genes whose copy numbers are between 10 and 20 according to mrCaNaVaR.



(a) NA12878



(b) 42S291210

Figure 3.7: Absolute copy numbers of genes whose copy numbers are greater than 20 according to mrCaNaVaR.

Chapter 4

Conclusion and Discussion

In this thesis, we present ParaCoND to discover paralog specific gene copy number within segmental duplications. Duplication-rich regions are mostly abandoned from structural variation discovery because of its complex nature. mrCaNaVaR was the first tool to address these problems, however its performance in differentiating paralogs from each other is poor and it uses a sequence alignment file where reads are mapped to multiple locations.

In our method, we utilized the singly unique nucleotides in order to differentiate the paralogs from each other. Our method was based on read depth and suffered from GC bias. We solved the problem using multiplicative version of LOESS method. We computed the average copy numbers of genes using read depth of singly unique nucleotides on that gene. And finally we sum the average copy numbers of genes residing in the same segmental duplication to find paralog specific copy number of genes.

Since read depth is limited to detecting duplications and deletions [20], we had to exclude other types of structural variations from our study. Furthermore, the absence of validated results makes us to compare our results with another. Therefore, it was difficult for us to determine ParaCoND's performance.

4.1 Future Directions

The main obstacle in genomic studies is that the input files are too large to process. In order to reduce storage and computational cost in large scale genome projects, the input files are kept in binary or compressed format (BAM/CRAM). Since multiple read mapping is the key element of mrCaNaVaR, a remapping process should be done with these files which would make duplication analysis costly. Therefore, it is highly likely that methods like ParaCoND that can work with readily available BAM/CRAM files will be regarded as enabling technique for duplication analysis due to its reduced computational cost.

In this thesis, we applied ParaCoND to human genomes. It can be extended to genomes from different organisms. Furthermore, it can also be extended to different structural variation types. The latter requires a rather big change since we need to change our main signature (read depth) for structural variation discovery.

The method can be improved in many ways, however, there is an urgent need of a validated data set.

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Appendix A

Data

- Segmental Duplication Database: `http://humanparalogy.gs.washington.edu/build37/build37.htm`

Appendix B

Code

- ParaCoND is available at `https://github.com/BilkentCompGen/paracond`